Evaluation of native *Trichoderma* isolates for the management of sugarcane smut (*Ustilago scitaminea*) in sugar plantations of Ethiopia

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Samuel Tegene¹, Mashilla Dejene², Habtamu Terefe²*, Girma Tegegn³, Esayas Tena⁴ and Amare Ayalew⁵

**Abstract:** Biological control of sugarcane smut (*Ustilago scitaminea* Syd.) using *Trichoderma* isolates is one of the untapped potentials in Ethiopian sugarcane plantations. To this end, this study was carried out with the objective to identify potential native *Trichoderma* isolates for the management of sugarcane smut. A total of 120 soil samples were collected from Kesem, Metehara, and Wonji sugarcane plantation fields. Consequently, 21 *Trichoderma* isolates were recovered from the samples, and *in-vitro* antagonist study of *Trichoderma* isolates against *U. scitaminea* was done using dual culture technique on fresh PDA. Among the native isolates, 12 of them revealed a pathogen suppression potential of >90% at 7 days after inoculation and most of them had higher pathogen suppression than the commercial isolate. However, five of the isolates were selected as potential candidates based on distinct suppression features, growth rate, and antagonistic performance. Identification of the five effective *Trichoderma* isolates (WI4-27, MKI2-k17, MI3-m33, WI12-25, and MKI3) was done based on macroscopic and

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Samuel Tegene is the research staff of the Ethiopian Sugar Corporation, Ethiopia. He has years of experience in various researches related to crop protection studies with regard to the biological control of diseases, weeds, and insects. Mr. Samuel Tegene has designed and executed many epidemiological studies under both controlled and natural conditions. He is involved in many national and international projects focusing on crop protection and climate-smart crop production systems, along with different teams. For instance, he has been working on an integrated striga management project implemented in Ethiopia funded by Melinda and Gates foundation and he has been serving as a focal person of the project at Eastern Hararghe; and he has been an innovation team member of CASCAPE project implemented by Haramaya University, and funded by The Netherlands government accomplishing a multidisciplinary research that focused on identifying problems of farmers, prioritization, and on scaling up of technologies generated through the project.

**PUBLIC INTEREST STATEMENT**

Sugarcane smut is a great threat to sugarcane production and productivity worldwide as well as in Ethiopia. The disease is reported from all Sugarcane Plantations of Ethiopia and is causing severe yield losses that reached 100% during severe infestations. However, lack of expertise and management options enhanced the rapid distribution of the disease to many parts of the country, which calls for the development of sustainable and environmentally friendly management schemes. Previous management practices focusing on fungicides were repeatedly reported to be inefficient. Therefore, native *Trichoderma* isolates were explored and tested for the management of smut pathogen, and the study revealed that *Trichoderma* isolates have a remarkable role in solving the problem. Moreover, the study has an impact on reducing the fungicide load that could enter the environment. Thus, the results could enable us to identify one option of management practices in designing effective and integrated smut management strategies.
microscopic characteristics. Thus, the isolates were identified as *T. harzianum*, *T. asperellum*, *T. viride*, *T. viride*, and *T. harzianum*, respectively. Accordingly, these isolates could be used as one component of integrated smut management practices once a study on compatibility with fungicides, *in-vivo* efficacy test against smut, and substrate selection for mass production of the isolates were commenced.

**Subjects:** Microbiology; Mycology

**Keywords:** antagonist; biological control; morphological features; suppression; *Trichoderma* isolate

### 1. Introduction

Sugarcane smut (*Ustilago scitaminea* Syd.) was identified as a priority threat to the sugar industry in Ethiopia, with a loss of 19% to 43% in cane and 29.5% to 42.8% in sugar yield (Firehun et al., 2009). A recent survey made on sugarcane smut distribution and intensity across plantations of the Ethiopian Sugar Estates revealed that the disease was widely distributed with different levels of intensity. Most of the fields were infested by smut with their incidence greater than the threshold level of 5% (Samuel et al., 2020). Management of sugarcane smut in Ethiopia used to involve cultural practices, use of resistant varieties, fungicide sett treatments, and the integrated use of different practices (Firehun et al., 2009). Besides the utilization of the above management options, this disease remained a menace to sugarcane production in the country. This is due to many factors, among which forced the planting of susceptible varieties, such as NCO334 and N14, because of good agronomic and varietal attributes (high yield, wider adaptation, tolerance to moisture stress, waterlogging, and good ratooning capacity).

Moreover, management of the sugarcane smut with fungicides has been found to be ineffective (Sundravadana et al., 2011; Wada & Anaso, 2016) mainly because of the formidable rind of setts, fibrous nature of nodes, presence of water in setts, and deep-seated buds in stubbles (ratoon) do not allow the fungicides to reach the site of infection to the desired concentration sufficient to suppress the embedded pathogen. Furthermore, sugarcane is a perennial plant with a long crop cycle of ratooning that can last for more than 10 years, which demands sustainable management practices that persist for longer periods without losing efficacy (Meyer et al., 2011). This quality of management practice is lacking in the country.

One of the most important environmentally safe, economically feasible, and sustainable options for the management of phytopathogens is the use of fungal biological control agents (Woo et al., 2006). In this regard, *Trichoderma* species have been successfully used as mycofungicides because of many essential features. Those features include fast-growth (Babu & Pallavi, 2013), high reproductive capacity (Kuzmanovska et al., 2018), inhibition of a broad spectrum of fungal pathogens (Manandhar et al., 2019), and have a diversity of suppression mechanisms (Woo et al., 2014). Suppression mechanisms were capacitated by being excellent competitors in the rhizosphere, modifying the rhizosphere, tolerant to soil-applied agrochemicals, survival under unfavorable conditions, efficient in utilizing any available soil nutrients, having strong aggressiveness against phytopathogenic fungi, and also promoting plant growth (Vinale et al., 2008).

Previous studies have documented that *Trichoderma* spp. are antagonistic to *U. scitaminea* and other sugarcane pathogens. For example, Singh et al. (2014) and Juma et al. (2018) demonstrated that *T. harzianum* and *T. viride* exhibited high suppression of mycelial growth and teliospore germination of *U. scitaminea* under-controlled conditions. Similarly, a field trial on the efficacy of *T. viride* as sett treatment revealed a 35 to 55% reduction in the incidence of smut as compared to the untreated check (Lal et al., 2009). Moreover, this study reported that treatment with *T. viride* improved germination (6%) increased number of millable canes (27%), and cane yield (38%) in plant crop and sprouting of clumps (13%), number of millable canes (51%) and yield (49%) in ratoon crop (Lal et al., 2009).
Despite the outstanding attributes of *Trichoderma* fungi in smut and other sugarcane disease management and sugarcane production, little effort has been made to explore and evaluate native *Trichoderma* isolates in smut management in the Ethiopian Sugar Estates. Recently, several commercial formulations of *Trichoderma* isolates exist in the world market that is easy to test and apply for the sugarcane smut disease. However, the demand for isolation of native *Trichoderma* isolates arises from better adaptation and colonization of the local environment by the native isolates (Chen et al., 2019; Ommati & Zaker, 2012). Moreover, it is also noted that all the *Trichoderma* isolates were not equally effective in the suppression of pathogens at different ecologies (Sundravadana et al., 2011). Consequently, isolation and identification of effective local antagonist isolate(s) are desirable for the successful management of a particular plant pathogen or disease. Therefore, the study was carried out with the objective to identify potential native *Trichoderma* isolates for the management of sugarcane smut in Sugar Plantations of Ethiopia.

2. Materials and methods

2.1. Description of the study areas

Soil samples were collected from three Ethiopian Sugar Estates (Figure 1), namely Wonji, Metehara, and Kesem. Wonji Sugar Estate is located at Boset District in Eastern Shoa. The Sugar Estate lies between 1223 and 1553 m a.s.l. with latitude and longitude that ranged from 8°23′ to 8°32′ N and 39°25′ to 39°33′ E, respectively. Metehara Sugar Estate is located in Fentale District, Eastern Shoa, within the Upper Awash Rift Valley. This Sugar Estate is located at altitude, latitude, and longitude that ranged from 946 to 1000 m a.s.l., 8°46′ to 8°52′ N and 39°46′ to 39°60′ E, respectively. Kesem Sugar Estate is found in the Dulecha District of Afar National Regional State. Kesem Sugar Estate is also located in the Rift Valley region of Ethiopia at the latitude of 9°08′ to 9°11′ N and longitude of 39°57′ to 40°03′ E with an altitude of 754–800 m a.s.l.

2.2. Soil samples collection

A total of 120 soil samples were collected using auger from Wonji (45 samples), Metehara (40 samples), and Kesem (35 samples) sugar estates during the 2018/2019 cropping year. Soil samples were taken from a depth of 30 cm in the upper soil profile at the root zone of sugarcane plants. The collection was made from five points from a given field in “X” fashion. Then, the composite and well-mixed soil samples from all sampled fields were brought to the Plant Pathology Laboratory of the Ethiopian Sugar Corporation Research Center. During soil sample collection, diverse sugarcane age, crop cycles, soil texture, and crop variety were visited intentionally to get widely diverse *Trichoderma* isolates.

2.3. Media preparation, isolation and maintenance of *Trichoderma* isolates

The standard laboratory techniques for the preparation of media, sterilization, isolation, and maintenance of fungal cultures were followed as described by Dhingra and Sinclair (1995) and Aneja (2005). The soil samples were air-dried at room temperature and ground into fine particles (1 mm) before isolation. Isolation of *Trichoderma* from the soil was done using serial dilutions and agar plating methods (Aneja, 2005). Briefly, 10 g of soil sample was suspended in 90 mL sterile-distilled water to make a microbial suspension. Three-fold serial dilutions: 10⁻², 10⁻³, and 10⁻⁴ were made by pipetting of 1 mL soil suspension into 9 mL sterile-distilled water. Finally, 1 mL of each various dilutions was added to sterile Petri dishes (triplicate for each dilution) containing 20–25 mL of the sterile, cool, and molten *Trichoderma* selective agar (TSA) medium.

The TSA medium was prepared based on procedures developed by Elad and Chet (1983): broad-spectrum antibacterial, chloramphenicol, and the fungicide benomyl were added after cooling the medium to 40°C at 1 g L⁻¹ and 2 mg L⁻¹ to suppress bacterial and fungal contaminants, respectively. Next, the prepared soil dilutions were streaked onto specific agar plates. The Petri plates were labeled and incubated in a growth chamber at 30°C for 10 days. The numbers of distinct colonies of *Trichoderma* isolate on TSA medium were counted and purified on PDA medium based on morphological variations, such as colony color, colony edge, colony diameter, and mycelial
growth habit as suggested by Kubicek and Harman (2002) and Williams et al. (2003). Purified cultures of *Trichoderma* isolates were maintained on PDA slants where a drop of glycerin was added to each slant and stored in the refrigerator at 4°C for further study (Amare, 2007).

2.4. Cultural and morphological characterization of *Trichoderma* isolates

Cultural and morphological observations of the colony of *Trichoderma* isolates were made based on growth on PDA at 30°C for 10 days. Colony diameters of the isolates were measured at three, seven, and 10 days after inoculation (DAI). Colony colors were determined visually by comparing and contrasting with color charts of standard color description (Ridgway, 1912). Moreover, colony edge, mycelial growth habit, colony cover (cm² day⁻¹), and other features that make isolates distinct were also studied. *Trichoderma* isolates showing nearly similar morphological characteristics were grouped under one isolate. And, 21 distinct *Trichoderma* isolates were recovered from the soil samples and upgraded to the evaluation study.
2.5. Collection, isolation and maintenance of teliospores of \textit{U. scitaminea}

Sugarcane smut spores were collected from commercial fields of Metehara, Wonji, and Kesem Sugar Estate following procedures of Tokeshi (1980). Accordingly, new smut whips were harvested with appropriate care to control spore dissemination in the field by covering with a plastic sheet, and the remaining stool was uprooted and buried into a dip pit. The harvested whips were taken to a dark processing room whereby the leaves and leaf sheaths were removed and exposing the whips for drying. The basal part of the whips, where the spores were not mature, was eliminated. Then, the whips were spread on a clean canvas with proper ventilation to allow proper drying. After 12–16 h, the dried spores were collected and sifted in a sieve of 100 meshes and stored in paper bags and placed in a desiccator at 4°C in a refrigerator.

Fresh PDA medium was used for the isolation of \textit{U. scitaminea}, which consisted of freshly sliced potato tubers (200 g), dextrose (20 g), agar (15 g), and 1000 mL of distilled sterile water as suggested by Amare (2007). Briefly, 200 g of potato slices were boiled in 500 mL distilled sterile water for 20 min and filtered through a muslin cloth. Agar powder was also melted in 500 mL distilled sterile water and mixed with dextrose. The mix was then added to filtrate of potato solution to make the volume 1000 mL and was autoclaved at 121°C and 15 psi for 20 min. The resultant substrate was then aseptically poured into sterile Petri dishes.

Smut spores were prepared according to procedures followed by Rocío et al. (2010) before inoculating onto fresh PDA. Briefly, teliospores of smut were added to 1 mL of streptomycin sulfate solution (0.5 g L\textsuperscript{−1}). After 10 min, the solution was stirred for 2 min to remove unnecessary decontaminants. The supernatant of the solution was discarded and spores were washed 3 times in the streptomycin sulfate solution, after which serial dilutions (10\textsuperscript{−1} to 10\textsuperscript{−4} spores mL\textsuperscript{−1}) were made using sterile-distilled water. Each serial dilution (100 µL) was used to inoculate fresh PDA. Sterile glass bead was placed on the agar surface and gently swirled to evenly spread the teliospore solution on the solidified surface of the PDA medium. Culture plates were properly packed using sterile Parafilm and maintained at 30°C in the incubator. Fungal growth was checked 18 h after incubation using a dissecting microscope and was allowed to grow for 7 days.

2.6. Evaluation of antagonistic potential of \textit{Trichoderma} isolates against \textit{U. scitaminea}

The dual culture method was utilized to evaluate the antagonistic potential of \textit{Trichoderma} isolates against \textit{U. scitaminea} (Leta & Selvaraj, 2013). Seven-days-old culture of \textit{U. scitaminea} was inoculated onto PDA medium, 12 h prior to inoculation of \textit{Trichoderma} isolates, to establish the growth of the pathogen. A 5 mm diameter mycelial disc from the periphery of the 7 days-old culture of \textit{Trichoderma isolate} was placed on the opposite side of \textit{U. scitaminea}, at equidistance from the center, on the same medium. In this trial, 21 native \textit{Trichoderma} isolates and one commercial formulation of \textit{Trichoderma} (Ecosom-TH) containing \textit{T. harzianum} imported from Agri-Life, India. The commercial isolate was imported with standard and legal procedures.

A total of 23 treatments including a pathogen control was arranged in a completely randomized design with three replications. The commercial isolate treated plates and untreated (the only pathogen inoculated) plates were used as a positive and negative standard check, respectively. All plates were allowed to grow at 30°C in the incubator for 10 days. The experiment was repeated twice. The growth rate of \textit{Trichoderma} isolates in dual culture was determined based on the radial growth of the isolates in two consecutive measurements following the formula used by Sharma and Singh (2014) and Sekhar et al. (2017):

\[ GR = \frac{R_s - R_f}{A_s - A_f} \]

where \( GR \) = growth rate of the isolate in cm day\textsuperscript{−1}; \( R_s \) = radial growth in the second measurement, \( R_f \) = radial growth in the first measurement, \( A_s \) = age of the colony at second measurement in days and \( A_f \) = age of the colony at the first measurement in days.

The radial growth of both \textit{U. scitaminea} and \textit{Trichoderma} isolates was measured at three, five, and 7 days after inoculation. The percentage of inhibition was calculated using procedures of Rita Tegene et al., Cogent Food & Agriculture (2021), 7: 1872853 https://doi.org/10.1080/23311932.2021.1872853
and Tricita (2004) and Imtiaj and Lee (2008). Best performing native Trichoderma isolates in the antagonistic test were followed for macroscopic and microscopic features to estimate their identity. Conventional techniques for estimation of the identity of Trichoderma colonies to the genus or species level were done based on the macroscopic characteristics (color, odor, physiological characters, and growth rate) and microscopic characteristics (mycelium, conidia, conidio- phore, phialides, and chlamydospores) (Kumar & Sharma, 2016).

Percentage inhibition (%) = \( \frac{C - T}{C} \times 100 \); where \( C \) = radial colony size of the pathogen in the control plates and \( T \) = radial colony size of the pathogen in the experimental plate.

2.7. Data analysis
Data on mean radial growth and mean percentage inhibition was subjected to analysis of variance (ANOVA) using SAS version 9.1 (SAS, 2014). The trial was repeated 2 times and combined data of the two trials were used for the analysis as two results were observed to be homogenous as checked through homogeneity test of goodness of fit (Gomez & Gomez, 1984). The growth rate of Trichoderma isolates and radial growth of U. scitaminea were analyzed after arcsine transformation of the values (Gomez & Gomez, 1984). The least significant difference (LSD) test was employed to make multiple comparisons among treatment means of radial growth of U. scitaminea and suppression by Trichoderma isolates (SAS, 2014).

3. Results

3.1. Prevalence, cultural and morphological variability of native Trichoderma isolates
In the study, 21 distinct Trichoderma isolates were extracted from the soil samples (Table 1). Among these, nearly one-third of the isolates was from Wonji and the other two-thirds of the isolates were from Metehara and Kesem Sugar Estates. Fields of sugarcane with ages ranging between 3 and 18 months were visited during sample collection. Of which, most (85.71%) of the Trichoderma isolates were found in sugarcane plants with their ages greater than 5 months (Table 1). The remaining (14.29%) isolates were recovered from sugarcane fields with their ages less than 5 months, irrespective of crop cycles (Table 1).

The prevalence of Trichoderma isolates also varied among crop cycles. Most (85.71%) of the Trichoderma isolates were obtained from soils of ratoon sugarcane fields, while the remaining (14.29%) isolates were found from plant cane fields. Of the 21 isolates explored, more than 90% of the isolates were recorded either from light or intermediate textured soils, while 10% of the isolates were recovered from heavy soils (Table 1). Isolates were found invariably with different varieties that were predominant in the studied areas despite their reaction to smut [susceptible (NCO 334), moderately susceptible (Mexico, N14) or resistant (B52, B59, Cuba, and B41)] (Table 1).

Purified colonies of Trichoderma isolates showed variable and distinct macroscopic characteristics, such as colony color, mycelium growth habit, colony diameter, rate of growth, colony edge, and some other features (Table 2). The analysis of variance revealed significant (P ≤ 0.05) variation among the isolates in their colony diameter and rate of growth at 7 DAI. The highest (10.33 cm) mean radial growth was recorded on isolate M14, followed by isolates M13-m33 (10.3 cm) and K12 (10.27 cm) colony diameter at 7 DAI. The shortest (6.47 cm) mean colony diameter was recorded on isolates MK12-m9 proceeded by the commercial isolate (7.07 cm) at 7 DAI. Similarly, the highest (0.75 cm day\(^{-1}\)) growth rate was noted on WI12-25, followed by WI4-24 (0.74 cm day\(^{-1}\)), while the least was computed from the commercial isolate which attained a mean radial growth rate of 0.18 cm day\(^{-1}\) at 7 DAI. All the native isolates had a faster growth rate as compared to the commercial isolate. The average growth rate that ranged between 0.18 cm day\(^{-1}\) for commercial isolate and 0.75 cm day\(^{-1}\) for WI12-25 was recorded during the study (Table 2 and 3).

Moreover, the isolates were characterized by three alternative colony edges. About 27.27%, 36.36%, and 36.36% of the isolates had smooth, toothed, and wavy colony edges, respectively.
| Isolate code | Sugar Estate (collection site) | Age of sugarcane (month) | Sugarcane variety | Crop cycle (ratoon frequency) | Soil type (textural group) |
|--------------|-------------------------------|--------------------------|-------------------|-----------------------------|--------------------------|
| WI3-4        | WSE                           | 8–12                     | B52 and N14       | 1st, 2nd ratoon             | Intermediate, light      |
| WI3-9        | WSE                           | 5–8                      | B41 and Cuba      | 1st and 2nd ratoon          | Heavy, intermediate      |
| WI4-24       | WSE                           | 3–7                      | NCO334 and C90    | Plant cane and 1st ratoon   | Heavy, intermediate      |
| WI4-27       | WSE                           | 10–13                    | NCO334 and B52    | 1st—3rd ratoon              | Light, intermediate      |
| WI10         | WSE                           | 11–14                    | NCO334 and N14    | 1st and 2nd ratoon          | Light, intermediate      |
| WI12-24      | WSE                           | 11–18                    | NCO334 and B59    | 1st ratoon                  | Light, intermediate      |
| WI12-25      | WSE                           | 7–8                      | B52, B59 and N14  | 1st—2nd ratoon              | Light                   |
| WI13         | WSE                           | 7–10                     | B41 and Cuba      | Plant cane                  | Light, intermediate      |
| MKI1- m26    | MHSE                          | 4–17                     | NCO334 and B52    | 2nd—4th ratoon              | Light, heavy             |
| MKI1- k21    | KSE                           | 8–12                     | NCO334            | 1st—3rd ratoon              | Light, intermediate      |
| MKI2- k9     | KSE                           | 5–9                      | NCO334 and B52    | 4th ratoon                  | Light                   |
| MKI2- k17    | KSE                           | 4–12                     | Mexico and B52    | 2nd ratoon                  | Light                   |
| MKI2- m7     | MHSE                          | 6–12                     | B52 and Mexico    | 1st—2nd ratoon              | Heavy, intermediate      |
| MKI2- m9     | MHSE                          | 8–17                     | B52 and Mexico    | 1st—4th ratoon              | Heavy, intermediate      |
| MKI3         | MHSE                          | 5–7                      | B52 and NCO334    | 1st—3rd ratoon              | Light, intermediate      |
| MKI4         | MHSE, KSE                     | 6–8                      | B52 and NCO334    | 4th—6th ratoon              | Intermediate, light      |
| MI3- m14     | MHSE                          | 8–10                     | B52 and NCO334    | 2nd—6th ratoon              | Intermediate, light      |
| MI3- m33     | MHSE                          | 5–9                      | NCO334 and B52    | Plant cane                  | Light, intermediate      |
| MI4          | MHSE                          | 6–9                      | B52 and NO334     | 1st—2nd ratoon              | Intermediate, light      |
| MKI5         | MHSE, KSE                     | 5–8                      | B41 and B52       | 1st—3rd ratoon              | Light, intermediate      |
| KI2          | KSE                           | 5–7                      | NCO334 and Mexico | 1st—4th ratoon              | Light, intermediate      |

*WI = Wonji isolate; MKI = Metehara and Kesem isolate; MI = Metehara isolate and KI = Kesem isolate. *WSE = Wonji Sugar Estate; MHSE = Metehara Sugar Estate and KSE = Kesem Sugar Estate. *Crop cycle refers to the sugarcane plant as newly planted (plant cane) or emerged after harvest (1st ratoon, if it was grown after 1st ratoon was harvested, 2nd ratoon, if it was grown after the 2nd ratoon was harvested; and so on). *Soil type is classified based on rough textural classification, i.e., light, intermediate and heavy referring to predominantly sandy, sand and clay fifty-fifty and predominantly clay.
Table 2. Cultural and morphological characteristics, and mean colony diameter of indigenous *Trichoderma* isolates on PDA medium at 7 days after inoculation, which was isolated from Sugarcane Plantations of Ethiopia, during the 2018/19 cropping year.

| Isolate code a | Colony diameter (cm) | Growth rate (cm day\(^{-1}\)) b | Colony color b | Colony edge | Colony growth habit | Other distinct features of the isolate |
|---------------|---------------------|-------------------------------|---------------|-------------|-------------------|--------------------------------------|
| WI3-4         | 8.83 cdef           | 0.59 (0.56)cdef              | Warbler green | Smooth     | Tufted hairy     | Small patches of green pigmented mycelium |
| WI3-9         | 9.33 bcde           | 0.64 (0.60)bcde              | Hay’s green   | Toothed    | Hairy             | Very small patches of green pigmented mycelium |
| WI4-24        | 9.50 bcd            | 0.74 (0.69)c                  | Roman green   | Smooth     | Cottony          | Small patches of white mycelium sometimes with green liquid |
| WI4-27        | 9.17 cdef           | 0.45 (0.44)cdgh              | Scheele’s green | Wavy     | Tufted hairy     | One grey hairy tipped mycelium encircled at the center |
| WI10          | 9.00 cdef           | 0.64 (0.60)bcde              | Peacock green | Wavy     | Cottony          | White tipped mycelium at the center of the culture |
| WI12-24       | 9.43 bcde           | 0.49 (0.47)cd                      | Cedar green   | Toothed    | Hairy             | Lots of very small scattered patches of green pigments |
| WI12-25       | 9.57 bc             | 0.75 (0.69)c                  | Light Helibore green | Wavy     | Cottony          | Greyish mycelium appear irregularly on green colony |
| WI13          | 7.42 h              | 0.32 (0.31)h                  | Callistic green | Wavy     | Hairy             | Scattered colony growth |
| MKI1-m26      | 7.03 h              | 0.29 (0.29)h                  | Diamine green | Smooth     | Tufted hairy     | Patches of white tipped mycelium appear irregularly |
| MKI1-k21      | 8.67 g              | 0.37 (0.36)g                  | Grassly green | Wavy     | Hairy             | Deep green tipped mycelium appears in concentric manner |
| MKI2-k9       | 9.17 cdef           | 0.53 (0.51)cdef              | Helibore green | Toothed    | Cottony          | Scattered white tipped mycelium and irregular green pigment |
| MKI2-k17      | 9.83 cd             | 0.60 (0.57)cde                | Light Bice-Bice green | Wavy     | Hairy             | Glassy appearance of mycelium |
| MKI2-m7       | 9.67 bc             | 0.53 (0.51)cdef              | Cedar green   | Smooth     | Hairy             | Scattered white tipped mycelium and irregular green pigment |
| MKI2-m9       | 6.47 i              | 0.19 (0.19)i                  | Dark Viridian green | Toothed    | Cottony          | Scattered white tipped mycelium |
| MKI3          | 9.83 db             | 0.49 (0.47)cde                | Hay’s Green   | Wavy     | Cottony          | Scattered white patches on the colony |
Table 2. Continued

| Isolate code | Colony diameter (cm) | Growth rate (cm day\(^{-1}\)) | Colony color | Colony edge | Colony growth habit | Other distinct features of the isolate |
|--------------|----------------------|-------------------------------|--------------|-------------|--------------------|--------------------------------------|
| MK14         | 7.13 \(^{h}\)        | 0.41(0.40) \(^{ehg}\)       | Olive green  | Toothed     | Hairy              | Intermediate scattering of deep green mycelium |
| MI3-m14      | 8.83 \(^{e}\)         | 0.45(0.44) \(^{e}\)         | Parrot green | Wavy        | Hairy              | Sprawling type of mycelium growth          |
| MI3-m33      | 10.30 \(^{p}\)        | 0.68(0.64) \(^{e}\)         | Cossack green| Toothed     | Cottony            | White tipped mycelium irregularly         |
| MI4          | 10.33 \(^{p}\)        | 0.51(0.49) \(^{ef}\)       | Light elm green| Wavy       | Hairy              | Concentric greyish tipped mycelium        |
| MI5          | 8.50 \(^{g}\)         | 0.42(0.41) \(^{ef}\)       | Cedar green  | Toothed     | Tufted hairy       | Scattered patches of mycelium            |
| KI2          | 10.27 \(^{p}\)        | 0.55(0.53) \(^{h}\)        | Grass green  | Smooth      | Hairy              | Sprawling type of mycelium growth         |
| CT           | 7.07 \(^{h}\)         | 0.18(0.18) \(^{j}\)        | Dark viridian green| Smooth | Tufted hairy      | Yellowish tipped mycelium                |
| LSD (0.05)   | 0.53                 | 0.13                          |              |             |                    |                                      |
| CV (%)       | 3.59                 | 16.04(8.35)                  |              |             |                    |                                      |

\(^{a}\)WI = Wonji isolate; MKI = Metehara and Kesem isolate; MI = Metehara isolate and IK = Kesem isolate. \(^{b}\) Colony color was determined based on color manual of Ridgway (1912). LSD = Least significant difference at 0.05 probability level. CV = Coefficient of variation. Means followed by different letter(s) within the same column are significantly different at P = 0.05. Figures in parenthesis refer to arcsine transformed values of each respective growth rate raw data.
Mycelial growth habit and colony cover greatly varied among the isolates studied. Mycelial growth habit varied within cottony and hairy types in which most (72.73%) of the isolates had hairy mycelial growth, while 27.27% of the isolates revealed cottony types of mycelial growth (Table 2).

3.2. Antagonistic potential and identity of native Trichoderma isolates
The mean colony radial growth and percentage inhibition of pathogen growth due to Trichoderma isolate antagonistic characteristic at 7 and 10 DAI are presented in Table 3. Radial growth of the pathogen in the dual culture was strongly influenced and showed significant (P < 0.05) variation among the isolates tested in the antagonism evaluation both at 7 and 10 DAI. The pathogen attained the highest radial growth of 3.83 cm in the untreated plate (negative control), followed by MKI2-m9 (2.72 cm) inoculated plates. Conversely, the lowest (0.63 cm) radial growth of the pathogen was recorded from the WI12-25 isolate dually inoculated plate at 7 DAI. Similarly, the lowest (0.08 cm) growth of smut pathogen was observed on plates inoculated with isolates MKI3 and MKI2-k17 along with the pathogen at 10 DAI. Comparably, the commercial isolate caused less suppression on pathogen growth and ranked 14th and 18th in suppression potential against the pathogen compared with native isolates tested at 7 and 10 DAI, respectively.

Most (80%) of the native Trichoderma isolates tested were found effective in inhibiting the mycelial growth of U. scitaminea in the dual culture experiment (Table 3). The test antagonists grew faster and occupying more space and showed dominance over the pathogen thereby limited the radial growth of the target pathogen. However, antagonists offered varying degrees of inhibition activity against the pathogen. Among the 21 test isolates, 12 of them (MKI3, MKI2-k17, MKI2-m7, MI4, WI12-24, WI12-25, WI4-27, KI2, MKI1-k21, M13-m14, WI3-9, and M13-m33) revealed a higher pathogen suppression potential of >90% than the other isolates at 10 DAI. Moreover, the highest (98.37%) and the lowest (60.77%) mycelium inhibition was recorded with isolates MKI3 and MKI2-k17, and MKI2-m9 at 10 DAI, respectively. Also, percentage suppression that ranged from 28.98% to 83.55% was observed due to the antagonists at 7 DAI. The highest (83.55%) suppression was recorded from isolate WI12-25, followed by WI4-27 (82.51%), while the lowest inhibition was noted on isolate MKI2-m9, which was only 28.98% suppression at 7 DAI.

Detail macroscopic and microscopic features of the five potential Trichoderma isolates (WI4-27, MKI2-k17, MI3-m33, WI12-25, and MKI3) identified in the study were further characterized to confirm the identity of the isolates as shown in Table 4 and Figure 4–8. Based on the morphological features observed, isolates were identified as T. harzianum (WI4-27), T. asperellum (MKI2-k17), T. viride (MI3-m33), T. viride (WI12-25), and T. harzianum (MKI3).

4. Discussion

4.1. Prevalence, cultural and morphological variability of native Trichoderma isolates
The findings of this study indicated that diverse Trichoderma isolates were present in the rhizosphere of sugarcane crop in Ethiopian sugarcane plantations. Many researchers also reported diverse populations of Trichoderma in the root zones of sugarcane and confirmed that the crop is able to host numerous Trichoderma species (Juma et al., 2018; Romão-Dumaresq et al., 2016; Shelake et al., 2019). The Trichoderma fungi prevailed in most of the soils studied at different proportions where Metehara having the highest prevalence and the least occurred at Kesem. Also, none of the isolates recorded at Wonji was found at both Metehara and Kesem and vice versa. This could be due to specific environmental preferences of the isolates and practices of organic fertilizer application, along with other factors. In line to this finding, the effects of several factors, including microclimate, the availability of substrates, as well as complex ecological interactions on the prevalence of different Trichoderma isolates was previously reported (Gupta et al., 2014; Hoyos-Carvajal & Bissett, 2011; Sun et al., 2011).
| Isolate code | Radial growth of *U. scitaminea* (cm) | Suppression of *U. scitaminea* (%) | Characteristics of suppression (mode of suppression) |
|-------------|--------------------------------------|----------------------------------|-----------------------------------------------|
|             | 7 DAI | 10 DAI | 7 DAI | 10 DAI |                                      |
| WI3-4       | 1.00(0.88) | 0.78(0.72) | 73.89 | 84.15 | Overgrowth |
| WI3-9       | 0.72(0.67) | 0.27(0.27) | 81.20 | 94.51 | Overgrowth |
| WI4-24      | 1.00(0.88) | 0.53(0.51) | 73.89 | 89.23 | Inhibition zone |
| WI4-27      | 0.67(0.63) | 0.18(0.18) | 82.51 | 96.34 | Inhibition zone |
| WI10        | 1.18(1.00) | 0.74(0.69) | 69.19 | 84.96 | Overgrowth and inhibition zone |
| WI12-24     | 0.68(0.64) | 0.12(0.12) | 82.25 | 97.56 | Overgrowth |
| WI12-25     | 0.63(0.59) | 0.15(0.15) | 83.55 | 96.95 | Overgrowth |
| WI13        | 1.50(1.19) | 0.67(0.63) | 60.84 | 86.38 | Overgrowth |
| MKI1-m26    | 1.27(1.06) | 1.00(0.88) | 66.84 | 79.67 | Inhibition zone |
| MKI1-k21    | 1.47(1.18) | 0.23(0.23) | 61.62 | 95.33 | Overgrowth |
| MKI2-k9     | 1.47(1.18) | 0.59(0.56) | 61.62 | 88.01 | Overgrowth |
| MKI2-k17    | 1.25(1.05) | 0.08(0.08) | 67.36 | 98.17 | Overgrowth |
| MKI2-m7     | 1.33(1.10) | 0.09(0.09) | 65.27 | 98.17 | Overgrowth |
| MKI2-m9     | 2.72(1.73) | 1.93(1.41) | 28.56 | 60.77 | Inhibition zone |
| MKI3        | 1.73(1.32) | 0.08(0.08) | 54.86 | 98.37 | Overgrowth and inhibition zone |
| MKI4        | 1.97(1.43) | 1.25(1.05) | 48.56 | 74.59 | Inhibition zone |
| MI3-m14     | 1.25(1.05) | 0.25(0.25) | 67.36 | 94.39 | Inhibition zone |
| MI3-m33     | 1.67(1.29) | 0.28(0.28) | 56.40 | 94.92 | Overgrowth |
| MI4         | 1.25(1.05) | 0.12(0.12) | 67.36 | 97.56 | Overgrowth |
| MKI5        | 1.75(1.33) | 1.25(1.05) | 54.31 | 74.59 | Overgrowth and inhibition zones |

(Continued)
Table 3. (Continued)

| Isolate code | Radial growth of *U. scitaminea* (cm) | Suppression of *U. scitaminea* (%) | Characteristics of suppression (mode of suppression) |
|--------------|--------------------------------------|----------------------------------|---------------------------------------------------|
|              | 7 DAI | 10 DAI | 7 DAI | 10 DAI |                     |
| KI2          | 1.33(1.10) | 0.18(0.18) | 65.27bcd | 96.34i | Inhibition zone |
| Comm. isolate | 1.38(1.13) | 1.18(1.00) | 63.97bcd | 76.02 imn | Inhibition zone |
| Pathogen     | 3.83(2.05) | 4.92(2.30) | 0 h | 0 a | - |
| LSD (0.05)   | 0.60 | 0.72 | 2.83 | 1.97 | - |
| CV (%)       | 12.65(7.81) | 22.35(5.87) | 7.64 | 3.90 | - |

*WI = Wonji isolate; MKI = Metehara and Kesem isolate; MI = Metehara isolate and KI/KI = Kesem isolate. LSD = Least significant difference at 0.05 probability level. CV = Coefficient of variation. Means followed by different letter(s) within the same column are significantly different at P = 0.05. Figures in parenthesis refer to arcsine transformed values of each respective radial growth of *U. scitaminea* raw data. Furthermore, the isolates followed two different modes of antagonism against the pathogen. The first group of the isolates inhibited the test pathogen through overgrowth (Figure 2 a and b) and the second category inhibited the test pathogen through forming a clear inhibition zone, secretion of inhibitory chemicals (Figure 3). In general terms, the study identified five potential isolates through growth rate and percent suppression of growth and sporulation of *U. scitaminea*. Of the five isolates, two isolates (WI4-27 and WI12-25) were from Wonji; two isolates (MI3-m33 and MKI3) were from Metehara and the other one isolate (MKI2-k17) was from Kesem Sugarcane Plantation. In their respective locations, the selected isolates have higher growth rate and percent suppression of the pathogen than the remaining isolates from the same location.
According to Gupta et al. (2014), several biotic and abiotic factors affect populations and diversity of microbial communities including *Trichoderma* in agricultural ecosystems. Some of the factors included plant species and their growth stage, total microbial competition, soil physicochemical properties, application of agronomic chemicals as well as the geographical region. Moreover, this study identified that there was variation in abundance of *Trichoderma* isolates with respect to growth stages, crop cycle, and soil types. As a result, a higher probability of getting *Trichoderma* isolates were associated with older sugarcane plants (>5 months) than younger growth stages. This could be attributed to the fact that young sugarcane plantations had relatively higher disturbance of the soil due to different agronomic practices. Similarly, Verbruggen and Kiers (2010) and López-Pineiro et al. (2013) reported the negative effects of crop management practices, such as tillage on the abundance of soil fungi.

In addition, the ratoon crop cycle hosted more number of *Trichoderma* isolates than the plant cane. This might be intimately associated with repeated tillage, inorganic fertilizer application, and pest management practices to plant cane before and after planting that would disturb the rhizosphere and affect the occurrence of the antagonists. Related to this finding, Vukicevich et al. (2016) noted the presence of diverse soil fungi with less disturbed soils. Soil texture in this study also had a strong influence on the prevalence of *Trichoderma* isolates. In this regard, *Trichoderma* isolates were recovered more abundantly on light and intermediate textured soils than on heavy soils. Although physicochemical properties of soils influence the survival of soil microflora, the principle that the better the soil to support the plant, the better it would be for *Trichoderma* for survival and flourishing according to Shelake et al. (2019) who reported diverse fungi community on soils that supported plant growth better. Of course, heavy-textured soils were suffering from water logging problems, which suppress both the plants and the microflora.

Furthermore, there were cultural and morphological variations among the isolates explored. That is, isolates were found to differ in colony color, mycelial growth habit, colony edge, colony radial growth, and growth rate. And, the isolates grew faster on PDA medium and covered the surface of the culture plate within 7DAI, with their colony diameter varying between 6 and 10 cm.
| Isolate Character and Identification | WI4-27 | WI12-25 | MKI2-k17 | MI3-m33 | MKI3 |
|-------------------------------------|--------|---------|----------|---------|------|
| Colony growth rate in 3 days        | Fast   | Fast    | Fast     | Intermediate | Fast |
| Colony color (front view)           | Light to dark green | White to green | Olive green to dark green | Creamy | Olive green to dark green |
| Colony color (reverse view)         | Creamy | Creamy  | Creamy   | Creamy  | Creamy |
| Colony edge                        | Wavy   | Smooth  | Wavy     | Wavy    | Smooth |
| Conidial shape and size             | Globose to ovoid | Globose to sub-globular | Globose to sub-globular | Globose to sub-globular | Globose to ovoid |
| Conidial wall                       | rough | smooth  | smooth    | smooth  | smooth |
| Culture smell                       | Sweet coconut | No odor | Sweet coconut | No odor | No odor |
| Mycelial form                       | Arachnoid | Ring-like zone | Pin-like | Solitary | Pin-like |
| Mycelial color                      | Green  | Green   | Green     | Green   | Green |
| Mycelial form                       | Whisked | Pored and at 90° | Ring-like | Solitary | Solitary |
| Conidophore branching               | Moderately branched | Highly branched | Moderately branched | Moderately branched | Moderately branched |
| Phialide                            | 2/3 whorls | Whorls | Whorls | Whorls | Whorls |
| Phialide shape                      | Sigmoid | 5.5 x 13.5 μm | Ampliform 3.5 x 12 μm | Whorls | Whorls |
| Chlamydomycetes spores              | Not observed | Not observed | Not observed | Not observed | Not observed |
| Isolate identity                    | T. harzianum | T. viride | T. viride | T. viride | T. asperellum |

Table 4. Morphological characteristics of the native Trichoderma isolates, which were identified as potential antagonists against U. scitaminea.

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This variation might probably be attributed to different factors that could be either intrinsic (nature of the fungi) or extrinsic (media, temperature, light, and humidity). Similar to the current study, other previous studies indicated the presence of differences in morphological and cultural features (fast growth, colony color, mycelial growth habit, and colony edge) as typical characteristics of different *Trichoderma* isolates and as preliminary differentiating criteria (Anwer et al., 2020; Naher et al., 2019).

4.2. Antagonistic potential and identity of native *Trichoderma* isolates

Findings of the study revealed that *Trichoderma* isolates subdued the growth of *U. scitaminea* at varying degrees of suppression (Table 3). The *Trichoderma* isolates grew faster than that of the
pathogen, considerably hindering the radial growth of the pathogen through overgrowth and forming inhibition zones in the dual culture. This could confirm that the native Trichoderma isolates had a remarkable antagonistic effect against the pathogen. Hence, they were found as potential biocontrol agents against the sugarcane smut pathogen. The antagonistic effect of the Trichoderma isolates against U. scitaminea and other sugarcane pathogens has already been well documented by different investigators (Juma et al., 2018; Romão-Dumaresq et al., 2016; Shailbala et al., 2019).

The Trichoderma isolates were found to inhibit the growth of U. scitaminea through three different mechanisms: overgrowth, secretion of chemicals that limit pathogen growth as manifested by the development of inhibition zone and combining the two modes at the same time. In
line with this, highly diverse mechanisms facilitating colonization of many pathogens and their niches by Trichoderma isolates were noted by Suresh and Nelson (2016) and Juma et al. (2018). Accordingly, the investigators confirmed that hyperparasitism, antibiosis, metabolite production, competition for available nutrients, and combinations of two or more of the described mechanisms as ways of suppression of the smut and other pathogens.

However, there was a significant variation among the isolates in limiting the radial growth of the smut pathogen and percent suppression potential of the Trichoderma isolates on U. scitaminea in the dual culture. The radial growth inhibition that ranged from 0.15 to 3.87 cm was recorded due to the isolates MKI3 and MKI2-m26 at 10 DAI. On the other hand, percent suppression varied between 60.12% and 98.48% at the same date of assessment. Though there were a few studies on in-vitro potential of Trichoderma isolates suppression against U. scitaminea, the current findings are in agreement with the investigations of Paramdeep et al. (2014) and Juma et al. (2018) who underlined high mycoparasitism and complete cover by T. harzianum over U. scitaminea under-controlled condition tests.

On the other hand, there were several investigations on the Trichoderma isolates in-vitro activity against other sugarcane pathogens, such as Fusarium moniliforme, Ceratocystis paradoxa, and Colletotrichum falcatum. For instance, a study by Gawade et al. (2012) showed that different strains of Trichoderma isolated from sugarcane rhizosphere inhibited the mycelial growth of F. moniliforme, causing wilt disease of sugarcane. Suresh and Nelson (2016) also reported 57% to 78% inhibition of mycelial growth of C. falcatum, causing red rot of sugarcane by different Trichoderma spp. Variable degrees of antagonistic activity against different pathogens in the various studies could be attributed to the assay methods followed, media, and environment required by the biocontrol agents (Prince et al., 2011). The degree of effectiveness of the antagonists also differs according to the growth rate and the nature, and quality and quantity of the inhibitory substances secreted by the antagonists (Alwathnani et al., 2012), which could serve as a criterion for the upgrading of the isolates for subsequent studies.

With regard to the identity of the five selected isolates (WI4-27, MKI2-k17, MI3-m33, WI12-25, and MKI3), these isolates were identified as Trichoderma harzianum, T. asperellum, T. viride,
T. viride, and T. harzianum, respectively. Similarly, isolates showing similar macroscopic and microscopic features were identified as T. harzianum, T. asperellum, and T. viride isolates by several authors (Kumar & Sharma, 2016; Sekhar et al., 2017; Siddiquee et al., 2017).

5. Conclusions
The study has confirmed that there were variations in the prevalence and types of Trichoderma populations in the rhizosphere of sugarcane crop with growth stages, crop cycle, and soil textural groups. Hence, fields of sugarcane crops with relatively old age, ratoon crops, light soils, and fields with less disturbed soils were found to support more Trichoderma isolates than others in an attempt to explore bioagents. Thus, the study demonstrated the presence of diverse Trichoderma isolates in the sugarcane rhizosphere and has a key implication for improving sugarcane growth and health. Most of the Trichoderma isolates had evident antagonistic activities and showed higher suppression potential against the growth of U. scitaminea than the commercial Trichoderma isolate and the controls. This witnessed the potential of the Trichoderma isolates as biocontrol agents against the pathogen. In this regard, the candidate Trichoderma isolates, namely WI4–27, MKI2–k17, MI3–m33, WI12–25, and MKI3, which was identified as T. harzianum, T. asperellum, T. viride, T. viride, and T. harzianum, respectively, could be recommended for use as potential bioagents against sugarcane smut in the Ethiopian Sugarcane Plantations after thorough investigations on substrate selection for mass multiplication, efficacy trials, and compatibility assay of the isolates with fungicides. However, future research should focus on the exploration of additional Trichoderma isolates with a broad-spectrum activities from all the Sugar Estates of Ethiopia, species-level identification through molecular techniques, and integration of the isolates with other smut management options for sustainable production of sugarcane.

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