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Genetic and morphological diversity of mono-spore isolates of Glomerella cingulata associated with coffee berry disease

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Abstract

In earlier experiments the perfect state, *Glomerella cingulata,* had been found associated with *Colletotrichum coffeanum.* As the linkage between *G. cingulata* and the coffee berry disease (CBD) pathogen had still been doubtful, further investigations were done. It was found that mono-spore cultures derived from *G. cingulata* perithecia from two different CBD isolates were not compatible. This would suggest genetic differences between these two CBD pathogens.

Preliminary tests with mono-ascospore isolates of one *G. cingulata* source resulted in a wide range of segregation types. It has not yet been possible to obtain a conidial form from *G. cingulata,* which has the morphological and pathogenic characteristics of *C. coffeanum.* The results of the experiments suggest, however, that a CBD pathogen may arise through genetic segregation from a *G. cingulata* state.

Additional keywords: Colletotrichum coffeanum, Coffea arabica.

Introduction

Glomerella cingulata (Stonem.) Spaulding & v. Schrenk is an ascomycete with a vegetative form of the *Colletrotrichum* type. The association of *G. cingulata,* however, with the coffee berry disease (CBD), caused by *Colletotrichum coffeanum* Noack *sensu* Hindorf (Hindorf, 1970) on *Coffea arabica* L. could not be established satisfactorily in recent CBD research (Firman and Waller, 1977).

In 1926 Small and McDonald both reported already the occurrence of *G. cingulata* in cultures of non-pathogenic *Colletotrichum* isolates from East African *C. arabica.* Shortly after the first reports on the rapidly increasing presence of CBD in Tanzania, Hocking et al. (1967) obtained indications that *G. cingulata* was associated with *C. coffeanum.* In Kenya Vermeulen (1970) could, however, only establish a link between *G. cingulata* and saprophytic strains of *Colletotrichum,* inhabiting the bark of coffee branches.

The diversity of the morphological characteristics of *Colletotrichum* spp. and *G. cingulata* found on arabica coffee has been described by Gibbs (1969) and Hindorf (1970). The differences between the isolates were based on colour and growth rate of the mycelium, shape and size of conidia or ascospores, type of reproduction and pathogenicity on green berries. In the *Colletotrichum* population isolated from coffee material only the CBD pathogen, *C. coffeanum,* produced conidia *in vitro* exclusively on solitary hyphae.

From 1974 until 1980 59 isolates, alleged to be *C. coffeanum,* from South and Central American coffee growing countries were tested in the Netherlands for their ability to induce CBD symptoms on coffee berries and seedlings. This material was compared with African CBD isolates (Gielink and Vermeulen, 1983). The association of *G. cingulata* not only with non-pathogenic *Colletotrichum* isolates but also with some CBD pathogens was repeatedly observed in the pathogenicity experiments.

This linkage between the pathogen and *G. cingulata* would imply a possible variability of the pathogenicity by genetic recombination in the perfect state. Further research on this association should therefore include the genetics of *G. cingulata.* This topic has been the subject of intensive and long-term investigations with isolates from other crops by Edgerton (1914), Edgerton et al. (1945), Lucas et al. (1944), Chilton and Wheeler (1949a, b), Wheeler (1950, 1954 and 1956) and Wheeler and McGahen (1952).

Using this considerable source of information, the association of *G. cingulata* with *C. coffeanum,* the causal organism of the coffee berry disease, has been studied. The main objective was to establish whether this association would possibly influence either the incidence of more pathogenic *C. coffeanum* strains in areas where CBD occurs or their emergence in hitherto disease-free, *C. arabica*-growing countries.

Materials and methods

The *Colletotrichum* and *G. cingulata* isolates viz. CBD Fyto, CBS 135.30, CBS 396.67, CBS 440.67 and 118 CC (Gielink and Vermeulen, 1983) were used in these experiments. Furthermore a *G. cingulata* isolate from Nicaragua (Nic), received from Mr H.D. Frinking was also included. This isolate produced perithecia on green berries, without showing CBD lesions.

Green berries (SL-28 or Bourbon varieties) were inoculated with droplets of conidial or ascospore suspensions (Bock, 1956). The berries were kept at room temperature (20-22 °C) in petri dishes (15 or 20 cm diameter) lined with moist filter paper. After completion of the recording of any CBD symptoms (10-15 days), the petri dishes were left until perithecia were observed on mummified berries. The inoculation of coffee seedlings (SL-28 variety) was done according to the technique described by Van der Vossen et al. (1976). The seedlings were grown in a greenhouse at a temperature of 22-23 °C. Appearance of perithecia on seedlings would take abut 6-8 months.

Perithecia of *G. cingulata* found on coffee seedlings and berries in conjunction with typical CBD symptoms, were considered to be the 'wild type' *G. cingulata.* In pure culture the wild type produces fertile perithecia in clumps on white mycelium.

The following methods of mono-ascospore isolation were used:

a. Perithecia on plant material (seedlings and berries) were excised under the dissecting microscope and then placed with the ostioli pointing upwards in the lids of inverted petri dishes with water agar or potato dextrose agar (PDA). These dishes were left overnight on the laboratory bench at ambient room temperatures (18-22 °C). The following morning ascospores, which had been ejected and often already had germinated, were observed on the agar surface above the perithecia. Mono-ascospore isolations were then made under low-power magnification of a compound microscope by cutting small agar blocks, each with one ascospore, with a sterile dissecting needle out of the agar. These blocks were then transferred aseptically to petri dishes with malt agar or PDA. It was attemped to produce as many mono-ascospore isolates as possible

from one perithecium.
b. Mono-ascospore isolations from perithecia growing in pure culture were made by carefully picking a single perithecium or clumped perithecia from the culture. After several rinses in sterile water to get rid of mycelium the perithecia were sqashed in sterile water. After proper dilution of the suspension mono-ascospore cultures could be obtained on malt agar or PDA.

Mono-ascospore isolates appeared to consist of two types: the wild type and a black mycelium type with sterile perithecia scattered through the colony. When grown together in one petri dish these types form a ridge of perithecia on the borderline of the two cultures (Edgerton, 1914). Previous research has conclusively shown that the two types mate in this ridge (Wheeler, 1950, 1954 and 1956). Because of this ridge of perithecia Edgerton (1914) called the wild type initially the 'plus type' and the black mycelium type the 'minus type'. Initially attention was focussed only on the incidence of plus and minus types from different sources and the incidence of a perithecial ridge between combinations of types from various origins.

As both the *G. cingulata* and a number of *Colletotrichum* spp. were known to inhabit *C. arabica*, it was necessary to make many mono-spore isolates in order to obtain sufficiently reliable information. The schematic segregation and recombination pattern set up by Chilton et al. (1945) was modified for the morphological and genetic classification of the characteristics of the mono-spore cultures. The modifications consisted of: firstly the introduction of a new notation as proposed by Wheeler and McGahen (1952) and secondly by incorporating Wheeler's conclusions (1954), i.e. changes occur by mutation and not by selection of the homozygotes (either homokaryotes or homothallic types) in the progeny of a heterozygote (either heterokaryote or heterothallic types). After completion of the preliminary series of experiments it was clear, however, that even the modified segregation and recombination scheme (Fig. 1) could not cover the whole range of mutants derived from one wild type source.
The following terminology has been used in this paper:
– the term *type* indicates the general nature of the mono-spore isolate when specific genetic information is lacking or when reference is made to groups of similar, but not always identical cultures (Edgerton et al., 1945). The various types are distinguished on the basis of several characters, e.g. colour of mycelium, perithecial formation and segregation pattern.
– the term *segregation* means the dissociation of internal and parental characters in mono-ascospore cultures and their subsequent subcultures. This term covers in this publication the dissociation of homozygotes (either homokaryotes or homothallic types) in the progeny of a heterozygote (either heterokaryote or heterothallic types). It also covers the dissociation of incomplete types arisen by mutation with certain genetic blocks, according to Wheeler's conclusion (1956).
– the term *compatibility* is used when two different types of the fungus when grown in the same petri dish, form a ridge of fertile perithecia on the borderline of the two cultures (Struble and Keitt, 1950), as occurs not only with the original plus and minus types, but also with some other types (Wheeler and McGahen, 1952).
– the terms *recombination* or *mating* are used when from the perithecia on the borderline of two compatible types a series of recombinant types can be obtained.

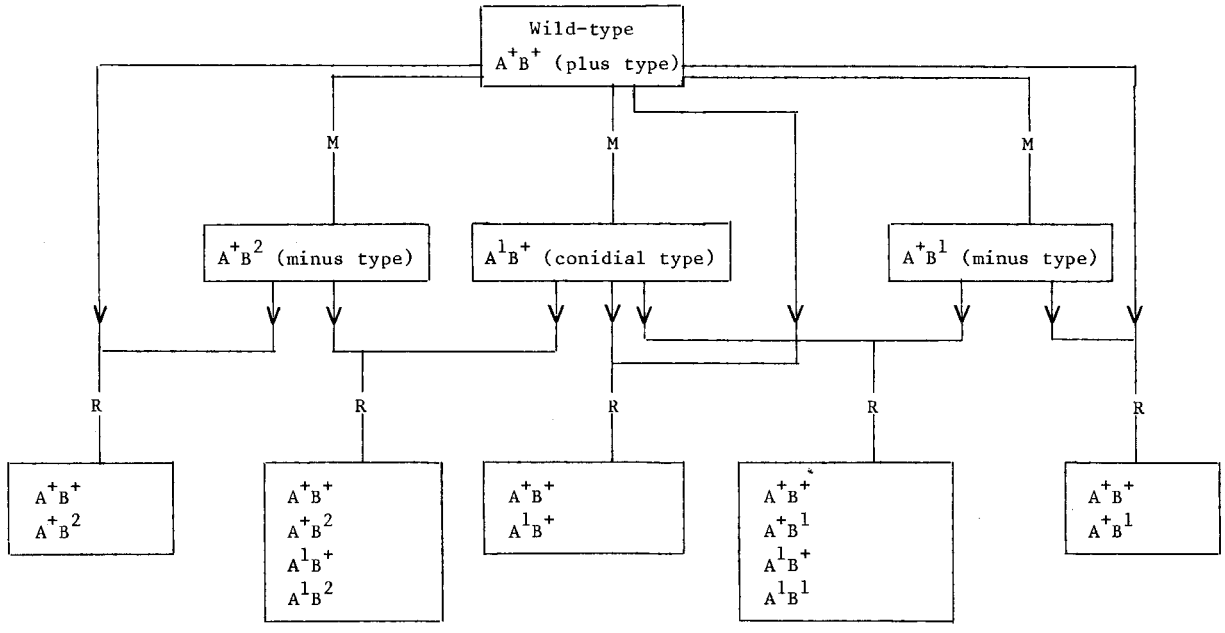

Fig. 1. Segregation and recombination types of *Glomerella cingulata* using tentative designations (based on schematic presentation by Chilton et al., 1945, and Wheeler and McGahen, 1952).

Results

Mono-ascospore isolates obtained in 1979 from perithecia found on mummified berries, inoculated with a conidial suspension of the CBD pathogen CBS 440. 67 (Gielink and Vermeulen, 1983) were initially only classified as 'plus' (whitish aerial mycelium, no conidia, clumped perithecia) and 'minus' (pinkblack appressed mycelium, pink masses of conidia, scattered mostly sterile perithecia) types. When grown together in one dish the two types of the 440.67 source formed on their borderline a ridge of fertile perithecia. Two plus types or two minus types grown together failed to induce the formation of a perithecial ridge.

In further tests the plus and minuts types derived from isolate CBS 440.67 were tried to be mated with conidial isolates of CBD Fyto, CBS 135.30, CBS 396.67 and CBS 440.67; they, however, did not induce a ridge of perithecia in any combination. The same negative result was obtained after combination with the plus and minus types derived from mono-ascospore isolates from the saprophytic Costa Rica culture (118 CC).

In 1980 perithecia of *G. cingulata* were found on the young leaves and growing-points of coffee seedlings, inoculated eight months before with isolate CBD Fyto. Mono-ascospore isolates from this source also yielded plus and minus types. Either combination with the opposite type of isolate CBS 440.67 did not induce perithecial ridges.

A new series of mono-ascospore cultures was obtained in 1980 from coffee berries inoculated with isolate CBS 440.67. This series, labelled as plus and minus CBS 440.67 (1980) types, was paired with the plus and minus CBS 440.67 (1979) types and subsequently also with the plus and minus 118 CC, plus and minus CBD Fyto and the new saprophytic plus and minus Nic types. The results of these combinations are presented in Table 1.

Table 1. Compatibility between plus and minus types derived from various *Glomerella cingulata* sources.

Type		118 CC		CBD Fyto		CBS 440.67 (1979)		CBS 440.67 (1980)		Nic	
		plus	minus	plus	minus	plus	minus	plus	minus	plus	minus
118 CC	plus	–	+	–	–	–	–	–	–	–	–
	minus	+	–	–	–	–	–	–	–	–	–
CBD Fyto	plus	–	–	–	+	–	–	–	–	–	–
	minus	–	–	+	–	–	–	–	–	–	–
CBS 440.67	plus	–	–	–	–	–	+	–	+	–	–
(1979)	minus	–	–	–	–	+	–	+	–	–	–
CBS 440.67	plus	–	–	–	–	–	+	–	+	–	–
(1980)	minus	–	–	–	–	+	–	+	–	–	–
Nic	plus	–	–	–	–	–	–	–	–	–	+
	minus	–	–	–	–	–	–	–	–	+	–

+ = ridge of perithecia on borderline: – = no.

Table 2. Morphological segregation in plus and minus types of mono-ascospore isolates.

Isolates	Total plus types	Total minus types	Total
CBD Fyto	20	23	43
CBS 440.67	105	116	221
118 CC	33	55	88
Nic	3	33	36

The segregation ratio is given in Table 2. The data are obtained by applying only the 'plus' or 'minus' criterion, i.e. mycelial colour white or pink/black, and compatibility. Based on these limited data it appears that the purely saprophytic isolates 118 CC and Nic give a higher segregation in minus types as compared to the pathogenic CBD Fyto and CBS 440.67 isolates.

With the perithecia of *G. cingulata,* found on berries inoculated with conidia of CBS 440.67, a more detailed series of segregation and recombination tests was carried

Table 3. Subtypes obtained from mono-ascospore isolates from perithecia of *Glomerella cingulata* found on berries inoculated with the CBD pathogen CBS 440.67 (designation based on Chilton et al., 1945 and Wheeler and McGahen, 1952).

Type	Description	Remarks
A^+B^+	Wild type	
A^+B^1	Perithecia scattered, fertile	
A^+B^2	Perithecia scattered, sterile	These types resemble *C. acutatum* but the colour of conidial masses is orange instead of pink
A^1B^+	Conidia in slimy masses, clumped in centre of colony	
A^1B^1 and A^1B^2	Conidia in slimy masses, scattered through colony	
A^3B^+	In centre of colony clumped large green-grey acervuli and along periphery clumped perithecia (like A^+B^+); similar to *cca* isolate (acervuli form) of *C. gloeosporioides* (Gibbs, 1969)	Type designations not used previously and without a genetic basis only for provisional classification to delineate genetic type development
A^3B^1	Grey acervuli scattered through colony, later also scattered perithecia	
A^3B^2	Green mycelium and conidial production on appressed mycelium (rudimentary acervuli?); colony becomes sterile and resembles then A^+B^2	
A^4B	Conidia production on appressed mycelium (rudimentary acervuli?), followed by white, aerial mycelium with conidiophores; at later stage perithecia are formed; resembles *ccm* isolate (mycelium form) of *C. gloeosporioides* (Gibbs, 1969)	

out. By using the dilution technique a great number of mono-ascospore subcultures from the wild type, designated as A^+B^+ (perithecia in clumps) was obtained. Some of these cultures could be roughly classified according to the schematic segregation pattern as established by Chilton et al. (1945) for *G. cingulata* found on *Ipomoea* (Fig. 1). Other types differed, however, considerably from those listed by Chilton et al. (1945). In Table 3 the main subtypes derived from source CBS 440.67 are listed.

Infection tests on detached green berries were carried out with the conidia or ascospores of the types listed in Table 3. Lesions were not observed after inoculation with an ascospore/conidia suspension of the wild type A^+B^+ or any of its segregation types. The A^+B^+ type stayed, however, alive on the berry and infected the berry through the stalk wounds. Ascospore isolations before the secondary infection yielded the A^+B^+, A^+B^1, A^+B^2 types; isolations after the secondary infection yielded the other types listed in Table 3. After 6-8 weeks perithecia were found on the mummified berries. Conidial suspensions of A^+B^1, A^+B^2 and A^1B^1 types did not cause any CBD-like symptoms on green berries.

Discussion

In the experiments described in this paper attention was given only to the incidence of *G. cingulata* on berries and seedlings showing clear CBD symptoms after inoculation with a conidial suspension of *C. coffeanum*. *G. cingulata* fruiting bodies found on coffee material, inoculated with alleged *C. coffeanum* isolates but not showing any CBD symptoms, were only used for comparative trials, as these perithecia were definitely associated with the secondary invasion by *C. gloeosporioides* (Vermeulen, 1970).

Initially the observations were mainly focussed on the incidence of plus and minus types, without detailing morphological differences within each type. Experimental evidence indicated that only a fertile perithecial ridge was formed between plus and minus types originating from one source, viz. from either isolate CBD Fyto, CBS 440.67, 118 CC or Nic. Combinations of the plus type from one source with the minus type from another source were not compatible and *vice versa*. These findings agree with those of Lucas et al. (1944) and Struble and Keitt (1950). Consequently this would mean, that the two CBD pathogens from Kenya – both associated with *G. cingulata* – are most likely genetically different strains of *C. coffeanum*.

More detailed research will be required, including not only the two other CBD isolates, viz. the McDonald isolate CBS 135.30 and the Angolan isolate CBS 396.67, but also more recent CBD isolates from African coffee areas. One of the immediate consequences of this finding – if confirmed and expanded to other isolates of the pathogen – would be the urgent need for renewed screening of the new coffee material which will be shortly made available in Kenya (Van der Vossen et al., 1976; Van der Vossen and Walyaro, 1980, 1981). This material should be subjected to pathogenicity tests including all CBD isolates with known different genetic backgrounds.

The preliminary segregation and recombination experiments with mono-ascospore isolates obtained from isolate CBS 440.67 indicate that – as with *G. cingulata* from *Ipomoea* (Wheeler 1950, 1954, 1956) and from apple (Andes and Keitt, 1950; Struble and Keitt, 1950) – the coffee *Glomerella* shows a wide variation of characteristics. It is not clear yet, why the incidence of plus and minus types in the coffee *Glomerella*

has not been reported previously (Vermeulen, 1970). It is possible that the occurrence of the *Glomerella* state on the typical nutritional stratum as provided by coffee bark, greatly affects the percentage of perithecia capable to produce the minus type, as was found for apple. This would explain why formerly only plus types were obtained, viz. white mycelium with clumped perithecia (Vermeulen, 1970). This one type only produced perithecia and no conidia (Hindorf, 1970), hence the wild-type A^+B^+. The other isolates then obtained from the bark-inhabiting *Colletotrichum* complex were respectively *C. gloeosporioides*-white (*ccm;* Gibbs, 1969) and *C. gloeosporioides*-acerv. (*cca;* Gibbs, 1969) with perithecia and acervuli, to be compared with our A^3B^+ and A^4B (Table 3).

With regard to the code designation used in Table 3 it should be stressed that there is no genetic basis yet for the applied code. It is used solely for the purpose of provisionally clarifying the rather confusing range of subtypes obtained. The code A^3 applied to the closed acervulus type as compared to the A^+ types (perithecial) and A^1 types (conidia in slimy masses). A^3B^+ (acervuli clumped in the colony centre, fertile perithecia in clumps in the periphery) resembles the A^+B^+ type. A^3B^2 (mycelium in agar, later almost sterile) is almost similar to A^+B^2 (perithecia scattered, sterile).

There are many intermediate types. The A^3 types are in fact intermediate between the perithecial and the closed acervulus type, as they produce two different fruiting bodies. Types were also found which could be considered to be intermediate between perithecial and the open acervulus types, as they formed conidia in slimy masses in the colony centre and perithecia at the colony periphery. Likewise the A^4B (Table 3) could be placed between the closed acervulus and the conidiophore types. All these intermediate types have one common characteristic, i.e. the less developed fruiting bodies are produced in the colony centre, while the more developed fruiting bodies are at the colony periphery.

It was shown conclusively in previous work (Chilton and Wheeler, 1949a, b; Wheeler, 1950, 1954; Wheeler and McGahen, 1952) that the diversity in morphological characters was genetically determined. Wheeler (1954) suggested that it was caused by mutation, inducing certain blocks in the sexual process. The development of acervuli may also be blocked in different ways. As there are many genes involved in these blockage processes, this would explain the wide range of intermediate types. In principle these mutations may not be related to pathogenicity.

Although not fully investigated it seems likely that nutrition can completely eliminate some of the effects of genetic blocks. The influence of nutrition was already suggested by Wheeler (1956).

Until now we failed to get *C. coffeanum* from mono-ascospore isolates of *G. cingulata,* but considering the results already obtained a logical relationship seems to be obvious. A model depicting the relationship between *G. cingulata* and *C. coffeanum* is presented in Table 4. The vertical sequence in Table 4 represents the change – as often found in fungi – of the wild type with fully developed sexual reproductive organs to a type with the most simple, asexual organs, viz. conidiophores. There is a subdivision in types with clumped reproduction organs and with scattered ones. Then the types can also be differentiated by fertility and sterility. The open acervulus (conidia in slimy masses) types do not show differentiation in fertility. Only by back crossing with the wild type it is possible to show the genetic difference between the A^1B^1 and A^1B^2.

Table 4. Provisional model depicting the association between *Glomerella cingulata* and *Colletotrichum coffeanum*.

Type of fruiting bodies or sporulation	Characteristics of fruiting bodies		
	clumped	scattered	
		fertile	sterile
Perithecia	*G. cingulata*[1, 4] A^+B^+[3]	A^1B^+[3]	A^+B^2[3]
Closed acervuli	*C. gloeosporioides* (acerv.)[1] *cca* isolate of *C. gloeosporioides*[2] A^3B^+[4]	A^3B^1[4]	A^3B^2[4]
Open acervuli	A^1B^+[3]	A^1B^1[3]	A^1B^2[3]
Conidiophores	No fruiting bodies; *C. coffeanum*[1], CBD[2]		

[1] Hindorf (1970).
[2] Gibbs (1969).
[3] Wheeler (1950, 1954, 1956).
[4] This paper.

Samenvatting

Genetische en morfologische verschillen van mono-spore isolaten van Glomerella cingulata geassocieerd met de koffiebesziekte

Zuid- en Midden-Amerikaanse *Colletotrichum*-isolaten, verkregen van koffiebessen, werden in Wageningen getoetst op koffiebessen en -zaailingen ter bepaling van de pathogeniteit. Hierbij werden *C. coffeanum* Noack *sensu* Hindorf-isolaten uit Afrika als vergelijkingsmateriaal gebruikt. Op materiaal geïnoculeerd met de Afrikaanse pathogenen trad in enkele gevallen de perfecte vorm van *C. coffeanum, G. cingulata* op, duidelijk gekoppeld aan de ziektesymptomen zoals bekend bij de koffiebesziekte.

Mono-ascospore isolaten van de gevonden *G. cingulata* leverden in eerste instantie cultures op, gelijkend op de plus- en min-culturen die reeds door Edgerton (1914) werden beschreven. Nader onderzoek leverde voorts duidelijke aanwijzingen op dat wij twee typen verkregen uit een *G. cingulata* van één CBD isolaat (CBS 440.67), die wèl onderling compatibel waren – er werd een rand fertiele perithecia gevormd op de grens van twee culturen – doch niet kruisbaar bleken te zijn met de twee types van isolaat CBD Fyto. Uitgaande van het diepgaande genetische *G. cingulata*-onderzoek dat reeds door anderen is verricht moest worden geconcludeerd dat deze twee CBD isolaten genetisch verschillend zijn. Verder onderzoek dient deze waarneming te bevestigen, terwijl voorts moet worden nagegaan of dit fenomeen voor méér isolaten van *C. coffeanum* van toepassing is. Indien zulks inderdaad het geval is, zal het nieuwe kruisingsmateriaal van koffie verkregen in Kenya dienen te worden getoetst met een

reeks van mogelijk verschillende *C. coffeanum*-isolaten om na te gaan of de verkregen resistentie tegen de koffiebesziekte inderdaad voor al deze vormen gehandhaafd blijft.

Verder onderzoek met de mono-ascospore isolaten verkregen uit de peritheciën van *G. cingulata* van CBD isolaat CBS 440.67 leidde uiteindelijk tot een reeks van segregatievormen. Uit compatibele kruisingen van deze isolaten werden voorts een groot aantal genetisch en morfologisch verschillende typen verkregen. Hierbij konden enerzijds vormen worden gevonden met uitsluitend peritheciën van *G. cingulata* en géén conidiën, terwijl anderzijds typen werden verkregen met morfologische kenmerken die bijna identiek zijn aan die van het CBD pathogeen *C. coffeanum.*

Verder onderzoek is nodig, maar het optreden van *G. cingulata* geassocieerd met *C. coffeanum* kan in elk geval inhouden, dat de genetische variabiliteit van *G. cingulata* in het veld onder invloed van een reeks van omstandigheden snel zou kunnen leiden tot het ontstaan van *Colletotrichum*-vormen, die in staat zijn koffiebessen te infecteren.

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