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Ontogenic changes in enzymes of carbon metabolism in relation to carbohydrate status in developing mungbean reproductive structures

Jyoti Chopra*, Narinder Kaur, Anil K. Gupta

Department of Biochemistry, Punjab Agricultural University, Ludhiana 141 004, India

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Abstract

The content of free sugars and the activities of enzymes involved in carbon metabolism-sucrose synthase, acid and alkaline invertase, phosphoenol pyruvate carboxylase, malic enzyme and isocitrate dehydrogenase were determined during seed development in mungbean pods. A decrease in carbohydrate content of pod wall from 10 to 25 days after flowering (DAF) and a concomitant increase in the seed till 20 DAF was observed. Sucrose remained the dominant soluble sugar in the pod wall and seed. In the branch of inflorescence and pod wall, the activities of sucrose metabolizing enzymes, viz. acid and alkaline invertase, sucrose synthase (synthesis and cleavage) and sucrose phosphate synthase were higher at 5–10 DAF, whereas in seed the maximum activities of these enzymes were observed at the time of maximum seed filling stage (10–20 DAF). High activities of sucrose synthase at the time of rapid seed filling can be correlated to its sink strength. Higher activities of phosphoenol pyruvate carboxylase in the branch of inflorescence and pod wall than in seed may indicate the involvement of the fruiting structure for recapturing respired CO_2. High activities of isocitrate dehydrogenase and malic enzyme in the seed at the time of rapid seed filling could provide NADPH and carbon skeletons required for the synthesis of various seed reserves.

Keywords: *Vigna radiata*; Leguminosae; Mungbean; Carbon metabolism; Sucrose metabolism; Sucrose synthase; Sucrose phosphate synthase; Invertase; Phosphoenol pyruvate carboxylase; Malic enzyme; Isocitrate dehydrogenase

1. Introduction

Starch is the major constituent of the mungbean seed (Sinclair & de Wit, 1975) and an important factor determining seed yield. In legumes, there are different views about how the sucrose entering the seed is metabolized. Sucrose cleavage is dominated by sucrose synthase pathway in broadbean and limabean (Hawker, 1971; Xu, Sung & Black, 1989) or by the sucrose synthase and alkaline invertase in soybean and pea (Lowell & Kuo, 1989; Edwards & ap Rees, 1986) or possibly solely by the invertase pathway in pea (Estruch & Beltren, 1991). Sucrose synthase and invertase activities have not been extensively studied in developing mungbean pods inspite of their importance in initial sucrose metabolism. The enzyme activity work presented here pivots around understanding sucrose metabolism in developing pods of mungbean.

During early fruit development in some legumes, the pod wall develops more quickly than the seed. Consequently, utilization of translocated photosynthate by the developing pod walls may be necessary for pod growth before the seed begins to develop. Since the relationships among pod growth, seed development and carbohydrate status during the pod development are not clear in mungbean, experiments were conducted to examine these hydrolytic enzymes in relation to the concentrations of sucrose, fructose and glucose.

The photosynthetic contribution of fruiting structure

* Corresponding author.

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to their own yield or to the yield of the seeds which they contain is reported in a number of legumes (Crookston, O'Toole & Ozbun, 1974; Singh & Pandey, 1980; Singh, Sheoran & Singh, 1986). In addition, its inner layer fixes CO_2 released in pod cavity by respiring seeds by PEP carboxylase, thereby conserving carbon economy (Luthra, Sheoran & Singh, 1983; Singal & Singh, 1986). Other enzymes of CO_2 assimilation like malic enzyme and isocitrate dehydrogenase may support PEP carboxylase for the generation of carbon skeletons utilized in the amino acid synthesis (Singh et al., 1986; Khanna-Chopra & Sinha, 1982; Meyer,

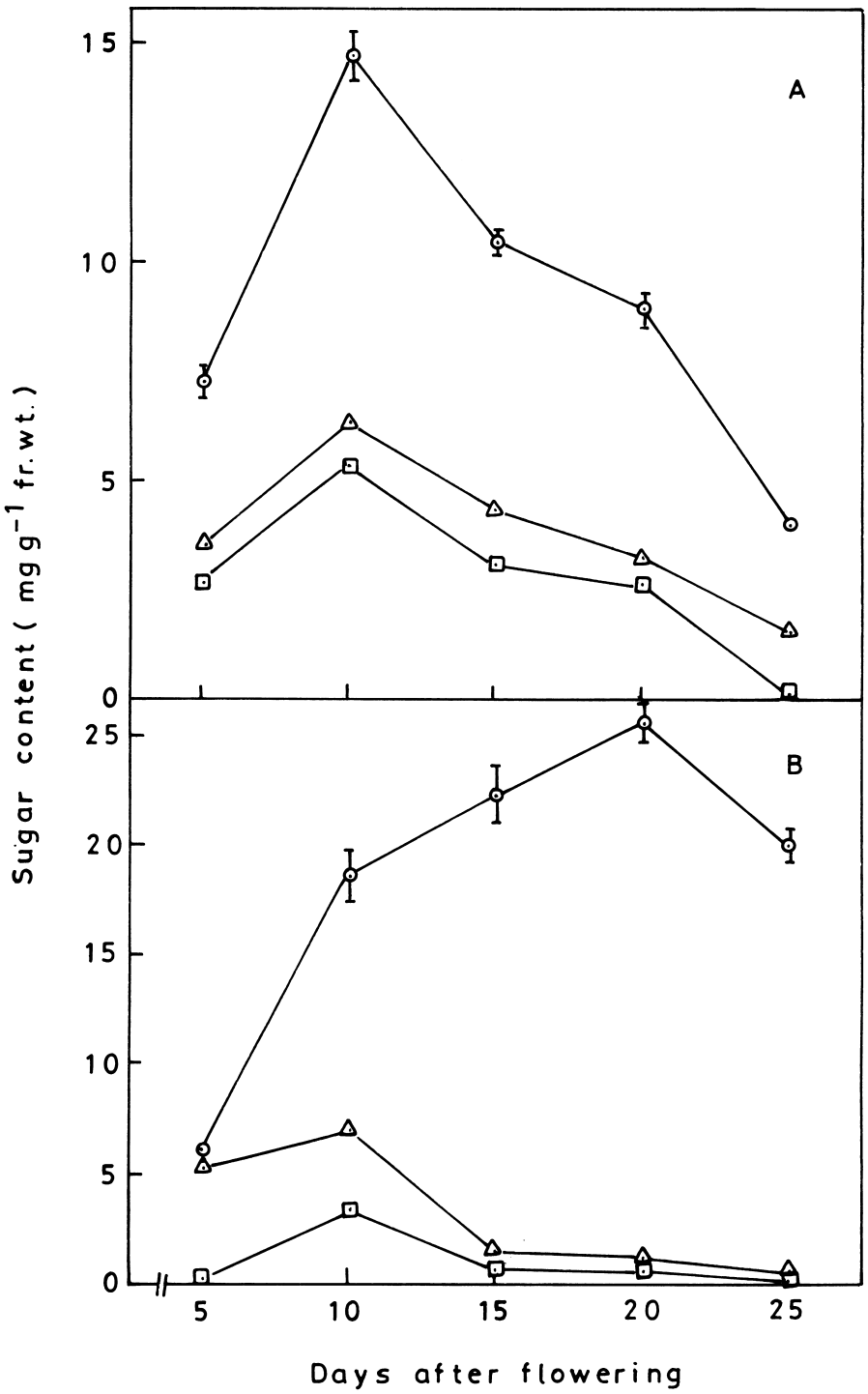

Fig. 1. Changes in the content of sucrose, glucose and fructose in pod wall and seeds of mungbean during development. (A) pod wall and (B) seed. ○: sucrose; △: glucose and □: fructose. The vertical bars show mean of three replicates. The vertical bars were not shown where SD was smaller than the symbol.

Kelly & Latzko, 1982; Latzko & Kelly, 1983; Aoyagi & Bassham, 1984). There is evidence that reserves of solutes are established in the pod wall of a number of legumes like field pea, fenugreek, cow pea, *Phaseolus vulgaris* and *Vigna mungo*, which provide later on the seed's requirement for starch, reducing sugars and nitrogenous materials (Flinn & Pate, 1968; Sauvaire, Girardon, Baccon & Risterucci, 1984; Peoples, Pate, Atkins & Murray, 1985a; Peoples, Atkins & Pate, 1985b; Peoples, Pate & Atkins, 1985c; Minamikawa, Yamauchi, Wade & Takeuchi, 1992). Hence, this aspect of the subject was also investigated in the pods. The present study could there-

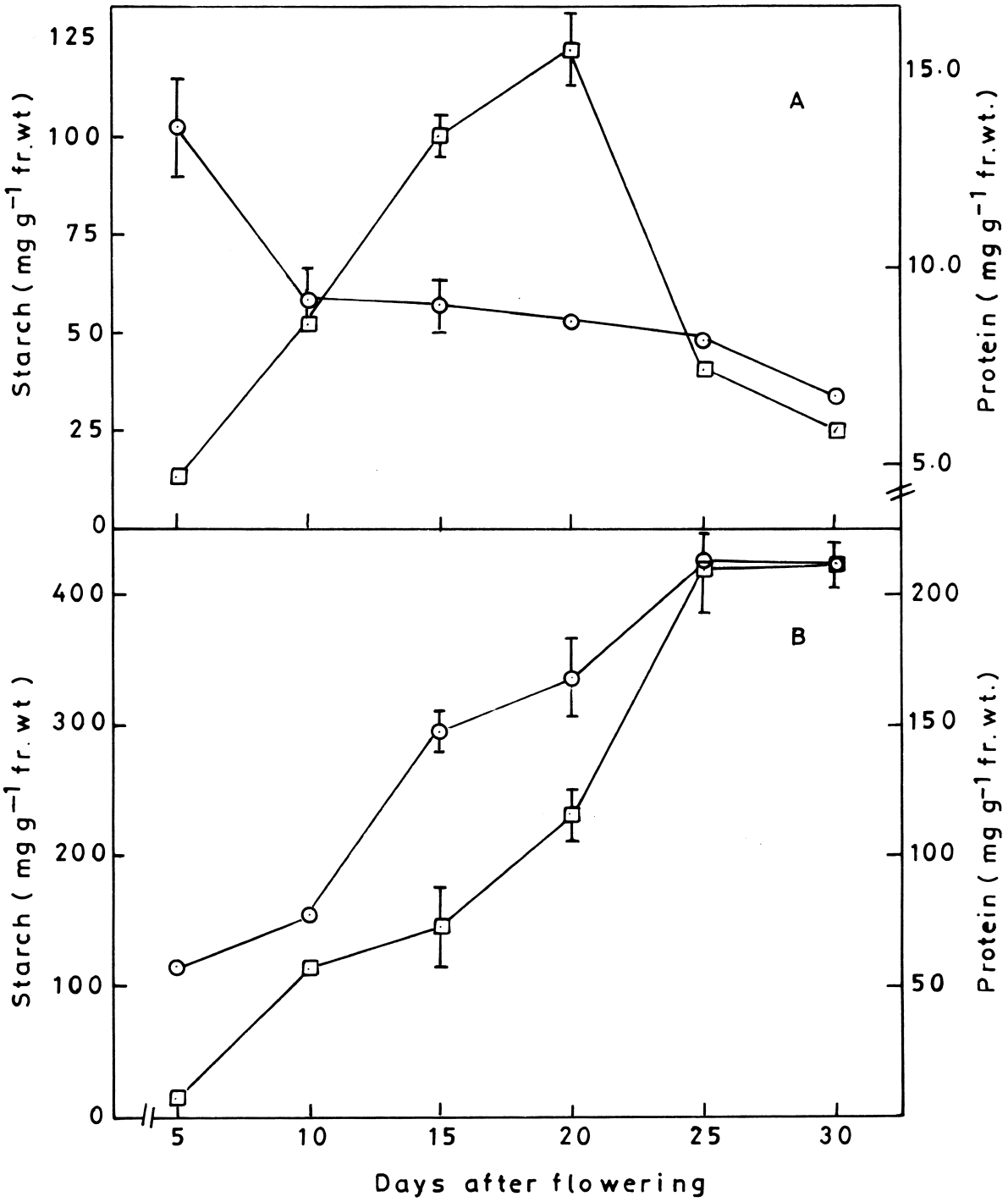

Fig. 2. Changes in starch and soluble protein content in pod wall and seeds. (A) pod wall and (B) seed. ○: protein and □: starch. The vertical bars were not shown where SD was smaller than the symbol.

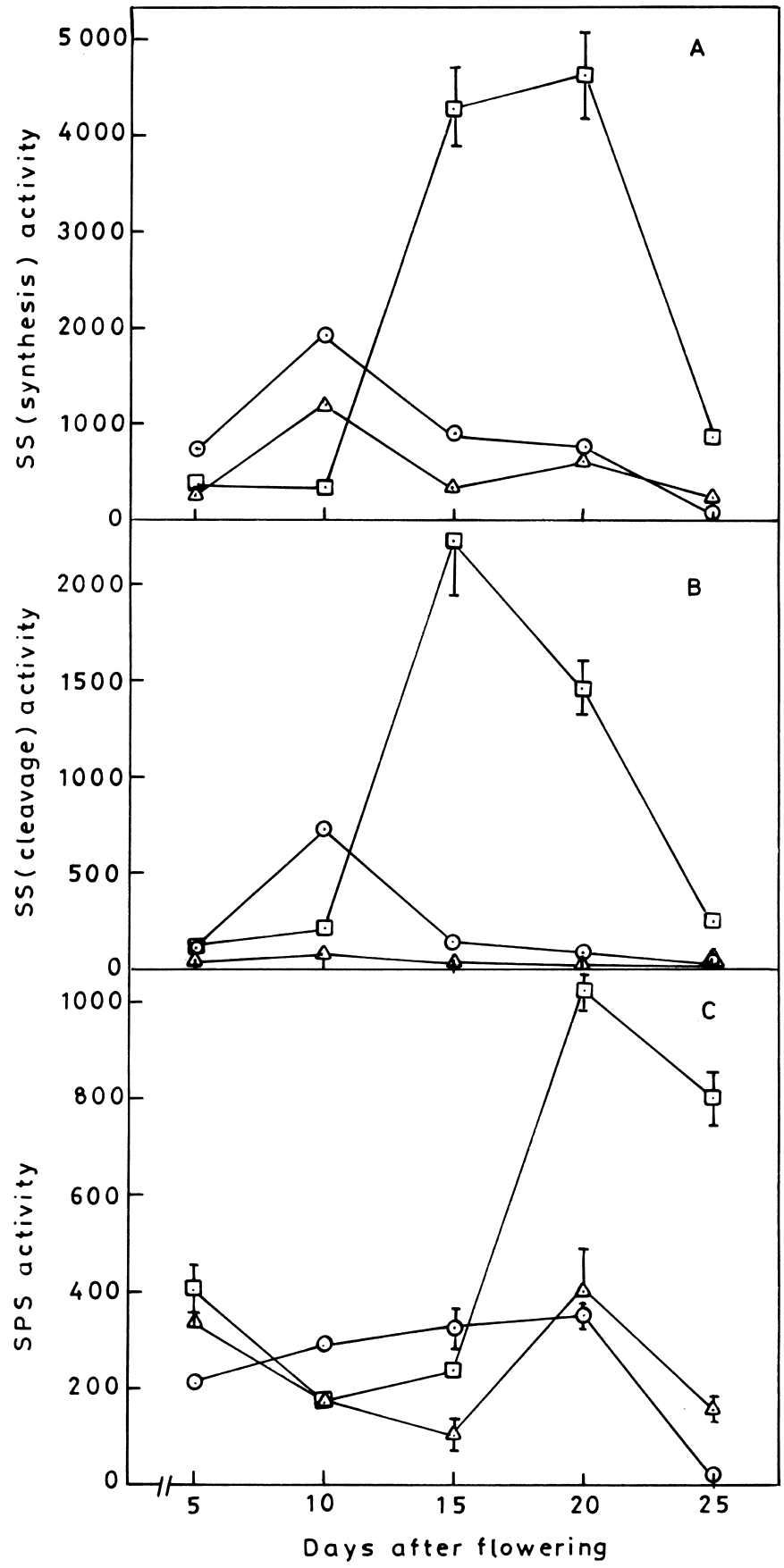

Fig. 3. Activity pattern of SS and SPS during development. (A) SS (synthesis); (B) SS (cleavage) and (C) SPS. ○: Branch of inflorescence; △: pod wall and □: seed. The vertical bars show SD from mean of three replicates. The vertical bars were not shown where SD was smaller than the symbol. Activity has been expressed as nmol product formed (min^{-1} g^{-1} fr.wt.).

fore lead to an information, suggesting the important enzymic steps regulating carbon metabolism and yield.

2. Results

2.1. Carbohydrates of pods

Changes in the content of glucose, fructose and sucrose in pod wall and seed at different developmental stages have been given in Fig. 1A and B. The contents of total soluble sugar decreased with development in pod wall and seed (data not given). At all stages of pod development, the level of sucrose was significantly higher than that of glucose and fructose in pod wall and seed. In seed, the sucrose content increased till 20 DAF and then declined. Sucrose was the main constituent of the seed soluble sugars and very low quantities of glucose and fructose were present (Fig. 1B). By using descending paper partition chromatography, raffinose, stachyose and verbascose were detected in the mungbean seeds at maturity. The starch content of pod wall increased from 5 to 20 DAF and thereafter declined till maturity (Fig. 2A).

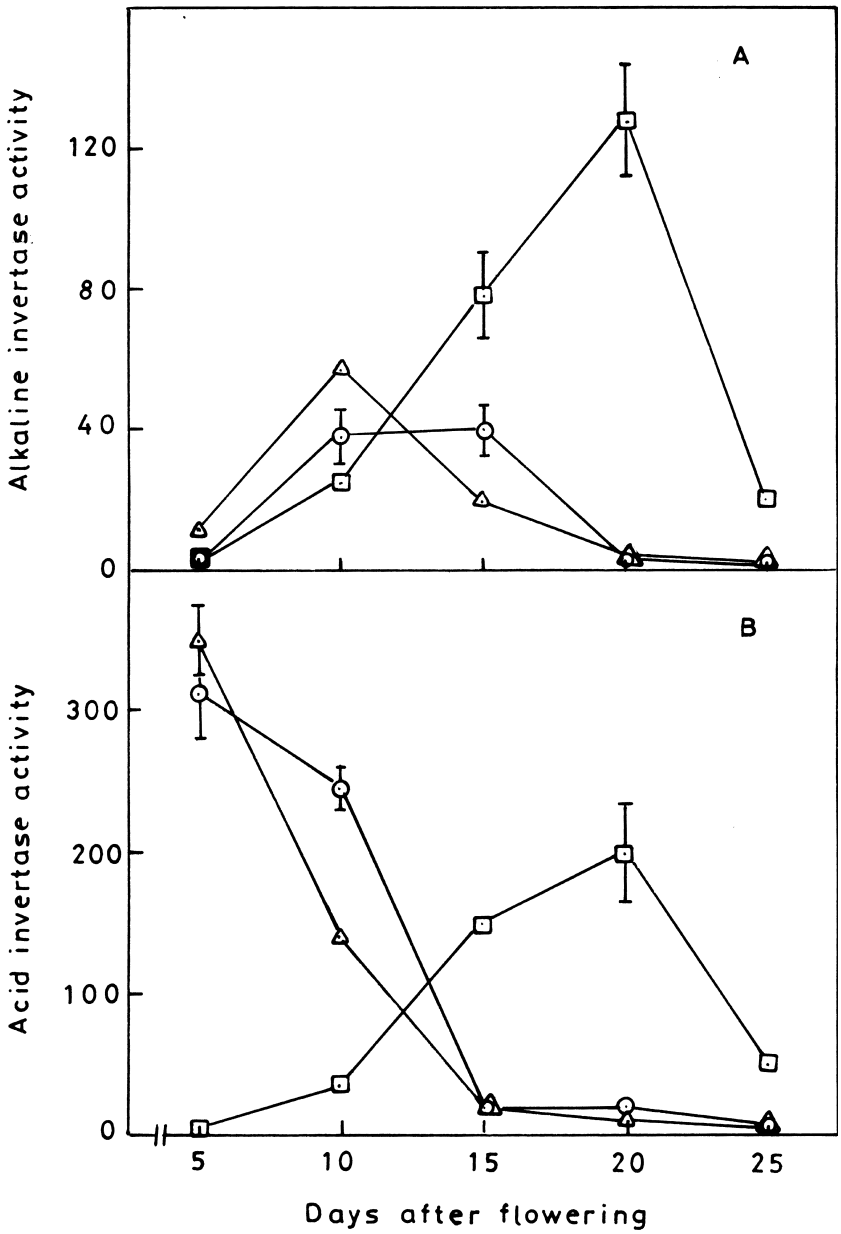

Fig. 4. Activity pattern of acid and alkaline invertase. (A) alkaline invertase and (B) acid invertase. ○: Branch of inflorescence; △: pod wall and □: seed. The vertical bars show SD from mean of three replicates. The vertical bars were not shown where SD was smaller than the symbol. Activity has been expressed as nmol product formed (min^{-1} g^{-1} fr.wt.).

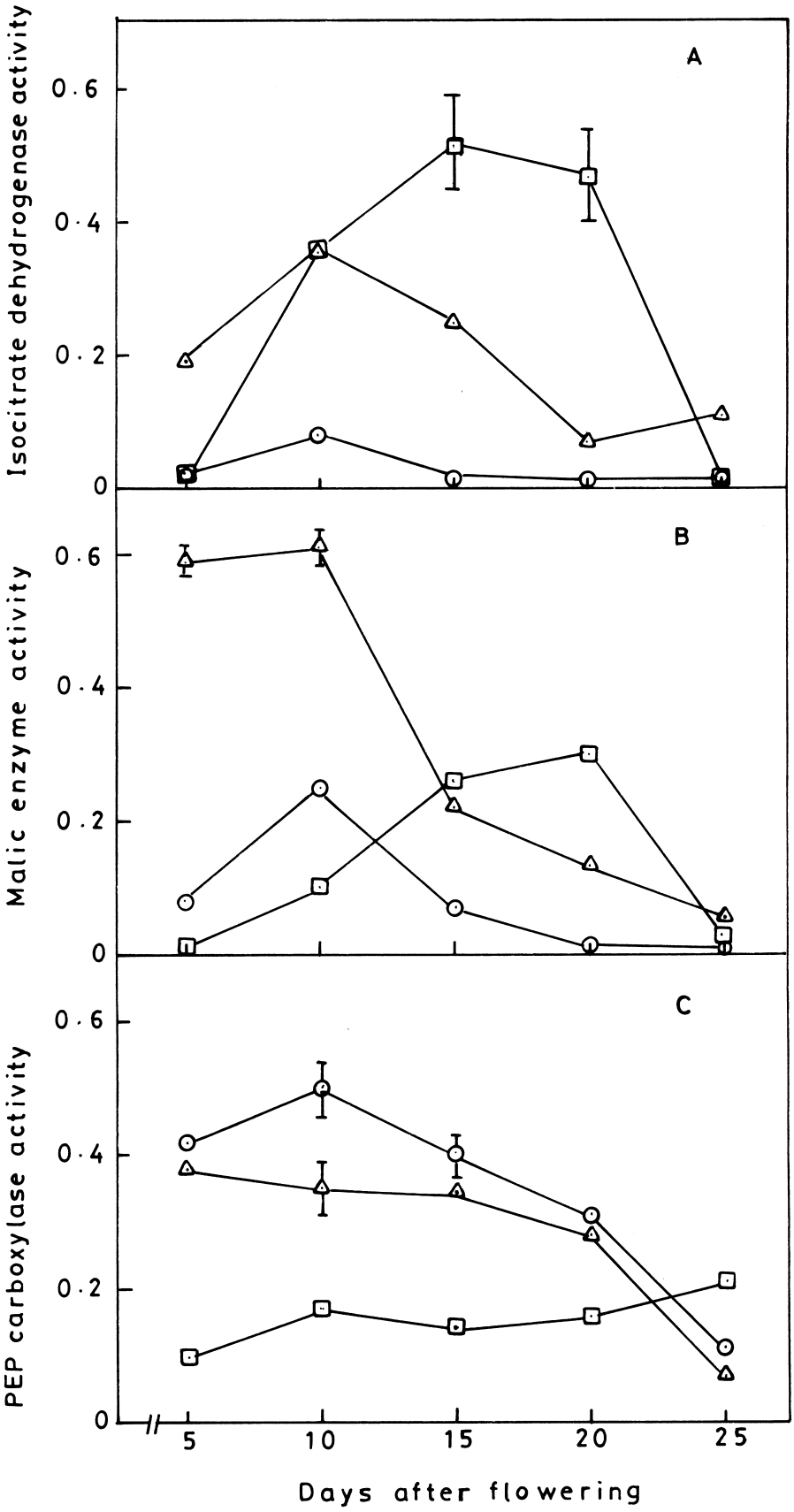

Fig. 5. Activity pattern of isocitrate dehydrogenase, malic enzyme and PEP carboxylase during development. (A) Isocitrate dehydrogenase; (B) malic enzyme and (C) PEP carboxylase. ○: Branch of inflorescence; △: pod wall and □: seed. The vertical bars show SD from mean of three replicates. The vertical bars were not shown where SD was smaller than the symbol. Activity has been expressed as nmol substrate utilized (min^{-1} g^{-1} fr.wt.).

However, in seed a continuous increase in starch content was observed from 5 to 25 DAF, after which it remained almost constant (Fig. 2B).

2.2. Activities of various enzymes during seed development

The activity of sucrose phosphate synthase (SPS) was lower than that of sucrose synthase (synthesis direction) in branch of inflorescence (BI), pod wall and seed at almost all stages of pod development (Fig. 3). Activity of SS (cleavage) in all the three tissues was very less at 5 DAF (Fig. 3B). From this point, activity increased rapidly reaching a peak at 10 DAF in BI and pod wall, however for seed the activity was higher during the phase of rapid seed filling (10–20 DAF) with a peak at 15 DAF (Fig. 3B).

Activities of alkaline and acid invertases are shown in Fig. 4A and B. Activity of alkaline invertase in all the three tissues was relatively lower than acid invertase throughout the course of pod development. In comparison with seed, very high acid invertase activity was detected in pod wall and BI at 5 DAF, after which it declined and was barely detectable at 15, 20 and 25 DAF stages (Fig. 4B). Compared with acid invertase, the activity of alkaline invertase in pod wall and BI was very low at 5 DAF (Fig. 4).

The PEP carboxylase was active in BI and pod wall at 5 and 10 DAF, and thereafter, a declining trend in its activity was observed (Fig. 5C). The activity of PEP carboxylase was higher in BI and pod wall than in seeds at all stages of pod development. High activity of malic enzyme was observed in BI at 5 and 10 DAF and thereafter it decreased till 25 DAF (Fig. 5B). In pod wall, the activity of malic enzyme was maximum at 10 DAF, thereafter it declined and was barely detectable at 20 and 25 DAF. However, in seed the activity was virtually undetectable at very young and mature stages of seed development (5 and 25 DAF) with an increased level of activity at 15 and 20 DAF. Activity of isocitrate dehydrogenase in pod wall was relatively low throughout the course of pod development (Fig. 5A). In BI, the activity increased from 5 DAF, reached a maximum at 10 DAF after which it declined. In seed, the activity of isocitrate dehydrogenase was substantial at the time of maximum seed filling (10–20 DAF).

3. Discussion

The decline in reducing sugar content of the pod wall with seed development, with increase in sucrose content from 5 to 10 DAF and its gradual decline thereafter suggests the conversion of hexoses into sucrose and its translocation in seed for its development. A very low level of sucrose, glucose and fructose in pod wall at 20 DAF gives support to the above view (Fig. 1A). Sucrose remained the principal free sugar during seed development and a fall in its concentration after 20 DAF may be due to its rapid conversion to raffinose and its higher analogues stachyose and verbascose. Raffinose, stachyose and verbascose were reported as quantitatively dominating sugars in mature mungbean seeds (Aman, 1979).

Young pod wall has the capacity to fix CO_2 in the presence of sunlight. Therefore, SPS, as it does in leaves, may play an important role in synthesizing some of the sucrose. However, the role of high activity of SS in synthesis direction especially in seeds is not clear at present. The high activity of SS in synthesis direction in seeds at 15 – 20 DAF stage may be just a reflection of the reversible reaction of high SS cleavage activity at these stages. Low activity of SS (cleavage) and alkaline invertase in BI as compared to pod wall and seed (Figs. 3B and 4A) suggested almost intact transport of sucrose without any significant cleavage from BI to reproductive structure. Since only SS has been related with the phloem associated sucrose hydrolytic activity (Hawker, 1971), the acid invertase detected in BI is likely not to be present in the direct path of sucrose translocation. Possibly it is localized either in the apoplast or vacuoles in non-vascular cells of BI. High activity of acid invertase and SS (cleavage) during initial stages of pod development suggested the possibility of hydrolysis of imported sucrose by these enzymes (Figs. 4B and 3B). As the pod wall develops more quickly than the seed, the utilization of transported photosynthate during initial stages by the pod wall may be necessary for pod growth before the seed begins to develop. Higher content of sucrose, glucose and fructose and high activity of acid and alkaline invertase and SS (cleavage) in pod wall at 10 DAF stage suggested the very active metabolic state of this tissue at this stage (Figs. 1A, 3B and 4). High activities of alkaline invertase and SS in seed around 10–15 DAF possibly indicate that seeds have the capacity to utilize sucrose irrespective of their site of unloading. The maximum activity of SS during the phase of rapid seed filling suggested that activity of sink strength could be correlated with that of sucrose synthase than any other hydrolytic enzyme (Fig. 6). The activity of SS has been correlated with starch synthesizing capacity of many sinks like cereals, potato tuber and egg plant (Echeverria & Humphreys, 1984; Sung, Xu & Black, 1989; Claussen, Hawker & Loveys, 1985).

The higher activity of PEP carboxylase in BI and pod wall suggested that the fruiting structure utilized PEP carboxylase for recapturing the respired CO_2, thus reducing the carbon losses to the atmosphere and playing a major role in fixing the internally generated CO_2. Oxaloacetate and malate, the sequential products

of PEP carboxylation are the means of storing CO_2 which is subsequently released within the cell by the action of malic enzyme and reduced by the Calvin cycle to give energy or used in the synthesis of carbon skeletons for amino acid synthesis (Singh et al., 1986; Khanna-Chopra & Sinha, 1982; Meyer et al., 1982; Latzko & Kelly, 1983; Aoyagi & Bassham, 1984). The substantial activity of isocitrate dehydrogenase in the seed from 10 to 20 DAF can be correlated with the maximum accumulation of starch and protein in seed from 10 to 20 DAF. In developing legume fruit, the primary suggested role of NADPH isocitrate dehydrogenase is to provide carbon skeletons and reducing power for the synthesis of amino acids. This enzyme has been proposed to be effectively linked to the deposition of protein reserves in the seeds of legumes (Murray, 1987). Decline in the starch and protein content in the pod wall during maturation phase with concomitant increase of these reserve constituents in seeds (Fig. 2) could indicate mobilization of carbohydrates and amino acids from pod wall for seed development (Flinn & Pate, 1968; Sauvaire et al., 1984; Peoples et al., 1985a, 1985b, 1985c; Minamikawa et al., 1992).

4. Experimental

4.1. Materials

Mungbean (*Vigna radiata* L. cv ML 267) was sown in the fields, in the month of July, situated at 247 m above sea-level at a latitude of 30°C 54′ N following recommended agronomic practices. Average temperature in the month of July, August and September was 30.3, 28.3 and 28.1°C, respectively. Fully opened flowers of the uniformly growing plants were tagged daily for 5 days at flowering stage, and plants at 5, 10, 15, 20 and 25 days were collected and brought under ice to the laboratory. In this way, tissue for a specific day after flowering was available for 5 continuous days. The branch carrying the inflorescence was cut from the base, at the point of attachment to the plant, and seeds were removed from the pods. Branch of inflorescence, podwall and seed were taken for preservation in 80% ethanol for determining carbohydrate composition. Enzymes were extracted immediately from the fresh tissue and activities were determined on the same day.

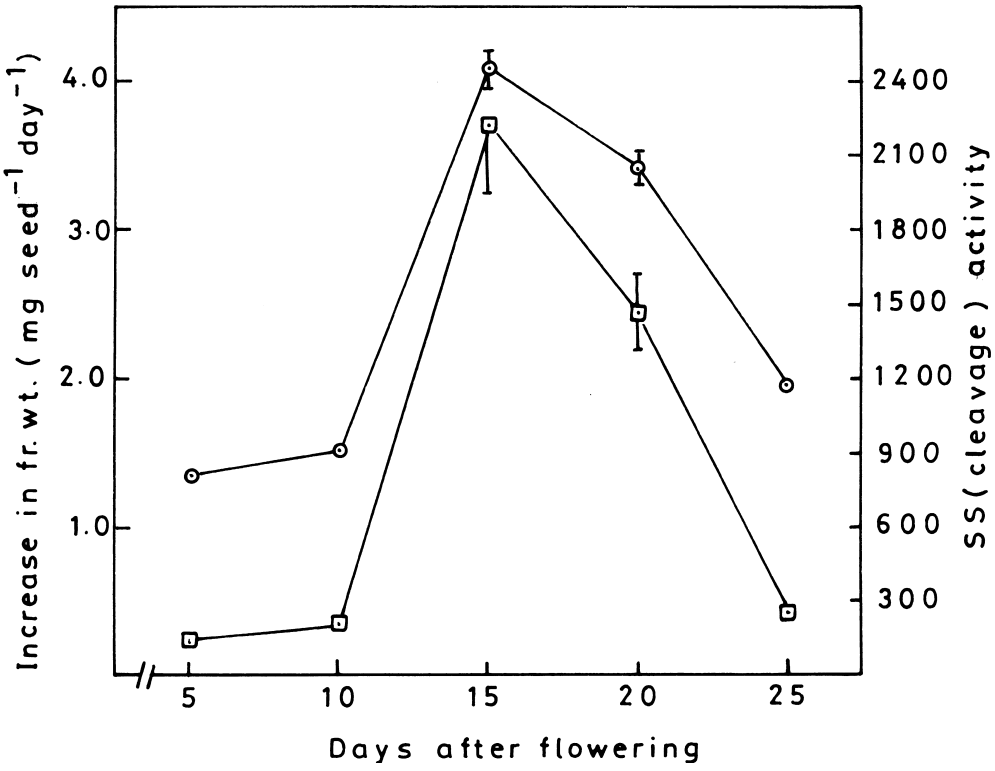

Fig. 6. Change in SS (cleavage) activity and fresh weight gain seed^{-1} day $^{-1}$. ○: fr.wt. and □: SS. Fresh weight data is mean of sample collected from six randomly selected plants and from each plant 80–125 seeds were taken. The vertical bars have not been shown where SD is smaller than the symbol.

4.2. Extraction and estimation of free sugars, starch and soluble proteins

Free sugars were quantitatively extracted twice with hot 80% EtOH and then twice with 70% EtOH. After these extractions, the tissue is free from extractable sugars. EtOH extracts of each sample were combined and concentrated by evaporation at 50°C under vacuum. Sucrose content was determined after hydrolysing the sucrose present in the extract with acid invertase (Sigma I 4504) and then estimating the glucose using the glucose oxidase and peroxidase reaction (Chopra, Kaur & Gupta, 1998). Quantity of glucose present before the invertase hydrolysis was also determined in the sugar extract. Free fructose was determined by subtracting bound fructose estimated after destroying free fructose with 30% NaOH from the total fructose determined by resorcinol-HCl procedure (Williard & Slattery, 1945).

Starch was estimated from the residue left after extraction of free sugars. To this residue, 5 ml of distilled water was added and the contents were kept in boiling water bath for 1 h. After bringing the contents to room temperature, 5 ml of amyloglucosidase (Sigma A7420, 10 units in 0.2 M NaOAc buffer pH 5.0) was added and contents incubated at 55°C for 6 h. Under these conditions, starch was completely hydrolysed and the glucose so formed was determined colorimetrically, using reaction with arsenomolybdate (Nelson, 1944).

For determining the soluble protein content, required tissue (0.5–1 g) was crushed in a pestle and mortar and was extracted with 6 ml of water and then twice again with same volume of 0.1 N NaOH. Supernatants obtained after centrifugation at 6000 g from each extraction were combined and proteins were precipitated at 4°C by raising the concentration of TCA to 10%. After 2 h, ppts were obtained by centrifugation (10,000 g) and were dissolved in 0.1 N NaOH (Lee & Takahashi, 1966). Protein was estimated using folin phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951).

4.3. Extraction of enzymes

For extracting sucrose synthase (EC 2.4.1.13) and sucrose-6-phosphate synthase (EC 2.4.1.14), the required tissues (0.5–1.0 g) were homogenized in cold (3–4°C) 100 mM HEPES buffer (pH 8.2) containing 10 mM EDTA, 15 mM KCl, 5 mM $MgCl_2$, 2 mM sodium diethyl dithiocarbamate and 5 mM β-mercaptoethanol. Insoluble polyvinylpolypyrrolidone (Sigma P2806, 100 mg g^{-1} tissue) was also added while extracting the enzyme. The supernatant after centrifuging at 10,000 g for 15 min was passed through sephadex G-25 column using 10 mM HEPES buffer (pH 7.0) to remove small molecular weight impurities. For extracting acid invertase (EC 3.2.1.26) and alkaline invertase (EC 3.2.1.27), 0.02 M sodium phosphate buffer (pH 7.0) was used.

For extracting isocitrate dehydrogenase (EC 1.1.1.42) and NADP-malic enzyme (EC 1.1.1.40), 10 mM HEPES buffer (pH 7.0) containing 2 mM mercaptoethanol was used. Insoluble polyvinylpolypyrrolidone (5 mg ml^{-1}) was also used at the time of extraction. The extract was centrifuged at 10,000 g and the supernatant was used for assaying enzyme activities. The PEP carboxylase (EC 4.1.1.31) was extracted by using the procedure described in Christeller, Laing and Sutton (1977). By using the above extraction procedures 92–97% of extractable enzyme activity could be extracted.

4.4. Enzyme assays

The assay system for sucrose synthase in the sucrose synthesis direction consisted of 2.2 μmol of UDP-glucose, 4.4 μmol of fructose, 70 μmol of HEPES buffer (pH 8.2) containing 3.5 μmol of $MgCl_2$ in a total volume of 40 μl. To this, 100 μl of enzyme was added to initiate the reaction. After incubation at 30°C for 20 min, the reaction was stopped by adding 20 μl of 30% NaOH and then kept in boiling water for 10 min. Sucrose was determined by using anthrone reagent (van Handel, 1968). Formation of products was linear for at least 25 min. For assaying the activity of SPS, fructose was replaced with fructose-6-phosphate along with 2 μmol of NaF. In control of SS and SPS, the enzyme was first denatured by keeping in boiling bath for 10 min before adding other constituents of the assay system.

The assay system for sucrose synthase in the direction of sucrose breakdown consisted of 100 μmol of HEPES buffer (pH 6.5) containing 2 μmol of UDP and 50 μmol of sucrose in a total volume of 0.4 ml. The reaction was initiated by adding 0.1 ml of enzyme. In control assays, UDP was absent. The assay mixture was incubated for 30 min at 37°C and fructose formed was determined (Nelson, 1944). Rate of product formation was linear for about 45 min.

Assay procedures for acid and alkaline invertases have been described earlier (Chopra et al., 1998). Activities of isocitrate dehydrogenase, NADP malic enzyme and PEP carboxylase was determined by procedures described in Tezuka, Yamamoto and Kondo (1990), Iglesias and Andreo (1990) and Christeller et al. (1977). All enzymes and carbohydrates were extracted from three different samples and assayed/determined in duplicate. Data are the mean ±SD of these values.

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