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Anticytokinin effect on apical dominance release in *in vitro* cultured *Rosa hybrida* L.

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Abstract

Effects of two cytokinin antagonists - 2-chloro-4-cyclobutyl-amino-6-ethylamino-1,3,5-triazine and N-(4-pyridyl)-O-(4-chlorophenyl)carbamate - on bud break and outgrowth in single nodes from two *Rosa hybrida* cultivars differing in their apical dominance were studied. The compounds were applied at three different concentrations separately or in combination with benzyladenine. Cytokinin antagonists reduced the number of the sprouted buds in both cultivars at different extent. Their effect was strongly dependent on both concentration applied and culture period duration. The replacement of anticytokinins with benzyladenine in the medium resulted in overcoming of the bud break suppression. Both compounds significantly inhibited bud outgrowth as well.

Additional key words: cytokinins, cytokinin antagonists, rose, single nodes.

Introduction

Plant micropropagation is often based on the apical or lateral bud formation and their growth into shoots. The control exerted by the shoot apex over the outgrowth of lateral buds is well documented (for review see Cline 1997). Recently, the distribution of endogenous cytokinins in relation to budbreak of *Rosa hybrida*, cv. Madelon have been demonstrated by Dieleman *et al.* (1997) and experiments with transgenic plants have also provided some support for the auxin to cytokinin ratio hypothesis (Beveridge *et al.* 2000, Madoka and Mori 2000). Under *in vitro* conditions the lateral or axillary buds can be activated by addition of 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 resistance of plants to various stresses (Kaminek 1992, McGaw and Burch 1995). Despite their importance, a little is known about the mechanism of their action. One of the promising tools in studying aspects of the cytokinin mode of action is the application of cytokinin antagonists. These compounds competitively inhibit cytokinin action and their effects are specific and reversible. Seven structural classes of cytokinin antagonists (anticytokinins) have been described (Sergiev 1999). Anticytokinin effects are studied mostly in relation to cytokinin receptors. A few reports are available about possibilities for application of these compounds (Yanosaka *et al.* 1989, Karanov *et al.* 1992). Triazine and carbamate derivatives used in our experiments have been characterized as cytokinin antagonists in tobacco callus bioassay (Shimizu *et al.* 1989, 1990), some other cytokinin test systems (Iwamura *et al.* 1979, Sergiev *et al.* 1995) and *Dactylis* leaf explants (Somleva *et al.* 1995, 2000).

The aim of this paper was to study the influence of cytokinin antagonists on bud break and outgrowth in single nodes of two cultivars of *Rosa hybrida* 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 - N^6-benzyladenine.
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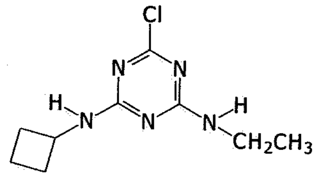
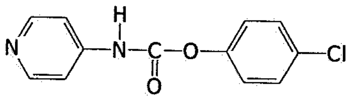
Materials and methods

The cytokinin antagonists 2-chloro-4-cyclobutyl-amino-6-ethylamino-1,3,5-triazine (ACK1, *on the left*) and N-(4-pyridyl)-O-(4-chlorophenyl) carbamate (ACK 2, *on the right*) were used in our experiments. The compounds were kindly provided by Prof. H. Iwamura from Kyoto University, Japan.





Two cultivars of *Rosa hybrida* L. differing in their apical dominance were used. Cultivar Madelon expresses strong apical dominance while cv. Motrea forms branches easily. The stock plants were maintained and subcultured every 5 weeks according to Van Telgen *et al.* (1992) on a standard MS medium supplemented with 1.5 mg dm^{-3} BA, 4.5 % (m/v) sucrose, and 7 g dm^{-3} agar. Growth conditions were temperature 22 °C and 16-h photoperiod (photosynthetic photon flux density 60 μmol m^{-2} s^{-1}, white fluorescent lamps).

Axillary buds from the 3rd and the 4th positions with a small piece of stem (single nodes) were transferred onto standard MS medium supplemented with 2.2 μM BA and/or 2.2 μM, 4.5 μM, and 10 μM of both cytokinin antagonists. The number of the sprouted buds as well as the explant fresh mass were determined every week during the following 4 weeks.

In order to obtain more detailed information on the interaction between cytokinins and their antagonists, pretreatment experiments were carried out. Explants cultured in the presence of 10 μM of both anticytokinins for 1, 2, and 3 weeks were transferred onto a medium containing 2.2 μM BA till the end of the culture period (4 weeks).

The data reported are means of the values obtained from 3 experiments in 10 replications each. LSD at a 5 % level of probability and SE are given.

Results and discussion

Apical dominance appears to be controlled by a balance between endogenous cytokinin and auxin levels. The difference in the apical dominance of both rose cultivars used in our experiments suggests that cv. Madelon has a greater auxin to cytokinin ratio than cv. Motrea. This suggestion was supported by the results for the number of sprouted buds from single nodes cultured on a hormone-free medium. Bud sprouting in cv. Motrea was 23 and 83 % after the first and second weeks of culture, respectively, and 90 % during the subsequent two weeks. After the same culture periods, 10, 50, and 53 % of the cultured Madelon explants had sprouted buds. However, no further development of the lateral buds from both cultivars was observed on a medium without any plant growth regulators. Exogenous purine-type cytokinins are proved to be essential for further growth of the sprouted buds and shoot formation in *in vitro* propagated roses (Lloyd *et al.* 1988, Campos and Salome 1990, Arnold *et al.* 1992, Van Telgen *et al.* 1992). More recently, the role of a phenylurea cytokinin in apical dominance release in *in vitro* cultured *R. hybrida* has been evaluated (Kapchina-Toteva *et al.* 2000).

In our experiments, application of 2.2 μM BA induced different response in both cultivars in dependance on their apical dominance. As a result of the cumulative effect of BA and the endogenous cytokinins the number of the sprouted buds in cv. Motrea was 40, 93.3, 96.7, and 100 % after 1, 2, 3, and 4 weeks, respectively, while it was lower in single nodes from cv. Madelon after the same culture periods (33.3, 77, 80, and 87 %, respectively). After the whole culture period, the increase in bud sprouting caused by BA was more than 30 % in Madelon cultures and 10 % in single nodes from cv. Motrea in comparison to the explants cultured on the hormone-free medium.

Anticytokinins influence various physiological processes presumably through changes in the endogenous cytokinin effects. Hence the application of cytokinin antagonists to the culture medium as sole plant growth regulators is an important approach for elucidation of their interaction with the endogenous cytokinins during apical dominance release. However, there are indications that anticytokinins do not affect physiological reactions typical for cytokinins in some plant systems. Cline *et al.* (1997) reported that the triazine and carbamate compounds had no effect on lateral bud break when added alone to the buds of isolated nodes in solution or directly to lateral buds of intact shoots of *Ipomoea nil.* Our data for the changes in the number of sprouted buds in *R. hybrida* suggest that the explant cells are susceptible to the compounds tested. The response of both *Rosa* cultivars to the range of anticytokinin concentrations tested was different (Fig. 1 and 2). The cytokinin antagonists applied at the lowest concentration affected lateral bud break almost at the same extent as BA did after the first week of culture initiation, while slight reduction in the number of the sprouted buds was observed during the subsequent culture. Most probably this concentration is too low for expression of the antagonistic properties of the compounds tested. When 4.5 µM of the compounds were added to the culture medium, significant suppression in bud sprouting was detected during the first two weeks of culture. The results presented here show that 10 µM is the most effective concentration in the range tested for expression of the antagonistic action of both triazine and carbamate anticytokinins during the whole culture period. These data confirm the concentration-dependent effect of the cytokinin antagonists, which has also been observed in other experimental systems (Shimizu *et al.* 1990, Sergiev *et al.* 1995, Somleva *et al.* 1995).

In our experiments, the antagonistic properties of both compounds were better expressed in the explants from cv. Madelon. Despite the differences in their chemical structure both cytokinin antagonists had similar influence on the lateral bud break in Motrea explants, while in cv. Madelon ACK2 exerted greater inhibition than the triazine derivative. Our previous results for the effects of

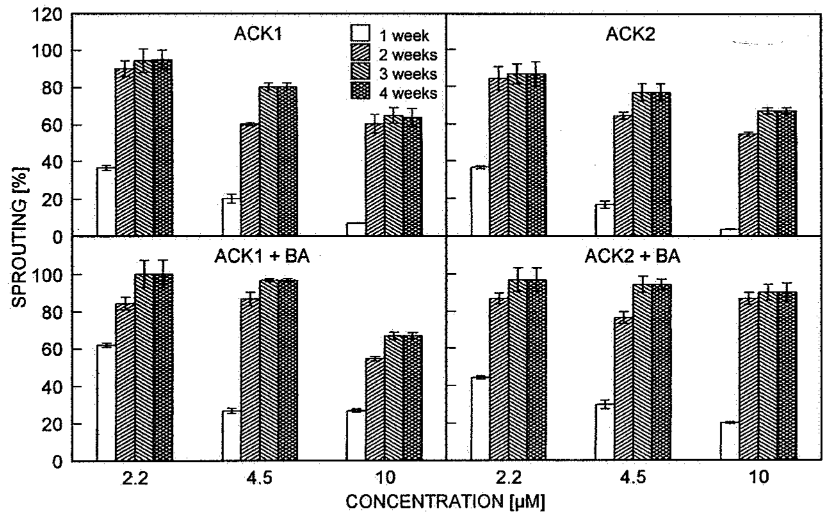


Fig. 1. Effect of anticytokinins on apical dominance release in single nodes of *R. hybrida*, cv. Motrea. ACK1 and ACK2 were added to the culture medium alone or in combination with 2.2 µM BA. Sprouting on hormone free medium in corresponding culture periods: 23, 83.3, 90 and 90 % and on only 2.2 µM BA: 40, 93.3, 96.7 and 100 %. *Bars* indicate means ± SE (*n* = 10).

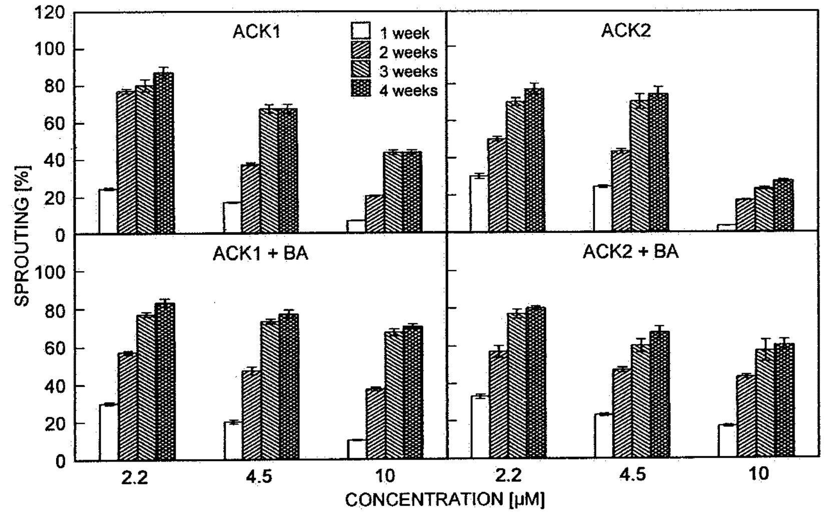


Fig. 2. Effect of anticytokinins on apical dominance release in single nodes of *R. hybrida*, cv. Madelon. ACK1 and ACK2 were added to the culture medium alone or in combination with 2.2 µM BA. Sprouting on hormone free medium in corresponding culture periods: 10, 50, 53.33 and 53.33 % and on only 2.2 µM BA: 33.33, 77, 80 and 87 %. *Bars* indicate means ± SE (*n* = 10).

these compounds on callus growth in two *Dactylis* genotypes showed that the carbamate anticytokinin was more effective as an antagonist in cultures initiated from the genotype with lower endogenous cytokinin content, while both compounds had similar action in the genotype possessing higher content of endogenous cytokinins (Somleva *et al.* 1995). On the basis of the data presented here, it is evident that both rose cultivars differ in their response to the antagonists applied, which most probably depends on their endogenous cytokinin levels.

The repression exerted by cytokinin antagonists was largely reversed by application of benzyladenine in the culture medium. When both types of plant growth regulators were present at a concentration of 2.2 μM, a synergistic effect was observed in single nodes cultured for one week (Fig. 1 and 2). This action was better expressed when BA was combined with its structural analogue ACK1. The exogenous cytokinin overcame the inhibitory effect of both compounds when they were applied at higher concentrations. These data are in agreement with the general notion that the effects of anticytokinins are reversible (Mok 1994). 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 *Ipomoea nil* (Cline *et al.* 1997). Our recent studies on anticytokinin effects on production of active oxygen species and antioxidant enzyme activities in embryogenic cultures of *D. glomerata* demonstrated that these compounds acted as cytokinin agonists or antagonists during the different stages of somatic embryogenesis (Somleva *et al.* 2000).

In another type of experiments the cytokinin antagonists were replaced with BA. The benzyladenine promotive effect on bud sprouting partially reversed the anticytokinin inhibitory effect (Table 1). The efficiency of this process is in inverse proportion to the duration of the preliminary culture in the presence of cytokinin antagonists. One-week-long treatment with anticytokinins did not cause a significant reduction in bud sprouting in comparison to the explants cultured in the presence of BA. The compensatory effect of the cytokinin was still well expressed in explants precultured with cytokinin

Table 1. Bud sprouting [%] in single nodes of *R. hybrida*, cv. Madelon cultured in the presence of 2.2 μM BA or 10 μM cytokinin antagonists (ACK 1 and ACK 2). Explants were precultured with anticytokinins for 1, 2 or 3 weeks followed by transfer onto the BA-containing medium (LSD at 5 % level of probability - 0.9).

Variant	Duration of the culture period [weeks]			
	1	2	3	4
Hormone free medium	10.00	50.00	53.33	53.33
BA (2.2 μM)	33.37	76.67	80.00	87.00
ACK 1 (10 μM)	6.67	20.00	43.33	43.33
ACK 1 (1 w) → BA (3 w)	-	33.33	63.33	73.33
ACK 1 (2 w) → BA (2 w)	-	-	60.00	67.00
ACK 1 (3 w) → BA (1 w)	-	-	-	50.00
ACK 2 (10 μM)	3.33	16.67	23.33	26.67
ACK 2 (1 w) → BA (3 w)	-	36.67	70.00	70.00
ACK 2 (2 w) → BA (2 w)	-	-	56.67	63.33
ACK 2 (3 w) → BA (1 w)	-	-	-	36.67

Table 2. Effects of 2.2 μM BA or 10 μM anticytokinins (ACK1 and ACK2) on R. *hybrida* explant fresh mass [mg plant^{-1}]. Fresh mass of the explants cultured on a hormone-free medium for one week is 19.4 ± 0.3 mg and 12.1 ± 0.2 mg for cv. Madelon and cv. Motrea, respectively. Each value is the mean of *ca.* 30 determinations ± SE. Values, significantly different from the control (single nodes cultured in the presence of BA for the same period) are indicated by * ($P < 0.05$) and ** ($P < 0.01$).

Cultivar	Plant growth regulator	Duration of the culture period [weeks]			
		1	2	3	4
Madelon	BA	32.2 ± 0.1	56.7 ± 0.4	59.3 ± 0.3	63.1 ± 0.7
	ACK 1	25.7 ± 0.2	26.3 ± 0.3**	28.2 ± 0.2**	31.0 ± 0.3**
	ACK 2	21.4 ± 0.1*	22.3 ± 0.4**	23.3 ± 0.2**	24.5 ± 0.3**
Motrea	BA	21.8 ± 0.2	34.2 ± 0.4	38.7 ± 0.5	44.1 ± 0.6
	ACK 1	16.4 ± 0.2	21.6 ± 0.3*	25.1 ± 0.3	27.2 ± 0.4*
	ACK 2	14.9 ± 0.1*	18.7 ± 0.2**	21.5 ± 0.2*	25.8 ± 0.3*

antagonists for two weeks. Prolongation of anticytokinin treatment resulted in a strong reduction of the number of sprouted buds. These data once again prove the reversible action of the anticytokinins in *Rosa* cultures. Sergiev *et al.* (1995) reported that the exchange of anticytokinin solutions with cytokinin ones resulted in overcoming of the inhibitory effect of ACK1 and ACK2 on growth of radish cotyledons. Our data are in agreement with the suggestion of these authors that the reversibility in the action of both cytokinins and their antagonists indicated a similarity in the mode of action of these two groups of plant growth regulators.

In general little is known concerning the effects of cytokinin antagonists on bud growth. It has been found that the antagonists, pyrrolo[2,3-d]pyrimidines, promoted bud outgrowth in peas whereas the triazine derivative retarded tillering of barley (Cline *et al.* 1997). Our results showed that when ACK1 and ACK2 were applied at the highest concentration tested (10 μM), they inhibited bud outgrowth in Madelon explants more significantly than in Motrea cultures (Table 2). The same tendency was established in relation to the explant dry mass (data not shown). Both compounds had greater antagonistic action in relation to bud outgrowth than to bud break. In addition, the presence of anticytokinins at a concentration of 10 μM provoked abnormal shoot development in some explants.

Anticytokinins are structural analogues of cytokinin-active compounds. Most authors believe that the cytokinin antagonists fit the cytokinin receptor but do not trigger the successive action leading to cell division and growth, and thus they are antagonists in tobacco callus assay. When act as agonists they are thought to fit the receptor in a fashion as do cytokinins (Iwamura 1994). Another suggestion is that the anticytokinins as structural analogues of cytokinins fit the active site of a cytokinin degrading enzyme (Cline *et al.* 1997). The results presented here demonstrate that cytokinin antagonists are useful tool for studying the role of cytokinins in apical dominance release in *in vitro* cultured *Rosa hybrida* L. as well as for elucidating some aspects of the mechanism of antagonist action.

References

Arnold, N., Binns, M., Barthakur, N., Cloutier, D.: A study of the effect of growth regulators and time of plantlet harvest on the *in vitro* multiplication rate of hardy and hybrid tea roses. - J. hort. Sci. **67**: 727-735, 1992.

Beveridge, C., Symons, G., Turnbull, C.: Auxin inhibition of decapitation-induced branching is dependent on graft-transmissible signals regulated by genes rms1 and rms2. - Plant Physiol. **123**: 689-697, 2000.

Campos, S., Salome, S.: Mass propagation of the dwarf rose cultivar "Rosamini". - Scientia Hort. **43**: 321-330, 1990.

Cline, M.: Concepts and terminology of apical dominance. - Amer. J. Bot. **84**: 1064-1069, 1997.

Cline, M., Wessel, T., Iwamura, H.: Cytokinin/auxin control on apical dominance in *Ipomoea nil*. - Plant Cell Physiol. **38**: 659-667, 1997.

Dieleman, J., Verstappen, F., Nicander, B., Kuiper, D., Tillberg, E., Tromp, J.: Cytokinins in *Rosa hybrida* in relation to bud break. - Physiol. Plant. **99**: 456-464.

Iwamura, H.: Cytokinin antagonists: synthesis and biological activity. - In: Mok, D., Mok, M. (ed.): Cytokinins Chemistry, Activity and Function. Pp. 43-55. CRC Press, London 1994.

Iwamura, H., Masuda, N. Koshimizu, K., Matsubara, S.: Cytokinin-agonistic and antagonistic activities of 4-substituted-2-methylpyrolo (2,3-d) pyrimidines, 7-deaza analogs of cytokinin-active adenine derivatives. - Phytochemistry **18**: 217-222, 1979.

Kaminek, M.: Progress in cytokinin research. - Trends Biotechnol. **10**: 159-164, 1992.

Kapchina-Toteva, V., Van Telgen, H.-J., Yakimova, E.: Role of phenylurea cytokinin CPPU in apical dominance release in *in vitro* cultured *Rosa hybrida* L. - J. Plant Growth Regul. **19**: 232-237, 2000.

Karanov, E., Alexieva, V., Sergiev, I.: Cytokinin antagonists - chemistry and action. - Bulg. J. Plant Physiol. **1**: 97-107, 1992.

Lloyd, D., Roberts, V., Short, K.: The induction *in vitro* of adventitious shoots in *Rosa*. - Euphytica **37**: 31-36, 1988.

Madoka, Y., Mori, H.: Two novel transcripts expressed in pea dormant axillary buds. - Plant Cell Physiol. **41**: 274-281, 2000.

McGaw, B., Burch, L.: Cytokinin biosynthesis and metabolism. - In: Davies, P.J. (ed.): Plant Hormones. Physiology, Biochemistry and Molecular Biology. Pp. 98-117. Kluwer Academic Publishers, Dordrecht 1995.

Mok, M.: Cytokinins and plant development - an overview. - In: Mok, D., Mok, M. (ed.): Cytokinins Chemistry, Activity and Function. Pp. 43-55, CRC Press, London 1994.

Sergiev, I.: Cytokinin antagonists: structure - activity relationships and physiological properties. - PhD Thesis, The Bulgarian Academy of Sciences, Sofia 1999.

Sergiev, I., Alexieva, V., Karanov, E.: Cytokinin and anticytokinin effects on growth and free polyamine content in etiolated and green radish cotyledons. - J. Plant Physiol. **145**: 266-270, 1995.

Shimizu, R., Iwamura, H., Matsubara, S., Fujita, T.: Development of s-triazine anticytokinins and their quantitative structure-activity relationship. - J. Agr. Food Chem. **37**: 236-240, 1989.

Shimizu, R., Iwamura, H., Fujita, T.: Anticytokinin activity of N-phenyl and N-pyridyl-carbamates. - Z. Naturforsch. **45c**: 89-95, 1990.

Somleva, M., Kapchina, V., Alexieva, V., Golovinsky, E.: Anticytokinin effects on *in vitro* response of embryogenic and non-embryogenic genotypes of *Dactylis glomerata* L. - Plant Growth Regul. **16**: 109-112, 1995.

Somleva, M., Kapchina-Toteva, V., Alexieva, V., Sergiev, I., Karanov, E.: Novel physiological properties of two cytokinin antagonists. - J. Plant Physiol. **156**: 623-627, 2000.

Van Telgen, H.-J., Ellagoz, V., Van Mil, A., Paffen, A., De Klerk, G.-J.: Role of plant hormones in lateral bud growth of rose and apple *in vitro*. - Acta Hort. **319**: 137-142, 1992.

Yanosaka, K., Iwamura, H., Fujita, T.: Flower induction in seedlings of *Asparagus officinalis* L. by N-phenylcarbamates. - Z. Naturforsch. **44c**: 226-232, 1989.