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Molecular characterization of plant growth-promoting *Trichoderma* from Saudi Arabia

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Fungi in the genus *Trichoderma* are widespread in the environment, mainly in soils. They are used in agriculture because of their mycoparasitic potential; *Trichoderma* have the ability to increase plant health and provide protection against phytopathogens, making them desirable plant symbionts. We isolated, identified, and characterized *Trichoderma* from different regions of Saudi Arabia and evaluated the ability of *Trichoderma* to promote plant growth. Morphological and molecular characterization, along with phylogenetic studies, were utilized to differentiate between *Trichoderma* species isolated from soil samples in the Abha and Riyadh regions, Saudi Arabia. Then, plant growth-promoting traits of the isolated *Trichoderma* species were assessed. Eight *Trichoderma* isolates were characterized via morphological and molecular analysis; six (*Trichoderma koningiopsis*, *Trichoderma lixii*, *Trichoderma koningii*, *Trichoderma harzianum*, *Trichoderma brevicompactum*, and *Trichoderma velutinum*) were from Abha and two (*T. lixii* and *T. harzianum*) were from Riyadh. The isolated *Trichoderma* strains belonged to three different clades (Clade 1: Harzianum, Clade 2: Brevicompactum, and Clade 3: Viride). The *Trichoderma* isolates varied in plant growth-promoting traits. Seeds treated with most isolates exhibited a high percentage of germination, except seeds treated with the T3-*T. koningii* isolate. 100% germination was reported for seeds treated with the T4-*T. harzianum* and T6-*T. brevicompactum* isolates, while seeds treated with the T1-*T. koniniopsis* and T5-*T. lixii* isolates showed 91.1% and 90.9% germination, respectively. Seeds treated with the T8-*T. velutinum*, T2-*T. lixii*, and T7-*T. harzianum* isolates had germination rates of 84.1%, 82.2%, and 72.7%, respectively. The *Trichoderma* isolate T5-*T. lixii* stimulated tomato plant growth the most, followed by T7-*T. harzianum*, T8-*T. velutinum*, T4-*T. harzianum*, T1-*T. koniniopsis*, T2-*T. lixii*, and T6-*T. brevicompactum*; the least effective was T3-*T. koningii*. A maximum fresh weight of 669.33 mg was observed for the T5-*T. lixii*-treated plants. The Abha region had a higher diversity of *Trichoderma* species than the Riyadh region, and most isolated *Trichoderma* spp. promoted tomato growth.

Keywords Plant growth promotion, *Trichoderma*, Seed germination, Tomato, Riyadh, Abha, Phylogenetic analysis

The agricultural systems of Saudi Arabia have significantly improved during the last 10 years. Despite the common perception that Saudi Arabia is a desert, there are several areas where cultivation is possible. The Kingdom of Saudi Arabia is moving forward with plans (Vision 2030) to develop the agricultural sector because of its direct impact on food security. By doing so, the Kingdom attaches importance to issues of food and water security, agricultural development, and environmental sustainability. According to the Food and Agriculture Organization (2009), the global population will reach 9.7 billion by 2050, and difficulties in meeting human food needs are expected due to the effects of climate change, a shrinking agricultural land area, and the degradation of the environment and natural resources, including the loss of numerous biodiversity components that are crucial to achieving sustainable agricultural production[1]. Research on fungal biodiversity within the genus *Trichoderma* and characterization of plant growth-promoting fungi is lacking in this region. Salinity and temperature are important factors in the agriculture of Saudi Arabia and plant growth-promoting fungi such as *Trichoderma* spp., which can tolerate high temperatures and salinities, are advantageous to agricultural production in this region. Considering the potential for *Trichoderma* species to increase plant growth and control phytopathogenic fungi, the present characterization of *Trichoderma* biodiversity was undertaken.

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Trichoderma is a globally dispersed, ubiquitous genus in the family Hypocreaceae, and *Trichoderma* fungi may be found in various soil types and root ecosystems, particularly those rich in organic materials. *Trichoderma* fungi reproduce asexually by producing conidia and chlamydospores and by producing ascospores in their natural habitats. Some of the most beneficial effects of *Trichoderma* spp. on plants include the control of minor infections, the delivery of dissolved nutrients, increased nutrient intake, increased glucose metabolism and photosynthesis, phytohormone production, bioremediation of heavy metals and environmental contaminants, and use in xenobiotic bioremediation[1–3] *Trichoderma* spp. can promote host plant resistance to a variety of biotic and abiotic stresses; they improve plant resistance to environmental challenges, including salt and drought, by stimulating plant growth, reprogramming gene expression in roots and shoots, maintaining nutritional uptake, and activating protective mechanisms to prevent oxidative damage[4–6].

Applying *Trichoderma* spp. to seeds, seedlings, and pathogen-free soils has been shown to stimulate plant growth[6]. Symbioses occur between crops and soil microorganisms, such as plant growth-promoting rhizobacteria (PGPR) and plant growth-promoting fungi (PGPF), which are both natural biostimulants. *Trichoderma* spp. that are PGPF have been used commercially to suppress phytopathogens such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Armillaria mellea*, and *Chondrostereum purpureum*[7]. However, the antagonistic capability and biostimulant action of *Trichoderma* vary greatly, resulting in strains with predominant biostimulant action and others with predominant agonistic action[8]. As a result, some *Trichoderma* strains are better suited for biological control as biopesticides, while others are better suited for boosting crop growth and nutrient uptake as biostimulants[9] *Trichoderma* species are effective mycoparasites that produce numerous secondary compounds, many of which have clinical significance[9]. Additionally, they have the ability to detect, penetrate, and destroy other fungi and certain nematodes, which contributes to their commercial success as biopesticides (more than 60% of all registered biopesticides contain *Trichoderma*).

Trichoderma species are distinguished by their rapid growth, ability to assimilate a wide range of substrates, and ability to produce a variety of antimicrobial agents. *Trichoderma* species synthesize siderophores as secondary metabolites with antibacterial properties that inhibit the growth of soil pathogens by scavenging iron and inactivating iron-dependent enzymes; thus, the fungi reduce plant disease and enhance plant growth[10,11]. Several *Trichoderma* species also have the ability to synthesize phytohormones and phytoregulators, including indole-3-acetic acid (IAA), which is important for plant growth and development[12,13]. Moreover, *Trichoderma* increase the bioavailability of phosphorus by breaking down insoluble phosphate in the soil via phytases, which facilitate and enhance the uptake of nutrients by plants. Previous studies have documented the strain-dependent growth-promoting effect of *Trichoderma* spp. on various plants, as well as the ability of different *Trichoderma* spp. to provide protection against plant diseases[14,15]. Attempts to understand the diversity and geographical distribution of *Trichoderma/Hypocrea* have resulted in global observations of the genus[16], but unfortunately, there have been few studies of *Trichoderma* in Saudi Arabia[7,17–21]. The goal of this study was to isolate and characterize *Trichoderma* strains from different regions of Saudi Arabia and to evaluate their growth-promoting effects on plants.

Materials and methods

Sample collection and *Trichoderma* isolation

Soil samples were collected at six sites each in the Abha and Riyadh regions, Saudi Arabia. Seventy-two soil samples were collected from all six sites in each region, Riyadh and Abha. Nineteen Trichoderma strains were obtained from these soil samples. The soil dilution plate method was used for the isolation of fungi[7,22]. The morphological and colony characteristics of *Trichoderma* isolates were studied in potato dextrose agar (PDA) (HIMEDIA, India), medium and *Trichoderma* selective medium (TSM), following previous studies[23,24]. The macro characteristics (colony radius, pigments, green conidia, odor, and colony appearance) and microcharacteristics (phialide, conidium, and presence of chlamydospores) were observed.

Determination of physical and chemical soil properties

A pH meter was employed to measure the soil pH. For pH measurement, a soil suspension with a ratio of 1:2.5 (soil to water) was prepared and shaken for an hour[25]. Additionally, an electric conductivity meter assessed the electrical conductivity (EC) in the soil's saturated paste extract. Meanwhile, soil moisture content (MC) was determined by oven drying at 103 °C for 12 h. The following equation calculates the percent moisture content in soil:

$$\mathrm{MC}\ (\%) = (\mathrm{Wf} - \mathrm{Wd}/\mathrm{Wd}) \times 100$$

where:

- MC (%) represents the moisture content in percentage.
- (Wf) is the weight of fresh soil.
- (Wd) is the weight of soil after drying in the oven.

To estimate organic matter (OM), the loss of ignition method was used[26]. The following equation calculates the percent organic matter in soil.

$$\mathrm{OM}\ (\%) = (\mathrm{W2} - \mathrm{W3})\,/\,(\mathrm{W2} - \mathrm{W1}) \times 100$$

where OM (%) is percent organic matter in soil, W1 = Weight of the crucible, W2 = Weight of the crucible + oven dry sample, W3 = Weight of the crucible + oven dry sample after ignition.

To determine whether the Trichoderma strains selected for the studies were nonpathogenic, a plant pathogenicity test was conducted. Twenty healthy two-week-old tomato plants were inoculated with a suspension of *Trichoderma* strain (1×10^7 CFU/ml), and the roots were inoculated via the root-dip method. Inoculated plants were transplanted singly into steam-sterilized peat moss soil and sand mixed at a 5:1 ratio. Seedlings

inoculated with sterilized water (1 ml per plant) served as controls. After one week, the plants were observed for any visible symptoms. The pathogenicity test was conducted twice.

Molecular identification of and phylogenetic analysis of isolated *Trichoderma* species

DNA extraction, PCR amplification, and molecular identification of species of *Trichoderma* were performed[27].

Phylogenetic analysis

For phylogenetic characterization of the isolated *Trichoderma* strains, the relevant downloaded sequences (Table 1) were aligned using Clustal W-pairwise sequence alignment of the EMBL nucleotide sequence database. The sequence alignments were trimmed and verified by the MUSCLE (UPGMA) algorithm using MEGA11 software, Auckland, New Zealand A phylogenetic tree was reconstructed, and the evolutionary history was inferred using the neighbor-joining method. The robustness of the internal branches was assessed with 500 bootstrap replications. Evolutionary distances were computed using the maximum composite likelihood method and were calculated in units of the number of base substitutions per site.

Biochemical characterization of plant growth-promoting *Trichoderma* spp.

The isolated *Trichoderma* strains were assessed for phosphate solubilization and IAA, ammonia, and siderophore production.

Phosphate solubilization activity

The ability of 8 Trichoderma isolates to solubilize and mineralize phosphate (P) in vitro was evaluated, and qualitative screening of phosphate solubilization was performed on Pikovskaya agar medium (HIMEDIA, India)[28].

Indole-3-acetic acid (IAA) production

For the quantitative estimation of IAA, DF salts in minimal media supplemented with L-tryptophan at a concentration of 1.02 g/l were prepared (HIMEDIA, INDIA)[29,30].

Ammonia production

Freshly grown *Trichoderma* isolates were cultured in peptone water (HIMEDIA, India) broth in test tubes at 28 °C for 2 days[31].

Siderophore production

Modified chrome azurol S (CAS) agar (HIMEDIA, India) with King's media (Kings Media, Kochi, Kerala) (pH 6.8) was used[11].

In vivo evaluation of the effect of *Trichoderma* isolates on tomato plant growth

The plant growth-promoting activity of the *Trichoderma* isolates was assessed by analyzing the seed germination and seedling growth of tomato plants[31].

Name	Code	Accession no.
1. *Trichoderma koningiopsis*	T1	Isolated species
2. *Trichoderma lixii*	T2	Isolated species
3. *Trichoderma koningii*	T3	Isolated species
4. *Trichoderma harzianum*	T4	Isolated species
5. *Trichoderma lixii*	T5	Isolated species
6. *Trichoderma brevicompactum*	T6	Isolated species
7. *Trichoderma harzianum*	T7	Isolated species
8. *Trichoderma velutinum*	T8	Isolated species
9. *Trichoderma viride*		MZ078774.1
10. *Trichoderma koningiopsis*		ON795065.1
11. *Trichoderma koningii*		EU280128.1
12. *Trichoderma virens*		KU729029
13. *Trichoderma atroviride*		JQ745258
14. *Trichoderma harzianum*		OL597950.1
15. *Trichoderma lixii*		JF923802.1
16. *Trichoderma brevicompactum*		KR094463.1
17. *Trichoderma velutinum*		HM176565.1
18. *Trichoderma longibrachiatum*		MH707326
19. *Fusarium oxysporum*		MT151384

Table 1. List of *Trichoderma* species used for phylogenetic analysis.

Effect of Trichoderma isolates on seed germination

Tomato seeds (Roma VF) were purchased from a local market (Salam Street) in Riyadh, Saudi Arabia. All methods were performed in accordance with the relevant guidelines/regulations/legislation.

Fifty tomato seeds of uniform size were surface sterilized with 0.5% NaClO (Xilong Scientific Co., Ltd., China), for 5 min and washed five times with sterile water. Ten seeds were transferred to Petri plates covered with a layer of cotton and filter paper. A spore suspension of the *Trichoderma* isolates (10^5 spores/ml) was poured over the seeds (100 µl/seed), and the plates were cultured for 7 d at 28 °C under 12 h/12 h light/dark conditions. Seeds treated with an equivalent volume of sterile water were used as controls. At the end of two weeks, the germination rate was calculated using the following equation:

$$\text{Percent seed germination } (\%)\ (\text{Gs}) = \text{Ts} \times 100$$

where Gs = the number of seeds germinated, and Ts = the total number of seeds[31].

Effect of Trichoderma isolates on tomato plant growth

Tomato seeds were surface sterilized, treated with *Trichoderma*, and sown in pots containing 5 g of sterile soil. The pots were watered as required with sterile water. After 2 weeks, the seedlings were treated (0.5 ml) with *Trichoderma* strains (10^5 spores/ml), allowed to grow for two weeks, treated again with *Trichoderma* strains (10^5 spores/ml), and grown for an additional 2 weeks. Seedlings were watered with sterile water as needed. Six weeks after germination, the experiment was terminated, and the plants were uprooted. The plants were removed from the soil, and the roots were washed carefully under running water. The plant shoot height, root length, and wet weight were measured. The plants were then dried at 105 °C for 30 min and subsequently at 50 °C for 24 h, and plant dry weight was recorded[32,33].

Results

After soil samples were collected from 6 locations in the Abha and Riyadh regions, PDA medium and TSM were used to isolate *Trichoderma* species. Twenty *Trichoderma* strains were isolated from soil samples collected from the Abha region, and 12 *Trichoderma* strains were isolated from soil samples collected from the Riyadh region. Subsequently, the isolates were purified and identified microscopically. The isolated *Trichoderma* strains were classified into six groups based on colony and morphological characteristics. A total of 16 isolates were selected for pathogenicity testing. Eight out of 34 isolates were selected for pathogenicity testing on tomato plants. From the PDA plates, a total of 8 *Trichoderma* strains were selected for further investigation. Soil from the Abha region had a higher diversity of *Trichoderma* species than soil from the Riyadh region. Among these 8 *Trichoderma* strains, six species (*T. koningiopsis*, *T. lixii*, *T. koningii*, *T. harzianum*, *T. brevicompactum*, and *T. velutinum)* were isolated from Abha, and two species (*T. lixii* and *T. harzianum*) were isolated from Riyadh (Figs. 1, 2, 3, 4, 5, 6, 7 and 8). The isolated *Trichoderma* species (Figs. 1 T1, 2 T2, 3 T3, 4 T4 and 5 T5, 6 T6, 7 T7 and 8 T8) were cultured at 28 °C for 7 days. Panels T1–T4 show (**A**) the anterior side of the PDA plate, (**B**) the superior side of the PDA plate, (**C**) chlamydospores, and (**D**, **E**, **F**, **G**, **H**, and **I**) conidiophores and phialides; (**D**) shows conidiation pustules on Pikorskaya agar after 4 days; (**E**, **F**) show conidia. The populations of fungi in both regions presented maximum CFU/g values of 45.3×10^2 and 83×10^2 for the Abha region and 14×10^2 and 47×10^2 for the Riyadh region. Moreover, soil from the Abha region exhibited greater fungal diversity than soil from the Riyadh region.

Molecular identification and phylogenetic analysis of the isolated *Trichoderma* species

The identities of the *Trichoderma* isolates were confirmed by molecular analysis. The internal transcribed spacer (ITS) region of fungal 18s rDNA was amplified using primers ITS4 and ITS. Searches using the Basic Local Alignment Search Tool (BLAST) (NCBI GenBank) were performed, and the results are presented in Table 2.

Phylogenetic analysis

A phylogenetic tree was constructed by analyzing nineteen sequences, including the sequences of the eight isolated *Trichoderma* species, 10 *Trichoderma* species from GenBank and a *Fusarium oxysporum* (MT151384) sequence as an outgroup (Fig. 9). MEGA11 was used for evolutionary analysis. The evolutionary history was inferred using the neighbor-joining method, and the evolutionary distances were computed using the maximum composite likelihood method. The results revealed a total of 464 positions in the final dataset. We observed that *T. harzianum*, *T. velutinum*, and *T. lixii* were closely related and belonged to the Harzianum clade (Clade 1), while *T. brevicompactum* belonged to the Brevicompactum clade (Clade 2), and *T. koningiopsis* and *T. koningii* belonged to the Viride clade (Clade 3).

Characterization of plant growth-promoting *Trichoderma* spp.

Biochemical analyses were performed to characterize the plant growth-promoting activity of *Trichoderma*. The isolates were assessed for phosphate solubilization, and IAA, ammonia, and siderophore production. The results are presented in Table 3.

Biochemical tests were performed to evaluate the promotion factors detected in *Trichoderma* species. The phosphate solubilization efficacy of the *Trichoderma* isolates was evaluated on Pikovaskaya agar by acidification, and all eight isolates utilized trisodium phosphate in Pikovaskaya agar and showed positive results (Fig. 10a,b). The ability to produce ammonia differed by isolate (Table 3). The highest production was exhibited by T4 (*T. harzianum*), T5 (*T. lixii*) and T7 (T. *harzianum*), and the other isolates, T1 (*T. koniniopsis*), T2 (*T. lixii*), T3 (T. *koningii*), T6 (*T. brevicompactum*), and T8 (*T. velutinum*), displayed moderate production (Table 3; Fig. 10c). Qualitative and quantitative analyses were conducted to determine IAA production by the eight *Trichoderma* isolates in culture media supplemented with tryptophan as a precursor. Interpolation of spectrophotometer readings using standard curves was used to quantify the amount of IAA produced by different isolates of

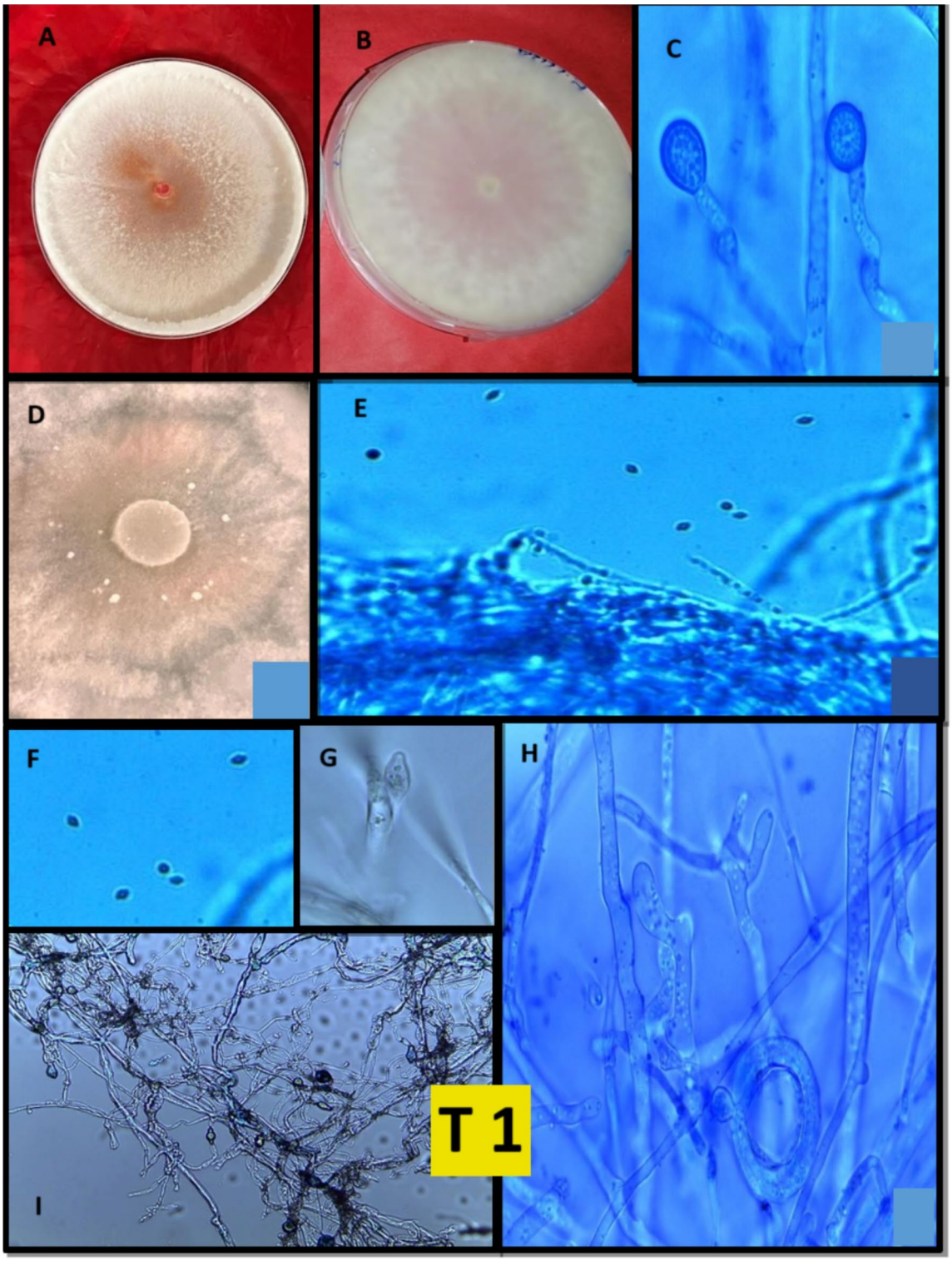

Fig. 1. The selected *Trichoderma* species (T-1) were cultured at 28 °C for 7 days. P (**A**) the anterior side of the PDA plate, (**B**) the superior side of the PDA plate, (**C**) chlamydospores, and (**D**–**I**) conidiophores and phialides; (**D**) shows conidiation pustules on Pikorskaya agar after 4 days; (**E**,**F**) show conidia.

Trichoderma. The production of IAA differed by isolate (Table 3). A high amount of IAA was produced by *T. brevicompactum* (51.24 ± 0.18 µg/ml) and *T. lixii* (50.82 ± 0.65 µg/ml), whereas *T. koniniopsis* (0.15 ± 0.052 µg/ml) was the lowest producer in media supplemented with tryptophan (Fig. 10d). Siderophore production also differed by isolate (Table 3). The ability to produce siderophores was demonstrated by the formation of orange halos around the colonies on the blue modified CAS agar plate (Fig. 10e,f). The T3 and T4 isolates (*T. koningii*

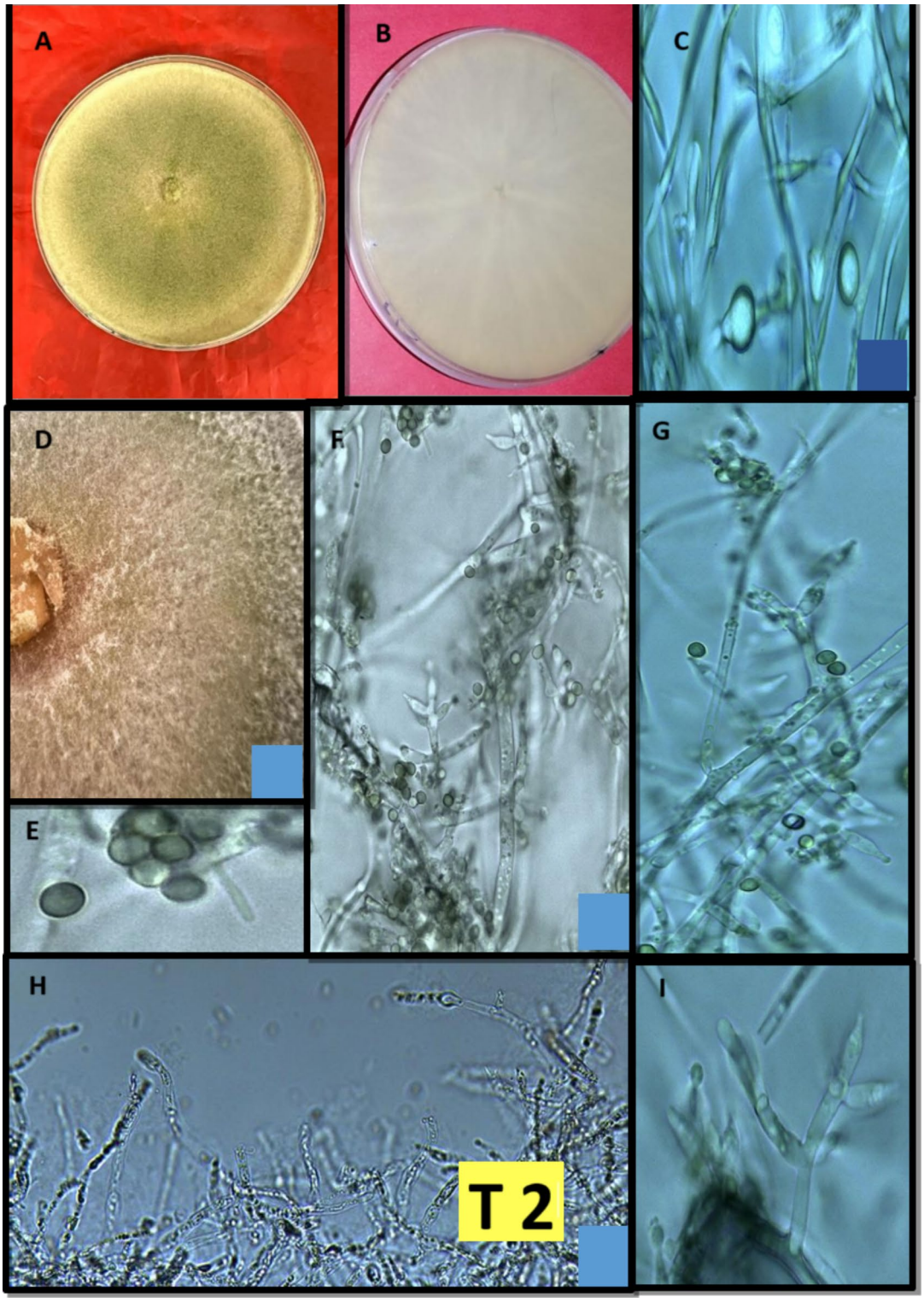

Fig. 2. The selected *Trichoderma* species (T-2) were cultured at 28 °C for 7 days. P (**A**) the anterior side of the PDA plate, (**B**) the superior side of the PDA plate, (**C**) chlamydospores, and (**D**–**I**) conidiophores and phialides; (**D**) shows conidiation pustules on Pikorskaya agar after 4 days; (**E**,**F**) show conidia.

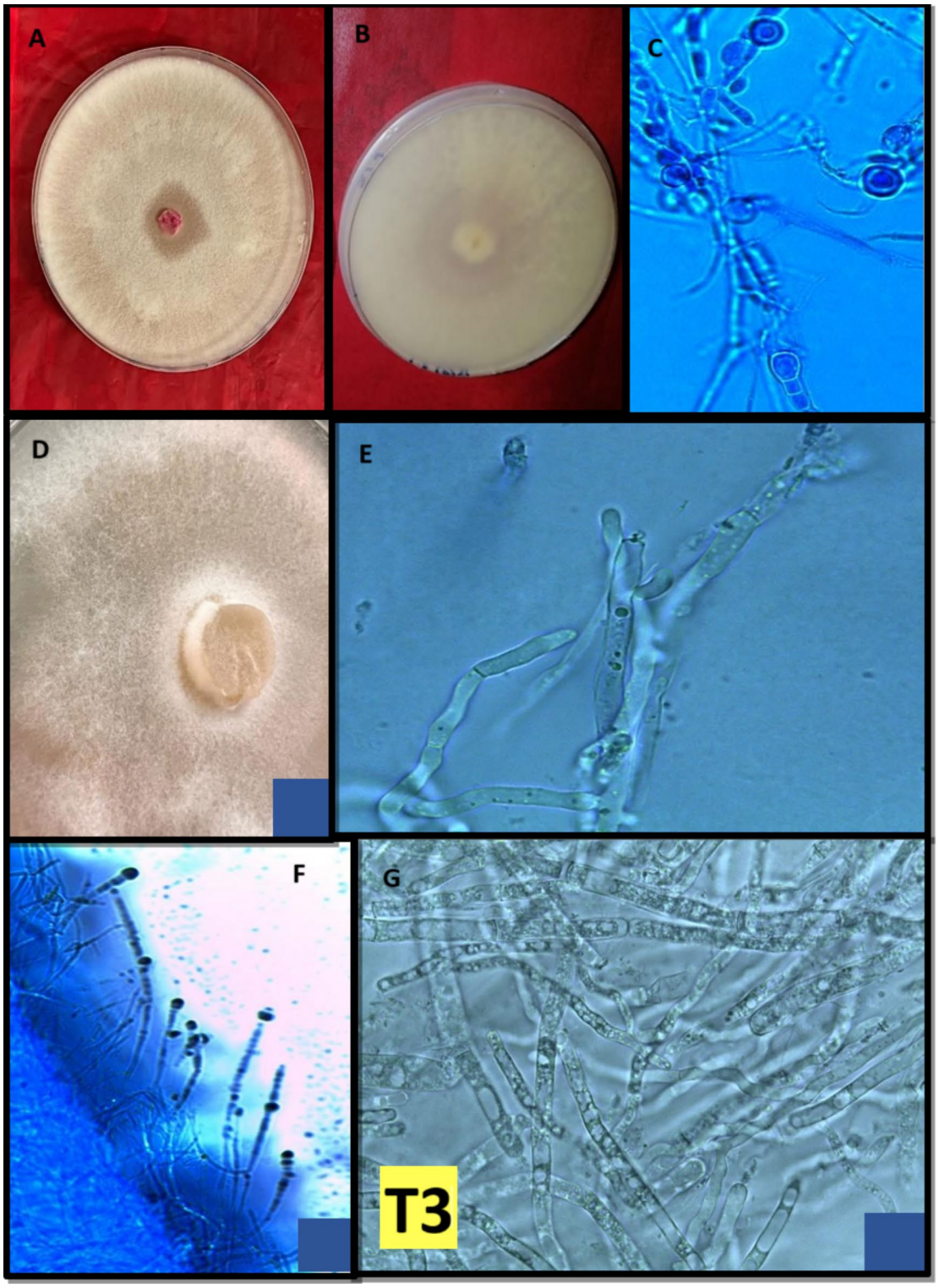

Fig. 3. The selected *Trichoderma* species (T-3) were cultured at 28 °C for 7 days. P (**A**) the anterior side of the PDA plate, (**B**) the superior side of the PDA plate, (**C**) chlamydospores, and (**D**,**E**) conidiophores and phialides; (**D**) shows conidiation pustules on Pikorskaya agar after 4 days; (**E**,**F**) show conidia.

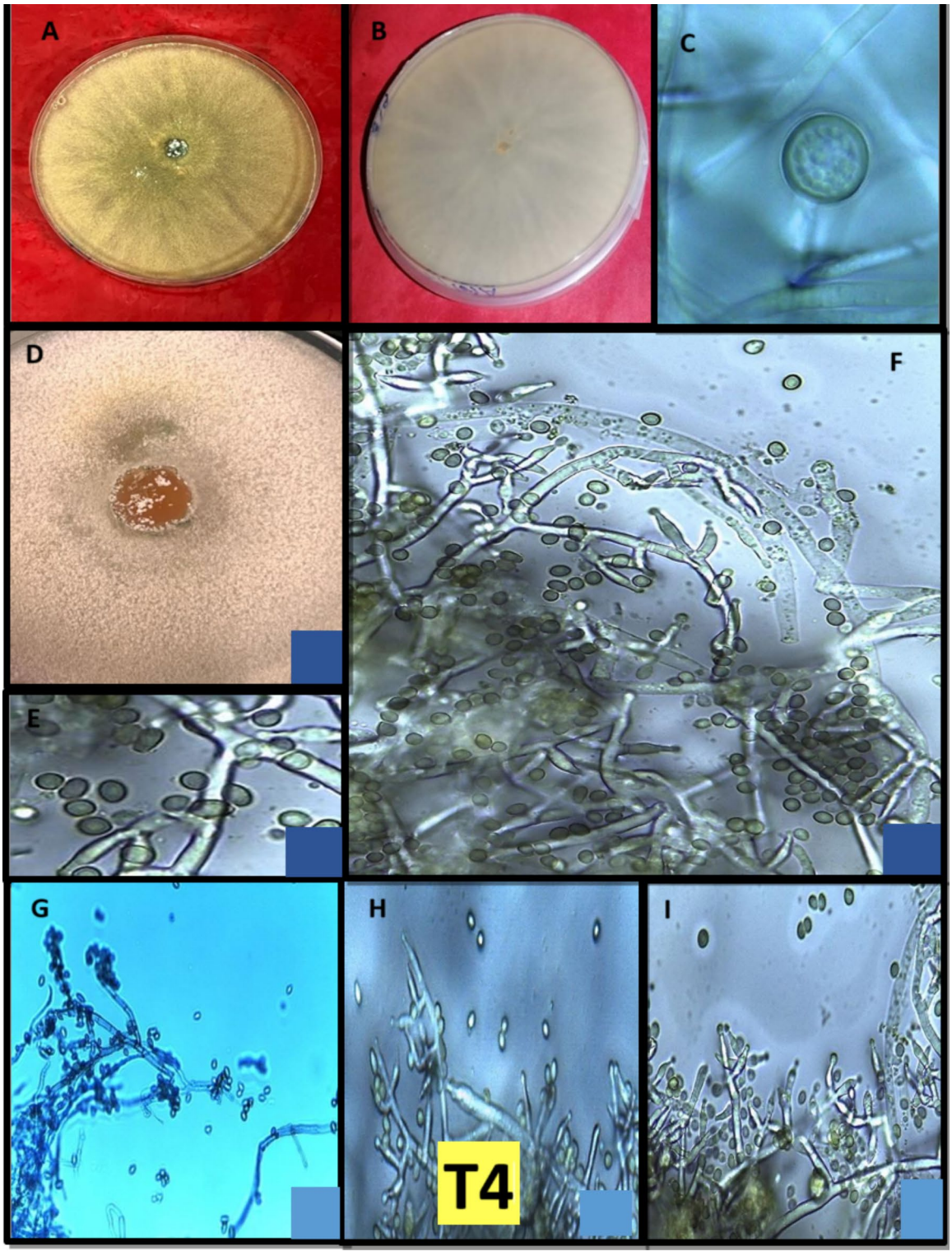

Fig. 4. The selected *Trichoderma* species (T-4) were cultured at 28 °C for 7 days. P (**A**) the anterior side of the PDA plate, (**B**) the superior side of the PDA plate, (**C**) chlamydospores, and (**D**,**E**) conidiophores and phialides; (**D**) shows conidiation pustules on Pikorskaya agar after 4 days; (**E**–**H**,**I**) show conidia.

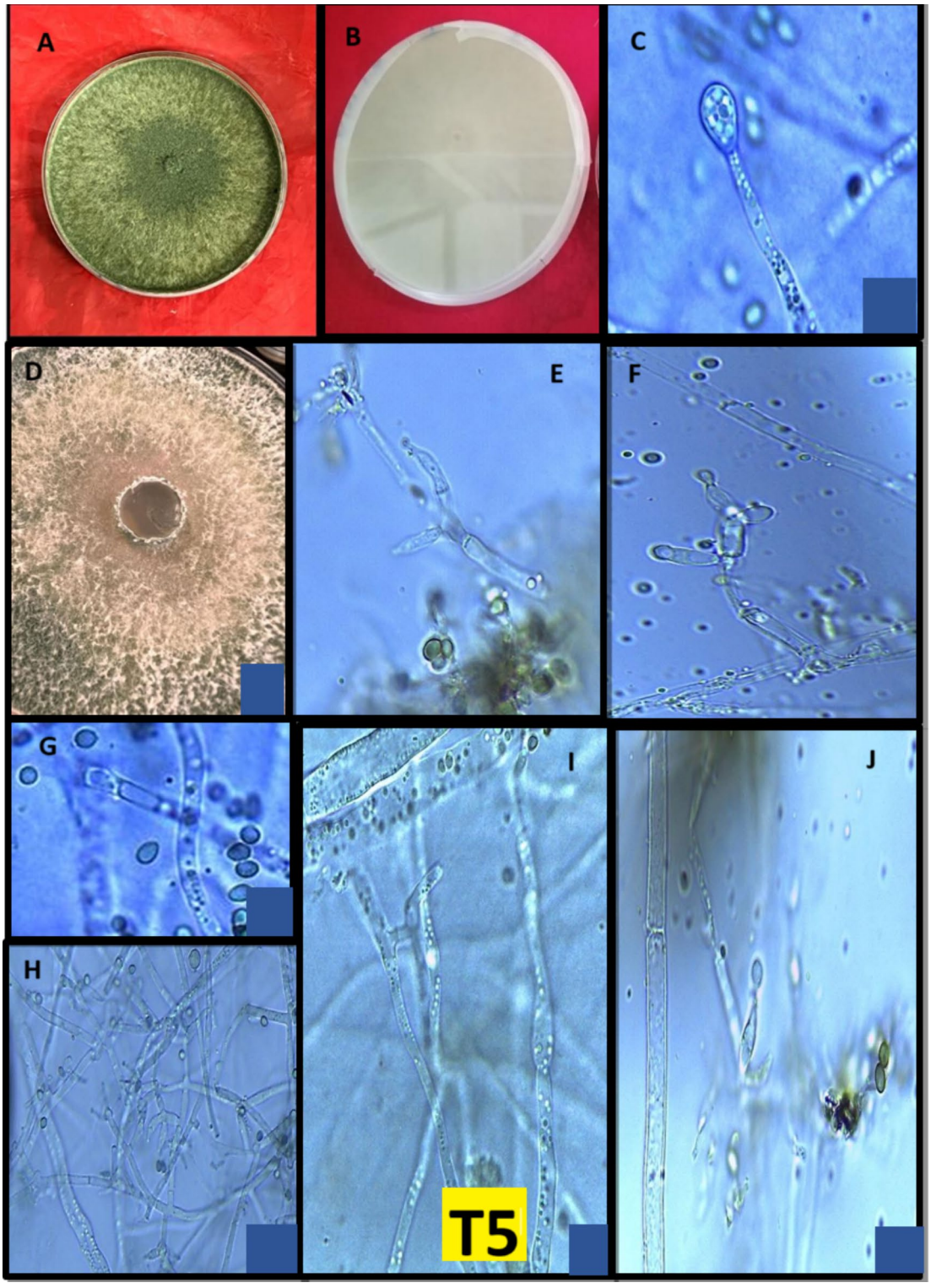

Fig. 5. The selected *Trichoderma* species (T-5) were cultured at 28 °C for 7 days. (**A**) the anterior side of the PDA plate, (**B**) the superior side of the PDA plate, (**C**) chlamydospores, and (**D**–**I**) conidiophores and phialides; (**D**) shows conidiation pustules on Pikorskaya agar after 4 days; (**E**,**F**) show conidia.

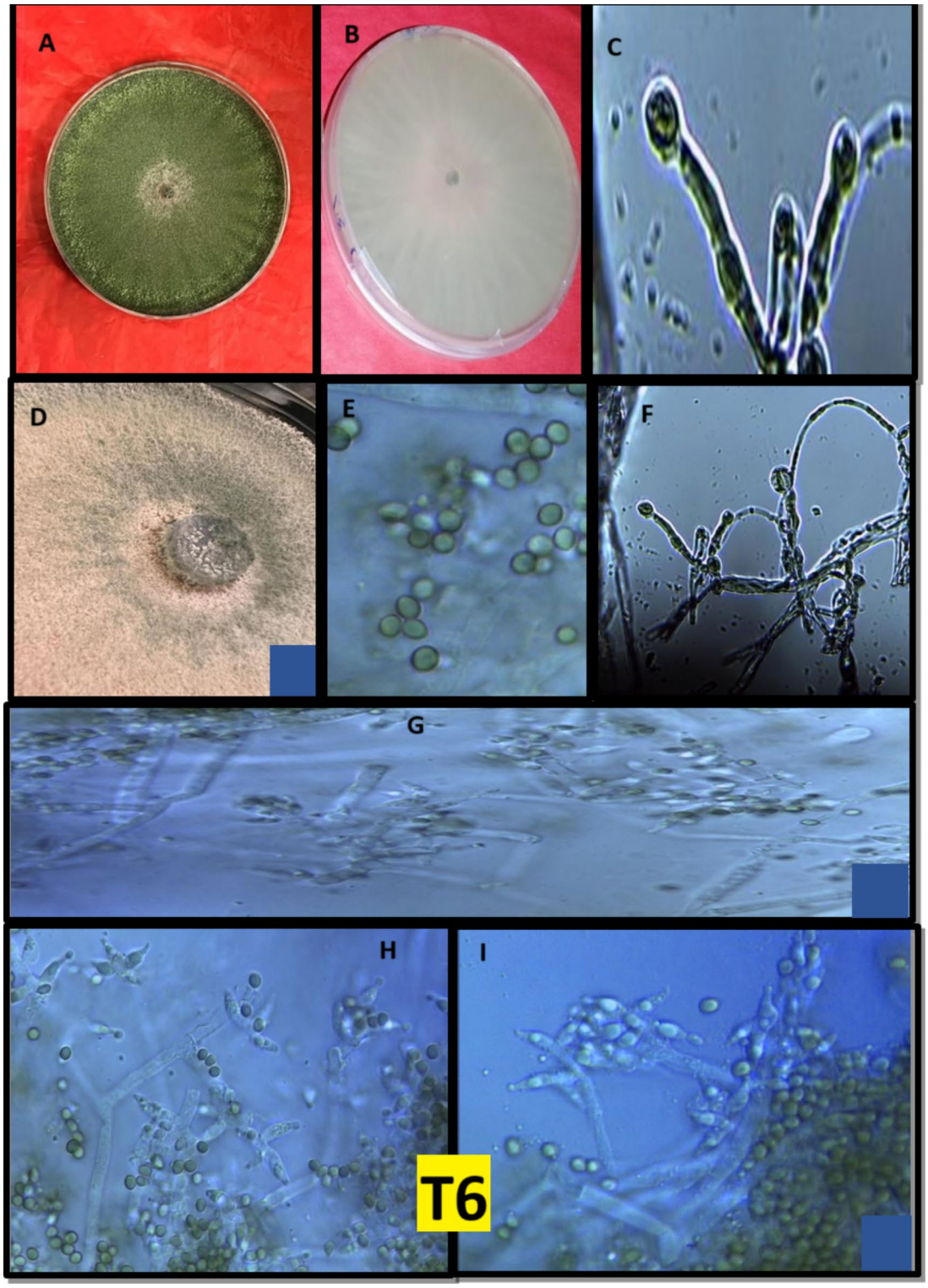

Fig. 6. The selected *Trichoderma* species (T-6) were cultured at 28 °C for 7 days. (**A**) the anterior side of the PDA plate, (**B**) the superior side of the PDA plate, (**C**) chlamydospores, and (**D**–**I**) conidiophores and phialides; (**D**) shows conidiation pustules on Pikorskaya agar after 4 days; (**E**,**F**) show conidia.

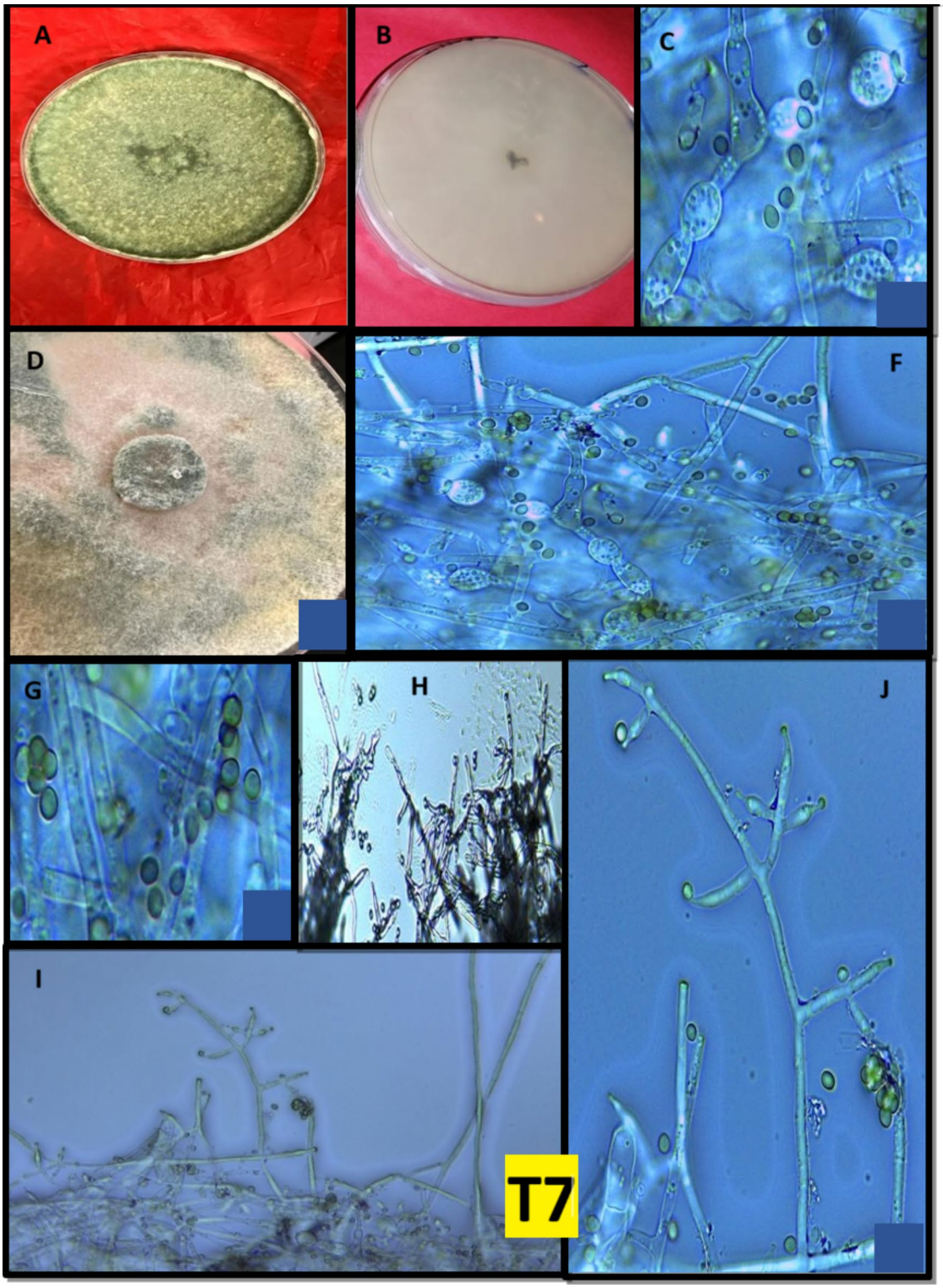

Fig. 7. The selected *Trichoderma* species (T-7) were cultured at 28 °C for 7 days. (**A**) the anterior side of the PDA plate, (**B**) the superior side of the PDA plate, (**C**) chlamydospores, and (**D**–**J**) conidiophores and phialides; (**D**) shows conidiation pustules on Pikorskaya agar after 4 days; (**E**,**F**) show conidia.

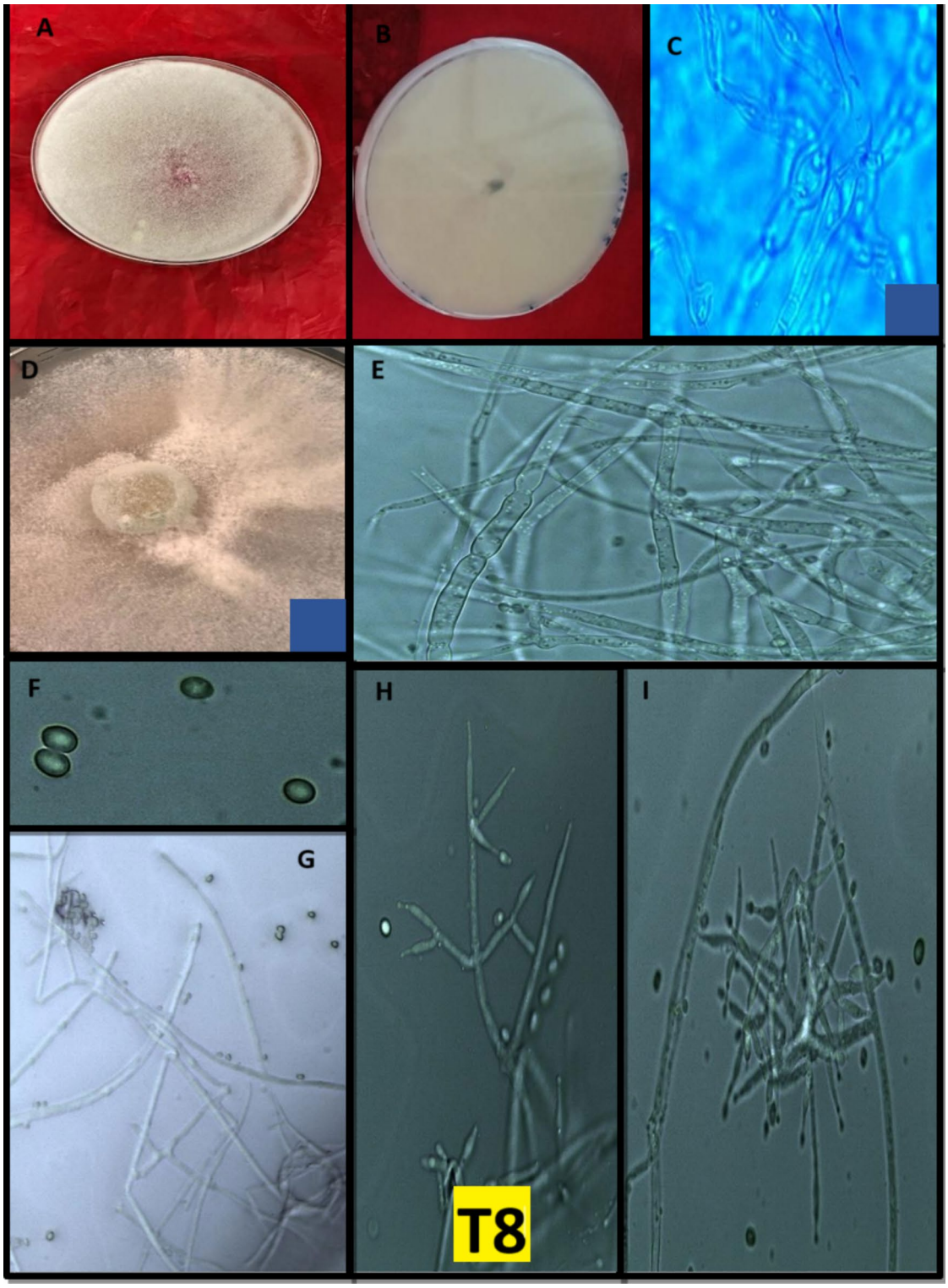

Fig. 8. The selected *Trichoderma* species (T-8) were cultured at 28 °C for 7 days. (**A**) the anterior side of the PDA plate, (**B**) the superior side of the PDA plate, (**C**) chlamydospores, and (**D**–**I**) conidiophores and phialides; (**D**) shows conidiation pustules on Pikorskaya agar after 4 days; (**E**,**F**) show conidia.

Code	Location of the soil sample	Scientific name	Similar organisms with accession number in GenBank	Percent similarity (%)	Accession number assigned to the isolates by NCBI
T1	A1 (Abha)	*Trichoderma koningiopsis*	ON795065.1	100	OQ513249
T2	R1 (Riyadh)	*Trichoderma lixii*	JF923802.1	99.84	OQ513250
T3	A6 (Abha)	*Trichoderma koningii*	EU280128.1	99.68	OQ513251
T4	A4 (Abha)	*Trichoderma harzianum*	OL597950.1	99.22	OQ513252
T5	A6 (Abha)	*Trichoderma lixii*	JF923802.1	99.84	OQ513253
T6	A3 (Abha)	*Trichoderma brevicompactum*	KR094463.1	99.2	OQ513254
T7	R6 (Riyadh)	*Trichoderma harzianum*	MT529404.1	100	OQ513255
T8	A6 (Abha)	*Trichoderma velutinum*	HM176565.1	99.21	OQ513256

Table 2. BLAST search results for *Trichoderma* isolates identified from ITS4 and ITS5 region sequences.

and *T. harzianum*, respectively) showed maximum zone formation, which was observed after 5 d. Approximately 25% of the isolates showed high siderophore production, and the remaining isolates were moderate producers. No zone formation was observed for isolates T5 (*T. lixii*) and T8 (*T. velutinum*).

In vivo evaluation of the effect of plant growth promoting *Trichoderma* on tomato plant growth

Effect of Trichoderma isolates on seed germination

Tomato seeds treated with *Trichoderma* isolates were observed for one week for seed germination. Compared with the control, priming with *Trichoderma* isolates significantly increased seed germination ($P \leq 0.05$), except for the T3 isolate. Seed germination was 100% in seeds treated with the T4 and T6 isolates, while seeds treated with the T1 and T5 isolates showed 91.1% and 90.9% seed germination, respectively. The seed germination rates for the T8, T2, and T7 isolates were 84.1%, 82.2%, and 72.7%, respectively. Seed germination after treatment with the T3 isolate was statistically equivalent to the control ($P \leq 0.05$) (Fig. 11).

Effects of Trichoderma isolates on tomato plant growth

Overall, *Trichoderma* isolates significantly ($P \leq 0.05$) increased tomato plant growth compared to that of untreated control plants (Fig. 12). Among the plants that received *Trichoderma* isolates, the greatest increase in shoot height was observed for the plants treated with T5-*T. lixii* (16.16 cm) followed by those treated with T7-*T. harzianum* (13.33 cm), T1-*T. koniniopsis* (11.33 cm), T2-*T. lixii* (10.83 cm), T4-*T. harzianum* (10.83 cm), T8-*T. velutinum* (10.16 cm), T6-*T. brevicompactum* (7.00 cm), and T3-*T. koningii* (5.70 cm). Post hoc analysis of shoot height indicated that most of the treatments were significantly different ($P \leq 0.05$) from each other, except for T3-*T. koningii*, which was equivalent to control plants. Conversely, the greatest increase in root length was recorded in the plants treated with T7-*T. harzianum* (7.23 cm), followed by those treated with T5-*T. lixii* (6.83 cm), T8-*T. velutinum* (5.16 cm), T4-*T. harzianum* (4.53 cm), T2-*T. lixii* (3.30 cm), T3-*T. koningii* (3.20 cm), T6-*T. brevicompactum* (2.76 cm) and T1-*T. koniniopsis* (2.40 cm). Post hoc analysis of plant root length revealed that there was no significant difference ($P \leq 0.05$) between T7-*T. harzianum* and T5-*T. lixii* or among the T2-*T. lixii*, T3-*T. koningii*, and T6-*T. brevicompactum* treatments. The greatest plant fresh weight was observed for the plants treated with T5-*T. lixii* (669.33 mg), followed by those treated with T7-*T. harzianum* (359.33 mg), T8-*T. velutinum* (299.67 mg), T3-*T. koningii* (284.33 mg), T4-*T. harzianum* (197.33 mg), T1-*T. koniniopsis* (193.0 mg), T2-*T. lixii* (146.33 mg), and T6-*T. brevicompactum* (73.00 mg). The maximum plant dry weight was observed for the plants treated with T5-*T. lixii* (28.7 mg), T7-*T. harzianum* (22.67 mg), T8-*T. velutinum* (13.4 mg), T1-*T. koniniopsis* (11.4 mg), T4-*T. harzianum* (10.37 mg), T2-*T. lixii* (9.67 mg), T3-*T. koningii* (7.7 mg), and T6-*T. brevicompactum* (5.7 mg). Analysis of plant fresh and dry weight revealed a significant ($P \leq 0.05$) difference between the control plants and the plants in the other treatment groups. However, there was no significant difference in the fresh weight of plants in the control and T6-*T. brevicompactum* groups or among the T1-*T. koniniopsis*, T2-*T. lixii*, and T4-*T. harzianum* groups. A significant difference ($P \leq 0.05$) in plant dry weight was detected among all treatments (Table 4).

Principal component analysis (PCA) for plant growth parameters and seed germination was performed to understand the effect of *Trichoderma* isolates on plant growth. The PCA biplot in Fig. 13 shows that shoot height, root length, plant fresh weight, and plant dry weight were highly correlated. Seed germination was moderately correlated with the other plant growth parameters. *Trichoderma* isolate T5-*T. lixii* had the greatest positive impact on plant growth; T7-*T. harzianum* and T8-*T. velutinum* also fell into the same quadrant, demonstrating a positive effect on plant growth. T4-*T. harzianum* also increased plant growth, as it fell into the second quadrant.

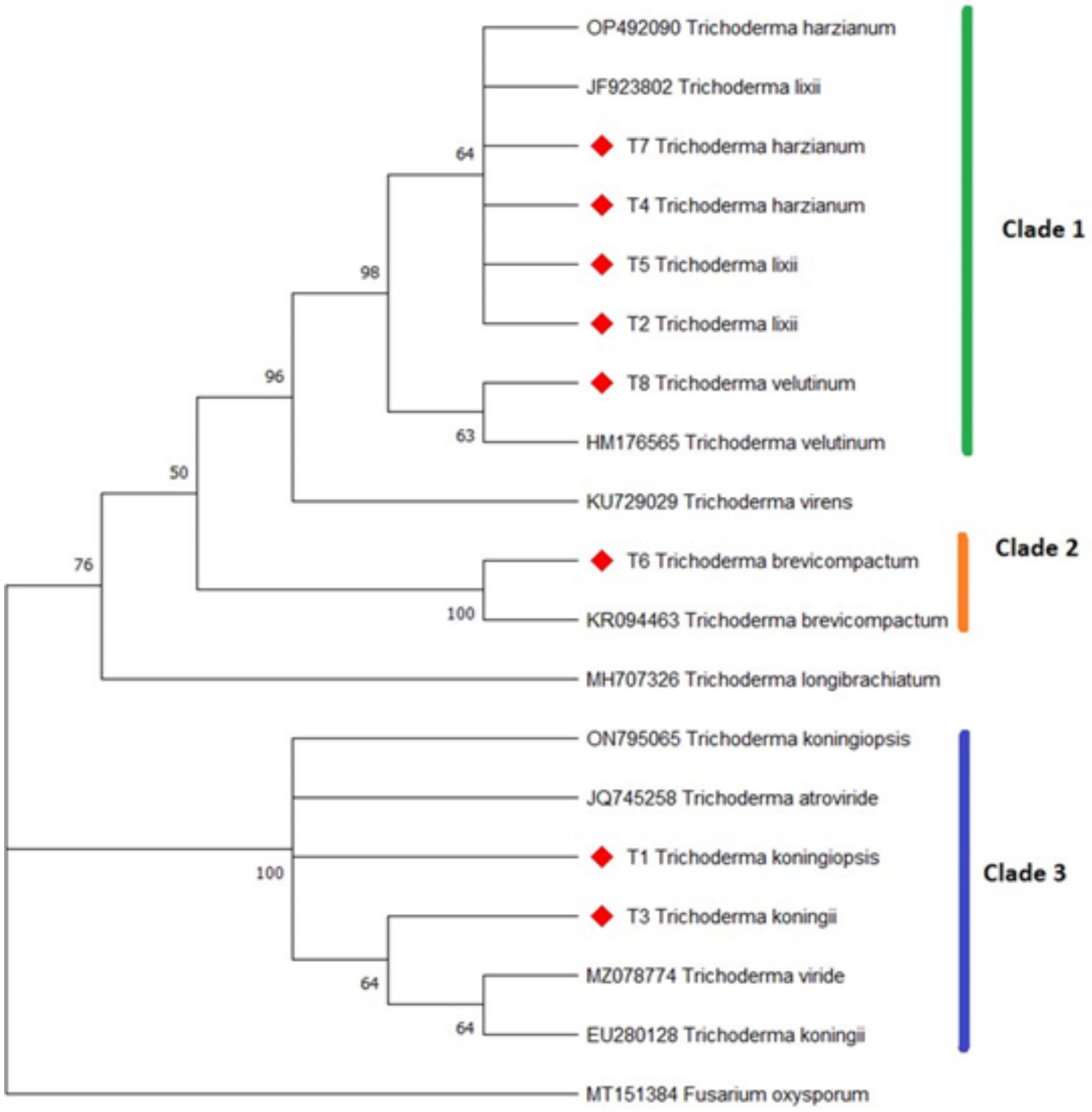

Fig. 9. Neighbor-joining phylogenetic tree based on ITS 4 and ITS 5 region sequences of eight isolated *Trichoderma* species, 10 *Trichoderma* species from GenBank, and *F. oxysporum* as an outgroup. The evolutionary distances were computed using the maximum composite likelihood method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. Clade 1: Harzianum, Clade 2: Brevicompactum, and Clade 3: Viride.

T1-*T. koniniopsis*, T2-*T. lixii*, and T6-*T. brevicompactum* moderately increased plant growth, whereas plants that received T3-*T. koningii* were similar in growth to control plants.

Discussion

Fungi from the genus *Trichoderma* have been widely used in agriculture because of their mycoparasitic potential and their ability to improve plant health and protect against phytopathogens, making them desirable symbionts. The goal of the current study was the isolation, molecular identification, and characterization of *Trichoderma* from Saudi Arabia[34] and the evaluation of their ability to promote plant growth.

Soil properties of the samples collected from Abha and Riyadh were also determined. In the Riyadh region, soil pH varied from mild to extremely alkaline, while soil in the Abha region had a neutral pH. The EC values also

Code	Strains	*Phosphate production	Ammonia production	*Siderophore production	IAA production	
						Conc. (µg/ml)
T1	*T. koniniopsis*	+++	++	+++	+	0.15 ± 0.052**
T2	*T. lixii*	+++	++	+	+	0.97 ± 0.052
T3	*T. koningii*	+++	++	+++	++	13.09 ± 0.84
T4	*T. harzianum*	+++	+++	+++	+	5.45 ± 0.22
T5	*T. lixii*	+++	+++	+	+++	50.82 ± 0.65
T6	*T. brevicompactum*	+++	++	+	+++	51.24 ± 0.18
T7	*T. harzianum*	+++	+++	++	+	0.39 ± 0.09
T8	*T. velutinum*	+++	++	-	++	13.42 ± 0.26

Table 3. Plant growth-promoting attributes exhibited by *Trichoderma* isolates. Production level was graded as + (low producer), ++ (moderate producer), or +++ (high producer) The amount of IAA was calculated from the standard curve. $y = 0.011x + 0.0057$, $R^2 = 0.997$ An * indicates 5 days incubation **Indicates that each value is the mean of 3 replicates ± SD

varied widely in both regions. Previous studies have reported different pH and EC values in the Riyadh region. Masoud & Aal reported an average pH of 7.64; however, their sample sites were different from those in the present study[34]. Al Barakah et al.[35] reported mean pH and EC values of 7.7 and 1.03 dS/m, respectively. Siham[36] reported a pH range of 7.58–7.76 and EC values of 22.5–32.0 from an industrial city in Riyadh. Irrigation water and dissolving soil minerals are some sources of salts in soil. Moreover, the Riyadh region is rich in weathered limestone, and the climate is exceedingly arid; therefore, evapotranspiration exceeds precipitation. These factors may contribute to higher pH and alkalinity[34,37]. The organic matter content of soil in the Abha region was greater than that in the Riyadh region. Since most soil in the Abha region was from a vegetation area, organic matter can be attributed to the decomposition of dead plant materials. As expected, the moisture content varied according to the sample collection site. The soil samples collected near a water body had a higher moisture content than the other samples. The Riyadh and Abha regions both had large populations of soil fungi. Recently, fungal populations were shown to range from 4.19 to 4.67 CFU/g in the Riyadh region, and the presence of *Trichoderma* was also observed[17,21,38–40].

The soil fungi of Saudi Arabia have been investigated previously, and although *Trichoderma* was not detected in desert soil samples[41], other studies reported *Trichoderma* in soils from other sources[42–44]. In the present study, we isolated eight *Trichoderma* species (*T. koningiopsis*, *T. lixii*, *T. koningii*, *T. harzianum*, *T. brevicompactum*, *T. velutinum*, *T. lixii*, and *T. harzianum*) from the Abha and Riyadh regions. Similarly, Hussein & Yousef isolated two species of *Trichoderma* (*T. harzianum and Trichoderma* sp.) from petroleum-contaminated soil[43]. Additionally, Abd-Elsalam identified two *Trichoderma* complex species *(T. harzianum/H. lixii* and *T. longibrachiatum/H. orientalis*) from soil collected from Rawdet Khuraim in Saudi Arabia using morphological criteria and DNA sequence analysis[18].

Molecular characterization based on multiple gene sequencing enables the accurate identification of *Trichoderma* species. Previous reports of *Trichoderma* isolation and characterization in Saudi Arabia depended on morphological methods, which may not distinguish closely related species. Globally, in 1998, Kindermann and others attempted to study the phylogeny of the whole *Trichoderma* genus based on sequencing of the ITS region, and they demonstrated that this approach was a powerful method of identifying *Trichoderma* species. Other researchers have since reported the importance of the ITS sequence in *Trichoderma* species identification[44–48]. However, our study aimed to provide an extra reliable method for the identification of Trichoderma species in Saudi Arabia We targeted the ITS region of rDNA, which is one of the most frequently targeted regions for the molecular characterization of *Trichoderma* species. We identified *T. koniniopsis*, *T. velutinum*, *T. brevicompactum*, two isolates of *T. harzianum*, two isolates of *T. lixii*, and *T. koningii.* This is the first time that such diverse *Trichoderma* species have been reported in Saudi Arabia.

Phylogenetic analysis revealed that the *Trichoderma* isolates belonged to three different clades. *T. harzianum*, *T. velutinum* and *T. lixii* were closely related and belonged to the Harzianum clade. This finding is consistent with that of Gherbawy[38], who identified 91 *Trichoderma* isolates based on ITS regions from the soil of Taif City, Saudi Arabia. A total of 78 isolates from the population were identified as *Trichoderma harzianum* (Tel. *Hypocrea lixii*). Additionally, Chaverri and others[24] reported that *T. harzianum* showed high similarity with *T. lixii*. Other molecular sequence data has demonstrated that *T. harzianum* is a genetically variable complex composed of one morphological species and several phylogenetic species[49].

Trichoderma species have beneficial effects on plants, including enhancing plant growth, root structure, seed germination, viability, photosynthetic efficiency, flowering, and yield quality, thereby promoting overall plant health[50]. In the present study, eight species of *Trichoderma* were investigated for plant growth-promoting traits, including phosphate solubilization and IAA, ammonia, and siderophore production. The *Trichoderma* isolates varied in terms of their plant growth-promoting traits. All the isolates were able to mobilize phosphate, while *T. harzianum* and *T. lixii* produced the greatest amount of ammonia. *T. koningii* and *T. harzianum* were superior in siderophore production, and *T. brevicompactum* and *T. lixii* produced the most IAA. Additionally, these eight isolates of *Trichoderma* were evaluated for their ability to stimulate tomato seed germination and plant growth in the early stages of seedling development. The results showed that all the *Trichoderma* isolates significantly increased seed germination and plant growth, especially the T5-*T. lixii*, T7-*T. harzianum*, and T8-*T. velutinum*

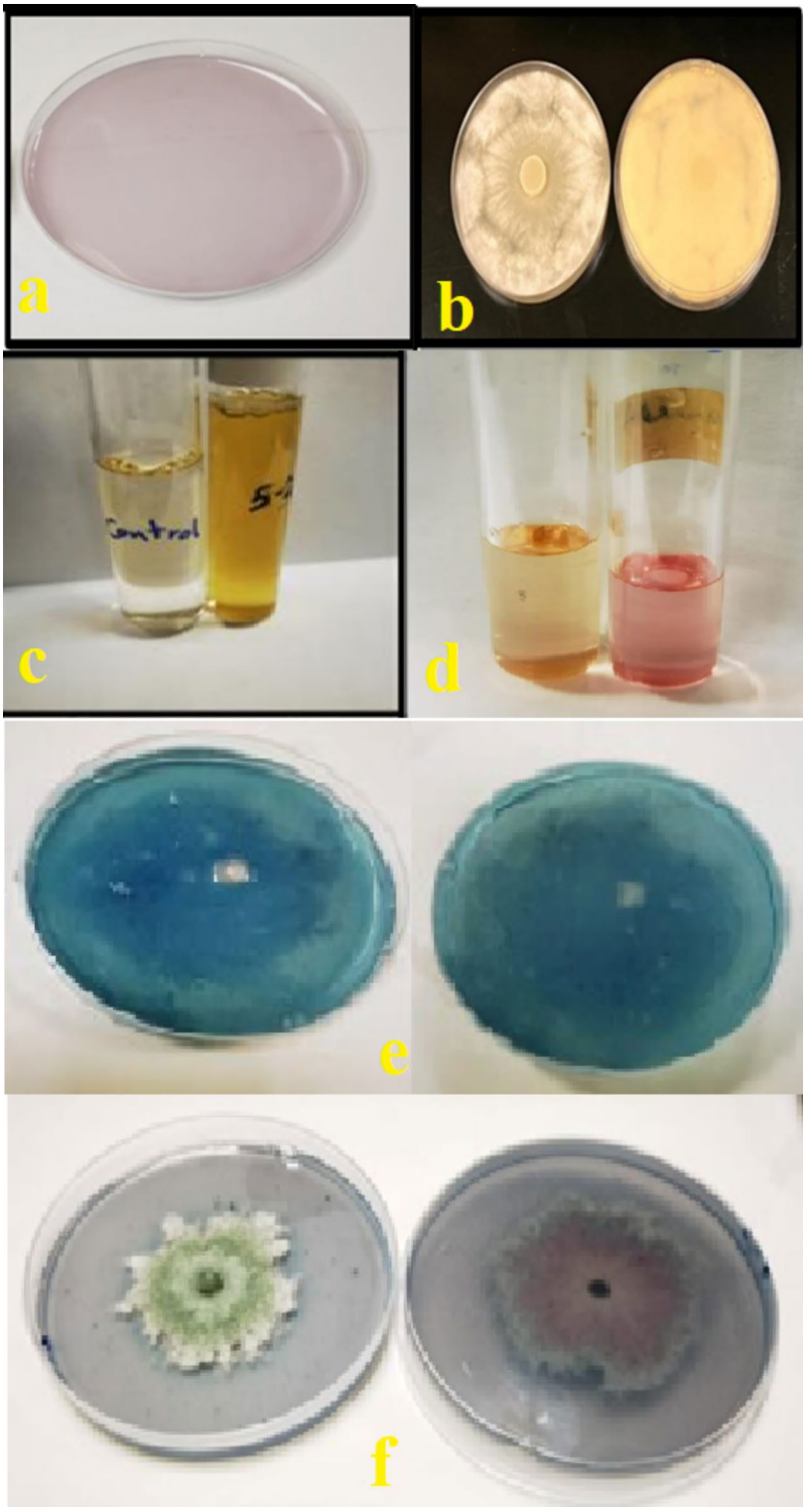

Fig. 10. Biochemical assays. Panel (**a**) shows the negative control (media without *Trichoderma*). Panel (**b**) shows *Trichoderma* species T1 was positive for phosphate solubilization. Panel (**c**) shows ammonia production of *Trichoderma* species T6. Panel (**d**) shows IAA production by *Trichoderma* species T6. Panel (**e**) shows the negative control for siderophore production in media without *Trichoderma*. Panel (f) shows the T3 and T4 species in the left and right images, respectively. A wide zone appears, which means that those species produce siderophores.

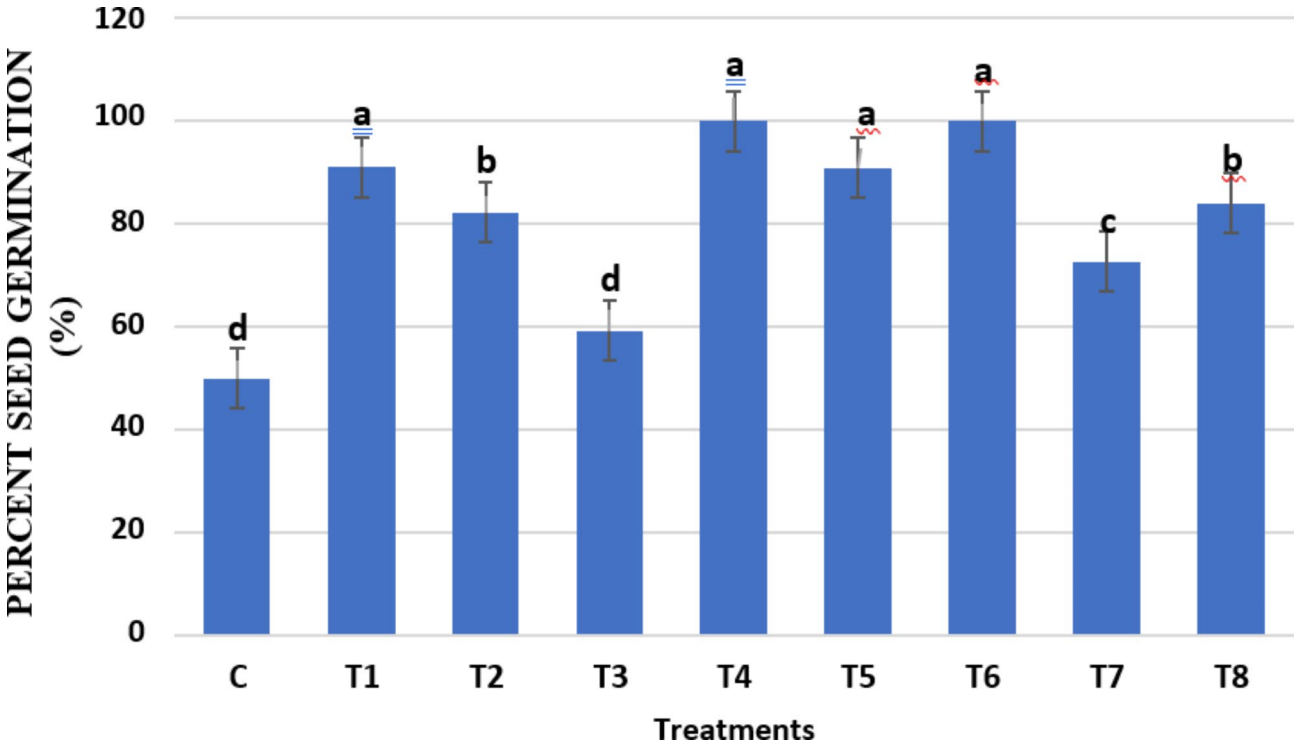

Fig. 11. The effects of *Trichoderma* isolates on seed germination. The data are presented as the mean ± SD ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$) according to a Tukey HSD test. C: Control, T1: *T. koniniopsis*, T2: *T. lixii*, T3: *T. koningii*, T4: *T. harzianum*, T5: *T. lixii*, T6: *T. brevicompactum*, T7: *T. harzianum*, and T8: *T. velutinum*.

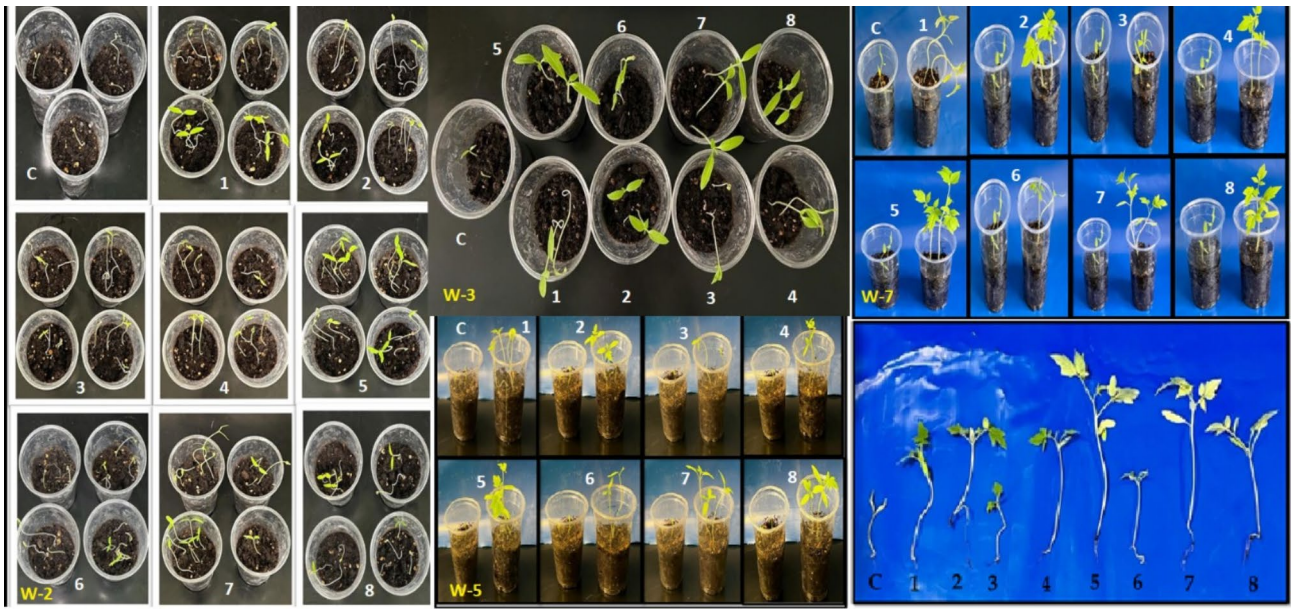

Fig. 12. Tomato plant growth after the first treatment and 2 weeks (W2), 3 weeks (W3), 5 weeks (W5), and 7 weeks (W7) after sowing. Control, **C**. Treatments included (**1**) T. *koniniopsis*, (**2**) *T. lixii*, (**3**) *T. koningii*, (**4**) *T. harzianum*, (**5**) *T. lixii*, (**6**) *T. brevicompactum*, (**7**) *T. harzianum*, and (**8**) *T. velutinum*.

isolates, which were highly effective at stimulating plant growth by increasing shoot and root length and the fresh and dry weights of shoots and roots. Our results are consistent with those obtained by Bader[15], who noted that a set of *Trichoderma* strains can produce IAA, solubilize phosphate, and promote tomato plant growth by increasing shoot length and the fresh and dry weights of shoots and roots.

Treatment	Shoot height (cm)	Root length (cm)	Plant fresh weight (mg/plant)	Plant dry weight (mg/plant)
TC	4.33 ± **2.08**^{a}	1.26 ± **0.252**^{a}	45.33 ± **0.0136**^{a}	3.73 ± **0.058**^{a}
T1	11.33 ± **1.528**^{c}	2.40 ± **0.529**^{ab}	193.0 ± **0.0775**^{ab}	11.40 ± **0.1**^{f}
T2	10.83 ± **2.363**^{bc}	3.30 ± **0.889**^{abc}	146.33 ± **0.063**^{ab}	9.67 ± **0.577**^{d}
T3	5.70 ± **0.985**^{a}	3.20 ± **0.985**^{abc}	284.33 ± **0.343**^{bc}	7.70 ± **0.1**^{c}
T4	10.83 ± **1.041**^{bc}	4.53 ± **0.950**^{bcd}	197.33 ± **0.030**^{ab}	10.37 ± **0.058**^{e}
T5	16.16 ± **0.764**^{d}	6.83 ± **1.041**^{d}	669.33 ± **0.112**^{e}	28.70 ± **0.1**^{i}
T6	7.00 ± **1**^{ab}	2.76 ± **0.666**^{abc}	73.00 ± **0.030**^{a}	5.70 ± **0.1**^{b}
T7	13.33 ± **1.528**^{cd}	7.23 ± **1.704**^{d}	359.33 ± **0.043**^{cd}	22.67 ± **0.058**^{h}
T8	10.16 ± **0.764**^{bc}	5.16 ± **0.764**^{cd}	299.67 ± **0.002**^{bc}	13.40 ± **0.1**^{g}

Table 4. Effect of *Trichoderma* isolates on tomato plant growth. Significant values are in [bold]. The data are presented as the mean ± SD ($n = 3$). Different letters indicate significant differences ($P \leq 0.05$) according to a Tukey HSD test. T1: *T. koniniopsis*, T2: *T. lixii*, T3: *T. koningii*, T4: *T. harzianum*, T5: *T. lixii*, T6: *T. brevicompactum*, T7: *T. harzianum*, and T8: *T. velutinum*

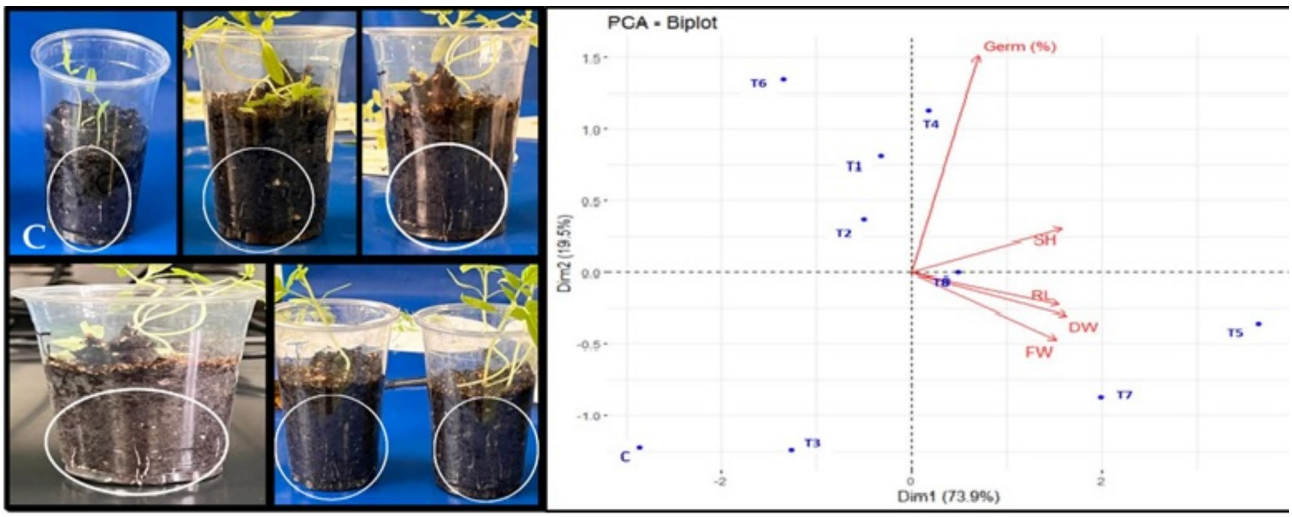

Fig. 13. The left images show root growth of tomato plants treated with *Trichoderma* isolates. Control, C. The right image shows the PCA biplot for tomato plant growth parameters. Treatments included the control (C), T. *koniniopsis* (1), *T. lixii* (2), *T. koningii* (3), *T. harzianum* (4), *T. lixii* (5), *T. brevicompactum* (6), *T. harzianum* (7), and *T. velutinum* (8). Germ (%): seed germination, Sh: shoot height, RL: root length, FW: plant fresh weight, and DW: plant dry weight.

Conclusion

The goal of the present study was to identify examples of the plant growth-promoting fungus *Trichoderma* in two regions of Saudi Arabia, Abha and Riyadh, by utilizing morphological and molecular tests. The soil properties of Abha and Riyadh differ significantly, as does the fungal population in these areas. Six diverse *Trichoderma* species were detected in Abha soil, while only two different species were isolated from Riyadh soil. Molecular identification and phylogenetic analysis confirmed the following six species: *T. koniniopsis*, *T. velutinum*, *T. brevicompactum*, *T. harzianum*, *T. lixii*, and *T. koningii.* Phylogenetic analysis based on ITS sequences grouped the strains into three clades. The *Trichoderma* isolates varied in phosphate solubilization and IAA, ammonia, and siderophore production, which are plant growth-promoting traits. In vivo experiments on tomato plants showed that all *Trichoderma* isolates except T3-*T. koningii* increased seed germination. The *Trichoderma* isolate T5-*T. lixii* had the greatest effect on tomato plant growth, followed by T7-*T. harzianum*, T8-*T. velutinum*, T4-*T. harzianum*, T1-*T. koniniopsis*, T2-*T. lixii*, and T6-*T. brevicompactum;* the least effective was T3-*T. koningii.* To our knowledge, this is the first characterization of plant growth-promoting *Trichoderma* and identification of *T. brevicompactum* from Saudi Arabia.

Data availability

The data presented in this study are available upon request from the corresponding author.

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Author contributions

Conceptualization, A.S.A., K.P. and M.S.A.; Data curation, A.S.A. and M.S.A.; Investigation, M.F.A. and K.P.; Methodology, A.S.A.; N.A.A.; Supervision, R.E.; Writing—original draft, A.S.A. and R.E.; Writing—review and editing, M.S.A. and K.P. The authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Sample availability

Samples of the compounds are not available from the authors.

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