

ISOLATION AND IDENTIFICATION OF TRICHODERMA SPECIES AND INVESTIGATING THEIR SEED TREATMENT EFFECT ON RAPESEED (*BRASSICA NAPUS* L.) GERMINATION

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ABSTRACT. *Trichoderma* fungus species are highly populations of fungi in world that they can colonize roots as plant symbiosis. Various types of *Trichoderma* are free-living fungi that are, generally, saprophytic on the remains of soil. In addition to its role in biological control, this fungus increases plant yield and growth. So far, many studies have been conducted to examine the ability of this agent to reduce biological tensions and biological control of plant pathogens. Thus, this study was conducted to isolate and identify species of *Trichoderma* fungus from rapeseed fields in Golestan and Qazvin province from Iran, and also to study isolated species on germination percentage and growth parameters of rapeseed seedlings in a randomized complete block design with three replications *in vitro*. Based on the results, three species of *T. harzianum*, *T. virens*, and *T. atroviride* were identified, which the isolate of *T. harzianum* T_{a19} showed a significant effect on the control group and other treatments in increasing germination

percentage, root length and stem; whereas, the seeds treated with the isolate *T. atroviride* T_{a11} showed no significant difference with the control group in spite of the increase in seed germination rate in comparison with the control and other treatments. According to the results, the use of *Trichoderma* fungus as a seed treatment like other researches on different products is recommended for increasing the growth of rapeseed.

Keywords: Fungus species; seed; root; stem; germination; colonization.

INTRODUCTION

Rapeseed is one of the world's oily seeds, which currently has the highest oil production in Europe among oily seeds. Increasing the production of oily seeds requires the introduction of new agro-systems and solving the problems of protecting the production of these products against

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various types of pests, insects, weeds, and plant pathogens (Dawidziuk *et al.*, 2014). Seed germination stage is important for determining the final plant density per unit. Sufficient plant density per unit is obtained when the cultivated seeds germinate fully and rapidly. Also, this stage is based on quantitative and qualitative performance. The seedling germination is an important step that plays an important role in the proper establishment of seedlings in the production process (Joodi and Sharifzadeh, 2009).

Various types of *Trichoderma* are free-living fungi that are, generally, saprophytic on the remains of soil. *Trichoderma* isolates show their bio-control activities through various mechanisms, such as competition for food and space, stimulating plant resistance mechanisms, and stimulating plant growth and development (Benitez *et al.*, 2004; Bjorkman *et al.*, 1998).

Creating induced resistance by *Trichoderma* species is identified by jasmonate and salicylates through production of pathogen-dependent proteins. These inducers include antifungal chitinases, gluconases, oxidative enzymes such as peroxidases and lipoxygenases.

Of course, low molecular weight compounds with microbial properties (phytooxins) are present in the process of induced resistance. Jasmonate and salicylic inducers provide systemic resistance to a variety of plant diseases. Root colonization with *Trichoderma* isolates significantly increased root

development, non-living stress resistance, absorption, and use of nutrients (Arora *et al.*, 1991).

Trichoderma isolates produce growth factors, such as auxin, cytokinin, ethylene, and cytokinin-like molecules such as zeatin and gibberellin (GA3), or GA3-dependent, which increase root growth and plant growth (Osiewacz, 2002).

In a research, after soaking the rice seeds in suspensions of *T. virens* and *T. harzianum*, it was found that the severity of the disease in the treated seeds decreased and the growth of these plants was effective against the control treatment (Anwar *et al.*, 2002). In a study, *Trichoderma* inoculum (*Zea mays*) affected the structure of the root system, which is dependent on increasing plant yield. The reported effects include increased production of root dry weight and increased hair root extensions (Bjorkman *et al.*, 1998; Harman *et al.*, 2004). Elad *et al.*, by adding *T. harzianum* to the potting soil, found a significant increase in bean plant growth, compared to non-*Trichoderma* treatment (Elad *et al.*, 1980). In a study conducted by Chet and Baker in greenhouse conditions, the production of the seeds stained with *Trichoderma* spores before cultivation increased (Chet and Baker, 1980).

Some *Trichoderma* isolates have the ability to colonize the plant's roots for a long time and penetrate into the epidermis. The best isolates are able to grow along with the roots of the plant and provide long-lasting beneficial effects. For example, corn

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seed treatment with *T. harzianum* T₂₂ resulted in higher growth and yield of the final plant, more vegetable leaves, increased rootstock in adult plants, and more effective nitrogen fertilizer application. *Trichoderma* seed treatment affects the plant's function in the long term, since T₂₂ is highly compatible with the extra-root space and can last longer as a symbiosis of the root (Harman *et al.*, 2008). In another study, the effect of four isolates of *Trichoderma* on seed germination showed that all used isolates had significant effect on germination, stem length, root length, and strength index of seed vigor (Tancic *et al.*, 2013). There are various reports of increased plant growth as a result of the association of *Trichoderma* (Harman *et al.*, 2004). In this research, the effects of several *Trichoderma* isolates on germination percentage and growth parameters of rapeseed seeds (root length, stem length) have been investigated.

MATERIALS AND METHODS

Sampling of soil

Average 100 g of soil from a depth of 10 to 30 cm from rapeseed fields in Golestan and Qazvin province from Iran were randomly sampled during a growing season of five points. The samples of each field were completely mixed and transferred to the laboratory in plastic bags (Ávila-Miranda *et al.*, 2006; Samuels *et al.*, 2011; Jakubíková *et al.*, 2006).

Isolation of *Trichoderma* isolates

Suspensions with dilution 0.1, 0.01, and 0.001 were prepared to isolate *Trichoderma* isolates from soil samples based on the dilution method

(Wijesundera *et al.*, 1991); 1000 µl of each concentration containing 1g of KH₂PO₄, 0.5 g of MgSO₄·7H₂O, 5 g of peptone, 10 g of glucose, 17 mg of Rose Bengal, and 20 g of agar was poured into the a culture medium, which after preparation of the above solution, its volume reached one liter using distilled water. After dissolving the materials, continuously stirring on the heater, the Arlon Meyer containers were sterilized at 120°C and at 105 Pa for 15-20 min. Then, in a sterile room conditions and in the vicinity of a gas flame, 0.2 ml of formaldehyde and 30 mg of streptomycin sulfate were added. At that time, about 15 ml of the culture medium was poured into 9 cm diameter Petri dishes and spread through a L-shaped glass rod. Petri pancakes were kept at 25 ± 1°C for 5 to 7 days (Samuels *et al.*, 2011; Davet and Rouxel, 2000).

Identification of *Trichoderma* isolates

After isolation and purification of the isolates, to identify them, firstly, the characteristics of the colony's shape and color (upper and lower surface), the growth rate on the malt-agar medium, morphological characteristics including the method of conidia production, then characteristics of conidiophores, such as phialides, cysts, and chlamydo spores were detected using the identification keys (Gams and Bissett, 1998; Bissett, 1991; Samuels *et al.*, 2002).

Investigating the effect of fungal growth factors on seed germination of rapeseed. In order to study the seed germination of rapeseed in laboratory conditions, 10 treatments with a control were carried out in a randomized complete block design with three replications. Treatments were three isolates of *T. virens* (T₁₆, T₅, T₂₄), *T. atroviride* (T₂, T_{a11}, T_{a6}) and *T. harzianum* (T_{a14}, T₁₉, T₉) and the control treatment (treated seeds with

arabic gum), which were randomly selected from identified isolates. For surface sterilization, rapeseed seeds were taken from a 1% sodium hypochlorite solution for 15 min and then washed several times using sterile distilled water (Javadi *et al.*, 2014). In the next step, rapeseed seeds were immersed in a 0.5% solution of carboxymethyl cellulose (arabic gum) for 15 min and then dried for 10 min under a hood. Spores suspensions at a concentration of 107 spores/ml were poured into a specific amount in Petri dish, and arabic gum-impregnated seeds were rolled in a suspension of spore and re-dried under a laminar hood.

A total of 15 inoculated seeds were grown in small and transparent pots containing sterilized soil and placed in a normal temperature at $25 \pm 1^\circ\text{C}$ for 7-10 days (Ahmad and Baker, 1987). After 7 and 10 days, seed germination percentage of rapeseed seeds and mean square of growth parameters (stem and root length

in mm) were calculated as the control treatment, respectively.

Statistical analysis of data

Laboratory tests were conducted in a randomized complete block design with three replications. Grouping of treatments was performed using Duncan test at 1% level and SAS 9.0 software.

RESULTS

Based on the macroscopic characteristics of the fungal colonies and morphological characteristics, including branching, shape, size, and specificity of the phialides, cysts and rhizomes were identified using the identification key of 25 isolates $T_{(1-25)}$ from Golestan province and 15 isolates of $T_{a(1-15)}$ of Qazvin province belonged to the three species of *Trichoderma* species. The important morphological characteristics of species are given in Table 1.

Table 1 - Morphological characteristics of *Trichoderma* isolates taken from soil of the rapeseed fields in Golestan and Qazvin province

Code	Isolate name	Colony		Conidio phore	Phialide	Conidia	
		Conidia production	Color	Branching	Shape	Shape	Size (micrometer)
$T_{(6-15)}$, $T_{(17-24)}$, $T_{a(2-5)}$, $T_{a(7-10)}$, $T_{a(12-15)}$	<i>T. harzianum</i>	Centered spores	Dark green	Pyramidal	Cylindrical to ampoule-shaped	Round to elliptical	2.5-4.5*4-6
T_5 , T_{16} , T_{25} , T_4 , T_2 4	<i>T. virens</i>	Scattered spores	Bluish dark green	A 3-6-clusted bunch	Jug-shaped	elliptical	3.12-6.24*2.34-3.9
$T_{(1-4)}$, T_{a1} , T_{a6} , T_{a11}	<i>T. atroviride</i>	Scattered spores	Yellowish and viridescent green	Low-clustered bunches with more than 3 clusters	Hook-shaped	Spherical	3.1-2.3*3.2-3.9

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Effect of fungal growth factors on seed germination of rapeseed

Based on the results of the comparison of the average germination percentage of the treatments, the average germination percentage of *T. harzianum* isolate (T₁₉) 82.22% was significant to the control treatment and other treatments.

The lowest germination percentage was observed for *T. atroviride* isolate (Ta₁₁) with 33.33%, which showed no significant difference at 1% level to the control. This isolate increased the seed germination rate on the 3rd and 4th day in comparison with other isolates and the control (Table 2).

Table 2 - Comparison of *Trichoderma* isolates effect on average of germination percentage and rapeseed seedling growth

Treatment	Average of root length (mm)	Average of stem length (mm)	Average of germination percentage
<i>T. virens</i> (T16)	9.7 ^{cde}	6.5 ^{bc}	44.44 ^{cde}
<i>T. virens</i> (T5)	19 ^{ab}	7.66 ^{bc}	75.56 ^{ab}
<i>T. virens</i> (T24)	15.33 ^{abc}	6.5 ^{bc}	57.78 ^{bcd}
<i>T. atroviride</i> (T2)	6 ^e	5.2 ^c	26.67 ^a
<i>T. atroviride</i> (Ta11)	8 ^{de}	6.4 ^{bc}	33.33 ^e
<i>T. atroviride</i> (Ta6)	6.33 ^e	6.2 ^{bc}	40 ^{de}
<i>T. harzianum</i> (Ta14)	14.33 ^{bc}	9 ^b	71.11 ^{ab}
<i>T. harzianum</i> (T19)	21 ^a	15 ^a	82.22 ^a
<i>T. harzianum</i> (T9)	12.7 ^{dc}	7.2 ^{bc}	66.67 ^{abc}
Control	11.2 ^{cde}	5.5 ^c	60 ^{abccd}

DISCUSSION

Identification of *Trichoderma* species from the fields of Golestan and Qazvin provinces indicates the ability to grow and propagate these biological agents in the rapeseed soils of these two provinces. The isolates included *T. harzianum* (75%), *T. virens* (12.5%), *T. atroviride* (15%) in this study. The presence of *Trichoderma* species in Mazandaran province farms was investigated in a study that more than 90% of the isolates were *T. harzianum* and *T. virens* (Naeemi *et al.*, 1999).

In a study, *T. atroviride* isolates appeared to be similar to the coconut

smell in the culture medium, which was contaminated by an anti-fungal antibiotic 6-Penthyne- α -Pyrone, which helped detect this fungus (Samuels *et al.*, 2002). The results of inoculation of *Trichoderma* isolates with studies performed on stimulating plant growth by *Trichoderma* isolates showed that the strength index of pepper seeds was increased in the treatment *T. harzianum*, which was effective in germination percentage (Asaduzzaman *et al.*, 2010). In another study, the colonization of corn roots with *T. virens* showed the growth rate of corn and also the rate of photosynthesis in corn leaves. In this study, *T. virens* isolate (T₅) also

increased germination percentage and seedling growth, but it was not significantly different from the control (Vargas *et al.*, 2009).

Another study showed that *T. harzianum*, at low concentrations, had the greatest effect on germination percentage and rate, but *T. atroviride*, despite increasing germination rate, reduced seed germination percentage in chickpea seeds. In this study, *T. harzianum* isolates showed a significant and acceptable difference, compared to the control and other treatments, but *T. atroviride* isolates, despite decreasing germination percentage, increased the seed germination rate of rapeseed seeds on the 3rd and 4th days (Qorbani *et al.*, 2011). According to the results of this study and other researches done so far, it can be said that the behavior of *Trichoderma* isolates varies from plant to plant and cannot be extended to all isolates, but by performing different tests to check their effect on different herbal products. In some ways, it increased the yield of plants, which over time helped to reduce the excessive use of chemical fertilizers.

CONCLUSIONS

Experiments performed in soils from two different provinces indicated important interactions between the seed treatments with fungus species, and showing that some fungus progress plant growth.

The response of germination percentage, root length and stem to seed treatment was dramatically

affected by *T. harzianum*. Despite *T. harzianum* had positive effect on seed treatment, different experiments must be design and applicate for investigation interactions between different *Trichoderma* species with different rapeseed cultivars in greenhouse and field conditions. If the results were good after many investigations, these can be recommended for farmers for application in cultivation

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