Basidiocarp Development on Mycelial Mats of *Crinipellis perniciosa*

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ABSTRACT

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Basidiocarps of *Crinipellis perniciosa* developed in 4–5 wk on mycelial mats supported by sterilized pieces of witches' brooms from cacao, and mats continued to produce basidiocarps for an additional 8 wk. Multibasidiospore and single-basidiospore isolates of the fungus from *Theobroma cacao, Solanum* spp., and lianas produced basidiocarps on mycelial mats.

Additional keywords: witches' broom, cacao, cocoa

Witches' broom of *Theobroma cacao* L. can be induced only by germinated basidiospores of *Crinipellis perniciosa* (Stahel) Singer (14); therefore a reliable source of these infective propagules is necessary to evaluate host responses to the pathogen. Basidiocarps, in which basidiospores are produced, develop most commonly in the field on necrotic witches' brooms after the brooms have been wet during rainy periods. Brooms can be removed from trees in the field, placed under a water sprinkler, and wetted intermittently, but basidiocarps may not develop for 2–16 mo (6). In a controlled laboratory environment, basidiocarps developed on witches' brooms after 8–12 wk (12). Basidiospores produced in either of these circumstances can be used to inoculate host plants. However, production of basidiospores was unpredictable; therefore, inoculum may be available when plant materials are not susceptible and, conversely, plant materials may be susceptible but basidiospores not available (7).

Basidiocarps developed after 10–13 mo on pieces of cacao in water agar inoculated with mycelia of the pathogen (10,11). Basidiospores produced in these basidiocarps were applied to cacao and induced symptoms of witches' broom. Pickering and Hedger (9) reported development of basidiocarps on pieces of brooms in water agar in flasks 7 wk after inoculation with agar disks from a culture of the fungus. Development of basidiocarps continued, and after 6 mo basidiocarps had developed in 45% of the flasks. The diameter of the pileus of these basidiocarps was 6–7 mm, whereas the pileus of basidiocarps from field collections ranged from 20 to 30 mm.

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Spore production in the absence of a discrete fruiting body has been reported for *C. perniciosa*. Delgado and Cook (4) observed chains of arthroconidia in paired cultures of the fungus, but witches' broom symptoms did not develop after application to cacao. Basidia and basidiospores developed after 6–8 wk in cultures of *C. perniciosa*, and basidiospores from these cultures induced symptoms of witches' broom in cacao (2). However, determination of the numbers of basidiospores in a suspension from an agar culture may be difficult.

Stahel (15) observed basidiocarp development on mycelial mats from agar cultures after the mats had been removed from the agar surface, dried for a short time, and then placed under a *Ficus* tree during 10 days of wet weather. Purdy (10) and Purdy et al (11) modified Stahel's methods to produce basidiocarps on mycelial mats supported by pine twigs or glass rods, but the basidiocarps were small and produced few basidiospores.

In this article, we report the reliable and predictable production of basidiocarps under semicontrolled conditions on mycelial mats of *C. perniciosa*.

MATERIALS AND METHODS

Source of isolates. Witches' brooms received from colleagues in countries where the disease occurs were hung in a moist chamber in the greenhouse to induce basidiocarp development. Single- and multiple-basidiospore cultures of the fungus were derived, and mycelial isolates were obtained directly from the brooms.

Isolate 550, used in the development of the method, was obtained as a multi-basidiospore isolate from a basidiocarp produced on a small piece of an autoclaved broom in water agar that had been inoculated with paired mycelial cultures of *C. perniciosa* isolated from brooms from Pichilingue, Ecuador, and Belem, Brazil (10). Thus, the exact origin of isolate 550 is unknown; it was selected for its ability to produce basidiocarps and infective basidiospores.

Table 1. Development of basidiocarps of *Crinipellis perniciosa* on mycelial mats of isolate 550 supported by pieces of old cacao witches' brooms, glass rods, or wood of five species[a]

Support	Number of mature basidiocarps by size (mm)[b]			
	2–3	4–6	7–15	Total
Witches' brooms	38	94	67	199
Balsa	4	1	0	5
Mahogony	6	8	0	14
Poplar	11	3	1	15
White oak	22	8	1	31
Yellow pine	13	3	0	16
Glass rods	14	4	0	18

[a] Basidiocarps produced in one experiment.
[b] V-8 juice medium inoculated on 2-20-86; mycelial mats hung on support materials and placed in moist chamber on 4-2-86; first mature basidiocarp on 5-12-86.

Mycelial mats. Mycelial mats of *C. perniciosa* were produced in 500-ml Erlenmeyer flasks, to each of which was added 100 ml of clarified V-8 juice liquid medium. (The liquid medium was made from 700 ml of V8 juice [Campbell Soup (Texas) Inc., Paris, TX 75460] plus 10.5 g of calcium carbonate, which were mixed on a magnetic stirrer for 5 min and centrifuged on a table-top centrifuge to remove solids. The liquid was saved, the pellet discarded. To 250 ml of this clarified V8 juice, 20 g of sucrose and 750 ml of deionized water were added.) Flasks with the culture medium were autoclaved. Two 6-mm disks from the advancing margin of yeast extract agar (16) cultures of *C. perniciosa* were put into each flask. The flasks were placed at 27 C in an incubator equipped with white light and black light fluorescent tubes (F20T12-BLB, Sylvania Electric Products, Inc., Sylvania Lighting Center, Danvers, MA 01923; peak emission 350 nm) that provided a light period for 12 hr/day. All flasks were shaken daily by hand during the first week to break up mycelial aggregates so that a uniform mycelial mat would develop. Mats were harvested after 5 wk.

During the period 1983–1985, mycelial mats were folded over glass rods and suspended in a plexiglass moist chamber (50 × 60 × 110 cm) in the laboratory, where the temperature was 21–24 C. Two blacklite blue fluorescent tubes that supplemented the normal laboratory light (9 hr/day) were located under the top cover of the moist chamber to stimulate formation of basidiocarps (1).

We used a daily moisture regime of (in hours) 2 wet, 5 dry, 1 wet, 6 dry, 1 wet, 4 dry, 1 wet, 4 dry. A humidifier centered at the bottom of the moist chamber atomized water upward into the chamber during the wet hours, when the relative humidity was 100%. During dry hours, when the humidifier did not operate, the relative humidity reached lows near 60%. Maintenance of a satisfactory moist condition for the mycelial mats was essential; if they were too wet, other microorganisms grew rampantly on the mats and interfered with normal development of basidiocarps. If the mats were too dry, they shriveled and did not produce basidiocarps. Heating and air conditioning of the ambient building air introduced problems in maintaining moisture conditions within the moist chamber. Their solution required daily observation of the moisture condition of mycelial mats and some modifications of the number of wet or dry hours.

Advantages of using natural media rather than artificial media (13) to induce sporulation of various fungi have been demonstrated. The most common natural substrate for basidiocarp development by *C. perniciosa* is dried witches' brooms. Pieces of dried brooms from cacao were autoclaved twice, 1 hr each at 24-hr intervals, to ensure that all propagules of *C. perniciosa* and other microorganisms were no longer viable. The sterile broom pieces were used to support mycelial mats in a manner similar to that of glass rods. Wood pieces of other species were also treated in the same way and evaluated as supports for mycelial mats.

RESULTS

In an experiment with 20 mycelial mats of isolate 550 of *C. perniciosa* on glass rods, basidiocarps developed 11 wk after inoculation of the V-8 juice culture medium, and basidiocarps continued to form on the mycelial mats for an additional 8 wk. A total of 299 basidiocarps were produced on the 20 mycelial mats, but one mat failed to produce any, whereas the most productive mat had 37. In another experiment with isolate 550, 15 mycelial mats on glass rods produced 171 basidiocarps. In this experiment, 40 basidiocarps developed on one mat, but five mats each produced fewer than five basidiocarps. Of 43 mycelial mats of isolate 550 on glass rods that were excessively wet for 2 wk, 83 basidiocarps developed on 22; only basidiocarp initials and developing stripes were observed on the other mats. Variability in numbers of basidiocarps per mat occurred in all experiments. Basidiocarps on mycelial mats on glass rods were small (pileus diameter 2–3 mm) and produced few basidiospores.

The advantage of using autoclaved broom pieces to support mycelial mats was obvious when compared with wood of five species and glass rods, using six mycelial mats per support material (Table 1). The experiment was repeated once. Sixfold more basidiocarps developed on mycelial mats supported by pieces of witches' brooms than on mats supported by white oak (the next highest number), and only 18 basidiocarps developed on mats supported by glass rods. The percentage of basidiocarps with a pileus of 7–15 mm or larger was higher on broom pieces (34%), than on white oak (3%), and there were no basidiocarps in this size range on glass rods. Basidiocarps were similar in size and basidiospore production to those on brooms in the field, and basidiocarp development continued for an additional 8 wk.

Basidiocarps of *C. perniciosa* similar in all respects to basidiocarps of isolate 550 developed on mycelial mats of 17 isolates or combinations of isolates from cacao supported by autoclaved broom pieces (Table 2). In addition, basidiocarps developed on mycelial mats of four isolates of the fungus from *Solanum* spp. collected near Manaus, Brazil, and on

mycelial mats of four isolates from lianas collected near Pichilingue, Quevedo, Ecuador. Basidiospores from basidiocarps produced by cacao isolates induced symptoms of witches' broom in cacao. Basidiospores of the isolates from *Solanum* spp. elicited responses in tomato suggestive of witches' broom but failed to induce symptoms of disease in cacao. In contrast, all inoculations of cacao and solanaceous plants with basidiospores of the liana isolates failed.

DISCUSSION

Evaluation of responses of cacao to the witches' broom pathogen requires at least two essential components; 1) basidiospores of *C. perniciosa*, and 2) cacao plants in a susceptible stage of development. The uncertain length of time needed for development of basidiocarps from naturally produced witches' brooms, 2–16 mo (6), makes timing of these events difficult, particularly for individuals who work where the disease does not occur. Production of basidiospores of *C. perniciosa* in mycelial cultures has been suggested (2) as an alternative method for inoculum production, but neither the frequency of this event nor the number of isolates that possessed this capability were mentioned. Therefore, basidiocarps of *C. perniciosa* are the most reliable source of basidiospores for inoculation of cacao.

Basidiocarps developed 8–12 wk after inoculation of the V-8 juice medium on mycelial mats supported by pieces of autoclaved witches' brooms from cacao, and basidiocarp production continued for an additional 8 wk or more. However, considerable variation occurred with respect to the number of basidiocarps produced per mycelial mat, even though the inoculum from which the mats developed was from the same culture of the fungus. This might have resulted from a differential moisture condition for mats in various locations within the moist chamber. We recognize that the environmental conditions within the moist chamber may not have satisfied all requirements for basidiocarp development, but they provided an environment within which basidiocarps were produced in sufficient numbers to provide inoculum. Variation in basidiocarp production within collections of brooms from locations where witches' broom occurs have been observed in Gainesville. These situations suggest that different requirements may exist for individual mats or brooms that affect basidiocarp development and are not being met by the methods employed.

Use of autoclaved broom pieces to support mycelial mats for basidiocarp production resulted in basidiocarps that were similar in all respects to those produced on brooms from the field. The fruiting bodies of agarics develop on or from a thallus consisting of hyphae that ramify a substrate. Mycelial mats of *C. perniciosa* on glass rods did not have a substrate to satisfy moisture and nutrient requirements, all of which had to be supplied externally. This situation resulted in considerable difficulty in maintenance of a moisture regime that supported basidiocarp development from mats on glass rods, and the small size of basidiocarps may be the result of limited nutrients within the mat to support basidiocarp growth. In contrast, the natural relationship between *C. perniciosa* and its common substrate, witches' brooms of cacao, was established when mycelial mats were supported by autoclaved broom pieces. Hyphae from the mat grew into the broom tissue and gained access to moisture and nutrients for the mycelial mat and developing basidiocarps. The fact that basidiocarps developed on the broom pieces beyond the limits of the mycelial mats supports this explanation.

Single-basidiospore and multibasidiospore isolates from cacao were equally productive with respect to basidiocarp development on mycelial mats. The basidiocarps that developed showed no morphological variation. Basidiocarps of isolates from *Solanum* spp. fit the description of the fungus by Bastos and Evans (3), and basidiocarps of isolates from lianas were as described by Griffith (8).

Table 2. Isolates of *Crinipellis perniciosa* that produced basidiocarps on mycelial mats supported by pieces of autoclaved witches' brooms from cacao

Isolate Number	Type[a]	Host	Source Location
4B	MS	*Theobroma cacao*	Quatra Bocas, Brazil
GH1	MS	*T. cacao*	Gainesville, FL
OP1	MS	*T. cacao*	Ouro Preto do Oeste, Brazil
540	MS	*T. cacao*	Gainesville, FL
541	MS	*T. cacao*	Gainesville, FL
549	MS	*T. cacao*	Quatra Bocas, Brazil
550	MS	*T. cacao*	Gainesville, FL
50 × 97[b]	MS	*T. cacao*	Gainesville, FL
550 × (GH1)	MS	*T. cacao*	Gainesville, FL
5550 × OP1	MS	*T. cacao*	Gainesville, FL
504	SS	*T. cacao*	Ouro Preto do Oeste, Brazil
509	SS	*T. cacao*	Ouro Preto do Oeste, Brazil
527	SS	*T. cacao*	Ouro Preto do Oeste, Brazil
767	SS	*T. cacao*	Caucagua, Venezuela
794	SS	Isolate 550	Gainesville, FL
797	SS	Isolate 550	Gainesville, FL
801	SS	Isolate 550	Gainesville, FL
87	SS	*Solanum* sp.	Manaus, Brazil
770	SS	Isolate 87	Manaus, Brazil
862	MS	*Solanum* sp.	Manaus, Brazil
866	MS	*Solanum* sp.	Manaus, Brazil
LA 52[c]	MS	Liana	Pichilingue, Ecuador
LA 58	MS	Liana	Pichilingue, Ecuador
LA 60	MS	Liana	Pichilingue, Ecuador
LC 17	MS	Liana	Pichilingue, Ecuador

[a] MS = multibasidiospore isolate, SS = single-basidiospore isolate.
[b] Isolate 97 is a mycelial isolate from brooms produced on *Herrania* sp. in Pichilingue, Ecuador, paired with isolate 550 to produce a mycelial mat. Isolate 97 was not evaluated alone for basidiocarp production.
[c] Liana isolates were kindly provided by Gareth Griffith, Department of Botany and Microbiology, School of Biological Sciences, The University College of Wales, Aberysthwyth, United Kingdom.

The methods we describe make it possible to produce inoculum of *C. perniciosa* repeatedly from the same isolate, and the new dimension of storing basidiospores for 1.5 yr or longer (5,7) allows scheduling of inoculation when plants are in a susceptible growth stage.

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