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Effect of Cytokinins on Photosynthetic Pigments and Chlorophyllase Activity in in Vitro Cultures of Axillary Buds of *Dianthus caryophyllus* L.

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Abstract. The effect of benzyladenine (BA) and two phenylurea cytokinins, *N*-phenyl-*N′*-(2-chloro-4-pyridyl)urea (4-PU-30) and thidiazuron (TDZ), on the growth, photosynthetic pigment content, and activity of chlorophyllase (chlorophyll-chlorophylliodhydrolase, EC 3.1.1.14) of in vitro cultures of carnations was studied. All cytokinins caused a rise in the fresh weigth and a drop in the dry weight of leaf mass produced by the explanted buds. Both 4-PU-30 and TDZ increased the chlorophyll content and this correlated with changes in chlorophyllase activity. The effect of 4-PU-30 and TDZ was similar to that caused by BA but at 10-fold or 100-fold lower concentrations. The application of higher concentrations of the phenylurea cytokinins caused an increase in the chlorophyll *a*/chlorophyll *b* ratio. However, at equimolar concentrations, the purine and both phenylurea cytokinins had opposite effects, probably indirect and related to some malformations caused by phenylureas. 4-PU-30 increased, but TDZ decreased, photosynthetic membrane stability, which argues for a different molecular organization of the chloroplast membranes.

Key Words. Cytokinin—Chlorophyllase—*Dianthus caryophyllus*—Photosynthetic pigments

Cytokinins are known to affect photosynthesis and to stimulate chloroplast biogenesis and chlorophyll biosynthesis, promoting the synthesis of RuBPCase, the differentiation of chloroplasts, and the expression of genes encoding the small subunit of RuBPCase and the light-harvesting chlorophyll *a*/*b*-binding proteins (Adedipe et al. 1971; Parthier 1989; Szweykowska 1992; Tetley and Thimann 1974).

Some synthetic phenylurea cytokinins were found to be more active than the purine cytokinins (Mok et al. 1982; Okamoto et al. 1978; Takahashi et al. 1978). Thidiazuron (TDZ) and *N*-phenyl-N^1-(2-chloro-4-pyridyl)urea (4-PU-30) have been reported to have high cytokinin activity at low concentrations (Karanov et al. 1992). Their cytokinin-like effects on several species include the promotion of growth and the stimulation of in vitro shoot proliferation (Fellman et al. 1987).

In our earlier work we showed that both TDZ and 4-PU-30 possessed higher activity than BA in relation to the micropropagation of carnations. At 0.4 μM, these phenylurea cytokinins caused not only a higher rate of multiplication but also abnormal growth and malformations. However, at concentrations 10 and 100 times lower, they were also effective without inducing side effects (Genkov and Ivanova 1995).

Using the same model system, we have studied the influence of BA, 4-PU-30, and TDZ on the growth, pigment content, and chlorophyllase activity (in acetone leaf powders and by examination of the resistance of chlorophyll to hydrolysis during the autolysis of isolated chloroplasts).

Materials and Methods

Axillary buds plus a small part of the adjacent tissues from the stem of virus-free plants of *Dianthus caryophyllus* cv. Red Lena were cultured on solid Murashige and Skoog medium (1962) which contained 0.8% BactoAgar. All media contained NAA at 0.5 μM and BA, 4-PU-30, or TDZ at the micromolar concentrations shown in the figures. Cultures were grown at 24–25°C for a 16-h photoperiod, provided by cool-white fluorescent tubes giving approximately 54 μmol $m^{-2}s^{-1}$.

Forty-day-old in vitro cultures of the axillary buds were used. About

Abbreviations: RuBPCase, ribulose-1,5-bisphosphate carboxylase; TDZ, *N*-phenyl-*N′*-1,2,3-thidiazol-5-ylurea (thidiazuron); 4-PU-30, *N*-phenyl-N^1-(2-chloro-4-pyridyl(urea); BA, benzyladenine; NAA, naphthaleneacetic acid; Chl, chlorophyll.

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40 such explanted buds per variant were treated, and only the leaves were used for measurements. Pigment content was determined by the method of Arnon (1949). Chlorophyllase activity was measured in acetone leaf powders, obtained after the extraction of endogenous Chl (Holden 1961; Sudyina et al. 1972). Enzyme activity during the autolysis of isolated chloroplasts was measured according to the method of Amir-Shapira et al. (1986). Chloroplasts were isolated in 0.1 M sodium phosphate buffer, pH 7.8, containing 0.4 M sucrose. After an osmotic shock, obtained by suspending the chloroplasts in the same buffer but without sucrose for 30 min at 4°C, Triton X-100 was added to the suspension at a final concentration of 0.4% (v/v). Autolytic destruction of chloroplast membranes was performed at 30°C with constant shaking of the chloroplast suspension in the detergent solution. After filtration, the filtrate was partitioned against petroleum ether, acetone, and 0.2 N KOH (6:4:1, v/v/v). The Chl, retained in the upper phase, was estimated at intervals of 1, 3, 20, and 48 h. Chlorophyllase activity was calculated as the percentage of the Chl destroyed.

The results were analyzed statistically using Fisher's criteria.

Results and Discussion

The results showed that the cytokinins used caused an increase in the fresh weight and a decrease in the dry weight of the leaf mass produced by the explanted buds (Fig. 1). The higher fresh weight correlated with the lower dry weight content. This is one of the features characterizing the vitrification caused by cytokinins in the medium during the micropropagation of carnations and other plant species (Leshem et al. 1988, Pasqualetto et al. 1986). *Vitrification* is the term generally used to describe the abnormal plant morphology of herbaceous and woody plants during their in vitro vegetative propagation (Gaspar 1991).

Both phenylurea cytokinins, depending on the concentrations applied, either stimulated or inhibited photosynthetic pigment content, but the effect of 4-PU-30 was more pronounced (Fig. 2). However, at concentrations equimolar to that of BA, 0.4 μM 4-PU-30 and TDZ decreased the pigment content. Moreover, the explants developed many short stems with shortened internodia. These plants had a higher multiplication coefficient but an altered morphology (a frutex-like form) and, to some extent, visible signs of vitrification.

The three cytokinins studied changed the ratio between the two kinds of Chl. Comparing their effects in equimolar concentrations, it was observed that the Chl *a*/Chl *b* ratio in 4-PU-30-treated material was almost equal to this ratio in BA-treated material but significantly lower than that after TDZ application. The effect of 4-PU-30 at a concentration of 4.0 μM corresponded to that achieved by TDZ at a concentration 10 times lower. The increase in the Chl *a*/Chl *b* ratio is the result of a decrease in the amount of Chl *b* produced in response to 4-PU-30 and TDZ treatments at the highest concentrations applied (4.0 μM for 4-PU-30 and 0.4 μM for TDZ). This could be a sign of some disruption in the photosynthetic apparatus and, in particular, of a reduction in the abundance of the

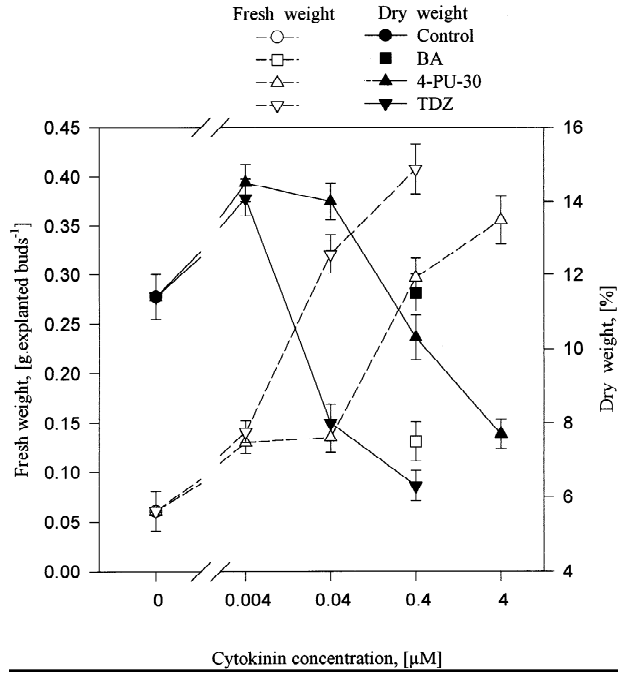

Fig. 1. Effect of cytokinins on the fresh and dry weight of leaves from cultivated carnation axillary buds. Statistical analysis data: LSD 5% for both parameters.

light-harvesting complex relative to the reaction centers in leaves with a decreased pigment content. However, other authors have suggested that the inversion in the Chl *a*/Chl *b* ratio may be because of the phenomenon of vitrification (Crevecoeur et al. 1987; Lavee and Messer 1969). Our data did not show such a drastic aberration. This discrepancy may be explained by the fact that there is no complete resemblance between the effect of cytokinins and the vitrification caused by environmental factors such as an increased humidity in the cultural glassware, and as a consequence of it, an abnormal function of the stomatal apparatus (Ziv and Ariel 1994).

The effects on Chl content (Fig. 2) which we noted may be because of reduced Chl destruction, increased Chl synthesis, or a combination of both. The enzyme(s) responsible for Chl destruction per se has not been determined, and the existing evidence is circumstantial (Drazkiewicz and Krupa 1991). Two enzymes have been shown to be capable of destorying Chl in vitro. Chlorophyllase, which cleaves the phytol chain with the production of free phytol and chlorophyllide, has been demonstrated (Holden 1961). Peroxidative degradation has been proposed as an alternative system (Martinoia et al. 1982). We have shown previously that TDZ and 4-PU-30 increased both superoxide dismutase and peroxidase activity significantly (soluble, ionically bound, covalently bound) in carnations cv. White Sim and Red Lena, the

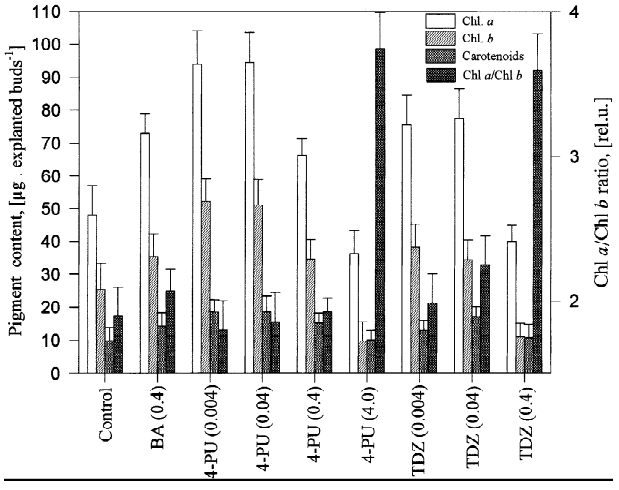

Fig. 2. Effect of cytokinins on the pigment content and Chl *a*/Chl *b* ratio in leaves from cultivated carnation axillary buds. Statistical analysis data: LSD 5% for all parameters.

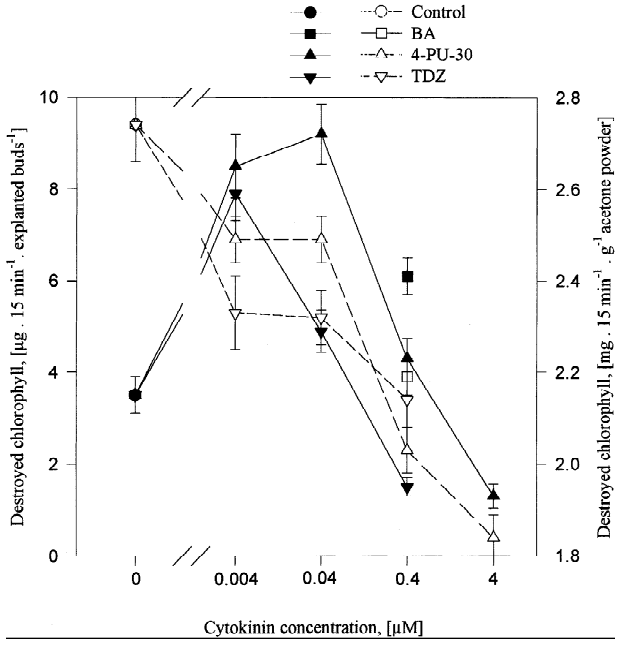

Fig. 3. Effect of cytokinins on the chlorophyllase activity in leaves from cultivated carnation axillary buds, expressed as destroyed Chl per plant (*black symbols*) and per g of acetone powder (*white symbols*). Statistical analysis data: LSD 5% for both parameters.

effect being concentration dependent (Genkov and Ivanova 1995). So, in this study, we examined the effect of the cytokinins on chlorophyllase activity. When chlorophyllase activity was expressed as mg of Chl degraded/mg of acetone powder/15 min (Fig. 3), it was observed that the lowest enzyme activity corresponded to the highest Chl amount (Fig. 2). By this action, cytokinins may retard the catabolism of the cell and cause a maintenance of the Chl content. Our results support the data of Sabater and Rodriguez (1978), Purohit and Chandra (1980), and Todorov et al. (1992) who demonstrated an inhibition of chlorophyll destruction in barley or maize leaves because of inhibited chlorophyllase activity. However, the accessibility of the enzyme and the substrate must also be taken into account as a part of the mode of action. Therefore, when chlorophyllase activity was expressed as the Chl amount degraded/plant/15 min, the changes observed were similar to those of the Chl content (Fig. 3). Probably such a high enzyme activity after 4-PU-30 and TDZ application (0.4 and 0.004 µM) could be caused by degradation of the photosynthetic membranes that contain both Chl and chlorophyllase.

However, as Amir-Shapira et al. (1986) and Sudyina et al. (1988) emphasize, the in vivo destruction of Chl by chlorophyllase depends not only on the quantitative enzyme-substrate relationships but also on the conditions of the photosystems and the light-harvesting complex, and their organizational integrity. To determine the physiologic effect of the three cytokinins on the stability of the photosynthetic membranes, an autolytic destruction of the photosynthetic pigments in isolated chloroplasts was carried out (Fig. 4). The highest resistance of the chloroplast membranes to autolysis was observed in bud leaves treated with 4-PU-30 at a concentration of 0.04 µM. However, TDZ (in both concentrations applied) and BA lowered the membrane resistance. Nevertheless, the compounds studied increased Chl hydrolysis, although its time course effects were different. The Chl destroyed in TDZ-treated material increased 1 h after the beginning of the experiment but during the last 28 h remained almost constant. The effect of BA was pronounced by the 3rd h, whereas the level of the destroyed Chl in 4-PU-30-treated material was influenced significantly later.

During the formation of the photosynthetic membranes, there is a specificity in the effect of cytokinins, which becomes apparent in comparing the differences in resistance to destruction by autolysis. The destruction is very strong in TDZ-treated material, weaker in BA-treated material, and the weakest in one that is 4-PU-30 treated. Significant differences between TDZ- and 4-PU-30-treated material, which argues for a different molecular organization of the chloroplast photosynthetic membranes, may indicate a different mode of action. However, additional investigations are needed to clarify these problems in detail.

The results presented in this paper give some additional information about the mode of physiologic action of the phenylurea cytokinins. Moreover, the effects observed on the growth of in vitro cultured carnation axillary buds are of great practical importance and may lead to gains in efficiency and precision in the use of external cytokinins.

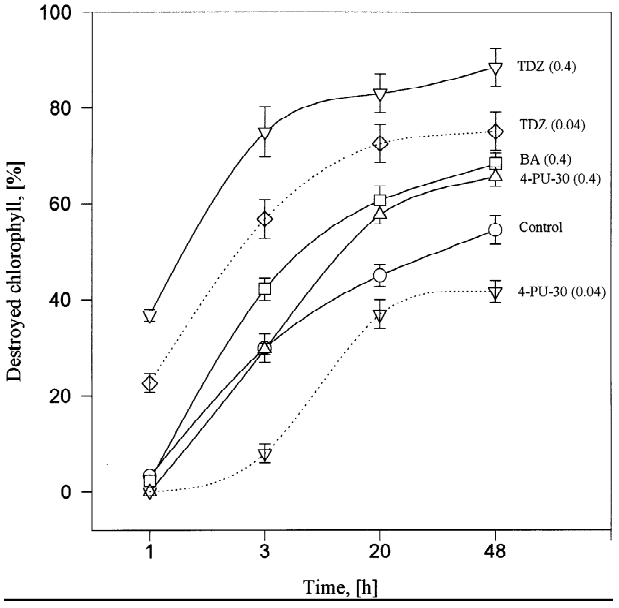

Fig. 4. Hydrolytic destruction of Chl during autolysis of isolated chloroplasts from leaves of cultivated carnation axillary buds. Statistical analysis data: LSD 5%.

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