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Short title: The fitness of *C. albifundus* in nature

Variation in growth rates and aggressiveness of naturally occurring self-fertile and self-sterile isolates of the wilt pathogen *Ceratocystis albifundus*

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Abstract

Ceratocystis albifundus is the most important fungal pathogen of black wattle (*Acacia mearnsii*) grown in plantations in Southern and Eastern Africa. It is a homothallic fungus but also undergoes uni-directional mating type switching. As a result, the ascospore progeny can be either self-fertile or self-sterile. The only apparent difference between these mating types is the deletion of the *MAT1-2-1* gene in self-sterile isolates. There is some evidence suggesting that self-sterile isolates grow more slowly than self-fertile isolates, but this has not been tested rigorously. The aim of this study was to determine whether self-sterile isolates are less fit by examining growth rate, relative germination rate and pathogenicity. Five self-sterile isolates were generated from each of five self-fertile isolates of *C. albifundus* and these 30 isolates were compared. The results showed that the self-sterile isolates grew consistently slower and were less pathogenic than the self-fertile isolates. The germination ratio of self-fertile to self-sterile isolates from single ascospores collected from the ascomata of five self-

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fertile isolates was on average 7:3. This could be a consequence of the self-sterile isolates having a lower germination rate. This observation, and the lower growth and pathogenicity levels, suggests that self-sterile isolates are not likely to compete effectively in nature, raising intriguing questions regarding their role and value to *C. albifundus* and other fungi having a similar mating system.

Introduction

Ceratocystis albifundus is an important fungal pathogen, posing a considerable threat to forestry plantations based on Australian *Acacia mearnsii* trees. This fungus is the causative agent of black wattle wilt disease in Southern and Eastern Africa (Morris *et al*., 1993; Wingfield *et al*., 1996; Roux & Wingfield, 1997; Roux *et al*., 2005). Disease symptoms that develop after infection by *C. albifundus* include the formation of cankers and lesions in the bark, gum exudation and streaked discoloration of the xylem associated with colonisation of the vascular tissue. Infected trees can wilt and die within six weeks after infection (Roux *et al*., 1999).

Mating in ascomycetes is strictly controlled by a single mating locus with two allelic or idiomorphic forms, *MAT1-1* and *MAT1-2,* which represent alternate alleles that differ in length, sequences and structure (Turgeon *et al*., 1993; Coppin *et al*., 1997). *Ceratocystis albifundus* is a homothallic fungus that is capable of selfing, enabling it to produce sexual offspring without having a partner of opposite mating type. This is possible because fertile isolates have all three *MAT* genes; *MAT1-1-1*, *MAT1-1-2* and *MAT1-2-1*. Furthermore, uni-directional mating type switching can occur during meiosis, giving rise to self-sterile mating type strains carrying only *MAT1-1-1* and *MAT1-1-2* genes as a result of the deletion of the *MAT1-2-1* gene. The *MAT1-2-1* gene is entirely lost from the genome and these isolates are thus unable to revert back to their self-fertile status (Perkins, 1987; Harrington & McNew, 1997; Witthuhn *et al*., 2000; Wilken *et al*., 2014). Wilken *et al*. (2014) further showed that self-fertile isolates of *C. fimbriata* are genetically identical to the parent strain, while the only apparent difference in the self-sterile isolates and the parent isolate is that they lack the *MAT1-2-1* gene from their entire genome.

Homothallic fungi do not require a compatible mating partner for sexual reproduction due to the ability to self-mate with their own mitotic descendants, giving rise to genetically identical progeny (Lin & Heitman, 2007; Billiard *et al*., 2011). In this regard, the haploid selfing in homothallic mating systems could result in low levels of genetic diversity and a likelihood of inbreeding depression. Nevertheless, many fungi are homothallic and this form of reproduction is common in nature (Nelson, 1996; McGuire *et al*., 2001; Gioti *et al*., 2012). In the case of uni-directional mating type switching, found in fungi such as *C. albifundus* where haploid selfing as well as outcrossing can occur, it might thus be argued that the self-sterile

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isolates are forced to mate with other isolates, in this way increasing genetic diversity within a population, although fungi having the homothallic mating system do have the potential to outcross. There is, however, some evidence that the self-sterile isolates are less fit than self-fertile isolates (De Beer, 1994; Roux, 1996; Harrington & McNew, 1995, 1997; Witthuhn *et al*., 2000). But this issue has not been tested for *Ceratocystis* species.

Fitness of microorganisms is characterised by phenotypic and genotypic traits heritable from generation to generation through successful reproduction, and consequently successful survival in a given environment (Pringle & Taylor, 2002). It has been suggested that growth rate is a simple and convenient means to measure the fitness of fungal pathogens (Pringle & Taylor, 2002). In addition, spore production rate and pathogenicity have been applied as quantitative traits to estimate the fitness of fungal pathogens (Pariaud *et al*., 2009). That is, based on the adaptive strategies of fungal pathogens, some individuals can be overshadowed by others having more competitive advantages. These would include faster growth and higher levels of pathogenicity, which ultimately facilitates more effective host colonization (Lockhart *et al*., 2005; Meyer *et al*., 2010).

There are many phenotypic differences between self-fertile and self-sterile mating type strains in filamentous ascomycetes. These, for example, include ascospore size and dimorphism, and formation of fruiting bodies, as well as more rapid linear growth rates (Webster & Butler, 1967; Uhm & Fujii, 1983; Perkins, 1987; Harrington & McNew, 1995, 1997; Witthuhn *et al*., 2000). Specifically, Witthuhn *et al.* (2000) reported that self-fertile mating strains in some *Ceratocystis* species have more rapid linear growth than the self-sterile isolates. In this study we considered the possible differences in fitness between self-fertile and self-sterile strains of *C. albifundus* by comparing growth rates, levels of aggressiveness in inoculation studies and germination percentage.

Materials and Methods

Collection and identification of isolates

All strains, CMW39129, CMW39130, CMW39131, CMW39132 and CMW39133, of the wilt pathogen *C. albifundus* used in this study were collected on the Bloemendal Experimental farm, near Pietermaritzburg in the KwaZulu-Natal Province of South Africa, where *Acacia mearnsii* trees are propagated commercially. Pieces of bark, bearing characteristic ascomata of *C. albifundus* were removed from bark flaps on tree stumps of recently felled trees. Cultures were made from the spore drops that were produced at the apices of the ascocarps collected from different trees. These were transferred directly onto separate Petri-dishes (65mm) containing 2% MEA (20g malt extract, Biolab, Midrand, South Africa, 20g agar, Difco Laboratories, Detroit, Mich) supplemented with 100mg/L Streptomycin sulphate (SIGMA-ALDRICH, Steinheim, Germany), and incubated at 25°C for

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2 weeks in the dark. All the isolates used in this study were deposited in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Five single ascospore isolates, from five different trees, producing prolific numbers of ascomata were chosen for study. These were identified based on morphology and using DNA sequence comparisons as described below. *Ceratocystis albifundus* can be distinguished from all other *Ceratocystis* species based on its characteristic light/cream coloured ascomatal bases, bearing black necks and producing hat shaped ascospores (Wingfield *et al*., 1996). The five selected isolates were then identified using sequences of the Internal Transcribed Spacer Regions (ITS1, ITS2) and 5.8S rDNA gene regions.

All *C. albifundus* cultures were incubated for two weeks to facilitate mycelial growth. Mycelium of the fungus was scraped from the surfaces of Petri dishes with sterilized surgical scalpel blades, transferred into 1.5ml Eppendorf tubes and ground to a fine powder with a pestle. Genomic DNA was extracted using PrepMan™ Ultra (Applied Biosystem, Foster City, CA) following the manufacturer's instructions. The quantity and quality of DNA extracted was assessed with a NanoDrop ND- 1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) to calibrate the concentration and purity of DNA as PCR templates.

The PCR amplification reactions were conducted on a Veriti 96-well Thermal Cycler (Applied Biosystems, Foster City, California, USA). The total volume of each PCR reaction mixture was 15 µl, containing 0.5 µl of genomic DNA, 0.3 µl (10 pM) of each primer (Forward and Reverse), 3 µl MyTaq PCR buffer (Bioline GmbH, Germany) and 0.09 µl of MyTaq DNA Polymerase (Bioline GmbH, Germany). The PCR cycling profile consisted of an initial denaturation at 97°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. A final extension was performed at 72°C for 10 min. Sequencing was done in both directions using primers, ITS1F and ITS4 (White *et al*., 1990) and the Big Dye® Terminator 3.1 cycle sequencing premix kit (Applied Biosystems, Foster City, California, USA) according to the instructions provided by the manufacturer. Sequences were determined using an ABI PRISIM 3100 Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Sequencing results were compared with published sequences of *C. albifundus*, obtained from Genbank, using BioEdit 7.0.9.0 Sequence Alignment Editor (Hall, 1999).

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Mating type proportion from single ascospore progenies

Single ascospore cultures were made directly from ascomata produced by the five self-fertile isolates confirmed to be *C. albifundus*. Ascospore masses transferred from the apices of the ascomata were suspended in 50 µl Isoparaffin solvent, Soltrol® 130 (Chemfit, Gauteng, South Africa) to facilitate dispersal (Whitney & Blauel, 1972) of the hydrophobic *Ceratocystis* ascospores. About 5 µl of spore suspension was streaked over the surfaces of 3.5% WA (35g Agar, Difco Laboratories, Detroit, Mich.) in 90mm Petri dishes using with a platinum wire loop and incubated at 25°C for 72 hours until germination of single ascospores was observed. Agar disks containing individual germlings were excised with an aseptic syringe needle and transferred onto 2% MYEA (20g malt extract, Biolab, Midrand, South Africa, 2g yeast extract, Biolab, Midrand, South Africa, 20g agar, Difco Laboratories, Detroit, Mich). Fifty single ascospore isolates were made from each of five parental isolates of *C. albifundus* and these represented self-fertile and self-sterile mating type progeny. The plates were incubated at 25°C for two weeks, and the ratio of self-fertile to self-sterile strains of *C. albifundus* was calculated.

Both morphological characters and molecular characterization was used to ensure the mating type identity of the self-fertile and self-sterile strains obtained from each of the five parental isolates. The presence or absence of ascomata was used as an initial indication of the mating type of the isolates (Webster & Butler, 1967). Two primer sets, Albi_MAT1-2_F, Albi_MAT1-2_R, and Albi_MAT2-1_F, Albi_MAT2-1_R were used to amplify the *MAT1-1-2* and *MAT1-2-1* genes in the *MAT* locus in five representative isolates from each of the mating types (Table 1). The amplification reactions were conducted on a Veriti 96-well Thermal Cycler. The total volume of the PCR reaction mixtures were 15 µl, containing 0.5 µl of genomic DNA, 0.3 µl (10 pM) of each primer (Forward and Reverse), 3 µl MyTaq PCR buffer (Bioline GmbH, Germany) and 0.09 µl of MyTaq DNA Polymerase (Bioline GmbH, Germany). The thermocycling profile for amplification of *MAT* genes consisted of an initial denaturation at 97°C for 1 min, followed by 35 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. A final extension was performed at 72°C for 7 min. The amplification products were analyzed by electrophoresis using a Mini-Sub Cell GT Cell (Bio-Rad, Hercules, CA, USA) and a Hoefer Scientific PS500X DC Power Supply (Hoefer Scientific, San Francisco, CA) on a 1.5% (w/v) agarose gel in 1 × TAE buffer (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA at pH 8.0), and then visualized using a Bio-Rad Gel Doc™ EZ imager and Image Lab™ software (version 3.0).

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Assessment of growth rates

Five confirmed self-sterile isolates from each of the five self-fertile parental strains, as well as the parental strains themselves, were compared for growth rate. Growth rates were assessed by transferring 5mm plugs from the edges of actively growing cultures onto the centres of 75mm Petri dishes containing 2% MYEA (20g malt extract, Biolab, Midrand, South Africa, 2g yeast extract, Biolab, Midrand, South Africa, 20g agar, Difco Laboratories, Detroit, Mich). The cultures were then incubated at 25°C for two weeks. Two measurements for colony diameter at right angles to each other were taken for each of the 25 self-sterile and five self-fertile isolates, daily for two weeks. The average growth rate and standard deviations were then calculated. The entire experiment was repeated once.

Assessment of aggressiveness

In order to detect possible differences in aggressiveness between self-fertile and self-sterile mating type stains of *C. albifundus*, inoculation trials were conducted on ~ two-year-old *A. mearnsii* trees located in a jungle stand near Pretoria East, Gauteng, South Africa. Trees of ~ 70mm diameter were inoculated at ~ 1.5m above ground. A total of 50 trees were inoculated with each of the five parental self-fertile strains (250 trees) and 20 trees were inoculated with each of 25 self-sterile isolates, five of which were derived from each of the five parental strains (total 500 trees). Twenty trees were used as controls by inoculating them with sterile MY agar.

Discs of agar bearing mycelium were taken from the margins of actively growing, 2-week-old cultures with a 7mm cork borer. For the inoculations, a wound was made on the stems of trees, using a 7mm cork borer, to expose the cambium and the agar discs overgrown with the test strain was inserted into the wounds with the mycelium facing the cambium. All inoculation points were covered with masking tape to prevent desiccation of the inoculum and cambium and to reduce contamination.

The inoculation study was conducted in two separate trials established in November 2012 and in December 2012, respectively. The results were assessed after six weeks during December 2012 and January 2013, respectively. Re-isolations were made from two trees for each treatment and the resulting fungi were identified to ensure that the inoculated fungi were responsible for the infections observed (Koch's postulates).

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Statistical Analyses

A chi-square analysis was used to test for statistical support of possible differences in the proportion of each mating type emerging from the single ascospore isolations. 95% confidence was accepted as an acceptable level of significance. The value for the degrees of freedom was established based on the number of mating types (self-fertile and self-sterile). The null hypothesis was that no significant differences would be observed in the production of self-fertile and self-sterile mating type progeny from single ascospore isolations. A test of independence was also conducted to determine whether there was a significant difference in each single ascospore isolation trial.

Welch's two-sample t-test and Analysis of variance (ANOVA) and Tukey's honest significance test (Tukey's HSD) were used to determine whether there were significant differences in the growth rates or aggressiveness of self-fertile and self-sterile mating types based on a P-value computed using the The R Stats Package (version 2.5.1; http://www.r-pro ject.org/) (R Core Team, 2012). In addition, General Linear Models (GLM) using the The R Stats Package (version 2.5.1; http://www.r-pro ject.org/) were applied to test the hypothesis that the ability of the self-fertile mating type isolates to grow faster is closely correlated with a higher level of aggressiveness compared to the self-sterile mating type isolates.

Results

Collection and identification of isolates

Ceratocystis albifundus isolates were successfully obtained from the stumps of recently (~four-week-old) harvested *A. mearnsii* trees. Fruiting bodies produced on MYEA medium had morphological features typical of *C. albifundus,* including light coloured ascomatal bases (Wingfield *et al*., 1996). This identification based on morphology was supported by ITS sequence data where the maximum percentage identity of the isolates was almost identical (99%) when blasted against those of *C. albifundus* strains in NCBI. All sequence data obtained in this study have been deposited in NCBI (accession numbers, KF147144 to KF147148).

Mating type proportion from single ascospore progenies

In total, 50 single ascospore progeny were recovered from each of the five parental self-fertile mating type isolates. The segregation ratio of self-fertile to self-sterile isolates was significantly biased towards the self-fertile mating type for four of the parental strains (CMW39129, CMW39130, CMW39131 and CMW39132). An exception was found for isolate CMW39131 in the second trial and CMW39133 in the both trials, which had a

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segregation ratio close to 1:1 for self-fertile and self-sterile mating types (Table 2). The greatest deviation from a 1:1 segregation ratio was found for isolates CMW39130 and CMW39131 in the first trial and CMW39132 isolate in the second trial, where this ratio was approximately 3:1 Overall, the segregation ratio of self-fertile to self-sterile was about 1.5:1 on average (Table 2). The biased segregation towards the self-fertile mating type was supported statistically based on chi-square analysis at a 95% confidence level (Table 2).

Assessment of growth rates

All the self-fertile isolates exhibited more rapid growth in comparison to the five self-sterile isolates derived from each of them, although isolates representing each of the two mating types had similar growth rates and produced similar growth patterns on MYEA at 25°C (Figure 1). These observations were also strongly supported statistically (Welch Two Sample t-test: P-value < 0.001).

Assessment of aggressiveness

In both inoculation trials, self-fertile isolates consistently produced longer lesions than the self-sterile isolates derived from them (Data shown only for the 2nd trial which were identical to those of the first trial; Figures 2, 3). Self-fertile strains resulted in distinct black lesions on the stems, gum exudation and streaking in the cambium six-weeks after inoculation. In contrast, self-sterile strains were not found to be associated with the same severe symptoms. Re-isolations from the lesions consistently yielded self-fertile and self-sterile isolates morphologically indistinguishable from the inoculated isolates. The hypothesis that there is a significant difference in aggressiveness between self-fertile and self-sterile isolates was statistically supported in the Analysis of variance (Probability = 2E-16) and Tukey's honest significance test (P-value = 0 at the 95% confidence level).

Correlation of growth and aggressiveness

Four measurements including growth rate and aggressiveness of isolates, representing both mating types, were applied to GLM tests available in the R Stats Package (version 2.5.1; http://www.r-pro ject.org/) (R Core Team, 2012). The results showed that self-fertile isolates have superior fitness based on growth rate as well as pathogenicity. This was evident from a significant correlation between growth rate and aggressiveness of self-fertile and self-sterile isolates based on GLM tests (Coefficient = 4.534, Standard error = 1.031, t-value = 4.397, Probability (>|t|) = 0.002).

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Discussion

In this study, we showed that there are significant differences in growth and aggressiveness between self-fertile parental and self-sterile offspring strains of the wilt pathogen *Ceratocystis albifundus*. These parental and offspring strains are essentially identical because this fungus is haploid, although we recognise that minor differences in the strains could have resulted as the consequence of sexual recombination. Self-sterile isolates of *C. albifundus* lack a single gene at the mating type locus. This deletion appears to have the intriguing outcome, resulting in self-sterile isolates being significantly less fit than their self-fertile counterparts.

Fungal pathogens that reproduce sexually would typically be expected to produce equal frequencies of progeny representing the two different mating types (Fisher, 1930). The same results have been reported for *C. coerulescens* (Harrington & McNew, 1997). In contrast, isolates of *C. albifundus* studied here showed a significant bias towards self-fertile mating. A similar bias is known in other fungal pathogens (Kwon-Chung & Bennett, 1978; Wickes *et al*., 1996; Nieuwenhuis & Aanen, 2012). The bias towards the self-fertile isolates could possibly arise from ascospores that have a reduced capacity to germinate or, alternatively, fewer ascospores representing the self-sterile form might be produced in each individual ascus. This, however, has not been tested.

Growth rate has been shown to represent a simple and efficient means to estimate the relative fitness of fungal populations (Pringle & Taylor, 2002; Schoustra *et al*., 2009). In this study, evidence for a lower level of fitness in self-sterile isolates was supported by the growth tests. All self-fertile mating type strains showed more rapid growth than self-sterile mating type strains. Although this has been suggested previously (De Beer, 1994; Roux, 1996; Harrington & McNew, 1995, 1997; Witthuhn *et al*., 2000), the current study is the first where molecular tools have been used to confirm the mating type of strains beyond doubt. The only apparent difference between the self-fertile and self-sterile isolates might be the deletion in the *MAT1-2-1* gene and this seems to be the most plausible reason for the reduction in growth although the mechanism is not understood.

Extensively repeated inoculation tests using a large number of isolates showed that self-fertile isolates of *C. albifundus* are significantly more aggressive than self-sterile isolates. Likewise, growth rate for the test isolates was positively correlated with aggressiveness of isolates. A correlation between growth rate and pathogenicity has been shown for various other ascomycete fungal pathogens (Belisario *et al*., 2008; Brasier & Kirk, 2010). Furthermore, it has also been suggested that differences in pathogenicity can be attributed to the involvement of mating type genes in fungi (Lockhart *et al*., 2005; Zhan *et al*., 2007). However, in both

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these examples, and unlike *C. albifundus*, the fungi were heterothallic and thus the mechanism underpinning the differences in pathogenicity are likely to be different.

There are very few fungi that undergo uni-directional mating type switching as is found in the tree pathogen *C. albifundus*. Although the evolutionary advantages for this irreversible switching are not understood, the fact that we have shown distinct differences in fitness of self-fertile and self-sterile isolates suggests that such an advantage exists. Uni-directional mating type switching is also a common feature in species of *Ceratocystis* as defined by De Beer *et al*. (2014) and related to *C. fimbriata* (Harrington & McNew, 1995, 1997; Wilken *et al*., 2014) and it is likely that similar differences in fitness of self-fertile and self-sterile isolates exist in species in this genus other than *C. albifundus.* Maintaining this system, which would require significant amounts of energy to be channelled into producing progeny with low levels of fitness, would logically be counterproductive. This is unless there were some advantages to be gained from this strategy. The most plausible explanation would be that the self-sterile isolates increase the genetic diversity by fully exploiting the benefits of sexual recombination in a given population. This might be particularly valuable in species having the potential to self, although homothallic isolates also have the potential to outcross. Future studies should thus consider the level of outcrossing that occurs in *Ceratocystis* species in nature.

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Figure 1. Schematic results of growth rate comparisons between self-fertile and self-sterile isolates of *Ceratocystis albifundus*. Bars represent standard deviation against means.

Figure 2. Lesion lengths on *Acacia mearnsii* trees associated with each mating type of *Ceratocystis albifundus* and sterile agar as control, six weeks after inoculation. The bar represents standard deviation against mean for each of the variables.

Figure 3. Disease symptoms on the stems of *Acacia mearnsii* trees caused by different mating type strains of *Ceratocystis albifundus*. A. Lesion caused by self-fertile mating strain. B. Lesion caused by self-sterile mating strain.

Table 1. Primers used for amplification of *MAT* genes.

Primers	Sequence	Size
Albi_MAT1-2_F	5' ATA GCA AAG GTA ATC GGT CT 3'	834bp
Albi_MAT1-2_R	5' GCC GTC GAA AGA ATC CTA 3'	
Albi_MAT2-1_F	5' CCC CTT CAT TTG GCC CAT 3'	596bp
Albi_MAT2-1_R	5' CAT CAA GTC TGT GCA TCC A 3'	

Table 2. Segregation ratios of self-fertile to self-sterile isolates of *Ceratocystis albifundus*.

Isolate	Mating type							
	Success to germinate						Failed to germinate	
	1st trial		Chi-square	2nd trial		Chi-square		
	Self-fertile	Self-sterile		Self-fertile	Self-sterile		1st trial	2nd trial
CMW39129	27	13	4.90	31	17	4.08	10	2
CMW39130	33	10	11.26	30	16	4.26	7	4
CMW39131	31	11	9.52	28	17	2.22	8	5
CMW39132	31	17	4.08	34	11	10.76	2	5
CMW39133	26	18	1.45	25	23	0.08	6	2

*0.05 probability level (3.841) df=1

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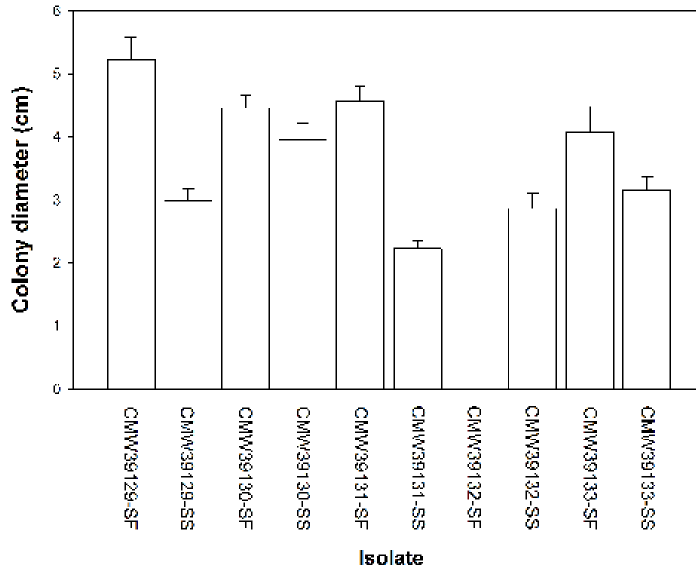

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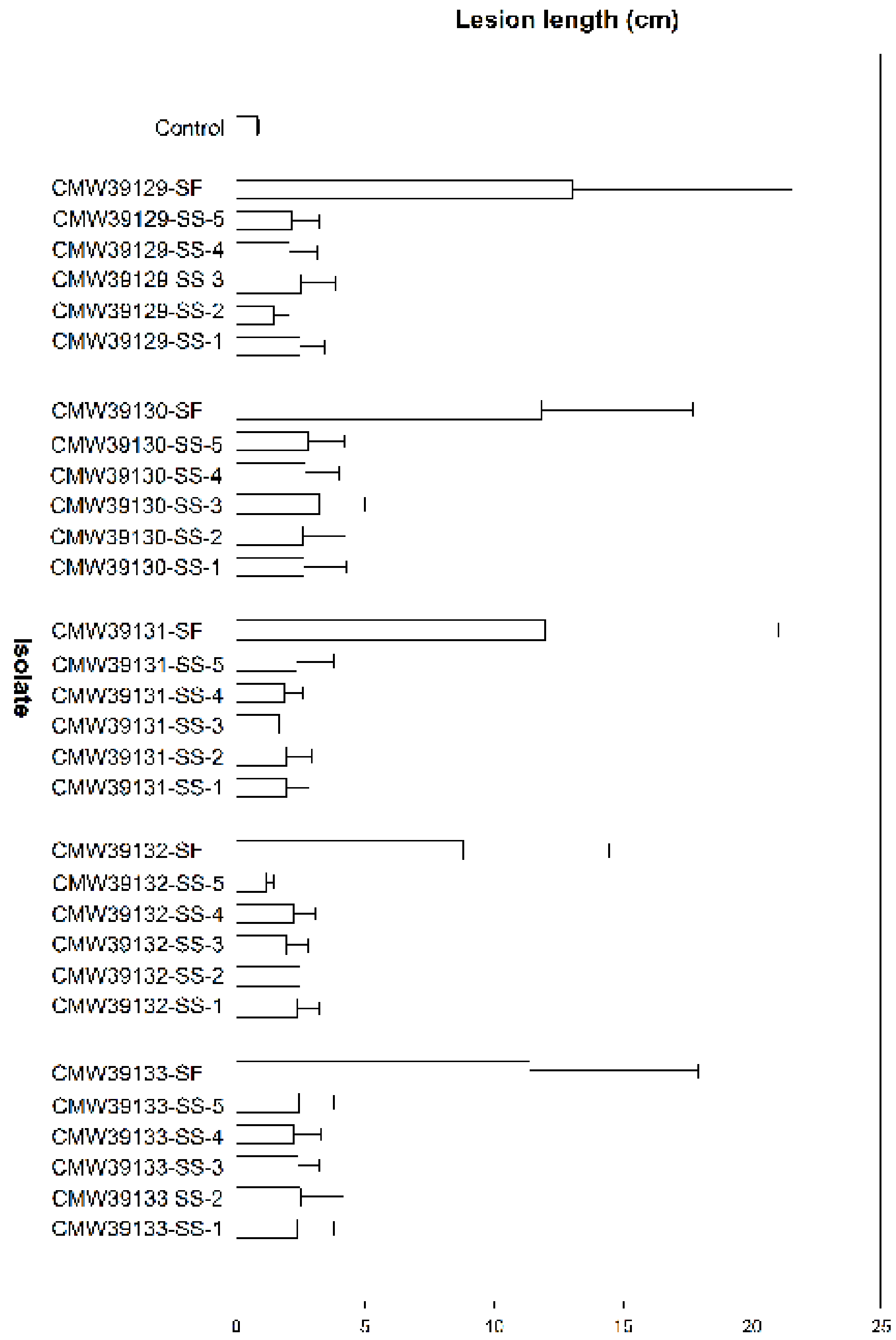

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