# Response of β-glucosidase to fungal infections in seed, ovary and fruit

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## Abstract

Localization and changes in the activity of β-glucosidase were investigated in wheat caryopsis and glumes infected with *Stagonospora nodorum* as well as in lily ovaries and harvested tomato fruits both inoculated with *Botrytis cinerea*. It was established that the pathogen invasion caused splitting of wheat seed coat, xylem blocking in lily carpel and decay in tomato fruits. *B. cinerea* invasion evoked disorders of the embryogenesis accompanied by a decreased activity of β-glucosidase in all ovules. The activity of the enzyme was not changed considerably in wheat seeds as the infection occurred in the late embryonal stages and the embryonal processes were not affected. In the seeds of harvested tomatoes distant from the invaded area the enzyme activity was not changed as well.

*Additional key words*: Botrytis cinerea, Lilium regale, Lycopersicon esculentum, Stagonospora (Septoria) nodorum, Triticum aestivum.

## Introduction

The investigations concerning biochemistry of the host-pathogen relationships revealed that hydrolytic enzymes play an important role in plant pathogenesis (Keen 1992). The increase in the activities of some hydrolases in invaded host tissues is considered as a component of the defense mechanisms and their role in plant resistance was analyzed (Pegg and Young 1981, Cline and Albersheim 1981, Mauch *et al.* 1988, Graham and Graham 1991). A special attention is paid to the role of β-glucosidase (β-GLU; E.C. 3.2.1.21) in the host response to fungal infection (Edreva and Georgieva 1980, Nichols *et al.* 1980, Edreva *et al.* 1986, Kozlowska 1993). Usually, these investigations are carried out on vegetative organs. The β-GLU response of floral organs and especially of ovaries and seeds is less investigated.

The results presented in this paper provide information about the localization and changes in the β-GLU activity of fruits and seeds in three host-pathogen systems: *Triticum aestivum - Stagonospora nodorum*, *Lilium regale - Botrytis cinerea* and *Lycopersicon esculentum - Botrytis cinerea*. Along with the biochemical approach a cytochemical one was applied to distinguish the reaction of pathogen hyphae and host cells.

## Materials and methods

**Fungi and inoculation:** A single pycnidiospore culture of *Stagonospora nodorum* (Berk.) Castellani & E.G. Germano, isolated from wheat leaf, was used in the study. The inoculation was made at the beginning of anthesis by dropping a suspension ($10^6$ spores $cm^{-3}$) supplemented with 0.2 % *Tween 20* on wheat flower stigma. The inoculated ears were enclosed in polyethylene bags for 96 h to maintain high relative humidity. The control ears received the same treatment except for the inoculation with *S. nodorum*.

The inoculation of lily ovaries was performed with *Botrytis cinerea* Pers. 10 d after pollination. The isolate was obtained from diseased lily plants grown in a greenhouse. The ovaries were inoculated with freshly

collected dry conidia produced on potato dextrose agar in Petri dishes with the aid of moist brush. The control ovaries were brushed with sterile distilled water. The control and inoculated ovaries were kept in polyethylene bags for 96 h.

Tomato fruits were inoculated with single-spore culture of *B. cinerea* originally isolated from mature tomato fruits. The inoculation was made by spraying with a conidial suspension of $10^6$ spores $cm^{-3}$. *Tween 20* (0.2 %) was added to the spore suspension to favour the penetration of the host cells by the fungus. Control fruits were sprayed with sterile distilled water supplemented with 0.2 % *Tween 20*. Both control and inoculated fruits were placed in clear plastic boxes with tightly fitting lids at 25 °C.

**Plants:** Wheat (*Triticum aestivum* L. cv. Slavianka), lily (*Lilium regale* Wils) and tomato (*Lycopersicon esculentum* Mill. cv. Ideal) were grown in a field. Wheat seeds and glumes were inoculated with *S. nodorum*. Lily ovaries and harvested green tomato fruits (3 to 4 cm in diameter) were inoculated with *B. cinerea*.

The infected plants were investigated at different times after inoculation. Wheat was studied after the appearance of brown stained spot necroses on the glumes and kernels (growth stage early-soft dough). The control and infected seeds were of similar maturity. The examination of lily (which develops dry fruits) extended till the 45th day after pollination when significant areas on the upper part of the pericarp were covered by necroses. Fleshy tomato fruits were investigated after decay incidence.

**Cytochemical localization of β-glucosidase:** The cytochemical reaction for the enzyme localization was carried out on 50 μm thick free floating frozen longitudinal sections of wheat seeds, cross-sections of wheat glumes and lily ovaries as well as cross-sections of pieces of tomato fruits containing pericarp, sheath and locular tissues and seeds of invaded and neighboring non-invaded areas. The material was fixed in Baker's calcium-formol (10 % neutral formol with 1 % $CaCl_2$) for 1 h at 0 °C. β-GLU was detected by the method of simultaneous azocopulation using 6-bromo-2-naphthyl-β-D-glucopyra-noside (*Serva*, Heidelberg, Germany) as a substrate and fast blue B (*Serva*) as a coupling dye in 0.1 M phosphate buffer pH 6.5 (Beneš *et al.* 1973). The reaction product is stained red-violet and is localized in cytoplasmic granules.

**Biochemical assay:** β-GLU activity was determined by the method of Giebel (1976). The enzyme was extracted with 0.05 M citric acid-sodium phosphate buffer pH 5.6. The extract was centrifuged at 20 000 $g$ for 50 min at 0 - 4 °C. The substrate was *p*-nitrophenyl-β-D-glucopy-ranoside (*Koch Light*, Colnbrook, England). The reaction medium contained 1 $cm^3$ enzyme extract and 1 $cm^3$ 5 mM substrate solution. After incubation at 37 °C the reaction was stopped by addition of 3 $cm^3$ 0.2 M NaOH. The same medium without incubation was used as a control. Protein estimation was made by the method of Lowry *et al.* (1951).

**Statistics:** Experiments were repeated three times with four replicates per experiment. Significance of differences was given by the Student's test at $P \leq 5$ % or 0.1 %.

## Results

**System *T. aestivum* - *S. nodorum*:** Initially *S. nodorum* hyphae grew on the surface of the wheat kernel, then got below the upper layer of the seed coat where large cavities were formed. The mycelium seldom colonized the embryo. In heavily damaged seeds, the embryo and endosperm cells became crushed, with compact content.

At the beginning of *S. nodorum* invasion when fungal hyphae grew on the surface of the wheat kernel a faint cytochemical reaction for β-GLU in the seed coat appeared beneath the mycelium. After fungal penetration into the seed a strong β-GLU staining arose in the seed coat as well as in large areas of the endosperm or embryo in the proximity of the hyphae (Fig. 1*A-C*). In glumes only tissues near the mycelium were strongly stained. The fungal hyphae also exhibited a positive cytochemical reaction for β-GLU. The biochemical experiments point that the inoculation induced a considerable enhancement of the enzyme activity in the glumes (464 %). Changes of

activity following the infection were not found in the seeds (Table 1).

**System *L. regale* - *B. cinerea*:** The first necroses appeared on the lower part of the ovary. The infection spread up in pericarp parenchyma and vascular bundles. Amorphous plugs appeared in the xylem vessels. The hyphae reached ovules mainly in the basal part of the ovary and clasped some of them. In some cases they penetrated between inner and outer integument. Regardless of the fact that we did not observe fungal hyphae in the embryo sac, the basal ovules were not fertilized and degenerated. The ovules of the upper and middle part of the ovary were with delayed embryogenesis. The detailed descriptions of the cytoembryological disturbances in *T. aestivum* seeds and *L. regale* ovaries after inoculation with *S. nodorum* and *B. cinerea*, respectively, were published elsewhere (Georgieva *et al.* 1999).
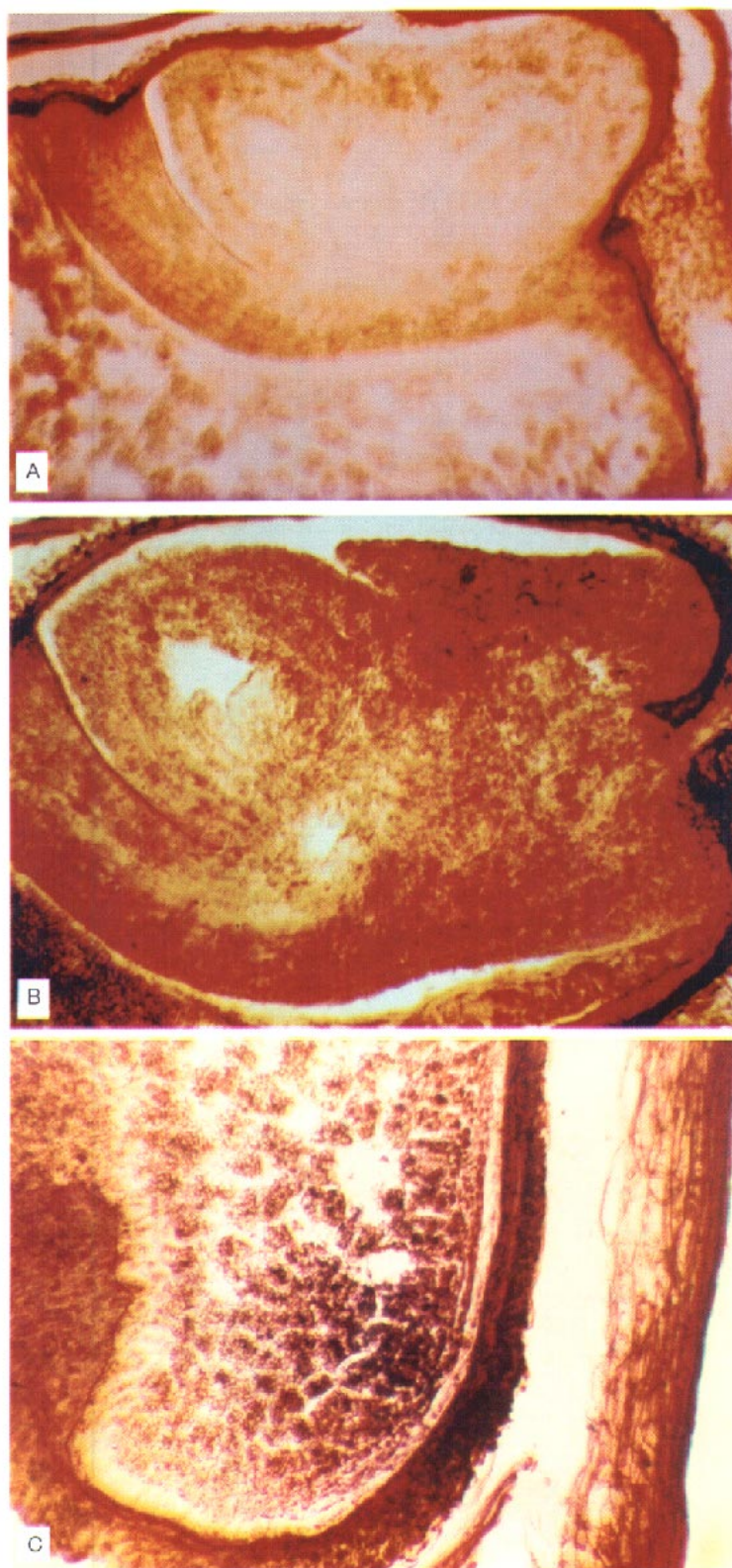
Fig. 1. Localization of β-glucosidase in *Triticum aestivum* seeds. *A* - Embryo and endosperm of non-infected seed with nearly negative reaction, ×45; *B* - Embryo of *Stagonospora nodorum* infected seed with enhanced β-glucosidase activity, ×88; *C* - Local rise of β-glucosidase activity in the seed coat and endosperm after *S. nodorum* invasion, ×45.
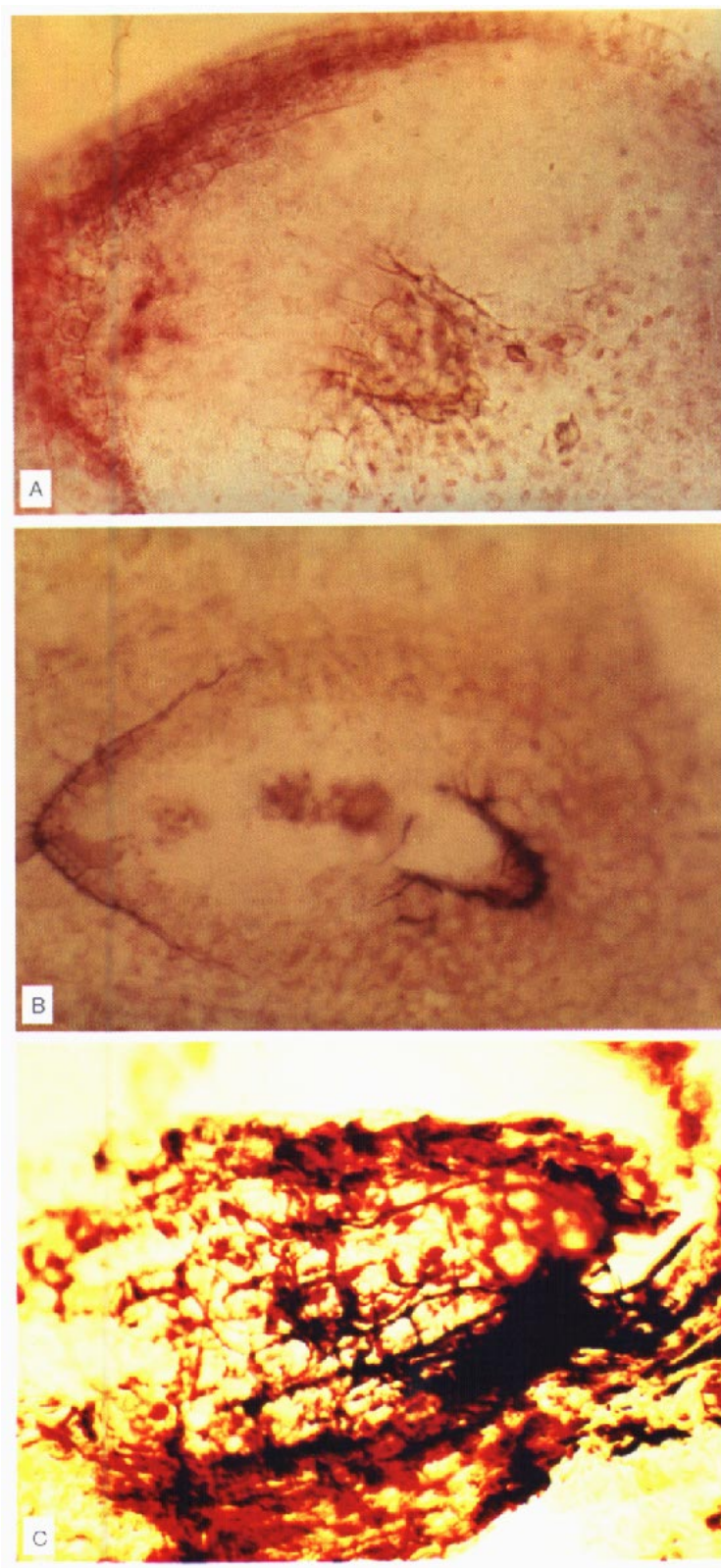
Fig. 2. Localization of β-glucosidase activity in *Lilium regale* ovules. *A* - Ovule of non-infected ovary with β-glucosidase activity in the micropylar region, ×52; *B* - Loss of β-glucosidase activity in ovule after *Botrytis cinerea* invasion, ×45; *C* - High enzyme activity around the hyphae growing on the surface of the ovule, ×45.

Table 1. β-glucosidase activity [μmol($p$-nitrophenol) mg$^{-1}$(protein) h$^{-1}$] in wheat seeds and glumes infected with *Stagonospora nodorum*, and lily ovaries and tomato fruits infected with *Botrytis cinerea*. Data are means of three experiments each with four replicates. Standard deviations are less than 10 % of the means. Significance of differences from the corresponding controls is given at 0.1 % (**) or 5 % (*) (Student's $t$-test).

| Plants | Organs | Control | Inoculated | [%] |
|---|---|---|---|---|
| Wheat | seeds | 0.570 | 0.550 | 96 |
| | glumes | 0.220 | 1.020** | 464 |
| Lily | pericarp | 0.041 | 0.050* | 122 |
| | seeds | 0.120 | 0.340** | 283 |
| Tomato | pericarp | 0.034 | 0.042* | 123 |
| | seeds | 0.041 | 0.052* | 127 |

As shown by the cytochemical tests, *B. cinerea* invasion was accompanied by a loss of β-GLU activity in the lily ovules that were not directly invaded (Fig. 2*A,B*). At the same time pronounced staining for β-GLU appeared in the invaded tissues. This response arose in the pericarp epidermis and parenchyma near the fungal hyphae as well as in the integuments of ovules adjacent to the necroses of the ovary wall (Fig. 2*C*).

According to the biochemical assays, the β-GLU activity in seeds of non-inoculated lily plants was higher than in the pericarp. The inoculation with *B. cinerea* caused an increase in enzyme activity near the necroses in both seeds and pericarp. The enhancement of the activity in the seeds was more significant (283 %) than in the pericarp (122 %) (Table 1).

**System *L. esculentum* - *B. cinerea*:** *B. cinerea* infection of harvested tomato fruits caused decay development in the pericarp and did not disturb the embryo and endosperm morphology.

The cytochemical staining for β-GLU of the parenchyma cells in the *B. cinerea* invaded tomato fruit pericarp was drastically enhanced around the mycelium. The seed coats of the seeds situated close to the pathogen hyphae were more deeply stained than the control ones. In the seeds distant from the invaded area the β-GLU staining was not changed. *B. cinerea* infection evoked a slight enhancement of the enzyme activity in the pericarp and in the seeds situated around the necroses as indicated by biochemical measurements (Table 1).

## Discussion

The results of the present investigation point that the degree of the metabolic disturbances in ovules and seeds depended on the embryological stage of the hosts during the pathogenic attack, the type of the fruit (dry or fleshy) and the morphological changes in the ovary evoked by the pathogen invasion.

*S. nodorum* succeeded to invade the wheat seed during the late embryogenesis but rarely penetrated deeply. The activity of β-GLU was weakly affected in the embryo and endosperm and only local enhancement around the fungal mycelium could be observed. Thus, the metabolic response of the wheat seed to *S. nodorum* invasion was similar to the response of the glumes, which are histologically identical to the leaves. Therefore the reaction of wheat seed to *S. nodorum* infection resembled the response of the vegetative organs. A similar local reaction was observed in the infected pericarp tissues and seeds of the fleshy tomato fruits having a thick pericarp.

On the contrary, in the system *L. regale* - *B. cinerea* the fungal infection was associated with more profound metabolic changes in the ovary expressed as a decrease and loss of β-GLU activity in ovary walls and ovules during the embryo development. The thin ovary walls of *L. regale* forming dry fruits facilitated the quicker and deeper fungal penetration to the ovules. This could explain that *B. cinerea* invasion especially in the lower parts of the lily pistil occurred in the early stages of the embryogenesis. Besides, *B. cinerea* invasion caused plug formation in xylem vessels of *L. regale* ovary. This affected the growth conditions of the embryo and endosperm more strongly as compared to the relatively minor effect of *S. nodorum* in the wheat seed coat and of *B. cinerea* in the pericarp of the fleshy tomato fruits.

It is known that glycosidases play a role in the processes of synthesis, elongation and degradation of cell walls and thus they are associated with cell growth (Johnson *et al.* 1974). They are also involved in the synthesis of phenol glycosides operating as growth regulators (Sembdner 1974). Therefore, the decrease in β-GLU activities in *L. regale* ovules after *B. cinerea* invasion may be related to different disturbances in the physiological processes during early embryogenesis.

Enhancement in activity of certain hydrolytic enzymes is considered to be a component of the defense mechanisms (Keen 1992). The data presented in this study demonstrate induction of local β-GLU response in the ovary and seeds in the three systems under study as it was reported for leaves in other host-fungal pathogen systems (Nichols *et al.* 1980, Edreva and Georgieva 1980, Edreva *et al.* 1986, Kozlowska 1993). The invasion of plants by pathogenic fungi is related to induction of β-GLU possibly by substrates available on the surface of the hyphae (Nichols *et al.* 1980). In its turn plant β-GLU may be involved in the processing and release of fungal

glucan elicitors, triggering a chain of reactions in the host (Nichols *et al.* 1980, Ham *et al.* 1995) including phytoalexin formation and biosynthesis of phenyl-propanoids and lignin - ingredients of chemical and mechanical defense barriers (Douglas 1996, Whetten *et al.* 1998). Moreover, by hydrolyzing β-phenyl-glucosides, β-GLU is responsible for the liberation of phenol aglucones with fungistatic and fungitoxic action (Pegg and Young 1981).

In conclusion, the fungal invasion of the wheat seeds and lily ovaries was associated with disturbances in the embryonal development that were more prominent in *L. regale* ovaries infected by *B. cinerea*. In *L. regale* ovules the disorders in the embryogenesis were accompanied by a decline of β-GLU. The enzyme activity was slightly affected in wheat seeds after *S. nodorum* attack as well as in seeds of harvested tomatoes infected with *B.cinerea*. The fungal invasion of fleshy tomato fruits evoked a local defense response in the seeds and ovaries resembling those of the vegetative organs.

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