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TRICHODERMA TOMENTOSUM AND T. AFROHARZIANUM AS POTENTIAL BIOCONTROL AGENTS FOR WHITE ROT DISEASE SCLEROTIUM CEPIVORUM BERK OF GARLIC IN AMBO, ETHIOPIA

[1&2] L.N, Ofodile, E. Ani[1] and A.A. Ayodeji[3].

[1]Department of Biological Science, Yaba College of Technology, Yaba, Lagos, Nigeria

[2]Mycology Department, Plant Protection Research Centre, Ambo, Ethiopia

[3]Department of Statistics, School of Science, Yaba College of Technology, Yaba, Lagos, Nigeria

Correspondence: nwannemka@yahoo.com

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ABSTRACT

Garlic (*Allium sativum*) is a favourite spice in Ethiopia that is widely cultivated and used as food and in medicine. This experiment was carried out for seven months in 2007. Some *Trichoderma* species were isolated from the soil in infected *Allium sativum* farms in three villages in Ambo, Ethiopia and identified. Internal transcribed spacer regions (ITS1-2) of rDNA and the translation elongation factor 1-α (eEF1a1) were generated from the isolates obtained using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). The species of fungi were further evaluated for their activity against *Sclerotium cepivorum* using Weller's and dual culture methods. AS1, AST1 and GS2 were identified as *Trichoderma tomentosum*, AS2 as *Hypocrea lixii* and GS1 as *Trichoderma afroharzianum*. AS1, AS2 and GS2 parasitised *S. cepivorum* and inhibited the growth of the pathogen while ATS1 and GS1 parasitised the pathogen only. AS1 showed the strongest significant antibiosis of 3.75 mm while the significantly least antibiosis was by GS2. GS1 showed 100% parasitism on the 7th day of incubation. These *Trichoderma* species inhibited the growth of the pathogens and can be useful in the production of fungicide for the control of white rot disease of garlic.

Keywords: *Trichoderma;* garlic; s*clerotium cepivorum;* biocontrol; Ethiopia

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INTRODUCTION

Allium sativum L. is a widely cultivated garlic in Ethiopia and other parts of the world and is preferred by most Ethiopians for its strong pungent culinary value (Bakonyi *et al*., 2011). It is used in the preparation of different foods and medicines in Ethiopia. Garlic is propagated by bulbs and exhibits high post-harvest and storage losses due to diseases (Gullino *et al*., 2010; Moustafa *et al*., 2013; Prahlad *et al.*, 2022).

Allium sativum is a multi-purpose herb with many ethnopharmacological values and has been proven to be a broad spectrum anti-microbial (Petrovska and

Cekovska, 2010; Bitew *et al.*, 2019 and Magryś *et al*., 2021). Garlic is used in the treatment of influenza, headache and chest pain in Ethiopia (Hailemeskel *et al.*, 2017). The possibility of incorporating garlic in animal feed to control its microbial content has been investigated with success (Bitew *et al.*, 2019). Garlic extracts also reduce the number of faecal eggs; they may be able to control parasite infections (Ding *et al*., 2023). Garlic can control aflatoxin-producing fungi and bacteria-producing exotoxin and endotoxin in food and feed. The insecticidal, anti-parasitic, anti-tumor and anti-microbial activity of garlic have been reported (Mikaili *et al*., 2013; Abiy and Berhe, 2016; Zeng *et al*., 2017).

The sulfur-containing compounds obtained from *Allium* species appear to have a wide range of activity, which shows up in various insecticidal, anti-fungal and nemacidal activities (Anwar *et al*., 2019). The absence of improved varieties of garlic, conventional method of production, pests and diseases that cause economic post-harvest losses exerted a negative impact on the production of garlic in Ethiopia. Among the diseases of garlic, the white rot has been identified as the most important especially in Ambo, Ethiopia. According to Dilbo *et al.* (2015), white rot is the most destructive disease of garlic throughout the world. White rot disease is caused by *Sclerotium cepivorum* Berk which causes heavy losses in onion and garlic (Amin *et al.*, 2014). The pathogen produces a great number of poppy seed-sized sclerotia, which can survive in soil for many years. Once established in a field, it permanently renders the field unsuitable for garlic and onion productions in Ethiopia for up to 40 or more years. The pathogen is particularly difficult to manage, as it can remain dormant in the soil for many years until the next crop is planted (Amin *et al*., 2014).

Conventional methods of disease control of garlic was based on the use of chemicals. Chemicals used against crop pathogens are toxic and have continued to pose increasing environmental threat. Organic and biological means and thorough integrated management efforts have been initiated by agricultural experts (Dilbo *et al*., 2015; Mahdizadehnaraghi *et al.*, 2015). The research for biocontrol agents as substitutes to these chemicals for control of crop diseases is also advancing (Ofodile *et al*., 2012). This paper reports the evaluation of *Trichoderma* spp. isolated from garlic farms in Ambo, Ethiopia, for their potential influence as biocontrol agents for white rot disease of garlic.

MATERIALS AND METHODS

Study Location

The experiments were carried out at the Mycology Department, Plant Protection Research Centre, Ambo, Ethiopia (Latitude 8^0 58’ 99”N).

Collection of infected seedlings of *Allium sativum*

Samples (30) of the infected seedlings of *Allium sativum* L. and bulbs showing the symptoms of white rot (rotten roots, bulb scales of garlic showed semi-watery decay, fluffy white growth around the base of the bulb and numerous small, spherical black bodies or sclerotia on mycelial mat by visual observation were randomly collected from garlic fields in Awaro, Al tuffa, Guder and Ambo, Ethiopia in February 2007. Soils were also collected from the infected fields. All samples were tagged with date and name of the area and substrates where they were collected as Awaro soil (AS), Al tuffa soil (ATS) and Guder soil (GS). The infected seedlings and bulb of *Allium*

sativum L. were conveyed to the Mycology Laboratory of the Plant Protection Research Centre (PPRC) Ambo, Ethiopia in labelled paper bags at room temperature and stored in the refrigerator at 4ºC, and relative humidity of 60^0C until use.

Isolation and Identification of *Sclerotium cepivorum* and *Trichoderma* species

Sclerotium cepivorum Berk and *Trichoderma* spp. used in the experiment were isolated from the collected infested soils and infected garlic roots and bulbs on potato dextrose agar (PDA) according to the method of Elad *et al*. (1981). The surfaces of the symptomatic tissues were disinfected with 70% ethanol for 1 min, rinsed 3 times in sterile water and dried with 5 layers of flame-sterilised filter papers (90 mm) and placed on PDA plates at pH 4.5 and incubated at 25ºC for five days for pathogen isolation from the roots and bulbs and 27ºC for seven days for the isolation from the soils. *S. cepivorum* that had grown on PDA were morphologically identified using colony characteristics, shape of mycelia, development of sclerotia and absence of spores. Diagnostic keys were used as supporting material for the identification of *Trichoderma* spp. (Barnett and Hunter, 1998; Gams and Bissett, 1998). Phylogenetic analysis was carried out on the isolates of *Trichoderma* spp. based on tef sequencing at Agricultural and Agri-Food Canada/Agriculture et Agroalimentaire, Canada.

Mycelia for DNA isolation were harvested from *Trichoderma* cultures and the genomic DNA was extracted using an E.Z.N.A. fungal DNA Kit (Omega Bio-Tek., Doraville, GA) following the manufacturer's protocol. Amplification and cycle sequencing reactions of DNA were performed on a GeneAmp 2700 thermocycler (Applied Biosystems, Foster City, CA). A region of nuclear rDNA, containing the ITS1 and ITS2 and the 5.8S rRNA gene, was amplified by PCR using the primer combinations NS5-fw (5'-TGG AAG TAA AAG TCG TAA CAA GG-3') and NS4-rev (5'-TCC TCC GCT TAT TGA TAT GC-3') [16] and the following parameters: 3 min initial denaturation at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 75 sec primer annealing at 58°C, 105 sec extension at 72°C, and a final extension period of 10 min at 72°C. A large 0.9-kb fragment comprising the 5' end of *tef1α* (eEF1a1) containing four introns was amplified by the primer pair tef71f (C AAA ATG GGT AAG GAG GAS AAG AC) and tef997R (CA GTA CCG GCR GCR ATR ATS AG) using the following 'touchdown' amplification protocol: 4 min initial denaturation at 94°C, 4 cycles each of 1 min at 94 °C, 90 sec at 60°C, and 90 sec at 72°C, followed by 17 cycles with the annealing temperature decreasing by 1˚C per cycle from 60˚C to 52˚C, followed by 14 cycles with annealing at 52˚C, and with a final extension period of 10 min at 72°C. Polymerase chain reactions were performed in 10 µL volumes as follows: 1.0 µL DNA, 1.0 µL 10x Titanium Taq buffer, 0.5 µL dNTP mix (2mM), 0.32 µL each primer (5mM), 6.76 µL water, 0.1 µL Titanium Taq Polymerase (Clontech, Mountain View, CA). Sequencing reactions were prepared using the ABI Prism® Big Dye™ Terminator reaction kit (v3.1 Applied Biosystems) in 5µL volume and 1/8 dilution using 5X Sequencing Buffer (Applied Biosystems). The PCR primers, used to amplify ITS, were also used for sequencing the ITS. The following interior primers were used in sequencing the tef: tef85f (AG GAC AAG ACT CAC ATC AAC G) and tef954r (AGT ACC AGT GAT CAT GTT CTT G) (Chaverri *et al*., 2003). The cycle sequencing reaction contained 1.0 µL (10-40 ng) PCR template, and employed the following amplification protocol: 25 cycles each of 30 sec denaturation at 96°C, 15 sec annealing at 50°C, and 4 min extension at 60°C.

Sequences were obtained using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Interaction between *Sclerotium cepivorum* and *Trichoderma* species

The evaluation of *Trichoderma* spp. as potential biocontrol agents of *S. cepivorum* was done according to *in vitro* dual culture method (Wellerm *et al*., 1985).

Mycoparasitism and antibiosis of *Trichoderma* spp. on *Sclerotium cepivorum*

Two species of *Trichoderma* were tested against *S. cepivorum* using the dual culture technique of Utkhede and Rahe (1980). Percentage of mycoparasitism by *Trichoderma* sp. was calculated using the formula below:

$$PMO\ \% = \frac{\text{Control Mean- Replicate Mean}}{\text{Control Mean}} \times 100$$

RESULTS

Sequencing the internal transcribed spacer regions (ITS1-2) of rDNA and the translation elongation factor 1-α (eEF1a1) AS1, ATS1, GS2 were identified as *Trichoderma tomentosum* occurring in the same clade in phylogenetic analyses although there are some sequence differences from the type of *T. tomentosum*. Phylogenetic significance of these differences is unknown. AS2 is close to *Hypocrea lixii* and, therefore, not referrable to *T. harzianum* s.s. which is a well-defined clonal species. GS1 corresponded to *afroharzianum* of the 'pseudoharzianum matrix' which appears to be another clonal species but differing significantly from *T. harzianum*. Figure 1 shows the tef sequence.

Results of mycoparasitism and antibiosis caused by *Trichoderma* spp. against *S. cepivorum* are shown in Table 1 and Figure 2. The species of *Trichoderma* completely suppresed the growth of *S. cepivorum* (Table 1). So *S. cepivorum* did not grow when inoculated after 48 hours of incubation of *Trichoderma* species. GS1 and AS1 growth covered the plates completely on the 5^{th} day of inoculation while the other isolates AS1, ATS and GS2 growth covered the plates on the 6^{th} day of inoculation. Meanwhile, in the control *S. cepivorum* growth covered the plates on the 7^{th} day of inoculation and started producing sclerotia on the 10^{th} day. *Trichoderma* species (AS1, AS2, ATS, GS1, and GS2) showed mycoparasitism against *S. cepivorum*. GS1 was a fast-growing isolate that completely covered the colony of the pathogen on the 7^{th} day. AS2 also completely overlapped the colony of *S. cepivorum* after the 12^{th} day but GS2 did not show any further parasitism after the 10^{th} day. AS1and ATS also stopped parasitising the pathogen after the 12^{th} day (Table 1). The mean overlapping percentages of AS1 and AS2 were above 90% while those of ATS and GS2 were below 90% by the 12^{th} day of incubation. Mycoparastism overlapping (%) of *S. cepivorum* after days of incubation was significantly different across the days at $p<0.05$. Likewise, the antagonists were significantly different from one another ($p<0.05$) and the effect of the interaction between *Trichoderma* spp and *S. cepivorum* was significant at $p<0.05$.

The mean zone of inhibition against *S. cepivorum* was 3.75 mm, 3.5 mm and 2.2 mm in diameter for AS1 AS2 and GS2, respectively. ATS and GS1 did not exhibit any inhibition zones against the pathogen (Fig 2).

Zones of inhibition of *Trichoderma* species against *S. cepivorum* after 12 days of incubation were all significantly different among the antagonists ($p<0.05$). AS1 was significantly stronger than AS2 and AS2 was significantly stronger than GS2 at $p < 0.05$.

DISCUSSION

GS1 identity corresponded to afroharzianum of the 'pseudoharzianum matrix' as defined by Druzhinina *et al*. (2010). *Trichoderma* species suppressed the growth of *S. cepivorum* Berk on PDA and masked their presence probably because the antagonist produced some enzymes, which were suppressive to the growth of the pathogen. This suggests that *Trichoderma tomentosum* and *T.* afro*harzianum* can effectively control *S. cepivorum* that causes white rot of garlic. According to Howell (2003), chitinases and/or glucanases enzymes produced by biocontrol agents are responsible for suppression of plant pathogens. Metcalf and Wilson (2001) reported that *T. koningii* (TrS) colonised onion roots infected with *S. cepivorum* through hyphal penetration into the infected epidermal and cortical tissues of the root to destroy *S. cepivorum* hyphae with little or no damage to uninfected tissues. This biocontrol phenomenon could be ascribed to production of endo- and exo-chitanase by *T. koningii.*

Many fungal antagonists have expressed ability to control many plant disease-causing fungi (Francisco *et al*., 2011; Kakvan *et al*., 2013; Naraghi *et al*., 2013; Blaszczyk *et al.*, 2014; Vinale *et al*., 2014). Some instances of successful biocontrol with *Trichoderma* have been ascribed to mechanism of mycoparasitism and /or antibiosis (Howell *et al*. 1993), competition and rhizosphere competence (Metcalf and Wilson, 2001), induction of defense response in plants, metabolism of germination stimulants released by seed (Howell, 2002) and adjunct mechanism which includes other characteristics with host plants which contribute to disease resistance and /or tolerance. Abd-El-Moity and Shatla as reported by Dilbo *et al.* (2015) showed that *T. harzianum* decreased the infection by *S. cepivorum* from 29% to 84% in greenhouse trials. Mycoparasitism and antibiosis were also implicated in the present work as shown by the overlapping of the pathogen's colony by the five isolates of *Trichoderma* and the formation of inhibition zones. Seven *Trichoderma* spp were tested for *in vitro* and *in vivo* anti-fungal activities against white rot of garlic and *T. hamantum, T. harzianum, T. oblongisporum* and *T. viride* had high percentage zones of inhibition of between 51.7 and 59.3% (Thoudam and Dutta, 2014). Ararsa and Thangavel (2013) reported the bio-control potential of seven *Trichoderma* species viz., *T. asperillum, T. atroviride, T. hamatum, T. harzianum, T. longibrachiatum, T. oblongisporum and T. viride* which were evaluated by *in-vitro* and *in vivo* experiments for their antagonistic and inhibition potential against garlic white rot disease caused by *S. cepivorum.* The species of *Trichoderma* showed 43.9 to 59.3% antagonism against the pathogen. *T. hamantum* had the highest inhibition effect (59.3%) followed by *T. harzianum* (53.3%), *T. oblongisporum* (52.7%), *T. viride* (51.8%), *T. asperillum* (50.2%), *T. longibrachiatum* (47.2%) and *T. atroviride* (43.9%).

Production of different enzymes have been implicated in the suppressive effect of *Trichoderma* spp against some species of fungal pathogens (De La Cruz *et al*.,

1992; Mohamed *et al*., 2010). De La Cruz *et al*. (1992) reported that chitinases appear to play nutritional role related to mycoparasitism exhibited by *Trichoderma*. *Trichoderma* spp. used in this study were collected from garlic bulbs, roots and soil of the garlic fields in Ambo. The best method of obtaining a potential biocontrol agent is to collect from the fields and soil where it would be used for disease-control and where it is thriving under moisture, temperature and available nutrients as in the natural environment (Metcalf and Wilson, 2001; Sharifi *et al*., 2010).

CONSCLUSION

The results obtained in this study showed that the five isolates of *Trichoderma* spp. (AS1, ATS1, GR and GS2) were. *T. tomentosum.* AS2 was a telemorph of *T. hazianum, Hypocrea lixii*, while GS1 was *T. afrohazianum.* These *Trichoderma* species can have significant antagonistic activity against garlic white rot pathogen. The antibiotic and enzymatic actions were implicated in activity of the biocontrol agents against white rot disease of garlic. Present work was laboratory experiment but further studies on the *in vivo* screening of the *Trichoderma* isolates against the white rot fungi in green house or field experiment is needed.

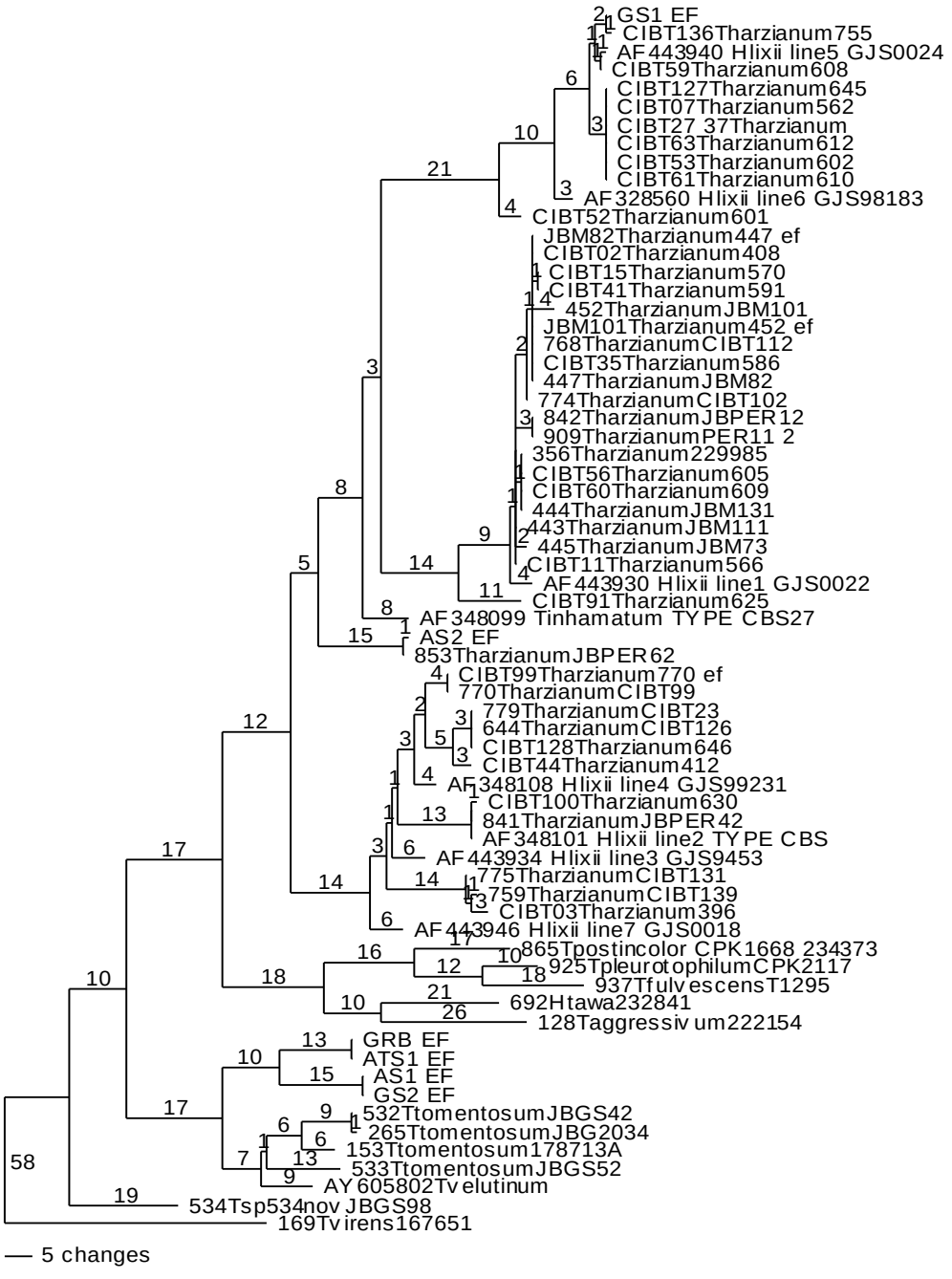

Fig. 1: Phylogenetic tree of the Tef sequence of *Trichoderma* species

Table 1: Interaction effect of *Trichoderma* species and *S. cepivorum* after days of incubation

DAY	ANTAGONIST (%)				
	AS1	AS2	ATS	GS1	GS2
4	3.03±0.66*	8.10±0.16*	6.73±0.17*	19.13±0.10*	4.38±0.29*
5	38.30±0.36*	25.50±0.08*	42.28±0.38*	80.53±0.33*	36.25±0.31*
6	64.03±0.10*	49.78±0.62*	63.78±0.17*	90.98±0.26*	59.03±0.82*
7	64.85±0.45*	53.50±0.42*	65.00±0.82*	100.00±0.0*	64.05±0.06*
8	71.43±0.43*	58.00±0.33*	68.40±0.44*	100.00±0.0*	66.38±0.48*
9	76.53±0.34*	65.63±0.43*	70.63±6.53*	100.00±0.0*	69.50±0.42*
10	76.43±0.57*	68.83±0.15*	75.00±0.82*	100.00±0.0*	74.33±3.28*
11	89.10±0.41*	72.50±0.41*	77.65±0.87*	100.00±0.0*	77.18±0.17*
12	90.08±0.19*	94.08±2.44*	81.48±1.74*	100.00±0.0*	77.13±0.78*

n=5 superscripts are significantly mycoparasitic at $p<0.05$

Key: AS1 = *Trichoderma* isolate from Awaro soil, AS2 = *Trichoderma* isolate from Awaro soil, ATS = *Trichoderma* isolate from Altufa soil, GS1=*Trichoderma* isolate from Guder soil, GS2 = *Trichoderma* isolate from Guder soil

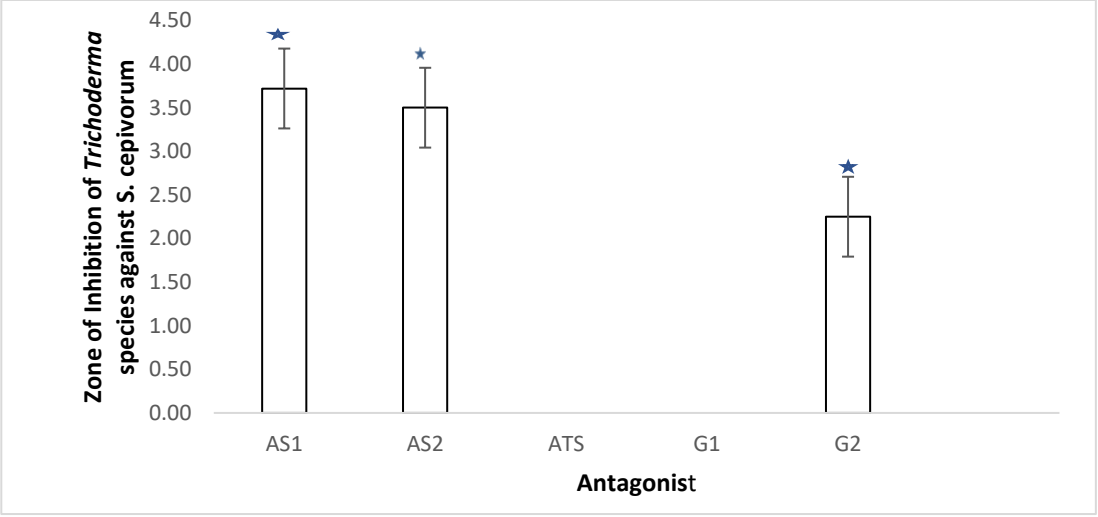

Figure 2: Zone of inhibition of *Trichoderma* species against *S. cepivorum* after days of incubation

Key: AS1 = *Trichoderma* isolate from Awaro soil, AS2 = *Trichoderma* isolate from Awaro soil, ATS = *Trichoderma* isolate from Altufa soil, GS1 = *Trichoderma* isolate from Guder soil, GS2 = *Trichoderma* isolate from Guder soil

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