

## Differences in the activity and distribution of peroxidases from three different portions of germinating *Brassica oleracea* seeds

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Peroxidase (POD, EC 1.11.1.7) activity, cellular localization and isozyme patterns were investigated in the seed integument, cotyledon and embryo axis of *Brassica oleracea* cv. Cappuccio during pregermination and seedling growth. Seeds started to germinate after 24 h of imbibition. POD activity was localized in the pigmented layer of the integument and in procambial strands of the cotyledon and embryo axis in the first 24 h of imbibition. It was localized in the integumental cells of palisade, pigmented and aleurone layers and in epidermal, meristematic, procambial cells and xylem elements of the root and hypocotyl after 48 h of imbibition. POD activity increased during germination and early seedling growth: in the integument, it reached a maximum value after 72 h of

imbibition, in the embryo axis and cotyledons, it increased up to 144 h of imbibition. The increase in peroxidase activity was accompanied by the appearance of new isozymes correlated with the development of seedling tissues. The isozyme profile was characterized by nine peroxidases: isoperoxidase of 50 kDa peculiar to integuments, that of 150 kDa to cotyledons and that of 82 kDa to the embryo axis. During pregerminative phase isozymes of 84 kDa were detected in the integument and cotyledons, of 48.5 kDa in the embryo axis. After germination, peroxidase activity and the complexity of the isozyme pattern increased, suggesting that they play a relevant role after rupture of the integument.

### Introduction

In plants, peroxidases (POD, EC 1.11.1.7) are reported as glycoproteins involved in many processes: plant growth and development (Fielding and Hall 1978b, Gibson and Liu 1978, Riquelme and Cardemil 1993); cell wall composition, i.e. lignin biosynthesis (Fukuda and Komamine 1982, Catesson et al. 1986, Bruce and West 1989), extensin polymerization (Fry 1987, van Hustee and Zheng 1995) and wall stiffening (Fry 1980); responses to a wide range of environmental stresses and stimuli (Gaspar et al. 1982, Castillo et al. 1984, Markkola et al. 1990, Cipollini 1998) and responses to the initial stages of mycorrhizal penetration (Spanu and Bonfante 1988). A partial explanation for the many roles of peroxidase is its multimolecular form, or isozymes, which are correlated with the activity of plant growth regulators during differentiation (Srivastava and van Huyssee 1977), involved in separate processes of organogenesis (Kay and Basile 1987) and related to specific developmental events in tissue culture (Whitmore 1978, Maldonado and van Huyssee 1980, Fukuda and Koma-

mine 1982, Kay and Basile 1987), and wound-healing (Angelini et al. 1990).

In seeds, POD have been localized in the integument of *Brassica juncea* (Le Beller et al. 1986) and soybean (Gillikin and Graham 1991, Gijzen et al. 1993, Schmitz et al. 1997). Little information is available on peroxidase isozymes in the integuments, cotyledons, and embryo axis of germinating seeds and seedlings, and differences between them. The aim of the present paper was to seek the relation between changes and differences in activity and distribution of peroxidases extracted from three different portions of *Brassica oleracea* seeds and seedlings.

### Materials and methods

#### Plant material

*Brassica oleracea* cv. Cappuccio seeds were sterilized, rinsed in distilled water and germinated in 9 cm Petri dishes on Whatman no. 1 filter paper moistened with 10

ml distilled water, in daylight, at 20°C. Fresh weight (FW) was evaluated at 3, 6, 12 and 24 h, every 24 h up to 96 h and at 144 h and expressed as percentage increase. Seeds with a radicle length of 1 mm were considered to have germinated.

### Sample preparation

Whole seeds, germinated as above, were collected after 12, 24, 48, 72, 96 and 144 h of imbibition, separated into integuments, cotyledons and embryo axis, and used for cytochemical determinations. For biochemical determinations, 500 mg of each tissue was placed in 2 ml 0.1 M K-phosphate buffer pH 6.1 and homogenized in a mortar for 2 min. The homogenates were centrifuged for 15 min at 13000 g at 4°C. Lipids floating on the supernatant were removed from the samples. The supernatant was used as an enzyme source for studies of soluble-POD activity.

### Cytochemical localization

Whole seeds, integument, cotyledon and embryo axis were frozen and sectioned with a cryostat. 10 µm thick unfixed sections were incubated for peroxidases in 20 ml 0.1 M Tris-HCl buffer pH 7.6, containing 10 mg *para*-phenylenediamine, 20 mg pyrocatechol and 0.2 ml fresh 1% H<sub>2</sub>O<sub>2</sub>; solution without H<sub>2</sub>O<sub>2</sub> was used as control (Gahan 1984). Sections were observed and photographed soon after incubation.

### Enzyme assay and protein determination

The peroxidase assay was performed at room temperature as follows: 3 ml distilled water were added to 1 ml 0.1 M phosphate buffer pH 6.2, 0.5 ml 2 M H<sub>2</sub>O<sub>2</sub>, 0.25 ml 10 mM 3,3'-dimethoxybenzidine dihydrochloride salt (Sigma *o*-dianisidine, Sigma Aldrich s.r.l., Milan, Italy) and 0.25 ml supernatant (Markkola et al. 1990). The reaction was run against a non-enzymic blank. Indirect tests of the effect of SDS on enzyme activity were performed by incubating *o*-dianisidine and H<sub>2</sub>O<sub>2</sub> with the enzyme standard (Sigma) in the presence of SDS. The absorbance was measured at 470 nm for 5 min using a Cary-1 Varian spectrophotometer and expressed as increase in absorbance g<sup>-1</sup> min<sup>-1</sup> ( $\Delta A g^{-1} min^{-1}$ ) of FW of sample during the first minute of incubation. The peroxidase standard from Sigma was dissolved in 3 ml phosphate buffer pH 6.1, diluted 1 : 1000 and measured with *o*-dianisidine.

The set of experiments was replicated three times. Data were analysed by the LSD test at a *P* = 0.05. Protein content of extracts was determined using the Bio-Rad dye reagent with bovine serum albumine as the standard (Bradford 1976).

### Electrophoresis

9% SDS polyacrylamide gels (pH 8.8) were prepared 24 h before the experiment and stored at 4°C to ensure a

complete polymerization. Gels were loaded with the supernatant from the integuments, cotyledon and embryo axis samples, with peroxidase enzyme and with Sigma prestained SDS-PAGE standard (broad range 180–26.6 kDa) to determine molecular masses. Electrophoresis was run at 4°C at 120 V for 60 min. Gels were reacted for peroxidases for 30 min with a freshly prepared solution of 10 mM 10% *o*-dianisidine in 0.1 M acetate buffer pH 4.5, or in 0.1 M phosphate buffer pH 6.2 or in ammonia buffer pH 8.0; 0.17% benzidine in 0.1 M phosphate buffer pH 6.2 (Gibson and Liu, 1978) and ammonia buffer pH 8.0 were also tested. The reaction solution was discarded and replaced with a 0.03% H<sub>2</sub>O<sub>2</sub> in the same buffers until POD bands were visible.

### Results

Water absorption during germination occurred in three stages (Fig. 1); the first lasted 3 h and was characterized by a sharp percentage increase in FW; the second lasted 24 h, was characterized by a constant percentage increase in FW and terminated with the rupture of the integuments and emission of the radicle. The third stage (seedling growth) was characterized by a further increase in FW.

### Histochemistry

Ripe seeds of *Brassica oleracea* have an external integument, consisting of mucilaginous cells, a crushed subepidermal parenchyma layer, a palisade layer and an inner integument consisting of a crushed pigmented epidermal layer. An aleurone layer one or two cells thick containing protein bodies and a hyaline compressed layer, are joined to the integument (Fig. 2A). Two oily cotyledons, folded along the mid-axis, envelop the embryo. The histochemical localization of peroxidases revealed activity in the pigmented layer of the integument after 12 (Fig. 2A) and

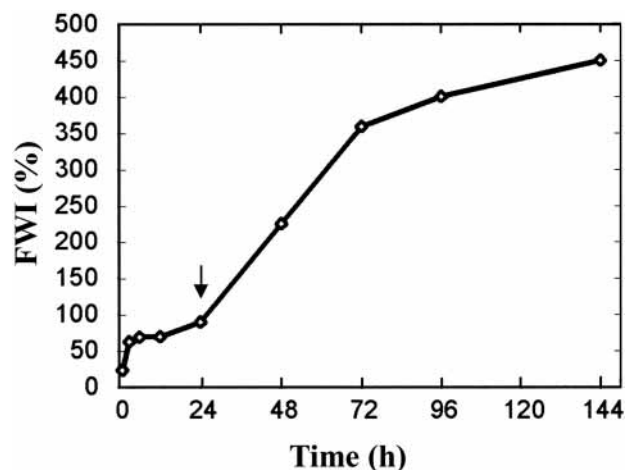


Fig. 1. Fresh weight increase (FWI) up to 144 h of germinating seeds of *Brassica oleracea* cv. Cappuccio. Arrow marks the time of visible radicle emergence.

24 h of imbibition, and several dark globular bodies in cells of the palisade, pigmented and aleurone layers after 48 h of imbibition (Fig. 2B). In the tissues of shoot and root apex of the embryo, no peroxidase activity was detected after 12 h of imbibition and low activity was detected after 24 h. Peroxidase activity became evident in the cytoplasm of epidermal and subepidermal cells, in tunica and corpus cells of the shoot apex (Fig. 2C), in meristematic cells of the root tip (Fig. 2D), in the cell walls of the hypocotyl (Fig. 2C) and in root procambial strands by 48 h imbibition. The intensity of the reaction product increased as differentiation of vascular elements proceeded. In cotyledons, enzyme activity was present in procambial cells at 24 h (Fig. 3A), in the cell walls and

cytoplasm of parenchyma cells (Fig. 3B) and differentiating tracheary elements, and in epidermal cells (Fig. 3C) at 48 h. High peroxidase activity was associated with the protoplasts of differentiating xylem elements and with the secondary wall thickenings of xylem elements (Fig. 3D) at 96 and 144 h.

#### Peroxidase activity

Peroxidase activity varied during seed germination (Table 1). It increased sharply in the integument between 24 and 72 h, when the integument breaks and the seedling starts to grow, and was essentially constant after 72

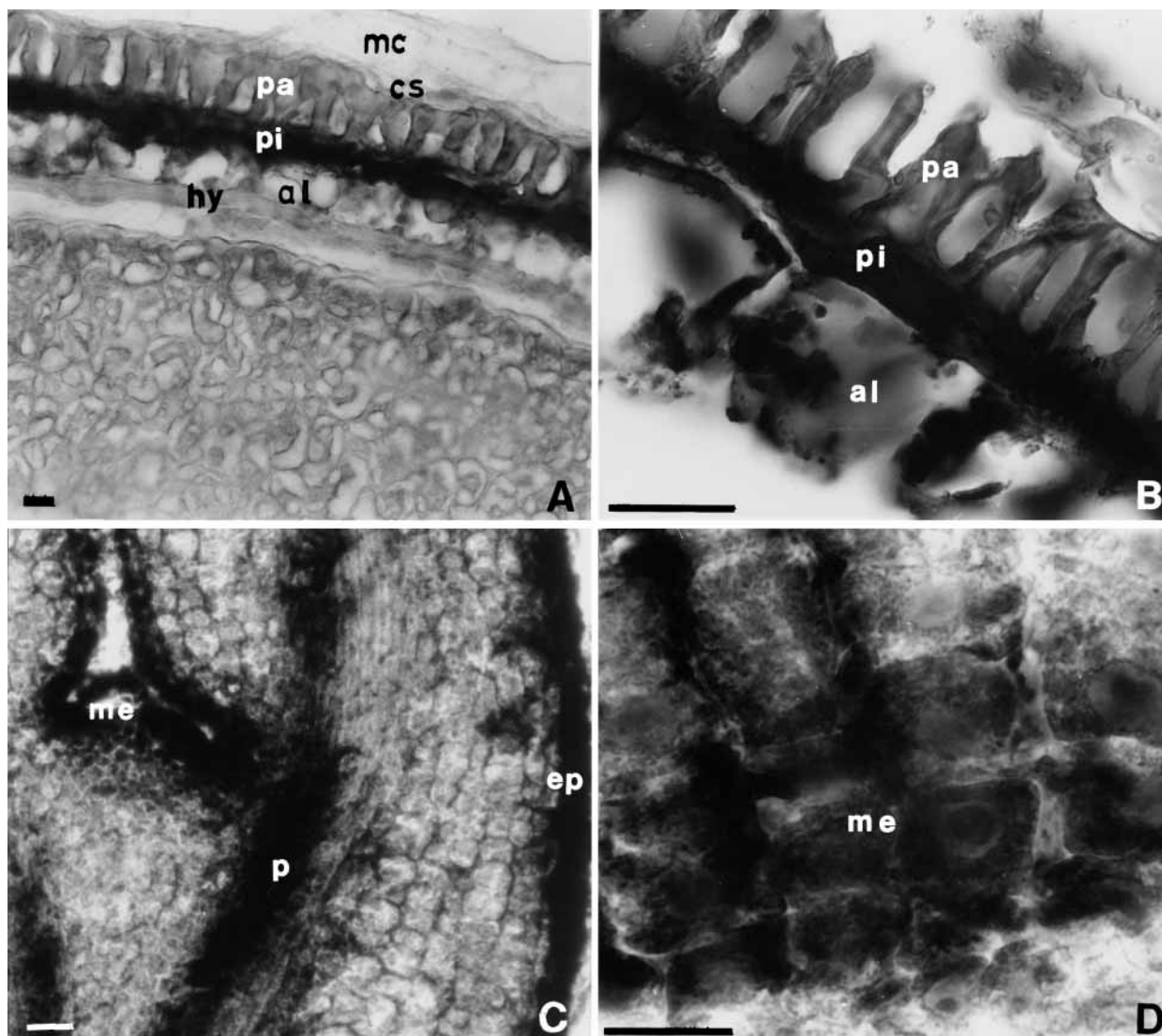


Fig. 2. (A)-(D). Histochemical localization of peroxidase activity in the integument of *Brassica oleracea* cv. Cappuccio. Transverse section of a whole seed imbibed for 12 h with the following layer: mucilaginous cells (mc), chushed subepidermal (cs), palisade (pa), pigmented (pi), aleurone (al), hyaline (hy); peroxidase activity in cells of the pigmented layer (pi) (A). Bar = 15  $\mu$ m. Seed imbibed for 48 h: peroxidase activity in the palisade (pa), pigmented (pi) and aleurone (al) layers (B). Bar = 15  $\mu$ m. Longitudinal sections of seed imbibed for 72 h: peroxidase activity in epidermal cells (ep), procambial strands (p) and meristem cells (me) of shoot (C) and root tip (D). C, Bar = 7.5  $\mu$ m. D, Bar = 15  $\mu$ m.



h. In the cotyledons and embryo axis, activity increased gradually as the embryo grew.

SDS did not affect enzyme activity (data not reported), but caused a shift in the colour of the reaction from brownish-red to green.

### Electrophoresis

Extracts of all seed tissues, separated on SDS electrophoresis gels, were stained by *o*-dianisidine or benzidine at different pHs to achieve the broader spectrum of isozymes and to determine eventual differences in isozyme expression. With benzidine and *o*-dianisidine at pH 6.1 or 8 it was not possible to see more than two bands in the tissues examined. The best results in this study were obtained with *o*-dianisidine at pH 4.5; the isozyme pat-

tern of peroxidases is indicated in Fig. 4. The results of the SDS electrophoretic analysis showed that only the 84 kDa peroxidase of the nine different molecular mass isoperoxidases is common to all three samples types. The integument sample (which includes endosperm tissue) and the cotyledons have 42.5 and 40.5 kDa in common. The cotyledons and embryo axis have 48.5, 46.5 and 45 kDa isoperoxidase in common. Each of the three remaining isoperoxidases of 50, 150 and 82 kDa, is peculiar to the integument, cotyledon, and embryo axis, respectively.

### Discussion

*Brassica oleracea* seeds began to germinate after 24 h of imbibition. In the present study the seeds were examined

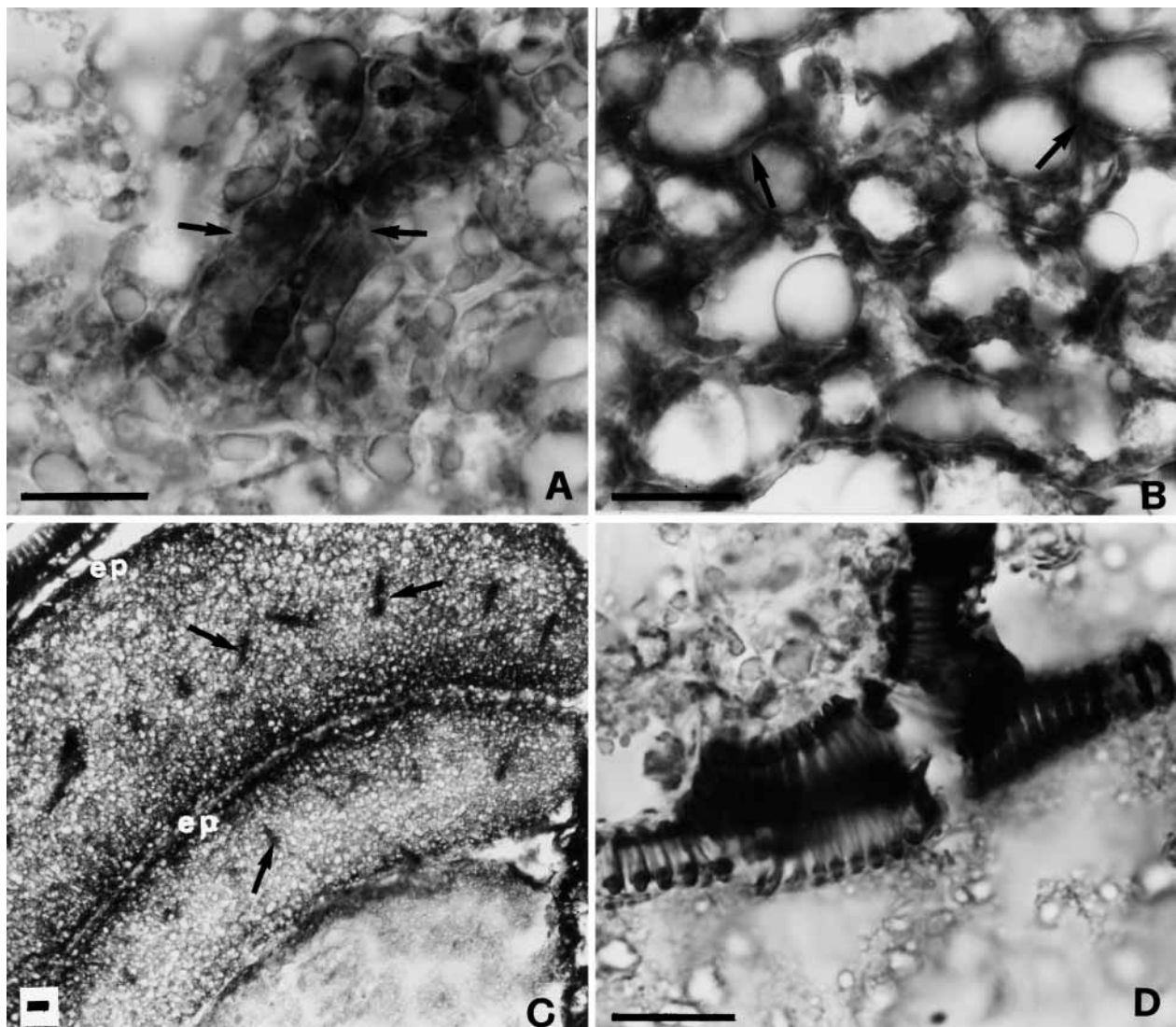


Fig. 3. (A)-(D). Histochemical localization of peroxidase activity in seeds of *Brassica oleracea* cv. Cappuccio. Cotyledon of seed imbibed for 24 h: peroxidase activity in procambial cells (arrows) (A). Bar = 15  $\mu$ m. Cotyledon of seed imbibed for 48 h: peroxidase activity in cell walls (arrows) and cytoplasm of parenchyma cells (B), in epidermal cells (ep) and in differentiating tracheary elements (arrows) (C), in xylem elements (D). B, Bar = 15  $\mu$ m. C, Bar = 7.5  $\mu$ m. D, Bar = 15  $\mu$ m

Table 1. Peroxidase activity ( $\Delta A \text{ g}^{-1} \text{ min}^{-1}$ ) of *Brassica oleracea* cv. Cappuccio seed tissues stained with *o*-dianisidine after different periods of imbibition. Means  $\pm$  SE ( $n = 3$ ). Within rows, the values followed by different letters are significantly different at  $P = 0.05$ .

Tissue	Time of imbibition									
	12 h		24 h		48 h		72 h		96 h	144 h
Integument	1.47	$\pm$	1.91	$\pm$	4.20	$\pm$	6.63 $\pm$ 0.22c		6.34 $\pm$ 0.95c	6.10 $\pm$ 0.35c
Cotyledon	1.81	$\pm$	2.18	$\pm$	2.45	$\pm$	3.34	$\pm$	4.08 $\pm$ 0.47c	6.16 $\pm$ 0.31d
	0.40a		0.21a		0.39a		0.49b			
Embryo axis	0.10	$\pm$	0.95	$\pm$	1.50	$\pm$	5.70 $\pm$ 0.81c		6.50	$\pm$
	0.10a		0.20b		0.23b				0.93d	9.50 $\pm$ 0.69e

for peroxidase activity, cytochemical localization and SDS electrophoresis from pregermination up to seedling formation. It is well known that SDS denatures enzymatic proteins by destroying the steric configuration responsible for enzyme activity. Chibbar et al. (1984) reported that 'the synthesis of peroxidase protein leads to a non-functional enzyme, because peroxidase activity is only re-established by association of the apoprotein with the heme moiety to form the holoenzyme'. According to Tams and Welinder (1991) 'deglycosylation of peroxidases generally results in the loss of enzyme activity'. In our case, indirect tests in which *o*-dianisidine and  $\text{H}_2\text{O}_2$  were incubated with the standard enzyme (Sigma) in the presence of SDS, showed that SDS did not inacti-

vate the protein but just caused a shift in the colour of the reaction.

The plant peroxidase system may be a family of closely related enzymes with different physiological functions in the cell, their expression being under developmental control (Gibson and Liu 1978). Peroxidase isozymes have shown specific affinity for different substrates (Fielding and Hall 1978a, Gibson and Liu 1978, Gahan et al. 1986). In the present study, *o*-dianisidine dissolved in acetate buffer at pH 4.5 gave the best reaction for detecting the electrophoretic pattern in different tissues of germinating *Brassica* seeds. Peroxidase activity has been correlated with seven processes related to plant development (Kay and Basile 1987) and located in unlig-

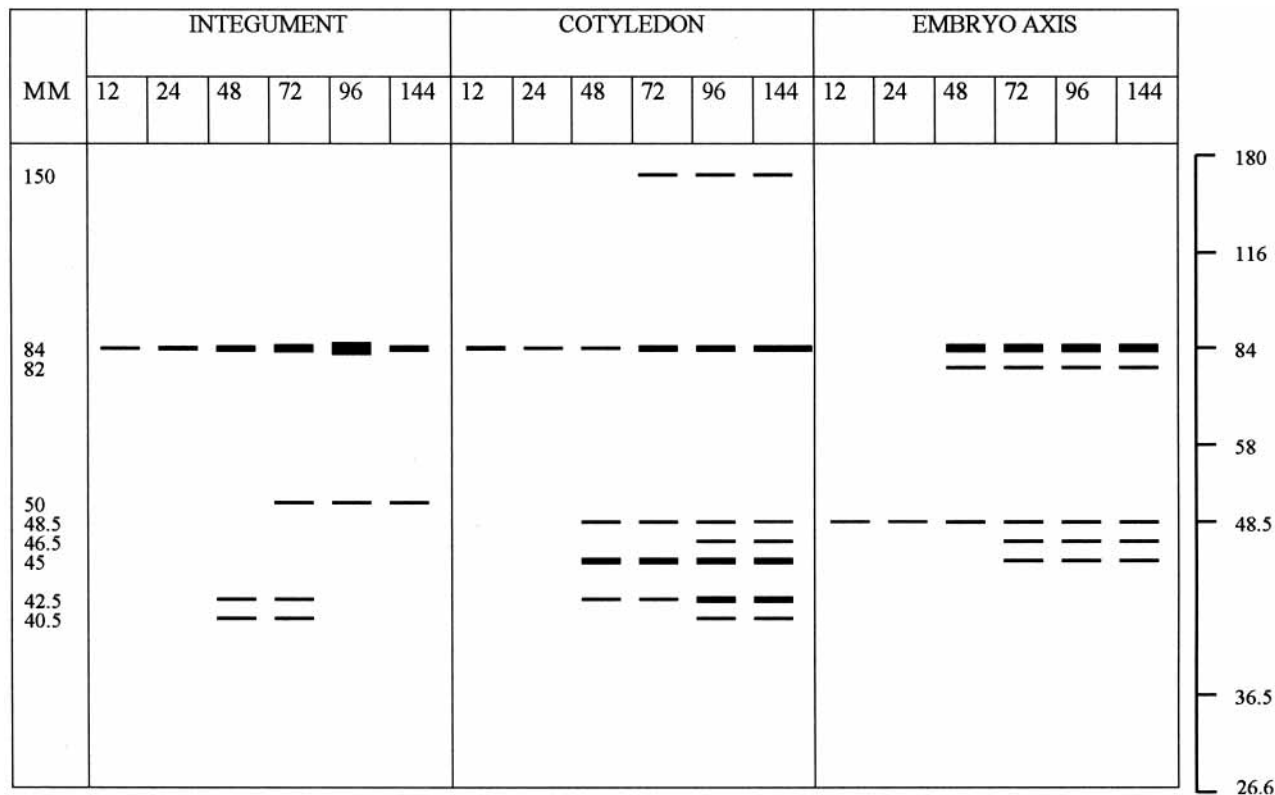


Fig. 4. Zymogram of peroxidase isozymes reacted with *o*-dianisidine from tissues of seeds of *Brassica oleracea* cv. Cappuccio imbibed for 12–144 h. The molecular masses of the bands are indicated on the left; the Sigma prestained standard (broad range 180–26.6 kDa) is on the right. Comparative banding intensity is indicated by the degree of thickness.

nified, growing cell walls of primary plant tissues, where phenolics are natural substrates for peroxidases (Fry 1986). Using *in vitro* cultures, Fukuda and Komamine (1982) demonstrated a parallel relationship between the increase in soluble peroxidase activity and the lignin content of xylem elements formed. In *Cicer arietinum*, soluble and wall fractions of peroxidase were observed to parallel the distribution of lignin deposition along the stem, higher in the basal zone and decreasing acropetally (Angelini et al. 1990). In *Pisum sativum* changes were observed in the detection of cell wall-bound and soluble peroxidases bands during root development, with the production of more complex physiological and structural aspects (Fielding and Hall 1978b). In *Brassica*, peroxidases are localized in meristematic cells of the root and shoot apex, in epidermal, subepidermal and parenchymatic cells, in procambial strands and lignified xylem elements. The increase in peroxidase activity during embryo axis growth and cotyledon expansion could be related to cell wall growth and lignification of xylem elements of growing seedlings. The nine POD isozymes with different molecular weights expressed during *Brassica* seed germination and seedling growth could be correlated to different developmental events. Isoperoxidases of 50, 150 and 82 kDa were peculiar to the integument, cotyledon and embryo axis, respectively. In the integument, a protein of 50 kDa appeared at 72 h and remained up to 144 h. This may be involved in integument degradation and metabolism of phenolic compounds observed in the cells of the palisade and pigmented layers of *Brassica oleracea* (Bellani et al. 2000). Phenols protect the germinating seed by an antimetabolic effect on soil microorganisms (Podbielkowska et al. 1994) or act as a deterrent to herbivores by reducing the nutritive value or palatability of the seed (Gijzen 1997). Isozymes with MM of 48.5, 46.5 and 45 kDa, detected in the cotyledons and embryo axis after germination and during seedling development, are probably involved in lignification of xylematic elements, which occurs at this stage. PODs of 42.5 and 40.5 kDa are peculiar of the postgerminative phase of temporary structures such as integument and cotyledons. The 150 kDa isozyme which appears after 72 h of imbibition, specific to the cotyledons, may be correlated to the advanced reserve mobilization occurring in this seed portion from this time. Peroxidases have been mainly associated to the maintenance of a correct size and shape of protoderm cells, apparently indispensable for normal embryo growth and to the catabolism of IAA in higher plants, thus suggesting their participation in the regulation of plant growth and development (Cella and Carbonera 1997). The same function can be hypothesized for the 82 kDa isoperoxidase peculiar to *Brassica* embryo axis. The band of 84 kDa, widespread in all the seed portions, was present in the integument and cotyledon before and after germination, in the embryo axis after germination, and can be correlated with cellular metabolic activity.

Peroxidase activity has been localized in the coat of the dry *Glycine max* seed (Gillikin and Graham 1991)

but not in that of *Sida spinosa* (Egley et al. 1983). In the *Brassica* integument, POD is localized in the cell walls of the palisade and pigmented layers and in the walls and cytoplasm of aleurone cells. A low level of peroxidase activity was detected up to 24 h of imbibition when the band of 84 kDa was the only one present. The increase in activity observed in the postgerminative phase was probably caused by the injury due to the rupture of the integument and matched with the appearance of the bands of 42.5 and 40.5 kDa. Wounding elicits the appearance of new isoforms of peroxidase and the increase in POD activity in soluble and in wall fractions (Angelini et al. 1990) with antioxidant function to rid cells of the H<sub>2</sub>O<sub>2</sub> produced by injury (Cipollini 1998).

In conclusion, before germination, the low POD activity is due to isozymes of 84 kDa localized in the integument and in the cotyledons and of 48.5 kDa localized in the embryo axis. After germination the activity of these isozymes is present and seven more isozymes appear, indicating that peroxidases are little represented in the imbibed seed, but play a significant role during the development of the seedling.

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