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Extracellular proteases of *Botrytis cinerea* and peculiarities of their secretion

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***Abstract* – The ability of ubiquitous necrotrophic plant pathogen *Botrytis cinerea* to secrete proteolytic enzymes in broth media was examined. It was found that protease secretion is mostly inducible and acidic proteases are responsible for the highest total extracellular proteolytic activity of the fungus. Results from analysis of substrate specificity and inhibitor assay have revealed the presence of aminopeptidases (leucyl and phenylalanyl), serine (trypsin- and subtilisin-like) and aspartic proteases in culture liquid. The pathogen produced identical spectrum of proteolytic enzymes in medium containing plant cell walls or casein. The data provide evidence that pH-, time course and biomass accumulation dependent regulatory mechanisms are essential for protease activity of *B. cinerea*. Putative functional roles of several groups of extracellular proteolytic enzymes are discussed in connection with the peculiarities of their secretion.**

Keywords – proteases; secretion; proteolytic activity; phytopathogenesis; Botrytis cinerea.

I. Introduction

Extracellular proteins of plant pathogenic fungi are assumed to be involved in different types of plant-pathogen interactions and development of versatile adaptive mechanisms during the course of evolution toward phytopathogenicity. It is notable that many of these proteins (including enzymes) are secreted by opportunistic fungal pathogens as the traits of saprophytic as well as parasitic lifestyle [1]. Thus, specifically developed tools required for infection process caused by this group of fungi can be based on biosynthetic regulation [1], releasing specific virulence molecules and regulation of secretion and activity of proteins.

Among a great complex of enzymes secreted by opportunistic necrotrophic ascomycete *Botrytis cinerea* [2], only part of them (β-glucosidase [3], pectin methylesterases [4]) are shown to be pathogenicity factors. Another category of *B. cinerea* extracellular enzymes (carboxypeptidase, aspartic protease, polygalacturonase, laccase), whose role in pathogenesis is inconclusive, is considered as potentially involved in infection process. Nevertheless, many of them have been reported to be capable of penetrating host membrane, dissolve cell wall components, and cause cell lysis («attack enzymes»). Apart from the multiplicity of activities, another essential feature of several *B. cinerea* attack enzymes was reported as subsequent secretion due to differential expression of responsible genes, which implies functioning of these compounds in individual stages of the infection process.

Interaction of *B. cinerea* with plants was also shown to involve fungal extracellular proteolytic system including aspartic proteases as the most extensively studied class of proteases produced by this pathogen. An attention of scientists to aspartic proteases as enzymes having possible role(s) in pathogenesis may be partially explained by the results of several researches demonstrated that they contribute the predominant proteolytic activity secreted by the fungus [5, 6]. These data allow to assume that the only proteolytic activity detected in *B. cinerea* culture medium represents aspartic proteases. Genomic analysis also indicated the importance of physiological role of these enzymes since the prevalence of genes encoding aspartic proteases was revealed [7]. Data on significance and possible functions of *B. cinerea* aspartic proteases during infection process have shown that they presumably possess multiple functional roles. According to the reports, they might be involved in conversion of host tissue into fungal biomass [8], releasing of phytotoxic cell wall components, facilitating of the hydrolysis of cell walls by endo-pectin lyase and decreasing of phytoalexin accumulation in plants [9].

It should be noted that remarkably enhanced interest of researchers to analysis of the compounds (in particular, secreted proteases) related to pathogenesis is largely caused by significant increase in the frequency of opportunistic fungal infections in immunocompromised patients. These infections may be triggered by the common micromycetes present in environment (water, soil, organic debris, etc.) or known plant pathogens never being considered as causal agents of human diseases. Therefore, proteases secreted by these opportunists can possess novel biological functions promoting the attack of host tissues or aimed on protection of fungal organism from components of the human immune system through the inactivation of proteinaceous defense substances.

The aim of this study was to conduct complex analysis of protease secretion by *B. cinerea* in order to evaluate potential roles of its exoproteases.

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II. Materials and methods

Fungal culture and growth conditions

B. cinerea strain was maintained on Czapek solution agar containing 30.0 g of sucrose, 2.0 g l^{-1} $NaNO_3$, 1.0 g l^{-1} K_2HPO_4, 0.5 g l^{-1} KCl, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 g l^{-1} $FeSO_4 \cdot 7H_2O$ and 15.0 g l^{-1} agar and stored at 4 °C. The conidia of 14-day-old cultures grown on Czapek solution agar slants were mechanically harvested in 2 ml distilled water and immediately transferred to one of the following medium: 58 ml of the standart Czapek solution, 58 ml of the modified Czapek solution lacking $NaNO_3$ but supplemented with 1% casein (w/v, dissolved in 0.1 M sodium phosphate, pH 7.4, buffer) or 18 ml of the modified Czapek solution lacking $NaNO_3$ but supplemented with 3% (fresh w/v) *Vigna radiata* cell walls. Cultures were subsequently growing in flasks at 22 °C on a rotary shaker at 180 rpm during 2–14 days. The cultivation was carried out with 3 replicates. The purity of the cultures was confirmed by microscopic examination. For examination of the dependence of the proteolytic activity on fungal age or nitrogen source, cultures were removed at 2 days intervals or after 7 days of cultivation and filtered through paper filters. For the prevention of microbial contamination, filtrates were supplemented with 0.02% NaN_3 (w/v). Collected filtrates were centrifuged for 10 min at 12000 rpm. The pH values of culture liquid were measured using a pH electrode. The culture supernatants were stored at –20 °C. For fungal biomass determination, harvested mycelia were dried at 80 °C until reaching constant weight. Cell-free supernatants were screened for the proteolytic activity and protease inhibitors using methods described below.

Assays

Proteolytic activity

Total proteolytic activity was measured using azocasein. 50 µl of culture supernatant and 150 µl of azocasein solution (0.5%, w/v, in distilled water) were added to 100 µl of 0.02 M citrate-phosphate, pH 4.0, 0.1 M sodium phosphate, pH 7.0, or 0.1 M Tris-HCl, pH 9.0, buffer. The mixture was incubated for 3 h at 37 °C in a microcentrifuge tube. The reaction was terminated by the addition of 300 µl of 10% trichloroacetic acid. Reaction in control sample was stopped immediately after the addition of azocasein solution. Solution was held at 4 °C for 30 min. The undigested substrate was removed by centrifugation for 15 min at 12000 rpm. Then 400 µl of 0.5 M NaOH was added to 400 µl of supernatant and the absorbance was read at 440 nm. One unit of the total proteolytic activity was defined as the amount of proteases which brought about an increase of 0.1 optical density units per minute under the assay conditions. The enzymatic activity, in arbitrary units (U), was then expressed in U min^{-1} g^{-1} dry mycelia. Each assay was performed in triplicate.

Class-specific proteolytic activity of culture liquid was measured with the synthetic chromogenic substrates dissolved in N,N-dimethylformamide. 20 mM BApNA (Nα-benzoyl-L-Arg-*p*NA) was used for measurement of trypsin-like, 20 mM GlpAAL*p*NA (Glp-Ala-Ala-Leu-*p*NA) – for subtilisin-like, 10 mM GlpP*p*NA (Glp-Phe-*p*NA) – for chymotrypsin-like and 20 mM GlpPA*p*NA (Glp-Phe-Ala-*p*NA) – for cysteine protease activity. For the determination of aminopeptidase activity 40 mM L-*p*NA (L-Leu-*p*-NA) and 35 mM P-*p*NA (L-Phe-*p*-NA) were also used. The reaction mixture contained 10 µl of culture supernatant, 5 µl of substrate solution and 185 µl of 0.01 M sodium phosphate, pH 6.9, buffer. To compare the activity of *B. cinerea* trypsin-like and subtilisin-like proteases under near-neutral and alkaline conditions, the reactions with BA*p*NA and GlpAAL*p*NA were also carried out in 0.1 M Tris-HCl, pH 9.0, buffer. For the measurement of cysteine protease activity culture supernatant was preincubated with dithiothreitol (DTT, final concentration 4 mM) at room temperature for 10 min. The reaction mixture was incubated at 37 °C in a 96-well microtitre plate. The change in absorbance at 405 nm was monitored in several hours until yellow coloration appeared. Specific proteolytic activity, in arbitrary units (U), was the change in absorbance units per minute multiplied by 100. The activity was given in U min^{-1} g^{-1} dry mycelia. Each assay was performed in triplicate.

Screening of protease inhibitors

Inhibitory activities of *B. cinerea* were determined against trypsin, subtilisin, chymotrypsin, papain and bromelain. Commercial preparation of trypsin (0.1 mg ml^{-1}) was dissolved in 0.001 M HCl solution, subtilisin (0.2 mg ml^{-1}), chymotrypsin (1.8 mg ml^{-1}), papain (5 mg ml^{-1}) or bromelain (5 mg ml^{-1}) – in 0.01 M sodium phosphate, pH 6.9, buffer. 50 µl of culture supernatant was preincubated with 5 µl of enzyme solution and 8 µl of 0.01 M sodium phosphate, pH 6.9, buffer at room temperature for 30 min. For papain and bromelain inhibition, enzymes were activated by DTT (final concentration 4 mM) at room temperature for 10 min. Then 132 µl of 0.01 M sodium phosphate, pH 6.9, buffer and 5 µl of the appropriate chromogenic substrate solution were added. The reaction mixture was incubated for 30 min at 37 °C in a 96-well microtitre plate. The absorbance was measured at 405 nm. For eliminating contribution of *B. cinerea* proteases, their activity was also assessed, and its level was deducted from resulting activity of enzyme preparation after its interaction with culture liquid. The activity of non-inhibited (for 6–14-day-old cultures) or preincubated with growth medium instead culture supernatant (for 2- and 4-day-old cultures) enzyme was taken as 100% activity. Inhibitory activity was expressed as the percentage of protease inhibition. Each assay was performed in triplicate.

Sensitivity of proteases to inhibitors

The sensitivity of proteases from culture liquid to inhibitors was examined by incubating 50 µl of culture supernatant with 0.2 mM PMSF (phenylmethylsulphonyl fluoride, in ethanol), 10 mM disodium ethylenediaminetetraacetate (EDTA, in 0.1 M sodium phosphate, pH 7.0, buffer), 6.5 mM DTT (in distilled water), 0.01 mM L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64, in ethanol), 0.01 mM pepstatin (in ethanol) and 0.1 mM 1,10-phenanthroline (in ethanol) in 0.1 M sodium phosphate, pH 7.0, buffer for all inhibitors except pepstatin, whose effect was assessed in 0.02 M citrate-phosphate, pH 4.0, buffer. The proteases from culture liquid were exposed to the inhibitors at room temperature for 20 min. After the addition of azocasein solution the reaction was

carried out for 3–5 h at 37 °C in a microcentrifuge tube. The total proteolytic activity was then measured as described above, and the percent of residual activity was determined.

Cell wall preparation from Vigna radiata leaves

Whole detached *Vigna radiata* leaves were rinsed with 70% acetone (v/v) until filtrates appeared free of pigment followed by 5 washes in distilled water. The material was then washed with 1% NaOH (w/v) and at least 5 times with distilled water. After that cell walls were rinsed with 1% HCl (v/v) and finally washed extensively with distilled water. Fresh *Vigna radiata* cell wall preparation in 0.1 M sodium phosphate, pH 7.4, buffer was added as a sole nitrogen source to modified Czapek solution lacking $NaNO_3$ for examination the ability of structural cell wall proteins to specifically induce the protease secretion by phytopathogen.

III. Results

The results showed that *B. cinerea* protease secretion is mostly inducible and its level depends on the type of nitrogen source present in culture medium (Table 1). Highest total proteolytic activity was observed in medium supplemented with casein as protease inducer compared with the medium containing *Vigna radiata* cell walls used as a sole nitrogen source. This activity of 7-day-old cultures increased under the acidic conditions (pH 4.0) and diminished with the growing of pH values reaching the lowest level at pH 9.0 in the presence of casein as well as plant cell walls. Maximum of protease secretion (in medium with casein) was detected on 4th dpi (onset of the exponential growth phase, Fig. 1). However, proteolytic activity displaying by culture at this growth stage was similar at acidic and neutral conditions.

The spectrum of class specific protease activity was identical in both modified media (Table 2). Protease assay with available *p*-nitroanilide substrates revealed that *B. cinerea* was able to secrete serine (trypsin- and subtilisin-like) proteases and aminopeptidases. It was shown that the fungus did not produce detectable levels of secreted chymotrypsin-like or cysteine proteases. The highest activity of trypsin- and subtilisin-like proteases was measured after fungal growth in medium with casein, while aminopeptidase production increased in the presence of plant cell walls.

TABLE I. TOTAL PROTEOLYTIC ACTIVITY OF *B. CINEREA* GROWN IN LIQUID MEDIA WITH DIFFERENT NITROGEN SOURCES FOR 7 DAYS

Nitrogen source	**Mycelial biomass, g dry mycelia l^{-1}**	**pH**	**Activity, U min^{-1} g^{-1} dry mycelia**		
			pH 4.0	***pH 7.0***	***pH 9.0***
$NaNO_3$	20.32	5.0	0.12	0.20	0.17
Vigna radiata cell walls	20.92	5.0	4.43	2.68	1.98
Casein	27.58	4.4	6.92	4.26	3.68

TABLE II. CLASS SPECIFIC PROTEOLYTIC ACTIVITY OF *B. CINEREA* GROWN IN LIQUID MEDIA WITH DIFFERENT NITROGEN SOURCES FOR 7 DAYS

Substrate	**Activity, U min^{-1} g^{-1} dry mycelia**		
	$NaNO_3$	***Casein***	***Vigna radiata cell walls***
BA*p*NA	0	210.87	129.46
GlpAAL*p*NA	2.11	31.97	23.90
GlpPA*p*NA	0	0	0
GlpP*p*NA	0	0	0
L-*p*NA	7.72	4.73	91.81
P-*p*NA	5.15	7.78	31.11

Low levels of subtilisin-like and aminopeptidase secretion were also detected in standart medium with $NaNO_3$. The fungus produced at first (on 4th dpi) subtilisin-like proteases and aminopeptidases (leucyl and phenylalanyl) and later (on 6th dpi) trypsin-like enzymes in medium supplemented with casein during the culturing period (Fig. 2, 3). After reaching a peak, subtilisin-like activity decreased dramatically constituting 28% of the maximal activity on 6th dpi, while the lowest activity (9% of the maximal activity) was detected on the 14th dpi. In contrast to subtilisin-like enzymes, the activity of trypsin-like proteases was sustained on significant level up to the end of cultivation. Subtilisin- and trypsin-like proteases were more active at near-neutral pH values compared with the alkaline conditions (pH 9.0) caused approximately 48 and 94% loss of maximal GlpAAL*p*NA and BA*p*NA activity, respectively (Fig. 2).

Total proteolytic activity of 4-day-old culture was inhibited approximately 83% by PMSF, indicating that serine proteases may represent the most prevalent class of *B. cinerea* proteases secreted during exponential phase and characterized

TABLE III. SENSITIVITY OF *B. CINEREA* PROTEASES FROM CULTURE LIQUID TO VARIOUS CLASS SPECIFIC INHIBITORS

Inhibitor	**Concentration, mM**	**pH**	**Residual activity, %**	
			4 dpi	***10 dpi***
PMSF	0.2	7.0	17.2	43.6
EDTA	10	7.0	98.3	104.3
DTT	6.5	7.0	62.7	64.2
E-64	0.01	7.0	100	100
Pepstatin	0.01	4.0	98.2	52.3
1,10-phenanthroline	0.1	7.0	95	100

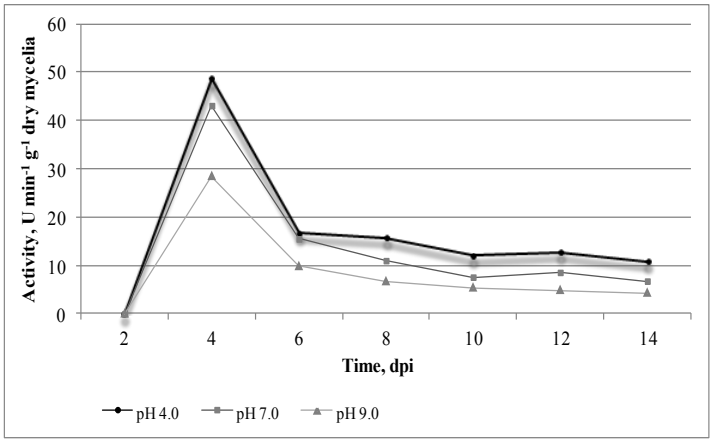

Fig. 1. Kinetics of the total protease production by *B. cinerea* grown in liquid medium with casein.

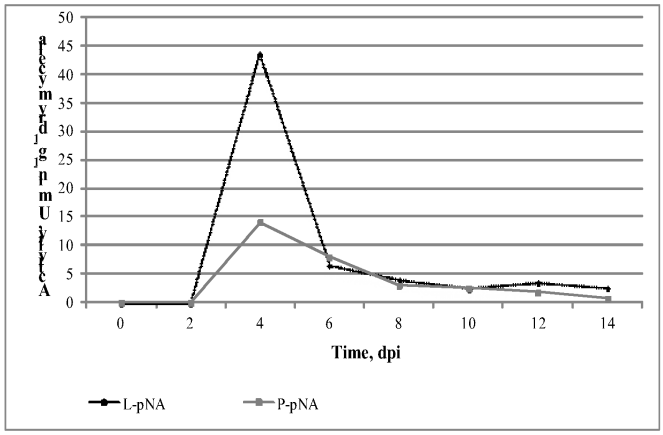

Fig. 3. Kinetics of aminopeptidase production by *B. cinerea* grown in liquid medium with casein.

by sufficient level of activity displayed during the whole period of cultivation (Fig. 4). Data on the effect of pepstatin showed that aspartic proteases are another class of *B. cinerea* extracellular proteases, which were actively secreted along with serine proteases in stationary growth phase. EDTA, E-64 and 1,10-phenanthroline had little or no effect on protease activity, which, however, was reduced on around 37 and 36% (depending on fungal age) by DTT (Table 3).

B. cinerea culture liquid was found to contain inhibitors of papain, bromelain and chymotrypsin (Fig. 5), but it was not active against subtilisin or trypsin. Secretion of inhibitors was detected on 6th dpi (onset of the stationary phase) and sustained on high level during the following period of cultivation.

IV. Discussion

B. cinerea generally infects a wide range of plant species [10], so this type of plant-pathogen interactions implies fungal metabolic versatility involving broad spectrum of enzyme activities. Revealed in the present study identical profiles of secretion of class specific proteases produced in medium supplemented with plant cell walls or casein also indicated a low specialized nutritional status of *B. cinerea* as opportunistic pathogen. According to the results, contribution of acidic proteolytic enzymes in total protease activity was the major irrespective of the type of inducer present in medium. This is consistent with known ability of the fungus to acidification of the environment through the production of oxalic [5], citric, malic and succinic acids [11] that could lead to adaptation of proteolytic system to low ambient pH values, which eventually became more permissive for its functioning. Identified in the present study aspartic proteases comprised substantial component of the group of *B. cinerea* acidic proteases that was confirmed by pepstatin inhibition. However, the fact that trypsin- or subtilisin-like activity detected at pH 6.9 was strongly reduced in acidic (pH 4.0, data not shown) and alkaline (pH 9.0) conditions suggests that, apart from the aspartic proteases, *B. cinerea* secretes proteases with near-neutral pH optimum of activity. Indeed, it is conceivable that total proteolytic activity displayed at neutral pH values may be partly contributed by activity of serine proteases revealed by inhibitor assay with specific serine protease

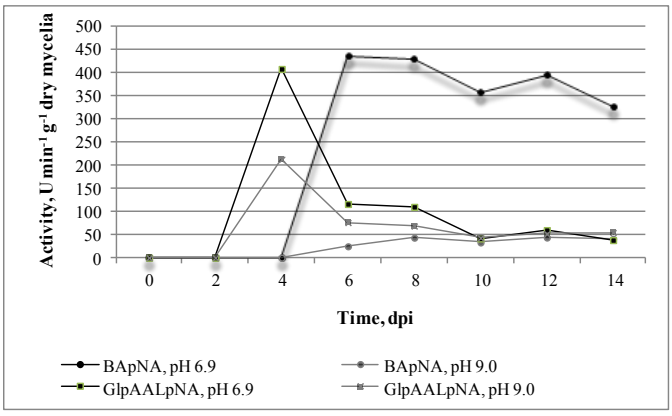

Fig. 2. Kinetics of trypsin- (measured with BA*p*NA) and subtilisin-like (measured with GlpAAL*p*NA) protease production by *B. cinerea* grown in liquid medium with casein.

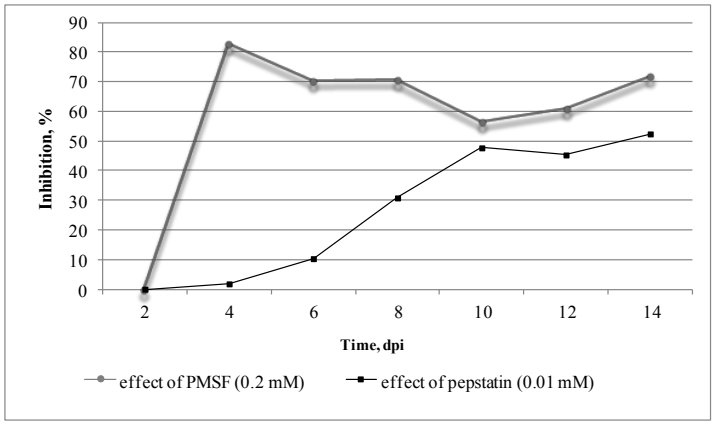

Fig. 4. Inhibition of *B. cinerea* proteolytic activity by PMSF and pepstatin during the cultivation in liquid medium with casein.

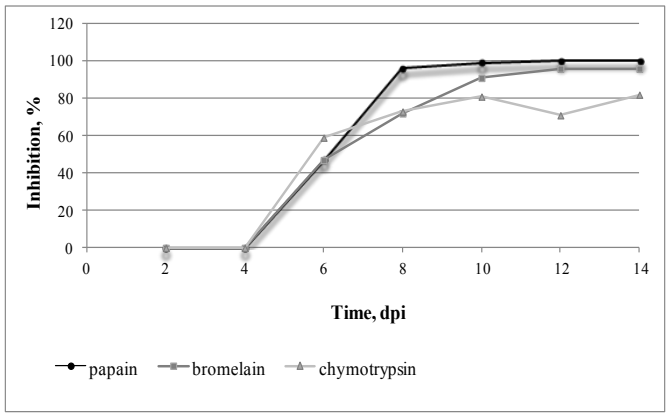

Fig. 5. Extracellular inhibitory activity of *B. cinerea* during the cultivation in liquid medium with casein.

inhibitor PMSF. Nevertheless, analysis of the secretion of trypsin-like proteases showed that low pH values were presumably required for its induction. This hypothesis is supported by the fact that, unlike subtilisin-like proteases, trypsin-like enzymes remain stable under the acidic conditions.

The ability of *B. cinerea* to secrete at first aminopeptidases and subtilisin-like and later trypsin-like proteases can be explained by different for these groups of proteases inducing pH values and biomass accumulation. Highest aminopeptidase and subtilisin-like activities obviously contributed to early peak of the total proteolytic activity observed under the conditions characterized by reaching fungal biomass level which is sufficient for protease secretion but not yet for strong acidification of the medium. This could lead to slight decrease of ambient pH (pH 6.5) at which these proteases were still stable. Further enhanced acidification of the medium (resulted in reduction of pH values of the cultures from 5.0 to 4.3) correlated with the steep loss of proteolytic activity due to possible acid inactivation of the early secreted proteases. Conversely, it is proposed that secretion of trypsin-like and aspartic proteases is induced under acidic conditions. It is not excluded that production of these proteins occurred only after the action of the early secreted proteases, which may serve as a signal of successful completion of the incipient stage of protein degradation performed by the fungus. This is consistent with the conclusion of Staples and Mayer [12] that there may be two categories of *B. cinerea* attack enzymes, those involved in the initial attack, and those which arise when the attack has succeeded. The data also demonstrated that no single factor (pH, time course, fungal biomass or nitrogen source) is likely to be responsible for the regulation of *B. cinerea* protease secretion and activity, but rather a combination of the factors is necessary. It is suggested that ambient pH values are of particular importance for functioning of *B. cinerea* proteolytic system due to their proposed ability to induce protease secretion and modulate the activity of the appropriate groups of proteolytic enzymes.

In this study, extracellular *B. cinerea* inhibitors of trypsin or subtilisin were not found, so regulation of the fungal exogenous proteolysis by trypsin- or subtilisin-like proteases does not appear to require the secretion of the inhibitors. The role of the revealed extracellular inhibitors of enzymes (papain, bromelain and chymotrypsin), which were not secreted by the fungus, may be expected in suppression of plant immune responses (effector function), however, precise molecular targets of these inhibitors remain to be elucidated.

Thus, differential protease secretion by *B. cinerea* suggests a significance of the consequent functioning of the groups of proteases. Early and short-term high aminopeptidase and subtilisin-like activity displayed at near-neutral pH values may participate in acquisition of the ecological advantages by the fungus in nutrient competition during saprophytic growth or be involved in initial steps of plant cell colonization resulted in breaching of the plant surface through the hydrolysis of structural cell wall proteins before fungal penetration of host tissues. Following active production of trypsin-like and aspartic proteases seems to be necessary for *B. cinerea* to carry out another functions, which are associated with pH lowering. According to the conclusions of Dunaevskii and coauthors [13], specific function of later secretion of stable trypsin-like proteases is important feature of plant pathogens. Moreover, results from analysis of predicted protease or homolog sequences with conserved regions similar to those in trypsins [14] indicate that the presence of trypsin or trypsin-like protease gene(s) represents a marker of fungal phytopathogenecity. Data of the present study allow to propose that trypsin-like as well as aspartic proteases are expected to be most needed and effective for fungal development after invasion in plant tissues often characterized by pH values ranging from 3.3 to 6.3 [5], demonstrating possible roles of these enzymes in phytopathogenesis.

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References

[1] W. Knogge, "Fungal infection of plants," The Plant Cell, vol. 8, pp. 1711-1722, October 1966.

[2] J.A.L. van Kan, "Infection strategies of *Botrytis cinerea*," in Proc. VIIIth IS Postharvest Phys. Ornamentals, vol. 669, Eds. N. Marissen et al.: Acta hort., 2005, pp. 77-90.

[3] I. Sasaki and H. Nagayama, "β-glucosidase from *Botrytis cinerea*: its relation to the pathogenicity of this fungus," Bioscience, Biotechnology and Biochemistry, vol. 58, pp. 616-620, 1994.

[4] A. Touzani, J.P. Muna, and B. Doneche, "Effet du pool enzymatique exocellulaire de *Botrytis cinerea* sur les cellules de *Vitis vinifera*. Application à la pellicule du raisin," J. Int. Sci Vigne Vin, vol. 28, pp. 19-31, 1994.

[5] S. Manteau, S. Abouna, B. Lambert, and L. Legendre, "Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*," FEMS Microbiology Ecology, vol. 43, pp. 359-366, 2003.

[6] A. ten Have, E. Dekkers, J. Kay, L.H. Phylip, and J.A.L. van Kan, "An aspartic proteinase gene family in the filamentous fungus *Botrytis cinerea* contains members with novel features," Microbiology, vol. 150, pp. 2475-2489, March 2004.

[7] J. Amselem, C.A. Cuomo, J.A.L. van Kan, M. Viaud, E.P. Benito, A. Couloux, P.M. Coutinho, R.P de Vries, P.S. Dyer, S. Fillinger, E. Fournier, L. Gout, M. Hahn, L. Kohn, N. Lapalu, K.M. Plummer, J.-M. Pradier, E. Quévillon, A. Sharon, A. Simon, A. ten Have, B. Tudzynski, P. Tudzynski, P. Wincker, M. Andrew, V. Anthouard, R.E. Beever, R. Beffa, I. Benoit, O. Bouzid, B. Brault, Z. Chen, M. Choquer, J. Collémare, P. Cotton, E.G. Danchin, C. Da Silva, A. Gautier, C. Giraud, T. Giraud, C. Gonzalez, S. Grossetete, U. Güldener, B. Henrissat, B.J. Howlett, C. Kodira, M. Kretschmer, A. Lappartient, M. Leroch, C. Levis, I. Mauceli, C. Neuvéglise, B. Oeser, M. Pearson, J. Poulain, N. Poussereau, H. Quesneville, C. Rascle, J. Schumacher, B. Ségurens, A. Sexton, E. Silva, C. Sirven, D.M. Soanes, N.J. Talbot, M. Templeton, C. Yandava, O. Yarden, Q. Zeng, J.A. Rollins, M.-H. Lebrun, and M. Dickman, "Genomic analysis of the necrotrophic fungal pathogens *Sclerotinia sclerotiorum* and *Botrytis cinerea*," Genomes of *Sclerotinia* and *Botrytis*, vol. 7, p. 27, August 2011.

[8] M. Nakajima and K. Akutsu, "Virulence factors of *Botrytis cinerea*," J Gen Plant Pathol, vol. 80, pp. 15-23, August 2013.

[9] S. Movahedi and J.B. Heale, "The roles of aspartic proteinase and endo-pectin lyase enzymes in the primary stages of infection and pathogenesis of various host tissues by different isolates of *Botrytis cinerea* Pers ex. Pers," Physiological and Molecular Plant Pathology, vol. 36, pp. 303-324, January 1990.

[10] Y. Elad, "Responses of plants to infection by *Botrytis cinerea* and novel means involved in reducing their susceptibility to infection," Biol. Rev., vol. 72, pp. 381-422, March 1997.

[11] K. Verhoeff, M. Leeman, R. van Peer, L. Posthuma, N. Schot, and G.W. van Eijk, "Changes in pH and the production of organic acids during colonisation of tomato petioles by *Botrytis cinerea*," J. Phytopathol., vol. 122, 327-336, 1988.

[12] R.C. Staples and A.M. Mayer, "Putative virulence factors of *Botrytis cinerea* acting as a wound pathogen," FEMS Microbiology Letters, vol. 134, pp. 1-7, October 1995.

[13] Y.E. Dunaevskii, T.N. Gruban, G.A. Belyakova, and M.A. Belozerskii, "Extracellular proteinases of filamentous fungi as potential markers of phytopathogenesis," Microbiology, vol. 75, 649-652, December 2006.

[14] A.G. Dubovenko, Y.E. Dunaevsky, M.A. Belozersky, B. Oppert, J.C. Lord, and E.N. Elpidina, "Trypsin-like proteins of the fungi as possible markers of pathogenicity," Fungal Biology, vol. 114, 151-159, January 2010.