THE EFFECT OF TWO DIFFERENT KILLING TECHNIQUES ON EARLY ^{14}C-LABELLED NITROGENOUS PHOTOSYNTHETIC PRODUCTS IN LEAVES OF *Zea mays* L.

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1977.

ABSTRACT

Alternative techniques of killing leaves with 80% ethanol or freeze killing with liquid nitrogen was found to affect the level of ^{14}C incorporation into the amino acids in leaves of Zea mays . The degree and nature of this effect , however , was markedly affected by both the light and the level of nitrate in which plants were . what ?

INTRODUCTION

During plant biochemical and physiological investigations, a number of techniques have previously been used for killing plant material at specific time intervals during investigation, in order to determine whether or not any differences in ^{14}C-labelled nitrogenous photosynthetic products are evident with different killing techniques.

Besides the significance of a specific method for killing plant material, time is also of importance when studying early labelling of photosynthetic products. In short term $^{14}CO_2$ feeding experiments, the exposure time to labelled carbon dioxide usually consists of approximately 5 to 15 seconds (Hatch & Slack, 1967; Kennedy & Laetch, 1973; Kennedy & Williams, 1977) and consequently, differences in even fractions of a second before killing procedures are brought about, may result in misleading data.

In a recent report, Kennedy & Williams (1977) investigated four different killing methods in two C_4-photosynthesising plants, namely Portulaca oleracea and Zea mays: These authors demonstrated significant alterations in the distribution of ^{14}C among early labelled photosynthetic products. Hatch & Slack (1966) used two killing techniques in their study on the C_4 pathway in sugar-cane (Saccharum sp.) and arrived at the conclusion that both methods gave similar results.

In the present study, two different killing techniques (plunging of material into cold 80% ethanol, and plunging into liquid nitrogen) were employed in order to determine whether significant changes in early ^{14}C-labelled nitrogenous compounds occurred in leaves of Zea mays L.

MATERIALS AND METHODS

Plant culture

Zea mays was selected for this investigation. Plants were grown under two different light regimes; a low light regime at 35000 lux light intensity, and a high light regime at ±95000 lux light intensity. Two different concentrations of nitrate (50 and 200 parts.10^{-6}) present in the feeding solution, were supplied to the root systems of maize plants grown under the different light regimes. Approximately 30 ml / pot of appropriate feeding solution was given every second day (see APPENDIX for composition of nutrient solutions).

Seed germination, growth and $^{14}CO_2$ feeding experiments performed on plants grown under a low light regime, were conducted in a growth chamber under controlled environmental conditions : 20°C day and 15°C night temperatures; day length of 14 hours at 35000 lux light intensity; and 80% relative humidity. Eight days after emergence, seedlings were transplanted into polystyrene pots (7,5 cm diameter x 9,5 cm height) containing vermiculite. Half of the experimental plants were placed in low light conditions in the growth chamber, while the other plants were placed in a greenhouse where they were subjected to sunlight conditions. $^{14}CO_2$ feeding experiments were also performed on maize plants grown under sunlight conditions (±95000 lux), at 76% relative humidity and a temperature of 22,6°C.

Supply of $^{14}CO_2$ to leaves

$^{14}CO_2$ feeding experiments were performed in duplicate, for each feeding level and at light intensity (low and

high) to which the plants had been subjected.

Third or fourth leaves of 27-day-old maize plants were used in all experiments. The fed leaf was introduced into the feeding chamber (Plate I#2) ; the feeding chamber was sealed, and the system was allowed an equilibration period of 10 minutes. After equilibration, the fed leaf was exposed to $^{14}CO_2$ by acidification of 100 µl sodium ^{14}C carbonate (activity 50 µ Ci) with 1 ml 10% v / v lactic acid. S.A.? Following a $^{14}CO_2$ assimilation period of 60 seconds, the fed leaf was immediately excised and removed from the feeding chamber. Fresh weight was recorded and $^{14}CO_2$ fixation was terminated using two different leaf-killing techniques :

i) Plunging into cold 80% ethanol (approximately 50 ml 80% ETOHETOH to 2 grams leaf material).

ii) The alternative killing technique was by plunging leaf material into liquid nitrogen before transferring to 80% ethanol.

Extraction of leaf material

Leaf samples in ethanol were homogenized with an ultra-Turrax homogenizer. The homogenate was allowed to stand in a cold-room (0^oC) to facilitate extraction of amino acids. The extract was filtered through Whatman No. 1 filter paper, and then reduced by evaporation using an air stream, in order to avoid all forms of heating which could alter or destroy the amino compounds. The extract was ev evaporated to below 10 ml and then made up to 10 ml with distilled water. Known volumes of this extract were then run on a BECKMAN 120 - C amino acid analyzer using a lithium buffer system (Lewis, 1975).

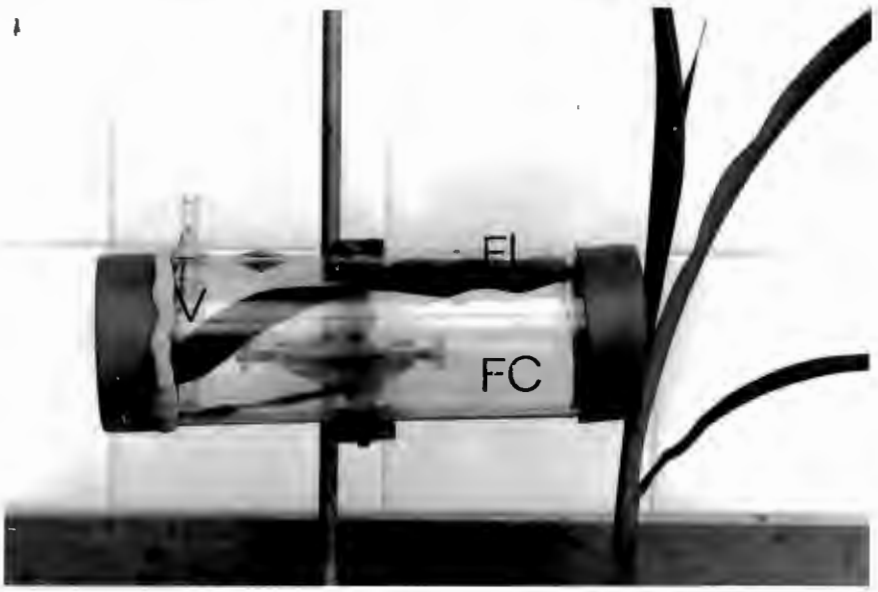

PLATE 1 : Apparatus used to expose leaves of *Zea mays L.* to $^{14}CO_2$

FC : Feeding chamber

FL : Fed leaf

V : Vial containing 100 µl $^{14}CO_3^=$

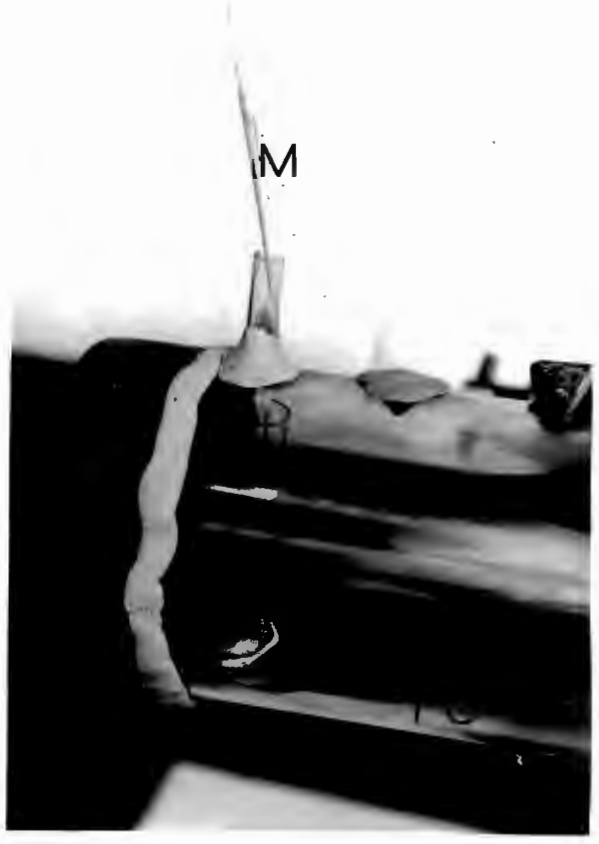

PLATE 2 : Illustrating the application of 10% lactic acid (v/v) in order to release $^{14}CO_2$ by acidification of sodium ^{14}C carbonate.

FC : Feeding chamber

FL : Fed leaf

V : Vial containing 100µl $^{14}CO_3^=$

M : Containing 1ml 10% lactic acid.
micro-pipette

^{14}C enrichments of the amino acids were estimated by splitting the effluent stream from the analyse column and passing the one half into a Beckman Discrete Sampler Unit, where the stream was mixed with a toluene-- Biosolve - PPO - POPOP cocktail, and then into a Beckman Beta - Mate liquid scintillation counter.

TABLE 1 The effect of killing with cold 80% ETHANOL under HIGH LIGHT INTENSITY (95 000 lux), on the incorporation of ^{14}C into early labelled amino compounds, in Zea mays L.

	200 Parts 10^{-6} Nitrate N				50 Parts 10^{-6} Nitrate N			
	DUPLICATE 1		DUPLICATE 2		DUPLICATE 1		DUPLICATE 2	
	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.
ASPARTIC ACID	0,18	4517,30	0,29	7150,08	0,04	4139,61	0,05	1375,05
THREONINE	0,03	-	0,05	-	0,03	-	0,03	-
SERINE	0,61	11,74	0,97	18,58	0,08	259,74	0,17	1944,12
GLUTAMIC ACID	0,65	54,70	1,04	86,58	0,41	96,67	0,57	1981,15
GLUTAMINE	0,47	-	1,23	-	0,01	-	0,02	-
GLYCINE	0,78	73,18	1,23	115,83	0,23	258,47	0,01	4283,85
ALANINE	0,70	44,91	1,11	710,80	0,85	132,45	1,03	10210,74

Leaf tissue was exposed to $^{14}CO_2$ for 60 seconds before harvesting. Experiments were conducted at a temperature of 22,6°C and 76% relative humidity.

TABLE 2 The effect of freeze killing with LIQUID NITROGEN under HIGH LIGHT INTENSITY (95 000 lux), on the incorporation of ^{14}C into early labelled amino compounds, in Zea mays L.

	200 Parts 10^{-6} Nitrate N				50 Parts 10^{-6} Nitrate N			
	DUPLICATE 1		DUPLICATE 2		DUPLICATE 1		DUPLICATE 2	
	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.
ASPARTIC ACID	0,02	3865,70	0,01	1198,10	0,06	1582,40	0,06	999,60
THREONINE	0,04	-	0,05	-	0,04	-	0,04	-
SERINE	0,44	-	0,33	-	0,02	-	0,25	-
GLUTAMIC ACID	0,56	-	0,68	-	0,55	-	0,60	-
GLUTAMINE	1,75	-	0,04	-	0,03	-	0,01	-
GLYCINE	0,97	28,30	0,81	-	0,65	24,20	0,82	20,90
ALANINE	0,11	139,93	0,03	162,20	0,09	46,10	0,05	112,20

Leaf tissue was exposed to $^{14}CO_2$ for 60 seconds before harvesting. Experiments were conducted at a temperature of 22,6°C and 76% relative humidity.

TABLE 3 The effect of killing with cold 80% ETHANOL under LOW LIGHT INTENSITY, on the incorporation of ^{14}C into early labelled amino compounds, in Zea mays L.

	200 Parts 10^{-6} Nitrate N				50 Parts 10^{-6} Nitrate N			
	DUPLICATE 1		DUPLICATE 2		DUPLICATE 1		DUPLICATE 2	
	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.
ASPARTIC ACID	0,25	67,08	0,27	41,62	0,05	740,74	0,01	3060,66
THREONINE	0,06	-	0,07	-	0,13	-	0,02	-
SERINE	4,94	-	0,05	-	0,19	-	0,12	-
GLUTAMIC ACID	1,53	-	1,08	-	0,63	69,39	0,48	70,01
GLUTAMINE	0,14	-	3,90	-	0,09	-	0,03	-
GLYCINE	0,16	-	0,25	-	-	-	0,05	-
ALANINE	0,15	35,12	2,14	7,65	1,17	297,75	0,78	322,42
ASPARAGINE	-	-	-	-	0,06	-	-	-

Leaf tissue was exposed to $^{14}CO_2$ for 60 seconds before harvesting. Experiments were conducted at a temperature of 20°C and 80% relative humidity.

TABLE 4 The effect of freeze killing with LIQUID NITROGEN under LOW LIGHT INTENSITY (34 000 lux), on the incorporation of ^{14}C into early labelled amino compounds, in Zea mays L.

	200 Parts 10^{-6} Nitrate N				50 Parts 10^{-6} Nitrate N			
	DUPLICATE 1		DUPLICATE 2		DUPLICATE 1		DUPLICATE 2	
	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.	u mol g^{-1} f.wt.	d $min^{-1}g^{-1}x10^{-3}$ f.wt.
ASPARTIC ACID	0,09	814,90	0,32	155,89	0,04	1990,61	o,05	1917,42
THREONINE	0,02	-	0,06	-	0,04	-	0,04	-
SERINE	0,22	155,84	0,34	27,12	0,20	-	0,20	-
GLUTAMIC ACID	0,61	65,07	1,86	4,84	0,80	-	0,90	-
GLUTAMINE	0,02	-	0,15	-	0,05	-	0,01	-
GLYCINE	0,16	660,18	0,22	88,38	0,14	49,20	0,14	33,58
ALANINE	0,32	1565,38	2,32	115,03	0,30	68,88	0,56	82,47
ASPARAGINE	-	-	0,04	-	-	-	-	-

Leaf tissue was exposed to $^{14}CO_2$ for 60 seconds before harvesting. Experiments were conducted at a temperature of 20°C and 80% relative humidity.

RESULTS

SECTION A

1. Comparison between the activity of early ^{14}C - labelled amino compounds (d min^{-1} g^{-1} f.wt x 10^{-3}) with differ ent killing techniques under HIGH LIGHT INTENSITY , (95000 lux) in maize plants fed with 200 part 10^{-6} and 50 parts 10^{-6} nitrate nitrogen supplied in the root systems.

The effects of killing leaf material of Zea mays L. with cold 80% ETHANOL, and LIQUID NITROGEN under HIGH LIGHT INTENSITY are displayed in TABLE 1 and TABLE 2 respectively.

1.1 Comparison between different nitrate feeding levels when leaf material was killed with cold 80% ETHANOL (TABLE 1).

An increase in the nitrate feeding level does not markedly alter the incorporation of ^{14}C - labelled into aspartate ; although greatest activity was observed at the nitrate nitrogen feeding concentration of 200 parts 10^{-6}. Conversely, ^{14}C- labelling decreased in serine , glutamic acid (glutamate) , glycine and alanine at a feeding level of 200 parts 10^{-6} as opposed to 50 parts 10^{-6} nitrate nitrogen.

1.2. Comparison between different nitrate nitrogen feeding levels when leaf material was killed with LIQUID NITROGEN (TABLE 2) .

With maize plants grown under both nitrate nitrogen feeding regimes , the amino acids serine and glutamic acid did not incorporate ^{14}C label when this method of terminating biological processes in the leaf material was used . No marked alteration in the incorporation of labelled carbon into

aspartate, glycine and alanine was evident ; however , activities for these amino acids were slightly higher when plants were fed with 200 parts 10^{-6} nitrate nitrogen .

1.3. Comparison between the effects of both killing techniques on maize plants treated with 200 parts 10^{-6} nitrate nitrogen.

It is evident that with both killing techniques the greatest quantity of labelled carbon is incorporated into aspartic acid (aspartate) . Ethanol treatment resulted in greater ^{14}C labelling into this amino acid than liquid nitrogen treatment. Glutamic acid and serine became labelled when leaf material was killed with ethanol , but not when killed by freezing . Glycine also incorporated labelled carbon more readily with ethanol killing than with liquid nitrogen killing . No conclusions can be drawn with different killing methods , due to variability in the results of replicate samples .

1.4. Comparison between the effects of both killing techniques on maize treated with 50 parts 10^{-6} nitrate nitrogen .

There is little difference between ^{14}C incorporation into aspartate when either one of the two killing techniques was used. Slightly higher levels of labelled carbon was incorporated into aspartate when ethanol treatment was used , as opposed to killing by freezing with liquid nitrogen . Glutamic acid and serine in plants fed with 50 parts 10^{-6} nitrate nitrogen , became labelled when leaf material was killed with cold 80% ethanol ; but not when killed by freezing with liquid nitrogen . Greater quantities of glycine and alanine were present in labelled form when et ethanol was used as opposed to freeze killing , in maize plants fed with 50 parts 10^{-6} nitrate nitrogen.

SECTION B

1. Comparison between activity of early ^{14}C-labelled amino compounds (d min $^{-1}$ g^{-1} f. wt. X 10^{-3}) with different killing techniques under LOW LIGHT INTENSITY (34000 lux) in maize plants fed with 200 parts 10^{-6} and 50 parts 10^{-6} nitrate nitrogen supplied via the erect systems .

The effects of killing leaf material of Zea mays L. with cold 80% ETHANOL , and LIQUID NITROGEN under LOW LIGHT INTENSITY , are displayed in TABLE 3 and TABLE 4 respectively .

1.1. Comparison between different nitrate nitrogen feeding levels when leaf material was killed with cold 80% ETHANOL(TABLE 3)

In plants grown under low light condition, an increase in the nitrate N feeding level appeared to reduce incorporation of ^{14}C into aspartate . Glutamic acid was labelled in plants fed with 50 parts 10^{-6} nitrate nitrogen , but absence of labelling was observed in plants fed with 200 parts 10^{-6} nitrate nitrogen . Greater amounts of ^{14}C-alanine were extracted from plants maintained on the low nitrate nitrogen feeding regime .

1.2. Comparison between different nitrate nitrogen feeding levels When leaf material was killed with LIQUID NITROGEN (TABLE 4)

With freeze killing of leaf samples [redacted] conditions of increased nitrate nitrogen also reduced ^{14}C incorporation into aspartate . Serine and Glutamic acid were labelled in plants fed with 200parts 10^{-6} nitrate N, but not in plants fed with 50 parts 10^{-6} nitrate N. After aspartate, alanine was the second amino acid that was most readily labelled with ^{14}C

Greater quantities of both ^{14}C alanine and ^{14}C glycine were extracted from 200 parts 10^{-6} nitrate N fed plants than from 50parts 10^{-6} nitrate N.

SECTION C

1. Comparison between the effects of early ^{14}C incorporation into amino compounds (d $min^{-1}g^{-1}$f.wt x 10^{-3}) of maize plants grown under different LIGHT intensities, and fed with 200 parts 10^{-6} and 50 parts 10^{-6} nitrate nitrogen supplied to the root systems.

1.1 Effects of HIGH and LOW LIGHT INTENSITIES when leaf samples were killed with cold 80% ETHANOL (TABLE 1 and TABLE 3)

Plants treated with both 200 parts 10^{-6} nitrate N and 50 parts 10^{-6} nitrate N incorporated greater quantities of ^{14}C activity, both qualitativily and quantitatively when under HIGH light intensity (95000 lux) as opposed to LOW light intensity (34000 lux).

1.2 Effects of HIGH and LOW LIGHT INTENSITIES when leaf samples were killed with liquid nitrogen (TABLE 2 and TABLE 4)

Contrasting with the qualitative increase under HIGH as opposed to LOW light intensity in ^{14}C incorporation observed in maize plants fed with 200 parts 10^{-6} nitrate N and killed with ETHANOL ; when the same nitrate feeding level was maintained

(200 parts 10^{-6}), but LIQUID NITROGEN was used to kill leaf material, it was found LOW light intensity,as opposed to HIGH light intensity, increased the range of amino acids that were labelled with ^{14}C . Quantitatively , HIGH light intensity increased ^{14}C incorporation into aspartic acid in plants fed with the higher nitrate nitrogen containing feeding solution. However , lower levels of ^{14}C-glycine and ^{14}C-alanine were observed in these plants ,when subjected to HIGH rather than LOW light conditions.

It is difficult to draw conclusions with regard to maize plants fed with 50 parts 10^{-6} nitrate nitrogen due to variability in the results of replicate samples . ^{14}C glycine content was greater , however , in both leaf samples killed by freezing with liquid nitrogen .

DISCUSSION OF RESULTS

When ETHANOL and LIQUID NITROGEN killing techniques were employed under HIGH LIGHT intensity, the most altered effect was the increase both qualitatively and quantitatively into the amino acids of ETHANOL killed leaf samples. Serine and glutamic acid became labelled with ^{14}C under HIGH LIGHT conditions, when biological activity was terminated with cold 80% ethanol, but not when freeze killing with liquid nitrogen was employed. Aspartic acid was the prime acceptor of newly fixed ^{14}C, derived from exogenous $^{14}CO_2$, in maize plants treated with both high and low nitrate nitrogen feeding levels, under HIGH LIGHT intensity. No definite conclusions can be drawn with regard to different ^{14}C incorporation into Alanine, when different killing techniques are used under HIGH LIGHT, due to the large discrepancy in results of replicate samples. However, there appears to be enhanced activity of ^{14}C-alanine in plants fed with 50 parts.10^{-6} nitrate nitrogen, when killed with ethanol as opposed to freeze killing with nitrogen.

Contrasting with what was observed in experiments conducted under the high light intensity regime (Table 1 and Table 2), the most altered effect under the LOW LIGHT regime was the OVERALL qualitative and quantitative increase in ^{14}C incorporation of leaf samples killed with LIQUID NITROGEN as opposed to killing with ethanol. Exceptions with regard to this finding, include the activity of ^{14}C-alanine which was higher in maize plants fed at the lower nitrate nitrogen feeding level (50 parts.10^{-6}). Under conditions of LOW LIGHT intensity, radioactivity incorporated into glycine also appears to be influenced by killing techniques, in that no glycine was labelled when killed with 80% ethanol. Since glycine WAS labelled when leaves of maize plants grown under HIGH LIGHT intensity were killed with 80% ethanol, it is evident that this difference can be attributed to the effect of the two light intensities to which the plants were subjected.

Freeze killing with LIQUID NITROGEN at high light intensity resulted in increased ^{14}C incorporation into aspartic acid in plants fed with 200 parts.10^{-6} nitrate nitrogen; however, all other ^{14}C levels of amino acids are reduced at high light

intensity and high nitrate feeding level. In freeze-killed plants fed with 50 parts 10^{-6} nitrate nitrogen there was greater ^{14}C activity in all labelled amino acids at the lower light intensity when compared with results obtained at the higher light intensity regime.

The effect of light intensity on ^{14}C incorporation into amino acids was more pronounced when leaf material was killed with 80% ethanol (Table 1 and Table 3): ^{14}C aspartate activity was greatly enhanced at the higher light intensity in plants fed with both 200 and 50 parts 10^{-6} nitrate nitrogen.

In a study made by Kennedy & Williams (1977), different killing techniques on early ^{14}C labelled photosynthetic products were also investigated in Zea mays L.: The alcohol treatments employed in this investigation included 80% boiling ethanol, or methanol at either 72°C or 23°C. The effect of killing with liquid nitrogen was also investigated. The above authors found that significantly more alanine was present in the tissue when killed with alcohol. They concluded that because the four-carbon acids were almost 100% labelled in position four, it was rather unlikely that alanine could be derived from the four-carbon acids after -decarboxylation or by randomization of label among carbon atoms 1 through 3. It was suggested that alanine could still be derived from PGA, the usual precursor of alanine in photosynthesis, by rapid turnover of a small PGA pool via phosphoglycerate mutase and enolase-mediated ractions.

However, the results discussed above, reported by Kennedy & Williams (1977) is in direct contradiction with certain observations in the present investigation. Killing of leaf material with cold 80% ethanol under LOW LIGHT conditions resulted in less incorporation of ^{14}C activity into alanine in plants fed with 200 parts 10^{-6} nitrate nitrogen. Whether or not the differences between these observations can be attributed to the difference in temperature of 80% ethanol solvents, remains to be determined.

No significant alteration in ^{14}C-alanine content of maize plants fed with 200 parts 10^{-6} nitrate under HIGH LIGHT intensity can be deduced, due to discrepancies between values obtained for replicate samples. However, changes in ^{14}C alanine contents in leaves of maize plants fed with 50 parts 10^{-6} nitrate nitrogen under LOW LIGHT intensity and HIGH LIGHT intensity are in agreement with the observations made by Kennedy & Williams (1977) in that ^{14}C activity of this amino acid is increased when killed with alcohol as opposed to freeze killing.

The present study reiterates the point made by Kennedy & Williams (1977) that care should be taken when comparing

ACKNOWLEDEGMENTS

I wouls like to thank Professor O.A.M. , my supervisor , for his assistance during the course of this project . I also wish to extend thanks to Jaqui Sommerville and Alma Bester for invaluable help with the typing .

APPENDIX

Preparation of Feeding Solutions :

Solution A : 50 ppm N-solution.

3,61 grams of KNO_3

6,69 grams of K_2SO_4

8,74 grams of $CaCl_2.6H_2O$ made up to 10 litres with water.

2,48 grams of $MgSO_4$

3,02 grams of $NaH_2PO_4.2H_2O$

Solution B : 200 ppm N-solution.

14,44 grams of KNO_3

16,67 grams of $CaCl_2.6H_2O$

2,48 grams of $MgSO_4$ made up to 10 litres with water.

3,02 grams of $NaH_2PO_4.2H_2O$

Solution C : Iron Chelate solution.

26,3 grams of Fe EDTA made up to 1 litre with water.

Solution D : Trace element solution.

2,00 grams of $MnSO_4.4H_2O$

0,24 grams of $CuSO_4.5H_2O$

0,45 grams of $ZnSO_4.7H_2O$ made up to 1 litre with water.

2,90 grams of H_3BO_3

1 ml of solution C and 1 ml of solution D was added to 1 litre of either solution A or solution B, before feeding plants.

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