Quantifying *Verticillium dahliae* in the soil profile using two methods in USA and Australian cotton soil

by

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ABSTRACT

Quantifying inoculum levels of *Verticillium dahliae* in field soil is essential for understanding potential disease pressure of Verticillium wilt in cotton and making informed management decisions. A number of semi-selective medium and several techniques for pathogen isolation have been developed for determining the inoculum levels of *V. dahliae* present in soil. Soil sampling methods, timing of sampling, media, and isolation techniques all influence the estimated inoculum levels in soil. The objective of this study was to determine the ideal combination of soil plating technique, media, sampling depth, and time of sampling for the detection and quantification of *V. dahliae* in field soils. Two soil plating techniques (dilution, or wet, plating and hand-spreading, or dry, plating) on four semi-selective media (Sorenson's NP-10, PDA, and acidified versions of each) were compared to quantify naturally occurring soil inoculum in germinated microsclerotia propagules per gram of soil. Soil from three depths was compared to examine the vertical distribution of the fungus in order to determine the ideal sampling depth. Sampling was conducted pre-planting and post-harvest to determine the ideal sampling time. The results suggest sampling soil prior to planting from 2-12 cm depth and using the dry-plating method on Sorenson's NP-10 media.

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CHAPTER 1

QUANTIFYING *VERTICILLIUM DAHLIAE* IN THE SOIL PROFILE USING TWO METHODS IN USA AND AUSTRALIAN COTTON SOIL

Introduction

Cotton is grown in 17 states of the USA, producing a crop worth over $75 billion USD in 2017 (National Cotton Council, 2017). Cotton is an economically important crop in Texas, which accounts for ~50% of total domestic production (National Cotton Council, 2016). Texas ranks number one in cotton production in the USA, producing a value of >$2 billion in 2018 (National Agricultural Statistics Service, 2019). Australia produces ~3% of the world's cotton and is the third largest exporter, behind the USA and India, averaging AUD $2 billion annually. Over the last 20 years, Australia's cotton yield has increased by 38% with more than 60% domestic cotton produced grown in New South Wales (NSW) (Cotton Australia, 2015).

Verticillium wilt caused by the soil borne fungal pathogen *Verticillium dahliae* (Kleb) has contributed to cotton yield loss in the USA and Australia. In the Southern High Plains of Texas and other parts of the United States, Verticillium wilt is a prominent yield-limiting factor for cotton (Sapkota et al., 2014). Following the 1984/1985 cotton disease surveys in NSW, Verticillium wilt was described as the first major disease in Australian cotton and remains a major disease in Australian cotton (Kirkby et al., 2013; Chapman et al., 2016).

The pathogen colonizes the vascular system, negatively impacting water and nutrient uptake. Symptoms include wilting, vascular discoloration, leaf mottling and necrosis. In Australia, Verticillium wilt has reduced yields by a range of 10-62% (Cotton Research & Development Corporation, 2016). In the USA, losses have ranged from 4.4% to 80%, and in severe instances the entire crop has been lost (Pegg & Brady, 2002). After infected plant tissue has died, multi-celled melonized resting bodies originating from hyphae are formed. These infectious propagules are known as microsclerotia (MS), which can survive in decaying plant tissue or in the soil as free units in the absence of a host for more than 10 years (Heal & Isaac, 1965; Evans et al., 1967; Pegg & Brady, 2002). Verticillium wilt is a monocyclic disease as the propagules formed in one season serve as the primary inoculum source in subsequent host crops (Rowe & Powelson, 2002).

Determining the inoculum levels in soil is essential for understanding potential disease pressure and making informed management decisions (Kabir et al., 2004). Inoculum levels in the soil can be quantified using many methods (Nicot & Rouse, 1987). Laboratory techniques using dilution plating, wet sieving, and dry plating with an Anderson sampler (Butterfield & DeVay, 1977; Nicot & Rouse, 1987) are widely used. Sorensen et al. (1991) used a known mass of soil spread onto petri plates with Sorensen's NP-10 semi-selective media using an Anderson sampler. Newer techniques are emerging, such as the use of real-time quantitative Polymerase Chain Reaction (PCR) (Wei, Fan et al., 2015; Wei, Shang et al., 2015), however, isolation of the fungal pathogen using laboratory plating methods requires no molecular expertise or

specialized equipment. Estimation of the number of MS in soil is commonly determined using renditions of the dry or wet plating techniques on semi selective media (Butterfield & DeVay, 1977; Goud & Termorshuizen, 2003) and reported as colony forming units (CFU) or propagules per gram (ppg) of dry soil.

Methods used in wet and dry plating, such as the use of an Anderson Sampler, sieves, soil milling, etc. vary by publication (Goud & Termorshuizen, 2003; Kabir et al., 2004). Numerous types of media have been used in studies quantifying *V. dahliae* inoculum (DeVay et al., 1974; Goud & Termorshuizen, 2003; Kabir et al., 2004). By comparing various components of soil isolation techniques, laboratories can augment practices to provide the most accurate estimation of *V. dahliae* populations in the soil and streamline laboratory processes for efficiency. Two plating methods, a hand-spreading of dry soil method and a soil dilution wet plating method, were chosen for comparison to determine which provided better estimates of *V. dahliae* inoculum isolated from cotton field soils.

Soil sampling procedures also vary among investigators (Goud and Termorshuizen, 2003). Work has been done examining the vertical distribution of *V. dahliae* in the soil profile (Taylor et al, 2005; Albers, 2013), but a consensus has not been reached concerning the depth of soil in which the fungus predominately congregates. Collecting soil from the optimum depth at which the inoculum is most densely populated provides the most accurate estimate of inoculum in the field and the potential for disease incidence and severity. Additionally, soilborne pathogen

populations fluctuate throughout the year (van Bruggen et al., 2006), it is crucial to determine the ideal time of sampling.

The objective of this study was to determine the ideal soil sampling and *V. dahliae* quantification methods by examining the vertical distribution of inoculum in cotton soil from various fields in Australia and the USA using two plating techniques: a modified method of dilution plating (wet) and a direct dry soil plating (dry) isolation techniques, alongside two sampling times (pre-planting and post-harvest) and three sampling depths (2-12, 13-24, 25-37 cm). If differences can be observed in comparisons of field sampling and *V. dahliae* isolation techniques, lab practices and sampling recommendations can be augmented for improvement.

Materials and Methods

Field selection and timing of sampling: Two separate studies were conducted in Texas, USA and NSW, Australia to compare wet and dry isolation techniques using different selective media and various sampling depths (Table 1.1). The fields sampled in the USA were research fields in the first study and commercial fields in the second study. Fields sampled in the USA are located in Swisher, Hale, Floyd, and Lubbock counties (Figure 1.2). The fields in Australia were all commercial cotton fields located in the Namoi and Gywdir Valleys (Figure 1.3). Prior to planting, each of the Australian fields (Lam 9, Lam 10, CSD and Mid) were prepared into permanent raised beds in Australia. In the USA, fields Quaker, Halfway, Aiken A, and Aiken B were prepared into raised beds while SWN and SWS were no-till systems without raised beds.

Soil sampling protocol: In the first study, in each of the two fields sampled in Australia composite soil samples were collected from three transect areas of 100 m x 100 m. Due to the smaller size of the two USA research fields, composite soil samples were collected from one transect area of 100 m x 100 m three times. A total of 20 individual soil samples from each transect at each depth of 2-12 cm and 13-24 cm were bulked and mixed thoroughly. From each field in Australia and the USA, a total of six composite samples from both 2-12 cm and 13-24 cm depth were obtained. In the second study, three replicate samples of 20 individual soil samples were taken from three 400 m x 400 m transects and an additional depth of 25-37 cm was included in all Australian and USA fields. In Australia and the USA, the total number of composite samples from each field was nine samples per depth.

Media preparation: In the first study, each bulk soil sample was isolated on four media: 25% Potato Dextrose Agar (PDA) plus Novobiocin, acidified 25% PDA plus Novobiocin, acidified Sorensen's NP-10 and Sorensen's NP-10 media. **Sorensen's NP-10** medium was prepared using a modified method published by Kabir et al. (2004). The media recipe included 2 g $NaNO_3$, 0.5 KCL, 0.5 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4$, 20 g of base agar, and 5 g PGA: Polygalacturonic acid from orange (P-3889) was used in Australia and Polygalacturonic acid sodium salt from citrus (P-3850) was used in the USA. The pH was adjusted to 5.5 with NaOH before adding 2 mL Tergitol NP-10. Post autoclave, 200 mg of Streptomycin sulphate salt was added. Note Chloramphenicol, Chlortetracycline and Potassium nitrate were omitted. Acidified Sorensen's NP-10 was made with the addition of lactic acid which adjusted

the pH to 4.8-5.0. **25% PDA plus Novobiocin** medium was prepared using 1-L bottles, pre-autoclave 9.75g/L Potato Dextrose Agar, 11.25g/L Base Agar with 1 L of TKA Water. Post-autoclave 6.65mL/L Novobiocin stock solution (0.0175g/mL). The acidified PDA was made with the addition of lactic acid.

Optimization of soil testing protocol: Results from a previous study comparing sieved and un-sieved rolled soil found un-sieved soil recovered significantly more ppg than sieved soil. For this previous study, one soil sample was air dried at room temperature for 14 days to kill viable conidia and hyphae. Once dry, the soil was mixed thoroughly and rolled before being divided into two samples. One of the samples was sieved through a 2 mm mesh screen and the other un-sieved. Sieved and un-sieved rolled soil was subsampled five times for dry plating and ten times for dilution plating. The optimum weight of soil applied to each plate was determined using similar methods described by Butterfield and DeVay (1977), Goud and Termorshuizen (2003), and Kabir et al. (2004). Ten replicate subsamples weighed into 0.1, 0.2, 0.5 and 1.0 g lots were taken from each of the sieved and un-sieved soil then dry plated onto Sorensen's NP-10 media. The plates were incubated for 14 days in the dark at 23 ± 1°C before the soil was gently washed from the surface under running tap water. Each plate was examined using a dissecting microscope, x 20 magnification. Colonies were counted and multiplied by the dilution factor and averaged across all replicates.

Field soil sampling: Soil samples from each field in both studies were collected on the same day and placed into labeled bags then air dried for 14 days at

25°C to kill present viable conidia and hyphae (Butterfield & DeVay, 1977). Once dry, the soil was thoroughly mixed and rolled to achieve uniform small particle size. Mixing the soil is important when working with the small subsample sizes needed for the direct dry plating technique (Goud & Termorshuizen, 2003). Five subsamples of 0.2 g of soil (for dry plating) and 10 subsamples of 20 g of soil (for wet plating) were weighed out from each bulk soil sample collected at each depth.

Soil isolations: For dry plating, 0.2 g of dry soil was hand-spread uniformly across the surface of five Petri plates using the method described in full by Goud and Termorshuizen (2003). Each of the 5 plates per bulk soil sample represented a replicate. For the wet plating, the method described by Wheeler et al. (2012) was modified as follows: 20 g of dry soil was suspended in 80 mL water and mixed thoroughly. In each of the 10 Petri plates, 1 mL of soil solution was pipetted and spread using a turntable. Each of the 10 plates per bulk sample represented a replicate. The plates were sealed with parafilm before being incubated for 14 days in the dark at 23 ± 1°C for both the wet and dry plating methods. The soil was gently washed from the surface under running tap water. Each plate was examined using a dissecting microscope, x 20 magnification. Colonies were counted and multiplied by the dilution factor and averaged across replicates. Results were reported as propagules per gram (ppg) of soil.

Statistical analysis: For the optimum weight of soil, the number of microsclerotia per plate were analyzed as a generalized linear model with a log link function (poisson regression) using the "glm" function in R (R Core Team, 2019). The

model had sample weight (0.1, 0.2, 0.5, 1 grams), treatment (sieved, un-sieved) and their interaction as testable factors. For study 1 and 2, the inoculum level (ppg) was analyzed as a linear mixed model using the "lme" function in the nlme R package (Pinheiro et al., 2018). For study 1, there were four types of media used, so fixed terms were media, plating method (wet, dry), and depth. For study 2, the fixed terms were plating method (wet, dry), depth (2-12, 13-24, and 25-37 cm). To make comparisons between studies 1 and 2, we analyzed only the data for the one media (Sorensen's NP-10) in study 1 (Table 1.8 and Table 1.9).

Each soil sample came from one of three transects within a field, and each soil sample was split into sub-samples for testing by using different media (study 1) and plating method. Random terms to account for this were included in the model. The ppg data was squared root transformed as inspection of residual plots on untransformed data showed variation increased with ppg. Predicted means from above models were obtained using the "predictmeans" function from R package predictmeans (Luo et. al. 2018).

Results

Soil preparation methods: Sieving and the weight of soil per plate had significant effects (p=0.010) on the number of microsclerotia observed per plate (Figure 1.1). Un-sieved soil samples at each weight had greater numbers of propagules than sieved soil samples, but only significantly higher when 0.1 g was plated. The optimum soil weight per plate was 0.2 g using un-sieved soil as it provided the highest

detection of microsclerotia and was used in all subsequent soil preparations for both the Australian and USA isolations in this study.

Media and plating technique: In the first study, in Australia there was an interaction of media and plating technique (p=0.0001) (Table 1.2). There was no significant difference in ppg isolated using NP-10 or acidified NP-10. The number of ppg observed using PDA and acidified PDA were minimal and omitted. In the USA, there was an interaction of media and plating technique (p<0.001) where the wet plating technique had lower ppg on Sorenson's NP-10 at 2-12 cm and 13-24 cm, but not for the acidified NP-10 media at 13-24 cm. There was no significant difference in ppg isolated using wet or dry plating technique on NP-10 or acidified NP-10 (Table 1.3). In the second study, in Australia isolating using the dry plating technique resulted in significantly (p<0.0001) higher ppg recovered than isolations using the wet plating technique (Table 4) for pre-plant soil. In USA, the dry plating technique isolations had significantly (p<0.0001) higher ppg than wet plating technique isolations (Table 1.5) for post-harvest soil. Using the dry plating technique resulted in higher ppg than using the wet method in the USA (Table 1.9) and Australia (Table 1.8) at all depths and both sampling times.

Sampling depth: In the first study, there was no significant difference in inoculum levels at depths 2-12 cm (24 ppg) or 13-24 cm (21 ppg) for the Australian pre-plant soil or USA post-harvest soil with 5 ppg at 2-12 cm and 3 ppg at the 13-24 cm depth. In the second study, in Australian post-harvest soils, there was a significant (p=0.0148) difference in inoculum levels, using both the wet and dry plating method,

within the depth profile (Table 1.6) as inoculum levels increased with depth. In the second study, in USA pre-planting soils, the opposite trend was observed as ppg levels decreased with depth using both the wet and dry plating methods (Table 1.9). One of the four Australian fields in the second study that was suspected to have high incidence of Verticillium wilt, in fact had zero *V. dahliae* recovered in the soil isolations. Further plant isolations revealed the disease in the field was Fusarium wilt. The results of this field (Mid) were not included in the analysis. In the USA pre-plant soils, depth was also significant (p=0.0132) with inoculum decreasing as depth increased (Table 1.7).

Sampling time: Comparisons between fields and timing of sampling (pre-plant and post-harvest) showed no interaction of plating technique with depth. In Australia, the pre-planting inoculum levels decreased with depth but not significantly. Australian post-harvest inoculum levels increased with depth (Table 1.8). Inoculum levels were much higher pre-plant compared to post-harvest. In the USA, the inoculum levels decreased with depth pre-planting and post-harvest (Table 1.9).

Discussion

Rolling the soil after drying is needed to release the microsclerotia from soil particles in clay soils (Goud and Termorshuizen, 2003) and to ensure that soil particle size is consistent. However, over-milling can break up the microsclerotia. Less microsclerotia were detected in the sieved dry soil than in the un-sieved. These are similar findings to the unpublished results cited by Goud and Termorshuizen (2003).

The amount of soil plated onto media can influence the number of colonies observed and therefore influence the estimated inoculum levels in soil samples. In preparation of the dry, hand-spreading plating method, the weight of soil deposited directly onto the Sorensen's NP-10 media had a significant effect on the number of ppg detected in dry soil with larger amounts of soil having a negative impact on the number of colonies observed. The optimum weight of soil was 0.2 g per plate. These findings are in agreement with Goud and Termorshuizen (2003) and Kabir et al. (2004) but contradict the findings of Butterfield and DeVay (1977). Kabir et al. (2004) reported the amount of soil plated on media can influence the estimation of MS.

The first study quantified the inoculum levels at 2-12 cm and 13-24 cm depths using four different media. The results show that Sorensen's NP-10 media had the highest recovery of ppg in both USA and Australian soils (Table 1.2 & Table 1.3). Less inoculum was detected using the acidified Sorenson's NP-10 than Sorenson's NP-10 for both dry and wet plating methods for all Australian soil samples (Table 1.2). Polygalacturonic acid (PGA) is an important component of Sorensen's NP-10 media for quantifying *V. dahliae* in soil. However, not all types of PGA equally favor the growth and recovery of *V. dahliae* (Kabir et al., 2004). In the Australian components of this study, Sorenson's NP-10 media with P-3889 amended with NaOH to adjust the pH to 5.5 had high recovery of MS, similar to the findings of Kabir et al. (2004). Similarly, in the USA study, Sorenson's NP-10 with P-3850 amended with NaOH to adjust the pH to 5.5 also recovered high numbers of ppg. However, direct comparisons of soil between countries in the same laboratory could not be done due to

biosecurity restrictions. As differences in observed ppg have been shown between Sorenson's NP-10 medium prepared with P-3850 and P-3889 (Kabir et al., 2004), it is possible that the differences in observed ppg in Australia and the USA could be explained by the difference in PGA used in each laboratory.

Both dry and wet plating techniques require very basic laboratory equipment to prepare medium and incubate cultures; only a dissecting microscope is required to quantify colonies to estimate the inoculum levels in soil. Dry plating methods exist in two general categories: with the use of the Anderson Air Sampler and the use of hand-spreading to apply soil to media. Methods using the Anderson Air Sampler are described by Butterfield and DeVay (1977), DeVay et al. (1974) and Harrison and Livingston (1966). The Anderson Air Sampler (Thermo Anderson, Smyrna, Georgia, USA) was designed by Anderson (1958) and evenly distributes soil onto medium. An alternative hand-spreading method was described by Goud and Termorshuizen (2003) in a study comparing hand-spreading of soil directly onto media to using an Anderson Air Sampler where no significant difference was observed between the two methods. Use of the Anderson Air Sampler was foregone for the hand-spreading technique, which requires no specialized equipment and requires less time to perform. First described by Easton et al. (1969), wet plating techniques include spreading wet sieved soil over plates or suspending soil in water and spreading aliquots of the soil suspension over plates. The wet plating technique used in this work had two critical differences that could account for lower ppg recovered compared to the dry method. In this work, Petri dishes were sealed with parafilm once all isolations were completed

without a specified amount of drying time after aliquoting the soil suspension. The moisture sealed in by the parafilm could have inhibited germination of MS through high relative humidity inside the Petri dish, leading to an underrepresentation of MS in the soil.

In this study, plating technique had a significant effect on observed ppg. Isolation using dry plating consistently recovered higher ppg than the wet plating technique in both studies and for all soil samples in both the USA and Australia. The dry method used in this study required only half the amount of materials as the wet method and took less time to perform. In addition to this cost advantage over the wet method, the dry hand-spreading method does not require the use and upfront cost of the Anderson Air Sampler. It can be performed in any laboratory, requires no specialized equipment and is more accessible to a larger number of researchers. By comparing quantification methods, the efficacy and efficiency of laboratory practices can be improved to provide the most accurate representations of inoculum in the soil.

Stages of the cotton season are temporally opposite between Australia and the USA. In Australia, inoculum levels were estimated for two fields pre-planting in 2017 and four fields post-harvest in 2018, although one field had no Verticillium. In the USA, inoculum levels were estimated for two fields post-harvest in 2017 and four fields pre-planting in 2018. The microsclerotia that serve as inoculum are formed in dying and dead plant tissue. There were significant differences in the number of ppg observed in soils collected at pre-planting and post-harvest in both countries. In both the USA and Australia, higher inoculum levels were detected in soils sampled prior to

planting compared to post-harvest. The higher inoculum density pre-plant and lower ppg post-harvest reported here are consistent with those published by Evans et al. (1967) and Evans and McKeen (1975) where inoculum levels in cotton fields naturally fluctuate throughout the season. MS are generally higher at planting, reducing through the growing season, then increasing post-harvest as infested plant material returns to the soil (Pegg & Brady, 2002). MS can survive without a plant host in the soil for more than 10 years (Pegg & Brady, 2002).

Inoculum levels differed in soil collected from various depths in the soil profile. In the first study, there was little difference in ppg observed at two sampling depths (2-12 cm and 13-24 cm) in Australia pre-planting, with slightly higher numbers found in the 2-12 cm depth. Significantly higher ppg were recorded in the USA soil in the 2-12 cm depth at both pre-plant and post-harvest. In the second study, higher numbers of inoculum were recorded at the 25-37 cm depth in post-harvest Australian soils. This opposite occurrence may be attributed to the movement of infected plant tissue from the surface to lower depths during the mechanical incorporation of plant matter into the soil following harvest. As inoculum levels fluctuate throughout the season, it is possible that the portion of the soil profile in which the highest concentration of inoculum can be found changes as well. This may be due to tillage practices, as the fields used in this study included no-till and traditional tillage systems. Irrigation practices may play a role in the vertical distribution of *V. dahliae* inoculum as well. The fields in this study included subsurface drip, furrow, and overhead pivot irrigation systems. Differences in cropping history among the fields

tested may account for some variation among quantified MS. In this study, soil was sampled to a depth of 35 cm. Other studies suggest a deeper sampling depth may be appropriate. A three-year study conducted in Texas, USA found that observed *V. dahliae* MS increased with depth, finding the highest concentrations of MS at 46-61 cm sampling depth and lowest at 0-16 cm and 16-31 cm in some instances (Albers, 2013). Additional studies examining the temporal and depth positioning of *V. dahliae* populations are warranted.

The soil types sampled differed between countries. Australian soils consisted of heavy grey vertosols and grey/red vertosols as described by The Australian Soil Classification (Commonwealth Scientific and Industrial Research Organisation, 2009). All soils sampled in the USA were Pullman clay loams as described by the United States Soil Taxonomy (Soil Survey Staff, 1999). While there may be an effect of soil type on *V. dahliae* MS survival and Verticillium wilt incidence and severity, it is "difficult to exclude" the possible effect of irrigation amount, frequency, and method of application as well as the effect of soil temperatures (Pegg & Brady, 2002).

The results of this study show that sampling for *V. dahliae* inoculum should be done prior to planting and from a depth of 2-12 cm in order to provide the best indication of the level of inoculum that poses a risk of infection throughout the growing season. Sorensen's NP-10 media amended with NaOH to adjust the pH to 5.5 using either wet or dry plating technique is suitable for quantifying inoculum in soil. Differences in inoculum detected are prevalent using different methods. Thus, laboratories doing repeat soil isolations to estimate inoculum levels should select one

method to use consistently which will allow comparisons over time and between locations.

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Tables and Figures

Table 1.1. Field name, irrigation type, soil type, and location of fields sampled in 2017 and 2018 at pre-planting and post-harvest in Australia and the USA.

Time of Sampling	Australia	USA
October 2017	Pre-planting	Post-harvest
	Lam 9: Furrow Irrigated Grey/Red vertosol Namoi Valley, NSW	Halfway: Pivot Irrigated Pullman clay loam Hale County, TX
	Lam 10: Furrow Irrigated Grey/Red vertosol Namoi Valley, NSW	Quaker: Subsurface drip irrigation Pullman clay loam Lubbock County, TX
AUS: June 2018 USA: April 2018	Post- harvest	Pre-planting
	Lam 9: Furrow Irrigated Grey/Red vertosol Namoi Valley, NSW	Aiken A: Furrow Irrigated Pullman clay loam Floyd County, TX
	Lam 10: Furrow Irrigated Grey/Red vertosol Namoi Valley, NSW	Aiken B: Furrow Irrigated Pullman clay loam Floyd County, TX
	CSD: Pivot Irrigated Grey/Red vertosol Namoi Valley, NSW	Swisher N: Pivot Irrigated Pullman clay loam Swisher County, TX
	Mid: Pivot Irrigated Heavy grey vertosol Gwydir Valley, NSW	Swisher S: Pivot Irrigated Pullman clay loam Swisher County, TX

Table 1.2. Average number of germinated *V. dahliae* microsclerotia in propagules per gram (ppg) of Australian pre-plant soil, isolated using two plating methods.

Media	Plating Technique	Australian pre-plant ppg [z]
NP-10	Dry	28.0 A
	Wet	18.0 B
acidified NP-10	Dry	0.2 C
	Wet	0.3 C

[z] Within a column, values marked with different letters are significantly different based on the least squared difference test ($p \leq 0.05$).

Table 1.3. Average number of germinated *V. dahliae* microsclerotia in propagules per gram (ppg) observed in USA post-harvest soil using two plating methods.

Media	Plating Technique	USA post-harvest ppg [z]
NP-10	Dry	7.9 A
	Wet	1.4 B
acidified NP-10	Dry	7.8 A
	Wet	0.6 C
PDA	Dry	0.1 D
	Wet	0.0 D
acidified PDA	Dry	0.0 D
	Wet	0.0 D

[z] Within a column, values marked with different letters are significantly different based on the least squared difference test ($p \leq 0.05$).

Table 1.4. Inoculum quantified in propagules per gram (ppg) in Australian post-harvest soil using wet and dry plating techniques.

Plating Technique	Australian post-harvest ppg [z]	USA pre-planting ppg [z]
Dry	8.6 A	39.9 A
Wet	3.4 B	16.1 B

[z] Within a column, values marked with different letters are significantly different based on the least squared difference test ($p \leq 0.05$).

Table 1.5. Inoculum quantified in propagules per gram (ppg) at three soil depths in USA pre-planting and Australian post-harvest cotton soils.

Sampling Depth ----------cm---------	USA pre-planting ppg [z]	Australian post-harvest ppg [z]
2-12	35.4 A	3.7 A
13-24	27.5 AB	5.3 A
25-37	18.5 B	8.8 B

[z] Within a column, values marked with different letters are significantly different based on the least squared difference test ($p \leq 0.05$).

Table 1.6. Inoculum quantified in propagules per gram (ppg) in pre-planting and post-harvest sampling times in Australian cotton soils using Sorenson's NP-10 media.

Plating Technique	Sampling Depth --------cm--------	Australian pre-planting ----------ppg [z]----------	Australian post-harvest ----------ppg [z]----------
Dry	2-12	30.4 A	5.4 BC
	13-24	25.7 AB	7.9 B
	25-37		13.6 A
Wet	2-12	18.5 BC	2.3 D
	13-24	17.4 C	3.2 CD
	25-37		5.1 BC

[z] Within a column, values marked with different letters are significantly different based on the least squared difference test ($p \leq 0.05$).

Table 1.7. Inoculum quantified in propagules per gram (ppg) in pre-planting and post-harvest sampling times in USA cotton soils using Sorenson's NP-10 media.

Plating Technique	Sampling Depth --------cm--------	USA pre-planting ----------ppg [z]----------	USA post-harvest ----------ppg [z]----------
Dry	2-12	51.2 A	10.8 A
	13-24	43.8 A	6.1 A
	25-37	27.0 B	
Wet	2-12	22.6 BC	2.2 B
	13-24	15.1 CD	0.7 C
	25-37	11.6 D	

[z] Within a column, values marked with different letters are significantly different based on the least squared difference test ($p \leq 0.05$).

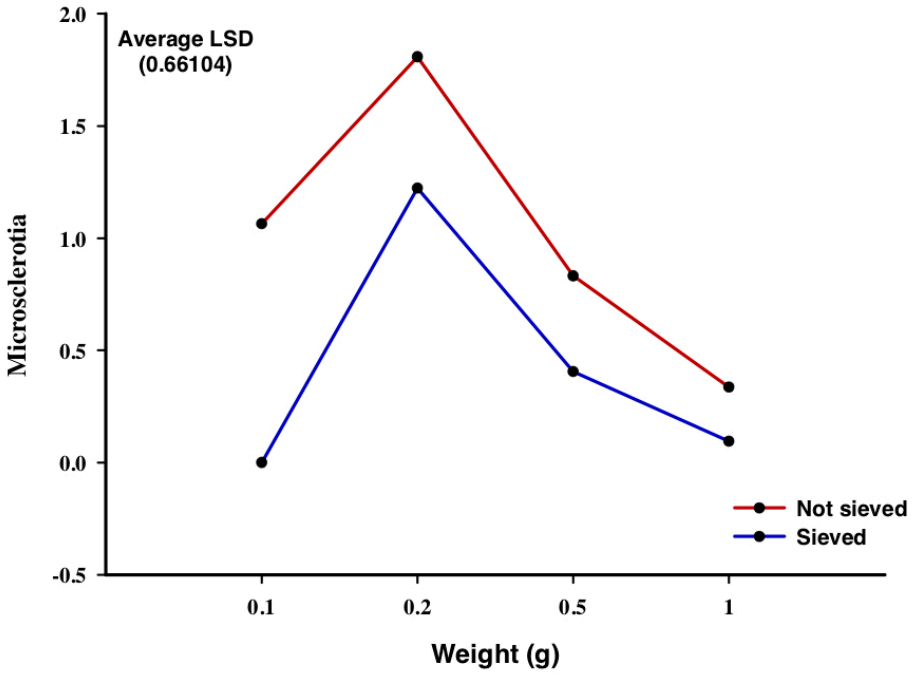

Figure 1. Number of microsclerotia (MS) log scale observed per plate using 0.1, 0.2, 0.5 and 1.0 g sieved and un-sieved soil per plate. Microsclerotia per plate log values are the average of 10 replicate plates.

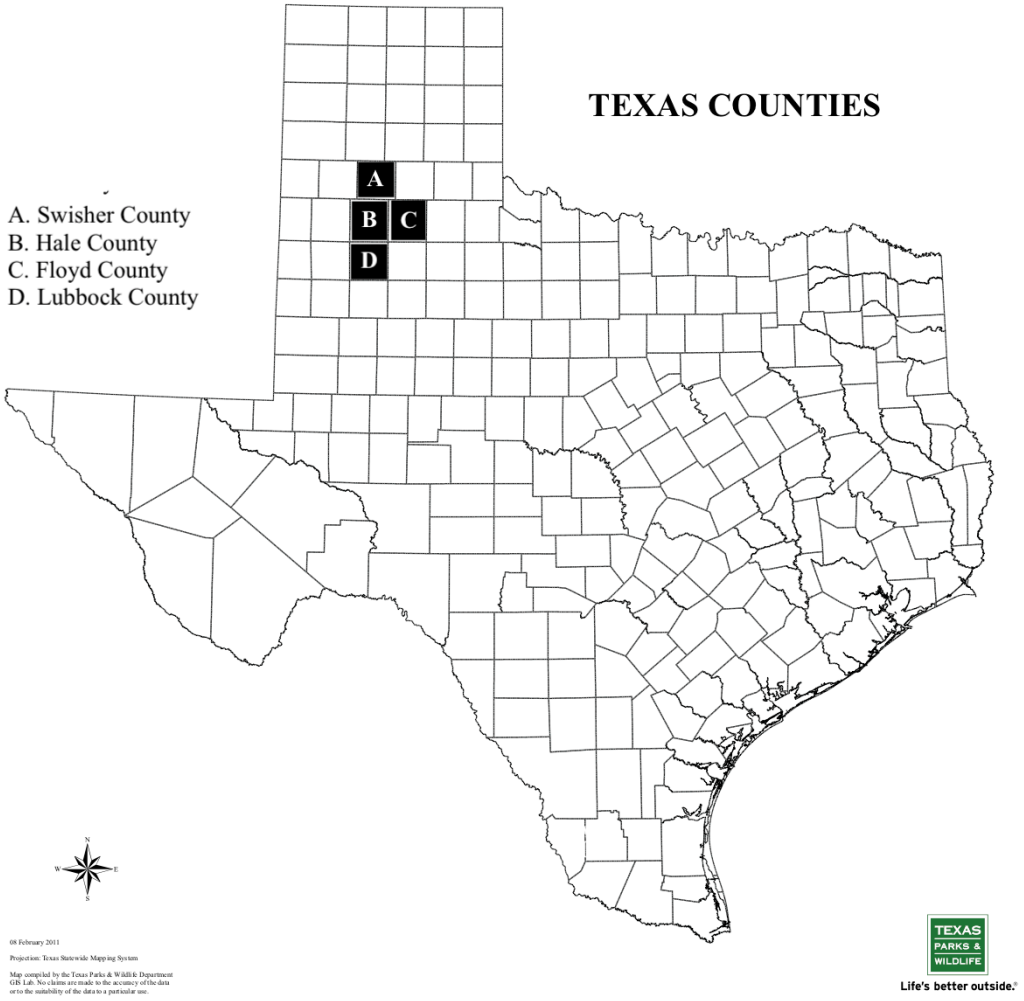

Figure 2. Map of county boundaries in Texas. Fields sampled are located in Swisher, Hale, Floyd, and Lubbock Counties, as indicated.

Note: Adapted from Texas Parks & Wildlife. (n.d.). "Texas - County Outline Map, without county names." Retrieved from https://legacy.lib.utexas.edu/maps/texas.html

Figure 3. Cotton growing regions of Australia. Fields sampled are located in the Gywdir and Namoi Valleys, as indicated.

Note: Adapted from Stiller, W. N., & Wilson, I. W. (2014, July 02). Australian Cotton Germplasm Resources. Retrieved from https://www.intechopen.com/books/world-cotton-germplasm-resources/australian-cotton-germplasm-resources.

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APPENDIX:

EFFECT OF PLANTING DATE, SEEDING RATE, AND CROP PROTECTION PRODUCT TREATMENTS AT PLANTING ON COTTON YIELD

Introduction

Determining the value of fungicide seed treatment combinations is vital in helping cotton producers of the Texas High Plains make the most informed, effective, and cost-efficient management decisions. This study aimed to see if we could enhance emergence on the small-seeded variety FM 1830GLT through the manipulation of planting date and seeding rate and the application of four treatments consisting of combined crop protection products from Bayer CropScience. Each treatment provided increasing levels of protection from pests including fungal pathogens, nematodes, and insects.

Materials and Methods

Field trials were conducted in 2017 and 2018 in the Texas High Plains to evaluate potential for improved emergence on FM 1830GLT with a combination of agronomic practices and Bayer crop protection products. Two fields in 2017 (Halfway and Quaker) and three fields in 2018 (Halfway, Quaker, and FBRI Drip) were selected, having a history of seedling disease caused by *Rhizoctonia solani.* Halfway is irrigated by overhead pivot; Quaker and FBRI Drip are irrigated through subsurface drip systems.

Treatments consisting of two planting dates (early and average), four chemical seed treatments (ranging from base to premium), and two seeding rates (2 and 4 seed per foot) were arranged in a split-split-split plot design with four replications in each field. The four crop protection product treatments were (1) base fungicide treatment, (2) base + CoPeO, (3) base + CoPeO + ATA, (4) base + CoPeO + ATA + Velum Total. Descriptions and application rates of the products in the four treatments are expressed in Table A.1.

Planting date served as whole plots, seed treatment served as sub-plots, and planting density as sub-sub-plots. Plots were 2-rows wide by 35 feet long on 40-inch centers; 5-foot alleys separated blocks. Premium seed treatments were applied with a modified Hege Seed Treater. Velum Total was applied as an in-furrow treatment. All other production practices were in accordance with local extension recommendations.

Seed cotton yields were estimated using a Case IH 95 stripper equipped with load cells. Plot harvest weights are expressed as pounds of seed cotton per acre.

Results and Discussion

No significant differences in yield were observed in year, location, and planting date for chemical treatment and the interaction of chemical treatment and planting rate (Table A.2). Planting rate alone had a significant effect on yield in every instance except the second planting date at the FBRI farm in 2017 (Table A.2). The base fungicide treatment, Treatment 1, provided ample protection against early season cotton pests as the additional products in Treatments 2, 3, and 4 showed no significant difference (Table A.2). Additional products should be added on a case-by-case basis,

depending on the pests present and at what pressure in the field. Additional studies further exploring the relationships between crop protection products, planting dates, and seeding densities are warranted.

Tables and Figures

Table A.1. Description of crop protection product treatments.

Entry No.	Entry/Treatment Description	Form. Type	AI Conc.	Dose Unit	Trans. Dose	Trans. Dose Unit
1	CALCIUM CARBONATE	WP	100	G/100 KG		
	SUSPENDING AGENT	WP		G/100 KG		
	COLOR COAT WHITE	FS		ML/100 KG		
	PRO-IZED BLUE COLORANT	FS		ML/100 KG		
	SPERA	FS	240	ML/100 KG		
	GAUCHO	FS	600	ML/100 KG		
	PROLINE 480 SC	SC	480	G A/100 KG		
	SECURE PLUS SEED GLOSS 661	FS		ML/100 KG		
	EVERGOL PRIME	FS	240	G A/100 KG		
	ALLEGIANCE FL	FS	318	ML/100 KG		
2	CALCIUM CARBONATE	WP	100	G/100 KG		
	SUSPENDING AGENT	WP		G/100 KG		
	COLOR COAT WHITE	FS		ML/100 KG		
	PRO-IZED BLUE COLORANT	FS		ML/100 KG		
	SPERA	FS	240	ML/100 KG		
	GAUCHO	FS	600	ML/100 KG		
	PROLINE 480 SC	SC	480	G A/100 KG		
	SECURE PLUS SEED GLOSS 661	FS		ML/100 KG		
	EVERGOL PRIME	FS	240	G A/100 KG		
	ALLEGIANCE FL	FS	318	ML/100 KG		
	FLUOPYRAM 600 FS	FS	600	MG A/SEED		
3	CALCIUM CARBONATE	WP	100	G/100 KG		
	SUSPENDING AGENT	WP		G/100 KG		
	COLOR COAT WHITE	FS		ML/100 KG		
	PRO-IZED BLUE COLORANT	FS		ML/100 KG		
	SPERA	FS	240	ML/100 KG		
	GAUCHO	FS	600	ML/100 KG		
	PROLINE 480 SC	SC	480	G A/100 KG		

Table A.1. Continued

	SECURE PLUS SEED GLOSS 661	FS		ML/100 KG		
	EVERGOL PRIME	FS	240	G A/100 KG		
	ALLEGIANCE FL	FS	318	ML/100 KG		
	AERIS SEED APPLIED SYSTEM	FS	600	MG A/SEED		
	TRILEX ADVANCED FS300	FS	300	ML/100 KG		
	FLUOPYRAM 600 FS	FS	600	MG A/SEED		
4	CALCIUM CARBONATE	WP	100	G/100 KG		
	SUSPENDING AGENT	WP		G/100 KG		
	COLOR COAT WHITE	FS		ML/100 KG		
	PRO-IZED BLUE COLORANT	FS		ML/100 KG		
	SPERA	FS	240	ML/100 KG		
	GAUCHO	FS	600	ML/100 KG		
	PROLINE 480 SC	SC	480	G A/100 KG		
	SECURE PLUS SEED GLOSS 661	FS		ML/100 KG		
	EVERGOL PRIME	FS	240	G A/100 KG		
	ALLEGIANCE FL	FS	318	ML/100 KG		
	AERIS SEED APPLIED SYSTEM	FS	600	MG A/SEED		
	TRILEX ADVANCED FS300	FS	300	ML/100 KG		
	FLUOPYRAM 600 FS	FS	600	MG A/SEED		
	VELUM TOTAL	SC	440	G A/HA	14	OZ/A

Table. A.2. P values for year, location, planting date, Type III Tests of Fixed Effects.

		2018		2017	
Location	Factor	Planting 1	Planting 2	Planting 1	Planting 2
Halfway	rate	<.0001*	<.0001*	<.0001*	<.0001*
	treatment	0.1546	0.2457	0.7572	0.4722
	rate*treatment	0.1052	0.2554	0.5706	0.3513
Quaker	rate	0.0026*	<.0001*	<.0001*	<.0001*
	treatment	0.4418	0.0577	0.7882	0.557
	rate*treatment	0.0763	0.2016	0.8361	0.0734
FBRI	rate	0.0019*	0.128		
	treatment	0.2765	0.6634		
	rate*treatment	0.0773	0.3414		

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