Variation in the Cultural Characteristics of Isolates of *Crinipellis perniciosa* in Trinidad[1]

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ABSTRACT

A sharp increase in the incidence of witches' broom disease on the progenies of two cocoa clones previously rated highly resistant to the causal pathogen *Crinipellis perniciosa* (Stahel), Singer prompted speculation that a severe strain of the fungus may have evolved in Trinidad. Comparisons were made in this study of the characteristics in culture of isolates of *C. perniciosa* from sites representative of the cocoa growing areas of the island as part of investigations to establish whether pathogenic variability exists within the Trinidad population of this fungus. Significant differences in growth rates between isolates were noted. The majority of the isolates were classified into two groups based on their mycelial densities. The isolates generally interacted positively with each other when grown in pairs on a plate and exhibited varying degrees of sensitivity to a range of chemicals incorporated in the growth media. The group of isolates from the various sites could not be differentiated in any of the trials. The results confirm other findings which suggested that the Trinidad population of *C. perniciosa* belongs to one pathotype.

COMPENDIO

Debido a un incremento considerable en la incidencia de la enfermedad escoba de bruja en los progenies de dos clones de cacao, que hasta el momento se clasificaban como altamente resistentes al patógeno causal *Crinipellis perniciosa* (Stahel) Singer. Se especuló que una raza severa del hongo había evolucionado en Trinidad. En este estudio se compararon las características en laboratorio de cultivos de *C. perniciosa*, representativos de las zonas productoras de cacao en la isla, como parte de investigaciones para determinar si existe la variabilidad patogénica dentro de la población de este hongo en Trinidad. Se observaron diferencias significativas en las tasas de crecimiento de los cultivos aislados. La mayoría de éstos se clasificaron en dos grupos, según su densidad micelial. En general, los cultivos aislados interactuaron entre sí de manera positiva cuando se cultivaron en pareja en un platillo, y mostraron diferentes grados de sensibilidad a una variedad de productos químicos incorporados al ambiente de crecimiento. En ninguna de las pruebas se lograron diferenciar. Los datos orientados confirman los resultados de otros estudios que sugerían que la población de *C. perniciosa* en Trinidad pertenecen a un solo patotipo.

INTRODUCTION

Witches' broom caused by *Crinipellis perniciosa* (Stahel) Singer is the second most important disease of cocoa (*Theobroma cacao*) in the Western hemisphere. The disease is presently confined to several South American countries and some Caribbean islands who between them produce about 35% of the world's cocoa. It is recognized that resistant genotypes offer the best prospects for controlling the disease, hense a search for such genetic materials and their incorporation into breeding programmes has been in progress since the disease was first discovered in Suriname in 1895. Two Upper Amazon selections, SCA 6 and SCA 12, introduced and established in northeast Trinidad where rainfall and witches' broom disease were high, consistently remained healthy in the field when inoculated, indicating that they may be immune to the pathogen (5). Both clones were extensively used in the hybridization programme to produce high-yielding cocoa types resistant to witches' broom disease in Trinidad. Infection of progenies of SCA 6 first noted in 1965 later increased in intensity, prompting suggestions that a mutation of *C. perniciosa* may have developed on the island (1).

Experimental evidence for the existence of pathotypes of *C. perniciosa* in South America was provided by Evans (6) and Wheeler and Mepsted (11). In the latter investigation, variation in the cultural characteristics of the isolates confirmed the existence of two populations of the pathogen previously established from the reactions of cocoa seedlings. Results also suggested that compatibility of the mycelia might be a more sensitive method than assessments of seedling reactions in determining genetic diversity among *C. perniciosa* isolates.

This study formed part of investigations aimed at ascertaining whether the "breakdown in the resistance" of the SCA clones is due to the development of a virulent strain of *C. perniciosa* in Trinidad

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It was undertaken in anticipation that differences in cultural characteristics might elucidate some information about variability within this fungal population.

MATERIALS AND METHODS

Source of inoculum

Isolates of *C. perniciosa* were obtained from green brooms collected from the following 10 locations, representative of the cocoa growing areas in Trinidad: Toco, El Reposo, Non Pareil, Marper, Rio Claro, Moruga, Las Hermanas, St. Augustine, St. Joseph and Santa Cruz. The isolates were maintained on V8 agar slants in culture tubes at 25°C and subcultured every six months.

Growth and characteristics in culture

The growth of five isolates from each of the 10 sites were compared. Five mm mycelial discs cut from the peripheral edges of five-day-old cultures of *C. perniciosa* were placed centrally on 9 cm Petri dishes, each containing 25 ml of V8 media. Each isolate was replicated on five dishes and incubated at 25°C. Colony growth (mean colony diameter less the diameter of the inoculum disc), was measured 13 days after inoculation along two perpendicular diameters and averaged. These measurements were compared by analysis of variance with the isolate means being tested for significance with LSD values ($P = 0.05$, $P = 0.01$).

The thickness, texture, border profile, colour and shape of the cultures were also recorded. The thickness and texture of the mycelial mat, recorded as mycelial density, were measured on the following scale, a modification of that used by Delgado (3).

\+ sparce mycelium
++ abundant mycelial mat
+++ abundant, thick mycelial mat.

Compatibility of the isolates

Pairs of 5 mm diameter discs from the growing edges of five-day-old cultures were placed on V8 agar in 9 cm Petri dishes. A total of 500 combinations between 50 isolates from 10 sites were systematically selected to determine interactions between isolates originating from similar and different locations, were each grown in duplicate dishes. Assessments of compatibility, based on whether hyphae of paired isolates intermingled at their common borders or did not, were made after 21 days.

Sensitivity of the isolates to chemicals *in vitro*

The sensitivity to chemicals of eight isolates of *C. perniciosa* obtained from El Reposo, Non Pareil, Marper, Moruga, Las Hermanas, La Reunion, St. Augustine and Santa Cruz were determined on V8 media in which test chemicals had been incorporated. The chemicals tested were the systemic fungicides benomyl, fenpropimorph, fosety-al, isoprothiolane, serinal, tridemorph, the triazole formulation E 969, the antibiotic cycloheximide and the growth regulator, Indole-3-Acetic acid. Each chemical was evaluated at eight concentrations. In each case, freshly prepared V8 media in 250 ml Ehrlenmeyer flasks was autoclaved at 104 kilopascals for 20 min and allowed to cool. To 198 ml of the melted agar at about 45°C, 2 ml aliquots of each concentration of a chemical dissolved in either acetone or distilled water were added and mixed throughly. Twenty ml of this media was dispensed per plate. Only distilled water was added to the control media.

Each plate was inoculated at the centre with a 5 mm mycelial disc from the peripheral area of a days-old colony. It was sealed and incubated at 25°C for 13 days. Mycelial growth at each chemical concentration was determined from two dishes with two measurements being taken at right angles on each plate and averaged. Linear growth in the presence of the chemicals was calculated as a percentage of growth in the control memia. The dosage-response regression was obtained by plotting the percentage inhibition of growth on a probit scale (2) against concentration of the fungicide on a logarithmic scale. The concentration of the fungicide required to inhibit growth by 50% (ED50) and the slope of the regression line were calculated for each isolate on each chemical.

RESULTS

Mycelial growth

A white colony was established on the medium within three days. After seven days, the majority of the cultures consisted of an inner ring of cream mycelium adpressed to the agar surface bordered by an outer layer of thick, cottony white mycelium. By the thirteenth day, the mycelium in many cases was differentiated into several concentric circles of dense and sparse growth. Mycelial growth in all cases proceeded in a uniform circular pattern with smooth borders.

Abundant mycelium adpressed to the agar surface was produced by 32 isolates, while 16 isolates had abundant and thick mycelial mats (Table 1). Both types of mycelia were produced by isolates from 8 of

the 10 areas. In contrast, an isolate each from Las Hermanas and Non Pareil, in the central and eastern districts respectively, produced sparse mycelia.

Differences in the growth of individual isolates after 13 days were highly significant ($P = 0.01$). However, variation in mycelial growth between groups of isolates from the different locations were not significant.

Compatibility of the isolates

Mycelia from paired discs of the same colony usually completely merged into each other. A visibly distinct border remained between the other isolates. Although there were differences in the degree of mixing at the lines of demarcation, positive interactions were observed in all cases. All isolates were therefore classified as compatible with each other.

Sensitivity of the isolates to chemicals *in vitro*

All isolates showed great sensitivity to cycloheximide, E 969, fenpropimorph and tridemorph with less than 5 ppm of each chemical causing 50% inhibition of mycelial growth (Table 2). Differences in the ED50 values of the chemicals were highly significant ($P = 0.001$) but the variation between the isolates not. Similarly, the slopes of the percentage inhibition-log dose curves indicated significant differences between the chemicals but not the isolates.

Table 1. The growth and cultural characteristics of *C. perniciosa* isolates incubated at 25°C for 13 days.

Origin of isolates		Colony diam (mm)	Mycelial density	Origin of isolates		Colony diam (mm)	Mycelial density
Santa Cruz	1	72	++	St Joseph	1	75	+++
	2	72	+++		2	74	++
	3	74	++		3	75	++
	4	73	++		4	75	+++
	5	73	++		5	75	++
Site mean		72.8		Site mean		74.8	
St Augustine	1	73	++	Las Hermanas	1	74	++
	2	74	+++		2	73	++
	3	75	+++		3	74	++
	4	73	++		4	72	+
	5	75	++		5	74	++
Site mean		74.0		Site mean		73.4	
Toco	1	74	++	Non Pareil	1	77	++
	2	73	+++		2	74	++
	3	72	+++		3	75	+++
	4	75	++		4	74	+
	5	74	++		5	74	++
Site mean		73.6		Site mean		74.8	
El Reposo	1	74	++	Marper	1	75	++
	2	73	+++		2	74	++
	3	72	++		3	73	++
	4	74	+++		4	72	++
	5	74	+++		5	74	++
Site mean		73.4		Site mean		73.6	
Rio Claro	1	76	++	Moruga	1	74	++
	2	73	+++		2	76	++
	3	73	+++		3	75	+++
	4	73	++		4	75	++
	5	74	++		5	74	+++
Site mean		73.8		Site mean		74.8	

Coefficient of variation 1.0%
S.E. isolate diameter means 0.3.

Table 2. Variation in the ED50 values and slopes of graphs showing the relationships between % inhibition of linear growth and the dosage concentration of isolates of *C perniciosa* growing on media amended with chemicals.

Origin of isolates	A*		B		C		D		E	
	ED50	Slope	ED50	Slope	ED50	Slope	ED50	Slope	ED50	Slope
Santa Cruz	190 9	0 61	0 4	1 43	1 3	1 66	3 041 1	2 23	9 1	1.46
St Augustine	196.0	0 61	0.4	1 42	1 3	1.64	3 038.2	2 26	8.9	1.47
La Reunion	178 2	0 62	0 4	1 46	1 3	1.64	3 041 6	2 25	9.1	1.45
Las Hermanas	183 7	0.62	0 4	1 42	1.3	1.62	3 031 8	2 27	9.2	1.46
El Reposo	186 6	0.61	0.4	1.44	1.3	1.64	3 034 3	2.26	9 0	1.49
Non Pareil	190 0	0.63	0.4	1 45	1.3	1.63	3 023 7	2.27	9.1	1.48
Marper	178.6	0 64	0.4	1.44	1.3	1.64	3 020 7	2.25	9.1	1.47
Moruga	191.5	0.64	0.4	1 43	1 3	1.66	3 012 1	2.26	9.1	1.47
Means	186 9	0 62	0.4	1 44	1.3	1.64	3 030 0	2.26	9.1	1.47
	c	h	f	f	f	d	a	a	e	e

LSD Chemical means (P = 0.001): ED50 7.2 Slope 0.02

*A Benomyl B Cycloheximide C Fenpropimorph D Fosetyl – Al E Indole – 3 – Acetic acid F Isoprothiolane G Serinal H E 969 (Triazole) I Tridemorph

DISCUSSION

Investigations with isolates from various countries of South America showed significant differences in growth rates, colony density and appearances exist between isolates of pathotypes A and B of *C perniciosa* (11). When grown in pairs on agar plates, mycelia of compatible isolates mingled freely whereas a distinct line of demarcation separated incompatible isolates. Generally no isolate in population A was compatible with those in population B, thereby endorsing the grouping of these isolates based on the reactions they caused on cocoa seedlings. Extrapolating from those results, the positive interactions between the Trinidad isolates suggest that genetic compatibility preponderates among the *C. perniciosa* population on the island. There is nevertheless some variation manifested in differences in colony growth and mycelial density. These differences are, however, typical of individual plates rather than of groups at various locations. Since a broom may arise from multiple basidiospore infections, some variation may be expected even among isolates from one broom. This was observed by Wheeler and Mepsted (11), who noted that the interchange of genetic material which occurs within a broom resulting from anastomoses of several mycelia may influence the interactions in culture of the new mycelia.

The possibility of using sensitivity to systemic fungicides as an aid in fungal taxonomy was first suggested by Edgington and Barron (4). Subsequently, the natural and artificially acquired tolerance of fungal strains to fungicides have been reported in many pathogens. Significant differences between *C. perniciosa* isolates in their sensitivity to 2 triazole fungicides, hexaconazole and triadimenol were reported by McQuilken *et al* (10). The amount of each formulation required to reduce fungal growth of an isolate from Trinidad was significantly less than that required by isolates from Manizales (Colombia) or Castanhal (Brazil). In contrast, differences between three isolates from Manizales were not significant. Previously isolates from Colombia were grouped as pathotype A and those from Brazil and Trinidad characterised pathotype B (11).

Wheeler and Mepsted (11) acknowledged the problem of linking information from the cultural studies with differences in pathogenicity of the isolates. In the present case, however, the lack of outstanding differences between the groups of isolates in their behaviour in culture confirms the results of other investigations in which comparisons of the morphological, histopathological and biochemical reactions of inoculated cocoa seedlings and clonal plants showed no significant differences between isolates of *C. perniciosa* in Trinidad (7, 8, 9). The need still exists for further investigations to ascertain whether there is any correlation between these cultural characteristics and the pathogenicity of the strains in the field. This would ameliorate the present logistic problems encountered in securing a

Continuation Table 2. Variation in the ED50 values and slopes of graphs showing the relationships between % inhibition of linear growth and the dosage concentration of isolates of *C perniciosa* growing on media amended with chemicals.

Origin of isolates	F		G		H		I		Means	
	ED50	Slope	ED50	Slope	ED50	Slope	ED50	Slope	ED50	Slope
Santa Cruz	48.4	1.67	210.8	2.00	1.3	1.08	3.1	0.61	389.6	1.42
St Augustine	48.6	1.67	209.8	1.99	1.3	1.09	3.2	0.61	389.6	1.42
La Reunion	48.3	1.65	212.2	1.92	1.3	1.09	3.1	0.60	388.4	1.41
Las Hermanas	48.5	1.64	212.0	1.96	1.3	1.09	3.2	0.61	387.9	1.41
El Reposo	48.4	1.65	213.7	1.97	1.3	1.09	3.2	0.60	388.7	1.42
Non Pareil	47.8	1.66	213.3	1.99	1.3	1.09	3.2	0.60	387.8	1.42
Marper	47.8	1.66	211.7	1.97	1.3	1.08	3.2	0.60	386.0	1.42
Moruga	48.0	1.67	208.5	1.98	1.3	1.08	3.1	0.60	386.1	1.42
Means	48.2	1.66	211.5	1.97	1.3	1.09	3.2	0.60	–	–
	d	c	b	b	f	g	f	i		

LSD Chemical means (P = 0.001): ED50 7.2 Slope 0.02

*A Benomyl B Cycloheximide C Fenpropimorph D Fosetyl – Al E Indole – 3 – Acetic acid F Isoprothiolane G Serinal H E 969 (Triazole) I Tridemorph

regular supply of inoculum and suitable host plants for comparative studies on the pathotypes of *C perniciosa*, which can only be performed outside cocoa-growing regions.

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