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Biochemical Changes Associated With *Brassica juncea* Seed Development. IV. Acid and Alkaline Phosphatases

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Abstract. Changes in acid and alkaline phosphatase activities in cytoplasmic and wall-bound fractions of developing mustard (*Brassica juncea*) seed were studied. Growth was measured by seed dry weight and water content. Seed dry weight data were fitted to a cubic polynomial equation. Seed water content and dry matter accumulation was significantly correlated. Cytoplasmic acid and alkaline phosphatase activities were substantially less in the cytoplasmic fraction than the wall-bound fraction. Wall-bound acid phosphatase activity was low initially, but high levels were maintained after day 25, indicating a relationship with dry matter accumulation. The results suggest that acid phosphatase plays an important role during mustard seed development.

Key Words. Acid phosphatase—Alkaline phosphatase—*Brassica juncea*—Seed development

One of the most important yet least available mineral nutrient for plant growth is phosphorus. It not only plays a vital functional role in energy transfer and metabolic regulation, but it is also an important component of many biomolecules, and a lack of this element is one of the factors that limits growth. Plants obtain phosphorous from the soil (Mengel and Kirkby 1987), from phytin (Bartnik and Szafranska, 1987), or from other phosphorylated molecules and store it in their vacuoles (Bieleski and Ferguson 1983). Efficient acquisition and use of phosphorous require a ubiquitous class of enzymes known as phosphatases that function to hydrolyze phosphorous from orthophosphate monoesters in a thermodynamically favorable process (Vincent et al. 1992). Phosphatases have been traditionally classified as being acid or alkaline phosphatases according to whether their optimal pH for catalysis is greater than or less than pH 7.0. Acid phosphatases are a group of enzymes that nonspecifically catalyze the hydrolysis of a variety of phosphate esters and are widely distributed in nature. They have been extensively studied in a number of plant systems (Kaneko et al. 1990, Thaker et al. 1996) and have considerable diversity with regard to their physical properties.

The alkaline phosphatases are a group of phosphomonoesterases that catalyze the hydrolysis of phosphate esters, releasing inorganic phosphorous (Angosto and Matilla 1990). They are widely distributed; their function has been studied in microorganisms but little data exist about their role in higher plants, and despite having broad specificity (Kaneko et al. 1990), relatively little is known about their physiologic roles. In some cases it has not been possible to quantify or even detect them in plants (Hota and Israelstam 1978). Recently these phosphatases have been purified to homogeneity in chick pea (Angosto and Matilla 1990, Angosto et al. 1988).

In this study we have examined the changes in acid and alkaline phosphatases during mustard seed development.

Materials and Methods

Mustard (*Brassica juncea* cv. Varuna T-59) seeds were sown in a farmer's field in black cotton soil rich in clay content (Vertisol) adjacent to the university (Saurashtra) campus. Ten rows, 1 m apart and 25 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. Fertilizer (urea, 90 kg/ha) was applied 40 and 70 days after sowing. Pollination was by insects. Flowers started appearing 50 days after sowing. Within 3–4 days pods started appearing. The flowering occurred acropetally (i.e. oldest flowers being at the base of rachis and youngest flowers being at the top). Although pods were present both on terminal and axillary raceme, in this study only pods of the terminal raceme were taken into consideration (morphologic details as given by

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Tayo and Morgan 1975). The length of the terminal rachis was approximately 80 cm. On the day of analysis, 20 such rachis of almost equal size were harvested and brought to the laboratory. Pods of different ages (difference being 4–5 days) were selected. Seeds from the selected pods were separated. Seeds from two pods of two rachis were pooled, and five seeds (each replicate) were taken for fresh and dry weight measurements. Ten such replicates were taken for growth and five replicates for biochemical analysis. The entire experiment was repeated twice. The fresh weight of these 10 replicate seeds was taken; then they were oven dried at 65°C to a constant weight, after which the dry weight was taken. Water content (in mg at each time) was calculated from the difference between the dry and wet weights. Dry weight and water content per seed were then calculated.

Biochemical Analysis

Fifteen to 30 seeds (depending on the growth stage) were chilled in a refrigerator (0–4°C), homogenized in a mortar with a pinch of sand in Tris-HCl buffer (0.3 M, pH 7.8) containing 1 mM EDTA and 5 mM mercaptoethanol, and centrifuged at 10,000 × *g* for 10 min. The supernatant was mixed with two parts of pure chilled acetone at −4°C to precipitate soluble proteins. The acetone-precipitated proteins were redissolved in 100 mM sodium-acetate buffer for acid phosphatase and in 40 mM buffer for alkaline phosphatase activity. After extraction of the soluble cytoplasmic enzymes, the residual wall material was thoroughly washed with distilled water and centrifuged until the washings were free of peroxidase reaction with guaiacol. The wall fraction was then kept in 10 mL of 1 M NaCl for 1 h at room temperature (25 ± 1°C) with regular shaking 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.

Various salt solutions of different molarities like NaCl, $CaCl_2$, KCl, and LiCl have been used to release ionically wall-bound enzyme (Dey and Del Campillo 1984, Thaker et al. 1987). Our preliminary standardization studies in mustard showed that higher concentrations did not improve the extraction of ionically wall-bound enzyme any further. Therefore 1M NaCl was used to extract wall-bound enzyme.

Acid Phosphatase Assay

The method of Thaker et al. (1996) was followed for the estimation of acid phosphatase activity. The reaction mixture consisted of 75 mM sodium acetate buffer (pH 4.2), 5 mM *p*-nitrophenylphosphate (in buffer), and the enzymatic solution. The mixture was incubated at room temperature (25 ± 2°C) for 30 min and then terminated by the addition of sodium carbonate solution (1 M). In the control, sodium carbonate was added before the addition of enzyme. The amount of *p*-nitrophenol released was measured at 400 nm. The enzyme activity is expressed as *n* moles *p*-nitrophenol released/h/seed.

Alkaline Phosphatase Assay

Alkaline phosphatase activity was measured spectrophotometrically by determining the increase in absorbance at 400 nm caused by the release of *p*-nitrophenol from *p*-nitrophenol phosphate. The modified method of Angosto and Matilla (1990) was followed for estimating alkaline phosphatase activity. The reaction mixture consisted of 100 mM glycine-NaOH (pH 10.5) buffer. 5 mM *p*-nitrophenol phosphate, and the enzymatic solution. The 0 min reading served as the control. The reaction mixture was kept in the dark, and absorbance was noted after 30 min incubation. The enzyme activity is expressed as *n* moles *p*-nitrophenol released/h/seed.

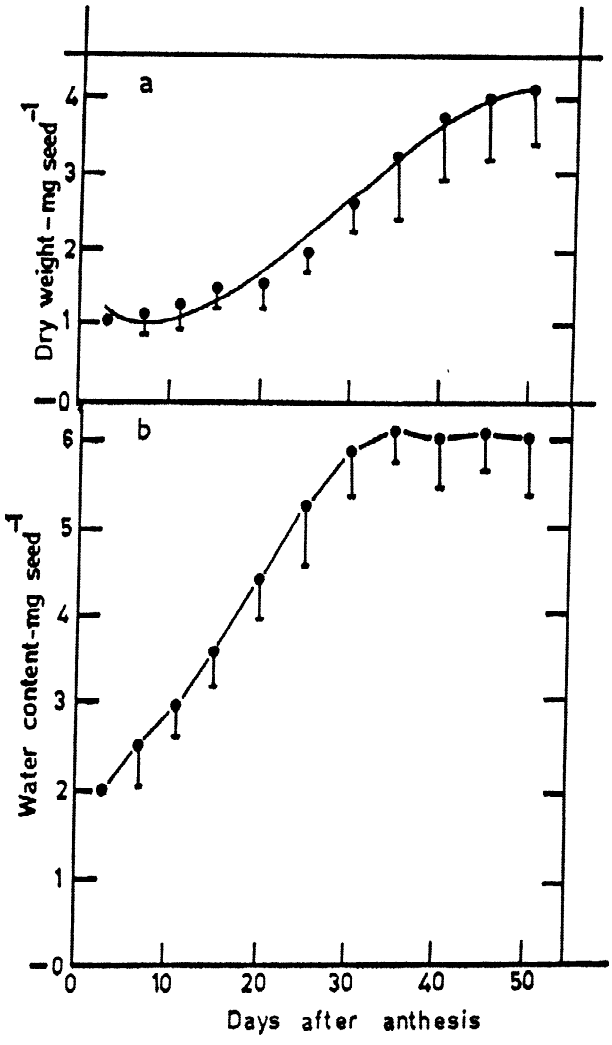

Fig. 1. Seed dry weight (*a*) and water content (*b*) versus days after anthesis. Vertical bars indicate ± SD.

Results and Discussion

Data of seed dry weight (mg/seed) was fitted to polynomial equations of different degrees, and the "best-fit" equation was determined statistically by performing the *t* test for different R^2 values (Nicholls and Calder 1973). The third degree polynomial was the best fit. The equation was of the following type: $Y = a + bx + cx^2 + dx^3$, where Y = seed dry weight, x = days after anthesis and *a,b,c,* and *d* are different regression coefficients. This

cubic polynomial adequately described the seed dry weight data ($r = 0.99$) (Fig. 1*a*). This agrees with the results of Gebeyehou et al. (1982) and Chanda and Singh (1998) on wheat, Rabadia et al. (1999) on cotton, and Thaker (1999) on *Hibiscus.* In general, the seed dry matter accumulation showed an initial lag phase (11 days). This was followed by a phase when seed dry weight increased linearly (40–45 days), and then a final lag phase before maximum weight was recorded on day 50. Similar results were obtained in mustard in our earlier work (Sant Saroop et al. 1996, 1998).

The water content was low on day 3, but increased steadily and attained maximum content at day 35, after which it maintained equilibrium until day 50 (Fig. 1*b*). Thus water content in this study showed a close correlation with dry matter accumulation, but maximum water content was achieved much before the attainment of maximum dry weight. A similar pattern of dry matter accumulation and water uptake has been reported (Martinez-Carrasco et al. 1988, Chanda and Singh 1997). Working with soybean seed development, Fraser et al. (1982) also showed that seed water content increased in parallel with dry weight but reached a maximum level much before the seed reached physiologic maturity. In several crop species, water relationships in developing seeds are independent of water relationships of maternal tissue and of external environmental factors (Barlow et al. 1980). Similarly, a lack of response of grain water relationships to imposed severe water deficits was observed in maize (Ouattar et al. 1987), and a number of authors have suggested that water status may play an important role in controlling seed development (Egli 1990, Chanda and Singh 1998, Rabadia et al. 1999).

A marked difference was found between soluble and bound acid phosphatase activity during the entire period of mustard seed development (Fig. 2). Soluble acid phosphatase activity was low throughout the study. In contrast, the wall-bound acid phosphatase activity was low initially; showed an increasing trend that sharply increased from day 25 to day 40; and later on, although the activity decreased slightly, high levels were maintained. Thus the wall-bound activity showed a close correlation with dry matter accumulation. It has been shown that the intracellular phosphatases play an important role in partitioning of photosynthetic carbon (Giaquinta, 1980). It is suggested that low inorganic phosphate concentration leads to a decrease in assimilate export (Machler et al. 1984), and at high levels chloroplasts are depleted of photosynthetic metabolites (Flugge et al. 1980). Furthermore, at this stage, low cytoplasmic acid phosphatase was present and high wall-bound acid phosphatase activity was present. It has been reported that activity of acid phosphatase changes from soluble to wall-bound fractions in bean hypocotyl (Mizuta and Suda 1980). It is also suggested that hydrolysis of phosphate esters is a critical process in energy metabolism and metabolic

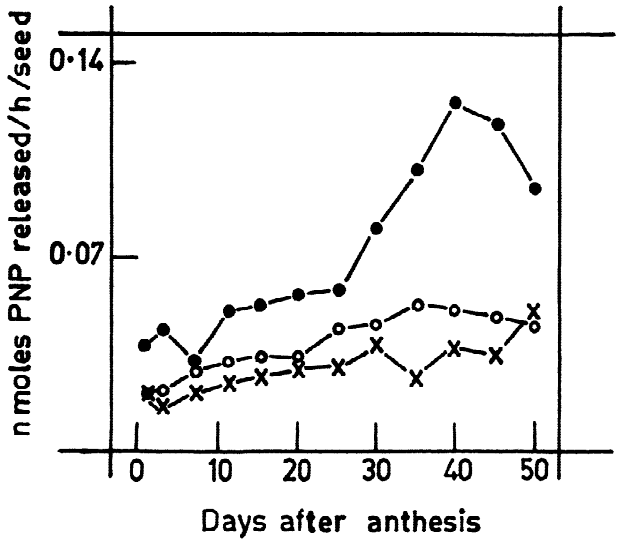

Fig. 2. Changes in acid and alkaline phosphatase activities during entire period of mustard seed growth. Cytoplasmic acid phosphatase (○—○); wall-bound acid phosphatase (●—●), and cytoplasmic alkaline phosphatase (x—x) activities.

regulation of plant cells (Duff et al. 1994). In this work, higher levels of acid phosphatase activity at the later part of the dry matter accumulation phase suggest the participation of this enzyme in the energetics of seed development and in fatty acid synthesis of developing mustard seed. Other developmental processes that cause acid phosphatase increase include flowering, senescence, and fruit ripening (Kanellis et al. 1989; Lal and Jaiswal 1988). Abscisic acid has been reported to accelerate the onset and enhance the magnitude of the increase in acid phosphatase activity that accompanies leaf senescence (De Leo and Sacher 1970).

Unlike acid phosphatase activity, no wall alkaline phosphatase activity could be detected. Changes in cytoplasmic alkaline phosphatase activity were similar to that of cytoplasmic acid phosphatase activity (Fig. 2). Thus low levels of cytoplasmic and absence of wall-bound alkaline phosphatase activity in this work suggest that they may not have any role in phosphate metabolism of mustard seed development. Low levels of alkaline phosphatases have been reported in other crop plants (Onofeghara and Koroma 1974; Thaker et al. 1996). In contrast, high levels of wall-bound acid phosphatase activity during the dry matter accumulation phase suggest that acid phosphatase may play a greater role in mustard seed development by enzyme activation and mobilization of nutrient reserves during this critical period of high metabolic activity. However, additional investigations on the immunologic and molecular characteristics of these enzymes and their differential expression within and between different plant tissues are needed. These studies

coupled with appropriate physiologic and biochemical analyses should yield additional insights about the cellular functions of this important class of enzymes.

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