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An optimized screening method for identifying levels of resistance to *Crinipellis perniciosa* in cocoa (*Theobroma cacao*)

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The effects of host age, leaf number, host type (clone or seedling), pathogen spore concentration and incubation time on inoculation with *Crinipellis perniciosa* (witches' broom disease of cocoa) were studied in greenhouse experiments using susceptible cocoa genotypes. Three methods of inoculation (agar-drop, water-drop and spray) were also tested. An optimized inoculation method was selected and tested for its repeatability as well as its ability to discriminate between various levels of resistance to *C. perniciosa* in cocoa. The optimized method (350 000 viable basidiospores per mL, 60 h incubation, agar-drop technique) produced 100% infection repeatedly, on both clonal and seedling plants of a susceptible genotype. Seedling age (2–12 months) and leaf number did not significantly affect the percentage of plants with symptoms or broom characteristics. This method discriminated effectively between the various levels of resistance in 14 cocoa genotypes and is recommended as an inoculation method to identify levels of resistance in germplasm collections. Symptom severity was shown to be a better measure of resistance than infection success.

Keywords: *Crinipellis perniciosa*, disease resistance, inoculation method, resistance screening, *Theobroma cacao*, witches' broom disease

Introduction

Witches' broom disease, caused by *Crinipellis perniciosa*, is a devastating disease of cocoa in Latin America and the Caribbean. The pathogen damages vegetative buds, flower cushions and pods (Baker & Holliday, 1957). The disease reportedly causes 30–50% crop loss in South American and Caribbean countries (Evans & Prior, 1987), but losses may be as high as 90% (Rudgard, 1986; Pereira, 2000). Possible future spread of the disease worldwide is considered a serious threat to cocoa production (Wood & Lass, 1985).

Control of the disease by phytosanitary and chemical means is very costly (Thorold, 1943; Zadoks, 1997) and often impractical (Laker & Rudgard, 1989). Genetic resistance therefore remains a long-term objective in managing the devastating effects of the disease in cocoa (Baker & Holliday, 1957; Luz *et al*., 1999). Although several large genetically diverse germplasm collections exist, identifying resistant genotypes through natural or artificial inoculations has produced inconsistent results (Andebrhan & Furtec, 1994). In addition, the utilization of identified resistance to witches' broom disease in resistance-breeding programmes has been hampered by the lack of reliable early screening techniques appropriate for segregating populations (Zadoks, 1997).

An automated spray technique (Purdy *et al*., 1997) using the inoculation method of Frias *et al*. (1995) was reported to be effective in screening for resistance to witches' broom disease among seedling populations, but has not yielded reproducible results with clonal genotypes in cocoa-producing countries (B. Eskes, Cirad-Cp, Programme Cacao, 34398 Montpellier Cedex 5, France, unpublished data). Furthermore, the level of precision obtained with this method, even with seedling populations, is insufficient to screen segregating populations.

Indirect selection methods, such as basidiospore germination using leaf extracts (Evans & Bastos, 1980) and phloem extracts (Bastos & Albuquerque, 2000), have not been developed sufficiently to be used as screening tools. Hence, despite a century of research work, witches' broom disease continues to have a crippling influence on many cocoa-growing areas in Latin America, such as Brazil (Pereira, 1999) and Ecuador (Aragundi *et al*., 1988).

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Standardization of factors that can affect infection success is critical to the development of a screening methodology that is consistent and unambiguous in distinguishing various levels of resistance. Variation in environmental (e.g. humidity, temperature, incubation time), host (e.g. genotype, age, size, leaf number, flush stage) and pathogen (e.g. pathotype, inoculum concentration and application technique) factors can affect the sensitivity and reproducibility of the screening method and these factors need to be carefully optimized. Several studies (Frias, 1987; Frias *et al*., 1991, 1995) have contributed to standardize inoculation techniques, but screening methods to date continue to yield inconsistent results.

This study aimed to develop an inoculation method capable of consistently achieving 100% infection in susceptible cocoa genotypes by considering factors that may affect symptoms. These factors include inoculation method, inoculum concentration, incubation time, host age and leaf number. Experiments to determine the repeatability of the optimized method, as well as its ability to discriminate between various levels of resistance, are described.

Materials and methods

Host genotypes

Seedlings from open-pollinated fruits of three cocoa genotypes (Amelonado, M 8 and Hybrid 19) and 14 clonal genotypes (SCA 6, SCA 12, ICS 1, ICS 84, ICS 95, IMC 57, IMC 67, JA 6/4, JA 5/19, JA 5/41, UF 11, Hybrid 19, M 8 and Amelonado) were used in the study. The clonal genotypes were micrografted onto 3-week-old TSH 919 seedlings, as described by Sreenivasan (1995). The plants were grown in potting bags and maintained in a 70% shadehouse. The plants were irrigated daily and fertilized weekly with foliar applications of Nutrex® 20-20-20 (Marman USA Inc., Tampa, FL, USA).

Inoculum preparation

Necrotic brooms were collected from cocoa trees on the San Juan Estate, Gran Couva, Trinidad. The brooms were surface-sterilized in sodium hypochlorite (0·5% v/v available Cl_2; 30 min), rinsed three times in sterile distilled water and hung in a moist chamber with a controlled temperature of about 25°C and subjected to alternating 8-h wet and 16-h dry periods (Suárez, 1977; Rocha & Wheeler, 1982). Basidiocarps were harvested at the fully turgid stage. Basidiospores were collected from the basidiocarps according to the method of Frias *et al*. (1991) and stored in liquid nitrogen as described by Frias *et al*. (1995). Numbers of basidiospores per mL of collection solution and germination percentages were determined according to Purdy *et al*. (1997). The inoculum was diluted to an appropriate concentration for each study.

Inoculation methods

The agar-drip method (Sreenivasan, 1987) involves placing a single drop (30 μL) of basidiospore suspension (final dilution prepared in 0·3% agar) on the apical bud at a single point using a micropipette. The water-drop method was the same as the agar-drop method, except that the final dilution of inoculum was prepared in sterile distilled water instead of agar. The spray method was a modification of the method of Frias (1987); the inoculum was prepared in the same way as for the water-drop method, but was delivered using a hand-held atomizer (Preval Sprayer, Precision Valve Co., Yonkers, NY, USA). A volume of 1 mL was sprayed from a distance of 10 cm from the flushes.

Inoculation and incubation conditions

Standardized induction of flushing of both seedlings and clones was obtained by pruning the terminal bud, followed by the application of 15 g Blaukorn® 12-12-17-2 fertilizer (BASF, Ludwigshafen, Germany) and regular watering. For seedling inoculation, emerging shoots with young leaves at the primary flushing-2 stage (Greathouse *et al*., 1971) were selected for inoculation as recommended by Frias *et al*. (1995). For inoculation of clonal plants, secondary flushing-2 stage (Greathouse *et al*., 1971) was used, since preliminary experiments using primary flushing-2 stage were unsuccessful.

Following inoculation using one of the methods described earlier, plants were incubated in sealed polythene bags containing moist tissue paper (100% RH) and kept in the dark for a defined period (incubation time). Inoculations were carried out in a 3 × 3 m room fitted with an air conditioner to maintain the temperature inside incubation bags at approximately 25°C. Following incubation, the polythene bags were removed and plants moved to a 70% shadehouse.

Experiments

Inoculum concentration

Six inoculum concentrations (125 000, 175 000, 225 000, 275 000, 325 000 and 375 000 basidiospores mL^{-1}) were tested on 3-week-old Amelonado seedlings (known to be susceptible to witches' broom disease). Inoculations were performed at the primary flushing-2 stage, using the agar-drop method. The experiment was arranged in a completely randomized design with three replications, with each replication consisting of 10 plants. Control plants (two plants per replication) were inoculated with 0·3% water agar only. Following inoculation, plants were incubated for 48 h in the dark and moved later to a 70% shadehouse.

Incubation time and inoculation technique

Nine incubation times (2, 4, 6, 8, 10, 12, 24, 36 and 48 h) were tested using two methods of inoculation (water-drop and agar-drop) on 3-week-old (primary flushing-2) seedlings of genotype M 8, with appropriate controls. The 18 treatment combinations were arranged in a completely randomized design with three replications (10 plants per

replicate). An inoculum concentration of 350 000 viable basidiospores mL^{-1} of *C. perniciosa*, determined from the previous experiment, was used. Incubation and postincubation treatment of seedlings was similar to that described before.

Inoculation techniques

Two inoculation techniques (spray and agar-drop) were tested on two clonal genotypes (UF 11, susceptible; and SCA 6, resistant) of cocoa (12 months old, secondary flushing-2 stage) to determine the method best able to discriminate between resistance levels. An inoculum concentration of 350 000 viable basidiospores mL^{-1} and an incubation time of 60 h were used. The four treatment combinations were arranged in a completely randomized design with four replications, with 10 plants per replication.

Host-plant age

Hybrid 19 seedlings (susceptible to witches' broom disease) at six different ages (2, 4, 6, 8, 10 and 16 months old) were inoculated to determine the effect of age on inoculation success and symptom expression (stem swelling and broom production). All plants were inoculated using the agar-drop method (inoculum concentration of 350 000 viable spores mL^{-1}; incubation time 60 h; primary flushing-2 stage), with appropriate controls. The experiment was arranged in a completely randomized design with three replicates (10 plants per replicate).

Host-leaf number

Ten-month-old-seedlings (Hybrid 19) were used in this study. Leaves were removed from the base upwards to obtain plants with 0, 5, 10, 15 or 20 leaves. The agar-drop technique with an inoculum concentration of 350 000 viable basidiospores mL^{-1} and an incubation time of 60 h was used to inoculate shoots at the primary flushing-2 stage. The experiment was arranged in a complete randomized design with three replicates, with 10 plants per replicate.

Repeatability of the optimized method

Ten-month-old seedlings (Hybrid 19) were inoculated using the standardized protocol (agar-drop method; inoculum concentration of 350 000 viable basidiospores mL^{-1}; 60 h incubation; primary flushing-2 stage) with controls. The experiment was repeated nine times over a 1-year period with three replicates (with each replicate containing 10 plants), to determine the consistency of the percentage of infected plants and symptom expression.

Discrimination of resistance

Ten-month-old micrografted plants of the 14 cocoa clones, described earlier, were inoculated using the standardized protocol to test the optimized method for its ability to distinguish various levels of resistance. The experiment was arranged in a completely randomized design with three replications. Each replication consisted of three plants.

Data collection and analysis

Inoculation success was based on the percentage of plants developing stem swellings and brooms. Susceptibility was measured by the degree of swelling (increase in stem girth) below the inoculation point, or by broom characteristics. Swellings were measured using a Vernier calliper over a 6-week period at weekly intervals. The following broom characteristics were recorded: number of active and inactive shoots; broom-base diameter; height of broom; length of longest active shoot; and broom fresh and dry weights. Brooms were harvested 12 weeks after inoculation (WAI) to obtain fresh and dry weights.

Analysis of variance was carried out on all the data collected to determine the significance of treatment effects, using Minitab© (Version 12; MINITAB Inc., PA, USA). Arcsine or $\log_{10}(X + 4)$ transformations were carried out on percentage data, when indicated. Comparison between genotypic means was done using Duncan's multiple range test (DMRT), and Friedman's test was carried out to determine the significance of the effect of clone on symptom and broom percentages.

Results

Optimum inoculum concentration

Symptoms started appearing 12–14 days after inoculation, initially as stem swellings, but subsequently developing into brooms. The percentage of plants showing symptoms (PPS) steadily increased over the 5-week period following inoculation and remained constant thereafter (Fig. 1). The effect of inoculum concentration on the arcsine percentage of plants with symptoms at 12 WAI was significant ($P < 0{\cdot}05$), and there was a linear increase ($b = 0{\cdot}228$; $R^2 = 96\%$) in the proportion of plants showing symptoms with increasing inoculum concentration up to 225 000 basidiospores mL^{-1}. PPS was greater than 95% at concentrations greater than 275 000 basidiospores mL^{-1} and there were no significant differences. The percentage of plants developing brooms (PPB) at 12 WAI mirrored the same trend, with almost 100% conversion of stem swellings into brooms (data not presented).

Although higher inoculum concentrations increased PPS and thus reduced the chance of escapes, they did not significantly ($P > 0{\cdot}05$) affect the severity of symptoms measured by stem swelling and broom characteristics. Furthermore, higher basidiospore concentrations resulted in a decrease in the standard errors associated with PPS, thereby improving experimental precision (data not presented).

Optimum incubation time and inoculation technique

Inoculation success, measured as $\log_{10}(X + 4)$PPS or $\log_{10}(X + 4)$PPB (12 WAI), was significantly ($P < 0{\cdot}05$) influenced by both incubation time and inoculation technique. The interaction was also significant ($P < 0{\cdot}05$), indicating that inoculation success varied differentially in

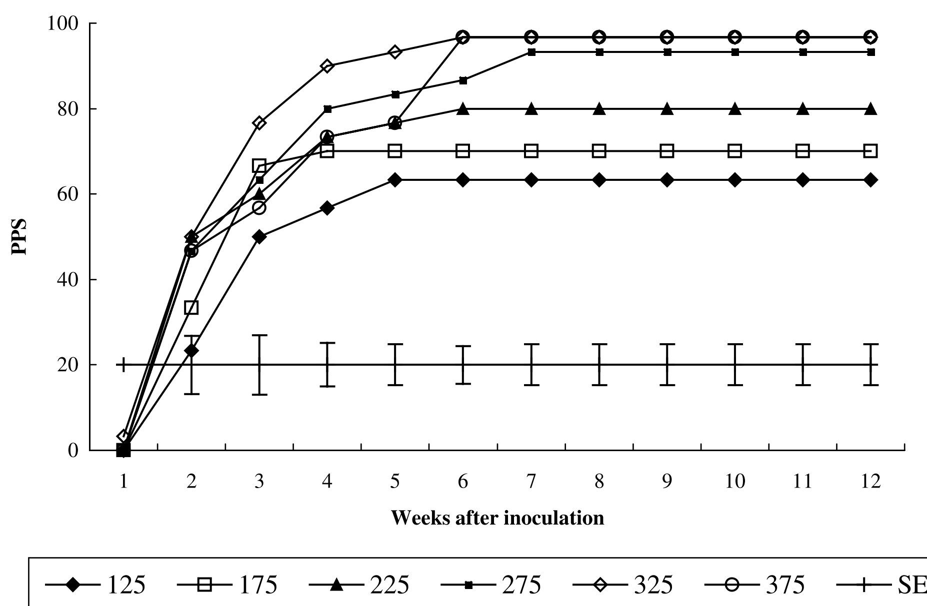

Figure 1 Effect of basidiospore concentration (× 10^3) on the percentage of plants showing symptoms (PPS) following agar-drop inoculation with *Crinipellis perniciosa* on seedlings of *Theobroma cacao* (genotype M 8; 10 seedlings per replicate; three replicates) (bars indicate standard error of means).

response to changes in incubation time for the two inoculation methods. PPS (12 WAI) increased with increasing incubation time, up to the longest time of 48 h, for both agar-drop and water-drop techniques (Fig. 2a), but the agar-drop method produced symptoms in 93% of the plants, compared with 80% for the water-drop method. PPB (Fig. 2b) showed a linear increase with increasing incubation time with the agar-drop method, with complete conversion of stem swellings into brooms. As a result, PPB was near 100% for the 48-h incubation treatment with the agar-drop inoculation method. For the water-drop method, however, the proportion of stem swellings which developed into brooms was small and variable at all incubation times, resulting in less than 20% PPB in all treatments.

The standard errors associated with the agar-drop method for measures of broom development decreased with increasing incubation time (Fig. 2a and b), indicating that experimental precision improved with increasing incubation time. This was not true of the water-drop method, however, where the standard errors remained relatively large, even at long incubation times. This showed that longer incubation times coupled with the use of the agar-drop inoculation method could improve precision and hence the ability of screening experiments to differentiate among resistance levels.

Incubation time did not significantly ($P > 0{\cdot}05$) affect symptom severity, measured as stem swelling or as any of the broom characteristics, although inoculation technique did ($P < 0{\cdot}05$). The inoculation technique × incubation time interaction was not significant ($P > 0{\cdot}05$) for any of the characteristics investigated. The effect of inoculation technique on stem swelling and broom characteristics (Table 1) could be explained by differences in the site of symptom induction. The agar-drop technique produced swellings at exactly the point of inoculation, which later developed into terminal brooms. In contrast, the water-drop method of inoculation often resulted in plants developing swellings along the cotyledonary node away from the point of inoculation, swellings which sometimes developed into axillary brooms. The swellings were larger with the agar-drop method than with the water-drop method. Similarly, broom-base diameter, broom length, broom nodes and broom dry weight were greater for the agar-drop technique than for the water-drop technique (Table 1).

Comparison of inoculation techniques

The effects of genotype, inoculation technique (agar-drop vs. spray) and the interaction between them (clone × inoculation method) on both arcsin(PPS) and arcsin(PPB) were significant ($P < 0{\cdot}05$). The agar-drop method was able to effectively discriminate between the resistant SCA 6 and the susceptible UF 11 based on PPS (Fig. 3a) as well as PPB (data not shown). Furthermore, in UF 11, stem swellings eventually developed terminal brooms in almost all plants, but in SCA 6, the proportion was only 0·62, indicating that PPS and the proportion of swellings developing into brooms may be components of resistance. Importantly, there were no escapes with UF 11 in any of the replicates when the agar-drop technique was used. The study also demonstrated that the inoculation conditions developed for susceptible seedling genotypes worked equally well with susceptible clonal plants.

In contrast to the agar-drop technique, the spray method induced only 50% infection among the susceptible clonal plants of UF 11 (Fig. 3a), showing that escapes can be

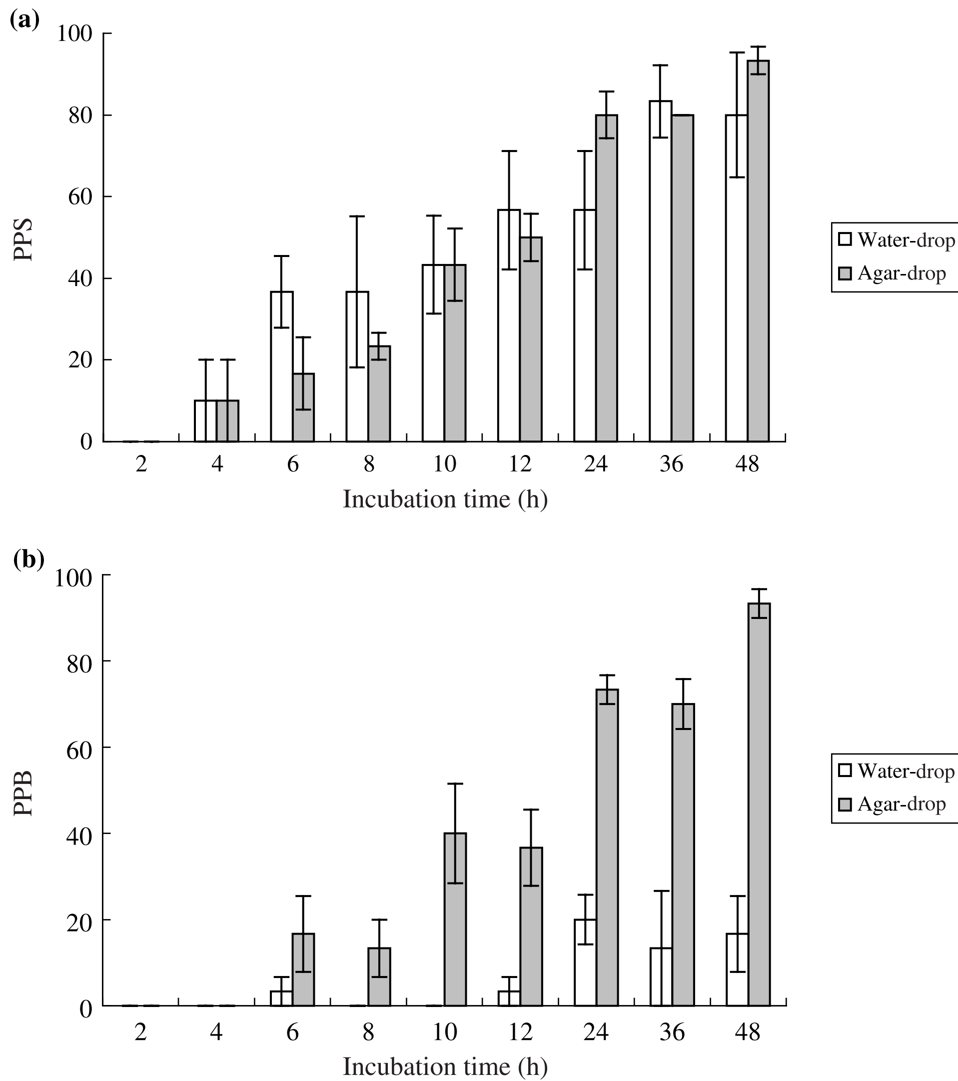

Figure 2 Effect of incubation time and inoculation technique on the percentage of plants developing (a) symptoms (PPS) and (b) brooms (PPB), following inoculation of *Theobroma cacao* seedlings (genotype Amelonado; 10 seedlings per replicate; three replicates) with *Crinipellis perniciosa* basidiospores (concentration 350 000 mL^{-1}) (bars indicate standard errors).

Table 1 Effect of inoculation technique on broom characters and stem swellings 12 weeks after inoculation of *Theobroma cacao* seedlings (genotype Amelonado; 10 seedlings per replicate; three replicates) with *Crinipellis perniciosa* basidiospores at a concentration of 350 000 mL^{-1}

Technique	ST-SW (mm)	BR-DIA (mm)	BR-LEN (mm)	ACT	INACT	LN-ACT (mm)	BR-DW (g)
Water-drop	2·1	3·3	22·0	1·6	0·5	6·2	0·3
Agar-drop	4·3	6·2	57·0	2·8	3·0	13·5	1·1
Pooled SE	2·3	3·6	24·3	1·7	1·6	10·0	0·5
Significance	*	*	*	*	*	*	*

ST-SW, stem swelling; BR-DIA, broom-base diameter; BR-LEN, broom length; ACT, number of active shoots; INACT, number of inactive shoots; LN-ACT, length of longest active shoot; BR-DW, broom dry weight.
*$P < 0.05$.

quite common with this method. In addition, the measures of PPS and PPB could not discriminate effectively between SCA 6 and UF 11, confirming that this method may not be useful to screen for witches' broom disease on the basis of disease incidence.

There were significant genotypic differences ($P < 0.001$) with respect to stem swelling (Fig. 3b), broom-base diameter, broom length and broom weight (Table 2). Interestingly, however, neither the effect of inoculation technique nor the interaction effect was significant ($P > 0.05$). This

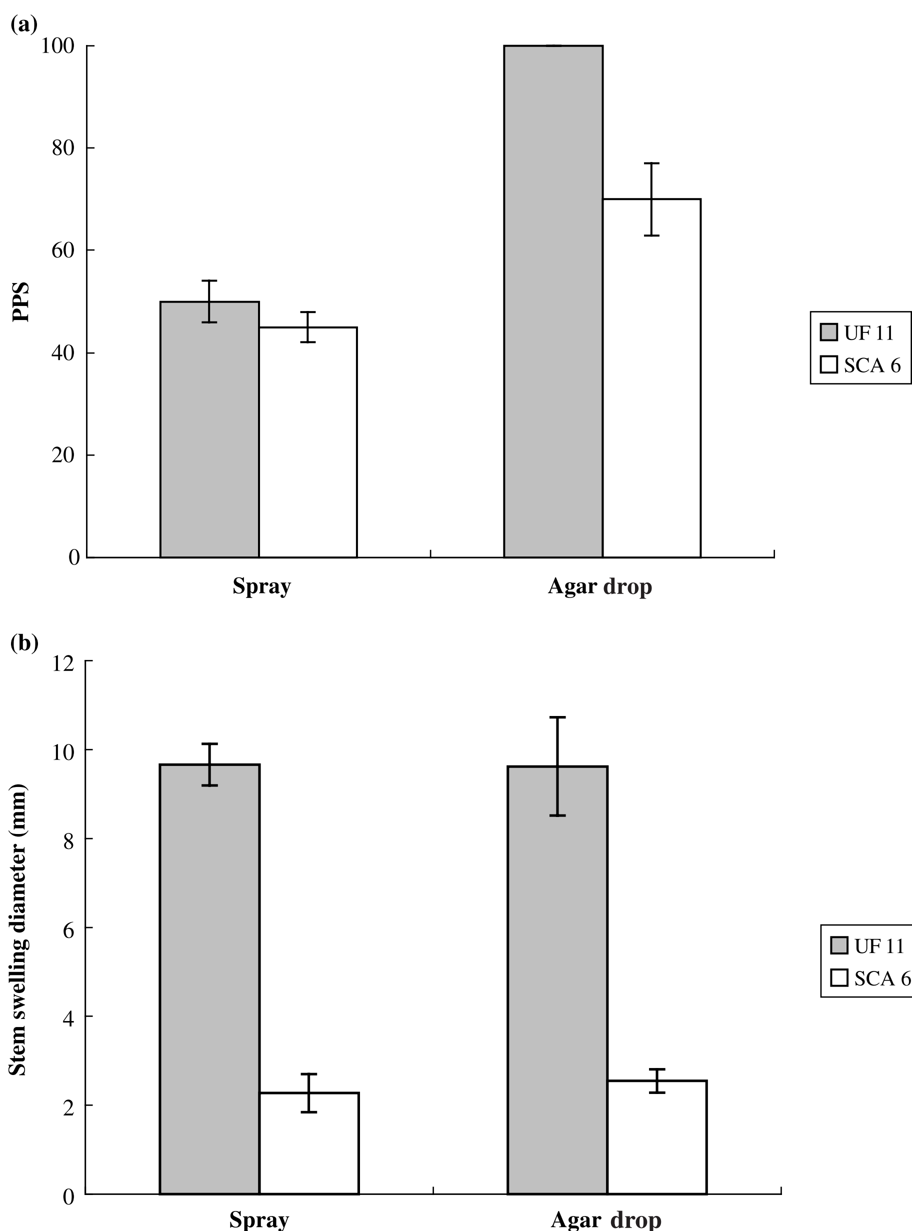

Figure 3 Effect of inoculation technique (spray vs. agar-drop) on the percentage of plants developing symptoms (PPS) (a) and stem swelling (b) 12 weeks after inoculation in two *Theobroma cacao* clones (10 plants per clone per replicate; four replicates) inoculated with *Crinipellis perniciosa* basidiospores at a concentration of 350 000 mL^{-1} (bars indicate standard errors).

suggests that both methods were equally discriminatory in separating the resistant clone from the susceptible clone based on symptomatology. Spray inoculation produced multiple symptoms at different positions on the same plant, while agar-drop inoculation elicited a single-location response at the inoculation site.

Effect of age of plants

Typical stem swellings appeared 14 days after inoculation, regardless of seedling age. The age of seedlings used did not significantly ($P > 0{\cdot}05$) influence arcsin(PPS) (93–100%) or arcsin(PPB) (90–100%), indicating that inoculation success was not affected by age (2–16 months). While host-plant age did not significantly ($P > 0{\cdot}05$) influence stem swelling, broom length or the number of active and inactive shoots on the broom, the age effect was significant ($P < 0{\cdot}05$) for broom-base diameter and broom dry weight (Fig. 4), although with respect to these latter characteristics, only the extreme age values (2 and 16 months old) were significantly different ($P < 0{\cdot}05$). Nevertheless, the study suggests that host-plant age should be standardized in disease-resistance screening exercises to eliminate effects on symptom severity.

Table 2 Effect of inoculation technique and genotypes of *Theobroma cacao* (10 plants per clone per replicate; four replicates) on broom characters and stem swelling 7 weeks after inoculation with *Crinipellis perniciosa* basidiospores at a concentration of 350 000 mL^{-1}

	ST-SW (mm)	BR-DIA (mm)	BR-LEN (mm)	BR-DW (g)
Genotype				
SCA 6	2·4	2·4	78·5	1·6
UF 11	9·6	9·6	183·2	4·8
SE	2·6	2·6	37·0	1·1
Significance	***	***	***	***
Technique				
Spray	6·0	6·0	125·1	3·4
Agar-drop	6·1	6·1	136·5	3·0
SE	0·4	0·4	4·0	0·1
Significance	NS	NS	NS	NS

ST-SW, stem swelling; BR-DIA, broom-base diameter; BR-LEN, broom length; BR-DW, broom dry weight.
***Significant ($P < 0·001$); NS, nonsignificant.

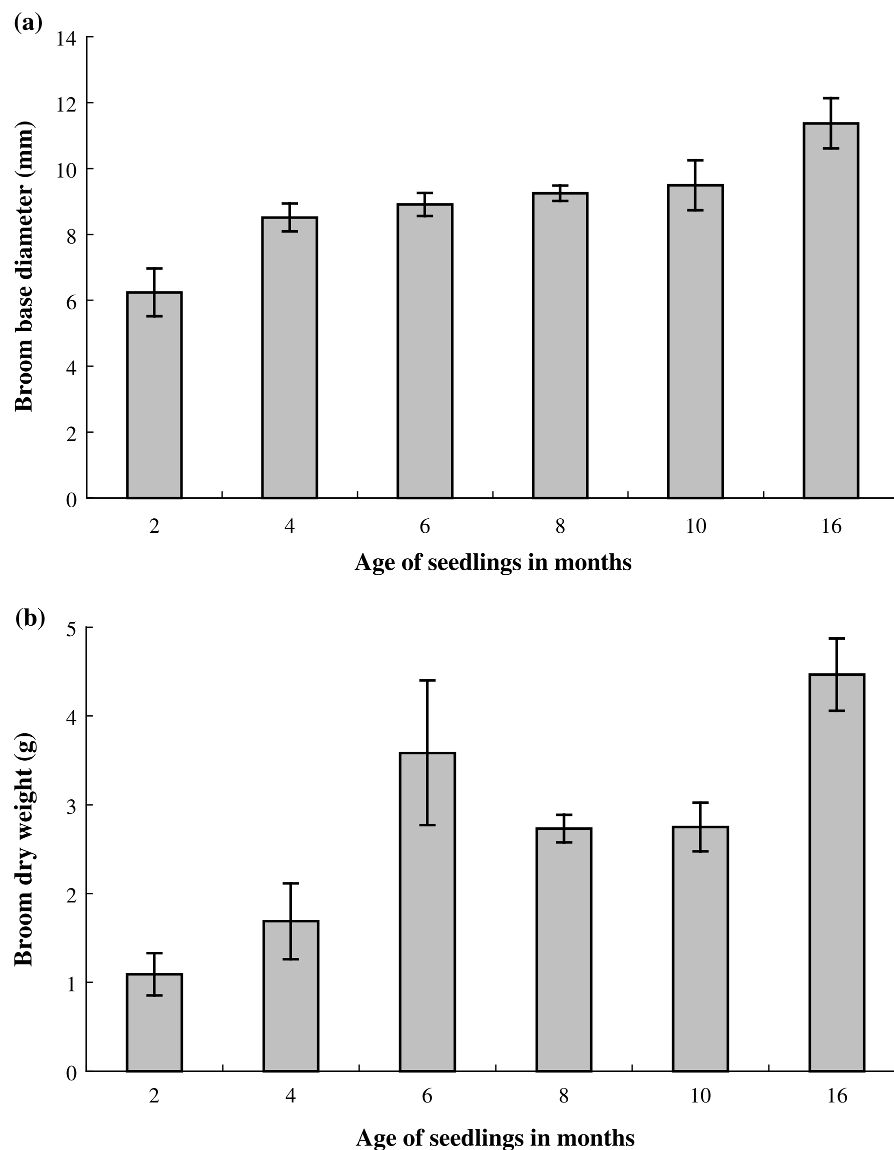

Figure 4 The effect of age on broom-base diameter (a) and broom dry weight (b) 7 weeks after agar-drop inoculation of *Theobroma cacao* seedlings (genotype Hybrid 19; 10 seedlings per replicate; three replicates) with *Crinipellis perniciosa* basidiospores at a concentration of 350 000 mL^{-1} (bars indicate standard errors).

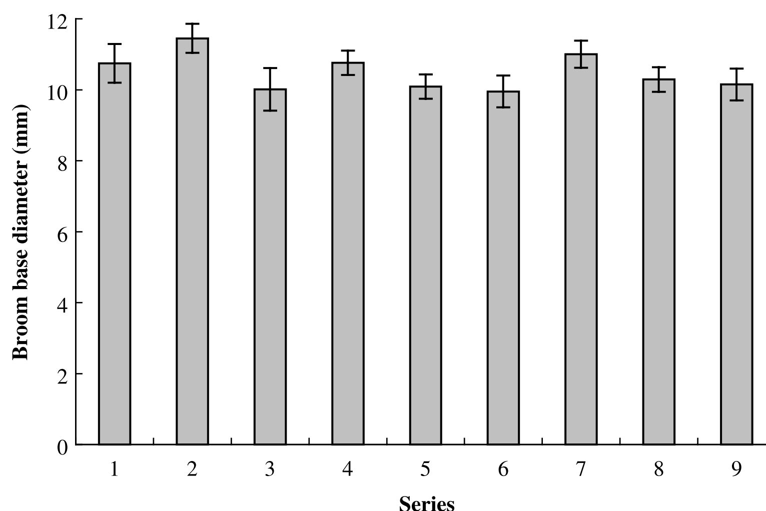

Figure 5 Repeatability of broom-base diameter over nine inoculations, in seedlings of susceptible *Theobroma cacao* (genotype Hybrid 19; 10 seedlings per replicate; three replicates) 7 weeks after agar-drop inoculation with *Crinipellis perniciosa* basidiospores at a concentration of 350 000 mL^{-1} (bars indicate standard errors).

Table 3 Measures of symptom severity in 14 genotypes of *Theobroma cacao* (three plants per replicate; three replicates), 7 weeks after agar-drop inoculation with *Crinipellis perniciosa* basidiospores at a concentration of 350 000 mL^{-1}

Genotype (parents)	PPS	PPB	ST-SW (mm)	BR-DIA (mm)	BR-LEN (mm)	BR-DW (g)
AMEL	9/9	9/9	7·4 bc	12·5 fg	177 efg	4·2 c
ICS 1	9/9	9/9	7·1 bc	12·2 f	162 cdef	3·5 b
ICS 84	9/9	9/9	5·7 bc	10·4 bcd	137 bc	4·3 c
ICS 95	9/9	9/9	6·1 bc	10·7 cde	142 bcd	4·2 c
IMC 57	9/9	7/9*	4·7 b	9·4 b	130 b	3·4 b
IMC 67	9/9	8/9	5·1 bc	9·6 bc	140 bcd	4·0 c
JA 6/4	9/9	9/9	9·5 bc	14·8 h	186 fg	5·0 d
JA 5/19	8/9	8/9	7·1 bc	12 ef	161 cdef	4 .0 c
JA 5/41	9/9	9/9	6 bc	10·8 cde	177 def	4·3 c
M 8	9/9	9/9	8·3 bc	13·6 g	151 bcde	5·0 d
SCA 6	6/9*	5/9*	3·1 a	6·9 a	94 a	1·5 a
SCA 12	7/9*	5/9*	3·1 a	6·8 a	77 a	1·2 a
Hybrid 19	9/9	9/9	5·8 bc	10·9 de	155 bcde	4·1 c
UF 11	9/9	9/9	10·2 c	15·3 h	199 g	5·8 e
Pooled SE			0·7	0·7	14·0	0·2
$LSD_{0·05}$			1·2	1·2	23·5	1·0

PPS, percentage of plants showing symptoms; PPB, percentage of plants showing brooms; ST-SW, stem swelling; BR-DIA, broom-base diameter; BR-LEN, broom length; BR-DW, broom dry weight.
*Significantly different from the rest based on Friedman's test.
Numbers followed by the same lower-case letters are not significantly different based on Duncan's multiple range test ($P < 0·05$).

Effect of leaf number

Differences in leaf number did not significantly ($P > 0·05$) affect arcsin(PPS) (93–100%) or arcsin(PPB) (93–97%). Similarly, stem girth and broom characteristics were not significantly affected ($P > 0·05$) by leaf number, within the range tested (data not presented).

Repeatability of inoculation methodology

The optimized inoculation method was able to produce repeatable estimates of PPS and PPB (90–100%) over nine series of inoculations, spanning 9 months, in seedlings of the susceptible genotype Hybrid 19 (data not shown). Similarly, broom-base diameter (Fig. 5) and other broom characteristics (data not shown) were also remarkably uniform over the nine series of inoculations.

Screening for resistance

There were significant differences ($P < 0·05$) between the 14 clones tested with respect to PPS, PPB, stem swelling, broom-base diameter, broom length and broom dry weight (Table 3). The study shows that the inoculation methodology developed can be used to discriminate effectively between various levels of resistance. Only the extremely resistant SCA clones were significantly different from the others with respect to PPS. Measures of symptom

severity (stem swelling and broom characteristics) were able to differentiate better between the clones.

Discussion

Investigations into the biology of the *C. perniciosa-T. cacao* host–pathogen system (Frias *et al*., 1991, 1995) led to the development of an automated spray inoculation system (Purdy *et al*., 1997) that delivers standardized concentrations of inoculum to susceptible, natural, infection courts under optimized environmental conditions. Inoculation conditions were optimized to use percentage infection to discriminate between resistant and susceptible clones of cocoa. It has since been applied to screen seedlings in several countries with reasonable success (Suárez & Delgado, 1993; Luz *et al*., 1999), while others have reported inconsistent results (Gramacho *et al*., 1996; Eskes, 2001).

This method, however, may not be ideal for screening segregating populations in breeding programmes for several reasons. Firstly, it allows escapes, which cannot be tolerated in screening segregating progenies in breeding programmes. Secondly, it produces multiple symptoms, which are difficult to score. Furthermore, the subjective nature of the scoring system can distort quantitative assessment of symptoms. Thirdly, the accuracy and repeatability of this system for assessment of resistance of individual plants has not been demonstrated.

This study confirms that basidiospore concentrations can be standardized and applied to a single infection court using the agar-drip technique of Sreenivasan (1987). This method is a modification of the agar-block method of Evans (1978), which was successfully adopted for screening by Laker (1987) and Wheeler & Mepsted (1988). The agar-drop method allows a simple progression of symptoms that can be easily assessed and quantified. The current study has demonstrated that infection of virtually 100% of inoculated plants could be achieved, on a repeatable basis, on a susceptible clone over a 1-year period using this technique. Given 100% infected plants, symptom severity measurements can be made on individual plants in screening breeding populations.

This study also showed that high inoculum concentrations (> 275 000 viable basidiospores mL^{-1}) and long incubation times (> 48 h) are required to avoid escapes, even with susceptible genotypes. Some infection occurred following a 4–6 h incubation time and the percentage infection continued to increase approximately linearly up to the 48-h incubation treatment. Disease escape occurs when the host, pathogen and a conducive environment do not coincide and interact at the right time or for sufficient duration (Agrios, 1997). The reduction in the number of escapes as incubation time and inoculum concentration were increased in this study supports the finding of Frias *et al*. (1995). However, Frias *et al*. (1995) obtained near 100% infection in both resistant (SCA 6 and 12) and susceptible (Catongo) seedlings with 75 000 basidiospores mL^{-1} and a 24-h incubation time using a spray inoculation technique. Hence, they recommended a much lower concentration of 12 500 basidiospores mL^{-1} to discriminate between resistant genotypes and susceptible ones. The larger percentage infection obtained with lower concentration and incubation times by Frias *et al*. (1995) may reflect greater coverage of infection courts (many flushes per plant) in their study than in the present study, where single flushes were targeted. Gramacho *et al*. (1996), however, reported highly variable results even at concentrations as high as 200 000 viable basidiospores mL^{-1}, using the spray inoculation method, which is consistent with this study.

These results provide evidence that associated standard errors decreased as inoculum concentration or incubation time was increased, thus improving the repeatability and precision of screening trials. Relatively low inoculum concentrations and/or short incubation times may account for the inconsistencies observed in many studies using spray inoculation (B. Eskes, unpublished data). A high level of repeatability is required when screening individual plants in segregating populations, since there is not the luxury of replications.

Suárez (1977) and, later, Sreenivasan & Dabydeen (1989) as well as Frias *et al*. (1991) demonstrated that the germ-tube of the germinating basidiospore ramifies and proliferates on wet leaf surfaces, resulting in multiple entries through stomatal openings, although it is not known whether the infection process is identical on apical meristems. However, any interruptions in the free moisture on the leaf surface would permanently stop the infection process (Frias *et al*., 1991). Hardwick *et al*. (1981) reported that during the ontogeny of the cocoa leaf, stomata on veins develop earlier and are larger than on the lamina. Hence, if stomatal entry is the only means of establishing successful infection, as demonstrated by Frias *et al*. (1991), and if successful entry into the young flush leaves is mainly through the few veinal stomata, it is quite possible that high basidiospore concentrations and longer incubation times may be required to increase the likelihood of entry by mycelial proliferation and coverage of leaf surface.

It is also conceivable that relatively long incubation times may result in multiple entries through the stomatal opening, thus increasing the chances of infection, especially in the more resistant genotypes. Frias *et al*. (1991) showed that yellow fluorescence associated with unsuccessful infections was delayed in successful infections, suggesting a delicate balance between host and pathogen. Long incubation times that favour multiple entries through the stomata may tilt the balance in favour of the pathogen, thus overcoming host resistance. This could account for the more repeatable infection establishment observed with longer incubation times. On the other hand, Agrios (1997) indicated that relative humidity plays an important function in increasing the succulence of host plants and, thus, their susceptibility to certain pathogens. Hence, the longer duration of surface moisture may be involved in delaying the host response, leading to more frequent infection success.

Among the inoculation techniques investigated, infection success was much greater for the agar-drop than for

the water-drop or the spray technique. Furthermore, the standard errors associated with percentage infection with the agar-drop method were much smaller than those for the spray and water-drop methods, and therefore results were highly repeatable. When basidiospore suspensions are atomized onto leaves and incubated in a mist chamber, free moisture may collect on leaves and form large drops, which either roll off, removing the spores that have not germinated, or collect at the leaf tips or leaf bases. This could account for variability in results. Similarly, with the water-drop method, inoculum run-off from the point of inoculation can produce multiple symptoms and, thus, variable results.

The effects of inoculum concentration, incubation time and inoculation technique (spray or agar-drop) affected infection success, but did not influence the severity of symptom expression in the susceptible genotypes in this study. This is consistent with the findings of Laker (1987), who pointed out that care should be taken in selecting an appropriate inoculum concentration when carrying out artificial inoculations, as resistant genotypes are likely to succumb to high pathogen pressures. Since pathogen establishment may be under delicate control (in favour of either the host or pathogen) and may be affected by pathogen pressures (Laker, 1987) or by the environment (Evans & Bastos, 1980), it may be more appropriate to breach this barrier and test resistance based on symptom severity. In the present study, results from the screening of 14 clones provided further evidence that symptom severity may be the most effective measure to distinguish between the various levels of resistance. The comparison between the agar-drop and spray techniques also showed that assessment of resistance based on symptom severity (calculated as an average over infected plants) may yield consistent results in screening clonal varieties, regardless of the inoculation method used, since escapes are not important. However, for the screening of segregating populations, a method that does not allow escapes, such as the agar-drop method, is essential, since replicates of genotypes are not available.

Wheeler (1999) expressed the opinion that broom size was an important component of resistance to witches' broom disease in cocoa among the various measures of symptom severity, since smaller brooms would have a low potential for basidiocarp production. Baker & Holliday (1957) also showed that smaller brooms produce fewer and smaller basidiocarps, with even fewer surviving to produce basidiospores, indicating the importance of broom size as a component of resistance. The present study showed clearly that broom weight and broom-base diameter were able to discriminate effectively between levels of resistance among the 14 clones.

While the percentage of stem swellings developing into brooms (with the agar-drop method) was always 100% for the susceptible genotype UF 11, it was only 62% for SCA 6, indicating that it may be a component of host-plant resistance. Cronshaw & Evans (1978) reported that terminal brooms developed from infections near the growing tip, where the fungus was able to colonize the meristematic tissue. However, when conditions were not favourable, swelling resulted, but no brooms were produced. The authors also suggested that if favourable conditions were to return in the next flush cycle, brooms might be produced. If this were the case, it suggests that even after pathogen establishment, the colonization of meristematic tissue may be inhibited in the resistant SCA 6. Resistance at the cellular level in SCA 6 has been reported in several studies using basidiospore germination on leaf extracts (Evans & Bastos, 1980) and phloem extracts (Bastos & Albuquerque, 2000), and growth on callus (Fonseca & Wheeler, 1990).

Neither the age of the host (2–16 months) nor the number of leaves on the plant (0–20 leaves) affected infection success or the percentage of plants showing broom development. Many researchers have indicated that cocoa needs to be in an active flush stage for infection success (Holliday, 1957; Cronshaw & Evans, 1978; Laker, 1987; Frias *et al*., 1995). Frias *et al*. (1995) showed that meristems with flushing leaves that ranged from 0·5 to 5 cm were equally susceptible. All inoculations were done at the primary flushing-2 (seedlings) and secondary flushing-2 (clonal plants) stages in the present study, which demonstrated that near 100% infection success can be obtained regardless of the age of the host plant or the number of leaves. These results are therefore consistent with previous work.

Although the age of the host plant did not significantly influence stem swelling, it affected broom-base diameter and broom dry weight. However, comparison of treatments showed that the effects were significantly different only for the extreme ages of 2 months versus 16 months. Pound (1943) and Baker & Holliday (1957) reported that the size of broom produced depends on the age and vigour of the tree. The present study shows that while age could affect broom size, host material can be effectively compared for resistance, provided there is not a wide difference in age. The results also indicate that resistance can be assessed on seedlings as young as 2 months of age. This will greatly reduce the time, space and resources required for breeding for resistance to witches' broom disease.

In conclusion, it is recommended that the agar-drop inoculation method be used to form the basis of an optimized technique for the inoculation of both cocoa seedlings and clonal varieties with basidiospores of *C. perniciosa*. The inoculation method should be applied with an inoculum concentration of 350 000 viable basidiospores mL^{-1} and an incubation time of 60 h, achieved by incubating the flush leaves (primary flushing-2 stage for seedlings; secondary flushing-2 stage for clones) in polythene bags containing moist tissue paper at 25°C. This method of inoculation avoids escapes, is repeatable over time, is capable of differentiating various level of resistance to witches' broom disease in cocoa, and indicated that symptom severity rather than incidence of disease is a better measure of resistance.

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