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Biocontrol Efficacy of Trichoderma Isolates in Controlling Aflatoxinogen Fungi (Aspergillus Flavus) and Growth Promoting of Maize in Cameroun

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Abstract

Maize is a significant cereal crop, serving as a dietary staple and an indispensable raw material for industrial use in Cameroon. Aflatoxinogen fungi (Aspergillus spp), an opportunistic plant microorganism, alter by mycotoxin the biochemical components in maize during processing and storage, causing post-harvest losses and compromising food safety. The use of Trichoderma spp as a biological control agent against aflatoxigenic fungi and as a fertilizer has long been proven in agriculture by numerous researchers. This study aimed to isolate and characterize Trichoderma spp from maize rhizosphere soil and assess its potential for biological control of Aspergillus Spp isolated from maize seeds and for promoting the growth of maize.

Isolation and sequencing showed that our isolates were Trichoderma harzianum for CR-TS1 and T. ghanense for CR-TS3, CR-TS4, and CR-TC1. When tested directly against A. flavus on a PDA medium, the Trichoderma spp inhibited growth with percentages of 78.83%, 77.01%, 75,88%, and 74,95% for CR-TS1, CR-TS3, CR-TC1, and CR-TS4 respectively. Similarly, to verify our isolates' capacity to secrete volatile compounds, a remote confrontation test was carried out by contrasting Trichoderma spp and Aspergillus spp. The results of this test demonstrated the secretion of volatile compounds by our Trichoderma spp with inhibition of 40.94%, 29.48%, 34.36, and 32.19% respectively for CR-ST1, CR-ST3, CR-TC1, and CR-TS4. Our study also showed that isolated Trichoderma spp can produce extracellular enzymes such as amylase, protease, and lignase.

Similarly, the phosphate solubilization test on Pikovskaya (PVK) medium and the siderophore test on chrome azurol S (CAS) agar medium using our Trichoderma spp isolates were all positive, with halo formation around the colony for phosphate solubilization and a color change from blue to yellow for the siderophore. IAA, one of the phytohormones essential not only for the microorganism but also for the plant, tested positive on PDB medium supplemented with L-tryptophan.

Similarly, the application of 107 spores/ml of suspension to maize seeds in vitro significantly ($p<0.05$) boosted maize germination from 90 to 100% compared with the control, which was at 80%. The results of pot experiments carried out to assess the growth promotion effect of our isolates showed a significant increase in plant size, root length, dry and fresh weight of plants and roots, as well as chlorophyll production. All these results show that our isolates can be used in agriculture as a biocontrol agent for aflatoxinogenic fungi and as a fertilizer.

Keywords: Biological Control, Trichoderma, Aspergillus, Aflatoxinogen, Growth Promotion, Maize, Cameroon

Introduction

Food security is a fundamental human right, and as the global population is expected to reach nearly 10 billion by 2050, it is the paramount concern of all countries to increase their agricultural production [1]. Maize is the most widely eaten staple food in sub-Saharan Africa (SSA) [2]. In Cameroon, maize is the most widely consumed cereal, far ahead of sorghum, rice, and wheat. But it's not just a staple crop that's eaten in the form of fermented, dry dough. It's also often roasted, used as maize porridge, and used for lots of other things.

Furthermore, it's a major source of raw materials for many local and sub-regional agro-industries (breweries, animal feed production). Maize is a key ingredient in animal feed, making up 65% of the inputs used to make poultry feed. Consequently, cultivation of maize represents a highly strategic undertaking in Cameroon, with implications for both food security and national sovereignty [3]. Cameroon produces over 2.1 million metric tons of maize, making it the 44th largest producer in the world and the 13th largest in Africa. However, this production is still insufficient to meet the country's ever-increasing demand, and it is therefore importing large quantities of maize and maize products [4].

Fungus species belong to the Aspergillus genus are, responsible for producing the toxic metabolite aflatoxin. This metabolite is known to induce cancer and alterations to the digestive tract, kidneys, blood, and nerves in humans and animals [5]. Furthermore, it's the cause of significant deterioration in the quality and quantity of maize, resulting in substantial economic losses [6]. The prevalence of aflatoxin contamination and associated infections is exacerbated under conditions of plant stress, such as those caused by high temperatures and drought. Consequently, the anticipated effects of climate change and global temperature increases are expected to enhance the likelihood of aflatoxin occurrence in temperate regions in the coming years. In this context, the management of aflatoxigenic fungi at both the pre- and post-harvest stages represents a crucial element of a comprehensive strategy aimed at reducing the risk of aflatoxin contamination at the consumer level [7].

To address this issue, a number of alternative approaches have been developed, one of which is the use of chemical inputs. However, the overuse of chemical fertilizers, despite their high nutrient content and ability to facilitate faster crop growth, has been found to be detrimental to human health and the environment. Furthermore, these fertilizers have been identified as a significant source of groundwater and atmospheric pollution [8]. However, ecologically sustainable agriculture, based on good agricultural practices and the use of beneficial micro-organisms as bio-fertilizers, can offer integral solutions to achieving a high-quality, sustainable food system.

Naturally, around 80% of higher plants including maize are associated with fungi and bacteria contained in bio-stimulant preparations that promote growth, increase yield, influence the biodiversity of plant species, contribute to fight against pathogens and pests, improve plant nutrition in polluted environments, stabilize soil aggregates by releasing numerous substances and have properties to ensure post-harvest protection. This has a positive ecological and economic impact, with a broad spectrum of action ensuring the stability of the natural environment [9]. It is therefore considered that the biological management of plant diseases with different bacterial and fungal biocontrol agents represents a safer option.

The fungus species Trichoderma (Hypocreales) are employed globally as a highly profitable agent for the biological control of plant diseases. The molecular-level interactions of Trichoderma spp. with host plants and pathogens are crucial for comprehending the diverse mechanisms by which the fungus establishes a symbiotic relationship with its plant host through its superior antifungal and antimicrobial activity. When working in synchrony, mycoparasitism, antibiosis, competition, and the induction of systemic acquired resistance (SAR)-like response are considered key factors in deciding the biocontrol potential of trichoderma [10].

Moreover, Trichoderma spp. have been demonstrated to enhance plant growth through a range of mechanisms, including the solubilization of insoluble phosphate, the production of siderophores, and the synthesis of plant hormones such as indole acetic acid [11]. It has been demonstrated that Trichoderma spp. possess the capacity to stimulate biological processes in a variety of vegetable crops. This includes the induction of resistance mechanisms, promotion of root development, and enhancement of overall growth [12-15].

In Cameroon, studies on Trichoderma spp. have shown satisfactory results in controlling soil pathogens [16-18]. To contribute to food security and improve farmers' livelihoods, it's interesting to assess the impact of Trichoderma spp. on aflatoxin production, pre-and post-harvest maize protection, and its potential to increase maize yields in Cameroon. The present study sought to assess the capacity for biological control in vitro and the plant growth-promoting (PGP) capabilities of four Trichoderma isolated from the soil of the Ntui cultivated department in the Center Region of Cameroon. Specifically, antagonist dual assays and the production of diffusible and volatile metabolites of Trichoderma isolates were conducted. The enzymes that are presumed to be involved in the antagonist activity of these fungi, including amylase, lignase, and protease, were also evaluated. The PGP activity was evaluated in vitro through the assessment of phosphate solubilization capacity, IAA, and siderophore production. In vivo assessment was conducted through the inoculation of a spore suspension of Trichoderma spp. on maize plants under greenhouse conditions.

Material and Methods

The experiment was conducted at the phytopathology laboratory of the Department of Crop Production and Technologies, Faculty of Agricultural Sciences and Technologies, Nigde Omer Halisdemir University (Nigde, Turkey) in 2024.

Sample Collection

The maize seed samples were obtained from a stock of maize from the first cropping season at a branch of the Agricultural Research Institute for Development (IRAD) in Cameroon which is the main research institute where many farmers obtain or buy their seed. The maize variety (CMS 8704) was selected for the study due to its prevalence among farmers in the Center Cameroun region. A total of 500 grams of seeds whit non visible cracks

or deformation was taken for Aspergillus isolates. To obtain antagonistic isolates, the rhizosphere zone of apparently healthy and vigorous maize plants was sampled at a depth of 5 to 10 cm after removal of the surface layer in a maize field in the Ntui area, Mbam-ekim Department, Central Cameroon Region. All samples were taken under aseptic conditions and recovered in sterile bags to avoid any risk of contamination between soil and seed samples.

Isolation of Aspergillus

Twenty (20) maize seeds were superficially disinfected with 70% alcohol for three minutes, and 2% sodium hypochlorite for two minutes, and were subsequently washed three times in sterile distilled water. The disinfected seeds were then dried on sterile paper towels, deposited on PDA medium, previously poured into Petri dishes, and supplemented with streptomycin (250 mg/L). Petri dishes were then incubated at 26±2°C for four to seven days until the fungi appeared [19]. The young fungal colony based on their morphological characteristics was aseptically picked up and transferred to fresh sterile PDA plates to obtain pure culture.

Isolation of Trichoderma Species

Trichoderma isolation was conducted on soil following the protocol. Ten (10) grams of soil sample were mixed with 90 ml of sterile distilled water in a conical flask, left for one hour in a shaker machine, and then a series of dilutions ranging from 10^{-1} to 10^{-7} was carried out. A total of 0.1 ml of each dilution was subsequently distributed on the surface of the PDA medium supplemented with the streptomycin (250 mg/L), which had been previously poured into the petri dish and the plates were then incubated at 26±2°C for 7 days [20]. Distinct colonies of Trichoderma spp. were picked and purified by culturing on PDA based on their morphological characteristics as described by Webster (1968) [21]. All Trichoderma spp. conidia were suspended in glycerol (15% v/v) and stored at −80 °C.

Molecular Characterization of Aspergillus and Trichoderma Isolates

DNA Extraction

The process begins with the cultivation of mycelial growth on a Potato Dextrose Broth (PDB) medium prepared according to the manufacturer's instructions thus (Add 200 g/L potato infusion and 20 g/L dextrose to 1 L distilled water and boil until dissolved. Autoclave at 121°C for 15 minutes) and incubated in a shaker incubator for 15 days. Following this, the mycelium is washed three times with sterile distilled water, with centrifugation at 6000 rpm for 30 minutes each time. The mycelium is then crushed using liquid nitrogen, with 200 mg placed in 2 ml Eppendorf tube and stored at - 80°C. DNA extraction was carried out as follows, the fungal mycelia (200mg) were treated with 1 ml of lysis buffer (2% CTAB buffer) containing 1 mM Tris HCl, pH 8, 0.1 mM EDTA, 2% sodium dodecyl sulfate w/v, 5 M NaCl, and mixed well using a lysser cloth until the mixture is homogenized.

The mixture was then incubated in a water bath at 65°C for 15 minutes. After incubation, the mixture was centrifuged at 14000 rpm at room temperature (RT) for 10 minutes. Subsequently, 700 µl of the supernatant was meticulously extracted and transferred to a fresh 1.5ml tube. An equal volume (700µl) of chloroform: isoamyl alcohol (24:1) was added and the emulsion was mixed well by carefully inverting the tubes from top to bottom. The tubes were centrifuged again at 14000 rpm under RT for 10 minutes. 550µl of the supernatant was removed and introduced into a new 1.5 ml tube, then an equal volume of chloroform: Isoamyl alcohol solution (550µl) was added and mixed well, then centrifuged again at 14000 rpm under RT for 10 minutes.

A 450µl of the supernatant was transferred to new 2ml tubes then 225µl of 5M NaCl pH 8.0 (half of the supernatant collected) was added followed by the introduction of an equal volume of ice-cold isopropanol (450µl) the tubes were well homogenized and incubated at - 20°C for 1h or overnight for DNA precipitation. Centrifuge the tubes at 14000 rpm at 4°C for a further 20 minutes. Then carefully drain off the salt and iso-propanol without disturbing the pellet and add 500 µl of ice-cold 70% ethanol. The tubes should be centrifugated at 14000 rpm at 4°C for 10 minutes. The ethanol should be added with care, taking care not to dislodge the pellet. The tubes should then be incubated in an oven at 34°C for approximately one hour to allow for the removal of residual ethanol. The pellet should be resuspended by the addition of 50 µl of sterile ultrapure water (molecular biology grade) [22, 23].

The quantity of DNA present in the samples was ascertained through the utilization of a Quawell Bio Spec Mini spectrophotometer. Absorbance A1 (260 nm), as well as the ratios A1/A2 (260/280 nm) and A1/A3 (260/230 nm), and DNA concentration, were determined to ascertain the success of the DNA extraction process and the purity of the samples produced. For the samples to be deemed "pure," the A1/A2 and A1/A3 ratios were required to be within the range of 1.8.

Amplification of the ITS Region

Molecular identification based on internal transcribed spacer (ITS) region of the ribosomal RNA gene and DNA-directed RNA polymerase II core subunit (RPB2) regions of Trichoderma isolates (Table 1). It was performed with polymerase chain reaction (PCR) using the 1 µl of extracted template DNA (20-30 ng/µl) in a 25 µl PCR mixture containing 12.5 µl of 2× Taq Master Mix, (Dream Taq Master Mix, Thermo ScientificTM), 1 µl (10Mm) forward primer, 1 µl 10 Mm reverse primer (final primer concentration are 0.4 Mm).

The polymerase chain reaction (PCR) conditions for the internal transcribed spacer (ITS) gene included an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, and extension at 72 °C for one minute. The conditions for PCR amplification of the RPB2 gene included an initial denaturation at 95 °C for three minutes, followed by 40 cycles of denaturation at 95 °C for 15 seconds, annealing at 56 °C for 15 seconds, and extension at 72 °C for one minute. The amplification process was concluded with a final extension at 72 °C for a period of five minutes [24, 25].

Identification of Aspergillus isolates to the species level was confirmed using only the sequence of the ITS gene. The amplified DNA was visualized in 1.2% agarose gels stained with ethidium bromide under ultraviolet light using the Gel Doc Biorad System (USA). The purified PCR products were subjected to sequenc-

ing. The sequenced data were then subjected to a comparison with the GenBank database, utilizing the BLASTn tool, which is accessible via the NCBI platform. The phylogenetic analysis of isolate Trichoderma spp and Aspergillus spp were performed using MEGA 11 software. Finally, the cross-ponding species for each isolate was identified.

Table 1: Primers used for amplification and sequencing

Locus	Definition	Primer Set	Sequence 5'-3'	Reference
ITS	Internal Transcribed Spacer	ITS1 F	TCCGTAGGTGAACCTGCGG	White et al., 1990
		ITS4 R	TCCTCCGCTTATTGATATGC	
RPB2	DNA-directed RNA polymerase II core subunit	fRPB2-5f	GAYGAYMGW-GATCAYTTYGG	Liu et al. 1999
		fRPB2-7cr	CCCATRGCTTGTYYRCCCAT	

In Vitro Antagonism Tests

The biocontrol potential of Trichoderma spp. isolates was assessed via three distinct inhibition tests: the first entailed a dual culture assay of the A. flavus isolate and each Trichoderma isolates on PDA dishes, the second a culture filtrate assay of Trichoderma isolates, and the third a volatile metabolite assay. The objective was to determine if the isolates could reduce aspergillus growth.

Dual-Culture Antagonistic Activity

This test was carried out in 90 mm diameter Petri dishes each containing 20 ml of PDA culture medium with streptomycin (250mg/L) added, which were inoculated 2 cm apart from the edge of the plate with a 6 mm-diameter mycelial plug from a fresh culture of Trichoderma isolates and on the opposite side, at 5 cm distance from the Trichoderma isolate inoculation point, with 6 mm-diameter mycelial plug from a fresh culture of aspergillus. Dual-culture petri dishes as well as the control petri dishes containing only aspergillus were incubated at 26° C ± 2 for 7 days. This test was carried out twice successively, with three (3) Petri dishes for each comparison [26]. The colony radial of Aspergillus on the control and test plates was measured, and the percentage of inhibition was calculated using the formula previously described by [27]. Percentage inhibition (%) = R1 − R2 R1 × 100, (1)

R1 = radial growth of aspergillus in control and R2 = radial growth of aspergillus with treatment.

Effect of Non-Volatile Compounds Produced by the Antagonists on the Growth of Aspergillus

The non-volatile compounds of our Trichoderma isolates were obtained by mixing in 250 ml Erlenmeyer conical flask 100ml of sterile PDB culture medium with a 1ml suspension containing 107 conidia of trichoderma spores. The mixture was incubated in a shaker machine at 600 rpm for 15 days at 26°C. The centrifuged mixture was sterilized by passing through a 0.22 µm cellulose membrane. A 50 ml of this filtrate was mixed with 50 ml of PDA cooled to 60°C and poured into Petri dishes. The mycelial disc (6 mm) of aspergillus was inoculated into the center of Petri dishes containing the mixture (PDA + culture filtrate) and incubated at 26±2°C until the pathogen completely covered the control Petri dishes containing only the PDA culture medium [28]. The test was carried out twice successively, with three (3) plates for each comparison.

Colony diameters of aspergillus were measured and converted to the percentage of inhibition through the following formula: Percentage inhibition (%) = Dc – Dt/ Dc × 100, (2)

Dc = mycelial growth of aspergillus on the control plate, and Dt = mycelial growth of aspergillus on the test plate.

Effect of Volatile Compounds Produced by the Antagonists on the Growth of Aspergillus

The production of volatile compounds and their inhibitory effects on the pathogen were demonstrated through the juxtaposition of two Petri dishes, each containing a PDA medium supplemented with streptomycin (250 mg/L). The bottom dish was inoculated at its center with a 6 mm disc of 7-day-old trichoderma, while the cover dish was inoculated at its center with a 6 mm disc of the pathogen.

The two dishes were hermetically sealed with parafilm and incubated at a temperature of 26±2°C. To establish control, a bottom plate comprising the medium alone was placed below a bottom plate containing the pathogenic fungus. Radial mycelia growth measurements were taken at 7 days [29]. The percentage growth inhibition was using the following formula: Percentage inhibition (%) = Dc – Dt/ Dc × 100, (3)

Dc = mycelial growth of aspergillus on the control plate, and Dt = mycelial growth of aspergillus on the test plate. Each treatment was performed in three (3) replicates.

Qualitative Determination of Enzymatic Activity, Amylase, Protease, and Ligase.

A qualitative enzyme assay of Trichoderma isolates was conducted using a plate assay on solid media to assess the production of extracellular enzymes. The assay employed the formation of clear zones, changes in coloration, and their intensity around fungal colonies as markers of amylase, protease, ligase phosphate solubilization, and siderophore production. The independent experiments were performed for this screening step with three replicates for each strain.

Amylase Production

Amylase production by Trichoderma isolates was demonstrated on a nutrient agar medium supplemented with 2g soluble starch at pH 6.0. After incubation at 26 ± 2° C for 72 h, the dishes were treated with 5 ml of diluted Lugol solution (once) for 15 min. Amylase production was evaluated through the observation of a light yellow halo formation around each isolate colony [30].

Lignase Production

Tannic acid medium (5.0 g tannic acid, 15.0 g malt extract agar, 20.0 g agar, and 1 L distilled water) was autoclaved at 121 ºC for 20 min and poured approximately 20 ml into each Petri dish. A 6 mm disc of trichoderma mycelium from a 5-day-old was placed in the center of the dish and incubated in the dark at room temperature (26±2ºC). The formation of a dark brown pigment around the inoculation point was used as an indicator of polyphenol oxidase (PPO) activity on the tannic acid medium [31].

Protease production

For protease screening, the Trichoderma isolates were cultivated on casein agar medium (comprising peptic digest of animal tissue 5.00 g, beef extract 1.50 g, yeast extract 1.50 g, sodium chloride 5.00 g, agar 15.00 g, casein 10.00 g, and distilled water to 1000ml) [12]. The medium was transferred to Petri dishes in a sterile manner and inoculated with an agar disc measuring 6 mm, cut from a 5-day-old fungal culture of each strain separately. The plates were then incubated at 26 ± 2°C in darkness for 3 to 5 days. Subsequently, the plates were flooded with bromocresol green dye. The clear zone around the colony indicates the proteolytic activity of microorganisms [32].

The Ability of Trichoderma Isolate to Solubilize Phosphate

The ability of the microbial isolates to solubilize phosphate was evaluated using a Pikovskaya Agar medium comprising 5 g Ca3(PO4)2, 5 g MgCl2.6H2O, 0.25 g MgSO4.7H2O, 0.2 g KCl, 0.1 (NH4)2SO4, 14 g agar, and 10 g glucose per liter of distilled water. Agar plates were inoculated with a plug of trichoderma and incubated for five days at 26 ± 2°C with constant illumination. The formation of clear zones around the colonies provided evidence of phosphate solubilization ability [33].

The ability of Trichoderma Isolates to the Production of Siderophore

The siderophore production detection test was carried out using the method described [34]. A 6 mm mycelial disc is inoculated into the previously prepared Blue Chromium Azurol S (CAS) Agar medium and subsequently incubated at 26°C for five days. The positive reaction is characterized by a change in coloration of the culture medium from blue to yellow [33].

Quantitative Determination of the Concentration of Indole-3-Acetic Acid (IAA)

The concentration of IAA was determined through spectrophotometric analysis. The efficiency of Trichoderma isolates in producing indole acetic acid (IAA) was evaluated using L-tryptophan as a precursor, by the methodology described by [35].

The assays were performed using a potato Dextrose Broth (PDB) solution, which was enriched with 0.5 g L-1 of L-tryptophan. The culture was incubated in a shaker with a rotation speed of 150 rpm at a temperature of 28 °C. Following a seven-day incubation period, a qualitative estimation of production was conducted via colorimetric assay. The filtrates were subjected to centrifugation, and 2 mL of each resulting supernatant was subsequently added to 2 mL of Salkowski reagent (comprising 1 mL of ferric chloride at a concentration of 0.5 M). This was followed by the addition of 50 mL of perchloric acid (35%) to the mixture. The development of a pink or red color indicated the production of IAA. The absorbance was subsequently measured at 530 nm using a spectrophotometer [36].

To determine the concentration of IAA produced the standard curve was plot follows the protocol described by: The range between the standard of IAA is -10 to 100 µg/ml. Different IAA concentrations are prepared as aqueous solutions of IAA, ranging from 10 µg/ml to 100 µg/ml [37]. Add 2 ml of Salkowski reagent and 2 ml of each serial dilution to each test tube. Subsequently, the test tubes are to be homogenized and incubated for 30 minutes at room temperature. This allows the solution to turn pink or red, indicating the production of IAA. The standard IAA solution is then measured for absorbance using a spectrophotometer at a wavelength of 530 nm. A standard graph is made by plotting the concentration of IAA (in micrograms/ml) versus optical density at 530 nm.

Effect of Trichoderma Spp. Inoculation in Seed Germination

Maize seeds were disinfected with 70% ethanol, 2% NaOCl, and sterile distilled water, then dried under a sterile air stream on autoclave blotting paper. The surface disinfected and dried seeds were divided into sterilized Petri dishes. The seeds were treated with 5ml of 107 spore suspension for four treatments (T) and three replicates of 10 seeds each: T1 - seeds inoculated with isolate CR-TS1; T2 - inoculation with isolate CR-TS3; T3 - inoculation with isolate CR-TC1; T4- inoculated with isolate CR-TS4 and T0 - control inoculate whit sterile water. The Petri dishes were put in an incubator at 26±2°c [38]. The germination of seeds was observed after 48 hours of germination, and the percent germination of seeds was calculated.

Promotion of Maize Growth by Trichoderma Isolates

Five-liter pots containing autoclaved soil were placed in a greenhouse with a humidity of between 70 and 80% and a temperature ranging from 23 to 40 ºC, with a 12-hour photoperiod. Maize seeds were disinfected with a (2%) solution of bleach, washed three times with sterile distilled water, and then immersed in a suspension of conidia 107 conidia/mL for 24 hours so that they adhered to the seed surface. Maize seeds for the control pots were soaked in sterile distilled water. Three (3) seeds were sown per pot, for each treatment with trichoderma. seven days after sowing, seedling emergence was noted and after three weeks under these conditions, measurements of the plant height, root length, chlorophyll rate, dry, and fresh roots length, and aerial part of the plant were obtained. The experiment was conducted with five replicates per treatment.

Statistical Analysis

We used Bartlett's and Shapiro-Wilk's tests to check for homogeneity of variances and normality. We fitted the data to linear models and used ANOVA to identify significant differences between the treatments (Tukey's test, $P < 0.05$). We did the statistical analyses using R version 4.3.2 (2023-10-31 ucrt).

Results

Species Identification and Phylogenetic Analysis

The fungi isolated in soil from the maize rhizosphere had pure circular cultures and filamentous margins. Colony growth was rapid, and by the third day, they had colonized the entire petri dish. Additionally, the emergence of greenish colonies was noted, with this phenomenon occurring 72 hours after initiating the process for isolates CR-TS1, CR-TS3, and CR-TC1, and 96 hours later for isolate CR-TS4.

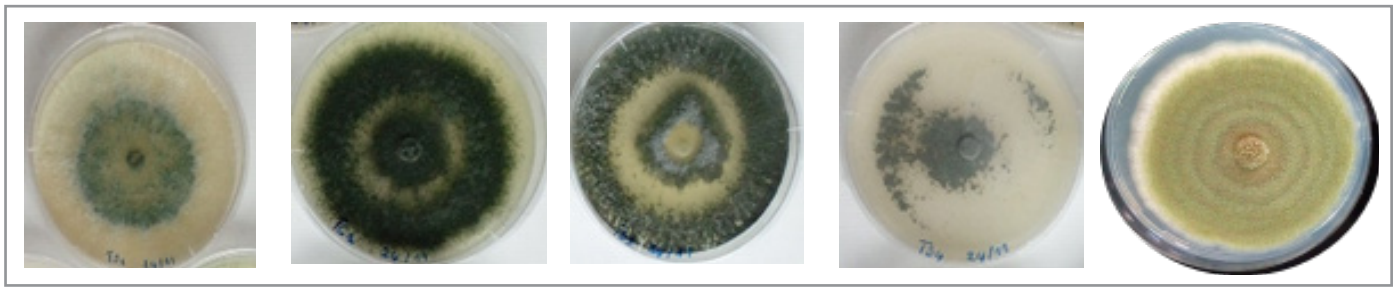


Figure 1: The morphology of the pure fungal colony on a potato dextrose agar (PDA) plate, cultivated via point inoculation, is presented herewith. (To view of the culture plate)

A full molecular identification was performed, and the amplified DNA fragment using ITS (1 and 4) and RPB2 primers resulted in a DNA fragment of around 600 -650 pb for ITS and 800 – 825 for RPB2 in 1.2% agarose. A database search conducted using the BLAST program revealed that the sequence of the PCR product exhibited identity, query coverage, and a 0.0 E value, as detailed in Table 3. In addition, the inferred phylogenetic tree indicated that the unknown sequence was clustered with different species sequences obtained from the GenBank database (Figure 2).

Table 2: Molecular identification of the four Trichoderma isolates CR-TS3, CR-TC1, CR-TS1, CR-TS4, and CR-MW based on Sanger sequencing of the two primers ITS, and Rpb2.

Isolate code	Gene region	Fragment Size (bp)	Organism	% Query cover	E. val-ue	% iden-tity	Accession number
CR-TS3	ITS	600 - 650	T. ghanense	97	0.0	99.3	
	RPB2	800 - 850	T. ghanense	94	0.0	95.63	
CR-TC1	ITS	600 - 650	T. ghanense	97	0.0	99.3	
	RPB2	800 - 850	T. ghanense	81	0.0	88.80	
CR-TS1	ITS	600 - 650	T.hazianum	100	0.0	98.66	
	RPB2	800 - 850	T.hazianum	86	0.0	99.88	
CR-TS4	ITS	600 - 650	T. ghanense	97	0.0	99.50	
	RPB2	800 - 850	T. ghanense	94	0.0	95.63	
CR-MW	ITS	500 - 550	A. flavus	93	0.0	98.14	

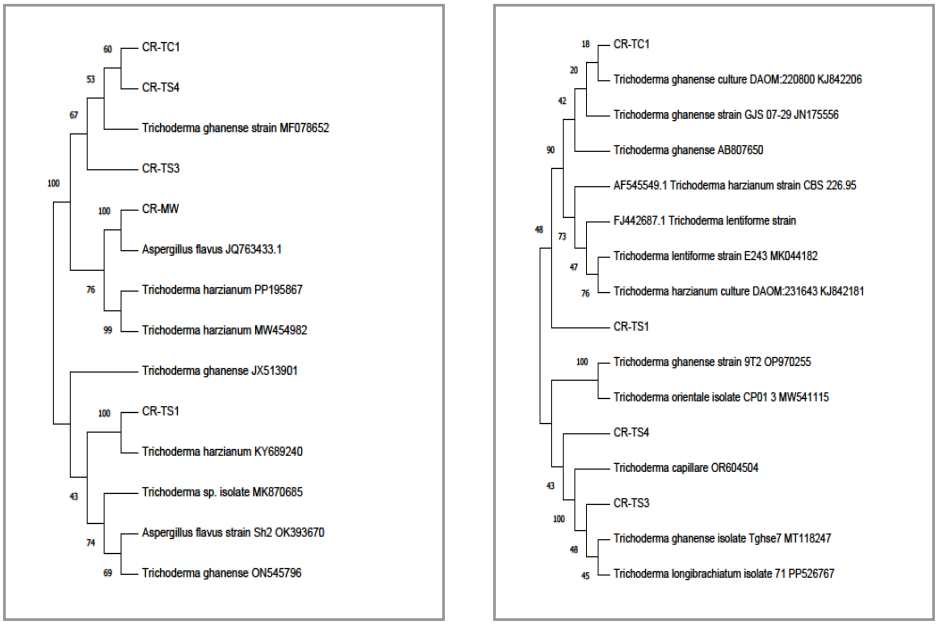


Figure 2: The resulting tree illustrates the phylogenetic relationship between the unknown sequence and the selected sequences from the GenBank database, 1000 bootstrap replications were used. (A: ITS phylogenetic, B: RPB2 phylogenetic)

Antifungal Activity of Trichoderma spp., Against Aspergillus Flavus in Dual Culture

Growth of the A. flavus isolate CR-MW was not significantly (P>0.05) inhibited by all four Trichoderma isolates (CR-TS1, CR-TC1 CR-TS3, and CR-TS4) in dual culture assay. The findings demonstrate that the inhibition rates observed in all isolated specimens exceeded 75%. Inhibition was demonstrated to be a highly stable phenomenon, as evidenced by the observation that A. flavus remained inhibited for periods exceeding 30 days (Table 2, Fig 3). Some observations show that trichoderma can grow above A. flavus and make spores. The color of the culture media also changes.

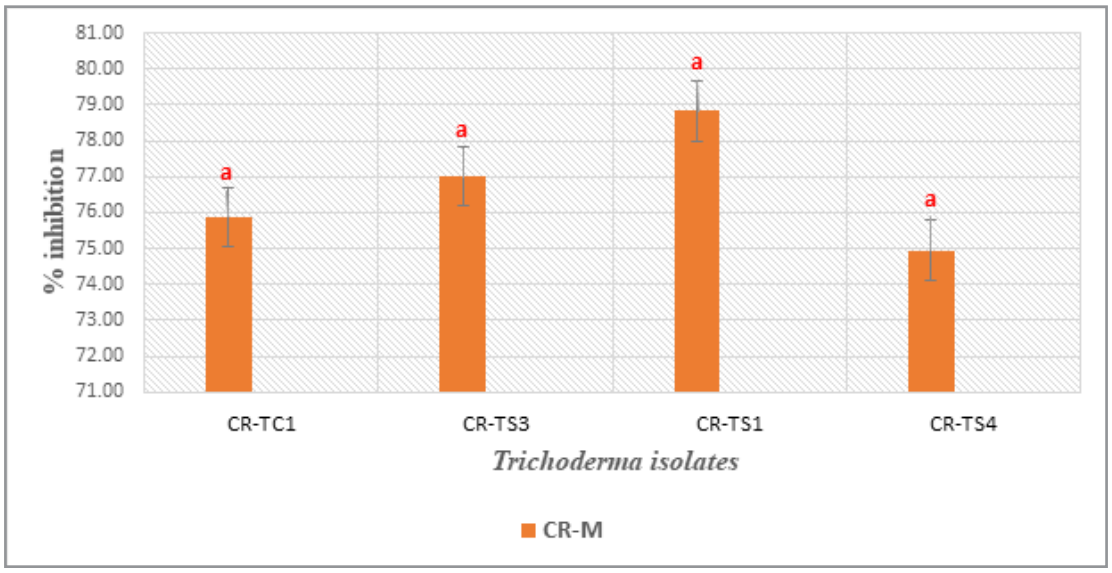


Figure 3: Percentage of Aspergillus flavus growth inhibition by Dual culture of Trichoderma isolates. Standard error is represented with bars. The letter above the bars with the same letter indicates no statistically significant difference between treatments, as determined by the Tukey's test ($p < 0.05$).

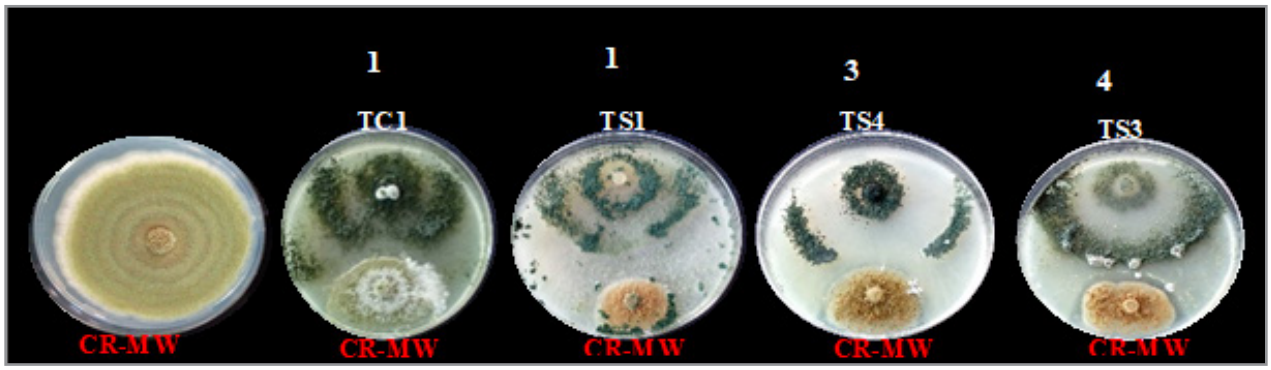


Figure 4: Photographs of PDA plates with Trichoderma isolates (CR-TC1, CR-TS1, CR-TS4, and CR-TS3) and Aspergillus flavus (CR-MW) after 30 days of the assay started. The interaction type, as defined by Whipps (1987), is represented by the following values: 1 = Trichoderma overgrowing A. flavus and A. flavus ceased to grow; 1/2 = Trichoderma overgrowing A. flavus, but A. flavus continued to grow; 2/1 = A. flavus overgrew Trichoderma, but Trichoderma continued to grow. The growth of Trichoderma is observed, yet Trichoderma continues to expand. In scenario 2, A. flavus overgrows Trichoderma, leading to the cessation of Trichoderma growth. In scenario 3, there is a slight mutual inhibition, indicated by an inhibition zone measuring ≤ 2 mm in width. In scenario 4, strong mutual inhibition is evident, with an inhibition zone measuring ≤ 4 mm in width.

Effect of Volatile Compounds Produced by the Antagonists on the Growth of Aspergillus

The inhibition of the A. flavus isolate CR-MW during a remote confrontation is evidence that our isolates produce volatile compounds. The inhibition rate was less than 50%. However, it was significantly different (P<0.05). The highest inhibition rate of 40.94% was found in isolate CR-TS1, followed by CR-TC1 (34.36 ± 1.91) and CR-TS4 (32.19 ± 2.53). The lowest rate was found in the isolate CR-TS3 (29.48 ± 2.46).

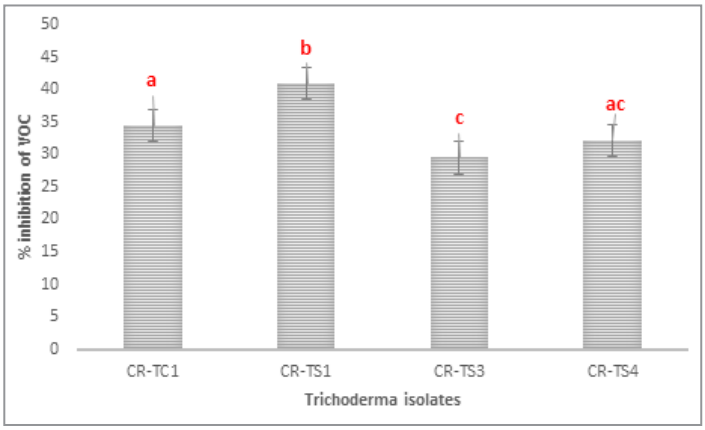


Figure 5: Percentage of Aspergillus flavus growth inhibition by volatile metabolites produced by Trichoderma isolates. The standard error is represented with bars. The letter above the bars with the same letter indicates no statistically significant difference between treatments, as determined by Tukey's test ($p < 0.05$).

Effect of Non-Volatile Compounds Produced by the Antagonists on the Growth of Aspergillus

The results showed that, non-volatile compounds produced by Trichoderma spp. significantly different ($P<0.05$) inhibited the growth of A. flavus isolate. The inhibition value, expressed as mycelial growth inhibition, ranged from 78.1 ± 2.6 % to 84.4 ±4.1% (Table). The highest inhibition rates were recorded by antagonist isolates CR-TS1 (84.4 ±4.01%) followed by CR-TS3 (81.4 ± 4.08 %), CR-TTC1 (79.8 ± 2.6 %), and the lowest rate was recorded by CR-TS4 (78.15 ± 2.6 %).

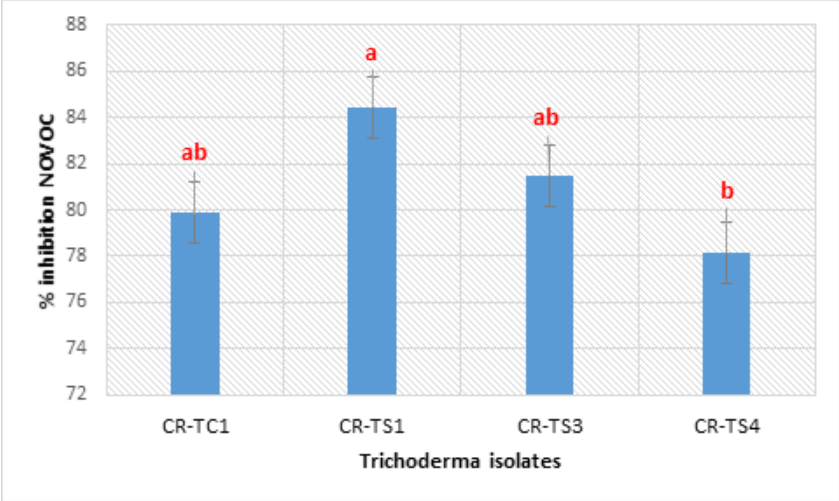


Figure 6: The inhibitory effect of non-volatile metabolites produced by Trichoderma isolates on Aspergillus flavus growth in percentage terms. The standard error is represented with bars. The letter above the bars with the same letter indicates no statistically significant difference between treatments, as determined by Tukey's test ($p < 0.05$).

Table 3: Inhibition effect of Dual culture, volatile compounds, and non-volatile compounds of Trichoderma isolates against Aspergillus flavus. In instances where means are followed by the same letter in a given column, it can be inferred that the statistical difference between treatments is insignificant., as determined by Tukey's test (P< 0.05).

T. isolates	% Dual culture inhi-bition	% Non-volatile compound (NoVoc)	% volatile compound (Voc)
CR-TC1	75.88 ± 3.22a	79.88±2.69ab	34.36 ± 1.91a
CR-TS1	78.83 ±4.41a	84.43±4.01a	40.94 ± 2.23b
CR-TS3	77.01 ±6.10a	81.48±4.08ab	29.48± 2.46c
CR-TS4	74.95 ±5.77a	78.15±2.67b	32.19± 2.53ac

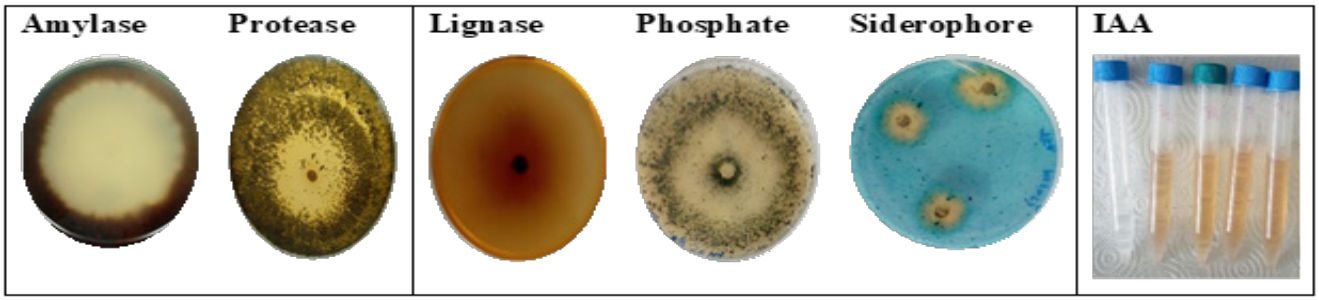
Qualitative Determination of Amylase, Protease, lignase, Phosphate Solubilization, Siderophore, and Indol-3- Acetic Acid (IAA)

Tests for the production of hydrolytic enzymes, phosphate solubilization, and siderophore production were conducted under the specifications of the respective medium. Upon completion of the aforementioned tests, it was determined that all Trichoderma isolates exhibited the capacity to produce amylase, lignase, siderophores, and IAA. However, among these isolates, only CR-TS4 was found to lack the ability to produce protease and solubilize phosphate. The results presented in the table below were estimated based on the diameter of the halo zone or the change in coloration of the medium.

(—) no change: isolates showing no enzyme activity or no halo zone, (+) diameter of media change or halo zone [1; 10] mm, (+ +) diameter of media change or halo zone] 10; 30] mm, (+ + +) diameter of media change or halo zone]30; 70] mm, and (+ + + +) diameter of media change or halo zone more than 70 mm (Table).

Table 4: Qualitative screening of Trichoderma isolates for active biomolecules

	Amylase	Ligninase	Protéase	Phosphate	Siderophore	IAA
CR-TS1	+ + + +	+ + +	+ +	+ + +	+ + +	++
CR-TC1	+ + + +	+ + +	+ +	+ +	+ +	++
CR-TS3	+ + +	+ + +	+	+	+	++
CR-TS4	+ +	+ + +	-	-	+ +	++



Quantitative Production of IAA

The present investigation revealed that all Trichoderma spp. were able to produce IAA. While, the quantity of IAA produced exhibited considerable variation, the highest levels were observed in CR-TS1, with 15 µg ml^{-1} of IAA, followed by CR-TC1, CR-TS4, and CR-TS3, which produced 13.63 µg ml^{-1}, 12.36 µg ml^{-1}, and 11.18 µg ml^{-1} of IAA, respectively.

Stimulating Germination of Maize Seeds by Trichoderma Spp

In the present investigation, seed germination was higher in treated seeds by Trichoderma spp as compared to control. The trichoderma conidial suspension showed a significant difference ($p<0.05$) increase in seed germination compared with the uninoculated control. As depicted in table, trichoderma conidial suspensions germinated maize seeds at rates ranging from 90 to 100%. In the present investigation, seedling emergence was higher in treated seeds as compared to control. The treatments also resulted in enhanced germination of seed. The results of the study indicated that the Trichoderma isolates CR-TS1, CR-TS3, and CR-TC1 were most effective in increasing seed germination, with a 100% germination rate observed in 4 days. On the other hand, the isolate CR-TS4 treatment, despite a germination rate of 90%, is not significantly different from the control (80%).

Table 5: Stimulating germination of maize seeds by Trichoderma spp. the same letter in a column means no significant difference between treatments, as determined by the Tukey's test ($p < 0.05$)

T. isolates	Jour 2 (%) P<0,05	Jour 3 (%) P<0,05	Jour 4 (%) P<0,05
CR-TS1	80 ± 1b	100 ± 00b	100 ± 00b
CR-TS3	76,6 ± 1,1b	83,3 ± 0,5b	100 ± 00b
CR-TC1	70 ± 1b	86,6 ± 0,5b	100 ± 00b
CR-TS4	66,6 ± 0,5b	83,3 ± 0,5b	90 ± 1ab
Contrôle	40 ± 1a	63,3 ± 1,1a	80 ± 1a

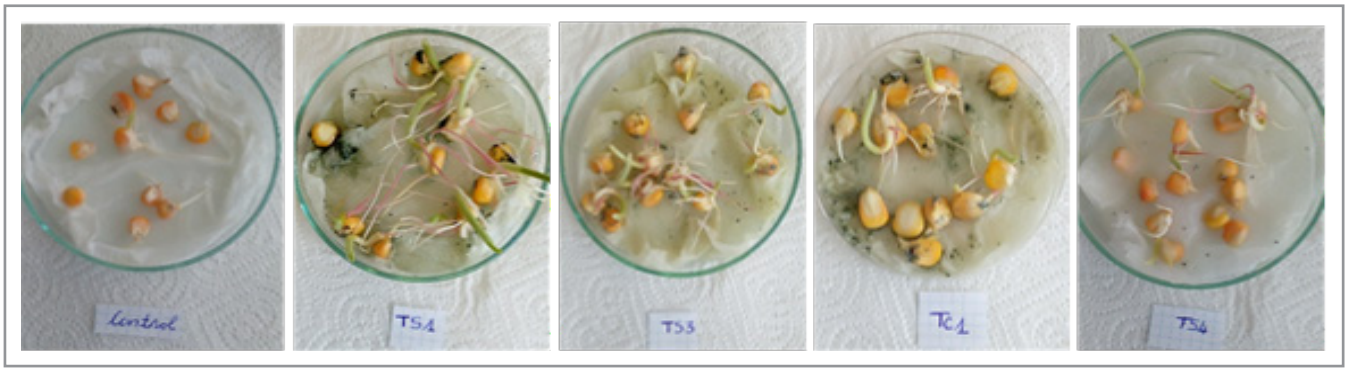


Figure 7: The effect of Trichoderma spp. on maize seed germination.

Growth promotion test

In this study, four Trichoderma isolates used in the test were able to improve plant growth throughout the greenhouse experiments. According to Table, the data obtained with seedling emergence of trichoderma did not differ significantly (P>0.05) from those verified with the control treatment based on the 5% Tukey test.

All the disinfected maize seeds sown in trichoderma-treated showed more than 85% increase in seedling emergence 8 days after sowing (Figure 8). Furthermore, an analysis of variance of means was carried out to compare the plant height, root length, fresh roots and shoot weight, dry roots, and shoot weight of control plants and plants treated with trichoderma on day 21. The data results were significantly different ($p< 0.05$) for each parameter (Table 5). The results analysis showed that the best results for this test were achieved with trichoderma CR-TS1 for which shoot height (73.6 ±4.6 cm), roots length (19.4 ± 0.8 cm), shoot fresh weight (7.2 ± 0.5 g) and dry shoot weight1(3.18 ± 0.02 g), root fresh weight (3.4 ± 0.1 g) and dry root weight (0.58 ± 0.1 g). The control plants had the least growth.

Table 6: Effect of Trichoderma spp on maize seedlings growth. control seedlings without Trichoderma; CR-TS1; CR-TS3; CR-TS4 CR-TC1: maize seedlings treated with Trichoderma spp. the mean ± standard deviation values followed by the same letters indicate that the groups in question do not differ from each other according to the Fisher's test ($p < 0.05$).

T. isolates	Seedling emergence %	Plant height (cm)	Roots length (cm)	Fresh Shoot (g)	Dry Shoot (g)	Fresh Root (g)	Dry Root (g)
CR-TS1	91.6±16.6a	73.6 ±4.6b	19.4± 0.8b	7.2 ± 0.5b	3.18 ± 0.02b	3.4± 0.1b	0.58± 0.01b
CR-TS4	74.9±31.9a	66.3 ±5.0ab	18.5 ±0.9b	6.6± 0.45bc	2.83 ± 0.11ac	3.1 ± 0.1b	0.44± 0.02b
CR-TS3	83.3±19.2a	58.3 ± 2.5ab	18.7± 0.5b	5.23 ± 0.58ac	3.01 ± 0.11bc	3.2± 0.1b	0.56 ± 0.03b
CR-TC1	91.6±16.6a	68.3 ± 1.2ab	18.4 ±0.5b	6.72± 1.14bc	3.17 ± 0.07b	3.2 ± 0.1b	0.57± 0.01b
CONTROL	91.6±16.6a	53.0 ± 6.1a	14.7 ±0.4a	4.75 ± 0.38a	1.92 ± 0.07a	1.7± 0.04a	0.16 ±0.01a

The findings demonstrate that the utilization of a trichoderma spore suspension significantly enhanced the growth of maize seedlings under greenhouse conditions.

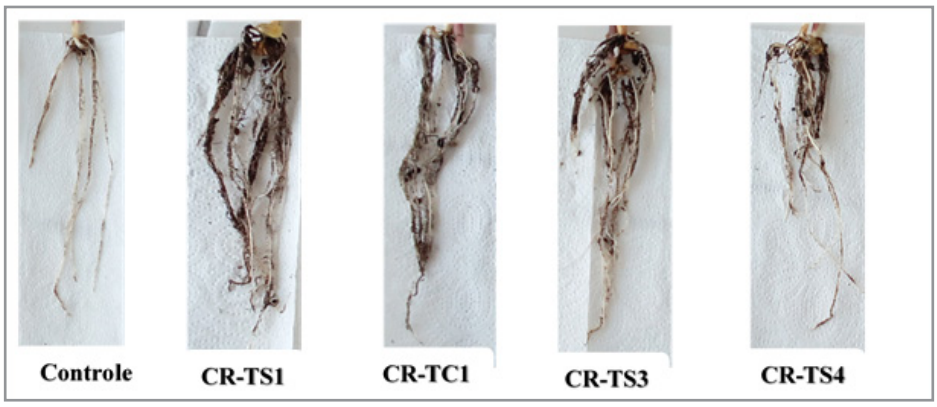


Figure 8: Effects of Trichoderma isolate on the growth of maize seedlings growth. control plants (plants without trichoderma); CR-TS1; CR-TS3; CR-TS4; CR-TC1 inoculated/treated plants

Discussion

In the present study, the rhizospheric soil of maize enabled the isolation of four Trichoderma isolates, designated CR-TS1, CR-TS3, CR-TS4, and CR-TC1. The isolates were identified macroscopically (colony color, colony growth, and texture) and molecularly (using ITS 1/4 and RPB2 sequences). The isolate CR-TS1 was identified as T. hazianum, the isolates CR-TS3, CR-TS4, and CR-TC1 were T. ghanense. Additionally, Aspergillus isolate from maize seed was identified as Aspergillus flavus. The four Trichoderma isolates demonstrated the capacity to inhibit the growth of Aspergillus flavus isolate CR-MW in vitro, utilizing the three methods employed for this purpose (Dual-culture, the preparation of the culture medium using extracts of non-volatile Trichoderma sp. compounds, followed by the inoculation of the pathogen onto this medium, and remote confrontation). The current in vitro study has elucidated the multifaceted activities of Trichoderma isolates, which exhibit ancestral attributes and are therefore valuable for managing phytopathogens. These attributes include mycoparasitism, competition for space and soil nutrients, antibiosis through the production of both volatile and non-volatile antifungal compounds and cell membrane-degrading enzymes [39, 40].

In these tests, it was observed the Trichoderma isolates grew faster than A. flavus, and in certain Petri dishes, they grew above A. flavus colonies while producing spores in the dual culture test. Due to their rapid growth and reproduction, Trichoderma isolate seized nutrients present in the culture medium while occupying space and consuming oxygen and air. Also, this process involves trichoderma utilizing the pathogen's mycelium as a source of nutrients. This mechanism is a common feature of several species of trichoderma, which are known to combat plant pathogens. Its efficacy has been demonstrated by [41-43]. This would have weakened the pathogen and caused its inhibition during dual-culture.

Furthermore, demonstrated that multiple Trichoderma isolates produce secondary antimicrobial metabolites, including trichomycin, gelatimycin, chlorotricomycin, pantaibol, flavonoids, phenolic compounds, and antibiotics [44, 45]. These metabolites have the potential to be associated with cell membrane degradation enzymes, facilitating the inhibition of pathogen growth. the various compounds produced by trichoderma could be responsible for inhibiting the pathogen when it is inoculated into the culture medium containing Trichoderma isolates extract and even during the dual culture. Similar outcomes were observed by in their investigation using different trichoderma, which showed that trichoderma has a high inhibition power against Aspergillus sp [15, 46, 26]. At the end, the results highlight the potential of the four Trichoderma isolates and have demonstrated effectiveness in the role of biocontrol agent against A. flavus.

The hydrolytic enzyme secretion test (amylase, protease, lignase) showed that the four Trichoderma isolates were able to produce hydrolytic enzymes at different levels. The secretion of hydrolytic enzymes by beneficial microorganisms is very important as it makes it easier to colonize plant rhizosphere, for the degradation of organic matter, enabling them to express their symbiotic effect and indirect biological control mechanisms by stimulating the host's immune response, providing not only the plant but also the beneficial microorganisms receive the nutrients essential for their growth and development. They also participate in the process of biocontrol by facilitating the degradation of the cell wall of the pathogen and inactivating the enzymes secreted by the pathogen to damage the plant [47-49]. This result is similar to that obtained by who reported that amylase production enables trichoderma to proliferate at a faster rate than phytopathogens in PDA, which may be a contributing factor to its superior growth [50].

The positive phosphate solubilization test, production of siderophore, and IAA provide evidence that the Trichoderma isolates can improve plant growth promotion. A significant portion of soil phosphate is not immediately accessible to plants. The capacity of microorganisms, such as trichoderma, to solubilize phosphate has garnered considerable attention, particularly in the context of the quest for environmentally-friendly agriculture [51]. Phosphate is widely regarded as a pivotal element in agricultural production, with a multitude of essential functions and a profound impact on plant growth. Its role in nodule formation, cell division, organization, and the development of flowers and fruits has been well documented [52].

Three potential mechanisms by which trichoderma could facilitate the conversion of phosphate into a soluble form were proposed. (i) acidification, (ii) production of chelating metabolites, and (iii) redox activity and concluded that chelation was the most likely mechanism for Phosphate solubilisation by trichoderma [53]. Microorganisms such as trichoderma secrete organic acids that dissolve phosphate minerals or directly chelate cations from the phosphate ion, thus releasing phosphate [54].

Production of siderophore by trichoderma exerts a pivotal influence on the process of biological control., as the trapped iron leads to the inactivation of the pathogen's enzymes by the formation of cofactors which supply it to the tall plant, thereby promoting its growth [55].

It has been reported that the synthesis of indole-3-acetic acid (IAA) by Trichoderma isolates is typically contingent upon the availability of its precursor, L-tryptophan. Additionally, the production of IAA is influenced by abiotic factors, such as temperature and pH [56-57].

The use of microbial agents to control plant diseases has been the subject of scientific investigation for a considerable period, with numerous research studies attesting to the ability of fungal-derived indole-3-acetic acid (IAA) to facilitate plant growth and development through direct physiological or biochemical mechanisms. This finding is supported by the research presented in reference [58]. In the same way, fungi are able to secrete plant growth-promoting substances, such as indole-3-acetic acid (IAA), which, in turn, induce systemic resistance mechanisms. in plants to suppress phytopathogenic strains, and disease development and to prevent pathogen attack has demonstrated that low concentrations of IAA stimulate root elongation, whereas high concentrations of IAA are responsible for the proper morphogenesis of lateral and adventitious roots [59, 56]. Similarly, the phosphate solubilization of both siderophore and indole-3-acetic acid (IAA), was observed in the research conducted by [60, 61, 55] which employed a distinct species of trichoderma.

The application of a suspension comprising 107 spores/ml Trichoderma isolates conidia to maize seeds yielded statistically significant ($p < 0.05$) results, with a notable augmentation of germination rates observed in the treated Petri dishes compared to the control Petri dishes. The current findings align with those previously published by who reported the greatest effect on the 1 × 107 spores / mL treatment of maize seeds with Trichoderma spp, observed that tomato seeds treated with T. harzianum (T22) exhibited enhanced germination rates compared to untreated tomato seeds [62, 63].

Moreover, the results of our study 21 days after inoculation with Trichoderma isolates in a pot showed enhanced growth ($P < 0.05$) seedling emergence, plant height, root lengths, fresh shoot and roots, dry shoot and roots, and chlorophyll matter under greenhouse conditions compared with control. The results suggest that the four trichoderma isolates have the potential for successful seed treatment applications and the capacity to establish beneficial interactions with plant roots. This could contribute to enhanced plant growth and protection against pathogens in agricultural ecosystems. Indeed, various species of trichoderma have been demonstrated to possess the potential for formulating biostimulants and biofertilizers, as evidenced by studies conducted by [58, 64, 14].

These findings are consistent with previous studies that demonstrated that the inoculation of a commercial Trichoderma sp. to the cultivation of Pisum sativum L. (Fabaceae) led to a notable enhancement in its growth and development [65]. This was observed to impact a range of physiological variables, including germination, leaf area, root dry weight, root fresh weight, root dry weight, leaf fresh weight, and root length, which collectively contributed to an increase in the yield of the crop when the same treatment was applied to the seed, observed that, in contrast to the growth of the control plants, the treatment of the plants with T. asperellum TC01 increased various growth characteristics [66].

These included shoot height, stem diameter, shoot, and root fresh weights, as well as shoot and root dry weights. The aforementioned observations were made 45 days after inoculation under greenhouse conditions [67]. demonstrated that inoculation with T. viride GT-8, T. reesei GT-31, and T. longibrachiatum GT-32 Trichoderma markedly enhanced growth parameters by over 45% relative to seedlings that did not receive inoculation. These results corroborate the hypothesis previously put forward by, which postulated that inoculation of plants with trichoderma leads to the manifestation of growth-promoting characteristics, including the synthesis of indole-3-acetic acid (IAA), which stimulates root growth and enhances water and nutrient absorption. Furthermore, the solubilization of phosphate and the production of siderophores in the soil are also facilitated by trichoderma. In addition to these direct effects, it can be hypothesized that this fungus possesses other abilities to indirectly promote plant growth [68].

Conclusion

The utilization of Trichoderma isolates as a biological agent for the control of plant pathogens and the enhancement of soil fertility can potentially replace or reduce the use of agrochemicals, thereby decreasing the negative ecological impact. As part of the study, the tested isolates of trichoderma were found to inhibit the growth of Aspergillus sp. using a variety of mechanisms involved in biological control and the properties of PGPs, including diffusible and volatile metabolite production, enzyme secretion (hydrolytic), siderophore production, IAA synthesis, and phosphate solubilization.

The findings of the in vitro and in vivo tests presented herewith demonstrated that the Trichoderma isolates under investigation are effective microorganisms for use in maize cultivation as a sustainable solution for inhibiting the growth of aflatoxin-producing fungi and improving performance. Nevertheless, further research is necessary to ascertain the fundamental interaction mechanisms involved in growth promotion and the biological control of aflatoxigenic agents. For this case, it is crucial to conduct fieldwork experiments to ascertain the efficacy of these isolates in controlling aflatoxigenic fungi, stimulating plant growth, and increasing agricultural yields. The findings of these studies will provide invaluable insight into the practical implementation and efficacy of biological inputs derived from our Trichoderma isolates, offering a sustainable and environmentally conscious approach to agricultural management.

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Author Contributions

MENYE was responsible for the initial drafting of the research project, sampling, laboratory work, and analyses, as well as the subsequent drafting of the manuscript. Sinem and Nida contributed to the drafting of the protocol and provided supervision with regard to laboratory handling. The BEGOUDE and BOYOMO were administered and conceptualized the projects. Cigdem provided essential reagents, supervised laboratory manipulations, and offered invaluable feedback on the manuscript. All authors have read and approved the final manuscript.

Conflict of Interests

The authors certify that they have no conflicts of interest.

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