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Identification of Races and in vitro *Sporulation of* Microcyclus ulei

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Cultural characteristics viz. *colony appearance, growth habits and* in vitro *sporulation of pure isolates of* Microcyclus ulei *from different* Hevea *clones were studied. The physiologic races of these isolates were identified by inoculating leaf discs and differential plants grown in polyethylene bags. The isolates could be classified into two morphological groups and four physiologic races* i.e. Races 2, 4, 5 *and* 6. *Generally, results of leaf discs were similar to those of plants in polyethylene bags.*

Microcyclus ulei (P. Henn.) v. Arx, the fungus causing South American leaf blight (SALB) of *Hevea* rubber forms dark slow-growing stroma on artificial medium. Variations in cultural characteristics *viz.* growth rate, colony appearance and sporulation among isolates had been observed[1-4] but how these characteristics relate to the race structure of the fungus has not been studied.

The exact number of races of *M. ulei* are not known. Experimentally, Langdon[5] identified *Races 1* and *2*. In addition, *Races 3* and *4* were differentiated by Miller[6]. Chee *et al.*[7] indicated the existence of nine races, eight of which were present in the state of Bahia, Brazil. They identified the races by inoculating field conidia from different sources (clones) onto leaf discs of various *Hevea* clones from which the differentiating clones were selected. The objective of this paper is to identify the physiologic races using pure isolates of *M. ulei* obtained from different *Hevea* clones by inoculating leaf discs and also plants grown in polyethylene bags, and also to compare the cultural characteristics of these isolates.

MATERIALS AND METHODS

Isolation of *M. ulei*

M. ulei was isolated by touching sporulating leaf lesions with the tip of an inoculating needle and transferring them into test tubes containing potato sucrose agar (PSA) amended with Panvit[R] (a commercial mixture of amino acids and vitamins) and chloroamphenicol[4]. When the colony had established, usually after two to three weeks, the stroma was transferred onto PSA amended with Panvit and Bonzo[R] dog-food, referred to as sporulation medium[4]. Fungal isolation was done at different times during 1985 to 1986.

In vitro Sporulation of *M. ulei* and Preparation of Inoculum

A piece (2 mm) of stroma of *M. ulei* was inoculated onto slants of PSA sporulation medium (10 ml per tube) in test tubes (2 × 20 cm). The tubes were incubated at 24°C under subdued light. After ten days, 1 ml of sterilised distilled water was pipetted into the tubes and the stroma was crushed against the side of the tube and spread evenly onto the surface of the medium. The tubes were incubated in the dark at 24°C. Commencing from the fourteenth day after spreading the stroma, the tubes were exposed to fluorescent light (2600 lux) for 90 min per day for three consecutive days. Spores were harvested on the seventeenth day by adding 5 ml of sterile distilled water and brushing off the conidia with an artist paint brush. The concentration of the

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spores in the suspension was determined with a Nurbauer hemocytometer.

In the preparation of conidial inoculum, the same procedure was followed except that instead of test tubes, 125 ml Erlenmeyer flasks were used and the cultures were exposed to light by the tenth day after spreading.

Inoculation of Leaf Discs and Plants in Polyethylene Bags

Leaf discs of differential clones were prepared and inoculated as previously described[8]. In addition, about seven-day-old leaves of plants grown in polyethylene bags (polybag plants) in a glasshouse were inoculated by spraying the lower surface of the leaves with conidia ($1 \times 10^{4-5}$ per millilitre) using an Atomist atomiser. The sprayed shoots were then covered with clear polyethylene bags for 16 - 24 h to maintain high humidity.

Assessment of Infection

Infection of leaf discs was assessed by determining the presence of lesions on the eighth day after inoculation. Infection of polybag plants was assessed fourteen days after inoculation based on leaf symptom diagrams of percentage of leaf area necrotic[8]. In addition, disease reaction was also classified by using the following scale:

0 = No symptoms
1 = Chlorotic flecks, no necrosis and no sporulation
2 = Chlorosis and necrosis with very little sporulation
3 = Necrosis with little to medium sporulation
4 = Necrosis with medium to heavy sporulation
5 = Necrosis with very heavy sporulation.

Races Differentiation

The clones used to differentiate races of *M. ulei* were IAN 710, IAN 717, FX 2804, FX 3925, FX 2261, FX 985 and FX 25[7]. FX 4098 was included for confirmation of *Race 6.*

RESULTS

Morphological Forms

On PSA, similar to Chee[1], two distinct morphological forms of the fungus were observed. The first was greenish black with smooth velvety appearance. The stroma was more flattened forming a crust on the agar surface with extensive mycelial growth occurring in the medium. Isolates of this group were mostly from progenies of *H. benthamiana* clone F 4542 (FX 2804, FX 567, FX 3899, FX 3925, IAN 3272, IAN 717 and IAN 3703). These isolates were identified to belong to *Race 2.* The other morphological form had dark raised carbonaceous stroma with little growth in the medium. They belong to *Races 4, 5* or *6.*

The appearance of the conidia produced in culture differed from those obtained from the field in that the 'twist' which is prominent in field conidia, was lacking in conidia from cultures. Further, they were smaller and a greater proportion of them were of single cell.

Sporulation in Culture

Most of the cultures had fully covered the surface of the medium two weeks after the stroma was crushed and spread except for isolates 25-1, 25-2, 2261-1 and 3864-3. The number of conidia produced varied with isolates *(Table 1).* Isolate 567-1 from FX 567 produced the highest number of conidia followed by isolates 2804-1, 985-1, 600-2 and 3899-2. Few spores were obtained from 2261-4, 25-1, 4163-2 and 3844-2 despite their comparatively good growth. When the isolates were grouped according to their respective races, *Race 2* produced significantly more conidia than *Races 4, 5* and *6* which were not significant among themselves.

Reaction of Isolates on Leaf Discs of Differential Clones

Leaf discs of certain differential clones developed distinct lesions when they were inoculated by certain isolates of *M. ulei* and not by others indicating that the isolates belong to different races. However, discs of clones FX 25

TABLE 1. SPORULATION OF ISOLATES OF *M. ULEI* IN CULTURE

Race	Source clone	Isolate no.	No. ($\times 10^5$) of conidia	
			Mean of 3 tubes	Race mean
2	FX 2804	1	11.802	9.392
		2	3.290	
	FX 3899	1	3.926	
		2	8.056	
	FX 567	1	20.167	
	RRIM 600	2	9.111	
4	FX 2261	4	0.524	2.545
	IAN 873	1	5.975	
		2	1.136	
5	FX 2261	1	2.944	2.303
	FX 3844	1	1.358	
		2	0.950	
	FX 3846	1	5.556	
	FX 25	1	0.395	
		2	4.883	
	FX 3864	1	1.741	
		3	0.593	
6	FX 985	1	9.178	3.870
		2	3.963	
	RRIM 600	1	1.833	
		3	2.543	
	FX 4163	1	3.210	
		2	0.951	
		3	2.605	
	FX 3864	4	6.673	
				F = 4.776*

*Significant at p = 0.05

and IAN 710 developed lesions when inoculated by all isolates including those found by Chee *et al.*[7] to be resistant. Some isolates formed few or small lesions on these clones.

The infection reaction of sixty-eight isolates by *M. ulei* could be differentiated into four races *i.e. Races 2, 4, 5* and *6 (Table 2).* Except for clones RRIM 600 and IAN 710, *Race 2* was isolated from clones which are progenies of *H. benthamiana* (F 4542, FX 516). *Race 4* was isolated from FX 2261 as reported by Chee *et al.*[7] and also from IAN 873. *Races 5* and *6* were isolated from clonal hybrids of many crosses of *H. brasiliensis.* Isolates from some clones e.g. IAN 710, RRIM 600, FX 985, FX 25, FX 2261, FX 3864 and FX 4163 were found to belong to more than one race.

Reaction of Isolates on Plants in Polyethylene Bags

The reaction of representative isolates of *Races 2, 4, 5* and *6* on differential clones grown in polyethylene bags was as indicated in *Table 3.* Generally, results obtained from these plants were similar to those of leaf discs *(Table 2).* However, clone FX 2261 when inoculated with *Race 5* isolates which formed lesions on leaf discs, formed nil or only chlorotic flecks or small lesions with nil or very little sporulation on polybag differential plants. For confirmation, some of the leaves of polybag plants following inoculations with these isolates were detached and incubated as for leaf discs. Subsequently, prominent lesions developed on the detached leaves while none or very small lesions

TABLE 2. REACTION OF ISOLATES OF *M. ULEI* ON LEAF DISCS OF DIFFERENTIAL CLONES

Hosts (parents)	No. tested	Reaction of differential clones		Race
		Susceptible	Resistant	
FX 2804 (F 4542 × Tjir 1)	13			2
FX 3899 (F 4542 × AVROS 363)	4			
FX 3925 (F 4542 × AVROS 363)	1	IAN 717		
FX 567 (F 4542 × AVROS 368)	1	IAN 710	FX 2261	
IAN 717 (F 4542 × PB 86)	4	FX 3925	FX 985	
IAN 3272 (FX 516 × PB 86)	1	FX 2804		
IAN 3703 (FX 516 × PB 86)	1	FX 25		
IAN 710 (F 409 × PB 86)	3			
RRIM 600 (Tjir 1 × PB 86)	1			
FX 2261 (F 1619 × AVROS 183)	4	IAN 710, FX 25	IAN 717, FX 3925	4
IAN 873 (FA 1717 × PB 86)	5	FX 2261	FX 2804, FX 985	
FX 3844 (B 45 × AVROS 183)	2			5
FX 3846 (B 45 × AVROS 183)	2	IAN 710	IAN 717	
FX 2261 (F 1619 × AVROS 183)	3	FX 25	FX 3925	
FX 985 (F 315 × AVROS 183)	3	FX 2261	FX 2804	
FX 25 (F 351 × AVROS 49)	2	FX 985		
FX 4163 (F 170 × Tjir 1)	1			
FX 3864 (F 38 × PB 86)	2			
FX 985 (F 315 × AVROS 183)	8			6
FX 3864 (F 38 × PB 86)	3	IAN 710	IAN 717	
FX 4163 (F 170 × Tjir 1)	3	FX 25	FX 3925	
FX 25 (F 351 × AVROS 49)	1	FX 985	FX 2804	
			FX 2261	

developed on polybag plants.. Other isolates which formed distinct lesions on leaf discs, also formed lesions on intact plants and *vice versa*. Similar to leaf discs, polybag plants of clones IAN 710 and FX 25 developed lesions when they were inoculated with the four races tested, but lesion size and sporulation differed between race-clone combinations.

Disease reaction of *Races, 1, 2, 3* and *4* on differential clones had earlier been described[5,6]. From this study, reaction of *Races 2, 4, 5* and *6* on a different set of differential clones are summarised in *Table 4*. *Race 2* infected *H. benthamiana* clones IAN 717, FX 3925 and FX 2804 while no symptoms developed on FX 2261 and FX 985. On IAN 710, *Race 2* formed small lesions with low to medium sporulation. FX 25 was rated as highly resistant to *Race 2* because the lesions possessed very little sporulation. These observations agree with Langdon[5] who indicated high susceptibility of IAN 717 and FX 3925 while IAN 717 and FX 25 were rated highly resistant (few non-sporulating lesions).

Race 4 did not infect IAN 717, FX 3925, FX 2804 and FX 985. FX 2261 was very susceptible, while IAN 710 was also susceptible but with less sporulation. FX 25 was rated marginally resistant since sporulation was low. Similar description of *Race 4* was given by Miller[6]. *Race 5* caused no symptoms on IAN 717, FX 3925 and FX 2804, while on IAN 710 and FX 25 necrotic lesions with low sporulation occurred. On FX 985, *Race 5* formed lesions with heavy sporulation. In this trial, *Race 5* formed no lesions or only small lesions with nil or little sporulation on FX 2261. *Race 6* formed no lesion on IAN 717, FX 3925, FX 2804 and FX 2261. IAN 710 inoculated with *Race 6* developed lesions with medium sporulation. *Race 6* formed lesions with very little conidia on FX 25, while on FX 985 it produced large lesions with very heavy sporulation *(Table 4)*.

TABLE 3. REACTION OF ISOLATES OF *M. ULEI* ON DIFFERENTIAL CLONES IN POLYETHYLENE BAGS

Race	Isolate	Reaction on differential clone																									
		IAN 717				FX 3925				IAN 710				FX 2804				FX 2261				FX 985				FX 25			
		LS	DA	SP	DR	LS	DA	SP	DR	LS	DA	SP	DR	LS	DA	SP	DR	LS	DA	SP	DR	LS	DA	SP	DR	LS	DA	SP	DR
2	567-1	2-4	4	4	5	2-4	4	4	5	1-3	3	3	3	1-3	4	4	5	<1	1	0	1	<1	1	0	1	1-3	4	2	2
	2804-10	2-3	3	4	5	2-3	4	4	5	1-2	2	1	2	4-6	4	5	5	<1	1	0	1	<1	1	0	1	2-3	3	2	3
	3899-2	2-5	3	5	5	2-9	3	5	5	2-4	3	3	3	2-6	3	5	5	0	1	0	0	—	—	—	—	2-3	2	1	2
	600-2	1-2	3	3	4	—	—	—	—	1-3	3	3	3	3-6	3	5	5	<1	1	0	1	<1	1	0	1	1-2	2	1	2
	710-1	1-3	4	4	4	3-6	3	4	4	3-8	3	2	3	—	—	—	—	<1	1	0	1	1-2	3	0	1	1-3	4	1	2
	710-2	1-3	4	4	4	3-7	5	4	5	1-3	4	3	3	—	—	—	—	0	1	0	0	1-2	3	0	1	1-2	3	1	2
4	873-3	<1	2	0	1	1-3	2	0	1	3-4	2	4	4	0	1	0	0	3-7	4	3	3	0	1	0	0	1-3	4	1	2
	873-4	<1	2	0	1	0	1	0	0	1-2	4	4	4	—	—	—	—	2-6	4	3	3	1-2	2	0	1	2-5	4	1	2
	2261-3	0	1	0	0	0	1	0	0	1-3	4	3	4	—	—	—	—	2-3	4	4	5	1-2	1	0	1	1-2	4	0	1
	2261-6	0	1	0	0	0	1	0	0	1-2	2	3	3	0	1	0	0	2-3	4	4	5	1-2	2	0	1	1-2	2	1	2
5	985-7	1-2	2	0	1	1-2	2	0	1	1-3	3	2	2	0	1	0	0	<1	3	0	2	1-3	4	5	5	2-4	4	2	3
	3846-1	0	1	0	0	<1	1	0	1	2-3	4	3	3	0	1	0	0	<1	2	0	1	4-7	3	4	5	1-2	3	1	2
	3846-2	0	1	0	0	—	—	—	—	2-4	2	3	3	0	1	0	0	0	1	0	0	—	—	—	—	2-4	3	3	3
	3846-3	0	1	0	0	0	1	0	0	2-4	3	3	3	0	1	0	0	1	2	0	1	2-5	4	5	5	1-3	3	2	3
	3844-3	0	1	0	0	0	1	0	0	1-3	4	2	3	0	1	0	0	0	1	0	0	2-4	4	4	5	1-2	3	1	2
	25-2	0	1	0	0	0	1	0	0	2-4	4	3	3	0	1	0	0	0	1	0	0	2-4	4	4	5	1-3	4	3	3
6	3864-4	0	1	0	0	—	—	—	—	1-3	2	3	3	0	1	0	0	0	1	0	0	4-9	2	5	5	2-3	2	1	2
	985-1	0	1	0	0	<1	1	0	1	1-2	2	2	3	0	1	0	0	0	1	0	0	2-4	2	4	5	1-3	3	1	2
	985-12	<1	1	0	1	—	—	—	—	1-2	4	1	2	<1	1	0	1	0	1	0	0	2-6	4	5	5	2-4	4	1	2
	4163-4	0	1	0	0	—	—	—	—	2-4	4	3	3	<1	1	0	1	0	1	0	0	2-4	4	5	5	2-4	4	2	3

LS = lesion size (mm); DA = leaf area necrotic, 1 = <1%, 2 = 1%–5%, 3 = 6%–15%, 4 = 16%–30%, 5 = >30%; SP = sporulation, 0 = none, 5 = very heavy; DR = disease reaction type, 0 = no symptoms, 1 = chlorotic flecks with no sporulation, 2 = chlorosis and necrosis with little sporulation, 3 = necrosis with little to medium sporulation, 4 = necrosis with medium to heavy sporulation, 5 = necrosis with very heavy sporulation; — = no data.

TABLE 4. DIFFERENTIAL *HEVEA* CLONES AND THEIR REACTION TO *RACES 2, 4, 5* AND *6* OF *M. ULEI*

Race	Reaction type on differential clone						
	IAN 717	FX 3925	IAN 710	FX 2804	FX 2261	FX 985	FX 25
2	S,5[a]	S,5	MR;2-3	S,5	R,0	R,0	HR,1-2
4	R,0	R,0	S,3-4	R,0	S,5	R,0	MR,1-2
5	R,0	R,0	MR,2-3	R,0	HR,0	S,5	MR,2-3
6	R,0	R,0	MR,2-3	R,0	R,0	S,4-5	MR,1-2

R = Very resistant to immune, no symptoms or chlorotic flecks or small necrotic lesions with no sporulation
HR = High resistance, small necrotic lesions with little sporulation
MR = Marginal resistance, larger lesions with little to medium sporulation
S = Large lesions with heavy sporulation
[a]Sporulation rating from zero (no spores) to five (very heavy)

DISCUSSION

In 1966, an isolate of *M. ulei* from Belem, Brasil was identified as *Race 4*[6]. Junqueira *et al.*[9] observed three main groups of isolates from cultures of different regions in Brazil but no attempts were made to classify them into races. It is certain that Brazil has more than one race of *M. ulei,* but rather than describing new races, *Races 4a, 4b* and *4c* were designated by SUDHEVEA[10] which Chee *et al.*[7] renamed as *Races 4, 5* and *6* respectively. However, it is to be noted that *Race 6* (Chee *et al.*[7]) did not infect FX 2261 and FX 4098 while *Race 4c* infected FX 4098 and not FX 2261[10]. In fact *Race 4c* was closer to the description of *Race 7* of Chee *et al.*[7] In the present study, isolates from clones FX 985, FX 3864, FX 4163 and FX 25 were recognised as *Race 6* on the basis that it did not infect FX 2261, and later inoculations indicated that these isolates infected FX 4098 thus agreeing with the description of *Race 4c. Races 1* and *3* as described by Miller[6] and *Races 6, 8* and *9*[7] were not encountered among the sixty-eight pure isolates studied. Chee *et al.*[7] indicated that *Race 1* infected clone IAN 873 while Miller[6] reported that most of the time IAN 873 was highly resistant to *Race 1* but sometimes *Race 1* infected this clone. Langdon[5] showed that this clone was resistant to *Race 1.* In the present study, the five isolates from IAN 873 were *Race 4.* Miller[6] also indicated that this clone was susceptible to *Race 4.*

Race 2 was isolated mainly from progenies of F 4542. *Races 4, 5* and *6* were isolated from clones which derived their resistance from many sources as indicated in *Table 2. Race 4* was isolated from progenies of F 1619 and FA 1717. Progenies of F 351, F 315, F 170 and B 38 yielded *Races 5* and *6. Race 5* was also isolated from progenies of B 45 and F 1619. Thus these results indicated the races which could break down the sources of resistance derived from respective parents e.g. *Race 2* breaks down resistance from F 4542.

Laboratory inoculation of lead discs, interpreted carefully, could be used as a quick method to differentiate races of *M. ulei.* Excised leaf discs had earlier been used to investigate the disease expression by races of *Melampsora* spp. on poplar cultivars *in vitro*[11]. The present study indicated that in laboratory tests, the differentials IAN 710 and FX 25 were not too useful to differentiate *Races 2, 4, 5* and *6* as these races formed lesions on these clones. In fact the results presented here on infection of these two clones by *Races 2* and *4* differ with the findings of Chee *et al.*[7] The difference could be due to the fact that the present authors gave a 'susceptible' rating whenever lesions were observed irrespective of sporulation while Chee *et al.*[7] took sporulation into consideration. One problem with FX 25 is that sporulation was not free and few conidia were detected even fourteen days after inoculation. Clone FX 2261 indicated contrasting

results on leaf discs and in polybag plants. It is possible that environmental conditions could have caused the difference. During the period when FX 2261 was inoculated with *Race 5,* the weather was hot and the relative humidity was low which could have inhibited the development of the fungus. In addition, FX 2261 was rated as susceptible in the laboratory although it has more resistance in the field[12]. On other clones simultaneously inoculated with FX 2261, lesions and sporulation developed. It was also observed that in the summer months in Bahia, Brazil, some infection occurred on clone FX 985 but was hardly detected on FX 2261. In his trial, Miller[6] indicated the influence of weather on clones IAN 873 and MDF 180. He concluded that IAN 873 was more sensitive to weather. Similar argument could be true for FX 2261.

The results presented here confirm the observation of Chee *et al.*[7] that a clone could be infected by more than a race in the field simultaneously. In fact, two races were isolated from different lesions of the same leaf. The use of field conidia randomly harvested from different leaves in the field in the study of races will not guarantee that a pure race is being used and the same race or races are used in the repeated experiments. This might explain differences in results observed by Chee *et al.*[7] and the present authors.

The isolates so far studied could be classified into four races, and morphologically into two groups based on differences in their growth form and sporulation in medium. This paper describes the reaction type of *Races 2, 4, 5* and *6* and confirms their existence in the state of Bahia, Brazil. The occurrence of races could account for the different morphology of the fungus observed earlier between isolates[1-4]. Further work is necessary to isolate and determine the reaction type of other races and only then could their existence be confirmed. If they do exist, it could be assumed that they are less predominant than *Races 2, 4, 5* and *6.*

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