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CHANGES IN THE MORPHOLOGY OF THE FUNGUS *ALTERNARIA ALTERNATA* IN THE TRANSMISSION FROM NATURAL IN ARTIFICIAL CONDITIONS OF CULTIVATION

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ABSTRACT

Ecological factors have great influence on the development of the pathogen, but they also influence it's morphology. The aim of these study was to make morphological measurements of the fungus *Alternaria alternata* - causing agent of the brown spot disease*,* which develops on infected tobacco leaves and than in a pure culture, in order to determine the changes in morphology created during the change of conditions for development.

With microscopic measurements it was determined that the fungus which develops upon an infected vegetative material forms hyphae with smaller width than when it develops in pure culture. During the transition in artificial conditions, the conidiophores get smaller width and lartger length. The conidia in a pure culture have smaller dimensions than those of the infected leaves and the maximum number of all septa is smaller.

Key words: *Alternaria* a*lternata*, tobacco, morphology, changes

ПРОМЕНИ ВО МОРФОЛОГИЈАТА НА ГАБАТА *ALTERNARIA ALTERNATA* ПРИ ПРЕНЕСУВАЊЕ ОД ПРИРОДНИ ВО ВЕШТАЧКИ УСЛОВИ НА ОДГЛЕДУВАЊЕ

Еколошките фактори имаат големо влијание врз развојот на патогенот, но исто така влијаат и врз неговата морфологија. Затоа, целта на овие истражувања беше да се извршат морфолошки мерења на предизвикувачот на болеста кафена дамкавост – габата *Alternaria alternata* која се развива на инфицирани тутунски листови, а потоа во чиста култура за да се утврдат промените во морфологијата што настануваат при промена на условите за развој.

Со микроскопските мерења беше утврдено дека габата која се развива врз инфициран растителен материјал формира хифи со помала ширина отколку кога се развива во чиста култура. При премин во вештачки услови конидиофорите добиваат помала ширина, но поголема должина. Конидиите во чиста култура имаат помали димензии отколку тие врз инфицираните листови, но и максималниот број на сите септи е помал.

Клучни зборови: *Alternaria alternata,* тутун, морфологија, промени

INTRODUCTION

The reduction of the yield in most of the diseases caused by the fungi of the gender *Alternaria* can be caused by reduction of the photosynthetic activity, direct attack of the pathogen, decrease of the quality of the product or a combination of those mechanisms (Rotem, 1994).

Alternaria alternata is a cause of the brown spot disease of tobacco. The combination of the ways of acting makes it one of the most important pathogens of tobacco. It attacks all types of tobacco, but the most susceptible are the large-leaf tobacco plants. The damages are mostly connected with the quality of tobacco leaves, but it also damages the smoking features of the tobacco raw material. Except of the time of vegetation, tobacco is a suitable substrate for development of this pathogen at the time of drying and processing of tobacco raw material. This fungus was isolated from the cigarettes, too (Kantor et al., 1979).

Its intensity of attack depends on the climate conditions, the sensitiveness of the type and a lot of other factors during the manufacturing (Gveroska, 2005). But, in the artificial inoculations, in a lot of cases, the intensity of attack depends on the choice of the isolate.

According to Otani and Khmoto (1992) and Rotem (1994), *A. alternata* unites species that create specific toxins which are very pathogenic to certain kinds. Because of that, they suggest for them to be qualified as specialized forms of *A. alternata*. For example, *A. alternata* f.sp. *tabaci* which creates AT toxin is a pathogenic of tobacco too.

The morphological variations are typical characteristic of *Alternaria* species, particularly *A. alternata* (Rotem, 1994; Slavov, 2004). But, AT toxin is a recognizable factor in patho-system species *Nicotiana - Alternaria alternata* (Kodama et al., 1990). All the isolates, as well as spores of the nonpathogenic, to which AT toxin was added, cause brown spot to inoculated plants.

Anyway, in field conditions, the resistant types show lower degree of resistance compared to the artificial inoculation with AT toxin, because Alternaria fungi can attack the cells during the penetration through the damages of wind, rain etc. That is why the *in vitro* selection is not always the best choice (Ishida, 1992). There is also difference in the conditions that influence sporulation and the size of the conidia too. The sporulation in natural conditions is created on green leaves, in the presence of water, and big influence has the relative humidity (Akimitsu, 2003).

The knowledge of morphology is important because there can be change in the pathogenicity of the isolates in cultivation of a culture, accompanied with a change in morphology of the conidia (Sobers and Doupnik, 1968). It is also necessary for the right methodological approach in all the investigations on the morphology, pathogenic features and the epidemiology of the disease.

The aim of this research was to give data for the morphology of the pathogen *A. alternate* in natural conditions, i.e. on infected tobacco leaves, and than in a pure culture, in order to confirm the possible changes of the morphology.

MATERIAL AND METHODS

Because of the fact that the large-leaf tobacco plants are characterized with higher susceptibility to brown spot disease, the isolates obtained from 5 large-leaf types, three of which were Virginia (V 12, V 13 and V 22) and two Burley (B 36 and B 98). The choice of the types was random. Infected tobacco leaves with characteristic symptoms of the disease were collected. Parts of the leaves with spots were kept in Petri dishes with wet filter paper for 48 hours.

The material from the surface of leaf spots was taken with a sterile needle and 5 microscopic preparations were prepared for every isolate. 25-30 samples randomly chosen during the microscoping were analyzed in each preparation. The same procedure was applied in investigation of the morphology of the fungus in a pure culture,

After all the measurements of the fresh material were made, it was approached towards getting pure cultures. First parts of the infected tissue were placed on wet agar. After the development of the colony, small fragments were sowed again on potato-dextrose agar. The received pure cultures were incubated in a thermostat of 25 degrees. Morphological measurements of the fungus were made on a 15-days pure culture.

The morphology of the fungus was studied by analyzis of microscopic measurements of the hyphae width and the conidiophores and conidia size (length and width). Determination (counting) of the diagonal, lengthwise and inclined septa was also made.

The measurements were made by ocular micrometer, after previously gauging and determination of the factor of appropriate increase according to the method of Ziberoski (1998).

RESULTS AND DISCUSSION

The pathogenic fungus A.alternata is a cause of the brown spot disease on tobacco in R. Macedonia (Gveroska, 2005) The disease got its name after the specific symptoms, i.e. appearance of brown spots on leaf surface. It can penetrate directly through the tissue. The secondary hyphae starts to develop after 49-72 hours mainly in substomic space, progresses towards the spongy parenchyma and continues to spread towards the palisade parenchyma, creating necrotic tissue (Sami Saad and Hagedorn, 1969). During the spreading of the infection, in the centre of the spots, the organs of the pathogen are developing, and concentric rings are created around them. A yellowish, chlorine belt is created around the spots (Figure 1).

By isolation of the pathogen from the spots of the infected tobacco leaves we got pure culture of the fungus (Figure 2).

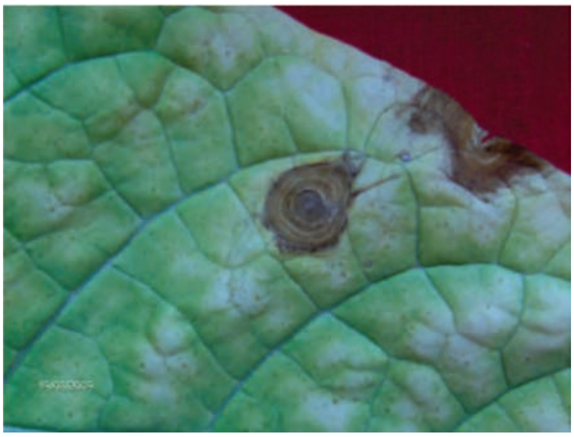

Figure 1. A. alternata - spots on infected tobacco leaf

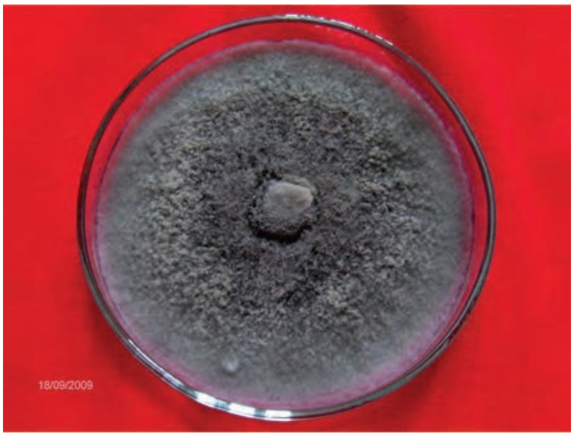

Figure 2. A. alternata - pure culture

The hyphae of the fungus A. alternata which develop in the spots of the infected tobacco leaves are 2.00-5.20 µm wide, or 3.19µm in average. When they develop in pure culture, their width is increased and ranges 2.80 - 6.00 µm, or 3.87 µm in average. The biggest increase of width can be noticed in the isolate B36 (Table 1).

In natural conditions, because of the larger conidia, the hyphae are less noticeable than in pure culture. They are satirized, branch laterally under different angle, they are colorless or they have light brown color (Figure 3).

The width of conidiophores of the fungus of the infected leaves is between 2.00 and 8.00 µm, and the length from 4.00 to 56.00µm, with an average value of the size 4.04 x 25.12µm. Conidiophores of the isolate B36 have the smallest, and those of the isolate V22 the biggest dimensions (Table 2).

Table 1. The width of the hyphae of *Alternaria alternata* (μm)

Isolate	Infected leaves		Pure culture	
	Width			
	From-to	Average	From-to	Average
V 12	2.80 - 3.20	2.93	2.80 - 6.00	3.65
V 13	2.80 - 5.20	3.67	4.00 - 4.70	4.59
V 22	2.00 - 4.00	3.12	3.20 - 4,23	3.55
B 36	2.00 - 4.00	2.68	3.20 - 4.70	4.14
B 98	2.80 - 4.00	3.53	3.20 - 4.23	3.44
Average	2.00 - 5.20	3.19	2.80 - 6.00	3.87

Table 2. Size of conidiophores of *Alternaria alternata* (μm) (infected leaves)

Isolate	Width		Length		Width x length
	From-to	Average	From-to	Average	
V 12	2.00-4.00	3.72	10.00-38.00	27.60	3.72 x 27.60
V 13	2.80 -4.80	3.84	4.00-56.00	26.00	3.84 x 26.00
V 22	4.00 -8.00	4.80	14.00-48.00	30.00	4.80 x 30.00
B 36	3.20 -3.90	3.90	8.00-32.00	19.50	3.90 x 19.50
B 98	2.80 -4.00	3.92	12.00- 36.00	24.00	3.92 x 24.00
Average	2.00- 8.00	4.04	4.00-56.00	25.42	4.04 x 25.42

Figure 3. *A. alternata* – Hyphae in a pure culture

The smallest width of conidiophores formed in the pure culture is 2.80 μm, and the largest 4.80 μm. Their length ranges from 8.00-60.00 μm. In average, the conidiophores size in the pure culture is 3.78 x 31.04 μm. In the isolate V12, the high value of the length is especially expressed (Table 3).

It can be concluded that the conidiophores of the fungus of the infected leaves during the transfer into artificial conditions get smaller width and larger length (Table 2 and 3).

The process of sporulation is induced by the sunlight and the lower frequency of ultraviolet rays and darkness in alternation. In the first phase there is a formation of conidiophores, which is stimulated by light, and than in the secondary phase, formatting of the conidia, which is inhibited by the light. The photoinduction is more active in higher temperatures (38 degrees), while the terminal in lower temperatures (Lacey, 1992). High temperatures like these, as well as photo period are present during the vegetation of tobacco, which affects both phases, and directly influences the size of conidiophores.

Table 3. Size of conidiophores of *Alternaria alternata* in pure culture (µm)

Isolate	Width		Length		Width x Length
	From-to	Average	From-to	Average	
V 12	2.80-4.00	3.72	16.00-60.00	36.20	3.72 x 60.00
V 13	3.20 -4.80	3.92	12.00-32.00	23.00	3.92x 23.00
V 22	3.20 -4.00	3.73	16.00-36.00	24.67	3.73x 24.67
B 36	3.20 -4.00	3.82	20.00 - 60.00	36.67	3.82 x 36.67
B 98	3.20 -4.00	3.73	8.00 - 44.00	34.67	3.73 x 34.67
Average	2.80- 4.80	3.78	8.00 -60.00	31.04	3.78 x 31.04

Conidiophores of the fungus of natural substrate in artificial conditions of cultivation are shown in Figures 4 and 5.

Figure 4. *A. alternata* – Conidiophores from spots of infected leaves

Figure 5. *A. alternata* – Conidiophores in pure culture

The width of conidia from the fungus formed on infected tobacco leaves is between 8.00 and 20.00 µм and the average value is 14.12 µm. The largest is the width of the conidia of B36 isolate. The length has the largest range between 28.00 and 84.00µm, and the average is 55.90µm. The conidia of the isolate V12 are characterized by their length, and they are almost twice the length of the conidia of other isolates (Table 4).

The average values of the conidia formed on the infected leaves are 14.12 x 55.90µm.

Su and Sun (1981) found polymorphic conidia in the infected tobacco leaves, with golden-brown body and light brown to transparent beak, long chain that are sometimes outspread, with diagonal, lengthwise and inclined partitions. Their size is bigger than the size of that from a culture grown on a surface.

Table 4. Size of the conidia of A. alternata (µm)
Infected leaves

Isolate	Width		Length		width x length
	From-to	Average	From-to	Average	
V 12	8.00- 16.00	13.36	48.00 -78.00	81.08	13.36 x 81.08
V 13	12.00 -20.00	15.04	42.00 -74.00	52.93	15.04 x 52.93
V 22	8.00- 16.00	12.68	44.00 -84.00	56.00	12.68 x 56.00
B 36	10.00 -20.00	15.88	28.00 - 54.00	41.07	15.88 x 41.07
B 98	10.00 - 20.00	13.64	28.00 - 68.00	48.40	13.64 x 48.40
Average	8.00 - 20.00	14.12	28.00 -84.00	55.90	14.12 x 55.90

In our investigations, also, the conidia formed in pure culture are smaller than those of the infected leaves (Table 5, Figure 6 and 7). Their average size is 11.05 x 30.16 µm. According to Misaghi et al (1978), the ecological factors largely change the size of its conidia. Slavov et al. (2004) say that the size of the conidia, the shape and the segmentation varies depending on the age of the spores, substrate, pH value, the temperature, the humidity and the light. The changes are noticeable even in the same isolate.

Table 5. Size of the conidia of A. alternate in a pure culture (µm)

Isolate	Width		Length		width x length
	From-to	Average	From-to	Average	
V 12	8.00 - 16.00	11.30	20.00- 44.00	31.45	11.30 x 31.45
V 13	8.00 -14.00	11.24	24.00 -48.00	33.16	11.24 x 33.16
V 22	8.00 - 16.00	11.60	17.20 -40.00	27.15	12.00 x 27.15
B 36	8.00 - 15.20	10.21	18.00 - 44.00	28.59	10.21 x 28.59
B 98	8.00 - 16.00	10.90	22.00 - 48.00	30.45	10.90 x 48.00
Average	8.00 -16.00	11.05	17.20 - 48.00	30.16	11.05 x 30.16

The conidia have lower upper limit of the width, and so the average value is smaller. The biggest change can be noticed in the isolate B36, where from the biggest (Table 4), now the conidia have the smallest width (Table 5).

The length is almost twice shorter than of the conidia upon the natural substrate, which can be noticed in the marginal and in the average value too. Significant change, i.e. shortening of the length of the conidia has the isolate V 12; it is shortened for 60%. According to Rotem (1994), the length of the spores is more relevant factor than the width and it contributes for the identification of the pathogen. The same author gives data for the morphological changes of A. alternata of the same tobacco leaf: the length of the conidiophores is 20 to 50 µm, the conidia have 4-6 septa, and they are 11-13 µm and long 30-86 µm. The values obtained in our investigations correspond with the given values.

According to Ritz (1995), besides the good development of substances with poorer nutritive mediums, reproductive structures of A. alternata have tendency to form on rich medium. The large leaf-tobaccos of the Virginia type are characterized with high content of sugars, which had an influence on the profuse sporulation, as well as on the size of conidia on the infected leaves. In the investigations of Hubballi, (2010), the surface with the extract of a leaf of the hoist had maximum influence on the development of all 15 tested isolates of A. alternate, followed by potato-dextrose surface (KDA). All the isolates sporulated on the KDA too, but they differ in the extent of sporulation, followed by different physiognomy of the colony.

Figure 6. A. alternate-conidia of the spots on infected leaves

Figure 7.A. alternate- conidia in a pure culture

The conidia formed on infected tobacco leaves have 2-8 diagonal septa, 0-4 lengthwise and 0-3 inclined septa. In a pure culture they have maximum 6 diagonal, 3 lengthwise and 2 inclined septa. The conidia of the isolate V 13 have the largest number of diagonal septa (8 i.e. 6) on the infected leaves as well as in the pure culture. The isolate V 22 has conidia with the largest number of inclined septa only on the infected leaves (Table 6).

Table 6. Number of septa in the conidia

Isolate	Infected leaves			Pure culture		
	Diagonal	Lengthwise	Inclined	Diagonal	Lengthwise	Inclined
V 12	3 - 7	0 - 3	0 - 2	2 - 4	1 - 2	0 - 2
V 13	2 - **8**	1 - 4	0 - 2	3 - **6**	1 - 2	0 - 2
V 22	3 - 7	0 - 3	0 - **3**	2 - 4	1 - 3	0 - 1
B 36	3 - 5	0 - 2	0 - 1	2 - 5	1 - 3	0 - 2
B 98	2 - 6	1 - 4	0 - 2	2 - 6	1 - 2	0 - 2
From-to	2 - 8	0 - 4	0 - 3	2 - 6	1 - 3	0 - 2

The highest percent of conidia formed on the infected leaves have 3 and 4 diagonal, 1 or 2 lengthwise and they do not have, or have only one inclined septum (Table 7a and b). The percentage of conidia with 3 diagonal septa is the biggest in the isolate B 36. Only the isolate V 13 has conidia with 8 diagonal septa (Table 7a). In the isolate B36, 71.43% of the conidia do not have inclined septa. The isolate V 22 even with only 10 % is the only one that has conidia with 3 inclined septa (Table 7b).

According to Simons (2007), the conidia have 1-7 (very often 3) diagonal and a small number or do not have lengthwise septa at all. In the examination of Kumar (2008), the conidia have 6-7 diagonal and 0-3 lengthwise septa.

Table 7. The percentage of conidia with different number septa (infected leaves)

a) diagonal

Isolate	Number of septa						
	2	3	4	5	6	7	8
	% of conidia						
V 12	0.00	8.33	16.67	25.00	25.00	16.67	0.00
V 13	6.67	26.67	20.00	13.33	13.33	6.67	**6.67**
V 22	0.00	20.00	40.00	20.00	0.00	0.00	0.00
B 36	0.00	**60.00**	26.67	6.67	0.00	0.00	0.00
B 98	6.67	26.67	26.67	26.67	6.67	0.00	0.00
Average	2.67	**28.34**	**26.00**	18.33	9.00	4.67	1.33

b) Lengthwise and inclined

Isolate	Lengthwise					Inclined			
	Number of septa								
	0	1	2	3	4	0	1	2	3
	% of conidia								
V 12	27.27	27.27	27.27	18.18	0.00	63.64	18.18	18.18	0.00
V 13	26.67	40.00	20.00	13.33	**13.33**	60.00	26.67	13.33	0.00
V 22	20.00	40.00	30.00	10.00	0.00	40.00	30.00	20.00	**10.00**
B 36	21.43	50.00	28.57	0.00	0.00	**71.43**	28.57	0.00	0.00
B 98	6.67	53.33	13.33	20.00	6.67	60.00	26.67	13.33	0.00
Average	20.41	**42.12**	**23.83**	12.31	4.00	**59.01**	**26.02**	12.97	2.00

Table 8. The percentage of conidia with different number septa (pure culture)
a) diagonal

Isolate	Number of septa				
	2	3	4	5	6
	% of conidia				
V12	13.33	**60.00**	26.67	0.00	0.00
V 13	0.00	33.33	33.33	0.00	**25.00**
V 22	13.33	6.67	20.00	0.00	0.00
B36	31.25	40.00	26.67	6.67	0.00
B 98	6.25	37.50	43.75	6.25	6.25
Average	12.83	**35.50**	**30.08**	2.58	6.25

b) Lengthwise and inclined

Isolate	Lengthwise				inclined		
	Number of septa						
	0	1	2	3	0	1	2
	% of conidia						
B 12	13.33	**66.67**	20.00	0.00	**80.00**	6.67	13.33
B 13	25.00	41.67	33.33	0.00	**75.00**	16.67	8.33
B 22	33.33	33.33	26.67	6.67	**80.00**	20.00	0.00
Б 36	18.75	56.25	18.75	6.25	**87.50**	6.25	6.25
Б 98	50.00	31.25	18.75	0.00	**50.00**	31.25	12.50
Average	28.08	**45.83**	23.50	2.58	**74.50**	16.19	8.09

The highest percentage of conidia have 3 and 4 diagonal septa even when they are formed in pure culture. The percentage of separation with 3 diagonal septa in the pure culture is the highest in the isolate V 12. Again, the isolate V 13 has the highest number (6) of diagonal septa (Table 8a).

The highest percentage, i.e. 45.83% of the conidia has lengthwise septum, and in relation to the isolates, the highest percentage was observed in the isolate V 12. The percentage of conidia without inclined septa is very high in all the isolates, as well as their average value (Table 8b).

CONCLUSION

- A. alternate in natural conditions (spots on tobacco leaves) forms hyphae with an average width of 3.19 µm, while in pure culture they are larger and are 3.87 µm wide.
- Conidiophores formed on infected tobacco leaves have an average size of 4.04x 25.42µm.
- In the transfer in artificial conditions, the conidiophores get smaller width, but larger length. Their dimensions are 3.78 x 31.04µm.
- The average values of the conidia formed on infected leaves are 14.12 x 55.90µm.
- The conidia formed in pure culture are smaller than those formed on the infected leaves. Their average size is 11.05 x 30.16µm.
- The conidia formed on the infected leaves have 2-8 diagonal septa, 0-4 lengthwise and 0-3 inclined septa. In a pure culture the septaration is smaller, i.e. they have maximum 6 diagonal, 3 lengthwise and 2 inclined septa.
- The highest percentage of conidia formed on the infected tobacco leaves have 3 and 4 diagonal and 1 or 2 lengthwise septae and they have none or only one inclined septum.
- In the pure culture, the highest percentage of the conidia also have 3 and 4 diagonal septa and 1 lengthwise septum. High percentage of them do not have inclined septa.

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