Assessment of Resistance to Witches'-Broom Disease in Clonal and Segregating Populations of *Theobroma cacao*

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ABSTRACT

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Fourteen clonal genotypes of cacao (*Theobroma cacao*) and their open-pollinated progenies were tested for their resistance to witches'-broom disease (*Crinipellis perniciosa*) using an agar-droplet inoculation method in separate, replicated greenhouse experiments. Nine measures of resistance were evaluated for precision, ability to discriminate between levels for resistance, and their correlation to field resistance. Correlations among the various measures of resistance were also ascertained. Data were re-examined to determine the accuracy and precision associated with individual plant measurement as well as the optimum sample size to assess segregating populations for resistance. The agar-droplet method was able to effectively discriminate various levels of resistance. Incubation period provided a precise, sensitive, valid, and heritable measure of resistance to *C. perniciosa*. Furthermore, incubation period was highly correlated with broom size and moderately correlated with broom frequency, two epidemiologically important components of resistance. The study also shows that 25 to 30 open-pollinated progeny are sufficient to provide a good estimate of a parent's resistance. The implications of the findings for identifying resistance among cacao germplasm collections as well as within segregating populations for resistance breeding are discussed.

Additional keywords: cocoa, parent-offspring regression, resistance screening

Witches'-broom disease caused by the basidiomycete fungus *Crinipellis perniciosa* (Stahel) Singer is a devastating disease of cacao (*Theobroma cacao* L.) in Latin America and the Caribbean. The pathogen causes damage to vegetative buds, flower cushions, and pods (1), usually resulting in 30 to 50% crop loss (6), but losses can be as high as 90% (17,22). Possible spread of the disease worldwide is considered a serious threat to the future of the cacao industry (31). Control of the disease through sanitation or chemical control measures is not only very costly (27,33) but also impractical (13). The most economically feasible long-term solution is thus the cultivation of resistant genotypes (1,15,21).

The limited progress in combining resistance to *C. perniciosa* into elite genetic backgrounds has been attributed to the lack of a suitable screening method (21) capable of revealing the maximum genotypic expression of major components of resistance and the poor understanding of the mechanism(s) and genetic basis of resistance (28). An ideal screening tool should not only effectively discriminate between levels of resistance on a quantitative basis, but it should also ensure that the purported resistance is valid, repeatable, and heritable.

Several inoculation methods have been reported in the literature, including seed inoculation (1,10), terminal bud inoculation using agar blocks (30), spray inoculation of whole plants (8), callus inoculation (7,16), and indirect methods based on characteristics of spore germination in leaf extracts or phloem sap (2,4,5). However, none of these have fulfilled the criteria required of a screening tool. Recently, an agar-droplet inoculation method (26), in which concentrated spores in a droplet of diluted agar are placed on a growing meristem, was shown to be repeatable using standard basidiospore concentrations and standardized incubation conditions in both clonal and seedling plants. The validity of this inoculation method as a screening tool has still not been tested thoroughly.

Vegetative brooms are epidemiologically more important than pod or flower cushion infections since they are the most productive and consistent source of inoculum in the field (23). Wheeler (29), citing the work of Baker and Holiday (1), concluded that broom number and broom size were important components of cacao resistance to witches'-broom disease, since they determine the pathogen's sporulation intensity and reproductive capacity. For instance, small brooms produce fewer basidiocarps than large brooms, and few basidiocarps on small brooms survive (1). Broom removal limits the impact of the disease through sanitation. Not surprisingly, many researchers (8,11,12,19,29) have therefore used broom frequency and intensity of broom development (broom size) as measures of resistance.

Wheeler (29) suggested that resistance to witches'-broom may be expressed in various ways and hence may need to be measured in various manners. He reasoned that proportion of plants with brooms, time to broom maturity, broom size, and ability of brooms to produce basidiocarps may all influence inoculum potential in the field and hence should be regarded as important components of resistance. However, very little is known of the interrelationships among these measures and, consequently, the mechanisms of resistance. Further, the validity, reproducibility, and heritability of these measures have not been ascertained.

This study utilizes the agar-droplet inoculation method as a screening tool for comparing the efficiency of various measures of resistance to *C. perniciosa* in clonal and segregating populations. The study also examines sample sizes required to assess resistance among progeny populations.

MATERIALS AND METHODS

Genotypes. Fourteen cacao genotypes (SCA 6, SCA 12, ICS 1, ICS 84, ICS 95, IMC 57, IMC 67, JA 6/4 [POU], JA 5/19 [POU], JA 5/41 [POU], UF 11, Hybrid 19, M 8, and West African Amelonado [WAA]), representing the various cacao populations held at the International Cacao Genebank, Trinidad (ICG,T), were micrografted (25) onto 3-week-old seedling stocks of TSH 919. The clonal scions were inoculated at 16 months of age.

Open-pollinated seedling progenies were obtained from 13 of the 14 genotypes (all genotypes except WAA). For each genotype, approximately five randomly selected pods, each containing approximately 30 seeds, were used to generate the open-pollinated progeny populations. Twelve-month-old seedlings were used for inoculation.

Inoculation method. The plants were pruned to induce young succulent growth, and the terminal meristem (flushing-2 stage, as described by Greathouse et al. [9]) of the emerging primary flush in the

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case of seedlings, and secondary flush in the case of clonal plants, was inoculated with basidiospores of *C. perniciosa* using the agar-droplet inoculation method (24,26). This method entails placing a drop (30 µl) of basidiospore suspension (350,000 viable basidiospores per ml in 0.3% agar) on the meristem using a micropipette, followed by incubation in polythene bags containing moist tissue paper at 25°C for a period of 60 h. Following incubation, the polythene covers were removed and the plants placed under 70% shade in a greenhouse. Plants were watered regularly and fertilized weekly for a period of 12 weeks with foliar applications of Nutrex (20:20:20 NPK) at a rate of 0.6 g/liter.

Experimental design. The 14 genotypes and their progenies were evaluated for resistance to witches'-broom disease in separate greenhouse experiments in a randomized complete block design with three replications. The replications contained three plants per replicate for the genotypes and 30 plants for the progeny populations, and the plants were inoculated over time at monthly intervals. Two plants per replicate were treated as the control for each genotype, using sterile distilled water in place of the basidiospore suspension.

Measures of resistance. The following indicators of host-plant interaction were recorded: the number of days until onset of stem swelling (incubation period), stem swelling at 5, 6, and 7 weeks after inoculation (increase in stem growth compared with the girth of control stems, measured to 0.1 mm, using a vernier caliper), percentage of plants showing swelling, percentage of plants developing brooms, broom characteristics (broom base diameter, broom length, broom dry weight, and number of active and nonactive shoots), and percentage of symptomatic plants developing brooms. Brooms were harvested 12 weeks after inoculation, dried to constant weight at 60°C, and weighed using an electronic balance (0.001 g resolution).

Data analysis. An analysis of variance (ANOVA) was carried out to determine the significance of differences among genotypes or among open-pollinated progenies representing the genotypes for each of the various measures of resistance using Minitab (Version 13; MINITAB Inc., State College, PA, USA) statistical software. Arcsine transformation was performed on percentage data.

The coefficient of variation and an index of differentiation were computed for the various measures of resistance. The latter is obtained by dividing the range of symptom expression for a particular variable by the $LSD_{0.05}$ for that resistance measure. The indices of differentiation were calculated on the full data set and also after removing the extreme levels of resistance represented by SCA 6, SCA 12, JA 6/4 [POU], and UF 11, the latter to determine the ability of various measures to differentiate among genotypes in the middle range.

Single plant analysis. Data from the clonal experiment were reanalyzed using single plant experimental units to determine the accuracy and precision of single plant estimates of the resistance measures. This simulated efficiencies one could expect with the various resistance measures when screening segregating populations for witches'-broom resistance. Standard errors, coefficients of variation, and indices of differentiation were calculated as before. Correlation between each single plant measurement for each resistance measure and the true mean averaged over nine plants provided an estimate of accuracy.

Heritability of resistance measures. The regression of progeny means for the various resistance measures on the parental means for the corresponding measures was investigated using linear regression analysis (parent-offspring regression analysis). Residual analysis was carried out to evaluate the fitted equations. The heritability was estimated by the coefficient of determination and the slope of the parent-offspring regression for each measure of resistance. The analyses were repeated after removing the extreme values to determine the heritability of the resistance measures in the middle range of resistance.

Previously published field observations of resistance to witches'-broom disease were available from the International Cacao Genebank, Trinidad (11,14). These were used to validate the various measures of resistance from the inoculation experiments using the Spearman's rank correlation method. A Pearson's correlation analysis was carried out to determine associations among the various measures of resistance separately for the genotypes and their progeny populations.

Optimum sample size for screening segregating populations. An iterative sampling approach was used to determine the optimum progeny size to obtain an accurate (valid) and precise (repeatable) measure of progeny means. Random samples of 5, 10, 15, 20, 25, or 30 plants from the 13 progeny families, each consisting of over 90 plants, were selected using random

Table 1. Comparison of various measures of resistance for heritability and their ability to differentiate among 14 clonal genotypes and their open pollinated progenies (n = 13) in *Theobroma cacao*

Genotypes in analysis	Measures of resistance[a]	Clonal genotypes				Progenies					
		Range	CV (%)	ID[b]	Sig.[c]	Range	CV (%)	ID	Sig.[c]	Slope[d]	R^2 for regression
Full data set	Inc. period (days)	14-31	1.28	38.6	***	16.1-30.3	3.5	35.5	***	0.75 (0.09)	0.86
	TBRM (days)	21-39	5.5	7.17	***	24.7-38.9	5.63	15.8	***	0.67 (0.08)	0.87
	PPS	67-100	7.52	2.6	***	78.9-100	3.38	12.34	***	0.35 (0.33)	0.59
	PPB	56-100	12.89	2.25	***	63.3-98.9	13.89	6.02	***	0.58 (0.10)	0.67
	PSB	14-31	13.97	0.78	***	76.9-98.9	5.94	7.48	***	0.46 (0.14)	0.5
	ST-SW (mm)	3.0-10.2	11.8	5.9	***	3.1-8.6	13.52	12.2	***	0.69 (0.13)	0.71
	BR-DIA (mm)	6.8-15.3	6.3	7.14	***	8.1-15.0	6.96	15.3	***	0.74 (0.14)	0.72
	BR-LEN (mm)	77-199	9.3	5.18	***	83.2-141.8	13.9	7.53	***	0.39 (0.14)	0.42
	BR-DW (g)	1.2-5.8	5.4	13.14	***	2.4-5.6	23.05	6.96	***	0.63 (0.16)	0.57
Genotype extremes removed[e]	Inc. period (days)	16.3-25.3	1.25	20.93	***	16.6-29.6	3.35	34.2	***	0.42 (0.17)	0.46
	TBRM (days)	22-33	4.17	5.95	***	26.2-38.8	6.61	11.89	***	0.41 (0.16)	0.48
	PPS	88.9-100	6.17	1.06	ns	94.4-98.9	19.25	0.46	ns	–0.28 (1.01)	0.13
	PPB	88.9-100	11.45	0.59	ns	78.9-97.8	5.87	6.49	*	0.27 (0.26)	0.13
	PSB	88.9-100	8.65	0.77	ns	83.7-98.9	5.69	5.15	ns	0.05 (0.24)	0.007
	ST-SW (mm)	4.73-8.32	10.37	3.15	***	4.7-8.2	12.11	8.14	***	0.58 (0.36)	0.28
	BR-DIA (mm)	9.4-12.5	5.99	2.72	***	10.1-14.5	6.82	8	***	0.58 (0.36)	0.24
	BR-LEN (mm)	130-177	7.65	2.33	**	76.6-127.4	12.88	7.06	**	0.07 (0.39)	0.005
	BR-DW (g)	3.4-5.1	3.71	6.53	***	2.6-3.7	22.97	2.44	*	1.26 (0.40)	0.58

[a] Inc. period, incubation period; TBRM, time taken to broom development; PPS, percentage of plants showing symptoms; PPB, percentage of plants showing brooms; PSB, percentage of plants showing symptoms developing into brooms; ST-SW, stem swelling; BR-DIA, broom-base diameter; BR-LEN, broom length; BR-DW, broom dry weight.

[b] Index of differentiation for each measure of resistance was calculated by dividing the range by $LSD_{0.05}$.

[c] ***, significant at $P < 0.001$; **, significant at $P < 0.01$; *, significant at $P < 0.05$; ns, not significant.

[d] Slope of parent-offspring regression. Estimates of slope (standard error in brackets) and coefficient of determination (R^2) were based on linear regression of progeny means on parental means.

[e] Extreme genotypes SCA 6, SCA 12, UF 11, and JA 6/4 and their respective progenies were removed from the analysis.

numbers generated with the RAND function in Excel (Microsoft Office 2000). Sampling was done in triplicate and subjected to ANOVA to determine the significance as well as the coefficient of variation associated with each sample size. The index of differentiation was calculated as before. Regression analysis was carried out to determine the relationships among the sample means, the true means (mean of 90 observations), and the parental means. The coefficient of determination provided an indication of the accuracy of the sample means. Based on the above analysis, the optimum progeny family size was determined for the most important resistance measures previously identified.

RESULTS

Analysis of variance showed that there were significant differences ($P < 0.001$) among the genotypes tested as well as among their progeny populations with respect to all the measures of resistance (Table 1).

Incubation period. The first symptom appeared as stem swelling at the site of inoculation in all the genotypes. Values for the time taken to symptom appearance (incubation period) showed (Fig. 1) a two-fold difference, both among genotypes (JA 6/4 [POU], 13.6 to SCA 6, 31.2 days) and among their respective progenies (JA 6/4 [POU], 16.1 to SCA 6, 30.2 days). This large difference coupled with relatively small coefficients of variation for incubation period provided excellent differentiation between genotypes/progenies (index of differentiation = 38.6). A strong parent-offspring regression for incubation period indicated that it was also highly heritable (Fig. 2A).

Vegetative brooms appeared 6 to 8 days after first evidence of stem swelling in all the genotypes tested, and hence the time taken to broom development (TBRM) showed a similar trend to that of the incubation period, varying from 21 days (JA 6/4 [POU]) to 40 days (SCA 6) in the genotypes. Although in most cases stem swellings developed into brooms, sometimes they remained as stem swellings with no further progression and occasionally disappeared or became less apparent as growth continued. This accounted for higher coefficients of variation for TBRM and, consequently, a lower index of differentiation when compared with incubation period (Table 1).

Disease incidence. Disease incidence measured as proportion of plants showing symptoms (PPS) or proportion of plants showing brooms (PPB) was only moderately heritable (0.35 to 0.58). Further, PPS was only able to separate the highly resistant genotypes, SCA 6 and SCA 12 (mean = 72.5%), from the rest (100%) (data not shown), resulting in a small index of differentiation. Although the genotypes fell into three groups based on PPB (SCA 6 and SCA 12 at 56%; IMC 57 and IMC 67 at 84%; others at 100%), the relatively high coefficient of variation associated with PPB accounted for a small index of differentiation. With the exception of SCA 6, SCA 12, IMC 57, and IMC 67, all genotypes showed 100% conversion of stem swellings to brooms (PSB) (data not presented).

Disease severity. Disease severity measured as stem swelling, broom-base diameter, broom length, and broom dry weight showed highly significant differences ($P < 0.001$) both among genotypes and among their respective progenies (Table 1). Among these measures of resistance, stem swelling and broom-base diameter showed the highest heritability

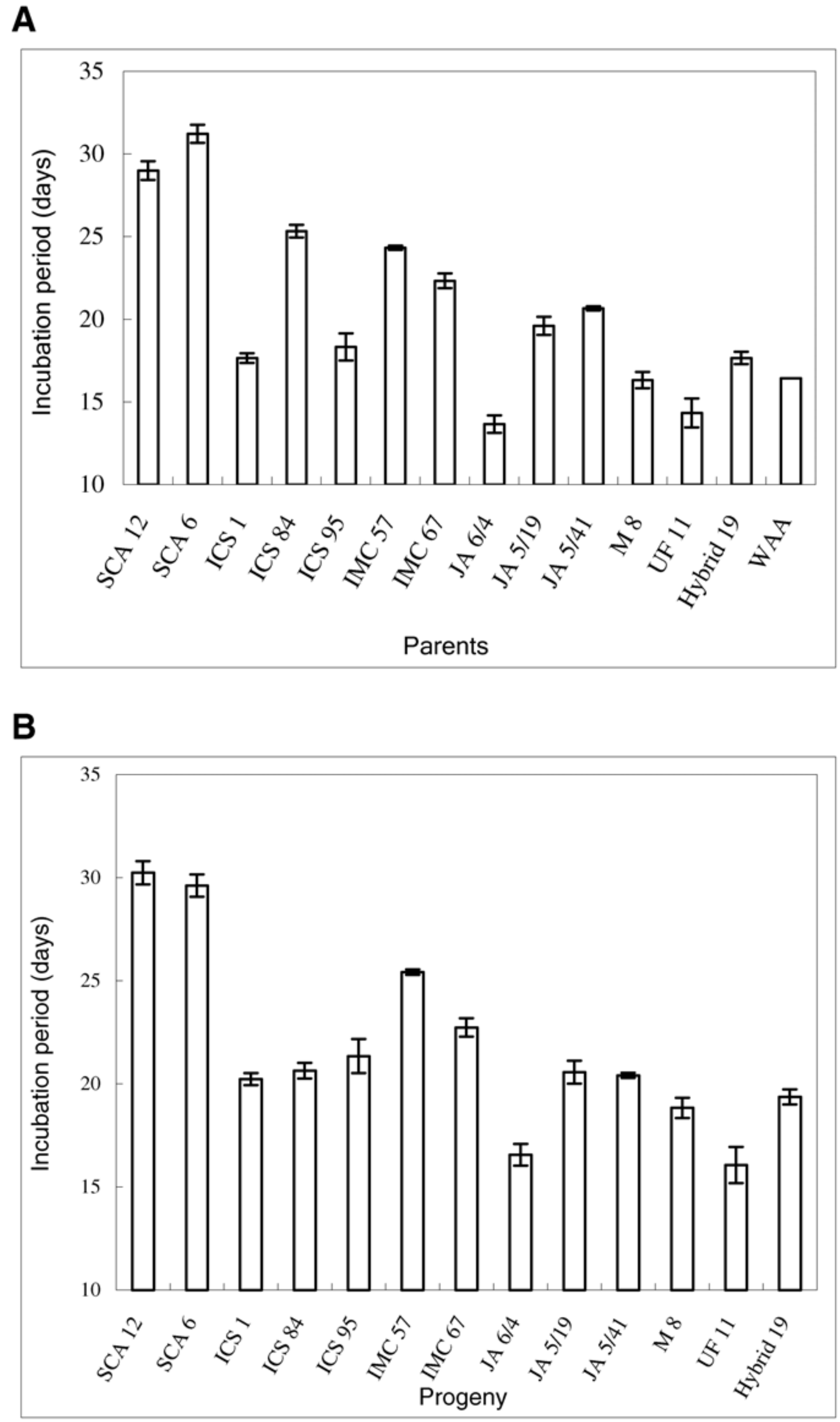

Fig. 1. Incubation period following inoculation with *Crinipellis perniciosa* for **A,** 14 genotypes (n = 9) and **B,** 13 open-pollinated progenies (n = 90) of *Theobroma cacao* (bars indicate standard error of means). Differences among parents or among progenies were highly significant ($P < 0.0001$) based on ANOVA.

(mean = 0.72) coupled with good differentiation among the parental genotypes and among progeny populations. In contrast, the heritability estimates of broom dry weight and broom length (Fig. 2B) were much smaller (0.42 to 0.57).

Differentiation of intermediate levels of resistance. When the parents and progenies with extreme levels of resistance or susceptibility (SCA 6, SCA 12, JA 6/4 [POU], and UF 11) were removed from the analysis, the genotypic effects on PPS, PPB, and PSB were not significant in the parents or in the progenies (Table 1). This indicates that these measures were only useful in differentiating genotypes with extreme levels of resistance or susceptibility. In contrast, incubation period had the highest index of differentiation for genotypes and progenies with intermediary levels of resistance, and incubation period had a moderate and significant ($P < 0.001$) R^2 for the parent-offspring regression when extreme genotypes were removed.

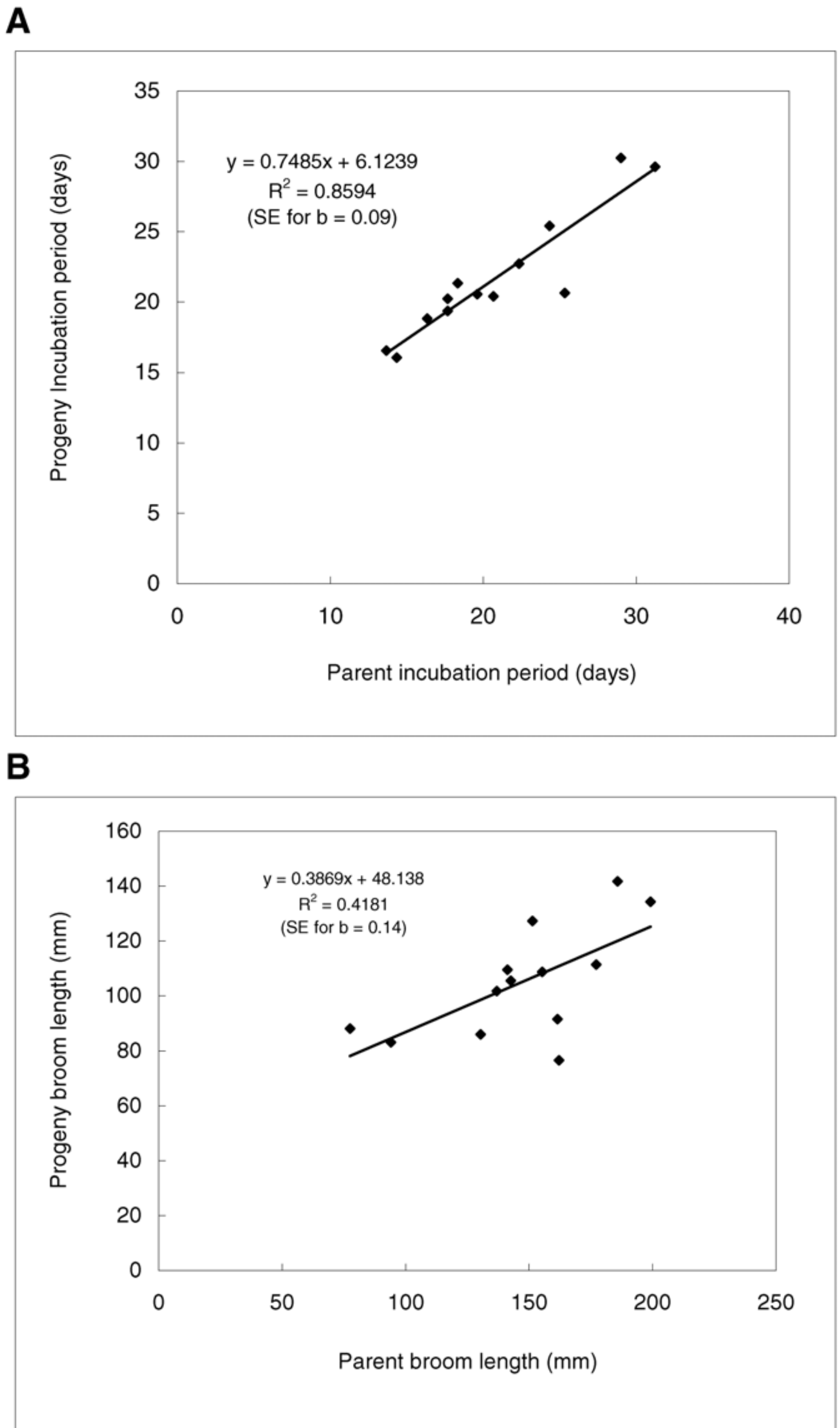

Fig. 2. Regressions between *Theobroma cacao* parents and their open-pollinated progenies inoculated with *Crinipellis perniciosa* for **A,** incubation period and **B,** broom length. Parent-offspring linear regression analysis was based on data from 13 parents and their open-pollinated progenies. Each data point is based on a mean of three observations.

Sample size for segregating populations. Since each genotype in a segregating population is represented by a single plant, reanalyzing the data using an experimental unit size of one will provide a simulation of screening segregating populations for resistance to *C. perniciosa*. In general, the coefficient of variation for the various measures increased 1.5- to 8.0-fold with single plant experimental units, thus reducing the ability of the test to differentiate among genotypes (Table 2). Nevertheless, incubation period continued to give the best index of differentiation among the measures investigated. Furthermore, the correlation between single values and means of nine observations over 10 iterations were consistently high for incubation period (r = 0.93 to 0.96) and broom weight (0.92 to 0.99).

Correlations among resistance measures. All the measures of resistance were highly correlated in the parental genotypes (r = 0.73 to 0.94) (data not provided). The correlations among incubation period, days to broom appearance, stem swelling, and broom-base diameter were high even when the extreme values were removed (Table 3). However, correlation of the above characters with PPS, PPB, and PSB were extremely small and not significant. Similarly, among the progeny means (Table 3), the correlation coefficients were high between incubation period, days to broom appearance, stem swelling and broom-base diameter, and among PPS, PPB, and broom size (length or weight), but not between characters from these two groups. The correlation between stem swelling and broom-base diameter was extremely high (r = 0.99) in both parents and progeny, indicating that only one of the two measures was required.

Correspondence between resistance measures and field resistance. The rank correlation between field resistance to *C. perniciosa* (14) and measures of resistance obtained from greenhouse screening was high for all resistance measures except PPS (Fig. 3). The strength of correlations between all measures and field resistance diminished when extreme genotypes (SCA 6 and 12; UF 11 and JA 6/4 [POU]) were removed from the analysis, and it became insignificant for PPS and only moderately significant for PPB and PSB. However, correlations remained high (0.8 to 0.9) for all other measures of resistance.

Optimum sample size for screening open-pollinated progenies. Strong parent-offspring regressions for certain resistance measures suggested that open-pollinated progenies can be conveniently used to predict parental performance. It is important, however, to determine optimal progeny sample sizes required to obtain a precise and accurate measure of parental performance. ANOVA of data generated through iterative random sampling of varying progeny sample sizes showed that the coefficient of variation (%) decreased with increasing sample size up to a sample size of 20 for incubation period and 15 for broom-base diameter (Table 4). Consequently, differentiation indices increased with increasing sample size up to 20 for both incubation period and broom-base diameter. When the extreme values were removed, optimum differentiation was achieved at n = 25 instead of n = 20 for incubation period.

The accuracy of progeny sample means was tested by the correlation of sample means to the estimate of the true mean based on all 90 observations (Table 4). The correlation over five iterative random samplings was high (0.96 to 0.99) for incubation period at n = 20 and did not improve further with increases in progeny sample size. Correlation with the true mean reached a plateau at n = 30 for incubation period and n = 20 for broom base diameter, when the extreme genotypes were removed from the analysis. The accuracy of means was also tested by regression on parental means. The R^2 values did not increase for sample sizes greater than 20 for incubation period nor for sample sizes greater than 15 for broom-base diameter.

DISCUSSION

Mechanisms of resistance. Resistance to witches'-broom disease may exist in various forms: successful pathogen entry and establishment may be manifested as percentage of plants showing symptoms (PPS) or brooms (PPB), rate of pathogen establishment or pathogen growth recorded as incubation period, or extent of pathogen ramification or growth and host response exhibited as symptom severity (stem swelling, broom size). Reproductive capacity manifested as sporulation intensity and/or sporulation period (broom size).

The strong correlation between PPS (successful pathogen establishment) and incubation period (rate of pathogen growth) when all the genotypes were included, but not when the extreme resistances were removed, suggests that these two manifestations of resistance are different but may also coexist, as in the SCA genotypes. The strong correlation between incubation period and stem swelling or broom size in the genotypes as well as progenies suggests that these are related and may be measures of pathogen growth, as suggested by Zadoks (33) and Wheeler (29).

Hence it appears that the cacao-*Crinipellis* pathosystem may have two components of resistance, broom frequency (PPB) and pathogen growth, which can be measured by incubation period, stem swelling, or any of the measures of broom size. Among these, incubation period and broom-base diameter were the most effective in differentiating various levels of resistance. Furthermore, they showed good heritability as revealed by strong parent-offspring regressions. It is therefore recommended that incubation period or broom base diameter should form the basis of assessment of pathogen growth.

The moderate correlation between PPB and incubation period even when the extreme susceptible and resistant genotypes were removed suggests that incubation period can give some indication of broom

Table 2. Various measures of resistance in *Theobroma cacao* for accuracy (correlation of single values to true mean) and ability to differentiate among clonal genotypes based on single plant experimental units

Measures of resistance[a]	Range	CV (%)	ID[b]	Sig.[c]	Correlation[d]
Inc. period (days)	14-29	10.4	8.1	***	0.93-0.96
TBRM (days)	21-40	13.5	5	***	0.81-0.92
PPS		–	–		–
PPB		–	–		–
PSB		–	–		–
ST-SW (mm)	2.8-10.2	21.1	5.5	***	0.78-0.96
BR-DIA (mm)	6.8-15.3	12.7	6.1	***	0.75-0.95
BR-LEN (mm)	76-199	18.1	4.6	***	0.61-0.92
BR-DW (g)	1.2-5.8	14.3	8.1	***	0.92-0.99

[a] Inc. period, incubation period; TBRM, time taken to broom development; PPS, percentage of plants showing symptoms; PPB, percentage of plants showing brooms; PSB, percentage of plants showing symptoms developing into brooms; ST-SW, stem swelling; BR-DIA, broom-base diameter; BR-LEN, broom length; BR-DW, broom dry weight.
[b] Index of differentiation for measures of resistance was calculated by dividing range over $LSD_{0.05}$.
[c] ***, highly significant ($P < 0.001$).
[d] Correlation of single values to true means. True means were based on nine observations.

Table 3. Correlations among various measures of resistance to *Crinipellis perniciosa* among progenies (full data set) and parental genotypes (extremes removed) in *Theobroma cacao*

	Measures of resistance[a]	Inc. period (days)	TBRM (days)	PPS	PPB	PSB	ST-SW (mm)	BR-DIA (mm)	BR-LEN (mm)	BR-DW (g)
Progenies	Inc. period (days)	1[b]								
	TBRM (days)	0.93	1							
	PPS	–0.08	0.08	1						
	PPB	0.04	0.05	0.57	1					
	PSB	–0.08	–0.25	–0.78	–0.01	1				
	ST-SW (mm)	–0.84	–0.79	–0.05	–0.12	0.09	1			
	BR-DIA (mm)	–0.85	–0.81	–0.04	–0.13	0.05	0.99	1		
	BR-LEN (mm)	–0.72	–0.6	–0.03	–0.25	0.08	0.54	0.53	1	
	BR-DW (g)	–0.66	–0.56	0.03	–0.39	–0.11	0.65	0.66	0.86	1
Parents	Inc. period (days)	1								
	TBRM (days)	0.81	1							
	PPS	0.29	0.09	1						
	PPB	–0.55	–0.35	0.3	1					
	PSB	–0.58	–0.41	–0.16	0.89	1				
	ST-SW (mm)	–0.77	–0.56	–0.25	0.52	0.66	1			
	BR-DIA (mm)	–0.8	–0.6	–0.2	0.55	0.66	0.99	1		
	BR-LEN (mm)	–0.62	–0.77	–0.17	0.5	0.6	0.56	0.58	1	
	BR-DW (g)	–0.35	–0.26	0.12	0.55	0.51	0.53	0.53	0.17	1

[a] Inc. period, incubation period; TBRM, time taken to broom development; PPS, percentage of plants showing symptoms; PPB, percentage of plants showing brooms; PSB, percentage of plants showing symptoms developing into brooms; ST-SW, stem swelling; BR-DIA, broom-base diameter; BR-LEN, broom length; BR-DW, broom dry weight.
[b] Correlation coefficients were calculated on the original scale for the progenies (3 replications; n = 39) and for the parents (after removing the extreme genotypes) based on 10 clones (3 replications; n = 30).

frequency as well. This moderate correlation could be explained by the nature of symptom development in response to *Crinipellis* infection. Cronshaw and Evans (3) concluded that a terminal broom developed if the infection site was close to the meristematic growing tip and if the fungus can colonize the meristematic tissue before it grows past the susceptible stage. Many studies, including those of Evans and Bastos (5) and Danquah (4), have shown that basidiospores have slower germ tube growth in extracts from SCA 6 than in extracts from susceptible genotypes. The reduced rate of conversion of swellings to brooms in SCA and IMC genotypes suggests that colonization of meristematic regions may have been delayed in these genotypes. It is equally possible that colonization may not have been sufficient to elicit broom development. Thus pathogen growth may account at least partly for broom frequency.

According to Evans and Bastos (5), greater ramification of the fungus in susceptible host tissues may cause greater cell leakiness or compartmentalization and elicit a greater hormonal response, which may account for the larger brooms. Evidence for limited hormonal response can be seen in the extremely small brooms produced in the highly resistant SCA 6 and SCA 12 and the moderately small brooms produced in the moderately resistant IMC 57 and IMC 67 as compared to susceptible genotypes.

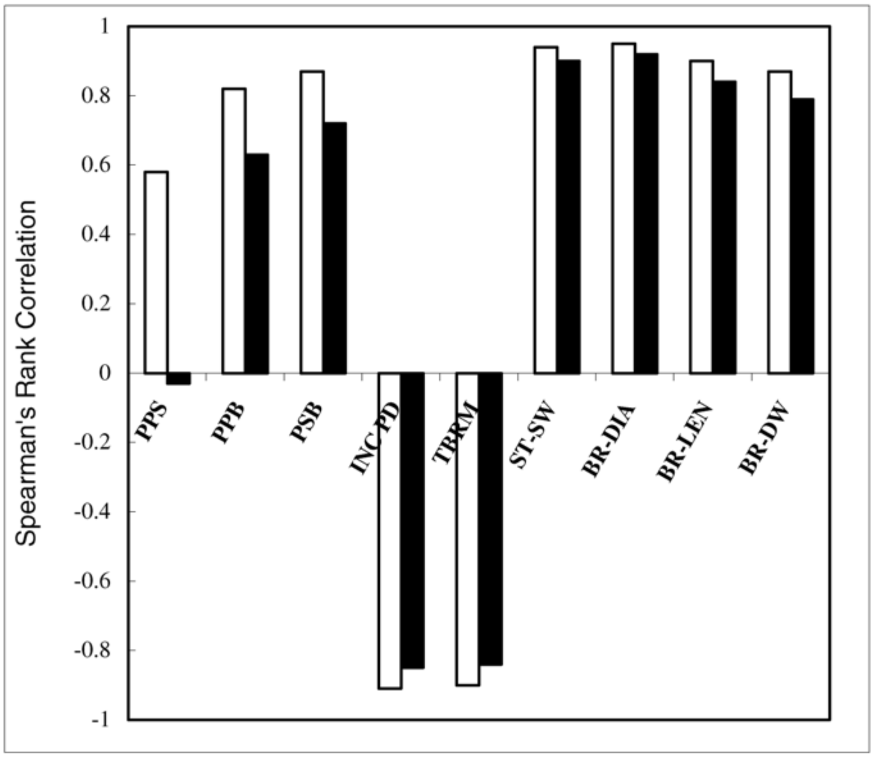

Fig. 3. Spearman's rank correlation for various measures of resistance (from this study) against field resistance (14) with the entire data set (open bars) and with extreme susceptible (UF11 and JA6/4) and extreme resistant (SCA 6 and SCA 12) genotypes removed (solid bars) from the analysis. INC PD, incubation period (days); TBRM, time taken to broom development; PPS, percentage of plants showing symptoms; PPB, percentage of plants showing brooms; PSB, percentage of plants showing symptoms developing into brooms; ST-SW, stem swelling; BR-DIA, broom-base diameter; BR-LEN, broom length.

Identifying resistance in breeding populations. Zadoks (32) suggested that identifying resistance to witches'-broom disease in segregating populations requires a screening method that does not allow escapes and a resistance measure that is precise, accurate, heritable (based on individual plant measurements), and capable of differentiating among intermediary levels of resistance. While most measures except PPS, PPB, and PSB had a good differentiation index and could be obtained from individual plants, only incubation period had high heritability and was able to differentiate intermediary levels of resistance. The high correlation between single-plant values of incubation period to the true mean and to progeny mean indicate that incubation period can be used effectively to screen seedling populations of cacao for witches'-broom resistance.

Evidence from this study suggests that there may be two mechanisms of resistance: one at the point of entry or establishment (PPS, PPB) and the other during pathogen growth. If true, a two-stage screening process will be effective in assessing and utilizing resistance. Incubation period is highly correlated to broom size and hence is most effective for identifying resistance to pathogen growth; however, it is only moderately correlated to mechanisms at the entry or establishment level, which determines broom frequency. It may be useful, therefore, to identify superior families based on PPS or PPB before selecting individual plants using incubation period. Such early screening methods could reduce cost of cacao breeding pro-

Table 4. Optimum sample sizes for assessing segregating populations of *Theobroma cacao* for resistance to *Crinipellis perniciosa* using incubation period and broom-base diameter

Character	Sample size	Correlation to true mean[a]: Range	Correlation to true mean[a]: Extreme removed	Regression to parental mean (R^2)	ANOVA (significance)[b]	CV (%)	Index of differentiation: All genotypes	Index of differentiation: Extremes removed[c]
Incubation period	5	0.71-0.85	0.00-0.63	0.59	***	10.41	3.55	0.84
	10	0.93-0.95	0.69-0.90	0.70	***	5.46	6.74	2.80
	15	0.88-0.98	0.74-0.85	0.74	***	5.49	6.21	2.77
	20	0.96-0.99	0.89-0.96	0.84	***	3.71	10.60	4.99
	25	0.96-0.99	0.78-0.98	0.85	***	3.18	12.28	6.35
	30	0.97-0.99	0.97-0.98	0.87	***	4.13	11.08	10.90
Broom-base diameter	5	0.85-0.88	0.81-0.83	0.65	***	8.43	4.58	3.43
	10	0.88-0.96	0.70-0.72	0.59	***	6.31	4.42	2.73
	15	0.88-0.98	0.77-0.96	0.76	***	5.24	5.93	3.85
	20	0.70-0.95	0.87-0.89	0.64	***	5.46	5.73	5.17
	25	0.94-0.96	0.84-0.95	0.66	***	4.81	7.67	4.35
	30	0.88-0.98	0.74-0.97	0.72	***	5.52	6.18	3.71

[a] Range of correlation to true mean was determined based on correlation of the true mean to sample means over 10 iterative samplings.
[b] ***, highly significant ($P < 0.001$).
[c] Genotypes/progenies representing SCA 6, SCA 12, UF 11, and JA 6/4 were removed from the analysis.

grams by allowing susceptible progeny to be discarded at the seedling stage.

The study also showed that most of the measures of resistance correlated well with field resistance. However, when the extreme levels of resistance to witches'-broom found in SCA 6 and SCA 12 were omitted from the analysis, there was no significant correlation between PPS and field resistance. This further confirms the previous suggestion that resistance to pathogen entry, manifested as PPS, may be peculiar to SCA genotypes. Bartley, as cited in Purdy and Schmidt (20), reported that although the clones and hybrids of SCA 6 and SCA 12 have performed differentially at various times and locations, they have shown reduced disease incidence in many situations.

Identifying resistance in germplasm collections. Currently, most germplasm collections are screened for resistance using field assessment/observations (14,18) or the automated spray inoculation system of Purdy et al. (21) using vegetatively propagated cacao plants. The latter system has been extensively tested in Brazil, Ecuador, Trinidad, and Venezuela with highly variable results (18). The attractiveness of the system is its high throughput of genotypes that can be inoculated within a short period of time. The limitations of this method include lack of repeatability, a complex nature of symptoms requiring a subjective scoring system, unknown heritability and poor differentiation between levels of resistance, and the high percentage of escapes that can result due to a number of undefined factors. Further, a major limiting step in the process is the grafting of plants, which requires space, time, and skill.

The agar-droplet method used in this study eliminates escapes (26), and the use of incubation period as a measure of resistance is simple, precise, accurate, and heritable. High heritability will allow the prediction of parental resistance based on screening of 20 open-pollinated seedling progeny, although maximum differentiation of intermediate levels may require a larger sample size of 30. Predictability can be further improved by developing top cross progenies. The results suggest that progeny screening using incubation period will provide a good prediction of parental resistance with less time, space, effort, and skill.

In conclusion, the results demonstrate that incubation period is a good measure of resistance. Most importantly, it is highly correlated to broom size and moderately correlated to broom frequency. Secondly, it provides the most precise and repeatable measure of resistance and consequently best differentiates between levels of resistance. Thirdly, it provides an accurate assessment of resistance even on a single plant basis. Fourthly, this measure shows a strong parent-offspring regression and a strong rank correlation with field resistance.

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