BIOLOGIA PLANTARUM 44 (3): 451-453, 2001

BRIEF COMMUNICATION

Growth and endogenous cytokinins in tobacco callus as affected by N-(2-chloro-4-pyridyl)-N'-phenylurea

L. ATANASOVA and L. ILIEV

Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Street 21, Sofia 1113, Bulgaria

Abstract

The effect of N-(2-chloro-4-pyridyl)-N'-phenylurea (4PU-30) on the growth and content of endogenous cytokinins of adenine type in tobacco (*Nicotiana tabacum* L.) callus was investigated. Biomass accumulation in calli grown on Murashige and Skoog (MS) medium with 4PU-30 was higher than that on MS medium with kinetin. The obvious presence of isopentenyladenine type cytokinins and traces of zeatin type cytokinins supposes modification in the endogenous cytokinin metabolism of the tobacco callus grown on 4PU-30.

Additional key words: *Nicotiana tabacum* L., phenylurea-adapted callus, endogenous adenine type cytokinins, indirect competitive ELISA.

An important effect of phenylurea cytokinins (CKs) is their ability to stimulate CK independence of callus tissues (Dyson *et al.* 1972, Mok *et al.* 1979, Capelle *et al.* 1983, Mok *et al.* 1987). This property may be due to their capacity to influence the biosynthesis or metabolism of endogenous CKs of adenine (Ade) type (Miller 1961, Capelle *et al.* 1983, Laloue and Fox 1985, Parker *et al.* 1986, Thomas and Katterman 1986) or to act directly like CKs as suggested from the similar binding properties between phenylureas and Ade CKs (Shudo 1994). The purpose of this study is to examine the content of Ade type CKs in tobacco callus tissue grown on medium supplemented with phenylurea CK 4PU-30.

Callus cultures of *Nicotiana tabacum* L., cv. Wisconsin 38, were grown at 26 °C and about 70 % relative humidity in dark on modified MS-medium supplemented with 2 mg dm^{-3} indole acetic acid, 0.025 mg dm^{-3} 4PU-30 or 0.2 mg dm^{-3} kinetin and subcultured every 4 weeks. Culture volume of 30 cm^3 was maintained in 100 cm^3-flasks.

For CK analyses 21-d-old callus tissue was used. The material was homogenized in ethanol at 4 °C. The homogenate was pelleted for 10 min at 10 000 *g* and the supernatant was collected. The pellet was re-extracted with 70 % ethanol and the combined supernatants were reduced *in vacuo* at 40 °C. The extract was applied onto *DEAE*-cellulose-column running directly into *Sepharose 4B*-column with linked antibodies against *trans*-zeatin riboside (ZR) and N^6-(Δ^2-isopentenyl)adenosine (iPA) (Atanassova *et al.* 1996). The tandem-columns were washed by a cycle of washings (Hansen *et al.* 1989) and CKs were eluted from the immunoaffinity column with ethanol. The eluate was evaporated *in vacuo* at 40 °C.

Aliquots of immunoaffinity-purified material in parallel with known quantities of synthetic *trans*-zeatin (Z) and N^6-(Δ^2-isopentenyl)adenine (iP) and their ribosides (ZR and iPA) were placed on plates with silica $60F_{254}$ (*Merck*, Darmstadt, Germany) and developed with solution of *n*-butanol : water : NH_4OH (6:2:1). The Rf of synthetic cytokinins were 0.35 for ZR, 0.48 for iPA, 0.67

Received 25 August 2000, *accepted* 5 December 2000.

Abbreviations: Ade - adenine; CK - cytokinin; iP - N^6-(Δ^2-isopentenyl)adenine; iPA - N^6-(Δ^2-isopentenyl)adenosine; MS medium - Murashige and Skoog medium; 4PU-30 - N-(2-chloro-4-pyridyl)-N'-phenylurea, TDZ - thidiazuron; Z - zeatin; ZR - zeatin riboside.

Acknowledgements: This study was supported by NFSI, projects K-440/95 and B-801/98. 4PU-30 was kindly provided by Prof. K. Shudo, Faculty of Pharmaceutical Sciences, University of Tokyo, Japan.

Fax (+35) 9 273 9952; e-mail: ljubka@obzor.bio21.bas.bg or iliev@obzor.bio21.bas.bg

for Z and 0.84 for iP. The spots for immunoassay were determined on the basis of UV_{254} absorbing areas of CK standards, scrapped off and eluted with 80 % ethanol. The eluates were evaporated and the levels of ZR, Z, iPA and iP were assayed by indirect competitive ELISA (Schwartzenberg 1989, Atanassova *et al.* 1996). The results are means of three ELISA determinations from two experiments. The estimates of CKs are corrected for a recovery of 50 - 70 % for Z and ZR and 35 - 50 % for iP and iPA.

In this study a strain of tobacco callus which grows well on a standard medium containing auxin and 4PU-30 as a sole CK source was used. The callus tissue is more friable than that grown on kinetin. The changes in fresh and dry masses of tobacco callus cultured on 0.025 mg dm^{-3} 4PU-30 and 0.2 mg dm^{-3} kinetin at 4-d intervals over 28-d period confirmed that 4PU-30 is effective CK for growing of tobacco tissue and its activity is higher than that of kinetin (Fig. 1).

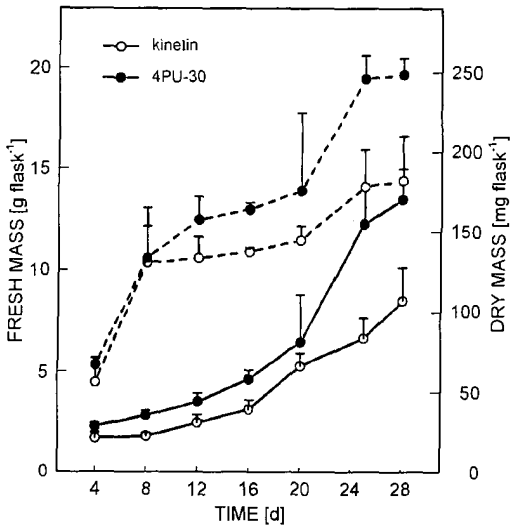

Fig. 1. Changes of fresh mass (*solid line*) and dry mass (*dotted line*) of tobacco callus grown on 0.2 mg dm^{-3} kinetin (*open circles*) or 0.025 mg dm^{-3} 4PU-30 (*solid circles*) during 28-d incubation period.

4PU-30 stimulates the callus to produce compounds showing immunorections similar to Ade Cks (Fig. 2). The prevailing part of these compounds demonstrated immunoreaction in anti-iPA-ELISA. The testing in anti-ZR-ELISA showed very low contents of ZR and Z.

The aim of the analysis of CKs extracted from 4PU-30-adapted tobacco callus was to examine how this phenylurea CK influenced the content of endogenous CKs of Ade type. The applied CK-specific antibodies demonstrated very low cross-activity with 4PU-30 and we expect that the determination of Ade CKs is not influensed by 4PU-30. The separation of purified extracts by thin layer chromatography compared to the CK standards followed by the testing of each spot in anti-ZR- and anti-iPA-ELISA give us the reason to suppose that endogenous CKs of Ade type were mainly determined. The obvious presence of iP type CKs and the traces of Z type CKs suppose modification in endogenous CK metabolism of the 4PU-30-adapted tobacco callus.

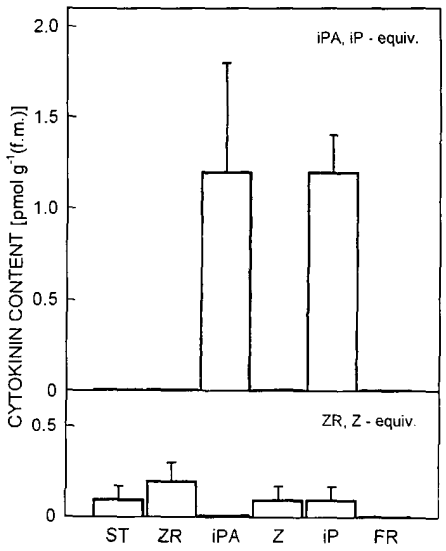

Fig. 2. TLC-ELISA profile of immunoaffinity-purified extract from tobacco callus grown on 4PU-30. ST - start; ZR - *trans*-zeatin riboside; iPA - N^6-(Δ^2-isopentenyl)adenosine; Z - *trans*-zeatin; iP - N^6-(Δ^2-isopentenyl)adenine; FR - front.

The interpretation of the data regarding the relations of phenylurea and Ade CKs is still speculative largely due to the insufficient knowledge on the mode of action of plant growth regulators in general.

The earliest speculation that the substituted phenylureas may serve as precursors for N^6-side chain of Ade derivatives (Miller 1961) was not confirmed. Further works showed that phenylurea CKs thidiazuron (TDZ) and diphenylurea were metabolized mainly as glucosyl derivatives and not converted to Ade type CKs (Dyson *et al.* 1972, Burrows and Leworthy 1976, Mok and Mok 1985). However, these CKs increased capacity of tissues to become CK independent. According to Meins (1994) the CK independence was maintained by positive feed-back loop in which CKs or physiologically related cell-division-promoting substances either induced their biosynthesis or inhibited their degradation. Phenylureas are known to influence both the interconversions between CK bases, nucleosides and nucleotides as well as their inactivation.

TDZ was found to affect iPA metabolism in *Phaseolus* callus. Its presence promoted the nucleotide conversion to biologically active nucleoside and inhibited the nucleotide formation (Capelle *et al.* 1983). Thus, TDZ might preserve iPA availability in the callus. Our results on the dominating of iPA and iP in the 4PU-30 adapted tobacco callus strengthen such a possibility.

The finding that phenylurea CKs inhibited the *in vitro* CK oxidase (Chatfield and Armstrong 1986, Burch and Horgan 1989, Laloue and Fox 1989, Kamínek and Armstrong 1990, Motyka and Kamínek 1992), glucosyl transferase and alanine synthase (Parker *et al.* 1986) raised the question that the biological activity of phenylurea CKs resulted from their ability to inhibit the degradation of the endogenous CKs. Thus, via inhibiting inactivation pathways phenylureas may protect endogenous CKs in the cell (Kamínek *et al.* 1997). Such a proposal provides an alternative explanation of the increase of endogenous Ade CKs observed in few studies (Thomas and Katterman 1986, Hutchinson and Saxena 1996) as well as the presence of iP type CKs observed by us. iP type CKs which are considered to be products of the early stages in CK biosynthesis, are preferrential substrates of CK oxidase (Laloue and Fox 1989, Armstrong 1994, Kamínek *et al.* 1997).

Summing up, we show that 4PU-30 allows the callus to grow and to produce compounds, that demonstrate chromatographic and immunochemical behaviour similar to isopentenyladenine type CKs.

References

Armstrong, D.J.: Cytokinin oxidase and the regulation of cytokinin degradation - In: Mok, D.W.S., Mok, M.C. (ed.): Cytokinins, Chemistry, Activity and Function. Pp. 139-154. CRC Press, Boca Raton 1994.

Atanassova, L.Y., Pissarska, M.G., Stoyanov, I.G.: Cytokinins and growth response of maize and pea plants to salt stress - Bulg. J. Plant Physiol. **22**: 22-31, 1996.

Burrows, W.J., Leworthy, D.P.: Metabolism of N,N'-diphenylurea by cytokinin-dependent tobacco callus: identification of the glucoside - Biochem. biophys. Res. Commun. **70**: 1109-1114, 1976.

Burch, L.R., Horgan R.: The purification of cytokinin oxidase from *Zea mays* kernels - Phytochemistry **28**: 1313-1319, 1989.

Capelle, S.C., Mok, D.W.C., Kirchner, S.C., Mok, M.C.: Effects of thidiazuron on cytokinin autonomy and the metabolism of N^6-(Δ^2-isopentenyl)[8-^{14}C]adenosine in callus tissues of *Phaseolus lunatus* L.- Plant Physiol. **73**: 796-802, 1983.

Chatfield, J.M., Armstrong, D.J.: Regulation of cytokinin oxidase in callus tissue of *Phaseolus vulgaris* L. cv. Great Northern - Plant Physiol. **80**: 493-499, 1986.

Dyson, W.H., Fox, J.E., McChesney, J.D.: Short term metabolism of urea and purine cytokinins. - Plant Physiol. **49**: 506-513, 1972.

Hansen, C.E., Kopperud, C., Heide, O.M.: Immunoaffinity chromatography for purification of cytokinins in plant extracts - Norw. J. agr. Sci. **3**: 73-77, 1989.

Hutchinson, M.J., Saxena, P.K.: Role of purine metabolism in thidiazuron-induced somatic embryogenesis of geranium (*Pelargonium* × *hortorum*) hypocotyl cultivars - Physiol. Plant. **98**: 517-522, 1996.

Kamínek, M., Armstrong, D.J.: Genotypic variation in cytokinin oxidase from *Phaseolus* callus cultures - Plant Physiol. **93**: 1530-1538, 1990.

Kamínek, M., Motyka, V., Vaňková, R.: Regulation of cytokinin content in plant cells - Physiol. Plant. **101**: 689-700, 1997.

Laloue, M., Fox, J.E.: Characterisation of an imine intermediate in the degradation of isopentenylated cytokinins by a cytokinin oxidase from wheat - In: Bopp, M., Knoop, B., Rademacher, W. (ed.): Abstracts of the 12th International Conference on Plant Growth Substances. P. 23. International Plant Growth Association, Heidelberg 1985.

Laloue, M., Fox, J.E.: Cytokinin oxidase from wheat. Partial purification and general properties - Plant Physiol. **90**: 899-906, 1989.

Meins, F.: Habituation of cultured cells for cytokinins - In: Mok, D.W.S., Mok, M.C. (ed.): Cytokinins, Chemistry, Activity and Function. Pp. 269-287. CRC Press, Boca Raton 1994.

Miller, C.: Kinetin and related compounds in plant growth - Annu. Rev. Plant Physiol. **12**: 395-408, 1961.

Mok, M.C., Mok, D.W.S.: The metabolism of [^{14}C]thidiazuron in callus tissues of *Phaseolus lunatus* L. - Physiol. Plant. **65**: 427-432, 1985.

Mok, M.C., Kim, S.-G., Armstrong, D.J., Mok, D.W.S.: Induction of cytokinin autonomy by N,N'-diphenylurea in tissue cultures of *Phaseolus lunatus* L. - Proc. nat. Acad. Sci. USA **76**: 3880-3884, 1979.

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** (Suppl.): 1194-1197, 1987.

Motyka, V., Kamínek, M.: Characterisation of cytokinin oxidase from tobacco and poplar callus cultures - In: Kamínek, M., Mok, D.W.S., Zažímalová, E. (ed.): Physiology and Biochemistry of Cytokinins in Plants. Pp 33-39. SBP Academic Publishing, The Hague 1992.

Parker, C.W., Entsch, B., Letham, D.S.: Inhibitors of two enzymes which metabolize cytokinins - Phytochemistry **25**: 303-310, 1986.

Schwartzenberg, K., von: [Der Cytokiningehalt in Nadeln unterschiedlich Stark von "neuartigen waldschäden betzoffener" Fichten (*Picea abies* [L.] Karst.), bestimmt mittels einer immunoenzymatischen Methode - ELISA.] - PhD Thesis. University of Bonn, Bonn 1989. [In German.]

Shudo, K.: Chemistry of phenylurea cytokinins - In: Mok, D.W.S., Mok, M.C. (ed.): Cytokinins, Chemistry, Activity and Function. Pp. 35-42. CRC Press, Boca Raton 1994.

Thomas, J.C., Katterman, F.R.: Cytokinin activity induced by thidiazuron. - Plant Physiol. **81**: 681-683, 1986.