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EFFECT OF PURINE AND PHENYLUREA CYTOKININS ON PEROXIDASE ACTIVITY IN RELATION TO APICAL DOMINANCE OF *IN VITRO* CULTIVATED *ROSA HYBRIDA* L.

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Summary. The formation of active oxygen species affects the morphogenesis of plant cells and tissues and inhibits the process of development during *in vitro* cultivation. Cytokinins have been found to be effective free radical scavengers. Peroxidase (EC 1.11.1.7) is among enzymes expressing antioxidative functions. The present study was focused on the effect of N^6-benzyladenine (BA), and N^1-(2-chloro-4-pyridyl)-N^2-phenylurea (4PU-30) on guaiacol-peroxidase activity regarding the break and growth of axilary buds in rose (*Rosa hybrida* L.) *in vitro*. The activity of peroxidase was determined by the method of Hart et al. (1971). Three concentrations of cytokinins (1.0 μM; 1.6 μM; 2.2 μM) were tested. An increase of peroxidase activity was established on day 1 after the transfer of single nodes on the media that was probably caused by a mechanical stress in the process of explant isolation or was contributed to the composition of nutrient media. When the explants were cultured on media with 1.0 μM BA a stimulation of enzyme activity was found later than in variants with 1.6 and 2.2 μM BA. The applied lower concentration of 4PU-30 (1.0 μM) caused opening of more axilary buds and obvious enhancement of peroxidase activity in comparison to the highest concentration of BA. These results supported the view that phenylurea cytokinins could be more active than purine cytokinins. Acceleration of peroxidase activity was associated with increased number of open axilary buds. Since the activity of peroxidase in our experiments was stimulated in response to cytokinin application we suppose that cytokinins might make this enzyme active thus control-

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ling the level of H_2O_2 and the rate of cell division. In addition, we suggest that the activity of peroxidase could be used as a biochemical marker of development in the plant object studied.

Key words: cytokinins, guaiacol-peroxidase, *in vitro* cultures, rose (*Rosa hydrida* L.), sprouting

Abbreviations: BA – N^6-benzyladenine, EDTA – ethylenediaminetetraacetic acid, PMSF – phenylmethanesulfonyl fluoride, PGRs – plant growth regulators, 4PU-30 – N^1-(2-chloro-4-pyridyl)-N^2-phenylurea; GDHP – guaiacol dehydrogenated product

Introducton

The formation of apical and lateral buds, their growth and the subculturing of mini-shoots are basic processes of *in vitro* propagation. The growth of buds can be inhibited by the development of dormancy in apical meristems or by domination from an actively growing apical bud (apical dominance) (Tucker, 1980; Cline, 1991; van Telgen et al., 1992). The type and concentration of plant growth regulators affect the process of *in vitro* propagation since they play a major role in cell division, differentiation and morphogenesis in plant tissue cultures. *In vitro* cultures are a good model system for studies on physiological effects of stimulators and inhibitors of plant growth and development (Zieslin et al., 1978; Hillman, 1984). Cytokinins have been found to be effective free radical scavengers (Leshem, 1988). In recent years it has been documented that phenylurea type cytokinins are more active in some model systems (Karanov et al., 1992), which directed their further application for propagation of some valuable agricultural varieties.

The formation of active oxygen species has been found to affect the morphogenesis of plant cells and tissues and to inhibit the process of development during *in vitro* cultivation. Moreover, the accumulation of such active oxigen forms has been established to suppress explant's development and H_2O_2 concentration as far as it is involved in detoxication of active oxigene species plays an important role in cell division (Benson and Roubelakis-Angelakis, 1994). The plant cells possess highly efficient defence systems for elimination of the harmful effect of oxidative stress. Guaiacol-peroxidase (EC 1.11.1.7), catalase (EC 1.11.1.6) and ascorbat-peroxidase (EC 1.11.1.11) are among enzymes expressing antioxidative functions. There are still limited data concerning the relation between peroxidase activity, cytokinin application and apical dominance release.

The present study was focused on the effect of N^6-benzyladenine (BA) and N^1-(2-chloro-4-pyridyl)-N^2-phenylurea (4PU-30) on guaiacol-peroxidase activity regarding the break and growth of axilary buds in rose (*Rosa hybrida* L.) *in vitro*.

Materials and Methods

Plant materials and cultivation

The experiments were carried out with shoot cultures of *Rosa hybrida* L., cultivars Madelon and Motrea. The growth of the two cultivars reflects their difference in degree of apical dominance. Cv. Madelon shows strong apical growth and branches with difficultly, whereas cv. Motrea is an easy branching variety. Plants were subcultured every five weeks according to van Telgen et al. (1992) on standard medium (MS at full strength, 4.5% (w/v) sucrose, 7 $g.l^{-1}$ agar) with 1.5 $mg.l^{-1}$ BA. Growth conditions were 20 °C and 16 hours of light (60 $\mu mol.m^{-2}.s^{-1}$ photosynthetic photon flux density, Philips TLD-33). Shoots were cut into axillary buds with a small piece of stem (single nodes). The single nodes were transferred to standard media and media containing growth regulators. The influence of three concentratons of cytokinins BA and 4PU-30 – 1.0 µM, 1.6 µM and 2.2 µM on peroxidase activity was tested.

Enzyme assay

Peroxidase (EC 1.11.1.7) activity was determined according to the method of Hart et al. (1971). The extract was prepared from 0.05–0.1 g material with 0.1 M phosphate buffer (pH 7.0) with addition of 1 mM EDTA and 10 µM PMSF (protease inhibitor). The homogenate was kept in a cold room for 30 min and than centrifuged at 15000 rpm. The supernatant was assayed for peroxidase activity. Incubation mixture contained enzyme extract, 20 mM guaiacol (H^+ donor), 0.1 M phosphate buffer and 10 mM H_2O_2.The absorption was measured at 470 nm for 5 min at 60 s intervals. Enzyme activity was defined as µmol GDHP.mg^{-1}protein.min^{-1}, using mmolar extinction coefficient of GDHP ($\varepsilon = 26.6\ mM^{-1}.cm^{-1}$)

Protein content was estimated by the Bradford method (1976) using bovine serum albumine as a standard.

The data were processed statistically by Student's t-criterium at $p \leq 0.05$.

Results and Discussion

The dynamics of guaiacol-peroxidase in explants of cv. Madelon and cv. Motrea roses cultured on media without PGRs (control) is shown on Fig. 1. The activity of peroxidase of cv. Madelon was established being higher in comparison to cv. Motrea. An increase of the activity was observed on day 1. Similar enhancement of peroxidase on day 1 was established in variants where plant growth regulators were included in the media and this did not depend on the type and concentrations of PGRs (Fig. 2, 3, 4 and 5). The effect was less expressed for cv. Motrea. On hormone free media cv.

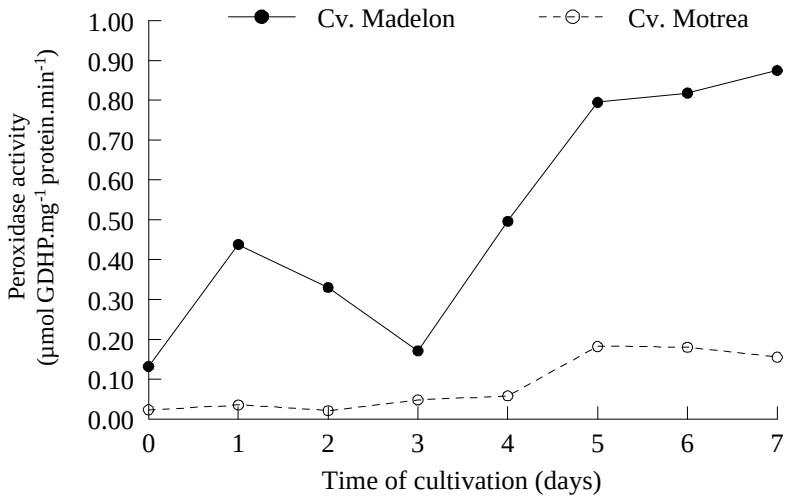

Fig. 1. Peroxidase activity in control plants of *in vitro* cultivated cv. Madelon and cv. Motrea roses

Madelon had highest activity of peroxidase on day 7 while cv. Motrea showed most obvious acceleration of the activity on day 5.

In these experiments we tested the effect of BA and 4PU-30 on peroxidase activity. Three different concentratons (1.0 µM, 1.6 µM and 2.2 µM) of these cytokinins

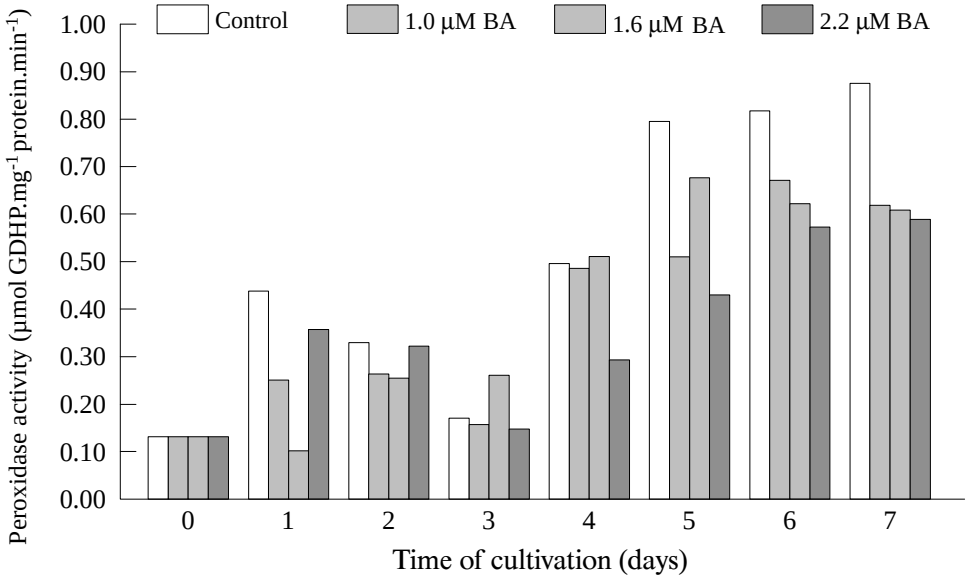

Fig. 2. Dynamic of peroxidase activity of cv. Madelon roses cultivated *in vitro* on media with different concentrations of BA

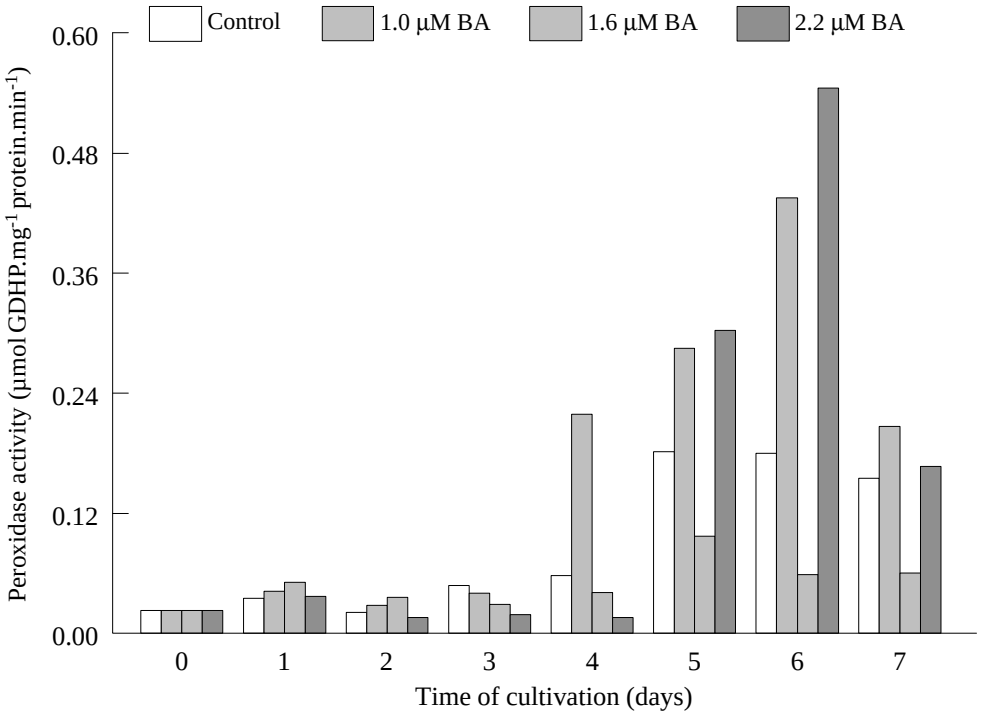

Fig. 3. Dynamic of peroxidase activity of cv. Motrea roses cultivated *in vitro* on media with different concentrations of BA

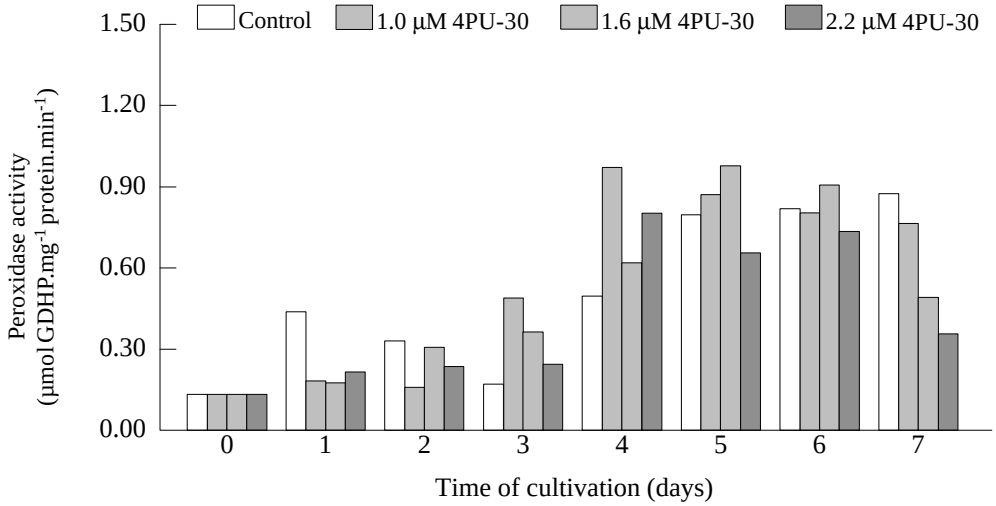

Fig. 4. Dynamic of peroxidase activity of cv. Madelon roses cultivated *in vitro* on media with different concentrations of 4PU-30

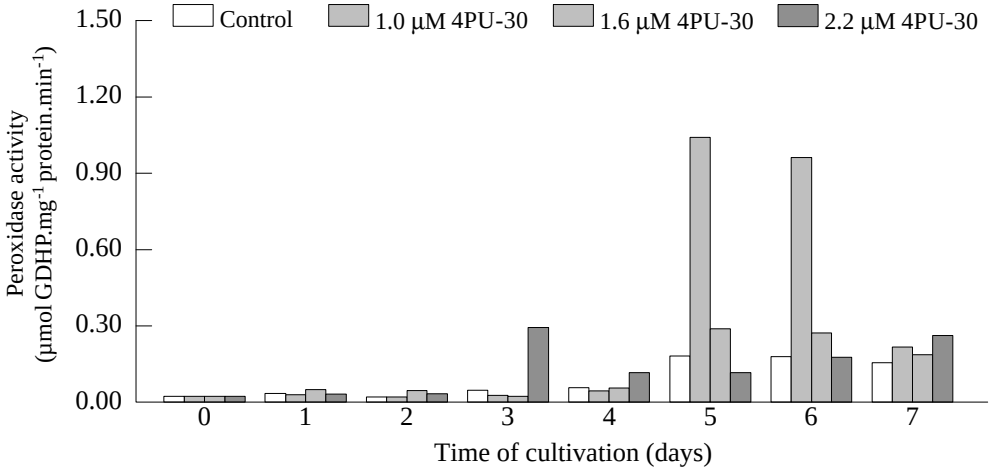

Fig. 5. Dynamic of peroxidase activity of cv. Motrea roses cultivated *in vitro* on media with different concentrations of 4PU-30

were included in the nutrient media. As presented on Fig. 2 and 3 the application of 1.0 μM BA caused maximum enhancement of peroxidase activity in cv. Madelon and cv. Motrea on day 4. According to the increase of BA concentration the rise of enzyme activity in cv. Madelon (Fig. 2) was pronounced earlier: on day 3 in response to 1.6 μM BA application and on day 2 when 2.2 μM BA was used. In cv. Motrea (Fig. 3) highest peroxidase activity was exhibited on day 2 on media with 1.6 μM BA and on day 6 on media with 2.2 μM BA.

The application of 1.0 μM and 1.6 μM 4PU-30 caused an acceleration of peroxidase activity on day 3 in explants of cv. Madelon roses followed by gradual decrease until day 7 (Fig. 4). When 2.2 μM 4PU-30 was used the enzyme activity expressed a maximum on day 4. In cv. Motrea explants (Fig. 5) the activity of peroxidase was of maximum value on day 5 in reaction to 1.0 μM 4PU-30, on day 2 in response to 1.6 μM 4PU-30 and on day 3 in the media with 2.2 μM 4PU-30.

In general, the application of 4PU-30 stimulated sprouting and development of higher number axillary buds in both studied rose cultivars in comparison to BA (Table 1). Obviously, the lowest concentration of 4PU-30 (1.0 μM) produced the best expressed effect on the two studied cultivars which gave us a reason to suggest that among studied concentrations of 4PU-30 1.0 μM was the limiting one. This was an indication for higher physiological activity of 4PU-30 in comparison to BA. Looking at tha data for the percentage of sprouting (Table 1) it was evident that the apical dominance release was suppressed with the increase of 4PU-30 concentration for cv. Motrea where even an inhibition of the axillary bud growth was noticed in response to 2.2 μM 4PU-30. After 4 weeks of cultivation the percentage of sprouting of cv. Madelon was 80% at 2.2 μM BA and 93% from axillary buds opened when 1.0 μM 4PU-30 was applied. For cv. Motrea 2.2 μM BA caused 86 % sprouting while 1.0 μM

Table 1. Effect of BA and 4PU-30 on the sprouting of *in vitro* cultivated *Rosa hybrida* L.

Variants	cv. Madelon	cv. Motrea
	% of sprouting after 7 days of cultivation	
Control	25.0	26.7
BA 1.0 µM	32.9[a]	27.6[a]
BA 1.6 µM	37.5[a]	33.3[a]
BA 2.2 µM	40.3[a]	46.6[a]
4PU-30 1.0 µM	75.3[b]	61.0[b]
4PU-30 1.6 µM	72.2[b]	56.7[b]
4PU-30 2.2 µM	70.3[b]	40.3[b]

Sprouting is calculated as a percentage of open buds to the initial number of single nodes (n=30). Data differ significantly from control values at $p \leq 5\%$ (a) and $p \leq 1\%$ (b)

4PU-30 stimulated 97% bud break (data not presented). In fact at the end of cultivation (4th week) both cytokinins were effective for almost full apical dominance overcoming but the highest number of open buds for the two cultivars in response to 1.0 µM 4PU-30 confirmed the initial (at the end of the first week) better physiological effect of the non-purine cytokinin. The results obtained in our experiments are concomitant with the view that phenylurea cytokinins pronounce higher activity at lower concentrations in comparison to purine cytokinins (Karanov et al., 1992) hence offering a possibility to use *in vitro* propagated roses as another model system for this sort of study. Present experimental data showed that the time of cultivation of cv. Madelon and cv. Motrea roses could be reduced to two weeks by using 4PU-30 which might be practically applicable.

According to Asada (1992) the physiological function of peroxidase is defensive and the same enzyme plays a role in the cell wall lignification. Since in our experiments an increase of guaiacol-peroxidase activity in the course of sprouting was established we suggest that cytokinin application might affect the apical dominance by stimulating the enzyme activity of *in vitro* studied rose cultivars. The enhancement of peroxidase activity found on day 1 of cultivation might be contributed to mechanical stress in the process of explant's isolation or might be caused by the composition of nutrient media. This suggestion supports the view of other authors that the change in hormonal balance is a factor inducing oxidative stress (Alexieva, 1993; Somleva, 1997).

The activity of peroxidase in our experiments was accelerated in response to cytokinin application which gave us a reason for the suggestion that cytokinins might make this enzyme active in our model system thus controlling the level of H_2O_2 and the rate of cell division. Furthermore, we suggest that the activity of peroxidase could be used as a biochemical marker of development in the studied plant object.

In control explants of cv. Madelon the highest level of peroxidase activity was demonstrated two days later that than of cv. Motrea. Basically, as the development of axilary buds depends on cytokinins and in this case the media did not contain PGRs it could be possible the earlier opening of cv. Motrea buds might be due to higher level of endogenous cytokinins moreover that the latest cultivar expresses very slight apical dominance. To verify this suggestion further studies are necessary. In addition, this is a discussible problem because some authors have found that the higher endogenous content of cytokinins is not always determinative for apical dominance release and bud sprouting (Jablanovic and Neskovic, 1977; van Staden et al., 1981).

Conclusions

Based on current experimental evidence we find it necessary to stress that the stimulation of peroxidase activity established on day 1 after the transfer on the media is possibly a response to mechanical stress in the process of explant isolation or might be caused by the composition of nutrient media. The subculturing of rose single nodes on media with 1.0 µM BA accelerated enzyme activity later than in variants with 1.6 and 2.2 µM BA. Lower applied concentration of 4PU-30 (1.0 µM) caused opening of more axilary buds and well exhibited enhancement of peroxidase activity in comparison to the highest concentration of BA. Our results support the view that phenylurea cytokinins could be more active than purine cytokinins. The augmentation of peroxidase activity was associated with increased number of open axilary buds. Since the activity of peroxidase in present experiments was stimulated in response to cytokinin application it could be hypothesized that cytokinins might make this enzyme active thus controlling the level of H_2O_2 and the rate of cell division. Other enzyme activities could also be among factors responsible for *in vitro* axillary bud growth and development of roses. Evidently further investigations are needed to make clear the connection between the process of sprouting and the dynamics of defence enzyme systems.

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