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PRELIMINARY STUDY RELATED HIGHLIGHTING THE INHIBITORY EFFECT OF *IN VITRO* FUNGUS GROWTH *MYCOSPHAERELLA GROSSULARIAE* (AUERS.) LIND. BY SAPROPHYTIC FUNGI

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Abstract

Mycosphaerella grossulariae (Auers.) Lind., one of the most important pathogenic fungi which affect the black currant ecological crops cultivated for alimentary supplements and phytotherapeutics drugs production. The present study brings the new data related to the possibility of „in vitro" vegetative growth inhibition of fungus mycelium using saprophytic fungi species. The species of fungi used for experimental trials was Trichoderma viride, Trichothecium roseum Link, Epicoccum nigrum Link. and Gliocladium roseum Bainier. The fungus was growth on several culture media for comparative testing and establishment of the most efficient medium for vegetative growth of the fungi. In the study we also follow the comparative approach doing by biometric measurements of the colonies in which work variant the pathogenic fungi had the high rate of growing. After the testing was made the PDA medium was selected for experimentation of inhibitory effect of the saprophytes. The method used for trials was the double cultures technique on the medium of agar which consists in inoculation of one media fragment of mycelium carrier separated from the agar medium of saprophytic fungi on some distance from pathogenic fungi. The study carried out had allowed the highlight of the fact that in all experimental variant used the saprophytic fungi had inhibits the Mycosphaerella grossulariae pathogen growing. The detailed analysis of the results we could reveal that the most inhibitory effect was done by T. viride followed by T. roseum and E.nigrum.

Key words: *blackcurrant crop, inhibitory effect, Mycosphaerella grossulariae, saprophytic fungi.*

INTRODUCTION

The biological resources and the sustainable exploit potential of aromatic and medicinal plants from our country are immense and represent an important sustainable component of Romanian agriculture (Manole, 2008). Romania has in his flora up to 3,600 species of plants and more than 1.000 are considered medicinal plants, spontaneous and cultivated (Alexan et al, 1988; Ardelean and Mohan, 2008; Bojor, 2003; Păun, 1995). In the classification of pharmacological industry are under different forms included − tea, medicinal and cosmetics products − almost 160 medicinal and aromatic species, among 110 are spontaneous collected and up 50 species are cultivated (Păun, 1995).

One of the species introduced in the cropping system, the blackcurrant crop (*Ribes nigrum* L.) are extremely economic important. The extension of ecological crops of this species are imposed the deeply acknowledgement of the pathogens in the purpose of finding rapidly and efficiently ways to biological control.

Mycosphaerella grossulariae (Auers.) Lind. is one of the most important pathogenic fungi which affect the black currant ecological crops cultivated for alimentary supplements and phytotherapeutics drugs production. In plantation of blackcurrant cultivated as medicinal plant in the south of Romania, *Mycosphaerella grossulariae* produces spots with picnidia on the blackcurrant's leaves (Petrescu and Oprea, 2012).

The present study brings the new data related to the possibility of *„in vitro"* vegetative growth inhibition of fungus mycelium using saprophytic fungi species. The fungal saprophytic species used for experimentation are already cited as antagonists of various pathogens of

cultivated plants (Şesan, 1997, Şesan and Oprea, 1995). The *in vitro* antagonistic activity of some fungi against other fungal pathogens of blackcurrant was previously investigated (Petrescu and Şesan, 2012; Petrescu et al., 2012). Biological control with antagonistic strains of fungi is an alternative and non polluting method for control the plant diseases produced by fungal pathogens (Fokkema, 1996).

Related to environmentally friendly alternative control methods of fungal pathogens of blackcurrant, a recent study analyzes the effect of some plant extracts on the development of the blackcurrant′s pathogen *Sphaerotheca mors-uvae* that produces American mildew, and on the pathogenic fungi isolated from phylloplane of blackcurrant in the South Eastern part of Romania, such as *Botrytis cinerea*, *Alternaria tenuissima* and *Fusarium oxysporum* (Enache et al., 2011).

The field experimental plots were located in the blackcurrant crops of S.C. Export-Import Hofigal S.A., which are playing the role of co-financing partner in the research consortium. The mentioned firm is a promoter of the systems for ecological agriculture in the case of some shrubs crops for alimentary supplements production.

MATERIALS AND METHODS

We are using for our study the biological material provided from reference isolate of the *Mycosphaerella grossulariae* (Auers.) Lind. CBS 235.37 pathogen, which was purchased from CBS culture collection of microorganisms, Utrecht, the Netherlands, and another 4 own isolates of saprophytes fungi obtained in 2010 from blackcurrant′s phylloplan, which were tested for their *in vitro* effect on the pathogen. These isolates are four strains of the fungi *Trichothecium roseum, Trichoderma viride, Gliocladiumroseum* and *Epicoccum-nigrum.*

In order to saprophytic fungi isolation the blackcurrant leaves were collected and introduced in plastic bags and brings to the RDIPP (Research Development Institute for Plant Protection) laboratory of Mycology for processing and analysis. In laboratory the leaves were divided into the small pieces with the help of sterile scissors. The leaves pieces were then placed on the water-agar media for sporulating stimulation and also on CGA media and incubated at the room temperature. The spores of different saprophytic fungi were observed and studied at the optical microscope. The saprophytic fungi spores or a little piece of fungi mycelium were being transferred on the culture media in sterile conditions. The culture media used for fungi growth and multiplication were PDA and MEA. The pathogenic strain of *Mycosphaerella grossulariae* was inoculated on different media and the colony diameter and colony characteristics were registereded. Four culture media were used, PDA (potato dextrose agar, MEA (malt extract agar) Czapek-Dox and Czapek). Each variant had 3 repetitions.

The saprophytic fungi and the pathogen were grown on PDA medium. On the specified time intervals biometrics measurements of the reverse side of the diameter of the colonies were performed in view to establish and compare the growing rate of the pathogenic fungus *Mycosphaerella grossulariae* with those of the saprophytic fungi. Looking for *in vitro* testing of the antagonic effect of the four saprophytic fungi against the strain of *Mycosphaerellagrossulariae* the double culture method were performed which mean the inoculation of both pathogen and saprophytic fungi on the same Petri dish at the same distance for the dish centre and the same distance one for another (Juan, 1964, Şesan and Oprea, 1995). The experimental design consists in 5 variant on 3 replicates each. The control medium culture had inoculated only with the saprophytic fungi. The Petri dishes selected for the experiment had a small diameter (60 mm) because of the length rhythm of pathogen growing. The medium used for testing was PDA. The diameter of the fungal inoculum, both pathogenic and saprophytic was of 3 mm and the distance between Petri dish centre and inoculum was of 10 mm, respectively. The distance between the pathogen inoculum and saprophytic one was of 20 mm. The saprophytic fungus was inoculated later, at the 5 days after pathogen inoculation in the moment when the colony characteristics of *Mycosphaerella grossulariae* are ready formed. The incubation was performed at room temperature (±24°C). The periodical measurements of internal radius of pathogen colony (the radius

oriented towards the centre of the Petri dish) on a period of 45 days were made and the values of these measurements were expressed by the media value of each variant. In the control case the internal radius which measures the colony growth was also the media of the periodical measurements values. On the basis of these media values of each variant, an inhibition degree were calculated after the follow relation, adapted and modified after Zivkovici et al. (2010):

$$I\% = \frac{I_C - I_V}{I_C} \quad (1)$$

Where:
I% = percent of growth inhibition;
I_C = internal radius of the colony of the fungus *Mycosphaerellagrossulariae* in control;
I_V = internal radius of the fungus *Mycosphaerellagrossulariae* in variant tested;
The inhibition scale used for values measurements and comparison between variants were adapted and modified after Zivkovici et al. (2010). This scale has 11 levels which permit to appreciate the inhibition degree (Table 1).
Periodically observation connected with colour of the colonies, back view of the colonies, contact line aspect and inhibition zone were made. Where I% <1 was considered 0 inhibition.

Table 1. Inhibition coefficient values and the corresponding inhibition level

Inhibition level	I%
0	0-1
1	1-10
2	11-20
3	21-30
4	31-40
5	41-50
6	51-60
7	61-70
8	71-80
9	81-90
10	91-100

Macroscopically, the relationship between the two fungal strains, a pathogenic one and a saprophytic one was made by the method described by Ana Hulea (1973) by observing the aspect of the contact line between the two colonies. Photos were taken after 7, 10 and 45 days.

RESULTS AND DISCUSSIONS

On the four media tested the colonies were circular, with hairy aspect, white colour and back view light brown (Figure 1).

Figure 1. Macroscopic in vitro aspect of the colony of the fungus M. grossulariae CBS 235.37 on MEA medium

After 45 days the colonies gets a more dark hue and the reverse side became dark brown. This result of the length vegetative growth of the colony was comparable with the observations of Stroe (1988) on the strains of another Mycosphaerella species, such as species *Mycosphaerella mori*, the pathogen which causes the antracnosis of *Morus alba*.
Among the four media tested, on the PDA medium *Mycosphaerella grossulariae* had a significant favorable growth. At 16 days after inoculation, the media value of colony diameter was of 19.3 mm, and the values are decreasing for MEA medium (19 mm), Czapek Dox (16.6 mm), and 15 mm on Czapek, respectively. At 24 day *Mycosphaerella grossulariae* had a better vegetative growth on PDA medium between the other media tested (Figure 2, Table 2).

Table 2. Diameters of the colonies of the fungus Mycosphaerella grossulariae (Auers.) Lind.CBS 235.37 on different culture media

variant	Culture medium	Colony diameter (mm)		
		after 16 days	after 24 days	after 45 days
1.	PDA	19.3	28.0	38.6
2.	Czapek-Dox	16.6	26.0	33.6
3.	Czapek	15.0	21.6	30.3
4.	MEA (Control)	19.0	26.3	32.3

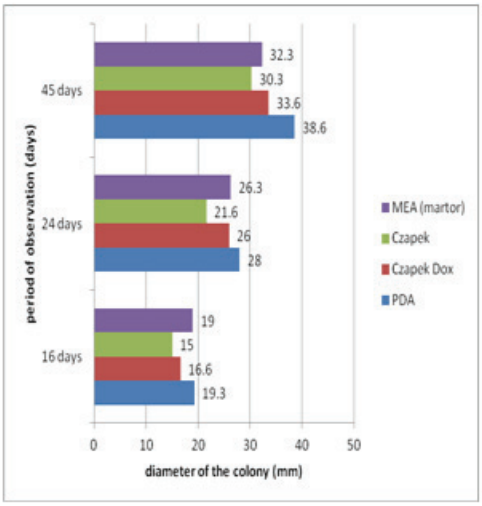

Figure 2. Vegetative growth of the colonies of the fungus Mycosphaerella grossulariae (Auers.) Lind.CBS 235.37 on different culture media

Among the five fungi grown on PDA medium, pathogenic fungi showed a significantly lower growth rate than saprophytic fungus *Trichoderma viride*. The other saprophytic fungi showed also significantly higher growth rates, but lower than *Trichoderma viride* (Figure 3, Table 3).

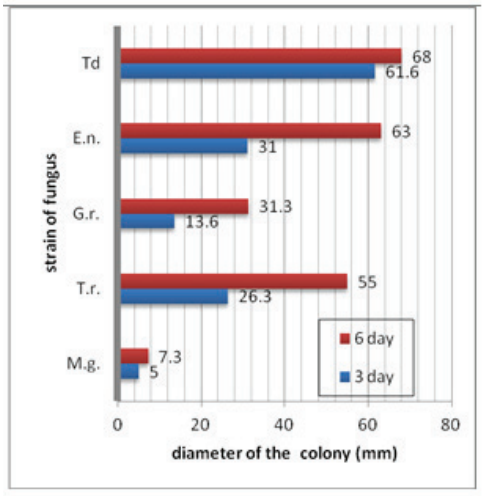

Figure 3. Diameter of the colonies of the strains of the fungi Trichoderma viride (Td), Epicoccum nigrum(E.n.), Gliocladium roseum (G.r.), Trichothecium roseum (T.r.) and the strain of the pathogenic fungus Mycosphaerella grossulariae (Auers.) Lind.CBS 235.37 (M.g.) on PDA medium

Table 3. The vegetative growth of the fungus Mycosphaerella grossulariae (Auers.) Lind.CBS 235.37 and the saprophytic fungi Trichoderma viride, Epicoccum nigrum, Gliocladium roseum and Trichothecium roseum grown on PDA medium

Variant	Colony diameter (mm)	
	after 3 days	after 6 days
Trichoderma viride	61.6	68.0
Epicoccumnigrum	31.0	63.0
Gliocladiumroseum	13.6	31.3
Trichothecium roseum	26.3	55.0
M. grossulariae	5.0	7.3

On the whole period of experimentation, in the control variant, fungus *Mycosphaerellagrossulariae* had a constantly growing rate and reaching a medium value of 5.91 mm after 7 days after inoculation of the saprophytic fungus in variant (Figure 1a), 7.3 mm after 10 days (Figure 1b), 15.8 mm after 30 days, 17.9 mm after 40 days and finally 18.7 mm after 45 days (Figure 1c). The shape of colonies was circular. The colour was white with the reverse of light brown (Figure 4).

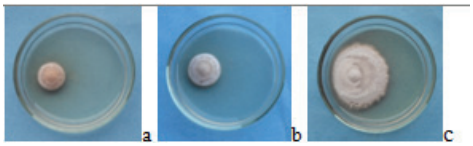

Figure 4. Macroscopic view of the colony of Mycosphaerella grossulariae (Auers.) Lind.CBS 235.37 on PDA medium after 7 days (a), after 10 days (b) and after 45 days (c) from pathogen inoculation in variants

The strain of *Trichothecium roseum* had a fast growth to the pathogen strain of *Mycosphaerellagrossulariae.* After 6 days from its inoculation, the mycelia of *Trichothecium roseum* began to cover all the area around the pathogen and blocked its expansion by the inhibitory effect under the internal radius which pointed the value of 3.95 mm read on the reverse side. After 7 days the *Trichothecium roseum* pink mycelium had developed around the white *Mycosphaerellagrossulariae* colony (Figure 5a). The division line had his concavity to pathogen fungi oriented who had a low rate of growing. The external radius continuosly growing against internal radius, the value registered being of 4.4 mm. After 10 days the mycelium of *T. roseum* had completely covered the pathogen colony which being totally inhibited (Figure 5b, Figure5c).

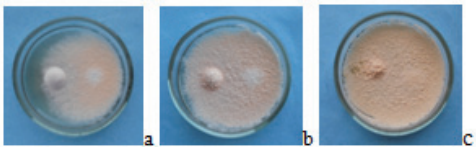

Figure 5. Macroscopic view of the colony of Mycosphaerella grossulariae (Auers.) Lind.CBS 235.37 on PDA medium after 7 days (a), after 10 days (b) and after 45 days (c) from inoculation of the strain of Trichothecium roseum

After 7 days from inoculation of the antagonistic fungus *Gliocladiumroseum*, internal radius of its colony became in the neighborhoods the phytopathogenic fungus colony (Figure 6a). After 10 days the contact line appeared as an obviously curve with the concavity oriented to the pathogen; this is characteristic of the relationship between the two fungal colonies among which there are differences in growth rate (Figure 5b). Between the two colonies being in the same Petri dish appeared a small area of sparse mycelium, which persisted throughout the 45 days of experimentation and it marked a inhibition of the pathogen exercises by saprophytic fungus (Figure 5b, Figure 5c). From this moment, the internal radius of the pathogen was inhibited in its growth and remained at the value of 5.35 mm, while the outer radius continued to grow until the colony of *Mycosphaerellagrossulariae* was completely surrounded by saprophytic fungus colony and the pathogen growth was completely inhibited (Figure 6c).

Figure 6. Macroscopic view of the colony of Mycosphaerella grossulariae (Auers.) Lind.CBS 235.37 on PDA medium after 7 days (a), after 10 days and after 45 days (c) from inoculation of the strain of Gliocladium roseum

After 7 days the contact zone between colony of the fungus *Epicoccumnigrum* and *Mycosphaerellagrossulariae* colony became a curve with the concavity oriented towards pathogen fungus which had a slower growth, and the inner radius was ceased growing (Figure 7a). After 10 days, the white mycelium of the pathogenic fungus was partially surrounded by the colony of the saprophytic fungus and continued to grow only by external radius (Figure 7b.). At the end of the experiment, phytopathogenic colony was covered by that of the antagonistic (Figure 7c).

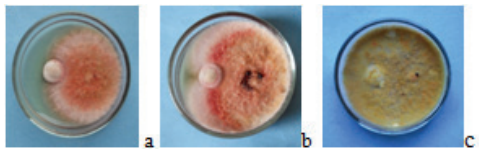

Figure 7. Macroscopic view of the colony of Mycosphaerella grossulariae (Auers.) Lind.CBS 235.37 on PDA medium after 7 days (a), after 10 days and after 45 days (c) from inoculation of the strain of Epicoccum nigrum

Trichoderma viride fungus grew very quickly over phytopathogenic fungus colony, so that at 7 days after inoculation antagonistic fungus invaded the colony, entirely covering the Petri dish and determine to cease growth both internal and external radius (Figure 8a, Figure 8b). Between the two colonies, pathogen′s and antagonist′s, the mycelium of fungus *Trichoderma viride* was sparse, sign that phytopathogenic fungus present in the vicinity may have an inhibitory effect on the fungus . At the end of the experiment, the Petri dish appeared totally covered by mycelium of the fungus *T. viride*, except for those inhibition zones that formed in the right of the two colonies that interacted (Figure 8c).

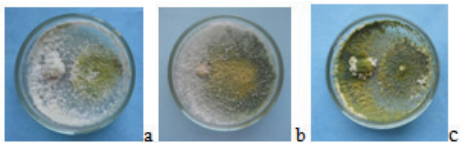

Figure 8. Macroscopic view of the colony of Mycosphaerella grossulariae (Auers.) Lind.CBS 235.37 on PDA medium after 7 days (a), after 10 days and after 45 days (c) from inoculation of the strain of Trichoderma viride

The average values of the percent of growth inhibition calculated after 7 days allowed assessment of inhibition as in the categories 4 for *Trichoderma viride*, 3 level for *Trichothecium roseum* and *Epicoccumnigrum* and only 1 level for the strain of *Gliocladiumroseum* (Table 4, Table 9, Figure 10).

Table 4. The in vitro effect exercised by some saprophytic fungi on M. grossulariae on PDA medium, expressed by inhibition percent (I%) and inhibition level (0-10), after 7 days

Variant	I%	level
M. grossulariae/ T. roseum	27,91	3
M. grossulariae/ G. roseum	9,47	1
M. grossulariae/ E. nigrum	24,53	3
M. grossulariae/ T. viride	30,11	4
Control (*M. grossulariae)*	0	0

At 10 days after inoculation, inhibition coefficient values increased, reaching category 5 for *Trichoderma viride* and *Trichothecium roseum*, 4 for *Epicoccumnigrum* and 3 for *Gliocladiumroseum* (Table 5, Table 9, Figure 10).

Table 5. The in vitro relationships between M. grossulariae and some saprophytic fungi on PDA medium, expressed by inhibition percent (I%) and inhibition level (0-10), after 10 days

Variant	I%	level
M. grossulariae/ T. roseum	41,64	5
M. grossulariae/ G. roseum	26,57	3
M. grossulariae/ E. nigrum	38,9	4
M. grossulariae/ T. viride	43,42	5
Control (*M. grossulariae)*	0	0

After 30 days from the inoculation, the inhibition values were significantly increased for all tested saprophytic fungi, being 73.86% in case of *Trichoderma viride*, 73.03 in case of *Trichothecium roseum*, 71.77 in case of the strain of *Epicoccumnigrum* and only 66.07% in case of *Gliocladiumroseum* (Table 6, Table 8, Figure 9). Inhibition level was 8 on our scale for three saprophytic fungal strains (*Trichoderma viride, Trichothecium roseum, Epicoccumnigrum*) and for one strain (strain of the fungus *Gliocladiumroseum*) the level of inhibition was a little lower, 7 respectively (Table 6, Table 9, Figure 10).

Table 6. The in vitro effect exercised by some saprophytic fungi on M. grossulariae on PDA medium, expressed by inhibition percent (I%) and inhibition level (0-10), after 30 days

Variant	I%	level
M. grossulariae/ T. roseum	73,03	8
M. grossulariae/ G. roseum	66,07	7
M. grossulariae/ E. nigrum	71,77	8
M. grossulariae/ T. viride	73,86	8
Control (*M. grossulariae)*	0	0

At the end of the experimentation, after 45 days of the antagonist inoculation we found that in case of *Trichoderma viride*, inhibition coe was 77.91, followed by *Trichothecium roseum* with 77.21 *Epicoccumnigrum*. *Gliocladiumroseum* showed the lowest coefficient of inhibition of 71.33 (Table 7, Table 8, Figure 9).

Table 7. The in vitro relationships between M. grossulariae and some saprophytic fungi on PDA medium, expressed by inhibition percent (I%) and inhibition level (0-10), after 45 days

Variant	I%	level
M. grossulariae/ T. roseum	77,21	8
M. grossulariae/ G. roseum	71,33	8
M. grossulariae/ E. nigrum	76,14	8
M. grossulariae/ T. viride	77,91	8
Control (*M. grossulariae)*	0	0

Table 8. Inhibition percent (I%) exercited by saprophytic fungi on M. grossulariae in experimentally variants

Variant (saprophytic strain fungus)	after 7 days	After 10 days	after 30 days	after 45 days
Trichothecium roseum	27,91	41,64	73,03	77,21
Gliocladium roseum	9,47	26,57	66,07	71,33
Epicoccum nigrum	24,53	38,9	71,77	76,14
Trichoderma viride	30,11	43,42	73,86	77,91
Control (*M. grossulariae)*	0	0	0	0

Related to degree of inhibition, however, for all saprophytic fungi tested for their inhibitory effect against the strain of phytopathogenic fungus *Mycosphaerella grossulariae*, the inhibition level was placed in category 8 (Table 7, Table 9, Figure 10).

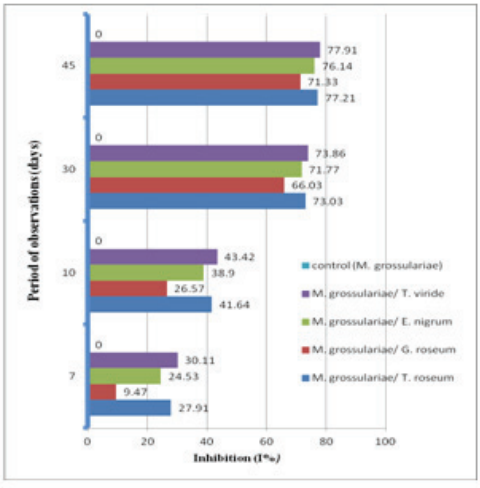

Figure 9. Evolution of inhibition percent (I%) in experimentally variants

Related to the process of the evolution of the inhibition percent values we observed that this is correlated with growth rate of the saprophytic fungi.

The fact the fungus *Trichothecium roseum* showed a higher inhibition than the fungus *E. nigrum* that had a faster growth, is probably due to the capacity of the species *T. roseum* to grow over and hyperparasitize the hyphae of the pathogens, while colony of the fungus *E. nigrum* was slowly surrounding the colony of *Mycosphaerella grossulariae.*

Table 9. Inhibition level exercited by saprophytic fungi on M. grossulariae in experimentally variants

Variant	Inhibition level			
	after 7 days	After 10 days	after 30 days	after 45 days
M. grossulariae/ T. roseum	3	5	8	8
M. grossulariae/ G. roseum	1	3	7	8
M. grossulariae/ E. nigrum	3	4	8	8
M. grossulariae/ T. viride	4	5	8	8
Control (*M. grossulariae)*	0	0	0	0

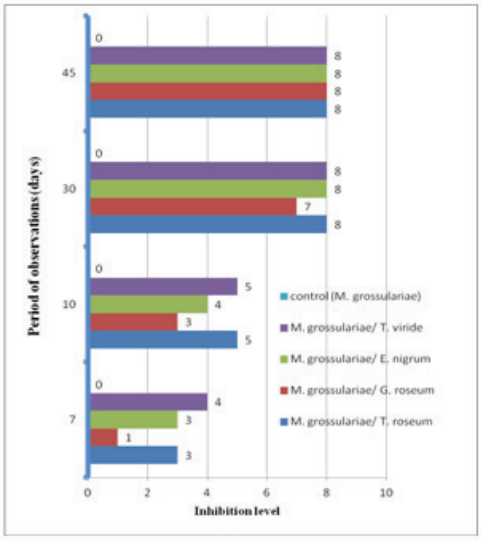

Figure 10. Evolution of inhibition level (0-10) in experimentally variants

CONCLUSIONS

The best vegetative growth of *Mycosphaerella-grossulariae* (Auers.) Lind. CBS 235.37 mycelia was registered on PDA medium, this type of medium being selected for growth fungi estimation used in this study and for inhibition effect of saprophytic fungi evaluation.

Among the 5 species incubated on PDA medium, the fast growing was observed in the case of the strain of *Trichoderma viride*, closely followed by the isolates of *Epicoccumnigrum* and *Trichothecium roseum.* Among saprophytic fungi the length growth was observed in the case of *Gliocladiumroseum* isolate.

The strain of *Mycosphaerellagrossulariae* had the lowest rate of growing, reaching a medium value of 7.3 mm of colony diameter after 6 days in comparison with the saprophytic fungi *Trichoderma viride* which totally covered the Petri dish surface.

All fungi tested related to their inhibitory capacity were influenced significantly the pathogenic fungus growth, but the stronger inhibitory effect was observed in the case of the strain of *Trichoderma viride,* followed by those of *Trichothecium roseum* and *Epicoccumnigrum.* The lowest inhibitory effect in the first 10 days after inoculation was observed in the case of *Gliocladiumroseum* strain, but after 45 days the inhibition value (I%) was closely to the other variants.

The inhibitory effect was direct proportional with the growth rate of both fungi: pathogenic one and the antagonistic with the exception of *Trichothecium roseum* and *Epicoccumnigrum.* Although *Trichothecium roseum* had a more length growth rate in comparison with *Epicoccumnigrum* it expresses a stronger inhibitory effect.

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