BIOLOGIA PLANTARUM 38 (2): 237-244, 1996

Structural and functional alterations in radish plants induced by the phenylurea cytokinin 4-PU-30

E.Z. STOYNOVA, L.K. ILIEV and G.T. GEORGIEV

M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia - 1113, Bulgaria

Abstract

Single treatment of expanding radish leaves with N_1-(2-chloro-4-pyridyl)-N_2-phenylurea (4-PU-30) lead to the stimulation of root cambial tissue activity and root growth. Leaf thickness, the volume of chlorophyll (Chl) containing cells per unit leaf area, starch content in the chloroplasts, and the Chl content increased simultaneously. These alterations were associated with increased leaf net photosynthetic rate and stomatal conductance in treated plants.

Additional key words: chlorophyll, chloroplast ultrastructure, fresh mass, leaf anatomy, net photosynthetic rate, plant age, root, shoot, starch, stomatal conductance.

Introduction

Some cytokinins of the phenylurea type possess higher activity than the purine ones (Takahashi *et al.* 1978, Okamoto *et al.* 1981). The treatment of flowers and of the growing ovary of grapes, kiwi, melon, apple and pear with 4-PU-30 resulted in significant stimulation of their growth and ripening (Nickell 1991). Cytokinin-active phenylureas, such as 4-PU-30, displayed biological properties qualitatively similar to those of adenine-type cytokinins in tissue culture systems; they promoted callus growth, induce organogenesis, and stimulated the production of ethylene. However, they showed one unusual effect, the tendency to enhance cytokinin autonomy of tissues (Mok *et al.* 1987). The treatment of radish cotyledons provided evidence that the 4-PU-30 stimulated cell division as well as cell and intercellular space enlargements (Stoynova and Iliev 1990). Application of 4-PU-30 to the radish plants increased 3 - 4 fold the root mass while the effect of kinetin was similar, but poorly manifested (Iliev *et al.* 1991). These results revealed high activity of some phenylurea cytokinins and substantiate necessity of cytological and physiological

Received 22 June 1995, *accepted* 7 September 1995.
Acknowledgements: This study was supported by the Bulgarian National Foundation for Scientific Researches (Contract B-413). 4-PU-30 was kindly provided by Prof. K. Shudo, Faculty of Pharmaceutical Sciences, University of Tokyo, Japan. We wish also to thank Dr. T. Tsonev for valuable advises.

investigations to clarify their mode of action. In many plant systems the exogenously applied purine cytokinins affected various photosynthetic characteristics and distribution of newly formed biomass (for references see Čatský *et al.* 1993). The functional alterations occurred along with some structural alterations in some sensitive to cytokinin plant tissues and organs including those at subcellular level (Lichtenthaler and Buschmann 1978, Bosselaers 1983, Rovenská *et al.* 1984, Iliev 1991). There are almost no data on the effects of phenylurea cytokinins. Thus, the aim of the present work was to study some physiological and morphoregulatory effects of 4-PU-30 on radish plants.

Materials and methods

"Red with white root" garden radish plants (*Raphanus sativus* L.) were grown in a greenhouse in pots containing leached diluvial soil. The plants were single sprayed (1.5 cm^3 per plant) with 4-PU-30 (50 g m^{-3}) during the fully expanded second leaf stage. The control plants were sprayed with tap water.

The net photosynthetic rate (P_N) and stomatal conductance (g_s) were determined by a Portable Photosynthesis System *LI-6000* (*Li-Cor*, Lincoln, USA). Chl content was determined according to Arnon (1949). Thin transversal segments of the thickest part of the roots and pieces of the central parts of the 4th leaf blade were used for microscopic investigations. They were double fixed with 3 % (m/v) glutaraldehyde (phosphate buffer, pH 7.2) and 1 % (m/v) OsO_4. The samples were included in resin (Spurr 1969). The ultrathin sections contrasted with uranyl acetate and lead citrate were observed with the transmission electron microscope *Zeiss EM-109* (*Zeiss*, Jena, Germany). The semi-thin sections from the same material for light-microscope observation were stained with toluidine blue. Stereological analyses of internal leaf structure were carried out using photomicrographs of leaf transections after Parkhurst (1982) by: (*a*) counting the number of randomly placed on the micrographs stereological sampling points for measuring fractions of the tissue volumes occupied by air space or by cells of various types. The amount of points covering each of the tissue compartment is proportional to its relative partial volume; (*b*) counting the intersections of perimeters (cell wall) by randomly placed on the micrographs stereological sampling lines to measure the cell surfaces.

The results of all experiments were presented as average value of five replications (five to ten plants per replication). The results were statistically analyzed using the Student's *t*-test.

Results

After the 4-PU-30 treatment the accumulation of biomass in the storage roots was significantly faster than in the control, while the growth of the shoots was slightly retarded (Table 1). The water content of the treated plants was not significantly altered.

The leaves of treated plants were darker green compared with the same leaves in the control. Chl *a+b* content, expressed per unit fresh mass increased in the leaves of treated plants (Table 2). The Chl *a* content was affected stronger than the Chl *b* content, and hence the Chl *a/b* ratio was higher in the treated plants. P_N and g_s during the experimental period increased in 4-PU-30 treated plants (Table 2).

Table 1. Effect of 4-PU-30 (50 g m^{-3}) on fresh mass and water content [g] or [% of control] of *Raphanus sativus* L. in the stages of 4th and 8th leaf developed (mean of 10 replicates ± S.E.).

	Control mass [g]		4-PU-30 mass [g]		mass [%]		water [%]	
	4	8	4	8	4	8	4	8
Shoot	2.505±0.160	5.403±0.170	2.236±0.084	4.941±0.239	89.3	91.4	100.52	99.80
Root	0.175±0.038	1.037±0.125	0.309±0.040*	2.515±0.333*	176.5	242.5	101.30	101.12

* - significantly different from control at $P < 0.01$

Table 2. Effect of 4-PU-30 on the chlorophyll (Chl) content [g kg^{-1}(f.m.)], net photosynthetic rate (P_N) [μmol(CO_2) m^{-2} s^{-1}] and stomatal conductance (g_s) [mmol(H_2O) m^{-2} s^{-1}] in the fourth leaf of *Raphanus sativus* in the stages of 4th and 8th leaf developed (mean of 3 experiments ± S.E.).

	4th leaf control	4-PU-30	[% of control]	8th leaf control	4-PU-30	[% of control]
Chl *a*	2.90± 0.11	3.20±0.05*	110.3	2.78±0.12	2.98±0.12	107.2
Chl *b*	0.98± 0.02	0.97±0.01	99.0	0.96±0.10	0.93±0.09	96.9
Chl *a+b*	3.88	4.17*	107.5	3.74	3.91	104.5
Chl *a/b*	2.96	3.30*	111.5	2.90	3.20*	110.3
P_N	14.47± 2.20	21.67±3.22*	149.6	4.88±0.43	7.35±1.55*	150.6
g_s	143.31±11.12	247.22±7.58*	172.51	60.11±1.76	72.58±1.26*	120.74

* - significantly different from control at $P < 0.05$

The transversal segments of the roots from the control plants (Fig. 1) revealed (normal for this stage of plant growth) increase in root thickness which was related to the existence of multi-layered cambium. Large vessels of secondary xylem were found on each side of the diarch primary xylem. The primary cortex was still present. The treatment with 4-PU-30 resulted in an increase in the secondary xylem and secondary phloem cells in which the storage function of the cells was more clearly expressed. The radially aligned large secondary xylem vessels produced by the cambium alternated with the rays of xylem parenchyma. The primary cortex was dead and the secondary cortex of the largely prevailing store phloem parenchyma developed. The rapid formation of cambial derivatives - secondary vascular tissues - as well as the rapid increase in root thickness illustrated the cambial activation by the 4-PU-30 treatment.

The leaves that developed after the treatment with 4-PU-30 were thicker than those of the control plants (Fig. 2). The alterations in leaf structure were due mainly

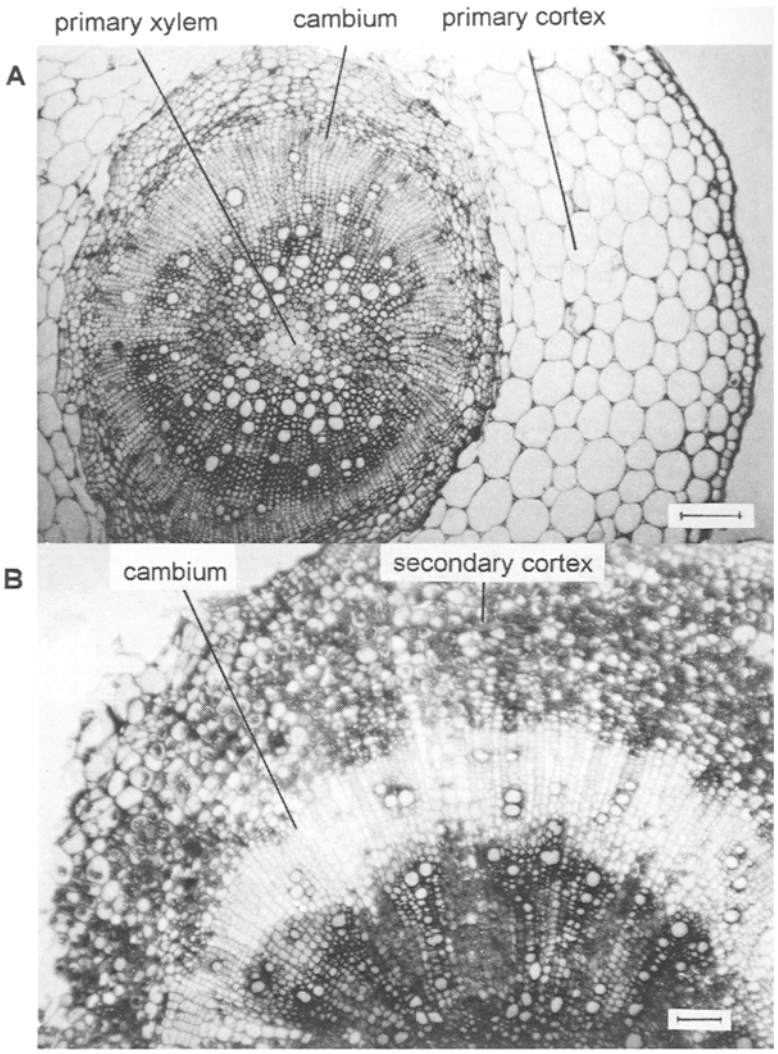

Fig. 1. Semi-thin transverse section of root in control (*A*) and 4-PU-30 treated (*B*) *Raphanus sativus* plants. *Bar* denotes 100 μm.

to the enlargement of cells, because the total number of cells per unit leaf area was not significantly altered. Only the Chl containing palisade and spongy mesophyll cells enlarged. The stereological analyses suggested that the density of cell material increased slightly in the palisade tissue and decreased in the spongy tissue of the treated plants (Fig. 3). Chlorenchyma cell surface area in contact with intercellular

air per unit volume of tissue was larger in the treated plants: that of the palisade tissue was 94 ± 12 in the control and 144 ± 29 cm^{-1} in the 4-PU-30 treated plants, and in the spongy tissue 120 ± 20 in the control and 291 ± 49 cm^{-1} in the treated plants. The chloroplasts of the 4-PU-30 treated plants produced more starch grains (both number and relative volume in the plastids) than those of the control plants (Fig. 4).

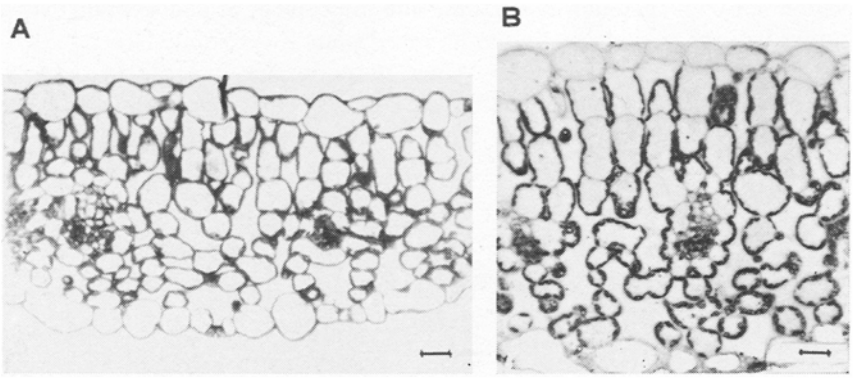

Fig. 2. Semi-thin transverse sections of fourth leaf in control (*A*) and 4-PU-30 treated (*B*) *Raphanus sativus* plants. *Bar* denotes 20 μm.

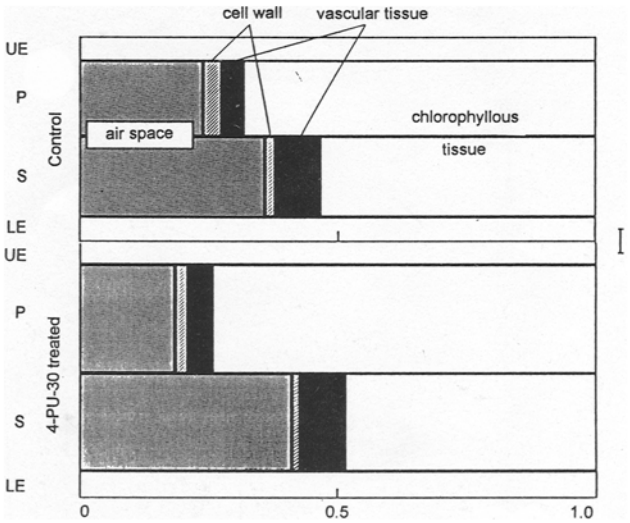

Fig. 3. Stereological measurements of internal structure of fourth leaf from control (*above*) and 4-PU-30 treated (*below*) *Raphanus sativus* plants. To the left, fractions of the tissue volume occupied by intercellulars, cell wall, vascular tissue and chlorenchyma, are shown for palisade (*P*) and spongy (*S*) mesophyll tissues. *UE* and *LE* are upper and lower epidermes. *Bar* denotes 20 μm.

Discussion

A similar stimulation of cell division and differentiation of cambium produced tissue such as obseved in this study after the 4-PU-30 treatment has been shown in other plants after application of purine cytokinins (see Kuang *et al.* 1991). The roots of many plant species, including radish, grow through formation and enlargement of cells by the cambium (Esau 1977). Radin and Loomis (1974) observed an *in vivo* correlation between cambium functioning and the content of endogenous cytokinins. They proposed the hypothesis that in an intact plant a cytokinins are supplied from the shoot, *i.e.* independent of the xylem flow. In our experiments, the visible stimulating effect of foliar application of 4-PU-30 on root growth was observed, accompanied by some retardation in the leaf mass growth. A possible interpretation

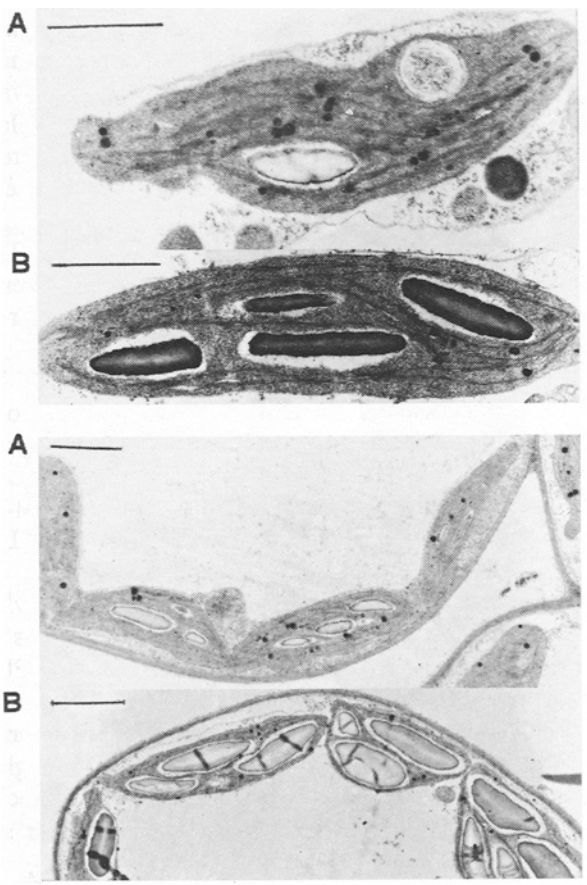

Fig. 4. *Raphanus sativus* palisade (*top*) and spongy (*bottom*) chloroplasts ultrastructure (*A* - control, *B* - 4-PU-30 treated plants). *Bar* denotes 1 μm.

of such delay of leaf expansion (but not of the leaf function) is given by Rapoport and Loomis (1985). They demonstrate competition between the leaves and the storage roots and substantiate the priority of the root in controling the distribution of assimilates. This control is realized by growth regulators or their derivatives that are translocated to the roots. The exogenously applied cytokinins of the purine type operate as mobilizing agents by directing the movement of diverse substances to the site(s) of their action (Mothes and Engelbrecht 1961) as well as to other "sinks" in the plant (Eriksen 1974, Kuang *et al.* 1991). We presume that functioning of the radish root as a powerful "sink" for assimilates is stimulated by the phenylurea cytokinin or by some derivative(s) of the latter which reach the root cambium and activate it. The significant increase in root size raises the question how do the leaves provide the photosynthates needed for the intensive root growth. During the later phases of plant development the stimulated root growth of 4-PU-30 treated plants (Georgiev *et al.* 1993) is maintained to a significant degree by the expanded leaf area. During the observed period when the increase of shoot fresh mass of the treated plants is slower than that of the control, the accelerated supply with assimilates is probably due to activated photosynthesis. P_N of the treated plants is possibly accelerated by the alterations in leaf structure. The increase in chlorenchyma volume and the cell surface area exposed to intercellular air (per unit leaf mesophyll volume) mediate the higher A_{mes}/A ratio in the treated plants, where A_{mes} is the exposed mesophyll cell surface area and A is the total leaf surface area. This ratio (Nobel 1974) characterizes the CO_2 diffusion inside the leaf and determines P_N. The stimulation of photosynthetic activity may be due also to the higher Chl content per unit leaf area in the 4-PU-30 treated plants.

The urea cytokinin used in this experiment increases the leaf blade thickness, Chl *a/b* ratio, and the starch content in the plastids. These are some of the alterations, the so called "sun-leaf" characteristics induced in the plants grown under high irradiance, as well as under the influence of purine cytokinins (Lichtenthaler and Buschmann 1978). The alterations of root structure caused by 4-PU-30 are also similar to those resulting from continuous irradiation (Torrey and Loomis 1967) or long day (Hayward 1938) treatment.

Our results confirm that the physiological effect of the phenylurea compound 4-PU-30 in radish plant resembles the physiological functions of the purine cytokinins, but they do not precise the primary site of its action. 4-PU-30 may either directly affect the cambium or more assimilates drawn to the cambium stimulate an increase in cambial cell division. However, very similar situation is found with regard to most of the known plant growth regulators. Thus, although the five major plant hormones have been known for many years, we still do not precisely know how they might be involved in plant development and which are their sites of primary action.

References

Arnon, D.I.: Cooper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. - Plant Physiol. **24**: 1-15, 1949.

Bosselaers, J.P.: Cytokinin effects on leaf architecture in *Phaseolus vulgaris* L. - J. exp. Bot. **34**: 1007-1017, 1983.

Čatský, J., Pospíšilová, J., Macháčková, J., Synková, H., Wilhelmová, N., Šesták, Z.: High level of endogenous cytokinins in transgenic potato plantlets limits photosynthesis. - Biol. Plant. **35**: 191-198, 1993.

Eriksen, E.N.: Root formation in pea cuttings. III. The influence of cytokinin at different developmental states. - Physiol. Plant. **30**: 163-171, 1974.

Esau, K.: Anatomy of Seed Plants. - J. Wiley and Sons, New York 1977.

Georgiev, G.T., Iliev, L.K., Karanov, E.N., Pulcheva, D.I.: Effect of phenylurea and purine type cytokinins on the growth and productivity of some root crop cultures. - Compt. rend. Acad. bulg. Sci. **46**: 85-87, 1993.

Hayward, H.E.: The Structure of Economic Plants. - Macmillan, New York 1938.

Iliev, L.K.: [Main physiological properties of cytokinins.] - Fiziol. Rast. (Sofia) **17**: 85-101, 1991. [In Bulg.]

Iliev, L.K., Georgiev, G.T., Karanov, E.N.: A new cytokinin effect of 4-PU-30 and dropp on root crops of radish (*Raphanus salivus* L.). - Comp. rend. Acad. bulg. Sci. **44**: 95-97, 1991.

Kuang, A., Peterson, C.M., Dute, R.R.: Pedicel abscission and rachis morphology of soybean as influenced by benzylaminopurine and the presence of pods. - Plant Growth Regul. **10**: 291-301, 1991.

Lichtenthaler, H.K., Buschmann, C.: Control of chloroplast development by red light, blue light and phytohormones. - In: Akoyunoglou, G., Argyroudi-Akoyunoglou, J.H. (ed.): Chloroplast Development, Pp. 801-816. Elsevier, Biomedical Press, Amsterdam 1978.

Mok, M.C., Mok, D.W.S., Turner, J.E., Mujer, C.V.: Biological and biochemical effects of cytokinin-active phenylurea derivatives in tissue culture systems. - HortScience **22**: 1194-1197, 1987.

Mothes, K., Engelbrecht, L.: Kinetin-induced directe transport of substances in excised leaves in the dark. - Phytochemistry **1**: 58-62, 1961.

Nickell, L.G.: Use of growth-regulating chemicals. - In: Raghavendra, A.S. (ed.): Physiology of Trees. Pp. 467-487. J. Wiley and Sons, New York 1991.

Nobel, P.S.: Introduction to Biophysical Plant Physiology. - Freeman and Co., San Francisco 1974.

Okamoto, T., Shudo, K., Takachachi, S., Kawachi, E., Isogai, Y.: 4-pyridylureas are surprisingly potent cytokinins. The structure-activity relationship. - Chem. Pharm. Bull. Tokyo **29**: 3748-3750, 1981.

Parkhurst, D.T.: Stereological methods for measuring internal leaf structure variables. - Amer. J. Bot. **69**: 31-39, 1982.

Radin, J.W., Loomis, R.S.: Polar transport of kinetin in tissues of radish. - Plant Physiol. **53**: 348-351, 1974.

Rapoport, H.F., Loomis, R.S.: Interaction of storage root and shoot in grafted sugarbeet and chard. - Crop Sci. **25**: 1079-1084, 1985.

Rovenská, B., Volfová, A., Chvojka, L.: The effect of cytokinin- and auxin-like substances on the anatomy and ultrastructure of vascular bundles in wheat. - Biol. Plant. **26**: 42-48, 1984.

Spurr, A.R.: A low-viscosity epoxy resin embedding medium for electron microscopy. - J. Ultrastruct. Res. **26**: 31-43, 1969.

Stoynova, E.Z., Iliev, L.K: [Cytological observations of radish cotyledons following treatment with cytokinins of purine and urea type.] - Fisiol. Rast. (Sofia) **16**: 10-19, 1990. [In Bulg.]

Takahashi, S., Shudo, K, Okamoto, T., Yamacha, K., Isogay, Y.: Cytokinin activity on N-phenyl-N'-(4-pyridyl)-urea derivatives. - Phytochemistry **17**: 1201-1207, 1978.

Torrey, J.G., Loomis, R.S.: Ontogenetic studies of vascular cambium formation in excised roots of *Raphanus sativus* L. - Phytomorphology **17**: 401-409, 1967.