New Phytol. (1977) **79**, 89–94.

SHIFTS IN PHOTOSYNTHETIC CARBON LABELLING PATTERN BY ETIOLATED RICE (*ORYZA SATIVA* L.) SEEDLINGS DURING GREENING

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(*Received 28 September 1976*)

SUMMARY

The carbon assimilation pattern in light by dark-grown rice (*Oryza sativa*) seedlings was studied during greening. After exposure of etiolated seedlings to light their capacity to synthesize the C_4-acids, malate and aspartate, increased for 12 h. The labelling of 3-phosphoglycerate, sugar phosphates, sucrose and insolubles, did not increase until after 12 h. Thereafter a continuous steep increase in synthesis of Calvin cycle intermediates, but not of C_4-acids, recurred. The levels of carboxylating enzymes (phosphoenolpyruvate and ribulose diphosphate carboxylases) in the seedlings correlated positively with the appearance of label in C_4 acids and in Calvin cycle compounds. We suggest that a β-carboxylation mechanism was activated immediately on illumination but was persistent for only 12 h. After an 8-h lag period following illumination, the Calvin cycle began to operate and continued during further growth of seedlings in light.

INTRODUCTION

Two distinct pathways of carbon fixation in light are known in green plants: the Calvin cycle of carbon assimilation and the C_4-dicarboxylic acid pathway of photosynthesis (Bassham and Calvin, 1957; Hatch and Slack, 1970; Black, 1973). Although the Calvin cycle is the major route for CO_2 entry in C_3-plants, these plants also synthesize C_4-acids during photosynthesis. Labelling of malate and aspartate during short-term exposure to $^{14}CO_2$ occurs in *Chlorella* (Calvin and Bassham, 1962) and in bean (Tamas and Bidwell, 1970). In fact C_3-plants possess the necessary enzyme systems for β-carboxylation, which is essential for C_4 photosynthesis. But the activities of these enzymes are much lower than those recorded in C_4-plants (Hatch and Slack, 1970; Black, 1973).

The photosynthetic capacities of leaves develop gradually during greening (Tolbert and Gailey, 1955; Rhoades and Yemm, 1966). During this process not only Calvin cycle intermediates such as 3-phosphoglycerate (PGA) and sugar phosphates but also compounds like malate, aspartate and glutamate are formed (Tolbert and Gailey, 1955).

Oryza sativa is a C_3-plant (Raghavendra, 1975). Yet etiolated rice seedlings synthesize predominantly C_4-acids, malate and aspartate, during carbon fixation. Hence the carbon labelling pattern by etiolated rice seedlings was followed during greening.

MATERIALS AND METHODS

Experimental material

Seeds of *Oryza sativa* L. var IR-22 were soaked in running tap water for 24 h. They were

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then sown in 15-cm Petri dishes on Whatman No. 1 filter paper moistened with water. They were allowed to grow for 7 days in darkness at 22 ± 2°C. After this time the seedlings were watered daily and were either illuminated at 22°C under a bank of fluorescent lamps giving light intensity of 100 W m^{-2} or maintained in darkness. At the end of the required period of illumination or of dark incubation the primary leaves were harvested and used as the experimental material.

$^{14}CO_2$ incorporation

Leaves were exposed to $^{14}CO_2$ by the dip method of Berry, Downton and Tregunna (1970) using 100 ml snap cap vials. The final concentration of carbon dioxide was 0.05% with a specific activity of 250 μCi $mmole^{-1}$ (light intensity 200 W m^{-2}, temperature 27 ± 1°C). After exposure to $^{14}CO_2$ for 60 s, the leaves were killed by immersing them in boiling 80% (v/v) ethanol and simultaneously the lights were turned off. The labelled compounds were separated and studied by conventional two dimensional paper chromatography and autoradiography (Benson *et al.*, 1950). The radioactive spots were located and the corresponding portions on chromatographic paper were cut out. Their radioactivity was measured on both sides by placing them directly in a continuous gas flow proportional counter (Raghavendra, 1975).

Carboxylation enzymes

Primary leaves were detached, sectioned, blotted dry and weighed. They were ground in a mortar with a pestle at 0°C with four volumes of 50 mM tris-HCl buffer, pH 7.8, containing 5 mM dithiothreitol (DTT); 1 mM EDTA; 2 mM $MgCl_2$; and 10 mM 2-mercaptoethanol. The extract was filtered through four layers of cheese-cloth.

The extracts were assayed for phosphoenolpyruvate (PEP) and ribulose diphosphate (RuDP) carboxylase activities by observing substrate-dependent radioactive bicarbonate incorporation. The enzyme extract was diluted so as to give a linearity for at least 10–15 min. The reaction mixture (2 ml) for PEP carboxylase (EC 4.1.1.31) contained 50 mM tris-HCl buffer, pH 8.0; 2 mM $MgCl_2$; 2 mM $NaH^{14}CO_3$ (1.6 mCi m $mole^{-1}$); 1.5 mM PEP and the enzyme. The reaction was stopped after 3 min with an equal volume of 1 N HCl saturated with 2,4-dinitrophenylhydrazine and an aliquot was examined for incorporated radioactivity (Raghavendra and Das, 1975). The assay mixture (2 ml) for RuDP carboxylase (EC 4.1.1.39) was 50 mM tris-HCl buffer, pH 7.8, with 10 mM $MgCl_2$; 3 mM DTT; 20 mM $NaH^{14}CO_3$ (0.3 mCi $mmole^{-1}$); 0.5 mM RuDP and the enzyme. The reaction was started with the addition of RuDP and was stopped after 5 min by 1 ml 4 N HCl. An aliquot was examined for incorporated radioactivity.

Chlorophyll

Leaf tissue was macerated with 80% (v/v) acetone and chlorophyll content was estimated by the method of Arnon (1949). Chlorophyll *a*/chlorophyll *b* ratio was determined by the method of Ogawa and Shibata (1965).

RESULTS

When etiolated seedlings were exposed to light the synthesis of malate and aspartate increased rapidly up to 8 h of illumination (Fig. 1). There was no further increase in the formation of these acids beyond 12 h. On the other hand PGA production was characterized by a lag period of up to 12 h. Similar lag periods were observed for the synthesis of alanine, sugar phosphates, sucrose and insoluble compounds. Thereafter, the labelling of these compounds (PGA, sugar phosphates, etc.) showed a steady steep increase with increasing periods of illumination. No remarkable changes were observed in the labelling pattern of malate, aspartate or PGA when the seedlings were retained in darkness (Fig. 2). Such seedlings did not incorporate any label into sugar phosphates, sucrose and insolubles. The amount of carbon fixed in darkness by etiolated seedlings accounted for less than 1% of that in light. The products of such dark fixation are shown in Table 1.

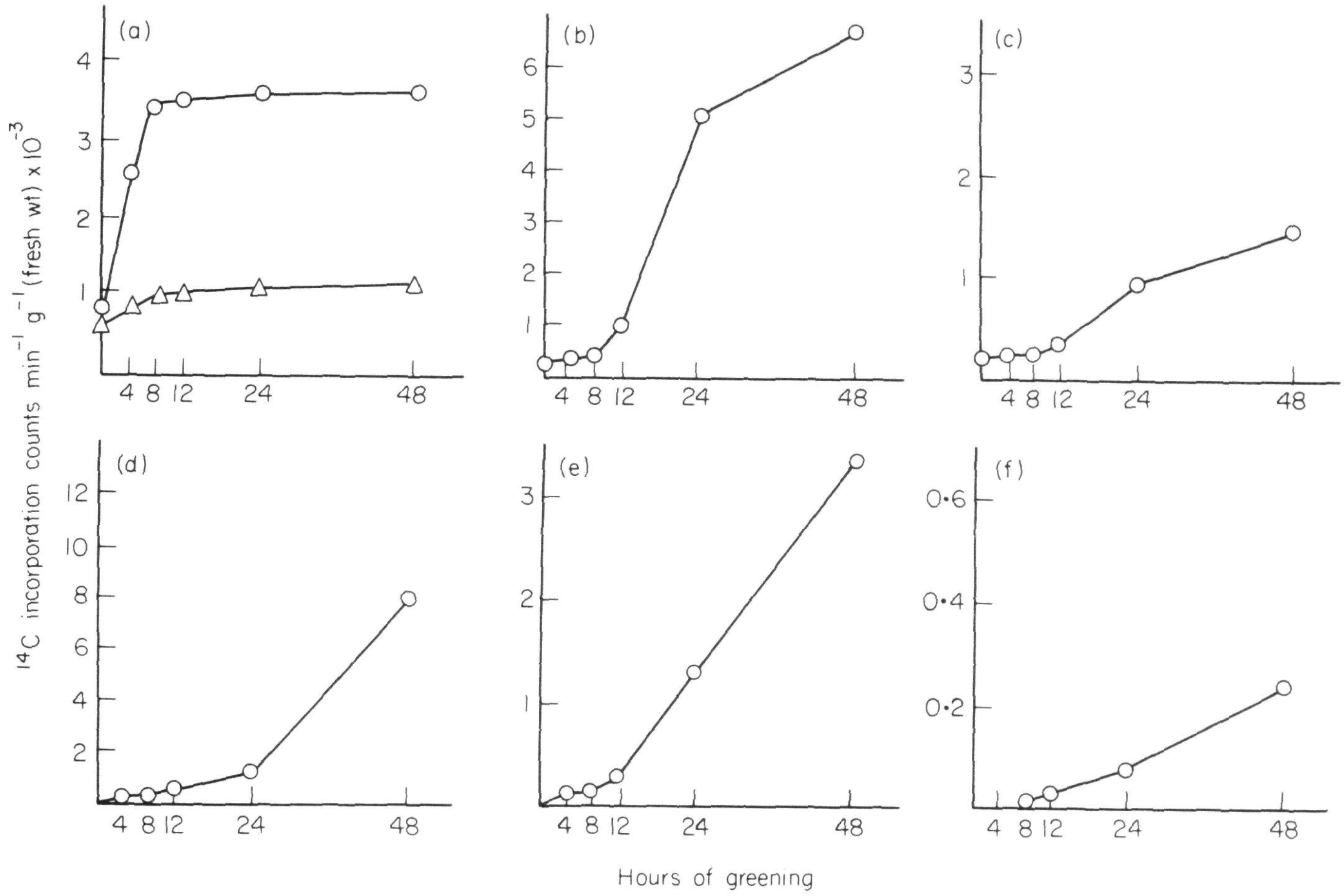

Fig. 1. Changes in photosynthetic carbon labelling pattern of various compounds during greening of rice seedlings. Seven day-old etiolated seedlings were transferred to light at zero time. (a) ○, Malate; △, aspartate; (b) PGA; (c) alanine; (d) sucrose; (e) sugar phosphates; (f) insolubles.

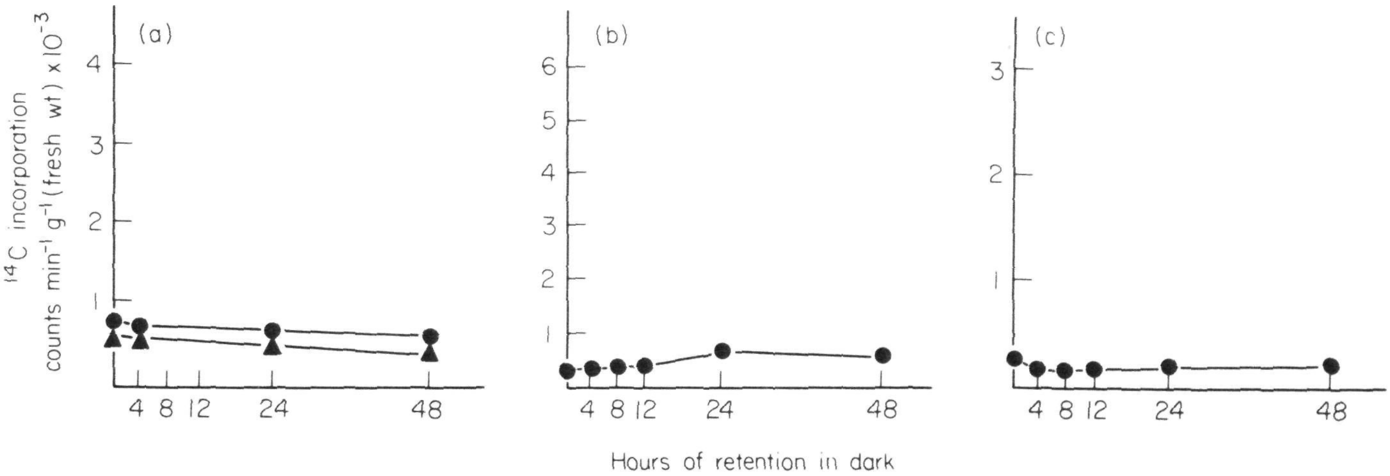

Fig. 2. Photosynthetic carbon labelling pattern by rice seedlings retained in darkness. Seven day-old etiolated seedlings were retained in darkness from zero time. (Contrast with Fig. 1). (a) ●, Malate; ▲, aspartate; (b) PGA; (c) alanine.

Table 1. *The pattern of carbon fixation in darkness by etiolated rice seedlings*

Compound	^{14}C incorporation counts min^{-1} g^{-1} (fresh weight) zero time	4 h after greening
Malate	86	225
Aspartate	43	84
PGA	8	16
Others (succinate, citrate, glutamate and alanine)	20	12

Total ^{14}C fixed during darkness was less than 1% of that fixed in light.

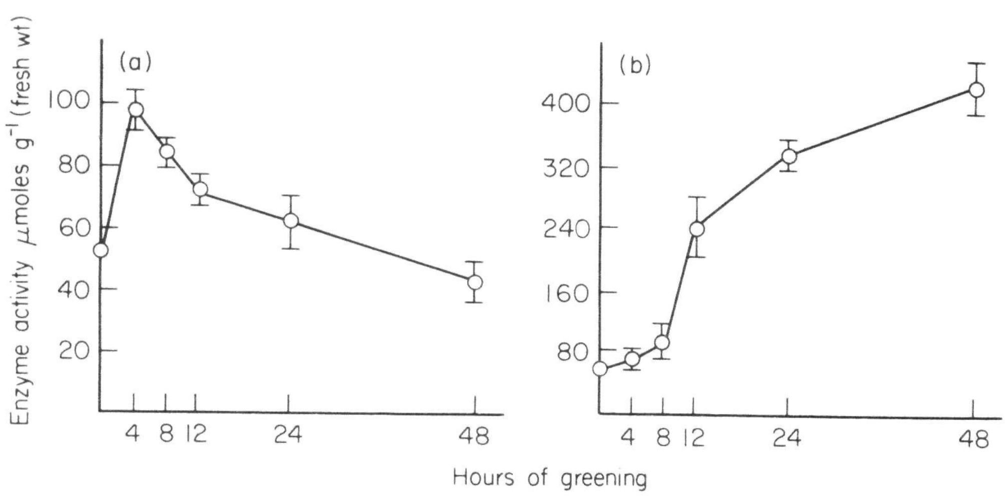

Fig. 3. Levels of carboxylation enzymes in etiolated rice seedlings on illumination. Seven day-old etiolated seedlings were transferred to light at zero time. The vertical lines represent the standard errors. (a) PEP carboxylase; (b) RUDP carboxylase.

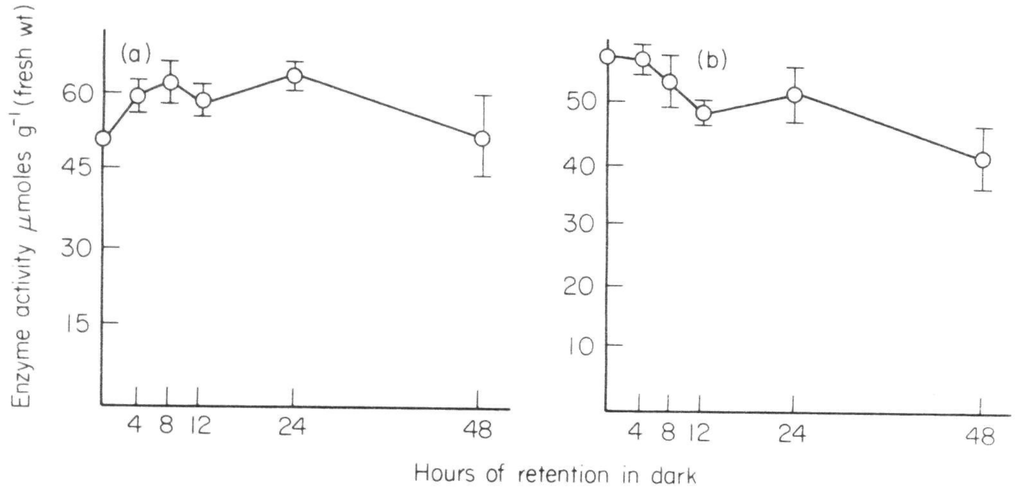

Fig. 4. Levels of carboxylation enzymes in etiolated rice seedlings when retained in darkness. Seven day-old etiolated seedlings were retained in darkness from zero time. The vertical lines represent the standard errors. (Contrast with Fig. 3). (a) PEP carboxylase; (b) RUDP carboxylase.

The activities of carboxylating enzymes were parallel to the carbon fixation pattern. PEP carboxylase activity showed a sharp rise after four hours of illumination and then decreased (Fig. 3). RuDP carboxylase was marked by a continuous increase after an initial lag period. When the seedlings were maintained in darkness there was little change in the levels of these enzymes except for a slight fall (Fig. 4).

Greening seedlings accumulated chlorophyll with time (Fig. 5). However the chlorophyll *a*/chlorophyll *b* ratio, after an initial increase decreased steadily.

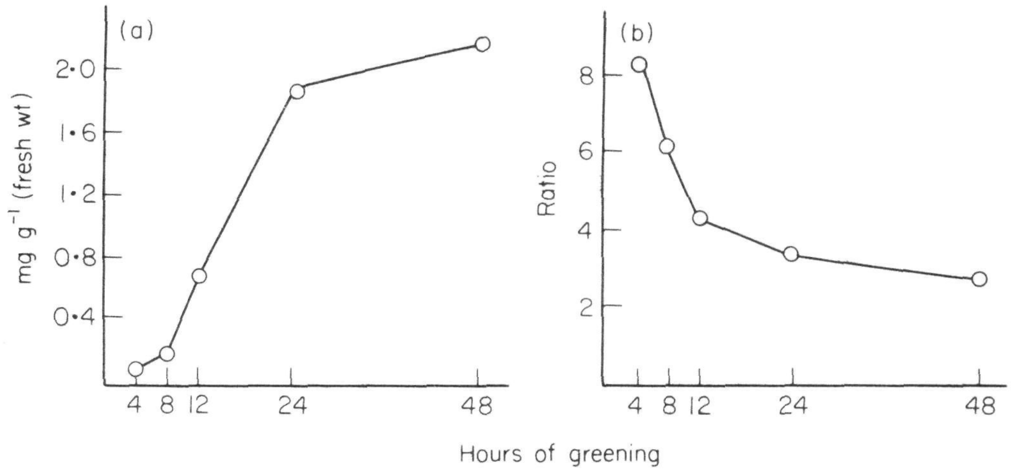

Fig. 5. Chlorophyll content (a) and chlorophyll *a*/*b* (b) ratio of etiolated rice seedlings during illumination. Seven day-old etiolated seedlings were transferred to light at zero time.

DISCUSSION

The increased formation of the C_4-acids, malate and aspartate, indicated a stimulation of β-carboxylation in rice seedlings during the early stages of greening. Increase in C_4-acid formation was noticed during the greening of etiolated barley seedlings (Tamas, Yemm and Bidwell, 1970). A number of enzymes associated with the Calvin cycle in seedlings show increased activity after light exposure (Margulies, 1964; Huffaker *et al*., 1966). Hall *et al.* (1959) reported that the enzymes of β-carboxylation did not increase on illumination in barley leaves. But Tamas *et al.* (1970) felt that the enhanced C_4-acid labelling in barley was definitely due to light stimulation of β-carboxylation. We also believe that a light stimulated β-carboxylation mechanism in rice gives rise to the increased synthesis of C_4-acids. The absence of any remarkable change in labelling pattern when seedlings were retained in darkness (Fig. 2) supports this view.

The high ratio of chlorophyll *a* to chlorophyll *b* of rice seedlings immediately after illumination, was followed by a decrease. This observation together with the chlorophyll accumulation with time, suggests that chlorophyll *a* synthesis precedes chlorophyll *b* formation and leads subsequently to a steady accumulation of total chlorophyll. Earlier investigations have also revealed a similar trend in chlorophyll synthesis (Argyroudi-Akoyunoglou and Akoyunoglou, 1970; DeGreef, Butler and Roth, 1971).

The light stimulation of C_4-acid formation persisted for only about 8 h. Such a short time reflects the relatively insignificant role of β-carboxylation in the life of a C_3-plant such as rice. On the other hand labelling of Calvin cycle intermediates, though initiated only after 12 h, continued to show a steady steep increase. Thus, there was a successive activation of two distinct components of photosynthetic carbon fixation on illumination: an immediate but short-lived stimulation of β-carboxylation and a more permanent Calvin cycle which developed after a lag period.

The C_4-pathway is generally considered to be a more advanced character than the C_3-pathway (Black, 1971). But the ontogenic development of β-carboxylation earlier than the Calvin cycle raises the question whether the reverse could be true. It is interesting that comparative photosynthetic studies with the leaves of C_3 plants (tobacco and *Cryptomeria*) of different age indicates that young leaves behave like C_4-types whereas older ones are typically C_3-types (Sugiwara and Fujiwara, 1969; Kisaki, Hirabayashi and Tano, 1973).

ACKNOWLEDGMENTS

This investigation was supported by an U.G.C. Junior Research Fellowship to A.S.R.

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