Epidemiology of *Sclerotinia sclerotiorum,* causal agent of Sclerotinia Stem Rot, on SE US *Brassica carinata*

by

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ABSTRACT

Brassica carinata is a non-food oil seed crop currently being introduced to the Southeast US as a winter crop. *Sclerotinia sclerotiorum*, causal agent of Sclerotinia Stem Rot (SSR) in oilseed brassicas, is the disease of most concern to SE US winter carinata, having the potential to reduce yield and ultimately farm-gate income. A disease management plan is currently in development which will include the use of a disease forecasting system. Implementation of a disease forecasting system necessitates determining the optimal environmental conditions for various life-stages of SE US isolates of this pathogen for use in validating or modifying such a system. This is because temperature requirements and optimums for disease onset and subsequent development may vary depending on the geographical origin of *S. sclerotiorum* isolates. We investigated the optimal conditions necessary for three life-stages; conditioning of sclerotia, germination of apothecia, and ascospore infection of dehiscent carinata petals. Multiple isolates of *S. sclerotiorum* were tested, collected from winter carinata or canola grown in the SE US.

In the conditioning test sclerotia of 4 isolates (HA, FH, TI, and BT) were subjected to 4 temperatures (15/3^{o}C, 18/6^{o}C, 21/9^{o}C day/night, and 12^{o}C constant), 3 moisture frequencies (moisture event every 1, 3, or 6 days), and 3 conditioning durations (4, 6, and 8 weeks). We found high variability amongst isolates in their response to temperature during conditioning and that a moisture event every day resulted in significantly less germination of sclerotia for most isolates. In the apothecia germination test we subjected uniformly conditioned sclerotia of 4 isolates (BR, BT, QC, and TI) to 3 light levels and 2 temperatures (18/6°C and 26/11°C) where we found a decrease in cumulative apothecia as light level increased and no significant difference between temperatures but significant differences between isolates with respect to

apothecia accumulation after a 4 week germination period. In the petal infection test ascospores of two isolates (FH and TI) inoculated onto carinata petals were subjected to 4 temperatures (15/3°C, 18/6°C, 21/9°C, and 26/11°C) and 4 humidity levels (about 97, 94, 84, and 74% RH). We found that infection increased as temperature and as RH increased, with no infection occurring below 84% RH at any temperature and no significant difference between isolates in terms of proportion of infection to non-infection of petals.

A management plan is currently being developed for controlling SSR in Southeastern US winter carinata which will include fungicide recommendations. An *in vitro* fungicide assay was done in order to supplement fungicide field trials in developing management recommendations for growers. Six fungicides were tested: chlorothalonil, boscalid [Endura®], pyraclostrobin [Headline®], fluxapyroxad + pyraclostrobin [Priaxor®], flutriafol [Topguard®], and metconazole + pyraclostrobin [Twinline®]. These fungicides were tested at varying rates, based on maximum recommended label rate, at 0.02, 0.05, 0.1, 0.5, and 1.0x, on three isolates (BR, FH, and TI) of *S. sclerotiorum* from the Southeast US. Significant differences among isolates' growth, relative to their sensitivity to fungicides ($P < 0.001$) were noted, though these differences in growth were only seen with less than 0.5x maximum recommended label rate, with growth on concentrations above 0.5x being similar. Only flutriafol, a demethylation inhibitor (DMI), and boscalid, a succinate dehydrogenase inhibitor (SDHI), were unable to prevent growth of any isolate regardless of fungicide concentration. This suggests that growers should not use DMI's or SDHI's to control SSR on winter carinata unless in combination with another fungicide chemistry.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

SSR	Sclerotinia Stem Rot
MRLR	Maximum Recommended Label Rate
SPARC	Southeast Partnership for Advanced Renewables from Carinata
NFOS	Non-Food Oil Seed
US	United States
USDA	United States Department of Agriculture
NASS	National Agricultural Statistics Service
PPFD	Photosynthetic Photon Flux Density
QoI	Quinone outside Inhibitor
SDHI	Succinate Dehydrogenase Inhibitor
MBC	Methyl Benzimidazole Carbamates
PPI	Percent petal Infection
DI	Disease Incidence
MPPI	Mean Percent Petal Infection
MDI	Mean Disease Incidence
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction

I. LITERATURE REVIEW

Carinata is a mustard that is grown as a Non-Food Oil Seed (NFOS); currently this crop is being commercially grown in the great plains of Canada along with Uruguay and Argentina and is being introduced to the Southeast US as a winter cash crop. In the Southeast US, the only winter cash crop commonly planted is winter wheat and planted acres fluctuate with selling price. For example, in Alabama when wheat price was about $7.25 per bu, 310,000 acres were planted in 2013 and this went down to 150,000 acres planted in 2017 when the price was $4.25 (USDA-NASS, 2018). With carinata's higher selling point, $10.50 per bu (Jeff Klingenberg, personal communication), and expected yields of 60 bu per acre, carinata may be a profitable alternative to winter wheat or fallow. Partly due to the decrease in winter wheat price, an estimated 1 million acres of winter fallow in the Southeast US, with at least 150,000 acres fallow in Alabama, is suitable for carinata production. Leaving acreage fallow during the winter increases the risk of erosion, but carinata would not only prevent erosion but would also improve soil health (Seepaul et al., 2016). Carinata will also provide farmers with income they may otherwise not have from those fields with the added benefit of not displacing a food crop.

The oil from carinata is considered highly suitable for the biofuel industry and can be easily refined into jet fuel along with a feedstock for other fuels and value-added organic molecules (Seepaul et al., 2016). This can help supply the demand the government has for biofuels, for example the US Navy has set a goal to transition to fuel use to 50% biofuel by 2020. However, carinata has never been grown in the Southeast US and there will be challenges in terms of how new diseases and a different climate will affect crop growth and yield potential. To help overcome these obstacles, a disease management plan for Southeastern carinata is needed.

Possibly the most economically damaging disease known to affect oil seed brassicas is Sclerotinia stem rot (SSR), caused by *Sclerotinia sclerotiorum*. However, little is known about the epidemiology or impact of this disease on brassica crops in the Southeast. An attempt to introduce canola to this region was made in the 1990's but failed due to a severe outbreak of SSR (Hagan, personal communication, 2017).

S. sclerotiorum is a necrotrophic plant pathogen that causes disease on over 400 plant species around the world (Boland and Hall, 1994), including many agriculturally important crops such as canola, sunflower, carrot, pea, peanut and lettuce. This pathogen has the potential to be devastating to carinata growth in the Southeastern US. The following literature review will be a summary of work that has been done on this pathogen in terms of understanding its biology, epidemiology, availability of resistant cultivars, and disease management strategies.

The fungus, *S. sclerotiorum*, is in the phylum Ascomycota, class Discomycetes, order Heliotales, family Sclerotiniaceae, and genus *Sclerotinia* (Bolton et al., 2006). This fungus is characterized *in-vitro* by hyaline, septate, branched, multinucleate hyphae. Mycelia appear white to tan in culture and is accompanied by the production of sclerotia which are survival structures and necessary for sexual reproduction. For plants infected with *S. sclerotiorum*, sclerotia usually form either inside plant tissue (i.e. in the pith of stems) or on stem lesions under high humidity (Bolton et al., 2006). Sclerotia can also form on flower or seed tissues and because of this can be found in harvested samples of crops, such as soybean (Bolton et al., 2006). Germination of sclerotia can occur after a dormant period either "myceliogenically" or "carpogenically" depending on environmental conditions (Bolton et al., 2006). A "conditioning" period of cool temperatures is often needed to break sclerotia out of dormancy. Carpogenic germination is the

formation of apothecia and production of ascospores, which are the main source of infection in most crops (Abawi and Grogan, 1979; Schwartz and Steadman, 1978). Though there is no production of asexual conidia, micro-conidia are present on hyphae; these micro-conidia do not germinate, however, and their function is not known.

Since *S. sclerotiorum* has such a broad host range, there are no unique symptoms attributed to this pathogen, diseases of which include a cottony soft rot of cabbage and carrots, stem rot in oilseeds and sunflowers, white mold in bean pods, damping off of young plants, and cottony soft rot of stored fruits (Bolton et al., 2006). In general, infection starts with the germination of an ascospore on a leaf or petiole. A water-soaked lesion enlarges down the petiole into the stem; water-soaked lesions will appear there as well. These lesions then become grey or bleached in color and the vascular tissue becomes shredded. Development of sclerotia occurs within and on the symptomatic plant tissue. This process often results in the girdling of the stem and lodging of the plant (Bolton et al., 2006).

Sclerotia are the defining structure of this pathogen and its namesake. *Sclerotinia sclerotiorum* is a model organism for the study of sclerotia as this fungus is easily grown in culture, the genome has been fully sequenced, and diseases caused by it have a high economic impact worldwide with annual loss estimates having exceeded $200 million in the US alone (Suszkiw, 2007). Sclerotia development occurs in three macroscopic phases: initiation, development, and maturation. During initiation, hyphae aggregate into a distinct mass referred to as a sclerotial initial. Development then occurs, characterized by further hyphal aggregation with an increase in size of the sclerotial initial. Lastly, during maturation, the sclerotia develop an outer rind which undergoes surface delimitation and melanization (Bolton et al., 2006). Sclerotial development usually occurs when mycelia encounter a nutrient limited environment. Oxalic acid

is involved in sclerotiogenesis which reduces ambient pH and sclerotia more readily develop in cultures with lower pH (Rollins, 2003). In fact, mutants that lack the ability to produce oxalic acid also lack the ability to produce sclerotia *in vitro* as well as being non-pathogenic *in planta* (Dickman and Mitra, 1992; Godoy et al., 1990). The mechanism behind sclerotiogenesis involves the Ras/MAPK pathway which is negatively regulated by Rap-1 in a PKA-independent cAMP signaling pathway (Bolton et al., 2006).

Sclerotia are vital in the disease cycle of *S. sclerotiorum.* They are the source of reproductive structures, inoculum, and allow long term survival in soil. Germination of sclerotia may begin an epidemic whether that germination be myceliogenic (i.e., mycelial growth) or carpogenic (i.e., apothecial production), though in most pathosystems, epidemics are driven by carpogenic germination and subsequent release of ascospores. Sclerotia that germinate myceliogenically can directly attack plant tissues (Bardin and Huang, 2001), whereas carpogenic germination creates apothecia, which forcibly release ascospores to infect above ground tissues. The most important environmental factors for carpogenic germination are temperature and moisture, but also the temperature at which sclerotia are produced.

Temperature during the production of sclerotia (in addition to the conditioning of sclerotia) is an important factor affecting carpogenic germination (Huang and Kozub, 1991). Huang and Kozub (1991) compared the carpogenic germination of sclerotia subjected to different temperatures during formation of sclerotia from 20 different isolates from around the world and different climates. Each isolate was subjected to either 10˚C or 25 ˚C during sclerotia formation and then those sclerotia were tested for germination with or without a conditioning period. Differences in scleortial germination between isolates was related to their geographical origin, e.g., sclerotia of isolates from warmer climates would not germinate when produced at

10˚C. In addition, most isolates were not able to germinate without a conditioning period regardless of the temperature at which sclerotia were produced. There was an exception in an isolate from Hawaii, which has a tropical climate where average temperatures stay between 23-27˚C year-round, that was able to germinate with no conditioning period of cool temperatures. Reported differences in optimal growth conditions noted by researchers working in different climates is likely due to differences between the geographical origins of the isolates used in those studies (Abawi and Grogan, 1979; Clarkson et al., 2007). It follows that the optimal conditions of other stages in the lifecycle (e.g., ascospore release, survival, infection) would also differ based on geographical origins of isolates.

Apothecia are often produced once the crop canopy closes as it coincides with shading and high soil moisture levels, which is conducive for carpogenic germination (Bolton et al., 2006). Apothecia have a receptacle which is flat to concave in shape with a hymenial layer on the upper surface. This cup or disc is born on a stipe that is connected to the sclerotia. Asci are produced in the hymenial layer in rows and are sac-like zygote cells which contain eight hyaline, ellipsoid, binucleate ascospores with dimensions of 4-6 x 9-14µm (Kohn, 1979).

Light, temperature, and moisture are the three environmental factors most important in the production of apothecia in *Sclerotinia sclerotiorum* (Abawi and Grogan, 1979; Letham, 1975). Sun and Yang (2000) sought to quantify the effect of these three environmental factors on apothecia development and analyzed three different variables in their study: total apothecia, total initials, and cumulative apothecia by cumulative degree days. For their isolates, collected from soybean fields in Iowa, they found an interaction between light intensity and temperature as well as moisture in the following way: in general, first apothecia appeared sooner at higher

temperatures but only at low light intensity. At low light intensity, the optimal temperature for apothecial development was 12-18°C regardless of moisture level, while at high light intensity the optimal temperature was shifted to 20°C when the moisture level was high. The degree day concept was also used as a continuous variable to compare against cumulative apothecia, where there was a difference when apothecia began to develop and when development ceased depending on the light intensity. At low light intensity, apothecia began to develop at 760 degree days and continued throughout the experiment which ended at 1720 degree days; at high light intensity, development began at 160 degree days and extended to 900. The stated levels of light intensity were 120-130 (high) and 80-90 (low) μmol m^2/s (a measure of photosynthetic photon flux density or PPFD). Sun and Yang's study (2000) on the effects of light, temperature, and moisture showed that there was an interaction between light intensity and temperature, as well as light intensity and moisture level in relation to apothecial development.

Ascospores are forcibly discharged from asci upon changes in humidity or mechanical disturbance of the apothecia (Hartill and Underhill, 1976). Discharge often happens in a "puff", where vast quantities of ascospores are simultaneously released. This can happen continuously for more than 10 days under optimum conditions and at a rate of 1600 spores/h (Clarkson et al., 2003). Though a majority of ascospores remain within the vicinity of their source point, travelling from as little as 2 to 20m (Wegulo et al., 2000), some can be picked up in turbulent wind conditions to travel several kilometers away from the source point (Li et al., 1994). Ascospores are covered in a sticky mucilage which helps them stick to substrates and can survive on plant tissues for up to 2 weeks depending on the environmental conditions, although high humidity, temperature and UV light will shorten their period of survivability (Clarkson et al.,

2003). In lab conditions, ascospores can survive on membrane filters for 24 months refrigerated or frozen in a sealed container with desiccant and retain 90% viability. These dried ascospores, the ones that remain viable, have no reduced efficacy in comparison to fresh ascospores in their ability to infect beans (*Phaseolus vulgaris*) (Hunter et al., 1981). This provides a convenient method of storing ascospores for later use as inoculum in a lab setting, such as testing fungicide efficacy.

Ascospores are unable to directly infect healthy plant tissue, requiring an exogenous nutrient source to initiate an infection (Bolton et al., 2006). Senescent material like fallen flower petals, as well as stamens and pollen, can be utilized as this nutrient source (Bardin and Huang, 2001). Epidemiologically this is convenient since the time when these nutrient sources become available is around the time of canopy closure, when the environment is conducive to apothecial germination and subsequent ascospore release.

Sclerotia can also germinate myceliogenically and this is important in several crops such as sunflower and carrot (Bardin and Huang, 2001; Holley and Nelson, 1986). Germination of this type is influenced by humidity and temperature (Huang et al., 1998). Mycelia produced directly attack plant tissue at and below the soil surface. In sunflower, infection begins in the roots and progresses up through the stem eventually reaching the raceme and causing head blight. A single large sclerotium can be produced on the sunflower head in the shape of a disc many centimeters in diameter and less than a centimeter thick (Bolton et al., 2006).

The mycelia of *S. sclerotiorum* can directly penetrate plant tissue using hydrolytic enzymes and mechanical pressure of appressoria; mycelium can also enter through stomata or wounds and avoid the need for penetration of epidermal cells. Furthermore, oxalic acid has been shown to deregulate stomatal guard cells and is produced in host tissues in advance of hyphae

(Guimaraes and Stotz, 2004). In addition, oxalic acid has been shown to commandeer the plants hypersensitive response and cause apoptosis, with plant cells dying several cells in advance of hyphae. Hyphae can also exit stomata to begin secondary infections and produce sclerotia on the host surface.

Del Rio et al. (2007) investigated the relationship between SSR incidence and yield loss in canola in North Dakota. For every percent increase of disease incidence there was an average decrease of 0.52% in yield. Percent incidence was quantified as number of diseased plants out of 50 from the center of research plots. At that time, this corresponded to a monetary loss of $3.06/ha for each percent increase in SSR incidence. Del Rio (2007) points out that, interestingly, this reported loss in yield is very similar to that reported by Morral et al. (1984) at 0.4-0.5%, 25 years earlier. Similar yield losses recorded for both studies are an indication of how little progress has been made in developing canola cultivars resistant to SSR over this 25-year period.

Disease caused by *S. sclerotiorum* has been historically difficult to manage due to the persistent nature of this pathogen and the general lack of resistance in target crops. However, partial resistance is available and, in the case of soybean, still provides economically significant control (Boland and Hall, 1987). Field screening for resistant cultivars has been historically difficult because of the sporadic nature of weather patterns favoring ascospore release and subsequent disease development, which creates uncertainty about whether the crop is exhibiting resistance via escape mechanisms (like altered flowering or canopy closure time) or if it is physiologically resistant. Therefore, realistic greenhouse studies are critical in screening for resistance. Kolkman and Kelly (2000) developed a greenhouse screening procedure using

oxalate, an important pathogenicity factor of *S. sclerotiorum*, to indirectly determine physiological resistance in common bean cultivars.

Uloth et al. (2015b) investigated the relative resistance to SSR of current and historical varieties of oilseed *Brassicas* in Australia. In Australia, SSR is a devastating disease reportedly causing yield losses of AU$59 million in Western Australia alone (Khangura et al., 2014) and yield losses up to 24% across all of Australia (Hind-Lanoiselet et al., 2008). Further-more, current management of SSR is cultural (e.g. crop rotation) and chemical based but such practices are not always effective and may be cost prohibitive due to the expense of spraying in Australian cropping systems.

Uloth et al. (2015a) tested the resistance of fifty-five cultivars of *Brassica napus* and *B. juncea*. The release year of these varieties varied from 1992 to 2013. Resistance was tested by affixing a mycelial mat of *S. sclerotiorum* onto stems of young plants and measuring the length of the lesion that developed after 72h. Mystic, a cultivar of *B. napus*, was found to have a high level of resistance, developing only a 3mm lesion as compared to a mustard cultivar which had the highest level of susceptibility on which developed a lesion 202.6mm in length. Overall, the average lesion length was around 50mm and there was no correlation between cultivar release date and disease resistance. For example, 'Mystic', which was developed in 1998, had high resistance but three of the newer *B. juncea* cultivars in this study (released in 2009 and 2013, and an experimental variety) proved highly susceptible. Study results show that minimal progress has been made in the development of disease resistance during the two decades of cultivar development looked at by this test.

Histological assessment of the cultivar Mystic showed that resistance is likely due to a high amount of lignin deposition in stem cortical cells, which forms a barrier to advancing *S.*

sclerotiorum hyphae. High lignin deposition in cortical cells has also been found in canola cultivars resistant to the black leg pathogen, *Leptosphaeria maculans* (Li et al., 2007). Recognition of this means of resistance may be useful in future breeding programs for other oilseed brassicas.

The lack of resistance makes the management of SSR more reliant on fungicide inputs, so choosing an efficacious fungicide is critical to protecting carinata from significant yield loss. Bradley et al. (2006) conducted field trials in North Dakota between 2002 and 2004 to determine the efficacy of foliar fungicides on SSR and canola yield. Registered fungicide active ingredients for control of SSR in canola in the US at this time were azoxystrobin, boscalid, thiophanate-methyl, and vinclozolin. Each of these fungicides represents a specific chemical group: quinone outside inhibitors (QoI), succinate dehydrogenase inhibitors (SDHI), methyl benzimidazole carbamates (MBC), and dicarboximides, respectively. All of these fungicides were effective in reducing disease (measured as percent disease incidence) when applied at label rates, but not all increased yield. Several non-registered fungicides provided significant control of SSR compared to a non-treated control: iprodione (dicarboximide), prothioconazole (a demethylation inhibitor fungicide (DMI)), tebuconazole (DMI), and trifloxystrobin (QoI). At the time of this study no fungicide resistant populations of *S. sclerotiorum* had been reported in North Dakota. However, resistant populations to the MBC fungicide benomyl had been reported in Canada (Gossen et al., 2001). Recently, *S. sclerotiorum* on rapeseed has developed resistance to fungicide chemistries in groups 2 (dicarboximides) and 12 (phenylpyrroles) in China (Duan et al., 2014; Zhou et al., 2014). This emphasizes the need to rotate fungicide chemical groups, or apply mixtures, to reduce the chance of resistance developing in *S. sclerotiorum* populations. Fungicide timing was also part of Bradley and Lamey's (2006) study with the timing of these fungicide applications

varied from 10-60% bloom in 10% increments. Results showed that no single timing was best; some years the timing did not play a role in disease control and in other years it did, likely due to environmental variables affecting the timing of inoculum presence. This is where disease forecasting systems can aid in predicting inoculum presence and help with correctly timing a fungicide application.

A review of available forecasting models for *S. sclerotiorum* diseases was done by Rothman and McLaren (2018) to select the most appropriate for use in South Africa. The types of forecasting systems evaluated were risk point systems, prediction models based on petal infestation, prediction models based on carpogenic germination, probability regression models, and crop-loss-related forecasting models for canola, dry bean, soybean, and lettuce. Management of SSR on canola in Europe has traditionally relied on preventative fungicide sprays at full bloom, in part because resistant cultivars are not available (Dunker and Tiedemann, 2004). However, field experiments conducted between 1984 and 2004 showed that these preventative sprays were only cost-effective 27-33% of the time (Dunker and Tiedmann, 2004). The introduction of risk assessment systems for *S. sclerotiorum* diseases has substantially increased the efficacy of fungicide sprays (Gugel and Morrall, 1986). These models vary in complexity from the relatively simple (e.g., risk point systems) to the more complex (e.g., crop-loss-related forecasting models).

Risk point systems use a survey where answers are allocated weighted points; these points are summed, and a management recommendation is given based on the total points. These questions can cover a variety of areas from agronomic characteristics of the crop to regional disease risk. Risk point systems have been developed for *Sclerotinina* diseases of canola in

Canada (Thomas, 1984), Germany (Ahlers, 1989), and Denmark (Jakobsen, 1991). The risk point system developed by Ahlers (1989) had a prediction accuracy of 60% with an overestimation of disease, which results in unnecessary fungicide applications. A similar risk point system was developed in Sweden for spring sown canola by Twengstrom et al. (1998) using field-specific weather parameters and agricultural production practices, years since last crop, disease in last host crop, crop density, precipitation in last 2 weeks, forecasted impact of weather, and regional risk of disease. Each of these parameters is allocated a risk point value based on field evaluations of how they contribute to disease risk. Point allocations for factors in this model were determined from data of 800 fields over a 10-year period. Evaluation of this system over a two-year period by Twengstrom et al. (1998) for accuracy determined that a management recommendation to spray was correct in 75% of fields. However, a 16% error rate where a recommendation to spray was made when not required was also noted. The relative simplicity of risk point models makes them easy to implement for growers, providing cost effective management (Twengstrom et al., 1998).

Gugel and Morrall (1986) developed a prediction model based on percent petal infestation (PPI) in canola. A relationship between PPI by ascospores and disease incidence (DI) in early bloom canola was established; however, the R^2 values for their models were relatively low. Ascospores were found infecting petals even when no apothecia were observed, suggesting that extrinsically produced inoculum was being recorded by this system (Gugel and Morrall, 1986; Morrall and Duek, 1982). Using this relationship between PPI and DI, they determined that a mean percent petal infestation (MPPI) below 45% was low risk and corresponded with less than 20% mean disease incidence (MDI). Moderate risk was between 45-90% MPPI (relating to 20-40% MDI), and high risk was over 90% MPPI (relating to over 40% MDI). This system was

re-evaluated by Turkington et al. (1991) and they found that crop canopy density and timing of flowering were useful variables in determining MPPI and MDI. Canopy density was classified as light, moderate, or heavy and inclusion of this variable in the model correctly forecast disease risk at 73% accuracy over a six-year period. The accuracy was best below 45% disease risk; however. Initial studies to determine petal infestation incidence relied on incubating petals on growth medium, a method which was time consuming (Morrall et al., 1989; Morton and Hall, 1989). Recently, the availability of serological (Jamaux and Spire, 1994; Lefol and Morrall, 1996) and PCR (Almquist & Wallenhammar, 2015; Freeman et al., 2002; Jones et al., 2015; Qin et al., 2011; Rogers et al., 2009; Yin et al., 2009) methods have allowed the detection and quantification of pathogens in a timely manner. The PPI model developed by Gugel and Morrall (1986) and modified by Turkington and Morall (1993) is a practical model which farmers or consultants can implement as *Sclerotinia* selective PDA kits are provided (Rothmann and McLaren, 2018). The practicality of such models can be further improved as serological and PCR tests are made available for growers, reducing the time it takes to confirm pathogen presence.

A prediction model based on carpogenic germination using independent variables of soil temperature and soil water potential was developed by Clarkson et al. (2007) for lettuce leaf drop disease (*S. sclerotiorum*) in North Dakota. This model assumes that sclerotia need a conditioning period to germinate; the more conducive temperatures are for sclerotia conditioning the higher the carpogenic germination, and thus higher disease risk (Clarkson et al., 2004). Two models were developed by Clarkson et al. (2007) which predicted germination of sclerotia, one of which was based on an exponential equation and the other an Arrhenius equation (which is used for temperature dependent reactions in physical chemistry). These models accurately predicted 80%

of germination or non-germination out of 64 sites studied (Clarkson et al., 2007). However, the models are not yet complete and there currently are no associated management recommendations. Nevertheless, Clarkson et al. (2007) believes that predicting the initial inoculum present in the field will be an effective means of forecasting lettuce leaf drop disease.

Mila et al. (2004) used probability regression models to calculate the prevalence and incidence of SSR on soybean. Prevalence was calculated with a logistic, and incidence with a Poisson regression model. Prevalence was described as percent infected fields in a region, and incidence as percent infected plants in a field. Logistic regression models are applied to qualitative outcomes, e.g., disease or no disease, and Poisson regression models are used for the study of rare events when the outcome takes the form of counts (Rothmann and McLaren, 2018). In the 1,545 fields sampled, only 85 had presence of SSR; thus, disease can be considered a rare event (Mila et al., 2004). The input variables used for these models were soil conditions, agronomic practices, and weather variables (i.e. temperature and rainfall). Tillage practices (conservation, minimum, conventional tillage) and soil texture were also recorded. The logistic prevalence model was split into two parts. Model 1 dealt with mid-western US spring conditions and model 2 dealt with summer conditions. These logistic analysis models were used to identify significant relationships between environmental and disease variables. Strong R^2 values were reported for both models indicating good predictive accuracy with $R^2 = 0.65$ and $R^2 = 0.71$ for model I and model II, respectively. According to Mila et al. (2004), model II would have better statistical reliability for predicting disease incidence on a regional scale based on the significant positive correlation between spring and summer temperatures with disease incidence. In contrast the R^2 values for the Poisson regression model were low, $R^2 = 0.065$. Speculation on the authors' part was that field specific information was not sufficiently integrated into the model, i.e.

selected cultivars may have differed in susceptibility, microclimate variables may not have been quantified, and ascospore inoculum was not quantified. The logistic regression method described was repeated by Harikrishnan and Del Rio (2008) to develop a probability model for white mold of dry bean in North Dakota. In this study, 250 fields were surveyed from 2003 to 2005 and included site specific weather data (collected from weather stations a maximum of 12.5 miles from fields). Data for weather parameters in the model were collected from May to August and included monthly total precipitation, number of rainy days, and mean minimum air temperature. With a spray threshold at greater than 20% disease incidence, the final model had a sensitivity of 97%, specificity of 85%, and R^2=0.85 associated with the overall accuracy of the model. This model was limited in accuracy when disease incidence was less than 20%; however, when disease incidence was above 20% it was very accurate in terms of a justifiable spray based on late season incidence (Rothmann and McLaren, 2018).

Koch et al. (2007) developed a crop-loss-related forecasting model for winter canola in Germany. Known as SkleroPro, this model has been adopted by many countries in Europe (Rothmann and McLaren, 2018). This model involved interactions between disease incidence and agronomic practices, and between disease incidence and the fluctuating price of the crop in terms of yield gains and fungicide application costs (Koch et al., 2007). The SkleroPro model runs from growth stage (GS) 55 (mid-bud stage) to GS68 (end of flowering, initiation of pod formation) at which point it would be too late for the possibility of yield gain from fungicide inputs (Dunker and von Tiedemann, 2004). Collected from the nearest weather station, variables for weather were hourly air temperature, precipitation, relative humidity, and sunshine hours. These data were used to calculate the temperature and relative humidity within the crop canopy. The date of bud stage (GS55) and crop rotation information need to be supplied by growers for

SkleroPro to give site specific recommendations. The crop rotation cycle (2, 3, or greater than 3 years) was pivotal for SkleroPro to make recommendations as it was determined to be the best site-specific variable influencing disease. After GS55, a crop-development-stage model is used to predict the onset of late bud stage (GS58) at which point a regional risk model is initiated. The prediction of late bud stage is meant to indicate that there is senescing tissue available for ascospores of *S. sclerotiorum* to colonize and use to infect the crop. At this point the model records the accumulation of hours during which environmental conditions (temperature, relative humidity) are conducive to disease, termed infection hours (Inh). Disease development was conducive at a lower threshold of 7-11 $^{\circ}$C with at least 86-80% RH (i.e. there was an interaction between temperature and RH where the temperature threshold decreased as RH increased), though optimal at 18°C. If conditions for disease were determined to be conducive for 23 hours after the critical growth stage, a regional disease risk was assumed and those infection hours after the 23h threshold are accumulated (InhSUM). Site specific information is then used to determine the economic damage threshold (Inh_i), which includes expected yield, commodity price, and fungicide application costs. If the value for Inh_i becomes greater than the value for InhSUM a recommendation to spray is delivered to the grower. A retrospective analysis of the SkleroPro model by Koch et al. (2007) determined an accuracy of 70%, with 24% of predictions overestimating DI and 6% underestimating DI. Compared to routine sprays (5 per season), which are economic 53% of the time, SkleroPro would have reduced unnecessary fungicide sprays by 39% if it had been implemented commercially. This model proved to be an effective crop-loss-related forecasting system with field, site and time specificity in terms of fungicide recommendations (Rothmann and McLaren, 2018).

Disease forecasting systems can have many variables, especially those that predict critical inoculum potential. Such systems may include very specific information such as pathogen isolate as well as environmental factors (air and soil temperatures, precipitation, etc.) (Rothmann and McLaren, 2018). It is important to consider the number of variables required for a model to work, which often relates to an increase in disease prediction accuracy, and what is practical to implement in the field. Many of these prediction models have helped reduce fungicide inputs while maintaining effective disease control, which helps in mitigating environmental damages as well as reducing risk of fungicide resistance. However, due to the sporadic nature of *Sclerotinia* diseases and the temperature adaptation of this fungus (Uloth et al., 2015b), these models often lose predictive accuracy when implemented in a different location (i.e., places with different climates and populations of *S. sclerotiorum*).

Recently, detection and quantification of inoculum has been used in aiding disease management systems. Many of these techniques use real-time PCR to quantify the amount of inoculum in environmental samples such as soil, petals and leaves, and in the air. There have even been a few real-time PCR assays developed for use in quantifying *S. sclerotiorum* diseases (Almquist and Wallenhammar, 2015; Rogers et al., 2009; Yin et al., 2009). Of these, the system developed by Rogers et al. (2009), which used a SYBR green reporter dye, was the most sensitive, being able to detect as few as 2 ascospores in air samples. It was also extremely specific to DNA of *S. sclerotiorum*, while other assays would also quantify DNA of closely related *Sclerotinia* species.

Almquist and Wallenhammar (2015) developed a real-time PCR assay for use in detection of *S. sclerotiorum* inoculum by adding a taqman probe for the primer set made by

Freeman et al. (2002). This RT-PCR assay was able to detect a minimum of 5 copies of target sequence in environmental samples such as petals, leaves, and in the air. Compared to the primer set alone that was developed by Freeman et al. (2002), the RT-PCR assay was twice as sensitive in detecting *S. sclerotiorum* DNA. Almquist and Wallenhammar (2015) used this assay in five field experiments to measure the development of SSR in canola from 2008 to 2010 in Sweden. In these experiments, petals and leaves were tested for presence of *S. sclerotiorum* DNA and airborne inoculum was quantified using a Burkard 7-day sampler. At one of their experimental sites they found no spores on petals, and spore release did not coincide with flowering, but spores were detected on leaves during flowering, with a corresponding 7% disease incidence. This suggests that it may be beneficial to sample leaves in such situations to predict disease incidence, or keep in mind that ascospore release from weeks earlier (before flowering) could still be viable inoculum on leaf tissues.

Rogers et al. (2009) developed a qPCR assay which amplified a region within the mitochondrial small subunit rRNA intron and ORF1 gene. The amplicon was 125bp and specific to *S. sclerotiorum* DNA in qPCR. This qPCR used the fluorescent based SYBR green system. Three seasons of different SSR severity on canola in England were monitored using this assay between 2003 and 2007. There was a severe outbreak of SSR in the 2007 season, but the 2003 and 2004 seasons had low incidence of SSR (Rogers et al., 2009). Interestingly, rainfall was not associated with ascospore presence during the severe 2007 season. In addition, severe disease was reported across the region, suggesting that inoculum from outside sources can play a major part in epidemics of SSR.

This primer set developed by Rogers et al. (2009) was the first to be used to quantify DNA of *S. sclerotiorum* in environmental samples using qPCR. Compared to the PCR assay

developed by Freeman et al. (2002), Rogers' assay was more sensitive. Being able to detect the equivalent of 1.4 ascospores worth of DNA. The sensitivity of this test makes it potentially useful in detecting low concentrations of inoculum in the air, because consistently low numbers of spores over a long enough period may cause an SSR epidemic and some PCR assays may not be sensitive enough to detect this build-up of inoculum.

The epidemiology of *S. sclerotiorum* differs depending on the geographical location of isolates. Because of this, disease management studies developed in certain locations may not work in others. This makes it necessary to validate previous work done when studying isolates from geographical regions without previous research. As such, this study will seek to determine the optimal conditions for *S. sclerotiorum* growth of isolates from the Southeast US. These optimal conditions will be determined using modified methods used in studies such as Sun and Yang (2000), Koch et al. (2007), and others. In addition, we will use real-time PCR to determine inoculum concentration and relate this to disease incidence and yield loss.

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II. DETERMINING OPTIMALL ENVIRONMENTAL PARAMETERS OF VARIOUS LIFE STAGES OF SE US ISOLATES OF *SCLEROTINIA SCLEROTIORUM*

Introduction

Brassica carinata is a cash crop being introduced to the Southeast US as a winter Non-Food Oil Seed (NFOS). Carinata has the potential to fit well in a double cropping system with summer crops, particularly soybean. Products from carinata include bio-jet fuels, bio-lubricants, and high protein seed meal for the beef cattle industry (Seepaul et al., 2016). Much of the farmable land in the Southeast remains fallow during the winter due to a lack of profitable winter crop options for growers. With carinata, growers could have a winter cash crop otherwise unavailable to them.

Sclerotinia sclerotiorum, known as white mold, causes Sclerotinia Stem Rot (SSR) in brassica oilseed crops (Boland and Hall, 1994; Bolton et al., 2006). In Western Australia, yield losses due to SSR have been estimated to reach $56 million in brassica oilseed crops (canola and carinata), and an estimated Australia-wide yearly yield loss reaching up to 24% (Hind-Lanoiselet et al., 2008; Khangura et al., 2014). In Canada, yield losses due to SSR have been reported between 11-15% in Saskatchewan summer canola (Morral et al., 1984). In North Dakota, average disease incidence in canola has been reported at 13.6% (Bradley et al., 2006). Due to this, SSR has the potential to impede the successful introduction of winter carinata to the SE US.

Sclerotinia sclerotiorum has a complex lifecycle. Apothecia form from survival structures called sclerotia and produce ascospores. Ascospores are the driving force behind epidemics of SSR in brassica oilseeds like rapeseed, to which carinata is closely related (Gugel

and Morrell, 1986). Sclerotia need conducive environmental conditions to produce apothecia and are only able to after being conditioned (Bolton et al., 2006). Sclerotia typically are conditioned by a period of cool temperatures, with optimal conditioning temperatures varying depending on the geographical origin of *S. sclerotiorum* isolates (Bolton et al., 2006; Huang and Kozub, 1991). In addition, optimal temperatures for infection of Australian carinata has been shown to differ between Northern and Southern Australian isolates of *S. sclerotiorum* (Uloth et al., 2016). This makes it necessary to validate the preferred environmental conditions of local isolates when implementing a disease forecasting system that was developed in a different location. This study aims to determine the preferred environmental conditions necessary for various life stages of SE US isolates of *S. sclerotiorum* in order to validate or modify an existing disease forecasting system. The life stages investigated in this study are conditioning of sclerotia, carpogenic germination of sclerotia, and ascospore infection of dehiscent carinata petals.

Materials and Methods

Conditioning of sclerotia

Three factors that influence conditioning of sclerotia were tested in this experiment (temperature, moisture frequency, and conditioning duration). Four temperature regimes were established at 15/3^{o}C, 18/6^{o}C, and 21/9^{o}C day/night (reflecting cold, average, and warm temperatures for a South Alabama winter), and a 12^{o}C constant. Three moisture frequency regimes were established by leaching conditioning sclerotia with 10cc of deionized water either every day, every three days, or every six days. Three conditioning durations of 4, 6, or 8 weeks were tested. With access to two growth chambers, each experimental replication was done in two parts (with two of the four temperature treatments). This experiment was done as a full factorial

design with two replicates (10 sclerotia per replicate) and the experiment was repeated once with data being pooled for analysis.

Four isolates of *S. sclerotiorum* (HA, FH, TI, and BT), collected from SE US grown carinata or canola, were used in this experiment (Table 1.). Sclerotia of each isolate were generated and harvested from sterile wheat grain as described by Pethybridge et al. (2015). Sclerotia were separated from wheat and sorted by size using sieves (no. 4, 6, and 20 sieves), with those sclerotia captured between the size 4 and size 6 sieves (4.5mm and 3.35mm apertures, respectively) used. Thirty of these similarly sized sclerotia were shallowly buried in pots of sterile sand such that each pot contained 30 sclerotia, which were placed into growth chambers and subjected to the previously mentioned conditions. After the appropriate conditioning period, 10 sclerotia were removed from each pot and placed on the surface of sterile sand in deep petri dishes (25mm depth) to begin a 4-week germination period on lab shelves under fluorescent lights (28-72 μmol $m^{-2}s^{-1}$) at room temperatures (22-28^{o}C). Each plate was randomly assigned a shelf and position on that shelf. During this period the sand in plates was kept moist to maintain a high level of humidity.

Conditioned sclerotia were monitored every two to three days, and mature apothecia counted and removed. At the end of the four-week germination period, the number of germinated sclerotia (out of 10) was recorded for each plate, where germination was defined as production of at least one stipe. Data from sclerotial germination, rather than apothecia production, was used to determine the effects of the conditioning factors tested in this trial. All data analysis was performed in R. Initial analysis on how conditioning factors affect germination of sclerotia was done using the lm function in R with all fixed effects and all interactions. Stepwise variable selection with criterion of 'lowest AIC' was done with the 'step' function in R to determine a

model of best fit for these data, using the forward and backward, or 'both', method for stepwise selection. Determining significance of fixed effects, interactions, and separation of means (according to Tukey's method) was done at a significance level of 0.05 on this model of 'best fit'.

Carpogenic germination of sclerotia

Two hundred sclerotia from each of four isolates (BR, BT, QC, and TI) (Table 1) were conditioned under uniform conditions of 18/6°C day/night (12h cycle, total darkness) for 6 weeks shallowly buried in pots of sand (40 sclerotia per pot). Following the uniform conditioning period, sclerotia were removed from pots and placed on the surface of sterile sand in deep petri plates (10 sclerotia per plate). Plates were distributed among shelves within two growth chambers (set at 18/6°C and 26/11°C day/night). Within each growth chamber were three shelves which related to 3 light levels, achieved by placing 0, 1, or 2 layers of mosquito netting between plates with sclerotia and lights. This resulted in 'high', 'medium', and 'low' light intensity levels for each growth chamber. Light intensity was measured as PAR in $\mu mol/m^2/s$ for each light level in each growth chamber. Due to lights in growth chambers being of different ages, intensity values differed between growth chambers; intensity readings for high, medium and low were 370, 160, 86, and 230, 100, 50, respectively, in the cooler and warmer growth chambers.

Conditioned sclerotia were germinated under these conditions for 4 weeks and accumulated numbers of apothecia were recorded every 2-3 days. Apothecia accumulation at the end of this four-week period was used for data analysis. Fixed effects of temperature, light intensity level, and isolate were analyzed for their effect on apothecia accumulation via linear regression with a model of best fit determined by step-wise AIC selection. Separation of means

was performed according to Tukey's least squared method (P=0.05). All data were analyzed using R statistical software.

Ascospore infection of dehiscent carinata petals

Fixed effects of temperature (15/3°C, 18/6°C, 21/9°C, and 26/11°C), relative humidity, and isolate (FH and TI) were assessed for their effect on ascospore germination on dehiscent carinata petals. Relative humidity levels (about 97, 94, 84, and 74%) were established using salt solutions of K_2SO_4, KNO_3, KCl, and NaCl, respectively (Greenspan, 1977). Carinata petals were placed into 'double decker' petri plates (one petal per plate) consisting of two plates glued together (one on top of the other) with 5 or 6 holes (made with a soldering iron) to allow movement of air between the lower and upper plates. A salt solution was contained in the bottom plate, and the carinata petal contained in the top plate. Carinata petals were inoculated with 10µl suspension of ascospores ($5x10^5$ spores per ml) such that about 500 spores were delivered to each petal.

Ascospores were collected from plates of mature apothecia (from the sclerotia conditioning or sclerotia germination tests) by vacuuming spore plumes onto an 1.0µm pore size mixed cellulose ester membrane filter (Advantec, Japan). Filters with ascospores were stored, sealed in glass tubes with desiccant beads, at -20°C until needed. Ascospore suspensions were made by washing filters with sterile distilled water. This suspension was quantified using a hemocytometer and then diluted with sterile distilled water to the previously mentioned concentration. Plates were then distributed amongst growth chambers and incubated for 4-9 days. Petals were observed every 2 days under a dissecting microscope and presence of mycelia was recorded. Petals were either recorded a '1' or '0' for germination dependent upon presence of

mycelia or a lack thereof on petals, respectively. Logistic regression was performed on these data to determine significance of fixed effects using R statistical software.

Results

Conditioning of sclerotia

According to lowest AIC, the model of best fit was as follows:

Sclerotia germination ~ Isolate + Temperature + Moisture Frequency + Conditioning Duration + Isolate:Temperature + Isolate:Moisture Frequency

Where sclerotia germination was the proportion of sclerotia (out of 10) which had germinated after a four-week germination period following the 4, 6, or 8-week conditioning period. This model of best fit had an R^2 of 0.3791 and *P*-value < 0.001. According to this best model analysis, all fixed effects, except temperature ($P = 0.296$), were significant ($P < 0.001$) and there were two significant interactions between moisture frequency and isolate ($P < 0.001$), and temperature and isolate ($P < 0.001$). Substantial variability among isolates in average germination and in germination response to temperature was noted (Fig. 1). For isolates TI and FH, germination at 15/3C was significantly lower than the other temperature levels, which did not significantly differ from each other. This pattern was reversed in isolate HA, where germination was significantly higher at 15/3°C than the other temperatures tested. For isolate BT there was significant variability in germination between temperature levels, with 21/8C having the highest germination and 18/6C the lowest. In general, moisture frequency event every day resulted in significantly lower germination than the other moisture levels (except for isolate TI) (Fig. 2), and germination increased as conditioning duration increased (data not shown).

Apothecia germination

Regression was done on all fixed effects and interactions in R using the 'lm' function. According to this full model, temperature was the only fixed effect that was not significant, and there was a significant interaction for isolate and light. Variable selection (forward and backward step-wise selection) was done using AIC to determine a model of best fit for these data. This resulted in the following linear model:

Apothecia ~ Isolate + Light + Isolate:Light

and this model had an adjusted R^2 of 0.77 and a *P*-value < 0.001. Separation of means was done to determine differences within isolate to their response to light. In general, as light levels decreased, apothecia accumulation increased (Fig. 3).

Ascospore Infection

Logistic regression (using the 'glm' function in R) determined that all main effects, except for isolate, significantly affected ascospore infection of carinata petals ($P < 0.001$). In addition, there were many significant interactions between main effects: RH:Hours ($P < 0.001$), Temp:RH ($P = 0.004$), and Temp:Hours ($P = 0.002$). Variable selection for a model of best fit was done according to AIC in R (using the 'step' function) in a 'both' ways stepwise algorithm on all main effects and interactions. This resulted in the following logistic regression model:

Infection ~ Temperature + Isolate + RH + Hours + RH:Hours + Temp:RH + Temp:Hours

To explain these interaction terms; infection occurred at lower RH with increasing incubation duration (RH:Hours). For Temp:RH, infection never occurred below 95% RH at the 15/3°C temperature treatment where at other temperatures infection occurred as low as 84% RH. Lastly, the rate of increase in infection (in terms of proportion of infected to non-infected petals) as

hours increased was significantly different among temperatures (Temp:Hours) (Fig. 4). In general, petal infection largely occurred at and above 94% RH, and infection did not occurr < 84% RH.

Discussion

In the sclerotia conditioning test, apothecia accumulation was not used to determine effect of conditioning parameters due to spatial effects on germination, possibly due to varying intensity between fluorescent lights (plates were set under 3 different lamps to germinate). The germination shelves in the lab were positioned one on top of the other with fluorescent lamps for each shelf. Variability in apothecia germination could have been due to variation in color/intensity of lights, with individual florescent tubes visibly differing in color. During the germination period, ambient temperatures fluctuated from 22°C to 29°C. This temperature fluctuation, coupled with the lighting variation, is a possible reason for the variation in apothecia production between shelves. Of the three shelves, the lowest shelf had the greatest apothecia production (data not reported) with the lowest intensity (about 28-42 umol $m^{-2}s^{-1}$) and the color of light was more of a warm yellow than the fluorescent white of the other lamps. By comparison, minimal apothecia production occurred on the other two shelves. In contrast to apothecia production, sclerotial germination did not significantly differ between shelves. This could be because stipe production (criteria for germination of sclerotia in this study) was already happening during the conditioning period and so was less influenced by the germination conditions.

Incidence of sclerotial germination was used because there were no significant spatial effects between lamps according to a spatial variability test, with respect to germination (defined as presence of at least one stipe) between lamps. The results from sclerotial conditioning indicate a high amount of variation in temperature preference for SE US isolates of *S. sclerotiorum* which is in line with previous reports of environmental adaptation based on geographical origin of isolate (Huang and Kozub, 1991; Uloth et al., 2015). Oddly, it appears that isolate BT had two optimal temperatures (at 15/3°C and 21/9°C) though this may have been due to differences between batches of sclerotia (only two temperature regimes were able to be tested at a time). A moisture event every day during conditioning resulted in significantly lower germination of sclerotia for all but one of the isolates tested (TI). Together, these results suggest that a management plan which uses a disease forecasting system to predict inoculum density, and thus disease severity, based on temperature during winter months may need to be specific to location. However, results suggest that lower disease severity would occur in years with extended periods of rainfall during the winter.

All isolates in the apothecia germination test had the same numerical trend in relation to light levels, where highest numbers of apothecia developed at the lowest light level, and fewest apothecia developed at the highest light level. However, a significant interaction between isolate and light was observed in our model. This is due to some isolates having significant differences between light levels while others did not and it is possible that with more replications those non-significant numerical trends could become significant, thus making an interaction between isolate and light non-significant.

Apothecia development typically occurs after canopy closure which would cause shading of the soil and retention of more soil moisture, a critical component of apothecia development (Clarkson et al., 2007). It also makes sense that there would be more development of apothecia at lower light intensity, as light intensity decreases as the crop canopy closes. However, in another study involving light intensity and apothecia development, with two light levels (80-90 and 120-130 µmol m^{-2} s^{-1}) as well as other environmental variables, the opposite was found to be true. Of the two light levels evaluated by Sun and Yang, 2000, more apothecia developed at the higher than the lower level. It is unclear why our isolates preferred lower light intensities compared to theirs.

Large differences in apothecial accumulation were observed between isolates. Ten sclerotia of isolate BT produced an average of about 40 apothecia at 'low light' and an average of about 19 apothecia at 'high light'. Compared to isolates QC and TI which accumulated fewer than 10 apothecia over the 4-week germination period even at the lowest light level, on average. This suggests that what is more important than light penetration of the crop canopy is the aggressiveness of the isolates that are present (in terms of possible ascospore production in a field relative to light intensity in the crop canopy). Even if a high light intensity (we tested intensities of up to 370 µmol m^{-2} s^{-1}) reaches the soil beneath the crop canopy (see appendix 1 for more reference to light intensity in a field setting), this could still result in high numbers of ascospores (in terms of disease development), assuming other essential environmental conditions are conducive to apothecial development.

From the results of the ascospore infection test, the optimal conditions for infection of dehiscent carinata petals by ascospores was at 26/11°C and RH above 94%. Although most infection occurred at RH above 94%, infection could occur as low as 84% RH given enough

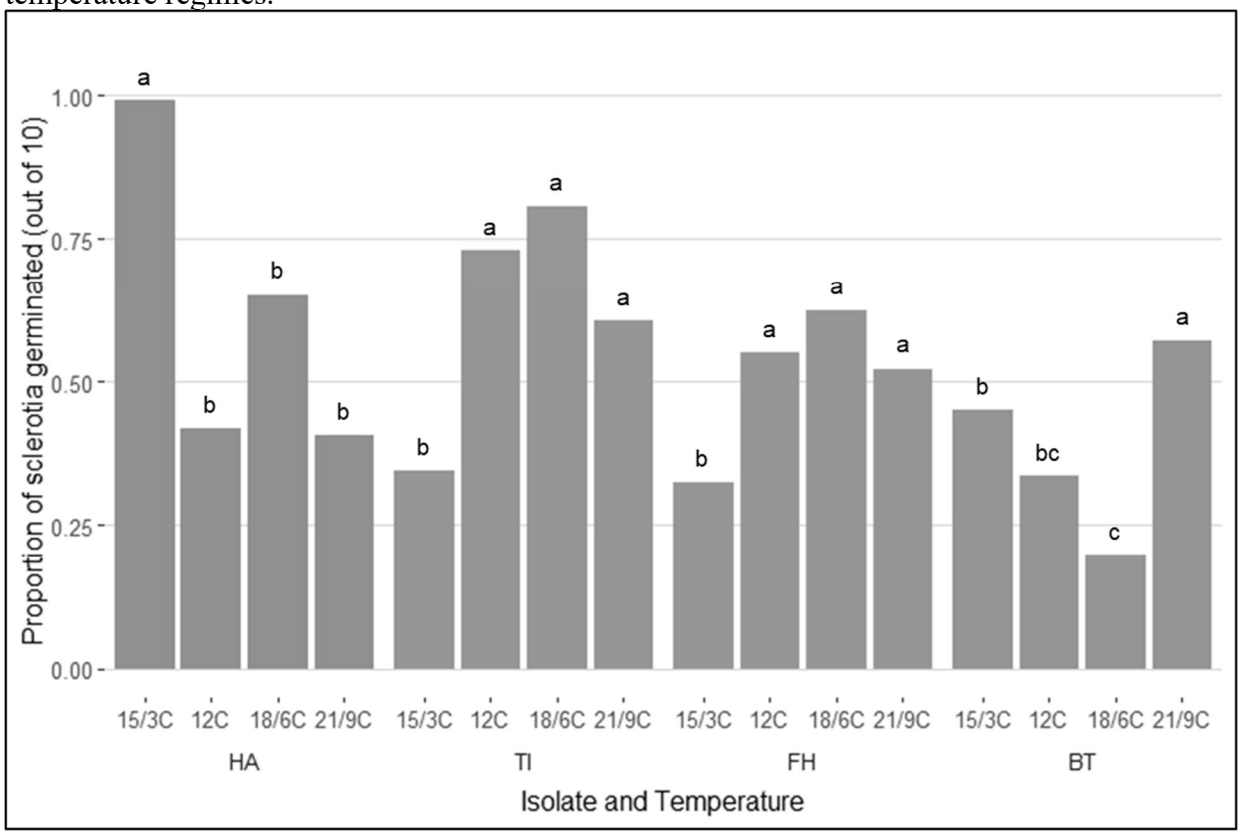
time. Mycelia was noted on almost all petals at 26/11°C after 280 hours of incubation when ≥ 84% RH. Though infection at 84% RH in other temperature treatments did occur, it was at a relatively low proportion. There is some uncertainty about whether a rating of no infection is because the ascospores failed to germinate, they failed to infect, or because the infection process on petals is very slow. While infection could occur at 84% RH, the amount of time needed for mycelium to develop might mean that such a low relative humidity would have little impact on disease development. Future trials should be aimed at finding upper infection thresholds in terms of temperature. These data could be useful for validation or modification of a disease forecasting system.

Tables and Figures

Table 1. Host and location origin of *Sclerotinia sclerotiorum* isolates used in this study

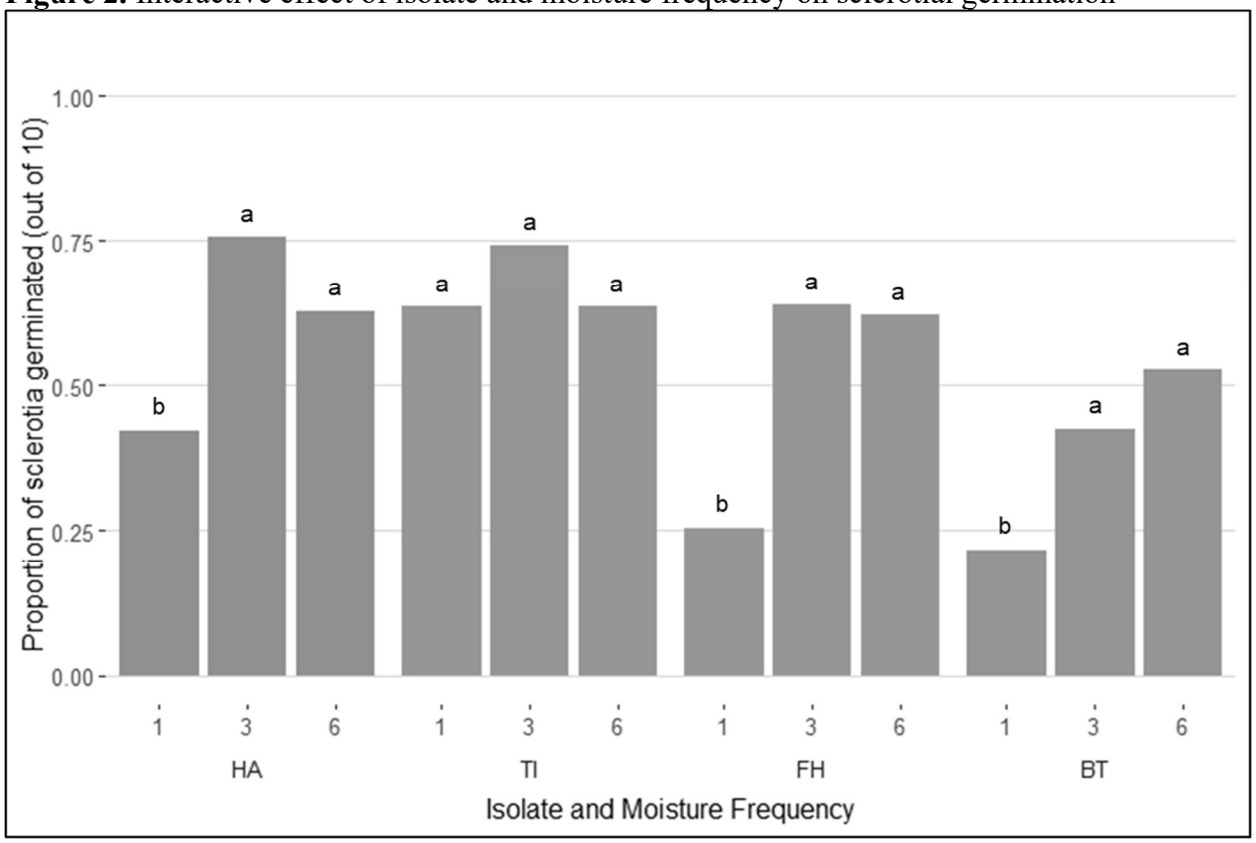
Isolate	Host	Source
HA	Canola	AL
FH	Carinata	Fairhope, AL
BT	Carinata	Brewton, AL
TI	Carinata	Tifton, GA
BR	Canola	BREC, Georgia
QC	Canola	Quincy, FL

Figure 1. Germination of sclerotia for each *Sclerotinia sclerotiorum* isolate at each of 4 temperature regimes.



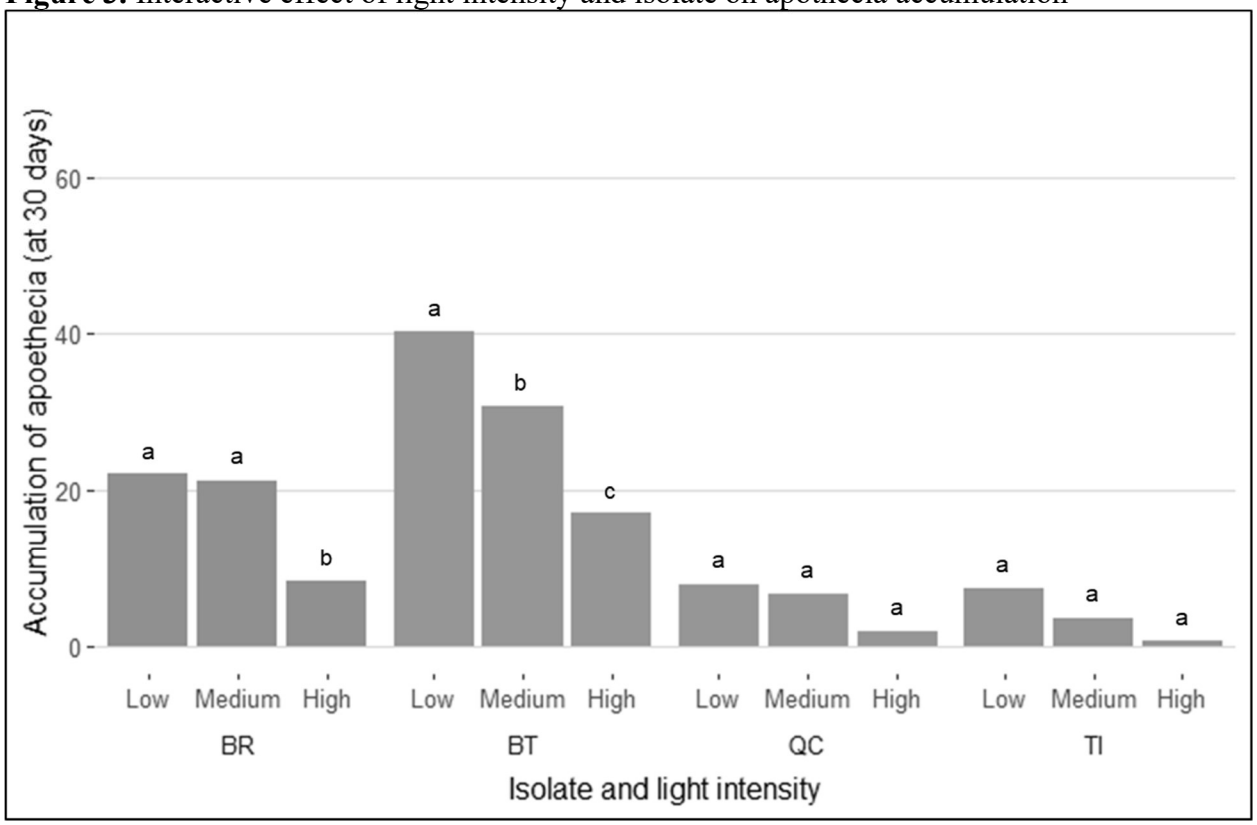
Note: Separation of means by isolate, where bars with matching letters within isolate are not significantly different ($P < 0.05$) according to Tukey's LSD.

Figure 2. Interactive effect of isolate and moisture frequency on sclerotial germination



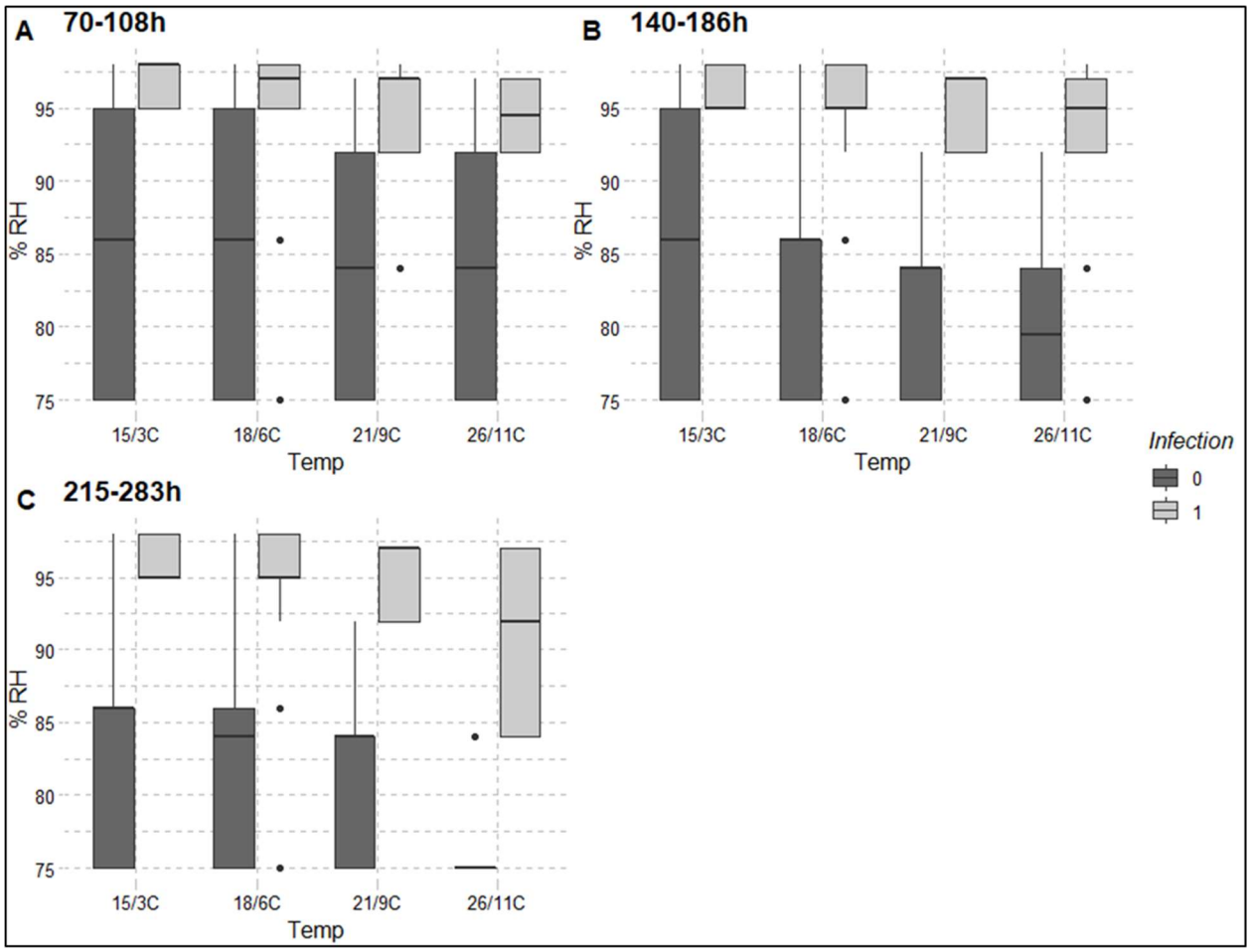
Note: Separation of means by isolate, where bars with matching letters are not significantly different ($P < 0.05$) according to Tukey's LSD.

Figure 3. Interactive effect of light intensity and isolate on apothecia accumulation



Note: Separation of means by isolate, where bars with matching letters within isolate are not significantly different ($P < 0.05$) according to Tukey's LSD.

Figure 4. Ascospore infection of carinata petals various temperatures and relative humidities over time.



Note: Side by side boxplots represent the distribution at which no infection on dehiscent carinata petals occurred (0) and where it did occur (1) relative to RH for each temperature. Data were grouped into three different periods to show the progression of non-successful and successful infection over the incubation period.

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III. *IN VITRO* FUNGICIDE EFFICACY OF SE US *SCLEROTINIA SCLEROTIORUM* ISOLATES

Introduction

Brassica carinata, commonly known as Ethiopian mustard, is currently being introduced to the Southeast US as a non-food oilseed (NFOS) crop grown over winter months. Oil pressed from the seeds of carinata is readily refined into jet fuel and the pressed seed is used as a high protein meal for beef cattle (Seepaul et al., 2016). Because carinata is a winter crop, it fits in to a double cropping system with summer crops such as soybean or cotton. *Sclerotinia sclerotiorum*, the causal agent of Sclerotinia Stem Rot (SSR), is an economically important pathogen known to cause significant yield losses in carinata and other brassica oilseed crops such as canola (e.g., Del Rio et al., 2007). In 2013, a survey of SSR in brassica crops was conducted in Western Australia with losses estimated at AU$59 million (worth about US$56 million at the time) (Khangura et al., 2014). SSR on winter carinata is the disease of greatest concern in the Southeast US, having the potential to impede the crop's successful introduction (Seepaul et al., 2016). As a new crop to the Southeast, a management plan is being developed which includes recommendations for fungicides that will control SSR. Fungicide use is necessary to control SSR due to a lack of resistant cultivars (Uloth et al., 2015). The objective of this study was to test the sensitivity of regional isolates of *S. sclerotiorum* to a variety of fungicidal chemistries that could be used on winter carinata and aid in management recommendations.

Materials and Methods

Isolates of *S. sclerotiorum* used in this study were collected from naturally infested carinata or canola grown in the SE US. Specifically, isolates were collected from Blairesville, GA canola (BR), Fairhope, AL carinata (FH), and Tifton, GA carinata (TI). Sclerotia were generated on sterilized wheat as described by Pethybridge et al. (2015). Wheat grain (25g in 500ml wide mouth flasks sterilized with 50ml of water) was inoculated with mycelium grown on PDA plates (which is what isolates were maintained on). After 4-6 weeks sclerotia would develop, which were harvested by washing the wheat and sclerotia through a series of sieves to separate sclerotia from wheat. Harvested sclerotia were then dried and stored in coin envelopes at room temperature until needed.

Sclerotia were conditioned at cool temperatures (between 15-21˚C day and 3-9˚C night) while shallowly buried in pots of sterile sand, which were periodically leached with deionized water (every 3 or 6 days) for 6 to 8 weeks. After conditioning, sclerotia were placed in deep dish petri plates (25x100mm) on sterile sand and incubated under lights at room temperature (~22°C) to germinate. Apothecia developed in as few as a couple days (dependent on whether stipes were already present from conditioning) and ascospores were collected from mature apothecia onto a 1.0µm pores size mixed cellulose ester membrane filter (Advantec, Japan). Filters with ascospores were stored, sealed in glass tubes with desiccant beads, at -20˚C until needed.

Six fungicides were tested, including quinone outside inhibitors (QOI's), demethylase inhibitors (DMI's), succinate dehydrogenase inhibitors (SDHI's), combinations thereof, and chlorothalonil (Table 1). PDA was amended with each fungicide at 0.02, 0.05, 0.1, 0.5, and 1.0 concentration of maximum recommended label rate (MRLR); these concentrations were converted from a.i. per acre to the area of a petri plate, which contained a volume of about 20ml

PDA (Table 1). A solution of fungicide product (in an amount according to the conversion calculations) was added to molten PDA prior to pouring into petri plates (15x100mm). Endura® concentrations were an exception; a miscalculation resulted in Endura concentrations at 5x more than stated (i.e., 0.1, 0.25, 0.5, 2.5, and 5x MRLR). Some fungicides in this study were not labeled for control of SSR on a brassica crop. In this case, MRLR was based on the highest rate recommended on the label for a different disease on a brassica crop, or a *S. sclerotiorum* incited disease on a different crop. For example, Twinline was not recommended for control of any *S. sclerotiorum* diseases nor for control of any disease on a brassica crop. In this case, the highest maximum recommended label rate was for Corynespora leaf spot on cotton at 12 fl ozs/A.

Ascospore suspensions were made by washing filters with 0.1% Tween solution (in sterile distilled water). This suspension was quantified using a hemocytometer and then diluted with sterile deionized water to $5x10^5$ ascospores per ml. Plates of fungicide amended PDA were inoculated with 10µl of this suspension such that about 500 ascospores were delivered to the center of each plate and incubated at room temperature for about 100 hours. After this period of incubation, the maximum diameter of resultant colonies was measured and recorded.

This experiment was done twice. In the first experiment, ascospores of two isolates (BR and TI) were used in a full factorial design (isolate x fungicide x fungicidal concentration) with three replicate plates. In the second run, an additional isolate was added (FH) and this experiment was done in a reduced factorial design with two replicate plates. Isolate FH was tested on all 6 fungicides, while isolates BR and TI were tested on only 4 of those fungicides (chlorothalonil, boscalid, fluxapryoxad+pyraclostrobin, and flutriafol). All fungicides used in the second experiment were used at rates from 0.2 to 1.0x MRLR (Table 1). Data from both

experimental repetitions were pooled and analyzed with the lmer function in R (R Core Team, 2018).

Differences among isolates of *S. sclerotiorum* in mycelial growth on varying rates of multiple fungicides were analyzed by creating a random slope-intercept model using the lmer function in R. The full model was described as:

Growth ~ Rate + Isolate + (Rate|Fungicide) + (Isolate|Fungicide)

Where 'Growth' is colony diameter after about 100 hours (94 and 113 hours in the first and second experiment, respectively), 'Rate' is the proportion of the maximum recommended label rate (MRLR) for each fungicide, '(Rate|Fungicide)' is a random slope term for rate relative to each fungicide, and '(Isolate|Fungicide)' is a random intercept term for isolate relative to each fungicide (to allow for differences in sensitivity to each fungicide). A likelihood ratio test (LRT) was used to test the significance of fixed effects in this model. A likelihood ratio test is a method of determining the significance of fixed effects in a linear mixed effects model whereby the difference between two nested models is tested using a chi square distribution. If the reduced model is not significantly different in fit to the full model, than the null hypothesis is retained.

Results

Chlorothalonil, a fungicide with multi-site action, was also tested and was able to prevent growth of mycelia from ascospores regardless of concentration or isolate (data not shown). The growth response of ascospores on varying rates of different fungicides was significantly different between isolates of *S. sclerotiorum* according to linear mixed effects model LRT ($P < 0.001$). This difference between isolates was mostly due to isolate growth at fungicide rates below 0.5 MRLR (Fig. 5). Where isolate BR did not grow on any concentration of Twinline® or

Headline®, and isolate FH had significantly more growth on Priaxor® than any other isolate. However, at and above 0.5 MRLR, all isolates grew on Endura® (boscalid, SDHI, group 7) and Topguard® (flutriafol, DMI, group 3), but not the other fungicides, with isolate mean growth between isolates being relatively similar on Endura® and Topguard®. Interestingly, no isolate was able to grow above 0.5 MRLR on either Twinline® or Priaxor®; these active ingredients belong to FRAC groups 3+11 and 7+11, respectively (Table 1.). Two of the three isolates (FH and TI) in this experiment were able to grow on Headline, but only below 0.5 MRLR (Fig. 5).

Discussion

Of the six fungicides tested in this study, only Endura (boscalid), Headline (pyraclostrobin), and Priaxor (fluxapryoxad+cyproconazole) are currently registered for control of SSR in carinata. While the other fungicides are not currently registered for SSR control, there are additional chemistries that belong to these same FRAC groups which are. For example, group 3 flutriafol is not registered but group 3 metconazole is a registered fungicide for control of SSR on carinata.

S. sclerotiorum has been reported to have developed resistance to fungicide chemistries in groups 2 (dicarboximides) and 12 (phenylpyrroles) on rapeseed in China (Duan et al., 2014; Zhou et al., 2014), and group 1 chemistry (benomyl) in Canada (Gossen et al., 2001). Growth of our isolates on group 7 chemistry boscalid (Endura®) from concentrations as low as 0.1 to 5x MRLR suggest that local isolates may be developing resistance to this fungicide group or that there is innate resistance present, though there was suppression of mycelial growth at all concentrations compared to the control. While the group 3 fungicide tested in this study (flutriafol) had little to no activity on *S. sclerotiorum* growth, another group 3 fungicide

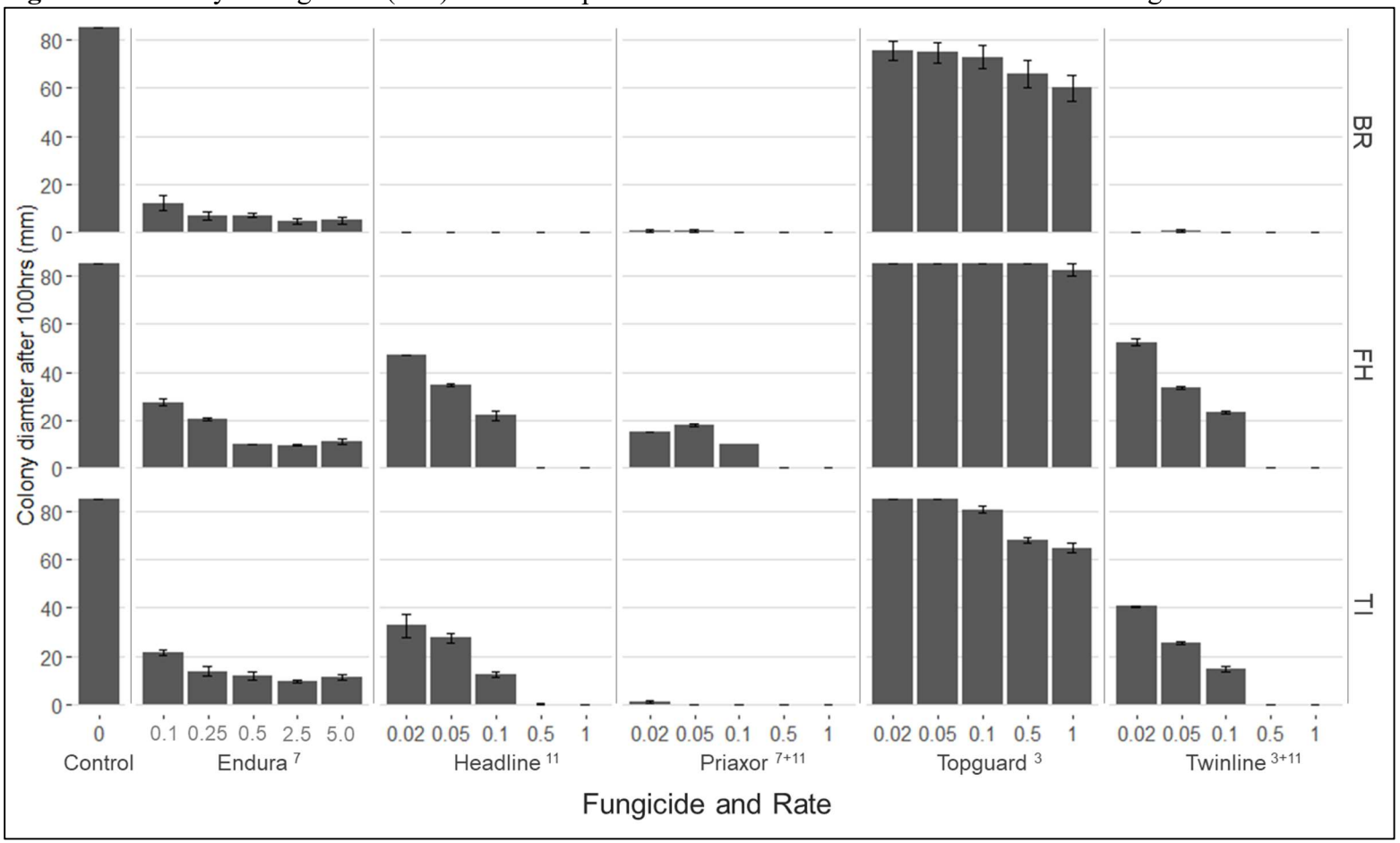
(tebuconazole) has been shown to be efficacious *in vitro* (Mueller et al., 2002). This suggests that while some chemistries within a FRAC group may not be efficacious, others within that same group may be.

The results from this study indicate that boscalid and flutriafol would not have good efficacy for management of SSR. However, field studies should be conducted to confirm that there is a correlation between *in vitro* efficacy and field efficacy before using these results in developing spray recommendations. As such, results from this test should not be used to recommend which fungicides a grower should use but instead should not use to control SSR in winter carinata. That is, there is no guarantee that a fungicide which is efficacious *in vitro* will retain that level of efficacy in the field (crop canopy structure, spray coverage, and weather may all influence efficacy of fungicide sprays); however, a fungicide which is not efficacious *in vitro* is likely to retain that lack of efficacy in a field setting.

Using combination fungicides is an important strategy in fungicide resistance management (FRAC, 2010). For instance, in this study we saw that combining a group 11 chemistry with a group 3 or 7 prevented growth of mycelia from ascospores on amended PDA at or above 0.5 MRLR when a group 3 or 7 fungicide was unable to do so alone. Likewise, using a multisite fungicide is almost always efficacious as they have virtually no risk to resistance development (Straub, 1991), although there is not currently a multisite chemistry registered for control of SSR in carinata. Based on the results of this test, farmers should not use the group 7 chemistry boscalid as there was mycelial growth of all isolates even at maximum label rate, which indicated possible resistance in local isolates of *S. sclerotiorum*. These results may also be useful in determining which fungicides to include, or not to include, in future fungicide field trials for developing a management guide for SSR on Southeastern US winter carinata.

Tables and Figures

Figure 5. Mean mycelial growth (mm) from ascospores of SE US isolates of S. sclerotiorum on fungicide amended PDA



Note: Bars represent mean growth with standard error on plates of fungicide amended media at the respective rate (ratio based on maximum recommended label rate for Sclerotinia Stem Rot on brassica crops) of each fungicide tested.

Table 2. Products and descriptions of fungicides used

Fungicide	Company	Country	a.i.	FRAC Group	Chemistry Group	MRLR[1]	Media conc.[2]
Daconil	Syngenta	Switzerland	Chlorothalonil	M	Chloronitrile	5.4 lbs/A	0.24 g/L
Headline	BASF	Germany	Pyraclostrobin	11	QOI	12 fl oz/A	34µl/L
Endura	BASF	Germany	Boscalid	7	SDHI	6 oz/A	0.08 g/L*
Priaxor	BASF	Germany	Fluxapryoxad + Pyraclostrobin	7 + 11	SDHI + QOI	8 Fl oz/A	23 µl/L
Topguard	FMC	United States	Flutriafol	3	DMI	8 Fl oz/A	23 µl/L
Twinline	BASF	Germany	Metconazole + Pyraclostrobin	3 + 11	DMI + QOI	9 Fl oz/A	25.8 µl/L

[1]Maximum Recommended label rate

[2]Concentration of product in amended PDA at 1x MRLR

*due to miscalculation in converting from oz/A to g/L, Endura concentrations in PDA were 5x more than stated (i.e., 0.1, 0.5, 1.0, 2.5, and 5x MRLR).

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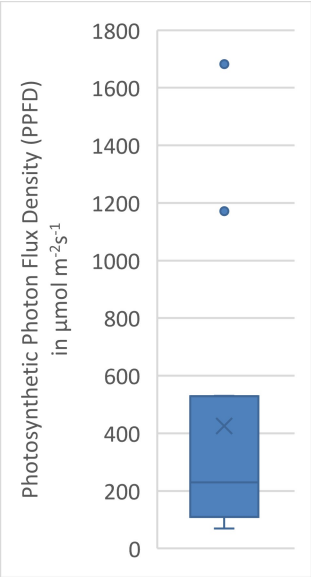
APPENDIX

1. Light intensity and canopy closure in a carinata research

To get an idea of canopy closure in a 2018-2019 carinata yield loss trial, light intensity readings were taken from within the canopies of some plots. Five plots were randomly selected from each of 3 reps (with 24 plots per rep). Plots contained four 14" rows which were 25' in length and readings were taken from a random spot along the length of each plot in between the second and third row. These readings were taken on the 5th of April 2019 at about 12:45pm (figure 6.). As a general observation, plots had very poor canopy closure. This may be due to a poor initial stand and lack of branching in plants as the season progressed.

As far as canopy closure relates to light intensity at soil level, a PPFD reading of about 100 umol $m^{-2}s^{-1}$ was a fully enclosed canopy. This was a fully shaded soil, with readings lower than this relating to the thickness of the canopy. For reference, light intensity on bare soil in the field next to the research trial was 1895 µmol $m^{-2}s^{-1}$, and light intensity under the bumper of a vehicle in the field was 20 µmol $m^{-2}s^{-1}$.

Figure 6. Light intensity within canopy of carinata yield loss trial.



Note: Readings were taken on 4/5/19 at about 12:45pm.

2. Identification of isolates

Isolates were identified using sanger-sequencing of the ITS1-ITS4 genomic regions. All isolates tested (QC, FH, BT, BR, HA, and TI) were confirmed to be *Sclerotinia sclerotiorum* (>99% identity) via BLAST of the sequences against the NCBI database. Isolate HA had only 90% identity with *S. sclerotiorum* possibly because of a low query cover of 83%.

Mycelium of isolates was grown in PDB in 5ml falcon tubes on shaker tables to generate enough genetic material to extract DNA from. DNA was extracted from mycelium using the Dneasy powersoil DNA extraction kit from Qiagen. Amplification of the ITS region was performed in lab using PCR with the standard ITS1 and ITS4 primers. PCR product was then sent to Eurofin for sequencing. Sequences received from Eurofin were then referenced against the NCBI fungal database using BLAST.

3. Airborne ascospore quantification and impaction spore trapping in a 2018-2019 carinata yield loss study

An attempt was made to quantify airborne ascospores in a carinata research plot using real-time PCR. However, this endeavor stalled out at establishing a robust spore standard curve series. Initially two primer probe sets were tested for specificity/accuracy of testing our *S. sclerotiorum* isolates while being insensitive to *Botrytis cinera*, *Sclerotinia minor*, and *Sclerotinia trifoliorum*.

The first real-time PCR primer/probe set, and thermocycler settings, were developed by Almquist and Wallenhammar (2015) who added a TaqMan MGB probe to a PCR primer set developed by Freeman et al. (2002). The target for the primer was within the ITS region of the *S. sclerotiorum* genome. The second primer/probe set was based on a SYBR real-time PCR primer set developed by Rogers et al. (2009) which targeted a *S. sclerotiorum* gene encoding for the mitochondrial small subunit rRNA intron and ORF1 protein. We slightly modified Rogers et al.'s assay, we used the same primer target but used a TaqMan MGB probe developed in lab. The reason for this is that the Rogers assay was sensitive only to *S. sclerotiorum* DNA and could detect lower amounts of DNA than the assay developed by Almquist and Wallenhammar which was sensitive to all previously mentioned *Sclerotinia* spp and could not detect as little DNA.

The primer/probe set developed by Almquist and Wallenhammar was ultimately the one chosen for use in detecting ascospores. Though the SYBR based qPCR (Rogers et al., 2009) was more sensitive (able to detect DNA representing as few as 2 ascospores) compared to the TaqMan probe based qPCR (able to detect as few as 10 ascospores), it was determined that developing a custom TaqMan probe for the primers used by Rogers et al. (2009) and the following optimization/validation of thermocycler settings would take too much time considering

the TaqMan probe-based qPCR was already optimized and validated by Almquist and Wallenhammar.

However, we could not establish good standard curves with the Almquist and Wallenhammar qPCR assay. A spore concentration series of 10, 100, 1000, and 10000 ascospores of *S. sclerotiorum* was used to try to establish a standard curve series to compare processed field samples to. Spore concentration samples were processed in triplicate but unfortunately every assay resulted in overlapping curves between concentrations. Because of this, there would be no way to relate back the C_T values of processed field samples to quantity of ascospores, hence why field samples were not processed.

The thermocycler used for our real-time PCR was different from the one used by Almquist and Wallenhammar (we used a Bio-Rad CFX86 Real-Time machine (Bio-Rad Laboratories CA, USA)). In addition, our thermocycler settings for the assay were slightly different from theirs because of this. The thermocycler settings as described by Almquist and Wallenhammar were, "The thermal cycling conditions were an initial denaturation for 10 min at 95°C, followed by a touchdown PCR consisting of a total of 54 cycles: 14 cycles of 95°C for 15s followed by annealing for 60s with temperature decreasing by 1°C every two cycles from 72-66°C; 40 cycles of 95°C for 15s followed by annealing for 60s at 65°C". Where they had an annealing step that decreased in temperature by 1°C every 2 cycles, our machine was only able to program a per cycle decrease (in this case, 0.5°C decrease per cycle). Other than this our assay was set-up the same, though run on a different machine.

A single impaction spore trap (built by Dr. Walter Mahaffee at the Corvallis, OR USDA-ARS HCRL), similar to a Rotorod® (IQVIA, Durham, NC), was set-up in the center of a

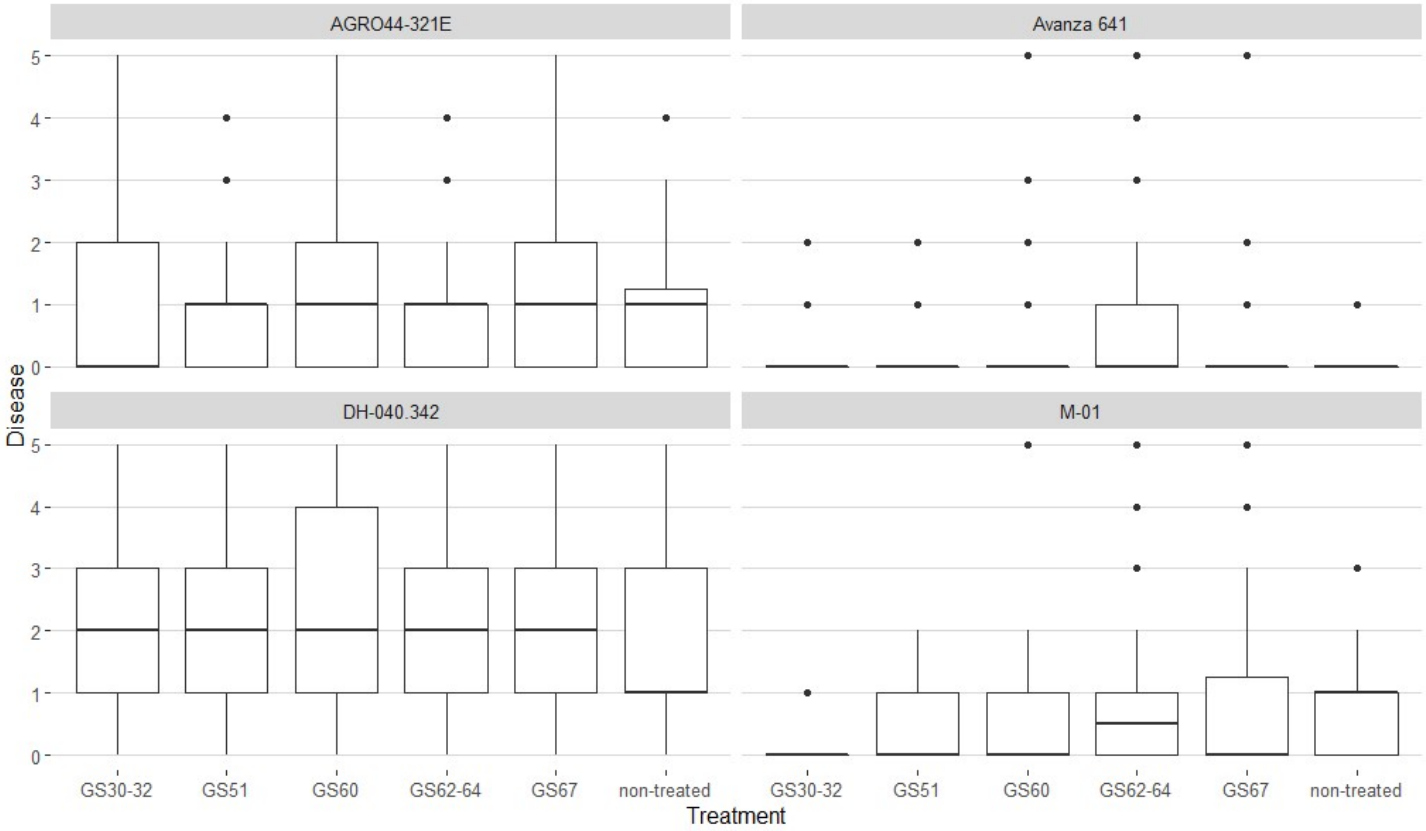
carinata yield loss study during the 2018-2019 season. This trap was operating 24/7 from the beginning of February to mid-May for 24 hrs per day. Sample rods from impaction traps were collected and replaced bi-weekly during this time. Collected sample rods were stored in -20°C until they could be analyzed for *S. sclerotiorum* DNA using a real-time PCR assay developed by Almquist and Wallenhammar (2015). Because we were unable to establish good spore concentration C_t standard curves, field samples were unable to be processed.

Collection rods were 36.5 mm (a size that fits in microcentrifuge extraction tubes) sections cut from 308L045X36T stainless steel TIG wire. This welding wire was used for collection rods because of their high purity and minimal PCR inhibitors, but still need to be cleaned well. Collection rods (i.e. sample rods) in impaction traps were cleaned by the follow procedure: washed in warm soapy (dish soap) water, placed in a 10% bleach solution and shaken at max speed on a table-top vortex for 15 min, rinsed 3x in DI water, dried, autoclaved for 25 min, and then finally dried in a laminar flow hood. Cleaned and dried rods were then lightly coated in vacuum grease to facilitate adhesion of ascospores to rods. Collection rods were stored in sterile falcon tubes with rods stabbed into plumber's putty (Oatey® stain-free plumber's putty) that was placed in the falcon tube cap. The assembly of the cleaned rods into falcon tubes was done aseptically in a biocontainment hood.

Though we were unable to quantify the number of spores on field samples, SSR severity in our field trial was very low during the 2018-2019 season (Figure 7). Most carinata varieties had average severity ratings of about 1 (on a 0-5 scale) across all treatments, except for variety DH-040.342 which had average ratings of 2. Most disease symptoms were found on pods, rather than on stems. Considering that there was so little disease, it is uncertain whether there would have been much detection of *S. sclerotiorum* ascospores on field samples since the sensitivity of

the assay had a threshold of 10 ascospores. Although it is possible that the reason for such low disease is not because of a lack of inoculum but of a lack of conducive conditions for infection. Indeed, canopy closure was poor in research plots which may have led to poor conditions for ascospore infection (see appendix 1 for more details.)

Figure 7. Disease ratings for Sclerotinia stem rot in a 2019 carinata yield loss study



Note: Study was conducted at EV Smith research station. Each section of boxplots belongs to a cultivar of carinata and within each section are boxplots which describe the disease severity Sclerotinia stem rot at the end of season relative to what growth stage fungicides were applied.