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EFFECT OF CYTOKININS ON *IN VITRO* CULTURED *EXACUM AFFINE* BALF.

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ABSTRACT. The effect of purine (BA) and phenylurea (CPPU) cytokinins on the development of *in vitro* cultured *Exacum affine* Balf. F. has been studied. We have shown that when the culture medium was supplemented with either BA or CPPU, the physiological features, such as the number of shoots and shoot fresh weight were stimulated. In contrast to our results for better effect of CPPU on *in vitro* cultured *Rosa hybrida* L. (Kapchina-Toteva *et al*., 2000), BA was found more efficient in improving the growth and development and in increasing the dry weight of micropropagated *Exacum affine*. Vitrification and lower number of shoots were established at application of 5 and 10 µM CPPU. 5 µM BA has been recommended for mass *in vitro* propagation of Persian violet. Activity of guaiacol-peroxidase was determined and discussed regarding the break and growth of axillary buds.

KEY WORDS. cytokinins, *Exacum affine*, micropropagation, peroxidase

INTRODUCTION

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 a process of apical dominance release, can be enhanced in response to exogenous cytokinins (Cline, 1994). Purine-type cytokinins have been most extensively studied and found to effectively release the apical dominance in *in vitro* propagated ornamentals (Lloyd *et al*. 1988; Bollmark *et al*. 1995; Arnold *et al*. 1992; van Telgen *et al*. 1992, Kapchina-Toteva et al., 2000). Also, phenylurea derivatives possessing cytokinin activity have been shown to express high activity and to exert positive physiological impact in a number of test systems for micropropagation (Takahasi *et*

al. 1978; Iwamura *et al*. 1980; Mok *et al*., 1982; Shudo, 1994). Supplementation of medium for *in vitro* apple and grapevine culture with thidiazuron (TDZ) or N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) causes an enhanced outgrowth of axillary buds (Gribaudo and Fronda, 1991; Sigiyama *et al*., 1993). In *in vitro* regeneration systems of *Pellargonium*, CPPU and TDZ induce somatic embryogenesis (Murthy *et al*., 1996; Mithila *et al*., 2001). Samyn *et al*. (2002) have reported an improvement of adventitious shoot regeneration of azalea (*Rhododendron simsii*) cultured on medium containing TDZ. Our study on *in vitro* cultured *Rosa hybrida* L. indicated that purine cytokinin N^6-benzyladenine (BA) and phenylurea cytokinin CPPU stimulated the axillary bud break and increased the number of open buds (Kapchina-Toteva *et al*., 2000). It has been demonstrated that in *in vitro* cultured *Gypsophila paniculata* L. the addition of CPPU to culture medium enhanced the physiological features such as bud sprouting and shoot fresh and dry weight (Kapchina-Toteva and Stoyanova, 2003).

Exacum affine, known commercially as Persian violet belongs to Family *Gentianaceae* and is grown for outdoor annual or indoor potted small bushy plant. It is normally propagated by seeds, and produces fragrant, blue, purple or white coloured flowers. The 40 known species of genus Exacum (Cronquist A., 1988) are distributed over a wide range from the central zone of Africa, including Madagascar, to the Arabian peninsula, Eastern India, the Himalayas and Sri Lanka, Malaysia, the eastern part of New Zealand (Riseman and Craig, 1995), Europe, Japan, USA (Serek and Trolle, 2000). Constituents of *E. affine* are p-coumaric acid, affinoside and glucosides (Kuvajima *et al*., 1996). Exacum is susceptible to a number of fungal diseases (*Botrytis, Fusarium, Nectria, Phytophthora, Pythium*) and virus infection (*Impatiens necrotic spot virus*). A survival mechanism, expressed as a zinc deficiency has been found in some crosses of Persian violet (Struwe *et al*., 2002). Micropropagation of *Exacum affine* provides an advantage for rapid multiplication of disease-free homogenous planting material, for obtaining a large number of cuttings and for effective control of plant growth and development through the use of plant growth regulators. *Exacum affine* is a good model system for testing cytokinins and for study on the physiological events, involved in the process of *in vitro* plant development. Our preliminary observations showed that at certain conditions *Exacum affine* goes to flowering *in vitro* and depending on the supplement with plant growth regulators produces various numbers of brunches (unpublished data).

Cytokinins have been found as effective free radical scavengers (Leshem, 1988). The formation of active oxygen species can affect the morphogenesis of plant cells and tissues and to inhibit the process of development in *in vitro* culture. Moreover, the accumulation of active oxygen species has been established to suppress explant development and, hydrogen peroxide (H_2O_2) concentration as far as it is involved in detoxication of active oxygen, plays an important role in cell division (Benson and Roubelakis-Angelakis, 1994; Marco and Roubelakis-Angelakis, 1996). The plant cells possess highly efficient defence systems for elimination 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.

The present study was undertaken to investigate the effect of purine (BA) and phenylurea (CPPU) cytokinins on the development of *in vitro* cultured *Exacum affine* Balf. F. Here we have shown that when the culture medium was supplemented with either BA or CPPU, the physiological features, such as the number of shoots and shoot fresh weight were stimulated. In contrast to our results for better effect of CPPU on *in vitro* cultured *Rosa hybrida* L. (Kapchina-Toteva *et al*., 2000), BA was found more efficient in improving the growth and development and in increasing the dry weight of micropropagated *Exacum affine*. Activity of guaiacol-peroxidase was determined and discussed regarding the break and growth of axillary buds.

MATERIAL AND METHODS

Plant materials and cultivation

The experiments were carried out with shoot cultures of *Exacum affine* Balf. F. Plants were subcultured every five weeks on standard medium (MS at full strength, 2.0 % (w/v) sucrose, 7 $g.l^{-1}$ agar, pH 5.8). Growth conditions were 22-23° 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 cytokinins, autoclaved with the medium.

Evaluation of cytokinin effect on plant growth and development

The synthetic cytokinins of purine-type N^6 benzyladenine (BA) and phenylurea-type N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) were tested in three concentrations - 1.0 µM; 5.0 µM and 10.0 µM. The bud break was determined as a percentage of open buds 4 weeks after the transfer. At the same duration after subculture, the number of shoots developed from the open axillary buds, shoot length, fresh and dry weight were measured.

Enzyme assay

Peroxidase (EC 1.11.1.7) activity was determined following 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 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 sec intervals. Enzyme activity was defined as µmol reduced guaiacol. mg $protein^{-1}$. min^{-1}, using molar extinction coefficient of reduced guaiacol (ε=26.6 mM^{-1}. cm^{-1})

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

Data evaluation

The statistical significance of the data was assessed by SEM which was the standard error of M_n; $n \geq 10$.

RESULTS AND DISCUSSION

In vitro growth and development

The addition of BA to culture medium considerably enhanced the number of shoots developed from single explant (Figure 1). Approximately 6 times increase was recorded in response to 5 µM BA in reference to control plants that were grown on standard media. 1 µM and 10 µM BA also significantly stimulated the development of new shoots. When the media was supplemented with 1 µM, 5 µM or 10 µM CPPU the number of brunches increased but to lesser extend in comparison to the effect of BA (Figure 1).

The results for fresh weight (FW) of plants are presented on Figure 2. Slight increase of FW was detected in BA treated plants. CPPU in concentration of 5 µM and 10 µM caused stronger magnification of FW. In contrast to this effect of latest two CPPU concentrations was the accumulation of dry mass (Figure 3). The established decrease of dry weight (DW) in comparison to control and BA cultured plants was accompanied with vitrification.

Vitrification is undesirable physiological process that appears only at *in vitro* culturing and is due to hormonal imbalance. It is suggested that at the process of subculture the level of cytokinins in plants increases which negatively affects the balance with the other hormones. Among the characteristics of vitrification are infiltration of water in the tissues (water space may increase up to 40% versus the normal 5%), expansion of cell size, elongation and curling of the leaves, decrease of dry weight in stems and leaves, reduced lignification of cell walls, diminished peroxidase activity, dilution of plastid pigments (lower amount per *g* FW), abnormal growth.

Vitrification was not observed in plants grown on media with 1 µM CPPU where percentage of DW from FW was higher in reference to the control. At medium supplemented with any of the three tested BA concentrations the accumulation of dry mass was better pronounce than on plants grown without cytokinins (Figure 3).

The increase of shoot number and DW and, the lack of vitrification in plants cultured in media containing BA resulted in good quality of micropropagated plants, which is an important reason to recommend BA for *in vitro* propagation of *Exacum affine*. Considering the number of in vitro developed brunches, which is of commercial interest, the addition of 5 µM BA was the most appropriate media composition.

Physiological status of plants, among a number of factors, depends on growth and climate conditions, light, nutrition and the balance of phytohormones. In *in vitro* plants cultivation conditions are controlled and plant development is influenced by the uptake and utilization of nutrients and plant growth regulators from the media and, to significant extend by endogenous concentration of hormones. The action of purine and phenylurea cytokinins is strongly dependent on their relation to the level of other plant growth regulators, plant species and cultivar peculiarity. Although in a number of model systems a higher activity and better expressed physiological effect of phenylurea cytokinins have been found (Gribaudo and Fronda, 1991; Sigiyama *et al.*, 1993; Mok *et al.*, 1982; Kapchina-Toteva *et al.*, 2000; Samyn *et al.*, 2002;

Kapchina-Toteva and Stoyanova, 2003), in present experiments with Persian violet we have established that purine type cytokinin BA affected more positively the growth, development and the appearance of microplants. This can be caused either by better uptake and more efficient utilization of the purine cytokinin or by the lower level of endogenous cytokinins. Since phenylurea cytokinins are reported to be more active, their negative influence on plant development might be due to overdose of cytokinins in the plant, which causes a suppression of the growth and vitrification thus harming the quality of microplants.

Effect of BA and CPPU on guaicol peroxidase activity

Peroxidase (EC 1.11.1.7) is among enzymes expressing antioxidative functions. In present study we have measure the activity of guaiacol-peroxidase regarding the effect N^6-benzyladenine (BA), and N_1-(2-chloro-4-pyridyl)-N_2-phenylurea (4-PU-30) on shoot development in *in vitro* cultured *Exacum affine*. In plants grown on medium containing 1 µM and 10 µM BA or 1 µM, 5 µM and 10 µM CPPU (Figure 4), the activity of peroxidase did not differ significantly from control plants. An increase was only found in plant supplemented with 5 µM BA. Such an enhancement of peroxidase activity can be caused by stress due to changes in media composition, but since it was not observed in the other variants of the media, most probably is attributed to the function of peroxidase in detoxification of active oxygen species that promoting growth of the explants. Moreover, the acceleration of enzyme activity was associated with formation of more shoots and accumulation of dry weight. We hare earlier reported an enhancement of peroxidase activity in response to cytokinins and in association with increased number of open axillary buds in *in vitro* cultured *Rosa hybrida* L (Kapchina-Toteva, V., Yakimova E. (1997)

These results supported the view that phenylurea cytokinins could be more active than purine cytokinins. The acceleration of peroxidase activity was associated with increased number of open axillary buds. The stimulation of peroxidase in present experiments in accordance to enhancement of shoot growth and development at 5 µM BA supports the view that cytokinins might make this enzyme active thus controlling the level of H_2O_2 and the rate of cell division. In addition, we suggested that the activity of peroxidase could be used as a biochemical marker of development in the studied plant object.

CONCLUSIONS

The supplement of the media for micropropagation of *Exacum affine* with BA exerts positive effect on the increase of shoot number and accumulation of dry mass in plants.

5 µM BA is recommended as a component of culture media for *Exacum affine*.

Stimulation of number of brunches and accumulation of dry weight are accompanied by an enhancement of guaiacol peroxidase activity.

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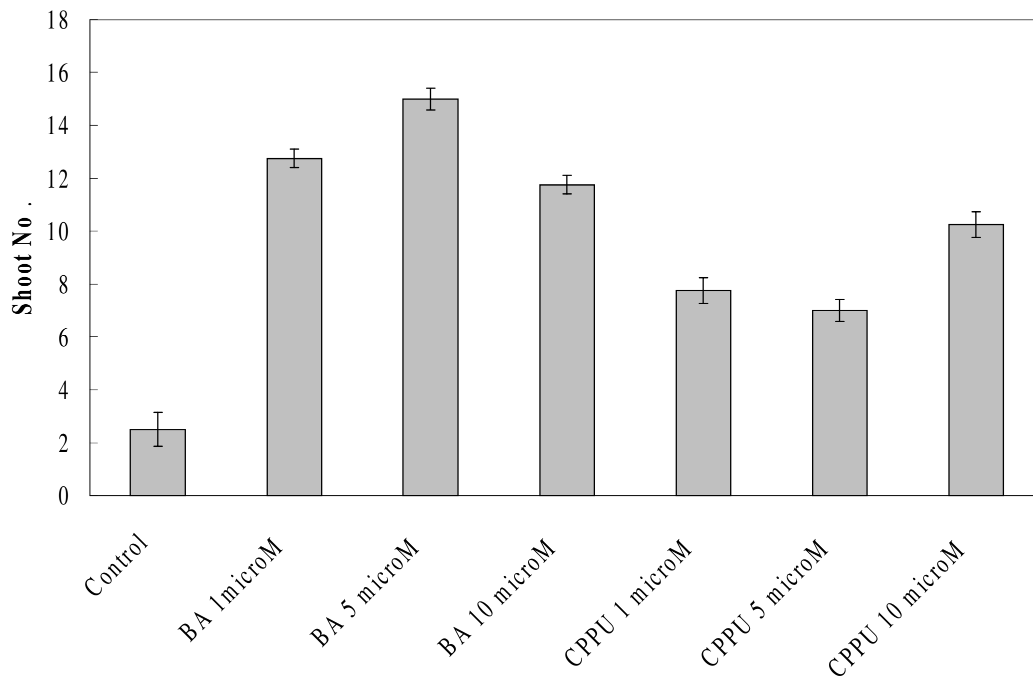

Figure 1. Effect of BA and CPPU on shoot number in *in vitro* cultured *Exacum affine* Balf.

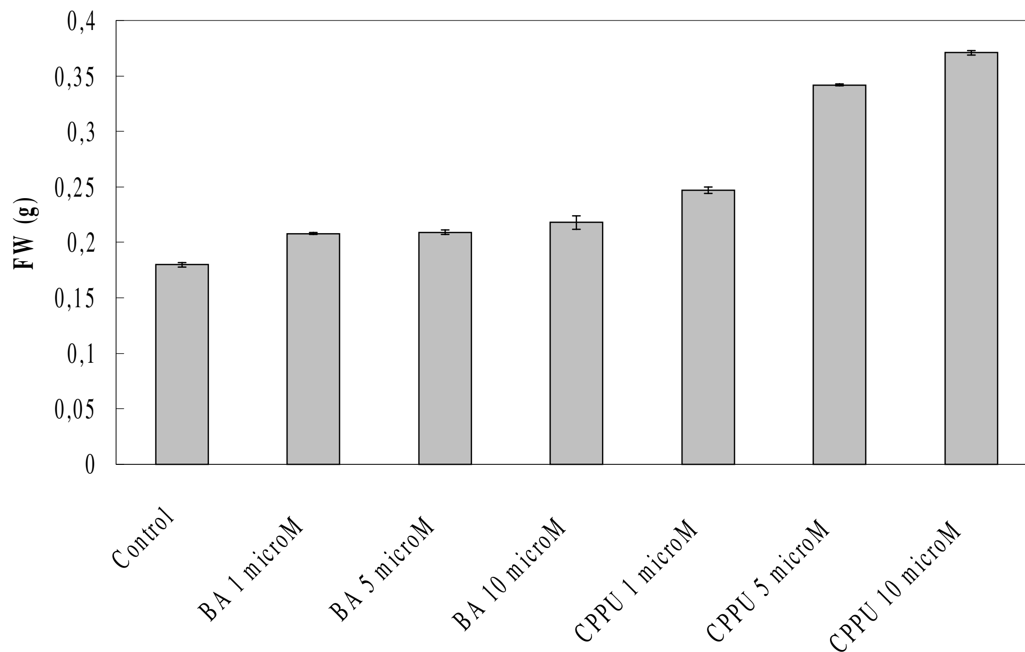

Figure 2. Effect of BA and CPPU on fresh weight (FW) of *in vitro* cultured *Exacum affine* Balf.

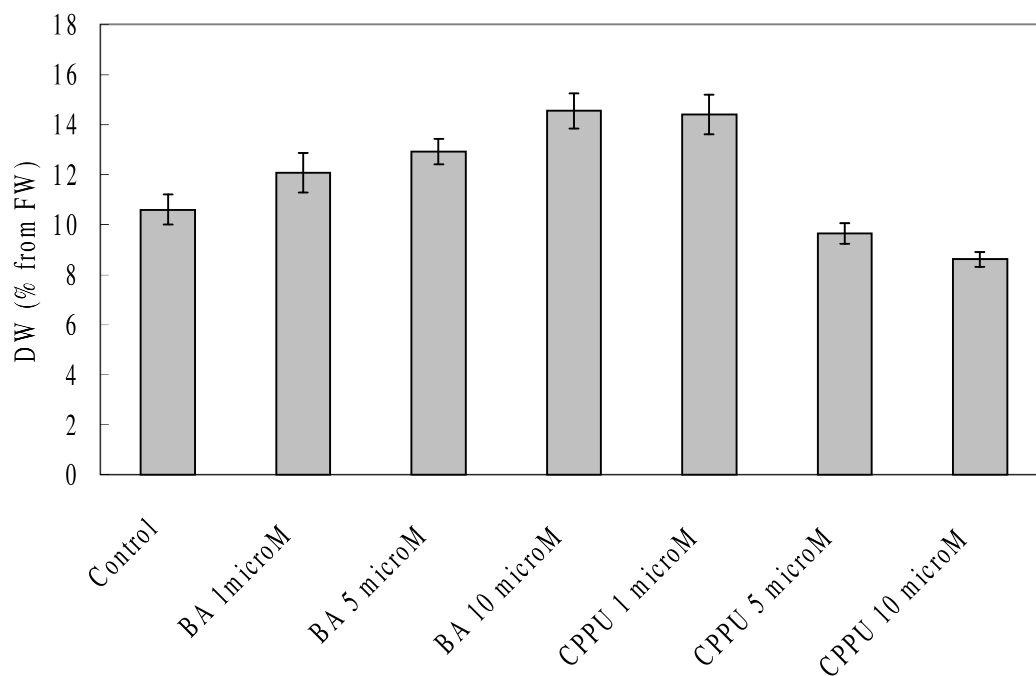

Figure 3. Effect of BA and CPPU on dry weight (DW % from FW) of *in vitro* cultured *Exacum affine* Balf.

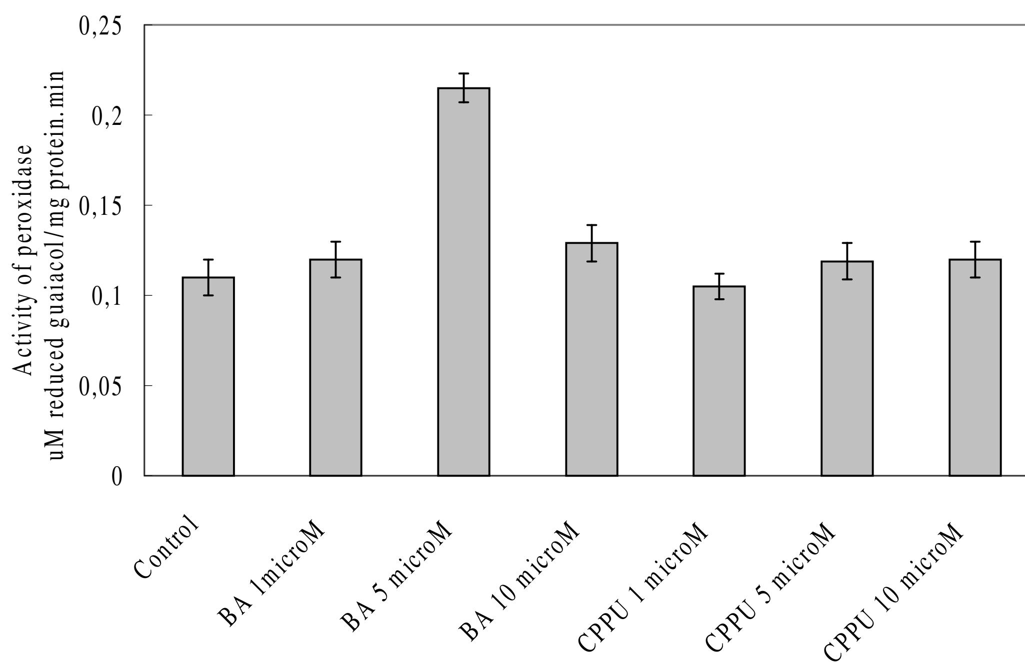

Figure 4. Activity of guaiacol peroxidase in *in vitro* cultured *Exacum affine* Balf.