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Table: 1, Figs: 3

Impact of endemic *Trichoderma asperellum Samuels, Lieckf and Nirenberg* strains on the yield and quality of garlic (*Allium sativum* L.)

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ABSTRACT

This study aimed to investigate the effects of 3-isolates of *Trichoderma asperellum* on the yield of pink garlic (*Allium sativum* L.) and its secondary metabolites. Yield parameters (bulb weight, clove weight, and number of cloves), were measured. Additionally, polyphenol flavonoids and antioxidant activity were determined, and functional components were identified using FTIR. The experiment consisted of 3 following treatments: T1 (TMSKOLDZ20), T2 (TMS11DZ15) and T3 (TMS5DZ150). The results indicated that the treatment T1 of *Trichoderma* isolate was the most effective in promoting garlic yield, while the treatment T3and T2 of *Trichoderma* isolates improve the total phenol, total flavonoid and antioxidant activity in garlic cloves. Further more only the treatment T3 showed additional absorption region compared to treatments. In conclusion, *Trichoderma* isolates tested in this study have the potential as bio-stimulants for promoting yield and secondary metabolites.

Key words: *Allium sativum*, FTIR analysis, Secondary metabolites, *Trichoderma* spp., pink garlic, yield quality

INTRODUCTION

Garlic (*Allium sativum*, *Alliaceae* family) is an aromatic herbaceous annual spice and is one of the oldest and most widely consumed bulbs after onion. The bulbs of *A. sativum* contain hundreds of phytochemical compounds, [allicin, ajoene, diallylsulphide, dithin, S-allylcysteine, enzymes, vitamin B, proteins, minerals, saponins, and flavonoids (6)]. Its total phenols and sulphur compounds possess several biological activities [antioxidant, antibacterial, antifungal, and anticarcinogenic properties (11)]. Chemical fertilizers are used to increase its yields. However, it has adverse effects, such as the accumulation of harmful chemical residues in the soil, which leads to environmental pollution and pose risks to ecosystems and human health (7).

Trichoderma is a globally distributed fungal genus that has significant ecological and agricultural importance. It is well known for its positive effects on plants as biofertilizers, biological agents and bio-stimulants (3). This effect is attributed to various mechanisms, including the solubilization of phosphates, micronutrients, and minerals. *Trichoderma* indirectly controls both major and minor root-infesting pathogens in the rhizosphere (14,26). Moreover, it enhances the nutritional quality of plants by activating their defence response, resulting in higher accumulation of secondary metabolites that promote human health (21).

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The effectiveness of *Trichoderma* in agriculture depends on several factors [environmental conditions, cultivation type, inoculum dosage and preparation, and the complex communication between plants and Trichoderma (5,29). However, there is limited information available on the interactions and effects of *Trichoderma spp*. on garlic growth promotion and secondary metabolism activation. This study aimed to investigate the impact of three specific *Trichoderma* species on enhancing garlic yield and secondary metabolites. The goal is to identify sustainable and economically viable approaches that can enhance crop efficiency through innovative agricultural practices.

MATERIALS AND METHODS

The experiment was conducted in greenhouse, Department of Biotechnology and Agroecology, University of Blida1, from November 2021 to July 2022 without controlled conditions. The experiment consisted of 3 following Treatments:

Treatment	***Trichoderma asperellum* strains**
T1	TMSKOLDZ20
T2	TMS11DZ15
T3	TMS5DZ15

These strains were obtained from Prof Saida Messgo-Moumene (15) Medicinal and Aromatic Plants Laboratory. Conidial suspensions of *Trichoderma* spp. were prepared by adding sterile distilled water to 15-days-old pure cultures of each isolate (grown on PDA medium and incubated at 28°C) the suspensions were collected separately in sterile test tubes. The spore concentrations were determined using a Malassez cell under an optical microscope at 400X magnification and adjusted to $3x10^7$ spores/ml with sterile distilled water.

Pink garlic cloves were sterilized with a 0.05 % sodium hypochlorite solution for 3 min, rinsed with sterilized water, and then planted individually in pots filled with 3 kg soil. Each clove was inoculated with a 20ml conidial suspension of *Trichoderma spp.* A completely randomized design was used with twelve pots serving as replicates for each treatment. Uninoculated pots served as the control. The plants were watered as necessary. During the harvest, six plants from each treatment were evaluated for bulb weight, clove weight and the number of cloves per bulb.

Spectroscopic analysis by FTIR:

Translucent sample discs were prepared by encapsulating 0.5 to 1.5 mg of peeled, sterilized and dried garlic powder within a100 mg KBr pellet and were loaded into the FTIR spectroscope. The FTIR spectral analysis was conducted through opus 6.5 software from TENSOR 27/BOKER(12).

Extract preparation:

Garlic samples from each treatment were peeled and rinsed with distilled water. They were then extracted separately using methanol according to Akullo *et al.* (4). Specifically, 25g of fresh garlic was mixed with 100 ml of methanol, and the mixture was shaken at 300 rpm on a mechanical shaker in the dark for 24 h. The solution was filtered

using Whatman filter paper No.1. The resulting filtrate was concentrated by rotary evaporation at 40 °C and stored at 4 °C for further analysis.

Phenolic compounds: The total polyphenol and flavonoid contents were determined from the methanolic extract of fresh garlic cloves.

Total polyphenols: The total phenol content in garlic cloves extracts was determined using the Folin-Ciocalteu assay, as described by Safdar *et al.* (24)

Methanolic solution of garlic cloves extract at 10 mg/ml concentration were prepared for analysis, 0.5 ml of the methanolic extract solution was mixed with 2.5 ml of 10 % Folin-Ciocalteu reagent that had been dissolved in distilled water and 2.5 ml of 7.5 % sodium carbonate, blank solution comprised 0.5 ml of methanol, 2.5ml of ten-fold diluted Folin-Ciocalteu reagent, and 2.5 ml of 7.5 % sodium carbonate, the samples were incubated at 25 °C for 30 min to elicit the development of blue colour. The absorbance was assessed at a wavelength of 765 nm through the application of a UV spectrophotometer; the calibration curve was prepared using varying concentrations of Gallic acid. The overall phenol content was quantified as mg Gallic acid equivalent GAE/g of extract. Similar procedure was followed for the Gallic acid standard solution.

Total flavonoids: The total flavonoids content was estimated using the method described by Woisky and Salatino (18). Specifically, 0.5 ml of the sample was mixed with 0.5 ml of 2 % ethanol solution of AlCl3. The resulting mixture was allowed to stand for 1 hour. At room temperature, the absorbance was measured at 420 nm to calculate the total flavonoid content as quercetin using a calibration curve.

Antioxidant activity: The DPPH assay was used to determine the antioxidant activity of garlic cloves extracts using the method proposed by Katalinic *et al.* (13). A dilution DPPH 1×10^{-4} M was prepared in methanol, 2 ml of DPPH solution were mixed with 1 ml of samples at different concentrations of 0.1, 0.5, 1 and 2 mg/ml with three replicates per sample and concentration. The mixture was incubated in dark at ambient temperature for 16 min, the absorbance was measured at 517 nm using a UV-30 spectrophotometer, and the blank simple used was methanolic dilution of DPPH. Standard ascorbic acid was also analyzed in similar way.

Statistical analysis

The study results were statistically analyzed using Minitab version19 software. A one-way analysis of variance (ANOVA) was conducted, followed by Tukey's test to determine significant differences ($P \leq 0.05$) (22). The results are presented as the mean ± standard deviation of four replicates (22).

RESULTS AND DISCUSSION

Yield promotion

The effect of treatments of *Trichoderma* on the weight of garlic bulb, clove and the number of cloves were presented in Figure 1. The statistical analysis demonstrated a significant variability ($P = 0.00$) between the treatments and the control, indicating that the treatments have a distinct effect on the measured parameters related to garlic yield. The treatment T1 significantly increased the weight of garlic bulb by 77 % compared to control. This suggested that the application of *Trichoderma asperellum* TMSKOLDZ20

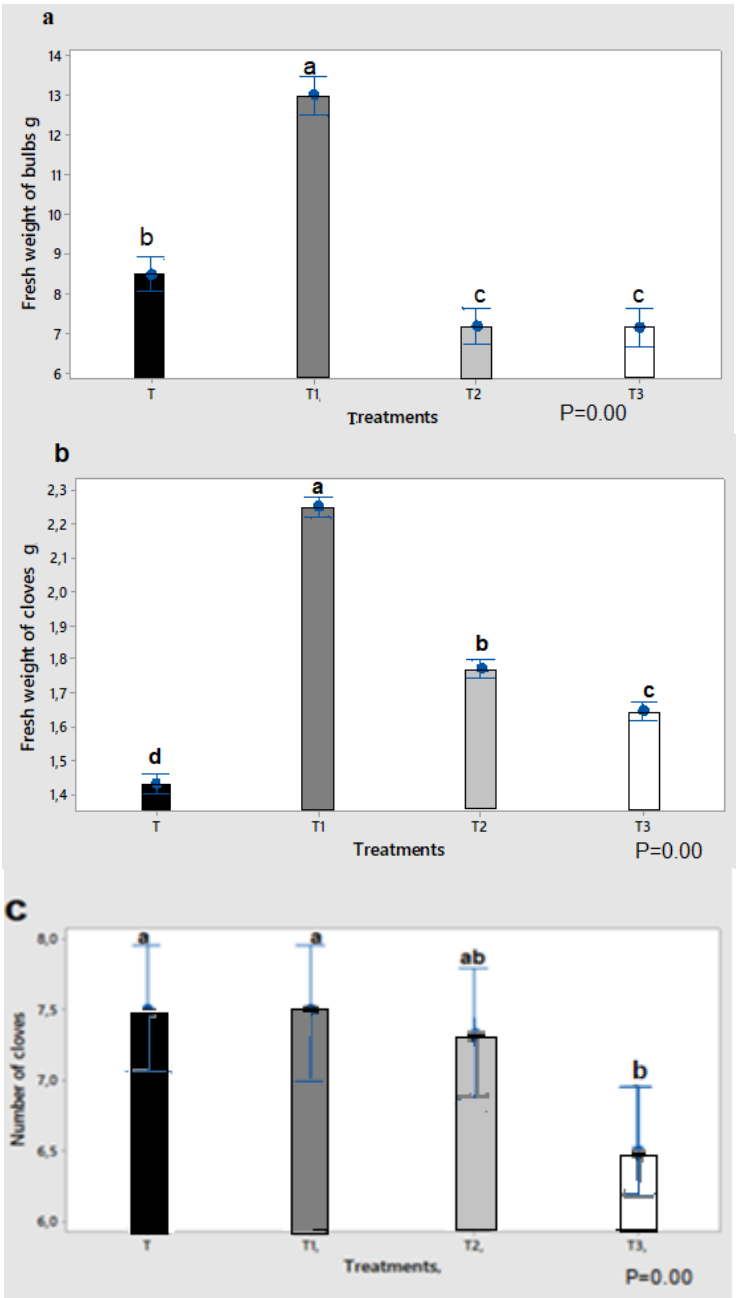


Figure 1. Effects of three isolates of *Trichoderma asperellum* on yield parameters of garlic: weight bulb (1a), cloves weight (1b) and cloves number (1c). Values followed by the same letter do not differ significantly. The results are expressed mean ± standard deviation. (T: control, T1,T2,T3 different treatments of *Trichoderma asperellum*).

effectively promoted the yield of garlic bulbs. Conversely, Treatments T3 and T2 decreased the garlic weight bulb than control.

In terms of cloves, treatment T1 showed no effect in the number of garlic cloves, while T2 and T3 treatments decreased cloves number compared to the control, all treatments T1, T2 and T3 exhibited an increase in cloves weight by 56.67 %, 23 % and 15 % respectively, over the control, indicating *Trichoderma* TMSKOLDZ20 (treatment T1) was the most effective in promoting garlic yield.

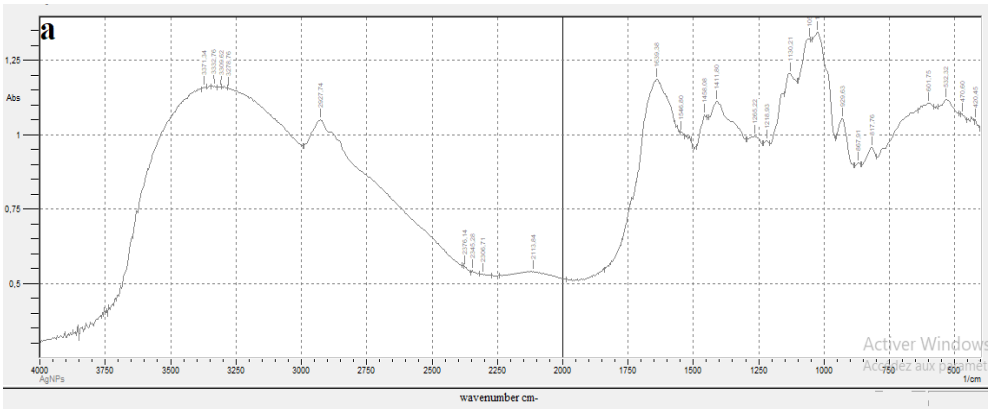
Fourier Transform Infrared (FTIR) analysis

The Fourier transform infrared analysis showed the presence of different functional groups, as evident from the distinct vibrations observed in the IR spectrum of garlic samples. The absorption region of functional groups exhibited spectral variability: shifts in band positions with appearance/disappearance of peaks, and variations in absorption intensity than control. The infrared region between 4000 and 400 cm^{-1} displays eight absorption regions for the T2 and T1 spectra of the treated samples, the samples T3 and the control group represent different vibrations, as shown in (Figure 2).

All treated samples displayed eight absorption ranges, except the control and T3 samples. The control sample showed an additional absorption between 2400-2000 cm^{-1}, while the T3 sample showed an additional absorption between 1870-1650 cm^{-1}.

In terms appearing and disappearing of peaks across different spectra, significant variability was observed in the spectra recorded for samples T3 and T2 in the absorption intervals between 3650 and 3200 cm^{-1}, sample T3 exhibited substantial variability in the absorption intervals between 3000-2800 cm^{-1}, 1870-1650 cm^{-1}, 1300-1100 cm^{-1}, and below the 500 absorption region. Considerable variability was observed in the absorption intervals of 2400-2000 cm^{-1}, 1500-1300 cm^{-1}, 600-700 cm^{-1} and 600-500 cm^{-1} in samples T3, T2 and T1. Furthermore, samples T2 and T1 exhibited significant variability in the absorption interval of 1650-1550 cm^{-1} compared to the control.

Similarity was recorded for sample T1 in the absorption interval of 3650-3200 cm^{-1} as well as, for samples T2 and T1 in the absorption intervals of 3000-2800 cm^{-1}, 1870-1650 cm^{-1}, 1300-1000 cm^{-1}, 700-600 cm^{-1} and below 500,similarity were observed also for samples T1, T2 and T3 in the absorption intervals of 2400-2000 cm-1, and for sample T3 in the absorption interval 1650-1550cm^{-1}.



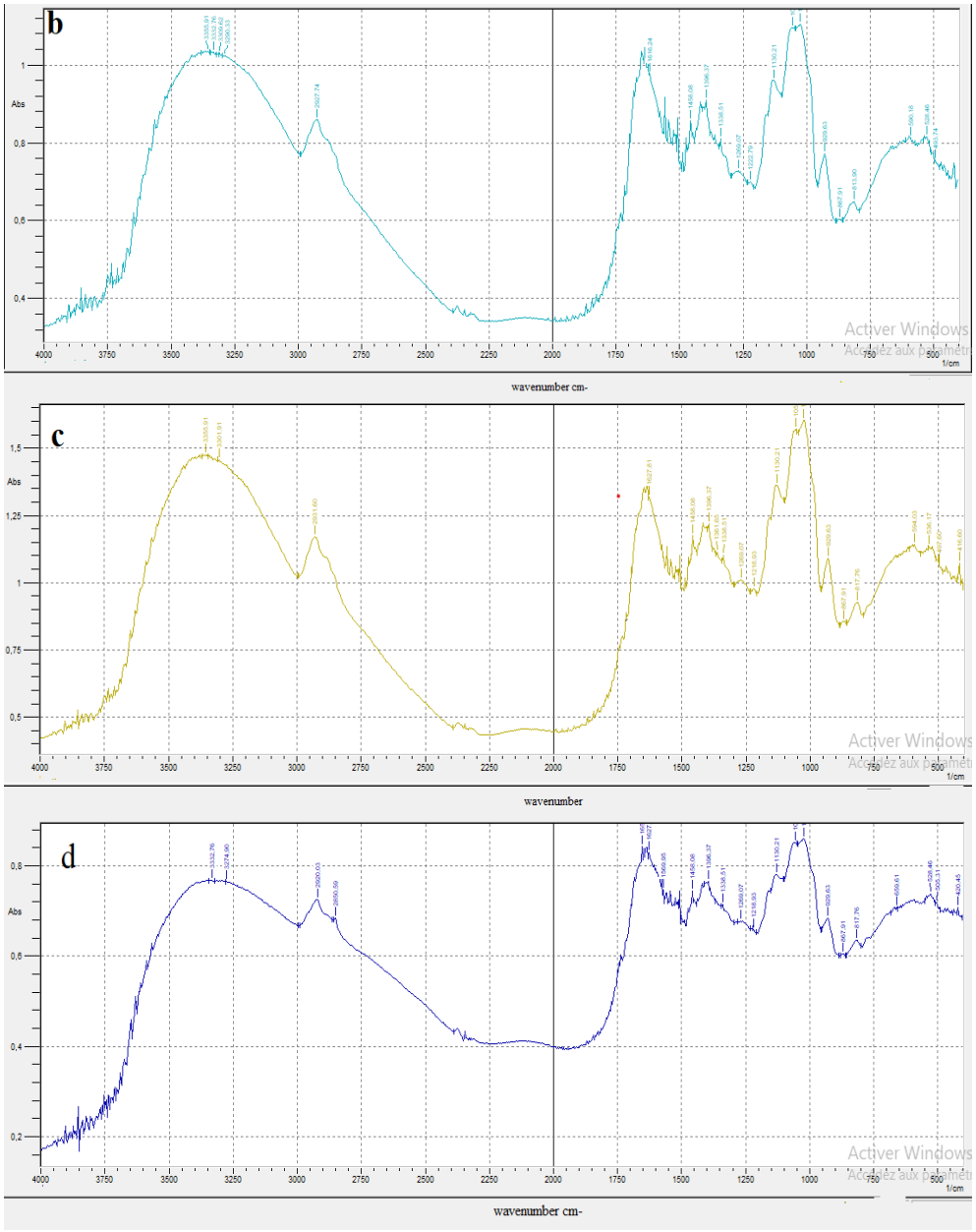


Figure 2. FITR Test Result of garlic powder samples treated with *Trichoderma* spp, (2a) control sample, (2b) sample treated with treatment T1, (2c) sample treated with treatment (T2), (2d) sample treated with treatment (T3).
(T: Control, T1,T2,T3 different treatments of *Trichoderma asperellum).*

The absorption intensity varied among the samples, with control sample showed the highest intensity, followed by the treated sample T3, and then the samples T2 and T1.

Total Phenolic and total Flavonoid Contents and Antioxidant Activity:

Total phenolic and total flavonoid Contents and Antioxidant activity were assessed to examine the impact of *Trichoderma* isolates on the quality of garlic. The statistical analysis indicated significant difference (P =0.00) in the levels of total polyphenol and total flavonoids between the *Trichoderma* isolates and the control sample. This difference was further confirmed by the Tukey test, which resulted in distinct alphabetical classifications for each group.

The results of the study indicate that the application of Treatments T2 and T3 to garlic plants led to a significant increase in their polyphenol and flavonoid contents. Specifically, the polyphenol content increased to 22.163mg/1g FW and 20.997 mg/1g FW, while the flavonoid content increased to 0.06 mg/1g FW and 0.056 mg/1g FW, respectively. Conversely, the application of T1 isolate significantly decreased the phenolic content 13.303 mg/1g FW, with no significant difference in flavonoid content 0.0386 mg/1g FW in garlic bulbs than control17.470 mg/1g FW, 0.042 mg/1g FW respectively (Figure 3a and 3b).

Our study suggested that methanolic extract of garlic biomolecules possess a significant ability to reduce DPPH free radicals, indicating strong antioxidant activity. *Trichoderma* isolates T3 and T2 increased antioxidant activity of garlic extracts compared to control, while the isolate T1 decreased the antioxidant activity of garlic extract compared to control. Garlic extracts treated by isolates T3, T2, T1 and control inhibited the DPPH radical by 60.5 % and 59 % 53 % and 55 % respectively at 2000 µg/ml however the ascorbic acid used as reference inhibited the DPPH radical by 97.5 % at 2000 µg/ml.

The analysis of variance revealed significant difference (P=0.00) in the IC_{50} values of the antioxidant activity of the methanolic extract between the treatments and the control. The garlic extract treated with T3 isolate showed the lowest and most important IC_{50} value of 0.43 µg/ml, compared to the control's value 0.9 µg/ml. This was followed by T2 isolate with 0.65 µg/ml, and T1 isolate with 1.65 µg/ml. however those values couldn't reach the value of ascorbic acid 0,01 µg/ml (Figure 3c).

Our results revealed that different treatment of *Trichoderma spp*. had varying effects on garlic yield parameters, among the treatments, only Treatment T1 showed significant positive effects. This is consistent with previous research that has demonstrated the diverse effects of different *Trichoderma* strains on various host plants. For instance, Stewart *et al*. (28) tested four strains of *T. longipile* for their ability to promote lettuce seedling growth and found varying growth responses, with one isolate failing to promote any growth, as well as both tested onion varieties exhibited an increase in bulb mass, when treated with *T. asperellum* isolates To and Tt. However, *T. asperellum* isolate Tm did not affect the bulb mass of either onion variety (18). Which confirmed that all isolates are not capable of promoting plant growth, and the degree of growth promotion achieved is also influenced by the genetic variability amongst *Trichoderma* isolates. The positive effects of *Trichoderma* probably due to its ability to improve nutrients uptake and transport in plants, produces growth hormones and other beneficial compounds that stimulate plant growth and development. Additionally, *Trichoderma* acts as a soil conditioning agent, improving the diversity and concentration of beneficial microorganisms in the soil (1,2).

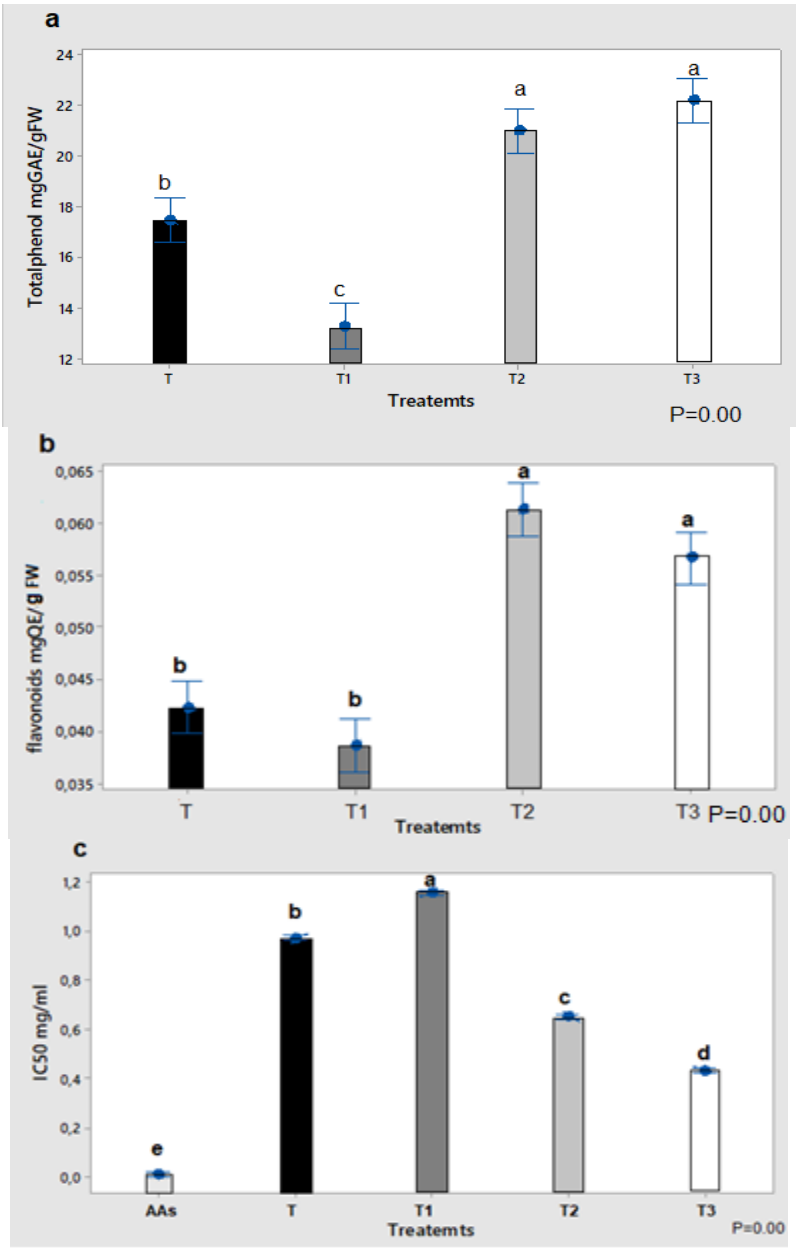


Figure 3. Impact of *Trichoderma spp* isolates on the levels of phenolic (3a) and flavonoids compounds (3b) and IC50 of antioxidant activity (3c) in garlic methanolic extracts. Values followed by the same letter do not differ significantly. The results are expressed mean ± standard deviation. (AAs: ascorbic acid, T: control, T1,T2,T3 different treatments of *Trichoderma asperellum*).

The preliminary phytochemical screening of the crude garlic powder revealed the presence of various phytochemicals, including phenolic compounds with alcohol bonds exhibiting O-H or N-H stretching vibrations, as well as proteins and polysaccharides containing aliphatic primary amine. These were detected in the region between 3650-3200 cm^{-1}. D'Souza *et al.* (9,33).

Between 3000-2800 cm^{-1}, symmetrical and asymmetrical C-H elongation vibrations of methylene (CH2) were also detected. These vibrations are mainly attributed to lipids, carbohydrates, and nucleic acids(33). -C≡N, -N=N+=N- and -C≡C- stretching vibrations were observed between 2400-2000 cm^{-1} region (32,33). The Amides stretching vibrations of the peptide and the vibrations of flavonoids and their derivatives (32,34) and (10) were detected between 1870-1650 cm^{-1}

The bending vibrations of C-H functional groups, including CH3, CH2 and CH, were observed between the range of 1500-1300 cm^{-1}(24). Additionally, the functional groups CH3, CH2, C-O-C, C-OH, S=O, P=O, and C-F were observed in the range of 1300-1100 cm^{-1} (34).

C=C, C=Nand NH stretching vibrations were observed between 1650-1550(34)

Elongation vibrations of bromo-aliphatic were observed between the region 700-600 cm^{-1} (22), according to Nandiyanto *et al.* (17) stretching vibration aliphatic iodinated alkyl halide compounds were observed between 600-500 cm^{-1}as well as C-C bending below 500 absorption region (33).

The IR spectrum of the treated sample T3 displayed an additional absorption region between 1870-1650 cm^{-1}, assigned to C=O stretching vibrations of the peptide (Amides I) and flavonoids and their derivatives which explains their high contents of phenolic compounds (10), which provides better antioxidant activity than other treatments and control

The garlic control sample also displayed an additional absorption region between 2400-2000 assigned to nitrile and azide groups (32). This could indicate the potential of Trichoderma strains in bioremediating these compounds, which explain the high radiation absorption in the control sample, despite the low antioxidant activity compared to the treated sample T3 (23).

The infrared spectra similarities indicate similarities in chemical composition, differences in band shapes and absorption intensities can be explained by changes in chemical characteristics resulting from the application of Trichoderma. These results are supported by Wei *et al*. (31), who reported changes in the FTIR spectra of treated wheat leaves attributed to the effect of static magnetic field (SMF) treatments on the molecular composition and structure of the leaves.

Inoculation with *Trichoderma* isolates T3 and T2 increased the synthesis of flavonoids and phenolic compounds in garlic bulbs. Many authors have reported the positive effect of *Trichoderma* on the accumulation of polyphenols and flavonoids in various plants, such as onion bulbs, *Passiflora caerulea* L., beans, grapes, tomatoes, and olive leaves (8,30). In contrast to the *Trichoderma* isolate T2 showed a decrease in phenolic compounds and no effect on flavonoid compounds than control. Vukelić *et al*.'s research (30) supports this finding, indicating that the use of *T. harzianum* reduces the total phenolic compounds in both types of tomato cultivars *Narvik* and *Gružanskizlatni* and explained this variations in polyphenol and flavonoid content of tomato fruit depend on the

tomato variety. This variation could be due to genetic differences, as well as different environmental stress conditions and agricultural practices that affect the chemical composition of plants. While Ortega-García (18) reported that the accumulation of phenolic compounds in response to Trichoderma species has been associated with biochemical protection against plant pathogens.

In garlic methanolic extracts of control sample and inoculated samples, DPPH free radical scavenging inhibition and IC_{50} were significantly lower than the standard. These results correlated to many researchers funding, including Akullo *et al.*'s (4) Otunola, and Afolayan (19).

Our results demonstrated significantly higher antioxidant activity compared to Akullo *et al*.'s (4) findings. In their study, garlic ethanolic extract inhibited free radical scavenging at 1mg/ml by 39.53 % and at 10 mg/ml by 81.24 %. However, our findings are consistent with Ourouadi *et al.* (20) research, where garlic cultivars Tetouan, Fez, and Ben Hamden inhibited DPPH free radical scavenging by 60.85 %, 59.79 % and 51 %, respectively. Several factors contribute to the variation in results, including genotype, regional effects, cultivation techniques, environmental conditions, and variations in methodology and experimental conditions used in different investigations (16).

The applied Trichoderma strains have a significant impact on the antioxidant activity of garlic. This finding is in line with previous research. For instance, Singh (27) reported that the extract of tomato fruit taken from Trichoderma-treated plants exhibited significantly higher scavenging activity on DPPH radicals than the control. Similarly, Șesan (25) found that the highest concentration of the Trichoderma plant bio-stimulant consortium (108 cfu/mL) resulted in the highest antioxidant activity in both assays (DPPH and TEAC).

Application of treatments T2 and T3 exhibited higher antioxidant activity than treatment T1 possibly due to increased total phenol and flavonoid accumulation. This result was supported by Vukelić (30) who reported that the use of *Trichoderma harzianum* as a biocontrol agent in tomato cultivation can have varying effects on tomato plant antioxidant activity depending on the specific compounds involved.

CONCLUSIONS

This study indicated that the evaluated isolates of *Trichoderma* spp. have the potential to serve as promising bio-stimulants for enhancing both the yield and quality of garlic in sustainable and organic farming systems. The mixture of these isolates can enhance garlic production sustainably and with high quality, supporting environmentally friendly agricultural practices and holding promise for the industrial valorisation of garlic.

DECLARATION

We declare that all authors of this manuscript made a significant contribution and we have not excluded any author that substantially contributed. We have followed the ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

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