BRIEF COMMUNICATION

Chlorophyll accumulation in black pine seedlings treated with 5-aminolevulinic acid

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Abstract

5-Aminolevulinic acid (10^{-5} M), a key precursor in chlorophyll biosynthetic pathway, stimulated chlorophyll accumulation in the dark grown black pine (*Pinus nigra* Arn.) seedlings and did not affect chlorophyll accumulation in the light.

Additional key words: embryo, *Pinus nigra*.

The ability of gymnosperm cotyledons to synthesise chlorophyll (Chl) in the dark offers a useful system for investigation of the regulatory mechanisms of Chl biosynthesis. Our previous studies show that the greening of black pine (*Pinus nigra* Arn.) cotyledons in the dark requires a contact with megagametophyte or exogenous cytokinin (Jelić and Bogdanović 1988, 1990).

We assumed that in gymnosperms synthesis of 5-aminolevulinic acid (5-ALA) is rate-limiting step in the biosynthetic pathway of Chl similarly as already shown for bacteria and any up to now investigated angiosperms (Castelfranco and Beale 1983, Kumar *et al.* 1996). We tested this assumption in black pine seedlings.

Pine seeds were imbibed in water during 24 h in the dark, and cultivated in Petri dishes in the light (irradiance of 220 μmol m^{-2} s^{-1}, powertube *72* cool white *1F72T12-CW-UHO, Sylvania* and incandescent bulb 25 W) or in the dark at 24 °C in distilled water (control plants) or in 10^{-6} to 10^{-2} M 5-ALA solution (ALA-fed plants). All manipulations of seedlings were performed in the dark or under a green safe irradiation (a photolamp with a 25 W bulb covered with a dark-green filter *Fotokemika TZ 35*). Chl was extracted in dimethylformamide and estimated

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spectrophotometrically according to Moran (1982). Results are mean values of five replications ± SD. Statistical evaluation of all measurements was made using Student's t-test.

Cotyledons of black pine grown in the dark synthesise Chl much slower than in the light. When the seeds germinated in the presence of exogenous 5-ALA, Chl accumulation was accelerated only in the dark, but these seedlings did not reach Chl concentration found in seedlings of corresponding age grown in the light (Table 1).

Table 1. Chlorophyll content [μg $embryo^{-1}$] in black pine embryos during germination in the light and in the dark. Means of five experiments ± SD. * - $P < 0.05$, ** - $P < 0.01$.

Germination [d]	Light control plants	ALA-fed plants	Dark control plants	ALA-fed plants
3	0.02 ± 0.00	0.02 ± 0.01	traces	traces
5	0.80 ± 0.07	0.09 ± 0.01	0.02 ± 0.00	0.06 ± 0.01 **
7	1.50 ± 0.09	1.47 ± 0.13	0.80 ± 0.00	0.14 ± 0.00 **
9	5.20 ± 0.96	4.64 ± 0.36	0.98 ± 0.02	1.43 ± 0.09 **
11	24.50 ± 1.32	18.40 ± 1.94	2.20 ± 0.11	3.47 ± 0.34 **
15	90.00 ± 7.89	67.32 ± 8.70	4.20 ± 0.25	5.22 ± 0.17 **

Seedlings grown in the light in the presence of exogenous 5-ALA in the early stages of development synthesised Chl at the same rate as the controls, and later on somewhat slower, although the differences were not statistically significant. Chl content in 5-ALA-fed black pine seedlings grown in the dark was concentration-dependent. Low concentration of 5-ALA stimulated Chl accumulation while higher concentrations inhibited it. The highest effect was on 10^{-5} M 5-ALA concentration (Table 2).

Table 2. The effect of different 5-ALA concentrations on chlorophyll (Chl) [μg $embryo^{-1}$] accumulation in embryos of black pine grown 7 d in the dark. Means of five experiments ± SD.

5-ALA concentration	Chl a+b	Effect	P
0	0.80 ± 0.04		
10^{-6} M	1.20 ± 0.08	stimulation	< 0.01
10^{-5} M	1.34 ± 0.10	stimulation	< 0.01
10^{-4} M	0.65 ± 0.05	inhibition	< 0.05
10^{-3} M	0.60 ± 0.04	inhibition	< 0.01
10^{-2} M	0.46 ± 0.02	inhibition	< 0.01

The results indicate that gymnosperm seedlings develop different mechanisms of regulation of Chl biosynthesis in the light and in the dark. In the light, exogenous 5-ALA does not have an expressive influence on Chl accumulation, which may be explained by optimal synthesis of the endogenous 5-ALA. In that case, regulation of greening would be the same as in angiosperms: in dark-light grown plants and in ALA-feeding experiments, ALA formation and protochlorophyllide accumulation showed an inverse correlation, may be due to protochlorophyllide regulation of ALA synthesis by a feedback mechanism (Beale and Castelfranco 1974).

When the seedlings green in the dark, exogenous 5-ALA in concentrations between 10^{-5} and 10^{-7} M (which are similar to the concentrations of endogenous 5-ALA in angiosperm seedlings after incubation with levulinic acid - Averina and Yaronskaya 1991, Kruse *et al.* 1997), stimulates Chl accumulation, probably due to compensation of insufficient production of endogenous 5-ALA. Higher 5-ALA concentrations decrease the accumulation rate of Chl in the dark which is in accordance with the results obtained for *Pinus jeffreyi* (Wolvertz and Brouers 1980) indicating that in the dark at 23 °C ALA-protochlorophyllide cannot be reduced into chlorophyll(ide) and that presence of ALA-protochlorophyllide inhibits any new Chl synthesis.

The available data on the influence of exogenous 5-ALA on Chl biosynthetic pathway are mostly concerned with angiosperms and are obtained under experimental conditions different from ours (comparatively short incubation in the presence of 5 mM 5-ALA in the dark) (Averina *et al.* 1996, Virgin 1996, Walter and Shalygo 1997). Angiosperms treated in darkness with 5-ALA accumulate exceptionally high amounts of Chl precursors, especially protoporphyrin IX, Mg-protoporphyrin-IX-monomethyl ester and protochlorophyllide, which act as photodynamic pigments and induce a generation of singlet oxygen after illumination followed by photodestruction of cell membranes (Toneva *et al.* 1997a). The level of this destruction depends most probably on the amount of accumulated tetrapyroles, the greening group of plants, as well as the chemical nature of the precursors (Toneva *et al.* 1997b).

The influence of 5-ALA on Chl synthesis in dark grown black pine cotyledons, may be interpreted by some not sufficiently investigated action of this molecule which is a consequence of its participation in a large number of metabolic pathways (Avissar and Moberg 1995).

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