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Potential of *Trichoderma harzianum* isolates in biocontrol of *Colletotrichum capsici* causing anthracnose of pepper (*Capsicum* spp.) in Nigeria

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ABSTRACT

Objective: To determine the potential of *Trichoderma harzianum* isolates as biocontrol agents of *C. capsici*, causing anthracnose of pepper.

Methodology and results: Four isolates of *T. harzianum* (Th-F, Th-G, Th-I and Th-N) obtained from CABI Biosciences, Egham, UK were evaluated. *In-vitro* interactions between *C. capsici* and *T. harzianum* isolates showed that *T. harzianum* isolates significantly (P≤0.05) reduced colony radius of *C. capsici* compared to the control. Seed treatment with *T. harzianum* isolates compared to benomyl showed that incidence of *C. capsici* was significantly (P≤0.05) lower with benomyl followed by Th-G treated seeds than with the other treatments. Effect on radial growth and sporulation of *C. capsici* was highest (P≤0.05) when the seeds were treated with Th-G followed by Th-I, benomyl and Th-F. Percent germination was significantly (P≤0.05) higher in control followed by benomyl treated seeds compared to other treatments. Seed treatment with Th-F, Th-G and Th-I resulted in significantly (P≤0.05) lower percent dead seedlings compared to seeds inoculated with *C. capsici* (CAF2) alone. Seeds treated with Th-N had significantly higher seedling length compared to the seeds treated with CAF2 alone. All the *T. harzianum* isolates tested against soil borne *C. capsici* significantly (P≤0.05) reduced colony forming units of the pathogen during the first week but subsequently, there was no significant difference.

Conclusion and application of findings: The *Trichoderma harzianum* isolates tested suppressed the growth of *C. capsici* and reduced the incidence of the pathogen on seeds and soil. Further work will be required towards formulating the organism (*Trichoderma harzianum*) for field and post harvest application to Control anthracnose of pepper.

Key words: Anthracnose, bio-control, *Colletotrichum capsici*, pepper, *Trichoderma*.

INTRODUCTION

Due to several adverse effects associated with chemical control of pest and diseases, attention is increasingly being given to development of alternative measures that are cost effective and environment friendly. Biological control based on myco-parasitism and hyper-parasitism between

some microbial organisms provides an alternative to chemical control. Several fungi such as *Aspergillus flavus*, *A. ochraceus*, *Penicillium aurantiogriseum*, *Coniothyrium minitans*, *Alternaria alternata*, *Epicoccum purpurascens*, *Coniothyrium olivaceum*, *Gliocladium* spp. and *Trichoderma* spp (Royse & Ries, 1978; Sinaga, 1986; Adebanjo & Bankole, 2004; Rabeendran *et al*., 2006) have been used as bio-control agents.

Amongst these fungi, *Trichoderma* spp are the most widely used. For example, *T. hamatum*, *T. harzianum*, *T. koningii* and *T. viride* are known to control damping off caused by *Rhizoctonia* and *Pythium* spp. in the laboratory, glasshouse and in the field (Papavizas, 1985). *Trichoderma hamatum* and *T. virens* effectively controlled *Sclerotinia* lettuce drop, reducing disease by 30 - 50% in New Zealand (Rabeendran *et al*., 2006), while *T. harzianum* controlled *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Pythium* spp. (Wells *et al*., 1972; Elad *et al*., 1980; Elad *et al*., 1981; Devaki *et al*., 1992). Jebessa and Ranamukhaarachchi (2006) showed that *T. harzianum*, *T. koningii* and *T. psedokoningii* could control *C. gloeosporioides* causing pepper anthracnose in Ethiopia. The possibility of applying *Trichoderma* spp. as a biocontrol agent of *C. capsici* causing anthracnose of pepper (Plate 1) in Nigeria has not been reported. This report describes experiments conducted to determine the effect of *Trichoderma harzianum* isolates on *C. capsici* isolated from diseased pepper in Nigeria.

MATERIALS AND METHODS

Fungal isolates: Experiments were conducted at the Crop Protection Research Laboratory of the University of Reading, UK from October 2006 to March 2007 to determine the potential of *T. harzianum* isolates as biological control agent against *C. capsici* causing anthracnose of pepper. The *T. harzianum* isolates used were Th-F, Th-G, Th-I and Th-N, coded based on their source. Isolate Th-F was a powder formulation obtained from a private company in the Netherlands, Th-G was a wild isolate from Greece, Th-I was a wild isolate from India and Th-N was a wild isolate from Nigeria, and all were obtained from CABI Biosciences, Egham, Surrey, UK., while *C.capsici* used was isolated from diseased sweet pepper fruits from Nigeria. *In-vitro* interactions between *C. capsici* and the *T. harzianum* isolates were studied. Subsequently, occurrence of *C. capsici* and its effect on seed germination was assessed on naturally infected seeds and on seeds treated with the *Trichoderma* isolates or with benomyl, a chemical fungicide used in management of anthracnose. The effect of *T. harzianum* isolates on *C. capsici* population in artificially inoculated soil was also studied.

Interaction between *C. capsici* and *Trichoderma harzianum*: The interaction between colonies of *C. capsici* (CAF2) and each of four isolates of *T. harzianum* (Th-F, Th-G, Th-I and Th-N) were studied using methods similar to those of Skidmore and Dickinson (1976), Royse and Ries (1978), Ahmed *et al*. (1999) and Ekefan *et al*. (2000). A mycelial plug (5mm) was taken from the edge of 14 day old culture of *C. capsici* using a cork borer and placed at the periphery of 9 cm culture plates containing PDA and incubated at 25 °C. Three days later, similar sized discs of *Trichoderma* isolates (7 day old) were placed on the same plates, 5.5 cm apart. The *Trichoderma* isolates were introduced later due to the slow growing nature of the *C. capsici* isolate used. Treatments in the experiment consisted of four *T. harzianum* isolates and a control where fresh agar plugs were placed opposite *C. capsici*. Each treatment was replicated eight times in a completely randomized design. Plates were incubated at 25 °C and examined daily after introducing *Trichoderma*. On the eighth day, the colony radius of *C. capsici* was measured and the nature of the interaction between the two organisms was described by the methods of Skidmore and Dickinson (1976). Percentage inhibition of the pathogen was estimated from the formula $[100 \times (r_1-r_2)/r_1]$, where: r_1 and r_2 are colony radius of *C. capsici* in control and in the dual cultures, respectively (Royse & Ries, 1978). Attempt was made to recover *C. capsici* from the area of interaction with *Trichoderma*.

Effect of *T. harzianum* and benomyl treatments on recovery of *C. capsici* from seed: 500 seeds of sweet pepper were artificially inoculated with *C. capsici* to ensure presence of the pathogen. The seeds were inoculated by soaking in 20 ml of spore suspension of *C. capsici* containing 10^6spores/ml for 1 hour and dried on the laminar airflow chambers for 24 hours after which they were incubated at 25 °C for 1 week. After this period, the seeds were treated by soaking in 10ml

spores suspension of *T. harzianum* isolates containing 2 x 10^6spores/ml, or benomyl solution containing 2 g of product per liter for 4 hours, dried and stored at 25 °C.

The treatments tested were (i) Th-F, (ii) Th-G, (iii) Th-I, (iv) benomyl and (v) control (seeds inoculated with *C. capsici* and soaked in 10ml deionised water for 4 hours). Each treatment consisted of 100 seeds which were divided into five replications (20 seeds per rep) and arranged in a completely randomized design. After 4 weeks of storage the seeds were plated on PDA, (five seeds per plate) and incubated at 25 °C. After 7 days, the seeds were assessed by recording the number of seeds infected with *C. capsici*. Radial growth of fungus around the seeds was measured using a meter rule while sporulation of *C. capsici* was scored subjectively using the following keys: 0 = No sporulation noticed, 1 = sporulation only on seeds, 2 = ≤ 5mm area round seeds covered with sporulation, 3 = >5mm area round seeds covered with sporulation, 4 = >10mm area round seeds covered with sporulation, 5 = >20mm area round seeds covered with sporulation.

Another experiment with similar treatments was set up, but the seeds were plated on blotter paper prepared by lining 9cm Petri dishes with three layers of moist sterile filter papers. After 14 days of plating, % germination, % seedling infected and dead, and seedling length were recorded.

Effect of *T. harzianum* on *C. capsici* in soil: Three isolates of *T. harzianum* (Th-F, Th-G and Th-I) were tested for antagonism against *C. capsici* in artificially inoculated sterile soil. Ten gram samples of sterilized loam (Roffey Brothers Ltd) was measured using an electronics precision balance (OHAUS; Model TS 120S), put into 50 ml round bottles and sterilized by autoclaving twice at 121°C and 1.05 Kg/cm^2 for 30 minutes at 24 hours intervals. The sterile soil was inoculated with a combination of *T. harzianum* isolates and *C. capsici* (CAF2). The treatments consisting of Th-F + CAF2, Th-G + CAF2, Th-I + CAF2 and CAF2 alone (control) were arranged in CRD and replicated 5 times.

A spore suspension of the *C. capsici* isolate was prepared from 12 day old cultures in SDW and adjusted to 1x10^6spores/ml. A spore suspension of each *Trichoderma* isolate was prepared from 6 day old cultures and adjusted to 7x10^5spores/ml using a haemocytometer. For treatments involving a combination of *T. harzianum* isolates and *C. capsici*, 10 g of soil was inoculated with 1ml each of the spore suspension of the respective fungi while for treatments involving *C. capsici* alone, 1ml each of spore suspension and deionised water was used. After inoculation, the soil was mixed thoroughly using a sterile metal rod and subsequently transferred to a glass house where it was kept at 25 °C.

One week after inoculation, and subsequently at two week intervals, the soils were assessed for population density of *C. capsici*. For assessment of survival of *C. capsici* in the soil, a 0.3 g sub-sample of each inoculated soil was suspended in 2ml of deionised water and serially diluted twice. A 200µl aliquot of the final dilution was added to ¼ strength PDA amended with Streptomycin sulphate to suppress bacterial growth. The suspension was spread on the medium using a sterile metal rod and then incubated at 25 °C. Each replicate consisted of two inoculated plates to account for variation between and within the replicates. Colony forming units of *C. capsici* were recorded on the 14th day after incubation.

Statistical analysis: All the data collected were subjected to analysis of variance (ANOVA) using Genstat (8th Edition) statistical software and means of treatments with significant F-test were separated using FLSD.

Plate 1: Healthy pepper fruit (far right) and fruits showing various levels of anthracnose lesions caused by *C. capsici*.

RESULTS

Interaction between *C. capsici* and *T. harzianum*: The *Trichoderma* isolates were all fast growing and on coming close to *C. capsici*, the mycelia of *Trichoderma* initially grew round the *C. capsici*, then into it, and eventually overgrew it (Plate 2). No inhibition zone was noticed between any of the *Trichoderma* isolates and *C. capsici*. Effect of the *Trichoderma* isolates on radius of the pathogen colony, % inhibition and recovery of *C. capsici* from the area of interaction with *Trichoderma* isolates are presented in Table 1. All the *T. harzianum* isolates tested significantly (P≤0.05) reduced colony radius of *C. capsici* compared to the control. Percent inhibition was highest for isolate Th-I, though not significantly different from isolates Th-G and Th-N. *C. capsici* was recovered from the interaction area in all the treatments assessed.

Plate 2: *C. capsici* (left) overgrown by *T. harzianum* (right). There was slight change in the colour of *T. harzianum* as it grew over *C. capsici*.

Table 1: Effect of *T. harzianum* isolates Th-F, Th-G, Th-I, Th-N on growth of *Colletotrichum capsici* in dual cultures on PDA.

Treatment	Colony radius (mm) of *C. capsici*	% inhibition of growth of *C. capsici*	Recovery of *C. capsici* from area of interaction
Th-F	20.86±0.70	44.00±2.27	+
Th-G	19.57±0.37	47.71±0.92	+
Th-I	19.14±0.70	48.71±1.70	+
Th-N	20.86±0.74	46.14±1.55	+
Control	36.00±1.39	-	-
FLSD (P≤0.05)	2.46	4.34	-

Th F, G, I and N are *Trichoderma harzianum* isolates. Values presented in this Table and subsequent Tables are means±SE.

Table 2: Effect of seed treatment with *T. harzianum* isolates and benomyl on % incidence, radial growth and sporulation of *C. capsici* on seed of sweet pepper artificially inoculated with the pathogen.

Treatments	% incidence	Radial growth (mm)	Sporulation (scores)
Th-F	100±0.00	7.22±0.48	3.6±0.24
Th-G	53±4.90	1.90±0.33	0.4±0.24
Th-I	96±2.45	2.92±0.12	1.0±0.00
Benomyl	40±3.87	3.32±0.28	1.0±0.00
Control	100±1.00	28.22±0.31	5.0±0.00
FLSD (P≤0.05)	8.9	0.1	0.5

Effect of *T. harzianum* and benomyl treatments on recovery of *C. capsici* from seeds: Percent incidence was significantly (P≤0.05) lower on seeds treated with benomyl followed by those treated with isolate Th-G when compared to the other treatments (Table 2). There was no significant difference in the incidence of the pathogen on seeds treated with isolates Th-F, Th-I and the control. Radial growth and sporulation of *C. capsici* were significantly reduced (P≤0.05) when the seeds were treated with isolates Th-G, Th-I, and benomyl.

Percent germination was significantly higher (P≤0.05) for the control and the benomyl treated seeds, compared to the other treatments (Table 3). There was

no significant difference between germination of seeds treated with the *T. harzianum* isolates and the seeds treated with pathogen (CAF2) alone. Percent dead seedling was significantly lower for the control and benomyl treated seeds compared to the other treatments.

Seed treatment with Th-F, Th-G and Th-I resulted in significantly (P≤0.05) lower percent dead seedlings compared with seeds treated with CAF2 alone

Seedling length was highest for the benomyl treated seeds and the control. Seeds treated with isolate Th-N had significantly higher seedling length compared to those treated with CAF2 alone. There was no significant difference in seedling length when seeds were treated with isolate Th-F, Th-G or Th-I compared to the seeds treated with the pathogen (CAF2) alone.

Table 3: Effect of *T. harzianum* isolates and benomyl treatments on % germination, % dead seedlings and seedling length of pepper *Capsicum annum* after seeds are artificially inoculated with *C. capsici* (CAF2).

Treatment	% germination	% dead seedlings	Seedling length (mm)
Th-F + CAF2	45±3.16	34±3.67	26.9±4.17
Th-G + CAF2	45±4.18	37±3.74	25.5±3.87
Th-I + CAF2	55±3.87	28±4.06	36.9±1.10
Th-N + CAF2	52±4.64	38±3.39	27.3±1.63
Benomyl	68±6.04	4±1.87	49.2±1.83
CAF2 alone	43±5.15	47±4.64	22.7±5.85
Control	82±1.22	0±0.00	56.7±3.09
FLSD (P≤0.05)	12.4	9.8	10.0

Effect of *T. harzianum* on *C. capsici* in soil: *C. capsici* survived in the soil for over 13 weeks (Fig. 1). All the *T. harzianum* isolates tested as biocontrol agents significantly (P≤0.05) reduced *C. capsici* population during the first week of assessment. The same trend was observed for week 3 except for isolate Th-I which did not differ significantly with the control. Subsequently, there was no significant difference between the *Trichoderma* treated soils and soils inoculated with *C. capsici* alone. However, data shows recovery of *C. capsici* was consistently higher in soil without *Trichoderma*.

DISCUSSION

T. harzianum isolates assessed in this investigation reduced colony growth of *C. capsici* when grown in dual culture. Members of *Trichoderma* spp. are known to be active hyperparasites of several fungi, and hence have been variously used as biocontrol agents. In studying the ability of antagonists to inhibit development of pathogenic fungi, the antagonist and the test fungi are often grown in dual cultures and the interactions observed. Skidmore and Dickinson (1976) presented five possible modes of interacting colony growth as follows: (a) mutually intermingling growth where both fungi grew into one another without any macroscopic signs of interaction, (b) intermingling growth where the fungus being observed is growing into the opposed fungus either above or below its colony, (c) intermingling growth where the fungus under observation has ceased growth and is being overgrown by another colony (d) slight inhibition where the fungi approached each other until almost in contact and a narrow demarcation line of about 1-2 mm between the colonies is clearly visible and (e) mutual inhibition at a distance of >2 mm.

In this study, each of the *T. harzianum* isolates assessed suppressed the growth of *C. capsici* eventually overgrowing it. In a similar experiment, Devaki *et al.* (1992) showed that *T. harzianum* suppressed the growth of *Pythium aphanidermatum* and *P. myriotylum* killing their mycelium within three days of inoculation as the test organism were not recovered in the area grown over by the antagonist. The antagonist is known to control plant diseases by antagonizing plant pathogens through mycoparasitism, by producing metabolites such as Beta 1-3 and 1-4 glucanases, directly competing with the pathogenic strain and inducing host resistance (Dennis & Webster, 1971a, 1971b; Campbell, 1989; Epavier & Alabouvette, 1994; Lorito *et al*., 1996; Duiff *et al*., 1998). The recovery of *C. capsici* in this investigation from the area of interaction with *Trichoderma* suggests that suppression of the growth of the pathogen might have

been due to competition rather than by antibiosis or mycoparasitism.

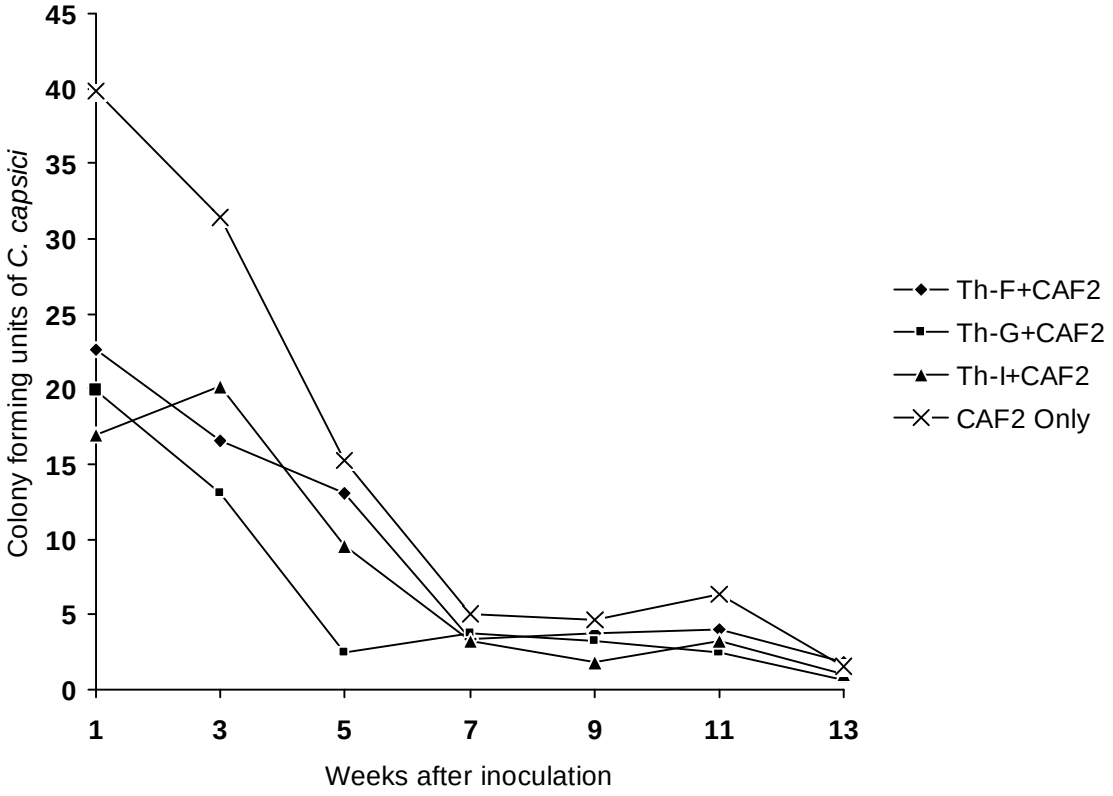

Figure 1: Survival of *C. capsici* (CAF2) in artificially inoculated soil and effect of *Trichoderma harzianum* isolates (Th-F, Th-G, Th-I) on recovery of the pathogen.

C. capsici was detected on naturally infected seeds, after four months of storage. This confirms earlier reports that seed-borne infection is an important means by which the pathogen is transmitted (Grover & Bansal, 1970; Mirdha & Siddique, 1998; Pathania *et al*., 2004). Higher % incidence of *C. capsici* was recorded on blotter compared to PDA and the same trend was observed for seed germination. This is likely because the high nutrient provided by PDA stimulated growth of other fast growing fungi, which inhibited the growth of *C. capsici* and also reduced seed germination.

Seed treatment with biocontrol agents has been found to be effective in controlling many fungal diseases. For example, Mao *et al*. (1997) showed that seed treatment with *Gliocladium virens*, *Trichoderma viride* and a bacterium, *Burkholderia cepacia* isolates increased seedling stand, plant height and fresh weight and decreased root rot severity in corn, giving results comparable to those of treating with a fungicide (captan). *Verticillium* wilt of cotton was effectively controlled by seed treatment with *Trichoderma virens* while *T.viride* and some bacteria species were effective against anthracnose of cowpea (Hanson, 2000; Adebanjo & Bankole, 2004). *T. harzianum* effectively also controlled *Sclerotium rolfsii* on blue lupines, tomato and peanuts (Wells *et al*., 1972).

In this investigation, result of seed treatment with *T. harzianum* isolates against *C. capsici* was inconsistent. Amongst the isolates tested, only Th-G reduced the incidence of the pathogen in artificially inoculated seeds but the same isolate was not consistent at increasing seed germination, and seedling length although these might be influenced by other factors besides pathogen presence. Adebayo and Ekpo (2004) showed that *T. harzianum* offered only moderate protection of tomato seedling against *Fusarium* wilt compared to *T. viride*. Pathania *et al*. (2004) found that seed treatment with *T. harzianum*

was less effective at reducing anthracnose of bell pepper compared to *T. hamatum*, indicating that species of *Trichoderma* are selective against different fungi.

The sources of inoculum of *C. capsici* were identified as plant debris, seeds and soil by Amusa *et al*. (2004). In this study, *C. capsici* survived in the absence of the host in soil for over 13 weeks, confirming that soil is an important means by which the pathogen survives during the non-cropping season. Given that the pathogen survives in both seed and soil, application of biocontrol agents could be targeted at seed and/or soil. In this investigation, *T. harzianum* isolates applied to soils artificially inoculated with *C. capsici* considerably reduced the population of the latter from soil, although it did not totally prevent the survival of the pathogen. This again suggests that competition rather than antibiosis could be involved in the mechanism of action of *T. harzianum* against *C. capsici*.

It could be concluded from the results of this study that *T. harzianum* affects the survival of *C. capsici* but may not be effective alone as a control agent for the disease. It is likely that when combined with other control measures, a better result could be obtained. Van Der Puije (2007) showed that combining *T. harzianum* isolates tested in this investigation, with plant extracts of *Azadirachta indica*, *Khaya senegalensis* and *Icacina* spp. was highly effective in controlling tomato wilt caused by *Fusarium oxysporum*. Future studies could target combining *T. harzianum* with plant extracts and also test more species of *Trichoderma* and other fungi for biocontrol control ability against *C. capsici*.

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