

A qualitative host-pathogen interaction in the *Theobroma cacao*-*Moniliophthora perniciosa* pathosystem

M. W. Shaw* and A. E. Vandenbon

School of Biological Sciences, University of Reading, Whiteknights, Reading RG6 6AJ, UK

The aim of this study was to test whether resistance of clones of *Theobroma cacao* (cocoa) varied between isolates of *Moniliophthora* (formerly *Crinipellis*) *perniciosa*, the cause of witches' broom disease. Developing buds of vegetatively propagated *T. cacao* grown in greenhouses in the UK were inoculated with 16 000 spores of *M. perniciosa* per meristem in water, under conditions where water condensed on the inoculated shoot for at least 12 h after inoculation. The proportion of successful inoculations varied between clones and was inversely correlated with time to symptom production or broom formation. A specific interaction was demonstrated among three single-spore isolates of *M. perniciosa* and the clone Scavina 6 (SCA 6) and a variety of susceptible clones. Isolates Castenhal-I and APC3 were equally likely to infect SCA 6 and the other clones, but isolate Gran Couva A9 never infected SCA 6, although it was as virulent on the other clones. The interaction was maintained when the wetness period was extended to 70 h. Offspring of SCA 6 × Amelonado matings were all susceptible to both Castenhal-I and GC-A5, with no evidence of greater variability in susceptibility to GC-A5 than Castenhal-I. This suggests recessive inheritance of a single homozygous factor conferring resistance to GC-A5, from SCA 6. The progenies were slightly more susceptible to Castenhal-I than GC-A5. The implications for managing the disease are discussed.

Keywords: co-adaptation, cocoa, *Crinipellis perniciosa*, R-gene, Scavina, vertical resistance, witches' broom of cacao

Introduction

Moniliophthora perniciosa, formerly *Crinipellis perniciosa* (Aime & Phillips-Mora, 2005), is a basidiomycete pathogen of cocoa trees, *Theobroma cacao* (Frias *et al.*, 1995). Host and pathogen are both native to the Amazon basin. Cocoa is a diploid species with complex incompatibility relationships; most clones are self-incompatible (Ford, 2003). The pathogen is a homothallic member of a group of species containing heterothallic members which attack or grow endophytically in a range of plants in other families (Griffith & Hedger, 1994). It infects developing shoots or flowers and grows internally in a biotrophic phase for some weeks, causing a proliferation of sideshoots and thickening and shortening of the main axis. After a further period of weeks the pathogen, while remaining internal, changes to a saprotrophic growth phase with distinctive mycelium, at the same time as the host broom tissue dies (Evans, 1980; Silva & Matsuoka, 1999; Kilaru & Hasenstein, 2005). The life-cycle is completed during phases of alternating wet and dry periods during which basidiocarps are produced externally on dead host tissue (Wheeler &

Suárez, 1993). The pathogen is genetically and morphologically variable (de Arruda *et al.*, 2003), but specific host-isolate interactions have not been previously demonstrated.

Broom formation damages trees and prevents normal growth, as well as directly destroying pods. The disease spread and intensified throughout South America during the 20th century and causes severe losses in several regions (Lass & Purdy, 1993). In favourable environments it can be managed by careful sanitation, but this is often uneconomic (Madison *et al.*, 1993). Resistant clones have been identified (Bartley, 2001; Marita *et al.*, 2001), but when planted over large areas, the resistance has proved inadequate or unstable, as, for example, in Rondônia in the Amazon basin and in Ecuador during the 1980s (Wheeler & Mepsted, 1988). Both de Arruda *et al.* (2003) and Andebrhan *et al.* (1999) suggested from molecular evidence that there are several geographically distinct clades of the pathogen in Brazil, and that selection of resistant clones should take this into account, testing against isolates representing each clade.

In 1997 a large international collaboration project to improve and disseminate cocoa germplasm (Cocoa Germplasm Utilisation and Characterisation: A Global Approach), funded by a consortium of donors, began. One aim of the project was to identify resistant clones suitable for inclusion in breeding work or direct dissemination.

*E-mail: m.w.shaw@reading.ac.uk

Accepted 23 August 2006

A conveyor belt with overhead sprays (Frias *et al.*, 1995; Purdy *et al.*, 1997; Surujdeo-Maharaj *et al.*, 2003) was adopted as a common method to assess clones for resistance to witches' broom in four regions within the cocoa-producing areas. This method did not use genetically pure isolates of the pathogen, and therefore left open the question of whether differences in resistance rankings between sites where testing was done were the result of environmental conditions or local differences in host-pathogen adaptation. To test this safely required work in a third country in which cocoa was not grown, to avoid the risk of spreading host-specific virulent pathotypes, should they exist, into new regions.

The work reported here had two aims. These were first, to determine whether clones of *T. cacao* had specific interactions with isolates of *M. perniciosa* from different producing regions, and secondly to improve knowledge of the resistance profile of clones to be tested. In the course of testing, a very strong host-isolate interaction ('vertical resistance' Deadman, 2006) was discovered, leading to a third aim, to determine the inheritance of resistance in the host. Attempts were also made to study the inheritance of virulence (host-specific pathogenicity) in the pathogen.

Materials and methods

Moniliophthora perniciosa inoculum

Isolates used were Castanhal-I (Cast-I; via Silwood Park, Imperial College, Ascot, UK/B. E. Wheeler, isolated from Rondônia, Brazil, in an area of Scavina-resistance breakdown), Gran Couva-A5 (GC-A5; via Silwood Park/B. E. Wheeler, isolated from Trinidad) and APC3 (via Aberystwyth, UK/G. C. Griffith, isolated from Bahia, Brazil).

Basidiospores of all pure cultures were produced by the method of Griffith & Hedger (1993). Cultures were subcultured on potato dextrose agar (PDA) in Petri dishes and incubated at 22°C in the dark until the culture covered the surface of the agar. Four 1-cm² squares of agar culture were placed mycelium downwards on bran/vermiculite substrate in a Phytocon dish (Sigma). The lids were replaced and the cultures incubated at 22°C in the dark until the mycelium covered the surface of the substrate. At this stage, usually after about 4 weeks, sterilized casing medium was added to the surface of the culture. Cultures were incubated until the surface was once again covered by the mycelium, a further 3–4 weeks. The casing material and lid were removed and the culture placed in a mist chamber on a wet sandbed at 23–25°C. Basidiocarps were produced in a total time of 72–148 days. Some isolates were consistently faster than others, but batches also differed for unknown reasons.

Collection and storage of basidiospores

Basidiospores were collected by attaching the cap of a basidiocarp to the lid of a Petri dish with Vaseline and suspending it over a beaker containing 16% glycerol in 0.01 M 2-morpholinoethanesulfonic acid (MES) + 10 μ L

Tween-20. Spores were collected overnight, counted using a haemocytometer, dispensed in approximately 1-mL aliquots into cryogenic vials (Nalgene) and stored over liquid nitrogen (Dickstein *et al.*, 1987). For use, a vial was thawed and diluted in equiosmotic stages to a final concentration of less than 1% glycerol and a concentration of approximately 1000 spores μ L⁻¹.

Inoculation of shoots

Budwood and seedlings were obtained from the collection at the Cocoa Quarantine Centre at Shinfield, Berkshire, UK. Plants were maintained in pots of a sand, gravel and vermiculite mix, with drippers supplying a balanced nutrient solution developed for cocoa (End, 1990). The glasshouse temperatures were designed to simulate the environment of a cocoa-growing region in Ghana, with both diurnal and annual fluctuations in temperature programmed in (Daymond & Hadley, 2004). Average annual temperature during months when inoculations were made was 25.3°C. Diurnal variation was to a minimum of 20°C and maximum of 31°C in January; a minimum of 22°C and maximum of 28°C in March and October. Sodium lighting was used as necessary to extend the daylength to 12 h.

Selected host clones (Table 2) were propagated by side-grafting buds onto Amelonado type seedlings, followed when the bud flushed by pruning of the seedling below the leaves, but well above the budding point. These plants were pruned to induce multiple growth points. Shoots were inoculated at the start of flushing, when the largest leaf was less than 1 cm long, with four 4- μ L droplets of spore suspension prepared from frozen suspensions of cultured basidiocarps, approximately 16 000 spores per shoot.

Inoculations were made in late afternoon in a polythene tent with plants standing on a wet sandbed containing heating coils at about 30°C, with the internal space humidified by a centrifugal or sonication-based humidifier; these conditions were maintained for 24 h. Successful inoculations took place when plants were radiatively cooling to the night sky and daytime temperatures were below those inside the greenhouse, essentially during spring, autumn and early winter. Only slight improvement in the frequency of infection occurred if the infection conditions were maintained for 70 h (cf. Surujdeo-Maharaj *et al.* (2003), who found more or less linear increases in infection with wetness period). After inoculation, plants were kept in enhanced humidity, provided by overhead misters, for 1 week before transfer to the main glasshouse. The minimum temperature during this post-inoculation period was set to 20°C, although in some winter inoculations, night-time temperatures in the bulk of the house dropped to 15°C.

Experimental design

For comparison of host susceptibilities, all suitable buds on a batch of plants were inoculated on one occasion. Each batch included Amelonado (plants coded G3 or B9)

Table 1 Analysis of deviance of a generalized linear model of the effects of inoculation date, host clone and isolate on the probability of a cocoa bud becoming infected with *Moniliophthora perniciosa*

Factor	d.f.	Deviance	Mean deviance	Deviance ratio ^a	F probability
Inoculation date	12	205.63	17.14	12.2	<0.001
Clone	21	219.22	10.44	7.4	<0.001
Isolate	2	67.03	33.52	23.9	<0.001
Clone.Isolate	31	106.99	3.45	2.5	<0.001
Residual	390	547.48	1.40		<0.001 ^b
Total	456	1146.35	2.51		

^aBecause the residual deviance is larger than expected by chance, it is more conservative to test the deviance ratio against *F* than the deviance against χ^2 .

^bAgainst χ^2 .

and Scavina 6 (SCA 6, Reading quarantine collection RUQ234) as standards. The particular clones and number of buds inoculated depended on availability. To improve comparison of reactions to different isolates, half (or one-third, where three isolates were used) of the buds on each plant were inoculated with each isolate under test, so the effects of variation between plants were balanced. In initial experiments symptoms were noted at approximately weekly intervals; in later work only the final number of successful infections was noted. Twelve batches of inoculations made between 1999 and 2002, at intervals of 1–3 months during suitable times of year, were included in the data analysed, representing 241 plants and approximately 2500 inoculations.

Inheritance of resistance and virulence

To test the patterns of inheritance in the host, crosses of SCA 6 and Amelonado clones were made in both directions by emasculating flowers before anthesis and pollinating artificially with ripe anthers from the other clone. Several pods were obtained. The resulting seedlings were tested with both GC-A5 and Castanhal-I, after pruning to obtain enough buds to inoculate both isolates simultaneously onto each seedling; 2–6 buds were inoculated with each isolate on each seedling. *Moniliophthora perniciosa* is known to be homothallic, but the possibility of crossing isolates by inoculating buds with an equiproportionate mixture of basidiospores of isolates GC-A5 and Castanhal-I was investigated. Basidiocarps were produced from the resulting brooms by hanging dry, necrotic brooms in a mist chamber with 12-h alternating wet/dry conditions until basidiocarps appeared, after 3–12 months.

Statistical analysis

The design was in effect a chained incomplete block design with a split-plot factor of isolate to improve precision in the estimation of host-pathogen interactions. However, sample sizes were irregular and the block arrangement very unbalanced because of experimental exigencies; the data were also binomial proportions. Infection frequencies were therefore analysed using generalized linear models in GENSTAT v7 (VSN International), with a logit link function

and binomial error. Main effects of differences between batches of inoculations were fitted first, followed by main effects of host clone, pathogen isolate and then their interaction. The residual deviance reflected inevitable binomial variation, differences between pots, and random differences between experimental units.

Results

Effects of clone and isolate on infection

The proportion of buds eventually producing brooms on the standard clones varied significantly between batches of inoculations. The differences between those made at the end of September and mid-May were reasonably small, although still the largest influence on the success of inoculations (Table 1, line 1). The incubation period varied more than the proportion of successful inoculations, and incubation periods in December inoculations were about 50% longer than in October or March.

The main effects of both host clone and pathogen isolate on symptom incidence were significant and the deviance associated was much larger than the interaction term (Table 1). Since the isolates were in effect a very small random sample of possible isolates, the main effects of host are of some interest for clone selection in breeding and improvement programmes. Averaged over the isolates used, clones RUQ3, CATIE 1000, Playa Alta 2 and SNK 413 were at least as susceptible as Amelonado B9, which was equivalent to Amelonado G3 (Table 2). Clones ICS 1, NA 33, PA 120, PA 150 and SIAL 339 appeared the most resistant, although sample sizes were small in some cases. The correlation between susceptibility and incubation period was negative and very significant, but not very strong ($r = -0.7$, $P < 0.001$) (Fig. 1). There was a good correlation between the incubation period to swellings and that to brooms (Fig. 2). The time taken to produce a broom was approximately 20% longer than that to produce a swelling.

The three isolates were almost identical in average infectiousness, when averaged over clones other than SCA 6 (Table 3). Brooms appeared significantly faster on Amelonado B9 infected with GC-A5 rather than Castanhal-I, and this trend was repeated in most clones (Fig. 3).

Clone	Accession number ^b	No. of plants tested	No. of inoculations	Standardized infection probability ^c	SE ^d
CATIE 1000	844	18	48	0.81	0.069
Playa Alta 2	232	8	27	0.69	0.106
B 9		73	263	0.64	0.028
G 3		39	90	0.61	0.057
RUQ3	3	6	21	0.55	0.119
T85/799	855	16	49	0.48	0.074
SNK 413	854	30	68	0.46	0.065
EET 59	791	14	51	0.46	0.050
Amaz 15-15	1	14	39	0.39	0.070
LCT EEN 46		16	37	0.38	0.083
GU 255P	846	25	50	0.33	0.066
SIC 6	49	2	7	0.28	0.116
SIAL 93	47	5	14	0.22	0.112
SCA 6	234	113	558	0.22	0.017
EQX 3360-3	152	23	47	0.21	0.068
ICS 1	847	57	149	0.15	0.033
IMC 47	849	14	52	0.14	0.047
SIAL 339	48	5	12	0.11	0.082
PA 120	852	8	23	0.05	0.050
NA 33	822	10	34	0.04	0.038
GU 307	772	2	12	0.00	0.002
PA 150		12	17	0.00	0.002

Table 2 Standardized probability of buds of clones of *Theobroma cacao* developing into a broom following inoculation with *Moniliophthora perniciosa*^a

^aAveraged over isolates GC-A5, Castanhal-I and APC3.

^bReading University quarantine facility accession code.

^cData corrected for differences between infection occasions using hosts common to occasions, using a generalized linear model with logit transform and binomial error distribution. Data are from the final scoring made in each batch. Generalized linear model contained terms for inoculation occasion, clone, isolate and clone.isolate interaction, estimated using the PREDICT command in GENSTAT, with marginal weights applied to the averages, adjusted to allow for missing factor combinations.

^dFrom the PREDICT command; given the unevenness of the numbers tested, these cannot be very accurate.

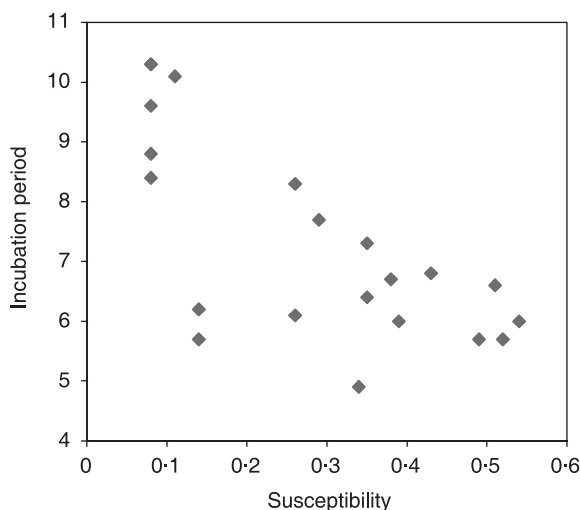


Figure 1 Relationship between susceptibility of *Theobroma cacao* to *Moniliophthora perniciosa* (standardized proportion of inoculations successful) and incubation period to broom production (corrected for differences between inoculations on the standard susceptibles). Data for *M. perniciosa* isolates Castanhal-I and Gran Couva-A5 and all host clones combined. Pearson correlation (r) = -0.7 on 19 d.f., $P < 0.001$.

Differential interactions between host clones and pathogen isolates

The interaction deviance in symptom incidence was significant. The biggest contribution to this was from the 'resistant' clone SCA 6, which was susceptible to Castanhal-I, but completely resistant to GC-A5 (Table 3). Without SCA 6, the interaction deviance in the remaining dataset was not significant ($F_{29,303} = 0.91$, $P > 0.5$). However, clone EET 59 was relatively resistant to APC3 and very susceptible to GC-A5, and clone ICS 1 appeared to be relatively resistant to both GC-A5 and Castanhal-I, but susceptible to APC3. Unfortunately, sample sizes were very small in both cases.

SCA 6 was susceptible to Castanhal-I (and APC3), but not at all to GC-A5 (Table 4). This host-pathogen interaction was significant at $P < 0.001$ using a chi-squared test. SCA 6 appeared to be less susceptible to APC3 than to Castanhal-I.

Surujdeo-Maharaj *et al.* (2003) reported higher incidences of infection with wetness periods up to 72 h. To test whether the differential interaction seen was the result of a greater general resistance of SCA 6 interacting with a weaker isolate, an inoculation was made with a 70-h

Table 3 Standardized probability of a bud of *Theobroma cacao* developing into a broom following inoculation with the *Moniliophthora perniciosa* isolates GC-A5, Castanhal-I and APC3

Clone ^a	APC3			Castanhal I			GC-A5		
	<i>i</i> ^b	SE	<i>n</i> ^c	<i>i</i>	SE	<i>n</i>	<i>i</i>	SE	<i>n</i>
CATIE 1000				0.76	0.10	25	0.87	0.08	23
Playa Alta 2				0.65	0.11	13	0.75	0.21	4
B 9	0.43	0.12	17	0.61	0.03	122	0.72	0.04	83
G 3	0.46	0.11	14	0.59	0.07	37	0.65	0.09	30
RUQ3				0.71	0.17	10	0.33	0.15	11
T85/799				0.56	0.10	24	0.37	0.10	25
SNK 413				0.49	0.09	33	0.41	0.08	35
EET 59	0.16	0.15	6	0.33	0.06	27	0.99	0.00	10
Amaz 15-15	0.50	0.35	2	0.31	0.07	19	0.58	0.14	12
LCT EEN 46				0.38	0.10	17	0.38	0.13	17
GU 255P	0.00	0.00	5	0.31	0.09	22	0.37	0.09	23
SIC 5				0.00	0.00	4	0.66	0.27	3
SIAL 93	0.54	0.17	7	0.20	0.16	6			
SCA 6	0.13	0.03	70	0.34	0.02	251	0.00	0.00	174
EQX 3360-3				0.27	0.10	22	0.13	0.08	19
ICS 1	0.38	0.09	29	0.14	0.04	60	0.09	0.05	36
IMC 47	0.00	0.00	7	0.30	0.12	14	0.09	0.04	23
SIAL 339	0.33	0.27	3	0.17	0.14	5	0.00	0.00	4
PA 120				0.09	0.08	12	0.00	0.00	11
NA 33				0.06	0.06	17	0.00	0.00	17
GU 307	0.00	0.00	6	0.00	0.00	6			
PA 150				0.00	0.00	9	0.00	0.00	8
Mean over clones ^d	0.44	0.06		0.41	0.02		0.39	0.02	

^aOrder follows Table 2.

^bProbability of an inoculated bud producing a broom, standardized for differences between occasions. Data corrected for differences between infection occasions using hosts common to a batch of inoculations, using a generalized linear model with logit transform and binomial error distribution. Data are from the final scoring made in each batch.

^cTotal number of buds inoculated.

^dExcluding SCA 6.

Table 4 Summary of inoculations of *Theobroma cacao* clones SCA 6 and Amelonado with *Moniliophthora perniciosa* isolates Castanhal-I and GC-A5^a

Inoculation date	SCA 6		Amelonado		
	Castanhal-I	GC-A5	Tree ^b	Castanhal-I	GC-A5
07/09/01	4/15	0/16	B9	0/14	1/14
26/09/01	12/18	0/14	B9	6/6	9/10
11/10/01	5/23	0/5	B9 + G3	3/5	5/7
24/10/01	6/15	0/14	B9 + G3	11/12	11/11
2/11/01	4/14	0/14	G3	9/9	7/9
16/11/01	10/16	0/16	B9 + G3	9/9	6/9
30/11/01	10/15	0/17	B9 + G3	9/12	10/11
14/12/01	10/16	0/19	B9 + G3	3/4	2/3
21/02/02	17/31	0/29	B9	11/15	10/14
13/03/02	2/21	0/20 ^c	G3	0/4	0/4

^aEach cell contains the number of shoots with symptoms divided by the number of surviving, developing inoculated shoots.

^bBudded material from two Amelonado trees, B9 and G3, in the Reading collection was used according to availability. There was no evidence of any difference in susceptibility if the data were examined separately.

^cTwo brooms developed on buds labelled as inoculated with GC-A5 in this experiment, which also included isolate APC3. DNA isolations from the brooms were made, DNA extracted, and ISSR profiles made. The band patterns appeared unrelated to those from either GC-A5 or Castanhal-I and were presumably APC-3.

wetness period. The incidence of infection was high or very high with all clones included and both isolates, apart from GC-A5 on SCA 6, which failed to infect, as in all previous inoculations (Table 5).

Inheritance of differential resistance

The progeny of crosses between SCA 6 and Amelonado were used to deduce the mode of inheritance of the differential

Table 5 Number of shoots of four clones of *Theobroma cacao* producing brooms or swellings out of total number of buds inoculated with an isolate of *Moniliophthora perniciosa* and given a 70-h wet period

Isolate	SCA 6	B 9	Amaz 15-15	Playa Alta 2	EET 59
Castanhal I	20/32 (2) ^a	12/15	6/11	4/4	9/11
GC-A5	0/29	10/14 (2)	7/12	3/4	9/9 (1)

^aFigure in brackets is number of buds that died post-inoculation.

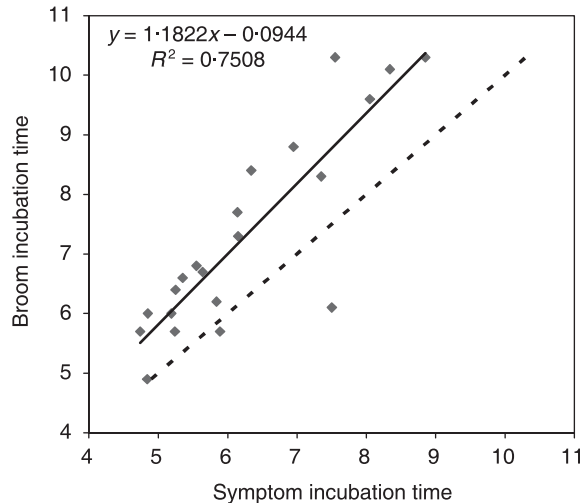


Figure 2 Relationship between average time to first observation of brooms and average time to first observation of any symptoms for *Moniliophthora perniciosa* growing on *Theobroma cacao*. Initial symptoms were swellings, unless broom production was very rapid and the swelling stage fell between observations. Each point represents the average for all observations on a clone-isolate combination; both Gran Couva-A5 and Castanhal-I tests are included. Solid line shows regression; dashed line shows the line of identity. The aberrant point is for ICS 1 and refers to a small number of meristems, one of which showed a swelling very late, which did not become a broom during the observation period.

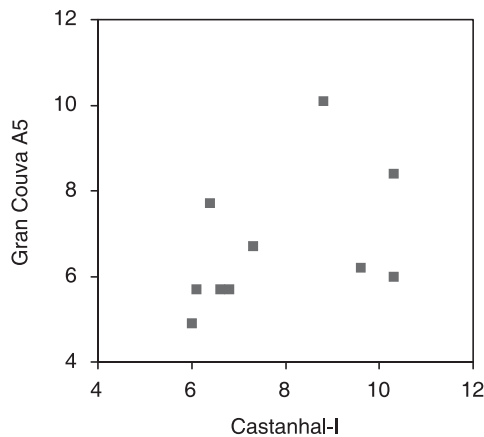


Figure 3 Incubation periods to brooms (weeks) of *Theobroma cacao* clones when inoculated with *Moniliophthora perniciosa* isolates Castanhal-I or Gran Couva A5.

Table 6 Tests of patterns of single-gene inheritance^a of resistance to *Moniliophthora perniciosa* isolate GC-A5 from *Theobroma cacao* clone SCA 6

Plant type	Plants tested ^b	Plants without GC-A5 infection	Expected: recessive ^c	Expected: heterozygous dominant ^d
B9	6	2		
SCA 6	6	6		
G36 × SCA 6	24	5	7.2	15.6
SCA 6 × B9	10	1	3.9	6.9
SCA 6 × G3	35	13	9.7	22.3
χ^2 (3 d.f.)			3.95 ^e	16.1 ^f

^aExpected values for two hypotheses are shown. The third hypothesis, homozygous dominant, was ruled out immediately because seedlings were mostly susceptible to GC-A5.

^bPlants with no symptoms on buds inoculated with Castanhal-I not included.

^cFor Poisson distribution and mean frequency of infection of GC-A5 on the inoculation date, excluding SCA6. The probability of a null result was calculated for each cross separately using the zero term of the Poisson distribution, depending on the number of buds inoculated, then summed over all inoculations in which Castanhal-I infected, in each batch of inoculations.

^dAssuming half the crosses had inherited a dominant resistance allele, and the remainder had a Poisson distributed number of successful infections.

^e $P = 0.3$.

^f $P = 0.001$.

resistance. The pruned seedlings were substantially more susceptible than grafts of the parental plants, but had a similar incubation period; however, this was probably a physiological effect, and it was the relative virulence of the two isolates which was important. *Theobroma cacao* is diploid, so if the resistance is governed by a single dominant allele, then the progeny should either be all resistant to GC-A5 if SCA 6 were homozygous for the allele, or 50% resistant and 50% susceptible if it were heterozygous. If the resistance is governed by a homozygous recessive allele, then the progeny should be all equally susceptible to GC-A5 and Castanhal-I. This appeared to be the case (Table 6).

The alternative is that the resistance is governed by several or many minor genes. In this case, the resistance of the progeny should be midway between the two parents, and variable, depending on how many factors are inherited by an individual seedling. GC-A5 was slightly, but not significantly, less infectious to the progeny than Castanhal-I,

with a log-odds difference in symptom incidence of 0.64 (SE 0.63). This was similar to the average difference on cloned material other than SCA-6, 0.28 (SE 1.6). The deviance between progeny plants was 1.76, allowing for differences between occasions. This was very slightly and non-significantly larger than the residual deviance of 1.63 in the bulk of the work with budded material. Both the mean difference in resistance level (similar to the susceptible parent) and the relatively low deviance in the resistance reactions of the progeny argue against polygenic inheritance.

There were slight and non-significant differences in the latent period, defined as time to 50% of final broom number, of GC-A5 and Castanhal-I on the progeny seedlings. GC-A5 was slightly faster on two occasions, Castanhal-I on one, and in the remaining batch the two were identical.

Brooms were produced on Amelonado hosts co-inoculated with GC-A5 and Castanhal-I in an equiproportionate mixture. They produced basidiocarps when exposed to suitable environmental conditions, and basidiospores [Correction added after publication 30 January 2007: 'basidia' changed to 'basidiospores'] were shed in abundance. Unfortunately, no spores [Correction added after publication 30 January 2007: 'basidia' changed to 'spores'] germinated, making it impossible to pursue the work. On other occasions, spores [Correction added after publication 30 January 2007: 'basidia' changed to 'spores'] produced from brooms produced on Amelonado and SCA 6 by single isolates, as well as brooms imported from producing countries, germinated easily, so the observation may be related to the co-inoculation.

Discussion

It was shown here that specific interactions, some very strong, exist between isolates of *M. perniciosa* and *T. cacao*. The average probability of a spore infecting under the conditions of this study was correlated to the time taken for swellings or brooms to appear, and an effective, if laborious, method was demonstrated for such studies in non-cocoa-growing areas.

The specific interaction between isolate GC-A5 and clone SCA 6 made infection so rare as to be undetectable under the conditions of this study, regardless of infection period. It was probably caused by a single recessive major gene, homozygous in SCA 6, because the progeny of crosses between SCA 6 and Amelonado clones susceptible to GC-A5 were all susceptible. Since the gene is recessive, it is not a classic R-gene, although the *T. cacao* genome does contain homologues to a number of genes behaving as R-genes in various pathosystems (Clement *et al.*, 2004; Lanaud *et al.*, 2004). It does not have the characteristics of the larger QTLs reported in progenies of SCA 6 × ICS1 by Queiroz *et al.* (2003) or Brown *et al.* (2005). The smaller QTL reported by Brown *et al.* (2005) showed recessiveness of resistance. Despite the strength of the interaction demonstrated in the present study, it might show up as a very minor resistance in an inoculation with a mixture of isolates, so it could be identical to the locus

suggested here. This is the first study of the resistance of cocoa clones to single-spore isolates of *M. perniciosa*, so there are no other reports of specific interactions in this pathosystem to compare it with.

The data also contained indications of more varied host-isolate interactions, such as that between clone EET 59, and isolates APC3 and GC-A5. This means that estimates of the relative resistance of host clones depend on the isolate composition of the pathogen population used for testing. In general, for high-throughput screening it is quite impractical to use defined spore sources; without detailed characterization of the variability present in natural populations of *M. perniciosa* it is not possible to choose a defined set of isolates to represent the variation present. In Table 2 a clone ranking is presented which is relative to the isolates used for testing; however, the clones shown to be very susceptible to both isolates were in many cases known to suffer badly from disease in the field (B. Eskes, IPGRI, Montpellier, France, personal communication).

Considering the results as a whole, the pathogen population seems to contain considerable variation in host specific virulence. The three isolates used were chosen to come from geographically widely separated areas, but it is likely that any given area contains a diversity of virulence types. This is supported by the successful infection of SCA 6 in Trinidad using mixed inocula from the estate in Gran Couva from which GC-A5 was isolated (Surujdeo-Maharaj *et al.*, 2003). Given the existence of such variation the pathogen population is likely to evolve to increased virulence in host populations with a narrow genetic base. It is plausible that this contributed to the breakdown of resistance in the plantations using Scavina germplasm in Rondônia in Brazil, where isolate Castanhal-I was obtained. Here, very large areas were planted during the 1970s with offspring of crosses among Scavina genotypes, all regarded as resistant. During the 1980s, a time of depressed cocoa prices, very severe attack by *M. perniciosa* developed, leading to massive losses in production (Wheeler & Mepsted, 1988). This was ascribed to poor maintenance of the plantations and cessation of hygiene measures because of a shortage of labour, but SCA 6 has retained a good level of resistance in other areas (Frias *et al.*, 1995; Surujdeo-Maharaj *et al.*, 2004).

The resistance of SCA 6 to natural field populations of *M. perniciosa* has never been complete in tests. This could be the result of a mixed pathogen population rather than or in addition to quantitative (horizontal) resistance factors. Unfortunately, this hypothesis is hard to test at present, since it would be very labour-intensive to isolate and test a large number of *M. perniciosa* isolates by the methods used here. In turn, this means that it is not feasible to predict how the pathogen population will evolve in response to a particular deployment of host germplasm.

Cocoa is a long-lived orchard crop. In such crops it takes a long time to breed resistant clones and is expensive and slow to replace hosts against which a pathogen has evolved virulence. It is clear that it would be unwise to drop quarantine precautions when moving cocoa germplasm

over large distances, even within the area in which *M. pernicioso* is endemic. However, testing of germplasm should take place in widely separated areas to ensure thorough sampling of the current *M. pernicioso* population.

Although classical biocontrol of *M. pernicioso* may now be in sight (Krauss & Soberanis, 2001; De Marco *et al.*, 2003), it would be very unwise to neglect the need for small- and large-scale diversity as a protection against unforeseen disease problems (Finckh & Wolfe, 2006). Although a few clones may have apparently superior agronomy, longer-term production stability may result from diverse plantings in which catastrophic resistance breakdown is less likely. In particular, the broadest possible genetic basis for improved host populations should be used, looking for geographically distinct parentages and incorporating multiple sources of resistance, even if they are not at the time of testing the best available. It would not be wise to deploy clonal mixtures of small numbers of genotypes, especially closely related genotypes, over large areas. Diversity of this kind is also useful in stabilizing pest problems, but in planning returns from a plantation it will always seem rational to plant only the clones giving maximum return at any one time. Some economic or educational incentive is needed to avoid this happening.

The related species, *Moniliophthora roreri*, the cause of the very damaging disease frosty pod rot, at present is expanding its range rapidly in central and south America. Both *M. pernicioso* and *M. roreri* seem to be members of a clade of biotrophic or endophytic fungi. The present results have some implications for the search for resistance to *M. roreri*, because the relatedness of the two pathogens suggests that specific resistance-virulence matching might be important in the frosty pod pathosystem also.

Because the gene segregating in the cross in this study appeared to be recessive, it is unlikely to be a classic R-gene, and genomics approaches to its identification (Kuhn *et al.*, 2003) are therefore problematic, simply because it is not obvious what to look for. Nonetheless, because it is extremely slow and expensive to screen large numbers of isolates and clones to find differential interactions, progress in studying this pathosystem must come from a collaboration between rapid molecular approaches and manageable experimental systems. The system here is workable but slow, and a large investment in facilities and labour would be needed for any large-scale population or mechanistic study conducted in a temperate country. It may be easier to do this kind of work in more isolated parts of producing countries with appropriate controls or molecular diagnostics to detect infection from background inoculum and prevent the escape of exotic isolates.

Acknowledgements

This work was funded by grants from the British Cacao, Chocolate and Confectionery Alliance and from the Common Fund for Commodities. We are grateful to Bertus Eskes for comment on the manuscript and helpful advice throughout the project, and to Bryan Wheeler and Gareth Griffith for invaluable advice and gifts of isolates.

References

- Aime MC, Phillips-Mora W, 2005. The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. *Mycologia* **97**, 1012–22.
- Andebrhan T, Figueira A, Yamada MM, Cascardo J, Furtek DB, 1999. Molecular fingerprinting suggests two primary outbreaks of witches' broom disease (*Crinipellis pernicioso*) of *Theobroma cacao* in Bahia, Brazil. *European Journal of Plant Pathology* **105**, 167–75.
- de Arruda MCC, Miller RNG, Ferreira M, Felipe MSS, 2003. Comparison of *Crinipellis pernicioso* isolates from Brazil by ERIC repetitive element sequence-based PCR genomic fingerprinting. *Plant Pathology* **52**, 236–44.
- Bartley BGD, 2001. The origin and compatibility relationships of the Scavina variety of *Theobroma cacao* L. *Ingenic Newsletter* **6**, 23–4.
- Brown J, Schnell RJ, Motamayor JC, Lopes U, Kuhn DN, Borrone JW, 2005. Resistance gene mapping for witches' broom disease in *Theobroma cacao* L. in an F2 population using SSR markers and candidate genes. *Journal of the American Society for Horticultural Science* **130**, 366–73.
- Clement D, Lanaud C, Sabau X, Fouet O, Cunff LI, Ruiz E, Risterucci AM, Glaszmann JC, Piffanelli P, 2004. Creation of BAC genomic resources for cocoa (*Theobroma cacao* L.) for physical mapping of RGA containing BAC clones. *Theoretical and Applied Genetics* **108**, 1627–34.
- Daymond AJ, Hadley P, 2004. The effects of temperature and light integral on early vegetative growth and chlorophyll fluorescence of four contrasting genotypes of cacao (*Theobroma cacao*). *Annals of Applied Biology* **145**, 257–62.
- Deadman ML, 2006. Epidemiological consequences of plant disease resistance. In: Cooke BM, Gareth Jones D, Kaye B, eds. *The Epidemiology of Plant Diseases*, 2nd edn. Dordrecht, the Netherlands: Springer, 139–58.
- De Marco JL, Valadares-Inglis MC, Felix CR, 2003. Production of hydrolytic enzymes by *Trichoderma* isolates with antagonistic activity against *Crinipellis pernicioso*, the causal agent of witches' broom of cocoa. *Brazilian Journal of Microbiology* **34**, 33–8.
- Dickstein ER, Purdy LH, Frias GA, 1987. *Crinipellis pernicioso*, the cacao witches' broom fungus-inoculum production and storage. *Phytopathology* **77**, 1747 (Abstract).
- End MJ, 1990. *A Study of the Effects of the Photo-Thermal Environment on Fruit and Seed Growth and Development in Theobroma cacao* L. Reading, UK: University of Reading, PhD thesis.
- Evans HC, 1980. Pleomorphism in *Crinipellis pernicioso*, causal agent of witches' broom disease of cocoa. *Transactions of the British Mycological Society* **74**, 515–23.
- Finckh MR, Wolfe MS, 2006. Diversification strategies. In: Cooke BM, Gareth Jones D, Kaye B, eds. *The Epidemiology of Plant Diseases*, 2nd edn. Dordrecht, the Netherlands: Springer, 269–308.
- Ford CS, 2003. *Investigations into the Genetic and Physiological Basis of Self-Incompatibility in Cocoa* (*Theobroma cacao* L.). Reading, UK: University of Reading, PhD thesis.
- Frias GA, Purdy LH, Schmidt RA, 1995. An inoculation method for evaluating resistance of cacao to *Crinipellis pernicioso*. *Plant Disease* **79**, 787–91.

- Griffith GW, Hedger JN, 1993. A novel method for producing basidiocarps of the cocoa pathogen *Crimipellis pernicioso* using a bran-vermiculite medium. *Netherlands Journal of Plant Pathology* **99**, 227–30.
- Griffith GW, Hedger JN, 1994. The breeding biology of biotypes of the witches' broom pathogen of cocoa, *Crimipellis pernicioso*. *Heredity* **72**, 278–89.
- Kilaru A, Hasenstein KH, 2005. Development and pathogenicity of the fungus *Crimipellis pernicioso* on interaction with cacao leaves. *Phytopathology* **95**, 101–7.
- Krauss U, Soberanis W, 2001. Biocontrol of cocoa pod diseases with mycoparasite mixtures. *Biological Control* **22**, 149–58.
- Kuhn DN, Heath M, Wisser RJ, Meerow A, Brown JS, Lopes U, Schnell RJ, 2003. Resistance gene homologues in *Theobroma cacao* as useful genetic markers. *Theoretical and Applied Genetics* **107**, 91–202.
- Lanaud C, Risterucci AM, Pieretti I, N'Goran JAK, Fargeas D, 2004. Characterisation and genetic mapping of resistance and defence gene analogs in cocoa (*Theobroma cacao* L.). *Molecular Breeding* **13**, 211–27.
- Lass RA, Purdy LH, 1993. Introduction to the international witches' broom project. In: Rudgard SA, Maddison AC, Andebrhan T, eds. *Disease Management in Cocoa: Comparative Epidemiology of Witches' Broom*. London, UK: Chapman & Hall, 1–7.
- Madison AC, Andebrhan T, Aranzazu F, Silva-Acuña R, 1993. Comparative phytosanitation studies. In: Rudgard SA, Maddison AC, Andebrhan T, eds. *Disease Management in Cocoa: Comparative Epidemiology of Witches' Broom*. London, UK: Chapman & Hall, 165–88.
- Marita JM, Nienhuis J, Pires JL, Aitken WM, 2001. Analysis of genetic diversity in *Theobroma cacao* with emphasis on witches' broom disease resistance. *Crop Science* **41**, 1305–16.
- Purdy LH, Schmidt RA, Dickstein ER, Frias GA, 1997. An automated system for screening *Theobroma cacao* for resistance to witches' broom. *Agrotropica* **9**, 119–26.
- Queiroz VT, Guimarães CT, Anherth D, Schuster I, Daher RT, Pereira MG, Miranda VRM, Loguercio LL, Barros EG, Moreira MA, 2003. Identification of a major QTL in cocoa (*Theobroma cacao* L.) associated with resistance to witches' broom disease. *Plant Breeding* **122**, 268–72.
- Silva SDVM, Matsuoka K, 1999. Histologia da interação *Crimipellis pernicioso* em cacauzeiros suscetível e resistente à vassoura-de-bruxa. *Fitopatologia Brasileira* **24**, 54–9.
- Surujdeo-Maharaj S, Umaharan P, Butler DR, Sreenivasan TN, 2003. An optimised screening method for identifying levels of resistance to *Crimipellis pernicioso* in cocoa (*Theobroma cacao*). *Plant Pathology* **52**, 464–75.
- Surujdeo-Maharaj S, Umaharan P, Butler DR, 2004. Assessment of resistance to witches' broom disease in clonal and segregating populations of *Theobroma cacao*. *Plant Disease* **88**, 797–803.
- Wheeler BEJ, Mepsted R, 1988. Pathogenic variability amongst isolates of *Crimipellis pernicioso* from cocoa (*Theobroma cacao*). *Plant Pathology* **37**, 475–88.
- Wheeler BEJ, Suárez C, 1993. The pathosystem. In: Rudgard SA, Maddison AC, Andebrhan T, eds. *Disease Management in Cocoa: Comparative Epidemiology of Witches' Broom*. London, UK: Chapman & Hall, 9–19.