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In Vitro Evaluation of Fungicides, Plant Extracts and Antagonists (*Trichoderma* Spp) on Chili Anthracnose (*Colletotrichum capsici* (Syd.))

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Abstract

Chili anthracnose caused by *Colletotrichum capsici* is one of the most devastating diseases that deter chili production in Southern Ethiopia. This study was conducted in 2014 with the aim to judiciously manage *Colletotrichum capsici* causing anthracnose of Chili. Six fungicides, namely, Benomyl, Tilt-250 EC, Vitavax-200, Rovral 50 WP, Dithane M-45 and Ridomil at concentration of 150, 250 and 300 ppm; and leaf extracts of garlic, ginger, onion and neem at three different concentrations (15%, 10% and 5%) were evaluated against the radial growth and mycelial dry weight of *Colletotrichum capsici*. Concurrently, 20 Trichoderma isolates formulated with residues of different crops were also tested against *Colletotrichum capsici* by using dual culture technique. In all these three experiments the treatments were arranged in CRD and data was analyzed through ANOVA. All the treatments were statistically different from the untreated check. From the six fungicides, all test concentrations of Tilt-250 EC significantly inhibited the mycelial growth of *Colletotrichum capsici*. Vitavax-200 and Royal 50WP gave 55.33-77.33 and 59.67-83.67; and 20.33-68.33mm and 20.67-73.67g, radial growth and mycelial dry weight of the test pathogen, respectively. Dithane M-45 and Cupravit were found to be significantly inferior to the rest of the fungicides.

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Among the tested plant extracts, garlic at the highest concentration (15 %) was found to be best in the reduction both the radial mycelial diameter (72.33) and mycelial dry weight (73.33) followed by Onion (15%) with 53.33 and 58% of radial mycelial diameter and mycelial dry weight, respectively. There was significant variation among isolates of *Trichoderma spp* and antagonistic activities ranged from 51 to 89% reduction of the mycelial radial growth of *Colletotrichum capsici* . Among the promising antagonists, the isolate Tri_3 of *Trichoderma harzinum* showed the highest, 89 %, inhibition of mycelial radial growth of *Colletotrichum capsici* . The wise use of these plant extracts, fungicides and antagonists must be enhanced to curtail chili anthracnose, In light with this study, there will be gradual shift from "routine fungicide use" to "apply when necessary" regime owing to these cheap and effective tactics ensuing in sustainable chili production.

Keywords: Anthracnose; *Colletotrichum capsici* ; Fungicides; Plant Extract and *Trichoderma harzianum.*

1. Introduction

Chili (*Capsicum frutescence* L.) has received a great deal of attention all over the world as important cash crop in general, and particularly in Ethiopia. Chili suffers from many diseases among them anthracnose caused by *Colletotrichum capsici* is the predominant one [1,2,3]. There are a few studies on yield losses due to diseases of Chili is available in the country. Though chemical fungicides have been extensively used worldwide to control various pathogens, their use has many attendant problems, such as environmental pollution, deteriorating human health, development of pathogen resistance to fungicides, and phytotoxicity [4,5]. To minimize these problems, researchers have sought to develop biological control agents for soil-borne plant pathogens that might be more environment- friendly. Phytopathogens have been used to control several antagonistic microorganisms, including *Trichoderma spp* [5,6]. As it has been reported, the inhibitory mechanisms of antagonistic microorganisms are antibiosis, competition, parasitism, and predation [7]. Methods for complete control of Chili seed borne diseases are yet to be developed. Management strategies for these diseases include use of presumed disease free seeds, resistant cultivars and fungicidal sprays. Seed treatment is one of the best methods to manage seed-borne diseases. The continuous and indiscriminate use of chemicals to manage the crop disease results in accumulation of harmful chemical residues in the soil, water and grains. Development of fungicide-resistant biotypes of the pathogen is a major constraint to control the major seed-borne pathogens of Chili [6,7]. In recent years, considerable success has been achieved by introducing antagonists to control seed-borne fungal pathogens [4,5,6]. A considerable work has been done in controlling seedling diseases of many crops caused by *Rhizoctonia solani*, *Botrytis gladiolorum*, *Botrytis fabae*, *Fusarium xylarioides* and *Sclerotium rolfsii* both in vitro and pot culture experiments by using Trichoderma [5,6,7,8]. Only a limited trials has been done in inhibiting the growth and development of *Colletotrichum capsici*, *F. oxysporum* and *Microphonoma phaseolina* by using Trichoderma [6]. Complete elimination of *Colletotrichum capsici* from Chili and other crops is very difficult by any single approach of control. Some plant extracts also found to be most effective in reducing the mycelial growth and development of many pathogens [8 9 10]. To develop a sustainable integrated control strategy against the anthracnose of chili, it is essential to ensure the in vitro efficacy of *Trichoderma spp*, some fungicides and some plant extracts. Considering the current situation, the present study has therefore been undertaken with the objective to evaluate the efficacy of fungicides, plant extracts and *Trichoderma spp* against the major anthracnose (*Colletotrichum capsici*) of chili.

2. Materials and Methods

2.1 The Study Area

The experiment was conducted to control *Colletotrichum capsici* causing anthracnose of chili by using fungicides, plant extracts and Trichoderma in the Department Microbial, Cellular and Molecular biology, College of Natural and Computational Sciences, Addis Ababa University, Addis Ababa, during 2013 and 2014 on-seasons.

2.2 Sources of plant extracts, Fungicides and Trichoderma spp isolates

Collectively, 20 isolates of *Trichoderma* spp (with 6 morphological categories in three formulations each) (Appendix 1) were isolated from rhizosphere and rhizoplane soils of bean, barley, tomato, sweet potato, chili, cauliflower, sweet pepper, and wheat field of SNNPR and Oromiya and farmer's fields of Amhara by soil dilution plate technique and root washing methods [4, 8] .

2.3 InVitro Evaluation of Fungicides on the Mycelial Growth of Colletotrichum capsici

Six fungicides namely Ridomil, Benomyl, Dithane M-45, Rovral 50WP, Tilt 250EC and Vitavax-200 were tested in an in vitro to evaluate their antagonistic effect on mycelial growth of *Colletotrichum capsici* following poison food technique [5]. All fungicides were used at 150 ppm, 250 ppm and 300 ppm. Fungicidal suspensions of different concentrations were prepared by dissolving required amount of each fungicide in warm PDA at 40-45 0C. The fungicides were thoroughly mixed with the medium by shaking with hands before autoclaving. Twenty ml of sterilized medium was poured in each 9 cm sterilized petridish. After solidification, the plates were inoculated by placing 5 mm discs of 3 days old PDA cultures of *Colletotrichum capsici*. Three replicated plates were used for each concentration of each fungicide. Three replicated PDA plates received without fungicides served as control. The inoculated plates were incubated at 28oC and data on the radial mycelial diameter was recorded after 4-5 days of incubation when the growth of the control plates completely covered the petri plate. Diameter of the colonies on PDA with and without fungicide was measured from the bottom side of the petri dishes. Inhibition of radial growth was computed based on mycelial diameter on control plate using the following formula as described by Fekadu and Tesfaye [6]; and Tesfaye and Kapoor [4,5].

% Inhibition = * 100; Where, X= Growth of control plate; Y= Growth of fungicide treated plate.

To determine the effect of the fungicides on mycelial dry weight of Colletotrichum capsici, Potato Dextrose Broth (PDB) was used, as recommended by Fekadu and Tesfaye [6]; Tesfaye and Kapoor [4,5]. The medium was prepared by mixing infusion of 200 g peeled potato; 20 g dextrose in 1000 ml distilled water. After cooking the medium was poured into 250 ml conical flask at the rate of 100 ml per flask. Required amount of each fungicide was added to the broth to have concentration of 150, 250, 300 ppm. Three replicated flasks were used for each dose of the six fungicides. The contents of the flasks were autoclaved at 1210C under 1kg/cm2 for 20 minutes. The flasks were placed inside a clean bench for cooling ambient temperature. The flasks were inoculated with mycelial discs of 5 day's old culture of *Colletotrichum capsici*. The discs were cut with a flame

sterilized cork borer (5 mm). Inoculation was done by putting one mycelial disc per flask with a flame-sterilized needle. Additional three flasks containing the broth (PDB) receiving no fungicides were used as control. The inoculated flasks were incubated at room temperature for 15 days. At the end of incubation, the cultures in all flasks were filtered separately through pre-weighted filter paper. Dry weight of mycelium was obtained by subtracting weight of filter paper from weight of filter paper and mycelium. Inhibition of mycelial dry weight was determined by comparing the growth in control flasks by using the formula described by Fekadu and Tesfaye [6] and Tesfaye and Kapoor [4,5].

2.4 In Vitro Evaluation of plant extracts on the mycelia growth of *Colletotrichum capsici*

In-vitro test was conducted to determine the effect of plant extracts on radial mycelial growth of Colletotrichum capsici following poison food technique as described by Fekadu and Tesfaye[6]; Tesfaye and Kapoor [4,5]); and Begum and Bhuiyan [12]. Water extracts of garlic (*Allium sativum*), ginger (*Zingiber officinale*), onion (Allium cepa) and neem (*Azadirachta indica*) leaves were tested. Stalk solutions of the materials were prepared by blending 100g of each plant material in 100 ml of sterilized water in a blender. PDA medium was amended with individual extract at 0, 5, 10 and 20% (v/v). Required amount of individual plant extract was added to the 100 ml conical flask with PDA medium to have concentrations of 0, 5, 10 and 20% (w/v) based on the procedure coined by Kabir et al. [13]. After thorough mixing with plant extracts the medium was autoclaved and approximately 15 ml of melted PDA mixed with extracts was poured into each 90 mm Petri dish. After solidification, the plates were inoculated by placing 5 mm discs of 3 days old PDA cultures of *C. capsici*. Similar procedure was followed as described under Serawit and Tesfaye [11] in an in vitro evaluation of fungicides against the growth of *Colletotrichum capsici*. Inhibition of radial mycelia growth was computed based on mycelial diameter on control plate using the same formula [4,5,6]. To determine the antagonistic effect of the plant extracts on mycelial dry weight of *Colletotrichum capsici*, potato dextrose broth (PDB) was used. Similar procedure was followed as described an in vitro the evaluation of fungicides against the hyphal dry weight of *Colletotrichum capsici*. Similar procedure was followed for taking the mycelial dry weight as described in Serawit and Tesfaye [11]; Fekadu, and Tesfaye [6]; Tesfaye and Kapoor [4,5].

2.5 InVitro Evaluation of T. harzianum Isolates against chili Anthracnose (Colletotrichum capsici)

Twenty isolates having different formulations and origins had been used for this experiment (Appendix 1). All the isolated Trichoderma isolates were identified as *Trichoderma harzianum*. The isolates of Trichoderma were purified in acidified agar (pH 4.5) using hyphal tip technique. After purification they were maintained as stock culture in PDA slants at 4 oC for future study 4. An in vitro screening experiment was conducted to find out the antagonistic effect of all the isolated 20 T. harzianum isolates against chili anthracnose (*Colletotrichum capsici*) on PDA by using dual culture technique [6]. Discs of mycelium (5mm diameter) of each of the selected fungal isolates were cut from the edge of an actively growing fungal mycelia with a cork borer. Test plates were prepared by pouring 20 ml of PDA per plate. After solidification, one mycelial disc of individual isolate of *T. harzianum* and one disk of test fungal pathogen was placed simultaneously on the edge of the each PDA Petri plate at opposite direction. Three replicated plates were used for each isolate of Trichoderma and test pathogen. The plates were arranged on the laboratory desks following completely randomized design. The plates received

only mycelial discs of the test pathogens served as control. The plates were incubated in the laboratory having ambient temperature of 25±3 C until mycelium growth of the test pathogens (*Colletotrichum capsici*) cover the whole control plate. Thereafter, inhibition percentages of *Colletotrichum capsici* was calculated based on the growth of the pathogen on PDA plates following the formula as suggested by Fekadu and Tesfaye [6].

2.6 Experimental Design and Data Analysis

The experiments were conducted following Completely Randomized Design (CRD) with three replications. Data were analyzed by using IBM SPSS version 20.0 program. The significant difference, if any, among the means were compared by Least Significance Difference (LSD).

3. Results and Discussion

3.1 InVitro Evaluation of Fungicide Against Anthracnose of Chili (Colletotrichum capsici)

In the present study, six fungicides, namely, Tilt-250 EC, Vitavax-200, Dithane M-45, Cupravit and Royal 50WP gave noticeable inhibition in mycelia radial growth and mycelial dry weight as compared to control (Table 1).

Table 1: In vitro Evaluation of Fungicides on the growth of chili anthracnose (*Colletotrichum capsici*)

Fungicides	Concentration (ppm)	% Inhibition		Fungi-cides	Concentration (ppm)	% Inhibition	
		Radial Growth (mm)	Mycelial dry weight (g)			Radial Growth (mm)	Mycelial dry weight (g)
Ridomil	150	18.67b	70.67e	Vitavax-200	150	76.33d	82.67f
	250	20.33b	71.33e		250	77.33d	83.67f
	300	23.33b	24.33bc		300	55.33c	59.67d
Benomyl	150	23.4b	28.67c	Royal 50WP	150	65.33cd	70.33e
	250	26.9b	83.33f		250	68.33d	73.67e
	300	99.33e	99.33g		300	20.33b	20.67b
Tilt-250EC	150	99.33e	99.33g	Dithane M-45	150	23.33b	24.33b
	250	99.33e	99.33g		250	26.67b	23.67b
	300	68.33d	75.67ef		300	20.33b	19.67b
Control		7.33a	0.37a	CV=15.8		LCD=10.6	LCD=6.4

*Values with same letter are not significantly different

Among six fungicides all concentrations Tilt-250 EC completely inhibit the mycelial growth of *Colletotrichum capsici* Vitavax-200 and Royal 50WP gave 55.33-77.33 and 59.67-83.67; and 20.33-68.33mm and 20.67-73.67g, radial growth and mycelial dry weight of the test pathogen, respectively. Dithane M-45 and Cupravit were

appeared to be significantly inferior in comparison to other fungicides in inhibiting the mycelial growth.

Nearly complete inhibition of mycelial dry weight *Colletotrichum capsici* was achieved with all the concentration of Tilt-250 EC. Mycelial dry weight was reduced by 83.33% and 73.67 with the second highest concentrations of Vitavax-200 and Royal 50WP, respectively. But Vitavax 200 was significantly superior to Rovral 50WP but significantly inferior to Tilt-250EC. Dithane M-45 at 300 ppm inhibited only 19.67 % mycelial dry weight and statistically similar to Royal 50% WP but superior to Cupravit which was appeared to be significantly inferior in comparison to all other fungicides. The trends in efficacy of all fungicides at all concentrations were almost similar as observed in case of inhibition of radial mycelial growth of the fungus. Among six tested fungicides, Tilt-250 EC appeared to be the best one in inhibiting the hyphal growth of *Colletotrichum capsici* which was followed by Vitavax-200, Rovral 50WP, Dithane M-45 and Cupravit. The present results are in partial agreement with other investigators who observed that Tilt 250 EC, Vitavax-200 and Rovral 50 WP were most effective against *Colletotrichum* capsici. in different crops[14]. Dithane M-45 was noted as poor performing fungicides against *Colletotrichum capsici*. Its poor performance against some *Colletotrichum* capsici. was also reported by Fekadu and Tesfaye [6]); Islam et al. [14] and Sharif [15]

3.2 InVitro Evaluation of Plant Extracts on the Mycelial Growth of Chili Anthracnose (*Colletotrichum capsici)*

Reduction of mycelial diameter and dry mycelium weight by anthracnose was found by all the treatments compared to control and the highest reduction was measured from the combined application of plant extracts of neem, Onion, ginger and garlic.

Table 2: In vitro evaluation of plant Extracts on chili anthracnose (*Colletotrichum capsici*)

Plant Extract	Concentration (%)	% Inhibition		Plant Extract	Concentration (%)	% Inhibition	
		Radial Growth (mm)	Mycelial dry weight(g)			Radial Growth (mm)	Mycelial dry weight(g)
1.Garlic	5	42e	44.67f		15	39.67e	42.67e
	10	51f	51.67g		5	21.67b	23.33b
	15	72.33g	73.33 i	4.Neem	10	25.67bc	28.67c
2.Onion	5	37.33de	40.33e		15	32.67d	34.33d
	10	48.67f	53.33gh	5.Control		7.67a	0.34a
	15	53.33f	58h				
3.Ginger	5	42e	41.67e				
	10	36.67d	39.33e		CV=14.7	LSD=5.5	LSD=4.2

*Values with same letter are not significantly different

From the tested plant extracts garlic at the highest concentration (15 %v/v) was found to be best in the reduction

both the radial mycelial diameter (72.33) and mycelial dry weight (73.33) followed by Onion (15 %v/v) that gave (53.33) and (58%), respectively. The rate of reduction was corroborated with its concentrations in case of all the tested plant extracts. Ginger at the highest concentration gave 39.67% and 42.67% reduction in mycelial diameter and dry mycelium weight, respectively, and significantly inferior to onion but superior to neem extract at all the concentrations. The results reported recently by Kabir *et al*. [13] with different botanicals in combination for controlling alternaria blight disease of broccoli are also in strong evidence with the present findings. Neem extract gave the highest 32.67% reduction in mycelial diameter and 34.33% reduction in mycelium dry weight at the highest 15% concentrations (Table 2) and significantly inferior to all other extracts. The result of the experiment showed that the most effective material is garlic, which was followed by onion, ginger and neem. The result was in conformity to the findings of Serawit and Tesfaye[11] who recommended that neem extract was very effective in controlling the anthracnose pathogen *Colletotrichum* capsici. in different crops. This result is also in corroboration with some previous research works [13, 16, and 17]. However, combinations of botanical extracts were more strong and effective even to a level that with minimum risk, such treatment can replace a highly effective chemical fungicidal treatment.

3.3 In Vitro Evaluation of Antagonistic Activities of *T. harzanum Against Chili Anthracnose (Colletotrichum capsici)*

The results of the screening of 20 isolates of *T. harzianum* against *Colletotrichum capsici* on PDA plates are presented in Table 3 and Figure 1. Among the selected isolates Tri_3 was appeared to be most effective against the test pathogens showing 89 % inhibition of mycelial growth and significantly higher compared to the other isolates. Identically higher inhibition of radial growth against *Colletotrichum capsici* was recorded with the isolates Tri-2 and Tri-5. The isolate Tri-8 inhibited 64 % radial growth of the pathogen but significantly inferior to the isolate Tri-2 and Tri-5. Isolate Tri-14, though, had shown the lowest inhibition in radial growth against *Colletotrichum capsici*, was statistically not different to Tri-9, Tri-10, Tri-11, Tri 16, Tri-19 and Tri-20. The finding was in conformity with the reports of D'Souza *et al*. [18]; and Rajathilagam and Kannabiran,[19] who observed significant reduction of the growth of chili anthracnose (*Colletotrichum capsici*) mycelia growth by using *T. harzianum*. All stuff considered, the effectiveness of the antagonist *Trichoderma* spp against Chili anthracnose (*Colletotrichum* capsici) was worth appreciation to design a control option. The results of the current in vitro evaluation of twenty Trichoderma isolates against *Colletotrichum capsici* of chili satisfied the required criteria for the selection of specific antagonist and isolate to pave the for the development of safer disease management of anthracnose of Chili.

Trichoderma species were found more effective in suppressing the mycelial growth of *Colletotrichum capsici* when compared to volatile compounds 6. It is observed that *Trichoderma* spp completely inhibits the radial mycelial growth of *Colletotrichum capsici* at a concentration of 15%. Further, reducing the concentration of culture inhibited mycelia growth and weight at a concentration between 5%-10%. As it is showing complete inhibition at a very low concentration invitro, there is need to give more importance for further studies in-vivo is in progress. It was also observed that with an increase in concentration of culture filtrates of all the Trichoderma species, the radial mycelial growth of test pathogen was proportionally decreased. It is reported and well documented that *T. viridae* were used as potential antagonist for controlling many fungal plant pathogens such

as *Colletotrichum acutatum, Colletotrichum falcatum, Fusarium oxysporum* [20,21,22]. Although species of Trichoderma is used extensively to control many plant pathogens but nosignificant work has been done to control anthracnose of bell pepper caused by *Colletotrichum capsici*. In the present study an attempt was made to test isolates of Trichoderma to control the growth of *Colletotrichum capsici* in-vitro. Based on the present investigation a new strategy will be developed for controlling anthracnose on *Capsicum* spp in vivo [6].

Table 3: Inhibition of radial Growth of *Colletotrichum capsici* by selected *Trichoderma harzanium* isolates in dual plate techniques

Isolate	**% inhibition of radial Growth(mm)**	**Isolate**	**% inhibition of radial Growth(mm)**	**Isolate**	**% inhibition of radial Growth(mm)**
Tri-1	62.33 e	Tri-8	64f	Tri-15	60de
Tri-2	67.33 g	Tri-9	52.67b	Tri-16	53.33bc
Tri-3	89 h	Tri-10	52.67b	Tri-17	61e
Tri-4	58d	Tri-11	52 b	Tri-18	63.33 ef
Tri-5	66.33 ef	Tri-12	64.67 f	Tri-19	51.33 b
Tri-6	55 c	Tri-13	57.33cd	Tri-20	51 b
Tri-7	62e	Tri-14	50.33 b	Control	10a
				CV=16.1%	LSD=3.5

*Values with same letter are not significantly different(p=0.05)

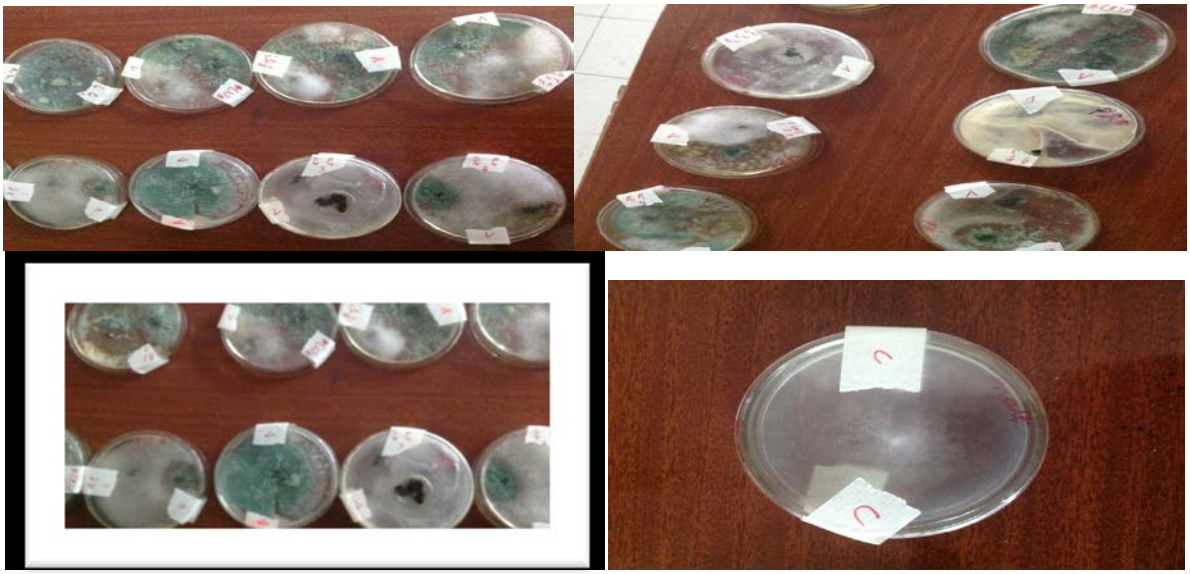

Figure 1: Dual culture of *Colletotrichum capsici* and *T. harzianum* isolate Tri3 on PDA plate (Upper left: Isolates showing high inhibitions of Mycelial growth of *Colletotrichum capsici* in presence of Trichoderma isolates; Upper right: Isolates showing low inhibition of mycelial growth of *Colletotrichum capsici*; Lower left:

Isolates showing moderate inhibition of mycelial growth of *Colletotrichum capsici*; Lower right: Control)

4. Conclusion and Recommendations

To continually renovate and implement an integrated control strategy, analyzing the efficacy of the individual component of an integrated measure against the test pathogen is vital. The results of the current in vitro evaluation of fungicides, plant extracts and Trichoderma isolates against *Colletotrichum capsici* of chili fulfilled the prerequisite criteria for the selection of appropriate dose of fungicide and plant extract and specific antagonist fungal isolate to develop an eco-friendly integrated disease management of anthracnose of chili in the field. Thus, it's is recommended for further use.

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Appendices

Appendix 1. List of Isolates of *Trichoderma* spp obtained from AAU, their designations and formulation (Recommended by: Tesfaye, 2015(unpublished); Tesfaye and Kapoor, 2007, 2010)

Table 4

Iso. name	Major gr. of the isolate	Formulation	Iso. name	Major gr. of the isolate	Formulation
Tri 1	37	Cotton seed	Tri 11	Th	Straw
Tri 2	37	Straw	Tri 12	Th	Coffee husk
Tri 3	37	Coffee husk	Tri 13	Th	Straw+husk
Tri 4	69	Cotton seed	Tri 14	47	Cotton seed
Tri 5	69	Straw	Tri 15	47	Straw
Tri 6	69	Coffee husk	Tri 16	47	Coffee husk
Tri 7	V	Cotton seed	Tri 17	Ad	Cotton seed
Tri 8	V	Straw	Tri 18	Ad	Straw
Tri 9	V	Coffee husk	Tri 19	Ad	Coffee husk
Tri 10	Th	Cotton seed	Tri 20	Ad	Straw+husk