ACTA ABP BIOCHIMICA POLONICA

Vol. 59, No 4/2012
567–573
on-line at: www.actabp.pl

Regular paper

# Effect of haloxyfop and cerulenin on *de novo* biosynthesis of lipids in roots of wheat and maize

Walentyna Banaś[1]✉, Tomasz Furmanek[2] and Antoni Banaś[3]

[1]Institute of Biology, University of Natural Sciences and Humanities, Siedlce, Poland; [2]Institute of Biology and Environmental Protection, Pomeranian University in Słupsk, Słupsk, Poland; [3]Intercollegiate Faculty of Biotechnology of the University of Gdańsk and the Medical University of Gdańsk, Gdańsk, Poland

**The study examines the effects of haloxyfop (herbicide) and cerulenin (antibiotic) on *de novo* biosynthesis of fatty acids and complex lipids in roots of two sensitive species: wheat and maize. Seedlings were grown in hydroponic cultures with addition of [1-$^{14}$C]acetate (control) and [1-$^{14}$C]acetate together with one of the tested substances. Neither haloxyfop nor cerulenin prevented the uptake of [1-$^{14}$C]acetate by the roots of tested species. In contrast, a strong inhibition of *de novo* biosynthesis of fatty acids was observed after a 4-h treatment. This phenomenon, however, tended to disappear with treatment time. After a 24-h incubation, the amount of radioactivity in *de novo* biosynthesized fatty acids in 1-cm-long root tips was up to three times higher than in the untreated control. In the "rest of roots", restoration of fatty acid biosynthesis capacity was less pronounced. Besides the effect on fatty acid biosynthesis, both tested inhibitors strongly suppressed the *de novo* biosynthesis of non-fatty acid-containing lipids. Analyses of radioactivity in individual lipid classes showed that after a 4-h treatment with haloxyfop or cerulenin the biosynthesis of most of the lipid classes was inhibited, although not to the same extent. After a 24-h treatment, an inhibition of *de novo* biosynthesis of some of the lipids was still observable, whereas the biosynthesis of others, especially phosphatidylethanolamine and phosphatidic acid, was strongly up-regulated. Contrary to the mainstream view that inhibition of fatty acid biosynthesis is the cause of haloxyfop and cerulenin phytotoxicity, the obtained results suggest multidirectional effects of both inhibitors.**

**Key words**: cerulenin, graminicides, haloxyfop, fatty acids

**Received**: 12 February, 2012; revised: 17 July, 2012; accepted: 29 October, 2012; available on-line: 08 November, 2012

## INTRODUCTION

Graminicides account for about 10% of the total herbicide market (Harwood, 1999). They are used for selective control of grass species in broad-leaved crops. Derivatives of aryloxyphenoxypropionic acid (e.g., haloxyfop) and derivatives of cyclohexanediones (e.g., alloxydim) belong to this herbicide family (Nestler, 1982; Cobb, 1992). Although they have been introduced into agriculture in mid-1980s, their plant annihilation mechanism remains controversial (De Prado *et al,* 1999; Shimabukuro *et al,* 2001; Luo *et al,* 2004). The mechanism of action of these herbicides was investigated most intensively during the last two decades of the twentieth century, when two competitive hypotheses were formulated. The first one (most popular) claims that the inhibition of lipid biosynthesis is the main reason of the phytotoxicity of graminicides. Acetyl-CoA carboxylase, the key enzyme in fatty acid biosynthesis, was regarded as a target for graminicide action. In contrast to most of dicotyledonous plants, which contain in their plastids a multiprotein complex of ACCase insensitive to graminicides, grasses contain a sensitive, multifunctional form of ACCase (Lichtenthaler, 1990; Cobb, 1992; Tardif *et al,* 1996; Heap & Morrison, 1986; Shukla *et al,* 1997). Alternative hypotheses claim that graminicides stimulate overproduction of free radicals causing plant death due to numerous damaging reactions known as "oxidative stress" (Banaś *et al,* 1993a; Shimabukuro & Hoffer, 1996; Shimabukuro *et al,* 2001; Luo *et al,* 2004). This hypothesis was supported by (among others) experiments with graminicide antagonists which could act as free radical scavengers. Banaś *et al* (1993a, 1993b) demonstrated that inhibition of wheat roots growth caused by haloxyfop or alloxydim was partly or completely reduced by addition of genistein, propyl gallate, or diphenylamine to the growth medium. Shimabukuro *et al* (2001) proved the antagonistic effect of vitamin E on diclofop-methyl activity, and Luo *et al* (2004) showed antagonistic effect of vitamin E and ethoxyquin on fluazifop-butyl activity. The body of evidence supporting each of the hypotheses is, however, only fragmentary.

In our earlier work, we demonstrated that it was not always possible to observe the inhibitory effect of graminicides on *de novo* lipid biosynthesis *in vivo*. Young parts of wheat leaves treated for 48 hrs with haloxyfop-ethoxyethyl incorporated $^{14}$C-labelled acetate into the lipid fraction at a similar rate as the control, despite the fact that the growth of the treated plants was completely inhibited (Banaś *et al,* 1990). In the root tips of wheat seedlings grown for 24 h with haloxyfop and $^{14}$C-labelled acetate, the amount of radioactivity in the lipid fraction was much higher than in the controls grown without the herbicide (Banaś *et al,* 1993c). In contrast to the mentioned results, when isolated plastids from wheat roots, or isolated chloroplasts from oat leaves were incubated with $^{14}$C-labelled acetate and the herbicide haloxyfop, a

✉e-mail: wbanas@uph.edu.pl

**Abbreviations**: ACCase, acetyl-CoA carboxylase; butyl PBD, 2-(tert-Butylphenyl)-5-(4-phenylphenyl)-1,3,4–oxadiazole; DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; FA, free fatty acids; LPE, lysophosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol; S, free sterols; SE, sterol esters.

**Table 1. Effect of haloxyfop and cerulenin on growth of wheat and maize roots**

| Tested species | Increase in root length [mm] | | | | | |
|---|---|---|---|---|---|---|
| | 4-h treatment | | | 24-h treatment | | |
| | C | H | Cer | C | H | Cer |
| Wheat | 4±1 | 3±1 (75) | 4±1 (100) | 24±2 | 12[a]±2 (50) | 13[a]±2 (54) |
| Maize | 3±1 | 2[a]±1 (67) | 3±1 (100) | 26±6 | 13[a]±2 (50) | 16[a]±2 (62) |

C, control; H, haloxyfop (wheat [$10^{-7}$ M], maize [$3\times10^{-8}$ M]); Cer, cerulenin [$10^{-5}$ M]. Means ±S.D. shown. Figures in parentheses show % of control values. a, significant difference between control and treated plants in mean difference two-sided test at $\alpha = 0.05$

clear inhibition of *de novo* biosynthesis of fatty acids was observed (Banaś *et al,* 1993c).

In the present experiments, the effects of a herbicide, haloxyfop and an antibiotic, cerulenin (two different potential inhibitors of fatty acid biosynthesis) were investigated. Roots of two sensitive grass species (wheat and maize) were treated with those inhibitors over different time periods and the effects of those inhibitors on *de novo* biosynthesis of fatty acids and complex lipids were studied in detail.

## MATERIALS AND METHODS

**Plant material.** The experiments were carried out on two species sensitive to graminicides: wheat (*Triticum aestivum* L. cv. Kadet) and maize (*Zea mays* L. cv. Concord). The grains were soaked in water for 18 h and sown on moist filter paper. After a 48-h germination in a dark growth chamber (wheat 21°C, maize 25°C), seedlings with 22–26 mm long central root were selected. The length of the central root was measured and subsequently, the seedlings were transferred to a nutrient solution (for composition see: (Nilsson, 1977)) with addition of $1.9\times10^{-4}$ M [1-$^{14}$C]acetate (sp. activity 0.1 μCi/63 nmol; Sigma-Aldrich) only (control), or to the nutrient solution with addition of [1-$^{14}$C]acetate and one of the tested substances — haloxyfop ($10^{-7}$ M for wheat and $3\times10^{-8}$ M for maize; supplied in the acid form by Dow Chemical Company), or cerulenin ($10^{-5}$ M in all experiments; Sigma-Aldrich). The seedlings were placed in holes drilled in cork discs floating on the solution and incubated in the dark (wheat 21°C, maize 25°C). At the end of the experiments, the roots were rinsed in distilled water and the length of the central root was measured again (the increase in length was used as a measure of root growth).

**Analysis of incorporation and distribution of labelled acetate.** For each single analysis 10 wheat or 5 maize roots were used. Each root was divided into 1-cm-long tip and the rest of the root. Both parts were analysed separately. They were homogenized in a glass homogenizer with 3.75 ml of chloroform/methanol (1:2, v/v) and the extracts were transferred to glass tubes to which 1.25 ml of 0.15 M acetic acid, 1.25 ml of chloroform, and 1.25 ml of water were added; tubes were vigorously shaken and then centrifuged for 3 min ($1000\times g$). The lower chloroform fraction contained lipids and the upper methanol/water fraction — polar compounds (Blight & Dyer, 1959). Aliquots of both fractions were subsequently used for the measurement of total radioactivity. In the next step, the rests of chloroform fractions were used for further analysis and the methanol/water fractions were discarded.

Each remaining chloroform fraction was further divided into two parts. One was evaporated to dryness and methylated for 60 min at 90°C with 2% $H_2SO_4$ in dry methanol. Next, 3 ml of hexane and 2 ml of water were added. After shaking and centrifugation, the hexane fractions were evaporated to dryness, dissolved in small amount of hexane and separated by TLC on precoated silica gel plates (Merck silica gel 60) with hexane:diethyl ether:acetic acid (70:30:1 by volume). The second part of each chloroform fraction, was also evaporated to dryness, dissolved in small amount of chloroform and directly separated by TLC with chloroform:methanol:acetic acid:water (170:30:20:7). The fatty acid methyl esters and other lipids were identified by comparing with standards run in parallel.

Total radioactivity in the chloroform and methanol/water fractions was determined on LKB Wallace 1214 Rackbeta scintillation counter using a toluene/ethanol scintillation cocktail (2:1 by volume + butyl PBD 4 g/l). The radioactive lipids were visualised and quantified on the TLC plates by electronic autoradiography (Instant Imager, Packard Instrument Co).

All experiments were repeated at least three times.

**Table 2. Effect of haloxyfop and cerulenin on [1-$^{14}$C]acetate uptake (sum of radioactivity in methanol/water and chloroform fractions) by wheat and maize roots**

| Tested species | Part of root | Total uptake [d.p.m. x$10^{-5}$ /10 roots (wheat) or /5 roots (maize)] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 4-h treatment | | | 24-h treatment | | |
| | | C | H | Cer | C | H | Cer |
| Wheat | 1 cm tip | 2.6±0.1 | 2.2±0.3 (85) | 2.3±0.3 (88) | 3.1±0.9 | 4.8[a]±0.2 (155) | 3.5±0.4 (113) |
| | rest of root | 2.5±0.6 | 2.6±0.9 (104) | 2.3±0.2 (92) | 11.7±1.6 | 6.3[a]±0.5 (54) | 7.0[a]±0.4 (60) |
| | average 1cm of rest of root | 1.2±0.3 | 1.3±0.5 (108) | 1.2±0.1 (100) | 2.9±0.4 | 2.3±0.1 (79) | 2.4±0.1 (83) |
| Maize | 1 cm tip | 1.3±0.2 | 1.4±0.2 (108) | 1.5±0.2 (115) | 2.3±0.4 | 3.1[a]±0.3 (135) | 3.0±0.6 (130) |
| | rest of root | 1.7±0.2 | 1.8±0.3 (106) | 1.7±0.4 (100) | 11.4±2.1 | 8.8±1.1 (77) | 8.4±1.9 (74) |
| | average 1cm of rest of root | 0.8±0.1 | 0.9±0.2 (113) | 0.9±0.2 (113) | 2.6±0.5 | 2.7±0.2 (104) | 2.7±0.6 (104) |

C, control; H, haloxyfop (wheat [$10^{-7}$ M]. maize [$3\times10^{-8}$ M]); Cer,. cerulenin [$10^{-5}$ M]. Means ±S.D. shown ($n\geq3$). Figures in parentheses show % of control values. a, significant difference between control and treated plants in mean difference two-sided test at $\alpha = 0.05$

**Table 3. Effect of haloxyfop and cerulenin on incorporation of [1-$^{14}$C]acetate into fatty acids of wheat and maize roots**

| Tested species | Part of root | Radioactivity in fatty acids [d.p.m. x $10^{-4}$ /10 roots (wheat) or /5 roots (maize)] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 4-h treatment | | | 24-h treatment | | |
| | | C | H | Cer | C | H | Cer |
| Wheat | 1 cm tip | 4.4±0.3 [17] | 1.7[a]±0.7 [9[a]] (37) | 1.9[a]±0.6 [8[a]] (43) | 3.5±1.3 [11] | 10.8[a]±0.8 [23[a]]; (309) | 5.5±0.8 [16] (157) |
| | rest of root | 4.4±1.0 [18] | 1.9[a]±0.9 [9[a]] (43) | 2.6[a]±0.8 [11[a]]; (59) | 28.4±1.8 [24] | 23.0[a]±1.2 [37[a]]; (81) | 19.8[a]±1.9 [28]; (70) |
| | average 1cm of rest of root | 2.2±0.25 | 1.2[a]±0.5 (55) | 1.5[a]±0.5 (68) | 6.3±0.6 | 9.1[a]±0.5 (144) | 6.5±0.6 (103) |
| Maize | 1 cm tip | 3.2±0.6 [26] | 2.4[a]±0.2 [17[a]] (75) | 2.0[a]±0.1 [14[a]] (62) | 3.3±1.6 [15] | 5.6[a]±0.3 [18] (170) | 5.7[a]±0.6 [22] (173) |
| | rest of root | 4.8±0.9 [27] | 2.2[a]±0.3 [13[a]] (46) | 1.9[a]±0.2 [12[a]] (40) | 32.7±8.2 [30] | 26.5±2.8 [30] (81) | 14.5[a]±2.0 [20[a]] (44) |
| | average 1cm of rest of root | 2.3±0.4 | 1.0[a]±0.1 (43) | 1.0[a]±0.1 (43) | 7.4±1.2 | 8.0±0.8 (108) | 4.7[a]±0.6 (64) |

C, control; H, haloxyfop (wheat [$10^{-7}$ M], maize [$3\times10^{-8}$ M]); Cer, cerulenin [$10^{-5}$ M]. Means ±S.D. shown ($n \geq 3$). Figures in square parentheses show % of total radioactivity in fatty acids; figures in parentheses show % of control values. a, significant difference between control and treated plants in mean difference two-sided test at $\alpha=0.05$

## RESULTS

The aim of preliminary experiments with non-radioactive acetate was to identify concentrations of haloxyfop and cerulenin that would be sufficient to cause about 50% inhibition of root growth during a 24-h treatment. It appeared that such growth inhibition of maize and wheat roots was achieved by using respectively $3\times10^{-8}$ M and $10^{-7}$ M haloxyfop. Cerulenin inhibited root elongation less efficiently and could only produce similar results as haloxyfop in at least 100 times higher concentrations ($10^{-5}$ M). The inhibition of root elongation by haloxyfop was observable already after a 4-h treatment. The inhibitory effect of cerulenin was observable only after longer periods. In experiments with [1-$^{14}$C]acetate the same/very similar inhibition of root growth (by haloxyfop and cerulenin) was observed as in experiments with non-labelled acetate (Table 1). During the first 4 h of treatment neither haloxyfop nor cerulenin significantly affected the uptake of $^{14}$C-labelled acetate by different parts of roots of both tested species. After 24 h of treatment, the amount of incorporated radioactivity in 1-cm root tips of haloxyfop- and cerulenin-treated plants was higher than in the control (however, only in the case of haloxyfop the difference was statistically significant). In the root parts remaining after cutting off the 1-cm-long tips, the incorporated amount of radioactivity was lower than in the control. However, the treated roots were shorter and the amount of incorporated radioactivity calculated *per* 1 cm of the "rest of root" did not differ significantly from the control (Table 2).

Only a fraction of the $^{14}$C-labelled acetate absorbed by the roots was used for *de novo* biosynthesis of fatty acids. The root tips of the control wheat and maize seedlings

**Table 4. Effect of haloxyfop and cerulenin on incorporation of [1-$^{14}$C]acetate into non-fatty acid-containing lipids of wheat and maize roots**

| Tested species | Part of root | Radioactivity in non-fatty acid-containing lipids [d.p.m. x $10^{-4}$ /10 roots (wheat) or /5 roots (maize)] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 4-h treatment | | | 24-h treatment | | |
| | | C | H | Cer | C | H | Cer |
| Wheat | 1 cm tip | 9.6±1.2 [37] | 5.9[a]±0.3 [26[a]] (61) | 6.9[a]±0.8 [30] (72) | 11.4±1.3 [37] | 17.6[a]±1.2 [37] (154) | 15.0±1.2 [43[a]] (132) |
| | rest of root | 4.9±1.0 [24] | 3.1[a]±0.7 [10[a]] (63) | 2.7[a]±0.3 [12[a]] (55) | 38.1±2.4 [33] | 12.4[a]±4.1 [20[a]] (33) | 15.1[a]±1.9 [22[a]] (40) |
| | average 1cm of rest of root | 2.5±0.5 | 1.6[a]±0.3 (64) | 1.4[a]±0.2 (56) | 9.3±0.6 | 4.6[a]±1.5 (49) | 5.2[a]±0.2 (56) |
| Maize | 1 cm tip | 4.5±0.8 [35] | 3.8±0.7 [29] (84) | 4.6±0.6 [31] (102) | 7.3±3.6 [31] | 12.0[a]±1.7 [38] (164) | 8.9±4.8 [31] (122) |
| | rest of root | 2.3±0.8 [12] | 1.3[a]±0.2 [8] (57) | 1.3[a]±0.4 [8] (57) | 33.0±6.6 [29] | 17.8[a]±3.5 [20[a]] (54) | 17.2[a]±5.4 [22[a]] (52) |
| | average 1cm of rest of root | 1.1±0.4 | 0.6[a]±0.1 (55) | 0.7±0.2 (64) | 7.4±1.8 | 6.9±0.9 (93) | 6.4±2.2 (86) |

C, control; H, haloxyfop (wheat [$10^{-7}$ M], maize [$3\times10^{-8}$ M]); Cer, cerulenin [$10^{-5}$M]. Means ±S.D shown ($n \geq 3$). Figures in square parentheses show % of total radioactivity in non-fatty acid containing lipids; figures in parentheses show % of control values. a, significant difference between control and treated plants in mean difference two-sided test at $\alpha=0.05$

**Table 5. Effect of haloxyfop and cerulenin on incorporation of [1-$^{14}$C]acetate into lipids (containing and not containing fatty acids) of wheat roots**

| Treatment time (hrs) | Part of root | Treatment | Radioactivity in different lipids (C, d.p.m. x $10^{-3}$ /10 roots; H and Cer, % of control values) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | SE TAG DAG | FA S | $X_1$ | $X_2$ | PA | $Y_1$ | $Y_2$ | PE | PG | PC DGDG | PS | PI LPE |
| 4 | 1 cm tips | **C** | **26.6** | **64.4** | **7.0** | **5.7** | **1.3** | **1.8** | **12.7** | **6.9** | **1.4** | **8.4** | **2.5** | **0.4** |
| | | H | 57 | 59 | 40 | 31 | 54 | 46 | 53 | 24 | 43 | 57 | 66 | 91 |
| | | Cer | 70 | 71 | 38 | 14 | 63 | 48 | 28 | 56 | 75 | 65 | 38 | 105 |
| | average 1 cm of rest of root | **C** | **6.6** | **17.4** | **5.6** | **3.5** | **0.5** | **0.3** | **2.8** | **4.0** | **0.9** | **2.9** | **1.4** | **1.2** |
| | | H | 59 | 57 | 45 | 32 | 40 | 47 | 59 | 65 | 72 | 46 | 64 | 90 |
| | | Cer | 41 | 64 | 30 | 48 | 62 | 57 | 35 | 61 | 86 | 69 | 57 | 137 |
| 24 | 1 cm tips | **C** | **23.8** | **81.9** | **3.7** | **1.0** | **2.5** | **1.9** | **9.4** | **8.9** | **2.1** | **5.2** | **1.6** | **0.6** |
| | | H | 203 | 118 | 114 | 109 | 494 | 131 | 139 | 667 | 489 | 218 | 294 | 331 |
| | | Cer | 198 | 105 | 126 | 177 | 365 | 74 | 39 | 298 | 235 | 177 | 88 | 172 |
| | average 1 cm of rest of root | **C** | **11.7** | **69.7** | **7.9** | **4.2** | **4.7** | **2.1** | **11.2** | **21.1** | **3.2** | **5.2** | **4.1** | **14.4** |
| | | H | 63 | 40 | 41 | 25 | 61 | 44 | 28 | 236 | 226 | 84 | 101 | 146 |
| | | Cer | 73 | 33 | 33 | 29 | 56 | 40 | 11 | 234 | 189 | 70 | 86 | 125 |

C, control; H, haloxyfop [$10^{-7}$ M]; Cer, cerulenin [$10^{-5}$ M]; standard deviation is not presented to improve readability of data (usually it was below 15% of the mean value) $X_1$, $X_2$, $Y_1$, $Y_2$, unidentified lipids

incorporated about 17 and 26% of the absorbed radioactivity, respectively, into fatty acids after 4-h incubation, with the amount falling to 11 and 15% respectively, after 24 h. The more mature parts of the roots (those remaining after cutting off the tips) utilised the $^{14}$C-labelled acetate for fatty acid biosynthesis more efficiently. In the case of wheat — about 18% and in the case of maize — 27% of the absorbed $^{14}$C-labelled acetate after a 4-h incubation, and 24 and 30%, respectively, after 24-h incubation were used for fatty acid biosynthesis. The addition of haloxyfop or cerulenin to the incubation medium strongly inhibited the incorporation of [$^{14}$C]acetate into fatty acids during the first 4 h of treatment. The inhibition was observable in both the absolute amount of radioactivity incorporated into *de novo* synthesised fatty acids, as well as in terms of the percentage of incorporated [$^{14}$C]acetate used for fatty acid biosynthesis. In the case of wheat roots, a stronger inhibition was detected in root tips, whereas in maize roots — in the mature parts of roots (Table 3).

The inhibition of fatty acid biosynthesis, which was clearly visible following the first 4 h of haloxyfop and cerulenin treatment, showed a tendency to revers with treatment time. After a 24-h incubation, the absolute amount of [$^{14}$C]acetate incorporated into fatty acids of 1-cm-long tips of haloxyfop-treated wheat roots was in

**Table 6. Effect of haloxyfop and cerulenin on incorporation of [1-$^{14}$C]acetate into lipids (containing and not containing fatty acids) of maize roots**

| Treatment time (hrs) | Part of root | Treatment | Radioactivity in different lipids (C, d.p.m. x $10^{-3}$ /5 roots; H and Cer, % of control values) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | SE TAG DAG | FA S | $X_1$ | $X_2$ | PA | $Y_1$ | $Y_2$ | PE | PG | PC DGDG | PS | PI LPE |
| 4 | 1 cm tips | **C** | **16.2** | **26.9** | **3.9** | **1.8** | **0.7** | **1.2** | **7.6** | **3.0** | **1.7** | **9.2** | **2.6** | **0.9** |
| | | H | 88 | 90 | 68 | 59 | 72 | 64 | 64 | 95 | 81 | 74 | 83 | 74 |
| | | Cer | 102 | 113 | 31 | 63 | 86 | 28 | 8 | 92 | 109 | 79 | 20 | 86 |
| | average 1 cm of rest of root | **C** | **6.8** | **5.4** | **2.8** | **1.8** | **0.6** | **0.3** | **3.4** | **2.5** | **1.4** | **6.1** | **1.6** | **0.4** |
| | | H | 49 | 62 | 48 | 28 | 32 | 31 | 45 | 44 | 45 | 54 | 46 | 33 |
| | | Cer | 55 | 81 | 33 | 31 | 42 | 27 | 14 | 67 | 62 | 47 | 22 | 73 |
| 24 | 1 cm tips | **C** | **22.3** | **42.4** | **4.1** | **1.6** | **2.1** | **1.9** | **6.4** | **9.2** | **1.4** | **9.0** | **1.7** | **0.7** |
| | | H | 190 | 162 | 77 | 111 | 540 | 111 | 144 | 185 | 319 | 127 | 155 | 143 |
| | | Cer | 190 | 114 | 106 | 101 | 358 | 84 | 9 | 285 | 148 | 96 | 69 | 158 |
| | average 1 cm of rest of root | **C** | **25.3** | **46.2** | **6.6** | **5.1** | **4.8** | **2.2** | **12.8** | **17.9** | **3.1** | **16.4** | **3.0** | **1.0** |
| | | H | 85 | 73 | 72 | 71 | 115 | 66 | 66 | 165 | 152 | 66 | 112 | 64 |
| | | Cer | 73 | 60 | 61 | 33 | 102 | 51 | 8 | 173 | 92 | 37 | 48 | 79 |

C, control; H, haloxyfop [$3\times10^{-8}$ M]; Cer, cerulenin [$10^{-5}$ M]; standard deviation is not presented to improve readability of data (usually it was below 15% of the mean value) $X_1$, $X_2$, $Y_1$, $Y_2$, unidentified lipids

fact 3 times higher than in the control, and 1.7 times higher in maize. The treatment with cerulenin resulted in an increase of radioactivity of root tip fatty acids of both tested species by slightly more than 50% over the values obtained for the control. In the rest of roots the absolute amount of radioactivity in fatty acids of the treated plants was always lower than in the control. However, when the absolute amount of incorporated radioactivity was divided by the length of the "rest of roots", only the cerulenin-treated maize roots showed a statistically significant inhibition of the *de novo* biosynthesis of fatty acids. The treatment of wheat roots with haloxyfop generated even a statistically significant increase (about 1.4 fold) of incorporation of [$^{14}$C]acetate into fatty acids (Table 3).

The 24-h treatment of wheat roots with haloxyfop resulted also in a statistically significant increase in utilisation of [$^{14}$C]acetate absorbed by roots for fatty acid biosynthesis. In the case of root tips, the utilisation increased from 11% (control) to 23% (treated) of absorbed [$^{14}$C]acetate, and in the "rest of roots" — from 24% to 37% respectively. Only the "rest of roots" of maize seedlings treated with cerulenin showed a statistically significant decrease in utilisation of [$^{14}$C]acetate absorbed by roots for fatty acid biosynthesis (Table 3).

Part of the [$^{14}$C]acetate incorporated by roots was used for biosynthesis of lipids not containing fatty acids. Contrary to the *de novo* biosynthesis of fatty acids, a higher percentage of [$^{14}$C]acetate was utilised for *de novo* biosynthesis of non-fatty acid-containing lipids in root tips (up to 43%) than in the "rest of roots". Strong inhibition of *de novo* biosynthesis of these types of compounds occurred in the tips and the "rest of roots" of wheat seedlings treated for 4 h with haloxyfop or cerulenin. When a 24-h treatment with the inhibitors was used, the inhibition occurred only in the "rest of roots". In the case of maize roots treated with haloxyfop or cerulenin for 4 or 24 h, the inhibition of *de novo* biosynthesis of non-fatty acid-containing lipids was visible only in the "rest of roots". However, only in the case of a 4-h treatment of maize roots with haloxyfop, the inhibition calculated *per* 1 cm length of the "rest of roots" was statistically significant (Table 4).

When chloroform fractions were separated on TLC and the radioactive lipids visualised and quantified by electronic autoradiography (as an example, see Fig. 1.), it appeared that the radioactivity was not distributed evenly among different lipid classes. In wheat up to 55%, and in maize up to 46% of the radioactivity was found in the "S + FA" fraction. These lipids were synthesised *de novo* in higher quantities in the root tips of both plants, during 4 h of incubation, compared to the quantities in an average 1 cm of the "rest of roots". The difference was less pronounced after 24 h of incubation, especially in the control plants. The second most radioactive lipid fraction after a 4-h incubation was "SE + TAG + DAG". However, after 24 h of incubation, the situation was not so clear. In some cases, other lipids (especially PE) contained more radioactivity than "SE + TAG + DAG", or even more than "S + FA" (average 1 cm of the "rest of roots" treated for 24 h with haloxyfop). The inhibitor used, as well as the incubation time, strongly influenced the distribution of radioactivity among different lipid classes localised on TLC below the "S + FA" fraction (polar lipids) (Tables 5 and 6).

Short treatment (4 h) with haloxyfop or cerulenin resulted in an inhibition of *de novo* biosynthesis of the majority of lipids. In wheat roots, the inhibition

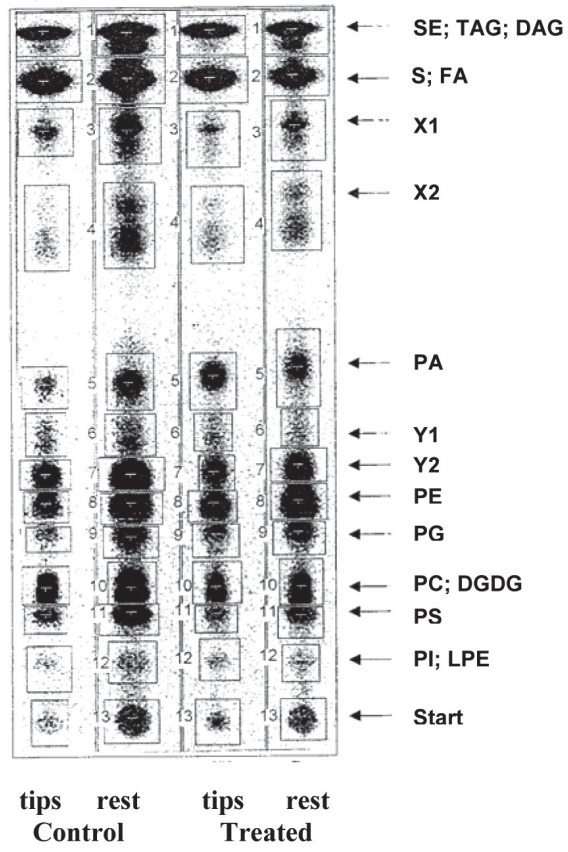


**Figure 1. Autoradiogram of lipids of chloroform fractions from "tips" and "rests" of maize roots separated on TLC with chloroform: methanol: acetic acid: water (170:30:20:7).**
The roots were grown for 24 hrs in nutrient solution with [1-$^{14}$C] acetate (control) and with [1-$^{14}$C]acetate + $3\times10^{-8}$ M haloxyfop (treated). $X_1$, $X_2$, $Y_1$, $Y_2$, unidentified lipids

caused by haloxyfop was similar in root tips and in an average 1 cm of the "rest of roots". In maize, the inhibition was stronger in the "rest of roots". Generally, a similar tendency could also be observed in the roots treated with cerulenin. However, cerulenin had a weaker effect on biosynthesis of neutral lipids (in the tips of maize roots there was no inhibitory effect at all), but very strongly inhibited the synthesis of some of the polar lipids, especially an unidentified lipid, $Y_2$ (probably cardiolipin) and PS (Tables 5 and 6).

After 24 h of treatment with haloxyfop or cerulenin, in the root tips of both wheat and maize seedlings the amount of radioactivity in the majority of lipids was higher than in the control. The strongest stimulatory effect was observed for the *de novo* biosynthesis of PE and PA. In contrast, in an average 1 cm of the "rest of roots", both haloxyfop and cerulenin inhibited the *de novo* biosynthesis of the majority of lipids, but the biosynthesis of some, especially PE and PG, was stimulated. In both plants, the average amount of radioactivity incorporated into polar lipids in an average 1 cm of the "rest of roots" was equal to or higher than in the control in the case of haloxyfop treatment and equal to or a bit lower than in the control in the case of cerulenin treatment. Similarly to the results of a 4-h treatment an incubation of the roots of both tested plants with cerulenine for 24 h caused a strong inhibition of an unidentified lipid, Y2 (Tables 5 and 6).

## DISCUSSION

In the present study, [$^{14}$C]acetate was used as a precursor for lipid biosynthesis. Already in 1982, Roughan and Slack (Roughan & Slack, 1982) showed that this radiolabelled compound is a suitable and effective precursor for plant lipids. It was also utilised in other studies addressing the effect of graminicides on lipid biosynthesis (e.g., Hoppe, 1985; Banaś *et al,* 1990, 1993c; Price *et al,* 2003). Therefore, the results obtained in this work can be compared with earlier research.

The potential of graminicides to inhibit acetyl-CoA carboxylase (ACCase) is well-documented (Lichtenthaler, 1990; Cobb, 1992). The experimental data that we present suggests, however, that the inhibition of *in vivo* fatty acid biosynthesis could be only temporary. The reasons why plants treated with haloxyfop rebuild their capacity for fatty acids biosynthesis remain unknown. One possible explanation could be an increase, with the time of treatment, of the ability to detoxify haloxyfop in plastids, where fatty acids are synthesized. If this could occur, the fatty acid biosynthesis would be restored, as the inhibition of ACCase is reversible (Rendina *et al,* 1988). Another possibility could be the synthesis of a haloxyfop-insensitive (or less susceptible) acetyl-CoA carboxylase. So far, there is no evidence that the plastids of grasses have more than one isoform of this enzyme. It has been shown, however, that grasses do have isoforms of ACCase with different sensitivity to graminicides (Herbert *et al,* 1997).

The rebuilding of the capacity for fatty acid biosynthesis occurred also in plants treated with cerulenin. However, this process was less pronounced than in those treated with haloxyfop. Cerulenin is an inhibitor of fatty acid biosynthesis with a different mechanism of action than graminicides. It inhibits activity of type I of β-keto-acyl synthase, which is responsible for the condensation reactions of malonyl group with the growing acyl chain until it reaches the length of 16 carbons (Packter & Stumpf, 1975; Feld *et al,* 1989). The reasons of why plants treated with cerulenin rebuild their capacity for biosynthesis of fatty acids could be similar as in the case of haloxyfop. Additionally, β-keto-acyl synthase type II and III could partially replace the type I inhibited by cerulenin. As fatty acids are absolutely essential for cell functioning, it seems reasonable to assume that in the course of evolution, several precautionary measures have been developed to prevent biosynthesis disruptions.

Regardless of the mechanism of restoration of fatty acid biosynthesis, the obtained results suggest that a shortage of *de novo* synthesised fatty acids is probably not the direct reason for haloxyfop's (and probably cerulenin's) phytotoxicity. However, one cannot exclude the possibility that the observed transient inhibition of fatty acid biosynthesis activates a cascade of events leading to accelerated ageing and subsequent death of susceptible plants.

Besides the above discussed impact on fatty acid biosynthesis, haloxyfop and cerulenin inhibited the incorporation of radiolabelled acetate into lipophilic compounds not containing fatty acids. To what extent the inhibition of biosynthesis of those compounds contributes to the phytotoxic effects of the tested inhibitors remains, however, open to further investigation.

The use of electronic autoradiography in this study allowed a detailed determination of the location of radioactive compounds on chromatograms. Additionally, it was possible to specify the utilisation of [$^{14}$C]acetate absorbed by roots for *de novo* synthesis of different classes of lipophilic compounds. These experiments have shown, for example, that in the mature parts of roots (the rest remaining after cutting off 1-cm-long tips), the inhibition of biosynthesis of compounds belonging, probably, to free sterols or free fatty acids, is not restored with the time of treatment. As the amount of free fatty acids in the extracts is usually small, it should be considered that the observed inhibition concerns mainly free sterols. The other significant difference observed between the control and the cerulenin-treated plants was a very strong inhibition (which did not disappear with time of treatment) of the biosynthesis of an unidentified lipid, $Y_2$, probably cardiolipin. It remains unknown whether the inhibition of the biosynthesis of this compound has a significant role in the phytotoxic effects of cerulenin on the treated plants. However, if the $Y_2$ lipid is cardiolipin (it was identified only by means of a co-chromatographed standard), the inhibition of its biosynthesis could cause significant disturbances in mitochondrial functions, as cardiolipin plays a crucial role in the functioning of these organelles (Christie, 2012). One cannot exclude that cardiolipin may also be involved in hexokinase II function. This protein plays a protective role in preservation of mitochondrial integrity, and it has recently been shown that cerulenin may affect its association with mitochondrial membranes in cancer cells, and thereby cause death of those cells (Jeong & Yoo, 2012). So far, no such effects have been demonstrated in plants. However, Kim *et al* (2006), after silencing the gene coding hexokinase I (associated with mitochondria) in *Nicotiana benthiana,* observed symptoms of programmed death in transformed cells.

A strong increase in the *de novo* biosynthesis of PE was one of the other significant changes in the biosynthesis of lipophilic compounds, caused by the treatment of the tested grass species with haloxyfop and cerulenin. A similar increase in the biosynthesis of PE occurs in plants exposed to cold (Steponkus *et al,* 1990; Palta *et al,* 1993) and is regarded as one of the mechanisms of the plants' acclimation to sub-zero temperatures. Whether the increase in PE synthesis in plants treated with the investigated inhibitors is an adaptive mechanism, or one of the causes of phytotoxic effects, remains unanswered. However, the fact that these changes occur only after a longer treatment may suggest the former.

In conclusion, it seems reasonable to suggest that inhibition of fatty acid biosynthesis is probably not the only (or maybe not even the main) reason of haloxyfop and cerulenin phytotoxicity. The results obtained for haloxyfop and the data from earlier experiments suggest that in order to explain its mechanism of action both competing hypotheses (the anabolic one, pointing to inhibition of fatty acid biosynthesis, and the catabolic one, postulating "oxidative stress" as the cause of plant death – see Introduction) should be considered. In the case of cerulenin's phytotoxic mode of action, at least its multidirectional effects on lipid metabolism (containing and not containing fatty acids) should be taken into account.

### Acknowledgements

This work was supported partially by KBN, grant no. 6 PO4C 072 12. The authors would also like to thank Professor Sten Stymne from the Swedish University of Agricultural Science for his comprehensive help throughout this research.

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