In-vitro Biocontrol of Fungi Associated with Leaf Diseases of Tomato (*Lycopersicon esculentum* Mill.) using *Trichoderma* Species

Tolulope S. Ewekeye, [1,2] Oyedamola A. Oke, [1] Oyinkansola B. Seriki, [1] Aminat T. Bello [1]

[1]Department of Botany, Lagos State University, Ojo, P. M. B. 0001, LASU Post Office, Ojo, Lagos, Nigeria
[2] Department of Botany, University of Ibadan, Oyo State, Nigeria
toluewekeye@gmail.com, damolaoke@gmail.com

Abstract: Biological control potential of fungi isolated from roots of diseased tomato plants against fungi associated with foliar diseases of tomato was investigated in this study. Fungi associated with the diseased tomato leaves included *Alternaria alternata, Fusarium solani, Phialophora melinii, Paecilomyces variotii,* and *Verticillium albo-atrium.* Each fungus was paired against *Trichoderma harzianum* and *Trichoderma koningii* which were isolated from the rhizophere of the diseased plants. The antagonists (*Trichoderma* species) were paired simultaneously against the pathogen, the pathogen before the antagonist and the antagonist before the pathogen. For the timing of inoculation, pairing of the antagonist before the pathogen was the most effective, followed by simultaneous pairing, while pathogen before antagonist gave the least antagonism after nine days of inoculation. The mode of antagonism was found to be by competition for space, antibiosis and mycoparasitism.
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1. Introduction

Biological control is the inhibition of growth, infection or reproduction of one organism using another organism (Baker, 1987; Cook, 1993). Biological control of plant diseases has been considered a viable alternative method to manage plant diseases as against the use of chemical pesticides and cultural practices (Cook, 1993, Agrios, 2005). Biocontrol is environmentally safe and in some cases it is the only option available to protect plants against pathogens (Cook, 1993).

Antagonists in biological control of plant pathogens are biological agents with the potential to interfere in the life processes of plant pathogens. (Cook and Baker, 1983). Antagonists of plant pathogen may be resident or introduced. Resident antagonists are part of the natural microbiota in soil or on roots, leaves or other plant parts, while introduced antagonists are those which are applied as cultures or prepared products to soil or plants (Dube and Podile, 1988).

Different mode of actions of biocontrol-active microorganisms in controlling fungal plant diseases include hyperparasitism, predation, antibiosis, cross protection, competition for site and nutrient and induced resistance (Heydari and Pessarakli, 2010).

The use of microorganisms that antagonize plant pathogens (biological control) is risk-free when it results in enhancement of resident antagonists. Biological control of fungal plant pathogens appears as an attractive and realistic approach and numerous microorganisms have been identified as biocontrol agents. Members of the *Trichoderma* genus are known as imperfect fungi, fast growing in culture and produce numerous green spores. They occur worldwide and are commonly associated with root, soil and plant debris. They have long been recognized as biological agents to control plant diseases. *Trichoderma* species have become popular biological agents to protect crops against plant pathogens all over the world. Researches have indicated that they can parasitize fungal pathogens and produce antibiotics (Tran, 2010).

Trichoderma species have been investigated for eighty years (Basim *et al.,* 1999). They have been used recently as biological control agents and their isolates have been commercially available of late (Loper and Buyer, 1991). *Trichoderma* affects a wide range of plant pathogens such as *Fusarium, Verticillium, Alternaria, Rhizopus, Venturia, Enthothia, Phytophthora, Fusicardium.* Living *Trichoderma* isolates produce metabolites which are antagonists to others in the soil environment. *Trichoderma* can also release compounds and extracellular enzymes known as trichodermin, which damage plant pathogen (Jones and Prusky, 2002). The colonies of *Trichoderma* have either loose or tuft colonies, which are correlated to structures of the conidiophores. *Trichoderma* are generally yellowish or light green in colour (Jones and Prusky, 2002).

The use of biocontrol agents is presently gaining momentum as a supplement to chemical treatment in integrated disease management module. The fungal antagonists may compete for an ecological niche by

consuming available nutrients and by secreting a spectrum of biochemicals effective against various fungal pathogens. These biochemicals may include cell wall degrading enzymes, siderophores, chelating irons, and a wide variety of volatile and non-volatile antibiotics (Dar *et al*., 2011).

Tomato fruits are among the most important vegetable products in the world. Tomatoes contribute to a healthy, well balanced diet. They are a rich source of minerals, vitamins, essential amino acid, sugars and dietary fibres (pectin). Tomatoes contain vitamin A and C, iron, folic acid and phosphorous (Poleman and Perkenpaugh, 1991). The production is however beset with many problems such as diseases, nematodes, insect pests, high flower drop, all these resulting in low yield and poor quality fruits (Denton and Swarup, 1983). Diseases of tomato include early blight, Late blight, Septoria leaf spot, Anthracnose, Fusarium wilt and Fusarium crown rot, Verticillium wilt among others.

This study was carried out to isolate fungi associated with foliar diseases of tomato and control the isolated organism(s) using *Trichoderma* species.

2. Material and Methods

2.1 Collection of samples

Diseased tomato leaves and soil around the roots of the diseased tomato plants were collected from farms in Olorunisola area of Alapoti village via Lusada, Ogun state, Nigeria. The diseased tomato leaves and the soil were collected in sterile black polythene bags and were taken to the laboratory for further processing.

2.2 Isolation of fungi

Diseased leaves were washed under running tap water, the infected parts were excised into small pieces and surface sterilised, they were then inoculated onto already sterilized Potato Dextrose Agar (PDA) and incubated. Repeated sub-culturing of mixed fungal cultures was carried out until pure fungal isolates were obtained. Isolation of fungi was carried out using direct plaiting and serial dilution methods. For direct plaiting, 1 gram of the collected soil sample was sprinkled on the agar after pouring and some was sprinkled on the plate directly before the agar was poured, the plates were then incubated at 28 ± 2^0C. For serial dilution method, 1g of the soil was weighed and suspended in 9mls of sterile distilled water in a test tube. The suspension was vortexed to extract the fungal spores, from each dilution; series of dilutions up to 10^6 were prepared. 1ml from each dilution was pipetted onto already prepared sterile PDA plates. The plates were rotated in a slow swirling motion to dispense the suspension on the medium.

2.3 Pathogenicity test

To confirm the pathogenicity of fungi isolated from diseased tomato leaves, spore suspension of each of the isolated fungus which was adjusted to about 3 X 10^4 spores/ml was used to inoculate healthy leaf samples detached from two month old seedlings. The inoculated leaves were then transferred into Petri dishes containing moistened sterile filter paper and incubated in a moist chamber.

2.4 Evaluation of biocontrol potential of antagonists against the pathogens

Pure isolates of the antagonists and the pathogen were used. The pathogens isolated from diseased tomato leaves were paired against the organisms (antagonists) isolated from the soils around the roots of diseased plants. The method of inoculation of the antagonist against the pathogen as described by Abou-Zeid *et al*., 2008 was used. Pairing of the antagonist against the pathogen on the plate was done simultaneously, the antagonist twenty four hours before the pathogen and the pathogen twenty-four hours before the antagonist. This was done to determine which pairing would be more effective.

The inhibition of the pathogens by the antagonists was measured. The grading of antagonistic activity as described by Sobowale (1994) was used:

0: the pathogen grew round the whole 9cm plate.
1: the antagonist only covered less than 1.5cm of the plate.
2: the antagonist only covered 1.50-3.00cm of the plate.
3: the antagonist covered 3.10-4.00cm of the plate
4: the antagonist covered between 4.10 and 4.90cm of the plate
5: the antagonist covered between 5.00 and 6.50cm of the plate
6: the antagonist covered between 6.60 and 7.90cm of the plate
7: the antagonist covered between 8.00 and 8.90cm of the plate
8: the antagonist grew round the whole 9cm plate.

3. RESULTS

The following organisms were isolated and identified on the diseased leaves of tomato: *Alternaria alternata, Aspergillus wentii, Aspergillus niger, Aspergillus ustus, Paecilomyces variotti, Fusarium solani, Aspergillus vesicolor, Aspergillus fumigatus, Phialophora melinii* and *Verticillium albo-atrium.* Five out of the isolated organisms: *Alternaria alternata, Fusarium solani, Phialophora melinii, Paecilomyces variotii* and *Verticillium albo-*

atrium were found to initiate disease symptoms when inoculated on healthy tomato leaves.

Trichoderma harzianum and *Trichoderma koningii* were isolated from the rhizosphere of the diseased tomato plants.

The results of the pairing of the antagonists against the pathogens after three days (Table 1) showed that inoculating the antagonists before the pathogens generally gave a higher grade of antagonism followed by simultaneous pairing and lastly pathogen before the antagonist. For most of the antagonists, there was progression in the grade of antagonism from the sixth to the ninth day (Tables 2&3).

Table 1: In vitro antagonistic effect of *Trichoderma harzianum* and *Trichoderma koniigii* on the causal pathogens after three days of inoculation.

Pairing	AT before P	P before AT	AT and P simultaneously
AT_h vs P_1	4	2	3
AT_k vs P_1	5	3	4
AT_h vs P_2	5	4	4
AT_k vs P_2	5	2	3
AT_h vs P_3	5	3	4
AT_k vs P_3	5	3	4
AT_h vs P_4	4	2	3
AT_k vs P_4	4	2	4
AT_h vs P_5	5	2	3
AT_k vs P_5	4	2	3

Key:
$AT_{h:}$ *Trichoderma harzianum*
AT_k: *Trichoderma koniigii*
P_1: *Alternaria alternata*
P_2: *Fusarium solani*
P_3: *Phialophora melinii*
P_4: *Paecilomyces variotii*
P_5: *Verticillium albo-atrium*

Grades
0: No antagonism
1: 10% antagonism
2: 30% antagonism
3: 40% antagonism
4: 50% antagonism
5: 60% antagonism
6: 80% antagonism
7: 90% antagonism
8: 100% antagonism

Table 2: In vitro antagonistic effect of *Trichoderma harzianum* and *Trichoderma koniigii* on the causal pathogens after six days of inoculation.

Pairing	AT before P	P before AT	AT and P simultaneously
AT_h vs P_1	4	3	4
AT_k vs P_1	5	2	3
AT_h vs P_2	6	4	5
AT_k vs P_2	6	3	5
AT_h vs P_3	5	4	4
AT_k vs P_3	5	3	4
AT_h vs P_4	5	4	4
AT_k vs P_4	6	4	5
AT_h vs P_5	5	3	4
AT_k vs P_5	7	5	6

Table 3: In vitro antagonistic effect of *Trichoderma harzianum* and *Trichoderma koniigii* on the causal pathogens after nine days of inoculation.

Pairing	AT before P	P before AT	AT and P simultaneously
AT_h vs P_1	6	4	5
AT_k vs P_1	6	5	5
AT_h vs P_2	7	4	5
AT_k vs P_2	7	5	5
AT_h vs P_3	7	4	6
AT_k vs P_3	6	4	5
AT_h vs P_4	7	3	5
AT_k vs P_4	6	3	5
AT_h vs P_5	7	4	6
AT_k vs P_5	7	5	6

4. DISCUSSION

Alternaria alternata, Fusarium solani, and *Verticillium albo-atrium* associated with foliar disease of tomato in this study have been previously isolated from diseased tomato (Booth, 1971; Van Dijk and Nelson, 2000; Glandorf *et. al.*, 2001).

The use of microbial antagonist to suppress plant disease has been reported (Lindsday, 1979). The mode of action of *T. harzianum* against *Alternaria alternata* indicates competition, hyperparatism and antibiosis. An antagonist can produce more than one mechanism of defense. The fact that *T. koningii* colonised as much space before *Alternaria alternata* was inoculated suggested competition for space, there was also a zone of inhibition between the pathogen and the antagonist. The growth of *T. harzianum* over parts of the mycelial mass of *Fusarium solani* suggests hyper parasitism, this agrees with the work of (Neilands,1981) who reported that the fungus

Trichoderma harzianum available commercially as seed treatment is a mycoparasite of several damping off pathogens including *Phytium* and *Rhizoctonia*. The mechanism of action of *T. koningii* against *Fusarium solani* indicates antibiosis as a clear zone of inhibition was maintained when P_2 was paired with AT_k for pathogen before the antagonist. *T. harzianum* colonized about 60% of the plate on the 6th day before *Phialophora melinii* was inoculated indicating competition for space and nutrient (Table 2). To successfully colonise the phytosphere, a microbe must effectively compete for available nutrient, nutrient which can be obtained from product of other organism and soil. There was no line of inhibition between *T. koningii* and *Phialophora melinii*, there was also strong competition for space. The action of *T. harzianum* against *Paecilomyces variotii* indicates antibiosis; there was clear zone of inhibition occurring between the two microorganisms when grown on the plate. The antagonist was found growing over the pathogen indicating hyperparsitism. The mode of action of *T. koningii* against *Paecilomyces variotii* suggested the former to be a stronger competitor. There was clear zone of inhibition maintained when AT_K was paired with P_3 simultaneously. There was also indication of antibiosis. The ability to produce multiple classes of antibiotics that differentially inhibit pathogens is likely to enhance biological control (Duchesne, 1994).

The action of *T. harzianum* against *Verticillium albo-atrium* is an indication of hyperparatism, there was direct contact of the antagonist with the pathogen. There was no production of line of inhibition. For simultaneous pairing of the antagonist against the pathogen, there was slow growth of the pathogen. The mechanism of action of *T. koningii* against *Verticillium albo-atrium* indicates competition for space and nutrients. Competition between non pathogens and pathogens for nutrient resources is important for limiting disease incidence and severity. There was total colonization of the pathogen by the antagonist likewise for pairing of pathogen before the antagonist and also the paring of antagonist before the pathogen. The pairing of the antagonist before the pathogen was the most effective method throughout the nine days of incubation; there was effective inhibition of the pathogen by the antagonists.

The mechanisms of antagonism exhibited by the two antagonists tested agrees with the work of McLaughin *et. al*., (1992) who stated that some antagonists may effect biocontrol against one pathogen by more than one mode of action and this broadens the antagonist's capability for biocontrol. Also, the overall behaviour of the antagonists against the pathogens could be said to be in line with the report of Chalutz *et. al*., (1988), which indicated that there is the possibility of an antagonist controlling more than one pathogen on a plant.

According to Singh and Paul (1988), it is often difficult to extrapolate from laboratory experiments to the field to know the exact biocontrol mechanism of an antagonist against a pathogen. The mode of action of an antagonist against a pathogen in-vitro might be also be different in-vivo (Janisiewicz, 1988) however, some antagonists such as *T. harzianum* have been known to control some pathogens both in the laboratory and in the field (Elad *et. al*., 1980).

Correspondence to:
Ewekeye, Tolulope S.
P. M. B 0001, Lagos State University Post Office,
Ojo, Lagos, Nigeria.
E-mail: toluewekeye@gmail.com

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