IN VITRO SCREENING OF ENDOPHYTIC *Fusarium oxysporum* AGAINST BANANA NEMATODE (*Helicotylenchus multicinctus*)

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Abstract

Experiments were conducted to investigate the antagonistic effects of secondary metabolites produced by endophytic *Fusarium oxysporum* against *Helicotylenchus multicinctus* spiral nematode of banana. Endophytic *F. oxysporum* were isolated from healthy bananas sampled from Kilifi district in Kenya. Five endophytic isolates of *F. oxysporum* were selected for the *in vitro* bioassays against all the motile stages of banana spiral nematode (*H. multicinctus*). Mortality and paralysis were recorded at 3, 6 and 24 hours of exposure to culture filtrates. The effect of the isolates on nematode paralysis and mortality depended on the time of exposure to the culture filtrates. Results from this study demonstrated that endophytic *F. oxysporum* has the potential as a biological control against *H. multicinctus* and their secondary metabolites can be harnessed as bio-nematicides.

Key words: Bio-nematicides, Nematode mortality, Secondary metabolites

INTRODUCTION

Bananas in East Africa are attacked by a complex of nematodes, which include burrowing nematodes (*Radopholus similis* (Cobb) Thorne), the root lesion nematode (*Pratylenchus goodeyi* (Sher and Allen)) and the spiral nematode (*Helicotylenchus multicinctus* (Cobb) Golden) (Gowen and Quénéhervé, 1990; Gold *et al.*, 1993; Karamura, 1993; Speijer *et al.*, 1999). The *H. multicinctus* nematode has been observed in every banana growing region in Kenya (Gichure and Ondieki, 1977; Gowen and Quénéhervé, 1990; Bridge *et al.*, 1997). The nematode has a wide host range and completes its life cycle in the plant and therefore is regarded as an endoparasite (Blake, 1966). Nematodes are spread from one farm to the other through infested planting materials, and can survive in the soil for over four months in the absence of the host-plant (Strich-Harari *et al.*, 1966). Damages caused by *H. multicinctus* lead to stunting, lengthened vegetative cycles, reduction in the number of leaves, bunch weight, and eventual toppling over, especially in soils with low nutrient content (Bridge *et al.*, 1997). Use of suckers from the same farm or from neighbouring farms is the main source of *H. multicinctus* infestation in banana fields. Substantial yield losses have been attributed to

this nematode within the first three years of banana plantation. However, estimates of crop loss attributed to this species of nematode are not available due to its association with other species and environmental factors.

Due to high cost of chemicals such as nematicides as well as related health and environmental hazards, chemical control of nematodes is a less feasible option to small-scale farmers. Cultural control measures such as corm-paring and hot water treatment, healthy suckers, clean planting material such as tissue-cultured ones only offer temporally control of nematodes since fields are normally re-infested highly with these nematodes (Stanton, 1994; Speijer *et al.*, 1995; Speijer *et al.*, 1999).

The use of affordable and sustainable alternative control measures against banana parasitic nematodes has elucidated increased research on the use of biological control agents (Kerry, 2005). Fungal endophytes have been demonstrated to control banana burrowing nematode (*Radopholus similis*) both *in vitro* and in screenhouse (Pocasangre, 2000; Niere, 2001). There are a number of ways through which endophytes protect host-plants against nematodes, including: improvement on plant physiology through enhanced tillering, root growth and increase in drought tolerance (Malinowski *et al.*, 1997; Elmi *et al.*, 2000), induction of systemic resistance (Kimmons *et al.*, 1990; Fuchs *et al.*, 1997), and production of nematicidal metabolites (antibiosis) (Hallman and Sikora, 1994; Alabouvette and Lemanceau, 1999; Cook and Lewis, 2001). Endophytic *Fusarium* species have been isolated from bananas in Uganda and shown to produce metabolites in culture which cause mortality and paralysis of *R. similis* (Pocasangre, 2000; Niere, 2001; Dubois *et al.*, 2004). Testing the culture filtrates of endophytic fungi *in vitro* is a rapid method of identifying endophytic fungal isolates with potential of biological control of banana nematodes (Nitao *et al.*, 1999). Therefore, this study screened endophytic *F. oxysporum* fungi antagonistic to *H. multicinctus* for production of metabolites, and compared the efficacy of the various isolates against *H. multicintus*.

MATERIALS AND METHODS

Nematodes and Preparation of Fungal Endophyte Isolates

Nematodes were isolated from necrotic banana roots using the modified Baermann dish. The number of living *H. multicintus* nematodes were counted in 100 µl of nematode suspension put on a nematode counting slide under a microscope (magnification X 20).

Endophyte isolates were obtained from a collection obtained from healthy bananas from Kilifi district, Coast province of Kenya. The isolates were

coded as 9SR25, 10MIPB14, 11SOC121, 11MOC353 and 10IJT51. The isolates were preserved on filter papers. Under aseptic conditions each isolate was cultured from a single filter paper onto fresh synthetic nutrient agar (SNA) medium (1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_47H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 0.6 ml NaOH (1 M) and 13.2 g agar in 1000 ml sterile distilled water) in 90 mm diameter Petri dishes. The Petri dishes were incubated in the laboratory at room temperature (±25^{o}C) with a photoperiod of ±12:12 L: D hours for 7 days to allow sporulation. For each strain three blocks of SNA, measuring 1 cm by 1 cm, were cut from each plate and aseptically inoculated into 80 ml sterile Pyrex bottles, containing potato dextrose broth (PDB) medium (12 g potato dextrose broth in 1 litre distilled water), and incubated for 7 days.

Culture Filtrates

Fungal culture filtrates were obtained from each strain by filtering the mycelia through cheese cloth into sterile centrifuging bottles and centrifuged at 6000 rpm for 15 minutes. Supernatant obtained was transferred aseptically under the laminar air flow hood into sterile standard bottles. The pH of the fungal filtrates was measured and recorded. Approximately 100 mixed species of *H. multicintus* suspended in 100 µl sterile distilled water were inoculated into sterile Bjorn® bottles. Sterile distilled water (SDW) at pH ~7 and uninnoculated PDB whose pH was adjusted to level of the average pH of the fungal culture filtrates were the two controls in the experiment.

The experiment was laid in a completely randomized design on a laboratory bench. All experiments were repeated thrice over time, with three replicates per isolate, giving a total of nine replicates per treatment. Mortality and paralysis was determined by counting the number of active, inactive and dead nematodes (males, females and juveniles) after 3, 6 and 24 hours of exposure to culture filtrate. Individual counts of each category were recorded for each time interval. The dead and paralyzed nematodes were counted as percentages of the initial total number of nematodes. After 24 hours, nematodes from each replicate were rinsed with SDW after concentrating them using 38-µm sieve and transferring them back into clean Bjorn® bottles containing SDW. The Bjorn® bottles were left on the bench under laboratory conditions for extra 24 hours. Nematodes were probed with a fine needle under the microscope and those which were straight in shape and remained immotile even after probing were considered dead.

Data Analysis

Abott's corrected mortality formula was used to calculate nematode mortality. Paralysis was calculated as the percentage of paralysed nematodes

out of the total original number of nematodes, at each time interval (Abbott, 1925). Corrected mortality and percentage paralysis data was used to evaluate the effects of individual isolates. Data were arcsine-square root transformed before analysis of variance (ANOVA). Laverne test was used to test for normality of distribution and homogeneity of variances. Analysis of Variance was carried to determine single factor effects and factor interactions. Where evident, effects of one factor were analysed at each time of the interacting factor. Where effects of the factors were significant, means were separated using the Tukey's test.

RESULTS

The effect of *F. oxysporum* isolates on nematode mortality depended on the time of exposure to the culture filtrates ($P = 0.0001$). *F. oxysporum* isolates varied in their effects in causing mortality on the nematodes across the three time intervals (Figure 1). All *F. oxysporum* isolates differed significantly from the control (PDB) ($P = 0.0001$).

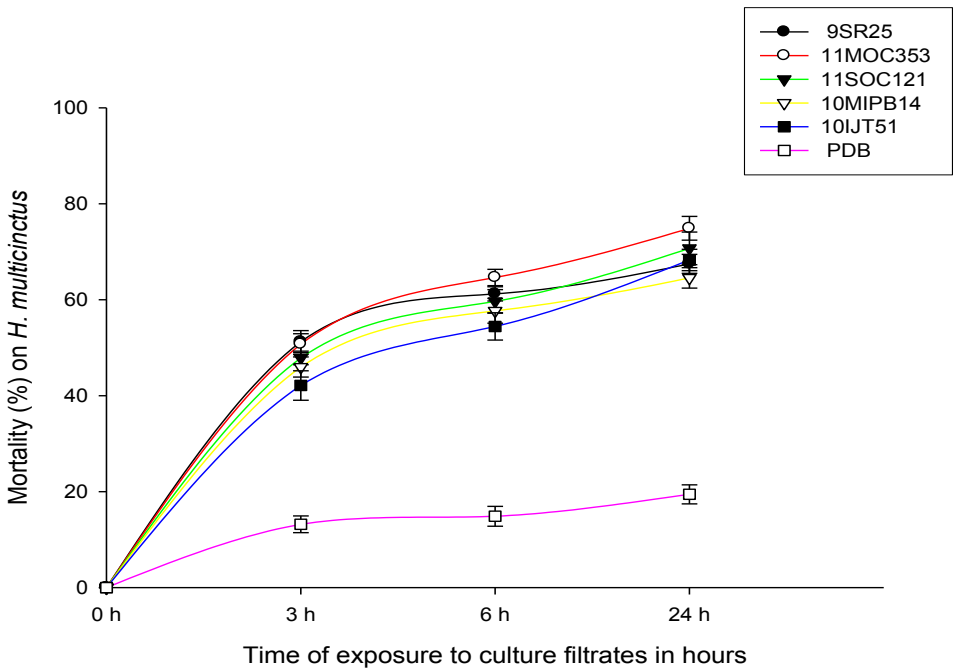

Figure 1: Corrected mortality of *H. multicintus* across three hours of exposure to culture filtrates of five different endophytic *F. oxysporum* isolates from Kilifi district, Kenya. Means and standard errors are for three experiments, each with three replicates.

The effect of *F. oxysporum* isolates on nematodes depended on the time of exposure in causing paralysis of the nematodes (P = 0.0001). Paralysis of *H. multicinctus* decreased with increase in the time of exposure (Figure 2). All *F. oxysporum* isolates had a low rate of paralysis on nematodes at 24 h of exposure compared to 3 h of exposure (Figure 2).

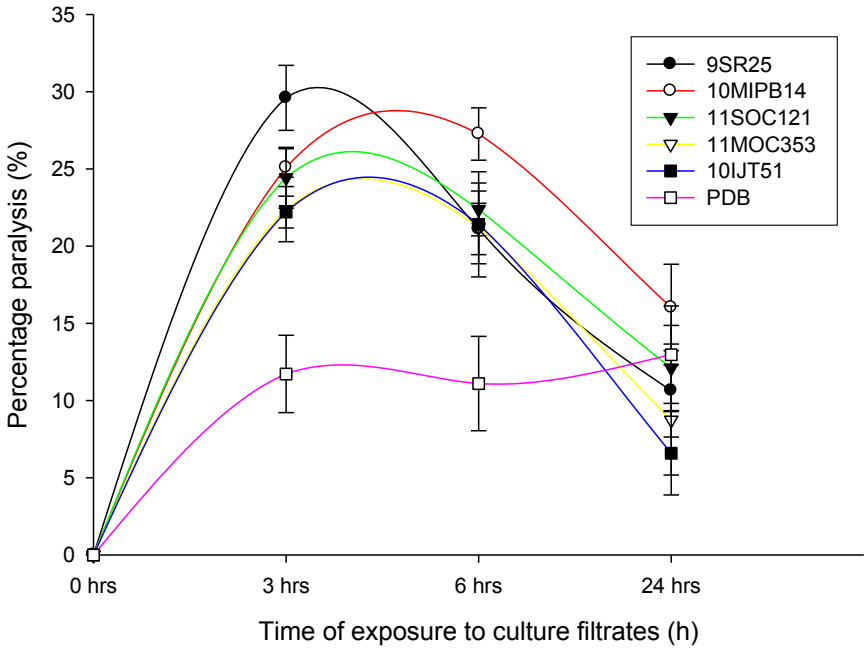

Figure 2: Paralysis of *H. multicintus* across three hours of exposure to culture filtrates of five different endophytic *F. oxysporum* isolates from Kilifi district, Kenya. Means and standard errors are for three experiments, each with three replicates.

DISCUSSION

Mortality and paralysis observed in the present study indicated that the *F. oxysporum* isolates produced metabolites that were antagonistic to the nematodes. Fungal isolates have been documented to cause antagonisim towards plant parasitic nematodes (Ciancio, 1995; Pocasangre, 2000; Niere, 2001; Dubois et al., 2004; Athman, 2006). The *F. oxysporum* isolate effect depended on the time of exposure to cause mortality and paralysis. Previous studies have also documented increased mortality of nematodes with increased duration of exposure to culture filtrates (Cayrol *et al.*, 1989). The

isolate 9SR25 produced consistent mortalities and paralysis towards the *H. multicintus* over the three time periods of exposure. The nematodes were rinsed with clean distilled water after the 24th hour of exposure to determine the effects of the isolates on the nematodes. Since after rinsing and probing the nematodes with a fine needle, none of the previously recorded dead nematodes showed any signs of life, the effects of the *F. oxysporum* isolates were presumed irreversible. However, toxic effects of culture filtrates of *P. lilacinus* have previously been shown to be reversed by different nematode species (Cayrol *et al.*, 1989).

The effect of *F. oxysporum* isolates on the nematodes was a function of time, with either mortality or paralysis depending on the length of exposure. Mortality of the nematodes was regarded as one of the best indicator of the best isolates. All the isolates differed from the control (non-inoculated PDB), indicating that the effect of the isolates was due to the metabolites produced by the endophytes and not due to the differences in the pH of isolates. However, previous studies show that toxin production by fungi is influenced by pH and the toxin produced acts in a wide range of pH and is, therefore, independent of the culture filtrates (Cayrol *et al.*, 1989).

CONCLUSIONS AND RECOMENDATIONS

Filtrates produced in the present study did not contain any spores, indicating that the *F. oxysporum* isolates used produced metabolites that were antagonistic to the nematodes. There is, therefore, great potential in utilization of endophytic fungi as biological control agent against the banana spiral nematode *H. multicintus*. The present study adds information on nematode-inhibiting effects of fungal culture filtrates. Further work should address the nature of the phytotoxins produced by *F. oxysporum* to determine if any of the previously documented ones such as zearalanone, fumonisins, tricothecenes and fusaric acids played a role (Vey *et al.*, 2001). Furthermore, molecular evaluation of the isolates should be undertaken.

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