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In vitro enzyme activities and products of $^{14}CO_2$ assimilation in flag leaf and ear parts of wheat (*Triticum aestivum* L.)

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Abstract. Activities of key enzymes of Calvin cycle and C_4 metabolism, rate of $^{14}CO_2$ fixation in light and dark and the initial products of photosynthetic $^{14}CO_2$ fixation were determined in flag leaf and different ear parts of wheat viz. pericarp, awn and glumes. Compared to the activities of RuBP carboxylase and other Calvin cycle enzymes viz. NADP-glyceraldehyde-3-phosphate dehydrogenase, NAD-glyceraldehyde-3-phosphate dehydrogenase and ribulose-5-phosphate kinase, the levels of PEP carboxylase and other enzymes of C_4 metabolism viz. NADP-malate dehydrogenase, NAD-malate dehydrogenase, NADP-malic enzyme, NAD-malic enzyme, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase, were generally greater in ear parts than in the flag leaf. In contrast to CO_2 fixation in light, the various ear parts incorporated CO_2 in darkness at much higher rates than flag leaf. In short term assimilation of $^{14}CO_2$ by illuminated ear parts, most of the ^{14}C was in malate with less in 3-phosphoglyceric acid, whereas flag leaves incorporated most into 3-phosphoglyceric acid. It seems likely that ear parts have the capability of assimilating CO_2 by the C_4 pathway of photosynthesis and utilise PEP carboxylase for recapturing the respired CO_2.

Introduction

Higher plants have been classified into three broad groups, namely, C_4, C_3 and CAM types on the basis of their leaf photosynthetic characteristics. However, all green organs or tissues on the same plant may not have the identical CO_2 fixation characteristics as has been observed for the pericarp of barley [6, 21], wheat [16, 25] and oats [25]. In cereals, dry matter entering grain is mainly from photosynthesis after ear emergence [1] and that various photosynthetic parts of the ear together with the flag leaf make the major contribution to grain carbohydrates [7]. The parts of the ear which contain chlorophyll and may contribute directly to grain carbohydrates, include awns, glumes, and pericarps. The pericarps of wheat and barley, the leaves of which have C_3 metabolism, possess very high activity of PEP carboxylase [6, 16] and also contain pyruvate orthophosphate dikinase- a key enzyme of C_4 metabolism [6, 19, 20] and thus may have C_4 metabolism. Compared to leaves, studies on the photosynthetic characteristics of the ear parts have not yet been carried out in detail during grain development. We have, therefore, investigated the photosynthetic characteristics of different ear parts of wheat

with a view to understand the nature of the CO_2 fixation pathway and the metabolic role played by these parts in grain growth.

Materials and methods

Plant material

A wheat crop (cv. WH-157) was raised under field conditions as described previously [15]. Ears and flag leaves were harvested randomly for further studies. Ear parts (awns, glumes and pericarps) were removed and used immediately. Samples from three replications were taken always at 10.00 a.m. (Average light intensity 1100 μE m^{-2} s^{-1}), first at the time of anthesis and thereafter at ten days interval.

Chemicals

All the biochemicals and enzymes were purchased from Sigma (St. Louis, MO, USA), $NaH^{14}CO_3$ (specific activity 50 mCi/mmol) was from BARC (India). All other chemicals were of analytical grade.

Determination of enzyme activities

Enzyme extraction. Five hundred mg of tissue was taken and the enzyme extract prepared in 50 mM imidazole-HCl buffer (pH 8.0) containing 10 mM DTT, 10 mM $MgCl_2$, 2 mMEDTA and 1% (w/v) PVP as described previously [22].

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 established conditions where linear reaction rates with respect to time and enzyme concentrations were obtained. All the enzymes from each replicate were assayed in duplicate and the average values are reported here. The activities of RuBP carboxylase (EC 4.1.1.39), NADP-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13), NAD-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) and ribulose-5-phosphate kinase (EC 2.7.1.19) were determined as described previously [22]. PEP carboxylase (EC 4.1.1.31) was assayed by following the method of Hatch [8]. The activities of NAD-malate dehydrogenase (EC 1.1.1.37) and NADP-malate dehydrogenase (EC 1.1.1.82) were measured according to the method of Hatch and Slack [11, 12]. NADP-malic enzyme (EC 1.1.1.40) and NAD-malic enzyme (EC 1.1.1.39) were assayed as described by Johnson and Hatch [13] and Hatch and Kagawa [9], respectively. Activities of glutamate oxaloacetate transaminase (EC 2.6.1.1.) and glutamate pyruvate transaminase (EC 2.6.1.2) were determined according to Bergmeyer [3] and Hatch and Mau [10]. The variations in replicated values was within 2%. Hence, average values are given in figures.

CO_2 fixation in light and dark

Rate of $^{14}CO_2$ fixation in light and dark was measured as per the method of Sinha et al. [23]. Fifty mg of the tissue was infiltered with 1 ml of $NaH^{14}CO_3$ (sp. activity, 50 mCi/mmol) in 0.1 M sodium phosphate buffer (pH 7.5) containing 5 μCi/ml of ^{14}C, using a vacuum pump for 30 sec. The tissue was then exposed to direct sunlight or in darkness for 15 min. After the feeding period, 2 ml of 2NHCl was added, the supernatant decanted and the tissue extracted as described in ref. [23].

Products of $^{14}CO_2$ fixation

Immediately after separation, the parts were incubated with $^{14}CO_2$ using a technique similar to that described by Coombs and Baldry [5]. One hundred mg tissue was quickly transferred to planchets having glass fibre soaked in 50 mM Tricine-KOH buffer (pH 7.5) containing 0.33 M sorbitol (to prevent dehydration). Five planchets were placed in perspex chamber (19 x 1.2 x 3.7 cm) with an effective volume of 50 ml and preilluminated in direct sunlight (light intensity 1100 $\mu E\, m^{-2}\, s^{-1}$) for 10 min. 0.1 ml of $NaH\ ^{14}CO_3$ (0.5 mCi/ml) was injected through a rubber seal into a well containing hydrochloric acid, to give the concentration of $^{14}CO_2$ in the chamber 500 $\mu l\, l^{-1}$. A gas mixing pump (PK Morgan Ltd., USA) circulated the air inside the chamber. After incubating for 20, 40, 60, 120 and 300 sec, the planchets were removed one at a time and the tissues killed (within 2 sec) and extracted in boiling 80% ethanol. The ethanol extract was evaporated to dryness. Chlorophyll was extracted from the solids by washing twice with chloroform. After evaporating off excess chloroform, the solids were taken up in water, an aliquot of which was analysed by paper chromatography using n-butanol-propionic acid-water (10:5:7) as the solvent [2]. Radioactive products were identified by co-chromatography and radioautography with authentic compounds. Areas containing ^{14}C were cut from the paper, extracted in 80% ethanol and radioactivity determined by liquid scintillation counting in a Beckman LS 100C liquid scintillation counter (efficiency 80%). The recovery of authentic compounds by paper chromatography was about 85–90%.

Estimation of protein and chlorophyll

Protein in the enzyme extracts was measured by the method of Lowry et al. [18] after TCA precipitation and chlorophyll according to Strain et al. [24].

Results

Enzymes of the Calvin cycle

The activity of RuBP carboxylase- a key enzyme of the Calvin cycle, was greater in the flagleaf than in awns, glumes or pericarps at any stage of grain

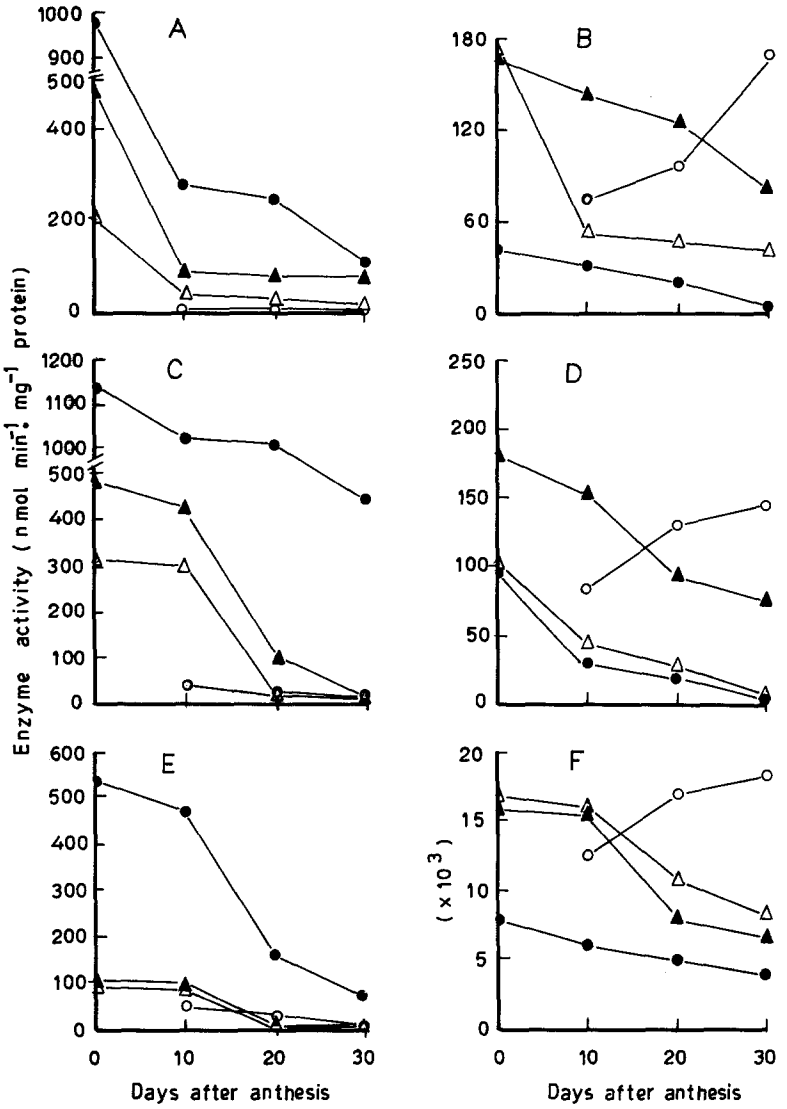

Figure 1. Enzyme activity of A, RuBP carboxylase; B, PEP carboxylase; C, NADP-glyceraldehyde-3-phosphate dehydrogenase; D, NADP-malate dehydrogenase, E, NAD-glyceraldehyde-3-phosphate dehydrogenase and F, NAD-malate dehydrogenase in flag leaf (●—●), pericarp (○—○), awns (▲—▲) and glumes (△—△) of wheat.

development (Fig. 1). Enzymes activity was greatest in younger parts and decreased as they matured. Among the ear parts, awns had greatest enzyme activity. The other Calvin cycle enzymes examined viz. NADP-glyceraldehyde-3-phosphate dehydrogenase, NAD-glyceraldehyde-3-phosphate dehydrogenase and ribulose-5-phosphate kinase, showed qualitatively similar patterns to that of RuBP carboxylase (Figs. 1 and 2).

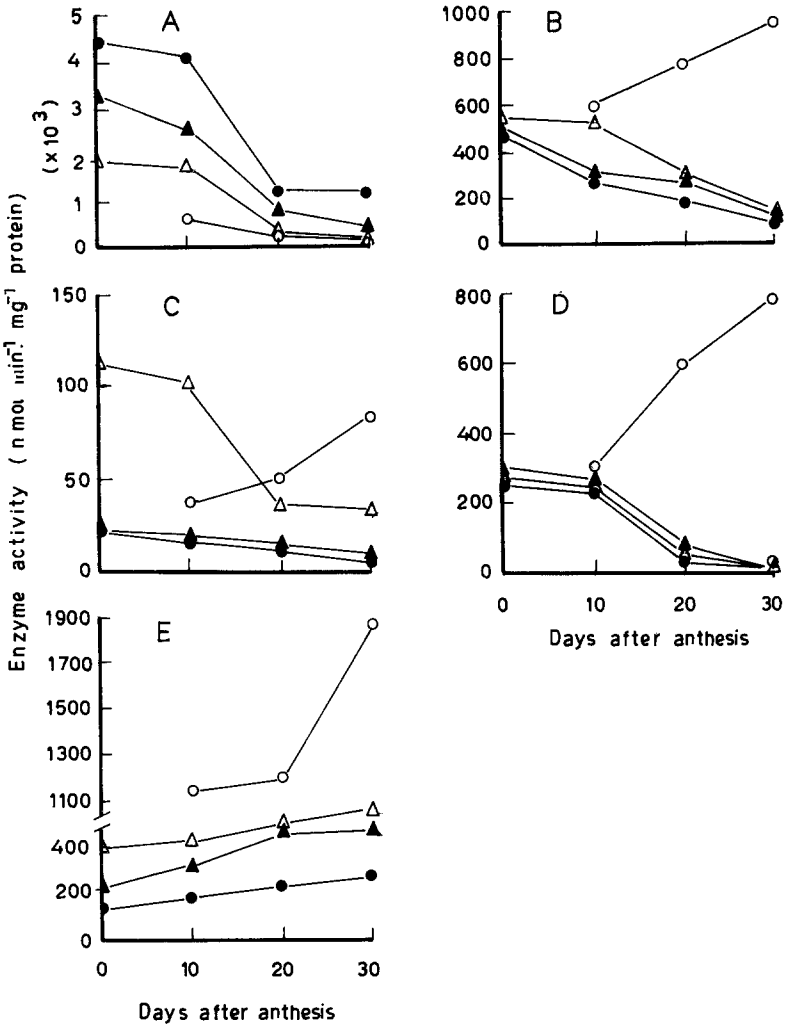

Figure 2. Enzyme activity of A, ribulose-5-phosphate kinase, B, NAD-malic enzyme, C, NADP-malic enzyme; D, glutamate oxaloacetate transaminase and E, glutamate pyruvate transaminase in flag leaf (●—●), pericarp (○—○), awns (▲—▲) and glumes (△—△) of wheat.

Enzymes of C_4 metabolism

In sharp contrast to RuBP carboxylase, PEP carboxylase was more active in ear parts than in flag leaves at every stage of grain development (Figure 1). Expressed on chlorophyll basis (data not given here), the level of PEP carboxylase was much more higher in ear parts than in flag leaf. In fact, in the case of awns, glumes and pericarps, the specific activity of PEP carboxylase exceeded that of RuBP carboxylase at all stages of grain development except at anthesis. Enzyme activity in pericarps increased with age whereas, the

reverse was observed in flag leaves, awns and glumes (Figure 1). Since there is no evidence to suggest that oxaloacetate or malate accumulates in parts surrounding wheat grain as they do in C_4 leaves, the enzymes concerned with further metabolism of oxaloacetate viz. NADP-malate dehydrogenase, NAD-malate dehydrogenase, NADP-malic enzyme, NAD-malic enzyme, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase were assayed. These enzymes qualitatively followed patterns similar to that of PEP carboxylase (Figures 1 and 2). However, the activity of glutamate oxaloacetate transminase increased with ageing in all the parts.

Rate of CO_2 fixation in light and dark

All the organs of wheat, studied here, were capable of carrying out CO_2 fixation in light and dark (Table 1). In the light, flag leaves and pericarps fixed CO_2 at rates of 47 to 145 and 248 to 372 μmol mg chl^{-1} h^{-1}, respectively, while awns incorporated $^{14}CO_2$ at lower rates. Glumes fixed CO_2 at a rate comparable to that of flag leaf. In flag leaves, awns and glumes, CO_2 fixation in the light decreased from anthesis onwards whereas, in pericarps it declined only 20 days after anthesis. In contrast ear parts incorporated CO_2 in dark at much higher rates than flag leaves (Table 1). Pericarps fixed 7 to 32 times more CO_2 than flag leaves and fixation in pericarps increased with ageing, whilst the reverse trend was observed in flag leaves, awns and glumes (Table 1). Ten days after anthesis, the ratio of light to dark CO_2 fixation was 19, 10, 4 and 3 in flag leaves, pericarps, awns and glumes, respectively. A similar trend in ratios was maintained throughout grain development.

Products of photosynthetic $^{14}CO_2$ fixation

The distribution of radioactivity between malate, 3-phosphoglyceric acid, sucrose and glucose-6-phosphate, obtained as products of photosynthetic $^{14}CO_2$ assimilation, was determined after 20, 40, 60, 120 and 300 sec of incubation. Of the $^{14}CO_2$ fixed after 20 sec photosynthesis, in pericarps, glumes, awns and flag leaves about 87%, 81%, 81% and 3% was in malate, whereas, 8%, 11%, 11% and 55% was in 3-phosphoglyceric acid, respectively

Table 1. CO_2 fixation in light and dark by flag leaf and different ear parts of wheat at various stages of grain development.

Days after anthesis		Flag leaf	Pericarp	Awn (n mol mgchl^{-1} h^{-1})	Glumes
0	light	144 ± 1.3	–	45.0 ± 0.5	142.3 ± 1.5
	dark	4.9 ± 0.00	–	11.6 ± 0.1	34.5 ± 0.2
10	light	97.1 ± 0.8	372.2 ± 4.5	39.9 ± 0.4	98.5 ± 1.0
	dark	4.9 ± 0.0	36.1 ± 0.4	10.4 ± 0.1	33.2 ± 0.2
20	light	63.8 ± 0.5	484.4 ± 7.8	30.9 ± 0.4	64.9 ± 0.7
	dark	4.3 ± 0.0	57.8 ± 0.7	9.5 ± 0.1	26.0 ± 0.3
30	light	47.5 ± 0.2	248.9 ± 4.1	17.4 ± 0.2	41.9 ± 0.2
	dark	3.5 ± 0.0	111.6 ± 1.2	7.5 ± 0.0	14.5 ± 0.1

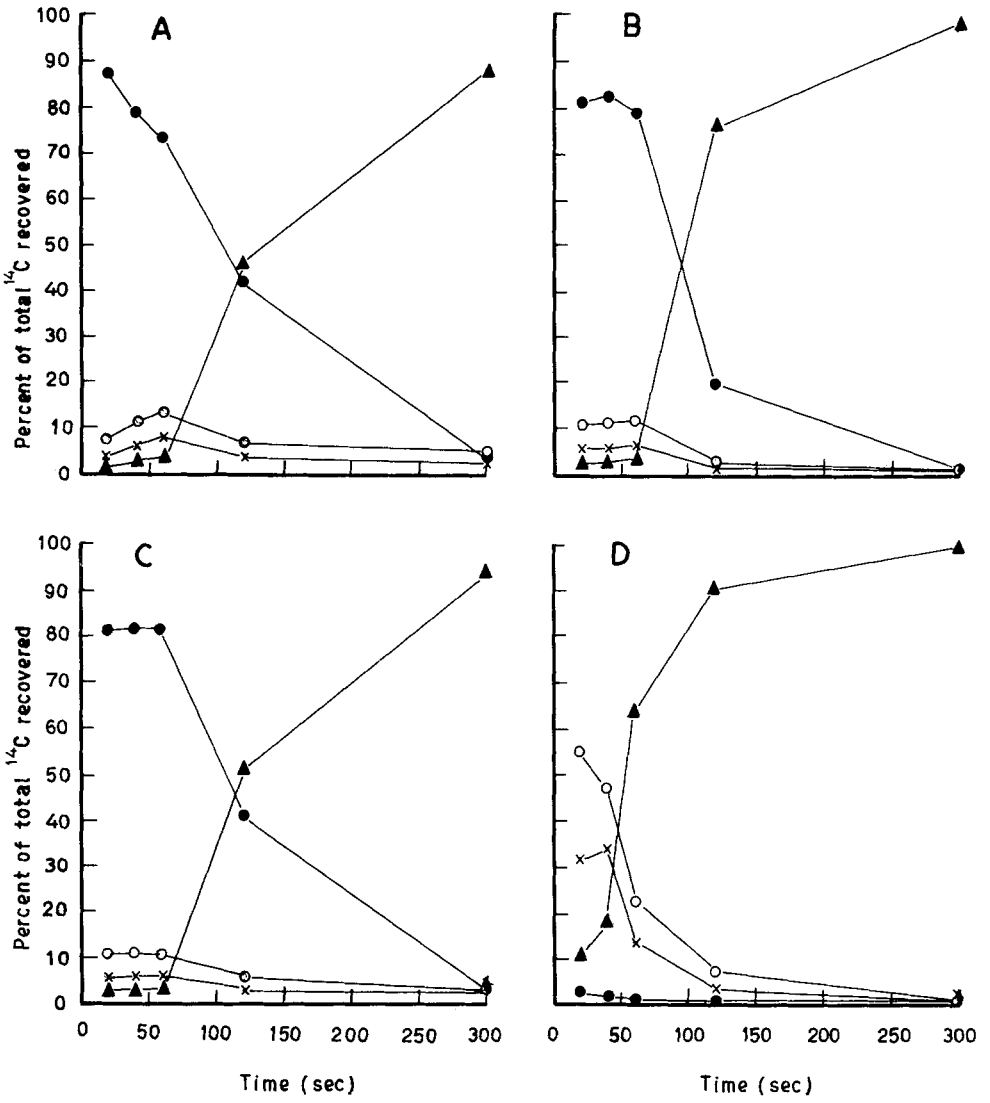

Figure 3. Time course distribution of ^{14}C in wheat ear parts assimilating $^{14}CO_2$ in light. A, pericarp; B, glumes; C, awns; D, Flag leaf; ●—●, malate; ○—○, 3-phosphoglyceric acid, ▲—▲, sucrose; X—X, glucose-6-phosphate.

(Figure 3 A–D). The remaining label was in sucrose and glucose-6-phosphate. Thus the ratio of C_4:C_3 products was 10.9, 7.6, 7.5 and 0.05 in pericarps, glumes, awns, and flag leaves, respectively. After 120 sec sucrose was the major labelled product and after 300 sec it accounted for 88–99% of total ^{14}C recovered (Figure 3 A–D).

Discussion

During light, pericarps fixed CO_2 at much higher rates than flag leaves, while awns incorporated CO_2 at lower rates at all stages of grain development (Table 1). Photosynthetic CO_2 assimilation by glumes, however, was similar to flag leaves. Wirth et al. [25], on the other hand, reported that photosynthetic CO_2 fixation by ear parts of wheat, and panical tissues of oat, was slower than that of flag leaves. Photosynthesis of pericarps was much faster than reported for barley pericarps [21]. In darkness, ear parts fixed CO_2 at much higher rates than did flag leaves. Similar observations have been reported earlier for various photosynthetic organs of wheat and oats [25].

In C_4 and CAM plant leaves, the first product of photosynthetic CO_2 fixation is mainly C_4 acid malate. Interestingly, in ear parts of wheat (C_3) also, the initial product of $^{14}CO_2$ fixation in short period (20 sec) photosynthesis was malate. Some 81–87% of radioactivity appeared in malate compared to 8–11% in 3-phosphoglyceric acid (Figure 3 A–C). However, flag leaves incorporated 55% of radioactivity in 3-phosphoglyceric acid and only about 3% in malate (Figure 3D). These results for ear parts of wheat resemble those obtained for photosynthesising leaves of C_4 plants [11]. Similar results have also been obtained for barley pericarps [21]. It therefore, indicates that the ear parts of wheat assimilate CO_2 by a C_4 type of metabolism. This is supported by the high activities of RuBP carboxylase and other enzymes of Calvin cycle in flag leaves compared to ear parts which had higher levels of enzymes of C_4 metabolism (Figures 1 and 2), at all stages of grain development especially when expressed on chlorophyll basis (data not given). Similarly, Latzko et al. [17] have reported that, on chlorophyll basis, RuBP carboxylase was three times more active in spinach than in maize, whereas, PEP carboxylase activity was about ten times higher in maize. The reported values of 350 μmol mg chl^{-1} h^{-1} [17] for maize PEP carboxylase are similar to ours for pericarps, but we obtained smaller values in awns and glumes.

The results of ^{14}C labelling studies and enzymatic analysis thus, show that malate is the initial product of photosynthetic CO_2 fixation in ear parts, whereas, 3-phosphoglyceric acid is the primary product of photosynthesis in flag leaf. However, time course studies of ^{14}C distribution in different metabolites revealed that in pericarps, ^{14}C-malate decreased with corresponding increase in ^{14}C-3-phosphoglyceric acid until 60 sec (Figure 3 A) indicating that this part might be assimilating CO_2 by C_4 pathway. It does not seem to be true in awns and glumes as the labelling patterns in malate and 3-phosphoglyceric acid were not similar to that of pericarps (Figure 3 B, C). Hence, the reaction sequence by which malate is converted to sucrose in these parts needs to be investigated in detail to prove the nature of pathway.

The CO_2 fixed by PEP carboxylase in pericarps is probably derived from

respiration of the endosperm rather than the atmosphere as very few stomata are known to be present on pericarps [4]. Light induced re-fixation of respiratory CO_2 by different parts of wheat ear has also been indicated earlier [14]. The possibility that pericarps, glumes and awns of wheat utilise PEP carboxylase to recapture respired CO_2 seems likely when the level of this enzyme and the ability to fix CO_2 in dark are considered for these parts.

From above we conclude that the ear parts of wheat, particularly the pericarp although without the anatomical and physiological properties characteristic of C_4 plants, is capable of assimilating CO_2 by C_4 pathway of photosynthesis with malate as the initial product of photosynthetic CO_2 assimilation.

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