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Summary of PhD Thesis

Biological control of the pathogenic fungus *Fusarium oxysporum* using *Trichoderma sp.* strains - molecular approach -

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Content

ACKNOWLEDGMENT

Now, at the end of the doctoral program, I am marked by feelings of happiness and fulfillment that come from the professional and personal satisfaction of the completed studies. The development, completion and defense of this thesis would have been impossible without the involvement and assistance of many special people to whom I want to say a few words of thanks.

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Chapter 1

The importance and history of research in the field

Pesticides, including fungicides, have been the subject of substantial criticism in recent years due to adverse effects on the environment with serious consequences for human health and for other non-target organisms. Therefore, the development of alternative, safer and more environmentally friendly control methods has become a top priority. In this context, biological pest control is becoming an important branch of agriculture.

The genus *Trichoderma spp.* was introduced into the mycological literature by Christian Hendrik Peerson (1794). Certain members of the genus *Trichoderma spp.* have various beneficial properties and are used as sources for a range of hydrolytic enzymes of industrial importance or as biocontrolling agents against plant phytopathogenic fungi. (Harman and Björkman, 1998; Howell, 2003; Harman et al., 2004; Howell, 2007; Kumar et al., 2008; Viterbo and Horwitz, 2010; Hermosa et al., 2012; Seiboth et al., 2012). Simultaneously, other species can even cause lethal infections in humans, especially in immunocompromised patients (Summerbell, 2003; Kredics et al., 2011; Hatvani et al., 2013) or are the causative agents of green mold in cultivated fungi (Samuels and et al., 2002; Park et al., 2006; Hatvani et al., 2007; Komon-Zelazowska et al., 2007).

Due to the multiple uses of *Trichoderma spp.* species, the correct identification of isolates is essential for all the groups mentioned above. In the past, *Trichoderma spp.* have been identified exclusively on the basis of morphological characteristics (Summerbell, 2003; Gams și Bissett, 1998).

However, identification based on morphological characters is difficult and requires expertise, and can easily lead to erroneous results. Therefore, the application of biochemical and molecular techniques is recommended to confirm the species-wide identification of *Trichoderma spp.*

Certain molecular techniques, such as DNA fingerprinting (Arisan-Atac et al., 1995) or ribosomal DNA sequence analysis of the ITS region (Internal Transcribed Spacer) [ITS 1 - between the genes for 18S and 5.8S rRNAs and ITS 2 - between the genes for 5.8S and 28S rRNAs (25S in plants)], as well as the gene fragments encoding elongation factor 1-α, endochitinase (*chi18-5*, formerly known as *ech42*), subunit II of RNA polymerase (*rpb2*), calmodulin 1 (*cal1*) (Kullnig-Gradinger et al., 2002; Druzhinina et al., 2008) are suitable to give an accurate diagnosis, thus eliminating morphology issues in the identification species.

To identify *Trichoderma* / *Hypocrea* species using the DNA barcoding technique, Druzhinina et al. (2005) introduced the first online database, **TrichOKey**, using oligonucleotide barcodes, available on the website of the International Taxonomy Subcommittee on Trichoderma and Hypocrea (http://www.trichoderma.info). Numerous strains of *Trichoderma* belonging to different species have been discovered as potential biocontrolling agents for agricultural pests.

The structural base of the fungal cell wall is composed of chitin, a homopolymer formed by N-acetyl-glucosamine units with β1 → 4 and β1 → 3 glycosidic bonds. Although chitin is located inside the cell wall, ie adjacent to the plasma membrane, its degradation seems to be a vital aspect in the interactions of fungi with plants.

Chitinases are secreted by plants in the defense response against pathogenic fungi (Huub et al., 1991; van Loon et al., 1998; Datta and Muthukrishnan, 1999; Leubner-Metzger and Meins Jr., 1999; van Loon and van Strien , 1999). Chito-oligosaccharides, released in this way from the cell wall of fungi, can in turn activate the defense response against fungi in plants.

The involvement of chitinases in mycoparasitic attack is an important mechanism that has been the subject of much research. A number of chitinase genes have been found to be strongly induced in mycoparasitism, e.g., *ech42*, *chit33*, and *chit36* (Carsolio et al., 1994; Mach et al., 1999; Zeilinger et al., 1999; de las Mercedes Dana and et al., 2001; Viterbo et al., 2002). N-acetyl-glucosaminidase Nag1 has also been shown to be strongly induced in mycoparasitism (Mach et al., 1999; Zeilinger et al., 1999).

Growth stimulation by the strains of *Trichoderma spp.* was observed in a large number of different groups of plants, including vegetables, field crops, ornamentals and forestry crops. Much of the research so far has focused on greenhouse vegetable crops, for example: cucumbers (*Cucumis sativus*), beans (*Phaseolus vulgaris*), eggplants (*Solanum melongena*), lettuce (*Lactuca sativa*), peas (*Pisum sativum*), radishes (*Raphanus sativus*), peppers (*Capsicum annuum*) and tomatoes (*Solanum lycopersicum*) (Chang et al., 1986; Paulitz et al., 1986; Baker, 1988; Lynch et al., 1991; Kleifeld and Chet, 1992; Ousley et al., 1993; 1994a; 1994b; El-Mohamedy and El-Baky, 2008). Seedlings treated with *Trichoderma spp.* were better developed, more vigorous and had a higher chlorophyll content.

Plant growth stimulation has been associated with strains of the following *Trichoderma spp.* species: *T. longipile* and *T. tomentosum* (Rabeendran et al., 2000), *T. harzianum* (Chang et al., 1986; Inbar and et al., 1994; Perveen and Bokhari, 2012), *T. viride*

(Ousley et al., 1993; 1994b; Perveen and Bokhari, 2012), *T. koningii* (Windham et al., 1986; Samuels et al., 2006) , *T. asperellum* (Li et al., 2018), *T. atroviride* (Colla et al., 2015) and *T. stromaticum* (De Souza et al., 2008).

However, in a single species of *Trichoderma spp.*, not all isolates are able to stimulate plant growth. For example, different strains of *Trichoderma harzianum* or *T. vir*ide have given different growth-stimulating effects on several host plants (Ousley et al., 1993, 1994b).

1.2. The aim and objectives of the PhD thesis

- Molecular identification of *Trichoderma spp.* strains based on *ITS* and *eEF1a1* sequences
- Study of the antagonism between *Trichoderma spp.* and different phytopathogenic fungi from the genus *Fusarium*
- Screening of some *Trichoderma spp.* strains for the production of hydrolytic enzymes
- Evaluation of gene expression responsible for chitinase activity in tomato crops
- Stimulating the growth of tomato plants with the help of some strains of *Trichoderma spp.* in the presence and absence of infection with *Fusarium oxysporum f. sp. radicis-lycopersici*

1.3. Thesis structure

The thesis is structured in two parts: study of literature (Chapter 1) and personal contributions consisting of 3 chapters (Chapter 2 - Chapter 4), each chapter representing one of the objectives of the thesis.

Each chapter of the second part of the thesis includes:

- an introduction regarding the analyzed subject;
- materials and methods used in the study;
- results and discussions;

The conclusions are drawn in a separate chapter (Chapter 5).

The complete list of references is presented at the end of the thesis.

Chapter 2

Research on the evaluation and selection of *Trichoderma spp.* strains used in the biological control of the phytopathogenic fungus *Fusarium oxysporum f. sp. radicis-lycopersici*

2.1 Molecular identification of *Trichoderma spp.*

The six strains of *Trichoderma spp.* collected for selection as biological control agents or plant growth stimulants, were identified at the species level (Samuels et al., 2009; Chaverri et al., 2015). For this, the sequences *ITS 1, ITS 2* and *eEF1a1* were obtained for all selected strains.

FASTA sequences were analyzed using *TrichOKey 2.0* (Druzhinina and Kubicek, 2005; Druzhinina et al., 2005; Druzhinina and Kopchinskiy, 2006) and *TrichoBLAST* (Kopchinskiy et al., 2005), tools available online at http: // www. trichoderma.info.

Figure 2.1. Alignment and analysis of *ITS* and *eEF1a1* sequences performed using *BioEdit, TrichOKey v2.0* and *TrichoBLAST (http://www.trichoderma.info).*

Sequence analysis of the *ITS 1* and *ITS 2* regions and the *eEF1a1* gene allowed the identification of strains belonging to two species: *Trichoderma asperellum* and *Trichoderma longibrachiatum*.

A phylogenetic analysis of the *eEF1a1* sequences, corresponding to the six strains, was performed using the MEGA6 package (Molecular Evolutionary Genetics Analysis version 6.0) by the UPGMA method (*Unweighted Pair Group Method with Arithmetic Mean*) (*Fig. 2.2*).

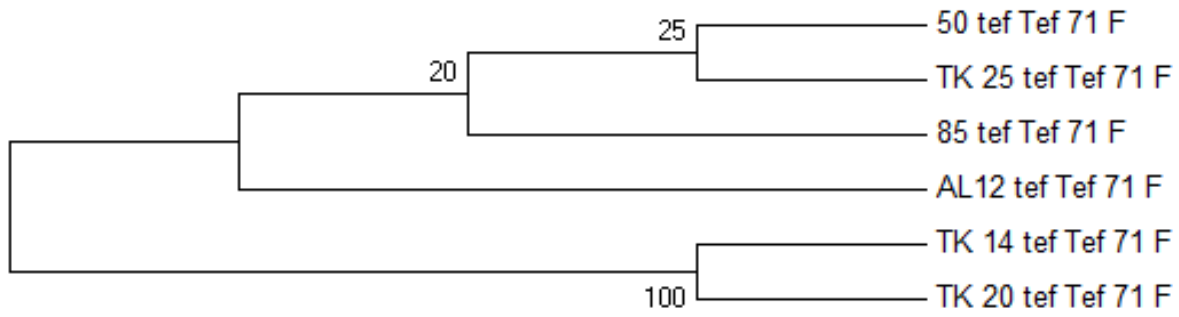

Figura 2.2. Phylogenetic tree of *Trichoderma spp.* strains based on *eEF1a1* sequences performed by UPGMA; Bootstrap 200.

2.2. *In vitro* interrelations of *Trichoderma spp.* and various phytopathogenic fungi, expressed as the coefficient *X*

In order to assess the degree of antagonism by the double culture method the behavior of *Trichoderma spp.* strains against three *Fusarium* strains (*Fusarium oxysporum f. sp. radicis-lycopersici, Fusarium tricinctum, Fusarium solani*) was tested on the PDA medium. The obtained results are presented below (*Fig. 2.3*).

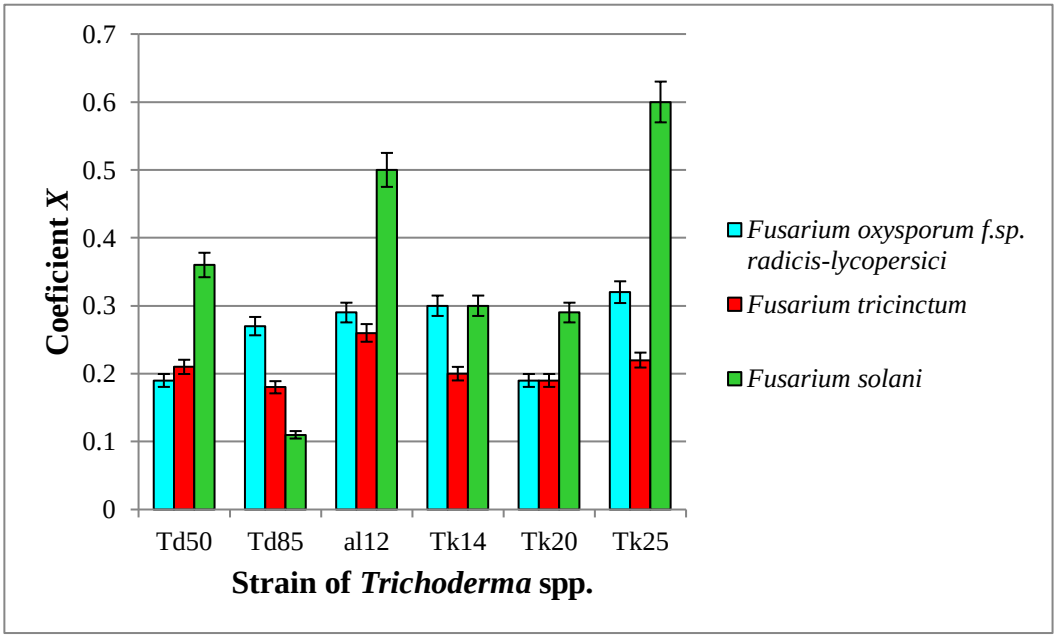

Figura 2.3. Antagonism between six strains of *Trichoderma spp.* and three species of phytopathogenic fungi of the genus *Fusarium*.

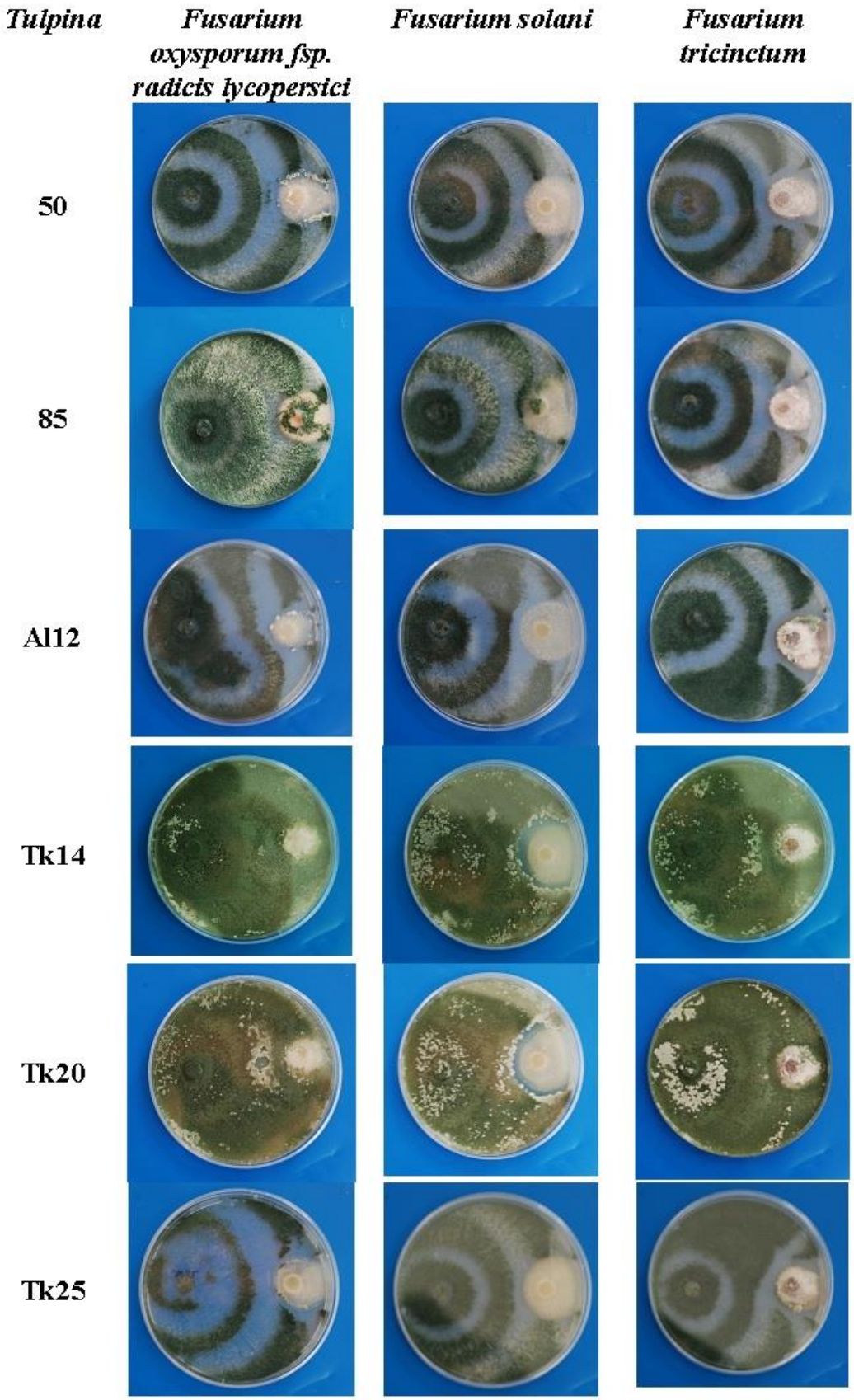

Figure 2.4. Testing the antagonistic capacity of *Trichoderma spp.* strains by the double culture method (Jouan,1964).

The value of the X coefficient calculated for *Fusarium oxysporum f. sp. radicis-lycopersici* is between 0.19 and 0.32 (at 8 days). Compared to *Fusarium tricinctum*, the values of the X coefficient varied between 0.19 and 0.26 (at 8 days). For the phytopathogenic fungus *Fusarium solani*, the value of the X coefficient varied between 0.11 and 0.60 (after 8 days). These results place the isolates of *Trichoderma spp.* in the antagonism class (the value of the coefficient X <1).

The results we obtained highlight the antagonistic character of the six strains of *Trichoderma spp.* studied by us against all three isolates subjected to tests.

The mechanism of action of *Trichoderma spp.* strains against phytopathogenic fungi is explained by the high sporulation capacity and competition for space and food that inhibited the development of colonies of *Fusarium oxysporum f. sp. radicis-lycopersici, Fusarium tricinctum* and *Fusarium solani* used in our experiments.

Numerous studies have highlighted the complexity of the mechanisms of action *Trichoderma* isolates: **antagonism** (inhibits the mycelial growth of the pathogen); **competition for food and space** (released spores grow faster than spores of pathogenic fungi and inhibit their development by colonizing them) and **plant-induced resistance** (Harman, 2006; Rojan et al., 2010).

2.3. Screening of some *Trichoderma spp.* strains for the production of hydrolytic enzymes

All six *Trichoderma* strains were qualitatively examined for the production of extracellular enzymes by the culture medium method. Our results show that all studied *Trichoderma* strains produce lytic enzymes, the level of production of these enzymes varying depending on the strain.

The method of highlighting on the medium was used to determine the chitinolytic activity (Agrawal and Kotasthane, 2012), which is based on the formation of a red-purple area around the fungal colony.

Td50, al12 and *Tk20* strains have a larger red area compared to strains *Td85, Tk14* and *Tk25*. These strains have a higher chitinase activity than *Td85, Tk14* and *Tk25* strains which have a lower chitinase activity (*Fig. 2.5*).

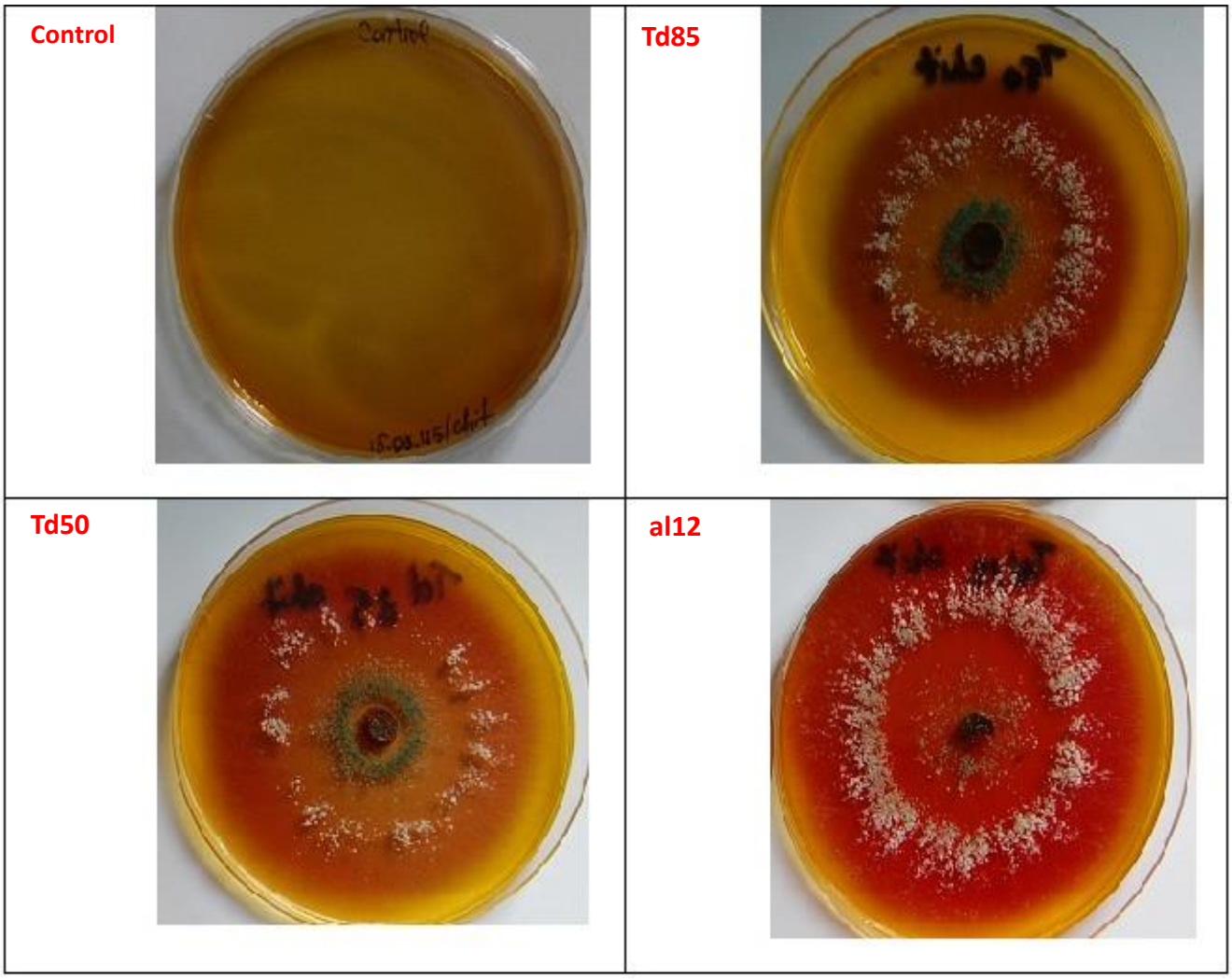

Figura 2.5. Evaluation of *Trichoderma* strains for the production of chitinases on colloidal chitin medium.

Chapter 3

Study of the chitinase activity of different strains of *Trichoderma spp.* in the presence of *Fusarium oxysporum f. sp. radicis-lycopersici*

3.1 Evaluation of the chitinase activity of *Trichoderma spp.*

The results presented below (*Fig. 3.1*) confirm that the strains of *Trichoderma spp.* under study show chitinase activity, which varies depending on the strain and species.

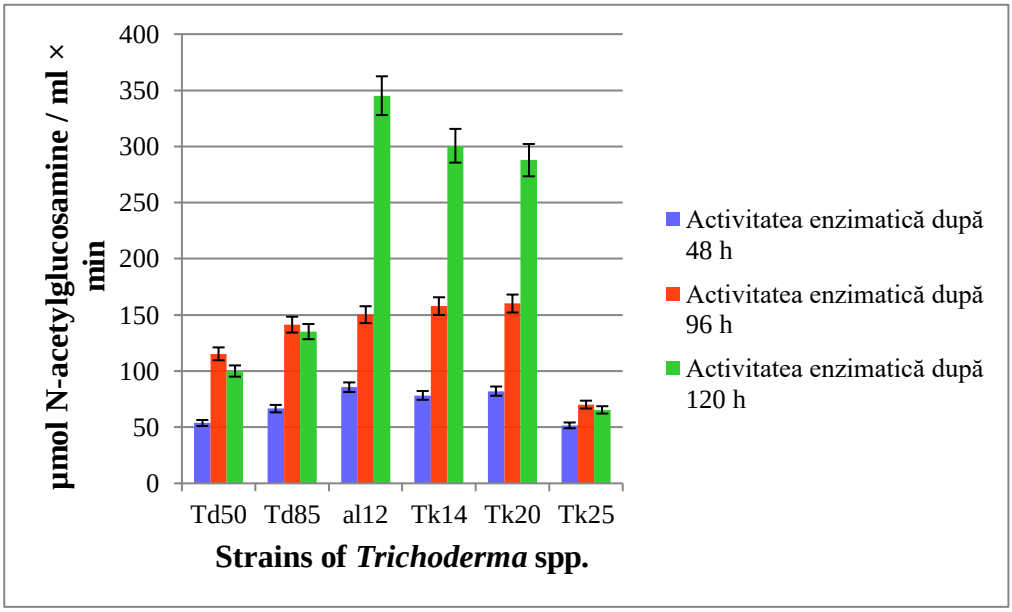

Figura 3.1. The variation of the enzymatic activity of chitinase 26 in the six strains of *Trichoderma spp.*, over 120 h.

There is an increase in the enzymatic activity of chitinase for strains *Td85, al12, Tk20* and *Tk25,* from 48 h to 120 h of incubation. The results are in agreement with those published by Parmar et al. (2015) who observed an increase in chitinase activity after 48 h, 72 h and 96 h of incubation, ranging between 5.62 U/mg and 8.9 U/mg. It should be noted that for strains *Td50, Td85, Tk25,* the chitinase activity begins to decrease slightly at 120 h after incubation (*Fig. 3.1*).

Among the studied strains, the following stand out:

- *al12* with a chitinase activity of 345.2 µmol NAG ml^{-1} min^{-1},
- *Tk14* with 300.6 µmol NAG ml^{-1} min^{-1} and
- *Tk20* with an activity of 287.8 µmol NAG ml^{-1} min^{-1}.

The strains studied by us were selected based on the qualitative method of detecting chitinase production, on a specific agar medium (Agrawal and Kotasthane, 2012). Among the studied strains (Petrișor et al., 2015), *Td50, al12* and *Tk20* have higher chitinase activity compared to *Td85, Tk14* and *Tk25* strains, by the plate method. However, these qualitative results are slightly different from the quantitative ones obtained with the spectrophotometric method. Also, Agrawal and Kotasthane (2012) observed that the chitinase activity highlighted on the medium is not always correlated with the chitinase activity quantified spectrophotometrically. They found that isolates with low and very low activity in agarized medium show a medium or high activity in the quantitative spectrophotometric determination. This could probably be due to the different incubation time of the two methods.

3.2 Evaluation of gene expression responsible for chitinase activity in tomato crop

The expression level of the *chit26* gene was determined by comparison with a *house-keeping gene (EF1α)* whose expression level does not depend on treatment with *Trichoderma spp.* Mean Ct values for each experimental variant (maximum 12) were used to calculate the expression fold of the tested gene (*expression fold*) by the ΔΔCt algorithm (Livak and Schmittgen, 2001).

The presence of specific reaction products was verified by analyzing the melting curve for each reaction. *Figure 3.2* shows examples of melting curves resulting for both genes (chit and *eEF1a1*).

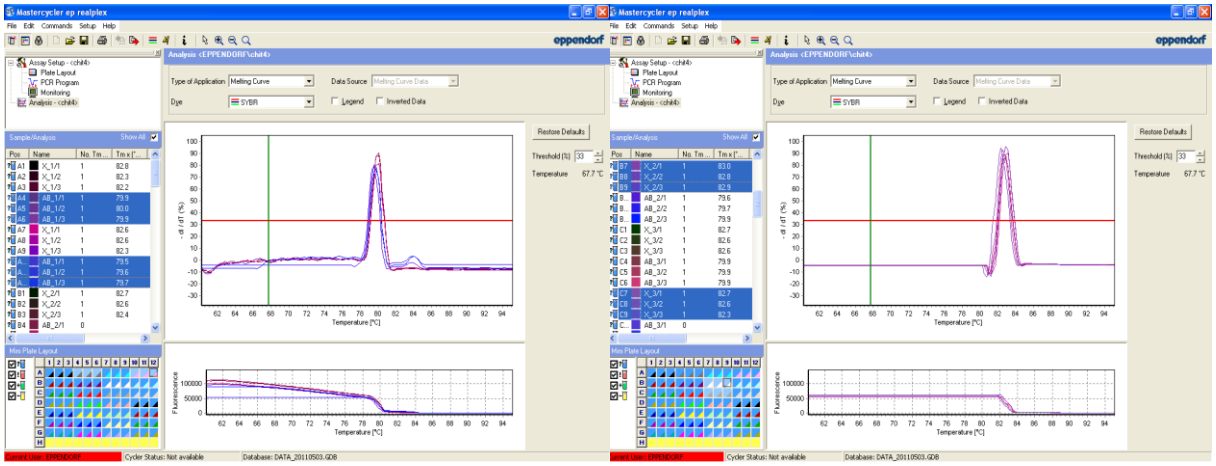

Figure 3.2. The analysis of the melting curve highlights the presence of a single reaction product with Tm specific for each set of primers (chit - left, *eEF1a1* - right).

In strains *al12*, *Tk14* and *Tk20*, the expression of chitinase 26 is significantly intesified in the presence of *FORL*. In the case of *Td50* and *Td85* strains, chitinase 26 expression is not significantly influenced by the presence of *FORL*. Exceptionally, chitinase 26 expression is inhibited in the *Tk25* strain in the presence of *FORL* (*Fig. 3.3*).

The high level of chitinase expression in strains *a12*, *Tk14* and *Tk20* is consistent with the previously determined increased chitinase activity. For isolates that exhibit chitinase activity, but without a significant increase expression level in chitinase 26, a plausible explanation is that the enzymatic activity is due to other chitinases encoded by genes that were not the subject of this analysis.

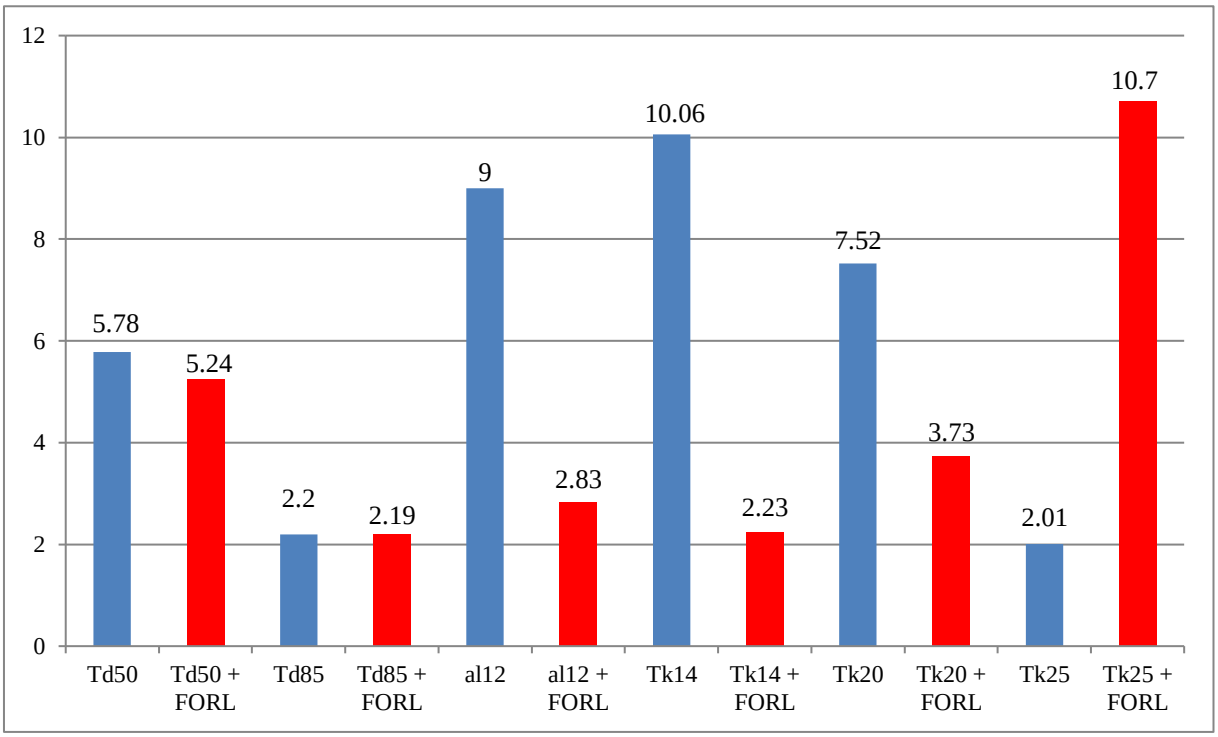

Figura 3.3. Variation in the *expression fold* of the chitinase 26 gene, reported in the control, in the presence or absence of *FORL*.

Chapter 4

Stimulating the growth of tomato plants using different strains of *Trichoderma spp.* in the presence and absence of infection with *Fusarium oxysporum f .sp. radicis-lycopersici*

The treatment of tomato plants with different strains of *Trichoderma spp.* had a beneficial effect on their growth and development both in the absence of the pathogen (*FORL*) and in its presence, leading to a delay in the onset of disease symptoms.

In the presence of *FORL* in the soil, all six strains of *Trichoderma spp.* used were able to increase the percentage of tomato emergence by 60%. In the presence of the pathogen alone, only 20% of the plants emerged. The effect of *Trichoderma spp.* (*Td85, al12, Tk14* and *Tk20*) strains on tomato plants was confirmed in cases where the seeds were inoculated only with the beneficial strains, which led to a significantly higher emergence.

The *Trichoderma spp.* strains used in this experiment decreased the severity of the disease produced by *FORL*, compared to the control (in which the biocontrol agent was not added0). The results of studies in the greenhouse indicate a 30% reduction in disease symptoms in FORL-infected tomato plants whose seeds were treated with *Tk20* and *Tk14*, while plants treated with *Td85, al12* show a significant 20% reduction in the disease. Plants treated only with *Trichoderma spp.* or *FORL* cause an incidence of the disease ranging from 0% to 70%, although the symptoms of the disease were not very severe.

The height of tomato plants treated with *Trichoderma spp.* varies depending on the strain of *Trichoderma spp.* applied. Plants treated with *al12* and *Td85* strains show a significant increase in height (29.8 cm and 28.3 cm) compared to the untreated control (21.8 cm). On the other hand, in the case of *Tk14* and *Tk20* strains tomato plants reached a similar height of about 25 cm. The lowest height of the plants (24.4 cm) was observed after treatment with *Tk25*, equivalent to the untreated control.

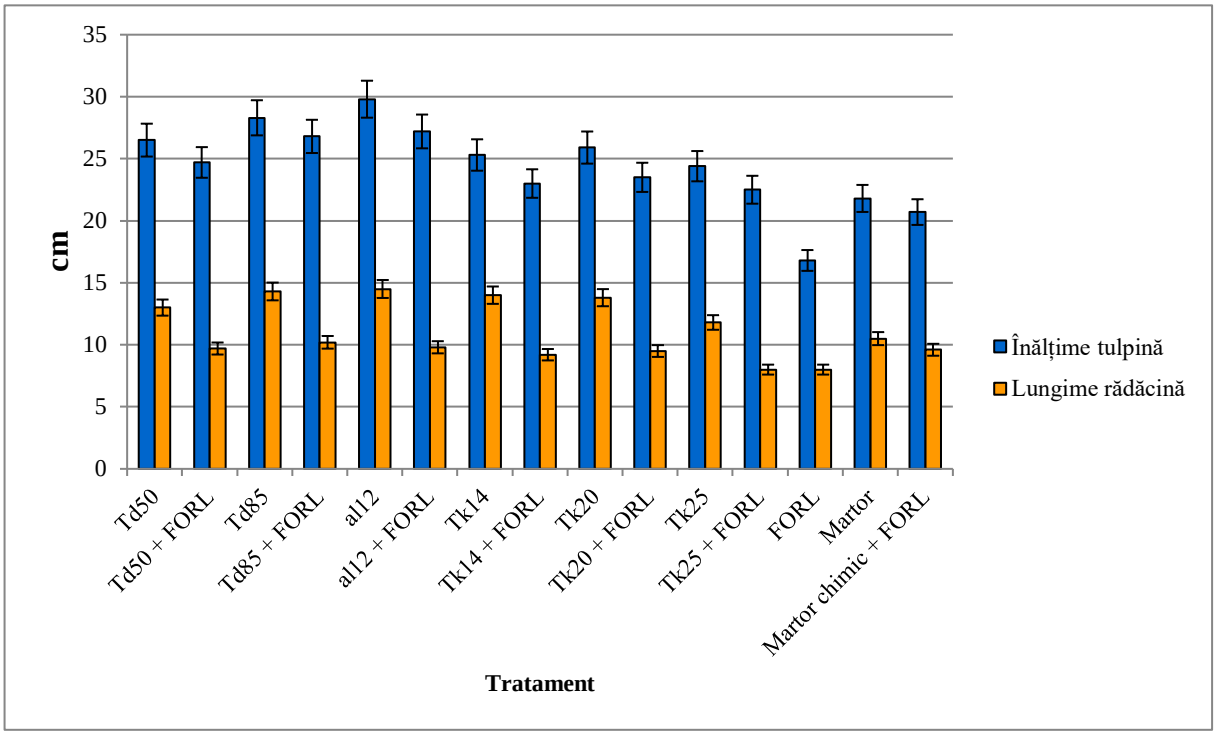

Figure 4.1. The effect of *Trichoderma spp.* treatment on stem height and root length in tomato plants infected or uninfected with *FORL*.

The lowest plant height was noted in tomatoes treated with *Tk25*, *Tk20* and *Tk14* and soil inoculated with *FORL*. However, the height of the plants in these variants is higher than the control infected with *FORL*. Also, the treatment of seeds with *al12* and *Td85* strains significantly increases the height of the plants, compared to the control inoculated only with *FORL*.

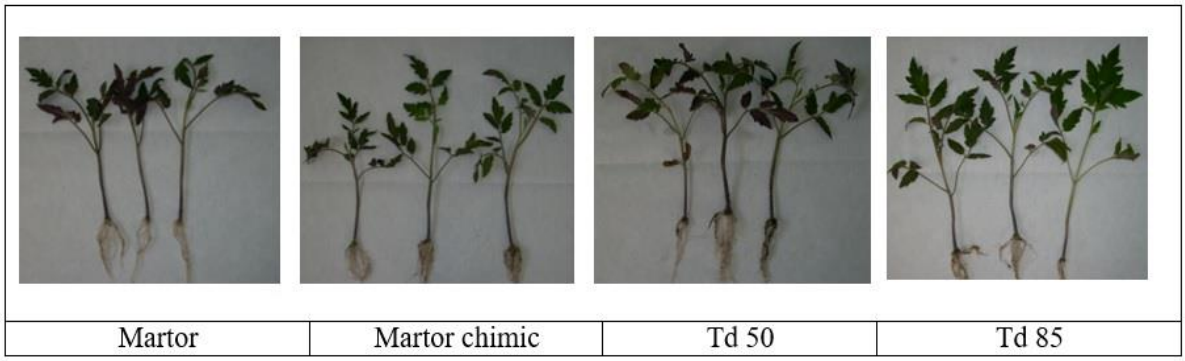

Figura 4.2. The influence of the application of the treatment with *Trichoderma spp.* on the growth parameters of the tomato plants.

The number of leaves/plant was different between the plants treated with *Trichoderma spp.* and control (*Fig. 4.3*). However, there was no pronounced difference between *Trichoderma spp.* strains applied to tomato plants. A slightly low number of leaves / plant is observed in plants treated with *Tk14* and *Tk20*, compared to plants where the other strains of *Trichoderma spp.*

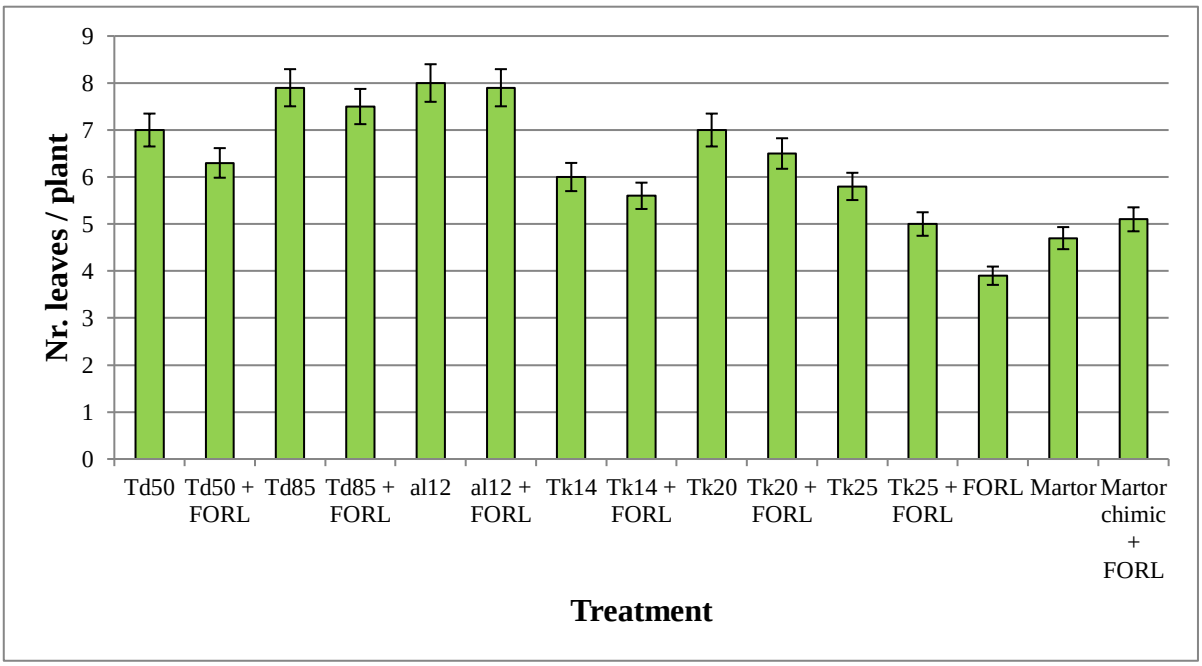

Figure 4.3. The effect of treatment with *Trichoderma spp.* on the average number of leaves in tomato plants infected or not infected with *FORL*.

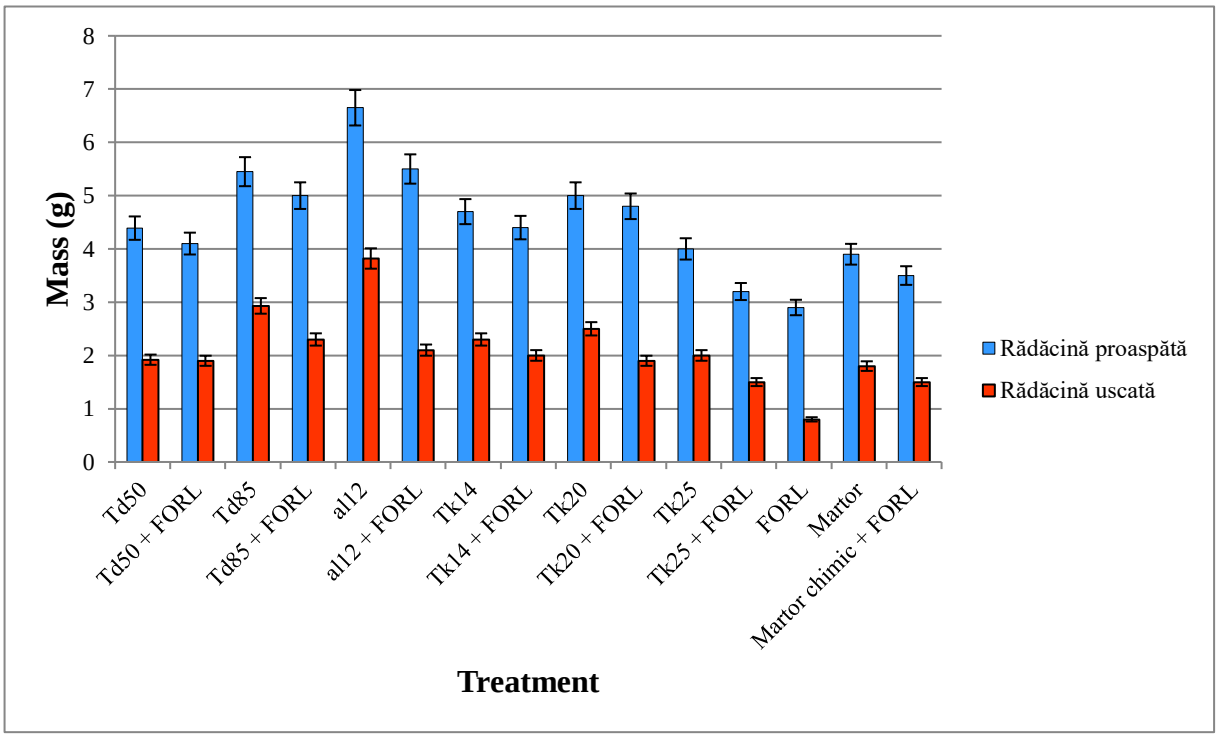

Figure 4.4. The effect of treatment with *Trichoderma spp.* on root mass in tomato plants infected or not infected with *FORL*.

Treatment with *al12, Td85* and *Tk20* significantly affects the **fresh and dry weight of the root of tomato plants** pretreated and inoculated with *FORL* compared to plants inoculated with FORL only (*Fig. 4.4*). Compared to the untreated control, tomato plants treated with *Tk14* and *Tk20* show an increase in the dry weight of the root, although there is no difference in the length of their roots. The results of this study demonstrate the beneficial effect of inoculation with *Trichoderma spp.* on the dry mass production of tomato seedlings.

The results of the present study demonstrate that all strains of *Trichoderma spp.* were able to stimulate plant growth parameters to varying degrees. This stimulation is the result of a better axial growth and a higher amount of biomass that are corroborated with those of other authors (Gravel et al., 2007; Contreras-Cornejo et al., 2009; Salas-Marina et al., 2011; Sofo et al., 2011). Biomass stimulation was observed not only in the aerial parts but also in the roots. The data obtained by us show that although all strains of *Trichoderma spp.* used in this study still synthesize IAA, the production of IAA varies depending on the strain tested. *Td85* and *al12* strains produce significant amounts of IAA ranging from 13.8 to 15.89 µg / ml (*Tab. 4.1*).

Table 4.1. IAA synthesis and phosphate solubilization in *Trichoderma spp.* strains *in vitro*.

Strains	**IAA (µg/ml)**		**Phosphate solubilization index**	**Phosphate concentration (mg/l)**
	With tryptophan	**Without triptofan**		
Td85	1,9	1,2	0	0,18
Td50	12,8	1,0	0	0,25
al12	13,8	1,3	0	0,30
Tk14	10,9	0,6	0	0
Tk20	11,2	0,9	0	0,35
Tk25	9,5	0,8	0	0

The significance of phosphate solubilization by fungi is to increase the absorption of phosphorus with a role in plant growth. From the data we obtained it is observed that none of the tested *Trichoderma spp.* strains is able to solubilize phosphate on Pikovskaya medium (*Tab. 4.1*). None of the strains gives a solubilization zone (halo), observable on Pikovskaya agarized medium, however, strains *Td85, Td50, al12* and *Tk20* show a slight solubilization of phosphates between 0.18 and 0.35 mg / l in the medium. liquid. Thus, the stimulation of

growth of the studied tomato plants is correlated with the production of IAA, but not with the solubilization of phosphates.

The production of phytohormones by the strains of *Trichoderma spp.* can lead to an increased intensity of photosynthesis which entails the stimulation of plant growth.

The content of chlorophyll a, chlorophyll b and total chlorophyll in the tomato leaves whose seeds were treated with *Trichoderma spp.* was increased (in all variants studied) compared to the untreated control, except for *Tk25* which has a lower content (*Fig. 4.6*).

This study indicates that seed treatment with *al12*, *Td85* and *Td50* strains results in a significant increase in assimilative pigments compared to untreated control.

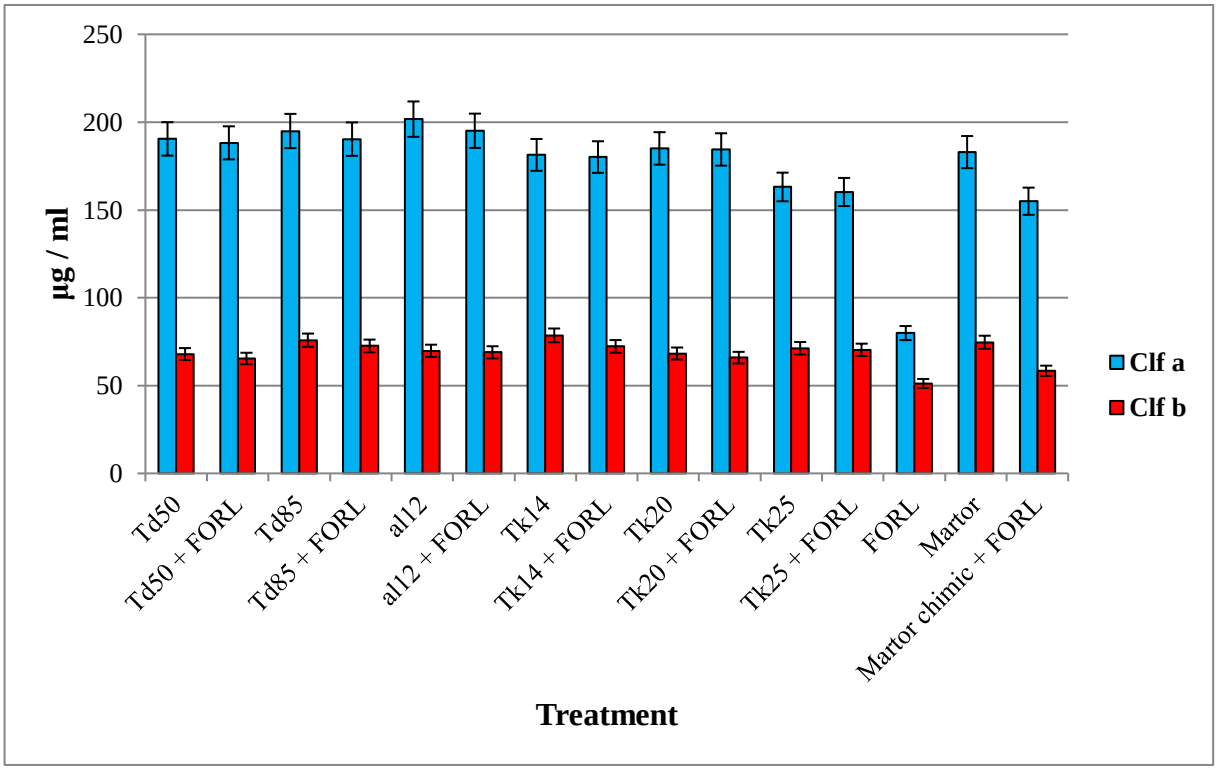

Figure 4.6. The effect of treatment with *Trichoderma spp.* strains on the content of photosynthetic pigments in uninfected and *FORL*-infected tomato plants.

Thus, the chlorophyll content of tomatoes inoculated with *al12* (201.71 µg / ml), *Td85* (194.95 µg / ml) and *Td50* (190.46 µg / ml) was much higher compared to the untreated control (182, 95 µg / ml). All these data support that the plants treated with *Trichoderma spp.* have an intense photosynthetic activity.

The decrease in the content of chlorophyll a and chlorophyll b is probably the result of an increase in the activity of chlorophyllase in the leaves of tomato plants infected with FORL.

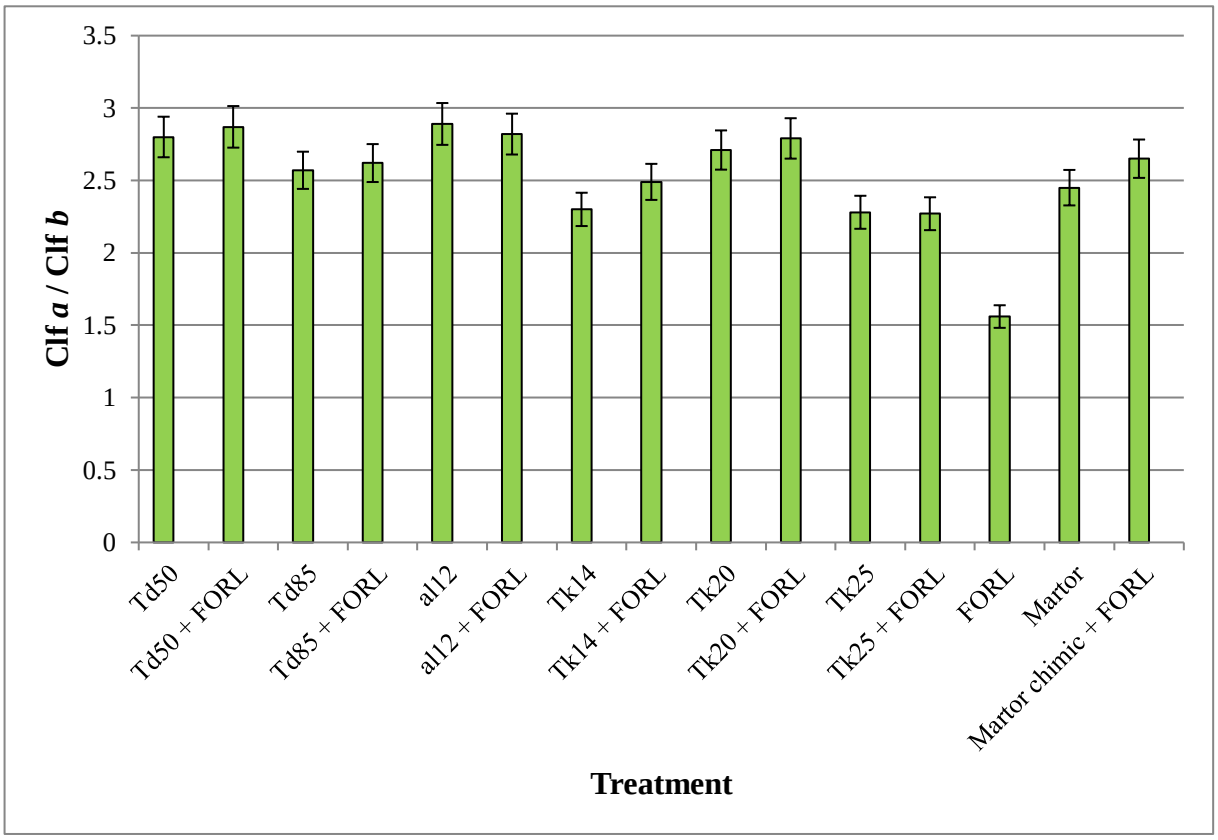

Figure 4.7. The effect of treatment with *Trichoderma spp.* strains on the Clf a / Clf b ratio in uninfected and *FORL*-infected tomato plants.

The Clf a/Clf b ratio is an indicator of stress. It decreases in all *FORL*-infected variants treated with *Trichoderma spp.* strains from 2.87 (in plants treated with *Trichoderma spp.* and infected with *FORL*) to 1.56 (in untreated plants infected with *FORL*) (*Fig. 4.7*).

Our results reveal that plants treated with *Trichoderma spp.* had a higher carotenoid content compared to the untreated control. Also, the results obtained by us do not show a significant difference in phenol content in plants infected with *FORL* compared to uninfected ones. However, the total phenol content decreases slightly in the leaves of infected plants compared to the untreated control, but this content increases compared to the variant that was infected with *FORL* and not treated with with *Trichoderma spp* (*Fig. 4.8* and *Fig. 4.9*). All these data, together with those on chlorophylls, confirm that in plants treated with *Trichoderma spp.* There is an intense photosynthetic activity.

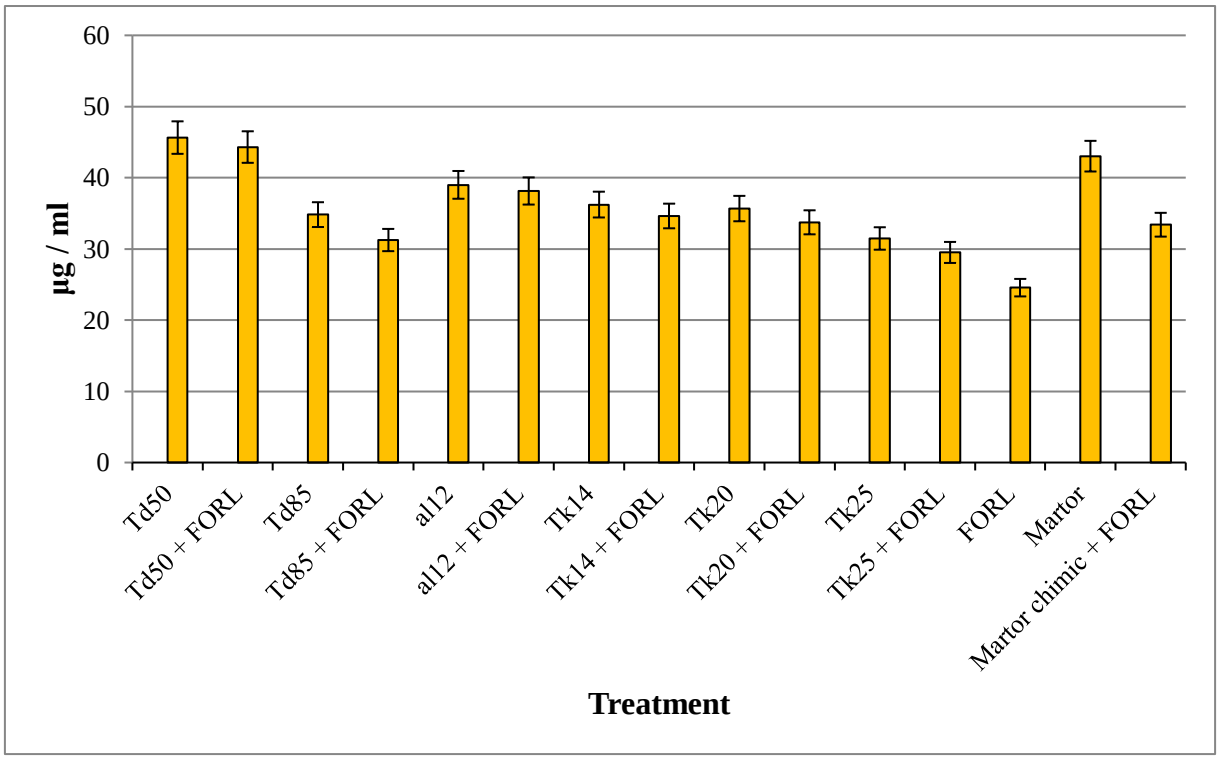

Figure 4.8. The effect of treatment with *Trichoderma spp.* strains on carotenoid and xanthophyll content (µg / ml) in uninfected and *FORL*-infected tomato plants.

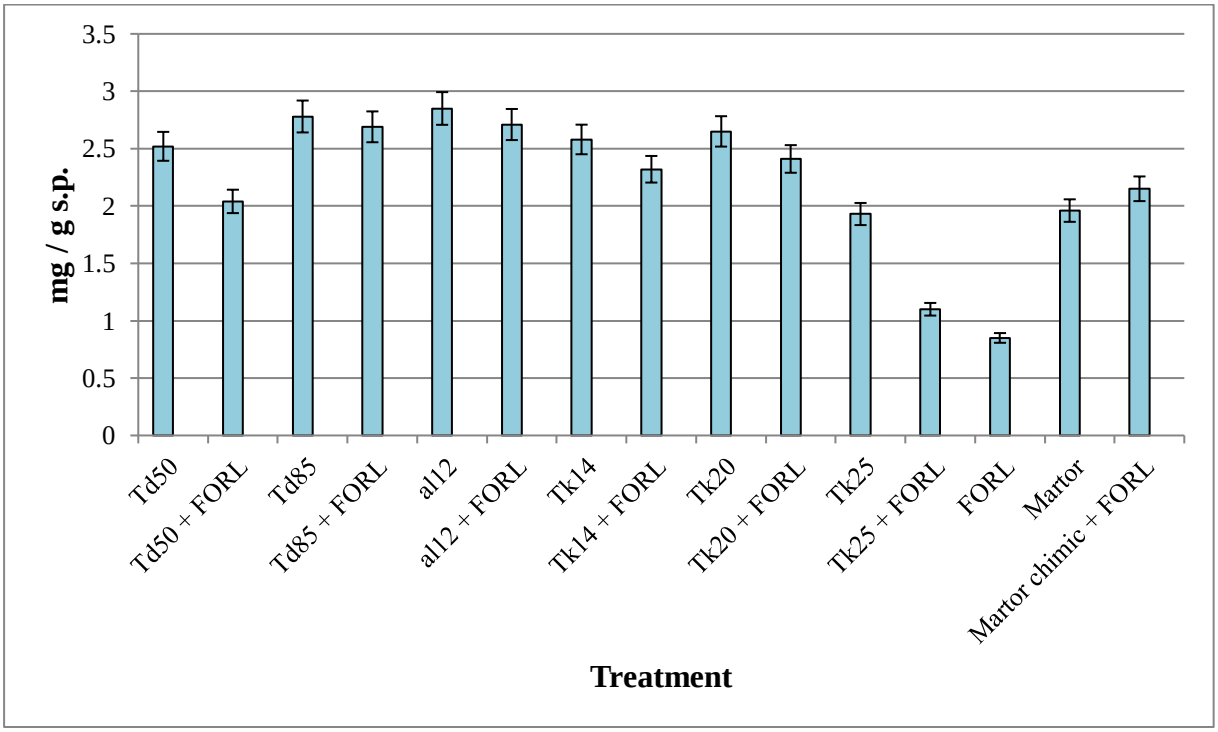

Figure 4.9. The effect of treatment with *Trichoderma spp.* strains on the total phenol content (mg / g fresh substance) in uninfected and *FORL*-infected tomato plants.

Chapter 5

5.1. Conclusions

The need to increase the productivity and quality of crops in agriculture justified the excessive use of chemical fertilizers, which led to serious problems of environmental pollution. The use of biofertilizers and biopesticides is an alternative for obtaining high yields with lower ecological impact. Soil microorganisms influence ecosystems by contributing to plant growth and nutrition, soil structure and fertility, and, implicitly, plant health. *Trichoderma spp.* species are able to colonize the surface of the roots and cause substantial changes in plant metabolism.

The results obtained by us emphasize the importance of the molecular approach for the identification of species that can also serve as a tool for understanding the phylogenetic relationships between different strains of *Trichoderma spp.*

Following the phylogenetic analysis based on the *ITS* and *eEF1a1* sequences from six isolates of *Trichoderma spp.*, two distinct species were identified. *Trichoderma asperellum* and *Trichoderma longibrachiatum*. The "dominant" species was *T. asperellum* with four of the six strains (*Td50, Td85, al12* and *Tk25*), followed by *T. longibrachiatum* with two strains (*Tk14* and *Tk20*).

The *Td85* and *Tk20* strains had the highest antagonistic activity compared to the three phytopathogenic strains of *Fusarium spp.*, These being followed, in order, by *Td50, Tk14, al12* and *Tk25*.

All studied *Trichoderma spp.* strains produce chitinase. Of the six strains studied, *Td50, al12* and *Tk20* stand out with the highest chitinase activity. Among the studied strains, the strains *al12* (345.2 µmol NAG / ml × min), *Tk14* (300.6 µmol NAG / ml × min) and *Tk20* (287.8 µmol NAG / ml × min) stand out in regard to the chitinase activity.

Chitinase activity is also demonstrated and supported by determinations of gene expression. The gene encoding chitinase was more strongly expressed in tomato plants

infected with *Fusarium oxysporum f. sp. radicis-lycopersici*, compared to healthy, uninfected ones. In this sense, the tomato plants treated with strains *Tk14*, *al12* and *Tk20* were highlighted.

Td85 and *al12* strains (identified as *Trichoderma asperellum*) significantly reduce the severity of fusariosis development and stimulate plant growth due to the production of IAA. These strains demonstrate numerous plant-stimulating characteristics, including the ability to produce IAA, cellulase, and chitinase.

In addition, *Td85* and *al12* strains induce an increase in root height and length in both healthy tomato plants and those infected with *Fusarium oxysporum f. sp. radicis-lycopersici*. Moreover, inoculation of tomato seeds with these two strains significantly reduces the development of fusariosis in tomato plants, which is consistent with the results obtained in *in vitro* tests with double cultures.

5.2. The originality of the thesis

- *Trichoderma spp.* strains used in these research were identified using the PCR technique to amplify *ITS* regions, as well as fragments of the *eEF1a1* gene encoding the elongation factor *1-α (tef1)*. The 6 strains of *Trichoderma spp.* were classified into two distinct species: *Trichoderma asperellum* and *Trichoderma longibrachiatum*.

- Regarding the degree of antagonism of the *Trichoderma spp.*: the strains *T. asperellum Td85* and *T. longibrachiatum Tk20* had the highest antagonistic activity, these being followed by *T. asperellum Td50*, *T. longibrachiatum Tk14*, *T. asperellum al12* and *T. asperellum Tk25*.

- All strains of *Trichoderma spp.* were qualitatively examined for the production of extracellular enzymes by the method of culture on a specific medium. *Td50, al12* and *Tk20* strains have a larger red colored area compared to strains *Td85, Tk14* and *Tk25*. These strains have higher chitinase activity compared to *Td85, Tk14* and *Tk25* strains which have lower chitinase activity.

- Evaluation of gene expression responsible for chitinase activity in tomato culture was analyzed by reverse transcription and qPCR. The gene encoding chitinase was more strongly expressed in tomato plants infected with *Fusarium oxysporum f. sp. radicis-lycopersici* compared to healthy ones. In this sense, the tomato plants treated with *T. longibrachiatum* strains *Tk14* and *Tk20* and *T. asperellum al12* stand out.

- Treatment of tomato plants with different strains of *Trichoderma spp.* had a beneficial effect on their growth and development both in the absence of phytopathogen (*Fusarium oxysporum f. sp. radicis-lycopersici*) and in its presence, leading to delayed symptoms of disease.

- All six strains of *Trichoderma spp.* were able to intensify the percentage of tomato emergence by 60%, in the presence of *Fusarium oxysporum f. sp. radicis-lycopersici* in soil. If the pathogen alone was present in the soil, only 20% of the plants emerged. The effect

of *Trichoderma spp.* (*Td85, al12*, *Tk14* and *Tk20*) strains on tomatoes was confirmed in plants where the seeds were inoculated only with beneficial strains which allowed a significantly higher emergence.

- *Td85* and *al12* strains, identified as *Trichoderma asperellum*, significantly reduce the severity of fusarium wilt and stimulate plant growth due to IAA production. The strains demonstrate numerous plant-stimulating characteristics, including the ability to produce IAA, cellulase, and chitinase.

- In addition, these two strains induce an increase in plant height and root length in both healthy tomato plants and those infected with *Fusarium oxysporum f. sp. radicis-lycopersici.*

5.3. Prospects for further development

Romanian farmers are looking for cleaner and more natural methods of supporting crops, especially vegetables. All the more so as, in most cases, producers are the first consumers of their own products.

Thus, the results obtained by us may be the basis for future research in order to obtain biopreparations based on *Trichoderma asperellum* (*Td85* and *al12* strains). These biopreparations can be used in phytosanitary treatments to control phytopathogens that cause significant damage and affect plant production.

We will also continue research to identify and characterize the molecular mechanisms involved in the interaction of *Trichoderma asperellum* with phytopathogens.

List of articles published in the field of thesis

Web of Science Journals

1. Petrișor Cristina, **Paica Alexandru**[*], Burnichi Floarea (2019), Physiological and growth response of tomato plants after *Trichoderma spp.* seed treatments, *Studia Universitatis Babeș-Bolyai, Chemia*, LXIV(2), 567-577, ISSN 1224-7154, (FI=0.305) – 1 citation

2. Petrișor Cristina, **Paica Alexandru**[*] (2019), Overview on utilities and analysis techniques of organic volatile compounds (VOCS) produced by fungi belonging to *Trichoderma spp.*, *Proceedings of the Romanian Academy*, Series B, 21(2), 91-98, ISSN 1454-8267; Indexat ISI

3.Petrișor Cristina, Paica Alexandru, Constantinescu, Florica (2017), Effect of secondary metabolites produced by different *Trichoderma spp.* isolates against *Fusarium oxysporum f.sp. radicis-lycopersici* and *Fusarium solani*, *Scientific Papers. Series B, Horticulture*, XVI, 407–411, ISSN 2285-5653, Indexat ISI – 5 citări

4. Petrișor Cristina; **Paica Alexandru**, Constantinescu Florica (2016), Influence of abiotic factors on in vitro growth of *Trichoderma* strains, *Proceedings of the Romanian Academy, Series B*, 18(1), 11-14, ISSN 1454-8267; Indexat ISI – 6 citări

5. Petrișor Cristina, **Paica Alexandru**, Constantinescu Florica (2016), Temperature and pH influence on antagonistic potential of *Trichoderma sp.* strains against Rhizoctonia solani, Scientific Papers. Series B, Horticulture, LX, 275-278; ISSN 2285-5653, Indexat ISI – 6 citări

6. **Paica Alexandru**, Petrișor Cristina, Constantinescu Florica (2015), Influence of abiotic factors on biological control ability of different *Trichoderma spp.* strains, *Annals of the University of Craiova*, XX(LVI), 543-550, Indexat ISI – 4 citări

BDI Journals

1. Petrișor Cristina, **Paica Alexandru**, Constantinescu Florica (2015), Screening of *Trichoderma sp.* strains for producing hydrolytic enzymes, *Romanian Journal for Plant Protection*, VIII, 7-10; ISSN 2248 129X.

2. **Paica Alexandru**, Sicuia Oana-Alina, Petrișor Cristina (2015), Comparative analysis of different DNA isolation methods for Trichoderma *spp.* strains used as biocontrol agents, *Journal of Horticulture, Forestry and Biotechnology*, 19(3), 22-25, ISSN 2066-1797

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