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Cytochemical investigation of *Botrytis cinerea (Hyphomycetes)* and *Septoria nodorum (Coelomycetes)*

Abstract

Georgieva, I. & Rodeva, R.: Cytochemical investigation of *Botrytis cinerea (Hyphomycetes)* and *Septoria nodorum (Coelomycetes)*. – Bocconea 5: 833-837. 1997. – ISSN 1120-4060.

A set of enzymes involved in various metabolic pathways in *Botrytis cinerea* and *Septoria nodorum*, two important necrotrophic plant pathogens, was studied cytochemically. Mycelia growing in ovaries of *Lilium regale* and seeds of *Triticum aestivum*, respectively, showed high activity of some hydrolases (α-esterase, AS-D-esterase, acid phosphatase, β-glucosidase, α-galactosidase), localized in cytoplasmic granules. Acid phosphatase was observed outside of the hyphae of *S. nodorum*, and ATPase within. Hydrolytic enzymes probably contribute both to the penetration of host cells and the utilization of their nutrient substances. The citric acid cycle and glycolysis occur in both fungi, as revealed by the presence of the dehydrogenases studied. *S. nodorum* has 6-phosphogluconate dehydrogenase, involved in the pentose phosphate pathway, whereas *B. cinerea* is characterized by the presence of peroxidase and polyphenoloxidase; the latter, which is probably related to cross-linking of matrix polymers in the cell wall, is also found in senescent hyphae of *S. nodorum*.

Introduction

Botrytis cinerea Pers. is an ubiquitous, host-unspecific plant pathogen. *Septoria nodorum* Berk. causes a foliage, glume and stem disease of wheat. Both parasites are important limiting factors for crop production.

Since *Septoria* and *Botrytis* diseases are of great economic importance, numerous studies on the physiology of pathogenicity and penetration of these two parasites have been published (King & al. 1983, Jarvis 1977, Salinas & Verhoeff 1995). However, the studies devoted to the biochemical characteristics of *B. cinerea* and *S. nodorum* are few as compared with those discussing host-parasite relationships and the response reaction of the host plant. Until now, it has been established that both pathogenic fungi produce a number of hydrolytic enzymes which are supposed to play an important role in penetration and colonization of plant tissues (McKeen 1974, Jarvis 1977, Magro 1984, Salinas & al. 1986, Leone 1990). In addition, it is known that *B. cinerea* possesses some oxidases that provoke local disturbances in the host tissue metabolism (Edlich & al. 1989).

Table 1. Enzyme activities observed in the mycelia of *Botrytis cinerea* and *Septoria nodorum*. –, no staining (negative reaction); +, weak staining; ++, moderate staining; +++, strong staining.

Enzyme:	*B. cinerea*	*S. nodorum*
α-esterase	+++	+++
AS-D-esterase	+++	+++
acid phosphatase	++	++
β-glucosidase	+++	+++
α-galactosidase	+	–
ATPase	–	++
isocitrate dehydrogenase	++	++
glutamate dehydrogenase	–	+
alcohol dehydrogenase	–	–
lactate dehydrogenase	++	++
6-phosphogluconate dehydrogenase	–	++
peroxidase	++	+
polyphenoloxidase	++	–

Verhoeff & Warren (1972) showed that enzymes in the *Botrytis cinerea* mycelium vary depending on its localization in different tissues and organs. The current study was made on fungal mycelia developed in the generative sphere of the host plants – ovaries of *Lilium regale* E. H. Wilson and seeds of *Triticum aestivum* L. – in an attempt to enquire further into the metabolism of two necrophytic fungi by the investigation of a set of enzymes involved in different metabolic pathways. Cytochemical reactions were used to prove the participation of several hydrolases in cell degradation; of some dehydrogenases involved in glucose breakdown; of two oxidases connected with cell wall metabolism; of glutamate dehydrogenase, a key enzyme connecting the metabolism of carbohydrates with that of amino acids; and ATPase triggering transportation processes.

Material and methods

Plant material. – The inoculation of *Lilium regale* flowers with *Botrytis cinerea* was carried out 10 days after pollination. The ovaries were brushed with freshly collected dry conidia with the aid of a moist hair brush. The inoculation with *Septoria nodorum* was made by dropping a 10^6 spores/ml suspension on the stigma of *Triticum aestivum* at incipient anthesis.

Cytochemical methods. – The cytochemical study was performed on 50 μm thick free-floating frozen sections of *Lilium regale* ovaries and wheat grains, respectively, at varying times after inoculation. The hydrolases, α-esterase, AS-D-esterase (pectinase), acid phosphatase, β-glucosidase and α-galactosidase were determined by the method of simultaneous azocopulation (Lojda & al. 1979), and ATPase by the method of Wackstein-Meisel. For the demonstration of dehydrogenases (isocitrate dehydrogenase, 6-phosphogluconate

dehydrogenase, alcohol dehydrogenase, lactate dehydrogenase, and glutamate dehydrogenase), the method of tetrazolium reductases as recommended by Lojda & al. (1979) was followed. The cytochemical determination of polyphenoloxidase was made with DOPA, following Becker & al. (see Lojda & al. 1979). Peroxidase activity was proved with diaminobenzidine tetrahydrochloride using the method of Graham & Karnovski (Burstone 1962).

Results and discussion

The network of *Botrytis cinerea* hyphae appeared on the outer integument of ovules, nearly 15 days after inoculation. The *Septoria nodorum* hyphae were observed on the surface of the wheat seed and/or in the cavities of the seed coat tissues.

As shown in Table 1, the mycelia of *Botrytis cinerea* and *Septoria nodorum* operate with a large number of enzymes. The hydrolases under study showed high activities, which essentially confirms earlier observation in similar studies on *Botrytis* and *Septoria* (Jarvis 1977, Magro 1984) and other pathogenic fungi (Edreva & al. 1980). Hydrolase activity was localized in cytoplasmic granules, but acid phosphatase activity was also found outside of the mycelium of *S. nodorum*. Extracellular activity of acid phosphatase supports the assumption that the penetration of the mycelium of *S. nodorum* into seed tissues may be achieved, not only by mechanical means but also biochemically. Our results expand the spectrum of hydrolytic enzymes found in *B. cinerea* (Jarvis 1977) and *S. nodorum* (Magro 1984).

It is known that *Botrytis cinerea* and *Septoria nodorum* are necrophytic pathogens with pertotrophic growth (Jarvis 1977, King & al. 1983, Zinkernagel & al. 1988). The hydrolytic enzymes found in their mycelia probably contribute to the lytic processes in cells that collapsed consequent to the action of the parasite toxins. In this way, not only is the penetration of both parasites through the dead host cells made possible, but also the utilization of the nutrient substances they contain. The acid phosphatase activity and ATP-hydrolysing activity connected with the transport mechanism of sugars (Sawdis 1991) may be of special importance in this process.

α-esterase plays a definite role in the regulation of small carbon chain levels in the cytoplasm (Rudolph & Stahmann 1966), and acid phosphatase participates in the control of the level of phosphate groups. In this way they both could be related not only with the lysis of the host tissues but also with anabolic processes in the fungal mycelium. On the other hand, AS-D-esterase may act as pectinase (McLean & Gahan 1970), while β-glucosidase is connected with the metabolism of di- and oligosaccharides, and α-galactosidase, with galactomannan deposition and galactose removal (Edwards & al. 1992). Thus, they all are involved in cell wall metabolism (Keegstra & Albersheim 1970, Gahan & Carmigang 1989) and probably contribute to the synthesis of cell walls and elongation of hyphal cells.

Peroxidase and polyphenoloxidase activities were found in the *Botrytis cinerea* mycelium, while *Septoria nodorum* hyphae lack polyphenoloxidase activity, and show peroxidase activity only in the senescent state. The investigations of Fry (1986) proved that

peroxidase is involved in the formation of cross-linkings of matrix polymers in the cell walls and in this way is of importance for their extensibility and growth.

The study of dehydrogenases has revealed that the processes of the citric acid cycle (isocitrate dehydrogenase) as well as glycolysis (lactate dehydrogenase) occur in the mycelium of both fungi. In addition, the enzyme of pentose phosphate pathway 6-phosphogluconate dehydrogenase was found in the *Septoria nodorum* mycelium. Hyphae of both parasites lack alcoholic fermentation, as was shown by the negative cytochemical reaction for alcohol dehydrogenase.

Conclusions

In *Botrytis cinerea* and *Septoria nodorum*, glucose degradation occurs through the citric acid cycle and glycolysis. The hyphae of both parasites lack alcoholic fermentation.

Hyphae of both necrophytic fungi show high hydrolase activities, which probably contributes to the penetration into the host tissues and the utilization of their nutrient substances.

The activities of esterases and glycosidases could both be involved in the synthesis of and cell walls elongation of hyphal cells.

ATPase triggering the transportation processes may be of special importance for the metabolism of *Septoria nodorum.*

Acknowledgements

The work presented in this paper was supported by the National Science Fund of the Ministry of Education, Science and Technology of Bulgaria (contract No. K 516).

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