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Biochemical Changes Associated with *Brassica juncea* Seed Development, II: Glycosidases

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Abstract. Changes in α- and β-galactosidase, glucosidase, and α-mannosidase and β-xylosidase activities were analyzed in developing mustard (*Brassica juncea*) seed. A cubic polynomial described the seed dry weight data adequately. A close parallelism between the water content and dry matter accumulation was shown (r = 0.991). Glycosidase activities were detected in both cytoplasmic and wall fractions. The trend was similar in both of the fractions, but the activity of α-mannosidase and α-galactosidase was considerably greater in the wall-bound fraction. The water content and the activity of glycosidic enzymes showed a significant linear correlation ($p < 0.001$). The results suggest that glycosidases have an important role in cell wall loosening during mustard seed development.

Key Words. *Brassica juncea*—Seed development—Glycosidases

Seed dry matter accumulation is one of the important components contributing to the yield of many crop plants. The final weight of the seed is a result of the rate at which the seed accumulates the dry matter and the duration over which this occurs. Increased seed fill rates and longer growth periods have been correlated with higher yields (Evans and Wardlaw 1976). However, very little is known about the factors controlling the rate and duration of seed growth. Seed growth from initiation to maturity follows a complex course of several phases which results from the integration of a wide range of morphogenetic and biochemical processes. It is well known that an increase in seed size during development is associated with an increase in seed volume which usually occurs only after the cell division phase is completed.

Plants increase in size mostly by increasing cell water content (Boyer 1988). Cell growth, an irreversible increase in cell volume, can occur by expansion or by elongation. During cell enlargement, the existing cell wall architecture must be modified to allow incorporation of new material, thus increasing the surface area of the cell and inducing water uptake by the protoplast (McCann and Roberts 1994). The transport of water to the expanding cell requires irreversible yielding of the cell wall that surrounds the cytoplasm to accommodate the inflowing water (Taiz 1984). The process of cell expansion requires the stretching of the longitudinal walls which occurs under the influence of turgor pressure, provided that the wall plasticity increases (Cleland 1977). Such an increase in wall plasticity is believed to involve the breaking and remaking of several different bonds between wall polysaccharides. Much direct and indirect evidence suggests that cell wall-degrading enzymes such as glycosidases and glucanases play an important role in cell wall loosening (Asamizu et al. 1981). Close correlations between the activities of wall glycosidic enzymes and the rate of cell elongation have been reported in different systems (Chanda et al. 1986, Tanimoto 1985).

Biochemical changes in the cell wall components that underlie loosening are controversial. Auxin-induced turnover of hemicellulosic glucans and xyloglucans has been regarded as the biochemical basis responsible for cell wall loosening in graminaceous plants (Nishitani and Masuda 1983). Correlation between β-glucosidase activity and the rate of cell elongation has been reported in other plants (Katsumi and Yamamoto 1979). It has been suggested that cell wall enzymes may contribute to the transport by hydrolyzing organic molecules in the extracellular fluid. Further, the importance of arabinogalactan in determining the mechanical properties of cell walls has been highlighted by Nishitani and Masuda (1981) and Monro et al. (1972). Labrador and Nicolas (1985)

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suggested that α-galactosidase may be an important enzyme in hydrolyzing arabinogalactan.

Thus it is assumed (from many reports in the literature) that the mechanism of cell elongation is determined by factors such as cell wall loosening, production of cell wall polysaccharides, and osmotic pressure (Cleland 1977). Considering the aforesaid, the main objective of the present work was to study changes in the activities of wall-loosening enzymes during the entire period of *Brassica juncea* seed development along with grain growth parameters in terms of fresh weight, dry weight, and water content (mg/seed).

Materials and Methods

The experiment was conducted at Rajkot, Gujarat, India. Rajkot is situated at N 20°58′ to 23°08′ latitude and N 70°20′ to 70°40′ longitude. The ecoclimate of Rajkot is arid to marginal semiarid. The mustard (*B. juncea* cv. Varuna T-59) seeds were sown in a farmer's field (black cotton soil-vertisol) adjacent to the university campus. Ten rows, 1 m apart and 30 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. Other cultural practices such as irrigation and weed control were conducted to maximize plant growth and yield. Irrigation was done at weekly intervals until maturity. Forty days after sowing, another dose of 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

On the day of anthesis, the seed pods were removed from the plants, packed in polyethylene 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 difference in these two weights was the water content in mg at each time. The data were fitted to an appropriate polynomial curve. Samples from three replicates were always taken at 9:00 a.m.

Biochemical Analysis

The required number of seeds were separated and chilled in a freezer (0–4°C) and then homogenized in a mortar with a pinch of sand in citrate phosphate buffer (100 mM, pH 5.0) and centrifuged at 10,000 ×*g*, for 10 min. The supernatant was mixed with pure chilled acetone, maintaining a ratio of 1:2 at −4°C to precipitate soluble proteins. The acetone-precipitated proteins were redissolved in citrate phosphate buffer (100 mM, pH 5.0), and the resulting solution served as the source of cytoplasmic enzymes. After the extraction of soluble cytoplasmic enzymes the residual wall material was washed thoroughly with distilled water and centrifuged until 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 at room temperature (25 ± 2°C) with regular shakings to release ionically wall-bound enzymes. After centrifugation at 10,000 ×*g*, for 10 min, the supernatant was used as the source of wall-bound enzymes.

Glycosidase Assay

Glycosidase activities were estimated with *p*-nitrophenyl α- or β-D-galactopyranoside, *p*-nitrophenyl α- or β-glucopyranoside, *p*-nitrophenyl α-mannopyranoside and *p*-nitrophenyl-β-D-xylopyranoside. To 1.0 mL of substrate (2 mg/mL) prepared in citrate phosphate buffer (50 mM, pH 5.0), 0.5 mL of enzyme was added. The reaction mixture was incubated at 30°C for 30 min, and then it was terminated by adding 1 M Na_2CO_3 solution; the amount of *p*-nitrophenol released was obtained as the increase in absorbance at 400 nm. Enzyme activities are expressed as nmol of *p*-nitrophenol released/h/seed. In biochemical analysis the standard deviation was very low.

Results and Discussion

The changes in seed dry weight vs days after anthesis were best fit to a cubic polynomial equation ($r = 0.99$). Initially, there was a short period of exponential growth (11 days) followed by a phase when seed growth was constant (40–45 days) and then a final lag phase before maximum weight was reached (Fig. 1*a*). Mustard seed thus followed a general pattern of growth as observed in other crop plants (Chanda and Singh 1996, Egli 1981). The water content was low initially but started increasing steadily and reached a maximum level at day 35; subsequently it maintained its level until the end of seed growth (Fig. 1*b*). In the majority of dicotyledon seeds, cell division in the cotyledon is usually complete early in seed development, and the increase in seed volume during development is primarily a result of cell expansion (Guldan and Brun 1985). It is reasonable to assume that the uptake of water and increase in seed volume, to accommodate dry weight, are passive processes following the accepted principles governing the water uptake and increase in volume by cells (Cosgrove 1986); it is also possible that the ability of the seed to take up water and the increase in volume may be important processes in determining the final seed size.

Cells grow in size mostly by increasing their water content. Enlargement occurs because solute concentrations are high enough inside the cell to extract water osmotically from its surroundings. As a result, the pressure in the cells (turgor) rises and extends the wall, irreversibly enlarging the cell compartment. In the present study, the developing mustard seed showed a continuous rise in water content up to 35 days after anthesis, indicating that the cell enlargement continued up to 35 days. Further, water content and dry matter accumulation showed a close correlation ($r = 0.991$; Table 1), which suggests that the rapid uptake of water may be required to support rapid dry matter accumulation. Pande et al. (1992) also showed that wheat cultivars having more water content had more dry weight and vice versa.

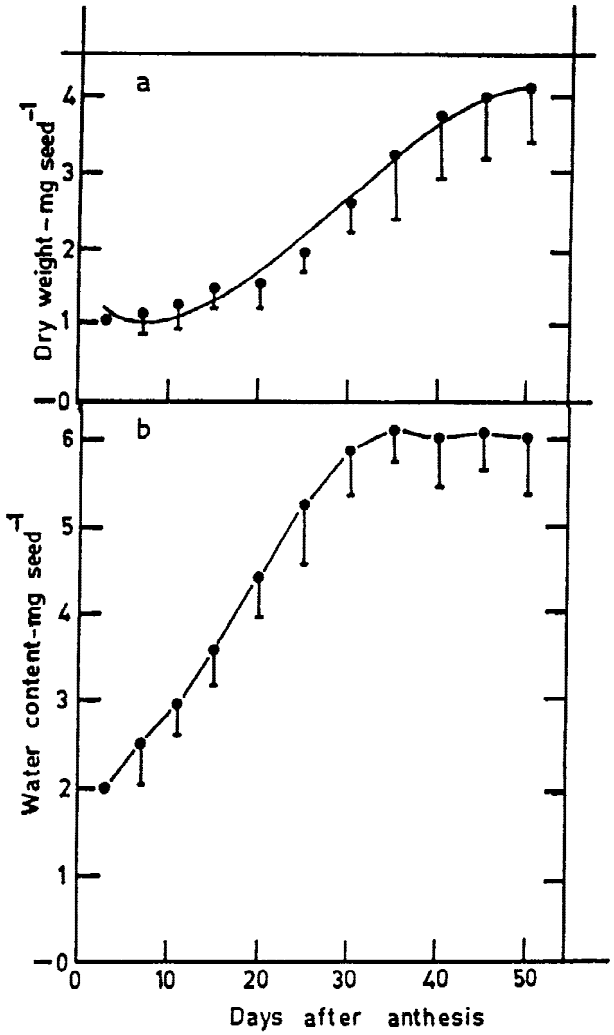

Fig. 1. *a,* seed dry weight vs days after anthesis predicted from a cubic polynomial equation and actual mean seed dry weight. *b,* water content of developing mustard seed during the entire period of seed growth. *Vertical bars* indicate ±S.D.

The physical theory of plant cell growth indicates that the rate of cell expansion is determined predominantly by the rate of two interrelated and simultaneous physical processes: the net rate of water uptake and the rate of the irreversible yielding of the cell wall. The changes in cell wall chemistry which underlie loosening are controversial. Pectic polysaccharides, which are major constituents of the primary cell walls, are interconnected covalently to the other carbohydrate polymers serving as structural elements of cell walls thus forming a tightly cross-linked matrix network. Plant growth and differentiation depend on the changes in the mechanical structural elements of cell walls, a process that could involve alteration in the amount and/or structure of pectic polysaccharides (Konno et al. 1986). It has been thought that glycosidases and glucanases are involved in the degradation of glycosidic linkages in polymers. However, it is also clear that the chemical structure of the primary cell walls of monocotyledon species differs markedly from those of dicotyledon species (Wilkie 1979).

Table I. Values of correlation coefficients between water content (mg/seed) and wall-bound enzymes and seed dry weight (mg) ($n = 12$).

Enzyme	Coefficient of correlation (r)
Dry weight	0.991**
α-Galactosidase	0.954**
α-Glucosidase	0.943**
α-Mannosidase	0.881**
β-Galactosidase	0.881**
β-Glucosidase	0.895**
β-Xylosidase	0.684*

Note. **, significant at 1% *p;* *significant at 5% *p.*

Changes in six glycosidase activities were detected in both cytoplasmic and wall-bound fraction (Fig. 2) but with different distributional patterns. In general, the activity was considerably higher in the wall-bound fraction than in the cytoplasm. Maximum wall-bound activity occurred in α-mannosidase followed by α- and β-galactosidases. The activity in the other three was almost equal. In the cytoplasmic fraction, also, α-mannosidase had the highest activity, whereas β-galactosidase activity was greater than that of α-galactosidase; β-xylosidase activity was the lowest. Thus, although they had different distributional patterns, the trend was similar. The water content and the activity of wall glycosidic enzymes showed a close correlation, indicating the important role of these enzymes in cell enlargement in the developing mustard seeds. The coefficient of linear correlation was significant at $p < 1\%$ (Table 1).

Correlation between different glycosidase activities and growth has been reported in various plant tissues and species. Masuda et al. (1985) reported that β-galactosidase activity was high in the growing cells and that the activity was related to polysaccharides such as galactan, arabinogalactan, and xyloglucan, which undergo changes during growth. Correlation between β-glucosidase and the rate of cell elongation has been reported by Murray and Bandurski (1975); Bucheli et al. (1985), working with cotton fibers, related glucanases to growth, and Tanimoto (1985) related growth to α-galactosidase and α-arabinosidase in *Pisum sativum.* Thus, the conclusion that these glycosidases are related to the elongation growth (cell wall loosening) and structure of the cell wall has been drawn by several authors. In the present work, changes in the activities of glycosidases in developing mustard seed showed close correlation with water uptake, indicating that these enzymes may be responsible for loosening the cell wall to accommodate inflowing water and subsequent cell elongation.

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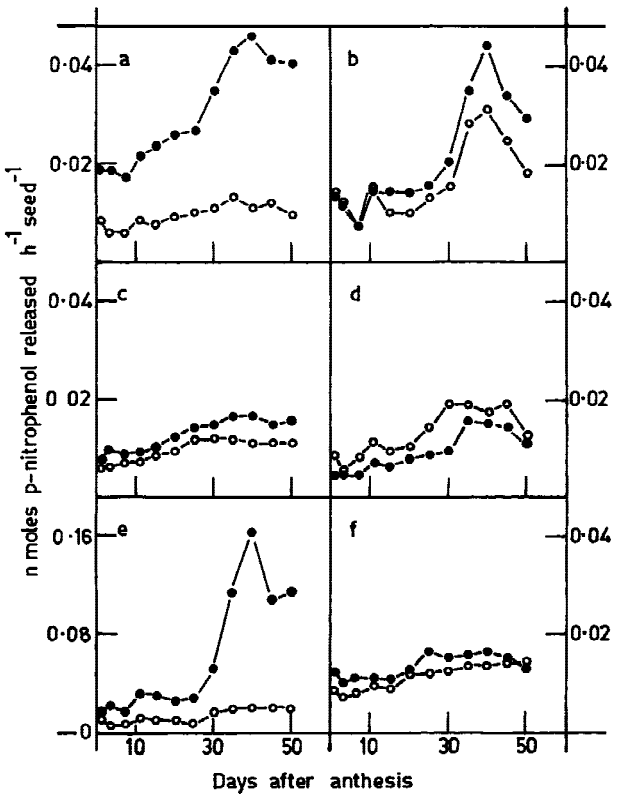

Fig. 2. Changes in glycosidase activities during the entire period of mustard seed growth. Cytoplasmic (○); wall-bound (●); *a*, α-galactosidase; *b*, β-galactosidase; *c*, α-glucosidase; *d*, β-glucosidase; *e*, α-mannosidase; *f*, β-xylosidase.

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