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Effect of cytokinins and cytokinin antagonists on *in vitro* cultured *Gypsophila paniculata* L.

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Abstract

This study deals with the effects of two cytokinins [kinetin (Kin) and N-(2-chloro-4-pyridyl)-N-phenylurea (CPPU)] and cytokinin antagonists [2-chloro-4-cyclobutyl-amino-6-ethylamino-1,3,5-triazine (ACK1) and N-(4-pyridyl)-O-(4-chloro-phenyl)carbamate (ACK2)] in concentration of 1 μM on *in vitro* cultured *Gypsophila*. The application of Kin and CPPU stimulated bud opening and increased fresh and dry masses. Cytokinin antagonists reduced the number of sprouted buds and bud fresh and dry masses. In plants treated with CPPU the chloroplasts possessed well developed membrane system, which covered almost the entire chloroplasts volume. In ACK2 treated plants, the plastid apparatus in each cell was represented by two types of chloroplast in which the inner membrane system was differently organized. Cell wall adjacent chloroplasts possessed structure similar to the controls. In inner located chloroplasts part of thylakoids were semi-concentrically arranged and partially destructed.

Additional key words: anticytokinins, axillary buds, chlorophyll *a+b*, chloroplast ultrastructure, *in vitro* propagation.

Introduction

Plant micro-propagation is often based on the apical or lateral bud formation and their growth into shoots. Type and concentration of plant growth regulators play a major role in cell division, differentiation, and morphogenesis in plant tissue cultures. Under *in vitro* conditions, the lateral or axillary buds can be activated by adding cytokinins to the culture medium. In addition to promoting bud break by reducing the dominance of the apical bud, cytokinins delay senescence, stimulate chloroplast development and nutrient metabolism, and enhance the plants resistance to various stresses (Kamínek 1992, McGaw and Birch 1995). There are more reports on *in vitro* response to 6-benzyladenine (BA) and kinetin (Kin), but a few are available about the application of phenylurea cytokinins. Phenylurea cytokinins have been more active and have produced a better effect in earlier test systems (Takahashi *et al.* 1978, Iwamura *et al.* 1979, 1980, Mok *et al.* 1982, Shudo 1994). Enhanced outgrowth of axillary buds including shoot proliferation and parthenocarpy has been observed in apple, grapevine and rose *in vitro* culture medium with thidiazuron or CPPU (Niewkera *et al.* 1986, Fellman *et al.* 1987, Gribaudo and Fronda 1991, Sugiyama *et al.* 1993, Yu 1999, Kapchina-Toteva *et al.* 2000). Despite cytokinins importance, little is known about the mechanism of their action. One of the instruments for studying cytokinin mode of action is the application of cytokinin antagonists (anticytokinins). These compounds competitively inhibit cytokinin action and their effects are specific and reversible. Triazine and carbamate derivatives used in our experiments have been characterized as cytokinin antagonists in tobacco callus bioassay (Shimizu *et al.* 1989, 1990), in *Dactylis* leaf explants (Somleva *et al.* 1995, 2000), in *Rose* single nodes (Kapchina-Toteva *et al.* 2002) and in some other test systems (Sergiev 1999).

The aim of present study was to investigate the influence of two types of cytokinins and cytokinin antagonists on the bud break, outgrowth of single nodes and ultrastructure of the chloroplasts of *in vitro* cultured *Gypsophila paniculata* L.

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Abbreviations: ACK1 - 2-chloro-4-cyclobutyl-amino-6-ethylamino-1,3,5-triazine; ACK2 - N-(4-pyridyl)-O-(4-chlorophenyl) carbamate; BA - 6-benzyladenine; CPPU - N-(2-chloro-4-pyridyl)-N-phenylurea, Kin - kinetin (N-6-furfurylaminopurine).

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Materials and methods

Gypsophila paniculata L. cv. Bristol Fairy stock plants were maintained and subcultured *in vitro* every 5 weeks on a standard full-strength medium (MS - Murashige and Skoog 1962), supplemented with 2.0 % (m/v) sucrose and 8 g dm^{-3} agar. Growth conditions were: temperature 22 °C and a 16-h photoperiod (photosynthetic photon flux density 60 μmol m^{-2} s^{-1}, white fluorescent lamps).

Axillary buds from the 3rd and 4th position with small piece of stem (single nodes) were transferred onto standard MS medium supplemented with 1 μM cytokinins: Kin (a cytokinin of the purine type), and CPPU (a cytokinin of phenylurea type) and cytokinin antagonists (anticytokinins): ACK1 (triazine type) and ACK2 (carbamate type), kindly provided by Prof. H. Iwamura from Kyoto University, Japan.

Bud sprouting, outgrowth of shoots and ultrastructure of the chloroplasts were determined using 30-d-old *in vitro* cultured plants. Bud sprouting was calculated as a percentage of open buds to the initial number of single nodes (n = 30). The data reported are the mean values obtained from 3 experiments in 10 replications each. Content of chlorophyll *a* and *b* was determined in 80 % (m/v) acetone extract according to Lichtenthaler (1987).

The samples for electron microscopy were taken from the middle part of lamina of the 4th leaf, fixed in 3 % (m/v) glutaraldehyde in phosphate buffer (pH 7.4) for 12 h at 4 °C and postfixed in 2 % (m/v) $KMnO_4$ for 4 h at room temperature. After dehydration the material was embedded in *Durcupan (Fluka*, Swizerland) and cut with *Tesla* (Prague, Czech Republic) ultramicrotome. Observations were carried out with *JEOL 1200 EX* (Tokyo, Japan) electron microscope.

Results

The application of Kin and CPPU stimulated bud opening in comparison to the control. When 1 μM CPPU was applied the percentage of open buds was greater than at the treatment with 1 μM Kin (Table 1). The cytokinin antagonists applied at the same concentration decreased the number of sprouted buds. Although both anticytokinins differ in their chemical structure they had similar effect on the lateral bud break - a decrease in the number of open buds. The fresh and dry masses accumulation of shoots was accelerated during culture on medium with either CPPU or kinetin (Table 1). Higher fresh mass was measured in response to CPPU. Dry mass was increased both by Kin and CPPU without significant differences. When 1 μM ACK1 or 1 μM ACK2 were applied, they reduced the fresh mass and dry mass of explants.

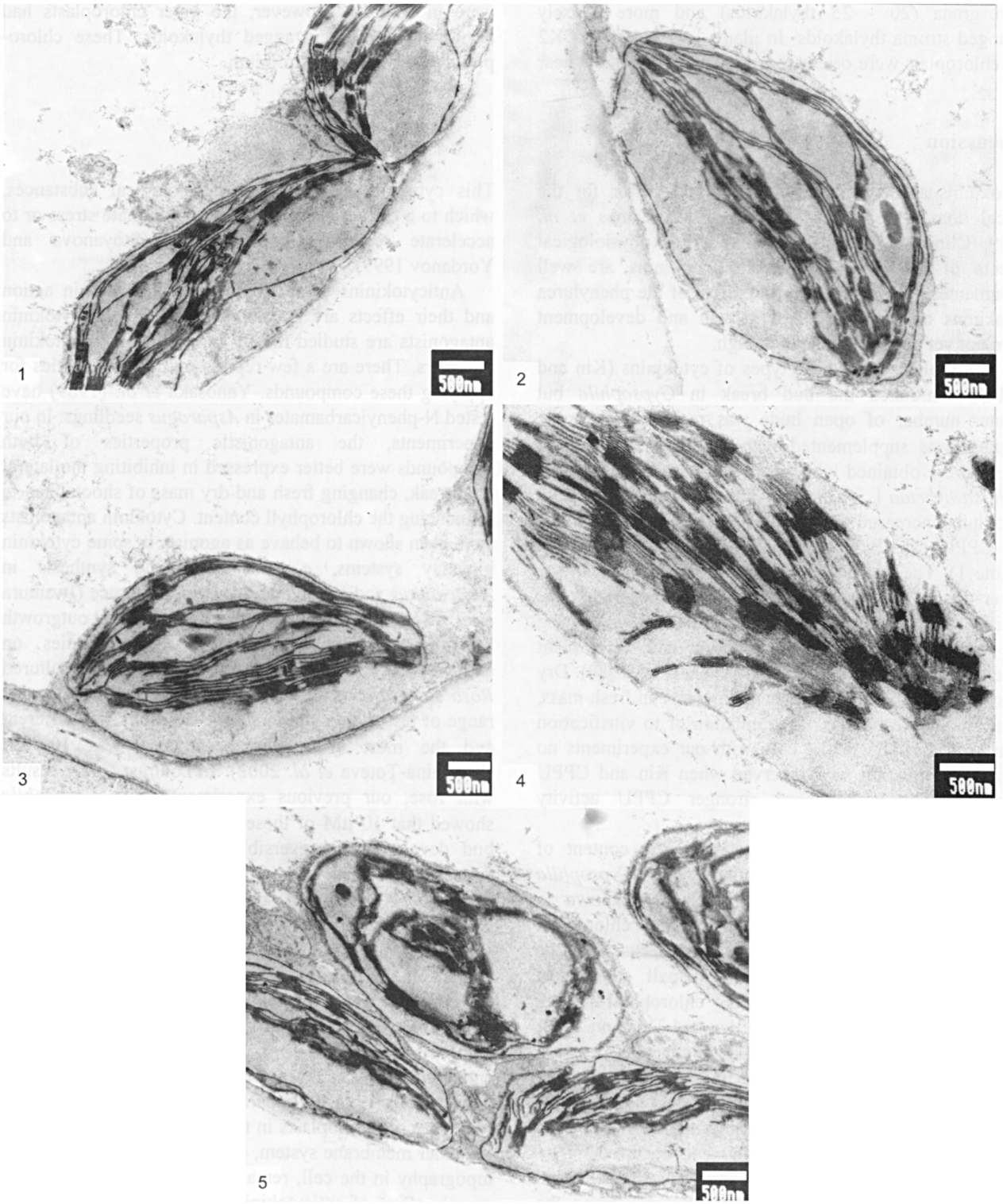
Chlorophyll (a+b) content in *G. paniculata* L. shoots decreased significantly in the presence of cytokinins and especially of CPPU and this was due to reduction of both chlorophyll *a* and *b* contents (Table 1). In contrast, the chlorophyll content of anticytokinin-treated single nodes, especially with 1 μM ACK2 was higher than in the control. The chlorophyll *a*/*b* ratio was not influenced by the cytokinin and cytokinin antagonists treatment.

The mesophyll chloroplasts in the 4th leaf of 30-d-old plants had a well-developed inner membrane system - grana and stroma thylakoids (Fig. 1). The grana were of different height (from 4 to 15 thylakoids) and were linked by comparatively long stroma thylakoids. Each chloroplast possessed a well developed peristromium (periferically structured stroma) adjacent to the cell wall. The chloroplasts and other organelles were localized in a relatively thin layer adjacent to the cell wall.

Structural organization of plastid apparatus in plants treated with Kin (Fig. 2) and ACK1 (Fig. 3) could not be distinguished from the one found in plants without plant growth regulators. In plants treated with CPPU the chloroplasts had even better developed membrane system,

Table 1. Effect of cytokinins (1 μM kinetin and 1 μM CPPU) and anticytokinins (1 μM ACK1 and 1 μM ACK2) on *in vitro* cultured *Gypsophila paniculata* plants. Sprouting is calculated as a percentage of open buds to the initial number of single nodes (n = 30). Chl - chlorophyll. Values significantly different from the control are indicated by * ($P \leq 0.05$) and ** ($P \leq 0.01$).

Variants	Sprouting [%]	Fresh mass [mg $plant^{-1}$]	Dry mass [mg $plant^{-1}$]	Chl (a+b) [μg g^{-1} f.m.]
Control	40.23 ± 0.6	233.9 ± 24	22.5 ± 0.6	567.0 ± 34
Kin	57.10 ± 0.3*	541.3 ± 33**	43.2 ± 0.7*	451.8 ± 24*
CPPU	70.43 ± 0.7**	1159.2 ± 42**	85.7 ± 0.4*	276.3 ± 14*
ACK1	24.80 ± 0.4*	156.4 ± 20*	13.7 ± 0.3*	600.9 ± 30
ACK2	30.25 ± 0.2*	154.4 ± 15*	15.4 ± 0.4*	673.1 ± 24*



Figs. 1 - 5. Ultrastructure of chloroplasts of 4th leaf of 30-d-old *Gypsophila* plants: control (Fig. 1), treated with 1 μM kinetin (Fig. 2), 1 μM ACK1 (Fig. 3), 1 μM CPPU (Fig. 4), or 1 μM ACK2 (Fig. 5).

encompassing almost the entire chloroplast volume (Fig. 4). It consisted of a large number of comparatively high grana (20 - 25 thylakoids) and more densely arranged stroma thylakoids. In plants treated with ACK2 the chloroplats were oriented in two layers - situated near the cell wall and near to the vacuole (Fig. 5). Chloroplasts adjacent to the cell wall were of similar structure than were in controls. However, the inner chloroplasts had semi-concentrically arranged thylakoids. These chloroplasts also lacked peristromium.

Discussion

Cytokinins are considered an important factor for the apical dominance release (Martin 1987, Tamas *et al.* 1989, Cline 1994, 1997). A number of physiological effects of natural and synthetic cytokinins are well documented, but particularly the effect of the phenylurea cytokinins on the process of growth and development have not yet been made clear enough.

The application of both types of cytokinins (Kin and CPPU) stimulated the bud break in *Gypsophila* but greater number of open buds was recorded when the medium was supplemented with 1 μM CPPU. Similar results were obtained with *in vitro* cultured single nodes of *Rosa hybrida* L. (Kapchina-Toteva *et al.* 2000). Both cytokinins increased the fresh and dry mass of the shoots, but CPPU caused more effective stimulation than Kin (Table 1). The higher fresh mass in correlation with the lower dry mass is one of the features characterizing the vitrification caused by cytokinins in the medium during the *in vitro* propagation of carnation and other plant species (Pasqualetto *et al.* 1986, Leshem *et al.* 1988). Dry mass changes indicate whether the increase in fresh mass, if any, is due to biomass accumulation or to vitrification (Gaspar 1991). As in the case with our experiments no sign of vitrification was observed when Kin and CPPU were applied, it supposed stronger CPPU activity compared to Kin.

Both kinetin and CPPU decreased the content of chlorophyll (*a+b*) in one-month old *Gypsophila paniculata* plants. Normally, cytokinins are known to affect photosynthesis and to stimulate chloroplast biogenesis, the differentiation of chloroplasts, and the expression of genes encoding the small subunit of RuBPCase and the light-harvesting chlorophyll-binding proteins (Adedipe *et al.* 1971, Tetley and Thimann 1974, Parthier 1989, Szweykowska 1992). In our model system, cytokinins promoted the increase in the number of open buds and in their fresh and dry masses. Therefore, we supposed that chlorophyll synthesis has not been inhibited, but the dilution of the pigments occurred.

The data from structural analyses showed that cytokinin from phenylurea type (CPPU) preserved the chloroplast structure. Only in this case the chloroplasts had better developed inner membrane system, encompassing the greater part of the organelle volume. This cytokinin is related to physiological substances, which to a certain extent are able to eliminate stress or to accelerate reconstructing processes (Stoyanova and Yordanov 1999).

Anticytokinins competitively inhibit cytokinin action and their effects are specific and reversible. Cytokinin antagonists are studied mostly in relation with cytokinin receptors. There are a few reports on the possibilities for applying these compounds. Yanosaka *et al.* (1989) have tested N-phenylcarbamates in *Asparagus* seedlings. In our experiments, the antagonistic properties of both compounds were better expressed in inhibiting the lateral bud break, changing fresh and dry mass of shoots than in influencing the chlorophyll content. Cytokinin antagonists have been shown to behave as agonists in some cytokinin bioassay systems, *e.g.* in betacyanin synthesis in *Amaranthus* and in seed germination in lettuce (Iwamura *et al.* 1979) as well as in the promotion of bud outgrowth in *Ipomea* (Cline 1997). Our recent studies on anticytokinin effects on bud sprouting in *in vitro* cultured *Rosa* showed that the response of both cultivars to the range of tested anticytokinin concentrations was different and the most effective concentration was 10 μM (Kapchina-Toteva *et al.* 2002). In contrast to the results with rose, our previous experiments with *Gypsophila* showed that 10 μM of these anticytokinins inhibited the bud development irreversibly (unpublished data). The concentration of 1 μM inhibited the bud development reversibly. Although the latest concentration reduced the fresh and dry mass, most probably, it was low for the expression of antagonistic properties on the chlorophyll content. It can be suggested that in such a concentration anticytokinins act as a cytokinin agonists.

In this particular investigation the structure of chloroplasts at the variant, with carbamate type anticytokinin (ACK2) was of interest. However, the issue why this plant plastid apparatus has been presented by two types of chloroplasts in the structural organization of the inner membrane system, directly connected with their topography in the cell, remains debatable. Although this exactly effect of anticytokinin ACK2 stayed obscure the obtained results provide a clue to better understanding of anticytokinin mode of action. To make the question clear further studies on other plant species are necessary.

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