Ife Journal of Agric. Vol. 16 & 17; 1994, 1995

Growth and Dhurrin Metabolism in Sorghum Seedling 2 : The effect of temperature, Gibberellic Acid, 2,4 Dinitrophenol and Glyphosate

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Abstract:

A small increase in temperature stimulated growth and dhurrin biosynthesis in green *Sorghum bicolor* (Linu) Moench Sudan 70 var. Redland x Greenleaf seedlings. Growth and dhurrin synthesis in the shoot are highly correlated with a coefficient r of 0.95. Gibberellic acid had no significant effect on either growth or dhurrin synthesis. 0.1 mM 2,4-dinitrophenol reduced growth rate by 50% but stimulated dhurrin biosynthesis by 60% while 0.2 mM reduced growth rate by 85% and dhurrin synthesis by 30%.

Low concentrations of glyphosate inhibited growth and dhurrin formation but this inhibition can be partially reversed by supplementation with aromatic amino acids. 2.0 and 4.0 mM glyphosate inhibited growth and dhurrin biosynthesis by 90% with a 24-hour lag period.

Glyhposate inhibited the conversion of ^{14}C-shikimic acid to dhurrin by 90% in whole seedlings with shikimic acid accumulating predominantly in the roots. Glyphosate also inhibited by 50-60% the reaction leading to the formation of p-hydroxyphenylacetonitrile by sorghum microsomal fraction while reducing the products by 20%.

GA_3 — Gibberellic acid
DNP — 2,4-dinitrophenol

Introduction

The cyanogenic glucoside of *Sorghum bicolor* — dhurrin — is synthesized from L-tyrosine (Conn 1979). There was evidence of its turn-over in etiolated (Bough and Gander 1971 and green sorghum seedlings (Blumenthal *et al.* 1963, Abrol and Conn 1966, Abrol *et al.* 1966) while the only quantitative study on the turn-over of dhurrin was carried out by Bough and Gander (1971) in etiolated seedlings using ^{14}C-tyrosine and Adewusi (1990) on green plants.

The use of chemicals in the study of metabolic processes in plants is not new. The only necessary condition is that the chemical should inhibit the target process without disturbing the plants' other metabolic functions. Thus, Gander (1960) observed some inhibition of dhurrin biosynthesis in *Sorghum vulgare* using 2,4-dinitrophenol and methylene blue. Nicola de Guidici et al (1974) employed 2,4-dinitrophenol to inhibit light induced betalain synthesis; while Stobart and Kinsman (1977) used gibberellic acid to inhibit amaranthin synthesis (another secondary product synthesised from tyrosine) in *Amaranthus caudatus*. Amrhein *et al.* (1980); and Steinrucken and Amrhein (1980) have shown that in buckwheat hypocotyl, glyphosate is a potent

inhibitor of the shikimic acid pathway which leads to the formation of tyrosine—the precursor of dhurrin.

The aim of this investigation was to assess the effect of temperature, gibberellic acid, DNP, and glyphosate on growth, dhurrin biosynthesis and other physiological processess in green sorghum seedlings and their potential utilization in dhurrin biosynthesis and turn-over studies. The effect of temperature was investigated as a result of some observed discrepancies due to variations intemperature in some growth chambers.

Materials and methods

Chemicals: GA_3 was a product of Calbiochem. and DNP was from Aldrich. High purity glyphosate (free acid) was a gift of Dr. E. G. Jaworski, Monsanto Agricultural Company. U^{14}C-tyrosine (specific activity 410 mCi/uM) was purchased from ICN while D-(2,3,4,5, (n)-^{14}C-)-snikimic acid (specific activity 84 mCi/uM) was obtained from Amersham. Shikimic acid and Amberlite IRA-410 were obtained from Sigma.

Plant Materials: Seeds of *Sorghum bicolor* (Linn) Moench Sudan 70 var. Redland x Greenleaf (Northrup King and Company Lubbock, Texas) were soaked in aerated water for about 24 hours and planted on a water saturated vermiculite in a growth chamber preset at 26: 22°C (day:night) temperature and a 16:8h (light:. dark) photoperiod.

Temperature Experiment: The seeds were planted in 3 plastic trays and transferred to growth chambers preset at 26, 28 and 32°C light, 22, 24 and 27°C dark temperatures, respectively. Temperature was monitored at 8 h intervals and deviation was not more than 0.5°C.

GA_3 Experiment: GA_3 solution was added to the vermiculite base of 4-day old seedlings such that concentrations were 2.8 and 5.6 uM. In other experiments, 2.8 and 5.6 uM GA_3-saturated vermiculite were used as the growth media throughout the experimental period.

DNP Experiment: 0.1 mM DNP-soaked vermiculite was either used as the growth medium or DNP solutions were added to the vermiculite base of 4-day old seedlings to give approximate concentrations of 0.1 and 0.2 mM.

Glyphosate Experiment: Glyphosate solution neutralised with twice its concentration of NaOH was used to irrigate the sorghum seedlings or added to the vermiculite base of 4-day old seedlings to give approximately the concentrations indicated. Where indicated, glyphosate treatment was supplemented with 1 mM aromatic amino acids. (L-tyrosine, L-phenylalanine and L-tryptophan).

Determination of Dhurrin: Dhurrin content was determined by the method of Gorz *et al.* (1977).

Radioactive labelling studies with U–^{14}C–Tyrosine: 4-day old seedlings were uprooted and placed in a 5 ml solution containing 1 mM phenylalanine, tryptophan and 2 mM glyphosate. After 5 hours, 20 *u*Ci^{14}C–tyrosine was added to the solution and mixed. The "feeding period" was 48 hours.

Hrdrolysis, Extraction, Chromatography and Analysis of Radioactive Dhurrin: After feeding, the roots were thoroughly washed in water. The dhurrin content of the

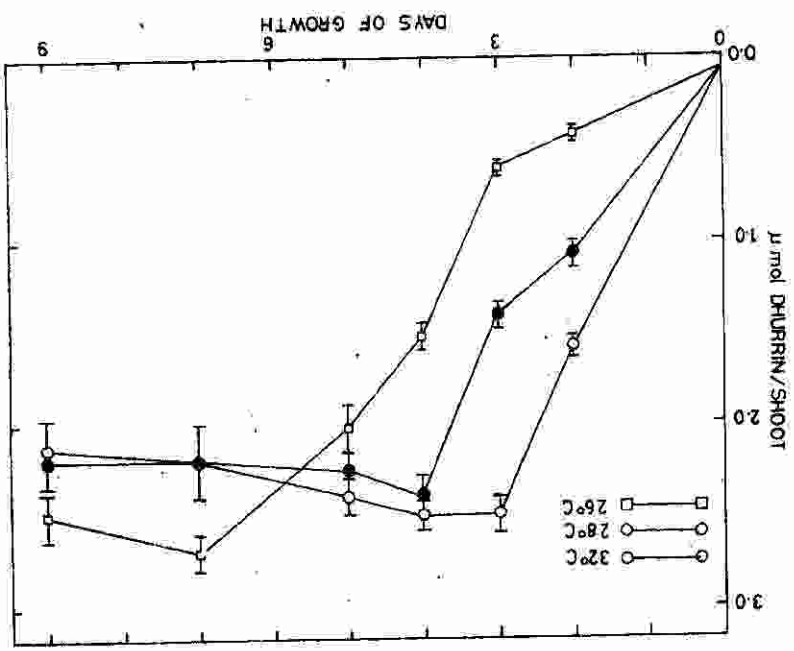

Fig.2:
The effect of temperature on dhurrin biosynthesis in green sorghum shoot.

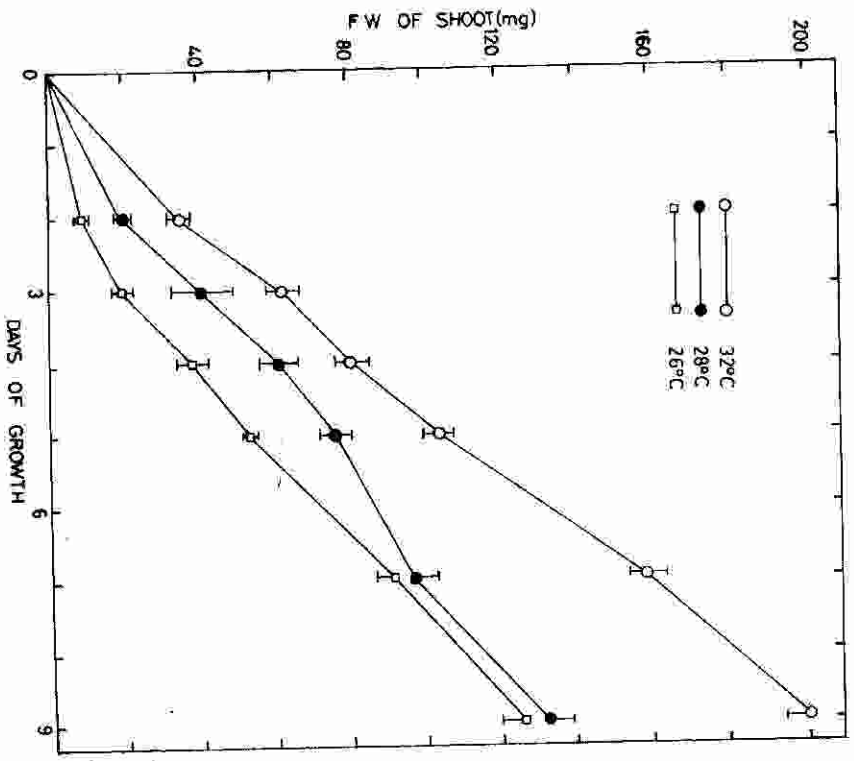

Fig.1:
The influence of temperature on the growth rate of sorghum shoot.

samples was hydrolysed as described by Gorz *et al.* (1977). The hydrolysate was reduced to 10 ml and 2ml aliquots were extracted 3 times with 2 ml diethyl ether. Chromatography of the ether extract was carried out on Bakerflex Silica gel 1B in a 5: 1 benzene: ethyl acetate solution for 3-4 hours. The plates were sprayed with 0.2% solution of 2'7 dichlorofluorescein and viewed under UV light. The radioactive bands were also located using a Packard model 7201 radiochromatogram, eluted with 1 ml water and counted in 10% aqueous scintillation fluid using a Beckman LS 230.

Estimation of U-^{14}-C-Tyrusine: The seedlings were hydrolysed after the feeding experiment and the hydrolysate concentrated. The amino acids were purified on a 1 x 5cm column of Dowex 50w x 8 as outlined by Hollander et al. (1979). The NH_4OH eluted amino acids were counted for radioactivity.

Feeding studies using ^{14}C-shikinic acid: 4-day old seedlings were placed in 2 ml 400 uM cold shikimic acid in the presence or absence of 2 mM glyphosate. After 5 hours 1 Çi ^{14}C-shikimic acid was added and mixed. After 48 hours, the plants were analysed for radioactive dhurrin. In one set of experiments, derooted seedlings were fed 1 Ci ^{14}C-shikimic acid and analysed for radioactive dhurrin.

Estimation of^{14}C-Chikimic Acid: Shikimic acid from the hydrolysate was purified on Amberlite IRA-410 by the method of Yoshida and Hasegawa (1957) and counted for radioactivity.

Preparation of Hicrosomal Fraction and the effect of glyphosate on tyrosene metabolism: Microsomal fraction from the leaves of 4-day old seedlings was prepared by the method of McFarlane *et al.* (1975). Glyphosate was added to 30 ui of the microsomal suspension to a final concentration of 10 and 333 uM. The reaction procedure, separation, identification and estimation of radioactivity in each product followed the method outlined by McFarlane *et al.* (1975) except that TLC was carried out using 4: 1 benzene: ethyl acetate.

Sampling: For each sampling point, 25 seedlings of about the same height were randomly selected and uprooted. The shoot, root and seeds were weighed and analysed for dhurrin. Experiments on temperature, GA_3, DNP and glyphosate were replicated 4 times while radioactive labelling studies were repeated once.

Results

In Fig. 1 it was shown that growth was faster at 32° than 28° and that of 28° was rapid compared to growth at 26°C. At 32°C, the shoot synthesized and accumulated dhurrin rapidly (Fig. 2) to reach a peak (2.5 umol dhurrin/shoot) on the 3rd day. At 28°C, dhurrin synthesis was not as rapid whereas at 26°C, dhurrin synthesis was slow in the first 3 days of growth and was faster thereafter to reach a peak of 2.7 umol/shoot on the 7th day. With a correlation factor (r) of 0.95, dhurrin biosynthesis seemed to be a function of the shoot's weight during the early stages of growth and this was irrespective of the growth temperature.

Effect of GA_3: GA_3, when added to 4-day old seedlings, had no effect on either growth or dhurrin biosynthesis. When used as the growth medium, there was a small but insignificant (P=0.05) increase in the weight of the shoots (115, 135 and 130 ± 10mg for 10-day old control, 2.8 and 5.6 uM GA_3 treated shoots, respectively.) The

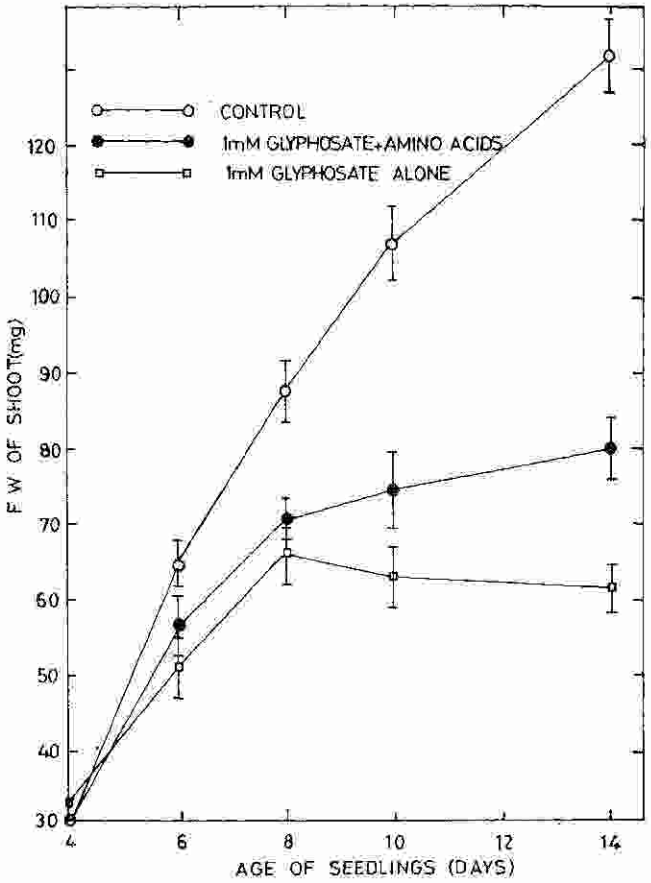

Fig.3:
The effect of 0.5 mM glyphosate and supplementation with 1 mM aromatic amino acids (tyrosine, phenylalanine and tryptophan) on the growth of green sorghum seedlings.

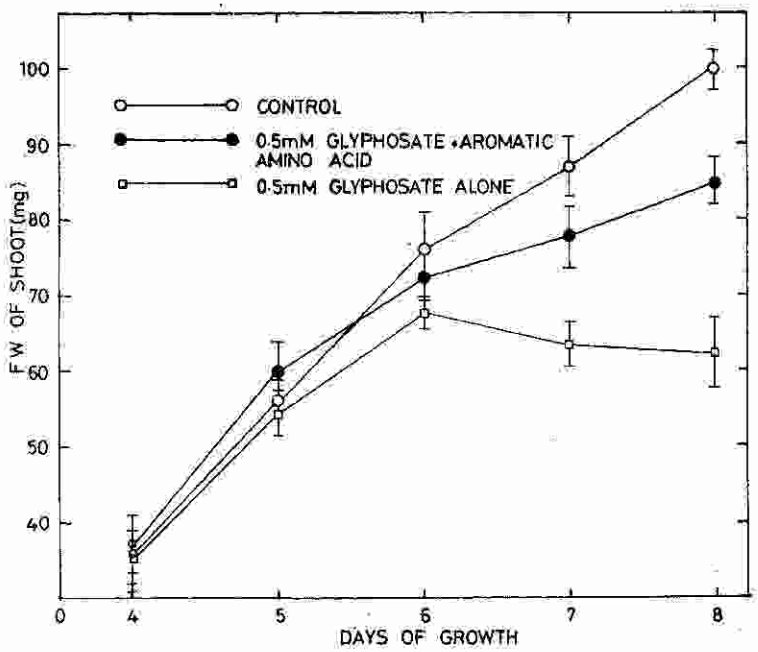

Fig.4:
The effect of 1 mM glyphosate and supplementation with 1 mM aromatic amino acids (tyrosine, phenylalanine and tryptophan) on the growth of green sorghum seedlings.

increase in the shoots weight was apparently reflected in the dhurrin content; thus the shoots contained 2.28 +0.06, 2.60 + 0.05 and 2.40 +0.08 umol dhurrin for the control, 2.8 and 5.6 uM GA_3 treatments, respectively.

Effect of DNP: Sorghum seeds planted in 0.1 mM DNP did not germinate but the same concentration reduced growth rate by 50% in 72 hours wh n added to the growth medium of 4-day old seedlings. Control seedlings gained 74+ 8 mg while 0.1 mM DNP treated seedlings gained 37 + 6 mg in weight over 72 hours. During this period, DNP stimulated dhurrin biosynthesis by 60%. Dhurrin content of DNP treated seedlings increased by 1.6 + 0.2 compared to 1.0 +0.2 umol/shoot in the control. 0.2 uM DNP inhibited growth of 4-day old seedlings by 85% and dhurrin synthesis by 30%.

The effect of glyphosate on the growth of sorghum seedlings: The presence of low concentrations (0.1 & 0.2 mM) of glyphosate in the media reduced growth rate by 35 and 50%, respectively (Table 1). At 0.5 mM, sorghum growth became stunted; for instance, the shoot of control seedlings weighed 94.1 mg on the 9th day compared to 27.1 mg for 0.5 mM glyphosate treated samples. Glyphosate concentrations above 0.5 mM in the medium inhibited sorghum growth entirely.

Glyphosate, when added to the vermiculite base of 4-day old sorghum seedlings, had no effect on growth at 0.1 mM while inhibition was 10-15% at 0.2 mM. At 0.5 mM, glyphosate inhibited growth substantially (Fig. 3) but this was partially reversed by the aromatic amino acids. In Fig. 4, it was shown that 1 mM glyphosate inhibited growth by more than 50% with and without aromatic amino acids' supplementation. At 2 and 4 mM, glyphosate inhibited growth by 90%; aromatic amino acids failed to alleviate the growth inhibition but prevented the treated seedlings from withering for some days. In all cases, there was about 48 hours' lag period between glyphosate treatment and growth inhibition.

Other effects of glyphosate on sorghum seedlings: With low concentrations, the first leaf expanded at the expense of other leaves and this was accompanied by the first leaf assuming a wider angle with high glyphosate concentrations.

The effect of glyphosate on dhurrin content of sorghum seedlings: 0.1 mM glyphosate had little or no effect ($\leq$ 10%) on dhurrin biosynthesis of 4-day old sorghum seedlings but inhibition was 30-50% at 1 mM. Dhurrin content of the control seedlings increased from 2.1 to 2.7 umol while in the 2 & 4 mM glyphosate treated samples, there was a 14% loss at the end of the experimental period (Fig. 5).

The effect of 2 mM glyphosate on shikimic acid metabolism: Table 2 showed that glyphosate did not seem to inhibit shikimic acid uptake but inhibited its metabolism to dhurrin. The radioactivity taken up in whole and derooted seedlings was 91-94% while its conversion to dhurrin varied substantially (Table 2). Glyphosate treatment resulted in the accumulation of 62% of the ^{14}C-shikimic acid taken up by whole seedlings while 8% accumulated in derooted seedlings. In whole seedlings 94% of the accumulated radioactivity was found in the roots (Table 3).

The effect of 2 mM glyphosate on the distribution of radioactivity from exogenous 14C-tyrosine: In Table 4, it was shown that glyphosate inhibited apparent ^{14}C-tyrosine uptake by 10% and reduced its conversion to dhurrin by 20%. Extractable ^{14}C-tyrosine accounted for 4.2% of the radioactivity taken up in the control and 6.7%

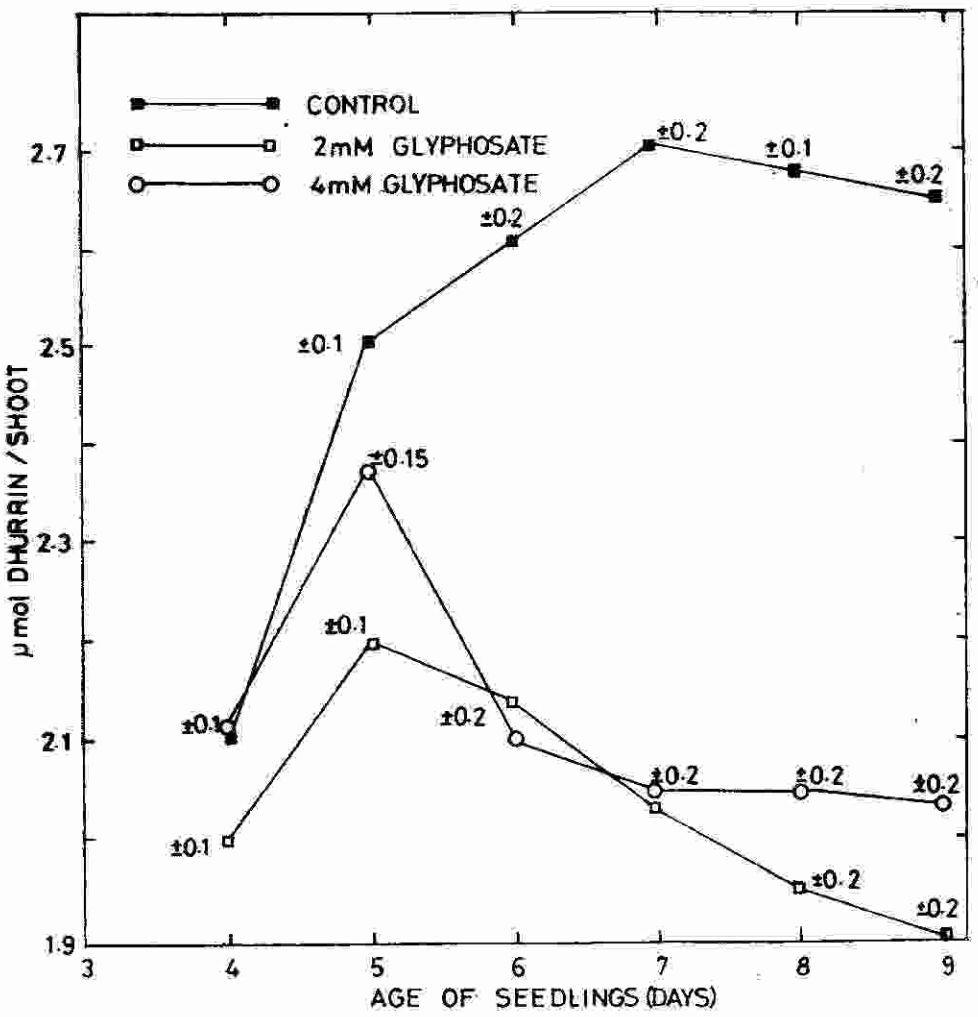

Fig.5:
The effect of 2.0 and 4.0 x 10^{-3} M glyphosate on *in vivo* dhurrin biosynthesis in the shoot of green sorghum seedlings.

Table 1: THE EFFECT OF DIFFERENT CONCENTRATIONS OF GLYPHOSATE ON GROWTH AND DHURRIN BIOSYNTHESIS IN GREEN SORGHUM SEEDLINGS.

Days sample	Assayed	Treatments Glyphosate Concentration ($\times 10^{-4}$M)			
		0	0.5	1.0	2.0
	FW of shoot (mg)	42.8±3.0	39.6±3.2	28.0±2.8	20.5±1.8
4	ppm cyanide/ shoot	1052±73	1262±55	1175±109	656±80
	umol cyanide/ shoot	1.67±0.17	1.85±0.12	1.22±0.2	0.50±0.2
	FW of shoot (mg)	55.4±2.9	49.5±3.1	42±2.1	27.4±0.8
6	ppm cyanide/ shoot	1108±113	1037±87	1069±101	659±73
	umol cyanide/ shoot	2.3±0.2	1.9±0.2	1.7±0.2	0.67±0.16
	FW of shoot (mg)	72.4±3.4	65.2±1.8	49.2±3.0	29.9±4.9
8	ppm cyanide/ shoot	886±67	880±39	851±35	626±52
	umol cyanide/ shoot	2.4±0.15	2.12±0.1	1.55±0.1	0.69±0.1
	FW of shoot (mg)	79.1±6.8	72.4±4.9	53.5±3.8	30.8±2.3
10	ppm cyanide/ shoot	852±37	745±54	721±118	554±50
	umol cyanide/ shoot	2.5±0.1	2.0±0.1	1.4±0.2	0.63±0.1

FW = Fresh weight

Table 2: THE EFFECT OF 2 mM GLYPHOSATE ON ^{14}C-SHIKIMIC ACID METABOLISM IN SORGHUM SEEDLINGS

		dpm/shoot/root/seed	
		Control	**Glyphosate**
	(Shoot	308	39
	(Root	2,103 + 38	202 + 42
Whole seedling	(Seed	–	–
	(cotyledon)	12 + 7	12 ± 6
% Radioactivity taken up		94	94
% Conversion to dhurrin		2.4	0.25
Derooted shoot		374	74
% Radioactivity taken up		91	92
% Conversion to dhurrin		0.4	0.007

Table 3: ^{14}C-SHIKIMIC ACID CONTENT OF SORGHUM SEEDLINGS

	dpm	**^{14}C-Shikimic acid**	
Tissue analysed	Control	Glyphosate	Ratio
Root	80,338	1,161,443	1:14
% Radioactivity recovered as ^{14}C-shikimic acid	5.2	62.0	1:8
Derooted shoot	72,471	158,333	1:2
% Radioactivity recovered as ^{14}C-shikimic acid	3.6	7.8	1:2

Table 4: THE EFFECT OF 2 mM GLYPHOSATE ON THE DISTRIBUTION OF RADIO-ACTIVITY (FROM EXOGENOUS ^{14}C-TYROSINE) IN SORGHUM SEEDLINGS

	Control	**Glyphosate**
% Radioactivity taken, u	78.5	70.0-
% ^{14}C-found in dhurrin	3.2	2.6
%Soluble ^{14}C-tyrosine in the shoot	0.59	0.84
% Soluble ^{14}C-tyrosine in the root	3.60	5.90

in glyphosate treated samples. Above 80% of this soluble tyrosine was again found in the roots.

The effect of glyphosate on dhurrin biosynthesis by microsomal particles: Table 5 indicated that the first biosynthetic step was not inhibited by glyphosate but the nitrile content was reduced by 48 and 61% with 10 and 333 uM glyphosate, respectively. The final products of this reaction (p-hydroxybenzaldehyde and p-hydroxybenzoic acid) were also reduced by 20%.

Table 5: THE EFFECT OF GLYPHOSATE ON DHURRIN BIOSYNTHESIS SORGHUM MICROSOMAL PARTICLES.

		Dpm	
Glyphosate concentration (um)	0	10	333
p-hydroxyphenylacetaldoxime	9,306	9,353	8,638
p-hydroxyhenylacetonitrile	917	479	357
p-hydroxybenzaldehyde	10,361	8,522	8,952
p-hydroxybenzoic acid	4,500	3,610	3,522

Discussion

In this study, it was found that a change in temperature of 2°C or more would appreciably affect the rate of sorghum growth and dhurrin biosynthesis. A high correlation factor (r = 0.95) between dhurrin content and shoot weight (which seemed independent of growth conditions) indicated a close genetic link between these two parameters (Hughes, 1973).

Stobart and Kinsman (1977) showed that 2.4 uM GA_3 inhibited amaranthin synthesis by 50% in 2-day old *Amaranthus caudatus* seedlings and suggested that GA_3 might exert this effect through a drain on available tyrosine by increasing protein synthesis and growth. In this study, GA_3 insignificantly increased both growth and dhurrin synthesis when used as the growth medium.

0.1 mM DNP partially inhibited growth but increased dhurrin biosynthesis while 0.2 mM inhibitied growth by 85% and dhurrin synthesis by 30%. DNP is an inhibitor of cyclic phosphorylation (Guidici de Nicola *et al*, 1974) and could explain the growth inhibition observed. However, dhurrin synthesis in sorghum seedlings is independent of photosynthesis (Akazawa et al 1960, Loyd and Gray 1970) and energy producing systems (Moller and Gonn) 1980). The increased dhurrin synthesis by 0.1 mM DNP could be the plant's response to a stress factor as observed earlier by Nelson (1953) while the observed 30% inhibition by 0.2 mM DNP could be attributed to an indirect effect through either reduced substrate synthesis or disruption of normal cell activities and structure.

0.5 and 1.0 mM glyphosate inhibited growth of sorghum seedlings but this inhibition can be partially reversed by supplementation with 1 mM tyrosine, phenylalanine and tryptophan. Many authors have reported growth inhibition by glyphosate and its reversal by aromatic amino acids (Jaworski 1972; Gresshoff 1979; Hollander and Amrhein 1980). The fact that high concentrations of glyphosate inhibited growth almost completely in the presence of aromatic amino acids would seem to suggest that glyphosate may be acting on processes other than the aromatic amino acid metabolism. For instance, Schaner (1976) indicated that glyphosate affected transpiration in

Phaseolus vulgaris while Tymonko and Foy (1978) also reported that glyphosate inhibited protein and RNA synthesis , photosynthesis and respiration in isolated cells. Rubin *et al.* (1982) furthermore raised the possibility that glyphosate may act at multiple enzyme targets in a given organism.

The result of the experiments with ^{14}C-shikimic acid indicated that sorghum seedlings are capable of metabolising exogenous shikimic acid to dhurrin with an apparent yield of 2.6%. Glyphosate (mM) did not inhibit apparent shikimic acid uptake but inhibited its metabolism to dhurrin by about 90% when fed through the roots and by 80% in derooted seedlings. This tends to show that glyphosate is active in both parts of the seedling. However, during glyphosate treatment, shikimic acid accumulated more in the root (94% of the accumulated acid) than in the shoot. This could mean (a) that glyphosate is more potent in the root than in the shoot as was observed by Duke and Hoagland (1978) and Hoagland *et al* (1979) or (b) that glyphosate inhibited translocation of ^{14}C-shikimic acid from the root to the shoot. Whatever system operated (either or both), it was clear that glyphosate inhibited the shikimic acid pathway in sorghum seedlings resulting in the accumulation of shikimic acid as already observed in buckwheat hypocotyls (Steinrucken and Amrhein, 1980).

When U-^{14}C-tyrosine was fed to sorghum seedlings, about 77% of the radioactivity was taken up but only 3.2% of this was found in dhurrin. This low incorporation was in agreement with earlier observations (Koukel *et al.* 1962; Bough and Gander 1971).

Glyphosate inhibited apparent ^{14}C-tyrosine uptake by about 10% comparable to the 14% inhibition of ^{14}C-shikimate uptake observed by Hollander and Amrhein (1980). Glyphosate also inhibited dhurrin biosynthesis from ^{14}C-tyrosine by about 20% (Table 4) but could be as much as 30-60% depending on the experimental conditions (McWorther and Azlin 1978; Adewusi and Conn, unpublished data). From Table 5, it seemd apparent that the step leading to the formation of p-hydroxyphenylacetonitrile was inhibited by 50-60%.

Extractable ^{14}C-tyrosine content was also low in the shoot (0.59% in the control and 0.84% in glyphosate treated samples) but in the roots, more radioactivity was accumulated (3.6% in the control and 5.9% in the test sample) This result, consistent with that obtained by Bough and Gander (1971) would tend to substantiate the hypothesis that glyphosate inhibited transport and/or translocation of materials in the roots.

Glyphosate out of the 3 chemicals tested has been shown to inhibit growth and dhurrin biosynthesis effectively in Sorghum seedlings. 2 mM glyphosate inhibited ^{14}C-shikimic acid metabolism to dhurrin by 90% and in addition, adversely affected tyrosine uptake and its conversion to dhurrin *in vivo* and *in vitro*. The importance of these findings lies in the fact that glyphosate can be used to monitor the biosynthesis, transport and turn-over of dhurrin in green sorghum seedlings.

Acknowledgements:

I am grateful to Dr. E. G. Jaworski, Monsanto Agricultural Chemical Company, St. Louis, Mo 63166 U.S.A. for providing high purity glyphosate. I acknowledge the help of Dr. E.S. Wurtele on microsomal particle preparation and the immense

contribution of Dr. E. E. Conn, Department of Biochemistry and Biophysics, University of California, Davis.

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