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Original article

Metabolism of isopentenyladenosine in the roots of Norway spruce seedlings exposed to nutritive stress

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Summary — Seedlings of Norway spruce (*Picea abies*) were grown on a low nutrient medium containing Al^{3+} ions (0.8 mM, stress) and a rich medium that was suitable for spruce and lacked Al^{3+} (control). After feeding with tritiated isopentenyladenosine *via* the roots, the metabolism of cytokinins in the roots of stressed and control plants was compared. HPLC radioactivity profiles of root extracts showed that isopentenyladenosine was mainly degraded to isopentenyladenine- and adenine/adenosine-like compounds. Stressed and non-stressed seedlings clearly differed with respect to the distribution of radioactivity for the different metabolites. The measurements showed that the degradation of isopentenyladenosine was strongly reduced in the roots of the stressed seedlings. Results are discussed with regard to the levels of endogenous cytokinins measured in spruce affected by the novel type of forest decline.

***Picea abies* / cytokinin / metabolism / radiolabelled cytokinin / forest decline**

Résumé — Métabolisme de l'isopentényladénosine dans les racines d'épicéa soumis à un stress nutritif. *De jeunes plantules d'épicéa (*Picea abies*) ont été plantés sur un milieu pauvre contenant des ions Al^{3+} (0,8 mM, stress) et sur un milieu sans Al^{3+} bien fourni en nutriments (témoin). Après marquage avec de l'isopentényladénosine tritiée,* via *les racines, le métabolisme des cytokinines dans les racines a été comparé chez les plants témoins et chez les plants stressés. Les profils de radioactivité, obtenus après HPLC, à partir des extraits racinaires, montrent que l'isopentényladénosine est principalement dégradé en isopentényladénosine et adénine ou adénosine. Les plantules*

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Ade: adenine; Ado: adenosine; AMP: adenosine 5'-monophosphate; BLF: synthetic soil solution (from German: Bodenlösung Fichte); Ck(s) cytokinin(s); iP: isopentenyladenine; [9R]iP: isopentenyladenosine; Z: zeatin; [9R]Z: zeatin riboside.

stressées et non stressées diffèrent clairement dans leur distribution radioactive pour les différents métabolites. Les marquages montrent que la dégradation de l'isopenténylad énosine est fortement retardée dans les racines des plantules stressées. Les résultats sont discutés en fonction des cytokinines endogènes chez l'épicéa, affecté par le nouveau type de dépérissement des forêts.

Picea abies / *cytokinine / métabolisme / cytokinine radiomarquée / dépérissement des forêts*

INTRODUCTION

In previous work it has been shown that Norway spruce trees affected by the novel type of forest decline in Germany exhibit large increases in the content of the endogenous cytokinin ribosides, zeatin riboside ([9R]Z) and isopentenyladenosine [9R]iP) (Schwartzenberg and Hahn, 1991). In trees that show specific yellowing of older needles the cytokinin (Ck) concentrations were clearly positively correlated to the extent of tree damage. The concentrations of the free Ck bases (zeatin (Z) and isopentenyladenine (iP)) also tended to be higher in needles of damaged trees.

Results from fertilisation experiments carried out at the Hils-site (Weserbergland, Germany) and experiments performed with spruce seedlings grown in hydroculture revealed that unfavourable conditions (nutrient shortage, low pH, or Al ions) can induce an increase of Ck ribosides in the upper part of the trees (Schwartzenberg, 1989). To date it is not clear which metabolic processes are responsible for this accumulation of Ck ribosides in stressed spruce trees.

Many investigations have shown that exogenously supplied Cks are actively metabolised by plant tissue (Letham and Palni, 1983; McGaw, 1986). Cks appear to be metabolised rapidly into the nucleotide forms and can be further converted into nucleosides and free bases. Nucleotides, nucleosides and free bases are interconvertible and this interconversion is mostly caused by enzymatic systems, which also have adenine (Ade), adenosine (Ado) and adenosine 5'-monophosphate (AMP) as substrates. It is as yet uncertain whether Ck-specific enzymes are also involved in the interconversion of Cks (Letham and Palni, 1983; McGaw, 1986).

The aim of this work is to investigate how far the metabolism of Cks in spruce roots becomes modified under unfavourable conditions. The metabolism of radiolabelled isopentenyladenosine ([9R][^{3}H]iP) in spruce seedlings stressed by nutrient shortage and phytotoxic Al^{3+} ions is compared with that of non-stressed plants.

MATERIALS AND METHODS

Plant material and culture conditions

Seeds of Norway spruce (*Picea abies* L Karst) were obtained from Staatliches Forstamt Nagold (Nagold, Baden-Württemberg, Germany); origin 84008, year of ripening 1990.

The seeds were germinated at 25°C on wetted filter paper. After germination the young seedlings were transferred to a hydroponic culture system using perlite (Caahmro, France) as a substrate. For the first 4 weeks after germination the perlite was wetted with Ingestad medium: $CaCl_2$ 1 mM; $FeCl_3$ 0.018 mM; $MgSO_4$ 0.61 mM; $MnSO_4$ 0.3 μM; NaCl 0.205 mM; NH_4NO_3 1.77 mM; KCl 0.95 mM; K_2HPO_4 0.32 mM; $CuSO_4$ 0.32 μM; H_3BO_3 16 μM; Na_2MoO_4 0.033 μM; $ZnSO_4$ 0.63 μM; pH 3.8.

At the end of the 4th week, half of the seedlings were transferred to a synthetic soil solution (BLF) medium (stress treatment): NH_4HSO_4 0.238 mM; $NaNO_3$ 0.099 mM; KNO_3 0.123 mM; NH_4NO_3 0.0762 mM; $FeSO_4$ 0.0197 mM; $MgSO_4$ 0.04 mM; $MnSO_4$ 0.236 mM; $CaCl_2$ 0.14 mM; K_2HPO_4 0.0161 mM; H_2SO_4 0.12 mM; H_3BO_3 0.0139 mM; $ZnSO_4$ 9.96 μM; $CuSO_4$ 0.016 μm;

Na_2MoO_4 0.165 μM; KI 0.722 μM; $CoSO_4$ 0.019 MM; pH 3.8.

The BLF medium mimics the nutrient concentrations found in a declining stand of Norway spruce in Germany. Al^{3+} ions, which represent a stress factor in combination with soil acidification and low nutrient supply, were added to the BLF medium in form of $AlCl_3$ (0.8 mM). The composition of the nutrient media Ingestad (control) and BLF (stress) was taken from Junga (1984).

The nutrient media were changed weekly. The seedlings were cultivated in a growth chamber at 20°C with light 80 μE m^{-2} s^{-1} for 16 h per day.

Synthesis of tritiated isopentenyladenosine

Tritiated [9R]iP was obtained after alkylation of (2) [^{3}H] adenosine with 4-bromo-2-methyl-2-butene as described by Laloue and Fox (1987). The radiochemical purity of the (2)-[^{3}H]-isopentenyladenosine ([9R][2-^{3}H]iP) as determined by HPLC and liquid scintillation counting was found to be 98%; the specific activity was 18 Ci/mmol.

Feeding with tritiated isopentenyladenosine

The roots of the 27-week-old seedlings were cleaned from the perlite and washed 3 times with sterile water.

The intact seedlings were transferred into hydroculture in order to be fed with the [9R]iP *via* the intact roots (2 seedlings per assay). The [9R][^{3}H]iP (34 000 Bq/seedling) was diluted in either Ingestad or BLF medium (sterile). The feeding solution (2 ml) was aerated with 80 ml/min air. Seedlings were incubated for 2, 6 and 24 h. The apparent uptake of [9R]iP was followed by determining the radioactivity in 50 μl aliquots of the feeding solution.

Extraction and prepurification of cytokinins

After feeding with [9R]iP, the roots were washed with water, dipped into liquid nitrogen and homogenized with a pestle and mortar. The powder was incubated for 15 h in Bieleski's reagent (methanol/chloroform/formic acid, 15:5:3, v:v:v; Bieleski, 1964) at –20°C.

The Bieleski's reagent was evaporated by rotary film evaporation. The residue was extracted with 80% methanol and was centrifuged at 500 *g*. The pellet was reextracted with 80% methanol and discarded. The supernatant was filtered (5 μm, cellulose acetate, Sartorius) and was passed through a Sep-Pak C18 cartridge (Waters) to remove lipophilic compounds. The effluent was filtered (0.45 μm, polypropylene, Sartorius) and was concentrated by rotary film evaporation prior to HPLC separation.

HPLC separation and detection of radiolabelled cytokinins

The HPLC separation of Cks and their metabolites was performed on a Beckmann system using a Merck LiChrospher 100 RP 18 column (250 x 4 mm).

The solvents were: (1) 40 mM acetic acid adjusted to pH 3.35 with triethylamine; and (2) 100% acetonitrile. The flow rate was 1.5 ml/min and the acetonitrile concentration raised from 0 to 100% within 50 min (for gradient see fig 4A insert). The HPLC effluent was fractionated (1.5 ml per fraction) and radioactivity was measured by liquid scintillation counting (Beckmann, LS 1801).

RESULTS

Morphological characteristics of seedlings

The morphology of stressed seedlings grown on the BLF medium differs from that of the Ingestad seedlings (control). The stressed seedlings exhibited a lower rate of shoot growth, tended to have yellower needles and brown roots. These seedlings produced a great number of lateral roots. Further characteristics of the BLF seedlings were a reduced length of the primary root and an increased ratio of the root to shoot fresh weight (table I).

Table I. Morphological characteristics of 27-week-old Norway spruce seedlings grown on Ingestad (control) and BLF medium (0.8 mM $AlCl_3$, stress).

	Ingestad	*BLF*
Length of primary roots (cm)	40 (± 11)	27 (± 2.5)
Number of lateral roots per cm primary root	2.6 (± 1.0)	5.3 (± 2.9)
Root / shoot ratio (FW/FW)	0.69 (± 0.11)	1.66 (± 0.49)

Data are mean values of 5 seedlings, standard deviation given in parentheses. FW = fresh weight.

Uptake and distribution of radioactivity in Norway spruce seedlings

The total radioactivity supplied in form of [9R][^{3}H]iP was measured by liquid scintillation counting and the apparent uptake of radioactivity was determined. The major part of the radioactivity was absorbed during the first 2 h. After 2 h the radioactivity uptake was low but constant. Within 6 h about 73% of the initial radioactivity was incorporated into the plants. BLF and Ingestad seedlings exhibited only small differences in the uptake kinetics (fig 1A).

Roots and shoots were extracted for Ck analysis. In order to protect Ck nucleotides from endogenous phosphatase activities, the homogenized root material was incubated in Bieleski's reagent (Bieleski, 1964) prior to the extraction with 80% methanol. Lipophilic compounds were retained by solid phase extraction (C18) and the total radioactivity in the purified extract was determined (fig 1B). The sum of total radioactivity detected in roots and shoots was found to be much less than the radioactivity that was apparently taken up by the plants. The radioactivity determined in the Ck extracts decreased over the course of incubation, while the apparent uptake of radioactivity increased. This suggests that the [9R]iP taken up was converted into non-extractable forms (fig 1).

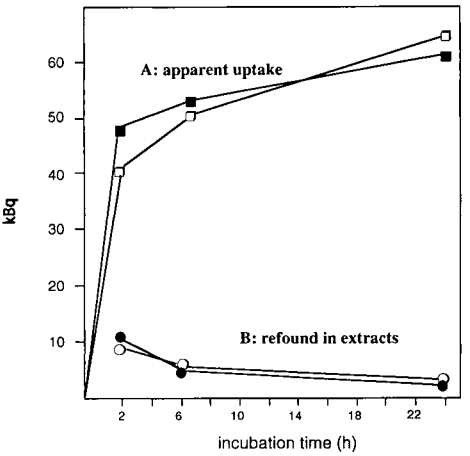

Fig 1. Time course of apparent radioactivity uptake (A) and total radioactivity refound in cytokinin extracts (B). Roots of intact Norway spruce seedlings (2 plants) were incubated in nutrient media containing 68 kBq of [9R][^{3}H]iP.□,O: Ingestad seedlings (control); ■,●: BLF seedlings (stress).

Figure 2 shows a comparison of the total radioactivity measured in the Ck extracts for roots *versus* shoots. The major part of the tritiated compounds was found in the roots, where the amount of extractable radioactivity was initially high and decreased over time. However, the radioactivity measured in the shoot extracts was very low throughout the entire course of the experiment.

Only for the Ingestad seedlings was even a small part of the radioactivity translocated into the shoot after 24 h. In the shoots of the BLF seedlings no increase in radioactivity was observed (fig 2).

HPLC analysis

Root extracts from the [9R]iP feeding experiments were submitted to HPLC separation and the radioactivity in the effluent was analyzed by liquid scintillation counting (fig 3). The radioactivity peaks separated by HPLC cochromatographed with the unlabelled standard substances AMP, Ade/Ado (not separated), iP and [9R]iP. Other unidentified peaks were detected (retention times 3–4 min and 33 min).

In roots of the Ingestad seedlings (fig 3A–C) only a minor peak of [9R]iP, which was used for the feeding, was found. However, a major radioactivity peak coelutes with iP.

In the roots of the BLF seedlings (fig 3D–F) the distribution of the radiolabelled metabolites was clearly different from that found in the Ingestad plants. The main difference was the reduced metabolism of [9R]iP. In roots of BLF seedlings, [9R]iP was found as a major tritiated compound up to 24 h after the start of feeding and its metabolite iP was only found in minor quantities (figs 3 and 4).

Interestingly, the roots of Ingestad and BLF seedling did not show any significant radioactivity detectable at the elution times for the hydroxylated Cks Z and [9R]Z (fig 3).

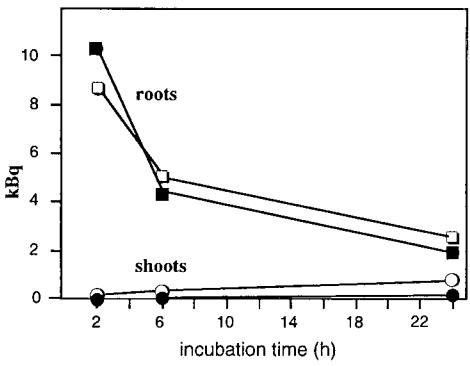

Fig 2. Time course of radioactivity in cytokinin extracts of roots (squares) and shoots (circles). Roots of intact Norway spruce seedlings (2 plants) were incubated with [9R] [^{3}H]iP.□,○: Ingestad seedlings (control); ■,●: BLF seedlings (stress).

DISCUSSION

Nutritive stress, including soil acidification, nutrient shortage and phytotoxic aluminium ions (Al^{3+}), have been suggested to be an important factor causing the phenomenon of the novel type of forest decline (Ulrich, 1983; Godbold *et al,* 1988; Klein and Perkins, 1988). In order to study possible effects of nutritive stress upon Ck metabolism in spruce, a low nutrient medium (BLF), which mimics the soil solution of an acidified, declining Norway spruce stand, was used to stress seedlings under laboratory conditions. Al ions, which can be considered as a stress factor in acidified, low nutrient soils, were added to the BLF medium. It is known that Al ions can disturb plant nutrition by inhibition of Ca and Mg uptake (Jorns and Hecht-Buchholz, 1985). What is important for the root damage, is not the absolute Al concentration but the molar ratio of the Ca and Mg ion concentrations to that of the Al ions. With a Ca/Al ratio of 0.77 and a Mg/Al ratio of 0.05, spruce roots meet a considerable Al stress in the BLF medium (Rost-Siebert, 1983).

The characteristics observed for the stressed BLF seedlings, such as enhanced formation of lateral roots, reduction of shoot growth and the yellowing needles of the stress-treated plants, are similar to proper-

ties of Al-treated spruce plants as described by Junga (1984) and Jorns and Hecht-Buchholz (1985).

During feeding with [9R][^{3}H]iP, the uptake kinetics and distribution of total radioactivity of the stressed and control seedlings were

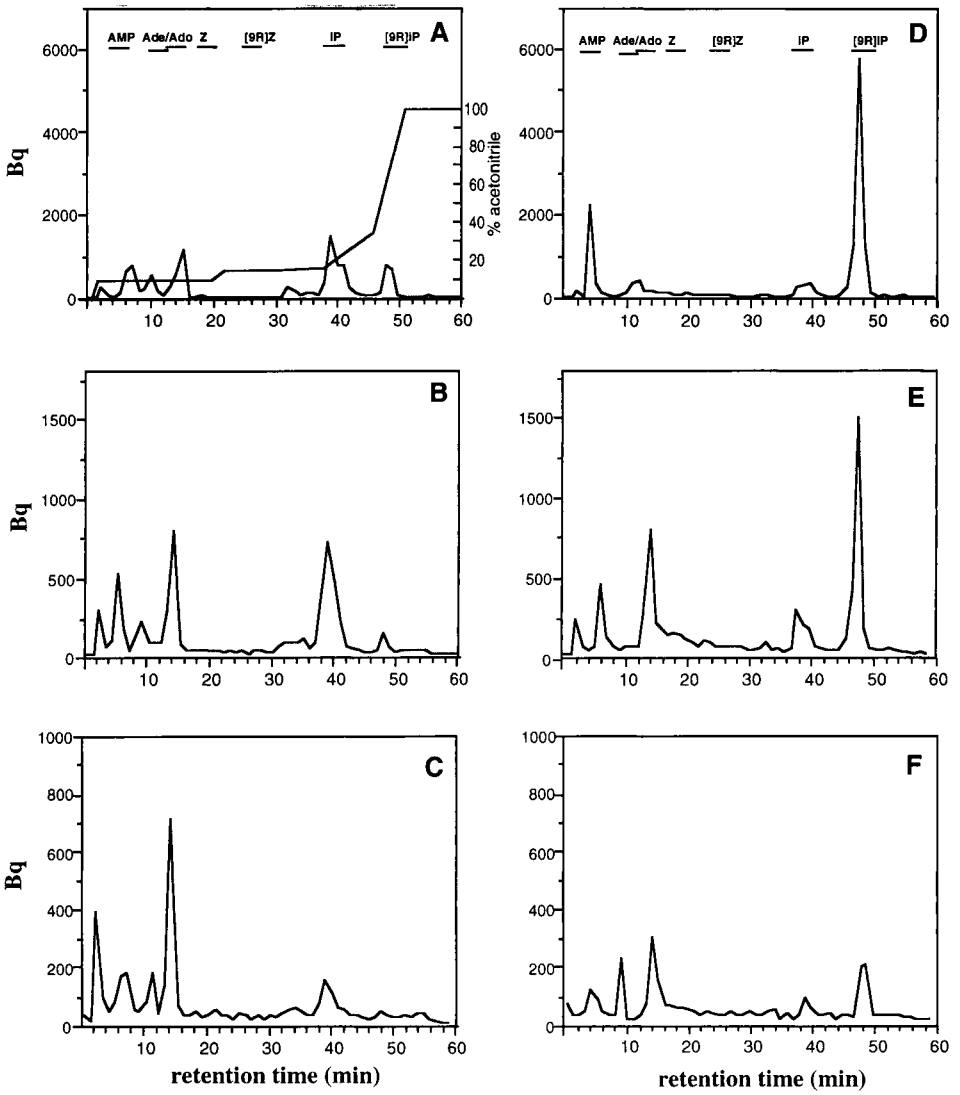

Fig 3. HPLC radioactivity profiles of root extracts (per 2 seedlings) after 2 h (**A**,**D**), 6 h (**B**,**E**) and 24 h (**C**,**F**) after feeding with [9R][^{3}H]iP in Ingestad medium (A, B, C) and BLF medium (D, E, F). The percentage of acetonitrile during the HPLC separation is inserted into **A**. Bars represent the elution times of standard substances.

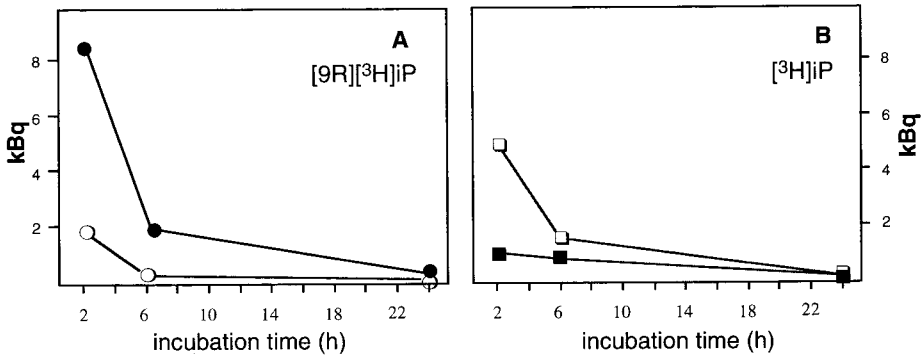

Fig 4. Time course of radioactivity detected in HPLC fractions of root extracts corresponding to [9R]iP (**A**) and iP (**B**). Roots of intact Norway spruce seedlings (2 plants) were incubated with [9R][^{3}H]iP. □ ,O: Ingestad seedlings (control); ■,●: BLF seedlings (stress).

found to be very similar. For both types of seedlings, it is remarkable that less than 28% of the radioactivity taken up could be detected in the Ck extract. Apparently a large part of the radioactivity had been converted to forms that are not extractable by the extraction/purification protocol used. A possible explanation could be that after degradation of Cks to adenine-like compounds by Ck-oxidase (fig 3), most of the radioactivity had been incorporated into the fraction of nucleic acids, which was not analysed.

During the entire feeding experiment (24 h), the seedlings absorbed about 200 µl liquid, which should have allowed a certain amount of radioactivity to be translocated into the upper parts of the seedlings, but very little extractable radioactivity was detected in the shoots (fig 2). The contradictory slopes of the radioactivity curves in roots and shoots indicate that losses of soluble radioactivity in the roots are only to a very limited extent due to transport into the shoot. This means that a very active metabolism of the [9R]iP takes place in the roots.

The HPLC radioactivity profiles confirm a rapid metabolism of [9R]iP in spruce roots. Although similar patterns of radiolabelled metabolites were detected in stressed and control plants, their quantitative distribution differs to a large extent (figs 3 and 4). A main effect of the stress treatment is that the metabolism of the [9R]iP is obviously retarded. After 2 h feeding, about 18% of the incorporated radioactivity in stressed seedlings was found in the [9R]iP fraction and 2.1% in the iP fraction compared to 4.4 and 8.5% in the Ingestad seedlings, respectively.

Despite the fact that the iP-type Cks are considered as precursors for the zeatin-type Cks (Letham and Palni, 1983) no significant amount of radioactivity was found for these hydroxylated forms. However, immunoenzymatic analysis of endogenous Cks has shown that zeatin-type Cks are present in the roots of spruce seedlings (Schwartzenberg *et al*, unpublished data). It can be assumed that either roots of spruce seedlings are not the primary sites for the hydroxylation of iP-forms to give the zeatin-forms or this hydroxylation is very slow. However, it should be taken into consideration that the metabolism of exogenously supplied Cks might differ from that of

endogenous Cks. The tritiated [9R]iP and its metabolites might have a different distribution in the cellular compartments in comparison to the endogenous forms.

Today it is widely accepted that roots are the main sites for Ck biosynthesis (Skeene, 1975; Torrey, 1976). Furthermore, in the conifer species *Pseudotsuga menziesii* it has been shown that Cks are transported in the xylem fluid (Doumas and Zaerr, 1988).

Considering our results concerning the concentration of endogenous CKs, we might assume that, in the roots of the stressed BLF plants, the enzymatic activities regulating Ck metabolism promote a high level of Ck ribosides in comparison to control plants.

When the endogenous Ck content was measured by means of immunotitration, the BLF seedlings showed an increase of [9R]iP and [9R]Z compared with the Ingestad plants. However, this increase of Ck ribosides was only strongly expressed for the shoots and less pronounced for the roots of the seedlings (Schwartzenberg, 1989; Schwartzenberg, unpublished data).

The picture of the regulation of endogenous Ck content remains incomplete as no data on Ck biosynthesis in spruce roots are available at present. Attempts to measure Ck biosynthesis (in spruce seedlings) by feeding large quantities of [^{3}H]adenine for 24 h *via* the roots revealed no considerable radioactivity in the fractions of the Cks E, [9R]Z, iP and [9R]iP (data not presented).

With regard to the previous measurements carried out in Germany on trees of forest stands with different degrees of damage, we think that unfavourable soil conditions could lead to a reduction of Ck riboside metabolism and thus change the cytokinin status of the trees (Schwartzenberg and Hahn, 1991). However, a direct comparison between the spruce trees from forest stands and the model system presented in this paper is not possible as plant material and growth conditions are too different. The presence of mycorrhiza in the spruce from the forest stands may be seen as the main difference to the seedlings used in this work. It seems likely that microorganisms associated with spruce roots, especially mycorrhizal fungi, have an influence on cytokinin status of the plants as they are capable of producing cytokinins and other plant hormones (Miller, 1966; Gogola, 1991; Kraigher *et al*, 1991).

There is also some evidence that microorganisms can interfere with the cytokinin metabolism of spruce roots. After incubation with [9R]iP or [9R]Z, we recently detected unknown Ck metabolites in the nutrient solution of spruce roots (Schwartzenberg *et al*, 1994). These metabolites were absent if sterile *in vitro* grown seedlings were used for incubation. For further work, we propose the study of Ck metabolism in sterile roots and in roots infected with microorganisms (mycorhizal fungi and/or soil bacteria). This seems important in order to show whether the delayed metabolism of Ck ribosides in the roots can cause an accumulation of [9R]iP and [9R]Z in needles or shoots, as has been found for spruce affected by the novel type of forest decline.

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