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ALTERNATIVE METHODS IN THE CONTROL OF *ALTERNARIA* DISEASES IN POTATO CULTURE (*SOLANUM TUBEROSUM*)

Carmen Mihaela Botea[1,2], *Cristina Petrișor*[1], *Viorel Fătu*[1], *Roxana Zaharia*[1], *Mali Sanda Manole* *[2]

[1]Research - Development Institute for Plant Protection, 8 Ion Ionescu de la Brad Blvd, PC013813, District 1, Bucharest, Romania
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Mărăști Blvd., District 1, Bucharest, Romania

*Correspondence address:
University of Agronomic Sciences and Veterinary Medicine of Bucharest
Faculty of Agriculture, 59 Mărăști Blvd., 011464, Bucharest, Romania, +40072357080
E-mail: mali.manole@agro-bucuresti.ro

Abstract: The potato (*Solanum tuberosum* L.) is a globally cultivated staple food crop of great importance, both as a food plant and as an industrial and fodder plant. Significant annual losses in potato production are reported due to soilborne phytopathogens. Therefore, effective control or management of plant diseases is an essential component of sustainable agricultural practices. *Alternaria* species synthesize dangerous toxins, which can accumulate in infected agricultural crops and persist in processed products. Fungi belonging to the genus *Trichoderma* have been widely used as effective biological control agents against fungal phytopathogens. The present study represents a continuation of this research, focusing on the prevention and control of *Alternaria* diseases in potato cultivation.

Keywords: *biological control, potato, Alternaria spp., Trichoderma spp.*

INTRODUCTION

The potato (*S. tuberosum*) is an herbaceous plant, widespread throughout the world, according to the I.N.S. (The National Institute of Statistics), being the fourth largest source of food energy after rice, wheat and corn. Around 341 million tons of potatoes are cultivated annually on 49 million hectares. Romania currently cultivates around 79.000 ha of potatoes with an average production of around 14.89 tons/ha, well below the European Union average (29.44 tons/ha) and a total production of 2.29 million tons.

Carrying out soil tillage at optimal times to obtain an adequate quality of production, using high-performance agricultural machinery that reduces the number of operations performed on the soil, planting plants at the optimal time, ensuring the right planting density, placing the crop on beneficial soil, and thus, growing potatoes in appropriate soil, ensuring balanced fertilization and phytosanitary products with low impact on the soil and the plants, these are the cultivation technologies that are intended to be used following the EU Green deal strategy.

Alternaria spp. is a genus of fungi belonging to the Phylum Ascomycota, Class Dothideomycetes and Order Pleosporales. Species of this genus are mainly plant pathogens. As a result of global warming, the specific climatic profile of potato growing areas allows a more aggressive attack of *Alternaria* spp. on potatoes. The abundance of the genus *Alternaria* increases with increasing temperature and its importance in climate change scenarios (Delgado-Baquerizo et al., 2020). The sudden alternation of wet and dry periods creates favorable conditions for the evolution of the disease. Maximum risk warnings occur in case of temperatures of 16 – 25°C accompanied by precipitation (Singh et al., 2015).

Alternaria species are mostly found as saprophytes in soil and plant debris. Sources of infection are mycelium and conidia stored in plant residues, soil and tubers (Thomma, 2003). Symptoms of the disease include leaf lesions that first present as brown spots, depending on the species, with its characteristic concentric rings, which eventually lead to severe defoliation and considerable production losses. Over time, researchers have described early blight (*Early blight*) caused by *A. solani* and brown spot (*Brown spot*) caused by *A. alternata*. Both pathogens occur together on the same plant and the initial stages of symptoms are difficult to differentiate (Schmey et al., 2024).

Several *Alternaria* species can produce secondary metabolites when they grow on different substrates. These metabolites cause plant diseases and can cause health disorders in humans and animals (Lopez & Cabral, 1999; Patriarca et al., 2018; Andrade Junior et al., 2018; Delgado-Baquerizo et al., 2020). In a global study of fungi present in the soil, *Alternaria* was the most abundant plant pathogen and the most dangerous disease of potatoes in all potato-growing countries of the world, especially in temperate climates (Yuldashova et al., 2023).

The most effective way to control disease is to use specific fungicides, but so far, only a few groups of fungicides are available. Many studies show the development of increased resistance to fungicides from the pathogen, which can lead to reduced disease control. Therefore, it is essential to find new ways to slow down this development of resistance and to seek alternative or complementary control strategies (Bellé & Hausladen, 2022).

To inhibit the development and spread of fungi from the *Alternaria* genus, and reduce damage and the use of pesticides, it is recommended to use biological preparations, inclusive based on fungi of the genus *Trichoderma* (Scerbacova & Crucean, 2021).

Filamentous fungi belonging to the genus *Trichoderma* are saprophytic microorganisms ubiquitous distributed, with dual activity, as they exhibit both plant growth-stimulating and biological control properties and are therefore used in many crops (Vinale et al., 2008; Adnan et al., 2019). They grow abundantly on different soil types and remain in the soil for a long time (Narasimhamurthy et al., 2018). Endophytic fungi live in symbiosis with the roots of many plant species and increase resistance to biotic and abiotic stress (Wen et al., 2022). *Trichoderma* species have a role in the mobilization and absorption of nutrients from the soil (Zin & Badaluddin, 2020).

Numerous researches have highlighted the complexity of the mechanisms of action of the isolates of the genus *Trichoderma*: antagonism (inhibits the mycelial growth of the pathogen); competition for food and space (the released spores grow faster than the spores of pathogenic fungi and inhibit their development by colonizing them) (Harman, 2006; Rojan et al., 2010). *Trichoderma* is considered a potent mycoparasite of various plant pathogenic fungi through competition, host hyperparasitism or antibiosis and can induce plant resistance (Yadav et al., 2021; El-Komy et al., 2015; Yao et al., 2023; Monfil & Casas-Flores, 2014). They grow well at temperatures between 25 °C and 30 °C (Samuels et al., 2010) and can be found in forest soils, gardens, decaying wood, compost grown by fungi, cereal grains, from different regions of the world and in marine environments (Druzhinina et. al., 2006; Blaszczyk et al, 2011).

The study investigated the effectiveness of two autochthonous *Trichoderma* spp. strains in the biological control of *Alternaria* spp. in potato plants, assessing in -vitro and in- vivo antagonistic activity and their ability to promote plant growth.

MATERIALS AND METHODS

In vitro experiment of antagonistic activity assessment

The *Trichoderma* strains, *T. viride* (Tv20) and *T. asperellum* (Td85) were obtained from the R.D.I.P.P. Bucharest collection.

Alternaria sp. isolate was obtained from infected potato leaves collected during preliminary infection monitoring studies conducted in the flowering period. The collected leaves were disinfected with 1% sodium hypochlorite for 1 minute, then washed three times with sterile distilled water. The isolation was performed by directly inoculating the leaves into PDA (potato-dextrose-agar) medium. Petri plates were incubated in the dark at 25 ± 2°C for 7 days. The *Alternaria* sp. isolate was then purified and morphologically identified at the genus level according to Samson et al. (1984).

To assess the antagonistic activity, a dual culture method was employed. Mycelial discs (6 mm diameter) from the *Alternaria* isolate were placed 1.5 cm from the edge of the Petri plate, with the *Trichoderma* strain positioned at the same distance, but in the opposite direction on the same plate. A control variant was also prepared, in which a 6 mm mycelial disc taken from a 7-day-old *Alternaria* culture was placed in the centre of the Petri plate. Each sample was tested, sealed with parafilm, and incubated at 25 ± 2°C in the dark for 14 days, with periodic observations to monitor phytopathogen growth. Measurements to evaluate the inhibition percentage of *Trichoderma* strains against *Alternaria* were performed at 3, and 7 days after the inoculation. The percent of mycelial growth inhibition was calculated according to the following formula:

$$I\,\% = (C-T)/C \times 100,$$

where: C = mycelial colony diameter in the control plate

T = mycelial colony diameter of the pathogen in the presence of the microorganism in double culture (Morton & Stroube, 1955).

In vivo experiment assessment of plant growth promotion and disease control

The Darilena potato variety, obtained from N.I.R.D.P.S.B. Brasov, was used in the experiment. This variety has a semi-early maturity period and is characterized by oval-shaped tubers, yellow skin, light yellow flesh, and moderately firm consistency, with a slightly floury texture. The starch content is 17.58%, classifying it in quality class B. The variety exhibits resistance to potato blackleg and potato stripe, and moderate resistance to late blight (on both leaves and tubers), potato virus Y, and potato leaf roll virus.

The *in vivo* experiment consisted of four treatment groups, each with nine replications (one pot per replication), with three tubers planted per pot. The tubers were cultivated in 16 L pot with sandy soil mixed with decomposed manure at a 3:1 ratio. The decomposed organic fertilizer provided essential nutrients to soil microbes, enhancing the effectiveness of the biocontrol agents and increasing their competitiveness in the rhizosphere (Liu et al., 2012).

The experiment utilized biopreparations based on *T. viride* (Tv20) and *T. asperellum* (Td85) strains, obtained from the R.D.I.P.P. Bucharest collection. The pathogenic strain used for inoculation was *Alternaria* sp., also obtained from the same collection.

Seven days before planting, the soil was inoculated with *Alternaria* sp. in three of the four experimental groups: V1 (Uninoculated soil (healthy control), V2 (Control *Alternaria*) – soil inoculated with *Alternaria* sp., V3 (*Alternaria* - Tv20) – soil inoculated with *Alternaria* sp. and treated with *T. viride* (Tv20) and V4 (*Alternaria* - Td85) – soil inoculated with *Alternaria* sp. and treated with *T. asperellum* (Td85).

The pathogen inoculum concentration was adjusted to 5×10^4 conidia/ml using a hemocytometer. At planting, the soil in V3 (*Alternaria* - Tv20) was treated with 0.5 g of biologically granulated preparation based on *T. viride* (Tv20) per pot. Similarly, the soil in V4 (*Alternaria* - Td85) was treated with 0.5 g of biologically granulated preparation based on *T. asperellum* (Td85) per pot. The biopreparations used were gradual-release granules on sodium alginate support, and the stock solution was adjusted to 2×10^7 spores/ml.

Observations and measurements were conducted at 15, 35, and 50 days after planting to assess the phytosanitary status of the crop and evaluate the effectiveness of the applied treatments in preventing and controlling the pathogen. Growth parameters were recorded, including the percentage of emergence and average plant length at 15 days, as well as shoot weight, number, and length at 35 days. At 50 days, shoot weight and length were further assessed. Additionally, the severity of the pathogen attack was quantified by determining the frequency, intensity, and degree of infection. Chlorophyll content measurements were also performed using a CCM-200 Plus chlorophyll meter, a portable device designed for non-destructive analysis.

The intensity and the degree of attack were calculated using the following formulas (Ivașcu, 2009).

$$\text{Attack Frequency } (F\ \%) = (n \times 100)/N,$$

where n = the total number of plants/organs attacked;
N = the total number of plants/organs analyzed;

$$\text{Attack intensity } (I\ \%) = \Sigma\ (i \times f)/\ n,$$

where i = the percentage of the attack or the grade awarded;
f = the number of plants (organs) noted with that note;
n = the total number of attacked plants (organs) analyzed;

$$\text{Degree of attack } (G\ \%) = (F\ \% \times I\ \%)/100,$$

where F % = the frequency of the attack and I % = the intensity of the attack.

RESULTS AND DISCUSSIONS

In vitro experiment of antagonistic activity assessment

The *in vitro* experiment demonstrated that both *Trichoderma* strains inhibited the mycelial growth of *Alternaria* sp. on a solid medium. In the dual culture assay, the inhibition rate ranged from 50% to 64%, confirming the antagonistic effect of *Trichoderma* spp. against the phytopathogen. At 3 days after inoculation, the inhibition of the mycelial growth was low, with very close values between tested strains. The largest reduction in mycelial growth was recorded at 7 days, for the Td85 strain, with a reduction in mycelial growth of the pathogen of 63.39%, followed by the Tv20 strain, with a reduction in mycelial growth of the pathogen of 50.98% (Table 1).

Table 1. Inhibition of the pathogen *Alternaria* sp. in double culture with *T. viride* (Tv20) and *T. asperellum* (Td85) at 3 days and 7 days

	Inhibition of mycelial growth %	
	at 3 days	**at 7 days**
***Alternaria* sp.- *T. viride* TV20**	**25.71 %**	**50.98 %**
***Alternaria* sp.- *T.asperellum* Td85**	**27.14 %**	**63.39 %**

After 14 days, in the *Alternaria*-Tv20 sample, the pathogen was completely covered by the biological strain Tv20, exhibiting a fungistatic (unidirectional) antagonism on the pathogen characterized by the expansion of the *Trichoderma* colony over the colony of the pathogen's mycelium. In the *Alternaria*-Td85 sample, the pathogen was surrounded by the mycelium of the Td85 strain, being covered in a proportion of 75% (Figure 1).

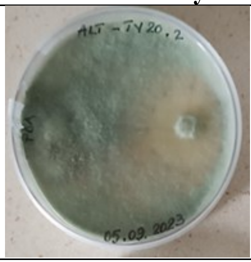
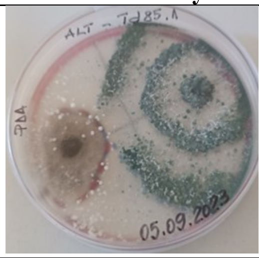
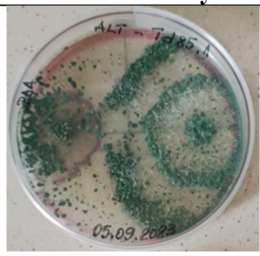
Tv20 at 7 days	Tv20 at 14 days	Td85 at 7 days	Td85 at 14 days
			

Figure 1. The antagonistic capacity of *T. viride* and *T. asperellum* against the pathogen *Alternaria* sp. using the double culture method

Because *Alternaria* species affect a variety of plant species, research on the impacts of beneficial *Trichoderma* strains is an important field of study globally. Researchers studying wheat and sunflower crops have found promising *in vitro* results with *Trichoderma* treatments against *Alternaria* spp. In tests of the effect of *T. viride* and *T. harzianum* strains against *A. alternata*, percentages of inhibition of the pathogen were 75.04% and 67.83%, respectively, in wheat (Yassin et al, 2022). In sunflower, *T. harzianum* T22 and *Trichoderma* sp. demonstrated that non-autoclaved 75 and 50% concentrations and undiluted (100%) autoclaved non-volatile cellular metabolites from *Trichoderma* sp. had the highest inhibitory effect of the pathogen (Arzanlou et al., 2013).

In comparison to the tested fungicide, *T. harzianum* demonstrated a significant reduction in onion purple blotch disease with a 73.12% inhibition rate as a biocontrol agent, according to *in vitro* study on onion culture that tested strains of *T. harzianum* and *T. longibrachiatum* against *A. porii* (Abo-Elyousr et al., 2013).

Six *Trichoderma* isolates were tested for their capacity to impede the growth of *A. alternata* mycelium to assess their effectiveness in treating tomato leaf spot disease. Notably, isolate TRI07 (Tri 1) of *T. afroharzianum* was the most successful in inhibiting the pathogen (76.66%), followed by Tri 2 (75.18%), Tri 5 (74.44%), Tri 6 (73.33%), and Tri 3 (71.11%). Tri 4 affected *A. alternata* the least (68.51%) (Philip et al., 2024).

Trichoderma species isolated from the pepper rhizosphere, *T. viride* and *T. harzianum*, have been discovered in other research to have an inhibitory effect on *A. alternata*. As a result, *T. viride* was found to reduce *Alternaria* growth by 70.10%, while *T. harzianum* was found to do so by 67.90% (Mandlik et al., 2022).

In vivo experiment to evaluate antagonistic activity and plant growth promotion capacity

In vivo, the sudden alternation of wet and dry periods during the vegetation period and the presence in the soil of the *Alternaria* sp. inoculum, have led to the appearance of the disease in the potato crop. The symptoms of the disease produced by *Alternaria* spp. were characterized by black congested necrotic lesions with typical concentric rings that gradually grow and cover the surface of the leaves (Figure 2).

Figure 2. Symptoms of the disease produced by *Alternaria* spp. in potato plants

At 15 days after planting, the results showed a 100% emergence percentage of the V3 sample (*Alternaria*-Tv20), followed by the V4 sample (*Alternaria*-Td85) with a sprouting percentage of 77.77% and the V1 sample (healthy control) with a sprouting percentage of 66.66% and the V2 sample (positive control *Alternaria*) with a sprouting percentage of 55.55% (Figure 3 - a). The highest growth was observed in potato plants treated with the Tv20 strain, reaching an average height of 4.57 cm, compared to the healthy control plants, which had an average height of 1.57 cm, highlighting its role as a plant growth stimulator. The Td85 strain also exhibited growth-promoting effects, though to a lesser extent, with an average plant height of 2.52 cm. The positive control sample inoculated with *Alternaria* sp. had an average height of 1.87 cm, indicating that under artificial inoculation conditions, *Alternaria* sp. did not negatively impact plant height compared to the healthy control. This may be attributed to the fact that during the early growth stages, the pathogen's development was limited due to unfavorable climatic conditions (Figure 3 - b).

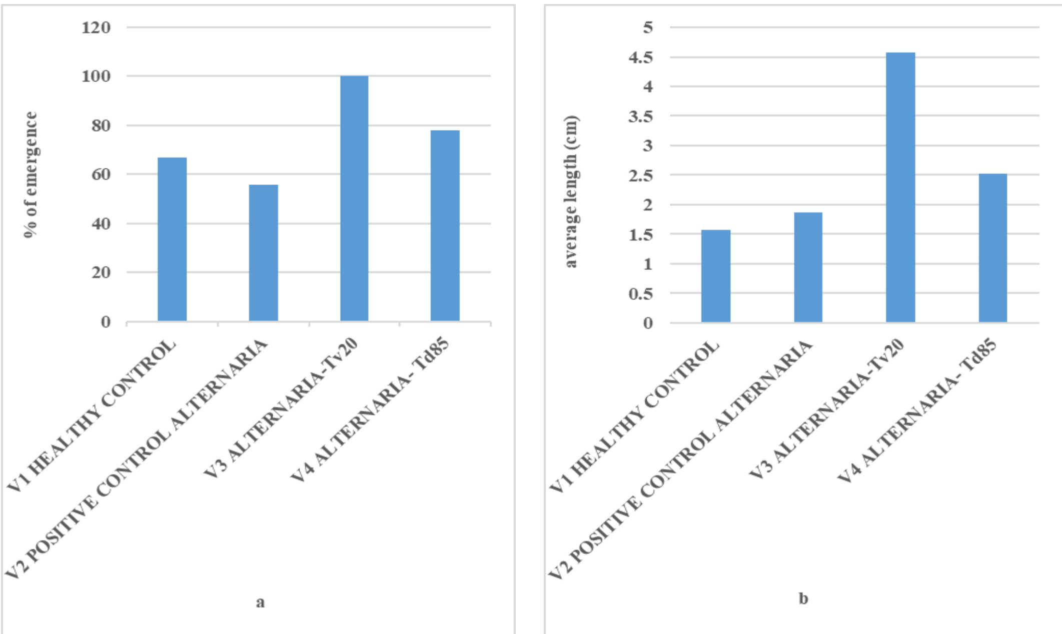


Figure 3. Effect of *Trichoderma* sp. on (a) percentage of emergence and (b) average length of potato plants, after 15 days

We note that the samples treated with *Trichoderma* strains help to accelerate the emergence by giving them a growth advance in vegetation, which comes with several advantages, especially resistance to the attack of weeds and diseases produced by pathogens (Essah & Honeycutt, 2004) (Figure 4).

V1 HEALTHY CONTROL	V2 POSITIVE CONTROL *ALTERNARIA*	V3 *ALTERNARIA*-Tv20	V4 *ALTERNARIA*- Td85
			

Figure 4. The emergence of potato plants following treatment with *T. viride* (Tv20) and *T. asperellum* (Td85) in soil already inoculated with *Alternaria* sp.

At 35 days after planting, growth parameters revealed that plants treated with the Tv20 strain developed the highest number of shoots, which were lighter but longer (Figure 5 – a,b,c). The average shoot weight in the Tv20-treated sample was 9.98 g, while the Td85-treated sample exhibited an average shoot weight of 17.96 g. In contrast, the healthy control plants had an average shoot weight of 12.78 g.

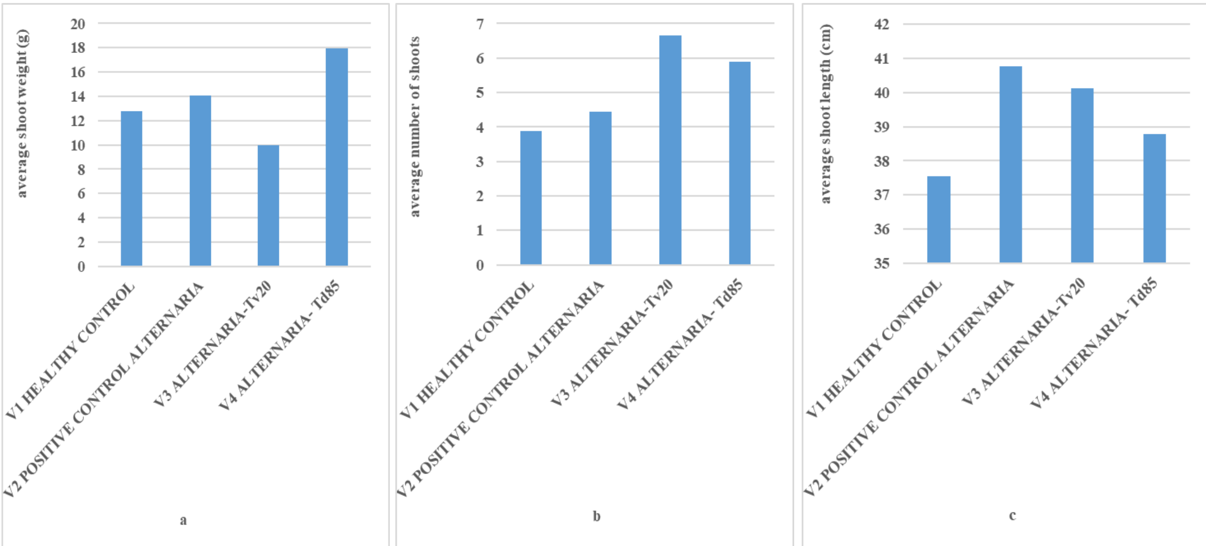


Figure 5. Effect of *Trichoderma* sp. on growth parameters, 35 days after planting: a) average shoot weight; b) average number of shoots; c) average shoot length

The quantification of the attack at 35 days indicates that the highest attack frequency was recorded in the V2 sample, inoculated only with *Alternaria* sp., reaching 69.33%, followed by the untreated control V1 sample, which had an attack frequency of 59.83%. In the treated samples, disease frequency was lower, measuring 58.74% for *Trichoderma* Tv20 and 57.27% for *Trichoderma* Td85.

The highest degree of attack, based on attack intensity, was observed in the V2 control sample inoculated only with *Alternaria* sp., with a percentage of 1.08%, followed closely by the V1 control sample, which had an attack percentage of 1.06%. In contrast, the biologically treated samples exhibited lower attack rates, with 1.04% for those treated with the Tv20 strain and 0.98% for those treated with the Td85 strain (Figure 6- a, b, c).

The results show a higher efficacy of the Td85 strain, compared to the protective effects offered to plants by the Tv20 strain against the pathogen taken in the study.

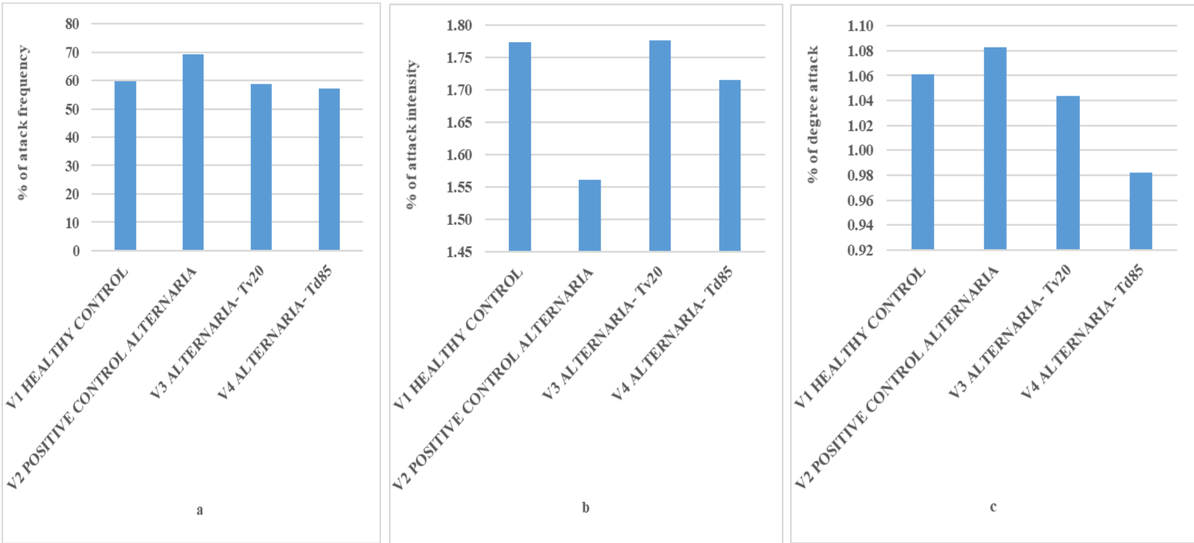


Figure 6. Assessment of the damage caused by *Alternaria* spp. on potato plants, 35 days after planting: a) attack frequency; b) attack intensity; c) degree of attack

At 50 days after planting, the monitoring of the growth parameters revealed that the plants in the sample treated with the Td85 strain were the best developed, with an average plant height of 38.78 cm and an average weight of 21.40 g. This is followed by the sample treated with the Tv20 strain with values of 36.67 cm and 20.94 g. The lowest values were observed in the sample infected with *Alternaria* sp., with values of 33.67 cm and 20.77 g (Figure 7- a, b).

Following the evolution of the treated samples it is observed that at the begining of vegetation, the plants treated with Tv20 had a faster emergence, a high number of shoots and higher height, which gives the biological strain the capacity to stimulate plant growth With the onset of the attack of *Alternaria* spp., the Td85 strain offered potato plants high protection against the pathogen, through an increased effectiveness of the treatment, which subsequently led to a better development of the plants in vegetation. The Tv20 strain also protected against the attack of the pathogen *Alternaria* spp., but with a lower efficacy than the other strain tested.

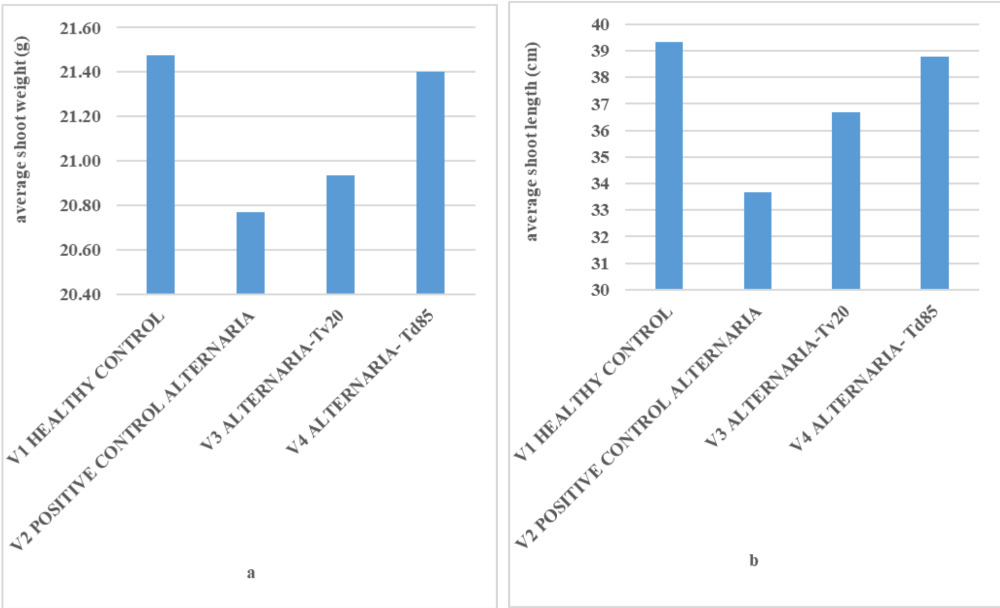


Figure 7. Evaluation of growth parameters, 50 days after planting: a) average shoot weight; b) average shoot length

The quantification of the attack at 50 days shows a high degree of attack on the control sample and *Alternaria* sample, with percentages of 1.88% and 2.10%, respectively. The lowest degree of attack was presented by the sample treated with the Td85 strain, with a percentage of 1%, followed by the sample treated with the Tv20 strain, with a percentage of 1.51% (Figure 8- a, b, c).

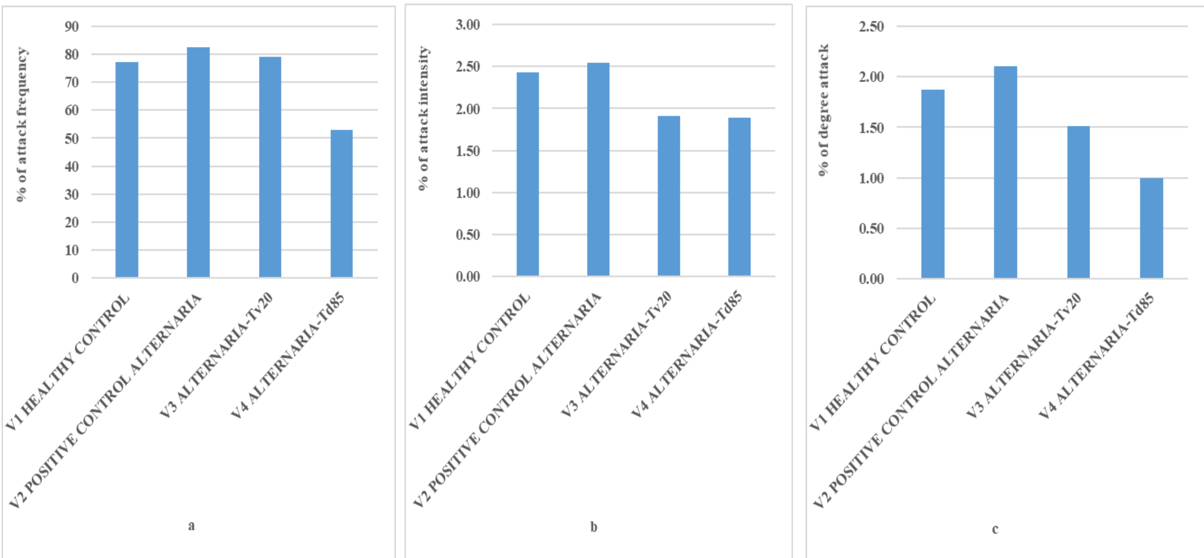


Figure 8 - Evaluation of the attack produced by *Alternaria* spp. on potato plants, 50 days after planting: a) attack frequency; b) attack intensity; c) degree of attack

In vivo, various studies have highlighted the beneficial effect of treatments with different strains of *Trichoderma* on different crops, both in combating pathogens and the plant growth process.

Strains of *T. pseuokoningii*, *T. virens*, and three strains of *T. harzianum* were evaluated for their ability to control *Alternaria tenuis* in pepper plants. The effectiveness of these strains was assessed both individually and in various combinations to combat the pathogen. The seeds treated with *T. harzianum* IMI-392432 (T4) exhibited the highest germination rate (85.56%) and a vigor index of 569.67, whereas the lowest values were found in the treatment with the pathogen alone (T1). These findings indicate that *T. harzianum* could enhance chili seed germination (Begum et al., 2010).

Research carried out in apple cultivation demonstrated that applying 6S-2 *Trichoderma* fertilizer to replanted soil enhanced fruit biomass development and elevated the bacterial-to-fungal ratio in the soil. Specifically, it raised the relative abundance of *Trichoderma, Bacillus*, and *Streptomyces* while decreasing the relative abundance of the detrimental *Fusarium* (Wang et al., 2022).

In a study on potatoes, tubers cultivated with a mix of three beneficial microbes demonstrated a significant reduction in disease occurrence and early blight symptoms compared to control plants infected with *A. solani* under greenhouse conditions. The lowest disease rate (10.61%) was found in the plants treated with a combination of *T. viride, B. subtilis,* and *P. fluorescens*, followed by plants treated with *T. viride* and *P. fluorescens* alone, which exhibited a disease rate of 20.93%. In contrast, control plants inoculated with pathogens displayed the highest disease incidence (Kumar et al., 2023).

Chlorophyll content is one of the internal factors that influence photosynthesis and at the same time, plant development. When the *Alternaria* attack occurs on potato plants it was observed that the photosynthetic activity decreases in the attacked plants compared to the healthy plants (Perseca et al., 1970) (Figure 9 - a, b). The attack of the pathogen influenced the two measurements, at 35 days when the attack was incipient, the chlorophyll content was higher than at 50 days when the measurements showed a lower chlorophyll content. The treatments with the two strains of *Trichoderma* positively influenced the content of assimilating pigments (total chlorophyll) of the V3 and V4 samples in the presence of the phytopathogen *Alternaria* spp. both 35 days and 50 days after planting.

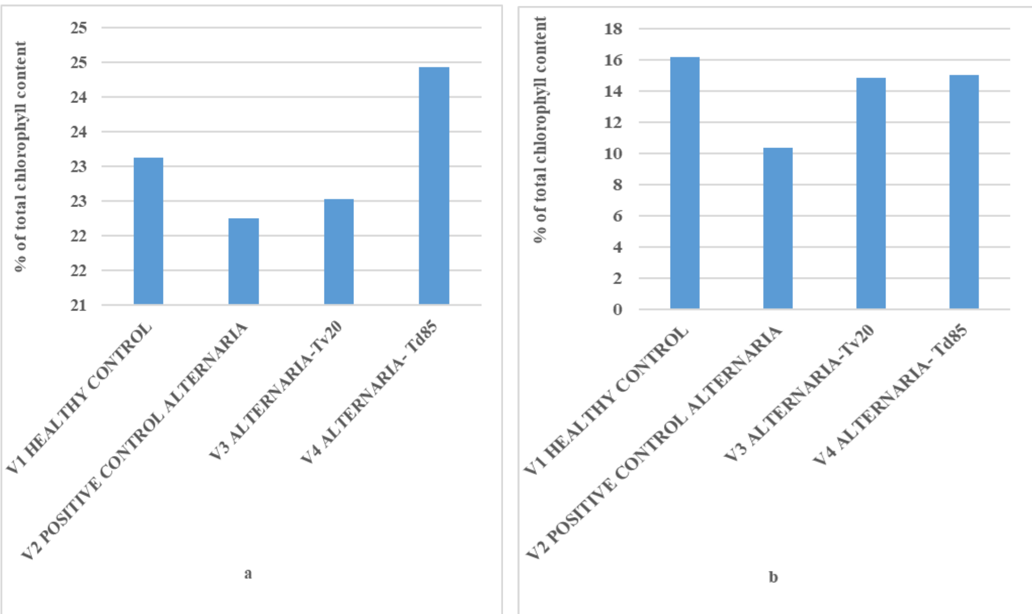


Figure 9. Total chlorophyll content at (a) 35 days after planting and (b) 50 days after planting

CONCLUSIONS

In vitro study showed that the *T. asperellum* (Td85) strain has a high antagonist potential against *Alternaria* sp., with an inhibition percentage of 63.39%. The *T. viride* (Tv20) strain has an inhibition percentage of 50.98%, having a medium antagonistic potential.

The *in vivo* study revealed that the efficacy of biological treatments with *T. viride* (Tv20) and *T. asperellum* (Td85) strains was lower than in the *in vitro* study. This discrepancy may be attributed to the influence of microorganisms naturally present in the potting soil, which could have interacted with both the antagonist and the pathogen, altering their activity and reducing the effectiveness of the treatments.

The study emphasizes the pronounced ability of *T. viride* (Tv20) to stimulate plant growth through rapid emergence and good development of shoots in vegetation, compared to the untreated control, supported by the increased values of total chlorophyll.

Studies carried out in pots, have highlighted the increased efficacy of the *T. asperellum* strain (Td85) compared to the *T. viride* strain (Tv20), in the biological control of the phytopathogen *Alternaria* spp., results supported by the low values of frequency and degree of attack.

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