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Original paper

Influence of hydrolyzed collagen and thyme oil on tomato seed germination and their use in controlling Alternaria alternata f.sp. lycopersici

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Abstract

This study presents some results regarding the treatment of *Lycopersicon esculentum* seeds with hydrolyzed collagen and thyme oil in different concentrations, in order to assess germination. Also, to evaluate the *in vitro* activity of collagen combined with antifungal substances on the tomato seeds infected with *Alternaria alternata* f.sp. *lycopersici*, this paper emphasizes the importance of using alternative non-polluting methods as essential oils in plant pathogens management.

A control (untreated) and experimental (pre-treated and treated) lots were used, and the observations focused on the development of the pathogen, as well as the occurrence of the necrosis and alternariosis symptoms.

Overall, three *in vitro* experiments were performed, as follows; seed germination; fungus prevention; infected seed treatment. In terms of growing, collagen alone delayed the germination of the tomato seeds, acting as a film on the seed surface. On the other hand, the mixture of collagen (stock solution and 50%) and thyme oil (0.5 ml, 0.25 ml respectively) inhibited germination. As for the preventive stage, the mixture of collagen and thyme oil 1 ml remained on the tomato seed surface, preventing the infection. Finally, the treatment of infected seeds with collagen and thyme oil 0.1 ml did not prevent the development of the pathogen, which was evidenced by the occurrence of necrotic radicles in tomatoes.

Keywords Hydrolyzed collagen, thyme oil, *Alternaria alternata*, tomato seeds.

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Introduction

Worldwide annual plants such as vegetables are affected by *Alternaria* diseases, with visible symptoms on their stems, leaves, flowers or fruit (A. ROCCO and L.M. PÉREZ [24]).

The long persistence of chemical products in soil and plants, the emergence of new pathogenic races with increased resistance to fungicides and environmental pollution are only several factors that have helped finding alternative strategies to control plant diseases.

Considering public concern about the use of pesticides in plant protection and the frequent occurrence of pesticide resistance, numerous researchers have investigated various compounds such as sodium bicarbonate (M. IVANOVIC & al [13]) or chitosan (B.H. OWNLEY and C.Y. HAMILTON [17]) as fungal biocompatible alternatives to plant disease control.

This paper presents some results regarding the treating of tomato seeds with hydrolyzed collagen and thyme oil in order to observe germination, suggesting the reduction of pesticides by using alternative non-polluting methods in pathogens management.

The presence of the micromycete *Alternaria* spp with a high incidence has been reported on seeds (L. M. BERCA and S. CRISTEA [2], M. S. CRISTEA (MANOLE) & al [5, 6], E.S MARDARE & al [15]). It was also noted that this fungus produces different brown spots on stems, petioles and leaves, which unite and form large necrotic areas (S. CRISTEA [4]).

The fungal pathogen *Alternaria alternata* f.sp. *lycopersici* has been the subject of concerns in many countries due to the fact that, so far, the theoretical and experimental results have not fully solved the problems related to the impact of these pathogens on crops (K.P. AKHTAR & al [1]; D. TSITSIGIANNIS & al [28]).

In *Lycopersicon esculentum*, the pathogen *Alternaria alternata* secretes a toxin responsible for leaf necrosis (V. PRASAD and R.S. UPADHYAY [22]). Numerous data from scientific literature provide recommendations on the biological and chemical control of *Alternaria alternata* f.sp. *lycopersici*. However, it is increasingly an issue of maintaining their efficiency and detecting alternative products that effectively prevent and control these pathogens (C. PANE & al [18]).

Several research papers suggested the use of botanical oils against *Alternaria* species in different concentrations *in vitro* and *in vivo* (W. FENG & al [8]; M. CĂLIN & al [7], A. PERELLÓ & al [20], A. PÉREZ-GONZÁLEZ & al [21]; T. PARVEEN and K. SHARMA [19], E. SĂNDULESCU & al [26]). Moreover, the fungicidal effect of the thyme essential oil against pathogens of tomatoes was well documented (I. HADIZADEH & al [9]; N.S. EL-MOUGY & al [16]). As for the hydrolyzed collagen, its biostimulant role in plant metabolism is already known (C. SÎRBU & al [27]).

The aims of this study were: (1) to assess the biocontrol activity of collagen and its effect on germination – alone or in combination with thyme oil of different concentrations; (2) to evaluate the *in vitro* activity of collagen combined with antifungal substances on the tomato seeds infected with *Alternaria alternata* f.sp. *lycopersici*.

Materials and Methods

Observations focussed on both tomato seed germination and seedling emergence in the control (untreated) and experimental (treated) lots, together with the occurrence of necrosis and alternariosis symptoms due to the development of the *Alternaria alternata* f.sp. *lycopersici* pathogen (C. RAICU and D. BACIU [23]).

1. Plant material and pathogenic fungi

This study used tomato seeds (*Lycopersycon esculentum*) from the Buzău 47 cultivar.

The fungus *Alternaria alternata* f.sp. *lycopersici* was isolated directly from various diseased tomato fruit. Inoculation was performed by removing small mycelium portions from the areas affected by pathogens; the detached mycelium was placed on a potato dextrose agar (PDA) culture medium. The influence of abiotic factors on the development of *Alternaria* species under laboratory conditions has been depicted before by E.Ş MARDARE & al [14].

Incubation was performed in the dark in the incubator at 22°C for 10 days; the developed colonies were purified by passing on another vessel with sterile culture medium. Isolates were identified based on sporal morphology, subsequently repaired and stored in pure cultures at 22°C (O. CONSTANTINESCU [3], A. HULEA [10]).

2. Biostimulator and antifungal products

The essential thyme oil (*Thymus vulgaris*: Lamiaceae) provided by Solaris was tested as a fungicide alternative in different concentrations: 0.5 ml, 0.25 ml, 0.1 ml (these volumes were diluted up to 10 ml). Hydrolyzed collagen Peptan type in two variants (stock solution and 50% – half of the stock solution concentration) was used both in germination and to evaluate its influence on prevention and treatment of the pathogen fungus (alone or in combination with thyme oil).

3. *In vitro* experiments

The three experiments (seed germination; fungus prevention; infected seed treatment) were tested through a protocol used by A. HULEA & al [11], C. RAICU and D. BACIU [23] and E. SĂNDULESCU and T. ŞCHIOPU [25], also recommended by the International Seed Testing Association. The seeds were placed between the folds of blotting paper moistened with sterile distilled water, followed by their incubation at temperatures between 22-24°C for 6-9-12 days.

3.1. Seed germination

Overall, 100 tomato seeds for collagen stock solution and 100 tomato seeds for collagen solution 50% were washed with sterile distilled water and dried in an oven using the blotch test method. They were subsequently moistened by immersion in collagen – stock solution and 50%, respectively, for 15 minutes.

In the second experiment carried out on germination, the same number of seeds underwent the above-mentioned protocol. This time they were immersed in a mixture of collagen stock solution-thyme oil and collagen 50% solution-thyme oil, respectively. Each type of experiment was prepared in three rehearsals (M. CĂLIN & al [7]).

The control group was composed from 100 seeds for each rehearsal, being washed with sterile distilled water and then dried. Observations were performed in dynamics (six, nine and twelve days) and followed seed germination and the emergence of tomato plantlets.

3.2. Assessing the prevention and treatment of fungal pathogen

Pre-treatment. For each of the three rehearsals, 200 seeds (100 for collagen – stock solution and 100 for collagen 50%) were disinfected with 70% ethyl alcohol, washed with sterile distilled water, dried in the oven and moistened by immersing them for 15 minutes in a mixture of collagen stock solution + thyme oil, 0.1 ml and collagen 50% + thyme oil 0.1 ml, respectively. Afterwards, the seeds were artificially contaminated by bathing in a calibrated spore suspension ($5*10^6$ spores/ml) for one hour and incubated at 22ºC.

Treatment. For each of the three rehearsals, 200 seeds (100 for collagen – stock solution and 100 for collagen 50%) were disinfected with 70% ethyl alcohol, washed with sterile distilled water, dried in the oven and artificially contaminated by bathing in a calibrated spore suspension ($5*10^6$ spores/ml) for one hour. The seeds were subsequently moistened by immersing them for 15 minutes in a mixture of collagen stock solution + thyme oil, 0.1 ml and collagen 50% + thyme oil 0.1 ml, respectively. The next step was represented by the incubation of infected tomato seeds at 22°C.

The seedlings development was scored by assessing the development stages, using a 0-3 grading scale (0, no germination; 1, root emergence; 2, cotyledon emergence; 3, well-developed seedling). The exhibited disease symptoms were evaluated by the necrosis index, using a 0-9 grading scale (0, no disease symptoms; 1, 1-2 mm necrosis on seedling stem; 3, extension of necrosis all around the stem; 5, a third of the radicular system or hypocotyl affected; 7, the whole radicular system or aerial part affected and covered with spores; 9, seedling killed and covered with spores) (B. IACOMI VASILESCU & al [12]).

4. Statistical analysis

The analysis of variance (ANOVA) was performed for the obtained data. The symbols "o, oo, ooo" were used to describe statistically difference between the control group and the experimental group: negatively significant, negatively distinct significant and negatively very significant, respectively.

Results and Discussions

1. First experiment: the influence of the treatment with collagen (stock solution, collagen 50%) on seed germination

In the control group, 60% of the tested seeds germinated on the sixth day of experiment, while 100% of germination process was noticed in the ninth day (Table 1, Figure 1).

Figure 1. Control group – Day 9

In the two variants of collagen concentrations (stock solution and 50%), results showed 50% germination when stock solution was used, and 70% when 50% collagen was used.

It was noticed that both concentration variants presented negatively significant differences (collagen 50%) and negatively distinct significant differences (collagen stock solution) in the 6^{th} day. Very significant differences between the control groups were noticed on the 9^{th} and the 12^{th} day (Table 1).

The computed correlation coefficient (r= -0.9933) and linear regression (R^2=0.9868) showed that seed germination was strongly negative influenced by the presence of collagen on the seminal skin of tomato seeds (Figure 2).

Table 1. Influence of collagen treatment on seed germination (No. pl. = number of tomato plantlets; Sign. = significance; Diff. = difference between control group and experimental group; % = percentage of germination; DF = degrees of freedom)

Experimental variants	Day 6				Day 9				Day 12			
	No.pl.	Diff.			No.pl.	Diff.			No.pl.	Diff.		
		Pl.	%	Sign.		Pl.	%	Sign.		Pl.	%	Sign.
Control group	60	-	100	-	100	-	100	-	100	-	100	-
Collagen 50%	50	-10	83.33	O	70	-30	70.00	ooo	70	-30	70.00	ooo
Collagen stock solution	40	-20	66.67	Oo	50	-50	50.00	ooo	50	-50	50.00	ooo
DF 5%		6.55				3.07				3.07		
DF 1%		10.84				5.09				5.09		
DF 0,1%		20.29				9.52				9.52		

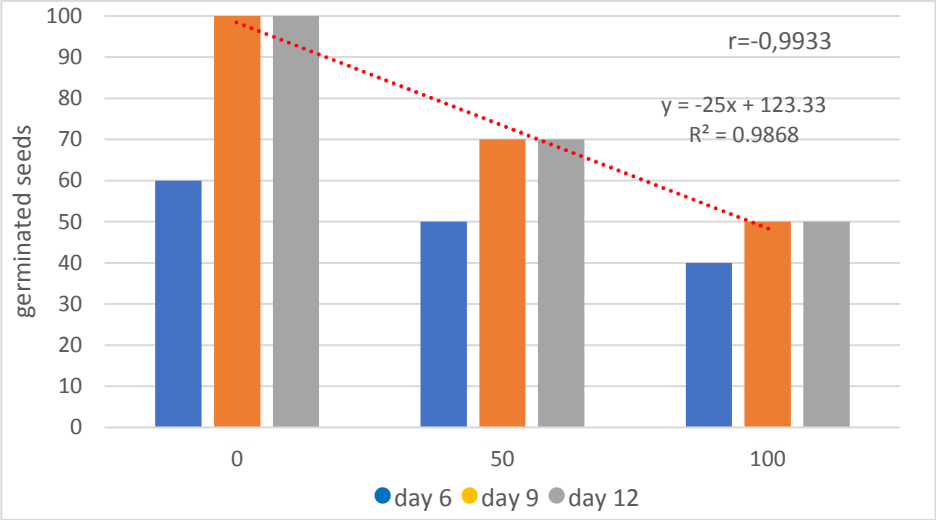

Figure 2. Influence of collagen treatment on seed germination and tomato breeding.

Hydrolyzed collagen delayed the germination of the tomato seeds, acting as a film on their surface. According to our observations, that effect was evident from the 9th day of germination.

2. Second experiment: the influence of treatment with the mixture of collagen (stock solution, 50%) and thyme oil in different concentrations on seed germination

2.1. The influence of treatment with the mixture of collagen and thyme oil 0.5 ml on seed germination

The results regarding the control group indicate that a 60% percentage of germination was noticed in the 6th day of observations, compared with the collagen-treated seeds in the experimental variants. The latter germinated in rates between 40% (collagen stock solution) and 50% (collagen 50%) and seeds treated with the mixture of collagen and thyme oil 0.5 ml, which inhibited seed germination.

On the 9th day, the seeds in the control group germinated at a rate of 100% and germination percentages were obtained in both experimental variants, 70% (collagen 50%) and 50% (collagen stock solution), respectively.

On the 12th day, observations showed that germination did not continue, but remained at the values of the 9th day reading, with very significant negative differences in all the analysed variants, compared with the control group (Table 2).

In the collagen stock solution, the germination rate was 50% and in the collagen 50% the germination percentage was 70% (Table 2, Figure 3). It can be noticed that hydrolyzed collagen delayed the germination of the tomato seeds, acting as a film on their surface. This effect was evident from the 9th day of germination.

The mixture of collagen (in both variants of concentrations) and thyme oil (0.5 ml) inhibited the germination of the tomato seeds. Collagen delayed seed germination and 0.5 ml thyme oil inhibited the process.

The computed correlation coefficient (r= -0.9717) and linear regression (R^2=0.94) indicated that seed germination was strongly negative influenced by both collagen (stock solution or 50%) and thyme oil 0.5 ml on the seminal skin of the tomato seeds (Figure 3).

Table 2. Influence of treatment with collagen (stock solution, 50%) and thyme oil 0.5 ml mixture on germination of tomato seed (No. pl. = number of tomato plantlets; Sign. = significance; Diff. = difference between control group and experimental group; % = percentage of germination; DF = degrees of freedom)

Experimental variants	**Day 6**				**Day 9**				**Day 12**			
	No. pl.	Diff.			**No. pl.**	Diff			**No. pl.**	Diff.		
		Pl.	**%**	Sign.		Pl.	%	Sign.		Pl.	%	Sign.
Control group	60	-	100	-	100	-	100	-	100	-	100	-
Collagen 50%	50	-10	83.33	ooo	70	-30	70.00	ooo	70	-30	70.00	ooo
Collagen stock solution	40	-20	66.67	ooo	50	-50	50.00	ooo	50	-50	50.00	ooo
Colagen stock solution + thyme oil 0.50 ml	0	-60	100	ooo	0	-100	100	ooo	0	-100	100	ooo
Collagen 50% +thyme oil 0.50 ml	0	-60	100	ooo	0	-100	100	ooo	0	-100	100	ooo
DF5%	2.77				2.35				2.35			
DF 1%	4.02				3.42				3.42			
DF 0.1%	6.03				5.12				5.12			

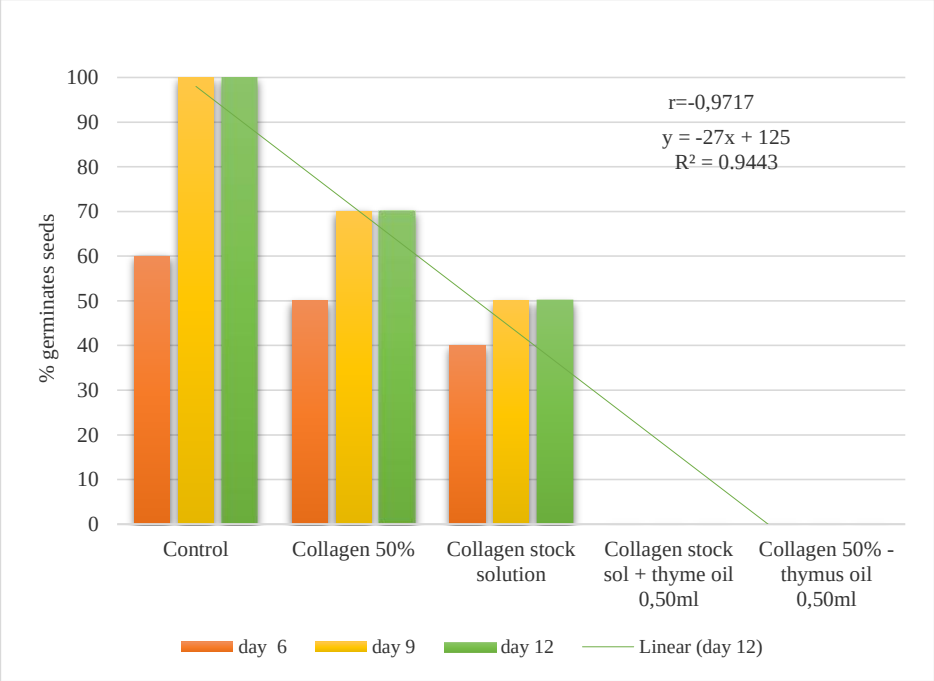

Figure 3. Influence of treatment with collagen (stock solution, 50%) and thyme oil 0.5 ml mixture on seed germination.

2.2. The influence of treatment with the mixture of collagen and thyme oil 0.25 ml on seed germination

On the 6th day of observations, germination in a percentage of 60% was found in the seeds belonging to the control group, compared with the seeds treated in collagen variants. The latter germinated in rates between 40% (collagen stock solution) and 50% (collagen 50%). The seeds treated with the mixture of collagen and thyme oil (0.25 ml) germinated in a percentage of 10%.

On the 9th day, the germination of the tomato seeds was 100% in the control group, 70% in the seeds treated with collagen 50%, and 50% in the seeds treated with collagen stock solution. In the variants where the mixture of collagen (stock solution or 50%) and 0.25 ml thyme oil was used, the seeds germinated only in percentages of 12% and 18% respectively, when the combination of substances inhibited germination by 88% and 82%, respectively.

On the 12th day, all seeds from the control group germinated, while in the collagen variants, the percentages of germination were 50% and 70%, in stock solution and collagen 50%, respectively. Collagen delayed the germination of the tomato seeds, acting as a film on their surface. According to our observations, this effect was evident from the 9th day of germination (Table 3, Figure 4).

On the 12th day, the observations showed that germination did not continue but remained at the values of the 9th day readings in all variants. The differences were negative, very significant compared to the control group, except for the collagen 50% + 0.25 ml thyme oil, where germination reached between 18% and 20% of the seeds (Table 3).

Table 3. Influence of collagen (stock solution, 50%) and thyme oil 0.25 ml mixture on the germination of tomato seed (No. pl. = number of tomato plantlets; Sign. = significance; Diff. = difference between control group and experimental group; % = percentage of germination; DF = degrees of freedom)

Experimental variants	Day 6				Day 9				Day 12			
	No. pl.	Diff.			No. pl.	Diff.			Nr pl	Diff.		
		Pl.	%	Sign.		Pl.	%	Sign.		Pl.	%	Sign.
Control group	60	-	100	-	100	-	100	-	100	-	100	-
Collagen 50%	50	-10	83.33	Ooo	70	-30	70.00	ooo	70	-30	70.00	ooo
Collagen stock solution	40	-20	66.67	Ooo	50	-50	50.00	ooo	50	-50	50.00	ooo
Collagen stock solution + thyme oil 0.25ml	10	-50	16.17	Ooo	12	-88	12.00	ooo	12	-88	12.00	ooo
Collagen 50% + tyme oil 0.25ml	10	-50	16.17	Ooo	18	-82	18.00	ooo	20	-80	20.00	ooo
DF 5%	3.75				2.15				1.89			
DF 1%	5.45				3.13				2.79			
DF 0.1%	8.18				4.69				4.12			

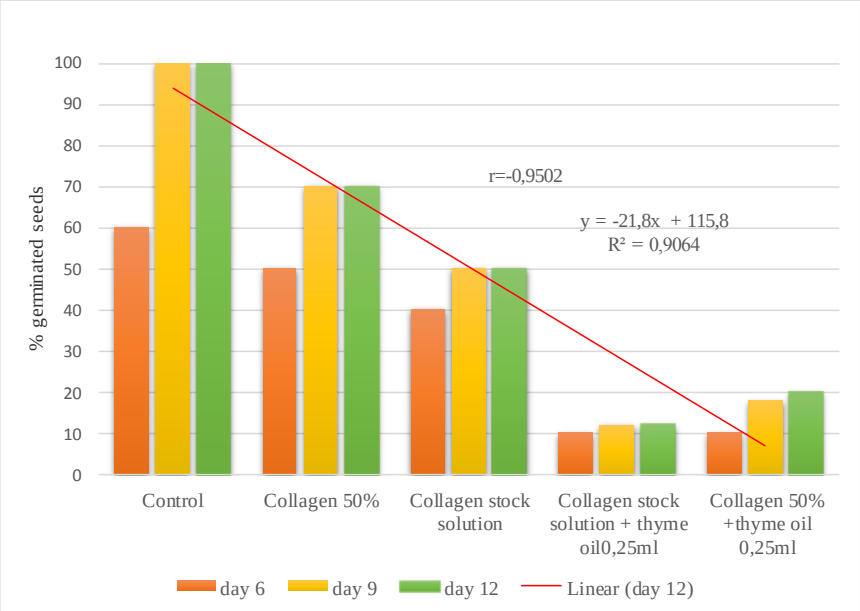

Figure 4. Influence of collagen (stock solution, 50%) and thyme oil 0.25 ml mixture on seed germination.

The computed correlation coefficient (r= -0.9502) and linear regression (R^2=0.90) indicated that the germination of the tomato seeds was strongly influenced by the presence of collagen (stock solution, 50%) and 0.25 ml thyme oil on the seminal skin (Figure 4).

The mixture of collagen (in both concentration variants) and thyme oil (0.25 ml) inhibited seed germination. Thus, on the 12th day, seeds germinated in percentages of 12% and 20% in the variants of stock solution mixed with thyme oil, and in the collagen 50% mixed with thyme oil, respectively.

Collagen delayed tomato seed germination and 0.25 ml thyme oil inhibited the process in rates of 88% in the variant treated with collagen stock solution (only 12 germinated seeds) and 80% in variant collagen 50%, (only 20 germinated seeds), respectively.

2.3. The influence of treatment with the mixture of collagen and thyme oil 0.1 ml on seed germination

Observations showed that the control group had high germination rates of 60% and 100%, on the 6th day, and on the 9th and 12th days, respectively.

The variants where the seeds were covered by the collagen film germinated in rates between 40% (collagen stock solution) and 50% (collagen 50%), while the seeds treated with the mixture of collagen (stock solution and 50%) and thyme oil 0.1 ml germinated at rates between 40% and 50%. It was found that on the 6th day, the addition of thyme oil (0.1 ml) to the stock solution and to the collagen 50%, did not cause any changes in the germination percentage.

On the 9th and 12th days, observations indicated that the computed germination values showed very significant negative differences compared with the control group. In the experimental variant collagen 50% stock solution + 0.1 ml thyme oil, germination was inhibited by 40%, while in the experimental variant collagen stock solution + 0.1 ml thyme oil, germination was inhibited by 50%.

Collagen delayed the germination of the tomato seeds, acting as a film on their surface, as noticed from the 9th day of germination (Table 4).

The analysis of the correlation coefficient (r = -0.76) and the linear regression (R^2 = 0.58) indicated that the collagen film (stock solution, 50%) with the addition of 0.1 ml thyme oil can have a slightly negative effect on the germination of the tomato seeds (Figure 5).

Table 4. Influence of collagen (stock solution, 50%) and thyme oil 0.1 ml mixture on germination of tomato seed (No. pl. = number of tomato plantlets; Sign. = significance; Diff. = difference between control group and experimental group; % = percentage of germination; DF = degrees of freedom)

Experimental variants	Day 6				Day 9				Day 12			
	No. pl.	Diff.			No. pl.	Diff.			No. pl.	Diff.		
		Pl.	%	Sign.		Pl.	%	Sign.		Pl.	%	Sign.
Control group	60	-	100	-	100	-	100	-	100	-	100	-
Collagen 50%	50	-10	83.33	Ooo	70	-30	70.00	ooo	70	-30	70.00	ooo
Collagen stock solution	40	-20	66.67	Ooo	50	-50	50.00	ooo	50	-50	50.00	ooo
Colagen stock solution + thyme oil 0.1 ml	40	-20	66.67	Ooo	50	-50	50.00	ooo	50	-50	50.00	ooo
Collagen 50% + thyme oil 0.1 ml	50	-10	83.33	Ooo	60	-40	40.00	ooo	60	-40	60.00	ooo
DL5%	3.96				2.68				2.68			
DL 1%	5.75				4.16				4.16			
DL 0.1%	8.63				6.24				6.24			

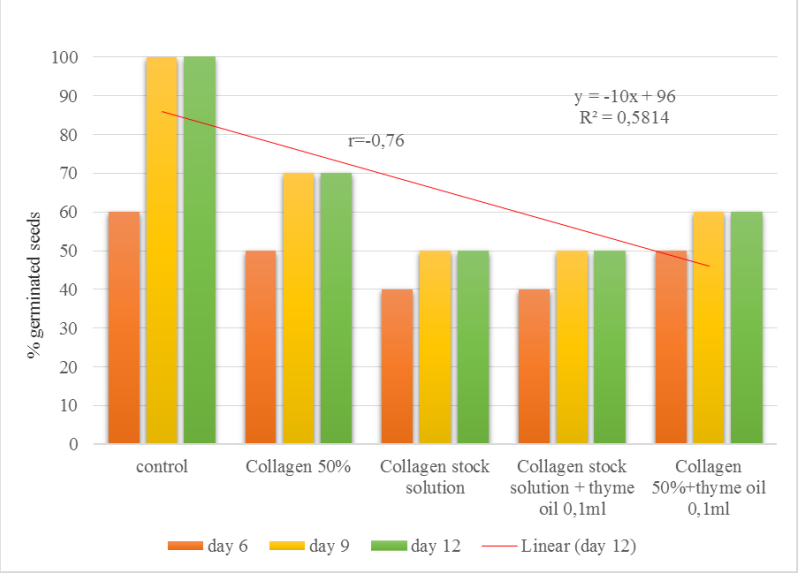

Figure 5. Influence of collagen (stock solution, 50%) and thyme oil 0.1 ml mixture on seed germination.

The collagen mixture (in both concentrations) and thyme oil (0.1 ml) inhibited the germination of tomato seeds. By comparing the results of the previous treatments, it can be noticed that thyme oil 0.1 ml did not inhibit germination. Hydrolyzed collagen, both in the stock solution and in the 50% variant delayed germination, as the data were similar to those previously obtained in first experiment when no thyme oil was used. Thus, germination was 50% in the experimental variant collagen stock solution + thyme oil (0.1 ml), and 60% in the variant collagen 50% + thyme oil.

It can be noticed that by increasing the concentration of thyme oil to 0.25 ml and 0.50 ml, respectively, seed germination is completely inhibited in stock collagen solution or 50% collagen solution (Figure 6).

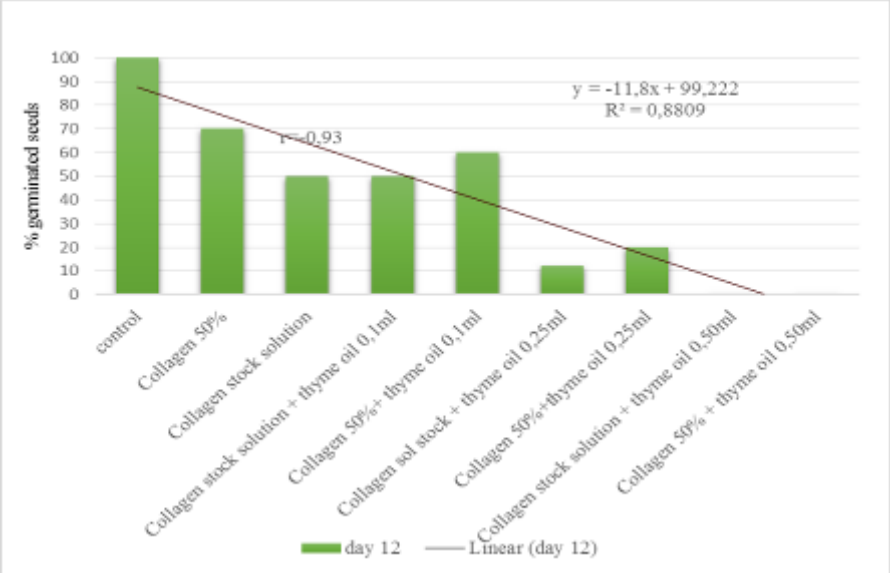

Figure 6. Influence of collagen and thyme oil mixture on seed germination.

The analysis of the correlation coefficient (r = -0.93) and the linear regression (R^2 = 0.88) indicated that the treatment with a simple collagen film (stock solution, 50%) and the addition of thyme oil (0.5 ml, 0.25 ml and 0.1 ml) can negatively influence the germination of the tomato seeds (Figure 6).

3.3. Third experiment: treatment effectiveness (preventive and curative) with collagen mixture (stock solution, 50%) and thyme oil 0.1 ml in control of pathogen *Alternaria alternata* f.sp. *lycopersici* in tomato

3.3.1. Preventive treatment

We supposed that the infection with *Alternaria* sp. can be prevented by washing the tomato seeds in alcohol and, to some extent, the collagen on the surface of the seeds. After immersion in spore suspension, the seeds germinated, while the mixture of collagen and thyme oil remained on the surface. Based on these data, we assumed that this was the result of the fine collagen film that was formed and remained on the seed skin (Table 5).

In the seeds of the control group, the presence of the pathogen *Alternaria alternata* f.sp. *lycopersici* on the seminal skin reduced the germination to a rate of 50%. On the 6th day, seeds presented a black mycelium. In the following days, when the radicles appeared, necroses were noticed. On the 12th day, the radicles and the hypocotyl of the tomato plants were necrotic, the cotyledons had necroses and the mycelium was black.

In the experimental variants preventively treated with collagen stock solution and collagen 50%, each mixed with thyme oil 0.1 ml, we assumed that alcohol was likely to soften seminal skin. This resulted in quick germination, before collagen film formation or resulting from the fact that collagen was removed to some extent after seed immersion in spore suspension.

On the 12th day of observation germination rate was 100% in the variant with collagen stock solution and 0.1 ml thyme oil (Figure 7). The radicles developed well, presenting a long hypocotyl, without necrosis on the cotyledons. The presence of the tested pathogen was not identified.

Table 5. Effectiveness of preventive treatment with collagen and thyme oil mixture for the control of the pathogen *Alternaria alternata* f.sp. *lycopersici* in tomato (GS= number of germinated seeds; Obs. = observations)

Experimental variants	**Day 6**		**Day 9**		**Day 12**	
	GS	**Obs.**	GS	**Obs.**	GS	**Obs.**
Control group	50	mycelium is present	50	**necrotic radicles**	50	radicles + necrotic hypocotyl; necrosis are present, black mycelium
Collagen stock solution + thyme oil 0.1ml	100	mycelium not developed	100	radicles emergence (3 cm), mycelium not developed	30 70	radicles + long hypocotyl; cotyledons are present, no necrosis or mycelium
Collagen 50% + thyme oil 0.1ml	100	mycelium not developed	100	radicles of 1.5-2 cm	100	cotyledons present, no necrosis or mycelium

In collagen 50% and 0.1ml thyme oil, on 6th (Figure 8), 9th (Figure 9) and 12th day (Figure 10), germination rate was 100%. The mycelium of the tested pathogen was not present and no necrosis occurred on the radicles or cotyledons.

Figure 7. Tomato seeds – *preventive* treatment with collagen stock solution and thyme oil mixture in controling the *Alternaria alternata* f.sp. *lycopersici* pathogen (Day 12).

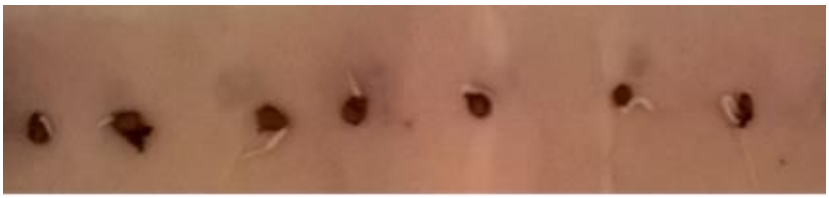

Figure 8. Tomato seeds – *preventive* treatment with collagen 50% and thyme oil mixture in controling the *Alternaria alternata* f.sp. *lycopersici* pathogen (Day 6).

Figure 9. Tomato seeds – *preventive* treatment with collagen 50% and thyme oil mixture in controling the *Alternaria alternata* f.sp. *lycopersici* pathogen (Day 9).

Figure 10. Tomato seeds – *preventive* treatment with collagen 50% and thyme oil mixture in controling the *Alternaria alternata* f.sp. *lycopersici* pathogen (Day 12).

The assessed development stages of the tomato plantlets in the preventive treatment are shown in Table 6. The mean value of 1.35 was reached on the 12th day. The necrosis index was 0.

Table 6. Development stages of tomato plantlets (preventive treatment)

Experimental variant	Development stages		
	6 days	9 days	12 days
Control group	0.5	0.5	0.5
Collagen stock solution + thyme oil	1	1	30 – 0.6 70 – 2.1 Mean 1.35
Collagen 50% + thyme oil	1	1	3

3.3.2. Curative treatment

We assumed that seed disinfection by alcohol, followed by their immersion in spore suspension of the pathogen *Alternaria* sp., led to the soaking of the seminal skin. Thus, the seeds germinated (Table 7, Figure 11) in a higher percentage (60% and 90%, respectively). However, collagen did not protect them by film formation, as showed by the occurrence of necrosis on the radicles and not only. For the control (untreated) group, where the seeds were infected only with the tested pathogen, the percentage of germination was reduced by 50%. The roots and the hypocotyl of the germinated seeds were necrotic and covered in a black mycelium. The radicles and the hypocotyl of the germinated seeds were necrotic and covered in a black mycelium.

On the 9th day, germination rate was 60% in the experimental variant treated with collagen stock solution (Figure 12). The tomato plantlets had necrotic radicles, with black mycelium.

On the 12th day, germination rate was 60% and the radicles were necrotic in the collagen stock solution. In the variant collagen 50%, in the 6th day, germination rate was 80% with necrotic radicles. In the variant collagen 50%, germination rate was 90% with necrotic radicles (Figure 13). The ungerminated seeds presented a developed mycelium.

The development stage of the tomato plantlets in the control group was 0.5 in all observations phases (Figure 14). For the tested variants, it reached the value of 1.2 in the collagen stock solution + thyme oil 0.1 ml and 2.3 in 50% collagen variant + thyme oil 0.1 ml.

The necrosis index increased from 2.5 on the 3rd day and 4.2 on the 12th day in the experimental variant collagen stock solution and 0.1 ml thyme oil. In the variant collagen 50% and 0.1ml thyme oil, the necrosis index of the tomato plantlets was 0.5 and 4.2 on the 3rd day and the 9th day, and 6.3 on the 12th of the observations (Figure 15).

Table 7. Effectiveness of curative treatment with collagen and thyme oil mixture for the control of the *Alternaria alternata* f.sp. *lycopersici* pathogen in tomato (GS = number of germinated seeds; Obs. = observations)

Experimental variants	Day 6		Day 9		Day 12	
	GS	Obs.	GS	Obs.	GS	Obs.
Control group	50	mycelium present	50	**necrotic radicles**	50	radicles and necrotic hypocotyl; black mycelium
Collagen stock solution + thyme oil 0.1ml	50	necrotic radicles, on half or at the top	60	necrotic radicles (1.5 cm)	60	30 with cotyledons (2.5 cm), necrotic radicles 30 germinated and necrotic
	10	not germinated, surrounded by mycelium				
Collagen 50% + thyme oil 0.1ml	70	germinated	20	germinated 0.5 cm	70	cotyledons present; necrotic radicles
	10	germinated with necroses	60	1.5 cm, necrotic radicles	20	necrotic radicles
					10	not germinated, with mycelium

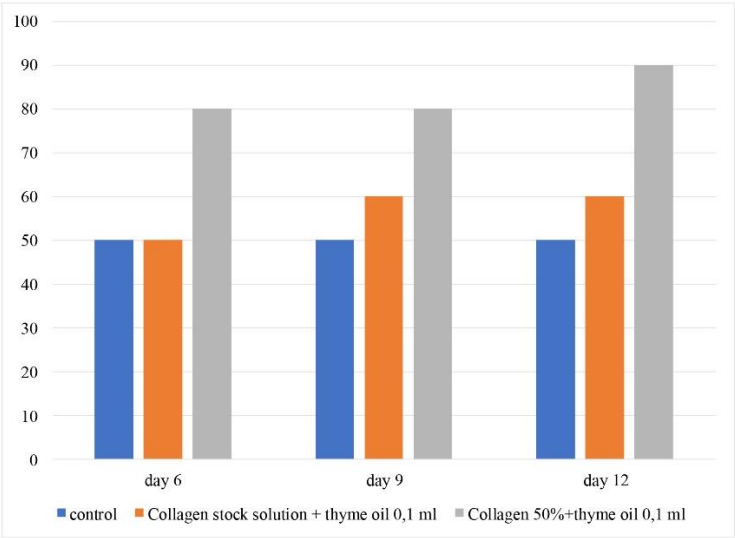

Figure 11. Effectiveness of curative treatment with collagen and thyme oil 0.1 ml mixture for the control of the *Alternaria alternata* f.sp. *lycopersici* pathogen in tomato.

A. **B.**

Figure 12. Left. Curative treatment with collagen stock solution and thyme oil mixture for the control of the *Alternaria alternata* f.sp. *lycopersici* pathogen in tomato (Day 9); Right. Control group.

Figure 13. Tomato seeds – curative treatment with collagen 50% and thyme oil mixture for the control of the *Alternaria alternata* f.sp. *lycopersici* pathogen in tomato (Day 12).

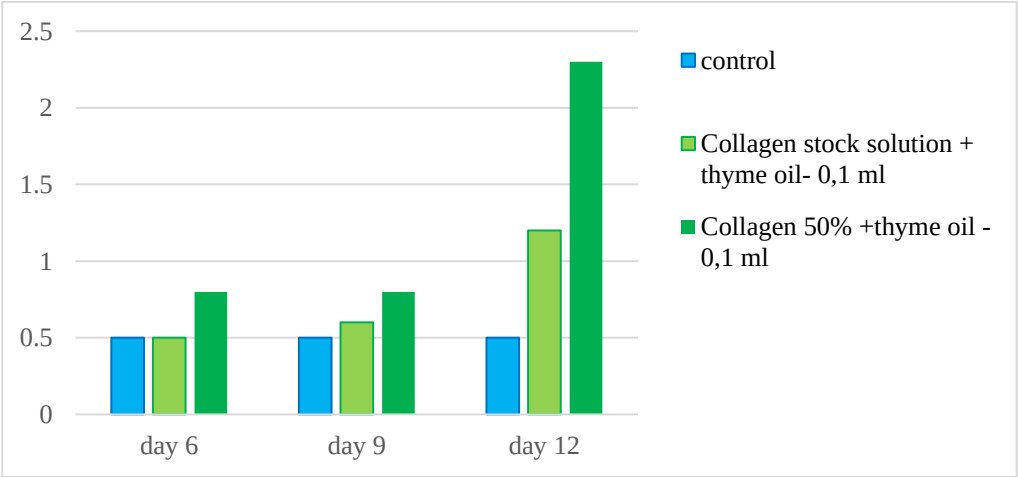

Figure 14. Development stages of tomato plants, curative treatment with the mixture of collagen 50% and thyme oil 0.1 ml.

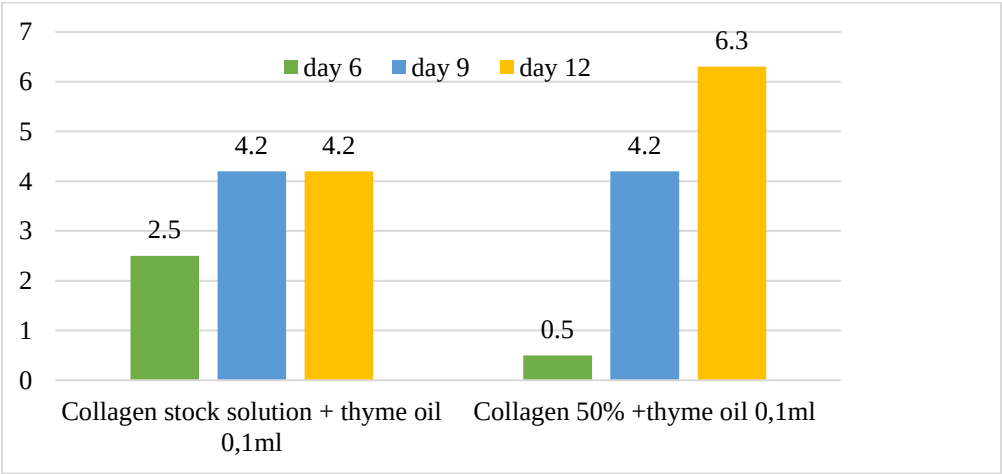

Figure 15. Necrosis index of tomato plantlets

Conclusions

Chemical fungicides are frequently used to provide protection against the pathogens of germinated seeds, despite their often toxicity and potential harmful effect on humans and the environment. Nevertheless, numerous biopesticides are available on the biocontrol agents market and their number continues to increase. In this paper we have developed a potential non-polluting alternative approach to the chemical products used in the control of the *Alternaria* pathogens.

The germination of the *Lycopersicon esculentum* seeds was strongly negatively influenced by the presence of collagen on their seminal skin, as it acted as a film on their surface.

The mixture of collagen (in both concentrations – stock solution and 50%) and thyme oil (0.5 ml, 0.25 ml respectively) inhibited the germination of the *Lycopersicon esculentum* seeds.

Thyme oil 0.1 ml did not inhibit or delay germination. Collagen in both variants recorded similar results to those obtained in the experiment that used no thyme oil.

Preventive treatments showed that the mixture of collagen and thyme oil 0.1 ml remained on the tomato seed surface, preventing the *Alternaria alternata* f.sp. *lycopersici* infection. Thus, it can be noticed that the findings of this study are similar to other previous works that have demonstrated the fungicidal effect of thyme essential oil against the tomato pathogens.

According to this study, it can be concluded that the mixture of collagen and thyme oil has an antifungal activity that can be potentially used in the control of the brown canckers caused by *Alternaria alternata* f. sp. *lycopersici*.

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