

***Trichoderma harzianum* IS005-12 promotes germination, seedling growth and seedborne fungi suppression in Italian ryegrass forage**

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Abstract: Potential of *Trichoderma harzianum* IS005-12 (TH-IS005-12) to promote seed germination and seedling growth of Italian ryegrass (IRG) forage was evaluated *in vitro*. Non-desiccated seeds and those pre-harvestly desiccated with total herbicide were treated 25 days (freshly harvested) and 178 days after harvest (mature) with TH-IS005-12 spore suspensions at 0 (T0), 1.8×10^7 (T1) and 1.2×10^9 (T2) spore/mL. TH-IS005-12 promoted the early and final germination and seedling growth in all non-desiccated and desiccated, freshly harvested as well as mature IRG seeds. It was more effective in pre-harvestly desiccated freshly harvested seeds where T2 treatment increased final germination rate for 24%, root number per seedling 1.6-fold and seedling vigour 1.9-fold compared to the untreated control. Moreover, TH-IS005-12 showed an inhibitory activity against seedborne fungi *Alternaria alternata* and *A. ventricosa* suppressing their growth *in vitro* by 82% and 77%, respectively.

Keywords: *Lolium multiflorum*; early dormancy; desiccation; biocontrol agent; biostimulant; fungi inhibitory activity

Lolium multiflorum Lam. (Italian ryegrass, IRG) is the fast growing grass, increasingly used as a forage and silage crop due to its favourable nutritive value and digestibility (Shao et al. 2002). Mixed with other forages, mostly legumes, IRG improves the quality of the pasture.

IRG is native of the Mediterranean area occupying about 23% of the 52 million ha under grassland in southern Europe (Humphreys et al. 2010). Since it provides satisfactory productivity through the mid-summer slump due to high yields, it is widely cultivated in temperate regions worldwide. In continental regions, forage grass seeds are harvested mainly in June and can be used to establish new crop by sow-

ing in autumn of the same year or in spring of the next year. The sowing in the harvest year resulted in satisfactory forage yields. Unfortunately, immediately after harvest IRG showed moderate levels of seed dormancy, indicating requirements for seed ripening to breakdown early dormancy (Stanisavljević et al. 2011). However, farmers usually want to achieve the desired number of plants per unit area under sowing in autumn with minor seed consumption to ensure efficient planting. Consequently, a study was carried out in attempt to improve early seed germination of IRG including scarification with sulfuric acid and addition of plant growth regulator substances (Veljević et al. 2018). Since germination and growth

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of mature IRG seeds can be also diminished by different environmental factors (Lin et al. 2018) as well as seed pathogens, enhancement of germination and growth of mature seeds is desirable too.

In the last decade, *Trichoderma* spp. have been recognised as suitable biocontrol agents for improving seed germination and plant growth (Contreras-Cornejo et al. 2016). Due to their biostimulating abilities and other potential benefits, these species have been widely used for agriculture application (Zin and Badaluddin 2020). Since a lower germination potential of the freshly harvested IRG seeds is a limiting factor for successful plantation in the autumn, the objective of the present study was to determine the possibility to use *T. harzianum* IS005-12 (TH-IS005-12), as low toxic and environmentally friendly tool for promoting germination, seedling emergence and growth of IRG plants. The effect of this biological agent on germination and growth of mature IRG seeds was also studied. Additionally, the effectiveness of *T. harzianum* IS005-12 to suppress IRG seedborne fungi in order to provide healthful seeds was investigated.

MATERIAL AND METHODS

Seed material. Seeds of IRG, the summer-active (continental) cv. K-13 were used. The seeds were harvested in summer (mid-June 2018) from non-desiccated field crop as well as from that desiccated with 3 L/ha of total herbicide Diquat (Reglone Forte Syngenta®). Harvested seed samples were cleaned manually and stored dry in paper bags at room temperature until use. Two experiments were carried out. The first one was performed at the end of June, 25 days after seed harvest (DAH) when the freshly harvested seeds showed diminished germination potential, and the second one in the middle of November including mature seeds (178 DAH).

Seed bio-treatment with *Trichoderma in vitro*. *T. harzianum* IS005-12 isolate was grown on potato dextrose agar (PDA) (Merck KGaA, Darmstadt, Germany) for 7 days at 25 ± 2 °C. The inocula were prepared by rubbing the colonies with a sterile loop and then colonies were covered with 5 mL sterile solution 0.85% NaCl and 0.1% Tween 80 (v/v). The isolates were shaken 10 s with a Vortex mixer, filtered and collected in a sterile tube. This procedure removed the plurality of the hyphae, producing an inoculum mostly constituted of spores. The spore suspension was adjusted with sterile saline to final

concentrations at 1.8×10^7 (T1) and 1.2×10^9 (T2) spores/mL, by microscopic enumeration with a cell-counting hemocytometer (Neubauer chamber; Paul Marienfeld, UK). The inocula were stored at 4 °C until use.

The seeds of IRG were surface disinfected in 70% ethanol for 1 min, rinsed five times with sterilised distilled water and dried under laminar air flow on sterile filter paper and then placed into filter paper moistened with 5 mL distilled water in Petri dishes (\varnothing 90 mm) (20 seeds per Petri dish). The seeds were exposed to a chilling treatment at 4 °C for 4 days. The prechilled seeds were treated by soaking in TH-IS005-12 spore suspensions for 30 min. Three inoculation treatments T0, T1 and T2 including 0, 1.8×10^7 and 1.2×10^9 spore/mL, respectively, were performed with five replicates per treatment. The seeds soaked in sterile distilled water were used as a control (T0). Treated seeds were placed in the filter paper moistened with 5 mL distilled water in Petri dishes (20 seeds per dish) that were sealed with parafilm to prevent evaporation and germinated in growth chamber at 26 ± 2 °C for 12 days under 16/8 h day/night photoperiod.

Germination and seedling growth test. In the first experiment the freshly harvested (25 DAH) seeds were treated by T0, T1 and T2 while in the second one mature seeds (178 DAH) were inoculated. The germination was recorded 2 days, 4 days and finally 12 days after treatment. Seed was considered germinated when the radicle has extended through the seed coat at least 1 mm. After 12 days, seedling, shoot and root length as well as the number of roots per seedling were counted. Germination percentage (GP) and seedling vigour index (SVI) were calculated according to the following equations:

$$GP (\%) = n/N \times 100$$

where: n – number of germinated seeds; N – total number of seeds, $SVI = GP \times$ seedling length where GP represents the germination rate.

***Trichoderma* TH-IS005-12 antifungal activity *in vitro*.** The dual culture technique (Rollán et al. 1999) was used to screen the antagonistic potential of TH-IS005-12 against two seedborne fungi. The *Alternaria* species and TH-IS005-12 were grown individually on PDA for 7 days at 26 ± 2 °C. Small blocks 5 × 5 mm of the mycelia of both *Alternaria* and TH-IS005-12 were cut from the surface of the fungal culture and placed into the same Petri dish with PDA medium on distance 2.0–2.5 cm. Petri

dishes without fungal confrontation for each fungus were used as control. All Petri plates were incubated at 26 ± 2 °C in darkness for 7 days. Antagonism activity was monitored by performing both daily measurements of fungal colony growth and direct observation of the plates after 3, 5 and 7 days of culture. Mycelia growth inhibition of the seedborne fungi was calculated by the formula:

$$I (\%) = (R1 - R2)/R1 \times 100$$

where: R1 – radial growth of mycelia of seedborne fungi on control plate without TH-IS005-12; R2 – radial growth of mycelia of seedborne fungi on plate with TH-IS005-12. All treatments were performed in triplicate and the experiment was performed thrice.

Statistical analysis. The experiments were set up in a completely randomised arrangement and a one-way analysis of variance (ANOVA) was used to evaluate the effects of TH-IS005-12 on germination and seedling growth, while the effects of seed maturation and TH-IS005-12 treatments were compared using two-way ANOVA. Percentage data were subjected to angular transformation and the root number data to square root transformation before statistical analysis, followed by inverse transformation for presentation. The means of TH-IS005-12

treatment and untreated control were compared by the Student's *t*-test at the confidence level $P \leq 0.05$ using the SAS software (SAS Institute, 2002; SAS/STAT, ver. 9.00. SAS Institute Inc., Cary, USA).

RESULTS AND DISCUSSION

The effect of TH-IS005-12 on germination and seedling growth of IRG. The effects of TH-IS005-12 spore solution at two concentrations, T1 and T2, on germination and growth of IRG freshly harvested seeds (25 DAH) and mature IRG seeds (178 DAH) were investigated. The experiments indicated that TH-IS005-12-treated seeds displayed a higher germination rate than untreated control seeds (Table 1, Figure 1). Thus, T2 treatment significantly accelerated germination rate, as early as 2 and 4 days after inoculation with TH-IS005-12 in non-desiccated freshly harvested seeds, and also increased their final germination for 10% (Table 1). Although T1 significantly enhanced early as well as final germination of desiccated, freshly harvested seeds, a more pronounced effect of T2 was observed on seed germination 4 days after treatment while their final germination was increased even by 24% (Table 1). Both T1 and T2 treatments significantly improved

Table 1. Effect of *Trichoderma harzianum* IS005-12 (TH-IS005-12) treatments on germination of the freshly harvested (25 DAH (days after harvest)) and mature (178 DAH), non-desiccated (–) and pre-harvestly desiccated (+) Italian ryegrass seeds

Pre-harvest seed treatment	TH-IS005-12 treatment	Freshly harvested seeds germination (%)		
		2 days	4 days	12 days
Desiccated –	T0	0.41 ± 0.03	0.81 ± 0.03	42.9 ± 0.3
	T1	2.7 ± 0.5*	5.7 ± 0.5*	45.0 ± 0.4
	T2	3.4 ± 0.6*	6.4 ± 0.6*	52.9 ± 0.4*
Desiccated +	T0	0.8 ± 0.04	24.9 ± 0.6	47.1 ± 0.1
	T1	5.9 ± 0.3*	35.6 ± 0.3*	59.1 ± 0.1*
	T2	2.4 ± 0.9	46.9 ± 0.2*	71.4 ± 0.2*
		Mature seeds germination (%)		
Desiccated –	T0	0.0 ± 0.0	51.1 ± 0.1	80.4 ± 0.3
	T1	0.8 ± 0.03*	61.2 ± 0.5*	87.6 ± 0.8*
	T2	12.0 ± 0.2*	58.5 ± 0.1*	90.7 ± 0.2*
Desiccated +	T0	22.6 ± 0.5	41.5 ± 0.04	75.5 ± 0.1
	T1	49.0 ± 0.2*	62.1 ± 0.1*	89.6 ± 0.8*
	T2	43.7 ± 0.1*	57.5 ± 0.05*	82.6 ± 0.1*

T0 – control treatment; T1 – 1.8×10^7 spore/mL; T2 – 1.2×10^9 spore/mL. Results are expressed as a means ± standard error of five replications ($n = 5$). Asterisk indicates significant differences of each treatment compared to control (*t*-test at $P < 0.05$)

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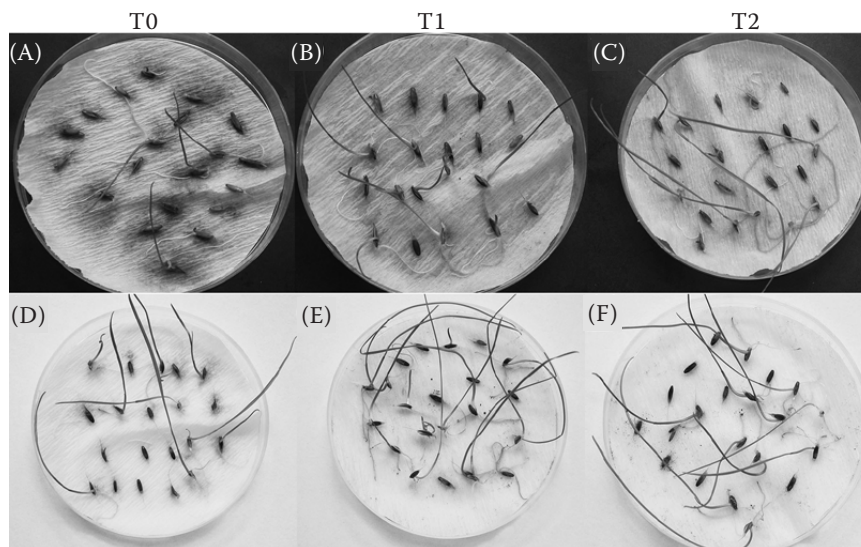


Figure 1. *Trichoderma harzianum* IS005-12 (TH-IS005-12) improved Italian ryegrass (IRG) seed germination, promoted seedlings growth and suppressed development of seedborne fungi. Seedlings developed from the freshly harvested non-desiccated (A–C) and pre-harvestly desiccated (D–F) IRG seeds. T0 – control without TH-IS005-12; T1 – 1.8×10^7 spore/mL and T2 – 1.2×10^9 spore/mL

early as well as final germination of mature IRG seeds (Table 1). Using TH-IS005-12, final germination of T2 treated non-desiccated mature IRG seeds and T1 treated desiccated mature seeds were 90.7% and 89.6%, respectively, compared to 80.4% and 75.5% of untreated controls, respectively (Table 1). According to ANOVA, in non-desiccated IRG seeds, early germination rate after 2 days was influenced by TH-IS005-12 as well as combination of TH-IS005-12 and seed maturation treatment, while TH-IS005-12 and maturation treatment equally influenced germination after 4 days. Maturation treatment had predominantly impact on final germination rate of these seeds. On the other hand, TH-IS005-12 and seed maturation treatment equally influenced early as well as final germination rates in desiccated IRG seeds. Seed dormancy is not a rare characteristics among grass species where the total germination of intact seeds can be significantly diminished. Since the germination potential is important for successful crop establishment, seed priming is becoming a usual practice to achieve satisfactory germination rate and seed uniformity (Moreno et al. 2018). The data of the present study provide useful information concerning enhancement of seed germination in both freshly harvested and mature IRG seeds, particularly in those subjected to desiccation treatment prior to harvest. For the agronomic practice point of view, such increase of germination can reduce the seeding rate, at least for the same percentage as germination was increased, enabling economic crop establishment. The seeding rate has also important impact on further performance of grass crops (Venuto et al. 2004). The obtained results are consistent

with previous reports indicating *Trichoderma* sp. as a promising agent for enhancing germination in various crop species including those from the Poaceae family – rice, maize and wheat (Okoth et al. 2011, Doni et al. 2014, De Oliveira et al. 2018).

Along with germination, TH-IS005-12 also promoted seedling's growth *in vitro* (Table 2, Figure 1). T2 treatment seems to be more effective than T1, facilitating most of seedling's traits at majority seeds batches (Table 2). T2 significantly improved shoot length, root length and seedlings' length in desiccated freshly harvested seeds as well as in both desiccated and non-desiccated mature seeds, while both T1 and T2 insignificantly increased shoot and root length and seedling length in non-desiccated freshly harvested seeds (Table 2). A prominent effect of T1 and T2 treatments was also shown in the number of roots per seedling (Table 2, Figure 2). Both treatments enhanced root numbers in all seed batches from 1.1–1.4 counted in untreated controls, to even 2.7 evidenced in seedlings developed from desiccated mature seeds treated by T2 (Table 2). The significant increase of seedling vigour index of desiccated freshly harvested seeds treated by T1 and T2 (1.4-fold and 1.9-fold, respectively) and 1.3-fold increase of non-desiccated ones treated by T2 compared to the control should be highlighted (Table 2). As both T1 and T2 significantly improved (1.3–1.4-fold, respectively) seedling vigour in non-desiccated mature seeds, significant seedling vigour improvement in desiccated mature seeds was achieved using T2 only (Table 2). According two-way ANOVA, SVI and shoot length in non-desiccated IRG seedlings were influenced by maturation treatment only, root and seedlings length by both maturation

Table 2. Growth promotion effect of *Trichoderma harzianum* IS005-12 (TH-IS005-12) on Italian ryegrass (IRG) seedlings developed from freshly harvested (25 DAH (days after harvest)) and mature IRG seeds (178 DAH)

Trait	TH-IS005-12 treatment	Freshly harvested seeds		Mature seeds	
		desiccation +	desiccation–	desiccation +	desiccation–
Shoot length (cm)	T0	4.1 ± 0.3	3.1 ± 0.3	4.9 ± 0.2	4.8 ± 0.2
	T1	5.0 ± 0.3*	3.3 ± 0.4	4.9 ± 0.3	5.4 ± 0.2
	T2	5.1 ± 0.3*	3.4 ± 0.4	5.8 ± 0.2*	5.7 ± 0.2*
Root length (cm)	T0	2.2 ± 0.2	3.3 ± 0.2	3.7 ± 0.2	4.6 ± 0.1
	T1	2.3 ± 0.2	3.9 ± 0.3	3.7 ± 0.3	5.5 ± 0.2*
	T2	2.8 ± 0.2*	4.00 ± 0.3	4.1 ± 0.2*	5.8 ± 0.2*
Seedling length (cm)	T0	6.2 ± 0.5	6.5 ± 0.5	8.5 ± 0.4	9.5 ± 0.3
	T1	7.2 ± 0.4	7.2 ± 0.7	8.6 ± 0.5	10.9 ± 0.4*
	T2	7.8 ± 0.5*	7.4 ± 0.7	9.8 ± 0.4*	11.5 ± 0.4*
Root number per seedling	T0	1.4 ± 0.005	1.1 ± 0.002	1.2 ± 0.002	1.1 ± 0.0001
	T1	2.5 ± 0.002*	1.9 ± 0.006*	2.5 ± 0.002*	1.5 ± 0.001*
	T2	2.3 ± 0.002*	1.3 ± 0.003*	2.7 ± 0.001*	1.6 ± 0.001*
Seedling vigour index	T0	293.3 ± 42.7	290.7 ± 38.8	642.7 ± 41.8	755.8 ± 45.6
	T1	422.4 ± 19.4*	325.5 ± 56.9	746.1 ± 83.4	923.8 ± 118.1*
	T2	555.8 ± 66.4*	390.9 ± 68.9*	809.9 ± 60.4*	1 037.7 ± 71.4*

T0 – control treatment; T1 – 1.8×10^7 spore/mL; T2 – 1.2×10^9 spore/mL. Results are expressed as a means ± standard error ($n = 44–93$). Asterisk indicates significant differences compared to control of each treatment (t -test at $P < 0.05$)

and TH-IS005-12 treatments. In desiccated IRG seeds all seedling characteristics were equally influenced by both treatments, except root number per seedling that was predominantly influenced by TH-IS005-12 treatment only in non-desiccated and desiccated seeds as well.

In general, the obtained results indicated multiple beneficial effects of TH-IS005-12 on IRG, including enhancement of germination of both freshly harvested and mature seeds as well as seedling growth and vigour improvement, particularly in seeds that were subjected to desiccation treatment prior to the harvest. In comparison to other *Trichoderma* species, *T. harzianum* displayed wider interaction potential associating with many various mono- and dicotyle-

donous species (Contreras-Cornejo et al. 2016). The most pronounced effect of TH-IS005-12 on IRG on enhancing the average root number per seedling can encourage their soil seating and facilitate water and mineral uptake potential. A study of Hermosa et al. (2012) indicated that seeds respond to *T. harzianum* before the radicle protrudes, while Harman (2000) reported that the treatment of soybean seeds by conidia of *T. harzianum* strain T22 enhanced cell elongation, followed by radicle protrusion inducing larger and more robust roots. The promoting effects of *T. harzianum* could be attributed to its ability to modulate metabolism of endogenous plant growth regulators (Martínez-Medina et al. 2011). Considering the fact that the *L. multiflorum* is mainly grown in mixture

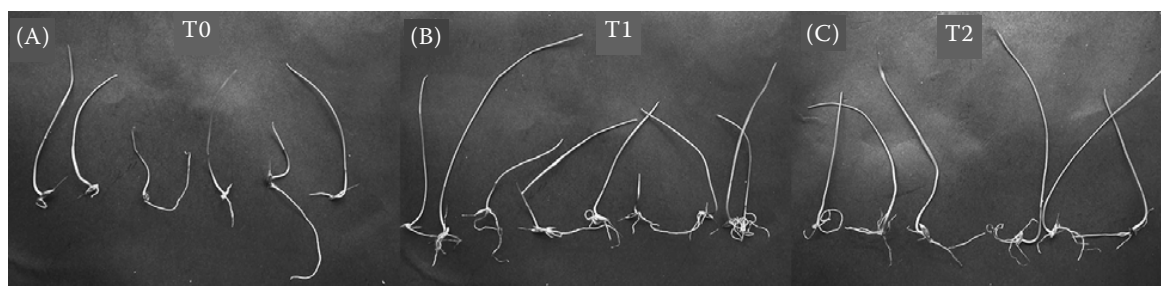


Figure 2. *Trichoderma harzianum* IS005-12 (TH-IS005-12) increased roots number in Italian ryegrass seedlings. (A) T0 – control treatment; (B) T1 – 1.8×10^7 spore/mL, and (C) T2 – 1.2×10^9 spore/mL

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with red clover, lower germination and seedling vigour can significantly impair competition with already developed plants and affect desirable density ratio of grass/leguminous mixture of the crop. Therefore, improved germination and growth rate of seedlings is important for establishing competitiveness in the initial growth, which in turn affects the length of use of the crop as well as the yield and quality of the forage.

Antagonistic activity of TH-IS005-12 versus seedborne *Alternaria* spp. The antagonistic activity of TH-IS005-12 isolate against two IRG seedborne fungi identified by molecular analysis as *A. alternata* (Figure 3A) and *A. ventricosa* (Figure 3B) (data not shown) was assessed using the dual culture system (Figure 3C,D). TH-IS005-12 displayed high antagonistic potential against both *Alternaria* species (Figure 3C–E). After 3 days of culture TH-IS005-12 suppressed *A. alternata* mycelia growth for 40%, while during 5 and 7 days of culture *A. alternata* growth was almost totally suppressed (77% and 82%, respectively, Figure 3E). A slightly weaker antagonistic potential of TH-IS005-12 against *A. ventricosa* was observed compared to *A. alternata*. Although after 3 days of

culture 45% of *A. ventricosa* mycelia growth was suspended, final antagonistic potential of 77% was counted after 7 days of culture (Figure 3E).

Alternaria species have a worldwide distribution as non-host-specific pathogens, infecting a range of agricultural plants, including those belonging to the Poaceae family (Kahl et al. 2015, Ramires et al. 2018). *T. harzianum* is indicated as a biocontrol agent in numbers of the plant species. Begum et al. (2010) reported the application of *T. harzianum* IMI 392432 to suppress *Alternaria* fruit rot disease in chili, *T. harzianum* UBSTH-501 induced systemic resistance on the spot blotch pathogen *Bipolaris sorokiniana* in wheat (Singh et al. 2019) while *T. harzianum* WKY1 controlled anthracnose disease in sorghum (Saber et al. 2017) and provided resistance against sheath blight in rice (Kumar et al. 2019). Since the biological control of fungal pathogens is an eco-friendly approach for crop protection, TH-IS005-12 could be applied as a fungicidal protective agent for healthy and organic IRG seeds production.

As IRG forage was found to be highly suitable as feed for organic dairy cows (Baldinger et al. 2011),

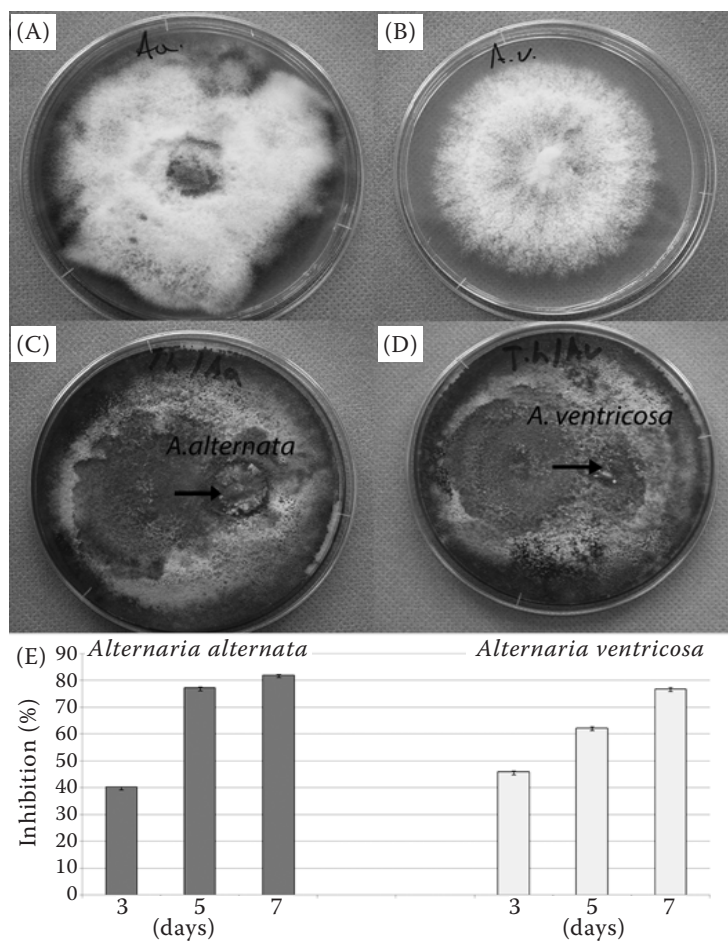


Figure 3. *In vitro* antifungal activity of *Trichoderma harzianum* IS005-12 (TH-IS005-12) against IRG seedborne fungi *Alternaria alternata* and *A. ventricosa* after 7 days. (A) *A. alternata* and (B) *A. ventricosa* (B). *T. harzianum* TH-IS005-12 mycelium overgrown (C) *A. alternata* and (D) *A. ventricosa*. Assessment of the TH-IS005-12 antagonistic activity after 3, 5 and 7 days (E). Results are expressed as a means \pm standard error of three replications ($n = 3$)

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TH-IS005-12 can be used as a non-toxic and environmentally friendly tool for promotion of IRG crop establishment by improving germination, seedling development and control of seedborne fungi. Further studies should be focused on the *ex vitro* effect of TH-IS005-12 to facilitate IRG crop establishment in competitive conditions.

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