Inheritance of phenotypic traits in the progeny of a *Ceratocystis* interspecific cross

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Highlights

- *Ceratocystis manginecans* is not pathogenic to *Ipomoea batatas*.
- Conidia production and growth rate are not associated with aggressiveness in *Ceratocystis*.
- Various phenotypic traits in *Ceratocystis* were confirmed to be quantitative.
- Host specificity is a heritable trait and regulated by a small number of genes.

Abstract

Ceratocystis fimbriata is a fungal plant pathogen that causes black rot on *Ipomoea batatas* (sweet potato). Based on inoculation studies on numerous tree species, the pathogen is known to be host specific. The closely related species, *C. manginecans,* causes severe wilt on a broad range of tree hosts, including *Mangifera indica* (mango), *Acacia mangium* and other leguminous tree species. The genetic factors underlying the pathogenicity and host specificity of *Ceratocystis* species have rarely been investigated. In this study, an F_1 population of 70 recombinant progeny from a cross between *C. fimbriata* and *C. manginecans* was generated and the inheritance of various phenotypic traits was

investigated. Results showed that colony colour, growth rate, asexual spore production and aggressiveness to *I. batatas* and *A. mangium* are all quantitative traits with high levels of heritability. However, traits such as conidia production and aggressiveness appeared to be regulated by a small number of genes. No correlation could be found between aggressiveness and other phenotypic traits, suggesting that these are inherited independently. This is the first study to consider genetic inheritance of pathogenicity and host specificity in *Ceratocystis* species and the results will contribute, in future, to the identification of quantitative trait loci (QTLs) and candidate genes associated with the traits investigated.

Keywords*:* *Ceratocystis fimbriata*; *Ceratocystis manginecans*; heritability; host specificity; quantitative traits

1. Introduction

The effective control of fungal plant pathogens is dependent on our understanding of the biology and life history traits of the pathogen. In fungal plant pathogens, most traits associated with pathogenicity and host specificity are quantitative and thus regulated by multiple genes. Examples of such traits in fungi include mycotoxin production (Cumagun et al. 2004; Talas et al. 2016), melanisation (Lendenmann et al. 2014), mycelial/hyphal growth rate (De Vos et al. 2011; Lee et al. 2015), hyphal length (Lin et al. 2006) and spore production (Lannou 2011; Milus et al. 2008). In some species, different mating types that differ in growth rate, have been shown to indirectly influence aggressiveness (Lee et al. 2015; Lin et al. 2006). However, to understand how these phenotypes are regulated, the genomic regions associated with each trait must be identified.

Sexual crosses between different strains or closely related species provide an effective approach to determine whether a trait is quantitative. This is achieved by investigating the segregation of traits in the progeny (Cumagun et al. 2004; Stewart and McDonald 2014). In *Ophiostoma ulmi*, for example, fitness traits such as host-colonising ability, toxin production

and growth rate were all determined to be complex, based on the non-Mendelian segregation in the progeny arising from a sexual cross (Kile and Brasier 1990). Interspecific crosses provide more divergent traits for comparison than intraspecific crosses and have been used to investigate the inheritance of traits associated with host specificity (De Vos et al. 2011; Lind et al. 2007; Olson et al. 2005). Although only established recently in fungi, quantitative studies have also been undertaken in natural populations, based on genome-wide association studies (GWAS), where genetic factors associated with virulence traits have been identified in several fungi (Dalman et al. 2013; Gao et al. 2016; Talas et al. 2016).

The genus *Ceratocystis* includes 36 species, most of which are plant pathogens (De Beer et al. 2014; Marin-Felix et al. 2017), that infect hosts such as forest and fruit trees (De Beer et al. 2014; Van Wyk et al. 2013) and various root crops (Harrington et al. 2014; Wingfield et al. 2013). Species in this genus infect their hosts through wounds, after which they occupy the xylem vessels that results in symptoms such as stem cankers, xylem staining, wilt and in many cases tree death (Van Wyk et al. 2013).

Knowledge of the factors associated with host specificity and aggressiveness in *Ceratocystis* species has been limited to studies focusing on host response during infection (Al-Sadi et al. 2010; Kojima and Uritani 1976; Trang et al. 2017) and studies on enzymes produced by *Ceratocystis* for the breakdown of host phenolics (Wadke et al. 2016). A recent *Ceratocystis* genome comparison identified potential pathogenicity genes in *C. cacaofunesta* and *C. fimbriata*, including putative effectors and a highly expanded phosphatidylinositol-specific phospholipase-C gene family (Molano et al. 2018). This provides various candidates for future functional studies.

Host specificity has been demonstrated in a number of *Ceratocystis* species. For example, *C. cacaofunesta* is pathogenic only on *Theobroma cacao* and *C. platani* only on *Platanus* spp. (Baker et al. 2003; Tsopelas et al. 2017). Similarly, the type species of *Ceratocystis, C. fimbriata sensu stricto,* has been reported only to cause black rot of *Ipomoea batatas* (sweet potato) (Halsted 1890). The host specificity of this species has been confirmed from inoculations on *Theobroma cacao*, *Platanus* spp (Baker et al. 2003) and *Acacia mangium* trees (Rauf M.R., personal communication 2016).

Contrary to the host specific *C. fimbriata*, the closely related species, *C. manginecans,* has a broader host range and has resulted in significant losses in *A. mangium* plantations (Brawner et al. 2015; Fourie et al. 2016; Tarigan et al. 2011) and *Mangifera indica* (mango) orchards (Al Adawi et al. 2006). Other reported hosts include *Eucalyptus* (Chen et al. 2013), *Punica granatum* (Harrington et al. 2014; Huang et al. 2003) and the leguminous trees, *Dalbergia sissoo* and *Prosopis cineraria* (Al Adawi et al. 2013). The pathogenicity of *C. manginecans* on *I. batatas* has not been determined.

Ceratocystis fimbriata s.s. and *C. manginecans* are morphologically very similar but phylogenetically distinct species (Fourie et al. 2014). They can, however, be induced to mate in a controlled laboratory environment (Ferreira et al. 2010; Li et al. 2016; Oliveira et al. 2015b). Mating studies have been used extensively in the Ceratocystidaceae to understand mating systems and the inheritance of mating type genes (Kile et al. 1996; Webster and Butler 1967), colony colour (Webster 1967), mycelial phenotypes, cycloheximide resistance (Harrington and McNew 1997) and microsatellite marker regions (Ferreira et al. 2010) within the family. This has established an ideal system to perform an interspecific cross that can be used to investigate the inheritance of other phenotypic traits in the progeny from such a cross (De Vos et al. 2011; Lind et al. 2007).

Ceratocystis species are haploid and homothallic, with a unidirectional mating-type switching system that results in both self-fertile (MAT2) and self-sterile (MAT1) progeny (Webster and Butler 1967; Wilken et al. 2014; Witthuhn et al. 2000). In MAT1 isolates one of the three MAT genes (*MAT1-2-1*) is absent. These isolates are used in sexual crosses in combination with self-sterile MAT2 isolates (Engelbrecht and Harrington 2005; Johnson et al. 2005). Isolates of the latter type have been produced only in laboratory conditions, they contain all three MAT genes but ascomata (sexual reproductive structures) and protoperithecia are absent, likely due to a mutation influencing protoperithecia formation (Ferreira et al. 2010; Webster 1967).

The aim of this study was to perform an interspecific cross between an isolate of *C. manginecans* and *C. fimbriata* and to investigate the inheritance of traits associated with host specificity and aggressiveness in the progeny. For this purpose, the recombinant

progeny from the cross was used to determine the frequency distribution and heritability of phenotypic traits. The primary trait investigated was lesion lengths induced on the respective plant hosts, *I. batatas* and *A. mangium*, after reciprocal inoculation. In addition, because colony pigmentation (Lendenmann et al. 2014), growth rate in culture (Zhan et al. 2016) and asexual spore production (Lannou 2011) has been associated with aggressiveness is other fungal pathogens, these traits were also considered in the current study.

2. Materials and methods

2.1. Parental isolates

Three *C. fimbriata s.s.* isolates (CMW14799 from USA and CMW42704 and CMW42705 from Malaysia) and two *C. manginecans* isolates (CMW46461 and CMW48940 from Malaysia) were used for mating experiments between the two species (Table 1). The parental isolates were selected, based on their specific pathogenicity on *I. batatas* and *A. mangium,* respectively. Cultures were grown on 2% malt extract agar (MEA), supplemented with 150 mg/L streptomycin (Sigma-Aldrich, Germany) and 1 mg/L thiamine (Sigma-Aldrich, Germany), at room temperature (22 °C) for 14 days.

Table 1
Information on the host and location of *Ceratocystis* isolates used in this study.

Species	CMW no.[a]	CBS no.[b]	Host	Location	Collector and date collected
C. fimbriata	14799	CBS114723	*Ipomoea batatas*	North Carolina, USA	D. McNew, 1998
	42704		"	Malaysia	R. Rauf, 2014
	42705		"	Malaysia	R. Rauf, 2014
C. manginecans	46461	CBS143454	*Acacia mangium*	Sabah, Malaysia	R. Rauf, 2014
	48940		"	Malaysia	R. Rauf, 2014

[a] Fungal culture collection at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa

[b] Culture Collection (CBS) of Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands

2.2. Mating between C. fimbriata and C. manginecans to establish an F_1 population

For each parental isolate used in the study, both MAT1 and MAT2 self-sterile cultures were generated. MAT1 individuals were produced from the self-fertile MAT2 parental isolates by dispersing spores from a single ascospore drop on MEA media, using Soltrol® 130 isoparaffin solvent (Chevron Phillips Chemical Company LP, Texas, US) as described by Wilken et al. (2014). Germinating single ascospores were isolated and cultures failing to produce ascomata were selected. MAT2 self-sterile isolates were produced by continuous sub-culturing of aerial mycelia of MAT2 self-fertile isolates until no ascomata were produced. The presence/absence of the *MAT1-2-1* gene in the putative MAT1 and MAT2 self-sterile isolates were confirmed using the primers MAT-121F and MAT-121R, following the PCR protocol as described by Wilken et al. (2014).

Interspecific crosses were performed by reciprocally crossing the MAT2 self-sterile and MAT1 isolate of each parent of *C. fimbriata* (CMW14799, CMW42704, CMW42705) and *C. manginecans* (CMW46461, CMW48940) with each other. MAT2 self-sterile mating types could only be produced for some isolates (CMW14799, CMW42705 and CMW46461) and thus in total, seven mating combinations were performed (Table 2). Crosses were performed by placing an agar plug of isolates of opposite species and opposite mating types on an MEA plate with a 2cm distance between them. Each cross had 10 technical repeats. These plates were incubated for two weeks at room temperature (22 °C). Ascomata with creamy spore drops, produced in the hybrid zone between the two isolates, indicated a successful cross. Only the two parental isolates for which both self-sterile mating types (MAT2 and MAT1) were successfully generated and where the mating experiment worked reciprocally (CMW14799 and CMW46461), were selected for the production of the hybrid F_1 population.

To obtain the F_1 progeny from successful crosses, two to three drops of ascospore masses were lifted from the apices of ascomata formed in the hybrid zone and dispersed on MEA plates. After 24 hours, a minimum of 100 germinating single spores were isolated and cultured from each reciprocal cross. Progeny isolates were labelled as MF if they were obtained from the *C. manginecans* (MAT2 self-sterile) and *C. fimbriata* (MAT1) cross and

labelled as FM when obtained from the *C. fimbriata* (MAT2 self-sterile) and *C. manginecans* (MAT1) cross. All progeny isolates produced in this study are deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria (Table S1).

Table 2
Mating success from the different mating types produced from the parental isolates.

Species		*C. manginecans*	*C. fimbriata*	
	Isolate and mating type	CMW46461 MAT2 self-sterile	CMW42705 MAT2 self-sterile	CMW14799 MAT2 self-sterile
C. manginecans	CMW46461 MAT1	N/A	Ascomata	Ascomata
	CMW48940 MAT1	N/A	Ascomata	Not performed
C. fimbriata	CMW42704 MAT1	No success	N/A	N/A
	CMW42705 MAT1	Ascomata	N/A	N/A
	CMW14799 MAT1	Ascomata	N/A	N/A

2.3. Fragment analyses to confirm recombinant progeny

To identify polymorphic microsatellite markers that could be used to screen for recombinant progeny, a set of 10 microsatellite markers, previously designed for *C. manginecans* (Fourie et al. 2016), were screened in the chosen parental isolates (CMW14799 and CMW46461). From the polymorphic markers identified, a subset of five were used for screening the 126 progeny obtained from the cross. DNA was obtained for each isolate using Chelex® 100 Molecular Biology Grade Resin (Bio-rad, California, USA). Mycelium was collected from the agar plates using a needle tip, then boiled in the Chelex resin for 30 min (100 °C) and centrifuged for 2 min at high speed. The supernatant was used for PCR reactions. The PCR amplification protocol was performed as described by Fourie et al. (2016), except that 3µl Chelex extract containing DNA was used per reaction. Fragment analyses were performed using GeneScan, including a Liz500(−250) size standard, on an ABI PRISM™ 3500xl Autosequencer (Thermo Fisher Scientific, Carlsbad, USA). Alleles were

scored using GeneMapper® v.4.1 (Applied Biosystems, Thermo Fisher Scientific, Carlsbad, USA), using the same protocol as described by Fourie et al. (2016).

A total of 70 isolates were selected for the F_1 population that included 35 recombinant individuals from each reciprocal cross. The segregation of all five microsatellite markers was investigated in the F_1 progeny by testing the deviation from a 1:1 mendelian segregation ratio, using a Chi-square test ($p<0.05$).

2.4. Colony colour and growth rate in progeny isolates

To determine whether colony colour was a heritable trait, its segregation in the progeny was considered. The two parental isolates (*C. fimbriata* CMW14799 and *C. manginecans* CMW46461) differ in colony colour and the amount of aerial versus submerged mycelia when grown on MEA. *Ceratocystis fimbriata* (CMW14799) had darker, more deeply olive green pigmentation than *C. manginecans* (CMW46461) and had significantly more fluffy, aerial mycelia. A representative colony for each of the parents and the 70 progeny, grown on MEA, was digitally photographed 14 days post inoculation (dpi) under controlled lighting, using an Epson Perfection™ V700 Photo scanner. The images of all isolates were sorted into categories, based on pigmentation, from light to dark.

The optimal growth temperature for the two parental isolates was determined by incubating the MAT2 self-fertile cultures at 20 °C, 25 °C and 30 °C on MEA. Additionally, to test whether nutrient acquisition might play a role in host specificity, the effect of host-specific plant extract media (*A. mangium* and *I. batatas*) on growth rate was tested for the two parental strains at the optimal growth temperature of 25 °C. The *A. mangium* extract agar (AEA) was obtained by boiling 110 g of plant material, and the *I. batatas* extract agar (IEA) by boiling 306 g of plant material in 1L distilled water in an autoclave. The liquid extract was combined with 15 g agar, filled with water to a final volume of 1L and autoclaved a second time. All media were supplemented with 150 mg/L streptomycin.

Plate inoculations of the MAT2 self-fertile parental isolates were performed on all three media types (MEA, AEA and IEA). As only the MEA and AEA media resulted in a significant difference in growth rate between the two parental isolates at 25 °C, these were selected

for further studies in the progeny. The growth rate of the MAT2 self-sterile and MAT1 parental isolates were also included with the progeny growth studies. Cultures used for the growth studies were incubated for 14 days on MEA after which 5mm diameter agar plugs were transferred to 90 mm agar plates. Ten plates were inoculated per isolate and per media type and incubated for 12 days at 25 °C. Plates were marked with two perpendicular lines on which colony size was recorded every second day. The data points from both axes on a plate were used to determine the average growth rate (mm/2 days) per plate and per isolate on the different media types.

2.5. Conidial production

The cultures grown on MEA media for the growth rate studies were used to determine conidial spore concentrations, approximately three weeks after the plates have been inoculated. Spore counts were performed for the self-fertile and self-sterile parental isolates (CMW14799 and CMW46461) as well as for all 70 progeny isolates. For each isolate, four mycelial agar plugs of 5mm diameter were cut from a single plate, combined, and agitated in an Eppendorf tube with 500µl distilled water, using a vortex mixer. Five plates were counted per isolate. A haemocytometer was used to count the number of spores in a 10ul spore suspension.

2.6. Pathogenicity screening on two host plants

Plant inoculations were undertaken with the parental isolates *C. fimbriata* (CMW14799) and *C. manginecans* (CMW46461), including the MAT2 self-fertile and MAT2 and MAT1 self-sterile isolates, and with the 70 progeny isolates. In this study we consider pathogenicity and aggressiveness to be distinct traits. Both pathogenicity toward a specific host (the ability to cause disease) and aggressiveness on the host (degree of pathogenicity) was investigated. An isolate was considered pathogenic, if it was able to produce a lesion on the host that was larger than the control. Aggressiveness was quantified by the length of the lesion induced on the host.

2.6.1. Inoculation trials on I. batatas

The sweet potato variety Blesbok was used in all inoculation experiments. Fourteen tubers were inoculated per isolate. Prior to inoculation, the tubers were surface disinfested with ethanol, washed with water and air-dried. Wounds were made, deeper than the cortex layer (±5mm), with a 5mm diameter cork borer and 20ul of inoculum, containing $1.5x10^6$ conidial cells/ml, was added. In cases where isolates did not produce conidia, mycelial agar plugs (5mm) were used in the inoculation. Clean MEA plugs were used as a negative control. Inoculated wounds were sealed with Parafilm M® (Bemis, Oshkosh). Inoculated tubers were stored in a greenhouse at 25 °C, with 90% humidity and direct external light was blocked with black plastic bags. After five weeks, two dimensions of the black rot lesions were measured, one along the length and one along the width of the surface of the tuber, to determine an average lesion size.

2.6.2. Inoculation trials on A. mangium

Acacia mangium seeds were germinated in a germination mix (Culterra, Johannesburg, South Africa), combined with potting soil, for up to 10 weeks. These were then transplanted into poly bags with potting soil, sand and 2:3:2 (22) fertilizer and grown for a further 12 months. The parental isolates (CMW14799 and CMW46461, MAT2 self-fertile, MAT1 and MAT2 self-sterile) and 70 progeny isolates were inoculated onto ten trees each and kept in a greenhouse at 28 °C. Trees of different sizes, ranging from 17.5cm–200.5cm in height and 0.4cm-1.4cm in width were distributed equally among all the isolates in the inoculation experiment. Wounds were made on the stems, on the second internode from the bottom, using a 4mm diameter cork borer. Mycelial agar plugs were used for inoculation after which wounds were sealed with Parafilm M® (Bemis, Oshkosh). Clean MEA agar plugs were used as negative controls. A fully randomised block design was used to store the trees during lesion development. Trees were maintained in a greenhouse at 28 °C for three weeks after which tree height and stem diameter as well as lesion lengths on the stems were measured for each tree. To obtain an accurate measurement of the lesion length, the bark and outer cortex layer of the stem were removed from the surface before measuring.

2.7. Inheritance of mating types in hybrid progeny

The mating type of all the progeny isolates was determined using primer sets Primer17/Primer25, to confirm the absence of the *MAT1-2-1* gene and Primer17/Primer30 to confirm the presence of the gene (Wilken et al. 2014). Both primer sets amplify an intergenic region between the *MAT1-1-1* and *MAT1-1-2* genes but primer30 binds to a region that is removed along with the *MAT1-2-1* gene and amplification is thus only possible if the gene is present. PCR reaction and amplification conditions were the same as that previously described for *Ceratocystis* (Wilken et al. 2014). Isolates lacking the *MAT1-2-1* gene were classified as MAT1 and those containing the *MAT1-2-1* gene, but not producing any ascomata, were classified as MAT2 self-sterile. To determine if the inheritance of the mating-types were consistent with a pattern of Mendelian inheritance, a Chi-square analysis ($p<0.05$) was performed on the mating-types (self-fertile vs self-sterile) and for the inheritance of the *MAT1-2-1* gene.

2.8. Statistical analyses

To construct a histogram of each trait, the frequency distribution among the progeny were calculated using the mean value of each individual for each trait. Traits included growth rate, conidial production, lesion length on *I. batatas* and lesion length on *A. mangium*. All subsequent analyses were performed in R version 3.3 (http://www.r-project.org/; R Core Team, 2016). A Shapiro-Wilk test was performed to confirm whether the data deviates from a normal distribution. A student's t-test was performed to compare the growth rates of a single isolate on two different media.

To determine whether any of the progeny isolates were significantly different from the parents for the five traits considered, an analysis of variance (ANOVA) test was performed, using either the lsmeans package (Lenth 2016) or agricolae (De Mendiburu 2014). The lsmeans package was used for the host inoculation studies because the data included an unequal number of samples. Due to the unbalanced nature of the inoculation data, the type III error sum of squares from the car package (Fox and Weisberg 2011) was used in the resulting ANOVA table. The null hypothesis assumed no differences between the means at a significance level of $\alpha=0.05$.

Tukey's honestly significance difference (Tukey's HSD) test was performed on all data to determine which isolates differed significantly from each other and from the parental isolates (P-value=0.05). A linear model was fitted to the data for the *A. mangium* inoculations in order to determine whether tree height or stem diameter influenced lesion length. Only the latter proved to contribute significantly, and an analysis of covariance (ANCOVA) model was subsequently used in comparing the means of all individuals by incorporating stem diameter as a continuous confounding variable.

To determine whether any of the phenotypic traits investigated in the progeny, including growth rate, conidial production and mating type (independent variables) are related to, or has an effect on lesion lengths on the host (two dependent variables), a multivariate analysis of covariance (MANCOVA) was performed. The average of each individual was calculated for each trait and these were used as dependent and independent variables. The analysis was performed using the car and lsmeans packages, based on a significance level of α=0.05. The lsmeans was used for the evaluation of multiple comparisons.

To investigate and visualise the relationship between all of the phenotypes considered (growth rate, spore production, pathogenicity to a specific host, colony colour and mating type), a multiple correspondence analysis (MCA) was performed in R, using the MCA function from the FactoMineR package (Lê et al. 2008). The first two dimensions, explaining most of the variance, were plotted on a MCA plot. Since some of the variables were continuous and others were categorical, all continuous data were divided into categories based on the four quartiles of the data set. To determine whether the association between any two of the seven variables were significant, a Fisher's exact test was furthermore performed and p-values adjusted with the Bonferroni correction, in order to account for multiple testing. This was performed using the base functions p.adjust and fisher.test in R version 3.3 (http://www.r-project.org/; R Core Team, 2016).

Broad sense heritability (H^2) of growth rate, conidial production and aggressiveness on the two hosts was determined using the equation $H^2 = V_G/V_P$. The sum of square means (MS), determined from ANOVA analyses, were used to calculate the genotypic variance (V_G) and phenotypic variance (V_P) of a trait (Akaffou et al. 2012). V_P was equal to the MS between

groups (MS_{BG}). The environmental variance (V_E) was based on the variation within the technical replicates of an isolate and this was equal to the MS within groups (MS_{WG}). To calculate V_G the formula used was $V_G = (MS_{BG} - MS_{WG})/n$ for which *n* equals the number of repeats per individual. The values calculated for V_P and V_G were then used to calculate H^2.

3. Results

3.1. Mating between C. fimbriata and C. manginecans to establish an F_1 population

MAT1 self-sterile isolates were obtained from all five potential parental isolates but MAT2 self-sterile isolates could only be produced for one *C. manginecans* (CMW46461) and two *C. fimbriata* (CMW14799, CMW42705) isolates. Therefore, the successfully obtained MAT2 self-sterile isolates (CMW14799, CMW42705, CMW46461) were crossed with all the MAT1 isolates of the opposite species (CMW14799, CMW42704, CMW42705, CMW46461, CMW48940) in all possible combinations (Table 2). Mating was not successful for all isolate combinations. The *C. manginecans* MAT2 self-sterile isolate (CMW46461) crossed successfully with two of the *C. fimbriata* MAT1 isolates (CMW14799 and CMW42705) but not with isolate CMW42704, suggesting this interspecific cross was not stable. Ascospore masses were also more watery than creamy in these crosses. The cross with CMW14799 MAT1 was selected for obtaining part of the F_1 population. For the reciprocal cross, both *C. fimbriata* MAT2 self-sterile isolates (CMW14799 and CMW42705) crossed successfully with the *C. manginecans* MAT1 isolate (CMW46461; Fig. 1), producing creamy ascospore drops. The cross with CMW14799 MAT2 self-sterile, however, produced the most viable and fertile progeny and was, therefore, selected for producing the other part of the F_1 population.

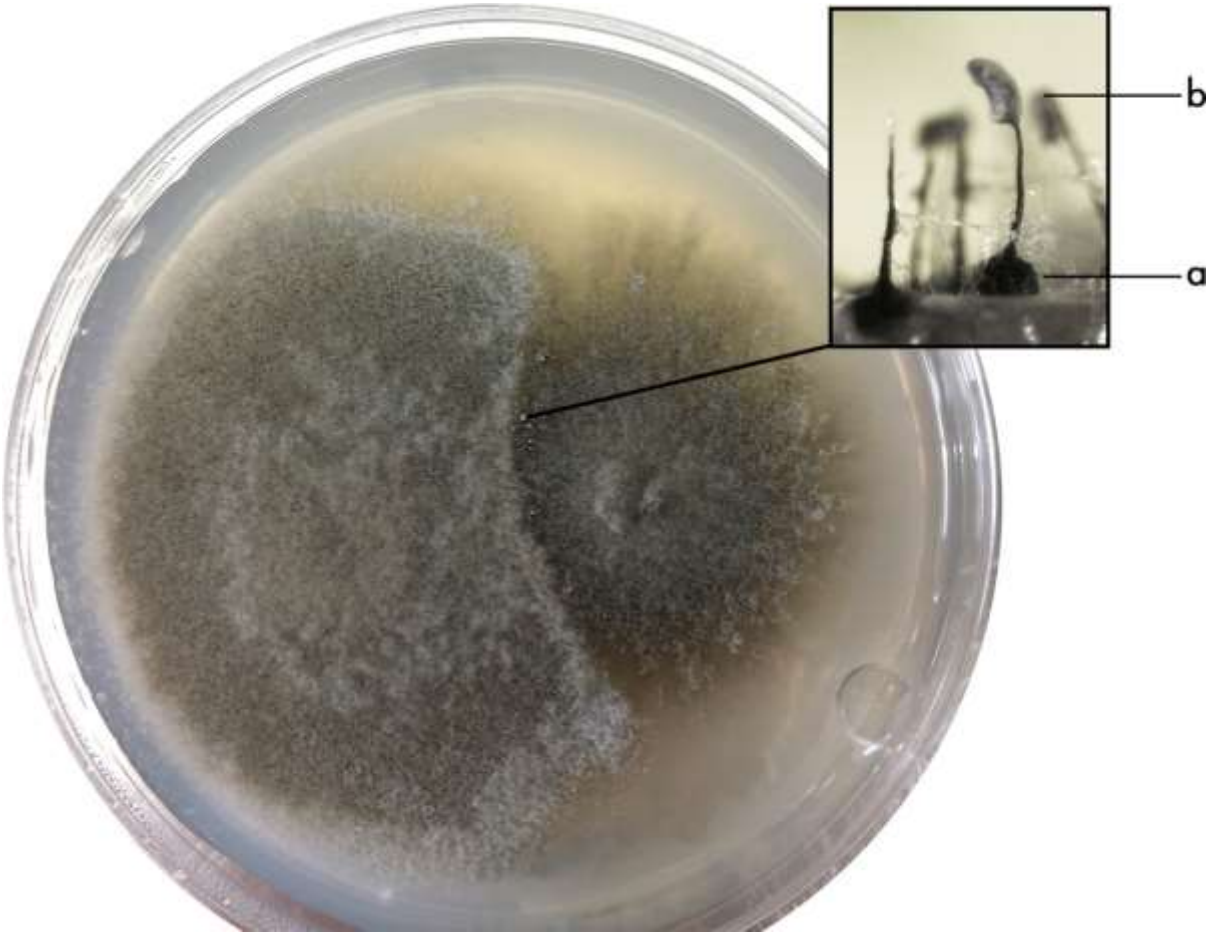

Fig. 1. Ascomata (a) with ascospore drops (b), obtained from the interspecific cross between *C. fimbriata* (MAT1), on the left, and *C. manginecans* (MAT2 self-sterile), on the right.

3.2. Fragment analyses to confirm recombinant progeny

All ten microsatellite markers tested in the parental isolates were polymorphic for the two species (Table S2). A subset of five markers (AF2, AF4, AF6, AF7 and AF9), that could be amplified in two PCR multiplex reactions, were selected for screening of the progeny isolates. From a total of 126 screened progeny isolates, 70 were single spore recombinant, 15 displayed double peaks at one to four of the markers and 41 were non-recombinant, representing either of the two parental genotypes. The 15 isolates with double peaks could be a mixed culture of two spores that only differ at some of the markers. Alternatively, this could be due to aneuploidy of some parental chromosomes, both present in a single isolate (Harrington and McNew 1998). Four of the five microsatellite markers segregated in a 1:1

ratio (X^2-value=3.2, p-value=0.5). Marker AF4 deviated significantly from a 1:1 ratio, with 78% of the progeny containing the *C. fimbriata* allele, suggesting a bias in the inheritance of some parts of the *C. fimbriata* genome. A BLAST analysis to the *C. fimbriata* genome (GenBank APWK03000000) (Fourie et al. 2017) showed that marker AF4 is in the coding region of a probable sucrose utilization protein (SUC1), which could have influenced the inheritance of this genomic region.

3.3. Colony colour and growth rate in progeny isolates

The mycelium of the *C. fimbriata* parent (CMW14799) was a very dark olive green and that of the *C. manginecans* parent (CMW46461) was a lighter olive. The colour of the MAT1 isolates of both species were similar to that of the *C. manginecans* parent, the MAT2 self-sterile of *C. fimbriata* was cream coloured, and that of *C. manginecans* was olive but lighter than the MAT1 isolate. The colony colour of the progeny varied significantly from the parents and among isolates (Fig. 2). Colour pigmentation showed a continuum from white to dark olive in the progeny. For descriptive purposes these could be classified into eight categories. These included white, off-white, light cream, cream, light olive, olive, darker olive and very dark olive (Fig. 3). The frequencies of these eight colour categories showed a bimodal distribution for the progeny isolates (Fig. S1A). Where the isolates were categorised broadly into light and dark colours, the ratio was 33:37 respectively. Although it resembles a 1:1 ratio, colony pigmentation is likely a quantitative trait, due to the range of colours observed. A significant association was, however, observed between light isolates and loss of pathogenicity (Fisher's exact test, p-value=0.01; Fig. S2, Table S3). In addition, since 96% of the pathogenic isolates were either olive or dark olive in colour (Table S4), a darker pigmentation might be associated with aggressiveness.

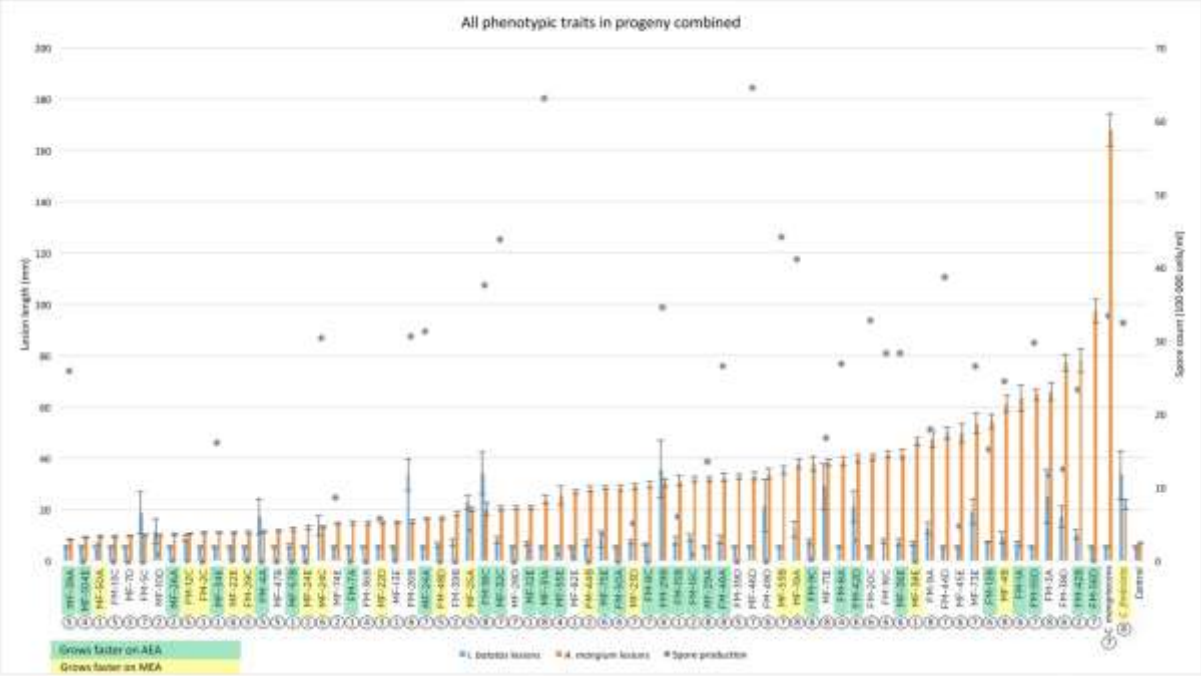

Fig. 2 Data distribution of the phenotypic traits in the 70 progeny from an interspecific cross. Aggressiveness toward *A. mangium* and *I. batatas*, measured as lesion length in mm, are indicated for each isolate, including the MAT2 self-fertile parental isolates. The average conidia spore count is indicated as grey dots. Isolates that grew significantly faster on one of the two media types (Student's t-test, $\alpha = 0.05$) are highlighted in green and yellow. Colony colour was coded from light to dark (1-8) and indicated underneath the label of each isolate.

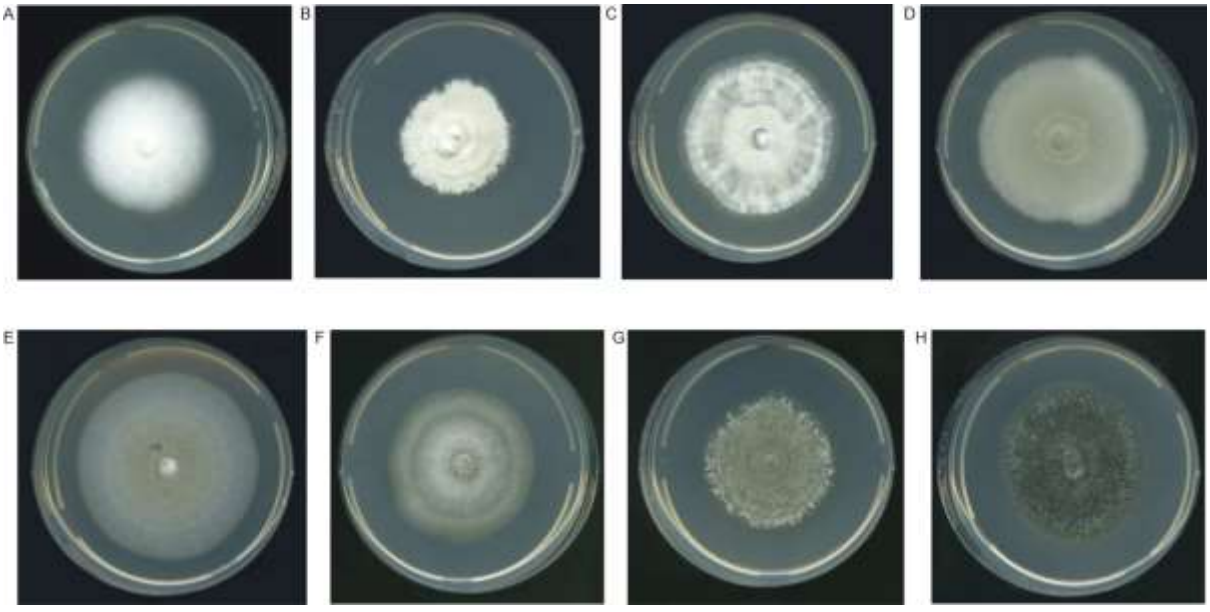

Fig. 3. Illustration of all eight categories of colony pigmentation observed in progeny culture morphology. Colours ranged from A) white, B) off-white, C) light cream, D) dark cream, E) light olive, F) olive, G) darker olive and H) very dark olive.

Both parental isolates grew optimally at 25 °C on MEA media and this temperature was used for all subsequent growth studies. *Ceratocystis manginecans* grew most rapidly on IEA (10.1 mm/2 days) and AEA (8,4 mm/2days) but less so on MEA (7,7 mm/2 days) (p-value < 0.05). The same tendency of preference for a specific media type was observed in the self-sterile *C. manginecans* isolates, although they grew faster than the self-fertile parent (Table S4). This suggests that the *C. manginecans* isolate's growth is improved by the nutrients provided by AEA, compared to MEA. In contrast, *C. fimbriata* did not have optimal growth on AEA but grew more slowly (7.8 mm/2days) than on MEA (9.4 mm/2 days) (p-value < 0.05). The *C. fimbriata* MAT2 self-sterile isolate grew significantly slower than the MAT2 self-fertile and MAT1 isolate (Table S4). There was a significant difference in growth rate between the two parents on all three media types (Fig. 4) but on MEA and IEA*, C. fimbriata* grew more rapidly while *C. manginecans* grew more rapidly on AEA and IEA.

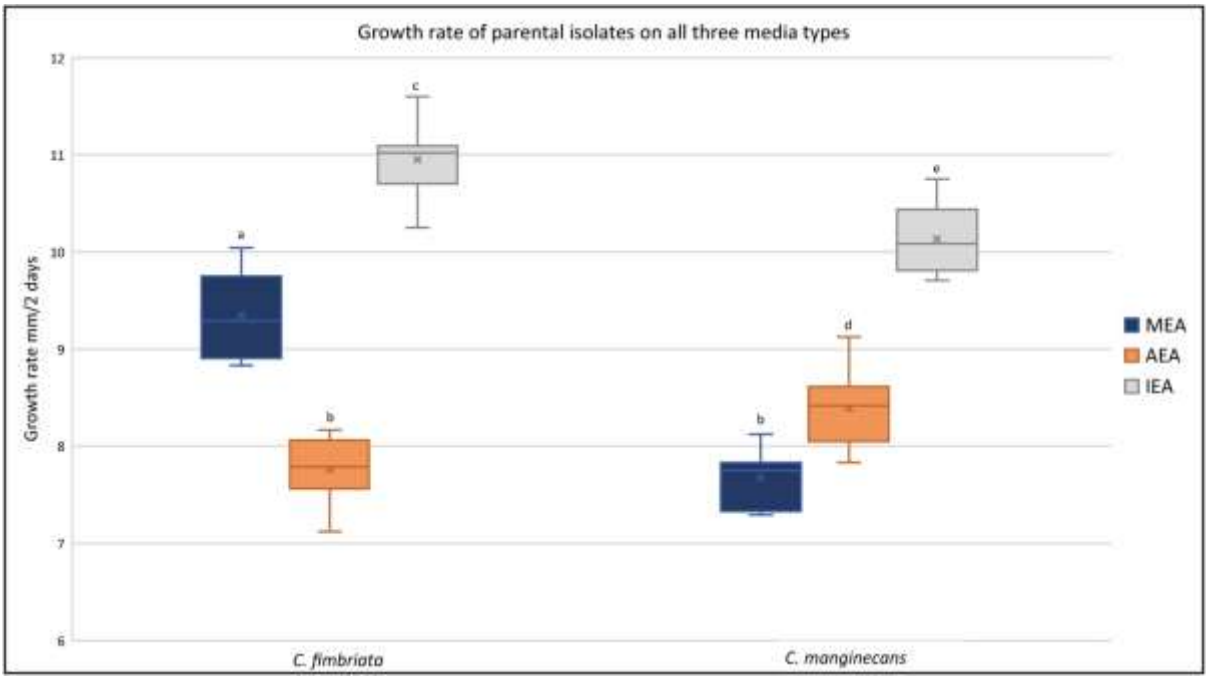

Fig. 4 Box and whisker plot representing the growth rates of the *C. fimbriata* and *C. manginecans* parental isolates on all three media types investigated (MEA (orange), AEA (blue) and IEA (grey)). Letters indicate Tukey HSD test results (p=0.05) and any datasets with the same letter are not significantly different from each other.

The significant increase in growth rate for *C. manginecans* and, in contrast, a reduction in growth rate for *C. fimbriata* on the AEA medium led to the choice of this medium, in

addition to MEA, to test the growth of the progeny isolates. Based on the average growth rate for each isolate, the progeny approached a normal distribution for growth both on MEA and AEA (Fig. S1), although this was not significant (Shapiro-Wilk test). For the broad-sense heritability, on both MEA and AEA media, high heritability values of H^2=0.935 (Table S5A) and H^2=0.835 (Table S5B), respectively, suggested a large genetic contribution to variation in growth rate. The MANCOVA analyses indicated no correlation between growth rate and aggressiveness on the host. Growth rate did also not show an association with any of the other phenotypes based on the MCA and Fisher's analyses (Fig. S2, Table S3).

Table 3

Comparison of growth rate, colony colour, aggressiveness and conidia production between the 70 progeny isolates and the two parental isolates

Phenotype	Data distribution[a]	Heritability	No. of progeny different from *C. manginecans*[b]	No. of progeny different from *C. fimbriata*[b]
MEA[c] medium	Normal	0.935	50 faster 5 slower	19 faster 27 slower
AEA[d] medium	Normal	0.835	47 faster 5 slower	57 faster 4 slower
Colony colour	Bimodal	-	63 lighter	50 lighter 9 darker
Agressiveness on *A. mangium*	Continuous, skewed right	0.51	70 less aggressive	11 (different from control)
Agressiveness on *I. batatas*	Continuous, skewed right	0.832	16 more aggressive	4 equally aggressive 66 less aggressive
Conidia production	Continuous, skewed right	0.764	2 higher concentration 47 lower concentration/ no spores	2 higher concentration 47 lower concentration/ no spores

[a] Normality was tested with Shapiro-Wilk, at a significance level of 0.05

[b] Differences based on Tukey HSD at a significance level of 0.05 by comparison to the MAT2 self-fertile parental isolate data

[c] Malt extract agar

[d] *Acacia mangium* extract agar

Compared to the parental isolates, 46 of the 70 progeny isolates had a different growth rate on MEA than the *C. fimbriata* parent and 55 of the progeny differed from the *C. manginecans* parent (p-value < 0.05)(Table 3). On AEA, 61 isolates had a different growth rate than the *C. fimbriata* parent and 52 isolates differed in growth rate from *C. manginecans* (p-value < 0.05). Comparison of growth rates of each progeny isolate on the

two media types showed that 35 isolates grew more rapidly on AEA than on MEA, 16 grew more slowly on AEA than on MEA and 19 isolates did not show any difference in growth on the two different media types (Fig. 5).

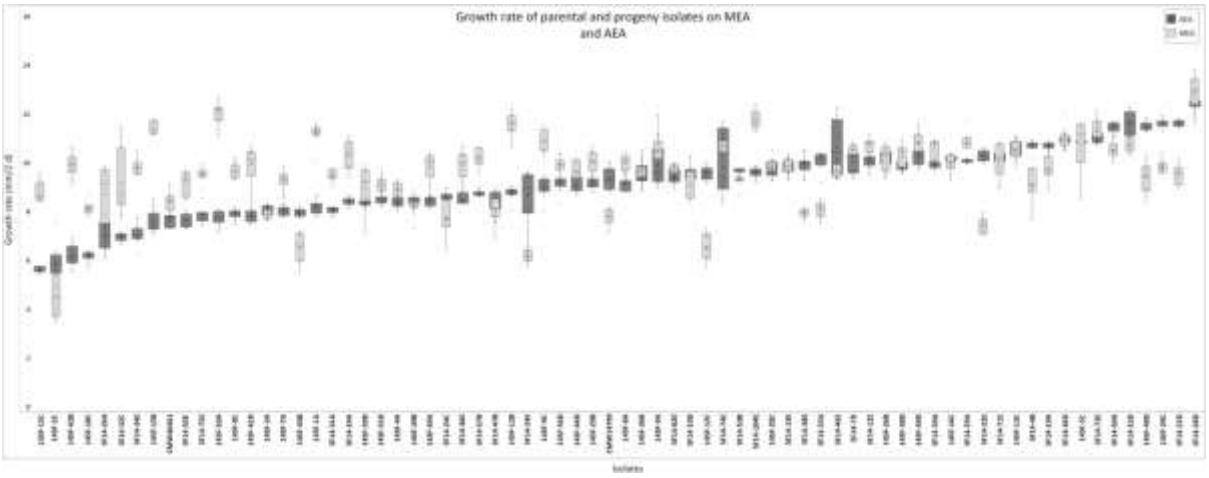

Fig. 5 Box and whisker plot representing the growth rates of the parents, *C. fimbriata* and *C. manginecans*, and all 70 progeny both on MEA (orange) and AEA media (blue).

3.4. Conidial production

The MAT2 self-fertile and MAT1 parental isolates did not differ from each other in the quantity of conidia produced (Table S4). The self-fertile *C. fimbriata* isolate produced 3.25 x 10^6 spores/ml and the MAT1 isolate 2.615 x 10^6 spores/ml. In the case of *C. manginecans* the self-fertile isolate produced 3.346 x 10^6 spores/ml and the MAT1 isolate 4.865 x 10^6 spores/ml. The MAT2 self-sterile parental isolates (*C. fimbriata* and *C. manginecans*), however, produced significantly fewer conidia at concentrations of 7.5 x 10^4 and 9.8 x 10^5 spores/ml, respectively.

The conidial production data for the progeny isolates was not normally distributed (Fig. S1F). This suggests a single or limited number of genes are involved in determining this trait. The progeny isolates varied significantly from one another and from the parents in conidial concentration (Table S4; Fig. 2), ranging from 1.2 x 10^5 to 6.455 x 10^6 spores/ml. Forty seven progeny isolates produced few or no conidia. Two isolates produced greater numbers of conidia than the parental isolates, one of which was MAT2 self-fertile and one MAT1. The

standard deviation was high for some of the isolates and an additional five technical repeats were included for those isolates to obtain a reliable average value. The broad-sense heritability of conidial production was H^2=0.764 (Table S5C), suggesting a high genetic contribution to this trait. There was no correlation between conidial production and any of the other phenotypic traits.

3.5. Pathogenicity screening on two host plants

3.5.1. Inoculations on I. batatas

The results of inoculations on *I. batatas* confirmed that *C. manginecans* is pathogenic to *A. mangium* but not to *I. batatas*. There was no significant difference between the lesion length induced by *C. manginecans* and the control (avg. 5.5 mm), while the lesions induced by *C. fimbriata* were significantly larger (avg. 33.5 mm) (Fig. S3). The MAT2 self-sterile strain of *C. fimbriata* was equally pathogenic on *I. batatas* than the self-fertile parent (α=0.05) and the MAT1 displayed slightly higher levels of aggressiveness (Fig. S4). Progeny isolates were considered pathogenic to *I. batatas* if they induced a lesion size larger than the control. The inheritance of pathogenicity to *I. batatas* in the progeny resembled a simple mendelian trait associated with two genes, with the ratio of non-pathogenic to pathogenic isolates being 54:16, close to a 3:1 ratio.

The aggressiveness of the 16 pathogenic and 54 non-pathogenic isolates was assessed by the lesion length induced on *I. batatas*. None of the progeny isolates were more aggressive than the parental *C. fimbriata* isolate. Four isolates were equally aggressive and the remaining isolates were all less aggressive than the *C. fimbriata* parental isolate (Fig. 2). The lesion length data displayed a continuous distribution but the frequency distribution was not normally distributed and was skewed toward lower aggressiveness (Fig. S1D). Aggressiveness thus resembled a quantitative trait in the progeny but the data distribution suggested the involvement of only a small number of genes. When the 16 pathogenic isolates were considered separately, no clear distribution of data was observed but it represented three data intervals (Fig. S1D). Thus, some of the pathogenic isolates displayed a low level of aggressiveness, some an intermediate and some high levels of aggressiveness.

This distribution of the data further supports the involvement of a small number of genes in aggressiveness. The broad sense heritability for aggressiveness on *I. batatas* indicated a high level of genetic contribution to the variation observed (H^2 =0.832; Table S5D).

3.5.2. Inoculations on A. mangium

Inoculation of the *C. fimbriata* parental isolate on *A. mangium* showed that the species is not pathogenic to this host, as the induced lesion lengths were similar to the control (avg. 25 mm). In contrast, lesion lengths arising from inoculation with *C. manginecans* were large (avg. 207.6 mm) and significantly longer than those induced by *C. fimbriata*. In two cases small lesions were produced on the trees inoculated with *C. fimbriata* but, statistically, the average lesion size produced by this isolate was not significantly different from that of the control (Fig. S5). Comparison between the self-fertile and self-sterile *C. manginecans* isolates showed that the MAT1 has a similar level of aggressiveness on *A. mangium* than that of the self-fertile parent (α=0.05). In contrast, the MAT2 self-sterile had a significantly lower level of aggressiveness (Fig. S6). The inheritance ratio in the progeny isolates of non-pathogenic to pathogenic was 5:1, suggesting the involvement of three to four genes in determining the pathogenicity of the isolates.

Similar to the situation with the *I. batatas* inoculations, aggressiveness of the progeny on *A. mangium* resembled a quantitative trait. This was evident where the data showed a continuous distribution, although the frequency distribution was skewed towards lower aggressiveness (Fig. S1E). A correlation analysis between tree height, stem diameter and lesion length showed that tree stem diameter has an influence on lesion length and this was adjusted for before comparisons were made between progeny isolates. None of the progeny isolates were less aggressive on *A. mangium* than the *C. manginecans* parent. Eleven of the progeny isolates were considered pathogenic as they produced lesion lengths significantly different from the control, although not significantly different from *C. fimbriata* (Fig. 2). The data for these pathogenic isolates displayed a frequency distribution that approached a normal curve (Fig. S1E). This suggests that a larger number of genes might be involved in determining aggressiveness on *A. mangium*.

The broad sense heritability of aggressiveness on *A. mangium* was not as high as that on *I. batatas* with a value of H^2=0.51 (Table S5D), although this can still be considered a significant genetic contribution. The lower value suggests that environmental variables, such as tree size variation and growth conditions, could have had some influence on the variation observed.

3.6. Inheritance of mating types

Only 12 of the 70 progeny isolates were MAT2 self-fertile and produced ascomata. This was much lower than the number of self-sterile isolates (58) arising from the cross. Of the 58 self-sterile isolates, 19 were of the MAT2 self-sterile mating type (containing the *MAT1-2-1* gene) and 39 were MAT1. The ratio of self-fertile: self-sterile was 1:3 ($p<0.05$) and not 1:1 as expected for a single mendelian trait. This confirms the involvement of a second gene in determining the phenotype, as suggested in previous studies (Webster 1967). Based on the MANCOVA analyses, the mating type of the isolates did not have an effect on the conidia production, growth rate or aggressiveness of the isolate. There was, however, a significant association between mating type and colony colour (Fischer's exact test, p=0.01, Table S3), as the lightly coloured isolates were all self-sterile (MAT1 or MAT2).

4. Discussion

In this study an interspecific cross between *C. fimbriata* and *C. manginecans* was successfully obtained in artificial laboratory conditions, allowing for the first study on the inheritance of pathogenicity and host specificity in recombinant progeny in *Ceratocystis*. By investigating the segregation of various traits in the F_1 progeny isolates we confirmed that colony pigmentation, conidia production, growth rate on MEA and AEA media and aggressiveness on *A. mangium* and *I. batatas* are quantitative traits with high heritability. Conidia production did not show a normal frequency distribution in the progeny and, although polygenic, is likely regulated by a small number of genes. Skewed frequency distributions were also observed for aggressiveness toward *A. mangium* and *I. batatas,* suggesting the association with a small number of genes.

During the mating experiments, not all isolate combinations of the attempted reciprocal crosses were equally successful, and some resulted in watery ascospore masses. This was also observed in other crosses between *Ceratocystis* isolates from different hosts, when *C. fimbriata* was used as the MAT1 partner (Engelbrecht and Harrington 2005; Ferreira et al. 2010). The lower success of the crosses could either be due to partial species barriers or influenced by the mating type of the isolate (Ferreira et al. 2010). Nonetheless, there was also a higher than expected number of individuals that displayed no recombination in the five SSR markers (41 of the 126 screened progeny) and one SSR maker (AF4) displayed segregation distortion. This suggests some bias in the inheritance of parental genetic material or possibly the presence of lethal isolates in the offspring produced.

Based on a biological species concept, some researchers consider *C. fimbriata* and *C. manginecans* to be the same species (Li et al. 2016; Oliveira et al. 2015b), whereas a phylogenetic species concept supports treating these species as distinct taxa (Fourie et al. 2014). The biological species boundary *C. fimbriata* and *C. manginecans* did not appear to be complete at a pre-zygotic stage but rather post-zygotic, influencing the viability and fertility of the progeny. This result is similar to that shown in an experimental cross between *C. fimbriata* and *C. eucalypticola* (Wilken 2015), between species in the closely related genus *Endoconidiophora* (Harrington and McNew 1998) and in other fungal interspecific crosses (Kao et al. 2010) as discussed by Stukenbrock et al. (2013).

A 1:3 ratio of self-fertile to self-sterile progeny was observed in the F_1 population from the interspecific cross. This is similar to that observed for intraspecific crosses between MAT1 and MAT2 self-sterile isolates of *E. coerulescens* (Harrington and McNew 1997) and interspecific crosses between *C. fimbriata* and *C. eucalypticola* (Wilken 2015). This ratio suggests that two genes, inherited independently, are involved in ascomatal formation. The mutation of an additional gene, likely involved in protoperithecia development (Ferreira et al. 2010; Webster 1967), resulted in the sterility of the MAT2 self-sterile parent, and this was inherited along with the MAT locus in the offspring. Even though the self-fertile : self-

sterile ratio deviated from a 1:1 ratio, the *MAT1-2-1* gene was inherited in a 1:1 ratio, suggesting no bias in the inheritance of the mating type locus.

The restricted host ranges of *C. fimbriata* and *C. manginecans* was confirmed in this study. As previously shown with cross inoculations on various other tree species (Baker et al. 2003), *C. fimbriata* from *I. batatas* is not pathogenic to tree species, including *A. mangium.* It is well known that *C. manginecans* is not specific to a particular tree host as isolates from mango can also infect, for example, *Schinus terebinthifolius* (Harrington 2011), *Dalbergia sissoo* and *Prosopis cineraria* trees (Al Adawi et al. 2013). However, the present study showed that *C. manginecans* from *A. mangium* is also not pathogenic to the root crop *I. batatas*. Inoculation studies on *I. batatas,* using other *Ceratocystis s.l.* strains from hosts such as coffee, prune, cacao, oak, taro and almond, also demonstrated a lack of pathogenicity on this host (Kojima and Uritani 1976).

The restricted host ranges of the investigated *Ceratocystis* species are not regulated by a single gene. When the progeny isolates, obtained from the interspecific cross, were inoculated onto *I. batatas* and *A. mangium* the inheritance of pathogenicity in the progeny deviated significantly from a 1:1 segregation of pathogenic vs non-pathogenic. The inheritance ratio observed for pathogenicity toward *I. batatas* (3:1) and *A. mangium* (5:1) suggests that a small number of genes are associated with determining their host range. Three individuals displayed pathogenicity to both hosts. This might indicate that the isolates either gained genes from the one parent, which enabled it to infect both hosts, or lost a gene that allowed it to evade host detection. The low number of genes associated with host specificity are consistent with what has been observed in similar studies on other fungal pathogens. For example, in an F_1 generation of *Magnaporthe oryzae*, generated from a cross between a wheat specific and rice specific parent, the inheritance ratio was consistent with three genes controlling host specificity (Tosa et al. 2006). Similar results were found for the related pathogen *M. grisea* that infects rice (Murakami et al. 2003; Murakami et al. 2000) and for the brown mustard plant pathogen *Leptosphaeria maculans* (Chen et al. 1996). Since a low number of genes are likely involved in host specificity in *Ceratocystis* species,

pathogen host interaction genes, such as avirulence or effector genes might play a role (Deng et al. 2017; Molano et al. 2018; Shirke et al. 2016) but would need further investigation.

Inoculation of the F_1 progeny isolates onto *I. batatas* and *A. mangium* showed that aggressiveness in *Ceratocystis* is a quantitative trait. This was reflected by a continuous distribution of lesion lengths induced by the progeny isolates on both plant hosts. The frequency distribution of the lesion lengths of the isolates pathogenic on *I. batatas* did not display a distinct distribution but three categories of increased aggressiveness were observed. This suggests the involvement of a small number of genes having a strong effect. Similarly, in a cross between two *Fusarium* species, both pathogenicity and aggressiveness was suggested to be associated with only a small number of genes (Cumagun et al. 2004). On *A. mangium* the pathogenic isolates displayed a normal distribution in aggressiveness and, in this case, it is likely that a greater number of genes are involved in determining aggressiveness on this host.

The differences in growth rate of *C. manginecans* and *C. fimbriata* on three different media suggests that these species differ in their ability to obtain nutrients from their respective hosts. Nutrient processing differences have been associated with host specificity in other fungal pathogens (Brunner et al. 2013; Van der Nest et al. 2015). The reduced growth rate of *C. fimbriata* on AEA could suggest that the tree plant nutrients were more accessible to *C. manginecans* than to *C. fimbriata*. This could be due to differences in degradative enzymes (Van den Brink and de Vries 2011). The fact that both species grew most rapidly on IEA suggests the simple sugars and starch from *I. batatas* (Ishida et al. 2000) were accessible and conducive for growth. Nutrient acquisition is unlikely to be the major factor determining host specificity in the *Ceratocystis* species, as the progeny pathogenic to *I. batatas* did not all display a slower growth rate on AEA. What was not tested on growth media, but could be an additional factor influencing host specificity, is the ability of the pathogen to process host response chemicals such as phenolics and terpenoids (Wadke et al. 2016). This should be investigated in future.

Growth rate was confirmed to be a quantitative trait in *C. fimbriata* and *C. manginecans.* This was evident from the continuous distribution and normal frequency distribution of the progeny growth rates both on MEA and AEA media. These results are similar to those for other filamentous fungi (De Vos et al. 2011; Van der Nest et al. 2009; Zhan et al. 2016). The genetic contribution to the regulation of growth rate was confirmed by the high broad-sense heritability values (H^2=0.935 and H^2=0.835). Although the *in vitro* growth rate of fungal pathogens has been associated with aggressiveness in several fungal species (Lee et al. 2015; Zhan et al. 2016), no clear correlation was observed in the isolates from this study. Similar results were found for *Ceratocystis* species pathogenic to *Eucalyptus* spp. (Oliveira et al. 2015a). Results of the present study indicate that aggressiveness and growth rate are traits regulated independently of each other.

The growth rate of an isolate could not be linked to a specific mating type in this study. The MAT1 mating type of *C. manginecans* grew more slowly than the MAT2 self-fertile isolate, which is similar to observations seen in *C. albifundus* (Lee et al. 2015). However, this trend was not observed for *C. fimbriata* where the MAT1 isolate was similar in growth rate to the MAT2 self-fertile isolate. In the case of the MAT2 self-sterile isolates, for which growth rate has never previously been investigated in *Ceratocystis*, the *C. fimbriata* MAT2 self-sterile isolate had a lower growth rate while in *C. manginecans* the growth rate did not differ from the MAT1 isolate. The mating type of a fungal species has been shown to be linked to growth rate in the yeast species *Cryptococcus neoformans* (Lin et al. 2006) and filamentous ascomycetes such as *C. albifundus* (Lee et al. 2015). However, in the present study the parents and F_1 progeny did not reflect a strong correlation between growth rate and a specific mating type.

Conidial production was shown to be a quantitative trait in *Ceratocystis*, most likely regulated by a few genes. This was evident from the continuous distribution of the phenotype in the progeny, ranging from sparse to abundant, and the non-normal frequency distribution observed. Although spore production has been linked to aggressiveness in some fungal species (Lannou 2011; Milus et al. 2008) studies in two different wheat pathogens

have found an independent regulation of spore production and lesion size induced on the host (Pariaud et al. 2009; Stewart et al. 2016). For the *Ceratocystis* species in the present study, no correlation was observed between these two traits. For example, one of the progeny isolates most aggressive to *A. mangium* (isolate FM-56D) produced very few conidia (Fig 4). This lack of correlation has also been observed for *Ceratocystis* species pathogenic to *Eucalyptus* spp. in Brazil (Oliveira et al. 2015a).

The *C. fimbriata* and *C. manginecans* parents and the F_1 progeny displayed a continuous variation in colony pigmentation, from light to dark. This continuous variation suggests that melanin production is a quantitative trait in *Ceratocystis*. This is in contrast to a previous inheritance study with *Ceratocystis sensu lato* isolates, which suggested the involvement of a single gene in colony and ascomatal pigmentation (Webster 1967). Melanin production, which influences colony pigmentation, has also been confirmed as a quantitative trait in other fungi (Butler and Day 1998; Langfelder et al. 2003). In *Zymoseptoria tritici*, for example, up to 16 putative genes were linked to this trait (Lendenmann et al. 2014). Melanin has also been strongly associated with virulence (Collemare et al. 2008; Langfelder et al. 2003). The correspondence between a lighter pigmentation and a lack of pathogenicity observed in the progeny in this study, suggests that melanin could play a role in the pathogenicity of *Ceratocystis* species. The lower fitness of white isolates has also been observed in other species of the Ceratocystidaceae (Van Wyk et al. 2004).

Numerous mating studies have been performed with species residing in the Ceratocystidaceae, where the inheritance of mating genes, other culture morphological traits and genetic markers have been considered (Ferreira et al. 2010; Harrington and McNew 1997, 1998; Johnson et al. 2005). The current study presents the first study considering the inheritance of host specificity and aggressiveness, using an F_1 population, for any species in this family. Results showed a high level of heritability and a quantitative nature of traits such as colony pigmentation, growth rate, conidial production, isolate aggressiveness and host specificity. The fact that a high level of aggressiveness in the pathogenic progeny could not be correlated with other life history traits such as mating

type, growth rate and conidial production, suggests that aggressiveness is inherited independently of the other traits. The hybrid population produced in this study provides a valuable resource for future studies to identify the genomic regions associated with the traits investigated in this study.

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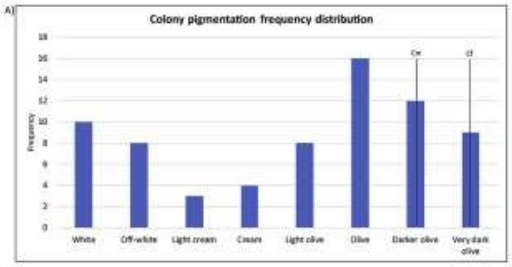
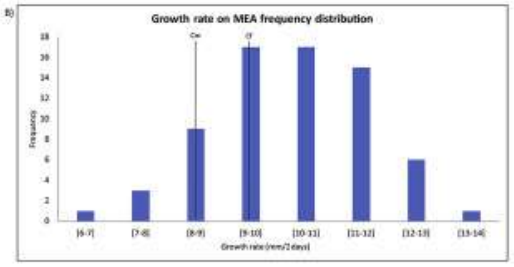
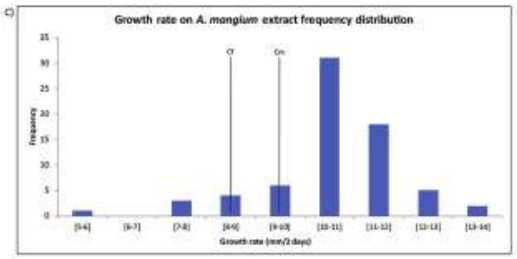
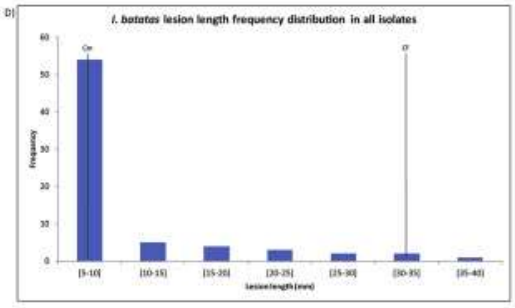
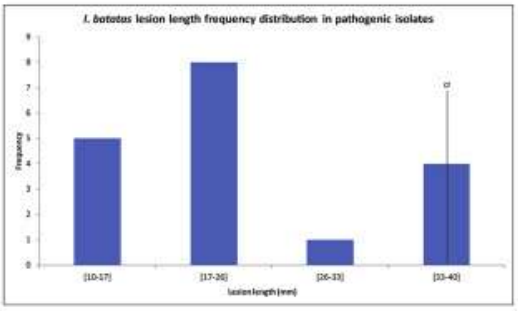
Supplementary material

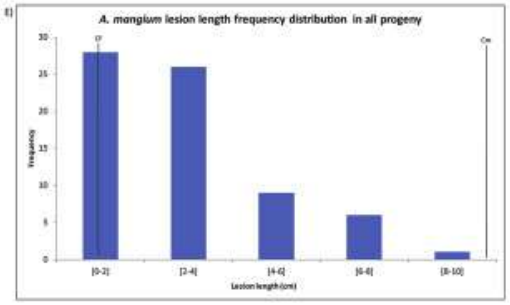


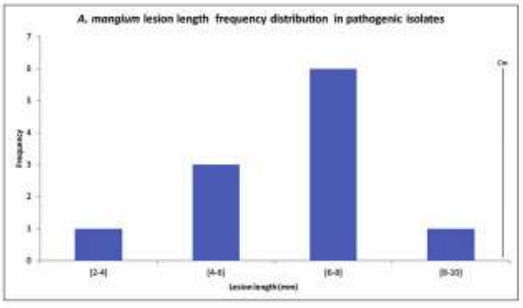


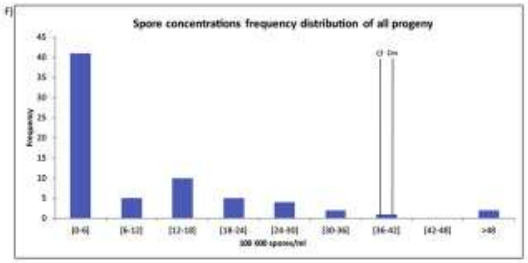












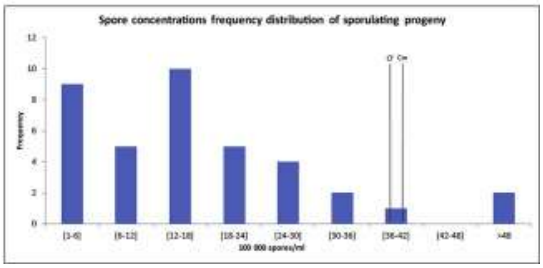


Fig. S1. Frequency distribution of the data observed in the 70 progeny in all phenotypes investigated, including A) Colony pigmentation, B) growth rate on MEA, C) Growth rate on *A. mangium* media, D) aggressiveness of all isolates (left) and only pathogenic isolates (right) on *I. batatas*, E) aggressiveness of all isolates (left) and only pathogenic isolates (right) on *A. mangium* and F) conidia spore production. The annotated lines on the bars indicate where the values of the parental isolates *C. fimbriata* (Cf) and *C. manginecans* (Cm) fit in the data distribution.

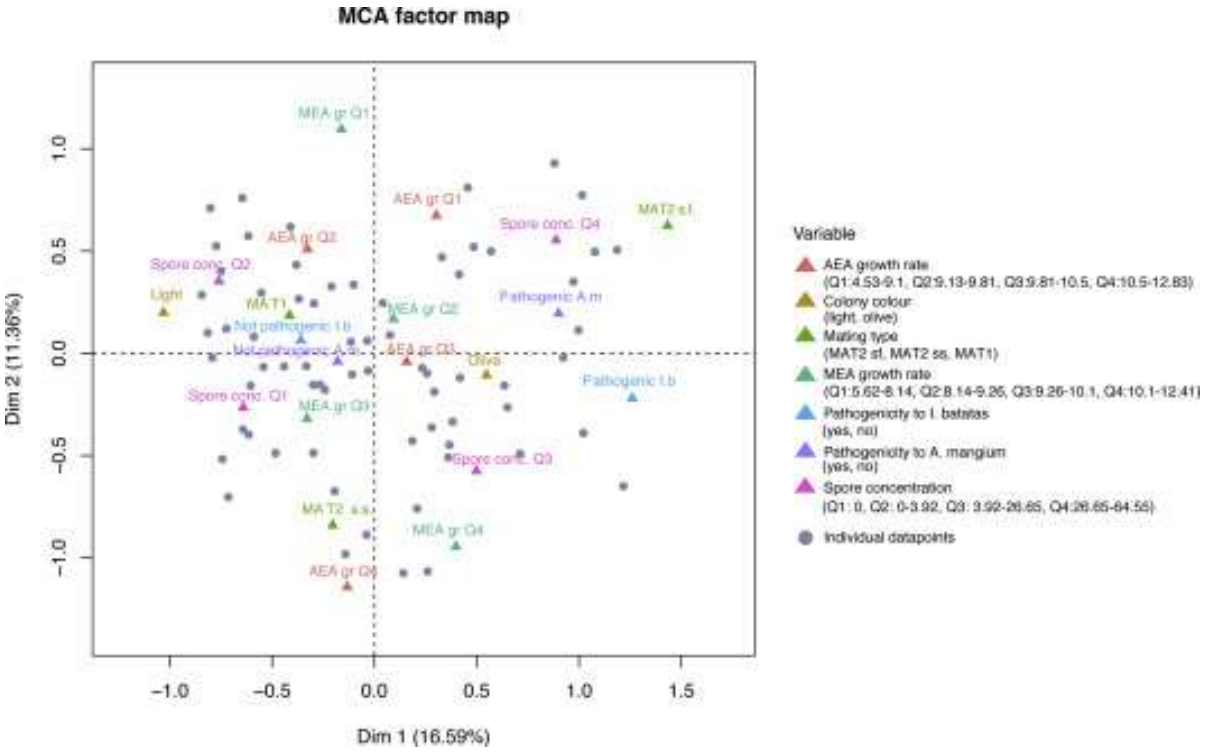


Fig. S2. Multiple correspondence plot, indicating the relationship between all the phenotypes investigated in this study. The first dimension explained 16.6% of the variance and the second dimension 11.4% of the variance in the data. The key indicates the colour assigned to each phenotype and the different categories each phenotype was placed into. The values on the axes indicate the contributions made by each category to the variation of the first and second component.

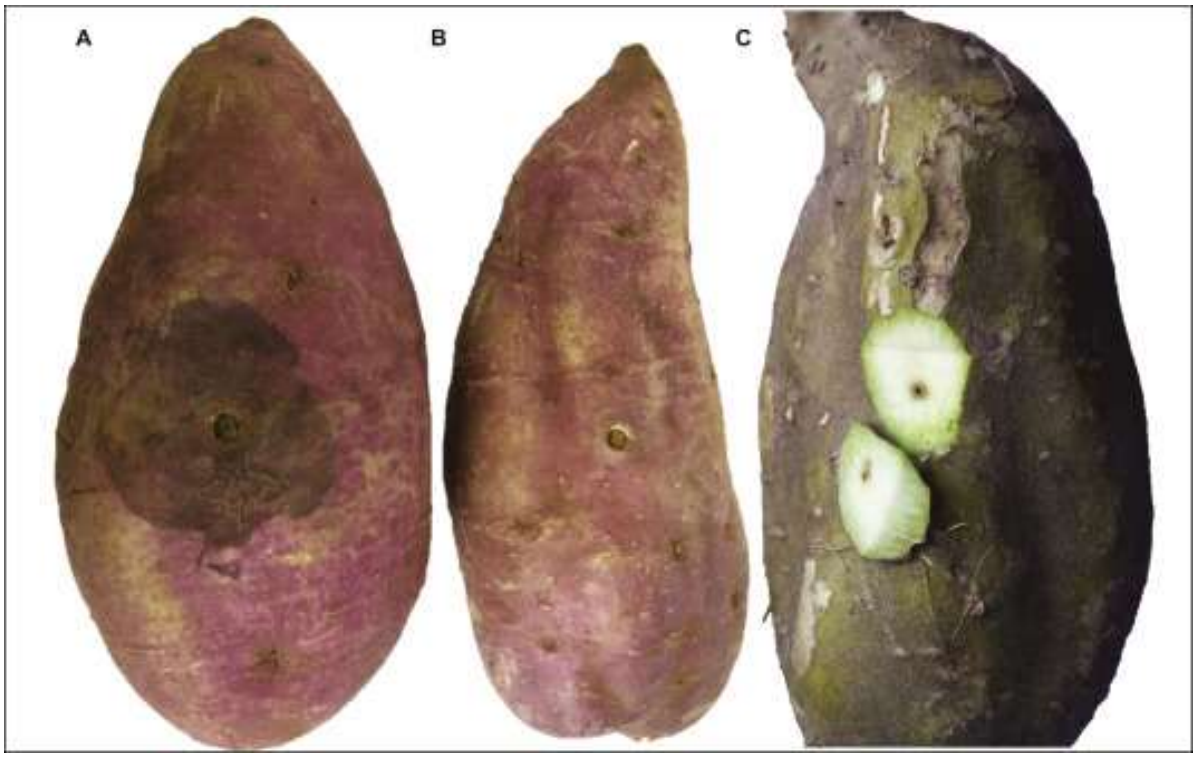


Fig. S3. Aggressiveness of *C. fimbriata* (A) and *C. manginecans* (B) compared to a negative control inoculation (C) on *I. batatas*.

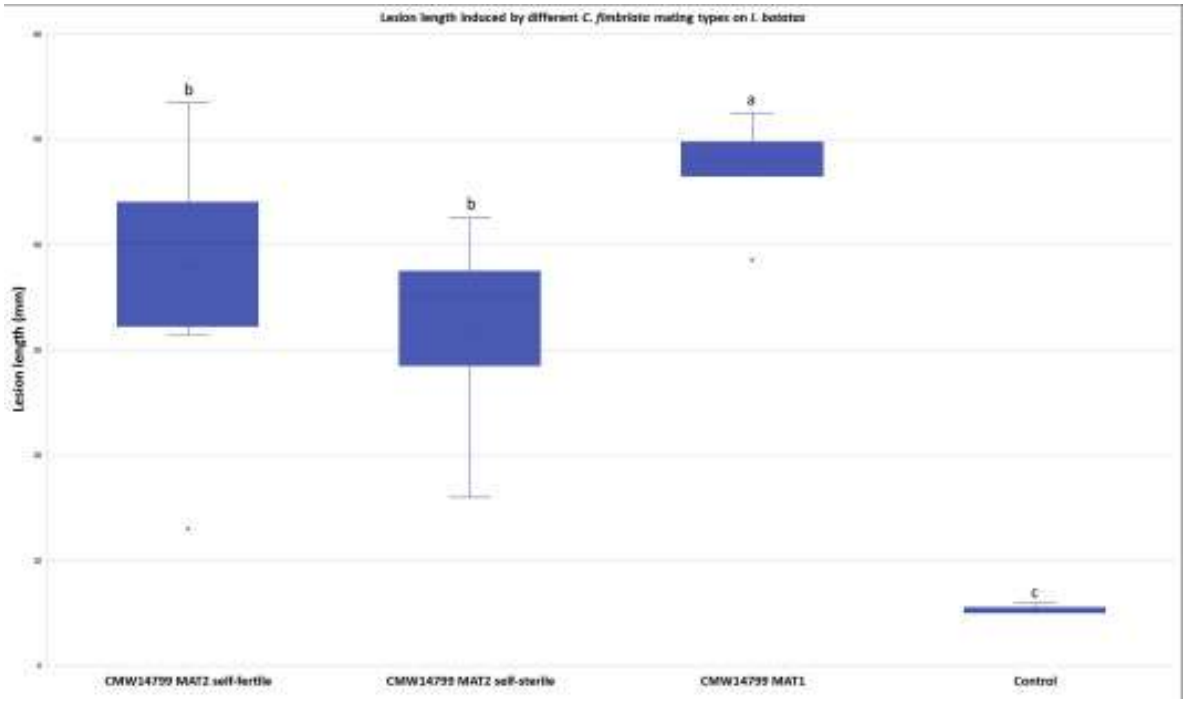


Fig. S4. Lesion length induced on *I. batatas* by different mating types of *C. fimbriata.*

Fig. S5. Aggressiveness of *C. fimbriata* (B) and *C. manginecans* (C) compared to a negative control inoculation (A) on *A. mangium*.

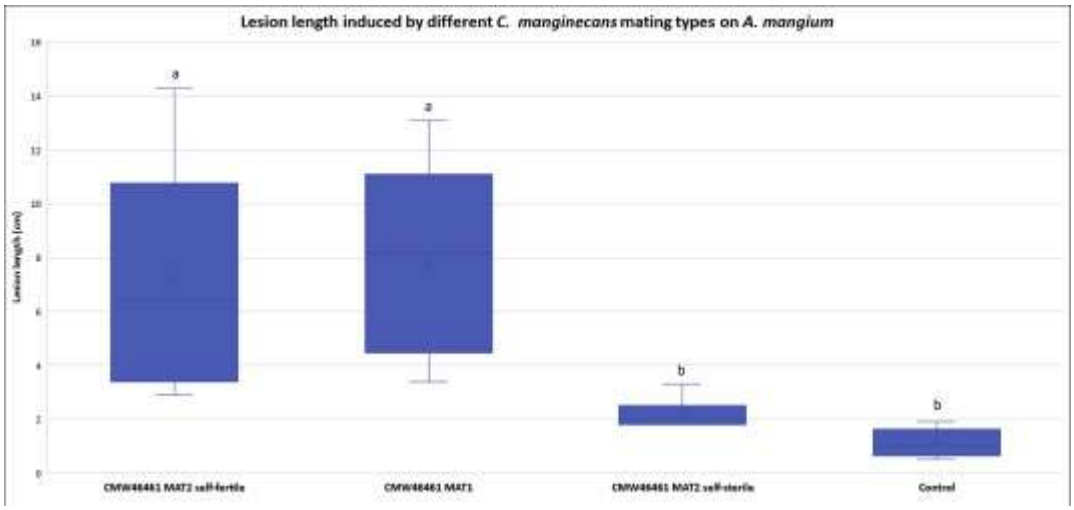


Fig. S6. Lesion length induced on *A. mangium* by different mating types of *C. manginecans.*

Table S1

CMW culture collection number and origin of all progeny produced from the interspecific cross between *C. fimbriata* and *C. manginecans* in this study.

Origin of hybrid isolate	CMW number	Personal collection number
CWM14799 MAT2 self-sterile x CMW46461 MAT1	**45834**	FM-1A
	45838	FM-2C
	45843	FM-3A
	45774	FM-4A
	45852	FM-5C
	45853	FM-7A
	45854	FM-8A
	45855	FM-8C
	45775	FM-9A
	45856	FM-9C
	45776	FM-12B
	45829	FM-12C
	45830	FM-13C
	45831	FM-15B
	45778	FM-15C
	45832	FM-16C
	45833	FM-18C
	45835	FM-20C
	45836	FM-26B
	45837	FM-29B
	45777	FM-29C
	45840	FM-36B
	45780	FM-38D
	45841	FM-39B
	45842	FM-39D
	45781	FM-42B
	45782	FM-42D
	45845	FM-44B
	45846	FM-44D
	45847	FM-48D
	45848	FM-49A
	45849	FM-49D
	45850	FM-50A
	45851	FM-55D
	45783	FM-56D
CMW46461 MAT2 self-sterile x CWM14799 MAT1	**46027**	MF-4B
	46032	MF-7D

	MF-10D
46464	MF-12E
46465	MF-13E
45857	MF-19A
46014	MF-22D
45787	MF-22E
46015	MF-23D
46016	MF-24A
45788	MF-24C
46017	MF-24E
45789	MF-25A
46467	MF-26A
46018	MF-28D
46019	MF-29A
46020	MF-31A
46021	MF-32C
46022	MF-34E
46023	MF-36E
46024	MF-38E
45790	MF-39A
46025	MF-45E
46026	MF-46D
46462	MF-47B
46463	MF-50A
46028	MF-53B
46029	MF-55E
46030	MF-57B
45791	MF-71E
45792	MF-73E
46031	MF-74E
45793	MF-75E
46033	MF-82E
46012	MF-104E

Table S2

Microsatellite marker allele sizes determined in the parental *C. fimbriata* and *C. manginecans* isolates and the 70 recombinant progeny isolates.

		Microsatellite marker[a]				
Relatedness	Isolate	AF2	AF4	AF6	AF7	AF9
C. manginecans parent	CMW46461	206	243	294	322	418
C. fimbriata parent	CMW14799	198	246	285	331	415
Progeny isolates	FM-1A	206	243	294	322	415
	FM-2C	206	246	285	322	415
	FM-3A	198	246	285	322	415
	FM-4A	198	246	285	322	418
	FM-5C	206	246	285	331	415
	FM-7A	198	246	294	331	415
	FM-8A	198	246	285	322	418
	FM-8C	206	243	294	322	415
	FM-9A	206	246	294	331	415
	FM-9C	206	246	285	331	418
	FM-12B	198	246	294	331	418
	FM-12C	198	246	294	322	415
	FM-13C	206	246	294	331	418
	FM-15B	206	246	285	322	418
	FM-15C	206	246	294	322	415
	FM-16C	206	246	285	322	418
	FM-18C	206	246	294	322	418
	FM-20C	206	246	285	322	415
	FM-26B	198	246	285	322	418
	FM-29B	206	246	285	331	418
	FM-29C	198	246	285	331	418
	FM-36B	198	243	294	331	418
	FM-38D	198	246	285	322	415
	FM-39B	198	246	285	322	418
	FM-39D	198	243	294	322	415
	FM-42B	206	246	285	322	415
	FM-42D	198	246	294	322	418
	FM-44B	206	243	285	322	418
	FM-44D	198	246	285	331	418
	FM-48D	198	246	294	331	415
	FM-49A	198	246	285	322	415
	FM-49D	198	246	294	331	418
	FM-50A	198	246	285	322	415
	FM-55D	206	246	294	331	418
	FM-56D	206	246	294	322	418
	MF-4B	198	246	285	331	418
	MF-7D	198	246	285	322	415
	MF-10D	206	246	285	322	415
	MF-12E	198	243	285	331	418
	MF-13E	198	246	285	322	415
	MF-19A	206	246	285	331	418
	MF-22D	206	246	285	331	415
	MF-22E	206	246	285	331	415

MF-23D	206	243	285	331	418
MF-24A	198	243	294	322	418
MF-24C	198	246	285	322	415
MF-24E	198	243	294	331	418
MF-25A	206	246	294	331	418
MF-26A	198	246	294	322	418
MF-28D	198	246	285	331	418
MF-29A	198	243	285	331	418
MF-31A	206	246	285	322	418
MF-32C	206	246	294	322	415
MF-34E	206	243	294	322	415
MF-36E	206	246	294	331	415
MF-38E	198	246	294	322	415
MF-39A	198	246	285	322	415
MF-45E	198	246	285	322	418
MF-46D	198	246	285	331	418
MF-47B	198	246	285	322	418
MF-50A	206	246	294	331	418
MF-53B	198	246	285	322	418
MF-55E	198	246	285	322	418
MF-57B	206	243	294	331	415
MF-71E	206	246	285	331	418
MF-73E	198	246	285	331	418
MF-74E	206	243	294	331	418
MF-75E	206	243	294	331	415
MF-82E	206	243	285	331	418
MF-104E	206	246	294	331	415

[a] Microsatellite markers developed by Fourie et al. (2016)

Table S3

Results from Fisher's exact test between all combinations of the phenotypes investigated in this study

	Pathogenic to *A. mangium*[a]	Pathogenic to *I. batatas*[a]	Colony colour[a]	MEA growth rate[a]	AEA growth rate[a]	Spore count[a]
Mating type	1	1	0.01	1	1	1
Pathogenic to *A. mangium*		1	0.01	1	1	1
Pathogenic to *I. batatas*			0.01	1	1	1
Colony colour				1	1	1
MEA growth rate					1	1
AEA growth rate						1

[a] P-value from Fisher's exact test, including the Bonferroni adjustment to correct for multiple comparisons

Table S4 (see Excel sheet)

Average values of all measured phenotypic data of the parental *C. fimbriata* and *C. manginecans* isolates and the 70 F_1 progeny isolates.

Table S5 (see Excel sheet)

Results obtained from ANOVA analyses of conidia production, growth rate and lesions on *I. batatas* and ANCOVA analyses of lesions on *A. mangium*.