Plant Physiol. (1987) 83, 1043–1047
0032-0889/87/83/1043/05/$01.00/0

Photosynthetic Carbon Fixation Characteristics of Fruiting Structures of *Brassica campestris* L.[1]

Received for publication August 29, 1986 and in revised form November 24, 1986

HARI R. SINGAL, INDER S. SHEORAN, AND RANDHIR SINGH*
Department of Chemistry and Biochemistry, Haryana Agricultural University, Hisar-125004, India

ABSTRACT

Activities of key enzymes of the Calvin cycle and C_4 metabolism, rates of CO_2 fixation, and the initial products of photosynthetic $^{14}CO_2$ fixation were determined in the podwall, seed coat (fruiting structures), and the subtending leaf (leaf below a receme) of *Brassica campestris* L. cv 'Toria.' Compared to activities of ribulose-1,5-bisphosphate carboxylase and other Calvin cycle enzymes, *e.g.* NADP-glyceraldehyde-3-phosphate-dehydrogenase and ribulose-5-phosphate kinase, the activities of phosphoenol pyruvate carboxylase and other enzymes of C_4 metabolism, *viz.* NADP-malate dehydrogenase, NADP-malic enzyme, glutamate pyruvate transaminase, and glutamate oxaloacetate transaminase, were generally much higher in seed than in podwall and leaf. Podwall and leaf were comparable to each other. Pulse-chase experiments showed that in seed the major product of $^{14}CO_2$ assimilation was malate (in short time), whereas in podwall and leaf, the label initially appeared in 3-PGA. With time, the label moved to sucrose. In contrast to legumes, *Brassica* pods were able to fix net CO_2 during light. However, respiratory losses were very high during the dark period.

The photosynthetic contribution of fruiting structures to their own yield or to the yield of the seeds which they contain has been the subject of considerable research. However, the relative contribution of these structures to seed yield varies considerably. In cereals, such as barley, rice, oats and wheat, the photosynthetic contribution of reproductive organs ranges from 10 to 75% (6). In legume's reproductive organs, little net CO_2 exchange has been shown to occur (6). However, in rapeseed (*Brassica* sp.) virtually 100% of the seed dry matter comes from photosynthetic CO_2 assimilation of the pod (2).

Although photosynthetic characteristics of fruiting structures of wheat (14, 19) barley (12), oat (19), chickpea (15), and pigeonpea (11) have been investigated in detail, no similar report is yet available on the photosynthetic characteristics of fruiting structures of oilseed crops. In our earlier studies with wheat (14) chickpea (15), and pigeonpea (11), we reported high activities of PEP[2]-carboxylase and other enzymes of C_4 metabolism in fruiting structures, suggesting that the primary assimilation of CO_2 in these parts occurs via PEP carboxylase. The present investigation was, therefore, aimed at carrying out detailed studies on the photosynthetic characteristics of fruiting structures of an oilseed crop (*Brassica campestris* L.) during the entire period of seed development with the purpose of understanding the nature of the CO_2 fixation pathway and the metabolic role played by these organs in seed development. The results reported here are contrary to those reported earlier for fruiting structures of cereals and legumes (14, 15).

MATERIALS AND METHODS

Chemicals. All biochemicals and enzymes used in these investigations were purchased from Sigma. NaH $^{14}CO_3$ was procured from Bhabha Atomic Research Centre (India).

Plant Material: *Brassica campestris* L. (cv Toria) was raised in the fields of the Oilseed Section of the Department of Plant Breeding of Haryana Agricultural University, Hisar during 1985. At the time of flower initiation, freshly opened flowers along with the leaf below that raceme were tagged. The pods formed from such flowers, together with leaves, were sampled at 10 d intervals until complete maturity. After removal, the pods were separated into podwall and seeds. Samples from three replications were always taken at 11:00 AM (average light intensity 1000 $\mu E\ m^{-2}\ s^{-1}$). The separated parts, *viz.* podwalls and seeds, were immediately used for further studies.

Enzymes Extraction. Five-hundred mg of each tissue was used and enzyme extracts prepared as described previously (14).

Enzyme Assays. Enzyme activities were determined spectrophotometrically at 340 nm by following the oxidation of NAD(P)H or reduction of NAD(P). All assays were carried out at 30°C. Preliminary assays were done for all the enzymes to determine optimum conditions where linear reaction rates with respect to time and enzyme concentration were obtained. The activities of various key enzymes of the Calvin cycle, *i.e.*, RuBP carboxylase (EC 4.1.1.39), NADP-GAP dehydrogenase (EC 1.2.1.13), and Ru5P kinase (EC 2.7.1.19), and C_4 metabolism, *i.e.* PEP carboxylase (EC 4.1.1.31), NADP-malate dehydrogenase (EC 1.1.1.82), NADP -malic enzyme (EC 1.1.1.40), glutamate oxaloacetate transaminase (EC 2.6.1.1), and glutamate pyruvate transaminase (EC 2.6.1.2), were determined by following the standard assay methods reported earlier (14). The assay mixture for RuBP carboxylase contained: 50 mM Hepes buffer (pH 7.8), 10 mM KCl, 1 mM EDTA, 15 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 0.2 mM NADH, 20 mM $NaHCO_3$, 5 mM phosphocreatine and 2 units each of GAP dehydrogenase, Ru5P isomerase, phosphocreatine kinase, phosphoglycerate kinase, and the enzyme extract; for NADP-GAP dehydrogenase: 100 mM Tris-HCl buffer (pH 7.8), 10 mM $MgCl_2$, 5 mM reduced glutathione (GSH), 5 mM ATP, 0.2 mM NADPH, 20 mM 3-PGA, 2 units of phosphoglycerate kinase, and the enzyme extract; for Ru5P kinase: 100 mM Tris-HCl buffer (pH 7.8), 10 mM Mg Cl_2, 20 mM KCl, 1 mM ATP, 1.5 mM PEP, 0.2 mM NADH, 10 mM DTT, 3 mM ribose 5-phosphate, 3 units each of ribose 5-phosphate isomerase and lactate dehydrogenase, 2 units of pyruvate kinase, and the enzyme extract; for PEP carboxylase: 50 mM Tris-HCl buffer (pH 8.1), 5 mM $MgCl_2$, 2 mM DTT, 0.2 mM NADH, 20 mM $NaHCO_3$,

[1] Financed from grants made by Haryana Agricultural University, Hisar under the project Chem-Biochem 6.

[2] Abbreviations: PEP, phosphoenolpyruvate; GAP, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; 3-PGA, 3-phosphoglyceric acid; Ru5P, ribulose 5-phosphate; RuBP, ribulose 1,5-bisphosphate.

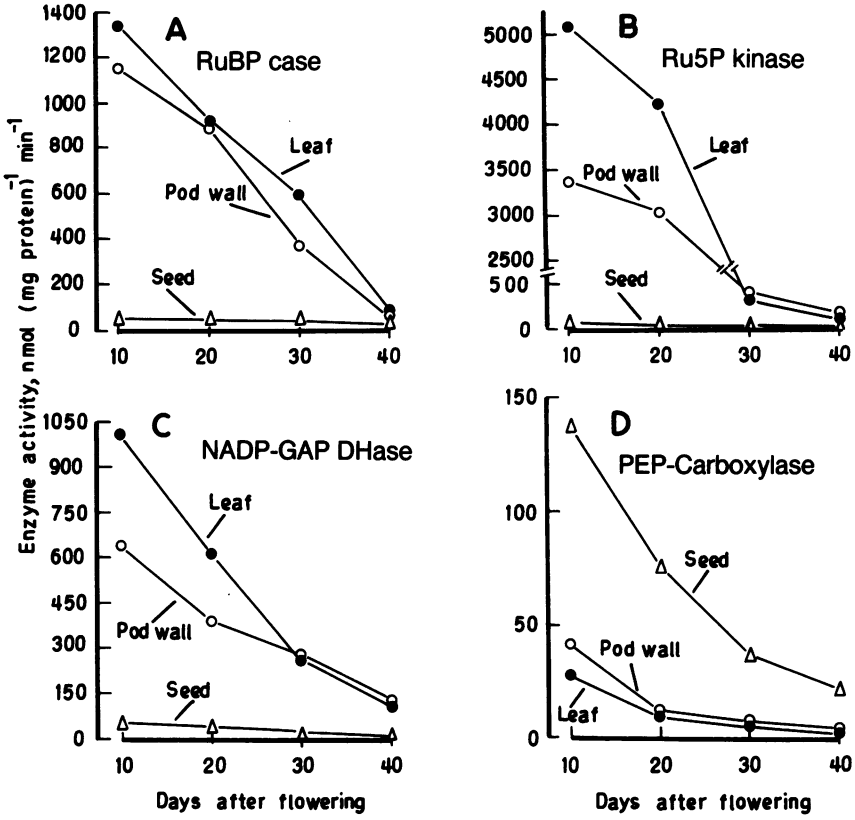

FIG. 1. Enzyme activity of A, RuBP carboxylase; B, Ru5P kinase; C, NADP-GAP dehydrogenase; and D, PEP carboxylase in leaf (●), podwall (○) and seed (△) of 'Toria.'

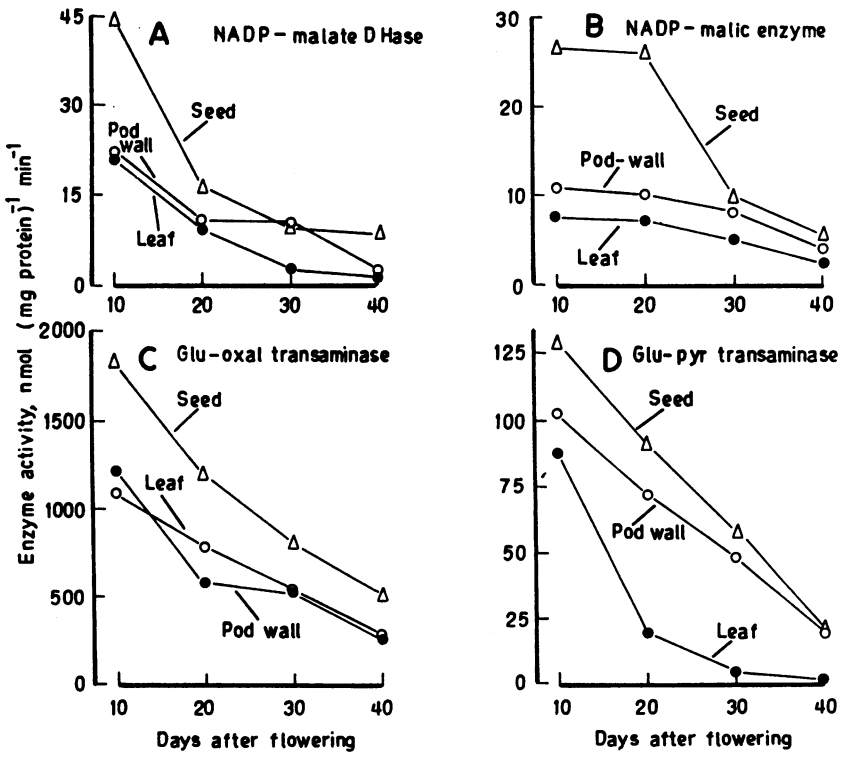

FIG. 2. Enzyme activity of NADP-malate dehydrogenase; B, NADP-malic enzyme; C, glutamate-oxaloacetate transaminase; and D, glutamate-pyruvate transaminase in leaf (●), podwall (○) and seed (△) or 'Toria.'

5 mM PEP, 2 units of malate dehydrogenase, and the enzyme extract; for NADP-malate dehydrogenase: 50 mM Tris-HCl buffer (pH 8.0), 1 mM EDTA, 0.2 mM NADPH, 0.5 mM oxaloacetate, and the enzyme extract; for NADP-malic enzyme: 50 mM Tricine buffer (pH 8.0), 1 mM EDTA, 0.25 mM NADP, 2.5 mM malate, 5 mM $MgCl_2$, and the enzyme extract; for glutamate oxaloacetate transaminase: 100 mM Tris-HCl buffer (pH 7.8), 35 mM aspartate (neutralized), 0.2 mM NADH, 7 mM 2-ketoglutarate, 2 units of malate dehydrogenase, and the enzyme extract; and for glutamate pyruvate transaminase: 100 mM Hepes buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADH, 10 mM alanine, 5 mM α-ketoglutarate, 4 units of lactate dehydrogenase, and the enzyme extract.

The final volume of assay mixture in each case was kept 1.5

Table I. *Changes in Protein and Chl Content in Leaf, Podwall and Seeds of 'Toria' during Seed Development*

Time after Anthesis	Chl			Protein		
	Leaf	Podwall	Seed	Leaf	Podwall	Seed
d	*mg·g⁻¹ fresh wt*					
10	1.78	0.39	0.06	27.5	15.2	16.7
20	0.82	0.34	0.39	15.1	12.4	34.9
30	0.64	0.25	0.30	12.9	11.2	62.1
40	0.44	0.21	0.18	10.2	9.2	75.8

Table II. *Dark Fixation of $^{14}CO_2$ by Leaf, Podwall, and Seeds of Toria*

Time after Anthesis	$^{14}CO_2$ Fixed		
	Leaf	Podwall	Seed
d	*nmol·mg⁻¹ Chl h⁻¹*		
10	169.4 ± 13.6	338.9 ± 15.5	757.1 ± 11.9
20	17.4 ± 0.2	21.9 ± 1.0	332.2 ± 4.4
30	4.6 ± 0.1	6.8 ± 0.1	159.7 ± 12.8
40	2.1 ± 0.1	4.6 ± 0.1	62.4 ± 6.0

ml. All the enzymes from each replicate were assayed in duplicate. The variations in replicated values were within 2%. Hence, average values are given.

CO_2 Fixation. Rate of $^{14}CO_2$ fixation in the dark was measured according to the method described earlier (16). Net CO_2 exchange of whole pods and isolated seeds (both in light and dark) was measured with an IRGA (ADC-Model 225) operating in the differential mode. Light intensity varied from 850 to 1000 μE m^{-2} s^{-2} during the period of these measurements. Four replicates of five pods each were taken for these measurements.

Pulse-Chase Experiment. For pulse chase studies, the tissues, immediately after separation, were incubated with $^{14}CO_2$ (500 $\mu l/l^{-1}$) in a perspex chamber for 15 s in light (1000 μE m^{-2} s^{-2}) using the technique described previously (14). After incubation, the tissues were taken out of the chamber. One set of tissues was immediately killed in boiling 80% (v/v) ethanol. In all other cases, tissues were exposed to atmospheric $^{12}CO_2$ for 15, 30, 45, and 120 s in the light and then killed and extracted in boiling 80% ethanol. The ethanol extract was evaporated to dryness. Chl was extracted from the solids by washing twice with $CHCl_3$ and solids were taken up in water. A suitable aliquot of the water soluble extract was then taken and radioactive compounds separated, identified, isolated and counted as described earlier (14).

Protein and Chl Estimation. Protein in the enzyme extracts was measured by following the method of Lowry *et al.* (10) after precipitation with TCA, Chl was estimated according to Strain *et al.* (18).

RESULTS

Enzymes of Calvin Cycle. The activity of RuBP carboxylase was much higher in leaf and podwall tissues than in seeds at all stages of seed development until d 40 (Fig. 1A). In general, the leaf and podwall had almost comparable activities of this enzyme. The enzyme activity was maximum when the pod was youngest and declined as maturity advanced. The other Calvin cycle enzymes examined, *i.e.* Ru5P kinase and NADP-GAP dehydrogenase showed qualitatively similar patterns to that of RuBP-carboxylase (Fig. 1, B and C, respectively).

Enzymes of C_4 Metabolism. In contract to RuBP carboxylase, PEP-carboxylase was more active in seed than in podwall and leaf tissues at each stage of seed development (Fig. 1D). In fact, in seeds the specific activity of PEP carboxylase exceeded that of RuBP carboxylase at all stages of development (Fig. 1, A and D). The enzyme activity in all the tissues, however, decreased as maturity advanced.

Since there is no evidence to suggest that oxaloacetate or malate accumulates in fruiting structures of oilseed plants as observed in leaves of plants possessing high activity of PEP-carboxylase, the enzymes concerned with further metabolism of oxaloacetate, *i.e.* NADP-malate dehydrogenase, NADP-malic enzyme, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase, were also monitored. These enzymes followed patterns of activity overtime similar to that of PEP-carboxylase (Fig. 2, A–D). Glutamate oxaloacetate transaminase had the highest specific activity at all stages of development.

Protein and Chl Content. Soluble protein content in leaf and podwall decreased with aging, whereas it showed the reverse trend in seeds (Table I). Chl content was much higher in leaf than in pod-wall and seeds. Again, the content in leaf and podwall decreased with maturity. In seeds, the maximum content was observed at d 20.

Rate of CO_2 Fixation in Dark. The rate of CO_2 fixation in the dark was about 15 to 35 times more in seeds than in leaf and podwall tissues except at d 10 where seeds fixed only 2 to 4 times more (Table II). Rate of CO_2 fixation decreased with aging in all the tissues. The pattern of dark CO_2 fixation, thus, correlated well with the activity of PEP-carboxylase (Fig. 1D).

Net CO_2 Exchange. In contrast to legumes, pods of *Brassica* fixed net CO_2 in presence of light which was maximum at d 20 (Table III). In dark, there was a net loss of CO_2 by pods which was much higher than the net fixation during light at all stages of seed development. Isolated seeds showed a net loss of CO_2

Table III. *Net CO_2 Exchange by 'Toria' Pod and Isolated Seeds*

Time after Anthesis	CO_2 Evolved			
	Whole pod		Seeds	
	Light	Dark	Light	Dark
d	*μg CO_2 organ⁻¹ h⁻¹*			
10	−2.84 ± 0.4[a]	159 ± 20.3	10.8 ± 1.1	15.1 ± 1.1
20	−26.9 ± 2.8	128 ± 9.1	61.9 ± 3.9	67.1 ± 11.2
30	−14.6 ± 2.1	147 ± 16.8	37.1 ± 4.1	47.6 ± 5.6
40	−16.9 ± 2.1	34.7 ± 4.3	20.3 ± 3.2	29.6 ± 3.3

[a] A negative value indicates net CO_2 fixation.

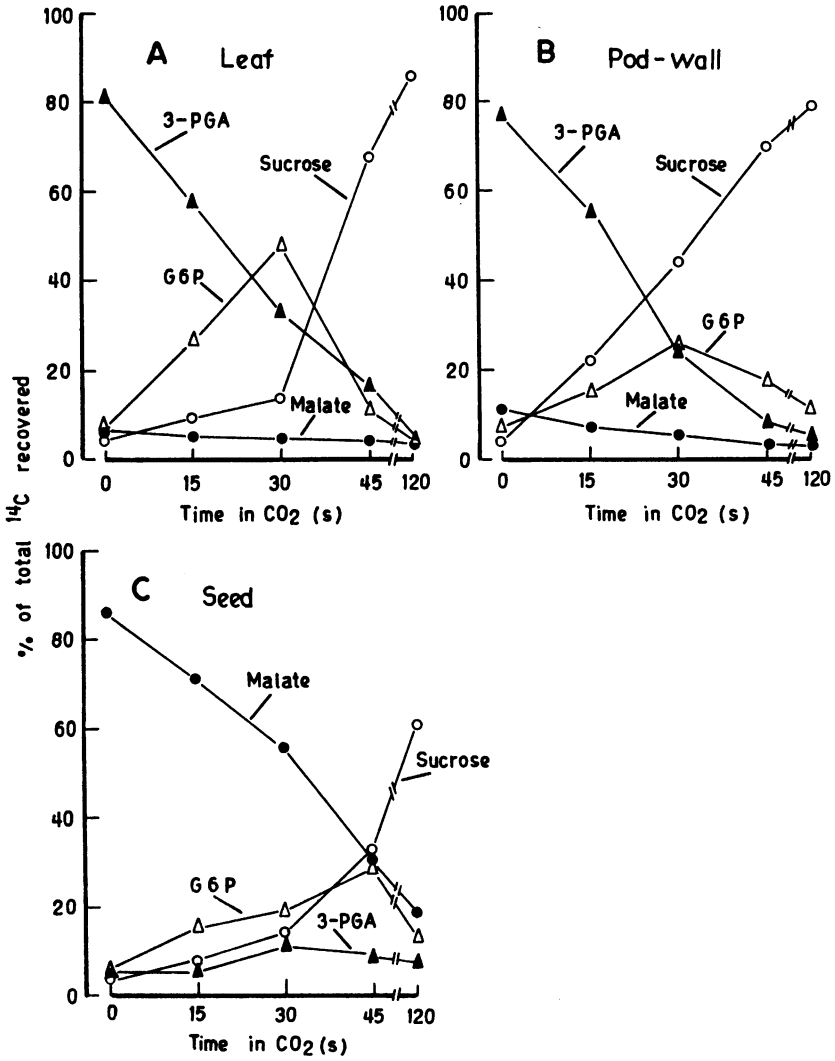

FIG. 3. Time course distribution (%) of ^{14}C during chase between malate (●), 3-phosphoglycerate (▲), glucose-6-P (△) and sucrose (○) following a 15-s pulse of $^{14}CO_2$ in A, leaf; B, podwall; and C, seed of 'Toria.'

both in light and dark. The losses were more in dark than in light at each stage of seed development.

Pulse-Chase Studies. Of the $^{14}CO_2$ fixed after 15 s photosynthesis by leaf, podwall and seed, about 82, 77, and 4% of the total radioactivity appeared in 3-PGA, whereas 7, 11, and 86%, respectively, was recovered in the C_4 acid-malate (Fig. 3). The remaining label was in sucrose and glucose-6-P. During a chase with $^{12}CO_2$, 3-PGA in leaf and podwall lost radioactivity more rapidly than malate in seeds. In all the tissues, however, the label in sucrose continued to increase with increasing time of chase with the rate being relatively slow in seeds. After 120 s of chase, sucrose accounted for about 86, 80, and 61% of the total radioactivity in leaf, podwall and seed, respectively.

DISCUSSION

Two experimental approaches, namely measurement of *in vitro* enzyme activities and kinetic studies with $^{14}CO_2$, were followed to determine the pathway of CO_2 fixation operating in fruiting structures of an oilseed crop 'Toria.' Interestingly, the activities of RuBP-carboxylase and other Calvin cycle enzymes were much high in leaf and podwall as compared to seed, which had higher activities of PEP-carboxylase and certain other enzymes of C_4-metabolism (Figs. 1 and 2) at all the stages of seed development. However, compared to leaf, very low activity of RuBP carboxylase has been reported in podwall tissues of different legumes (6, 7, 11, 13, 15, 17). While the podwall had a high RuBP-carboxylase: PEP-carboxylase ratio, a reverse trend was observed in case of seed (Fig. 1). A lower RuBP-carboxylase: PEP-carboxylase ratio has, however, been reported in podwall of chickpea (9, 15). These results suggest that in podwall of 'Toria' the primary carboxylation reaction assimilating CO_2 is catalyzed by RuBP-carboxylase and not by PEP-carboxylase as is the case in fruiting structure of cereals (14, 19) and legumes (15).

The above observations were further confirmed when the ^{14}C-labeling pattern of various intermediate metabolites was investigated. After 15 s of photosynthesis in $^{14}CO_2$, in leaf and podwall about 80% of total radioactivity appeared in 3-PGA as compared to about 10% in malate (Fig. 3). Contrary to this, the seed showed 86% of total radioactivity in malate and only 4% in 3-PGA. During further exposure to $^{12}CO_2$, in both the leaf and podwall, the radioactivity decreased in 3-PGA and increased in sucrose with the increase in time of chase (Fig. 3). After 2 min, at least 80% of the radioactivity moved to sucrose in leaf and podwall. However, in case of seed, about 20% of the radioactivity was still left in malate.

In contrast to the CO_2 exchange studies with legumes (6, 11, 13, 15), pods of 'Toria' fixed net CO_2 in presence of light (Table III). However, in dark, there was a net loss of CO_2 by the pods which was about 2- to 50-fold more than the daytime fixation. Isolated seeds showed a net loss of CO_2 both in light and dark, although they have Chl. The loss of CO_2 was less in light compared to dark, thereby suggesting that some CO_2 is refixed

photosynthetically by isolated seeds.

The results of ^{14}C-labeling experiments, enzymic analysis and CO_2 exchange studies thus clearly establish that like leaf, pod wall of 'Toria' assimilates CO_2 via the Calvin cycle, with RuBP carboxylase catalyzing the initial carboxylation reaction and producing 3-PGA as the primary product of photosynthesis. It is now well known that net incorporation of CO_2 into carbohydrates for plant growth is achieved solely by the Calvin cycle. The observed net fixation of CO_2 during light by the podwall (Table II) further supports the above view point and is suggestive of an important role of podwall in seed yield of 'Toria.' The report of Khanna-Chopra and Sinha (8) that podwall of mustard translocates current photosynthate to the developing fruit and that the translocation is more compared to leaf during later stages of development, further reveals the importance of fruitwall in seed yield.

In seeds, however, the rapid labeling and relatively high proportion of ^{14}C in malate (Fig. 3) suggest that CO_2 is incorporated into oxaloacetate via PEP-carboxylase which is rapidly converted to malate by malate dehydrogenase. Malate after decarboxylation by NADP-malic enzyme, may play a significant role in generating NADPH for biosynthesis of fatty acids as reported in soybean seeds (1). The CO_2 evolved after decarboxylation may be reduced to carbohydrate by the enzymes of the Calvin cycle. This is supported by the observation that less CO_2 is lost during light compared to dark by the isolated seeds (Table III).

High activity of PEP-carboxylase and higher rates of dark CO_2 fixation by seeds suggest that PEP carboxylase in seeds fixes CO_2 released during either decarboxylation of malate by malic enzyme or seed respiration. Gluconeogenic tissues of fatty seeds have been reported to catalyze appreciable incorporation of $^{14}CO_2$ into sugars in dark (4, 5). Nonphotosynthetic fixation of CO_2 by PEP-carboxylase has been reported to occur both under light and dark conditions (3). This enzyme in seeds may thus, help in reducing respiratory CO_2 losses, thereby contributing to seed dry matter in addition to the dry matter deposited as a result of net CO_2 fixation by podwall.

Acknowledgments—One of the authors (H.R.S.) is thankful to Council of Scientific and Industrial Research, New Delhi, for financial assistance in the form of Senior Research Fellowship of the Council. Dr. Hari Singh, Breeder (Oilseeds) is thanked for providing the plant material.

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