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RESEARCH PAPER

Effects of norflurazon on CO_2 fixation and Rubisco activity in some C_3 and C_4 plants under different light intensity

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ABSTRACT

Norflurazon (NF) is a herbicide that is known to inhibit carotenoid biosynthesis, destroy chlorophylls, desintefrate chloroplasts, and ruin chloroplast ribosome. In this study, we clarify how light intensity modulates effects of NF on CO_2 fixation and activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in some C_3 and C_4 plants. We investigate effects of NF on maize, barley, triticale, and radish under low light intensity (0.12 and 0.4 W/m^2), and on maize, wheat, bean, and radish under high light intensity (100 W/m^2). We show that the effects are modulated by light intensity and species peculiarities. Specifically, C_3 plants, with no mechanism for CO_2 recycling, loose carbon compounds easier compared to C_4 plants, resulting in earlier manifestation of photodestruction under NF treatment. Besides, we show that Rubisco activity increases as far as its content decreases.

Keywords: CO_2 fixation, Rubisco activity, light intensity, norflurazon, C_3 plants, C_4 plants, carotenoids, chlorophylls

РЕЗЮМЕ

Косогова Т.М., Русинова Н.Г., Карапетян Н.В. Влияние норфлуразона на фиксацию CO_2 и активность Rubisco у некоторых C_3- и C_4-растений при различной освещенности. Обработка растений гербицидом норфлуразоном (НФ) приводит к подавлению биосинтеза каротиноидов, дезинтеграции хлоропластов, разрушению в них рибосом и хлорофиллов. Данное исследование посвящено особенностям влияния НФ на фиксацию CO_2 и активность Rubisco у некоторых C_3- и C_4-растений при различной освещенности. Исследовано влияние НФ на кукурузу, ячмень, тритикале и редис при низкой освещенности (0,12 и 0,4 Вт/м^2), и на кукурузу, пшеницу, бобы и редис – при высокой (100 Вт/м^2) Показано, что влияние НФ зависит от освещенности и видовых особенностей растений. В частности, C_3-растения, не имея механизма дополнительного связывания CO_2, легче теряют соединения углерода, вследствие чего вызываемые НФ фотодеструктивные процессы проявляются у них раньше. Установлено также, что активность Rubisco обратно пропорциональна ее содержанию.

Ключевые слова: фиксация CO_2, активность Rubisco, освещенность, норфлуразон, C_3-растения, C_4-растения, каротиноиды, хлорофиллы

INTRODUCTION

Norflurazon (NF), also known as SAN 9789, is a trademark of 4-chlor-5-methylamino-2-(3-trifluormethyl-phenyl)-pyridazin-3-on, a herbicide long known for its capability to inhibit carotenoid biosynthesis (Bartels & Watson 1978). Enzyme-kinetics studies explain NF action by its targeting phytoene desaturase, the enzyme responsible for phytoene transformation into phytofluene in the carotenoid-biosynthesis pathway (Sandmann et al. 1989); the molecular mechanism is based on competition with the enzyme cofactors (Breitenbach et al. 2001). Recent molecular-genetics studies indicate that the herbicide also differentially affects expression of carotenoid-pathway genes (Rivera-Madrid et al. 2013). The carotenoid deficiency facilitates formation of singlet oxygen, resulting in oxidative stress (Golfred & Karapetyan 1989). The latter strongly affects, inter alia, the photosynthetic apparatus: typical manifestations include chlorophyll degradation, photosystem II (PSII) inhibition, chloroplast disintegration, and chloroplast 70S ribosomes destruction (Bolychevtseva et al. 1987, 1988, Ezhova et al. 1997, Jung 2004), although chloroplast proteins vary in their vulnerability to photooxidation (Mayfild et al. 1986).

Under high light intensity, NF inhibits chloroplast enzymes including ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Feierabend & Schubert 1978, Frosch et al. 1979). In barley, NF effects are less pronounced under low light intensity regime compared to high (Bolychevtseva et al. 1987). Electrophoresis results did not show changes in soluble protein content, including Rubisco subunits, under

NF (Bolychevtseva et al. 1988). In the present study, we aim at further clarifying how light intensity modulates effects of NF on CO_2 fixation and Rubisco activity in C_3 and C_4 plants.

MATERIAL AND METHODS

Plant material and treatment

In the first set of experiments, effects of NF were studied under low light intensity, either 0.12 or 0.4 W/m^2. Seedlings of maize, barley, triticale (cultivar AD-206), and radish were sprouted from the seeds in Petri dishes with wet filter paper within a luminostate, either with (treatment) or without (control) 10^{-4} M NF solution added. During the experiment, temperature was 23°C.

In the second set of experiments, effects of NF were studied under high light intensity, 100 W/m^2. Seedling of maize, wheat, bean, and radish were sprouted from the seeds in containers with soil (d=0.43 m), either with (treatment) or without (control) 75 ml of 10^{-4} M NF added per container on the 14th day. Then, the seedlings were cultivated for additional 12 days.

CO_2 fixation

CO_2 fixation rate was measured using radioisotope method, based on $^{14}CO_2$ absorption by leaves (Rusinova et al. 1992). 20–500 g of leaves was put into a chamber with $^{14}CO_2$ (V=150 ml), and kept there for 1 min, either in darkness, or under different light intensity (0.12, 0.4, and 4 W/m^2). $^{14}CO_2$ content in the chamber was 0.9 %, equal to 19 Ci/mol. Then, the leaves were fixed by 80 % ethanol. After centrifuging the plant samples, radioactivity was measured using the SL-30 and SL-4000 liquid scintillation counters (France).

Rubisco content and activity

To extract Rubisco, 1 g of leaves were milled with 5 ml of 50 mM Tris-HCl buffer (pH=8.0) containing 10 mM of $MgCl_2$, 5 mM of dithiothreitol, and 1 mM of EDTA (T=2–4°C). The homogenate was filtered, and the filtrate was centrifuged for 40 min under 23 000 g. In the supernatant, two characteristics were measured: (i) total soluble proteins (TSP) contents – on sedimentation by 3 % trichloracetic acid solution (Lowry et al. 1951), and (ii) Rubisco carboxylase activity – based on velocity of $NaH^{14}CO_3$ incorporation into acid-resistant products in the presence of ribulose-1,5-biophosphate under 30°C, using radioisotope method (Ezhova et al. 1997).

The enzyme mixture contained: 10 mM of $MgCl_2$, 10 mM of dithiothreitol, and 50 mM of Na_2CO_3 in 0.05 M Tris-HCl buffer (pH=8.0); 0.5 mM of ribulose-1,5-bisphosphate. The enzyme mixture was incubated for 2 min. Protein content did not exceed 50–100 μg. The control enzyme mixture contained the same ingredients except ribulose-1,5-bisphosphate; the ingredients were incubated for 10 min under 30°C.

To stop the reaction, an equal volume of 2 N HCl solution was added to the enzyme mixture. Protein sediment was removed by centrifuging. To get rid of $^{14}CO_2$ excess, supernatant was kept for 30 min under 70°C. Then, 0.025 ml of the sample was put into scintillation liquid (100 ml of SL 108 and 30 ml of ethanol), and radioactivity was measured using the SL-30 liquid scintillation counter (France). Rubisco activity was expressed in μM of $^{14}CO_2$, fixed by 1 mg of protein per 1 min.

Rubisco was separated from the extract using electrophoresis; its content was measured according to Dean & Leech (1982). Disc electrophoresis was carried out in polyacrilamide gel with linear concentration gradient 5 to 15 % (pH=8.9) (1964). To build the calibration curve, 0 to 240 μg of Rubisco from bean leaves was used. The method precision is 20 %.

Pigment content

Pigments were extracted by 80% acetone; their content was measured according to Bolychevtseva et al. (1987).

RESULTS AND DISCUSSION

Effects of NF on CO_2 fixation, TSP and Rubisco content, and Rubisco activity in leaves of 7-day maize, barley, triticale, and radish seedlings under low light intensity (the first set of experiments) are reported in Table 1. Under 0.12 W/m^2, NF treatment intensified CO_2 fixation in all the plants, up to 2 times. TSP content increased in all the plants (1.4–2 times). Rubisco content increased in barley, triticale, and radish (1.2–1.9 times), while substantially decreased in maize (3.6 times). Rubisco activity per its own content considerably increased in maize (2.3 times). At the same time, Rubisco activity per TSP contents decreased in all the plants (1.2 times – in barley, radish, and triticale, 2.3 times – in maize), possibly as a result of NF-activated biosynthesis of other proteins. Under 0.4 W/m^2, NF treatment intensified CO_2 fixation in barley, triticale, and radish, while inhibited it in maize. TSP content decreased in maize, barley, and triticale, while rose in radish (1.3 times). Rubisco content drastically decreased (up to 30 times) in all the plants except radish. The treated maize and barley seedlings showed higher Rubisco activity per its own content (1.6–2 times) compared to control. At the same time, all the plants demonstrated lower Rubisco activity per TSP contents under the treatment.

Effects of NF on TSP and Rubisco content, and Rubisco activity in leaves of 26-day maize, wheat, bean, and radish seedlings under high light intensity (the second set of experiments) are reported in Table 2. There, the same consistent pattern holds, i.e. Rubisco activity per its own content increases (1.5–2 times) while the enzyme content in contrast decreases (1.4–2.7 times). At the same time, Rubisco activity per TSP content did not change significantly. The latter can be explained by indirect effects of NF on the enzyme: 10^{-4} M NF solution did not affect Rubisco activity *in vitro*, which is in line with literature data (Mayfield et al. 1986).

Effects of NF on pigment content in leaves of 7-day maize, barley, and radish seedlings under low light intensity (the first set of experiments) are reported in Table 3. NF treatment reduced pigment content, with more pronounced effect under 0.4 compared to 0.12 W/m^2. The highest NF-resistance under low light intensity (0.12 and 0.4 W/m^2) was observed in sciophytic radish, which demonstrated less decrease in carotenoid and chlorophyll content compared to barley and maize. This can be explained by species peculiarities, e.g. by metabolic pathways (C_3 vs C_4). In radish,

NF treatment under low light intensity (0.12 and 0.4 W/m^2) did not reduce Rubisco content and activity, while did raise CO_2 fixation and TSP content (Table 1). On the other hand, relatively high chlorophyll content together with carotenoid deficiency under NF treatment in radish created preconditions for fast chloroplast photodestruction in a shifting experiment: when radish seedling grown under low light intensity (0.12 W/m^2) were exposed to high light intensity (100 W/m^2), total necrosis of leaves developed within 1 day. This fast photodestruction may be associated with overloading of pigment systems by excitation and consequent structural changes in PSII components (Fedtke 1979). In contrast, heliophilous maize seedlings, although containing less carotenoids and chlorophylls both in control and under NF treatment, did survive in the same shifting experiment, with no significant changes in pigments and TSP content, as well as in Rubisco activity (Table 4).

Earlier, Bolychevtseva et al. (1988) showed that NF treatment does not cause photodestruction of 70S ribosome under relatively low light intensity, although carotenoid and chlorophyll content significantly decreases. Thus, it seemed reasonable to expect no changes in chloroplast proteins biosynthesis. However, NF treatment did result in both TSP and Rubisco content in barley, triticale, and radish under 0.12 W/m^2 (Table 1). The small Rubisco subunit is synthesized in cytoplasm, while the large one – in chloroplasts; one may confer from this that NF activates protein biosynthesis in both cell departments. Under high light in-

Table 1. Effects of norflurazon on TSP and Rubisco content, Rubisco activity, and CO_2 fixation in 7-day maize, barley, triticale, and radish seedlings under low light intensity (the first set of experiments)

Light intensity, W/m^2	Plant	TSP content, mg/g of fresh mass of leaves		Rubisco content, mg/g of fresh mass of leaves		Rubisco activity, units				CO_2 fixation rate, nmol $^{14}CO_2$/min per 1 g of fresh mass of leaves	
						per 1 mg of TSP		per 1 mg of Rubisco			
		Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
0.12	Maize	13.0±0.8	18.0±1.2	1.1±0.2	0.3±0.1	0.31±0.04	0.13±0.10	3.66±1.20	7.80±1.00	20.0±1.0	30.0±1.1
	Barley	7.0±0.9	14.0±1.9	2.2±0.1	3.6±0.2	0.73±0.01	0.62±0.01	2.32±0.02	2.40±0.01	8.0±1.0	16.0±1.4
	Triticale	7.0±1.0	14.0±1.7	4.7±1.1	9.0±1.0	0.73±0.02	0.62±0.01	2.30±0.02	2.40±0.02	8.0±1.4	16.0±0.9
	Radish	21.0±0.7	30.0±1.5	5.0±0.2	6.0±0.2	0.46±0.01	0.38±0.01	1.93±0.01	1.92±0.00	4.0±0.5	6.0±0.6
0.4	Maize	16.0±1.4	11.0±0.7	1.2±0.2	0.1±0.0	0.36±0.06	0.07±0.04	4.00±0.02	7.70±0.02	100.0±4.7	30.0±2.4
	Barley	12.0±0.9	8.0±1.2	3.5±0.5	0.2±0.1	0.65±0.15	0.09±0.01	2.23±0.30	3.60±0.24	10.0±1.4	15.0±1.2
	Triticale	9.8±1.7	7.5±1.0	3.3±0.9	0.1±0.0	0.74±0.20	0.02±0.01	2.20±0.01	2.25±0.00	1.2±0.9	18.0±2.0
	Radish	21.0±1.9	29.0±1.7	4.0±0.0	4.0±0.0	0.40±0.02	0.30±0.01	2.10±0.02	2.20±0.02	6.0±0.9	15.0±1.8

Table 2. Effects of norflurazon on TSP and Rubisco content, and Rubisco activity in 26-day maize, wheat, bean, and radish seedlings under high light intensity (the second set of experiments)

Light intensity, W/m^2	Plant	TSP content, mg/g of fresh mass of leaves		Rubisco content, mg/g of fresh mass of leaves		Rubisco activity, units			
						per 1 mg of TSP		per 1 mg of Rubisco	
		Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
100	Maize	7.6±0.1	8.3±0.1	1.3±0.1	0.9±0.1	0.72±0.02	0.92±0.01	4.21±0.75	8.30±0.90
	Wheat	12.7±0.1	12.2±0.2	5.1±0.3	3.0±0.1	1.10±0.01	0.98± 0.01	2.70±0.17	3.98±0.25
	Bean	16.9±0.2	14.1±0.2	7.7±1.2	2.8±0.2	1.02±0.01	0.84±0.01	2.23±0.03	4.20±0.50
	Radish	20.9±0.5	18.4±0.4	8.4±1.3	4.0±0.9	0.80±0.01	0.78±0.00	2.00±0.20	3.78±0.40

Table 3. Effects of norflurazon on pigment content in 7-day maize, barley, and radish seedlings under low light intensity (the first set of experiments)

Light intensity, W/m^2	Plant	Pigment content, µg/g of fresh mass of leaves					
		Chlorophyll a		Chlorophyll b		Carotenoids	
		Control	Treatment	Control	Treatment	Control	Treatment
0.12	Maize	307.0±3.3	10.0±2.0	55.0±2.4	3.0±0.7	60.0±0.9	2.0±0.3
	Barley	460.0±3.3	87.0±2.7	84.0±2.1	10.0±0.7	122.0±2.7	10.0±0.3
	Radish	812.0±4.4	336.0±6.1	82.0±2.1	14.0±0.6	284.0±2.8	20.0±0.2
0.4	Maize	1091.0±4.1	3.0±0.1	299.0±1.4	3.0±0.3	414.0±2.0	2.0±0.3
	Barley	735.0±6.4	4.0±0.2	157.0±2.7	4.0±0.1	247.0±3.0	1.0±0.1
	Radish	1310.0±3.5	181.0 ±4.6	280.0±1.7	9.0±0.4	292.0±2.1	20.0±0.3

Table 4. Effects of norflurazon on pigments and TSP content, and Rubisco activity in maize seedlings under change in light intensity (shifting experiment)

Light intensity, W/m^2	Pigment content, µg/g of fresh mass of leaves						TSP content, mcg/g of fresh mass of leaves		Rubisco activity per 1 g of TSP, units	
	Chlorophyll a		Chlorophyll b		Carotenoids					
	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
0.4	1248.0±5.6	3.0±0.0	341.0±2.2	3.0±0.4	521.0±3.6	2.0±0.0	15.4±0.3	9.2±0.6	0.52±0.01	0.04±0.00
0.4.+ 1 day of 100.0	1295.0±7.3	3.2±0.0	356.0±2.7	5.4±0.4	550.0±7.8	2.0±0.0	16.6±0.3	11.8±0.6	0.64±0.03	0.05±0.00

tensity (100 W/m^2) photodestruction is more pronounced, regardless of age and regime of treatment and growing, which reduces Rubisco content but simultaneously raises Rubisco activity per its own content (Tables 1, 2).

Comparing radish and maize cases indicate that chloroplast photodestruction under NF is sensitized by chlorophylls due to carotenoid deficiency, which is in line with Bolychevtseva et al. (1988). Possibly, maize and other C_4 plants show decrease in CO_2 usage due to its recycling by phosphoenolpyruvate carboxylase, which biosynthesis is not affected by NF (Bolychevtseva et al. 1987). C_3 plants do not have such mechanism and therefore loose carbon compounds easier compared to C_4 plants, resulting in earlier manifestation of photodestruction.

Thus, light intensity differentially modulates effects of NF on CO_2 fixation and Rubisco activity in C_3 and C_4 plants. In maize, a C_4 plant, Rubisco content decreases under 0.12 W/m^2, despite carotenoid and chlorophyll deficiency. At the same time, it appears more resistant under high light intensity, possibly due to the mechanism for CO_2 recycling. This conclusion is in line with the results of Bolychevtseva et al. (1987, 1988) showing that Rubisco keeps on functioning under severe PSII inhibition caused by NF. Rubisco activity increases under decrease in its content – due to either intermediates formed under photodestruction or activated biosynthesis of some chloroplast enzymes (e.g. Rubisco activase which raises Rubisco affinity to CO_2) (Streusand & Portis 1987).

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