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Compatibility Studies of Fungicides with Combination of *Trichoderma* Species under *In vitro* Conditions

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Abstract

Fungicides viz., curzate and sancozeb were used at different concentrations that is., 100, 200, 400, 600, 800 and 1000 ppm active ingredient to evaluate *Trichoderma* species viz., *Trichoderma harzianum* (AUT1) and *Trichoderma viride* (AUT2) in favour of tolerance to fungicides. By increasing the fungicide concentrations to 400 ppm (sancozeb) and 600 ppm (curzate), the *Trichoderma* species tolerate the fungicides 50% and slightly incompatible at higher concentrations of 800 and 1000 ppm, and completely inhibited beyond 1000 ppm compared to the control for both fungicides. The highest 97.8% was recorded at 100 ppm for curzate fungicides when combined with AUT2 and 96.7% of compatibility was recorded at concentration of 100 ppm when AUT1 is combined with the same fungicides. But, in the case of sancozeb the highest compatibility (97.8%) was recorded when combined AUT2, and 95.5% with AUT1 at 100 ppm. Therefore, the present compatibility study assist in the selection of bio control agents, which can be used with reduced amount of preferred fungicides for the control of plant pathogenic fungi.

Keywords: *Trichoderma* species; Fungicides; Compatibility; *In vitro*.

Introduction

Different biological control agents (BCAs) can be used for the control of plant diseases. These include fungi, bacteria and actinomycetes. The most important BCAs belong to the genus *Trichoderma* species, *Bacillus* species, *Pseudomonas* species and *streptomycetes*. Biological control of plant pathogens is an eye-catching alternative to decrease heavy dependence of modern agriculture on costly chemical fungicides, which not only cause environmental pollution but also lead to the development of resistant strains [1].

A recent list of mechanisms are viz., mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, solubulization and sequestration of inorganic nutrients, induced resistance and inactivation of the pathogens enzymes [2]. Apart from biocontrol ability, the BCAs possess other traits such as rhizosphere competence, tolerance of fungicides, saprophytic competitive ability, ability to tolerate high and low temperatures, adaptability to different edaphic conditions, good searching ability, host specificity, high reproduction rate, short life cycle, adaptability, well adapted to different stages of life cycle of target host, able to maintain itself after reducing host population [1,3] have showed that *Trichoderma viride* displaced the naturally occurring mycoflora on the surface of the yam tuber.

To develop an effective disease management programme, the compatibility of potential bio agents with fungicides is essential. Combinations of fungicides and compatible bio agents in an IDM strategy protects the seeds and seedlings from soil borne and seed borne inoculum [4]. Integration of compatible bio agents with fungicides may enhance the effectiveness of disease control and provide better management of soil borne diseases [5]. The combination of BCAs with fungicides would provide similar disease suppression as achieved with higher fungicide use [6]. Combining antagonists with synthetic chemicals eliminates the chance of resistance development and reduces the fungicide application. It is therefore, proposed to identify the compatibility of the potential bio agents with commonly used fungicides for the eco-friendly management of the tea diseases. As fungicides should have inhibitory effect on the pathogen but should not have deleterious effect on the antagonists, an understanding of the effect of fungicides on the pathogen and the antagonists would provide information for the selection of fungicides and fungicide resistant antagonists, through compatibility studies *in vitro*. In addition, this strategy may display even better control of resistant strains of fungal pathogens and may help the commercial growers to reduce the amount of fungicide use, thus lowering the amount of chemical residue in the marketed products. Combined applications of BCAs followed by small quantities of fungicides may help the antagonists and the relative cost of the formulations [7].

Trichoderma species are known to suppress infection of root by soil borne pathogens like *Macrophomina phaseolina, Rhizoctonia solani*, *Fusarium* species and *Pythium* species on various crops [8,9,10]. Species of *Trichoderma* also have growth promoting capabilities that may or may not be integral to biological control [9,11,12]. *Trichoderma harzianum* has shown effective control of root infecting fungi and root-knot nematodes [13,14]. *Trichoderma harzianum* isolated from rhizome rot suppressive soils reduced the disease and increased plant growth and yield [15]. It has been reported that many *Trichoderma* species has an innate and/or induced resistance to many fungicides but the level of resistance varies with the fungicide [16]. The combined use of BCAs and chemical pesticides has attracted much attention in order to obtain synergestic or additive effects in the control of soilborne diseases [17].

In view of this, investigation was conducted to test the possiblity of combining *Trichoderma* species with fungicides under laboratory condition. The long term goal is to develop an effective IDM package for managing soil borne plant disease as well as to prevent the

resistence development in pathogens to fungicides. Integrating chemical resistant *Trichoderma* species has an importance in the framework of integrated disease management. Disease prevention can be increased by using such tolerant species that keeps pathogens under sufficient pressure so that they cannot thrive. Keeping the above in view, the present work was designend to observe the compatibility of different fungicides (curzate and sancozeb) with the BCA that is., *Trichoderma harzianum* (AUT1) and *Trichoderma viride* (AUT2) *in vitro*.

Materials and Methods

Collection of *Trichoderma* species and fungicides

Two species of *Trichoderma* (*Trichoderma harzianum* and *Trichoderma viride*) were used to study its compatibility with fungicides under *in vitro* conditions and they were designated as AUT1 and AUT2, respectively. The culture was obtained from Mycology Laboratory, Department of Microbial, Cellular and Molecular Biology, Addis Ababa University which were isolated from the soil samples collected from Gera, Gomma, Mana, Kossa and Seka Chokersa woredas of Jimma Zone, Ethiopia by [18]. They are further checked for purity and are used for experimentation. The fungicides used were curzate (43.95%WP) and sancozeb (80%WP). Sancozeb and curzate fungicides were obtained from Mycology Laboratory Research, Addis Ababa University.

The poisoned food technique

The purpose of this experiment was to evaluate the efficacy of curzate and sancozeb fungicides at different concentrations against *Trichoderma* species which were available currently on market to control fungal pathogens. Evaluation and screening was employed according to [19]. The fungicide concentrations were prepared as follows, if the formulated product (fungicide) has, 50% active ingredient, for 1 ppm solution 2 mg of the formulated product should be dissolved in a liter of solvent [19]. Therefore, curzate (Copper oxychloride 39.75%+Cymoxanil 4.2%) has 43.95% WP, for 100 ppm solution 0.175 g, 200 ppm (0.35 g), 400 ppm (0.7g), 600 ppm (1.05 g), 800 ppm (1.4 g) and 1000 ppm (1.75 g) was added in a liter of solvent. For preparation of sancozeb (mancozeb 80% WP) 0.32 g, 0.64 g, 1.28 g, 1.92 g, 2.56 g and 3.2 g were used for 100, 200, 400, 600, 800 and 1000ppm, respectively and dissolved in a litre of distilled sterilized water. The fungicides were added to the autoclaved Potato dextrose agar (PDA) medium to prevent denaturation of the fungicides, cooled to 45°C with the amount of 2 ml per plate, so that the required concentrations were obtained. Triplicate culture plates, each containing 20 ml of the test medium, were used to test each *Trichoderma* species at different concentration. Potato dextrose agar plates inoculated with *Trichoderma* species without fungicide were used as control. Mycelial plugs of 5 mm in diameter were cut from 7 days actively growing margins of the fungal culture by sterile cork borer and transferred aseptically into the centre of the Petri dish containing PDA medium with different concentrations of fungicide. Inoculated plates were incubated at 25°C for 10 days. Growth of *Trichoderma* species at each concentration was determined by measuring mycelia growth diameters in two perpendicular directions on each culture plate. Measurements were averaged in triplicates, and the diameters of the plugs used to inoculate the plates were subtracted from each measurement. The relative growth reduction for each fungicide was calculated by the equation below.

$$L = \frac{C - T}{C} \times 100$$

Where L is percentage of inhibition; C is radial growth of the *Trichoderma* species in control; T is radial growth of the fungus in the presence of the fungicides [20].

Tolerance of *Trichoderma* species to fungicides

Species of *Trichoderma harzianum* (AUT1) and *Trichoderma viride* (AUT2) were evaluated for tolerance to fungicides (curzate, 43.95% WP and sancozeb, 80% WP) by using food poison method [19] at 100, 200, 400, 600, 800 and 1000 ppm concentrations. Fungicides (curzate and sancozeb) were added to PDA to get final concentration of 100, 200, 400, 600, 800 and 1000 ppm active ingredient. Potato dextrose agar medium without fungicide served as control. A 5mm inoculum disc of *Trichoderma* species was cut from the margin of actively growing colony and placed in centre of each Petri plate. Petri plates were incubated at 25 ± 1°C. Three replications were maintained for each treatment. Per cent reduction in radial growth over control was calculated by using the following formula:

$$L = \frac{C - T}{C} \times 100$$

Where, L=Percentage reduction in growth of *Trichoderma* species C=Radial growth (mm) of *Trichoderma* species in control T=Radial growth (mm) *Trichoderma* species in treatment.

Combination of *Trichoderma* species with fungicides

The combined use of *Trichoderma* species and fungicides were applied by the method of [19]. In this technique, the growth medium was poisoned with fungal toxicants. The fungicide concentrations of 600 ppm for curzate and 400 ppm for sancozeb were prepared and added to the autoclaved PDA medium after cooled to 45°C, so that the required concentration was obtained for both fungicides. Triplicate culture plates, each containing 20 ml of the test medium was poured and after solidification of medium, the test *Trichoderma* species was inoculated. Potato dextrose agar plates without *Trichoderma* species and fungicides were used as a control. The growth of *Trichoderma* species at 600 ppm and 400 ppm fungicides combination were determined by measuring mycelia growth diameters and percentage inhibition of radial growth was calculated following the formula suggested by [20]:

$$L = \frac{C - T}{C} \times 100$$

Where L is mean inhibition per cent of radial mycelial growth; C is radial growth measurement of the test *Trichoderma* species in control; T is radial growth of the test *Trichoderma* species in combination with fungicides.

Methods of Data Analysis

The statistical analysis of mycelia growth diameters of *Trichoderma* species and per cent of inhibition were tested. Mean comparisons of different parameters were conducted using the procedures of SPSS statistical analysis software version 16. Mean separation was determined according to Duncan's multiple range test ($P<0.05$).

Results

In vitro screening of *Trichoderma* species for tolerance to curzate and sancozeb

Results of Tables 1 and 2 showed that, *Trichoderma* spp. AUT1 and AUT2 were screened for tolerance to fungicides like curzate and sancozeb. Incorporation of curzate and sancozeb in growth medium did not affect the growth of *Trichoderma* spp. instead fungicides favoured the growth of antagonistic fungi at lower concentrations of 100 and 200 ppm. However, by increasing the fungicidal concentrations to 400 and 600 ppm, the antagonists tolerate the fungicides to some extent and reduced slightly at higher concentrations of 800 and 1000 ppm compared to control. The highest (97.8%) was recorded for curzate fungicides when combined with AUT2 and 96.7% of compatibility was recorded at concentration of 100 ppm when AUT1 is combined with the same fungicides. But, in the case of sancozeb the highest compatibility (97.8%) was recorded when combined AUT2 and 95.5% with AUT1.

Concentration (ppm)	*Trichoderma harzianum* (AUT1)		*Trichoderma viride* (AUT2)		Mean ± SD
	Growth (mm)	Percent of compatibility	Growth (mm)	Percent of compatibility	
100	87.0 ± 0.57a	96.7b	88.0 ± 0.57f	97.8a	45.1
200	71.0 ± 0.57b	78.9c	81.0 ± 0.57e	90.0b	45.7
400	62.6 ± 0.66c	69.6d	71.0 ± 0.57d	78.9c	46.2
600	44.3 ± 0.57d	49.3e	42.3 ± 1.20c	47.1d	47.5
800	31.0 ± 0.57e	34.5f	31.0 ± 0.57a	34.5f	48.3
1000	23.3 ± 0.88f	25.9a	28.6 ± 0.88b	31.9e	48.4
Control (mm)	90.0 ± 0.0g	100.0g	90.0 ± 0.0f	100.0a	90
Mean ± SD	53.2 ± 5.43	40.84	57.0 ± 5.80	63.3	53.02

Table 1: Screening of *Trichoderma* species for tolerance to curzate at different concentration after seven days of incubation at 25°C. Each value is an average of three replicates ± Standard deviation. Means followed by the same letters within a column are not significantly ($p<0.05$) different, according to Duncan's multiple range test.

Concentration (ppm)	*Trichoderma harzianum* (AUT1)		*Trichoderma viride* (AUT1)		Mean ± SD
	Growth (mm)	Percent of Compatibility	Growth (mm)	Percent of compatibility	
100	85.0 ± 0.57f	95.5b	88.0 ± 0.57e	97.8a	45.2
200	66.6 ± 0.88e	74.1c	71.6 ± 0.88d	79.7b	46.1
400	43.0 ± 0.57d	47.8d	43.0 ± 1.52c	52.2c	47.6
600	30.0 ± 0.33c	33.7e	33.0 ± 1.15b	47.8d	48.2
800	22.0 ± 0.57b	24.5f	26.6 ± 0.88a	28.6e	48.6
1000	19.0 ± 0.57a	21.1g	25.6 ± 0.33a	29.7e	48.7
Control (mm)	90.0 ± 0.0g	100.0a	90.0 ± 0.0e	100.0a	47.4
Mean ± SD	50.8 ± 5.85	49.3	54.0 ± 5.75	53.3	47.4

Table 2: Screening of *Trichoderma* species for tolerance to sancozeb at different concentration after seven days of incubation at 25°C. Each value is an average of three replicates ± Standard deviation. Means followed by the same letters within a column are not significantly ($p<0.05$) different, according to Duncan's multiple range test.

In both curzate and sancozeb, the lower concentrations of 100 and 200 ppm, they well tolerated with both *Trichoderma* species and hence they are effective in managing plant pathogens. In addition using such combinations at lower concentration decreases resistance activity and

soil pollution. From comparison of means of means, AUT1was more inhibited than AUT2 by both fungicides. AUT2 was more tolerate to both fungicides than AUT1 as Tables 1 and 2. Since both fungicides may have poisonous effect on AUT1 and AUT2, when concentration increased and increased Tables 1 and 2, an appreciative of the effect of fungicides on antagonists would afford information on the selection of selective fungicides and fungicides resistant antagonists for compatibility studies.

Discussion

In the present study, laboratory experiments were conducted to observe the compatibility of *Trichoderma* species with fungicides. The result revealed that at the selected concentrations of curzate (400 ppm) and sancozeb (600 ppm), both *Trichoderma* species was 50% compatible with both fungicides (Tables 1 and 2). This showed that the AUT1 and AUT2 were able utilize the fungicides as a source of nutrient, but above these concentrations it may weaken the efficacy of *Trichoderma* species (AUT1 and AUT2). The percent of compatibility decreased with an increase in the concentration of fungicide. Reduced amount of fungicide can stress and weaken the pathogen and render its propagules more susceptible to subsequent attack by the antagonist [21].

A progressive increase in per cent inhibition of radial growth in AUT1 and AUT2 was observed as the concentration of both fungicides increased. Both fungicides were able to completely suppress the growth of both *Trichoderma* species at above the concentration (1000 ppm) used in the present study. Former reports suggest that bio control agents that can tolerate a certain level of fungicides were mixed with agrochemicals, resulting in eradication of diseases [22]. Similarly, [23] reported that thiram, copper oxychloride and Mancozeb at 0.2 % are compatible with *Trichoderma harzianum* and *Trichoderma viride* [24], also reported compatibility of *Trichoderma* species with Dithane, Bavistin and Ridomil at any level of selected concentration that is., 50 ppm, 100 ppm, 200 ppm, 300 ppm and highly insensitive to blue copper and captaf [25] reported that the *Trichoderma* isolate GRHF4 was more compatible with mancozeb followed by copper oxychloride. Similar results were also obtained by [26]. They observed that mancozeb was compatible with *Trichoderma* species. Similar results were also observed by [27], who reported copper oxychloride and copper hydroxide to be highly compatible with *Trichoderma harzianum.* of the time, fungicides produce undesirable effects on non-targeting organisms, so the use of microorganisms that antagonize plant pathogenic fungi is risk free [9]. Moreover, the combination of fungicide tolerant biological control agents with reduced levels of fungicide integrated control strategies would promote the degree of disease suppression similar to that achieved with full dosage of fungicides [6]. There are reports where the biocontrol agents, which can tolerate fungicides up to a certain level, were mixed with fungicides and resulted in eradication of diseases [22].

Therefore, rather than applying these chemicals alone, it is very important to use *Trichoderma* species (AUT1 and AUT2) in combination with fungicides at lower concentration for effective management of fungal pathogens since they do not have side effect on the environments. Similarly, [28] have reported that integration of biological control agents and commonly used fungicides showed positive association by reducing the seed infection compared to fungicide and the fungal antagonists individually [29] have reported that the efficiency of the biological control agent could further be improved when it was applied with the recommended fungicide and used at a lower concentration. Thus, the antagonistic potential of *Trichoderma* species in terms of enhanced modes of action as increased hyper parasitism activity in the present study. The result of the present screening would help in the selection of biological control agents, which can be used, with reduced dose of selected fungicides for the control of plant pathogenic fungi.

Conclusion

Present findings indicated that treatment of AUT1 and AUT2 would be high compatable with both curzate and sancozeb at 100 ppm concentration, followed by 200 ppm concentration. High incompatibility was observed at the concentration of (800 ppm, 1000 ppm and above for curzate, while 600 ppm, 800 ppm, 1000 ppm and above in the case of sancozeb). As BCAs cannot handle the disease entirely when bulky size infection is already recognized in the field, farmers prefer fungicides for managing the crop diseases. But fungicides are harmful to the environment and also injurious for the soil, efficiency and human and animal health. Due to the disadvantages of fungicides, IDM programs (100 and 200 ppm for both fungicides) with BCAs are recommended, in which judicious use of fungicides and their integration with BCAs is favored. As fungicides may have harmful effect on antagonists, an indebted of the effect of fungicides on antagonists would provide information on the selection of selective fungicides and fungicides resistant antagonists for compatibility studies as has been suggested in the present paper.

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