An Inoculation Method for Evaluating Resistance of Cacao to *Crinipellis perniciosa*

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ABSTRACT

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Methods are described to collect and store basidiospores of *Crinipellis perniciosa* and to inoculate seedlings of cacao (*Theobroma cacao*) for evaluation of resistance to witches'-broom disease. Basidiospores of *C. perniciosa* collected overnight in a 16% glycerol solution (containing buffer and Tween 20) remained viable but did not germinate until the collecting solution was diluted to 3% glycerol. Basidiospores freshly collected or stored in the collecting solution for 1 week at 4°C, or stored in liquid nitrogen for up to 18 months remained viable and, after application to cacao seedlings, caused witches'-broom. Witches'-broom developed on inoculated susceptible seedlings placed in a dew chamber for 4 to 6 h at 25°C, but did not develop on seedlings after 2 to 4 h of wetness. Maximal percentages of seedlings became diseased with 15 h of wetness. Differences in percentage of witches'-broom were evident among susceptible and putatively resistant cacao families inoculated with basidiospore concentrations of 10,000 and 12,500 spores per ml; however, higher concentrations (25,000 to 100,000 per ml) resulted in nearly 100% disease. Likewise, inoculation of flushes with large succulent leaves resulted in high percentages of disease in all families. Discrimination among families by percentages of diseased seedlings was achieved when small (0.3 to 1.5 cm) leaves were inoculated. Procedures for basidiospore collection and storage, inoculation of cacao seedlings, and control of pre- and postinoculation conditions support the effective and efficient screening of cacao for resistance to witches'-broom disease.

Additional keyword: cocoa

Witches'-broom disease of cacao (*Theobroma cacao* L.), caused by the basidiomycete *Crinipellis perniciosa* (Stahel) Singer occurs in all countries of South America where cacao is grown, and also occurs in Grenada, Panama, Trinidad-Tobago, and St. Vincent (5). The disease causes great losses in some of these areas and poses a threat to cacao in other locations currently free of witches'-broom disease. Phytosanitation and chemical control offer some potential for control of witches'-broom (6), but both are costly and labor intensive, and least likely to be employed when cacao bean prices are low. Also, the efficacy of both practices is greatly subject to weather conditions, especially rain. Potentially, host resistance offers a biologically sound, economically feasible, and environmentally safe management practice to control witches'-broom.

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Breeding for resistance to witches'-broom has been inhibited by the lack of an appropriate inoculation procedure for a large-scale resistance screening program. Two methods of inoculation have been reported: the Holliday method (4), in which germinated seedlings are immersed in a basidiospore suspension; and the agar-block method (2), in which basidiospores are collected on the surface of agar, blocks of which are then affixed to susceptible cacao plant parts, covered with moist cotton, and wrapped in foil or plastic. These methods result in infection of cacao, but both methods lack attributes to develop a discriminative program designed to screen large numbers of progeny rapidly and dependably. In addition, neither method is designed to simulate natural inoculation, facilitate control of inoculum concentration, or effectively solve the logistics of inoculum collection, storage, coordination of susceptible tissue and inoculum availability, and quality control required in a large-scale screening program.

The objective of this study was to develop an inoculation method that would support a large-scale resistance screening program for cacao/witches'-broom. This method must be efficient and dependable, allow for standardization and flexibility of inoculum concentration, and be compatible with quality control and automation. A successful system has been developed at the Rust Screening Center of the U.S. Department of Agriculture, Forest Service in Asheville, N.C., for fusiform rust of southern pines, caused by *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Hedge. & N. Hunt) Burdsall & G. Snow (1). The concentrated basidiospore spray (CBS) method of inoculating seedlings (10) is used in a system that provides control of (i) plant growth, before and after inoculation, (ii) inoculum concentration, (iii) incubation conditions during and after inoculation, and (iv) data collection and analyses.

We sought to adapt the CBS-rust procedures to an inoculation method for *Crinipellis perniciosa*/cacao. If successful, this method would greatly enhance the testing of cacao for resistance to *C. perniciosa* and aid in the search for resistant materials.

METHODS AND MATERIALS

Plant material. All plant materials were obtained from CATIE, Turrialba, Costa Rica, and INIAP, Estación Experimental Tropical Pichilingue, Quevedo, Ecuador. Included were seed from open-pollinated clones as well as seed from clones or crosses of clones that are considered to be resistant (SCA 6 × SIL 1, SCA 12 × SIL 1, and EET 233), or susceptible (EET 19, EET 381, EET 382, EET 400, and Catongo) to *Crinipellis perniciosa*. Seeds were planted in 6-cm^2 peat pots with Metromix 500 Potting Mix (Grace Horticultural Products, Cambridge, Mass.). Plants were fertilized once a week with a complete fertilizer, Peter's General Purpose Fertilizer (Peter's Fertilizer Products, Fogelsville, Pa.). Seedlings were grown at 21 to 30°C in a greenhouse with 73% shade provided by woven plastic cloth. Typical flushes (2 to 4 leaves ≤1.5 cm) on 3- to 4-week-old seedlings and axillary flushes induced by pruning 2- to 4-month-old seedlings above the first two leaves were shown previously to be susceptible tissues (3), and were selected for inoculation unless otherwise stated. On older seedlings, the distal portion of the two mature, dark green leaves left on the pruned seedlings were removed perpendicular to the midrib 1 day before inoculation. Flushes induced by pruning 5- to 6-week-old seedlings above the cotyledons were also inoculated.

Inoculum source, preparation and application. Basidiocarps of *C. perniciosa*

developed on brooms from Ecuador, Trinidad, Venezuela, and Colombia in a Plexiglas moist chamber located in a greenhouse with alternating periods (8 to 12 h) of wetting and drying (12,13). Pilei were removed from basidiocarps and affixed with petroleum jelly to the inside cover of a petri plate placed over a 250-ml beaker containing 50 to 60 ml of a collection solution (described below) and placed on a magnetic stir plate. The solution was maintained at 21 to 24°C and stirred continuously during the basidiospore collecting period, usually overnight.

The spore concentration in the collection solution was determined by 10 hemacytometer counts. The collected basidiospore suspension was diluted with additional collection solution to prepare a stock suspension containing 65,000 to 750,000 basidiospores per ml. To allow basidiospores to germinate, 4.33 parts 2-(*N*-morpholino)ethanesulfonic acid (MES) with Tween 20 was added to each part of stock suspension to reduce the glycerol concentration to 3%. To reduce the number of basidiospores to a predetermined concentration, 3% glycerol in MES with Tween 20 was added. Basidiospore germination was consistently higher than 90% using this dilution method.

Germination percentages of basidiospores stored in the 16% glycerol collection solution were determined by placing a drop of diluted basidiospore suspension on 2.0% water agar in 60-mm-diameter petri plates and counting germinated basidiospores in a random sample of 200 basidiospores.

Approximately 0.6 to 0.8 ml of the inoculum suspension was sprayed on each new flush of cacao seedlings with a handheld, CO_2-powered atomizer. Inoculated seedlings were immediately transferred to a dew chamber (100% relative humidity [RH]) and incubated at 25°C. Unless otherwise noted, inoculated plants were incubated for 24 h, and then placed in a greenhouse at 21 to 30°C.

Basidiospore collection solution. A collection solution for basidiospores of *C. perniciosa* was developed by trial and error and was composed of 16% glycerol in 0.01 M MES buffer, pH 6.1, and 0.01% Tween 20. Tween 20 was added to the solution after autoclaving to reduce surface tension on inoculated plant surfaces and to aid uniform distribution of basidiospores in the suspension. Basidiospore viability was significantly reduced when Tween 20 was added prior to autoclaving. Appropriate concentrations of glycerol, MES buffer, and Tween 20 were determined from germination and germ tube elongation of basidiospores collected in solutions. Suspensions were incubated in the laboratory for 6 to 9 h at 24 to 29°C, stained with lactophenol-trypan blue, and placed on 1% water agar. At least 200 spores per suspension with four replications were observed under the microscope to estimate the percentage of germination. Twenty germ tubes were measured for each suspension and replication.

Basidiospore storage. Apical flushes on seedlings of susceptible and resistant families were inoculated with suspensions of basidiospores (75,000 viable spores per ml, Ecuador source) stored for 0, 2, 3, and 7 days in the collecting solution at 4°C. Groups of 22 to 99 seedlings per family were inoculated on four dates.

Basidiospores were collected as described, filtered through a 0.25-mm Millipore filter, mixed 1:1 with 8.5% autoclaved skim milk in 10% glycerol, and placed in 2-ml cryogenic vials that were submerged in liquid nitrogen. Suspensions were thawed at 37°C immediately prior to inoculation. Cotyledon flushes on EET 400 seedlings were inoculated on three dates with freshly collected basidiospores (Ecuador source), and with basidiospores (Trinidad and Venezuela sources) stored for 18 months in liquid nitrogen. On each date, 14 to 20 seedlings (20 to 40 flushes) were sprayed with fresh or stored suspension of 10,000 and 25,0000 spores per ml.

Wetness duration. To determine the optimal wetness duration for inoculated seedlings, axillary flushes of open- and self-pollinated Catongo, open-pollinated EET 400, SCA 6, and SCA 12 were inoculated (200,000 spores per ml, Trinidad source) and immediately placed in a dew chamber at 25°C with 100% RH. Additional seedlings of SCA 6 and SCA 12 were inoculated with 100,000 spores per ml (Trinidad source). Eighty inoculated seedlings of each family were placed in the chamber and 10 seedlings containing 15 to 20 flushes were removed every 2 to 3 h up

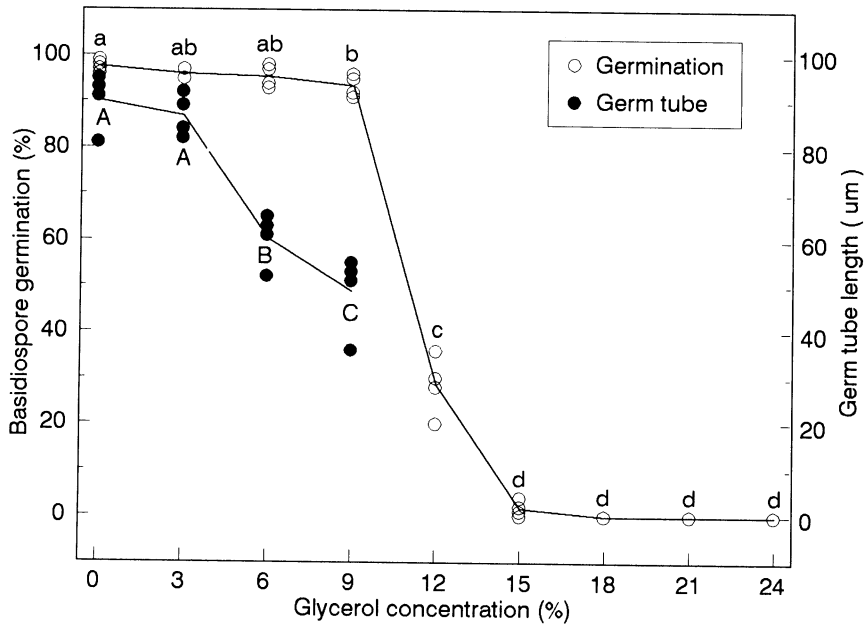

Fig. 1. Germination percentage and germ tube length after 9 h of basidiospores of *Crinipellis perniciosa* in solutions of 0 to 24% glycerol with 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. Curve is fit to treatment means. Germination percentages with the same lower case letter and germ tube length with the same upper case letter are not significantly different ($P = 0.05$).

Table 1. Numbers and percentages of seedlings of susceptible and resistant cacao families, inoculated with two different concentrations of basidiospores of *Crinipellis perniciosa*

	Susceptible families				Resistant families		
Basidiospore concentrations[x]	**EET 19**	**EET 381**	**EET 382**	**EET 233**	**SCA 12 × SIL 1**	**SCA 6 × SIL 1**	**Total**
25,000 per ml[y]	88 (100%)	128 (100%)	143 (99.3%)	20 (100%)	142 (100%)	156 (100%)	677 (99.8%)
75,000 per ml[z]	267 (100%)			160 (100%)			427 (100%)
Total							1,104 (99.9%)

[x] Spores were collected overnight in the collection solution (16% glycerol with 2-(*N*-morpholino)ethanesulfonic acid (MES) 0.01, pH 6.1, buffer and Tween 20) and diluted to 3% glycerol for inoculations.
[y] Average of five inoculation dates (replications) except for families EET 19 and EET 233, which had four and two replications, respectively.
[z] Average of four and three inoculation dates (replications) for families EET 19 and EET 233, respectively.

to 15 h and at 24 h after inoculation. Upon removal from the chamber, leaf surface moisture dissipated rapidly and plants were placed in the greenhouse.

Inoculum concentration. Axillary flushes on seedlings of families SCA 6, SCA 12, and Catongo were inoculated with fresh basidiospore suspensions (Ecuador source) of three concentrations: 12,500, 37,500, and 75,000 spores per ml. Fifteen to 25 seedlings (20 to 40 flushes) per family per basidiospore concentration were inoculated on four dates. Mature leaves were removed as previously indicated 1 day before inoculation.

Leaf length. Apical flushes on seedlings from SCA 6, SCA 12, and EET 400 were inoculated with fresh suspensions (Ecuador source) at a concentration of 10,000 basidiospores per ml. During the development of the first flush, seedlings were divided into three groups: seedlings with leaf lengths between 0.3 and 1.5 cm, 1.8 and 3.0 cm, and 3.3 and 5.0 cm. Sixteen to 25 seedlings per family per flush size were inoculated on four different dates.

Data collection and analysis. Disease incidence (the percentage of seedlings or flushes that developed witches'-brooms or exhibited swellings on the pulvini or petioles) was evaluated 3 months after inoculation. Seedlings were examined twice a week to identify flush mortality unassociated with witches'-broom disease. Infection percentage was transformed to arcsine (7) prior to analyses of variance.

The experimental design used in all inoculation experiments was a randomized complete block, in which inoculation dates were considered as blocks (replications). Means were compared with Duncan's multiple range test using the error mean square from the analysis of variance. A monomolecular curve (8) was fitted to the data from the incubation time experiments.

RESULTS

Basidiospore collection and storage. Glycerol at concentrations of 0 to 9% in MES plus 0.01% Tween 20 had no detrimental effect on basidiospore germination (Fig. 1). Germination decreased significantly as glycerol concentration was increased above 9% and spores did not germinate in solutions with more than 15% glycerol. Germ tube growth was not significantly inhibited by 3% glycerol, but as glycerol concentrations increased above 3%, germ tube growth was inhibited (Fig. 1).

Tween 20 at concentrations of 0 to 0.03% in MES plus 3% glycerol had no effect on basidiospore germination; concentrations >0.03% reduced germination (data not shown). Basidiospore germ tube growth was gradually reduced as the Tween 20 concentration was increased above 0.01%. Tween 20 added prior to autoclaving reduced germination significantly (data not shown).

Basidiospore suspensions collected overnight, diluted, and immediately sprayed on succulent apical flushes of cacao seedlings induced symptoms of witches'-broom on nearly 100% of both susceptible and resistant families at concentrations of 25,000 and 75,000 viable spores per ml (Table 1). Similarly, the same basidiospore suspensions (75,000 per ml) stored for 0, 2, 3, and 7 days prior to inoculation produced 97 to 100% disease on a susceptible (Catongo) and the putatively resistant (EET 233) family, regardless of the length of storage (Table 2). Basidiospores stored for 18 months in liquid nitrogen caused symptoms of witches'-broom in 86 and 95.3% of flushes at the cotyledonary nodes of seedlings of the susceptible family (EET 400) inoculated with 10,000 and 25,000 viable spores per ml, respectively (Table 3). Storage of basidiospores had no effect on subsequent disease incidence compared with freshly collected (unstored) basidiospores.

Wetness duration. Symptoms of witches'-broom did not appear on inoculated cacao seedlings after 2 and 4 h of wetness, but disease symptoms developed on inoculated seedlings after 6 h of wetness. A monomolecular curve fitted to the data shows that an average of 6 h of wetness at 25°C was required for infection to take place (Fig. 2). However, according to the model, infection may occur earlier or later depending on the variety, as illustrated by the susceptible Catongo Self and the resistant SCA 12. The percentage of seedlings exhibiting symptoms (diseased flushes) increased rapidly as wetness duration increased, reaching a maximum at 12 to 15 h. At the high inoculum concentrations of 100,000 (data not shown) and 200,000 spores ml, 85 to 95% of the flushes became infected in resistant and susceptible varieties subjected to 24 h of wetness.

Inoculum concentration. The percentages of diseased flushes on seedlings of three cacao families increased as the inoculum concentration increased from 12,500 to 37,500 to 75,000 viable basidiospores per ml (Table 4). Differences in disease percentages between susceptible and resis-

Table 2. Effect of short-term inoculum storage on the percentages of seedlings that became diseased in a susceptible (S) and a putatively resistant (R) cacao family spray-inoculated with a high concentration (75,000 per ml) of viable basidiospores of *Crinipellis perniciosa*

	EET 19 (S)		EET 233 (R)	
Storage time, days[z]	No. inoculated	Percent infected	No. inoculated	Percent infected
0	71	100	34	100
2	56	100	50	100
3	34	100	34	097
7	25	100	22	100

[z] Spores were collected overnight in the collection solution (16% glycerol with 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer 0.01 M, pH 6.1, and Tween 20) and then stored at 4°C, prior to dilution to 3% glycerol for inoculations.

Table 3. Percentages of infection by basidiospores of *Crinipellis perniciosa* after storage in liquid-nitrogenand inoculation of cotyledonary flushes of susceptible cacao family EET 400

	Inoculum concentration (viable basidiospores per ml)			
	10,000		25,000	
Storage time, months[x]	Plants inoculated	Percent infected	Plants inoculated	Percent infected
0 (fresh)	44	95.0 a[y,z]	90	95.4 a
18 (in liquid nitrogen)	45	86.0 a	76	95.3 a

[x] Spores collected overnight in a collecting solution (16% glycerol, 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, Tween 20) prior to dilution for inoculations or storage in liquid nitrogen.
[y] Average of four inoculation dates (replications).
[z] Means within columns followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 4. Effect of inoculum concentration on the percentages of diseased axillary flushes in susceptible (S) and resistant (R) cacao families spray-inoculated with basidiospores of *Crinipellis perniciosa*

	Inoculum concentration (basidiospores per ml)		
Family	12,500	37,500	75,000
Catongo (S)	88.8 a[z]	89.7 a	98.5 a
SCA 12 (R)	48.7 b	88.4 a	95.7 a
SCA 6 (R)	49.4 b	85.7 a	96.5 a

[z] Percentages are averages of four inoculation dates (replications). In each replication 20 to 40 flushes on 15 to 25 seedlings per family were evaluated. Means within columns followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

tant families were detected only at the lowest inoculum concentration. At 37,000 and 75,000 spores per ml, disease percentages were uniformly high in all families.

Leaf length. The percentage of diseased seedlings increased significantly as leaf length on inoculated flushes increased from 0.3 to 1.5 to 3.3 to 5.0 cm. Seedlings of the susceptible family, EET 400, developed high percentages of disease regardless of the flush leaf length when the seedlings were inoculated (Table 5). However, the percent disease on the resistant family (SCA 6) was significantly less than that on the EET 400 seedlings with small (0.3 to 1.5 cm) and intermediate size (1.8 to 3.0 cm) leaves. There was no difference in the percentage of diseased seedlings between susceptible and moderately resistant families when larger (3.3 to 5.0 cm) leaves were inoculated.

DISCUSSION

Cacao seedlings of susceptible and putatively resistant families were successfully infected with *Crinipellis perniciosa* when succulent flushes were sprayed with fresh basidiospores. However, fresh basidiospores are not always available when plant materials are in a stage of growth susceptible to infection. A method was needed to ensure that spores would be available whenever inoculation of plant materials was necessary; thus, our attention focused on collection and storage of basidiospores.

Procedures used to collect and store basidiospores of *Cronartium quercuum* f. sp. *fusiforme* (10) in a dilute acid solution were not successful with basidiospores of *C. perniciosa*. The dilute acid solution appeared to prevent germination of basidiospores even after dilution and rinsing. The 16% glycerol solution that contained MES buffer and Tween 20 prevented germination of basidiospores of *C. perniciosa* during collection, and basidiospore viability was maintained 0 to 7 days at 4°C and up to 18 months in liquid nitrogen storage. When the 16% glycerol/basidiospore suspension was diluted to 3% glycerol, basidiospores germinated normally and infection subsequent to inoculation occurred as if the basidiospores had been freshly collected. This information provides the flexibility needed to coordinate availability of susceptible cacao host tissue and inoculum required to screen large numbers of seedlings in a disease resistance screening program.

Following inoculation, successful infection occurred on seedlings exposed to as few as 4 to 6 h of wetness (100% RH) and reached a maximum after 12 to 14 h of wetness (100% RH). However, it is recommended that seedlings be kept in a moisture chamber at 25 to 27°C and 100% RH for 24 h to maintain leaf surface moisture to insure maximal germination and germ tube penetration. Frias et al. (3) reported that basidiospores of *C. perniciosa* required plant surface moisture for germination and penetration, and that interruptions in the free moisture period permanently stopped the infection process.

Inoculations with high concentrations of basidiospores (37,500 to 200,000 viable basidiospores per ml) resulted in nearly 100% infection of the seedlings in all families tested. However, differences in the percentage of diseased flushes and diseased seedlings were detected between susceptible and resistant families with inoculum concentrations of 12,500 spores per ml. Similar results have been reported for fusiform rust, i.e., discrimination among families in percentage of seedlings infected by *C. quercuum* f. sp. *fusiforme* is dependent on the inoculum concentration, and at very high spore concentrations almost all seedlings are infected (9).

Inoculations of large, succulent flushes on cacao resulted in uniformly high infection percentages in all families; discrimination between susceptible and resistant families occurred only when small flushes (leaves 0.5 to 1.3 cm in length) were inoculated. The reasons for reduced infection percentages on small flushes are unknown, but may be related to the trophic responses (3) of germ tubes toward infection sites (stomata) on flushes with fewer infection sites on small leaves, inoculum run-off and retention on flushes, small leaves may intercept fewer basidiospores, or other influences. Whatever the case, successful inoculation of small flushes is an advantage in a screening program because smaller plants are easier to handle and require less space.

Flush size, basidiospore concentration, and wetness duration significantly affected responses of cacao families to *Crinipellis perniciosa*. The procedures described to control these variables allow the rapid, efficient, and dependable inoculation and infection of cacao seedlings. These methods can be adapted to a large-scale screening program. The procedures can be automated to increase efficiency, but, more

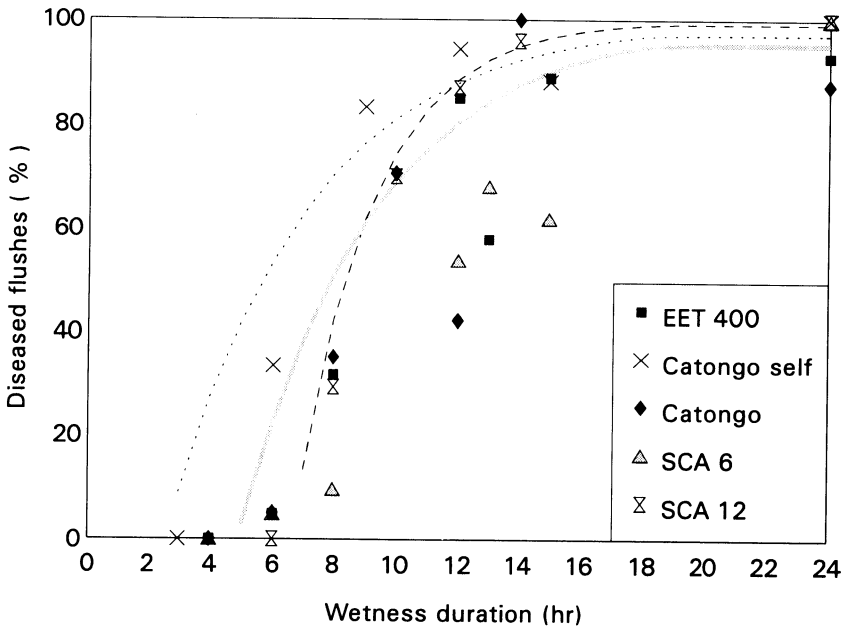

Fig. 2. Percentages of diseased flushes on seedlings of susceptible (EET 400, Catongo self, Catongo), and resistant cacao families (SCA 6, SCA 12) inoculated with basidiospores (200,000 per ml) of *Crinipellis perniciosa* and incubated in a dew chamber with 100% relative humidity for 0 to 24 h at 25°C.

Table 5. Effect of leaf length on the percentage of diseased seedlings in susceptible (S) and resistant (R) cacao families spray-inoculated with a basidiospore suspension (10,000 spores/ml) of *Crinipellis perniciosa*

Family	Leaf length (cm)		
	0.3 to 1.5	1.8 to 3.0	3.3 to 5.0
EET 400 (S)	97.2 a[z]	95.8 a	97.5 a
SCA 12 (R)	92.9 ab	96.1 a	97.5 a
SCA 6 (R)	80.8 b	85.8 b	95.0 a
Average	90.3 A	92.5 AB	96.8 B

[z] Percentages are means of four inoculation dates (replications), which were not significantly different from one another. Total seedlings inoculated (16 to 25 seedlings per family per leaf size) were 251, 235 and 221 for leaf sizes 0.3 to 1.5, 1.8 to 3.0, and 3.3 to 5.0, respectively. Means within columns followed by the same lower case letter, or means within row followed by the same upper case letters, are not significantly different according to Duncan's multiple range test ($P = 0.05$).

important, they provide quality control. It may be necessary in a practical screening program to manipulate the plant growth environment and the inoculum concentration to discriminate resistant and susceptible families; more detailed investigations will be needed to perfect quality control.

Subsequent to the completion of this research, and based in part on the results presented here, an automated system for inoculating seedlings, or vegetatively propagated plants, of cacao was installed at the Instituo Naciónal de Investigaciones Autonoma Agropecuarias, Estación Experimental Tropical "Pichilingue," Quevedo, Ecuador, and at the Comissão Executiva do Plano da Lavoura Cacaueira, Centro de Pesquisas do Cacau, Itabuna, Bahia, Brazil. At both locations, the automated system is being used to screen germ plasm of cacao for resistance to *Crinipellis perniciosa* to enhance their respective cacao improvement programs.

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