

Association between isoenzymatic phenotypes and resistance to the fungus *Microcyclus ulei* P. Henn. (v. Arx.) in rubber tree clones*

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ABSTRACT

Electrophoretic analysis of 78 rubber tree clones was carried out in starch gel in order to detect associations between enzyme phenotypes and resistance to South American Leaf Blight caused by *Microcyclus ulei*. Seven enzymatic systems were studied: Adh, Pgi, 6Pgd, Mdh, Lap, Skd and Acp.

Associations between specific enzymatic phenotypes (Pgi 4-3, 6Pgd 2-1 and Mdh₁ 4-2) and resistance indicate that selection for resistance to *M. ulei* would be possible, using these markers.

INTRODUCTION

South American Leaf Blight (SALB) is the most important disease of the rubber tree and the greatest limiting factor for the development of natural rubber production in Brazil. It is caused by *Microcyclus ulei* P. Henn. (v. Arx.), which attacks the plantation at an epidemic level, preventing rubber exploitation in the humid Amazon. Valois (1983) pointed out four possible ways to control this fungus: planting rubber trees outside of the humid Amazon region; genetic control, which involves research to obtain resistant and productive clones; chemical control and crown budding. Until now none of these alternatives has

succeeded well under climatic conditions favorable to *M. ulei*.

Research on biochemical mechanisms and substances involved in the resistance to this pathogen have been carried out since the 1960s. Many enzymes which participate either directly or indirectly in the hypersensitivity reaction to *M. ulei* have been studied (Lieberei, 1981, 1986; Lieberei *et al.*, 1985, 1989; Selmar *et al.*, 1989). Isoenzymatic characterization of *Hevea* sp has been conducted in order to quantify the genetic variability of Malasian and Amazonian samples (Soleille, 1984; Chevallier, 1988).

MATERIAL AND METHODS

Samples were prepared from 15 or more leaflets, predominantly at phenological state B (Hallé *et al.*, 1978), from 78 rubber tree clones collected at CPAA/EMBRAPA - Manaus (55 clones), ESALQ/USP, Piracicaba (21 clones) and IAC - Campinas (two clones). The clones analyzed electrophoretically and their respective progenitors are identified in Table I.

* Part of a thesis presented by A.N.K.F. to Departamento de Genética e Matemática Aplicada à Biologia, FMRP, USP, Ribeirão Preto, in partial fulfillment of the requirements for the Doctoral degree.

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Table I - Identification and progenitors of rubber tree clones (*Hevea* sp) analyzed by electrophoresis (Bahia *et al.*, 1985).

Clone	Progenitors	Clone	Progenitors
<i>H. camporum</i>	species	AM/87/944/C	IAN 6158 x CNS BT 7838
<i>H. rigidifolia</i>	species	AM/87/1022/C	IAN 6158 x CNS BT 7838
<i>H. guian.</i> var. <i>guian.</i>	species	polyploid clones	
<i>H. nitida</i>	species	CNSAM 7704P1	native clone
<i>H. spruceana</i>	species	FX 4098 P1	PB 86 x B 110
<i>H. brasiliensis</i> clones		FX 985 P1	F 315 x AV 183
MDF 180	native clone ¹	FX 3295 P1	F 4542 x AV 363
LCB 510	native clone	FX 3899 P1	F 4542 x AV 363
PFB 5	native clone	IAN 6158 P1	FX 43-655 x PB186
PB 86	native clone	IAN 6323	Tjir 1 x FX 3810
PB 235	PB 5/51 x PBS 38	IAC 222	PB 86 x FA 1717
FX 3864	PB 86 x B 38	<i>H. pauc.</i> x <i>H. brasiliensis</i>	
FX 985	F 315 x AV 183	IAN 6486	P 10 x PB 86
FX 2261	F 1619 x AV 183	<i>H. brasiliensis</i> x <i>H. bent.</i>	
FX 25	F 351 x AV 49	IAN 3087	FX 516 x PB 86
FX 3844	PB 183 x B 45	IAN 2878	FX 516 x PB 86
RRIM 527	PILB 50 x PILB 84	IAN 2880	FX 516 x PB 86
RRIM 600	Tjir 1 x PB 86	IAN 717	PB 86 x F 4542
IAN 873	PB 86 x FA 1717	IAN 3193	FX 516 x PB 86
IAN 2388	FX 2025 x FX 25	IAN 2909	FX 516 x PB 86
<i>H. pauciflora</i> clones		IAN 6323	Tjir 1 x FX 3810
P10	native clone	IAN 3044	FX 516 x PB 86
PA 31	native clone	IAN 710	PB 86 x F 409
CNS BT 7831	native clone	IAN 6158	FX 43-655 x PB 186
CNS BT 7835	native clone	FX 3899	FX 4542 x AV 363
CNS BT 7838	native clone	FX 3810	FX 4542 x AV 363
CNS AM 7745	native clone	FX 2804	FX 4542 x Tjir 1
<i>H. benthamiana</i> clones		FX 3925	FX 4542 x AV 363
F 4512	native clone	FX 567	FX 4542 x AV 368
CNS AM 8203	native clone	AM/86/14	FX 4098 x IAN 6158
CNS AM 8204	native clone	AM/86/24	FX 4098 x IAN 6158
native clones		AM/86/148	FX 4098 x IAN 6158
CNS AM 7752	native clone	AM/86/984	IAN 6158 x FX 985
CNS AM 7701	native clone	AM/86/117	FX 4098 x IAN 6158
CNS AM 7718	native clone	AM/86/18	FX 4098 x IAN 6158
CNS AM 7907	native clone	AM/86/120	FX 4098 x IAN 6158
CNS AM 7731	native clone	AM/86/1062	IAN 6158 x FX 985
CNS AM 7665	native clone	AM/86/415	FX 4098 x IAN 6158
CNS AM 7623	native clone	PL 2	IAN 6158 x FX 985
AM/86/1296/C	native clone	PL 6	IAN 6158 x FX 985
illegitimate clones		PL 7	IAN 6158 x FX 985
AM/86/41	IAN 6158 ill. ²	PL 8	IAN 6158 x FX 985
AM/86/315	IAN 6158 ill.	PL 10	IAN 6158 x FX 985
AM/86/150	IAN 6158 ill.	PL 11	IAN 6158 x FX 985
AM/86/197	IAN 6158 ill.	PL 12	IAN 6158 x FX 985
AM/86/433	IAN 6158 ill.	PL 13	IAN 6158 x FX 985
AM/86/2106/C	IAN 6158 ill.	PL 15	IAN 6158 x FX 985
AM/86/116	IAN 6158 ill.	AM/86/354	FX 4098 x IAN 6158
AM/86/2206/C	IAN 6158 ill.	AM/86/29	FX 4098 x IAN 6158
AM/87/832/C	IAN 6158 ill.	AM/86/126	FX 4098 x IAN 6158
<i>H. brasiliensis</i> x <i>H. benth.</i> x <i>H. pauc.</i> hybrids		AM/86/106	FX 4098 x IAN 6158
AM/87/1056/C	IAN 6158 x CNS BT 7838	AM/86/229	FX 4098 x IAN 6158
AM/86/1906/C	IAN 6158 x CNS BP 08	AM/86/1241	IAN 6158 x FX 985
AM/87/1196/C	IAN 6158 x CNS BT 7831	AM/86/318	FX 4098 x IAN 6158
AM/87/932/C	IAN 6158 x CNS BT 7835	AM/86/271	FX 4098 x IAN 6158
AM/87/923/C	IAN 6158 x CNS BT 7835	AM/86/1514	IAN 6158 x FX 985
AM/87/955/C	IAN 6158 x CNS BT 7838	AM/86/37	FX 4098 x IAN 6158

1 - native clone (undomesticated material); 2 - unknown parental male.

The leaflets from the clones grown in Manaus were lyophilized at -52°C for 72 hours under 0.1 mbar pressure. The enzyme extraction methods for electrophoretic analysis in starch gel were described by Lebrun and Chevallier (1988). Seven enzymatic systems were studied: shikimate dehydrogenase (Skd - EC 1.1.1.25), alcohol dehydrogenase (Adh - EC 1.1.1.37), phosphoglucose isomerase (Pgi - EC 5.3.1.9), leucine aminopeptidase (Lap - EC 3.4.11.1), 6-phosphogluconate dehydrogenase (6PgD - EC 1.1.1.44) and acid phosphatase (Acp - 3.1.3.2). Table II shows the buffers used for electrophoretic analysis of these enzymes and their respective migration conditions.

Table II - Enzyme systems, buffers and migration conditions used for electrophoretic analysis of extracts of leaves of rubber tree clones.

Enzyme	Locus	No. of alleles	Buffers		Migration mA/cm
			Electrode	Gel	
Skd	<i>Skd</i>	6	A	C	1.5
Adh	<i>Adh</i>	3	A	C	1.5
Mdh	<i>Mdh</i> ₁	4	A	C	1.5
Pgi	<i>Pgi</i>	4	A	C	1.5
Lap	<i>Lap</i> ₁	5	A	C	1.5
6PgD	<i>6PgD</i>	3	B	D	3.0
Acp	<i>Acp</i>	2	A	A	1.5

A - 0.15 M tris + 0.05 M citric acid, pH 6.6.

B - 0.1 M tris + 0.028 M citric acid, pH 7.5.

C - 6.8 mM histidine, adjusted to pH 6.0 with 1 M tris.

D - 12.5 mM tris + 3.5 mM citric acid, pH 7.5.

Evaluations for resistance to *M. ulei* were carried out using two isolates (I and II) in the different clones. A system of scores ranging from 0 to 10 which considered fungus sporulation (RI and RII) and the leaf lesion diameter (LDI and LDII) was used (Junqueira *et al.*, 1988, 1990; Junqueira and Araújo, personal communication). Clones with scores lower than or equal to 3.5 were considered resistant.

Group I isolates sporulate on all the clones carrying genes from clone F4542, an ancestor of *Hevea benthamiana*, from which they were derived, and on some clones of *H. brasiliensis*. Group II isolates sporulate on the majority of the *H. brasiliensis* and on a few of the clones carrying F 4542 genes from *H. benthamiana* (Junqueira *et al.*, 1989).

For statistical tests, rare enzyme phenotypes were grouped into classes. The association between the phenotypic classes and resistance to the pathogen was

checked with χ^2 tests. The Woolf chi-square method was used to analyze the association between a specific enzyme phenotype and resistance (Woolf, 1955).

The association between activity of Acp and resistance was analyzed by linear correlation. The association between average heterozygosity (\bar{H}), calculated according to Tanksley *et al.* (1981), and resistance was estimated by chi-square tests and linear correlations. The significance of the correlations was verified using the t-test. Correlation analysis between the resistance parameters (resistance and lesion diameter for the two groups of isolates of the pathogen) was carried out.

RESULTS

Table III shows the association between phenotypic classes of the rubber tree clones and resistance to *M. ulei* given by RI, RII, LDI and LDII. For example, Pgi x RII expresses the relationship between the phenotypic classes of the enzyme phosphoglucose isomerase (Pgi) and the resistance to isolates of group II of *M. ulei* (RII) in this table. The chi-square values suggest significant association between Pgi x RII, Lap₁ x RI, Lap₁ x RII, Pgi x LDI and Pgi x LDII.

Table IV shows the association between specific enzymatic phenotypes and resistance. The Woolf chi-square values indicate the existence of associations between Pgi 4-3, 6PgD 2-1 and Mdh₁ 4-2, with resistance.

The analysis of association between the activity of Acp and resistance to *M. ulei* gave non-significant (t-test) correlation values (Acp x RI = 0.0565; Acp x RII = 0.1744; Acp x LDI = 0.0015 and Acp x LDII = 0.0658).

Table V indicates no evidence of association between average heterozygosity (\bar{H}) and the resistance parameters.

Table VI shows the correlations between average heterozygosity (\bar{H}) and resistance parameters and correlations between resistance parameters. Correlations between RI-RII and LDI-LDII were non-significant, but correlations between RI-LDI and RII-LDII were significant.

DISCUSSION

Some of the enzymes that showed association with resistance, such as Pgi and Lap, also had specific enzymatic phenotypes associated with resistance (for example, phenotypes Pgi 4-3 and Lap₁ 3-2).

Table III - Analysis through chi-square test between classes of enzyme phenotypes and resistance (R) to *Microcyclus ulei*. R was a score according to fungus sporulation.

Enzyme	x	Resist.	χ^2	df
Skd	x	RI	3.02	1
Skd	x	RII	1.51	1
Adh	x	RI	0.59	1
Adh	x	RII	1.21	1
Pgi	x	RI	3.56	1
Pgi	x	RII	12.16**	1
6PgD	x	RI	1.04	1
6PgD	x	RII	2.01	1
Lap ₁	x	RI	11.14**	4
Lap ₁	x	RII	10.32**	4
Mdh ₁	x	RI	2.23	2
Mdh ₁	x	RII	3.16	2
Skd	x	LDI	0.48	1
Skd	x	LDII	0.37	1
Adh	x	LDI	0.08	1
Adh	x	LDII	2.40	1
Pgi	x	LDI	4.16*	1
Pgi	x	LDII	11.00*	1
6PgD	x	LDI	0.02	1
6PgD	x	LDII	1.61	1
Lap ₁	x	LDI	0.34	1
Lap ₁	x	LDII	1.57	1
Mdh ₁	x	LDI	0.25	1
Mdh ₁	x	LDII	0.05	1

*P < 0.05

**P < 0.01

RI - Resistance to the isolates of the group I of *M. ulei*; RII - resistance to the isolates of group II of *M. ulei*; LDI - diameter of lesions caused by the isolates of group I of *M. ulei* and LDII - diameter of lesions caused by the isolates of group II of *M. ulei*.

On the other hand, the phenotypic classes 6PgD and Mdh, whose chi-square values indicated that they were not associated with resistance, did have some individual phenotypes associated with resistance (6PgD 2-1 and Mdh₁ 4-2) according to the Woolf chi-square (Table IV). Discrepancies in the associations between phenotypic classes and resistance, and between specific enzymatic phenotypes and resistance require analysis of a larger number of clones.

Associations are usually explained by gene linkage, pleiotropy or by the heterozygosity level. The gene linkage hypothesis requires a low rate of recombination between markers and resistance. Stuber (1987) reported that the distance between the marker-locus and the target-locus should be minimal

Table IV - Association through Woolf chi-square between enzymatic phenotypes and resistance to *Microcyclus*.

Enzymatic phenotype	x	Resist.	χ^2
Skd	2	RI	2.92
Skd	2	RII	1.48
Adh	1	RI	0.03
Adh	2	RI	2.28
Adh	2-1	RI	2.45
Adh	1	RII	0.09
Adh	2	RII	1.63
Adh	2-1	RII	3.64
Pgi	3-2	RI	0.01
Pgi	4-3	RI	0.38
Pgi	4-1	RII	1.51
Pgi	4-3	RII	9.58**
6PgD	2	RI	2.21
6PgD	2-1	RI	0.20
6PgD	2	RII	2.91
6PgD	2-1	RII	7.06**
Lap ₁	4	RI	0.61
Lap ₁	4-3	RI	1.89
Lap ₁	3	RII	0.24
Lap ₁	4	RII	2.82
Lap ₁	3-2	RII	0.90
Lap ₁	4-3	RII	0.00
Lap ₁	5-3	RII	2.78
Lap ₁	3	RI	1.98
Lap ₁	3-2	RI	6.27**
Mdh ₁	2	RI	2.30
Mdh ₁	3-2	RI	0.36
Mdh ₁	4-2	RI	0.03
Mdh ₁	2	RII	0.82
Mdh ₁	4-2	RII	8.23**
Adh	2-1	LDI	1.54
6PgD	2	LDI	0.09
Mdh ₁	2	LDI	0.12
Mdh ₁	4-2	LDI	0.62
Mdh ₁	4-2	LDII	2.17

**P < 0.01. Abbreviations in Table III.

Table V - Evaluation of the association between average heterozygosity (\bar{H}) and resistance (RI, RII, LDI and LDII) to *Microcyclus ulei*.

\bar{H}	x	Resist.	χ^2	df	P
\bar{H}	x	RI	3.19	2	0.3-0.2
\bar{H}	x	RII	4.29	2	0.2-0.1
\bar{H}	x	LDI	0.39	1	0.7-0.5
\bar{H}	x	LDII	0.91	1	0.5-0.3

Table VI -Correlations between average heterozygosity (\bar{H}) and resistance to *Microcyclus ulei* (RI, RII, LDI and LDII), and between parameters of resistance to *M. ulei*.

	\bar{H}	RI	RII	LDI	LDII
\bar{H}	---				
RI	0.2832	---			
RII	-0.2192	0.1931	---		
LDI	0.1281	0.8290**	0.0823	---	
LDII	-0.3046	-0.0776	0.7539**	0.0800	---

**t-test probability $P < 0.01$.

(probably less than five centimorgans) for an effective manipulation of markers. The markers should also be distributed all along the genome.

Thus the enzymatic phenotypes Pgi 4-3, 6Pgd 2-1 and Mdh₁ 4-2 would be coded by alleles that are close to main genes responsible for the specific vertical resistance to group II of *M. ulei* isolates (Junqueira *et al.*, 1992), belonging to the *H. benthamiana* and *H. brasiliensis* germplasm.

In the case of horizontal resistance of clones with *H. pauciflora* germplasm (Junqueira *et al.*, 1992), many markers are required to obtain at least two flanking genes for each of the several loci involved in the resistance, allowing a selection efficiency of 99% (Tanksley and Orton, 1983). The RFLP technique (Helentjaris *et al.*, 1985; Tanksley and Hewitt, 1988) has been a valuable tool in the search for these markers as it allows the detection of a larger number of polymorphisms than isoenzymatic techniques.

One of the greatest difficulties in finding association between molecular markers and resistance is due to the difficulty in obtaining clones with horizontal resistance. Horizontal resistance was developed in the evolution of *H. pauciflora*, a species subject to introgressive hybridization. However, many natural hybrids with other susceptible species have lost this character (Gonçalves *et al.*, 1990).

It is possible, according to Seibert (1948), that the native Indians using the Amazon rivers during many centuries have changed the natural habitat of *Hevea*. The Indians may have provided conditions for interspecific hybridization which contributed to the many cases of introgression, leading to the loss of species identity in certain places (Baldwin Junior, 1947). According to Wycherley (1977), Indian influence was relevant only in the elimination of the barriers between species by clearing planting areas and by changing the course of creeks, being limited and conjectural as far as the domestication of the rubber tree is concerned.

Heterozygosity level has been reported to be correlated to plant adaptation. This hypothesis is based on the commonly accepted idea that heterozygosity confers homeostasis in development (Lerner, 1954) and has been proved in plant breeding by crossing of inbred lines. This hypothesis, however, does not satisfactorily explain the associations between specific enzymatic phenotypes and resistance since the correlations between heterozygosity and resistance were not significant (Table VI). The absence of significant correlations does not exclude, however, the possibility of its existence. The estimate of heterozygosity based on the small number of loci studied in this work may not represent the true heterozygosity of the loci linked to resistance in these clones.

The non-significant correlations between RI and RII and between LDI and LDII (Table VI) show that the isolates of the groups I and II act differently, as Junqueira *et al.* (1989) suggested. The significant correlations between RI and LDI and between RII and LDII, on the other hand, suggest that both diameter of the lesions and sporulation degree are adequate parameters to determine resistance (Junqueira *et al.*, 1988).

The associations detected suggest a selection strategy for resistance to the isolates of group II. The clones which presented the phenotypes Pgi 4-3, 6Pgd 2-1 and Mdh₁ 4-2 could be selected and submitted to resistance tests.

ACKNOWLEDGMENTS

We thank Elisabete M.S. Barreto Beira for advice and help with lab procedures, Ricardo P. Rebello, for his cooperation in the collecting of data, Prof. José Dias da Costa, and Dr. Paulo de S. Gonçalves for permission to collect the material and Dr. Nelcimar R. Souza, for preparing the seedlings.

Research supported by CENARGEN, EMBRAPA. Publication supported by FAPESP.

RESUMO

Análises eletroforéticas de extratos de folíolos de 78 clones de seringueira foram conduzidas com o objetivo de verificar a presença de associação entre fenótipos enzimáticos e resistência ao *Microcyclus ulei*. Foram estudados os seguintes sistemas enzimáticos: Adh, Pgi, 6Pgd, Mdh, Lap, Skd e Acp.

Foram detectadas associações entre os fenótipos Pgi 4-3, 6Pgd 2-1 e Mdh₁ 4-2 e resistência, o que aponta para a possibilidade de seleção para estes fenótipos visando resistência ao patógeno em programas de melhoramento.

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(Received July 7, 1994)