Research Article

Yüksel Gezgin*, Derya Maral Gül, Seçil Sözer Şenşatar, Can Uraz Kara, Sayit Sargın, Fazilet Vardar Sukan and Rengin Eltem

Evaluation of *Trichoderma atroviride* and *Trichoderma citrinoviride* growth profiles and their potentials as biocontrol agent and biofertilizer

Trichoderma atroviride ve Trichoderma citrinoviride'nin büyüme profilleri ve biyogübre ile biokontrol ajanı olarak potansiyellerinin değerlendirilmesi

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Abstract

Background: Two *Trichoderma* species (*T. atroviride* and *T. citrinoviride*) were evaluated for their potential to have *Trichoderma*-based biological control agent and biofertilizer properties.
Materials and Methods: Eight *Trichoderma* spp. were identified by molecular methods. The mycoparasitic activities against different phytopathogenic fungi and their capacity to produce extracellular lytic enzymes were investigated. Furthermore, indole-3-acetic acid production and phosphate solubilization capabilities of *Trichoderma* spp. were evaluated together with the effect of some physical parameters and different carbon:nitrogen sources on mycelial growth and conidium production.
Results and Discussion: All strains exhibited lytic enzymes and indole-3 acetic acid production as well as phosphate solubility characteristics. *Trichoderma citrinoviride* demostrated more mycoparasitic activity against *Fusarium oxysporium* than *T. atroviride* whereas *T. atroviride* was found more effective against *Rhizoctonia solani*. In particular, at lower temperatures, conidium production of *T. atroviride* strains were significantly higher than *T. citrinoviride* strains. Both strains grew well on all carbon sources tested. The effect of organic nitrogen sources on growth were notably higher than inorganic nitrogen sources.
Conclusion: The results provided valuable insight in both the highest mycelial growth and conidia production conditions of these cultures for further similar studies related to development of *Trichoderma*-based new commercial biological control and biofertilizer formulations in different agro-climatic regions.

Keywords: *T. atroviride*; *T. citrinoviride*; biological control agent; biofertilizer; plant growth-promoting fungi.

Öz

Amaç: İki *Trichoderma* (*T. atroviride* ve *T. citrinoviride*) türünün*Trichoderma*-tabanlı biyolojik kontrol ajanı potansiyelleri ve biyogübre özellikleri değerlendirilmiştir.
Gereç ve Yöntemler: Sekiz adet *Trichoderma* spp. moleküler metotlar kullanılarak tanılanmıştır. Farklı fitopatojenik mantarlara karşı mikoparazitik aktiviteleri ve ekstraselüler litik enzimleri üretme potansiyelleri incelenmiştir. Bunun yanısıra *Trichoderma* spp. lerinin indol asetik asit üretimi ve fosfat çözünürleştirme kapasiteleri misel büyümesi ve konidium üretimi üzerine farklı karbon:azot kaynaklarının ve bazı fiziksel parametrelerin etkisi ile birlikte değerlendirilmiştir.
Bulgular ve Tartışma: Tüm suşlar litik enzim, indol asetik asit üretimi ve fosfat çözündürme özellikleri sergilemişlerdir. *Trichoderma citrinoviride* suşları *Fusarium oxysporium*'e karşı *T. atroviride*'den daha fazla mikoparazitik aktivite göstermiştir oysa *T. atroviride* suşlarının *Rhizoctonia solani*'ye karşı daha etkili olduğu görülmüştür. Özellikle düşük sıcaklıklarda *T. atroviride* suşlarının konidium üretimleri *T. citrinoviride* suşlarına göre daha yüksektir. Her iki suş da test edilen tüm karbon kaynaklarında büyümüştür. Büyüme üzerine organik nitrojen kaynakları inorganik nitrojen kaynaklarına göre yüksektir.

***Corresponding author: Yüksel Gezgin,** Department of Bioengineering, Faculty of Engineering, Ege University, 35100 Bornova-Izmir, Turkey, Phone: +90 232 3115811; Fax: +90 232 311 58 80, e-mail: yukselgezgin@gmail.com.
https://orcid.org/0000-0001-5812-1882
Derya Maral Gül, Seçil Sözer Şenşatar, Can Uraz Kara, Sayit Sargın, Fazilet Vardar Sukan and Rengin Eltem: Department of Bioengineering, Faculty of Engineering, Ege University, Bornova-Izmir, Turkey

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Sonuç: Sonuçlar farklı iklim bölgelerinde *Trichoderma*-temelli yeni ticari biyolojik kontrol ve biyogübre formülasyonlarının geliştirilmesiyle ilgili benzer çalışmalar için bu kültürlerin hem en yüksek misel büyümesi ve hem konidium üretimleriyle ilgili değerli bilgiler sağlamaktadır.

Anahtar Kelimeler: *T. atroviride*; *T. citrinoviride*; biyolojikkontrolajanı; biyogübre; bitki büyümesini teşvik eden mantarlar.

Introduction

Trichoderma spp. have been studied extensively with respect to many characteristics and are known as successful habitats of the soil ecosystem competing against other fungi [1–4]. Biocontrol process may be performed as indirectly (i.e.: by competition for nutrients and space, chancing the environmental conditions, or promoting plant growth and plant defense mechanisms and antibiosis) or direct mechanisms, as in mycoparasitism [1]. *Trichoderma* spp. demonstrate considerable mycoparasitic activity due to the production of lytic enzymes such as chitinases, β-1,3-glucanases and various proteases [5, 6]. The production of several antibiotics by *Trichoderma* spp. helps to improve the plant immune system thereby preventing possible plant infections. Compared to bacterial biological control agents (BCAs), fungal based BCAs exhibit a broader spectrum of activity in terms of disease management and therefore they have gained wide acceptance in the recent years [7–9]. *Trichoderma* spp. have an important potential as plant growth promoting fungi [6], comprising of almost 50% of the fungal BCAs market, mostly as plant growth enhancers [7]. *Trichoderma* spp. have the ability to solubilise valuable nutrients such as phosphate compounds by secreting different organic acids. They also produce different hydrolytic enzymes, contributing to the enrichment of the soil composition. *Trichoderma* spp. are also effective bioremediation agents due to their ability to degrade xenobiotics, removing potential hazards from the soil. Besides their plant growth enhancement properties, *Trichoderma* spp. are important candidates for use as BCAs [4].

Some fungal genera such as *Trichoderma, Aspergillus, Fusarium, Penicillium* are most frequently reported plant growth-promoting (PG-P) fungi and owing to their multiple beneficial effects on plant quantity and quality have attracted considerable interest as bio-fertilisers [10].

Trichoderma-based commercial biofertilizers (*T*-bcb) have been widely studied for plant growth and plant diseases control [5]. There are many *T*-bcb available in the market such as Bioorganic Plus (NovaScience Co. Ltd, Thailand), BioVam (T&J Enterprises, USA), PLantmate® (Agrimms Technologies Ltd), Superzyme (JH Biotech, Inc., Ventura, CA. USA), Tricho® (Agrimms Technologies Ltd) [11].

In this study, eight *Trichoderma* spp. isolates were evaluated in respect of their potential as BCAs and biofertilizer. The mycoparasitic activity, lytic enzyme productions indole-3-acetic acid (IAA) production and phosphate solubilization (P-S) capabilities of the species were tested together with the effects of temperature in light/dark, different carbon and nitrogen sources on mycelial growth and conidia production (M-G and C-P).

Materials and methods

Fungal isolates

Eight *Trichoderma* spp. (EGE-K-65, 71, 67, 72, 128, 129, 130, 131) were isolated from lumbering industry in Turkey.

For the isolation of *Trichoderma* spp., the soil dilution plating method was used [2]. The diluted samples were directly plated onto Rose Bengal Chloramphenicol agar as a selective medium. After 6 days of incubation at 28°C, fungal colonies were transferred to Malt Extract Agar (MEA) slants and they were stored +4°C for further investigations.

Determining the optimal medium for *Trichoderma* spp.

Five different growth media were used for evaluation of M-G and C-P at 28°C for 6 days. Tested growth media were as follows; Malt Yeast Glucose agar (MYGA) medium, MEA, Czapek Dox Agar (CDA), Modified CDA and Mendel's Medium [7, 12]. Since optimum pH values for the growth of the isolates were found as between pH 5.0 and 6.0 previously [13], it was kept constant at pH 5.50 in further experiments performed in this study.

DNA extraction, PCR amplifications and sequencing

For DNA extraction, fungal isolates were cultivated on MYGA at 28°C for 6 days. DNA extraction was carried out as described previously by Chen et al. [14]. The ITS region of the nuclear rDNA gene cluster was amplified from DNA using primers *ITS*1 (TCCGTAGGTGAACCTGCGG) and *ITS*4

(TCCTCCGCTTATTGATATGC) [15]. PCR amplifications and sequencing were performed by REFGEN (Turkey). The sequences were aligned in Molecular Evolutionary Genetics Analysis (MEGA) v.6 using Clustal W. The Phylogenetic trees were established with Maximum Likelihood method in MEGA v.6. For determining each clade, a bootstrap analysis was performed with 1000 replication.

Screening of *Trichoderma* spp. as a BCAs in vitro

Analysis of mycoparasitic activity against some phytopathogenic fungi in vitro

Three phytopathogenic fungi (*Verticillium* sp., *R. solani* and *F. oxysporium*) were obtained from the culture collection of Ege University Department of Plant Protection. The mycoparasitic activities of *Trichoderma* spp. were tested by dual culture techniques against phytopathogenic fungi after co-cultivation on PDA at 28°C for 10 days. Antagonism was also carried out according to the classification proposed by Bell et al. [2, 16, 17].

Determination of lytic enzyme activities

β-1.3 Glucanase activity (EC 3.2.1.39), chitinase activity (EC 3.2.1.14) and protease activity (EC3.4.21.4) were determined [18, 19] by using modified *Trichoderma* Liquid Enzyme (TLE) [20]. The components of the medium used for lytic enzyme production were as follows: 0.1% bactopeptone, 1.43% corn steep solids (CSS), KH_2PO_4 0.2%, 0.03% $MgSO_4 \cdot 7H_2O$, 0.03% glucose, and 0.1% (v/v) trace elements solution containing Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+} [20].

This medium (50 mL), in 250 mL flask, was inoculated with 1.0×10^8 (microscopic count) spores of *Trichoderma* spp., and incubated on rotary shaker (180 rpm) at 27°C and 30°C, respectively, for 4 and 7 days. Supernatant was separated and stocked at –20°C until enzyme activity measurement. All experiments were performed in duplicate.

Analysis of β-1, 3-glucanase activity

β-1, 3-glucanase activity (EC 3.2.1.39) was measured using 25% (w/v) suspension of laminarin that dissolved in 50 mM acetate buffer (pH 5.0) at 40°C for 30 min. Reducing sugars liberated from the substrate were determined with dinitrosalicylic acid (DNS) method described by Miller [18]. One unit of β-1, 3-glucanase activity was defined as the amount of enzyme that released 1 µmol reducing sugar in 1 min under assay conditions.

Determination of chitinase activity

Chitin (0.5%) obtained from crab shells (Sigma C7170) was used as substrate in modified TLE medium for chitinase activity. Chitinase activity was measured in terms of the amount of N-acetyl-D-glucosamine sugars released from chitin. Reducing sugars liberated from the substrate were determined with DNS method described by Miller [18]. One unit (U) of chitinase activity was defined as the amount of enzyme necessary to produce 1 µmol of *N*-acetylglucosamine (NAG) in 1 min [18].

Analysis of protease activity

Protease activity (EC3.4.21.4) was measured using azocasein (Sigma, A2765) as the substrate as described by Chun et al. [19]. One unit (U) protease activity was defined as the amount necessary to increase the absorbance at the rate of 0.01 at 440 nm in 1 min [19].

Screening of *Trichoderma* spp. as biofertilizers in vitro

Analysis of IAA production by *Trichoderma* spp.

Flasks (250 mL) containing 50 mL of half-strength Tryptic Soy Broth (TSB, Sigma-Aldrich) supplemented with 200 µg/mL L-tryptophan (Fluka, 22092) were inoculated with three agar plugs of 6 mm from 6 days old *Trichoderma* spp. and the flasks were incubated at 27°C at 150 rpm [21]. After 7 days of incubation, the cultures were filtered. IAA (mg/L) was measured by mixing 1 mL of the filtrate with 2 mL of Salkowski reagent (150 mL of $HClO_4$, 250 mL of distilled water and 7.5 mL of 0.5 M $FeCl_3 \cdot 6H_2O$) [21]. Reaction mixtures were incubated at room temperature for 20 min and the absorbances were measured at 535 nm [21].

Analysis of P-S capacity of *Trichoderma* spp.

P-S was determined using the method previously described by Murphy and Riley [22]. The 250 mL flasks containing 50 mL National Botanical Research Institute's Phosphate growth medium (NBRIP) were inoculated with 1.0×10^8 spores per mL.

The medium contained L^{-1}: glucose, 10 g, $Ca_3(PO_4)_2$, 5 g, $MgCl_2 \cdot 6H_2O$, 5 g, $MgSO_4 \cdot 7H_2O$, 0.25 g, KCl, 0.2 g and $(NH_4)_2SO_4$, 0.1 g [23]. All flasks were incubated for 10 days at 27°C, 150 rpm. After incubation, cultures were filtrated and P-S (mg/L) was measured by mixing 40 mL of the filtrate with 8 mL of ascorbic acid solution. Reaction mixtures were allowed to stay at room temperature for 10 min and the absorbances were measured at 700 nm.

Effect of temperature, carbon and nitrogen sources on M-G and C-P

Highest M-G and C-P at different conditions were evaluated, including different incubation temperature in light and dark conditions, the use of different carbon and nitrogen sources [12, 24–26].

The growth of each strain was measured by colony diameter [17]. The conidia production of *Trichoderma* spp. was graded as (–), 1(+), 2(+), 3(+), 4(+), 5(+).

Analysis of different temperatures in light and dark conditions

The effect of different temperatures on M-G and C-P under light (130 lux) [27] and dark conditions were investigated using 12 different temperatures (4, 10, 15, 20, 24, 27, 30, 33, 35, 40, 43 and 45°C). An agar plug of 6 mm, from 6 days old *Trichoderma* spp. was centrally inoculated on MYGA and the plates were incubated at temperatures listed above.

Effect of different carbon sources on growth

Sixteen different carbon sources (glucose, fructose, galactose, maltose, saccharose, lactose, raffinose, arabinose, cellulose, chitin, glycerol, mannitol, sorbitol, xylitol, trehalose and xylose) were used to determine their effect on growth. Each carbon source at a final concentration of 1% (w/v) was added into MCMA (carbon source, 10.0 g/L; yeast extract, 7.0 g/L; $NaNO_3$, 2 g/L; KCl, 0.5 g/L; $MgSO_4$, 0.5 g/L; $FeSO_4$, 0.01; K_2HPO_4, 1.0 g/L; agar, 20.0 g/L) [12] and all plates were incubated at 28°C for 3 days.

Effect of different nitrogen sources on growth

Eight different nitrogen sources (yeast extract, corn steep solid, soybean flour, peptone, urea, sodium nitrate, ammonium sulfate and diammonium hydrogen phosphate) were used to test their effect on growth. Each nitrogen source at a final concentration of 0.7% (w/v) was added into MCMA (nitrogen source, 7.0 g/L; glucose, 10.0 g/L; $NaNO_3$, 2 g/L; KCl, 0.5 g/L; $MgSO_4$, 0.5 g/L; $FeSO_4$, 0.01; K_2HPO_4, 1.0 g/L; agar, 20 g/L) [12]. All plates were incubated at 28°C for 3 days.

Statistical analysis

The experiments were carried out in triplicate. The results were statistically analyzed and Tukey test was used to compare the differences and to determine the standard deviations and standard errors of the means and to test the significance of treatment at $p \leq 0.05$.

Results and discussion

Determination of the optimal medium for *Trichoderma* spp.

Among the tested media, MYGA was determined as a optimal medium for the cultivation of *Trichoderma* spp. after observational comparison (data not shown) [7]. Further studies were carried out by using MYGA.

Identification of *Trichoderma* strains by molecular methods

The ITS rDNA region of *Trichoderma* spp. was sequenced and the fungal isolates were identified as *T. atroviride* (n = 3), *T. citrinoviride* (n = 5). The GenBank accession numbers are shown on Table 1 and the phylogenetic trees were constructed with MEGA v.6 as seen on Figure 1.

Screening of *Trichoderma* spp. as a BCAs in vitro

Analysis of mycoparasitic activity of *Trichoderma* spp. against some phytopathogenic fungi in vitro

Trichoderma spp. vary considerably in their mycoparazitic activity against phytopathogenic fungi [8, 28]. For instance, it has been reported that *T. atroviride* caused significant decrease in growth rate of *Leptosphaeria* spp. [3], and *R. solani* [8, 28].

Table 1: Genbank accession numbers of DNA sequences, Length of Sequence (bp), isolation source of *Trichoderma* spp. used in phylogenetic analyses, *T. atroviride* (n = 3) and *T. citrinoviride* (n = 5).

Trichoderma spp.	Isolates names	Length of sequence (bp)	Genbank accession number	Isolation source	Country of origin
T. atroviride	EGE-K-65	588	JX119037	Lumber	Turkey
T. atroviride	EGE-K-71	598	JX119036	Lumber	Turkey
T. atroviride	EGE-K-131	712	JX119035	Lumber	Turkey
T. citrinoviride	EGE-K-67	799	JX125613	Lumber	Turkey
T. citrinoviride	EGE-K-72	639	JX125614	Lumber	Turkey
T. citrinoviride	EGE-K-128	823	JX125615	Lumber	Turkey
T. citrinoviride	EGE-K-129	818	JX125616	Lumber	Turkey
T. citrinoviride	EGE-K-130	597	JX125617	Lumber	Turkey

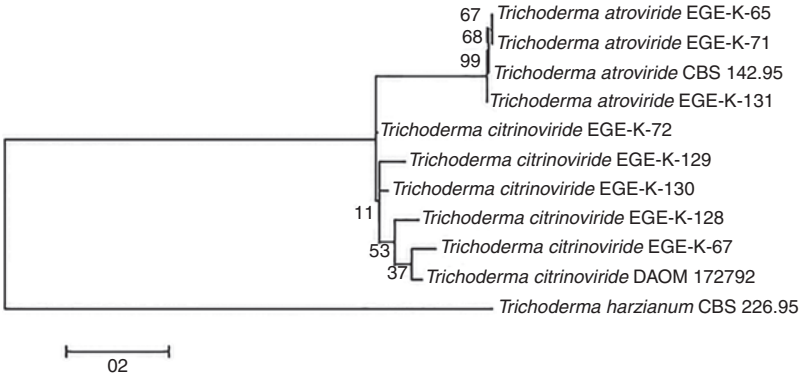

Figure 1: The phylogenetic tree for *T. citrinoviride* and *T. atroviride* and relative species based on the internal transcribed spacer (ITS) ribosomal DNA (rDNA) gene sequences.

In this study, all of the *Trichoderma* spp. overgrew on the mycelia of *Verticillium* sp. Interestingly, *T. citrinoviride* demostrated strong effect against *F. oxysporium* and *T. atroviride* was very effective against *R. solani*. This is particularly important to improve the antagonistic effects of BCAs on phytopathogenic fungi and particularly important for the biocontrol of phytopathogenic fungi such as *F. oxysporium* and *R. solani* [1].

Mycoparasitism also involves some morphological changes [9]. As seen on Figure 2, significant antagonist action leading to morphological changes in vitro was achieved by *T. citrinoviride* (EGE-K-72 and EGE-K-128) against *F. oxysporium*.

Lytic enzyme activities

Chitinase, protease and β-1,3-glucanase are known as mycoparasitism-related enzymes (Table 2), since they are degrading the cell wall and inhibiting the growth of phytopathogenic fungi. Furthermore, β-1,3-glucanase inhibits spore germination of phytopathogen together with chitinases [1]. In the present study, all *Trichoderma* spp. produced lytic enzymes such as chitinase, protease and β-1,3-glucanase. The chitinase, 1,3-glucanase and protease activities for *T. citrinoviride* and *T. atroviride* species were determined as 0.17–0.21 and 0.14–0.17 U/mL, 0.12–0.21 and 0.07–0.25 at U/mL and, 0.64–2.29 and 1.76–1.22 U/mL, respectively (Table 2).

Screening of *Trichoderma* spp. in respect of biofertilizer properties in vitro

Analysis of IAA production by *Trichoderma* spp.

Several *Trichoderma* species can produce PG-P substances such as the auxin phytohormone IAA. Its production has been suggested to promote root growth, and resulting in increased root mass [29].

IAA production levels in this study were determined to be a minimum of 5.65 ± 0.05 and 11.56 ± 1.92 mg/L and a maximum of 8.81 ± 0.03 for *T. atroviride* and 57.01 ± 3.11 mg/L for *T. citrinoviride* (Table 2).

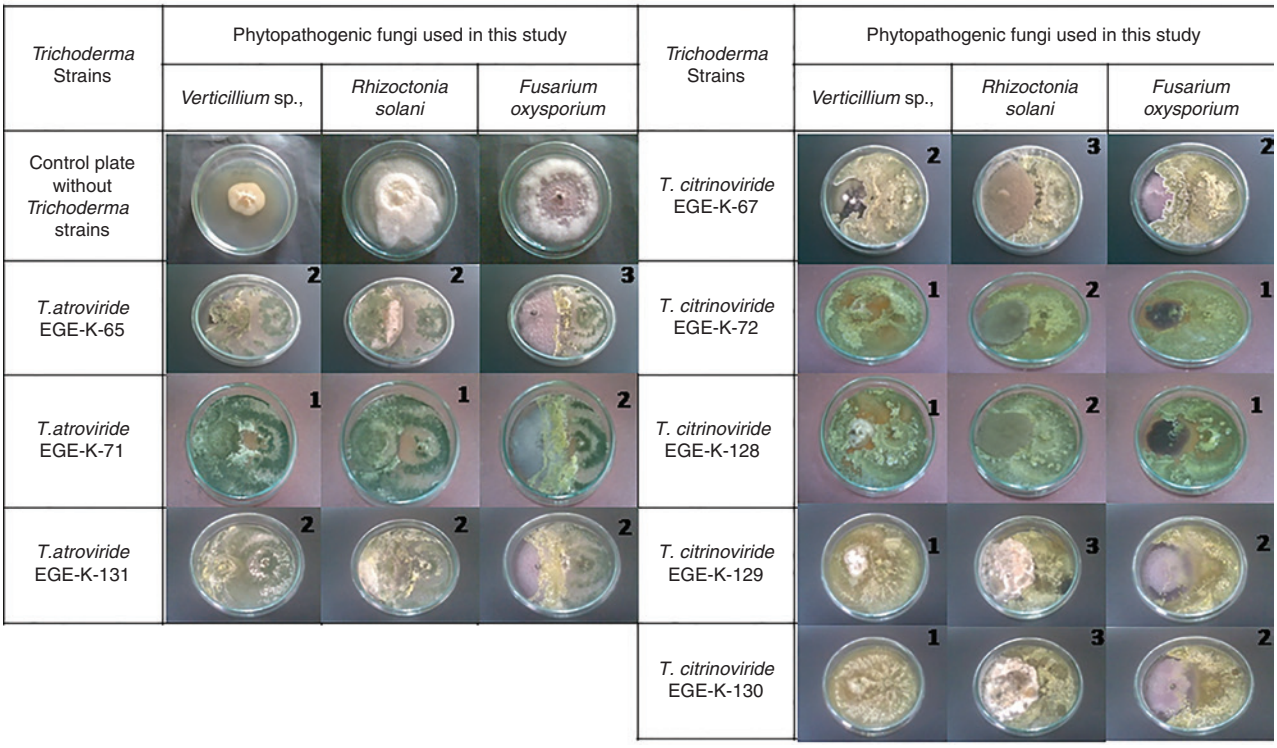

Figure 2: Dual growth *Trichoderma* spp. and three phytopathogenic fungi (*Verticillium* sp, *Rhizoctonia solani* and *Fusarium oxysporium*) at 27°C for 10 days.
According to Bell's classification each number represents a different growth pattern as follows: (**1**) *Trichoderma* completely overgrew the pathogen and covered the entire medium surface, (**2**) *Trichoderma* overgrew at least two-thirds of the medium surface, (**3**) *Trichoderma* and the pathogen each colonized approximately one-half of the medium surface and neither organism dominated the other, (**4**) the pathogen colonized at least two-thirds of the medium surface and withstood encroachment by *Trichoderma* and (**5**) the pathogen completely dominated *Trichoderma*, overgrew it, and occupied the entire medium surface.

Table 2: Lytic enzymes, Indole-3-acetic acid production and phosphate solubilization of *T. atrorivide* and *T. citrinoviride* species at 27°C and 30°C, respectively.

Trichoderma spp.		Lytic enzymes[a] (U/mL)						IAA[a] production (mg/L)	Phosphate solubility[a] (mg/L)
		Chitinase		Protease		β-1,3-Glucanase			
		4 days	7 days	4 days	7 days	4 days	7 days	7 days	10 days
T. atroviride (n = 3)	Min	0.12 ± 0.01	0.05 ± 0.01	0.56 ± 0.10	ND	0.05 ± 0.04	0.16 ± 0.07	5.65 ± 0.05	2.94 ± 0.03
	Max	0.16 ± 0.01	0.29 ± 0.03	2.7 ± 0.10	2.27 ± 0.93	0.10 ± 0.01	0.40 ± 0.27	8.81 ± 0.03	8.69 ± 0.04
	Mean	0.14	0.17	1.76	1.22	0.07	0.25	7.23	6.01
T. citrinoviride (n = 5)	Min	0.06 ± 0.02	0.19 ± 0.07	1.13 ± 0.33	ND	0.05 ± 0.04	ND	11.56 ± 1.92	9.07 ± 0.50
	Max	0.24 ± 0.08	0.29 ± 0.02	2.99 ± 0.82	1.73 ± 0.73	0.23 ± 0.02	0.37 ± 0.07	57.01 ± 3.11	16.24 ± 2.73
	Mean	0.17	0.21	2.29	0.64	0.12	0.21	23.50	12.54

[a]Incubation period is 4–7 days for enzymes, 7 days for IAA production and 10 days for phosphate solubility, ND, Not detected.

Analysis of P-S capacity of *Trichoderma* spp.

Trichoderma species are potential phosphate solubilizing microorganisms and play an important role in providing phosphorus to plants [22, 23, 30, 31]. Studies related to several *Trichoderma*-based biofertilizers are available and they are used for controlling plant diseases and promoting plant growth [10, 30]. Therefore, *T. atroviride* and *T. citrinoviride* were screened for in-vitro P-solubilization. The average concentrations of phosphate (mg/L) were determined as 6.01 mg/L for *T. atroviride* and 12.54 mg/L for *T. citrinoviride* (Table 2). Although this value is higher

than that obtained in Gravel et al. [21], there are some reports mentioning much higher amounts of phosphate solubility (up to 404.07 $\mu g \cdot mL^{-1}$) values as compared to this study [30, 31]. Since the main aim was not to optimize phosphate solubilisation, further optimization need to be performed to be able to obtain higher values especially considering the incubation period with time course productions.

Effect of temperature, carbon and nitrogen sources on M-G and C-P

Analysis of different temperatures in light and dark conditions

Studies indicated that there were significant differences on the fungal M-G and C-P due to physical growth conditions such as incubation temperature together with light and dark conditions and nutritional requirements using different carbon and nitrogen sources [9, 24, 32].

The mycelia growth and conidia production of all *T. atroviride* and *T. citrinoviride* species in the present study were affected by different temperatures as well as light and dark conditions. The differences are significant ($p < 0.05$) between *T. citrinoviride* and *T. atroviride* species at different temperatures in light/dark conditions.

Optimum growth temperatures were found to be 27°C for *T. atroviride* and between 27 and 35°C for *T. citrinoviride* (Figures 3A,B, Figure 4A,B).

Although strains of all five *T. citrinoviride* grew well between at 15°C and 40°C, no growth was observed above 40°C and below 15°C after 3 days of incubation under light conditions (Figure 3A). However, all *T. citrinoviride* strains were able to grow at 43°C for 3 days under dark condition (Figure 3B). Interestingly, *T. citrinoviride* EGE-KL-128 grew at 10°C for 3 days under both light and dark conditions (Figure 3A,B).

Strains of *T. atroviride* grew well between 15°C and 30°C after 3 days of incubation under light conditions (Figure 4A) and they were able to grow at 15°C–33°C for 3 days under dark conditions (Figure 4B). Previous studies

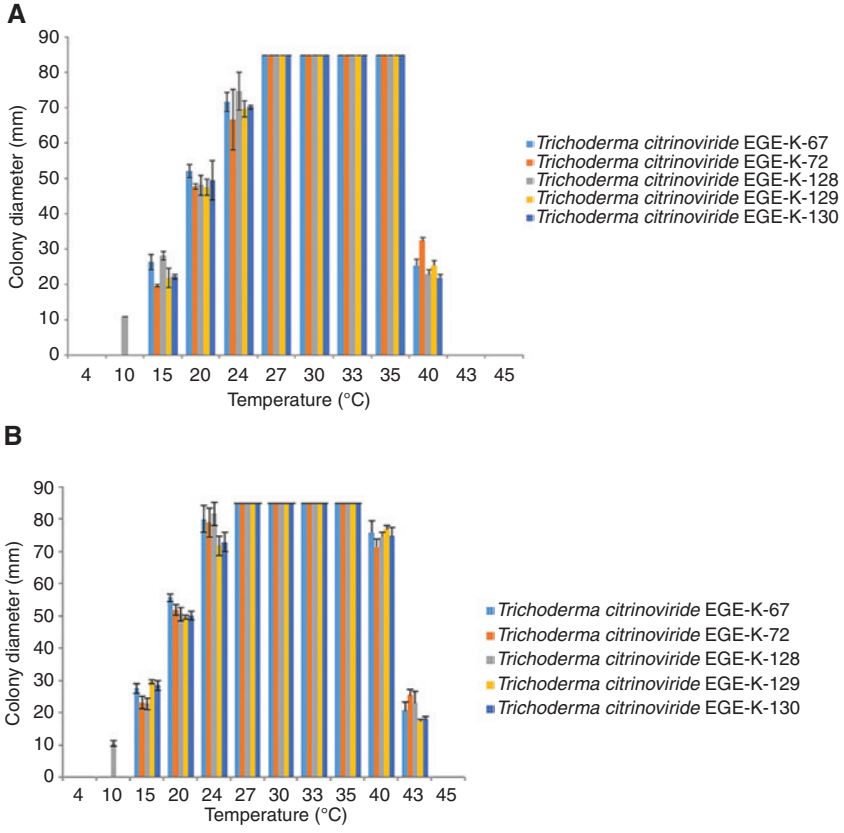

Figure 3: (A) Effect of different temperature (4, 10, 15, 20, 24, 27, 30, 33, 35, 40 and 45°C) on mycelia growth profiling of *T. citrinoviride* in light (130 lux) for 3 days. (B) Effect of different temperature (4, 10, 15, 20, 24, 27, 30, 33, 35, 40 and 45°C) on mycelia growth profiling of *T. citrinoviride* in dark condition for 3 days.
The differences are significant ($p < 0.05$) between *T. citrinoviride* and *T. atroviride* species at different temperatures in light.

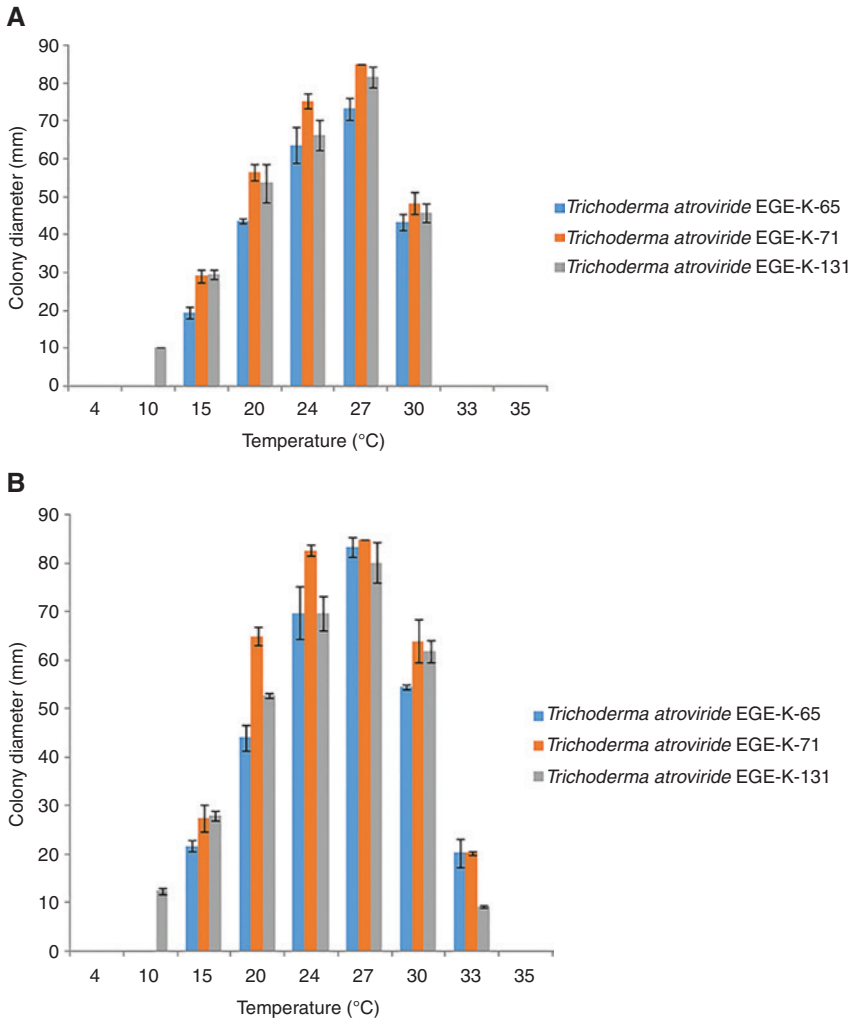

Figure 4: (A) Effect of different temperature (4, 10, 15, 20, 24, 27, 30, 33, 35, 40 and 45°C) on mycelia growth profiling of *T. atroviride* in the light (130 lux) condition for 3 days. (B) Effect of different temperature (4, 10, 15, 20, 24, 27, 30, 33, 35, 40 and 45°C) mycelia growth profiling of *T. atroviride* in the dark condition for 3 days.
The differences are significant ($p < 0.05$) between *T. citrinoviride* and *T. atroviride* species at different temperatures in dark.

by Daryaei et al. [25] have shown that conidia production was enhanced by *T. atroviride* LU132 colonies with the exposure of dark conditions. All strains grew at 10°C after 1 week in both light and dark conditions except for *T. atroviride* EGE-K-131 which grew at 10°C for 3 days (Figure 4A,B).

In general, conidia production temperature ranges were determined as 15–30°C and 20–35°C for *T. atroviride* and *T. citrinoviride* (Table 3). These results are comparable with the study reported by Daryaei et al. [24] where lower amount of conidia produced at 30°C. They reported that the best conidia production for *T. atroviride* was produced at 25°C.

None of the strains produced conidia at 4°C, 10°C, 43°C and 45°C for 10 days in the present study. It is also worth mentioning that, at lower temperatures, conidia production of *T. atroviride* was significantly higher than *T. citrinoviride* (Table 3). On the other hand, the highest conidia production for *T. citrinoviride* was achieved between 33°C and 35°C and it declined at 40°C (Table 3).

Overall evaluation of temparature effect on growth suggested that *T. atroviride* produced higher amount of conidia at lower temperatures as compared to *T. citrinoviride*. Likewise, the conidia production pattern of *T. citrinoviride* seemed to increase as the temperature increased. Significant differences were found when growing both species at different temperatures under the effect of light and dark. Thus, the highest conidia production perfomances of both *Trichoderma* species in the wide temperature range provided that it is possible to take this advantage for the production of BCAs in different climatic conditions around the world.

Table 3: Effect of temperature on conidium production of *Trichoderma* spp. in dark and light conditions at 10 days.

Trichoderma spp	Isolate name	Temperatures															
		15°C		20°C		24°C		27°C		30°C		33°C		35°C		40°C	
		D	L	D	L	D	L	D	L	D	L	D	L	D	L	D	L
T. atroviride	EGE-K-65	4(+)	4(+)	5(+)	5(+)	5(+)	5(+)	4(+)	5(+)	3(+)	3(+)	–	–	–	–	–	–
	EGE-K-71	4(+)	4(+)	5(+)	5(+)	4(+)	5(+)	4(+)	5(+)	4(+)	5(+)	–	–	–	–	–	–
	EGE-K-131	3(+)	3(+)	5(+)	5(+)	5(+)	5(+)	5(+)	5(+)	5(+)	4(+)	–	–	–	–	–	–
T.citrinoviride	EGE-K-67	2(+)	4(+)	3(+)	4(+)	3(+)	4(+)	4(+)	4(+)	4(+)	5(+)	5(+)	5(+)	5(+)	5(+)	1(+)	1(+)
	EGE-K-72	2(+)	1(+)	4(+)	5(+)	4(+)	5(+)	4(+)	5(+)	5(+)	5(+)	5(+)	5(+)	5(+)	5(+)	1(+)	1(+)
	EGE-K-128	1(+)	2(+)	3(+)	5(+)	4(+)	5(+)	4(+)	4(+)	5(+)	5(+)	5(+)	5(+)	5(+)	5(+)	1(+)	1(+)
	EGE-K-129	1(+)	2(+)	4(+)	4(+)	4(+)	5(+)	4(+)	5(+)	4(+)	5(+)	5(+)	5(+)	5(+)	5(+)	1(+)	1(+)
	EGE-K-130	–	1(+)	4(+)	5(+)	4(+)	5(+)	4(+)	5(+)	5(+)	5(+)	5(+)	5(+)	5(+)	5(+)	1(+)	1(+)

The conidium production of *Trichoderma* spp. was observed during 10 days and was graded as (–), 1(+), 2(+), 3(+), 4(+), 5(+). D, Dark condition; L, light condition. None of species produced conidia at 4°C, 10°C, 43°C and 45°C for 10 days.

Effect of different carbon sources on growth

One of the most important factor that effect production of *Trichoderma*-based BCAs is the carbon source and several scientists have tried different substrates as a carbon source to obtain high sporulation [33–35].

Both species (*T. atroviride* and *T. citrinoviride*) grew well on all carbon sources tested in the present study. No significant differences in mycelial growth were observed with respect to different carbon sources used tested (Figure 5A and B). In a similar study, sucrose, mannose, glucose, xylose, and starch were found effective for the growth of *T. harzianum* as compared to maltose and D-galactose [33] whereas glucose [36] sucrose [35] and cellobiose [37] were found favourable in other studies.

It is worth mentioning that no conidia production was observed after 10 days of incubation with some carbon sources tested in this study. These carbon sources are maltose, raffinose, cellulose, chitin, sorbitol for *T. atroviride* EGE-K-65; saccharose, raffinose, cellulose for *T. citrinoviride* EGE-K-72; maltose, saccharose, raffinose, cellulose, chitin, xylitol for *T. citrinoviride* EGE-K-129; and saccharose, lactose, raffinose, cellulose, chitin, xylitol for *T. citrinoviride* EGE-K-130 (data not shown).

It has been reported that the Carbon:Nitrogen (C:N) ratios of media had significant effects on the spore yield of fungal BCAs to optimize nutritional conditions for mass production [38]. Daryaei et al. [24] reported that different C:N ratio and carbon contents for maximal conidia production of *T. atroviride* LU132 were 5:1 and 4.2 g/L, respectively. The C:N value for maximal M-G and C-P was found as 2:1 in our experimental conditions, which is quite similar to Daryaei et al. [24]. They reported that the greatest inhibition on *R. solani* growth was achieved from conidia produced at high concentration of trehalose. Trehalose accumulation was induced by stress conditions which is required for stress tolerance like dehydration of fungal cells under high temperatures [8, 24, 26, 28]. The highest mycelial growth for *T. citrinoviride* in this study was found not only when mannitol and trehalose were used but also with glucose, galactose glycerol, mannitol and xylose containing media (Figure 5A).

Effect of different nitrogen sources on growth

All *Trichoderma* spp. demonstrated pronounced growth with CSS (Figure 6A and B). *T. citrinoviride* strains, with the exception of *T. citrinoviride* EGE-KL-72, exhibited good mycelium growth with yeast extract and soybean flour (Figure 6A). The highest mycelial growth was achieved by *T. citrinoviride* EGE-K-129 on pepton (Figure 6A).

The results showed that the effect of organic nitrogen sources (corn steep solids > yeast extract > soybean flour) on the growth of *T. atroviride* and *T. citrinoviride* species were significantly higher than those observed with inorganic nitrogen sources with the exception of *T. citrinoviride* EGE-KL-72 (Figure 6A and B). The effect of organic nitrogen sources on growth was notably higher than inorganic nitrogen sources.

T. citrinoviride EGE-K-72 showed very weak growth on sodium nitrate and ammonium sulfate and did not grow in the medium containing diammonium hydrogen phosphate. Among the *T. citrinoviride* strains, *T. citrinoviride* EGE-K-129 demostrated better growth with inorganic nitrogen sources as compared to the other species (Figure 6A).

T. atroviride strains revealed similar growth patterns on sodium nitrate and ammonium sulfate and their

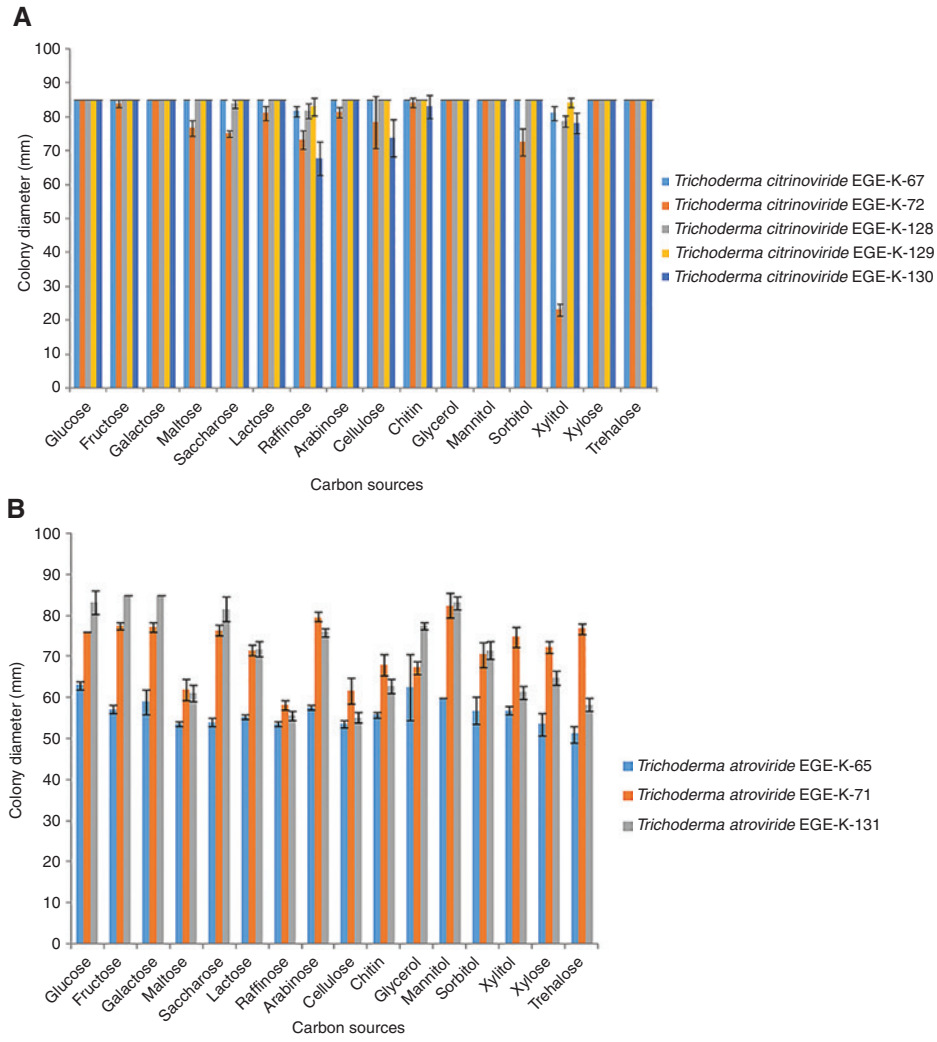

Figure 5: (A) Effect of different carbon sources on mycelia growth profiling of *T. citrinoviride* at 28°C for 3 days, (B) Effect of different carbon sources on mycelia growth profiling of *T. atroviride* at 28°C for 3 days.
The differences are significant ($p < 0.05$) between *T. citrinoviride* and *T. atroviride* species with different carbon sources.

effect on growth was significantly higher than the one obtained by diammonium hydrogen phosphate. Our results were in agreement with other results obtained by other researchers in which the best growth and sporulation of *T. viride* and *T. harzianum* were favored by ammonium forms of nitrogen as compared to nitrite or nitrate forms [35, 39].

As seen on Figure 6A and B, none of the *Trichoderma* species can grow on urea up to 3 days of cultivation. The minimum mycelial growth was initiated after 4 days of incubation. Towards the end of the incubation period differences between the colony diameters were very high ranging between 10 and 40 mm.

All species produced significant amounts of conidia on all organic nitrogen sources. But, none of the *Trichoderma* species produce conidia on urea which is in accordance to those reported previously [33]. *Trichoderma citrinoviride* EGE-K-72 did not produce conidia on inorganic nitrogen sources during 10 days. Exceptionally, *T. citrinoviride* EGE-K-129 produced conidia on diammonium hydrogen phosphate (data not shown).

Conclusion

The results provided valuable data for achieving highest M-G and C-P of *Trichoderma* species in terms of their portential as for the preparation of new commercial biological control and biofertilizer formulations under different agro-climatic regions since they exposed growth in a wide temperature range.

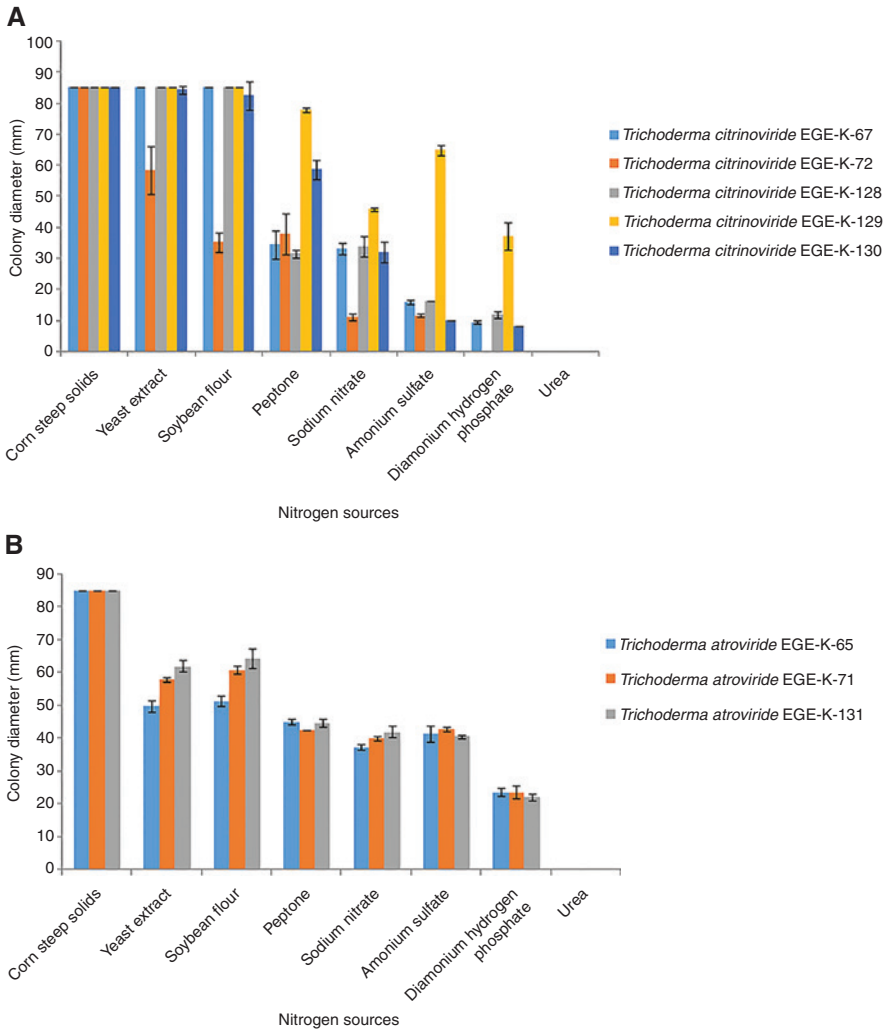

Figure 6: (A) Effect of different nitrogen sources on mycelia growth profiling of *T. citrinoviride* at 28°C for 3 days. (B) Effect of different nitrogen sources on mycelia growth profiling of *T. atroviride* at 28°C for 3 days.
The differences are significant ($p < 0.05$) between *T. citrinoviride* and *T. atroviride* species with different nitrogen sources.

In particular, to the best of our knowledge it was the first study concerning the *T. citrinoviride* in respect of BCAs and biofertilizer properties.

According to the results obtained, it was clearly seen that *T. citrinoviride* strains might have potential for use as efficient BCAs and PG-P fungi.

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