

AN INOCULATION METHOD TO EVALUATE RESISTANCE TO
WITCHES' BROOM DISEASE OF CACAO

BY

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To my parents, Arnolde and Consuelo,
to Minerva, Gustavo, and Roger

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TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGMENTS.....	iii
ABSTRACT.....	vi
CHAPTERS	
ONE INTRODUCTION.....	1
TWO THE INFECTION PROCESS OF <u>Crinipellis</u> <u>perniciosa</u> ON VEGETATIVE FLUSHES OF CACAO.....	7
Introduction.....	7
Materials and Methods.....	9
Results.....	16
Discussion.....	26
THREE DEVELOPMENT OF AN INOCULATION METHOD FOR EVALUATING RESISTANCE TO WITCHES' BROOM DISEASE OF CACAO.....	33
Introduction.....	33
Materials and Methods.....	36
Results.....	48
Discussion.....	59
FOUR EVALUATION OF RESISTANCE TO WITCHES' BROOM DISEASE IN CACAO SEEDLINGS AND TREES IN ECUADOR.....	69
Introduction.....	69
Materials and Methods.....	71
Results.....	78
Discussion.....	83
FIVE SUMMARY AND CONCLUSIONS.....	93
APPENDIX.....	98
LITERATURE CITED.....	104
BIOGRAPHICAL SKETCH.....	111

Abstract of Dissertation Presented to the Graduated School
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Previously, evaluation of cacao (Theobroma cacao L.)
resistance to the witches' broom disease, by artificial
inoculations with basidiospores of Crinipellis pernicioso
(Stahel) Singer, produced unreliable results. The objective
of this study was to develop a reliable inoculation method.

Basic aspects of the infection process important to
inoculation procedures were studied. Vegetative flushes of
cacao inoculated with C. pernicioso basidiospores were
examined using fluorescence microscopy. Basidiospores
deposited on dry plant surfaces plasmolyzed. Germination
and host infection occurred when spores were deposited on
wet flushes and wetness was maintained for a minimum of 4-6
hr. Basidiospore germ tube tropism towards stomata and the
base of fallen or collapsed multicellular trichomes was
observed. Host penetration and subsequent formation of
subepidermal vesicles occurred within 12 hr after

inoculation. Intercellular hyphae developed from substomatal vesicles in some infection sites, while in others the pathogen failed to colonize host tissue. This failure was associated with a change in fluorescence of host cells at and around the infection sites.

Basidiospores were collected overnight in 15% glycerol solutions and stored for 0 (fresh) to 18 months. Basidiospore suspensions of known concentration were sprayed on cacao plants with young vegetative flushes and incubated for 24 hr in a dew, mist, or humidity chamber. Inoculations conducted in Gainesville FL produced consistent results. Differences among progeny from putative resistant and susceptible clones were detected with suspensions containing 10,000-12,500 basidiospores/ml, higher spore concentrations resulted in similar disease incidence on resistant and susceptible families. Fresh and stored basidiospore suspensions produced similar percentages of disease on inoculated seedlings.

Significant differences in resistance among cacao families inoculated in Ecuador were detected with inoculum concentrations of 25,000-150,000 basidiospores/ml. The disease percentages varied among inoculation dates. Differences in resistance among 4-year-old trees inoculated in the field were also detected. Approximately 50 % of the trees with low percentages of diseased flushes (<6%) under field conditions were highly susceptible to artificial inoculation.

The inoculation method has the potential to be incorporated into breeding programs to select trees, evaluate cacao families and screen large populations of seedlings for resistance to witches' broom disease.

CHAPTER ONE
Introduction

Production of cacao (Theobroma cacao L.) is severely affected by witches' broom disease (WB) in northern South American countries and some Caribbean Islands. The rapid decrease in production and exportation of cacao in Ecuador, Surinam, and Trinidad after the appearance of WB illustrates the economic importance of this disease (Baker and Holliday 1957). In Trinidad, pod losses of 10-68% due to WB have been reported in experimental plots (Baker and Dale 1944). Evans et al. (1977) sampled several cacao producing areas in Ecuador and found that the combined pod losses produced by WB and monilia pod rot vary from 27-60%.

The pathogen that causes WB disease, Crinipellis pernicioso (Stahel) Singer, affects young vegetative flushes and flower cushions by inducing abnormal proliferation (hyperplasia and hypertrophy) and necrosis, which give rise to the characteristic witches' broom symptoms. Young pods affected by the disease develop abnormally and/or become necrotic, losing their commercial value. Five to eight months after infection, basidiocarps are produced on the necrotic tissues; air-borne basidiospores are released and transported to young flushes or pods where infection occurs (Evans 1982).

There is no efficient and economic method to control WB. Rapidly growing tissues, which are most susceptible to the disease, can not be continuously covered with protective fungicides. Reduction of disease incidence can be achieved by frequent applications and then only of easily accessible plant parts, such as seedling flushes or hand pollinated pods set on the trunk of the tree (Cronshaw 1979, Watson and Mulligan 1986). Coverage of all susceptible sites on adult trees seems impractical, but promising results have been obtained with systemic fungicides (Cifuentes et al. 1981, Laker et al. 1987a). Researchers in several countries are screening both systemic and protective fungicides to improve this potential method of control (Lass and Rudgard 1985).

Management of WB is limited practically to cultural practices. Removal of brooms from the plantation maintains the disease at low levels (Stahel 1919, Aranzazu and Jaramillo 1984, Rudgard 1983). However, this method is laborious, expensive, and impractical in areas of high WB incidence (Watson and Mulligan 1986, Thorold 1943a, 1943b). In addition, aerial inoculum from unmanaged areas may negate the effect of this practice. Hand pollination timed to have fruit set in the dry season has also reduced disease incidence in pods, but adoption of this practice by cacao growers seems unlikely (Watson and Mulligan 1986).

Host resistance is thought to be potentially the best long term alternative to reduce WB incidence (Stahel 1915, Bartley 1986). However, there has been little progress

towards the development of resistant cacao varieties. In the late 1940s, progeny from cacao populations that showed variability in their response to the disease were tested for resistance (Desrosiers et al. 1955, Ampuero 1960). Seedlings that survived natural infection and inoculation with basidiospores of *C. perniciosus* were grown in experimental plots. Progeny from these plants had significantly fewer diseased seedlings than progeny from non-screened plants when exposed to natural infection and inoculation (Desrosiers et al. 1955). Based on these results, Desrosiers et al. suggested recurrent selection to improve cacao resistance to WB. However this strategy was not adopted in breeding programs.

The selection of clones that showed little or no infection in the field (Pound 1938, Soria 1970) prompted the initiation of interclonal hybridization programs in Trinidad and Ecuador in the 1940s and 1950s to improve WB resistance in commercially acceptable clones (Ampuero and Alvarado 1960, Bartley 1977). Resistant clones such as SCA 6 and 12; Silecia 1, 5, and 8; and IMC 67 produced progeny that had low disease incidence during their first years in the field, but disease incidence increased with time; 10 years after planting, over 80% of SCA 6 progeny were infected in Trinidad cacao fields (Bartley 1977, 1986). In Ecuador, disease incidence on SCA 6 and 12 clones and their progeny increased within 7 years to a level at which the materials were no longer considered resistant (INIAP 1973, Chalmers

1972). A similar situation is occurring in other countries where putative resistant materials are used (Abdu-F et al. 1984, Restrepo 1981, Capriles 1977).

Two hypotheses have been proposed to explain the "breakdown" in resistance to WB. One proposed the presence of pathogenic races capable of infecting the resistant material (Bartley 1967a, 1986); the other hypothesis suggests the increase in amount of inoculum and availability of infection sites (vegetative and reproductive flushes) as the main factors responsible for the change in disease incidence (Robinson 1978). Experimental evidence has not been obtained to support either of these hypotheses.

Differences in the type of WB symptoms produced by inoculation of seedlings with C. perniciosa isolates from different countries have been reported (Wheeler and Mepsted 1981). However, interaction between isolates and cacao varieties or clones has not been observed. In recent inoculation experiments conducted in Trinidad, ranking of clones according to their resistance to WB was the same as that reported in 1950s (Laker et al. 1987b). This provides some evidence to suggest that the pathogen population is similar to that previously encountered by the host.

Inoculation of seedlings with C. perniciosa basidiospores, according to Holliday's method (Holliday 1955), was used to evaluate the performance of progeny from resistant clones. Expectations were that artificial inoculations would facilitate selection of resistant trees

by replacing the long term (5-10 years) field evaluations. However, results obtained with this method were inconsistent. Repeated inoculations of the same material gave variable responses. Differences in the percentages of diseased seedlings between progenies from resistant and susceptible clones were sometimes detected, but nearly 100% disease in both progenies was also common (INIAP 1982/83). In addition, seedlings that survived inoculation became infected in the field (Bartley 1977). These observations limited the use of Holliday's inoculation method in breeding programs. Other inoculation methods for the evaluation of resistance to WB are not reliable (Stahel 1915, Briton-Jones and Cheesman 1931, Baker and McKee 1943, Desrosiers et al. 1955) or have not been sufficiently tested to determine whether they can be incorporated into breeding schemes (Cronshaw and Evans 1978, Andebrhan 1983).

Inconsistent results obtained with artificial inoculation methods to date are probably the result of varying inoculum concentrations and suboptimal incubation conditions. Holliday's method (Holliday 1955) requires the use of suspensions with 200,000 basidiospores/ml; however, spores used to prepare the suspensions are collected on dry surfaces and may have only 10-50% germination (Medeiros and Rodrigues 1975, Baker and McKee 1943). Therefore, the concentration of viable basidiospores may vary between 20,000 to 100,000/ml. This variation could produced the inconsistent results obtained with Holliday's inoculation

method (Bartley 1958, 1959). In addition, Holliday's method requires dipping germinated seeds in basidiospore suspension and immediately sowing them in moist soil; in this case, physical and biological characteristics of the soil may affect basidiospore germination and thus chances for infection.

Breeders can not afford to spend the time, space, and labor required for field evaluations of WB resistance on large cacao populations. Therefore, development of more reliable inoculation methods seem essential to assure the production of resistant varieties. The inoculation technique used to screen pine seedling for resistance to fusiform rust (Anderson et al. 1982) was adapted in this study to evaluate WB resistance in cacao seedlings and trees. Modifications to the inoculation technique were based on the study of the infection process of C. perniciosa on vegetative flushes of cacao, and on the development of methods to collect and store basidiospores. The efficiency of the modified method was then evaluated in inoculations of selected cacao families and clones.

The objective of this research was to develop a reliable inoculation method with the potential to be incorporated into cacao breeding programs.

CHAPTER TWO
THE INFECTION PROCESS OF Crinipellis pernicios ON VEGETATIVE
FLUSHES OF CACAO

Introduction

Detailed knowledge of the infection process of Crinipellis pernicios (Stahel) Singer on cacao (Theobroma cacao L) is essential for the development of standard inoculation methods to evaluate the resistance of cacao varieties to the witches' broom disease. Such knowledge is also essential in clarifying the epidemiology of the disease. However, several aspects of the infection process are not well understood. For example, optimal conditions for in vivo germination of basidiospores have not been determined, and the mechanism of penetration has been observed only on leaves and pods (Stahel 1919, Cronshaw and Evans 1978, Sreenivasan 1987, Sreenivasan and Dabydeen 1987), even though petioles, pulvini, and stems are also susceptible to infection (Cronshaw and Evans 1978).

Factors affecting basidiospore germination have been studied only in vitro. Sparsely distributed basidiospores collected on dry glass surfaces immediately lost their viability, whereas more concentrated numbers of basidiospores survived for 15-20 hr (Stahel 1919). Basidiospores collected in buffer solutions or on agar had optimal germination at pH 6, and 25 C, respectively

(Medeiros and Rodrigues 1975b, Baker and Holliday 1957, Rocha 1983). The requirements for basidiospore germination on plant surfaces and, therefore, optimal incubation conditions for infection of inoculated plants are uncertain.

The mechanism of penetration has received the most attention. Germ tubes entering stomata on leaves and pods and colonizing the epicarp of young pods were first observed by Stahel (1919). Suarez (1977) and Aragundi (1982) failed to substantiate Stahel's observations. Cronshaw and Evans (1978) reported that *C. pernicios* penetrated the leaf epidermis directly, but occasional entrance through stomata was also observed. However, recent observations on penetration and colonization of young cacao leaves indicate that stomata are important infection sites (Sreenivasan 1987, Sreenivasan and Dabydeen 1987).

Penetration of vegetative flushes of cacao by *C. pernicios* has been observed only on leaves (Stahel 1919, Cronshaw and Evans 1978, Sreenivasan 1987, Sreenivasan and Dabydeen 1987). However typical WB symptoms were not obtained when leaves were inoculated with the agar block method (Cronshaw and Evans 1978). Thus, it is questionable that the penetration mechanisms observed on leaves occur on the other parts of the flush.

Observation of spore germination on plant surfaces, penetration mechanisms, and colonization of plant tissues by fungal pathogens is facilitated by the use of fluorescence microscopy (Patton and Johnson 1970, Rohringer et al. 1977,

Yigal et al. 1987). This technique has been used to study host responses to infection and how these responses may be related to disease resistance (Niks and Kuiper 1983, Luke et al. 1984). In this research, fluorescence microscopy was used to study germination of C. pernicioso basidiospores on plant surfaces, the penetration mechanisms on the different parts of vegetative flushes, and the reaction of the host to infection. The objective of this study was to generate basic knowledge for the development of standard inoculation techniques to evaluate resistance in cacao to the witches' broom disease.

Materials and Methods

Inoculum Production and Plant Material

Diseased material (brooms) was imported from Colombia, Venezuela, Brazil, and Trinidad.¹ The brooms were placed in plexiglass chambers in which dry and wet periods were regulated with humidifiers to stimulate the production of C. pernicioso basidiocarps (Rocha and Wheeler 1982).

Cacao seeds from open pollinated Catongo and SCA 12 clones were obtained from Costa Rica,² planted in plastic pots containing Metromix 500 Potting Mix (Grace Horticultural Products, Cambridge, Mass. 02146) and

¹ Brooms were kindly provided by Ing. Fabio Aranzazu (ICA, Colombia), Dr. Lilian Capriles de Reyes (Estacion Experimental Caucagua, Venezuela), Dr. Teklu Andebrhan (CEPLAC, Brazil), and Dr. Cheryl Gonsalves (Ministry of Agriculture Trinidad).

² Seeds were kindly provided for CATIE, Costa Rica through Dr. Gustavo Enríquez.

fertilized weekly with Peter's general purpose fertilizer (20:20:20 N P K, Peters Fertilizer Products, Foyelsville, Pennsylvania 18051). Plants were grown for 2-6 mo at 21-30 C in a greenhouse with 50% shade, which was provided by woven cloth (V. J. Growers, Apopka, FL 32703). Attached or detached vegetative flushes or flush parts were used at three stages of development described by Greathouse et al. (1971) as flushing 2 (F-2), interflush 1 (I-1), and interflush 2 (I-2). The F-2 begins when expanding leaves become visible in the shoot tip and ends when the last expanding leaf of a particular growth flush is greater than 1.5 cm in length; I-1 follows F-2 and ends before all leaves of the flush attain the deep green color of a mature leaf; flushes at I-2 have fully expanded and are dark green leaves.

Basidiospore Germination

Percentage of basidiospore germination on cacao tissue was quantified under different incubation conditions. Leaves and stems from Catongo I-1 flushes were placed on the bottom of a Petri plate lined with a piece of paper towel; a set of plant parts was sprayed with water to form a continuous film over their surfaces ("wet"), and another set was not sprayed ("dry"). Basidiocarps of C. perniciosa produced on brooms were stuck to the lid of a Petri plate with Vaseline and placed over the stems and adaxial surface of leaves. Unless otherwise stated, basidiocarps were removed after 5 minutes of active spore deposition. A third

set of plant parts was sprayed with a basidiospore suspension containing about one million spores/ml. The suspension was prepared by collecting basidiospores in a solution of 0.01 M MES (2[N-Morpholino] ethanesulfonic acid, Sigma Chem. Co., St. Louis, MO 63170) at pH 6.1 and 0.01% Tween 20 (Fisher Chem. Co., Fair Lawn, NJ. 07410). Basidiospores were also collected on dry slides to provide a comparison between in vitro and in vivo germination.

Inoculated plant parts were incubated at 25 C in a dew chamber, which produced and/or maintained a water film on plant part surfaces, or at room temperature (24-29 C) in closed plastic containers to which glycerol at specific gravities of 1.137 and 1.082 or water were added to obtain relative humidities of 80, 90, and 100%, respectively (Braun and Braun 1958). After 6 hr of incubation, leaves and stems were removed from the dew chamber or from the plastic containers; water was allowed to evaporate from the surface of the tissue, which was then sprayed with a solution of Cellufluor 0.03% in 0.01 M Tris/Hcl buffer at pH 8. The samples were examined at 125 and 312 X under a Leitz 20 microscope with epifluorescence. A random sample of 200 basidiospores was examined to estimate the percentage of germination. This experiment was carried out five times, twice using spores from basidiocarps produced on brooms from Venezuela and once with spores from each of the other sources of basiocarps (Trinidad, Colombia, and Brazil).

Basidiospore Survival

Survival of *C. pernicios* basidiospores deposited for 5 min on dry leaves from Catongo I-1 flushes was studied. Some of the inoculated leaves were immediately sprayed with water and incubated at 25 C in a dew chamber for 24 hr. Other inoculated leaves were incubated at room temperature (24-29 C) and RH of 80, 90, and 100% for 0.5, 1 and 2 hr. The leaves were then sprayed with water and incubated in the dew chamber. Basidiospores deposited on dry leaves and on slides for 20-25 min and placed in a dew chamber were also examined. A control in which basidiospores were deposited on wet tissue provided a comparison. Cellufluor 0.03% was sprayed on the inoculated leaves and the percentage of basidiospore germination was estimated. This experiment was carried out twice, once using inoculum from Trinidad and once with inoculum from Venezuela.

The ability of basidiospores to survive intermittent wet and dry periods was assessed. Basidiospores were deposited on wet leaves from Catongo I-1 flushes. The inoculated leaves were incubated at 25 C in a dew chamber for 2-3 hr to allow formation of germ tubes one to two times the length of the basidiospore. The leaves were removed from the dew chamber to allow evaporation of water from their surfaces. Five minutes after the leaf surface was dried, the inoculated leaves were sprayed with water, returned to the dew chamber and incubated for 24 hours. Cellufluor 0.03% was sprayed on the inoculated leaves and

the percentages of 200 basidiospores with germ tubes longer than the length of the spores was estimated. The experiment was conducted twice using inoculum from Venezuela.

Germ Tube Development

Germ tube development of C. pernicioso on Catongo flushes at different stages of development was examined to determine whether pathogen growth on plant surfaces occurred randomly or followed a specific pattern. Detached and attached, F-2 and I-1 Catongo flushes were placed horizontally in a dew chamber at 25 C. Flushes were sprayed with water and inoculated by placing basidiocarps on stems and the adaxial surfaces of leaves for 5 min. Hardened (mature, deep green colored) leaves from I-2 flushes were also inoculated. After 3, 12 and 24 hr of incubation, the inoculated tissue was removed from the dew chamber, kept under laboratory conditions to allow plant surface water to evaporate, and sprayed with 0.03% cellufluor. Specimens were examined under the microscope with epifluorescence.

Germ tube growth on leaf surfaces was also studied with a method designed to avoid any disturbance of the position of germ tubes on the inoculated tissue. Basidiospores of C. pernicioso were collected in a solution containing 0.01 M MES buffer at pH 6.1, 15% glycerol and 0.01 % Tween 20 (see Chapter Three). One drop of a 0.1% cellufluor solution was added to every 5 ml of spore suspension and mixed for 10 min. The suspension was filtered through a 0.45-micron Millipore filter (Millipore Corp., Bedford, MA 01730) to

eliminate the glycerol and the excess cellufluor. The surface of the filter was kept wet to avoid spore damage. Basidiospores were washed off the filter with 0.2% agar; the resulting suspension was homogenized and sprayed onto leaf pieces from Catongo F-2 and I-1 flushes placed on slides. Some leaves were wounded with a razor blade before inoculation. A cover slip was placed over the inoculated leaf pieces to facilitate observations under the microscope. Cellufluor did not affect basidiospore germination and was translocated to the germ tube; this allowed microscopic observations without further handling of the inoculated leaves. Observations were made after 3, 12, and 24 hr of incubation at 25 C in a dew chamber. This experiment was carried out twice using inoculum from Colombia.

Host Penetration and Post-penetration Development

Penetration mechanisms were observed on attached and detached Catongo F-2 and I-1 flushes and on hardened leaves. Basidiospores were deposited on all organs of the flush, as described before, and incubated for 12, 24 and 48 hr at 25 C in a dew chamber. Some inoculated plants were transferred to the greenhouse where they were kept for 5 additional days.

Inoculated leaves were cut in 1-cm² pieces and placed in chloroform:methanol (2:1) until chlorophyll was dissolved (10-15 min for young leaves). Specimens were transferred to lactophenol-methanol (1:3), boiled for 10 min and left overnight in this solution. Cleared leaves were washed

twice for 15 min each with 100% methanol; passed through 10 min soakings of 95, 90, 80, 70, 50, 30, and 0% methanol in 0.01 M Tris/HCL buffer at pH 8.0; and transferred to a 0.1% solution of the brightener tinopal BHS (Ciba-Geigy) in Tris/HCL buffer at pH 8.0 until mesophyll cells were observed with fluorescence microscopy (5-20 min). Specimens were washed twice for 5 min each time with Tris/HCL buffer and were placed in 1 M KOH at 60 C until leaves became transparent, which generally took from 4 hr for small leaves to overnight for fully developed leaves. The flaccid leaf pieces resulting from the KOH treatment were transferred to water and then to slides using a spatula. The specimens were mounted in lactophenol and examined under fluorescence microscopy.

Inoculated stems, petioles and pulvini were soaked in a 0.1% solution of tinopal for 5 min and then washed in buffer for 1 min. Free hand sections, cut perpendicular to the epidermis, were placed in 1 M KOH at ca. 60 C overnight, transferred to lactophenol and left overnight or until tissues were clear. Specimens were mounted in lactophenol for observation.

The ability of C. perniciosa to incite broom formation after leaf infection was evaluated by inoculating open pollinated SCA 12 seedlings with axillary flushes bearing leaves no longer than 1.5 cm. Agar blocks with fresh basidiospore prints were applied to the adaxial surface of

the leaves. Inoculated seedling were incubated at 25 C in a dew chamber for 24 hours and transferred to the greenhouse.

Results

Basidiospore Germination

Basidiospores deposited on dry slides and host tissue for 5 min did not germinate under any of the incubation conditions (Table 2-1, Fig 2-1A). Germination occurred when basidiospores were deposited on wet flushes or when basidiospore suspensions were sprayed on dry flushes (Table 2-1, Fig. 2-1B). Only the dew chamber provided the incubation conditions required for optimal basidiospore germination (83-100%). When inoculated, wet plant parts were incubated at 100% RH, a low percentage of basidiospores began to germinate but did not develop further (Table 2-1). Incubation at 90 and 80% RH did not result in basidiospore germination (Table 2-1). Basidiospores that failed to germinate on both plant surfaces and slides were plasmolyzed (Fig. 2-1A, C).

Basidiospore Survival

Basidiospores deposited from basidiocarps onto dry leaves for 5 min. did not survive under any of the conditions used in this experiment. However, when basidiospore deposition on dry leaves was extended to 20-25 min. and immediately incubated in a dew chamber, some germination occurred (ca. 5%)(Fig 2-1C). Germination was 80-100% in the controls (Fig 2-1B).

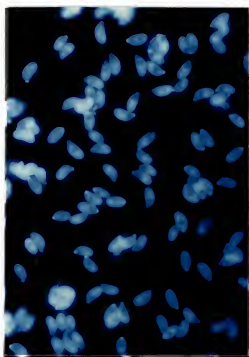
Table 2-1. Germination of *Crinipellis pernicioso* basidiospores on cacao leaves, using several inoculation methods and incubation conditions.

Inoculation Method	Trial**	Incubation Conditions			
		Dew	100% RH	90% RH	80% RH
Basidiospore Germination (%)					
Basidiospore deposition	1	0	0	0	0
on dry leaves* and	2	0	0	0	0
slides	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
Basidiospore deposition	1	94	7.2***	0	0
on wet leaves*	2	98	5.0	0	0
	3	100	1.3	0	0
	4	98	4.5	0	0
	5	98	7.0	0	0
Bsaidiospore suspensions	1	90	5.5	0	0
sprayed onto dry	2	91	4.0	0	0
leaves*	3	89	0.7	0	0
	4	83	7.9	0	0
	5	84	2.5	0	0

* Germination on stems was very similar to that on Leaves

** Basidiocarps for trial 1, 2, and 3 were produced on brooms from Trinidad, Brazil, and Colombia, respectively. Brooms from Venezuela were used in trials 4 and 5.

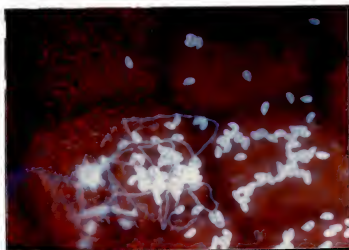
*** Germinated basidiospores on Leaves incubated at 100% RH had short germ tubes, two to four times the length of the basidiospore, that did not developed further.



A



B



C

Fig. 2-1. Fluorescence photomicrographs of basidiospores of *Crinipellis perniciosa* deposited from basidiocarps onto dry slides (A) or wet cacao leaf surfaces (B) for 5 min and incubated in a dew chamber for 6 hr. C, Basidiospores deposited for 25 min on dry leaf surfaces and incubated for 24 hr. Note the darker center of plasmolyzed basidiospores.

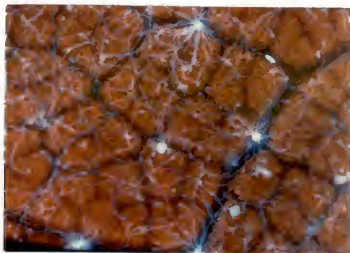
Basidiospores did not survive intermittent wet and dry periods. Germ tube growth initiated on wet tissue was irreversibly terminated when the surface of the tissue was allowed to dry for 5 min.

Germ Tube Growth

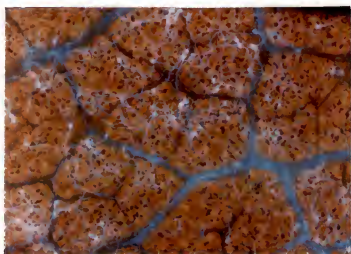
Basidiospore germ tubes had a marked tropic response towards stomata on leaves and stems of young F-2 flushes; many germ tubes were observed entering a single stoma (Fig 2-2A, 2-4A). This tropic response was weaker on leaves from I-1 flushes and undetectable on fully hardened leaves (Fig 2-2B). However, some germ tubes, apparently growing randomly on hardened leaves, were also observed entering stomata. Germ tubes were also attracted towards the bases of fallen or collapsing, multicellular trichomes present on fully developed but non-hardened I-1 flushes (Fig 2-3A, B). Mechanical wounds on young leaves also induced the tropic response. No differences were evident between attached and detached flushes regarding growth of the germ tubes towards the penetration sites.

Host Penetration and Post-penetration Development

Penetration of cacao tissue by *C. pernicios*a germ tubes occurred through stomata. Penetration was observed on all the parts of young F-2 flushes and on fully developed, but non-hardened, I-1 flushes. No appressoria or other specialized penetration structures were observed on stomata through which the germ tubes entered (Fig 2-4A, C, Fig. 2-5A). Substomatal vesicles were formed after penetration

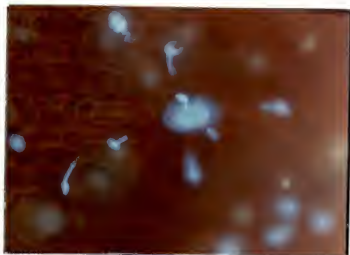


A

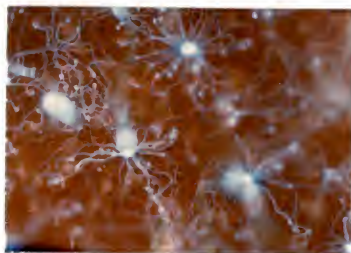


B

Fig. 2-2. Fluorescence photomicrographs of Crinipellis pernicioso germ tubes on cacao leaves 24 hr after inoculation. A, Directional growth of germ tubes towards stomata on leaf veins of young flushes. B, Random growth of germ tubes on hardened leaves.



A



B

Fig. 2-3. Fluorescence photomicrographs of Crinipellis pernicioso germ tubes on cacao stems from fully developed, but non-hardened, flushes. Directional growth of germ tubes towards trichome bases 3 hr (A), and 24 hr (B) after inoculation.

**A****C****B****D**

Fig. 2-4. Fluorescence photomicrographs of cacao leaves infected with Crinipellis perniciosa. Germ tubes entering a stoma on a leaf vein from a young F-2 flush (A) and forming substomatal vesicles (B) 24 hr and 7 days after inoculation, respectively. Penetration of stomata (C) and formation of substomatal vesicles (D) on leaves from fully developed, but non-hardened, flushes 12 hr after inoculation.

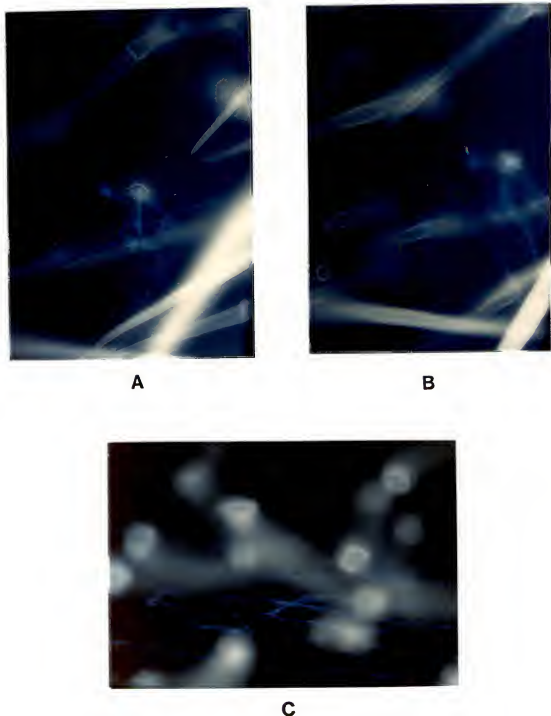
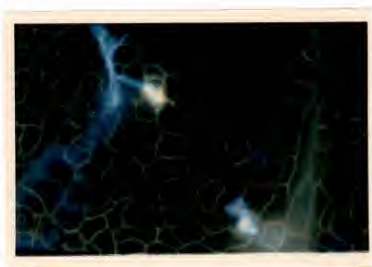


Fig. 2-5. Fluorescence photomicrographs of cacao stems infected with *Crinipellis pernicios*. Germ tubes entering a stoma (A), forming substomatal vesicles (B), and colonizing cortex tissue (C) in stems from young flushes, 7 days after inoculation.

(Fig 2-4B, Fig 2-4D, Fig. 2-5B). Intercellular hyphae developed from these vesicles and increased from sparse to abundant from 1 to 7 days after inoculation.

Fully developed, but non-hardened, I-1 flushes were also penetrated through the bases of fallen or collapsed, multicellular trichomes. Germ tubes gaining access to the plant through these infection sites formed vesicles, but colonization was not observed. On fully hardened leaves, germ tubes were observed entering stomata before the leaves were cleared, but neither substomatal vesicles nor colonizing hyphae were observed after clearing. No differences in the penetration mechanisms were evident between attached and detached flushes.

Growth of *C. pernicioso* after penetration varied among infection sites in the same flush and within the same organ. In some cases, hyphae grew freely throughout the infected tissue, whereas in others, mycelial growth stopped shortly after penetration. This failure to colonize cacao tissue was associated with the development of a golden colored fluorescence ("golden fluorescence") of host cells at and around the sites of infection (Fig 2-6A, B). The intensity of this fluorescence varied from site to site and was apparently correlated negatively with the extent of colonization 24 and 48 hr after inoculation. However, 7 days after inoculation, many of the successfully colonized infection sites also showed some degree of golden fluorescence (Fig 2-4B).



A



B

Fig. 2-6. Response of cacao tissue to the infection by *Crinipellis pernicios*a 48 hr after inoculation of leaves from non-hardened flushes. Three penetrated stomata (A), and surrounding mesophyll (B) with different degrees of golden fluorescence and pathogen growth. Note the profuse hyphal growth in infection sites with no golden colored fluorescence, contrasting with the growth in sites with golden fluorescence. The white bluish fluorescence of the fungus was imparted by the brightener Tinopal. Fluorescence of the guard and mesophyll cells is natural.

Inoculation of leaves on young F-2 flushes resulted in broom formation (Fig 2-7). Nine of sixteen of the inoculated SCA 12 flushes developed typical witches' broom symptoms. The petiole and pulvini of the inoculated leaf were always swollen. In many cases the axillary bud closest to the inoculated leaf developed into a broom, and the hypertrophy did not extend below the petiole associated with the inoculated leaf (Fig 2-6).

Discussion

Basidiospores lost their viability immediately after contacting dry surfaces. Plasmolyzed basidiospores observed on the surface of inoculated dry flushes and on slides suggest that an osmotic shock is responsible for this rapid loss in viability. Prolonging basidiospore deposition on dry leaves or slides from 5 to 25 min. allowed a low percentage of basidiospores to survive, probably because basidiospore layers closer to the dry surface served as a buffer for those subsequently deposited. Basidiospore germination required free water on the surface of the tissue. When wet, inoculated plant parts were incubated at 80-100% RH and room temperature (24-29 C), surface water evaporated soon after inoculation because the dew point (20-23 C) was never reached (Braun and Braun 1958). Water on the plant parts incubated at 100% RH took longer to evaporate, which explains the low percentage of basidiospores that initiated germination but did not develop further (Table 2-1). These observations have important



Fig. 2-7. Broom development on cacao seedlings 2 months after the application of Crinipellis pernicioso basidiospores to leaves, no longer than 1.5 cm, on young axillary flushes. The petioles of inoculated leaves are labeled with a metallic ring.

implications on the efficiency of inoculation methods. For example, a high proportion of basidiospores collected on dry glass surfaces will plasmolyze. Thus, when this collection method is used, inoculum concentration would vary according to the extent of plasmolysis. Basidiospores can also lose viability if they are exposed to dry surfaces during basidiospore germination. Therefore, inoculated tissue should be maintained wet until infection occurs.

Basidiospores of *C. perniciosa* were not able to survive for any period of time on dry flushes. Germ tube growth on wet plant surfaces was irreversibly terminated when the water on the tissue was allowed to evaporate. The stringent requirements for liquid water on any surface as a prerequisite for germination are met in nature, since the peak of basidiospore release occurs between 22:00-04:00 hr (Baker and Holliday 1957, Solorzano 1977, Evans and Solorzano 1981) when dew is prevalent. Basidiospores released early in the afternoon, when dew is not present, have a reduced probability for infection. Chances for infection would also be reduced if the basidiospores are released early in the morning (04:00-06:00 hr), because, in most of the cases, dew will not persist through the time required for germination and penetration. Very few, if any basidiospores are released during the day (07:00-18:00 hr) when temperature rises and when free water (from rain) on the surface of the flush would probably evaporate before infection takes place.

The evident tropic response of C. perniciosa germ tubes increases the chances for infection. The intensity of this tropic response decreased as flush development progressed. Perhaps the reduction in carbohydrate availability during the development of cacao flushes (Machado and Hardwick 1987) may produce changes in the quality and quantity of exudates at the infection sites and thus affect the intensity with which they attract C. perniciosa germ tubes. Robinson and Hodges (1977) demonstrated that growth of germ tubes of Dreschlera sorokiniana (Sacc) Subram and Jain and subsequent formation of preinfection structures on the leaves of Poa pratensis were correlated with the content of sugars and amino acids in the leaves.

Basidiospores of C. perniciosa germinated and penetrated through stomata on all of the organs of non-hardened flushes. Substomatal vesicles and colonization hyphae developing from these vesicles were observed in every part of the flush, indicating that stomata are common infection sites on vegetative flushes. This infection mechanism is remarkably similar to that reported by Patton and Johnson (1970) on white pine needles inoculated with Cronartium ribicola J. C. Fisher ex Rabenh. The role of C. perniciosa substomatal vesicles in the infection process was not studied. However, their common occurrence 12 hr after inoculation (Fig 2-4D) suggests that they could play an important role by protecting the pathogen from desiccation. Wetness on plant surfaces may be essential for the infection

process only until substomatal vesicles are formed. On hardened leaves, substomatal vesicles and colonization hyphae were never observed. Probably, the nutritional requirements of the pathogen for post-penetrational growth are not met in this type of tissue.

Colonization hyphae were not observed developing from penetrated multicellular trichome bases. These infection sites are probably less important than stomata because multicellular trichomes do not occur at the more susceptible early stages of flush development. However, the strong attraction of germ tubes towards trichome bases on fully developed, but non-hardened, I-1 flushes (Fig 2-3B), on which the orientation of germ tubes towards stomata is greatly reduced, suggests that trichome bases may be important ports of entry for the pathogen before flush hardening. Although colonization of I-1 flushes apparently does not occur soon after penetration, vesicles formed under the epidermis could remain dormant until the next flush cycle. The position of these dormant infections would probably determine the type of symptoms that would develop. Infections relatively distant from the apical meristem may produce swellings or cankers; brooms could be produced when the infections occur close to axillary or apical buds.

Direct penetration was never observed in this study. Other researchers have observed *C. perniciosus* germ tubes penetrating leaf epidermis directly (Cronshaw and Evans 1978, Sreenivasan and Dabydeen 1987). Penetration through

small wounds, produced on the leaves as they are processed for inoculation (surface sterilized), could result in apparent direct penetration. In this study, no treatment was given to the leaves before inoculation.

Infection sites (stomata) within a flush were not colonized equally. The degree of colonization 24-48 hr after inoculation apparently decreased as the intensity of the golden fluorescence at and around the penetrated stomata increased. This fluorescence could be correlated with the degree of cacao resistance to WB. In other crops, host resistance to diseases has been positively correlated with the proportion of infection sites in which a change in fluorescence is observed after penetration. (Niks and Kuiper 1983, Luke et al. 1984, Gangadin 1987). In preliminary observations of inoculated cacao leaves from an open pollinated EET 233 seedling, previously selected for its resistance to artificial inoculation, the frequency of penetrated stomata with golden fluorescence was apparently higher than on leaves from an open pollinated seedling from the susceptible EET 19 clone.

Successfully colonized infection sites that showed golden fluorescence 7 days after inoculation could be the result of a late reaction of the plant to infection. However, the importance of this late reaction on subsequent colonization was not established. Although the nature of the golden fluorescence was not elucidated, some phenolic compounds that could have this type of fluorescence have

been extracted from cacao tissues and proved to inhibit germination of *C. perniciosa* basidiospore (Bastos 1987).

Inoculation of leaves (5-15 mm long) on young flushes gave rise to the development of brooms. Cronshaw and Evans' (1978) lack of success in obtaining brooms from leaf inoculation may have been due to the age of the leaves and/or the type of flush they used. Results obtained here indicate that the efficiency of inoculation methods in which vegetative flushes are used as susceptible tissue could be improved by covering all parts of the flush with inoculum, rather than by applying basidiospores to specific sites as is required in some inoculation methods (Cronshaw and Evans 1978, Baker and Mckee 1943, Briton-Jones and Cheesman 1931).

The results of this research indicate that a standardized method of inoculation would require keeping plant surfaces wet during inoculation and basidiospore germination. When the objective of inoculating plants is to evaluate their resistance to witches' broom, inoculum concentration should play a very important role. The probability of a germ tube entering a stoma without the potential to stop pathogen growth would increase as inoculum concentration is raised, so even plants with high proportions of "resistant sites" would become diseased if sufficient inoculum pressure is applied.

CHAPTER III
DEVELOPMENT OF AN INOCULATION METHOD FOR EVALUATING RESISTANCE
TO WITCHES' BROOM DISEASE OF CACAO

Introduction

Efficient inoculation methods facilitate evaluating perennial crops for disease resistance (Van der Graaff 1985, FAO 1986). In the case of cacao (Theobroma cacao L), breeding for resistance to witches' broom disease, incited by Crinipellis perniciosa (Stahel) Singer, has been a long and inefficient process; this has been due in part to the attempted evaluation of resistance under uncontrolled field conditions (Bartley 1986, Watson and Mulligan 1986). At best, evaluation of a clone or progeny from a specific cross requires 5-10 years, large experimental plots, and extensive labor. Using artificial inoculation methods could reduce the need for field tests and increase the efficiency of breeding cacao for resistance to this disease.

Several methods have been developed for evaluating resistance to witches' broom (WB), but their use in breeding programs has met with limited success. Inoculation of germinated seeds by dipping them into basidiospore suspensions (INIAP 1950, Holliday 1955) was used in Ecuador, Trinidad, and Venezuela to evaluate resistance in progeny from crosses of promising clones (INIAP 1982/83, Bartley 1977, Capriles and Rojas 1977). Inconsistent results or

failure to detect differences between families from putative susceptible and resistant clones discredited the usefulness of this method (INIAP 1982/83, Bartley 1958, 1959, 1986, Capriles and Rojas 1977). Application of agar blocks with basidiospore prints to the stems of developing cacao flushes has also been used to evaluate resistance (Cronshaw and Evans 1978, Abud et al. 1984, Laker et al. 1987). However, this method is not suitable for inoculation of large quantities of material and also produces inconsistent results (Rocha 1983).

Other inoculation methods for the evaluation of resistance to WB are not reliable or usefull to mass testing or have not been sufficiently explored to determine whether they can be incorporated into breeding schemes (Stahel 1915, Briton-Jones and Cheesman 1931, Baker and McKee 1943, Desrosier et al. 1955, Andebrhan 1983).

Factors contributing to the lack of success of inoculation methods to evaluate resistance to WB have not been determined. However, two important components in a standard inoculation technique, inoculum viability and inoculum concentration, are not easily controlled. For example, the commonly used Holliday (1955) method requires suspensions containing 200,000 basidiospores/ml. Basidiospores used to prepare the suspension are collected on glass surfaces and may have 10-50% germination (Medeiros and Rodrigues 1975, Baker and McKee 1943). Therefore the concentration of viable basidiospores could vary between

20,000 to 100,000/ml; such a variation could affect reproducibility. In contrast, basidiospores collected on agar have consistently resulted in 90-100% germination. However, no practical method has been designed to adjust the number of spores on the agar, and thus the inoculum density applied to the plant is unknown.

The inoculum dosage for evaluating resistance to WB has been established based on the concentration of basidiospores needed to produce 100% disease on progeny from susceptible clones (Holliday 1955). Thus, escapes, such as uninfected susceptible plants are avoided. However, if resistance is expressed quantitatively (levels of resistance), the escapes should be the seedlings with the highest levels of resistance within the family. Perhaps the inoculum concentration necessary to infect 100% of the progeny from susceptible clones overcomes the level of resistance in most or all the progeny from resistant clones.

Having inoculum and susceptible plants available simultaneously is another critical element for inoculation methods. Basidiospores are the only infective propagule produced by *C. perniciosus* (Stahel 1915). Therefore, a system to consistently produce basidiocarps would provide inoculum when required. Although methods have been developed to stimulate basidiocarp production on brooms and mycelial mats (Rocha and Wheeler 1985, Purdy et al. 1983), basidiocarp flushes can not be predicted accurately. Inoculum storage is an alternative strategy. However, no

successful method to store basidiospores has been developed. The objective of this research was to develop an efficient and reliable inoculation method to evaluate resistance to witches' broom disease of cacao.

Materials and Methods

The following aspects of the inoculation procedure were investigated: basidiospore collection and storage, inoculum concentration, seedling growth stage, and incubation time. Conditions used to incubate inoculated plants were established in previous studies (see Chapter Two). The technique used to screen pine seedlings for resistance to fusiform rust (Anderson et al. 1982) served as a guideline for the development of the inoculation method.

Inoculum Production

Basidiocarps were produced on cacao brooms imported from Ecuador, Trinidad, Venezuela, and Colombia¹. The brooms were placed in plexiglass chambers in which 8-to 12-hr wet periods were provided with humidifiers to stimulate inoculum production (Rocha and Wheeler 1982). The inoculum source used in each experiment is specified in the corresponding methodology.

Basidiospore Collection

The solution formulated to collect basidiospores of C. pernicioso ("collecting solution") contains MES buffer (0.01

¹ Brooms were kindly provided by Ing. Jaime Aragundi (EET Pichilingue, Ecuador), Dr. Cherly Gonsalves (Ministry of Agriculture, Trinidad), Dr. Lilian Capriles (Estacion Experimental Caucagua, Venezuela), Fabio Aranzazu (ICA, Colombia).

M (2[N-Morpholino] ethanesulfonic Acid, Sigma Chem. Co., St. Louis, MO. 63178) at pH 6.1, 15-16% glycerol, and 0.01% Tween 20 (Fisher Scientific Co., Fair Lawn, NJ 07410). This last component was added after autoclaving the solution, otherwise basidiospore viability was reduced as much as 30%. The pKa of MES is 6.1 and thus the maximum buffer capacity is at pH 6.1, which is near the optimal pH of 6.0 for basidiospore germination in suspensions (Medeiros and Rodrigues 1975b). Tween 20 facilitated the homogeneous distribution of basidiospores in the suspension and reduced surface tension on inoculated tissues. Glycerol at a concentration of 15-16% inhibited basidiospore germination but did not affect spore viability; reducing glycerol concentration to 3% allowed normal germination. Basidiospores collected in the dilute acid solution used to collect spores of Cronartium fusiform (Anderson et al. 1982) had low percentages of germination.

Basidiospore suspensions were prepared as follows: basidiocarps were stuck to the lid of a Petri plate with vaseline and placed over a 250-ml beaker containing 50-60 ml of the collecting solution. The beaker was set on a stirring plate and the solution was stirred continuously with a magnetic bar during basidiospore collection. The period of basidiospore collection was 12-20 hr. Viable basidiospores with subsequent germination rates of 94-100% were consistently obtained with this collecting method.

The formulation of the collecting solution was determined from trial and error experiments. In order to confirm whether this formulation was the best, basidiospore germination and germ tube growth in suspensions with different concentrations of Tween 20 and glycerol were examined. The effect of glycerol concentration on germination was studied by collecting basidiospores in MES solutions with 0.01% Tween 20 and 0 to 21% glycerol (MES-Tween-glycerol). Three milliliters of each solution were placed in 60-mm-diameter Petri plates; one to three basidiocarps produced on brooms from Venezuela were stuck to the lid of the plate with vaseline and placed over each of the solutions for 3 min to allow basidiospore deposition. All spore suspensions were homogenized, by repeatedly collecting and expelling the suspensions with a Pasteur pipet, and incubated at lab temperature (ca 25 C) for 24 hr. The percentage of basidiospore germination was evaluated in each plate by adding a drop of lactophenol-typan blue and homogenizing the suspension; several drops were transferred to a 1% agar plate; agar blocks containing the basidiospores were placed on a slide and covered with a cover slip. At least 200 basidiospores from each suspension were examined under the microscope to estimate the percentage of germination.

The effect of glycerol concentration on germ tube growth rate was also evaluated. Basidiospore suspensions collected overnight in the collecting solution were diluted

with 0.01 M MES plus 0.01% Tween 20 (MES-Tween) to reduce glycerol concentration from 15% to 9, 6, and 3%; the diluent was added gradually to prevent basidiospore plasmolysis. A control without glycerol was prepared by diluting 5 ml of the suspension with 20 ml of MES-Tween; the resulting suspension was filtered through a 0.45-micron Millipore filter (Millipore Corp., Bedford, MA 01730) without allowing the surface of the filter to dry, then the basidiospores were resuspended in MES-Tween. The suspensions were incubated at room temperature (24-29 C) for 9 hr, transferred to agar plates, and processed for microscopic observations as previously described. Twenty germ tubes were measured for each suspension. These measurements were facilitated by moving the cover slip on the agar block towards one of the sides until the germ tubes were uncoiled.

The effect of Tween 20 concentration on basidiospore germination and germ tube growth was evaluated. Basidiospore suspensions, collected overnight in the collecting solution, were gradually diluted with MES-Tween in order to reduce glycerol concentration from 15 to 3% and allow germination. The diluted suspensions were filtered through 0.45-microns Millipore filters and resuspended in MES plus 3% glycerol solutions containing 0 to 0.05% Tween 20. The suspensions were incubated in the lab for 9 hr and processed, as described before, for evaluation of germ tube growth and percentage of germination.

The experiments were replicated four times using basidiocarps produced on brooms from Ecuador.

Basidiospore Storage

Basidiospores were collected overnight in 50-60 ml of collecting solution; the resulting suspension was stored for 1 to 7 days at 4 C or was fractioned into 1-to 2-ml aliquots, transferred to cryogenic tubes, submerged in liquid nitrogen (-320 C) and stored for as long as 18 months. Concentrated basidiospore suspensions mixed 1:1 with autoclaved 8.5% skim milk (BBL Microbiology Systems, Cockeysville, MD 21030) in MES plus 15% glycerol were also stored in liquid nitrogen. Concentrated suspensions were obtained by filtering 50-60 ml of suspension through a 0.45-micron Millipore filter without allowing the surface of the filter to dry, preventing spore plasmolysis. The basidiospores were washed off the filter with 3 ml of MES plus 15% glycerol without Tween 20, homogenized and transferred to cryogenic tubes.

Stored basidiospores were induced to germinate by diluting the suspension with MES-Tween solution to reduce the concentration of glycerol from 15 to 3%. Suspensions stored in liquid nitrogen were thawed at approximately 37 C before dilution. Viability of stored basidiospores varied from 37-95%. Inconsistent percentages of germination were common even within a storage treatment. For example, one concentrated basidiospore suspension stored in liquid

nitrogen for 18 months had 53% germination while another had 88%.

Inoculum Preparation

Fresh basidiospore suspensions. The concentration of basidiospores collected overnight in the collecting solution ranged from 1 to 3 million basidiospores/ml. A sample of 10-30 ml of this suspensions was diluted with collecting solution to reduce basidiospore concentration to 350,000-500,000/ml, which allowed a more accurate estimate of the number of basidiospores/ml. Ten samples, taken immediately after shaking the suspension vigorously, were counted on a hemacytometer and averaged to calculate the number of basidiospores/ml. This suspension subsequently will be referred to as the "stock suspension". When inoculum concentrations higher than 75,000 basidiospores/ml were needed for inoculation, a stock suspension containing approximately one million basidiospores/ml was prepared.

The stock suspension was diluted to calibrate the number of basidiospores/ml and to reduce glycerol concentration from 15 or 16% to 3% to allow basidiospore germination. This was achieved simultaneously by using MES-Tween and MES-Tween-3% glycerol as diluents. The total volume of diluents needed to adjust the number of basidiospores/ml to the desired concentration was calculated as follows:

$$Vd = \frac{(Vsk)(Csk)}{(IC)}$$

where V_d is the total volume of diluents, V_{sk} is the volume of stock suspension, C_{sk} is the concentration of basidiospores in the stock suspension, and IC is the desired inoculum concentration. The MES-Tween diluent had to be added to the stock suspension (V_{sk}) at a ratio of 4:1 to reduce glycerol concentration from 15% to 3%. Thus the volume of MES-Tween-3% glycerol needed to complete the dilution was the difference between the total volume of diluents (V_d) and the volume of MES-Tween [$V_d - (4 \times V_{sk})$ or $V_d - (4.33 \times V_{sk})$ for stock suspensions with glycerol 15 or 16%, respectively].

Diluents were added gradually, with a burette, while the stock suspension was stirred; MES-Tween-3% glycerol diluent was added first to allow a more gradual change in osmotic pressure, and thus maintain optimal basidiospore viability.

Stored basidiospore suspensions. A small sample (0.5-4 ml) of the stored suspension was gradually diluted with MES-Tween solution at a ratio of 1:4 to reduce glycerol concentration to 3%, and the suspension was then incubated for 5 hr at room temperature. The percentage of germination was evaluated and used to calculate the number of viable basidiospores/ml; inoculum calibration then proceeded as described for fresh suspensions.

Plant Material

Cacao seeds from open pollinated clones were received from Costa Rica and Ecuador, planted in 6-cm² peat pots with

Metromix 500 Potting Mix (Grace Horticultural Products, Cambridge, Mass 02146), and fertilized weekly with Peter's general purpose fertilizer (20:20:20 N P K, Peter's Fertilizers products, Foyelsville, Pennsylvania 18051). Seedlings were grown at 21-30 C in the greenhouse with 50% shade, which was provided by woven cloth (V. J. Growers, Apopka FL. 32703). Mites and aphids were controlled with Pentac (Zoecon Corp.) and Isotox (Chevron Chemical Co.), respectively, but young flushes were never sprayed before inoculation.

The first flush of 3- to 4-week-old cacao seedlings was the material preferred for inoculation. However, it was not always possible to coordinate availability of these seedlings with inoculum production. In some cases, axillary flushes, induced by pruning 2-to 4-month-old seedlings above the first two leaves, were inoculated. The two hardened leaves (mature, dark green) left on the seedlings were cut perpendicular to the mid rib just before inoculation or one day in advance; only 1/5 of each leaves were left attached to the petioles. Buds at the cotyledonary node which flushed following the pruning of 5-to 6-week-old seedlings above the cotyledons, were also used in one of the experiments. Unless otherwise stated, seedlings selected for inoculation had flushes with two to four leaves no longer than 1.5 cm.

Inoculation and Disease Evaluation

Seedlings were inoculated with basidiospore suspensions of known concentration. Approximately 0.6-0.8 of suspension was sprayed on each flush with a chromist atomizer (Gelman Scientific Inc., Ann Arbor, MI 48106). Inoculated seedlings were immediately transferred to a dew chamber and incubated at 25 C. The water and wall temperatures of the dew chamber were adjusted to 40 and 5 C, respectively, to maintain plant surfaces wet; unless otherwise stated, inoculated plants were incubated for 24 hr. After incubation, 3-to 4-week-old seedlings were covered with a plastic bag for a few hours to prevent wilting. Inoculated seedlings were maintained in a greenhouse for observation.

The percentage of seedling flushes with brooms 3 months after inoculation was used to evaluate disease incidence in each of the experiments. Seedling were examined at least monthly to assure that mortality due to WB would not be confused with other causes. Swellings at the petioles or pulvini were also consider as diseased, even though brooms did not develop during the evaluation period.

Incubation Time

Axillary flushes on seedlings from open and self pollinated Catongo (Susceptible), open pollinated EET 400 (moderately resistant), SCA 6, and SCA 12 (resistant) were inoculated with basidiospore suspensions calibrated to 200,000 basidiospores/ml (Trinidad inoculum source). Seedlings of SCA 6 and SCA 12 were also inoculated with

100,000 basidiospore/ml. The seedlings were incubated in a dew chamber for 2-24 hr and transferred to the greenhouse. Groups of 15-21 flushes on 10 seedlings of each family per incubation time per inoculum concentrations were inoculated. In most of the cases only one combination of variety plus inoculum density was tested at a time. This provided a good estimate of the incubation time required for infection to take place, but invalidated statistical comparison among varieties.

Efficiency of the Inoculation Method

Fresh inoculum. Axillary flushes on open pollinated seedlings from three susceptible clones (EET 19, EET 382, and EET 381), two resistant hybrids (SCA 12 X Sil 1, and SCA 6 X Sil 1), and one resistant clone (EET 233) were inoculated with suspensions calibrated to 25,000 basidiospores/ml (Ecuador inoculum source). Groups of 10-37 seedlings per family were inoculated on five different dates. The experiment was designed to evaluate the reproducibility of the inoculation method and determine whether differences in resistance among the cacao families could be detected with this inoculum concentration.

Short-term stored inoculum. Seedlings of clones EET 19 and EET 233 were inoculated with fresh and stored suspensions calibrated to 75,000 viable basidiospores/ml (Ecuador inoculum source). Groups of 22-99 seedlings per family were inoculated on eight dates. Fresh inoculum was applied to the seedlings on dates one to five. Inoculum

stored in the refrigerator for 2, 3, and 7 days was applied on dates six, seven and eight, respectively. The effect of short-term storage on basidiospore infectivity and the reproducibility of the inoculation method were evaluated.

Long-term stored inoculum. Cotyledonary flushes of EET 400 seedlings were inoculated with fresh and liquid nitrogen-stored suspensions calibrated to 10,000 and 25,000 basidiospores/ml. Inoculum was prepared from two basidiospore suspensions from the Trinidad inoculum source and one from Venezuela, both of which had been stored for 18 months in liquid nitrogen. Each of these suspensions was considered as a replication of the long-term storage treatment. Three fresh basidiospore suspensions from the Ecuador inoculum source were used as controls. Groups of 20-40 flushes on 14-19 seedlings were inoculated on three dates with each storage treatment and each inoculum concentration. On every inoculation date, one fresh and one stored suspension were used for inoculation. This factorial experiment was designed to evaluate the effect of long-term storage of basidiospores on infectivity and the reproducibility of the inoculation method.

Inoculum Density and Evaluation of Resistance

Axillary flushes on seedlings of SCA 6, SCA 12, and Catongo were inoculated with fresh suspensions (Ecuador inoculum source) at six different inoculum concentrations ranging from 12,500 to 75,000 basidiospores/ml. Groups of 20-40 flushes on 15-25 seedlings per family per inoculum

level were inoculated on five dates. On dates two to five, mature hardened leaves were cut off the plant one day before inoculation, while on date one they were cut just before inoculation. Due to limited availability of seedlings from the SCA clones, these families were inoculated only at three levels of inoculum and on four of the dates. This unbalanced, incomplete factorial experiment was still appropriate to determine whether differences among the families could be detected, whether the inoculum density affected these differences, and how reproducible the inoculation method was.

Flush Size and Evaluation of Resistance

Seedlings from SCA 6, SCA 12 and EET 400 were inoculated with fresh suspensions (Ecuador inoculum source) at a concentration of 10,000 basidiospores/ml. During the development of the first flush, seedlings were divided into three groups: seedlings with leaves between 0.3-1.5 cm long, 1.8-3.0 cm long, and 3.3-5.0 cm. Groups of 16-25 seedlings per family per flush size were inoculated on four different dates. This factorial experiment was designed to evaluate the effect of flush size on seedling susceptibility to WB, the resistance of the families, and the reproducibility of the inoculation method.

Data Analysis

The experimental design used in the inoculation experiments was a randomized complete block, in which inoculation dates were considered as blocks. The arcsine

transformation (Little and Hills 1978) was applied to the data; analyses of variance of the transformed data were done with the GLM procedure in SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC). Means were statistically compared with Duncans' multiple range test; the error term used for this purpose was the error mean square in the analysis of variance table obtained with the GLM procedure.

Results

Basidiospore Collection

Glycerol at concentrations of 3-9% in MES plus 0.01% Tween 20 had no effect on basidiospore germination as compared with solutions without glycerol (Fig 3-1). When glycerol concentration was increased above 9%, germination decreased rapidly until no germination occurred at glycerol concentrations higher than 15%. Germ tube growth was not affected by 3% glycerol (Fig 3-1). However, as glycerol concentration was increased above 3%, germ tube growth rate decreased (Fig 3-1).

Tween 20 at concentrations of 0.005-0.03% in MES plus 3% glycerol had no effect on basidiospore germination as compared with suspensions without Tween 20 (Fig 3-2). However, the germ tube growth rate was gradually reduced as the Tween 20 concentration was increased above 0.01% (Fig 3-2).

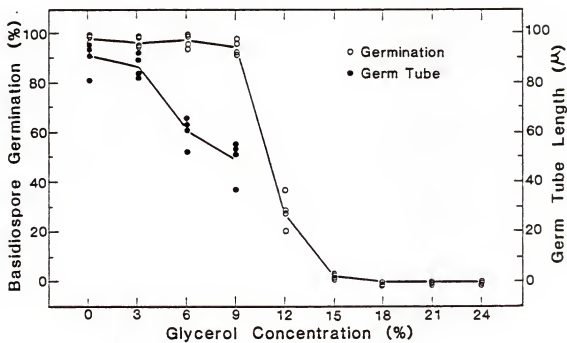


Fig. 3-1. Effect of glycerol concentration in solutions of 2[N-Morpholino]ethanesulfonic acid buffer on germination and germ tube length of *Crinipellis perniciosus* basidiospores. Germination was evaluated after 24 hr of incubation; germ tube length was measured after 9 hr of incubation.

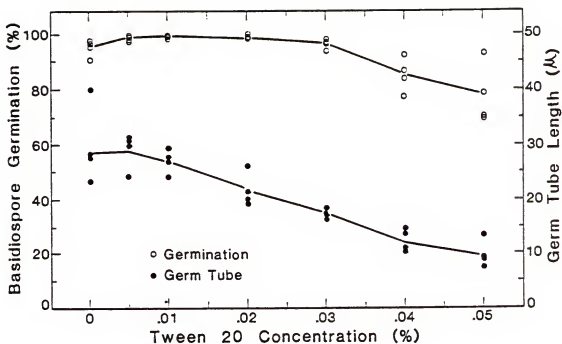


Fig. 3-2 Effect of Tween 20 concentration in solutions of 2[*N*-Morpholino] ethanesulfonic acid buffer plus 3% glycerol on germination and germ tube length of *Crinipellis perniciosia* basidiospores. Germination was evaluated after 24 hr of incubation; germ tube length was measured after 9 hr of incubation.

Incubation Time

The first diseased flushes were detected on seedlings inoculated with suspensions calibrated to 200,000 basidiospores/ml and incubated in the dew chamber for 6 hr (Fig 3-3A). Inoculated seedlings incubated for 2-4 hr did not develop brooms. Therefore, at least 4-6 hr were needed for infection under the incubation conditions used in this experiment. Maximum percentages of disease in all cacao families but SCA 6 were achieved after 14-15 hr of incubation. When SCA 6, and SCA 12 families were inoculated with suspensions calibrated to 100,000 basidiospores/ml, the first diseased flushes were detected on seedlings incubated for 8 hr, and maximal percentages of diseased flushes were achieved at 14-15 hr of incubation (Fig 3-3B). Seedlings incubated for 2-6 hr did not develop symptoms.

Differences among the families were evident when the seedlings were incubated for 6-12 hr but not when they were incubated for 24 hr; generally, the Catongo self pollinated family appeared to be the most susceptible (Fig 3-3A). However, statistical comparisons among the families used in this experiment were not possible because they were not inoculated at the same time.

Efficiency of the Inoculation Method

A consistent 97-100% disease was obtained when 3-to 4-week-old seedlings of several cacao families were inoculated with fresh or short term-stored (1-7 days) basidiospore suspensions calibrated to 25,000 or 75,000/ml (Table 3-1,

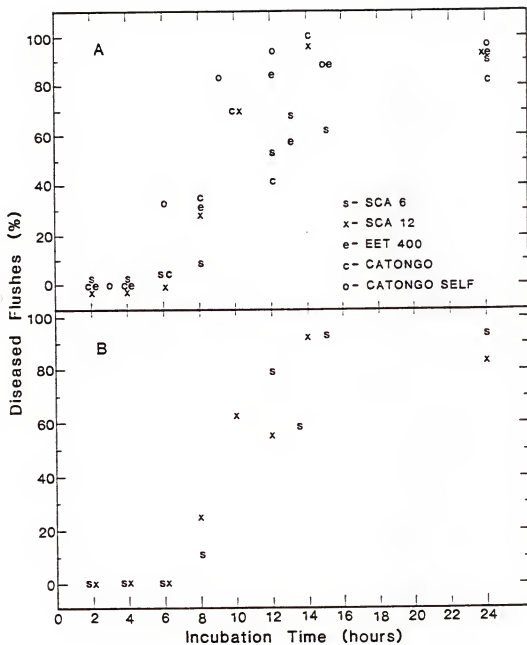


Fig. 3-3. Effect of incubation time on the percentages of diseased flushes inoculated with suspensions of Crinipellis perniciosa basidiospores. A, 200,000 basidiospores/ml, B, 100,000 basidiospores/ml.

Table 3-1. Percentage of diseased seedlings in two cacao families inoculated with fresh and stored suspensions of Crinipellis perniciosa basidiospores calibrated to 75,000/ml.

Inoculation Date*	% of Seedlings Diseased (Inoculated Seedlings)	
	EET 19	EET 233
I	100 (60)	100 (40)
II	100 (49)	- -
III	100 (59)	100 (49)
IV	100 (99)	100 (71)
V	100 (71)	100 (34)
VI	100 (56)	100 (50)
VII	100 (34)	97 (34)
VIII	100 (25)	100 (22)

* Seedlings used in dates V, VI, VII, and VIII were inoculated with the same basidiospore suspensions stored in the refrigerator for 0, 2, 3, and 7 days, respectively.

3-2). At these inoculum concentrations, no differences among families were detected.

Basidiospores stored for 0 and 18 months in liquid nitrogen and calibrated to 10,000 and 25,000 viable basidiospores/ml induced similar percentages of disease on cotyledonary flushes of EET 400 seedlings (Table 3-3; Table A-1, appendix).

Inoculum Density and Evaluation of Resistance

Significant differences in the percentages of flushes diseased on SCA 6, SCA 12, and Catongo families inoculated on five dates with basidiospore suspensions at a concentration of 12,500, 35,000 and 75,000 basidiospores/ml (Table 3-4A; Table A-2, appendix). When data from the first date of inoculation was not considered to calculate the mean percentages of diseased flushes, significant differences were detected among families inoculated with basidiospore suspensions at a concentration of 12,500/ml but no differences were observed when inoculum concentration was raised to 35,000 or 75,000/ml (Table 3-4B). At this latter inoculum concentration all families had nearly 100% disease (Table 3-4B). The average percentage of flushes diseased on the first date of inoculation was significantly lower than the other four inoculation dates (Table 3-5). Mature leaves of the seedlings inoculated on date one were removed just before inoculation, while on the other inoculation dates the leaves were removed a day before inoculation.

Table 3-2. Percentage of diseased seedlings in six cacao families inoculated with fresh suspensions of *Crinipellis pernicioso* basidiospores calibrated to 25,000/ml on five dates.

Date	Family					
	<u>EET 19</u>	<u>EET 381</u>	<u>EET 382</u>	<u>EET 233</u>	<u>SCA12 X SIL1</u>	<u>SCA6 X SIL1</u>
	% of Seedlings Diseased (Inoculated Seedlings)					
I	100 (30)	100 (30)	100 (30)	--	100 (36)	100 (30)
II	--	100 (25)	100 (23)	--	100 (36)	100 (23)
III	100 (17)	100 (28)	100 (23)	--	100 (34)	100 (36)
IV	100 (11)	100 (25)	100 (36)	100 (10)	100 (18)	100 (30)
V	100 (30)	100 (20)	97 (31)	100 (10)	100 (18)	100 (37)

Table 3-3. Effect of long-term storage of basidiospore of Crinipellis perniciosa on infection of EET 400 cotyledonary flushes inoculated with suspensions containing 10,000, and 25,000 basidiospores/ml.

Storage (Months)	<u>Inoculum Density (Basidiospores/ml)</u>	
	10,000	25,000
	% of Flushes Diseased* (Inoculated Flushes)	
0 (fresh)	95 a** (44)	95.4 a (90)
18	86 a (45)	95.3 a (76)

* Average of four replications (inoculation dates)

** Means followed by the same letter are not statistically different according to Duncan's multiple range test ($p=0.05$).

Table 3-4. Effect of *Crinipellis pernicioso* inoculum concentration on the percentages of diseased flushes in cacao families from resistant (SCA 6 and SCA 12) and susceptible (Catongo) clones.

Family	Inoculum Concentration (Basidiospores/ml)					
	12,500	25,000	37,500	50,000	62,500	75,000
% of Flushes Diseased						
A*						
Catongo	77.5 a**	74.9	83.7 a	88.8	100	92.8 a
SCA 12	41.9 b	-***	68.6 ab	-	-	79.9 b
SCA 6	41.0 b	-	61.2 b	-	-	82.8 b
B						
Catongo	88.8 a	88.0	89.7 a	100	100	98.5 a
SCA 12	48.7 b	-	88.4 a	-	-	95.7 a
SCA 6	49.4 b	-	85.7 a	-	-	96.5 a

* A, average of five inoculation dates. B, average of dates two to five.

** Means, within columns, followed by the same letter are not statistically different according to Duncan's multiple range test ($p=0.05$).

*** The families SCA 6, and 12 were not inoculated at some of the inoculum concentrations.

Table 3-5. Comparison among inoculation dates in a factorial experiment in which three cacao families (SCA 6, SCA 12, and Catongo) were inoculated with Crinipellis perniciosa basidiospore suspensions at three different concentrations.

Date	Inoculated Flushes	% of Flushes Diseased*
IV	244	85.5 a***
II	243	85.4 a
III	90	83.4 a
V	148	80.2 a
I**	338	40.2 b

* Date means (averaged over all families and inoculum concentrations). All inoculum concentration were applied to the three cacao families in dates I and II. Some inoculum concentrations and/or families are missing in inoculation dates III, IV, V.

** Hardened leaves of the seedlings used in date I were removed just before inoculation, while in the other dates hardened leaves were removed a day in advance.

*** Means followed by the same letter are not statistically different according to Duncan's multiple range test ($p=0.05$)

Flush Size and Evaluation of Resistance

Percentages of disease on inoculated seedlings 3-4 weeks old were significantly affected by flush size (Table 3-6; Table A-3, appendix). The percentage of disease increased as the length of the leaves in the flush increased from 0.3-1.5 cm to 3.3-5.0 cm (Table 3-6). At the smallest leaf size (0.3-1.5 cm), the SCA 6 family had significantly less disease than did the EET 400 family (Table 3-7) and SCA 12 was no different than SCA 6 or EET 400. At the intermediate leaf size (1.8-3.0 cm), SCA 6 family had fewer diseased seedlings than did the SCA 12 and EET 400 families. However, no significant differences were detected among families when flushes with the largest leaves (3.3-5.0 cm) were inoculated. No significant differences in disease among inoculation dates were detected (Table 3-8).

Discussion

The formulated collecting solution was successfully used to obtain a highly concentrated suspension of nongerminated, viable basidiospores of C. pernicios. A consistent 94-100% germination was readily obtained by gradually diluting the concentration of glycerol in the suspension. Solutions with 15-16% are probably isotonic; thus they prevent basidiospore plasmolysis and germination. The solution would therefore become hypotonic when glycerol concentration is reduced to 3%, allowing the germination process to proceed.

Table 3-6. Effect of seedling flush size on the percentage of disease in a factorial experiment in which three cacao families (SCA 6, SCA 12 and EET 400) at three stages of flush development were inoculated with Crinipellis perniciosa basidiospore suspensions calibrated to 10,000/ml.

Leaf size range in the flush	Inoculated Seedlings	% of Seedlings* Diseased
3.3-5.0	221	96.8 a**
1.8-3.0	235	92.5 ab
0.3-1.5	251	90.3 b

* Flush size means (averaged over all families and inoculation dates). A nonsignificant interaction between family and flush size allowed comparisons among flush size means.

** Means followed by the same letter are not statistically different according to Duncan's multiple range test ($p=0.05$).

Table 3-7. Effect of seedling flush size on the percentage of diseased seedlings in three cacao families inoculated with Crinipellis perniciosa basidiospore suspensions at a concentration of 10,000 basidiospores/ml.

Family	<u>Leaf Size Range in the Flush (cm)</u>		
	0.3-1.5	1.8-3.0	3.3-5.0
	(% of Seedlings Diseased*)		
EET 400	97.2 a**	95.8 a	97.5 a
SCA 12	92.9 ab	96.1 a	97.5 a
SCA 6	80.8 b	85.8 b	95.0 a

* Means of four inoculation dates.

** Means, within columns, followed by the same letter are not statistically different according to Duncan's multiple range test ($p=0.05$).

Table 3-8. Comparison among inoculation dates in a factorial experiment in which three cacao families (SCA 6, SCA 12, and EET 400) were inoculated at three stages of flush development with Crinipellis perniciosa basidiospore suspensions at a concentration of 10,000 basidiospore/ml.

Date	Inoculated Seedlings	% of Seedlings Diseased*
I	182	95.7 a**
II	182	93.3 a
III	180	93.3 a
IV	160	89.8 a

* Date means (averaged over all families and flush sizes).
 ** Means followed by the same letter are not statistically different according to Duncan's multiple range test (p=0.05).

Inoculations with fresh and stored basidiospores produced similar percentages of disease. Therefore, the storage methods reported here can be used to coordinate availability of inoculum and susceptible cacao tissue. Short-term storage in the refrigerator would be particularly useful when differences in the timing of inoculum production and seedling availability is a matter of days. Long-term storage in liquid nitrogen can be used in places or times of the year in which brooms and basidiocarp production are limited. Availability of inoculum from different C. perniciosa isolates can also be coordinated with this method

Small flushes with leaves no longer than 1.5 cm were significantly less susceptible than flushes with leaves 3.3-5.0 cm long when inoculated with suspensions calibrated to 10,000 basidiospores/ml. These differences may be the result of physiological changes during the development of the flush, such as availability of carbohydrates (Machado and Hardwick 1987). These changes could affect the tropic response of C. perniciosa germ tubes towards infection sites (see Chapter Two) or the rate of colonization. Differences in disease susceptibility among flush sizes could also be explained by the frequency of infection sites (stomata per cm^2).

Seedlings bearing their first flush seemed to be more susceptible (Table 3-7) than seedlings with cotyledonary or axillary flushes (Table 3-4) and required less growing time in the greenhouse before inoculation. Flushes with leaves

no longer than 1.5 cm were the easiest to identify during seedling growth and to cover uniformly with inoculum. These characteristics may be important to obtain consistent results with the inoculation method, especially for large scale experiments. Therefore, seedlings bearing their first flush with leaves no longer than 1.5 cm seem to be the best material for inoculation. Although this flush size was not the most susceptible, 100% disease was readily obtained when concentrations higher than 10,000 basidiospores/ml were used for inoculation (Table 3-1, 3-2).

Longer incubation time was required for infection of seedlings inoculated with suspensions calibrated to 200,000 basidiospores/ml than for seedlings inoculated with 100,000 basidiospores/ml (Fig 3-3), but maximal infection was achieved at 14-15 hr with both inoculum concentrations. In this study, 24 hr of incubation time were considered appropriated to evaluate the resistance of cacao families inoculated with 10,000 to 75,000 basidiospores/ml; this incubation time exceeded considerably the requirement for maximal infection.

The responses of cacao families to inoculation were highly reproducible (Table 3-1, 3-2, 3-5, 3-8). This was probably achieved by controlling viable inoculum concentration, incubation conditions, and inoculating seedling at the same stage of development. However, a slight variation in the methodology may give rise to inconsistent results. For example, the percentages of

diseased axillary flushes were higher on seedlings from which hardened leaves were removed a day before inoculation than on seedlings in which hardened leaves were removed just before inoculation (Table 3-5). This suggests that changes in the physiology of the flush produced by pruning the leaves may significantly affect the susceptibility of cacao seedlings. Hardened leaves are believed to serve as sources of growth regulators involved in the control of cacao flushing (S.ABU-Hamed et al. 1981).

Inoculation of cacao seedlings with suspensions calibrated to 25,000 to 75,000 basidiospores/ml consistently resulted in 100% disease on families from both susceptible and resistant clones (Table 3-1, 3-2). Significant differences among families were detected only when seedlings were inoculated with basidiospore concentrations that produced less than 100% disease in progeny of susceptible clones such as Catongo. The SCA 6 and SCA 12 families were consistently differentiated from the Catongo family when axillary flushes bearing leaves no longer than 1.5 cm were inoculated with 10,000 or 12,500 basidiospores/ml; at this inoculum density, 77-88% of the seedlings from the susceptible clone (Catongo) were diseased (Table 3-4). When the inoculum density was increased to produce nearly 100% disease in Catongo, the differences were no longer detected (Table 3-4B). This suggests that resistance is overcome at high inoculum concentrations and that a low percentage of the progeny from Catongo may have similar levels of

resistance to those in the progeny from SCA 6 and SCA 12. Although inoculum concentrations that produce nearly 100% disease are not useful to detect differences among families, such concentrations may be useful for screening large cacao populations to select the seedlings with the highest levels of resistance.

Increasing inoculum concentration in cacao plantations in Ecuador was suggested by Suarez and Robinson (Robinson 1982) as one of the main factors conducive to the "break down" of SCA 6 resistance in that country. An alternative hypothesis implies the presence of a pathogenic race capable of infecting this clone and its progeny. Data obtained in this study supports the hypothesis of Suarez and Robinson because SCA 6 progeny showed some resistance to inoculation with the Ecuador source of inoculum (Table 3-4, 3-7) at concentrations of 10,000 and 12,500 basidiospores/ml but responded as susceptible to the inoculation with 75,000 basidiospore/ml.

The developed inoculation method has several practical advantages in regard to its suitability for integration into cacao breeding programs. No specific schedule for inoculation is required since basidiospores can be collected for periods of time as long as 24 hr, permitting inoculum preparation at any time during the day. This characteristic is a practical requirement for an inoculation method that is to be used as a routine procedure in breeding programs.

Efficiency in the use of greenhouse space and the relative short time required to evaluate resistance of cacao seedlings to WB are two characteristics that would facilitate the use of the developed inoculation method in breeding programs. Fifty days after planting, approximately 95% of the infected seedlings displayed symptoms. This implies that greenhouse space could be reused every 2 months. Since each seedling occupies only 6 cm² of space, an average sized greenhouse would provide enough space to evaluate several thousand seedlings every 2 months.

The number of seedlings that can be inoculated using the developed inoculation method would probably be limited only by space available for incubation. In the experiments conducted here, seedlings were incubated in a dew chamber with a capacity for 200 seedlings. However, chambers with more capacity can be used. The main requirement that the incubation chamber has to meet is to maintain a water film on the surface of the flushes during the incubation time. This could be achieved in mist chambers in which mist is regulated to prevent washing-off the inoculum, or in humidity chambers (100% RH) in which the temperature can be maintain below the dew point. When these types of chambers are used, basidiospore suspensions in 0.04% agar may facilitate keeping the flush properly wet during incubation. Such suspensions were effective when used to inoculate trees in the field where incubation conditions could not be standardized (see Chapter Four).

The method developed here is efficient, highly reproducible and could be used in breeding programs to evaluate resistance and screen large seedling populations in a short period of time.

CHAPTER FOUR
EVALUATION OF RESISTANCE TO WITCHES' BROOM DISEASE IN CACAO
SEEDLINGS AND TREES IN ECUADOR

Introduction

Attempts to breed cacao (Theobroma cacao L) for resistance to witches broom disease (caused by Crinipellis perniciosa (Stahel) Singer in Ecuador have been unsuccessful. After years of effort, no resistant variety or clone has been released to control this devastating disease. A similar situation occurs in most of the South American countries where the disease is severe.

Several factors have contributed to the limited progress in breeding cacao for resistance to witches broom (WB), but probably the most important factor is the lack of an efficient and reliable method to evaluate resistance. Field evaluations have produced inconsistent results from one year to another, (INIAP 1973, Chalmers 1972, Bartley 1977, 1986, Baker and Crowdy 1942, Baker and Dale 1944). The time (5-10 years) and space required for field evaluations have further complicated any breeding work. Artificial inoculation of seedlings with basidiospores of C. perniciosa is an alternative method to evaluate resistance to this disease. However, this approach has also failed to provide reliable results (INIAP 1982/83, Bartley 1958, 1959, 1977, 1986, Bartley and Amponsah 1966, Capriles and Rojas

1977). Apparently, variable inoculum concentrations applied to the plant and the conditions used to incubate the inoculated plants were responsible for this failure. When these factors are controlled, differences in the progeny from resistant and susceptible clones can be consistently detected (see Chapter Three).

Improvement of cacao resistant to WB in Ecuador was initiated with the selection of seedlings from cacao populations that showed variability in their response to the disease (Desrosiers et al 1955, Ampuero 1960). Seedlings that survived natural infection and inoculation with *C. pernicioso* basidiospores were grown in experimental plots; progeny from these screened trees had significantly fewer diseased seedlings than progeny from nonscreened plants (Desrosiers et al 1955). Based on these results, Desrosiers et al (1955) suggested a recurrent selection strategy to breed cacao for resistance to WB in Ecuador.

The recurrent selection strategy was discontinued in the early 1950s in favor of an interclonal hybridization program. Clones that showed little or no infection in the field, such as SCA 6, SCA 12, Silecia 1, and Silecia 5, were crossed with commercially acceptable clones (Ampuero and Alvarado 1960). Several of these hybrids showed some degree of resistance, but disease incidence increased with time, and by the early 1970's the hybrids were no longer considered resistant (INIAP 1973, Chalmers 1972). The clones also developed high incidences of the disease.

Two hypotheses have been proposed to explain the change in response in the clones and hybrids originally classified as resistant in Ecuador. One implies the presence of pathogenic races capable of infecting the clones previously termed resistant (Bartley 1967a, 1986 Chalmers 1972); the other hypothesis suggests an increase in the amount of inoculum as the main factor responsible for the change (Robinson 1974).

The resistance of progeny from clones or hybrids that were classified as resistant in 1950s and 1960s was evaluated with the inoculation method described in chapter three. In addition, trees evaluated in the field from 1983 to 1985 were inoculated to examine the relationship between the response of cacao to natural infection and artificial inoculation. The objectives of this study were to determine whether resistance to WB can be detected in these materials using inoculum from the present population of *C. perniciosa*, and further to assess the potential of this inoculation method to evaluate cacao in breeding programs for resistance to this disease.

Materials and Methods

This research was conducted in laboratories, greenhouses and experimental plots at Estacion Experimental Tropical Pichilingue, Instituto Nacional de Investigacion Agropecuaria, Ecuador in 1986.

Plant Material

Cacao seeds, from which the testae were removed, were planted in bamboo cane-node halves or in 7-X 15-cm perforated plastic bags containing soil collected at the station and mixed 3:1 (vol/vol) with balsa wood dust. Seedlings were grown in the greenhouse at 20-38 C.

Seedlings (3-4 weeks old) with their first flush bearing leaves no longer than 1.5 cm or with flushes at the cotyledonary node, induced by pruning 5-to 6-week old seedlings above the cotyledons, were selected for inoculation. Twenty seeds were planted in each bamboo pot, but only the seedlings at the same stage of development (leaves between 0.3-1.5 cm long) were left in the pot for inoculation. Seedlings in plastic bags were selected individually from the batch as they reached the stage of flush development required for inoculation.

Trees (4 years old) grown in the field were induced to flush by pruning. In addition, terminal buds, and the four to six leaves closest to the bud were removed to stimulate the production of axillary flushes; the remaining leaves were cut perpendicularly to the mid rib, leaving only 1/5 attached to the petiole. Axillary flushes selected for inoculation had two to four leaves no longer than 2.0 cm.

Inoculation Method

Inoculum production. Basidiocarps of *C. pernicios* were produced on brooms collected from cacao plots at Pichilingue in which several clones were planted. One end

of the broom was tied with metallic wire and hung vertically from chicken wire tables in a screen house. Irregular wet and dry periods were provided with a sprinkler system to stimulate basidiocarp production (Rocha and Wheeler 1982).

Inoculation of seedlings. Seedlings were inoculated with basidiospore suspensions at concentrations of 25,000-150,000 basidiospores/ml. These inoculum concentrations produced 30-95% infection in a preliminary inoculation test made with open pollinated seedlings from a susceptible clone (Tenguel 15). Basidiospore suspensions were prepared as detailed in Chapter Three. Flushes of the seedlings selected for inoculation were sprayed with approximately 0.6-0.8 ml of suspension with a chromist atomizer (Gelman Sciences Inc. Ann Arbor, MI 48106), placed immediately into a mist chamber, and incubated for 24 hr.

The mist chamber was a canvas tent, 5 m long by 4 m wide by 3.5 m high, placed under the canopy of trees (Teca sp). The tent was supported with a bamboo cane frame; inside the tent, cane posts and metallic wire were used to construct four palm-leaf walls and an access door. Along the top of the walls water was supplied with a perforated plastic hose to create a water curtain. Two nozzles on top of the tent, one in the front and one in the back, delivered water to the surface of the tent during incubation of inoculated plants. In addition, a humidifier in the tent provided free water to the atmosphere to assure that inoculated tissue remained wet. When water was not

available to supply the palm leaf walls and the surface of the tent, the humidifier was essential to maintain adequate moisture for infection. Temperature in the chamber varied from 18-28 C.

Inoculation of trees. Trees (4 years old) were inoculated with suspensions containing 150,000 basidiospores/ml in 0.04% agar. A stock suspension of 750,000 basidiospore/ml was prepared according to the method detailed in Chapter Three and diluted 1:4 with 0.2% autoclaved water agar to obtain suspensions with 150,000 basidiospores/ml and 0.04% agar. Approximately 0.6-0.8 ml was sprayed onto axillary flushes with a chromist atomizer; the flushes were covered for at least 36 hr with plastic bags lined with wet paper towels. The open end of the bag was sealed to the plant stem with parafilm.

Evaluation of Resistance

The levels of resistance of progenies or individual trees were estimated based on the percentages of inoculated flushes that developed brooms and swellings. Unless otherwise stated, inoculated plants were examined every month for 3 months. Flushes with swellings at the petioles and pulvini were considered as diseased, even when brooms did not develop during the evaluation period.

Progeny Evaluations in The Greenhouse:

Crosses among resistant and susceptible clones.

Seedlings from three interclonal crosses and one open pollinated clone were grown in bamboo cane-node halves.

Cotyledonary flushes were inoculated with basidiospore suspensions at five different inoculum concentrations ranging between 25,000 to 150,000 spores/ml. Groups of 10-40 flushes per inoculum density per variety were inoculated on four different dates.

The crosses evaluated were ICS 6 X Silecia 1, Tenguel 33 X Silecia 1; and IMC 67 X Tenguel 15. Open pollinated seedlings from Tenguel 15 were used as a susceptible control. According to evaluations made in the 1950s, Silecia 1 and ICS 6 are highly resistant and IMC 67 is moderately resistant. Tenguel 15 and 33 are highly susceptible.

Silecia 1 was selected in Ecuador in 1949 for its resistance to WB (Soria 1970); from 1956-1959, this clone showed a level of resistance even higher than SCA 6, and SCA 12 (Ampuero and Desrosiers 1960). The clones with which Silecia 1 was crossed, ICS 6 and Tenguel 33, were classified as 4th (resistant) and 63rd (susceptible) among 77 clones evaluated in Pichilingue in 1951 (Desrosiers et al 1955). IMC 67 is a clone with intermediate levels of resistance; in a evaluation of 50 clones, IMC 67 was classified as the 19th best in 1954 and the 5th best in 1956 (Muntzing 1958). Tenguel 15 has good agronomic characteristics, but it is very susceptible to WB. The inoculated Silecia crosses were evaluated in 1963 (Enriquez 1963); ICS 6 X Silecia 1 was significantly more resistant than Tenguel 33 X Silecia 1.

Open pollinated resistant trees. Open pollinated seeds were harvested from from four experimental plots planted at Pichilingue in 1966 with interclonal crosses of SCA 6, SCA 12, Silecia 1 and Silecia 5. Trees grown in these plots survived inoculation with C. perniciosa at the seedling stage (INIAP 1982/83). Open pollinated progeny from Tengel 15 were used as susceptible controls. Groups of 32-155 cotyledonary flushes on seedlings grown in plastic bag pots were inoculated with 75,000 basidiospores/ml on four different dates. The percentage of flushes diseased in the first two inoculation dates was quantified as described before (see Evaluation of Resistance); on the third and fourth dates, the percentage of disease was based on the number of seedlings that survived and showed no symptoms one year after inoculation.

Reinoculation of Selected Seedlings

Open pollinated seedlings from Tengel 15, grown in plastic bags, and bearing their first flush, were inoculated with 75,000 basidiospores/ml. Seventeen seedlings out of 150 inoculated seedlings were selected because they did not show symptoms during the development of the first flush. These seedlings were reinoculated when the second flush had two leaves no longer than 1.5 cm. The same type of flush on non-selected seedlings of the same age and family were also inoculated in order to determine whether differences in resistance between selected and non-selected seedlings could be detected.

Evaluation of Trees in The Field

Eighteen trees (4 year old) belonging to four different interclonal hybrids were inoculated in the field with 150,000 basidiospores/ml. These trees were selected based on their similar vigor, but contrasting differences in the percentage of diseased flushes registered from 1983-1985 by Mongrovejo and Maddison¹ (1987). Nine to 65 axillary flushes were inoculated to evaluate the response of each tree.

Trees from the following hybrids were evaluated: UF 29 X Santa Rosa 34, Tenguel 33 X Silecia 1, Pandora 3 X Pichilingue 1, and Pandora 1 X Pound 12. Silecia 1, Pandora 3, Pichilingue 1 and Pound 12 are clones selected for their resistance to WB. Susceptible clones with good agronomic characteristics include UF-29, Santa Rosa 34, and Tenguel 33.

It was not possible to coordinate flushing in all of the trees or to inoculate more than two to four trees per day; therefore, at least two trees, one with a history of high WB incidence and another with low incidence, were inoculated every day to assure that lack of infection was due to the resistance of the tree and not to failure of the inoculation technique. In addition, some of the trees were inoculated on two different dates to confirm their response

¹ Data on individual trees within the experimental plot and assistance in the development of this experiment was kindly provided by Ing. Eduardo Mongrovejo, and Dr. Alan Maddison, Programa de Cacao, and Departamento de Fitopatología, EET Pichilingue, Ecuador.

and to determine whether results obtained with the inoculation method were reproducible. Linear correlation analysis was applied to examine the relationship between the response of the trees to inoculation and the percentage of naturally infected flushes registered during 1983-1985 by Mongrovejo and Maddison (1987).

Analysis of Data

Greenhouse experiments were designed as a randomized complete block in which inoculation dates were considered as blocks. The arcsine transformation (Little and Hills 1978) was applied to the data; analyses of variance of the transformed data were done with the GLM procedure in SAS (Statistical Analysis System, SAS Institute Inc., Cary, NC). Means of disease percentages were statistically compared with Duncan's multiple range test; the error term used for this purpose was the error mean square in the analysis of variance table obtained with the GLM procedure.

Disease incidence on trees inoculated the same day were compared with a Z test, while trees inoculated on two dates were compared with Duncan's multiple range test as described above. The relationship between the susceptibility of cacao trees to artificial inoculation and natural infection was evaluated with a correlation analysis.

Results

Progeny Evaluations in The Greenhouse:

Crosses among resistant and susceptible clones.

Progeny from the cross between two resistant clones, ICS 6 X

Silecia 1, had the lowest average percentage of seedlings diseased, except at the lowest level of inoculum (Fig 4-1). In contrast, progeny from the susceptible clone Tenguel 15, either open pollinated or crossed with a clone with intermediate level of resistance (IMC 67), had the highest percentage of disease (Table 4-1; Table A-4, appendix). An intermediate response was observed in the progeny from the cross between the susceptible Tenguel 33 with the resistant Silecia 1. Data from all inoculum levels were pooled to compare the overall response of the hybrids (Table 4-1). Three groups were differentiated according to Duncan's multiple range test: ICS 6 X Silecia 1 had significantly fewer diseased flushes than the other crosses; Tenguel 33 X Silecia 1 had significantly less disease than the susceptible control but was not different from the cross IMC 67 X Tenguel 15. This latter cross could not be differentiated from the susceptible control (open pollinated Tenguel 15).

Open pollinated resistant-trees. Open pollinated seedlings from resistant hybrid trees had significantly less disease than the susceptible control Tenguel 15 (Table 4-2; Table A-5, appendix). Differences among the progeny of the resistant hybrids were not significant.

Reinoculation of Selected Seedlings

Open pollinated Tenguel 15 seedlings, selected for their resistance to inoculation with *C. pernicios*a basidiospores, and reinoculated, had significantly less

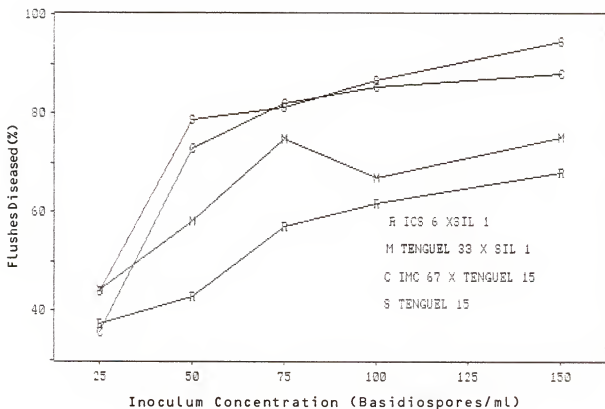


Fig. 4-1. Responses of cacao seedlings from three interclonal crosses and one open pollinated clone to inoculation with suspensions of *Crinipellis pernicioso* basidiospores at five concentrations. Each point represent the average percentage of flushes diseased from three or four inoculation dates.

Table 4-1. Responses of cacao seedlings from three interclonal crosses and one open pollinated clone to inoculation with basidiospore suspensions of Crinipellis perniciosa at concentrations ranging from 25,000 to 150,000/ml.

Family	Inoculated Flushes	% of Flushes Diseased*
Tenguel 15***	455	77.1a**
IMC 67 X Tenguel 15	311	73.7ab
Tenguel 33 X Silecia 1	302	64.4 b
ICA 6 X Silecia 1	759	53.2 c

* Family means (averaged over all inoculum concentrations and inoculation dates). A nonsignificant interaction between family and inoculum concentration allowed comparison among family means).

** Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p=0.05$).

*** Open pollinated seedlings from Tenguel 15 were used as susceptible control.

Table 4-2. Responses of open pollinated progeny from four selected cacao hybrids and one susceptible clone (Tenguel 15) to inoculation with suspensions of Crinipellis perniciosa basidiospores at a concentration of 75,000 basidiospores/mL.

Parental* Material	Inoculated Flushes	% of Flushes Diseased**
Tenguel 15***	304	81.5a ³
SCA 12 X desconocido	383	50.8 b
SCA 6 X Silecia 1	202	50.5 b
SCA 12 X Silecia 1	279	42.5 b
SCA 6 X Silecia 5	333	42.4 b

* Hybrid trees, from which open pollinated progeny were obtained, survived inoculation at seedling stage in 1966 (INIAP 1966).

** Means of four inoculation dates (replications). Tenguel 15, and SCA 6 X Silecia 1 were inoculated only on three and two inoculation dates, respectively. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p=0.05$).

*** Open pollinated susceptible control.

disease than non-selected seedlings of the same age and family (Table 4-3).

Evaluation of Trees in The Field

Significant differences in the percentages of diseased flushes were observed among trees inoculated the same day and among the average response of trees inoculated twice (Table 4-4, Table 4-5; Table A-5, appendix). These differences were observed among trees of different hybrids and among trees of the same hybrid. The response of trees inoculated twice was consistent; no significant differences between the first and second inoculations were detected (Table 4-6). During the development of this experiment (June-September 1986) only two of 300 flushes used to evaluate natural infection developed brooms.

The percentage of diseased flushes per tree after artificial inoculation and the percentage of naturally infected flushes registered by Mongrovejo and Maddison (1987) during 1983-1985 were not correlated; the correlation coefficient "r" was not significantly different for zero (Fig 4-2).

Discussion

Results from the percentages of infected flushes in interclonal hybrid seedlings are in agreement with field evaluations made in the 1950s and 1960s (Desrosiers et al 1955, Ampuero and Desrosiers 1960, Enriquez 1963). The percentage of disease flushes varied according to the level of resistance of the parental clones. The hybrids between

Table 4-3. Responses of selected and nonselected, open pollinated cacao seedlings from tenguel 15 to inoculation with suspensions of Crinipellis perniciosa basidiospores at a concentration of 75,000 basidiospores/ml.

Type of seedlings	Inoculated Seedlings	% of Seedlings Diseased
Nonselected	44	38.6a**
Selected*	17	17.6 b

* Seedlings that did not develop symptoms after inoculation of their first flush with 75,000 basidiospores/ml.

** Percentages followed for the same letter are not significantly different according to a Z test ($p=0.05$).

Table 4-4. Responses of interclonal hybrid trees located in the field to natural infection and to inoculation with suspensions of *Crinipellis pernicios*a basidiospores at a concentration of 150,000 basidiospores/ml.

Interclonal Hybrid	Tree*	Natural** Infection (%)	Artificial Infection (%)	Inoculated Flushes	
UF 29 X EET 48	II-1	18.7	100.0a***	33	
	IV-10	28.2	88.2	34	
Tenguel 33 X	I-12	8.2	89.2b	28	
Silecia 1	IV-2	9.9	95.0	20	
	II-15	7.4	100.0	10	
Pandora 3 X	I-1	6.0	97.3c	38	
Pichilingue 1	I-15	5.6	22.2b	18	
	IV-4	4.7	93.4	46	
	IV-12	4.5	23.8	21	
	IV-3	2.8	62.5	32	
	II-3	1.8	100.0	9	
	III-7	1.3	91.3d	23	
	II-10	0.6	64.6d	65	
	IV-10	0.6	76.7	43	
	Pandora 1 X	II-5	1.4	80.0	10
	Pound 12	III-6	1.6	20.0ac	25
I-15		1.2	84.2	19	
IV-14		0.5	5.9	17	

* Block and number assigned to the tree in the experimental plots.

** The percentage of naturally infected flushes was evaluated from 1983-1985 by Mongrovejo and Maddison (1985), who kindly provided us with data on the selected trees.

*** Percentages followed by the same letter indicate trees that were inoculated the same day and are significantly different according to an approximated Z test ($p=0.05$).

Table 4-5. Mean percentage of flushes diseased on hybrid cacao trees in the field inoculated on two different dates with suspensions of Crinipellis perniciosa basidiospores at a concentration of 150,000/ml.

Interclonal Hybrid	Tree	Inoculated Flushes	% of Flushes Diseased*
Pandora 3 X Pichilingue 1	I-1	38	98.0a*
Pandora 1 X Pound 12	I-15	19	83.0ab
Pandora 3 X Pichilingue 1	II-10	65	64.5 b
Pandora 3 X Pichilingue 1	I-15	18	27.7 c
Pandora 1 X Pound 12	III-6	25	18.0 c

* Mean of the percentage of diseased flushes from two inoculation dates. Means followed by the same letter are not significantly different according to Duncan's multiple range test ($p=0.05$)

Table 4-6. Response of cacao trees (4 years old) to a second inoculation of axillary flushes with basidiospore suspensions at a concentration of 75,000 basidiospores/mL.

Inoculation No.	Inoculated Flushes	% of Flushes Diseased*
First	93	61.7a
Second	72	54.7a

* Average of the response of five inoculated trees. Percentages followed by the same letter are not significantly different according with Duncan's multiple range test.

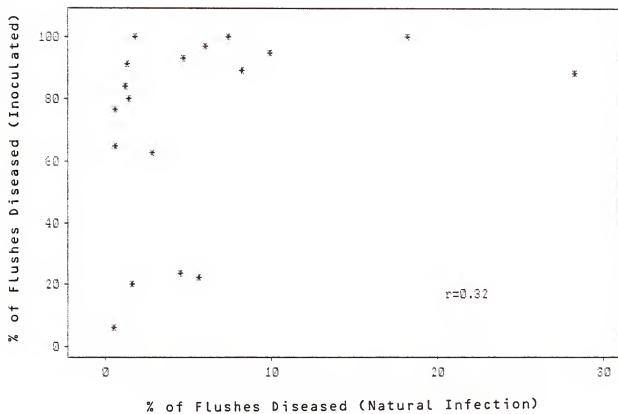


Fig. 4-2. Relationship between the percentage of flushes diseased on 4 year old trees by natural infection and by artificial inoculation with Crinipellis pernicios basidiospores at a concentration of 150,000/ml. The correlation coefficient $r=0.32$ was not significantly different from 0.

two clones reported as resistant (Silecia 1 and ICS 6) had the lowest percentage of disease. Intermediate or high percentages of disease were observed in hybrids between susceptible X resistant (Tenguel 33 X Silecia 1) and susceptible X moderately resistant (Tenguel 15 X IMC 67), respectively. The response of the two Silecia hybrids inoculated here agrees with field evaluations made on the same hybrids in 1963 by Enriquez (1963).

Hybrids between resistant clones, such as Silecia 1, Silecia 5, SCA 6, and SCA 12, that survived inoculation with C. perniciosa at seedling stage in 1966 (INIAP 1982/83), produced progeny more resistant than progeny from a susceptible clone (Tenguel 15).

These data do not support the hypothesis that a new pathogenic race is responsible for the increased incidence of WB in clones previously considered resistant in Ecuador. Similar observations were recently reported in Trinidad (Laker, Sreenivasan, and Kumar 1987). On the other hand, evidence to support the hypothesis that an increase in inoculum concentration is responsible for this change in disease incidence was presented in Chapter Three.

The resistance of progeny from trees that survived inoculation at the seedling stage (Table 4-2) and the response of selected seedlings to reinoculation (Table 4-3) suggest that artificial inoculation can improve the selection of cacao clones with resistance to WB. However, high incidence of WB on selected trees planted in 1966 at

Pichilingue, Ecuador has been reported (INIAP 1982/83). Probably more than one selection cycle or higher inoculum concentration for the selection is needed to obtain resistance levels effective against the inoculum concentration present at Pichilingue.

The inoculum density needed to produce maximal infection in progeny from a susceptible clone was much higher under the experimental conditions in Ecuador than those that we have used in Gainesville, Florida (Chapter Three). Nearly 100% infection was obtained in Gainesville with a inoculum density of 25,000 basidiospores/ml, whereas, in Ecuador, inoculated progeny from the same clone needed more than 75,000 basidiospores/ml from the same inoculum source (brooms from Pichilingue, Ecuador) to produce nearly 100% infection. This difference may be due to seedling growing conditions such as soil type, temperature, and light or to differences in temperature and leaf wetness during incubation of the plants after inoculation.

Hybrid trees (4 years old) with relatively high percentages of naturally diseased flushes during 1983-1985 (Mongrovejo and Maddison 1987) were very susceptible to inoculation with 150,000 basidiospores/ml (Table 4-4). A few trees with low percentages of naturally infected flushes also had relatively low percentages of flushes diseased after artificial inoculation. Lack of correlation between natural and artificial infection (Fig 4-2) was mainly due to trees that were uninfected by natural inoculation (less than

5% of diseased flushes) but susceptible to artificial inoculation. These trees may have escaped natural infection or they may have levels of resistance that were overcome by the basidiospore concentration used in artificial inoculations.

According to the results, of this research, artificial inoculation could play an important role in the selection of clones for breeding programs because it would facilitate the elimination of trees that escape natural infection but are not resistant to WB. In addition, the inoculation method used here allowed evaluation of trees in situ. Thus, cloning would not be necessary for selection of resistant trees; and the time and effort needed to select WB resistant trees would be minimized.

Important factors that have contributed to the limited progress in breeding cacao for resistance to WB in Ecuador are the breeding strategies and lack of reliable inoculation methods. Expectations from the interclonal hybridization program initiated in 1951 (Ampuero 1960) were based on the belief that some clones were immune to the disease and that this character could be transferred to other clones as a qualitative trait. However, the response of progeny from resistant clones has shown that this character may be a quantitative trait; for example, most of SCA 6 and SCA 12 progeny in Trinidad are less resistant than the parental (Bartley 1977). This phenomenon, known as regression (Simmonds 1979), occurs with quantitatively inherited traits

when the progenitor tree is among the best in its family, as is the case with SCA 6 resistance to WB (Bartley 1967b). Regression also occurs in SCA 6 resistance to black pod disease (Phytophthora spp), which has been determined to be a quantitatively inherited trait (Rodriguez-R. 1983).

Regardless of the mechanism of inheritance of WB resistance, a breeding strategy can be designed to increase resistance to WB at the same time that cacao production and quality characteristics are improved. Selection of the parental material (genetic base) may be facilitated by inoculation of promising trees with C. perniciosa basidiospores. Polycrosses among the selected trees and screening of the progeny for resistance to WB may result in hybrid vigor and high levels of resistance. Selection of trees could then be emphasized in field evaluations of agronomic characters. Polycrosses among selected trees, screening for WB resistance and field selection for production and quality may be repeated several times to further improve desirable traits and to reach levels of resistance to WB that are effective in the field.

The method for inoculation of trees described here and the one developed for inoculation of seedlings (see Chapter Three) are potentially useful in cacao breeding programs since they can be used to evaluate cacao families, select trees, and screen progeny for resistance to the witches' broom disease.

CHAPTER FIVE SUMMARY AND CONCLUSIONS

A reliable inoculation method was developed to test cacao seedlings and trees for resistance to witches' broom disease. Knowledge gained about the infection process of Crinipellis perniciosa on cacao and development of new techniques for collecting and storing basidiospores were incorporated into an inoculation procedure modeled after a system for evaluating resistance of pine seedlings to fusiform rust. The efficiency of the method and its potential use in cacao breeding programs was evaluated in Gainesville, FL and Ecuador by repeated inoculation of open pollinated and hand pollinated cacao families and by inoculation of trees under field conditions. The conclusions of this research are summarized.

Infection Process

1. Optimal basidiospore germination and host penetration occurred when spores were deposited onto wet plant surfaces and the tissue was maintained wet while these processes took place.

2. Germ tube tropism towards stomata on young flushes and towards trichome bases on fully developed, but non-hardened, flushes were commonly observed on inoculated plants.

3. Basidiospore germ tubes entered stomata on young flushes. Entrance was also observed through the base of fallen or collapsing trichomes on fully developed, but non-hardened, flushes. The fungus produced subepidermal vesicles within 12 hr.

4. Intercellular hyphae developed from the vesicles in some infection sites, while in others the pathogen failed to colonize host tissue. This failure was associated with a change in the fluorescence of host cells at and around the infection sites. The change in fluorescence may be related to resistance.

Development of The Inoculation Method

5. Basidiospores collected overnight in a solution containing 0.01 M MES, 15-16% glycerol, and 0.01% Tween 20 did not germinate in the solution but were 93-100% viable. Basidiospores collected in this solution and stored in the refrigerator for 3-4 days or in liquid nitrogen for as long as 18 months were as infective as freshly collected basidiospores, although the percentage of germination after storage varied from 37 to 95%.

6. Four to six hours of dew were required for germination and penetration which subsequently resulted in broom development on axillary flushes of 2-month-old seedlings inoculated with 200,000 basidiospores/ml and incubated in a dew chamber at 25 C. Maximal infection was obtained with a dew period of 9-15 hr.

7. The size of the flush on 3- to 4-weeks old seedlings inoculated with 10,000 basidiospores/ml and incubated in a dew chamber at 25 C for 24 hr significantly affected the percentage of diseased seedlings. Flushes with leaves 3.3-5.0 cm long were more susceptible than flushes with leaves 0.3-1.5 cm long. Seedlings with the latter flush size were selected for all other experiments because this type of flush was easier to identify and inoculate than were the larger flushes.

Efficiency of The Inoculation Method

8. Highly reproducible results were obtained in Gainesville, Florida when 3-to 4-week-old seedlings or axillary flushes on 2-month-old seedlings were inoculated with 10,000-75,000 basidiospores/ml and incubated in a dew chamber at 25 C for 24 hr. Differences in resistance among open pollinated cacao families were detected only with inoculum concentrations of 10,000-12,500 basidiospores/ml. At higher inoculum concentrations, the response of resistant and susceptible families were similar with respect to the percentage of flushes that develop witches broom symptoms.

9. Poorly-reproducible results were obtained in Ecuador when cotyledonary flushes were inoculated with 25,000-150,000 basidiospores/ml and incubated in a mist chamber at temperatures varying from 18-28 C. Despite this variation, significant differences in the percentages of infected flushes were detected in hybrid seedlings from crosses

between clones classified as resistant and susceptible in Ecuador during the 1950's and 1960's.

10. The basidiospore concentration required to produce nearly 100% disease was higher in experiments conducted in Ecuador than in those conducted in Gainesville, FL. This probably was due to the different growing conditions and/or variable temperatures and tissue wetnesses during incubation of inoculated plants.

11. Open pollinated progeny from trees that survived inoculation with Holliday's method at the seedling stage in 1966 had significantly less disease than progeny from a clone designated susceptible.

12. Seedlings selected for their resistance to inoculation with 75,000 basidiospores/ml had significantly less disease upon reinoculation, when compared with non-selected seedlings of the same family and age.

12. Significant differences in the response of 4-year-old trees inoculated in the field were observed. Some of the trees with low disease incidence when exposed to natural infection during 1983-1985 were highly susceptible to artificial inoculation. These trees may have escaped natural infection or may have levels of resistance that were overcome by the inoculum concentration applied (150,000 basidiospores/ml).

Basic information obtained in this study on the infection process, the response of cacao plants to infection, and the relationship between inoculum

concentration and resistance is a contribution to the knowledge of the C. perniciosa-I. cacao interaction, which could be used to develop effective breeding strategies. In addition, the methods developed here, are useful tools for selection of resistant plants and evaluation of large quantities of seedlings in short periods of time.

APPENDIX

Table A-1. Summary for the Anova f-test for the factorial experiment in which cotyledonary flushes of cacao on open pollinated EET 400 seedlings were inoculated with *Crimipellis perniciosae* basidiospore suspensions stored for 0 and 18 mo in liquid nitrogen. Flushes were inoculated with suspensions at inoculum concentrations of 10,000 and 25,000 basidiospores/ml.

Dependent Variable: Y (aresin % of flushes diseased/100)

Source	DF	Sum of Squares	Mean Square	F value	PR > F	R-Square	C.V.
Model	5	315.31	63.06	0.98	0.5205	0.55	10.34
Error	4	256.17	64.04		Root MSE		Y mean
Corrected Total	9	571.49			8.00		77.41
Source	DF	Anova SS	F value	PR > F			
Storage Time (ST)	1	54.14	0.85	0.4099			
Inoculation Date	2	108.29	0.85	0.4940			
Inoc. Concentration (IC)	1	42.30	0.66	0.4620			
ST * IC	1	45.57	0.71	0.4564			

Table A-2. Summary for the Anova f-test for the factorial experiment in which three cacao families (SCA 6, SCA 12, and Catongo) were inoculated with Crinipellis perniciosa basidiospore suspensions at concentrations ranging from 12,500 to 75,000/mL.

Source		DF	Sum of Squares	Mean Square	F value	PR > F	R-Square	C.V.
Model		15	17621.55	1174.77	35.09	0.0001	0.95	8.96
Error		29	971.04	33.48	33.48		Root MSE	Y mean
Corrected Total		44	18592.56				5.79	64.59

Source		DF	Anova SS	F value	PR > F
Family (Fam)		2	1204.76	17.99	0.0001
Inoc. Concentration (IC)		5	5117.89	30.57	0.0001
Inoculation Date		4	7912.98	59.08	0.0001
Fam * IC		4	557.14	4.16	0.0088

Table A-3. Summary for the Anova f-test for the factorial experiment in which seedlings of three cacao families (SCA 6, SCA 12, and EET 400) at three stages of development (flush size) were inoculated with Crinipellis pernicioso basidiospore suspensions at a concentration of 10,000/ml.

Dependent Variable: Y (arcsin % to flushes diseased/100)

Source	DF	Sum of Squares	Mean Square	F value	PR > F	R-Square	C.V.
Model	11	2194.89	199.54	3.56	0.0049	0.63	9.55
Error	23	1287.92	56.00	Root MSE		Y mean	
Corrected Total	34	3482.80		7.48		78.32	

Source	DF	Anova SS	F value	PR > F
Family	2	1185.83	10.59	0.0005
Flush Size	2	375.54	3.35	0.0527
Inoculation Date	3	334.40	1.99	0.1435
Family * Flush Size	4	162.24	0.72	0.5843

Table A-4. Summary for the Anova *t*-test for the factorial experiment in which seedlings from three interclonal crosses and one open pollinated clone were inoculated with *Crinipellis verniciosa* basidiospore suspensions at concentrations ranging from 25,000 to 75,000/ml.

Dependent Variable: Y (arcsin % of flushes diseased/100)

Source	DF	Sum of Squares	Mean Square	F value	PR > F	R-Square
C.V.						
Model	22	14118.17	641.73	5.50	0.0001	0.7218.93
Error	47	5481.34	116.62		Root MSE	Y mean
Corrected Total	69	19599.51			10.80	57.05

Source	DF	Anova SS	F value	PR > F
Family	3	2789.17	7.97	0.0002
Inoc. Concentration (IC)	4	6785.55	14.55	0.0001
Inoculation Date	3	3135.29	8.96	0.0001
Fam * IC	12	962.20	0.69	0.7547

Table A-5. Summary for the Anova f-test for the experiment in which five open pollinated cacao families were inoculated with Crinipellis perniciosa basidiospore suspensions at a concentration of 75,000/ml.

Dependent Variable: Y (arcsin % of flushes diseased/100)						
Source	DF	Sum of Squares	Mean Square	F value	PR > F	R-Square
C.V.						
Model	7	0.4389	0.0627	22.82	0.0001	0.9510.02
Error	9	0.0247	0.0027		Root MSE	Y mean
Corrected Total	16	0.4637			0.052	0.523
Source	DF	Anova SS	F value	PR > F		
Family	4	0.2520	22.93	0.0001		
Inoculation Date	3	0.1040	12.62	0.0014		

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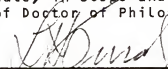
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.




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