African Journal of Biotechnology Vol. 5 (5), pp. 419-424, 1 March 2006
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2006 Academic Journals

Full Length Research Paper

Field evaluation of selected formulations of Trichoderma species as seed treatment to control damping-off of cowpea caused by *Macrophomina phaseolina*

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Accepted 9 September, 2005

The experiment was carried out between 1997 and 1998 at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria to test the efficacy of biological seed treatment of cowpea against *Macrophomina phaseolina* infection in the field. *Trichoderma* sp., *T. koningii* Oudem (IMI 361600) and *T. harzianum* Rifai (IMI 361601) were selected from soil dilutions and tested *in vitro* for their antagonistic behaviour against cowpea pathogen *M. phaseolina* before use in the field. The *in vitro* tests in dixenic cultures, showed all three *Trichoderma* species growing fast and stopping the growth of the pathogen by the third day of pairing. Two varieties IT86D-2120, and Ife Brown were tested and two formulations of each *Trichoderma* species (mycelial suspension and suspension + starch) were also tested. The analysis of variance showed that there were significant differences between stands from the two trials and varieties. Treatment effect was also significant. The variety IT86D-2120 had significantly better stands in both trials. The highest plant stand of 53.8% at 7 days after planting (DAP), from the first trial, was from IT86D-2120 seeds treated with *T. koningii* + starch, which decreased to 49.3% by 21 DAP due to post emergence damping off. In the second trial, IT86D-2120 seeds treated in *T. harzianum* suspension had percentage stand of 55% at 7 DAP, which reduced to 45.8% at 21 DAP.

Key words: *Trichoderma harzianum*, *T. koningii*; biological control, field trial, *Macrophomina phaseolina*.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is the legume of choice of many people in Africa. Cowpea is high in protein as well as other essential nutrients providing more than half the plant protein in human diets in some areas of the semi humid tropics and is a key staple of many developing countries (Rachie, 1985). Cowpea production worldwide in 1994 was estimated at 11.29 million metric tonnes from 17.68 million hectares. Africa produced 3.35 million metric tonnes, out of which Nigeria produced 1.75 million metric tonnes or 15.5% of world production (FAO, 1994). More than 70% of the world production is concentrated in three countries; Nigeria, Brazil and Niger with Nigeria being the world's largest producer.

In the rain forest the greatest losses in cowpea production occur because of seed decay and seedling damping-off (Emechebe and Shoyinka, 1985). Damping-off occurs in seedlings before and after emergence and is induced by a number of pathogens including *Macrophomina phaseolina* and species of *Colletotrichum* (Emechebe and Shoyinka, 1985). Disease management practices include the use of chemical methods, resistant

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varieties and biological treatments. A mixture of control methods, including biological control, will reduce the risk of chemical residue build-up in the food chain and will also reduce the possibility of pathogens becoming resistant to fungicides.

In Nigeria, most of the studies on biological control of plant pathogens have been restricted to greenhouse and laboratory studies (Omoifo and Ikotun, 1987; Adekunle, 1988; Ikotun and Adekunle, 1990). In one reported field study, Wokocha et al. (1986) used *Trichoderma sp.* to control the basal stem rot disease of tomato caused by *Sclerotium (Corticium) rolfsii* Sacc. in the northern parts of Nigeria.

Different formulations have been used to control soil borne pathogens, these are: suspensions of bacteria (Paulitz et al., 1992), fungal spores (Harman et al., 1980), and powdery preparations of fungal mycelium (Latunde-Dada, 1993). Strashnov et al. (1985) showed that *Trichoderma harzianum* (Rifai) limited fruit rot of tomatoes caused by *Rhizoctonia solani* (Kuhn) to about 43% when applied to the soil and 85% when coated on the seeds. We have also reported control of *M. phaseolina* in the screen house using formulations of *Trichoderma* species, *T. koningii*, and *T. harzianum*. Treatment of cowpea seeds with *T. harzianum* suspension + starch and planted in soils amended with sclerotia of *M. phaseolina* resulted in stands of 76% at 7 DAP, compared to 29% for non treated seeds planted in soils amended with *M. phaseolina* (Adekunle et al., 2001).

In a previous study (Adekunle et al., 2001), we isolated and identified active antagonistic microorganisms, as well as conducted an evaluation of three formulations of these antagonists as seed treatment in pot trials. In this study, we hope to provide more information on the performance of two formulations (antagonist suspension and suspension + starch) of three *Trichoderma* antagonists as well as evaluate the varietal responses of two cowpea varieties in a field situation.

MATERIALS AND METHODS

Isolation of antagonists and *in vitro* study

The *Trichoderma* species used in this study were isolated using the soil dilution technique. The experiments were laid out as a completely randomised design. All pairings with the pathogen were done on acidified potato dextrose agar (APDA), with the antagonists placed at the center of the plates and the pathogen at four peripheral positions 1ncm from the plate wall. The dixenic cultures in five replicates were incubated at 28-30°C for two weeks and observations on colony growth of both pathogen and antagonist were taken every three days.

A test of competition between the *Trichoderma* species and the pathogen was also carried out. One ml of spore suspension each of an antagonist and a pathogen was introduced into sterile Petri dishes and molten agar at 45°C was poured over them. The plates were swirled round in a clockwise direction to ensure mixing of the two suspensions. Plates were incubated at 28-30°C and after four days the plates were observed for the presence of the pathogen colonies.

Preparation of rice inoculants for *M. phaseolina*

Seeds of the rice variety TOX 3081-36-1-4-1-3-2 were moistened (1 g rice seeds: 1 ml water) and placed in conical flasks. The mouth of each flask was plugged with cotton wool and wrapped in aluminum foil before autoclaving at 1.05 kg/cm^2 and 121°C for 3 h. After cooling for 12 h, the flasks were again autoclaved at 121°C for another 3 h. Following further cooling, the flasks were inoculated with one 2-cm mycelial plug from a 7-day-old culture of *M. phaseolina* and incubated at 28-30°C for 15 days by which time the rice seeds were colonized with the pathogen. The inoculum was stored at 4°C before use in the field.

One hundred colonized rice seed were plated on APDA at 28-30°C and after five days the cultures were examined under the microscope for the presence of the pathogen. The number of seeds showing the mycelial presence of the pathogen were counted and divided by 100. This gave the percentage recovery of *M. phaseolina* from the inoculants. Ten colonized rice seeds were introduced per planting hole to induce *M. phaseolina* infection in the field.

Biomass production of *Trichoderma* species

The biomass production of the three different species *Trichoderma harzianum, T. koningii* and *Trichoderma* sp. was done in a static liquid culture of the modified Richards medium as used by Harman et al. (1991) which consists of 10 g KNO_3, 5 g KH_2PO_4, 1.3 g anhydrous $MgSO_4$, 8 g sucrose, 20 mg $FeCl_3$ and 150 ml of tomato juice in 1 litre of distilled water.

The medium (100 ml) was put in 250-ml Erlenmeyer flasks, and was inoculated with a 1-cm^2 agar plug from a 7-day-old culture on APDA and incubated for 21 days at approximately 28°C. The mycelia were harvested and dried at 30°C for 24-48 h in an oven and stored at 4°C.

Seed treatment

For the field experiment testing the efficacy of the biological agents, the three *Trichoderma* species were applied as suspension of ground mycelial mat with or without cassava starch as an adhesive at the following concentrations: *T. koningii*, 6.8 x 10^7 cfu/ml; *T. harzianum*, 2.0 x 10^{10} cfu/ml; and *Trichoderma* sp., 1.8 x 10^7. Two cultivars of cowpea were used (Ife Brown and IT86D-2120) and all seeds were treated for 30 min. Control seeds were planted in soils to which *Macrophomina*-infested seeds were introduced.

Field experimental design

The experimental design was a randomized complete block with eight treatments, two cultivars, and four replicates for each treatment. The initial stand at 7 DAP and final stand at 21 DAP were recorded and analysis of variance was carried out using the general linear models procedure of the Statistical Analysis Software (SAS, 1985). Means separation was done using orthogonal coefficients. The number of healthy stands per treatment was recorded at 7, 14 and 21 DAP. Percentage stand was calculated from the number of expected stands (which was two stands per hill). Percentage stand = (no of observed stands/ no of hills x 2) x100.

RESULTS

In vitro study

The growth of the pathogen, *M. phaseolina*, was 4.5 cm

in three days and by the 6th day of inoculation, the whole plate was covered. *T. koningii* was the slowest of the three antagonistic species investigated; it covered 5.2 cm in 3 days and the whole plate was covered in 5 days. *T. harzianum* covered the whole plate in 4 days while it took 3 days for the *Trichoderma* sp. to cover the whole plate.

Three days after pairing with the pathogen, *T. koningii* and *T. harzianum* had diameters of 4.5 cm and 4.8 cm, respectively and had come in contact with the cultures of the pathogen which was about 2.2 cm stopping all further growth by it. When paired with the *Trichoderma* sp., the antagonists grew to 4.8 cm on the first day and by the second day had started to overgrow the cultures of the pathogen.

The test of competition carried out showed whole plates covered exclusively by the antagonists and the pathogen could not be re-isolated from these plates.

Table 1. Effects of treatment, trial and variety on 7-, 14- and 21-day plant stand of cowpea planted in soils amended with rice inoculant of *M. phaseolina*.

Main effects	DF	F	Sig
%stand 7 day combined	9	60.85	.000
Treat	7	21.88	.000
` Trial	1	71.51	.000
Variety	1	323.37	.000
Covariates combined	2	779.63	.000
%stand 14day	1	21.83	.000
%stand 21day	1	17.63	.000
2 way interaction	15	0.96	.507
Treat*Trial	7	0.83	.562
Treat *Variety	7	1.13	.353
Trial*Variety	1	0.84	.402
3 way interaction			
Treat*Trial*Variety	7	0.84	.556
Model	33	64.46	.000
Residual	94		
Total	127		

Table 2. Effect of trial on 7-, 14- and 21-day stands of cowpea treated in formulations of *Trichoderma* sp., *T. koningii* and *T. harzianum* and planted in soil amended with rice inoculum of *M. phaseolina*.

Main effects	DF	F	Sig
7-day stand	between groups 1	4.191	.043
14-day stand	between groups 1	3.214	.075
21-day stand	between groups 1	3.312	.071

Field study

Statistical analysis showed that treatment, variety and trials had significant effect on stands (Table 1). Table 2 shows that the differences between the trials were only significant at 7 DAP and further differences were insignificant for the readings at 14 and 21 DAP. Treatment of seeds with bioagents resulted in percentage stands that varied for the different antagonists as well as for the two varieties used in the two trials. IT 86D-2120 variety had stands that were significantly better than those for the Ife Brown variety (Table 3).

The highest percentage stand for Ife Brown was from the control, i.e. 39.8% at 7 DAP in soil inoculated with the pathogen. The percentage stand latter dropped to 38.4% by 14 DAP and further still to 37% by 21 DAP. The treatment with *Trichoderma* sp. suspension had the lowest percentage stand. At 7 DAP, plant stand was 14.8% and it increased slightly to 15.8% by 21 DAP (Table 3).

With IT 86D-2120, the highest stand was from the seeds treated with the *T. koningii* + starch. At 7 DAP, the initial stand was 53.8% and this dropped to a final stand of 49.3% at 21 DAP. The lowest percentage stands were from the seeds treated in *Trichoderma* sp. with stands of 24.8% at 7 DAP and 25.5% at 21 DAP (Table 3).

In the second trial, for Ife Brown, treatment in *T. koningii* suspension gave the highest percentage stands from the 7 to 21 DAP. Seeds treated in *Trichoderma* sp. had the lowest percentage stands. For IT 86D-2120, the highest stands were from the seeds treated with the *T. harzianum*; 55% 7 DAP, 46.6% 14 DAP and 45.8% for 21 DAP. The lowest percentage stands of 20.6% 7 DAP, 19.8% 14 DAP and 18.9% 21 DAP were from the *Trichoderma* sp. + starch-treated seeds.

In both trials, the highest percentage stands were from variety IT 86D-2120 variety. The *Trichoderma* sp., and *Trichoderma* sp. + starch seed treatments gave the lowest percentage stands for variety Ife Brown in both trials. For variety IT 86D-2120, seeds treated in *Trichoderma* sp. gave lowest stand for the first experiment and *Trichoderma* + starch the lowest in the second trial.

Pairwise comparisons of the percentage stands from all the different treatments were used to detect significant treatment effects. Comparisons were made between the same treatments for the two different varieties. In the first trial (Table 4), seeds treated with *T. harzianum* and *T. koningii* + starch for the variety IT 86D-2120 were significantly better in all the days of observation than those of Ife Brown.

In the second trial, as in the first, IT 86D-2120 had better stands than Ife Brown. When the percentage stands were compared in the pairwise comparison, control seeds, *Trichoderma* sp. treated seeds and *T. harzianum* treated seeds of the variety IT 86D-2120 had stands that were significantly better than those from

Table 3. Percentage plant stand from treated with cowpea seeds planted in fileds infested with M . phaseolina (at the rate of ten infested rice seeds per hole).

Treatment	Cowpea Variety					
	Ife Brown			IT 86D-2120		
	Day-7	Day-14	Day-21	Day-7	Day-14	Day-21
First Trial						
Benlate 26.09 26.09	26.8	27.5		44.4	39.8	41.5
Control 39.84	38.4	37.1		47.4	46.7	44.6
Trichoderma sp.	19.70	17.8	19.4	24.8	27.3	25.0
T.harzianum	20.14	18.4	19.2	50.2	46.6	43.8
T.h+starch	19.85	17.0	17.0	43.8	42.0	36.0
T. koningii	16.75	18.0	17.5	44.9	37.9	36.5
T.k+starch	23.92	23.0	21.7	53.8	48.5	49.3
T+starch	14,76	12.6	15.8	41.0	39.4	35.8
Second trial						
Benlate 24.7 24.7	22.1	20.3		26.4	24.1	21.9
Control 3.0 3.0	13.8	14.1		34.9	34.9	33.2
Trichoderma	11.1	10.4	10.7	29.4	30.7	33.4
T.harzianum	26.8	25.1	23.8	55.0	46.6	`45.8
T.h+starch	19.5	20.8	21.1	26.1	27.4	22.6
T. koningii	21.4	33.0	29.0	32.4	29.6	32.1
TK+starch	28.4	25.6	24.9	21.1	19.3	21.0
T+starch	13.0	18.3	15.7	20.6	19.8	18.9

LSD day 7, 14 and 21 in first experiment = 25.8, 27.9 and 26.2.
LSD day 7, 14 and 21 in second experiment = 27.3, 25.4 and 26.3.
T, *Trichoderma* sp.; T.h, *T. harzianum*; T.k, *T. koningii.*

Table 4. Pairwise comparison of percentage stands from two varieties of cowpea seeds treated with bioagents and planted in soils infested with *M. phaseolina*.

IT86D-2120 vs IFE BROWN	Probabilities of significance		
	7-day	14-day	21-day
Benlate	0.11	0.25	0.20
Control	0.50	0.46	0.45
Trichoderma sp.	0.65	0.40	0.60
T. harzianum	0.01	0.01	.03*
T. harzianum + starch	0.04	0.03	0.09
T. koningii	0.02	0.08	0.09
T. koningii + starch	0.01	0.03	0.01*
Trichoderma sp. + starch	0.02	0.02	0.07
Second trial			
Benlate	0.86	0.82	0.62
Control	0.03	0.02	0.04*
Trichoderma sp	0.07	0.03	0.02*
T. harzianum	0.01	0.02	0.02*
T. harzianum + starch	0.51	0.47	0.88
T. koningii	0.92	0.71	0.74
T. koningii + starch	0.46	0.48	0.60
Trichoderma sp. + starch	0.45	0.87	0.72

* pairing showing significant differences till the 21-st day.

Table 5. Effects of variety, trial and treatment on plant stand from cowpea seeds treated and planted in soil amended with rice inoculants of *M. phaseolina* using one way analysis of variance.

Variety	% day 7 (se)	% day 14 (se)	% day 21 (se)
IT86D	37.33 (2.940)	35.03 (2.57)	33. 94(2.56)
Ife Brown	21.82 (1.92)	21.31 (2.00)	20.92 (1.87)
Trial			
1	33.22 (2.58)	31.25 (2.62)	30.47 (2.44)
2	25.93 (2.46)	25.09 (2.22)	24.40 (2.28)
Treatment			
Benlate	30.40 (5.34)	28.19 (4.99)	28.53 (5.0)
Control	34.05 (5.61	33.45 (5.68)	32.23 (5.06)
Trichoderma sp	21.24 (5.09)	21.55 (5.24)	22.11 (5.13)
T. harzianum	38.03 (5.15)	34.14 (5.15)	33.14 (4.62)
Th. + starch	27.30 (5.02)	26.81 (5.03)	24.17 (4.90)
T. koningii	31.42 (5.08)	29.59 (4.56)	28.79 (4.88)
Tk. + starch	31.80 (4.63)	29.09 (4.48)	28.97 (4.47)
T. + starch	22.35 (4.51)	22.52 (4.29)	21.53 (4.14)

(se) = standard error.

variety Ife Brown in all the days of observation.

DISCUSSION

Results obtained from the *in vitro* part of this study showed that the three *Trichoderma* species could be used effectively to control the pathogen. They achieved total control of the pathogen by growing fast and stopping further growth of the pathogen. In this study, there was observed coiling of the mycelia of the *Trichoderma* species and lysing of the pathogen mycelia. This ability of the *Trichoderma* as a biocontrol agent was also reported by Upadhyay and Mukhopadhyay (1986). They reported that *T. harzianum* isolate IMI 238493 lyses the mycelia and sclerotia of *Sclerotium rolfsii*. Inbar et al. (1996) also observed hyphal interaction between the mycoparasite, *T. harzianum*, and the soilborne pathogen, *Sclerotinia sclerotiorum*.

The highest stand at 21 DAP was 49.3% from the IT86D-2120 seeds treated with *T. koningii* + starch in the first trial. This was not significantly different from the stands from all the other treatments at 21 DAP. It however showed that the variety was more tolerant to infection by the pathogen. A weakness in this experiment was that the study was not carried out in fields with known infection of the pathogen, but rather on fields in which the pathogen was introduced in the form of inoculum. Because of this, the seeds had to contend with the introduced inoculum as well as a number of other pathogens and saprophytes in the field. The poor performance of all treatments from this study can be attributed to the poor growth of the antagonists in the field, environmental factors, and competitive mycoflora. Germinating seeds and roots of seedlings are known to excrete exudates, and they are the major force behind spermosphere and rhizosphere activities of soil borne plant pathogens as well as those associated microorganisms (Nelson, 1992). Competitive native microflora can also be a major deterrent in the effectiveness of biological agents applied to the soil and to seeds. Papavizas (1981) reported that *T. harzianum* did not survive well in the rhizosphere of bean and pea seedlings when the seeds were coated with the conidia and when the conidia were applied directly to the soil one day before planting. Hubbard et al. (1983) also found *Trichoderma* species to be susceptible to competition from *Pseudomonas* sp. when used as a biological control agent in natural soil.

The treatment of the seeds with the *Trichoderma* species resulted in stands that were not significantly different from the Benlate treatment (Table 5). It is probable, that the level of inoculum introduced in the field were well above that which would be seen in a field with natural infection. As such there was low-level performance by both the antagonist treatments and the chemical treatment. The continuous use of chemical treatments has resulted in control failure when the pathogens become resistant to the active ingredient (Williams and Gisi, 1992). We may be looking at the possible development of resistance to benomyl by *M. phaseolina*. This development emphasizes the need for intensification of research in the field of alternative control that may be used as part of a control management strategy.

A combination of well-selected variety as well an effective use of seed dressing in the field might prove to be the most effective means of combating the menace of soil borne pathogens in the African set up. Farmers in African countries do not enjoy the kind of subsidy there

counterparts in developed countries have. A well researched and formulated biopesticide using the *Trichoderma* species that can be produced locally with a variety of cheap substrates such as corn husk and oil palm fruit husk can be an economically viable project, providing jobs as well as cheaper control of soil borne pathogens.

ACKNOWLEDGEMENT

The International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, funded this project.

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