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Clonality in South African isolates and evidence for a European origin of the root pathogen *Thielaviopsis basicola*

Maria M. GELDENHUIS[a], *Jolanda ROUX*[a], *André J. CILLIERS*[b], *Brenda D. WINGFIELD*[c,*], *Michael J. WINGFIELD*[a]

[a]*Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa*
[b]*14 Field Road, Lilianton, Boksburg, Gauteng, South Africa*
[c]*Department of Genetics, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, South Africa*

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ABSTRACT

Thielaviopsis basicola is a soil-borne fungal pathogen with a wide host range and a cosmopolitan distribution. It causes disease on many agricultural crops, and in South Africa is the causal agent of black pod rot of groundnuts and black root rot on chicory. Knowledge of the population diversity of *T. basicola* could provide valuable information regarding management strategies, the possible movement, origin, and reproductive strategies of the fungus. The objective of this study was to determine the population diversity of *T. basicola* isolates from groundnuts and chicory in South Africa using co-dominant polymorphic markers. These markers were also used to compare isolates from South Africa with those from other hosts and geographic regions. Seven loci revealed nine alleles and two genotypes, one on groundnut and one on chicory, differing at only two loci. *T. basicola* isolates from eight different countries and ten different hosts revealed 17 genotypes across the seven loci with 39 different alleles. The lack of diversity for the two South African host-related populations of isolates suggests that *T. basicola* was introduced into South Africa. Some evidence is provided for a European origin of the pathogen, possibly linked to trade in root crops.

Introduction

Thielaviopsis basicola (syn. *Chalara elegans*) is a soil-borne fungus with a worldwide distribution (Nag Raj & Kendrick 1975). It has a wide host range and has been found on more than 137 plant genera (Yarwood 1981). *T. basicola* causes root rot disease on many agricultural crops including chicory (Prinsloo *et al.* 1991), bean (Tabachnik *et al.* 1979), cotton (Mathre *et al.* 1966), groundnut (Tabachnik *et al.* 1979), tobacco (Gayed 1972), and tomato (Koike & Henderson 1998) and is an important post harvest pathogen on fresh market carrots (Punja *et al.* 1992).

No sexual state has been observed for *T. basicola*, although DNA-based phylogenetic studies indicate that it resides with *Ceratocystis* in the *Microascales* (Paulin-Mahady *et al.* 2002). The mating system has not been studied in this fungus and

* Corresponding author.
E-mail address: brenda.wingfield@fabi.up.ac.za
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it is not known whether more than one mating type occurs in isolates. *T. basicola* is haploid and reproduces via endoconidia and thick-walled chlamydospores enabling it to survive in the soil for long periods of time (Nag Raj & Kendrick 1975). The pathogen adheres to root surfaces and can be transferred to new areas on seed or by insects (Labuschagne & Kotzé 1991; Stanghellini *et al.* 1999). *T. basicola* is an important pathogen in South Africa where it causes black root rot of chicory (*Cichorium intybys*) and black pod rot of groundnuts (*Arachis hypogaea*) (Prinsloo 1980; Prinsloo *et al.* 1991).

Very little is known regarding the origin of *T. basicola* in South Africa, or elsewhere in the world. The objective of this study was to use co-dominant polymorphic markers recently developed for *T. basicola* (Geldenhuis *et al.* 2004) to study the population diversity of *T. basicola* from groundnuts and chicory in South Africa. We also used these markers to compare isolates representing the two South African populations with those available to us from other hosts and geographic origins.

Materials and methods

Fungal cultures and isolations

Thielaviopsis basicola isolates from groundnuts and chicory in South Africa were collected in several areas. Isolates from groundnuts were obtained from 205 groundnut samples randomly taken from an infected crop of groundnuts near Jan Kempdorp and an infested field in Potchefstroom (Fig 1). One hundred and forty-five diseased plant samples were taken from Jan Kempdorp and 60 samples from Potchefstroom. The groundnut fields were smaller than 12 ha in size and corn had previously been planted on the land. The chicory isolates were from 50 diseased chicory plants randomly taken from different delivery trucks at the chicory processing mill at Alexandria in the Eastern Cape Province. The diseased plants originated from chicory farms in the vicinity of Alexandria one of the few chicory growing areas in South Africa (Fig 1).

Isolations from groundnuts and chicory were performed using carrots as bait (Moller & De Vay 1968). The diseased tissue was placed tightly between two surface sterilised carrot slices and incubated at 25 °C in moist chambers. After 5–6 d, the carrots were examined for fungal growth and the groundnut pods were cracked open to find *T. basicola* sporulating on the kernels and hull tissue. Conidial masses were transferred to 2 % malt extract agar (MEA; 20 g l^{-1} malt extract and 20 g l^{-1} agar, Biolab Diagnostics, Midrand, SA) containing streptomycin sulphate (0.4 g l^{-1}; SIGMA St Louis, MO, USA).

Single conidial cultures were made for all isolates used in the study. Conidia were scraped from the MEA surface with a needle and placed in 8 ml sterile distilled water. The spore suspension (1 ml) was transferred to and spread on the surface

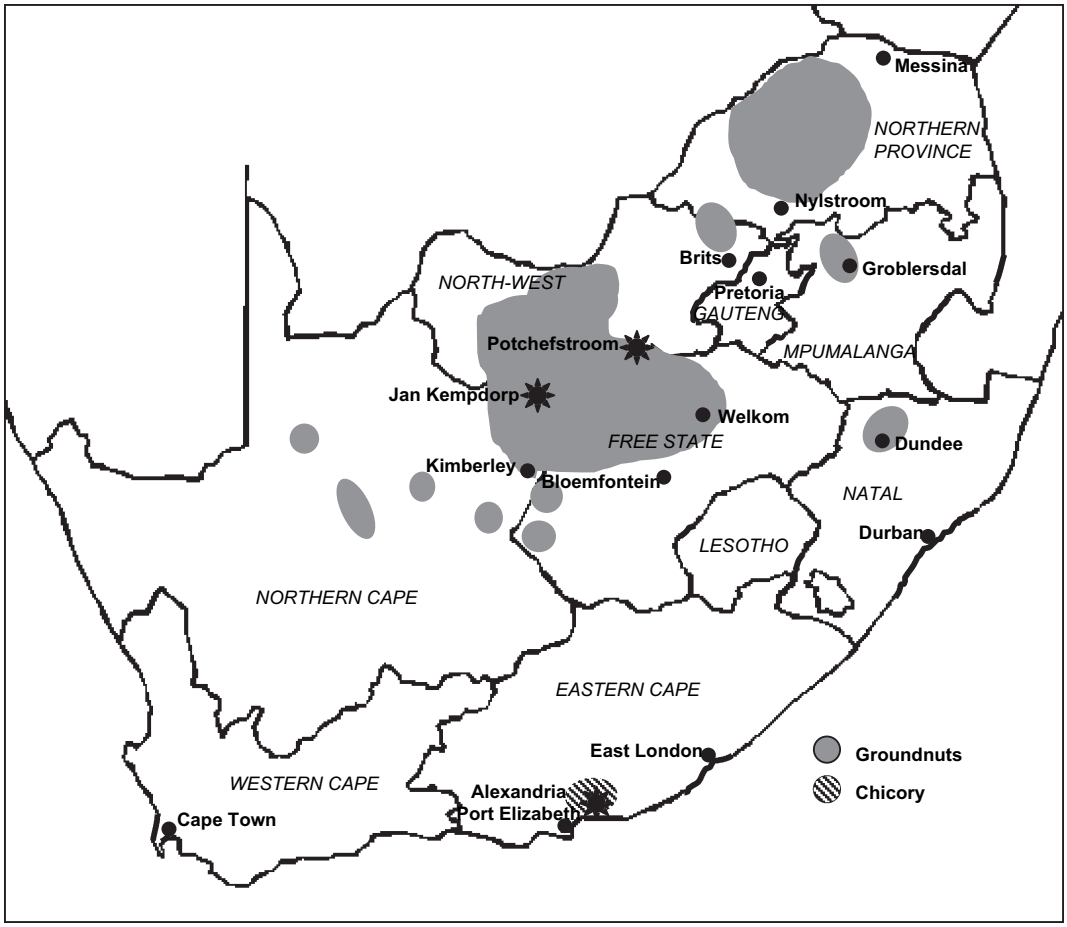

Fig 1 – A map of the main groundnut and chicory producing areas in South Africa as provided by the Agricultural Research Council, Grain Crops Institute, Potchefstroom.

of 2 % water agar plates (20 g l^{-1} agar; Biolab). Excess water was removed and the plates were incubated at 25 °C for 16 h. Single germinating conidia were transferred to 2 % MEA, incubated at 25 °C and resulting cultures were stored at 4 °C.

Isolates of *T. basicola* from other countries were from a wide variety of hosts, geographic areas and some were specifically from international culture collections (Table 1). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria and representative South African isolates have been deposited in CBS (Centraalbureau voor Schimmelcultures, Utrecht).

Population genetic analyses

Isolates were transferred to fresh 2 % MEA in Petri dishes and incubated at 25 °C. After 10 d, DNA was extracted from the isolates using the protocol described by Barnes *et al.* (2001). Seven fluorescently labelled SSR primers designed for *Thielaviopsis basicola* (Geldenhuis *et al.* 2004) were used in PCR amplification of all isolates (Table 1). The same PCR reaction mixtures and conditions were used as described by Geldenhuis *et al.* (2004).

Differences in PCR product sizes were determined by separating the fluorescently labelled PCR products using polyacrylamide gel electrophoresis (PAGE) on an ABI Prism 377 DNA sequencer. Results were analyzed using the GeneScan® 2.1 program (Applied Biosystems, Foster City, USA.) and Genotyper® (Applied Biosystems, Foster City, USA.). Samples for GeneScan analysis were prepared as described by Burgess *et al.* (2001b).

The gene diversity of each locus of the South African population as a whole (both groundnut and chicory isolates) was calculated using the equation

$$H = 1 - \sum_{k} x_k^2$$

where H is the gene diversity and x_k is the frequency of the k^{th} allele (Nei 1973). The gene diversity of the population was then calculated by adding the gene diversities of each locus and dividing it by the number of loci. The gene diversity for the remaining isolates used in this study could not be determined due to the small sample sizes.

Representative isolates from the different hosts and geographic regions were selected for distance analysis. The total nucleotide length for each allele was used to calculate distances (D_{AD}) using the MICROSAT program (http://human.stanford.edu/microsat). The distance matrix obtained from these calculations was then analyzed with MEGA version 2.1 (Kumar *et al.* 2001), using the neighbour joining option to produce a distance tree (Fig 2).

Results

Fungal cultures and isolations

Bacteria from the diseased plant samples contaminated more than 50 % of the carrots that were used as bait to isolate *Thielaviopsis basicola* and this reduced the number of plants from which isolates were retrieved. A total of 79 *T. basicola* isolates were obtained from diseased groundnuts and 15 isolates were obtained from chicory. Each of these isolates originated from a different plant. Fifty-three groundnut isolates were obtained from Jan Kempdorp and 26 from Potchefstroom. All isolates fitted the colony morphology description of *T. basicola* given by Nag Raj and Kendrick (1975). The colonies were effuse with a white colour when young, while older cultures become brownish black and powdery. Isolates produced cylindrical conidia from phialides as well as characteristic aleurioconidia produced in chains, which are typical of *T. basicola*.

Table 1 – *Thielaviopsis basicola* isolates used in this study

Isolate number[a]	Alternative designation[b]	Country	Host	Number of isolates	Genotype profile
CMW 5463–5541		South Africa	Groundnuts	79	2314671
CMW 7622–7633			Chicory	15	1314681
CMW 4098, 4100, 4381, 4685, 4689		Ecuador	Carrots	5	5213353
CMW 4684		"	"	1	5214363
CMW 4686		"	"	1	4231114
CMW 4457		"	"	1	3241124
CMW 5451–5453		USA	Lettuce	3	1414582
CMW 5896		Uganda	Carrots	1	3212434
CMW 5916		"	Carrots	1	3212445
CMW 6714–6716, 6718–6723		Australia	Carrots	9	5222234
CMW 7065	CBS 341.33	Netherlands	*Primula* sp.	1	3212444
CMW 7066	CBS 342.33	"	*Euphorbia pulcherrima*	1	1314681
CMW 7068	CBS 413.52	"	*Lathyrus odoratus*	1	1314582
CMW 7069	CBS 414.52	"	*Primula* sp.	1	3212441
CMW 7071	CBS 430.74	"	*Betula* sp.	1	32224(10)4
CMW 7067	CBS 487.48	Belgium	*Paphiopedilum* sp.	1	6113394
CMW 7070	CBS 150.67	Switzerland	*Nicotiana tabacum*	1	1314671

[a] CMW refers to the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[b] CBS refers to the culture collection of the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

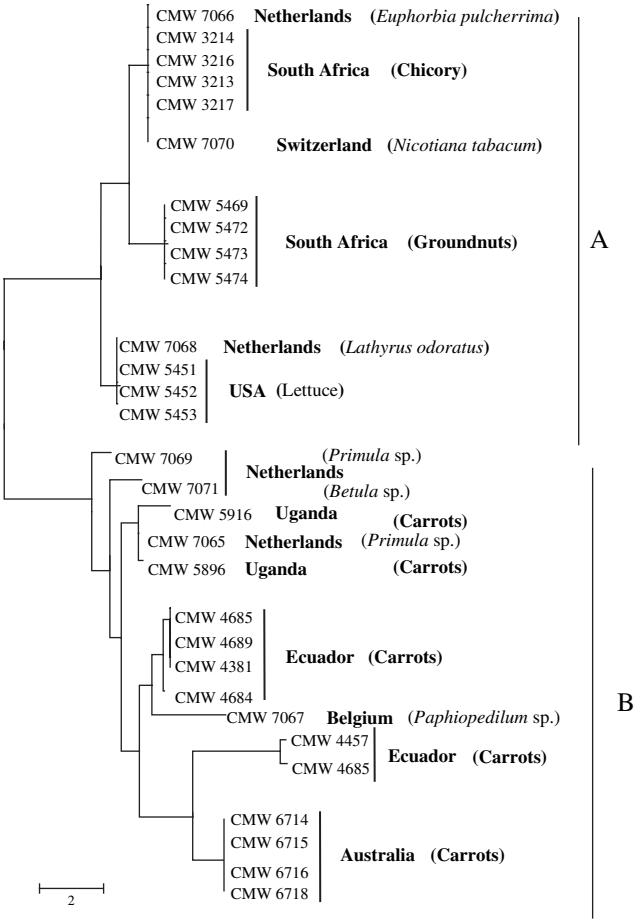

Fig 2 – Neighbour joining distance tree compiled for isolates of *Thielaviopsis basicola* representing different hosts, countries and genotypes. The genetic distances were calculated using D_{AD} on total nucleotide length for each allele at the seven loci.

Population genetic analyses

The seven fluorescently labelled polymorphic primers successfully amplified all of the isolates used in the study. GeneScan analysis for the South African isolates revealed nine alleles across the seven loci (Table 2). Only two genotypes were observed for this population (Table 1). All the groundnut isolates represented the same genotype, with no differences between those from the two different areas. The chicory isolates also represented a single genotype, but it was different to that from groundnut (Table 1).

The worldwide collection of *T. basicola* isolates had 39 alleles across the seven loci (Table 2), resulting in 17 genotypes (Table 1). The number of alleles per locus ranged from four to ten. There were four genotypes from eight isolates from carrots in Ecuador; however, five isolates from the Netherlands all had different genotypes. Only one genotype was observed for the nine Australian isolates and one for the three isolates from the USA. Single isolates from Switzerland and Belgium all represented different genotypes, and these were also different to those from all other countries tested (Table 1). No genotypes were shared between any of the non-South African isolates available for this study.

GeneScan analysis revealed no variation within the groundnut and chicory isolates (Table 1). For this reason, no separate analyses were performed on these two populations, separately. The gene diversity (H) of the South African *T. basicola* population (groundnut and chicory) was 0.077.

The distance tree compiled for all representative isolates revealed two distinct clades (Fig 2). The first of these (clade A) included three subclades, one of which included all groundnut isolates. A second subclade included the chicory isolates together with one isolate from Switzerland and one from the Netherlands. The third subclade consisted of three isolates from lettuce in the USA and one isolate from the Netherlands. The second major clade (clade B) included isolates from Ecuador, Australia, Uganda, the Netherlands, and Belgium. Most of the isolates in this clade originated from carrots.

Discussion

Results of this study using codominant polymorphic markers have shown that the important root pathogen *Thielaviopsis basicola*, from three different regions in South Africa is represented by a genetically uniform population. Thus, a relatively large collection of isolates included only two different genotypes. Furthermore, these were relatively closely related and subdivided based on their host of origin. These results provide strong evidence to suggest that *T. basicola* has been introduced into South Africa. Furthermore, a comparison with isolates from other parts of the world suggests that the fungus originated in Europe.

T. basicola isolates from groundnuts were collected in two geographic regions of South Africa. Groundnuts are produced in many parts of the country (Fig 1), but two regions, where disease outbreaks were reported at the time, were chosen: Jan Kempdorp and Potchefstroom. Samples were specifically from diseased plant material and the sampling areas are known for high disease incidence annually. In this respect, the isolates of *T. basicola*, originated from areas representative of the black pod rot disease in South Africa.

The gene diversity of the South African *T. basicola* population was very low when compared with that of other fungi (Goodwin *et al.* 1992; Wikler & Gordon 2000). Recently established populations would be expected to have low gene diversity, while a pathogen would typically have the highest gene diversity in its country of origin (Gordon *et al.* 1996; McDonald 1997). Multiple introductions of a fungus into a country could also result in a high gene diversity (Burdon & Roelfs 1985; Correll *et al.* 1992; Burgess *et al.* 2001a). The low gene diversity of *T. basicola* from groundnut and chicory in the present study supports the view that it has been introduced into South Africa and that there have only been a few introductions.

Two major clades emerged from distance analysis for the *T. basicola* isolates used in this study. One clade included groundnut and chicory isolates from South Africa, clustering together with isolates from The Netherlands, Switzerland, and the USA. Chicory isolates were especially close to isolates from the Netherlands and Switzerland. Although additional isolates from these countries would be required to clarify their

Table 2 – Alleles observed at seven loci for *Thielaviopsis basicola* isolates from South Africa (SA), Ecuador (ECU), USA, Uganda (UGA), Australia (AUS), the Netherlands (NET), Belgium (BEL) and Switzerland (SWI)

Locus	Allele	SA	ECU	USA	UGA	AUS	NET	BEL	SWI
NG3/4	395	-	2	-	-	-	-	-	-
	396	-	-	-	-	9	-	-	-
	405	-	6	-	-	-	-	1	-
	408	-	-	-	2	-	3	-	-
	427	-	-	3	-	-	1	-	-
	435	94	-	-	-	-	1	-	1
NG5/6	433	-	1	-	-	-	-	-	-
	434	-	1	-	-	-	-	-	-
	445	-	-	-	1	9	-	-	-
	446	-	-	-	1	-	2	-	-
	448	-	5	-	-	-	-	-	-
	449	-	1	-	-	-	-	-	-
	451	79	-	-	-	-	-	-	1
	452	15	-	3	-	-	2	-	-
	454	-	-	-	-	-	-	1	-
	457	-	-	-	-	-	1	-	-
NG 13/14	300	-	2	-	-	-	-	-	-
	301	-	-	-	2	9	3	-	-
	303	-	5	-	-	-	-	1	-
	304	94	1	3	-	-	2	-	1
NG15/16	377	-	-	-	-	-	-	1	-
	378	-	8	-	2	9	3	-	-
	385	94	-	-	-	-	2	-	1
	386	-	-	3	-	-	-	-	-
NG17/18	341	94	6	3	2	-	4	1	1
	342	-	-	-	-	9	1	-	-
	346	-	1	-	-	-	-	-	-
	347	-	1	-	-	-	-	-	-
NG19/20	316	15	-	3	-		2	-	1
	324	79	-	-	-	-	-	-	-
	331	-	1	-	2	-	3	-	-
	332	-	1	-	-	-	-	-	-
	341	-	6	-	-	9	-	-	-
	351	-	-	-	-	-	-	1	-
NG21/22	378	94	-	-	-	-	2	-	1
	379	-	-	3	-	-	1	-	-
	382	-	6	-	-	-	-	-	-
	385	-	2	-	1	9	2	1	-
	392	-	-	-	1	-	-	-	-
Total isolates		94	8	3	2	9	5	1	1

Allele sizes are indicated in the number of base pairs.

relatedness to those from South Africa, intercontinental spread, probably originally from Europe seems likely to have occurred. Isolates from Ecuador, Australia, Uganda, the Netherlands, and Belgium resided in the second major clade. Isolates in this clade included those from carrots in Ecuador, Uganda, and Australia, and it seems likely that they also originated in Europe.

The number of *T. basicola* isolates from plants other than groundnuts and chicory (South Africa), was low and insufficient to support statistical calculations relating to populations. However, the fact that four genotypes were present in a small collection of isolates from a single host (carrots) in Ecuador, suggests a high level of genetic diversity in that country. Similarly, the fact that five isolates from the Netherlands all had different genotypes also indicates high levels of diversity in that country. These results could be misleading due to the small number of isolates, but in comparison with results form South Africa, they indicate clear trends. Furthermore, the fact that distance analysis shows isolates from Europe reside within both major clades together with isolates from countries such as Ecuador, suggests that the fungus is native to Europe and that it has been distributed to other

countries. This could easily have occurred through the widespread distribution of bulbs and root crops from Europe to other parts of the world, over long periods of time. An alternative hypothesis is that the pathogen was introduced into Europe from an unknown destination through the international trade in crop plants and subsequently reexported to other countries.

The *T. basicola* isolates from groundnuts all represent the same genotype, even though they were collected from different areas. Jan Kempdorp and Potchefstroom are separated by a distance of approx. 300 km. This suggests that the pathogen was introduced to the different areas from the same source, probably via infected seed. Labuschagne and Kotzé (1991) demonstrated that black pod rot could be transmitted through contaminated seed, and they found that even seed that appears to be healthy can be infected.

Our results based on distance analysis suggest that *T. basicola* has been subjected to host specialisation. Isolates residing in one of the two major clades, represent those from carrots. In contrast, isolates from chicory, groundnuts, and lettuce all resided in the second major clade. The isolates from the Netherlands originated from different hosts, and they all represent a different genotype. This appears to represent host selection by genotypes. These results are consistent with those of Punja and Sun (1999) who showed, using RAPDs, that isolates from the same host and geographical region tend to group together. Evidence of some degree of host specificity in *T. basicola* has also been reported for isolates that are pathogenic to one host but not to another (Keller & Shanks 1955; Lloyd & Lockwood 1963).

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