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FINAL REPORT

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Dr. GLORIA C. MOLINA
Dept. of Crop Protection
Honduran Foundation for Agricultural Research (FHIA)
P.O. Box 2067
San Pedro Sula, Honduras, C.A.

"Production, extraction and purification of mycotoxin produced by *Mycosphaerella fijiensis* var. *difformis* and evaluation of its use for screening for resistance to Black Sigatoka".

Summary of the report

Studies were carried out to demonstrate the production of mycotoxin by *Mycosphaerella fijiensis* and its involvement in the pathogenesis of Black Sigatoka disease of banana. Different culture media and solvents for extraction of the phytotoxic component were evaluated. Coconut and soybean broths proved to be the best media among those evaluated. The phytotoxic component was best extracted by either chloroform or ethyl acetate. The biological activity of the toxin was observed on meristem cultured plantlets, inoculated with *M. fijiensis* culture extracts. Phytotoxic activity was expressed by necrosis from the point of inoculation. Necrosis was evident 24 hours after introduction of the extracts from *Mycosphaerella fijiensis* inoculated broths. No necrosis was observed on plants treated with extracts from uninoculated broths. Banana and plantain varieties with known levels of susceptibility and resistance in the field to *M. fijiensis* were subsequently treated with the phytotoxic extracts. Results showed that resistant varieties developed smaller necrotic areas compared to susceptible ones. This indicates the potential use of the phytotoxic extract as a tool for early screening of bananas and plantains against Black Sigatoka.

Purification of the toxin has been done and led to identification of the specific fraction causing necrosis on infected leaves.

Introduction

Black Sigatoka is the major disease problem of bananas and plantain world wide (1,5,6). Cost of chemical control to this disease runs to tens of millions of dollars annually among commercial plantations. Yield losses among subsistent banana and plantain growers is significant although accurate quantitative data is unavailable (7). The use of Black Sigatoka resistant variety is an ideal and long sought control strategy. The impact of resistant variety will be felt mostly by small banana and plantain growers who can not afford to use the costly chemical control (2,3,4).

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However, breeding for resistance in banana is a very tedious process due to long generation cycle of the crop and seed infertility related to polyploidy of the crop. The traditional screening process involves a long waiting period in the field as well as requires large space.

The discovery of phytotoxic specific toxin produced by *Mycosphaerella fijiensis* provided a promising alternative of an early and rapid screening for resistance to *M. fijiensis*.

The objectives of the study are the following:

1. To improve in-vitro culture/incubation procedure to increase yield of toxin.
2. To evaluate solvents in which toxin could be extracted for better recovery.
3. To purify toxin.
4. To evaluate different techniques of inoculating the toxin to differential varieties.
5. To evaluate dosage series of the toxin on differential varieties.

Materials and Methods

1. Evaluation of various media. The following media were tested:

    a. banana decoction broth
    b. coconut water
    c. soybean broth

2. Evaluation of incubation conditions.

    a. Duration of light: continuous light, 12 hr dark/12 hr light.
    b. Temperature: 20, 25 and 30°C.
    c. Length of incubation: 14, 21 and 28 days.

3. Incubation conditions

    a. Continuous agitation or shaking
    b. Stationary condition

Development of methods to increase yield of toxin also included development of techniques to extract toxin from broth media. The following solvents were tested: chloroform, ethyl acetate, ethyl either and methanol. Extraction of toxin using these solvents were done in fume hood for safety purposes.

Toxin yield was assessed by taking weight of crude extracts after extraction by solvent. Phytotoxicactivity was evaluated by inoculating toxin at various concentrations to meristem cultured plantlets of a susceptible variety of banana, *Musa acuminata* Gran Nain.

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Purification of fraction was done to improve sensitivity of toxin. Purification was done with the use of thin layer chromatography using silica gel G as absorbent. Different fractions separated from this method were tested for their biological activity.

Another aspect of this study was to develop the toxin as a tool in screening for disease resistance to Black Sigatoka, hence a standard technique such as standard quantity and method for inoculating the toxin to test plants were established.

The following quantity were tested:

a. 5 *ug*/plantlet
b. 10 *ug*/plantlet
c. 20 *ug*/plantlet

The following methods to inoculate the plantlets were tested:

a. Deposition of the toxin in an open leaf.
b. Injection of toxin in the leaf.
c. Mixing toxin in media for culturing banana plantlets.
d. Submerging the cut leaf tip of plantlets in diluted toxin.

Once above techniques were established on a susceptible variety, the activity of the toxin was evaluated on differential varieties of bananas and plantains with known resistance and susceptibility to Black Sigatoka. The activity of the toxin was assessed on the degree of necrosis on the leaves of the plantlets. The following varieties were tested:

1. *Musa acuminata* 'Gran Nain'
2. *Musa acuminata* x burmanica 'Horn Plantain'
3. *Musa acuminata* Cavendish
4. *Musa acuminata* 3A-105-7
5. *Musa acuminata* 3A-106-6
6. *Musa acuminata* 'Saba'
7. *Musa acuminata* IV-9
8. *Musa acuminata* 3437

Results and Discussion

1. Evaluation of media

Coconut and soybean broth were identified to be the best media to produce the phytotoxic compound. However, the level of toxic activity in diluted crude extracts showed that the concentration of the phytotoxic principle needs to be increased for better sensitivity. In this study, efforts had been done to evaluate media that would give good yield of toxin.

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The fungus, *Mycosphaerella fijiensis* grew in all the media tested. However, more profuse growth was observed in the coconut and soybean broth compared with the media prepared from banana broth and Fries medium. It was also observed that growth was more rapid on cultures incubated with continuous agitation. *M. fijiensis* grew in numerous single velvety gray colonies. Based on the amount of growth 28 days after inoculation, coconut and soybean broths were observed the best media for toxin production.

Extraction with organic solvents were proved the best method to obtain culture filtrate. The extracts from coconut and soybean broths inoculated with *M. fijiensis* resulted in necrosis when injected on plantlets, while extracts from uninoculated broth did not show any necrosis. Chloroform and ethyl acetate were proved to be the best solvents to extract toxin from coconut and soybean broth. The test plants treated with toxin showed necrosis 24 hours after treatment. The necrosis was characterized by appearance of water soaked lesion from the point of inoculation. The lesions rapidly developed from brown to black necrosis, similar to normal symptom of Black sigatoka natural infection.

The extracts, both from inoculated and uninoculated cultures extracted with methanol and ethyl demonstrated toxic activity. This is interpreted as phytotoxicity produced by toxic components of the solvents.

Using coconut and soybean broths as media and chloroform and ethyl acetate as solvents the experiment was repeated. The results os the subsequent tests were consistent with previous tests. The characteristic necrosis was noted on plantlets treated with coconut broth and soybean broth. This result demonstrated that the fungus of Black Sigatoka produces a compound that causes necrosis on banana plantlets.

Techniques to inoculate the toxin were evaluated. All techniques tested produced characteristic necrosis except the addition of toxin in media to grow meristem culture. The injection of test metabolite on the leaves and deposition of toxin on unfurled leaf seem to be the best method. With these 2 methods, the amount of toxin to introduce in plantlets can be measured with precision.

Toxic activity of extracts can be observed up to dilution of 1:1 to 1:100, however, 1:10 dilution was used because necrosis produced were more evident. The dilutions 1:1 to 1:5 caused severe damaged to plantlets.

The different varieties of bananas tested showed varied reaction to the toxin, Table I. Using injection as inoculation technique and 1:10 dilution, the toxin clearly separates susceptible and resistant varieties to Black Sigatoka.

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Table 1 Index of necrotic lesions developed in leaves of test plants with ranging levels of resistance/susceptibility to Black Sigatoka with three toxin preparations.

Toxin Preparation	G.N.	Plantain	Saba	IV-9
Purified toxin	++++	++++	+	-
Partially purified toxin	++	++	-	-
Crude extract	+++	+++	-	-

0 = No necrosis
+ = Degree of necrosis

Conclusions

Results obtained from this study indicate that a toxic principle produced by *Mycosphaerella fijiensis* is involved in the pathogenesis of banana/plantain disease Black Sigatoka. This demonstrates that a host specific toxin can be utilized to differentiate host genotypes with varying levels of resistance of susceptibility to Black Sigatoka.

When developed as a tool for mass selection, it could be a great help to plant breeders. Furthermore the use of toxin instead of the pathogen as a selection pressure, opens up possibility of evaluating various international collection of genotypes of bananas against various races of *M. fijiensis* all over the world without danger of introducing the pathogen in different geographical areas.

The toxin may can also be used to characterize the variability of various populations of M. fijiensis by measuring and comparing toxin yield, which indirectly determines virulence of the pathogen.

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gm/gm
PV.02\Morep

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