BIOLOGIA PLANTARUM (PRAHA)

33 (5) : 337–344, 1991

The Activity and Localization of Some Hydrolytic Enzymes During Early Embryogenesis of *Lilium regale* after Pollination with γ-Irradiated Pollen

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Abstract. Changes in the activity and localization of nonspecific esterase, acid phosphatase, α-galactosidase and β-glucosidase in *L. regale* pistils after pollination with γ-irradiated pollen were studied. In the embryo sac and in the ovule reduction of AS-esterase and α-galactosidase and, at the same time, enhancement of α-esterase, acid phosphatase and β-glucosidase activities were observed. The changes in hydrolytic enzyme activities are discussed as manifestations of lethal factors resulting from structural disturbances of DNA in the generative nucleus and in sperms caused by irradiation.

The effect of γ-rays on pollen and the influence of irradiated pollen on the embryonal processes were investigated many times. It was found as a result of cytological and cytoembryological observations that γ-irradiation of pollen caused significant changes in the structural organization of the sperm nuclei (Grant *et al.* 1980), as well as disorders in both the process of fertilization and the structure of embryo and endosperm.

Various species of the genus *Lilium* were used in similar studies. In *L. formosanum* Cave and Brown (1954) observed the absence of double fertilization and cell division disturbances during embryogenesis which led to the formation in the embryo sac of micronuclei and lobed nuclei. Vassileva-Dryanovska (1966) described in *L. speciosum* haploid embryos produced by stimulation of the egg cell by the sperm pycnotic chromatin or by pseudogamy under the influence of the developing endosperm.

In our earlier cytochemical investigations on anomalies of embryogenesis and on embryo sac death in *Lilium* (Georgieva 1987) a drastic increase of peroxidase was observed in the chalazal region after pollination with irradiated pollen. Disturbances in normal embryo sac metabolism were also observed concerning glutamate dehydrogenase activity (Georgieva 1984). In the present work attention was focused on investigating the effect of pollination with γ-irradiated pollen on some hydrolytic processes in the pistil.

Received May 28, 1989; accepted June 7, 1990

MATERIAL AND METHODS

The flowers of *Lilium regale* Wils. were emasculated and isolated on the day of anthesis. Three days later, when their stigmas had reached the highest receptivity they were pollinated with the treated pollen immediately after its irradiation. The anthers, picked on the day of anthesis, were γ-irradiated with 10, 25, 50 and 100 kR, at an irradiation rate of 1480 R min^{-1}. Pistils pollinated with non-irradiated pollen were used as controls.

Histochemical determinations of the investigated enzymes were made on pistils fixed in Baker's Ca-formol, taken at five-day intervals from 5 to 40 d after pollination. Free-floating cryostat sections were used. The simultaneous azocoupling procedures were applied. Non specific esterase was assessed with the aid of two substrates: α-naphthyl acetate (α-esterase) and naphthol-AS-acetate (AS-esterase) (Beneš 1962). Acid phosphatase was visualized with 1-naphthyl phosphate (Lojda *et al.* 1979). α-Galactosidase was localized with 2-naphthyl-α-D-galactopyranoside (Lojda and Papoušek 1979), while β-glucosidase with 6-bromo-2-naphthyl-β-D-glucopyranoside (Beneš *et al.* 1973). Sections incubated in media without substrates were used as controls. We consider here the results of the enzyme reaction *in situ*. Speaking about staining or colour intensity we consider here the results of the enzyme reaction *in situ*.

RESULTS

α-Esterase

The cells in the unfertilized embryo sac revealed a very slightly positive reaction. In fertilized controls, the enzyme activity in the zygote cytosol increased, but the proembryo and nuclear endosperm showed an almost negative reaction (Table I, Fig. 1; Table III, Fig. 1). On the other hand, the content of nonfertilized and degenerating embryo sacs had a highly positive reaction (Table III, Fig. 2). The cells of the nucellus, the inner integument and the phloem of the funiculus revealed α-esterase activity during the whole embryogenesis.

The process of fertilization with irradiated pollen enhanced slightly the activity around the pronuclei. Enzyme activity of the pronuclei which could not fuse up to the 20th day after pollination was very high. Early after fertilization α-esterase activity was well manifested in the zygote and in the primary endosperm nucleus. In cases when the zygote could not initiate proembryo up to 15 d after pollination, it acquired a very deep, almost black enzyme staining. The nuclear endosperm, produced following application of pollen irradiated with more than 25, was characterized by increased α-esterase activity, and the higher the pollen irradiation dose was, the denser were the black stained cytosolic granules observed (Table I, Fig. 2; Table III, Figs. 3 and 4). Sometimes the nuclei looked pycnotic and deeply coloured. When an embryo was formed, the α-esterase activity recorded in its cells was also higher (Table III, Figs. 3 and 4).

With the rise in pollen irradiation dose, α-esterase activity in the cells of the inner integument and of the nucellus increased progressively. It must be noted that ovules which did not differ from the controls were also found in the pistil. A rise in the pollen irradiation dose reduced the number of this type of ovules.

AS-Esterase

During the normal embryogenesis the elements of a mature embryo showed a slightly positive reaction. Scarce blue granules were observed in the cytosol. During fertilization, AS-esterase activity gradually increased. The cytosol of the zygote was full of dark blue granules. The proembryo and nuclear endosperm also possessed active AS-esterase. The nucellus, the inner integument and the vascular bundles revealed a positive reaction as well.

After the irradiated pollen tube penetrated into the embryo sac, *i.e.* at the time of pronuclei union, the enzyme activity in the egg cell and in the central cell did not rise. The enzyme activity of the zygote was low. The proembryo and the nuclear endosperm had a negative reaction. Colour intensity, characterizing the enzyme activity in the ovules, was reduced parallel to the increase in the pollen irradiation dose.

Acid Phosphatase

Before fertilization acid phosphatase was found in the cytosol of embryo sac cells. During fertilization of the control plants the enzyme activity in the embryo sac increased. The enzyme was found in the cells of the embryo. In the course of growth the colour characterizing acid phosphatase activity became slowly reduced. The nuclear endosperm was active during the entire period of its existence. An increase of enzyme activity was observed in the case of unfertilized embryo sac degeneration.

Fertilization with irradiated pollen led to a rise of acid phosphatase activity in the embryo sac. In the case of no fusion of pronuclei and of inability of the zygote and primary endosperm nucleus to develop, their acid phosphatase activity was high and the cell nuclei were pycnotic. The same was observed in 3–4 cell embryos with disrupted development. The nuclear endosperm possessed a much higher enzyme activity than normal. The nuclei revealed particularly deep staining. The proembryo and the nuclear endosperm of embryo sacs which did not increase in size during the 20 d after pollination, were highly positive. The activity in the nucellus and in the inner integument was higher. At the same time, embryo sacs with growing embryos, though considerably inhibited in their development, differed only slightly from the controls. With the rise of the pollen irradiation dose, the degeneration processes in the ovules were enhanced and the enzyme activity of the embryo sacs was more pronounced. The contrary holds for the integuments, where the enzyme staining was greatly reduced.

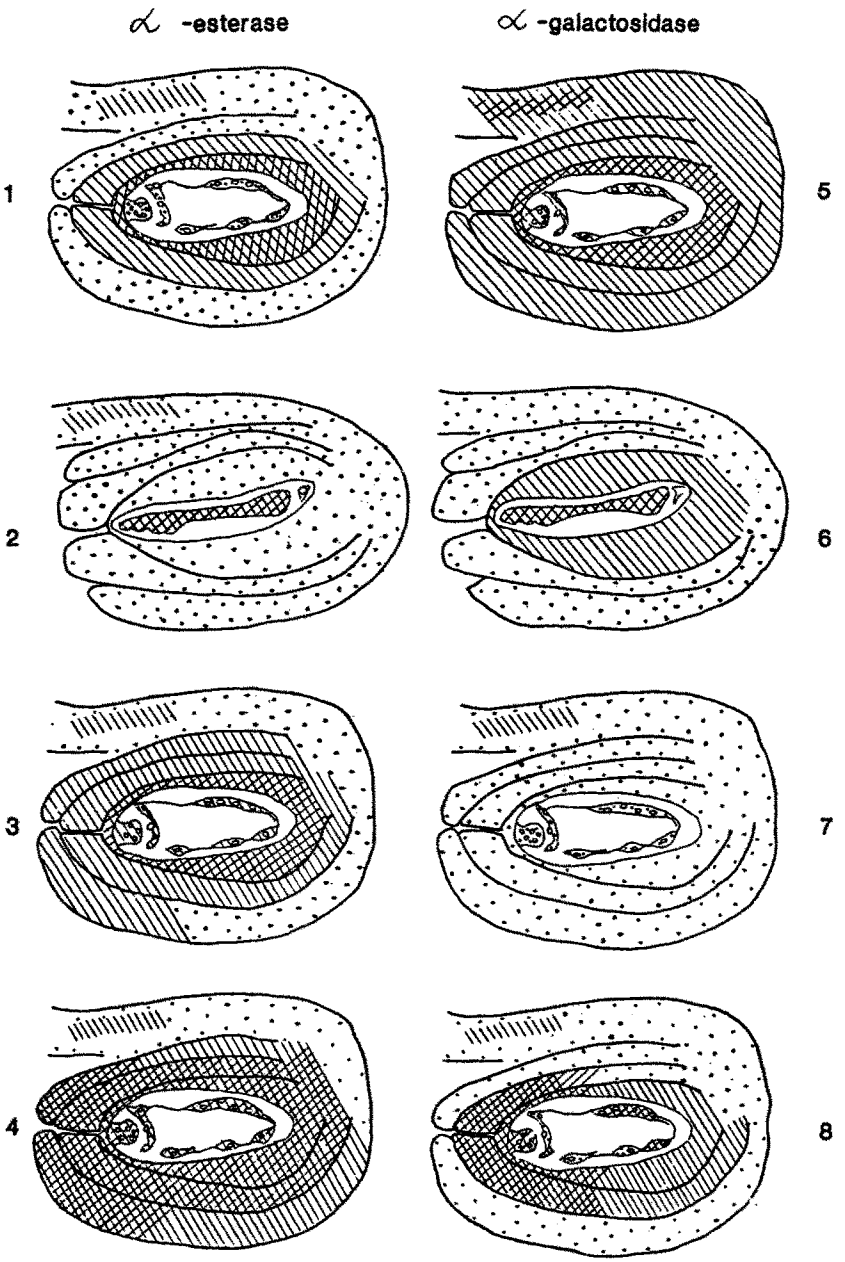

Table III. Histochemical localization of α-esterase (Figs. 1–4) and α-galactosidase (Fig. 5–8) in cross sections of *L. regale* pistil.

Figs. 1,5 – Embryo sacs with normal embryos.

Figs. 2,6 – Abortive unfertilized embryo sacs of control plants.

Figs. 3,7 – Early embryogenesis following pollination with 50 R. γ-irradiated pollen.

Figs. 4,8 – Embryo sacs at the stage of free nuclear endosperm after pollination with 100 R. γ-irradiated pollen.

α-Galactosidase

All cells that compose the embryo sac exhibited well expressed α-galactosidase activity. Enzyme activity in embryo sacs of control plants increased in the process of fertilization and remained at a relatively high level in the zygote, the proembryo and the nuclear endosperm. Also the integuments, the nucellus and the funiculus vascular bundle became more heavily stained (Table II, Fig. 1; Table III, Fig. 1). An increased enzyme activity was shown in the abortive embryo sacs, too (Table III, Fig. 2).

Regardless of the applied pollen irradiation dose *i.e.* of the possibility of normal fertilization, the egg cell and the central cell of the embryo sac contained an abundance of deeply stained granules during the coupling process (as in the controls). Diffuse staining of the cytosol occured in the frequently encountered cases of zygote degeneration. Enzyme reaction in the proembryos with considerably retarded growth and with abnormal morphology, was almost negative (Table II, Fig. 2; Table III, Figs 3 and 4). However, the degenerated embryo sacs had a densely stained content. The embryo and endosperm of ovules with no significant disturbances in the embryonal development were as coloured as the controls.

β-Glucosidase

No β-glucosidase activity could be observed in the embryo sacs of control plants during early embryonic development. The enzyme was localized in the inner integument only. Its activity increased here in the micropylar region after the proembryo passed the sphaerical stage. Degenerative changes in unfertilized embryo sacs were accompanied by greatly enhanced enzyme activity.

After pollination with irradiated pollen the zygote, as well as the proembryo and nuclear endosperm were rather intense in colour. Degenerative changes of the embryo and endosperm became more pronounced along with the increase in the pollen irradiation dose. They were accompanied by greatly enhanced β-glucosidase. In cases when the cell endosperm developed in the embryo sac and the proembryo grew, no β-glucosidase activity could be detected in them nor in the controls.

DISCUSSION

Studies conducted to determine the effect of irradiated pollen on the reproduction of different lily species revealed that several aberrant seed types (twin or triplet embryoed seeds, seeds with small embryos, seeds with endosperm but no embryo and chaff) developed following pollination with X- and γ-irradiated pollen (Price 1957). Later an evidence was presented by several authors that cytological disturbances of the fertilization (Vassileva-Dryanovska 1966) and cytoembryological abnormalities (Cave and Brown 1954) were

responsible for the appearance of these abnormal seeds. In the mentioned studies only the results concerning the behavior of nuclei in the embryo sac during fertilization and embryogenesis were described. The present observations revealed that after pollination with irradiated pollen distinct changes in the metabolism of the cytosol occured as well, which also contribute to the development of abnormal seeds and to chaff formation.

The enzymes under study take part in the normal metabolism of the embryo sac and their activities show several changes throughout the embryogenesis (Georgieva 1977). The present study does not establish a considerable deviation from the normal behavior of these hydrolases in embryo sacs which formed seeds with twin or small embryos after the application of irradiated pollen. The significant shift in the hydrolytic processes, observed mainly in embryo sacs with substantial disturbances in early embryogenesis, lead ultimately to a chaff formation as well as to seeds containing only endosperm. The number of abnormal embryo sacs of this kind increased and the deviations from the norm of the hydrolytic processes in them became more prominent with increased doses. Most probably the enhanced number of dominant lethals (Price 1957) carried by irradiated pollen was the cause of such deviations.

It must be pointed out that the changes in the activities of all hydrolases studied are not similar. AS-esterase and similar deviations in metabolism occurred not only after direct radiation treatment but also after pollination with irradiated pollen.

The present results as well as the results of our previous investigations (Georgieva 1984) brought evidence that the death of the cells of the proembryo and of free nuclear endosperm after pollination with irradiated pollen was a prolonged process with several successive steps of metabolic disturbances. The initial anomalies appeared almost immediately after the beginning of fertilization. They comprised on the one hand – the inhibition of constituent enzymes of certain metabolic pathways including a few hydrolases and – on the other hand – the activation of some other hydrolases. All these changes probably lead to a fall of cell viability. The terminal phase was connected with the increased activity of all hydrolases studied and lead to autolysis.

This suggestion was supported by our observations on the pattern of intracellular enzyme distribution during abnormal embryogenesis. At the beginning the enhanced activity of – esterase, acid phosphatase and β-glucosidase was manifested cytochemically as an increase in the number of intensively stained cytosolic granules corresponding probably to lysosomes (Fawcett 1981) or vacuoles which are lysosomal in nature (Matile 1975). Later a diffused staining of the collapsed cell content occurred in the degenerating embryo sacs which shows - apparently – a mixing of hydrolases with cytosol and the beginning of uncontrolled autolysis. That is why it could be assumed that drastic enhancement of α-esterase, acid phosphatase and α-galactosidase

activities in the ovules were reduced at the time of early embryogenesis as compared with the control and increased during the last stages of embryo sac degeneration. As to the α-esterase and acid phosphatase, their activities were considerably enhanced, beginning with zygote formation and ending at the stage of proembryo and free nuclear endosperm development. β-Glucosidase showed similar behaviour, its activity being manifested in the proembryo and endosperm. All these anomalies led to embryo or endosperm death at the phase of free nuclear endosperm.

During the last few years many authors have investigated the biochemical events which accompany cell deactivation, damage and death of different cell systems. The analysis of data obtained from evaluation of the loss of cell viability suggests that the process of cell death is not a simple one: cells first pass through a deactivated state before true death can occur (Jones 1987). On the other hand the examination of cell damage and death (Maleck *et al.* 1986) showed that cell damage and killing agents provoke abnormal accumulation and progressive leakage of numerous hydrolytic enzymes leading to cell death.

Investigations, concerning mainly animals (Hanson and Komar 1985), have shown that changes in the metabolism connected with cell destruction following irradiation are manifested biochemically first as inhibition of some metabolic pathways and then - at the final stages – as increased production or enhanced activities of certain hydrolytic enzymes. The present study demonstrated that in the embryogenesis of *L. regale* β-glucosidase in early embryogenesis could be considered as a marker for the presence of lethal factors.

It can be accepted that the main reason for the observed changes in hydrolytic processes during early embryogenesis after pollination with irradiated pollen, was the disrupted structures of the generative nucleus and the sperms and their DNA, respectively. Calgari *et al.* (1987) have assumed that X- and γ-rays damaged pollen DNA to such an extent, that only fragments of it could be effectively transmitted to the progeny. It remains to collect relevant experimental data of this kind concerning our material.

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Tables I and II at the end of the issue