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The effects of glyphosate on the development and cell ultrastructure of white mustard (*Sinapis alba* L.) seedlings

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Summary: Résumé: Zusammenfassung

The phytotoxicity of glyphosate (N-(phosphonomethyl glycine) to seedlings of white mustard (*Sinapis alba*) cultivated indoors was studied. Yellowing and wrinkling of leaves was observed, necrotic spots appeared and the elongation of the seedlings was significantly reduced at doses 0·49 kg ai/ha and above. Only when sprayed at 4·97 kg ai/ha was the effect of glyphosate 100% lethal (5–7 days after spraying). At the highest concentration of herbicide a marked decrease in chlorophyll content was found but with 0·49 kg ai/ha the chlorophyll content was found to be higher than that in the leaves of control plants.

Two and fourteen days after spraying with glyphosate and the commercial product samples of leaf and stem were harvested for electron microscopy. Cellular defects in the leaves ranging from slight swelling to complete disruption of the chloroplasts were detected at the two highest herbicide doses 48 h after spraying. These defects were intensified with time and in addition other sub-morphological changes occurred; decrease in starch grain content, an increase in the number of dictyosomes and mitochondria, disruption of tonoplasts and increase of plastoglobuli. In the more central parts of stem segments the commercial product resulted in greater cellular effects than did glyphosate. It is suggested that the differences may be due to the surfactant.

Effets du glyphosate sur le développement et l'infrastructure cellulaire de plantules de moutarde blanche (Sinapis alba *L.*)

La phytotoxicité du glyphosate (N-(phosphonométhyle glycine), pour des plantules de moutarde blanche (*Sinapis alba*) cultivées à l'intérieur, a été étudiée.

Il a été observé un jaunissement et un gaufrage des feuilles, l'apparition de points de nécroses, et l'élongation des plantules a été significativement réduite à la dose de 0,49 kg/ha (m.a.) et au-dessus. C'est seulement avec un traitement à 4,97 kg/ha (m.a.) que le glyphosate a été létal à 100% (5 à 7 jours après le traitement).

A la concentration la plus élevée de l'herbicide, une diminution marquée du taux de chlorophylle a été observée, mais à 0,49 kg/ha (m.a.), la teneur en chlorophylle des feuilles de plantes traitées a été trouvée supérieure à celle des plantes témoins.

Deux et 14 jours après le traitement avec du glyphosate et du produit commercial, des échantillons de feuilles et de tiges ont été récoltés pour examen au microscope électronique. Des anomalies cellulaires ont été décelées dans les feuilles aux doses d'herbicide les plus élevées, anomalies qui allaient d'un léger gonflement à la disparition complète des chloroplastes. Ces anomalies se sont intensifiées avec le temps et, en outre, sont apparues avec le temps d'autres modifications sub-morphologiques consistant en une diminution de la teneur en grains d'amidon, un accroissement du nombre des dictyosomes et des mitochondries, une perturbation des tonoplastes et une augmentation des plastoglobules. Dans la partie la plus centrale des fragments de tiges, le produit commercial a provoqué plus de réactions cellulaires que le glyphosate. Il est suggéré que ces différences peuvent être dues à l'agent de surface.

Die Wirkung von Glyphosat auf die Entwicklung und die Zellultrastruktur von Keimlingen des Weissen Senfs (Sinapis alba *L.*)

Es wurde die phytotoxische Wirkung von Glyphosat [N-(Phosphonomethyl)-glycin] auf Keimlinge des Weissen Senfs (*Sinapis alba*) unter kontrollierten Bedingungen untersucht.

An den Blättern waren Vergilbungen und Verkrüppelungen zu beobachten, nekrotische Flecken traten auf und das Längenwachstum der Pflänzchen war bei 0,49 kg AS ha^{-1} und höheren Dosierungen signifikant verringert. Nur wenn 4,97 kg AS ha^{-1} gespritzt wurden, wirkte Glyphosat letal (5–7 Tage nach der Spritzung).

Bei der höchsten Herbizidkonzentration war der Chlorophyllgehalt der Blätter deutlich erniedrigt, bei 0,49 kg AS ha^{-1} war er aber höher als bei den unbehandelten Kontrollpflanzen.

Zwei und 14 Tage nach der Spritzung mit Glyphosat und seinem Handelsprodukt wurden Blatt- und Stengelproben für elektronenmikroskopische Untersuchungen geerntet. In den Blättern wurden 48 h nach der Spritzung der zwei höchsten Aufwandmengen zelluläre Defekte wie leichte Anschwellungen, bis hin zur vollständigen Zerstörung der Chloroplasten, festgestellt. Diese Defekte verstärkten sich im Laufe der Zeit und zusätzlich traten weitere submorphologische Veränderung auf: abnehmender Gehalt an Stärkekörnern, zunehmende Zahl an Dictyosomen und Mitochondrien, Aufreissen des Tonoplasts und eine Zunahme an Plastoglobuli. Im zentralen Stengelbereich wurden durch das Handelsprodukt mehr Veränderungen hervorgerufen als durch die Aktivsubstanz. Es wird angenommen, dass diese Unterschiede auf den Gehalt des Handelsprodukts an Netzmittel zurückzuführen ist.

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Introduction

The broad action spectrum of glyphosate (Baird

et al., 1971), the lack of soil residual effects and ready translocation in plants have made this compound unique as a post-emergence herbicide (Sprankle, Megitt & Penner, 1975a & b). Few attempts have been made to identify its possible sites of action at the molecular and cellular level. Jaworski (1972) suggested that glyphosate inhibited aromatic amino acid biosynthesis by repressing chorismate mutase and/or prephenate dehydratase. Boyle & Evans (1974) in an attempt to detect a possible mutagenic effect, found that glyphosate induced meiotic chromosome abberrations in autumn rye. The effect of glyphosate on chloroplast ultrastructure in quackgrass mesophyll cells has been studied by Campbell, Evans & Reed (1976). The enzyme myrosinase (a thioglucoside glucohydrolase) which catalyses the hydrolysis of glucosinolates found in cruciferous plants, is also affected by glyphosate (Riiser, Engesaeth & Iversen, 1978).

The object of the present study was to investigate the effects of glyphosate on cell ultrastructure of white mustard seedlings and to attempt to correlate these observations with the morphological development of the plants. Chlorophyll content after glyphosate treatment was also measured.

Materials and methods

Seeds of white mustard (*Sinapis alba* L.) were germinated in plastic boxes filled with potting soil and kept either in the greenhouse or in a special cultivation-room throughout the experimental period. The mean temperature in the greenhouse was 25°C and relative humidity 60%; in the cultivation room the conditions were 25°C and 70%, respectively. In the latter case the plants were constantly illuminated with fluorescent light with a spectral intensity distribution as described previously (Iversen & Siegel, 1976). Plants were sprayed with herbicide 14 days after germination.

The herbicide glyphosate (pure glyphosate from Monsanto Company, St Louis, Missouri) was applied in water at rates of 0·005, 0·10, 0·49 and 4·97 kg ai/ha using a laboratory sprayer. For comparison the commercial formulation of glyphosate* was used in the same concentration range for the electronmicroscopical examinations. The control plants were sprayed with water. There were four replications.

* Roundup®, 360 g ai/l

The morphological development of control and glyphosate sprayed plants was followed by visual observation. The initial length of the seedlings was determined before spraying and followed for 2 weeks except in the cases where the dosage proved to be lethal for the plants. At the same time the content of chlorophyll per unit leaf area was estimated for the living leaves.

For chlorophyll determinations 0·5 g of the youngest leaves without petioles were cut and homogenized in darkness in 80% aqueous acetone at 4°C for 2 h. After centrifugation and removal of cell debris, the chlorophyll content was determined using a Beckman UV 25 spectrophotometer at 663, 652, 645, and 475 nm. The different chlorophyll components were determined as follows (Bruinsma, 1963): chl.a; $12{\cdot}7 \cdot A_{663} - 2{\cdot}7 \cdot A_{645}$, chl.b; $22{\cdot}9 \cdot A_{645} - 4{\cdot}7 \cdot A_{663}$ and chl. a + b as the mean of the following determinations: $20{\cdot}2 \cdot A_{645} + 8{\cdot}0 \cdot A_{663}$ and $27{\cdot}8 \cdot A_{652}$.

The areas of twenty leaves selected from the same part of the plants as the leaves for the chlorophyll determinations, were determined from weighed paper replicas of the fresh leaves. Both the chlorophyll and area values given are the means of three replicates from each group.

For the electron microscopical study mature leaves or stem pieces were selected at random from each group of treated plants 2 and 14 days after spraying. The material was prefixed at 21°C in 3% glutaraldehyde in 0·1 M phosphate buffer (pH 7·3) for 2 h followed by postfixation in 2% osmium tetroxide in the same buffer at 4°C. After dehydration in increasing concentrations of ethanol the specimens were embedded in Epon. The sections were cut using glass and diamond knives and stained in 2% uranyl acetate for 15 min followed by 2% lead citrate for 5 min. The sections were examined in a JEOL 100 B electron microscope at 80 KV.

Results

Morphological development and elongation

Two days after the application of the most concentrated solution of glyphosate, mild chlorosis and wrinkling of the treated leaves was observed. After 3–4 days, chlorosis was intensified, necrotic spots were frequently observed, and occasionally dehydration occurred in the basal part of the seedling stem and roots.

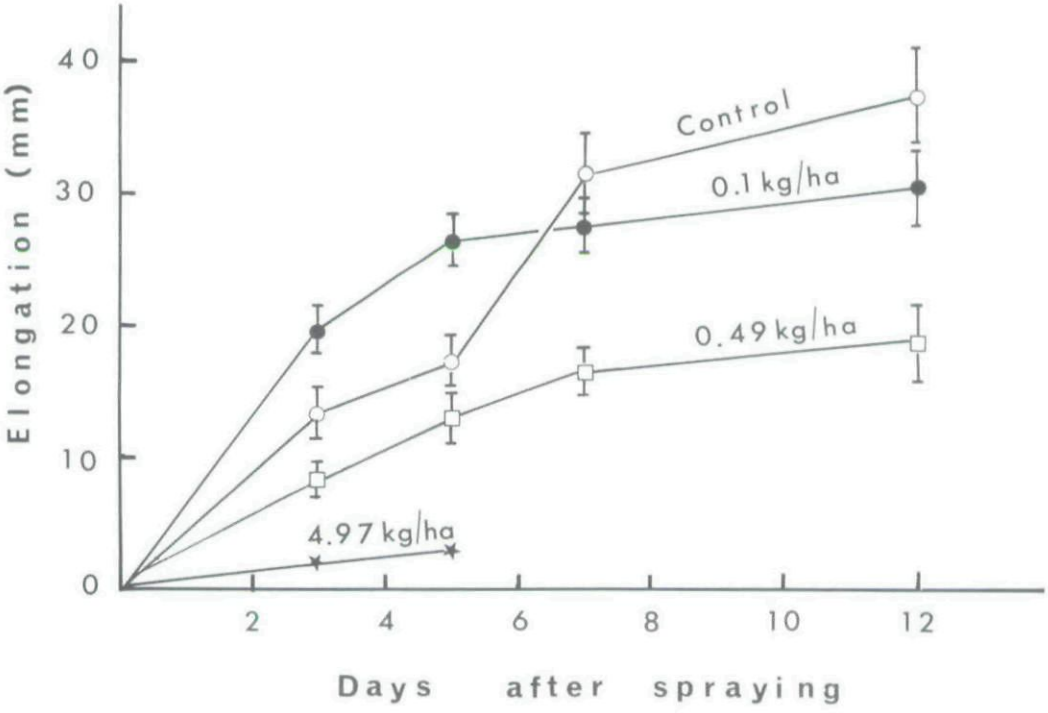

Fig. 1. The effect of glyphosate on the elongation of the intact plant. The initial average height of the seedlings was 72 mm. Vertical bars are standard errors of the mean. Glyphosate concentrations: ●: 0·10 kg ai/ha, □: 0·49 kg ai/ha, ★: 4·97 kg ai/ha; ○: control.

The visual symptoms which developed in the seedlings treated with 0·49 kg ai/ha, were initially the same as those for the highest concentration of glyphosate but usually delayed in time. In comparison with the control the leaf areas were restricted in growth and they appeared a more intense green 6 days after spraying. After 12 days anthocyanins occurred in the basal parts of the stems.

The seedlings treated with the lowest dose of glyphosate showed mild chlorosis which intensified with time in the younger leaves. After 12 days necrotic spots occurred in the older leaves and anthocyanins in the seedling stems.

At 0·1 kg ai/ha, glyphosate increased the total height of white mustard seedlings 5 days after spraying (Fig. 1). At the highest dose (4·97 kg ai/ha) a large decrease in the rate of elongation was observed immediately after spraying (Fig. 1).

Twelve days after spraying 100%, 55% and 15% of the seedlings had died in the high, medium and low dose treatments, respectively (Table 1).

Table 1 Effect of glyphosate on seedlings of white mustard, sprayed with herbicide after appearance of two mature leaves. The numbers of dead plants are given as a percentage of the total number of plants sprayed with the herbicide

Dose (kg ai/ha)	Days after spraying			
	3	5	7	12
0 (control)	0	0	0	0
0·10	0	0	15	15
0·49	0	20	40	55
4·97	15	20	100	100

Chlorophyll content

Treatment with the highest dose of glyphosate caused a decrease in the chlorophyll content when determined 3 days after spraying and estimated per unit leaf area (Table 2). After treatment with 0·49 kg ai/ha glyphosate the chlorophyll content per unit leaf area was found to be higher than that of the control and it increased with time after spraying (Table 2).

The ultrastructure of the cells

When observed at the submicroscopic level the cellular defects in the leaves ranged from slight swelling to complete disruption of the chloro-

Table 2 Effect of glyphosate on chlorophyll content (chl. a + b) per unit leaf area (g/m^2) of living leaves of white mustard

Dose (kg ai/ha)	Days after spraying			
	0	3	6	10
0(control)	0·98	0·80	0·88	0·53
0·10		0·65	0·82	0·67
0·49		1·04	1·17	1·24
4·97		0·70	—	—

plasts. In the latter case residual and disorganized thylakoids were observed (Fig. 2c); in the same cells the tonoplast was broken and the cellular content scattered in the cell. Cells taken from leaves treated with 0·05 kg ai/ha appeared normal when compared with the control material (Fig. 2 a & b).

When leaf mesophyll cells were examined 2 weeks after treatment with 0·05 kg ai/ha of the commercial product only one effect was detectable; the starch grains in the chloroplasts had disappeared (Fig. 3b). Leaf cells treated with 0.49 kg ai/ha or more of the commercial product appeared to be increasingly affected. Tonoplasts and cristae membranes in the mitochondria were generally disrupted and swelling of chloroplasts was frequently seen in material collected 2 weeks after spraying. The glyphosate-treated material showed submicroscopic defects.

In stem segments taken from plants 2 weeks after spraying with the highest concentration the effects from the commercial product were different from those of the pure compound. With the commercial product some totally disrupted chloroplasts were found in cells near the vascular system. More frequently chloroplasts were swollen with disorientation of the grana. The number of dictyosomes and mitochondria seemed to have been increased in the same cells in comparison with the control. The more peripheral cells in the cortical region of the stem contained intact chloroplasts without any obvious symptoms of disruption. Neither did the cytoplasmic content in these cells appear to differ from the control plant cells from the same region. On the other hand in stem segments treated with pure glyphosate the cells near the vascular region were only slightly affected by the herbicide. The chloroplasts in a few cells showed swelling and a number of plastoglobuli which were not observed in the control cells were found (Fig. 4). In a few other cells the tonoplast was disrupted and the

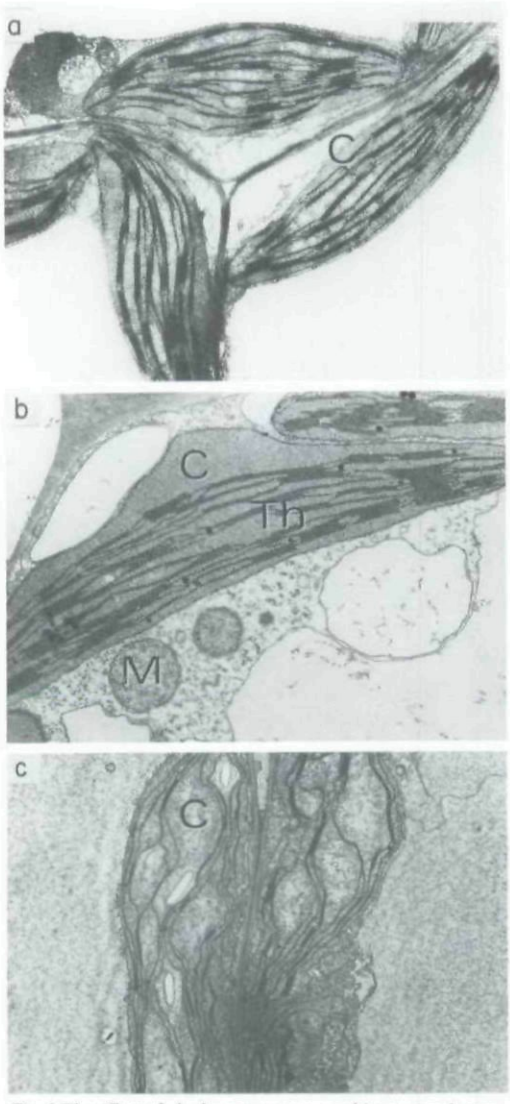

Fig. 2 The effect of glyphosate on young white mustard mesophyll cells collected 48 h after spraying. (a) control (× 11,300); (b) 0·05 kg ai/ha (× 10,000); (c) 4·97 kg ai/ha (× 10,500). The following organelles are indicated: chloroplasts, C; mitochondria, M; thylakoid membranes, Th.

cellular organelles were scattered in the vacuole. The peripheral cells in the cortex were only occasionally affected by pure glyphosate.

Discussion

The results showed that postemergence applications of low rates of glyphosate on 2-week-old seedlings of white mustard were phytotoxic. In

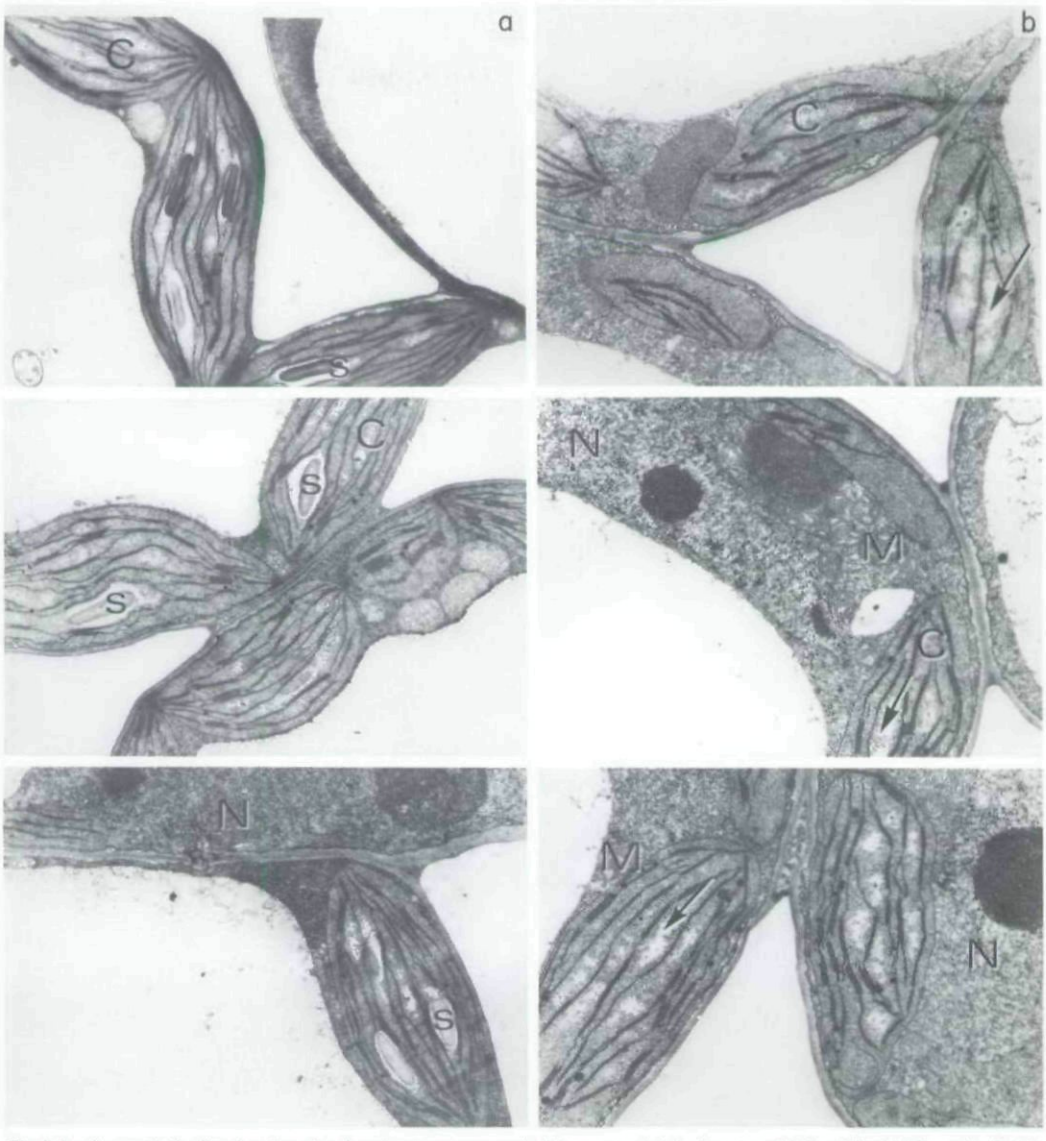

Fig. 3. Leaf mesophyll cells taken 2 weeks after plants were sprayed with commercial glyphosate at 0·05 kg ai/ha (b). Control cells (a); starch grains, s; nucleus, N; chloroplasts, C; mitochondrion, M. Note the starch-depleted areas in the chloroplasts (arrow). Magnifications (from top to bottom); (a): ×8300, ×8300, ×10,000. (b): ×11,300, ×11,300, ×11,300.

general, the symptoms (chlorosis, wrinkling of leaves and wilting) were greater at the higher doses. The elongation of the stems was decreased at the highest doses. However, at the lowest dose a temporary stimulation was observed. The phytotoxic effects are consistent with those described by other authors (e.g. Segura, Bingham & Foy, 1978).

The visual observation of chlorosis after the herbicidal treatments could be correlated with the analysis of chlorophyll content in the same plant material. When treated with 0·49 kg ai/ha glyphosate the chlorophyll content per unit leaf area was found to be higher than that of control leaves (Table 2). It is assumed that at this concentration of glyphosate senescence in white mustard is delayed.

Campbell *et al.* (1976) observed yellowing of the leaves of *Agropyron repens* (L.) Beauv. at doses of 2·24 and 4·49 kg ai/ha 72 h after spraying.

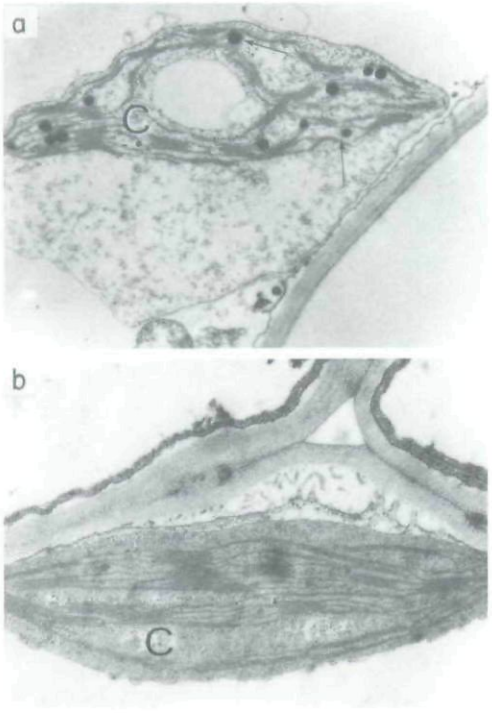

Fig. 4 Stem cortex cells taken 2 weeks after spraying. (a) glyphosate at 0·49 kg ai/ha (× 11,000). (b) control cells (× 12,000). Note the osmiophilic globoids (arrows).

Although they did not observe any morphological symptoms 24 h after treatment with glyphosate they did find cellular symptoms ranging from partial to complete disruption of the chloroplast envelope and swelling of rough endoplasmic reticulum (r-ER) with the subsequent formation of vesicles. In the present study submorphological analyses of the plant material took place 48 h after spraying. In general the chloroplasts were the organelles initially most affected and the observations of the destructive processes occurring in these organelles with time and dose are consistent with the description given by Campbell *et al.* (1976). In white mustard, swelling of r-ER was not observed as an initial phenomenon. Although in ultrastructural analysis in general it is important to beware of artefacts, the consistency of the observations made by Campbell *et al.* (1976) and the present results using different plant materials and preparation procedures makes it most likely that the ultrastructural changes were due to glyphosate.

In a detailed study by Wyrill & Burnside (1977) different surfactants were evaluated for their ability to enhance glyphosate toxicity to *Asclepias syriaca* L. and *Apocynum cannabinum* L. They concluded that nonionic ether and ester ethoxylates combined with a dimethyl amine quaternary ammonium salt were the most effective surfactants. With the present results in mind it would be interesting to study the interaction between glyphosate and surfactants further.

Acknowledgment

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