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Lack of evidence for sexual reproduction in field populations of *Colletotrichum lentis*

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ABSTRACT

Species in the genus *Colletotrichum* have mating systems that deviate from those of other genera in the Ascomycota, and for many species only the anamorph is known. The teleomorph of *Colletotrichum lentis* is inducible *in vitro*, but has not been reported in nature. A molecular population study based on amplified fragment length polymorphisms was conducted on 179 field isolates from lentil fields in the Canadian province of Saskatchewan. More than 94% similarity was observed among isolates, and 130 of 131 isolates sampled from a single field shared the same haplotype. Genotypic diversity was equal to 0.47, and association indices I_A and $\bar{r}_d$ were significantly different from zero. A linkage between mating incompatibility group (mIG) 1 and race 0 was observed among field isolates, but F_1 isolates from a cross between an mIG-1/race 0 and an mIG-2/race 1 isolate revealed free recombination of both traits. Results indicate that this fungus does not, or very rarely, sexually reproduces under field conditions.

1. Introduction

Sexual reproduction is costly to fungi, as it is for all organisms, but has the advantage of generating sexual structures vital for the survival of some species in adverse environmental conditions, and more importantly, of creating genotypic variation through recombination which provides opportunity for loss of harmful genes whilst combining genes beneficial for survival (Brown, 1999). In the case of fungi that are pathogens of agricultural crops, virulence is a major trait that determines the competitiveness of an individual in the population. The durability of resistance in agricultural crops very much depends on the biology of the pathogen, and sexually reproducing pathogen populations with high variability may pose a major constraint to the success of resistance breeding programs (McDonald and Linde, 2002).

In ascomycetes, mating systems are diverse but most often species are either self-compatible (homothallic) or self-incompatible (heterothallic) (Kronstad and Staben, 1997). Mating in nearly all heterothallic species described to date is controlled by a single locus/two alleles system which determines a two-mating-type polarity. The two alleles have been described as idiomorphs due to the complete lack of sequence identity, and are commonly referred to as *MAT1-1* and *MAT1-2* (Turgeon et al., 1993; Turgeon and Yoder, 2000). Homothallic species usually possess both idiomorphs in tandem in the same nucleus (Yun et al., 1999), but other mechanisms exist that can lead to a homothallic phenotype (reviewed in Billiard et al., 2011).

The genus *Colletotrichum* is an exception, with all species studied to date having unusual mating systems that do not conform to these two categories. For *Colletotrichum gloeosporioides*, a system of unbalanced heterothallism was proposed, where each partner of a fertile cross carries mutated fertility gene(s) that either can complement each other (cross fertility) or not (cross sterility) (Edgerton, 1914; Wheeler and McGahen, 1952; Wheeler, 1954). This system was also considered for *Colletotrichum graminicola*, where the presence of another isolate was thought to induce selfing (induced homothallism) (Vaillancourt et al., 2000).

The anthracnose pathogen from lentil, previously identified as *Colletotrichum truncatum*, but recently distinguished as the new species *Colletotrichum lentis* (Damm et al., 2014) can be induced to mate under laboratory conditions (Armstrong-Cho and Banniza, 2006) , with perithecia being formed within 10–14 d by pairing isolates on sterile lentil stems. No selfing was observed, but two mating incompatibility groups (mIG1 and mIG2) were identified, all isolates of which have the HMG box characteristic for the *MAT1-2*

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idiomorph, but not the alpha domain of the *MAT1-1* (Armstrong-Cho and Banniza, 2006; Menat et al., 2012). Mating conditions in the laboratory were not very different from those potentially encountered in the field hence raising the possibility for mating of *C. lentis* to occur in nature. However, sexual structures have never been reported from lentil plants infected with *C. lentis* under natural conditions. One specific concern in this species is the existence of two pathogenic races with different levels of aggressiveness, the more aggressive race 0 and the less aggressive race 1 (Buchwaldt et al., 2004; Armstrong-Cho et al., 2012). Lentil varieties partially resistant to the less aggressive race 1 are available (Vandenberg et al., 2002; Buchwaldt et al., 2004), and germplasm is in development with partial resistance to the more aggressive race 0 (Tullu et al., 2006; Fiala et al., 2009; Vail and Vandenberg, 2011). The emergence of any new race with different modalities of aggressiveness and different responses to available resistant cultivars could have important implications for lentil cultivation.

Detecting perithecia or ascospores in field settings is difficult, primarily due to the small size of structures and contamination of plants with other fungi. Instead, the population structure and diversity of field isolates of *C. lentis* was investigated here by amplified fragment length polymorphism (AFLP) fingerprinting, to estimate the extent of linkage disequilibrium. Further evidence was collected by determining whether isolates of the two mating incompatibility groups required for mating coexisted on a small scale in lentil fields that enabled physical contact, and if co-existing isolates were effectively cross-fertile.

2. Materials and methods

2.1. Isolates

A total of 230 monoconidial isolates were utilized, 192 of which were field isolates of *C. lentis* collected from across the lentil growing area of the province of Saskatchewan, Canada, between 2001 and 2008, two were received from Agriculture and Agri-Food Canada, Saskatoon Research Centre, and had been isolated in Manitoba in 1995, and 36 were ascospore-derived isolates from a cross developed previously from CT-21 (race 1, mIG2) × CT-30 (race 0, mIG1) (unpublished data) (Table 1).

Fifty-nine of the above 192 Saskatchewan field isolates were obtained from infected lentil plant material collected in 39 arbitrarily identified, commercial fields to capture diversity at the provincial level. A single lentil field near Dysart in south-eastern Saskatchewan, Canada, with severe anthracnose infestation was intensively sampled in 2008. Infected plant material was collected from 3 transects approximately 10 m apart, with 6 sampling points approximately 10 m apart on each transect. At each sampling point, samples from five neighbouring plants were collected, and up to four isolates per plant were isolated for a total of 134 isolates. The exact location of each isolate in the field was recorded.

For all field isolates, stem lesions were excised from the plant and were surface-sterilized for 3 min in a 10% sodium hypochlorite solution, washed in sterile water and placed in Petri dishes containing potato dextrose agar (PDA, DifcoTM, Becton, Dickinson & Co., Sparks, Maryland, U.S.A.) amended with streptomycin. After a few days of incubation at 22 °C, colonies were examined under the microscope for morphological features characteristic of the species and mycelium or spores were transferred onto fresh PDA amended with antibiotics. Once sporulation was observed, spores were dislodged, diluted in sterile water and streaked onto fresh PDA. For some tissues heavily contaminated by other fungal species, conidial masses from acervuli characteristic for *C. lentis* were dislodged with a scalpel from the plant tissue and streaked onto PDA amended with antibiotics. After 24 h, three germinated conidia were transferred individually to new plates with the help of a dissecting microscope and the species was confirmed based on conidial morphology. Isolates were routinely cultured on oatmeal-agar medium (OMA: 30 g oatmeal flour [Quick Oats Robin Hood, Smucker Food of Canada, Markham, Ontario, Canada], 8.8 g granulated agar [Difco™, Becton, Dickinson & Company, Sparks, MD, USA], 1 L distilled water) and incubated at 22 °C with a 12 h photoperiod.

Different sets of isolates from this collection of 230 isolates were used for the molecular population study, for determining mating incompatibility groups and for race identification. These sets of isolates are described in the relevant section below.

2.2. Molecular population study

A molecular population study based on amplified fragment length polymorphisms was conducted on 179 isolates, 48 of which came from 33 fields, and 131 originated from the single lentil field at Dysart (Table 1).

Liquid cultures were obtained by inoculating 40 ml centrifuge tubes containing 25 ml glucose yeast medium (1 g $NH_4H_2PO_4$ [EM Science], 0.2 g KCl [OmniPur®, EMD™], 0.2 g $MgSO_4 \cdot 7H_2O$ [EM Science], 10 g glucose [BDH®], 5 g yeast extract, 0.01 g $ZnSO_4 \cdot 7H_2O$ [EM Science], 0.005 g $CuSO_4 \cdot 5H_2O$ [EM Science], 1 l distilled water) with small amounts of mycelium from 5 to 7 d cultures and incubating in a shaker (Model SI-600, Lab Companion, Jeio Tech, Seoul, Korea) at 23 °C and 130 rpm for 5–7 d. Mycelia were harvested by centrifugation, stored at −80 °C for 48 h in 2 ml microcentrifuge tubes and subsequently lyophilized for 2 d in a Labconco cryo-freezer (Labconco Corp., Kansas City, MO, USA). Freeze-dried mycelium was coarsely powdered with a pipette tip and DNA was extracted following a protocol modified from Raeder and Broda (1985). In a microcentrifuge tube, 1 ml of extraction buffer constituted of 200 mM Tris HCl pH 8.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS was added to approximately 1 ml of powdered mycelium. The tubes were manually shaken before being incubated for 30 min at 37 °C. After adding 500 μl of 24:24:1 phenol/chloroform/isoamyl alcohol, the tubes were shaken again and centrifuged for 30 min at 13,000 g. The upper aqueous phase was transferred to a new microcentrifuge tube and 25 μl of RNAse A were added followed by incubation at 37 °C for 10 min. An equal volume of chloroform was added and the solution was centrifuged for 10 min at 13,000 g. The upper aqueous phase was transferred into a new microcentrifuge, and DNA was precipitated with × 0.5 that volume of ice-cold isopropanol. The solution was then kept at −20 °C for at least two hours to allow DNA to settle. After a short centrifugation, the liquid was discarded carefully to avoid disturbing the pellet. The pellet was washed with 70% ethanol, dried overnight and re-suspended in 100 μl sterile 10 mM Tris HCl at pH 8, and 1 mM EDTA.

To identify primer pairs that generated strong polymorphic markers, eight isolates of *C. lentis* (CT-20, CT-21, CT-30, CT-31, CT-39, CT-58, CT-59, CT-60) were chosen as they represented a highly variable set in terms of geographical origin and crop year to maximize the potential for genetic variability and to represent every available combination of mating incompatibility and race identity available. AFLP analysis was performed according to Vos et al. (1995) with some modifications. Digestion and ligation of DNA were performed with the IRDye Fluorescent AFLP kit for Large Plant Genome Analysis (LI-COR Biosciences). A 1:10 dilution of the PCR products was performed for use in pre-amplification.

For pre-amplification, the oligonucleotides primers EcoRI + A and MseI + C were used. PCR was performed as follows: for a 50 μl reaction, 2.5 μl of diluted digested/ligated mixture were added to 5 μl 10X PCR reaction buffer (100 mM Tris–HCl (pH 8.3), 150 mM Mg-acetate, 500 mM K-acetate)), 1.5 μl of 50 mM $MgCl_2$, 1 unit of