RESEARCH ARTICLE

Investigation of anthraquinone-producing Trichoderma reesei by high-performance liquid chromatography (HPLC)

Zeyad Khalaf Hussein[1], Ghada Basil Alomashi[2], Khalied Yassen Zakair[3]

Abstract

Objective: To investigate the isolates of *Trichoderma* that produce anthraquinones by High-performance Liquid Chromatography (HPLC).

Methods: *Trichoderma* specimen were collected from Wassit Soil and identified dependent on morphological features and tested for production of anthraquinones by HPLC, *Trichoderma* isolate which produced a high concentration of anthraquinones diagnosed for the species using PCR-ITS region.

Results: Ten isolates were identified as *Trichoderma* according to morphological and microscopic features. Results three *Trichoderma* isolates show differences between the concentrations of anthraquinones among the ten isolates. The total concentration of this compound in the extracts of specimens 1, 2 and 3 were (7.765µg/ml), (2.308µg/ml), and (4.977µg/ml) respectively. At the final concentration of *Trichoderma* isolates, genomic DNA have been extracted (400 to 600 µg) / (2 to 3g) fresh mycelium, and with a concentration of (1.6 to1.8), and the results of amplifying *Trichoderma* DNA samples by using ITS-1 and ITS-4 showed a single unique band consistent with *T. reesei F48-03*, Which with other isolates of the *Trichoderma* were missing, were identified successfully.

Conclusion: For identification and phylogenetic classification of *Trichoderma*, DNA-based methods that provide useful classification information are presently used. For several years, most *T. spp*. is regarded as a single species due to their morphological similarity. This research used ITS markers to distinguish genotypes within *T. spp.* because of amplifying a distinct, naturally determined locus with a couple of *T.reesei* -specific oligonucleotide primers. This research was carried out to provide supporting evidence for the long-standing antimicrobial use of anthraquinone.

Keywords: DNA, Primers, Trichoderma, Phylogeny, Soil, Chromatography, Liquid, Polymerase, Reaction, Anthraquinones, Mycelium, Plant, Genomics. **DOI: https://doi.org/10.47391/JPMA.IQ-25**

Introduction

Many crop pathogens have been successfully managed through the use of Trichoderma species in field trials. These fungi are free-living and highly interactive in root, soil, and foliar environments.[1-5] The fungus Trichoderma has been identified as a possible source of bioactive molecules. There are different mechanisms that have been reported as being responsible for their biocontrol activity. These mechanisms include chitinolytic enzyme secretion, resource competition, mycoparasitism, and inhibitory compound production are all possible outcomes. *Trichoderma* species produced chemicals that can either kill bacteria or prevent their growth such as antibiotics and anthraquinone.[6-9] However, Trichoderma spp. are categorized according to the morphology of their colony structures and is not enough to divide and isolate, necessitating many molecular techniques for verification of organisms, like sequencing tests.[10,11] These are (RAPD) random amplification of polymorphic DNA and (ISSR) inter-simple sequence repeat.[3,12] *Trichoderma* species have evolved pathways that create a special secondary metabolite which can generate a wide range of bioactive molecules.[2] Canvassers can produce naturally derived products for pharmaceutical, agricultural, and industrial applications by leveraging the diverse chemical profiles of *Trichoderma Spp.*[2] The present study was planned to evaluate the *Trichoderma* species to productiveness of Anthraquinone compounds.

Material and methods

Identification of *Trichoderma harzianum* isolates: Twenty-five samples of soil were collected from various depths (4-5 cm) of Wassit soil using a metal spatula that was repeatedly sterilized in 70% alcohol. New polythene bags were used to transport the isolates to the research laboratory for the mycological analysis as soon as they were collected. A conical bottle (250 ml) containing (100 ml) of distillated sterile water was filled with ten-gram soil samples. In order to obtain a homogenous suspension, a series of dilutions of the soil isolate (10^{-1}, 10^{-2}, and 10^{-3}) were prepared by shaking the flask on an electric shaker. Each sample was diluted by adding 1 ml to a petri dish of potato dextrose agar medium at a ratio of 10^{-3}. (OXOID, England). Seven days were spent in an incubator set to 28.2°C.[13] Fungi were grown in multiple colonies on PDA plates before being subcultured and transferred to ensure a genetically

[1,2] Department of Medical Microbiology, University of Al Qadisiyah, Iraq;
[3] Kut Technical Institute, Middle Technical University, Baghdad, Iraq.
Corrospondience: Zeyad Khalaf Hussein email: med.ph.20.18@qu.edu.iq

uniform strain. Tobacco molds (Trichoderma spp. colonies were examined under a microscope and given a diagnosis based on their morphology (morphological characteristics).[14,15] These included conidiophore, pallid and conidium arrangements, while cultural structures included linear development, growth patterns, colony colour, and hyphae pigmenting. The fungus has successfully detected numerous habitats through cultivation media because of genetic, nutritional, and environmental factors.

Detection of anthraquinones producing by *Trichoderma* using HPLC: To test the production of anthraquinones in crude extracts, modified Czapek's Dox Liquid medium (Oxoid, UK); Sugarcane bagasse (150 mg per litter) was used. Isolates of Trichoderma were first grown on PDA for seven days. Next, two blocks of Trichoderma agar culture (10 mm) were added to the modified czapek's media (500 ml) in flasks and incubated at 30⁰ in the dark with shaking for ten days. Antifungal compounds were extracted from modified Czapek's media by adding 500 ml of the organic solvent ethyl acetate (EtOAc) and shaking the mixture at 121 revolutions per minute (overnight). After a 24-hour period of inactivity, the extraction was successful. Considering the evaporation temperature of the solvents (EtOAc, 77.1°C) and the boiling point of the extracts (37°C), a rotary evaporator (Gallen hamp., England) was used to extract the antifungal compounds.[16]

High-performance liquid chromatography (HPLC) test has been used to detect the production of anthraquinones and for the samples (*Trichoderma* isolates) that produce a higher concentration of anthraquinones. The samples of the extract were loaded with an anthraquinones reference standard (Sigma-Aldrich, chemie Gmb H, USA). The operation of (H.P.L.C) was, stationary phase: pursuit xfs 3µ C-18 250*4.6mm diameter particles 5 µm, varian, mobile phase: acetonitrile: water (50:50), rates of flow (0.8ml/min), temperature:35°C, wavelength: 280nm, sample volume : 20 µl.

DNA extraction: *Trichoderma* isolate selected to diagnose for the species using PCR-ITS region based on anthraquinones production. The mycelia of *Trichoderma* isolate were inoculated and cultured into PDA broth at (28±2°C) for five days. The DNA genomes extracted from *Trichoderma* isolate with (Purification of genomic DNA was performed using the Wizard Genomic DNA Purification Kit (Promega, USA).

Amplifying the *ITS* Gene by PCR: Both the ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) primers are known to be effective (Integrated DNA Macrogen, Korea) applying to the amplification of the internal transcribed spacer (ITS) gene. The PCR reaction volume was (25l) and contained (3l) of DNA, (12.5l) of Master Mix (Promega, Madison, WI, USA), (1 µl) of each primer (20 p mol.), and deionized DW. up to (25 µl). A C1000 TouchTM Thermal Cycler was used to warm the contents of the tubes (Bio-Rad, Munich, Germany) at 95⁰C for 5 min, for DNA amplification. Consequently, Taq polymerase has been added, followed by thirty cycles of the 30 second at 95⁰C, 30 second at 55⁰C, and 30 second at 72°C, with a final extension at 72°C for 7 minutes. The use of Tris-borate-EDTA buffer for electrophoresis1% agarose gel was analyzed for amplified DNA products. Ethidium bromide (10 µg−ml−1) was used for stained gels. DNA Ladder (1500 bp.) RTU (Gene Dire X ®, München, Germany) have been used as a standard. UV illumination conceived of DNA

Aligning and Sequencing Data: The PCR products' sequences were all sent to a Sanger sequencer (ABI3730XL, Automated DNA Sequencer; Macrogen Corporation, Korea) for analysis. The data was analyzed using genius software, The nucleotide BLAST programme was used to find sequence homology between the PCR fragments and the Gene Bank database, and the sequencing data was correlated with this resource (http://www.ncbi.nlm.nih.gov/BLAST/). All sequences were submitted to the Gene Bank at the National Center for Biotechnology Information (NCBI) and are available there with the accession number AB13730XL.

Results

Identification of *Trichoderma* isolates: Initially, from a total of 25 soil samples cultures in this study, 10 samples tested positive for *Trichoderma* isolates, according to morphological and microscopic features.[17] The result of High-performance Liquid Chromatography showed that three *Trichoderma* isolates produced anthraquinones differences between the concentrations of secondary metabolic compound (anthraquinones). Among the three isolates, specimen 1, specimen 2, and specimen 3 were (7.765µg/ml), (2.308µg/ml), and (4.977µg/ml) respectively.

Measurement of the total content of anthraquinones extracts, using the HPLC method revealed that the highest total concentration was present in sample 1 (7.765µg/ml), sample 3 (4.977µg/ml), while sample 1 (2.308µg/ml) was the minor compound. HPLC peaks of this compound for *Trichoderma* showed in the (Figure 1).

For this Trichoderma isolate, the nuclear DNA region encompassing the ITS1, ITS4, and 18S rRNA gene was amplified for a thorough molecular characterization. The ITS regions amplified have been successfully in strain, resulting in PCR a unique product size of approximately 650 base pairs, which have been specifically sequenced. The size of the resulting amplicon from this region was between (650bp), (Figure 2).

The PCR and gene sequence data suggest that *Trichoderma reesei* is present. The BLAST programme's alignments of this species showed 99.9 percent identification and 0.0 percent gaps with the universal isolate (Figure 3). Differences between strains, as well as the locations of the ITS sequences, are exposed (Figure 4).

A phylogenetic tree has been developed using MEGA 5.1 software to create *Trichoderma reesei* ITS nucleotide sequences that have been cloned. The strains were divided into one different sub-clades of phylogenetics (Figure 5). Group 1 excluded isolates of *Trichoderma* 8 (*Trichoderma reesei)*, *T. orientale* MK 020687, *T. reesei* KY031342, *T. longibrachiatum KY568698*, and *T. longibrachiatum* MF164178.

Discussion

Initially, from a total of 25 soil samples cultures in this study,

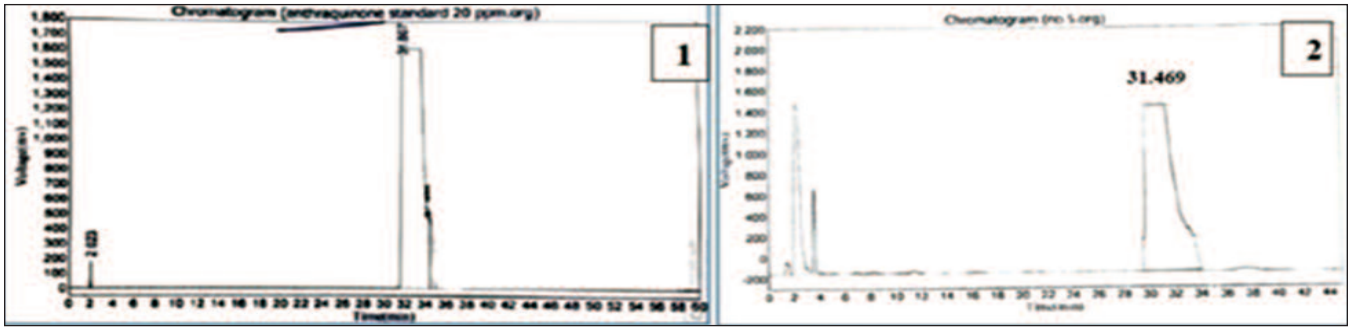

Figure 1: HPLC profile of Anthraquinones 1. Anthraquinones standard. 2. Anthraquinones extract.

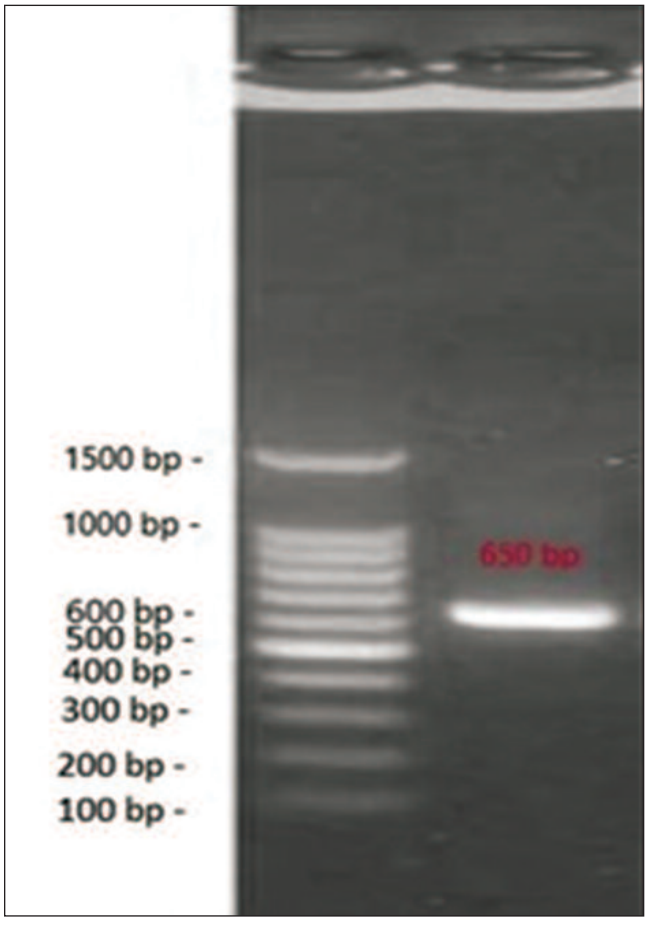

Figure 2: Agarose gel electrophoresis of PCR product for Trichoderma isolates the band size (~650 bp.). The product was electrophoresis on 2% L: DNA ladder (1500), lane 1: *Trichoderma sp*

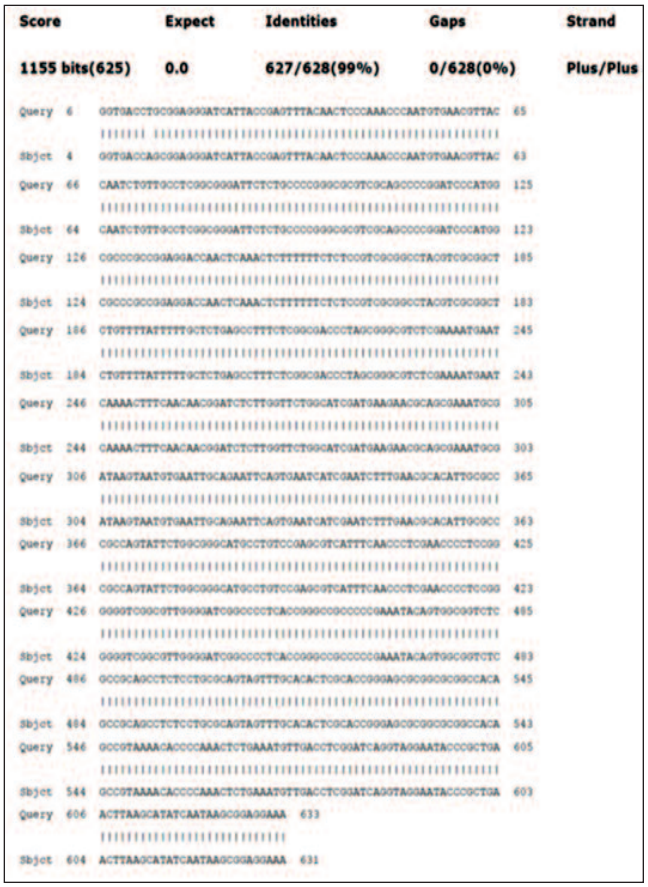

Figure 3: Trichoderma reesei strain F48-03 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene,and internal transcribed spacer2,complete sequence; and large subunit ribosomal RNA gene.

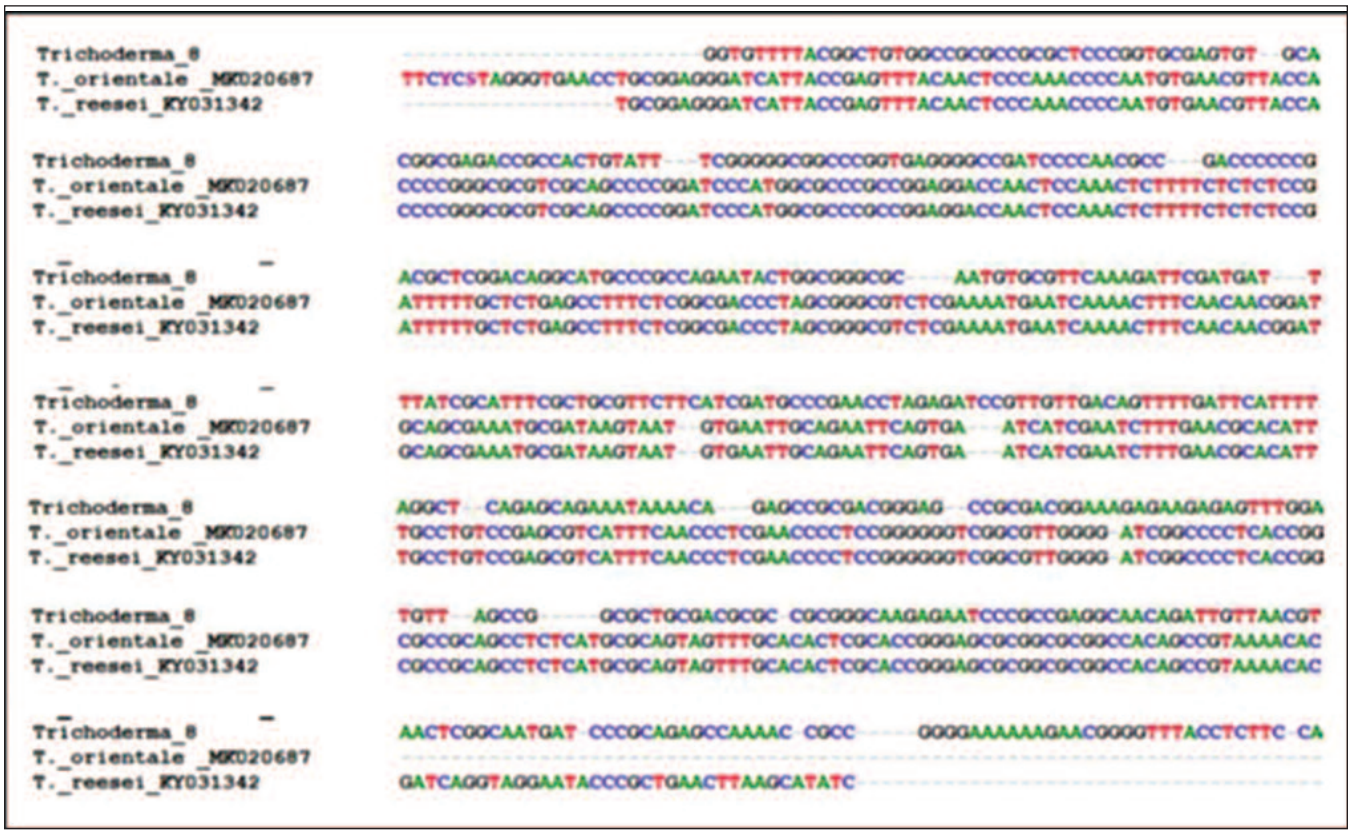

Figure 4: The polymorphic nucleotide sites within Trichoderma reesei isolates identified by ITS gene.

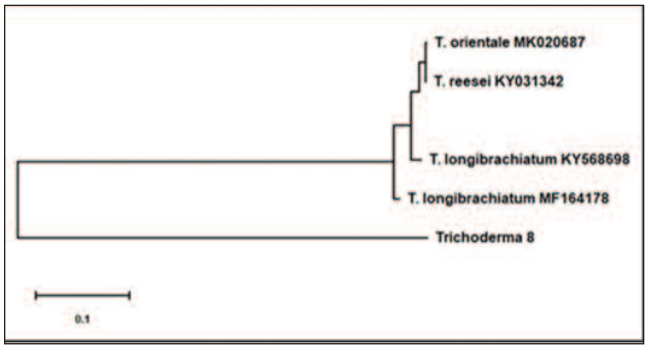

Figure 5: Neighbour-joining phylogeny based on ITS gene sequences of two selected Trichoderma isolates and related Trichoderma reesei obtained from a BLAST search of the NCBI.

10 samples tested positive for *Trichoderma* isolates, according to morphological and microscopic features.[17]

T. reesei is one of the most researched *Trichoderma* species[18,19] predominantly due to its broad application in biotechnology.[20,21] In these results, the *Trichoderma reesei* ITS sequences region, which were described morphologically as identified in a study[22] have been confirmed. The amplicon obtained of this region was in size range of (~650 bp.). This result is in agreement with that of Shamkhi et. al.[23] Using the ITS nucleotide sequences, a phylogenetic tree was constructed that distinguished *T. reesei* in contrast to other strains. Similarly, Kuhls and colleagues in their study used sequence analysis to differentiate between *T. reesei* and *T. longibrachiatum*.[24] In order to classify the various species of Trichoderma, ITS sequences have been utilized by several other researchers.[17,11]

Conclusion

For identification and phylogenetic classification of *Trichoderma*, DNA-based methods that provide useful classification information are presently used. For several years, most *T. spp*. were regarded as a single species due to their morphological similarity. This research used ITS markers to distinguish genotypes within *T. spp*. as a result of amplifying a distinct, naturally determined locus with a couple of *T. reesei* -specific oligonucleotide primers.

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References

1. Cao ZJ, Qin WT, Zhao J, Liu Y, Wang SX, Zheng SY. Three new Trichoderma species in harzianum clade associated with the contaminated substrates of edible fungi. J Fungi 2022;8:1154. doi: 10.3390/jof8111154.
2. Ayyandurai M, Akila R, Manonmani K, Mini ML, Vellaikumar S, Brindhadevi S, et al. Combined application of Trichoderma longibrachiatum T (SP)-20 and Trichoderma asperellum T (AR)-10 in the management of stem rot of groundnut. Legum Res 2023;46:215-21.
3. Šašić Zorić L, Janjušević L, Djisalov M, Knežić T, Vunduk J, Milenković I, et al. Molecular Approaches for Detection of Trichoderma Green Mold Disease in Edible Mushroom Production. Biology 2023;12:299. doi: 10.3390/biology12020299.
4. Stracquadanio C, Quiles JM, Meca G, Cacciola SO. Antifungal Activity of Bioactive Metabolites Produced by Trichodermaasperellum and Trichodermaatroviride in Liquid Medium J Fungi (Basel) 2020;6:263. doi: 10.3390/jof6040263.
5. Mohan PN. Studies on antagonistic potential of Trichoderma spp. from saline soils. Agricultural University; 2017. [Online] 2017 [Cited 2023 August 30]. Available from URL: http://krishikosh.egranth.ac.in/handle/1/5810030745
6. Tyśkiewicz R, Nowak A, Ozimek E, Jaroszuk-Ściseł J. Trichoderma: The Current Status of Its Application in Agriculture for the Biocontrol of Fungal Phytopathogens and Stimulation of Plant Growth. Int J Mol Sci 2022;23:2329. doi: 10.3390/ijms23042329.
7. Khan RAA, Najeeb S, Mao Z, Ling J, Yang Y, Li Y, et al. Bioactive Secondary Metabolites from Trichoderma spp. against Phytopathogenic Bacteria and Root-Knot Nematode. Microorganisms 2020;8:401. doi: 10.3390/microorganisms8030401.
8. Keswani C, Singh HB, Hermosa R, García-Estrada C, Caradus J, He YW, et al. Antimicrobial secondary metabolites from agriculturally important fungi as next biocontrol agents. Appl Microbiol Biotechnol 2019;103:9287-303. doi: 10.1007/s00253-019-10209-2.
9. Khan RAA, Najeeb S, Hussain S, Xie B, Li Y. Bioactive Secondary Metabolites from Trichoderma spp. against Phytopathogenic Fungi. Microorganisms 2020;8:817. doi: 10.3390/microorganisms8060817.
10. Mendoza JL, Pérez MI, Prieto JM, Velásquez JD, Olivares JG, Langarica HR. Antibiosis of Trichoderma spp strains native to northeastern Mexico against the pathogenic fungus Macrophomina phaseolina. Braz J Microbiol 2015;46:1093-101. doi: 10.1590/S1517-838246420120177.
11. Fahmi AI, Eissa RA, El-Halfawi KA, Hamza HA, Helwa MS. Identification of Trichoderma spp. by DNA barcode and screening for cellulolytic activity. J Microb Biochem Technol 2016;8:202-9. doi: 10.4172/1948-5948.1000286
12. Rezaee Danesh Y, Pellegrini M, Kariman K, Boyno G, Djebaili R, Farda B, et al. Genetic Diversity of Trichoderma harzianum Isolates in Sunflower Rhizosphere: The Application of the URP Molecular Marker. Sustainability 2022;14:15111. doi: 10.3390/su142215111
13. Dwiastuti ME, Fitriasari PD. Exploration of Trichoderma spp. and fungal pathogen that causes a strawberry anthracnose and examination of in vitro antagonistic activity. Biotika 2017;18:58-68.
14. Natsiopoulos D, Tziolias A, Lagogiannis I, Mantzoukas S, Eliopoulos PA. Growth-promoting and protective effect of Trichoderma atrobrunneum and T. simmonsii on tomato against soil-borne fungal pathogens. Crops 2022;2:202-17. Doi:10.3390/crops2030015
15. Iqbal S, Ashfaq M, Malik AH, Khan KS, Mathew P. Isolation, preservation and revival of Trichoderma viride in culture media. J Entomol Zool Stud 2017;5:1640-6.
16. Soliman IA, Hasanien YA, Zaki AG, Shawky HA, Nassrallah AA. Irradiation impact on biological activities of Anthraquinone pigment produced from Talaromyces purpureogenus and its evaluation, characterization and application in beef burger as natural preservative. BMC Microbiol 2022;22:325. doi: 10.1186/s12866-022-02734-4.
17. Guzmán-Guzmán P, Kumar A, de Los Santos-Villalobos S, Parra-Cota FI, Orozco-Mosqueda MD, Fadiji AE, et al. Trichoderma species: Our best fungal allies in the biocontrol of plant diseases—A review. Plants 2023;12:432. Doi: 10.3390/plants12030432
18. Schmoll M, Seiboth B, Druzhinina I, Kubicek CP. Genomics Analysis of Biocontrol Species and Industrial Enzyme Producers from the Genus Trichoderma. In: Nowrousian M, eds. Fungal genomics, 2nd ed. Berlin, Germany: Springer, Berlin, Heidelberg, 2014; pp 233-64.
19. Schmoll M, Dattenböck C, Carreras-Villaseñor N, Mendoza-Mendoza A, Tisch D, Alemán MI, et al. The genomes of three uneven siblings: footprints of the lifestyles of three Trichoderma species. Microbiol Mol Biol Rev 2016;80:205-327. doi: 10.1128/MMBR.00040-15
20. Gudynaite-Savitch L, White TC. Fungal biotechnology for industrial enzyme production: focus on (Hemi) cellulase production strategies, advances and challenges. In: Schmoll M, Dattenböck C, eds. Gene Expression Systems in Fungi: Advancements and Applications, 1st ed. Switzerland: Springer Cham, 2016; pp 395-439.
21. Arnau J, Yaver D, Hjort CM. Strategies and challenges for the development of industrial enzymes using fungal cell factories. In: Nevalainen H, eds. Grand Challenges in Fungal Biotechnology, 1st ed. Switzerland: Springer Cham, 2020; pp 179-210.
22. Zhao R, Mao LJ, Zhang CL. Three new species of Trichoderma (Hypocreales, Hypocreaceae) from soils in China. MycoKeys 2023;97:21-40. doi: 10.3897/mycokeys.97.101635.
23. Shamkhi IJ, AL-araji AM. Investigation of Emodin-producing Trichoderma isolates by High-Performance Chromatography (HPLC). Biochem Cell Arch 2021;21:4737-42.
24. Kuhls K, Lieckfeldt E, Samuels GJ, Meyer W, Kubicek CP, Börner T. Revision of Trichoderma sect. Longibrachiatum including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. Mycologia 1997;89:442-60. Doi: 10.2307/3761038