Effect of Norflurazon on Resorcinolic Lipid Metabolism in Rye Seedlings

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Norflurazon is a selective pyridazinone herbicide excessively employed in the control of many annual grasses and broad-leaved weeds. This chemical causes plant bleaching due to the inhibition of the carotenoid pigment biogenesis as well as induces irreparable changes to chloroplasts, which are considered the organelles where the biosynthesis of resorcinolic lipids takes place. Resorcinolic lipids, a group of phenolic compounds, constitute not only an essential part of the plant antifungal defense system, but also are an important component of the human cereal diet. The aim of this study was to investigate the effect of norflurazon on the biosynthesis of resorcinolic lipids in 5-day-old rye plants (*Secale cereale* L.) that were grown at three different temperatures under light or dark conditions. At all tested temperatures, norflurazon decreased the fresh biomass of light-grown rye seedlings and increased the weight of plants grown in darkness. Compared with respective controls, this herbicide caused an increase in total content of alkylresorcinols in both green and etiolated plants with the exception of dark-grown norflurazon-treated rye at 29 °C. The general level of saturated homologues was markedly decreased by norflurazon in all etiolated plants and in light-grown seedlings at 15 °C. Independent of thermal and light conditions, in all norflurazon-treated samples two alkylresorcinol derivatives predominated: 1,3-dihydroxy-5-*n*-heptadecylbenzene and 1,3-dihydroxy-5-*n*-nonadecylbenzene. Thus, our results suggest that norflurazon affected the metabolism of alkylresorcinols in rye seedlings and its action was dependent on external stimuli.

Key words: 5-*n*-Alkylresorcinols, Herbicide, Norflurazon

Introduction

Norflurazon [SAN 9789, 4-chloro-5-methylamino-2-(3-trifluoromethylphenyl)-pyridazin-3(2*H*)one] is a selective pyridazinone herbicide that is excessively employed in the control of many annual grasses and broad-leaved weeds (Tomlin, 1994). This pre-emergent pesticide is readily taken up and absorbed by plant roots and transported upwards in the xylem to the growing plant parts, where it causes extensive damage to the photosynthetic apparatus (Mersie and Singh, 1987). Norflurazon is a non-competitive inhibitor of phytoene desaturase, a major enzyme of the carotenoid biogenesis pathway, which blocks the accumulation of carotenoids (Bartles and Watson, 1978; Eder, 1979). Interestingly, the effect of norflurazon on the level of carotenoids in tomato leaves was found dependent on light conditions (Simkin *et al.*, 2003). Treatment of plants with this herbicide also decreased the level of chlorophyll, which was a result of its rapid photooxidation in the absence of carotenoids. In the presence of norflurazon, plants accumulated only short chromophores that cannot protect the plants against photodegradation (Henningsen and Stumman, 1982; Jung *et al.*, 2000). Lack of those fundamental pigments also impairs the formation of photosynthetic membranes, which results in the concerted bleaching of plants. That is why plants grown in the presence of norflurazon are whitish or pinkish although otherwise normally differentiated (Simpson *et al.*, 1986).

The inhibition of carotenogenesis led to a decline in the ratio of ribosomes to plastids, but also

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affected rRNA synthesis in plastids (Reib *et al.*, 1983). In addition, the expression of many nuclear genes encoding for plastid-localized proteins was suppressed in norflurazon-treated seedlings. This phenomenon occurs due to the impairment of signaling from destroyed chloroplasts to nuclei (Ernest and Schelfbeck, 1988; Tamada *et al.*, 2003). In the photobleached plant cells, the inhibiting effect was noticed at both the transcription or translation gene level (Batschauer *et al.*, 1986; Ernest and Schelfbeck, 1988). Moreover, the synthesis of phytohormone abscisic acid was reduced in plants after treatment with this chemical (Henson, 1984; Feldman and Sun, 1986). Norflurazon also exerted some effects on both content and composition of extra- and intra-chloroplastic lipids, especially glycolipids (Abrous *et al.*, 1998; Di Baccio *et al.*, 2002). This phenyl-pyridazinone compound modulated the activity of a specific Δ^{15}-desaturase, which uses monogalactosyldiacylglycerols as substrates (Ohlrogge and Browse, 1995).

Plastids are the subcellular organelles where the biosynthesis of resorcinolic lipids has been described (Deszcz and Kozubek, 2000). This group of phenolic lipids consists of derivatives of 1,3-dihydroxy-5-alk(en)ylbenzene. Their presence has been demonstrated especially in cereals where they play a key role in the defense system against fungal pathogens (Verdeal and Lorenz, 1977; Zarnowski and Kozubek, 2002; Zarnowski *et al.*, 2004). Alkylresorcinols also are an important component of the human cereal diet (Chen *et al.*, 2004; Ross *et al.*, 2004; Parikka *et al.*, 2006). In our previous work, we showed the significant effect of herbicides on the biosynthesis of 5-*n*-alkylresorcinols in rye seedlings (Magnucka *et al.*, 2001).

Resorcinolic lipids are polyketide pathway products because their phenolic ring is biosynthesized from acetate (Kozubek and Tyman, 1999, 2005). The direct precursors of the side-chain of these compounds are fatty acids (Suzuki *et al.*, 2003; Funa *et al.*, 2006). It has been postulated that the synthesis of 5-*n*-alkylresorcinols is the result of the convergence of the fatty acid biosynthetic pathway and the activity of polyketide synthase-type enzymes. The first pathway is responsible for the synthesis of the aliphatic tail and the second pathway for the formation of the phenolic head (Kozubek and Tyman, 1999, 2005; Suzuki *et al.*, 2003; Funa *et al.*, 2006). Therefore, the hypothetical pathway for the biosynthesis of these compounds begins with the formation of a starter unit, a fatty acid-CoA originating from fatty acid synthesis. Next, the sequential aldol condensation of acetyl units, derived from 3 moieties of malonyl-CoA, to the starter unit takes place. The Claisen cyclization of the formed intermediate yields 6-alkylresorcinolic acid attached to the acyl carrier protein. The liberation of this protein is accompanied by the decarboxylation of acid yielding 5-*n*-alkylresorcinol (Kozubek and Tyman, 1999, 2005).

In this paper we investigate how norflurazon, a chloroplast-destroying agent, affects the biosynthesis of resorcinolic lipids in rye seedlings (*Secale cereale* L.) grown under various thermal and light conditions.

Materials and Methods

Chemicals

Norflurazon was obtained from Novartis Crop Protection AG (Basel, Switzerland). Solvents and reagents were from Polskie Odczynniki Chemiczne (Gliwice, Poland) and from Chempur (Piekary Slaskie, Poland). Diazonic dye Fast Blue $B \times BF_4$ was obtained from Chemapol (Prague, Czech Republic).

Plant material

Qualified grains of winter-crop rye (*Secale cereale* L. cv. Dankowskie Zlote) were used. Fully mature grains were released from "Danko" Plant Breeding Farm (Choryn, Poland), in 2000. A complete cultivar voucher is available from the Central Laboratory for Studies of Cultivable Plants "COBORU" (Slupia Wielka, Poland).

Treatments and growth conditions

Grains of rye (35 g) were surface-sterilized by immersing in 0.1% (v/v) Tween 80 for 15 min, followed by a 15-min-long incubation in 5% (w/v) chloramine and three additional washes in sterile distilled water. Disinfected seeds were germinated in growth chambers padded with a sterile tissue paper soaked with water (control plants) or a solution containing 10 mg l^{-1} norflurazon. Seedlings were grown at three different temperatures: (15 ± 2), (22 ± 2) and (29 ± 2) °C under continuous light or in darkness. Shoots of rye seedlings were collected after 5 d of culturing and their fresh (FW) and dry weight (DW) were determined gravimetrically.

Isolation and determination of alkylresorcinol content

Alkylresorcinols were extracted from dried shoots of rye with acetone according our method previously described (Magnucka *et al.*, 2001). Then the solvent from the acetone fraction was removed by vacuum evaporation. The oily residue was redissolved in chloroform and applied on Si60 TLC plates (Merck, Darmstadt, Germany). Separation was carried out in *n*-hexane/ethyl ether/formic acid (70:30:1, v/v). Afterwards, narrow strips on both sides of the gel were sprayed with aqueous 0.05% (w/v) Fast Blue $B \times BF_4$. Part of the gel containing these compounds was scraped off the plate and reextracted with a mixture of acetone/methanol (4:1, v/v). After centrifugation ($7500 \times g$, 10 min), the supernatant was concentrated *in vacuo* and then redissolved in *n*-propanol. The microcolorimetric method was used for quantitative determination of alkylresorcinols (Tluscik *et al.*, 1981). All determinations were carried out in triplicate.

Chromatography analyses

Composition of alkylresorcinol homologues was evaluated using gas chromatography coupled with mass spectrometry (GC/EI-MS) according to the method described elsewhere (Zarnowski and Suzuki, 2004). 70 μl from the 100 μl alkylresorcinol mixture redissolved in ethyl acetate were added to a glass capillary-tube (∅ *ca.* 2 mm × 5 cm). After removal of the solvent, 5 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetimide were added. The tube was sealed and allowed to stand at 70 °C for 30 min. 1 μl of the derivatized sample was injected into a HP 5890 Series II gas chromatograph connected to a JEOL SX-102A mass spectrometer, at 70 eV with a helium flow rate of 1 ml/min. A DB-1 column (∅ *ca.* 0.25 mm × 15 m, 0.25 μm film thickness; G&L Science, Tokyo, Japan) was used. The column oven temperature was programmed as follows: 130 °C for 1 min, 30 °C/min to 250 °C, 15 °C/min up to 320 °C and 320 °C for 2 min. The sample injection port temperature was set at 250 °C. Identification of each alkylresorcinol homologue was obtained from the molecular ion and common base ion peak at *m*/*z* 268, which is characteristic of those molecules. The retention time of each saturated homologue was 9.3 min ([M^+] 464, $C_{15:0}$), 10.4 min ([M^+] 492, $C_{17:0}$), 11.6 min ([M^+] 520, $C_{19:0}$), 12.7 min ([M^+] 548, $C_{21:0}$), 13.8 min ([M^+] 576, $C_{23:0}$) and 14.9 min ([M^+] 604, $C_{25:0}$), respectively. The relative composition and total amount of each homologue were estimated on the basis of the area of the ion peak at *m*/*z* 268.

Additional identification of resorcinolic lipids was carried out using a set of thin layer chromatography techniques. Pattern of the homologues according to the length of the side-chain was determined on the basis of reversed-phase thin layer chromatography on RP18 HPTLC plates (Kozubek, 1985). The composition of homologues according to their unsaturation was determined by argentation chromatography on silica gel impregnated with 5% (w/v) $AgNO_3$ (Kaczmarek and Tluscik, 1984). After development, all chromatograms were sprayed with aqueous 0.05% (w/v) Fast Blue $B \times BF_4$ and alkylresorcinols were identified as described above.

Results

Effect of norflurazon on fresh and dry weights of green and etiolated rye seedlings grown at various temperatures

The analysis of the influence of various temperatures upon fresh biomasses of both green and etiolated control rye seedlings demonstrated that 22 °C was the optimal temperature for growth of these plants. In turn, the smallest yields of rye biomass were gathered at 15 °C (Table I).

The application of norflurazon decreased the fresh weight of green seedlings at all tested temperatures in relation to respective controls. The most noticeable reduction in rye biomass was observed at 15 °C; herbicide reduced the weight of these plants by 23% compared to untreated control plants. In turn, the dry matter content of rye seedlings grown under light conditions was markedly decreased at both 22 °C and 15 °C, by 18% and 31%, respectively. At the highest temperature norflurazon caused a slight increase in weight of these seedlings.

In the case of dark-grown plants the herbicide increased their fresh biomass at all growth temperatures. The most considerable increase, by 94% with reference to control sample, was noticed at 29 °C. Also, the dry weight of norflurazon-treated rye was the highest at this temperature. Compared

Table I. Effect of norflurazon on fresh and dry biomass of green and etiolated rye seedlings grown at various temperatures.

Sample	Temperature [°C]	Green seedlings		Etiolated seedlings	
		FW [g]	DW [g]	FW [g]	DW [g]
Control	29 ± 2	16.3 ± 0.4	2.3 ± 0.1	16.0 ± 1.2	2.1 ± 0.1
Norflurazon		13.7 ± 0.5	2.5 ± 0.2	31.0 ± 1.7	3.4 ± 0.2
Control	22 ± 2	20.8 ± 1.4	3.3 ± 0.0	21.4 ± 0.6	2.5 ± 0.1
Norflurazon		19.6 ± 1.0	2.7 ± 0.1	24.6 ± 0.9	3.1 ± 0.2
Control	15 ± 2	10.7 ± 0.5	1.6 ± 0.1	7.6 ± 0.2	1.1 ± 0.0
Norflurazon		8.2 ± 0.3	1.1 ± 0.0	8.5 ± 0.7	1.3 ± 0.1

Mean values express weight of seedlings obtained after germination of 35 g of rye grains. Mean values ± SD from three independent experiments.

with the control, it was increased by 62%. In addition, the effect of this chemical on water retention capacity of rye shoots was slight. The differences in water content between norflurazon-treated seedlings and control plants did not exceed 4.5%.

Effect of herbicide and growth conditions upon alkylresorcinol content in rye plants

We found that 5-day-old rye seedlings produced detectable amounts of alkylresorcinols. Moreover, our results indicate that temperature, light conditions and herbicide treatment affected the total resorcinolic lipid content of rye seedlings. The content of resorcinolic lipids in control rye grown in darkness was about 2 times higher than that in green seedlings, independent of thermal conditions. Moreover, the increase in the level of alkylresorcinols was negatively correlated with temperature. Thus, light and high temperatures seem to have an inhibiting action on the synthesis of those phenolic lipids.

Compared with control plants, norflurazon caused a considerable increase in the amount of alkyresorcinols in green seedlings at all tested temperatures. The contents of alkylresorcinols in these seedlings were negatively correlated with the temperature. The highest, 4-fold increase in the level of resorcinolic lipids was noticed at 29 °C. In turn, seedlings kept in dark conditions biosynthesized a higher level of alkylresorcinols than that in respective control only at 29 °C and 15 °C. However at 29 °C the content of these lipids was affected by above 263%, whereas only about 17% increase at 15 °C was observed. At 22 °C norflurazon caused a significant decrease in concentration of these phenolic compounds, by about 19%, compared with dark-grown control plants.

Effect of norflurazon and growth conditions upon alkylresorcinol homologue pattern

Ten resorcinol homologues differing in side-chain length and unsaturation were found in most of the analyzed samples. The qualitative compositions of these derivatives in controls and herbicide-treated plants were similar.

In green control seedlings grown at both 15 °C and 29 °C the major compounds were 1,3-dihydroxy-5-*n*-heptadecylbenzene (AR $C_{17:0}$) and 1,3-dihydroxy-5-*n*-nonadecylbenzene (AR $C_{19:0}$), whereas at 22 °C only the first homologue dominated (Table II). Both of these compounds were also predominant homologues in plants exposed to norflurazon at all tested temperatures. We also observed that the herbicide applied at 15 °C caused a considerable decrease in the general content of saturated homologues, whereas at the other temperatures the level of these compounds was very slightly increased. Despite these results, the slight decrease in the percentage of saturated, mainly long-chain derivatives along with a decline of temperature was observed. This effect was compensated by the slight increase in AR $C_{17:1}$ and AR $C_{19:1}$. The opposite correlation was observed in the case of AR $C_{19:1}$ in control plants.

Saturated AR $C_{19:0}$ and 1,3-dihydroxy-5-*n*-heneicosylbenzene (AR $C_{21:0}$) homologues dominated in etiolated control plants grown at both 29 °C and 22 °C (Table III). At 15 °C AR $C_{17:0}$ was also a major compound. In turn, at the lowest temperature AR $C_{17:0}$ and AR $C_{19:0}$ were the predominant derivatives. These two compounds also dominated in all norflurazon-treated seedlings grown in darkness.

The general level of saturated homologues in dark-grown plants supplied with norflurazon was

Table II. Effect of norflurazon on content and homologue composition of alkylresorcinols in green rye seedlings under various thermal conditions.

Sample	Temperature [°C]	Content[a] [mg/kg]	Homologue composition[b] (% of total alkylresorcinol content) R									
			$C_{15:0}$	$C_{17:1}$	$C_{17:0}$	$C_{19:1}$	$C_{19:0}$	$C_{21:1}$	$C_{21:0}$	$C_{23:1}$	$C_{23:0}$	$C_{25:0}$
Control	29 ± 2	1.9 ± 0.1	5.5	4.1	38.5	8.7	24.7	2.3	10.2	n.d.	4.2	1.8
Norflurazon		7.6 ± 0.4	5.6	5.1	33.4	7.3	27.3	2.3	10.5	n.d.	5.6	3.0
Control	22 ± 2	3.1 ± 0.2	7.8	6.8	29.6	6.5	18.0	2.8	7.5	n.d.	17.4	3.6
Norflurazon		12.0 ± 0.6	4.4	5.1	39.0	7.8	28.1	2.3	9.7	n.d	2.1	1.5
Control	15 ± 2	4.1 ± 0.1	5.8	4.0	35.2	5.6	23.9	*t*	9.8	n.d.	14.3	1.4
Norflurazon		13.6 ± 0.8	4.5	7.8	36.8	8.5	27.5	3.0	8.5	t	2.0	1.0

[a] Dry weight.
[b] R = C_{15}–C_{25} saturated or monounsaturated side-chain.
n.d., not detected; t, trace (below 0.5%).
Mean values expressing content of alkylresorcinols ± SD obtained from three independent experiments.

Table III. Effect of norflurazon on content and homologue composition of alkylresorcinols in etiolated rye seedlings under various thermal conditions.

Sample	Temperature [°C]	Content[a] [mg/kg]	Homologue composition[b] (% of total alkylresorcinol content) R									
			$C_{15:0}$	$C_{17:1}$	$C_{17:0}$	$C_{19:1}$	$C_{19:0}$	$C_{21:1}$	$C_{21:0}$	$C_{23:1}$	$C_{23:0}$	$C_{25:0}$
Control	29 ± 2	3.0 ± 0.1	3.1	1.8	16.0	3.0	35.1	1.4	31.7	n.d.	5.9	2.0
Norflurazon		10.9 ± 0.6	4.5	6.3	39.4	9.9	26.3	2.3	7.8	t	2.0	1.2
Control	22 ± 2	6.2 ± 0.3	9.6	1.7	21.7	3.8	29.5	1.9	23.9	0.6	6.0	1.3
Norflurazon		5.0 ± 0.1	3.8	5.5	36.8	6.8	24.2	2.7	13.0	0.6	5.1	1.5
Control	15 ± 2	8.1 ± 0.4	5.3	6.3	40.0	6.7	21.8	4.2	9.6	0.7	4.0	1.4
Norflurazon		9.5 ± 0.4	3.7	9.3	34.9	9.4	24.9	3.0	9.5	0.6	3.1	1.6

[a] Dry weight.
[b] R = C_{15}–C_{25} saturated or monounsaturated side-chain.
n.d., not detected; t, trace (below 0.5%).
Mean values expressing content of alkylresorcinols ± SD obtained from three independent experiments.

lower than in respective control seedlings at all tested temperatures. Moreover, in both control and herbicide-treated seedlings the negative correlation between percentage of AR $C_{21:1}$ and temperature was noticed. In the case of AR $C_{17:0}$, the relationship was different in control plants and after treatment with norflurazon.

Discussion

Compared to the green control plants, the norflurazon-treated seedlings exhibited completely pinkish shoots as they became depigmented due to photobleaching of the photosynthetic pigments. The fresh biomass of these plants was lower than the weight of respective controls at all tested temperatures. This fact is in agreement with the results presented by Dalla Vecchia *et al.* (2001) who observed that maize plants grown in the presence of norflurazon were shorter than green controls incubated at different temperatures. By contrast, the pesticide caused an increase in the fresh matter content of dark-grown seedlings at all temperatures. Increasing temperature also increased the

weight of seedlings supplied with norflurazon. These observation confirmed results reporter by Irving and coworkers (1999) who showed that norflurazon significantly increased the elongation growth of etiolated maize coleoptiles in comparison with untreated control. Thus, the effect of this chemical on plants is highly dependent on the light conditions and the differences concern mainly the structure of plastids (Pardo and Schiff, 1980; Reib *et al*., 1983; Di Baccio *et al*., 2002). Reib *et al*. (1983) showed that cotyledons of norflurazon-treated mustard seedlings grown in strong white light did not contain normal chloroplasts, but only small chlorophyll-free rudiments with completely destroyed internal structure. Treatment of etiolated bean leaves with this herbicide had only little effect on the formation of normal prothylakoids and prolamellar bodies (Pardo and Schiff, 1980). The effect of fluridone, an herbicide also inhibiting the carotenoid synthesis, was markedly stronger on barley plastid ultrastructure in plants grown in the presence of light than in darkness (Popova, 1996). Di Baccio *et al*. (2002) showed that norflurazon affects the level and composition of etioplast lipids. Our result proved that the metabolism of resorcinolic lipids is also under the influence of this herbicide. Norflurazon caused a considerable increase in the total amounts of these lipids in plants grown in the presence of light at all tested temperatures. Also in darkness, rye seedlings grown at 29 °C and 15 °C with norflurazon possessed a higher concentration of alkylresorcinols. This chemical also determined changes in the composition of resorcinolic lipids. In dark-grown seedlings it caused a notable decrease in the general level of saturated derivatives at all tested temperatures. Significant changes in these homologues were noticed only in green plants grown at the lowest temperature. The decrease observed in the level of saturated homologues and simultaneous increase in the total percentage of monounsaturated derivatives in norflurazon-treated seedlings does not exclude the inhibiting effect of this herbicide treatment on plastid desaturases. The pattern of ten resorcinolic homologues was affected by this herbicide, but its effect was markedly dependent on both thermal and light conditions. This observation is probably due to a strong co-operative organization of lipid metabolism in plants, which are able to compensate for desaturases deficiency in one compartment by using unsaturated fatty acids from anaffected compartment, *e.g.* from cytoplasm. Because resorcinolic lipids have an antifungal activity (Verdeal and Lorenz, 1977; Zarnowski and Kozubek, 2002; Zarnowski *et al*., 2004), such modifications in both content and homologue composition of these compounds may improve the defense/resistance system of plants to fungal pathogens.

In conclusion, our study showed that norflurazon markedly affected the biosynthesis of resorcinolic lipids in rye seedlings. We also assume that seedlings of monocotyledons, which survived the application of this herbicide, might become less susceptible to microbial pathogen infections.

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