Asian Plant Research Journal

1(2): 1-8, 2018; Article no.APRJ.45064

Rapid Pathological Characterisation of *Deightoniella torulosa* by Artificial Inoculation of Three Varieties of Banana and Plantain

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/APRJ/2018/v1i226272
Editor(s):
(1) Dr. Langa Tembo, Lecturer, Department of Agriculture Production, Makerere University, Kampala, Uganda and Department of Plant Science, University of Zambia, Lusaka, Zambia.
Reviewers:
(1) Andrei Ana-Maria, Research Development Institute for Plant Protection, Romania.
(2) Amit Kumar, Rama University, India.
(3) Clint Magill, Texas A&M University, USA.
Complete Peer review History: http://www.sciencedomain.org/review-history/27407

Original Research Article

Received 05 September 2018
Accepted 16 November 2018
Published 26 November 2018

ABSTRACT

Aims: Banana and plantain contribute to food security in Côte d'Ivoire. However, one of the major difficulties arising in its production remains parasitic constraints causing the loss of production. Among these constraints, leaf diseases cause more damage.
Methodology: This study was initiated to analyse the nature of host-pathogen interactions (resistance or sensitivity) between the fungus *Deightoniella torulosa* and certain varieties of banana and plantain in Côte d'Ivoire. The pathogenicity of the fungus was measured from artificial inoculation on leaf fragments that were kept alive on a specific culture medium in Petri dishes. This medium consisted of agar amended with Benzimidazole. Leaf' fragments that survived inoculation of spore suspensions, exhibited the characteristic symptoms of the fungus *Deightoniella torulosa*. The impact of the fungus on the degradation of chlorophyll was noted.

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Results: The results showed that the strains of *Deightoniella torulosa* have a pathogenic activity on the banana trees. The concentration of 75 µM of Benzimidazole incorporated into the survival medium can be used for inoculation tests on leaf fragments.
Conclusion: The Grande Naine cultivar could be used as a control indicator for *D. torulosa*.

Keywords: Banana trees; Deightoniella torulosa; pathogen; inoculation; Côte d'Ivoire.

1. INTRODUCTION

Banana (*Musa* spp.), originate in Southeast Asia, is a perennial monocotyledon herb belonging to the family of Musaceae [1]. Its large leaves, about 1 m^2, curl on each other and form a pseudo-trunk. This pseudo-trunk has the appearance of tree [2,3]. Banana is the fourth most important food crops after rice, wheat and maize [4,5]. In Africa, particularly in Côte d'Ivoire, the dessert banana is an important element in Ivorian economy. This sector contributes about 3 % of national GDP and 8-10 % of agricultural GDP. The production of dessert bananas is about 260 000 ton per year, 80 % of this production is exported to the EU market and 10 % to the sub-Sahara region [6].

The banana economy is a source of employment and source of income for producers. The banana sector absorbs a large labour force requiring minimal training, which reduces poverty [7,8]. Despite the importance of dessert banana and plantain, it is under phytosanitary constraints, particularly those related to nematodes, the fungus that causes Sigatoka, viruses and weevils. The damage caused by these pests and parasites results in yield losses of up to 100 % if no control method is applied [9,10].

The rigorous observation of the necrosis due to concern over Sigatoka diseases brings out other symptoms which are different from those described in the literature. It seems likely that the fungal species *Deightoniella torulosa* may play a role in the early senescence of leaves when they are attacked [11]. This hypothesis will be address.

The selection of new banana and plantain genotypes requires appropriate methods to evaluate the response of the host plant to *Deightoniella torulosa*. Several methods of artificial inoculation based on improved laboratory techniques have been described [12,13]. However, these methods are laborious and expensive because they require laboratory equipment and a greenhouse.

In order to overcome the unavailability of banana vitroplants and to better the control of the environmental conditions associated with the large working spaces required a use of whole plants, it will be necessary to develop an artificial inoculation technique under standardized controlled conditions: To do this, an experimental inoculation technique on leaf fragments has been developed based on the fact that certain molecules, such as benzimidazole, depending on their concentrations, delay leaf senescence by stimulating the conversion of proplasts to chloroplasts, resulting in an extension of the life of the leaf fragments [14]. Thus, by inoculations of different cultivars of dessert banana and plantain, *in vitro* pathological characterisation of *Deightoniella torulosa* was performed.

2. MATERIALS AND METHODS

2.1 Plant Material

Plant material consists of banana varieties Grande Naine (*Musa* AAA), Poyo (*Musa* AAA) and Orishele (*Musa* AAB).

2.2 Fungal Material

The fungal material consists of a strain of *Deightoniella torulosa* (DTOA) isolated from banana leaves in Aboisso area in the south-eastern of Côte d'Ivoire.

2.3 Pathological Characterisation of Isolates of *Deightoniella torulosa*

2.3.1 Isolation of the fungus

The culture medium was composed of Potato Dextrose Agar (PDA). Banana' leaves and those of plantain with lesions presenting typical symptoms of the disease were applied on the underside on the agar medium to trap conidia. These conidia were then collected under a microscope equipped with a camera (Magnification: 100x) using a sterile needle and transferred to Petri dishes containing PDA medium. The plates were sealed and incubated

for 3 weeks at 25 ± 2 °C. After spore's germination, the mycelium produced was also removed and transplanted onto a new PDA culture medium.

2.4 *In vitro* Inoculation of Leaf Fragments of Different Banana Cultivars by *Deightoniella torulosa*

2.4.1 Preparation of leaves

The second youngest leaf of each cultivar of banana and plantain obtained from *in vitro* culture in the Plant Physiology Laboratory of University Félix HOUPHOUËT-BOIGNY was cut at the base of the petiole and then immersed in distilled water contained in jars in order to avoid a rapid drying out of it.

Foliar fragments (explants) of 2.5 cm per side were cut with a scalpel, cleaned with sterile distilled water and placed on survival media contained in Petri dishes.

2.4.2 Preparation of the survival medium

The survival medium was composed of agar (4 g), mannitol (5 g) and sucrose (5 g) per 1 liter of preparation.

Benzimidazole was added to the survival medium in order to obtain final concentrations of 25 mg/l; 50 mg/l and 75 mg/l. The media thus obtained were sterilised by autoclaving at 121 °C for 30 min at a pressure of 1 bar. After cooling to 45 °C, these media were poured into sterile Petri dishes under a laminar flow hood and near a flame.

2.5 Investigation of the Concentration of Benzimidazole that Delays Senescence of Leaf Fragments of Different Banana Cultivars

The effect of the different doses of benzimidazole on each leaf' fragments chlorophyll was determined after a stay of 13 days on the survival medium in petri dishes.

A leaf fragment of each of the three cultivars such as Orishele, Grande Naine and Poyo was placed on the survival medium in Petri dishes containing an identical concentration of Benzimidazole. A control was made without Benzimidazole. A total of 20 Petri dishes were prepared at 5 repetitions per treatment. All the Petri dishes were placed in a culture room during a 12-hour photoperiod. Daily observations were made during 13 days. The benzimidazole concentration which delayed the senescence of the leaf fragments the most, was selected for subsequent experiments.

2.6 Production of Inoculums and Inoculation of Leaf Explants

The conidial suspension was prepared on modified V8 medium from 10-day old colonies on PDA culture medium at a temperature of 25 ± 2 °C. The spore suspension was recovered using a spatula and was adjusted to the concentration of 3.10^5 spores / ml using a double Malassez dialing cell. To evaluate the pathogenic activity of *Deightoniella torulosa*, leaf fragments of Orishele, Grande Naine and Poyo cultivars were placed on the survival medium retained in Petri dishes and inoculated. In the same Petri dish, two fragments of each of the three cultivars were arranged. On both fragments, one was inoculated by depositing in its middle 10 µl of a spore solution calibrated at 3.10^5 spores / ml using a micropipette and the other serving as a control was treated with 10 µl of sterile distilled water. A total of 5 petri dishes were prepared at a rate of 5 repetitions per benzimidazole concentration. All boxes were placed in a culture chamber under a 12-hour photoperiod. Daily observations were made during 13 days. The experiment was repeated 3 times.

2.7 Follow-up of Leaf Explants in Petri Dishes after Inoculation

Observations were made daily after inoculations. The growth of fungus-induced spots was evaluated every 24 h for 13 days by measuring the average of two perpendicular diameters passing through the middle of the spot. Five repetitions were performed for each concentration.

2.8 Investigation of the Impact of the Presence of *Deightoniella torulosa* on the Degradation of Chlorophyll of Leaf Fragments

The impact of *D. torulosa* on chlorophyll degradation of leaf fragments was demonstrated by the determination of total chlorophyll content at 13^{th} day after inoculation. For the extraction of pigments and monitoring of the degradation of chlorophyll, a sample from leaves of each cultivar

was tested at different concentrations of benzimidazole.

Pigment extraction consisted of grinding previously weighed samples in acetone followed by a spectrophotometric assay at the wavelengths of 645 nm and 663 nm. The total chlorophyll content (T Ch1) was calculated according Mc Kinney's formula (1941). This formula is the following.

T ChI (mg/l of solution) = 20.2 OD_{645} + 8.2 DO_{663}. This content was reduced to mg/g of leaf.

2.9 Statistical Analyzes

The data were calculated as averages of 3 repetitions using the Excel 2013 software and the graphs were also done using the same software. The results obtained were submitted to analysis of variance of the means to evaluate the significance of the threshold effect p <0.05 compared to the smallest significant difference with the Newman-Keuls test. All these analyzes were carried out with the software STATISTICA version 7.1.

3. RESULTS AND DISCUSSION

3.1 Effect of Benzimidazole Doses on Total Chlorophyll Content of Leaf Fragments of Banana Cultivars

The total chlorophyll content in the leaf fragments of Orishele, Grande Naine and Poyo cultivars at time T_0 was respectively 0.593; 0.389 and 0.557 mg/g MF (Figure 1). After a stay for 13 days on a survival medium without benzimidazole, this content increased to 0.279; 0.178 and 0.333 mg / g MF.

Within this same period, the fragments from the 25 µM survival media of Benzimidazole had 0.476 mg / g MF for the cultivar Orishele; 0.593 mg / g MF for the cultivar Grande Naine and 0.808 mg / g MF for the cultivar Poyo. In 50 Mm of Benzimidazole, the total chlorophyll content was 0.534 mg / g MF; 0.540 mg / g MF and 01,051 mg / g MF respectively for the varieties Orishele, Grande Naine and Poyo. In the presence of 75 µM Benzimidazole, the cultivar Orishele had a total chlorophyll content of 0.590 mg / g MF; Grand Naine had 0.748 mg/g MF and Poyo had 1.246 mg / g MF. The total chlorophyll content of the different cultivars increased significantly (P <0.05) at all concentrations of Benzimidazole (Fig. 1).

3.2 Sensitivity of Leaf Fragments of Different Cultivars to *Deightoniella torulosa*

Symptoms appeared 48 h after inoculation. Signs were revealed at the level of the spore deposition area in the form of a small circular discoloration spot forming a yellowish halo whose center is rusty brown in color (Fig. 2).

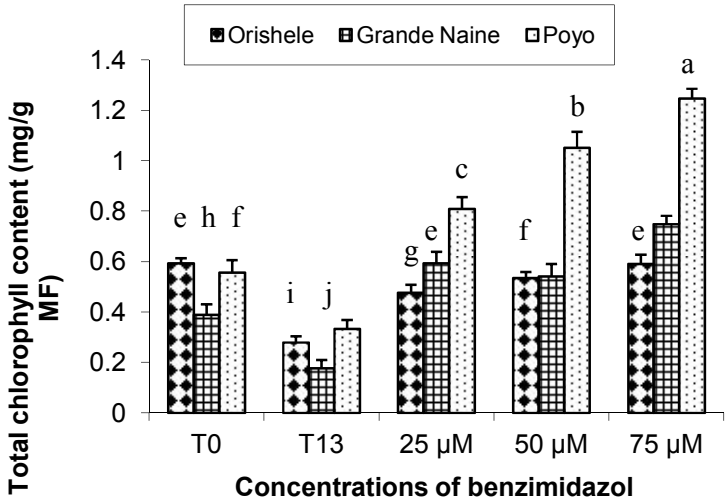

Fig. 1. Effect of benzimidazole dose on total foliar fragments' chlorophyll of the cultivars Orishele, Grande Naine and Poyo after 0 (T0) and 13 (T13) days

Means of stain diameters followed by same letters are not significantly different (Newman-Keuls test at 5% threshold)

On the 2nd day of the experiment, the stain diameter was identical for all cultivars. From this day, the diameter of *D. torulosa* spots on leaf fragments in survival increased steadily with time in all cultivars of banana. It has increased on average from 5.3 mm to 19.70; 23.15 and 19.95 mm respectively for the cultivars Orishele, Grande Naine and Poyo (Fig. 3).

This stain diameter was statistically greater for the cultivar Grande Naine from day 7 (13.30 mm) compared to the Orishele (11.10 mm) and Poyo (11.46 mm) until the 13th day. The experiment has shown that there was no difference in stain diameter between Orishele and Poyo cultivars.

3.3 Effect of *Deightoniella torulosa* on the Degradation of Chlorophyll

The total chlorophyll content of leaf fragments after inoculation with spores of *Deightoniella torulosa* varies with cultivars. It was 0.456; 0.322 and 1.154 mg/g MF respectively for the cultivars Orishele, Grande Naine and Poyo (Fig. 4). The control leaf fragments had in this same order content of 0.590; 0.748 and 1.246 mg/g MF. These chlorophyll levels decreased significantly for Orishele and Grand Naine ($P < 0.05$). Total chlorophyll levels were statistically identical in both control and inoculated Poyo cultivars.

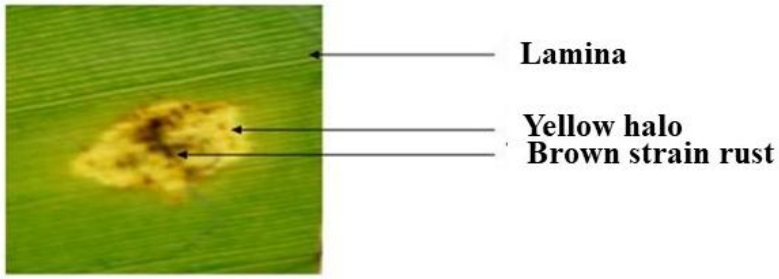

Fig. 2. Appearance of symptoms due to the presence of *Deigtoniella torulosa* on a fragment of plantain leaf (Orishele, *Musa* AAB) 7 days after inoculation G x 10

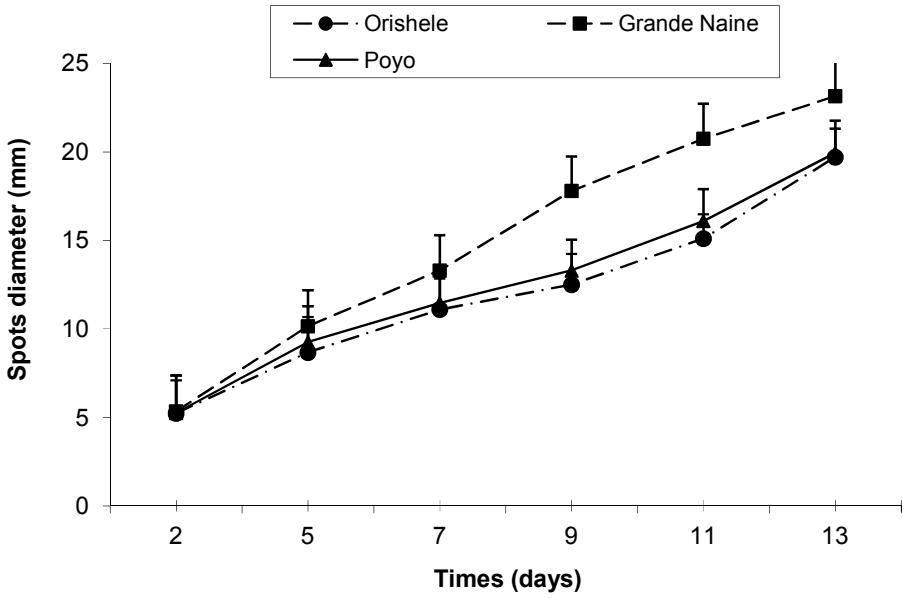

Fig. 3. Lesion diameter evolution caused by *Deightoniella torulosa* on banana leaf fragments in regards of time

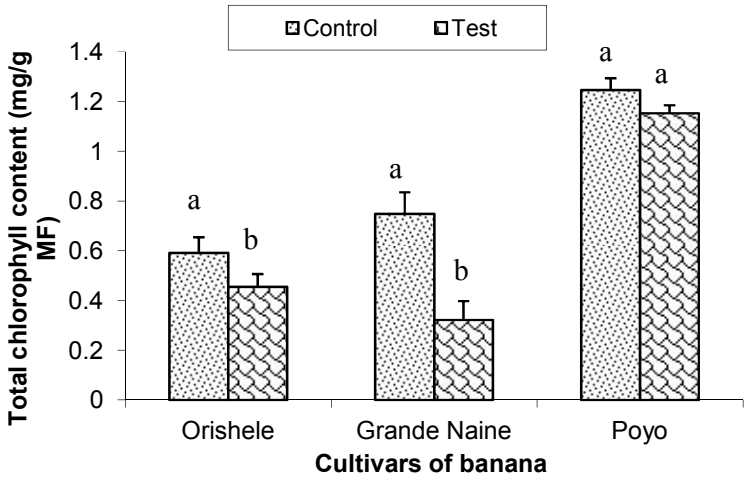

Fig. 4. Effect of *Deightoniella torulosa* on the total chlorophyll content of the foliar fragments of banana trees surviving on medium containing benzimidazole at a dose of 75 µM

4. DISCUSSION

4.1 Dose-response Effect of Benzimidazole on Chlorophyll Content of Leaf Fragments of Different Banana Cultivars

The chlorophyll content decreased with time when the leaf fragments were put on a survival medium without Benzimidazole. This drop in the mass of chlorophyll results from the degradation of chlorophyll following by the senescence of the leaf fragments. Our results were consistent with those of [14] who showed that without Benzimidazole, the survival time of the banana leaf fragments does not exceed 15 days. The chlorophyll content has varied from one cultivar to another showing that some cultivars have more chlorophyll pigments than others. This difference in content is varietal, therefore genetic.

Different concentrations of Benzimidazole delay the senescence of the leaves. This experiment showed that all the concentrations of this molecule used delayed the senescence of the banana leaf fragments at the 13th day of the experiment compared to the control at the same time. On the other hand, when the comparison was made with the control T_0, it was observed that the concentrations of Benzimidazole (25 µM) decreased significantly the chlorophyll content in the cultivar Orishele while it increased in the cultivars Grand Naine and Poyo. It was the same with the dose of 50 µM. Optimal levels of total chlorophyll were obtained with the dose of 75 µM for all cultivars. These levels were significantly higher than those of the beginning (T_0).

These results have shown that the Benzimidazole concentration has an effect on the stimulation of chlorophyll synthesis of plants. Indeed, it has been shown that certain benzimidazole derivatives have a cytokinin-like activity [15]. Cytokinins significantly slow down leaves senescence and in particular chloroplasts senescence.

The action of Benzimidazole is thought to be due to its greater or lesser structural similarity with the N6-substituted adenines, as shown by [16,17,18]. For inoculation tests of leaf fragments, the dose of 75 µM of benzimidazole in the survival medium can be retained.

4.2 Impact of *Deightoniella torulosa* on Chlorophyll Degradation of Leaf Fragments

The pathogenic activity of *D. torulosa* was evaluated by determining the stain diameter on banana leaf fragments. Symptoms on leaf fragments showed that *Deightoniella torulosa* is a pathogenic fungus of banana. From this point of view, our results were consistent with those of [19]. The first symptoms characterized by spots of the same diameter appeared 2 days after the inoculations of cultivars Orishele, Grand Naine and Poyo. These results show that the *D. torulosa* strain is pathogenic for all these cultivars. Spots diameter increased in over time,

but without significant differences between cultivars until the 5th day. From 7 to 13 days after inoculation, stain was significantly higher in Grande Naine than in Orishele and Poyo. This result has shown that the variety Grande Naine is more sensitive to *D. torulosa* than the Orishele and Poyo varieties. Our results are different from those of [20] who showed that Orishele was more susceptible to *D. torulosa* than Grande Naine.

This result could be explained by the difference in pathogenesis linked to the origin of the strains; our strain was isolated in Aboisso, while that of these authors was isolated in Abidjan. The 13-days period can be used as the time to evaluate the susceptibility of banana trees to *D. torulosa*.

The chlorophyll content of the leaf fragments after inoculation with *D. torulusa* and maintained in survival on 75 µM of Benzimidazole varied in regard of cultivar.

For the cultivar Grande Naine, the decrease in total chlorophyll content was greater than those of the cultivars Orishele and Poyo. This observation is related to the size of *D. torulosa* spots that were larger for Grande Naine, confirming its high sensitivity to the fungus. This cultivar can be used as a sensitive reference in banana assessments against *D. torulosa*, as it's the case with *Mycosphaerella fijiensis*.

4. CONCLUSION

This study showed that the concentration of 75 ppm of benzimidazole can be used for inoculation tests on leaf fragments. *In vitro* inoculations of banana leaf fragments showed that *Deightoniella torulosa* is able to infect and cause lesions that expand over time. These fungus attacks caused a significant reduction in total chlorophyll content in two cultivars. The cultivar Grande Naine can be used as a control indicator for *D. torulosa*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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