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C_4 PHOTOSYNTHESIS IN *Euphorbia degeneri* AND *E. remyi*: A Comparison of Photosynthetic Carbon Metabolism in Leaves, Callus Cultures and Regenerated Plants

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(Ph.D. Thesis)

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C_4 Photosynthesis in *Euphorbia degeneri* and *E. remyi*
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Abstract

Based on analysis of $^{14}CO_2$ fixation kinetics and assays of enzymes related to C_4 metabolism (NAD-ME, NADP-ME, NAD-MDH, NADP-MDH, AST,ALT), leaves and regenerated plants of *Euphorbia degeneri* exhibit a modified NADP-ME-type photosynthesis. Apparently, both aspartate and malate are used for transport of CO_2 to bundle sheath cells. Callus grown on either non-shoot-forming or shoot-forming media fixes CO_2 into RPP-cycle intermediates and sucrose, as well as malate and aspartate. $^{14}CO_2$ pulse/chase kinetics show no significant loss of label from C_4 acids throughout a one minute chase. Analysis of PEPCase revealed the presence of 2 isoenzymes in both leaf and regenerated plant tissues (K_m [PEP]=0.080 and 0.550) but only one isoenzyme in callus (K_m=0.100). It appears that C_4 photosynthesis does not occur in callus derived from this C_4 dicot but is regenerated concomitant with shoot regeneration, and β-carboxylation of PEP in callus, mediated by the low K_m isoenzyme of PEPCase, produces C_4 acids that are not involved in the CO_2 shuttle mechanism characteristic of C_4 photosynthesis.

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C_4 PHOTOSYNTHESIS IN *EUPHORBIA DEGENERI* AND *E. REMYI*.

A COMPARISON OF PHOTOSYNTHETIC CARBON METABOLISM IN LEAVES, CALLUS CULTURES AND REGENERATED PLANTS.

INTRODUCTION

The goal of this research was to study the C_4 pathway of photosynthesis in the Hawaiian C_4 dicotyledonous plants Euphorbia degeneri and E. remyi. Callus cultures capable of regenerating shoots were used to study aspects of photosynthesis in tissues with no mesophyll/bundle sheath cell organization, but that were derived from a C_4 plant. Results of experiments that used mature leaf tissue were compared to those that used callus tissue and young regenerated plantlets to develop a better understanding of the intercellular relationships between the different leaf cell types in the role of C_4 photosynthesis.

C_4 photosynthesis is characterized by specific $^{14}CO_2$ fixation kinetics (Hatch, 1971). Incubating leaves with radiolabelled carbon dioxide results first in the labelling of four carbon acids (malate and aspartate) followed later by the labelling of reductive pentose phosphate (RPP)-cycle intermediates. With very short periods of incubation in $^{14}CO_2$, C_4 acids contain most (>50%) of the ^{14}C-label. Pulse-chase experiments designed so that a short pulse of $^{14}CO_2$ is followed by a long period of $^{12}CO_2$ incubation shows the following. In sugar cane (Hatch and Slack, 1966), malate loses label rapidly during the chase (much more rapidly than aspartate) and is followed by an increase in label in 3-phosphoglycerate (PGA) and RPP-cycle intermediates. The significance of these results is that PGA label increases only after label in C_4 acids diminishes, and then concomitant with the increase of label in sucrose and starch. The

results demonstrate that labelled carbon (in maize) moves from CO_2 to malate to PGA to sucrose and starch.

In other C_4 plants however (Gutierrrez et al, 1974), the predominant C_4-acid is aspartate (ASP), and the loss of label from ASP during a $^{12}CO_2$ 'chase' is equivalent to that of malate in maize. Both C_4 acids are formed in all C_4 plants, but in different species one C_4-acid usually predominates (Hatch, 1971; Gutierrez et al, 1974). It is not clear whether the predominance of one C_4-acid is fixed or whether environmental conditions could cause a switch from one C_4 acid to the other.

Another characteristic of C_4 photosynthesis is the spatial segregation of the two carboxylating reactions. The primary carboxylation step occurs in the mesophyll cells (MC) to form malate and aspartate (through oxaloacetate, OAA) from phosphoenolpyruvate (PEP). The generated four carbon acid then diffuses to the bundle sheath cells (BSC) where it is decarboxylated to regenerate CO_2 via one or more of at least three different pathways (for review see Edwards and Walker, 1983). The CO_2 then enters the RPP-cycle to be reduced to carbohydrate, and pyruvate or alanine diffuses back into the mesophyll cells to complete the cycle (Hatch, 1971; Hatch, 1979). Pyruvate re-enters the C_4 acid cycle in the mesophyll cells of the leaf by phosphorylation to form PEP.

A salient feature of C_4 photosynthesis is the presence of two biochemically and morphologically distinct leaf cell types, ie., mesophyll- and bundle sheath cells, or so-called 'Kranz anatomy'. An intriguing question in the study of C_4 photosynthesis is whether Kranz anatomy is necessary or if the C_4 photosynthetic pathway can exist in

its absence.

C_3/C_4 intermediate species may provide evidence for the relationship between Kranz anatomy and C_4 photosynthesis. In species of Panicum reputed to be C_3/C_4 intermediates (ie. P. miliodies), a gradation exists (based on chloroplast ultrastructure) that corresponds to the degree of C_3/C_4 intermediacy between those species exhibiting Kranz anatomy and others that have only a weakly developed bundle sheath (Brown et al., 1983). Rathnam and Chollet (1979) suggesed that the intermediacy of photosynthetic metabolism in P. miliodies was due to the RPP-cycle being present in both leaf cell types (MC and BSC) rather than only in the BSC. At least in the intermediate species of Panicum then, there seems to be a correlation between the degree of anatomical differentiation and the expression of C_4 photosynthesis, but that Kranz anatomy alone does not necessarily imply that C_4 photosynthesis is operating.

A similiar situation exists in hybrids between Atriplex patula ssp. hastata (C_3) and A. rosea (C_4). In this example, Kranz anatomy is present but radiotracer kinetics show that C_4 photosynthesis is not functional (Bjorkman et al., 1971). Thus, in this hybrid as well as P. miliodies, it seems that the presence of a Kranz complex as well as certain biochemical pathways are essential for a completely functional C_4 photosynthetic pathway.

Plant development provides other evidence for the direct relationship between Kranz anatomy and C_4 photosynthesis. For example, maize seedlings initially exhibit only C_3 photosynthesis (leaves 1-3) but as new leaves are produced they gradually shift toward the C_4 pattern up to and beyond day 7 (Delèens and Brulfert,

1983). Crespo et al. (1979) demonstrated that all leaves of maize seedlings had kranz anatomy, but that bundle sheath chloroplasts of leaf 1 exhibited ultrastructural characteristics that tended towards a C_3-type. In these and other experiments (Crespo et al., 1979), functional C_4 photosynthesis was related to the expression in the leaves of certain C_4-related enzymes rather than just the presence of Kranz anatomy.

Examples that refute the hypothesis that Kranz anatomy and C_4 photosynthesis are obligately related are the reports that show that undifferentiated callus of the C_4 plants Froelichia gracillus (Laetsch and Kortschak, 1972) and Portulaca oleraceae (Kennedy et al., 1977) may exhibit C_4 photosynthesis. The data, however, may be subject to a different interpretation since the callus $^{14}CO_2$ labelling kinetics are not strictly equivalent to kinetics obtained with intact C_4 leaves. Kennedy et al. (1977) demonstrated an early labelling of malate in P. oleraceae callus, however, the accumulation of malate in callus cultures, and even the loss of label from the total malate (or C_4 acid pool) is not necessarily indicative of a C_4 photosynthetic system. For example, malate is the first and predominantly labelled compound in shoot-forming and non-shoot-forming callus cultures of the C_3 plant Nicotiana tabacum (Plumb-Dhindsa et al, 1979). Other biochemical criteria, such as rapid loss of label from C_4 acids, expression of a C_4-type PEPC and other C_4 pathway related enzymes (eg. aspartate-, alanine aminotransferase) are essential to definitively demonstrate the existence of a functional C_4 photosynthetic system. To date no research has unequivocally demonstrated the presence of the C_4 photosynthetic pathway in plant tissue cultures.

These data suggest that C_4 photosynthesis is a complex phenomenon that involves a cooperation of intra- and intercellular activities (Hatch and Osmond, 1976). Other experiments are needed, however, to further demonstrate such a relationship.

It is interesting, therefore to study the expression of C_4 photosynthesis as a function of carbon metabolism and enzyme expression over the course of development of Kranz anatomy; not in developing seedlings and unorganized callus tissues, but in a tissue culture system that was capable of regenerating plants from callus. A tissue culture system that is able to regenerate whole plants may provide a unique system to help answer whether the anatomical relationship of bundle sheath and mesophyll cells is necessary for the expression of a complete C_4 photosynthetic system. By following the development of the biochemical pathways concomitant with the formation of anatomical features associated with C_4 photosynthesis, one may be able to draw conclusions regarding the relationship between these two cell types. The interplay between mesophyll and bundle sheath cells during the dedifferentiation that occurs during callus induction in culture and the re-establishment of a BSC/MC organization during organogenesis may be used as a system to study the development of the C_4 photosynthetic pathway.

It was thought that a tissue culture system composed of undifferentiated tissues during one phase of growth and organogenic (shoot forming) tissues during an alternate phase of growth would complement a similiar study using mature, whole leaves. Callus cultures of C_4 plants would not be expected to exhibit C_4 photosynthesis since Kranz anatomy or any discernible mesophyll/bundle

sheath cell organization is lacking. If such a cellular relationship did develop in callus, then the likelihood of C_4 photosynthesis could be increased and could be measured, for example, as heightened PEPC levels or the expression of the C_4-type PEPC (Ting and Osmond, 1973). Conversely, C_4 photosynthesis in unorganized callus would indicate that C_4 photosynthesis is not strictly dependent on Kranz anatomy.

The research in this dissertation is based on the study of two species of Euphorbia (E. degeneri and E. remyi) and on a tissue culture system with cultures derived from leaf and stem explants from E. degeneri. E. degeneri and E. remyi have been reported in the literature as being C_4 species based mainly on leaf anatomy, CO_2 compensation point (Pearcy et al, 1982) and recently on quantum yield values (Ehleringer and Pearcy, 1984). I have investigated the C_4 photosynthetic pathway in whole leaves of both species and callus cultures of Euphorbia degeneri. No tissue culture had previously been done on either species. Photosynthetic carbon metabolism as well as assays of certain photosynthetic enzymes were the major tools of the research. I have demonstrated that the C_4 pathway of photosynthesis is present in both species of Euphorbia studied but not in callus, and that the C_4 pathway is regenerated along with the regeneration of shoots.

The dissertation is divided into three sections. Chapter one describes the culture conditions used to induce callus and subsequent shoot and root regeneration in E. degeneri. Chapter two gives a description of the photosynthetic pathways in Euphorbia degeneri and E. remyi. The discussion of C_4 photosynthesis in these two species sets a baseline for comparison to the tissue culture system. Chapter

three describes the carbon fixation pathways found in cultures and regenerated plants of E. degeneri tissues and gives the conclusions reached regarding the metabolic functions of the carboxylating enzymes found in whole plant and callus.

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CHAPTER ONE

TISSUE CULTURE OF *EUPHORBIA DEGENERI*

INTRODUCTION

Tissue culture, when used as a model system to study aspects of plant metabolism, has proved to be an important technique that augments research on whole plants. Researchers have used tissue or cell culture to study aspects of plant metabolism or development that would otherwise be less easily interpreted when taken as a component of the whole plant. Examples of such an approach include the study of the role of plant growth regulators on development of tissues in culture (Aloni, 1980; Strauss et al., 1981; Watson and Halperin, 1981;) and metabolic changes occurring during organogenesis (Grady and Bassham, 1983). A tissue culture system can also be used to study such metabolic processes as the production of secondary plant products (Misawa and Suzuki, 1982; Shimazaki and Ashihara, 1982; Biesboer and Mahlberg, 1979; Tideman and Hawker, 1982; Vakkari, 1980), and cell wall biosynthesis (Asamizu and Nishi, 1979; for review see Barz and Ellis, 1981). In this investigation, tissue culture was used as a means to study the C_4 pathway of photosynthesis in Euphorbia degeneri during the differentiation of plants from unorganized callus. Photosynthetic carbon metabolism was investigated in whole plant, callus and regenerated plants.

In some aspects of the study of metabolism it is desirable to study the organism over a series of developmental stages. A plant tissue culture system, under some conditions, may offer an opportunity to follow the expression of a certain metabolic

pathway from the dedifferentiation stages of culture initiation through callus maintenance and into the redifferentiation stages of organ regeneration. For example, a tissue culture system that is capable of regenerating plants would allow the researcher to study regulation and expression of photosynthetic enzymes in tissues that have no normal leaf anatomy but that may be photosynthetic (ie. green callus). Developmental studies that use young leaves to investigate certain pathways of photosynthesis (Hayakawa et al., 1981; Goatly et al., 1975) do not have the advantage of being able to investigate unorganized tissues. As plants regenerate from unorganized callus, the normal leaf anatomy is reformed and any changes in photosynthetic metabolism that may ensue can be correlated with the developing anatomy. A study like this is not possible with normally developing embryos since embryos are not easily accessible and are (usually) not photosynthetic. Plants regenerated from callus have the advantage of being both readily accessible and photosynthetic. A study of photosynthetic metabolism as it may change during plant development, then, is only practical using a tissue culture based system.

I have developed a tissue culture system using the C_4 Hawaiian dicotyledenous plant Euphorbia degeneri to study the development of the C_4 pathway of photosynthesis. Under the appropriate culture conditions callus cultures can be initiated and either maintained as callus or be induced to form shoots and roots. In this investigation I was interested in studying the expression of photosynthetic carbon metabolism in callus as the

tissue regenerated into the "normal" bundle sheath/mesophyll cell anatomical relationship of C_4 plants. This is the first such study that used a tissue cultured C_4 plant to study the development of photosynthetic metabolism during the regeneration of plants.

MATERIALS AND METHODS

Plant Material

Euphorbia degeneri plants (Hawaiian, subgenus Chamaesyce) were obtained from Dr. Robert Pearcy (University of California, Davis). They were maintained in growth chambers set for 12:12 L:D; 27 C; and a light intensity combined from incandescent and fluorescent lights of 600 uE m^{-2} s^{-1}.

Explant Culture

Tissue from the nodes and leaves of young shoots were used as explants for tissue culture. Whole branches (5-10 cm long) were sterilized first by rinsing in 70% EtOH for 30 seconds then by immersion in 10% Clorox/detergent (three drops Tween 40) solution for 15 minutes and followed by three rinses in sterile, deionized water. Leaves and nodes were then cut into small sections and placed on semi-solid medium (either T.C. Agar, KC Biological, Lenexa, KA 66215 8g l^{-1}; or GELRITE, Kelco, San Diego, CA 92123, 2.5g l^{-1}). Cultures were maintained either under continuous light (150 uE m^{-2} s^{-1}) or 12:12 L:D (100 uE m^{-2} s^{-1}). In both environments the temperature varied from 23 C (D) to 27 C (L).

Culture Media

The basic culture medium used was Murashige and Skoog (MS) macro- and micronutrients (Murashige and Skoog, 1962) (Grand Island Biological Co., Santa Clara, CA 95050) supplemented with

th_ plant growth regulators 2,4-D (2,4-dichlorophenoxyacetic acid), 2iP (N^6-[Δ^2-Isopentenyl]adenosine) and Picloram (4-amino 3,5,6-trichloropicolinic acid) (Table 1). Media were designated POIN or P10 dependent on the growth regulator used and the ratio of auxin to cytokinin (Table 1). Shoots regenerated from calli cultured on P10 medium and were then transferred to vermiculite in Magenta GA7 vessels (Magenta Corp. Chicago IL 60641) and watered with half-strength Hoagland's solution until roots formed. Plants were then processed for low humidity acclimation as described below. Other culture media were tried (Table 2) but were found to be unsatisfactory.

Plant Recovery

Rooted plants were transferred to two inch clay pots containing pumice gravel and were maintained on a misting bench at the Botanical Garden of the University of California at Berkeley for one month until new growth of the shoot and roots indicated that the plants had successfully acclimatized. The plants were then transplanted into four or six inch pots and maintained in ambient humidity, but low light, for two to three weeks before being exposed to full growth chamber or glasshouse conditions.

Light Microscopy

Tissues for sectioning were fixed in 10% acrolein overnight at 4 C, dehydrated in methyl cellosolve/ethanol and embedded in glycol methacrylate according to the methods of Feder and O'Brien (1968). Sections were made at 2-5 uM thickness on a Sorval JB-4

microtome and stained with 0.05% Toluidine Blue plus 0.1% BHT for 2-5 minutes. Photomicrographs were made on an Olympus microscope equipped with a Nikon Microflex Model EFM camera.

Chlorophyll and protein determination

Chlorophyll content of leaves and callus was determined in 80% acetone according to the methods of Bruinsma (1963). Protein was determined according to the method of Bradford (1976) using reagents obtained from Bio-Rad (Richmond, CA).

RESULTS AND DISCUSSION

Callus formed from both leaf and node tissues on 5 of the 8 media tested. Except for two isolated events (discussed below) shoots regenerated only from callus originally isolated from node explants and induced and maintained on P10 medium (Table 2). In two node-derived calli lines that were originally isolated on POIN medium, shoots formed after four passages followed by nine passages on P10' (one year total time in culture). Only a few shoots developed, but the fact that there was regeneration demonstrates that in at least these two lines, culture on POIN had no deleterious effect on organogenesis. Callus formed from both leaf and node explants maintained on both POIN and P10 media. Since callus maintained on POIN medium had a growth rate greater than other media tried (data not shown), and callus maintained on P10 regenerated shoots, POIN and P10 media were chosen for all subsequent cultures and experiments.

It is significant that P10 medium contained picloram as the auxin component. Picloram is a powerful synthetic auxin (Muller et al., 1983) that has been used to stimulate organogenesis (shoot formation) in a number of tissue culture systems (Sinha et al., 1983; Phillips and Collins, 1979, 1980). The experiments in this report demonstrate that E. degeneri cultures respond to picloram in a similiar manner. Very low concentrations (on the order of 10^{-8} M) induce shoot formation, whereas concentrations of IAA, NAA or 2,4-D that have been shown to induce shoot

formation in Brassica (Bhattacharya and Sen, 1980), Panicum (Lu et al., 1981) and other cultured plants elicited no organogenic response in E. degeneri callus (Table 2).

Growth of callus as a function of fresh weight verses time in culture shows sigmoidal kinetics. Callus grown on POIN medium gained fresh weight at approximately twice the rate of callus grown on P10 (Fig 1). On the basis of dry weight, callus grown on P10 medium was 78% more dense than callus grown on POIN (Table 3) so that when growth curves for callus are recalculated to be a function of dry weight, POIN and P10 callus are seen to have almost equivalent rates of growth (Fig 2) even though morphology and organogenic potential are much different; POIN medium produced a light green, friable callus and P10 medium produced a dark green, non-friable callus that was capable of regenerating shoots. Picloram therefore had little effect on growth rate but mainly seemed to affect organogenic potential.

Density of P10 grown callus is reflected in the chlorophyll and soluble protein per unit fresh weight values (Table 4). P10 grown callus is very green throughout. Cells that make up POIN callus are smaller than cells of P10 grown callus (cf. Fig 3a,b). POIN callus in section shows little or no tissue differentiation as the cells are uniformly parenchymatous although tracheoids are found scattered throughout the tissue (Fig. 3b,d). P10 callus in section shows differentiated tissues including localized areas of meristematic activity (Fig. 3c).

It has been shown in other tissue culture systems that varying the amount of carbon source and hormones (usually sucrose, auxin and cytokinins) in the medium on which homogenous calli are grown, can elicit differentiation of xylem, phloem and a vascular cambium (Wetmore and Rier, 1963; Yeoman and Aitchison, 1973). When E. degeneri cultures isolated on POIN medium are transferred to P10 medium, the decrease in auxin/cytokinin ratio (POIN: 1:4; P10: 1:40) elicits cellular differentiation in a pattern similiar to that seen by other workers (Halperin, 1969). Figure 3c shows the cellular differentiation that occurs in calli maintained on P10 medium. Numerous meristematic centers ('meristemoids', Fahn, 1982) are found throughout the callus. It is likely that the ratio of auxin to cytokinin is the only factor that causes the tissue differentiation in this case since sucrose and the concentration of salts is the same in both POIN and P10 medium. Cellular differentiation within callus followed by organogenesis is a frequently observed response to decreased auxin concentration (Skoog and Miller, 1957; Halperin, 1969).

Cellular origin of callus

When biochemical or developmental pathways in callus are studied it is important to know from where on the plant body the original explant was taken. Tran Thanh Van et al. (1974) for example, demonstrated in tobacco that cultured epidermal peels regenerated different organs depending on the original location on the plant body of the explant. De Jimenez and Fernandez (1983) demonstrated that differences in glutamine synthase levels

exist between root and leaf derived callus of Bouvardia ternifolia, Constabel et al. (1981) demonstrated that there was a difference in alkaloid types synthesized in Catharanthus roseus cultures derived from individual leaf protoplasts, and Arinson and Boll (1975) showed isozymic differences between different suspension cultures derived from a single bush been seedling. These examples of tissue-derived differences in metabolic or organogenic expression in callus cultures are important to consider if comparisons are to be made between callus and the plant from which it is derived. It is conceivable that genetic or epigenetic potential of cells may be altered during tissue differentiation, and if so, callus derived from only certain cell or tissue types may not express genes representative of the parent plant genome, but rather genes that make up a subset of the whole plant genome which are expressed only in the cell or tissue type or even only in the callus. In such a case, comparison of certain metabolic pathways found in intact plant tissues and derived callus would not be valid. However, if the callus were derived from all cell types of the explant tissue or if callus has the known potential to regenerate organs, than a comparison between the organized and unorganized callus would be possible. To determine the origin of callus in Euphorbia degeneri, I have studied the development of callus from leaf and node explants grown on P0IN medium. Node explants isolated and maintained on P10 medium regenerate whole shoots so no such developmental study was made on P10 grown callus.

The pattern of development of callus from leaf explants is shown in Figure 4. Initially, there is a swelling of mesophyll cells of the leaf (cf. Figs. 4a,b) that is followed by division of all cell types (ordinary epidermal cells, mesophyll cells and bundle sheath cells; Fig. 4c,e). Within several weeks, the original organization of the leaf is obscured by a callus formation (Fig 4c,e) that disrupts the normal leaf morphology by producing localized ulcerations ('U' Fig. 4e). Callus isolated from leaf tissue can be grown and maintained on POIN medium.

Development of callus from stem tissue arises from divisions of cortical and phloem parenchyma cells (Fig. 4d). Maintenance on POIN medium induces cell division in pith parenchyma and outer cortical cells as well, but no division of epidermal cells was seen to occur. Division of cells through the second week on POIN medium produce callus tissue that extends into the cortex as localized areas of cell division (Fig. 4f, arrow). It can be seen then, that during initiation of callus from leaf explants, all leaf cell types divide and eventually contribute to the callus. Initiation of callus from stem explants involves cell division in all stem parenchyma cell types except the epidermis. E. degeneri callus therefore, fulfills the requirements for comparison to the whole plant as stated above. That is, that all cells of the callus are derived from all living cell types of the original explant.

Shoot development from callus

Shoot development from P10 callus appears to be of endogenous origin in *E. degeneri* callus (Fig. 5). Meristemoids are found throughout the callus in subepidermal (subsurface) positions (Fig. 3c,5a), which eventually form a cambium-like zone in transection (Fig. 5b). Eventually the regenerated shoot expands through the surface of the callus (Fig. 5c) and elongates to form a typical regenerated shoot (Fig. 5d). Shoot regeneration from callus of other species has been reported to arise from surface initials (Dunstan et al., 1978) or from subsurface initials (Ozias-Akins and Vasil, 1982; Thorpe, 1978). Thorpe noted that in tobacco callus the earliest histological events leading to shoot initiation usually occurred in the lower half of the callus and resulted in the production of meristemoids and later shoots. In rice however, Nakano and Maeda (1974) noted that both surface and subsurface meristemoids produced shoots. In *E. degeneri* callus it appears that shoots arise only from subsurface initials, and unlike Thorpe's (1978) tobacco callus, shoots arise from all sectors of the callus, not just the portions in contact with solid medium. (For review see Halperin, 1969.)

Euphorbia degeneri as a tissue culture system.

A tissue culture system has been developed to successfully culture *Euphorbia degeneri* shoot explants to form callus and regenerate shoots from callus. It was possible with this system to study the pathway of C_4 photosynthesis during the

dedifferentiation of tissues into callus and the subsequent regeneration of shoots that possess the typical C_4 anatomy. This developmental study complements research done on whole plants (both C_3 and C_4) and is the first report of callus formation and plant regeneration in E. *degeneri* and of research on photosynthetic carbon metabolism on whole plant, callus and the regenerated plant of a C_4 dicot.

It was desirable, in light of previous demonstrations of C_4 pathway variability in some plants (Crespo et al., 1979;, Kennedy, 1976, Ludlow and Wilson, 1971), to study the gain of the C_4 pathway during whole plant development. The *Euphorbia degeneri* tissue culture system provided a method whereby the development of the C_4 photosynthetic pathway could be studied under the controlled conditions of a tissue culture system.

Table 1

Composition of P0IN (non shoot-forming) and P10 (shoot-forming) media. (Values in g/l).

COMPONENT	P0IN	P10
MS salt mix	4.3	4.3
myo-Inositol	0.10	0.10
NaH_2PO_4	0.17	0.17
Nicotinic acid	.001	.003
Pyridoxine HCl	.001	.003
Thiamine HCl	.010	.020
Sucrose	20.0	20.0
2iP	.002	.004
2,4-D	.0005	---
Picloram	---	.0001
TC agar	8.0	8.0
pH*	5.8	5.8

*: measured before autoclaving

Table 2

Organogenic potential of *E. degeneri* tissues.

MEDIUM	LEAVES	NODES
POIN	%	%
MS1	-	-*
MS2	-	-*
MS5	-	-
P10	0	+#
SH	0	0
MSBN	X	0
MSBD	X	0

-:no development; 0:callus; %:profuse callus;

+:shoots; ++:prolific shoots; X:not done.

#: Shoots developed after callus had been on medium >3 weeks.

*: Axillary bud development only.

MS1: MS salts, no hormones

MS2: MS salts + 2iP (0.3mg/l) + IAA (0.17mg/l)

MS5: MS salts + 2iP (30mg/l) + 2,4-D (3mg/l) + IAA (0.3mg/l)

MSBN: MS salts + BA (2.3mg/l) + NAA (0.9mg/l)

MSBD: MS salts + BA (2.3mg/l) + 2,4-D (0.09mg/l)

SH: Schenk and Hildebrandt (1972) initial medium (minus pCPA)

Table 3

Dry weight as a percent of fresh weight

	DAY 0	DAY 39
POIN**	3.38 +-0.35	2.95 +-0.44
P10***	6.08 +-0.60	5.18 +-0.62

*: Start of experiment

: n=5 *: n=10

Table 4

Chlorophylls a plus b and soluble protein in mgch gFW^{-1} and mgch gDW^{-1} after 39 days in culture for E. degeneri callus.

	gFW^{-1}		gDW^{-1}	
	mgch	protein	mgch	protein
POIN	.084	2.00	2.65	63.8
P10	.166	2.50	2.95	44.4

Figure 1. Growth of *E. degeneri* POIN (○) and P10 (△) callus as a function of increase in fresh weight verses days in culture.

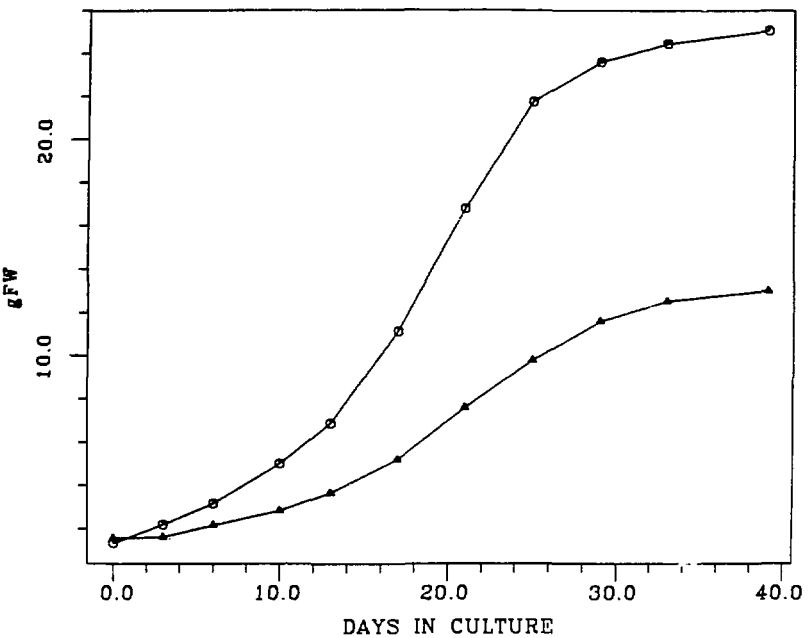

gFW
10.0
20.0
0.0
10.0
20.0
30.0
40.0
DAYS IN CULTURE

Figure 2. Growth of E. degeneri P0IN (O) and P10 (Δ) callus as a function of increase in dry weight verses days in culture.

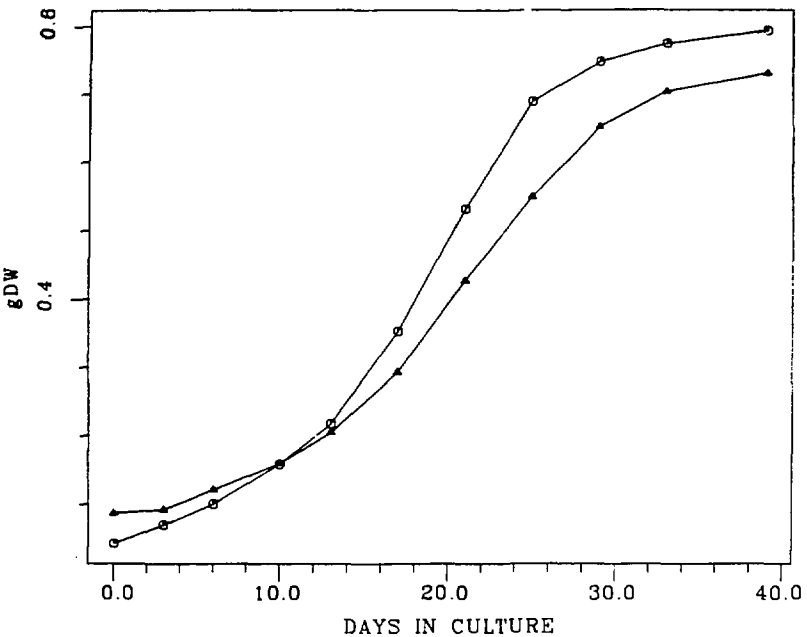
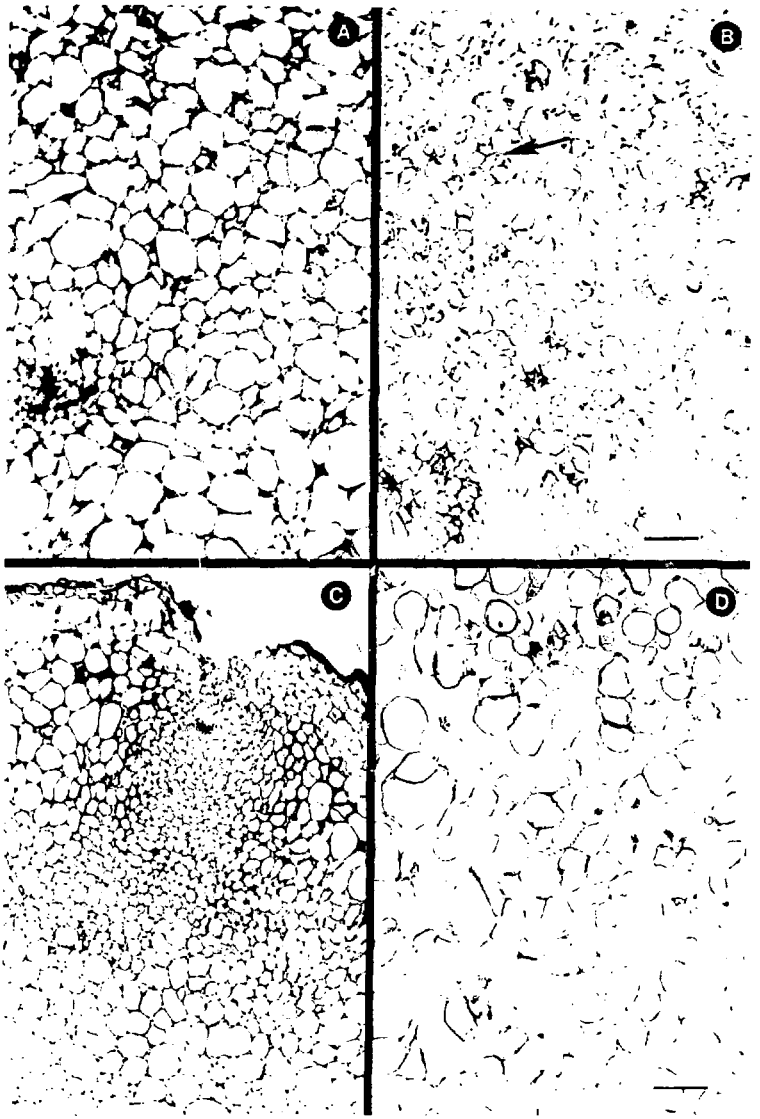
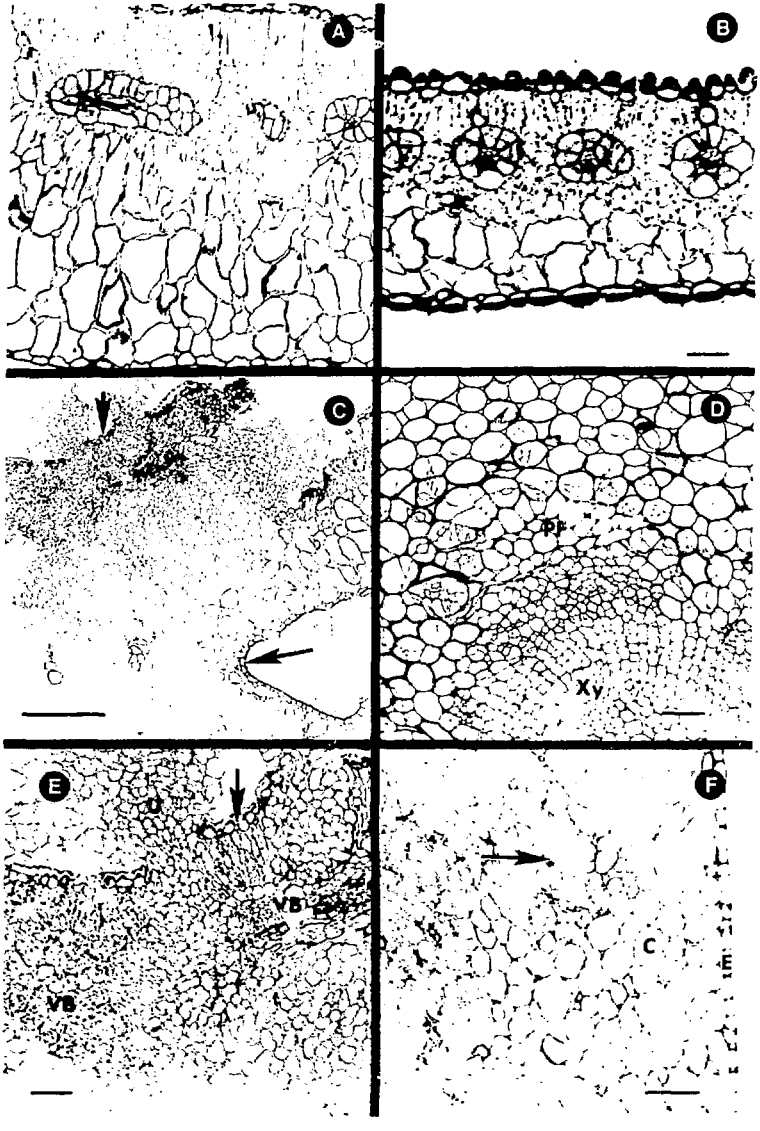

0.8
0.4
gDW
0.0
10.0
20.0
30.0
40.0
DAYS IN CULTURE

Figure 3. Light micrographs of transections of P10 (3a,c) and POIN (3b,d) callus. Scale bars in Fig. 3a,b,c= 50um. Scale bars in Fig. 3d= 25um. Arrow in Fig. 3b shows tracheoid.



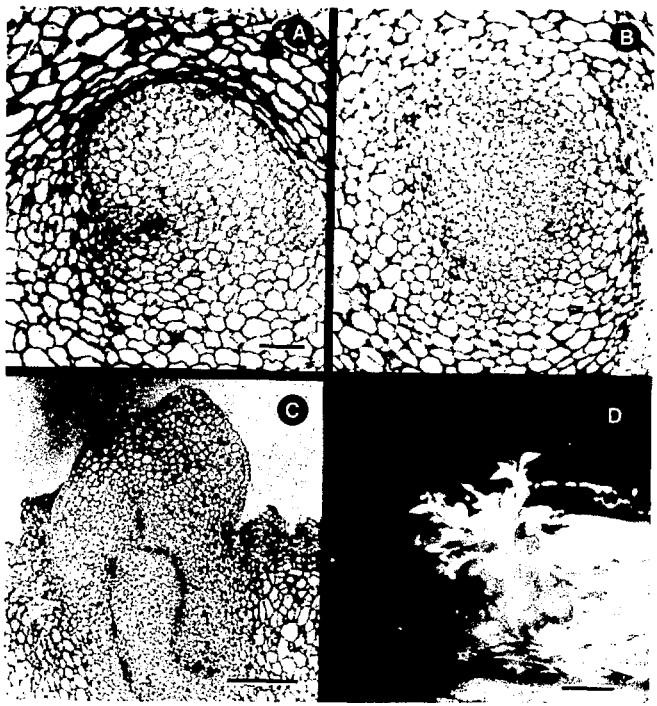
XBB 845-3519

Figure 4. Series of cellular events leading to callus formation from E. degeneri leaves (Figs. 4a,b,c,e) and stems (Figs. 4d,f). Arrows in 3c,e show leaf ad- and abaxial epidermis. Arrow in Fig. 3f shows localized area of cell division in outer cortex of stem. PF, phloem fibers; Xy, xylem; VB, vascular bundle; U, localized ulcerations emerging through the leaf epidermis; C, stem cortex; E, stem epidermis. Figs. 3a,b scale bar (in 3b)= 100um; Fig. 3c scale bar = 0.5mm; Figs. 3d scale bar = 50um; Fig. 3e scale bar = 100um; Fig. 3f scale bar = 40um.



XBB 845-3520

Figure 5. Cellular events leading to shoot initiation from E. degeneri P10 callus. Figs. 5a,b scale bar = 100um. Fig. 5c scale bar = 0.5mm. Fig. 5d scale bar = 2cm.



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CHAPTER TWO

C_4 PHOTOSYNTHESIS IN THE HAWAIIAN *EUPHORBIA* SPECIES: *E. DEGENERI* AND *E. REMYI*.

INTRODUCTION

The presence of the C_4 pathway of photosynthesis has been demonstrated in at least 840 spp. including 14 families of both monocots and dicots (12 dicot and 2 monocot families) (Hesla et al., 1982; Downton, 1975; Krenzer et al., 1975). The essential aspects of C_4 photosynthesis, namely $^{14}CO_2$ labelling kinetics has been studied in only a fraction of the species thought to exhibit C_4 photosynthesis. The remaining majority of C_4 plants have been typed as such based only on the presence of 'Kranz anatomy' (Kemp et al., 1983; Gutierrez et al., 1974; Laetsch, 1974), and/or $^{13}C/^{12}C$ ratios (Bender, 1968,1971; Tregunna et al.,1970; Smith and Brown, 1973; Pearcy and Troughton, 1975) or the presence and distribution in the leaf of certain carboxylating and decarboxylating enzymes (Ku et al., 1974; Harrison and Black, 1982; Waghmode and Joshi, 1982). A definitive demonstration of the C_4 photosynthetic pathway depends primarily on characterizing the pathway of radiolabelled carbon during photosynthesis (Hatch et al., 1967). All such experiments to date have shown that $^{14}CO_2$ is initially fixed in the mesophyll cells into oxaloacetate (OAA) from phosphoenolpyruvate (PEP) via the reaction catalyzed by phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) (Hatch and Slack, 1966; Hatch and Osmond, 1976). The resulting C_4-acid diffuses to the bundle sheath cells (Hatch and Osmond, 1976), is decarboxylated along one or more of three different, species specific pathways (Hatch and Osmond, 1976) to eventually regenerate PEP and thus complete the C_4 cycle. Some species

show a high NADP-'malic enzyme' (EC 1.1.1.40) and NADP-malate dehydrogenase (MDH, EC 1.1.1.82) activity in leaves, and subsequently decarboxylate malic acid in the bundle sheath cells (Andrews et al. 1971; Chen et al., 1971; Edwards and Black, 1971; Hatch, 1971; Downton, 1970; Hatch and Slack, 1970; Slack and Hatch, 1967) and thus use malate as the primary transport C_4-acid. Other species, in contrast, show a low NADP-'malic enzyme' and NADP-MDH activity that is compensated for by a high alanine- (ALT, EC 2.6.1.2) and aspartate aminotransferase (AST, EC 2.6.1.1) activity (Hatch and Slack, 1970; Chen et al., 1971; Hatch, 1971; Andrews et al., 1971) and thus these species seem to use aspartate as the primary transport metabolite and CO_2 donor. At least one species (Gomphrena celosoides) appears to have intermediate values for MDH, AST and ALT and therefore may possibly use both malate and aspartate for transport of CO_2 from the mesophyll cells to the bundle sheath cells. (Hatch et al., 1967; Andrews et al., 1971). For review see Hatch and Osmond, 1976.

In the present studies evidence from $^{14}CO_2$ radiotracer experiments, and assays of certain enzymes involved with the C_4 cycle definitively demonstrate that the C_4 pathway of photosynthesis is present in two species of Euphorbia; species previously described as C_4 plants based solely on anatomy and $^{13}C/^{12}C$ ratios. By using the ^{14}C label, it was possible to detect high levels of labelled malic and aspartic acid after very short periods of photosynthesis and to conclude that E. degeneri and E. remyi are C_4 plants, but that based on ^{14}C-labelling

kinetics of malate and aspartate and on AST and ALT activity they seem to exhibit properties of a modified NADP-ME-type C_4 pathway. The apparent role of both malate and aspartate in transporting CO_2 from the mesophyll cells to the bundle sheath cells has been suggested only once before (Andrews et al., 1971) and it is to this question that the present paper is directed. Evidence is presented that strongly suggests a role for both malic and aspartic acid as being alternate CO_2-transporting C_4-dicarboxylic acids in these two Euphorbia species.

MATERIALS and METHODS

Euphorbia degeneri and E. remyi plants were obtained from Dr. Robert Pearcy, University of California-Davis and maintained under growth chamber conditions (25 C; 12:12 L:D) and under average light conditions of 600 uE m^{-2} s^{-1}. $^{14}CO_2$ and $NaH^{14}CO_3$ were prepared from $Ba^{14}CO_3$ as described in Bassham (1961).

^{14}C tracer studies: leaf disc experiments

For the determination of the first products of ^{14}C fixation leaf discs were used. E. degeneri or E. remyi plants were preincubated for one hour at a light intensity equal to the experimental level. Light intensity was approx. 2000 uE m^{-2} s^{-1} but absolute values varied from experiment tc experiment. Leaf discs were punched out of the center of E. degeneri leaves (4th fully expanded leaf pair) or from the middle, sides of E. remyi leaves (2nd fully expanded leaf pair) with a #3 cork borer (7mm diameter). Discs were cut in preincubation medium (MOPS, 50mM; carbonic anhydrase, 8 Units; $NaH^{12}CO_3$, 16mM; pH 7.5) and preincubated under experimental conditions for 2 minutes. Experiments were commenced by transferring the leaf disc to 0.20 ml incubation medium (MOPS, 50mM; CA, 8 Units; pH 7.5). At T_0, 0.025 ml $NaH^{14}CO_3$ (0.141M; specific activity approx. 50 uCi/uM) was added to the incubation flask (#24 Dupour tissue grinder; Kontes Scientific Glasswear) to make a final concentration of $NaH^{14}CO_3$ of approx. 17mM. The incubation vessel was supported by

a ringstand and illuminated by a GE DXB 500W photo lamp. A 6 l water-filled jar was placed between the lamp and the incubation vessel. For the light intensity experiments successive layers of screening were placed between the lamp and vessel to attenuate the intensity to the determined values and the discs were allowed to photosynthesize for 2 minutes. At the times specified liquid nitrogen (LN_2) was poured into the incubation vessel. Previous experiments showed that the discs would be frozen against the side of the vessel within 1-2 seconds. Thus frozen, the vessel was capped with aluminum foil and partially submersed into LN_2 for storage until extraction.

Soluble radioactive components were extracted from the leaf discs as follows. LN_2 frozen samples were ground in 1 ml 80% (aq. v/v) methanol in a dry ice/isopropanol bath, transferred to centrifuge tubes and centrifuged at 1000 RPM for 10 min (IEC centrifuge). The supernatant was collected and the pellet was further extracted at room temperature with successively 0.5 ml 20% (v/v) MeOH and 80% (v/v) acidified MeOH. The supernatants were pooled and a 0.1 ml sample was processed to determine total ^{14}C fixation into soluble components by acidification with acetic acid followed by partial drying under a nitrogen stream. Scintillation solution (Aquassure, New England Nuclear) was added for liquid scintillation counting on a Packard Tri-Carb 460C LSC. To determine the ^{14}C-fixation into methanol insoluble compounds (starch) the pellet was washed with acetic acid, dried overnight under vacuum and oxidized in a Packard Automatic Combustion Apparatus. $^{14}CO_2$ from the combusted sample was collected in

Carbo-Sorb and scintillation counted in *Permafluor* (Packard Instruments Co.).

^{14}C tracer studies: whole leaf experiments

For the determination of the flow of radiolabelled carbon through photosynthetic intermediates, 'pulse-chase' experiments using $^{14}CO_2$ were performed on attached leaves of E. degeneri and E. remyi. Attached branches (1-4 leaf pairs) were preincubated for 5 minutes in a 600 ml Pyrex chamber. The incubation chamber was fitted with a gas inlet and outlet tube to a closed steady-state gas circulation system as described by Platt et al. (1976) but modified so as to allow preincubation and post incubation of the leaves with humidified air lacking $^{14}CO_2$. Preincubation and postincubation ('chase') was with air level CO_2 and O_2. The light level was approx. 1500 uE m^{-2} s^{-1} with UV radiation filtered out by a water cooled UV-absorbing glass filter. Temperature within the chamber remained a constant 25 C throughout the experiment. A rubber gasket was fitted around the branch so that the gas system remained closed with the plant in place. Incubation with $^{14}CO_2$ commenced when $^{14}CO_2$ was allowed to flow into the steady-state apparatus. The final concentration of $^{14}CO_2$ was approx. 40 ppm and the total concentration of CO_2 during incubation was approx. 400 ppm, thus the specific activity of all $^{14}CO_2$ experiments was approx. 5.8uCi/umole. Absolute values varied from experiment to experiment.

E. degeneri leaves were incubated in the presence of $^{14}CO_2$ for 1 min and E. remyi for 2 min, after which the rubber gasket

was removed and the chamber was rotated out and away from the branch, which allowed the branch to remain in the same orientation and distance from the light source. Photosynthesis was continued in room air for a certain period of time. At the times specified, individual leaves of E. degeneri were removed and plunged into LN_2. For experiments on E. remyi a paper punch was used to punch leaf disc pairs out of individual leaves (disc diameter 6mm) and into LN_2. Leaves and discs were stored in LN_2 until processing for radiolabelled metabolites. Radiolabelled compounds were processed as described below except that extraction of soluble compounds was with 80% MeOH (v/v), 20% MeOH (v/v), and water as described by Platt et al. (1977).

Analysis of $^{14}CO_2$ metabolites

The supernatant was subjected to two dimensional paper chromatography in the standard system previously described (Pederson et al., 1977). First dimension solvent was phenol:H_2O:acetic acid (84:16:1) pH 4.1; second dimension solvent was butanol:propionic acid:H_2O (74:36:49). After location by radioautography on Kodak SB-5 film (3 weeks exposure), the labelled metabolites on the paper were analyzed as described in Larsen et al. (1981). The labelled areas were cut into small pieces, shaken for 1 h with 2.5 ml of water in scintillation vials, and the amount of activity was determined by liquid scintillation counting after the addition of 18 ml Aquassure. Values of ^{14}C-labelling were obtained for hexose monophosphates, 3-P-glycerate, malate, aspartate, glycerate, alanine, sucrose,

glucose, and fructose.

CO_2 compensation point

The CO_2 compensation point for E. degeneri and E. remyi was determined on the steady-state apparatus. The apparatus was configured as described above except that no $^{14}CO_2$ was injected into the system. After preincubating the leaves (attached as described above) for 10 minutes with the steady-state system in a flow-through mode (air-level CO_2 and O_2) the system was closed and the plant was allowed to photosynthesize for 2 h or until the rate of decrease of CO_2 concentration in the system reached 0. CO_2 level was followed using a Beckman Model 865 Infrared CO_2 Analyzer.

Detection of radiolabelled oxaloacetic acid

With the standard procedures used, labelled oxaloacetate (OAA) would not be detected due to its spontaneous decarboxylation and release of $^{14}CO_2$ (Arnoff, 1956; Hatch and Slack, 1966; Hatch et al., 1967). Therefore the following procedure was used. Leaf discs from E. degeneri were incubated in the presence of $NaH^{14}CO_3$ in incubation medium (light intensity 1500 uE m^{-2} s^{-1}) for 1-3 seconds and frozen as before in LN_2. Extraction was in 1) 80% (v/v) EtOH containing 0.2M HCl, 2,4-dinitrophenylhydrazine (DNPH; 5mg/ml) at -70 C followed by room temperature incubation for 30 min. 2) centrifuge and re-extraction 20% (v/v) EtOH, 3) centrifugation and re-extraction with water. The supernatant was further extracted with chloroform to obtain 2,4-dinitrophenylhydrazones (Arnoff, 1956).

The radioactivity in the chloroform extract was determined by liquid scintillation counting and compared to the radioactivity in the aqueous phase to calculate the percent OAA. This procedure fixes other keto acids as well, but with an incubation in $^{14}CO_2$ of less than 3 seconds, it is probable that OAA would be the only ^{14}C-labelled keto acid.

Enzyme assays

Phosphoenolpyruvate carboxylase was assayed by following spectrophotometrically the oxidation of NADH at 340nm in a coupled reaction with added excess malate dehydrogenase (MDH) (Smith, 1968; Mukerji and Ting, 1971; Ting and Osmond, 1973a,b). The assay mixture was TRIS, 50mM; $MgSO_4$; KCl, 50mM; NADH, 0.1mM; PEP, 1.0mM; $NaHCO_3$, 1.3mM. Plant material was homogenized in a #24 Dupour tissue grinder (Kontes Scientific Glasswear) at 4 C in solution 'A' (TRIS, 50mM; $MgSO_4$, 10mM; ethylene glycol, 20% (v/v)) plus DTT (5mM) and insoluble PVP (1%, w/v) and centrifuged at 20,000 X g for 30 min. The supernatant was then assayed immediately for PEPC activity.

For the assay of other enzymes, leaf proteins were extracted in HEPES (50 mM), EDTA (2 mM), insol-PVP (1% w/v), Na Ascorbate (20 mM) and centrifuged at 20,000 x g for 30 min. The supernatant was assayed immediately for enzymatic activity. For the assay of AST and ALT, pyridoxal phosphate (20 ug ml^{-1}) was added to the extraction mixture.

Aspartate- and alanine aminotransferase activity were measured in crude extracts (prepared as described above) by coupling with added MDH in excess (4 Units ml^{-1} for AST or LDH in excess (4 Units ml^{-1}) for ALT. The assay mixture consisted of HEPES, 50mM, pH 8.0; EDTA, 2 mM, NADH, 0.2 mM; pyridoxal phosphate, 10ug ml^{-1}; 2-oxoglutarate, 2.5 mM. For the assay of AST, aspartate (2.5 mM) was added to the reaction mixture. For the assay of ALT alanine (10 mM) was added. Final volume of the reaction mixture was 1 ml. Activity of both AST and ALT was assayed spectrophotometrically by following the oxidation of NADH at 340nm. Leaves of the same age from two individual plants from both species were assayed for AST activity. Activity is expressed as umole NADHox (mg protein)$^{-1}$ min^{-1} or umole NADHox (mg chlorophyll)$^{-1}$ min^{-1}.

NADP-'malic enzyme', NAD-ME, NAD-MDH and NADP-MDH were assayed in crude leaf extracts obtained in the same manner as for the aminotransferase reactions. Assays were performed in the same basic medium (HEPES, 50 mM; EDTA, 2 mM; pH 8.0) with the following additions. For NADP-ME assays $MgCl_2$ (5 mM), malate (2.5 mM) and $NADP^+$ (0.25 mM) were added. For NAD-ME assays $MnSO_4$ (5 mM), malate (5 mM), NAD^+ (0.2 mM) and Co-A (75 uM) were added. Assays of both malic enzymes followed the increase in absorbance at 340nm. NAD-MDH was assayed with the addition of oxaloacetic acid (OAA, 0.5 mM) and NADH (0.2 mM) by following the decrease in absorbance at 340nm. NADP-MDH was assayed with the addition of OAA (0.5 mM) and NADPH (0.2 mM) by following the decrease in absorbance at 340nm (Johnson and Hatch, 1970).

Chlorophyll, protein, and microscopy

Chlorophyll content of leaves was determined in 80% (v/v) acetone according to the methods of Bruinsma (1963). For comparison to $^{14}CO_2$ experiments, identical non-labelled leaves or leaf discs were used for chlorophyll determinations. Protein was determined according to the method of Bradford (1976) using reagents obtained from Bio-Rad (Richmond, CA).

Tissues for sectioning for light microscopy were fixed in 10% (aq.) acrolein overnight at 4 C, dehydrated in methyl cellosolve/ethanol and embedded in glycol methacrylate according to the methods of Feder and O'Brien (1968). Sections were made at 2-5 uM thickness on a Sorvall JB-4 microtome and stained with 0.05% Toluidine Blue plus 0.1 %BHT for 2-5 minutes. Photomicrographs were made on an Olympus microscope equipped with a Nikon Microflex Model EFM camera.

All experiments were performed at least twice with each experimental point done in duplicate. Data points for kinetics experiments are averages of duplicates and are derived from individual experiments. That is, though replicate experiments showed equivalent trends, each graph represents only one experiment.

RESULTS

Effect of light intensity on incorporation of ^{14}C

The rate of total incorporation of $^{14}CO_2$ under 6 different light intensities was determined for E. degeneri and E. remyi leaf discs (Fig. 1). The plants used were previously grown in a growth chamber under a light intensity of 400-600 uE m^{-2} s^{-1}. It can be seen that the rate of ^{14}C incorporation increased linearly with increasing light intensity, but that the rate remained constant at and above a light intensity approximately equal to that under which the plants were originally grown (arrows, Figs. 1a,b). Some photoinhibition of ^{14}C-fixation was observed in E. degeneri at the highest light intensities used. Photoinhibition at high light intensities is thought to reflect a saturation of photosynthetic units rather than a limitation of CO_2 fixation since the CO_2 concentration at which the experiments were performed was approx. 65 X ambient.

Incorporation of radioactivity from $^{14}CO_2$ as a function of time

The total fixation of $^{14}CO_2$ (Fig. 2) and ^{14}C label in individual compounds (Figs. 3,4; Tables 2,3) were determined for leaf discs exposed to $NaH^{14}CO_3$ for periods of up to 60 seconds at a light intensity of approx. half full sunlight. Over this period the rate of ^{14}C fixation remained linear (Fig. 2). Plots of radioactivity in individual compounds as a function of time of

incubation are shown for E. degeneri (Fig. 3) and E. remyi (Fig. 4). These plots show several salient phenomena ascribed to C_4 plants. First, the initial products of $^{14}CO_2$ fixation are C_4-acids (either malate or aspartate) and with increased time of incubation, the percentage of label in C_4-acids decreases. Secondly, RPP-cycle intermediates (namely 3-phosphoglycerate and hexose phosphates) comprise only a small percentage of the total label at the beginning of $^{14}CO_2$ incubation and increase in percentage with increased incubation time. Also, the kinetics of the decrease in %-label in C_4-acids mirrors the increase in %-label in RPP-cycle intermediates. Although the absolute percentage of malic acid differs between the two species, the pattern of very early labelling of this C_4-acid followed by a later labelling of aspartate implys that the transport C_4-acid is malic acid. Alanine labelling increased with time for E. degeneri leaf discs (Table 2), but remained unchanged in E. remyi discs (Table 3). In both species, starch increased in %-label over time (Tables 2,3).

Using the killing and extraction procedure described above, but with the addition of DNPH, demonstrated that with an incubation time of 1-3 seconds an additional 3-4% label was located in 2,4-dinitrophenylhydrozones (Table 1), presumed to be oxaloacetic acid.

Changes in the distribution of ^{14}C label after transfer from $^{14}CO_2$ in air to $^{12}CO_2$ in air

When attached leaves were transferred from $^{14}CO_2$ in air to

air containing no $^{14}CO_2$ but maintained at the same light intensity, the total amount of radioactivity remained constant but the percent label in individual components changed greatly with time in the chase (Figs. 5 and 6). Both species showed a decrease with time in the percent labelled C_4-acids concomitant with an increase in percent label in 3-PGA, HMP, and sugars (sucrose, glucose and fructose). However, the %-label in specific C_4-acids varied depending on the plant used and the experiment performed. In E. remyi (Fig. 6) aspartate was the most predominantly labelled C_4-acid at the termination of the 'pulse' period and appeared to lose ^{14}C-label at a higher initial rate than malate (based on change in percent of total ^{14}C-label. In contrast, E. degeneri (Fig. 5a,b) showed both patterns, depending on the experiment. In one (Fig. 5a) aspartic acid appeared to be the primary transport C_4-acid and in another (Fig. 5b) malate showed kinetics characteristics of being the transport C_4-acid.

CO_2 compensation point

Plots of the net CO_2 uptake as a function of environmental CO_2 concentration clearly demonstrate the CO_2 exchange characteristics of C_4 photosynthesis (Fig. 7). Both species showed a CO_2 compensation point of approx. 2 PPM with the rate of net CO_2 uptake increasing linearly to a point of CO_2 concentration that is a fraction of that found in the atmosphere. E. degeneri showed no increase in net CO_2 uptake above 250-300 ppm CO_2 whereas E. remyi appeared to have a more linear response

that did not saturate at the highest CO_2 concentration tested (300 ppm). The response of E. remyi to CO_2 concentration in these experiments was similiar to what Robichaux and Pearcy (1980) reported for glasshouse-grown plants. Their results also showed an apparent CO_2 compensation point near 0 for both Euphorbias studied in this report.

Enzyme activities

It has been previously reported (Andrews et al., 1971; Hatch et al., 1975) that one plant (Gomphrena celosoides) has more active AST and ALT enzymes than is typically found in NADP-ME-type C_4 plants, and based on these data the authors suggested that both aspartate and malate may make equal contributions to CO_2 transport. The apparent utilization of more than one transporting C_4-acid by both E. degeneri and E. remyi prompted a similiar study of AST and ALT in the plants of both species. In Table 4 the activities of AST, ALT and other enzymes implicated in the metabolism of C_4-acids are listed. Values obtained from leaf extracts of E. degeneri, E. remyi and Z. mays are compared to published values for representative NADP-ME, NAD-ME and PKC-type C_4 plants (Andrews et al., 1971; Hatch et al., 1975). The data presented in Table 4 demonstrate clear divisions of C_4-species based on the activity of enzymes related to the C_4 pathway. Aspartate-transporting C_4 plants (NAD-ME and PCK-ME type) exhibit high activities of AST and ALT whereas malate transporters (NADP-ME type) show low activities. E. degeneri and E. remyi also show high AST and ALT activity (with the possible

exception being a low level of AST in E. degeneri) that are similiar in value to those found by Hatch et al. (1975) for Gomphrena celosoides but intermediate in value between NADP-ME- and PCK-type C_4 plants. The values for NADP-ME for the Euphorbias are equivalent to those found in Zea mays and G. celosoides but are approximately 50 times greater than activities for these enzymes found in plants of either the PCK- or NAD-ME-type C_4 pathways. NAD-MDH activities in leaf extracts of all plants were high, with the highest activities in the NAD-ME-type species (240 umole (mg chlorophyll)$^{-1}$ min^{-1}) and the lowest in maize (41 umole (mg chlorophyll)$^{-1}$ min^{-1}) and intermediate values for E. degeneri (80.8 umole (mg chlorophyll)$^{-1}$ min^{-1}) and E. remyi (82 umole (mg chlorophyll)$^{-1}$ min^{-1}). Values for both Euphorbias were intermediate between those found in NADP-ME- and either PCK- or NAD-ME-types. Notably, the values were somewhat lower than those reported for G. celosoides.

Leaf anatomy

Both species show 'Kranz'-type leaf anatomy with prominent bundle sheath cells that contain centripetally located chloroplasts (Fig. 8). The cell walls of the bundle sheath cells of both species are more deeply staining than the cell walls of the surrounding mesophyll cells.

DISCUSSION

In C_4 plants radiotracer studies indicate that OAA formed via PEPC in mesophyll cells is rapdily converted to malate and aspartate (Hatch, 1971; Chen et al., 1971). The dominance of one C_4-acid or another differs between species relative to the amount of label after short periods in $^{14}CO_2$ (Downton, 1970), and also with respect to which C_4-acid is directly involved in transfer of CO_2 to RPP metabolites in the bundle sheath cells (Chen et al., 1971; Hatch, 1971). The predominance of one C_4-acid and the relative activities of various enzymes related to C_4 photosynthesis, as well as the apparent position of chloroplasts in the bundle sheath cells (Hatch and Osmond, 1976) provides the basis for grouping C_4 plants into three defined sub-groups: 'NADP-ME-type', 'PCK-type', 'NAD-ME-type' (Hatch et al., 1975).

It is clear from the data presented in this report that Euphorbia degeneri and E. remyi are C_4 plants that exhibit a variation of the NADP-ME type pathway. Previous workers (Pearcy and Troughton, 1975; Webster et al., 1975) had demonstrated that these and other dicot species were C_4 plants based on CO_2 compensation point, delta ^{13}C, and leaf anatomy but no work had previously been done using analysis by ^{14}C labelling or enzyme kinetics to trace the specific pathways present. Erlingher and Pearcy (1984) presented data that suggested that both E. degeneri and E. remyi were NADP-ME type C_4 plants but their conclusions, based only on quantum yield values, are equivocal since the values obtained overlap those values obtained for NAD-

ME type plants. Also, CO_2 usage curves from which Pearcy et al. (1982) obtained CO_2 compensation point values showed an unusual slope for E. remyi that may imply a different or modified CO_2-dependent utilization by this species. Measurement of other photosynthetic factors has shown that E. remyi possesses unusual characteristics for a C_4 plant. It has a lower maximum photosynthetic rate and an unusual g_m'/g_l' ratio (mesophyll/leaf conductance; Pearcy et al., 1982) when compared to the other members of the Hawaiian C_4 Euphorbias and values that are similiar to those found in C_3 plants.

Although the existence of C_4 photosynthesis in these Euphorbias has been reasonably well characterized, a conclusive demonstration of the $^{14}CO_2$ fixation kinetics and assays of the relevant carboxylating and decarboxylating enzymes had not been undertaken before the present study. With such an extensive background of information it seemed appropriate to investigate the specifics of the C_4 pathway(s) of photosynthesis present in these two species of the Hawaiian Euphorbias.

The present studies provide evidence that unlike (almost) all other C_4 plants studied (Hatch, 1976; Hatch and Osmond, 1976), both malic and aspartic acid contribute to the transfer of CO_2 to the RPP cycle. Labelling leaf discs with ^{14}C-bicarbonate at concentrations approximately 65 times greater than air level yield kinetics of malate and aspartate labelling that demonstrates an apparent initial fixation of ^{14}C into malic acid in both species studied. E. degeneri has slightly different kinetics in that aspartate labelling represents approximately

10% of the total ^{14}C-label extrapolated to zero time, whereas for E. remyi, aspartate labelling extrapolates to zero % at T_0. The data obtained from these short-term ^{14}C-labelling experiments suggests that malic acid acts in both species as the primary transport C_4-acid.

On the other hand, 'pulse-chase' experiments designed to show the decarboxylation of the specific transport C_4-acid implicate aspartic acid as the specific CO_2 transporting metabolite. Furthermore, experiments using $^{14}CO_2$ labelling at air level (330 ppm) of intact E. degeneri leaves yielded data consistent with primary labelling of aspartate or malate, depending on the experiment. In all pulse-chase experiments the percentage of total label in individual C_4-acids was quite high at the onset of the $^{12}CO_2$ chase period. Although the percentage of ^{14}C found in C_4-acids and 3-PGA after a period of labelling is dependent on the duration of exposure to $^{14}CO_2$ (Hatch and Osmond, 1976), two identical pulse-chase experiments with E. degeneri yielded kinetics patterns that were alternately predominant in malate or aspartate but that had similiar percentages of label in C_4 compounds. Pulse-chase experiments using attached E. remyi leaves, on the other hand, always exhibited kinetics consistent with aspartate as being the primary transport C_4-acid since during the 'chase' label was lost more rapidly from it than from malate. The ^{14}C-kinetics presented in this paper however suggest that either malate or aspartate can function as the transport C_4-acid.

Results of analysis of enzyme activity in other species

suggests that, although there is no evidence for the exclusive operation of either malate or aspartate as a CO_2 donor in any species (Hatch, 1971) one pathway is predominant in any given species (Hatch et al., 1975; Ku et al., 1974; Hatch and Kagawa, 1974; Hatch, 1973; Hatch and Mau, 1973; Gutierrez et al., 1974; Andrews et al., 1971; Downton, 1970). In one case, Andrews et al. (1971) suggested that malate and aspartate may contribute equally in Gomphrena celosoides (Amaranthaceae). Andrews et al. (1971) assayed for activity of four enzymes ('malic' enzyme, NADP-MDH, ALT and AST) in leaf extracts and reported values that were intermediate between malate transport C_4 plants (NADP-ME-type, eg. Zea mays) and aspartate transport types (PCK-type, eg. Chloris gayana, or NAD-ME-type, eg. Amaranthus edulis). Hatch et al. (1975) further characterized plants representing these C_4 sub-groups by assaying for the activity of PEP-CK and NAD-MDH as well as those listed above and also reported values of AST and ALT for G. celosoides that were intermediate between those found in aspartate- and malate-transport types. Hatch et al. (1975) suggested that the higher aminotransferase activities, compared with other NADP-ME species may indicate that this species could transport aspartate as well as malate to bundle sheath cells, and that both malate and aspartate would act as CO_2 translocators. However, no $^{14}CO_2$ labelling of G. celosoides was performed, so no unequivocal demonstration of the ability to utilize malate and aspartate equally was made.

The two Euphorbia species studied in this research show values for AST and ALT intermediate between those reported for

NADP-ME and other C_4 plants and similiar to those found in G. celosoides (Andrews et al., 1971; Hatch et al., 1975). However, activities of other enzymes associated with the C_4 pathway were found to be at levels similiar to those found in NADP-ME type species. The conclusion therefore, based on enzymological evidence and $^{14}CO_2$ kinetics analysis is consistent with the hypothesis that these Euphorbia species show characteristics of a modified NADP-ME-type C_4 photosynthetic pathway in that they appear to use aspartate as well as malate for CO_2 translocation.

Anatomical evidence may also provide a basis for comparing Euphorbia degeneri and E. remyi to G. celosoides and its reputed intermediacy. For all C_4 species studied (Gutierrez et al., 1974; Hatch and Osmond, 1976) the position of the chloroplasts in the bundle sheath cells relative to tracheary elements in the vascular bundle (centrifugal verses centripetal) seems to be related to the specific C_4-type pathway. NAD-ME-types have centripetal, PCK- and NAD-ME-types seem to have centrifugally located chloroplasts. However, Hatch et al. (1975) reported that G. celosoides (a NADP-ME-type) has centripetally located chloroplasts. Fisher and Evert (1982) have shown that Amaranthus retroflexis, a C_4 dicot with the NAD-ME-type pathway (Gutierrez et al., 1974) also has centripetal chloroplasts, but Raghavendra and Das (1976) have shown that in two species of C_4 Euphorbias (E. hirta and E. thymifolia) the chloroplasts have no defined arrangement but rather are distributed throughout the bundle sheath cell. So it appears that C_4 dicots have chloroplasts in all possible positions and that a specific

dependence of chloroplast position on C_4-type in dicots does not exist. In the present study both species of Euphorbia were found to have centripetally located chloroplasts and thus are similiar in this respect to G. celosoides.

In summary, radiotracer studies and analysis of specific C_4-related enzymes emphasize the existence of the NADP-ME-type pathway of photosynthesis in Euphorbia degeneri and E. remyi but demonstrate the apparent ability of both plants to use aspartate as well as malate to transport CO_2 from the mesophyll to the bundle sheath cells. The position of the chloroplasts in the bundle sheath cells of both species also implicates the presence of either the NAD-ME- or NADP-ME-type pathway but does not discriminate between the two. Electron microscopy of bundle sheath chloroplasts may be useful in making such a discrimination, but has not yet been undertaken.

The ability of these species to use aspartate as well as malate for CO_2 transport may be important in the balance of nitrogen in the plant since Pearcy et al. (1982) reported that photosynthesis was strongly related to leaf nitrogen content. Other workers have also reported the relationship of aspartate-forming C_4 photosynthesis to nitrogen metabolism and the TCA-cycle (Chapman and Osmond, 1974; Raghavendra and Das, 1976). Chapman and Osmond (1974) reported that a high activity of AST in bundle sheath cells of Atriplex spongiosa resulted in an increased flow of carbon into TCA-cycle intermediates (via PEP carboxylation) as well as the RPP-cycle. This suggests that in some C_4 plants an increase in cytoplasmic ammonia (from

photorespiration or environmental sources) could stimulate PEP carboxylation and thus the synthesis of aspartate (from OAA). In other systems, ammonia has been shown to stimulate increased carbon flow from photosynthetic intermediates to amino acid skeletons (Platt et al., 1977). The increase in cytoplasmic pH by the influx of ammonia causes an increase in activity of PEPC, apparently due to the sharp pH dependence of the enzyme (Platt et al., 1977; Smith and Brown, 1981). Also, aspartate may serve to maintain intercellular balance of amino groups as well as coupling for transamination reactions (Hatch and Osmond, 1976). Thus, with the capability of transamination in bundle sheath cells, aspartate could perform a dual role of transfer of nitrogen (and stimulation of amino acid synthesis) and transfer of CO_2. The presence of ammonia could regulate both amino acid synthesis and the CO_2 shuttle mechanism of C_4 photosynthesis.

Table 1.

OAA determination in E. degeneri leaf discs.

		DPM/0.1ml	% Total DPM	%OAA*
+ DNPH	Aq	1703	93.62	
	CHCl3	116	6.38	
				3.58
- DNPH	Aq	1599	97.20	
	CHCl3	46	2.80	

*: ^{14}C-labelled 2,4-dinitrophenylhydrozones.

Table 2.

Distribution of labelled intermediates over time after $Na^{14}CO_3$ incubation in the light by E. degeneri leaf discs in Experiments 1 and 2.

	% of total ^{14}C-incorporation					
	30*	45	60	90	120	180
ALA	16.1	15.5	16.0	8.3	8.0	5.7
ALA_2	8.7	10.2	11.7	11.7	15.1	16.9
STARCH	3.7	5.8	2.9	4.3	4.5	7.0
$STARCH_2$	4.6	5.4	3.2	6.2	6.2	7.5

*= time in chase in seconds

$_2$= Data from Experiment 2

Table 3.

Distribution of labelled intermediates over time after $Na^{14}CO_3$ incubation in the light by E. remyi leaf discs.

	% of total ^{14}C-incorporation				
	10*	30	60	120	180
ALA	20.0	19.3	19.2	19.7	18.6
STARCH	5.3	7.4	9.1	11.6	12.2

*= time in chase in seconds

Table 4.

Comparison of activities of certain C_4 enzymes.

Enzyme activity expressed as *umoles NADHox/mg chlorophyll/min*

Type		AST	ALT	NADP-ME	NADP-MDH	NAD-MDH	NAD-ME
%	E.d+	5.92	17.7	10.9	7.9	80.8	<.01
%	E.r+	25.3	28.8	11.9	6.6	82.0	NA
1	G.c+*	14.5	18	13.5	11.5	NA	0.4
1	Z.m+	7.3	3.9	NA	4.2	81.6	NA
1	Z.m+*	5.7	1.4	10.7	9.5	41	0.2
2	P.m+*	53	42	0.20	1.2	83	0.5
3	A.s+*	42	56	0.25	2.1	240	8.9

Type 1= NADP-ME-type; Type 2= PCK-type; Type 3= NAD-ME-type

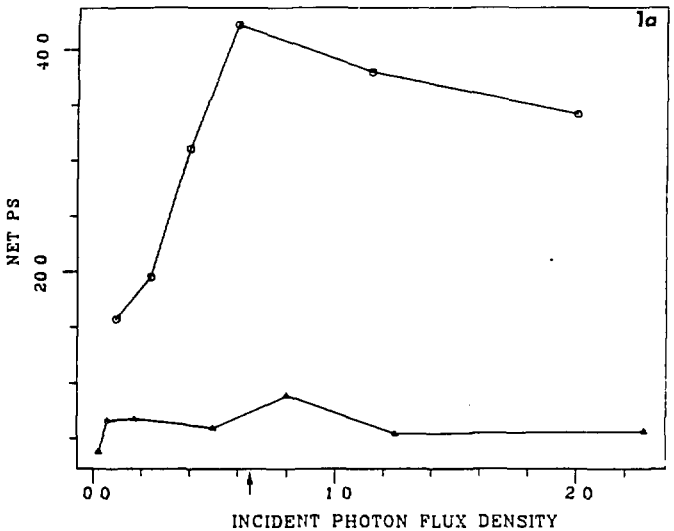
*: Data taken from Hatch et al. (1975)

+: E.d, Euphorbia degeneri; E.r, Euphorbia remyi;
G.c, Gomphrena celosoides; Z.m, Zea mays;
P.m, Panicum maximum; A.s, Atriplex spongiosa

%: The possible significance of higher AST and ALT plus lower NADP-MDH is discussed in the text.

NA= not assayed

Figure 1. Photosynthesis as a function of light intensity for E. degeneri (O) and E. remyi (Δ). Figure 1a is photosynthesis in methanol soluble compounds; figure 1b is photosynthesis into methanol insoluble compounds (starch). Incident photon flux density expressed as uE m^{-2} s^{-1}. Net PS (photosynthesis) expressed as umole ^{14}C (mg chlorophyll)$^{-1}$ hr^{-1}. Arrow indicates ambient light intensity. Leaf discs were allowed to photosynthesize for 2 minutes at the specified light intensity.


1a
NET PS
40 0
20 0
0 0
1 0
2 0
INCIDENT PHOTON FLUX DENSITY

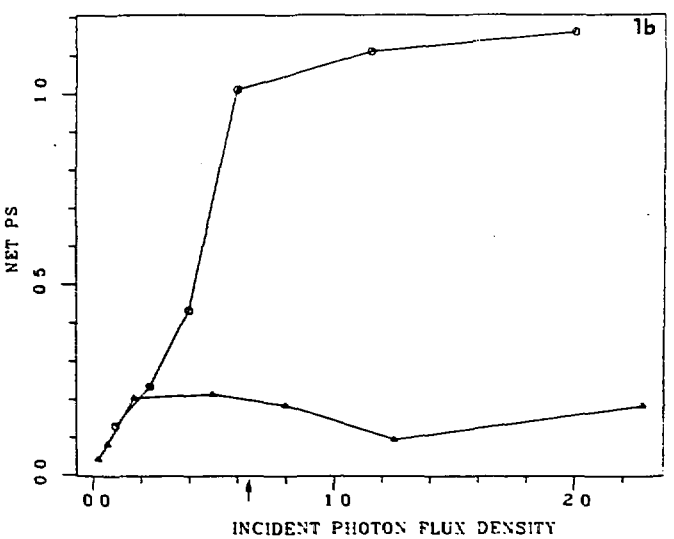

1b
NET PS
1 0
0 5
0 0
1 0
2 0
INCIDENT PHOTON FLUX DENSITY

Figure 2. Total ^{14}C incorporated into E. remyi leaf discs as a function of time. (O)= methanol soluble; (Δ)= methanol insoluble (starch). Total fixation expressed as umoles ^{14}C (mg chlorophyll)$^{-1}$. Methods of analysis described in Methods section of text.

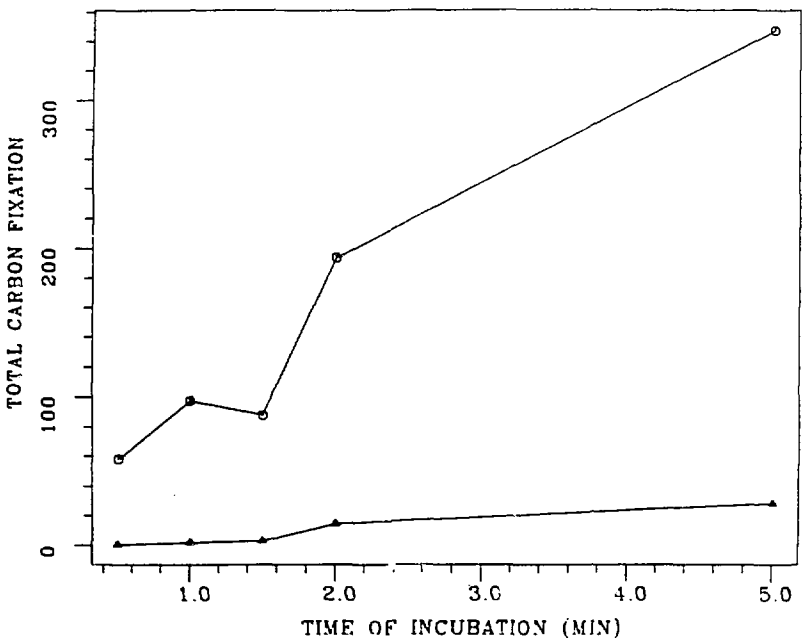

TOTAL CARBON FIXATION
0
100
200
300
1.0
2.0
3.0
4.0
5.0
TIME OF INCUBATION (MIN)

Figure 3. Proportion of total radioactivity in individual compounds for periods in $^{14}CO_2$ up to 60 sec. for E. degeneri leaf discs. Details of procedures for identifying and counting individual compounds are described in the Methods section. (O), malate; (X), 3-PGA; (Δ), aspartate; (★), sucrose.

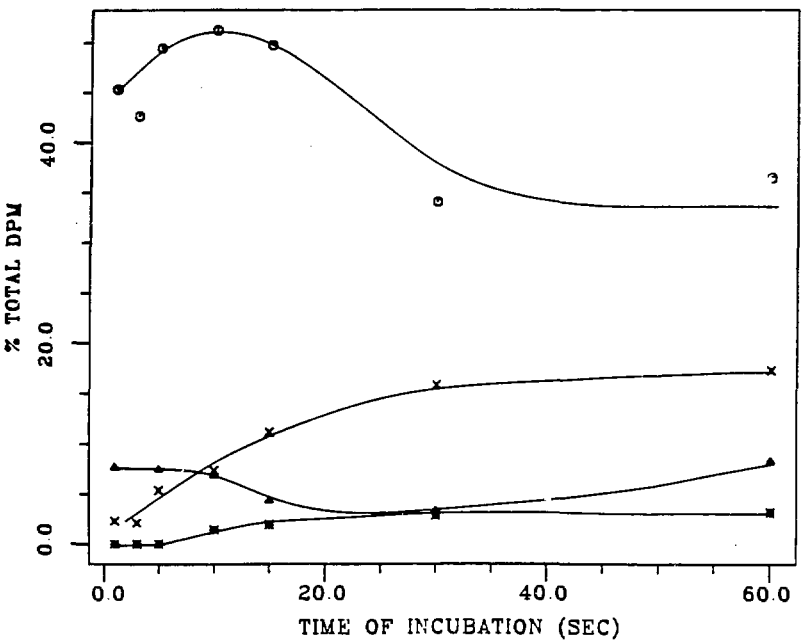

% TOTAL DPM
40.0
20.0
0.0
0.0
20.0
40.0
60.0
TIME OF INCUBATION (SEC)

Figure 4. Proportion of total radioactivity in individual compounds for periods in $^{14}CO_2$ up to 60 sec. for E. remyi leaf discs. Details of procedures for identifying and counting individual compounds are described in the Methods section. (O), malate; (X), 3-PGA; (Δ), aspartate; (★), sucrose.

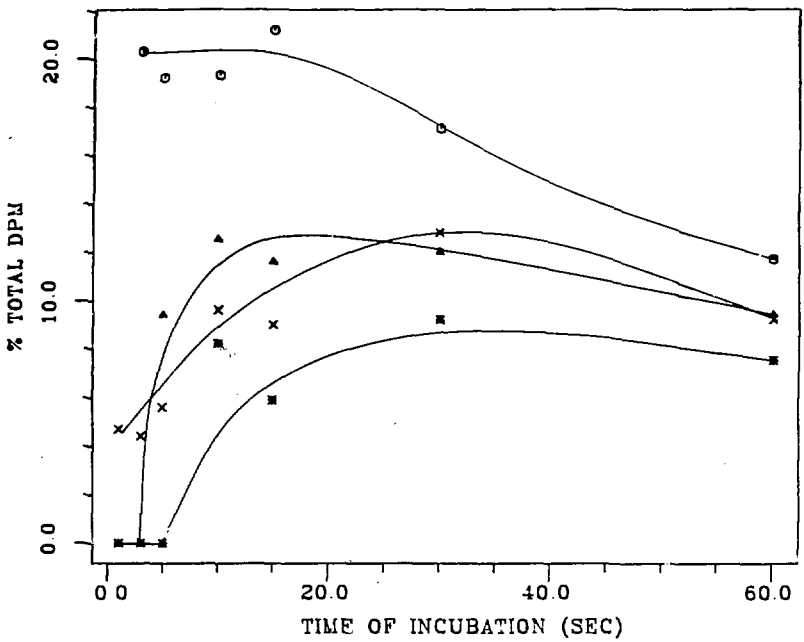
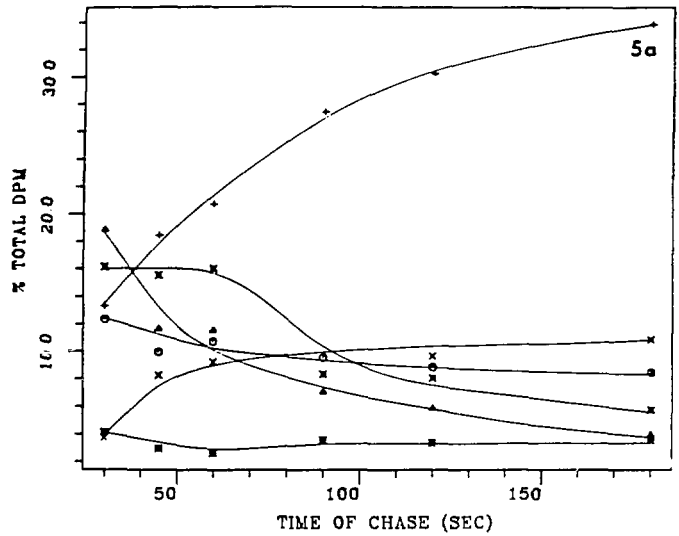

% TOTAL DPM
0.0
10.0
20.0
0 0
20.0
40.0
60.0
TIME OF INCUBATION (SEC)

Figure 5. Changes in the distribution of radioactivity in Experiment 1 (Fig. 5a) and Experiment 2 (Fig. 5b) after transfer of leaves of E. degeneri from $^{14}CO_2$ to carbon dioxide. After 1 min. in $^{14}CO_2$ the leaves were removed from the chamber without changing the distance from the light source. At intervals individual leaf pairs were removed and processed for radioactive intermediates as outlined in the Methods section. (Δ), aspartate; (O), malate; (X), 3-PGA; (★), hexose monophosphates; (⊹), sugars.


5a
% TOTAL DPM
30.0
20.0
10.0
50
100
150
TIME OF CHASE (SEC)

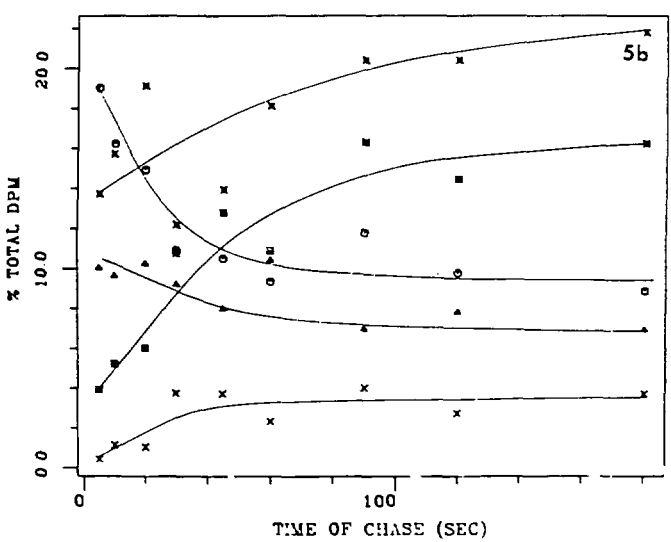

5b
% TOTAL DPM
20.0
10.0
0.0
0
100
TIME OF CHASE (SEC)

Figure 6. Changes in the distribution of radioactivity after transfer of leaves of E. remyi from $^{14}CO_2$ to carbon dioxide. After 1 min. in $^{14}CO_2$ the leaves were removed from the chamber without changing the distance from the light source. At intervals individual leaf pairs were removed and processed for radioactive intermediates as outlined in the Methods section. (Δ), aspartate; (O), malate; (X), 3-PGA; (★), hexose monophosphates; (✱), sugars.

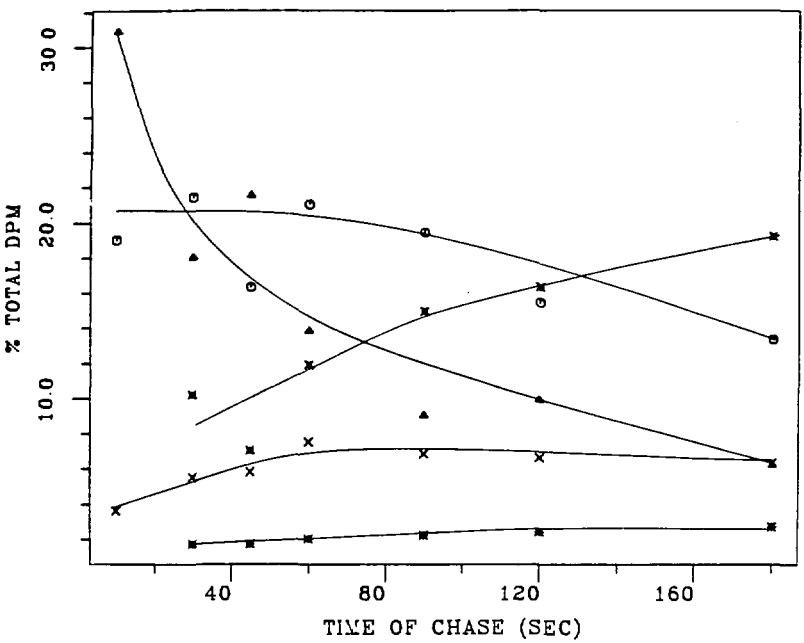

% TOTAL DPM
30.0
20.0
10.0
40
80
120
160
TIME OF CHASE (SEC)

Figure 7. Responses of photosynthetic CO_2 uptake to external CO_2 concentration of E. degeneri (O) and E. remyi (Δ). Net CO_2 uptake expressed as ul CO_2 m^{-2} s^{-1}. Chamber CO_2 concentration is expressed as ppm.

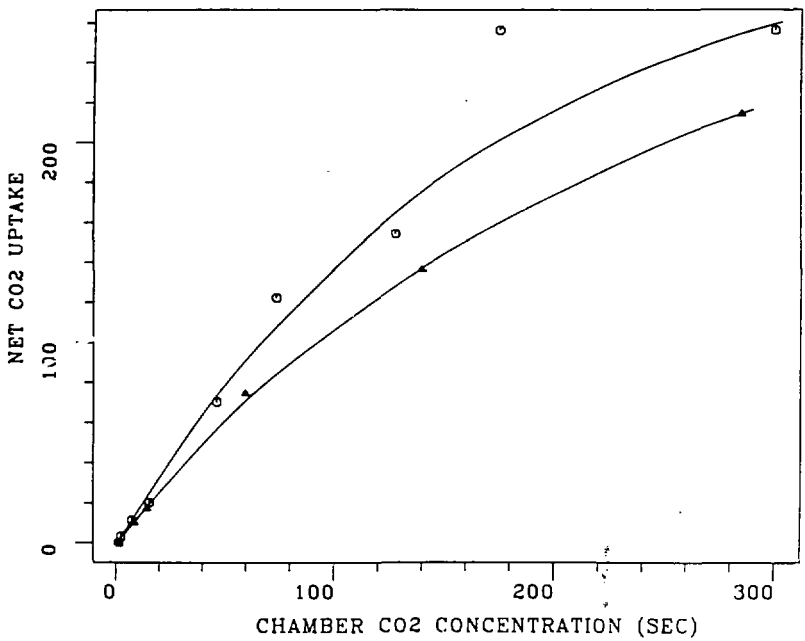
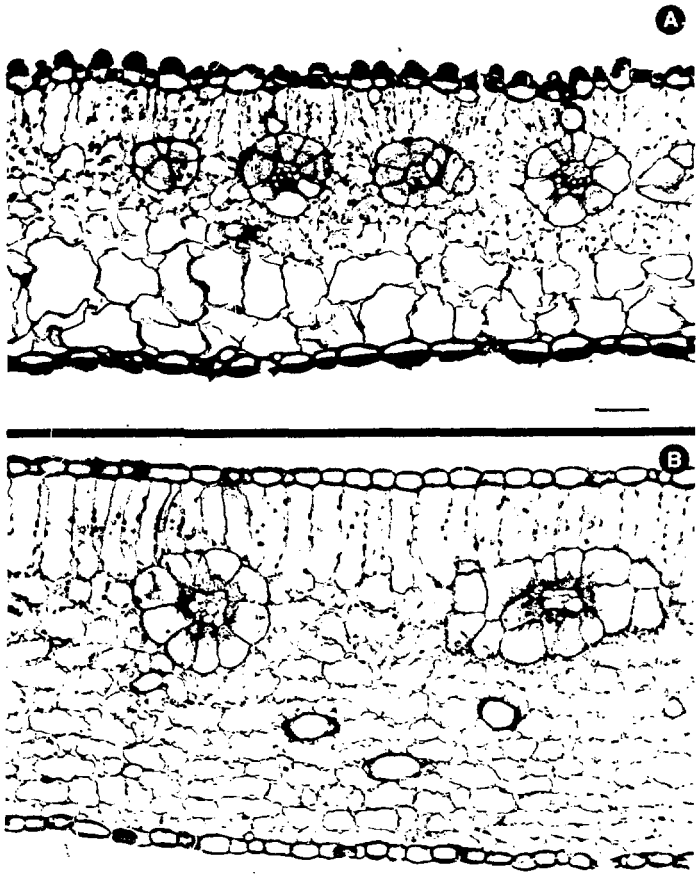

NET CO2 UPTAKE
0
100
200
0
100
200
300
CHAMBER CO2 CONCENTRATION (SEC)

Figure 8. Light micrographs of leaf transections of *E. degeneri* (8a) and *E. remyi* (8b). Scale bar = 100um.



XBB 845-3522

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CHAPTER THREE

PHOTOSYNTHETIC CARBON METABOLISM IN CALLUS CULTURES AND REGENERATED PLANTS OF *EUPHORBIA DEGENERI*

INTRODUCTION

The prevailing view of C_4 photosynthesis is that CO_2 is transferred via the C-4 carboxyl of C_4-acids to the site of the RPP-cycle where it is released and subsequently fixed and reduced to carbohydrate (Hatch and Slack, 1966; Hatch and Osmond, 1976). In all cases studied, the initial fixation of CO_2 occurs in leaf mesophyll cells and is mediated by PEPC (phosphoenolpyruvate carboxylase) and the fixation of CO_2 leading to carbohydrate occurs in the bundle sheath cells where it is mediated by RuBPC (ribulosebisphosphate carboxylase) (Hatch and Osmond, 1976). There has been no definitive demonstration, however, that this 'division of labor' is a necessary component of C_4 photosynthesis (for review see Hatch and Osmond, 1976).

Tissue culture has provided a system for some workers (Usada et al., 1971; Kennedy et al., 1977; Sprey and Laetsch, 1978; Seeni and Gnanam, 1983) to evaluate the importance of the bundle sheath/mesophyll cell organization ('Kranz anatomy') for the complete functioning of the C_4 pathway of photosynthesis. In most cases studied, callus or suspension cultured cells were shown to exhibit both a C_3- and C_4-like photosynthesis. On that basis, some workers have even questioned the necessity of Kranz anatomy for the functioning of C_4 photosynthesis. Kennedy (1976) and Kennedy et al. (1977), citing evidence derived from callus cultures of Portulaca oleracea, suggested that C_4 photosynthesis was present even though the cultures had no Kranz-type organization. Their suggestion was based on kinetics of ^{14}C-

fixation that showed a high percentage of label in C_4-acids after short periods of incubation. Although they do state that caution should be made in considering the importance of the C_4 system to the overall physiology of the callus cells. Nishida et al. (1980) did similiar experiments with photoautotrophic and photomixotrophic tobacco callus and found that the RPP-cycle operated in both types of callus, but that a high percentage of label was also localized in C_4-acids within the first 10 seconds of incubation. These data are similiar to those reported by Paul and Bassham (1977) for cultured Papaver somniferum (a C_3 plant) leaf mesophyll cells, as well as those reported for callus derived from the C_4 plants Amaranthus retroflexus L. (Usada et al., 1971), and Gisekia pharnaceoides (Seeni and Gnanam, 1983) and the CAM plant Chamaecereus sylvestrii (Seeni and Gnanam, 1980). In contrast to Kennedy et al. (1977), Seeni and Gnanam (1983) concluded that C_4 photosynthesis was not present in callus cultures derived from the C_4 plant, but that photosynthetic carbon fixation occurred via RPP-cycle reactions only.

It appears then, that the central question is not whether synthesis of C_4-acids occurs in cultured tissues and cells, but whether the C_4 pathway of photosynthesis, as defined as a mechanism of CO_2 transport to the site of the RPP-cycle, is present in tissue cultures derived from C_4 plants and if so, whether Kranz anatomy is present in those cultures.

This paper describes photosynthetic carbon metabolism in callus cultures and regenerated plants of the C_4 Hawaiian dicot Euphorbia degeneri. The goal of the research was to compare the

patterns of photosynthesis in plants and callus cultures and to evaluate the relationship between Kranz anatomy and pathways of photosynthesis. This is the first report that describes the study of photosynthetic carbon metabolism in regenerated plants as well as in callus derived from the same C_4 plant species.

MATERIALS AND METHODS

Euphorbia degeneri plants were obtained from Dr. Robert Pearcy, University of California-Davis and maintained under growth chamber conditions (25 C; 12:12 L:D) and under average light conditions of 600 uE m^{-2} s^{-1}. $^{14}CO_2$ was prepared from $Ba^{14}CO_3$ as described in Bassham (1961).

Tissue culture

Calli and regenerated plants were obtained as described in Ruzin (1984a). Calli were maintained on either non-shoot-forming (P0IN: MS salts; 2iP, 2 mg/l; 2,4-D, 0.5 mg/l) or shoot-forming (P10: MS salts; 2iP, 4 mg/l; picloram, 0.1 mg/l). $^{14}CO_2$ experiments were performed on either callus or rooted, sterile plants that had from 6 to 8 fully expanded leaf pairs. These are designated 'older regenerated plants' (ORP) in contrast to plants having fewer than 3 leaf pairs ('young regenerated plants'; YRP).

^{14}C tracer studies: regenerated plants

For the determination of the flow of radiolabelled carbon through photosynthetic intermediates, 'pulse-chase' experiments using $^{14}CO_2$ were performed on attached leaves of older regenerated plants of E. degeneri. Attached branches (6-8 leaf pairs) were preincubated for 5 minutes in a 100 ml plastic chamber. The incubation chamber was fitted with a gas inlet and outlet tube to a closed steady-state gas circulation system as described by Platt et al. (1976) but modified so as to allow

preincubation and post-incubation of the leaves with humidified air lacking $^{14}CO_2$. Preincubation and postincubation ('chase') was with air level CO_2 and O_2. The light level was approx. 800 uE m^{-2} s^{-1} with UV radiation filtered out by a water cooled UV-absorbing glass filter. Temperature within the chamber remained a constant 25 C throughout the experiment. The chamber was slotted to allow the branch to remain attached to the plant and not be damaged when the plastic cover was fitted over the open top. Finally, plastic sealer was applied around the slot so that the gas system remained closed with the branch in place. Incubation with $^{14}CO_2$ commenced when $^{14}CO_2$ was allowed to flow into the steady-state apparatus. The final concentration of $^{14}CO_2$ was approx. 40 ppm and the total concentration of CO_2 during incubation was approx. 400 ppm, thus the specific activity of all $^{14}CO_2$ experiments was approx. 5.8 uCi/umole. Absolute values varied from experiment to experiment.

^{14}C tracer studies: callus

For determining the flow of radiolabelled carbon through photosynthetic intermediates in callus, pulse-chase experiments were done according to the following procedure. $^{14}CO_2$ in air was administered to P10 calli using the same closed steady-state gas circulation system and methods as described above, but with the following modifications. Solid P10 medium was placed on the bottom of the incubation chamber to act as a moist base for calli. Light level was approx. 1500 uE m^{-2} s^{-1}. The final concentration of $^{14}CO_2$ was approx. 47ppm and the total

concentration of CO_2 was approx. 500ppm, thus the specific activity of $^{14}CO_2$ was approx. 5.5 uCi/umole. Absolute values varied from experiment to experiment.

E. degeneri leaves were incubated in the presence of $^{14}CO_2$ for 1 min and callus for 2 to 4 min, after which the flow of $^{14}CO_2$ was stopped and the chamber cover was removed. This allowed the branch or callus to remain in the same orientation and distance from the light source. Photosynthesis continued in room air for a certain peroid of time. At the times specified, individual leaf or calli pairs were removed and plunged into liquid nitrogen (LN_2). Leaves and calli were stored in LN_2 until processing for radiolabelled metabolites. Radiolabelled compounds were processed as described below (Platt et al., 1977).

Analysis of $^{14}CO_2$ metabolites

Soluble radioactive components were extracted from the leaf discs or calli as follows. Samples frozen in LN_2 were ground in 1 ml 80% (v/v) methanol in a dry ice/isopropanol bath, transferred to centrifuge tubes and centrifuged at 1000 RPM for 10 min (IEC centrifuge). The supernatant was collected and was further extracted at room temperature with 0.5 ml 20% (v/v) MeOH followed by a third extraction in 0.5 ml water. The supernatants were pooled and a 0.1 ml sample was processed to determine total ^{14}C-fixation into soluble components by acidification with acetic acid followed by partial drying under a nitrogen stream. Scintillation solution (Aquassure, New England Nuclear) was added for liquid scintillation counting on a Packard Tri-Carb 460C LSC.

To determine the ^{14}C fixation into methanol insoluble compounds (starch) the pellet was washed with acetic acid, dried overnight under vacuum and oxidized in a Packard Automatic Combustion Apparatus. $^{14}CO_2$ from the combusted sample was collected in Carbo-Sorb and scintillation counted in Permafluor (Packard Instruments Co.).

The supernatant was subjected to two dimensional paper chromatography in the standard system previously described (Pederson et al., 1977). First dimension solvent was phenol:H_2O:acetic acid (84:16:1) pH 4.1; second dimension solvent was butanol:propionic acid:H_2O (74:36:49). After location by radioautography on Kodak SB-5 film (3 weeks exposure), the labelled metabolites on the paper were analyzed as described in Larsen et al. (1981). The areas containing labelled metabolites were cut into small pieces, shaken for 1 h with 2.5 ml of water in scintillation vials, and the amount of activity determined by liquid scintillation counting after the addition of 18 ml Aquassure. Values of ^{14}C-labelling were obtained for hexose monophosphates, 3-P-glycerate, malate, aspartate, glycerate, alanine, sucrose, glucose, and fructose.

Enzyme assays

Phosphoenolpyruvate carboxylase (PEPC) was assayed by following spectrophotometrically the oxidation of NADH at 340nm in a coupled reaction with added excess malate dehydrogenase (MDH) (Smith, 1968; Mukerji and Ting, 1971; Ting and Osmond, 1973a,b). The assay mixture was TRIS, 50mM; $MgSO_4$; KCl, 50mM;

NADH, 0.1mM; PEP, 1.0mM; $NaHCO_3$, 1.3mM. Plant material was homogenized at 4 C in solution 'A' (TRIS, 50mM; $MgSO_4$, 10mM; ethylene glycol, 20% (v/v)) plus DTT (5mM) and insoluble PVP (1%, w/v) and centrifuged at 20,000 X g for 30 min. The supernatant was then assayed immediately for enzyme activity. PEPC isoenzymes were determined according to the methods of Ting and Osmond (1973a,b) but with the following modifications. Crude extract (5-7 ml) was added to a 1 X 25 cm DEAE-sephadex (A-25-120; Sigma Chemical Co.) ion exchange column previously equilibrated with solution 'A' plus DTT (5mM). The column was eluted with a linear KCl gradient of from 0 to 0.3M. Fractions (0.4 ml) were collected and analyzed for PEPC activity. Apparent Michaelis constants for PEP were calculated according to the procedure of Wilkinson (1961) using a FORTRAN program developed for the VAX 11/780.

For the assay of other enzymes, proteins were extracted in HEPES (50 mM), EDTA (2 mM), insol-PVP (1% w/v), Na Ascorbate (20 mM) and centrifuged at 20,000 x g for 30 min. The supernatant was assayed immediately for enzymatic activity. For the assay of AST and ALT, pyridoxal phosphate (20 ug ml^{-1}) was added to the extraction mixture.

Aspartate- and alanine aminotransferase activity (AST and ALT respectively) were measured in crude extracts (prepared as described above) by coupling with added MDH in excess (4 Units ml^{-1}) for AST or lactate dehydrogenase (LDH) in excess (4 Units ml^{-1}) for ALT. The assay mixture consisted of HEPES, 50mM, pH 8.0; EDTA, 2 mM, NADH, 0.2 mM; pyridoxal phosphate, 10ug ml^{-1}; 2-

oxoglutarate, 2.5 mM. For the assay of AST, aspartate (2.5 mM) was added to the reaction mixture. For the assay of ALT, alanine (10 mM) was added. Final volume of the reaction mixture was 1 ml. Activities of both AST and ALT were assayed spectrophotometrically by following the oxidation of NADH at 340nm.

NADP-'malic enzyme' (-ME), NAD-ME, NAD-MDH and NADP-MDH were assayed in crude extracts obtained in the same manner as for the aminotransferase reactions. Assays were performed in the same basic medium (HEPES, 50 mM; EDTA, 2 mM; pH 8.0) with the following additions. For NADP-ME assays $MgCl_2$ (5 mM), malate (2.5 mM) and $NADP^+$ (0.25 mM) were added. For NAD-ME assays $MnSO_4$ (5 mM), malate (5 mM), NAD^+ (0.2 mM) and Co-A (75 uM) were added. Assays of both malic enzymes followed the increase in absorbance at 340nm. NAD-MDH was assayed with the addition of oxaloacetic acid (OAA, 0.5 mM) and NADH (0.2 mM) by following the decrease in absorbance at 340nm. NADP-MDH was assayed with the addition of OAA (0.5 mM) and NADPH (0.2 mM) by following the decrease in absorbance at 340nm (Johnson and Hatch, 1970). Activity is expressed as umole NADHox (mg protein)$^{-1}$ min^{-1} or umole NADHox (mg chlorophyll)$^{-1}$ min^{-1}. (For -ME assays substitute NADred for NADHox.)

Chlorophyll and microscopy

Chlorophyll content of leaves and callus was determined in 80% (aq. v/v) acetone according to the methods of Bruinsma (1963). For comparison to $^{14}CO_2$ experiments, identical non-

labelled leaves or calli were used for chlorophyll determinations. Protein was determined according to the method of Bradford (1976) using reagents obtained from Bio-Rad (Richmond, CA).

Tissues for sectioning for light microscopy were fixed in 10% acrolein overnight at 4 C, dehydrated in methyl cellosolve/ethanol and embedded in glycol methacrylate according to Feder and O'Brien (1968). Sections were made at 2-5 uM thickness on a Sorvall JB-4 microtome and stained with 0.05% Toluidine Blue plus 0.1 %BHT for 2-5 minutes. Photomicrographs were made on an Olympus microscope equipped with a Nikon Microflex Model EFM camera.

RESULTS

$^{14}CO_2$ fixation studies

Analysis of pulse-chase experiments with mid-log phase callus tissues (Fig. 1) shows that at the beginning of the chase period approximately 44% of the total fixation was in sugars and sugar phosphates, and 9% was in the C_4-acids malate and aspartate. The amount of label in the C_4-acid pool did not decrease substantially over the 3 minute chase. Alanine contained about 15% of the label at the beginning of the chase and decreased to 11% after 3 minutes. Label in sucrose increased from 7.2% to 11%. Citrate and glutamate remained unchanged throughout the chase (Table 1).

Analysis of pulse-chase experiments with older regenerated plants (Fig. 2) shows a different pattern of photosynthetic $^{14}CO_2$ labelling. C_4-acids made up the predominant percentage of label at the beginning of the chase (44.1%), decreasing to 35.2% after 3 min. Sugars (glucose and sucrose) increased from about 5% to 9% of the total. Sugar phosphates increased only slightly from 11.5% to 13.9%. Alanine increased from 6 to 10.1%. Other intermediates decreased slightly during time in the chase (Table 2). The distribution of label in TCA cycle intermediates was negligible.

PEPC isoenzymes

PEPC of parent and young regenerated E. degeneri plant leaves exists in at least two forms as defined on DEAE-sephadex (Fig. 3,4,5). One form elutes at a low ionic strength and has a high K_m [PEP] (0.50 mM) (Fig. 5; designated PEPC I), another form elutes at a higher ionic strength and has a low K_m [PEP] of approx. 0.10 mM (PEPC II) (Fig 5). It can be seen from the coincidence of elution profiles that both leaves and young regenerated plants contain both forms of PEPC (Fig. 4). Callus tissue however, contains only one form of PEPC as defined on DEAE-sephadex (Fig. 4). K_m [PEP] (Fig 5) was determined to be 0.10 mM. Based on activity, the amount of PEPC I in leaves is approx. 19 X the amount of PEPC II.

Enzyme assays

Tables 3 and 4 list the activities of specific C_4-related enzymes as a function of chlorophyll or protein (respectively) for P10 callus, young regenerated plant leaves ('YRP'; shoots with fewer than 3 leaf pairs), old regenerated plant leaves ('ORP'; shoots with 8 to 10 leaf pairs), and adult plant leaves. When related to the amount of chlorophyll in the tissue (Table 3) the following trends can be seen. Aminotransferase (aspartate and alanine) activity varied little between callus, regenerated plants and adult leaves. Older regenerated plants showed a lower ALT level compared to adult leaves (4.38 vs 17.7). NAD-MDH activity was high in callus and young regenerated leaves but was considerably lower in older regenerated leaves and adult leaves.

NADP-MDH increased in activity from callus through young regenerated plants to adult leaves, but as for ALT, it was lower in activity in older regenerated plants. NADP-ME increased from a very low activity in callus (0.26) to a high value in adult leaves (10.9). Little or no detectable NAD-ME activity was found in any tissue.

When expressed as a function of protein content (Table 4), the same trends for enzymatic activity can be seen, but with the following exceptions. ALT activity was not lower in older regenerated plants. AST in adult leaves shows almost a 4-fold increase in activity compared to P10 callus. NAD-MDH has the same activity in callus, regenerated leaves and adult leaves.

Anatomy

The callus tissue of E. degeneri is composed mostly of parenchymatous cells surrounding 'islands' of meristematic cells (Ruzin, 1984a). Numerous chloroplasts are present, as well as seemingly isolated vascular bundles. Notably, no Kranz anatomy, bundle sheath cells, or dimorphic chloroplasts were found in callus grown on either shoot-forming or non-shoot-forming media.

In leaves of older regenerated plants Kranz anatomy is present (Fig. 6b,c), but several features are different from that found in adult leaves. There are fewer chloroplasts in bundle sheath cells of older regenerated plant leaves compared to adult leaves, and at the level of the light microscope, the chloroplasts of ORP bundle sheath cells have a different morphology than those of adult plant leaves. Dark-staining

bodies were found within some chloroplasts of bundle sheath cells of ORPs (Fig. 6c, arrow). Such objects were not found within chloroplasts of bundle sheath cells of adult leaves (cf. Fig. 6c,d). Bundle sheath chloroplasts in adult leaves are obviously different in morphology from mesophyll cell chloroplasts and are centripetally located (Fig. 6d). development and extent of palisade parenchyma and Kranz mesophyll (Fisher and Evert, 1982) in regenerated leaves is less than in mature leaves (cf. Fig. 6a,b).

DISCUSSION

It is generally believed that C_4 photosynthesis is dependent on, among other things, the unique physical arrangement of the leaf cells of C_4 plants, ie. Kranz anatomy (Hatch and Osmond, 1976, Laetsch, 1974). The localization of carboxylating and decarboxylating enzymes in either leaf mesophyll or bundle sheath cells (Chen et al., 1973; Gutierrez et al., 1974; Hatch and Osmond, 1976), the presence of 'specialized' chlorophast structures (peripheral reticulum) in bundle sheath cells (Laetsch, 1971, 1974) and the lack of an unequivocal demonstration of a C_4 plant without Kranz anatomy, all suggest that the C_4 pathway is dependent on Kranz anatomy. Some workers, however (Kennedy, 1976; Kennedy et al., 1977) have stated that the C_4 pathway was present in the homogeneous cells of callus tissues derived from C_4 plants and thus proposed that C_4 photosynthesis may not be dependent on Kranz anatomy.

The purpose of this research was to study the pathways of photosynthetic carbon metabolism in callus cultures of a C_4 plant, and to relate that to the observed photosynthesis in the parent and regenerated plants. The assumption that since callus cultures lacked Kranz anatomy then they would also lack true C_4 photosynthesis was shown to be correct for the Hawaiian C_4 plant Euphorbia degeneri. It was also shown that the C_4 pathway of photosynthesis was absent from callus but was 'regenerated' along with regeneration and maturation of shoots from callus. The hypothesis that C_4 photosynthesis is obligately related to the

presence of Kranz anatomy is strengthened by this research.

Definitive proof of C_4 photosynthesis is to demonstrate the transfer of radiolabelled carbon from C_4-acids to RPP-cycle intermediates (Hatch and Osmond, 1976; Hatch and Slack, 1966). In E. degeneri callus, C_4-acids contain 5-10% of the total ^{14}C-label after 2 minutes of incubation with $^{14}CO_2$, however, this amount of label does not decrease with time during incubation in 'cold'CO_2. Since loss of label from C_4-acids concomitant with increased label in RPP-cycle intermediates is indicative of transfer of label from C_4-acids (Hatch and Slack, 1966) no such transfer occurs in E. degeneri callus. Kennedy et al. (1977) stated that green callus of the C_4 dicot Portulaca oleracea does exhibit the C_4 cycle. They based their conclusions on the high percentage of label in C_4-acids after short incubation times in $^{14}CO_2$ and the apparent loss of label from C_4-acids during the chase peroid. However, in P. oleracea the loss of label from C_4-acids decreases from about 37% to 27% of the total labelled compounds with no further decrease throughout the two minute chase. Even after a two minute chase, substantial amount of label remained in C_4-acids in P. oleracea callus indicating that transfer of label from C_4-acids to RPP-cycle intermediates was incomplete. This is in sharp contrast to what Hatch and Osmond (1976) reported for Zea mays leaves and I have reported for Euphorbia degeneri leaves (Ruzin, 1984b). In these and other cases (Hatch, 1976), the percent of label in C_4-acids decreases from an initial value of 30-40% to less than 5% during a 1 to 2 minute chase indicating almost total transfer of label to RPP-

cycle intermediates.

Furthermore, other workers have demonstrated a pattern of $^{14}CO_2$ labelling in callus cultures derived from C_3 plants that is similiar to that seen in C_4 plant tissue cultures. Seeni and Gnanam (1982) and Nishida et al. (1980), demonstrated labelling of C_4-acids (respectively 15% and 43% of the total label) after incubating green tobacco cultures with $^{14}CO_2$. Both research groups however, concluded that the RPP-cycle as well as β-carboxylation (catalyzed by PEPC) were present in suspension cultured cells. Tissue cultures of CAM plants have also been reported to fix a significant percentage of $^{14}CO_2$ into C_4-acids. Seeni and Gnanam (1980, 1983) using Chamaecereus sylvestrii and Gisekia pharnaceoides L. suspension cultures showed (respectively) 18% and 22% of the total label in the C_4-acid malate after short periods of $^{14}CO_2$ incubation. In all cases though, the majority of label was found in RPP-cycle intermediates indicating, as suggested by Seeni and Gnanam (1982), that C_3 photosynthesis as well as β-carboxylation exist simultaneously in callus and suspension cultures of CAM plants.

The pattern of loss of label from regenerated plants of E. degeneri suggests that an intermediate pattern of photosynthetic carbon metabolism exists in leaves of older plantlets compared to either callus or (significantly) adult plant leaves. As in adult leaves, the primary labelled intermediates after one minute of $^{14}CO_2$ incubation are C_4-acids (malate particularly), but the loss of label from malate is not nearly as rapid or to the same extent as that which occurs in leaves of adult plants. In older

regenerated plants, label in C_4-acids decreases from 55% to only 35% even after 3 minutes of chase. The loss of label from C_4-acids in leaves of older regenerated plants is simultaneous with an increase in label in RPP-cycle intermediates (primarily hexose monophosphates) and sugars. In comparison to adult plant leaves (Ruzin, 1984b) older regenerated plant leaves exhibit characteristics of a 'sluggish' C_4 cycle in that transfer of carbon from C_4-acids to RPP-cycle intermediates exists, but at an attenuated rate and extent.

The pattern of photosynthetic carbon labelling in callus and regenerated plant leaves of E. degeneri may be explained by studying the activities of enzymes related to the C_4 pathway. Of particular interest are the activities of NADP-ME and NAD-MDH. NADP-ME is the malate decarboxylating enzyme found in the bundle sheath cells of NADP-ME-type C_4 plants (Gutierrez et al., 1974; Hatch et al., 1975; Hatch and Osmond, 1976) of which E. degeneri is one (Pearcy et al., 1982; Ruzin, 1984b). In callus the activity of NADP-ME is very low compared to that found in the adult plant leaf. In regenerated plants NADP-ME activity varies from 1/4 to 1/2 of that found in the adult leaf (based as a function of amount of chlorophyll). The low activity of NADP-ME present in callus cultures precludes the decarboxylation of malate via the usual way found in C_4 plant leaves. In fact, as was demonstrated by the ^{14}C-labelling experiments in this report, malic acid (plus aspartate) seem not to be decarboxylated at all (over a three minute chase) and thus C_4 photosynthesis would not be functional in callus tissues. The lower activity of NADP-ME in regenerated

leaves compared to adult plant leaves suggests that a lower amount of C_4-acid decarboxylation is present in those leaves. This is consistent with the $^{14}CO_2$-fixation kinetics since they also showed a lower rate of C_4-acid decarboxylation.

The activity of NADP-MDH in callus is very low. This is interesting since NADP-MDH mediates the formation of malate from OAA in mesophyll cells of NADP-ME-type C_4 plants (Gutierrez et al., 1974; Hatch and Osmond, 1976). The low activity of NADP-MDH would suggest that a low rate of malate formation would be present in callus but, as was shown, malate is synthesized rapidly and in significant amounts. NAD-MDH however shows a very high activity in callus and a decreased activity in regenerated plants and adult leaves. It is known (Lehninger, 1975) that NAD^+-specific MDH is an important enzyme in the oxidation of carbohydrates via the TCA-cycle, so it is probable that in E. degeneri callus the malate formed during $^{14}CO_2$ incubation is mediated by this 'metabolic' MDH rather than the C_4-photosynthesis form. Seeni and Gnanam (1982) found a similarly high activity of NAD-MDH in cultured peanut mesophyll cells but a very low activity in leaves. They also concluded that the high percentage of C_4-acid labelling was related to the metabolism of malate via the TCA-cycle. An increased activity of NADP-ME was reported by Holtum and Winter (1982) in the CAM plant Mesembryanthemum crystallinum L. after transition from C_3 photosynthesis to CAM. This CAM plant will photosynthesize using only the RPP-cycle if treated with low salt (<100 mM) or CAM if treated with high salt (200 mM). Holtum and Winter (1982)

reported, that under the conditions of low salt, both malic enzymes and malate dehydrogenases decreased to levels that are approximately 10% of the level found during the CAM phase (NAD-MDH halves in activity). The decrease in NADP-ME in callus and regenerated plants of E. degeneri is similiar to the above example, and supports the contention that callus is strictly C_3 and young regenerated plants exhibit both the C_3 and C_4 pathways.

The absence of the C_4 form of PEPC (PEPC I) in callus is further evidence for the lack of C_4 photosynthesis in that tissue. It has been demonstrated that C_4 plants synthesize at least two forms of PEPC (Ting and Osmond, 1973a,b; Goatly et al., 1975; Mukerji, 1977; Hague and Sims, 1980; Hayakawa et al., 1981; Holaday and Black, 1981) and that each form has different kinetic properties. Ting and Osmond (1973b) surveyed C_3, C_4, and CAM plants and concluded that the isoenzymes of PEPC contribute to different metabolic pathways. One form (high K_m [PEP]; PEPC I) is found only in C_4 plants and is responsible for the synthesis of C_4-acids as photosynthetic intermediates (ie. for transfer of CO_2 from mesophyll to bundle sheath cells) and another form (low K_m [PEP]; PEPC II) is responsible for the synthesis of C_4-acids as photosynthetic products (discussed below). These two forms are present in E. degeneri leaves (Fig. 3), but only one form (PEPC II) is present in callus (Fig. 4). Given the evidence described above, it seems likely that PEPC found in callus is 'metabolic' PEPC and is not involved in C_4 photosynthesis.

Regenerated plants of any age synthesize both forms of PEPC. PEPC I and II were identified from young regenerated shoots and

older regenerated plants, as well as adult plant leaves (data not shown). The elution profiles and K_m[PEP] values are consistent with what Ting and Osmond (1973a,b) characterize as the C_4- and C_3-form. The C_4 form appears to be synthesized concomitant with the development of leaf form and not necessarily with the development of Kranz anatomy, since young regenerated plant leaves lack Kranz anatomy (data not shown). The appearance of the C_4 form of PEPC during greening has been reported for sugar cane (Goatly and Smith, 1974) and maize (Hague and Sims, 1980; Hayakawa et al., 1981). The appearance of PEPC I during greening is the result of de novo synthesis (Sims and Hague, 1981; Hayakawa et al., 1981) rather than a modification or activation of PEPC II as was suggested by Goatly and Smith (1974). Since a 'new form' of PEPC is synthesized during transition from C_3 to CAM in Mesembryanthemum crystallinum L. (von Willert et al., 1976), PEPC I seems to mark the transition to CAM as well. It is probable, therefore, that the appearance of PEPC I during the development of E. degeneri shoots from callus is the result of de novo synthesis of a specific C_4 isoenzyme of PEPC involved in C_4 photosynthesis.

The presence of PEPC I does not necessarily mean that a functional C_4 photosynthetic pathway would be present. As was discussed above, enzyme and $^{14}CO_2$ fixation kinetics of older regenerated shoots suggests that an impared C_4 photosynthesis was present. Anatomical observation may provide some insight. Leaves of regenerated shoots (with fewer than 10 leaf pairs) have an anatomy that is somewhat modified from adult leaves.

Mesophyll not closely related to vascular bundles (non-Kranz mesophyll) is highly developed, while Kranz mesophyll and bundle sheath cells seem to be underdeveloped (relative to adult leaves). At the level of the light microscope, the bundle sheath cells are somewhat like mesophyll cells in that they contain chloroplasts that are similiar to those found in mesophyll cells. Although causality is unknown, it appears that leaves of older regenerated plants exhibit an intermediate anatomy and an intermediate photosynthetic pathway. The appearance of PEPC I simultaneous with the regeneration of leaves would be expected since PEPC (I) synthesis in C_4 plants is restricted to mesophyll cells (Hatch and Osmond, 1976) and mesophyll cells of older regenerated leaves seem to be fully developed. However, specialization of bundle sheath cells of older regenerated plants did not appear to be complete, and thus the intermediate C_4 photosynthetic cycle that would be predicted was present.

Despite the operation of both the RPP-cycle and β-carboxylation in callus cultures and regenerated plants of E. degeneri, it appears likely that true C_4 photosynthesis is not operational in callus and only partially so in leaves of young regenerated plants. In callus, malate formation via β-carboxylation of PEP is not indicative of C_4 photosynthesis but rather appears to be involved in metabolic events leading to either cell division or organogenesis. Plumb-Dhindsa et al. (1979) have argued that PEP carboxylation leading to malate formation is a response of the tissue to a requirement for NADPH (via malate oxidation) for reductive biosynthesis of cellular

components during organogenesis. Other workers (Nesius and Fletcher, 1975; Nato and Mathieu, 1978; Seeni and Gnanam, 1982) elaborated on this hypothesis and suggested that malate formation in rapidly dividing tissue cultures was a response to depletion of TCA-cycle intermediates via amino acid synthesis, and thus PEP carboxylation functions anapleurotically. Yet another function of PEPC in tissue cultures may be for the synthesis of malate as an osmoticum (Plumb-Dhindsa et al., 1979; Dhindsa et al., 1975) or as a pH-stat (Davies, 1973,1979).

Older regenerated leaves, on the other hand, do show C_4 photosynthesis. However, the transfer of CO_2 from C_4-acids to RPP-cycle intermediates is slow, possibly due to insufficient amounts of NADP-ME and other 'factors' normally present in bundle sheath cells. The bundle sheath cells of regenerated leaves are incompletely developed and thus leaf anatomy is somewhat like a C_3 plant. A phenomenon similiar to regenerated plants is found in C_3-C_4 intermediate species of Panicum. Rathnam and Chollet (1979) studied the photosynthetic intermediate species Panicum milioides and concluded that the intermediacy of photosynthesis was due to the localization of the RPP-cycle in both bundle sheath and mesophyll cells. Furthermore, there is a gradation of Kranz anatomy to non-Kranz anatomy among Panicum species that corresponds to the degree of C_3-C_4 intermediacy (Brown et al., 1983). In Panicum as in E. degeneri regenerated plants there seems to be a strong correlation between C_4 photosynthesis and Kranz anatomy.

A gradation exists in the C_4 dicot E. degeneri from C_3

photosynthesis and non-Kranz anatomy in callus cultures to a photosynthetic and anatomically intermediate state in young regenerated plants to true C_4 photosynthesis and Kranz anatomy in adult leaves. These correlative phenomena strongly support the hypothesis that C_4 photosynthesis is obligatorily related to the presence of Kranz anatomy. Although Kranz anatomy seems to be a necessary component of C_4 photosynthesis, its presence should not be construed as being definitive proof of its presence.

Table 1.

Distribution of labelled intermediates over time after $^{14}CO_2$ incubation in the light by callus of E. degeneri

	% of total ^{14}C-incorporation				
	10*	30	60	90	180
CIT	1.0	0.7	0.8	0.7	1.0
GLU	8.6	12.4	9.7	9.1	7.6
ALA	14.7	13.9	15.7	11.4	11.1
SUC	7.2	7.4	8.2	9.0	11.0
STARCH	1.0	0.7	0.9	0.8	0.7

*= time in chase in seconds

Table 2.

Distribution of labelled intermediates over time after $^{14}CO_2$ incubation in the light by leaves of regenerated E. degeneri.

% of total ^{14}C-incorporation

	15*	30	60	120	180
GLU	9.5	8.9	6.7	3.9	3.1
ALA	6.0	5.8	9.0	7.7	10.1
SUC	1.6	0.8	1.5	3.3	6.0
STARCH	7.7	5.0	6.8	9.1	10.9

*= time in chase in seconds

Table 3

Comparison of activities of certain C_4 enzymes in callus, regenerated plants and parent plants of E. degeneri.

Enzyme activity expressed as umoles NADHox (mg chlorophyll)$^{-1}$ min^{-1}

*	AST	ALT	NAD-ME	NADP-ME	NADP-MDH	NAD-MDH
P10	4.74	25.1	0	0.26	0.62	268.4
YRP	1.76	37.4	0.07	6.04	3.60	259.2
ORP	4.72	4.38	0	2.29	0.13	48.1
APP	5.92	17.7	NA	10.9	7.9	80.8

*:P10= P10 Callus (shoot forming medium); YRP= young regenerated plant having fewer than 3 fully expanded leaf pairs; ORP= older regenerated plant having between 8 and 10 leaf pairs; APP= adult parental plant.

NA= not assayed.

Table 4

Comparison of activities of certain C_4 enzymes in callus, regenerated plants and parent plants of E. degeneri.
Enzyme activity expressed as umoles NADHox (mg protein)$^{-1}$ min^{-1}

*	AST	ALT	NAD-ME	NADP-ME	NADP-MDH	NAD-MDH
P10	0.244	1.11	0	0.011	0.27	11.68
YRP	0.250	1.52	0.003	0.245	0.146	10.51
ORP	1.05	0.97	0	0.507	0.028	10.69
APP	0.90	0.48	NA	0.469	4.23	10.09

*:P10= P10 Callus (shoot forming medium); YRP= young regenerated plant having fewer than 3 fully expanded leaf pairs; ORP= older regenerated plant having between 8 and 10 leaf pairs; APP= adult parental plant.
NA= not assayed.

Figure 1. Changes in the distribution of radioactivity of metabolites over time after transfer of P10 calli from $^{14}CO_2$ to air. After 2 minutes in $^{14}CO_2$ the calli were allowed to photosynthesize in air minus radioactivity without changing the distance from the light source. At intervals, individual calli were removed and analyzed as described in the Methods section. (*), glucose; (X), hexose monophosphates; (O), malate; (Δ), aspartate.

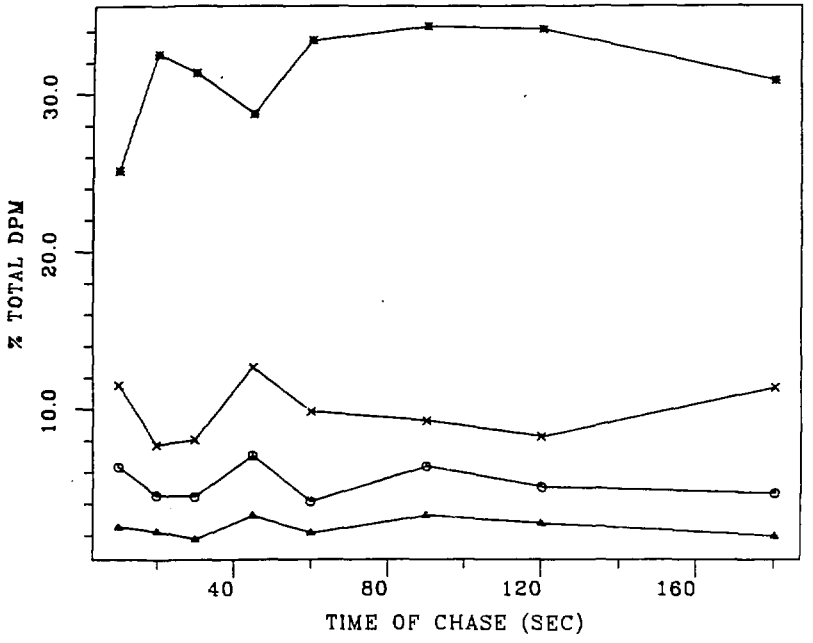

% TOTAL DPM
30.0
20.0
10.0
40
80
120
160
TIME OF CHASE (SEC)

Figure 2. Changes in the distribution of radioactivity of metabolites over time after transfer of leaves of older regenerated plants from $^{14}CO_2$ to air. After 1 minute in $^{14}CO_2$ the leaves were allowed to photosynthesize in air minus radioactivity without changing the distance from the light source. At intervals, leaf pairs were removed and analyzed as described in the Methods section. (✱), C_4-acids; (△), hexose monophosphates; (✳), asparate; (○), 3-phosphoglycerate, (X), sugars.

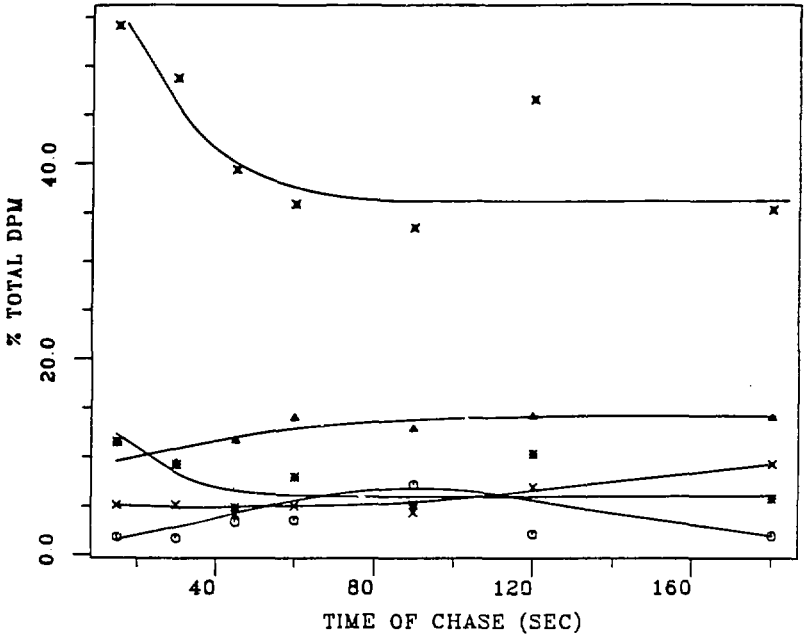

% TOTAL DPM
0.0
20.0
40.0
40
80
120
160
TIME OF CHASE (SEC)

Figure 3. The elution profile of PEP carboxylase activity in the adult leaf (O) of E. degeneri on DEAE-sephadex using a linear KCl gradient (0-0.3M). The profile shows both PEPC I and II. (Δ), KCl gradient. PEPC activity expressed as umoles NADHox $.05ml^{-1}$ 5 min^{-1}.

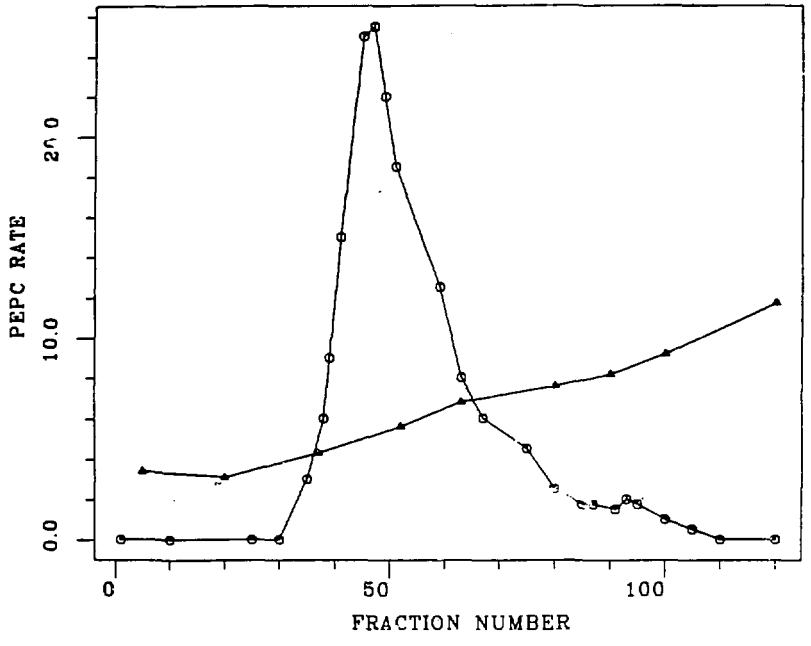

PEPC RATE
0.0
10.0
0
50
100
FRACTION NUMBER

Figure 4. The elution profiles of PEP carboxylase activity in adult leaf, young regenerated plant leaf, and P10 callus on DEAE-sephadex using a linear KCl gradient (0-0.3M). Values for regenerated plant leaf and callus are multiplied X 8. (O), leaf; (x), regenerated plant leaf; (*), P10 callus; (Δ), KCl gradient

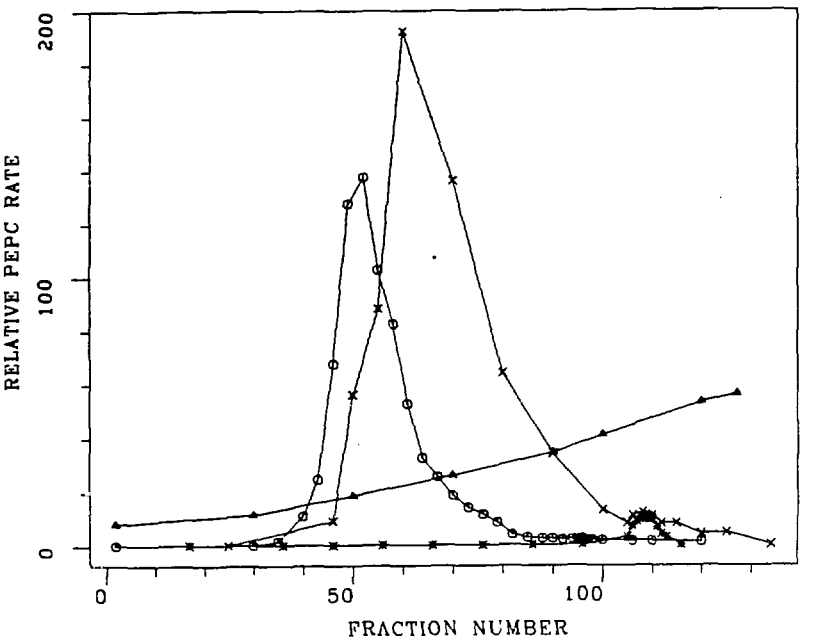

RELATIVE PEPC RATE
200
100
0
0
50
100
FRACTION NUMBER

Figure 5. Double reciprocal plots for major PEPC peaks of adult leaf, young regenerated leaf, and P10 callus with respect to PEP. (Δ), adult leaf PEPC; (X), young regenerated leaf PEPC; (O), P10 callus.

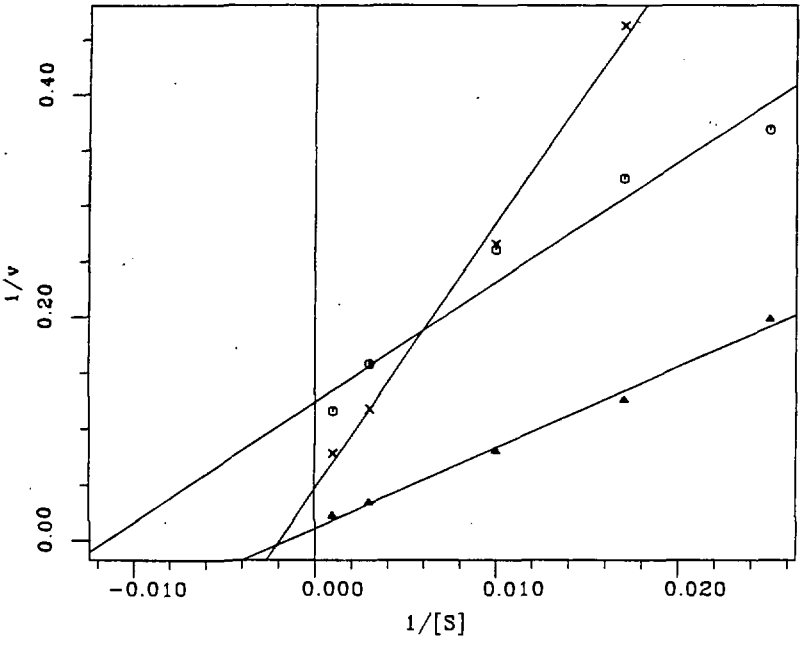
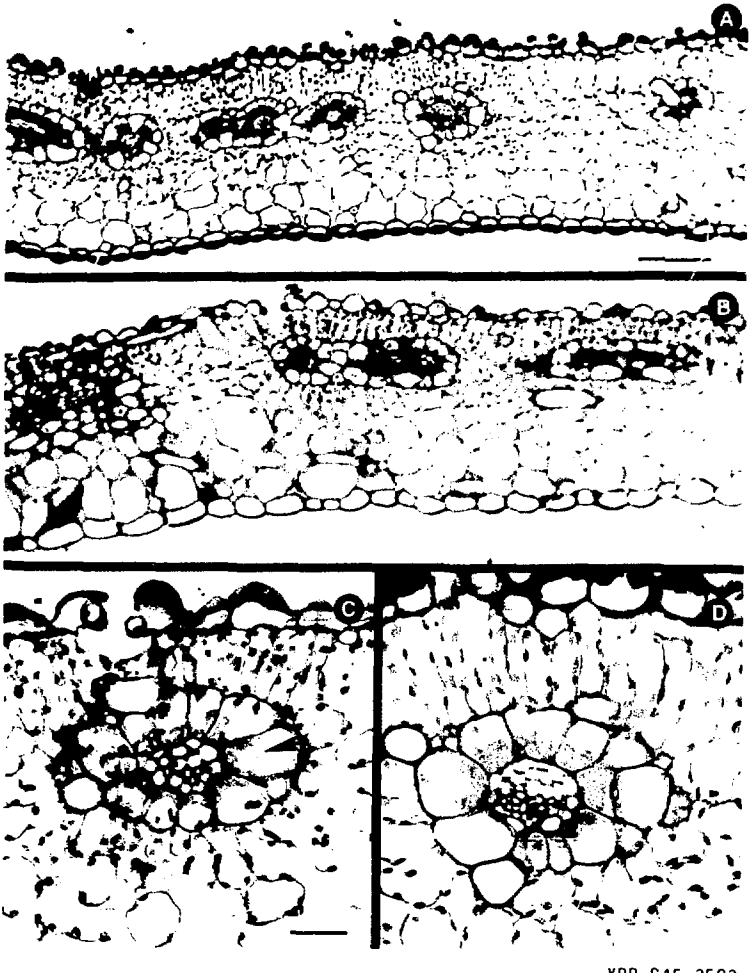

0.40
0.20
0.00
1/v
-0.010
0.000
0.010
0.020
1/[S]

Figure 6. Transections of adult (A,D) and older regenerated plant leaf (B,C). Scale for Figs.5a,b (in a) = 100um. Scale for Figs. c,d (in c) = 25um. Arrow in 5c discussed in text.



XBB 845-3523

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All in all it's just another

brick in the wall

-Pink Floyd-