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Action of Photosynthetic Diuron Herbicide on Cell Organelles and Biochemical Constituents of the Leaves of Two Soybean Cultivars

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Two varieties of soybean (*Glycine max* L. cv. Clark and Crawford) showed a high sensitivity to low concentrations of diuron herbicide [3-(3,4-dichlorophenyl)-1,1-dimethyl urea]. Depending on the application dose of diuron, chlorosis, necrosis, and wilting of leaves ending in death after 7 to 10 days were observed. These symptoms were more obvious in the Clark variety even at the lowest concentration of diuron (1 ppm, 4.29 μM). Electron microscopic observation of treated plants revealed disorganization in the ultrastructure of mesophyll cell organelles. Disappearance of starch grains, decrease of plastoglobuli, and swelling and disarrangement of grana thylakoid of chloroplasts were observed in comparison to untreated plants. The guard cells of stomata of the Clark variety were seriously affected. The chlorophyll content of diuron-treated plants was sharply lowered. On the other hand, proline, soluble protein, and carbohydrate contents of leaves were increased in diuron-treated plants. The protein pattern of treated leaves of the two cultivars was altered, and the most profound change is the appearance of a new ~207-kDa polypeptide. © 2000 Academic Press

INTRODUCTION

Herbicides have been selected for their weed-killing characteristics; however, they also affect growth, cell ultrastructure, and metabolism of economic crops (1–3). Herbicide groups (triazines, phenylureas, pyridazinones, biscarbamates), often termed as classical diuron-type herbicides, have been developed against the photosynthetic apparatus that acts as a PSII inhibitor (4). They compete for a common binding site of the D1 protein on thylakoid membranes (5). The redox components required for the function of PSII are localized on the heterodimer of the D1 and D2 proteins (6).

Diuron is the prevalent herbicide and has been shown to inhibit PSII electron flow (7, 8) and hence prevent the reduction of $NADP^+$ required for CO_2 fixation. Oettmeier and Soll (9) reported that the short-chain plastoquinol analogues competed with diuron for a binding site on chloroplast thylakoids. Other reports have provided evidence that PSII-inhibiting herbicides displace plastoquinone at the Q_B-binding site on the D1 protein and specifically block electron flow from Q_A to Q_B (10–12). Photobleaching of chlorophylls and carotenoids in chloroplasts was induced by diuron (13, 14) and atrazine (15).

Many groups of herbicides target amino acid biosynthesis (16). Chlorsulfuron herbicide (sulfonylureas) induced the increase of proline content (3) and inhibited the biosynthesis of branched-chain amino acids (17). Inhibition of total leaf protein by herbicides was also reported by Singh *et al.* (1).

One of the primary limitations in this research has been the lack of tolerance to the herbicides by one of the major crops of the world (soybean). Therefore, the aim of this work was to study the action of diuron on the ultrastructure of photosynthetic and stomatal apparatus and some biochemical constituents of two soybean cultivars.

MATERIALS AND METHODS

Seeds of soybean (*Glycine max* L. cv. Clark and Crawford) were sterilized by dipping in 7% Chlorox solution for 5 min and then washed several times with distilled water. Seeds were germinated in clean plastic containers in a mixture of sand–clay soil. On the 15th day, seedlings of the two varieties having the same size were

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chosen and treated with 1 ppm (4.29 μM), 2 ppm (8.58 μM), or 5 ppm (21.46 μM) diuron. Additions of diuron were according to the field capacity of soil experiments (26%). On the 22nd day, fresh leaves were taken for the determination of pigment content, ultrastructure study, and the separation of protein on gel-electrophoresis. The remaining fresh leaves were harvested and dried for the determination of proline, carbohydrate, and protein contents. Plants which were treated with 5 ppm diuron were wilted after 7 days and thus were omitted from the study. The soil of the experimental plants (1- and 2 ppm diuron), kept wet for 12 months and then used again to cultivate soybean, produced no symptoms in the plants.

Ultrastructural Studies

Plant samples for TEM (about 1 mm^2) were fixed in 2.5% glutaraldehyde, in 0.05 M phosphate buffer (pH 7), for 3 h at room temperature. Samples were rinsed several times with 0.05 M phosphate buffer (pH 7) and then post-fixed with 1% OsO_4 in 0.05 M phosphate buffer for 2 h at room temperature. Samples were then rinsed several times with 0.05 M phosphate buffer, dehydrated in a gradient acetone series, and embedded in Spurr's medium (18). Ultrathin sections (60 nm thick) were stained with uranyl acetate and lead citrate. Specimens were viewed 15 times with a Jeol-1010 transmission electron microscope at 100 kV.

Pigment Content Estimation

The pigment contents (chlorophyll (Chl)-*a*, Chl-*b*, and carotenoids) of control and herbicide-treated plants were measured when the leaves (treated plants) showed signs of chlorosis (7 days). A known fresh weight of leaves was homogenized in 85% aqueous acetone solution. Pigment concentration was spectrophotometrically determined according to Arnon (19) and Metzner *et al.* (20). Measurements of chlorophyll *a* and *b* and total carotenoid contents were performed three times for control and each treatment. Mean values and standard deviations were calculated in relation to fresh weight basis.

Proline Content Estimation

Proline content of leaves was determined according to Bates *et al.* (21). A known dry weight (0.1 g) was extracted in 10 ml of aqueous 3% sulfosalicylic acid over-night. The extract was centrifuged at 1500-*g* for 10 min. Two milliliters of the supernatant was mixed for reaction with 2 ml of fresh acid ninhydrin solution and 2 ml glacial acetic acid in a test tube for 1 h at 100°C. The reaction was terminated in an ice bath, and the mixture was extracted with 4 ml toluene. The extract was vigorously stirred for 20 s using a test tube stirrer. Thereafter, the chromophore-containing toluene was aspirated from the aqueous phase, and its absorbency was measured at 520 nm. The proline content was determined from a standard curve and calculated on a dry weight basis.

Carbohydrate Content Estimation

Soluble carbohydrate content was determined in the aqueous solution with anthrone sulfuric acid reagent according to Fales (23), using glucose as a standard. The anthrone sulfuric reagent consists of 0.2 g anthrone, 8 ml absolute ethyl alcohol, 30 ml distilled water, and a 100-ml concentration of sulfuric acid ($D = 1.84$) A Spekol Carl-Zeiss spectro-colorimeter was used, and the blue–green color that developed was measured as the absorbance of 620 nm.

Protein Content Estimation

Soluble protein content was determined according to Lowry *et al.* (22). Tissue samples (0.1 g) were extracted in 10 ml distilled water for 2h at 90°C. The extract was centrifuged and the supernatants were pooled. The water-soluble protein was estimated by the Folin–phenol reagents. Bovine serum albumin was used as a standard.

Protein Extraction and Electrophoretic Analysis

Leaves (1 g) of fresh weight were ground in 1 ml extraction buffer in a mortar at 4°C. The extraction buffer (pH 8.5) consisted of 0.2 M

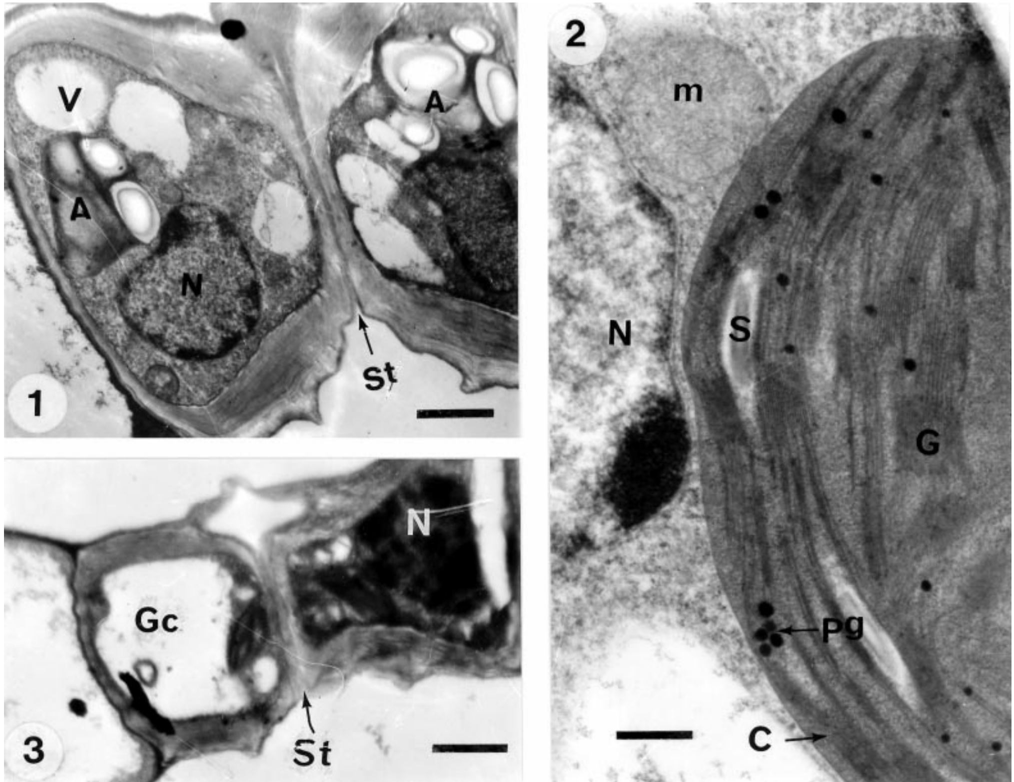


FIG. 1. Guard cells contain many starch grains and have a thick cell wall (CW) in the side adjacent to the stomatal pore (St). Amyloplast (A) Bar, 1.3 μm

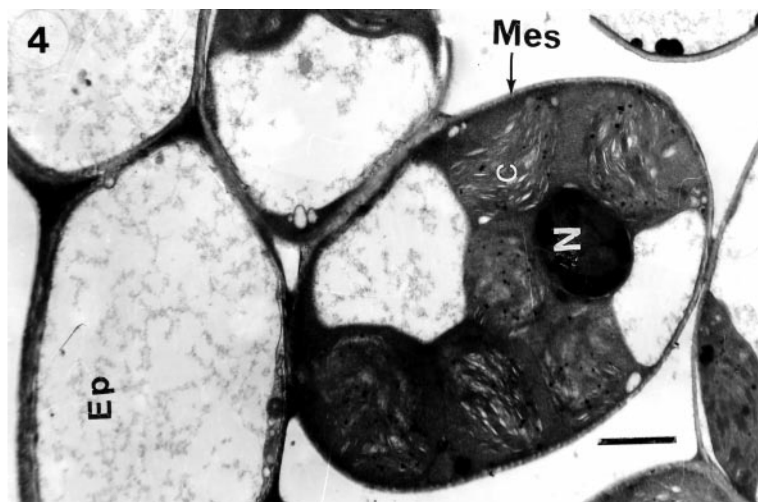
FIG. 2. Electron micrograph of the control soybean leaves (L. cv. Clark). The fine structure of mesophyll chloroplast, mitochondria (M), and nucleus (N). Bar, 0.4 μm.

FIG. 3. Electron micrograph of the treated (1 ppm diuron) soybean leaves (L. cv. Clark). Guard cells were degenerated and their organelles disappeared. Bar, 1.55 μm.

Tris–HCl, 1M sucrose, and 0.3% (v/v) 2-mercaptoethanol (24). The homogenate was centrifuged at 15,000 *g* for 15 min. SDS–PAGE was performed according to the procedure of Laemmli (25) using 7.5 to 15% continuous gradient acrylamide slab gels (1.5 mm thick). The supernatant of protein samples was mixed with an equal volume of buffer containing 0.125 M Tris–HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and bromophenol as a tracking dye and immersed in a boiling water bath for 3 min. Supernatants (0.3 ml) containing about 45 μg of protein were loaded onto 7.5% acrylamide slab gels and subjected to electrophoresis (Bio-Rad, Protean II xi cell). Gels were run at 18 mA for 10 h at 10°C in 0.025 Tris–HCl + 192 mM glycine + 0.1% SDS (pH 8.3). The gels were stained with Coomassie blue.

RESULTS AND DISCUSSION

Irreversible damage occurs when the defense system is overwhelmed. Signs of wilting of 5 ppm diuron-treated leaves started to appear after 7 days of treatment. In plants treated with 1 and 2 ppm diuron, chlorosis and necrosis of leaves were observed in the two varieties after 7 days of treatment. These symptoms were much more obvious in the Clark variety. The symptoms were more obvious on plants with increasing doses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of diuron. However, recultivation of the experimental soil of 1 and 2 ppm diuron kept wet for 12 months produced no symptoms on soybean plants.

Electron Microscopic Observations

As shown in Figs. 1–7, the ultrastructure observations of diuron-treated soybean revealed much subcellular disorganization in comparison to the control plants. Stomata (Fig. 1) and mesophyll chloroplast (Fig. 2) of untreated Clark variety contained starch grains while the stomata and chloroplasts of the treated plants lacked starch grains (Figs. 3–5). The mean plastoglobuli number/profile of chloroplast was 20.66 $\pm$ 1.52 for the control and 12.33 $\pm$ 2.08 for 1 ppm diuron-treated plants. In diuron-treated leaves of the Clark variety (Figs. 3–5), the cells and their organelles, in most cases, were degenerated. Grana stacks and stroma lamellae lost their arrangements and aggregated within the chloroplast (Fig. 5). Swelling of thylakoid membranes occurred; consequently, the intrathylakoid space was increased and many vesicles were formed (Figs. 4 and 5). Figures (6–8) show the cell ultrastructure of control and diuron-treated leaves of the Crawford variety. Chloroplast grana stacks of the control plants (Fig. 6) were well-developed. The mean plastoglobuli number/profile of chloroplast was 29 $\pm$ 3.05 for the control and 14.33 $\pm$ 1.58 for 1 ppm diuron. In diuron-treated plants (Fig. 8), the chloroplasts became spherical shaped. Moreover, chloroplast grana (Fig. 8) were dispersed and the grana thylakoid was weakly stained (less electron opaque). In contrast, nuclear materials of treated plants were relatively more electron opaque in comparison with those of the control plants. Starch grains of guard cells of treated leaves disappeared (Fig. 7). Chloroplast residuals (Fig. 7) in guard cells were observed (Fig. 7).

From the ultrastructural view, electron microscopic observations indicated that the chloroplasts of two varieties contain starch grains and had a lower proportion of thylakoid grana stacking (Figs. 2 and 6). In contrast to chloroplasts in control plants, the disappearance of starch grains and disorganization of grana stacking of chloroplasts were noted in diuron-treated plants (Figs. 4, 5 and 8). Thylakoid grana stacking and plastoglobuli number in chloroplasts of the Crawford variety were greater than that of Clark. Both of those characteristics seem to illustrate the less sensitivity in Crawford than that of Clark to the action of diuron. However, both varieties are very sensitive to diuron. Many herbicides including diuron inhibit photosynthesis by blockage of photosystem II (26, 27). The net effect of blocking electron transport is the destruction of the photosystem II reaction center and photooxidation of lipid and chlorophyll molecules (14). Chloroplasts have various mechanisms to scavenge toxic oxygen species, but in the present study they are overwhelmed by the level of oxidative stress generated. The herbicide-resistant chloroplasts are characterized by increased grana stacking, decreased starch content, and lower chlorophyll *a*/*b* ratio (5, 28).

Biochemical Activities

After 7 days of growth in the presence of diuron, the herbicide-treated plants showed considerably high differences in the pigment contents in comparison with that of the untreated plants (Table 1). Chl-*a*, Chl-*b*, and carotenoids

FIG. 4. Electron micrograph of the treated (1 ppm diuron) soybean leaves (L. cv.Clark). A portion of leaf shows the epiderms (Ep) and mesophyll cells (Mes). Nucleus (N) and chloroplasts (C) of mesophyll cells were degenerated. Bar, 2.2 μm.

FIG. 5. Electron micrographs of the treated (1 ppm diuron) soybean leaves (L. cv. Clark). The fine structure of chloroplast (C). The grana stacks (G) lost their arrangement and aggregated on one side within the chloroplast. Starch grains disappeared and many vesicles were formed. Bar, 0.4 μm.

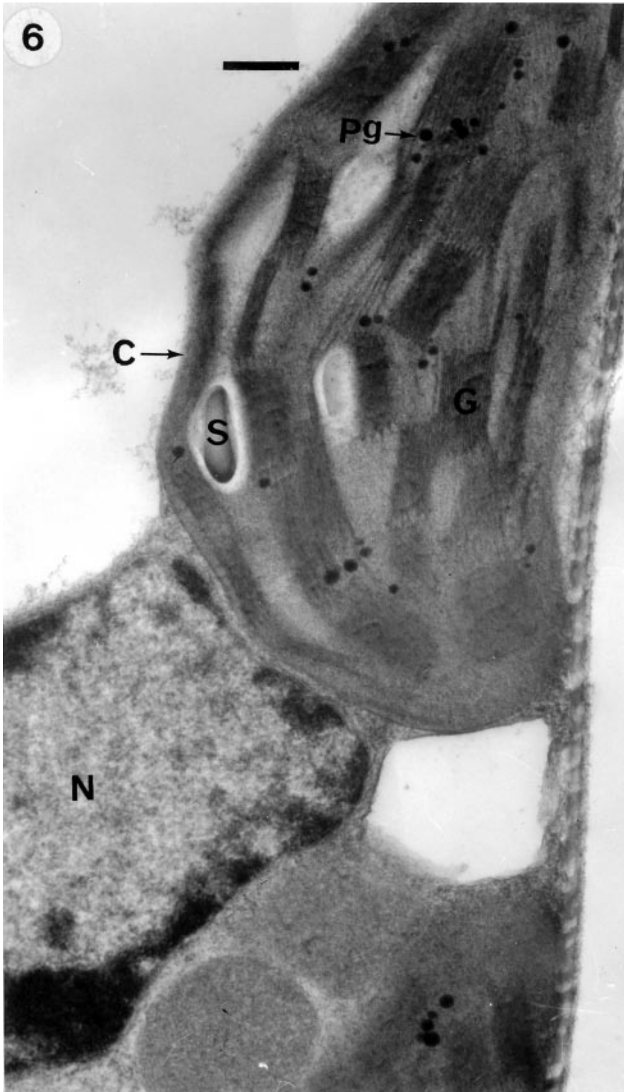
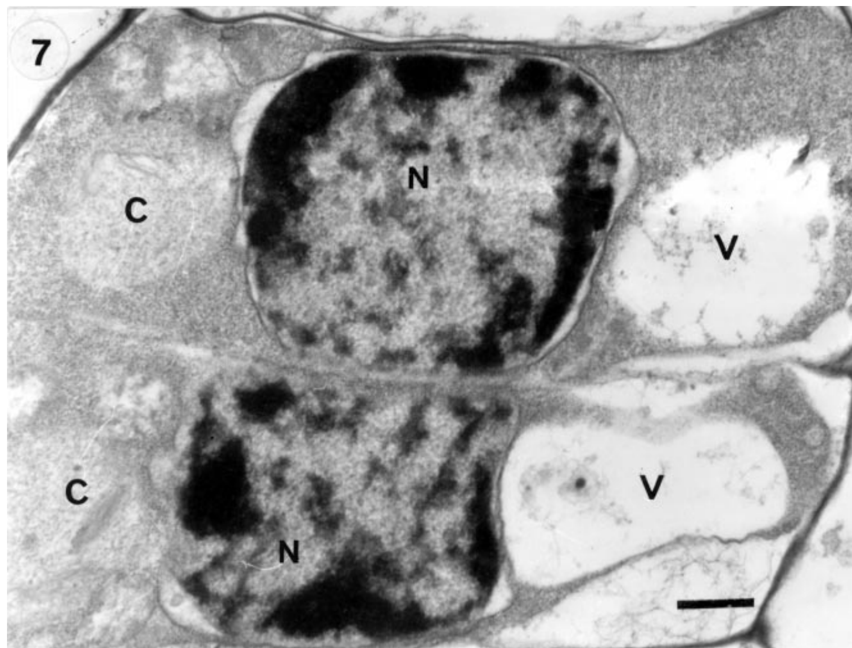


FIG. 6. Electron micrographs of the control soybean leaves (L. cv. Crawford). The fine structure of the nucleus (N) and chloroplast (C) of a leaf mesophyll cell. Well-arranged grana (G) and plastoglobuli (Pg) are clearly observed in the chloroplast. Bar, 0.45 μm.

were decreased significantly with herbicide treatment. Carotenoid content of the two varieties was more affected with increasing diuron treatments. The mean carotenoid contents of Clark or Crawford were 0.49 or 0.45 mg/g^{-1} for the control plants and 0.12 or 0.19 mg/g^{-1} for 2 ppm diuron-treated plants, respectively. Pigment deficiency can be caused either by photobleaching (14) or by inhibition of biosynthesis of either chlorophylls or carotenoids (8). Boger and Sandmann (29) reported that diuron and other inhibitors of photosynthetic electron flow inhibit formation of protoporphyrin IX in autotrophic *Scenedesmus* as well as autotrophic soybean cell cultures and *Lemna*. However, the diuron herbicide activity is due to the oxidative stress generated when photosynthetic electron transport is blocked (14).



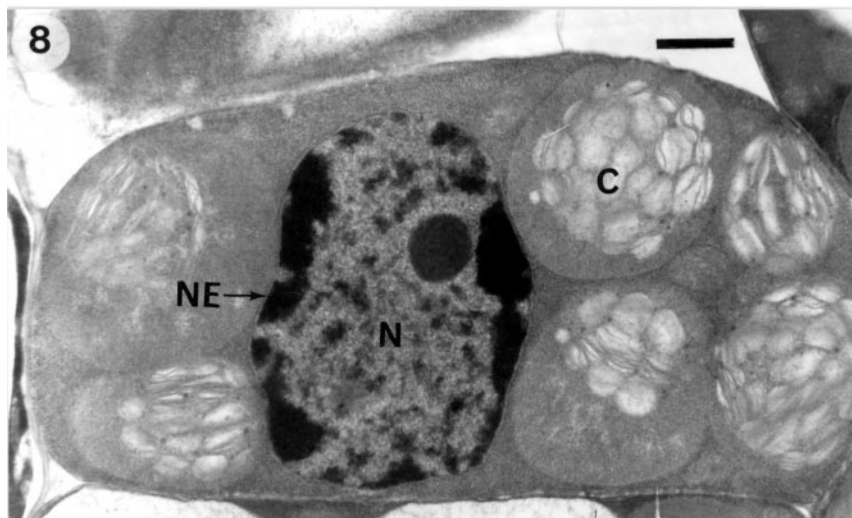


FIG. 7. Electron micrographs of the treated (1 ppm diuron) soybean leaves (L. cv. Crawford). Both chloroplast residuals (C) and the cytoplasm of guard cells (Gc) do not contain starch grains. Nuclear material separated from nuclear envelope. Bar, 0.62 μm.

FIG. 8. Electron micrographs of the treated (1 ppm diuron) soybean leaves (L. cv. Crawford). Mesophyll cell chloroplasts (C) were spherical shaped and their grana were swollen and weakly stained. Bar, 1 μm.

TABLE 1
Pigment Contents (mg/g fresh wt^{-1}) of Treated and Untreated Soybean Leaves (L. cv. Clark and Crawford)

Treatment	Chlorophyll *a*	Chlorophyll *b*	Carotenoid
Clark (control)	1.48 ± 0.10	0.59 ± 0.02	0.49 ± 0.26
1 ppm diuron	1.11 ± 0.065	0.48 ± 0.04	0.31 ± 0.031
2 ppm diuron	0.53 ± 0.087	0.26 ± 0.031	0.12 ± 0.020
Crawford (control)	1.58 ± 0.12	0.67 ± 0.10	0.45 ± 0.031
1 ppm diuron	1.04 ± 0.061	0.45 ± 0.36	0.27 ± 0.044
2 ppm diuron	0.73 ± 0.060	0.36 ± 0.25	0.19 ± 0.025

Note. Values are means of three replicates ± SD.

Proline content of treated plants was markedly increased in comparison with that of the untreated plants (Fig. 9). This increase, in most cases, was concomitant with an increase in diuron dose treatments. Therefore, the metabolic action of the diuron probably represents a severe stress situation comparable to various environmental stress factors such as drought (30) and salt stress (31). The results in the present work show that the diuron leads to plant wilting and structural perturbations in the cells. It is more probable that the proline increase reflected stress due to herbicide action. An increase in proline content of roots of *Pisum* and *Vicia* was observed after chlorsulfuron herbicide treatments (3). On the other hand, high levels of NaCl induced biochemical changes related to the metabolism of activated oxygen, producing $O_2^{\cdot -}$ radicals that can damage enzymes and membranes (32, 33). Under drought and saline conditions, proline accumulates for osmoregulation and protect proteins from the inhibitory effects of ions (30, 31).

Soluble carbohydrate (Fig. 10) and soluble protein (Fig. 11) were increased with increasing diuron application. However, total protein and carbohydrate were decreased under diuron stress (data not shown). Inhibition of total leaf protein of pea following triazole, metrization, or pyrazon herbicides was noted by Singh *et al.* (1). However, photosynthetic metrization herbicide increased protein content in leaves of *Lupinus albus* (2).

The electrophoretic pattern of leaf proteins from soybean plants treated with diuron showed changes in the protein profiles in comparison with that of the untreated soybean plants (Fig. 12). In higher plants, photosystem II is a multiprotein complex and consists of at least 23 different subunits located primarily in the grana or

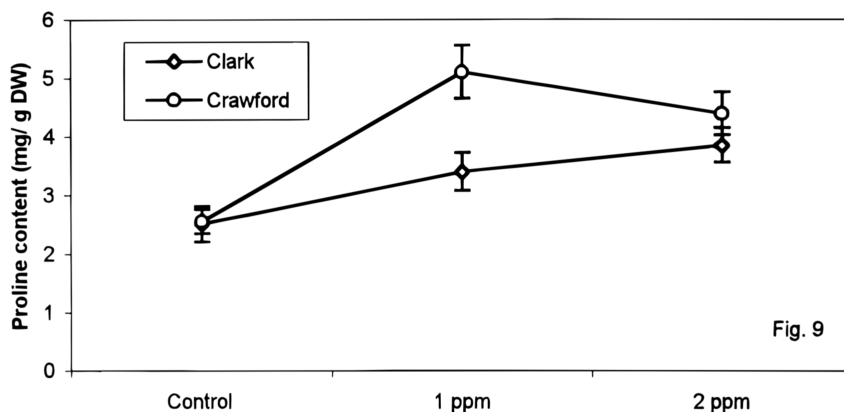


FIG. 9. Proline content (mg/g dry wt^{-1}) of treated and untreated soybean leaves.(L. cv. Clark and Crawford). Values are means of three replicates ± SD.

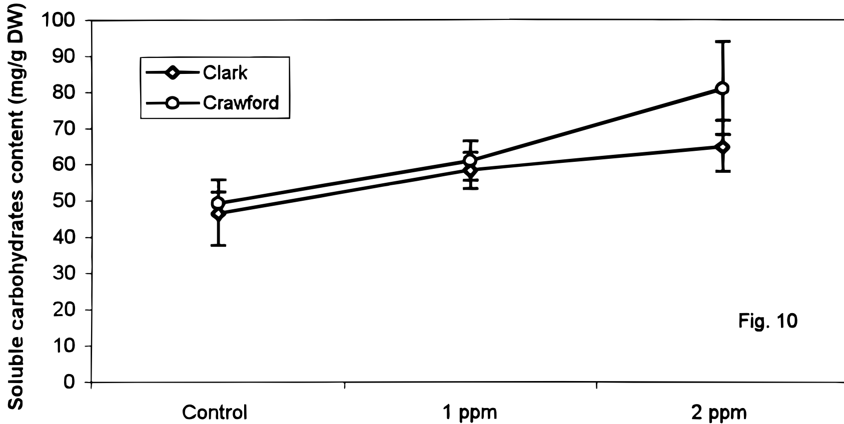


FIG. 10. Soluble carbohydrate content (mg/g dry wt^{-1}) of treated and untreated soybean leaves (L. cv. Clark and Crawford). Values are means of three replicates ± SD.

appressed lamella (32). The changes in the protein pattern (Fig. 12) were as follows: (i) A new polypeptide of about 207 kDa was formed in both varieties in diuron-treated plants. (ii) The polypeptides of about 67, 52, 46, 42, 32, and 11 kDa in two varieties of soybean were accumulated. Accumulation of these polypeptides was increased with increasing diuron concentration. Induction of the formation of a 41-kDa adduct of the D1 protein by photosynthetic herbicides has been previously reported (34, 35). (iii) A polypeptide of about 63 kDa disappeared in diuron-treated plants of the Crawford variety (Fig. 12). Therefore, changes in polypeptides (synthesis, accumulation, or disappearance) of treated plants probably lead to repair of oxidative damage of membranes around the photosystem II. Finally, it can be concluded that the protein pattern and other analysis in this study indicated that the stomatal and photosynthetic apparatus and leaf metabolism of the two soybean cultivars were seriously affected by a low concentration of diuron.

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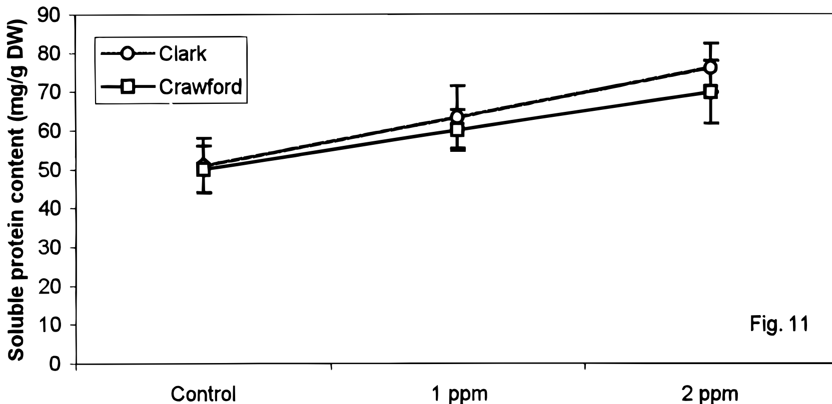

FIG. 11. Soluble protein content (mg/g dry wt^{-1}) of treated and untreated soybean leaves (L. cv. Clark and Crawford). Values are means of three replicates ± SD.

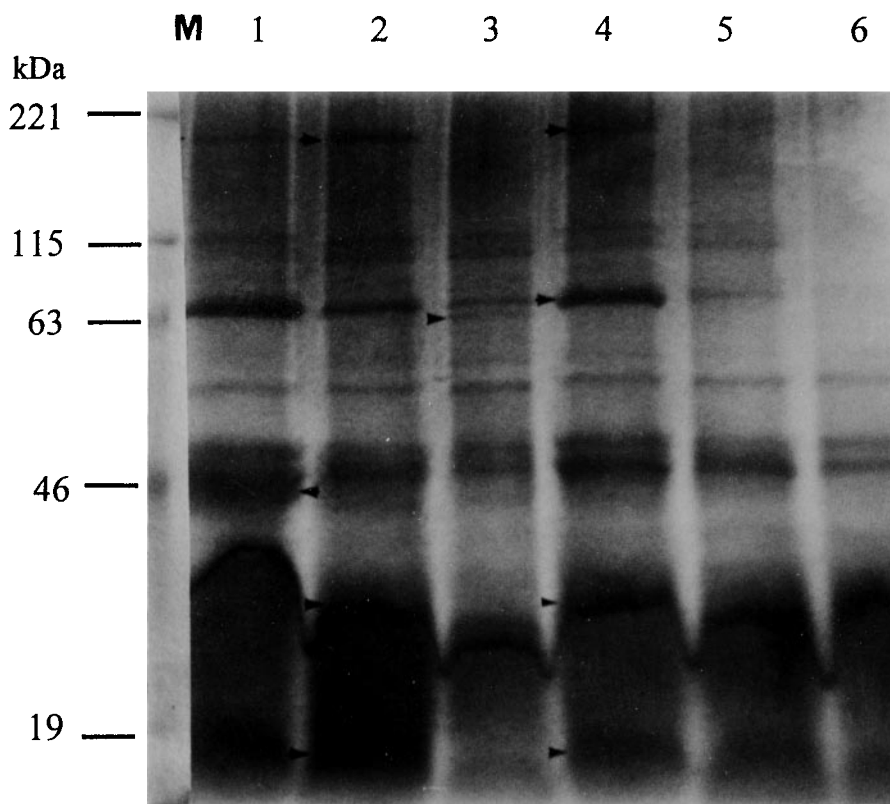

FIG. 12. Electrophoretic profiles of the total soluble proteins extracted from leaves of soybean (*Glycine max* L. cv. Clark and Crawford) after 7 days of being treated with or without diuron. Clark control (lane 6). Herbicide treatments: 1 ppm diuron (lane 5); 2 ppm diuron (lane 4). Crawford control (lane 3). Herbicide treatments: 1 ppm diuron (lane 2); 2 ppm diuron (lane 1). The supernatant of protein samples containing about 45 μg protein was loaded onto gel wells. Molecular masses of marker proteins; myosin (221 kDa), β-galactosidase (115 kDa), bovine serum albumin (63 kDa), ovalbumin (46 kDa) and lysozyme (19 kDa). Polypeptide bands were visualized by Coomassie blue. Acrylamide slab gel was dried in gel dryer (Bio-Rad Model 543) and then photographed. Arrow-heads refer to polypeptides that were altered.

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