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CHANGES IN SOLUBLE AND IONICALLY BOUND PEROXIDASE ACTIVITIES DURING *BRASSICA JUNCEA* SEED DEVELOPMENT

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Summary. Changes in peroxidase activity was analyzed in developing mustard (Brassica juncea) seed. The activity in soluble and bound fraction was assayed with three hydrogen donors viz. ferulic acid, caffeic acid and chlorogenic acid. Growth was measured in terms of dry weight and water content. Seed dry weight data was fitted to a cubic polynomial equation. The rate of dry matter accumulation was calculated by differentiating the best-fit cubic polynomial. A close parallelism between water content and dry matter accumulation was observed in developing mustard seed. The peroxidase activity was less during initial phase of seed development and showed a rising trend after d 20. Thus the activity showed an inverse correlation with water content. This inverse correlation suggests an important role of peroxidase in termination of the process of cell elongation of mustard seed.

***Key words*:** *Brassica juncea*, seed development, peroxidases, cell walls

Introduction

Indole-3-acetic acid (IAA) is the principal auxin found in the seeds of higher plants (Schneider and Wightman, 1974). To function as a regulator of cell expansion, its own concentration in the target tissue must be controlled (Pilet and Saugy, 1982). Auxin affects cell elongation, probably, through its influence on cell wall extensibility. However, the modifications of cell wall architecture that confer extensibility to the walls, are poorly understood. The cell wall is sometimes considered as the site of primary action of plant peroxidases.

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Since long, peroxidases are known to be involved in growth regulation, and different biochemical pathways have been proposed to examine their mechanism of action (Goldberg et al., 1987). Peroxidases play a key role in the stiffening of the cell wall and in processes associated with plant growth through the formation of phenolic cross-link (Fry, 1986). This process is similar to that occurring in lignifying cell walls where peroxidases are known to catalyze the oxidative polymerization of cinnamic alcohols. In many of the studies, guantitation and identification of peroxidases was based on measuring the enzyme activity, using various hydrogen donors. Valero et al. (1991) indicated that cell wall peroxidase activity in epicotyl of *Cicer arietinum* is related to cell wall stiffening. There are numerous studies to suggest inverse correlation between peroxidase activity and IAA content and/or elongation growth (Thaker et al., 1986; Zheng and van Huystee, 1992). Involvement of peroxidases in termination of elongation phase in wheat grain development was reported by Chanda and Singh (1997). In an earlier study, the role of glycosidases in mustard seed development was reported (Saroop et al., 1998). In the present work, we studied changes in soluble and bound peroxidases during Brassica juncea seed development.

Materials and methods

Mustard seeds (*Brassica juncea* cv. "Varuna" T-59) were sown in a farmer's field in black cotton soil (vertisol). Normal cultural practices including irrigation, weeding, fertilizer application were conducted to optimize growth and yield. Ten rows, 1 m apart and 40 m long were prepared, and a basal dose of fertilizer (diammonium phosphate) was provided at the rate of 100 kg/ha. Plant to plant spacing was 0.5 m. Irrigation was done at weekly intervals until maturity. Forty days after sowing another dose of urea fertilizer (90 kg/ha) was provided. A similar dose of urea fertilizer was given after another 30 days. Flowering took place 50 days after sowing. Seed samples were harvested at an interval of 4–5 days after fertilization until maturity for growth and biochemical analysis. The age of the seed was ascertained by tagging the flowers at anthesis.

Growth analysis

Growth was measured in terras of fresh weight, dry weight and water content. On the day of anthesis, the seed pods were removed from the plants, packed in polythene bags, and brought to the laboratory. Seeds were separated immediately from the pods and subjected to growth measurements and biochemical analyses on the same day. Ten seeds in ten replicates at each harvest were used for fresh and dry weight measurements. The seeds were oven dried at 65 °C till constant weight was achieved, after which the dry weight was taken. The difference in fresh and dry weight measurements

was the water content in nig at each time. The seed dry weight data was fitted to polynomial functions and the selection of the appropriate polynomial regression model was made statistically by the Slack of fits method (Nicholls and Calder, 1973). The instantaneous rate of grain filling dw/dt was calculated as the derivative of the appropriate polynomial.

Biochemical analysis

The required number of seeds (the number varied from 100–230 depending on the age of the seed) were separated and chilled in a freezer (0–4 °C). The chilled material was homogenized in pre-chilled Na-phosphate extraction buffer (0.02 M, pH 6.4) in a cooled mortar with a pinch of sand at 4 °C in a cold room. The homogenate was filtered through 4–5 layers of muslin cloth and then centrifuged at 15 000×g for 20 min. The supernatant was mixed with pure chilled acetone (1:2) at -4 °C to precipitate soluble proteins. The acetone-precipitated proteins were redissolved in Na-phosphate buffer (0.02 M, pH 6.4) which was used for estimating soluble enzyme. The residue was thoroughly washed with distilled water and centrifuged till the washings were free of peroxidase reaction with guaiacol. The wall fraction was then kept with 10 ml of 1 M NaCl for 1 h to release ionically bound enzymes. After centrifugation at 10 000 g for 10 min, the supernatant was used as the source of wal ionically bound enzyme.

Peroxidase assay

The peroxidase activity was measured by recording the change in absorbance at 400 nm (ΔA_{400}) due to the oxidation of the hydrogen donor in the presence of H_2O_2. The reaction mixture consisted of 12 mM Na-phosphate buffer (pH 6.4), 4 mM either ferulic acid, caffeic acid or chlorogenic acid, 1 mM H_2O_2 and the enzyme. The reaction was initiated by the addition of H_2O_2 and the increase in absorbance was monitored. The activity is expressed as $\Delta A_{400}.min^{-1}.seed^{-1}$. The complete biochemical analysis was done in two independent preparations (each time with three replicates) and the mean values are presented.

Results and Discussion

Changes in seed dry weight and water content

The changes in seed dry weight versus days after anthesis was fitted to polynomial equations and the best fit curve was determined statistically. A third degree polynomial adequately described the seed dry weight data (Fig. la). Soon after anthesis, there was a short period of exponential growth (11 days) which was followed by a phase when seed growth rate was constant and this phase was maintained up to 40–45 days and

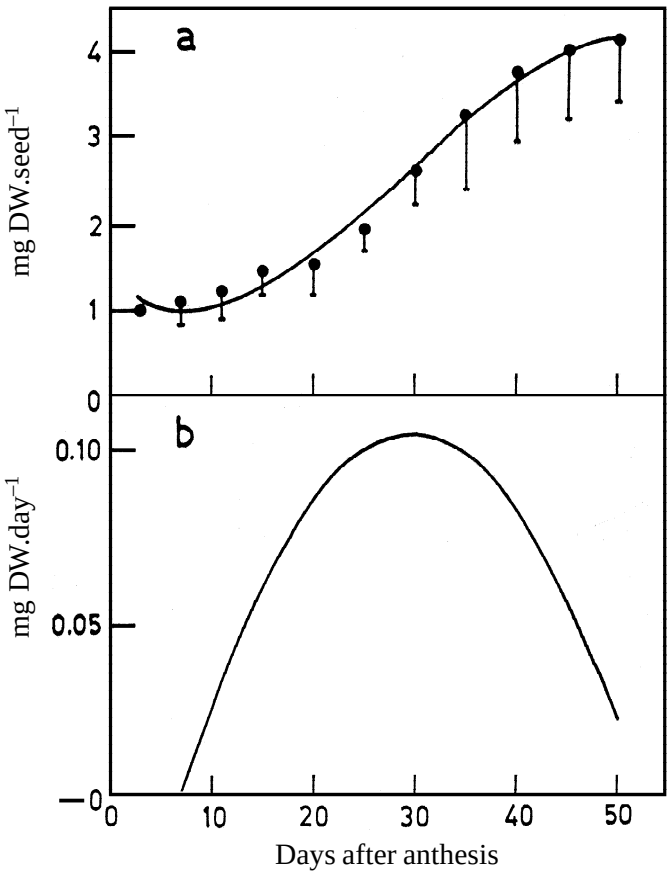

Fig. 1.a. Seed dry weight versus days after anthesis predicted from a cubic polynomial equation and actual mean seed dry weight. **b**. Rate of dry matter accumulation obtained by differentiating the cubic polynomial. Vertical bars indicate ±S.D.

then a final lag phase was observed before maximum weight was recorded at day 50. The growth of mustard seed thus, followed a general pattern of growth that has been observed in other crop plants including mustard (Chanda and Singh, 1996, Saroop et al., 1998).

The rate of dry matter accumulation was calculated by differentiating the cubic polynomial and is presented in Fig. 1b. The developing seed started accumulating dry matter after 7 days of anthesis. It reached maximum level at day 30 after which it had a low but positive rate even at day 50 after anthesis. The water content, on the other hand, started increasing from day 3 onwards and attained maximum content at day 35, after which it maintained it level till day 50 (Fig. 2). Thus maximum water content was. achieved before the attainment of maximum dry weight content. Similar pattern of dry matter accumulation and water uptake have been reported (Martinez-Carrasco et al., 1988; Chanda and Singh, 1997).

Plant cell wall plays a critical role in water uptake. A wide range of growth regulating agents, including both hormones and environmental factors, apparently affect

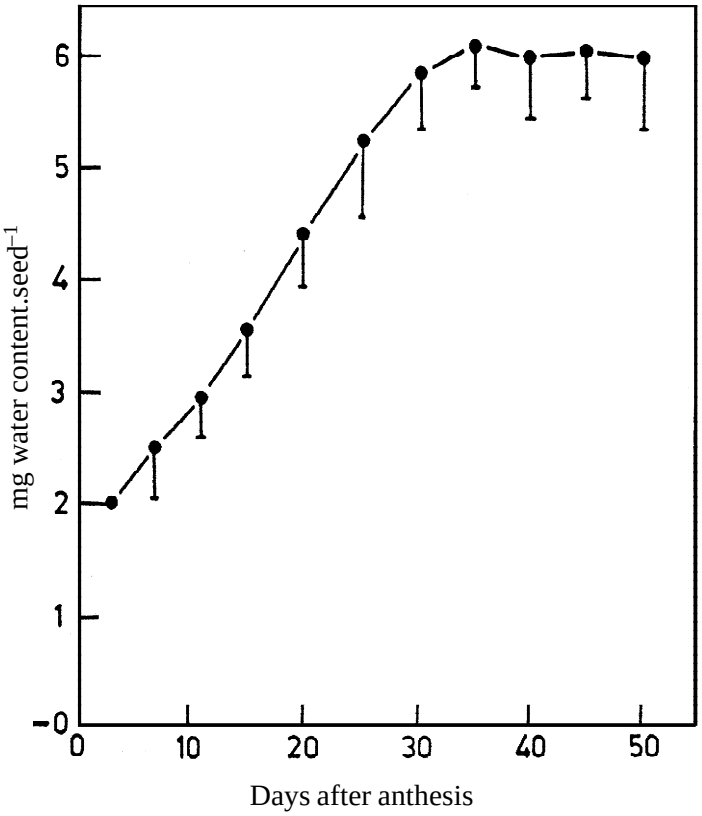

Fig. 2. Water content (mg.seed^{-1}) of developing mustard seed during entire period of seed growth.

water uptake by modifying the ability of the primary wall to extend irreversibly. Further, it is also dependent upon turgor pressure, generated by the accumulation of solutes. Although, the turgor potential in developing seed is not high, but could be adequate driving force for cell enlargement in developing seed. It has been also shown that cell turgor can affect phloem loading (Wolswinkel, 1990) and sucrose and amino acid transport in developing seeds (Wolswinkel and Ammerlan, 1989). The close parallelism between water content and dry matter accumulation of developing mustard seed therefore suggests that a rapid uptake of water may be required to support rapid rate of dry matter accumulation.

Changes in peroxidase activity

Changes in soluble peroxidase activity using ferulic acid, caffeic acid and chlorogenic acid as phenol substrates are presented in Fig. 3. In all the 3 substrates, the soluble peroxidase activity increased slightly up to 20 d after anthesis, while between d 20–30

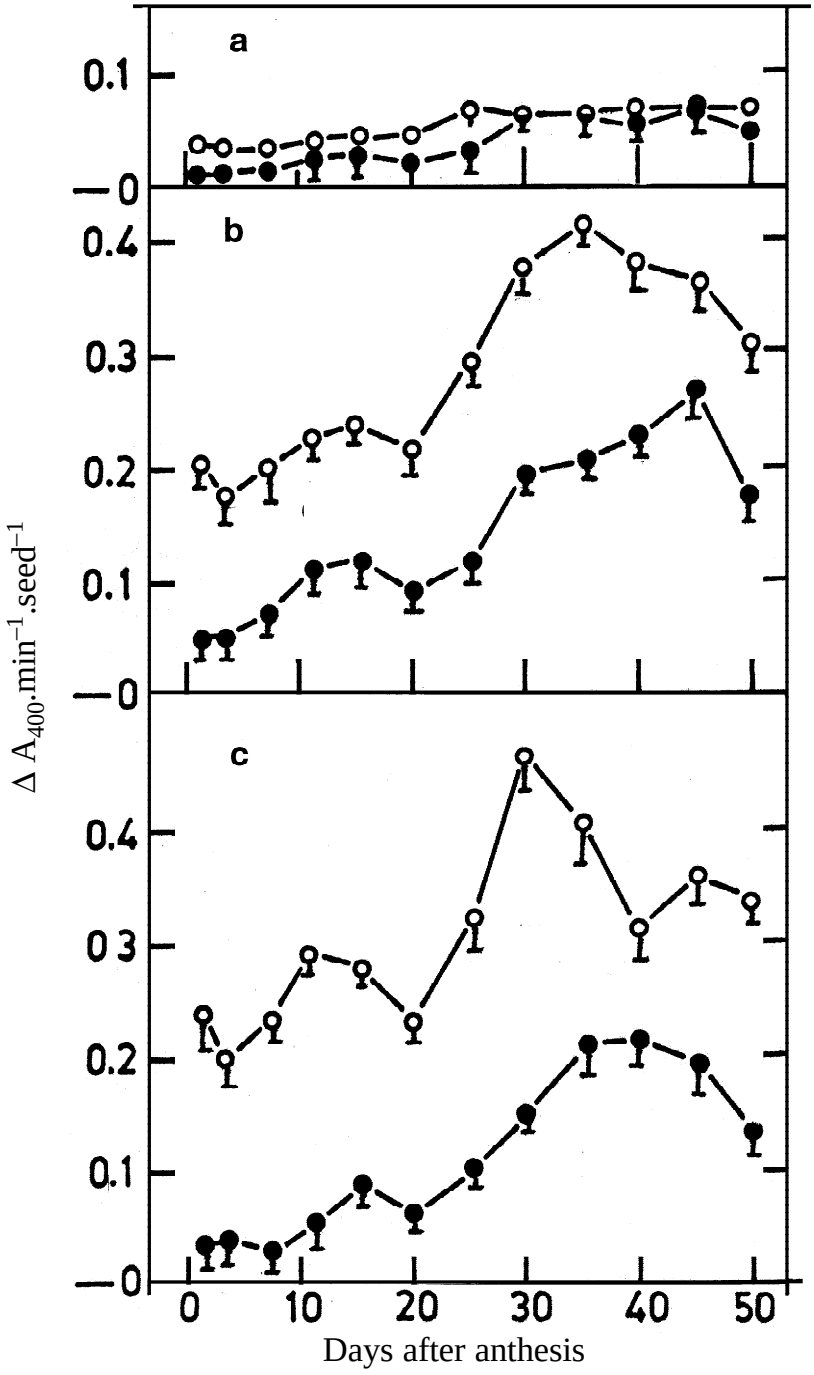

Fig. 3. Changes recorded in soluble (o–o) and bound (•–•) peroxidase activity using (**a**) ferulic acid, (**b**) caffeic acid and (**c**) chlorogenic acid as hydrogen donors, during the entire period of mustard seed growth.

a sharp increase in activity was observed and during later stages, the activity decreased slightly. In spite of the similar trends in all the 3 substrates used, the levels were different. The activity was minimum when ferulic acid was the hydrogen donor and caffeic and chlorogenic acid had almost equal activity. The activity, however, showed an inverse correlation with water content. An iverse ralationship was observed between water uptake level and peroxidase activity. The ionically bound peroxidase activity was comparatively less than that of cytoplasmic activity when caffeic or chlorogenic acid were the hydrogen donors. The activity with ferulic acid was the same in both fractions. It is known that most peroxidase activity can be fractioned into large number of isozymes, the precise role of which often remains uncertain. Working with tobacco epidermal explants, Kay and Basile (1987) resolved 47 isoperoxidases from cytoplasmic and bound fractions and could correlate 25 of them with specific developmental events. However a definite role of these isoenzymes remains obscure. Nevertheless there are numerous reports in literature with respect to peroxidase involvement in oxidation of endogeous IAA (Beffa et al., 1990).

Further, a considerable variation between histochemical and biochemical data have been noted. Histochemical observations of different species revealed that only lignifying cells were able to react with syringaldazine, while most peroxidases can oxidize a wide range of H2 donor including syringaldazine *in vitro* (Pang et al., 1989). Based on these studies, it has been suggested that solubilization of peroxidases from non-lignifying cell walls undergo changes at some recognition site (s) which enable the enzymes to react with a wide range of H_2 donors. Modifications of cell wall peroxidase specificity during extraction process have been postulated by Ros Barcelo et al. (1987). In the present work, the ionically bound peroxidase activity showed an inverse correlation with water content which suggests an important role of these peroxidases in the overall mechanism of cell elongation of developing mustard seed. Indeed, such an inverse correlation is well documented in diverse tissues (Chanda et al., 1986; Thaker et al., 1986; Bagatharia and Chanda, 1998). There are data that peroxidase catalyzes the oxidative coupling of phenols, thus which binding molecules of polysaccharides and glycoproteins (Fry, 1983). This results in changes of the physical properties of cell walls. Peroxidases are able to reduce cell wall extension rate by catalysing the formation of covalent bonds, such as isodityrosine or diferulic acid bridges (Fry, 1986) and by oxidizing cinnamic alcohols to radicals which polymerize to lignin (Bolwell, 1988). Such changes in the mechanical properties are related to an increase in cell wall rigidity (Sakurai et al., 1982). Further, Fry (1984) suggested that feruloylated matrix may suppress the metabolism of matrix polysaccharides by hydrolases. Metabolic turnover of matrix polysacharides is generally considered to be the cause in changing mechanical properties of cell wall (Kamisaka et al., 1990). It may be concluded that cell wall peroxidase may be the controlling site for cessation of elongation growth in mustard seed development as reported earlier in wheat grain development (Chanda and Singh, 1997).

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