CHAPTER 5

INFLUENCE OF DIMETHENAMID, FLUMETSULAM + METOLACHLOR, IMAZETHAPYR, METAZACHLOR AND METOLACHLOR ON DRY BEAN MORPHOLOGY AND CELL ULTRASTRUCTURE

Introduction

Ultrastructural studies with herbicides are typically limited to observing or detecting structural changes caused by several biochemical alterations originating from one primary site of action (Bartels, 1985). If one step (primary site) of a process is inhibited by a herbicide, subsequent steps in that pathway or in others of the integrated, complex system may not be able to operate or to remain stable.

The majority of herbicides have been developed to act on respiration and photosynthesis due to the importance of these life processes in plant growth (Black, 1985). Those herbicides inhibit various reactions of the respiration and photosynthesis processes and can also have an indirect influence on these plant functions *via* their effect on chloroplasts and mitochondria (Bartels, 1985).

Herbicides that inhibit the enzyme acetyl-CoA carboxylase include: fluazifop-P-butyl, haloxyfop-R-methyl ester, propaquizafop, quizalofop-P-ethyl, cycloxydim and

sethoxydim. Those that inhibit acetolactate synthase are, *inter alia*, flumetsulam and imazethapyr. Pendimethalin and trifluralin inhibit tubulin formation; EPTC disrupts mitosis; and acetochlor, alachlor, dimethenamid, metazachlor and metolachlor are regarded as having multiple sites of action (Zimdahl, 1993). Four chemical classes of herbicides inhibit acetolactate synthase (ALS): sulfonylureas (e.g. chlorsulfuron), imidazolinones (e.g. imazethapyr), triazolopyrimidine sulfonanilides (e.g. flumetsulam) and pyrimidinyl thiobenzoates. ALS is an essential enzyme in the biosynthesis pathway of the branched-chain amino acids: valine, leucine and isoleucine in plants (Shaner, 1991). ALS inhibition stops protein synthesis and causes decreased photosynthate translocation to meristems, which leads to rapid cessation of cell division and plant growth.

The acetanilides (metolachlor, metazachlor and dimethenamid) inhibit early seedling growth. This effect is most evident on root growth. These responses appear to be associated with an interference with both cell division and cell enlargement. They do not appear to inhibit seed germination but they usually kill or affect susceptible plants before they emerge from the soil (Ashton & Monaco, 1991). Reinhardt & Nel (1986) reported that the "non-photosynthesis-inhibiting" herbicide alachlor, which belong to the acetanilide group, disrupts the chloroplast membranes of grain sorghum plants *(Sorghum bicolor* L.). This and other changes in membrane integrity were observed when alachlor-treated plants were studied on an ultrastructural level by means of electronmicroscopy. There are more apparently contradictory evidence on the effect of

the acetanilides on *de novo* fatty acid biosynthesis and thus on membrane integrity. The primary mechanism of action of the acetanilides has not been determined and their classification could change (Zimdahl, 1993).

The purpose of this study was to compare the effects of certain "non-photosynthesis-inhibiting" herbicides on the ultrastructure of chloroplasts, mitochondria and starch grains in a dry bean cultivar. Growth responses of the crop were also monitored.

Materials and Methods

The dry bean cultivar Helderberg was used in this glasshouse trial because it was identified in field trials (Fouché, 1996) as one of the least tolerant cultivars to the registered herbicides used. The growth medium was washed river sand in 7.5-L pots with plastic linings to prevent leaching of the herbicides. Six seeds were planted per pot and upon emergence plants were thinned to five seedlings. Dimethenamid (Frontier® 900 EC, 1350 g a.i. ha^{-1}), flumetsulam + metolachlor (Bateleur® 816 EC, 2202 g a.i. ha^{-1}), imazethapyr (Hammer® 100 SL, 90 g a.i. ha^{-1}), metolachlor (Dual-S® 930 EC, 2100 g a.i. ha^{-1}) and metazachlor (Pree® 400 SC, 900 g a.i. ha^{-1}) were applied to the soil surface at three times the recommended rate to force injury symptoms. Excessive amounts of herbicide in practice could be due to over application or conditions that promote high concentrations in the plant (e.g. climate, soil type, activity enhancers, etc.).

Water levels were replenished on alternate days to a level approximating the water-holding capacity of the growth medium. All pots received a commercial nutrient solution (Chemicult® and Multifeed-P®) after seedling emergence. There were three replications and pots were arranged according to the completely randomized design. Positions of pots were changed on alternate days.

The temperature regime in the mechanically heated and cooled glasshouse was 25/18 ± 2^0C day/night at 12 hours daylength. Seedling emergence, plant height and dry mass were measured 14 days after planting. Data was subjected to analysis of variance and means were compared at the 5% level of significance using the LSD test of Tukey.

One of the completely unfolded leaves at the primary (unifoliate) leaf node from one replication was sampled for each treatment 14 days after planting. These leaves were used to prepare ultra-thin sections to study cell structure by means of transmission electronmicroscopy.

Fresh tissue segments (1 mm by 2 mm) were cut from the leaves of control and treated seedlings with a surgical blade. The tissue was fixed in 2.5% glutaraldehyde in 0.075 M phosphate buffer (pH 7.5) for one hour. Another fixation with 0.25% aqueous osmium tetroxide in the same buffer followed for two hours. Specimens were dehydrated in a graded series of acetone (from 30 to 100%) whereafter specimens were infiltrated with 30 and 60% quetol in acetone, each for one hour. Another infiltration with pure quetol

followed for four hours. The specimens were then polimerised at 65 °C for a 24-hour period (van der Merwe & Coetzee, 1992).

Sections were cut on an ultramicrotome and stained with 4% aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963). The specimens were observed and photographed with an electronmicroscope equipped with a 35-mm camera.

Results and Discussion

Data for seedling emergence, plant height and aboveground dry mass (DM) are given in Table 11.

Emergence. Imazethapyr was the only herbicide that caused a significant reduction in the emergence of cv. Helderberg (Table 11).

Plant height. Significant reductions in plant height were caused by all herbicides. Imazethapyr caused the smallest reduction; and dimethenamid, metazachlor and metolachlor the largest.

Aboveground dry mass. All the herbicides, except imazethapyr, caused a significant reduction in DM. Dimethenamid, metazachlor and metolachlor were responsible for the largest reduction. These findings are in accordance with that in Chapter 2 where aboveground DM was reduced by all the herbicides at the 2x-rate, except for imazethapyr and metolachlor.

Table 11 Influence of excessive amounts (3x-rate) of herbicides on emergence (number of seedlings), plant height (cm) and aboveground dry mass (g) of cv Helderberg (ANOVA in Appendix B: Tables 6B, 7B & 8B)

Treatment	**Emergence**	**Plant height (cm)**	**Plant height (% of control)**	**Aboveground DM (g)**	**Aboveground DM (% of control)**
Control	4.67	8.00		0.327	
Dimethenamid	4.00	3.33	41.6	0.044	13.5
Flumetsulam + metolachlor	5.00	4.67	58.4	0.077	23.5
Imazethapyr	3.00	6.50	81.3	0.239	73.1
Metazachlor	4.67	2.67	33.4	0.047	14.4
Metolachlor	5.00	3.97	49.6	0.063	19.3
Mean	4.39	4.86	52.9	0.132	40.4
LSDT (P=0.05)	Emergence = 1.23	Plant height = 1.40	DM = 0.10		

Morphological changes

Except for imazethapyr, all the herbicides altered the morphological appearance of the dry bean plants. The acetanilides (dimethenamid, metolachlor and metazachlor) stunted the plants severely. Main stems of all the treated plants were shorter and had more and shorter internodes than those of the control plants. The most prominent effect was the crinkling of primary leaves. Leaves started dying back from the leaf tips. No chlorosis, typical of photosynthetic inhibitors, was observed. Flumetsulam + metolachlor showed similar results but were less prominent. Visual damage varied from high to low in the order: metazachlor > dimethenamid > metolachlor > flumetsulam + metolachlor > imazethapyr. De Beer (1988) found similar results with excessive amounts of alachlor and metolachlor. He concluded that alachlor caused more pronounced phytotoxic symptoms than metolachlor on dry beans.

The acetanilides inhibit early seedling growth, and the effect is most evident on root growth (Ashton & Monaco, 1991). These responses appear to be associated with interference in both cell division and cell enlargement. Acetanilides do not appear to inhibit seed germination but they usually kill or affect susceptible plants before emergence from the soil. The imidazolinones (imazethapyr), on the other hand, inhibit acetolactate synthase with subsequent inhibition of protein synthesis and decreased photosynthate translocation to meristems. This leads to rapid cessation of cell division and plant growth (Shaner, 1991). Findings in this study are in accordance with Fouché

(1996) who concluded that the small white cultivar Helderberg varied in tolerance to pre-emergence herbicides from tolerant to less tolerant in the order: dimethenamid > imazethapyr > metolachlor > metazachlor > flumetsulam + metolachlor.

Ultrastructural changes

The chloroplasts and mitochondria of the treated plants differed in some respects from those of the control plants. These differences are shown in electronmicroscope photographs presented as Figures 5.1-5.12.

Chloroplasts. None of the herbicides caused drastic changes in the structure of chloroplasts. However, all the herbicides except imazethapyr (Figure 5.3), caused a reduction of the number of stroma and granum lamellae (metolachlor: Figure 5.5; flumetsulam + metolachlor: Figure 5.6; dimethenamid: Figure 5.7; metazachlor: Figure 5.8). Starch granules of treated plants, with the exception again of imazethapyr-treated plants (Figure 5.3), appeared depleted and were rounder in shape than those of control plants (Figure 5.1). The chloroplast is surrounded by a limiting double membrane. Its internal structure is a complex membrane system embedded in a granular matrix, the stroma (Grunning & Steer, 1975). The pigments involved in photosynthesis are found in the membranes, whereas the enzymes involved in carbon fixation, 70S ribosomes, strands of naked DNA fibrils, and often starch grains and lipid globules, are located in

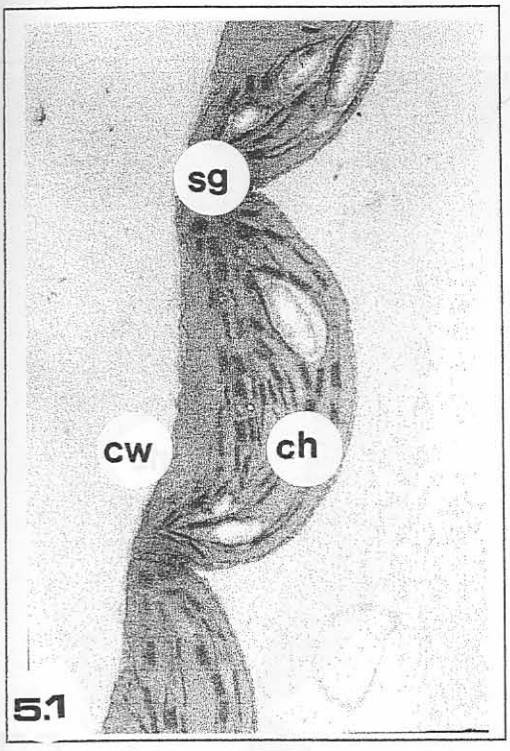
the stroma. The membranes form flattened discs or sacs (lamellae or thylakoids) that may be layered into stacks called grana.

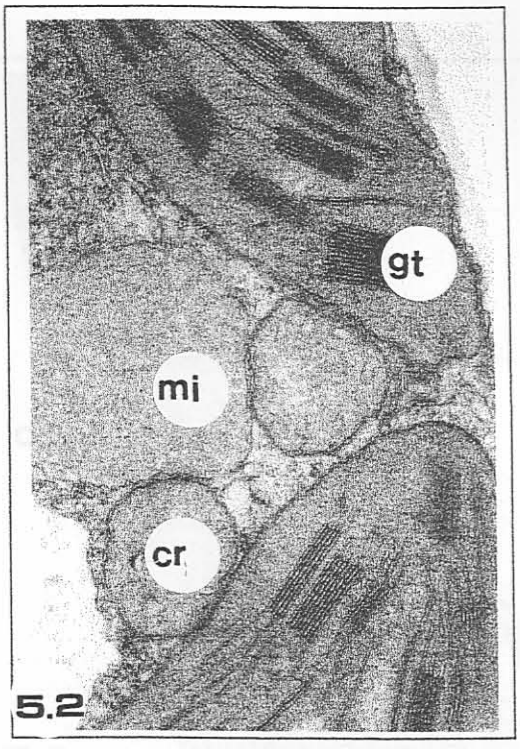
Mitochondria. A comparison of mitochondria of control plants (Figure 5.2) with those in treated plants (metolachlor: Figure 5.9; flumetsulam + metolachlor: Figure 5.10; dimethenamid: Figure 5.11 and metazachlor: Figure 5.12) revealed signs of degradation of the mitochondria, with imazethapyr-treated plants again the exception (Figure 5.4). Mitochondria move freely in streaming cytoplasm, appear to divide by fission, and coalesce with transient adherence to chloroplasts and other organelles (Tzagoloff, 1982). Mitochondria tend to be spherical in shape, but size may vary and transient changes in shape are exhibited (Tzagoloff, 1982). Mitochondria have a sophisticated membrane system within which are incorporated the enzymes that mediate cellular respiration. Cristae of mitochondria in control plants are attached to the inner wall and appear even-sized. The arrangement of cristae in treated plants (Figures 5.9, 5.10, 5.11, 5.12) was chaotic and they were swollen.

The electronmicroscope photographs show definite differences in chloroplasts and mitochondria between treated and control plants. Similar effects were reported by De Beer (1988) for dry beans treated with alachlor and metolachlor. These ultrastructural effects are probably manifestations of acetyl-Co enzyme A (CoA) inhibition as reported by Molin, Anderson & Porter (1985). According to Bidwell (1974) CoA is needed in the formation of chlorophyll, and is an important part of the Krebs cycle that is based in the

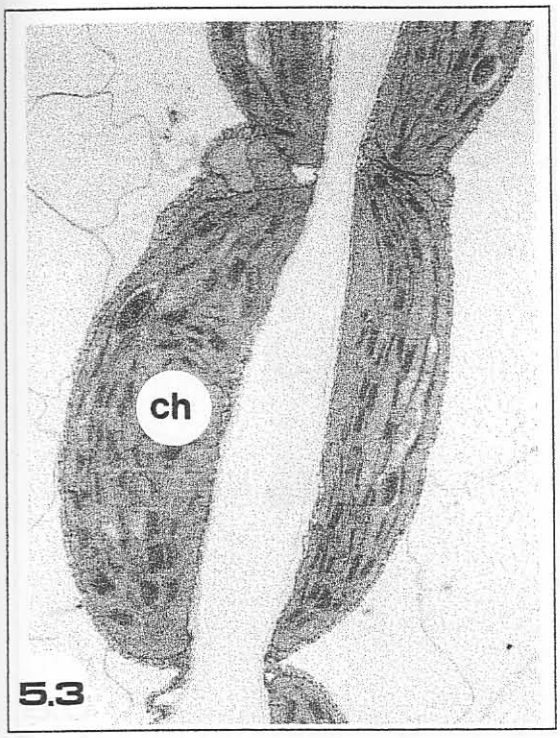
mitochondria. Changes in cristae suggest a slower respiration tempo in affected plants. Reinhardt & Nel (1986) worked with grain sorghum and reported that alachlor not only influences the stroma lammellae but also causes disintegration of cell vacuole membranes, double membranes of chloroplasts and membranes of nuclei in grain sorghum. The present study did not show any influence on chloroplast membranes.

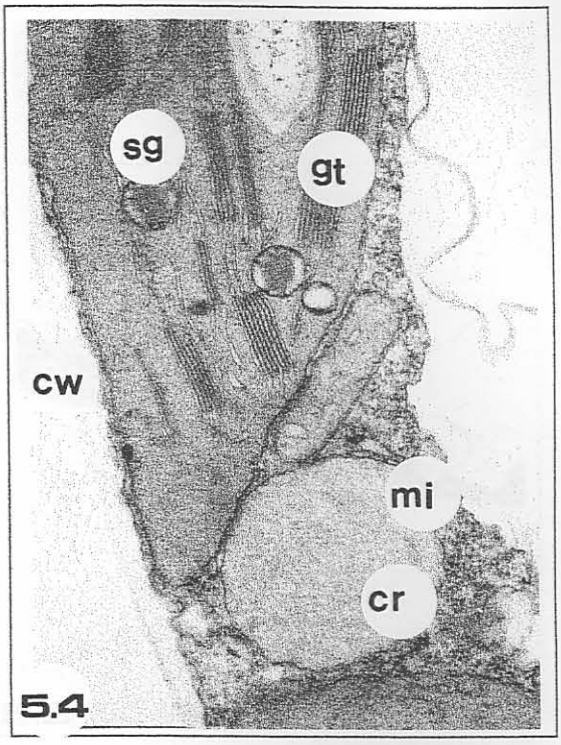
Effects on cloroplasts and mitochondria suggest that both photosynthesis and respiration efficiency will be influenced negatively, which in turn will have a negative influence on plant growth and yield.



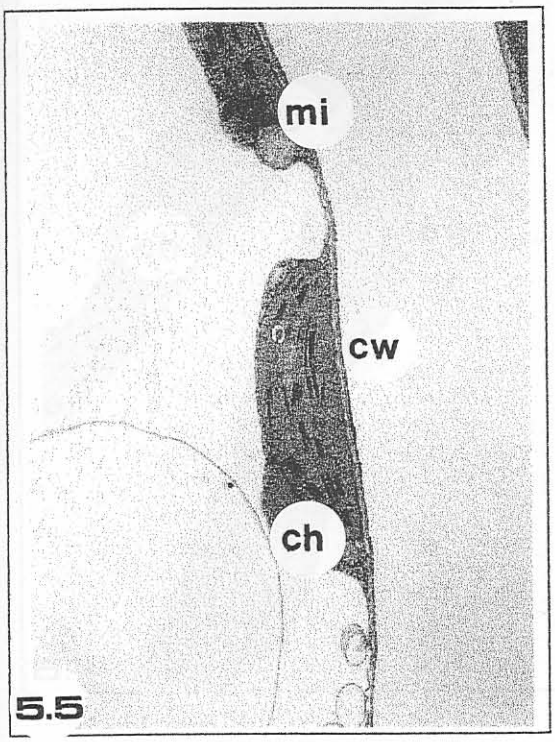


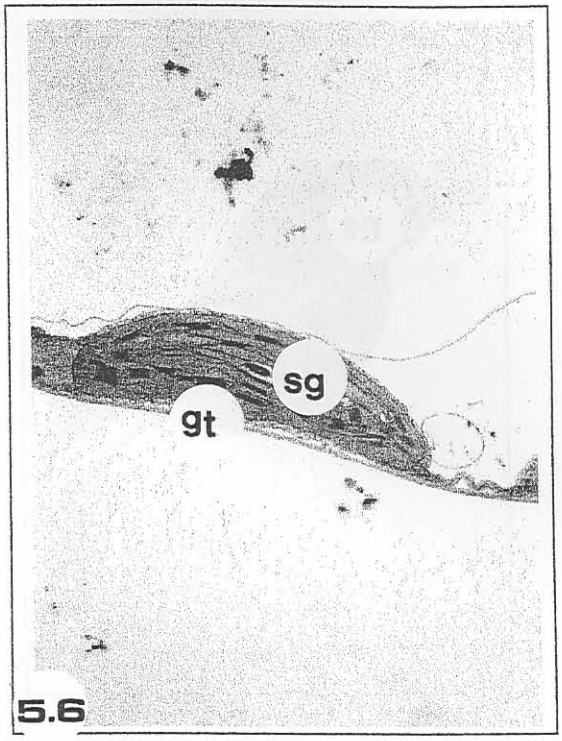
Figures 5.1 & 5.2 Micrographs of cross sections through the leaf of an untreated dry bean plant. (5.1) A chloroplast (7500 x) with starch granules and thylakoids, mitochondria and cell wall. (5.2) Mitochondria (36 000 x). Note that cristae of mitochondria of control plants are attached to the inner wall and appear even-sized. (ch - chloroplast, cr - cristae, cw - cell wall, gt - grana with thylakoids, mi - mitochondria, sg - starch granule)



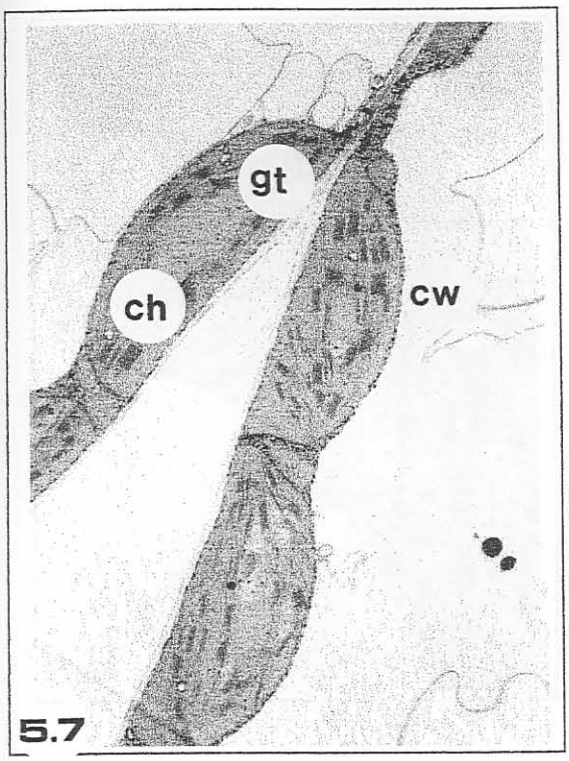


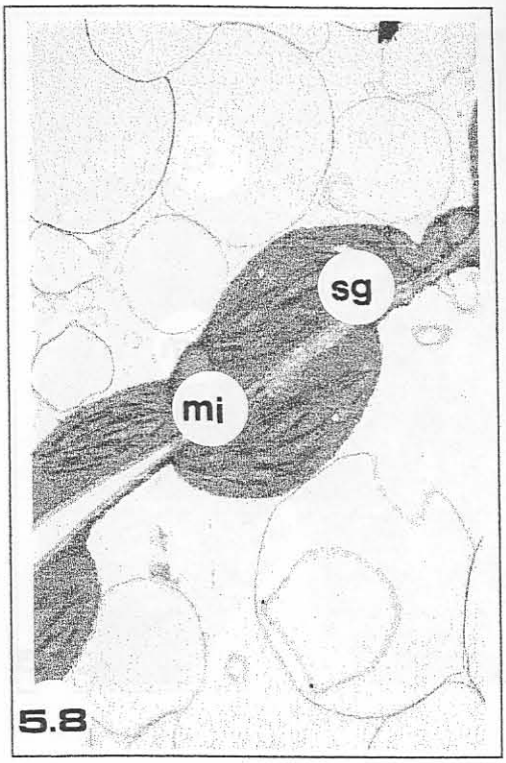
Figures 5.3 & 5.4 Micrographs of cross sections through the leaf of imazethapyr-treated dry bean plants. (5.3) A chloroplast (7 500 x) with normal starch granules and the same quantity of thylakoids, mitochondria and cell wall. (5.4) Mitochondria (36 000 x). Note that cristae of mitochondria of imazethapyr-treated plants are attached to the inner wall and appear even-sized. (ch - chloroplast, cr - cristae, cw - cell wall, gt - grana with thylakoids, mi - mitochondria, sg - starch granule)



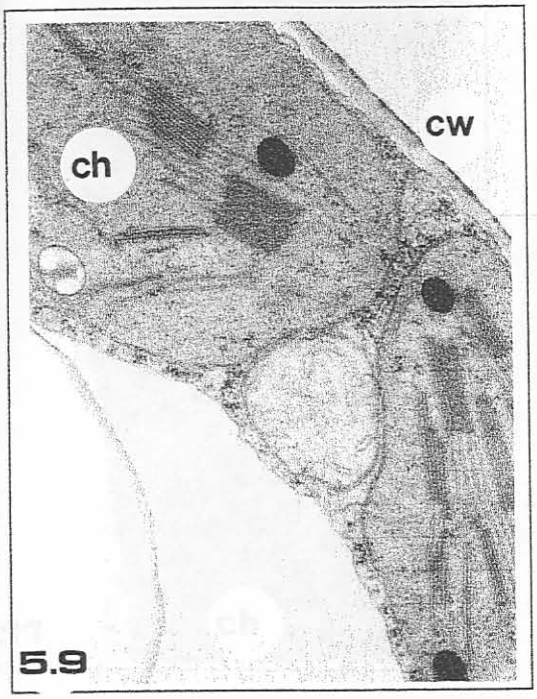


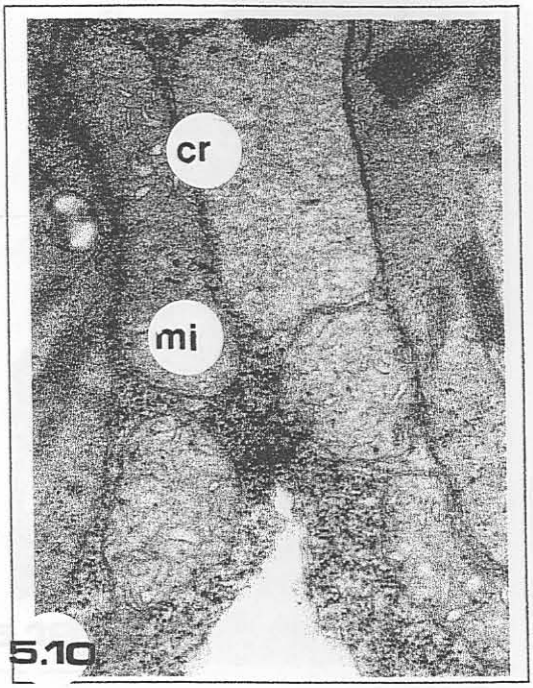
Figures 5.5 & 5.6 Micrographs of cross sections through the leaf of herbicide-treated dry bean plants demonstrating effects on chloroplasts (7 500 x). (5.5) Metolachlor, (5.6) flumetsulam + metolachlor. Starch granules appear depleted and are rounder in shape than control plants and show a reduced number of stroma and granum lamellae. (ch - chloroplast, cw - cell wall, gt - grana with thylakoids, mi - mitochondria, sg - starch granule)



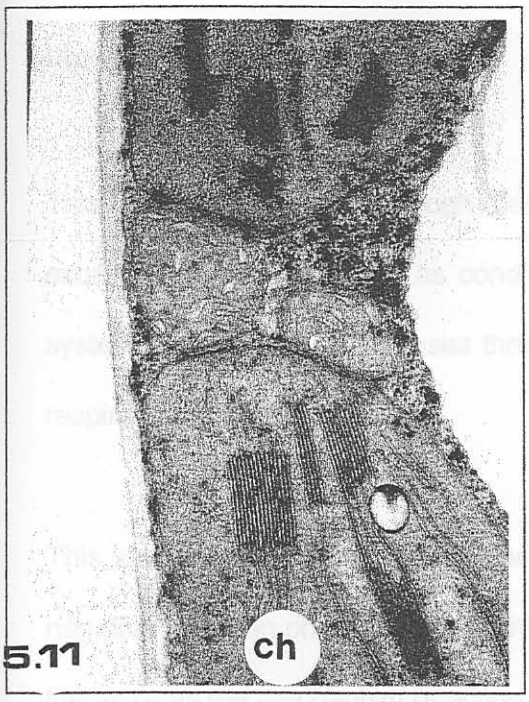


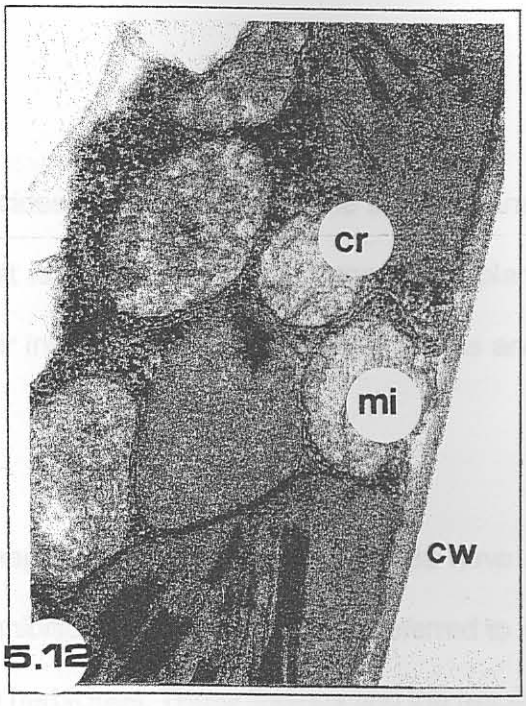
Figures 5.7 & 5.8 Micrographs of cross sections through the leaf of herbicide-treated dry bean plants demonstrating effects on chloroplasts (7 500 x). (5.7) Dimethenamid, (5.8) metazachlor. Starch granules appear depleted and are rounder in shape than control plants and show a reduced number of stroma and granum lamellae. (ch - chloroplast, cw - cell wall, gt - grana with thylakoids, mi - mitochondria, sg - starch granule)





Figures 5.9 & 5.10 Micrographs of cross sections through the leaf of herbicide-treated dry bean plants demonstrating effects on the mitochondria (36 000 x). (5.9) Metolachlor, (5.10) flumetsulam + metolachlor. Note that the arrangement of cristes in treated plants is chaotic and they are swollen. (ch - chloroplast, cr - cristae, cw - cell wall, mi - mitochondria)





Figures 5.11 & 5.12 Micrographs of cross sections through the leaf of herbicide-treated dry bean plants demonstrating effects on the mitochondria (36 000 x). (5.11) Dimethenamid, (5.12) metazachlor. Note that the arrangement of cristes in treated plants is chaotic and they are swollen. (ch - chloroplast, cr - cristae, cw - cell wall, mi - mitochondria)

Conclusions

This study showed that although these herbicides are registered for use in dry beans, excessive amounts as well as conditions that favour high concentrations in the plant system could cause yield losses through their interference in both photosynthesis and respiration efficiency.

This study indicated that, except for imazethapyr, the herbicides tested could have a negative effect on photosynthesis, although inhibition of this process is not referred to in literature as the mechanism of action of these herbicides. These findings and the results in Chapter 4 indicate that some of the tested herbicides may have an effect (probably secondary) on photosynthesis. According to Zimdahl (1993) the classification of the acetanilide group of herbicides according to their mechanism of action could change since the primary mechanism of action has not yet been determined.