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EFFECT OF BA AND CPPU ON PROTEASE AND a-AMYLASE ACTIVITY OF *IN VITRO* CULTURED EXPLANTS OF *ROSA HYBRIDA* L.

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Summary. The present study was focused on the effect of N^6-benzyladenine (BA) and N_1-(2-chloro-4-pyridyl)-N_2-phenylurea (CPPU) on protease and α-amylase activity regarding the *in vitro* break and growth of lateral buds of rose (*Rosa hybrida* L.), cvs. Madelon and Motrea. Cv. Madelon shows strong apical growth and suppressed branching, whereas cv. Motrea is an easy branching variety. The explants were subcultured every five weeks on standard medium and then transferred onto media containing growth regulators. The impact of cytokinins BA and CPPU on protease and α-amylase activity was traced within a period of one month. On day 1st of culture cv. Motrea showed lower protease activity than that in the control in response both to CPPU and BA while in cv. Madelon an opposite effect of enhanced protease activity was found. After 4 days a decrease of protease activity occurred for cv. Madelon in media with either BA or CPPU. On the same day an acceleration of enzyme activity was observed in cv. Motrea when cultured with BA. In comparison to hormone free media much lower protease activity was detected in one-month-old plants of cv. Madelon when treated with cytokinins and a decline was established in cv. Motrea in respect to the tendency within the first 7 days. We suggest that keeping the activity of proteases at lower level cytokinins are able to support the growth and development of lateral shoots. The α-amylase activity was enhanced on day 1st most probably due to mechanical stress at explant isolation. A stronger increase of α-amylase activity was observed upon treatment with CPPU and this coincided

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with the higher number of open buds at the end of the first week of culture. It may be assumed that the active degradation of starch that occurred on day 4th in response to CPPU application is a necessary tool for stimulation of bud outgrowth thus helping the apical dominance release.

Key words: α-amylase, apical dominance, BA, CPPU, protease, rose

Abbreviations: BA – N^6-benzyladenine; CPPU – N_1-(2-chloro-4-pyridyl)-N_2-phenylurea, HF – hormone free; IBA – γ-indole butiric acid

Introduction

Apical dominance can be defined as the control exerted by the shoot apex over the outgrowth of the lateral buds mainly via suppressed branching as an effect of auxins from the apical meristem (Cline, 1994, 1997). Hence, the type and concentration of plant growth regulators affect the capacity of *in vitro* propagation since they play a major role in cell division, differentiation and morphogenesis in plant tissue cultures. Axillary bud outgrowth, which is considered as a process of apical dominance release, can be enhanced in response to exogenous cytokinins (Philips, 1975; Bollmark, 1995). In *in vitro* propagated roses the effects of BA and kinetin on apical dominance have been extensively studied (Davies, 1980; Hasegawa, 1980; Skirvin et al., 1984; Lloyd et al., 1988; Campos et al., 1990; Arnold et al., 1992; Van Telgen et al., 1992), but no data are yet available concerning the application of phenylurea cytokinins. Higher activity and better effect of phenylurea cytokinins have already been demonstrated in various test systems (Takahasi et al., 1971; Okamoto et al., 1978; Iwamura et al., 1980; Karanov et al., 1992; Shudo, 1994). Enhanced outgrowth of axillary buds is established when medium for *in vitro* apple and grapevine culture has been supplemented with thidiazuron and CPPU (Niewkera et al., 1986; Fellman et al., 1987; Gribaudo and Fronda, 1991). In the process of apical dominance release different metabolic functions are altered and the effect of purine cytokinins is discussed (Cline, 1994), but there is still limited information concerning the impact of phenylurea cytokinins on hydrolytic and proteolytic enzymes in *in vitro* cultures.

This study was undertaken to investigate the effects of purine (BA) and phenylurea (CPPU) cytokinins on apical dominance release and activity of α-amylase and proteases in *in vitro* cultured *Rosa hybrida* L. We demonstrated that when CPPU was added to the culture medium, the physiological features such as bud sprouting were considerably enhanced and the activity of α-amylase and proteases were affected in response both to BA and CPPU. Our results indicated differences in the cultivar behaviour.

Materials and Methods

Plant material and growth conditions

The experiments were carried out with shoot cultures of *Rosa hybrida* L., cv. Madelon and cv. Motrea. Both cultivars are known to express a different degree of apical dominance and *in vitro* (Van Telgen et al., 1992) cv. Madelon shows strong apical growth and less number of outgrowing shoots than cv. Motrea (Scheme 1). Rose plants cultured on a standard MS medium supplemented with 1.5 mg.l^{-1} BA, 4.5% (w/v) sucrose, and 7 g.l^{-1} agar were subcultured every five weeks according to Van Telgen et al. (1992). The explants isolated from these cultures were grown on the same medium containing reduced amount of BA (1.0 mg.l^{-1}). For shoot elongation liquid basal MS medium (2 ml per plant) was added one week later. Axillary buds from 3rd and 4th position with a small part of elongated shoots (single nodes) were used as an explant source (30 explants per treatment). Growth conditions were 20 °C and 16 hours of light (60 μmol.m^{-2}.s^{-1} photosynthetic photon flux density, Philips TLD-33).

The single nodes with buds already present in the explant were transferred to standard medium supplemented with 1.0 μM of the synthetic cytokinins of purine-type N^6-benzyladenine (BA) or phenylurea-type N-(2-chloro-4-pyridyl)-N′-phenylurea (CPPU). The budbreak was determined as the percentage of open buds 1 and 4 weeks after the transfer. For statistical significance the data were processed and assessed by LSD at 5% level.

Rosa hybrida “Madelon” *Rosa hybrida* “Motrea”

Scheme 1. Plants of *Rosa hybrida* L.

Protease assay

Protease activity was determined according to Sarath (1990). The leaves were ground on ice with extraction buffer containing 25 mM Tris-HCl pH 7.5, centrifuged twice at 10 000×g for 15 min and the supernatant was used for the assay. A substrate azoalbumin (2% in the extraction buffer) and an aliquot of the extract were added to the reaction mixture. The samples were incubated for 45 min at 37 °C and the reaction terminated with 10% TCA. After a centrifugation at 10 000 rpm for 5 min the pellet was discarded and 1 M NaOH was added to the supernatant. The absorbency was measured at 440 nm with reference to the blank. Also enzyme blank with azoalbumin, TCA and extract (added in this order) was used.

a-Amylase assay

The activity of α-*amylase* (EC 3.2.1.1.) was estimated according to the modified method of Plummer (1988) The method was based on the interaction of 3,5-dinitrosalicylic acid with reducing sugars and the amount of the resulting substance was measured colorimetrically at 530 nm. The specific activity was expressed in mg maltose per mg protein. *Protein* content was determined by the method of Bradford (1976) with bovine serum albumine as a standard.

Results and Discussion

Apical dominance represents an inhibition from apically produced auxin on the outgrowth of axillary buds. Cytokinins are considered as an important factor in controlling and breaking the dormancy and apical dominance (Martin, 1987; Tamas et al., 1989; Cline, 1994, 1997). Recently, the distribution of endogenous cytokinins in relation to budbreak of *Rosa hybrida*, cv. Madelon has been demonstrated by Dieleman et al. (1997). A number of physiological effects of natural and synthetic cytokinins are well documented, but the mechanism through which these plant growth regulators, and in particular the phenylurea cytokinins control the processes of growth and development are not yet quite clear.

In our experiments in both rose cultivars Madelon and Motrea, the application of 1.0 µM CPPU stimulated the bud opening to a greater extent than 1.0 µM BA. This effect was clearly exhibited in the 1st week of culture when the number of sprouted buds was nearly two and three times greater in cv. Motrea and cv. Madelon, respectively, in comparison to BA treatment (Fig. 1). Higher percentage of open buds was also established at 1.0 µM CPPU after 4 weeks of subculture. In our earlier study we demonstrated that cv. Motrea responded differentially to higher concentrations of the tested cytokinins during the 1st and 4th weeks: enhanced budbreak with the increase

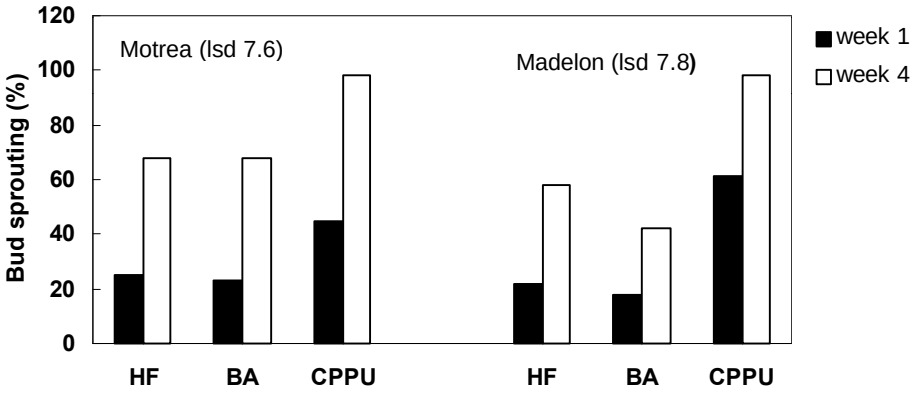

Fig. 1. Effect of 1.0 µM BA and 1.0 µM CPPU on bud sprouting of *in vitro Rosa hybrida* L.

of BA concentration and diminished outgrowth with the increase of CPPU concentration as for cv. Madelon, the percentage of sprouted buds did not change with the increase of CPPU concentration both after 1 and 4 weeks (Kapchina-Toteva et al., 2000).

The activity of α-amylase was studied to obtain information whether the bud opening in our model may contribute to the degradation of starch. In addition, a function of α-amylase in response to mechanical stress was suggested. Cv. Motrea, that expressed lower degree of apical dominance, showed higher initial activity of α-amylase. An increase of α-amylase activity was recorded on day 1st in both cultivars independently of the treatment applied (Fig. 2). It can be suggested that as α-amylase is a stress indicator, this acceleration of the activity might be due to mechanical stress at explant isolation. This is in accordance with the findings that in tobacco and barley leaves a shift of the activity of amylolitic enzymes in response to temperature, wounding (Dreier et al., 1995), water stress (Jacobsen et al., 1986) and pathogenic infection (Heitz et al., 1991) has been assessed. On day 1st in cv. Motrea and also in cv. Made-

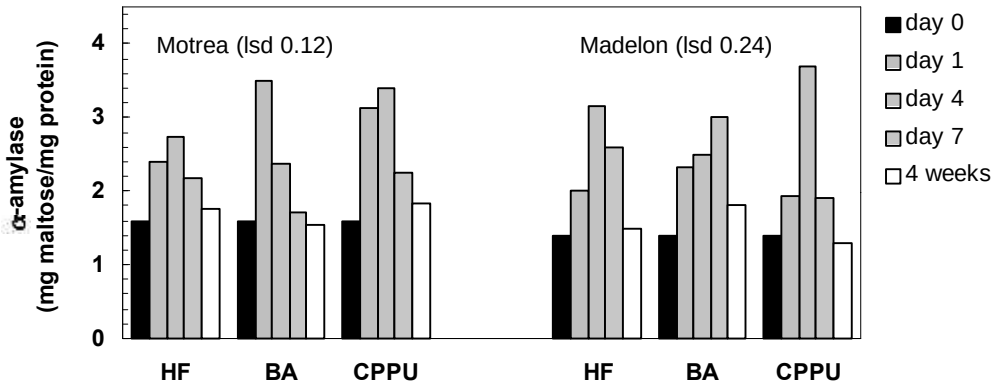

Fig. 2. Effect of 1.0 µM BA and 1.0 µM CPPU on α-amylase activity in *in vitro Rosa hybrida* L.

lon, BA caused a higher increase of α-amylase activity in comparison to CPPU while on day 4th stronger acceleration of the activity of α-amylase was measured in the explants cultured on media with CPPU in comparison to the effect of BA. An enhancement of α-amylase activity occurred in CPPU treated explants of cv. Motrea on day 7th when the activity exceeded that in BA-treated explants. An opposite effect was observed on day 7th in cv. Madelon. In 4-week-old rose plants the activity of α-amylase diminished to the level close to that on day 0 (before explant's isolation). These results indicated that α-amylase played a role in bud opening of the *in vitro* cultured rose plants. The stimulation of the activity by CPPU was exerted later than by BA, but the enhancement of α-amylase activity correlated with higher number of open buds at the end of 1st week of culture. It may be assumed that the active degradation of starch that occurred on day 4 in response to CPPU application is a necessary tool for stimulation of bud outgrowth thus helping the apical dominance release. Recent studies have shown that the addition of kinetin to culture media strongly reduces the IBA-stimulated starch accumulation during root formation in *in vitro* cultured hypocotyl cuttings of *Pinus radiata* (Li and Leung, 2000). This might help the explanation of the effect of cytokinins on starch breakdown during the outgrowth of axillary buds *in vitro*.

The impact of cytokinins BA and CPPU on protease was traced within a period of one week and in 1-month-old plants. On day 1st of culture cv. Motrea showed lower protease activity than that in the control in response both to CPPU and BA while in cv. Madelon an opposite effect of enhanced protease activity was found (Fig. 3). After 4 days a decrease in protease activity in comparison to culture on hormone-free media was registered in cv. Madelon on media with either BA or CPPU. On the same day enzyme activity acceleration was observed in cv. Motrea when cultured with BA. In comparison to hormone-free media much lower protease activity was detected in one-

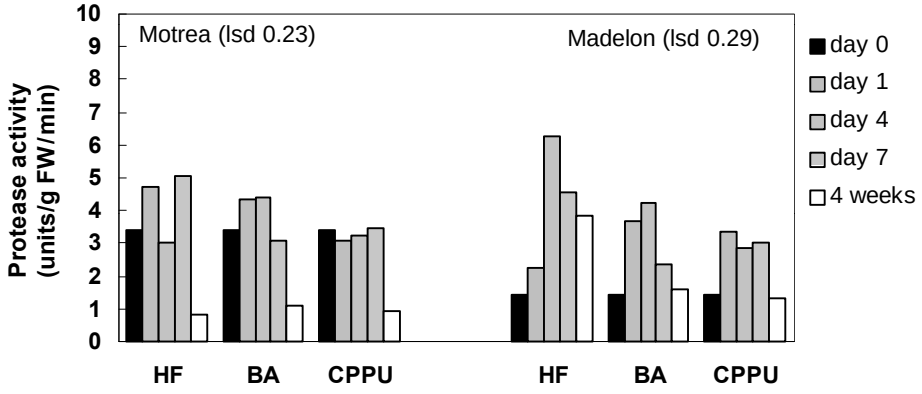

Fig. 3. Effect of 1.0 µM BA and 1.0 µM CPPU on protease activity in *in vitro Rosa hybrida* L.

month-old plants of cv. Madelon when treated with cytokinins and a decline was established in cv. Motrea in respect to the tendency within the first 7 days.

When the explants were cultured on hormone-free media, some of the buds opened, but the development of the plants was suppressed. For a normal *in vitro* culture of roses the addition of cytokinins is a necessary condition. Significantly lower protease activity was detected in cv. Madelon at the end of 4th week in comparison to culture on HF media and this was in correlation with better development of the plants. It could be suggested that keeping the activity of proteases at lower level cytokinins are able to support the growth and development. On the other hand, the increase in protease activity in cytokinin-treated explants of cv. Madelon on day 1st might be an indication for apical dominance release, i.e. cytokinins might regulate the process of apical dominance release via an indirect mechanism acting through proteases. Cv. Madelon which was characterized by stronger apical dominance, formed less number of shoots and showed higher protease activity than cv. Motrea even in hormone-free media. Although it is not easy to find a clear correlation between protease activity and the effect of cytokinins in our model it could be suggested that lowering the activity of proteases, CPPU and BA may delay the destructive processes including the early senescence of the explants, thus promoting the outgrowth of axillary buds.

The role of proteases in plant development is still an object of discussions. In different plant organs different proteases are activated. In young leaves aminopeptidases are prevailing, whereas in senescing tissues mainly carboxypeptidases and endopeptidases are actively functioning (Peoples and Dalling, 1988). In *in vitro* cultures it is suggested that the activation of different proteases is due to different compounds in the nutrition media and in this context an activation of carboxypeptidases has been proposed (Fisher et al., 1998). In our experiments we did not study the activity of different groups of proteases and only a total protease activity was determined. As in the earlier periods of culture senescence is not an expected event, the lower protease activity in cytokinin-treated explants may be considered as a marker for development stimulation.

The reported results support the view that phenylurea cytokinins possess higher activity in comparison to purine cytokinins (Karanov et al., 1992) and provide information about the physiological effects of BA and CPPU on α-amylase and protease activities showing that the changes in these enzymes are involved in the mechanism of bud opening in *in vitro* cultured rose plants and might be a sequence of apical dominance release.

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