Understanding the impacts of temperature and soil moisture on the germination of *Sclerotinia sclerotiorum* sclerotia

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List of Abbreviations

μm	Micrometre
ANOVA	Analyses of variance
°C	Celsius
C	Cortex
CA	Canada
CE	Controlled environment
CG	Carpogenic germination
CR	Conditioning rate
d.f.	Degrees of freedom
DE	Dissection experiment
EPR	Early percentile range
EtOH	Ethyl alcohol
EU	Experimental unit
F	F statistics
FAA	Formalin–acetic acid–alcohol
FD	Feret´s diameter
FTE	Fluctuating temperature experiment
G	Gram
GR	Germination rate
HTT	Hydrothermal time model
IA	Iowa
IDR	Interdecile range
IPM	Integrated pest management
IQR	Interquartile range
l.s.d.	Least significant difference
LPR	Late percentile range
M	Medulla
MG	Myceliogenic germination
Mm	Millimetre
p	Probality value
PCNB	Pentachloronitrobenzene fungicide
PDA	Potato dextrose agar
R	Rind
RH	Relative humidity
s.e.d.	Standard error of difference

S1, S2, S3	Stage 1, Stage 2, Stage 3
SD	Standard deviation
SME	Soil Moisture Experiment
T	Temperature
T1, T2	Temperature at S1, S2
T_{a-b},	a,b = x = population percentile Difference between time to germination of b and a % population
TE	Temperature Experiment
TE1, TE2	Temperature experiments
T_x	x = population percentile Time to germination of x percentile of population (i.e. T10)
UK	United Kingdom
v.r.	Variation ratio
w/w	Weight by weight

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Declaration

This thesis is presented in accordance with the regulations for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work in this thesis has been undertaken by the author except where otherwise stated.

Summary

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal pathogen causing Sclerotinia disease in a variety of crops all over the world. Infection of most host plants is through airborne ascospores released from mushroom-like apothecia, produced following carpogenic germination of soilborne sclerotia. Disease control relies mostly on fungicides to kill the ascospores and but efficacy is dependent on the correct timing of the limited number of allowed sprays.

Temperature and soil moisture are critical factors affecting carpogenic germination of *S. sclerotiorum* sclerotia with previous work suggesting a two-stage process; a "conditioning" phase (Stage 1, S1) requiring cold temperatures followed by a "germination" phase (Stage 2, S2) requiring warmer temperatures. Little is known about the conditioning process, and isolates within and between different geographic locations, vary in their individual temperature requirements. This study aimed to further investigate the effect of temperature and moisture on carpogenic germination of *S. sclerotiorum* sclerotia and model these underlying processes.

Extensive controlled environment experiments identified clear differences in the temperature requirements of sclerotia from two UK *S. sclerotiorum* isolates (L5, L6) for carpogenic germination. Differences in optimum conditioning requirements were apparent in S1 and S2. Furthermore, a two temperature optima, "spring" and "summer" were identified for isolate L6, explaining a possible adaptation of an UK isolate to initiate further cycles of infection within a single year.

Examination of the effect of a dry period during the S1 conditioning phase significantly limited and delayed germination, while dry periods introduced in S2 arrested germination but germination quickly resumed after moist conditions were restored.

Sclerotia of *S. sclerotiorum* isolate L5, exposed to various temperatures regimes were also examined microscopically for the presence of primordia (apothecial initials). Primordia were observed in sclerotia incubated at 11 - 20°C as early as 7 days, and the number increased with time and temperature; however, no germination was observed in sclerotia at 20°C without conditioning. In contrast, primordia were not observed until after 70 days at 4°C. When sclerotia were conditioned at 4°C for 28 days and transferred to 17°C, primordia and stipe germination was observed 14 days after transfer.

Data from these controlled environment experiments led to the formulation of a new model for carpogenic germination of *S. sclerotiorum* sclerotia, whereby conditioning and germination phases run in parallel rather than sequentially with each associated with low and high temperature respectively. Rates of conditioning and germination for times to 10, 25, 50, 75 and 90% sclerotial germination were determined for 8 temperatures in a first step of model parameter optimisation, which informed the shape of the temperature response curve with rate functions combining linear and logistic parts of the curve fitted to times to 10% germination.

The utility of a potential model to forecast germination of *S. sclerotiorum* sclerotia in the field is discussed.

1 Introduction

1.1 Biology of *Sclerotinia sclerotiorum*

Sclerotinia sclerotiorum (Lib.) de Bary is a necrotrophic fungal pathogen causing Sclerotinia disease (Figure 1.1) in more than 400 plant species, including a wide variety of important crops (Boland and Hall, 1994) all over the world (Figure 1.2) . A variety of names is used to describe the symptoms on different crops e.g. lettuce drop; cottony rot, white mold or watery soft rot of bean, cabbage, carrot, peanut, potato; stem rot of sunflower, cauliflower, bean, potato, tomato, soybean; rot of cauliflower, broad bean, beet, cabbage; damping off of celery (Agrios, 1997).

Figure 1.1 Sclerotinia disease on lettuce caused by *S. sclerotiorum* (infection observed at Wellesbourne, UK, 2017).

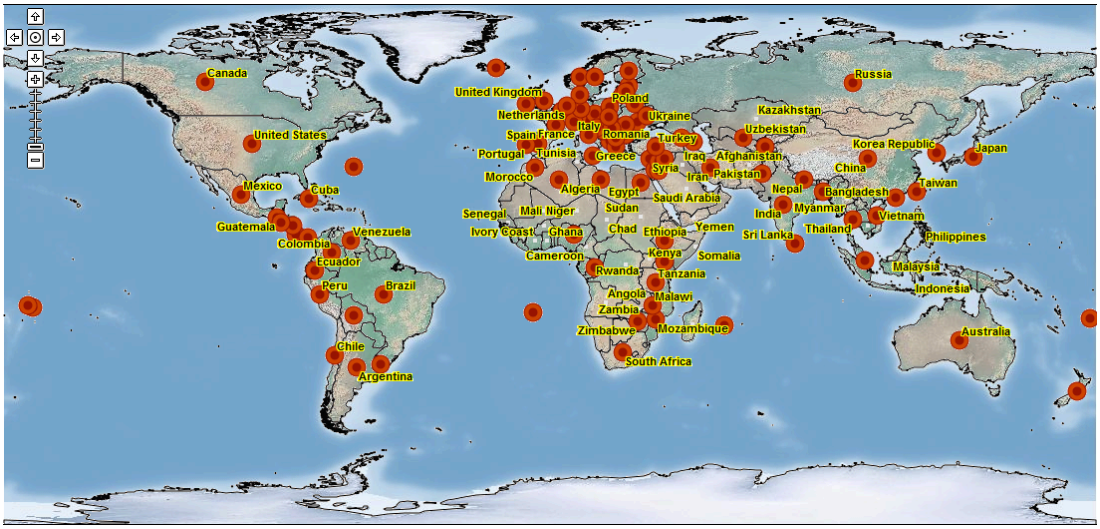

Figure 1.2 Distribution map of *Sclerotinia sclerotiorum*. Red circles indicate countries where Sclerotinia disease has been reported (Plantwise, (2005).

Carpogenic germination of *S. sclerotiorum* sclerotia

S. sclerotiorum infection of most host plants is by airborne ascospores released from mushroom-like apothecia, produced following carpogenic germination of soilborne sclerotia. The germination of soilborne sclerotia is hence a key phase in the life cycle of *S. sclerotiorum* (Figure 1.3). Sclerotia can survive in soil for several years (Adams, 1979) but must be close to the soil surface for carpogenic germination and apothecial production to occur. Infected plants are rapidly colonized by the pathogen, causing wilting and plant death, with further sclerotia formed on the dead plant tissue and subsequently returned to the soil.

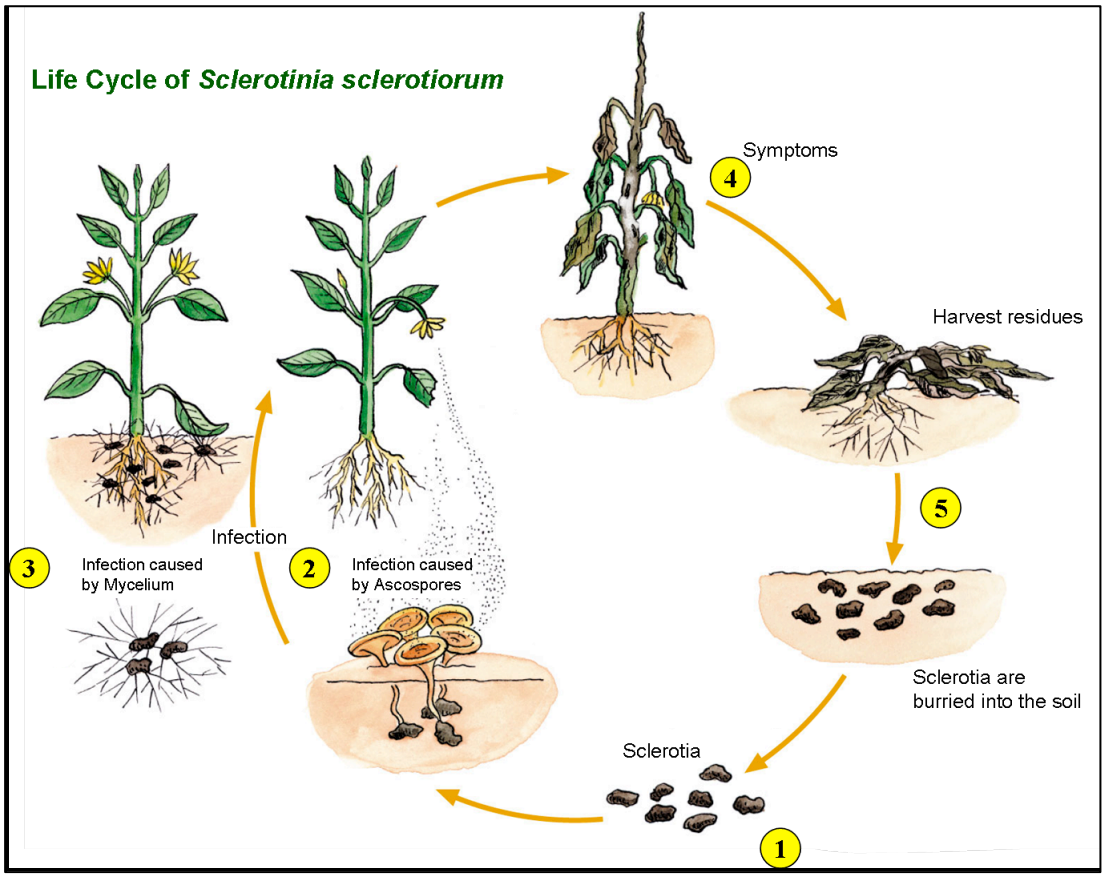

Figure 1.3 Life cycle of *Sclerotinia sclerotiorum* (www.prophyta.de)

Myceliogenic germination of *S. sclerotiorum* sclerotia

Sclerotia of *S. sclerotiorum* can also germinate mycelially by individual hyphae emerging through the rind (Coley-Smith and Cooke, 1971, Adams and Tate, 1976, Huang, 1985, Huang and Sun, 1989) with the ability to develop into mycelial colonies and produce new small daughter sclerotia (Huang and Kozub, 1994). Saito (1977) reported sclerotia germinating either by myceliogenic or carpogenic germination but with the coexistence of these two modes of germination being rare. Furthermore

myceliogenic germination is generally more associated with certain stresses applied to sclerotia, such as freezing temperatures of -10 and -20°C (Huang, 1991), high temperatures (20-25°C) and the desiccant drying of sclerotia prior to transfer to high humidity (Huang et al., 1998). Myceliogenic germination of sclerotia is often used as a way of testing viability where cut sclerotia are placed on agar media and observed for mycelial production (Grogan and Abawi, 1975, Coley-Smith and Javed, 1970).

1.2 Effect of environmental factors on processes involved in carpogenic germination of sclerotia

Temperature

Previous studies have shown that sclerotia from some *S. sclerotiorum* isolates require a '*conditioning phase*' of cold temperatures before rapid, high levels of carpogenic germination can occur (Dillard et al., 1995, Huang and Kozub, 1991, Phillips, 1987, Clarkson et al., 2004, Clarkson et al., 2007). Different 'standard' treatments for conditioning of sclerotia have been used. For instance, Sun *et al.* (2000) preconditioned sclerotia at 4-5˚C for 2 months prior to germination experiments while Clarkson *et al.* (2007) reported that sclerotia of a UK *S. sclerotiorum* isolate were fully conditioned after 2-6 days at 5˚C in soil, but took up to 30-80 days at 15˚C. Clarkson *et al.* (2004) indicated that a conditioning period is required for most UK isolates to ensure carpogenic germination at 20˚C, and standard treatments were either 5˚C or 10˚C for approximately 4 weeks.

Temperature also affects the rate of apothecial production for pre-conditioned sclerotia with a range of temperatures and optima reported. Sun and Yang (2000) suggested a range of 12˚C to 30˚C (optimum around 20˚C) while Hao et al. (2003) reported an optimal range of 10˚C to 20˚C. The same optimum range was reported by Wu and Subbarao (2008) with maximum germination at 15˚C, and the fastest rate at 20˚C. Clarkson et al. (2004) reported temperature having a significant effect on both rate of germination and the final number of germinated sclerotia, with the optimum temperature ranging from 15°C to 20°C; however germination also occurred between 5°C and 25°C with sufficient moisture being required in all cases.

The effect of temperature fluctuation on carpogenic germination of *S. sclerotiorum* sclerotia has rarely been considered, and experiments designed to explore responses at constant temperatures may as suggested by Mila and Yang (2008) not reflect what occurs in the field environment. Mila and Yang (2008) tested temperature fluctuations

of 4, 8, 12, and 16°C around a median of 20°C, and a constant 20°C. The highest and fastest germination was observed for daily temperature fluctuations of 8°C, the slowest germination was at 12°C fluctuation and generally the shorter fluctuations produced more apothecia compared to higher fluctuations. Clarkson et al. (2004) compared germination rates derived from sclerotia buried in controlled laboratory environment with a constant temperature with sclerotia buried in field under fluctuating temperature conditions and thermal time analyses suggested a similar response in both cases. Moreover, in the field, a synchronized germination of mixed populations is common (Caroline Young, ADAS, personal communication), despite *S. sclerotiorum* isolates appearing to differ in their conditioning (and temperature) requirements.

Additionally Liu and Paul (2007) reported repeat germination of sclerotia left in a growth chamber for 5 to 8 months indicating that they remain viable as long as sufficient nutrients remain to support further germination to produce stipes/apothecia.

Several authors have reported that sclerotia from *S. sclerotiorum* isolates originating from warm climates do not need a period of cold temperatures for conditioning at all (Huang and Kozub, 1991, Wu and Subbarao, 2008), and also that differences in the germination temperature range may depend on the origin of isolates and on the temperature at which the sclerotia were formed (Hao et al., 2003, Huang and Kozub, 1991, Uloth et al., 2015).

Moisture

Morrall (1977) reported that *S. sclerotiorum* sclerotia germinate over a wide range of soil moisture levels (in a heavy clay soil) and at water potentials close to saturation, and researchers agree that moist (not saturated) soil is required (Phillips, 1987). Clarkson et al. (2004) found that germination occurred for soil water potentials ≥-100kPa but that there was little or no germination at -300kPa. Ferraz et al. (1999), reported apothecial production when soil potential was above -54kPa, with high production above -25kPa while Hao et al. (2003) also reported little germination at -300kPa. Wu and Subbarao (2008) examined effects of interrupted soil moisture (dry periods) on carpogenic germination of *S. sclerotiorum* and reported that no apothecia were produced during the dry periods or immediately after restoring moist conditions. Furthermore, they reported that a 10 to 20 days period of low soil moisture can completely arrest carpogenic germination and it took up to 35 days between rewetting

and the appearance of new apothecia, regardless of when and for how long (10+ days) the dry period was applied.

Other factors

Sun and Yang (2000) demonstrated an interaction between temperature, light intensity and moisture level such that optimal temperature and temperature range for germination of *S. sclerotiorum* sclerotia were affected by both light intensity and moisture level.

Wu and Subbarao (2008) summarised the effects of burial depth on the carpogenic germination of sclerotia, concluding that sclerotia buried at 0 - 2 cm produce more apothecia at a faster rate than those buried deeper and that sclerotia could produce apothecia from as deep as 4 - 5 cm.

The substrate on which sclerotia are incubated also appears to have an effect on the germination process; for instance, sclerotia produced on wheat grain in a flask failed to germinate at all even after extended periods of time (Clarkson et al., 2007, Phillips, 1986).

Overall, there are therefore some diverse results from different studies investigating effects of environmental factors on germination of *S. sclerotiorum* sclerotia. This could be because of differences in the experimental methodology (Foley et al., 2016) but may also be due to variation between and within populations from different geographical origins (Huang and Kozub, 1991). These factors make it challenging to predict the behaviour of any particular population, whether a single isolate or a mixture.

1.3 Disease management

Losses instigated by Sclerotinia disease include the direct loss of yield, reduction in crop quality, and the increased cost of production due to crop protection and disease management interventions as well as abandonment of heavily infected fields (Saharan, 2008).

Sclerotinia disease control includes integrated disease and agronomic management strategies implemented in various stages of life cycle of both host and *S. sclerotiorum*. These include:

- Crop rotation: introducing a non-host crop into affected fields prevents the build-up of sclerotia in the soil although even low numbers of sclerotia can initiate disease due to the apothecia producing large numbers of airborne ascospores (Saharan, 2008). Because of the wide host range of *S. sclerotiorum*, suitable rotation crops are limited and monocot crops such as corn, winter wheat (Neumann et al., 2002), and rice (Gupta et al., 1987, Singh, 1987) are recommended.
- Cultivar selection and resistance: Breeding for resistance is problematic due to Sclerotinia disease outbreaks being highly dependent on environmental conditions, making screening for resistance under field conditions challenging as it is difficult to determine which portion of the resistance in the field is the result of physiological resistance or escape mechanisms associated with disease severity (Saharan, 2008). Approaches to identify resistance under controlled conditions are generally more successful but widespread implementation of *Sclerotinia* resistance is lacking in any crop.
- Tillage practices: *S. sclerotiorum* sclerotia buried deeper in the soil are reintroduce to the surface where they can germinate to produce apothecia or infect plants by mycelial germination more readily.
- Irrigation management, plant density, row orientation and canopy clipping/pruning: These management practices all influence the microclimate conditions where moist and shaded conditions as well as contact between infected and uninfected tissue promote Sclerotinia disease outbreaks and spread (Blad et al., 1978, Rotem and Palti, 1969, Huang and Hoes, 1980).
- Soil solarization: If *S. sclerotiorum* sclerotia (in soil) are exposed to temperatures ranging from 35°C to 45°C for several days, viability is reduced along with their ability to produce apothecia (Phillips, 1990, Sharma et al., 2005, Swaminathan et al., 1999, Wu, 1991). Phillips (1990) explains that as well as the direct effect of heat, viability of sclerotia is also decreased by microbial colonization and degradation following exposure to sub-lethal temperatures during solarization.
- Biological control: Many studies have investigated the potential of microbial biological control agents to reduce the concentration of initial inoculum by killing *S. sclerotiorum* sclerotia or inhibiting their germination. Mycoparasites predominantly fungi include *Coniothyrium minitans* (commercial product), *Trichoderma viride*, *Gliocladium catenulatum* which kill sclerotia and/or

vegetative hyphae as a result of direct penetration of hyphae of *S. sclerotiorum* (Hoes and Huang, 1975, Huang, 1977, Huang and Hoes, 1976).

- Application of fungicides: Since soil applied fungicides (e.g. Vinclozolin, Procymidone, Benomyl, Dichlozoline, Thiram, Quintozene) are now largely banned, control of Sclerotinia disease is largely focussed on the application of foliar fungicides where proper coverage of leaves and susceptible tissues must be achieved to kill the ascospores. Timing is crucial as these must be applied before infection occurs and not too early such that the concentration of fungicide has decreased. Efficacy of fungicides is therefore variable between crops and years because of the difficulty in determining disease risk (i.e. presence of ascospores) which results in inaccurate timing of the limited number of allowed sprays (Bolton et al., 2006b, Clarkson et al., 2004, Clarkson et al., 2007, Twengström et al., 1998b, Young et al., 2004, Saharan, 2008).

To reach a maximum disease control a combination of several of these disease management strategies must be combined into a single programme (Saharan, 2008).

Forecasting Sclerotinia disease in field

To optimise fungicide applications among other disease control strategies, forecasting models have been developed relating pathogen, host and environmental conditions conducive to Sclerotinia disease and integrating relationships among these three aspects of the disease triangle (Agrios, 1997). These forecasting models aim to provide estimates of the timing of initial infection or disease occurrence, and subsequent development (McDonald, 2004).

1.4 Modelling approaches

There are number of different modelling approaches used to describe biological processes which have been utilised for disease forecasting.

Physiological time models

Physiological time according to Lovell et al. (2004) enables to quantify process of development in terms of the accumulation of physiological time units above a threshold and thus effects of fluctuating environmental conditions on processes such as germination or growth can be explained.

Thermal time as summarized by Lovell et al. (2004) is used by researchers in entomology and plant physiology commonly, to measure growth and development in relation to thermal time. Thermal time accumulates heat units above a threshold (base) temperature over a period of time. The assumption about a shape of the response to temperature is vital, as a simple linear response is not always appropriate. However, for a more complex shapes there are often insufficiently detailed observations to allow estimation of model parameters. The simplest thermal time approach is the *accumulated day degree* approach, where the time in days is multiplied by the average day temperature. Sun and Yang (2000) used this approach for apothecial production of *S. sclerotiorum*. Identification and application of temperature thresholds, above or below which biological processes cease and there is not further physiological change are important especially for linear thermal time models (Lovell et al., 2004).

Hydrothermal time (HTT) models consider the effects of temperature and water potential simultaneously and are used as unifying models to describing the patterns of germination in seed populations as they enter and leave environmentally induced dormant or inhibited states (Allen, 2003). Similar to thermal time, the HTT models suggest that germination rates are proportional to how much temperature and water potential exceed base/threshold values (Bradford, 2002). This approach is widely explored for seed germination, modelling for example carrot and onion seed germination (Allen, 2003) but could be potentially applied to gemination of *S. sclerotiorum* sclerotia.

Stage models

These models represent a widely used approach in population ecology modelling, where organisms go through different life stages. Plant disease cycles represent pathogen biology as a series of development stages (e.g. *S. sclerotiorum* life cycle Figure 1.3) including dormancy, reproduction, dispersal, and pathogenesis. Each of the stages is determined by an interaction among host, pathogen, and environment and this forms the base of many plant disease prediction models (De Wolf and Isard, 2007).

A number of thermo-dynamical models concerning the effect of temperature effect on development of powdery mildew pathogens have been published. The study of Xu (1999a) explored a one stage model, which represented fungal development in the latent period in relation to temperature, and this was further subdivided in to a two-

stage model with an incubation and post-incubation period where a different response to temperature for each stage was observed. A further development of the two-stage model was carried out with extended temperature regimes and for different powdery mildew pathogens (Xu, 1999b, Xu and Robinson, 2000).

1.5 Modelling approaches previously used for *Sclerotinia sclerotiorum*

Dispersal models

S. sclerotiorum has been suggested previously as a potential biocontrol of giant thistle (*C. arvense*) in New Zealand pastures. Due to the possible risk associated of infecting important crop plants, a methodology for risk analysis was introduced. The relative disease risk from *S. sclerotiorum* ascospores was defined compared with naturally occurring background inoculum. An empirical model for sclerotia decay in soil over time and mechanistic models quantifying escape and dispersal of airborne ascospores were developed which resulted in a risk evaluation using cartographic techniques. Mechanistic models were proposed for assessing the escape and dispersal of ascospores in the air, from the treated pasture (De Jong et al., 2002).

Forecasting models

A number of forecasting models for Sclerotinia disease have been proposed, which have used various types and levels of risk assessment through combining biological and ecological knowledge of *S. sclerotiorum*.

A forecasting model for oilseed rape was produced based on a risk point table of field specific data and precipitation based on the analysis of data from about 800 fields collected during a 10-year period. Six factors assessed as affecting *Sclerotinia* infection were evaluated; the number of years since last oilseed rape crop, disease incidence in last *Sclerotinia* affected host crop, crop density, rain in the last 2 weeks before flowering, weather forecast and regional risk for apothecium development. Based on this, a point scale was allocated based on the risk of heavy infestation (Twengström et al., 1998b).

Algorithms for predicting outbreaks of Sclerotinia blight of peanut were evaluated by Langston et al. (2002) using indices of moisture, soil temperature, vine growth, and canopy density consistently improved the timing of fungicide applications in comparison with weekly scouting or calendar-timed sprays.

A forecasting system model based on crop phenology, the presence of ascospores or apothecia, and field history of disease was developed for Sclerotinia disease of carrots. Infection of carrots occurs at two different life stages as examined by Kora et al. (2003). Epidemics in the field are associated with infection of senescing leaves by ascospores, and forecasting was based on soil moisture, canopy closure, senescing leaves, air and soil temperature, as well as the presence and number of apothecia. Epidemics in storage were associated with spread of disease from carrot to carrot and forecasting was based on air temperature, rate of cooling, surface wetness, and the level pre-existing infection.

McDonald (2004) summarized a number of forecasting models for White mold of bean, used combinations of the number of apothecia, canopy density, bloom, and rainfall and emphasized the influence of these variables on disease.

A study from New Zealand Kiwifruit used a systems dynamics approach to model Sclerotinia disease development based on interactions between the pathogen's life cycle, crop phenology and the environment. A positive relationship between apothecial density and disease incidence was reported, with a series of biological clocks and environmental thresholds, to predict disease potential at the end of flowering (Hoyte et al., 2003)

A crop-loss related forecasting model for Sclerotinia stem rot (ScleroPro) on oilseed rape (*Brassica napus*) was developed and widely used in Germany (Koch et al., 2007). The model consisted of a two-part approach. The first part provided a regional assessment of disease risk based on four weather variables (air temperature, relative humidity, rainfall and sunshine duration). The second part provided a field-site specific, economy-based recommendation to spray, based on costs of spray, expected yield and price of rapeseed. Crop rotation was also included in the model as a field-site specific risk factor. http://www.phytopathology.uni-goettingen.de/index.php?id=159

Forecasting models to predict germination of *S. sclerotiorum* sclerotia

Ecological models based on understanding of the biology and ecology of *S. sclerotiorum* have also been produced, and some have particularly examined the environmental factors affecting conditioning and germination of sclerotia in order to predict carpogenic germination and production of apothecia. Examples of this approach and the inspiration/motivation for this study are the published papers by

(Clarkson et al., 2004, Clarkson et al., 2007) (further described in Chapter 3 and Chapter 6). In a first modelling attempt, Clarkson et al. (2004) examined the feasibility of developing a forecasting system for carpogenic germination of *S. sclerotiorum* sclerotia by using an experimental approach investigating the key relationships amongst temperature, soil water potential and carpogenic germination for two UK *S. sclerotiorum* isolates. In a second study (Clarkson et al., 2007), further controlled environment data were collected leading to development of a forecasting model based on a mathematical simulation which critically included the requirement for a cold "conditioning" phase to be completed before subsequent germination could occur, with both process rates being dependent on temperature when soil moisture was not limiting (further detail is presented in Chapter 7). When applied to a field situation, the model included a moisture threshold estimate, whereby for air temperatures of between 12 – 20˚C, a total rainfall of >4mm in the past 4 days was required for germination to proceed (the soil was considered moist enough at temperatures <10˚C). However, some model limitations were identified:

- Insufficient data was collected for temperature treatments of < 30 days and <10°C to properly assess conditioning requirements;
- A failure of the model to predict germination of sclerotia from isolates with different temperature requirements;
- Only the time to 50% germination was modelled rather than the whole distribution of germination times;
- Incorporation of the arbitrary moisture threshold to mimic the delay in germination in less favourable conditions;

Overall as summarized by McDonald (2004), even when there are a number of forecasting methods for *S. sclerotiorum*, because of the variable severity of epidemics, a lack of registered fungicides, little or no infrastructure to deliver disease-forecasting systems and declining prevalence of integrated pest management (IPM) programs the development and implementation of such forecasting models still fails to be widely exploited.

1.6 Project aims

This project aimed to further investigate the effect of temperature and other factors on germination of *S. sclerotiorum* sclerotia and model the processes of conditioning and germination. This would then enable future investigation of the effect of other environmental factors and climate change scenarios on Sclerotinia risk in the UK.

The main objectives of the project were to:

- Evaluate the effect of different temperature regimes on carpogenic germination of *S. sclerotiorum* sclerotia and determine the physiological changes leading to formation of stipes and apothecia;
- Evaluate the effect of moisture on carpogenic germination of *S. sclerotiorum* sclerotia;
- Produce a model to simulate germination of *S. sclerotiorum* sclerotia with emphasis on modelling the whole distribution of germination times for different *S. sclerotiorum* isolates.

To achieve these objectives, the initial part of the project focused on further examining and quantifying the effect of temperature on carpogenic germination of sclerotia from two *S. sclerotiorum* isolates (L5, L6) selected based on their different requirements for a cold conditioning phase in preliminary experiments. These experiments were designed to address some of the data issues and gaps associated with previous experiments (Clarkson et al., 2004, Clarkson et al., 2007) including a larger sample size, lower temperatures and shorter durations of low temperature exposure. Furthermore, experiments investigating the effects of different soil moisture conditions were also introduced, examining various durations of dry periods on sclerotial germination. To increase the understanding of the physiological processes underlying the conditioning and germination phases, microscopic observation of dissected sclerotia was performed prior to germination to identify any structural changes that might precede the formation of stipes and apothecia. A range of modelling approaches were considered for the new data generated in this project as informed by an understanding of the biological processes underlying conditioning and germination.

1.7 Thesis structure

Chapter 2. *S. sclerotiorum* isolates, methods for sclerotia production, experiment organization and statistical methods common for all the experimental work included in this study.

Chapter 3. Main experiments to examine the effect of different temperature regimes on germination of sclerotia from two *S. sclerotiorum* isolates (TE1 & TE2).

Chapter 4. Dissection experiment examining physiological changes of sclerotia from one *S. sclerotiorum* isolate (DE) for different temperature regimes and timepoints.

Chapter 5. Soil moisture experiments (SME_S1 & SME_S2) examining the effect of different durations of dry periods introduced at different times during the germination process of two *S. sclerotiorum* isolates.

Chapter 6. Model development, with further information on the published forecasting model on which this project is based, followed by description of new approaches as the result of this study leading to formulation of a new model. The description of the new model is provided along with the process of parameter estimation. A brief showcase of germination data observed in field and model application is also discussed.

Chapter 7. General discussion, which aims to summarise the main results of this research and discuss in the context of other work as well as the development of a practical forecasting model for *Sclerotinia*.

2 General methods

2.1 Selection of *S. sclerotiorum* isolates for this study based on re-analysis of data from previous research

To enable successful examination of temperature effects on the processes involved in carpogenic germination, it was important to select *S. sclerotiorum* isolates with different biological and genetic characteristics and in particular with contrasting responses to temperature. Characteristics considered were:

- Genotype;
- Reliable production of sclerotia;
- High levels of carpogenic germination;
- Germination response to temperature (in particular requirement / non-requirement for cold conditioning).

To enable selection of *S. sclerotiorum* isolates, data from previous published and unpublished research was evaluated and re-analysed. A previous Defra-funded research project (Defra Project code: HH3230SFV (DEFRA, 2009)) conducted by Dr J. P. Clarkson (The University of Warwick, HRI) examined the extent of *S. sclerotiorum* diversity by characterising UK isolates from different crops using agar-plate based tests to assign them to mycelial compatibility groups (MCGs), and also established whether sclerotia from different isolates differed in their germination response to environmental factors. *S. sclerotiorum* sclerotia were collected over two years from three crop types and four field sites in the UK and comprised carrot (Blyth, Nottinghamshire, 2005), lettuce (Petworth, Sussex, 2005) oilseed rape (Preston Wynn, Herefordshire, 2005 and 2007). Sclerotia from 46 *S. sclerotiorum* isolates selected from these crops, as well as two historic UK isolates from lettuce (TM, IMI390054; 13, IMI 390053; Clarkson, 2007), and two isolates collected from oilseed rape in the USA (LMK211 and LMK199; personal communication, John Clarkson), which represented 28 MCGs, were buried in pasteurised compost under moist conditions (as described later in this chapter, 2.3) and were observed for carpogenic germination to produce apothecia following a 'standard' conditioning period of 5°C for 20 days prior to transfer to 15°C. The new ANOVA analyses showed a significant difference between isolates in mean time to germination ($F_{48,94}$=6.54, $p<0.001$) and the level of germination achieved ($F_{49,98}$=5.16, $p<0.001$) (Figure 2.1). The mean germination times ranged from 76 (LMK211) to 191 (O23) days, with the fastest

germinating UK isolate being L6 with 85 days. Germination level varied from 0% (L44) to 92% (L6). Hence, the *S. sclerotiorum* isolates demonstrated a wide range of germination abilities under these controlled conditions, and there was also some evidence that germination times were similar for isolates from the same MCG (data not shown).

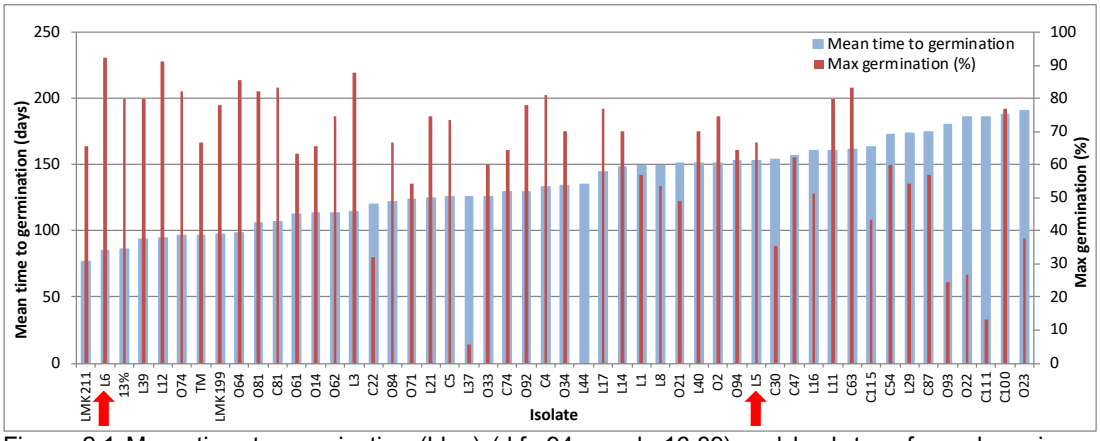

Figure 2.1 Mean time to germination (blue) (d.f.=94, s.e.d.=16.89) and back transformed maximum germination percentage (red) (Angular transformation, d.f.=98, s.e.d.=9.555) for sclerotia from 50 *S. sclerotiorum* isolates incubated initially for 20 days at 5°C followed by transfer to 15°C. Red arrows indicate isolates L5 and L6, selected for this PhD study.

In a second experiment in the same Defra project, sclerotia of five of the 50 *S. sclerotiorum* isolates originating from lettuce plants (13, L5, L6, L17 and L21), were buried in pasteurised compost under moist conditions (as described later in this chapter, 2.3) and initially conditioned at five temperatures ranging from 5°C to 20°C for 4 durations (5, 20 and 40 days) followed by subsequent transfer to 15°C. Control treatments at a constant 15°C were also included for each isolate, and apothecia production was observed for 250 days. Reanalysis of these data showed a significant effect of isolate, control treatment and conditioning temperature on maximum percentage germination ($F_{8,100}$=8.84, $p<0.001$), where isolate L5 achieved significantly lower germination when initially incubated at 20°C (dark blue) compared to other isolates, the control treatment (orange) and the other conditioning temperatures, irrespective of the duration of the initial incubation (Figure 2.2).

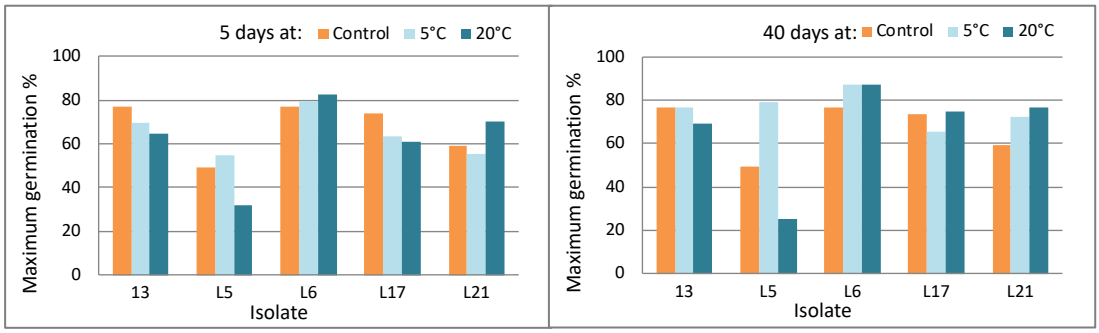

Figure 2.2 Comparison of maximum % germination (back transformed ANOVA) for five *S. sclerotiorum* isolates after 5 and 40 days incubation at 5 and 20°C followed by transfer to 15°C and control treatment (orange) at constant 15°C (Angular transformation, d.f.=100, s.e.d.=2.883).

Reanalysis of the data for mean time to germination of the five *S. sclerotiorum* isolates showed a significant interaction between isolate, control treatment, conditioning temperature and duration ($F_{16,100}$=3.09, p<0.001). The isolates responded differently to the treatments, where for the constant temperature of 15°C (control) isolate L6 germinated fastest (94 days) and L5 the slowest (220 days). Varying conditioning temperature resulted in different germination rates, with longer durations making these effects more distinct. Isolate L5 was most affected by the different conditioning treatments, such that 40 days at 5°C resulted in a 121 day decrease in mean germination time (99 days) when compared to the control treatment (220 days) while a conditioning treatment of 40 days at 20°C resulted in a 20 day increase in mean germination time compared to the control (240 days). In contrast, the mean time to germination after 40 days at 5°C and 40 days at 20°C was 91 and 112 days respectively for isolate L6 (Figure 2.3).

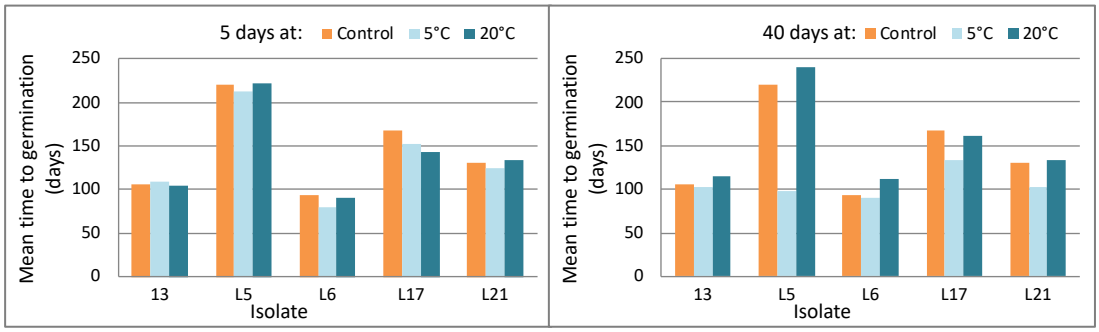

Figure 2.3 ANOVA calculated mean time to germination for five *S. sclerotiorum* isolates after 5 and 40 days incubation at 5°C (light blue) and 20°C (dark blue) followed by transfer to 15°C and control treatment (orange) at constant 15°C (d.f.=100, s.e.d.=13.46).

The *S. sclerotiorum* isolates L5 and L6 therefore showed a distinct and contrasting response to conditioning temperature and duration, whereby the level and time to germination for isolate L5 was limited at shorter durations and high temperatures in compared to isolate L6.

Based on this re-analyses of previous data, *S. sclerotiorum* isolates L5 and L6 (both from lettuce from Petworth, Sussex in 2005) were selected for experiments in this PhD project based on their ability to produce large numbers of sclerotia, to achieve a high germination under controlled conditions and importantly because they showed a contrasting response to conditioning temperature. The *S. sclerotiorum* isolate L6 represents an isolate with either no or little requirement for cold conditioning or the ability to condition at a higher temperature and/or to complete conditioning in a very short period of time at low temperature. In contrast, L5 represents an isolate requiring a longer period of cold conditioning to achieve high and fast germination. Although L5 achieved a lower percentage germination with long germination times in the first experiment compared to some other isolates, its ability to produce sclerotia (general observation from earlier experiments) and the results from the second experiment made it a good selection. Furthermore, both isolates were used in a previous field experiment, where sclerotia of both isolates show a synchronized germination in spring when buried the previous autumn (John Clarkson, personal communication).

2.2 Production of *S. sclerotiorum* sclerotia for experiments

2.2.1 Methods

Sclerotia from the two selected *S. sclerotiorum* isolates L5 and L6 were taken from frozen storage, dissected and placed on potato dextrose agar (PDA) plates and subsequently placed at 18°C to initiate mycelial growth for 3 to 5 days. The resulting colonies were sub-cultured to produce actively growing colonies. Sterile/autoclaved wheat grain (100g wheat grain, 140ml water) in 1L flasks were inoculated with 4 agar plugs of actively growing mycelium from each isolate (Figure 2.4A). Flasks were incubated at 20°C in the dark and shaken two times per week to achieve an even growth and maturation of sclerotia (Figure 2.4B). After approx. 6 weeks (or after reduction of white mycelium), sclerotia were washed out onto a 2mm sieve, and the wheat grain separated from mature sclerotia by flotation (Figure 2.4, C&D).

Figure 2.4 A) Inoculation of wheat grain flask with agar plugs of actively growing mycelium of *S. sclerotiorum* isolate; B) Inoculated wheat grain flask after 5-6 weeks at 20°C in the dark (*S. sclerotiorum* isolates L5 & L6). Isolate L5 produced white "fluffy" mycelium longer than isolate L6; C &D) Separated wheat grain and sclerotia during the washing out.

To ensure prompt drying, washed out sclerotia were spread in a thin layer on paper tissue on a plastic tray, and the paper was exchanged for a dry one several times. The trays containing sclerotia were placed in an airflow cabinet at room temperature (approx. 20°C) for 1-3 days. Some of the sclerotia produced for the first Temperature experiment and Dissection experiment (TE1 & DE) were initially left to dry in an unheated room (temperature fluctuation 5-18°C) over 3 days, after which they were transferred to an airflow cabinet and dried at room temperature (approx. 20°C) until completely dry. All subsequent sclerotia produced were only dried at room temperature to avoid any period of cold temperature which could have affected results in TE1 (further discussed in Chapter 3.2).

Dried *S. sclerotiorum* sclerotia were separated into different size groups by further sieving (mesh size 2.0, 2.8, 3.3, 4.0 and 5.6mm; Figure 2.5) and subsequently used in experiments or stored dry at room temperature until needed.

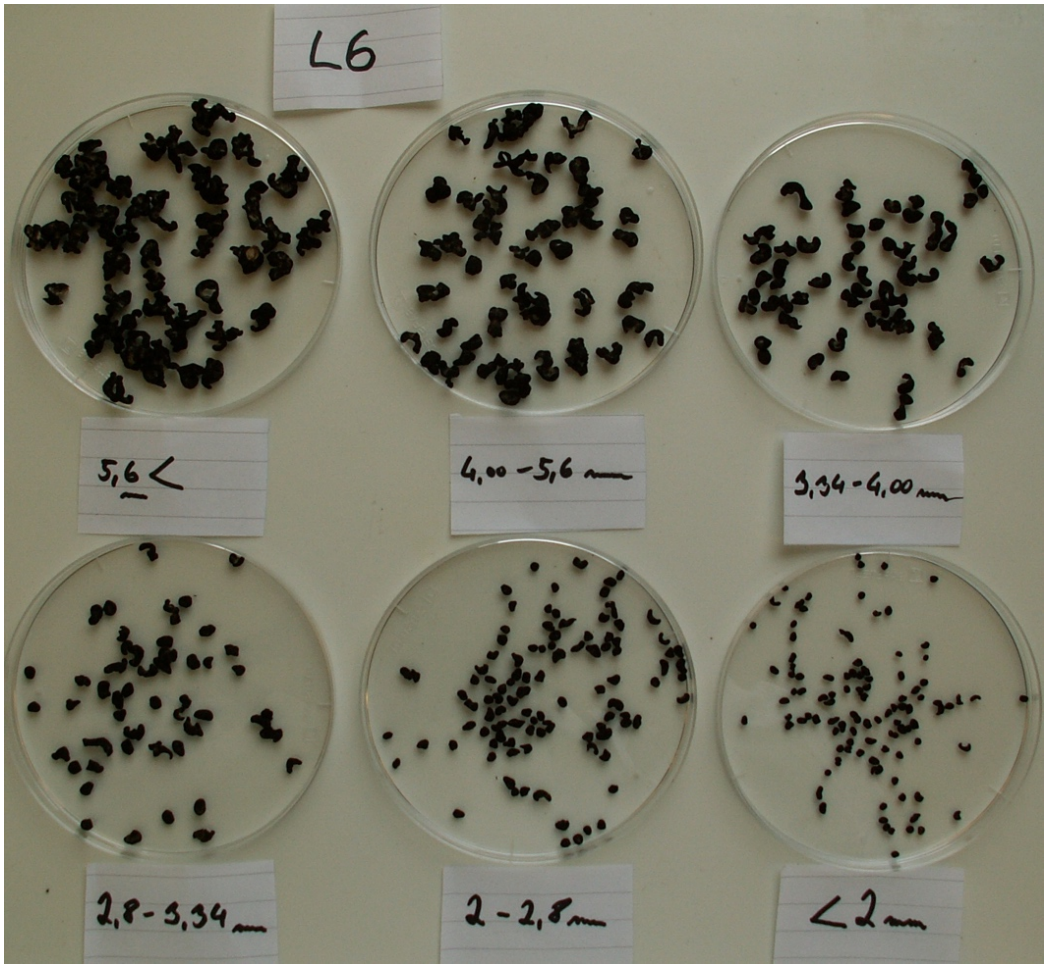

Figure 2.5 Illustration of different size groups of sclerotia for *S. sclerotiorum* isolate L6.

The following statistics were calculated for each isolate at of the five production runs (Table 2.1) and summary values presented in the results are the means across these five production dates (if not stated otherwise):

- The number of sclerotia produced in each size group, estimated based on a sample of 50 to 100 sclerotia counted and weighed to obtain the mean weight per sclerotium, with the total weight of sclerotia produced (per size group) then divided by the mean weight per sclerotium from the sample (per size group);
- The mean number of sclerotia produced per 1L flask, calculated by dividing the total number of sclerotia produced (the sum of the number of sclerotia produced in each size group) by the number of flasks;
- The mean weight of individual sclerotium, calculated by dividing the total weight of produced sclerotia by the total number of produced sclerotia (the sum of the number of sclerotia produced in each size group);
- The percentage of sclerotia produced in each size group, calculated by dividing the number of sclerotia produced for each size group by the total number of sclerotia produced, and multiplied by 100.

2.2.2 Results

Sclerotia of *S. sclerotiorum* isolate L6 formed and matured more rapidly than those from isolate L5, and the cotton like appearance of growing mycelium disappeared by the end of the six-week period. For five different production dates, a similar pattern of sclerotia development occurred, where for isolate L6, 124 out of 126 flasks provided mature sclerotia (Figure 2.6. A), in contrast to isolate L5 where sclerotia were obtained from only 110 out of 124 flasks (Table 2.1). This was mostly due to isolate L5 producing large clumps of mycelium which were extremely difficult to break up by shaking during the maturation process, and these failed to produce large numbers of healthy looking mature black sclerotia (Figure 2.6. B).

Figure 2.6 Examples of a wheat grain flasks inoculated with *S. sclerotiorum* isolate L5 A) flask with normally maturing sclerotia; B) flask with big clumps of mycelium and wheat grain, failing to produce large numbers of healthy looking mature sclerotia.

Production date	No. of flasks washed		Estimated number of sclerotia / 1L flask		Experiment
	L5	L6	L5	L6	
January 2015	70	74	2,307	3,273	TE1, DE
Jun 2015	14	20	1,896	3,541	TE2
September 2015	3	7	2,127	3,036	Field
May 2016	16	18	1,599	3,010	Field
December 2016	7	5	2,076	3,271	SME, Field
Total	110	124	2,001*	3,226*	

Table 2.1 Production of *S. sclerotiorum* isolates L5 & L6 sclerotia on wheat grain in 1L flasks. Estimated total number of sclerotia for each run are based on measured dry weight of a subset of sclerotia for each size category and a total dry weight of sclerotia produced for each size category for each production date. *mean of estimated numbers of sclerotia for all production dates. Experiments: TE1 & TE2 = Temperature experiments (1st & 2nd), DE = Dissection experiment, SME = Soil Moisture Experiment, Field = Field burials.

The mean number of sclerotia produced per 1L flask for isolates L5 = 2,001 (SD = 268) and for isolate L6 = 3,226 (SD = 216) (Table 2.1). The mean weight of individual sclerotium was for isolate L5 = 23.34 mg (SD=3.73), and for isolate L6 = 12.16 mg (SD=3.13).

For each isolate at each production date the percentage of sclerotia in each size interval was calculated. ANOVA was used, with production date included as a block effect, to test for differences between isolates in the percentage for each size interval. For the size interval <2.00mm the percentage of sclerotia produced for isolate L6 was significantly greater than that for isolate L5 ($F_{1,4}$=31.08, p=0.005). For the size interval 2.00 – 2.80mm there was no significant difference between the isolates ($F_{1,4}$=4.13, p=0.112). For the remaining size groups the percentage of sclerotia produced for isolate L6 was significantly smaller than for isolate L5 (2.80 - 3.34mm: $F_{1,4}$=62.21, p<0.001; 3.34 - 4.00mm: $F_{1,4}$=65.46, p<0.001, 4.0 - 5.6mm: $F_{1,4}$=54.56, p=0.002, 5.60<mm: $F_{1,4}$=23.88, p=0.008) (Figure 2.7).

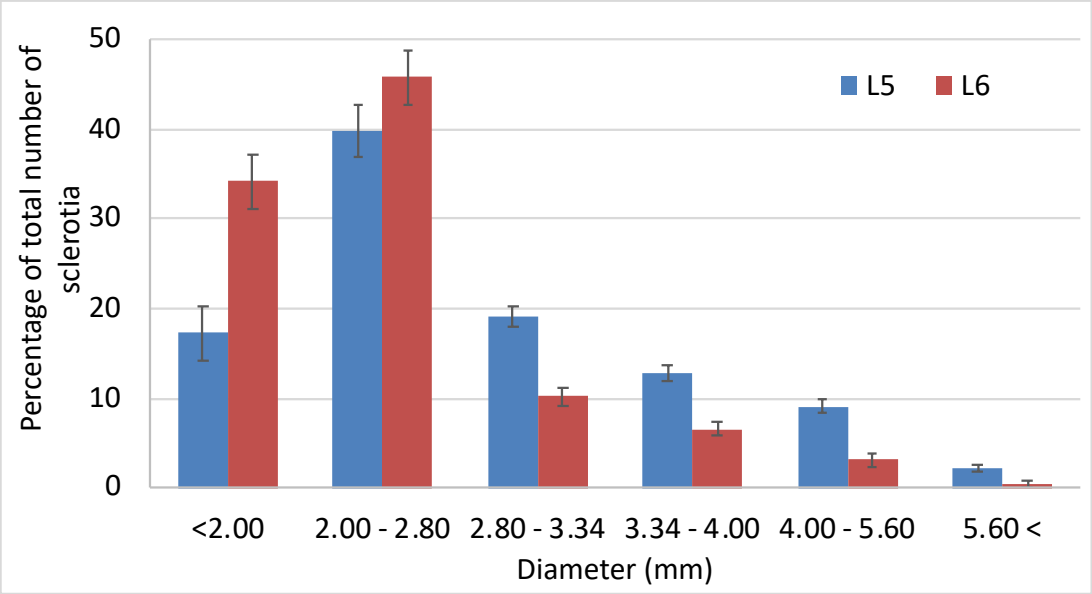

Figure 2.7 ANOVA calculated means of the percentage of sclerotia (%) for the different size categories obtained over the five production dates (block) in years 2015/2016, for the *S. sclerotiorum* isolates L5 and L6. Error bars represent the s.e.d. calculated for each size groups.

2.2.3 Discussion

Isolate L6 generally produced more sclerotia compared to isolate L5 (when produced on wheat grain). Regarding the size distribution, both isolates produced the highest proportion of sclerotia in the size interval 2.00 – 2.80mm (~ 45%). Isolate L6 produced a higher proportion of sclerotia smaller than 2.00mm compared to isolate L5, while for size intervals greater than 2.80mm, isolate L6 consistently produced smaller proportions of sclerotia compared to isolate L5. Similar differences in the size

of sclerotia produced for various *S. sclerotiorum* isolates (including L5 and L6) were observed previously by Taylor et al. (2018). He reports that isolate L6 produced greater numbers of smaller sclerotia compared to other isolates (including isolate L5), when produced on potato dextrose agar. Furthermore, the same trend was also observed when sclerotia of L6 and other isolates were produced on five different crop plants (bean, carrot, lettuce, oilseed rape and potato). Taylor et al. (2018) also compared the production of apothecia for differently sized sclerotia, assessing the level and time to germination. Here, for isolate L6, larger sclerotia showed higher and faster germination, irrespective of the host plant on which they were produced. This effect of size could influence germination experiments if the size of sclerotia is not carefuly considered.

2.3 Experimental setup for experiments on carpogenic germination of *S. sclerotiorum* sclerotia

For all laboratory experiments in this thesis which examined the effect of different environmental factors on carpogenic germination of *S. sclerotiorum* sclerotia, a consistent approach was used where sclerotia were placed in compost. Compost (John Innes No.1; J. Arthur Bower's, UK) was sieved (4mm), pasteurized (110°C for 30min) and moisture content calculated following oven drying of three samples at 80°C overnight. For the two Temperature (TE1, TE2), and Dissection (DE) experiments 150g of compost was dispensed into 300ml clear plastic food containers (Bunzl UK Ltd., London), tamped down and sclerotia partially buried by pushing them down such that the top edge was level with the soil surface (Chapter 2.1, Figure 2.8). Water was added to adjust the moisture content to approximately 30% (w/w). For the Soil moisture experiment (SME) only, Petri dishes were used instead of the plastic food containers and filled with 50g of moist/dry compost as described further in Chapter 5.2. Compost was rewetted by spraying with water as required during the experiments so a suitable moist environment was maintained in which sclerotia should be able to germinate. In all experiments, plastic boxes with buried sclerotia were randomized and each replicate unit was located in opaque plastic container (® Really Useful Box) of capacity either 35L (internal dimensions: 370 x 310 x 280; fit max. 48 (6x8) food containers) or 18L (internal dimensions: 395 x 335 x 170, fit max. 24 (6x4) food containers) to keep the sclerotia in dark while in CE rooms/cabinets. To monitor temperature at 30-minute intervals, Thermochron® iButtons® (DS1921G,) were placed in the centre of each large plastic opaque container, in between the

boxes with buried sclerotia. Data were downloaded using the OneWireViewer freely available software (http://www.maximintegrated.com/en/app-notes/index.mvp/id/4373).

For all experiments the size of sclerotia and the size variation within and between the two *S. sclerotiorum* isolates was taking in account when designing the experiments either by selecting the most appropriate sclerotia size for the assessment (DE - Chapter 4.2, SME - Chapter 5.2, Field trial) or by choosing representative categories across the size range to account for possible effect of the sclerotial size on carpogenic germination (TE1 & TE2, different size used for each replicate Chapter 3.2, Table 3.2).

2.4 Statistical methods

Software used for calculations, design of experiments, statistical analyses, graphs and modelling were GenStat (GenStat 17th / 18th edition, VSN International Ltd, Hemel Hempstead), Excel (Microsoft Office 2011-2016) and R (R version 3.3.0 (2016-05-03)).

Data summaries (as defined in Table 2.2) were calculated for each treatment/rep combination. The main summary statistics considered further were: maximum germination percentage ($G_{Max\%}$), calculated based on viable sclerotia, (number germinated by the end of S3; (Table 2.2), Chapter 3.2), including myceliogenic and carpogenic germinated sclerotia; mean time to germination (*M*) calculated based on the number of sclerotia germinated by the end of S2 - for correct calculation of the mean time to germination a midpoint of each observation interval was used as representative of the germination time for all sclerotia germinated in that time period; time to germination of various percentiles of population based on germinated sclerotia (T_x, *x = population percentile*), where for statistical analyses only germination times until the end of S2 were used. For modelling purposes, S3 data provided further information to support estimation of model parameters, therefore the germination until the end of S3 was used; standard deviation (*SD*). These statistics were chosen to provide a comprehensive understanding of the carpogenic germination of sclerotia.

In order to satisfy the ANOVA assumption of homogeneity of variance it was necessary to apply an angular transformation to the $G_{Max\%}$ values prior to analysis, using the ANGULAR function in GenStat. This transforms a percentage p (0 <p < 100) to an angle, a (0 <a <90) using formula a = (180/pi) × arcsin(sqrt(p/100)). To aid

interpretation of the analyses of this transformed variable, tables of back-transformed means were calculated from the tables of means produced from each ANOVA model using the IANGULAR function in GenStat.

Analysis of variance was used to assess for effects of selected treatment factors. Generally for the effects described in the results the F statistics including the appropriate degrees of freedom (d.f.) for the corresponding factor (f) and residual term (r) are shown as subscript ($F_{f,r}$) and the p value is reported. Furthermore, where appropriate the standard error of the difference (s.e.d.) and least significant difference at p = 0.05 (l.s.d.) are reported.

For all parameters, a general ANOVA directive was used in GenStat with “Rep” as a blocking factor to account for the different size of sclerotia and “Isolate” either as a crossed factor common for all treatments (SME) or the datasets for each isolate were tested separately (TE1, TE2).

Summary statistics from observed data			Expression/gen stat directive
Notation	Units	Description	
S1	Abbreviation	Initial incubation (conditioning) period for sclerotia at T1 temperature	
S2	Abbreviation	Second incubation (germination) period for sclerotia at T2 temperature	
S3	Abbreviation	Third incubation period to check for viability of sclerotia not germinated during S1 & S2 at 11°C	
Rep	number	Replication	
N	number	Buried sclerotia	
z	number	Number of intervals	
f_i	number	Number of germinated sclerotia in each interval	
t_i	days	Observation time point; i = observation number	
x_i	days	Midpoint of observation interval	$(t_i + t_{i-1})/2$
N_G	number	Number of germinated sclerotia (S1, S2)	
N_{NG}	number	Number of ungerminated sclerotia (S1, S2, S3)	
N_{MG}	number	Number of sclerotia germinated myceliogenically	
N_{INF}	number	Number of sclerotia removed due to infection	
N_V	number	Number of viable sclerotia	$N - N_{INF} - N_{NG}$
$G_{Max\%}$	%	Maximum germination	$\frac{\boldsymbol{N_G}}{\boldsymbol{N_V}} \times \mathbf{100}$
$G_{Max\%}$_a	angl	Maximum germination - transformed	ANGULAR (beck-transformed - IANGULAR)
M	days	Mean time to germination	$\frac{\sum_{i=1}^{z} \boldsymbol{x_i} \times \boldsymbol{f_i}}{\boldsymbol{N_G}}$
VAR	$days^2$	Variance of germination times	$\frac{\sum_{i=1}^{z}(f_i \times x_i^2) - N_G \times M^2}{N_G - 1}$
SD	days	Standard deviation of germination times	$\sqrt{\boldsymbol{VAR}}$
$T_{y;}$	days	Time to germination of population % (y=10…90%) of germinated sclerotia	INTERPOLATE (linear)
T_a-T_b	days	Difference in times to germination of a and b percentile of population	

Table 2.2 Showing an overview of statistics and expressions used for calculation in GenStat for the 1st and 2nd temperature experiment. The Rep, n, z, f_i, t_i, x representing inputs for calculations of further statistics. The rather high number of statistic shell aid the better understanding of overall distribution of the germination times.

3 Main temperature experiments

This experimental chapter aims to further investigate the effects of temperature on the germination processes of two contrasting *S. sclerotiorum* isolates with distinctive temperature requirements. The chapter starts with an introduction describing the most relevant studies to this experimental work. A description of the experimental methods, the statistical design and the statistical analyses approaches follows. Results are analysed based on the topics they aim to address and to assist with understanding the complexity of outcomes of these two temperature experiments. At the end a summary of the results is presented.

3.1 Introduction

Temperature is thought to be one of the main factors affecting germination of *S. sclerotiorum* sclerotia. As reported in Chapter 1.2,1.2 there are several studies from various parts of the world examining the effect of temperature on germination and identifying a number of favourable conditions for both conditioning and germination or no need for conditioning at all. These differences are generally associated with the geographic origin of the isolates, however differences in temperature requirements can also be observed for isolates originating from the same country (Clarkson et al., 2007). This variability of pathogen characteristics makes it difficult to fully understand and quantify the processes involved in carpogenic germination of sclerotia, and to produce forecasting tools associated with variability in the environmental conditions.

This study aims to extend previous research of temperature requirements for UK *Sclerotinia sclerotiorum* isolates (Clarkson et al., 2004, Clarkson et al., 2007, Young et al., 2014). Clarkson et al. (2004) examined two UK isolates (TM, 13) to determine the relationships between temperature and carpogenic germination for sclerotia initially incubated at 4°C for 4 to 20 weeks (stored in wheat grains flasks) followed by transfer to constant temperatures ranging from 5°C to 25°C when water potential ≥ -100kPa for isolate TM and ≥-30kPa for isolate 13.

Optimum temperatures for both fast and high levels of germination (production of stipes) were identified as 15°C for isolate TM and 18-22°C for isolate 13. First sclerotia appeared after 9-19 days after transfer to temperature of 15-18°C for isolate TM and 18-22°C for isolate 13, with time to germination increasing at lower temperatures and levels of germination decreasing at higher temperatures. Both isolates were studied further in Clarkson et al. (2007) in both control environments

and field conditions. These laboratory experiments explored the effects of different temperatures and durations of the initial (conditioning) process as well as the effect of different substrates, before transfer to a range of subsequent (germination) temperatures:

- Exp. 1. Sclerotia were incubated at 4°C in wheat grain flasks for durations from 0 to 417 days, after which they were harvested, dried and subsequently buried in soil at 10, 13, 15 and 18°C and observed for germination.
- Exp. 2. Fresh produced and dried sclerotia were incubated in bulk batches in soil for period of 30, 50, 75 and 100 days at 4, 7, 10, 13, 15 and 20°C, subsequently retrieved and reburied in soil at 15°C to observe for germination.
- Exp. 3. Fresh produced and dried sclerotia were incubated in bulk batches in soil at 4°C for period of 0, 15, 30, 50, 75 and 100 days, subsequently retrieved and reburied in soil at 10, 13, 15 and 18°C to observe for germination.

For each experiment 30 sclerotia were used as a sample size and germination was recorded for 195 days. In summary mean time to germination (time after transfer to soil) decreased with increased duration of low temperature incubation and increased with increasing subsequent transfer temperature for both isolates. For Exp. 1, mean germination times were ranging from 196 days for “unconditioned” sclerotia to 19 days for sclerotia incubated at 4°C for 417 days for all subsequent temperatures, with a little decrease in time to germination after incubation longer than 100 days. For Exp. 2, the shortest germination times were observed after 100 days of incubation period at 4 to 10°C where it took 13 to 40 days for isolate 13 and 53 to 72 days for isolate TM. Furthermore, for isolate 13, germination occurred after incubation of 100 days at temperatures of 13 and 15°C and isolate TM showed low germination (<50%) after incubation at 15°C for 30 and 50 days. In Exp. 3, after incubation at 4°C, independently of the duration of incubation, the mean time to germination decreased significantly with all subsequent temperatures. Shortest times, 22 to 28 days for isolate 13 and 47 to 50 days for isolate TM, were observed for 100 days incubation periods and >15°C subsequent temperatures. With no incubation at 4°C germination was either very long, >75 days, or final germination was <50% (10 and 25°C for isolate 13 and 10, 13 18 and 25°C for isolate TM). Final germination below 50% was also observed at 25°C after incubation at 4°C for 15 and 30 days for isolate 13 and 15, 30, 50 and 75 days for isolate TM. The two UK isolates showed variation in the response to temperature, where isolate 13 achieved faster and higher germination even with either shorter duration of low temperature or higher initial temperature

compared to isolate TM. Clarkson et al. (2007) formulated an assumption that a cold conditioning period has to be completed before rapid and high-level germination can occur at higher temperatures, as the basis for the forecasting model developed.

Although considerable research was done to examine the effect of temperature on carpogenic germination there are still questions and issues to be addressed that were not fully resolved by these previous experiments (Clarkson et al., 2004, Clarkson et al., 2007) including the model limitation as described in Chapter 1.5. Lower temperatures and shorter durations of low temperature exposure were introduced in the design of the initial experiment as the lack of information on early responses at low temperatures proved limiting for correct estimation of rates associated with either of the two previously identified processes. Temperatures were further adjusted in the second experiment to identify the rate limiting temperatures for both processes. A larger sample size is used to provide a better depiction of the distribution of germination times.

The work described in this chapter aims to investigate the effect of temperature on processes involved in carpogenic germination of two UK *S. sclerotiorum* isolates with emphasis on the whole population:

- Effect of various single constant temperatures.
- Effect of various constant temperatures with a transfer from initial low temperature to a subsequent higher temperature; highlighting tree different aspects two stages treatments: initial temperature; duration of initial temperature and final temperature.
- Variation in response to temperature for two selected *S. sclerotiorum* isolates with distinctive temperature requirements.

The following objectives to test emerged from previous research, about the effects of temperature on the level, speed and uniformity of germination:

1. Under constant temperature regime (no transfer).

Hypothesis 1. The germination response changes with temperature.

- This would allow the identification of optimum temperature for each isolate.

2. The initial temperature (conditioning) prior to higher temperature promotes high, rapid and uniform germination.

Hypothesis 2. The germination response for each temperature changes with the introduction of an initial lower temperature and with the duration of this initial period.

- This would allow the identification of the optimum initial temperature and optimum duration of initial temperature for each isolate.
- Additionally, more specific hypothesis could be answered for each isolate.

  ⇒ The initial low temperature of 0°C further improves germination compared to other temperatures (e.g. 4 and 8°C).

  ⇒ For the fast conditioning (or not requiring conditioning) isolate (L6) duration of 7 days of the initial low temperatures 0 to 11°C is sufficient to promote high, rapid and uniform germination.

3. The final temperature promotes high, rapid and uniform germination.

Hypothesis 3. The germination response for each initial temperature changes with the final higher temperature, subsequently transferred to, and with the duration of the initial temperature period.

- This would allow the identification of optimum duration of initial temperature and optimum final temperature followed, for each isolate.

4. As the result from earlier objectives and hypothesis, the optimum conditions for each isolate will be identified, where peak carpogenic germination happens as result of experience of the optimum low temperature for the optimum duration and followed by optimum high temperature.

- The optimum conditions are affected by isolate, where we would expect variation in requirements for initial temperature and duration, however the subsequent temperature requirements should be ferly similar.

5. Additionally, to above mentioned hypothesis, we were interested in possible effects of the temperature treatments on myceliogenic germination and overall viability of sclerotia.

Hypothesis 4: The myceliogenic germination level changes with temperature.

Hypothesis 5: The sclerotia viability level changes with temperature.

3.2 Methods

3.2.1 Experiment set-up

In both experiments two *S. sclerotiorum* isolates, L5 and L6, were used and sclerotia produced as described in Chapter 2.

The experiment setup was informed by previous studies and consisted of transfer treatments exposing sclerotia to an initial "conditioning" temperature (T1) in Stage 1 (S1) followed by transfer to a higher "germination" temperature (T2) in Stage 2 (S2) after a range of S1 durations (Table 3.1). Control treatments with no transfer, a single temperature treatment where sclerotia were maintained at a constant temperature, were included in both experiments. In order to balance the large number of temperature regimes with the feasibility of setting up the whole experiment and subsequent regular germination assessments, only transfers of sclerotia from lower (T1) to higher (T2) temperatures were tested (based on the assumption formulated by Clarkson et al. (2007).

The first temperature experiment (TE1) examined a wider range of S1 "conditioning" temperatures, 0, 4, 8, 11, 14, 17, 20°C, and S1 durations, 0, 7, 14, 29, 56 and 84 days. To reduce the number of treatments, S1 duration of 7 days was introduced only for T1 of 0 to 11°C and S1 duration of 84 days was introduced only for higher T1 14 to 17°C (Table 3.1), assuming that 7 days is too short an incubation time for temperatures >11°C and similarly that 84 days incubation for temperatures <14°C is an unnecessarily long period and including these treatments would not add further information compared to the treatments selected.

In the second temperature experiment (TE2) temperatures 0°C and 8°C were omitted and a new maximum temperature, 25°C was introduced (informed by early observations in TE1 suggesting the previous maximum temperature of 20°C to be not limiting for germination for the isolate L6 (Table 3.1). In addition, a range of treatments was repeated in both experiments for consistency.

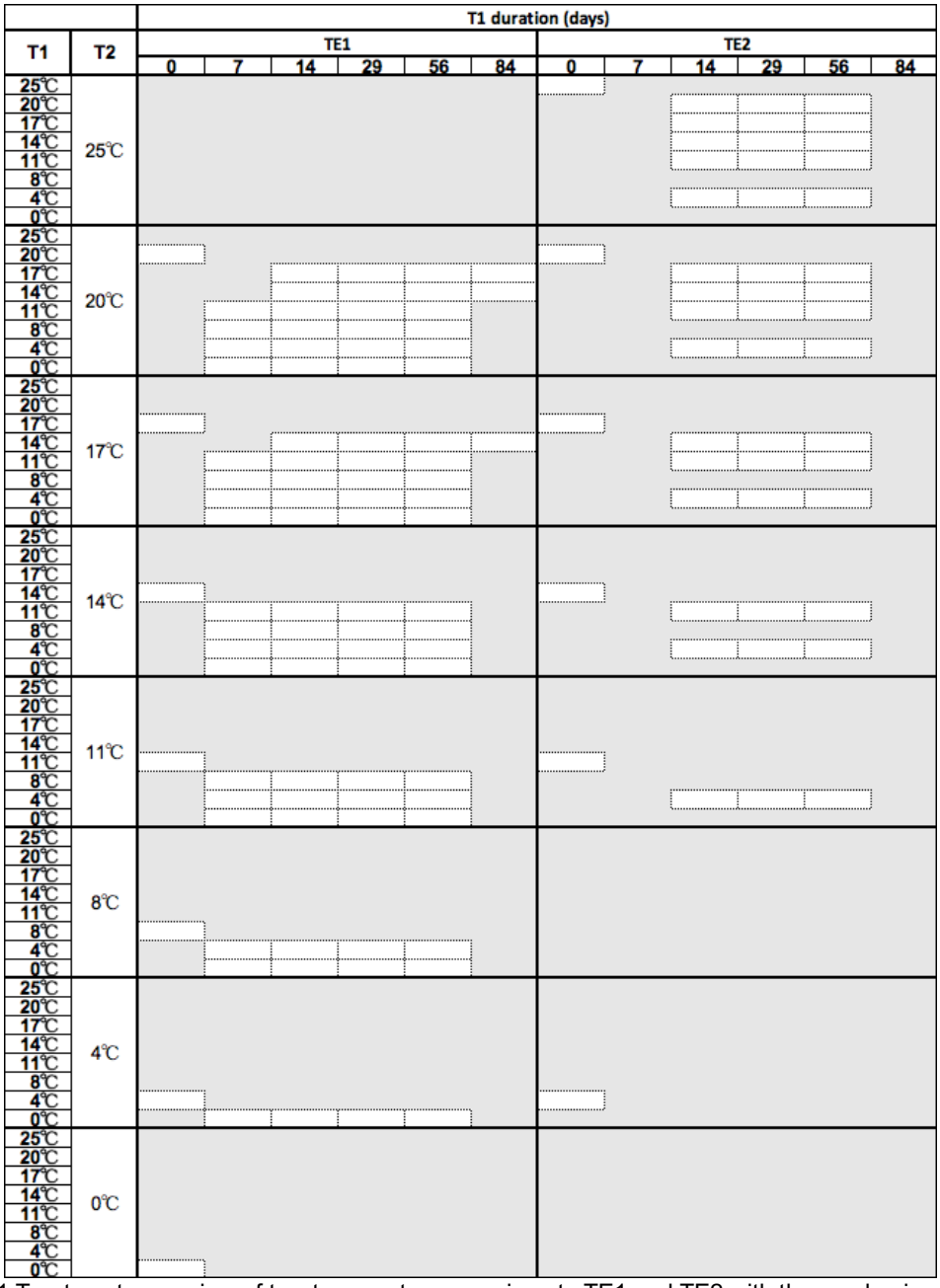

Table 3.1 Treatments overview of two temperature experiments TE1 and TE2 with the emphasis on S2 temperatures, cells representing combination of Stage 1 temperatures (T1), Stage 1 durations and Stage 2 temperatures (T2); Grey = no treatment.

To address any impact of the size distribution of sclerotia produced for both isolates (see Chapter 0), and to minimize any bias caused by a tendency to select bigger sclerotia for burial, sclerotia of different size range were used for each replicate (Table 3.2). Differences in the size distribution in sclerotia produced for TE2 compared to TE1 (Chapter 2, Figure 6) required an adjustment in the size interval used for two replicates containing larger sclerotia. The implementation of the differently sized sclerotia in the experimental design assumes no interaction between treatments and sclerotial size for both *S. sclerotiorum* isolates.

	TE1		TE2	
	Size (mm)	Sclerotia / box	Size (mm)	Sclerotia / box
Rep 1	4.00 < < 5.60	48	3.35 < < 5.60	56
Rep 2	2.80 < < 4.00	56	2.80 < < 3.35	56
Rep 3	2.00 < < 2.80	56	2.00 < < 2.80	56

Table 3.2. Size and number of sclerotia per box used for the two temperature experiments.

Sclerotia buried in soil (see Chapter 2) were arranged in a grid of 8 x 7 rows, however, because of the size of sclerotia used for the Rep 1 in TE1 only 8 x 6 rows were possible to fit into the plastic box used (Figure 3.1).

Figure 3.1 Examples of sclerotia arranged in soil boxes for the three replicates for TE1 (from left to right: Rep 1, Rep 2, Rep 3)

In total, each experiment consisted of 91 and 51 (Table 3.1) treatment combinations with 29,120 and 17,136 sclerotia used in total in TE1 and TE2 respectively.

Soil boxes, prepared as described in Chapter 2, were for the duration of the experiment allocated in large plastic containers with the location of each box being randomized and identified prior to the experimental setup for each transfer occasion and temperature (Figure 3.2). This allocation was maintained throughout the duration of the experiment. Soil boxes with 100% sclerotia germination were removed from the containers.

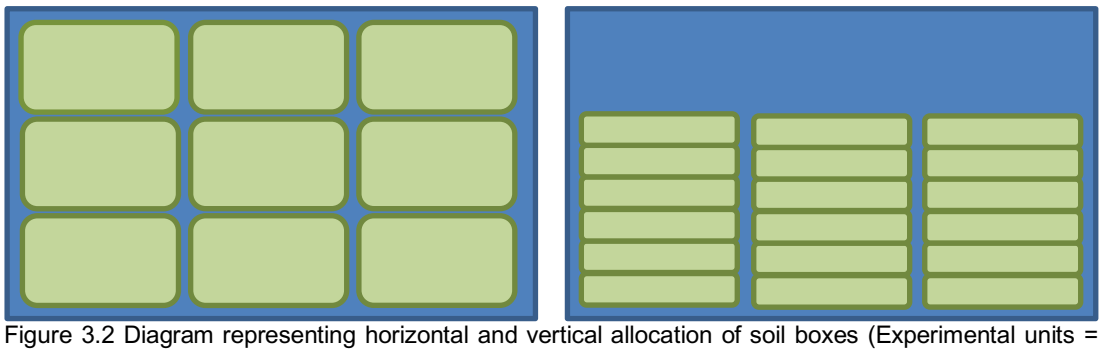

Figure 3.2 Diagram representing horizontal and vertical allocation of soil boxes (Experimental units = green box) inside of large opaque plastic containers (blue box). The number of rows was determined by the total number of soil boxes at given temperature and time/date and each box location was determined in order with randomized design prior the experimental setup.

Germination was indicated by the presence of a stipe (Figure 3.3) and was recorded once a week or more frequently in periods with increased germination, for the duration of 280 days (S1, S2). All remaining, ungerminated sclerotia were moved to 11°C and

assess for viability by observing for germination for an additional period of approximately 150 days in Stage 3 (S3). The observations in S3 for TE1 were occasional with only four recordings, starting after approximately 40 days after transfer to S3. However, this was improved in TE2 with 14 recording occasions starting approximately 7 days after transfer to S3.

Figure 3.3. Germination of *S. sclerotiorum* sclerotia to produce stipes

Additionally, to germination of stipes, myceliogenic germination (MG) (Chapter 1) of sclerotia was observed and recorded. Sclerotia were assumed to show myceliogenic germinated when producing a large amount of white cotton-like mycelium (Figure 3.4) and subsequently become soft and squashy (degraded), and these were removed from soil boxes.

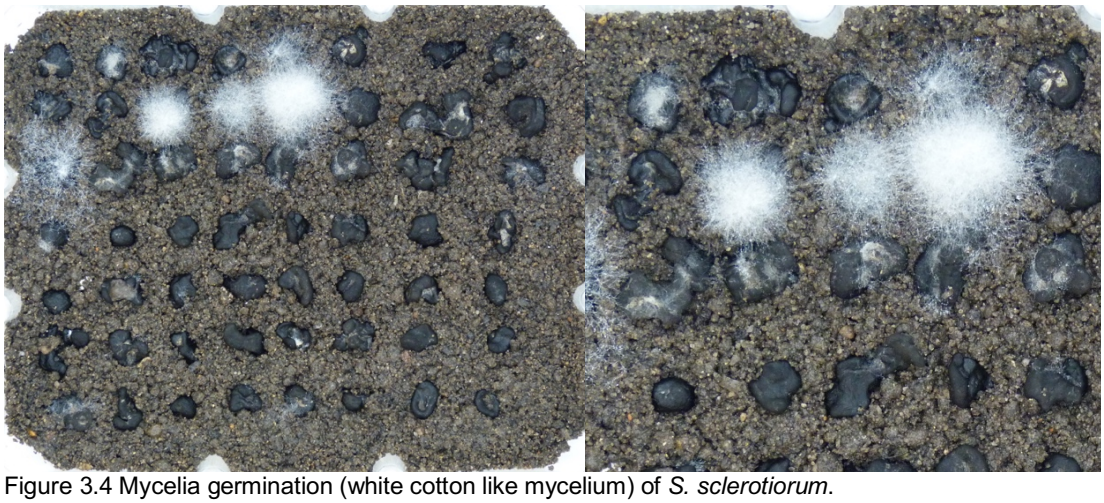

Figure 3.4 Mycelia germination (white cotton like mycelium) of *S. sclerotiorum*.

3.2.2 Statistical analyses

Observed germination responses are presented in the form of cumulative germination curves (where relevant in the text, or in the appendix). In order to characterize the

complex responses to temperature and understand the effect of temperature (and temperature exposure duration) on carpogenic germination as a whole, analyses focused on three aspects of these cumulative curves (Figure 3.5):

Maximum germination – total number of sclerotia germinated in each treatment by the end of S2. Although this attribute is not included in the existing model (Clarkson et al., 2007) it would be interesting to implement this component in the form of a risk indicator along with germination time.

Time to germination – represented either by mean time to germination or time to germination of any percentage of the population. This feature is the most relevant as the one being modelled.

Shape of response – representing variation in germination times characterized by differences between selected population germination times. This characteristic, along with detailed examination of germination times of different percentiles of the population, enables prediction of the distribution of germination times for the whole population.

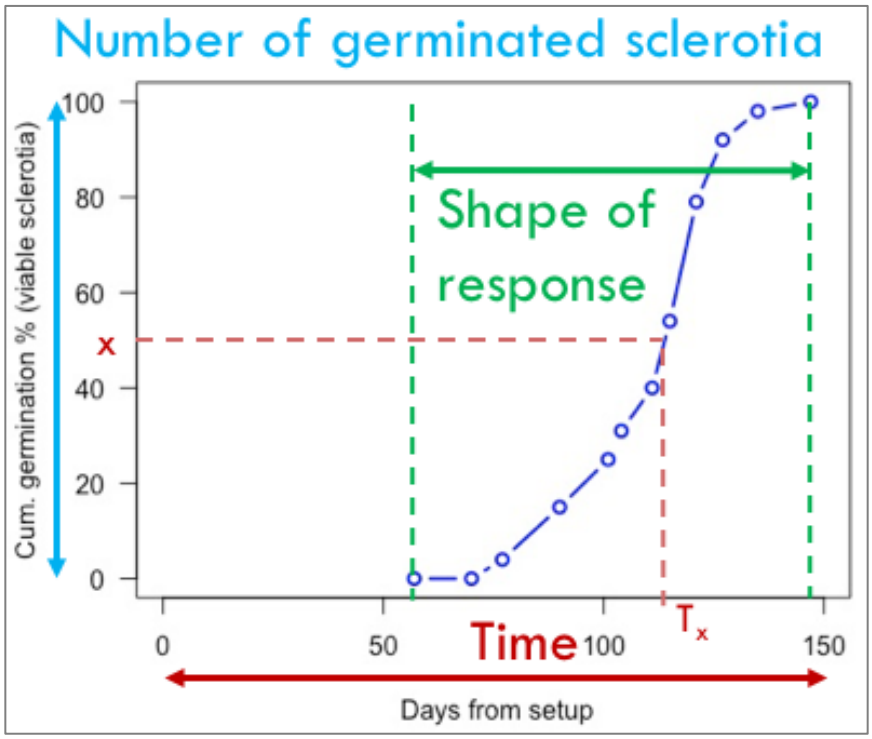

Figure 3.5 Representation of cumulative germination curve and the tree aspects of characterizing the germination response to experimental treatment. Light blue – Number of germinated sclerotia %; Red – Time to germination, where x (e.g. 50%) is population percentile and T_{50} is time to germination of this percentile; Green – Shape of response representing variation in the germination times by difference between selected population germination times.

These characteristics were captured using a number of summary statistics: Maximum percentage germination (viable sclerotia, Table 3.3), Mean time to germination (S3), Time to 10, 25, 50, 75 and 90% of population of germinated sclerotia (T_{10}, T_{25}, T_{50}, T_{75}, T_{90}), standard deviation of time to germination (SD)(S3), difference in times to germination of various % of population (T_{75}-T_{25} (interquartile range - IQR), T_{25}-T_{10} (early percentile range - EPR), T_{90}-T_{75} (late percentile range - LPR), T_{90}-T_{10} (interdecile range - IDR)), calculated as described in Chapter 2 (2.?), and these summery statistics were analysed using ANOVA. The maximum percentage germination is assessed from sclerotia germinated by the end of S2, where the total number of sclerotia is the number of viable sclerotia, sclerotia germinated by the end of S3 including sclerotia germinated myceliogenicly (Table 3.3). The time to different germination percentiles was calculated by linear interpolation between observed times during S1 and S2. Values are not available for some treatments where germination did not achieve the required percentile for any of the replicates.

<table>
<tr><td colspan="5">Set up</td></tr>
<tr><td colspan="3">Viable sclerotia</td><td rowspan="5">Infection</td><td rowspan="5">Non-viable: Not germinated by the end of S3</td></tr>
<tr><td colspan="2">Germinated sclerotia (whole duration of experiment 420 days)</td><td rowspan="4">Myceliogenic germination</td></tr>
<tr><td colspan="2">Germinated sclerotia (main part of experiment 280 days)</td><td rowspan="3">Germination S3</td></tr>
<tr><td>Germination S1</td><td>Germination S2</td></tr>
</table>

Table 3.3 The schematic overview of grouping germination observations for the purpose of later analyses.

The data recorded in S3 for TE1 were infrequent compared to recordings in S1 & S2 and S3 in TE2, where the first record was made after ~ 35 days with high germination incidence. Control treatments for 20°C transferred after 280 days to 11°C where TE2 germination did not start before ~20 days. As we do not have earlier recordings for TE1 in S3 and our best estimate is still to use the original observations, we are aware that linearly interpolated values for the early percentiles for TE1 are underestimated and for the late percentiles are overestimated (Figure 3.6).

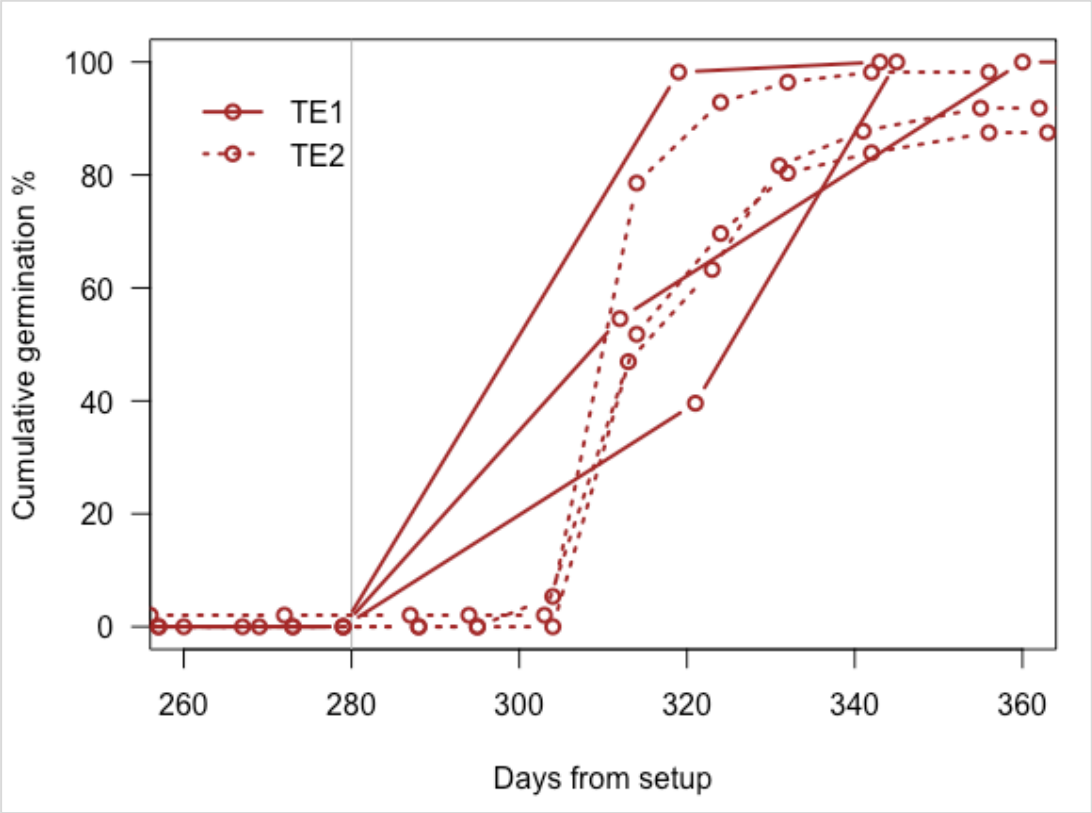

Figure 3.6 Cumulative germination (%) of isolate L5 control 20°C after transferor to 11°C after 280 for experiment TE1 & TE2. The severity of recordings in TE1 effect the linear interpolation for times to germination of different percentiles of population

The summary of S3 germination is only descriptive (no statistical test) as this was not originally designed as a part of the main experiment, however data collected provided additional information. Statistics were calculated as replicate averages (3 reps) and include proportion (%) of non-viable sclerotia (sclerotia not germinated by the end of S3/number of sclerotia at the set up*100), proportion (%) of sclerotia germinated in S3 (sclerotia germinated in S3/ number of sclerotia viable*100), and mean time to germination in S3 (only in S3).

To assess the impact of the exposure to cold temperatures during sclerotia production ("preconditioning") for TE1 (Chapter 2.2.1) and the effect of the differently sized sclerotia used in each replicate (Table 3.2) correlation and linear regression were used to examine relationships for the two *S. sclerotiorum* isolates L5 & L6:

Preconditioning – Summary statistics for Maximum germination % and Mean time to germination for common treatments in TE1 & TE2 (Table 3.1) were plotted against each other. The results are summarized in Appendix 1.

Replicate differences – Summary statistics for Maximum germination % and T_{50} for TE1 & TE2 with values for each replicate plotted against the means across replicates.

Linear regression lines were fitted to the plotted data and information on *slope*, *intercept* and R^2 (Coefficient of determination) along with *r (*Pearson correlation coefficient) were obtained and assessed for deviation from the 1:1 line. The results are summarized in Appendix 2.

To enable the identification of the impacts of the three different treatment components (T1, T2, S1 duration) and combination of these components, analyses of single temperature treatment (control) on their own, and comparison of transfer treatments with the (single temperature) treatments, three different ANOVA models were constructed (TE1 - Table 3.4, TE2 - Table 3.5). In each model an extra term was included to account for the variation due to treatment combinations not included in the main treatment structure, so that all models resulted in the same residual mean squares for a particular response variable (and hence consistent values of s.e.d. and l.s.d. (p=0.05) for comparing the treatment means):

- Control – This approach allows us to address Hypothesis 1 and identify the optimum temperature for each isolate for the single temperature treatments. Temperature (term *T_control*) is nested within the control (term *control*), together with the transfer treatments (term *treatment*), included to ensure a consistent residual mean square for all analysis models (TE1 - Table 3.4, TE2 - Table 3.5).
- Approach T2 – This approach evaluates how the response changes with the initial T1 and duration of S1 for a given T2. Furthermore, it allows us to address Hypothesis 2 identifying for each isolate the optimum T1 and duration of S1 at each T2. Analyses contain comparison of control and transferred treatments (terms *t2_x_con*) and, for transferred treatments, effects of S1 temperature (terms *t2_x_t1*), S1 duration (terms *t2_x_tra*) and the interaction between them, nested within each level of S2 temperature (term *temp2*), where *x* represents the S2 temperature (TE1 - Table 3.4, TE2 - Table 3.5). For the T2 = 17°C and 20°C in TE1 additional terms were needed to reflect the different transfer times included for different S1 temperatures nested in the combining factor for three S1 durations groups of (terms *t2_x_tra1)*: S1 temperatures included in S1 duration of 7 days (term t2_x_t1_7d); S1 temperatures included in S1 durations of 14, 29 and 56 days (term t2_x_tra2 crossed with term t2_x_t1_main); S1 temperatures included in S1 duration of 84 days (term t2_20_t1_84d) (Table 3.4).

- Approach T1 – This approach evaluates how the response changes with the final T2 and duration of S1 for a given T1. Furthermore, it allows us to address Hypothesis 3 identifying for each isolate the optimum final temperature T2 and duration of S1 for each initial T1. Analyses contain comparison of control and transferred treatments (terms *t1_x_con*) and, for transferred treatments, effects of S2 temperature (terms *t1_x_t2*), S1 duration (terms *t1_x_tra*) and the interaction between them, nested within each level of S1 temperature (term *temp1*), where *x* represents the S1 temperature (TE1 - Table 3.4, TE2 - Table 3.5).

Factor *Isolate* was initially included in the ANOVA analyses as a main factor but because of the great number of significant interaction terms and complexity of reporting such analyses, it was concluded to analyse each isolate independently.

Exp	Approach	Blocking	ANOVA model
TE1	Control	Rep	control/(T_control + treatment)
	T2	Rep	temp2/((t2_20_con/(t2_20_tra1/(t2_20_t1_7d + t2_20_tra2*t2_20_t1_main + t2_20_t1_84d)) + (t2_17_con/t2_17_tra1/(t2_17_t1_7d + t2_17_tra2*t2_17_t1_main)) + (t2_14_con/(t2_14_t1*t2_14_tra)) + (t2_11_con/(t2_11_t1*t2_11_tra)) + (t2_8_con/(t2_8_t1*t2_8_tra)) + (t2_4_tra))
	T1	Rep	temp1/((t1_0_con/(t1_0_t2*t1_0_tra)) + (t1_4_con/(t1_4_t2*t1_4_tra)) + (t1_8_con/(t1_8_t2*t1_8_tra)) + (t1_11_con/(t1_11_t2*t1_11_tra)) + (t1_14_con/(t1_14_t2*t1_14_tra)) + t1_17_tra)

Table 3.4 ANOVA models used for the analyses of first temperature experiment data with additional factors created to aid the model specification based on the experiment design, where, for example t1_0_con/(t1_0_t2*t1_0_tra) represents the sum of four components: t1_0_con, t1_0_con.t1_0_t2, t1_0_con.t1_0_tra, and t1_0_con.t1_0_t2.t1_0_tra.

Exp	Approach	Blocking	ANOVA model
TE2	Control	Rep	control/(T_control + treatment)
	T2	Rep	temp2/((t2_25_con/(t2_25_t1*t2_25_tra)) + (t2_20_con/(t2_20_t1*t2_20_tra)) + (t2_17_con/(t2_17_t1*t2_17_tra)) + (t2_14_con/(t2_14_t1*t2_14_tra)) + (t2_11_tra))
	T1	Rep	temp1/((t1_4_con/(t1_4_t2*t1_4_tra)) + (t1_11_con/(t1_11_t2*t1_11_tra)) + (t1_14_con/(t1_14_t2*t1_14_tra)) + (t1_17_con/(t1_17_t2*t1_17_tra)) + (t1_17_con/(t1_17_t2*t1_17_tra)) + t1_20_tra)

Table 3.5. ANOVA models used for the analyses of second temperature experiment data with additional factors created to aid the model specification based on the experiment design, where, for example t1_4_con/(t1_4_t2*t1_4_tra) represents the sum of four components: t1_4_con, t1_4_con.t1_4_t2, t1_4_con.t1_4_tra, and t1_4_con.t1_4_t2.t1_4_tra.

3.3 Results

3.3.1 Viability of sclerotia and myceliogenic germination

Viability of sclerotia (Hypothesis 5)

Treatments which failed to achieve 100% germination during the S1 and S2, the main part of the temperature experiment, were transferred for an additional 150 days to 11°C for viability testing.

For the isolate L5 a greater number of non-viable sclerotia was observed compared to L6. Generally, the viability of isolate L5 sclerotia was reduced for T1 = 0°C and T2 = 17, 20 and 25°C (Figure 3.7). The highest proportion of non-viable sclerotia was observed for the single temperature of 0°C, 46 % (s.e. = 7.22) and T1 = 0°C affected viability of sclerotia transferred to T2 = 4 and 20°C, levels ranging from 3 to 10% and 6 to 15%, respectively. Most treatments with T2 =11°C (all T1, including single temperature) and T2= 14°C (except T1 = 11°C, S1 duration = 14 days) reached 100% viability.

For isolate L6 the maximum percentage of non-viable sclerotia, 6%, was observed at T1 = 20°C, T2 = 25°C and S1 durations of 14 and 56 days (Figure 3.8). Generally, the viability of isolate L6 was little affected by the treatments examined, with some reduction in viability for T2 = 20 and 25°C.

Figure 3.7 Proportion (%) of non-viable sclerotia (out of the total sclerotia buried), *S. sclerotiorum* isolate L5, in TE1 and TE2 (not germinated during the S1, S2 and S3), average of three replicates. Empty cells represent treatments with 0 non-viable sclerotia. Colour intensity represents the increase in non-viability.

Figure 3.8 Proportion (%) of non-viable sclerotia (out of the total sclerotia buried), *S. sclerotiorum* isolate L6, in TE1 and TE2 (not germinated during the S1, S2 and S3), average of three replicates. Empty cells represent treatments with 0 non-viable sclerotia. Colour intensity represents the increase in non-viability.

Myceliogenic germination (Hypothesis 4)

S. sclerotiorum isolate L5 showed higher myceliogenic germination compared to isolate L6. For both isolates the myceliogenic germination observed was higher for TE2 and was highest for Rep 3 (smallest sclerotia) with the total number of observed myceliogenic germination across all treatments (for isolate L5) being 4, 7 and 29 sclerotia in TE1 and 31, 60 and 77 sclerotia in TE2 for Reps 1, 2 and 3 respectively. Isolate L6 showed much lower myceliogenic germination with only 1 sclerotium recorded in TE1 (Rep 3) and only 3, 5 and 6 sclerotia in TE2 for Reps 1, 2 and 3 respectively.

For both isolates myceliogenic germination seems to occur more frequently at high (T2) temperatures: 17-25°C for isolate L5 (Table 3.6) and 20-25°C for isolate L6 (Table 3.7). Initial temperature (T1) and duration of S1 seem to have little effect as myceliogenic germination is observed across various T1 and S1 durations, without a distinctive pattern.

However, the overall amount of myceliogenic germination observed was small with a highest number of myceliogenic germinated sclerotia for isolate L5 of 8 sclerotia, observed for Rep 3 (Rep1 = 0 and Rep 2 = 2) initially kept at 11°C and transferred to 25°C after 29 days with mean myceliogenic germination across replicates 6.1% (Table 3.6) out of the viable sclerotia. The treatment with the second highest myceliogenic germination for isolate L5 was initially kept at 20°C and transferred to 25°C after 56 days also with mean myceliogenic germination across replicates 6.1% (Rep1 = 2, Rep 2 = 3 and Rep 3 = 3) out of viable sclerotia (Table 3.6).

MG germ %_v_L5		T1 duration (days)											
Rep mean		Exp 1.						Exp 2.					
T1	T2	0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							2.8					
20℃										3.6	2.8	6.1	
17℃										2.5	5.3	4.4	
14℃										3.0	4.2	1.8	
11℃										2.0	6.1	1.8	
8℃													
4℃										3.9	2.4	1.8	
0℃													
25℃	20℃												
20℃		0						4.2					
17℃				1.4	0.6	0	1.2			4.2	3.7	1.8	
14℃				0	0	0	0			2.4	1.2	1.8	
11℃			0.7	0.6	1.9	0				3.0	2.5	1.2	
8℃			0.7	0.7	4.2	0							
4℃			1.2	0	0	0				3.0	3.1	3.0	
0℃			0	0	0	0.7							
25℃	17℃												
20℃													
17℃		0						2.2					
14℃				2.0	0	0	0			0	1.2	0	
11℃			0	0	0	1.2				0.7	0.6	0	
8℃			0	0.6	0	0							
4℃			0.7	0	0	0				3.3	1.2	1.2	
0℃			0	1.2	0.6	0.6							
25℃	14℃												
20℃													
17℃													
14℃		0						1.2					
11℃			0	1.2	0	0				0	0.6	0.6	
8℃			0	0	0	0.6							
4℃			0	0	0	0				0.6	0	0.6	
0℃			0	0	0	0							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		0						1.8					
8℃			0	0	0	0							
4℃			0	0	0	0				0	0	0	
0℃			0	0.6	0	0							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		0											
4℃			0	0	0.6	0							
0℃			0	0.6	0	0							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		1.2						0.6					
0℃			0	0	0	0							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		0											

Table 3.6 Replicate mean (3 replicates) of observed myceliogenic germination in TE1 and TE2 with percentage calculated as a proportion of viable sclerotia for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration.

MG germ %_v_L6		T1 duration (days)											
Rep mean		Exp 1.						Exp 2.					
T1	T2	0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							0					
20℃										0	1.2	0	
17℃										0	0	0	
14℃										0.7	0.6	0.6	
11℃										0.6	0	0	
8℃													
4℃										0	0	0.6	
0℃													
25℃	20℃												
20℃		0						1.2					
17℃				0	0	0	0			1.2	0.6	0	
14℃				0	0	0	0			0	0.6	0	
11℃			0	0	0	0				0	0	0.6	
8℃			0	0	0	0							
4℃			0	0	0.6	0				0	0	0	
0℃			0	0	0	0							
25℃	17℃												
20℃													
17℃		0						0					
14℃				0	0	0	0			0	0	0	
11℃			0	0	0	0				0	0	0	
8℃			0	0	0	0							
4℃			0	0	0	0				0	0	0	
0℃			0	0	0	0							
25℃	14℃												
20℃													
17℃													
14℃		0						0					
11℃			0	0	0	0				0	0	0	
8℃			0	0	0	0							
4℃			0	0	0	0				0	0	0	
0℃			0	0	0	0							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		0						0					
8℃			0	0	0	0							
4℃			0	0	0	0				0	0	0	
0℃			0	0	0	0							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		0											
4℃			0	0	0	0							
0℃			0	0	0	0							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		0						0					
0℃			0	0	0	0							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		0											

Table 3.7 Replicate mean (3 replicates) of observed myceliogenic germination in TE1 and TE2 with percentage calculated as a proportion of viable sclerotia for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration.

3.3.2 Effect of single temperature exposure (Hypothesis 1)

Control treatments represent the simplest treatment with sclerotia exposed to a single temperature for the duration of the main part of the experiment (280 days) and therefore these treatments provide a baseline for comparison with the transfer treatments, including T1, duration of S1 and T2.

Isolate L5

S. sclerotiorum isolate L5 germination showed a distinct response to temperature, represented in the form of cumulative germination curves (Figure 3.9). At temperatures of 0°C (green), 20°C (brown) and 25°C (black) no germination was observed. Temperatures 8°C (dark blue) and 11°C (purple) showed a high, fastest and uniform germination. The germination at 14°C (pink) was similarly high, however the germination was somewhat delayed especially for the higher percentiles and in TE2. At 4°C (light blue) and 17°C (orange) the level of germination was reduced and further delayed especially in TE2 where the germination at 4°C was considerably delayed.

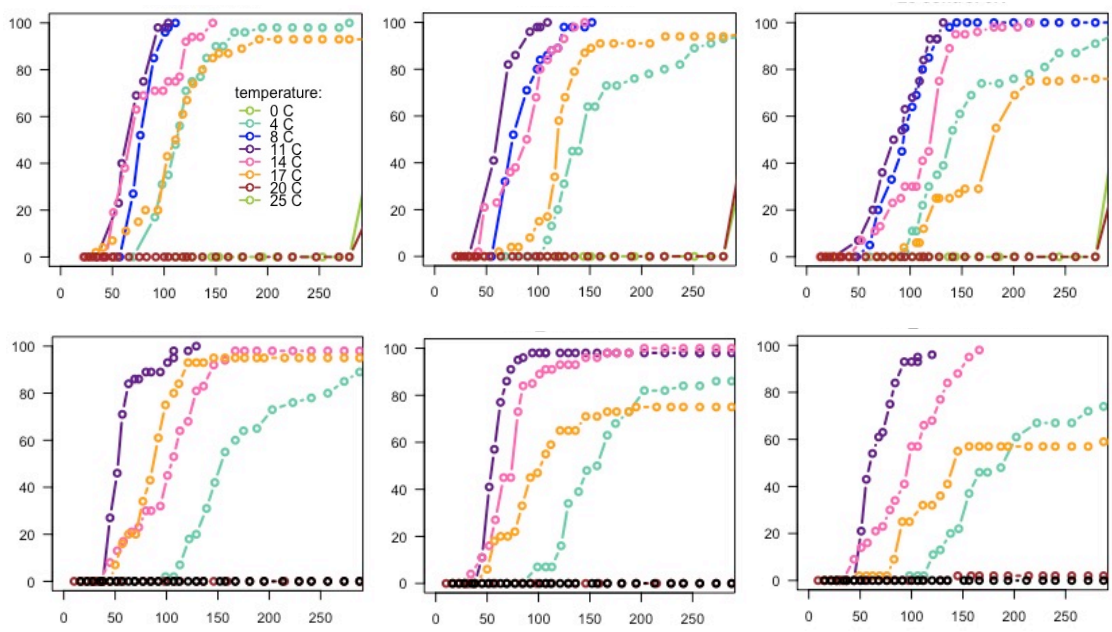

Figure 3.9 Cumulative germination curves for *S. sclerotiorum* isolate L5 control treatment observed for tree replicates (from left to right: Rep1, Rep2, Rep3) for TE1 (top) and TE2 (bottom).

For the isolate L5 analyses showed a significant effect of single temperature on the maximum percentage germination (TE1: $F_{6,180}$=132.58, p<0.001, TE2: $F_{5,100}$=106.34, p<0.001). Extreme high and low temperatures, 0°C & 20°C in TE1 and 25°C in TE2, resulted in no germination (Table 3.8). Temperatures in the range of 8 to 14°C in TE1

achieved 100% germination and both 11 and 14°C achieved 99% germination in TE2, with a significant reduction of germination for lower and higher temperatures.

Exp.	Maximum germination (%)	Temperature °C							
		0	4	8	11	14	17	20	25
TE1	Back transformed (%)	0	96	100	100	100	89	0	-
	Angular transformation	0^{c}	79^{b}	90^{a}	90^{a}	90^{a}	71^{b}	0^{c}	-
	s.e.d.	5.107							
	l.s.d.	10.077 (t=1.973, p=0.05, d.f.=180)							
TE2	Back transformed (%)	-	83	-	99	99	78	0	0
	Angular transformation	-	66^{b}	-	84^{a}	85^{a}	62^{b}	3^{c}	0^{c}
	s.e.d.	5.308							
	l.s.d.	10.531 (t=1.984, p=0.05, d.f.=100)							

Table 3.8 Isolate L5 ANOVA obtained table of the angular transformed means for Maximum percentage germination (viable) for TE1 and TE2. Alongside the back-transformed values are presented with transformed s.e.d. and l.s.d. and corresponding values for t - t statistics, p - probability and d.f. - degrees of freedom. Different superscript letters represent significant differences between means (p=0.05).

Analyses of time to germination for T_{10}, T_{25}, T_{50}, T_{75}, T_{90} showed significant effects of single temperature, with a consistent trend across all examined percentiles for both experiments (TE1: $F_{4,148}$=11.01, $F_{4,143}$=14.16, $F_{4,137}$=15.27, $F_{4,130}$=15.50, $F_{4,115}$=21.92; TE2: $F_{3,56}$=54.76, $F_{3,51}$=7.81, $F_{3,47}$=20.57, $F_{3,37}$=29.77, $F_{3,30}$=102.79 all $p<0.001$). Times to germination for all percentiles were shortest at 11°C with the germination times of 45, 57, 69, 86 and 97 days (TE1, Table 3.9) and 44, 48, 55, 66 and 84 days (TE2, Table 3.10), respectively. The times to germination increased with both decreasing and increasing temperatures around 11°C for both experiments, with significant delays at 4 and 17°C, where the germination times frequently doubled (17°C) or tripled (4°C) compared to germination times observed at 11°C.

TE1	Temperature (°C)							s.e.d.	l.s.d.	d.f.
	0	4	8	11	14	17	20			
*T_{10}	-	98^{b}	61^{a}	45^{a}	52^{a}	89^{b}	-	9.90	19.57	148
*T_{25}	-	110^{b}	69^{a}	57^{a}	69^{a}	111^{b}	-	9.53	18.83	143
*T_{50}	-	130^{c}	82^{a}	69^{a}	92^{b}	136^{c}	-	10.81	21.37	137
*T_{75}	-	170^{b}	113^{a}	86^{a}	96^{a}	162^{b}	-	13.75	27.21	130
*T_{90}	-	227^{c}	112^{a}	97^{a}	126^{a}	173^{b}	-	15.96	31.61	115

Table 3.9 ANOVA estimated means for times to germination for 10% (T_{10}), 25% (T_{25}), 50% (T_{50}), 75% (T_{75}), and 90% (T_{90}) of population for *S. sclerotiorum* isolate L5 in TE1 (control treatment). Some of the values could not be obtained as the germination was lower than required percentile. Different superscript letters represent significant differences between means (p=0.05) (individual rows); * features statistics with significant results (<0.05).

TE2	Temperature (°C)						s.e.d.	l.s.d.	d.f.
	4	11	14	17	20	25			
*T_{10}	117^{c}	44^{a}	46^{a}	63^{b}	-	-	6.54	13.09	56
*T_{25}	136^{b}	48^{a}	68^{a}	81^{a}	-	-	18.95	38.05	51
*T_{50}	166^{c}	55^{a}	92^{b}	109^{b}	-	-	14.44	29.04	47
*T_{75}	204^{d}	66^{a}	110^{b}	150^{c}	-	-	15.23	30.86	37
*T_{90}	293^{c}	84^{a}	131^{b}	-	-	-	12.83	26.21	30

Table 3.10 ANOVA estimated means for times to germination for 10% (T_{10}), 25% (T_{25}), 50% (T_{50}), 75% (T_{75}), and 90% (T_{90}) of population for *S. sclerotiorum* isolate L5 in TE2 (control treatment). Some of the values could not be obtained as the germination was lower than required percentile. Different superscript letters represent significant differences between means (p=0.05) (individual rows); * features statistics with significant results (<0.05).

Analyses of differences in germination times for various percentiles show no significant effect of temperature for the early percentiles range T_{25}-T_{10} (EPR) for both experiments (TE1: $F_{4,143}$=1.49, p=0.208, TE2: $F_{3,51}$=0.50, p=0.683), with the smallest differences at 8°C (TE1) and 11°C (TE2), 8 and 4 days, respectively, and greatest differences at 17°C (TE1) and 14°C (TE2), 22 and 23 days, respectively (TE1, Table 3.11, TE2, Table 3.12).

For the late percentiles range T_{90}-T_{75} (LPR) the effect of temperature was significant (TE1: $F_{4,115}$=9.26, TE2: $F_{3,30}$=15.27, both p<0.001) with the smallest differences at 11°C, 11 (TE1) and 18 (TE2) days. The greatest differences (significant) were observed at 4°C, 65 (TE1) and 81 (TE2) days, respectively (Table 3.11, Table 3.12). The late percentile range at 17°C in TE2 could not be examined because the final level of germination was below 90%.

For both experiments the differences in germination times for the EPR and LPR were similar for temperatures of 11°C (TE1) and 14°C, and increased for LPR for 4°C and 17°C (TE1). Therefore, the distribution of germination times for 11°C (TE1) and 14°C was rather symmetrical, while for the remaining temperatures it was faster for the earlier percentiles and the distribution of germination times was skewed to the left.

Interdecile range T_{90}-T_{10} (IDR) was significantly affected by temperature in both experiments (TE1: $F_{4,115}$=8.23, p<0.001, TE2: $F_{3,30}$=46.07, p<0.001). Smallest differences in germination times (narrowest distributions of germination times) were observed at 8°C and 11°C in TE1, both 51 days and at 11°C in TE2, 40 days. The greatest differences in germination times (widest distributions of germination times) were at 4°C, 128 days (TE1) and 177 days (TE2) (Table 3.11, Table 3.12). The interdecile range at 17°C in TE2 could not be examined because the final level of germination was below 90%.

TE1	Temperature (°C)							s.e.d.	l.s.d.	d.f.
	0	4	8	11	14	17	20			
T_{25}- T_{10}	-	12^{ab}	8^{a}	12^{ab}	17^{ab}	22^{b}	-	6.34	12.54	143
*T_{90}- T_{75}	-	65^{c}	16^{a}	11^{a}	13^{a}	39^{b}	-	10.66	21.11	115
*T_{90}- T_{10}	-	128^{c}	51^{a}	51^{a}	75^{ab}	95^{b}	-	16.08	31.86	115

Table 3.11 ANOVA estimated means for differences in germination times for: T_{75}-T_{25} (interquartile range - IQR), T_{25}-T_{10} (early percentile range - EPR), T_{90}-T_{75} (late percentile range - LPR), T_{90}-T_{10} (interdecile range - IDR) for *S. sclerotiorum* isolate L5 in TE1 (control treatment). Some of the values could not be obtained as the germination was lower than required percentiles. Different superscript letters represent significant differences between means (p=0.05) and * features statistics with significant results (<0.05).

TE2	Temperature (°C)						s.e.d.	l.s.d.	d.f.
	4	11	14	17	20	25			
T_{25}- T_{10}	19^{a}	4^{a}	23^{a}	18^{a}	-	-	16.24	32.60	51
*T_{90}- T_{75}	81^{b}	18^{a}	20^{a}	-	-	-	10.93	22.33	30
*T_{90}- T_{10}	177^{c}	40^{a}	85^{b}	-	-	-	16.08	31.86	30

Table 3.12 ANOVA estimated means for differences in germination times for: T_{75}-T_{25} (interquartile range - IQR), T_{25}-T_{10} (early percentile range - EPR), T_{90}-T_{75} (late percentile range - LPR), T_{90}-T_{10} (interdecile range - IDR) for *S. sclerotiorum* isolate L5 in TE2 (control treatment). Some of the values could not be obtained as the germination was lower than required percentiles. Different superscript letters represent significant differences between means (p=0.05) and * features statistics with significant results (<0.05).

Isolate L6

Cumulative germination curves generated based on the observed data for isolate L6 (Figure 3.10) showed germination at temperatures as high as 20°C (brown) for both experiments. The increased germination at 20°C in TE1 is assumed to be due to possible preconditioning during sclerotia production (Chapter 2.2) as addressed in Appendix 1. Cumulative germination curves for the middle temperatures 8-14°C (dark blue, purple, pink) follow similar pattern across both experiments and three replicates with a sigmoidal shape. Germination at 17°C (orange) was in line with these mid temperatures in TE1, but showed a delay in germination in TE2, where the cumulative germination curve aligned with germination observed at 4°C (light blue). The cumulative germination for 4°C was flatter compared to other temperatures, especially in TE1. At 0°C (green) and 25°C (black) no germination was observed over the 280 days duration of the experiment.

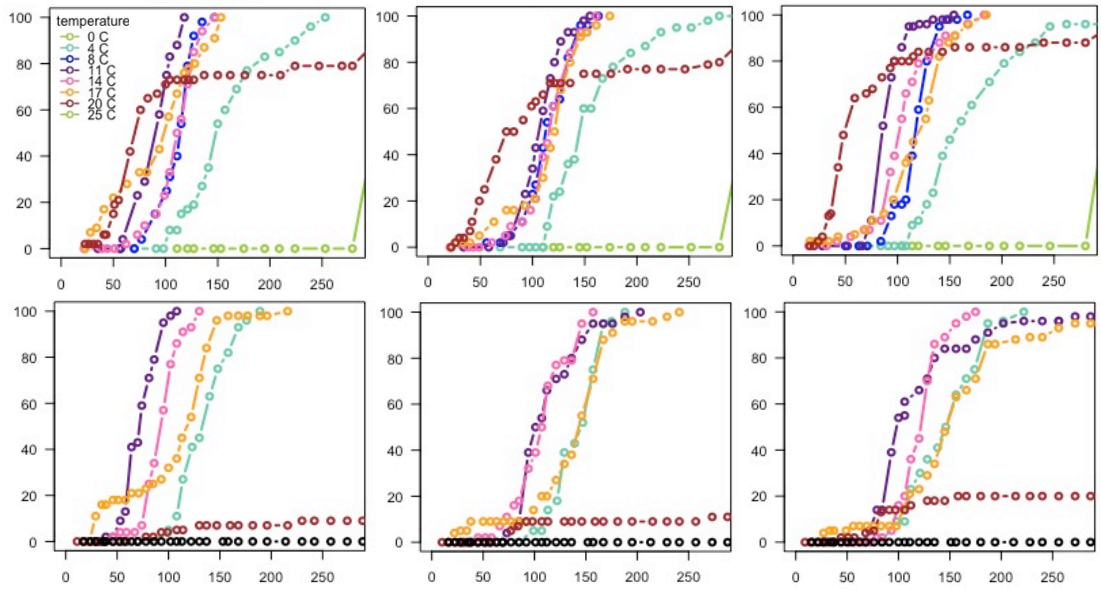

Figure 3.10 Cumulative germination curves for *S. sclerotiorum* isolate L6 control treatment observed for tree replicates (from left to right: Rep1, Rep2, Rep3) for TE1 (top) and TE2 (bottom).

For isolate L6 analyses showed a significant effect of single temperature on the maximum percentage germination (TE1: $P_{6,180}$ = 409.22, TE2: $P_{5,100}$ = 134.90, both p<0.001). In both experiments ~100% germination was achieved for single temperatures ranging from 4 to 17°C (Table 3.13). A significant decrease in germination was observed for 20°C, with 83% (TE1) and 13% (TE2), where this difference is a cause of concern and ambiguity in the TE1 data, which were possibly corrupted by exposure to low temperatures during sclerotia production (4-10°C for 3 nights during drying, this is further examined in Appendix 1. Extreme high and low temperatures, 0°C and 25°C, resulted in no germination.

Exp.	Max germination %	Temperature °C							
		0	4	8	11	14	17	**20	25
TE1	Back transformed (%)	0	100	100	100	100	100	**83	-
	Angular transformation	0^c	86^a	90^a	90^a	90^a	90^a	65^b	-
	s.e.d.	2.340							
	l.s.d.	4.618 (t=1.973, p=0.05, d.f.=180)							
TE2	Back transformed (%)	-	100	-	100	100	99	13	0
	Angular transformation	-	90^a	-	87^a	90^a	86^a	21^b	0^c
	s.e.d.	4.961							
	l.s.d.	9.842 (t=1.984, p=0.05, d.f.=100)							

Table 3.13 Isolate L6 ANOVA obtained table of the Angular transformed means for max germination % (viable) for TE1 and TE2. Alongside the back-transformed values are presented with transformed s.e.d. and l.s.d. and corresponding values for t - t statistics, p - probability and d.f. - degrees of freedom); ** data possibly corrupted by preconditioning.

Analyses of time to germination for T_{10}, T_{25}, T_{50}, T_{75}, T_{90} showed significant effects of temperature in both experiments (TE1: $F_{5,178}$=34.35, $F_{5,178}$=27.51, $F_{5,178}$=25.86, $F_{5,176}$=9.30, $F_{5,164}$=15.77 all p<0.001; TE2: $F_{4,90}$=12.42, p<0.001, $F_{3,88}$=2.96, p=0.037, $F_{3,80}$=2.80, p=0.045, $F_{3,67}$=3.40, p=0.023, $F_{3,61}$=4.54, p=0.006). Similarly, to the analysis of the level of germination above, the fast germination observed at 17 and 20°C in TE1, T_{10} = 57 and 40 days were considered unreliable and will be overlooked (Table 3.14), particularly as in TE2 the T_{10} = 74 and 169 days respectively (Table 3.15). The remaining data show consistent trends where the fastest germination was generally observed at 11°C, with the germination times of 74, 83, 94, 105, and 116 days in TE1 (Table 3.14) and 72, 79, 90, 115 and 142 days in TE2 (Table 3.15), respectively, except in TE2 the T_{90}, where fastest germination was observed at 14°C, 134 days. Germination was significantly slowed with both decreasing and increasing temperature around 11°C. The greatest delay was observed at 20°C, for T_{10} in TE2, more than twice the time observed at 11 - 17°C.

TE1	Temperature (°C)							s.e.d.	l.s.d.	d.f.
	0	4	8	11	14	17	**20			
*T_{10}	-	115^{e}	88^{d}	74^{c}	82^{cd}	57^{b}	40^{a}	6.24	12.32	178
*T_{25}	-	132^{e}	104^{d}	83^{b}	98^{cd}	87^{bc}	50^{a}	7.23	14.27	178
*T_{50}	-	150^{d}	115^{c}	94^{b}	110^{c}	114^{c}	67^{a}	7.52	14.84	178
*T_{75}	-	181^{b}	126^{a}	105^{a}	123^{a}	129^{a}	158^{b}	12.76	25.18	176
*T_{90}	-	226^{c}	134^{ab}	116^{a}	140^{ab}	147^{b}	-	15.14	29.89	164

Table 3.14 ANOVA estimated means for times to germination for 10% (T_{10}), 25% (T_{25}), 50% (T_{50}), 75% (T_{75}), and 90% (T_{90}) of population for *S. sclerotiorum* isolate L6 in TE1 (control treatment). Some of the values could not be obtained as the germination was lower than required percentile. Different superscript letters represent significant differences between means (p=0.05) and * features statistics with significant results (<0.05); ** data possibly corrupted by preconditioning.

TE2	Temperature (°C)						s.e.d.	l.s.d.	d.f.
	4	11	14	17	20	25			
*T_{10}	108^{b}	72^{a}	81^{a}	74^{a}	169^{c}	-	16.38	32.55	90
*T_{25}	117^{b}	79^{a}	93^{ab}	109^{b}	-	-	13.84	27.51	88
*T_{50}	142^{b}	90^{a}	107^{ab}	135^{b}	-	-	20.45	40.70	80
*T_{75}	160^{b}	115^{a}	117^{a}	156^{b}	-	-	18.65	37.23	67
*T_{90}	171^{ab}	142^{a}	134^{a}	186^{b}	-	-	16.25	32.49	61

Table 3.15 ANOVA estimated means for times to germination for 10% (T_{10}), 25% (T_{25}), 50% (T_{50}), 75% (T_{75}), and 90% (T_{90}) of population for *S. sclerotiorum* isolate L6 in TE2 (control treatment). Some of the values could not be obtained as the germination was lower than required percentile. Different superscript letters represent significant differences between means (p=0.05) and * features statistics with significant results (<0.05).

Analyses of differences in germination times for various percentiles show a significant effect of temperature for early percentiles range T_{25}-T_{10} (EPR) for both experiments (TE1: $F_{5,\ 178}$=5.40, p<0.001, TE2: $F_{3,88}$=4.51, p=0.005), with smallest differences at

11°C, 9 days and 8 days, and greatest (significantly) differences at 17°C, 30 days and 35 days (TE1, Table 3.16 and TE2, Table 3.17, respectively).

The late percentile range T_{90}-T_{75} (LPR) showed no significant effect of temperature for both experiments (TE1: $F_{4,\ 164}$=2.07, p=0.087, TE2: $F_{3,61}$=1.40, p=0.251). Furthermore, the results for both experiments were contrasting, where in TE1 the smallest difference in germination times was observed at 8°C, and increased with decreasing and increasing temperature, ranging from 45, 9, 12, 16 and 19 days (4, 8, 11, 14 and 17°C, respectively (Table 3.16)). In contrast, in TE2 there was no pattern observed and differences were 12, 27, 17 and 28 days (4, 11, 14 and 17°C, respectively (Table 3.17)). Only at 14°C the results for both experiments were comparable.

For both experiments the differences in germination times for the EPR and LPR were similar for temperatures of 14°C (both TE1 and TE2), and generally increased for LPR for 4°C and decreased for 17°C. The distribution of germination times for <14°C was therefore skewed to the left in contrast to 17°C, skewed to the right.

The interdecile range T_{90}-T_{10} (IDR) was significantly affected by temperature in both experiments (TE1: $F_{4,164}$=6.80, TE2: $F_{3,61}$=5.99, both p<0.001), however the pattern was inconsistent between experiments. In TE1 the smallest difference, and therefore the narrowest distribution of germination times, was observed for 11°C, and the difference significantly increased with both decreasing and increasing temperatures, towards 111 days at 4°C and 91 days at 17°C (Table 3.16). In TE2 no pattern was observed and the differences were 64, 70, 53 and 112 days at 4, 11, 14 and 17°C, respectively (Table 3.17).

TE1	Temperature (°C)							s.e.d.	l.s.d.	d.f.
	0	4	8	11	14	17	**20			
*T_{25}- T_{10}	-	17^{a}	16^{a}	9^{a}	16^{a}	30^{b}	10^{a}	4.57	9.01	178
T_{90}- T_{75}	-	45^{b}	9^{a}	12^{a}	16^{a}	19^{ab}	-	14.34	28.32	164
*T_{90}- T_{10}	-	111^{b}	46^{a}	43^{a}	57^{a}	91^{b}	-	16.24	32.06	164

Table 3.16 ANOVA estimated means for differences in germination times for: T_{75}-T_{25} (interquartile range - IQR), T_{25}-T_{10} (early percentile range - EPR), T_{90}-T_{75} (late percentile range - LPR), T_{90}-T_{10} (interdecile range - IDR) for *S. sclerotiorum* isolate L6 in TE1 (control treatment). Some of the values could not be obtained as the germination was lower than required percentiles. Different superscript letters represent significant differences between means (p=0.05) and * features statistics with significant results (<0.05); ** data possibly corrupted by preconditioning.

TE2	Temperature (°C)						s.e.d.	l.s.d.	d.f.
	4	11	14	17	20	25			
*T_{25}- T_{10}	10^a	8^a	12^a	35^b	-	-	8.39	16.68	88
T_{90}- T_{75}	12^a	27^a	17^a	28^a	-	-	10.06	20.12	61
*T_{90}- T_{10}	64^a	70^a	53^a	112^b	-	-	14.90	29.79	61

Table 3.17 ANOVA estimated means for differences in germination times for: T_{75}-T_{25} (interquartile range - IQR), T_{25}-T_{10} (early percentile range - EPR), T_{90}-T_{75} (late percentile range - LPR), T_{90}-T_{10} (interdecile range - IDR) for *S. sclerotiorum* isolate L6 in TE2 (control treatment). Some of the values could not be obtained as the germination was lower than required percentiles. Different superscript letters represent significant differences between means (p=0.05) and * features statistics with significant results (<0.05).

Summary and isolate comparison - Effect of single temperature exposure (Hypothesis 1.)

In the single temperature treatments, isolate L5 showed a preference for 11°C where it displayed the highest, fastest and most uniform germination in both experiments (Table 3.18). The level of germination was almost 100% in temperatures 8 to 14°C and for temperatures 0, 20 and 25°C no germination was observed. Fastest germination was for T_{10} = 45 and 46 days and increased to T_{90} = 97 and 84 days (at 11°C, TE1 and TE2, respectively). The distribution of germination times was narrowest, most uniform, for 8 and 11°C and skewed to the left when further from the optimum temperature. Results observed for the single temperature treatments for isolate L6 were conflicting (Appendix 1) for the two experiments because of high germination at 20°C, 83%, in TE1 and only 13% in TE2. However, isolate L6 (Table 3.19) proved to be able to respond to a wider range of temperatures compared to isolate L5. A level of germination of almost 100% was achieved for a range of temperatures from 4 to 17°C in both experiments. For temperatures 0 and 25°C, no germination was observed, similarly to L5. For isolate L6, controversially fastest germination was observed at 17 and 20°C in TE1 where T_{10} = 57 and 40 days, respectively, however these results were not repeated in the TE2 and were assumed to be unreliable because of possible preconditioning during the sclerotia production. Ignoring these, the fastest germination was observed in TE1 at 11°C, T_{10} = 74 days and T_{90} = 116 days and in TE2 at 11°C, T_{10} = 72 days and at 14°C, T_{90} = 134 days. Generally, the germination times at the optimum temperature (11°C) were faster for isolate L5 compared to isolate L6, by ~ 30 days for T_{10} and 25 – 68 days for T_{90}. The distribution of germination times for isolate L6 was narrowest, most uniform for 8 and 11°C in TE1 and 14°C in TE2. The distribution of germination times was skewed to the left for lower temperatures (4°C) in contrast to high temperatures (17°C) where a small skewness to the right was observed (in contrast to isolate L5).

Temp.	Max germination	Time to germination (all %)	Shape of response	EPR	LPR	Skewness	IDR
0°C	Base*						
4°C	Sub - optimum	Sub - optimum	Sub - optimum	Sub - optimum	Sub - optimum	Left	Sub - optimum
8°C	Optimum	Sub - optimum	Optimum	Optimum	Sub - optimum	Normal /Left	Optimum
11°C	Optimum	Optimum	Optimum	Optimum	Optimum	Normal	Optimum
14°C	Optimum	Supra - optimum	Supra - optimum	Supra - optimum	Supra - optimum	Normal	Supra - optimum
17°C	Supra - optimum	Supra - optimum	Supra - optimum	Supra - optimum	Supra - optimum	Left	Supra - optimum
20°C	Ceiling*						
25°C	Ceiling*						

Table 3.18 Summary of the effect of a single constant temperature on carpogenic germination of *S. sclerotiorum* isolate L5; * approximate base/ceiling temperature, it is our best estimate based on treatments/temperatures selected for the experiment.

Temp.	Max germination	Time to germination (all %)	Shape of response	EPR	LPR	Skewness	IDR
0°C	Base*						
4°C	Sub - optimum	Sub - optimum	Sub - optimum	Sub - optimum	Sub - optimum	Left	Sub - optimum
8°C	Optimum	Sub - optimum	Optimum	Sub - optimum	Optimum	Normal/ Left	Optimum
11°C	Optimum	Optimum	Optimum	Optimum	Optimum	Normal / Left	Optimum
14°C	Optimum	Supra - optimum	Optimum	Supra - optimum	Optimum	Normal	Optimum
17°C	Optimum	Optimum** (supra)	Supra - optimum	Supra - optimum	Supra - optimum	Right	Supra - optimum
20°C	Supra - optimum	Optimum** (supra)					
25°C	Ceiling*						

Table 3.19 Summary of the effect of a single constant temperature on carpogenic germination of *S. sclerotiorum* isolate L6; * approximate base/ceiling temperature, it is our best estimate based on treatments/temperatures selected for the experiment;** Uncertain because of conflicting result in TE1 and TE2 (possible effect of preconditioning, Appendix 1)

3.3.3 The effect of initial low temperature (Hypothesis 2)

3.3.3.1 Level of germination.

Isolate L5

For isolate L5, constant single temperatures of 17 - 25°C were identified as supra-optimum and ceiling temperatures, causing a reduction in germination and limiting germination completely (Figure 3.11, Table 3.18). In the transfer treatments, a number of initial S1 temperatures and durations of S1 were introduced resulting in an increase in germination levels (e.g. cumulative germination curves presented for T2 = 20°C, T1 = 0 – 20°C (various colours), and S1 duration 7 to 84 days (grey background, left to right) in TE1, Figure 3.11).

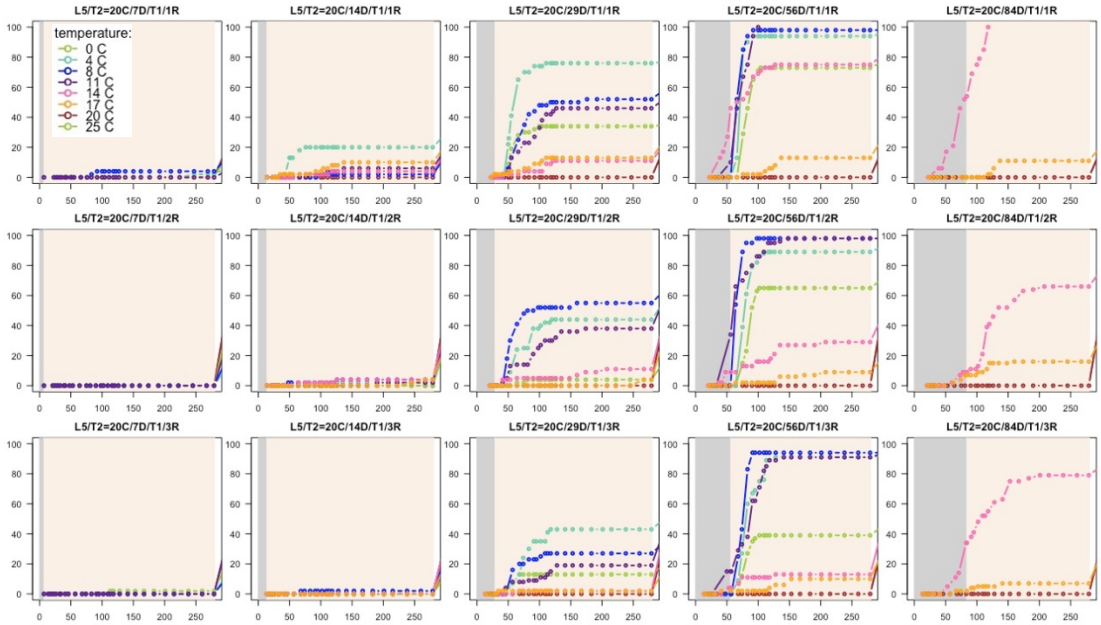

Figure 3.11 Cumulative germination curves for *S. sclerotiorum* isolate L5 observed in TE1, T2=20°C; T1: green = 0°C, light blue = 4°C, dark blue = 8°C, purple = 11°C, pink = 14°C, orange = 17°C, brown = 20°C; S1 duration (left to right, represented by grey are on each graph): 7, 14, 29, 56 and 84 days (only T1 = 14 and 17°C); for tree replicates (top to bottom: Rep1, Rep2, Rep3).

The T2 approach analyses of maximum germination showed a significant effect of the main factor T2 in both experiments (TE1: $F_{6,180}$=871.78, TE2: $F_{5,100}$=779.76, both p<0.001). The temperatures T2 = 4 to 14°C showed no effect of initial low temperature or duration of S1, with almost 100% germination observed, in both single temperature and transfer treatments. In contrast, at T2 temperatures of 17, 20 and 25°C significant differences were observed between the single temperature treatment and transfer treatments, as well as significant interactions between T1 and S1 duration for transfer treatments. Summarising the T2 approach analyses results for 17, 20 and 25°C temperatures (back transformed means are presented in Table 3.20):

- Inside of T2 = 25°C (TE2) a significant difference between single temperature and transfer treatments ($F_{1,100}$=5.59, p=0.02) and for transfer treatments a significant interaction between T1 and S1 duration was observed ($F_{8,100}$=12.18, p<0.001).
- Inside of T2 = 20°C a significant difference between single temperature and transfer treatments was observed (TE1: $F_{1,180}$=57.03, TE2: $F_{1,100}$=30.14, both p<0.001). In TE1 a significant effect of S1 durations of 7, main (14-56 days) and 84 days ($F_{2,180}$=130.00, p<0.001). For transfer treatments a significant interaction between T1 and S1 duration was observed in TE2 ($F_{6,100}$=9.05, p<0.001) and for durations of S1 longer then 7 days in TE1 (for S1 durations

of 14, 29 and 56 days: $F_{10,180}$=12.76, p<0.001 and for 84 days: $F_{1,180}$=93.64, p<0.001).

- Inside of T2 = 17°C in TE2 a significant difference between single temperature and transfer treatments was observed ($F_{1,100}$=5.82, p=0.018) and for transfer treatments a significant effect of S1 duration ($F_{2,100}$=29.00, p<0.001). In TE1 a marginally non-significant difference between single temperature and transfer treatments was observed ($F_{1,180}$=3.82, p=0.052), with a significant effect of S1 duration ($F_{2,180}$=4.76, p=0.010) and, for S1 durations of 14, 29 and 56 days, a significant interaction between T1 and S1 duration ($F_{8,180}$=3.18, p=0.002).

Max germ %_L5 viable		T1 duration (days)											
		Exp 1.						Exp 2.					
T1	T2	0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							0					
20℃										0	0	0	
17℃										0	0	5	
14℃										0	4	24	
11℃										0	0	76	
8℃													
4℃										0	0	8	
0℃													
25℃	20℃												
20℃		0						0					
17℃				3	5	10	11			0	6	10	
14℃				2	7	37	87			1	18	38	
11℃			0	2	34	98				0	29	92	
8℃			0	3	44	97							
4℃			0	6	55	91				0	26	52	
0℃			0	0	15	59							
25℃	17℃												
20℃													
17℃		89						78					
14℃				54	95	90	100			69	86	98	
11℃			91	94	91	99				81	88	99	
8℃			93	88	96	100							
4℃			94	94	100	100				77	93	100	
0℃			95	95	100	100							

Table 3.20 ANOVA table of T2 approach with back transformed means for Max germination % (based on viable sclerotia) for TE1 and TE2 for isolate L5 for combination of treatments with T2 = 17, 20 and 25°C, T1 ranging from 0°C to 20°C, and T1 duration ranging from 0 to 84 days; Colour gradient from 100% (green) to 0 % (red) germination; Grey = no treatment combination tested; Transformed (angular) TE1: d.f. = 180, s.e.d. = 5.107, l.s.d. = 10.077; TE2: d.f. = 100, s.e.d. = 5.308 l.s.d. = 10.531.

Overall the application of lower temperatures and longer durations of S1 increased germination. The significant interaction between T1 and S1 duration was largely represented by a significant increase in germination with longer duration of S1 and, where with increased T2 a longer period of T1 was required, the optimum T1 shifted from T1 = 0-4°C towards 11°C. T1 > 11°C increased germination as well, however the increase was smaller than T ≤ 11°C, irrespective of the S1 duration. The duration of S1, 56 days and T1 temperature of 11°C generally produced the greatest increase in germination level, where the germination increased from 89 - 78% (17°C) and 0% (20 and 25°C) for the single temperature treatments to 100% and 99% (17°C), 97% and 92% (20°C) and 76% (25°C) (TE1 and TE2, respectively, Table 3.20). Only at T2

= 17°C, for T1 = 0 and 4°C, a duration of 29 days at S1 sufficed to increase the germination level to 100% (TE1) and 93% (TE2) (Table 3.20).

Isolate L6

For isolate L6 constant single temperatures of 20°C and 25°C were identified as a supra-optimum and ceiling temperature, causing reduction in germination or limiting germination completely. In the transfer treatments a number of initial S1 temperatures and durations of S1 were introduced resulting in an increase in germination levels at these limiting temperatures (e.g. cumulative germination curves presented for T2 = 25°C, T1 = 0 – 20°C (various colours), and S1 duration 14 to 56 days (dark grey background, left to right) in TE2, Figure 3.12).

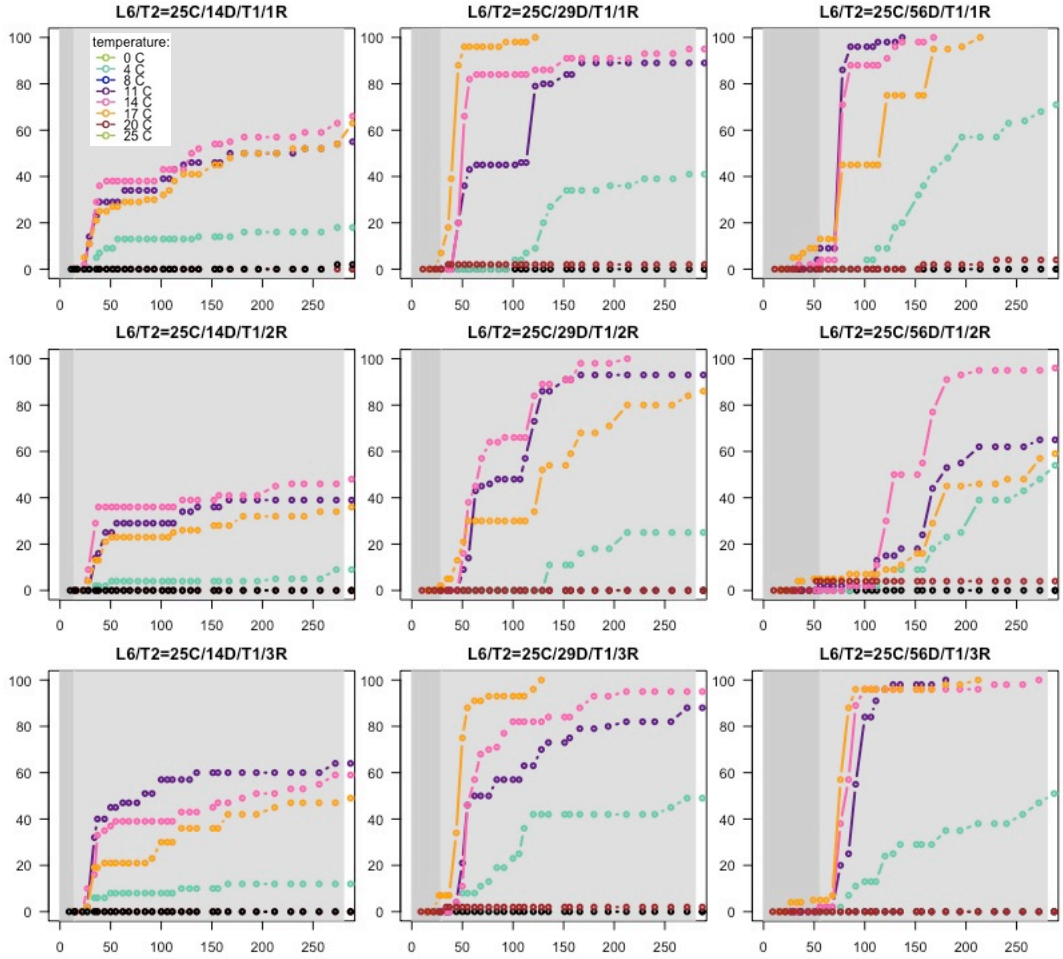

Figure 3.12 Cumulative germination curves for *S. sclerotiorum* isolate L6 observed in TE2, T2 = 25°C; T1: green = 0°C, light blue = 4°C, dark blue = 8°C, purple = 11°C, pink = 14°C, orange = 17°C, brown = 20°C, black = 25°C; S1 duration (left to right, represented by dark grey on each graph): 14, 29 and 56 days; for tree replicates (top to bottom: Rep1, Rep2, Rep3).

The T2 approach analyses of maximum germination showed a significant effect of the main factor T2 in both experiments (TE1: $F_{6,180}$=590.92, TE2: $F_{5,100}$=284.37, both $p<0.001$). Interestingly analyses showed contradictory effects of lower temperatures

in S1 where for T2 ≤ 20°C germination increased and for T2 = 4 and 8°C germination decreased (although only by 6% at the extreme). Summarising the T2 approach analyses results for T2 = 4, 8, 20 and 25°C temperatures (back transformed means for T2 = 20 and 25°C are presented in Table 3.21):

- Inside of T2 = 25°C (TE2) a significant difference between single temperature and transfer treatments ($F_{1,100}$=186.04, p<0.001) and, for transfer treatments, a significant interaction between T1 and S1 duration was observed ($F_{8,100}$=5.30, p<0.001).
- Inside of T2 = 20°C a significant difference between single temperature and transfer treatments was observed (TE1: $F_{1,180}$=65.51, TE2: $F_{1,100}$=199.38, both p<0.001). In TE2 a significant effect of T1 and S1 duration was observed (T1: $F_{3,100}$=43.70, S1 duration: $F_{2,100}$=63.85, p<0.001) and in TE1 a significant effect of S1 durations of 7, main (14-56 days) and 84 days ($F_{2,180}$=124.16, p<0.001). For transfer treatments in TE1 a significant interaction between T1 and S1 duration was observed for S1 duration <84 days (for S1 duration 7 days: $F_{3,180}$=13.44, and for 14, 29 and 56 days: $F_{10,180}$=3.66, both p<0.001).
- Inside of T2 = 8°C significant effect of T1 was observed (TE1: $F_{1,180}$=4.38, p=0.038).
- Inside of T2 = 4°C significant effect of S1 duration (including single temperature treatment) (TE1: $F_{4,180}$=10.77, p<0.001).

For T2 ≤ 20°C overall the application of lower T1 (except 0°C) and longer durations of S1 increased germination. The significant interaction between T1 and S1 duration was largely represented by germination improvements seen for higher T1 (14 and 17°C) and with increasing T1 shorter S1 durations were required (29 days). Duration of S1 of 29+ days and T1 = 14 and 17°C increased germination to ~100% compared to single temperature treatments at 20°C (83% in TE1 and 13% in TE2) and 25°C (0% in TE2) (Table 3.21). In contrast T1 = 4°C and S1 duration of 56 days increased germination to 94% (TE1) and 79% (TE2) for T2 = 20°C and to 59% (TE2) for T2 = 25°C (Table 3.21). For T2 = 4 and 8°C the introduction of T1 = 0 and 4°C resulted in significant but small decreased in germination with the smallest germination observed for 56 days at 0°C followed by 4°C (94%).

Max germ %_L6 viable		T1 duration (days)											
		TE1						TE2					
T1	T2	0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							0					
20℃										0	1	2	
17℃										49	98	95	
14℃										58	98	100	
11℃										53	90	96	
8℃													
4℃										13	38	59	
0℃													
25℃	20℃												
20℃		83						13					
17℃				96	100	100	100			77	100	100	
14℃				98	99	100	100			83	100	100	
11℃			93	96	98	100				73	99	100	
8℃			87	94	98	98							
4℃			89	93	98	94				42	66	79	
0℃			76	79	88	82							

Table 3.21 T2 approach ANOVA table of back transformed means for Max germination % (based on viable sclerotia) for TE1 and TE2 for isolate L6 for combination of treatments with T2 = 20 and 25°C, T1 ranging from 0°C to 20°C, and T1 duration ranging from 0 to 84 days; Colour gradient from 100% (green) to 0 % (red) germination; Grey = no treatment combination tested; Transformed (angular) TE1: d.f. = 180, s.e.d. = 2.340, l.s.d. = 4.618; TE2: d.f. = 100, s.e.d. = 4.961, l.s.d. = 9.842.

Summary and isolate comparison - The effect of initial low temperature on level of germination (Hypothesis 2.)

Generally, for both isolates, where a decrease of germination was observed for the extreme single temperature treatments (T = T2) conditioning in S1 (T1<T2) increased germination in S2 (T2). Furthermore, a duration of 7 days of lower temperatures can improve germination, however for best results 29 days was required and this requirement further increased for less optimal T1 and T2 temperatures (L5 - Table 3.22, L6 - Table 3.23). For both isolates, temperatures ≤ 4°C (single and in combination - T1 = 0°C and T2 = 4°C for transfer treatments) generally decreased germination.

The isolates differ in their temperature preference, where isolate L5 seemed to be more sensitive to temperature (narrow optimum temperature range for the single temperature treatments, 8-14°C) and the germination level was reduced by temperatures ≥17°C, this was improved by introduction of low temperatures especially T1 = 4-11°C (Table 3.22). In contrast, isolate L6 showed ~100% germination for a wide range of single temperature treatments (4 – 17°C) with germination levels reduced by temperatures ≥20°C but this was improved by introduction of temperatures T1 = 11 -17°C (Table 3.23).

L5	Maximum germination	
T2	Single temperature (T = T2)	The effect of initial low temperature (T1) and S1 duration (T1<T2)
0°C	Base*** 0%*	N/A
4°C	Sub – optimum 96%*, 83%**	No effect, ~100%, generally small increase (+/-3%)
8°C	Optimum 100%*	*No effect, ~100%
11°C	Optimum 100%*, 99%**	No effect, 100%
14°C	Optimum 100%*, 99%**	No effect, 100%
17°C	Supra – optimum 89%*, 78%**	**Significant increase, longer S1 was required for higher T1** T1=0-4°C, 29 days S1 achieved ~100%, 95-100%* at T1=0°C, 94-100%*, 77-100%** at T1=4°C, T1=8-11°C, 56 days S1 achieved ~100% 88-100%* at T1=8°C, 91-99%*, 81-99%** at T1=11°C, T1=14°C, 84 days S1 achieved ~100%, 54-100%*, 69-98%** at T1=14°C
20°C	Ceiling*** 0%	**Significant increase after 29 days at S1,** **longer S1 was required for higher T1** T1 = 0-11°C, 56 days S1 achieved >50%, 8-11°C best increase 0-59%* at T1=0°C, 0-91%*, 0-52%** at T1=4°C 0-97%* at T1=8°C, 0-98%*, 0-92%** at T1=11°C T1 = 14 - 17°C, 84* days S1 achieved 87% and 11% 2-87%*, 1-38%** at T1=14°C, 3-11%*, 0-10%** at T1=17°C
25°C	Ceiling*** 0%**	**Significant increase after 56 days at S1 and T1<20°C**** 0-8% at T1=4°C, 0-76% at T1=11°C, 0-24% at T1=14°C, 0-5% at T1=17°C

Table 3.22 Summary of the effects of the single temperature and transfer treatments on level of carpogenic germination of *S. sclerotiorum* isolate L5; * TE1, ** TE2, *** approximate base/ceiling temperature, it is our best estimate based on treatments/temperatures selected for the experiment.

L6	Maximum germination	
T2	Single temperature (T=T2)	The effect of initial low temperature (T1) and S1 duration (T1<T2)
0°C	Base*** 0%*	N/A
4°C	Optimum 100%	No effect for S1 duration <56 days, 100% Significant decrease at T1 = 0°C, 56 days, 96%*
8°C	Optimum 100%	No effect, 100% except significant decrease, for T1 = 4°C, 7 days, 99%*
11°C	Optimum 100%	No effect, 100%
14°C	Optimum 100%	No effect, 100%
17°C	Optimum 100%*, 99%**	No effect, 100%
20°C	Supra – optimum 83%*, 13%**	**Significant increase after 7 days at S1,** **longer S1 was required for lower T1** 76-82%* at T1=0°C, 89-98%*, 42-79%** at T1=4°C 87-99%* at T1=8°C, 93-100%*, 73-100%** at T1=11°C 98-100%*, 83-100%** at T1=14°C, 96-100%*, 77-100%** at T1=17°C
25°C	Ceiling*** 0%**	**Significant increase after 14+ days at S1 and T1<20°C**** 13-59% at T1=4°C, 53-96% at T1=11°C, 58-100% at T1=14°C, 49-95% at T1=17°C, 0-2% at T1=20°C

Table 3.23 Summary of the effects of the single temperature and transfer treatments on level of carpogenic germination of *S. sclerotiorum* isolate L6; * TE1, ** TE2, *** approximate base/ceiling temperature, it is our best estimate based on treatments/temperatures selected for the experiment.

3.3.3.2 Time to germination

Isolate L5

Time to germination of 10% population

The analysis of the time to 10% germination for isolate L5 showed significant effects of the main factor T2 in both experiments (TE1: $F_{5,148}$=99.67 and TE2: $F_{5,56}$=40.03, both p<0.001), and for each T2 the following significant effects of T1 and S1 duration were observed (Table 3.24):

- Inside T2 = 25°C, (TE2) a significant effect of T1 ($F_{2,56}$=42.15, p<0.001.
- Inside of T2 = 20°C, in TE1 only, for the transfer treatments a significant effect of S1 duration (7, main (14-56 days) and 84 days) ($F_{1,148}$=9.99, p=0.002) and of T1 temperature for S1 duration of 84 days ($F_{1,148}$=35.88, p<0.001). A significant interaction between T1 and S1 duration for both experiments (TE1 (main=14-56 days): $F_{6,148}$=20.67, p<0.001, TE2: $F_{3,56}$=5.36, p=0.003).
- Inside of T2 = 17°C in TE1 only, a significant difference between single temperature and transfer treatments ($F_{1,148}$=6.55, p=0.012), for the transfer treatments a significant effect of S1 duration (7, main (14-56 days) and 84 days) ($F_{2,148}$=11.01, p<0.001) and of T1 temperature for S1 duration of 7 days ($F_{3,148}$=4.95, p=0.003). A significant interaction between T1 and S1 duration for both experiments (TE1 (main=14-56 days): $F_{8,148}$=4.23, TE2: $F_{4,56}$=16.75, both p<0.001).
- Inside of T2 = 4 to 14°C, in TE1 for the transfer treatments a significant effect of S1 duration (T1=14°C: $F_{3,148}$=4.04, p=0.008; T1=11°C: $F_{3,148}$=11.86, p<0.001; T1=8°C, $F_{3,148}$=5.71, p=0.001; T1=4°C: $F_{4,148}$=4.44, p=0.002). In TE2 for T2 = 14°C a significant interaction between T1 and S1 duration ($F_{2,56}$=3.43, p=0.039) and for T2 = 11°C a significant effect of S1 duration ($F_{3,56}$=8.59, p<0.001).

A general response for transfer treatments when initial lower temperature was introduced was similar to the single temperature response, where the fastest germination times (T_{10}) were observed for T2 = 11 and 14°C and T_{10} increased with both increasing and decreasing T2. The effect of T1 and S1 duration often showed a significant interaction, and the pattern observed for different T2 could be divided as follows (Table 3.24):

The T2 ≥ 17°C generally required at least 29 days at T1 = 0-11°C to achieve fast germination (in TE1 a reduction in T_{10} by almost ~50% compared to single temperature treatment (T=T2)). With increasing T1 (> 11°C) and T2 (> 17°C) generally long germination times were observed (or importantly, germination did not achieve 10%), where at least 56 days at S1 was required to achieve relatively fast germination times.

For T2 = 11 and 14°C generally the fastest germination times for T_{10} were observed. Acceleration (not significant) of germination, compared to the single temperature treatments (T =T2), was observed for most of the S1 durations ≤ 29 days. The effect of duration of S1 interacted with T1, where generally with increasing T1 a longer period of S1 was required to achieve fast germination. For T1 ≤ 4°C only 7 days at S1 would produce the fastest germination times observed (~39 days).

For T2 ≤ 8°C the T_{10} was shortest for the single temperature treatments (T=T2), and a longer duration of S1 and lower T1 resulted in delayed germination.

The fastest germination times could be aligned along a diagonal drawn across treatments (black dotted arrow, Table 3.24), starting at 7 days at T1 = 0°C followed by T2 = 11°C, with increasing T2 a longer S1 duration is preferred, towards 29 days at T2 = 17°C where T1 = 4°C becomes the best performing T1 temperature. Generally, the S1 duration of 56 days was found already too long and where germination of 10% of the population was often completed either at S1 (T_{10} > 56 days) or the observed T_{10} was longer compared to S1 durations of 7, 14 and 29 days.

T_{10}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃													
14℃												46	
11℃												43	
8℃													
4℃												74	
0℃													
25℃	20℃												
20℃													
17℃				138	108	136	125				66	59	
14℃					162	58	66				50	64	
11℃					67	46					54	44	
8℃					50	59							
4℃				53	54	66					49	70	
0℃					57	71							
25℃	17℃												
20℃													
17℃		89						63					
14℃				117	81	70	77			97	47	42	
11℃			102	87	46	52				62	48	43	
8℃			93	87	47	59							
4℃			75	45	47	65				50	50	68	
0℃			68	75	48	71							
25℃	14℃												
20℃													
17℃													
14℃		52						46					
11℃			54	42	49	39				41	42	45	
8℃			45	45	45	60							
4℃			38	42	45	65				41	50	69	
0℃			47	47	51	74							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		45						44					
8℃			44	43	45	62							
4℃			39	39	46	69				46	55	74	
0℃			38	45	57	79							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		61											
4℃			64	65	68	82							
0℃			61	68	77	97							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		98						117					
0℃			103	110	114	136							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Table 3.24 T2 approach ANOVA table of means for time to germination of 10% of the population (T_{10}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 10%; Grey = no treatment combination tested; TE1: d.f. = 148, s.e.d. = 9.904, l.s.d. = 19.572; TE2: d.f. = 56, s.e.d. = 6.536, l.s.d. = 13.093.

Time to germination of 25%, 50%, 75% and 90%

Full tables with ANOVA estimated means for isolate L5 are shown in the appendices: T_{25} (Appendix 3), T_{50} (Appendix 4), T_{75} (Appendix 5), T_{90} (Appendix 6).

For isolate L5 the common result for the analyses of germination times for increased levels of germination (i.e. T_{25}, T_{50}, T_{75} and T_{90}), showed similarity in response to T2 temperature conditions to T_{10} (similar three patterns). The main change in requirements was observed for T2 $\geq$ 11°, where for fast germination times with increasing percentile, longer S1 duration and T1 temperatures of T1 = 0-8°C were required and this was further emphasised when T2 was further (higher) from 11°C.

For example, for T2 = 14°C (Table 3.25) for T_{10} the fastest germination time was observed for T1 = 4°C, S1 duration 7 days, 38 days (TE1), which was significantly faster compared to single temperature treatment at 14C, 52 days (TE1). Furthermore, only the germination times observed for S1 duration of 56 days, T1 = 0-8°C were significantly longer (in both experiments) as for T1 = 11°C the 10% germination was completed already in S1. With increased percentiles the fastest germination times for T1 = 0-8°C in TE1 shifted towards S1 duration of 29 days (T_{50}, T_{75}, T_{90}) and in TE2 were consistent for 14 days. For the T1 = 11°C, S1 duration 29 days, the time to germination became significantly longer for ≥50% (TE1) and 90% (TE2) percentiles compared to T1 = 0-8°C. For T1 = 11°C generally a longer S1 duration was required for fast germination compared to lower T1 and this become more evident with increasing percentiles of germinated sclerotia.

Statistical analyses for increased percentiles for T2 = 14°C showed the following significant effects of T1 and S1 duration:

- T_{25}: In TE1 only, for the transfer treatments a significant effect of S1 duration ($F_{3,143}$=4.97, p<0.001) and a marginally non-significant interaction between T1 and S1 duration ($F_{9,143}$=1.92, p=0.053).
- T_{50}: For both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,137}$=6.15, TE2: $F_{3,47}$=6.58, both p=0.014). In TE1 only, for transfer treatments a significant effect of T1 ($F_{3,137}$=4.76, p= 0.003) and S1 duration ($F_{3,137}$=3.51, p=0.017).
- T_{75}: For both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,130}$=6.32, p=0.013, TE2: $F_{1,37}$=7.64, p=0.009). In TE1 only, for transfer treatments a significant effect of T1 ($F_{3,130}$=3.04, p=

0.031) and S1 duration ($F_{3,130}$=5.72, p=0.001), and in TE2 a significant interaction between T1 and S1 duration ($F_{2,37}$=3.77, p= 0.032).

- T_{90}: For both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,115}$=4.70, p=0.032, TE2: $F_{1,30}$=11.15, p=0.002). In TE1 only, for transfer treatments a significant effect of S1 duration ($F_{3,115}$=8.69, p<0.001), and in TE2 only a significant interaction between T1 and S1 duration ($F_{2,30}$=5.10, p= 0.012)

L5: T2 = 14℃		T1 duration (days)											
%	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T10	25℃												
	20℃												
	17℃												
	14℃	52						46					
	11℃		54	42	49	39				41	42	45	
	8℃		45	45	45	60							
	4℃		38	42	45	65				41	50	69	
	0℃		47	47	51	74							
T25	25℃												
	20℃												
	17℃												
	14℃	69						68					
	11℃		79	61	60	51				50	50	48	
	8℃		59	53	49	63							
	4℃		48	47	49	69				46	55	74	
	0℃		61	55	55	77							
T50	25℃												
	20℃												
	17℃												
	14℃	92						92					
	11℃		100	82	91	63				65	65	52	
	8℃		83	68	57	70							
	4℃		69	58	56	75				55	63	80	
	0℃		79	67	61	82							
T75	25℃												
	20℃												
	17℃												
	14℃	113						110					
	11℃		119	98	108	78				90	91	60	
	8℃		104	90	65	77							
	4℃		90	91	65	85				66	72	89	
	0℃		96	88	66	88							
T90	25℃												
	20℃												
	17℃												
	14℃	126						131					
	11℃		132	115	119	92				130	105	93	
	8℃		124	111	72	85							
	4℃		109	109	71	92				77	84	99	
	0℃		113	109	72	95							

Table 3.25 ANOVA table of means for T2 = 14°C times to germination to 10%, 25%, 50%, 75% and 90% of the population (T_{10}, T_{25}, T_{50}, T_{75}, T_{90}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1 and S1 duration; Colour gradient from longest (green) to shortest (red) germination time for each percentile individually; Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{10} – d.f. = 148, s.e.d. = 9.904, l.s.d. = 19.572, T_{25} – d.f. = 143, s.e.d. = 9.530, l.s.d. = 18.838, T_{50} – d.f. = 137, s.e.d. = 10.806, l.s.d. = 21.369, T_{75} – d.f. = 130, s.e.d. = 13.75, l.s.d. = 27.21, T_{90} – d.f. = 115, s.e.d. = 15.96, l.s.d. = 31.61; In TE2: T_{10} – d.f. = 56, s.e.d. = 6.536, l.s.d = 13.093, T_{25} – d.f. = 51, s.e.d. = 18.95, l.s.d. = 38.05, T_{50} – d.f. = 47, s.e.d. = 14.44, l.s.d. = 29.04, T_{75} – d.f. = 37, s.e.d. = 15.23, l.s.d. = 30.86, T_{90} – d.f. = 30, s.e.d. = 12.83, l.s.d. = 26.21.

Isolate L6

Time to germination of 10% population

The analysis of the time to 10% germination for the isolate L6 showed a significant effect of the main factor T2 in both experiments (TE1: $F_{5,178}$=400.12 and TE2: $F_{5,90}$=6.05, both p<0.001), and for each T2 the following significant effects of T1 and S1 duration were observed:

- Inside T2 = 25°C, (TE2) a significant effect of T1 ($F3_{,90}$=20.77, p<0.001) and S1 duration ($F_{2,90}$=20.31, p<0.001).
- Inside of T2 = 20°C, in TE2 only, a significant difference between single temperature and transfer treatments ($F_{1,90}$=109.07, p<0.001). In TE1 only, a significant effect of T1 (for all S1 duration: 7 days: $F_{3,178}$=10.10, main (14, 29 and 56 days): $F_{5,178}$=6.64, 84 days: $F_{1,178}$=28.29, all p<0.001). In both experiments a significant effect of all S1 duration (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,178}$=53.93, main (14, 29 and 56 days): $F_{2,178}$=92.76, TE2: $F_{2,90}$=7.20, all p<0.001).
- Inside of T2 = 17°C, in TE1 only, a significant effect of all S1 durations (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,178}$=29.45, main (14, 29 and 56 days): $F_{2,178}$=21.14, all p<0.001) and of T1 temperature for S1 durations of 14, 29 and 56 days ($F_{4,178}$=4.63, p=0.001).
- Inside of T2 = 14°C, in TE1 only, a significant difference between single temperature and transfer treatments ($F_{1,178}$=10.75, p=0.001) and a significant effect of S1 duration ($F_{3,178}$=8.63, p<0.001).
- Inside of T2 = 8°C, in TE1 only, a significant effect of S1 duration ($F_{3,178}$=9.70, p<0.001).
- No significant differences for treatments in TE1 for T2 = 11°C and in TE2 for T2 = 11, 14 and 17°C.

The general response for transfer treatments for isolate L6, when an initial lower temperature was applied, was similar to the single temperature response in TE1, where the fastest germination times (T_{10}) were observed for T2 = 20°C and T_{10} increased with both increasing and, even more, with decreasing T2. A significant difference between the single temperature treatment and transfer treatments was only observed for T2 = 14°C (TE1) and 20°C (TE2), where, in both cases, application of initial lower temperature accelerated germination irrespective of S1 durations. The

effect of T1 and S1 duration often showed a significant interaction, and the pattern observed for different T2 could be divided as follows (Table 3.26):

The T2 = 20 and 25°C the fastest germination times were observed for the transfer treatments consistently across experiments, where the shortest S1 durations and T1 = 8 to 17°C resulted in fastest germination times. Interestingly for T2 = 25°C S1 durations of 56 days (for all T1) and T1 = 4°C (for all S1 durations) resulted in significantly delayed germination compared to other S1 durations and T1s.

For T2 = (11°C in TE2) 14 and 17°C, generally the application of lower temperatures significantly accelerated germination, where the duration of S1 = 29 days for T2 = 11 (TE2) and 14°C, and for T2 = 17°C, 14-29 days S1 duration, resulted in the shortest germination times for these T2 temperatures. The T1 resulting in the fastest T_{10} was in the range of 4 to 11°C.

For T2 $\leq$ 8°C (11°C in TE1) the T_{10} was regularly shortest then (or comparable with) the single temperature treatments (T=T2), and a longer duration of S1 and lower T1 resulted in delayed germination.

The fastest germination times could be aligned along a diagonal drown across treatments (black dotted arrow, Table 3.26), starting at T2 = 20°C (25°C), 7 (14) days and T1 = 17°C, followed by T2 = 17°C, and with decreasing T2 a longer S1 duration is preferred, towards 29 days at T2 = 14°C where T1 = 4-11°C become the best performing T1 temperatures. Generally, the S1 duration of 56 days was found already too long and the observed T_{10} was generally (significantly for T1>11°C, TE1) longer compared to S1 durations of 7, 14 and/or 29 days.

T_{10}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃										30	37	85	
14℃										28	47	84	
11℃										30	47	84	
8℃													
4℃										109	108	123	
0℃													
25℃	20℃												
20℃		40						169					
17℃				20	34	54	47			21	40	52	
14℃				25	43	62	80			24	42	67	
11℃			13	25	40	62				25	39	58	
8℃			17	26	38	61							
4℃			25	27	39	62				48	42	63	
0℃			45	42	54	72							
25℃	17℃												
20℃													
17℃		57						74					
14℃				65	59	69	89			61	59	72	
11℃			50	51	50	64				48	48	58	
8℃			51	44	47	64							
4℃			49	43	45	64				41	45	66	
0℃			58	51	48	69							
25℃	14℃												
20℃													
17℃													
14℃		82						81					
11℃			70	79	60	72				66	63	62	
8℃			69	64	56	71							
4℃			72	60	58	74				60	56	72	
0℃			69	66	59	77							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		73						72					
8℃			75	71	71	78							
4℃			71	75	75	81				59	58	74	
0℃			77	76	74	85							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		88											
4℃			90	82	85	100							
0℃			88	83	95	111							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		115						108					
0℃			124	119	117	137							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Table 3.26 T2 approach ANOVA table of means for time to germination of 10% of the population (T_{10}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 10%; Grey = no treatment combination tested. Light blue border = shortest germination time for control treatment (0 days at T1) and transfer treatments; Dark blue border = Highlighting transfer treatments with times shorter than control treatment; TE1: d.f = 178, s.e.d. = 6.242, l.s.d. = 12.318; TE2: d.f = 90, s.e.d. = 16.38, l.s.d. = 32.55.

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Time to germination of 25%, 50%, 75% and 90%

Full tables with ANOVA estimated means for isolate L6 are shown in the appendices: T_{25} (Appendix 7), T_{50} (Appendix 8), T_{75} (Appendix 9),T_{90} (Appendix 10).

For isolate L6 the common result for the analyses of germination times for increased levels of germination (i.e. T_{25}, T_{50}, T_{75} and T_{90}), showed similarity in response to T2 temperature conditions as for T_{10} (similar three patterns). The main change observed was for faster germination times with increasing percentiles, a requirement for longer S1 durations, and T1 temperatures diverging towards T1 = 17°C for T2 ≥ 20°C and T1 = 0 - 8°C (11°C in TE2) for T2 = 14-17°C (marginally 11°C).

For example, for T2 = 20°C (Table 3.27) for T_{10}, the fastest germination time was observed for T1 = 11°C (T1 ≤ 11°C included for 7 days S1 duration), S1 duration of 7 days, 13 days (TE1) and T1 = 17C, S1 duration of 14 days, 21 days (TE2), which, for both experiments, was significantly faster compared to the single temperature treatment at 20°C, 40 (TE1) and 169 (TE2) days. In both experiments a delay in germination times was observed with longer S1 duration (significant) and decreasing T1 (significant in TE1). With increased percentiles the fastest germination times shifted towards a S1 duration of 29 days and T1 = 14-17°C. Furthermore, the T1 and S1 duration showed an interaction (TE1 significant) for T_{75} and T_{90}, where the germination times were significantly delayed for T1 = 0-4°C (8°C for T_{90}) and were inconsistent with higher T1, and where germination level was lower and therefore for some treatments T_{90} and T_{75} (TE2) could not be obtained. Statistical analyses for increased percentiles for T2 = 20°C showed the following significant effects of T1 and S1 duration:

- T_{25}: for transfer treatments in TE1 only, a significant effect of T1 for all S1 durations except 84 days (7 days: $F_{3,178}$=19.72, main (14, 29 and 56 days): $F_{5,178}$=9.05, all p<0.001). In both experiments a significant effect of all S1 durations (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,178}$=69.36, main (14, 29 and 56 days): $F_{2,178}$=73.35, TE2: $F_{2,88}$=9.82, all p<0.001).
- T_{50}: in both experiments for transfer treatments a significant effect of T1 for all S1 durations except 84 days (TE1:7 days: $F_{3,178}$=26.72, main (14, 29 and 56 days): $F_{5,178}$=17.66, all p<0.001, TE2: $F_{3,80}$=3.16, p=0.029). In TE1, and marginally in TE2, a significant effect of all S1 durations was observed (TE1:

7, main (14, 29 and 56 days) and 84 days: $F_{2,178}$=46.12, main (14, 29 and 56 days): $F_{2,178}$=65.78, all p<0.001, TE2: $F_{2,80}$=2.80, p=0.067).

- T_{75}: in TE1 only, a significant difference between single temperature and transfer treatments ($F_{1,176}$=67.82, p<0.001). In both experiments for transfer treatments, a significant effect of T1 for all S1 durations except 84 days (TE1:7 days: $F_{3,176}$=21.21, main (14, 29 and 56 days): $F_{5,176}$=45.29, TE2: $F_{3,67}$=26.28, all p<0.001) and S1 durations (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,176}$=6.38, p=0.002, main (14, 29 and 56 days): $F_{2,176}$=17.95, p<0.001, TE2: $F_{2,67}$=7.44, p<0.001). Furthermore, in TE1, and marginally in TE2, a significant interaction between T1 and S1 durations of 14 to 56 days (TE1: main (14, 29 and 56 days): $F_{10,176}$=2.49, p=0.008, TE2: $F_{4,67}$=2.24, p=0.074)
- T_{90}: in both experiments for transfer treatments a significant effect of S1 duration (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,164}$=45.96, p<0.001, main (14, 29 and 56 days): $F_{2,164}$=22.62, p<0.001, TE2: $F_{2,61}$=3.48, p=0.037). In TE1 a significant effect of T1 for 7 days S1 duration ($F_{1,164}$=33.03, p<0.001) and a significant interaction between T1 and S1 duration for 14, 29 and 56 days ($F_{8,164}$=2.38, p=0.019).

L6: T2 = 20℃		T1 duration (days)											
%	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T10	25℃												
	20℃	40						169					
	17℃			20	34	54	47			21	40	52	
	14℃			25	43	62	80			24	42	67	
	11℃		13	25	40	62				25	39	58	
	8℃		17	26	38	61							
	4℃		25	27	39	62				48	42	63	
	0℃		45	42	54	72							
T25	25℃												
	20℃	50											
	17℃			24	41	62	89			25	44	71	
	14℃			27	45	66	89			26	45	70	
	11℃		18	28	43	65				29	42	61	
	8℃		26	30	41	64							
	4℃		39	31	41	65				73	49	68	
	0℃		70	55	61	83							
T50	25℃												
	20℃	67											
	17℃			30	46	74	95			36	50	75	
	14℃			31	48	72	93			33	48	75	
	11℃		33	33	47	69				39	46	65	
	8℃		43	38	46	69							
	4℃		53	47	50	72				93	74	77	
	0℃		95	73	74	101							
T75	25℃												
	20℃	158											
	17℃			46	49	79	100			71	54	80	
	14℃			49	53	76	97			67	52	79	
	11℃		54	53	53	73				122	51	70	
	8℃		72	60	59	74							
	4℃		86	70	76	102						154	
	0℃		150	167	104	172							
T90	25℃												
	20℃												
	17℃			135	56	84	105				59	84	
	14℃			77	58	79	103			69	55	82	
	11℃		126	77	72	76					62	74	
	8℃			129	84	87							
	4℃		213	169	111	128							
	0℃				198								

Table 3.27 ANOVA table of means for T2 = 20°C times to germination to 10%, 25%, 50%, 75% and 90% of the population (T_{10}, T_{25}, T_{50}, T_{75}, T_{90}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1 and S1 duration; Colour gradient from longest (green) to shortest (red) germination time for each percentile individually; Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{10} – d.f. = 178, s.e.d. = 6.242, l.s.d. = 12.318, T_{25} – d.f. = 178, s.e.d. = 7.230, l.s.d. = 14.268, T_{50} – d.f. = 178, s.e.d. = 7.522, l.s.d. = 14.843, T_{75} – d.f. = 176, s.e.d. = 12.756, l.s.d. = 25.175, T_{90} – d.f. = 164, s.e.d. = 15.14, l.s.d. = 29.89; In TE2: T_{10} – d.f. = 90, s.e.d. = 16.38, l.s.d = 32.55, T_{25} – d.f. = 88, s.e.d. = 13.84, l.s.d. = 27.51, T_{50} – d.f. = 80, s.e.d. = 20.45, l.s.d. = 40.70, T_{75} – d.f. = 67, s.e.d. = 18.65, l.s.d. = 37.23, T_{90} – d.f. = 61, s.e.d. = 16.25, l.s.d. = 32.49.

For example, for T2 = 17°C (Table 3.28) for T_{10} the fastest germination time was observed for T1 = 4°C, S1 duration of 14 days, 43 and 41 days (TE1, TE2 respectively). For both experiments there was no significant difference between single temperature and transfer treatments and in TE2 no significant effects for transfer treatments either. Generally, the T_{10} was delayed (significantly in TE1) with T1 spreading from T1 = 11°C and S1 duration 14 days. With increased percentiles the fastest germination times shifted towards a S1 duration of 29 days and T1 = 4-8°C. Furthermore, in both experiments a significant difference between single temperature and transfer treatments was observed for T_{25}, T_{50}, T_{75} and T_{90}, with the single temperature T_{90} being twice (147 days, TE1) and triple (186 days, TE2) the

fastest germination time observed for transfer treatments, 71 and 69 days (TE1, TE2 respectively). Statistical analyses for increased percentiles for T2 = 17°C showed the following significant effects of T1 and S1 duration:

- T_{25}: significant difference between single temperature and transfer treatments (TE1: $F_{1,178}$=15.18, TE2: $F_{1,88}$=19.32, both p<0.001). For transfer treatments in TE1 a significant effect of S1 duration (7, main (14, 29 and 56 days) and 84 days: $F_{2,178}$=17.83, main (14, 29 and 56 days): $F_{2,178}$=9.04, all p<0.001) and of T1 (marginally in TE2) for S1 duration of 14, 29 and 56 days (TE1: $F_{4,178}$=7.28, p<0.001, TE2: $F_{2,88}$=2.96, p=0.057).
- T_{50}: significant difference between single temperature and transfer treatments (TE1: $F_{1,178}$=37.12, TE2: $F_{1,80}$=15.68, p<0.001). For transfer treatments in TE1 a significant effect of S1 duration (7, main (14, 29 and 56 days) and 84 days: $F_{2,178}$=31.58, main (14, 29 and 56 days): $F_{2,178}$=10.64, all p<0.001) and of T1 (marginally in TE2) for S1 duration of 14, 29 and 56 days (TE1: $F_{4,178}$=11.45, p<0.001, TE2: $F_{2,80}$=3.01, p=0.055).
- T_{75}: significant difference between single temperature and transfer treatments (TE1: $F_{1,176}$=14.85, TE2: $F_{1,67}$=23.92, p<0.001). For transfer treatments a significant effect of S1 duration (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,176}$=16.41, main (14, 29 and 56 days): $F_{2,176}$=7.52, all p<0.001, TE2: $F_{2,67}$=5.24, all p=0.008) and of T1 for S1 duration of 14, 29 and 56 days (TE1: $F_{4,176}$=5.95, p<0.001, TE2: $F_{2,67}$=5.93, p=0.004).
- T_{90}: significant difference between single temperature and transfer treatments (TE1: $F_{1,164}$=14.80, TE2: $F_{1,61}$=52.14, p<0.001). For transfer treatments a significant effect of S1 duration (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,164}$=12.99, main (14, 29 and 56 days): $F_{2,164}$=9.28, TE2: $F_{2,61}$=10.61, all p<0.001) and T1 for S1 duration of 14, 29 and 56 days (TE1: $F_{4,164}$=5.77, TE2: $F_{2,61}$=10.21, all p<0.001).

L6: T2 = 17℃		T1 duration (days)											
%	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T10	25℃												
	20℃												
	17℃	57						74					
	14℃			65	59	69	89			61	59	72	
	11℃		50	51	50	64				48	48	58	
	8℃		51	44	47	64							
	4℃		49	43	45	64				41	45	66	
	0℃		58	51	48	69							
T25	25℃												
	20℃												
	17℃	87						109					
	14℃			84	73	75	94			82	66	76	
	11℃		73	63	57	68				59	51	62	
	8℃		65	54	51	68							
	4℃		73	56	50	68				54	51	70	
	0℃		72	63	54	75							
T50	25℃												
	20℃												
	17℃	114						136					
	14℃			105	88	82	99			113	81	83	
	11℃		104	82	66	74				75	57	66	
	8℃		88	66	58	73							
	4℃		96	70	58	74				73	57	77	
	0℃		96	84	62	80							
T75	25℃												
	20℃												
	17℃	129						156					
	14℃			120	107	94	105			134	102	93	
	11℃		123	101	75	79				94	62	70	
	8℃		110	79	65	79							
	4℃		107	85	66	83				96	62	82	
	0℃		118	107	70	86							
T90	25℃												
	20℃												
	17℃	147						186					
	14℃			132	126	111	111			146	115	107	
	11℃		138	115	89	88				109	69	74	
	8℃		126	99	71	85							
	4℃		121	102	74	91				115	67	85	
	0℃		131	128	76	93							

Table 3.28 ANOVA table of means for T2 = 17°C times to germination to 10%, 25%, 50%, 75% and 90% of the population (T_{10}, T_{25}, T_{50}, T_{75}, T_{90}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1 and S1 duration; Colour gradient from longest (green) to shortest (red) germination time for each percentile individually; Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{10} – d.f. = 178, s.e.d. = 6.242, l.s.d. = 12.318, T_{25} – d.f. = 178, s.e.d. = 7.230, l.s.d. = 14.268, T_{50} – d.f. = 178, s.e.d. = 7.522, l.s.d. = 14.843, T_{75} – d.f. = 176, s.e.d. = 12.756, l.s.d. = 25.175, T_{90} – d.f. = 164, s.e.d. = 15.14, l.s.d. = 29.89; In TE2: T_{10} – d.f. = 90, s.e.d. = 16.38, l.s.d = 32.55, T_{25} – d.f. = 88, s.e.d. = 13.84, l.s.d. = 27.51, T_{50} – d.f. = 80, s.e.d. = 20.45, l.s.d. = 40.70, T_{75} – d.f. = 67, s.e.d. = 18.65, l.s.d. = 37.23, T_{90} – d.f. = 61, s.e.d. = 16.25, l.s.d. = 32.49.

Summary and isolate comparison - The effect of initial low temperature on time to germination (Hypothesis 2.)

For both isolates, transfer treatments (combinations of T2, T1 and S1 duration) could be identified which resulted in accelerated germination response and also which delayed germination.

In optimum conditions (combinations of T1, T2 and S1 duration), individual for each isolate, germination times were longer for isolate L5 (Table 3.29) compared to isolate L6 (Table 3.30).

Isolates considerably differ in their T2 and T1 requirements for a fast germination, where for T_{10} optimum temperatures for L5 were T2 = 11 – 14°C and T1 = 0-4°C (Table 3.29) and for L6 were T2 = 20°C and T1 = 17°C (Table 3.30). The optimum S1 duration for T_{10} is similar for both isolates, 7 days, although for L6 this is more an assumption as at T1 = 17°C the shortest S1 duration used was 14 days, though data for the surrounding temperature treatments support this assumption.

For the increased percentiles for isolate L5 generally longer S1 germination was required, gravitating towards 29 days of S1 duration, when deviating from optimum T1, and towards 56 days, when deviating from optimum T2. For isolate L6 this was not so straight forward, where for increased percentiles also an extended S1 duration was required, 29 days, however for T1 temperatures a split in trend could be observed, where for T2 ≥ 20°C the T1 = 14 – 17°C (~56 days) and for T2 = 17 - 11°C the T1 = 0 - 11°C (~ 67 days) resulted in fastest germination times.

For both isolates, T2 ≤ 8°C generally resulted in longer germination times (although L5 (Table 3.29) was faster compared to L6 (Table 3.30)) and there was no improvement when initial lower temperatures were introduced. Furthermore, the delay in germination generally increased with longer S1 duration. This trend was consistent also for the increasing percentiles of germinated sclerotia.

Interestingly there were treatments where both isolates showed similar and relatively fast germination times, ranging from 41 to 50 days (treatments where the imaginary trendlines cross, shown as black arrows: L5 - Table 3.24 and L6 - Table 3.26): 14 days at T1 = 4°C or 29 days at T1 = 4-11°C followed by T2 = 17°C. Therefore, it is reasonable to assume there is a specific combination of T1, S1 duration, followed by T2 where both isolates show identical and moreover fast germination times. This was consistent also for the time to increased percentiles of germinated sclerotia. T_{90} for T2 = 17°C, T1 = 0 – 11°C (8°C in TE1) and where the S1 duration preference shifted towards 59 days, observed germination times were ranging from 79 to 95 for isolate L5 and 74 to 93 days for isolate L6.

L5	Time to germination (all %)		
T2	Single temperature (T = T2)	The effect of initial low temperature (T1) and S1 duration (T1<T2)	
	T_{10} (days)		T_{10} range (days)
0°C	N/A	N/A	N/A
4°C	Sub - optimum T_{10} =98*, 117**	**T_{10}: Significant delay** with increasing S1 duration **Increased %** – consistent with trend observed for T_{10}	T_{10} =103* - T_{10} =136*
8°C	Sub - optimum T_{10} =61*	**T_{10}: Significant delay** with increasing S1 duration **Increased %** – consistent with trend observed for T_{10}	T_{10} =61*- T_{10} =97*
11°C	Optimum T_{10} =45*, 44**	**T_{10}: Fastest = 7 days S1 duration and T1 = 0°C** With increasing S1 duration, the higher T1 produced faster germination times compared to lower T1 S1 duration 56 days caused significant delay for all T1 **Increased %** – consistent with trend observed for T_{10}, with S1 duration marginally shifted towards longer duration	T_{10} =38*, 46** - T_{10} =79*, 74**
14°C	Supra - optimum T_{10} =52*, 46**	**T_{10}: Fastest = 7 days S1 duration and T1 = 4°C** For S1 duration 14-29 days, generally similar for all T1, T1=0°C marginally longer times S1 duration 56 days caused significant delay for T1=0-8°C and at 11°C germination was completed already in S1 **Increased %** - shift in trend towards longer S1 durations ≥29 days accelerate germination for T_{90}	T_{10} =38*, 41** - T_{10} =74*, 69**
17°C	Supra - optimum T_{10} =89*, 63**	**T_{10}: Fastest = 14 days S1 duration and T1 = 4°C** **Generally, significantly shorter times** were observed for S1 duration 29 days, T1=0-11°C (14°C in TE2) Significant acceleration for S1 duration 29 days S1 duration <29 days, shorter time with lower T1 S1 >29 days generally shorter time with higher T1, at T1=11°C (11-14°C in TE2) germination completed in S1 **Increased %** - shift in trend towards longer S1 durations 29 days for T1=0-4°C and 56 days T1= 0-11°C accelerate germination for T_{90}	T_{10} =45*, 47** - T_{10} =117*, 97**
20°C	N/A	T_{10}**: Fastest time = 14 days S1 duration and T1 = 4°C** Generally higher T1, requires longer S1 duration, T1=17°C long delay (TE1) **Increased %** - reduction in germination, shift in trend towards longer S1 durations, 56 days and T1= 4-11°C for T_{90}	T_{10} =50*, 49** - T_{10} =162*, 70**
25°C	N/A	**T_{10}:** Obtained only for 56 days S1 duration, T1=4-14°C For 11-14°C, 56 days at S1, germination completed at S1 **Increased % -** reduction in germination, only T1=11°C achieved 75% T_{75} = 86** days	T_{10} =74**

Table 3.29 Summary of the effects of the initial low temperature and S1 duration for the single temperature and transfer treatments on time to germination of *S. sclerotiorum* isolate L56; * TE1, ** TE2.

L6	Time to germination (all %)		
T2	Single temperature (T = T2)	The effect of initial low temperature (T1) and S1 duration (T1<T2)	
	T_{10} (days)		T_{10} range (days)
0°C		N/A	N/A
4°C	Sub – optimum T_{10} =115*, 108**	**T_{10}: Significant delay** with increasing S1 duration **Increased %** – consistent with trend observed for T_{10}	T_{10} =117* - T_{10} =137*
8°C	Sub – optimum T_{10} =88*	**T_{10}: Significant delay** with increasing S1 duration **Increased %** – consistent with trend observed for T_{10}	T_{10} =82*- T_{10} =111*
11°C	Optimum T_{10} =73*, 72**	**T_{10}: Fastest = 14 days S1 duration and T1 = 4°C** **No significant effects**, where with S1 duration 56 days showing longest germination times **Increased %** – consistent with trend observed for T_{10}, with S1 duration marginally shifted towards longer duration, with 29 and 56 days showing shorter germination times	T_{10} =71*, 58** - T_{10} =85*, 74**
14°C	Supra – optimum T_{10} =82*, 81**	**T_{10}: Fastest = 29 days S1 duration and T1 = 4-8°C** Germination times increased around the fastest treatments **Increased %** - shift in trend towards longer S1 durations, ≥29 days accelerate germination for T_{90}	T_{10} =56*, 56** - T_{10} =79*, 72**
17°C	Optimum (supra) T_{10} =57*, 74**	**T_{10}: Fastest = 14 days S1 duration and T1 = 4°C** **Generally, significantly shorter times** were observed for S1 duration 7-29 days, T1=0-11°C (14°C in TE2) Significant acceleration for S1 duration 29 days S1 duration 56+ days showing longest germination times **Increased %** - significant shorter compared to single temp. treatments, shift in trend towards longer S1 durations 29 days for T1=0-4°C and 56 days T1= 0-11°C significantly accelerate germination for T_{90}	T_{10} =43*, 41** - T_{10} =89*, 72**
20°C	Optimum (supra) T_{10} =40*, 169**	T_{10}**: Fastest time = 7 days S1 duration and T1 = 11°C** **Generally, fastest germination time** for the shortest S1 duration and highest T1 **Increased %** - shift in trend towards S1 duration 29 days and T1= 14-17°C for T_{90} reduction in germination (T1 = 4°C)	T_{10} =13*, 21** - T_{10} =80*, 67**
25°C		T_{10}**: Fastest time = 14 days S1 duration and T1 = 17°C** **Generally, fastest germination time** for the shortest S1 duration and highest T1 **Increased % -** shift in trend towards S1 duration 29 days and T1= 17°C for T_{90}reduction in germination	T_{10} =30** T_{10} =123**

Table 3.30 Summary of the effects of the initial low temperature and S1 duration for the single temperature and transfer treatments on time to germination of *S. sclerotiorum* isolate L6; * TE1, ** TE2.

3.3.3.3 Variation in germination times - uniformity

Isolate L5

T_{90}-T_{10} - interdecile range

The T2 approach analysis for the differences in germination time between 90% and 10% germination (interdecile range, IDR) for the isolate L5 showed a significant effect of the main factor T2 in both experiments (TE1: $F_{5,115}$=67.75 and TE2: $F_{3,30}$=77.13, both $p<0.001$), and, for each T2, the following significant effects of T1 and S1 duration were observed (Table 3.31):

- Inside T2 = 25°C, (TE2) germination <90%.

- Inside of T2 = 20°C, limited germination in both experiments. In TE1 a significant effect of S1 duration (7, main (14, 29 and 56 days) and 84 days: $F_{1,115}$=20.69, main (14, 29 and 56 days): $F_{2,115}$=11.11, both p<0.001).
- Inside of T2 = 17°C in TE1 only, a significant effect of S1 duration (7, main (14-56 days) and 84 days: $F_{2,115}$=7.39, p<0.001) and a significant interaction between T1 and S1 duration was observed for both experiments (TE1 (main=14-56 days): $F_{7,115}$=4.83, TE2: $F_{2,30}$=12.60, both p<0.001).
- Inside of T2 = 14°C, in TE2 only, a significant difference between single temperature and transfer treatments (TE1: $F_{1,115}$=3.70, p=0.057, TE2: $F_{1,30}$=14.33, p<0.001). For both experiments a significant effect of T1 (TE1: $F_{3,115}$=4.01, p=0.009, TE2: $F_{1,30}$=23.20, p<0.001) and S1 duration (TE1: $F_{3,115}$=13.73, p<0.001, TE2: $F_{2,30}$=3.59, p=0.040).
- For T2 $\leq$ 11°C, in both experiments no significant effects.

Introduction of initial lower temperature T1 = 0-8°C (11°C in TE2) significantly improved the uniformity of germination (T_{90}-T_{10}, IDR) for T2 = 14 to 17°C. For T2 = 14°C 29 to 56 days at S1, IDR = 21 days at T1 = 0°C(Table 3.31) and for T2 = 17°C 56 days at S1, IDR = 20 days at T1 = 0°C (Table 3.31) were required to promote uniform germination. Similarly, for T2 = 11°C an improvement in germination uniformity was observed after an initial low temperature was applied, mostly for T1 = 0°C, S1 duration 29 days, IDR = 25 days (Table 3.31), however these were not significant.

For T2 $\geq$ 20°C limited germination was observed and therefor the IDR could not be assessed.

T_{90} - T_{10}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	20℃												
20℃													
17℃													
14℃							81						
11℃						66						42	
8℃						22							
4℃						40							
0℃													
25℃	17℃												
20℃													
17℃		95						73					
14℃					110	90	77				102	90	
11℃			122	103	99	63				106	123	38	
8℃			97	106	129	20							
4℃			96	118	67	25					36	27	
0℃			117	115	45	20							
25℃	14℃												
20℃													
17℃													
14℃		75						85					
11℃			78	73	71	53				89	64	49	
8℃			80	66	27	26							
4℃			71	67	26	27				36	34	30	
0℃			66	62	21	21							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		51						40					
8℃			54	50	45	30							
4℃			40	37	33	33				38	35	33	
0℃			39	36	25	27							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		51											
4℃			60	47	59	51							
0℃			51	54	52	50							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		128						177					
0℃			122	122	107	89							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Table 3.31 T2 approach ANOVA table of means for difference in times to germination for IDR – T_{90}-T_{10} in TE1 and TE2 for isolate L5 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: d.f. = 115, s.e.d. = 16.08, l.s.d. = 31.86; TE2: d.f. = 30, s.e.d. = 12.20, l.s.d. = 24.92.

Early percentile range, T_{25}-T_{10} and late percentile range, T_{90}-T_{75}

The pattern described for IDR is consisted with observations for EPR (early percentile range, T_{25}-T_{10}) and LPR (late percentile range, T_{90}-T_{75}). Furthermore, for the single temperature treatments there was a skewness to left (EPR < LPR) observed for temperatures further from the optimum (8 - 11°C), meaning that the germination slows down towards the later percentiles. The skewness was reduced as well as the differences in germination times, becoming similar for early and late percentiles, for the transfer treatments after 56 days S1 duration of T1 = 0-8°C.

For example, for T2 = 17°C there was no significant difference in the EPR and LPR between the single temperature and transfer treatments. Generally, the EPR < LPR for S1 durations of 7 to 29 days. For the transfer treatments, the EPR and LPR were both significantly reduced with application of initial lower temperatures. For EPR, T1 = 0-4°C with S1 duration of 29 days and T1 =0-11°C with S1 duration of 56 days reduced differences in germination times to 3-10 days (Table 3.32). For LPR T1 = 0-8°C with a S1 duration of 56 days reduced differences in germination times to 5-8 days (Table 3.32). Statistical analyses for EPR and LPR for T2 = 17°C showed the following significant effects of T1 and S1 duration:

- EPR: in both experiments for transfer treatments a significant (TE2 marginally) effect of S1 duration of 14, 29 and 56 days (TE1: $F_{2,143}$=12.95, p<0.001, TE2: $F_{2,51}$=3.10, p=0.053) and in TE1 a significant effect of T1 for S1 duration of 14, 29 and 56 days ($F_{4,143}$=9.20, p<0.001)
- LPR: in both experiments for transfer treatments a significant effect of S1 duration (TE1: 7, main (14-56 days) and 84 days: $F_{2,115}$=15.82, p<0.001, TE2: $F_{2,30}$=4.38, p=0.021) and T1 (TE1: 7 days S1 duration: $F_{3,115}$=4.13, p=0.008, TE2: $F_{7,115}$=4.83, p<0.001). In TE1 a significant interaction between T1 and S1 duration of 14, 29 and 56 days ($F_{7,115}$=2.56, p=0.018).

This trend is similar for further temperatures and full tables with ANOVA estimated means (T2 approach) for the various differences in germination times for isolate L5 are shown in the appendices: T_{25}-T_{10}, early percentile range (EPR) (Appendix 11), T_{90}-T_{75} - late percentile range (LPR) (Appendix 12).

L5: T2 = 17℃		T1 duration (days)											
diff	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T_{25}-T_{10}	25℃												
	20℃												
	17℃	22						18					
	14℃			36	26	19	20			20	38	19	
	11℃		15	19	22	10				43	20	4	
	8℃		16	22	10	4							
	4℃		15	16	5	3				38	7	7	
	0℃		23	17	4	4							
T_{90}-T_{75}	25℃												
	20℃												
	17℃	39						23					
	14℃				24	19	19				34	19	
	11℃		68	27	20	16				22	42	14	
	8℃		48	39	58	5							
	4℃		31	42	31	8					15	6	
	0℃		49	39	19	5							
T_{90}-T_{10}	25℃												
	20℃												
	17℃	95						73					
	14℃				110	90	77				102	90	
	11℃		122	103	99	63				106	123	38	
	8℃		97	106	129	20							
	4℃		96	118	67	25					36	27	
	0℃		117	115	45	20							

Table 3.32 ANOVA table of means for T2 = 17°C for difference in times to germination for EPR - T_{25}-T_{10}, LPR - T_{90}-T_{75} and IDR - T_{90}-T_{10}, in TE1 and TE2 for isolate L5 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red) difference statistics separately; Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{25}-T_{10} – d.f. = 143, s.e.d. = 6.343, l.s.d. = 12.539, T_{90}-T_{75} – d.f. = 115, s.e.d. = 10.656, l.s.d. = 21.108, T_{90}-T_{10} – d.f. = 115, s.e.d. = 16.08, l.s.d. = 31.86; TE2: T_{25}-T_{10} – d.f. = 51, s.e.d. = 16.24, l.s.d. = 32.60, T_{90}-T_{75} – d.f. = 30, s.e.d. = 10.93, l.s.d. = 22.33, T_{90}-T_{10} – d.f. = 30, s.e.d. = 12.20, l.s.d. = 24.92.

Isolate L6

T_{90}-T_{10} - interdecile range

The T2 approach analysis for the differences in germination time between 90% and 10% germination (interdecile range, IDR) for the isolate L6 showed a significant effect of the main factor T2 in both experiments (TE1: $F_{5,164}$=58.13 and TE2: $F_{5,61}$=13.50, both p<0.001), and for each T2 the following significant effects of T1 and S1 duration were observed:

- Inside T2 = 25°C, (TE2) for the transfer treatments a significant interaction between T1 and S1 duration ($F_{2,61}$=19.71, p<0.001).
- Inside of T2 = 20°C, in both experiments, a significant effect of S1 duration (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{1,164}$=88.83, TE2: $F_{2,61}$=7.86, both p<0.001). In TE1 a significant effect of T1 for the 7 and 84 days S1 duration (7 days: $F_{1,164}$=19.66, p<0.001, 84 days: $F1_{1,164}$=4.73, p=0.031) and a marginally significant interaction between T1 and S1 duration for the main S1 duration (14, 29 and 56 days) ($F_{8,164}$=1.96, p=0.055).
- Inside of T2 = 17°C, in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,164}$=12.64, TE2: $F_{1,61}$=38.06, both p<0.001). For the transfer treatments a significant effect of

S1 duration (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,164}$=16.75, main (14, 29 and 56 days): $F_{2,164}$=13.63, TE2: $F_{2,61}$=19.27, all p<0.001). In TE2 additionally a significant main effect of T1 ($F_{2,61}$=5.12, p=0.009).

- Inside of T2 = 14°C, for transfer treatments in TE1 a significant effect of S1 duration ($F_{3,164}$=4.73, p=0.003), and in TE2 a significant effect of T1 ($F_{1,61}$=4.18, p=0.045).
- Inside of T2 = 11°C, in TE2 only, a significant effect of S1 duration ($F_{3,61}$=3.05, p=0.035).
- Inside of T2 = 8°C, no significant effects.
- Inside of T2 = 4°C, in (TE1) a significant effect of S1 duration ($F_{4,164}$=3.40, p=0.011).

Application of initial lower temperature for 29 to 56 days significantly improved the uniformity of germination, with the shortest IDR (T_{90}-T_{10}) for T2 = 11 to 20°C. For T2 = 20°C most uniform germination was observed for T1 = 14 and 17°C. For T1 = 17°C, 29 and 56 days of S1, IDR = 15, 18 (TE1) and 13, 16 (TE2) days (respectively) and for T1 = 14°C 56 days at S1, IDR = 14 (TE1) and 16 (TE2) days. For T2 = 11 – 17°C, S1 duration of 29 and 56 days and T1 = 0-8°C (11°C for T2 = 17) were required, where with decreasing T2 lower T1 would perform marginally better. For T2=8°C, no effect of initial low temperature was observed and the distribution of germination time became wider with longer duration of S1 for T2 = 4°C.

T_{90} - T_{10}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃											18	64	
14℃											106	46	
11℃											99	24	
8℃													
4℃													
0℃													
25℃	20℃												
20℃													
17℃				116	22	30	58				20	32	
14℃				53	15	18	23			40	13	16	
11℃			113	51	32	14					23	16	
8℃				103	45	26							
4℃			185	142	73	66							
0℃					145								
25℃	17℃												
20℃													
17℃		91						112					
14℃				68	66	42	22			86	56	35	
11℃			87	63	39	24				61	22	16	
8℃			75	55	25	21							
4℃			72	58	29	26				74	22	19	
0℃			74	76	27	25							
25℃	14℃												
20℃													
17℃													
14℃		57						53					
11℃			60	51	49	40				58	49	57	
8℃			55	53	37	27							
4℃			60	51	36	26				62	27	22	
0℃			55	53	34	26							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		43						70					
8℃			50	50	40	35							
4℃			47	43	33	28				57	46	26	
0℃			39	36	25	27							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		46											
4℃			58	56	53	46							
0℃			47	47	50	43							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		111						64					
0℃			97	88	135	134							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Table 3.33 T2 approach ANOVA table of means for difference in times to germination for IDR – T_{90}-T_{10} in TE1 and TE2 for isolate L6 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: d.f. = 164, s.e.d. = 16.24, l.s.d. = 32.06; TE2: d.f. = 61, s.e.d. = 14.90, l.s.d. = 29.79.

Early percentile range, T_{25}-T_{10} and late percentile range, T_{90}-T_{75}

The pattern described for IDR was consistent with observations for EPR (early percentile range, T_{25}-T_{10}) and LPR (late percentile range, T_{90}-T_{75}). Generally, for T2 $\geq$ 11°C, the differences in germination times were reduced after 29 and 56 days S1 duration at T1 = 0-17°C, consistently for early and late percentiles for the transfer treatments and with it the distribution of germination times became normal (EPR $\approx$ LPR), symmetric and uniform. Furthermore, for the single temperature treatments there was a marginal skewness to right (EPR > LPR) observed for temperature 17°C, meaning the germination accelerated for later percentiles compared to early percentile (Table 3.34). In contrast for 20°C single temperature treatments no LPR could be determined, however for the transfer treatments a skewness to left (EPR < LPR) was observed for the S1 durations 7 and 14 days and with lower T1 this observation was consistent also for longer S1 durations, meaning the early percentiles showed a faster germination compared to late percentile (Table 3.35).

For example, for T2 = 17°C, a marginal skewness to left was observed for transfer treatments, especially for short S1 durations. For both percentile ranges, S1 duration of 29 and 56 days, T1 = 0-11°C improved uniformity of germination for the EPR (significantly) and LPR, by reducing the differences between germination times, up to IDR = 3 days (Table 3.34), and where the distribution of germination become more symmetric. Statistical analyses for EPR and LPR for T2 = 17°C showed the following significant effects of T1 and S1 duration:

- EPR: in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,178}$=36.68, TE2: $F_{1,88}$=17.87, both p<0.001). For the transfer treatments a significant effect of S1 duration (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,178}$=17.53, main (14, 29 and 56 days): $F_{2,178}$=9.02, both p<0.001, TE2: $F_{2,88}$=3.24, p=0.044). In TE1 additionally a significant effect of T1 for S1 duration of 7 days ($F_{3,178}$=2.90, p=0.037).
- LPR: only in TE2 a significant difference between single temperature and transfer treatments ($F_{1,61}$=6.72, p=0.012).

L6: T2 = 17℃		T1 duration (days)											
diff	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T_{25}-T_{10}	25℃												
	20℃												
	17℃	30						35					
	14℃			20	13	6	5			21	7	4	
	11℃		23	12	7	4				11	4	3	
	8℃		14	11	5	4							
	4℃		24	12	5	4				13	6	4	
	0℃		14	12	6	6							
T_{90}-T_{75}	25℃												
	20℃												
	17℃	19						30					
	14℃			13	18	17	6			12	14	14	
	11℃		15	13	14	9				14	7	4	
	8℃		16	20	6	6							
	4℃		15	16	7	8				19	4	3	
	0℃		13	21	6	7							
T_{90}-T_{10}	25℃												
	20℃												
	17℃	91						112					
	14℃			68	66	42	22			86	56	35	
	11℃		87	63	39	24				61	22	16	
	8℃		75	55	25	21							
	4℃		72	58	29	26				74	22	19	
	0℃		74	76	27	25							

Table 3.34 ANOVA table of means for T2 = 17°C for difference in times to germination for EPR - T_{25}-T_{10}, LPR - T_{90}-T_{75} and IDR - T_{90}-T_{10}, in TE1 and TE2 for isolate L6 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red) difference statistics separately; Empty cell = germination less than required percentile; Grey = no treatment combination tested; TE1: T_{25}-T_{10} – d.f. = 178, s.e.d. = 4.567, l.s.d. = 9.013, T_{90}-T_{75} – d.f. = 164, s.e.d. = 14.34, l.s.d. = 28.32, T_{90}-T_{10} – d.f. = 164, s.e.d. = 16.24, l.s.d. = 32.06; TE2: T_{25}-T_{10} – d.f. = 88, s.e.d. = 8.391, l.s.d. = 16.675, T_{90}-T_{75} – d.f. = 61, s.e.d. = 10.62, l.s.d. = 20.120, T_{90}-T_{10} – d.f. = 61, s.e.d. = 14.90, l.s.d. = 29.79.

For example, for T2 = 20°C showed different response for the short S1 durations in form of faster germination for the early percentiles, ranging from 2 to 25 days (Table 3.35) compared to late percentiles, ranging from 19 to 129 days (Table 3.35), resulting into strongly left skewed distribution of germination times. For the longer duration of S1 (29 and 56 days) and T1 ≥ 11°C the distribution of germination become narrow, symmetrical and uniform (EPR ≈ LPR). Statistical analyses for EPR and LPR for T2 = 20°C showed the following significant effects of T1 and S1 duration:

- EPR: in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,178}$=36.68, TE2: $F_{1,88}$=17.87, both p<0.001). For the transfer treatments a significant effect of S1 duration (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,178}$=17.53, main (14, 29 and 56 days): $F_{2,178}$=9.02, both p<0.001, TE2: $F_{2,88}$=3.24, p=0.044). In TE1 additionally a significant effect of T1 for S1 duration of 7 days ($F_{3,178}$=2.90, p=0.037).
- LPR: germination bellow 90% observed for single temperature treatments. For transfer treatments for both experiments a significant effect of S1 duration (TE1: 7, main (14, 29 and 56 days) and 84 days: $F_{2,164}$=70.84, main (14, 29 and 56 days): $F_{2,164}$=45.24, both p<0.001, TE2: $F_{2,61}$=6.29, p=0.003). In TE1 additionally a significant effect of T1 for S1 duration of 7 days ($F_{1,164}$=15.53,

p<0.001) and significant interaction between T1 and S1 duration for the main S1 duration (14, 29 and 56 days) ($F_{8,164}$=2.77, p=0.007).

L6: T2 = 20℃		T1 duration (days)											
		TE1						TE2					
diff	T1	0	7	14	29	56	84	0	7	14	29	56	84
T_{25}-T_{10}	25℃												
	20℃	10											
	17℃			4	6	8	42			4	5	19	
	14℃			2	3	4	9			3	3	4	
	11℃		5	3	3	3				4	3	3	
	8℃		9	4	3	4							
	4℃		14	4	3	4				25	7	5	
	0℃		25	13	7	12							
T_{90}-T_{75}	25℃												
	20℃												
	17℃			89	7	5	6				6	4	
	14℃			29	5	4	6			19	3	3	
	11℃		72	25	19	3					11	4	
	8℃			69	25	12							
	4℃		129	99	35	32							
	0℃				103								
T_{90}-T_{10}	25℃												
	20℃												
	17℃			116	22	30	58				20	32	
	14℃			53	15	18	23			40	13	16	
	11℃		113	51	32	14					23	16	
	8℃			103	45	26							
	4℃		185	142	73	66							
	0℃				145								

Table 3.35 ANOVA table of means for T2 = 20°C for difference in times to germination for EPR - T_{25}-T_{10}, LPR - T_{90}-T_{75} and IDR - T_{90}-T_{10}, in TE1 and TE2 for isolate L6 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red) difference statistics separately; Empty cell = germination less than required percentile; Grey = no treatment combination tested; TE1: T_{25}-T_{10} – d.f. = 178, s.e.d. = 4.567, l.s.d. = 9.013, T_{90}-T_{75} – d.f. = 164, s.e.d. = 14.34, l.s.d. = 28.32, T_{90}-T_{10} – d.f. = 164, s.e.d. = 16.24, l.s.d. = 32.06; TE2: T_{25}-T_{10} – d.f. = 88, s.e.d. = 8.391, l.s.d. = 16.675, T_{90}-T_{75} – d.f. = 61, s.e.d. = 10.62, l.s.d. = 20.120, T_{90}-T_{10} – d.f. = 61, s.e.d. = 14.90, l.s.d. = 29.79.

This trend is similar for further temperatures and full tables with ANOVA estimated means (T2 approach) for the various differences in germination times for isolate L6 are shown in the appendices: T_{25}-T_{10}, early percentile range (EPR) (Appendix 13), T_{90}-T_{75} - late percentile range (LPR) (Appendix 14).

Summary and isolate comparison - The effect of initial low temperature on the uniformity of germination (Hypothesis 2.)

For both isolates transfer treatments (combinations of T2, T1 and S1 duration) could be identified which resulted in promotion of rapid and uniform germination, assessed by the shortest IDR (interdecile range - T_{90}-T_{10}).

For isolate L5 the most uniform germination was observed at T2 = 17°C, T1 = 0 and 8°C for S1 duration 56 days, IDR = 20 days and T2 = 14°C, T1 = 0 for S1 duration 29 and 56 days, IDR = 21 days (Table 3.31).

For isolate L6 the most uniform germination was observed at T2 = 20°C, T1 = 14°C for S1 duration 29 days, IDR = 13 days (Table 3.33).

Generally, for both isolates the uniformity of germination improved for T2 = 11 – 17°C (significantly for 14 and 17°C) after applying lower T1 and S1 duration at least 29 days (L5-Table 3.36, L6-Table 3.37). For isolate L5 at higher T2 (17°C) longer S1 duration was required for all T1, 56 days.

For lower T2 = 8 – 11°C both isolates showed no significant reduction for differences in germination times, however for T2 = 11°C after 29 days in S1 the IDR was reduced by 26 and 18 days for isolate L5 and L6, respectively, and for T2 = 8°C no change was observed for both isolates.

The distribution of germination times for T2 = 4°C was considerably wider compered to higher T2 for both isolates and contrastingly the distribution of germination times for isolates L5 further widened and for isolate L6 contracted with longer S1 duration (L5-Table 3.36, L6-Table 3.37).

For the T1 response there was marginal difference between isolates, where isolate L5 preferred T1 = 0 - 8°C and isolate L6 T1 = 0 - 14°C, where longer S1 was required for the higher T1 (14°C) for isolate L6.

The main difference between the isolates appeared for T2 = 20°C, where for isolate L5 only few treatments achieved germination of 90% and therefore the IDR could not be fully assessed, however IDR for T1 = 8°C for 56 days was only 22 days (Table 3.31). For isolate L6 at T2 = 20°C improvement in IDR was observed for T1 = 11 – 17°C after 29 days where longer S1 would increase the range of T1 towards 8°C (Table 3.33). Also, a distinct response was observed for early percentile range for isolate L6 T2 = 20°C (IPR) where a rapid germination was observed for treatments for all T1 after S1 duration of 29 days, ranging from 3 to 7 days, and where for T1=14°C already 7 days at S1 would result in short IDR, 5 days (Table 3.35). However, for later percentile the fast germination was observed for the longer S1 durations and T1 = 11 to 17°C (Table 3.35).

Furthermore, the application of initial lower temperature for 29-56 days of S1 duration generally increased the symmetry of the distribution of germination times, especially for the isolate L5.

L5	Differences in germination times - uniformity		
T2	Single temperature (T = T2)	The effect of initial low temperature (T1) and S1 duration (T1<T2)	
	T_{90} - T_{10} (days)		T_{90} - T_{10} range (days)
0°C	N/A	N/A	N/A
4°C	Sub - optimum 128*, 177**	**Smallest IDR: 56 days S1 duration and T1 = 0°C** Increasing uniformity with increasing S1 duration	min =89* - max =122*
8°C	Optimum 51*	**Smallest IDR: 14 days S1 duration and T1 = 4°C** No effect	min =47* - max =60*
11°C	Optimum 51*, 40**	**Smallest IDR: 29 days S1 duration and T1 = 0°C** Increasing uniformity with lower T1 and S1 duration of 29 to 56 days	min =25*, 33** - max =54*, 38**
14°C	Supra - optimum 75*, 85**	**Smallest IDR: 29/56 days S1 duration and T1 = 0°C** Significant effect of T1 and S1 duration where T1 = 0-8°C and S1 duration 29 and 56 days increased uniformity	min =21*, 30** - max =80*, 89**
17°C	Supra - optimum 95*, 73**	**Smallest IDR: 56 days S1 duration and T1 = 0 & 8°C** Significant interaction of T1 and S1 duration, where T1 = 0-8°C and S1 duration 56 days increased uniformity	min =20*, 27** - max =129*, 123**
20°C	N/A	**Smallest IDR: 56 days S1 duration and T1 = 8°C** Reduction in germination	min =22*, 42** max =81*, 42**
25°C	N/A	reduction in germination	N/A

Table 3.36 Summary of the effects of the initial low temperature and S1 duration for the single temperature and transfer treatments on differences in germination times T_{90} – T_{10} (interdecile range - IDR) - uniformity of *S. sclerotiorum* isolate L5; * TE1, ** TE2.

L6	Differences in germination times - uniformity		
T2	Single temperature (T = T2)	The effect of initial low temperature (T1) and S1 duration (T1<T2)	
	T_{90} - T_{10} (days)		T_{90} - T_{10} range (days)
0°C	N/A	N/A	N/A
4°C	Sub – optimum 111*, 64**	**Smallest IDR: 14 days S1 duration and T1 = 0°C** Declining uniformity with increasing S1 duration	min = 88* - max = 135*
8°C	Optimum 48*	**Smallest IDR: 56 days S1 duration and T1 = 0°C** No effect	min = 43* - max = 58*
11°C	Optimum 43*, 70**	**Smallest IDR: 29 days S1 duration and T1 = 0°C** Increasing uniformity with lower T1 and S1 duration of 29 to 56 days	min = 25*, 26** - max = 50*, 57**
14°C	Optimum 57*, 53**	**Smallest IDR: 56 days S1 duration and T1 = 4°C** Significant effect of T1 (TE2) and S1 duration (TE1), where T1 = 0-8°C and S1 duration 29 and 56 days increased uniformity	min = 26*, 22** - max = 60*, 62**
17°C	Supra – optimum 91*, 112**	**Smallest IDR: 56 days S1 duration and T1 = 11°C** Significant effect of T1 (TE2) and S1 duration, where T1 = 0-11(14)°C and S1 duration 29 and 56 (84) days increased uniformity	min = 21*, 16** - max = 87*, 86**
20°C	N/A	**Smallest IDR: 29 days S1 duration and T1 = 14°C** Significant effect of T1 (TE1) and S1 duration, where T1 = 8-17(14)°C and S1 duration 29 and 56 (84) days increased uniformity, reduction in germination (T1 = 4°C)	min =14*, 13** - max =185*, 40**
25°C	N/A	**Smallest IDR: 29 days S1 duration and T1 = 17°C** reduction in germination	min = 18** max =106**

Table 3.37 Summary of the effects of the initial low temperature and S1 duration for the single temperature and transfer treatments differences in germination times T_{90} – T_{10} (interdecile range - IDR) - uniformity of *S. sclerotiorum* isolate L6; * TE1, ** TE2.

3.3.4 The effect of final high temperature (Hypothesis 3)

3.3.4.1 Level of germination

Isolate L5

The T1 approach analyses of maximum germination showed a significant effect of the main factor T1 in both experiments (TE1: $F_{6,180}$=222.92, TE2: $F_{5,100}$=289.07, both p<0.001), and for each T1 the following significant effects of T2 and S1 duration were observed (Table 3.38):

- Inside of T1 = 0°C, (TE1) a significant difference between single temperature and transfer treatments ($F_{1,180}$=418.31, p<0.001). For the transfer treatments a significant interaction between T2 and S1 duration ($F_{15,180}$=6.03, p<0.001).
- Inside of T1 = 4°C, in TE2 only, a significant difference between single temperature and transfer treatments (TE2: $F_{1,100}$=5.21, p=0.025). For the transfer treatments a significant interaction between T2 and S1 duration in both experiments (TE1: $F_{12,180}$=14.93, TE2: $F_{8,100}$=6.39, both p<0.001).
- Inside of T1 = 8°C, (TE1) a significant difference between single temperature and transfer treatments ($F_{1,180}$=21.31, p<0.001). For the transfer treatments a significant interaction between T2 and S1 duration ($F_{9,180}$=22.02, p<0.001).
- Inside of T1 = 11°C, in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,180}$=42.31, TE2: $F_{1,100}$=55.51, both p<0.001). For the transfer treatments a significant interaction between T2 and S1 duration (TE1: $F_{6,180}$=30.20, TE2: $F_{6,100}$=23.97, both p<0.001).
- Inside of T1 = 14°C, in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,180}$=100.91, TE2: $F_{1,100}$=159.02, both p<0.001). For the transfer treatments in TE1 a significant interaction between T2 and S1 duration ($F_{3,180}$=12.25, p<0.001) and in TE2 significant effect of T2 ($F_{1,100}$=183.54, p<0.001) and S1 duration ($F_{2,100}$=45.19, p<0.001).
- Inside of T1 = 17°C, in TE2 a significant difference between single temperature and transfer treatments ($F_{1,100}$=180.77, p<0.001). For the transfer treatments a significant effect of T2 in TE2 ($F_{1,100}$=5.13, p=0.026) and S1 duration in both experiments (TE1: $F_{4,180}$=48.31, TE2: $F_{2,100}$=8.82, p<0.001).
- Inside of T1 = 20°C, (TE2) no germination.

Generally, applying a higher T2 temperature significantly reduced germination levels for all T1, except 0°C, compared to the single temperature treatments (T = T1) (Table 3.38). For the T1 temperature 0°C, germination significantly increased with transfer to higher temperature, except for S1 duration <29 days and T2 = 20°C, where similar to the single temperature treatment, no germination was observed.

Transfer treatments for combination of temperatures T1 = 0 – 11°C and T2 = 8 – 14°C achieved 100% germination irrespective of S1 duration, comparable to the germination observed for the single temperature treatments (T = T2) (Table 3.38).

However, when T2 ≥ 17°C was applied to transfer treatments, for all T1 a significant reduction in germination level was observed, and germination was severely reduced with higher T2, however a longer duration of S1 significantly increased the level of germination, where the increase was reduced for T1 ≥ 14°C.

The 0°C T1 temperature did significantly increase germination levels at T2 ≥ 17°C, but this improvement was small compared to T1 ranging from 4°C to 11°C.

Max germ %_L5 viable		T1 duration (days)											
		TE1						TE2					
T1	T2	0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							0					
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃									0	0	0	
	20℃	0						0					
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃									0	0	5	
	20℃			3	5	10	11			0	6	10	
	17℃	89						78					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃									0	4	24	
	20℃			2	7	37	87			1	18	38	
	17℃			54	95	90	100			69	86	98	
	14℃	100						99					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃									0	0	76	
	20℃		0	2	34	98				0	29	92	
	17℃		91	94	91	99				81	88	99	
	14℃		100	99	100	100				100	100	100	
	11℃	100						99					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃		0	3	44	97							
	17℃		93	88	96	100							
	14℃		100	100	100	100							
	11℃		100	100	100	100							
	8℃	100											
	4℃												
	0℃												
4℃	25℃									0	0	8	
	20℃		0	6	55	91				0	26	52	
	17℃		94	94	100	100				77	93	100	
	14℃		100	100	100	100				100	100	100	
	11℃		100	100	100	100				100	100	100	
	8℃		100	100	100	100							
	4℃	96						83					
	0℃												
0℃	25℃												
	20℃		0	0	15	59							
	17℃		95	95	100	100							
	14℃		100	100	100	100							
	11℃		100	100	100	100							
	8℃		100	100	100	100							
	4℃		98	93	98	99							
	0℃	0											

Table 3.38 ANOVA table of T1 approach with back transformed means for Max germination % (based on viable sclerotia) for TE1 and TE2 for isolate L5 for combination of treatments with T1 ranging from 0°C to 20°C, T2 = ranging from 4°C to 25°C, and T1 duration ranging from 0 to 84 days; Colour gradient from 100% (green) to 0 % (red) germination; Grey = no treatment combination tested; Transformed (angular) TE1: d.f. = 180, s.e.d. = 5.107, l.s.d. = 10.077; TE2: d.f. = 100, s.e.d. = 5.308, l.s.d. = 10.531.

Isolate L6

The T1 approach analyses of maximum germination showed a significant effect of the main factor T1 in both experiments (TE1: $F_{6,180}$=63.23, TE2: $F_{5,100}$=376.29, both p<0.001), and for each T1 the following significant effects of T2 and S1 duration were observed (Table 3.39):

- Inside of T1 = 0°C, (TE1) a significant difference between single temperature and transfer treatments ($F_{1,180}$=2526.57, p<0.001). For the transfer treatments a significant interaction between T2 and S1 duration ($F_{15,180}$=3.43, p<0.001).
- Inside of T1 = 4°C, in TE2 only, a significant difference between single temperature and transfer treatments ($F_{1,100}$=25.28, p<0.001). For the transfer treatments in TE1 a significant effect of T2 ($F_{1,100}$=55.61, p<0.001) and in TE2 a significant interaction between T2 and S1 duration ($F_{8,100}$=4.25, p<0.001).
- Inside of T1 = 8°C, (TE1) for the transfer treatments a significant interaction between T2 and S1 duration ($F_{9,180}$=4.80, p<0.001).
- Inside of T1 = 11°C, in both experiments for the transfer treatments a significant interaction between T2 and S1 duration (TE1: $F_{6,180}$=4.90, TE2: $F_{6,100}$=7.27, both p<0.001).
- Inside of T1 = 14°C, in TE2 only, a significant difference between single temperature and transfer treatments ($F_{1,100}$=5.76, p=0.018). For the transfer treatments in both experiments a significant interaction between T2 and S1 duration (TE1: $F_{3,180}$=3.14, TE2: $F_{4,100}$=8.39, both p<0.001).
- Inside of T1 = 17°C, in TE2 a significant difference between single temperature and transfer treatments ($F_{1,100}$=10.00, p=0.002). For the transfer treatments a significant effect of T2 in TE2 ($F_{1,100}$=15.42, p<0.001) and S1 duration in both experiments (TE1: $F_{4,180}$=9.03, TE2: $F_{2,100}$=52.41, both p<0.001).
- Inside of T1 = 20°C, (TE2) a significant effect of S1 duration ($F_{3,100}$=5.45, p=0.002).

Generally, transferring to higher T2 temperatures reduced germination levels for T1 ≥ 14°C (significantly in TE2), compared to the single temperature treatments (T = T1), where the reduction was mainly caused by the lower germination level observed for 14 days S1 duration in TE2 (Table 3.39). There was a difference between experiments in germination levels observed at T2 = 20°C, where the difference was more distinct at T1 = 4°C (irrespective of S1 duration) and S1 duration of 14 days (irrespective of T1). For these treatments in TE1 a considerably higher germination was observed than in TE2, where this could be caused by preconditioning during sclerotia production as discussed in Appendix 1.

For the T1 temperature 0°C, germination significantly increased with transfer to higher temperatures, where generally for all T2 except T2 = 20°C, ~100% germination was achieved for all S1 durations (Table 3.39).

Transfer treatments for combination of temperatures T1 = 0 – 14°C and T2 = 4 – 17°C achieved generally ~100% germination, irrespective of S1 duration, comparable to germination levels observed for the single temperature treatments (T = 4-17°C) (Table 3.39).

When T2 = 20 and 25°C was applied to transfer treatments, for all T1 a significant reduction in germination level was observed (≥76%, ≥42% at T2 = 20°C in TE1 and TE2, respectively, and ≥ 13% at T2 = 25°C in TE2, Table 3.39), however T1 = 4-17°C and increasing S1 duration resulted in increased germination levels, up to ~100% (less at T1 = 4°C in TE2), and up 88% for T1 = 0°C (TE1) (Table 3.39).

Max germ %_L6 viable		T1 duration (days)											
		TE1						TE2					
T1	T2	0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							0					
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃									0	1	2	
	20℃	83						13					
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃									49	98	95	
	20℃			96	100	100	100			77	100	100	
	17℃	100						99					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃									58	98	100	
	20℃			98	99	100	100			83	100	100	
	17℃			100	100	100	100			100	100	100	
	14℃	100						100					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃									53	90	96	
	20℃		93	96	98	100				73	99	100	
	17℃		100	100	100	100				100	100	100	
	14℃		100	100	100	100				100	100	100	
	11℃	100						100					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃		87	94	99	98							
	17℃		100	100	100	100							
	14℃		100	100	100	100							
	11℃		100	100	100	100							
	8℃	100											
	4℃												
	0℃												
4℃	25℃									13	38	59	
	20℃		89	93	98	94				42	66	79	
	17℃		100	100	100	100				100	100	100	
	14℃		100	100	100	100				100	100	100	
	11℃		100	100	100	100				100	100	100	
	8℃		99	100	100	100							
	4℃	100						100					
	0℃												
0℃	25℃												
	20℃		76	79	88	82							
	17℃		100	100	100	100							
	14℃		100	100	100	100							
	11℃		100	100	100	100							
	8℃		100	100	100	100							
	4℃		100	100	100	94							
	0℃	0											

Table 3.39 T1 approach ANOVA table of back transformed means for Max germination % (based on viable sclerotia) for TE1 and TE2 for isolate L6 for combination of treatments with T1 ranging from 0°C to 20°C, T2 ranging from 4°C to 25°C, and T1 duration ranging from 0 to 84 days; Colour gradient from 100% (green) to 0 % (red) germination; Grey = no treatment combination tested; Transformed (angular) TE1: d.f. = 180, s.e.d. = 2.340, l.s.d. = 4.618; TE2: d.f. = 100, s.e.d. = 4.961, l.s.d. = 9.842.

Summary and isolate comparison - The effect of final high temperature on level of germination (Hypothesis 3.)

Similar responses to high final temperature (T2) and S1 duration were observed for both isolates, where the isolates differ in the range of optimum temperatures.

For isolate L5 an optimum range of temperatures (T) was identified for single temperature treatments 8 to 14°C, where the germination level was reduced with lower and higher temperature around the optimum range and with no germination for 0, 20 and 25°C (Table 3.40). For the T2 temperature the response was less consistent. Applying a final T2 temperature (T=T1, T1<T2, Table 3.40) T2 $\leq$ 14°C resulted in an increase at T1 = 0 - 4°C and no effect at T1 = 8 - 11°C in germination level. The T2 $\geq$ 17°C increased germination levels at T1 = 0°C and reduced germination levels at T1 = 4 - 17°C. The reduction was greater for higher T2. Generally, the increase in duration of S1 resulted in an increase in germination level and a longer S1 duration was required for higher T1 and T2, where an S1 duration of at least 29 days was required to significantly increase germination level at T2 = 20°C. T1 = 20°C resulted in no germination.

For isolate L6 an optimum range of temperatures (T) was identified for single temperature treatments 4 to 17°C, where the germination level was reduced with higher temperature and there was no germination for 0 and 25°C (Table 3.41). Applying a final T2 temperature (T=T1, T1<T2, Table 3.41) T2 $\leq$ 17°C resulted in an increase in germination at T1 = 0°C and no effect at T1 = 4 - 14°C. Applying final T2 temperature T2 $\geq$ 20°C increased the germination level at T1 = 0°C and reduced the germination level at T1 = 4 - 20°C. Generally, the increase in duration of S1 resulted in an increase in germination level and a longer S1 duration was required for higher T1 and T2, where an S1 duration of at least 14 days was required to significantly increase germination level at T2 = 25°C. Isolate L6 managed to show some germination, 2%, at T2 = 25°C after 56 days at T1 = 20°C (Table 3.41).

L5	Maximum germination	
T1	Single temperature (T = T1)	The effect of final high temperature (T2) and S1 duration (T1<T2)
0°C	Base*** 0%*	**Significant increase, longer S1 duration was required for higher T2,** 100%* at T2 = 8-14°C, irrespective S1 duration, 93*-100%* at T2 = 4 and 17°C, 0*-59%* at T2=20°C
4°C	Sub – optimum 96%*, 83%**	**No effect at T2 = 8-14°C**, irrespective S1 duration, 100% **Significant reduction for T2 ≥ 17°C, increase with longer S1 duration,** 94-100%*, 77-100%** at T2 = 17°C, 0-91%*, 0-52%** at T2=20°C, 0-8%** at T2=25°C
8°C	Optimum 100%*	**No effect at T2 = 11-14°C**, irrespective S1 duration, 100% **Significant reduction for T2 ≥ 17°C, increase with longer S1 duration,** 88-100%* at T2 = 17°C, 0-97%* at T2=20°C
11°C	Optimum 100%*, 99%**	**No effect at T2 = 14°C**, irrespective S1 duration, ~100% **Significant reduction for T2 ≥ 17°C, increase with longer S1 duration,** 91-99%*, 81-99%** at T2 = 17°C, 0-98%*, 0-92%** at T2=20°C, 0-76%** at T2=25°C
14°C	Optimum 100%*, 99%**	**Significant reduction, increase with longer S1 duration (84 days*),** 54-100%*, 69-98%**at T2 = 17°C, 2-87%*, 1-38%** at T2=20°C, 0-24%** at T2=25°C
17°C	Supra – optimum 89%*, 78%**	**Significant reduction, increase with longer S1 duration (84 days*),** 3-11%*, 0-10%** at T2=20°C, 0-5%** at T2=25°C
20°C	Ceiling*** 0%	Ceiling*** 0%
25°C	Ceiling* 0%**	N/A

Table 3.40 Summary of the effects of final high temperature (T1<T2) and S1 duration on Transfer treatments, compared to the single temperature (T = T1) treatments, on level of carpogenic germination of *S. sclerotiorum* isolate L5; * TE1, ** TE2, *** approximate base/ceiling temperature, it is our best estimate based on treatments/temperatures selected for the experiment.

L6	Maximum germination	
T1	Single temperature (T=T1)	The effect of final high temperature (T2) and S1 duration (T1<T2)
0°C	Base*** 0%*	**Significant increase**, **longer S1 duration was required for higher T2**, 100%* at T2 = 4-17°C, irrespective S1 duration (except 56 days, T2=4°C), 76*-88%* at T2=20°C
4°C	Optimum 100%	**No effect at T2 = 8-17°C** irrespective S1 duration, 100% **Significant reduction for T2 ≥ 20°C, increase with longer S1 duration,** 89-98%*, 42-79%** at T2=20°C, 13-59%** at T2=25°C
8°C	Optimum 100%	**No effect at T2 = 11-17°C**, irrespective S1 duration, 100% **Significant reduction for T2 ≥ 20°C, increase with longer S1 duration,** 87-99%* at T2=20°C
11°C	Optimum 100%	**No effect at T2 = 14-17°C**, irrespective S1 duration, 100% **Significant reduction for T2 ≥ 20°C, increase with longer S1 duration,** 93-100%*, 73-100%** at T2=20°C, 53-96%** at T2=25°C
14°C	Optimum 100%	**No effect at T2 = 17°C**, irrespective S1 duration, 100% **Significant reduction for T2 ≥ 20°C, increase with longer S1 duration,** 98-100%*, 83-100%** at T2=20°C, 58-100%** at T2=25°C
17°C	Optimum 100%*, 99%**	**Significant** reduction, germination level increased with longer S1** duration 96-100%*, 77-100%** at T2=20°C, 49-95%** at T2=25°C
20°C	Supra – optimum 83%*, 13%**	**Significant** reduction, germination level marginally increased with** **longer S1 duration**, 0-2%** at T2=25°C
25°C	Ceiling*** 0%**	N/A

Table 3.41 Summary of the effects of final high temperature (T1<T2) and S1 duration on Transfer treatments, compared to the single temperature (T = T1) treatments, on level of carpogenic germination of *S. sclerotiorum* isolate L6; * TE1, ** TE2, *** approximate base/ceiling temperature, it is our best

3.3.4.2 Time to germination

Isolate L5

Time to germination of 10% population

The T1 approach analysis of the time to 10% germination for the isolate L5 showed a significant effect of the main factor T1 in both experiments (TE1: $F_{5,148}$=93.60 and TE2: $F_{5,56}$=29.76, both p<0.001), and for each T1 the following significant effects of T2 and S1 duration were observed:

- Inside T1 = 0°C, (TE1) for transfer treatments, significant effects of T2 ($F_{5,148}$=45.05, p<0.001) and duration of S1 ($F_{3,148}$=17.58, p<0.001).
- Inside T1 = 4°C, in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,148}$=34.30, TE2: $F_{1,56}$=161.41, both p<0.001) and for the transfer treatments a significant effect of S1 duration (TE1: $F_{3,148}$=8.80, TE2: $F_{2,56}$=38.96, both p<0.001). In TE1, also a significant effect of T2 ($F_{4,148}$=6.57, p<0.001).
- Inside T1 = 8°C, (TE1) a significant interaction between T2 and S1 duration ($F_{7,148}$=4.43, p<0.001).
- Inside T1 = 11°C, in TE1 only, a significant difference between single temperature and transfer treatments (TE1: $F_{1,148}$=5.30, p=0.023, TE2: $F_{1,56}$=0.68, p=0.412). For transfer treatments a significant interaction between T2 and S1 duration in TE1 (TE1: $F_{4,148}$=5.31, p<0.001, TE2: $F_{3,56}$=2.61, p=0.060). In TE2 a significant effect of S1 duration ($F_{2,56}$=3.93, p=0.025).
- Inside T1 = 14°C, in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,148}$=35.50, TE2: $F_{1,56}$=17.16, both p<0.001) and for the transfer treatments a significant interaction between T2 and S1 duration (TE1: $F_{2,148}$=28.66, p<0.001, TE2: $F_{1,56}$=4.24, p=0.044).
- Inside T1 = 17°C, in TE1 a significant effect of S1 duration ($F_{4,148}$=8.67, p<0.001).
- Inside T1 = 20°C, (TE2) germination was <10% thus no T_{10} were obtained.

For isolate L5, the time to germination for each initial temperature (T1) changes with the final higher temperature (T2), subsequently transferred to, and with the duration of the initial temperature period (S1 duration). The response to final T2 temperature and S1 duration for each T1 and could be divided as follows:

Generally, T1 $\leq$ 8°C and T2 = 4-14°C and S1 duration 7-29 days resulted in a significant reduction in T_{10} compared to the single temperature treatments (except 0°C, no germination) (T=T1). For the transfer treatments T2=11-14°C, 7 days at S1 showed the best improvement, T_{10} = 38-41 days (Table 3.42) and 56 days S1 duration resulted in a significant increase in T_{10}. Furthermore, T2 = 4-8°C resulted in a significant increase in T_{10} for each S1 duration compared to T2 = 11-14°C, and the T_{10} increased with lower T1 and longer S1. For T2 = 17 - 25°C longer S1 duration was required, at least 14 days at T1= 4°C and 29 days at T1= 0 and 8°C, for fast T_{10}, ranging from 45 to 57 days (Table 3.42).

For the T1 = 11°C in TE1 a delay in germination, and in TE2 no effect, for transfer treatments compared to single temperature treatments was observed (T=T1). The delay in germination in TE1 was caused by higher T2 and shorter S1 duration (<29 days), where longer S1 duration and lower T2 would result in similar germination time as single temperature treatment.

Generally, a significant delay in T_{10} was observed for T1 $\geq$ 14°C, for transfer treatments compared to single temperature treatments (T=T1), where the germination time for transfer treatments would decrease with lower T2 and for lower T2 with longer S1 duration.

$T1_{10}$_L5		T1 duration (days)											
		TE1						TE2					
T1	T2	0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃												
	20℃			138	108	136	125				66	59	
	17℃	89						63					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃											46	
	20℃				162	58	66				50	64	
	17℃			117	81	70	77			97	47	42	
	14℃	52						46					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃											43	
	20℃				67	46					54	44	
	17℃		102	87	46	52				62	48	43	
	14℃		54	42	49	39				41	42	45	
	11℃	45						44					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃				50	59							
	17℃		93	87	47	59							
	14℃		45	45	45	60							
	11℃		41	43	45	62							
	8℃	61											
	4℃												
	0℃												
4℃	25℃											74	
	20℃			53	54	66					49	70	
	17℃		75	45	47	65				50	50	68	
	14℃		38	42	45	65				41	50	69	
	11℃		39	39	46	69				46	55	74	
	8℃		64	65	68	82							
	4℃	98						117					
	0℃												
0℃	25℃												
	20℃				57	71							
	17℃		68	75	48	71							
	14℃		47	47	51	74							
	11℃		38	45	57	79							
	8℃		61	68	77	97							
	4℃		103	110	114	136							
	0℃												

Table 3.42 T1 approach ANOVA table of means for time to germination of 10% of the population (T_{10}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 10%; Grey = no treatment combination tested; TE1: d.f. = 148, s.e.d. = 9.904, l.s.d. = 19.572; TE2: d.f. = 56, s.e.d. = 6.536, l.s.d. = 13.093.

Time to germination of 25%, 50%, 75% and 90%

Full tables with ANOVA estimated means for isolate L5 are shown in the appendices: T_{25} (Appendix 15), T_{50} (Appendix 16), T_{75} (Appendix 17), T_{90} (Appendix 18).

For isolate L5 the analyses of germination times for increased levels of germination (T_{25}, T_{50}, T_{75} and T_{90}) showed similar patterns in response to final T2 temperature and S1 duration for each T1 as observed for T_{10}. Generally, with increasing percentile for each T1 the time to germination was more sensitive to T2 and a longer S1 duration was required (example of T1 = 4°C shown below, Table 3.43). The identified optimum T2 = 11 - 14°C for T_{10} showed a change in response to S1 duration towards T_{90}. The fastest T_{90}, 71-72 (TE1) and 77 (TE2) days, were observed for T2 = 14°C, T1 = 0-8°C and at least 14 (TE2) - 29 (TE1) (significant difference compared to shorter S1 duration) days at S1 were required (Appendix 18). At T2 = 11°C, T1 = 0-4°C for T_{90} for S1 duration of 7 - 29 days, comparably fast germination times were observed (to T2 = 14°C), ranging from 75 (TE1) to 91 (TE2) days and where the 56 days S1 duration resulted in a considerable (non-significant) increase in T_{90}, ranging from 102 to 107 days (Appendix 18).

Statistical analyses for increased percentiles for T1 = 4°C showed the following significant effects of T2 and S1 duration:

- T_{25}: In both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,143}$=44.67, TE2: $F_{1,51}$=22.24, both p<0.001). In TE1 only, for the transfer treatments a significant interaction between T2 and S1 duration ($F_{10,143}$=2.23, p= 0.019).
- T_{50}: For both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,137}$=47.75, TE2: $F_{1,47}$=67.90, both p<0.001) and for transfer treatments a significant interaction between T2 and S1 duration (TE1: $F_{10,137}$=4.24, p<0.001, TE2: $F_{4,47}$=3.34, p=0.017).
- T_{75}: For both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,130}$=42.68, TE2: $F_{1,37}$=111.94, both p<0.001) and for transfer treatments a significant interaction between T2 and S1 duration (TE1: $F_{10,130}$=4.90, p<0.001, TE2: $F_{4,37}$=5.23, p=0.002).
- T_{90}: For both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,115}$=102, TE2: $F_{1,30}$=476.88, both p<0.001). for transfer treatments in TE2 only, a significant effect of S1 duration

($F_{2,30}$=7.34, p=0.003), and in TE1 only a significant interaction between T1 and S1 duration ($F_{9,115}$=4.08, p<0.001)

L5: T1 = 4℃		T1 duration (days)											
%	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T10	25℃											74	
	20℃			53	54	66					49	70	
	17℃		75	45	47	65				50	50	68	
	14℃		38	42	45	65				41	50	69	
	11℃		39	39	46	69				46	55	74	
	8℃		64	65	68	82							
	4℃	98						117					
	0℃												
T25	25℃											85	
	20℃				66	71					89	75	
	17℃		90	61	52	68				88	57	75	
	14℃		48	47	49	69				46	55	74	
	11℃		49	49	55	76				50	58	78	
	8℃		71	72	75	88							
	4℃	110						136					
	0℃												
T50	25℃												
	20℃				67	76					88	108	
	17℃		124	101	62	73				112	65	81	
	14℃		69	58	56	75				55	63	80	
	11℃		59	59	62	82				57	64	85	
	8℃		83	80	84	102							
	4℃	130						166					
	0℃												
T75	25℃												
	20℃				121	89							
	17℃		151	140	83	83				141	84	89	
	14℃		90	91	65	85				66	72	89	
	11℃		70	67	69	91				66	75	97	
	8℃		100	91	99	113							
	4℃	162						204					
	0℃												
T90	25℃												
	20℃					107							
	17℃		165	165	114	91					84	95	
	14℃		109	109	71	92				77	84	99	
	11℃		79	75	78	102				84	91	107	
	8℃		124	112	127	133							
	4℃	227						293					
	0℃												

Table 3.43 ANOVA table of means for T1 = 4°C times to germination to 10%, 25%, 50%, 75% and 90% of the population (T_{10}, T_{25}, T_{50}, T_{75}, T_{90}) in TE1 and TE2 for isolate L5 for combination of treatments with different T2 and S1 duration; Colour gradient from longest (green) to shortest (red) germination time for each percentile individually; Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{10} – d.f. = 148, s.e.d. = 9.904, l.s.d. = 19.572, T_{25} – d.f. = 143, s.e.d. = 9.530, l.s.d. = 18.838, T_{50} – d.f. = 137, s.e.d. = 10.806, l.s.d. = 21.369, T_{75} – d.f. = 130, s.e.d. = 13.75, l.s.d. = 27.21, T_{90} – d.f. = 115, s.e.d. = 15.96, l.s.d. = 31.61; In TE2: T_{10} – d.f. = 56, s.e.d. = 6.536, l.s.d = 13.093, T_{25} – d.f. = 51, s.e.d. = 18.95, l.s.d. = 38.05, T_{50} – d.f. = 47, s.e.d. = 14.44, l.s.d. = 29.04, T_{75} – d.f. = 37, s.e.d. = 15.23, l.s.d. = 30.86, T_{90} – d.f. = 30, s.e.d. = 12.83, l.s.d. = 26.21.

Isolate L6

Time to germination of 10% population

The T1 approach analysis of the time to 10% germination for the isolate L6 showed a significant effect of the main factor T1 in both experiments (TE1: $F_{6,178}$=87.19 and TE2: $F_{4,90}$=85.68, both p<0.001), and for each T1 the following significant effects of T2 and S1 duration were observed:

- Inside T1 = 0°C, (TE1) for transfer treatments significant effects of T2 ($F_{5,178}$=146.29, p<0.001) and duration of S1 ($F_{3,178}$=22.70, p<0.001).
- Inside T1 = 4°C, in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,178}$=125.79, p<0.001, TE2: $F_{1,90}$=10.96, p=0.001). For the transfer treatments a significant interaction between T2 and S1 duration in TE1 ($F_{12,178}$=2.14, p=0.017) and significant effects of T2 and S1 duration in TE2 (T2: $F_{4,90}$=15.07, p<0.001, S1 duration: $F_{2,90}$=3.61, p=0.031).
- Inside T1 = 8°C, (TE1) a significant difference between single temperature and transfer treatments ($F_{1,178}$=48.42, p<0.001) and for the transfer treatments a significant interaction between T2 and S1 duration ($F_{9,178}$=4.43, p<0.001).
- Inside T1 = 11°C, in TE1 only, a significant difference between single temperature and transfer treatments (TE1: $F_{1,178}$=19.61, p<0.001, TE2: $F_{1,90}$=2.63, p=0.108). For the transfer treatments a significant interaction between T2 and S1 duration in TE2 ($F_{6,90}$=7.57, p<0.001) and a significant effect of S1 duration in TE1 ($F_{6,178}$=7.57, p<0.001).
- Inside T1 = 14°C, in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,148}$=19.64, p<0.001, TE2: $F_{1,90}$=5.00, both p=0.028) and for the transfer treatments a significant interaction between T2 and S1 duration in TE1 ($F_{3,178}$=5.80, p<0.001) and a significant effect of S1 duration in TE2 ($F_{2,90}$=7.92, p<0.001).
- Inside T1 = 17°C, in TE2 a significant difference between single temperature and transfer treatments ($F_{1,90}$=5.78, p=0.018) and in both experiments a significant effect of S1 duration (TE1: $F_{4,178}$=12.03, TE2: $F_{4,178}$=7.26, both p<0.001).
- Inside T1 = 20°C, (TE2) germination was <10% thus no T_{10} were obtained.

For isolate L6, the time to germination for each initial temperature (T1) changes with the final higher temperature (T2), subsequently transferred to, and with the duration of the initial temperature period (S1 duration).

Generally, for T1= 0-17°C a significant reduction in T_{10} was observed compared to the single temperature treatments (T=T1) (except 0°C, no germination) after applying T2 (T1<T2). For transfer treatments at each T1 temperature the fastest T_{10} was consistently observed for T2 = 20°C and shortest S1 duration, i.e. 13 days (TE1) at T1 = 11°C, S1 duration 7 days and T2 = 20°C or 20 (TE1) and 21 (TE2) days at T1 = 17°C, S1 duration 14 days and T2 = 20°C (Table 3.44). Generally, for T2 = 20 and

25°C with longer S1 duration T_{10} increased. For T2 = 4-17°C lower T1 increased T_{10} with the shortest germination times for S1 duration of 14 - 29 days and where 56 days at S1 commonly delayed germination significantly. Additionally, the longest T_{10} were observed for transfer treatments T1= 0°C to T2 = 4°C (TE1) and T1 = 4°C to T2 = 25°C (TE2) where germination was significantly delayed when compared to the remaining transfer treatments, with T_{10} ranging from 117 to 137 days (TE1) and 109 to 123 days (TE2), respectively, and these germination times were comparable with T_{10} observed at single temperature 4°C, 115 (TE1) and 108 (TE2) days (Table 3.44).

T_{10}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃	40						169					
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃									30	37	85	
	20℃			20	34	54	47			21	40	52	
	17℃	57						74					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃									28	47	84	
	20℃			25	43	62	80			24	42	67	
	17℃			65	59	69	89			61	59	72	
	14℃	82						81					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃									30	47	84	
	20℃		13	25	40	62				25	39	58	
	17℃		50	51	50	64				48	48	58	
	14℃		70	79	60	72				66	63	62	
	11℃	73						72					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃		17	26	38	61							
	17℃		51	44	47	64							
	14℃		69	64	56	71							
	11℃		75	71	71	78							
	8℃	88											
	4℃												
	0℃												
4℃	25℃									109	108	123	
	20℃		25	27	39	62				48	42	63	
	17℃		49	43	45	64				41	45	66	
	14℃		72	60	58	74				60	56	72	
	11℃		71	75	75	81				59	58	74	
	8℃		90	82	85	100							
	4℃	115						108					
	0℃												
0℃	25℃												
	20℃		45	42	54	72							
	17℃		58	51	48	69							
	14℃		69	66	59	77							
	11℃		77	76	74	85							
	8℃		88	83	95	111							
	4℃		124	119	117	137							
	0℃												

Table 3.44 T1 approach ANOVA table of means for time to germination of 10% of the population (T_{10}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 10%; Grey = no treatment combination tested; TE1: d.f = 178, s.e.d. = 6.242, l.s.d. = 12.318; TE2: d.f = 90, s.e.d. = 16.38, l.s.d. = 32.55.

Time to germination of 25%, 50%, 75% and 90%

Full tables with ANOVA estimated means for isolate L6 are shown in the appendices: T_{25} (Appendix 19), T_{50} (Appendix 20), T_{75} (Appendix 21), T_{90} (Appendix 22).

For isolate L6 the analyses of germination times for increased levels of germination (T_{25}, T_{50}, T_{75} and T_{90}) showed a common change for all T1 with a shift in S1 duration towards longer, 29 days S1 duration, for the fastest germination times (T_{90}) (Appendix 22). The response in time to germination for higher percentiles (T_{75} and T_{90}) showed a difference in pattern in response to final T2 temperature between T1 = 0 - 8°C and T1 = 11 – 25°C. At T1 = 11 – 25°C the time to germination response to T2 was similar to T_{10} for all levels of germination, generally the fastest germination time was observed at T2 = 20°C, i.e. T_{90} for T1 = 14°C and 29 days S1 duration, 58 (TE1) and 55 (TE2) days (Table 3.45). At T1 = 0 – 8°C, there is a difference in optimum T2, where the fastest germination times are observed for T2 = 17°C, i.e. T_{90} for T1 = 4°C and 29 days S1 duration, 74 (TE1) and 67 (TE2) days (Table 3.46).

Statistical analyses for increased percentiles for T1 = 14°C (Table 3.45) showed the following significant effects of T2 and S1 duration:

- T_{25}: for both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,178}=28.37$, $p<0.001$, TE2: $F_{1,88}=10.24$, $p=0.002$). For transfer treatments a significant interaction between T2 and S1 duration (TE1: $F_{3,178}=10.91$, $p<0.001$, TE2: $F_{4,88}=2.73$, $p=0.034$).
- T_{50}: in TE1 a significant difference between single temperature and transfer treatments ($F_{1,178}=32.61$, $p<0.001$). For transfer treatments in both experiments a significant interaction between T2 and S1 duration (TE1: $F_{3,178}=17.74$, TE2: $F_{4,88}=5.35$, both $p<0.001$).
- T_{75}: in TE1 a significant difference between single temperature and transfer treatments ($F_{1,176}=13.86$, $p<0.001$). For transfer treatments in TE1 a significant interaction between T2 and S1 duration ($F_{3,176}=5.48$, $p<0.001$) and in TE2 a significant effect of T2 ($F_{2,67}=10.03$, $p<0.001$).
- T_{90}: In TE1 a significant difference between single temperature and transfer treatments ($F_{1,164}=12.27$, $p<0.001$). For transfer treatments in both experiments a significant interaction between T2 and S1 duration (TE1: $F_{3,164}=3.03$, $p=0.031$, TE2: $F_{3,61}=2.61$, $p=0.050$).

L6: T1 = 14℃		T1 duration (days)											
%	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T10	25℃									28	47	84	
	20℃			25	43	62	80			24	42	67	
	17℃			65	59	69	89			61	59	72	
	14℃	82						81					
	11℃												
	8℃												
	4℃												
	0℃												
T25	25℃									35	51	88	
	20℃			27	45	66	89			26	45	70	
	17℃			84	73	75	94			82	66	76	
	14℃	98						93					
	11℃												
	8℃												
	4℃												
	0℃												
T50	25℃									160	58	95	
	20℃			31	48	72	93			33	48	75	
	17℃			105	88	82	99			113	81	83	
	14℃	110						107					
	11℃												
	8℃												
	4℃												
	0℃												
T75	25℃										87	111	
	20℃			49	53	76	97			67	52	79	
	17℃			120	107	94	105			134	102	93	
	14℃	123						117					
	11℃												
	8℃												
	4℃												
	0℃												
T90	25℃										153	130	
	20℃			77	58	79	103			69	55	82	
	17℃			132	126	111	111			146	115	107	
	14℃	140						134					
	11℃												
	8℃												
	4℃												
	0℃												

Table 3.45 ANOVA table of means for T1 = 14°C times to germination to 10%, 25%, 50%, 75% and 90% of the population (T_{10}, T_{25}, T_{50}, T_{75}, T_{90}) in TE1 and TE2 for isolate L6 for combination of treatments with different T2 and S1 duration; Colour gradient from longest (green) to shortest (red) germination time for each percentile individually; Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{10} – d.f. = 178, s.e.d. = 6.242, l.s.d. = 12.318, T_{25} – d.f. = 178, s.e.d. = 7.230, l.s.d. = 14.268, T_{50} – d.f. = 178, s.e.d. = 7.522, l.s.d. = 14.843, T_{75} – d.f. = 176, s.e.d. = 12.756, l.s.d. = 25.175, T_{90} – d.f. = 164, s.e.d. = 15.14, l.s.d. = 29.89; In TE2: T_{10} – d.f. = 90, s.e.d. = 16.38, l.s.d = 32.55, T_{25} – d.f. = 88, s.e.d. = 13.84, l.s.d. = 27.51, T_{50} – d.f. = 80, s.e.d. = 20.45, l.s.d. = 40.70, T_{75} – d.f. = 67, s.e.d. = 18.65, l.s.d. = 37.23, T_{90} – d.f. = 61, s.e.d. = 16.25, l.s.d. = 32.49.

Statistical analyses for increased percentiles for T1 = 4°C (Table 3.46) showed the following significant effects of T2 and S1 duration:

- T_{25}: for both experiments significant difference between single temperature and transfer treatments (TE1: $F_{1,178}$=120.68, TE2: $F_{1,88}$=11.07, both p<0.001). For transfer treatments in TE1 a significant interaction between T2 and S1 duration ($F_{12,178}$=2.21, p=0.013), and in TE2 a significant effect of T2 ($F_{4,88}$=48.20, p<0.001).
- T_{50}: for both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,178}$=137.60, p<0.001, TE2: $F_{1,88}$=4.09, p=0.047). For transfer treatments in TE1 a significant interaction between T2 and S1 duration ($F_{12,178}$=2.44, p=0.006) and in TE2 a significant effect of T2 ($F_{4,88}$=79.77, p<0.001).

- T_{75}: for both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,176}$=75.25, TE2: $F_{1,67}$=17.57, both p<0.001). For transfer treatments a significant effect of T2 (TE1: $F_{4,176}$=18.64, TE2: $F_{1,67}$=18.55, both p<0.001) and in TE1 a significant effect of S1 duration ($F_{3,176}$=3.68, p=0.013).
- T_{90}: for both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,164}$=86.90, TE2: $F_{1,61}$=37.65, both p<0.001). For transfer treatments in TE1 a significant interaction between T2 and S1 duration ($F_{12,164}$=2.60, p=0.003) and in TE2 a significant effect of S1 duration ($F_{2,61}$=11.31, p<0.001).

L6: T1 = 4℃		T1 duration (days)											
%	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T10	25℃									109	108	123	
	20℃		25	27	39	62				48	42	63	
	17℃		49	43	45	64				41	45	66	
	14℃		72	60	58	74				60	56	72	
	11℃		71	75	75	81				59	58	74	
	8℃		90	82	85	100							
	4℃	115						108					
	0℃												
T25	25℃										151	154	
	20℃		39	31	41	65				73	49	68	
	17℃		73	56	50	68				54	51	70	
	14℃		89	71	65	80				72	64	75	
	11℃		84	82	82	88				69	70	80	
	8℃		100	99	104	115							
	4℃	132						117					
	0℃												
T50	25℃											247	
	20℃		53	47	50	72				93	74	77	
	17℃		96	70	58	74				73	57	77	
	14℃		99	84	74	88				85	71	82	
	11℃		94	91	90	97				80	79	89	
	8℃		116	113	118	125							
	4℃	149						142					
	0℃												
T75	25℃												
	20℃		86	70	76	102						154	
	17℃		107	85	66	83				96	62	82	
	14℃		113	98	87	95				99	77	89	
	11℃		106	103	100	104				91	87	94	
	8℃		135	125	131	136							
	4℃	181						160					
	0℃												
T90	25℃												
	20℃		213	169	111	128							
	17℃		121	102	74	91				115	67	85	
	14℃		132	111	94	100				123	83	94	
	11℃		119	119	108	110				116	104	100	
	8℃		149	138	138	146							
	4℃	226						171					
	0℃												

Table 3.46 ANOVA table of means for T1 = 4°C times to germination to 10%, 25%, 50%, 75% and 90% of the population (T_{10}, T_{25}, T_{50}, T_{75}, T_{90}) in TE1 and TE2 for isolate L6 for combination of treatments with different T2 and S1 duration; Colour gradient from longest (green) to shortest (red) germination time for each percentile individually; Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{10} – d.f. = 178, s.e.d. = 6.242, l.s.d. = 12.318, T_{25} – d.f. = 178, s.e.d. = 7.230, l.s.d. = 14.268, T_{50} – d.f. = 178, s.e.d. = 7.522, l.s.d. = 14.843, T_{75} – d.f. = 176, s.e.d. = 12.756, l.s.d. = 25.175, T_{90} – d.f. = 164, s.e.d. = 15.14, l.s.d. = 29.89; In TE2: T_{10} – d.f. = 90, s.e.d. = 16.38, l.s.d = 32.55, T_{25} – d.f. = 88, s.e.d. = 13.84, l.s.d. = 27.51, T_{50} – d.f. = 80, s.e.d. = 20.45, l.s.d. = 40.70, T_{75} – d.f. = 67, s.e.d. = 18.65, l.s.d. = 37.23, T_{90} – d.f. = 61, s.e.d. = 16.25, l.s.d. = 32.49.

Summary and isolate comparison - The effects of final high temperature and S1 duration on time to germination (Hypothesis 3.)

Fastest germination times (T_{10}) for isolates L5 and L6 were already described in Chapter 3.3.3.2.

For various initial temperature (T1), there was a distinct variation between the isolates in respect of the effects of high final temperature (T2) on T_{10}, where both isolates have different T2 optimum, T2 = 11-14°C for isolate L5 (Table 3.47) and T2 = 20°C for isolate L6 (Table 3.48). In regards to S1 duration for both isolates, when optimum T2 and T1<T2 applied, 7 days of S1 duration resulted in the fastest germination times. Generally, germination time increased with longer S1, except for isolate L5 when T2 was above optimum and for isolate L6 when temperature was below optimum, and the effect was more prevailing for isolate L5 along with an interaction with limited germination. S1 duration of 56 days was for both isolates unnecessary long for T1< optimum T2 and for T1 ~ optimum T2, resulted in T_{10} shorter than S1 duration, where the germination at S1 was more common for isolate L5.

With respect to germination times for the increasing percentiles the described effects for T_{10} become more distinct and were best observed near the optimum T2.

For isolate L5 for T_{90} the optimum T2, 11°C and 14°C showed a difference in response (Table 3.47). For T2 = 11°C, still 7 days S1 duration along with lower T1 (0-4°C) showed fastest germination times, where with higher T1 longer S1 was preferred (29 days at T1 = 8°C). For T2 = 14°C, at least 14-29 days was required at T1 (T1<T2) for fast germination times. Generally, a shift towards longer S1 durations was observed.

For isolate L6 also a shift in response for germination times for the increased percentiles was observed, mainly a shift in S1 duration towards 29 days. The shortest times to germination were consistently observed for T2 = 20°C, however interesting development for T_{90} was observed for lower T1, where at T1 = 0-8°C the optimum T2 moved towards 17°C (Table 3.48).

L5	Time to germination (all %)		
T1	Single temperature (T = T1)	The effects of final high temperature (T2) and S1 duration (T1<T2)	
	T_{10} (days)		T_{10} range (days)
0°C	N/A	**T_{10}:** fastest for T2=11°C, 7 days at S1, Optimum: T2=11-14°C S1 duration 7-14 days at S1, T2<11°C longer T_{10}, further delay with longer S1 duration, T2>14°C longer T_{10}, shortest after 29 days at S1, Generally, delay in T_{10} for 56 days S1 duration, T2>17°C, delayed/limiting germination for <29 days at S1, **Increased %** - more sensitive towards optimum T2 (11°C), and generally a longer S1 duration was required	T_{10} =38* T_{10} =136*
4°C	Sub – optimum T_{10} =98*, 117**	**T_{10}: Significant reduction,** fastest at T2=14°C, 7 days at S1, Optimum: T2=11-14°C S1 duration 7-29 days at S1 T2<11°C longer T_{10}, further delay with longer S1 duration, T2>14°C longer T_{10}, shortest after 14 -29 days at S1, Generally, delay in T_{10} for 56 days S1 duration, T2>17°C, delayed/limiting germination for <29 days at S1, **Increased %** - more sensitive towards optimum T2 (11°C), and generally a longer S1 duration was required	T_{10} =38*, 41** T_{10} =82*, 74**
8°C	Sub - optimum T_{10} =61*	**T_{10}: Reduction,** fastest for T2 = 11°C, 7 days at S1, Optimum: T2=11-14°C S1 duration 7-29 days at S1, T2>14°C longer T_{10}, shortest after 14 -29 days at S1, Generally, delay in T_{10} for 56 days S1 duration, T2>17°C, delayed/limiting germination for <29 days at S1, **Increased %** – more sensitive towards optimum T2 (11°C), and generally a longer S1 duration was required	T_{10} =41* T_{10} =93*
11°C	Optimum T_{10} =45*, 44**	**T_{10}: Significant delay*, no effect**,** fastest for T2=14°C, 56 days at S1, no effect of S1 at T2 = 14°C, For higher T2, delay in germination or limiting germination for 7-14 (29**) days S1 duration, **Increased %** - limited germination for T2 = 20 and 25°C, higher T2 and duration <56 days delay germination for T_{90}	T_{10} =39*, 41** T_{10} =102*, 62**
14°C	Supra - optimum T_{10} =52*, 46**	**T_{10}: Significant delay = 7 days S1 duration and T1 = 4°C** **Increased %** - limited germination	T_{10} =58*, 42** T_{10} =162*, 97**
17°C	Supra - optimum T_{10} =89*, 63**	**T_{10}: Fastest = 14 days S1 duration and T1 = 4°C** **Increased %** - limited germination	T_{10} =108*, 59** T_{10} =138*, 66**
20°C	N/A	No germination	N/A
25°C	N/A	N/A	N/A

Table 3.47 Summary of the effects of the initial low temperature and S1 duration for the single temperature and transfer treatments on time to germination of *S. sclerotiorum* isolate L56; * TE1, ** TE2.

L6	Time to germination (all %)		
T1	Single temperature (T = T1)	The effect of final high temperature (T2) and S1 duration (T1<T2)	
	T_{10} (days)		T_{10} range (days)
0°C	N/A	**T_{10}:** fastest for T2 = 20°C and 14 days at S1, delay with lower T2 and longer S1 duration, **Increased %** - fastest at T2 = 17°C, 29 days at S1	T_{10} =42* - T_{10} =137*
4°C	Sub – optimum T_{10} =115*, 108**	**T_{10}: Significant reduction**, fastest for T2 = 20°C and 7 days at S1, delay with lower T2 and longer S1 duration, **Increased %** - fastest at T2 = 17°C, 29 days at S1	T_{10} =25*, 41** - T_{10} =100*, 123
8°C	Sub – optimum T_{10} =88*	**T_{10}: Significant reduction**, fastest for T2 = 20°C and 7 days at S1, delay with lower T2 and longer S1 duration, **Increased %** - fastest at T2 = 17°C, 29 days at S1	T_{10} =17*- T_{10} =78*
11°C	Optimum T_{10} =73*, 72**	**T_{10}: Significant reduction**, fastest for T2 = 20°C and 7 days at S1, delay with lower T2 and longer S1 duration, **Increased %** - consistent with trend observed for T_{10}, preferred S1 duration shifted towards 29 days	T_{10} =13*, 25** - T_{10} =79*, 84**
14°C	Supra – optimum T_{10} =82*, 81**	**T_{10}: Significant reduction**, fastest for T2 = 20°C and 14 days at S1, delay with lower T2 and longer S1 duration, **Increased %** - consistent with trend observed for T_{10}, preferred S1 duration shifted towards 29 days	T_{10} =25*, 24** - T_{10} =89*, 84**
17°C	Optimum (supra) T_{10} =57*, 74**	**T_{10}: Significant reduction**, fastest for T2 = 20°C and 7 days at S1, delay with lower T2 and longer S1 duration, **Increased %** - consistent with trend observed for T_{10}, preferred S1 duration shifted towards 29 days	T_{10} =20*, 21** - T_{10} =54*, 85**
20°C	Optimum (supra) T_{10} =40*, 169**	Reduction in germination	N/A
25°C	N/A	N/A	N/A

Table 3.48 Summary of the effects of the initial low temperature and S1 duration for the single temperature and transfer treatments on time to germination of *S. sclerotiorum* isolate L6; * TE1, ** TE2.

3.3.4.3 Variation in germination times – uniformity

Isolate L5

T_{90}-T_{10} - interdecile range

The T1 approach analysis for the differences in germination time between 90% and 10% germination (interdecile range) for the isolate L5 showed a significant effect of the main factor T1 in both experiments (TE1: $F_{5,115}$=23.88 and TE2: $F_{3,30}$=82.18, both $p<0.001$), and for each T1 the following significant effects of T2 and S1 duration were observed:

- Inside T1 = 0°C, (TE1) for transfer treatments a significant interaction between T2 and duration of S1 ($F_{12,115}$=2.88, p=0.002).
- Inside T1 = 4°C, in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,115}$=39.99, TE2: $F_{1,30}$=258.84, both $p<0.001$) and in TE1 for the transfer treatments a significant interaction between T2 and duration of S1 ($F_{9,115}$=3.09, p=0.002).

- Inside T1 = 8°C, (TE1) a significant interaction between T2 and S1 duration ($F_{6,115}$=5.29, p<0.001).
- Inside T1 = 11°C, for both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,115}$=8.03, p=0.005, TE2: $F_{1,30}$=17.17, p<0.001). For transfer treatments a significant interaction between T2 and S1 duration in TE2 (TE2: $F_{2,30}$=8.32, p<0.001) and significant effects of T2 ($F_{2,115}$=6.64, p=0.002) and S1 duration ($F_{3,115}$=6.94, p<0.001).
- Inside T1 = 14°C, in TE1 a significant effect of S1 duration (TE1: $F_{2,115}$=3.86, p=0.024, TE2: $F_{1,30}$=2.71, p<0.001),
- Furthermore, in both experiments a limited number of treatments reached germination of 90% in T1≥14°C.

For isolate L5, the differences in germination times between 90% and 10% germination (IDR) for each initial temperature (T1) changed with the final higher temperature (T2), subsequently transferred to, and with the duration of the initial temperature period (S1 duration) (Table 3.49). The observed responses could be divided based on the initial T1.

For T1 = 11 - 14°C, the IDR observed for transfer treatments was larger (significantly for T1 = 11°C), compared to single temperature treatments (T = T1) and the IDR was reduced for lower T2 and with longer S1 duration.

For T1 = 0 - 8°C generally a reduction (T1 = 4°C significant) in IDR was observed for transfer treatments compared to single temperature treatments. For the transfer treatments the T2 = 11°C showed fastest germination times across all S1 durations and these were further reduced with lower T1, i.e. 39 days after 7 days at T1 = 0°C (Table 3.49). For T2 > 11°C at least 14-29 days S1 duration were required for a fast and uniform germination and with increasing T2 longer S1 was required. With T2 < 11°C IDR increased, rapidly with lower T2 (4°C), where longer S1 duration improved uniformity.

The shortest IDRs, and therefore most uniform germination, were observed for T2 = 17°C and after 56 days at T1 = 0 - 8°C, 20 (0°C), 25 (TE1), 27 (TE2) (4°C), 20 (8°C) days (Table 3.49).

For T1 ≥ 17°C limited germination was observed and therefore the IDR could not be assessed.

T_{90} - T_{10}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃												
	20℃												
	17℃	95						73					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃												
	20℃						81						
	17℃				110	90	77				102	90	
	14℃	75						85					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃												
	20℃					66						42	
	17℃		122	103	99	63				106	123	38	
	14℃		78	73	71	53				89	64	49	
	11℃	51						40					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃					22							
	17℃		97	106	129	20							
	14℃		80	66	27	26							
	11℃		54	50	45	30							
	8℃	51											
	4℃												
	0℃												
4℃	25℃												
	20℃					40							
	17℃		96	118	67	25					36	27	
	14℃		71	67	26	27				36	34	30	
	11℃		40	37	33	33				38	35	33	
	8℃		60	47	59	51							
	4℃	128						177					
	0℃												
0℃	25℃												
	20℃												
	17℃		117	115	45	20							
	14℃		66	62	21	21							
	11℃		39	36	25	27							
	8℃		51	54	52	50							
	4℃		122	122	107	89							
	0℃												

Table 3.49 T1 approach ANOVA table of means for difference in times to germination for IDR – T_{90}-T_{10} in TE1 and TE2 for isolate L5 for combination of treatments with different T2 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: d.f. = 115, s.e.d. = 16.08, l.s.d. = 31.86; TE2: d.f. = 30, s.e.d. = 12.20, l.s.d. = 24.92.

Early percentile range, T_{25}-T_{10} and late percentile range, T_{90}-T_{75}

The pattern described for IDR was consistent with observations for EPR (early percentile range, T_{25}-T_{10}) and LPR (late percentile range, T_{90}-T_{75}), where the

observed effects were generally larger (more significant) for the LPR. This is predominantly caused by greater sensitivity towards T2 for LPR (similar to IDR), where for the T2 further from optimum (11°C) the LPR increased (significantly) and this resulted in left skewed distribution of germination times (EPR < LPR). This effect was reduced by longer S1 durations for T2 > 11°C, higher T2 required longer S1, where the LPR was reduced to similar values observed for EPR and the distribution of germination times become more uniform, and was not affected by longer S1 durations for T2 $\leq$ 11°C where the distribution continued to show skewness to left (i.e. example of T1 = 4°C, Table 3.50).

Statistical analyses for EPR and LPR for T1 = 4°C showed the following significant effects of T2 and S1 duration:

- EPR: no significant effects,
- LPR: in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,115}$=34.58, TE2: $F_{1,30}$=73.86, both p<0.001) and for the transfer treatments in TE1 a significant effect of T2 ($F_{1,115}$=34.58, p=0.003).

This trend is similar for further temperatures and full tables with ANOVA estimated means (T1 approach) for the various differences in germination times for isolate L5 are shown in the appendices: T_{25}-T_{10}, early percentile range (EPR) (Appendix 23), T_{90}-T_{75} - late percentile range (LPR) (Appendix 24).

L5: T1 = 4℃		T1 duration (days)											
diff	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T_{25}-T_{10}	25℃											10	
	20℃				12	4					40	6	
	17℃		15	16	5	3				38	7	7	
	14℃		10	5	4	4				5	5	4	
	11℃		10	10	9	6				4	3	4	
	8℃		7	7	7	6							
	4℃	12						19					
	0℃												
T_{90}-T_{75}	25℃												
	20℃					16							
	17℃		31	42	31	8					15	6	
	14℃		19	17	6	8				12	12	9	
	11℃		10	8	9	11				18	16	10	
	8℃		24	21	28	20							
	4℃	65						81					
	0℃												
T_{90}-T_{10}	25℃												
	20℃					40							
	17℃		96	118	67	25					36	27	
	14℃		71	67	26	27				36	34	30	
	11℃		40	37	33	33				38	35	33	
	8℃		60	47	59	51							
	4℃	128						177					
	0℃												

Table 3.50 ANOVA table of means for T1 = 4°C for difference in times to germination for EPR - T_{25}-T_{10}, LPR - T_{90}-T_{75}, IDR - T_{90}-T_{10}, in TE1 and TE2 for isolate L5 for combination of treatments with different T2 and S1 duration; Colour gradient from large (blue) to small (red) difference statistics separately; Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{25}-T_{10} – d.f. = 143, s.e.d. = 6.343, l.s.d. = 12.539, T_{90}-T_{75} – d.f. = 115, s.e.d. = 10.656, l.s.d. = 21.108, T_{90}-T_{10} – d.f. = 115, s.e.d. = 16.08, l.s.d. = 31.86; TE2: T_{25}-T_{10} – d.f. = 51, s.e.d. = 16.24, l.s.d. = 32.60 , T_{90}-T_{75} – d.f. = 30, s.e.d. = 10.93, l.s.d. = 22.33, T_{90}-T_{10} – d.f. = 30, s.e.d. = 12.20, l.s.d. = 24.92.

Isolate L6

T_{90}-T_{10} - interdecile range

The T1 approach analysis for the differences in germination time between 90% and 10% germination (interdecile range) for the isolate L6 showed a significant effect of the main factor T1 in both experiments (TE1: $F_{5,164}$=60.08, p<0.001 and TE2: $F_{3,61}$=4.23, both p=0.009), and for each T1 the following significant effects of T2 and S1 duration were observed:

- Inside T1 = 0°C, (TE1) for transfer treatments a significant interaction between T2 and duration of S1 ($F_{12,164}$=2.93, p=0.001).
- Inside T1 = 4°C, in both experiments a significant difference between single temperature and transfer treatments (TE1: $F_{1,164}$=19.32, p<0.001, TE2: $F_{1,61}$=4.82, p=0.032) and in TE1 for the transfer treatments a significant interaction between T2 and duration of S1 ($F_{12,164}$=3.38, p<0.001). In TE2 for the transfer treatments a significant effect of S1 duration ($F_{1,61}$=21.70, p<0.001)
- Inside T1 = 8°C, (TE1) significant effects of T2 ($F_{3,164}$=3.07, p=0.029) and S1 duration ($F_{3,164}$=11.39, p<0.001).

- Inside T1 = 11°C, In TE1 a significant difference between single temperature and transfer treatments ($F_{1,61}$=4.80, p=0.032). In both experiments for transfer treatments a significant interaction between T2 and S1 duration (TE1: $F_{6,164}$=2.49, p=0.025, TE2: $F_{4,61}$=5.78, p<0.001).
- Inside T1 = 14°C, in TE2 a significant interaction between T2 and S1 duration ($F_{1,61}$=3.27, p=0.027). In TE1 for transfer treatments a significant effect of T2 ($F_{1,164}$=7.42, p=0.007) and S1 duration ($F_{3,164}$=4.09, p=0.008).
- Inside T1 = 17°C, in TE2 only, a significant interaction between T2 and S1 duration ($F_{1,61}$=47.43, p<0.001). In both experiments for transfer treatments a significant effect of S1 duration (TE1: $F_{4,164}$=11.95, p<0.001, TE2: $F_{1,61}$=7.50, p=0.008)
- For T1 = 20°C, (TE2) germination under 90%.

The differences in germination times between 90% and 10% germination (IDR) for isolate L6, showed a differential response to high final temperature (T2), subsequently transferred to, and quite uniform response to S1 duration for the range of initial temperatures (T1) (Table 3.51). The observed response could be divided based on the initial T1.

For the T1 ≥ 11°C, the germination uniformity was best for T2 = 20°C and with increasing T1 and T2, a shorter S1 duration was required, 29 days. Overall the shortest IDRs, and therefore most uniform germination, were observed for T2 = 20°C and after 29 days at T1 = 14°C, 15 (TE1), 13 (TE2) days (Table 3.51).

For T1≤ 8°C generally a reduction (T1 = 4°C significant) in IDR was observed for transfer treatments compared to the single temperature treatments (T = T1). For all T1 longer S1 durations, 29 - 56 days, and T2 = 11 - 17°C, resulted in significant reductions in IDR, and therefore the most uniform germination, IDR ranging from 19 to 40 days (Table 3.51). For the transfer treatments for T1 = 0°C the T2 = 11°C showed fastest germination times for S1 duration 7 - 29 days, i.e. 39 days after 7 days at T1 = 0°C (Table 3.51) and for higher T1 this remained consistent for S1 durations of 7 and 14 days. For T2 ≤ 8°C an increase in the IDR was observed and this was more rapid for lower T2. Furthermore, for T2 = 4°C the IDR increased with longer S1 duration leading to a wider distribution of germination times. For combination of T1 = 4°C and T2 = 20°C, the overall longest IDR was observed, 185 days (S1 duration of 7 days) and a longer S1 duration improved the germination uniformity, to 66 days (S1 duration 56 days) (Table 3.51).

T_{90} - T_{10}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃										18	64	
	20℃			116	22	30	58				20	32	
	17℃	91						112					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃										106	46	
	20℃			53	15	18	23			40	13	16	
	17℃			68	66	42	22			86	56	35	
	14℃	57						53					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃										99	24	
	20℃		113	51	32	14					23	16	
	17℃		87	63	39	24				61	22	16	
	14℃		60	51	49	40				58	49	57	
	11℃	43						70					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃			103	45	26							
	17℃		75	55	25	21							
	14℃		55	53	37	27							
	11℃		50	50	40	35							
	8℃	46											
	4℃												
	0℃												
4℃	25℃												
	20℃		185	142	73	66							
	17℃		72	58	29	26				74	22	19	
	14℃		60	51	36	26				62	27	22	
	11℃		47	43	33	28				57	46	26	
	8℃		58	56	53	46							
	4℃	111						64					
	0℃												
0℃	25℃												
	20℃				145								
	17℃		74	76	27	25							
	14℃		55	53	34	26							
	11℃		39	36	25	27							
	8℃		47	47	50	43							
	4℃		97	88	135	134							
	0℃												

Table 3.51 T1 approach ANOVA table of means for difference in times to germination for IDR – T_{90}-T_{10} in TE1 and TE2 for isolate L6 for combination of treatments with different T2 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: d.f. = 164, s.e.d. = 16.24, l.s.d. = 32.06; TE2: d.f. = 61, s.e.d. = 14.90, l.s.d. = 29.79.

Early percentile range, T_{25}-T_{10} and late percentile range, T_{90}-T_{75}

The pattern described for IDR was consistent with observations for EPR (early percentile range, T_{25}-T_{10}) and LPR (late percentile range, T_{90}-T_{75}), where the

observed effects were generally larger (more significant) for the LPR for T2 = 20C. Occasionally, larger LPR compared to EPR resulted in left skewed distributions of germination times. This effect was reduced by longer S1 durations, where the LPR was reduced to similar values observed for EPR and the distribution of germination times became more uniform, (i.e. example of T1 = 4°C, Table 3.52).

Statistical analyses for EPR and LPR for T1 = 4°C showed the following significant effects of T2 and S1 duration:

- EPR: In TE1 a significant difference between single temperature and transfer treatments ($F_{1,178}$=4.25, p=0.041) For transfer treatments in TE1 a significant interaction between T2 and S1 duration ($F_{12,178}$=2.23, p=0.012) and in TE2 significant effects of T2 ($F_{4,88}$=16.13, p<0.001) and S1 duration ($F_{2,88}$=4.35, p=0.016).
- LPR: In TE1 a significant difference between single temperature and transfer treatments ($F_{1,164}$=4.35, p=0.039). For transfer treatments in TE1 a significant interaction between T2 and S1 duration ($F_{12,164}$=3.74, p<0.001) and in TE2 a significant effect of S1 duration ($F_{2,61}$=8.27, p<0.001).

L6: T1 = 4℃		T1 duration (days)											
		TE1						TE2					
diff	T2	0	7	14	29	56	84	0	7	14	29	56	84
T_{25}-T_{10}	25℃										43	31	
	20℃		14	4	3	4				25	7	5	
	17℃		24	12	5	4				13	6	4	
	14℃		17	11	7	7				12	8	4	
	11℃		12	7	7	7				10	12	7	
	8℃		9	17	18	14							
	4℃	17						9					
	0℃												
T_{90}-T_{75}	25℃												
	20℃		129	99	35	32							
	17℃		15	16	7	8				19	4	3	
	14℃		19	13	8	6				23	5	6	
	11℃		13	16	8	6				25	17	6	
	8℃		14	13	7	10							
	4℃	45						12					
	0℃												
T_{90}-T_{10}	25℃												
	20℃		185	142	73	66							
	17℃		72	58	29	26				74	22	19	
	14℃		60	51	36	26				62	27	22	
	11℃		47	43	33	28				57	46	26	
	8℃		58	56	53	46							
	4℃	111						64					
	0℃												

Table 3.52 ANOVA table of means for T1 = 4°C for difference in times to germination for EPR - T_{25}-T_{10}, LPR - T_{90}-T_{75}, IDR - T_{90}-T_{10}, in TE1 and TE2 for isolate L6 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red) difference statistics separately; Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{25}-T_{10} – d.f. = 178, s.e.d. = 4.567, l.s.d. = 9.013, T_{90}-T_{75} – d.f. = 164, s.e.d. = 14.34, l.s.d. = 28.32, T_{90}-T_{10} – d.f. = 164, s.e.d. = 16.24, l.s.d. = 32.06; TE2: T_{25}-T_{10} – d.f. = 88, s.e.d. = 8.391, l.s.d. = 16.675, T_{90}-T_{75} – d.f. = 61, s.e.d. = 10.62, l.s.d. = 20.120, T_{90}-T_{10} – d.f. = 61, s.e.d. = 14.90, l.s.d. = 29.79.

These trends were similar for further temperatures and full tables with ANOVA estimated means (T1 approach) for the various differences in germination times for

isolate L6 are shown in the appendices: T_{25}-T_{10}, early percentile range (EPR) (Appendix 25), T_{90}-T_{75} - late percentile range (LPR) (Appendix 26).

Summary and isolate comparison - The effects of final high temperature and S1 duration on time to germination (Hypothesis 3.)

The differences in germination times between 90% and 10% germination (interdecile range - IDR) were assessed, to inform about uniformity of germination times. For each initial temperature (T1) a change with the higher final temperature (T2), subsequently transferred to, and with the duration of the initial temperature period (S1 duration) was observed for both isolates. A range of T1 was identified where both isolates showed similar response to T2 and S1 duration and where a difference in responses for the isolates was observed.

A generally similar response to T2 and S1 duration was observed for both isolates for T1 = 0-8°C, where longer S1 durations, 29 - 56 days, and T2 = 11 - 17°C resulted in a significant reduction in IDR, and therefore the most uniform germination, except for S1 duration of 29 days and T2 = 17°C for isolate L5 (Table 3.53). Furthermore, for both isolates for the short S1 durations, 7 – 14 days, T2 = 11°C showed consistently lower IDR (compared to other T2) and IDR values for both isolates were almost equal. A difference at these T1 temperature was in response to S1 duration for T2 = 4°C, where for both isolates large IDR was observed, however for isolate L5 the IDR reduced, and for isolate L6 increased, with longer S1 duration.

For the T1 = 11 - 17°C a major difference in response was observed for the isolates. The uniformity of germination significantly reduced for isolate L5 (Table 3.53), where the IDR increased with higher T1 and T2, and an increasing duration of S1 improved uniformity at these temperatures. In contrast for isolate L6 (Table 3.54), the most uniform germination was observed at these T1, where IDR reduced with high T2 for S1 durations of 29 and 56 days and inside these S1 durations with increasing T1 the 29 days at S1 was preferred.

Generally, for T2 $\geq$ 20°C limited germination was observed for isolate L5 and for isolate L6 the short duration of S1 and/or low T1 resulted either in a limited germination or a large IDR.

Regarding the skewness, for both isolates when optimum T1 and T2 temperatures were applied with S1 durations of 29 to 56 days, generally a symmetric distribution of

germination times was observed. For isolate L5 when further from optimum conditions, consistently a left skewed distribution of germination times was observed. This is less apparent for isolate L6, except at high T2.

L5	Differences in germination times - uniformity		
T1	Single temperature (T = T1)	The effects of final high temperature (T2) and S1 duration (T1<T2)	
	T_{90} - T_{10} (days)		T_{90} - T_{10} range (days)
0°C	N/A	**Smallest IDR: 56 days S1 duration and T2 = 14°C,** T2>11°C - significant increase in uniformity after 29-56 days at S1, for higher T2 longer S1 is required, T2=11°C - high uniformity also for 7-14 days at S1, with longer S1 duration advance by ~10 days, T2≤8°C – reduction in uniformity, longer S1 increased uniformity, this was more severe for lower T2 Reduction in germination for T2≤20°C	min =20* max =122*
4°C	Sub - optimum 128*, 177**	**Smallest IDR: 56 days S1 duration and T2 = 17°C,** Significant increase in uniformity compared to control, longer S1 and T2 ~ 11°C increase uniformity (significantly), for higher T2 longer S1 is required, Reduction in germination for T2≤20°C	min =25*, 27** max =118*, 38**
8°C	Optimum 51*	**Smallest IDR: 56 days S1 duration and T2 = 17°C,** No significant difference for transfer and control, longer S1 and lower T2 increase uniformity (significantly), Reduction in germination for T2≤20°C	min =20* - max =129*
11°C	Optimum 51*, 40**	**Smallest IDR: 56 days S1 duration and T1 = 14°C,** Significant reduction in uniformity compared to control, longer S1 (56 days) and lower T2, both significantly increase uniformity, Reduction in germination for T2≤20°C	min =53*, 38** - max =122*, 123**
14°C	Supra - optimum 75*, 85**	**Smallest IDR: 84 days S1 duration and T1 = 17°C,** Reduction in uniformity, where longer S1 (significantly) and lower T2 increase uniformity, Reduction in germination for T2≤20°C	min =77*, 90** - max =110*, 102**
17°C	Supra - optimum 95*, 73**	Reduction in germination	N/A
20°C	N/A	Reduction in germination	N/A
25°C	N/A	Reduction in germination	N/A

Table 3.53 Summary of the effects of the initial low temperature and S1 duration for the single temperature and transfer treatments on differences in germination times T_{90} – T_{10} (interdecile range - IDR) - uniformity of *S. sclerotiorum* isolate L5; * TE1, ** TE2.

L6	Differences in germination times - uniformity		
T1	Single temperature (T = T1)	The effects of final high temperature (T2) and S1 duration (T1<T2)	
	T_{90} - T_{10} (days)		T_{90} - T_{10} range (days)
0°C	N/A	**Smallest IDR: 29 days S1 duration and T2 = 11°C and 56 days S1 duration and T2 = 17°C,** T2>11°C - significant increase in uniformity after 29-56 days at S1, for higher T2 longer S1 is required, T2=11°C - high uniformity also for 7-14 days at S1, with longer S1 duration advance in uniformity by ~10 days, T2≤8°C – reduction in uniformity, and where for T2=4°C longer S1 decreased uniformity (significantly), Reduction in germination for T2≤20°C	min =25* max =145*
4°C	Sub – optimum 111*, 64**	**Smallest IDR: 56 days S1 duration and T1 = 17°C** Significant increase in uniformity compared to control, 29 and 56 days at S1 and T2 = 11-17°C increase uniformity (significantly), T2 = 20°C*, 7 days at S1 extremely high IDR observed, where longer S1 duration improved the uniformity, after 56 days at S1 by 114 days, Reduction in germination for T2≤20°C**	min = 26*, 19** max = 185*, 74**
8°C	Optimum 48*	**Smallest IDR: 56 days S1 duration and T1 = 17°C** longer S1 and T2~17°C increased uniformity (significantly), Reduction in germination for T2=20°C, 7 days at S1	min = 21* max = 103*
11°C	Optimum 43*, 70**	**Smallest IDR: 56 days S1 duration and T1 = 20°C** Significant increase in uniformity compared to control** Significant increase in uniformity for T2 = 20°C and S1 duration of 29 to 56 days, Reduction in germination/uniformity for T2≤20°C and short S1 duration	min = 14*, 16** - max = 113*, 99**
14°C	Optimum 57*, 53**	**Smallest IDR: 29 days S1 duration and T1 = 20°C** Significant increase in uniformity of T1** and S1 duration where T1 = 0-8°C and S1 duration 29 and 56 days increased uniformity	min = 15*, 13** - max = 68*, 106**
17°C	Supra – optimum 91*, 112**	**Smallest IDR: 29 days S1 duration and T1 = 20°C** Significant effect of T1 (TE2) and S1 duration, where T1 = 0-11(14)°C and S1 duration 29 and 56 (84) days increased uniformity Reduction in germination/uniformity for T2≤20°C and short S1 duration	min = 22*, 18** - max = 116*, 64**
20°C	N/A	Reduction in germination	N/A
25°C	N/A	Reduction in germination	N/A

Table 3.54 Summary of the effects of the initial low temperature and S1 duration for the single temperature and transfer treatments differences in germination times T_{90} – T_{10} (interdecile range - IDR) - uniformity of *S. sclerotiorum* isolate L6; * TE1, ** TE2.

3.3.5 S3 germination

3.3.5.1 Level of germination in S3

A number of treatments showed low germination during the S1 + S2. However most of the germination could be observed after more favourable conditions were re-introduced (T = 11°C) in S3. The S3 temperature of 11°C proved to be a good choice for the viability test where both isolates completed germination to high levels (isolate L5 - Table 3.55, isolate L6 - Table 3.56). Treatments affected were generally the extreme T1 and T2 conditions for both isolates as described earlier in this chapter (3.3.2, 3.3.3.1 and 3.3.4.1), for isolate L5: single temperature 0, 4, 17, 20 and 25°C, and transfer treatments to final T2 = 4, 17, 20 and 25°C. For isolate L6: single temperature 0, 20 and 25°C, and transfer treatments to final T2 = 20 and 25°C.

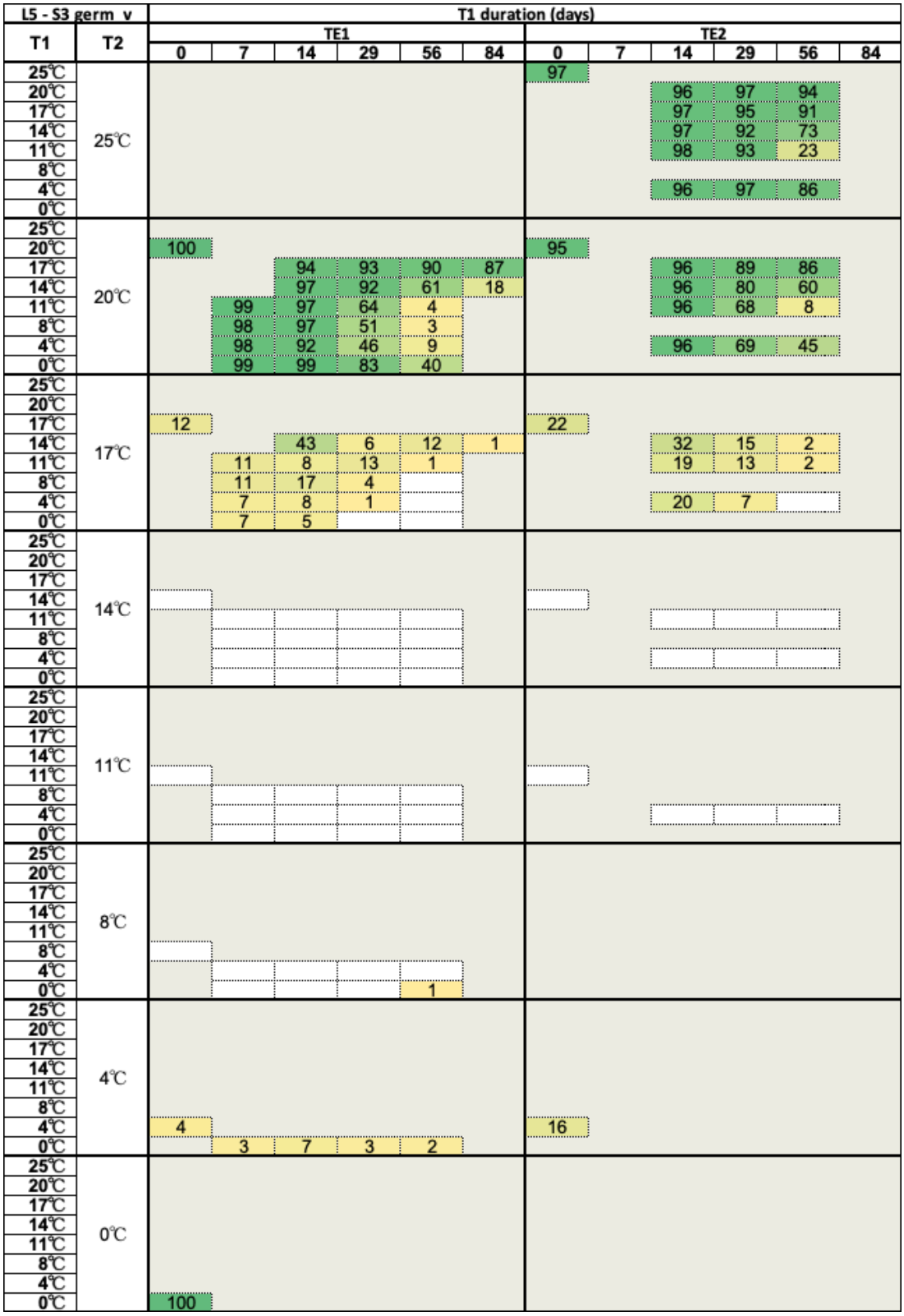

Table 3.55 Proportion (%) of *S. sclerotiorum* sclerotia, isolate L5, germinated in S3 (transferred to 11°C for viability test after 280 days (S1+S2), for additional 150 days) (calculated out of the total viable sclerotia) in TE1 and TE2, average of three replicates. Empty cells represent treatments with 0 germination in S3. Colour gradient represents the increase in germination: yellow – low germination, green – high germination.

L6 - S3 germ v		T1 duration (days)											
		TE1						TE2					
T1	T2	0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							100					
20℃										99	98	98	
17℃										51	5	14	
14℃										42	3	1	
11℃										46	10	12	
8℃													
4℃										87	61	41	
0℃													
25℃	20℃												
20℃		18						86					
17℃				4						22	1		
14℃				3	1					18			
11℃			7	5	3					28	2		
8℃			13	6	1	3							
4℃			11	7	3	7				58	34	21	
0℃			25	21	12	18							
25℃	17℃												
20℃													
17℃								2					
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	14℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	11℃												
20℃													
17℃													
14℃													
11℃								1					
8℃													
4℃													
0℃													
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃			2	1	1								
0℃													
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		1											
0℃			1		1	6							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		100											

Table 3.56 Proportion (%) of *S. sclerotiorum* sclerotia, isolate L6, germinated in S3 (transferred to 11°C for viability test after 280 days (S1+S2), for additional 150 days) (calculated out of the total viable sclerotia) in TE1 and TE2, average of three replicates. Empty cells represent treatments with 0 germination in S3. Colour gradient represents the increase in germination: yellow – low germination, green – high germination.

3.3.5.2 *Time to germination in S3*

The recordings for TE1 were infrequent compared to TE2, as S3 was not initially considered as a possible addition to the temperature experiment, outside of testing for sclerotial viability. However, for most of the ungerminated sclerotia, after transfer to 11°C (S3) germination re-started and cumulative germination curves for this post experimental period were produced similar to S1 and S2 (Figure 3.13).

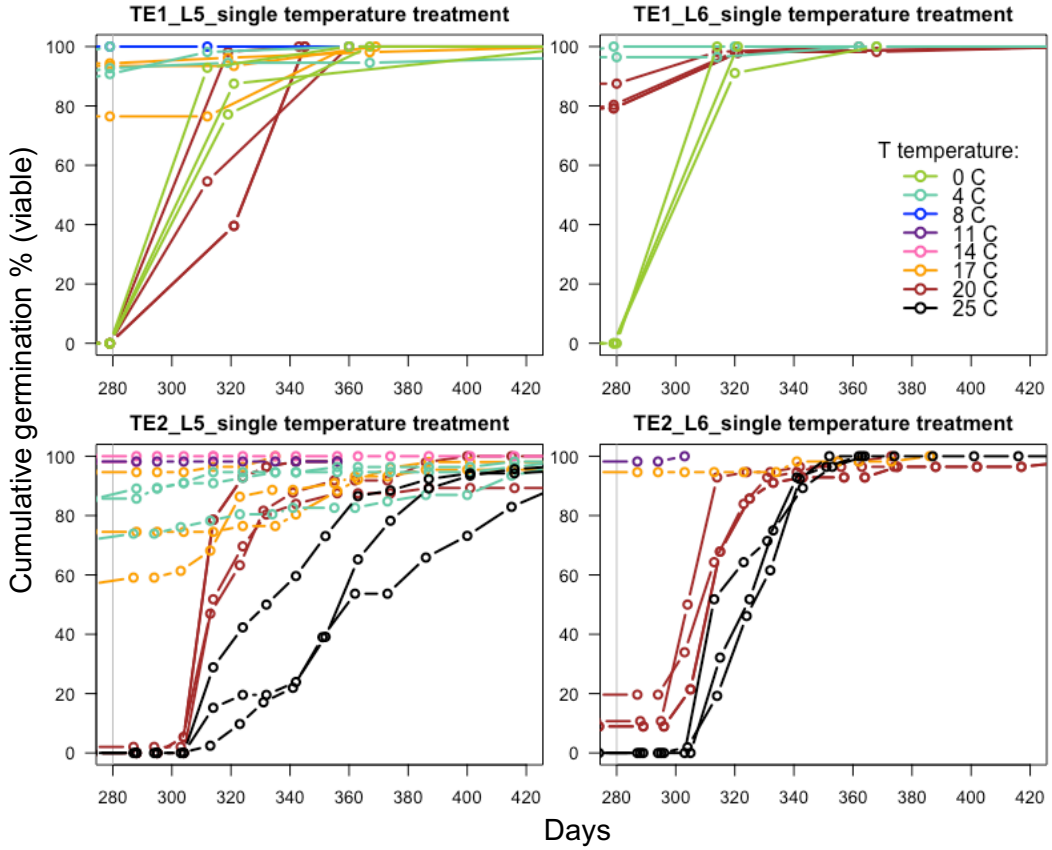

Figure 3.13 Cumulative germination curves of viable sclerotia (including S1 and S2 accumulated germination) for single temperature treatments emphasizing S3 (showing 280 to 420 days).

Mean time to germination for sclerotia germinated in S3 was obtained for both isolates in TE1 and TE2 (L5 - Table 3.57, L6 - Table 3.58), where mean time was calculated from the midpoints of the observation intervals and, where replicates were available, mean across replicates was calculated and SD obtained (L5 - Appendix 27, L6 - Appendix 28).

Germination times to 50% of population (T_{50}) observed for treatments including 11°C in the main part of experiment (S1+S2) (L5 - Appendix 27, L6 - Appendix 28) were considered as a good comparison for germination times observed in S3.

The mean times to germinations were generally longer for isolate L5 (Table 3.57) compared to isolate L6 (Table 3.58), except at lower T2 temperatures.

The T_{50} observed for treatments including 11°C ranged for isolate L5 from 55 to 89 days (Appendix 27) and for isolate L6 from 79 to 98 days (Appendix 28).

For isolate L5 for each T2 temperature the following range of the mean time to germination for each T2 was observed: 0°C – 27 days (TE1); 4°C – 17 to 48 days (TE1), 67 days (TE2); 8°C – 17 days (TE1); 17°C – 20 to 66 days (117 days extreme) (TE1), 37 - 55 days (TE2); 20°C – 32 to 54 days (TE1), 18 - 30 days (TE2); 25°C – 20 to 65 days (TE2) (Table 3.57).

For isolate L6 for each T2 temperature the following range of the mean time to germination for each T2 was observed: 0°C – 21 days (TE1); 4°C – 17 to 58 days (TE1); 8°C – 17 – 44 days (TE1); 11°C – 12 days (TE2); 17°C – 65 days (TE2); 20°C – 19 to 39 days (TE1), 21 - 49 days (TE2); 25°C – 12 to 44 days (TE2) (Table 3.58).

L5 m.t.g_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							65					
20℃										63	60	62	
17℃										59	26	24	
14℃										37	23	20	
11℃										44	24	22	
8℃													
4℃										54	44	35	
0℃													
25℃	20℃												
20℃		32						29					
17℃				42	43	45	43			26	24	24	
14℃				36	35	36	36			24	18	18	
11℃			41	40	38	46				22	19	30	
8℃			49	42	46	54							
4℃			48	45	47	37				27	28	26	
0℃			40	46	52	47							
25℃	17℃												
20℃													
17℃		64						45					
14℃				53	59	39	20			45	44	53	
11℃			51	54	44	117				53	50	55	
8℃			35	50	34								
4℃			34	56	64					48	37		
0℃			66	58									
25℃	14℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	11℃												
20℃													
17℃													
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8℃													
4℃													
0℃							17						
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		48						67					
0℃			18	26	18	17							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		27											

Table 3.57 Mean time to germination observed for *S. sclerotiorum* isolates L5 in TE1 and TE2 for treatments which did not achieve 100% germination at the end of S2 (280 days) and were transferred to 11°C for viability test (S3) for additional 150 days; Mean time was calculated from midpoints of observation intervals and where replicates were available, mean across replicates was calculated and SD obtained (Appendix 27).

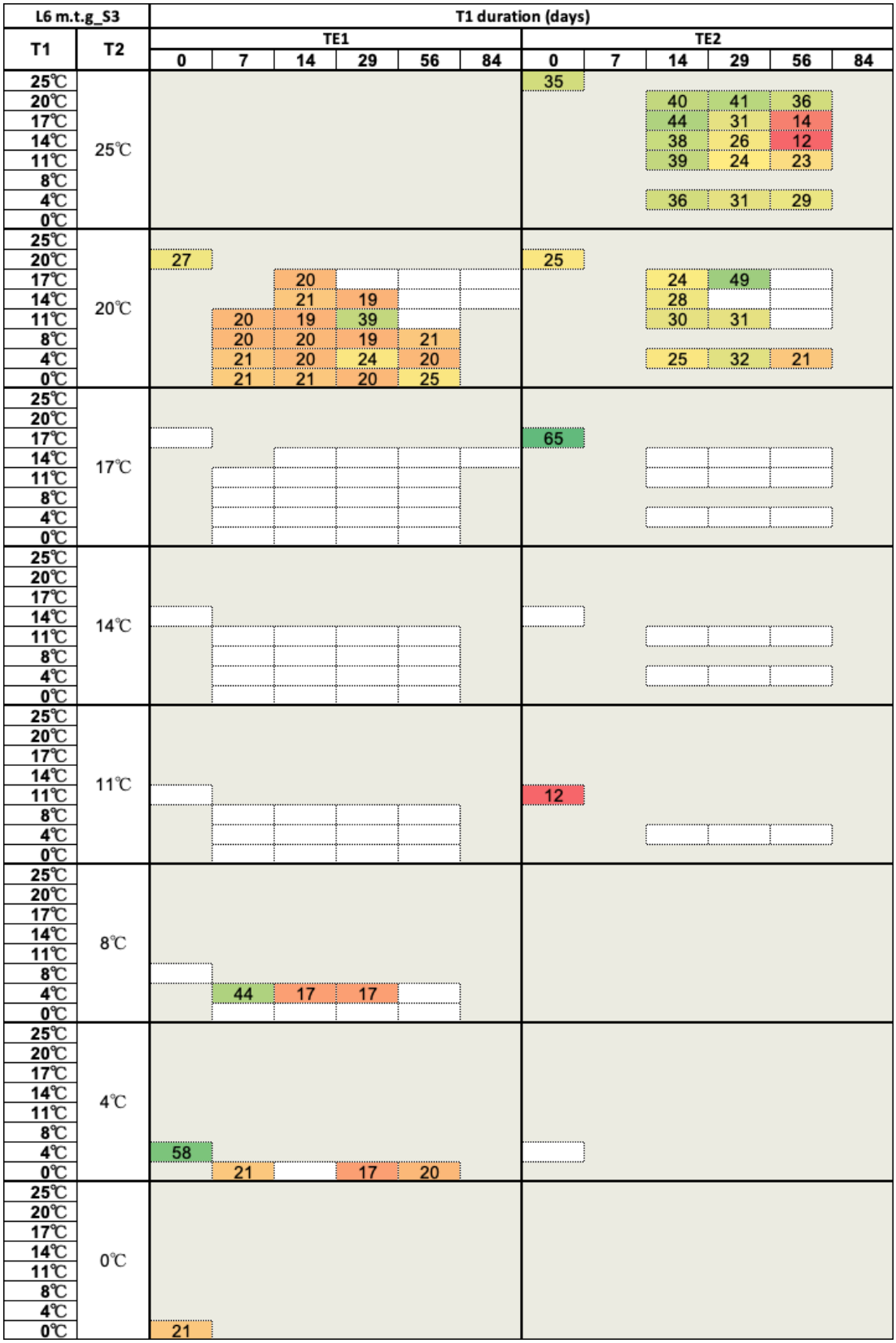

Table 3.58 Mean time to germination observed for *S. sclerotiorum* isolates L6 in TE1 and TE2 for treatments which did not achieve 100% germination at the end of S2 (280 days) and were transferred to 11°C for viability test (S3) for additional 150 days; Mean time was calculated from midpoints of observation intervals and where replicates were available, mean across replicates was calculated and SD obtained (Appendix 28).

3.4 Discussion

Temperature is thought to be one of the main factors affecting germination of *S. sclerotiorum* sclerotia. Several studies from various parts of the world have examined the effect of temperature on germination and identified a number of favourable conditions for both conditioning and germination (Phillips, 1987, Huang and Kozub, 1991, Dillard et al., 1995, Clarkson et al., 2004, Clarkson et al., 2007) or no requirement for conditioning at all (Liu and Paul, 2007, Wu and Subbarao, 2008). Generally, the differences in temperature requirements for carpogenic germination are associated with the geographic origin (Huang and Kozub, 1991, Hao et al., 2003, Uloth et al., 2015), although evidence for these dissimilarities was reported also for isolates originating from the same country (Clarkson et al., 2007, Clarkson et al., 2017). Additionally, there is great variability in the methods used in these studies, making a comparison of the outcomes more challenging. Although numerous researchers have investigated the temperature effects on sclerotia germination, none have carried out such a complex, detailed and comprehensive study as presented in this chapter, with larger sample size and addressing conditioning and germination simultaneously (equally important), was not done previously. This study aimed to investigate the effect of temperature on the processes involved in carpogenic germination for two UK *S. sclerotiorum* isolates (L5 and L6), for which previous studies (DEFRA, 2009) have reported different temperature requirements (Chapter 2.1), with emphasis on the whole population. The following were tested:

- Effect of various single constant temperatures.
- Effect of various constant temperatures with a transfer from an initial low temperature to a subsequent higher temperature; highlighting three different aspects of the two-stage treatments: initial temperature; duration of initial temperature and final temperature.
- Variation in response to temperature for two selected *S. sclerotiorum* isolates with distinctive temperature requirements.

Hypothesis 1 was concerned with whether the germination response of sclerotia changes with constant single temperatures for *S. sclerotiorum* isolates L5 and L6. Although the optimum temperature for the level, speed and uniformity of germination was 11°C for both isolates, there was variation in the response to single temperature treatments.

The optimum single temperature identified for isolate L5 was 11°C where T_{10}= 44-45 days, T_{90} = 84-97 days, IDR = 40-51 days. With temperatures further from the optimum, there was observed a reduction in the level of germination at 4°C and 17°C and no germination at 0°C and >20°C (base and upper ceiling temperature). Furthermore, there was a delay in early and late percentiles (T_{10}, T_{90}) and a spread in the distribution of germination times (increased IDR). The delay in the start of germination (T_{10}) was smaller towards 14°C and the spread of the germination times distribution (IDR) was smaller for 8°C.

The optimum single temperature identified for isolate L6 was 11°C where T_{10}= 72-73 days, T_{90} = 116-142 days, IDR = 43-70 days. With reducing temperature there was no effect on the level of germination, except at 0°C (base temperature) where no germination was observed, a delay in early and late percentile germination times (T_{10}, T_{90}) was observed and the distribution of germination times becomes wider (increased IDR). With increasing temperature, the germination response was variable. The level of germination was reduced at 20°C (especially in TE2) and completely arrested at 25°C (ceiling temperature). With the increasing temperature in TE1 an earlier start of germination (T_{10}) was observed, except for 14°C, a delay in late percentile (T_{90}) and a wider distribution of germination times (IDR). In TE2 there was observed a severe delay in early germination (T_{10}) at 20°C, comparable at 17°C and again delayed at 14°C. The faster germination for the late percentile was observed at 14°C and it was severely delayed at 17°C (T_{90}). The uniformity of germination was best for 14°C and the distribution of germination times was noticeably stretched at 17°C. The variation between TE1 and TE2 was likely due to the cold period which some of the sclerotia (mainly isolate L6) experienced during the initial production. This mainly affected the level of germination and T_{10} observed for isolate L6 at temperature 20°C (Appendix 1).

Overall, we would expect the *S. sclerotiorum* isolates to show a difference in germination pattern in the single temperature regimes as the main criteria for the isolate selection was a variation in temperature response, and this was assumed to be due to different conditioning requirements (not present in the single temperature treatment with high temperatures). At <17°C isolate L5 showed earlier and faster germination, with a wider distribution of germination times compared to isolate L6. At temperatures ≥ 17°C the germination level of isolate L5 was limited and stopped by 20°C in contrast to 25°C for isolate L6.

Generally, the optimum and ceiling temperatures for the single temperature treatments described were similar to temperatures reported by other authors (Sun and Yang, 2000, Hao et al., 2003, Clarkson et al., 2004, Clarkson et al., 2007, Wu and Subbarao, 2008). Furthermore, the level of germination observed was very high, including for temperatures as low as 4°C. This could be due to the length of the experiment (280 days), allowing for enough time to complete germination for temperatures outside the optimum range. Clarkson et al. (2007) reports very long mean germination times of 175, 75 and 83 days for unconditioned sclerotia of *S. sclerotiorum* isolate 13 (at 13, 15 and 18°C, respectively) and 242 days at 15°C for isolate TM, (germination level <50% and mean time to germination could not be derived for 10 and 25°C for isolate 13 and 10,13, 18 and 25°C for isolate TM). Thus the germination levels reported by Clarkson et al. (2007) were considerably lower, the optimum temperature was higher (compared to 11°C observed here) and the germination times were somewhat comparable for isolate 13, but considerably longer for TM (although different statistics used).

Hypothesis 2 was concerned with whether the germination response for each temperature changes with the introduction of an initial lower temperature and with the duration of this initial period while Hypothesis 3, was concerned with whether the germination response for each temperature changes with the final higher temperature, subsequently transferred to, and with the duration of the initial temperature period. Although these two hypotheses were addressed with separate analyses, and so presented separately in the results, the germination response of sclerotia to the combination of two temperature regimes is a combination of these two hypotheses.

Application of a two-stage experimental approach, where sclerotia were initially exposed to an initial lower temperature for various durations, followed by higher temperatures, resulted in an improvement in the level, speed and uniformity of germination compared to the single temperature treatments. Optimum combinations of temperatures in stage 1 and stage 2 of the transfer treatments were identified for both isolates as follows.

For isolate L5 these were T1 = 4-8°C, S1 duration 29 days and T2 = 14°C, where level of germination was 100%, T_{10} = 45 days, T_{90} = 71-72 days, and therefore IDR = T_{90}-T_{10} = 26-27 days. A shorter S1 duration improved the early germination (T_{10}) for these temperatures, but the distribution of germination times becomes wider, the uniformity decreased and this resulted in later germination for the higher percentiles

(T_{90}). On the other hand, a longer S1 duration improved the uniformity of germination, however, the start of germination was delayed (T_{10}). Reduction of T2 = 11°C in combination with the short S1 duration of 7-14 days resulted in similar or slightly improved start of germination (T_{10}), considerably improved late percentile germination and germination uniformity (compared to short S1 durations at optimum T2). With increasing S1 duration a delay in early germination was observed and a similarly improved germination uniformity especially for T1 = 0°C at optimum T2. For treatments with T1 below optimum, 0°C and T2 $\leq$8°C also, a high germination was observed, however, the start of the germination was delayed (T_{10}), even more so with longer S1 duration and lower T2, and the distribution of germination times widened with lower T2, although less so for longer S1 durations. These effects become stronger with temperatures further below the optimum. Treatments with T1 and T2 above optimum, reduced germination level, delayed the beginning of germination and decreased uniformity of germination. Longer S1 duration (56 days) at the optimum T1 improved germination also at supra optimum T2, however <100% germination was achieved and the start of germination (T_{10}) was delayed compared to the optimum conditions (T2).

Isolate L6 showed more complex behaviour, where actually two optima for the combination of T1, S1 duration and T2 were identified. The “summer” optimum, where a high level, earliest, fastest and most uniform germination was observed. The “spring” optimum showed a clear decline (mainly in uniformity of germination), and therefore it could be seen as a reaction towards conditions further from the “summer” optimum, however the response to the changing conditions across the parameter space of the examined factors was driving both optima further from each other (disconnected).

The “summer” optimum conditions were T1 = 14°C, S1 duration 29 days and T2 = 20°C, where the level of germination was ~100%, T_{10} = 42-43 days, T_{90} = 55-58 days, and therefore IDR = T_{90}-T_{10} = 13-15 days. Treatments with T1 close to optimum and shorter S1 duration, noticeably improved the start of germination (T_{10}) for the optimum and supra-optimum T2, however the level of germination was severely reduced (especially in TE2) and the distribution of germination times became extremely wide, resulting in a delayed germination for the later percentiles (T_{90}). This was further intensified for the T1 further from optimum and shortest S1 durations combined (i.e. T1 = 0°C, S1 duration of 7 days). The longer S1 durations for the optimum

temperatures, improved the uniformity of germination, and germination level was 100%, however, the beginning of the germination was delayed (T_{10}).

For the sub-optimum T2, a change in T1 optimum was observed, where this was shifted towards lower T1= 4-8°C forming the "summer" optimum. The "spring" optimum was observed at T1 = 4-8°C, S1 duration 29 days and T2 = 17°C, where level of germination was 100%, T_{10} = 45-47 days, T_{90} = 67-74 days, and therefore IDR = T_{90}-T_{10} = 22-25 days. The lower T2 delayed the start of the germination (T_{10}) for short and long S1 durations and this was intensified by low T2. Furthermore, for the T2 = 8-17°C the uniformity of germination times increased with longer S1, 29 and 56 days, and increased for T2 = 4°C. The germination for late percentile increased when further from "second" optimum and distinctively for T2 = 4°C. 100% germination was observed at the sub-optimum T2 except for 7 days at T1 = 4°C followed by T2 = 8, 99% and T1 = 0°C for 56 days followed by T2 = 4°C, 94%.

Interestingly there was a combination of T1, S1 duration and T2 where both *S. sclerotiorum* isolates showed similarity in their germination response, which can be associated with the "spring" optimum for isolate L6 and these relate to the spring conditions in field. At T1 = 0°C for 29 days followed by T2 = 17°C, the isolates showed a similar start of germination, 48 days (T_{10}), with the distribution of germination times wider for isolate L5, IDR = 45 (L5), 27 (L6) days, resulting in less uniform germination and slightly delayed germination for late percentiles of isolate L5, T_{90} = 94 (L5), 76 (L6) days. Increased T1, had no effect on isolate L6 and T1<4°C significantly reduced the germination uniformity of isolate L5 resulting in delayed germination for late percentiles. However, while increasing the S1 duration to 56 days, delayed the start of germination for both isolates, it improved the uniformity of germination for both isolates (including T1= 0-8°C for isolate L5), and resulted in similar late percentile germination times (T_{90}), 79 – 91 (L5) and 85 – 93 (L6) days.

Additionally, a more specific hypothesis could be answered for each isolate.

- The initial low temperature of 0°C further improves germination compared to other temperatures (e.g. 4 and 8°C). There is not a simple answer to this hypothesis, because there are treatments where we can see some improvement and treatments where not. However, there is not a significant improvement when 0°C is used for conditioning compared to other low temperatures.

  ⇒ The 0°C was assessed as a sub-optimum conditioning temperature because both isolates showed in some combination of T1 and T2 a limiting

effect of 0°C on the level of germination. For example, the germination level was reduced for isolate L6 for T1=0°C S1 duration 56 days followed by T2 = 4°C, 94%, and by T2 = 20°C, 76-88%. In contrast isolate L6 showed predominantly 100% germination including high T1 and T2 combinations, i.e. T1=17°C followed by T2 = 20°C, 77-100%. Similarly, for isolate L5 the 0°C conditioning temperature showed lower germination levels in combination with T2 = 20°C, compared to T1 = 4 – 11°C. I.e. germination level at T2 = 20°C when conditioned 56 days at T1 = 0°C was 59% in contrast to 91-100% (4°C), 97% (8°C), 92-98 (11°C).

⇒ In regards to time to germination and germination uniformity there were treatments combinations for isolate L5, when T1 = 0°C performed similarly or better to 4°C, generally the shortest S1 duration (7 days) followed by 8-11°C, i.e. T_{10} = 61 and 38 days at T1 = 0°C for 7 days followed by 8°C and 11°C, respectively and T_{10} = 64 and 39 days at T1 = 4°C for 7 days followed by 8°C and 11°C, respectively.

⇒ For the germination uniformity there were treatments combinations for isolate L5, when T1 = 0°C performed somewhat better to 4°C, i.e. for T1 = 0°C, T_{90} -T_{10} = 39, 36, 25, and 27 days at T2 = 11°C and T_{90} -T_{10} = 66, 62, 21, and 21 days at T2 = 14°C compared to T1 = 4°C, T_{90} -T_{10} = 40, 37, 33, and 33 days at T2 = 11°C and T_{90} -T_{10} = 71, 67, 26, and 27 days at T2 = 14°C, for S1 duration 7, 14, 29 and 56 days, respectively.

- For the fast conditioning isolate L6, a duration of 7 days for the initial low-temperature treatments of 0 to 11°C is sufficient to promote high, rapid and uniform germination.

  ⇒ The short duration of S1, for both isolates, resulted in an early start of germination for the optimum T1 and T2, however, the uniformity of germination was worse compared to 29 days S1 duration.

It is challenging to compare these results with previous research as there is variation in approach, methods and the final statistics investigated. Furthermore, germination times assessed in this chapter are the times to germination combined across S1 and S2 of freshly produced sclerotia. Some authors (Clarkson et al., 2007) refer to germination times only as times observed in germination temperature (S2), use sclerotia with a standard pre-treatment (incubation/conditioning) (Hao et al., 2003), use sclerotia retrieved from field (Sun and Yang, 2000), or use a logistic function to model the response (Wu and Subbarao, 2008). The various methods and approaches of investigation of sclerotial germination use different assumptions for the

experimental design, data collection and final analyses; i.e. the logistic model approach could not be used because of the cumulative nature of the recording of times to germination. The germination time as a combination of S1 and S2 were more suitable for this work, as for some treatments with T1 temperatures between 8 to 14°C and longer durations of S1 (56 days) germination was observed already in S1, and these observations would be otherwise lost. Furthermore, the aim of this study was to investigate the processes associated with germination and these can proceed or be finished during the initial "conditioning" phase.

A further complication would be adding data from the S3 (viability test) period to the analyses. It was not possible to statistically analyse data obtained from S3 observations because, first of all the S3 was not originally designed as a part of the main temperature experiment, beyond the viability test. Furthermore, the inclusion of an additional stage would further complicate already complex analyses of the treatment's effects. Also, a correct association of observed effects with examined treatments and stages could prove difficult. For both isolates the observed mean times to germination in S3 were considerably shorter than germination times to 50% of population (T_{50}). This suggest that germination was stopped in S2 (unfavourable conditions) and more likely restarted after more favourable conditions were reintroduced, rather than that a new cycle of the germination process was initiated. There is a considerable difference between responses for TE1 and TE2 for both isolates. This is most likely due to the differences in recording intervals during data collection. In TE1 the intervals are much larger, and as the calculation of mean time to germination at the midpoint of recording intervals was taken, this results in great imprecision in the final time to germination. However, this is the best available estimate. Finally, there was information which could be usefully obtained from this approach and these observations were used in the sclerotia germination model development in Chapter 6.

Generally, the results reported here are similar and/ or in the range of temperatures reported by other researchers. Moreover, the previous assumption that a conditioning is required (the initial low temperature) before rapid uniform and high-level germination is reached is confirmed by the observations reported here (Phillips, 1987, Huang and Kozub, 1991, Dillard et al., 1995, Clarkson et al., 2004, Clarkson et al., 2007). Additionally, there are two aspects of the conditioning requirements which should be taken in the account, depending on how we use the information (i.e. we are interested in T_{10}, mean time, level of germination and so on). Shorter durations of

conditioning result in an earlier start of germination, but although longer S1 durations delay the start of germination, the level and the uniformity of germination is improved. This is true for both isolates (L5, L6) although they have different optimum temperatures, in both T1 and T2. The short duration of cold conditioning was initially introduced for isolate L6 and the long S1 durations for isolate L5 (based on an assumption that L6 requires less conditioning prior to germination at high temperatures compared to L5). As already stated the duration of S1 has an impact on the level of germination, especially for isolate L5 (but not exclusively), where the germination in supra-optimum temperatures increased with longer duration of lower temperatures in T1. However, for the S1 duration of 56 days germination regularly started in this stage; therefore, it is more complicated to assess this germination, where it is uncertain whether this is actually only a residual of germination at S1 where the process of germination progressed so far that even unfavourable conditions in S2 did not prevent the stipe production, or whether the conditioning improved the germinability of sclerotia, so that they can produce stipes at the supra-optimum temperatures. A further investigation of the process involved in carpogenic germination of *S. sclerotiorum* sclerotia, with additional tools (i.e. chemical analyses, microscopy, molecular analyses) would be required to expand on the ability to discriminate between conditioning and germination process.

The detailed analysis presented provided a better understanding of the variation between *S. sclerotiorum* isolates, and the sources of variation (Huang and Kozub, 1991, Dillard et al., 1995, Clarkson et al., 2007, Wu and Subbarao, 2008). The general thought is that the variation between isolates is mainly due to the conditioning requirements, where both isolates show preference for various temperatures 9both T1 and T2), isolate L5 prefers lower temperatures in contrast to isolate L6. A more interesting outcome of the analyses is the identification of two optima for isolate L6. This explains an adaptation of *S. sclerotiorum* isolate to enable carpogenic germination more than once a year. Generally, it has been thought that because of a cold conditioning requirement, UK *S. sclerotiorum* isolates only germinate once a year to produce ascospore inoculum (in spring). The result from L6 suggests that sclerotia produced in spring and summer can germinate without cold temperature winter conditioning and therefore initiate further cycles of infection within a single year. Cold conditions trigger response associated with “spring” optimum (lower T1 and T2), while absence of low temperature, and generally higher temperatures allow for response which is defined by the “summer” optimum (higher T1 and T2).

While producing sclerotia of *S. sclerotiorum* isolates L5 and L6, a difference in the size and in the size distribution of produced sclerotia was observed. The isolate L5 produced in average larger sclerotia compared to isolate L6, furthermore isolate L5 produced significantly less of <2.00 mm and significantly more of >2.80 mm sclerotia (for each subsequent size interval). A similar trend was observed by Taylor et al. (2018), where *S. sclerotiorum* isolates produced different numbers and sizes of sclerotia even though production was done on identical substrates. Larger sclerotia were found to germinate at a higher level, faster and produce a greater number of apothecia (stipes). The size effect was addressed in the design of replication and as a blocking factor in analyses but was not further examined. It is important to point out that there were differences in response between sclerotia of different size, however, populations existing in the field comprise of differently sized sclerotia and therefore the use of replicate means is a suitable representation of such variance. Various authors report variation in sclerotial size and suggest a controlled way of the use of sclerotia of different size in experiments (Sun and Yang, 2000, Mila and Yang, 2008, Wu and Subbarao, 2008).

Additionally, to the above-mentioned hypothesis, we were interested in the possible effects of the temperature treatments on myceliogenic germination and on the overall viability of sclerotia.

Hypothesis 4: The myceliogenic germination level changes with temperature.

Isolate L5 showed increased levels (max 6%) of mycelial germination compared to isolate L6 (max 1%) where this was associated with high T2 temperatures ≥17°C, irrespective of T1 and S1 duration. Similarly, high temperatures (20-25°C) and the desiccant drying of sclerotia prior to transfer to high humidity increasing mycelial germination was reported by Huang et al. (1998). This is in contrast to the study by Huang (1991), who demonstrated an induced myceliogenic germination (100%) by applying -20 and -10°C incubation and 0% for 0.5 - 30°C incubation period for four weeks prior to germination observations at 20°C for a single *S. sclerotiorum* isolate originating from western Canada. Incubation at 10°C showed 80-100% carpogenic germination of sclerotia of the same isolate. Furthermore, he observed production of daughter sclerotia (secondary sclerotia) after myceliogenic germination, also without the presence of a crop, where similar observations were made in experiments completed for this project. Infection through both types of germination were reported in fields, where the prevalence of each type of germination was associated with crop and growth conditions (Huang, 1985).

Hypothesis 5: The sclerotia viability changes with temperature.

For the isolate L5, a greater number of non-viable sclerotia was observed compared to L6. Generally, the viability was reduced by T1 = 0°C and T2 = 17, 20 and 25°C (max 17%) for isolate L5 where the highest proportion of non-viable sclerotia was observed for the single temperature of 0°C, 46 % (s.e. = 7.22) and by T2 = 20 and 25°C (max 6%) for isolate L6. Viability of sclerotia is often not reported, or the term is associated with not germinated sclerotia during the experiment (Gupta and Singh, 2017). However, in this study, the germination observations were divided following different stages of the experiment (S1, S2, S3) and further divided into carpogenic and myceliogenic germination. The non-viable sclerotia were those which did not germinate even after additional time in S3 (11°C, 150 days) and sclerotia removed due to infection. Sclerotia which germinated by the end of S3, carpogenic or myceliogenicly were assumed viable, and the level of germination was calculated from these viable sclerotia. An additional option to test the viability of sclerotia was to dissect the remaining sclerotia and plate them on PDA plates to observe for growth of mycelial colony (Coley-Smith and Javed, 1970, Grogan and Abawi, 1975). This method was rejected in favour of the introduction of S3, because of possible inconsistency in the outcome, where sclerotia which germinated myceliogenicly could still not be viable for carpogenic germination. This is something that would need to be addressed in further studies and identifying a standardized method and reporting to allow for improved research reproduction and a systematic interpretation of results across published studies. In favour of testing viability of sclerotia for carpogenic germination by applying more favourable germination (conditioning) temperatures was the collection of additional, post-experimental germination data, which could be used to draw conclusions about amount of germination (conditioning) progress completed during the main part of experiment and this could be later used in modelling of germination times in Chapter 6. It is unfortunate that the PDA test of sclerotia viability was not applied for the non-viable sclerotia from the main temperature experiment as this could have possibly allowed for a comparison between these methods and the formulation of further conclusions and recommendations for sclerotia viability assessment.

The following observations on experimental design and data analyses used in this project were made and possible recommendation for further research were formulated:

- Standardised methods should be used in the production of sclerotia, pre-experiment storage, sclerotia size selection, conditioning and incubation. These are vital for producing results which are mutually comparable. Researchers used various conditions for sclerotia conditioning, i.e. bulk butches in soil (Clarkson et al., 2007, Phillips, 1986), wheat grain flasks (Clarkson et al., 2007, Clarkson et al., 2004), sterile paper towel (Sun and Yang, 2000, Mila and Yang, 2008), on PDA containing mycelial cultures, after sclerotia where formatted and maturated (Huang, 1991, Foley et al., 2016), batch of sclerotia placed in cheesecloth bag in distilled water (Dillard et al., 1995).
- The number of sclerotia used in experiments was ranging from 5 (Gupta and Singh, 2017) to 30 (Clarkson et al., 2007) per experimental unit. An increased number of sclerotia used in experiments (48-56 sclerotia/per replicate used in this study) provides an improved understanding of the response of the whole distribution of the sample/population and reduces the error compared with a smaller sample size.
- Importance of statistics used in experiment analyses. Level of germination, mean time to germination and start of germination are commonly used statistics, where the mean time to germination should be directly associated with the achieved level of germination. It is important to realize and be able to identify to what proportion of germinated, or viable, sclerotia the mean time relates. There is a different amount of information included in the mean time derived for lower levels of germination. Therefore, a use of time to a certain percentage of sclerotia germinated could provide a more precise description of germination/conditioning times/rates and compare the various treatments in a standardized manner. Additionally, when assessing the time to germination it is vital whether we refer to a whole time (including conditioning, S1+S2) or only to time in S2. Interpretation of results associated only with S2 can be misleading, where with longer S1 duration a part of the germination already progressed in S1 (i.e. germination observed for T1 as low as 4°C), which is omitted from analyses. Finally, the issue of evaluating the viability of sclerotia was already discussed earlier.
- Experimental design, where the availability of the three-factor grid (T1, S1 duration and T2) enabled identification of the specific behaviour of isolate L6. This would not be the case if a simpler experimental design would be used, i.e. one S1 temperature followed by various S2 temperatures, or vice versa,

with varying S1 durations (basically all previous studies). A limitation of the experimental design for this study was to include only transfers from lower to higher temperature (T1<T2), where this was proposed based on the original assumption that conditioning has to be completed first, prior to a high, fast and uniform germination and to reduce the number of treatments. However, for further studies it would be beneficial to include transfers both ways. Regarding the S1 duration, the results are suggesting including S1 duration of 56+ days is generally not adding any further improvement. Yes, the germination levels at the supra optimum and ceiling temperatures (T2) were improved with longer S1 duration, however the germination often happened at S1 (especially for isolate L5). Therefore, it is problematic to decide whether with longer duration of S1 we actually observe an effect of conditioning or whether the germination (stipe production) proceeded so far in S1, that it continues also at less favourable temperatures, and is later arrested. Inclusion of control treatments with no transfer is also vital, where this provides a reference point for transfer treatments (both processes, conditioning and germination, proceed at a single temperature).

- Plausibly, the analyses in the form presented could look excessive and too comprehensive, however as long as we can't clearly distinguish between conditioning and germination, processes involved in the carpogenic germination of sclerotia, the conclusions derived could be misleading as we can't decisively identify how and which process was actually affected, especially whilst these processes have contrasting responses to temperature. The three-factor grid in combination with a number of statistics used to describe the response to treatments for the whole population, together, allowed a more dynamic and complex understanding of the effects observed. Generally, when any of the factors examined has changed it affected the response for the other factors and it could be associated with specific statistics. Furthermore, independence between the processes associated with carpogenic germination is something that could be further investigated. The current assumption is that germination is promoted by conditioning (conditioning is prior to germination, hence the experiment design), however without a method clearly distinguishing between the process, such investigations are limited. This also provides the motivation behind the dissection experiment described in Chapter 4.
- Information on the origin of *S. sclerotinia* isolates should always be provided, since by now it is well established that there is variation in temperature

requirements between isolates of different geographic origin (Liu and Paul, 2007, Dillard et al., 1995, Foley et al., 2016), although some recent studies show variation in temperature for isolates originating from the same country (Clarkson et al., 2004, Clarkson et al., 2007), and even the same field in the case of this study. It is vital to take the origin of the isolates into account; however, this is a common standard between researchers. This is furthermore vital for an understanding and characterisation of *S. sclerotinia* population structure globally and locally.

Finally, the data collected in the scope of the main temperature experiments and the described responses to temperature for both isolates, provide a key foundation for model development and parameter estimation described in Chapter 6.

4 Developmental changes in *S. sclerotiorum* sclerotia at different temperatures.

4.1 Introduction

As described previously in Chapter 1.2, it is generally perceived that carpogenic germination of *S. sclerotiorum* sclerotia involves two processes: “conditioning” and “germination”, with both processes requiring contrasting temperature conditions. Conditioning is the less understood process, where the main difficulty lies in the inability to identify and directly observe this phase. Carpogenic germination is distinguished by initiation of stipes on the top of the sclerotia (Figure 4.1, A) and is differentiated from the later process of apothecia formation (Figure 4.1, B)(Saharan, 2008).

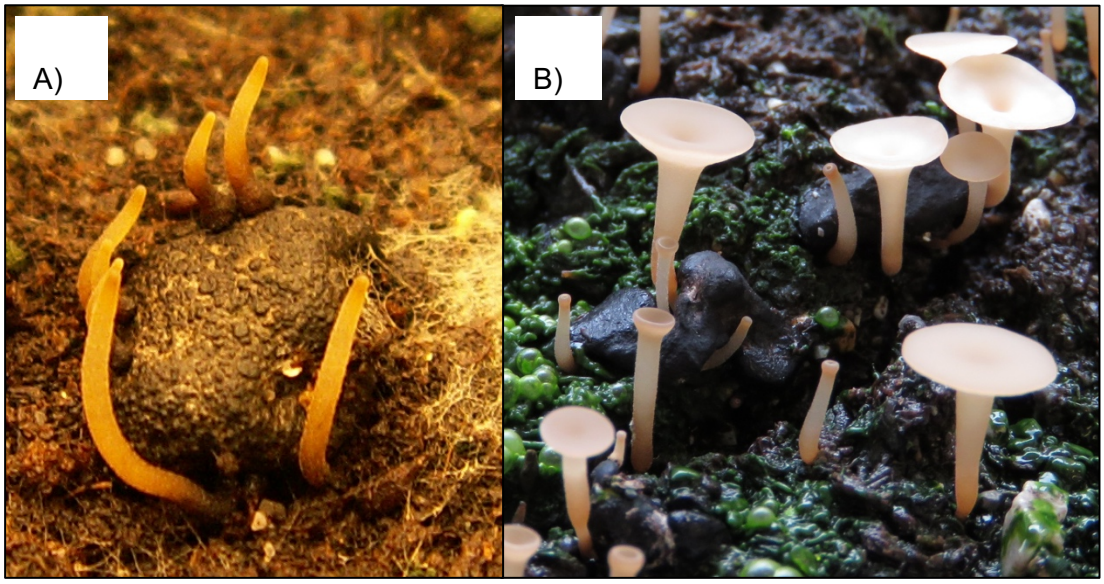

Figure 4.1 A) *Sclerotinia sclerotiorum* stipes germinated from sclerotia; B) *Sclerotinia sclerotiorum* apothecia and stipes germinating from sclerotia.

Sclerotia are a hyphal aggregates consisting of three layers of tissue: rind; cortex, medulla (Deacon, 2006) (Figure 4.2). Willetts and Bullock (1992) describe the rind as a dark outer layer of thickened, pigmented cells (containing melanin) from one to several cells thick and a cortex consisting of close-fitting rounded cells and the medulla. The medulla is the centre of the sclerotium consisting of hyphae imbedded in a fibrillar matrix (Colotelo, 1974, Saito, 1974a in Deacon (2006) and contains substantial nutrient storage reserves of glycogen and lipids.

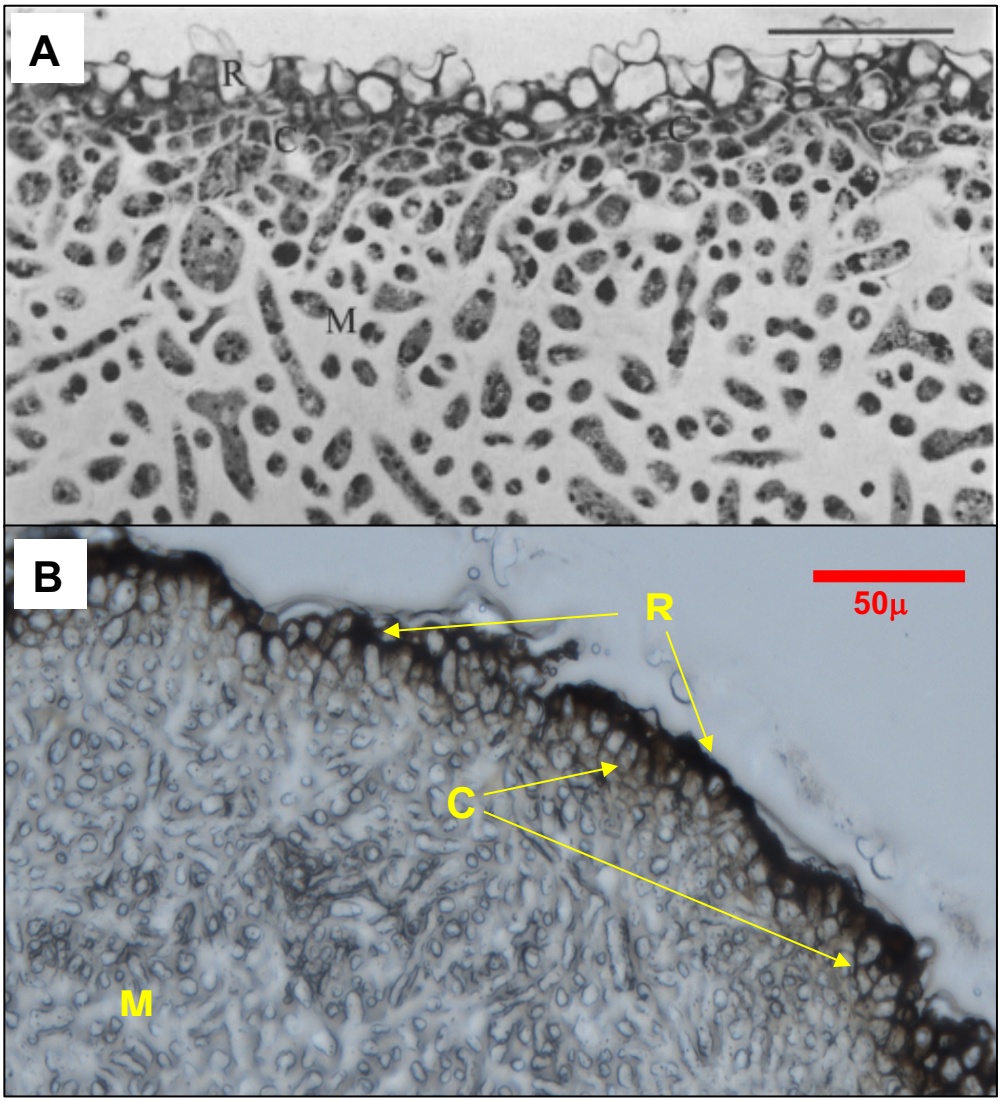

Figure 4.2 Light microscope image of dissected sclerotium picturing R – Rind, C – Cortex, M - Medulla for: A) *Sclerotinia minor* (Deacon, 2006), scale bar = 50µm; B) *Sclerotinia sclerotiorum* (own observation).

Developmental studies carried out by Kosasih and Willetts (1975) on two-month-old *S. sclerotiorum* sclerotia, incubated at 14˚C, showed that stipes appeared on the surface after about 3 weeks and mature apothecia 10-15 days later. They also described, under microscope observed, “centra” or “nests” (<40 µm diameter) of interwoven dense hyphae in dissected sclerotia, predominantly located in the cortical region as apothecia initials. Further development led to active division of the hyphae forming a knot and raised dark, brown and shiny areas at the surface of sclerotia as the first external evidence of stipe formation observed a few days later.

Another study carried out by Saito (1973) examined *S. sclerotiorum* sclerotia incubated at 3˚C for four weeks followed by transfer to 15°C. Primordia giving rise to stipes were identified which represented distinct structures compared to the medullary tissue and these structures could not be found in sclerotia before the

transfer. Four stages of primordial development into stipes were described (Figure 4.3):

- Stage I. - Deeply stained areas in the medulla near the rind of the sclerotium, comprising a centralized cluster of narrow cells surrounded by broad, thin-walled hyphal cells (Figure 4.3, 1-2). This first stage of primordial development appeared after 2 days and reached a maximum 5 days after transfer to 15°C (Figure 4.4_B).
- Stage II. - Primordia enclosed by irregular thick-walled and dark pigmented cells (Figure 4.3, 3-4), started to appear on the 4th day after transfer, and increased subsequently with decreasing numbers of Stage I. primordia (Figure 4.4_B).
- Stage III. - A mass of thin-walled hyphae clearly distinguished from the medulla located close to the rind of the sclerotia (Figure 4.3, 5). These appeared 5 days after transfer, but in fewer numbers compared to earlier primordial stages and remained almost constant for the duration of experiment.
- Stage IV. - Primordia ruptured the sclerotial rind and developed into stipes that were only visible under the microscope after approximately 7 days after transfer (Figure 4.3, 6). The presence of these was expressed in numbers of sclerotia "germinated" (Figure 4.4_A, dotted line) and correlated well with visually observed stipes 3 days later (Figure 4.4_A, full line). Stipes were observed after 9 days and reached a maximum after 16 days after transfer to 15°C (Figure 4.4_A, full line).

The diameter of primordia of Stage I. reached 20.8 – 98.8 μm with a mean of 44.5 μm 7 days after transfer, and this diameter range overlapped with Stage II. primordia which ranged from 20.8 to 46.8 μm (mean 37.8 μm). The Stage I. and II. primordia were more distinguished by the dark pigmented cells rather than by their size. The diameter of Stage III. primordia at first ranged 65.0 – 85.8 μm (mean 78.0 μm) and then gradually increased. The location of primordia was reported to be predominantly close to the rind (within 55μm).

Saito (1973) concludes that the described cell clusters likely constitute the initials of the vegetative hyphae of the apothecial stipes and establishes the term "stipe primordia". A single sclerotium can produce a great number of primordia but not all will develop into apothecial stipes. It was suggested that the production of Stage II.

primordia negatively regulate production of further Stage I. primordia and that only a few develop into stipes.

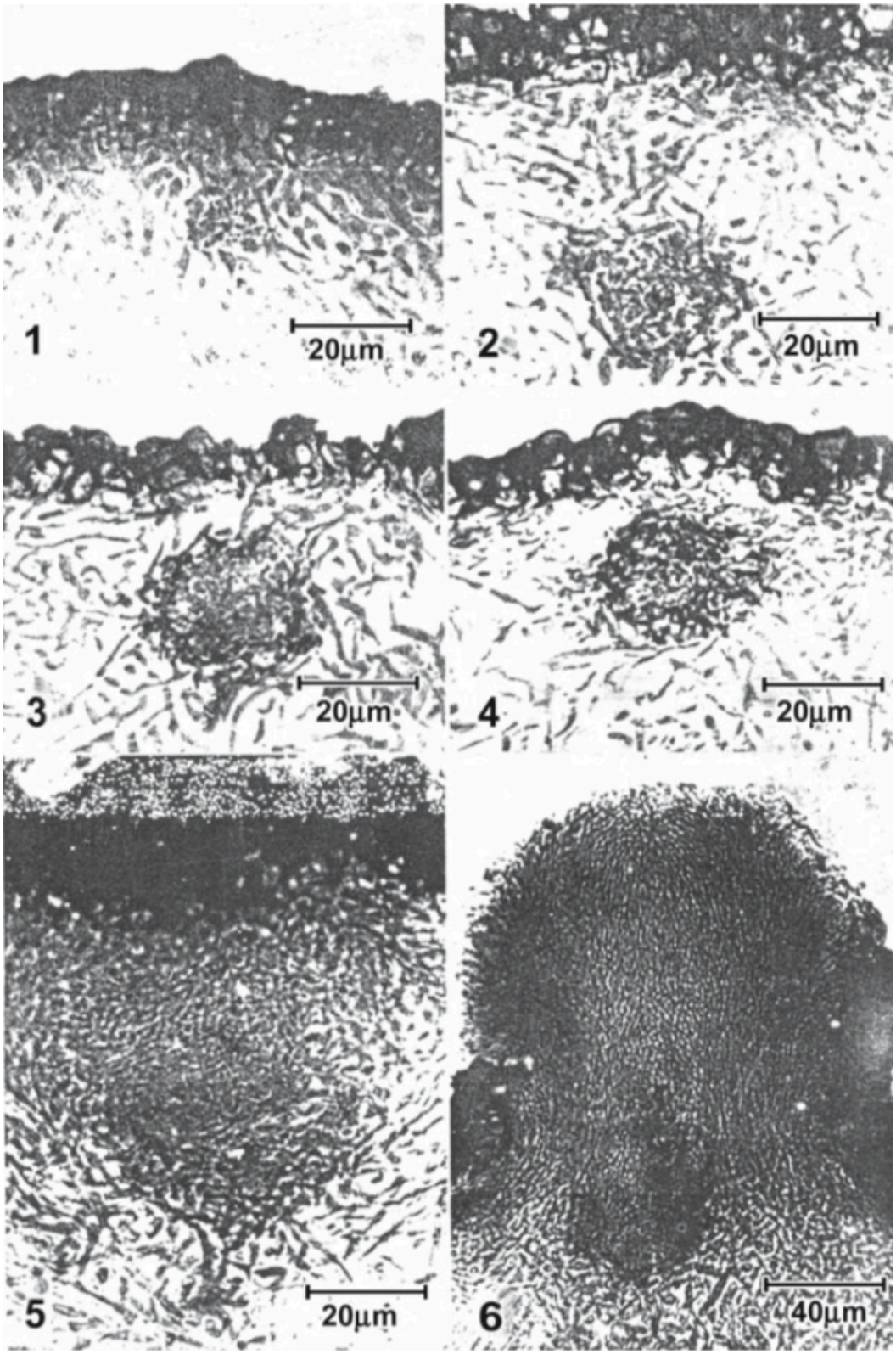

Figure 4.3 Vertical sections of sclerotium of *S. sclerotiorum* showing successive stages of apothecial stipe development; (1) Stage I primordium showing deeply stained meristematic structure; (2) Stage I primordium increased in size, but pigmentation not yet occurred; (3) Stage II primordium with dark pigmentation around the primordium; (4) Stage II primordium infiltrated with pigmentation; (5) Stage III primordium; (6) Stage IV stipe (Saito, 1973).

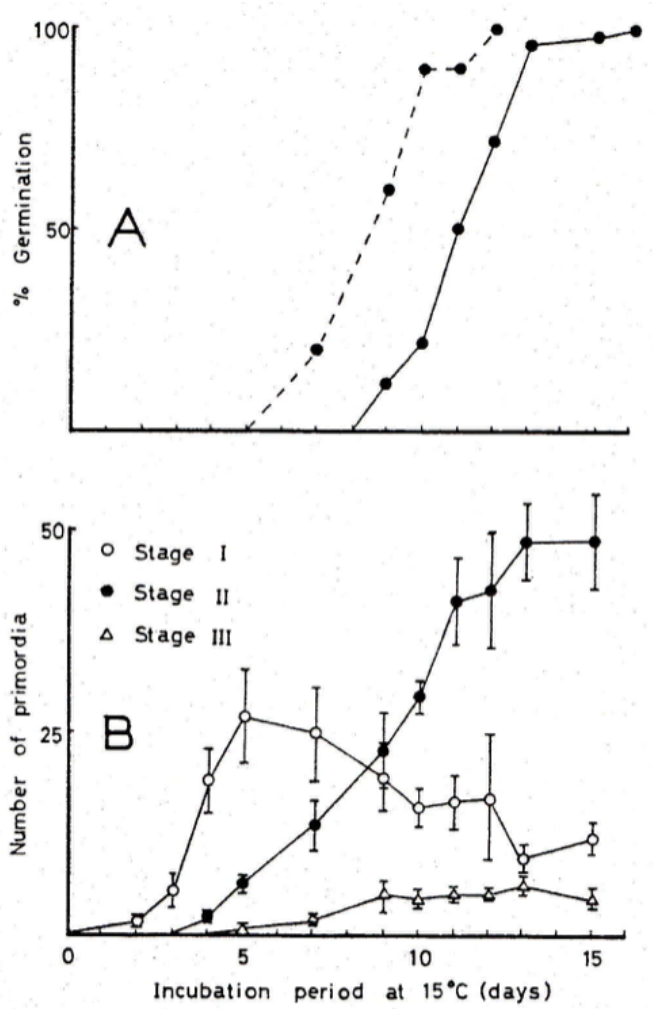

Figure 4.4 Development of primordia stages and germination of *S. sclerotiorum* sclerotia over time; A) Germination of sclerotia as determined by externally visible stipe protrusions (solid line), and by formation of stage IV-primordia in sclerotia (dotted line). The former determination was made for 100 sclerotia and the latter was made microscopically for 10 sclerotia collected at each time point; B) Number of stipe primordia at different developmental stages. Data indicates the number of primordia found in 100 sections of sclerotia at each time point. The vertical line at each point indicates standard error. Reproduced from Saito (1973).

In a study by Phillips (1986), primordia were not detected in *S. sclerotiorum* sclerotia conditioned in soil (in field for various durations) irrespective of their age or source (crop). He reported that primordia were likely formed in conditions suitable for germination (rather than for conditioning) and proposed that conditioning and germination are two separate phenomena. He also suggests that a resting period of 6-8 days is required for fully conditioned sclerotia for development and maturation of stipe primordia as described by Saito (1973).

Saharan (2008) summarized further work of Saito (1977) and introduced the term “functional maturity” which characterized newly produced sclerotia capable of germination and apothecia formation. These were distinguished from “mature-looking” sclerotia which were recognised by dark pigmentation of the rind (melanisation) and disappearance of liquid droplets from the sclerotial surface.

Although “functionally mature” sclerotia are not easily recognised, it is suggested this phase of maturation is different from vegetative growth as it is not inhibited by adding vitamins to a medium unlike for mycelial germination.

There have been very few studies investigating the development of *S. sclerotiorum* sclerotia during carpogenic germination, although the process of primordia development was well described by Saito (1973). All of these studies have been carried out using conditioned sclerotia (various cold treatment) and transfer to one selected temperature (supporting germination). Furthermore, a subsequent development of stipes delayed by several days followed primordia observation. However, no previous study provided insight into the effect of various temperatures on primordial development.

From the previous work described above we can conclude that primordia are:

- initials of vegetative hyphae that develop into apothecial stipes; therefore, they are directly associated with the final phase of carpogenic germination;
- likely formed in conditions suitable for germination, as early stage of stipe development;
- have not been observed during conditioning at low temperature, prior to transfer to higher germination temperature;

The work in this chapter aimed to study the development of primordia in *S. sclerotiorum* sclerotia for a number of temperature treatments selected in consistency with the main temperature experiment (TE1, TE2, Chapter 3), to further understand processes involved in carpogenic germination of sclerotia at an early stage, prior the stipe production visible on the surface of a sclerotium.

An assumption was formulated that primordia are initials of stipes (although not all primordia develop into stipe) and are associated with the “germination” process of carpogenic germination. Furthermore, it is assumed the primordia appear after sufficient cold incubation “conditioning” is provided; after sclerotia are placed to temperature suitable for germination; and after several days some of the primordia develop into stipes.

Following hypothesis were tested:

- Primordia are produced at germination temperature, prior stipe production.
- Primordia are produced after “conditioning” at low temperature is completed.

4.2 Material and methods

4.2.1 Experimental setup and data collection

Dry *S. sclerotiorum* sclerotia (size 2.8 - 3.3 mm) of isolate L5 and L6, stored at room temperature at 20°C for one month, were soaked in water for 24h and planted in soil-based compost as described previously (Chapter 2.3). Experiment setup and the treatment selection was similar to the main temperature experiment (TE) (Chapter 3.2), including temperatures 4, 11, 17, and 20°C and where sclerotia were either kept at a single temperature or were transferred after 28 days to T2 = 17˚C (Table 4.1). The temperatures selected aimed to provide a good temperature range and include "conditioning" and "germination" supporting temperatures, hence the production of primordia at various temperatures could be assessed. Preliminary results from TE1 suggested that T2 = 20°C could be limiting for isolate L5 germination. Since the visual observation of stipes was vital part of this experiment, the T2 = 17°C was chosen as more suitable S2 temperature compared to 20°C, although it meant to transfer from higher T1 to lower T2 (for T1 = 20°C), in contrast with the design used in the TE (Chapter 3.2).

Compost filled boxes were setup as described previously (Chapter 2.3) and sclerotia were pushed into the compost similarly to TE (Figure 3.1). Inside the box sclerotia were organized corresponding with number of collection dates (Table 4.1), for the single temperature treatments sclerotia were arranged in 10 rows, for the temperature transfer treatments in 7 rows and each row contained 10 sclerotia representing a sample. These samples of 10 sclerotia were collected in a randomized order (one row at each time point), whereby the first sample was taken on the day of the experiment set-up, followed by sample collection every 7 days for 10 weeks (Table 4.1). For the single temperature treatment (no transfer to S2) the first sample was collected on 7th day from set-up and for the transfers treatments the first sample was collected on 42nd day from setup and 14th day from transfer to S2 (28th day). Prior collection of sclerotia samples, at each timepoint (weekly), a visual assessment of stipe occurrence on sclerotia present in each box was recorded (alike the TE germination assessment, Figure 3.3).

As the preparation of samples for the microscopy was extremely challenging and time-consuming process, only a subset of sclerotia samples originally collected was finally used for microscopy. Sclerotia samples collected and not used for dissection

were stored for possible future work. The restriction of final sample collection for microscopy was done as follows:

- Four timepoints were selected with respect to provide sufficient coverage of possible time effect on primordia development at various temperatures: 7, 28, 42 and 70 days (red boxes, Table 4.1).
- Samples where stipe germination was once observed (visually), following samples were excluded from microscopy analyses (i.e. 11 and 17°C, the 70th day sample, Table 4.1), since the experiment aimed to asses primordia development prior to stipe production.

Days from set-up	0	7	14	21	28	35	42	49	56	63	70
Set - up											
4°C constant											
11°C constant											
17°C constant											
20°C constant											
4°C 28D transfer to 17°C											
11°C 28D transfer to 17°C											
20°C 28D transfer to 17°C											

Table 4.1 Overview of samples of sclerotia selected for dissection experiment for *S. sclerotiorum* isolate L5. red boxes indicate timepoints selected for dissection and for the final image analyses.

- Only for isolate L5 microscopy images were produced, since it was selected as the potentially more informative isolate, because of its requirement for cold conditioning to achieve high and fast germination in higher temperatures (Chapter 2.1). Assuming there are microscopic physiological changes associated with conditioning and germination inside of the sclerotia, there is a greater potential to observe these for isolate with a stronger response to cold conditioning.
- Finally, the number of sclerotia dissected was three per treatment (from originally 10 available).

4.2.2 Preparation of sclerotial samples for microscopy

Following sampling, *S. sclerotiorum* sclerotia were placed straight into FAA fixative (50 ml ethanol, 5 ml acetic acid, 10 ml 37% formaldehyde, 35 ml distilled water) for 24-48h to ensure complete penetration (Saito, 1973).

After the fixation phase was completed, sclerotia were washed twice for 10 min in phosphate buffer (0.1M pH7.2) and successively dehydrated by washing twice for 10 minutes in an increasing ethanol concentration series (EtOH 10%, 20%…100%).

After the 100% EtOH concentration sclerotia were transferred to Histoclear clearing agent in four one-hour long steps of increasing concentrations (by 25%) to 100% Histoclear. Samples where then left in 100% Histoclear overnight at room temperature with a number of Paraplast chips (embedding agent similar to wax, Sigma-Aldrich, UK) added. Next the samples were placed into an oven at 55-60°C to ensure complete melting of Paraplast. Over the next couple of days, the Paraplast concentration was increased by removing some of the melted Paraplast/Histoclear solution and by adding further melted 100% Paraplast to achieve 100% Paraplast solution and complete penetration of sclerotia. Sclerotia were then moved into a mold which was filled with melted Paraplast, and then covered with a stub holder and placed in fridge for the Paraplast to harden. Such prepared samples of sclerotia were cut into 10 µm thick slices using microtome sectioning equipment and placed on glass slide. All sections of sclerotia collected were dewaxed (Paraplast removed) in a reverse process, and were subjected to a decreasing concentration of Histoclear/water solution: 100%, 75%, 50%, 25% Histoclear series, to 0% distilled water (each step repeated 2x for 1 min duration) and samples were subsequently stained with Aniline Blue (0.1g Aniline blue dissolved in 10ml distilled H2O, then 50ml lactic acid (85%) (Edited by Mueller et al. (2004), p615) for 1.5 min and washed with distilled water.

As indicated from the literature (Saito, 1973), primordia are located predominantly close to the sclerotial rind and therefore sections of sclerotia with the longest circumference provided the best chance of observing primordia. Following this assumption, the selection/cutting process concentrated on the inner 60% of each sclerotial specimen, with every 10^{th} slice being selected and placed on a glass slide. The number of slices selected then increased to every 5^{th} slice near the approximate centre of the sclerotium. This approach allowed a number of sections (up to 50 sections/per sclerotium, depending on sample quality) to be collected from each sclerotium, with all slices counted to acquire the approximate size of the sclerotium. For each individual sclerotium a set of 10 sections was finally selected for assessment of primordial presence/absence, which best represented the inner 60% (equally distributed) and provided sufficient quality for the image analyses.

The selected sections of *S. sclerotiorum* sclerotia were examined under the microscope at x100 magnification and subsequent digital images obtained were evaluated for presence and abundance of primordia. ImageJ software (https://imagej.nih.gov/ij/index.html) was used to draw ellipse around sclerotia

(circular shape) and measure Feret diameter (Max – Feret Diameter, Figure 4.5) (FD) - the longest distance between any two points along the selection boundary (ImageJ User Guide IJ 1.43) was recorded. Where a stipe was observed (microscopic observation), the width of the base of stipe growing out of sclerotia was measured.

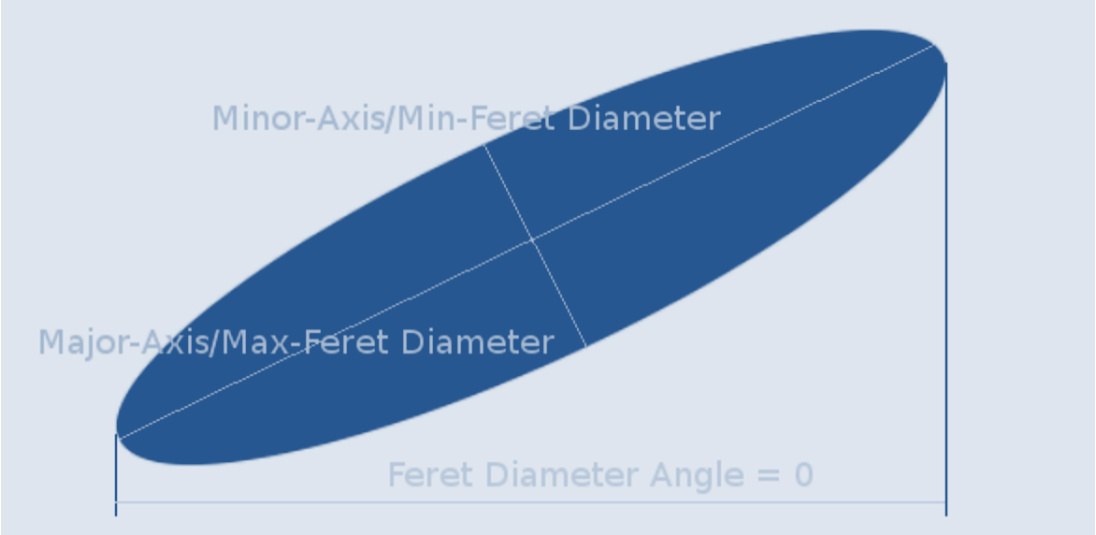

Figure 4.5 Feret Diameter with Max and Min Feret diameter referring to major and minor eclipse axis and Feret Diameter Angle referring to the angle of Max ferret diameter rotation (ImageJ User Guide IJ 1.43).

On a number of initial samples, the methodology of dissection samples collection was tested, as described earlier in this chapter. The number and location of sections, staining protocol and images digitalization process was established. The delivery of the complete set (except initial samples) of dissection samples, from wax imbedding, sectioning to digitization, was outsourced by The Rothamsted Bioimaging, CAS Department, Rothamsted Research, following the instructions provided.

4.3 Results

4.3.1 Identification of primordia, stipe initials and stipes

Primordia

Primordia observed where stained a distinctive blue colour and where composed of dense, narrow and thin-walled cells without the presence of air gaps between hyphae as observed in the surrounding medulla (Figure 4.6). All primordia observed where located close to the rind. Although there were differences in the depth of staining of primordia, adjustments in illumination or software meant that primordia as described could be clearly identified from across the different temperature treatments. The classification of primordia as proposed by Saito (1973), was not possible as the observed structures did not show as many distinct features as described by the author.

Examples of primordia detected for each treatment are in Appendix 29 to 43.

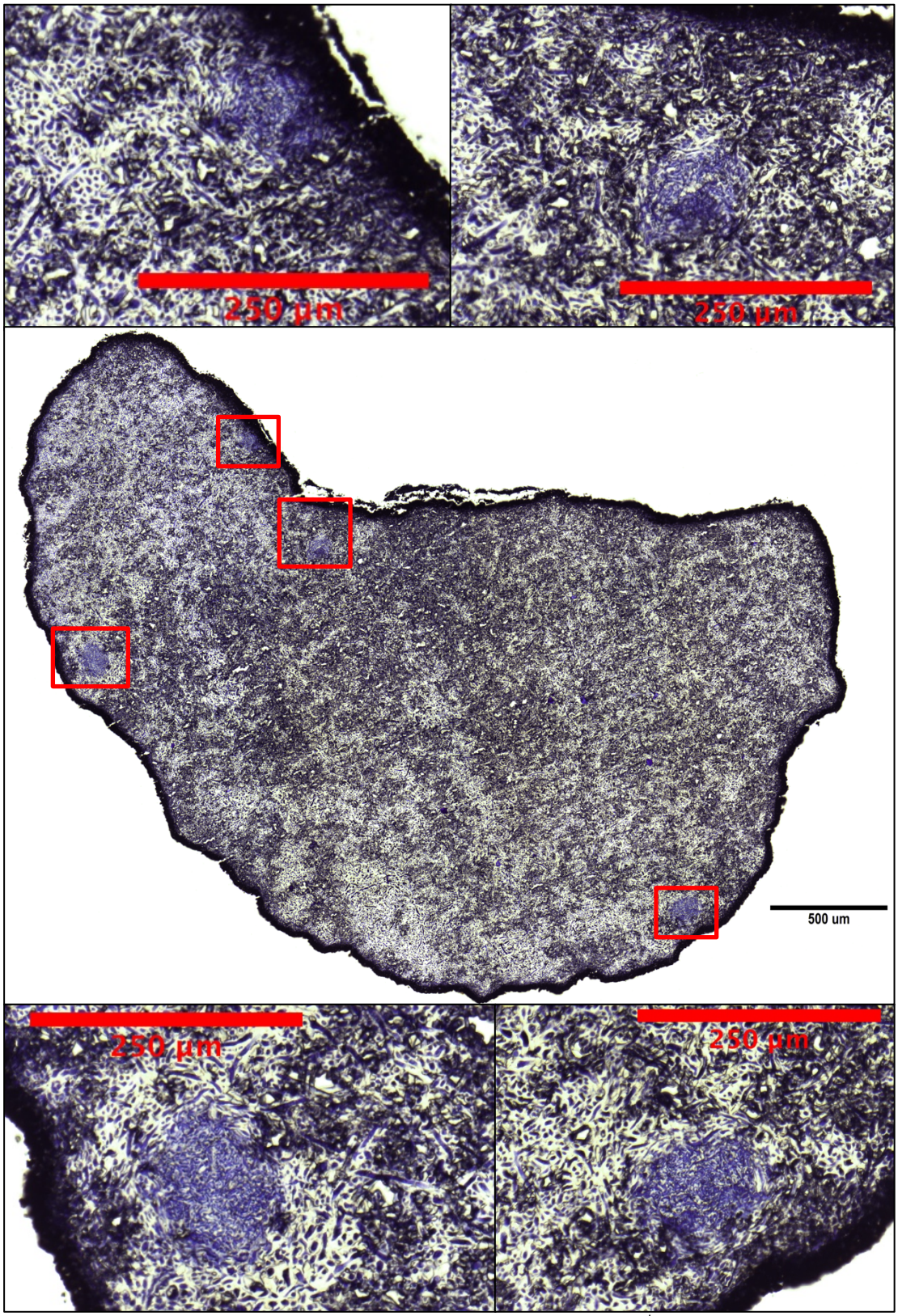

Figure 4.6 Microscopic section of *S. sclerotiorum* sclerotium at the 42nd day showing presence of primordia (4 in total); Sclerotium was treated by incubation for 28 days at T1 = 20°C followed by transfer to T2 = 17°C.

Active hyphae

Dense-stained, thin-walled hyphal cells, lacking air gaps were observed that were distinct to regular hyphae, but compared to primordia cells were less densely packed, and these were termed “active hyphae” (Figure 4.7) as these aggregations have been not previously characterized in the literature. Aggregated cells of “active hyphae” were always located close to the rind, similar to primordia but these structures lacked a distinct spherical form in the centre. Likewise, they may be a stipe initials at a very early stage as they were largely associated with “knobs” observed in the rind, distinct from regular elevations in the rind which comprised of regular medullar hyphae. Identification of this tissue is very subjective; however, it was worthwhile to consider.

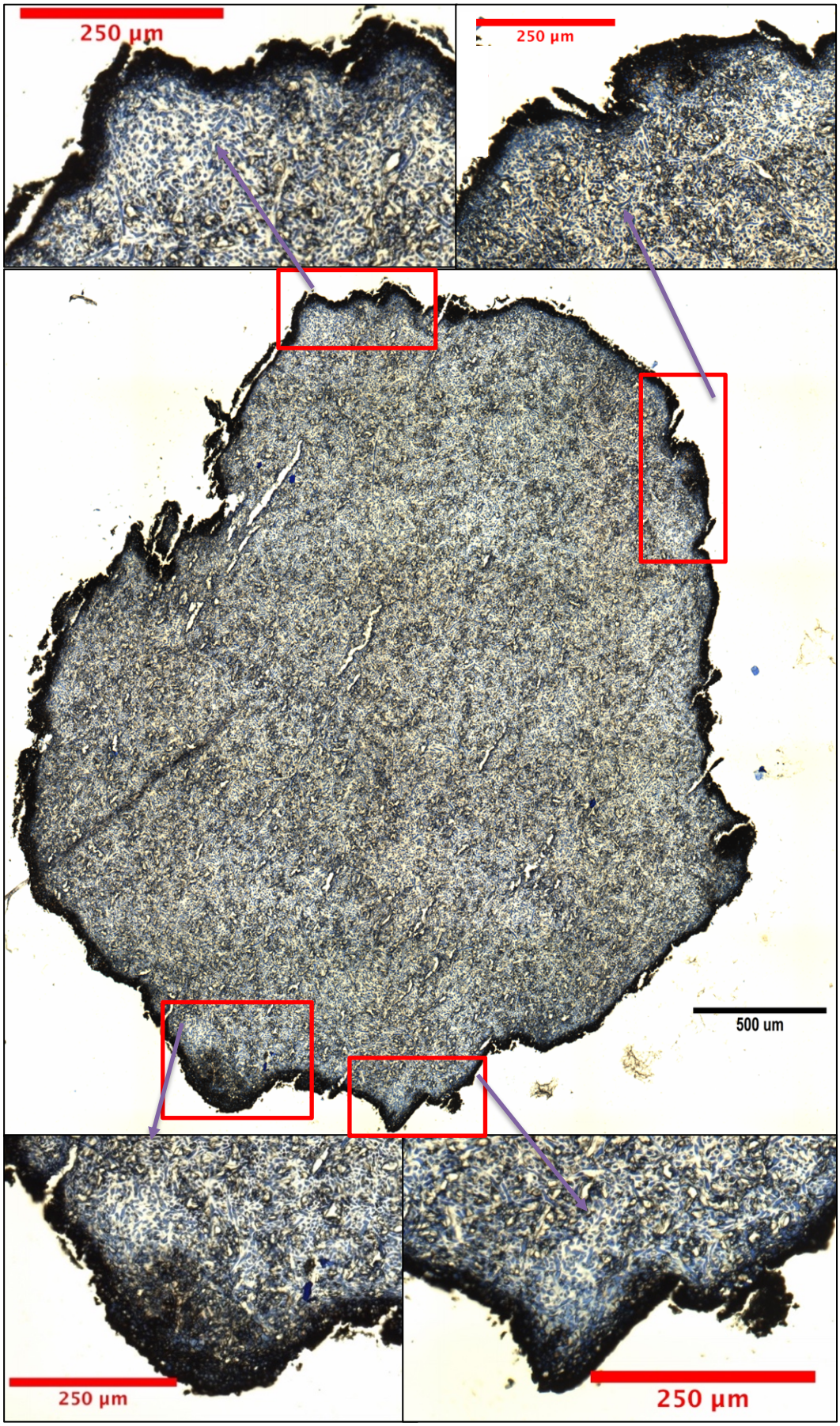

Figure 4.7 Microscopic section of *S. sclerotiorum* sclerotium after incubation for 28 days at 4°C and 14 days at 17°C showing detail of stipe initials and "active hyphae".

Stipes

The formation of hyphae which erupted through the rind as a stipe, was observed microscopically in two dissected sclerotia for treatments: after 42 days at constant 17°C (Figure 4.9); and after incubation for 28 days at 11°C and 14 days at 17°C (Figure 4.9). The stain colour and cell structure resembled the cells comprising primordia, but a deep stained centre as described by Saito (1973) was absent (Figure 4.3).

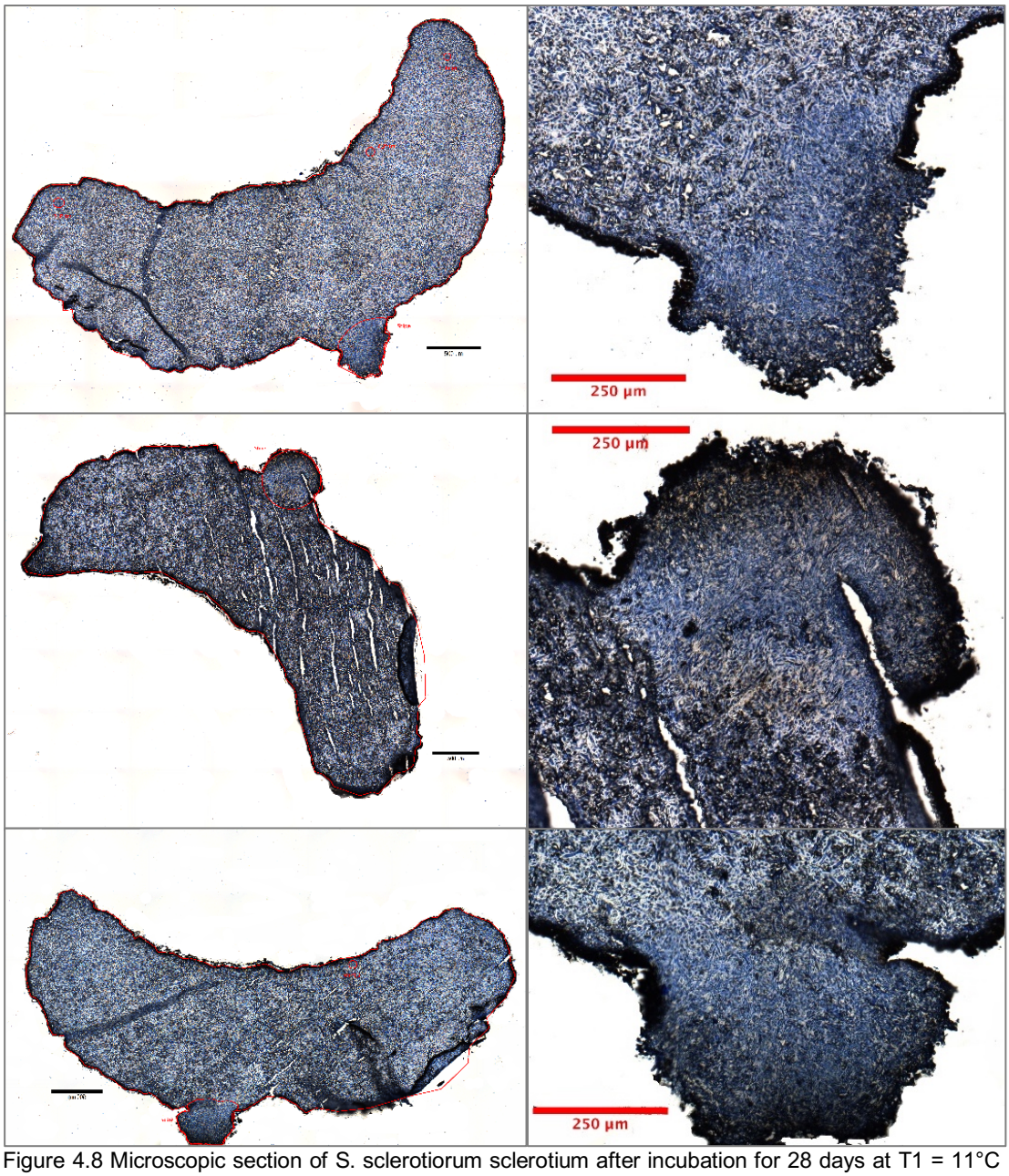

Figure 4.8 Microscopic section of S. sclerotiorum sclerotium after incubation for 28 days at T1 = 11°C and 14 days at T2 = 17°C showing detail of stipe, in subsequent slices 100µm apart.

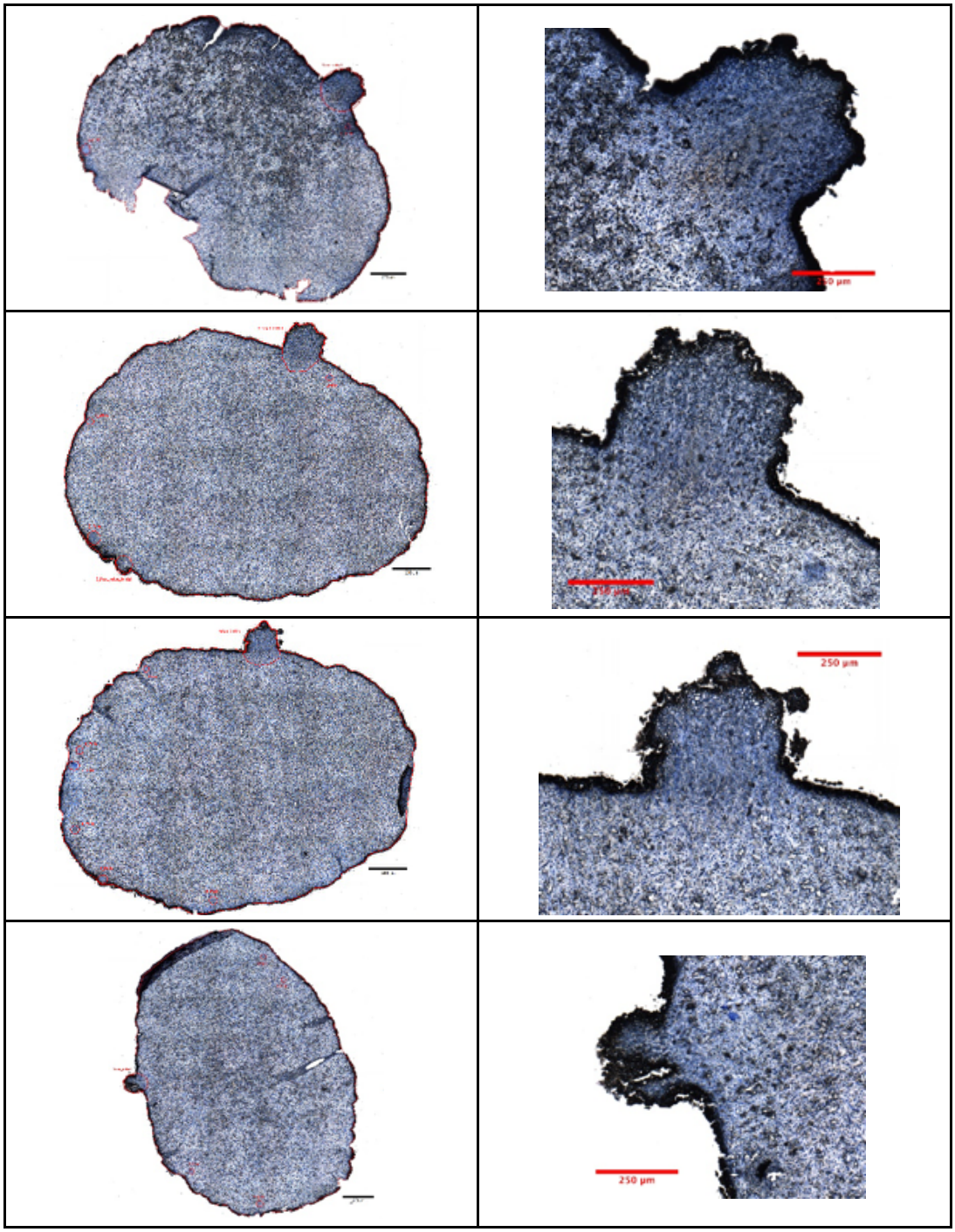

Figure 4.9 Microscopic section of S. sclerotiorum sclerotium after incubation for 42 days at 17°C (single temperature) showing detail of stipe and primordium (2nd picture from top), in subsequent slices 50µm apart.

More structures that resembled stipes were observed (Figure 4.10) however these lacked differentiated 'active' cells observed in more clearly defined stipes (Figure 4.8, Figure 4.9). These were captured on five sequential sections for the sample originating from 70 days at 4°C.

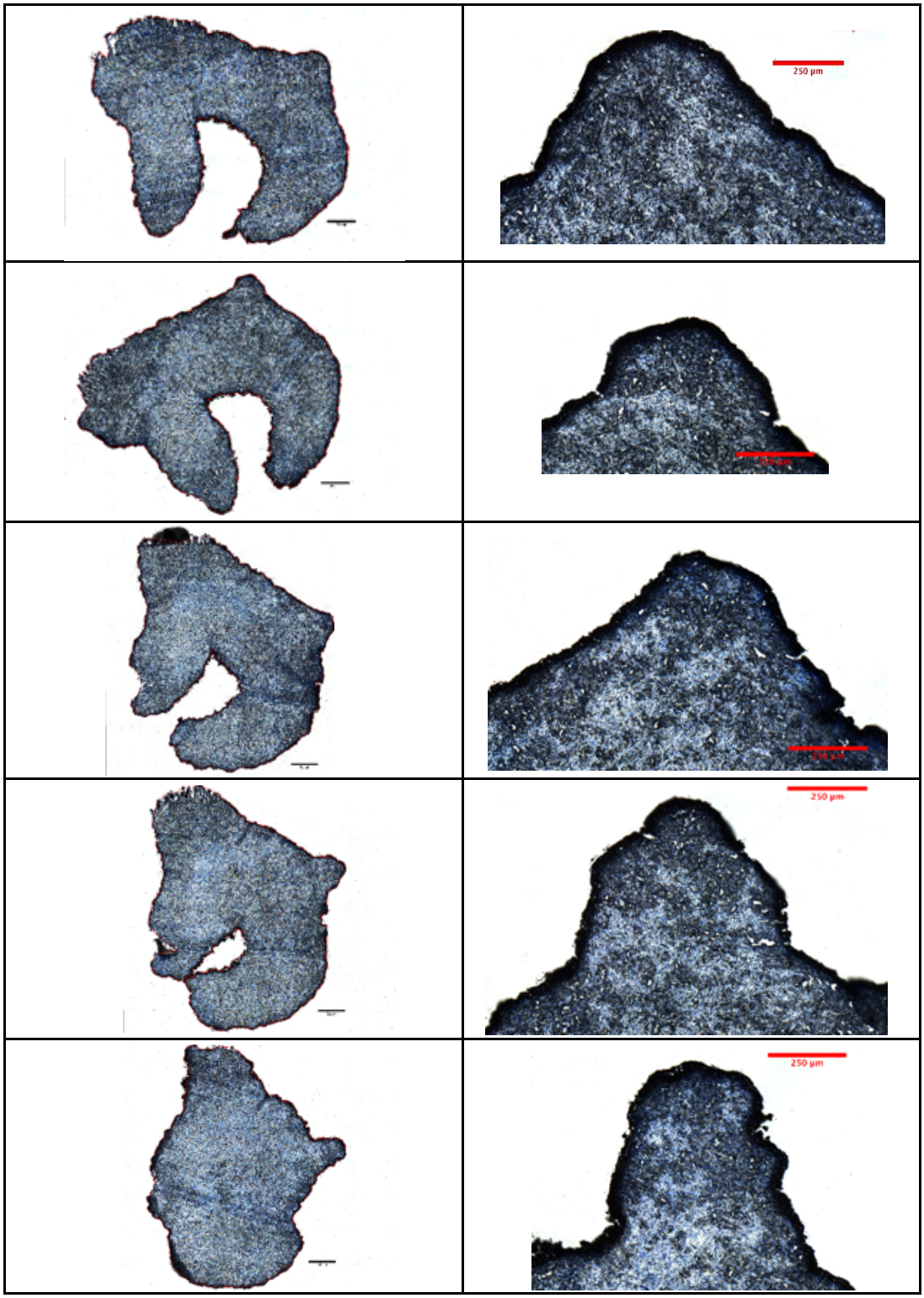

Figure 4.10 Microscopic section of S. sclerotiorum sclerotium after incubation for 70 days at 4°C (single temperature) showing stipe resembling structure, however cells are not distinct to surrounding hyphae, in subsequent slices 100 - 150µm apart.

4.3.2 Development of primordia, stipe initials and stipes for different temperature treatments

Dissection of sclerotia at the start of the experiment (24h of after soaking in water at 20°C) provided no evidence for the presence of primordia or any other morphological structures.

The presence of primordia was observed for sclerotia of *S. sclerotiorum* isolate L5 after 7 days at single temperature treatments of 11°C (two sclerotia), 17°C (two sclerotia) and 20°C (four sclerotia). In general, for the single temperature treatment the number of primordia observed increased with increasing temperature and incubation time, where after 28 days, 5, 39, 39 primordia and after 42 days 20, 60, 84 primordia were observed at 11°C, 17°C and 20°C, respectively (Figure 4.11). Furthermore after 70 days at constant 20°C, 82 primordia were observed.

Transfer of *S. sclerotiorum* sclerotia from 11°C to 17°C after 28 days promoted primordia production compared to single temperature treatment at 11°C, such that the number increased from 20 to 72 primordia after 42 days (from experiment start). The transfer from 20°C to 17°C after 28 days, slightly decreased an already high number of primordia present after 42 days, from 84 at single 20°C to 73 for transfer treatment (Figure 4.11).

At a constant temperature of 4°C first primordia (two) were detected after 70 days. However, transfer to 17°C after 28 days promoted earlier development of primordia, where 5 primordia and 17 “active hyphae” (Figure 4.7) were observed after 42 days from start (14 days from transfer). The presence of “active hyphae” and “nodulated” surface of sclerotia was also observed for temperature transfer treatment from 11°C to T2 = 17°C, after 42 days (14 days after transfer) (Figure 4.11).

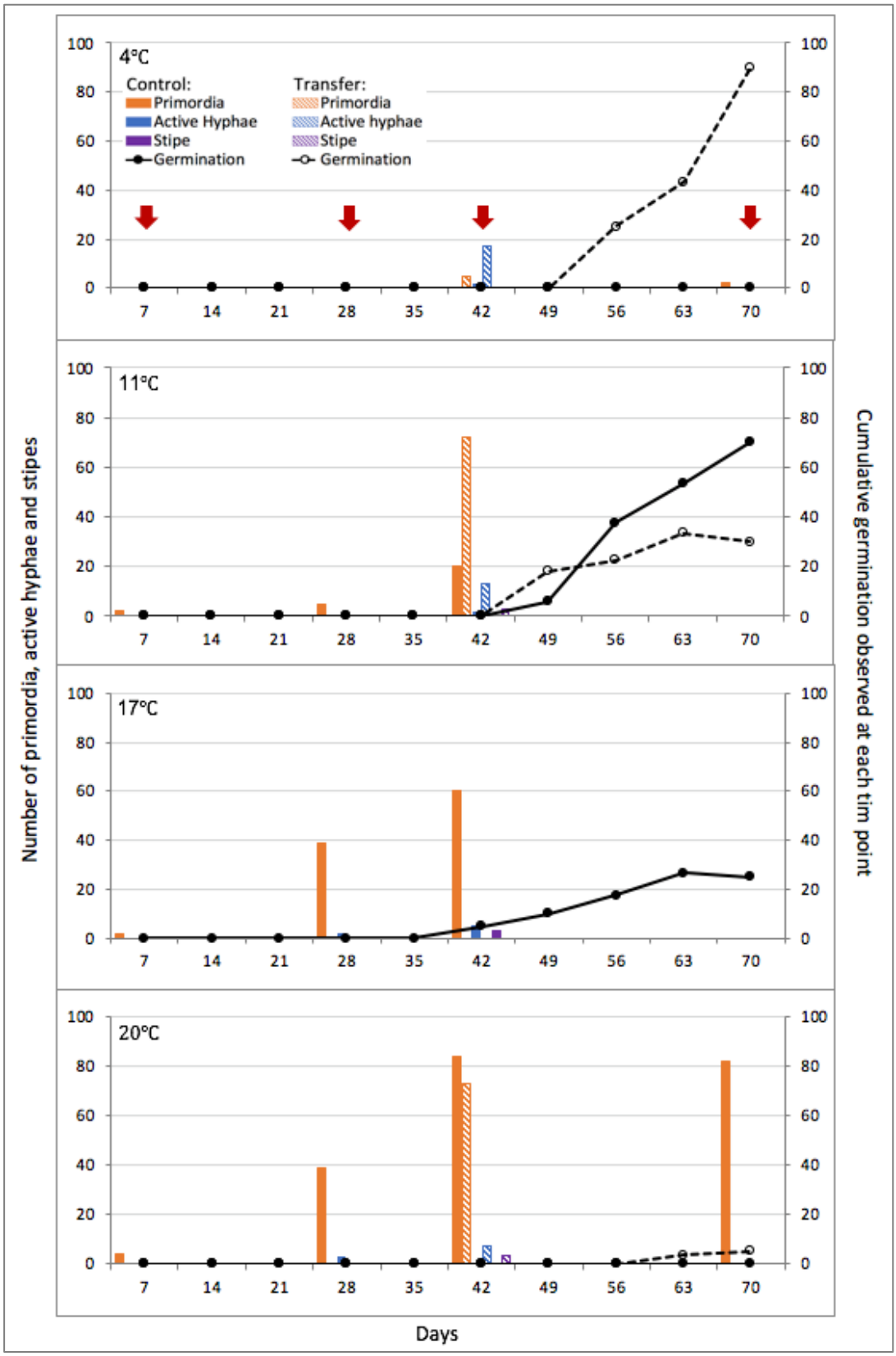

Figure 4.11 Number of primordia, stipe initials and stipes (bars) detected microscopically at four time points and percentage germination, by visual assessment (lines) for *S. sclerotiorum* sclerotia isolate L5 for constant temperature treatments of 4,11,17 and 20˚C (full colour bars / solid line) and temperature transfer treatments to 17˚C after 28 days (striped bars / dotted lines (mean % of sclerotia after sample for microscopy taken at each timepoint); Arrows indicate timepoints where sclerotia were sampled and dissected.

Number of primordia observed in *S. sclerotiorum* sclerotia

The maximum number of primordia observed in a single section of a *S. sclerotiorum* sclerotium was nine, recorded after 70 days at constant 20°C (Figure 4.12). Generally, the number of primordia per section for a single temperature treatment increased with increasing temperature and time since burial. A maximum of six primordia per sclerotium were observed after 42 days (14 days after transfer to T2 = 17°C) within the temperature transfer treatments for T1 = 11°C and 20°C; similarly, to the single temperature treatment at 17°C. For an initial temperature of 4°C transferred to 17°C after 28 days, a maximum of one primordium per sclerotium was observed after 42 days (14 days from transfer); by comparison, a single primordium per section was observed after 7 days at single temperature at 17°C and 20°C, and after 70 days at 4°C.

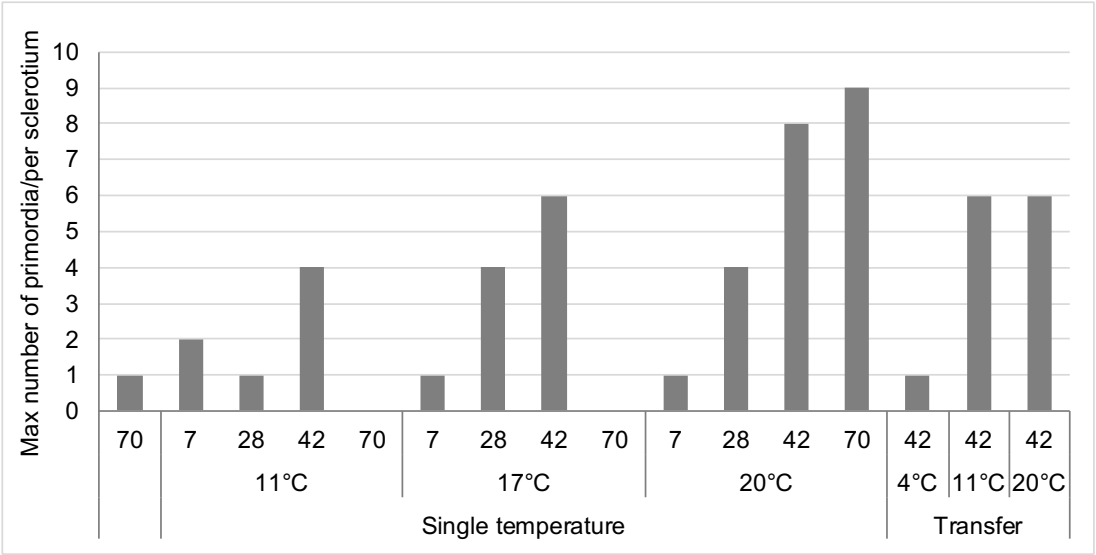

Figure 4.12 Maximum number of primordia observed in a single section for *S. sclerotiorum* isolate L5 sclerotia.

Size of primordia observed in *S. sclerotiorum* sclerotia

Primordia varied in their FD value and ranged from 46 to 270.8 μm (mean 114.5 μm). Furthermore a trend was observed in the mean size distribution of primordia for the FD, which increased with increasing temperature and time from burial (Figure 4.13).

For the single temperature treatment of 4°C, only two primordia were detected after 70 days with sizes of 93.4 and 113.7 μm and these were slightly larger compared to the five primordia found in sclerotia transferred from 4 to 17°C where FD ranged from 77.5 to 98.6 μm (mean = 83.4μm).

For the single temperature of 11°C, two primordia were observed after 7 days with a size of 99 & 102 μm, and those were larger than the primordia found after 28 days, ranged from 50.6 μm to 85.6 μm (mean 72.9μm), and after 42 days, ranged from 58.4 to 121.5 μm, (mean 87.1 μm). Primordia observed after 42 days for the temperature transfer treatment from 11°C to 17°C showed noticeable increase in size with FD ranging from 46 to 270.7 μm (mean 140.5 μm) compared to the single temperature treatment at 11°C.

Primordia observed at single temperatures of 17°C (2) and 20°C (5) were similar in size and consistently larger than those produced at 11°C at the same time points, except 7 days (Figure 4.13). After 7 days the size of the observed primordia was 78.2 and 84.7 at 17°C and ranged from 57.8 to 105.9 μm (mean 77.1 μm) at 20°C. After 28 days, primordia FD increased to 64.9 to 195.9 μm (mean 107.8μm) and 57.2 to 209.7 μm (mean 114 μm) for 17°C and 20°C, respectively. After 42 days, the increase in mean FD of primordia was minor compared to 28 days for both temperatures. The largest primordia were observed after 70 days at constant 20°C with FD ranging from 46 to 270.1 μm (mean 140.5 μm). The transfer treatment from 20°C to 17°C showed comparable size of primordia to the 17 and 20°C single temperature treatments after 42 days, with FD ranging from 54.4 to 238.1 μm (mean 129.8 μm).

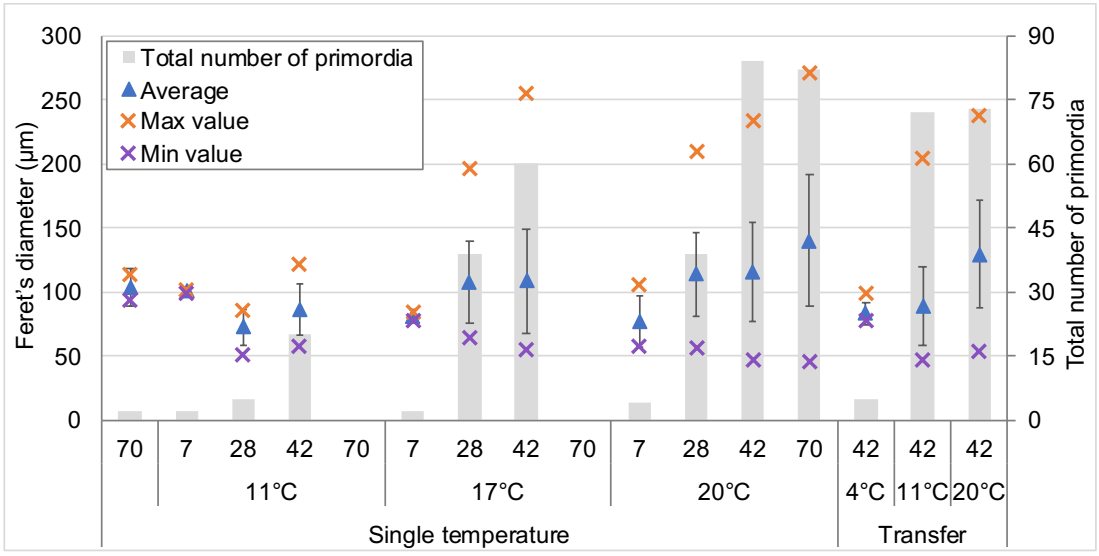

Figure 4.13 Ferret's diameter (FD) of primordia observed in dissected *S. sclerotiorum* sclerotia collected from different temperature treatments after 7, 28, 42 and 70 days. Symbols represent mean (error bars = standard deviation), maximum and minimum values per treatment. On the secondary axes, bars (grey) represent the total number of primordia observed in 10 sections of 3 sclerotia for each treatment.

Germination of *S. sclerotiorum* sclerotia to produce stipes

While collecting samples for dissection at each time point, germination of the remaining sclerotia to produce visible stipes was also recorded. Germination was first

observed for single temperature treatments of 11°C and 17°C after 49 and 42 days and reached a total germination of 70% and 25%, respectively, after 70 days (Figure 4.11). Germination was also observed for the temperature transfer treatment of 11°C to 17°C after 49 days (21 days after transfer) and reached 30% after 70 days. The transfer treatment from 11°C to 17°C showed similar time to first appearance of stipes compared to the single temperature of 11°C, but the final percentage germination achieved was smaller. Comparing the transfer treatment from 11°C to 17°C to the single temperature of 17°C, there was a 7-day delay in the first appearance of stipes and a slight increase in the final germination (24% for the single temperature treatment at 17°C).

For sclerotia transferred from 4°C, first germination was recorded after 56 days (28 days after transfer) with total germination of 90% (highest observed germination) by the end of the experiment. For the 20°C transfer treatment only one sclerotium produced stipes after 63 days (35 days from transfer). Constant temperature treatments of 4°C and 20°C did not produce stipes for the 70 days duration of the experiment.

4.4 Discussion

Overall message here is that primordia are not produced in the low temperature conditioning phase. Primordia are produced in higher temperatures (T>11°C) and more importantly, also in sclerotia without low temperature pre-treatment. The primordia were observed as early as after 7 days, and the number and size of the primordia increased with time and temperature. However, although an abundant presence of primordia was confirmed for temperature like T=20°C, sclerotia failed to produce stipes during the 70 days experiment duration, without exposure to lower temperatures.

Dissection of *S. sclerotiorum* sclerotia (isolate L5) identified the presence of structures distinctive from surrounding medulla hyphae that resembled the primordia described by Saito (1973). However, classification into different stages of primordial development as described by Saito (1973) was not possible due to the lack of any consistent pigmentation in the centre of primordia. Additionally, further developmental structures that included “active hyphae”, stipe initials and stipes were observed.

The size variation in observed primordia systematically increased with time and temperature for both single temperature and temperature transfer treatments. The

large variation in size of primordia observed for each treatment and timepoint is most likely due to the location where primordia were dissected as the distance between slices varied between 40 to 400 μm depending on sclerotium size and sample quality. Therefore, predominantly each primordium or stipe initial observed were only present in a single slice, while in contrast, the larger stipe structures, could be traced across a number of sections of the same sclerotium.

It was evident from the results that an increase in the number of primordia present in sclerotia was associated with increasing temperature and time from burial, irrespective of whether the sclerotia were conditioned at low temperature. Furthermore, the low temperatures of 4°C had an inhibitory effect on primordial production as only two primordia were observed after 70 days. For temperatures of 11, 17 and 20°C the first primordia were observed as early as 7 days without prior cold conditioning. These observations agree with previous studies, where Saito (1973) reported presence of primordia as early as 2 days after transfer to 15°C from 3°C but none prior to transfer. Similarly, Phillips (1986) reported that no primordia were present in sclerotia after conditioning in soil (field). Both authors therefore conclude that primordia are produced in temperatures favouring germination rather than conditioning as this study also indicates. Additionally, this work for the first time confirmed the presence of primordia in unconditioned sclerotia (i.e. not exposed to low temperatures); this is especially interesting as *S. sclerotiorum* isolate L5 was selected for this study particularly because it has a high requirement for cold conditioning to achieve germination (see Chapter 2.1).

Interestingly, the number of observed primordia for sclerotia transferred from 4°C to 17°C, 14 days after the transfer, showed very low numbers of primordia (5) but the highest number of "active hyphae" (17). Furthermore, 28 days after transfer (56th day from set up) the first appearance of stipes was observed, delayed by 7 and 14 days compared to the single temperature treatments at 11°C and 17°C, respectively, but reached the highest percentage germination recorded by the end of the experiment, 90% (70th day). The term "active hyphae" is new and aims to highlight observation of mobilized "active" cells collections close to rind, which show some staining and differentiation to regular medullar hyphae, but do not have the spherical shape which is well defined for primordia observation. These active hyphae were predominantly found in treatments after transfer from low to high temperature, and where stipes production was observed in increased numbers 7 to 14 days after. It is possible that the active hyphae serve as a platform where enzymatic and metabolic activities are

concentrated and where metabolites are transferred to, to facilitate a prompt development of apothecial stipes. However, more work would be required here, starting with analyses of the omitted data points, to allow for a more detailed time frame of the developmental changes to be constructed. Furthermore, various staining methods could be used for identification of different chemical compounds and metabolites. Chemical analyses of sclerotia using methods like mass spectrometry were also considered, however the rigid construction of sclerotia makes it difficult to brake sclerotia into powder.

The germination of *S. sclerotiorum* to produce stipes, in response to the different temperature treatments observed in this dissection experiment was consistent with the response in the main temperature experiments (Chapter 3), where the isolate L5 achieved 96% germination (Table 3.22) and the time to germination for 10% (T_{10}, Table 3.27) was 98 days for the single temperature treatment at 4°C. No germination was observed for 20°C. This provides additional information, beyond the 70 days of dissection experiment, that Isolate L5 sclerotia are capable of high germination at temperature as low as 4°C. Plus although they do not produce large number of primordia (at least not in the first 70 days), they do facilitate development of the few primordia into stipes when more time is provided at 4C. When transferred from 4C to a higher temperature smaller number of primordia is observed which develop fast into stipes. In contrast sclerotia maintained at a constant 20°C, are unable to germinate in significant numbers despite the presence of primordia from day 7 and the increase in numbers and size over the 70 days. The implication here is although primordia are present in sclerotia at high temperatures, these do not guarantee that germination will occur (not for 280 days). In contrast small and delayed number of primordia are produced at low temperature, but sclerotia are capable of germination when more time is allowed (~90 days). The best results for germination are achieved for transfer from low to high temperature (the opposite was not tested!), where the “active hyphae” observed after transfer seems to be facilitating a fast development of few recently developed primordia into stipes. Therefore, another factor is required, where the originally described conditioning process at low temperatures can be applicable, with a crucial distinction in process description. The discrepancy to original assumptions about the processes involved in carpogenic germination is about the subsequent order of the processes. As primordia are directly associated with sclerotia germination into stipes, their very early presence in unconditioned sclerotia and availability for the whole duration of dissection experiment is in contrast with our initial assumption of two subsequent processes where conditioning has to be completed for

subsequent rapid and high germination to occur (Clarkson et al., 2007). A parallel organization of the processes accruing at favourable conditions independently of each other progress would explain dissection experiment observations better. However, to test the independency of the two process a reverse order of the temperatures, including transfer from high to low, could be designed.

Finally, the dissection experiment was completed only for isolate L5 which was identified as the isolate requiring cold conditioning to achieve high and fast germination in higher temperatures. In contrast the isolate L6 is capable of fast germination at high temperatures without any or little conditioning, and furthermore time to germination at lower temperatures is generally longer than for the isolate L5 (Chapter 3.4). For a complete picture it would be vital to complete the microscopy analyses for isolate L6 sclerotia, to see whether structures identified for isolate L5 would be found in sclerotia for isolate L6 as well. Particularly the presence of “active hyphae” could be of interest, where eventually these could be observed in abundant numbers in single high temperature treatments. Generally, we would assume to see primordia presence from as early as 7 days, with increasing number and size with increasing temperature and time (similar to isolate L5, maybe even more promptly), unless the development of primordia into stipes could be observed, resulting in inhibition of primordia development as described by Saito (1973).

5 Soil moisture experiment

5.1 Introduction

Overall researchers agree that moist (not saturated) soil is required for carpogenic germination of *S. sclerotiorum* sclerotia (Phillips, 1987). Clarkson et al. (2004) observed germination for soil water potentials ≥-100kPa but that there was little or no germination at -300kPa.

Nepal and del Río Mendoza (2012) examined the effect of sclerotial water content on carpogenic germination of *S. sclerotiorum* reporting that smaller sclerotia imbibed water significantly faster compared to larger sclerotia, both in water and in the soil regardless of the saturation level and were able to fully saturate by 25h (large sclerotia). Furthermore, the level of sclerotial moisture content had a significant effect on the level of germination, with highest levels observed for fully saturated sclerotia while germination was arrested below 70-80% water content.

The effect of moisture along with light intensity and temperature on the production of *S. sclerotiorum* apothecia was examined by Sun and Yang (2000) for an isolate originating from Gilmore, IA, USA. Sclerotia were incubated at 4.5°C for 2 months and subsequently exposed to two light intensities (high and low), five temperatures (6 to 30°C at low light intensity and 10 to 30°C at high light intensity) and three levels of soil moisture (free water visible on surface of sand, sand near saturation, 50% of near saturation). Results showed that at low light the optimum temperature range for germination was 12-18°C irrespective of sand moisture, but at high light intensity the optimal temperature shifted to 20°C when sand moisture level was high.

Huang et al. (1998) reported that desiccation of *S. sclerotiorum* was an important factor that resulted in myceliogenic germination and hyphal growth for sclerotia from three *S. sclerotiorum* isolates collected on sunflower. Different degrees of dryness were attained by the sclerotia prior to incubation for 2 weeks at 25°C or room temperature at different RH levels. For fresh sclerotia, myceliogenic germination was observed only for 100% RH, while for desiccated sclerotia myceliogenic germination was more vigorous and occurred at 85 -100 % RH, but reduced with declining RH.

Foley et al. (2016) examined the effect of incubation temperature (4 weeks) of hydrated and desiccated sclerotia and the duration of sclerotia desiccation (1-21 days at 20°C after production) on germination of *S. sclerotiorum* isolates Sun-87 (Canada)

and 1980 (Nebraska) with the aim of identifying treatments that resulted in either myceliogenic or carpogenic germination. It was concluded that solely varying conditioning temperature (-20°C to 30°C) was not sufficient to trigger myceliogenic germination (in contrast to carpogenic germination). However, incubation of sclerotia in a desiccated state limited carpogenic germination and increased mycelial germination (up to 34%). Furthermore when 4 to 8-week-old sclerotia were desiccated for 1-21 days prior to placing at a temperature suitable for germination (16°C), a small but significant increase in myceliogenic germination was observed but with no significant difference between different durations of desiccation.

Mila and Yang (2008) studied the effect of fluctuating soil moisture on carpogenic germination of *S. sclerotiorum* (isolate originating from IA, USA) and concluded that soil water potential fluctuations were detrimental to germination and apothecia production. The germination of sclerotia under fluctuating moisture conditions was less than a tenth of the germination observed under constant saturation and the time for germination to occur was almost doubled.

Wu and Subbarao (2008) examined effects of interrupted soil moisture (dry periods) on carpogenic germination of *S. sclerotiorum* (isolate originating from CA, USA) where no apothecia were produced during the dry periods or immediately after restoring moist conditions. They also reported that a period of 10 to 20 days of low soil moisture completely arrested carpogenic germination and it took up to 35 days between rewetting and the appearance of new apothecia, regardless of when and for how long (10+ days) the dry period was applied.

Germination of *S. sclerotiorum* sclerotia predominantly occur in the upper soil level, near the surface. Soil temperature near the soil surface is affected more by the weather conditions, with considerable fluctuations in temperature and moisture on a daily basis compared to deeper levels. Rather than examining the effects of different levels of soil moisture, the aim of this chapter was to understand the effect of dry conditions applied at different times on carpogenic germination of sclerotia.

The soil moisture experiment (SME) consisted of two sub experiments run simultaneously. The first sub experiment (SME_S1) aimed to evaluate the effect of a dry period applied during the whole conditioning phase in S1, prior to the transfer to germination temperature in S2, while the second sub experiment (SME_S2) assessed the effect of dry periods of various lengths and timings during the

germination phase in S2, after a moist, cold incubation in S1 (S1 and S2 as explained in Chapter 3.2).

The following hypotheses were tested in SME_S1:

- Dry conditions during S1 will increase myceliogenic germination.
- Dry conditions during S1 will decrease carpogenic germination.
- Dry conditions during S1 will delay carpogenic germination.

The following hypotheses were tested in SME_S2:

- Dry conditions during S2 will increase myceliogenic germination.
- The duration of dry conditions during S2 will affect the level and time to carpogenic germination to produce stipes.
- The timing of dry conditions during S2 will affect the level and time of carpogenic germination to produce stipes

5.2 Methods

Sclerotia of two *S. sclerotiorum* isolates L5 and L6 (Chapter 2.1.) were produced as described in Chapter 2.2. Air dried sclerotia (size range 2.8 – 4.0mm) were soaked in water for 24h after which they were partially buried, by pressing into the surface of 50g of pasteurised compost contained in Petri dishes (9 cm diameter, 2.7 mm deep; Chapter 2). Oven dry compost was used as a dry treatment, while approx. 15ml of water was added for a moist treatment (approx. 50%, w/c) (Figure 5.1). Each Petri dish contained 25 sclerotia, with three replicates per treatment. Experimental units (EU) were randomized in opaque plastic boxes and kept under different controlled temperature conditions rooms in the dark. After sclerotia were buried, Petri dishes were sealed with Parafilm to minimize the loss of moisture for the 28 days duration of S1.

Figure 5.1 Petri dishes containing dry (left) and moist compost (right).

Both SME_S1 and SME_S2 were set up to include two temperature regimes similar to the previous temperature experiments (Chapter 3.2) with a transfer from S1 temperature T1, also referred to as “conditioning”, to S2 with temperatures T2. To ensure high and rapid carpogenic germination during the SME, these temperature regimes were selected close to the optimum for conditioning (T1 = 5°C) and germination T2 = 15°C (L5) and T2 = 17°C (L6) in respect of isolate differences observed in the temperature experiment (Chapter 3). A single temperature treatment at T2 (no conditioning) with constant moist conditions is included in SME_S1. The control treatment, which is shared by both sub experiments, represents the transfer treatment with constantly moist conditions

- SME_S1: *S. sclerotiorum* sclerotia (isolates L5 & L6) were exposed to both dry and moist conditioning periods of 28 days in the S1 phase (T1 = 5°C) followed by transfer to S2 (T2 =15 and 17°C) under constantly moist conditions, and to a single temperature treatment (only T2 = 15 and 17°C) with constantly moist conditions (Figure 5.2).

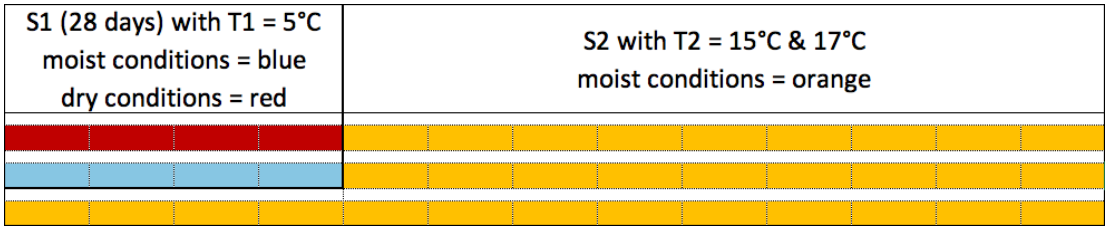

Figure 5.2 *S. sclerotiorum* sclerotia subjected standard S1 conditioning phase (T1 = 5°C for 28 days) under moist (blue bars) and dry (red bars) conditions before transfer into S2 phase (T2 = 15 and 17°C) with constant moisture level (yellow bars). A single temperature treatment (T2 = 15 and 17°C) with a constant moisture level was included (continuous yellow bars). Each cell represents one week.

- SME_S2: *S. sclerotiorum* sclerotia (isolates L5 & L6) were exposed to moist conditioning in S1 (T1 = 5°C, 28 days) and transferred to S2 (T2 = 15 and 17°C). In S2, dry periods of 7, 7+7, 14 and 28 days were introduced at the beginning of S2 and after 14 days from the transfer to S2 (Figure 5.3). A transfer treatment with constant moist conditions in S2 was included as a "*control moist*".

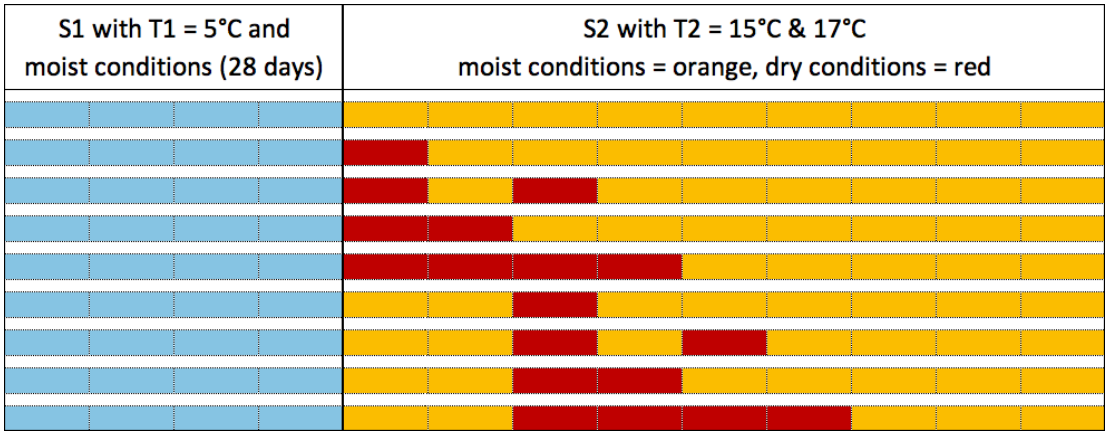

Figure 5.3. *S. sclerotiorum* sclerotia subjected standard S1 conditioning phase (28 days) under moist conditions (blue bars) before transfer into S2 phase at 15°C and 17°C. In S2 sclerotia were exposed to moist (yellow bars) and dry (red bars) periods. Dry periods were 7, 7+7, 14, and 28 days long and were applied immediately or 14 days after transfer. Each cell represents one week.

Sclerotia were observed for myceliogenic germination to produce mycelial hyphae (Figure 5.4, left) (soft degraded sclerotia as a result of myceliogenic germination and daughter sclerotia produced anew were removed from the experiment) and carpogenic germination to produce stipes (Figure 5.4, right) (germinated sclerotia were removed from experiment) once a week for 153 days after which all ungerminated sclerotia, were transferred to constant 11°C under moist conditions for an additional 160 days to check for viability.

Figure 5.4 Myceliogenic germination of *S. sclerotiorum* sclerotia (left), showing white mycelium with a daughter sclerotium of smaller size produced (bottom left); Carpogenic germination of *S. sclerotiorum* sclerotia (right) with production of stipes (bottom right).

Statistical analyses

Relevant statistics were calculated as described in Chapter 2. (Table 2.2): Maximum germination percentage (including angular transformation), myceliogenic germination percentage (including angular transformation), where both statistics are calculated from the number of viable sclerotia as described in Chapter 3. (Table 3.3) and time to various % of population germination, T_{25}, T_{50} and T_{75} (estimated from total number of viable sclerotia). All statistical analyses were by Analysis of Variance (ANOVA) and carried out in Genstat® (18th edition, VSN international Ltd.).

- SME_S1 used a randomized factorial design with 3 replicates used as a blocking factor, the main factors considered were "*Isolate*" (2 levels – L5 and L6), "T2" (2 levels - T2 = 15°C and 17°C), "*S1 conditions*" (3 levels – dry, moist, single T2 temperature = no conditioning) (Figure 5.2).
- SME_S2 also used a randomized design with 3 replicates as a blocking factor, with factors considered "*Isolate*" (2 levels – L5 and L6), "T2" (2 levels - T2 = 15°C and 17°C), "Timing of dry period" (2 levels - dry periods start immediately at transfer or 14 days later) and duration of dry period where the first test was comparing the total duration of dry period (7, 14, 28 days) and second test was comparing the interrupted and uninterrupted 14 day periods (Figure 5.3).

Important treatment terms were identified from the ANOVA table, with the relevant F-test statistics presented with degrees of freedom and probability values. For significant terms ($p<0.05$) treatments means were compared using standard errors of differences (s.e.d.) and least significant differences (l.s.d.) at the 5% significance level.

5.3 Results

5.3.1 Effect of dry period during S1 on germination of *S. sclerotiorum* sclerotia

Cumulative germination curves for *S. sclerotiorum* isolate L5 sclerotia (Figure 5.5) with moist conditioning (full line) at 5°C for 28 days showed high and fast germination for both T2 temperatures. Sclerotia exposed to the dry period in S1 performed worst, with delayed and decreased germination for both T2 temperatures, and with T2 = 15°C performing slightly better compared to T2 = 17°C, achieving higher level of germination at the end of the S2. However, the cumulative germination curves for dry "conditioned" sclerotia continue to increase (although slowly) to the end of the experiment, suggesting possible continual increase in level of germination if the experiment duration was longer. Sclerotia exposed to moist single temperature treatment (T = T2) showed erliest germination (by 14 and 21 days compared to moist and dry S1, respectively), however the distribution of germination times was wider (flatter cumulative germination curve) compared to moist conditioned and similar compared to dry conditioned sclerotia. Level of germination for the single temperature treatment with T = 17°C was lower compared to 15°C. The dry S1 conditioned treatements showed a much lower level of germination compared to the other two treatments by the end of the experimental period (153 days). However, the cumulative germination curves for dry "conditioned" sclerotia continue to increase (although slowly) to the end of the experiment (similar to Single temperature T=17°C), suggesting that if the experiments had been longer, germination level would most likely continue to increase.

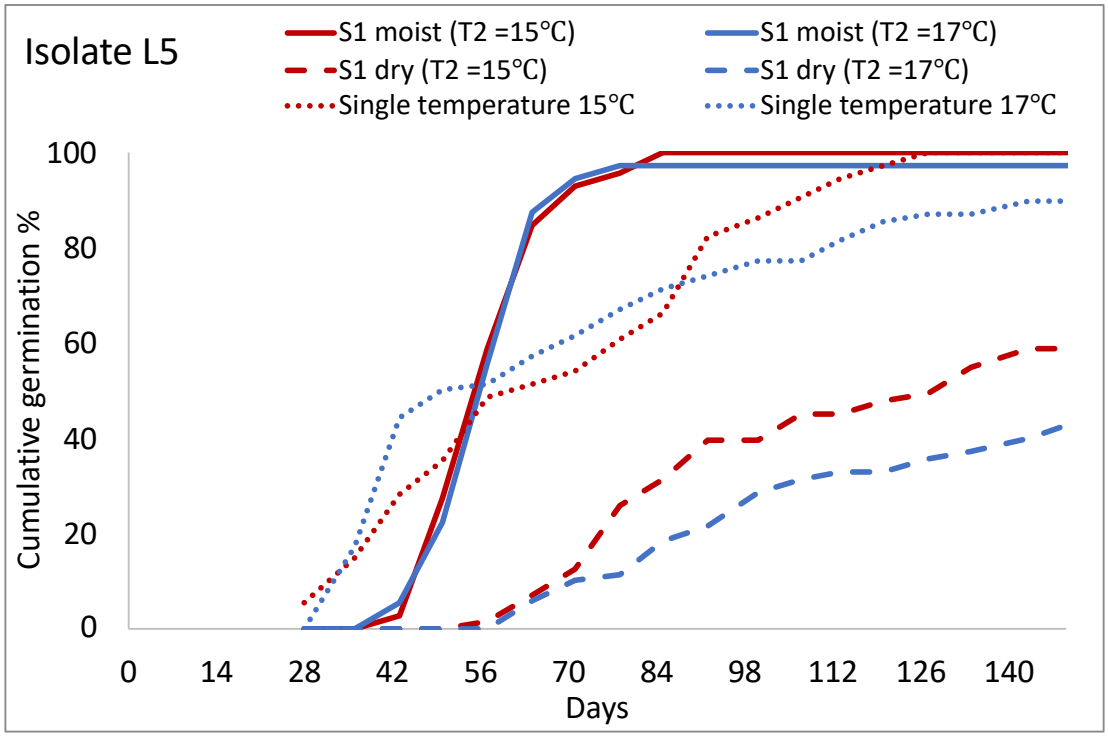

Figure 5.5 Cumulative germination curves produced for observed replicate means (3 rep) for *S. sclerotiorum* isolate L5. T2 = 15°C (red) and 17°C (blue) following incubation at T1 = 5°C (28 days) with either dry (dashed lines) or moist (solid lines) conditions at S1 and a single temperature treatment with only T2 (dots).

In contrast to *S. sclerotiorum* isolate L5, cumulative germination curves for isolate L6 sclerotia (Figure 5.6) indicated much less of an effect of the dry period in S1 or of the absence of S1 for T2 = 17°C. The moist conditioned sclerotia (full line) at 5°C for 28 days showed high and fast germination for both T2 temperatures. Sclerotia exposed to moist single temperature treatment (T = T2) showed erliest germination (by at least 7 and 21 days compared to moist and dry S1, respectively (first observation at the 28th day, end of S1)), however the distribution of germination times was wider (flatter cumulative germination curve) with 100% germination achieved with aproximately 42 days delay for both T2, compared to moist conditioned sclerotia. Sclerotia exposed to the dry period in S1 showed delayed start of germination for both T2 (by 14 days compared to moist conditioned sclerotia), where for T2 = 17°C, relatively fast germination followed and 100% germination was achieved with 42 days delay (compared to moist conditioned sclerotia, similar to single temperature treatmts). For T2 = 15°C, dry conditioned sclerotia cumulative germination curve was flatter, with continual increase towards the end of S2 but not reaching 100% and resulting in large delay towards the late percentiles (aproximately 80 days, compared to moist conditioned sclerotia at T2 = 15°C).

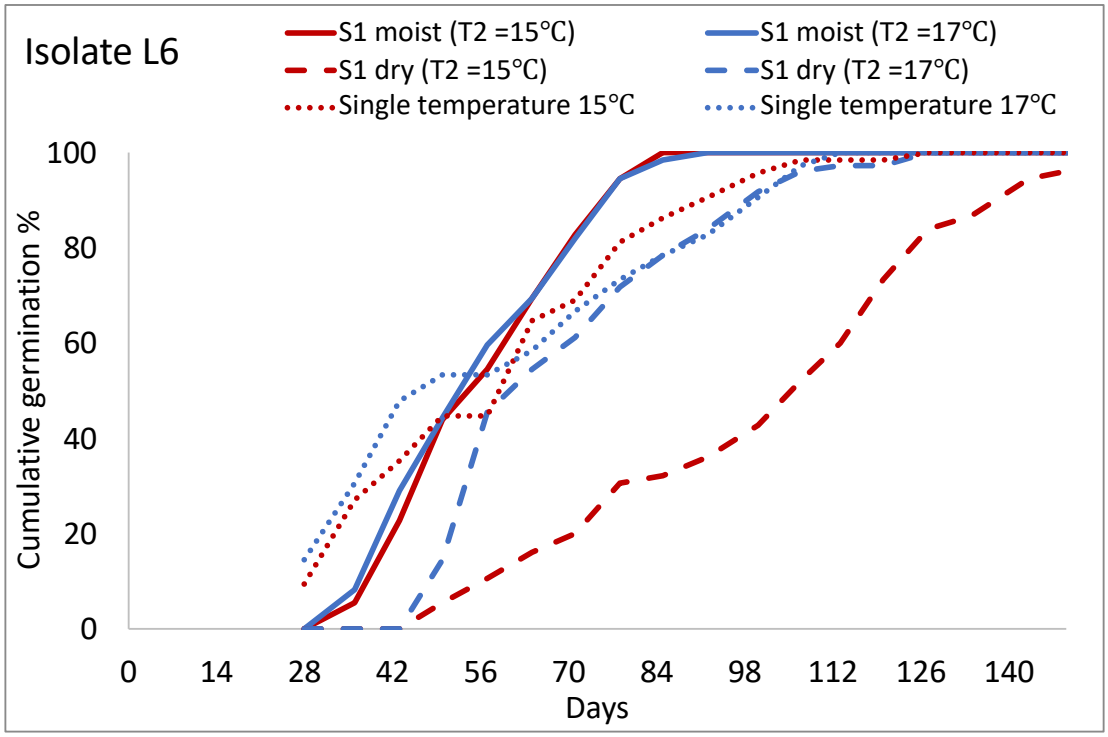

Figure 5.6 Cumulative germination curves produced for observed replicate means (3 rep) for *S. sclerotiorum* isolate L6. T2 = 15°C (red) and 17°C (blue) following conditioning at T1 = 5°C (28 days) with either dry (stripes) or moist (full) conditions at S1 and a control treatment with only S2 (no conditioning) (dots).

Myceliogenic germination

The analyses of the effect of S1 conditions on myceliogenic germination showed a significant effect of the main factor isolate ($F_{1,22}$=85.40, p<0.001), and significant interactions between isolate and S1 conditions ($F_{2,22}$=34.93, p<0.001), and between isolate and S2 temperature ($F_{1,22}$=5.56, p=0.028). For the isolate L5 only, a significantly higher mycelial germination was observed when dry conditioning in S1 was introduced, 17% and 18% (T2 = 15 and 17°C, respectively), compared to 0% and 2% (T2 = 15 and 17°C, respectively) for both moist conditioning in S1 and for the single T2 temperature (Figure 5.7**).**

Carpogenic germination

The maximum germination percentage analyses showed significant effects of main factors isolate ($F_{1,22}$=76.88, p<0.001) and S1 conditions ($F_{2,22}$=49.35, p<0.001) and significant interactions between isolate and S1 conditions ($F_{2,22}$=28.92, p<0.001), and between isolate and S2 temperature ($F_{1,22}$=13.61, p=0.001). The isolate L6 reached 100% germination except for the dry conditioning in S1 and T2 = 15°C, where 97% germination was observed (Figure 5.7). In contrary, for the isolate L5 the dry conditioning in S1 significantly decreased the level of carpogenic germination, 62%

and 51% (T2 = 15 and 17°C, respectively). For the remaining treatments and T2 = 15°C, isolate L5 achieved 100% germination. For isolate L5 when exposed to moist conditioning in S1 (T1 = 5°C) and transferred to T2 = 17°C, only a small decrease in germination was observed, 2%, and for the single temperature treatment a significant decrease, 9%, in germination was observed.

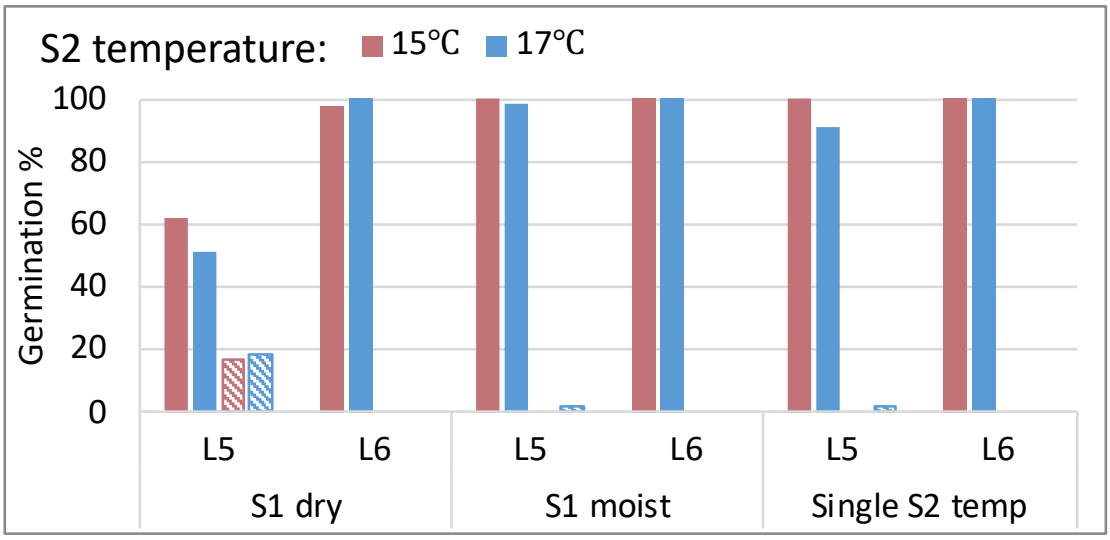

Figure 5.7. Maximum percentage of carpogenic (CG, full colour) and myceliogenic (MG, strips) germination (beck-transformed ANOVA means) after 153 days at S2 temperatures 15°C (red) and 17°C (blue) following incubation at T1 = 5°C (28 days) with either dry or moist conditions at S1 conditioning and a control treatment with only S2 (no conditioning) for two S. sclerotiorum isolates (L5 & L6) (angular transformed ANOVA results: CG: d.f. = 22, s.e.d.= 4.585, l.s.d.= 9.510, MG: d.f. = 22, s.e.d.= 2.888, l.s.d.= 5.990).

Time to carpogenic germination

To the asses the distribution of germination times, three percentiles were selected for analyses T_{25}, T_{50}, T_{75}.

For the T_{25}, analyses showed a strong significant effect of two main factors: isolate ($F_{1,21}$=15.53, p<0.001) and S1 conditions ($F_{2,21}$=39.02, p<0.001) along with significant interaction of these two factors ($F_{2,21}$=5.63, p=0.011). This was displayed by a delayed germination for both isolates when exposed to dry conditions in S1 compared to moist conditions in S1 or the single temperature treatment and longer T_{25} for isolate L5. For the dry conditions in S1 the T_{25} for isolate L5 was significantly longer compared to L6 for both T2, 21 and 47 days at T2 = 14 and 17°C, respectively (Figure 5.8, upper). The isolates showed contrasting responses to T2 when conditioned at dry S1 (although not significant), where T_{25} was longer for isolate L5 and shorter for L6 at T2 = 17°C compared to germination times at T2 = 15°C.

The analyses of T_{50} showed complex interactions where again the main factors had significant effects (isolate: $F_{1,20}$=20.58, p<0.001, S1 conditions: $F_{2,20}$=75.31, p<0.001) and there was a significant interaction between isolate, S1 conditions and S2 temperature ($F_{2,20}$=12.54, p<0.001). Similarly, to T_{25}, the T_{50} was delayed for treatment with dry S1 conditions compared to moist S1 conditions and the single temperature treatment. However, the contrasting responses between the isolates under dry S1 conditions to the T2 temperature was much more profound, where at T2 = 15°C T_{50} was similar for both isolates, 112 (L5) and 107 (L6) days, while at T2 = 17°C T_{50} was delayed by 41 days for isolate L5 and accelerated by 46 days for isolate L6 (Figure 5.8, middle)

The analyses of T_{75} showed a significant effect of the main factors S1 conditions ($F_{2,18}$=37.68, p<0.001), S2 temperature ($F_{1,18}$=6.76, p=0.018) and a significant interaction of these two factors ($F_{2,18}$=13.59, p<0.001). The T_{75} was fastest for the treatment with moist S1 conditioning irrespective of the isolate and T2, with germination times of 61 days for isolate L5 (both T2) and 67 and 64 days for isolate L6 (T2 = 15 and 17°C, respectively). The single temperature treatment showed a delay in germination times for isolate L5 by 17 and 25 days, and by 3 and 18 days for isolate L6 (T2 = 15 & 17°C, respectively). The dry S1 conditioning almost doubled the time for T_{75} at T2 = 15°C, with a delay of 61 (L5) and 54 (L6) days compared to moist S1 conditions. For isolate L6 at T2 = 17°C germination was delayed by 21 days. For the T_{75}, isolate L5 no replicate reached 75% germination for S1 = dry and T2 = 17°C during the 150 days of experiment duration. A missing value was therefore included in the analyses for this treatment and the value estimated by ANOVA of T_{75} = 84 days (delay by 23 days) was clearly an underestimation, especially compared to the earlier percentiles (T_{25} = 100 and T_{50} = 153 days). Furthermore, the cumulative germination curve (Figure 5.5) was consistently increasing to the end of the experiment duration, suggesting isolate L5 would continue germination if more time had been allowed. An arbitrary value of T_{75} = 160 days was therefore suggested and included in graphical presentation of ANOVA results (Figure 5.8, bottom, light blue bar) to depict the response more realistically, showing a similar trend as observed for T_{25} and T_{50}, where the dry S1 conditioning caused delay in germination with a comparable response for both isolates at T2 = 15°C, and a contrasting response at T2 = 17°C, where for isolate L5 germination was further delayed and for isolate L6, contrary, the delay in germination is smaller.

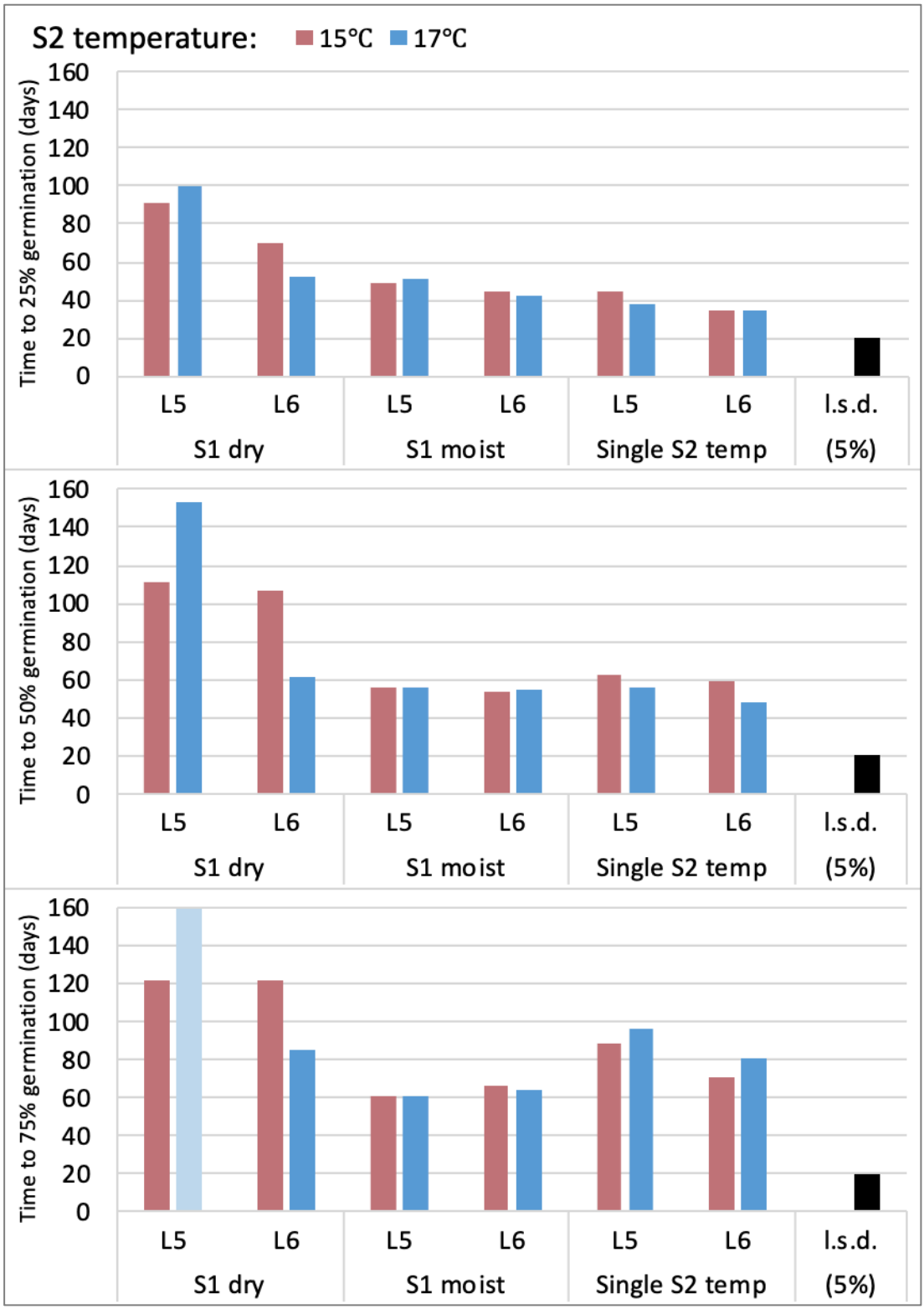

Figure 5.8 ANOVA means for the time to germination of 25% (top), 50% (middle) and 75% (bottom) of sclerotia for transfer treatments with conditioning at 5°C for 28 days in S1 at either dry or moist conditions followed by S2, and a single temperature treatment with only S2 temperatures T2 = 15°C (red) and 17°C (blue) for two *S. sclerotiorum* isolates, L5 & L6; For isolate L5, S1 = dry and T2 = 17°C the T_{75} was not observed (75% germination was not reached in the 153 days of the experiment duration), therefore an arbitrary value of 160 days is presented in the graph (light blue), representing a more realistic value compared to ANOVA estimate (84 days not shown) and where the T_{25} and T_{50} were taken in account; (T_{25}: d.f. = 21, s.e.d.= 9.68, l.s.d. = 20.14, T_{50}: d.f. = 20, s.e.d.= 9.83, l.s.d. = 20.50, T_{75}: d.f. = 18, s.e.d.= 9.28, l.s.d. = 19.50).

5.3.2 Effect of dry period in S2 on germination of *S. sclerotiorum* sclerotia

Cumulative carpogenic germination curves for *S. sclerotiorum* isolates, L5 (Figure 5.9) and L6 (Figure 5.10), showed a comparable response to all dry periods imposed in S2 for both isolates, with a high, fast and relatively uniform germination. However, during the dry periods no germination was recorded and therefore the germination curves were flat during these periods. When the dry periods were omitted from the plots, generally the curves aligned well with the control treatment (constant moisture), irrespective of the duration or timing of dry period as well as irrespective the isolate or T2 temperature (bottom figures: Figure 5.9 (L5), Figure 5.10 (L6)). The flat parts of the cumulative curves, corresponding with no germination in the dry periods, were more evident for isolate L6 and T2 = 17C, because of an earlier germination start, shortly after transfer.

For the isolate L5, the 28 days dry period treatment applied 14 days after transfer to S2, seemed to result in the longest delay when combined with T2 = 17C. This could be explained by the supra-optimum germination temperature for isolate L5 introducing additional stress for sclerotia (Figure 5.9), For isolate L6, almost 40% germination occurred in the first 14 days after transfer to S2, therefore, the introduction of the delayed dry periods came in the later stage of the germination process compared to other treatments. Interestingly even after rewetting the germination was renewed, and the 28 days dry period introduced 14 days after S2 transfer caused some delay in germination times (Figure 5.10).

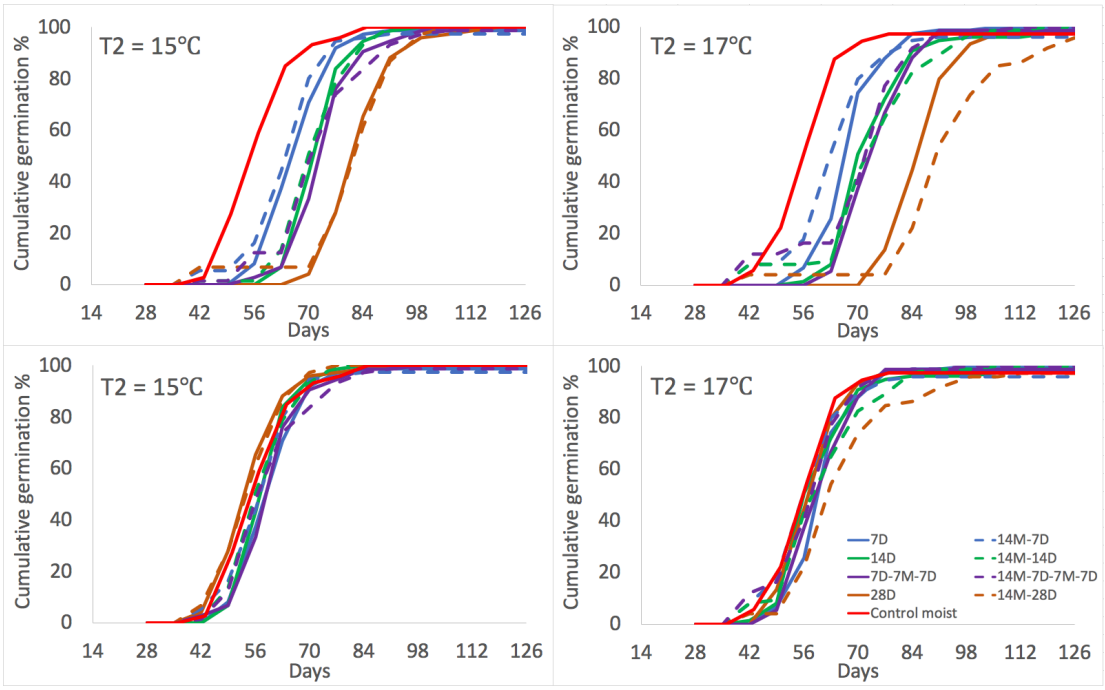

Figure 5.9 Cumulative germination curves observed for *S. sclerotiorum* isolate L5 (top) and with dry periods removed from observation (bottom). Sclerotia were incubated at 5°C for 28 days and transferred to T2 = 15°C and 17°C with dry periods of 7, 7+7, 14 and 28 days introduced at the beginning of S2 and after 14 days at S2. Control moist – constant moist condition in S2. Curves are replicate means (3 rep).

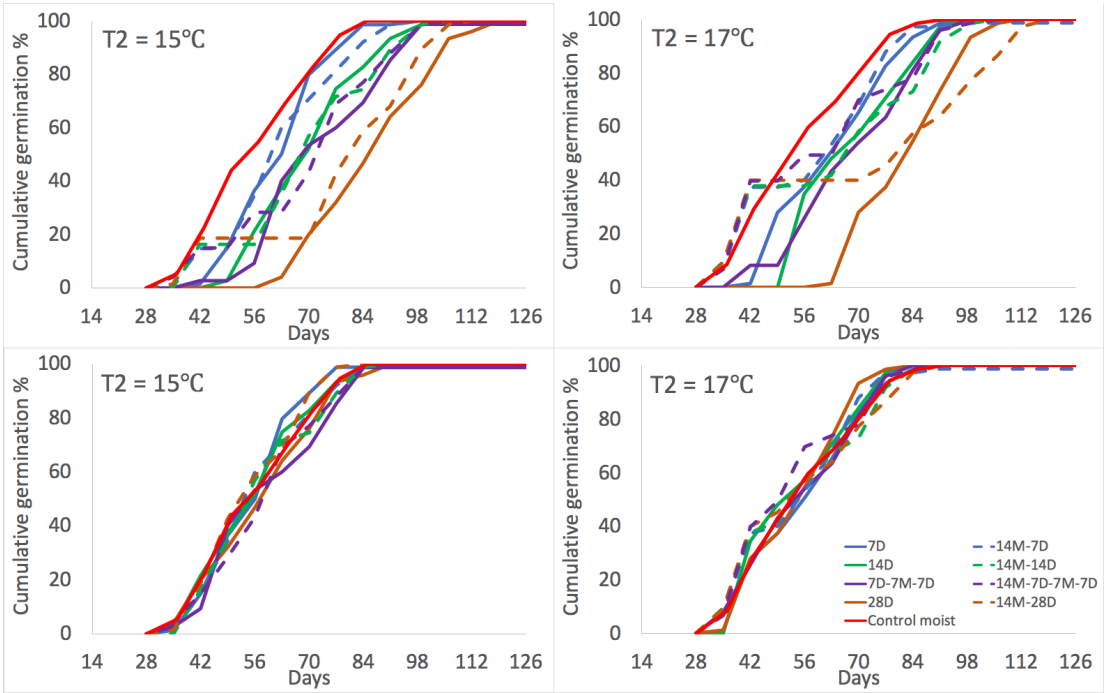

Figure 5.10 Cumulative germination curves observed for *S. sclerotiorum* isolate L6 (top) and with dry periods removed from observation (bottom). Sclerotia were incubated at 5°C for 28 days and transferred to T2 = 15°C and 17°C with dry periods of 7, 7+7, 14 and 28 days introduced at the beginning of S2 and after 14 days at S2. Control moist – constant moist condition in S2. Curves are replicate means (3 rep).

Myceliogenic germination

The analyses of the level of myceliogenic germination showed a significant effect of main factor isolate ($F_{1,70}$=4.29, p=0.042) and a significant interaction between factors isolate, T2, dry period and the duration of dry period ($F_{2,70}$=3.71, p=0.03), although the general level of MG was very low (<2%, Table 5.1). Isolate L6 was almost unaffected by the treatments examined (only 2 sclerotia), while the isolate L5 showed significantly higher MG for the moist control treatment at T2 =17°C (Table 5.1).

MG %	T2	Dry timing	Dry period duration (days)				Control moist
			7	7+7	14	28	
Back transf. L5	15°C	28	0.45	0	0	0	0
		42	0.91	0.45	0	0	
	17°C	28	0	0.45	0	0.45	1.91
		42	0	0	0	0.45	
Back transf. L6	15°C	28	0	0.45	0	0	0
		42	0	0	0	0	
	17°C	28	0	0	0	0	0
		42	0.45	0	0	0	
Angular transf. L5	15°C	28	3.85	0	0	0	0
		42	5.48	3.85	0	0	
	17°C	28	0	3.85	0	3.85	7.94
		42	0	0	0	3.85	
Angular transf. L6	15°C	28	0	3.85	0	0	0
		42	0	0	0	0	
	17°C	28	0	0	0	0	0
		42	3.85	0	0	0	
s.e.d.			2.841				
l.s.d.			5.666 (t=1.994, p=0.05, d.f.=70)				

Table 5.1 ANOVA estimated means for angular transformed and back-transformed myceliogenic germination observed for *S. sclerotiorum* isolates L5 and L6 (total viable sclerotia).

Carpogenic germination

Generally, a high level of germination (~100%) was observed for both isolates and all treatments. The maximum carpogenic germination percentage analyses (Table 5.2) showed similar results to myceliogenic germination, a significant effect of the main factor isolate ($F_{1,70}$=9.24, p=0.003) and a significant interaction of *S. sclerotiorum* isolate, T2, dry period and the duration of dry period ($F_{2,70}$=3.94, p=0.024). The carpogenic germination for isolate L5 was significantly lower (however only by ~2%) for T2 = 17°C for constantly moist S2 and for 28 days dry period in S2, irrespective of timing, compared to other treatment combinations. The level of germination for isolate L6 was unaffected by factors introduced with ~100% germination for all treatments. For both isolates no germination was observed during dry periods.

Maximum germination %	T2	Dry timing	Dry period duration (days)				Control moist
			7	7+7	14	28	
Back transf. L5	15°C	28	100	100	100	100	100
		42	99	100	100	100	
	17°C	28	100	100	100	98	98
		42	100	100	100	98	
Back transf. L6	15°C	28	100	100	100	100	100
		42	100	100	100	100	
	17°C	28	100	100	100	100	100
		42	100	100	100	100	
Angular transf. L5	15°C	28	86	90	90	90	90
		42	85	86	90	90	
	17°C	28	90	86	86	82	82
		42	86	90	90	82	
Angular transf. L6	15°C	28	90	86	90	90	90
		42	90	90	90	90	
	17°C	28	90	90	90	90	90
		42	90	90	90	90	
s.e.d.			3.146				
l.s.d.			6.275 (t=1.994, p=0.05, d.f.=70)				

Table 5.2 ANOVA estimated means for angular transformed and back-transformed carpogenic germination (values are rounded) observed for S. sclerotiorum isolate L5 (total viable sclerotia).

Time to carpogenic germination

For both isolates and all examined percentiles, there was no significant evidence for a difference between 14 days dry period applied continuously or interrupted (7 days dry + 7 days moist + 7 days dry)(T_{25}: Table 5.3, T_{50}: Table 5.4, T_{75}: Table 5.5), therefore duration of dry period is generally more important than timing. The effect of timing and interrupted dry period would possibly become more relevant if the middle (in-between) moist period(s) would stimulate sufficient germination to pass a particular percentile.

Analyses of time to 25% germination showed complex responses to factors examined with a significant effect of the main factors (Isolate: $F_{1,70}$=297.80, T2: $F_{1,70}$=36.86, and dry conditions in S2: $F_{1,70}$=168.71, all $p<0.001$) and significant interaction between isolate, T2, dry conditions, timing and duration of dry period (7+7 and 14 grouped) ($F_{2,70}$=5.59, p=0.006). Generally, for T_{25} isolate L5 germinated slower compared to L6. The T2 response was contrasting for both isolates where L5 germinated faster and L6 slower at T2 = 15°C than at T2 = 17°C. Both isolates responded to dry period with significantly delayed germination time, where the delay was principally corresponding with the duration of dry period applied (for values on difference between T_{25} control moist and dry treatments see Appendix 44). The timing of dry period showed a significant effect for isolate L6 at T2=17°C, where the T_{25} was

predominantly reached before the 42nd day (*39 days, Table 5.3), therefore the delayed dry period was not applied to the earlier percentiles and the T_{25} observed was comparable with control moist treatment (no dry period), 43 days.

Isolate	T2	Dry timing (day)	Control moist	Dry period duration (days)			
				7	7+7	14	28
L5	15°C	28	49	61	68	66	76
		42		57	65	65	76
	17°C	28	51	62	70	66	80
		42		58	67	67	85
L6	15°C	28	44	53	61	57	73
		42		53	59	60	72
	17°C	28	43	51	56	54	69
		42		44	*39	*39	*39
s.e.d.			3.034				
l.s.d.			6.051 (t=1.994, p=0.05, d.f.=70)				

Table 5.3 ANOVA estimated means for T25 observed for S. sclerotiorum isolates L5 and L6 comparing two S2 temperatures (T2 = 15 and 17°C) and various durations of dry period initiated directly after transfer (28th day) and 14 days after transfer (42nd day) to S2 and a control moist treatment with no dry period in S2; Colour gradient shows the variation in germination times: red=shortest, yellow = middle, green = longest T25; *25 % germination achieved prior the introduction of dry period.

Analyses of time to 50% and 75% germination had significant main factors: isolate (T_{50}: $F_{1,70}$=11.93, p<0.001, T_{75}: $F_{1,70}$=7.31, p=0.009) and dry conditions in S2 (T_{50}: $F_{1,70}$=115.64, p<0.001, T_{75}: $F_{1,70}$=116.05, p<0.001), a strong significant interaction between dry conditions in S2 and duration of dry period (T_{50}: $F_{2,70}$=116.05, p<0.001, T_{75}: $F_{2,70}$=107,78, p<0.001) and a significant interaction between isolate and T2 which is weaker with the later percentile (T_{50}: $F_{1,70}$=4.14, p=0.046, T_{75}: $F_{1,70}$=3.94, p=0.051). Generally, for the response to dry period and the duration of dry period, both T_{50} and T_{75} are consistent with T_{25}, where both isolates responded to dry period with significantly delayed germination time, and the delay was principally corresponding with the duration of dry period applied (for values on T_{50} and T_{75} difference between control moist and dry treatments see Appendix 45 and Appendix 46, respectively). The difference between isolates and the effect of the isolate by S2 temperature interaction was changing with the later percentile, where for the T_{50} generally L5 germinated slower compared to L6 and the T2 response was different for both isolates where L5 germinated faster and L6 slower at T2 = 15°C than at T2 = 17°C (Table 5.4). Contrastingly, for the T_{75}, the isolate L5 germinated generally faster compared to L6 and L5 germinated faster and L6 slower at T2 = 15°C than at T2 = 17°C (Table 5.5).

Isolate	T2	Dry timing (day)	Control moist	Dry period duration (days)			
				7	7+7	14	28
L5	15°C	28	56	65	73	71	81
		42		64	71	70	81
	17°C	28	56	66	74	70	85
		42		63	72	72	91
L6	15°C	28	54	61	70	68	85
		42		60	72	71	80
	17°C	28	55	62	67	65	83
		42		59	61	67	82
s.e.d.			3.984				
l.s.d.			7.947 (t=1.994, p=0.05, d.f.=70)				

Table 5.4. ANOVA estimated means for T_{50} observed for S. sclerotiorum isolates L5 and L6 comparing two S2 temperatures (T2 = 15 and 17°C) and various durations of dry period initiated directly after transfer (28th day) and 14 days after transfer (42nd day) to S2 and a control moist treatment with no dry period in S2; Colour gradient shows the variation in germination times: red=shortest, yellow = middle, green = longest T_{50}.

Isolate	T2	Dry timing (day)	Control moist	Dry period duration (days)			
				7	7+7	14	28
L5	15°C	28	61	70	77	76	86
		42		69	78	76	87
	17°C	28	60	71	78	78	91
		42		71	77	81	103
L6	15°C	28	67	69	86	79	97
		42		72	81	80	94
	17°C	28	64	73	82	79	91
		42		72	75	83	97
s.e.d.			4.365				
l.s.d.			8.706 (t=1.994, p=0.05, d.f.=70)				

Table 5.5 ANOVA estimated means for T_{75} observed for S. sclerotiorum isolates L5 and L6 comparing two S2 temperatures (T2 = 15 and 17°C) and various durations of dry period initiated directly after transfer (28th day) and 14 days after transfer (42nd day) to S2 and a control moist treatment with no dry period in S2; Colour gradient shows the variation in germination times: red=shortest, yellow = middle, green = longest T_{75}.

5.4 Discussion

Imposing dry periods on sclerotia of *S. sclerotiorum* generally caused an increase in myceliogenic germination for isolate L5 (up to 18% at T2 = 17°C), similarly to results presented by Foley et al. (2016) where incubation of sclerotia in a desiccated state limited carpogenic germination, and increased myceliogenic germination up to 34%. Huang et al. (1998) hypothesized that increased myceliogenic germination after cycles of dry and wet conditions was due to severe injury to top layers of sclerotial tissue, resulting in release of nutrients supporting the growth of hyphae. However, the increased myceliogenic germination was not consistent for both isolates, suggesting

an isolate specific response rather than a result of physical (mechanical) damage to sclerotial structures.

Carpogenic germination was significantly influenced by dry conditioning introduced in S1 for *S. sclerotiorum* isolate L5, where considerably lower germination (62%) was achieved by the end of the experimntal period (153 days), however the cumulative germination curve was persistently increasing, suggesting a continuation of germination if more time was allowed.

The time to germination for the earlier percentile was shortest for the single temperature treatment and longest for the dry conditioned sclerotia in S1. For T_{50} the difference in germination times for the single temperature and moist conditioned in S1 treatments was negligible and for the T_{75} the germination was fastest for the moist conditioned sclerotia in S1. The dry conditioning in S1 significantly delayed germination times, consistently for all percentiles and both isolates. Furthermore, the effect of T2 temperature following dry conditioning in S1 affected germination times for the isolates in different ways. At T2 = 15°C germination times achieved for both isolates where comparable, T_{50} = 112 (L5) and 107 (L6) days, however for T2 = 17°C germination time for isolate L5 was further delayed to 153 days and for isolate L6 the delay became reduced, only 61 days. The interaction between isolate and T2 temperature observed for various conditions introduced at S1, where isolate L5 generally performed better at T2 = 15°C and isolate L6 at T2 = 17°C, was consistent with the results presented in Chapter 3.3 (Main Temperature experiment), where isolates showed different temperature requirements. Association between optimum temperature and moisture level was reported by Sun and Yang (2000), where increased moisture (and light intensity) increased the range of optimum temperatures.

The impact of dry period introduced in S2 had a small effect on the level of mycelial and carpogenic germination on isolate L5 and none on isolate L6 at S2 temperature close to optimum. Although results were significant, the observed myceliogenic germination was very small 0-2% for isolate L5 and 0-0.45% for isolate L6 and the decrease of carpogenic germination was 0-2% for L5 and ~0% for isolate L6.

Arrested germination was observed during the dry periods similarly to Wu *et al.* (2008),and both isolates responded to dry periods in S2 with a delayed germination time compared to constantly moist conditions. Germination resumed after moist conditions were reintroduced and the delay was principally corresponding (L5=-2/+15, L6=-8/+5 days) with the duration of the dry period applied. The largest

deviation from the duration of the dry period, 15 days (next close was +6 days), was observed for isolate L5, T_{75}, T2 = 17°C and 28 days dry period applied after 14 days from transfer to S2. This is in contrast to the observations of Wu *et al.* (2008), where he reports that 10 to 20 days of low soil moisture can completely arrest carpogenic germination of *S. sclerotiorum* sclerotia, with up to 35 days between rewetting and the appearance of new apothecia, regardless of when and for how long (10+ days) the dry period was applied. Furthermore, the large delay observed for this particular treatment could be a result of a combination of two factors: a longer period of dry and the supra-optimum germination temperature (17°C for isolate L5), where both conditions could function as stresses on carpogenic germination of sclerotia. Examination of the impacts of further temperatures could bring insight for a possible interaction of T2 temperatures and episodes of dry conditions affecting *S. sclerotiorum* sclerotia germination, as the observation for isolate L5 and T2= 17°C implies.

However, the observation of arrested and renewed germination of *S. sclerotiorum* sclerotia after moist conditions were reintroduced after dry periods of 7 to 28 days in S2, with the germination times generally delayed only by the period of dry conditions, suggesting that germination rates at the corresponding temperatures were unaffected (predominantly), is positive. A similar assumption was used to account for changing moisture conditions in the field by ADAS to predict *S. sclerotiorum* sclerotia germination in field (Clarkson et al., 2007), where a threshold model was used if the average 24 hour temperature was between 12°C to 20°C, and with germination accumulation arrested unless at least 4mm of rain was observed in the past 4 days. Use of such a threshold model is more practical than the use a soil moisture model for germination rates (similar to the rates model for temperature) as the soil moisture is a more complex factor, more challenging to measure and control either in field or in controlled environment. Furthermore, the facility to collect accurate soil moisture data representing whole fields and/or regions is a major challenge.

6 Model Development

6.1 Introduction

The existing forecasting model (Clarkson *et al.*, 2007) assumes that a cold conditioning phase must be completed before subsequent rapid germination can occur with both process rates being dependent on temperature when soil moisture is not limiting. This model requires a subsequent order of processes leading to final production of apothecia (Figure 6.1).

Figure 6.1 Schematic drawing of Clarkson's sequential model for processes involved in carpogenic germination of S. sclerotiorum sclerotia.

The model proposed by Clarkson at.al (2007) predicts the mean time to germination by numerical integration of two functions representing temperature-dependent rates for both processes. Over the range of observed temperatures, 5 to 20°C, an exponential curve was used to model the relationship between conditioning rate (CR) and temperature (Equation 6.1), including three parameters *a*, *b*, and *k* (Table 6.1), and an Arrhenius curve was proposed to model the germination rate (GR) (Equation 6.2), including two parameters d_0 and d_1 (Table 6.1).

$$\boldsymbol{CR = a + be^{-kT}}$$

Equation 6.1 Equation describing exponential relationship between conditioning rate (CR) and temperature (T), where a, b and k are estimated parameters (Clarkson et al., 2007).

$$\boldsymbol{GR = exp\left(\frac{d_0 + d_1}{T + 273}\right)}$$

Equation 6.2 Arrhenius equation describing relationship between germination rate (GR) and temperature (T), where d_0 and d_1 are estimated parameters (Clarkson et al., 2007).

Isolate	Value/ s.e.	Conditioning			Germination	
		a	b	k	d_0	d_1
13	value	0.03273	1.000	1.498	31.12	-10,138
	s.e.	0.00395	…	0.398	4.36	1.236
TM	value	0.01056	1.28	0.435	24.8	-8.422
	s.e.	0.001	1.61	0.118	3.38	961

Table 6.1 Model parameters estimated for *S. sclerotiorum* isolates 13 and TM, including standard errors (s.e.) (Clarkson et al., 2007).

The fitted curves for two *S. sclerotiorum* isolates, 13 and TM, as used in the published study, exhibit a continual decrease in conditioning rate with increasing temperature and a continual increase in germination rate with increasing temperature (Figure 6.2).

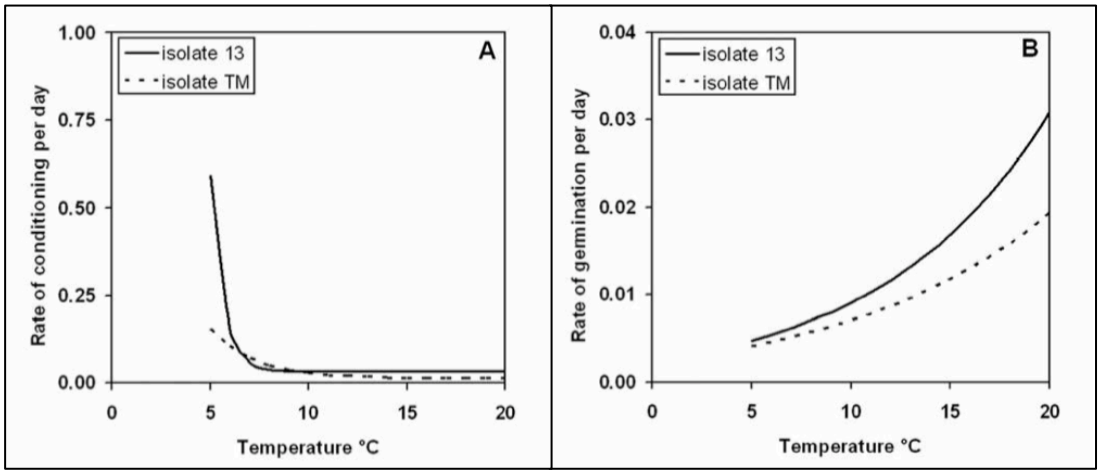

Figure 6.2 Fitted conditioning (A) and germination (B) rates for two *S. sclerotiorum* isolates 13 and TM (Clarkson *et al.*, 2007).

The model calculates the time to germination of 50% population by accumulation of a portion of conditioning and germination process for a period of time (depending on the frequency of temperature recordings) until the process is completed (accumulation reaches one). The time to germination for a certain percentage of sclerotia is then estimated assuming a lognormal distribution for times to germination, with a fixed standard deviation (s = 0.1417, for both isolates) of the corresponding normal distribution. Additionally, the model includes a moisture threshold amendment to account for the changes in field environmental conditions (moisture) where for the air temperatures between 12 – 20°C a total rainfall of 4mm in the past 4 days is required for the accumulation of the germination rate to proceed. Outside of this range the model does not have moisture requirement condition.

6.2 Field Germination

S. sclerotiorum isolates L5 and L6 were also used in field trials set up at Wellesbourne, UK to observ sclerotia germination under natural conditions. This data could be potentially used for model validation.

Sclerotia of *S. sclerotiorum* isolates L5 and L6 produced as described in Chapter 2.2 were buried in bare soil (no shading or canopy cover) at different timepoints (16 in total), from October 2015 to March 2017, and assessed for production of apothecia. Sclerotia of each isolate (25) were buried within 5x5 grids (mesh size 1.5 cm), approximately 1 cm deep and covered with soil (Figure 6.3).

Figure 6.3 A, Plastic grid with sclerotia just before burial; B, Apothecia produced by germinating sclerotia.

For each *S. sclerotiorum* isolate and burial time there were four replicate grids that were arranged in different locations over four blocks giving a total of 48 locations per block (organized in 12 rows x 4 columns). Burials were randomly allocated to these locations (1-48) (Figure 6.4). Environmental conditions (rainfall, air temperature, soil temperature and soil moisture were monitored at 1-hour intervals using a DL2e Data Logger (Delta-T, UK).

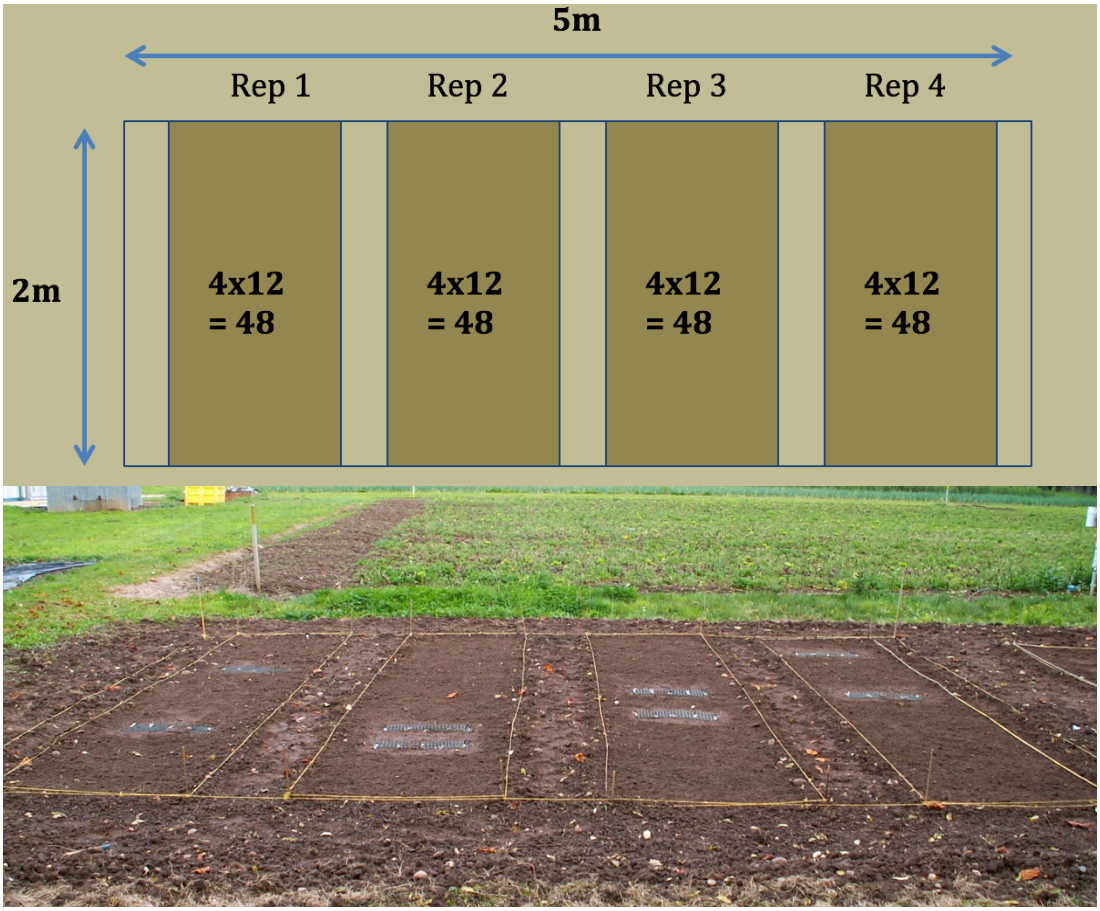

Figure 6.4. Wellesbourne field experiment location plan (top) and reality (bottom). Field was divided into 4 replicate blocs with 48 locations (organized in 12 rows and 4 columns) for possible sclerotia burial.

During the field experiments two contrasting springs were experienced (Figure 6.5):

- A warm spring in 2016 - soil temperature (orange line, Figure 6.5) after 25/04/2015 increased up to 20°C and fluctuated between 16 to 19°C till the end of May. During May and June, a substantial drop in soil temperature (<15°C) occurred for a couple of days, followed by repeated increases in temperature at the beginning of June up to 25°C, followed by substantial rain (blue line, Figure 6.5) and decrease in temperature to 17 – 20°C fluctuation for couple of days. Shortly after heavy rain first germination was observed in the middle of June, with a peak in the second half of June, at soil temperatures fluctuating around 20°C.
- A considerably colder spring in 2017 (compared to 2016) - where by the end of April soil temperature dropped below 10°C (air temperature <5°C) and started to rise up to 16°C (fluctuating around 15°C) by the time of the first germination (orange line, Figure 6.5), observed mid-May following a substantial amount of rainfall in the first half of May (blue line, Figure 6.5).

These two contrasting spring weather conditions were even more interesting as the buried *S. sclerotiorum* isolates showed a distinctive pattern in germination. Isolate L6 germinated readily in both years, where isolate L5 germinated only in spring 2017 (Figure 6.5). This observation corresponds with the observations and outcomes from the main temperature experiment (Chapter 3.4), where optimum germination temperatures identified for isolate L5 ranged from 11 to 14°C and for isolate L6 from 14 to 20°C. Furthermore, for isolate L6 a second germination later in year was observed (2016 and 2017). However, for both isolates the germination was always associated with substantial period of rain prior germination.

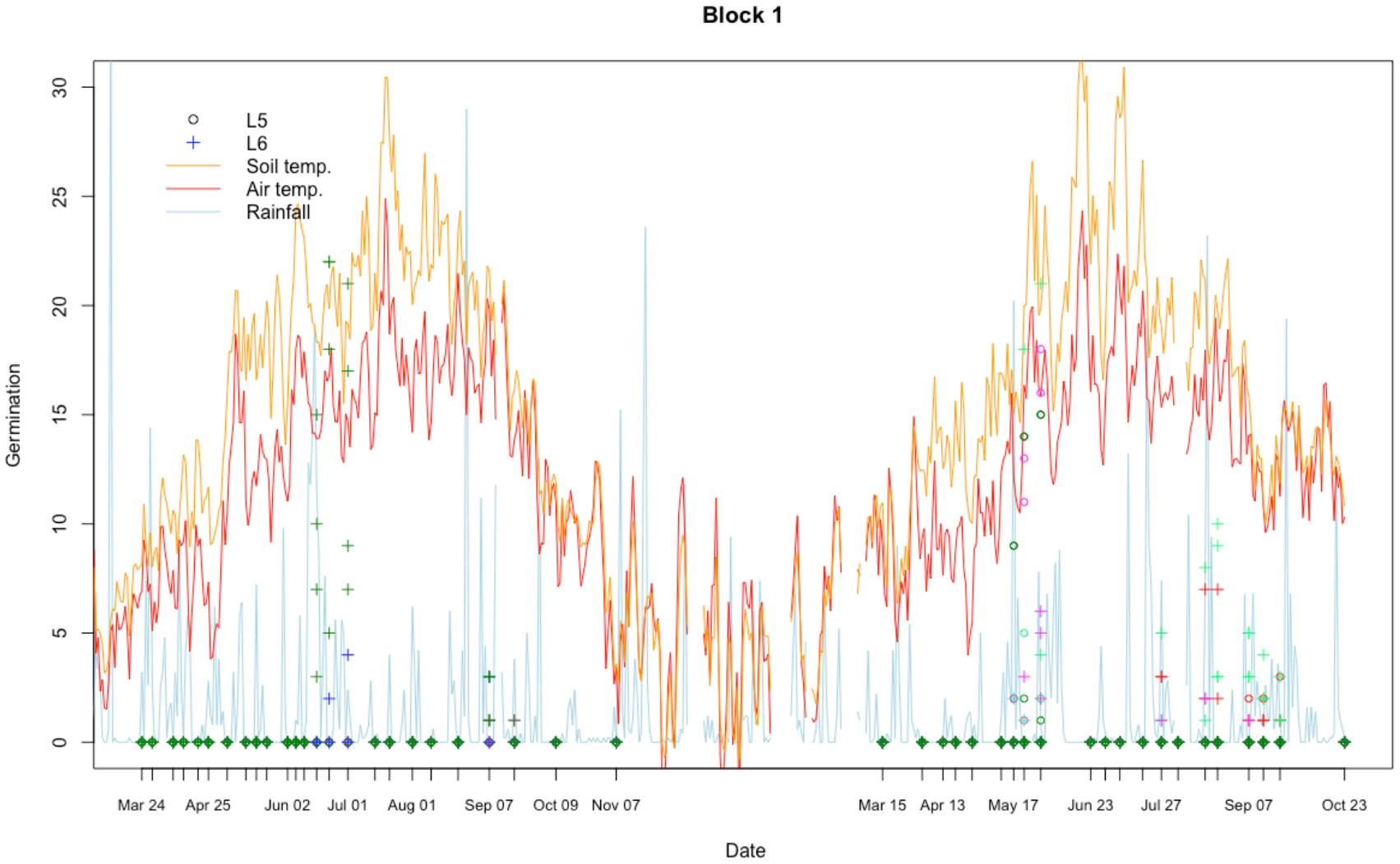

Figure 6.5 Field germination observed at Wellesbourne 2016-2017, Block 1.

6.3 New model formulation

Results revealed in the Dissection experiment (Chapter 4.3) for the isolate L5 led to the formulation of a new model. As revealed in previous studies by Saito (1973), primordia which have been previously observed only after transfer to a germination temperature (15°C) for sufficiently "conditioned" (4 weeks at 3°C) sclerotia were now observed in unconditioned sclerotia at 11,17 and 20°C (constant temperature), as early as after one week. For the constant 11 and 17°C treatments, germination of sclerotia to produce stipes was observed after 49 and 42 days, respectively, while the constant 20°C treatment failed to produce any stipes in the 70 days duration of the experiment. Furthermore, an increased number of primordia remained present up to 70 days (whole duration of experiment) in sclerotia at 20°C. In contrast, at the constant 4°C very few primordia (1 primordium at 70th day) and no germination were observed for the 70 days duration of the experiment. For treatments transferred after 28 days to 17°C primordia were observed after 42 days (14 days after transfer) for all S1 temperatures and germination of stipes was recorded after 49 ,56 and 63 days following transfer from 11°C, 4°C and 20°C respectively. The germination recorded in the temperature experiment (Chapter 3.4) was similar to germination observations for the dissection experiment (Figure 6.6). For the single temperature treatments at 4°C and 20°C the first observed germination (in TE1 and TE2) was beyond 70 days (duration of DE), where for constant 4°C total germination was ~ 90% compared to ~ 0% for constant 20°C (1 sclerotium germinated after 145 days in TE2) (Figure 6.6).

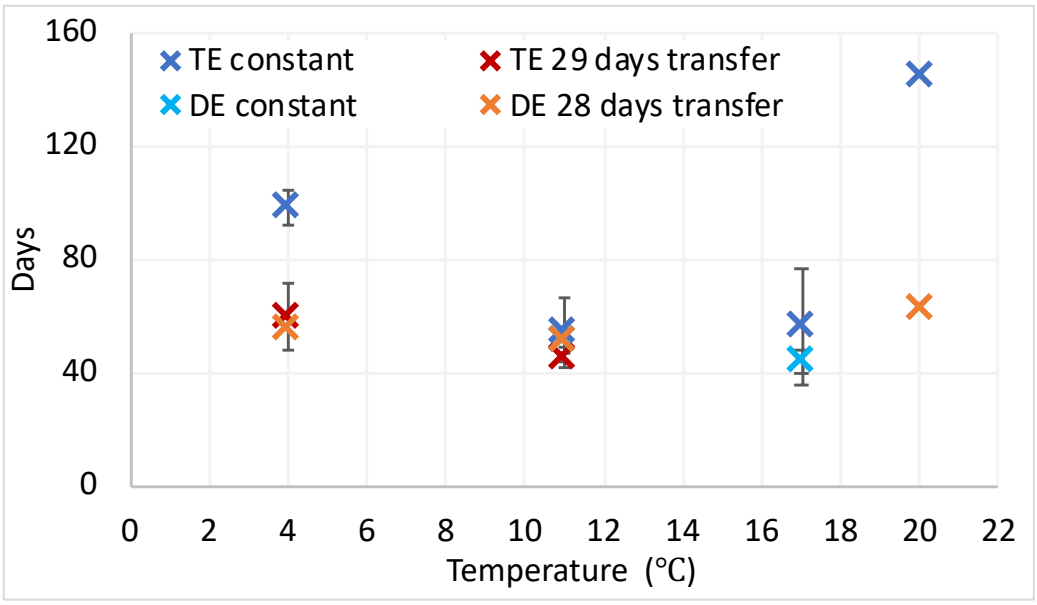

Figure 6.6 Average days of 1st germination observation in TE (Temperature experiment, Chapter 3.4) and DE (dissection experiment) for constant and transfer (to 17°C) treatments with temperatures 4,11,17 and 20°C.

The previous assumption was that primordia are an early stage of stipe development and therefore directly associated with sclerotia germination/stipe production. Following the published model, if primordia are associated with the germination process they should not be observed prior to full conditioning of sclerotia. Therefore, a new Parallel model is suggested (Figure 6.7) based on new assumptions (when moisture is not limiting) where the two processes:

- are considered to be independent;
- proceed simultaneously (in parallel) with different effects of temperature on the process rates;
- both need to be completed for stipe / apothecia production to occur.

Figure 6.7 Schematic drawing of new Parallel model for processes involved in carpogenic germination of *S. sclerotiorum* sclerotia.

6.3.1 Initial rates calculation – Parallel model

The temperature experiments (TE1, TE2) provide a further insight into temperature effects on carpogenic germination of sclerotia and deliver a strong data base for model parameter estimation. Assuming the new parallel model structure we can use the single (constant) temperature treatments for calculation of initial rates prior to formal model fitting. Assuming the two processes are proceeding independently and simultaneously, following separate temperature dependent rates, the observed time to germination (time to the appearance of stipes) is ***the time needed for the slower process to be completed, either "conditioning" or "germination",*** under the treatment specific conditions. Single temperature treatments are the simplest treatments where both processes are progressing at one constant temperature. By applying these assumptions to the estimated times to germination obtained in TE1 and TE2 for the single and transfer treatments, initial rates (CR and GR) for the two parallel processes were estimated for each temperature and for the different percentiles of the populations (Chapter 3.4).

The calculation of initial rates is explained in the following steps for the example of T_{50} (TE1, Table 6.6, Appendix 4, Appendix 50) for isolate L5:

6. Identify the observed "Critical Temperature" (T^C) – the temperature at which the fastest germination is observed for the single temperature treatments.

   ⇒ For the T_{50}, the single temperature treatments show a "V" shape response to temperature, with the shortest time to germination at 11°C (Figure 6.8, Table 3.10, Table 6.6).

   ⇒ T^C = 11°C, T_{50} = 69 days

7. Identify condition rates (CR) for single temperature treatments where conditioning is expected to be the slower process:

   ⇒ Assuming that the conditioning time increases with increasing temperatures, all times observed for the single temperatures greater than T^C were accounted for by the time taken for conditioning at the given temperature (Blue arrow, Figure 6.8).

   ⇒ T = 14°C, T_{50} = 92 days, **CR_{14} = 1/92 =0 .0109**

   ⇒ T = 17°C, T_{50} = 136 days, **CR_{17} = 1/136 = 0.0073**

8. Identify germination rates (GR) for single temperature treatments where germination is expected to be the slower process:

   ⇒ Assuming that the germination time increases with decreasing temperatures, all times observed for the single temperatures smaller than T^C were accounted for by the time taken for germination at the given temperature (Orange arrow, Figure 6.8).

   ⇒ T = 8°C, T_{50} = 82 days, **GR_8 = 1/82 = 0.0122**

   ⇒ T = 4°C, T_{50} = 131 days, **GR_4 =1 /131 = 0.0077**

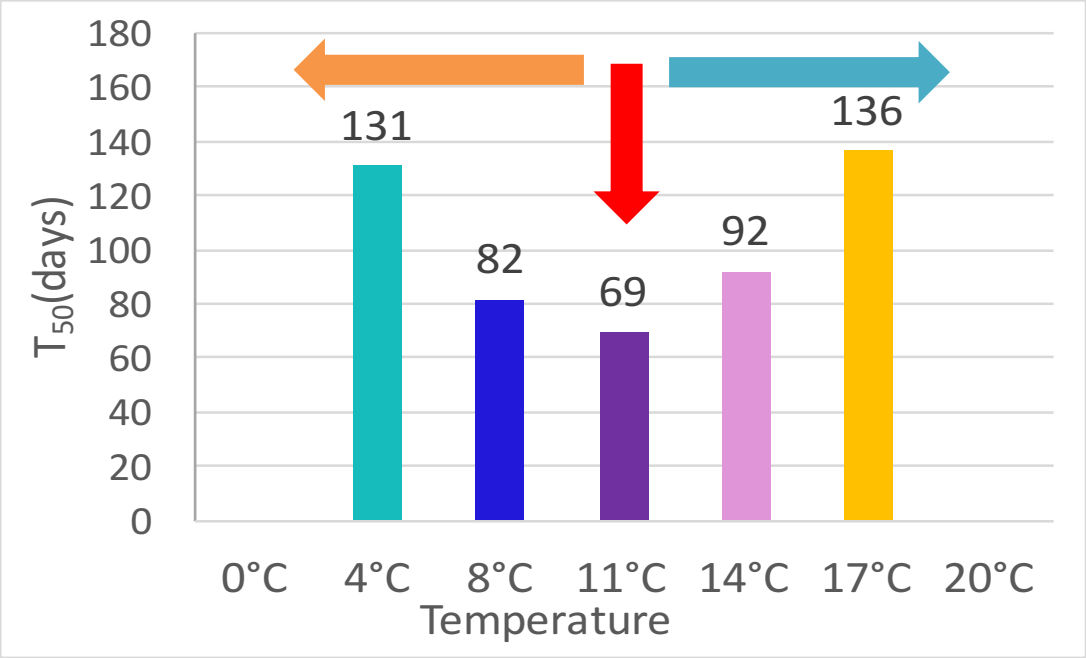

Figure 6.8 Time to 50% germination (T_{50}) estimated (ANOVA table of means) for the single temperature treatments in TE1 (Chapter 3.4) for isolate L5. Arrows indicating: Red - the critical temperature T^C; Orange – temperatures where germination is expected to be taking longer than conditioning; Blue - temperatures where conditioning is expected to be taking longer than germination.

9. Estimate process rates for the T^C (11°C) based on germination times observed for transfer treatments including T^C in S1 or S2 (Table 6.2):

   ⇒ Faster T_{50} (<69 days) was predominantly observed for treatments including S1 temperatures <T^C (T1 = 0, 4 and 8°C, S1 duration of 7, 14 and 28 days), where 59 days was the fastest T_{50} observed. Based on earlier assumptions about the effect of temperature on the different process rates, these temperatures support a more rapid conditioning rather than germination process. So, the reduction in time was caused by quicker conditioning for T<T^C. Consequently, the T_{50} observed at T^C is the duration of the conditioning process and the fastest observed T_{50} (59 days) germination is the maximum time (our best estimate) needed for completion of the germination process at T^C. This was the maximum time because the transfer treatments with T1<T^C reduced the time at T^C by the duration of S1, and these conditions were hostile for the germination process and were more likely to slow it down. Therefore:

   ⇒ T=11°C, **GR_{11}=1/59=0.0169** and **CR_{11}=1/69=0.0144**

T_{50}_L5		S1 - T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
11℃	25℃											54	
	20℃					72						55	
	17℃		146	127	116	84				129	88	54	
	14℃		100	82	91	63				65	65	52	
11℃		69						55					
8℃	11℃		61	62	64	74							
4℃			59	59	62	82				57	64	85	
0℃			59	62	68	89							

Table 6.2 ANOVA table of means for time to germination of 50% of the population (T_{50}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration, including 11°C (Chapter 3.4); Colour gradient from longest (green) to shortest (red) germination time; Empty cell = germination less then 50% (at the end of S2); Grey = no treatment combination tested.

10. Calculate further CR and GR for the remaining temperatures, based on estimated germination times for the transfer treatments and the CR and GR values estimated in steps 1. to 4. Calculations use previously obtained rates to calculate the proportion (rate multiplied by time) of the process (either conditioning or germination) completed in S1 (T1) or S2 (T2), and estimate the rate at the other temperature so that the cumulative proportions sum to 1 (i.e. the process is completed) (Equation 6.3).

$$\mathbf{1} = \boldsymbol{S1} * \boldsymbol{R_{T1}} + (\boldsymbol{T_{50}} - \boldsymbol{S1}) * \boldsymbol{R_{T2}}$$

Equation 6.3 Accumulation of either conditioning or germination process; S1 = S1 duration, R_{T1} = rate at S1 (T1), R_{T2} = rate at S2 (T2), T_{50} = Time to 50% germination.

The Equation 6.3 was rearranged depending on whether we were identifying rate for a process progressing at the temperature T1 = R_{T1} (Equation 6.5) or T2 = R_{T2} (Equation 6.4).

$$\boldsymbol{R_{T1}} = \frac{\mathbf{1} - (\boldsymbol{T_{50}} - \boldsymbol{S1}) * \boldsymbol{R_{T2}}}{\boldsymbol{S1}}$$

Equation 6.4 Reordered Equation 6.3, so the rate R_{T1} can be identified; S1 = S1 duration, R_{T1} = rate at S1 (T1), R_{T2} = rate at S2 (T2), T_{50} = Time to 50% germination.

$$\boldsymbol{R_{T2}} = \frac{\mathbf{1} - \boldsymbol{S1} * \boldsymbol{R_{T1}}}{\boldsymbol{T_{50}} - \boldsymbol{S1}}$$

Equation 6.5 Reordered Equation 6.3, so the rate R_{T2} can be identified; S1 = S1 duration, R_{T2} = rate at S2 (T2), R_{T1} = rate at S1 (T1), T_{50} = Time to 50% germination.

⇒ For the single temperature treatments of 0 and 20°C no germination was observed during the main part of the experiment (S1+S2). Sclerotia which did not germinate by the end of the S2 (279 days) were transferred to 11°C for additional 150 days. In the case of the single temperature treatments, the germination (times to germination) observed after transfer to S3 (Appendix 50, including S3), T3 = 11°C (S2+S3), could be treated similarly

to the data from the transfer treatments (S1+S2) and the CR_{11} and GR_{11} could be used to calculate corresponding rates CR_{20} and GR_0 (Equation 6.5):

For 20°C T_{50} = 311.25 days (Replicate mean (3)), S1 = 279 days:

$$CR_{20} = \frac{1 - (311.25 - 279) * 0.0169}{279}$$

CR_{20} = 0.0016

For 0°C T_{50} = 301.57 days (Replicate mean (3)), S1 = 279 days:

$$GR_0 = \frac{1 - (301.57 - 279) * 0.0144}{279}$$

GR_0 = 0.0024

⇒ For the calculation of initial conditioning rates for 0, 4 and 8°C (CR_0, CR_4 and CR_8) were used: Equation 6.4, T_{50} (Table 6.6, Appendix 4) for transfer treatments where T1 = 0, 4 or 8°C, S1 = 7, 14, 28 and 56 days, T2 ≥ 11°C and the initial conditioning rates CR_{11}, CR_{14}, CR_{17} and CR_{20} identified earlier (i.e. T1 = 8°C, calculation of CR_8, Table 6.3)

$T_{50} - S1$					
T1	T2	S1 duration (days)			
		7	14	28	56
8°C	11°C	54.33	47.93	35.45	17.86
	14°C	75.58	53.60	28.40	14.15
	17°C	124.10	124.58	57.87	11.67
	20°C	N/A	N/A	100.85	12.19
$1 - (T_{50} - S1) * R_{T2}$					
T1	T2	S1 duration (days)			
		7	14	28	56
8°C	11°C	0.2166	0.3089	0.4888	0.7425
	14°C	0.1793	0.4180	0.6916	0.8463
	17°C	0.0905	0.0870	0.5759	0.9145
	20°C	N/A	N/A	0.8066	0.9766
CR_8					
T1	T2	S1 duration (days)			
		7	14	28	56
8°C	11°C	0.0309	0.0221	0.0169	0.0133
	14°C	0.0256	0.0299	0.0238	0.0151
	17°C	0.0129	0.0062	0.0199	0.0163
	20°C	N/A	N/A	0.0278	0.0174
Mean		0.0199 (s.d. = 0.0072)			

Table 6.3 Calculation of initial conditioning rate at 8°C (CR_8) for isolate L5, based on T_{50} estimated for transfer treatments (Chapter 3.4), T1 = 8°C, T2 > 8°C, S1 = 7, 14, 28 and 56 days and where the proportion of germination completed in S1 (T1) and S2 (T2) add to 1 (process completed) (Equation 6.5)

⇒ For the calculation of initial germination rates for 14, 17 and 20°C (GR_{14}, GR_{17} and GR_{20}) were used: Equation 6.5, T_{50} (Table 6.6, Appendix 4) for transfer treatments where T1 ≤ 8°C, S1 = 7, 14, 28 and 56 days including the corresponding T2 (i.e. T2 = 14°C, calculation of GR_{14}, Table 6.4) and the initial germination rates GR_0, GR_4 and GR_8 identified earlier. The temperature combination of T1 = 11°C and T2 = 14°C was excluded from calculations as it was difficult to determine whether conditioning or germination was the longer taking process at this stage.

$T_{50} - S1$					
T1	T2	S1 duration (days)			
		7	14	28	56
0°C	14°C	71.67	53.40	31.50	26.06
4°C		62.16	43.90	26.69	19.33
8°C		75.58	53.60	28.40	14.15
$1 - S1 * GR_{T1}$					
T1	T2	S1 duration (days)			
		7	14	28	56
0°C	14°C	0.9831	0.9661	0.9299	0.8646
4°C		0.9463	0.8926	0.7776	0.5704
8°C		0.9144	0.8287	0.6452	0.3148
GR_{14}					
T1	T2	S1 duration (days)			
		7	14	28	56
0°C	14°C	0.0137	0.0181	0.0295	0.0332
4°C		0.0152	0.0203	0.0291	0.0295
8°C		0.0121	0.0155	0.0227	0.0222
Mean		0.0218 (s.d. = 0.0071)			

Table 6.4 Calculation of initial germination rate at 14°C (GR_{14}) for isolate L5, based on T_{50} estimated for transfer treatments (Chapter 3.4), T1<14°C, T2 = 14°C, S1 = 7, 14, 28 and 56 days and where the proportion of germination completed in S1 (T1) and S2 (T2) add to 1 (process completed) (Equation 6.5).

Following the steps 1 to 5 a set of initial rates for isolate L5 was calculated for the T_{50} (Table 6.5, Figure 6.9). Similar approach was used to estimate rates for different percentiles for isolate L5, and these estimates were used to identify appropriate rate functions to be fitted to the observed germination data allowing for rates to vary in a systematic way with temperature.

Temp	Conditioning (days)	Conditioning rate (1/days)	Germination (days)	Germination rate (1/days)
0°C	46	0.0217	415	0.0024
4°C	40	0.0250	130	0.0077
8°C	50	0.0199	82	0.0122
11°C	69	0.0144	59	0.0169
14°C	92	0.0109	46	0.0218
17°C	136	0.0073	57	0.0176
20°C	615	0.0016	48	0.0210

Table 6.5 Times to conditioning and germination along with corresponding rates calculated from T_{50} (ANOVA table) observed in TE1 for isolate L5. Colour – times and rates derived directly from the single temperature treatments.

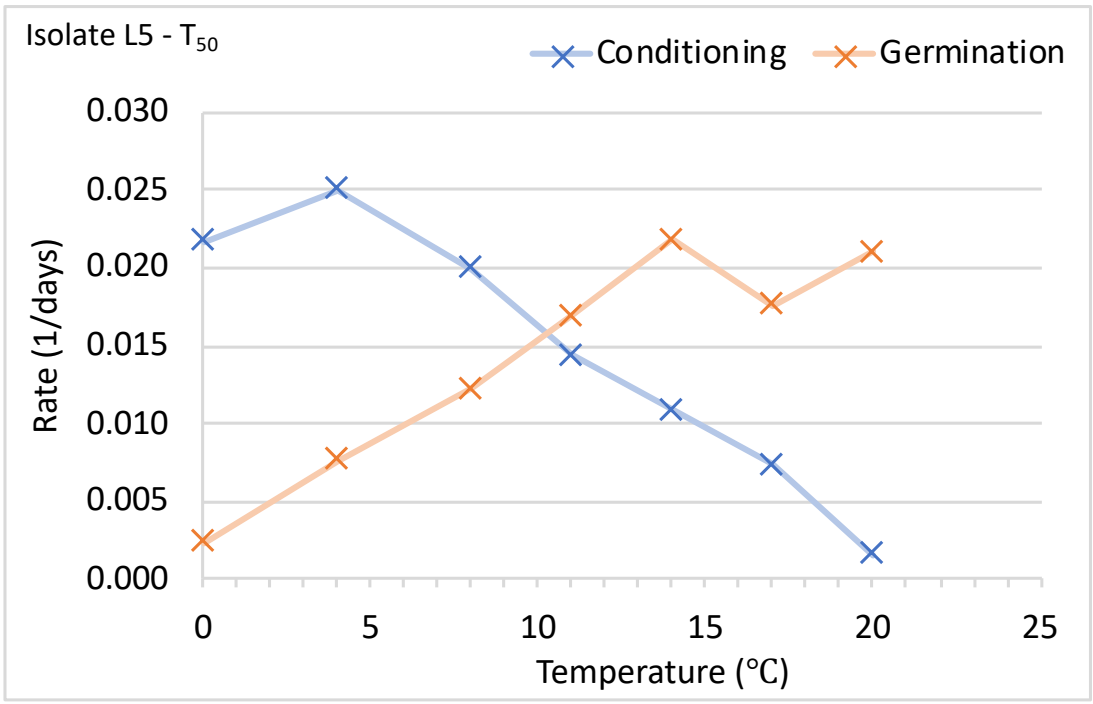

Figure 6.9 Initial rates calculated for conditioning and germination process for Isolate L5 TE1 T_{50} data based on the parallel model.

As a proof of concept and to identify treatments where the proposed model fails to fit the observed data, the T_{50} was recalculate for the TE1 treatments, isolate L5, based on the initial rates (Table 6.5) for both processes (conditioning and germination) in following steps (Table 6.6):

1. Calculate fraction of process completed at S1:

   ⇒ If fraction of process completed at S1 was >=1 the process was completed at S1;

   Time S1 = $1/R_{T1}$

   ⇒ If fraction of process completed at S1 was <1 the process was not completed at S1 and therefore continues at S2;

2. Calculate fraction of process to be completed at S2:

   ⇒ Fraction of process completed at S2 = 1- fraction of process completed at S1;

   Time S2 = fraction of process completed at S2/R_{T2}

3. Calculate the total time:

   ⇒ **Time = Time S1 + Time S2**

4. Steps 1 to 3 were completed for both processes, using the corresponding rates.

   ⇒ The final time is identified as time taken for the **slower process**.

The recalculated T_{50} based on estimates of initial rates generally fit the observed data (Figure 6.10, Table 6.6), where the fastest germination times (red colour, Table 6.6) were recalculated for treatments T1 = 4°C, S1 duration of 7 days and T1 = 8°C, S1 duration of 14 days, followed by T2 = 11°C, 64 days, compared to 59 and 62 days in TE1, respectively. The fastest T_{50} in TE1 was observed for T1 = 4°C, S1 duration of 29 days, T2 = 14°C, 56 days, compared to 65 days recalculated based on initial rates. The slowest (green colour, Table 6.6) germination times, over 280 days, overlap with the treatments where T_{50} was not observed by the end of the main part of the experiment in TE1 (S1 + S2), except for T1 = 0°C for 29 days followed by T2 = 20°C, where T_{50} was estimated to 258 days. These treatments included single temperature treatments for 0 and 20°C and most of the transfer treatments at T2 = 20°C, S1 duration < 56 days. The greatest discrepancies between the recalculated and observed T_{50} for TE1 generally occurred for T2 = 20°C transfer treatments (overestimated) and for T2 = 17°C after 14 days at T1 = 14°C (underestimated) as follows:

T2 = 20°C, S1 = 56 days, T1 = 14°C, T_{50} = 74 days, estimated for 260 days;
T2 = 20°C, S1 = 56 days, T1 = 11°C, T_{50} = 72 days, estimated for 174 days;
T2 = 20°C, S1 = 29 days, T1 = 8°C, T_{50} = 130 days, estimated for 288 days;
T2 = 20°C, S1 = 29 days, T1 = 4°C, T_{50} = 67 days, estimated for 199 days;
T2 = 17°C, S1 = 14 days, T1 = 14°C, T_{50} = 211 days, estimated for 130 days.

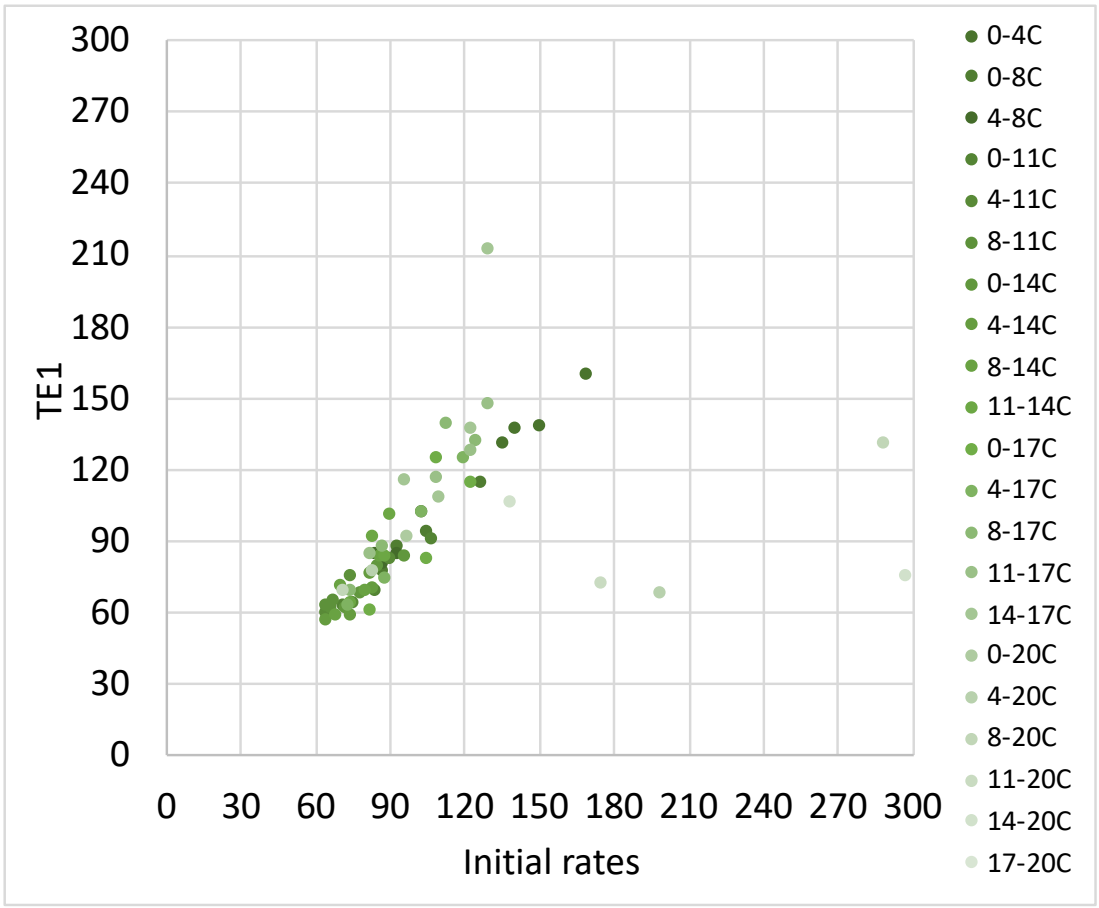

Figure 6.10 Scatterplot showing T_{50} for transfer treatments in TE1, for isolate L5, comparing the time to germination (days) calculated based on the estimated initial rates and T_{50} ANOVA estimates (TE1).

T_{50}_L5		T1 duration (days)											
T1	T2	TE1						Estimates based on initial rates					
		0	7	14	29	56	84	0	7	14	29	56	84
20℃	20℃							615					
17℃										566	514	419	321
14℃						74	105			536	451	297	138
11℃						72			560	505	387	174	
8℃					130	68			536	458	288	71	
4℃					67	76			515	414	199	83	
0℃						91			529	443	258	97	
20℃	17℃												
17℃		136						136					
14℃				211	136	107	114			130	122	109	96
11℃			146	127	116	84			130	123	108	82	
8℃			131	139	87	68			124	112	87	74	
4℃			124	101	62	73			120	103	73	88	
0℃			113	124	59	81			123	109	82	105	
20℃	14℃												
17℃													
14℃		92						92					
11℃			100	82	91	63			90	87	83	74	
8℃			83	68	57	70			86	80	68	70	
4℃			69	58	56	75			83	74	65	82	
0℃			79	67	61	82			85	78	72	96	
20℃	11℃												
17℃													
14℃													
11℃		69						69					
8℃			61	62	64	74			67	64	67	75	
4℃			59	59	62	82			64	67	75	90	
0℃			59	62	68	89			66	71	84	107	
20℃	8℃												
17℃													
14℃													
11℃													
8℃		82						82					
4℃			83	80	84	102			84	87	93	103	
0℃			77	87	93	114			87	93	105	127	
20℃	4℃												
17℃													
14℃													
11℃													
8℃													
4℃		130						130					
0℃			130	137	137	159			135	140	150	169	
20℃	0℃												
17℃													
14℃													
11℃													
8℃													
4℃													
0℃								415					

Table 6.6 Comparison of time to germination for isolate L5, T_{50}: ANOVA estimates (TE1, Chapter 3.4) and recalculated for TE1 treatments based on initial rates (Table 6.5). T1 = Temperature at S1, T2 = temperature at S2, S1 duration = T1 duration; Colour scale represents shortest time = red, middle time = yellow, longest time = green.

This approach was used to identify where the later models failed to predict the observed data.

6.3.2 Incorporation of S3 data

The parallel model simplified the parameter optimisation algorithm and allowed for observations in S3 to be used for treatments where no germination was observed at the end of S2 and therefore gather further information than from previous experiments.

As we were interested in modelling the germination times of successfully germinated sclerotia, germination times for all deciles where interpolated from germinated sclerotia, including germination recorded in S3 (viability test, Chapter 3) to fill the missing data (no germination by the end of S1+S2, 280 days (i.e. T_{25}, grey colour, isolate L6 - Figure 6.11, isolate L5 - Figure 6.12)). For both isolates the germination process was reactivated after transfer to 11°C in S3 (i.e. T_{25}, orange colour, isolate L6 - Figure 6.11, isolate L5 - Figure 6.12). This approach is vital for the isolate L5 where the higher T2 temperatures (20-25°C) resulted in low or no germination. In contrast for isolate L6 (i.e. T_{25},) only a few treatments showed no or low germination in S2 (25°C). As the missing values for time to germination became abundant for the higher percentiles so did the importance of using the S3 data. This approach enabled to use the full set of treatments and therefore to use information beyond the main part of experiment, which would normally be lost on missing values or by excluding treatments from optimization process.

Data used for model fitting are shown in Appendix: Isolate L5 – Appendix 47 – 53; Isolate L6 - Appendix 54 – 60.

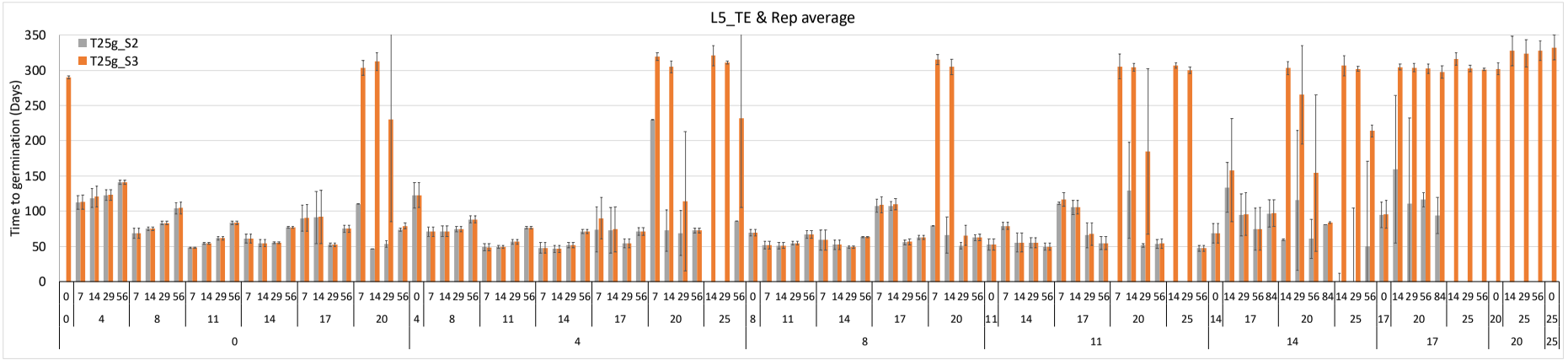

Figure 6.12 Replicate and TE1 & TE2 experiments average of Time to germination for 25% of population for isolate L5 for observations by the end of S2 (grey) and observations including S3 (orange). Error bars represent SD.

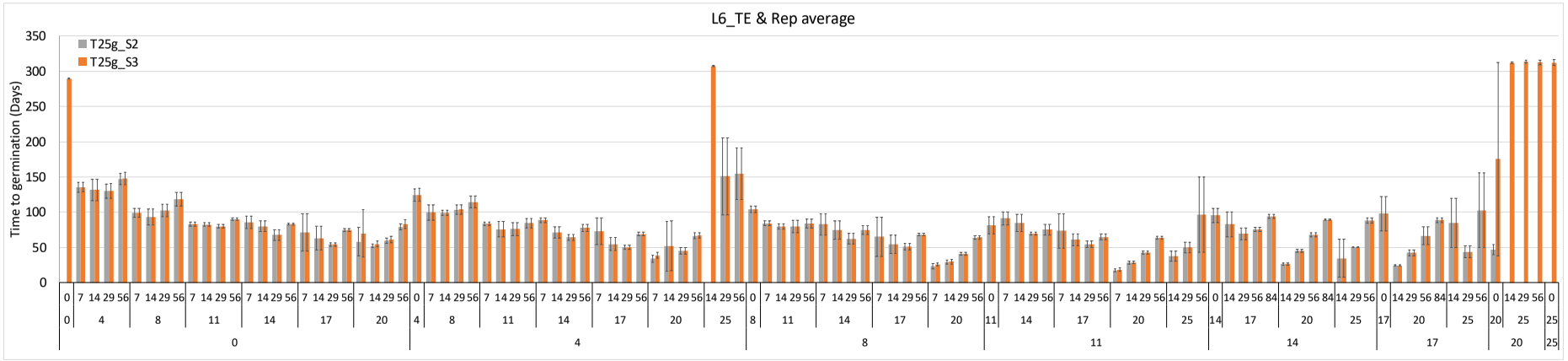

Figure 6.11. Replicate and TE1 & TE2 experiments average of Time to germination for 25% of population for isolate L6 for observations by the end of S2 (grey) and observations including S3 (orange). Error bars represent SD

6.4 Model fitting

6.4.1 Introduction

For the model fitting the FITNONLINEAR directive in GenStat software (Genstat 64-bit Release 19.1, VSN International Ltd.) was used. The optimization program is an iterating process searching for the best combinations of model parameters fitting the chosen variable. It is designed to search for the minimum value of a function as the parameters vary; for nonlinear regression models, the function involved is the deviance, or minus twice the log-likelihood, so the algorithm searches for the maximum-likelihood solution. The optimization process relies on having good initial estimates of the parameters (as calculated in the process for initial rates described in section 6.3.1) so that that search process is focussed on an appropriate part of the parameter space, and the GenStat implementation provides various alternative optimization algorithms (GenStat Manual, https://genstat.kb.vsni.co.uk/knowledge-base/online-documentation/):

- modified Gauss-Newton method – expressing the likelihood function as a sum of squares.
- modified Newton method
- modified Fletcher-Powell method - similar to the Newton method, with an occasional step in the search being determined by the Fletcher-Powell algorithm rather than by the Newton algorithm

Another way to manipulate the search for the best parameter values is to modify the initial step length for the search, or, as last resource, to set the step length to zero to fix the corresponding parameter at its initial value. This allows complex problems to be tackled in stages, optimizing some parameters with others fixed, and then optimizing the others in turn (GenStat Manual).

The various fitted models were compared based on the ability of the optimization process to converge (competition of search), F-test statistics = variance ratio (v.r.), corresponding degrees of freedom (d.f.), percentage variance accounted for (R^2) and the condition to fix a parameter for the search to converge.

The model fitting and parameter optimization was divided into three successive parts, based on the fitting approach and the set of parameters to be estimated simultaneously using all of the observed germination times for number of percentiles

separately for the isolate L5 (and L6 initially) from the germination data from both TE1 and TE2. Values included in parameter optimizations were T1, T2, T1 duration (S1 duration) and the observed values are T_x (time to germination to x% population). This was extended by adding T3 and T2 duration (S2 duration) values for the parameter optimization including S3 germination data. The set of equation in the optimization process is further specified for each model fitting, where for the parallel model this was similar to logical process described for the recalculation of T_{50} from initial rates, earlier in this chapter (page 198).

Part 1: Rate fitting – 16 rates (parameters) for conditioning and germination were estimated for each isolate and for each of the temperatures (0, 4, 8, 11, 14, 17, 20 and 25°C) included in the experiments (TE1 & TE2). This approach does not provide information on rates at temperatures other than those included in experiments. In this part an initial direct comparison between sequential and parallel model was made for data including S1 and S2 germination only. As the parallel model provided a simplification (independence) of the processes this allowed for inclusion of the data from S3 into the parameter estimation by adjusting the optimisation to include S3. The parameter optimization steps are explained later in this chapter for S2 data (6.4.2.2) and including S3 data (6.4.2.3).

Part 2: Curve fitting – based on rate fitting the most appropriate curves (rate functions) were identified and parameters fitted for each isolate combining TE1 & TE2 data for germination times for various population percentiles (number of parameters varied depending on the used rate functions, 10 to 12 parameters). On top of reduction in number of parameters, based on the temperature depending rate functions CR and GR can be calculated for any temperature. The parameter optimization steps are explained later in this chapter (6.4.3.2).

Part 3: Distribution fitting – population model – after exploring the development of rate functions for various population percentiles, identify trends in the parameter values across percentiles and suggest possible solutions to modelling the whole distribution of germination times for the observed sclerotial population at once. This step was not attempted because of continual work on Part 2. and its possibilities are discussed later in this chapter (Discussion 6.5).

6.4.2 Rate fitting

6.4.2.1 Model specification - Sequential and Parallel model (S2 data)

The following calculations (1 to 7) were included in each iterative step of the parameter optimisation for the sequential model (S1+ S2), with the values of CR and GR updated at each step:

1. Specification of CR and GR for 8 temperatures (total of 16 parameters) for each S1 and S2 temperature (T1 and T2):

   ⇒ CR_{T1}, CR_{T2}, GR_{T1}, GR_{T2}

2. Calculation of conditioning and germination daily progress at S1 and S2:

   ⇒ C_progress_T1 = $(1/CR_{T1})$

   ⇒ C_progress_T2 = $(1/CR_{T2})$

   ⇒ G_progress_T1 = $(1/GR_{T1})$

   ⇒ G_progress_T2 = $(1/GR_{T2})$

3. Calculation of fraction of conditioning process at S1:

   ⇒ C_fract_T1 = T1 duration * CR_{T1}

   C_fract_T1 < 1 – conditioning continues in S2 (Step 4. and Step 6.1)

   C_fract_T1 ≥ 1 – conditioning completed in S1, germination follows (Step 5. and Step 6.2)

4. Calculation of time for conditioning process at S2:

   ⇒ C_time_T2 = (1 - C_fract_T1) * C_progress_T2

5. Calculation of fraction of germination process at S1:

   ⇒ G_fract_T1 = (T1 duration - C_progress_T1) * GR_{T1}

   G_fract_T1 ≥ 1 - germination completed in S1 (Step 7.1.)

   G_fract_T1 < 1 - germination continues in S2 (Step 6 and 7.2.)

6. Calculation of time for germination process at S2.

   6.1. Conditioning continued in S2:

   ⇒ G_time_T2 = G_progress_T2

6.2. Conditioning completed in S1:

⇒ G_time_T2 = (1 - G_fract_T1) * G_progress_T2

7. Calculation of total time to germination (i.e. T_{50}):
   7.1. Germination completed in S1:

   ⇒ Fitted = C_progress_T1 + G_progress_T1

   7.2. Germination continued in S2:

   ⇒ Fitted = S1 duration + C_time_T2 + G_time_T2

The following calculations (1 to 7) were included in each iterative step of the parameter optimisation for the parallel model (S1+ S2), with the values of CR and GR updated at each step. The calculations in steps 2 – 6 were identical for conditioning and germination:

1. Specification of CR and GR for 8 temperatures (total of 16 parameters) for each S1 and S2 temperature (T1 and T2):

   ⇒ CR_{T1}, CR_{T2}, GR_{T1}, GR_{T2}

2. Calculation of conditioning time (progress) at S1 and S2 and germination progress at S1 and S2:

   ⇒ C_progress_T1 = $(1/CR_{T1})$

   ⇒ C_progress_T2 = $(1/CR_{T2})$

   ⇒ G_progress_T1 = $(1/GR_{T1})$

   ⇒ G_progress_T2 = $(1/GR_{T2})$

3. Calculation of fraction of conditioning and germination at S1:

   ⇒ C_fract_T1 = T1 duration * CR_{T1}

   C_fract_T1 ≥ 1 – conditioning completed in S1 (Step 4.1.)

   C_fract_T1 < 1 – conditioning continues in S2 (Step 4.2., Step 5)

   ⇒ G_fract_T1 = T1 duration * GR_{T1}

   G_fract_T1 ≥ 1 – germination completed in S1 (Step 4.1.)

   G_fract_T1 < 1 – germination continues in S2 (Step 4.2., Step 5)

4. Calculation of time for conditioning and germination process at S1.
   4.1. Conditioning and/or germination completed in S1:

   ⇒ C_time_T1 = C_progress_T1

⇒ G_time_T1 = G_progress_T1

4.2. Conditioning and/or germination continues in S2:

⇒ C_time_T1 = T1 duration

⇒ G_time_T1 = T1 duration

5. Calculation of time for conditioning and germination process at S2

⇒ C_time_T2 = (1 - C_fract_T1) * C_progress_T2

⇒ G_time_T2 = (1 - G_fract_T1) * G_progress_T2

6. Calculation of total time for conditioning and germination:

⇒ C_time = C_time_T1 + C_time_T2

⇒ G_time = G_time_T1 + G_time_T2

7. Calculation of total time to germination (i.e. T_{50}), where the total time is identified as time taken for the longer process (hence max) and both processes have to be completed (hence > 0):

⇒ Fitted = max (C_time, G_time); C_time ∧ G_time > 0

6.4.2.2 Results - Sequential versus Parallel model (S2 data)

The original sequential model first calculates progress of conditioning in S1 and S2 (if applicable) and allocates the remaining time for the germination process identifying germination rates at S1 (if applicable) and S2 temperature. The parallel process model as specified above, identifies rates and calculates the time for both processes following an identical set of equation. The longer taking process is taken in account as the final time to germination with a concluding condition that both processes have to be completed for successful germination/stipe production. To test the original sequential model versus the new parallel model, both were fitted to T_{25}, T_{50} and T_{75} for each isolate individually and for the TE1 & TE2 combined data (only germination completed by the of S1 and S2 was included).

For both isolates' convergence was achieved only with the parallel model approach for T_{75}. Estimated parameters for the isolate L5 resulted in 70.1 percentage variance accounted for with the standard error of observations estimated to be 21.6 and the regression results $F_{16,250} = 427.29$, compared to the sequential model approach where GR_{25} out of bonds ended parameter optimization. For isolate L6 percentage variance accounted for 55.5, with the standard error of observations estimated to be 23.8 and

the regression results $F_{16,359}$ = 446.76. Although, it was not possible to finalize the parameter optimization for most of the times compared, generally the parallel model approach showed higher percentage variance accounted, 63 - 70% for L5 (Table 6.7) and 55 - 69% for L6 (Table 6.8) compared to the sequential model, 6 - 18% for L5 (Table 6.7) and 8 - 36% for L6 (Table 6.8), and therefore the parallel model explained the experimental data better.

L5	T_{25}		T_{50}		T_{75}	
	S.M.	**P.M.**	**S.M.**	**P.M.**	**S.M.**	**P.M.**
d.f. (reg/res)	16/288	16/288	16/270	16/270	16/250	16/250
F (v.r.)	116.32	382.01	142.85	459.01	147.52	427.29
R^2	Residual variance > response variance	63.3	6.4	68.6	18.8	70.1
S.e. of observation	29.9	17.3	31.6	18.3	35.6	21.6
Optimization issue	CR_0 out of bounds	CR_{25} out of bounds	not converg.	CR_{25} out of bounds	GR_{25} out of bounds	-

Table 6.7 Rate fitting outcome for T_{25}, T_{50} and T_{75} for isolates L5, based on TE1 and TE2 combined data (S1 + S2), using two different model approaches: Sequential (S.M.) and Parallel model (P.M.).

L6	T_{25}		T_{50}		T_{75}	
	S.M.	**P.M.**	**S.M.**	**P.M.**	**S.M.**	**P.M.**
d.f. (reg/res)	16/389	16/389	16/396	16/380	16/380	16/359
F (v.r.)	273.98	592.96	207.00	488.45	204.55	446.76
R^2	36.3	69.2	24.0	65.7	8.0	55.5
S.e. of observation	22.8	15.9	30.6	20.5	34.3	23.8
Optimization issue	CR_4 out of bounds	no progress	CR_8 out of bounds	no progress	CR_8 out of bounds	-

Table 6.8 Rate fitting outcome for T_{25}, T_{50} and T_{75} for isolates L6, based on TE1 and TE2 combined data (S1 + S2), using two different model approaches: Sequential (S.M.) and Parallel model (P.M.).

The conditioning and germination rates estimated for various time to germination can be found in Appendix 61 and are plotted in Figure 6.13.

For the sequential model at low temperatures the estimated conditioning rates (Figure 6.13, light blue) went out of bounds (0.1) and a similar problem was observed in the published study (Clarkson *et al.*, 2007). To address the issue lower temperatures and short period of conditioning where introduced in the TE1, however this issue persists and therefore it could be an intrinsic issue of the model form. The out of bounds values for both conditioning and germination rates for isolate L5 are the consequence of lack of germination (by the end of S2) and therefore no germination times recorded for these treatments (missing values). The increase in conditioning rates for 20°C for

isolate L6 could be explained by short germination times observed, what is a specific behaviour of this isolate.

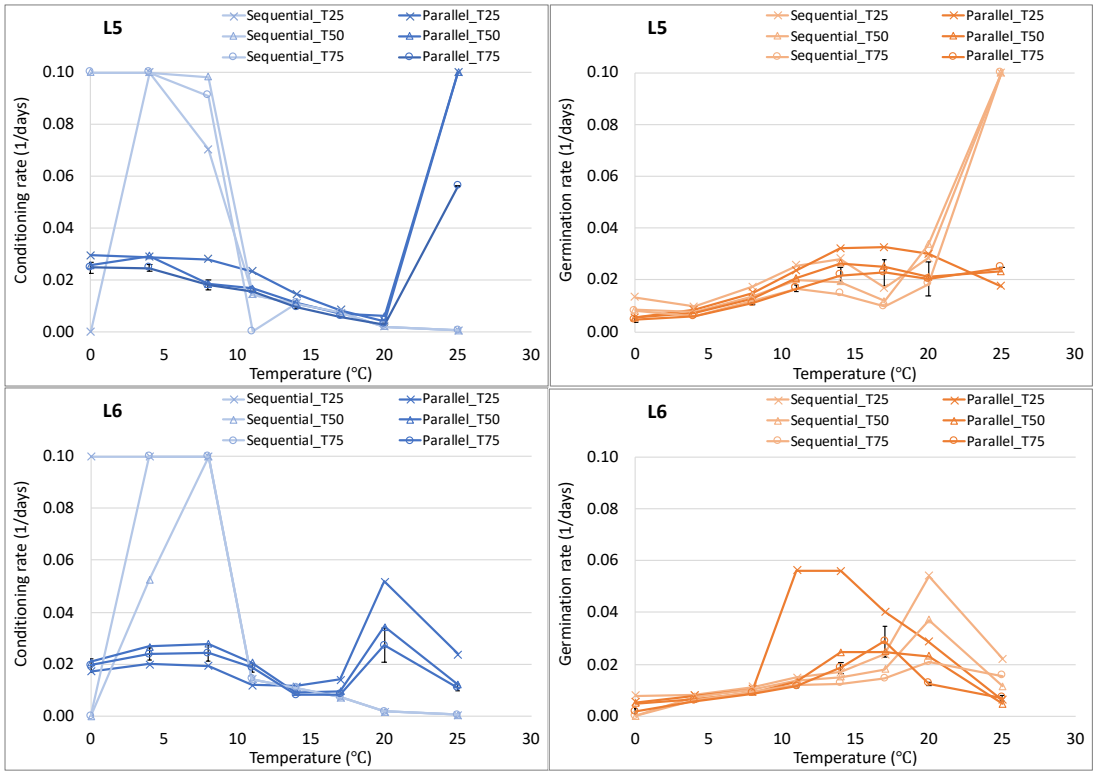

Figure 6.13 Estimated conditioning and germination rates for the combined TE1 & TE2 data (S1 + S2 germination) for germination times T_{25}, T_{50} and T_{75} (various symbols) for isolates L5 and L6 using sequential (light colour) and parallel (dark colour) model approach; Error bars = standard error of estimate, where applicable.

Overall the initial outcomes from rate estimation are positive. The parallel model performs better compared to the sequential model and the estimated parameters follow the anticipated shapes (conditioning rate decreases, and germination rate increases, with increasing temperature). As the parallel model algorithm does not distinguish between conditioning and germination rates during the rate fitting process this is affected by the initial rates and it supports the importance of well-chosen initial values.

6.4.2.3 Model specification – Parallel model including S3 data

The following calculations (1 to 10) were included in each iterative step of the parameter optimisation for the parallel including S3 data (S1 + S2 + S3), with the values of CR and GR updated at each step. The calculations in steps 2 – 9 were identical for conditioning and germination:

1. Specification of CR and GR for 8 temperatures (total of 16 parameters) for each S1, S2 and S3 temperature (T1, T2 and T3):

   ⇒ CR_{T1}, CR_{T2}, CR_{T3}, GR_{T1}, GR_{T2}, GR_{T3}

2. Calculation of conditioning and germination time (progress) at S1, S2 and S3:

   ⇒ C_progress_T1 = $(1/CR_{T1})$

   ⇒ C_progress_T2 = $(1/CR_{T2})$

   ⇒ C_progress_T3 = $(1/CR_{T3})$

   ⇒ G_progress_T1 = $(1/GR_{T1})$

   ⇒ G_progress_T2 = $(1/GR_{T2})$

   ⇒ G_progress_T3 = $(1/GR_{T3})$

3. Calculation of fraction of conditioning and germination at S1:

   ⇒ C_fract_T1 = T1 duration * CR_{T1}

   C_fract_T1 ≥ 1 – conditioning completed in S1 (Step 4.1.)

   C_fract_T1 < 1 – conditioning continues in S2 (Step 4.2., Step 5)

   ⇒ G_fract_T1 = T1 duration * GR_{T1}

   G_fract_T1 ≥ 1 – germination completed in S1 (Step 4.1.)

   G_fract_T1 < 1 – germination continues in S2 (Step 4.2., Step 5)

4. Calculation of time for conditioning and germination process at S1.

   4.1. Conditioning and/or germination completed in S1:

   ⇒ C_time_T1 = C_progress_T1

   ⇒ G_time_T1 = G_progress_T1

   4.2. Conditioning and/or germination continues in S2:

   ⇒ C_time_T1 = T1 duration

   ⇒ G_time_T1 = T1 duration

5. Calculation of time for conditioning and germination process at S2:

   ⇒ C_time_T2 = (1 - C_fract_T1) * C_progress_T2

   C_time_T2 ≤ T2 duration - conditioning completed in S2 (Step 6.1.)

   C_time_T2 > T2 duration - conditioning continues in S3 (Step 6.2., Step 7, Step 8)

⇒ G_time_T2 = (1 - G_fract_T1) * G_progress_T2

G_time_T2 ≤ T2 duration - germination completed in S2 (Step 6.1.)

G_time_T2 > T2 duration - germination continues in S3 (Step 6.2., Step 7, Step 8)

6. Calculation of total time for conditioning and germination process at S2.

   6.1. Conditioning and/or germination completed in S2:

   ⇒ Tot_C_time_T2 = C_time_T2

   ⇒ Tot_G_time_T2 = G_time_T2

   6.2. Conditioning and/or germination continues in S3:

   ⇒ Tot_C_time_T2 = T2 duration

   ⇒ Tot_G_time_T2 = T2 duration

7. Calculation of fraction of conditioning and germination process at S1 + S2:

   ⇒ C_frac_T1_T2 = C_fract_T1 + T2 duration * CR_{T2}

   ⇒ G_frac_T1_T2 = G_fract_T1 + T2 duration * GR_{T2}

8. Calculation of time for conditioning and germination process at S2:

   ⇒ C_time_T3 = (1 - C_frac_T1_T2) * C_ progress _T3

   ⇒ G_time_T3 = (1 - G_frac_T1_T2) * G_ progress _T3

9. Calculation of total time for conditioning and germination:

   ⇒ C_time = C_time_T1 + tot_C_time_T2 + C_time_T3

   ⇒ G_time = G_time_T1 + tot_G_time_T2 + G_time_T3

10. Calculation of total time to germination (i.e. T_{50}), where the total time is identified as time taken for the longer process (hence max) and both processes have to be completed (hence > 0):

    ⇒ Fitted = max (C_time, G_time); C_time ∧ G_time > 0

6.4.2.4 Results - Parallel model including S3 data

Conditioning and germination rates for 8 temperatures were obtained for isolate L5 (Appendix 62) and L6 (Appendix 63) based on combined TE1 and TE2 data for time to germination based on 7 percentiles: T_{10}, T_{25}, T_{40}, T_{50}, T_{60}, T_{75}, T_{90}. For the T_{90} isolate L5 (Table 6.9) and T_{50} isolate L6 (Table 6.10) two optimizations runs are shown (1st

and 2nd), because of the large number of parameters required to be fixed for a successful optimization. The second run identifies the s.e. of the parameters estimates fixed in the 1st run (best possible optimization obtained and smallest number of fixed parameters), and is done by fixing all parameters successfully optimized in the first run, to their corresponding estimated values.

For the isolate L5 model fitting was generally successful, where only for the high percentiles, T_{75} and T_{90}, parameters needed to be fixed for the optimization process to converge. The percentage variance accounted for was generally high, 83.7 to 92.9 with considerably high s.e. of observations 29.6 to 46.9 (Table 6.9). The best fit was obtained for T_{50} with the highest percentage variance accounted for 92.9 % and 29.6 s.e. of observations.

L5	**T_{10}**	**T_{25}**	**T_{40}**	**T_{50}**	**T_{60}**	**T_{75}**	**T_{90}_1st**	**T_{90}_2nd**
d.f. (reg/ res)	16/410	16/410	16/410	16/410	16/410	15/411	12/414	10/416
F (v.r.)	413.49	749.22	840.75	1162.1	952.8	838.45	936.02	1123.3
R^2	85.8	90.8	90.9	92.9	90.8	87.7	83.7	83.7
S.e. of observ.	39.1	32.9	33.1	29.6	34.0	39.9	46.9	46.9
Opt. issue	-	-	-	-	-	no progr	no progr	no progr
Par. fixed	-	-	-	-	-	GR_{25}	GR_{14}, GR_{17}, GR_{20}, GR_{25}	CR_8, CR_{11}, CR_{14}, CR_{17}, CR_{20}, GR_{25}

Table 6.9 Rate fitting outcome for T_{10}, T_{25}, T_{40}, T_{50}, T_{60}, T_{75} and T_{90} for isolates L5, based on TE1 and TE2 combined data (S3), using Parallel model approach.

Generally, for isolate L6 it was more difficult to reach convergence in the optimization process, and for all times to germination at least one parameter needed to be fixed (usually rates associated with conditioning). The percentage variance accounted for ranged from 66.9 to 82.8 with s.e. of observations ranging from 22.4 to 46.9 (Table 6.10). The best fit was obtained for T_{10} with the highest percentage variance accounted for, 82.8 % and s.e. of observations 22.4. Additionally, the parameters CR_{11}, CR_{25} needed to be fixed for the optimization process to converge. Generally, the main reason for the unsuccessful parameter optimization was the inability to progress in the parameter search, suggesting a flat surface of the error function.

L6	T_{10}	T_{25}	T_{40}	T_{50}_1st	T_{50}_2nd	T_{60}	T_{75}	T_{90}
d.f. (reg/ res)	14/412	14/412	15/411	12/414	4/422	14/412	15/411	14/412
F (v.r.)	469.7	460.1	303.8	439.7	1344	395.9	282.4	400.6
R^2	82.8	80.6	72.0	75.1	75.6	75.8	66.9	71.3
S.e. of observ.	22.4	25.5	33.5	33.5	33.2	35.1	45.0	46.9
Opt. issue	no prog.	no prog.	no prog.	no prog.	no prog.	no prog.	no prog.	no prog.
Par. fixed	CR_{11}, CR_{25}	CR_{11}, CR_{25}	GR_0	CR_0, CR_4, CR_8, CR_{20}	all except T_{50} 1st	CR_{17}, CR_{25}	CR_{25}	CR_{17}, CR_{25}

Table 6.10 Rate fitting outcome for T_{10}, T_{25}, T_{40}, T_{50}, T_{60}, T_{75} and T_{90} for isolates L6, based on TE1 and TE2 combined data (S3), using Parallel model approach.

The estimated rates for conditioning and germination for isolate L5 (top, Figure 6.14) are described in a greater detail later (Curve fitting 6.4.3, Figure 6.15). Large standard errors for the estimated germination rates for isolate L5 (Figure 6.14) (Appendix 62), associated with the higher temperatures, are caused by a limited germination observed in S2 for these treatments and where the observed germination times showed a greater variation between replicates, especially for early percentiles.

The estimated conditioning rates for the isolate L6 (bottom left, Figure 6.14) (Appendix 63) were much higher compared to L5 (max. for T_{10} CR_8 = 0.03114), where the maximum rates were estimated for T_{10} and T_{25} for T = 8 and 11°C, 0.129 and 0.1286 (equivalent of 7.8 days to complete conditioning), respectively. For the remaining percentiles the CR_8 is consistently fastest rate, except for T_{50} (T_{50}_1) where again CR_8 and CR_{11} are almost identical (0.1073 and 0.1077, respectively, equivalent to 9.3 days) and for T_{90} where CR_{14} is the fastest rate, 0.03618 (27.6 days). With the increasing temperature CR was declining up to CR_{25}, ranging between 0.002641 (T_{50}_1) to 0.000015 (T_{60}) (equivalent of 406 to 6666 days). For the temperatures below 8°C, except for T_{90}, CR_4 was consistently smaller the CR_0, suggesting slower conditioning at 4°C compared to 0°C and 8°C, which does not make sense biologically. However, for many of the CR parameters the s.e. could not be determined or these needed to be fixed to successfully complete optimization (Appendix 63). This is implying that the proposed model is possibly not explaining the processes correctly for isolate L6 (as anticipated). This is due to the different temperature requirements as described in Chapter 3. The estimated rates for

germination for isolate L6 (bottom right, Figure 6.14) (Appendix 63) are much more sensible, with consistently fastest germination at 20°C, GR_{20} ranging from 0.0562 (T_{10}) to 0.01861 (T_{75}) (equivalent of 17.8 to 53.7 days). The germination rates have consistent shape for all percentiles, where from T=20°C GR decrease for both decreasing and increasing temperature.

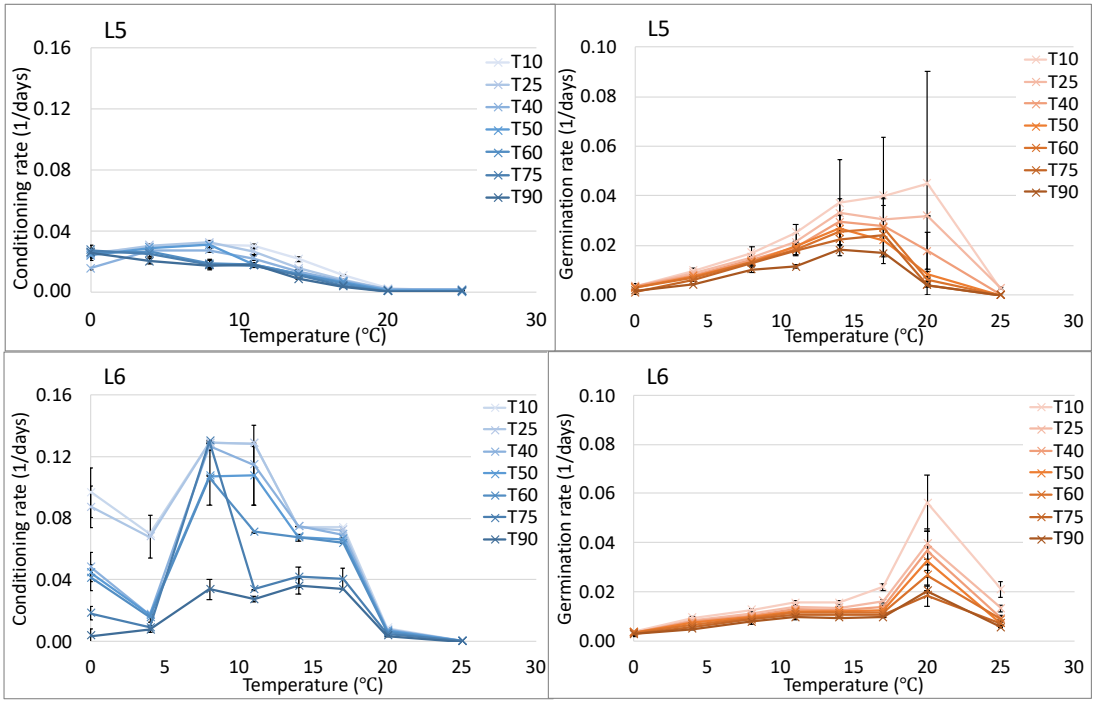

Figure 6.14 Parallel model fitted rates for 8 temperatures 0, 4, 8, 11, 14, 17, 20 and 25°C were obtained for isolate L5 and L6 based on combined TE1 and TE2 data (including S3) for statistics T_{10}, T_{25}, T_{40}, T_{50}, T_{60}, T_{75}, T_{90}.

The estimated rates for the selected percentiles follow similar pattern (Figure 6.14), except for the conditioning rates for isolate L6 which show extremely fast conditioning. The disagreements with fitting the isolate L6 were anticipated, as the isolates show variation in response to S1 and S2 temperatures (as described in Chapter 3.). Furthermore, the parallel model, as well as the initial rates calculation, is based on understanding the response of isolate L5 (following the dissection experiment outcomes, Chapter 4.3).

The estimated rates (Figure 6.14) for various percentiles do not always follow the increase or decrease in percentile, where ideally, we would like to see consistent increase or decrease in estimated rates for subsequent percentiles. If this is not the case (i.e. where lines are crossing) the possibility of producing a model for the whole distribution of germination times could be limited. To answer this issue at this stage a repeated parameter optimization could be done, where the upper and lower bounds

for the parameters could be set so a consistent increase or decrease in rates with increasing percentiles is achieved.

Further work on examining these fits and the source of variation or error needs to be well identified and appropriately addressed. However, for the purpose of deciding on the possible response shape, curve, describing relationship between conditioning and germination rate and temperature, these rate estimates suffice.

6.4.3 Curve fitting

6.4.3.1 Introduction

This part of model fitting was completed for isolate L5 only. The fitted rates for the individual temperatures (Figure 6.14) (Appendix 62) were divided into two groups, where a difference in response shape for the CR (more distinctive compared to GR) was observed for the time to germination for 10-50% (top, Figure 6.15) and 60 to 90% (bottom, Figure 6.15).

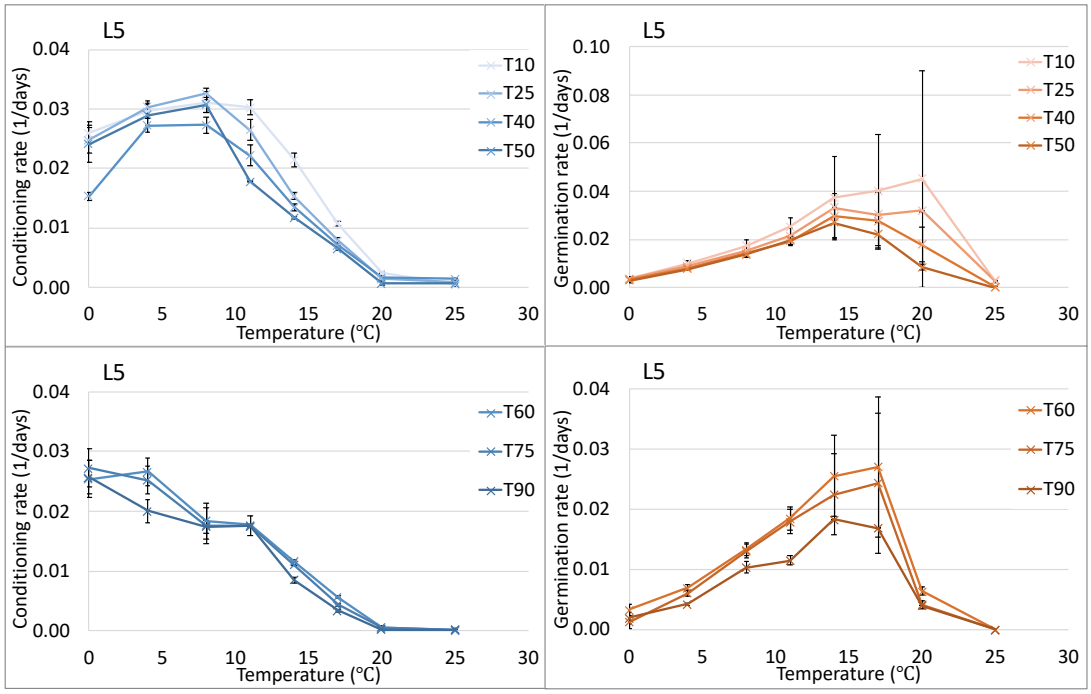

Figure 6.15 Parallel model fitted rates for 8 temperatures 0, 4, 8, 11, 14, 17, 20 and 25°C were obtained for isolate L5 based on combined TE1 and TE2 data (including S3) for statistics T_{10}, T_{25}, T_{40}, T_{50} (top) and T_{60}, T_{75}, T_{90} (bottom).

For the earlier percentiles a hill shaped response to temperature was shown for CR, with the fastest CR_8, ranging from 0.03114 (T_{10}) to 0.02731 (T_{40}) (corresponding to 32.1 to 36.6 days to complete conditioning). The CR declined with both declining and increasing temperature. The smallest conditioning rates were estimated for 20 and 25°C, below 0.00236 (CR_{20} for T_{10}, corresponding to over 423.7 days). At the low

temperature end the CR_0 was consistently smaller then CR_4 and greater than CR_{14}, ranging from 0.02595 (T_{10}) to 0.015342 (T_{40}) (38.5 to 65.2 days). Similarly, the GR showed a hill-shaped response to temperature with the fastest GR_{14}, ranging from 0.03253 to 0.03069 (30.7 to 32.6 days), except for T_{10} where the fastest GR was estimated for 20°C, 0.00451 (22.2 days).

For the later percentiles the conditioning response to temperature changes, where the CR for T_{60} shows only a small hill shape with fastest CR_4, 0.02661 (37.6 days) (small decline towards CR_0), and for T_{75} and T_{90} the CR consistently declines with increasing temperature, with fastest CR_0, 0.0272 (T_{75}) and 0.02571 (T_{90}) (36.8 and 38.9 days, respectively). The slowest CR was estimated for 20 and 25C, below 0.00054 (CR_{20} for T_{75}, corresponding to over 1851,9 days). The germination response is hill shaped and the fastest GR shifted from GR_{17} (T_{60} and T_{75}), 0.027 and 0.0243 (37 and 41.2 days, respectively), towards GR_{14} for T_{90}, 0.0183 (54.6 days). The slowest GR was estimated for 0 and 25°C, below 0.00185 (GR_0 for T_{90}, corresponding to over 540.5 days)

Generally, the estimated conditioning and germination rates declined with the increasing proportion of the population, however this decline was not consistent across all temperatures.

Curves and respective functions in consideration were: Constant rate (Equation 6.6), linear increasing (Equation 6.7) and declining (Equation 6.8); and logistic increasing (Equation 6.9) and declining (Equation 6.10) and a combination of these functions would be considered to describe the rate functions for conditioning and germination process on temperatures ranging from 0 to 25°C (Figure 6.16).

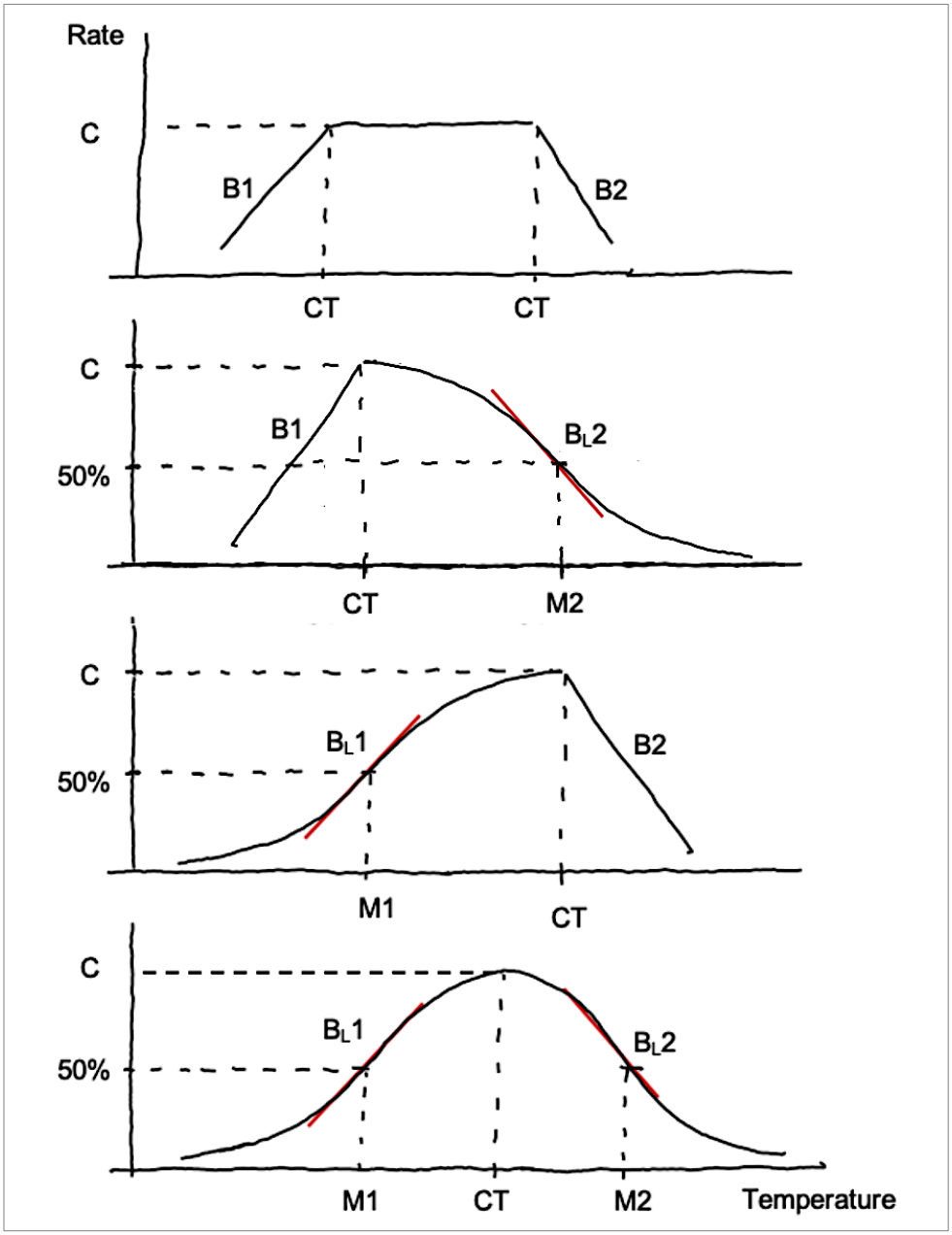

Figure 6.16 Proposed shapes describing pattern observed by fitting individual rates to isolate L5; from top to bottom: linear + constant + linear, linear + logistic, logistic + linear, logistic + logistic; C - max rate; CT - temp at max rate; B – increase (B1)/decrease (B2) in rate below/above CT (slope/linear regression); B_L- increase (B_L1)/decrease (B_L2) in rate below/above CT for logistic function (first derivate); M - temperature at the midpoint of increasing (M1)/declining (M2) part for logistic function, where the rate is at 50%

$$R_x = C;\ for\ x = CT$$

Equation 6.6 Relationship between the rate at temperature x (R_x) and maximum rate (C) achieved at CT

$$R_x = \big(C - B1 * (CT - x)\big);\ for\ x < CT$$

Equation 6.7 Linear increase in rate at the temperature x (R_x), for temperatures below CT (temperature at maximum rate).

$$R_x = \big(C + B2 * (x - CT)\big);\ for\ x > CT$$

Equation 6.8 Linear decrease in rate at the temperature x (R_x), for temperatures over CT (temperature at maximum rate).

$$R_x = \frac{C}{1 + e^{-B_L1(x-M1)}};\ for\ x < CT$$

Equation 6.9 Logistic increase in rate at the temperature x (R_x), for temperatures below CT (temperature at maximum rate).

$$R_x = \frac{C}{1 + e^{-B_L2(M2-x)}};\ for\ x > CT$$

Equation 6.10 Logistic decrease in rate at the temperature x (R_x), for temperatures over CT (temperature at maximum rate).

Where the parameters to estimate were as follows (Figure 6.16, and as used in Equation 6.6 – 6.10):

⇒ C – Maximum rate;

⇒ CT – Temperature at maximum rate (max and min);

⇒ B1 - Increase in rate below CT (linear function, slope);

⇒ B2 – Decrease in rate below CT (linear function, slope);

⇒ B_L1- Increase in rate for logistic function (T<CT) (first derivative);

⇒ B_L2 - Reduction in rate for logistic function (T>CT) (first derivative);

⇒ M1 – Temperature at the midpoint of increasing logistic, rate at 50% of the maximum (C);

⇒ M2 - Temperature at the midpoint of declining logistic, rate at 50% of the maximum (C);

6.4.3.2 Model specification and fitting results

A decision on shape of the observed curves was made and appropriate functions were selected for the T_{10} and T_{25}.

- CR = linear (increasing) (Equation 6.7) + logistic (decreasing) (Equation 6.10), (5 parameters)
- GR = logistic (increasing) (Equation 6.9) + logistic (decreasing) (Equation 6.10), (6 parameters)

For the curve fitting an identical set of calculations (2 to 10) as for the rate fitting (6.4.2.3) was used and where the difference between these two approaches was incorporated in the first step – parameter specification. Again, all calculations were

included in each iterative step of the parameter optimisation for the parallel including S3 data (S1 + S2 + S3), with the values of CR and GR updated at each step. The calculations in steps 2 – 9 were identical for conditioning and germination:

1. Specification of CR and GR based on selected temperatures depending functions (total of 11 parameters) for each S1, S2 and S3 temperature (T1, T2 and T3):

$\Rightarrow$ T1, T2, T3 < CCT; $CR_{T1} = CC-CB1*(CCT-T1)$

$CR_{T2} = CC-CB1*(CCT-T2)$

$CR_{T3} = CC-CB1*(CCT-T3)$

$\Rightarrow$ T1, T2, T3 $\geq$ CCT; $CR_{T1} = CC/(1+\exp(-CB_L2*(CM-T1)))$

$CR_{T2} = CC/(1+\exp(-CB_L2*(CM-T2)))$

$CR_{T3} = CC/(1+\exp(-CB_L2*(CM-T3)))$

$\Rightarrow$ T1, T2, T3 < GCT; $GR_{T1} = GC/(1+\exp(-GB_L1*(T1-GM1)))$

$GR_{T2} = GC/(1+\exp(-GB_L1*(T2-GM1)))$

$GR_{T3} = GC/(1+\exp(-GB_L1*(T3-GM1)))$

$\Rightarrow$ T1, T2, T3 $\geq$ GCT; $GR_{T1} = GC/(1+\exp(-GB_L2*(GM2-T1)))$

$GR_{T2} = GC/(1+\exp(-GB_L2*(GM2-T2)))$

$GR_{T3} = GC/(1+\exp(-GB_L2*(GM2-T3)))$

For both percentiles, optimization was successful only after a number of parameters were fixed (Table 6.11). The percentage variance accounted for was high 85.8 and 89.7% and with high s.e. of observations 39.0 and 34.7. The curve and rate fitting outcomes are similar and also the plotted curves follow the rates estimated in the first step (Figure 6.18).

L5	**T_{10}**	**T_{25}**
Curve combination	CR = Linear + Logistic GR = Logistic + Logistic	CR = Linear + Logistic GR = Logistic + Logistic
d.f. (reg/ res)	9/417	8/418
F (v.r.)	737.37	1340.37
R^2	85.8	89.7
S.e. of observ.	39.0	34.7
Opt. issue	No progress	No progress
Par. fixed	GCT, GB_L2	CB_L2, GCT, GB_L2

Table 6.11 Curve fitting outcome for T_{10}, T_{25}, for isolates L5, based on TE1 and TE2 combined data (S3), using Parallel model approach.

Parameters with large s.e. (uncertainty) were the temperature at the maximum CR (CCT), the slope of the increasing linear line (CB1) (for T_{10}), and the temperature at 50% GR (GM1) (Table 6.12). This was mostly due to relatively large interval between temperatures selected for the experiment and fairly small changes in CR in the temperature range 4 to 11°C for T_{10}.

L5	T_{10}		T_{25}	
Parameters	**Estimated**	**s.e.**	**Estimated**	**s.e.**
CC	0.0313	0.00112	0.03068	0.00141
CCT	5.97	2.47	5.374	0.981
CB1	0.000913	0.000543	0.001897	0.000436
CB_L2	0.5969	0.0278	0.4253	*
CM2	15.811	0.185	14.01	0.19
GC	0.0369	0.00798	0.03082	0.00821
GCT	20	*	21	*
GB_L1	0.2796	0.031	0.2684	0.0581
GB_L2	0.9	*	1	*
GM1	7.91	1.62	7.57	2.27
GM2	22.24	0.269	22.761	0.295

Table 6.12 Parameters estimated for conditioning and germination rates for T_{10}, T_{25}, for isolates L5, based on TE1 and TE2 combined data (S3).

During the optimization, the germination curve parameters for the declining logistic curve (GCT, GB_L2) were causing problems and needed to be fixed for a successful optimization. This was mostly due to lack of data for that region, as the maximum germination rate was estimated at 20°C (T_{10}) and 21°C (T_{25}) (Table 6.12) and, consequently, the parameters of the proposed logistic curve had to be estimated based on two (max) available data points (temperatures), 20 and 25°C. Therefore, only a simpler, linear response for the declining part of the GR curve could be included (similar to the increase in conditioning rate).

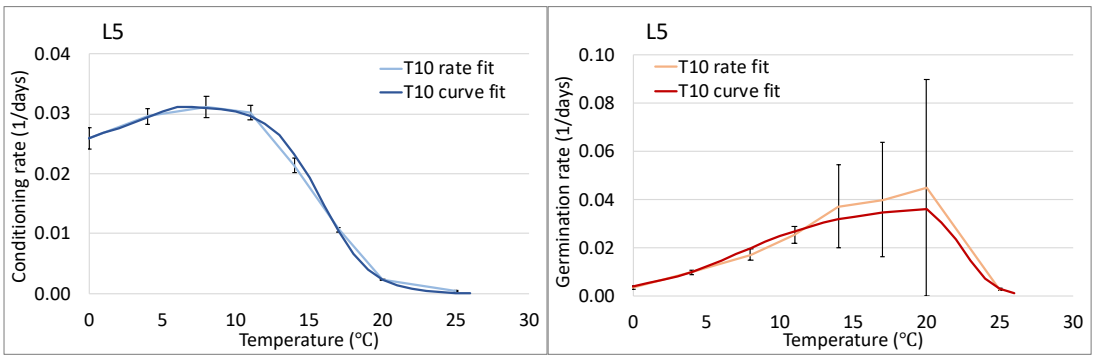

Figure 6.17 Fitted curves/ functions for T10 for isolate L5. Function describing rate change with temperature as combination of: conditioning = increasing linear + decreasing logistic function; germination = increasing logistic function + decreasing logistic function.

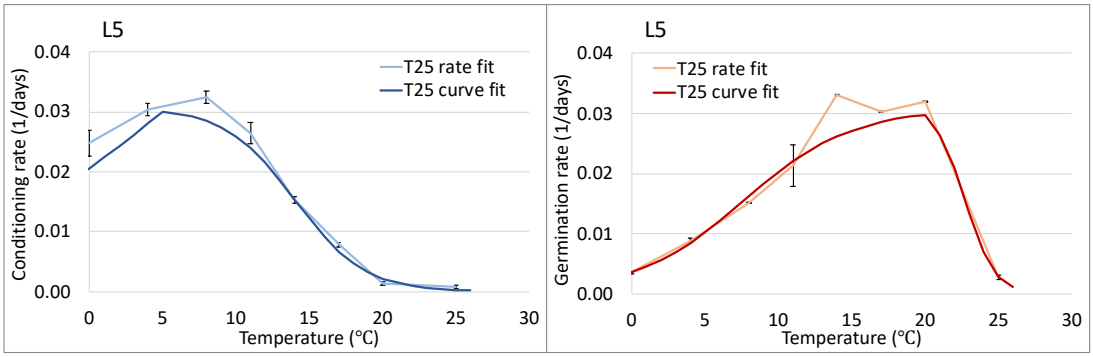

Figure 6.18 Fitted curves/ functions for T25 for isolate L5. Function describing rate change with temperature as combination of: conditioning = increasing linear + decreasing logistic function; germination = increasing logistic function + decreasing logistic function.

Repeated optimization was done for T_{10} with simplification on the decreasing part of the germination curve, as suggested:

- CR = linear (increasing) + logistic (decreasing), (5 parameters)
- GR = logistic (increasing) + linear (decreasing), (5 parameters)

The first step of optimization process – parameter specification was therefore changed accordingly:

$\Rightarrow$ T1, T2, T3 < CCT; $CR_{T1} = CC\text{-}CB1*(CCT\text{-}T1)$

$CR_{T2} = CC\text{-}CB1*(CCT\text{-}T2)$

$CR_{T3} = CC\text{-}CB1*(CCT\text{-}T3)$

$\Rightarrow$ T1, T2, T3 $\geq$ CCT; $CR_{T1} = CC/(1+\exp(-CB_L2*(CM\text{-}T1)))$

$CR_{T2} = CC/(1+\exp(-CB_L2*(CM\text{-}T2)))$

$CR_{T3} = CC/(1+\exp(-CB_L2*(CM\text{-}T3)))$

$\Rightarrow$ T1, T2, T3 < GCT; $GR_{T1} = GC/(1+\exp(-GB_L1*(T1\text{-}GM1)))$

$GR_{T2} = GC/(1+\exp(-GB_L1*(T2\text{-}GM1)))$

$GR_{T3} = GC/(1+\exp(-GB_L1*(T3\text{-}GM1)))$

$\Rightarrow$ T1, T2, T3 $\geq$ GCT; $GR_{T1} = GC+CB2*(T1\text{-}GCT)$

$GR_{T2} = GC+CB2*(T2\text{-}GCT)$

$GR_{T3} = GC+CB2*(T3\text{-}GCT)$

Additionally, when implementing the linear response for the declining part of the GR curve, a further problem occurred. The asymptote of the logistic curve and the linear response did not meet at the temperature estimated to be the change point between the two responses shapes (T_{10}_1, T_{10}_2). To address this issue, an additional element

was introduced into the optimization for the T_{10}_3 run in the first step for GR – parameter specification, coupling the maximum rate from the logistic curve into the linear curve calculation (highlighted):

$\Rightarrow$ T1, T2, T3 < GCT; $GR_{T1} = GC/(1+\exp(-GB_L1*(T1-GM1)))$

$GR_{T2} = GC/(1+\exp(-GB_L1*(T2-GM1)))$

$GR_{T3} = GC/(1+\exp(-GB_L1*(T3-GM1)))$

$\Rightarrow$ T1, T2, T3 $\geq$ GCT;

$GR_{T1} = GC/(1+\exp(-GB_L1*(GCT-GM1)))+CB2*(T1-GCT)$

$GR_{T2} = GC/(1+\exp(-GB_L1*(GCT-GM1)))+CB2*(T2-GCT)$

$GR_{T3} = GC/(1+\exp(-GB_L1*(GCT-GM1)))+CB2*(T3-GCT)$

These changes resulted in a successful optimization (Table 6.13) without the necessity to fix any parameters, and the percentage variance and s.e. of observations remained almost identical with the previous optimization (Table 6.11).

L5	T_{10}_1	T_{10}_2	T_{10}_3
Curve combination	CR = Linear + Logistic GR = Logistic + Linear	CR = Linear + Logistic GR = Logistic + Linear	CR = Linear + Logistic GR = Logistic + Linear
d.f. (reg/ res)	10/416	10/416	10/416
F (v.r.)	660.41	658.31	660.74
R^2	85.7	85.7	85.7
S.e. of observation	39.1	39.1	39.1
Opt. issue	-	No progress	-
Par. fixed	-	none	-

Table 6.13 Curve fitting outcome for T_{10}, for isolates L5, based on TE1 and TE2 combined data (S3), using Parallel model approach, using various combination of conditioning and germination rate curves; T_{10}_3 fit the equation for germination rate calculation was altered to couple the meeting point of logistic and linear curve.

Parameters with large s.e.s (uncertainty) were the temperature at the maximum CR and GR (CCT and GCT), the slope of the increasing linear line (CB1), temperature at 50% GR (GM1) and the slope for the linear decreasing line (GB2), for which no s.e. could be obtained (Table 6.14, Figure 6.19). These parameters were affected by the total number and the interval size between temperatures used in the experiments TE1 and TE2, and as well as the large s.e. from the rate fitting of GR in the first step already suggested, by limited germination observed in S2 and large variation in replicates response for T $\geq$ 14°C.

L5	T_{10}_1		T_{10}_2		T_{10}_3	
Param.	**Estimate**	**s.e.**	**Estimate**	**s.e.**	**Estimate**	**s.e.**
CC	0.03128	0.00111	0.0311	*	0.03142	0.00111
CCT	5.88	2.4	5.793	*	5.95	2.39
CB1	0.000923	0.000534	0.0009153	*	0.000936	0.000541
CB_L2	0.5973	0.0278	0.5996	*	0.5977	0.0278
CM2	15.815	0.185	15.84	*	15.801	0.184
GC	0.0478	0.0215	0.04507	*	0.02993	0.00459
GCT	18.07	3.28	15.89	*	20.051	0.758
GB_L1	0.2499	0.0424	0.2395	*	0.316	0.0439
GM1	9.86	3.37	10	*	6.29	1.23
GB2	-0.0065	*	-0.004628	*	-0.005432	*

Table 6.14 Parameters estimated for conditioning and germination rates for T_{10}, for isolates L5, based on TE1 and TE2 combined data (S3); T_{10}_3 fit the equation for germination rate calculation was altered to couple the meeting point of logistic and linear curve.

The quite dramatic change in GR parameters when comparing T_{10}_1 and T_{10}_3 (Table 6.14, Figure 6.19), showed no impact on the optimization outcome (Table 6.13). A repeated optimization including the T_{10}_3 setting would be required along with analyses of the residuals and change in residuals with various parameters, to better understand the sources of errors in the model fitting.

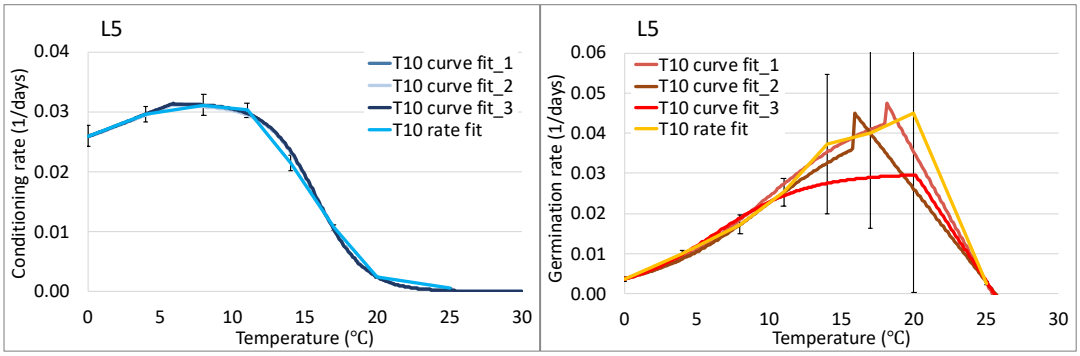

Figure 6.19 Fitted curves/ functions for T10 for isolate L5. Function describing rate change with temperature as combination of: 1st and 2nd fit, conditioning = increasing linear + decreasing logistic function; germination = increasing logistic function + decreasing linear function; 3th fit the equation for germination rate calculation was altered to couple the meeting point of logistic and linear curve.

6.5 Discussion

Generally, the parallel model as proposed for *S. sclerotium* isolate L5, explains the experimental data well. To properly test the model a validation against earlier experiments and field germination would be vital.

As an alternative for isolate L6, given the potential to germinate without conditioning, an option would be to construct a model without the conditioning phase or to restrict the conditioning requirements for certain temperatures. However, for a better understanding of the L6 conditioning requirement, a similar experiment to dissection of sclerotia for isolate L5 would be crucial.

Limitations of the model were associated with:

- Observation data - i.e. in-between replicate and experiment variation.
- Choice of temperatures - where a smaller interval between temperatures would allow for more detailed parameter optimisation, and allow more complex shapes to be used, i.e. logistic function, where linear function was used.
- Duration of experiments - this was already improved by the availability of S3 data. The decision to test the viability of sclerotia by introducing more favourable conditions and the genuine ability of sclerotia to reassume germination after time in unfavourable conditions was very positive. However, further experiments could be designed to test the effect of such arrest on conditioning and germination rates, or aid to identify periods and conditions where the process are prevented, so further differentiation between the condition and germination can be introduced.
- Reverse transfer between temperatures - as main temperature experiment designed in Chapter 3 used only transfer from lower to higher temperature. This would allow to further test the assumption about independence of the conditioning and germination process. Although noticeable work was done in the scope of this thesis to enable distinction between conditioning and germination processes the possibility of interaction between them cannot be ruled out. A potential suggestion would be to incorporate association (link, loop) between the processes into model, i.e. completed conditioning process accelerates germination rate. We could speculate about this by looking at the dissection experiment where the presence of the “active hyphae” could represent a mobilisation of metabolites in the outskirt of sclerotia (close to rind), which would quicken germination and final stipe production. Furthermore, a third (temperature-dependent) integrating process could be proposed resulting in the final apothecia production (Figure 6.20).

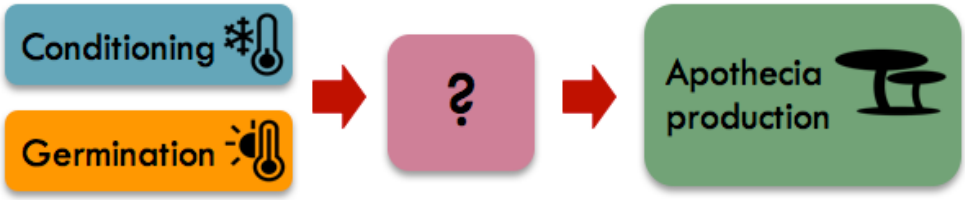

Figure 6.20 Schematic drawing parallel model and additional 3th integrating (temperature dependent) process as addition to conditioning and germination for *S. sclerotiorum* sclerotia.

- Modelling work described in this chapter provides a ground for further work outside the scope of this thesis. Finalization of the curve fitting for remaining percentiles would allow for the next step, distribution fitting to be explored. This would allow to predict germination time for any proportion of sclerotia population at any temperature.

7 General discussion

Sclerotinia sclerotiorum causing Sclerotinia disease is a major problem in many vegetable crops causing yield losses due to disease epidemics in plants in fields or post-harvest in storage. *S. sclerotiorum* presence is confirmed all over the world, across various geographic locations from hot and moist conditions in Brazil, Central America and India to colder and more seasonal climates in Europe, North America and Canada. The modern age cultural practices (i.e. monocrops, large fields, non-removal of infected crop residue, irrigation, agriculture intensification) and globalisation, have resulted in favourable conditions for an increase in disease incidence, the accumulation of inoculum in soil over the years, and the spread of *S. sclerotinia* isolates between different geographic locations. This has resulted in a critical situation and a need for a fast and sustainable solution for Sclerotinia disease control. There have been a number of studies (introduced in Chapter 1) examining the life cycle of the fungal pathogen and its relationship with plants and environmental conditions. The survival of *S. sclerotiorum* sclerotia in soil, and subsequent carpogenic germination are crucial parts of the life cycle, that directly lead to plant infection through ascospores released by apothecia. Several studies as described in Chapter 1 and in the introduction of this chapter (Rotem and Palti, 1969, Hoes and Huang, 1975, Huang and Hoes, 1976, Huang, 1977, Blad et al., 1978, Huang and Hoes, 1980, Wu, 1991, Twengström et al., 1998a, Bardin and Huang, 2001, Clarkson et al., 2004, McLaren et al., 2004, Sharma et al., 2005, Bolton et al., 2006a, Clarkson et al., 2007, Wu and Subbarao, 2008, Saharan, 2008, Zhang and Xue, 2010, Young et al., 2014, Derbyshire and Denton-Giles, 2016, Foley et al., 2016) have examined cultural practices and integrated disease and agronomic management strategies to reduce infection incidence and the survival of sclerotia inoculum in soil, including the development of forecasting systems and models for the timing of fungicide applications to kill spores which predict the presence of apothecia or ascospores and hence infection risk (incidence) in the field. However, there are largely no attempts to reduce sclerotial inoculum in the soil. Temperature (Coley-Smith and Javed, 1970, Phillips, 1986, Phillips, 1990, Huang and Kozub, 1991, Sansford and Coley-Smith, 1992, Dillard et al., 1995, Sun and Yang, 2000, Bardin and Huang, 2001, Clarkson et al., 2004, Marinelli et al., 2004, Matheron and Porchas, 2005, Clarkson et al., 2007, Liu and Paul, 2007, Wu and Subbarao, 2008, Mila and Yang, 2008, Foley et al., 2016, Gupta and Singh, 2017) and moisture (Ferraz et al., 1999, Hao et al., 2003, Clarkson et al., 2004, Matheron and Porchas, 2005, Wu and Subbarao, 2008, Mila and Yang, 2008, Nepal and Mendoza, 2009, Nepal and del Río Mendoza, 2012) have been

identified as the most relevant environmental factors affecting carpogenic germination of *S. sclerotiorum* sclerotia. Temperature affects carpogenic germination in two ways; previous studies have shown that sclerotia from some *S. sclerotiorum* isolates require a short 'conditioning phase' at cold temperatures before a rapid and high level of carpogenic germination can occur. Temperature also affects the rate of apothecial production for the (pre-conditioned) sclerotia with a range of optimal temperatures reported. Sun and Yang (2000) suggested a range of 12˚C to 30˚C (optimum around 20˚C) while Hao et al. (2003) reported an optimal range of 10˚C to 20˚C. The same optimum range was reported by Wu and Subbarao (2008) with maximum germination at 15˚C, and the fastest rate at 20˚C. Clarkson et al. (2004) reported temperature having a significant effect on both rate of germination and the final number of germinated sclerotia, with the optimum temperature ranging from 15°C to 20°C; however germination also occurred between 5°C and 25°C with sufficient moisture being required in all cases.

Researchers agree that *S. sclerotiorum* isolates from different geographic locations (Huang and Kozub, 1991, Hao et al., 2003, Uloth et al., 2015) and also from within one geographic location (Clarkson et al., 2004, Clarkson et al., 2007) differ in their germination response to temperature. The between-isolate variation in the germination response to temperature and limited knowledge about the process of conditioning complicate the possibility of developing a unified forecasting model for *S. sclerotiorum* sclerotia germination in the field.

The aims of this research, as outlined in Chapter 1, were therefore to further investigate the effect of temperature and moisture on the germination of *S. sclerotiorum* sclerotia and to model the processes of conditioning and germination based on the temperature response.

The main objectives of the project were to:

- Evaluate the effect of different temperature regimes on the carpogenic germination of *S. sclerotiorum* sclerotia, and determine the physiological changes leading to the formation of stipes and apothecia;
- Evaluate the effect of moisture on the carpogenic germination of *S. sclerotiorum* sclerotia;
- Produce a model to simulate germination of *S. sclerotiorum* sclerotia with an emphasis on modelling the whole distribution of germination times for contrasting *S. sclerotiorum* isolates.

Assessing the impact of temperature on germination of S. sclerotiorum sclerotia – design of experiments and treatments

To achieve the first of these objectives, the initial part of the project focused on a very detailed examination of the effect of temperature on carpogenic germination of sclerotia from two UK *S. sclerotiorum* isolates (L5, L6) selected based on their different requirements for a cold conditioning phase, as identified in previous experiments. These new experiments were designed to address some of the data issues and gaps associated with the previous experiments (Clarkson et al., 2004, Clarkson et al., 2007) including a larger sample size, exposure to lower temperatures and shorter durations of low-temperature exposure. The complex analyses of data from these temperature experiments showed variation in trends and temperature requirements between isolates L5 and L6, although not entirely as originally hypothesised. The general thought was that variation between isolates was mainly due to variation in conditioning requirements. However, the results of these temperature experiments suggest this not to be the case, as both isolates showed an improved germination response when sclerotia were exposed to an initial low temperature, compared to the response under constant single temperature conditions. For both isolates, an earlier start of germination was observed after the shortest duration in the low temperature phase (S1), 7 or 14 days. However, to ensure fast germination throughout the whole population and the most uniform germination, at least 29 days at a low temperature was required, even when then exposed to higher optimum temperatures. A duration of 56+ days of S1 was too long, as although fast germination for the highest percentiles was observed, the delay of the start of germination resulted in an overall delay in germination compared to the response with only 29 days S1 duration. Furthermore, germination was observed for the longer S1 durations, especially for isolate L5 indicating that the conditioning phase was already complete.

Regarding the level of germination, generally, a longer initial duration at a lower temperature supported subsequent germination at supra-optimum temperatures when sclerotia were transferred to warmer temperatures (S2), although it is unclear how to interpret these observations. Sclerotia which germinate during S1 were only experiencing one temperature, as for the constant single temperature treatments and, therefore they should be excluded from the analyses of S2 temperature effects. However, this then changes the experimental design (introducing missing observations), as well as the consistency of the S2 assessment (varying numbers of

sclerotia available to germinate at the start of S2, and the “fast” sclerotia already having responded), where excluding the germinated sclerotia potentially introduces a bias, as a part of the population is omitted from the experiment for these treatments. To complicate this even more, the germination of sclerotia at S2 supra-optimum temperatures could be assigned to the S1 period as well, where potentially the sclerotia germination progressed so far in S1, that, despite unfavourable conditions in S2, stipe development would continue. This is something to contemplate further when designing experiments involving more than one stage.

Temperature experiments conducted for this project were large and time-consuming, and although the results obtained are important for understanding the variation between *S. sclerotiorum* isolates, repeating these for a large number of isolates would be extremely challenging. However, it would be vital to produce similar studies for a greater number of isolates and possibly identify groups of isolates with similar behaviours. Possible reductions in the size of the experimental design could be achieved by using: fewer S1 durations, e.g. 7 days and 29 days at only a single temperature; fewer S2 temperatures (unless supporting model development) where the extreme temperatures of 0 and 25°C could be omitted and a temperature interval of 5°C could be sufficient. A possible recommendation for analysis of various isolates for the optimum T1 and T2 along of S1 duration already suggested, could be a set of lower and higher T1 (4, 11, 14 and 17°C) followed by set of medium and high T2 (11, 14, 17 and 20°C). The main amendment to suggest is the way in which such experiments are analysed. It is evident from the main temperature experiment analyses that different statistical summaries address different questions and are affected in slightly different ways. Therefore, if the germination response is not assessed as whole (as presented in Chapter 3) misinterpretation or overlooking of some effects could occur. This is further related to the difficulty to compare results from different studies because of a variation in the materials, methods and analysed statistics.

Assessing the impact of temperature germination of S. sclerotiorum sclerotia – isolate differences

The variation in germination between *S. sclerotiorum* isolates indicated in the results of these experiments is due to their differential temperature requirements for both processes, conditioning and germination. Isolate L5 is more sensitive to temperature and shows a narrower range of optimum temperatures, and has one optimum combination. In contrast isolate L6 is more robust to temperature changes (especially

to higher temperatures), has a wider range of optimum temperatures and has two distinct optimal combinations, a “summer” optimum and a “spring” optimum. The two optima reported for isolate L6 suggests that sclerotia produced in spring and summer can germinate without cold temperature winter conditioning and therefore initiate further cycles of infection within a single year. Cold conditions trigger response associated with “spring” optimum (lower T1 and T2), while absence of low temperature, and generally higher temperatures allow for response which is defined by the “summer” optimum (higher T1 and T2). Such behaviour was described first time and furthermore, it explains how isolates can adapt to different geographic location or changing climatic conditions. Furthermore, for the UK isolates it was assumed that cold conditioning is generally required.

For isolate L5 the overall optimum conditions were T1 = 4-8°C, S1 duration 29 days and T2 = 14°C, where level of germination was 100%, T_{10} = 45 days, T_{90} = 71-72 days, and therefore IDR =T_{90}-T_{10} = 26-27 days. For isolate L6 the “summer” optimum conditions were T1 = 14°C, S1 duration 29 days and T2 = 20°C, where level of germination was ~100%, T_{10} = 42-43 days, T_{90} = 55-58 days, and therefore IDR =T_{90}-T_{10} = 13-15 days and the “spring” optimum were T1 = 4-8°C, S1 duration 29 days and T2 = 17°C, where level of germination was 100%, T_{10} = 45-47 days, T_{90} = 67-74 days, and therefore IDR =T_{90}-T_{10} = 22-25 days. The "spring" optimum for isolate L6 is close to the optimum identified for isolate L5, where the generally higher final temperature is preferred for isolate L6 compared to isolate L5. The motivation for recognising two sets of optimal conditions for isolate L6 is that there is a distinct difference in the conditioning temperature preference between the final optimum temperatures identified. For T2 = 20°C, germination improves with increasing T1, whereas for T2 = 17°C preference moves towards T1 = 4-8°C. This behaviour suggests that sclerotia from isolate L6 could germinate in spring and that sclerotia produced following subsequent infection could then germinate in late Summer / early Autumn hence producing a second wave of inoculum. This behaviour was not described in such detail in previous studies, for any isolate, and although it is known that isolate L6 (as well as many others) can and does germinate without conditioning, a detailed description of this response was not published previously.

For both *S. sclerotiorum* isolates, no germination was observed at a constant single temperature of 0°C, however conditioning at 0°C improved germination at higher temperatures, although generally less than for 4-8°C for isolate L5. For isolate L6 this initial low temperature caused some disturbance in both germination level and the

uniformity of germination when followed by a high temperature, possibly because the combination of T1 and T2 with a large amplitude was more disturbing compared to temperatures closer to each other. Identifying 0°C as the limiting temperature for germination as well as the sub-optimum conditioning temperature is important to address the inference from the Clarkson model (2007), where the fitted conditioning rate function suggested an increase in conditioning rates for temperatures <4°C although these were not examined as 0°C was not included in the experiments supporting the model construction. In addition, a freezing temperature is not commonly used by researchers to induce carpogenic germination, but it was reported as temperature inducing myceliogenic germination (Huang, 1991). The ceiling temperatures were identified for isolate L5 as 20°C and for isolate L6 as 25°C, although longer S1 durations resulted in some germination also at these temperatures. Again, it is problematic to conclude whether this germination is a residual effect from the progress towards germination during S1, where for some treatments for isolate L5 germination occurred during S1. The identified ceiling temperatures are in the range reported by other authors (as described earlier).

Understanding the physiological processes involved in germination of S. sclerotiorum sclerotia

To increase the understanding of the physiological processes underlying the conditioning and germination phases, microscopic observation of dissected *S. sclerotiorum* sclerotia was performed prior to germination to identify any structural changes that might precede the formation of stipes and apothecia. Identification of a possible separation of the two processes (conditioning and germination) is particularly important when investigating the progress of two processes with an opposite response to temperature assessed on a single joint indicator (stipe production). The presence of primordia, described as early developmental stages of apothecial stipes by Saito (1973), was confirmed in sclerotia of isolate L5 prior to the observation of germination. Furthermore, this was first time that the presence of primordia was assessed in both conditioned (initial temperature of 4, 11, 17, 20°C then transferred to 17°C after 28 days) and unconditioned (exposed to 4, 11, 17, 20°C throughout) sclerotia and at various temperatures to examine possible temperature responses, and where the assumption was tested that primordia, as the initial developmental stage of stipes, would form only after conditioning is completed, shortly prior to actual stipe formation in the germination-favourable conditions. Results of microscopic dissection of sclerotia for isolate L5, which was identified to be the isolate requiring

cold conditioning for high level, fast and uniform germination, showed primordia presence in unconditioned sclerotia as early as 7 days at a temperature of 11-20°C. Furthermore, the number and size of primordia increased over time and with increasing temperature. However, although primordia were present in high numbers at 20°C, unconditioned sclerotia failed to produce apothecial stipes. At a lower temperature, 4°C, no primordia were observed over the duration of 70 days (experiment duration) and also no stipes were produced during this period. For the transfer treatments, sclerotia conditioned at 4°C achieved the highest and most uniform germination, although somewhat delayed compared to 11 and 17°C. In addition to observing primordia stipe initials and stipes, developmental structures i.e. newly described “active hyphae”, were also observed. The term "active hyphae" is new and aims to highlight the observation of mobilized "active" cell collections close to the rind, which show some staining and differentiation to regular medullar hyphae but are not as structurally developed as primordia. These active hyphae were predominantly found in treatments after transfer from low (4-11°C) to high temperature, and where stipe production was observed in increased numbers between 7 to 14 days after transfer. It is possible that the active hyphae serve as a platform where enzymatic and metabolic activities are concentrated and where metabolites are transferred to, to facilitate a prompt development of apothecial stipes. For successful germination both need to be present, active hyphae and primordia, however, the development of each of these structures seem to be independent processes, which show an individual response to temperature. These are very promising results, where possibly we were able to distinguish between the conditioning and germination processes. However, more work would be required to establish a more detailed time frame of the developmental changes, where various staining methods could be tested, and, more importantly, more isolates should be assessed especially as isolate L6 showed such a different response to temperature. Besides, if we do not understand the processes thoroughly it is very difficult to describe and compare the behaviour of different isolates (local or from different geographic origins) because of possible misinterpretation of observations, and furthermore to design appropriate experiments to address scientific questions adequately.

Assessing the impact of moisture on germination of S. sclerotiorum sclerotia

Experiments investigating the effects of a dry period on germination of *S. sclerotiorum* sclerotia were introduced, examining the impacts of various durations of dry periods

during both conditioning (S1) and germination (S2) phases on sclerotial germination. Imposing dry periods on sclerotia of *S. sclerotiorum* generally caused an increase in myceliogenic germination, although predominantly for isolate L5. Carpogenic germination was significantly influenced by dry periods introduced in S1 for *S. sclerotiorum* isolate L5, where considerably lower carpogenic germination (62% compared with xxx%) was achieved by the end of the experimental period (153 days), though this was generally associated with delayed germination. However, for both isolates dry conditions in S1 delayed germination, and this was further intensified for temperatures outside the optimum range (i.e. 17°C for isolate L5).

The dry periods introduced in S2 had a small effect on the level of mycelial and carpogenic germination for both isolates. Germination was arrested during the dry periods, however, it resumed after moist conditions were reintroduced and the delay was principally corresponding with the duration of dry period applied, even for a dry period as long as 28 days. This suggests that germination rates at the corresponding temperatures, when sufficient moisture was provided, were unaffected by dry periods. This is in contrast with observations reported by Wu and Subbarao (2008) that a period of 10 to 20 days of low soil moisture completely arrested carpogenic germination and it took up to 35 days between rewetting and the appearance of new apothecia, regardless of when and for how long (10+ days) the dry period was applied. Understandably, germination in the field will then be highly dependent on there being sufficient moisture, and a forecasting model ignoring soil moisture/ rainfall cannot be realistic. Moisture seems to be a limiting factor, as it has the capacity to arrest germination completely, whereby the temperature predominantly affects the timing, by either delaying or accelerating germination. The results from moisture experiments suggest a relatively simple relationship of germination to moisture - moist = germination proceeds, dry = germination arrested, and therefore the use of a threshold adjustment to the germination forecasting model, as suggested (and used) previously by ADAS (personal communication) is reasonable. Soil moisture is a complex factor, more challenging to measure and control either in the field or in a controlled environment. Furthermore, the facility to collect accurate soil moisture data representing whole fields and/or regions is a major challenge. Naturally, this is further affected by a number of other factors affecting microclimate, like canopy closure, sunlight, wind conditions and soil type which can contribute to faster desiccation of the soil surface, where the germinating sclerotia are predominantly located and furthermore when irrigation is applied continuous sufficient moisture can be assumed. That's when the relevance of temperature-driven germination and temperature

dependent rates comes to the foreground, to forecast the timing of application of fungicides by forecasting sclerotial germination.

Developing a new model for carpogenic germination of S. sclerotiorum sclerotia

The controlled environment experiments provided a good understanding of the germination response of *S. sclerotiorum* sclerotia for isolates L5 and L6 in relation to temperature, while the microscopy work done for isolate L5 provided an important insight into the organisation of the processes involved. A parallel model was developed for these two processes in response to temperature and compared with the original sequential model proposed by Clarkson et al (2007). The parallel model assumes that conditioning and germination are independent processes, each following its own response to temperature, where conditioning proceeds faster at lower and germination at higher temperatures. For a successful sclerotia germination (stipe production), both processes need to be completed and the time observed at the completion is the time taken for the longer process at the given conditions. Additionally, the parallel model allowed for the effective inclusion of observations made in S3, where the viability of sclerotia which did not germinate in the main part of the temperature experiments was assessed. A series of modelling approaches was considered for the new data generated in this project, as informed by an understanding of the biological processes underlying conditioning and germination. However, not all the three steps proposed for model development were completed. In the scope of this project, only the rate fitting and (partially) the curve fitting (for T_{10} and T_{20} percentiles only) was possible to accomplish. However, these initial steps showed promise, which suggests that the final step of modelling the time to germination for the whole population distribution of sclerotia can be achieved. As was anticipated, the rate model fitting showed a systematic reduction in rates with the increasing percentile modelled, for both processes and across the range of temperatures, which could be incorporated as further parameters when modelling the whole distribution of germination times.

Additionally, the parallel model was developed based on isolate L5 and an extension or incorporation of isolate L6 would be a vital step for the development of a model for a mixed population of *S. sclerotiorum* sclerotia, as frequently observed in the field. Isolate L6 showed a different response to temperature, especially for the high temperatures. The analyses reported in this Chapter 3 revealed two optima for isolate L6, one for T2 $\geq$ 20°C and the second for T2 $\leq$ 17°C. These two optima show a contrasting response to temperature, especially for the conditioning temperatures,

and therefore an alteration to the parallel model would be necessary to take this variation into account. The “summer” optimum is a distinct behaviour of isolate L6 (and possibly other isolates thought as not requiring germination, where this needs further investigation), and the “spring” optimum shows similar response to conditioning temperatures to isolate L5. This could be addressed in model formulation, by allowing for isolate L6 a temperature range (≥ 20°C) where conditioning response to temperature is adjusted for an anew defined temperature response, higher conditioning temperature are preferred, or where no conditioning is required and only the germination time is considered. However, to further develop this it is vital to better understand the isolate L6 temperature response, similar to isolate L5 (i.e. a microscopy study). In addition, the following model limitations were identified: the model is based on only one isolate; there was substantial between-replicate and between-experiment variation; the experimental temperature intervals were potentially too wide and so important changes in response were potentially missed; there was possibly an insufficient duration of experiments (for extreme temperatures); there was a need for the inclusion of transfers from high to low temperature (to test the parallel order and independence of the two germination processes).

Finally, there is also the potential for the model to be used to forecast sclerotial germination, and therefore Sclerotinia disease occurrence, in the field. However, further work is required to finish the optimization of the proposed model, (so far only parameters for T_{10} and T_{20} have been derived), to allow the application of this model to field data and test how well the model performs in real conditions. Furthermore, if the rate functions for the remaining percentiles could be identified, the possibility of modelling the whole population could be evaluated, allowing the prediction of germination times for any percentile of the sclerotia population at any temperature.

A mixed *S. sclerotiorum* isolate/population model is an inevitable requirement for practical use, especially as the sclerotia can survive in the soil for several years and sclerotia of various isolates, with different temperature requirements, are available for germination when suitable conditions occur. This mixed inoculum represents a vast pool of possibilities for the pathogen to survive and develop. Furthermore, if environmental conditions are not conducive to carpogenic germination of sclerotia, myceliogenic germination may also occur (usually under increased stress and depending on isolate), which can result in the production of daughter sclerotia.

As mentioned previously, further work on understanding the processes associated with carpogenic germination of *S. sclerotiorum* sclerotia is required. This should include chemical analyses and genetic work to further explore the mechanisms behind the "conditioning" of sclerotia. After having detailed information about the response to temperature for these two isolates, a genetic or chemical association relating to development over time could be studied, although chemical analyses may prove a challenge as the sclerotia are very hard structures.

Assessing disease risk

The final question is whether, when compared with the conceptually simpler method of risk tables, the construction of a complex model as proposed is necessary or not. The original idea for a forecasting model for a mixed population of *S. sclerotiorum* isolates assumed that isolates differ only in their conditioning requirements and therefore that the germination pattern (assuming there was enough cold conditioning for all isolates over-winter) would be similar for all isolates, and we could apply a single function to predicting germination in the field even for a mixed population. However, this is not entirely the case, as the results for isolate L5 and L6 show variation in optimum temperatures. In addition, the presence of two sets of optimal conditions for isolate L6 suggest that we could differentiate conditions when isolates behave similarly and when not. One solution to modelling these different responses could be to run the forecast with parameters for several isolates at once, after classifying the isolates and selecting representatives, which would be used as model isolates. Based on the environmental conditions, the prediction of the first germination among the isolates would then indicate Sclerotinia disease risk in the field. Conversely, this could involve quite a complex forecasting model with a large information input (further work would be required to classify and standardize sclerotia isolates based on their temperature requirements if other factors are omitted). Additionally, the temperature effect on the level of germination could be incorporated in the forecasting model/system in the form of "risk", where when optimum germination temperatures are observed a high and uniform germination, apothecia production, is to be expected (when sufficient moisture is available).

Generally, it is easier to adapt to environmental changes with a better understanding of the underlying processes, rather than rely on risk tables based on years of observations in the field, in relatively limited environments. This is becoming more important with rising concerns about the impacts of climate change and with the global expansion of Sclerotinia disease, where historic data could be limiting with regards to

both isolate diversity and weather conditions. There are numerous studies on climate change scenarios, where some were focused on the implications for *S. sclerotiorum* species (Siebold and Von Tiedemann, 2012) and Sclerotinia disease (Uloth et al., 2015), suggesting a positive impact of potential warmer climate scenarios on disease incidence, isolate adaptation and infection potential.

Finally, a preference would be to use the obtained knowledge in a sustainable way and view the agriculture and food production as a part of a greater system rather than only individual businesses trying to achieve high income by unsustainable intensification of production (as witnessed in many cases). Use of integrated disease management approaches, i.e. incorporating crop rotation, plant density, crop residue, harvest storage, cultivar selection, irrigation management, soil solarization, biological and finally chemical control practices, as well as a reflection on past observations, should be a vital part of future Sclerotinia disease control and model development.

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Appendixes

Appendix 1 Potential “preconditioning” for TE1.

Additionally, to providing further observations for examination of the effect of temperature on sclerotia germination, TE2 provided a rectification for the TE1. Some of the sclerotia (mostly isolate L6) used in TE1 had experienced temperatures below 10°C through production (Chapter 2.2). This period at cold temperature could cause some “preconditioning” prior the start of the experiment.

When comparing the common treatments for the TE1 and TE2, for the statistic Maximum germination percentage there was obvious inconsistency in the responses for isolate L6 between the two experiments, where the fitted regression line was significantly different from a 1:1 relationship in contrast to isolate L5 (Pearson correlation coefficient R^2 = 0.95 (intercept = 0.7181, slope = 0.9302), (Top - left, Figure 0.1). For isolate L6 an increased germination in TE1 compared to TE2 was observed for number of treatments, mostly S1 durations 14 days (all T1), T1 = 4°C (all S1 durations), T2 = 20°C and single temperature of 20°C. Those observations resulted into significant deviation from 1:1 relationship line (R^2 = 0.87 (intercept = -337.405, slope = 4.361) (Top – right, Figure 0.1).

When comparing the common treatments for the TE1 and TE2, for the statistic Mean time to germination there was an obvious inconsistency in the responses for isolate L5 between the two experiments, where the fitted regression line was significantly different from a 1:1 relationship in contrast to isolate L6 (R^2 = 0.87 (intercept = 9.8083, slope = 0.8675)), (Bottom - right, Figure 0.1). For the isolate L5 a few observations are pulling the line more horizontally, therefore the smaller R2 and different values for slope and intercept (R^2 = 0.58 (intercept = 43.0232, slope = 0.4397)) (Bottom - left, Figure 0.1). However, these observations were made for treatments with low germination levels and therefore this were more likely outlier than effect related to possible preconditioning (T1=14°C, 17°C, S1 duration = 14 days, T2=17°C, 20°C, max germination = 7%, 3.5%, respectively).

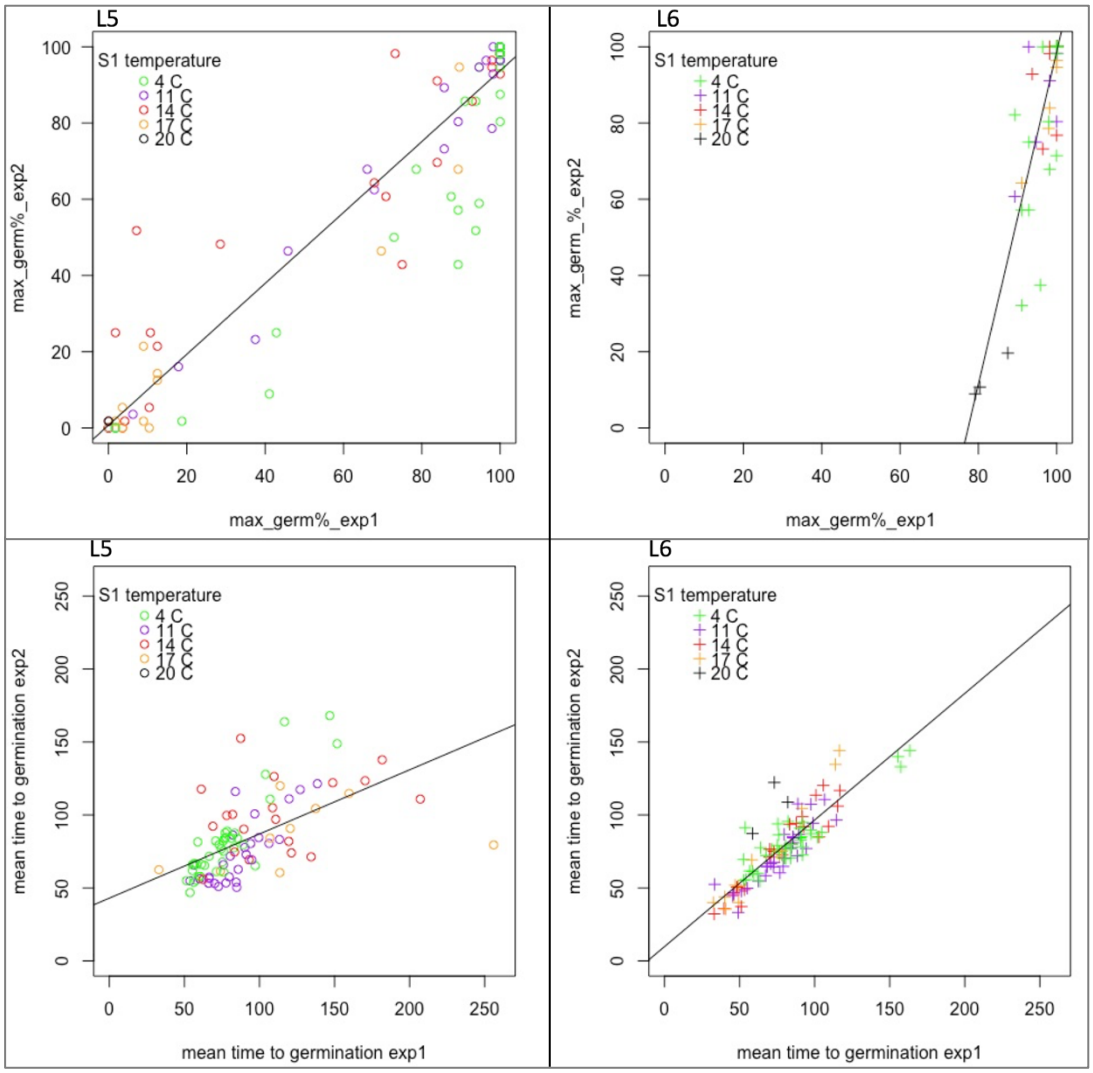


Figure 0.1 Scatterplot shoving Maximum germination (%) - top and Mean time to germination (days) - bottom for isolate L5 - left and isolate L6 – right, for the common treatments in TE1 & TE2 with a fitted linear regression line (maximum germination: L5 - R^2 = 0.95 (intercept = 0.7181, slope = 0.9302), L6- R^2 = 0.87 (intercept = -337.405, slope = 4.361; mean time: L5 - R^2 = 0.58 (intercept = 43.0232, slope = 0.4397), L6- R^2 = 0.87 (intercept = 9.8083, slope = 0.8675)). Different colours represent conditioning temperatures (S1 = T1).

Although there was some effect of preconditioning observed for isolate L6 in TE1, this is not a critical situation, because “preconditioning” mainly affected the level of germination rather than time to germination. This is important conclusion because the mean time to germination is the parameter we aim to model, while the maximum germination parameter is more informative.

Appendix 2 Sclerotia size effect.

Examination of the relationship between different size of sclerotia integrated into experimental design for different replicates shows a strong correlation between replicate and replicate mean values irrespective isolate and temperature experiment for both examined statistics. The fitted linear regression lines explain the data very well with R^2 (Table 0.1) greater than 0.97 for maximum germination % (Figure 0.2) and ranging from 0.77 to 0.95 for T_{50} (Figure 0.3). The relationship between the rep mean values compared to each of the replicate is very strong for Maximum germination % with the fitted lines marginally varying from 1:1 relationship r = 0.98 - 0.99 and with the slope values ranging from 0.9586 (TE1, L5, Rep 1) to 1.0350 (TE1, L5, Rep 3). T_{50} shows a deviation for both isolates in TE1, where longer germination observed for Rep 2 (L5), Rep 3 (L6) are pulling the fitted line up with the increasing time, with the slope values ranging from 0.8914 (TE1, L5, Rep 2) to 1.2653 (TE2, L6, Rep 2) (Table 0.1). The values of intercept vary from -6.2105 (TE1, L5, Rep 3 (Figure 0.2, A, yellow line)) to 6.5786 (TE1, L5, Rep 1 (Figure 0.2, A, green line)) for maximum germination % and from -14.2530 (TE1, L5, Rep 3 (Figure 0.3, A, yellow line)) and -12.9370 (TE1, L6, Rep 2 (Figure 0.3, B, blue line)) to 10.5430 (TE1, L5, Rep 2 (Figure 0.3, A, blue line)) for T_{50} (Table 0.1).

Exp	Isolate	Rep	Maximum germination %				T50			
			Slope	Intercept	R2	r	Slope	Intercept	R2	r
TE1	L5	1	0.9586	6.5786	0.97	0.99	0.9316	-1.5870	0.89	0.94
		2	1.0064	-0.3681	0.98	0.99	0.8914	10 .543	0.89	0.94
		3	1.0350	-6.2105	0.97	0.99	1.2461	-14.253	0.92	0.96
	L6	1	0.9956	0.7385	0.99	0.99	1.0058	-4.7445	0.90	0.95
		2	0.9828	1.8259	0.98	0.99	1.0314	1.2780	0.95	0.97
		3	1.0215	2.5644	0.98	0.99	0.9629	3.4665	0.95	0.97
TE2	L5	1	1.0242	0.3721	0.98	0.99	1.0030	-6.1866	0.80	0.89
		2	1.0117	0.7715	0.99	0.99	0.9171	5.7494	0.78	0.89
		3	0.9641	-1.1435	0.97	0.99	1.0880	0.1246	0.90	0.95
	L6	1	0.9836	3.2617	0.98	0.99	0.8956	1.5194	0.83	0.91
		2	1.0199	-3.4987	0.96	0.98	1.2653	-12.937	0.85	0.92
		3	0.9965	0.2370	0.98	0.99	0.9371	4.3897	0.77	0.88

Table 0.1 Summary of fitted linear regression for maximum germination percentage (Figure 0.1) and time to germination of 50% of sclerotia T_{50} (Figure 0.3) for both temperature experiments (TE1 & TE2) and two *S. sclerotiorum* isolates (L5 & L6).

Overall, there was only a small deviation (Rep 3 (L5), Rep 2 (L6), TE1) observed in the change of slope or intercept of fitted linear regression lines, and these do not

directly associate with the increase of the size of sclerotia (Rep1 = large sclerotia, R3 = small sclerotia), rather are seem to be caused by few late germinating outliers. However, the relationship between size and germination response could be more complex, including interaction with temperature, where in optimum conditions sclerotia potentially behave similarly, independently of the size, while in more challenging conditions size could proof as a positive (increasing) or negative (limiting) factor for some of the statistics. However, these assumptions would require a further examination across temperature treatments so a greater replication of size treatments is obtained. For the purpose of this work and use of the germination data in further analyses, it was concluded that for both temperature experiments there was sufficient evidence for the effect of different size of sclerotia to be included as a blocking term in ANOVA analyses, assuming no interaction between treatments and sclerotial size and providing a controlled representation of variation in size of sclerotia for both *S. sclerotiorum* isolates.

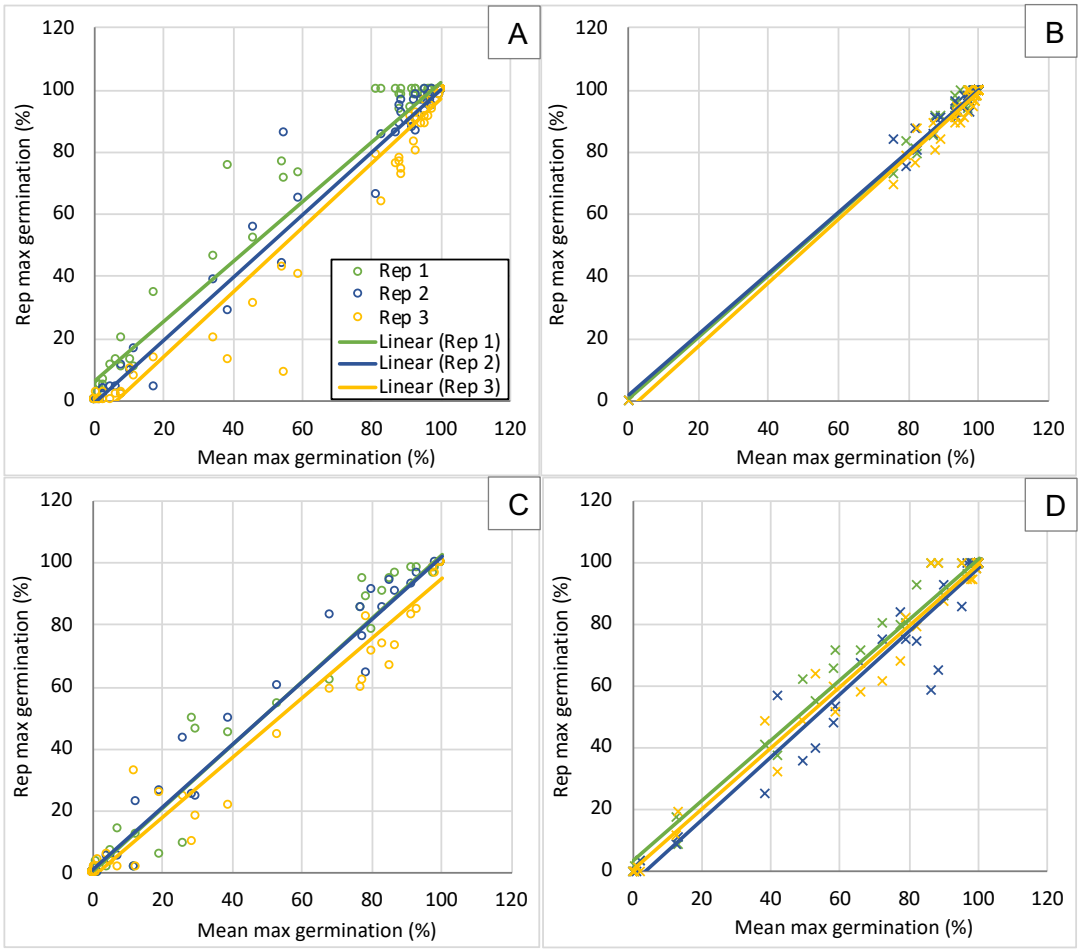


Figure 0.2 Scatterplot shoving maximum percentage germination for isolate L5 (circle symbol, A – TE1, C – TE2) and isolate L6 (cross symbol, B – TE1, D – TE2) with values for each replicate (Rep1 = Green, Rep 2 = Blue, Rep 3 = Yellow) plotted against the means across replicates. Linear regression lines fitted for each replicate with slope, intercept and R^2 provided in Table 0.1.

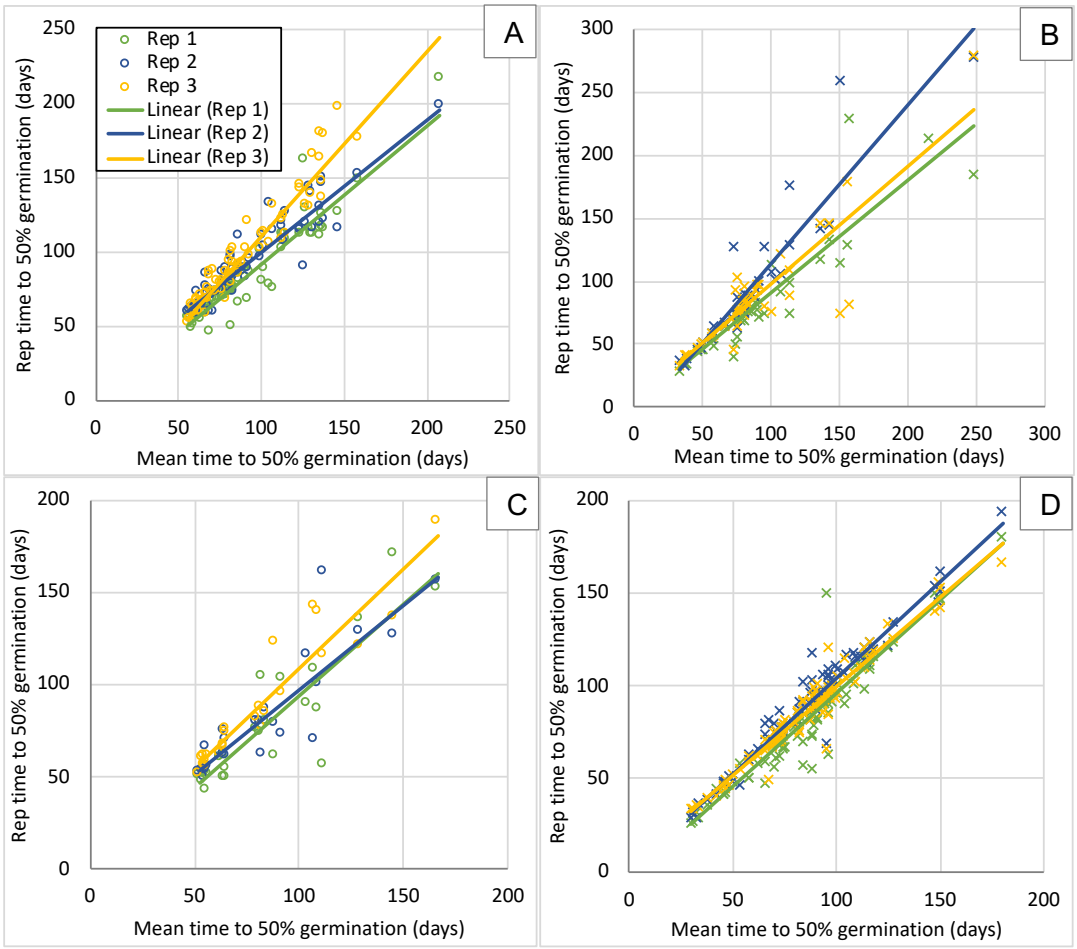


Figure 0.3 Scatterplot shoving T_{50} germination times for isolate L5 (circle symbol, A – TE1, C – TE2) and isolate L6 (cross symbol, B – TE1, D – TE2) with values for each replicate (Rep1 = Green, Rep 2 = Blue, Rep 3 = Yellow) plotted against the means across replicates. Number of observations decreased of TE2, because of low germination (<50%) for several treatments, especially for isolate L5. Linear regression lines fitted for each replicate with slope, intercept and R^2 provided in Table 0.1.

Appendix 3 T2 approach ANOVA table of means for time to germination of 25% of the population (T_{25}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 25%; Grey = no treatment combination tested; TE1: d.f. = 143, s.e.d. = 9.530, l.s.d. = 18.838; TE2: d.f. = 51, s.e.d. = 18.95, l.s.d. = 38.05.

T_{25}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃													
14℃												48	
11℃												47	
8℃													
4℃												85	
0℃													
25℃	20℃												
20℃													
17℃													
14℃						93	84				203	81	
11℃					94	58					55	49	
8℃					65	63							
4℃					66	71					89	75	
0℃					69	79							
25℃	17℃												
20℃													
17℃		111						81					
14℃				153	107	89	97			116	85	61	
11℃			117	106	68	62				105	67	47	
8℃			109	110	57	63							
4℃			90	61	52	68				88	57	75	
0℃			91	92	53	75							
25℃	14℃												
20℃													
17℃													
14℃		69						68					
11℃			79	61	60	51				50	50	48	
8℃			59	53	49	63							
4℃			48	47	49	69				46	55	74	
0℃			61	55	55	77							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		57						48					
8℃			52	51	55	67							
4℃			49	49	55	76				50	58	78	
0℃			48	55	62	84							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		69											
4℃			71	72	75	88							
0℃			68	75	83	104							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		110						136					
0℃			113	121	123	141							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 4 T2 approach ANOVA table of means for time to germination of 50% of the population (T_{50}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 50%; Grey = no treatment combination tested; TE1: d.f. = 137, s.e.d. = 10.806, l.s.d. = 21.369; TE2: d.f. = 47, s.e.d. = 14.44, l.s.d. = 29.04.

T_{50}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃											54	
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	20℃												
20℃													
17℃													
14℃						74	105					82	
11℃						72						55	
8℃					130	68							
4℃					67	76					88	108	
0℃						91							
25℃	17℃												
20℃													
17℃		136						109					
14℃				211	136	107	114			146	108	82	
11℃			146	127	116	84				129	88	54	
8℃			131	139	87	68							
4℃			124	101	62	73				112	65	81	
0℃			113	124	59	81							
25℃	14℃												
20℃													
17℃													
14℃		92						92					
11℃			100	82	91	63				65	65	52	
8℃			83	68	57	70							
4℃			69	58	56	75				55	63	80	
0℃			79	67	61	82							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		69						55					
8℃			61	62	64	74							
4℃			59	59	62	82				57	64	85	
0℃			59	62	68	89							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		82											
4℃			83	80	84	102							
0℃			77	87	93	114							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		130						166					
0℃			130	137	137	159							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 5 T2 approach ANOVA table of means for time to germination of 75% of the population (T_{75}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 75%; Grey = no treatment combination tested; TE1: d.f. = 130, s.e.d. = 13.75, l.s.d. = 27.21; TE2: d.f. = 37, s.e.d. = 15.23, l.s.d. = 30.86.

T_{75}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃											63	
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	20℃												
20℃													
17℃													
14℃						292	134						
11℃						90						79	
8℃						75							
4℃					121	89							
0℃													
25℃	17℃												
20℃													
17℃		170						150					
14℃				216	167	147	135			162	117	113	
11℃			155	164	164	100				156	130	67	
8℃			140	175	118	74							
4℃			151	140	83	83				141	84	89	
0℃			127	144	75	87							
25℃	14℃												
20℃													
17℃													
14℃		113						110					
11℃			119	98	108	78				90	91	60	
8℃			104	90	65	77							
4℃			90	91	65	85				66	72	89	
0℃			96	88	66	88							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		86						66					
8℃			76	75	78	83							
4℃			70	67	69	91				66	75	97	
0℃			68	72	74	96							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		96											
4℃			100	91	99	113							
0℃			86	101	112	126							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		162						204					
0℃			165	168	170	186							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 6 T2 approach ANOVA table of means for time to germination of 90% of the population (T_{90}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 90%; Grey = no treatment combination tested; TE1: d.f. = 115, s.e.d. = 15.96, l.s.d. = 31.61; TE2: d.f. = 30, s.e.d. = 12.83, l.s.d. = 26.21.

T_{90}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	20℃												
20℃													
17℃													
14℃							131						
11℃						112						86	
8℃						81							
4℃						107							
0℃													
25℃	17℃												
20℃													
17℃		173						126					
14℃					182	138	154				149	132	
11℃			221	208	147	115				164	169	81	
8℃			186	181	176	79							
4℃			165	165	114	91					84	95	
0℃			173	169	94	91							
25℃	14℃												
20℃													
17℃													
14℃		126						131					
11℃			132	115	119	92				130	105	93	
8℃			124	111	72	85							
4℃			109	109	71	92				77	84	99	
0℃			113	109	72	95							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		97						84					
8℃			95	93	91	93							
4℃			79	75	78	102				84	91	107	
0℃			77	81	82	106							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		112											
4℃			124	112	127	133							
0℃			112	122	129	147							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		227						293					
0℃			225	233	221	226							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 7 T2 approach ANOVA table of means for time to germination of 25% of the population (T_{25}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 25%; Grey = no treatment combination tested; TE1: d.f. = 178, s.e.d. = 7.230, l.s.d. = 14.268; TE2: d.f. = 88, s.e.d. = 13.84, l.s.d. = 27.51.

T_{25}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃										85	44	103	
14℃										35	51	88	
11℃										38	52	105	
8℃													
4℃											151	154	
0℃													
25℃	20℃												
20℃		50											
17℃				24	41	62	89			25	44	71	
14℃				27	45	66	89			26	45	70	
11℃			18	28	43	65				29	42	61	
8℃			26	30	41	64							
4℃			39	31	41	65				73	49	68	
0℃			70	55	61	83							
25℃	17℃												
20℃													
17℃		87						109					
14℃				84	73	75	94			82	66	76	
11℃			73	63	57	68				59	51	62	
8℃			65	54	51	68							
4℃			73	56	50	68				54	51	70	
0℃			72	63	54	75							
25℃	14℃												
20℃													
17℃													
14℃		98						93					
11℃			91	91	69	80				80	70	71	
8℃			83	75	62	75							
4℃			89	71	65	80				72	64	75	
0℃			86	80	68	83							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		83						79					
8℃			84	80	79	84							
4℃			84	82	82	88				69	70	80	
0℃			83	83	80	90							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		104											
4℃			100	99	104	115							
0℃			99	93	102	119							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		132						117					
0℃			136	132	130	148							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 8 T2 approach ANOVA table of means for time to germination of 50% of the population (T_{50}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 50%; Grey = no treatment combination tested; TE1: d.f. = 178, s.e.d. = 7.522, l.s.d. = 14.843; TE2: d.f. = 80, s.e.d. = 20.45, l.s.d. = 40.70.

T_{50}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃										222	72	150	
14℃										160	58	95	
11℃										161	99	114	
8℃													
4℃												247	
0℃													
25℃	20℃												
20℃		67											
17℃				30	46	74	95			36	50	75	
14℃				31	48	72	93			33	48	75	
11℃			33	33	47	69				39	46	65	
8℃			43	38	46	69							
4℃			53	47	50	72				93	74	77	
0℃			95	73	74	101							
25℃	17℃												
20℃													
17℃		114						136					
14℃				105	88	82	99			113	81	83	
11℃			104	82	66	74				75	57	66	
8℃			88	66	58	73							
4℃			96	70	58	74				73	57	77	
0℃			96	84	62	80							
25℃	14℃												
20℃													
17℃													
14℃		110						107					
11℃			100	100	82	88				90	81	80	
8℃			96	88	71	82							
4℃			99	84	74	88				85	71	82	
0℃			98	96	76	90							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		94						90					
8℃			98	93	89	91							
4℃			94	91	90	97				80	79	89	
0℃			92	90	87	97							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		115											
4℃			116	113	118	125							
0℃			112	109	116	128							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		149						142					
0℃			150	148	150	180							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 9 T2 approach ANOVA table of means for time to germination of 75% of the population (T_{75}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 75%; Grey = no treatment combination tested; TE1: d.f. = 176, s.e.d. = 12.756, l.s.d. = 25.175; TE2: d.f. = 67, s.e.d. = 18.65, l.s.d. = 37.23.

T_{75}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃											99	123	
14℃											87	111	
11℃											133	90	
8℃													
4℃													
0℃													
25℃	20℃												
20℃		158											
17℃				46	49	79	100			71	54	80	
14℃				49	53	76	97			67	52	79	
11℃			54	53	53	73				122	51	70	
8℃			72	60	59	74							
4℃			86	70	76	102						154	
0℃			150	167	104	172							
25℃	17℃												
20℃													
17℃		129						156					
14℃				120	107	94	105			134	102	93	
11℃			123	101	75	79				94	62	70	
8℃			110	79	65	79							
4℃			107	85	66	83				96	62	82	
0℃			118	107	70	86							
25℃	14℃												
20℃													
17℃													
14℃		123						117					
11℃			118	112	97	97				107	96	97	
8℃			109	103	83	89							
4℃			113	98	87	95				99	77	89	
0℃			112	106	84	96							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		105						115					
8℃			112	104	101	100							
4℃			106	103	100	104				91	87	94	
0℃			104	99	95	106							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		126											
4℃			135	125	131	136							
0℃			124	118	129	138							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		181						160					
0℃			183	181	197	222							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 10 T2 approach ANOVA table of means for time to germination of 90% of the population (T_{90}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 90%; Grey = no treatment combination tested; TE1: d.f. = 164, s.e.d. = 15.14, l.s.d. = 29.89; TE2: d.f. = 61, s.e.d. = 16.25, l.s.d. = 32.49.

T_{90}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃											54	126	
14℃											153	130	
11℃											148	96	
8℃													
4℃													
0℃													
25℃	20℃												
20℃													
17℃				135	56	84	105				59	84	
14℃				77	58	79	103			69	55	82	
11℃			126	77	72	76					62	74	
8℃				129	84	87							
4℃			213	169	111	128							
0℃					198								
25℃	17℃												
20℃													
17℃		147						186					
14℃				132	126	111	111			146	115	107	
11℃			138	115	89	88				109	69	74	
8℃			126	99	71	85							
4℃			121	102	74	91				115	67	85	
0℃			131	128	76	93							
25℃	14℃												
20℃													
17℃													
14℃		140						134					
11℃			130	130	109	112				124	112	119	
8℃			124	117	93	99							
4℃			132	111	94	100				123	83	94	
0℃			125	119	93	103							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		116						142					
8℃			124	121	111	113							
4℃			119	119	108	110				116	104	100	
0℃			117	106	103	114							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		134											
4℃			149	138	138	146							
0℃			135	130	145	154							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		226						171					
0℃			221	207	252	270							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 11 T2 approach ANOVA table of means for difference in times to germination for EPR - T_{25}-T_{10} in TE1 and TE2 for isolate L5 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{25}-T_{10} – d.f. = 143, s.e.d. = 6.343, l.s.d. = 12.539; TE2: T_{25}-T_{10} – d.f. = 51, s.e.d. = 16.24, l.s.d. = 32.60.

T_{25}-T_{10}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃													
14℃												5	
11℃												5	
8℃													
4℃												10	
0℃													
25℃	20℃												
20℃													
17℃						21	21						
14℃						40	18				152	16	
11℃					40	13					19	5	
8℃					15	4							
4℃					12	4					40	6	
0℃					13	8							
25℃	17℃												
20℃													
17℃		22						18					
14℃				36	26	19	20			20	38	19	
11℃			15	19	22	10				43	20	4	
8℃			16	22	10	4							
4℃			15	16	5	3				38	7	7	
0℃			23	17	4	4							
25℃	14℃												
20℃													
17℃													
14℃		17						23					
11℃			25	20	11	12				9	9	3	
8℃			15	8	4	4							
4℃			10	5	4	4				5	5	4	
0℃			14	8	4	3							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		12						4					
8℃			11	8	10	5							
4℃			10	10	9	6					4	3	4
0℃			10	10	5	5							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		8											
4℃			7	7	7	6							
0℃			7	7	6	8							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		12						19					
0℃			10	10	9	5							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 12 T2 approach ANOVA table of means for difference in times to germination for LPR – T_{90}-T_{75} in TE1 and TE2 for isolate L5 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{90}-T_{75} – d.f. = 115, s.e.d. = 10.656, l.s.d. = 21.108; TE2: T_{90}-T_{75} – d.f. = 30, s.e.d. = 10.93, l.s.d. = 22.33.

T_{90}-T_{75}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	20℃												
20℃													
17℃													
14℃							20						
11℃						22						23	
8℃						6							
4℃						16							
0℃													
25℃	17℃												
20℃													
17℃		39						23					
14℃					24	19	19				34	19	
11℃			68	27	20	16				22	42	14	
8℃			48	39	58	5							
4℃			31	42	31	8					15	6	
0℃			49	39	19	5							
25℃	14℃												
20℃													
17℃													
14℃		13						20					
11℃			13	17	12	14				40	14	33	
8℃			20	21	7	8							
4℃			19	17	6	8				12	12	9	
0℃			17	20	7	7							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		11						18					
8℃			19	18	13	10							
4℃			10	8	9	11				18	16	10	
0℃			9	10	8	10							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		16											
4℃			24	21	28	20							
0℃			26	21	17	21							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		65						81					
0℃			60	65	50	40							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													

Appendix 13 T2 approach ANOVA table of means for difference in times to germination for EPR - T_{25}-T_{10} in TE1 and TE2 for isolate L6 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{25}-T_{10} – d.f. = 178, s.e.d. = 4.567, l.s.d. = 9.013; TE2: T_{25}-T_{10} – d.f. = 88, s.e.d. = 8.391, l.s.d. = 16.675.

T_{25}-T_{10}_L6		T1 duration (days)											
diff	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T_{25}-T_{10}	25℃												
	20℃												
	17℃									54	7	18	
	14℃									7	4	4	
	11℃									8	6	20	
	8℃												
	4℃										43	31	
	0℃												
T_{25}-T_{10}	25℃												
	20℃	10											
	17℃			4	6	8	42			4	5	19	
	14℃			2	3	4	9			3	3	4	
	11℃		5	3	3	3				4	3	3	
	8℃		9	4	3	4							
	4℃		14	4	3	4				25	7	5	
	0℃		25	13	7	12							
T_{25}-T_{10}	25℃												
	20℃												
	17℃	30						35					
	14℃			20	13	6	5			21	7	4	
	11℃		23	12	7	4				11	4	3	
	8℃		14	11	5	4							
	4℃		24	12	5	4				13	6	4	
	0℃		14	12	6	6							
T_{25}-T_{10}	25℃												
	20℃												
	17℃												
	14℃	16						12					
	11℃		21	12	9	7				14	7	9	
	8℃		14	10	6	4							
	4℃		17	11	7	7				12	8	4	
	0℃		16	14	8	6							
T_{25}-T_{10}	25℃												
	20℃												
	17℃												
	14℃												
	11℃	9						8					
	8℃		10	9	8	6							
	4℃		12	7	7	7				10	12	7	
	0℃		6	6	5	5							
T_{25}-T_{10}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃	16											
	4℃		9	17	18	14							
	0℃		11	10	7	7							
T_{25}-T_{10}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃	17						9					
	0℃		12	13	13	11							
T_{25}-T_{10}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												

Appendix 14 T2 approach ANOVA table of means for difference in times to germination for LPR – T_{90}-T_{75} in TE1 and TE2 for isolate L6 for combination of treatments with different T1 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In In TE1: T_{90}-T_{75} – d.f. = 164, s.e.d. = 14.34, l.s.d. = 28.32; TE2: T_{90}-T_{75} – d.f. = 61, s.e.d. = 10.62, l.s.d. = 20.120.

T_{90}-T_{75}_L6		T1 duration (days)											
diff	T1	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T_{90}-T_{75}	25℃							14					
	20℃												
	17℃										5	4	
	14℃										67	19	
	11℃										31	7	
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃												
	17℃			89	7	5	6				6	4	
	14℃			29	5	4	6			19	3	3	
	11℃		72	25	19	3					11	4	
	8℃			69	25	12							
	4℃		129	99	35	32							
	0℃				103								
T_{90}-T_{75}	25℃												
	20℃												
	17℃	19						30					
	14℃			13	18	17	6			12	14	14	
	11℃		15	13	14	9				14	7	4	
	8℃		16	20	6	6							
	4℃		15	16	7	8				19	4	3	
	0℃		13	21	6	7							
T_{90}-T_{75}	25℃												
	20℃												
	17℃												
	14℃	16						17					
	11℃		12	18	12	15				17	16	22	
	8℃		15	14	10	10							
	4℃		19	13	8	6				23	5	6	
	0℃		13	13	9	7							
T_{90}-T_{75}	25℃												
	20℃												
	17℃												
	14℃												
	11℃	12						27					
	8℃		13	16	10	13							
	4℃		13	16	8	6				25	17	6	
	0℃		14	7	8	9							
T_{90}-T_{75}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃	9											
	4℃		14	13	7	10							
	0℃		11	12	16	16							
T_{90}-T_{75}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃	45						12					
	0℃		38	26	55	49							
T_{90}-T_{75}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												

Appendix 15 T1 approach ANOVA table of means for time to germination of 25% of the population (T_{25}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 25%; Grey = no treatment combination tested; TE1: d.f. = 143, s.e.d. = 9.530, l.s.d. = 18.838; TE2: d.f. = 51, s.e.d. = 18.95, l.s.d. = 38.05.

T_{25}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T25	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T25	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T25	25℃												
	20℃												
	17℃	111						81					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T25	25℃											48	
	20℃					93	84				203	81	
	17℃			153	107	89	97			116	85	61	
	14℃	69						68					
	11℃												
	8℃												
	4℃												
	0℃												
T25	25℃											47	
	20℃				94	58					55	49	
	17℃		117	106	68	62				105	67	47	
	14℃		79	61	60	51				50	50	48	
	11℃	57						48					
	8℃												
	4℃												
	0℃												
T25	25℃												
	20℃				65	63							
	17℃		109	110	57	63							
	14℃		59	53	49	63							
	11℃		52	51	55	67							
	8℃	69											
	4℃												
	0℃												
T25	25℃											85	
	20℃				66	71					89	75	
	17℃		90	61	52	68				88	57	75	
	14℃		48	47	49	69				46	55	74	
	11℃		49	49	55	76				50	58	78	
	8℃		71	72	75	88							
	4℃	110						136					
	0℃												
T25	25℃												
	20℃				69	79							
	17℃		91	92	53	75							
	14℃		61	55	55	77							
	11℃		48	55	62	84							
	8℃		68	75	83	104							
	4℃		113	121	123	141							
	0℃												

Appendix 16 T1 approach ANOVA table of means for time to germination of 50% of the population (T_{50}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 50%; Grey = no treatment combination tested; TE1: d.f. = 137, s.e.d. = 10.806, l.s.d. = 21.369; TE2: d.f. = 47, s.e.d. = 14.44, l.s.d. = 29.04.

T_{50}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃												
	20℃												
	17℃	136						109					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃												
	20℃					74	105					82	
	17℃			211	136	107	114			146	108	82	
	14℃	92						92					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃											54	
	20℃					72						55	
	17℃		146	127	116	84				129	88	54	
	14℃		100	82	91	63				65	65	52	
	11℃	69						55					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃				130	68							
	17℃		131	139	87	68							
	14℃		83	68	57	70							
	11℃		61	62	64	74							
	8℃	82											
	4℃												
	0℃												
4℃	25℃												
	20℃				67	76					88	108	
	17℃		124	101	62	73				112	65	81	
	14℃		69	58	56	75				55	63	80	
	11℃		59	59	62	82				57	64	85	
	8℃		83	80	84	102							
	4℃	130						166					
	0℃												
0℃	25℃												
	20℃					91							
	17℃		113	124	59	81							
	14℃		79	67	61	82							
	11℃		59	62	68	89							
	8℃		77	87	93	114							
	4℃		130	137	137	159							
	0℃												

Appendix 17 T1 approach ANOVA table of means for time to germination of 75% of the population (T_{75}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 75%; Grey = no treatment combination tested; TE1: d.f. = 130, s.e.d. = 13.75, l.s.d. = 27.21; TE2: d.f. = 37, s.e.d. = 15.23, l.s.d. = 30.86.

T_{75}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃												
	20℃												
	17℃	170						150					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃												
	20℃					292	134						
	17℃			216	167	147	135			162	117	113	
	14℃	113						110					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃											63	
	20℃					90						79	
	17℃		155	164	164	100				156	130	67	
	14℃		119	98	108	78				90	91	60	
	11℃	86						66					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃					75							
	17℃		140	175	118	74							
	14℃		104	90	65	77							
	11℃		76	75	78	83							
	8℃	96											
	4℃												
	0℃												
4℃	25℃												
	20℃				121	89							
	17℃		151	140	83	83				141	84	89	
	14℃		90	91	65	85				66	72	89	
	11℃		70	67	69	91				66	75	97	
	8℃		100	91	99	113							
	4℃	162						204					
	0℃												
0℃	25℃												
	20℃												
	17℃		127	144	75	87							
	14℃		96	88	66	88							
	11℃		68	72	74	96							
	8℃		86	101	112	126							
	4℃		165	168	170	186							
	0℃												

Appendix 18 T1 approach ANOVA table of means for time to germination of 90% of the population (T_{90}) in TE1 and TE2 for isolate L5 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 90%; Grey = no treatment combination tested; TE1: d.f. = 115, s.e.d. = 15.96, l.s.d. = 31.61; TE2: d.f. = 30, s.e.d. = 12.83, l.s.d. = 26.21.

T_{90}_L5		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃												
	20℃												
	17℃	173						126					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃												
	20℃						131						
	17℃				182	138	154				149	132	
	14℃	126						131					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃												
	20℃					112						86	
	17℃		221	208	147	115				164	169	81	
	14℃		132	115	119	92				130	105	93	
	11℃	97						84					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃					81							
	17℃		186	181	176	79							
	14℃		124	111	72	85							
	11℃		95	93	91	93							
	8℃	112											
	4℃												
	0℃												
4℃	25℃												
	20℃					107							
	17℃		165	165	114	91					84	95	
	14℃		109	109	71	92				77	84	99	
	11℃		79	75	78	102				84	91	107	
	8℃		124	112	127	133							
	4℃	227						293					
	0℃												
0℃	25℃												
	20℃												
	17℃		173	169	94	91							
	14℃		113	109	72	95							
	11℃		77	81	82	106							
	8℃		112	122	129	147							
	4℃		225	233	221	226							
	0℃												

Appendix 19 T1 approach ANOVA table of means for time to germination of 25% of the population (T_{25}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 25%; Grey = no treatment combination tested; TE1: d.f. = 178, s.e.d. = 7.230, l.s.d. = 14.268; TE2: d.f. = 88, s.e.d. = 13.84, l.s.d. = 27.51.

T_{25}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T25	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T25	25℃												
	20℃	50											
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T25	25℃									85	44	103	
	20℃			24	41	62	89			25	44	71	
	17℃	87						109					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T25	25℃									35	51	88	
	20℃			27	45	66	89			26	45	70	
	17℃			84	73	75	94			82	66	76	
	14℃	98						93					
	11℃												
	8℃												
	4℃												
	0℃												
T25	25℃									38	52	105	
	20℃		18	28	43	65				29	42	61	
	17℃		73	63	57	68				59	51	62	
	14℃		91	91	69	80				80	70	71	
	11℃	83						79					
	8℃												
	4℃												
	0℃												
T25	25℃												
	20℃		26	30	41	64							
	17℃		65	54	51	68							
	14℃		83	75	62	75							
	11℃		84	80	79	84							
	8℃	104											
	4℃												
	0℃												
T25	25℃										151	154	
	20℃		39	31	41	65				73	49	68	
	17℃		73	56	50	68				54	51	70	
	14℃		89	71	65	80				72	64	75	
	11℃		84	82	82	88				69	70	80	
	8℃		100	99	104	115							
	4℃	132						117					
	0℃												
T25	25℃												
	20℃		70	55	61	83							
	17℃		72	63	54	75							
	14℃		86	80	68	83							
	11℃		83	83	80	90							
	8℃		99	93	102	119							
	4℃		136	132	130	148							
	0℃												

Appendix 20 T1 approach ANOVA table of means for time to germination of 50% of the population (T_{50}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 50%; Grey = no treatment combination tested; TE1: d.f. = 178, s.e.d. = 7.522, l.s.d. = 14.843; TE2: d.f. = 80, s.e.d. = 20.45, l.s.d. = 40.70.

T_{50}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃	67											
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃									222	72	150	
	20℃			30	46	74	95			36	50	75	
	17℃	114						136					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃									160	58	95	
	20℃			31	48	72	93			33	48	75	
	17℃			105	88	82	99			113	81	83	
	14℃	110						107					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃									161	99	114	
	20℃		33	33	47	69				39	46	65	
	17℃		104	82	66	74				75	57	66	
	14℃		100	100	82	88				90	81	80	
	11℃	94						90					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃		43	38	46	69							
	17℃		88	66	58	73							
	14℃		96	88	71	82							
	11℃		98	93	89	91							
	8℃	115											
	4℃												
	0℃												
4℃	25℃											247	
	20℃		53	47	50	72				93	74	77	
	17℃		96	70	58	74				73	57	77	
	14℃		99	84	74	88				85	71	82	
	11℃		94	91	90	97				80	79	89	
	8℃		116	113	118	125							
	4℃	149						142					
	0℃												
0℃	25℃												
	20℃		95	73	74	101							
	17℃		96	84	62	80							
	14℃		98	96	76	90							
	11℃		92	90	87	97							
	8℃		112	109	116	128							
	4℃		150	148	150	180							
	0℃												

Appendix 21 T1 approach ANOVA table of means for time to germination of 75% of the population (T_{75}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 75%; Grey = no treatment combination tested; TE1: d.f. = 176, s.e.d. = 12.756, l.s.d. = 25.175; TE2: d.f. = 67, s.e.d. = 18.65, l.s.d. = 37.23.

T_{75}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃	158											
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃										99	123	
	20℃			46	49	79	100			71	54	80	
	17℃	129						156					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃										87	111	
	20℃			49	53	76	97			67	52	79	
	17℃			120	107	94	105			134	102	93	
	14℃	123						117					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃										133	90	
	20℃		54	53	53	73				122	51	70	
	17℃		123	101	75	79				94	62	70	
	14℃		118	112	97	97				107	96	97	
	11℃	105						115					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃		72	60	59	74							
	17℃		110	79	65	79							
	14℃		109	103	83	89							
	11℃		112	104	101	100							
	8℃	126											
	4℃												
	0℃												
4℃	25℃												
	20℃		86	70	76	102						154	
	17℃		107	85	66	83				96	62	82	
	14℃		113	98	87	95				99	77	89	
	11℃		106	103	100	104				91	87	94	
	8℃		135	125	131	136							
	4℃	181						160					
	0℃												
0℃	25℃												
	20℃		150	167	104	172							
	17℃		118	107	70	86							
	14℃		112	106	84	96							
	11℃		104	99	95	106							
	8℃		124	118	129	138							
	4℃		183	181	197	222							
	0℃												

Appendix 22 T1 approach ANOVA table of means for time to germination of 90% of the population (T_{90}) in TE1 and TE2 for isolate L6 for combination of treatments with different T1, T2 temperature and T1 duration; Colour gradient from longest (green) to shortest germination (red) time; Empty cell = germination less then 90%; Grey = no treatment combination tested; TE1: d.f. = 164, s.e.d. = 15.14, l.s.d. = 29.89; TE2: d.f. = 61, s.e.d. = 16.25, l.s.d. = 32.49.

T_{90}_L6		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
20℃	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
17℃	25℃										54	126	
	20℃			135	56	84	105				59	84	
	17℃	147						186					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
14℃	25℃										153	130	
	20℃			77	58	79	103			69	55	82	
	17℃			132	126	111	111			146	115	107	
	14℃	140						134					
	11℃												
	8℃												
	4℃												
	0℃												
11℃	25℃										148	96	
	20℃		126	77	72	76					62	74	
	17℃		138	115	89	88				109	69	74	
	14℃		130	130	109	112				124	112	119	
	11℃	116						142					
	8℃												
	4℃												
	0℃												
8℃	25℃												
	20℃			129	84	87							
	17℃		126	99	71	85							
	14℃		124	117	93	99							
	11℃		124	121	111	113							
	8℃	134											
	4℃												
	0℃												
4℃	25℃												
	20℃		213	169	111	128							
	17℃		121	102	74	91				115	67	85	
	14℃		132	111	94	100				123	83	94	
	11℃		119	119	108	110				116	104	100	
	8℃		149	138	138	146							
	4℃	226						171					
	0℃												
0℃	25℃												
	20℃				198								
	17℃		131	128	76	93							
	14℃		125	119	93	103							
	11℃		117	106	103	114							
	8℃		135	130	145	154							
	4℃		221	207	252	270							
	0℃												

Appendix 23 T1 approach ANOVA table of means for difference in times to germination for EPR - T_{25}-T_{10} in TE1 and TE2 for isolate L5 for combination of treatments with different T2 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{25}-T_{10} – d.f. = 143, s.e.d. = 6.343, l.s.d. = 12.539; TE2: T_{25}-T_{10} – d.f. = 51, s.e.d. = 16.24, l.s.d. = 32.60.

T_{25}-T_{10}_L5		T1 duration (days)											
diff	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T_{25}-T_{10}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃												
	20℃					21	21						
	17℃	22						18					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃											5	
	20℃					40	18				152	16	
	17℃			36	26	19	20			20	38	19	
	14℃	17						23					
	11℃												
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃											5	
	20℃				40	13					19	5	
	17℃		15	19	22	10				43	20	4	
	14℃		25	20	11	12				9	9	3	
	11℃	12						4					
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃												
	20℃				15	4							
	17℃		16	22	10	4							
	14℃		15	8	4	4							
	11℃		11	8	10	5							
	8℃	8											
	4℃												
	0℃												
T_{25}-T_{10}	25℃											10	
	20℃				12	4					40	6	
	17℃		15	16	5	3				38	7	7	
	14℃		10	5	4	4				5	5	4	
	11℃		10	10	9	6				4	3	4	
	8℃		7	7	7	6							
	4℃	12						19					
	0℃												
T_{25}-T_{10}	25℃												
	20℃				13	8							
	17℃		23	17	4	4							
	14℃		14	8	4	3							
	11℃		10	10	5	5							
	8℃		7	7	6	8							
	4℃		10	10	9	5							
	0℃												

Appendix 24 T1 approach ANOVA table of means for difference in times to germination for LPR – T_{90}-T_{75} in TE1 and TE2 for isolate L5 for combination of treatments with different T2 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{90}-T_{75} – d.f. = 115, s.e.d. = 10.656, l.s.d. = 21.108; TE2: T_{90}-T_{75} – d.f. = 30, s.e.d. = 10.93, l.s.d. = 22.33.

T_{90}-T_{75}_L5		T1 duration (days)											
diff	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T_{90}-T_{75}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃												
	17℃	39						23					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃						20						
	17℃				24	19	19				34	19	
	14℃	13						20					
	11℃												
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃					22						23	
	17℃		68	27	20	16				22	42	14	
	14℃		13	17	12	14				40	14	33	
	11℃	11						18					
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃					6							
	17℃		48	39	58	5							
	14℃		20	21	7	8							
	11℃		19	18	13	10							
	8℃	16											
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃					16							
	17℃		31	42	31	8					15	6	
	14℃		19	17	6	8				12	12	9	
	11℃		10	8	9	11				18	16	10	
	8℃		24	21	28	20							
	4℃	65						81					
	0℃												
T_{90}-T_{75}	25℃												
	20℃												
	17℃		49	39	19	5							
	14℃		17	20	7	7							
	11℃		9	10	8	10							
	8℃		26	21	17	21							
	4℃		60	65	50	40							
	0℃												

Appendix 25 T1 approach ANOVA table of means for difference in times to germination for EPR - T_{25}-T_{10} in TE1 and TE2 for isolate L6 for combination of treatments with different T2 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{25}-T_{10} – d.f. = 178, s.e.d. = 4.567, l.s.d. = 9.013; TE2: T_{25}-T_{10} – d.f. = 88, s.e.d. = 8.391, l.s.d. = 16.675.

T_{25}-T_{10}_L6		T1 duration (days)											
diff	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T_{25}-T_{10}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃												
	20℃	10											
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃									54	7	18	
	20℃			4	6	8	42			4	5	19	
	17℃	30						35					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃									7	4	4	
	20℃			2	3	4	9			3	3	4	
	17℃			20	13	6	5			21	7	4	
	14℃	16						12					
	11℃												
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃									8	6	20	
	20℃		5	3	3	3				4	3	3	
	17℃		23	12	7	4				11	4	3	
	14℃		21	12	9	7				14	7	9	
	11℃	9						8					
	8℃												
	4℃												
	0℃												
T_{25}-T_{10}	25℃												
	20℃		9	4	3	4							
	17℃		14	11	5	4							
	14℃		14	10	6	4							
	11℃		10	9	8	6							
	8℃	16											
	4℃												
	0℃												
T_{25}-T_{10}	25℃										43	31	
	20℃		14	4	3	4				25	7	5	
	17℃		24	12	5	4				13	6	4	
	14℃		17	11	7	7				12	8	4	
	11℃		12	7	7	7				10	12	7	
	8℃		9	17	18	14							
	4℃	17						9					
	0℃												
T_{25}-T_{10}	25℃												
	20℃		25	13	7	12							
	17℃		14	12	6	6							
	14℃		16	14	8	6							
	11℃		6	6	5	5							
	8℃		11	10	7	7							
	4℃		12	13	13	11							
	0℃												

Appendix 26 T1 approach ANOVA table of means for difference in times to germination for LPR – T_{90}-T_{75} in TE1 and TE2 for isolate L6 for combination of treatments with different T2 and S1 duration; Colour gradient from large (blue) to small (red); Empty cell = germination less than required percentile; Grey = no treatment combination tested; In TE1: T_{90}-T_{75} – d.f. = 164, s.e.d. = 14.34, l.s.d. = 28.32; TE2: T_{90}-T_{75} – d.f. = 61, s.e.d. = 10.62, l.s.d. = 20.120.

T_{90}-T_{75}_L6		T1 duration (days)											
diff	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
T_{90}-T_{75}	25℃							14					
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃												
	17℃												
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃										5	4	
	20℃				89	7	5	6			6	4	
	17℃	19						30					
	14℃												
	11℃												
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃										67	19	
	20℃				29	5	4	6		19	3	3	
	17℃				13	18	17	6		12	14	14	
	14℃	16						17					
	11℃												
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃										31	7	
	20℃		72	25	19	3					11	4	
	17℃		15	13	14	9				14	7	4	
	14℃		12	18	12	15				17	16	22	
	11℃	12						27					
	8℃												
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃			69	25	12							
	17℃		16	20	6	6							
	14℃		15	14	10	10							
	11℃		13	16	10	13							
	8℃	9											
	4℃												
	0℃												
T_{90}-T_{75}	25℃												
	20℃		129	99	35	32							
	17℃		15	16	7	8				19	4	3	
	14℃		19	13	8	6				23	5	6	
	11℃		13	16	8	6				25	17	6	
	8℃		14	13	7	10							
	4℃	45						12					
	0℃												
T_{90}-T_{75}	25℃												
	20℃				103								
	17℃		13	21	6	7							
	14℃		13	13	9	7							
	11℃		14	7	8	9							
	8℃		11	12	16	16							
	4℃		38	26	55	49							
	0℃												

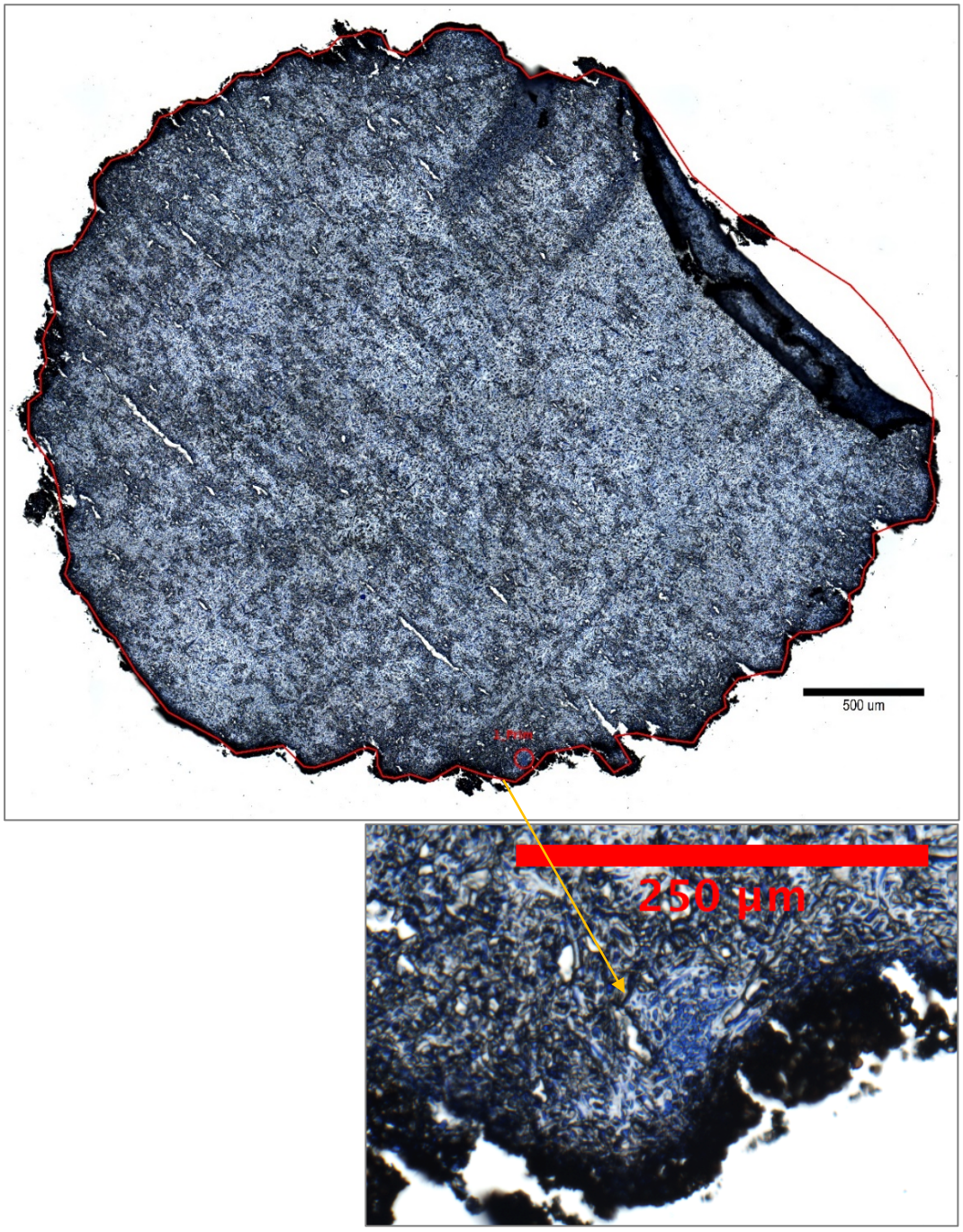
Appendix 27 Standard deviation of the Mean time to germination observed for *S. sclerotiorum* isolates L5 in TE1 and TE2 for treatments which did not achieve 100% germination at the end of S2 (280 days) and were transferred to 11°C for viability test (S3) for additional 150 days; Mean time was calculated from midpoints of observation intervals and where replicates were available, mean across replicates was calculated and SD obtained.

L5 m.t.g_S3_s.d.		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							17.7					
20℃										19.8	19.0	10.0	
17℃										13.5	11.4	3.1	
14℃										9.6	5.5	3.3	
11℃										9.8	5.0	5.0	
8℃													
4℃										12.9	8.7	7.1	
0℃													
25℃	20℃												
20℃		10.5						6.4					
17℃				4.8	8.6	2.4	3.4			4.2	2.2	3.7	
14℃				9.2	9.4	1.4	1.3			3.0	1.7	2.9	
11℃			10.9	7.8	7.6	7.9				2.7	1.0	11.4	
8℃			4.6	5.3	4.5	2.5							
4℃			3.7	7.1	5.6	10.2				4.5	5.1	3.4	
0℃			8.3	9.7	4.1	3.2							
25℃	17℃												
20℃													
17℃		6.3						16.6					
14℃				8.4	56.1	16.4	0.0			2.9	5.8	34.1	
11℃			13.1	21.2	10.8	0.0				1.2	5.4	47.4	
8℃			5.4	15.3	27.6								
4℃			19.6	15.0	0.0					9.4	14.7		
0℃			8.4	11.1									
25℃	14℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	11℃												
20℃													
17℃													
14℃													
11℃								0.0					
8℃													
4℃													
0℃													
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃						0.0							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		33.3						20.0					
0℃			2.5	12.9	2.5	0.0							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		6.5											

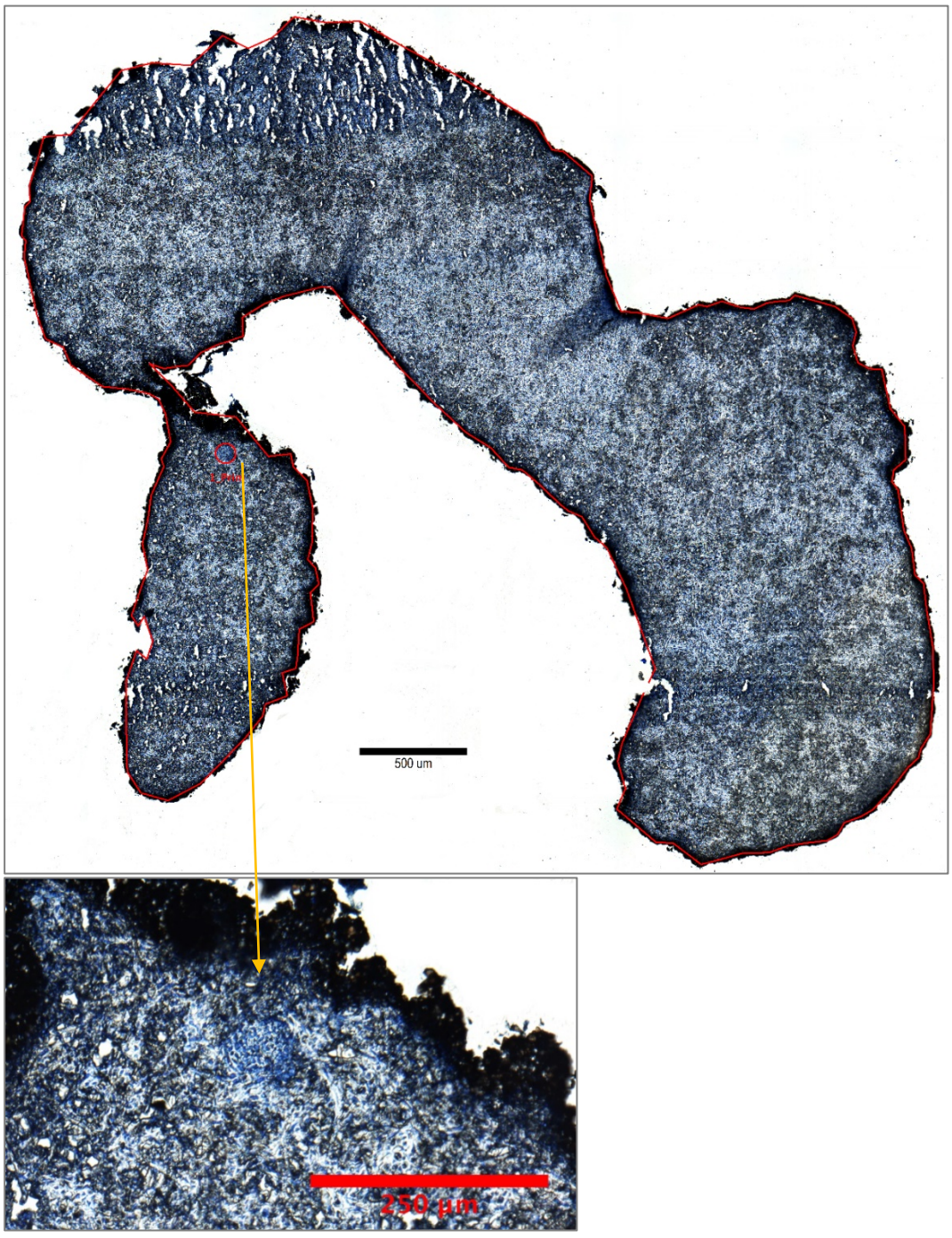
Appendix 28 Standard deviation of the Mean time to germination observed for *S. sclerotiorum* isolates L6 in TE1 and TE2 for treatments which did not achieve 100% germination at the end of S2 (280 days) and were transferred to 11°C for viability test (S3) for additional 150 days; Mean time was calculated from midpoints of observation intervals and where replicates were available, mean across replicates was calculated and SD obtained.

L6 m.t.g_S3_s.d.		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							2.7					
20℃										4.9	5.0	6.1	
17℃										4.3	0.0	0.0	
14℃										2.5	14.3	0.0	
11℃										7.5	5.8	0.0	
8℃													
4℃										8.9	10.3	11.1	
0℃													
25℃	20℃												
20℃		3.9						6.9					
17℃				2.2						7.8	0.0		
14℃				0.4	2.5					7.8			
11℃			2.2	2.5	26.5					2.1	0.0		
8℃			2.2	2.2	2.5								
4℃			0.5	2.2	4.8	2.2				5.0	7.0	6.3	
0℃			0.7	0.3	2.2	4.7							
25℃	17℃												
20℃													
17℃								0.0					
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	14℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃													
25℃	11℃												
20℃													
17℃													
14℃													
11℃								0.0					
8℃													
4℃													
0℃													
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃			0.0	0.0	0.0								
0℃													
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		0.0											
0℃			0.0		0.0	2.2							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		3.7											

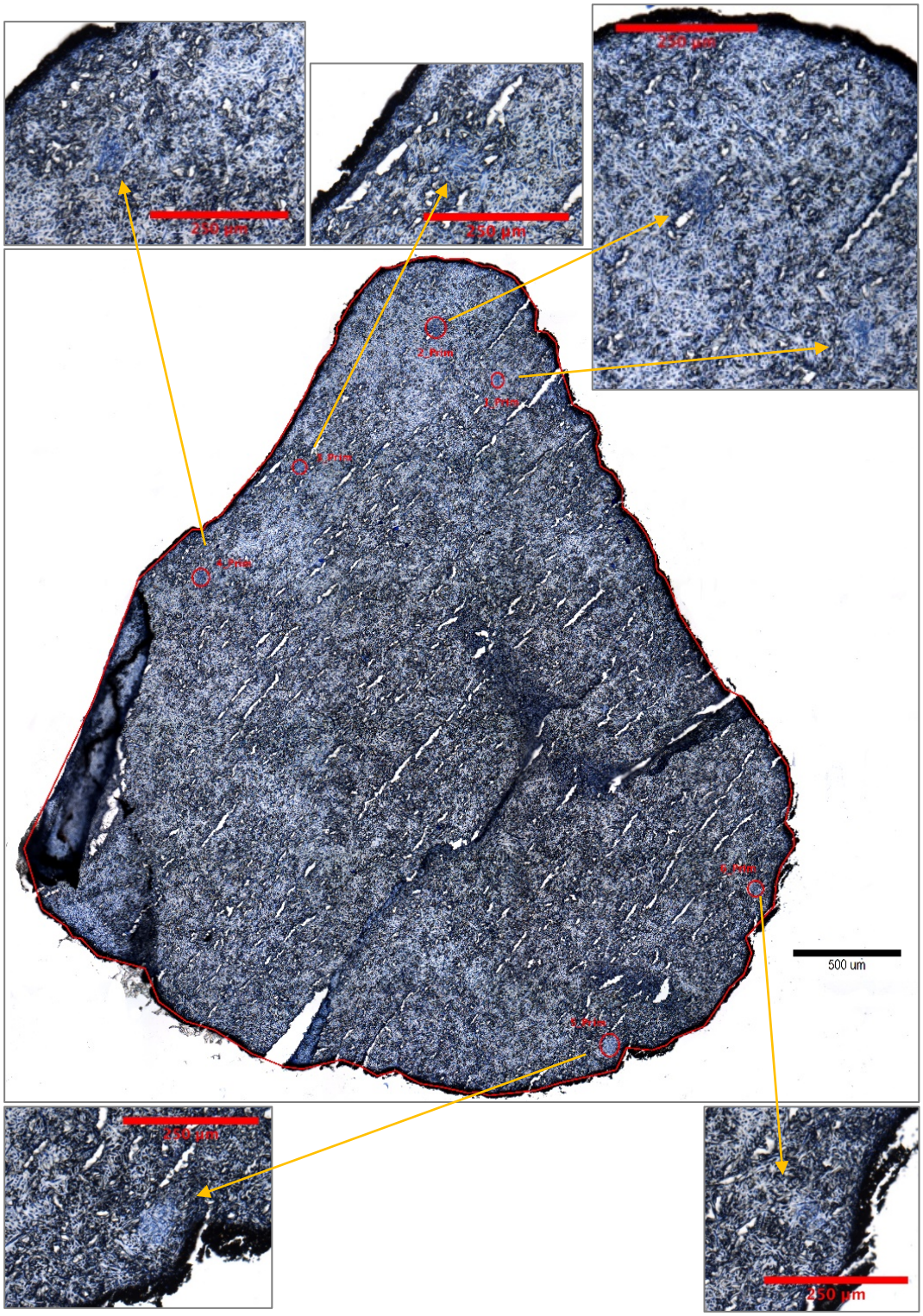
Appendix 29 Microscopic section of *S. sclerotiorum* sclerotium at the 42nd day showing presence of primordia (1 in total); Sclerotium was treated by incubation for 28 days at T1 = 4°C followed by transfer to T2 = 17°C.



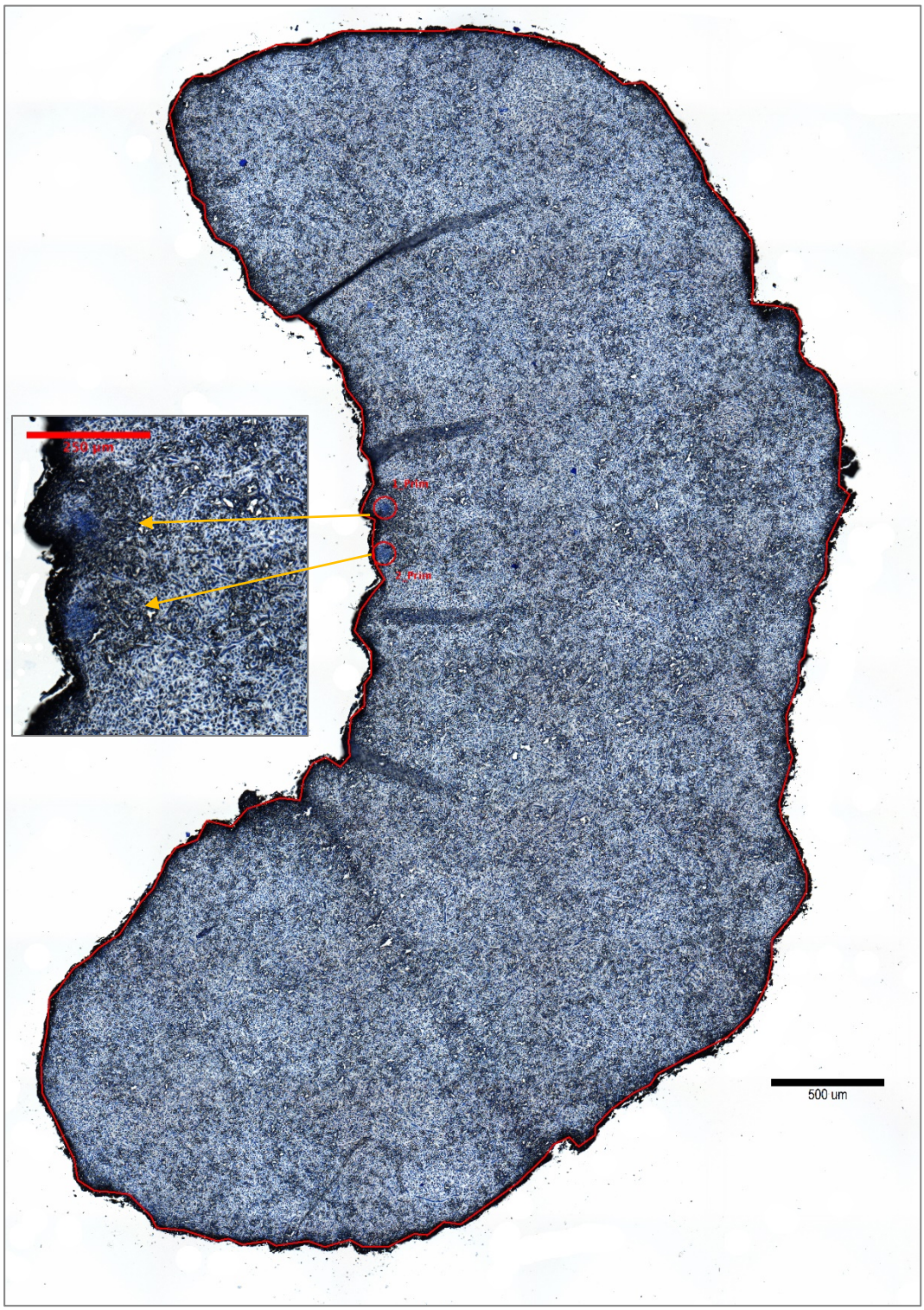
Appendix 30 Microscopic section of *S. sclerotiorum* sclerotium at the 70th day showing presence of primordia (4 in total); Sclerotium was treated by single temperature at T1 = 4°C.



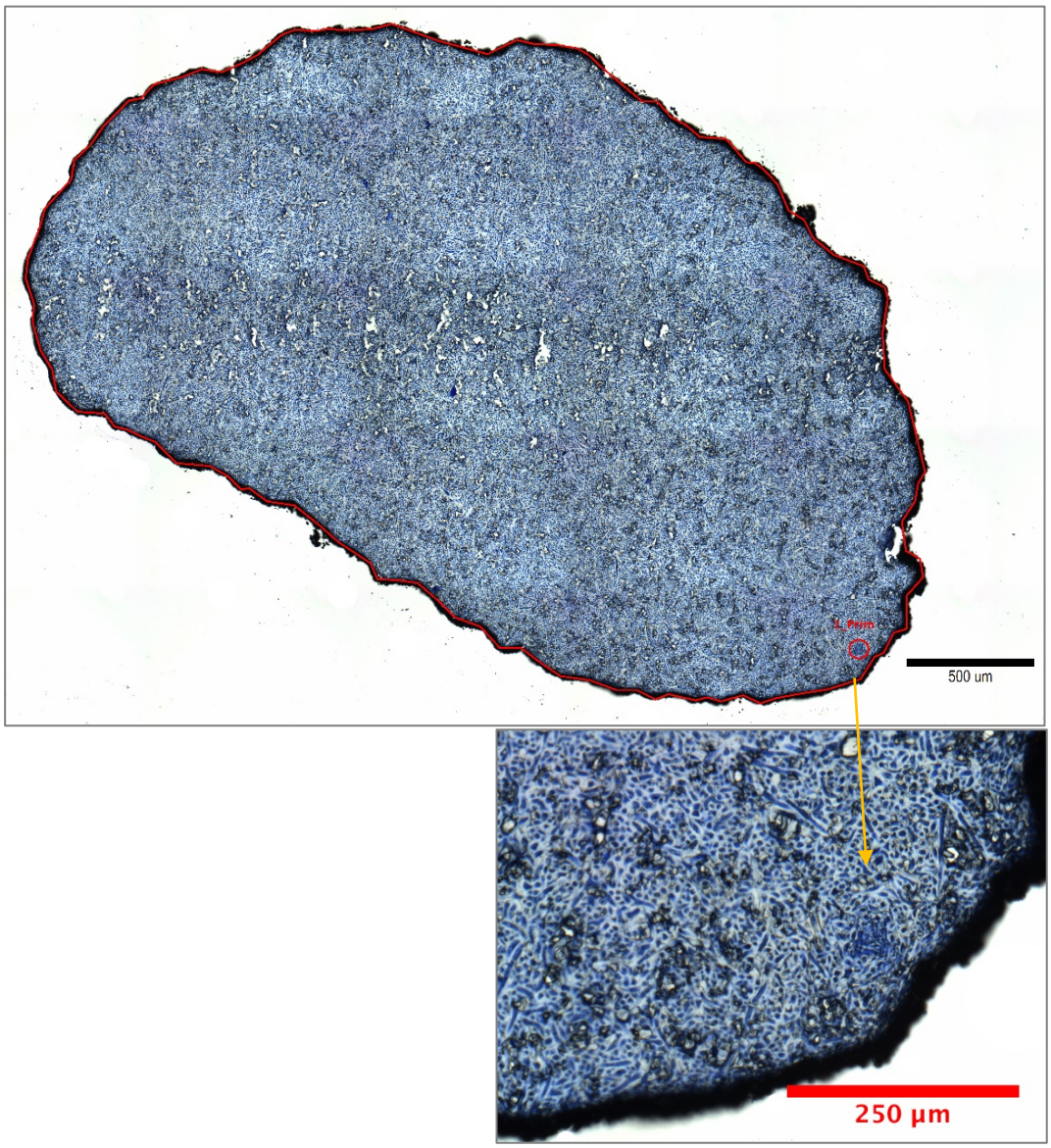
Appendix 31 Microscopic section of *S. sclerotiorum* sclerotium at the 42nd day showing presence of primordia (6 in total); Sclerotium was treated by incubation for 28 days at T1 = 11°C followed by transfer to T2 = 17°C.



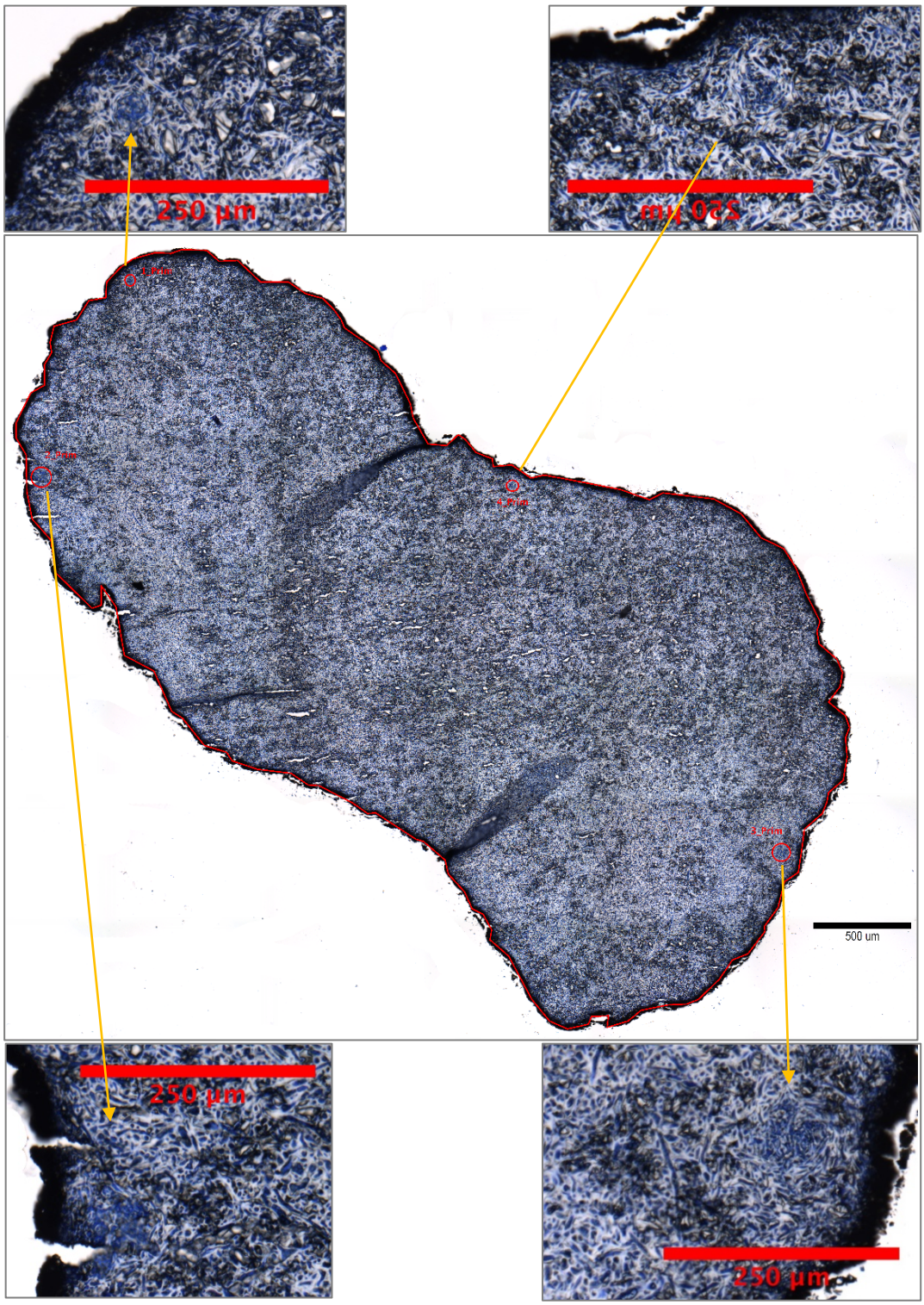
Appendix 32 Microscopic section of *S. sclerotiorum* sclerotium at the 7th day showing presence of primordia (2 in total); Sclerotium was treated by single temperature at T1 = 11°C.



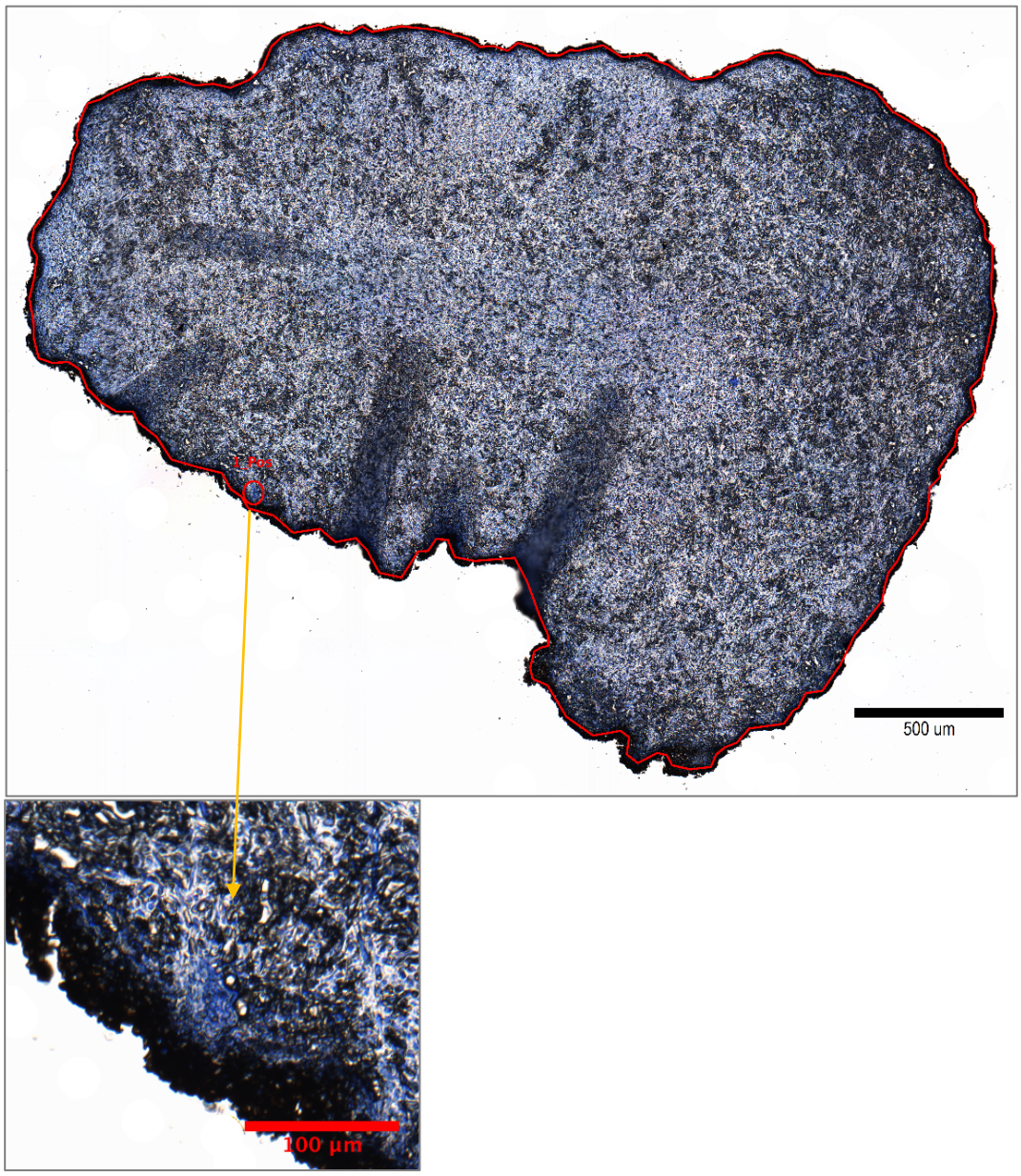
Appendix 33 Microscopic section of *S. sclerotiorum* sclerotium at the 28th day showing presence of primordia (1 in total); Sclerotium was treated by single temperature at T1 = 11°C.



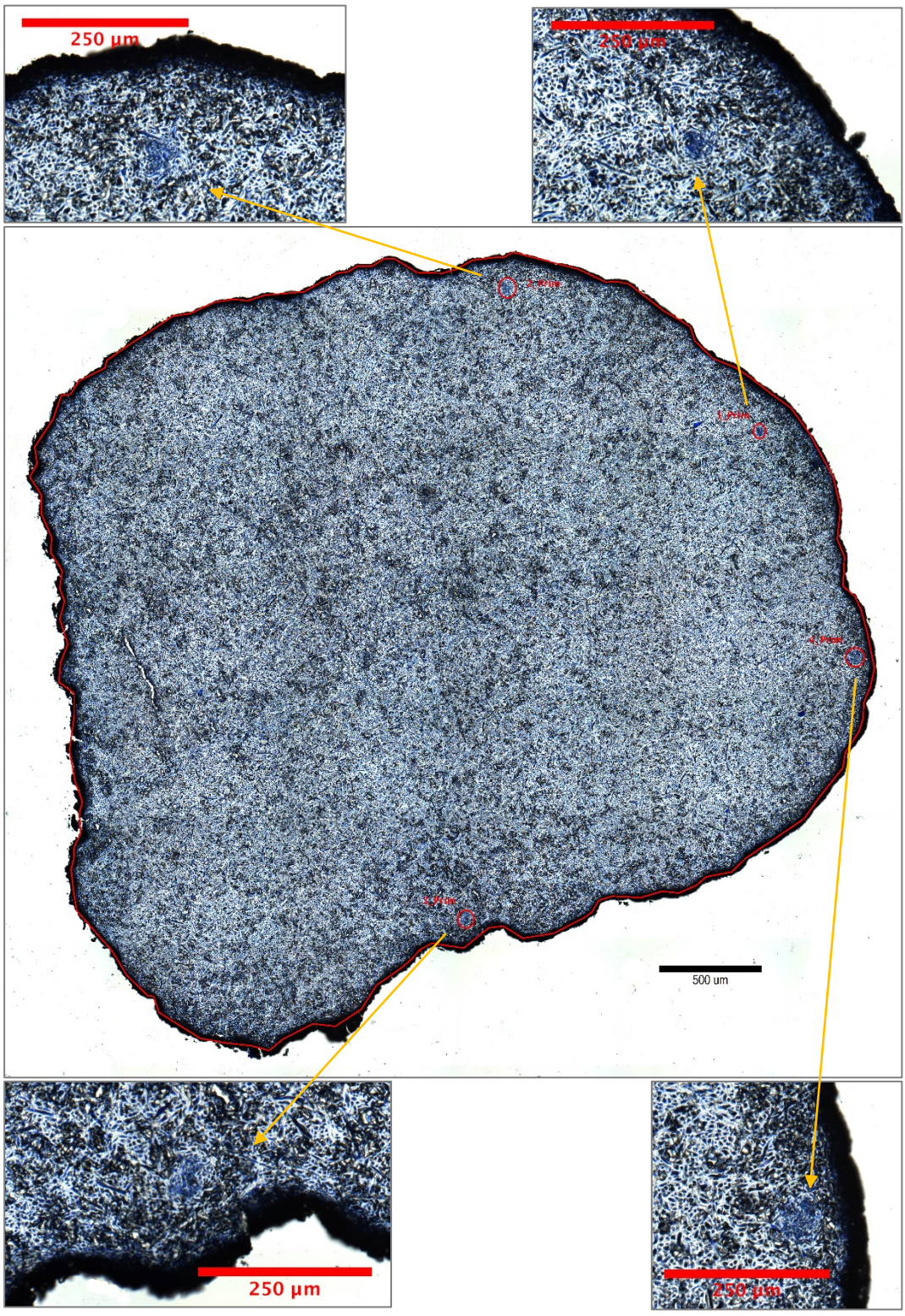
Appendix 34 Microscopic section of *S. sclerotiorum* sclerotium at the 42nd day showing presence of primordia (4 in total); Sclerotium was treated by single temperature at T1 = 11°C.



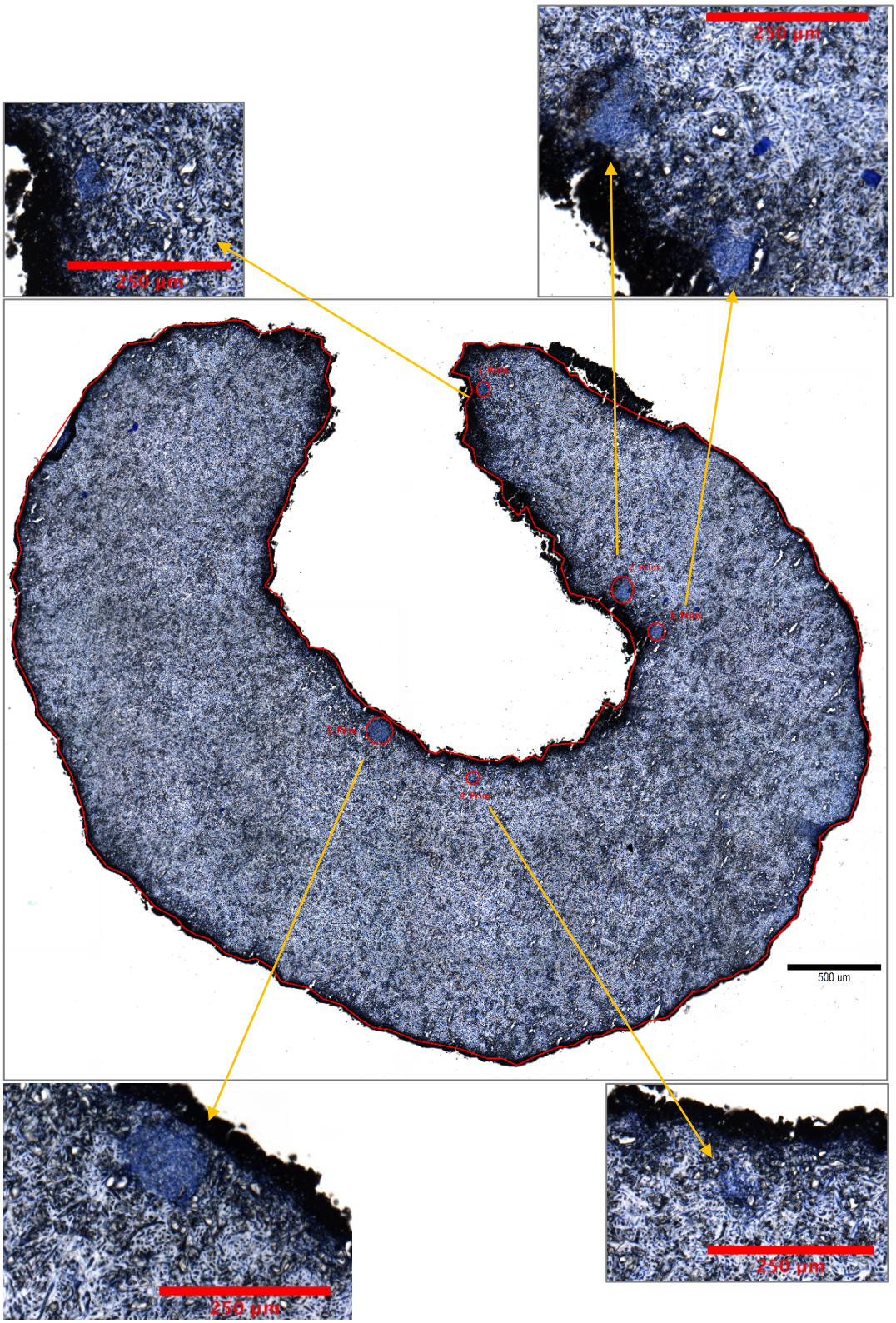
Appendix 35 Microscopic section of *S. sclerotiorum* sclerotium at the 7th day showing presence of primordia (1 in total); Sclerotium was treated by single temperature at T1 = 11°C.



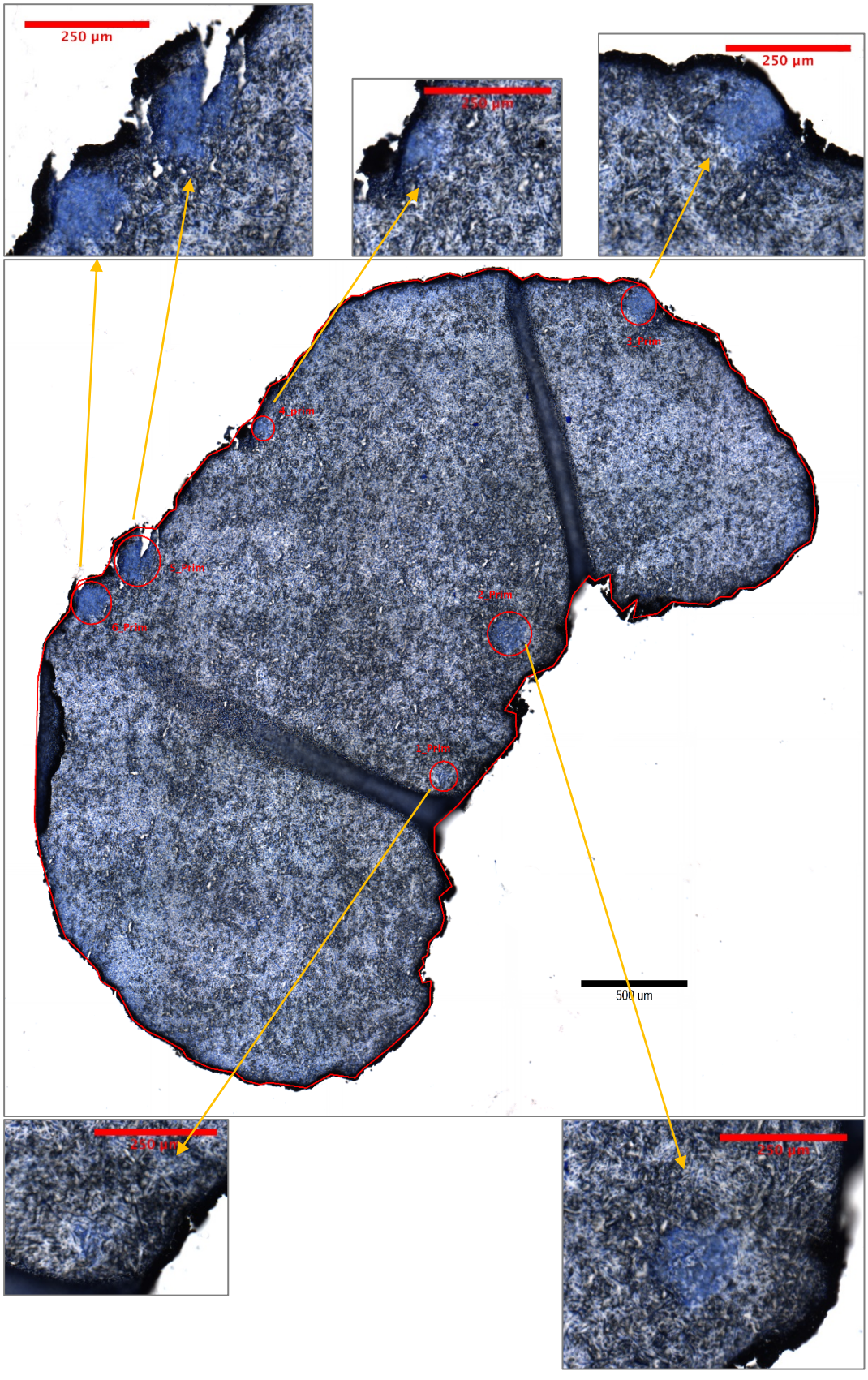
Appendix 36 Microscopic section of *S. sclerotiorum* sclerotium at the 28th day showing presence of primordia (4 in total); Sclerotium was treated by single temperature at T1 = 17°C.



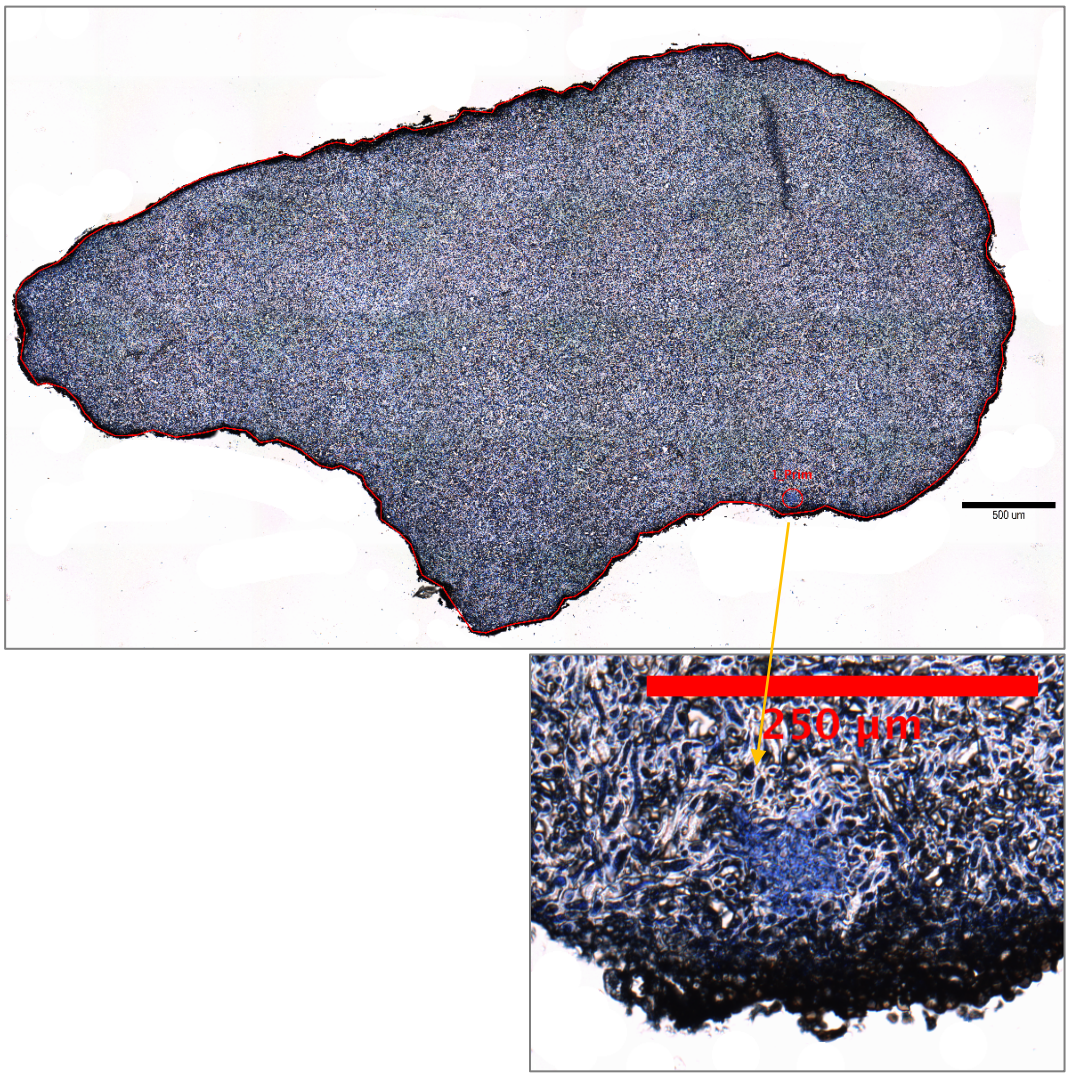
Appendix 37 Microscopic section of *S. sclerotiorum* sclerotium at the 42nd day showing presence of primordia (5 in total); Sclerotium was treated by single temperature at T1 = 17°C.



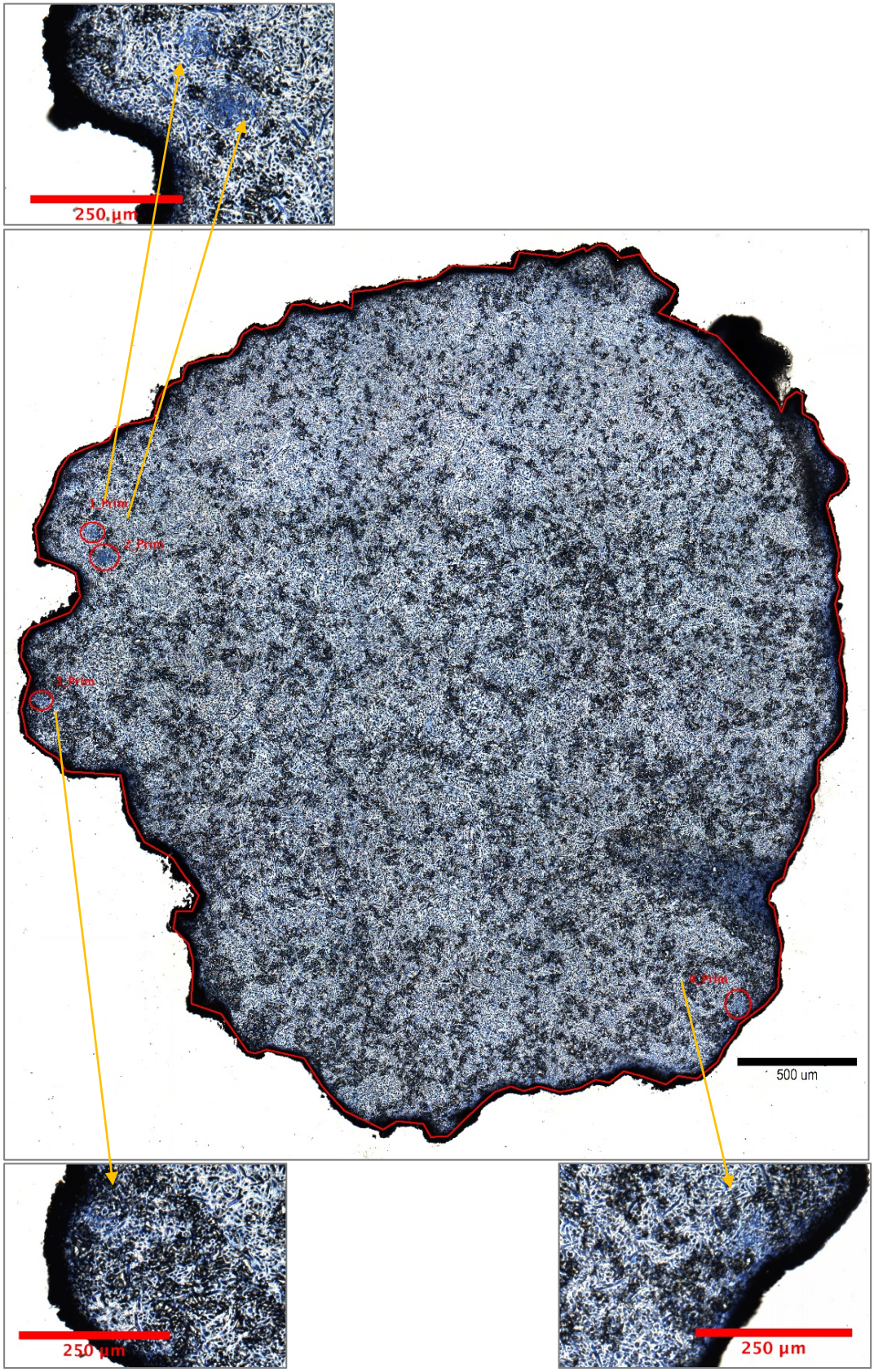
Appendix 38Microscopic section of *S. sclerotiorum* sclerotium at the 42nd day showing presence of primordia (6 in total); Sclerotium was treated by incubation for 28 days at T1 = 20°C followed by transfer to T2 = 17°C.



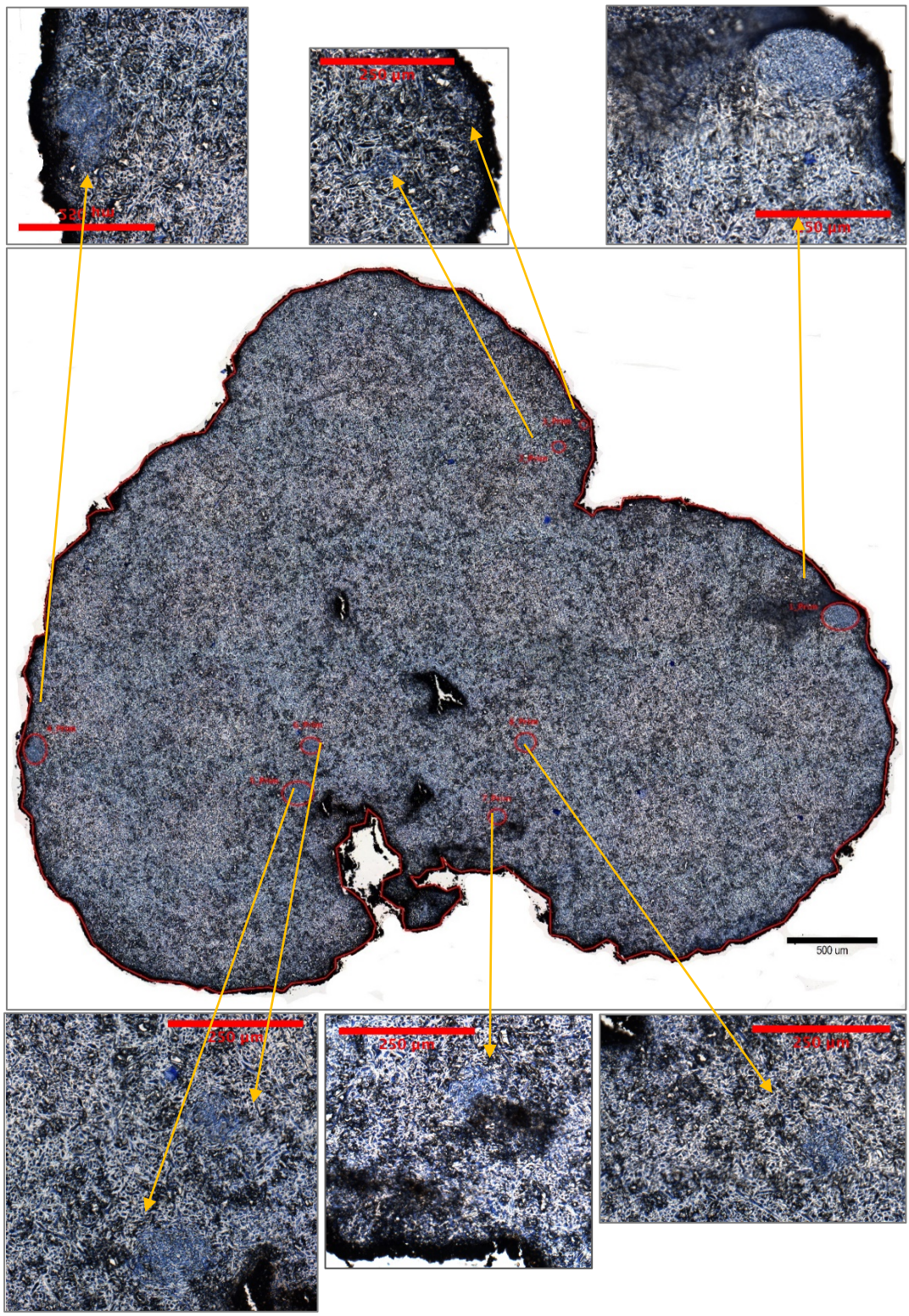
Appendix 39 Microscopic section of *S. sclerotiorum* sclerotium at the 7th day showing presence of primordia (1 in total); Sclerotium was treated by single temperature at T1 = 20°C.



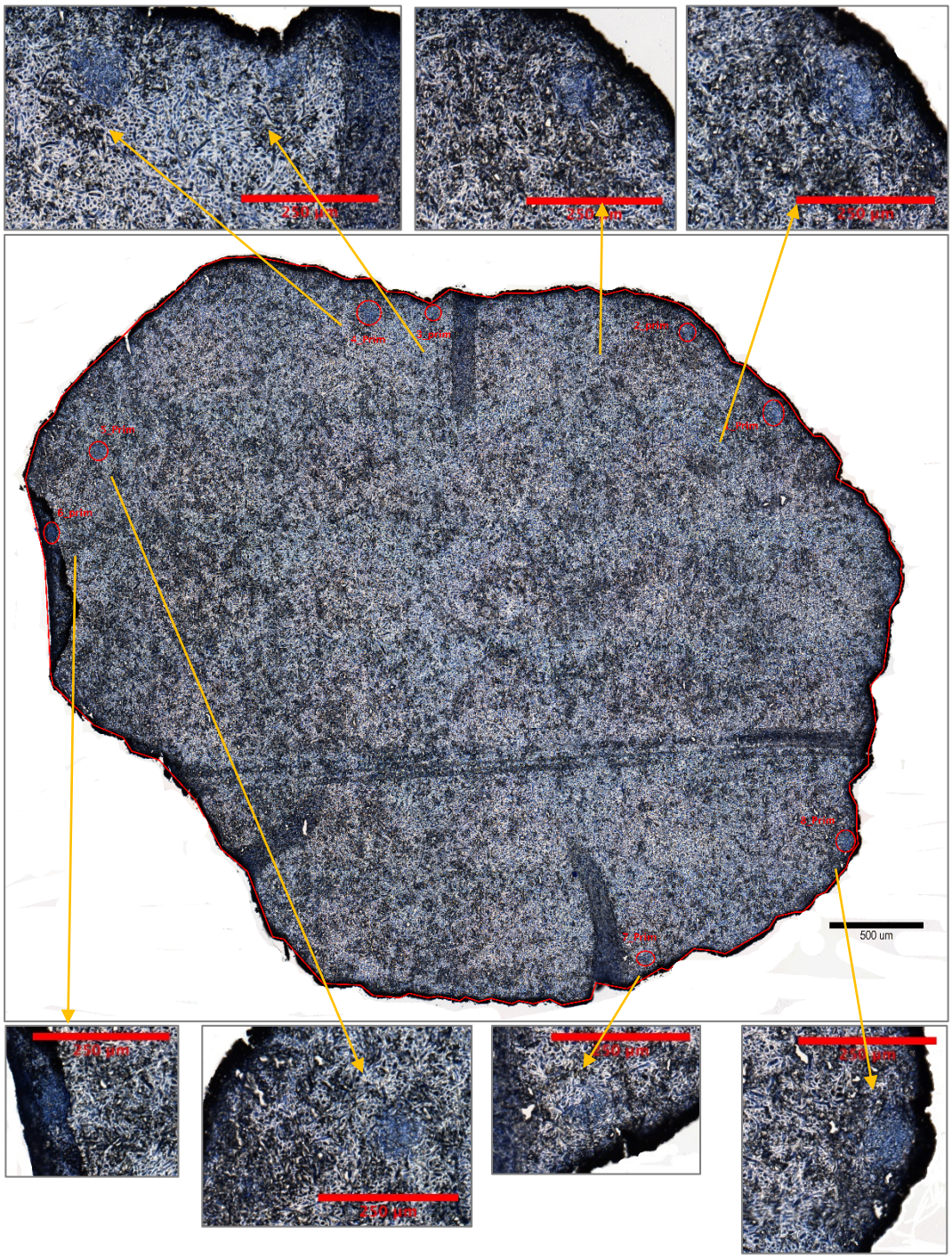
Appendix 40 Microscopic section of *S. sclerotiorum* sclerotium at the 28th day showing presence of primordia (4 in total); Sclerotium was treated by single temperature at T1 = 20°C.



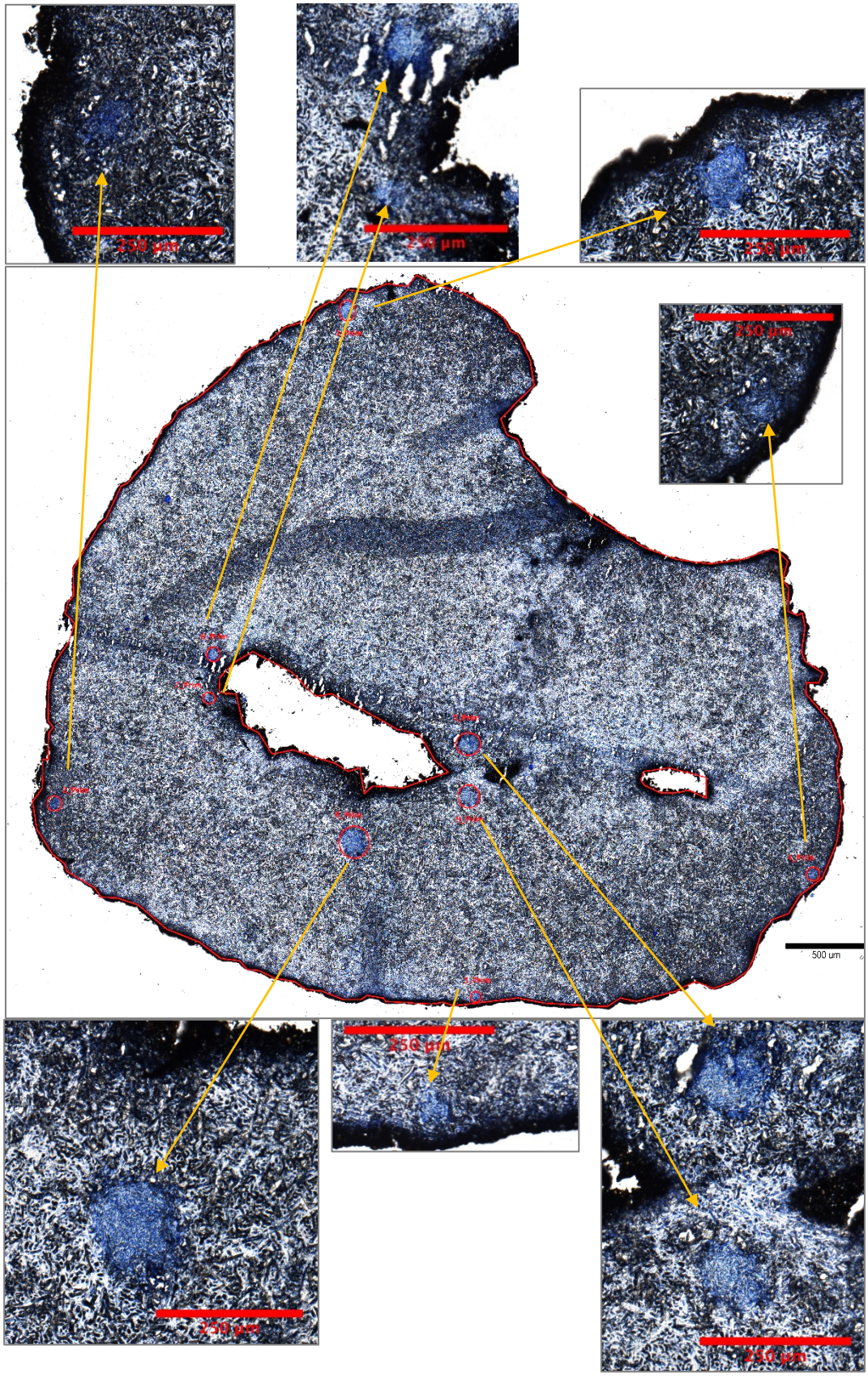
Appendix 41 Microscopic section of *S. sclerotiorum* sclerotium at the 42nd day showing presence of primordia (8 in total); Sclerotium was treated by single temperature at T1 = 20°C.



Appendix 42 Microscopic section of *S. sclerotiorum* sclerotium at the 42nd day showing presence of primordia (7 in total); Sclerotium was treated by single temperature at T1 = 20°C.



Appendix 43 Microscopic section of *S. sclerotiorum* sclerotium at the 70th day showing presence of primordia (8 in total); Sclerotium was treated by single temperature at T1 = 20°C.



Appendix 44 The difference between ANOVA estimated means for T_{25} control moist treatment with no dry period in S2 and T_{25} estimated for various durations of dry period (7, 7+7, 14, 28 days) initiated directly after transfer (28th day) and 14 days after transfer (42nd day) to S2, observed for *S. sclerotiorum* isolates L5 and L6 at two S2 temperatures (T2 = 15 and 17°C); Colour gradient shows the variation of difference in germination times: red=shortest, white = middle, blue = longest.

Isolate	T2	Dry timing (day)	Dry period duration (days)			
			7	7+7	14	28
L5	15°C	28	12	19	17	27
		42	8	16	16	27
	17°C	28	12	19	15	30
		42	7	16	16	34
L6	15°C	28	9	16	13	29
		42	8	15	16	28
	17°C	28	8	13	12	27
		42	2	-4*	-4*	-4*

Appendix 45 The difference between ANOVA estimated means for T_{50} control moist treatment with no dry period in S2 and T_{50} estimated for various durations of dry period (7, 7+7, 14, 28 days) initiated directly after transfer (28th day) and 14 days after transfer (42nd day) to S2, observed for *S. sclerotiorum* isolates L5 and L6 at two S2 temperatures (T2 = 15 and 17°C); Colour gradient shows the variation of difference in germination times: red=shortest, white = middle, blue = longest.

Isolate	T2	Dry timing (day)	Dry period duration (days)			
			7	7+7	14	28
L5	15°C	28	10	17	15	26
		42	9	15	14	26
	17°C	28	10	17	14	28
		42	7	15	16	34
L6	15°C	28	7	16	15	31
		42	7	18	17	26
	17°C	28	7	11	9	28
		42	4	6	12	27

Appendix 46 The difference between ANOVA estimated means for T_{75} control moist treatment with no dry period in S2 and T_{75} estimated for various durations of dry period (7, 7+7, 14, 28 days) initiated directly after transfer (28th day) and 14 days after transfer (42nd day) to S2, observed for *S. sclerotiorum* isolates L5 and L6 at two S2 temperatures (T2 = 15 and 17°C); Colour gradient shows the variation of difference in germination times: red=shortest, white = middle, blue = longest.

Isolate	T2	Dry timing (day)	Dry period duration (days)			
			7	7+7	14	28
L5	15°C	28	10	17	15	26
		42	8	17	16	26
	17°C	28	11	17	18	30
		42	10	16	20	43
L6	15°C	28	2	19	12	30
		42	5	14	13	27
	17°C	28	9	18	15	27
		42	8	11	19	33

Appendix 47 Time to germination of 10% of sclerotia (T_{10}) for isolate L5 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{10}_L5_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							314					
20℃										314	308	312	
17℃										307	299	296	
14℃										302	297	129	
11℃										303	299	43	
8℃													
4℃										309	306	225	
0℃													
25℃	20℃												
20℃		286						305					
17℃				235	226	230	176			305	223	139	
14℃				286	202	58	66			303	132	64	
11℃			297	286	67	46				302	54	44	
8℃			293	288	50	59							
4℃			302	208	54	66				306	133	70	
0℃			288	293	134	71							
25℃	17℃												
20℃													
17℃		89						63					
14℃				171	81	70	77			97	47	42	
11℃			102	87	46	52				62	48	43	
8℃			93	87	47	59							
4℃			75	45	47	65				50	50	68	
0℃			68	75	48	71							
25℃	14℃												
20℃													
17℃													
14℃		52						46					
11℃			54	42	49	39				41	42	45	
8℃			45	45	45	60							
4℃			38	42	45	65				41	50	69	
0℃			47	47	51	74							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		45						44					
8℃			41	43	45	62							
4℃			39	39	46	69				46	55	74	
0℃			38	45	57	79							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		61											
4℃			64	65	68	82							
0℃			61	68	77	97							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		98						117					
0℃			103	110	114	136							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		284											

Appendix 48 Time to germination of 25% of sclerotia (T_{25}) for isolate L5 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{25}_L5_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							332					
20℃										328	324	328	
17℃										316	302	301	
14℃										306	302	214	
11℃										309	303	47	
8℃													
4℃										321	311	232	
0℃													
25℃	20℃												
20℃		296						308					
17℃				302	302	302	298			307	305	302	
14℃				300	295	156	84			306	236	152	
11℃			305	302	156	58				306	214	49	
8℃			315	305	65	63							
4℃			319	301	66	71				309	162	75	
0℃			304	312	230	79							
25℃	17℃												
20℃													
17℃		111						81					
14℃				200	107	89	97			116	85	61	
11℃			117	106	68	62				105	67	47	
8℃			109	110	57	63							
4℃			90	61	52	68				88	56	75	
0℃			91	92	53	75							
25℃	14℃												
20℃													
17℃													
14℃		69						68					
11℃			79	61	60	51				50	50	48	
8℃			59	53	49	63							
4℃			48	47	49	69				46	55	73	
0℃			61	55	55	77							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		57						48					
8℃			52	51	55	67							
4℃			49	49	55	76				50	58	77	
0℃			48	55	62	84							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		69											
4℃			71	72	75	88							
0℃			68	75	83	104							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		110						136					
0℃			113	121	123	141							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		290											

Appendix 49 Time to germination of 40% of sclerotia (T_{40}) for isolate L5 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{40}_L5_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							341					
20℃										338	337	340	
17℃										329	305	305	
14℃										310	305	226	
11℃										314	306	51	
8℃													
4℃										333	318	307	
0℃													
25℃	20℃												
20℃		307						310					
17℃				317	316	319	314			310	308	307	
14℃				309	307	214	94			309	299	189	
11℃			313	315	228	68				308	238	53	
8℃			325	316	148	66							
4℃			325	319	90	74				312	227	94	
0℃			314	321	323	149							
25℃	17℃												
20℃													
17℃		131						100					
14℃				238	125	96	108			132	96	69	
11℃			138	116	108	75				119	78	51	
8℃			118	121	77	66							
4℃			114	85	57	71				99	61	78	
0℃			104	117	57	79							
25℃	14℃												
20℃													
17℃													
14℃		85						84					
11℃			91	77	74	58				57	58	51	
8℃			71	61	54	67							
4℃			60	53	53	73				51	59	77	
0℃			73	62	58	80							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		64						52					
8℃			57	58	60	71							
4℃			55	56	60	79				54	61	81	
0℃			56	59	66	87							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		78											
4℃			78	77	79	95							
0℃			74	81	89	109							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		123						148					
0℃			125	133	131	150							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		297											

Appendix 50 Time to germination of 50% of sclerotia (T_{50}) for isolate L5 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{50}_L5_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							348					
20℃										351	345	348	
17℃										340	308	308	
14℃										313	307	303	
11℃										318	308	54	
8℃													
4℃										339	326	310	
0℃													
25℃	20℃												
20℃		311						312					
17℃				323	322	324	322			312	310	308	
14℃				314	312	224	105			310	301	299	
11℃			319	320	296	72				309	300	55	
8℃			329	323	189	68							
4℃			329	325	217	76				313	307	171	
0℃			320	327	328	162							
25℃	17℃												
20℃													
17℃		136						109					
14℃				245	136	107	114			146	108	82	
11℃			146	127	116	84				129	88	54	
8℃			131	139	87	68							
4℃			124	101	62	73				112	65	81	
0℃			113	124	59	81							
25℃	14℃												
20℃													
17℃													
14℃		92						92					
11℃			100	82	91	63				65	64	52	
8℃			83	68	57	70							
4℃			69	58	56	75				55	62	80	
0℃			79	67	60	82							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		69						55					
8℃			61	62	64	74							
4℃			59	59	62	82				57	64	84	
0℃			59	62	68	89							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		82											
4℃			83	80	84	102							
0℃			77	87	93	114							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		130						166					
0℃			130	137	137	159							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		302											

Appendix 51 Time to germination of 60% of sclerotia (T_{60}) for isolate L5 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{60}_L5_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							359					
20℃										358	351	356	
17℃										351	311	311	
14℃										318	309	306	
11℃										334	310	134	
8℃													
4℃										345	335	312	
0℃													
25℃	20℃												
20℃		317						316					
17℃				329	328	329	327			314	312	310	
14℃				319	317	237	127			312	303	302	
11℃			324	326	312	75				311	303	60	
8℃			334	328	307	71							
4℃			333	330	230	79				315	312	257	
0℃			325	331	332	169							
25℃	17℃												
20℃													
17℃		143						160					
14℃				252	146	119	123			231	123	100	
11℃			158	135	125	90				138	101	61	
8℃			144	177	99	70							
4℃			137	124	74	75				146	70	84	
0℃			118	131	63	83							
25℃	14℃												
20℃													
17℃													
14℃		96						98					
11℃			110	87	99	69				71	78	54	
8℃			91	75	60	73							
4℃			74	64	59	78				59	66	83	
0℃			85	73	63	84							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		75						59					
8℃			67	67	70	77							
4℃			63	62	65	86				60	68	89	
0℃			62	65	71	92							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		87											
4℃			87	83	89	106							
0℃			80	91	100	118							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		136						177					
0℃			135	146	144	168							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		306											

Appendix 52 Time to germination of 75% of sclerotia (T_{75}) for isolate L5 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{75}_L5_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							374					
20℃										370	366	366	
17℃										375	318	316	
14℃										339	313	309	
11℃										348	315	141	
8℃													
4℃										360	347	329	
0℃													
25℃	20℃												
20℃		326						321					
17℃				337	336	337	336			319	315	314	
14℃				329	327	311	188			317	308	306	
11℃			334	335	326	90				313	308	79	
8℃			340	336	330	75							
4℃			339	337	256	89				319	315	312	
0℃			334	339	339	313							
25℃	17℃												
20℃													
17℃		170						202					
14℃				293	167	147	135			268	180	113	
11℃			196	164	164	100				205	184	67	
8℃			183	213	118	74							
4℃			151	140	83	83				208	84	89	
0℃			126	144	75	87							
25℃	14℃												
20℃													
17℃													
14℃		113						110					
11℃			119	98	108	78				90	91	60	
8℃			104	90	65	77							
4℃			90	91	64	85				66	72	89	
0℃			96	88	66	88							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		86						66					
8℃			76	75	78	83							
4℃			70	67	69	91				66	75	97	
0℃			68	72	74	96							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		96											
4℃			100	91	99	113							
0℃			86	101	112	126							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		162						233					
0℃			165	168	170	186							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		313											

Appendix 53 Time to germination of 90% of sclerotia (T_{90}) for isolate L5 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{90}_L5_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							392					
20℃										390	393	390	
17℃										395	336	329	
14℃										368	326	315	
11℃										377	327	307	
8℃													
4℃										378	365	352	
0℃													
25℃	20℃												
20℃		335						334					
17℃				345	345	345	344			327	321	320	
14℃				341	340	337	251			324	314	314	
11℃			344	343	340	112				318	317	160	
8℃			347	344	342	81							
4℃			345	345	340	163				324	324	318	
0℃			344	346	347	339							
25℃	17℃												
20℃													
17℃		225						266					
14℃				338	213	248	153			335	214	132	
11℃			255	268	255	115				282	225	81	
8℃			229	275	176	79							
4℃			214	210	114	91				330	162	95	
0℃			218	211	94	91							
25℃	14℃												
20℃													
17℃													
14℃		126						131					
11℃			132	115	119	92				129	105	93	
8℃			124	111	72	85							
4℃			109	109	71	92				77	84	99	
0℃			113	109	72	95							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		97						84					
8℃			95	93	91	93							
4℃			79	75	78	102				84	91	107	
0℃			77	81	82	106							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		112											
4℃			124	112	127	133							
0℃			112	122	129	147							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		226						330					
0℃			225	233	221	226							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		334											

Appendix 54 Time to germination of 10% of sclerotia (T_{10}) for isolate L6 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{10}_L6_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							307					
20℃										307	308	307	
17℃										30	37	85	
14℃										28	47	84	
11℃										30	47	84	
8℃													
4℃										172	108	123	
0℃													
25℃	20℃												
20℃		40						214					
17℃				20	34	54	47			21	39	52	
14℃				25	43	62	80			23	42	66	
11℃			13	25	40	62				25	39	58	
8℃			17	26	38	61							
4℃			25	27	39	62				48	42	63	
0℃			45	42	54	72							
25℃	17℃												
20℃													
17℃		57						74					
14℃				65	59	69	89			61	59	72	
11℃			50	51	50	64				48	48	58	
8℃			51	44	47	64							
4℃			49	43	45	64				41	45	66	
0℃			58	51	48	69							
25℃	14℃												
20℃													
17℃													
14℃		82						81					
11℃			70	79	60	72				66	63	62	
8℃			69	64	56	71							
4℃			72	60	58	74				60	56	72	
0℃			69	66	59	77							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		73						72					
8℃			75	71	71	78							
4℃			71	75	75	81				59	58	74	
0℃			77	76	74	85							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		88											
4℃			90	82	85	100							
0℃			88	83	95	111							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		115						108					
0℃			124	119	117	137							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		283											

Appendix 55 Time to germination of 25% of sclerotia (T_{25}) for isolate L6 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{25}_L6_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							312					
20℃										312	313	313	
17℃										85	44	103	
14℃										35	51	88	
11℃										38	52	104	
8℃													
4℃										307	151	154	
0℃													
25℃	20℃												
20℃		50						300					
17℃				24	41	62	89			25	44	71	
14℃				27	45	66	89			26	45	70	
11℃			18	28	43	65				29	42	61	
8℃			26	30	41	64							
4℃			39	31	41	65				73	49	68	
0℃			70	55	61	83							
25℃	17℃												
20℃													
17℃		87						109					
14℃				84	73	75	94			82	66	76	
11℃			73	63	57	68				59	51	62	
8℃			65	54	51	68							
4℃			73	56	50	68				54	51	70	
0℃			72	63	54	75							
25℃	14℃												
20℃													
17℃													
14℃		98						93					
11℃			91	91	69	80				80	70	71	
8℃			83	75	62	75							
4℃			89	71	65	80				72	64	75	
0℃			86	80	68	83							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		83						79					
8℃			84	80	79	84							
4℃			84	82	82	88				69	70	80	
0℃			83	83	80	90							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		104											
4℃			100	99	104	115							
0℃			99	93	102	119							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		132						117					
0℃			136	132	130	148							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		289											

Appendix 56 Time to germination of 40% of sclerotia (T_{40}) for isolate L6 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{40}_L6_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							317		321	321	317	
20℃										196	69	109	
17℃										121	54	92	
14℃										149	57	109	
11℃													
8℃										311	229	218	
4℃													
0℃													
25℃	20℃												
20℃		59						305					
17℃				28	44	71	93			31	48	74	
14℃				29	47	69	92			30	47	73	
11℃			26	31	45	68				33	45	64	
8℃			36	33	44	67							
4℃			47	39	46	69				221	58	73	
0℃			83	64	68	94							
25℃	17℃												
20℃													
17℃		106						129					
14℃				100	82	79	97			100	76	81	
11℃			95	72	63	72				70	54	64	
8℃			80	62	55	71							
4℃			86	65	54	72				67	54	74	
0℃			89	76	59	78							
25℃	14℃												
20℃													
17℃													
14℃		105						102					
11℃			97	96	77	85				86	76	77	
8℃			91	83	67	79							
4℃			95	80	71	85				80	69	79	
0℃			93	91	73	87							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		90						84					
8℃			93	88	85	88							
4℃			91	87	87	94				76	76	86	
0℃			88	87	84	94							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		111											
4℃			108	107	113	121							
0℃			107	104	110	125							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		143						130					
0℃			144	143	141	160							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		295											

Appendix 57 Time to germination of 50% of sclerotia (T_{50}) for isolate L6 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{50}_L6_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							321					
20℃										327	326	320	
17℃										277	72	150	
14℃										202	58	95	
11℃										205	99	114	
8℃													
4℃										316	304	247	
0℃													
25℃	20℃												
20℃		67						308					
17℃				30	46	74	95			36	50	75	
14℃				31	48	72	93			33	48	74	
11℃			33	33	47	69				38	46	65	
8℃			43	38	45	69							
4℃			53	47	50	72				237	74	77	
0℃			95	73	74	101							
25℃	17℃												
20℃													
17℃		114						136					
14℃				105	88	82	99			113	81	83	
11℃			104	82	66	74				75	57	66	
8℃			88	66	58	73							
4℃			96	70	58	74				73	56	77	
0℃			96	84	62	80							
25℃	14℃												
20℃													
17℃													
14℃		110						107					
11℃			100	100	82	88				90	81	80	
8℃			96	88	71	82							
4℃			99	84	74	88				85	71	82	
0℃			98	96	76	90							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		94						90					
8℃			98	92	89	91							
4℃			94	91	90	97				80	79	88	
0℃			92	90	87	97							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		115											
4℃			116	113	118	125							
0℃			112	109	116	128							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		149						141					
0℃			150	148	150	180							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		300											

Appendix 58 Time to germination of 60% of sclerotia (T_{60}) for isolate L6 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{60}_L6_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							326					
20℃										332	331	324	
17℃										310	83	163	
14℃										289	62	107	
11℃										248	113	125	
8℃													
4℃										324	310	281	
0℃													
25℃	20℃												
20℃		77						310					
17℃				33	47	76	97			46	51	77	
14℃				34	49	73	95			39	49	76	
11℃			39	37	49	71				55	48	67	
8℃			49	44	49	71							
4℃			61	54	60	76				305	163	84	
0℃			113	88	84	115							
25℃	17℃												
20℃													
17℃		120						143					
14℃				110	93	87	101			125	90	86	
11℃			113	90	69	75				82	59	67	
8℃			95	70	60	75							
4℃			101	76	61	77				82	59	79	
0℃			106	91	65	82							
25℃	14℃												
20℃													
17℃													
14℃		114						110					
11℃			105	105	87	92				97	86	84	
8℃			102	94	76	84							
4℃			105	88	78	91				89	73	84	
0℃			105	100	79	92							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		98						97					
8℃			103	97	93	94							
4℃			99	96	94	100				84	82	91	
0℃			98	94	90	100							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		119											
4℃			123	117	124	129							
0℃			116	113	121	131							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		161						147					
0℃			159	158	166	199							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		304											

Appendix 59 Time to germination of 75% of sclerotia (T_{75}) for isolate L6 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{75}_L6_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							334					
20℃										338	340	332	
17℃										327	99	179	
14℃										318	87	111	
11℃										322	133	158	
8℃													
4℃										331	317	310	
0℃													
25℃	20℃												
20℃		158						316					
17℃				46	49	79	100			148	54	80	
14℃				49	53	76	97			141	52	79	
11℃			54	53	53	73				240	51	70	
8℃			72	60	59	74							
4℃			86	70	76	102				311	305	199	
0℃			241	167	104	172							
25℃	17℃												
20℃													
17℃		129						156					
14℃				120	107	94	105			134	102	93	
11℃			123	101	75	79				94	62	70	
8℃			110	79	65	79							
4℃			107	85	66	83				96	62	82	
0℃			118	107	70	86							
25℃	14℃												
20℃													
17℃													
14℃		123						117					
11℃			118	112	97	97				107	96	97	
8℃			109	103	83	89							
4℃			113	98	87	95				99	77	88	
0℃			112	106	84	96							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		105						115					
8℃			112	104	101	100							
4℃			106	103	100	104				91	87	94	
0℃			104	99	95	106							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		126											
4℃			135	125	131	136							
0℃			124	118	129	138							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		181						160					
0℃			183	181	197	222							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		310											

Appendix 60 Time to germination of 90% of sclerotia (T_{90}) for isolate L6 (rep means), including the germination at S3 (yellow). These data were used in parameter optimisation in Chapter 6.

T_{90}_L6_S3		T1 duration (days)											
T1	T2	TE1						TE2					
		0	7	14	29	56	84	0	7	14	29	56	84
25℃	25℃							342					
20℃										348	355	345	
17℃										345	136	185	
14℃										337	153	130	
11℃										336	248	166	
8℃													
4℃										343	331	327	
0℃													
25℃	20℃												
20℃		297						324					
17℃				135	56	84	105			310	59	84	
14℃				77	58	79	103			225	54	82	
11℃			126	143	72	76				315	62	74	
8℃			289	129	84	86							
4℃			238	169	111	177				322	324	308	
0℃			302	300	228	297							
25℃	17℃												
20℃													
17℃		147						186					
14℃				132	126	111	111			146	115	107	
11℃			137	115	89	88				109	69	74	
8℃			126	98	71	85							
4℃			121	102	73	91				115	66	85	
0℃			131	128	76	93							
25℃	14℃												
20℃													
17℃													
14℃		139						134					
11℃			130	130	109	112				124	112	119	
8℃			124	117	93	98							
4℃			132	111	94	100				123	83	94	
0℃			125	119	93	103							
25℃	11℃												
20℃													
17℃													
14℃													
11℃		116						142					
8℃			124	121	111	113							
4℃			119	119	108	110				116	104	100	
0℃			117	106	103	114							
25℃	8℃												
20℃													
17℃													
14℃													
11℃													
8℃		134											
4℃			149	138	138	146							
0℃			135	130	145	154							
25℃	4℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃		226						171					
0℃			221	207	252	270							
25℃	0℃												
20℃													
17℃													
14℃													
11℃													
8℃													
4℃													
0℃		316											

Appendix 61. Conditioning and germination rates estimated for T_{25}, T_{50} and T_{75} for isolates L5 and L6, based on TE1 and TE2 combined data (S1 + S2), using two different model approaches: Sequential (S.M.) and Parallel model (P.M.); * no Standard error obtained.

L5 - T_{25}	Sequential model				Parallel model			
	Estimated parameters		Standard error		Estimated parameters		Standard error	
Temp.	CR	GR	CR	GR	CR	GR	CR	GR
0°C	0.00001	0.0134	*	*	0.02938	0.005582	*	*
4°C	0.1	0.009718	*	*	0.02872	0.008531	*	*
8°C	0.07038	0.01737	*	*	0.02805	0.01493	*	*
11°C	0.0144	0.02568	*	*	0.02317	0.02362	*	*
14°C	0.0109	0.02817	*	*	0.01451	0.03206	*	*
17°C	0.0073	0.01695	*	*	0.008433	0.03262	*	*
20°C	0.0019	0.02841	*	*	0.004142	0.0301	*	*
25°C	0.00054	0.1	*	*	0.1	0.01755	*	*

L6 - T_{25}	Sequential model				Parallel model			
	Estimated parameters		Standard error		Estimated parameters		Standard error	
Temp.	CR	GR	CR	GR	CR	GR	CR	GR
0°C	0.1	0.007824	*	*	0.01738	0.005308	*	*
4°C	0.1	0.00807	*	*	0.01995	0.007831	*	*
8°C	0.1	0.01118	*	*	0.01948	0.01053	*	*
11°C	0.0144	0.01488	*	*	0.0121	0.05608	*	*
14°C	0.0109	0.01708	*	*	0.01159	0.05596	*	*
17°C	0.0073	0.0237	*	*	0.01407	0.04019	*	*
20°C	0.0019	0.05407	*	*	0.0519	0.02871	*	*
25°C	0.00054	0.02205	*	*	0.02378	0.00617	*	*

L5 - T_{50}	Sequential model				Parallel model			
	Estimated parameters		Standard error		Estimated parameters		Standard error	
Temp.	CR	GR	CR	GR	CR	GR	CR	GR
0°C	0.1	0.00838	*	*	0.02564	0.00546	*	*
4°C	0.1	0.007606	*	*	0.02933	0.007217	*	*
8°C	0.09833	0.01372	*	*	0.01832	0.01291	*	*
11°C	0.0144	0.02012	*	*	0.01668	0.02078	*	*
14°C	0.0109	0.01927	*	*	0.01139	0.02647	*	*
17°C	0.0073	0.01178	*	*	0.006822	0.02528	*	*
20°C	0.0019	0.03361	*	*	0.006016	0.02106	*	*
25°C	0.00054	0.1	*	*	0.1	0.0233	*	*

L6 - T_{50}	Sequential model				Parallel model			
	Estimated parameters		Standard error		Estimated parameters		Standard error	
Temp.	CR	GR	CR	GR	CR	GR	CR	GR
0°C	0.00001	0.0000389	*	*	0.02095	0.005008	*	*
4°C	0.05239	0.007383	*	*	0.02681	0.00655	*	*
8°C	0.1	0.01052	*	*	0.0279	0.009226	*	*
11°C	0.0144	0.01384	*	*	0.02061	0.01311	*	*
14°C	0.0109	0.01506	*	*	0.009346	0.02465	*	*
17°C	0.0073	0.01813	*	*	0.009713	0.02489	*	*
20°C	0.0019	0.037	*	*	0.03451	0.02315	*	*
25°C	0.00054	0.01144	*	*	0.01214	0.004767	*	*

L5 - T_{75}	Sequential model				Parallel model			
	Estimated parameters		Standard error		Estimated parameters		Standard error	
Temp.	CR	GR	CR	GR	CR	GR	CR	GR
0°C	0.1	0.007984	*	*	0.02476	0.00472	0.00215	0.00109
4°C	0.1	0.006135	*	*	0.02453	0.005906	0.00133	0.000226
8°C	0.09105	0.01188	*	*	0.01798	0.010947	0.00196	0.000562
11°C	0.00001	0.01661	*	*	0.015645	0.01674	0.000404	0.00114
14°C	0.0109	0.01458	*	*	0.009333	0.02186	0.000287	0.00312
17°C	0.0073	0.009712	*	*	0.005733	0.02283	0.000146	0.00519
20°C	0.0019	0.01846	*	*	0.002895	0.02041	0.00031	0.00674
25°C	0.00054	0.1	*	*	0.05608	0.02485	*	*

L6 - T_{75}	Sequential model				Parallel model			
	Estimated parameters		Standard error		Estimated parameters		Standard error	
Temp.	CR	GR	CR	GR	CR	GR	CR	GR
0°C	0.00001	0.000015 63	*	*	0.01968	0.00177	0.00256	0.00101
4°C	0.1	0.006039	*	*	0.02391	0.005739	0.00239	0.00021
8°C	0.1	0.009267	*	*	0.02433	0.008717	0.00326	0.000383
11°C	0.0144	0.01182	*	*	0.01868	0.011397	0.00189	0.000472
14°C	0.0109	0.01259	*	*	0.008216	0.01867	0.000328	0.00202
17°C	0.0073	0.01445	*	*	0.008405	0.02876	0.000306	0.00598
20°C	0.0019	0.02083	*	*	0.02722	0.012517	0.00651	0.000579
25°C	0.00054	0.0155	*	*	0.01097	0.006941	0.0013	0.000928

Appendix 62. Conditioning and germination rates estimated for T_{10}, T_{25}, T_{40}, T_{50}, T_{60}, T_{75} and T_{90} for isolates L5, based on TE1 and TE2 combined data (S3), Parallel model; * no Standard error obtained.

L5	Estimated parameters - Conditioning Rate							
	0°C	4°C	8°C	11°C	14°C	17°C	20°C	25°C
T_{10}	0.02595	0.0296	0.03114	0.03024	0.02144	0.010681	0.00236	0.000474
T_{25}	0.02481	0.03037	0.03253	0.02645	0.015348	0.007876	0.001418	0.000864
T_{40}	0.015342	0.02722	0.02731	0.02215	0.013475	0.007045	0.001636	0.001403
T_{50}	0.024080	0.029000	0.030690	0.017795	0.011725	0.006448	0.000674	0.000601
T_{60}	0.025430	0.026610	0.018380	0.017723	0.011607	0.005605	0.000520	0.000096
T_{75}	0.027200	0.025200	0.017600	0.017500	0.011000	0.004400	0.000540	0.000240
T_{90}_1	0.025710	0.020010	0.017430	0.017560	0.008492	0.003353	0.000233	0.000246
T_{90}_2	0.025680	0.019870	0.017400	0.017500	0.008490	0.003290	0.000210	0.000250
S.e. - Conditioning Rate								
T_{10}	0.001810	0.001270	0.001700	0.001230	0.001230	0.000431	0.000106	0.000167
T_{25}	0.002130	0.001070	0.001030	0.001820	0.000588	0.000414	0.000296	0.000220
T_{40}	0.000645	0.001180	0.001380	0.001710	0.000596	0.000316	0.000218	0.000240
T_{50}	0.003140	0.001770	0.001270	0.000116	0.000145	0.000228	0.000215	0.000394
T_{60}	0.003190	0.002310	0.002990	0.000144	0.000183	0.000199	0.000249	0.000105
T_{75}	0.002990	0.002170	0.002890	0.000150	0.000145	0.000143	0.000041	0.000029
T_{90}_1	0.002750	0.001950	0.001050	0.001600	0.000494	0.000119	0.000398	0.000066
T_{90}_2	0.002610	0.001770	*	*	*	*	*	0.001130

L5	Estimated parameters - Germination Rate							
	0°C	4°C	8°C	11°C	14°C	17°C	20°C	25°C
T_{10}	0.003570	0.009929	0.017190	0.025280	0.037200	0.040000	0.045100	0.002583
T_{25}	0.003576	0.008904	0.015210	0.021320	0.033024	0.030334	0.031899	0.002784
T_{40}	0.003250	0.008098	0.014050	0.019080	0.029580	0.027700	0.017790	0.000182
T_{50}	0.002909	0.007579	0.013520	0.019620	0.026860	0.022050	0.008380	0.000010
T_{60}	0.003210	0.006932	0.013250	0.018410	0.025460	0.027000	0.006371	0.000001
T_{75}	0.001200	0.006000	0.013000	0.017900	0.022400	0.024300	0.004100	0.000011
T_{90}_1	0.001850	0.004172	0.010268	0.011418	0.018300	0.016800	0.003931	0.000011
T_{90}_2	0.001941	0.004259	0.010223	0.011577	0.018150	0.016700	0.003759	0.000011
S.e. - Germination Rate								
T_{10}	0.000630	0.000938	0.002400	0.003390	0.017400	0.023600	0.044900	0.000450
T_{25}	0.000173	0.000354	0.000048	0.003440	0.000050	0.000085	0.000064	0.000312
T_{40}	0.001130	0.000547	0.001340	0.001620	0.009380	0.011600	0.007330	0.000524
T_{50}	0.000960	0.000419	0.001030	0.001740	0.006320	0.004820	0.001050	*
T_{60}	0.001010	0.000405	0.001090	0.002000	0.006740	0.011700	0.000688	*
T_{75}	0.001200	0.000358	0.001070	0.000592	0.005700	0.010100	0.000376	*
T_{90}_1	0.000934	0.000206	0.000986	0.000733	*	*	*	*
T_{90}_2	0.000948	0.000217	0.000985	0.000750	0.004470	0.004790	0.000347	*

Appendix 63. Conditioning and germination rates estimated for T_{10}, T_{25}, T_{40}, T_{50}, T_{60}, T_{75} and T_{90} for isolates L6, based on TE1 and TE2 combined data (S3), Parallel model; * no Standard error obtained.

L6	Estimated parameters - Conditioning Rate							
	0°C	4°C	8°C	11°C	14°C	17°C	20°C	25°C
T_{10}	0.0966000	0.0697090	0.1290000	0.1286000	0.0745700	0.0742500	0.0085800	0.0001531
T_{25}	0.0872000	0.0679000	0.1290000	0.1286000	0.0743100	0.0722300	0.0061860	0.0001531
T_{40}	0.0482400	0.0174179	0.1269000	0.1146000	0.0747700	0.0694420	0.0074470	0.0001531
T_{50}_1	0.0445800	0.0169500	0.1073000	0.1077000	0.0678770	0.0664600	0.0070010	0.0002641
T_{50}_2	0.0445800	0.0169518	0.1073000	0.1019000	0.0678400	0.0664300	0.0070010	0.0002670
T_{60}	0.0412800	0.0154900	0.1064000	0.0711770	0.0675200	0.0638900	0.0061220	0.0000150
T_{75}	0.0183700	0.0090600	0.1300000	0.0339410	0.0418100	0.0406700	0.0050410	0.0001500
T_{90}	0.0036000	0.0074700	0.0339500	0.0275300	0.0361800	0.0340100	0.0035410	0.0001065
S.e. - Conditioning Rate								
T_{10}	0.0162000	0.0001410	*	*	*	*	0.0006320	*
T_{25}	0.0135000	0.0138000	*	*	*	*	0.0003980	*
T_{40}	0.0095300	0.0000528	*	0.0257000	*	0.0002110	0.0004990	*
T_{50}_1	*	*	*	0.0191000	0.0005120	0.0006200	*	0.0000266
T_{50}_2	0.0089500	0.0000914	0.0193000	*	*	*	0.0004320	*
T_{60}	0.0084500	0.0030800	0.0179000	0.0000217	0.0022700	*	0.0004040	*
T_{75}	0.0042500	0.0016400	*	0.0001340	0.0067700	0.0067900	0.0002730	*
T_{90}	0.0044600	0.0012700	0.0065300	0.0021100	0.0050600	*	0.0001550	*

L6	Estimated parameters - Germination Rate							
	0°C	4°C	8°C	11°C	14°C	17°C	20°C	25°C
T_{10}	0.003603	0.009430	0.012658	0.015564	0.015554	0.021960	0.056200	0.020960
T_{25}	0.003585	0.008272	0.011091	0.013994	0.013463	0.016141	0.039890	0.013360
T_{40}	0.003575	0.007884	0.010131	0.012910	0.012439	0.013783	0.036810	0.009300
T_{50}_1	0.003528	0.007314	0.009646	0.012241	0.012135	0.012636	0.032390	0.007201
T_{50}_2	0.003501	0.007315	0.009650	0.012240	0.012150	0.012640	0.032170	0.007199
T_{60}	0.003408	0.006749	0.009288	0.011695	0.011396	0.011796	0.026780	0.008780
T_{75}	0.002993	0.005865	0.008746	0.010817	0.010644	0.010782	0.018610	0.007170
T_{90}	0.002852	0.004786	0.007919	0.009549	0.009351	0.009570	0.020360	0.005454
S.e. - Germination Rate								
T_{10}	0.000169	0.000463	0.000743	0.000816	0.000751	0.001390	0.011300	0.003180
T_{25}	0.000266	0.000411	0.000652	0.000734	0.000625	0.000871	0.006140	0.001340
T_{40}	0.000406	0.000494	0.000719	0.000814	0.000688	0.000823	0.007890	0.001030
T_{50}_1	0.000681	0.000434	0.000654	0.000728	0.000640	0.000684	0.006220	0.000692
T_{50}_2	*	*	*	*	*	*	*	*
T_{60}	0.000733	0.000391	0.000631	0.000706	0.000614	0.000643	0.004360	0.001220
T_{75}	0.000869	0.000383	0.000709	0.000772	0.000675	0.000674	0.003450	0.001140
T_{90}	0.000799	0.000269	0.000601	0.000628	0.000541	0.000541	0.005420	0.000627