Ontogenetic Variation of Four Cytokinins in Soybean Root Pressure Exudate

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ABSTRACT

Cytokinins exported from the root may be involved in the correlative control of plant development. To test this hypothesis in soybean (Glycine max [L.] Merr. cv. McCall, cv Chippewa 64, and cv Hodgson 78), cytokinins were intercepted en route from the root to the shoot by collecting root pressure exudate from detopped roots. The quantities of four cytokinins in the exudate were studied throughout the development of plants grown in the field and in controlled environment chambers. Zeatin, zeatin riboside, and their dihydro derivatives, dihydrozeatin and dihydrozeatin riboside, were isolated and quantitated using high-performance liquid chromatography.

Cytokinin fluxes (pmoles per plant per hour) were independent of exudate flux (grams per plant per hour). All fluxes are averages for a 6- or 8-h collection period. The ribosides accounted for the majority of the observed cytokinin transport. The fluxes of zeatin riboside and dihydrozeatin riboside increased from low levels during vegetative growth to maxima during late flowering or early pod formation. Before the seeds began rapid dry matter accumulation, zeatin riboside and dihydrozeatin riboside fluxes decreased and remained at low levels through maturation. The fluxes of zeatin and dihydrozeatin were low throughout development.

No correlation was found between cytokinin fluxes and nodule dry weight or specific nodule activity (acetylene reduction).

The timing of distinct peaks in zeatin riboside and dihydrozeatin riboside fluxes during flowering or pod formation suggests that cytokinins exported from the root may function in the regulation of reproductive growth in soybean.

The roles of cytokinins in plant growth and development are not fully understood, but evidence supports the hypothesis that cytokinins are produced by the root system and transported to the shoot, where they are involved in the regulation of shoot processes (3, 22, 27). Transport in the xylem sap of materials with cytokinin-like activity (determined by bioassay) has been demonstrated in numerous species including sunflower (22), tomato (7), and lupin (8, 9). Cytokinin-like materials have also been reported in the root nodules of Vicia faba (13) and Alnus glutinosa (14). Henson and Wheeler (15) proposed that cytokinins are exported from nodules to the xylem and there supplement the supply moving from the roots to the shoot. Within 24 h of injecting [14C]zeatin into the nodules, they detected Z2 and a number of its metabolites throughout the plant.

If cytokinins from the root system are involved in regulating shoot growth and function, then their supply to the shoot should fluctuate over the course of development. Seasonal variations of cytokinin-like activity in root pressure exudate have been reported in a number of genera (2, 3, 7-9, 22, 24). In Perilla, there was a 5-fold increase in cytokinin-like activity in root pressure exudate after floral induction (2, 3). Conversely, the root sap of Xanthium plants which had been induced to flower contained less than one-third as much cytokinin-like activity as did the sap of vegetative plants (24).

Although cytokinins were not specifically identified as the active regulating component, several studies have shown that roots or factors from the roots are required for maintaining leaf Chl content (18) and photosynthesis (4, 5, 18, 27). In Phaseolus, foliar applications of BA delayed Chl loss and photosynthetic decline associated with senescence (1).

In A. glutinosa, cytokinin-like activity in the root nodules increased prior to the onset of nitrogenase activity (acetylene reduction) and declined by the time N2-fixing capacity had been fully established (14). Likewise, Newcomb et al. demonstrated that cytokinin-like activity in pea nodules was greatest early in nodule development and decreased thereafter (20). Henson and Wheeler hypothesized that cytokinins exported from nodules affected N2-fixation through either local action in the nodule or by a remote effect on the shoot (14).

Presence of cytokinins in soybean root pressure exudate has not been demonstrated. Therefore, it was not known whether or not these hormones could be involved in the regulation of soybean development. Hence, the objectives of our research were to (a) establish the presence of cytokinins and (b) describe profiles of cytokinin flux in root pressure exudate in relation to the ontogeny of soybean plants grown in the field and in growth chambers. Root pressure exudate was collected and acetylene reduction activity was measured from late in vegetative growth through maturity. Four cytokinins for which biological activity has been shown (7, 17), Z, diZ, ZR, and diZR, were isolated and quantitated by HPLC. The cytokinin profiles are discussed in relation to several measures of shoot and nodule development.

MATERIALS AND METHODS

Plant Culture. Two indeterminate soybean (Glycine max [L.] Merr.) genotypes having different maturities were planted in field plots in St. Paul, MN, on a Waukegan silt-loam soil. The field was treated with 1.1 kg ha–1 of preplant incorporated trifluralin

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2 Abbreviations: Z, zeatin; diZ, dihydrozeatin; ZR, zeatin riboside; diZR, dihydrozeatin riboside; TMS, trimethylsilyl; SNA, specific nodule activity.
for weed control. Plots were seeded on July 9, 1979 with cv McCall (maturity group 00) and on May 28, 1980 with cv Chippewa 64 (maturity group 1). Rows were 7 m long and spaced 0.75 m apart. At the first trifoliate leaf stage, plants were thinned to a uniform spacing of 7.5 cm within the rows. Each sample plot consisted of six adjacent plants in a row, and bordered sample plots were arranged in a randomized, complete block design with four replications.

A similar experiment was also conducted in plant growth chambers (Conviron model E15). Seeds of cv Hodgson 78 (maturity group 1) were planted in 4-L plastic pots in a 1:1 (v/v) mixture of silt-loam and Zorball (IMC Chem., Des Plaines, IL). At the time of planting, seeds were inoculated with a peat slurry of Rhizobium japonicum, USDA strain 123, and all plants developed a well-nodulated root system. Twelve d after planting, plants were thinned to one plant per pot. The plants were arranged in a randomized, complete block design, with 10 replicate plants for each sampling date.

Plants in the growth chambers received a 12-h photoperiod and a 27/21°C day/night temperature regime. A combination of fluorescent and incandescent lamps supplied an average photosynthetic photon flux density of 500 μE m⁻² s⁻¹ at the top of the canopy. Three times per week, plants received 250 ml of half-strength low N (50 μM N as NH₄NO₃) Hoagland solution (16). Plants were irrigated with tap water as required.

**Collection of Root Pressure Exudate.** Root pressure exudate was collected every 7 to 14 d, from just prior to flowering until stage R6 (full seed) (11) in 1979, stage R5 (beginning seed) in 1980, and stage R7 (beginning maturity) in the growth chambers. In the field, exudate was collected for a 6-h period beginning approximately 3 h after sunrise; in the growth chamber experiment, it was collected for 8 h, beginning 4 to 5 h after the lights were turned on. Plants were kept well watered prior to exudate collection.

On the day of collection, plants were detopped just below the unifoliate leaf node. The first few drops of exudate from the stump were probably contaminated with cell debris and were discarded. A reduced pressure of 37 cm Hg was used to draw exudate from the cut surface of each stump, through latex and capillary glass tubing, into separate foil-covered, silicated (Aquasil; Pierce Chem Co., Rockford, IL) 18-mm culture tubes. The collection tubes were held on ice. At the end of the collection period, the exudate was weighed, frozen, and subsequently lyophilized. The lyophilized exudate was stored at −20°C prior to analysis for cytokinin content.

The developmental stage (11) was recorded for each plant being sampled. In the growth chamber experiment, dry weights of individual plant parts were recorded after drying to constant weight at 60°C. Abscised leaves and petioles were not included in the measurements. In 1980, the detached root systems used for the acetylene reduction assay were used for the measurement of nodule dry weight.

**Acetylene Reduction Assay.** In the 1980 field experiment, acetylene reduction and nodule dry weight were measured through stage R6. At each sampling date, six adjacent plants were detopped at their cotyledonary nodes and their root systems excavated. Root nodule activity was determined as described by Finn and Brun (12). Specific nodule activity was expressed as pmol C₂H₄ ng⁻¹ nodule dry weight h⁻¹.

**Cytokinin Analysis.** HPLC was used to isolate and quantitate four cytokinins, Z, diZ, ZR, and diZR, in root pressure exudate. Preliminary studies on exudate from single plants revealed quantities of cytokinins approaching the lower limits of detectability (<5 ng/plant). Therefore, in the field experiments, the lyophilized exudate from the six plants within a replication was pooled prior to hormone analysis. In the growth chamber experiment, exudate from three or four replicate plants was combined.

Figure 1 and Table I summarize the sequence and specifications of the chromatographic systems used. Absorbance of the column eluate was monitored at 280 nm. In the preparative steps, fractions were collected according to the retention times of authentic standards. In the analytical steps, peaks which co-chromatographed with authentic standards were calibrated against these standards by their respective A₂₈₀ pmol peaks. Suitable radioactive cytokinin standards were not available; consequently, internal standards were not used and percent recovery of the hormones was not calculated.

The length of the exudate collection period and the number of plants sampled were not the same in all experiments. Therefore, hormone fluxes were expressed as pmol plant⁻¹ h⁻¹ and exudate flux as g plant⁻¹ h⁻¹ so that the results of the three experiments could be compared. The cytokinin content and exudate weight were only measured at the end of the exudation period; hence, the reported fluxes are averages and may not represent the actual rates of transport of cytokinins and exudate.

**GC-MS.** After cation-exchange HPLC, samples were run on HPLC system 5 (Table I) to remove salts and other impurities which had accumulated from the solvent in HPLC system 4. Trimethylsilyl derivatives of dry standards and samples were prepared just prior to injection on the GC-mass spectrometer. The reagent was N,O-bis(trimethylsilyl)trifluoroacetamide-trimethylchlorosilane (99:1, 8 μl; Regis Chemical Co., Morton Grove, IL), with 2 μl pyridine as solvent. The reaction mixture was heated at 55°C for 0.5 h. Mass spectra were obtained using an LKB 9000 gas chromatography-mass spectrometer operating at an ionization potential of 70 eV and interfaced to a PDP 8e computer. The samples were run on a 60 cm × 2 mm (i.d.) glass column packed with 3% OV-1 on 100 to 120 mesh Supelcoport. Temperature was programmed from 150 to 290°C at 8°C/min, and the He flow rate was 30 ml/min.

**RESULTS**

Analysis of selected ZR and diZR samples by GC-MS verified that the HPLC procedures used for hormone isolation and quantification were reproducible.
titration had in fact yielded pure ZR and diZR. Samples of Z and diZ were not analyzed by GC-MS because they were not present in significant quantities at any sampling date. Mass spectra of TMS-ZR samples contained ions (relative intensities in parentheses) of m/z 639 (M+, 3.1), 624 (3.9), 549 (7.1), 536 (9.5), 320 (7.0), 230 (8.5), 201 (17.9), and 73 (TMS, 100) which were characteristic of TMS-ZR standards and corresponded to published spectra (6). Major ions in mass spectra of TMS-diZR samples included (relative intensities in parentheses) m/z 641 (M+, 1.6), 626 (4.3), 322 (32.9), 294 (6.9), 230 (35.9), 162 (10.1), and 73 (TMS, 100). Ions with these m/z ratios were also characteristic of TMS-diZR standards and were used by Wang and Horgan to identify diZR from Phaseolus leaves (25).

Sample quantitation was by selected ion current monitoring. Samples were calibrated against standards on the basis of peak intensity of specific ions, m/z 320 and 201 for ZR and m/z 322 and 230 for diZR, while also monitoring the other ions listed above for identification. Within experimental limits, the resulting levels of ZR and diZR were in agreement with those which previously had been determined by peak height analysis using HPLC system 4. Therefore, the results of analysis by GC-MS not only verified that the isolated compounds were ZR and diZR, but they also demonstrated the validity of our HPLC procedures for quantitation of these compounds from soybean root pressure exudate.

The patterns of ZR and diZR fluxes throughout development were similar in these experiments. In each case, ZR and diZR fluxes were initially low, increased to maxima, and then rapidly declined and remained low throughout the rest of development. However, the maximum fluxes occurred at different times with respect to developmental stage and days after sowing. In the field, maximum fluxes occurred at approximately stage R2 (full bloom); this stage corresponded to 36 d after sowing in 1979 and 55 d after sowing in 1980 (Figs. 2 and 3). In the growth chamber, maximum fluxes were observed when the plants reached stage R4 (full pod), 40 d after sowing (Fig. 4). The fluxes of ZR and diZR were approximately equal in field-grown plants, but diZR flux was 75% greater than ZR flux until stage R5 in plants grown in growth chambers.

The fluxes of the free base cytokinins, Z and diZ, were significantly lower than the fluxes of the corresponding ribosides until stage R5 (1979) or stage R3, beginning pod, (1980), by which time ZR and diZR fluxes had declined to low levels comparable to Z and diZ. The fluxes of Z and diZ only showed significant variation with development under field conditions. However, in 1980, Z flux did not differ significantly from zero except at the first sampling date.

Dry weights of individual plant parts are shown for the growth chamber experiment in Figure 5. At 43 d after sowing, rapid seed growth began, and accumulation of dry matter in vegetative parts
of the shoot ceased. Little additional growth of the roots occurred after this time, but nodule mass continued to increase until 75 d after sowing. Decreasing shoot dry matter subsequent to 54 d after sowing was due to leaf abscission, which began at the lower nodes.

The cytokinin flux in the growth chamber experiment was greatest at 40 d after sowing (Fig. 4); hence, the fluxes of ZR and diZR from the root to the shoot had reached maxima and had already begun to decline at a time when the shoot was still adding new vegetative tissues and when rapid seed growth had not yet commenced. Similarly in the field (Figs. 2 and 3), ZR and diZR fluxes were maximal at full bloom (stage R2), when these indeterminate genotypes were still producing vegetative growth, and decreased to low levels by stage R5, when rapid seed growth began.

The flux of root pressure exudate changed significantly during development, but the patterns were different in the three experiments (Figs. 2–4). In the field, cytokinin flux did not coincide with exudate flux in either year. In the growth chamber, the profile of exudate flux was similar to those of ZR and diZR. However, the exudate flux dropped approximately 50% between 46 and 62 d after sowing, whereas the fluxes of ZR and diZR declined from relatively high levels to almost nil in the same period. In each of the experiments, the concentration of cytokinins in the exudate varied with sampling date. As a consequence, the amount of these hormones recovered in the exudate was a function of the concentration as well as of the volume of the exudate collected.

Specific nodule activity and nodule dry weight for the 1980 experiment are shown in Figure 6. Specific nodule activity was maximal 78 d after sowing, and nodule dry weight continued to increase throughout the experiment, while ZR and diZR fluxes had maxima 55 d after sowing (Fig. 3). There were no significant positive correlations of ZR and diZR fluxes with SNA or nodule mass.

**DISCUSSION**

Our results demonstrate the presence of cytokinins in root pressure exudate and establish a pattern for significant changes in cytokinin flux during soybean development. The fluxes of ZR and diZR from the root to the shoot were low at the onset of flowering (R1), reached maxima late in flowering (field) or during pod formation (growth chamber), and abruptly dropped to low levels before the seeds began rapid dry matter accumulation (R5). Except for the inexplicable rise in diZR flux with the onset of maturity in the growth chamber experiment (Fig. 4), cytokinin flux in root pressure exudate remained very low throughout seed growth and maturation. Our data are similar to those of Beever and Woolhouse (2, 3) for cytokinin-like activity in the root pressure exudate of *Perilla frutescens*. They found activity in the sap was low during the vegetative phase, reached a maximum during fruit development, and returned to low levels at maturity. In contrast, Davey and van Staden (8, 9) found a somewhat different developmental pattern of cytokinin-like activity in the bleeding sap of white lupin. Cytokinin-like activity decreased through the vegetative stage until 2 weeks after anthesis, increased through fruiting (8 weeks after anthesis), and dropped to minimal levels at 10 weeks after anthesis.

Sitton et al. (22) and Wareing et al. (27) have proposed that a decrease in cytokinin flux to the leaves after flowering is responsible, in part, for the leaf senescence which accompanies fruiting in monocarpic species. The rapid decline in cytokinin flux during pod formation and the continued low levels during seed development which we observed in soybean are consistent with this interpretation. However, the cytokinin flux in the exudate was also low at initial flowering, when the leaf canopy was probably

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**Fig. 2.** Mean flux of cytokinins and of root pressure exudate for the 1979 field experiment. Stage of development (after Fehr et al. [11]) on each sampling date is shown at the top of the figure. Bars indicate ± se of four replicates.

**Fig. 3.** Mean flux of cytokinins and of root pressure exudate for the 1980 field experiment. Stage of development as in Figure 1. Bars indicate ± se of four replicates.
at its functional peak (21). Therefore, the cytokinin supply to the soybean shoot may be more directly related to reproductive development and may only influence leaf senescence indirectly through interactions with other hormonal, nutritional, or environmental factors.

Davey and van Staden (10) detected cytokinin-like activity in the sap (presumably xylem and phloem) entering white lupin fruits. Further, cytokinin-like activity was also present in developing seeds and pod walls. Although it is well known that cytokinins promote cell division in tissue cultures, Davey and van Staden (10) suggested that the cytokinins in these tissues affected seed growth by attracting nutrients to the developing fruits. Neising and Morris (19) reported a correspondence between cytokinin levels and rates of cell division in Phaseolus embryos. Nevertheless, they also proposed an indirect role for cytokinins, i.e., via protein synthesis, in the regulation of seed growth. We have not determined the destinations of cytokinins which are transported in soybean xylem sap. However, perhaps a portion of these hormones ultimately reach young reproductive structures. There they could stimulate seed growth by triggering cell division or by one of the mechanisms mentioned above.

The relationship between SNA and the levels of cytokinins we found in root pressure exudate of soybean is similar to a relationship between total nodule activity and cytokinin-like activity present in the root nodules of Alnus glutinosa (14). The temporal separation of the cytokinin and SNA profile maxima in soybeans (Figs. 3 and 6) is consistent with reports that the cytokinin content of nodules is high early in nodule ontogeny and decreases as the nodules grow and total nodule activity develops (14, 20). Our data indicate that the export of cytokinins from roots was not required for maximal SNA because ZR and diZR fluxes were greatest 22 d before maximal SNA was attained. Nodules may contribute to the total cytokinin flux; however, it cannot be assumed that the cytokinin flux from the whole root system is a reflection of either nodule cytokinin content or export. At this point, it would be appropriate to devise a method for studying cytokinin export from the nodules directly.

At their maximum level of transport, ZR and diZR were several-fold more abundant in the exudate than were their free base counterparts. Even when all of the cytokinins were at low levels, the ribosides were generally present in greater quantities than the...
free bases. The close parallels of Z withZR and diZ with diZR, especially in the 1979 field experiment, suggest that the free base cytokinins which were detected may have resulted from the hydrolysis of ZR and diZR during collection, storage, and analysis. Although ZR has been reported to be responsible for the majority of cytokinin-like activity in the root pressure exudate of many species including lupin (8, 9) and tomato (7), activity due to Z and ZR was approximately the same in the sap of a number of other herbaceous species (23). In any case, the ribosides were the major cytokinins recovered from soybean root pressure exudate.

The significance of the ratio of ribosides to free bases is not known. Although Leonard et al. (17) found that on a molar basis Z and diZ were more active than their ribosides in the tobacco callus bioassay, Davey and van Staden (7) determined that the free bases and their ribosides were equally active in the soybean bioassay. If the free bases and their ribosides are in fact equally active in the soybean plant, then the reason for the predominance of the ribosides in the root pressure exudate is not clear. Perhaps the ribose moiety facilitates solubility and transport or in some way protects the hormone molecule from degradation during transport.

The occurrence in soybean root pressure exudate of cytokinins with a saturated side chain (diZ and diZR) in amounts equal to or greater than those with an unsaturated side chain (Z and ZR) is of interest. Davey and van Staden (10) observed similar amounts of the saturated cytokinins and their unsaturated counterparts in lupin seeds. There is no data concerning their relative activities in the soybean bioassay, but in the tobacco callus bioassay, Leonard et al. (17) showed that the cytokinins with saturated and unsaturated side chains had approximately the same activity. Wareing et al. (26) suggested that the saturated side chain is less susceptible to attack by cytokinin oxidase enzymes which readily cleave the side chains of Z and ZR. Thus, the significance of the saturated side chain may lie in its metabolic properties rather than in its physiological activity.

Distinct profiles of root pressure exudate flux were observed during development. Exudate profiles similar to those in the growth chamber and the 1979 field experiments have been reported for lupin (8, 9), tomato (7), and Perilla (3). Unlike the cytokinin profiles, the patterns of root pressure exudate flux were not uniform across experiments. Profiles in the 1979 field and growth chamber experiments were similar (Figs. 2 and 4). Exudate flux rose to a maximum between stages R4 and R5 and then declined through the rest of development. In contrast exudate flux in the 1980 field experiment (Fig. 3) was maximal at stage R1 and followed a general declining trend through the sampling period. In each experiment, exudate flux was greatest earlier in development and decreased substantially as the plants matured. Maximal exudate flux in field grown plants was more than 50% greater than in plants grown in growth chambers. This may be related to unrestricted root growth in the field. These results support our observations in preliminary experiments (data not shown) that the volume of exudate was influenced by such environmental conditions as the volume of pots in which plants were grown and the water status of the plants.

Changes in cytokinin flux with plant development were at least partially independent of changes in exudate flux. Although there were some qualitative similarities between cytokinin flux and exudate flux in each of the experiments, no quantitative relationship was observed. Lack of similarity of cytokinin flux and volume of root pressure exudate has also been shown in Perilla (2, 3), tomato (7), lupin (8, 9), and sunflower (22). This may be indicative of regulated synthesis and transport of cytokinins to the shoot in response to physiological and environmental stimuli. Little is known of the function of cytokinins in plant development in vivo. However, inasmuch as the volume of exudate was itself subject to developmental and environmental fluctuations, the fact that the cytokinin flux to the shoot was independent of exudate volume supports a regulatory role for cytokinins.

In summary, we have shown that the fluxes of ZR and diZR in the transpiration stream fluctuate significantly during soybean development. These cytokinins may be involved in the control of reproductive development, but they do not seem to be directly related to SNA or leaf senescence. It seems that cytokinins serve some function in regulating the coordinated development of the plant, but the precise nature of this regulation is not clear.

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