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TOBACCO REACTION TO TOXIC METABOLITES OF THE PATHOGEN *PHYTOFTHORA PARASITICA VAR. NICOTIANAE*

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ABSTRACT

In phytopathological laboratory of Tobacco Institute-Prilep *in vitro* investigations were made on the presence of toxic matters in inoculum of the pathogen *Phytophthora parasitica var. nicotianae* and their impact on tobacco plant, to determine whether these toxic metabolites cause necrotic reactions and other damages on the host-plant. Comparison was made between symptoms resulting from the effect of toxic metabolites and symptoms caused by the pathogen itself. Inoculum was prepared from suspension of fungus mycelium and filtrate as selective agent obtained from the fungus grown on liquid medium and after that filtrated through filter paper and bacteriological filter of 0.22 μ porosity. Leaves of *Nicotiana nesophila* species and leaves of two oriental and one Burley variety of *Nicotiana tabacum* were used in the investigation. Inoculation was made by two methods: adding a drop of the inoculum on leaf surface and submerging the base of the leaf in the inoculum. In leaves inoculated by both methods, differences in necrosis development on leaf surface could be noticed. The necrosis developed more rapidly in leaves inoculated with a suspension of pathogen's mycelium, where percentage of damaged leaf tissue was also higher compared to the leaves inoculated with selective agent. There were no differences, however, between leaf symptoms caused by toxic metabolites and those caused by the pathogen itself. The aim of the investigation was to determine the infectivity of toxic compounds released by the pathogen and the effects of two methods of inoculation on the percentage of damaged leaf tissue.

Key words: tobacco, pathogen, toxins, inoculum, suspension

РЕАКЦИЈА НА ТУТУНОТ СПРЕМА ТОКСИЧНИТЕ МЕТАБОЛИТИ ОД ПАТОГЕНОТ *PHYTOFTHORA PARASITICA VAR. NICOTIANAE*

Во фитопатолошката лабораторија како и во *in vitro* лабораторијата при Научниот институт за тутун од Прилеп, вршени се испитувања за присуството на токсични материи во инокулумот од патогенот *Phytophthora parasitica var. nicotianae* како и на нивното влијание врз тутунското растение, односно дали овие токсични метаболити причинуваат одредени некротични реакции или други оштетувања кај растението-домаќин. Направена е споредба на симптомите коишто се појавија како резултат на влијанието на токсичните метаболити и симптомите причинети од самиот патоген. Како инокулум беа користени суспензија подготвена од мицелија на габата и филтрат како селективен агенс добиен од габата одгледувана на течна хранлива подлога, кој потоа е филтриран низ филтер-хартија и бактериолошки филтер со порозност од 0,22 микрона. За испитување беа користени листови од видот *N. nesophila* и листови од две ориенталски и една берлејска сорта од *N. tabacum*. Инокулирањето е извршено

со поставување на капка од инокулумот на површината од листот и со потопување на основата од листот во користениот инокулум. Кај инокулираните листови по двете методи (со капка и со потопување) и со двата инокулума, можеше да се забележи разлика во развојот на некрозата која се појавуваше по површината на листовите. Некрозата се шириеше многу побрзо кај листовите кои беа инокулирани со суспензија подготвена од мицелија на патогенот, каде и процентот на оштетено лисно ткиво беше повисок, а побавно кај листовите инокулирани со селективниот агенс. Симптомите појавени по површината на листовите причинети од токсичните метаболити на патогенот не се разликуваа од оние кои беа причинети од самиот патоген. Целта во ова истражување беше да се провери инфективноста на токсичните материи што ги ослободува патогенот и разликата во оштетената лисна површина причинета од двата користени инокулума.

Клучни зборови: тутун, патоген, токсини, инокулум, суспензија

INTRODUCTION

Black shank is one of the most devastating diseases in tobacco and a serious threat to tobacco production all over the world. It can be found in all tobacco producing countries and, depending on climate conditions and host resistance, it can cause severe losses of tobacco yield. The causing agent of the disease is phytopathogenic fungus *P. parasitica var. nicotianae*. It is a soilborn pathogen which infects the plant through its root, and symptoms occur along the stem. In the beginning, infected plants suddenly lose their turgor and wilt, and later necrosis appears at basal stem region, spreading along its length. The root system becomes dark, leaves turn to yellow, wilt and shrivel along the stem which becomes brown-black in color, with only few green leaves remaining on the top of the plant (Taskoski, 2003). Losses of tobacco can be more severe in rainy years and in irrigating conditions.

According to Wolf (1954, cit. Lucas 1975), symptoms of wilting are caused by systemic effect of toxins created by the pathogen itself. According to Powers (1954, cit. Lucas 1975), wilting is a result of prevention of water and nutrients movement through plant vessels, as well as of the presence of rubber matters and tylosis, created during decomposition of cells attacked by the pathogen. Subject of our investigation was to find which of these factors have a stronger impact on the process of tobacco wilting .

During development of pathogens, smaller or greater pathological changes occur in leaf tissue. They are usually manifested through necroses or wilting of certain parts of the plant or even of the whole plant. According to Cutova (1983), toxins themselves can cause disease symptoms similar to those of the natural infections and reaction of some varieties to the toxins does not differ from the reaction to the pathogen.

Toxins are metabolitic products of the pathogenic microorganisms which can cause death of cell protoplasts. The process of cell intoxication itself has a harmful effect upon living parts of the cell, having a greater influence on its biochemical and physiological activities than on its structure. Phytopathology literature presents data on the existence of high number of toxins. Some of them are specific, but the importance of non-specific toxins is of equal importance (Sutic, 1986).

The main task of our investigation was to find out whether the pathogenic fungi *P. parasitica var. nicotianae* produce metabolites which would be capable of infecting plant tissue just as the pathogen itself, to study the mode of their activity and to identify eventual differences in symptoms obtained by both inoculation methods.

MATERIAL AND METHODS

Leaves of *N. nesophila* species, which is highly resistant to the pathogen, and leaves of two oriental (P 12-2/1 and P 23) and one Burley (B2) varieties were used in the trial, while non-inoculated leaves of P 12-2/1 were used as a check. Suspension made of fungal mycelium and selective agent obtained from the fungus by filtration was used as inoculum. Inoculation was performed in two variants: by adding a drop of the inoculum on leaf surface and by submerging the base of the leaf in the inoculum. Four replications were made for each variant. Two of the variants were inoculated with suspension and the other two with selective agent. Five healthy middle belt leaves from each variety were prepared for each variant. Prior to inoculation, each leaf area was measured by the formula P=a.b.K, where the middle value (0.63) was used as correction factor (K) (Saric et co., 1990).

Pure *P. parasitica var. nicotianae* culture was used for inoculum preparation. The fungus was previously isolated from infected tobacco plants on oat agar medium, by standard laboratory method. The culture was grown 15-20 days at 25^{0}C.

Suspension of the fungal mycelium was prepared from the culture on Petri dish and blended in 100 ml distilled water. The suspension prepared in this way was used for inoculation of tobacco leaves.

In the first variant, leaf surface was injured with sterile needle prior to inoculation and a drop of the suspension was added in the injured area. Inoculated leaves were stored 15 days in a wet chamber. Leaves which served as a check were inoculated in the injured area byadding a drop of sterile distilled water.

In the second variant, the base of tobacco leaves was submerged in 20 ml mycelial suspension, and check leaves were submerged in 20 ml sterile distilled water.

For investigation of the selective agent infectivity, Ppn was grown on liquid nutrient medium by the method of Slavov (2002). The liquid medium was passed through filter paper and bacteriological filter of 0.22 μ porosity, followed by filtrate concentration and another filtration through bacteriological filter. The concentrated filtrate of the culture presented a selective agent.

The mode of work with selective agent was the same as that with mycelial suspension.

In the first variant with drop inoculation the selective agent was used undiluted, and in the second variant the selective agent was diluted with sterile distilled water in 1:10 ratio, where the leaves were placed.

Estimation and measurement of necrosis development on leaf surface was made on the 2nd, 4th, 9th, 11th and 14th day of inoculation. In leaves inoculated with drop, the necrosis diameter was measured, and in leaves submerged in the inoculum the length of necrosis was measured from the leaf base, i.e from the starting point of necrosis to its end. Based on measurements of necrotized leaf tissue, the percentage of damaged leaf tissue was estimated. Results of the investigations are presented as a mean value of all replications.

RESULTS AND DISCUSSION

Necrosis was observed on leaf surface in both types of inoculation, with certain differences in its growth and size, depending on the type of inoculum.

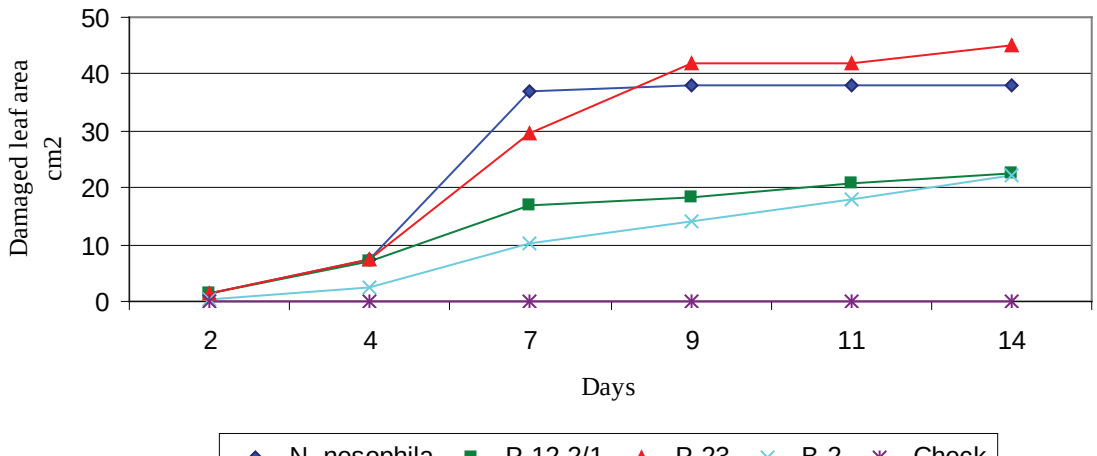
Results on occurrence and growth of necrosis in leaves inoculated with drop of suspension on leaf surface are presented in Table 1 and Figure 1. As soon as the second day of inoculation, necrosis of about 1.50 cm in size was observed in all inoculated leaves, which developed with different intensity during the period of observation. It should be emphasized that necrosis developed quite rapidly in inoculated leaves of *N. nesophila*, a species which is otherwise known as resistant to this pathogen (Taskoski, 2005).

Table 1 - Leaves inoculated with a drop of mycelium suspension

Species-Tob. variety	Leaf size, l/w	Leaf area cm^2	Spread of necrosis by days, in cm^2					
			2	4	7	9	11	14
N. nesophila	9.5/6.5	38.90	1.50	7.30	36.85	37.95	37.95	38.11
P 12-2/1	9.0/6.0	34.02	1.50	7.05	17.01	18.27	20.79	22.52
P 23	13.2/7.2	59.87	1.50	7.56	29.69	41.89	41.89	45.21
B 2	12.9/6.6	53.64	0.51	2.31	10.29	13.91	17.91	22.28
Check P 12-2/1	15.1/7.5	71.34	-	-	-	-	-	-

On the 14th day of observation, the following level of necrosis was observed: 45.21cm^2 of leaf tissue was damaged in variety P 23 , 38.11 cm^2 in *N. nesophila* species, 22.52cm^2 in P12-2/1 and 22.28 in B2 cm^2. No symptoms of necrosis were observed in leaves that were used as a check.

Fig.1 Necrosis development



On the basis of damaged leaf tissue, the level of infection was estimated, and it ranged fro 41.53 in B2 variety to 97.96% in *N. nesophila*. Varieties P 23 and P 12-2/1 (Table 2) also showed high level of infection of leaf tissue, ranging from 75.51% and 66.19% (Table 2).

Table 2 - Level of infection in leaves inoculated with a drop of mycelium suspension

Species-tob. variety	Leaf size, l/w cm	Leaf area cm^2	Damaged leaf area cm^2	Infected leaf tissue %
N. nesophila	9.5/6.5	38.90	38.11	97.96
P 12-2/1	9.0/6.0	34.02	22.52	66.19
P 23	13.2/7.2	59.87	45.21	75.51
B 2	12.9/6.6	53.64	22.28	41.53
Check P 12-2/1	15.1/7.5	71.34	0.00	0.00

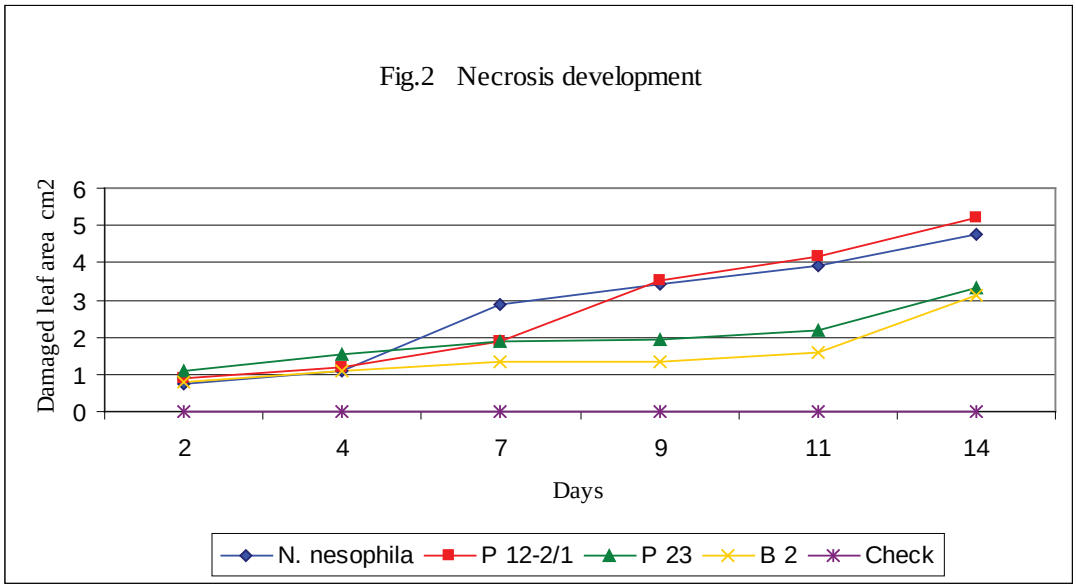
Tissue of the leaves inoculated with a drop of the selective agent was also damaged. Necrosis was observed even on the second day of inoculation, only to somewhat lesser extent (Table 3, Fig. 2). During all days of observation, necrosis was spreading almost identically in all inoculated leaves, and on the 14th day the heaviest damage was measured in P 12-2/1 (5.23 cm^2), N. nesophila (4.762 cm^2) and P 23 and B2 (3.12 cm^2).

In this case, too, the check leaves remained healthy and free of damage.

Table 3 - Leaves inoculated with a drop of selective agent

Species-Tob. variety	Leaf size l/w cm	Leaf area cm^2	Spread of necrosis by days, in cm^2					
			2	4	7	9	11	14
N. nesophila	8.8/5.6	31.05	0.73	1.07	2.88	3.41	3.93	4.76
P 12-2/1	14.3/7.1	63.96	0.89	1.20	1.87	3.50	4.17	5.23
P 23	13.0/7.0	57.33	1.10	1.56	1.86	1.92	2.19	3.30
B 2	9.9/5.3	33.05	0.81	1.08	1.34	1.36	1.60	3.12
Check P 12-2/1	15.5/9.3	90.81	-	-	-	-	-	-

Fig.2 Necrosis development



Considering that in this variant lower damage of leaf surface was observed, the infection percentage was lower, too. The highest percentage of infection was measured in *N. nesophila* species (15.33%), and the lowest in P 23 variety (5.75%).

Table 4 - Level of infection in leaves inoculated with a drop of selective agent

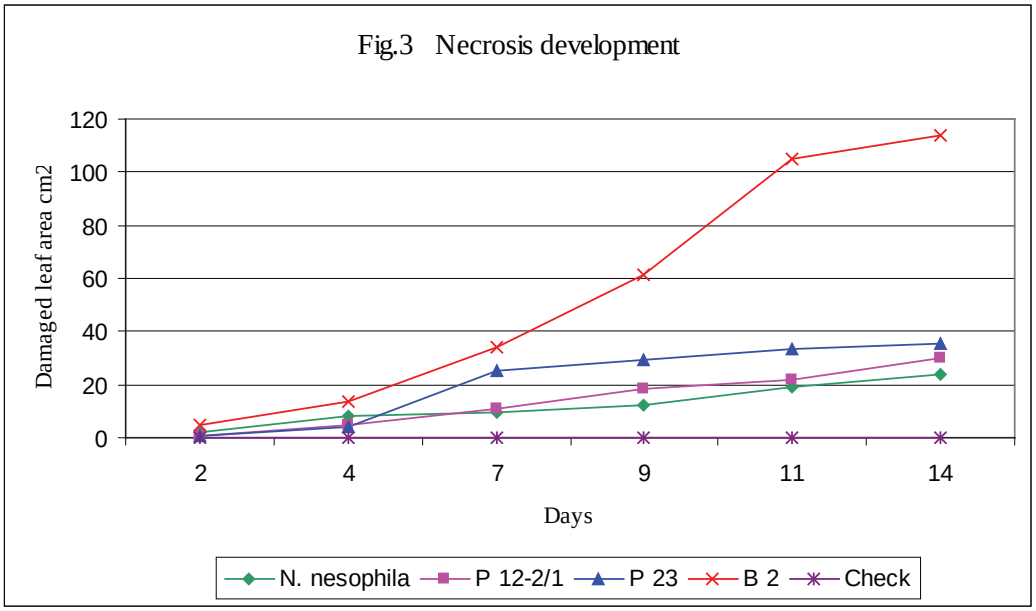
Species-Tob. variety	Leaf size l/w cm	Leaf area cm^2	Damaged leaf area cm^2	Infected leaf tissue %
N. nesophila	8.8/5.6	31.05	4.76	15.33
P 12-2/1	14.3/7.1	63.96	5.23	8.17
P 23	13.0/7.0	57.33	3.30	5.75
B 2	9.9/5.3	33.05	3.12	9.44
Check-P 12-2/1	15.5/9.3	90.81	0.00	0.00

Similar results were obtained in leaf inoculation by the second method, i.e. by submerging the leaf base in the inoculum. Results of investigation on necrosis in leaves inoculated with suspension of fungus mycelium are presented in Table 5, Figure 3. On the second day of observation, the infection intensity was similar to the one observed in inoculation with a drop selective agent and ranged between 1.00 cm^2 in P 12-2/1 and P 23 to 4.75 cm^2 in B2. Higher intensity of necrosis during observation was measured in B2, reaching 113.66 cm^2 on the 14th day of observation. The lowest intensity in this mode of inoculation was measured in the leaves of *N. nesophila* - 23.56 cm^2.

Table 5 - Leaves inoculated by submerging in mycelium suspension

Species-Tob. variety	Leaf size l/w cm	Leaf area cm^2	Spread of necrosis by days, in cm^2					
			2	4	7	9	11	14
N. nesophila	10.3/6.0	38.93	1.83	8.25	9.28	12.46	18.98	23.56
P 12-2/1	15.5/6.1	59.56	1.00	5.10	10.60	18.23	21.73	30.12
P 23	12.6/6.5	51.59	1.00	4.11	25.45	29.40	33.07	35.33
B 2	21.8/9.9	135.96	4.75	13.80	33.93	61.26	104.67	113.66
Check P 12-2/1	17.6/8.4	93.13	-	-	-	-	-	-

Fig.3 Necrosis development



Damages and percentage of infection, estimated on the basis of total and infected leaf surface are presented in Table 6. As could be seen from the Table, there are no major differences in the level of infection of investigated varieties and the wild species. The lowest percentage of infection was assessed in P 12-2/1 (50.57%), and the highest in B2 (83.59%).

Table 6 - Level of infection in leaves submerged in mycelium suspension

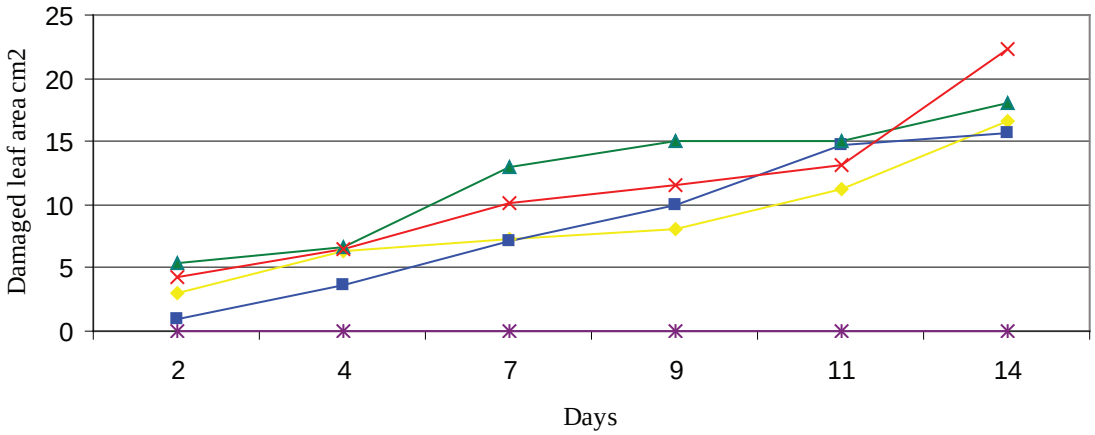
Species-Tob. variety	Leaf size l/w cm	Leaf area cm^2	Damaged leaf area cm^2	Infected leaf tissue %
N. nesophila	10.3/6.0	38.93	23.56	60.51
P 12-2/1	15.5/6.1	59.56	30.12	50.57
P 23	12.6/6.5	51.59	35.33	68.48
B 2	21.8/9.9	135.96	113.66	83.59
Check P 12-2/1	17.6/8.4	93.13	0.00	0.00

In leaves submerged in selective agent of the pathogen, higher damage, compared to other variants, was observed on leaf surface on the second day of inoculation, ranging from 1.00 cm^2 in P 12-2/1 to 5.40 cm^2 in P 23. On the last day, the highest damage was measured in B2 (22.30 cm^2) and the lowest in P12-2/1 (15.68 cm^2). (Table 7, Fig. 4). In this investigation, too, no symptoms of disease were observed in the check leaves.

Table 7 - Leaves inoculated by submerging in a selective agent

Species-Tob. variety	Leaf size l/w cm	Leaf area cm^2	Spread of necrosis by days, in cm^2					
			2	4	7	9	11	14
N. nesophila	9.5/5.0	29.92	3.00	6.40	7.20	8.00	11.20	16.60
P 12-2/1	13.3/6.0	50.27	1.00	3.63	7.10	9.98	14.71	15.68
P 23	10.0/5.5	34.65	5.40	6.60	13.00	15.00	15.00	18.00
B 2	9.8/5.2	32.10	4.20	6.50	10.10	11.50	13.10	22.30
Check P 12-2/1	14.9/6.3	59.13	-	-	-	-	-	-

Fig.4 Necrosis development



Percentage of infected leaf surface in this variant ranged between 31.19 % in P 12-2/1 and 69.47% in B2 (Table 8).

From the four variants investigated it could be seen that in both inoculation methods, daily growth of necrosis was highest in the leaves inoculated with suspension prepared from fungus mycelium. The daily growth is much lower in the leaves inoculated with selective agent.

Percentage of infected area differs significantly. It is much lower in the leaves inoculated with a drop of the selective agent and higher in the leaves inoculated with a drop of the suspension. The difference in percentage of infected leaf tissue was very small when leaves were inoculated by the second method, i.e. by submerging the base of the leaf in the inoculum.

Table 8 - Level of infection in leaves submerged in a selective agent

Species-Tob. variety	Leaf size l/w cm	Leaf area cm^2	Damaged leaf area cm^2	Infected leaf tissue %
N. nesophila	9.5/5	29.92	16.60	55.48
P 12-2/1	13.3/6.0	50.27	15.68	31.19
P 23	10.0/5.5	34.65	18.00	51.94
B 2	9.8/5.2	32.10	22.30	69.47
Check- P 12-2/1	14.9/6.3	59.13	0.00	0.00

The increased necrosis in leaves inoculated with suspension is due to the prolonged active metabolism of the pathogen, which thereby becomes more infective. According to Kutova (1983), cells affected by toxins do not die immediately, but are partially damaged by decrease of their physiological functions. More obvious changes appear in cells that are closer to the damage. Under the influence of toxins, the ability of wilted leaves placed in pure water to regain their turgor is very poor, unlike the mechanically provoked wilting, where turgor recovers very quickly.

As it was also reported, many varieties that are resistant to the pathogen show high susceptibility to the toxins. These statements are in compliance with investigations of Sutic (1986), who described toxins as low-molecular-weight compounds which spread easily from the place of infection, causing necrosis of healthy neighboring cells. This type of toxic effect is characterized by chlorotic zone around necrosis.

CONCLUSIONS

The investigations confirm the negative effect of toxic metabolites of the pathogen on the cells of plant tissue. The results obtained lead to the following statements:

- The fungus of *P. parasitica var. nicotianae* creates metabolites that are toxic to tobacco plant cells.
- 2. As a result of toxic metabolites, necrosis appears on leaf surface or along the vessels of tobacco inoculated with a drop of selective agent or a drop of suspension of the fungus mycelium, i.e. when they are submerged in selective agent or in suspension prepared with mycelium of the pathogen.
- Inoculated leaves from different tobacco varieties had a different reaction to the toxic metabolites of the pathogen.
- Higher necrosis level and higher percentage of damaged leaf tissue in almost all varieties were observed in leaves inoculated with suspension of the fungus mycelium in both investigated variants.
- Significantly lower percentage of infection was observed in leaves inoculated with selective agent in both variants, but it was particularly low in leaves inoculated with a drop of this inoculum.
- Selective agent infects the cells of tobacco leaves, which can be observed as a lesion surrounded by a chlorotic ring.

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