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Evaluation of Fungal Bio-Agents Against Tea Root Rot (*Fusarium oxysporum*) in South-Western Ethiopia

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ABSTRACT

Background and Objective: The most often consumed stimulant plant, tea (*Camellia sinensis*), is a major contributor to the nation's economic output. However, its production is decreased by fungal diseases such as *Fusarium oxysporum* induced root rot. The objective of this study is to evaluate the efficacy of fungal bio-agents in managing tea root rot in the tea-growing regions of Southwestern Ethiopia. **Materials and Methods:** Samples were taken from the Gumero and Wish-Wash tea plantation areas in Southwest Ethiopia. Using the dual culture method five bio agents namely Tg01, Tg02, Tg03, Tg04, Tg05, and three known *Trichoderma* spp. (*Trichoderma harzianum*, *Trichoderma asperellum*, and *Trichoderma viride*) were characterized and evaluated. These bio-agents were identified as fungal species. The ANOVA was conducted using SAS software (version 9.4), with arcsine transformation for data normalization and mean separation via the least significant difference at $p = 0.05$. **Results:** The results revealed significant differences ($p<0.01$) between the isolates. The range of radial growth was 1.76 to 4.41 mm/day. The lowest (1.76) and the highest (4.41) mm/day radial growth were recorded from Tg01 and *T. asperellum*, respectively. Additionally, the isolates' inhibition percentages against the examined pathogen varied. Likewise, Tg01 and *T. asperellum* revealed the lowest (43.1%) and highest (92.9%) inhibitory percentages. The *Trichoderma* species, specifically *T. harzianum*, *T. asperellum*, and *T. viride*, exhibited the highest degree of inhibition against the target pathogen and the fastest radial growth when compared to the other isolates. **Conclusion:** The next stage of the study should be to choose and identify the best possible bio-control agents, followed by evaluation in the field and a greenhouse. Overall, the BCAs of the investigated isolates demonstrated positive and promising outcomes under *in vitro* evaluation of the target pathogen in this study.

KEYWORDS

Antagonist, biocontrol, fungal species, *Fusarium oxysporum*, *Trichoderma* spp

INTRODUCTION

Tea (*Camellia sinensis* (L.)) is one of the most economically important nonalcoholic beverage crops widely consumed in large quantities. Its consumption exceeds three billion cups/day around the world. Initially, it was consumed as a medical drink and as a refreshment later. In terms of its contribution to the GDP, health benefits (like preventing tooth decay, cancer, lowering blood pressure, cholesterol, and clotting,

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preventing heart disease, normalizing diabetes, and lowering blood glucose), poverty alleviation, and job opportunities, tea industry is crucial[1,2]. Tea is produced in China, India, Kenya, and Sri Lanka more than in other countries[3,4].

Initially, tea (Assam type) was introduced in 1927 and grown in the Southwestern part of Ethiopia. The first garden was established at Ilu Aba Bora Zone (Gore town) of the Oromia Region. Currently, tea is produced by our growers around plantation companies in Ethiopia. Its production is mainly by private farms and contributes a lot to generating income for the country[5,6]. Also, it becomes an essential crop to shift through the product options to define investment opportunities for the producers.

However, its production is declining due to biotic and abiotic constraints[7]. Among the biotic pressures, fungal diseases such as Fusarium wilt (*Fusarium oxysporum*), black rot, blight (brown blight, grey blight, blister blight, etc.), and anthracnose (*Colletotrichum* spp.) are commonly reported from tea growing areas across the world and Ethiopia in particular[8-10]. Sniha *et al.*[11] have reported yield loss due to the disease estimated to be about 5-10%, and the loss is increasing at the rate of 10-20% per year on average. In addition, yield losses of up to 100% can also be recorded under dramatic environmental changes that favor disease epidemics[12,13]. As tea is a perennial crop grown under monoculture; it offers an opportunity for various diseases epidemic development. In several tea-producing countries, leaf, stem, and root diseases are the most serious threats that affect the yield and quality of tea.

Among the diseases, root rot is economically an important factor in tea production, since it kills irrespective of tree age or vigor[14,15]. From their nature, soil-borne pathogens like *F. oxysporum* are very difficult to manage once they occur and can exist for several years by causing huge damage, especially on perennial crops. Arefin and Hossain[5] reported that tea root rot disease is becoming severe, significant, and epidemic in different tea-producing areas, mainly in large plantations such as Wish-Wash, Gumero, and Chewaka, with an average incidence of 16-20% in Southwestern Ethiopia.

To reduce the impacts of this disease, having effective management options is unquestionable and quite crucial. Foremost control, the initial approach is disease avoidance, and in situations where it does not succeed, the use of chemicals is mandatory to tackle the yield losses[16]. However, managing such soil-borne pathogens by using the usual methods like cultural practices and chemicals is quite difficult. Also, recommended fungicides are very expensive and highly toxic to the users and the environment[17], and indiscriminate use of fungicides considerably increases hazards to human beings and the environment. Hence, searching for the easiest, most economical, reliable, and most effective management approach like biological control is very crucial. Bio-agents can be used as part of integrated disease management strategies to reduce the pest population in the way of their antagonism behavior[18]. These antagonistic microorganisms can include various groups of organisms, mainly fungi and bacteria. The most effective alternatives to reduce the effects of diseases that restrict the growth of soil-borne pathogens, including *F. oxysporum*, are various bio-control agents (BCAs) or bio-fungicides[19]. The importance of BCAs was cited in numerous research investigations.

For instance, Singh *et al.*[20] said that the three antagonists (Ch. Cupreum CC3003Ch Ch, Globosum CG05, and Ch. Lucknowense CL01) reduced the formation of conidia by 67-76% and hindered the mycelial growth of *F. oxysporum* by up to 32-34%. Additionally, recent research by Bastakoti *et al.*[21] has shown that the antagonistic activities of *Trichoderma* spp. offered maximal suppression (about 94%) on *Fusarium solani* and *R. solani*. In Ethiopia, research on the efficacy of bioagents against tea root rot is lacking. With this concern, this study was initiated to isolate, characterize, and evaluate the antifungal potential of bio-agents against *F. oxysporum* from tea-producing areas of Southwestern Ethiopia.

MATERIALS AND METHODS

Description of the study area: The activity was conducted from the 2021-2023 cropping year in the Jimma Agricultural Research Center Phytopathology Laboratory.

Soil sample collection and isolation: The tea farms (Wish-Wash and Gumero) were used as the sample sources for soil. Ten soil samples (from the top 10 to 15 cm of the soil) from each plantation were taken, bulked up, and brought to the JARC Phytopathology Laboratory. The bulked samples were sieved using a 0.5 mm mesh size after being allowed to air dry for 4-5 days at room temperature on a lab bench. Using sterile distilled water, successive dilution procedures were used for the isolation process. Following that, cultures were cultured at 27±2°C using 0.5 mL of the diluted sample that had been put onto the prepared PDA medium. Colonies that appeared on the culture plate after 5 days of incubation and had distinct morphologies were separated and purified once more on PDA. To maintain the isolates for later usage, pure cultures were then kept at 4°C[11]. The activity was conducted from the 2021-2023 cropping year. All the collected samples (the pathogen and soil) were taken with the permission of the landowners.

Isolates characterization and identification: Following isolation, the isolates were distinguished based on cultural and morphological properties of the fungus, such as mycelia development, colony traits, pigmentation/color, size, form, conidial density, etc. Here, the isolates were identified and characterized using a color chart, hemocytometer (Neubauer, Hui Zhou City, China), light microscopy (Olympus, Tokyo City, Japan), and identification manual. Comparison was used to determine the species status of the fungus[22].

Pathogen (*Fusarium oxysporum*) isolation: The Wish-Wash and Gumero tea estates' afflicted areas were where the pathogen that causes tea root rot was sampled. Following the prescribed methods, isolation was carried out on Potato Dextrose Agar (PDA) medium, and to prevent contamination, sub-culturing was carried out after 5 days. Then, for the upcoming experimental works, pure culture was kept at 4°C.

Antagonistic evaluation of the dual culture: Five isolates (Tg01-05) and three *Trichoderma* spp (*T. viride*, *T. harzianum*, and *T. asperellum*) a total of eight treatments (Table 1) were used and evaluated antagonistically against (*F. oxysporum*) using a dual-culture approach following the Gent *et al.*[3] method. On a 9 cm diameter Petri plate on PDA separately, 5 mm diameter mycelial disc of each antagonist was positioned on the side opposing the target pathogen (*F. oxysporum*). It was plated alone (as a control) and incubated in a Completely Randomized Design (CRD) with three replications at 25±2°C for comparison. The pathogen development was then monitored every three days until the isolate's maximum potential was reached.

Statistical analysis: The revised data was summarized using the Microsoft Excel 2021 version. The SAS software (version 9.4) was employed for the Analysis of Variance (ANOVA). Arcsine transformation was used to normalize the data and mean separation between treatments was determined using the least significant difference with a p-value of 0.05.

Table 1: Treatments used for *in vitro*/dual culture/evaluation

Treatment	Description
T1	*Trichoderma harzianum*
T2	*Trichoderma asperellum*
T3	*Trichoderma viride*
T4	Tg_{01}
T5	Tg_{02}
T6	T_{03}
T7	T_{04}
T8	T_{05}

RESULTS AND DISCUSSION

Isolation and identification: From soil samples collected from the tea-growing areas of Gumero and Wish-Wash, five antagonists known as Tg01, Tg02, Tg03, Tg04, and Tg05 (Table 2) were isolated. They displayed various pigmentation on potato dextrose agar culture medium. The majority of them indicated, per shape, elevation, and color, filiform, filamentous, flat, and flowery white margins. Each isolate is a member of a different fungus species based on its physical characteristics Table 2 presents the cultural characteristics and pigmentations of various *Trichoderma* spp. isolates. Most isolates, including *Trichoderma harzianum*, *Trichoderma asperellum*, and *Trichoderma viride*, exhibited filiform margins and irregular shapes with a flat elevation, along with ghost white front and floral white reverse pigmentations. In contrast, the Tg isolates, such as Tg01 and Tg05, displayed convex elevations, with Tg01 having a white front and light golden rod reverse, while Tg05 exhibited a white front and light golden rod reverse. Overall, the isolates demonstrated consistent morphological traits, with variations in pigmentations noted primarily on the reverse side.

The results showed that the radial expansion of the isolates varied significantly ($p<0.01$) from another. All the newly isolated BCAs and the three *Trichoderma* spp., *T. viride*, *T. harzianum*, and *T. asperellum*, displayed the quickest radial growth rate. Also, the isolates exhibited considerable variation in conidial density and length ($p<0.01$) among the isolates, except for their width (no significant variance). Conidial width, however, did not significantly differ amongst the isolates. The Tg03 showed the highest conidial density (236 m/mL), while Tg04 indicated the lowest (49 m/mL). Except for Tg05, which had a substantial and highest (3.24 µm) spore length, none of the isolates displayed a significant variation in spore length (Table 3).

***In vitro* evaluation of dual culture:** The results of the *in vitro* evaluation showed that the bio agents' ability to inhibit pathogen growth varied, within 43.1 to 92.9% inhibition potential ranging. Also, the newly isolated BCAs showed encouraging results of their inhibition potential against the target pathogen (*F. oxysporum*). The pathogen development is inhibited by more than 50% in three of these, namely Tg03 (T_6), Tg04 (T_7), and Tg05 (T_8), with respective inhibition levels of 55.6, 50.7, and 57.5% (Fig 1; Table 4). This shows that the fungal antagonist efficiently slows the colony growth of the pathogen under the dual culture test.

Among the tested BCAs, the maximum inhibition percentage was observed from the three *Trichoderma* spp. (*T. viride*, *T. harzianum*, and *T. asperellum*). The highest inhibition (92.9%) was recorded from *T. harzianum*, followed by *T. asperellum* (85.9%) and *T. viride* (84.6%). Among the tested BCAs, the maximum inhibition percentage was observed from the three *Trichoderma* spp. (*T. viride*, *T. harzianum*,

Fig. 1: Isolates inhibition potential against *Fusarium oxysporum* on dual culture plates

Table 2: Cultural characteristics of the isolates and the *Trichoderma* spp

Isolate /BACs/	Cultural feature			Pigmentation	
	Margin	Form/shape	Elevation	Front	Reverse
Trichoderma harzianum	Filiform	Irregular	Flat	Ghost white	Floral white
Trichoderma asperellum	Filiform	Irregular	Flat	Ghost white	Floral white
Trichoderma viride	Filiform	Irregular	Flat	Ghost white	Floral white
Tg01	Filiform	Irregular	Convex	White	Light golden rod
Tg02	Filiform	Filamentous	Flat	Floral white	papaya whip
Tg03	Filiform	Filamentous	Flat	Floral white	papaya whip
Tg04	Entire	Filamentous	Flat	Floral white	papaya whip
Tg05	Undulate	Irregular	Convex	White	Light golden rod

Tg (01-05): Isolates gained from the sampled areas

Table 3: Microscopic features of the isolates evaluated on potato dextrose agar medium

Isolate	Conidial density 10,000 mL	Spore size (µm)		Altitude (m.a.s.l)	Co-ordinates altitude	
		Length	Width		Longitude (E°)	(N°)
Tg01	172.67^{b}	2.75^{b}	2.02^{a}	1822	36.1313'	7.3527'
Tg02	135.33^{c}	2.80^{b}	2.02^{a}	1847	36.1329'	7.3547'
Tg03	236^{a}	2.96^{ba}	2.00^{a}	1853	36.1332'	7.3469'
Tg04	49.33^{d}	2.69^{b}	2.00^{a}	1875	36.1263'	7.3370'
Tg05	148.33^{cb}	3.24^{a}	2.00^{a}	1892	36.1238'	7.3329'
p-value	0.001	0.012	0.235			
SE	30.206	0.098	0.005			

The result with similar letters showed no significant difference, m.a.s.l: Meter above sea level, Tg (01-05): Isolates gained from the sampled areas and the results shared similar letters showed non-significant difference

Table 4: Radial growth and inhibition percentage of the isolates and *Trichoderma* species

Isolate	Radial growth (mm)	Inhibition (%)
Trichoderma harzianum	4.31^{a}	92.9
Trichoderma asperellum	4.41^{a}	85.9
Trichoderma viride	4.34^{a}	84.6
Tg01	1.76^{e}	43.1
Tg02	3.34^{c}	49.2
Tg03	3.39^{bc}	55.6
Tg04	3.56^{b}	50.7
Tg05	2.13^{d}	57.5
p-value	0.012	-
SE	0.318	6.185

Mm: Millimeter, SE: Standard error, the result with similar letter have no significant difference statistically and Tg (01-05): Isolates gained from the sampled areas

and *T. asperellum*). The highest inhibition (92.9%) was recorded from treatment 1 (*T. harzianum*), followed by treatment 2; *T. asperellum* (85.9%) and treatment 3; *T. viride* (84.6%). This shows the strongest and most effective bio-control activities of *Trichoderma* species against *F. oxysporum*. In another way, the lowest inhibition percentage (43.1%) was recorded from isolate Tg01 (treatment 4) under *in vitro* evaluation (Fig. 2; Table 4).

The biggest global hazard to crop productivity is plant diseases to date. Among these, the main agricultural difficulties include soil-inhibiting fungal diseases such as *F. oxysporum* and *Verticillium dahliae*[7]. Therefore, creating the most cost-effective, efficient, and ecologically safe management system is necessary to prevent such diseases to supply enough food for the world's expanding population. Many possible fungal bio-controls are effective in preventing these serious diseases in major crops, and they have drawn a lot of attention[23]. Potential bio-pesticides for field or greenhouse research, these fungus-based bio-control agents show strong antagonistic action against a range of soil and airborne plant diseases.

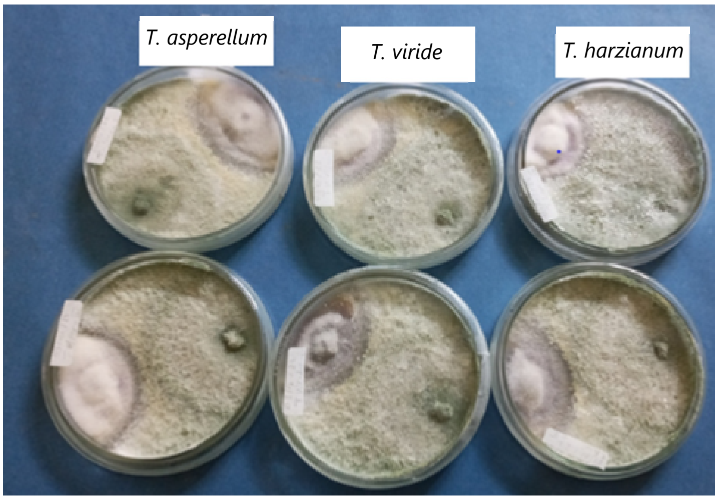

Fig. 2: Inhibition potential of the three *Trichoderma* spp., against *Fusarium oxysporum* on the dual culture plate

Additionally, helpful fungi generate a lot of bioactive substances that can be utilized as agrochemicals for crop protection[24,25]. Since fungal bio-agents (BCAs) prevent the formation of many plant pathogens and have received a lot of attention at the moment. Among the most widely employed fungal BCAs to combat bacterial and fungal plant diseases, are *Trichoderma*, *Aspergillus*, and *Penicillium*. Primarily, *Trichoderma* species are well-known for their diverse range of roles in the soil as saprotrophs, inhabitants, plant symbionts, and mycoparasites, as well as their antagonistic actions against a variety of phytopathogens[26].

The dual culture data from this investigation showed that, in contrast to the other isolates, *Trichoderma* isolates grew quickly and effectively against *F. oxysporum*. The greatest option to lower the risk of soil-borne diseases is bio-control agents' potentially harmful bio-agents, which can colonize plant diseases that invade the vascular system, including *F. oxysporum* and *verticillium* species[27,28]. The current difficulty in most of the agricultural production of tea in many different areas is this[29]. Thus, the greatest (68%) and most efficient suppression of *T. harzianum* against these soil pathogens (*Fusarium solani*) under *in-vitro* evaluation was reported by Nakaew *et al.*[7], which is consistent with our findings. Similar to this, Ayaz *et al.*[24] showed that *T. viride* gave the highest level of inhibition (about 94%) against *Fusarium solani* and *R. solani*. The BCAs can use a variety of strategies to suppress themselves in this situation. This includes the release of medicines or chemicals that are similar to antibiotics, as well as hyphal parasitism, which inhibits pathogen growth directly by penetrating the hyphal wall and absorbing and lysing the mycelium[7].

CONCLUSION

The Tg01, Tg02, Tg03, Tg04, and Tg05, five isolated bio-agents, were isolated, described, and assessed in a lab setting. Conidial density ranged from 49.33 to 236 mL, while other morphological traits, such as radial growth, showed significant variation (1.76 to 4.34 mm). On a dual culture test, their antagonistic potential against the *F. oxysporum* that was indicated varied from 43.1 to 92.9% inhibition. As a whole, BCAs are potentially the best alternatives to chemicals; these tested bio-agents under this experiment offer promising results for controlling the target pathogen (*F. oxysporum*). However, further validation work under greenhouse and field conditions is very crucial and should be the next research plan.

SIGNIFICANCE STATEMENT

This study highlights the potential of fungal bio-agents as effective biocontrol agents for managing *F. oxysporum*, a soil-borne pathogen causing root rot in tea production. The bio-agents exhibited promising antagonistic activity. Given their affordability and effectiveness, these bio-agents present a viable alternative to costly cultural and chemical management strategies. The activity needs further greenhouse and field validation studies for future use as disease control option.

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