

Full Length Research Paper

Evaluation of some bioagents and botanicals in *in vitro* control of *Colletotrichum destructivum*

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Collectotrichum destructivum has not been effectively controlled. This led to trials on the use of bio-agents and botanicals to control the pathogen. The bio-agents such as *Pseudomonas fluorescens*, *Bacillus subtilis*, *Trichoderma harzianum* and *Trichoderma pseudokoningii* were inoculated as dixerenic culture with the pathogen to monitor antagonistic effect. In another experiment, botanicals of tobacco (*Nicotiana tabacum*) and castor plant (*Ricinus communis*) were incorporated as poison in a growth media. Of all the four bio-agents used, only *P. fluorescens* was able to inhibit the growth of the pathogen mycelia significantly by 52%. Though *B. subtilis* also inhibited the growth of the pathogen, the effect on the pathogen was not significant when compared with that of *P. fluorescens*. The extracts of *R. communis* at higher concentration acted as growth “promoter” to the pathogen; its mycelia had a better sporulation and fluffiness than that of the controlled plate. The extract of *N. tabacum* significantly controlled the growth of the pathogen at the highest concentration when compared with other extracts and the control. In comparison, bio-agents were found to have better control of the pathogen than the botanicals. *P. fluorescens* was the best bio-agent in the control of *C. destructivum*.

Key words: Anthracnose, bioagents, botanicals, inhibitory effect.

INTRODUCTION

Cowpea, *Vigna unguiculata* (L.) Walp, is a primary and commonly cultivated legume crop by many farmers in Africa. It is grown for its seed, as a vegetable crop, for fodder, green manure, as a cash crop and cover crop (Prasanna, 1985; Kormawa et al., 2000; Singh and Rachie, 1985; IITA, 1985). They are highly compatible as companion crop with a wide range of food and fibre crops. Cowpea is grown in many agro-ecological zones of Nigeria (Emechebe and Shoyinka, 1985).

This crop, however, suffers disease damage which has greatly reduced its production and yield potentials. Anthracnose disease of cowpea affects the above ground parts with the production of water – soaked lesions in all tissues of cowpea plant, and is induced by *Colletotrichum destructivum* O’Gara (Onesirosan and Baker, 1971; Allen et al., 1998). It has been described as a seed-borne fungus and found on soil surface or plant debris (Prasanna, 1985; Amusa et al., 1994; Fokunang et al., 1997) surviving for at least two years on diseased stem

tissues either on the soil surface or buried (Onesirosan and Sagay, 1975; Singh and Rachie, 1985; Bailey et al., 1990).

Efforts geared towards the control of *C. destructivum* have taken various forms which include the application of chemical e.g. fungicides and an integrated pest and disease control (Emechebe and Shoyinka, 1985). However, weekly or biweekly applications of benomyl are effective against this disease (IITA, 1985) and the application of phosphorus fertilizer gave lowest severity of the disease.

These control methods are more expensive, requiring skilled labour, and in addition fungicides may be phytotoxic to cowpea. Consequently, it is desirable to search for an alternative by using the natural biological balance to control the disease. Biological control of plant diseases through the use of antagonistic micro-organisms is very promising (Baker and Cook, 1982; Amusa et al., 1994; Adekunle et al., 2001). The problems mostly faced with this method of control and in the development of environmentally friendly protection strategy of crop plants are what to use, how to use and when to use the method. This generates the idea on the use of antagonistic orga-

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nisms in the control.

Trichoderma harzianum has been efficient in control of several pathogens (Adekunle et al., 2001). The potential values of *Trichoderma* spp. as bioagents were reported by Bankole and Adebajo (2004) and Howell, (2003) for the protection of several seedlings, potted outdoor and field diseases of crops. Mohammed and Amusa (2003) observed that *T. harzianum* grew over all the pathogens tested in their study. Mechanisms of antagonism suggested by other researchers were biois (Fravel, 1988), mycoparasitism (Elad et al., 1983) and rhizosphere competence (Howell, 2003), which are the factors responsible for hyperparasitism. Bourah and Kumar (2003) reported that the secondary metabolites produced by a strain of *Pseudomonas fluorescens* produce antibiotics phenazine (PHE) 2, 4-diacetyl phloroglucinol (PHL) and siderophore phyoverdin (PYO) in king's B and succinic acid media respectively. Pukall et al. (2005) identified four different types of *Bacillus* spp. namely *B. pumilis*, *B. fusiformis*, *B. subtilis* and *B. mojavensis* with toxin producing strains outside *B. cereus*.

The fungi toxicity of four plant extracts was compared with the synthetic benomyl fungicide by Obi (1991), and found that the fungitoxic effect of foliar sprays of plant oil extracts was greater than those associated with benomyl. Ukpabe (2002) conjectured that the antifungal activities of *Ricinus communis* leaf extract, which were due to chemical contents of ricin and other active ingredients, showed inhibitory effect on *Fusarium oxysporum*. The narcotic and stimulating properties of tobacco are due chiefly to the presence of 1 - 5% of alkaloid nicotine and was formerly much used as an insecticide (Sigmund and Gustav, 1991; Onwueme and Sinai, 1999). One of the requirements for implementation of botanical is the development of appropriate formulation with high potency even at the end of processing and application.

In this study, we have identified fungitoxic active plants, conducted an evaluation of six different concentrations of the plant extracts and compared their effectiveness. Also identification of the microorganisms that are antagonistic to growth and development of *C. destructivum* was carried out.

MATERIALS AND METHODS

Collection and Isolation of the organisms

The organisms used as antagonistic micro-organisms in this study were *T. harzianum*, *T. pseudokoningii*, *B. subtilis* and *P. fluorescens*. Pure cultures of the bacteria and fungi isolates were obtained from the soil using facilities provided by the Department of Botany and Microbiology, University of Ibadan and the Pathology Laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan. The cultures were maintained and multiplied by sub-culturing on Nutrient Agar (NA) and Potato Dextrose Agar (PDA). Samples of infected cowpea stem pieces were cut, rinsed and sterilized in 10% Sodium hypochlorite NaOCl and five changes of sterile distilled water before being plated on Potato Dextrose Agar (PDA), prior to the incubation. It was further stored at about 4°C before use.

Extraction of the plant materials

The leaves of tobacco (*N. tabacum*), wild (purple) and domesticated (green) types of *R. communis*, were used as the botanicals. The leaves were well packed in envelopes and oven-dried at 80°C for 48 h separately. One hundred grams (100 g) of the dried leaves were weighed and blended into powder. Distilled water of 100 ml was added to it and the suspension was heated over water bath at 70°C for 20 min. The content was filtered using a piece of muslin cloth, and autoclaved for 20 min at 1.05 kg²cm.

The plant extracts were first poured into the Petri dishes. Then, molten PDA at 45 - 50°C was poured aseptically on the plant extract in the Petri plates and swirled round five times for even dispersion of the extract into the agar. The extracts were incorporated at different concentrations: 100,000, 10,000, 1,000, 100, 10 and 0 ppm. A 5 mm disc of *C. destructivum* was released into the poisoned agar. The treatments were replicated three times incubated at room temperature and measurement of the growth of the fungus was taken at several different time intervals after inoculation. The experiment was set up in a 4 x 5 factorial experiment in a Completely Randomized Design (CRD); analysis of variance was carried out on the growth rate data by using SAS (1985).

To investigate the effects of these micro-organisms on target pathogen, a dual cultural technique reported by Ikotun and Adekunle (1990) and Singh (1991) was used while the mode of parasitism was evaluated after the methods of Haung and Hoes (1976). One week old cultures in Potato Dextrose Agar (PDA) were used for this experiment. Culture discs (5 mm) of each of the potential antagonists and target pathogens were taken and transferred to 90 mm diameter PDA culture plates. The 5 mm mycelia plug of the antagonistic microorganism were plated at the middle of the Petri dishes and agar discs of the target were simultaneously inoculated at four equidistant points. The treatments were replicated three times. The control was without antagonists. The dioxenic cultures were incubated at room temperature of about 28 - 30°C for 5 days (minimum) and were placed upside down. The cultures were later observed for interaction between the target organism and the various antagonists.

Observation started 48th h of inoculation on the inhibition zone. Comparisons of the different treatments were carried out with Statistical Analysis Software (SAS), 1985) and treatment means were separated using Duncan's Multiple Range Test (P = 0.05).

RESULTS

The four antagonistic micro-organisms (bio-agents) were separately paired with target pathogen (*C. destructivum*) to determine the best antagonist and its effectiveness on the pathogen. When the fungi species of *Trichoderma* were paired with the pathogen, *T. pseudokoningii* was found to exhibit hyperparasitic effect on *C. destructivum* at 72 and 96 h of incubation when compared with *T. harzianum* and *B. subtilis* (Table 1). *T. harzianum* was unable to exhibit any parasitic effect on *C. destructivum*; instead the pathogen suppressed its growth at 48 and 72 h of incubation but, slightly inhibited the growth when compared with the control at the later hours of incubation.

The two bacteria used in the experiment suppressed the growth of *C. destructivum*. The antagonist *P. fluorescens* exhibited greater parasitic action by suppressing the pathogen by 52% while *B. subtilis* was less, with 32.56% at 120 h of incubation (Table 2). The results showed significant difference (P = 0.05) among the anta-

Table 1. Results of bio-agents inhibitory rate on the pathogen, *C. destructivum* s growth (cm) with respect to time of incubation (h).

Antagonist	48	72	96	120
<i>T. pseudokoningii</i>	4.3a	5.2c	5.8cd	6.3b
<i>T. harzianum</i>	5.3a	6.5ab	6.6bc	7.4a
<i>B. subtilis</i>	5.1a	6.3bc	7.0b	7.7a
<i>P. fluorescens</i>	4.8a	5.4bc	5.6cd	5.5c
Control	5.3a	6.9a	7.5a	8.9a

Means in a column with similar letter(s) are not significantly different at 0.05 level according to Duncan Multiple Range Test.

Table 2. Inhibitory rate in percentage (%) with the respect to incubation times (h).

Antagonist	48	72	96	120
<i>T.pseudokoningii</i>	18.90	23.50	22.70	44.70
<i>T. harzianum</i>	0.00	4.40	12.60	35.10
<i>B. subtilis</i>	3.80	7.40	6.70	32.46
<i>P. fluorescens</i>	19.90	1.40	26.70	51.75

gonists; there was an exceptional inhibitory effect by *P. fluorescens* on the pathogen at 120 h of incubation.

The results showed that there was no significant difference in the mycelial growth of *C. destructivum* on poisoned agar of the plant crude extract of *N. tabacum*, green and purple varieties of *R. communis* at 48 and 72 h after inoculation. But, significant differences were noticed on the mycelia extension as from 96 h of incubation Table 3. The purple, *R. communis* at 100,000 ppm had highest inhibitory effect on the pathogen; the other concentrations (10,000; 1,000; 100; 10 ppm) slightly inhibited the growth. There was no significant difference between 96 and 120 h of incubation on the growth of the pathogen. The highest concentration (100,000 ppm) of *N. tabacum* inhibited the mycelial extension significantly at 120 h.

The leaf extract of purple, *R. communis* at concentration of 100,000 ppm inhibited the growth of *C. destructivum* when compared with the control which had the highest growth (normal growth). It could be deduced from this that the highest concentration of purple *R. communis* extract had good inhibitory effect on the pathogen, *C. destructivum*. This is in support with Ukpabe (2002) findings that leaf extracts of *R. communis* have inhibitory effect on *Fusarium oxysporum* due to the fungi-toxic substance present in the extract. The green *R. communis* was able to inhibit the growth of *C. destructivum* at a lower concentration while its highest concentration (100,000 ppm) was not able to inhibit *C. destructivum* indicating that it could serve as a growth promoter of the pathogen.

DISCUSSION

Results obtained from the *in-vitro* study show that *P. fluorescens* could be used effectively against the seed-borne pathogen, *C. destructivum*. When the pathogen was paired with *P. fluorescens* a remarkable and almost total control of the pathogen was achieved. Though it was not a fast-growing organism, it was able to stop further growth of the pathogen by the third day after pairing. It is conjectured that, its activity may be due to metabolite secreted by the bacterium into the medium. This metabolite could have brought about the change in colour of the whitish fluffy mycelia of *C. destructivum* to purple.

Although, it was not determined if the release of the metabolite was due to the presence of the antagonist or it was a normal secretion by the target pathogen in reaction to the presence of the antagonist, it was probably the ability of *P. fluorescens* to produce antibiotics that enhanced its usefulness as bio-control agent. The antibiotics might have caused growth inhibition of the target organism. *Pseudomonas* species are known to produce siderophore (Pseudobactiri) which may deprive the pathogen of iron (Upadhayay and Rai, 1988). *Pseudomonas* species have effectively controlled *Pythium aphanidermatum*, *Verticillium dahliae* and *Alternaria* specie (Ajibade, 2002).

Also, a strain of *P. fluorescens* was reported to carry the gene of *B. thuringiensis* toxin (Prescott et al., 2002). *Pseudomonas* is capable of producing an intracellular protein toxin crystal (the papasporal body) during sporulation which can act as a microbial insecticide for specific insect groups (Prescott et al., 2002). Junyeop et al. (2003) and Pedras et al. (2003) reported aerugine and Pseudophomins A and B antibiotics, respectively, are biologically active against some fungal pathogens. Our findings, therefore, suggest that *P. fluorescens* serves as an important bio-agent and can be used to control *C. destructivum*, as its effectiveness was revealed at reducing the mycelial growth of the pathogen.

T. pseudokoningii controlled the growth of *C. destructivum* by colonization and hyper-parasitism. This was observed as a result of fast growth rate of its mycelia which helped it in colonizing the growth substrate to the detriment of the pathogen. The pathogen mycelia later overgrew the mycelia of the antagonist after 2 weeks of pairing. This suggests that the *T. pseudokoningii* does not have inhibitory effect on the target pathogen. The existence of direct competition often exhibited by antagonists might be competing with target organisms for nutrients and space (Prescott et al., 2002). The value of Trichoderma spp. as bioagents has been reported by Howell (2003) for the protection of several diseases of crops. The *C. destructivum* was able to overcome the antagonist due to the fact that it is hemibiotrophic in nature (Prescott et al., 2002).

B. subtilis was the least effective organism used as an antagonist in the control of *C. destructivum* because of its

Table 3. Effect of different concentrations of plant crude extracts (of tobacco and castor plant leaves) on the growth diameter (cm) of *C. destructivum*.

Concentrations (ppm)	Purple <i>R. communis</i>				Green <i>R. communis</i>				<i>Nicotiana tabacum</i>			
	48	72	96	120	48	72	96	120	48	72	96	120
100,000	2.6a	3.6a	4.6a	6.3ab	2.8a	4.2a	5.5a	8.0a	2.6a	3.6a	5.2a	5.3ab
10,000	2.7a	3.5a	5.3b	6.5b	2.8a	4.2a	5.5a	7.3a	2.7a	3.9a	5.4b	6.4ab
1,000	2.9a	4.1a	5.1b	6.7bc	2.9a	4.1a	5.4a	7.0a	2.7a	3.7a	5.5b	7.0ab
100	2.8a	3.7a	5.1ab	5.8a	2.6a	3.7a	5.2a	6.8a	2.7a	3.7a	5.7bc	7.1ab
10	2.8a	4.2a	5.4b	6.7bc	2.8a	4.0a	5.3a	7.5a	2.9a	4.1a	6.1c	7.4ab
0	2.7a	4.0a	5.7bc	6.4ab	3.1a	3.9a	5.8b	6.9a	2.6a	3.9a	5.2a	7.2ab

Means in a column with similar letter(s) are not significantly different at 0.05 level according to Duncan's Multiple Range Test.

*Time (h).

inability to significantly inhibit the growth of the later. *B. subtilis* is generally known to inhibit growth of other microbes by antibiosis (Prescott et al., 2002). The restriction observed on the growth of *C. destructivum* in this *in vitro* study might be due to the rapid colonization of the growth substrate by *B. subtilis*, which had a faster growth rate than the *P. fluorescens* into the growth medium. It stopped the hyphal growth of *C. destructivum* even though the effect of the antibiotics was not noticed in the *B. subtilis*.

Bowers and Locke (1997) observed that the botanical extract of 1% aqueous emulsion did not significantly reduce the growth of *Fusarium oxysporum* f. sp. *chrysanthemi*. *Nicotiana tabacum* could not inhibit the growth of the pathogen as much as other plant extracts. Aver'yavnov (2000) reported that diffusate from plant materials inhibited the growth of fungal pathogens. It, however, reduced the growth of *C. destructivum* significantly at the highest concentration of 100,000 ppm. It is therefore concluded that *P. fluorescens* can be used to inhibit growth and development of cowpea anthracnose disease caused by *C. destructivum*. Also, high concentration of tobacco leaf extract, without being diluted gave the inhibitory effect on the growth of *C. destructivum*.

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