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EFFECT OF CYTOKININ-ACTIVE PHENYLUREA DERIVATIVES ON SHOOT MULTIPLICATION, PEROXIDASE AND SUPEROXIDE DISMUTASE ACTIVITIES OF *in vitro* CULTURED CARNATION

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Summary. In the present study examined the effects of different types of cytokinins on the growth of *in vitro* cultured carnation. NAA alone produced neither multiplication nor elongation. Combined with BAP, NAA increased multiplication. The biological activity of 4-PU-30 and especially that of thidiazuron was considerably higher than BAP's activity. In equal concentrations with BAP thidiazuron increased more than 6 times the number of shoots and about 5 times the number of explants per culture in cv. White Sim. All tested concentrations of 4-PU-30 and thidiazuron increased the fresh weight per culture in cv. Red Lena, more than BAP. Application of 4-PU-30 and thidiazuron induced changes in all fractions of peroxidase but soluble peroxidase had highest effect. New bands only be seen after application of both 4 μM and 0.4 μM 4-PU-30. SOD activity was stimulated by BAP. All studied concentrations of thidiazuron and 4-PU-30 also increased SOD activity. Strongest increase in SOD activity is in the highest concentration of thidiazuron.

Key words: carnation, multiplication, thidiazuron, 4-PU-30, peroxidase, SOD.

Abbreviations: NAA – naphthalenacetic acid; BAP – benzylaminopurine; TDZ – thidiazuron; SOD – superoxide dismutase

Introduction

Plant growth regulator studies and plant tissue culture research are closely related and mutually supported. The manipulation of plant cells, tissues and organs in culture, with important applications in propagation and genetic modification of plants, is

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highly dependent on the use of appropriate growth regulator regimes. On the other hand tissue culture systems are useful as bioassays to define the growth-regulating activity of many compounds. The discovery of cytokinin N-(2-furanylmethyl)-1H-purin-6-amine (kinetin) by Miller et al. (1956) was particularly relevant in this respect.

Naturally occurring cytokinins, as well as many synthetic ones, contain adenine and a side chain at the N^6 position. However, as early as 1955, an unrelated compound, N,N'- diphenylurea (DPU), was found to possess growth-promoting properties (Shantz and Steward, 1955). Although the cytokinin activity of DPU was generally low compared to that of adenine derivatives, highly active phenylurea compounds were subsequently discovered. Some examples are N-phenyl-N′-(2-chloro-4-pyridyl)urea (4-PU-30), (Takahashi et al., 1978), and N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuron, Dropp), which exibit activities higher than the activity of (E)-2-methyl-4-(1H-purin-6-ylamino)-2-buten-1-ol (zeatin) in several bioassays (Mok and Mok, 1985; Alexieva and Karanov, 1992; Karanov et al., 1992; Karanov et al., 1992).

Vitrification is a morphological and physiological disorder frequently affecting both herbaceous and woody plants during *in vitro* vegetative regeneration (Leshem, 1983; Meira et al., 1983). The role of growth factor imbalance as an inducer of vitrification has been discussed. It has been shown that, in carnation, high concentration of NAA in the culture medium increases the proportion of shoots that turn into vitrified plantlets, while BAP has the opposite effect (Leshem et al., 1988). Gaspar et al. (1987) have found that BAP availability in the culture medium induces vitrification in apple.

Peroxidase is a multifunctional enzyme. As said by Edreva (1988) the different molecular forms of peroxidases take part in growth control, development, differentiation and morphogenesis. That is why the tissue, organ and ontogenetic specifications of peroxidases (iso-peroxidases) could be used as a markers for the plant physiological status.

The role of peroxidases in vitrification is specific and important for *in vitro* cultures. It cannot be said that vitrified carnation tissues lose peroxidases in general since guaiacol activity was high in soluble and membrane fractions, low in wall ionic ones and high in wall covalent ones (Kevers and Gaspar, 1985; Kevers et al., 1987).

Superoxide dismutases (SODs) are metaloproteins that catalyze the dismutation of superoxide radicals to hydrogen peroxide and oxygen. In plants, environmental adversity often leads to increased generation of reduced oxygen species and consequently, SOD has been proposed to be important in plant stress tolerance. In some cases the changes in activity of peroxidase and SOD go parallelly. For example no difference was found in the enzymatic activities of SOD and peroxidase in healthy and damaged spruce needles (Osswald et al., 1992).

In the present study we examined the effects of different types of cytokinins on the growth of *in vitro* cultured carnation. As markers for the physiologycal status of plants we used the enzymatic activities of peroxidase and superoxide dismutase.

Materials and Methods

Axial buds of virus-free plants of *Dianthus caryophyllus* cv. White Sim and Red Lena were cultured on solid Murashige and Skoog's medium which contained 0.8% Bacto agar, 0.5 µM NAA and 0.4 µM BAP according Leshem (1983) or different thidiazuron and 4-PU-30 concentrations. Dropp (50% thidiazuron) from "Schering" was used. Plant material was previously *in vitro* propagated on a solid MS medium without addition of cytokinins. Plantlets on a medium free of growth regulators were used as a control. The pH was adjusted to 5.7 before autoclaving. Cultures were grown at 24–25 °C in 16 h photoperiod, provided by cool-white fluorescent tubes giving approx. 3 000 lx. One month after culture initiation the shoots were removed, their number and weight were recorded, and the enzyme activity was measured.

Enzyme Extraction and Measurement

Soluble peroxidase. Stems with leaves were homogenized in 0.1 M phosphate buffer (KH_2PO_4, Na_2HPO_4) at pH 6.0 (tissue : buffer ratio 1:4, wt/vol) in the presence of Dowex resin (anion exchange 1×8, 200/400 mesh) with the aim of removing phenolic inhibitors of peroxidase. The supernatant was used for determination of soluble peroxidase activity.

Ionically bound peroxidase. Residue was extracted overnight at 4 °C with 1 M KCl in 0.1 M phosphate buffer at pH 6.0. The extract was then centrifuged, dialized against 0.05 M phosphate buffer at pH 6.0 and assayed for peroxidase activity. This was considered the ionically bound fraction.

Covalently bound peroxidase. The residue, after extraction of the ionically bound fraction, was incubated for 5 h at room temperature with 0.2 M phosphate buffer, pH 5.25. It contained peroxidase-free cellulase (2.5 mg/ml) and pectinase (1.5 mg/ml).

Peroxidase activity. Guiacol (20 mM) was used as the hydrogen donor in the assay for peroxidase activity, with 0.1 M phosphate buffer at pH 6.0, and 10 mM hydrogen peroxide substrate. Enzyme activity was measured by following the increase in absorbance at 470 nm per minute per unit fresh weigth or mg protein.

Isoenzyme separation and gel staining. The procedure described by Davis (1964) was used with 7.5 % polyacrylamide for the separating gel and 4% for the stacking gel. Gel was incubated in the presence of 10 mM benzydine for visualisation and 10 mM hydrogen peroxide as a substrate.

Enzyme extraction, incubation of the extract and determination of superoxide dismutase activity are according to Beauchamp and Fridovich (1970). Enzyme activity was defined in terms of the quantity extract giving 50% inhibition of the reduction of NBT.

Protein was determined according to Bradford (1976).

Results and Discussion

The effects of tested cytokinins on shoot formation have been determined in two cvs. White Sim and Red Lena. Auxin (0.5 µM NAA) alone produced neither multiplication nor elongation of the explants. When combined with BAP, NAA increased the multiplication coefficient to 7.23. The biological activity of 4-PU-30 and especially that of thidiazuron was considerably higher than BAP's activity. In equal concentrations with BAP thidiazuron increased more than 6 times the number of shoots and about 5 times the number of explants per culture in cv. White Sim. The effect of 4-PU-30, but in higher concentrations, was similar.

Thidiazuron (0.04 µM) was more active than BAP even in 10 times lower concentration. Also the lowest examined concentrations of phenylurea derivatives increased more the length of stems in comparison with BAP (Table 1). Similar are the results in

Table 1. Effects of cytokinins on shoot multiplication in carnation cv. White Sim

Treatment concentrations µM		Number of shoots per culture	Length of shoots	Number of explants per culture
1	Control	1.05 ±0.15	6.90 ±1.29	4.17 ±1.24
2	NAA 0.5	1.53 ±0.66	6.83 ±1.03	5.90 ±0.98
3	BAP 0.4 + NAA	2.22 ±0.12	5.12 ±0.74	7.23 ±0.76
4	4-PU-30 4 + NAA	14.19 ±0.63	3.62 ±0.88	42.49 ±9.23
5	4-PU-30 0.4 + NAA	4.71 ±1.11	6.58 ±0.56	10.21 ±1.25
6	4-PU-30 0.04 + NAA	2.17 ±0.05	7.49 ±0.39	9.11 ±1.06
7	4-PU-30 0.004 + NAA	2.00 ±0.24	6.83 ±0.75	8.19 ±1.41
8	TDZ 0.4 + NAA	13.57 ±0.76	3.16 ±0.65	35.47 ±1.82
9	TDZ 0.04 + NAA	3.75 ±0.36	6.35 ±0.36	14.96 ±2.04
10	TDZ 0.004 + NAA	2.19 ±0.06	8.03 ±0.83	10.43 ±1.33

cv. Red Lena. The highest concentrations – 4 µM for 4-PU-30 and 0.4 µM thidiazuron increased the number of shoots per culture but the stems were shorter and with large leaves (Fig. 1 and 2). Shoot growth was strongly dependent on cytokinin supply to the medium. The mean number of shoots per culture was rising with the increase in cytokinin concentrations. Maximum multiplication rate (42.49 and 35.47 shoots per culture) was obtained with 4 mM 4-PU-30 and 0.4 mM thidiazuron but those shoots failed to elongate, often assuming the forms of tiny rosettes.

Effects of 4-PU-30 and thidiazuron on the multiplication coefficient in carnation are closely related to the alteration of fresh and dry weight. All tested concentrations of 4-PU-30 and thidiazuron increased more the fresh weight per culture in cv. Red Lena, compared to BAP. Similar were the effects in cv. White Sim except one concentration (4-PU-30). No significant dry weight changes were observed after application of BAP. But in all concentrations of thidiazuron and 4-PU-30 the dry weight per one gram fresh weight

Fig.1. Effects of 4-PU-30 on shoot multiplication in carnation cv. Red Lena
1. NAA 0.5 µM
2. BAP 0.4 µM + NAA 0.5 µM
3. 4-PU-30 4 µM + NAA 0.5 µM
4. 4-PU-30 0.4 µM + NAA 0.5 µM
5. 4-PU-30 0.04 µM + NAA 0.5 µM
6. 4-PU-30 0.004 µM + AA 0.5 µM

Fig 2. Effects of thidiazuron on shoot multiplication in carnation cv. Red Lena
1. NAA 0.5 µM
2. BAP 0.4 µM + NAA 0.5 µM
3. TDZ 0.4 µM + NAA 0.5 µM
4. TDZ 0.04 µM + NAA 0.5 µM
5. TDZ 0.004 µM + NAA 0.5 µM

decreased. This effect was stronger in high concentrations. However if we calculate the dry weight per one plant we can see that it was increased by all cytokinin treatments (Table 2 and 3).

Table 2. Effect of cytokinins on shoot growth in carnation cv. White Sim

	Treatment µM	Fresh weight per culture	Dry weight per g.fr.w.	Dry weight per culture
1	Control	0.175 ±0.013	0.125 ±0.009	0.021 ±0.009
2	NAA 0.5	0.234 ±0.021	0.117 ±0.007	0.028 ±0.007
3	BAP 0.4 + NAA	0.312 ±0.018	0.135 ±0.005	0.042 ±0.008
4	4-PU-30 4 + NAA	1.003 ±0.047	0.058 ±0.011	0.058 ±0.003
5	4-PU-30 0.4 + NAA	0.474 ±0.033	0.059 ±0.008	0.028 ±0.004
6	4-PU-30 0.04 + NAA	0.268 ±0.030	0.118 ±0.009	0.032 ±0.005
7	4-PU-30 0.004 + NAA	0.353 ±0.024	0.128 ±0.006	0.045 ±0.007
8	TDZ 0.4 + NAA	1.036 ±0.035	0.049 ±0.010	0.051 ±0.006
9	TDZ 0.04 + NAA	0.540 ±0.022	0.081 ±0.012	0.044 ±0.010
10	TDZ 0.004 + NAA	0.307 ±0.012	0.120 ±0.008	0.037 ±0.006

Table 3. Effect of cytokinins on shoot growth in carnation cv. Red Lena

	Treatment µM	Fresh weight per culture	Dry weight per g.fr.w.	Dry weight per culture
1	Control	0.181±0.044	0.133±0.010	0.024±0.008
2	NAA 0.5	0.216±0.025	0.121±0.006	0.026±0.005
3	BAP 0.4 + NAA	0.205±0.022	0.134±0.009	0.027±0.003
4	4-PU-30 4 + NAA	1.081±0.059	0.060±0.008	0.065±0.004
5	4-PU-30 0.4 + NAA	0.349±0.012	0.068±0.007	0.027±0.005
6	4-PU-30 0.04 + NAA	0.347±0.022	0.120±0.008	0.042±0.006
7	4-PU-30 0.004 + NAA	0.368±0.041	0.128±0.011	0.047±0.005
8	TDZ 0.4 + NAA	1.098±0.093	0.055±0.008	0.060±0.007
9	TDZ 0.04 + NAA	0.644±0.038	0.090±0.005	0.058±0.006
10	TDZ 0.004 + NAA	0.248±0.017	0.128±0.009	0.032±0.002

Thidiazuron and 4-PU-30 effects in micropropagation systems are not well examined. However, it is known that thidiazuron is metabolized by bean callus tissues (Mok et al., 1985). Also Garelkova and Alexieva (1992), Marinova and Iliev (1992) studied the response of oriental beech *(Fagus orientalis* Lips*.)* to 4-PU-30 and the effect of thidiazuron on adventitious bud formation in *Cordyline terminalis*. Thidiazuron also caused profound stimulation of shoot formation in apple *(Malus* sp*.)* cultured *in vitro*, in hackberry *(Celtis occidentalis* L*.)* and in a difficult to propagate *Acer clone*. Meristem formation in *Torenia* cultured *in vitro* was increased by 4-PU-30 which also stimulated shoot formation in mulberry *(Morus* sp*.)* hypocotyls cultured *in vitro* at a greater rate than when BAP was used. In micropropagation of azaleas zeatin induced highest microshoot production at 50 µM. The same effect with 4-PU-30 was reached at its lowest concentration – 0.5 µM. As Mok et al. (1987) showed 0.3 µM thidiazuron was very effective in broccoli *(Brassica oleracea* L*.)* multiplication and 10 µM already induced abnormal growth and vitrification. In our experiments with carnation the effective concentrations were about 10 and 100 times lower than these for broccoli (Fellman et al., 1987; Mok et al., 1987).

Peroxidase activity in carnation shoots was stimulated by BAP while NAA was ineffective. Highest activity in control plants was determined for ionically bound peroxidase. Application of 4-PU-30 and thidiazuron induced changes in all fractions of peroxidase, but soluble peroxidase led to strongest rise. In many cases ionically bound peroxidase decreased. The effect of most concentrations on ionically bound peroxidase is opposite to the effect of BAP (Table 4 and 5). If peroxidase activity is calculated per mg protein similar effect may be seen. Cytokinins increase soluble peroxidase. This effect is stronger in the highest 4-PU-30 and thidiazuron concentrations. Thus, it can be concluded that growth effect of thidiazuron and 4-PU-30 is closely connected, more or less, with changes in all studied peroxidase fractions (Table 6).

Table 4. Effect of cytokinins on soluble, ionically and covalently bound peroxidase activity in carnation cv. White Sim

	Treatment Conc. µM	Peroxidase activity (ΔOD.min^{-1}.g fresh weight^{-1} Soluble	Ionically bound	Covalently bound
1	Control	53.8±7.6	108.2±3.1	26.6±4.3
2	NAA 0.5	55.9±4.3	130.1±0.9	27.4±5.3
3	BAP 0.4 + NAA	76.5±7.9	116.0±5.8	29.3±2.1
4	4-PU-30 4 + NAA	232.2±9.0	98.4±4.9	56.4±9.8
5	4-PU-30 0.4 + NAA	126.4±4.3	84.5±2.4	31.2±8.1
6	4-PU-30 0.04 + NAA	65.2±3.6	49.6±7.5	38.2±7.6
7	4-PU-30 0.004 + NAA	60.1±2.1	54.1±3.1	12.3±6.7
8	TDZ 0.4 + NAA	113.4±7.1	97.1±6.8	33.6±8.6
9	TDZ 0.04 + NAA	98.4±5.7	104.5±4.3	39.6±4.6
10	TDZ 0.004 + NAA	90.4±4.2	126.1±5.7	41.6±7.8

Table 5. Effect of cytokinins on soluble, ionically and covalently bound peroxidase activity in carnation cv. Red Lena

	Treatment Conc. µM	Peroxidase activity (ΔOD.min^{-1}.g fresh weigh^{-1}) Soluble	Ionically bound	Covalently bound
1	Control	42.7± 1.3	91.3± 5.6	22.6±1.3
2	NAA 0.5	40.2± 0.3	92.8± 3.3	23.5±3.2
3	BAP 0.4 + NAA	86.7± 0.7	126.7± 1.9	35.7±1.3
4	4-PU-30 4 + NAA	257.8± 6.1	135.2±18.8	51.8±2.4
5	4PU-30 0.4 + NAA	118.9± 1.1	83.2± 7.0	33.8±4.5
6	4-PU-30 0.04 + NAA	105.9± 4.9	91.8± 2.9	49.6±3.5
7	4-PU-30 0.004 + NAA	87.9± 1.6	88.5± 2.7	25.3±4.3
8	TDZ 0.4 + NAA	121.1± 4.2	84.0± 1.4	21.2±3.8
9	TDZ 0.04 + NAA	106.1± 3.6	86.4± 2.6	21.6±1.8
10	TDZ 0.004 + NAA	77.5± 2.2	94.9± 2.1	22.1±2.3

Distinct differences were found in the peroxidase isoenzyme profile of the soluble fraction when an identical amount of protein was loaded per lane. Generally three anionic isoenzymes were distinguished. New bands could be seen after application of both 4µM and 0.4µM 4-PU-30 (Fig. 3). Reports of the effect of different types of cytokinins on enzyme activities are limited. Miller (1985) suggested that cytokinins might modify reactions that produce hydrogen peroxide or affect peroxidases directly. Kuroda et al. (1990) showed that C_4 (R_f 0.40) chloroplast peroxidase was suppressed by kinetin treatment. The growth-promoting activity of thidiazuron was accompanied by high acid phosphatase levels shown by using polyacrilamide gel electrophoresis (Mok et al., 1987).

Table 6. Effect of cytokinins on soluble and ionically bound peroxidase activity in carnations cvs. White Sim and Red Lena (calculated per mg protein)

		Peroxidase activity (ΔOD.min^{-1}.mg prot^{-1})			
		White Sim		Red Lena	
	Treatment Conc. µM	Soluble	Ionically bound	Soluble	Ionically bound
1	Control	11.6±2.6	83.2±3.1	8.2±1.3	17.6±2.1
2	NAA 0.5	14.3±4.3	100.0±5.3	8.7±0.3	20.2±1.0
3	BAP 0.4 + NAA	27.3±2.9	89.2±5.8	29.6±0.7	145.3±8.2
4	4-PU-30 4 + NAA	192.6±9.0	75.6±4.9	184.1±6.1	96.6±8.5
5	4-PU-30 0.4 +NAA	78.9±4.3	65.0±8.4	72.9±1.1	52.2±6.3
6	4-PU-30 0.04+NAA	25.5±3.6	38.1±7.5	51.1±4.9	42.7±5.6
7	4-PU-30 0.004+NAA	13.2±2.1	41.6±6.1	21.3±1.6	21.6±5.3
8	TDZ 0.4 + NAA	141.8±7.1	74.6±6.8	152.1±3.5	105.5±8.7
9	TDZ 0.04 + NAA	56.2±5.7	80.3±4.3	60.2±8.6	49.4±6.3
10	TDZ 0.004 +NAA	28.5±4.2	97.1±5.7	27.9±2.2	34.5±5.4

Fig. 3. Detection in polyacrylamide gels of peroxidase activities in extracts prepared from carnation grown *in vitro* cv. Red Lena

1. Control
2. NAA 0.5 µM
3. BAP 0.4 µM + NAA 0.5 µM
4. 4-PU-30 4 µM + NAA 0.5 µM
5. 4-PU-30 0.4 µM + NAA 0.5 µM
6. 4-PU-30 0.04 µM + NAA 0.5 µM
7. TDZ 0.4 µM + NAA 0.5 µM
8. TDZ 0.04 µM + NAA 0.5 µM

Thus, it can be concluded that the growth effect of cytokinins may be related to changes in the isoenzyme profiles.

The activity of SOD in carnation shoots was promoted by BAP and slightly increased by NAA. All concentrations of thidiazuron and 4-PU-30 studied also increased SOD activity. Strongest increase in SOD activity was observed at the highest concentration of thidiazuron (Table 7).

Table 7. Effect of cytokinins on superoxide dismutase in carnations grown *in vitro*

		Units. mg prot.$^{-1}$	
	Treatment μM	cv. White Sim	cv. Red Lena
1	Control	6.76 ± 0.87	5.99 ± 1.33
2	NAA 0.5	7.85 ± 0.66	6.64 ± 1.78
3	BAP 0.4 + NAA	10.32 ± 1.90	8.92 ± 1.98
4	4-PU-30 4 + NAA	9.23 ± 0.85	7.85 ± 1.62
5	4-PU-30 0.4 + NAA	10.65 ± 1.67	9.30 ± 1.75
6	4-PU-30 0.04 + NAA	12.86 ± 1.91	11.15 ± 1.44
7	4-PU-30 0.004 + NAA	8.30 ± 1.44	8.93 ± 1.67
8	TDZ 0.4 + NAA	13.27 ± 1.41	19.63 ± 2.31
9	TDZ 0.04 + NAA	8.80 ± 2.09	10.40 ± 1.32
10	TDZ 0.004 + NAA	8.81 ± 1.32	11.03 ± 2.76

Cytokinin-active phenylureas, such as thidiazuron and 4-PU-30, show many biological properties qualitatively similar to those of adenine-type cytokinins but also some quite different properties. Similarly thidiazuron and 4-PU-30 are resistant to oxidases, they are stable, but biologically more active at low concentrations than the adenine-type cytokinins. These properties may enhance their future use in tissue culture manipulations. These findings may promote further investigations of the physiological properties and selectivity of phenylurea cytokinins.

As implied by these results substanses other than purine-type cytokinins can elicit cytokinin-like responses in carnation tissue culture systems (multiplication coefficient, growth, peroxidase and superoxide dismutase activities). Thidiazuron and 4-PU-30 are biologically more active at low concentration than BAP. Meanwhile, the results discussed here suggest that there is a great potential in employing thidiazuron and 4-PU-30 to obtain the desired cytokinin-type (and possible other) response. More research is necessary to gain greater efficiency and precision in their use.

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