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Detection and Characterisation of Benomyl Resistant Strains of Microcyclus ulei

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Benomyl resistant isolates of Microcyclus ulei *were detected by inoculating potato sucrose agar medium amended with 1 mg per litre benomyl.* Race 6 *and to a lesser extent* Race 2 *of the fungus had a higher frequency of yielding resistant isolates. Resistant isolates developed longer germtubes on benomyl-treated cellophane and required higher concentrations of benomyl to control infection. Benomyl resistant isolates were also cross-resistant to thiophanate methyl and carbendazim. Sporulation of a benomyl resistant isolate was not significantly reduced by the presence of low concentrations of benomyl or a benomyl sensitive isolate.*

Benzimidazole fungicides, specifically benomyl and thiophanate methyl have been used for more than ten years to control South American leaf blight (SALB)[1] of *Hevea* rubber caused by *Microcyclus ulei* (P. Henn.) v. Arx. Good control with benomyl and thiophanate methyl had been reported previously[2-5]. Later reports however, indicated relatively lower effectiveness[6-8]. *M. ulei* might have developed resistance to these fungicides although it had not been confirmed experimentally. Instead Zhang and Chee[9] demonstrated that races of *M. ulei* had different sensitivities to these fungicides based on differences in conidia germination, infection of leaf discs and disease control on different clones.

It is less common now to use benomyl and thiophanate methyl to control SALB. Other systemic fungicides found effective against SALB are the ergosterol biosynthesis inhibitors triadimefon and triforine which are now being used to control SALB[7]. Fenarimol and bitertanol are also effective[6]. Benzimidazole fungicides were also recommended to treat other diseases of rubber such as black crust *(Phyllacora huberi)* and target leaf spot *(Thanatephorus cucumeris)*[10]. In areas where these diseases occur together with SALB, the treatment of black crust and target leaf spot would also subject *M. ulei* to selection pressure for benzimidazole resistant strains.

MATERIALS AND METHODS

Detection of Resistant Strains

Isolates of four races *(Races 2, 4, 5* and *6)* of *M. ulei* were inoculated onto potato sucrose agar (PSA) amended with Panvit[R] (a commercial preparation of a mixture of vitamins and minerals[11]) and 1 mg per litre benomyl (Benlate 50 WP) contained in test tubes. The number of isolates used for each race is shown in *Table 1.* The test tubes were incubated at

TABLE 1. SENSITIVITY OF ISOLATES OF FOUR RACES OF *MICROCYCLUS ULEI* TO BENOMYL

Race	Total	No. resistant	Percentage resistant
2	19	5	26.32
4	5	0	0.00
5	10	1	10.00
6	11	6	54.55
Total/Average	45	12	26.70

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24°C in darkness for forty days. Isolates which grew on this medium were termed resistant to benomyl. Resistance was confirmed by further inoculation onto medium containing 5 mg per litre benomyl.

Degree of Resistance

The degrees of resistance of isolates were compared by either determining germtube length on cellophane or infection of leaf discs, a method suitable for such study with systemic fungicides[12,13]. For determination of germtube length, squares (1 cm^2) of cellophane (Dupont) were dipped for 2 h in fungicide solution and after free-water was shaken off they were brushed with dry conidia obtained from lesions on plants previously inoculated with isolate 25-2 (sensitive) or 985-10 (resistant). The squares placed on glass slides were incubated at 24°C in a petri dish lined with wetted filter paper. Leaf discs of *Hevea* (clone FX 25) were prepared and incubated as described previously[14]. These leaf discs were suspended on solutions of different concentrations of benomyl and 5 h later were inoculated with conidia of *M. ulei*. Lesions were counted eight days after inoculation. Whenever leaf discs were used in later experiments, it was referred to as the leaf disc test.

Stability of Resistance

An isolate sensitive to 1 mg per litre benomyl was sequentially transferred onto PSA medium containing increasing concentrations (0, 0.5, 1, 2, 2.5, 5, 10 and 15 mg per litre) of benomyl. On each medium, the fungus was allowed to grow for at least one month.

A resistant isolate (567-1) preserved under oil for more than a year was inoculated onto benomyl-free rubber plants. The conidia subsequently produced were inoculated onto new sets of plants. The process was repeated until four consecutive sets of plants were inoculated. The conidia from the fourth set of plants were isolated and tested for resistance to benomyl by inoculating PSA medium amended with 1 mg per litre benomyl.

Cross Resistant to Other Benzimidazoles

Isolates resistant to benomyl were inoculated onto PSA medium amended with 5 mg per litre thiophanate methyl. The test tubes were incubated at 24°C and mycelial growth determined forty days after incubation.

In the leaf disc test, leaf discs suspended on solutions of either thiophanate methyl, carbendazim or triforine, each at 12.5 mg per litre, and 1 mg per litre chlorothalonil were inoculated with *M. ulei* isolates resistant (985-10, 567-1) or sensitive (25-2, 3846-3) to benomyl. In the case of chlorothalonil, it was also sprayed onto the discs and allowed to dry before being inoculated. Lesions were counted eight days after inoculation.

Pathogenicity Studies

Young leaves (about seven days old) of plants grown in polyethylene bags placed in a glasshouse were sprayed until run-off with 50 mg per litre benomyl in the presence of 0.05% Triton X 114. When dry, the lower surfaces of these leaves were inoculated with conidial suspensions (1×10^5 conidia per millilitre) of either a benomyl sensitive or benomyl resistant isolate of *M. ulei* using an Atomist atomiser. The leaves were then covered for 12 h with clear polyethylene bags. Sporulating lesions were counted on the eighth day after inoculation.

To evaluate the effect of benomyl on sporulation of a benomyl resistant isolate, plants were sprayed with benomyl and subsequently inoculated with a benomyl resistant isolate as described above. Eight days after inoculation, the conidia produced were brushed into a known volume of water using an artist brush and its concentration determined using a haemocytometer. The effect of benomyl on sporulation was also studied using leaf discs suspended on solutions of different concentrations of benomyl. After fourteen days of incubation, the discs were agitated in water with a drop of diluted Triton X 114. Spore concentration was determined using a haemocytometer.

Competition Between Benomyl Resistant and Sensitive Isolates

Young leaves of *Hevea* growing in polyethylene bags were inoculated as previously described with a mixture of conidial suspension of a benomyl resistant isolate and a benomyl sensitive isolate (2×10^5 conidia per mililitre each). The conidia subsequently produced were then inoculated on a new set of plants and also on leaf discs suspended on solutions of benomyl to check for resistant populations. The process was repeated until five consecutive sets of plants were inoculated.

Preparation of Fungicides

A wettable powder formulation of the test fungicide was first dissolved in ethyl alcohol before being made up to the dosage level required; the concentration of the alcohol in the final dilution never exceeded 1%.

RESULTS

Detection of Resistant Isolates

In tests where PSA medium amended with 1 mg per litre benomyl was inoculated with pure isolates of known races of *M. ulei,* sensitive isolates failed to grow while the resistant isolates grew to more than 1 cm after forty days. All except two isolates which were resistant to 1 mg per litre benomyl were also resistant to 5 mg per litre benomyl. The growth of some isolates on PSA medium amended with 5 mg per litre benomyl is shown in *Figure 1.* Among the isolates tested, 26.7% of them were resistant to 1 mg per litre benomyl *(Table 1).* The occurrence of resistant isolates varied with races. Most of the resistant isolates were from *Race 6;* 54.6% of this race was resistant. This was followed by *Race 2* (26.3%) and *Race 5* (10.0%). The five isolates of *Race 4* were sensitive to benomyl *(Table 1).*

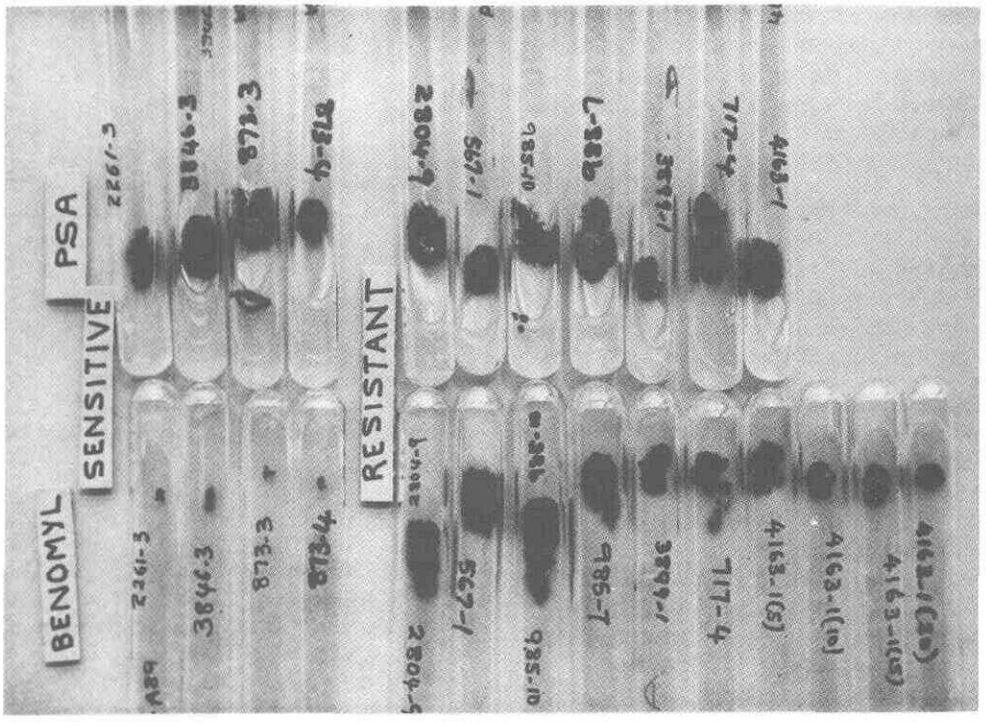

Figure 1. Growth of benomyl resistant and benomyl sensitive isolates of Microcyclus ulei *on potato sucrose agar (PSA) and PSA amended with 5 mg per litre benomyl except in isolate 4163–1 with 5, 10, 15 and 20 mg per litre benomyl.*

TABLE 2. EFFECT OF BENOMYL ON GERMTUBE LENGTH OF BENOMYL SENSITIVE AND RESISTANT ISOLATES OF *MICROCYCLUS ULEI*

Isolate	Germtube length in benomyl (μm)							
	0.25 mg/litre	0.50 mg/litre	1.00 mg/litre	4.00 mg/litre	6.25 mg/litre	12.50 mg/litre	25.00 mg/litre	Mean
Sensitive	4.63	6.53	—	3.69	7.03	9.95	6.90	6.46
Resistant	36.81	51.2	29.78	24.06	24.80	29.80	13.80	30.04
	$t = 4.87$, $P<0.001$							

Treatments with 0.25–4.0 mg per litre benomyl were incubated for 24 h while treatment with 6.25 mg per litre benomyl and above were incubated for 36 h.

Degree of Resistance

The lengths of germtubes of an isolate resistant, and another sensitive to 1 mg per litre benomyl on cellophane treated with benomyl are given in *Table 2*. Germtube length of the sensitive isolate was significantly shorter than that of the resistant isolate. In addition, on cellophane treated with 0.25 mg per litre benomyl or a higher concentration of benomyl, the germtubes of the sensitive isolate curled while no such curling was observed in germtubes of the resistant isolate even on cellophane dipped in 25 mg per litre benomyl.

In leaf disc tests, the concentration of benomyl which would effect 95% reduction in lesion number (EC 95) also varied according to the isolates, the values were about 1 mg per litre or less for sensitive isolates and more than 30 mg per litre for resistant isolates *(Table 3)*.

Leaf discs of clone FX 3864 suspended on solutions of either 25 mg per litre benomyl or 50 mg per litre thiophanate methyl were inoculated with field conidia of *M. ulei* obtained from five clones of *Hevea*. Results showed that there was approximately 80% reduction in lesion number on fungicide-treated discs suggesting that about 20% conidia obtained from clones FX 3864 or FX 985 were resistant to benomyl and thiophanate methyl. The population of conidia resistant to these fungicides was lower in the other clones tested *(Table 4)*.

In tests on PSA medium, when one of the resistant isolates was used, it still grew on PSA medium amended with 20 mg per litre benomyl *(Figure 1)*.

TABLE 3. TOXICITY OF BENOMYL TO ISOLATES OF *MICROCYCLUS ULEI*

Isolate	EC 95 (mg/litre)[a]
25-2	< 3.12[b]
3846-3	< 0.78[b]
3846-21	0.43
2804-29	1.17
985-7	35.50
985-10	44.30
2804-9	36.89
567-1	36.56

[a]Concentration effecting 95% control calculated from linear regression of benomyl concentration *versus* percentage control.

[b]Lowest concentration tested

Stability of Resistance

An isolate resistant to 1 mg per litre benomyl was successfully trained to grow on medium supplied with 10 mg per litre benomyl. However, growth was very slow when stroma obtained from PSA with 10 mg per litre benomyl was transferred to a medium amended with 15 mg per litre benomyl. The stroma from 10 mg per litre benomyl after being transferred onto four changes of pure PSA

TABLE 4. POPULATION OF BENOMYL AND THIOPHANATE METHYL RESISTANT ISOLATES IN FIELD CONIDIA OF *MICROCYCLUS ULEI*

Source of conidia	Population[a] (%) resistant to	
	Benomyl (25 mg/litre)	Thiophanate methyl (50 mg/litre)
FX 3864	23.59	20.11
FX 985	20.67	37.79
FX 2261	12.40	9.62
IAN 873	11.41	15.29
FX 2804	9.65	7.61
F =	0.813^{NS}	3.993*
$LSD_{0.05}$ =	21.596	18.640

The data were averages of two experiments with two replicates per experiment

*Significant at 5%

NS — Not significant

[a] $\frac{\text{Lesion number of treated}}{\text{Lesion number of control}} \times 100$

grew on PSA amended with 1 mg per litre benomyl indicating that its resistance to benomyl was 'acquired resistance' and therefore was stable.

In another test where a resistant isolate was inoculated onto several consecutive sets of *Hevea* plants not treated with benomyl, pure isolate obtained from the fourth set of plants still grew on PSA medium containing 1 mg per litre benomyl indicating that the isolate retained its resistance to benomyl.

Cross-resistance to Other Benzimidazoles

On PSA medium, all tested isolates which were resistant to benomyl were also resistant to 5 mg per litre thiophanate methyl. In leaf disc tests, thiophanate methyl and carbendazim were not effective against benomyl resistant isolates while they were effective against the benomyl sensitive isolates *(Table 5)*. Chlorothalonil and triforine were effective against both sensitive and resistant isolates.

Pathogenicity of Benomyl Resistant Isolates

Benomyl resistant isolates isolated from the field sporulated normally on culture medium and the spores produced on this medium caused normal infection of plants. When pathogenicity of resistant and sensitive isolates was tested on plants sprayed and not sprayed with benomyl, 50 mg per litre benomyl effectively controlled lesion formation of the sensitive isolates but had little effect on the resistant isolates *(Table 6)*. In addition, the difference in the amount of conidia produced by the resistant isolates on leaves treated with benomyl compared to the untreated leaves was not significant, both in leaf disc tests and on plants in planting bags *(Table 7)*. Conidia produced on benomyl-treated leaves germinated (54.0%) equally well compared to conidia obtained from untreated leaves (57.0%).

Competition Between Benomyl Resistant and Benomyl Sensitive Isolates

When the competitiveness of a resistant isolate was tested, benomyl resistant population still existed among conidia obtained from the fifth set of plants in which the first set was inoculated with a mixture of a resistant isolate and a sensitive isolate *(Table 8)*.

DISCUSSION

Fungal resistance to fungicides is a continuing challenge to crop protectionists[13]. Resistance

TABLE 5. EFFECT OF THREE BENZIMIDAZOLE FUNGICIDES, TRIFORINE AND CHLOROTHALONIL ON INFECTION OF *HEVEA* LEAF DISCS INOCULATED WITH BENOMYL SENSITIVE OR RESISTANT ISOLATES OF *MICROCYCLUS ULEI*

Fungicide	Rate (mg/litre)	Disease control[a] (%)	
		Sensitive	Resistant
Benomyl (Benlate 50 WP)	12.5	98.05	63.10
Thiophanate methyl (Cercobin M 70 WP)	12.5	91.05	33.15
Carbendazim (Delsene 75 WP)	12.5	97.10	31.00
Triforine (Saprol 190 g/litre)	12.5	97.35	96.75
Chlorothalonil (Daconil 75 WP)	1.0	95.75	90.75
		$LSD_{0.05}$ = 21.38	

[a]Mean of two resistant and two sensitive isolates

TABLE 6. EFFECT OF BENOMYL ON INFECTION OF *HEVEA* LEAVES BY BENOMYL SENSITIVE AND RESISTANT ISOLATES OF *MICROCYCLUS ULEI*

Isolate	Benomyl (mg/litre)	Lesion number/cm^2 leaf
Sensitive	0	5.80
	50	0.08
Resistant	0	3.36
	50	3.26
		$LSD_{0.05}$ = 2.31

TABLE 7. EFFECT OF BENOMYL ON PRODUCTION OF CONIDIA BY A BENOMYL RESISTANT ISOLATE OF *MICROCYCLUS ULEI*

Benomyl (mg/litre)	Conidia production ($\times 10^3$)
Leaf disc test	
0.0	20.81
0.1	23.48
0.2	15.32
0.4	12.93
0.8	21.61
Polybag plants	$F = 0.68^{NS}$
0.0	11.20
50.0	10.08
	$F = 0.53^{NS}$

NS — Not significant

TABLE 8. CHANGES IN POPULATION OF A BENOMYL RESISTANT ISOLATE OF *MICROCYCLUS ULEI* IN COMPETITION WITH A BENOMYL SENSITIVE ISOLATE

Generation of conidia	Population (%) resistant to benomyl at	
	0.8 mg/litre	1.6 mg/litre
First	49.34	40.12
Second	54.33	32.20
Third	32.99	31.70
Fifth	33.05	27.92

to benzimidazole fungicides especially benomyl had been reported in many pathogens[13,15,16]. Benzimidazole fungicides especially benomyl and thiophanate methyl had been extensively used in Brazil to control SALB since 1974

particularly in the PROMASE project (special project to control SALB[1,17]). The potential of *M. ulei* to develop resistance to benomyl is demonstrated by its ability to acquire resistance by training. In the field, the nature of fungicide treatment could have offered a strong selection pressure for resistant strains. The dosage rates used were relatively low (100-150g per hectare)[1] and the exposure of the fungus to the fungicides was long as seven weekly-rounds of spraying or more were conducted[1,18,19]. Therefore it is not surprising to find isolates of *M. ulei* resistant to benomyl and thiophanate methyl. Though resistant isolates were obtained from the field, it is not certain whether these resistant isolates were the consequence of benomyl usage. However, since benomyl was previously more effective against the pathogen, there is a possibility that benomyl usage contributed to the build-up of resistant strains. Unfortunately, there is no published data to indicate the baseline sensitivity of wild isolates to benomyl. Various percentage inhibition of germination of conidia of *M. ulei* have been quoted ranging from complete or near complete inhibition by benomyl at 25 mg per litre[5], 74% inhibition at 10 mg per litre[6], 96% inhibition at 10 mg per litre[20] and 50% inhibition at 12.5 mg per litre[9]. These differences could be due to the presence of different amounts of benomyl resistant strains in the test samples.

Zhang and Chee[9] indicated differences in sensitivities of races of *M. ulei* to benomyl and thiophanate methyl. The results presented here indicated that the differences in sensitivity between races could be due to the differences in frequency of occurrence of resistant isolates in different race groups. There might be race differences in tolerant potential, rate of dominance and parasitic fitness of tolerant strains. In Bahia state, Brazil, chemical control was commercially performed mostly on clones which are hosts of *Race 6* (FX 985, FX 25, FX 3864, FX 3844, FX 4163, *etc.*) and *Race 2* (*Hevea benthamiana* hybrids) and less commonly on FX 2261, the host of *Race 4*[17]. This could have contributed to the higher frequency of resistant isolates in *Race 6* and to a lesser extent in *Race 2*.

Epidemiologically, it is important to note that the benomyl resistant isolate was parasitically fit as it caused infection and produced as much viable conidia on benomyl-treated leaves as on benomyl-free leaves. Moreover, the results also indicated that resistance to benomyl was stable. These characteristics imply that a longer period should elapse before benomyl can be reused to treat SALB once the fungus has developed resistance to it.

Presently, many ergosterol biosynthesis inhibitor fungicides are being recommended to treat SALB[6-8]. Bearing in mind that resistance to these fungicides had been reported in some pathogens[21-23], their use should be carefully managed to avoid the build-up of resistant strains. Alternating and/or mixing protective fungicides with systemic fungicides are generally adopted to overcome this problem[13,15,16]. It is also important to monitor the occurrence and build-up of resistant strains.

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