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Research Article

Effect of temperature and culture medium on the growth and sporulation of eight *Trichoderma* species

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Abstract

Trichoderma species are important agents of biological control of soil-borne plant pathogens. The growth and reproduction of these fungi are influenced by the culture medium and temperature. This study was conducted to determine the effect of temperature and culture medium on the growth and sporulation of *Trichoderma* species. Ten isolates of *Trichoderma* species were isolated from agricultural soils of different regions of Lorestan Province, Iran. The study of morphological characteristics and sequencing of ITS-rDNA, and *tef1α* gene regions showed that they are belong to eight species of *Trichoderma*. Investigating the effect of four types of culture medium and five temperatures to determine optimum culture medium and temperature for the growth and reproduction of these fungi, showed that the Potato/Dextrose/Agar (PDA) medium is the best, and the temperature of 20 to 30 degrees Celsius is optimal for the growth and reproduction of these fungi. Evaluation of their ability to inhibit the growth of the soil-borne plant pathogenic fungus *Rhizoctonia solani* in vitro, showed that *T. harzianum* LT8 has the most inhibition ability. Therefore, this isolate can be used as a potential biocontrol agent for this plant pathogenic fungus in future research.

Keywords: ITS-rDNA, Mycelial growth inhibition, *Rhizoctonia solan*

مقاله پژوهشی

تاثیر دما و محیط کشت بر رشد و اسپورزایی هشت گونه *Trichoderma*

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میرزایی‌پور ز، بازگیر ع، ظفری د، درویش‌نیا م (۱۴۰۲) تاثیر دما و محیط کشت بر رشد و اسپورزایی هشت گونه *Trichoderma* . دانش بیماری‌شناسی گیاهی ۱۲(۲): ۱۱۶-۱۰۵.

چکیده

گونه‌های *Trichoderma* از عوامل مهم مهارزیستی بیمارگرهای خاک‌زی گیاهان هستند. رشد و

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تکثیر این قارچ‌ها تحت تأثیر محیط کشت و دما قرار می‌گیرد . این پژوهش به منظور تعیین تأثیر دما و محیط کشت بر رشد و هاگ‌زایی گونه‌های *Trichoderma* انجام شد. ده جدایه گونه‌های *Trichoderma* از خاک‌های زراعی مناطق مختلف استان لرستان، ایران جداسازی شدند. مطالعه مشخصات مرفولوژی و توالی‌یابی ناحیه‌های ژنی ITS-rDNA, *tef1α* آنها نشان داد که متعلق به هشت گونه *Trichoderma* هستند. بررسی تاثیر چهار نوع محیط کشت و پنج دما برای تعیین محیط‌کشت و دمای بهینه برای رشد و تکثیر این قارچها، نشان داد که محیط سیب‌زمینی/دکستروز/آگار (PDA) بهترین محیط و دمای ۲۰ تا ۳۰ درجه سلسیوس برای رشد و تکثیر این قارچ‌ها بهینه هستند. بررسی توانایی آنها در بازدارندگی از رشد قارچ خاک‌زی بیمارگر گیاهی *Rhizoctonia solani* در شرایط آزمایشگاهی، نشان داد که *T. harzianum* LT8 بیشترین توانایی بازدارندگی را دارد. بنابراین از این جدایه می‌توان به عنوان یک عامل بالقوه مهارزیستی برای این قارچ بیمارگر گیاهی در پژوهش‌های آینده استفاده کرد.

واژگان کلیدی: بازدارندگی از رشد میسلیوم، ITS-rDNA ، *Rhizoctonia solani*

Introduction مقدمه

Trichoderma species are one of the most abundant soil-borne fungi commonly found in plants rhizosphere (Sharma et al. 2019). These fungi are distributed worldwide thanks to their rapid growth, their ability to use different substrates and tolerate the presence of different pollutants and environmental conditions (Harman et al. 2004, Sharma et al. 2019, Hu et al. 2020). A large volume of scientific studies and reports show that the success of *Trichoderma* species as bio-control agents is based on the activation of several direct mechanisms such as hyper-parasitism, competition and antibiosis or indirectly by improving plant growth and vigor and enhancing stress tolerance. Therefore, these fungi not only protect plants by eliminating pathogens, but also enhance plant resistant to biotic and abiotic stresses (Contreras-Cornejo et al. 2016, Zin and Badaluddin, 2020, Hu et al. 2020, Thapa et al. 2020, Sood et al. 2020)

Abiotic environmental parameters may adversely affect the biological control by *Trichoderma* species (Kredics et al. 2003). Several studies have shown the temperature, pH and culture media are important factors affecting spore germination, germ tube growth, mycelia growth, and competitive saprophytic ability, on volatile and non-volatile metabolites production of *Trichoderma* species (Kredics et al. 2003, Malathi and Doraisamy 2003, Gade et al. 2009, Shahid et al. 2011, Zehra et al. 2017).Temperature is a prominent factor that determines natural distribution of *Trichoderma* species in soil and its antagonistic activity. The optimum temperature for growth differs among the *Trichoderma* species (Kredics et al. 2003). This study was conducted to determine the effect of temperature and culture medium on the growth and sporulation of isolates of *Trichoderma* species.

Materials and Methods مواد و روش‌ها

Isolation, purification and identification of Trichoderma species

Soil samples were collected in plastic bags, from 5-30 cm depth of rhizosphere soil of

different geographical regions of the Lorestan Province, Iran. The collected samples were stored at 4°C before use. *Trichoderma* species isolation carried out by serial dilution technique of soil samples in water and application of *Trichoderma* species selective media (TSM) (Elad and Chet 1983, Laila et al. 2019). The purified isolates were maintained on Potato/Dextrose/Agar (PDA) slants at 4 °C for further use. Isolates of *Trichoderma* species were first identified morphologically based on macro-morphological (colony, color, pattern and growth rate) and micro-morphological (conidial, phialide and chlamydospore shape and size) characteristics (Bissett 1984, Gams and Bissett 2002, Sharma and Singh 2014) and finally accomplished by combination of morphological, morphometric and molecular identification using the ITS-rDNA and *tef1α* regions.

Testing the effect of culture media and temperature on the growth and sporulation of Trichoderma species

Four different sterilized solid media *viz.* PDA, CMD, MEA and SNA were used for this experiment. A 5mm plug of the periphery of growing colony of the each isolate on PDA culture was removed using a sterile cork borer and placed in the center of each petri plate. This experiment carried out adopting a completely randomized design with three replications (petri plate). The plates were then incubated at 15, 20, 25, 30 and 35 °C. Growth rate, sporulation, and colony color of each treatment was recorded at 24 hours intervals until complete petri dish colonization by them.

Effect of temperatures 15, 20, 25, 30 and 35°C on the growth rate of the each isolate were studied on PDA medium. From actively growing culture of each isolate a 5mm in diameter plug, using a sterile Cork borer was placed at the center of petri plates. The inoculated Petri plates were kept in the dark at 15, 20, 25, 30 and 35 °C in the BOD incubator and diameter of mycelial colony (mm) of these isolates were recorded at 24 h intervals until the petri plates were completely colonized by them. This experiment was performed adopting a completely randomized design with three replications. Spore production was evaluated for these isolates from their 7-day-old PDA cultures grown at 25±1 °C. Spore suspensions were prepared from these cultures by scrubbing the spores with a sterile loop in distilled water and removing mycelium fragments using a filter paper. The spores were counted using a haemocytometer. For each isolate three replicates were evaluated (Carro-Huerga et al. 2021).

Testing biological activity of Trichoderma species

The biological activity of these isolates was evaluated against the soil-born plant pathogen *Rhizoctonia solani* (OQ436050) using dual-culture test by Dennis and Webster (1971) method *in vitro*. The percentage of *R. solani* mycelial growth inhibition by *Trichoderma* isolates for each replicate was calculated using I%= (R1- R2)/R1×100 formula in which: I%= percentage of *R. solani* mycelial growth inhibition by *Trichoderma* isolate, R1= *R. solani* mycelial growth (mm), R2= *R. solani* mycelial growth (mm) in dual-culture.

The SAS software was used for data statistical analysis of all tests, and means were compared using Tukey's range test.

Results یافته‌ها

Identified Trichoderma species

ITS-rDNA and *tef1α* genes sequencing and analysis using the BLAST tool revealed that

جدول ۱. لیست جدایه‌های مورد استفاده، گونه آنها و کد دستیابی آنها در بانک ژنNCBI .

Table 1. List of used *Trichoderma* isolates, their species and accession numbers in NCBI Gen Bank.

Isolates Code	Species	NCBI Gen Bank accession number	
		ITS	*Tef1*
LT7	*T. atroviride*	OQ469323	OQ525911
LT8	*T. harzianum*	OQ469750	OQ504831
LT9	*T. virens*	OQ469480	OQ525910
LT20	*Trichoderma* sp.	OQ422163	OQ504832
LT33	*T. atrobrunneum*	OQ469486	OQ525908
LT35	*T. longibrachiatum*	OQ469493	OQ504829
LT101	*T. harzianum*	OQ422955	OQ504830
LT140	*T. harzianum*	OQ422164	OQ525912
LT148	*T. guizhouense*	OQ469500	OQ525909
LT200	*T. citrinoviride*	OQ469772	OQ525913

all of the 10 isolates show an identity ranging from 97% to 100% with the available sequences in NCBI Gen Bank. Furthermore, the phylogenetic analysis revealed that these 10 isolates represent eight different species, namely three isolates of *T. harzianum* (LT8, LT101 and LT140), one isolate each of *T. atrobrunneum* (LT33), *T. atroviride* (LT7), *T. citrinoviride* (LT200), *T. virens* (LT9), *T. longibrachiatum (*LT35*), T. guizhouense* (LT148) and *T.* sp. (LT20), (Table 1).

The effect of culture media and temperature on the growth and sporulation of Trichoderma species

The isolates were grown at 15, 20, 25, 30 and 35°C on PDA, CMD, MEA and SNA media. It was found that the isolates had different phenotypic characteristics depending on the media and temperature. They also had different growth patterns on each medium. Most of the isolates grew faster on PDA medium than other media at all tested temperatures (Table 2, Fig.1).

LT35 and LT20 had the highest and lowest growth rates, respectively, on PDA, CMD and MEA at 25°C. On SNA, LT9 and LT20 had the highest and lowest growth rates, respectively. At 15°C on PDA, LT35 and LT20 had the highest and lowest growth rates, respectively, after 48h. At 20°C, all isolates colonized the petri dishes after 3 days incubation. LT200 was the fastest growing isolate at 20°C after 48h. LT35 was the fastest isolate to colonize the petri dishes at 25°C after 2 days. Most isolates grew well at 25°C, but poorly at 35°C. Some isolates did not grow at all or produced abnormal colonies and few spores at 15°C or 35°C. LT8 and LT35 had the highest growth rates at 35°C after 48h.

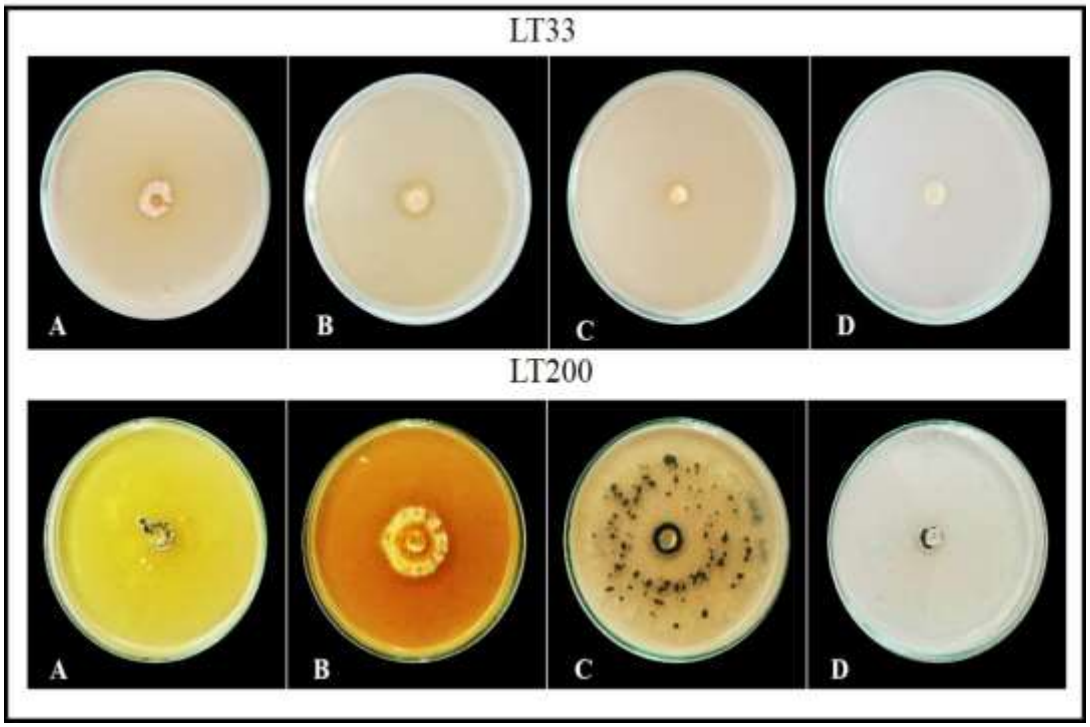
The result of spore production of isolates at 25°C experiment, showed that LT140, by 2.5×10^7 spore/ml produced the the highest spore production , followed by LT20 and LT8

جدول ۲. میزان رشد جدایه‌های گونه‌های *Trichoderma* در محیط کشت‌های مختلف پس از ۴۸ ساعت در دمای ۲۵ درجه سلسیوس.

Table 2. The growth rate (mm) of isolates of *Trichoderma* species on different culture media at 25°C after 48 hours.

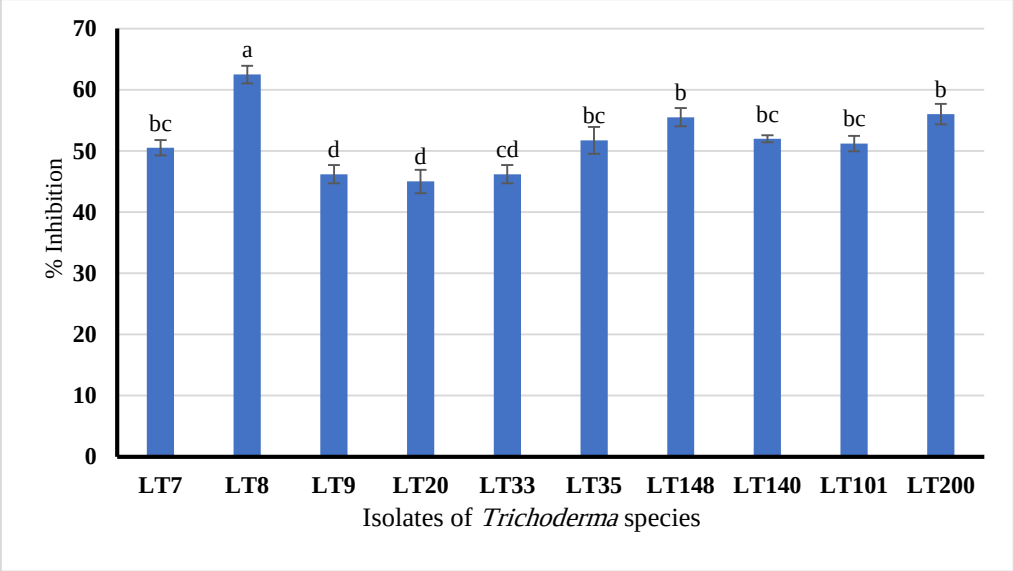
Isolate	PDA	CMD	MEA	SNA	*p*
LT7	27.50±0.28 a	22.50±0.50 b	24.66±0.88 ab	24.00±1.00 ab	0.000
LT8	35.66±0.33 a	32.00±1.00 b	32.66±0.88 ab	27.66±0.66 b	0.000
LT9	29±1.00 ab	28.16±0.44 a	31.16±1.00 ab	33.33±0.88 b	0.015
LT20	18.33±0.88 a	17.33±0.66 ab	13.50±0.50 ab	6±1.00 c	0.000
LT33	36.50±0.50 a	36.66±0.33 a	37.50±0.50 a	26.33±0.88 b	<0.000
LT35	42.50±0.00 a	42.50±0.00 a	42.50±0.00 a	28.33±0.88 b	<0.000
LT101	25.50±0.50 a	30.00±0.57 b	27.00±0.57 a	8.50±0.76 c	<0.000
LT140	30.66±0.66 a	34.50±0.50 a	34.50±0.50 a	28.67±1.86 a	0.043
LT148	25±0.57 a	23.00±1.00 a	23.33±0.33 a	16.50±0.50 b	0.000
LT200	31±1.00 a	29.00±0.57 a	28.50±0.76 a	22.50±1.50 b	0.003

isolates, respectively and the lowest amount of spore production was related to LT33 isolate. LT7 and LT20 isolates did not produce any spores after 7 days incubation.



شکل۱. رشد جدایه های LT33 در دمای ۱۵ درجه سانتی‌گراد و LT200 در دمای ۳۵ درجه سانتی‌گراد روی PDA (A)، CMD (B)، MEA (C)و SNA (D). پتری‌ها ۹ سانتی‌متری.

Figure 1. Growth of the isolates LT33 at 15 ºC and LT200 at 35 ºC on PDA (A), CMD (B), MEA (C) and SNA (D). The 9 cm petri plates.



شکل۲. بازدارندگی از رشد میسلیومی *Rhizoctonia solani* توسط ۱۰ جدایه هشت گونه *Trichoderma*.

Figure 2. Mycelial growth inhibition of *Rhizoctonia solani* by 10 isolates of *Trichoderma* species.

Biological activity of Trichoderma species

Results of *in vitro* evaluation of the antagonistic activity of different isolates of *Trichoderma* species against *R. solani* showed that the percentage of inhibition of mycelial growth (IMG) of *R. solani* by these isolates varied from 45 to 62.5% (Fig. 2). LT8, LT200, and LT35 isolates with 62.5% ,and 56%, 51.6%, showed higher *R. solani* IMG than the other, respectively.

Discussion بحث

In this study, we evaluated the effect of different temperatures and culture media on the growth and sporulation of 10 isolates of *Trichoderma* species. The growth rate of the 10 tested isolates on PDA, CMD and MEA media after 48 hours was almost in the same range, but on SNA culture media most isolates had lower growth rate and sporulation, with the exception of LT9 isolate which was faster than other isolates. It was found that among the tested culture media, PDA was the best solid media for growth and sporulation of the tested isolates, which may be due to rich and very diverse components of potato extract used in preparation of PDA, which provides more essential elements for the growth of fungi. Shahid et al. (2011), and Singh et al. (2014) reported that the best solid media for growth and sporulation of *Trichoderma* species was PDA. Several scientific reports showed that PDA media is the best choice for the growth and sporulation of *Trichoderma* fungi. Mustafa et al. (2009), proved that among the tested media, including PDA, wheat bran media was the best media for the growth of different isolates of *Trichoderma* species. Sporulation rate was another important parameter that was considered in our study. *Trichoderma* species are known for their ability to produce spores in large quantities and at fast rates. This is a crucial characteristic for these fungi, since they are often sold as wettable powders that contain a certain amount of dried fungal

conidia. The spore production can vary depending on different factors, such as temperature and culture media (Woo et al. 2014, Carro-Huerga et al. 2021). It was found that LT140 isolate produced the highest spores (2.5×10^7 spore/mL) at 25°C after 7 days, while LT33 isolate produced the least spore production. LT20 and LT7 isolates did not produce any spores at all, regardless of the temperature or time. Our results on spore production agree with those of other researchers (Carro-Huerga et al. 2021). Some of our isolates had remarkably high sporulation rates, which is a typical feature of this genus (Gams and Bissett 2002). High spore production can help to quick colonization of the substrates, and thus make them useful as BCA due to their high reproductive activity (Benítez et al. 2004). Numerous scientific studies and reports show that the growth rate and sporulation of different isolates of *Trichoderma* species are strongly influenced by temperature and temperature as a physical factor plays the most critical role in enhancing fungal growth. The temperature range in which *Trichoderma* species can grow is relatively wide. The lowest temperature was zero degrees Celsius for *T. polysporum* and the highest temperature was 40 ºC for *T. koningii* (Limón et al. 2004, Galarza et al. 2015). All *Trichoderma* isolates used in this study showed different adaptations to changing temperature conditions. Optimal temperature for the growth and sporulation of *Trichoderma* isolates is in the temperature range of 25-30°C (Kredics et al. 2003, Singh and Kumar 2009, Maurya et al. 2017). The results of present study also showed that temperature has a great effect on the growth rate of different species of *Trichoderma*. In the present research it was found that the optimum range for the growth of *Trichoderma* isolates was 20–30 ºC. In most isolates the growth rate was primarily increased with increasing temperature from 20 to 30 °C and it was decreased afterward, The same results for different isolates such as *Trichoderma harzianum*, *T. viride*, *T. atroviride*, *T. asperellum*, *T. pseudokoningii* were obtained by researchers including (Jackson et al. 1991, Santamarina et al. 2006, Ali et al. 2012, Domingues et al. 2016). According to the report of Hewedy et al. (2020), 25 ºC was the best incubation temperature for all *Trichoderma* isolates regardless of the medium used for their growth. Domingues et al. (2016) reported an increase in the mycelial growth of all *Trichoderma* isolates at temperatures ranging from 12 ºC to 27 ºC, and then decreased up to 37ºC, being inhibited at 42ºC. The report of Singh et al. (2014), deduced that all *Trichoderma* species grew at four temperature (20, 25, 30 and 35 °C) but grew best at a temperature range of 25 ºC to 30 ºC. According to Ali et al. (2012), the optimum range for the growth of *T. harzianum* and *T. viride* was 20–30 ºC. Also in agreement with our findings Shahid et al. (2011) found that the optimal temperature for growth and sporulation of *T. longibrachiatum* is 30°C. In this research the total growth rate of isolates of *Trichoderma* species, increased as the temperature increased from 20 °C to 30 °C and beyond this most isolates grew very slowly. It was also found that among the tested isolates, the LT35 and LT20 isolates had the highest and lowest growth rate in all of the tested temperatures and cultures media, respectively. These two isolates both belong to the *Longibrachiatum* section.

In this study it was found that all of the 10 tested *Trichoderma* isolates reduced the growth rate of *R. solani* by 45-62.5% in the dual culture tests. From this part of the experiment it can be concluded that all of these isolates of eight *Trichoderma* species are more competitive than *R. solani* through further growth and competition for space and nutrients and inhibited the mycelia growth of *R. solani* which is exerted by antibiosis mechanism through toxin, antibiotics and cell wall lytic enzymes released by *Trichoderma* isolates against *R. solani*. These results indicate that there is a significant difference in terms of antagonistic activity against *R. solani* among the isolates of

Trichoderma species, from different ecological regions of Lorestan province, Iran. Previous studies claimed that different *Trichoderma* isolates are effective bio control agents against *R. solani* (Kamala and Devi 2012, Chao and Zhuang 2019, Silva deOliveira et al. 2021). It is noteworthy that significant differences were observed in terms of antagonistic behavior of different isolates of *Trichoderma* species against *R. solani* which may be due to different activities and difference in production of compounds such as volatile and non-volatile compounds, antibiotics, enzymes such as cellulase, protease and chitinase and other compounds (Kannangara and Dharmarathna 2017). One study, also has reported antagonistic activity and percentage of inhibition of growth of different pathogens including *R. solani* by different isolates of *Trichoderma* species from different parts of the world (Contreras-Cornejo et al. 2016).

Conclusion نتیجه‌گیری

Based on our tests; between four types of culture media, and five temperatures, the PDA medium is the best, and the temperature of 20 to 30 degrees Celsius is optimal for the growth and reproduction of these 10 isolates of eight *Trichoderma* species. Evaluation of their ability to inhibit the growth of the soil-borne plant pathogenic fungus *Rhizoctonia solani* in vitro, showed that *T. harzianum* LT8 has the most inhibition ability. Therefore, this isolate can be used as a potential biocontrol agent for this plant pathogenic fungus in future research.

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