# Metabolism of [8-$^{14}$C] *trans*-zeatin by intact and decapitated tap roots of *Pinus pinea*

N. Atzmon[†] and J. van Staden[*]

NU Research Unit for Plant Growth and Development, Department of Botany, University of Natal, Pietermaritzburg, 3200
Republic of South Africa

[†] Present address: Department of Horticulture, The Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel

Intact and root tip-decapitated seedlings of *Pinus pinea* were treated with [8-$^{14}$C]zeatin. In intact plants, most of the applied radioactivity remained in the roots. Root tip removal resulted in greater transport of radioactivity to the shoot. The relative distribution of radioactivity along the tap root was non-linear. High amounts of radioactivity associated with zeatin-like compounds were recovered in the elongation zone of intact tap roots. HPLC separation of extracts from the different sections from intact roots showed that the applied zeatin was rapidly metabolized. Very little radioactivity was detected in the upper sections of the root where lateral root formation and lateral root elongation occurred. This radioactivity was not associated with the retention times of authentic cytokinin standards. In the tap root elongation zone, peaks of radioactivity which co-chromatographed with authentic zeatin and *iso*-pentenyladenine were detected. The uneven distribution of applied cytokinins in the tap root, and particularly the higher levels recovered from the root elongation zone, could explain why lateral roots do not develop in this region.

Intakte saailinge van *Pinus pinea* en saailinge waarvan die wortelpunte verwyder is, is met [8-$^{14}$C]seatien behandel. In intakte plante het die meeste van die aangewende radioaktiwiteit in die wortels agtergebly. Wortelpuntverwydering het groter vervoer van die radioaktiwiteit na die stingels tot gevolg gehad. Die relatiewe verspreiding van die radioaktiwiteit in die saailinge was nie eenvormig nie. 'n Groot hoeveelheid radioaktiwiteit wat met seatien-agtige verbindings geassosieer is, is in die verlengingsone van die intakte wortels gevind. HPLC skeiding van ekstrakte van die verskillende seksies van intakte wortels het aangetoon dat die seatien vinnig gemetaboliseer is. Min radioaktiwiteit is in die boonste seksies van die wortels waar laterale wortelvorming en laterale wortelverlenging voorkom, waargeneem. Hierdie radioaktiwiteit was nie geassosieer met die retensietye van outentieke sitokinienstandaarde nie. In die wortelverlengingsone is pieke van radioaktiwiteit wat met seatien en *iso*-pentenieladenien gekochromatografeer het, waargeneem. Die oneweredige verspreiding van aangewende sitokiniene in die hoofwortel, en meer spesifiek die herwinning van groter hoeveelhede in die wortelverlengingsone, kan moontlik verklaar waarom laterale wortels nie in hierdie sone ontwikkel nie.

[*] To whom correspondence should be addressed.

## Introduction

It is well documented that root tips produce substances that strongly inhibit lateral root development (Torrey 1959; Böttger 1974; Wightman *et al.* 1980). Cytokinins are believed to be synthesized in root tips (Van Staden & Davey 1979), and are known to inhibit root formation (Torrey 1967; Van Staden & Harty 1988). Torrey (1962) proposed that lateral root induction is controlled by a gradient of cytokinins which is high in the root tip and declines acropetally towards the base of the primary root. Circumstantial evidence supporting the existence of such a gradient was provided by Short and Torrey (1972) and Wightman *et al.* (1980). There is, however, little information concerning the distribution of cytokinins in plant roots. Recently it was shown that [$^3$H]*iso*-pentenyladenine (iP) is rapidly transported to the shoots of *Pinus pinea* (Atzmon & Van Staden 1993b). The radioactivity detected in the root was distributed unevenly, most being detected in the elongating zone of the tap root where lateral roots normally do not develop. This distribution is significant if viewed in the context that several reports indicated that roots are differentially sensitive to the application of different cytokinins (Goodwin & Morris 1979; Wightman *et al.* 1980; Stenlid 1982; Atzmon & Van Staden 1993a). This factor may have an important influence on root development and thus on root morphology.

While many cytokinin-like compounds have been isolated from xylem sap (Palmer & Wong 1985), and, in conifers specifically, considerable amounts of iP-type cytokinins have been found (Von Schwartzenberg *et al.* 1988; Doumas *et al.* 1989), zeatin and its metabolites are apparently the most common cytokinins in plants (Letham & Palni 1983). Doumas and Zaerr (1988) demonstrated the presence of both the iP and Z groups of cytokinins in the xylem extrudate of Douglas-fir. These authors showed different patterns of transport for individual cytokinins during spring and suggested that the distribution pattern of the two groups of cytokinins may be different within the roots of conifers.

In the present study the relative distribution of [8-$^{14}$C] *trans*-zeatin in the root and its metabolism along the tap root of *Pinus pinea* seedlings were investigated. This was done to test the hypothesis that a varying gradient of cytokinin exists along a tap root with well-defined zones of development (Atzmon 1991).

## Materials and Methods

### Plant material

Three weeks after sowing in trays filled with vermiculite, seedlings of *Pinus pinea* L. were transferred to half-strength nutrient solution (Hoagland & Snyder 1933). Seedlings, 9 per treatment, were grown in 10-l buckets. The culture vessels were aerated continuously and kept in a greenhouse maintained at 25±4°C under a 14-h photoperiod. One, 4 and 7 days before the application of [8-$^{14}$C]Z, the root tips (3 mm) of three groups of plants (9 per group) were removed. At the first root tip-decapitation, which

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Abbreviations: iP, *iso*-pentenyladenine; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; Z, zeatin.

occurred 7 days before radio-label application, the average tap root length was 15 cm. Intact and root tip-decapitated seedlings of the same physiological age were submitted to radio-labelling using 1-ml Eppendorf tubes containing [8-$^{14}$C]Z. The seedlings were held in the tube with a polystyrene holder so that only the first 1 cm of the tap root was in the cytokinin solution. The tubes were covered with aluminium foil to prevent illumination of the roots. Each Eppendorf tube contained distilled water (100 μl) in which [8-$^{14}$C]Z was dissolved (10 000 Bq) (specific activity 441.1 MBq mmol$^{-1}$). After 3 h the roots were rinsed with distilled water and the seedlings transferred back to the culture buckets. Three, 12 or 24 h later, seedlings were harvested for extraction and analysis.

## Cytokinin extraction

At the completion of each treatment, the nine treated seedlings were divided into three replicates. Each replicate was divided into roots and shoots. The roots of intact seedlings were further divided into four sections (Figure 1): I, first 1 cm of the tap root apex; II, section containing the elongating tap root section without visible lateral roots; III, middle section, including the lateral root emergence zone where lateral roots were visible but not longer than 0.5 cm; and IV, an upper section, containing well-developed lateral roots. The root tip-decapitated seedlings were divided into sections II – IV. After having been weighed, all sections were frozen in liquid nitrogen and stored at –20°C until they were analysed. Each sample (500 mg) was ground with 10 ml 80% ethanol. The samples were centrifuged for 10 min at 1000g, 1.0 ml of each extract was transferred to scintillation vials and 4 ml Beckman Ready Solv was added to each vial. Radioactivity was determined using a Beckman LS 3800 Scintillation Counter. The radioactivity within each section of the seedling was calculated and the results expressed as the percentage of the total radioactivity extracted in the ethanolic extracts.

## HPLC analysis of plant extracts

HPLC analysis was performed on the extracts of root sections. The remaining 9 ml of each extract (obtained as described above) was filtered through Whatman's No. 1 filter paper, which was washed twice more with 80% ethanol. The filtrates were combined and dried *in vacuo*. The residues were resuspended in 1 ml 80% ethanol and filtered through 0.22-μm Millipore filters. The filtrate
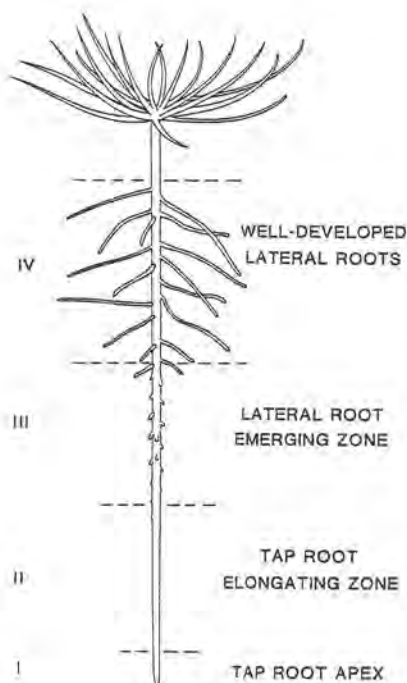
was dried by vacuum centrifugation and resuspended in 300 μl 80% HPLC grade methanol. A 100-μl aliquot of each extract was separated by reverse phase HPLC as previously described (Van Staden *et al.* 1990). A Varian 5000 instrument fitted with a Supelcosil LC-DB (C$_{18}$, 5 μ, 250 × 4.6 mm) was used at a flow rate of 1 ml min$^{-1}$. The buffer system used was 0.2M acetic acid buffered to pH 3.5 with triethylamine : methanol (5 – 50% methanol over 90 min). Ninety 1-ml fractions were collected, taken to dryness and then resuspended in 1 ml methanol, 4 ml Beckman EP Ready Solv scintillation cocktail was added, and radioactivity was determined with a Beckman LS 3800 spectrometer.

## TLC analysis

Aliquots of certain radioactive peaks detected following HPLC separation were dried, resuspended in 40 μl of 80% ethanol and chromatographed by TLC. Silica gel (Merck$_{254}$) plates were used and the extracts were separated using n-butanol : 25% NH$_4$OH : water (6:1:2, v/v/v upper phase). Relevant authentic cytokinin standards were run simultaneously. The chromatograms were divided into 10 R$_f$ units, the silica gel was scraped from the plates into scintillation tubes, 2 ml methanol and 4 ml Beckman EP Ready Solv were added to each and the radioactivity was measured.

## Results

After 24 h only about 17% of the recovered radioactivity was present in the shoots of the *Pinus pinea* seedlings. When the root tips were removed, transport was enhanced greatly (Figure 2).

The relative distribution of radioactivity following [8-$^{14}$C]Z application was not linear (Figure 3). Three hours after applying [8-$^{14}$C]Z, 90% of the radioactivity was found in the elongating zone of the tap root (section II), while the remainder was divided equally between the other three root sections analysed. This pattern did not change much with time. The relative amount of radioactivity recovered in the elongating zone decreased with time while it increased slightly in the other parts of the tap root.

In decapitated seedlings most of the radioactivity was also detected in the elongating zone (section II) of the tap root (Figure 4). The relative amount of radioactivity that was detected in sections III and IV increased most in the roots decapitated 7 days prior to zeatin application.

Separation of ethanolic extracts from intact root sections extracted for the different times of application by HPLC
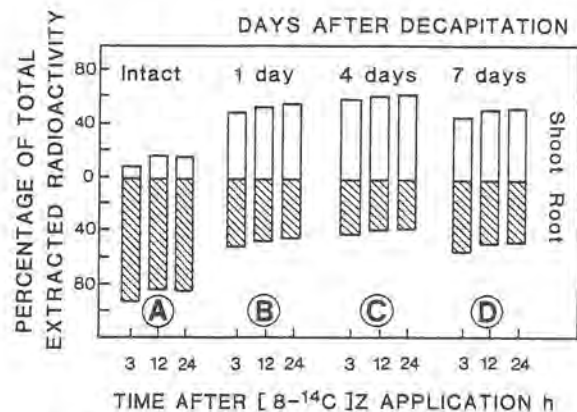


**Figure 1** Diagram showing how the root system was divided into various sections for analyses.



**Figure 2** Relative distribution of the radioactivity detected in the 80% ethanolic extracts of roots and shoots of intact *P. pinea* seedlings (A) and seedlings where the root tips were removed respectively 1, 4 and 7 days prior to treatment (B – D). Sampling was done 3, 12 and 24 h after treating the apical 1 cm of the intact or decapitated tap roots with [8-$^{14}$C]Z.

showed noticeable changes in the radioactivity profiles between the different sections (Figure 5). In the root tip (section I), 3 h after treatment with [8-$^{14}$C]Z, the major peak detected co-chromatographed with Z. After 12 h, Z was still detected but the major peak now co-chromatographed with iP. Twenty-four hours after applying [8-$^{14}$C]Z, no major radioactive peaks co-chromatographed with cytokinin standards. The radioactive profile in the root elongation zone (section II) was similar to that recorded for the root tip, except that no peak coincident with iP was detected. In section III, two peaks that co-chromatographed with Z and iP were detected, and in the lateral root elongation zone (section IV) no clear peaks that co-chromatographed with authentic standards were recorded.

## Discussion

In the present study with intact *Pinus pinea* seedlings where [8-$^{14}$C]Z was applied to the root tips, only about 17% of total radioactivity detected was found in the shoots. When [$^3$H]iP was applied, most (59%) of the recovered radioactivity was present in the shoot within 24 h (Atzmon & Van Staden 1993b). This indicates that the Z and iP-type cytokinins are not necessarily transported at the same rate by plant roots. This information is in agreement with the finding that in Douglas-fir different cytokinins showed different patterns of transport during spring (Doumas & Zaerr 1988).

The relative accumulation of zeatin-like radioactivity in the elongating zone (section II) of the root, while poor transport to the upper section occurred, suggests that zeatin may be involved in apical dominance and the inhibition of lateral root formation in this section of the root system. This hypothesis was put forward earlier (Van Staden & Harty 1988) and is supported by the fact that in this study most of the radioactivity following [8-$^{14}$C]Z application not only remained in the roots but specifically in that part where lateral roots do not develop. Support for this hypothesis was also provided by a study which showed that zeatin application increased tap root dry weight while lateral root dry weight was decreased (Atzmon & Van Staden 1993a).

The fact that root tip removal resulted in an increase of the relative radioactivity in the shoots supports the suggestion (Atzmon & Van Staden 1993b) that the root tip is not only responsible for cytokinin synthesis but also affects its mobility and distribution in the plant. It is well known that removal of the root tip, which is the site of cytokinin synthesis, stimulates lateral root development (Wightman & Thimann 1980; Biddington & Dearman 1982). It was suggested that this stimulatory effect is due to a decrease in inhibitors (Böttger 1974).

From a comparison of the distribution of zeatin-like substances along intact and decapitated roots with that of iP-like compounds (Atzmon & Van Staden 1993b), it would appear that not only the absolute amount but also the ratio between zeatin and iP in each of the root sections might play a role in lateral root development. While iP tended to accumulate in sections in which lateral root development was most pronounced (Atzmon & Van Staden 1993b), zeatin tended to accumulate in sections where lateral roots did not form.

Letham and Palni (1983) indicated that iP acts as a precursor for zeatin. However, in the present study, a peak which co-chromatographed with iP was found in the root tip following application of [8-$^{14}$C]Z. As this was found only in the apex (section I) of the tap root, it is possible that the relatively high amount of zeatin in the apex following exogenous application caused changes in the metabolic pathway. The fact that 24 h after application, no radioactive peaks coincident with cytokinin-like compounds were detected, suggests that zeatin was very rapidly metabolized. This is in agreement with other studies (Letham & Palni 1983). Very little radioactivity coincident with cytokinins was detected in the upper root sections (III and IV). This suggests that the metabolism of zeatin was high in these root zones or, alternatively, it was metabolized lower down the root and thus not transported to the upper parts of the root. This again differed from the distribution pattern found for iP (Atzmon & Van Staden 1993b).

## Acknowledgements

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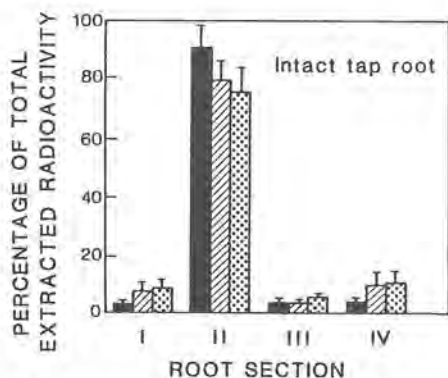


**Figure 3** Relative distribution of radioactivity along the tap roots of intact *Pinus pinea* seedlings 3 (solid columns), 12 (hatched columns) and 24 h (dotted columns) after the application of [8-$^{14}$C]Z to the tips of the tap roots.
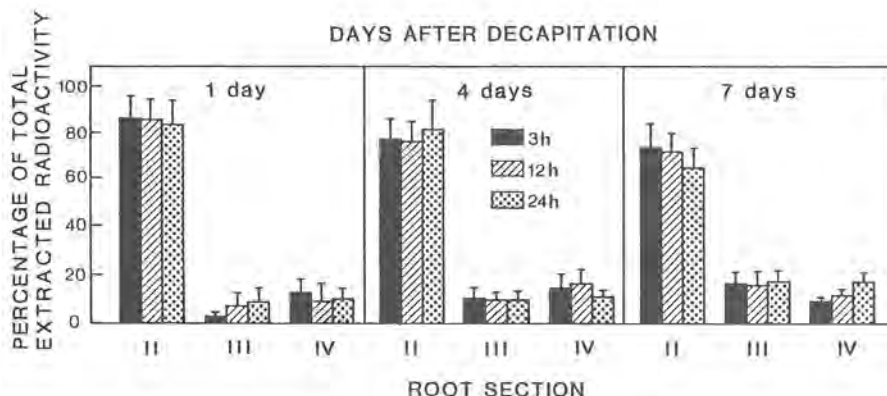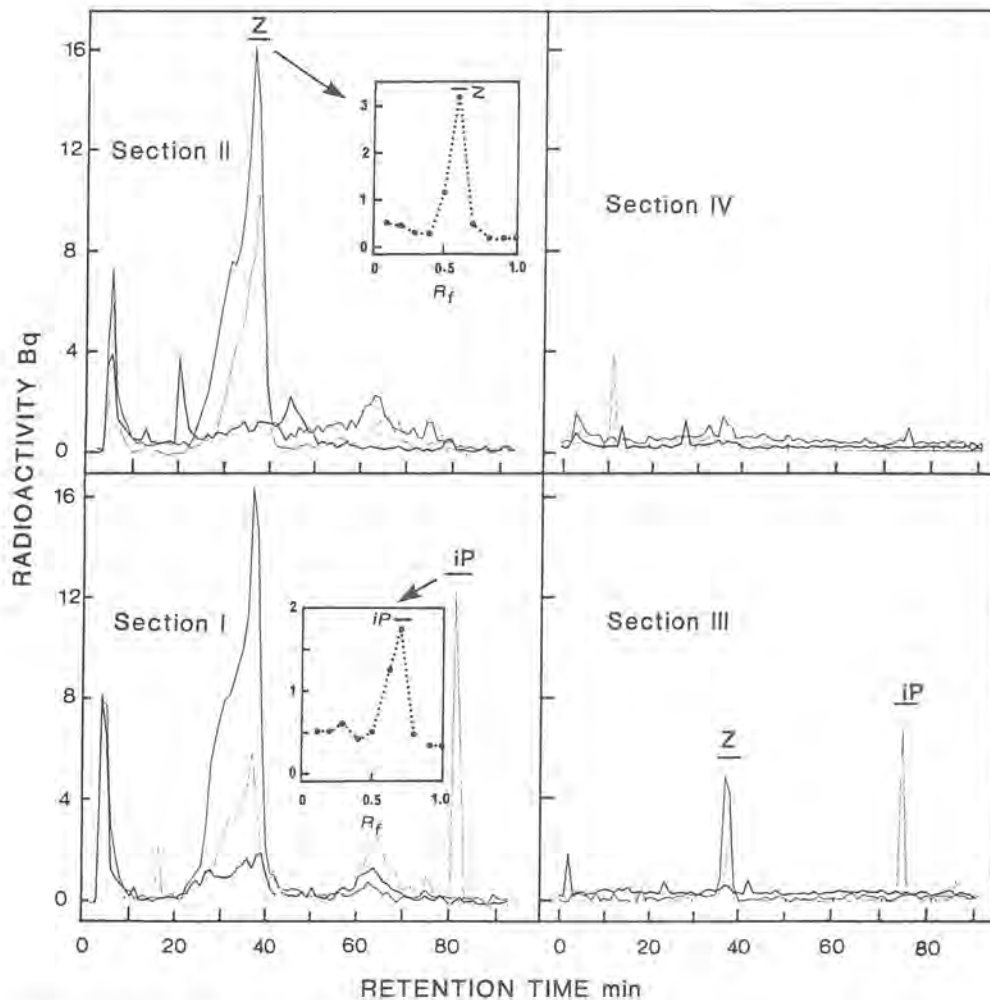


**Figure 4** Relative distribution of radioactivity along the tap roots of *P. pinea* seedlings following [8-$^{14}$C]Z application. The [8-$^{14}$C]Z was supplied 1, 4 and 7 days after decapitation of the root tips.

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**Figure 5** HPLC separation of extracts from different sections of the intact root 3 (thin line), 12 (dotted line) and 24 h (thicker line) after the application of [8-¹⁴C]Z to the root tip. Inserts represent TLC separations of aliquots of relevant peaks.

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