Selcuk Journal of Agriculture and Food Sciences

http://sjafs.selcuk.edu.tr/sjafs/index

Research Article

Selcuk J Agr Food Sci

(2023) 37(1), 133-144

e-ISSN: 2458-8377

DOI: 10.15316/SJAFS.2023.014

Efficacy of Some *Trichoderma* Isolates as Biocontrol Agents Against *Rhizoctonia solani* Kühn in Bean *(Phaseolus vulgaris L.)*

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HIGHLIGHTS

- *Rhizoctonia solani* is a disease agent that causes significant crop losses in beans in our country. Due to the adoption of environmentally friendly approaches in the world in recent years, it has become necessary to find different solutions for this disease, which is difficult to control.
- This study, it was aimed to determine the in vitro and in vivo effectiveness of *Trichoderma,* which is an important part of biological control, against *R. solani* in beans.

Abstract

This study was carried out to determine the *in vitro* and *in vivo* activities of *Trichoderma* isolates isolated from soil and plants collected from different provinces in 2020-2021 against *Rhizoctonia solani*, which causes root rot in beans. Using the pathogen as a trap, 61 *Trichoderma* isolates were obtained from 65 soil samples from 20 provinces. In addition, 8 *Trichoderma* isolates previously obtained from different plants were included in the experiment. *Trichoderma* isolates showed very strong (4 *Trichoderma* isolates), strong (1 *Trichoderma* isolate), moderate (18 *Trichoderma* isolates) and low level (18 *Trichoderma* isolates) hyperparasitic effects against *R. solani* in *in vitro* experiments with dual culture method. In comparison, some isolates (28 *Trichoderma* isolates) were found to be ineffective. As a result of *in vivo* tests with 10 *Trichoderma* isolates selected according to the effect results *in vitro*, it was determined that *Trichoderma* isolates were 8-89% effective against *R. solani*. The most effective *Trichoderma* isolates against *R. solani* was *Trichoderma virens-130* with an 89% effect, followed by *Trichoderma-106* and *Trichoderma-162.1* with 82% and 75% effect, respectively. According to these results, it was observed that the isolates of *Trichoderma*, which were moderately and highly effective *in vitro*, significantly reduced the severity of the disease *in vivo*.

Keywords: Bean; *Rhizoctonia solani*; Biocontrol; *Trichoderma*

1. Introduction

Beans (*Phaseolus vulgaris* L.) are one of the most important legumes in human nutrition worldwide. Root rot diseases are the main problem limiting bean production in many parts of the world (Ceyhan 2004; Naseri and Hemmati 2017; Tamüksek and Ceyhan 2022; Tekin and Ceyhan 2022). *Rhizoctonia solani* Kühn (Telemorph: *Thanatephorus cucumeris*), one of the critical factors causing root rot diseases, has been reported in every region of the world where beans are produced, and it can cause significant losses ranging from 20-100%. *R. solani* causes thin, long, flattened, and reddish-brown lesions of different sizes on the hypocotyl and roots of bean plants and softening of the roots. In the later stages of the infection, it has been reported that it causes yellowing

Citation: Salman Ö, Boyraz N (2023). Efficacy of some *trichoderma* isolates as biocontrol agents against *Rhizoctonia solani* Kühn in bean *(Phaseolus vulgaris l.). Selcuk Journal of Agriculture and Food Sciences*, 37(1), 133-144. https://doi.org/10. 15316/SJAFS.2023.014

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Received date: 10/01/2023

Accepted date: 20/03/2023

of the leaves, stagnation in plant growth, and ultimately the death of the plant before it matures. In the presence of moisture, the pathogen can infect leaves (web blight), petioles, flowers, capsules, and grains. When it infects the capsules, the disease also transfers to the seeds, causing significant damage to both yield and quality. The fungus survives as mycelium or sclerotia in plant remains in the soil (Akarca 2013; Palacıoğlu et al. 2019).

Binucleic (Telemorph: Ceratobasidium) and multinucleic *R. solani* (Telemorph: *Thanatephorus cucumeris*) and *Rhizoctonia zeae* (Telemorph: *Waitea circinata* var. *zeae*) have been reported as pathogens in beans (Godoy-Lutz et al. 2003; Nerey et al. 2010). *R. solani* and binucleic *Rhizoctonia* spp. isolates are divided into anastomosis groups according to their hyphal anastomosis reactions. Until now, 13 AG of *R. solani*, named AG 1-AG 13, and 16 binucleic AG have been described (Carling et al. 2002; Sharon et al. 2008). Root and collar rot in beans is caused by *Rhizoctonia solani* AG 4, AG 2-2, and AG 5 subgroups. The most common group seen in beans in the world is AG-4 (Godoy-Lutz 2003; Gogoy-Lutz 2008; Çebi Kılıçoğlu 2009; Valentin Torres et al. 2016).

Many fungal microorganisms living in the soil are known as potential biological control agents, and the most well-known among them are *Trichoderma* species (Punja and Utkhede 2003; Ting and Choong 2009). Thanks to *Trichoderma*'s practical biocontrol abilities; many of the commercial products are marketed in Asia, Europe, and the USA. The mechanisms involved in the biocontrol activity of *Trichoderma* spp. against plant pathogens (such as mycoparasitism, competition for space and food, and antibiosis) are essential for planning effective and safe biocontrol strategies (Wolska et al. 2012). *Trichoderma* spp. also produces different antibiotic substances; gliotoxin, gliovirin, viridin, and trichoviridin. It is also known that *Trichoderma* spp. prevent the development of pathogenic fungi by changing the rhizosphere. In addition, *Trichoderma* spp. in the rhizosphere helps the plant to support nutrient/fertilizer uptake, seed germination, and photosynthetic rates (Yedidia et al. 2003).

There are many studies on using *Trichoderma* spp. against *Rhizoctonia solani*, causing stem and root canker in beans. Yobo et al. (2011) investigated the effects of 6 *Trichoderma* (*T. atroviride* 3A, *T. atroviride* 6, *T. harzianum* SY, *T. pseudokoningii*, unidentified *Trichoderma* sp. 2F and *T. harzianum* kmd) and 3 *Bacillus subtilis* (*B. subtilis* B69, *B. subtilis* B77 ve *B. subtilis* B8) isolates seperately and in combination against *Rhizoctonia solani* which causes damping off disease in bean and cucumber, *in vivo*. Fungal and bacterial isolates obtained in the study related to yield applied to the seed in greenhouse and rhizotron studies. In greenhouse experiments, it was determined that seedling dry-weight of beans yielded the highest results when *T. atroviride* strain 6 and *Bacillus subtilis* B69 were applied together. Rhizotron studies have also been found to give results in support of this. In biological control experiments, it was determined that the survival rate of the plants was high when *T. harzianum* kmd, *T. atroviride* 3A and *T. harzianum* SY have applied alone under greenhouse conditions. It has been observed that the efficacy of the combination of *B. subtilis* B69 and B81 is increased when used together with *T. atroviride* 3A, *T. atroviride* 6, or *T. harzianum* kmd. According to the results of this study, the efficacy of each of the *Trichoderma* and *Bacillus* combinations gave better results than the application of *Bacillus* isolates alone.

According to the results obtained, it was determined that the best application for suppressing the disease was the combination of *B. pumilus* INR7 + *T. harzianum*. This application reduced the disease by 54% (Nasir et al. 2018).

Bozdeveci et al. (2019) determined the effects of *T. harzianum* ID11C obtained from tea soil against *R. solani* B227 on biocontrol activity and bean growth. It has been observed that this biocontrol agent is tolerant to heavy metals, has a plant growth-promoting effect and suppresses pathogen growth.

Rhizoctonia solani is a plant pathogen causing significant crop losses in beans in our country. Due to the adoption of environmentally friendly approaches in the world in recent years, it has become necessary to find different solutions for this disease, which is difficult to control. For this reason, this study aimed to determine

the *in vitro* and *in vivo* effectiveness of *Trichoderma* spp., an important part of biological control, against *R. solani* in beans.

2. Materials and Methods

2.1. Materials

2.1.1. Fungal isolates used in tests

In the experiment, an isolate belonging to the AG 4HGI anastomosis group of *Rhizoctonia solani*, isolated from a bean and determined to be pathogenic and anastomosis group, was used.

A total of 69 *Trichoderma* was isolated from different soil samples (61 isolates) and plant materials (8 isolates) as bioagents.

2.1.2. Plant material

To determine the *in vivo* efficacy of *Trichoderma* isolates against *R. solani* AG 4HGI, Üstün 42 variety was used.

2.1.3. Plant growing media and chemicals

Potato Dextrose Agar medium (PDA, Merck) containing streptomycin sulfate was used to obtain fresh cultures of *R. solani* and *Trichoderma*. PDA medium containing Rose Bengal was used to obtain *Trichoderma* from soil samples by trapping method (Table 1).

Table 1. Chemical content of PDA containing Tolchlofos methyl-rose bengal

Name of the ingredients	Concentration
Rose Bengal	32 mg
PDA	39 g
Streptomycin sulfate solution	100 ml
Tolclofos Methyl	6 mg
Distilled Water	1000 ml

In addition, PDA medium amended with antibiotics was used in the dual culture tests of *Trichoderma* spp.-*R. solani*. 2% sodium hypochlorite (NaOCl) was used for the surface disinfection of bean seeds, and water agar (WA) was used to germinate seeds. *Trichoderma* isolates were preserved in 30% glycerol. The culture of *R. solani* has stored in barley grains long-term preservation. A mixture of peat/soil/perlite prepared in a ratio of 2:1:1 (v/v/v) was used in *in vivo* experiments.

2.2. Methods

2.2.2. Fresh cultures of Rhizoctonia solani

Firstly, new cultures of *Rhizoctonia solani* isolates stored in barley culture were prepared. For this purpose, PDA medium containing 100 ml/1000 ml Streptomycin sulfate was prepared. One barley grain covered by the fungus was taken with the help of forceps, placed in the prepared PDA medium and incubated at 25 ^{0}C for 7 days to obtain fresh cultures.

2.2.2. Isolation and long-term storage of trichoderma

To obtain *Trichoderma* isolates used in the experiments, soil samples were obtained from regions with different ecological characteristics, especially from some districts of Konya (Karatay, Çumra, Altınekin, Meram, Sarayönü, Ilgın). Samples were taken from the rhizosphere of well-developed plants in healthy or

pathogen-infested fields. These soil samples were sieved using fine-meshed sieves. Then, a PDA medium containing Rose Bengal was used to trap *Trichoderma* from these soil samples. The medium prepared as given in Table 1 was autoclaved at 121 ^{0}C for 20 minutes and when the temperature of the medium fell to 45 ^{0}C, tolclofos methyl and streptomycin sulfate were added to it (Aydın 2008).

Rhizoctonia solani was used to obtain *Trichoderma* cultures. First, a 7-day fresh culture of *Rhizoctonia solani* was obtained, and then soil samples selected from different regions were covered with soil and incubated at 25 ^{0}C for 14 days. After this waiting period, the soil on the surface of the *Rhizoctonia solani* culture was thoroughly cleaned with sterile distilled water and 5 pieces of agar disc were transferred to a PDA medium containing improved rose bengal and incubated at 25 ^{0}C. Petri dishes were checked frequently and different *Trichoderma* isolates growing in each petri dish were transferred to a PDA medium to obtain a pure culture. According to their microscopic features (conidiophore, branching shape of the conidiophore, number and arrangement of phialides, shape and color of conidia, chlamydospore formation and location) genus level identification of *Trichoderma* were determined, and these samples were stored in 30% glycerol at -20 ^{0}C for long term preservation. 61 *Trichoderma* isolates were obtained using the trapping method from 65 soil samples selected from the 20 provinces where the soil samples were taken. A total of 69 *Trichoderma* isolates were obtained and 8 isolates spontaneously grown on plant materials.

2.2.3. Determination of in vitro efficacy of Trichoderma isolates

In dual culture tests, 7-day-old fresh cultures of *Trichoderma* and *R. solani* were used. Agar plates cut from fresh cultures were placed opposite each other on PDA medium with antibiotics. The evaluation was made after 7 days of incubation at 25 °C. Information on the scale values and definitions used in the evaluation are given in Table 2.

Table 2. Scale values were used to determine the efficacy of *Trichoderma* isolates.

Scale Value	Definition
1	*Trichoderma* completely develops on the pathogen and completely covers the medium.
2	*Trichoderma* covers two-thirds of the media surface.
3	*Trichoderma* and pathogens both cover almost half of the media surface and neither can dominate the other.
4	The pathogen covers two-thirds of the environment and is based on the pressure of *Trichoderma*.
5	The pathogen develops completely on *Trichoderma* and covers the surface.

Scale values of *Trichoderma* isolates evaluated according to the scale ≤2 indicate a high level of hyperparasitic effect against the pathogen (1: Very strong, 2: Strong), and a score of ≥3 indicates low or no hyperparasitic effect (3: Moderately effective, 4 : Less effective, 5: Ineffective) (Bell et al. 1982; Durak 2011). Accordingly, 10 *Trichoderma* isolates with the best effect were used in *in vivo* studies.

2.2.4. Preparation of the Inoculum

A barley culture medium was used to prepare the *R. solani* inoculum. For this, 1/3 of the test tube is filled with barley grain. It was sterilized at 121^{0}C for 60 minutes by adding 2 times its water weight to the barley grain. Autoclaving was carried out twice. Excess moisture was then removed from the tubes. An agar plate from fresh *Rhizoctonia solani* cultures was transferred to the prepared barley cultures and incubated at 25 °C for 3 weeks. One barley grain containing pathogen was used for each bean seed from these prepared barley grains (Carling and Summer 1992).

2.2.5. Determination of in vivo Efficacy of Selected Trichoderma Isolates against R. solani

1 lt pot were used in the experiment. These pots were filled with peat/soil/perlite mixture and sterilized at 121 ^{0}C for 60 minutes. To determine the efficacy of *Trichoderma* isolates, the experiment was set up as three replications for each isolate. 3 seeds (Üstün 42 bean varieties) were planted in a pot.

Pre-germination was done in bean seeds. For surface disinfection, the seeds were first kept in 2% sodium hypochlorite for 1 minute, then passed through sterile distilled water twice for one minute and left to dry on sterile papers at room temperature. Then, it was transferred to petri dishes containing water agar and kept at 25 ^{0}C for 3-4 days.

Trichoderma cultures were first incubated at 25 ^{0}C for 7 days to determine the effectiveness of *Trichoderma* işletesi selected for use in *in vivo* experiments against *R. solani*. A spore suspension of 15 ml was prepared from each *Trichoderma* to isolate to apply to the seeds from these cultures. The density of the spore suspension was adjusted to 10^8 spores/ml by counting on a haematocytometer (Thoma slide). Then the seeds were kept in the solution for 15 minutes. Then, the seeds were placed in pots and one of the barley grains covered with the mycelium of the pathogen was placed next to it with forceps. In the pots used as the control group, a sterile barley grain was placed next to the germinated seed instead of pathogenic barley and covered with soil. The remaining spore suspension was equally divided into pots and poured. Experiments were carried out with 3 replications.

After inoculation, the plants were expected to grow in a climate room with 12 hours of light and 12 hours of darkness (65% humidity and 25 °C) for four weeks before being evaluated for disease. (Buhur 2014; Başbağcı et al. 2019).

2.2.6. Evaluation of in vivo Efficacy of Trichoderma Isolates against R. solani

The activities of *Trichoderma* isolates against *R. solani* were compared by calculating the disease severity. For this purpose, the uprooted plants were thoroughly cleaned under tap water, and then all plants were examined for disease. A 0-4 scale was used to evaluate the severity of the disease (Table 3) (Muyolo et al. 1993).

Table 3. 0-4 scale used in the evaluation of *R. solani* infection in bean plants.

Scale Value	Definition
0	Healthy seedling
1	Very small brown superficial lesions on roots or stem
2	Deep and extensive lesions on the roots or stem, regression in root development
3	Severe root rot, deep lesions surrounding the main root or stem, significantly reduced root length
4	Dead plant

After the evaluation was made according to the scale, the disease severity index was calculated according to the Towsend-Hauberger formula (1943).

$$\% \text{ Disease Severity Index} = [\Sigma(SD \times BS)/(ESD \times TB)] \times 100 \quad (1)$$

According to the formula; SD: Scale value, BS: Number of plants on the same scale, ESD: Highest scale value TB: Total number of plants

The data obtained from the evaluations were compared with the Duncan Multiple Comparison tests in the SPSS 17.0 statistical program (SPSS Inc, Chicago, IL, USA) at a $p<0.05$ significance level. The most effective isolate statistically was sent to BM Labosis for molecular characterization.

3. Results and Discussion

3.1. Trichoderma isolates

To obtain the *Trichoderma* isolates we used as bioagents in the experiments, the province where the soil samples were taken, and the number of *Trichoderma* isolates obtained are given in Table 4. As a result, 61 *Trichoderma* isolates were obtained from 65 soil samples from 20 provinces. In addition, 8 *Trichoderma* isolates that developed spontaneously in isolations made from plants in previous studies were also used in the tests.

Table 4. Soil samples used to obtain *Trichoderma* and the number of *Trichoderma* isolates obtained

Province	Number of Samples	Soil/Plant Organs	Number of *Trichoderma* Isolates
Konya	29		25
Afyonkarahisar	1		2
Van	1		-
Muğla	2		3
Eskişehir	2		2
Adana	1		-
Amasya	2		2
İzmir	4		-
Aydın	3		4
Erzurum	2		2
Kayseri-Develi	2	Soil	2
Çanakkale-Batakovası	2		3
Nevşehir	2		2
Tokat	2		4
Şanlıurfa	2		-
Niğde	1		3
Antalya	1		-
Mersin	2		3
Samsun	2		4
Karaman	2		0
Muğla	3	Root (*Brassica oleracea*)	3
Niğde	3	Tuber (*Solanum nigrum*)	3
Konya	2	Root (*Phaseolus vulgaris*)	2
Total	**73**		**69**

3.2. In vitro efficacy of obtained Trichoderma isolates against R. solani

In dual culture tests, it was observed that a lytic zone was formed in some of the *Trichoderma* isolates and the mycelial growth of the pathogen was disrupted. Other *Trichoderma* isolates that we used in our study showed a hyperparasitic effect and quickly covered the pathogen. Dual culture studies were evaluated at 7 days of age. In some of the Petri dishes that were observed to be effective, *Trichoderma* did not fully develop conidial growth, although it covered *R. solani* mycelium. As a result, different developments were observed in *R. solani-Trichoderma* dual cultures (Table 5).

Table 5. *In vitro* Efficacy of *Trichoderma* Isolates against *R. solani*

No	Code of *Trichoderma* Isolates	Efficacy Level of *Trichoderma* Isolates against *R. solani*	No	Code of *Trichoderma* Isolates	Efficacy Level of *Trichoderma* Isolates against *R. solani*
1	Lahana fet1	VSH	22	108.2	LH
2	Lahana Fet.2	MH	23	113	MH
3	Lahana Fet3	MH	24	118.2	LH
4	Pa Niğde1	VSH	25	126.1	LH
5	Pa Niğde2	MH	26	130	VSH
6	T	MH	27	133	MH
7	TH4K2a	MH	28	138	LH
8	T-2.1	LH	29	153.1	MH
9	T-2.2	LH	30	153.3	LH
10	33	MH	31	160	MH
11	39.1	LH	32	162.1	MH
12	39.2	LH	33	162.2	MH
13	64	VSH	34	187	LH
14	74.1	LH	35	204.1	MH
15	90	MH	36	204.2	LH
16	99	MH	37	216	MH
17	99.2	SH	38	217.1	LH
18	103.2	LH	39	217.2	LH
19	104.1	MH	40	218.1	LH
20	104.2	LH	41	219.1	LH
21	106	MH			

VSH: very strong hyperparasitic, SH: strong hyperparasitic, MH: moderate hyperparasitic, LH: low-level hyperparasitic.

As a result of dual culture studies, 4 of the *Trichoderma* isolates against *R. solani* were determined as very strongly hyperparasitic, 1 strongly hyperparasitic, 18 moderately hyperparasitic, 18 mildly, 28 ineffective. Considering these results, 10 isolates thought to be effective in *R. solani-Trichoderma* dual cultures are as seen in Figure 1.

Similar to our *in vitro* test results, Mayo et al. (2015) determined the efficacy of *Trichoderma* isolates against root rot caused by *R. solani* in beans. The inhibition rate of T003, T004, T006, T020, T022, T012, T013, T025, T016, T007, T024, T005 and T010 is 75-86.70%, while 86.70% of T003, T004, T006, T020 and T022 isolates reported to have the highest inhibition percentage with.

El-Benawy et al. (2020) determined the efficacy of 6 *Trichoderma* isolates against *R. solani in vitro*. The efficacy of these isolates T19, T20 and T22 according to the 5-day dual culture results, respectively; 67.02%, 67.57% and 68.00%. According to the dual culture results we obtained, 4 *Trichoderma* isolates (La-Fet1, Pa-Niğ, 130 and 64) showed a very strong hyperparasitic effect by completely covering *R. solani* in the petri dish.

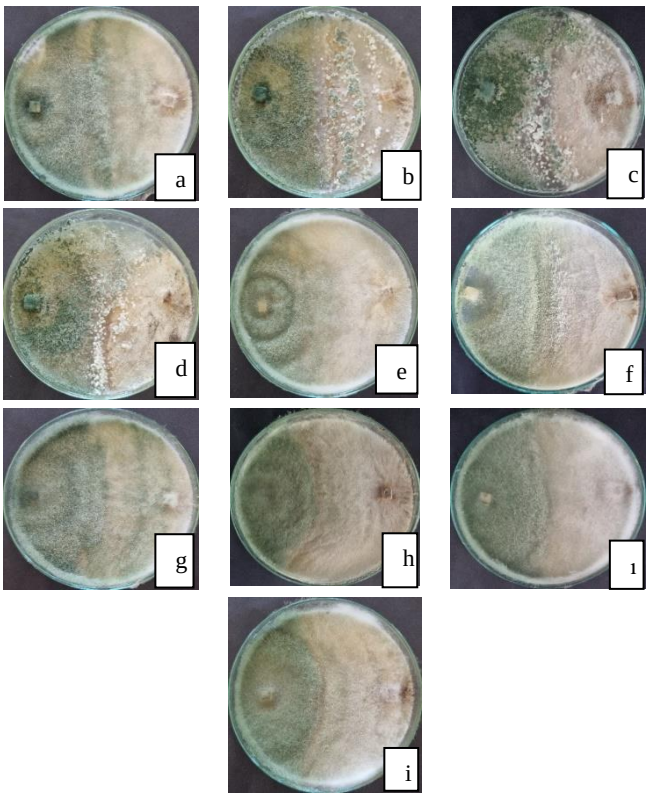

Figure 1. The hyperparasitic effects of different *Trichoderma* isolates against *R. solani* in dual culture tests: **(a)***Trichoderma* Pa-Niğ 1; **(b)** *Trichoderma* La-Fet1; **(c)** *Trichoderma* 64; **(d)** *Trichoderma* 99.2; **(e)** *Trichoderma* 104.1; **(f)** *Trichoderma* 106; **(g)** *Trichoderma* 130; **(h)** *Trichoderma* 133; **(ı)** *Trichoderma* 153.1; **(i)** *Trichoderma* 162.1.

3.3. In vivo efficacy of Trichoderma isolates against Rhizoctonia solani

Trichoderma isolates (10) used against *R. solani* showed an inhibitory effect of 8-89%. The most effective isolate was *Trichoderma virens* 130 with 89% efficiency. This isolate was followed by *Trichoderma* isolates 106 and 162.1, with efficacy rates of 82% and 75%, respectively (Table 6 and Figure 2).

Table 6. *In vivo* Efficacy of *Trichoderma* Isolates against *Rhizoctonia solani*

Code of *Trichoderma* Isolate	Disease Incidence (%)	Disease Severity (%)	Efficiency Rate (%)
153.1	83bc	47d	53f
Lahana Fet 1	100a	44de	56ef
99.2	100a	92b	8h
64	100a	86c	24g
106	78c	18h	82b
162.1	63d	25g	75c
133	100a	39ef	61de
130	44e	11ı	89a
Pa Niğde 1	88b	33f	67d
104.1	100a	33f	67d
+K	100a	100a	0ı

P<0.05 (There is no statistical difference between the means expressed with the same letter in the same column.

In the positive control of *R. solani*, pre-emergence damping-off symptoms occurred. From this point of view, although disease incidence is high, there is a significant decrease in disease severity, which is also seen in *in vivo* tests.

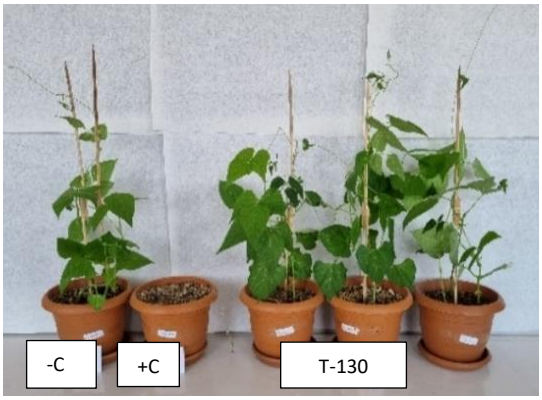

Figure 2. The effect of *Trichoderma virens* 130 against *R. solani in vivo*. (-C: negative control, +C: positive control, T-130: *Trichoderma virens* isolate)

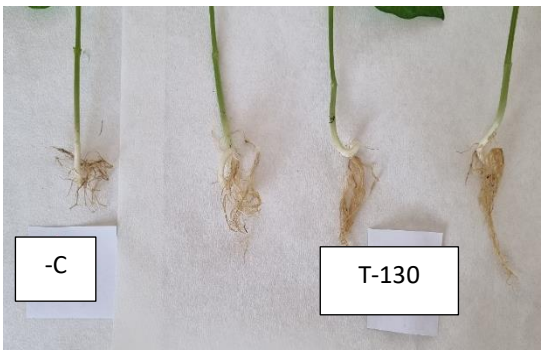

Figure 3. Appearances in the root crown of plants that are effective against *R. solani* against *Trichoderma virens* 130 *in vivo.*

In parallel with our research, it has been reported that *Trichoderma* is effective against *R. solani* in some other studies. Kamala and Devi (2012) determined the effectiveness of *Trichoderma* T10, T17 and T83 isolates, which were effective *in vitro*, in pot experiments, in their study on the effectiveness of 114 *Trichoderma* isolates against *R. solani*. Accordingly, T83 gave the most effective results. The disease was reduced by 11-76.6% in plants inoculated with *R. solani* and T83.

Nofal et al. (2021) described *Trichoderma* species isolated from samples taken from the rhizosphere of healthy beans in 23 locations in Egypt. It was determined that all *Trichoderma* spp. obtained had a positive effect on the development of *R. solani*. The T5 *Trichoderma* isolate was identified as *T. koningii* and inhibited the growth of the pathogen 100%. *T koningii* inhibited *R. solani* mycoparasitic ally. It was also observed that *T. koningii* produced a high amount of chitinase and protease enzymes that hydrolyze chitin.

According to our evaluation results regarding the *in vitro* efficacy of *Trichoderma* isolates against *R. solani*, it was determined that 41 isolates were effective at different levels and 28 were ineffective. Successful results were also obtained from *in vivo* trials of isolates with very strong or moderate efficacy in *in vitro* trials. The most effective isolate was *Trichoderma virens* 130. Although these results do not entirely suppress the disease,

they show that it significantly reduces the severity of the disease and that it can be used. In addition, it should be considered that the most effective isolate can give more effective results with different *Trichoderma* isolates or bacterial isolates.

Making these fungal bioagents, which are naturally present in our country's soil, usable as biofertilizers or biopreparations against diseases will significantly decrease the inoculum level of pathogenic microorganisms in the soil. Obtaining such products from naturally occurring microorganisms in our country's soil flora is important in eliminating fundamental problems that may be encountered and ecologically adapting these microorganisms to the environment in which they are inoculated.

Author Contributions: Özden SALMAN planned, established and conducted the study. Nuh BOYRAZ analyzed the data obtained in the study and Özden SALMAN wrote the article. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: This study was derived from Özden SALMAN's doctoral thesis. We would like to thank Selçuk University ÖYP Coordinatorship (Coordinatorship of Faculty Member Training Program) for their support (Project no. 2019-ÖYP-011).

Conflicts of Interest: The authors declare no conflict of interest.

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