PROTEIN, ENZYME AND AMINO ACIDS MODIFICATION IN COWPEA (Vigna unguiculata (L.) Walp.) SEED WITH GLYPHOSATE

By

ANTONIO LUIZ CERDEIRA

A Dissertation
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Plant Pathology
and Weed Science

Mississippi State, Mississippi

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DEDICATION

To my children Cesar and Marcos

and to my wife Rita

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my major professor, Dr. A. Wayne Cole, and to Dr. Dawn S. Luthe for their immense help, guidance, and friendship during all periods of this work. Special thanks are also extended to Dr. C. H. Andrews, Dr. James M. McGuire, Dr. G. E. Coats, and Dr. R. B. Koch.

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Finally, my deepest recognition is to my parents, Jose and Julieta, who always shared this time with love and understanding.

ABSTRACT

Antonio Luiz Cerdeira, Doctor of Philosophy, 1985

Major: Weed Science, Department of Plant Pathology and Weed Science

Title of Thesis: Protein, Enzyme and Amino Acids Modification in Cowpea (Vigna unguiculata (L.) Walp.) Seed with Glyphosate

Directed by: Dr. A. Wayne Cole, Professor of Weed Science

Pages in Thesis: 61 Words in Abstract: 225

ABSTRACT

Glyphosate [N-(phosphonomethyl) glycine] was used as a preharvest desiccant on cowpea (Vigna unguiculata (L.) Walp) in greenhouse and field studies in 1982, 1983 and 1984. Various stages of pod development were selected and tagged at the time of herbicide application in order to make comparative studies of the effect of the herbicide on biochemical components of the seeds.

Seed protein content did not change with glyphosate if field produced pods were treated 14 or more days after flowering (DAF). Seed from pods 10 DAF at plant desiccation with 1.12 kg/ha of glyphosate had significantly less total protein than the control harvested on the same day. Electrophoretic analysis of those seeds confirmed these results. Free amino acid analysis showed a four-fold increase in histidine content of seeds treated at 10 DAF.

The accumulation of storage proteins in seeds from greenhouse produced plants was analyzed by sodium dodecyl polyacrylamide gel electrophoresis. A large increase in protein content per seed occurred between 10 and 11 DAF. Polypeptides with molecular weights of 54, 47, and 41 kilodaltons (kD) appeared to be the major polypeptides and accumulated first. Treatment of those plants with glyphosate when pods were 7 and 10 DAF prevented accumulation of the major storage protein polypeptides. Growth as measured by pod width, seed dry weight, and seed length were also inhibited by glyphosate when plants bearing pods 7, 10, and 11 DAF were treated.

Field produced seeds were analyzed for lipoxygenase activity and it was shown to be optimally activated by 0.68 mM calcium at pH 7.5. Seeds from plants treated with glyphosate showed a significant increase in the lipoxygenase activity.

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Total Protein and Free Amino Acid Modification in Southernpea (*Vigna unguiculata* (L.) Walp.) with Glyphosate

Total Protein and Free Amino Acid Modification in Southernpea (*Vigna unguiculata* (L.) Walp.) with Glyphosate

Antonio L. Cerdeira, A. Wayne Cole, and Dawn S. Luthe

Additional index words. Kjeldahl, electrophoresis, polypeptide.

Abstract. Total seed protein content, amino acids and polypeptides were examined following desiccation of southernpea (*Vigna unguiculata* (L.) Walp.) plants with glyphosate [N-(phosphono-methyl) glycine]. Seed protein weight of mature seeds was not reduced by the desiccation process, however, protein content of more immature seeds was reduced. Polypeptides were reduced in the same way. Free amino acid evaluation indicated that histidine increased up to four times on those seeds that were immature at the time of treatment. No differences were found in bound amino acid content. Electrophoretic analysis during germination indicated that the proteins reduced in immature seeds with glyphosate desiccation were storage class proteins.

Indeterminate growth of southernpea results in various stages of seedpod maturity throughout the season, so that the use of a desiccant such as glyphosate to facilitate mechanical harvesting would desiccate immature seedpods. Southernpea seeds which were immature at the time of desiccation with glyphosate [N-(phosphonomethyl)glycine] did not germinate as rapidly as those which were mature (5). Glyphosate was more effective than sodium chlorate or paraquat (1,1'-Dimethyl-4,4'-bipiridinium ion) in reducing the moisture content of grain, stem, and leaves of grain sorghum [Sorghum bicolor (L.) Moench.] (2). Additional responses were inhibition of axillary buds and reduced germination of seed treated at greater than 30% moisture content (2). A 10% (v/v) solution of glyphosate applied to the inflourescence of wild oat (Avena fatua L.) at the "soft and hard cheese" stage of development prevented the production of any viable seed (11). Preharvest desiccation of cotton (Gossypium hirsutum L.) with glyphosate resulted in significant injury to those seeds which were immature (9). Soybean (Glycine max (L.) Merr.) response to glyphosate varied among varieties and with the stage of development at the time of application (8). Control studies on several perennial weed species (6, 14, 15) showed that glyphosate was translocated extensively in various plants, and that it was more effective as the plants matured.

Biochemical studies of glyphosate mode of action indicate that it is an inhibitor of a specific reaction in the shikimic acid pathway leading to the biosynthesis of aromatic amino acids (13), and could consequently affect seed storage protein.

The objective of this study was to determine the response of southernpea seed protein to parent plant desiccation with glyphosate.

Southernpea plants were grown in the field during the summer of 1982. Production was using ordinary cultural practices, and flowers were tagged at bloom opening so that four stages of pod maturity, 10, 12, 14, and 16 days after flowering (DAF), could be identified. When a sufficient number of pods reached these stages of maturity, plants were sprayed with glyphosate at 1.12; 0.56; and 0.28 kg/ha in 234 l/ha of water. The tagged pods from treated plants were harvested one week after spraying, shelled, freeze-dried, and seed at each maturity stage were combined. Seeds from control plants were harvested in the same manner, at the same stages of maturity and on the day of treatment. Four lots of 10 seeds for each treatment were analyzed for total protein content by the Kjeldahl method (1). The experimental design was completely randomized with four replications.

Seed polypeptides were characterized by SDS-polyacrylamide gel electrophoresis (PAGE). In this procedure, freeze-dried seeds were homogenized in 1 ml

of buffer containing 63 mM Tris HCl (pH 7.8) and 10 mM B-mercaptoethanol for 10 sec. After homogenization, 1 ml of sample buffer (10) containing 63 mM Tris HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 5% (v/v) B-mercaptoethanol, 0.001% (w/v) bromphenol blue, and 1 mM of phenylmethyl sulfonyl fluoride was added to the homogenate, and the sample was boiled for 5 min. Cell debris was removed from the samples by centrifugation in a microcentrifuge at 13000 x g. Aliquots of the supernatants were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS - PAGE) as described by Laemlli (10). On these discontinuous, denaturing gels, polypeptides were separated according to their molecular weights. The stacking gel contained 125 mM Tris HCl (pH 6.8), 0.1% (w/v) SDS, and 5% (w/v) acrylamide; the separating gel contained 375 mM Tris HCl (pH 8.8), 0.1% (w/v) SDS, and 10% (w/v) acrylamide. The electrode buffer (pH 8.3) contained 25 mM Tris HCl, 192 mM glycine, and 0.1% (w/v) SDS. Electrophoresis was conducted at 40 ma per slab until the tracking dye (bromphenol blue) reached the bottom of the gel. Gels were stained for 30 min. in a solution containing 45% methanol, 9% (v/v) acetic acid, and 0.2% (w/v) Coommassie brilliant blue R. Gels were destained in a solution containing 5% (v/v) methanol and 7.5% (v/v) acetic acid.

Free and bound amino acids were also analyzed. Free amino acids were extracted from seeds pulverized with a mortar and pestle using a solution (12:5:3) (v/v/v) of

methanol:chloroform:water. The solution was then centrifuged for 5 min. at 14000 x g. After centrifugation 10 ml of extracting solution were added to the pellet and it was reextracted and centrifuged as described above. Five ml of chloroform and 5 ml of water were added to the combined supernatants, and this mixture was centrifuged again. The aqueous layer was retained, evaporated to dryness at 45°C in a water bath and the residue was analyzed with an amino acid analyzer (Beckman 120-C). Bound amino acids were extracted by digesting the pellet remaining with a solution of 3N mercaptoethanesulfonic acid, concentrated by centrifugation and evaporation and analyzed in the same manner as with free amino acids. The analysis was repeated twice.

Seed protein content did not change with treatment when the pods were 14 or 16 DAF at the time of plant desiccation (Table 1). Seed from pods 12 DAF at plant desiccation continued to increase in protein content after treatment and were equal to the control harvested at the same time as those from treated plants. However, the control harvested on the day of treatment contained less total protein. Seed from pods 10 DAF at plant desiccation also continued to increase in protein content, however, the 1.12 kg/ha rate resulted in significantly less total protein than the control which was harvested at the same time (Table 1).

Table 1. Average protein per seed of southernpea from pods at four mature stages when plants were desiccated with glyphosate.

DAF[a]		Rate		Weight
Treated	Harvested	(kg/ha)	% Protein	(mg)
10	10	Control	20[b]	9.2 h[b]
10	17	Control	21	29.8 bcde
10	17	0.28	21	27.3 def
10	17	0.56	21	27.0 ef
10	17	1.12	22	20.1 g
12	12	Control	21	18.3 g
12	19	Control	20	27.4 cdef
12	19	0.28	21	27.3 def
12	19	0.56	20	25.9 f
12	19	1.12	20	25.7 f
14	14	Control	22	29.7 bcde
14	21	Control	22	32.4 ab
14	21	0.28	21	30.3 bc
14	21	0.56	21	29.9 bcde
14	21	1.12	20	30.1 bcd
16	16	Control	21	33.0 ab
16	23	Control	20	33.2 a
16	23	0.28	20	33.4 a
16	23	0.56	20	33.2 a
16	23	1.12	21	34.4 a

[a]Days after flower opened.

[b]All means within a column followed by the same letter do not differ significantly according to DNMRT at the .05 level of probability.

Polypeptide accumulation was affected in the seeds when plants were treated at 12 DAF even though total protein was not (Figure 1). The accumulation in the seeds from treated plants was less than in the untreated, but greater than in those harvested on the day of treatment. The changes in accumulation were quantitative and not qualitative; that is, no specific polypeptides were absent. Other studies (3, 4, 7, 12) have indicated that the 49 and 54 kd polypeptides are globulins which are the predominant storage protein class in these seeds. Rapid loss of this group of polypeptides during germination (Figure 2) suggests that they are in the storage proteins and are probably essential for a source of reduced N. A reduced supply of these proteins, as would be the case in seeds from plants desiccated at 12 DAF and younger, may influence the rate of emergence and seedling vigor.

Analysis of free amino acids was done to determine if there was a reduction in tyrosine and phenylalanine supply which could limit the synthesis of storage proteins. Histidine was up to four times greater in the treated seed than in the control when pods were 10 and 12 DAF (Table 2). However, there was no indication of a reduction in the other aromatic amino acids. No effect was found in bound amino acid content.

Depending upon the stage of southernpea development, glyphosate affected the protein and histidine content when used as a preharvest desiccant. Other studies (5) indicate

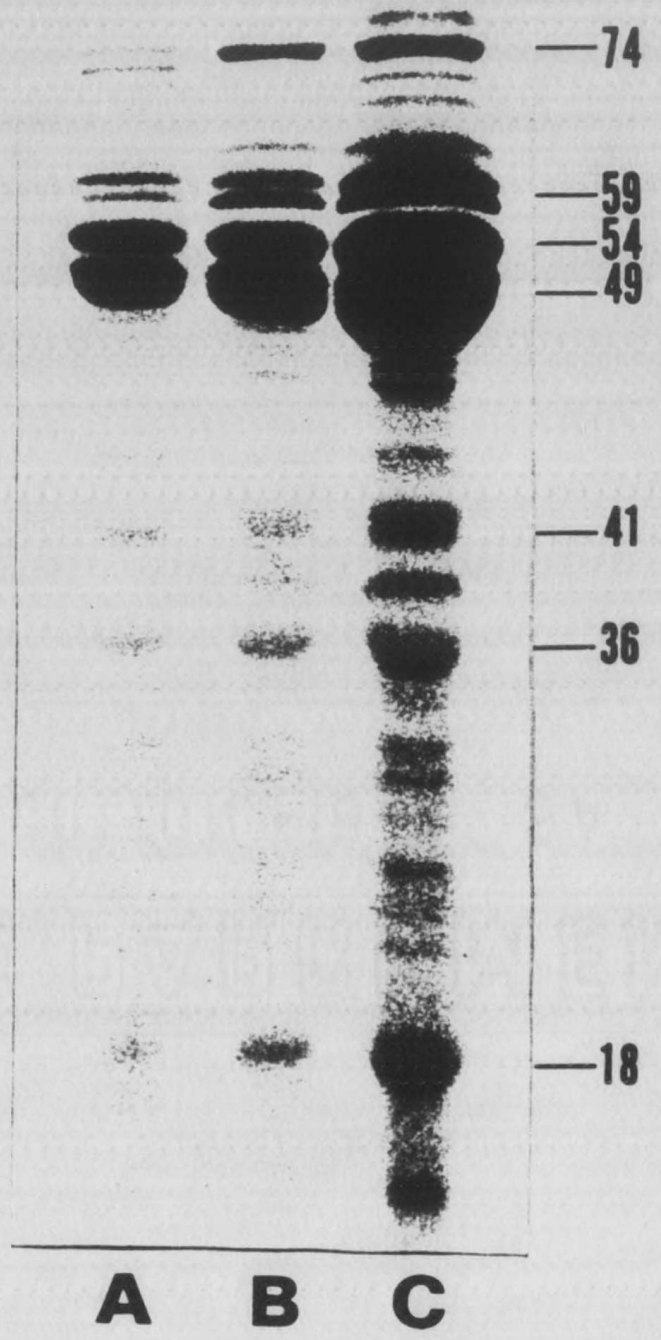

Fig. 1. Sodium dodecyl polyacrylamide gel electrophoresis of seed extracts from southernpea plants treated with glyphosate at 12 DAF and harvested 7 days later (B). Control with 19 DAF (C). Control with 12 DAF (A). The amount of each extract applied to the gel was 1 seed per lane. Numbers in the margin refer to the molecular weight in kilodaltons of the major polypeptides.

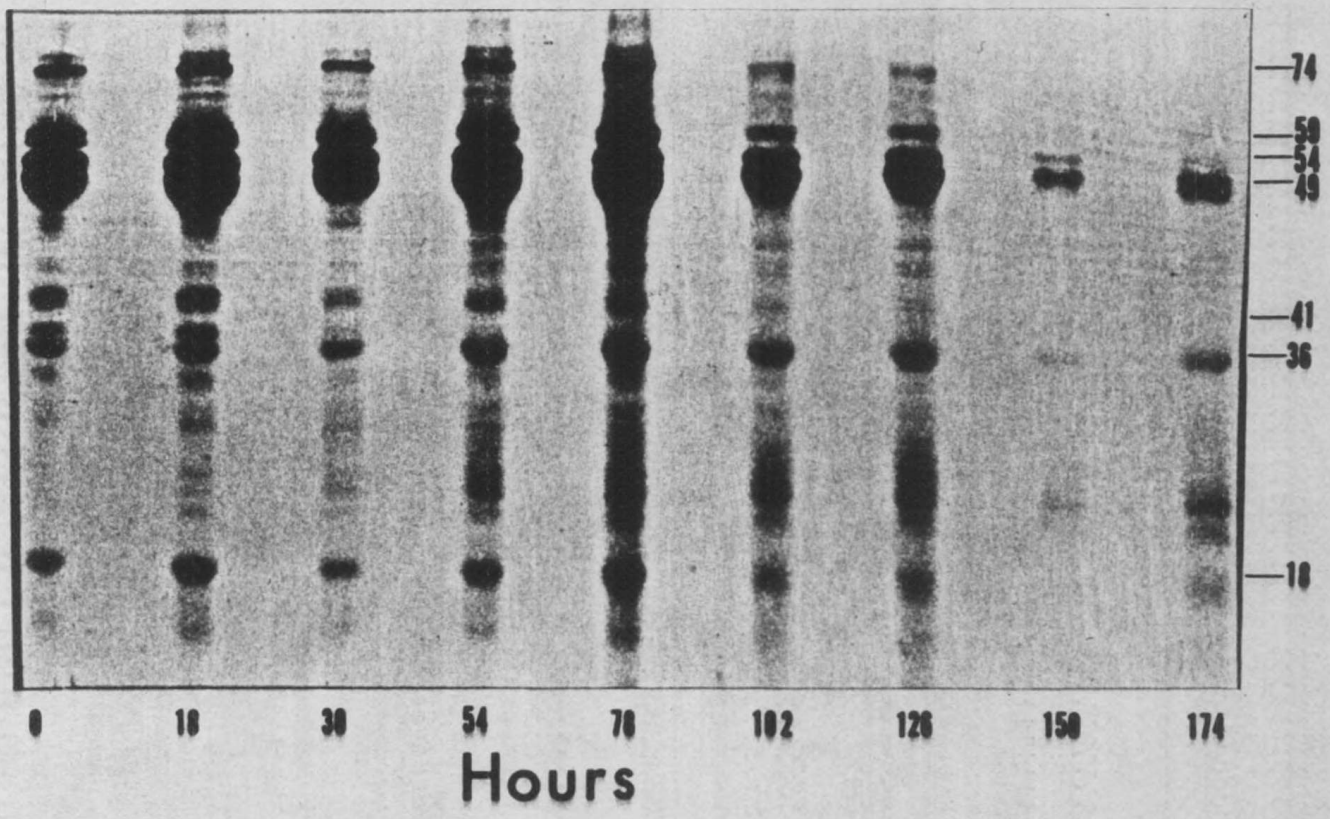

Fig. 2. Sodium dodecyl polyacrylamide gel electrophoresis of southernpea seed extracts submitted to a standard germination test from 9 to 174 hours of inhibition. The amount of each extract applied to the gel was 1 seed per lane. Numbers in the margin refer to the molecular weight in kilodaltons of the major polypeptides.

H10
H9
H8
H7
H6
H5
H4

Table 2. Effect of glyphosate on southernpea seeds free amino acids content (ppm).

DAF[a]	Tyrosine		Phenylalanine		Histidine	
	T[b]	C[b]	T	C	T	C
10	74	34	135	133	962	238
12	72	24	88	135	456	269

[a]Days after flowering at treatment. Seeds were harvested seven days later.

[b]T = Parent plants treated with glyphosate at 1.12 kg/ha.
C = Control.

that for mechanical harvesting, treated plots could be harvested without difficulty with a field combine; whereas, the untreated plots could not because of the bulk of green plant material. Seed maturity appears to be a major factor in determining the feasibility of using glyphosate as a desiccant because of the modification of the amount and possibly structure of seed protein.

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Chapter 2. Written according to requirements for Weed Science Journal.

Cowpea (*Vigna unguiculata*) Seed Protein Response to Glyphosate

INTRODUCTION

Cowpea is an important crop in the southern United States as well as in tropical regions throughout the world. Mechanical harvesting of dry cowpea is complicated because of a growth habit in which fruit set and maturity occur at a time when there is an abundance of green stems and leaves (4). The use of glyphosate for defoliation and desiccation aided mechanical harvesting procedures, but it also resulted in less viable seeds when it was applied to the plant at some developmental stages (2, 6).

It has been noted that some seeds from glyphosate-treated cowpea plants germinated poorly, and that the seedlings were less vigorous than those from control plants (4). This may indicate that the amount of storage materials needed to support seedling growth was reduced in seeds from treated plants. Previous data indicated that this may be the case when the plants with pods in the early stages of development were treated (4). This reduction of storage materials (especially seed proteins) also has negative aspects for those who depend on cowpea as a diet staple.

Glyphosate is believed to inhibit 5-enolpyruvyl-shikimic-acid-3-phosphate-synthase, the enzyme which catalyzes the reaction forming 5-enolpyruvyl-shikimic-acid-3-phosphate (1, 9). Inhibition of this enzyme by

glyphosate (9) may result in reduced amounts of the aromatic amino acids, which may in turn, limit the amount of proteins that accumulate in these seeds.

Previous work (4) suggested that once the dry pod development stage was reached, glyphosate application to pod bearing plants did not affect the accumulation of storage materials within the seed. However, when pods were at younger stages of development, the application of glyphosate appeared to impair the ability of seeds to complete development. The objective of this study was to determine the earliest stage of seed development that would withstand glyphosate plant desiccation without decreasing seed protein content. An additional objective was to determine if any particular class of protein was affected by the herbicide.

MATERIALS AND METHODS

Cowpeas were grown in 15 by 20 cm pots filled before planting with a 2:2:1 (v/v/v) soil (Oktibbeha sandy loam; fine, mixed, thermic, Typic Hapludalfs):sand: peat mix. After emergence, plants were thinned to two plants per pot and each individual plant was staked. Plants were grown in a greenhouse with no supplemental light or fertilization. Aldicarb [2-methyl-2-(methylthio) propionaldehyde-O-(methylcarbamoyl) oxime) at approximately 50 mg ai/pot was added at the first true leaf stage of plant development for insect control. Pots were watered overhead on a daily

basis as needed, and greenhouse temperature was maintained at approximately 25 C. Pod age was determined by tagging the flowers as they opened. Under these growing conditions, time from flowering to seed and pod maturity was approximately 26 days.

Pods ranging from 5 to 19 DAF were harvested on the same day and pod length and width were measured on three randomly selected pods from each DAF. Seeds within each DAF were combined, and 4 groups of 5 seed each were selected at random to determine seed fresh weight and length. The seeds were then frozen in liquid nitrogen and freeze-dried for dry weight determinations and protein analysis. Each experiment was conducted twice in a completely randomized design, and the results are reported as a combined analysis of the means of two experiments. Protein analysis by electrophoresis was done two or more times for each DAF and the data presented are typical of the patterns obtained.

Pod-bearing plants were sprayed with glyphosate at a rate of 1.12 kg ai/ha in 234 L/ha of water. Seven days after treatment, which is the approximate time required for plant desiccation, pods that were at 7, 10, 11, 12, and 13 DAF at the time of treatment were harvested from glyphosate-treated and control plants.

Freeze-dried seeds were pulverized with a mortar and pestle prior to homogenization with a mechanical homogenizer. For seeds harvested 6 to 8, 9 to 12, and 13 to 19 DAF; 5, 2 and 1 seed, respectively, were homogenized in 1 ml

of buffer containing 63 mM Tris HCl, pH 7.8 and 10 mM B-mercaptoethanol for 10 sec. After homogenization 1 ml of sample buffer (7) containing 63 mM Tris HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 5% (v/v) B-mercaptoethanol and 0.001% (w/v) bromphenol blue, and 1 mM of phenylmethyl sulfonyl fluoride was added to the homogenate, and the sample was boiled for 5 min. Cell debris was removed from the samples by centrifugation in a microcentrifuge at 13,000 xg. Aliquots of the supernatants were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (7). On these discontinuous, denaturing gels, polypeptides were separated according to their molecular weights. The stacking gel contained 125 mM Tris HCl (pH 6.8), 0.1% (w/v) SDS, and 5% (w/v) acrylamide; the separating gel contained 375 mM Tris HCl (pH 8.8), 0.1% (w/v) SDS, and 10% (w/v) acrylamide. The electrode buffer (pH 8.3) contained 25 mM Tris HCl, 192 mM glycine, and 0.1% (w/v) SDS. Electrophoresis was conducted at 40 ma per slab until the tracking dye (bromphenol blue) reached the bottom of the gel. Gels were stained for 30 min in a solution containing 45% (v/v) methanol, 9% (v/v) acetic acid, and 0.2% (w/v) Coommassie brilliant blue R. Gels were destained in a solution containing 5% (v/v) methanol and 7.5% (v/v) acetic acid.

RESULTS AND DISCUSSION

From 5 to 19 DAF the average cowpea pod length and width increased from 6.2 to 16.5 cm, and 0.4 to 1.1 cm, respectively (Figure 1, 2). The average fresh and dry weight of seeds increased from 3 to 413 mg/seed, and 0.7 to 182 mg/seed respectively (Figure 3), while seed length increased from 0.3 to 1.2 cm (Figure 4). The increase in seed fresh and dry weight accumulation is similar to that described by Carasco et al. (3). These authors (3) divided the cowpea seed development period into four phases: I - cell division, II - growth, III - maximum growth, and IV - desiccation. Using the data for fresh and dry seed weight (Figure 3) we assigned the growth phases to the following time intervals: phase I, 5 to 8 DAF; phase II, 9 to 12 DAF; phase III, 13 to 15 DAF; and phase IV, 16 DAF to maturity.

Analysis of seed proteins by SDS-PAGE (Figure 5) showed that very few proteins were present in the seeds harvested at 10 DAF; at 11 DAF there was a marked increase in the number and intensity of polypeptides as visualized on the gel. Polypeptides with molecular weights of 54, 49, and 41 kD were the first to appear at 11 DAF. At 13 DAF polypeptides with molecular weights of 59 and 36 kD appeared. Similar polypeptides with molecular weights in the general range of 52, 54, and 56 kD have been identified in other

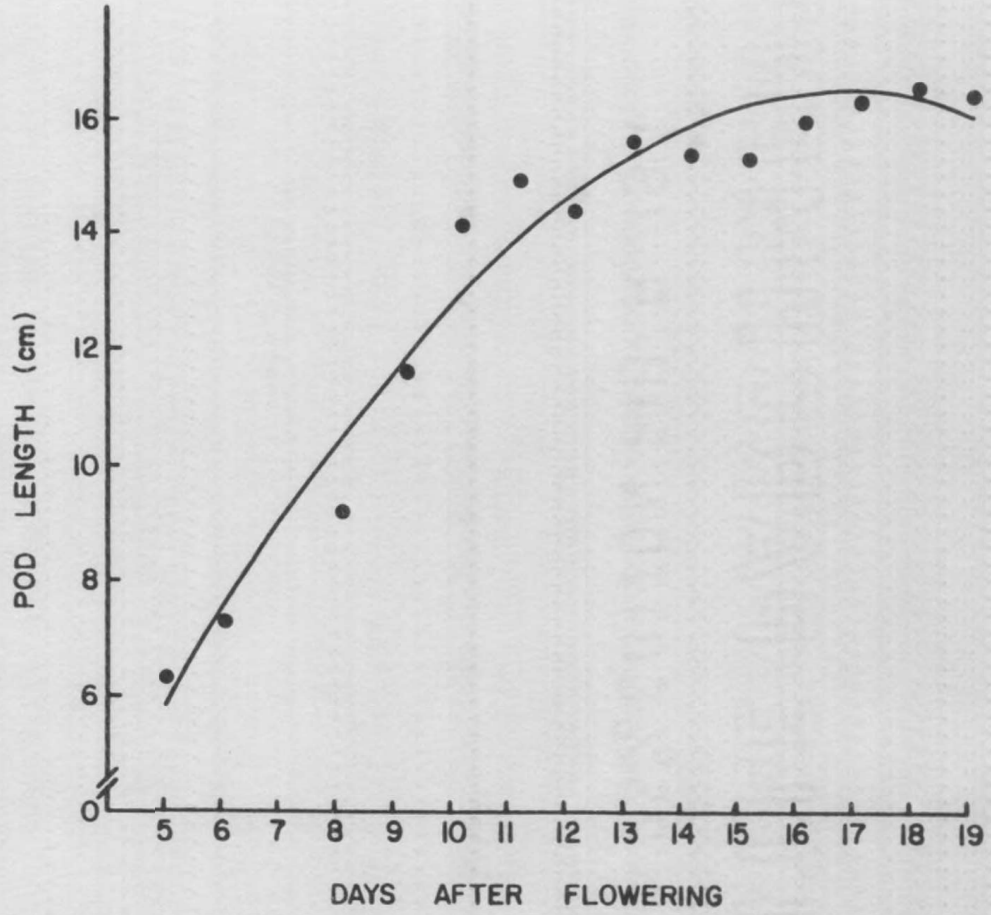
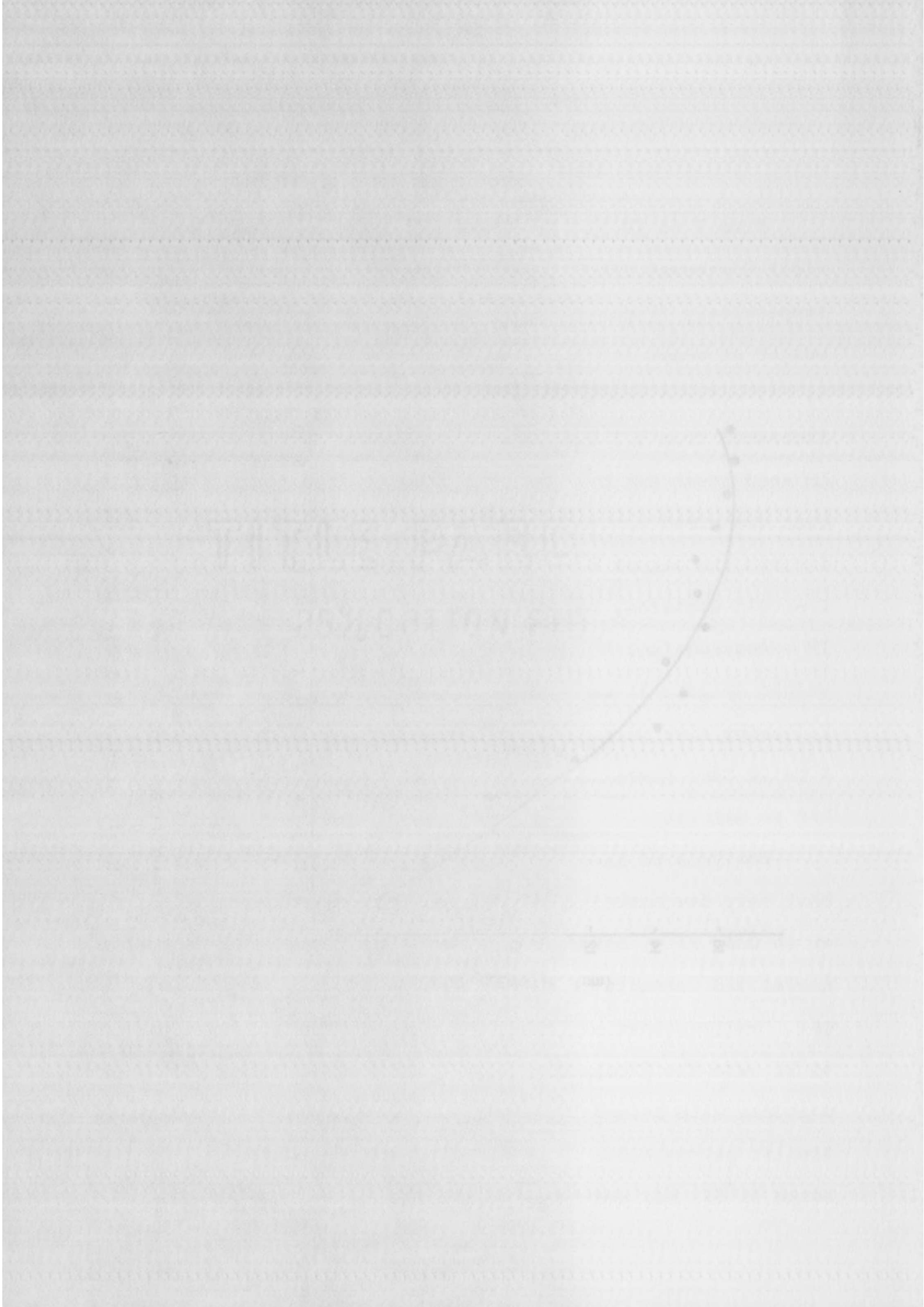


Fig. 1. Predicted and observed values of cowpea seedpod length from 5 to 19 days after flowing. $Y = -4.95810962 + 2.562939X^2 - 0.076499X^2$, $r = 0.97$, standard deviation = 0.78 cm.



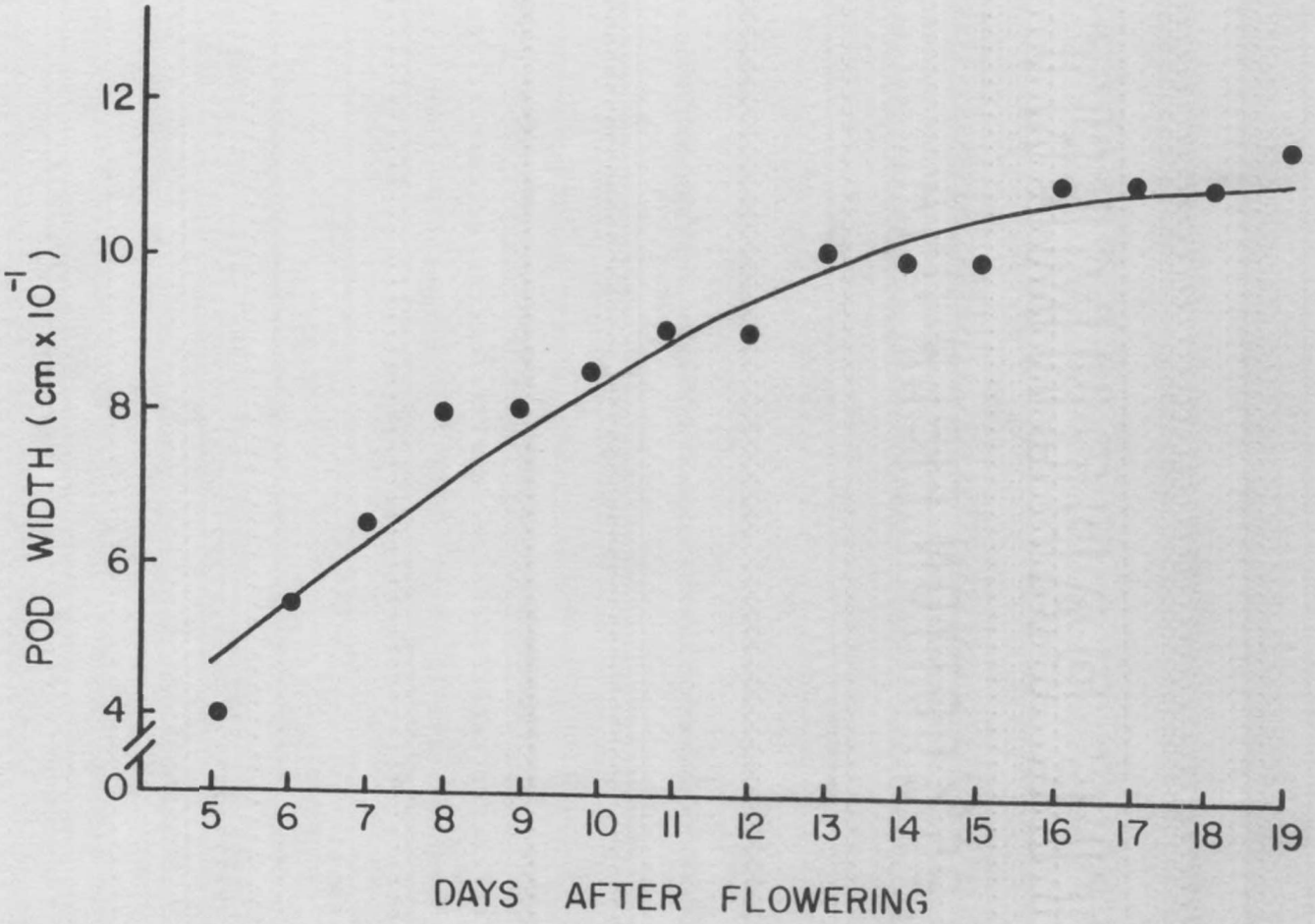


Fig. 2. Predicted and observed values of cowpea seedpod width from 5 to 19 days after flowering. $Y = 0.07353578 + 0.123610X - 0.003216X^2$, $r = 0.96$, standard deviation = 0.06 cm.

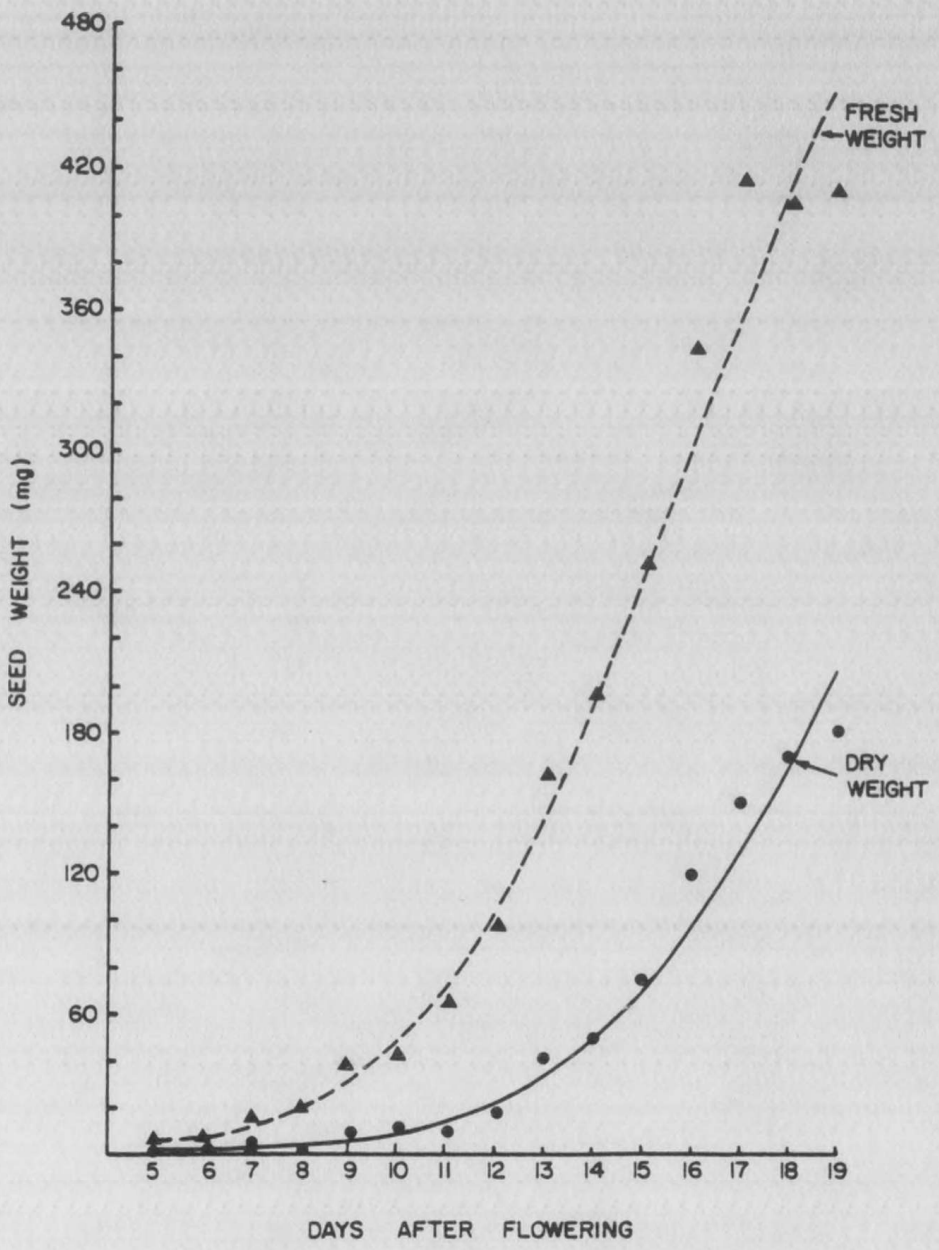


Fig. 3. Predicted and observed values of cowpea seed fresh and dry weight from 5 to 19 days after flowering. Fresh weight: $\log Y = 0.9063125 + 0.375986X - 0.008985X^2 - 0.261683 \log X$, $r = 0.99$, standard deviation = 1.18 mg. Dry weight: $\log Y = 0.21090686 + 0.690079X - 0.013259X^2 - 4.86425310gX$, $r = 0.99$, standard deviation = 1.20.

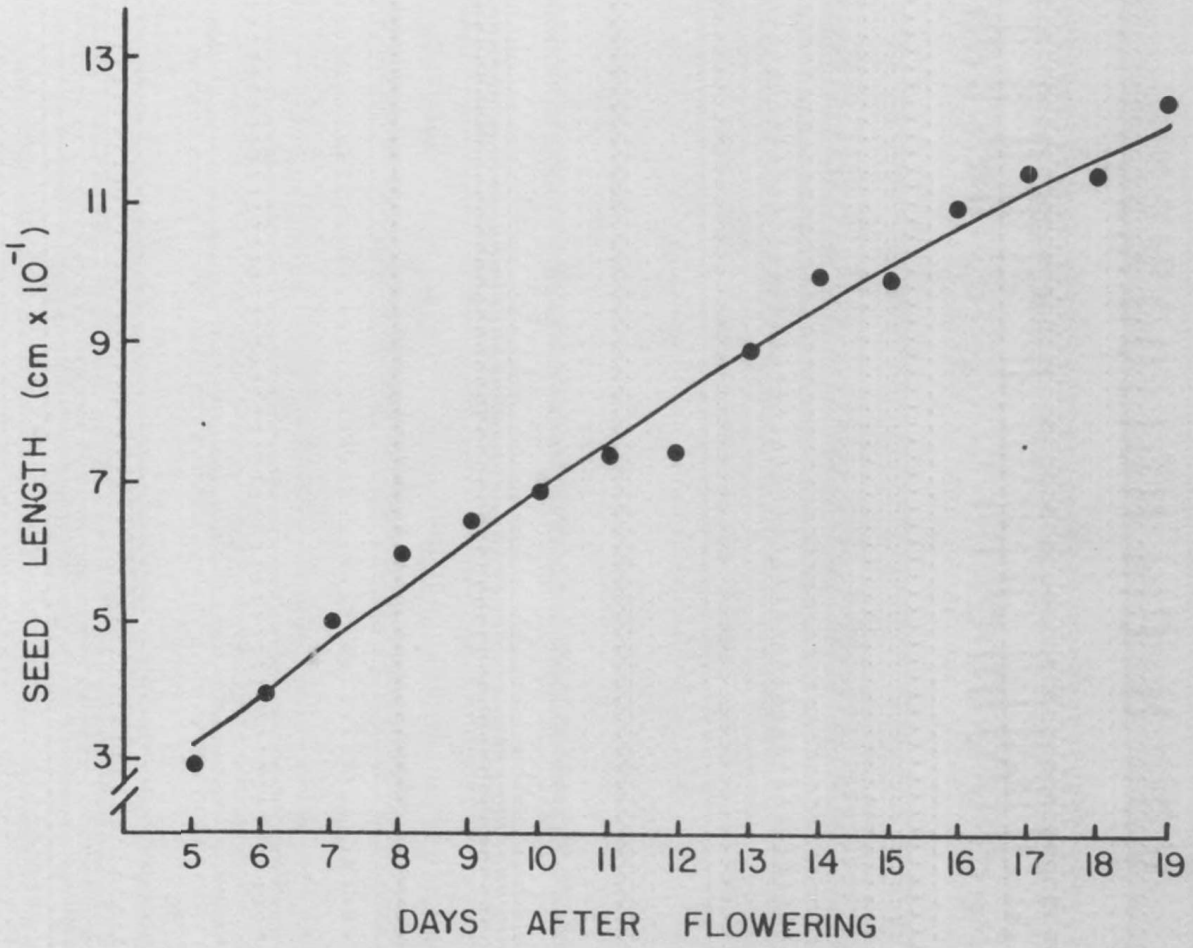


Fig. 4. Predicted and observed values of cowpea seed length from 5 to 19 days after flowering. $Y = 0.09528315 + 0.089629X - 0.001026X^2$, $r = 0.99$, standard deviation = 0.05 cm.

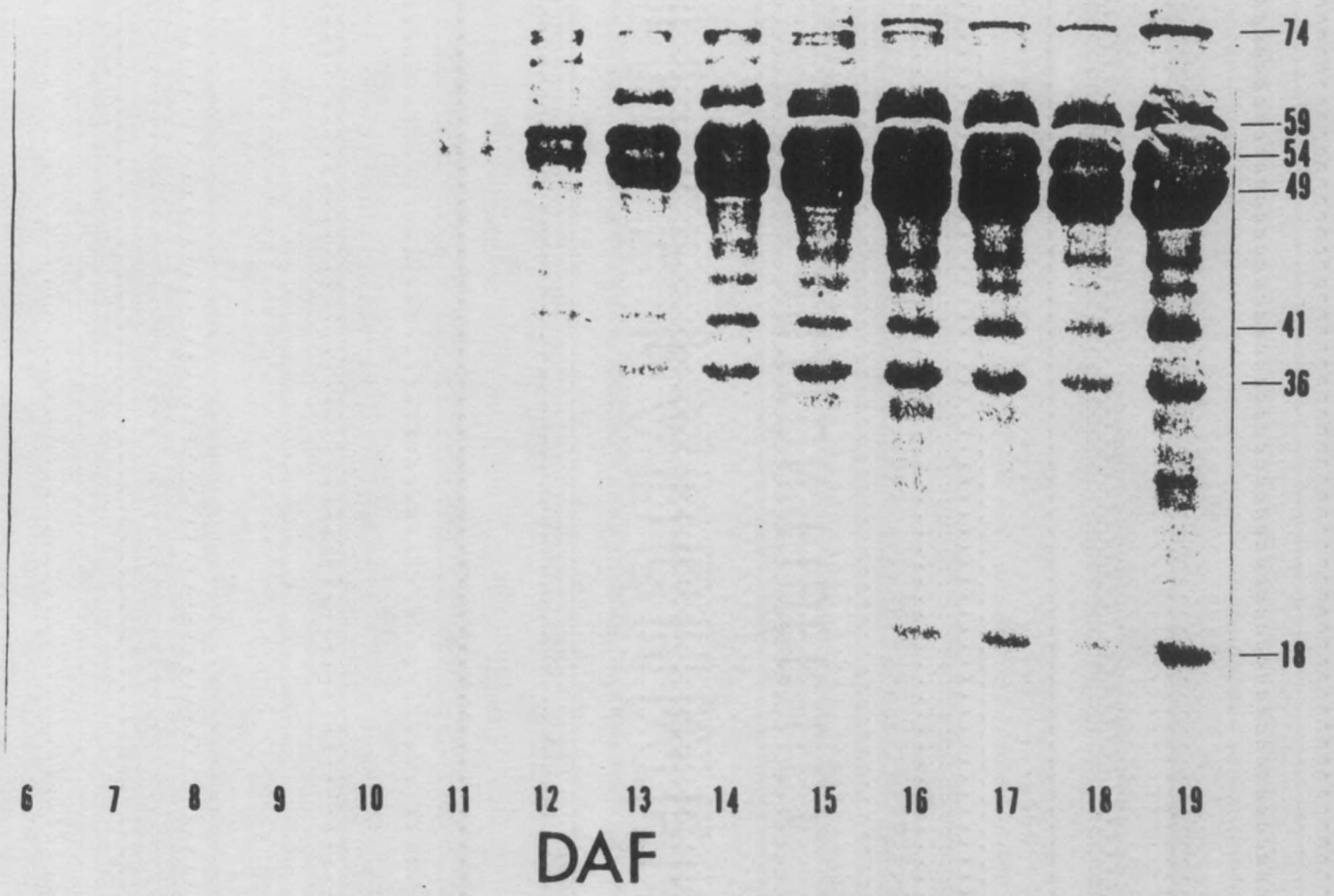


Fig. 5. Sodium dodecyl polyacrylamide gel electrophoresis of seed extracts from cowpeas aged 6 to 19 days after flowering. Relative number of seeds: 6 to 8 DAF-5 seeds per lane; 9 to 12 DAF-2 seeds per lane; and 13 to 19 DAF-1 seed per lane. Numbers in the margin refer to the molecular weight in kilodaltons of the major polypeptides.

studies of cowpea seed proteins (3, 5, 8). Since the most abundant storage protein class in cowpea is the globulin fraction, and because the 7S "vicilin-like" fraction predominates over the 11S or "legumin-like" fraction (5), it seems likely that the 54 and 49 kD polypeptides are the major 7S globulin, vignin (5, 8). The 59 and 18 kD polypeptides, which begin to accumulate at 13 DAF, are most likely the reduced subunits of a large (approximately 82 kD) polypeptide which is "legumin-like" (8). The 41 kD polypeptide, which is present from 11 DAF until maturity, may correspond to the 43 kD polypeptide described as an albumin by Murray et al. (8).

In general, these polypeptides accumulated in a time-frame similar to that previously described (3). Vignin synthesis began in phase II (11 DAF) and most of the protein accumulation occurred during phase III (13-15 DAF).

Glyphosate reduced pod and seed size when compared to untreated plants (Table 1), especially when the herbicide was applied in phase I (7 DAF). During this growth phase, there was an approximate 90% reduction in both seed fresh and dry weight. Glyphosate application during phase II inhibited seed fresh weight accumulation by 88, 72, and 51% for 10, 11, and 12 DAF, respectively. The inhibition of dry weight accumulation was 82% at 10 DAF, 53% at 11 DAF, and although not significant, 22% at 12 DAF. Application in phase III (13 DAF), reduced fresh weight by 32%; dry weight was not affected.

Table 1. Developmental characteristics of cowpea pods and seeds treated with glyphosate.[a]

Treatment State DAF[b]	Maturity phase[c]	Rate of application	Pod		Seed		
			Length	Width	Dry wt	Fresh wt	Length
		(kg/ha)	(cm)	(cm)	(mg)	(mg)	(cm)
7	I	Untreated	16.1	1.0	90.5	271.4	0.8
7	I	1.12	9.5 *	0.5 *	10.7 *	27.0 *	0.3 *
10	II	Untreated	16.7	1.0	123.1	339.3	0.9
10	II	1.12	11.4 *	0.5 *	22.4 *	39.6 *	0.4 *
11	II	Untreated	16.9	1.0	141.3	359.0	0.9
11	II	1.12	11.1 *	0.6 *	65.9 *	99.7 *	0.6 *
12	II	Untreated	17.6	1.0	142.8	349.2	0.9
12	II	1.12	15.1 *	0.9	11.5	171.6 *	0.9
13	III	Untreated	16.5	1.0	142.6	344.5	1.0
13	III	1.12	16.0	0.9	140.2	233.5 *	0.9

[a]Values for glyphosate treatments followed by a "*" are significantly different from the respective control according to the LSD test (P = 0.05).

[b]Days after flowering. Pods were harvested 7 days later.

[c]Phase I (cell division), phase II (growth), phase III (maximum growth).

Table 1. Developmental characteristics of cowpea pods and seeds with different degrees of physiological maturity.

Treatment	Pod length (cm)	Pod width (cm)	Pod thickness (cm)	Pod weight (g)	Seed weight (g)	Seed length (cm)	Seed width (cm)	Seed thickness (cm)	Seed weight (g)	Seed moisture content (%)	Seed germination (%)
0 DAP	4.15	0.95	0.85	1.61	0.41	0.75	0.55	0.45	0.15	85.0	0
5 DAP	5.25	1.05	0.95	2.05	0.55	0.85	0.65	0.55	0.25	75.0	0
10 DAP	6.35	1.15	1.05	2.55	0.75	0.95	0.75	0.65	0.35	65.0	0
15 DAP	7.45	1.25	1.15	3.05	0.95	1.05	0.85	0.75	0.45	55.0	0
20 DAP	8.55	1.35	1.25	3.55	1.15	1.15	0.95	0.85	0.55	45.0	0
25 DAP	9.65	1.45	1.35	4.05	1.35	1.25	1.05	0.95	0.65	35.0	0
30 DAP	10.75	1.55	1.45	4.55	1.55	1.35	1.15	1.05	0.75	25.0	0
35 DAP	11.85	1.65	1.55	5.05	1.75	1.45	1.25	1.15	0.85	15.0	0
40 DAP	12.95	1.75	1.65	5.55	1.95	1.55	1.35	1.25	0.95	5.0	0
45 DAP	14.05	1.85	1.75	6.05	2.15	1.65	1.45	1.35	1.05	0.0	0

When plants were treated during phases II (12 DAF) and III (13 DAF) glyphosate inhibited the dry weight accumulation less than the fresh weight accumulation (Table 1). At these later stages of development it is possible that the seeds had begun to dry, hence the inhibitory effect of the herbicide was less.

SDS-PAGE analysis of seed proteins from treated and control plants showed that glyphosate prevented most of the 49, 54 and 59 kD polypeptide accumulation in seeds from pods sprayed 7 and 10 DAF (Figure 6). Although the pods remained on the plants for 7 days after treatment, no vignin polypeptides were detected. Seeds from untreated plants developed normally and the polypeptide patterns for the controls at 7 and 10 DAF (Figure 6) were similar to those from seeds with the same chronological ages of 14 and 17 DAF (Figure 5).

When plants were treated with pods at 11 DAF and seeds were harvested 7 days later (chronological age of 18 DAF), the polypeptide pattern of seeds from treated plants (Figure 6) was similar to that of 11 DAF seeds from untreated plants (Figure 5). Accumulation of the three major polypeptides was reduced when pods were 11 DAF at the time the plants was treated (Figure 6). In the early stages of development (7, 10, and 11 DAF) protein accumulation in seeds from treated plants appeared to be arrested at the day of spraying. This is paradoxical because glyphosate is believed to be a slow acting herbicide. In perennial species, visible

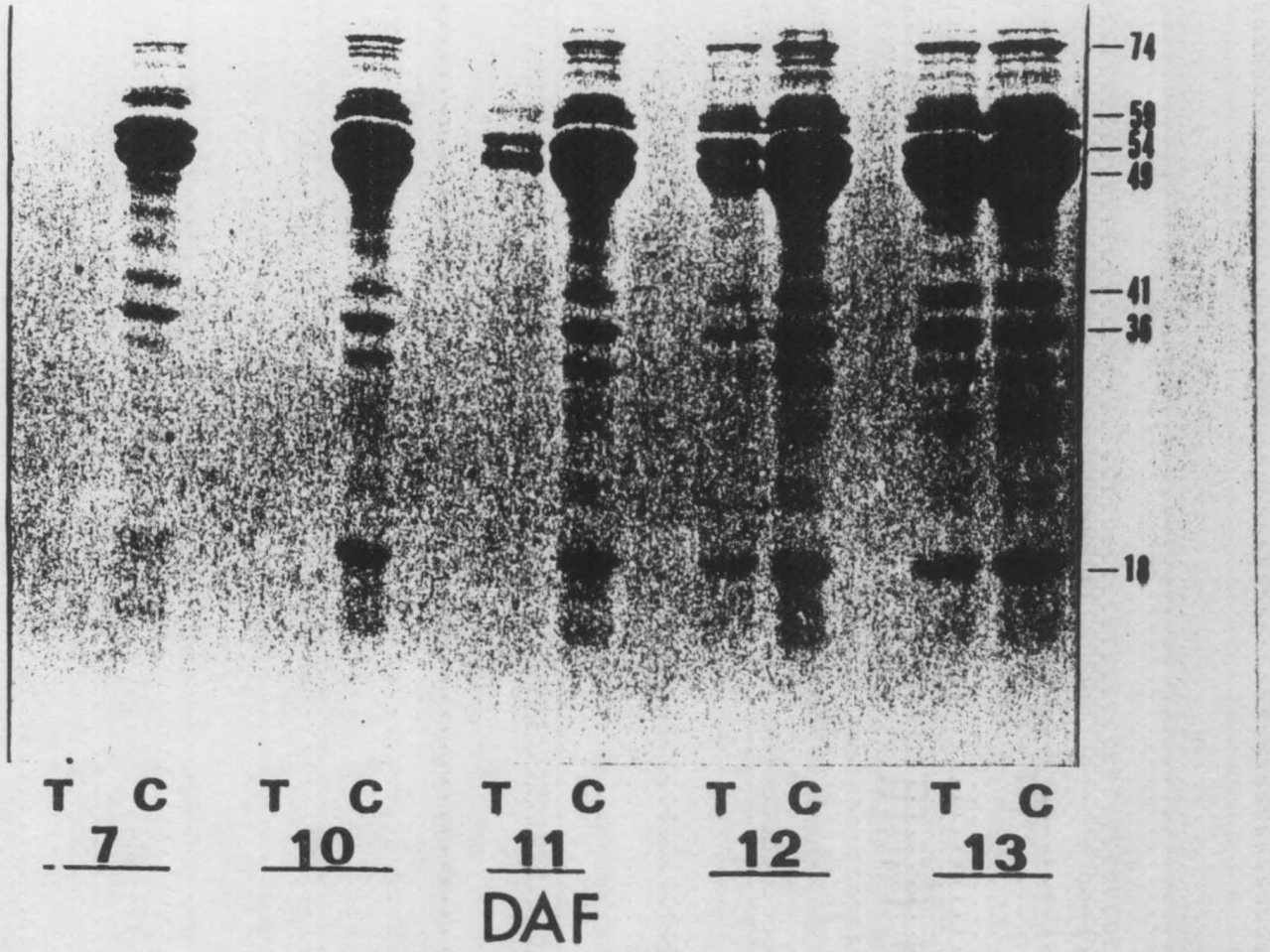


Fig. 6. Sodium dodecyl polyacrylamide gel electrophoresis of seed extracts from cowpea plants treated with glyphosate (T) and control (C) when the pods were 7, 10, 11, 12 and 13 DAF and harvested 7 days after treatment. Relative number of seeds per lane: 7(T), 4 seeds; 7(C), 2 seeds; 10(T), 2 seeds; 10(C), 1 seed; 11(T), 2 seeds; 11(C), 1 seed; all others 1 seed. Numbers in the margin refer to the molecular weight in kilodaltons of the major polypeptides.

effects of glyphosate generally occur 7 to 10 days after spraying (10).

The difference between polypeptides in seed from treated and control plants was less when glyphosate was applied as pods were in late phase II (12 DAF). In this case, the accumulation of the three major polypeptides was reduced less than at the earlier stages (Figure 6). The 49, 54, and 59 kD polypeptides were present in the seed extracts from pods of plants treated at 12 DAF and harvested 7 days later. However, the presence of the 36 and 59 kD polypeptides was representative of seeds harvested at 13 rather than 12 DAF (Figure 5). It is not known why the synthesis of these two polypeptides was not arrested at the time of spraying as was the case in pods 7, 10, and 11 DAF at the time of plant treatment.

Although the amount of the three major polypeptides was less in seeds from pods of plants treated at 13 DAF, (Figure 6), the polypeptide pattern was quite similar to that of seeds from the untreated plants (Figure 5). Glyphosate appeared to arrest the production of the major cowpea polypeptides when it was applied to plants with pods at 7, 10, and 11 DAF. The amount of inhibition was less as the pod matured although there was a decrease in the amount of the three major polypeptides when pods were treated at 12 or 13 DAF. The inhibition was much greater when the herbicide was applied one or two days earlier (Figure 6).

The data presented here indicated that if plants were treated with glyphosate when the pods were 7 to 10 DAF (a time period which precedes the onset of vignin synthesis), they did not initiate synthesis of the 49, 54 and 59 kD polypeptides in the 7 days they remained on the plant. If this effect were caused by plant desiccation alone, one would not expect it to occur so rapidly. The rapidity of the response suggested that desiccation probably was not the only process involved in preventing vignin accumulation. In any case, it seems advantageous to wait until the youngest pods on the plant are 13 DAF before applying glyphosate as a desiccant.

The primary effect of glyphosate on the accumulation of vignin is not known, but it should be noted that cowpea globulins contain relatively large amounts of the aromatic amino acids phenylalanine and tyrosine (3). The total amount of these two amino acids in the albumin and globulin fractions is 5.43 and 8.89 g/100 g protein, respectively (3). The 52 kD polypeptide (which corresponds to the 49 kD band in this study) contains 10.17 g/100 g protein of phenylalanine and tyrosine combined (3). Inhibition of 5-enolpyruvyl-shikimic-acid-3-phosphate synthase by glyphosate may reduce or deplete the concentration of the aromatic amino acids needed to synthesize vignin. From the data reported here it is difficult to determine if this effect is specific for vignin, because the large proportion of vignin, 72% of total seed protein (8), in

the seed may mask effects on the other storage protein fractions. Reduction of phenylalanine and tyrosine may also affect the other storage protein fractions, but to a smaller extent.

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Chapter 3. Written according to requirements for Journal of Agricultural & Food Chemistry.

Cowpea Seed Lipoxygenase Modification with Glyphosate

Cowpea Seed Lipoxygenase Modification with Glyphosate.

ABSTRACT

The predominant cowpea seed lipoxygenase was optimally activated by calcium at 0.68 mM and at pH 7.5. Seeds were analyzed during germination and showed a decrease in lipoxygenase specific activity with time. Seeds from plants treated with the herbicide glyphosate [N-(phosphonomethyl) glycine] showed a significant increase in lipoxygenase content. This could have been a result of senescence, or the effects of the herbicide on those plants. Such a conclusion is based on findings that (a) lipoxygenase, the activity of which was enhanced by glyphosate, has been reported to promote formation of 12-oxo-phytodienoic acid, and (b) 12-oxo-phytodienoic acid has been proposed to be a precursor of jasmonic acid, a growth regulator which promotes senescence of plants. However, we have not found lipoxygenase activity in mature leaves which would question its role in the synthesis of jasmonic acid in the green tissues of plants.

The cowpea (Vigna unguiculata (L.) Walp.) is an important crop in tropical regions throughout the world. Mechanical harvest of this crop is complicated because of a growth habit which gives fruit set and maturity at a time when there is an abundance of green stems and leaves. It has been suggested that the herbicide glyphosate [N-(phosphono-methyl) glycine] could be used to desiccate the plants, and therefore, facilitate mechanical harvesting (Cole and Cerdeira, 1982). However, when glyphosate was used as a desiccant, Cole and Cerdeira (1982) found that cowpea seeds from treated plants germinated and grew more slowly than those from untreated plants. Further studies (Cerdeira et al., 1985) showed a reduction of proteins in seeds from treated plants. This reduction is consistent with the proposed mode of action of glyphosate which is believed to inhibit the enzyme, 5-enolpyruvil-shikimic-acid 3-phosphatesynthase, which catalizes the reaction forming 5-enolpyruvilshikimic-acid-3-phosphate (Amrhein et al., 1980). Inhibition of this enzyme by glyphosate resulted in reduced amounts of aromatic amino acids (Amrhein et al., 1980; Steinrucker and Amrhein, 1980). It has been postulated that the reduced amounts of these amino acids limit the amount of proteins that accumulate in seeds from plants treated with glyphosate (Cerdeira et al., 1985).

The desiccating effect of glyphosate requires several days and appears to have some similarity to natural senescence. It is possible that treating the plants with

glyphosate induces the synthesis of a senescence hormone in the plant. Vick and Zimmerman (1983) have suggested that jasmonic acid could be formed from linolenic acid catalyzed by lipoxygenase; and in further studies (Ueda and Kato, 1980; Yamane et al., 1981; and Dathe et al., 1981) jasmonic acid was identified as a growth regulator which promotes senescence of plants. Therefore, it is possible that lipoxygenase may be involved in the synthesis of jasmonic acid and that jasmonic acid synthesis may be triggered by glyphosate treatment.

Lipoxygenase (LOX) catalyzes the oxidation of polyunsaturated fatty acids containing cis, cis-1,4 pentadiene systems to form hydroperoxides (Galliard and Chan, 1980). This enzyme is widely distributed in the plant kingdom especially in legumes, in which soybean was reported as being the richest source (Scott, 1975; Koch, 1958). Lipoxygenase activity has been found in seeds (5, 9, 11) and also in green leaves (7). Three different types of lipoxygenase have been identified in soybean (3). LOX-1 (3) is non-calcium stimulated with the highest activity when assayed at pH 9.0 with linoleic acid as substrate. According to Koch (11) soybean Ca-stimulated lipoxygenase is activated by Ca and has its optimal specific activity when assayed at pH 7.5 with linoleic acid as substrate. Koch (11) also reported a third soybean isoenzyme, which has the highest activity when assayed with trilinolein at pH 5.5. However, it should be noted that trilinolein

activity is observed at higher pH levels, and may be due to the LOX enzymes active on linoleic acid.

Truong and Mendoza (1982) reported two lipoxygenase isoenzymes from cowpea seeds, with one enzyme being inhibited and the other activated by calcium. The same authors also found 94% of the total lipoxygenase activity in the cotyledons. The pH range for optimal enzyme activity for both isoenzymes was between 5 and 8.

The present study was conducted to determine if glyphosate had an effect on the content of the calcium stimulated lipoxygenase of seeds from treated plants. Preliminary investigations indicated that LOX activity may increase after glyphosate treatment. These studies were also conducted to determine the changes of lipoxygenase activity in developing and germinating cowpea seeds.

EXPERIMENTAL SECTION

Plant Material. Cowpea plants cv. Mississippi Purple were grown in the field during the summer of 1982, in the greenhouse during the fall of 1983 and the summer of 1984. In the greenhouse plants were grown in 15 by 20 cm pots filled with a 2:2:1 (V/V/V) soil (Oktibbeha Sandy Loam; Fine, Mixed, Thermic, Typic Hapludalfs): sand: peat mix. After emergence, plants were thinned to two plants per pot and each plant was staked. No supplemental light or fertilization was used. For insect control aldicarb

(2-methyl-2-(methylthio)-propionaldehyde-o-methyl-carbamoyl)oxime) at approximately 50 mg ai/pot was added at the first true leaf stage of development. Pots were watered overhead on a daily basis as needed and greenhouse temperature was maintained at approximately 25 C. Pod age for both field-grown and greenhouse plants was determined by tagging the flowers as they opened. When a majority of the pods reached the appropriate ages the plants were sprayed with glyphosate at 1.12 kg/ha in 234 l/ha of water. One week after spraying the tagged pods from treated and control plants were harvested, shelled, and freeze-dried. Seeds at each maturity stage were pooled and analyzed for lipoxygenase activity. For developmental studies, pods 5, 7, 9, 13, 16, 17, and 19 days after flowering (DAF) were harvested, seeds were lyophilized and analyzed for lipoxygenase activity.

For analysis of LOX activity during germination, cowpea seeds from untreated plants were germinated in an incubator at 30 C in sterilized sand under light and dark conditions. Whole seedlings were harvested at 2, 4, 6, and 8 days after planting, freeze-dried and analyzed for lipoxygenase content. To determine the effect of glyphosate on LOX activity in green tissue, greenhouse-grown seedlings were sprayed with glyphosate at 1.12 kg/ha at five and 17 days after planting. Leaves from control and treated plants were harvested during eight days, at two day intervals, and analyzed for lipoxygenase activity.

Enzyme Extraction and Assays

Lyophilized cowpea seeds were homogenized with a mortar and pestle. The cowpea proteins were extracted by stirring 1 g of ground seeds in 10 ml of buffer (10 mM Tris HCl, pH 7.0) for 30 min. The mixture was filtered at room temperature through filter paper (qualitative medium porosity), centrifuged at 10,000 xg for 10 min. at room temperature and the supernatant was used for enzyme assays.

Lipoxygenase activity in the supernatant was measured in a 3 ml assay solution containing either 0.35 mM linoleic acid, 2.7 mM trilinolein, or in some cases EGTA (Ethylene glycol-bis-B-amino-ethyl-ether) was added at a final concentration of 0.83 mM in order to chelate any calcium ions. The pH of the buffer was either 5.5, 7.5, or 9.0 depending on the assay. The lipoxygenase assay was initiated by adding 20 µl of the enzyme extract and measuring the O_2 consumption polaragraphically at 25°C using a Yellow Springs oxygen monitor with a Clark electrode. Specific activities were calculated by multiplying the total activity expressed as M of O_2 by 0.78 to adjust for the amount of O_2 in distilled water, multiplied by 100, and divided by the amount of protein in the assay. The amount of protein in the enzyme extract was determined by the Lowry method (7).

RESULTS AND DISCUSSION

The highest LOX activity was found when the cowpea extract was assayed at pH 7.5 in the presence of Ca and linoleic acid (Table 1). This activity was eliminated when EGTA was added to chelate the available Ca. This activity in the presence of Ca, was probably due to Ca stimulated LOX as described by Koch et al., 1968. Another type of LOX activity was found when the extract was assayed in the presence of trilinolein (Table 1). This activity was not as high as the Ca stimulated activity and appeared to be less affected by pH of the assay mixture. It appeared that there were two LOX isoenzymes in this cowpea extract, one stimulated by Ca at pH 7.5 and another which had a broad pH range for activity. These results are similar to those of Koch (1968) and Dillard et al. (1961) who found differences in physical properties of lipoxygenase preparations from soybeans, peanuts, and navy beans. No activity was found at pH 9.0. Because the primary LOX activity was the Ca stimulated LOX, we decided to concentrate our studies on it.

To learn more about LOX activity during plant development two experiments were done to determine LOX activity in both developing and germinating seeds. Cowpea seeds were germinated under dark and light conditions and the activity of the enzyme in seed extracts decreased with

Table 1. Comparison of variations of lipoxygenase activities with pH and substrate.

pH	Substrate (with 3 ml, pH 7.5 imidazole buffer)	Specific Activity (μM O_2/mg protein/minute)
7.5	Ca[1] + L.A.[2]	3.1 a[3]
7.5	EGTA[4] + L.A.	0.0 f
7.5	EGTA + Trilinolein	0.5 c
7.5	L.A.	0.6 bc
9.0	Ca + L.A.	0.2 de
9.0	EGTA + L.A.	0.1 ef
9.0	EGTA + Trilinolein	0.8 b
9.0	L.A.	0.0 f
5.5	Ca + L.A.	0.2 de
5.5	EGTA + L.A.	0.6 bc
5.5	EGTA + Trilinolein	0.8 b
5.5	L.A.	0.4 cd

[1]5 μl of calcium ($CaCl_2$) at 0.45 M, final concentration 0.75 mM.

[2]5 μl of linoleic acid at 0.21 M, final concentration 0.35 mM.

[3]Means not followd by a common letter differ significantly at the probability .05 by Duncan's New Multiple Range test.

[4]5 μl ethylene glycol-bis-B-amino-ethyl-ether at 0.3 M, added to block any possible effect of Ca. Final concentration 0.83 mM.

[5]50 μl of trilinolein at 0.13 M, final concentration 2.7 mM.

increased in time of germination (Table 2). A greenhouse experiment was conducted to prepare a developmental time-table of cowpea lipoxygenase activity as the seed developed. The specific activity of the enzyme increased from zero to 2.9 from five to 19 DAF (Fig. 1).

Studies of cowpea seed protein accumulation during seed formation and protein utilization during seed germination (Cerdeira et al., 1985) showed a pattern similar to that of lipoxygenase in seeds. Thus, lipoxygenase might have a possible role in germination. A possible role of LOX on germination was also suggested by Gerritsen et al. (8).

Cowpea seedlings treated with glyphosate five and 17 days after planting and harvested at two day intervals after treatment had no lipoxygenase activity in the leaf tissue. Holden (1969) reported the enzyme to be very active on leaf tissues of other species such as peanuts (Phaseolus vulgaris). The reason for the lack of agreement is not understood and is possibly due to different assay techniques. In the laboratory of R. Koch, numerous germination studies on soybean, navy beans and blackeyed pea seeds failed to show any LOX activity in seedling extracts after green leaf formation.

To test the effect of glyphosate on LOX in developing seeds, plants were treated with glyphosate at 1.12 kg/ha at five DAF. Treated and control developing seeds were harvested at two-day intervals and LOX was measured. The

Table 2. Calcium-stimulated lipoxygenase activities of seeds germinated under light and dark conditions.

D.A.I.[1]	Specific Activity (μO_2/mg prot./min.) Light	Dark
0	3.1 a[2]	3.5 a[2]
2	2.3 b	3.1 b
4	1.7 c	2.2 c
6	0.8 d	1.3 d
8	0.0 e	0.0 e

[1]Days After Inbibition.

[2]Means not followed by a common letter differ significantly at the probability .05 by Duncan's New Multiple Range test.

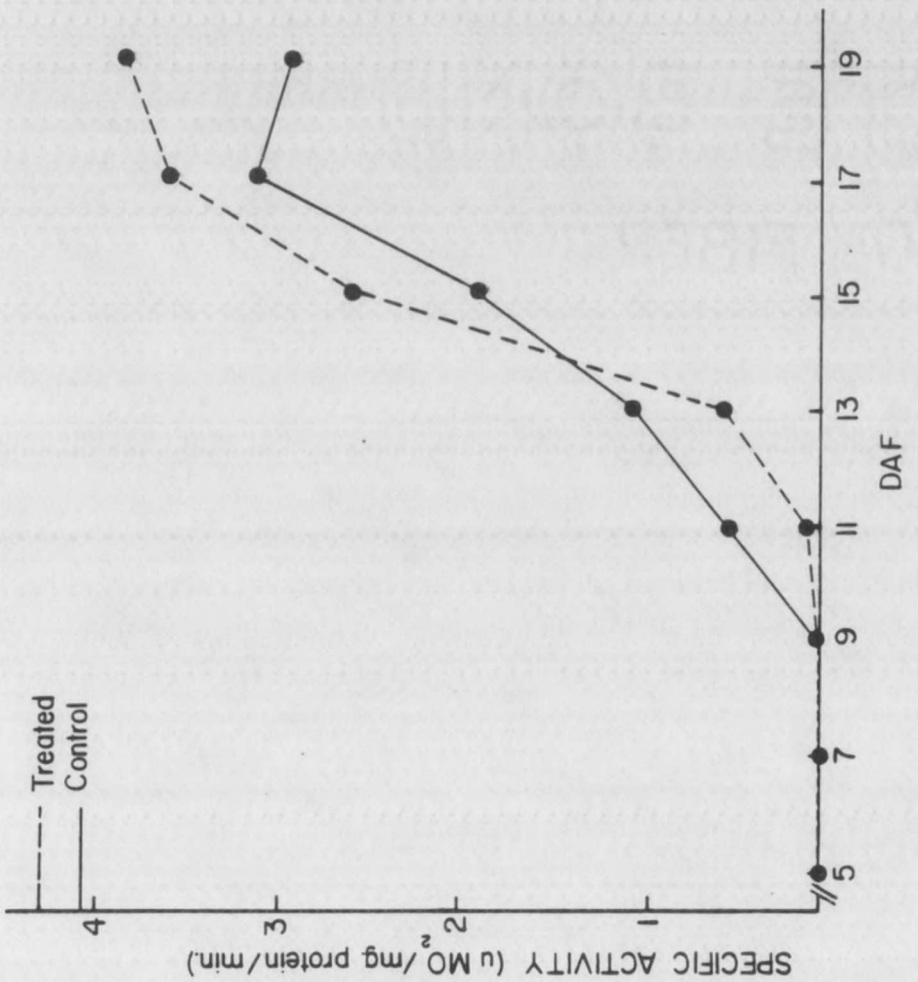

Fig. 1. Specific activity of lipoxygenase in seed from glyphosate treated and untreated plants 2, 4, 6, 8, 10 and 12 days after treatmert.

amount of the enzyme from treated seeds was slightly higher at the later phase of seed development and slightly lower until 13 DAF (Fig. 1).

Seeds from plants treated with glyphosate in the field when pods were 10, 12, 14, and 16 DAF and harvested a week later showed a significant increase on the specific activity of lipoxygenase (Table 3). The seeds from the treated plants appeared to be less developed (smaller, wrinkled) than those seeds from untreated plants.

It is not known why glyphosate promoted an increase in cowpea seed lipoxygenase (Table 3). Vick and Zimmerman (1983) found that linolenic acid was converted to 12-oxo-phytodienoic acid by lipoxygenase and hydroperoxide cyclase enzymes present in Vicia faba pericarp. The same authors also found 12-oxo-phytodienoic acid to be a precursor of jasmonic acid. One of the possible explanations of glyphosate action on plants might be its relationship with lipoxygenase and jasmonic acid. Jasmonic acid has been reported to be a growth regulator which promotes senescence of plants (Ueda and Kato, 1980; Yamane et al., 1981; Dathe et al., 1981). Since glyphosate enhanced the activity of lipoxygenase, an enzyme reported to be important for formation of jasmonic acid, it could also be formed as a final effect of glyphosate treatment, and then the plant would senesce and die. However, it should be noted that in the present studies, no LOX activity was found in mature plants; LOX was only found in the seeds.

Table 3. Effect of glyphosate on lipoxygenase content of cowpea seeds.

DAF[1]	Specific Activity ($\mu M\ O_2$/mg prot./min.)
10C[2]	1.1 b[3]
10T	2.5 a
12C	1.4 b
12T	2.8 a
14C	1.0 b
14T	2.4 a
16C	3.1 a
16T	2.9 a

[1]Days after flowering.

[2]T,treated with glyphosate; C,control. Seeds were harvested one week after treatment.

[3]Means not followed by a common letter differ significantly at the probability level of .05 by Duncan's New Multiple Range test.

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SUMMARY AND CONCLUSIONS

The use of glyphosate as a desiccant on whole plants resulted in inhibition of pod width, seed dry weight, length, protein, and polypeptides when those seeds were at younger stages of maturity at the time of plant desiccation with glyphosate. Those young seeds also showed an increase in histidine content and lipoxygenase activity. More mature seeds were less affected.

Seeds at the older stages of maturity were not affected by glyphosate application. At the time when a dry harvest would be conducted these stages would account for a large percentage of the total crop, therefore, this practice appears to be potentially useful in this particular crop for more efficient mechanical harvesting. This procedure would need to be employed for desiccation of green stem and leaf tissue when a majority of the pods were dry in order to have only a small percentage of the seeds affected.

APPENDIX

Table 1. Average dry weight of 10 southernpea seeds from pods at four ages when plants were desiccated with glyphosate.

DAF[a]		Rate	Dry Weight
Treated	Harvested	(kg/ha)	(gm)
10	10	Control	0.45 g[b]
10	17	Control	1.43 cdefg
10	17	0.28	1.33 fgh
10	17	0.56	1.30 gh
10	17	1.12	0.91 i
12	12	Control	0.86 i
12	19	Control	1.36 efgh
12	19	0.28	1.49 defgh
12	19	0.56	1.28 h
12	19	1.12	1.28 h
14	14	Control	1.33 efgh
14	21	Control	1.50 cd
14	21	0.28	1.46 cde
14	21	0.56	1.45 cdef
14	21	1.12	1.42 cdefg
16	16	Control	1.54 bc
16	23	Control	1.69 a
16	23	0.28	1.63 ab
16	23	0.56	1.68 a
16	23	1.12	1.63 ab

[a]Days after flower opened.

[b]All means within a column followed by a common letter do not differ significantly at the probability of .05 by Duncan's New Multiple Range test.

Table 2. Average lipid weight of 10 southernpea seeds from pods at four ages when plants were desiccated with glyphosate.

DAF[a]		Rate	Weight
Treated	Harvested	(kg/ha)	(mgs)
10	10	Control	5.72 i[b]
10	17	Control	17.58 def
10	17	0.28	16.86 def
10	17	0.56	16.12 fg
10	17	1.12	10.83 h
12	12	Control	10.79 h
12	19	Control	16.69 defg
12	19	0.28	16.12 fg
12	19	0.56	16.21 fg
12	19	1.12	14.94 g
14	14	Control	16.59 efg
14	21	Control	18.34 cde
14	21	0.28	17.04 def
14	21	0.56	17.43 def
14	21	1.12	17.23 def
16	16	Control	18.56 cd
16	23	Control	21.76 a
16	23	0.28	19.63 bc
16	23	0.56	19.69 bc
16	23	1.12	20.50 ab

[a]Days after flower opened.

[b]All means within a column followed by a common letter do not differ significantly at the probability of .05 by Duncan's New Multiple Range test.

Table 3. Average "total available carbohydrate" weight of 10 southernpea seeds from pods at four ages when plants were desiccated with glyphosate.

DAF[a]		Rate	Weight
Treated	Harvested	(kg/ha)	(mgs)
10	10	Control	195.7 i[b]
10	17	Control	693.5 bc
10	17	0.28	638.4 cdef
10	17	0.56	619.2 ef
10	17	1.12	449.3 g
12	12	Control	376.2 h
12	19	Control	686.8 bcd
12	19	0.28	655.0 bcde
12	19	0.56	632.4 def
12	19	1.12	628.8 def
14	14	Control	585.2 f
14	21	Control	699.4 b
14	21	0.28	706.3 b
14	21	0.56	683.3 bcd
14	21	1.12	674.5 bcde
16	16	Control	702.6 b
16	23	Control	811.6 a
16	23	0.28	806.8 a
16	23	0.56	770.7 a
16	23	1.12	784.4 a

[a]Days after flower opened.

[b]All means within a column followed by a common letter do not differ significantly at the probability of .05 by Duncan's New Multiple Range Test.

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