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EFFECT OF AGE ON THE FIXATION OF $^{14}CO_2$ IN SUGARS, ORGANIC ACIDS AND AMINO ACIDS OF BEAN LEAVES

A. RAAFAT* and W. HÖFNER

Justus Liebig-Universität, Giessen, Hesse, Germany

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Abstract-Fixation of $^{14}CO_2$ in sugars, organic acids and amino acids of primary bean leaves varying in age was determined after 12 hr photosynthesis. The period of leaf expansion was characterized by nearly equal amounts of activity in both glucose and fructose. Thereafter, fructose had relatively higher values than glucose at full expansion and remained so during the chlorophyll deteriorative phases towards senescence. The activity in a-ketoglutarate, succinate and fumarate increased gradually till the full expansion stage then decreased with advancing age. On the other hand, no substantial increase in ^{14}C fixation in both citric and malic acids was noticed by the full expansion stage. The drop in ^{14}C fixation in the amino acids as the primary leaves attained their full growth mainly resulted from a drop in labeling of nearly all the amino acids studied with the exception of r-amino butyric acid, proline, alanine and serine, all of which recorded an increase. On the appearance of the first visible signs of senescence an increased activity was found in glutamine, asparagine, arginine, glutamic acid, serine and glycine which suggests a sudden direction of the nitrogen metabolism in the leaf towards forming the important translocatory as well as the temporary storage forms of nitrogen by this stage. Results showed the importance of the amino acid synthesis at the early stages of growth for the development of maximum photosynthetic capacity which occurs in a subsequent stage. The changes in $^{14}CO_2$ fixation in the amino acids of primary bean leaves of different age appeared to be more characterized to the specific amino acids themselves as affected by age rather than due to a general effect of age as in the case of sugars and organic acids.

Zusammenfassung-In verschieden alten Primärblättern von Bohnen wurde der $^{14}CO_2$-Einbau in Zucker, organische Säuren und Aminosäuren nach 12 Std Photosynthese untersucht. Die Periode der Blattentwicklung war gekennzeichnet durch annähernd gleiche Radioaktivität sowohl in Glukose als auch in Fruktose. Später während der vollen Blattentfaltung erreichte die Fruktose gegeniiber der Glukose relativ höhere Werte, die sich auch während der Abbauphase des Chlorophylls mit beginnender Seneszenz nicht veränderten. Die ^{14}C-Aktivität in α-Ketoglutarsäure, Bernsteinslure und Fumarsäure nahm bis zum Stadium der Vollentwicklung allmählich zu, urn dann mit fortschreitendem Alter kontinuierlich abzunehmen. Dagegen konnte in Citronen- und Apfelsäure keine gesicherte Erhöhung des ^{14}C-Einbaus während des Stadiums der vollen Blattentwicklung festgestellt werden. Die Abnahme des ^{14}C-Einbaus in Aminosäuren nachdem die Primärblätter ihre Hauptwachstumsphase erreicht hatten, war vorwiegend durch eine geringere ^{14}C-Markierung fast aller untersuchten Aminosluren bedingt. Eine Ausnahme bildeten γ-Amino-Buttersäure, Prolin, Alanin und Serin, die zu diesem Zeitpunkt eine Zunahme der ^{14}C-Aktivität zeigten. Mit dem Auftreten der ersten sichtbaren Zeichen der Seneszenz ließ sich eine erhöhte ^{14}C-Aktivität in Glutamin, Asparagin, Arginin, Glutaminslure, Serin und Glycin nachweisen, was eine verstärkte Ausrichtung des Stickstoffmetabolismus des Blattes auf Bildung von Transportformen und kurzlebigen Vorratsformen des Stickstoffs in diesem Entwicklungsstadium vermuten läßt. Die Ergebnisse zeigten die Bedeutung der Aminosäuresynthese in den frühen Wachstumsstadien für die Ausbildung der maximalen photosynthetischen Kapazitlt in den folgenden Stadien. Die Änderungen der $^{14}CO_2$-Fixierung in Aminosäuren von primären Bohnenblttern verschiedenen Alters scheint mehr vom Einfluß des Alters auf spezifische Aminosluren als durch einen allgemeinen Alterungseffekt wie im Falle der Zucker und organischen Säuren bedingt zu sein.

INTRODUCTION

AGEING is undoubtedly a varied process.[1] The deteriorative processes of ageing gradually decrease the functional capacity of the system and lead in most cases to the irreversible changes of senescence.

* Permanent address: Department of Botany, Faculty of Agriculture, Ain Shams University, Cairo U.A.R.

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Considering foliar senescence, emphasis has been placed on the photosynthetic apparatus because it shows the earliest physiological and biochemical changes during senescence and is of course the dominant metabolic system in the leaf.[2] However, there have been only few reports on the variation in the distribution of carbon among the products of photosynthesis as affected by leaf age.

Ongun and Stocking,[3] found that after 15 min of photosynthesis in $^{14}CO_2$ the soluble amino acids of tobacco leaf had most activity in serine, glycine, aspartic acid and alanine. A higher percentage of ^{14}C was found in the soluble amino acids and the proteins in young leaves compared to (fully expanded) older ones. The data of fixation of radiocarbon obtained by Carr and Pate[4] suggested that in very young field pea leaves a relatively large proportion of the primary photosynthetic products are used for the synthesis of protein and other insoluble materials. Later in the life of the leaf, the photosynthetic process showed specialization towards the production of water-soluble substances, some 90 per cent of this activity being recovered in the single compound, sucrose. In the case of amino acids, the same authors pointed out that carbon dioxide was fixed largely into serine, glycine, alanine and other compounds not translocated in quantity from the roots. This seemed to be a fairly stable feature of the photosynthetic apparatus, there being no significant changes in the percentage distribution of radiocarbon between these amino acids throughout the photosynthetic life of the leaf. A similar set of amino acids was found to feature prominently in photosynthesis of other plants.[5-7] This communication deals with the changes in $^{14}CO_2$ fixation products during photosynthesis as affected by leaf age, starting with young expanding stage up to the approach of senescence. Knowledge of these changes should shed more light upon the nature of the biochemical processes accompanying ageing.

RESULTS AND DISCUSSION

Activity of the Different Fractions

The distribution of ^{14}C activity in the different fractions of the primary leaves varying in age after 12 hr photosynthesis is illustrated in Fig. 1. Except for the youngest plants (age 9 days) the main activity was located in the sugar fraction. On the other hand, the organic acid and amino acid fractions had relatively low values. The highest ^{14}C fixation in the sugar and insoluble fractions was observed in the 21-day age (full expansion stage). Both the 14- and 21-day ages were characterized by the highest fixation in the organic acid fraction. In the case of amino acid fraction, maximum ^{14}C fixation occurred at the 14-day age, 1 week earlier than that of sugars.

These results suggest that shifting to different metabolic cycles can occur at an early stage of leaf growth before full maturity is attained.[8] In this regard Osborne[9] also has stated that, during growth and differentiation, the many biochemical processes that occur in the leaf reach maximal activity at different stages of development.

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[3] A. ONGUN and C. R. STOCKING, *Plant Physiol. 40,819* (1965a).
[4] D. J. CARR and J. S. PATE, *Symp. Soc. Exptl. Biol.* 21, 559 (1967).
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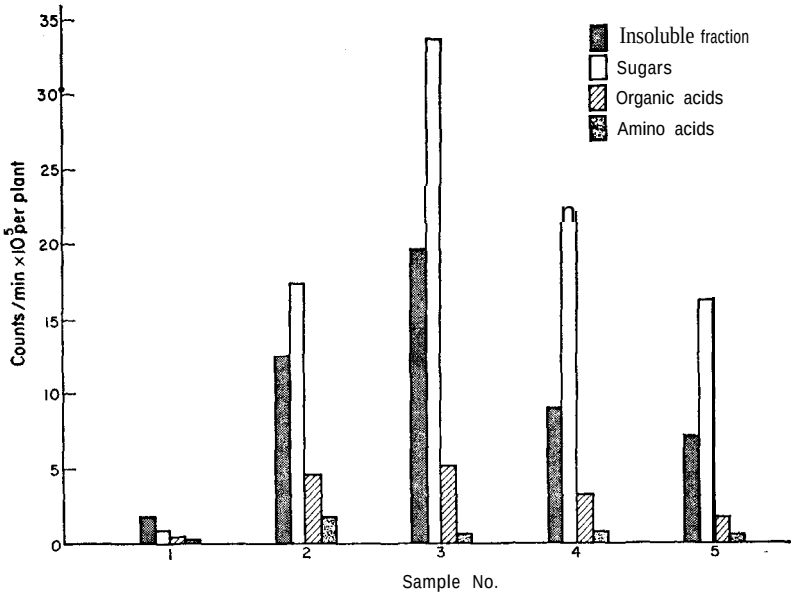

FIG. 1. DISTRIBUTION OF ^{14}C RADIOACTIVITY IN THE INSOLUBLE FRACTION, SUGARS, ORGANIC ACIDS AND AMINO ACIDS OFPRIMARY BEANLEAVES OF VARYING AGE AFTER 12 hr PHOTOSYNTHESIS.

Labeling of Sugars after 12 *hr Photosynthesis*

The period of leaf expansion was characterized by nearly equal amounts of activity in both glucose and fructose (Fig. 2). The activity in these sugars appeared to increase rapidly accompanying the increase in leaf area and total chlorophyll content (Table 1). After the

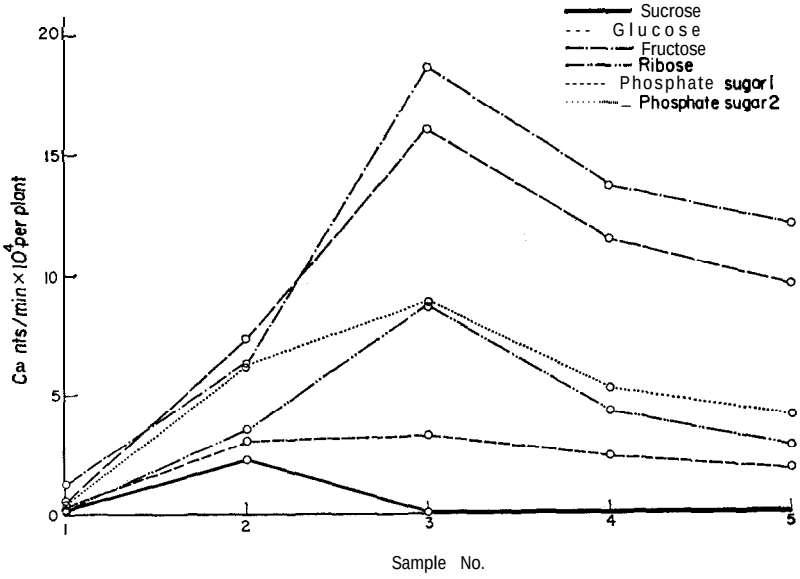

FIG. 2. DISTRIBUTION OF ^{14}C ACTIVITY IN THE SUGARS OF PRIMARY BEAN LEAVES OF DIFFERENT AGE AFTER 12 hr PHOTOSYNTHESIS.

TABLE 1. GROWTH AND CHLOROPHYLL CONTENT OF PRIMARY BEAN LEAVES (PER PLANT) AS AFFECTED BY AGE

Sample no.	Days after sowing	Fr. wt. (g)	leaf area (cm^2)	Chlorophylls	
				ug/cm^2	mg/leaves
1	9	0·10	7·9	57.1	0·45
2	14	0·69	45.5	63.6	2·89
3	21	1·22	77·3	52.6	4·07
4	28	1.27	79.2	40·9	3.24
5	35	1·29	81.4	33·7	2.74

14-day stage most of the activity was located in the two sugars. Fructose was relatively more active than glucose at the full expansion stage and remained so as chlorophyll was lost towards senescence. This may point to an important role of fructose in the carbohydrate metabolism.[10] On the other hand, incorporation of ^{14}C into sucrose was at its maximum at the 14-day stage, then decreased to negligible values thereafter. The activity in ribose showed nearly a similar trend to those of hexoses. These results may indicate that as the primary bean leaf ages, there is an increased tendency for ^{14}C to be fixed into hexose sugars. If we consider translocation in the bean plant as light independent,[11] we may relate the high activity incorporated into glucose and fructose with the main type of sugars translocated. In this regard, other investigations concluding that glucose and fructose are more important than sucrose as translocatory compounds have already been reviewed by Leonard.[12]

Coupled with the foregoing results, it can be clearly seen that the decreasing capability of primary bean leaf for CO, fixation in sugars noticed after the leaf attained full growth is of a quantitative rather than qualitative nature. Similar conclusions have already been drawn with the soluble proteins of excised barley leaves during senescence.[13]

Incorporation of ^{14}C into Organic Acids

The fixation of ^{14}C in the organic acids of primary leaves as affected by age is illustrated in Fig. 3. The highest values of activity appeared in glucoronic and gluconic acids. The activity in these acids as the leaves aged showed a similar trend to that obtained with hexose sugars. The increasing activity in these acids during leaf expansion can be correlated with the synthesis of non-cellulosic polysaccharides needed for the building up of cell material during this active period of growth. Immediately after full growth was attained, the capability of the primary leaves to incorporate photosynthetic CO_2 into these acids decreased suddenly and reached a relatively low value by the start of visible senescence.

Changes in activity of the organic acids of the Krebs cycle analysed in this study (α-ketoglutaric, succinic and fumaric acids) showed, more or less, one characteristic trend; increasing gradually till full expansion then decreasing continuously with advancing age. In this connection, Geronimo and Beevers,[14] found that the values of QO_2 of intact pea

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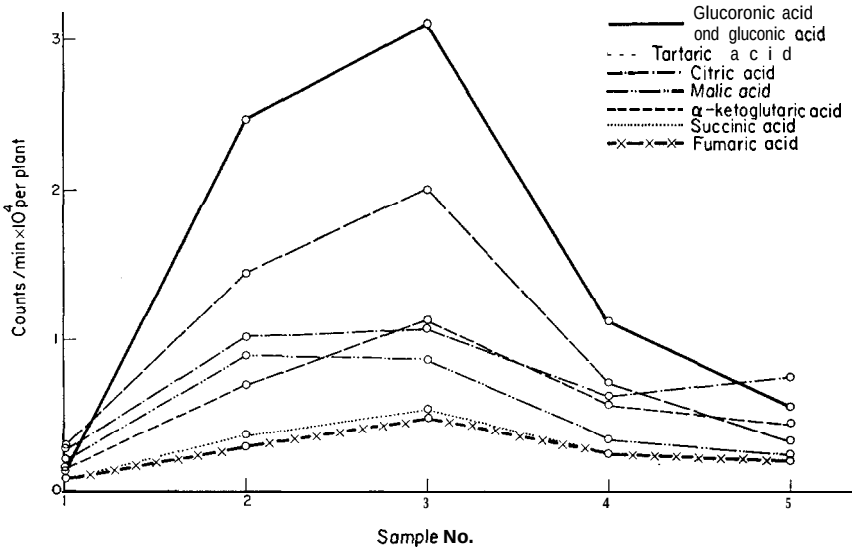

FIG. 3. DISTRIBUTION OF ^{14}C ACTIVITY IN THE ORGANIC ACIDS OF PRIMARY BEAN LEAVES OF DIFFERENT AGE AFTER 12 hr PHOTOSYNTHESIS.

leaves decreased 40-60 per cent with increasing age, and at the same time the oxidation of 7 Krebs cycle intermediates by mitochondrial preparation from these leaves decreased 60-100 per cent. Varner[15] also indicated that the cellular respiratory capacity decreases during senescence which is probably caused by gradual loss of mitochondrial function with the loss of phosphorylative activity preceding that of electron transport capacity.

The above results may also indicate that respiration is somewhat closely correlated with photosynthetic activity in the ageing primary bean leaves. Such a conclusion is in accord with Larson and Gordon,[16] who obtained with *Populus* leaves a similar trend concerning net photosynthesis and dark respiration. Moreover, Smillie[17] showed that as the assimilative powers deteriorate in pea primary leaves so also does the respiratory ability.

Neither citric and malic acids showed any substantial increase in ^{14}C fixation by the full expansion stage, similar to that observed with the other carboxylic acids. Here, we have to recall that although most of the amino acids showed a severe drop in ^{14}C incorporation in the latter stage, aspartic acid and asparagine seemed to retain a relatively high level of activity. The latter observation may throw some light on the behaviour of the two above mentioned organic acids. In this connection, results of $^{14}CO_2$ studies have already emphasized the important relationships between carboxylic acids and amino acids.[18]

Incorporation of ^{14}C into amino acids

The relative amounts of activity which appeared in the amino acids of ageing primary leaves are given in Table 2. The youngest stage (g-day-old seedling) was characterized by a

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[16] P. R. LARSON and J. C. GORDON, *Am. J. Bot. 56, 1058 (1969).*
[17] R. M. SMILLIE, *Plant Physiol.* 37, 716 (1962).
[18] H. BEEVERS, M. L. STILLER and V. S. BUTT, in *Plant Physiology A Treatise* (edited by F. C. STEWARD), Vol. IV B, p. 119. Academic Press, New York (1966).

TABLE 2. DISTRIBUTION OF ^{14}C RADIOACTIVITY IN THE AMINO ACIDS OF PRIMARY LEAVES OF BEAN PLANTS VARYINGIN AGE

Amino acid	Activity counts/min per plant				
	9-day age	14-day age	21-day age	28-day age	35-day age
Glutamic acid	—	603	tr.*	176	565
Glutamine	—	862	2200	tr.	161
NH,-butyric acid	133	1607		342	188
Proline	83	751	852	240	tr.
Arginine	—	6580	1589	256	573
Aspartic acid	199	3511	1840	416	398
Asparagine	—	4130	2553	372	592
Methionine sulphoxide	—	1013	tr.	tr.	tr.
Lysine	—	398	—	—	—
Serine	67	502	610	104	188
Glycine	tr.*	151	102	40	113
Cysteine	—	125			—
Alanine	65	1500	1862	424	257

* tr. = traces.

relatively small amount of fixed $^{14}CO_2$ in alanine, aspartic acid, serine, glycine, proline and y-amino butyric acid after 12 hr photosynthesis. On the other hand, no activity was present in glutamine, glutamic acid, asparagine and arginine in spite of their presence as detected by the ninhydrin reaction (Fig. 4). During the course of leaf expansion, and coinciding with the maximum ^{14}C incorporation into the total amino acid fraction noticed at the 14-day age, labeling appeared in nearly all the individual acids with the highest amounts in arginine, asparagine and aspartic acid. The drop noticed in ^{14}C incorporation into the amino acids as the primary leaves attained their full growth may give an indication for a corresponding decrease in the rate of protein synthesis during ageing of the leaf." This drop mainly resulted from a drop in the activity of nearly all the amino acids with the exception of y-amino butyric acid, alanine serine and proline, which recorded an increase. The earlier drop in the content of aspartic acid, methionine sulfoxide and cysteine, was not observed in the case of the other amino acids (Fig. 4). With progressive ageing, the degree of labeling in the different amino acids generally decreased. On the appearance of the first visible signs of senescence, however, increased activity was recorded in glutamine, asparagine, arginine, glutatamic acid, serine and glycine. This behaviour was almost the reverse of that noticed in the youngest juvenile stage and may indicate a sudden direction of the nitrogen metabolism in the leaf towards forming the important translocatory as well as temporary storage forms of nitrogen on the onset of senescence. This can also be seen when the specific activity is calculated for the last stage of growth. The latter conclusion is in accord with those obtained by other investigators. Specht-Jürgensen[20] indicated that the main soluble nitrogen compounds in the yellow leaves of ***Ginkgo biloba*** were amides (especially glutamine), ammonia and valine. Similar results were obtained with excised leaves by Pucher and Vickery[21] and Yemm.[22] Moreover, the results of Simon,[23] indicated that the loss of protein from lower

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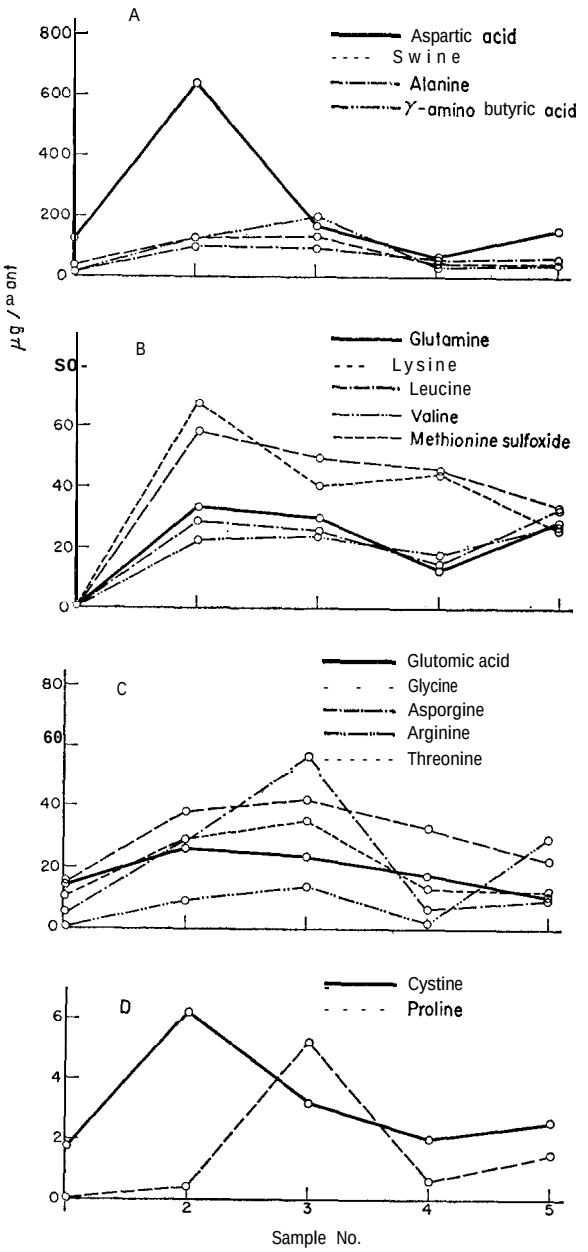

FIG. 4. THE CONTENTOF AMINO ACIDS IN THE PRIMARY BEAN LEAVES AS AFFECTED BY AGE.

senescing leaves on the plant could be due to translocation out of the leaf of amino acids and amides to regions of growth and development rather than due to defect of protein synthesis within the leaf. The same author has argued that the senescence of attached leaves is due to the translocation of metabolites, especially amino acids, to the growing parts of the plant, so that the leaves can no longer synthesize proteins.

Similar results concerning the behaviour of the non protein amino acid y-aminobutyric acid, obtained in this study, were reported by Noguchi *et al.*[24] with tobacco leaves varying in age.

The above results clearly show that $^{14}CO_2$ fixation in nearly all the amino acids was at its maximum while the leaf was still expanding (16day-age). Thereafter, ^{14}C fixation dropped corresponding to a similar though less sharp drop in the total amino acid content at the full expansion stage where maximum photosynthetic activity was attained. This might indicate the importance of amino acid synthesis at the early stages of growth for the development of the maximum photosynthetic capacity which occurs in a subsequent stage.

From the foregoing results it appears also that the changes in $^{14}CO_2$ fixation in amino acids by the photosynthesizing primary bean leaves of different age are more characteristic of the specific amino acids themselves, as affected by age, rather than being due to a general effect of age as in the case of sugars and organic acids. In this regard, Carr and Pate.[4] mentioned that during ageing of the leaf there is an equally interesting and highly reproducible sequence in the build-up of individual amides and amino acids in the soluble phase of the leaf. Maximum concentrations are attained in a definite order, with most of the events in the sequence having been passed several weeks before the leaf becomes noticeably senescent. Moreover, Steward *et al.*[25] outlined that some amino acids are present in several pools which may be turning over at different rates and not equally involved in protein synthesis.

Moreover it should be noted that the maximum ^{14}C activity in serine coincided with the maximum ^{14}C-fixation in sugars, which may indicate a light dependent sequence between serine and carbohydrates.[26]

EXPERIMENTAL

In order to obtain a series of primary bean leaves of different ages for exposing to $^{14}CO_2$ at the same time, five sowing dates were chosen. The seeds (*Phaseolus vulgaris* L. c.v. Schreiber's Grandimmuna) were sown in sand culture using Hoagland solution and the growth was maintained under controlled conditions at 20° and 5000 lx for 12-hr daily. Experimental seedlings were 9, 14, 21, 28 and 35 days old. The primary leaves were still expanding at the first two stages, fully expanded at the third stage, and showed the first visible symptoms of senescence (yellowing) by the last stage. Exposure of the plants to $^{14}CO_2$ was done in controlled-environment chamber for 12 hr. Details of the procedure, preparation of the samples for analyses, extraction of photosynthates and separation of the products on ion exchange resins and by paper chromatography are described elsewhere.* For determining the activity of the individual sugars and organic acids, the spots were burnt and the $^{14}CO_2$ evolved absorbed and measured using Packard Tri-Carb liquid scintillation spectrometer." In the case of amino acids the total content was estimated colorimetrically after elution of the ninhydrin spots.[28] The activity in the individual spots was located by autoradiography on parallel chromatograms and counted as well, using a Berthold-DC-Scanner with peak integrator. All the activity measurements were made in duplicate from parallel chromatograms and values representing an average from 10

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plants were expressed as counts/min/plant. For chlorophyll determination, the frozen primary leaves were extracted by 80 per cent acetone[29] and the absorptivity of the extracts measured at 663 and 645 nm.[30]

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