Genetic Variation Among Populations of *Thielaviopsis basicola*, the Causal Agent of Black Root Rot.

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Abstract

Thielaviopsis basicola is a ubiquitous soilborne fungal plant pathogen with a wide host range. It is the causal agent of black root rot on many agriculturally important crops such as cotton, tobacco and legumes. Twenty-five *T. basicola* isolates collected from three cotton-growing regions, and peat and lettuce soils from a range of locations were examined for genetic variation using the RAPD-PCR technique with 10 arbitrary primers. DNA polymorphisms were detected among isolates from the cotton-growing regions of Goondiwindi, Qld, and Narrabri and Warren, NSW. A phenogram was constructed using the unweighted pair-group method with arithmetic averages (UPGMA) for cluster analysis. Isolates from two cotton-growing regions each clustered into a distinct group based on RAPD-PCR profiles suggesting independent evolution of *T. basicola* between these regions. Isolates from the third cotton-growing region did not cluster and were distributed between the other two regions, suggesting migration and gene flow between these regions. Lettuce isolates clustered with peat isolates providing more evidence that peat is a source of *T. basicola* found in lettuce soils. The results indicated that RAPD-PCR is a useful tool in detecting genetic variability in populations of *T. basicola*.

Introduction

Thielaviopsis basicola Berk. & Br. (syn *Chalara elegans* Nag Raj & Kendrick) is a soilborne plant fungal pathogen with a wide host range including various agricultural and horticultural crops such as cotton, lettuce, tobacco and legumes. *T. basicola* is widely distributed on agricultural and non-cultivated soils and being found on all continents except Antarctica (Subramanian, 1968) is able to infect over 100 plants in 33 families (Yarwood, 1981). The common name, black root rot arises from the characteristic black lesions which develop on the cortical tissue of susceptible roots.

Black root rot was first recorded on cotton in Australia in 1990, when cotton plants with diseased crown tissue and stunted cotton seedlings with blackened roots were collected from Moree, New South Wales (Allen, 1990). Since then, the distribution and severity of black root rot has increased exponentially resulting in a widespread epidemic. The first record of *T. basicola* on lettuce was in Queensland in 1983, however it was not until 1990-92 when it was consistently isolated from lesions on lettuce roots from field and nursery seedlings. Infected lettuce seedlings have been associated with peat-based media prior to field transplanting (O'Brien and Davis, 1994). Peat is a major component of media in nursery production of seedlings and most peat used in Australia for seedling production is

imported. *T. basicola* has been isolated from peat originating from Canada, (Graham and Timmer, 1990), New Zealand, Ireland and other northern European countries.

While the pathogenicity of *T. basicola* varies greatly among many crops, the genetic basis for this is poorly understood. Different physiological races of *T. basicola* were reported by Allison (1938), while morphological variation was described by Stover (1950) referring to grey and brown cultural wildtypes. Few reports on molecular studies of *T. basicola* have been described. Random Amplified Polymorphic DNA (RAPD) (Williams, et. al., 1990) analysis has been used to study genetic variability within populations of many plant pathogens (Bentley et. al., 1994; Kekemu et. al., 1997; Barasubiye, 1995). RAPDs are a useful tool in studying populations as no sequencing information of either primer or genome of interest is required.

The objective of this preliminary study was to test the hypothesis that documented differences in host specificity of *T. basicola* isolates affecting lettuce and cotton could be partially explained by genetic variation within and among populations of the pathogen due to different evolutionary histories.

Materials & methods

Fungal cultures

A total of 25 isolates of *T. basicola* were used in this study (Table 1). Fifteen samples of *T. basicola* were baited from cotton soils from New South Wales (NSW) and Queensland (Qld). They were chosen in order to be diverse in geographic origin and representative of cotton populations. Cotton fields from Warren and Narrabri (NSW) and Goondiwindi (Qld) were known to be infested with the pathogen at the time of sampling. Replicated soil samples were taken 30-400m apart from each site with a soil corer, then oven dried for long term storage. *T. basicola* was isolated from 200g soil sub-samples using carrot baits (modified from Yarwood, 1946). *T. basicola* chlamydospores growing on the cut surface of the carrot discs were transferred directly to potato dextrose agar (PDA)(Difco Laboratories, Detroit) amended with streptomycin (120ug/ml) and incubated at 25°C for 3-4 days. Pure cultures were grown from single spores and cultures exhibiting sectoring were not used.

Lettuce and peat isolates were grown from cultures stored under sterile distilled water. Agar cubes were plated on PDA amended with streptomycin (120ug/ml) and pure cultures grown from single spores.

DNA isolation

For each isolate duplicate cultures were grown in 100ml potato dextrose broth (PDB) (Difco Laboratories, Detroit).in 250ml flasks for up to 7 days with gentle agitation once per day. Mycelium

was harvested by straining cultures through four layers of Miracloth (Calbiochem Inc.) and then lyophilised for short-term storage at –20°C prior to DNA extraction. The DNA extraction method of Drenth and Govers (1994) was used and a duplicate set of DNA was produced. The concentration of DNA was determined using a DyNAQuant 200 Fluorometer (Hoefer) and DNA integrity was checked by electrophoresis on a 0.8% agarose gel.

Table 1 Geographic and host origin of 25 isolates of *T. basicola* analysed in this study.

UQ Accession No.*	Original Designation**	Region	Host
UQ4962, UQ4963, UQ4965, UQ4966, UQ4967	C-01, C-02, C-04, C-05, C-06	Warren, NSW	Cotton soil
UQ4968, UQ4969, UQ4971, UQ4973, UQ4974	C-08, C-09, C-11, C-12A, C-12B	Narrabri, NSW	Cotton soil
UQ4975, UQ4976, UQ4977, UQ4978, UQ4979	C-13, C-15, C-1, C-17, C-18	Goondiwindi, Qld	Cotton soil
UQ4980	2864	Rochedale, Qld	Lettuce soil
UQ4986	4150	Bundaberg, Qld	Lettuce soil
UQ4988	4237	Stanthorpe, Qld	Lettuce soil
UQ4989, UQ4990, UQ4991	4247, 4249, 4250	Cambooya, Qld	Lettuce soil
UQ499, UQ4994	4279, 4280	Ireland	Peat
UQ4995	4289	Unknown	Lettuce soil
UQ5005	T-01	Myrtleford, Vic	Tobacco soil

* denotes The University Of Queensland Accession number
** denotes original accession used for the purpose of this study

Primer sequences

Sixty-eight arbitrarily designed 10-mer primers (Operon) were tested for their ability to reveal polymorphisms among a preliminary set of three *T. basicola* isolates. Ten primers that yielded repeatable polymorphic amplification products from a representative subset of three isolates were selected.

RAPD-PCR amplification conditions

The RAPD method of Williams et. al., (1990) using 10-mer primers was used. The PCR was done in 500µl tubes containing 11.2µl dH_2O, 2.5µl 10xPCR Buffer, 4µl $MgCl_2$, 4µl dNTPs, 1.5µl primer, 0.3µl *Tth*+ polymerase (Fisher Biotec) and 1.5µl DNA template. A negative control containing all

reaction reagents except for template DNA was included in each set of PCR reactions. PCR was performed in a Perkin Elmer/Cetus DNA Thermal Cycler. Conditions were programmed as follows: 1 cycle of denaturation for 5 minutes followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 37°C for 1 minute and extension at 72°C for two minutes. Amplification was completed by a final extension at 72°C for 7 minutes.

The PCR products were separated by electrophoresis in 1.5% agarose gels in 1 x TBE buffer and visualised by EtBr staining. A DNA ladder of 100 base pairs was used as a size reference and a *T. basicola* isolate from the mycological herbarium (QDPI) was used as the standard on all gels.

A DNA polymorphism was identified when a band was present in the agarose gel from one isolate and absent in another. Bands were scored as present (1) or absent (0). A data matrix combined all data pooled from the ten RAPD primers for the twenty-five isolates. Data analysis was performed using the Numerical Taxonomy System (NTSYS-pc) Version 2.0 programs (Rohlf, 1990). The SIMQUAL program was used to convert the data matrix into a similarity matrix using the Jaccard co-efficient (Rohlf, 1990). The samples were compared by scoring the number of common bands divided by the total number of bands present (Bentley et. al., 1994). Genetic relatedness among the twenty-five isolates was assessed using the unweighted pair-group method, arithmetic average (UPGMA) of the SAHN program and a phylogenetic tree was constructed using the TREE program (Rohlf, 1990).

Results

Of the sixty-eight primers screened with three isolates of *T. basicola*, 15 failed to produce amplification products under the PCR conditions used and 20 produced monomorphic amplification products. Ten primers with repeatable polymorphisms were selected from the remaining 33. The 10 selected primers revealed useful DNA polymorphisms and generated a total of 38 monomorphic and 92 polymorphic markers among the 25 isolates tested.

Data obtained for genetic relatedness of the duplicate set of isolates were identical. A similarity matrix comparing genetic relatedness (data not shown) was used to construct a phenogram (Figure 1) using the UPGMA option of NTSYS (Rolhf, 1990) to establish the level of relatedness among *T. basicola* isolates in this study.

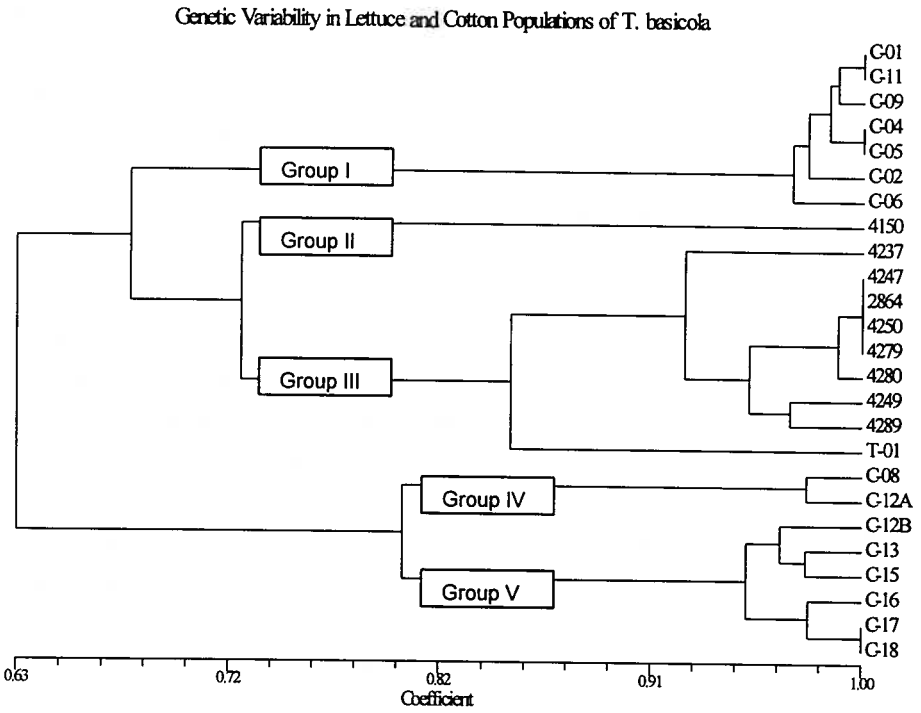

Figure 1 Phenogram of 25 isolates of *T. basicola* clustered by the UPGMA method based on all bands generated using 10 Operon primers. The value of 1.00 indicates 100% genetic similarity.

Five distinct clusters were differentiated by the UPGMA cluster analysis using genetic similarity of 80% as the threshold (Fig. 1). All cotton isolates from Warren, NSW clustered into Group I and all cotton isolates from Goondiwindi, Qld clustered into Group V. Isolates from Narrabri separated into 3 groups: Group I - Warren (2 isolates), Group IV - Narrabri (2 isolates) and Group V - Goondiwindi (1 isolate). *T. basicola* isolates from lettuce soils and peat clustered into Group III. Group II consisted of a single peat isolate.

Discussion

Genetic variation within populations of *T. basicola* from lettuce and cotton soil was examined using RAPD-PCR. RAPD-PCR proved to be a useful technique for assessing genetic relatedness among *T. basicola* populations. Cluster analysis of the RAPD-PCR data differentiated geographically separate populations of the pathogen. The cotton isolates formed distinct groups (or clades) based on geographic origin. Lettuce and peat isolates clustered separately and were most closely related to Group I isolates from Warren (genetic similarity of 65%). This initial analysis however was based on small populations and RAPD primers were selected for those showing polymorphisms.

Genetic differentiation in populations of most fungal species is known to occur between geographic regions (McDonald and McDermott, 1993). The evidence from this study suggests that the Warren and Goondiwindi populations may have evolved independently due to geographic separation. In addition, selection pressure from different cotton cultivars may have an additive effect. Evidence of population variation driven by strong selection pressure by the host genotype has also been recorded for other plant pathogens eg.. (McDermott et al., 1989, McDonald and McDermott, 1993). This study suggests that within the limits of sampling and primers used, the Warren clade is least genetically similar to all other cotton isolates analysed.

The absence of clustering in cotton isolates obtained from Narrabri may be indicative of gene flow that is not restricted locally (Milgroom and Lipari, 1995). *T. basicola* chlamydospores are soilborne and may be dispersed via machinery and infected plant material. Inference can be drawn that genetic similarity between the Narrabri population and Warren and Goondiwindi populations may have occurred by man-induced migration and gene flow.

The occurrence of lettuce as a new host may be the result of a change in plant production methods (ie transplanting nursery-grown plants instead of direct seedling) and novel exposure to a distinct genetic form of the pathogen. Clonal lineages occurring within the lettuce and peat population distributed across geographic regions suggests the possibility that this line was introduced from a single source.

RAPD-PCR profiles were useful in delineating populations from lettuce and cotton soils and in future may provide information that is useful for breeding resistant cultivars of cotton and/or lettuce. Given that gene flow has been identified in this study, plant breeders must be concerned with pathotypes present within the local pathogen population and importantly, the potential for new pathotypes to migrate from other populations (McDonald and McDermott, 1993). Knowledge of the genetics of *T. basicola* populations and their potential to evolve in response to different control strategies will lead to better management of the disease.

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