

A Phytotoxic Activity in Extracts of Broth Cultures of *Mycosphaerella fijiensis* var. *difformis* and Its Use to Evaluate Host Resistance to Black Sigatoka

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ABSTRACT

Molina, G. C., and Krausz, J. P. 1989. A phytotoxic activity in extracts of broth cultures of *Mycosphaerella fijiensis* var. *difformis* and its use to evaluate host resistance to black Sigatoka. *Plant Disease* 73:142-144.

Crude extracts from 28-day-old coconut (*Cocos nucifera*) and soybean (*Glycine max*) broth cultures of *Mycosphaerella fijiensis* var. *difformis* caused tissue necrosis on treated banana (*Musa acuminata*) and plantain (*Musa acuminata* × *M. balbisiana*) plants. The phytotoxic activity could be extracted by either chloroform or ethyl acetate. Phytotoxic activity was evaluated by either dipping cut leaf tips of banana and plantain plants developed from meristem cultures into a 1:10 aqueous dilution of the extract or by puncturing the leaf with a sharp needle through a 20- μ l droplet of the diluted extract placed on the leaf surface. Extract from uninoculated broth cultures showed no phytotoxic activity. Relative levels of field resistance to *M. fijiensis* var. *difformis* in seven banana and plantain differential cultivars were strongly inversely correlated with the area of necrotic tissue in extract-treated plants. The diluted extract failed to cause a phytotoxic reaction on eight treated plant species that were not of the genus *Musa*.

Black Sigatoka, caused by *Mycosphaerella fijiensis* var. *difformis* Mulder & Stover, is a very destructive disease of banana (*Musa acuminata* Colla) and plantain (*Musa acuminata* × *M. balbisiana* Colla) in Central America, northern South America, West Africa, and Asia (1,2,6,8,9). The disease is spreading rapidly in West Africa and South America.

The cost of chemical control of the disease in bananas grown for export in Central and South America is approximately \$100 million annually (7). In Central America production of plantains for local consumption and exportation has suffered drastic reductions due to the disease (2,6). The socioeconomic importance of both plantains and bananas to many of the areas affected by black Sigatoka is substantial. The rapid dissemination of this disease will undoubtedly cause increasing social and economic hardships.

The development of cultivars of bananas and, especially, plantains resistant to black Sigatoka is imperative to control the disease economically. However, breeding plantains and bananas is a very slow, tedious process due to serious problems with polyploidy and seed and pollen infertility. Improved techniques are needed for rapid screening of resistance to black Sigatoka in young plants and for testing the available resistant germ plasm against a worldwide collection of isolates of the pathogen without international transport of plants or fungal isolates.

The presence of a distinct, elongated

chlorotic zone surrounding lesions caused by *M. fijiensis* var. *difformis* suggests the possible effect of a pathogen-induced phytotoxin. This paper describes efforts to extract a phytotoxic principle from broth cultures of *M. fijiensis* var. *difformis* and to test the extracts for phytotoxic activity on banana and plantain cultivars with varying levels of resistance to black Sigatoka. The potential use of the extract for screening for resistance to black Sigatoka and for studying the nature of available sources of resistance and of the pathogenic variability of *M. fijiensis* var. *difformis* is discussed.

MATERIALS AND METHODS

Fungal cultures were obtained from ascospores discharged from infected banana leaves using the technique described by Stover (5). Single germinating ascospores were transferred to soybean agar slants prepared from 20 g of finely ground soybeans, 20 g of dextrose,

and 18 g of agar per liter of distilled water. After 18 days of incubation at room temperature, 10 ml of sterile, distilled water were added to each culture tube. The surface of the cultures was scraped with a sterile glass rod and a 1-ml sample of the fungal suspension was used to inoculate each 250-ml flask containing 50 ml of either banana decoction, coconut broth, soybean broth, or Fries medium (4). To prepare banana decoction, coconut broth, and soybean broth, 200 g of banana leaves, grated coconut, or soybeans, respectively, were added to 1 L of distilled water in 2,000-ml Erlenmeyer flasks. The flasks were steamed for 30 min. The contents were filtered through cheesecloth and diluted to 2 L with distilled water. Dextrose and yeast extract (20 g each) were added, and the mixtures were autoclaved. The pH of each broth was adjusted to 6.0 with hydrochloric acid before autoclaving. The inoculated 250-ml flasks were incubated at 28 C for 28 days under constant agitation. Uninoculated broths served as controls.

Chloroform, ethyl acetate, ethyl ether, and methanol were evaluated as solvents for extracting phytotoxic activity from the culture filtrates of *M. fijiensis* var. *difformis*. The organic solvents were used at a ratio of 1:2 (v/v) (culture filtrate to organic solvent). The extraction process was repeated twice and each extraction lasted 48 hr. The combined extract from the two extractions was evaporated to 2 ml in a rotary evaporator. The extracts then were dried by blowing a stream of nitrogen gas onto their surface. Extractions were also done from uninoculated broth.

Table 1. Severity of necrosis on meristem-cultured banana plantlets treated with extracts from various media with or without *Mycosphaerella fijiensis* var. *difformis* and extracted with two organic solvents

Media	Solvent	Reaction ^a	
		Without <i>M. fijiensis</i> var. <i>difformis</i>	With <i>M. fijiensis</i> var. <i>difformis</i>
Banana decoction	Chloroform	—	+
	Ethyl acetate	—	+
Coconut extract	Chloroform	—	+++
	Ethyl acetate	—	+
Fries medium	Chloroform	+	++
	Ethyl acetate	+	++
Soybean broth	Chloroform	—	++
	Ethyl acetate	—	++

^a Average distance of advance of the necrotic reaction from the treated cut leaf tip 24 hr after treatment; + = trace to 1.5 mm, ++ = 1.6 to 3.0 mm, +++ = more than 3.0 mm.

The crude extracts were diluted at a ratio of 1:10 (w/v) (crude extract to sterile distilled water) and tested for phytotoxic activity on 10–12.5 cm tall, meristem-cultured plantlets of banana and plantain cultivars and breeding lines with varying levels of field-observed resistance to black Sigatoka. Treatments consisted of cutting the leaf tips of the plantlets and dipping them for 1 min in the diluted extract or by placing a 20- μ l droplet of the diluted extract onto the leaf and piercing the leaf with a sterile needle through the droplet. The plantlets were incubated in the test tubes at 27 C for 24 hr. Phytotoxic activity was quantified by measuring the distance from the cut leaf tip to the farthest margin of necrotic tissue with the cut-leaf method or by measuring perpendicular diameters of the area of necrotic tissue surrounding the pin prick with the needle-piercing method.

To determine the host specificity of the possible toxic metabolite, four of each of the following plants were grown in pots for 4 wk and treated with the dilute crude extract using the needle-piercing method: cucumber (*Cucumis sativus* L.), watermelon (*Citrullus vulgaris* Schrad.), cowpea (*Vigna sinensis* (Torner) Savi.), soybean (*Glycine max* (L.) Merr.), tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana tabacum* L.), heliconia (*Heliconia caribaea* Lam.), and banana (*Musa acuminata*).

RESULTS AND DISCUSSION

The fungus grew in all the media tested, but grew more abundantly in the coconut and soybean broths. The fungal growth consisted of numerous, individual, velvety, grayish-to-black mycelial colonies. Chloroform and ethyl acetate extractions of all inoculated broth cultures were toxic to plantlets (Table 1), whereas those of the uninoculated controls, except from Fries medium, were not.

Extracts from coconut and soybean broths had the most phytotoxic activity. The phytotoxic response was characterized by a water-soaked appearance of tissue progressing from the point of treatment. The tissue turned dark brown to black within 24 hr (Fig. 1).

Methanol and ethyl ether extracts of both inoculated and uninoculated broth media were phytotoxic on treated plants. Therefore, these solvents were not used for subsequent extractions. In contrast, in the separate bioassays of chloroform and ethyl acetate extracts of inoculated coconut and soybean broth, severe phytotoxicity was observed in treated leaf tips of the susceptible cultivar Grand Naine.

In the subsequent tests in which the needle-piercing method of treatment was used, the relative phytotoxicity caused by the extract, as measured by the diameter of the area of necrotic tissue, clearly

Table 2. Phytotoxic response of differential cultivars of banana and plantain treated with diluted extract from culture of *Mycosphaerella fijiensis* var. *difformis*

Cultivar	Diameter of necrotic area ^a (mm)	Field-observed susceptibility ^b
Grand Naine	5.5 a	5
Platano 'Cuerno'	4.5 b	4
3A-105-7	4.2 b	4
Sabá	3.6 c	3
3A-106-6	3.0 d	3
SH 3437	2.0 e	2
IV-9	1.0 f	1

^a Average of perpendicular diameters from two trials each replicated three times on two leaves per replication and observed 24 hr after treatment with 20 μ l of diluted extract using the needle-piercing method. Averages followed by different letters are significantly different according to Duncan's multiple range test ($P = 0.01$).

^b Relative susceptibility to black Sigatoka based on field observation over several years; 1 = very resistant, 5 = very susceptible.

varied between the black Sigatoka-susceptible cultivar Grand Naine and the line IV-9 reported as highly resistant to black Sigatoka (3) (Table 2). Among the other plants with varying levels of resistance, good correlation exists between the relative severity of phytotoxicity caused by the extract and the observed level of susceptibility as determined by field observation over many years. Moreover, the extract appears to be toxic only to banana and plantain, because no phytotoxicity was observed on any other treated plant species, including the taxonomically related heliconia. Thus, host-specific phytotoxic activity was produced in vitro by the causal organism of the black Sigatoka disease of plantain and banana. The toxic principle appears to be related to the infection process in the host-pathogen interaction, because banana and plantain cultivars with well-recognized levels of resistance to black Sigatoka are less affected than are cultivars with known low levels of resistance or high susceptibility to the disease.

The purification and characterization of the metabolite(s) responsible for the phytotoxicity is being planned. The toxic activity apparently can be used to screen banana and plantain breeding material rapidly and at a very young age of the plant. This should prove very useful to hasten and to quantify the resistance screening process. Breeding programs active in areas where black Sigatoka has not yet arrived (i.e., Jamaica, Brazil) could use the toxic activity to screen their breeding material for resistance and avoid sending plant material to other countries for testing. Resistant germ plasm could also be screened for resistance to numerous isolates of *M. fijiensis* var. *difformis* collected world-

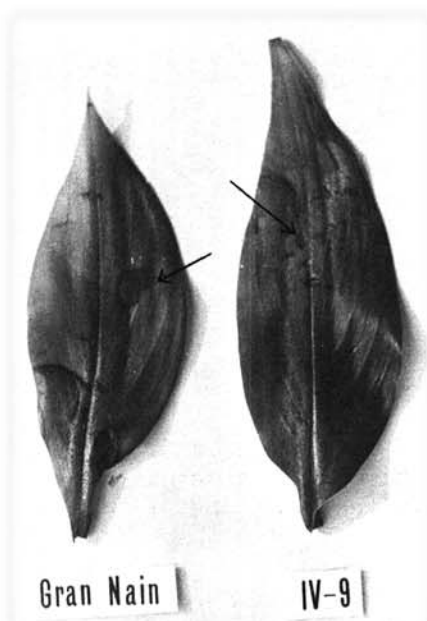


Fig. 1. Characteristic water-soaked, necrotic symptoms on leaves of young, meristem-cultured banana plants treated with a 1:10 (w/v) aqueous dilution of an abstract from broth culture of *Mycosphaerella fijiensis* var. *difformis*. Very susceptible reaction is shown on cultivar Grand Naine and highly resistant reaction on line IV-9. The treatment involved piercing the leaf with a sterile needle through a 20- μ l droplet of the diluted extract. The arrows indicate points of treatment.

wide by using the toxic activity and thus avoid the danger of international exchange of fungal cultures or plant materials.

The use of the toxic activity may prove to be a very beneficial tool for study of the host-pathogen interaction in the black Sigatoka disease, the nature of the available resistance, and the potential pathogenic variability of the causal organism.

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