Serine Proteinases Secreted by Two Isolates of the Fungus *Alternaria solani*

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Abstract: It is well-known *Alternaria solani* Sorauer is the causative agent of alternariosis. In this paper, serine proteinases secretion by two genetically related isolates of the fungus, collected from potato and tomato plants grown in central Russia have been studied. The data clarify functions of these enzymes in the process of pathogenesis in which they can play a pivotal role. Also, the data should allow classifying *Alternaria*'s strains more precisely. It was found that the two isolates produced trypsin-like and subtilisin-like proteinases during growth both in synthetic culture medium and in medium containing heat-stable vegetable proteins. There were significant differences in the influence of the environment on the serine proteinase secretion by the potato and tomato isolates of *A. solani*. The proportion of such serine proteinases as trypsin-like and subtilisin-like enzymes depends on the composition of the growth medium, especially on the available organic nitrogen form, as well as features both of the pathogenic fungus and of the host plant. So, the tomato isolate demonstrated weak growth and low level or absence of serine proteinase excretion on cultivation with the medium containing proteins extracted from potato tubers and pea seeds. The potato isolate secreted many more serine proteinases, among which the trypsin-like enzymes dominated. Our data suggest that the tomato isolate, when grown on medium with proteins extracted from potato tubers, lost pathogenicity and became to behave as a saprophyte, while the potato isolate retained its pathogenic properties on growth on any tested medium.

Keywords: Fungus, trypsin-like, subtilisin-like, proteinase.

1. INTRODUCTION

The genus *Alternaria* is widespread and has great economic importance because it causes rotting of flowers, leaves, and roots of plants, destructive leaf spots, and damages products or seed of numerous hosts, especially *Solanaceae*, during storage [1]. This genus includes a heterogeneous group of saprophytic and pathogenic fungi, that belong to the division Ascomycota, class Dothideomycetes, order Pleosporales, and family Pleosporaceae [2]. The fungus *Alternaria solani* Sorauer, which is the causative agent of alternariosis of potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.), can be attributed to one of the most famous and malicious members of this genus [1]. The fungus infects the plant leaves and fruits [3]. The damage symptoms do not depend on the type of the parasite, but sometimes they vary depending on host plant distinctions. In recent years a taxonomic revision of the genus *Alternaria* has taken place, and many new species has been described, including some occurring on the Solanaceae [4]. It is known that the distribution area of large-spore species of *A. solani* is restricted to European Russia center, North Caucasus, the south of Western Siberia, and the regions of Baikal and the Russian Far East. Such distribution is due to their narrow specialization and considerable sensitivity to climatic conditions, because distinct *Alternaria* species can differ greatly in physiological and ecological properties [5]. Heterotrophic nutrition type, which is characteristic for these fungi, determines their ability to form biotic relationships with representatives of different kingdoms of living organisms [6, 7].

Isolates of the *A. solani* large-spore species form two large clusters and belong to 18 haplotypes in total. There is rigorous proof that the subpopulation of *A. solani* causing potato rot is genetically distinct from the one that causes tomato rot [5]. Morphological identification of species has a number of difficulties related to the dependence of morphology on the composition of the medium, temperature, mode of illumination, etc. Large-spore strains often do not form a conidial sporulation on composed growth medium. The high level of polymorphism is associated with the high degree of variability of physiological, pathogenic, and genetic properties of *A. solani* populations. Therefore, special importance is attached to species features that do not depend on the morphology. These include, for example, the structural and biochemical characteristics of the fungal genome and their proteins, in particular proteinases.

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It is known that hydrolytic enzymes secreted by fungi play a pivotal role in the maintenance of biotic relationships. The hydrolytic enzymes of the fungus control the processes of the pathogenesis. They break down the host plant cell walls and thus provide penetration of the pathogen into the plant tissue [8-10]. Also, they participate in the destruction of the protein shell of spores during germination and penetration of hyphae through the cell wall, hydrolyzing both protective proteins and exogenous nutrients providing the cell with monomers. Thus extracellular proteinases of fungi not only play a special role in pathogenesis, but they also decompose required food substrates [11, 12]. It was shown that nonpathogenic mutants of the fungus *Pyrenopeziza brassicae*, a leaf pathogen of host plants of the crucifer family (Cruciferae), do not have the ability to produce extracellular cysteine proteinase. Recovery of pathogenicity in these mutants was accompanied by the recovery of their ability to form this proteinase [10].

Synthesis and secretion of proteinases of the fungus is an energy-intensive process, which leads to the formation of enzymes most adapted to the environmental conditions of the mycelium. Therefore, differences in the composition of proteinases secreted by the mycelium developing in various systems indirectly reflect the environmental and metabolic characteristics of the fungi.

Many pathogenic fungi produce proteolytic enzymes, and their activity determines their ability to use proteins of the host plant [13-15]. Among them serine proteinases are dominant, although there are enzymes belonging to other mechanistic classes. All known serine proteinases of pathogens can be divided into trypsin-like and subtilisin-like enzymes. It has been shown that the genome of the majority of fungal plant pathogens contains genes encoding trypsin-like proteinases. Analysis of available fungal genomes has shown that 95% of species whose genome sequences have been found to be similar to trypsin were pathogens [16, 17]. It is very likely that the trypsin-like enzymes can be considered as markers of fungal pathogenicity.

In this paper, the composition and dynamics of secretion of serine proteinases during the growth of two isolates of the fungus *Alternaria solani* Sorauer have been studied. The data should allow these organisms to be classified more precisely and to determine not only the degree of their pathogenicity, but their dependence on the nutrient substrate.

2. MATERIALS AND METHODS

2.1. Materials

The substrates: azocasein, N,α-benzoyl-L-Arg-pNa (BAPNA), N-Succinyl-Gly-Gly-L-Phe-pNa (Suc-GGFPNa), and N-carbobenzyloxy-L-Ala-L-Ala-L-Leu-pNa (Z-AALPNA) were purchased from Sigma-Aldrich (USA). Low-molecular weight protein markers were from Pharmacia (Sweden). All other reagents were of the highest grade commercially available.

2.2. Microbiological Material

Two isolates sampled from potato (isolate 043-021) and tomato (isolate A7AKTL125) leaves in the central part of Russia were studied. These isolates belong to the large-spore species of *Alternaria solani* Sorauer [18]. The cultures were maintained on oatmeal agar and stored at room temperature (21°C). Ten milliliters of mycelial suspension in distilled water was used to inoculate 200 ml of the liquid growth medium. The isolates were cultured on three mediums. Medium A contained modified Czapek's medium in which instead of the mineral nitrogen, 1% casein was added. Medium B was a potato-carrot broth that contained heat-stable proteins and carbohydrates from potato tubers and carrot roots. Medium C was pea-carrot broth that contained heat-stable proteins and carbohydrates from pea seeds and carrot roots. Basing on the chemical composition of the vegetable components [19-21] used for the preparation of B and C media we can assume that the medium B was to be rich in carbohydrates whereas the medium C was more balanced in ratio of proteins and carbohydrates. These mediums were tested for suitability to give good growth as well as for adequate enzyme production. Mycelium was harvested on a weighed Whatman No. 41 filter paper after 6, 12, and 17 days of the growth of the microorganism. It was washed with a small quantity of warm distilled water, heated overnight in an oven at about 90°C, cooled in a desiccator, and weighed. Further loss in weight was not obtained by longer periods of drying. Crude culture filtrate obtained after harvesting mycelium was used for enzyme assays.

2.3. Enzyme Assays

Proteolytic enzyme activity was determined by method [22], using as substrate 0.5% azocasein in 0.1 M Tris-HCl buffer, pH 7.5. One unit of proteolytic activity (U) was the amount of enzyme that increased optical density at 366 nm in the supernatant by 0.1 per

min after precipitation by TCA of the reaction mixture proteins. Amidase enzyme activity was determined by the method of Erlanger *et al*. [23], using *p*-nitroanilide substrates: BAPNA, Suc-GGFPNa, and Z-AALPNA. The substrate concentration was 0.5 mM. One unit of amidase activity (AU) was the amount of enzyme that hydrolyzed 1 nmol of the substrate in min.

2.4. SDS-PAGE

SDS-PAGE was performed in the presence of copolymerized substrate (0.1% gelatin) by the method of Heussen and Dowdle [24]. Samples consisted of 30 µg of freeze-dried culture liquid, which were applied to each lane after dissolving in the sample buffer. Gels were stained with 0.1% Amido black solution in ethanol: acetic acid: water (3:1:6) for 1 h and washed with the same solution without the dye. Proteins with proteolytic activity were detected as colorless bands on the dark blue background of the colored gel.

2.5. Statistical Analysis

Experiments were performed in 3-4-fold repetition. Data are presented as means ± standard error of the mean. Significant difference was defined as $p < 0.05$.

3. RESULTS

3.1. Accumulation of Mycelium Biomass and Proteolytic Exoenzymes

It is known that one of the most important factors determining the secretion of fungal extracellular enzymes is the availability of the optimal substrate at the optimal concentration in the growth medium. In this regard, the effect of the composition of medium nutrients on exoproteinases production of two *A. solani* Sorauer isolates, that were sampled from potato and tomato leaves, respectively, and that hereafter are referred to as potato (043-021) and tomato (A7AKTL125) isolates, were studied in controlled batch cultures.

For each experiment, one of three growth mediums was used. Medium A contained a modified Czapek's medium with 1% casein as the protein source; medium B contained heat-stable water-soluble proteins of potato tubers only, and medium C contained heat-stable water-soluble proteins of pea seeds only. It is known that proteins extracted from the pea seeds and potato tubers contain all of the essential amino acids, so they are characterized by high biological value.

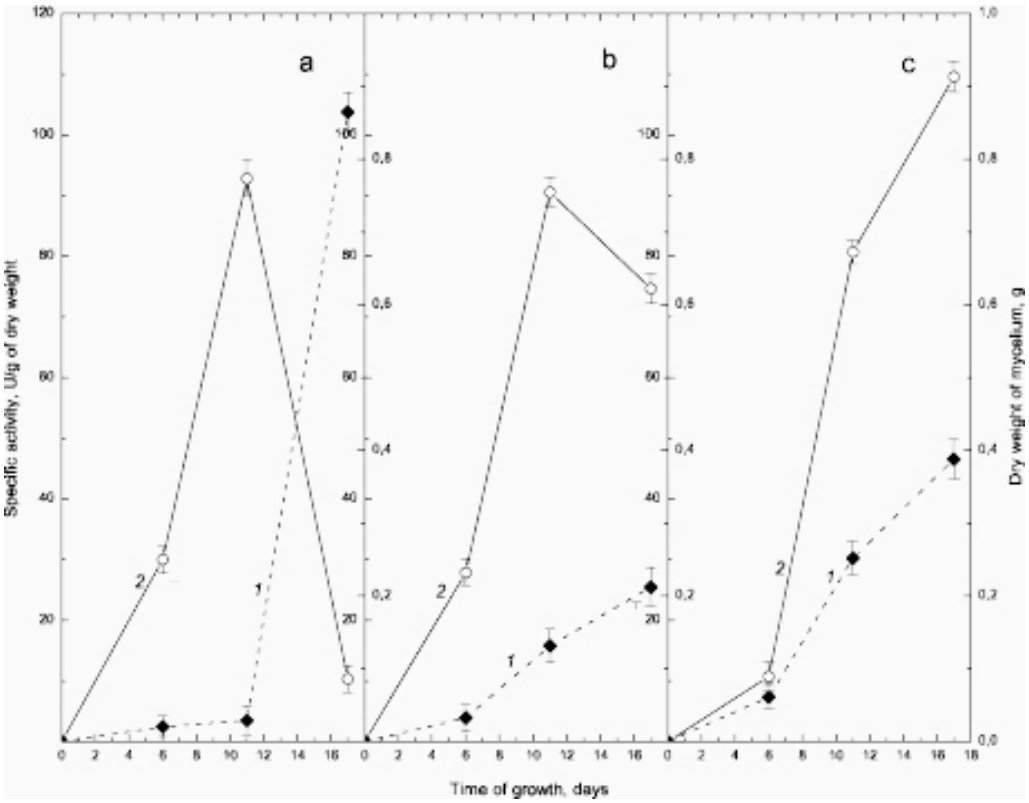

Figure 1: Dynamics of the dry weight of mycelium (1) and exoproteolytic activity (2) of *Alternaria solani* 043-021 during the growth of the fungus on different nutritional mediums: **a** – modified Czapek's medium containing 1% casein instead of mineral nitrogen, **b** – potato-carrot broth, and **c** – pea-carrot broth; azocasein used as a substrate.

Although the amino acids can be used as the sole source of carbon and nitrogen for the fungi, the absence of carbohydrates can reduce exoproteinase production. It was shown that the presence of carbohydrates (over 1%) in the growth medium of the microorganisms increased (from 2 to 15 times) the proteolytic enzyme secretion, whereas their lack (less than 0.1%) in some cases it suppressed [25]. So carrot roots were additionally used as a source of carbohydrates for the growth medium.

Figures **1** and **2** show dynamics of growth of the *A. solani* isolates at 20°C with all mediums tested. Mycelium growth of both isolates was observed up to 17 days regardless of the medium chosen, but after 6 days the biomass grew increasingly. One can see that the most active mycelium growth of the potato isolate occurred with medium A, and the greatest increase in biomass was observed after 12 days (Figure **1a**, *curve 1*). The active mycelium growth of the potato isolate started after 6 days of growth with medium B (Figure **1b**, *curve 1*). A similar increase in the biomass of this isolate was observed with medium C, but its growth rate was much higher (Figure **1c**, *curve 1*). The mycelium growth of the tomato isolate was significantly lower with all chosen mediums. When mediums A and C were used, the active growth of the biomass of the tomato isolate began after 6 days of cultivation (Figures **2a** and **2c**, *curves 1*), while biomass accumulation in medium B began after 12 days of cultivation (Figure **2b**, *curve 1*). At the same time, the biomass growth of this isolate with medium C was slowed after 11 days of cultivation, and it remained at a constant level up to 17 days (Figure **2c**, *curve 1*). It should be noted that the tomato isolate accumulated significantly lower amounts of biomass compared with the potato isolate.

Thus, it can be assumed that the growth of pathogenic fungal mycelium is defined both by the form of available organic nitrogen and peculiarities of the pathogens. The data indicated clear relationship between nitrogen source and its concentration. Based on the observed relationships, the selection of environmental factors to increase secreted proteinase activity is not straightforward, as unexpected antagonistic or synergistic effects can occur.

The secretion of extracellular proteolytic activity into the culture fluid accompanied the mycelium growth of these two isolates in all used mediums. The level of the

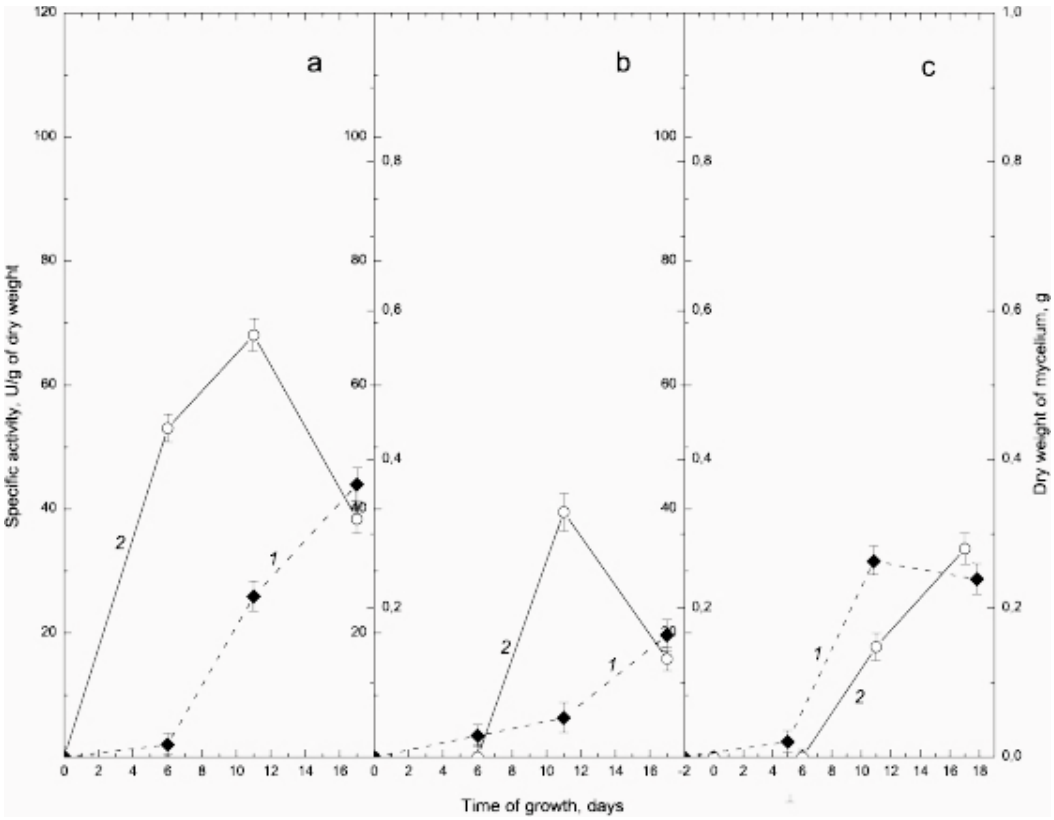

Figure 2: Dynamics of the dry weight of mycelium (1) and exoproteolytic activity (2) of *Alternaria solani* A7AKTL125 during the growth of the fungus on different nutritional mediums: **a** – modified Czapek's medium containing 1% casein instead of mineral nitrogen, **b** – potato-carrot broth, and **c** – pea-carrot broth; azocasein used as a substrate.

secretion depended on the amount of the available protein. The increase in proteolytic activity in the cultural fluid observed in the exponential phase of the culture growth and its maximum level also depended on the selected culture medium (Figures **1** and **2**, *curves 2*). So, when medium A was used, the maximum activity of the enzymes secreted by both isolates was reached by day 11 of culture growth. Then there was a sharp decrease in the proteinase secretion on the background of increasing biomass (Figures **1a** and **2a**, *curves 2*). Thus, with the synthetic modified Czapek's medium the character of the change in the proteolytic activities secreted by both isolates was almost the same.

The proteolytic activity secreted by the potato isolate increased from the first days of culture growth on medium B. This activity achieved a maximum at days 10-11 of the development of the culture and then followed a sharp decrease (Figure **1b**, *curve 2*). When the potato isolate was cultivated with medium C, the exoproteolytic activity in the culture increased during the entire development cycle. The proteolytic activity was considerably increased after 6 days of culture growth and reached a maximum on day 17 (Figure **1c**, *curve 2*).

The proteolytic activities of the tomato isolate grown on medium B and C were significantly lower and appeared only by day 6 of development of the culture (Figures **2b** and **2c**, *curves 2*). A sharp increase in the secreted proteolytic activity was observed on days 10-11 of culture growth if medium B was used, and then it decreased sharply (Figure **2b**, *curve 2*). During cultivation of the tomato isolate on medium C the exoproteolytic activity was growing evenly throughout the development cycle, reaching a maximum at 17 days of culture growth (Figure **2c**, *curve 2*).

Thus, the proteolytic activity of the enzymes secreted by the isolates in medium A containing casein quickly reached a maximum and then decreased quite sharply. An increase in the exoproteolytic activity under growth of both isolates on mediums B and C containing vegetable proteins without any additional additives occurred gradually. The data suggest a direct link between the amount of available organic substrate and the level of the exocellular proteolytic activity secreted by the fungus. It is very likely that the rapid drop in measured activity after reaching a maximum is a consequence of depletion of the protein substrate in the culture medium, and therefore there is no need to maintain a certain level of enzyme protein hydrolysis. This indicates that the enzyme secretion is not directly related to the accumulation of the fungal biomass, and it is more dependent on the productivity of the mycelium. It is possible that the decrease in the exoproteolytic activity level in the culture medium with pure protein (1% casein) may be due to catabolite repression of the enzyme synthesis by products of proteolysis of the proteins.

The data indicated that *A. solani* exoproteinases are produced as the primary metabolites that are necessary to sustain of the fungal cells.

3.2. Specificity Some Exoproteinases of A. Solani

The exoenzymes secreted by both isolates of *A. solani* were the most effective at neutral and slightly alkaline pH values and were characterized by a maximum at pH 8.0-8.5 (data not shown).

A comparative study of the substrate specificity of extracellular proteinases secreted by two isolates of *Alternaria* is shown in Figures **3** and **4**. The results of the study of the activity of the enzymes secreted by the two isolates of the fungus confirmed that exoproteinases of the ascomycete *A. solani* efficiently hydrolyze BAPNA (a substrate for trypsin-like proteinases) and to a lesser extent Z-AALPNA (a substrate for subtilisin-like proteinases) regardless of whether the pathogen infects leaves of potato or tomato. They did not affect Suc-GGFPNa (a substrate for chymotrypsin-like proteinases). But the ratio of the trypsin-like and subtilisin-like activities depended on both the composition of the culture medium and the nature of the isolate.

On growth of potato isolate 043-021 on medium A, an increase in the secretion of trypsin-like proteinases, which started at 6 days, was observed. This increase reached a maximum at days 10-11 of cultivation. Then the activity of the trypsin-like proteinases began to decrease abruptly (Figure **3a**, *curve 1*). Subtilisin-like proteinases were secreted initially and their activity reached a maximum at 6 day, and then decreased to 12 day and then remained constant (Figure **3a**, *curve 2*).

Secretion of subtilisin-like and trypsin-like proteinases by the potato isolate in medium B with high content of carbohydrates started from the first days of the development of the culture (Figure **3b**, *curves 1,2*). The trypsin-like activity increased rapidly and reached a maximum in 11-12-day culture (Figure **3b**, *curves 1*). Then it decreased sharply, and on day 17 of cultivation

it was not detected. The subtilisin-like activity increased exponentially, and at day 17 of the culture growth reached a maximum value (Figure **3b**, *curve 2*).

In medium C relatively balanced in protein and carbohydrates, the trypsin-like and subtilisin-like proteinases were secreted together by isolate 043-021 starting from 6 days of growth and reached a maximum on day 17 of culture growth (Figure **3c**, *curves 1,2*).

Trypsin-like and subtilisin-like proteinases were secreted by tomato isolate A7AKTL125 simultaneously from the first day of culture growth on medium A. The increase in both activities was similar to 6 day, reaching a maximum (Figure **4a**, *curves 1,2*). Thereafter, there was a sharp drop in the secretion of the trypsin-like proteinases. Their secretion began to increase again after 11-day culture (Figure **4a**, *curve 1*). The activity of the subtilisin-like proteinases produced by the tomato isolate decreased fractionally and remained constant until the end of the cultivation (Figure **4a**, *curve 2*).

Secretion of serine proteinases by this isolate in medium B was not detected. The tomato isolate did not secrete trypsin-like proteinases in medium C. However, the activity of subtilisin-like proteinases appeared after 6 days of growth and increased until day 17 (Figure **4c**, *curve 2*).

It can be assumed that in the early stages of growth both isolates secreted mainly subtilisin-like proteinases. Furthermore, it should be noted that the composition of simple carbohydrates present in the culture medium had no significant effect on the qualitative and quantitative characteristics of the extracellular enzymes.

3.3. Gel Electrophoresis

SDS-PAGE showed that these isolates secreted three or more proteins with proteolytic activity differing in molecular mass (Figures **5**, **6**). Both isolates in medium A secreted proteinases with molecular mass values of 90, 50, and 40-30 kDa to 6 day growth, and change in the secreted enzyme composition was not observed, only an increase in their content (Figures **5** and **6**, lanes *1-3*). This may indicate that the full range of proteinases was secreted from the first stages of development of the microorganism in medium A

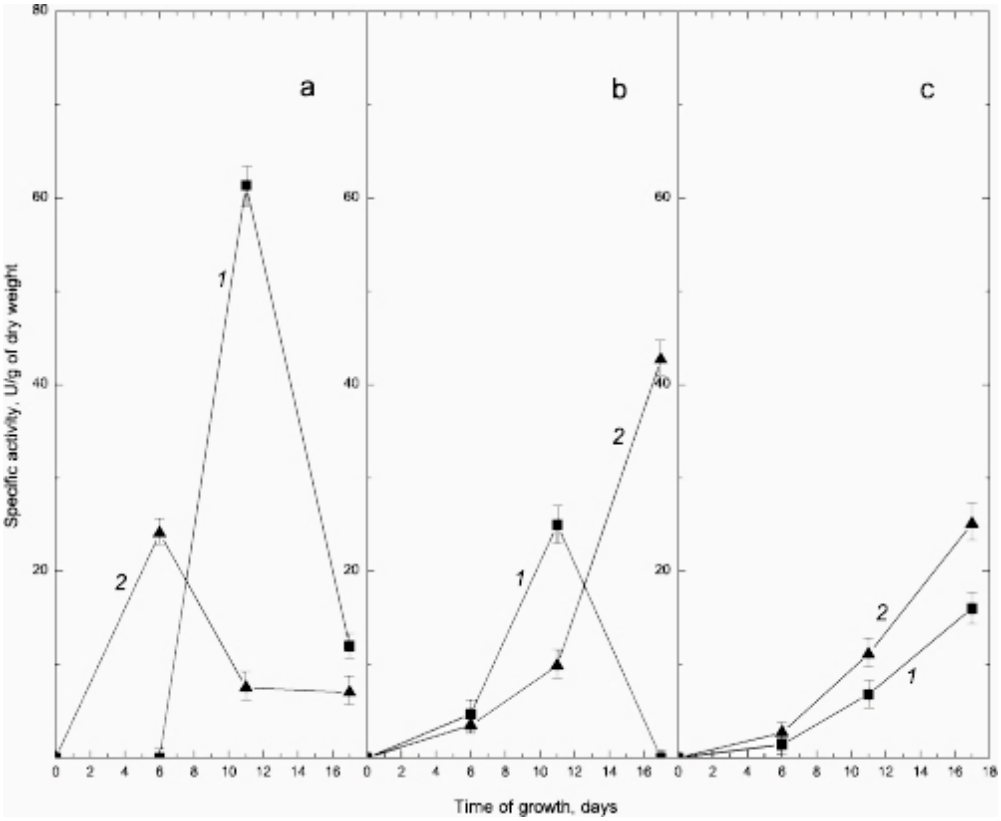

Figure 3: Dynamics of changes in trypsin-like (1) and subtilisin-like activities (2) of *Alternaria solani* 043-021 during the growth of the fungus on different nutritional mediums: **a** – modified Czapek's medium containing 1% casein instead of mineral nitrogen, **b** – potato-carrot broth, and **c** – pea-carrot broth; BAPNA used as a substrate for trypsin-like proteinases, Z-AALPNA - a substrate for subtilisin-like proteinases

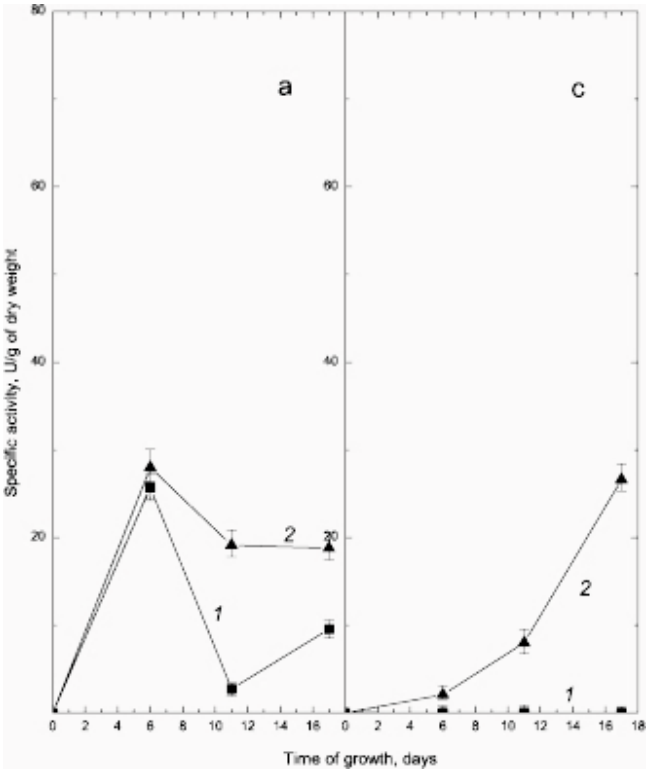

Figure 4: Dynamics of changes in trypsin-like (1) and subtilisin-like activities (2) of *Alternaria solani* A7AKTL125 during the growth of the fungus on different nutritional mediums: a – modified Czapek's medium containing 1% casein instead of mineral nitrogen, c – pea-carrot broth (the activities were practically not found during the growth on potato-carrot broth); BAPNA used as a substrate for trypsin-like proteinases, Z-AALPNA - a substrate for subtilisin-like proteinases.

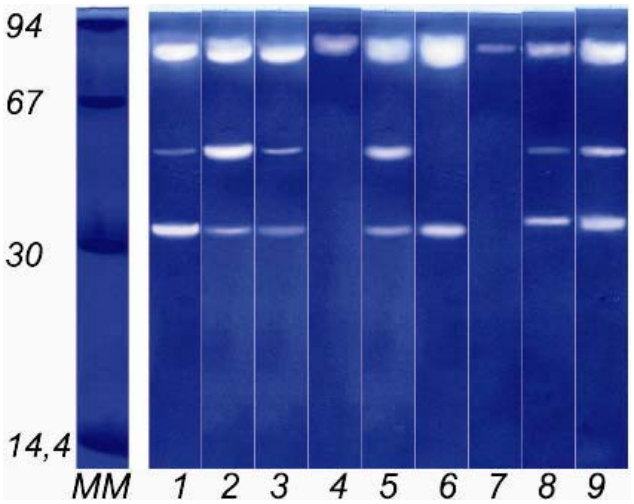

Figure 5: Zymograms of exoproteinases of *Alternaria solani* 043-021 during growth on different nutritional mediums: lanes 1-3 – modified Czapek's medium containing 1% casein, lanes 4-6 – potato-carrot broth, and lanes 7-9 – pea-carrot broth. Lanes 1, 4, and 7 correspond to the 6thday of fungus growth, lanes 2, 5, and 8 correspond to the 11th day, and lanes 3, 6, and 9 correspond to the 17th day of fungus growth. Lane MM represents the molecular mass (kDa) markers as follows: phosphorylase b (94), bovine serum albumin (67), carboanhydrase (30), and lactalbumin (14.4). The zymograms were obtained after SDS-PAGE in the presence of gelatin.

balanced in protein and carbohydrate content. However, the composition of the exoproteinases of both isolates changed significantly during the growth of the microorganisms. During the first 6 days of development of the potato isolate, exoproteinases with molecular masses of about 90 and 30 kDa predominated (Figure **5**, lane *1*). At 12 days of development, there was a noticeable predominance of exoproteinases with molecular masses of about 50 kDa (Figure **5**, lane *2*). At 17 days, the content of proteins with molecular masses of 50 and 30 kDa were approximately the same (Figure **5**, lane *3*). The composition of the proteins secreted by the tomato isolate was constant throughout the cycle of the development of the fungus on medium A. The tomato isolate A7AKTL125 secreted high (90 and 65 kDa) as well as lower molecular mass (about 25-30 kDa) proteinases (Figure **6**, lanes *1-3*).

Potato isolate 043-021 started to secrete 90-kDa proteinases in mediums B and C (Figure **5**, lanes *4, 7*). It should be noted that the content of the proteinases secreted by the potato isolate varied depending on the incubation medium. Thus, the 50-kDa proteinase was not detected in medium B on the 6th and 17th days of growth, but it appeared on the 12th day (Figure **5**, lanes *4, 5, 6*). The content of this protein was increased in medium C to the 17th day of growth (Figure **5**, lanes *8, 9*). The component with molecular mass 30 kDa was determined on the 11th and 17th day both in medium B and in medium C (Figure **5**, lanes *5, 6, 8, 9*).

It was found that the dynamics of exoproteinase accumulation by tomato isolate A7AKTL125 depended on the growing medium also (Figure **6**). The proteins with high molecular masses (90 and 60 kDa) were present in medium B, but proteins with a molecular mass of about 30 kDa were absent in this environment (Figure **5**, lanes *5, 6*). In medium C the component with molecular mass of 60 kDa was absent, and components with molecular mass of about 30 kDa were found.

It is interesting to compare these data with data obtained in the study of changes in the activity of the exoproteinases during growth of both isolates in different cultural environments. Comparison of Figures **3** and **5** suggests that the 50-kDa protein secreted by the potato isolate possesses trypsin-like activity, and the 30-kDa protein has subtilisin-like activity. The 50-

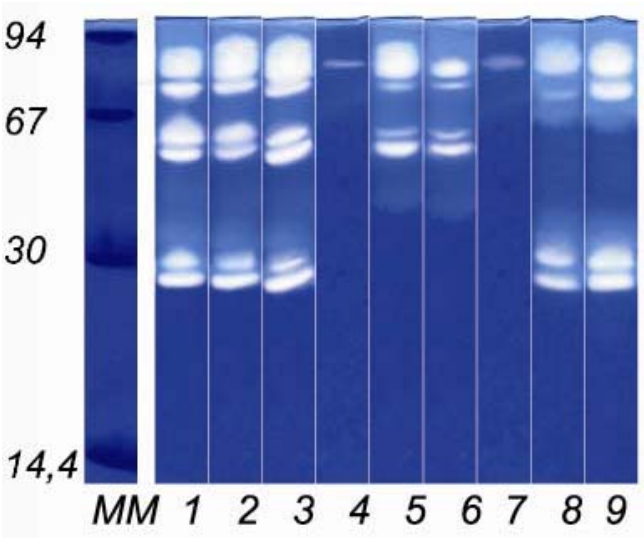

Figure 6: Zymograms of exoproteinases of *Alternaria solani* A7AKTL125 during growth on different nutritional mediums: lanes 1-3 – modified Czapek's medium containing 1% casein, lanes 4-6 – potato-carrot broth, and lanes 7-9 – pea-carrot broth. Lanes 1, 4, and 7 correspond to the 6th day of fungus growth, lanes 2, 5, and 8 correspond to the 11th day, and lanes 3, 6, and 9 correspond to the 17th day of fungus growth. Lane MM represents the molecular mass (kDa) markers as follows: phosphorylase b (94), bovine serum albumin (67), carboanhydrase (30), and lactalbumin (14.4). The zymograms were obtained after SDS-PAGE in the presence of gelatin.

kDa protein is not produced by the tomato isolate with growth in all tested environments (Figures **4** and **6**). However, the growth of the isolate with mediums A and C is accompanied by the secretion of at least two proteins with molecular masses of about 30 kDa. It can be assumed that these proteins have subtilisin-like activity.

4. DISCUSSION

The differences in the composition of peptidases secreted by the mycelium reflect the environmental and metabolic characteristics of the fungi indirectly. Despite this fact, several thousand peptidases secreted by fungi have been isolated and characterized. Studies dedicated to finding connections between different ecological and taxonomic groups of fungi and the composition of secreted peptidases is still insufficiently investigated.

It is known that microorganisms produce proteolytic enzymes in response to the depletion of readily available nutrients in the medium. The consequence of this can become either a decrease in catabolite repression or activation of synthesis and/or exoenzyme secretion, for example, by the mechanism of the substrate-specific induction [26-28].

Many enzymes secreted by pathogenic fungi can affect their relationships with plant hosts. It can be assumed that differences in the properties of these enzymes provide fungi selective advantages in different habitats. A number of studies have shown that fungi affecting agricultural plants produce serine proteinases [13-15, 29, 30]. The enzymes belonging to this class vary significantly in substrate specificity, which can correspond to the requirements of ecological niches of the fungi [26].

We have shown the extracellular proteinases produced by potato and tomato isolates of the fungus *A. solani*, which refers to the large-spore species *A. solani* Sorauer [18]. It was previously shown that the production of extracellular proteinases is subject to at least four regulatory mechanisms: carbon, nitrogen, and sulfur metabolite repression and pH control [30-32]. The influence of several environmental factors on the production of extracellular proteinases of both isolates of *A. solani* has been studied systematically in controlled batch cultures. Both isolates produced proteolytic enzymes on all mediums tested and all of them supported fairly good growth. We have shown that culturing both isolates of *A. solani* with medium containing casein resulted in secretion of higher levels and significant differences of the secreted proteinase composition compared to cultivation with a medium containing vegetable proteins. Changes were observed only in the dynamics of accumulation of serine proteinases. Thus, the secretion of the proteolytic enzymes in the synthetic medium commonly used for the cultivation of the microorganism had almost no dependence on the source of the pathogen isolation (host plant). It is known that proteinases secreted by plant pathogen *in vitro* can significantly differ from those secreted *in vivo* during fungal infections [33]. It was possible to infer that synthetic modified Czapek's medium can be used for obtaining highly active proteinases secreted by the pathogen, but this medium is not suitable for studying features of the dynamics of exoproteinase secretion by different pathogens.

Our data suggest a direct link between the amount of available organic substrate and the level of extracellular proteolytic activity secreted by the fungus. It is very likely that the rapid drop in measured proteolytic activity after reaching a maximum is a consequence of depletion of the protein substrate in the culture medium. This indicates that the enzyme secretion is not directly related to the accumulation of the fungal biomass, and it is more dependent on the productivity of the mycelium. It is possible that the

decrease in the proteolytic activity in culture medium containing pure protein (1% casein) can be due to catabolite repression of the synthesis enzymes by the products of proteolysis of proteins.

When using mediums containing no synthetic additives that are similar to the natural habitat of the pathogen, the proteinase secretion by these isolates defined both their specificity and growing conditions. Thus, in medium containing heat-stable proteins of potato tubers, high proteolytic activity was secreted by the potato isolate during the entire cycle of the development of the fungus. Proteinase secretion by the tomato isolate was significantly lower in the same medium and was not observed during the whole life cycle of the fungus. In medium containing heat-stable proteins of pea seeds, exoproteinase activity secreted by the potato isolate was much higher than the tomato isolate. This result is consistent with the earlier obtained data for the phytopathogenic fungus *Rhizoctonia solani* Kühn [30]. It has been shown that a change in the fungal growing condition, especially available nitrogen sources, led to a change not only in the quantity of produced proteinases, but their nature and the specificity of their action. We have shown that cultivation of both isolates of *A. solani* in the medium containing casein was accompanied by a higher level of secretion of proteolytic activity compared with medium without any synthetic additives, and the composition of the proteinases varied significantly.

Based on the analysis of the substrate specificity of the extracellular proteinases, it was established that the potato isolate secreted serine proteinases in the culture medium containing heat-stable proteins of both potato and pea seeds. These proteinases can be attributed to subtilisin-like and trypsin-like enzymes. The changing of the medium composition led to a change in the ratio of these enzymes, but the activity of the trypsin-like proteinases was significantly higher than that of the subtilisin-like proteinases. At the same time, the tomato isolate did not produce the serine proteinases in the medium containing the heat-stable potato proteins. This isolate produced the subtilisin-like enzymes only in the environment containing the heat-stable pea seed proteins. It can be assumed that the absence of production of serine proteinases by *A. solani* isolate from tomato in medium, containing heat-stable potato proteins, due to the presence among these proteins of a large amount trypsin and subtilisin inhibitors [34, 35]. It is likely that potato isolate was adapted to them and tomato isolate was not. This suggests that the exoproteinase secretion, the serine type in particular, is determined by the habitat, and especially the available nutrient source.

It is also known that high production of trypsin-like exoenzymes is characteristic of pathogenic forms of fungi [29]. It can be assumed that the potato isolate acts as a pathogen in both growing mediums. In turn, the tomato isolate of the fungus *A. solani* completely loses pathogenic properties and becomes a saprophyte in the medium enriched with the organic nitrogen sources. Specificity of the action, time of appearance, and the presence of trypsin-like activity in the pathogens indicate its possible involvement in the phytopathogenesis.

Thus, we have demonstrated that proteinases produced during growth of environmental fungi on such substrates as plants are likely to differ both in quality and in quantity from those produced in a synthetic medium. The production of extracellular proteinases in response to nitrogen and carbon starvation is reduced. Thus, it can be assumed that both the mycelial growth of a pathogenic fungus and production of extracellular proteinases are simultaneously determined by the state of available organic nitrogen, peculiarities of the pathogen, and the host plant.

Our data suggest that different nutritional sources can be important for differential production of serine proteinases. The serine proteinases produced by pathogenic fungi may play a different role in pathogenesis, increasing adaptation to a large range of hosts, or having different functions in their survival in different ecological habitats outside the host. Like the subtilisins, the trypsins are inducible by environmental cues [27, 28]. Thus, there are several mechanisms available for the different strains to adapt the enzyme activities to their specific needs on their particular hosts. Fungi normally produce a wide range of proteolytic enzymes to degrade protein substrates. However, the differences in the properties of the proteinases found in the studied microorganisms were unlikely to be caused by variations in the food substrate composition only. It seems likely, therefore, that the difference in the proteinase compositions that we observed has a significant genetic component.

It is known that there is no evidence of geographic differentiation and no correlation between geographic location and genetic affinity, but there is evidence of population subdivision according to the host plant [5]. The same we can say about the exoproteinases composition, which largely depends on the host plant.

This suggests that differences in the properties of the enzymes provide selective advantages in different habitats. The subtilisin-like proteinases most likely have the same functions in pathogenesis as trypsin-like proteinases. Thus, there are several mechanisms that are available for different strains of microorganisms to adapt to both their needs and the specific plant hosts.

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