Acta Bot. Croat. 60 (2), 211–218, 2001

CODEN: ABCRA 25
ISSN 0365–0588
UDC 581.3:582.47

Dedicated to Prof. dr. sc. ZVONIMIR DEVIDÉ on the occasion of his 80th birthday

The effect of 2,4-D on the photosynthetic apparatus in cotyledons of spruce (*Picea abies* L. Karst.) seedlings grown in the dark

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Growth regulating substance, 2,4-dichlorophenoxyacetic acid (2,4-D) is widely used in plant cell cultures and also in agriculture as a herbicide. The effect of 2,4-D on photosynthetic pigment content and chloroplast ultrastructure in spruce seedlings grown in the dark was studied. Seedlings were grown for 16 days in the dark at room temperature in Petri dishes moistened either with tap water (control) or 4.5, 45 and 450 μM 2,4-D. The lowest concentration (4.5 μM) of 2,4-D induced both chlorophyll and carotenoid biosynthesis as compared to the control treatment. There were no statistically significant differences in any of the measured parameters between the control and the 45 μM 2,4-D treatment. A herbicidal concentration of 2,4-D (450 μM) induced a decline in chlorophyll content, while carotenoids were not significantly influenced. In contrast to the low concentration treatments, a herbicidal concentration of 2,4-D caused extensive changes in chloroplast shape and ultrastructure.

Key words: *Picea abies*, seedling, pigments, chloroplast, ultrastructure, 2,4-D, herbicide.

Introduction

2,4-dichlorophenoxyacetic acid (2,4-D) is widely used in plant cell culture as a growth regulating substance. It has been reported that 2,4-D induces DNA synthesis during callus formation, and also regulates RNA synthesis by binding to the chromatin proteins (JACOBSEN 1983). It is well known that the auxin level in the culture medium has a strong effect on the level of DNA methylation in tissue explants (LOSCHIAVO et al. 1989, LAMBE et al. 1997). A recent study by LELJAK-LEVANIĆ (2000) showed that 2,4-D increases the level of DNA methylation during the somatic embryogenesis of pumpkin (*Cucurbita pepo* L.).

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In higher concentrations, 2,4-D is used in agriculture and forestry as a herbicide. PAVLICA et al. (1991, 1998) showed that herbicidal effect of 2,4-D on plant cells has been expressed through the induction of chromosome abnormalities and microtubule disruption.

The capability of spruce seedlings to synthesize photosynthetic pigments in the dark (BARTLETT and DODGE 1980, WRISCHER et al. 1998) was used to study the effect of 2,4-D on chlorophyll and carotenoid content and etiochloroplast ultrastructure in cotyledons.

Material and methods

Norway spruce (*Picea* abies L. Karst.) seeds were imbibed in tap water for 24 hours at +4 °C before further procedure. The germination of the seeds was attained on filter-paper underlined with cotton in Petri dishes moistened with tap water (control), 4.5, 45 and 450 μM 2,4-D, respectively. Seedlings were grown for 16 days in the dark at room temperature. The support (cotton and filter-paper moistened with the water and the previously named solutions of 2,4-D) was changed every two days. Experiment was done in duplicate.

For quantitative analysis of photosynthetic pigments, cotyledons from three seedlings were arranged in one sample. Pigments were extracted with ice-cold absolute acetone. The absorbance was measured at 661.6, 644.8 and 470 nm. The concentration of chlorophyll *a*, *b* and total carotenoids was calculated using the absorption coefficients given by LICHTENTHALER (1987). All measurements were done in triplicate. The data attained were subjected to t-test.

For ultrastructural studies, tissue was fixed following the standard procedure (1% glutaraldehyde, postfixation with 1% OsO_4), dehydrated and embedded in Araldite (WRISCHER et al. 1998). Ultrathin sections were contrasted using uranyl acetate and lead citrate, and examined with a Zeiss EM 10 electron microscope.

Results

The mean values of photosynthetic pigments content are given in Figs. 1, 2. The lower concentration (4.5 μM) of 2,4-D induced chlorophyll *a* biosynthesis (Fig. 1A) and the concentration was significantly higher than in cotyledons grown on tap water (Tab. 1). A medium concentration (45 μM) tends to increase chlorophyll *a* content (Fig. 1A), but this was not statistically significant (Tab. 1). The herbicidal concentration of 2,4-D (450 μM) caused a decline in chlorophyll *a* content (Fig. 1A), its concentration being significantly lower than in cotyledons grown on tap water (Tab. 1). The differences measured for chlorophyll *b* content (Fig. 1B) were not statistically significant, considering all the treatments (Tab 1). Since the chlorophyll *a* content was dominant in relation to chlorophyll *b*, the significance in total chlorophyll concentrations (Fig. 2A) showed the same relationship as for chlorophyll *a* (Tab. 1). The lower concentration of 2,4-D (4.5 μM) significantly increased total carotenoid content, while the medium (45 μM) and the highest (450 μM) concentrations did not cause significant changes (Tab. 1, Fig. 2B).

Ultrastructural studies of etiochloroplasts from cotyledons grown on tap water showed a well-developed thylakoid system with distinguished stroma and grana thylakoids, abundant prolamellar bodies and some starch grains (Fig. 3). The etiochloroplasts from cotyle-

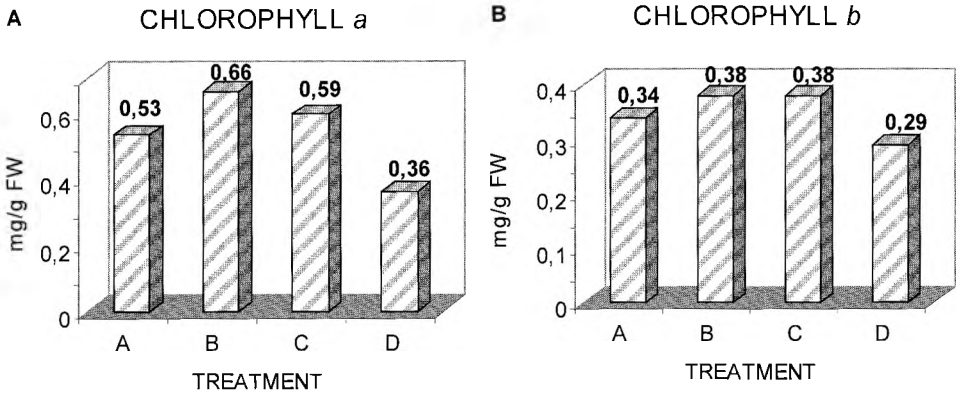

Fig. 1. The average values of chlorophyll *a* (A) and chlorophyll *b* (B) concentrations (in mg/g fresh weight – FW) in cotyledons of spruce seedlings grown in the dark: **A** – tap water; **B** – 4.5 μM 2,4-D; **C** – 45 μM 2,4-D; **D** – 450 μM 2,4-D.

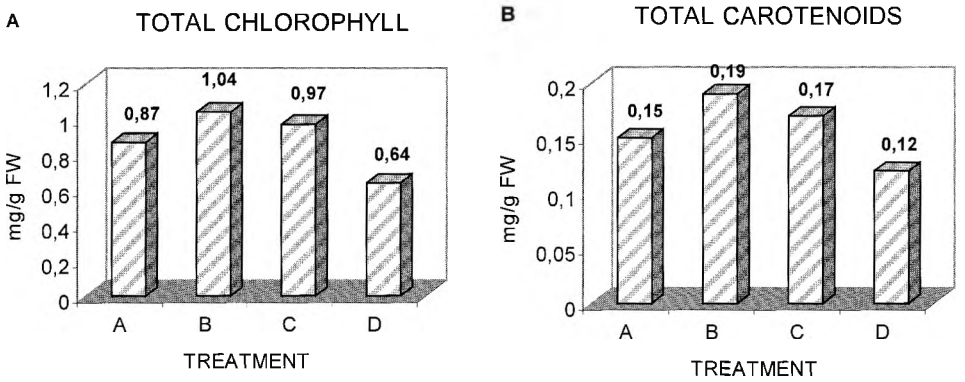

Fig. 2. The average values of total chlorophyll (A) and total carotenoids (B) concentrations (in mg/g fresh weight – FW) in cotyledons of seedlings grown in the dark: **A** – tap water; **B** – 4.5 μM 2,4-D; **C** – 45 μM 2,4-D; **D** – 450 μM 2,4-D.

Table 1. Statistical significances (P(t)<5% – significant, NS – nonsignificant) in photosynthetic pigments (Chl *a* – chlorophyll *a*; Chl *b* – chlorophyll *b*; Chl *a*+*b* – total chlorophyll, Car – total carotenoids) content for treatments: A – tap water; B – 4.5 μM 2,4-D; C – 45 μM 2,4-D; D – 450 μM 2,4-D.

TREATMENT	Chl a	Chl b	Chl a+b	Car
A vs B	P(t)<5%	NS	P(t)<5%	P(t)<5%
A vs C	NS	NS	NS	NS
A vs D	P(t)<5%	NS	P(t)<5%	NS

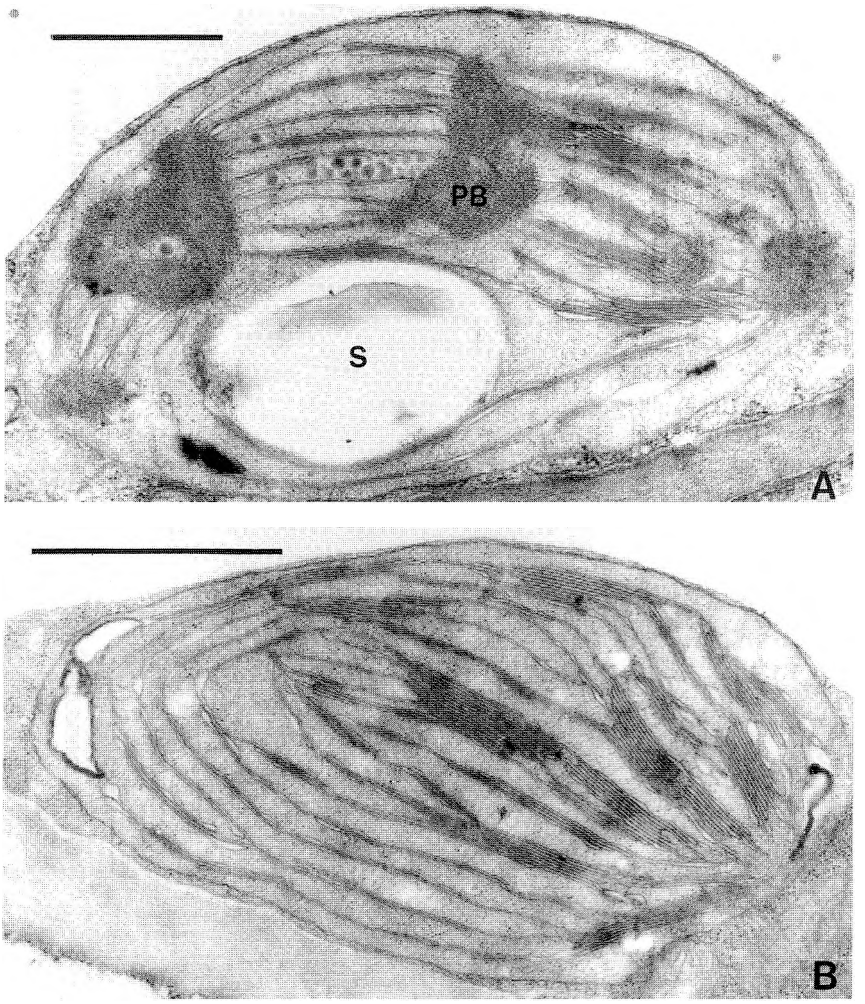

Fig. 3. Etiochloroplasts from tap water grown cotyledons. Bar = 1 μm. **A** – Some starch grains (S) and abundant prolamellar bodies (PB) are visible. **B** – A well developed thylakoid system with distinguished stroma and grana thylakoids was generally present.

dons grown on 4.5 μM 2,4-D were characterized with a well developed thylakoid system and visible prolamellar bodies as in control (Fig. 4A). The concentration of 45 μM 2,4-D caused changes in the shape of etiochloroplasts (Fig. 4B). They became plump and some swelling of thylakoids as well as some starch grains could be noticed. Herbicidal concentration of 2,4-D (450 μM) had influenced both, the chloroplasts shape and ultrastructure (Figs. 4C, D). Chloroplasts were plump, almost roundish with bigger starch grains. The thylakoid system was less developed, the grana system was specially reduced and more swelling was present.

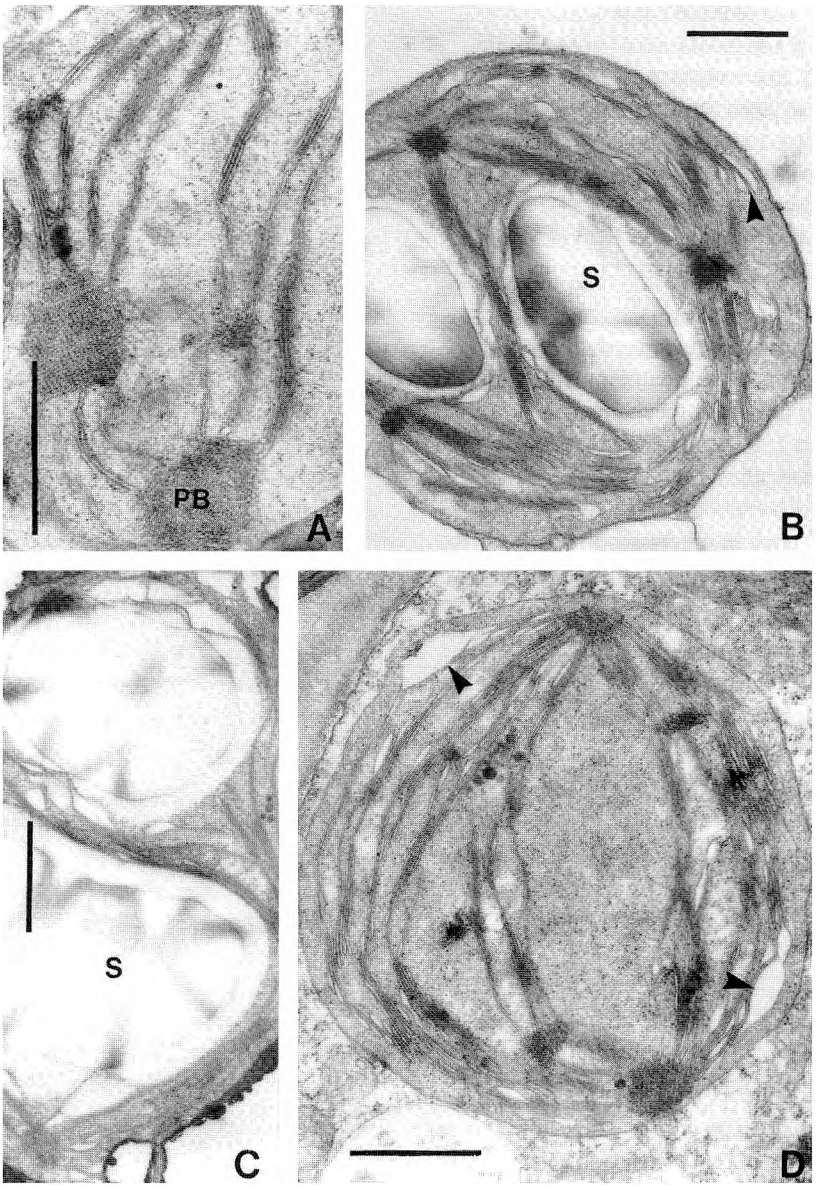

Fig. 4. The effects of 2,4-D on cotyledonary etiochloroplast shape and ultrastructure. Bar = 1μm. **A** – The ultrastructure of etiochloroplasts from cotyledons grown on 4.5 μM 2,4-D was characterized by a well-developed thylakoid system and visible prolamellar bodies (PB) as in control ones. **B** -The concentration of 45 μM 2,4-D caused changes in etiochloroplast shape. They became plump and some thylakoid swelling (arrowhead) could be noticed. Starch grains (S) were present. **C** and **D** -The herbicidal concentration of 450 μM 2,4-D had an increased influence on both chloroplast shape and ultrastructure. Chloroplasts were plump, almost roundish with big starch grains (S) inside. The thylakoid system was less developed and more swelling was present (arrowhead).

Discussion

The measurement of photosynthetic pigment content showed that some chlorophyll was present in the cotyledons of untreated spruce seedlings grown in the dark (Figs. 1, 2). Although a number of enzymes are involved in chlorophyll biosynthesis (von WETTSTEIN et al. 1995, SUZUKI et al. 1997), the critical point seems to be the reduction of protochlorophyllide to chlorophyllide. In contrast to angiosperms, gymnosperm seedlings utilize the light-independent NADPH-protochlorophyllide oxidoreductase (POR), which makes them capable of chlorophyll biosynthesis in the dark (BOGDANOVIĆ 1973, BARTLETT and DODGE 1980, FORREITER and APEL 1993, WRISCHER et al. 1998).

Significantly increased chlorophyll *a*, *b* and total carotenoids were observed in the cotyledons upon the treatment with the lowest concentration of 2,4-D (4.5 μM) (Tab. 1, Fig. 2). The proposed mechanism of auxin action on gene expression (JACOBSEN 1983, MACDONALD 1997) suggested that application of 2,4-D in low concentrations should stimulate protein synthesis and thus enhanced photosynthetic pigments biosynthesis as well. Such an effect is present only in a specific concentration range, which was manifested by a loss of significance in chlorophylls and carotenoid content (Tab. 1) as compared to the medium 2,4-D concentration (45 μM) with tap water treatment.

It is proposed that chlorophyll *b* is synthesized by an oxygenase enzyme that directly modifies chlorophyll *a* to chlorophyll *b via* a hydroxymethyl intermediate (von WETTSTEIN et al. 1995, SUZUKI et al. 1997). Since no significant differences in chlorophyll *b* content were detected for all treatments (Tab. 1) it is quite certain that 2,4-D had no influence on such oxygenase activity.

Two different ways of chlorophyll degradation in green tissues are suggested (HEATON et al. 1996, LOUDA et al. 1998). The degreening enzymes included in those processes were characterized as chlorophyllases and peroxidases (HUFF 1982, JOHNSON-FLANAGAN and MCLACHLAN 1990, DRAZKIEZWICZ and KRUPA 1991, JOHNSON-FLANAGAN and SPENCER 1996). The highest concentration of 2,4-D (450 μM) induced a significant decrease of chlorophyll *a* and total chlorophyll content (Tab. 1, Figs. 1, 2). It is possible that the induction of ethylene biosynthesis could be achieved through the action of the herbicidal concentration of 2,4-D (JACOBSEN 1983, DRAZKIEWICZ 1994). Ethylene in turn increases peroxidase (INGEMARSSON 1994) and chlorophyllase activity (DRAZKIEWICZ 1994) and in that way triggers chlorophyll degradation.

An interesting observation from this study was the fact that total carotenoid content was not significantly influenced (Tab. 1) by the action of the highest 2,4-D concentration (450 μM). The role of carotenoids in protecting the photosynthetic membranes from destruction is well established (SIEFERMANN-HARMS 1987). Accordingly, we could speculate that carotenoid presence in the etiochloroplasts of dark-grown spruce seedlings prevented more dramatic functional and structural changes upon treatment with herbicidal concentration of 2,4-D.

The ultrastructural studies (Figs. 3, 4) showed that the herbicidal concentration of 2,4-D had a damaging effect on thylakoid membranes as well as pronounced effect on starch synthesis. SEGURA-AGUILAR et al. (1995) demonstrated a similar effect on thylakoid membranes upon the action of a herbicidal concentration of 2,4,5-trichlorophenoxyacetic acid

(2,4,5-T). In their investigation it was shown that the reduction in the number and dimensions of grana thylakoids was pronounced in some chloroplasts to such a level that it was no longer possible to distinguish grana. A strong reduction of thylakoid number was also observed in cotyledons of spruce seedlings grown in the dark treated with herbicide norflurazon (WRISCHER et al. 1998). The occurrence of large starch grains inside the etiochloroplasts (Fig. 4C) further indicated stress situation. A similar effect could be seen in chloroplasts of damaged spruce needles affected by different abiotic factors (FINK 1989, 1993, 1999).

We could conclude that the impact of 2,4-D on the photosynthetic pigment content was stimulative in the low concentration range, but also caused a decline in chlorophyll content and changes in chloroplast ultrastructure when applied in a herbicidal concentration.

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