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EFFECT OF NORFLURAZON ON LIPID METABOLISM IN SOYA SEEDLINGS

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Abstract—Norflurazon (4-chloro,5-methylamino-2-α-α-α,trifluoro-methyl-phenyl-3-(2H)pyridazinone) (San 9789) was applied before sowing to soya seeds (*Glycine max*., var. Weber). At concentrations between 10^{-6} M and 10^{-4} M a strong effect on the morphology and the physiology of the seedlings was observed. The seedlings became depigmented due to photobleaching of the photosynthetic pigments. A 40% reduction of the total lipid content was observed at 10^{-4} M. The chloroplastic contents of MGDG, DGDG and PG decreased of 41%, 63% and 22%, respectively. In contrast, the content in extra-chloroplastic lipids (mainly PC) increased. Only a few changes in total fatty composition was observed in the treated plants with a slight increase in the percentage of $C_{16:0}$ and a concomitent decrease in the $C_{18:3}$ percentage. The fatty acid composition of MGDG and DGDG were almost totally unaffected. In PG a decrease in the percentage of $C_{16:1}$ *trans* (from 33.6 to 21.1%) was observed, while the percentage of $C_{16:0}$ and $C_{18:3}$ increased. In the three extra-chloroplastic classes (PC, PE and Pl) a large increase in the unsaturation level was observed resulting from an increase in the percentage of $C_{18:3}$.

Studies of the lipid metabolism using radioactive labelling with [1-^{14}C]acetate as a precursor led to the conclusion that, in plants treated with norflurazon, the intra-chloroplastic pathway of desaturation was inhibited while a stimulation of the extra chloroplastic desaturation pathway was occuring, partly compensating the deficiency of the chloroplastic desaturases.

INTRODUCTION

Four lipids are always found in all photosynthetic membranes including those of cyanobacteria [1]. These are: the two galactolipids mono-(MGDG) and digalactosyldiacylglycerol (DGDG), which in eukaryotic photosynthetic organisms represent more than 80% of the total polar lipids; a glycosulpholipid which is largely represented in photosynthetic bacteria and cyanobacteria, but found only in small amount (less than 5%) in eukaryotic photosynthetic organisms; sulphoquinovosyldiacylglycerol (SQDG); and finally a phospholipid: phosphatidylglycerol, which represents between 10% (eukaryotic green cells) and 20% (cyanobacteria) of the total lipid content.

Chloroplastic galactolipids, especially MGDG, are very rich in polyunsaturated fatty acids. In the chloroplast of higher plants, triunsaturated fatty acids are predominant: mainly linolenic acid ($C_{18:3}$ $\Delta_{9,12,15}$) but also, in certain plants, hexadecatrienoic acid ($C_{16:3}$ $\Delta_{7,10,15}$) which can represent more than 30% of the fatty acids in MGDG.

In all eukaryotic photosynthetic organisms, *trans* Δ_3 hexadecenoic acid ($C_{16:1}t$) an highly unusual fatty acid, is found esterifying specifically the *sn*-2 position of PG. Both the *trans* configuration and the Δ_3 position of the double bound are very unusual for a natural fatty acid. An important set of experiments has focused on the possible role played by PG containing $C_{16:1}t$ in the photosynthetic membrane [2, 3].

In plant cells, the biosynthetic pathway for gly-

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Abbreviations—$C_{16:0}$, palmitic acid; $C_{16:1}c$, Δ_7 hexadecenoic acid; $C_{16:1}t$, Δ_3*trans* hexadecenoic acid; $C_{16:2}$, Δ-$_{7,10}$ hexadecadienoic acid; $C_{16:3}$, $\Delta_{7,10,15}$ hexadecatrienoic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, Δ_9 octadecenoic acid (oleic acid); $C_{18:2}$, $\Delta_{9,12}$ octadecadienoic acid (linoleic acid); $C_{18:3}$, $\Delta_{9,12,15}$, octadecatrienoic acid (linolenic acid); MGDG, mono+galactosyldiacylglycerol; DGDG, diagalactosyldiacyl+glycerol; PA, phosphatidic acid; PC; phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; TL, total lipid.

cerolipid biosynthesis involves a complex set of interactions between the chloroplastic and the extra-chloroplastic compartments. *De novo* fatty acid synthesis takes place in the chloroplast which also contains a set of desaturases and acylases allowing it to produce all of the chloroplastic lipid classes rich in polyunsaturated fatty acids. Because of the selectivity of the chloroplastic lyso-phosphatidic acid acylases, which only esterify the *sn*-2 position of glycerol with C_{16} acyl chains, this pathway is called the intra-chloroplastic biosynthetic pathway or prokaryotic pathway. The chloroplast exports fatty acids toward the cytoplasm where these fatty acids are used by the acylases and the desaturases linked to the endoplasmic reticulum to build the extra-chloroplastic lipid classes (PC, PE and PI). This is the extra-chloroplastic or eukaryotic pathway for glycerolipid synthesis. Furthermore, unsaturated fatty acids produced in the cytoplasm can return to the chloroplast with PC or lyso PC as shuttle to build the chloroplastic lipidic classes containing C_{18} acyl chain at the 2-position of *sn*-glycerol. This is the co-operative pathway for chloroplast lipid biosynthesis.

The chloroplast envelope is involved in the biosynthesis of many components of the chloroplasts such as carotenoids, protochlorophylls and lipids. It is now well established that it contains several desaturases [4]. This envelope is the target of a number herbicides including the pyridazinones. The phytotoxicity of pyridazinones depends on the degree of methylation of the amine function and also of the presence or absence of a trifluoromethyl group on the aromatic ring. The effects of this family of herbicide include: alteration of chloroplast structure [5]; a decrease in photosynthetic pigment content [6]; a decrease in photosynthetic activity [7]; and inhibition of the desaturation of 18:2 MGDG [8, 9].

It is presently accepted that the main target of substituted pyridazinone, excepting BASF 13-338 [10], is phytoene desaturase, a key enzyme in the biosynthetic pathway of carotenoids [11, 12] which is localized in the chloroplast envelope. However, it seems possible that the herbicide can also inhibit the other desaturases found in the envelope.

The present work was undertaken to study all the effects of norflurazon on the general lipid metabolism of soya seedlings. Particular attention was paid to the effects of the herbicide on both the intrachloroplastic and the extrachloroplastic pathways of glycerolipid synthesis.

RESULTS

Effect of norflurazon on chlorophylls, carotenoids and total lipid content

Norflurazon from a concentration of 10^{-6} M inhibited chlorophyll and carotenoid accumulation (Table 1). At 10^{-4} M, chlorophyll and carotenoid accumulation were inhibited up to 83% and 92%, respectively.

No effect on the lipid content of the leaves was observed below a concentration of 10^{-4} M. But at a concentration of 10^{-4} M induced a strong "photobleaching" of the leaves, the lipid content of which was reduced by 40%.

Effect of the herbicide on lipid class composition

At 10^{-4} M, the lipid class composition (Table 2) was markedly affected with a large decrease in the percentage of MGDG and DGDG and a small decrease in the percentage of PG, while the percentages of PC and PE increased markedly. The percentage of PI remained unaffected. SQDG percentages (data not shown) remained around 5% in the treated as well as in the control plants.

Effect of the herbicide treatment on lipid class fatty acid composition

At 10^{-4} M, the total fatty acid composition was only slightly affected with a small decrease in the percentage of $C_{18:3}$ and a concomitent increase in the $C_{16:0}$ percentage.

The fatty acid compositions of MGDG and DGDG were unaffected by herbicide treatment (Table 3). In PG, the treatment induced a significative decrease in the percentage of $C_{16:1}t$ with the concomitent increase in the $C_{16:0}$ percentage. The percentage of $C_{18:3}$ also increased. In lipid classes characteristic of the extra chloroplastic compartment, the most important chan-

Table 1. Effect of norflurazon on total lipid, carotenoid and chlorophyll content. (Results are the average of three experiments)

Norflurazon (M)	Total lipid content (mg g^{-1} MVF)	Carotenoids (mg g^{-1} MVF)	Chlorophylls (mg g^{-1} MVF)
0 (Control)	5.7	0.70	3.0
10^{-6}	6.1	0.61	2.33
10^{-5}	6.2	0.30	1.88
10^{-4}	3.2	0.05	0.35

Table 2. Effect of norflurazon (10^{-4} M) on the lipid class composition. (% Total lipid composition) (results are the average of three experiments)

	MGDG	DGDG	PG	PC	PE	PI	LN
Control	30.3±2.8	20.0±2.5	7.1±1.0	14.0±2.0	2.5±1.0	3.4±0.9	18.9±0.7
Treated	17.9±0.6	7.3±1.4	5.5±0.8	25.4±5.1	7.9±0.7	3.5±0.3	24.6±0.7

Table 3. Effect of norflurazon (10^{-4} M) on the fatty acid composition of lipid classes (% total fatty acid composition)

	$C_{16:0}$	$C_{16:1}$ *t*	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
LT						
Control	14.9±1.2	2.2±0.5	8.7±1.1	5.0±0.5	10.3±1.7	55.2±7.2
Treated	19.2±0.8	1.5±0.3	7.9±1.3	3.8±0.1	11.1±1.6	49.3±2.3
MGDG						
Control	3.2±0.5	—	2.4±1.6	0.2±0.0	2.8±0.6	89.7±1.3
Treated	4.5±1.6	—	1.5±0.3	0.7±0.3	1.4±0.0	89.8±3.8
DGDG						
Control	12.3±0.6	—	3.5±0.3	0.7±0.3	2.0±0.1	79.7±1.2
Treated	12.0±0.8	—	6.0±1.7	2.6±0.3	1.1±0.0	76.4±2.3
PG						
Control	32.6±0.8	33.6±2.1	9.4±1.5	6.5±0.9	4.2±0.2	7.8±1.0
Treated	42.9±1.1	21.1±1.8	10.9±0.5	3.5±0.3	2.1±0.1	15.1±2.6
PC						
Control	22.1±0.3	—	7.8±0.9	2.8±0.4	30.5±1.4	35.4±1.8
Treated	20.9±0.6	—	7.8±0.8	1.5±0.3	12.5±0.8	55.7±2.0
PE						
Control	30.5±2.8	—	19.1±1.1	2.6±0.3	26.4±3.9	12.8±1.4
Treated	30.6±1.6	—	19.7±1.5	2.3±0.7	15.3±3.5	27.8±0.7
PI						
Control	39.1±3.6	—	30.6±1.4	2.5±0.8	15.0±1.0	16.1±1.6
Treated	37.8±2.1	—	18.1±2.1	2.7±0.7	8.2±0.5	31.6±1.2

ges concerned PC and PE where a very large increase in the percentage of $C_{18:3}$ was observed with a decrease in the percentage of $C_{18:2}$.

Effect on the herbicide treatment on the incorporation of radioactive acetate in lipids. The incorporation of radioactive acetate into total lipids was very similar in control and treated plants up to and including 24 h but after this time the radioactivity decreased more rapidly in the treated plants (Fig. 1). The incorporation of radioactive acetate into total fatty acids, 6, 24 and 48 h after the deposition of radioactive droplets on the leaves is shown in Fig. 2. The main significant differences concerned the labelling of $C_{18:1}$ and $C_{18:2}$. In fact, $C_{18:1}$ was always significantly more labelled in treated plants. $C_{18:2}$ was largely more highly labelled at 6 h in the control plants, while in the treated plants the labelling of this fatty acid increased more slowly to reach a slightly higher level than in the control plant after 24 and 48 h of acetate incorporation. The labelling of $C_{18:3}$ was quite similar in control and treated plants. The labelling of the chloroplastic lipids DGDG and SQDG (not shown in the figure) were comparable in control and treated plants; it was clear that labelling of MGDG and PG was reduced in treated plants after 48 h of incorporation.

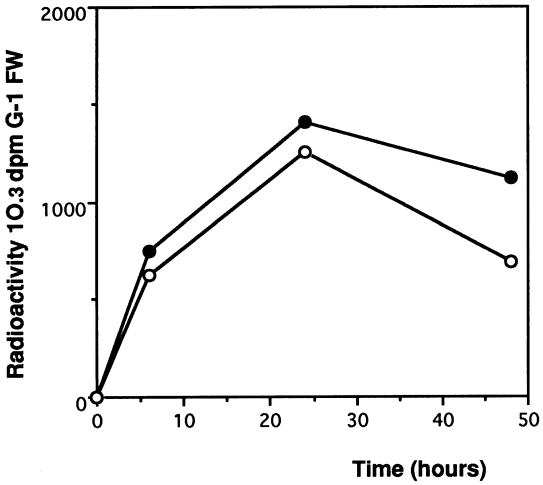

Fig. 1. Distribution of radioactivity in total leaf lipids from control plants and plants treated with norflurazon (10^{-4} M). ●—●, Control; ○—○, treated.

In the extrachloroplastic lipid classes, labelling was always significantly reduced in PC and PE (Fig. 3A).

The labelling of fatty acids was followed for each

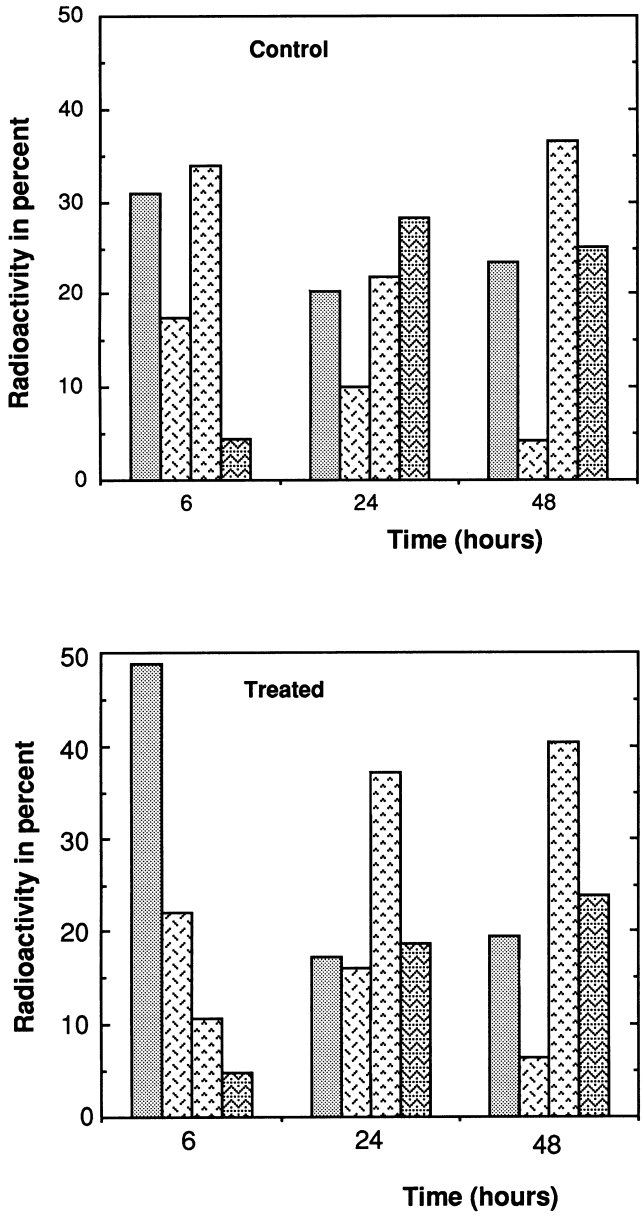

Fig. 2. Incorporation of [1-^{14}C]acetate into fatty acids of total leaf lipids in control plants and plants treated with norflurazon (10^{-4} M). ▩, C16:0; ▩, C18:0; ▩, C18:2; ▩, C18:3.

of the main lipidic classes. No difference were found in lipid classes in which the fatty acid composition was unaffected by the treatment with herbicide, particularly DGDG.

The labelling of fatty acids after 48 h in the four polar lipid classes in which the fatty acid composition was affected by the treatment is shown in Fig. 3B. In the case of PG $C_{16:0}$ was more highly labelled after 48 h of incubation while $C_{16:1t}$ was slightly less labelled in treated plants as compared to the controls. Slight differences in the kinetics of labelling of $C_{18:1}$ and $C_{18:2}$ were also observed between treated and control plants. On the other hand, despite the fact that the mass percentage of $C_{18:3}$ was two times higher in the treated than in the control plants (Table 2), the percentage of radioactivity incorporated into this fatty acid became significantly lower after 48 h of incorporation in the treated plants. In MGDG, the percentage of labelling in $C_{18:3}$ decreased slightly in the treated plants while $C_{18:2}$ increased.

In PC and PE, despite the large differences in the mass percentages of $C_{18:2}$ and $C_{18:3}$ induced by the herbicide treatment, only slight differences in the pattern of labelling of these fatty acids was observed. In

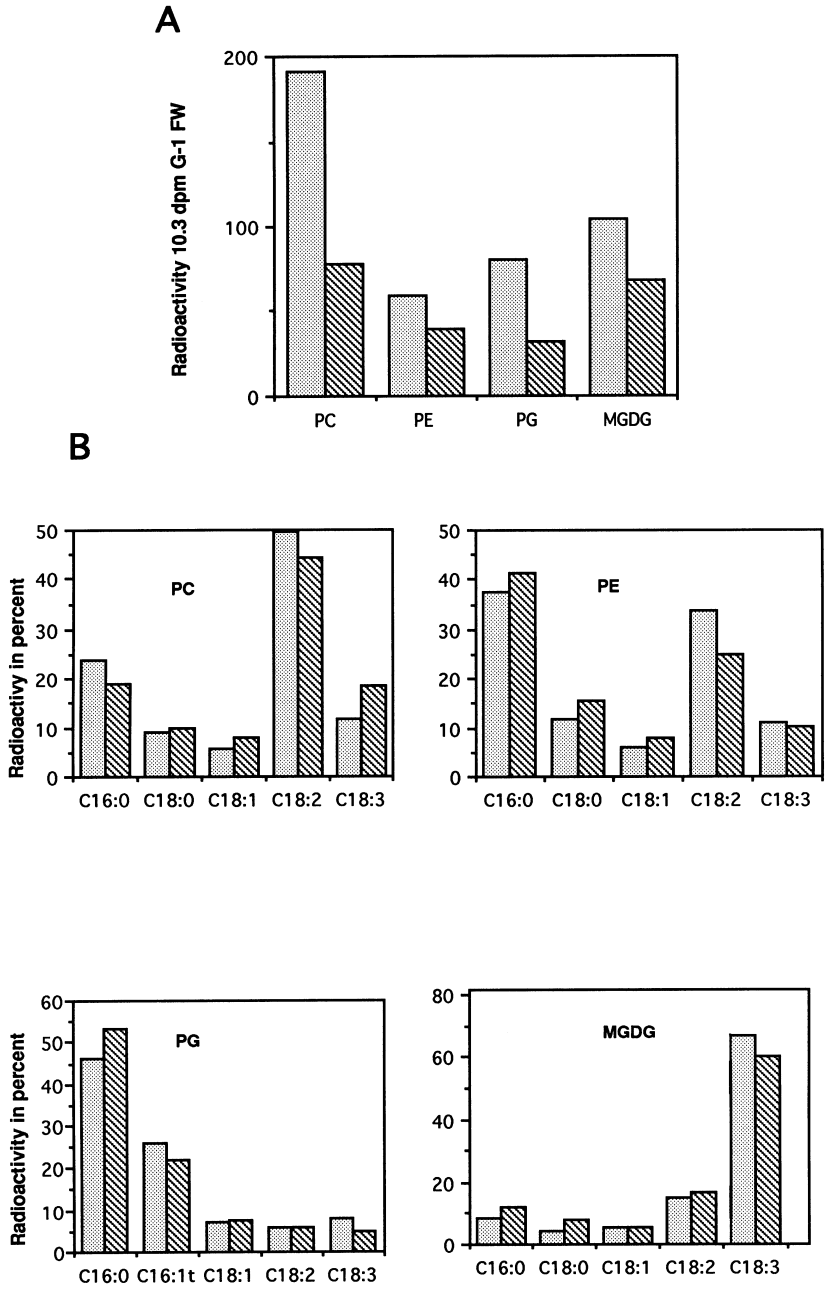

Fig. 3. Incorporation of radioactivity 48 h after deposition of radioactive droplets on the leaves into: A: The polar lipids from control and treated plants; B: The fatty acids of polar lipids in control plants and plants treated with norflurazon. □, Control; ▨ treated.

both PC and PE, $C_{18:1}$ remained always slightly more labelled and $C_{18:2}$ significantly less labelled in treated plants as compared to controls. The radioactivity found in $C_{18:3}$, after 48 h of incorporation was significantly higher in treated plants but in PE, despite the fact that the mass percentage of $C_{18:3}$ was more than two times higher in the treated plants than in the control as compared to the controls (Table 3), no differences in the relative labelling of this fatty acid were found.

CONCLUSIONS

Treatment of soya bean seedlings with 10^{-4} M norflurazon has a marked effect on the lipid composition of the leaves with a marked decrease in the level of intra chloroplastic lipid species which, despite a concomitant increase in the level of PC and PE, results in a decrease in the total lipid content. Despite the large decrease in the content of the two main galactolipids MGDG and DGDG, a perfectly normal fatty acid

composition characterized by a high mass percentage of $C_{18:3}$ is found for these lipidic classes. In contrast, the fatty acid composition of PG is significantly affected; the mass percentage of $C_{16:1t}$ decreased while the percentages of $C_{16:0}$ and $C_{18:3}$ increased. On the other hand, in the extrachloroplastic lipid classes, PC and PE, a large increase in the mass percentage of $C_{18:3}$ is observed.

Radioactivity from [1-^{14}C]acetate was readily incorporated in the leaves of treated plants (after 6 h of incorporation, the radioactivity recovered in the total lipid fraction was almost the same in the treated and control plants) showing that *de novo* fatty acid synthesis located in the chloroplast was not affected. But after 24 or 48 h the radioactivity incorporation in the lipids decreased more rapidly in the treated plants, probably indicating that lipid turnover was accelerated by the herbicide. A study of the kinetic of labelling in the main lipid classes showed that this decrease in radioactivity occured in PC, PE and PG of both treated and control plants, but occured more rapidly in the treated plants. The radioactivity incorporated into MGDG continued to increase until 48 h, but more slowly in treated plants. The level of radioactivity remained very stable in DGDG (with only a slight reduction of the level of labelling in the treated plants).

The labelling of $C_{18:3}$ MGDG decreased slightly (11%) while the $C_{18:3}$ PC increased markedly (58%). In PG, the labelling of $C_{16:1t}$ decreased also.

All these results can be tentatively explained by an effect of the herbicide treatment on the chloroplastic desaturases pathway. However, it is possible that this effect does not result from the direct action of norflurazon on the desaturase located in the envelope but indirectly as a consequence of the action of the herbicide on the carotenoid biosynthetic pathway.

Nevertheless, it is also possible that an inhibitor such as norflurazon in addition to inhibiting the desaturation of phytoene could also have an effect on the MGDG-18:2 desaturase located in the same chloroplast envelope. This study also suggests, as previously reported in several physiological or genetical situations, that because of the strong co-operative organization of lipid metabolism, plants are able to compensate for desaturase deficiency in one compartment (chloroplastic compartment) by using polyunsaturated fatty acids produced in the unaffected compartment (cytoplasmic compartment). The increase in the percentage of $C_{18:3}$ in PC and PE of treated plants could result from an increase in cytoplasmic desaturase activities induced by the inhibition of the intrachloroplastic desaturase pathway. Through this co-operative pathway, the plants are able to regulate fatty acid composition in a number of different physiological or stress conditions.

On the other hand, the decrease in the percent of $C_{16:1t}$ observed in the PG could indicate that the treatment induces some disorganization of the photosynthetic membranes, since this lipid has been shown to be implicated in the stabilization of the LHCII antennae [13].

EXPERIMENTAL

Materials

Norflurazon (4-chloro,5-methylamino-2-α-α-α,trifluoromethyl phenyl-3-(2H)pyridazinone) was applied before sowing to soya seeds.

Growth conditions

Soya seeds (*Glycine max*. var. Weber) were germinated and grown on a soil wetted with the water (control) or with the herbicide soln (treated), in field conditions.

Lipids analysis

The first leaves of the seedlings were cut and fixed in boiling water. Lipid were extracted with $CHCl_3$–MeOH according to Ref. [14]. Lipid classes were separated on TLC according to Refs [15, 16].

For fatty acid analysis, aliquots of the total lipid extract, or spots corresponding to lipid classes separated on TLC, were transmethylated as described [17] and analyzed by capillary GC, isothermally at 170°, using a Girdel 30 apparatus equipped with a 50 m long, 0.25 mm diameter carbowax column. Heptadecanoate was added in known amount as a standard for quantitative determinations of fatty acids.

Labelling experiments

Microdroplets of sodium [1-^{14}C]acetate (2.1 GBq mmol^{-1}) were put on the first leaves of control and treated plants. Radioactivity in the lipid classes was determined by scraping the corresponding spots off the TLC plate and counting them by liquid scintillation spectrometry. Radioactivity in fatty acids was determined by radio-GC using a Girdel 300 apparatus equipped with a 25 m long, 0.5 mm diameter carbowax column at 170°, coupled to a radiomatic gas proportional counter.

Radioactive labelling experiments were repeated three times. In the paper, we report the results of one typical experiment, because the quantitative level of incorporation was different in each experiment.

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