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Correlation of Xylem Sap Cytokinin Levels with Monocarpic Senescence in Soybean[1]

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ABSTRACT

Cytokinins (CKs) coming from the roots via the xylem are known to delay leaf senescence, and their decline may be important in the senescence of soybean (*Glycine max*) plants during pod development (monocarpic senescence). Therefore, using radioimmunoassay of highly purified CKs, we quantified the zeatin (Z), zeatin riboside (ZR), the dihydro derivatives (DZ, DZR), the O-glucosides, and DZ nucleotide in xylem sap collected from root stocks under pressure at various stages of pod development. Z, ZR, DZ, and DZR dropped sharply during early pod development to levels below those expected to retard senescence. Pod removal at full extension, which delayed leaf senescence, caused an increase in xylem sap CKs (particularly ZR and DZR), while depodding at late podfill, which did not delay senescence, likewise did not increase the CK levels greatly. The levels of the O-glucosides and the DZ nucleotide were relatively low, and they showed less change with senescence or depodding. The differences in the responses of individual CKs to senescence and depodding suggest differences in their metabolism. Judging from their activity, concentrations and response to depodding, DZR and ZR may be the most important senescence retardants in soybean xylem sap. These data also suggest that the pods can depress CK production by the roots at an early stage and this decrease in CK production is required for monocarpic senescence in soybean.

CK[2] appears to be the major senescence-retarding hormone in plants, and its role in leaves is particularly important (30). Nonetheless, there is little integrated information on the CK hormone systems regulating senescence or other processes (21). A wide variety of studies have shown that leaf senescence is usually correlated with a decrease in CK activity levels in the leaves and have implicated roots as the major sources of CKs in mature leaves (30). These root-produced CKs are carried through the xylem into the leaves with the transpiration stream.

In soybean, the developing pods, specifically the seeds, cause the plant to degenerate (monocarpic senescence) and die (14, 15, 19, 20). Removal of the pods before, but not during, late podfill can prevent the dramatic yellowing and death of the plant (15, 19). How does CK fit into this correlative control picture? Early in reproductive development, the foliar CK-like activity (16) declines. This decrease is due neither to diversion of the flux from the leaves to the pods (22, 23) nor to an increase in the metabolism of CKs (Z and ZR and their metabolites), which is quite rapid anyhow in mature leaves (22, 23, 27). Thus, a decline in CK production by the roots could account for the decrease in foliar CK levels. In order to test further the connection between CKs and leaf senescence and to fill in a gap in our understanding of the role of CK in the control of senescence, this study examines the xylem sap levels of CK as a relative index of CK flux in plants allowed to develop fruit and senesce normally as well as in depodded plants.

MATERIALS AND METHODS

Plant Culture and Treatments

Seeds (*Glycine max* [L.] Merrill cv 'Anoka') were germinated in vermiculite and then transplanted to pots with a 2:1 (v/v) mixture of soil and perlite as described elsewhere (24). After 4 weeks on a greenhouse bench under extended days, the plants were placed in environmental control chambers with SDs (10 h) at 27°C, nights 22°C and about 75% RH. These plants flowered after about 15 SDs, and the most advanced pods reached a length of 1 cm at about 22 SDs.

Some plants were depodded as the most advanced pods approached full extension, before podfill (SD 28), and pods were removed thereafter as they became fully extended. This depodding stimulated new pod formation only slightly. Others were depodded as the pods entered late podfill (SD 47). These treatments were chosen because the former clearly prevents leaf yellowing and abscission and death of the plants, whereas the latter does not (15, 19).

Xylem Sap Collection

Xylem sap was collected during midday from five plants at each stage as described elsewhere (24). At key developmental stages, the plants were cut off about 8 cm above the soil level, the cut surface was wiped with methanol/formic acid/H_2O (14:1:2, v/v) in order to inhibit phosphatase action and thereby prevent loss of the nucleotides (4, 22), the rootstock was placed in a sealed chamber with the stem sticking out,

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[2] Abbreviations: CK, cytokinin; cytokinins: IPA, isopentenyladenine; Z, zeatin; ZR, zeatin riboside; DZ, dihydrozeatin; DZR, dihydrozeatin riboside; OGZ, etc., the corresponding O-glucosides; IPMP, isopentenyladenine riboside 5′-monophosphate; ZMP, zeatin riboside 5′-monophosphate; DZMP, dihydrozeatin riboside 5′-monophosphate; RIA, radioimmunoassay; SD, short day.

and the rootstock was subjected to 100 kPa of pressure for 50 min. The first two drops of exudate were discarded and the cut stem was connected to low-volume plastic tubing which ran into a glass tube with 0.10 mL concentrated formic acid in an ice bath. The volume of the sap samples was then determined, and the samples were frozen, freeze-dried, and stored in darkness at −13°C.

Purification of the CKs

The samples were fractionated on a phosphocellulose column as described by Badenoch-Jones *et al.* (1). The runoff (PC runoff) was saved to prepare the nucleotides. The adsorbed CKs were eluted with 0.5 N NH_4OH and partitioned into *n*-butanol. The organic phases were evaporated under vacuum and the samples were chromatographed on 1 mm-thick silica gel (Merck 60 PF_{254}) layers and developed in *n*-butanol, concentrated acetic acid, H_2O (450:113:188, v/v). The O-glucoside and the Z-DZ-ZR-DZR zones were eluted (22). The glucoside fraction was hydrolysed with β-glucosidase, and the CKs were partitioned into *n*-butanol (9). The dried PC runoffs (nucleotides) were redissolved in H_2O and extracted with *n*-butanol. The aqueous phases were reevaporated, redissolved in H_2O, hydrolyzed with alkaline phosphatase, and partitioned into *n*-butanol (25). The butanol extracts from the glucosidase and the phosphatase digests were then evaporated under vacuum.

The evaporated eluate of the Z-DZ-ZR-DZR zone was dissolved in 0.2 M NH_4OH and separated into a base (Z + DZ) fraction and a riboside (ZR + DZR) fraction using a dihydroxyboryl polymer column (13). Radioactive markers (^{3}H-DZ, ^{3}H-DZR, and ^{14}C-AR) were added to monitor column performance and recovery. It should be noted that samples of the boryl polymer obtained recently (Sigma Chemical Co.) tended to retain CKs by adsorption in addition to retention of ribosides by complex formation, but this absorption was eliminated by including methanol (25% v/v) in all solutions.

Quantification of the CKs

The purified CKs were determined by RIA as described by Badenoch-Jones *et al.* (1, 2). Losses were estimated by running similar amounts of Z, DZ, ZR, and DZR through the above purification procedures in parallel with the sap samples to determine the correction factors which were applied to the sap samples. Percentage recovery values for Z, DZ, ZR, and DZR were 54, 60, 39, and 42, respectively. These values were also applied to correct approximately for losses of O-glucosides. Nucleotide values were not corrected for loss in purification; however, we expect these losses to be less than those cited above.

The hydrolysed O-glucoside eluate could contain Z, ZR, DZ, and DZR derived from their respective O-glucosides. Since the antibodies raised against ZR and DZR cross-react with Z and DZ, respectively, the values for OGZ+OGZR are expressed as ZR equivalents, and those for OGDZ+OGDZR as DZR equivalents.

After RIA, the amounts of CKs remaining from any one sample were too low for verification by GC-MS. Accordingly, all the free base-containing fractions (Z + DZ) were combined after RIA, and deuterium-labeled Z and DZ (29) were added. The combined fractions were then further purified by TLC on cellulose and silica gel (13), converted to *t*-butyldimethylsilyl derivatives and subjected to GC-MS (12). The values found by GC-MS for Z (20.9 ng) and DZ (28.2 ng) were in accord with the totals (23.9 and 31.4 ng, respectively) calculated from the content of individual samples determined by RIA. Similarly, the remaining riboside fractions from podded plants at SD 47 and 57 were all combined as were the corresponding fractions from depodded plants. The amounts of ZR and DZR determined by RIA in the bulked fractions were 3.70 and 2.94 (podded) and 15.2 and 33.7 ng (depodded), respectively, while the corresponding values determined by GC-MS were 3.11, 2.49, 13.0 and 29.7 ng, respectively. Converting these values to sap concentrations, the ZR and DZR levels (ng/mL) were 6.36 and 4.69, respectively, in podded plants, and 9.63 and 22.0 in depodded plants. Thus, GC-MS not only confirms the identity and quantities of the material measured by RIA, but it verifies the dramatic increases in CKs induced by depodding.

RESULTS

Changes in Xylem Sap Volume Collected and Effects of Depodding

The amounts and patterns of change in xylem sap volumes collected over 50 min in these studies (Table I) resembled those reported in the studies on xylem sap mineral levels (24) which employed the same conditions and procedures. The volume yields rose as the pods progressed from 1 cm to full extension and then declined. Depodding at full extension delayed but did not prevent the decrease in sap volume yield, while depodding at late podfill had no effect. The possible significance of these changes is discussed elsewhere (24).

Changes in Xylem Sap CKs during Pod Development and Monocarpic Senescence

In the xylem sap from normal plants with 1-cm pods, ZR and DZR (Figs. 1 and 2) appeared to be the most concentrated CKs at 64 and 75 nM, respectively, while Z and DZ (Figs. 3 and 4) were fairly close at 40 and 51 nM, respectively. As the pods elongated and before the start of podfill, these CKs dropped dramatically to 7, 13, 10, and 15 nM, respectively. Thereafter, these CKs rose slowly (ZR and DZR) or more rapidly (Z and DZ) with DZ reaching a level of 49 nM, essentially equal to the original level, by the time the leaves had become mostly yellow.

The O-glucosides followed a similar pattern (Fig. 5). When the pods were 1 cm, OGZR+OGZ and OGDZR+OGDZ were each about 17 nM. Their concentrations were less at early-midpodfill but rose to 21 to 22 nM when the leaves were yellow.

The nucleotide DZMP was present in very low levels, 1.7 to 6.2 nM (Fig. 5C), while ZMP was not detectable (less than 1.1 nM). DZMP levels changed in a pattern similar to the O-glucosides but did not rise much.

Table I. *Changes in the Volume of Xylem Sap Collected (50 min at 100 kPa) from Soybean Rootstocks during Pod Development and Effects of Depodding*

Same plants as in Figure 1.

Stage of Pod-Bearing Plants	Sap Collected on Short Day	Sap Volume Collected		
		Pod-bearing plants	Depodded at full extension	Depodded at late podfill
		μL ± SE		
Pods 1 cm	22	518 ± 85		
Pods at full extension	28	1,925 ± 170		
Early-mid podfill	36	1,162 ± 144		
Late podfill	47	550 ± 215	1,200 ± 28	
Leaves mostly yellow, pods yellow	57	400 ± 56	812 ± 209[a]	438 ± 51

[a] These plants did not yellow.

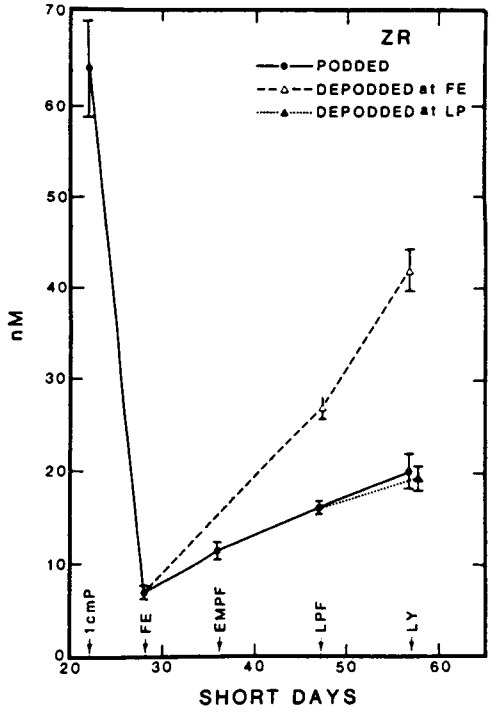

Figure 1. Changes in the xylem sap concentrations of ZR during pod development and monocarpic senescence and the effects of depodding. The symbol 1 cmP signifies that the most advanced pods were 1 cm long; FE, full pod extension; EMPF, early-mid-podfill; LPF, late podfill; LY, leaf yellowing. Depodding involved continuous removal of all pods as they reached full extension or late podfill. The data are from the same plants as in Table I. The standard error bars are for values from at least three and generally four different rootstocks.

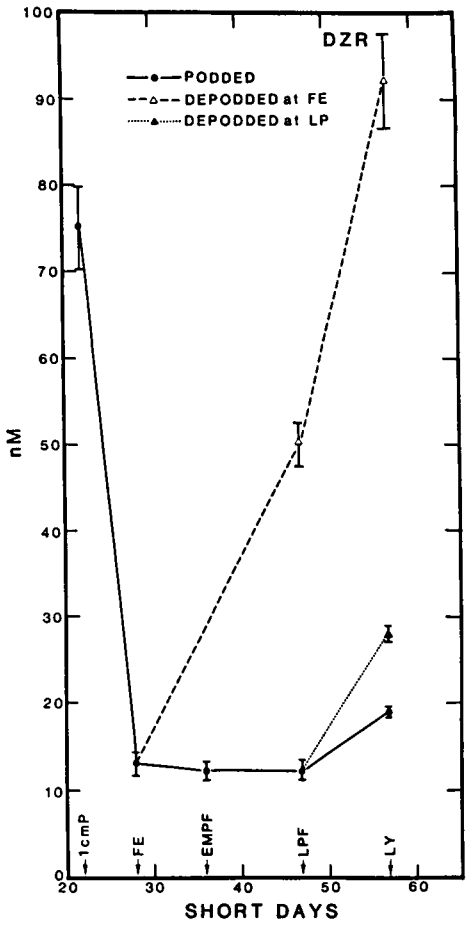

Figure 2. Changes in the xylem sap concentrations of DZR during pod development and monocarpic senescence and the effects of depodding. Abbreviations and other details as in Figure 1.

Effects of Depodding on Xylem Sap CKs

Compared to podded plants at late podfill, removal of the pods as they reached full extension produced a dramatic increase (5X) in the levels of DZR and a substantial rise (about 2×) in ZR (Fig. 1, Fig 2). This depodding also increased Z twofold by late podfill (Fig. 3); however, Z in the normal podded plants also rose to these levels later when the leaves were yellow. Depodding at full extension exerted quite different effects on DZ levels; unlike DZR, ZR, and Z, DZ increased greatly in podded plants and depodding diminished that rise (Fig. 4). This depodding did not significantly affect DZMP (Fig. 5C) or OGZR+OGZ (Fig. 5B). As with DZ, this depodding decreased OGDZR+OGDZ levels (Fig. 5A). De-

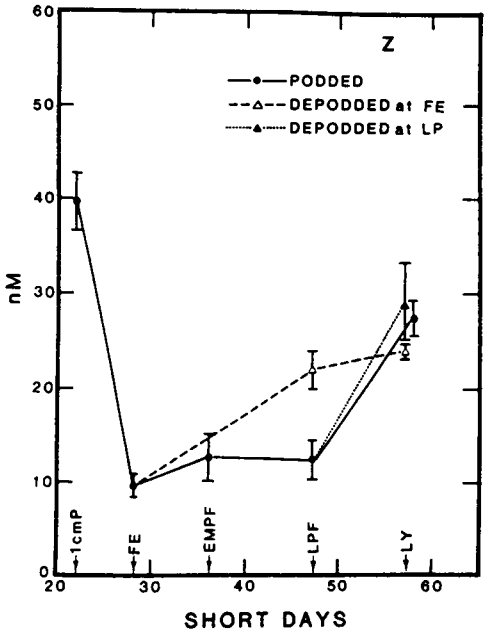

Figure 3. Changes in the xylem sap concentrations of Z during pod development and monocarpic senescence and the effects of depodding. Abbreviations and other details as in Figure 1.

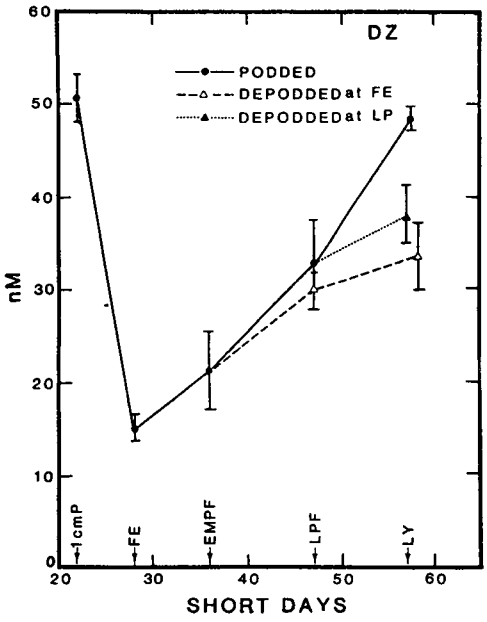

Figure 4. Changes in the xylem sap concentrations of DZ during pod development and monocarpic senescence and the effects of depodding. Abbreviations and other details as in Figure 1.

podding at late podfill had no effect on ZR and Z levels (Fig. 1, Fig. 3), while it increased DZR levels somewhat (Fig. 2) and decreased (suppressed the rise in) DZ levels (Fig. 4).

DISCUSSION

Xylem Sap CKs

Z, DZ, ZR, and DZR are the predominant CKs in the xylem sap of other legumes, *i.e.* two species of lupine, during fruit development (7, 8, 13). They are also present in the xylem sap of fruiting soybean plants (11). In addition, lesser quantities of O-glucosides and nucleotides occur in lupine. We found ZR, DZR, Z, and DZ were major CK constituents in soybean xylem sap; however, their O-glucosides and DZMP were also present in sufficient quantities to warrant considering their possible physiological activity. Little or no IPA or its riboside occur in lupine xylem sap (13), but there may be small amounts (1 nM) of IPMP (13). Similarly, IPA has been reported to be only a very minor constituent in soybean xylem sap, but no data are available for the riboside or IPMP (5). In any case, IPA and its riboside would be relatively inactive in this system (10). Since other CK nucleotides are less active than the bases or ribosides when supplied through the xylem of soybean explants (21), IPMP should be even less active than IPA, so we have not pursued IPA and its derivatives. It is, of course, not possible to be sure that other, unknown CKs are not present, but the Z, DZ, ZR, and DZR in the xylem sap seem sufficient to account for the CK activity of the xylem sap (21).

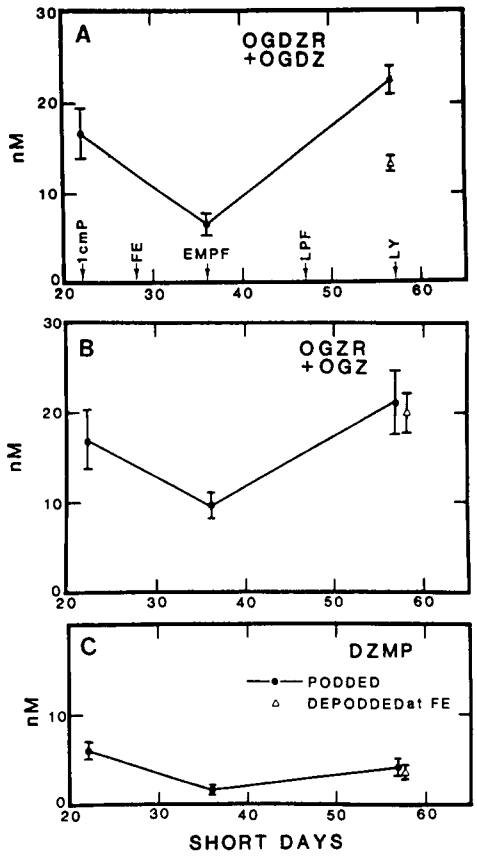

Figure 5. Changes in the xylem sap concentrations of (A) OGDZR + OGDZ, (B) OGZR + OGZ, and (C) DZMP during pod development and monocarpic senescence and the effects of depodding. Abbreviations and other details as in Figure 1.

Changes in Xylem Sap CKs during Reproductive Development

Sooner or later, CK activity in the xylem sap coming from the roots decreases during reproductive development, though

there may be some differences depending on the species and conditions (3, 6–8, 11, 28, 33). Thus, a decline in CK production by the roots may be important in monocarpic leaf senescence (18, 30).

Because our observations on the soybean xylem sap CK levels differ significantly from those reported by Heindl *et al.* (11), some discussion is warranted. The timing of the decline relative to pod development is different (comparing chamber-grown plants); ours occurred earlier, during pod elongation or before. These differences may be due to differences in environmental conditions (1, 11, 30). Since we designed our sampling times based on Heindl *et al.* our first sample may have been past the peak CK concentration. Thus, the earlier levels may have been higher. Other differences exist in the behavior of Z, DZ, ZR, and DZR, but in general, they decrease. Besides these four CKs, we found considerable amounts of DZMP and O-glucosides, but they changed less as pod development progressed.

Our observations differ more significantly from those of Heindl *et al.* (11) with respect to CK concentrations. We found a total of 229 nM for ZR, DZR, Z, and DZ when the pods were 1-cm long and 44 nM later at full pod extension, whereas their chamber-grown plants had a maximum of about 20 nM at full bloom and full pod extension. Losses during purification would contribute as the values of Heindl *et al.* (11) were not corrected for such losses. The main cause was probably their long, 8 h, collection period, which would be expected to lower CK production due to altered root metabolism (24, 26) in the decapitated plants. In addition, prolonged collection (*e.g.* 48 h [3]) of root sap at low flow rates, as in root exudate without pressure or suction, increases the likelihood of metabolism of the CKs by tissues around the xylem (22, 27) and in the sap itself. Indeed, it was shown a long time ago (32) that root xylem sap collected by suction contains more CK activity than bleeding sap. Thus, xylem sap collected with suction or pressure over a short period will give a more representative sample of the sap flowing in the xylem than that collected over long periods and/or at very low flow rates.

Still, another important factor which could influence sap CK concentrations is variation in dilution resulting from changes in transpiration. From the completion of the increase in leaf surface area early in reproductive development until very late in senescence, the stomatal aperture and transpiration rate are fairly constant in normal, podded soybean plants under the relatively low stress conditions employed here (18, 19). As the most advanced pods progressed from 1 cm to full extension, the transpiration rate per plant rose from about 7.5 mL/h (averaged over 24 h) to about 9.2, apparently due to increased leaf area, and then remained constant until leaf yellowing at which time it declined to 7.1 mL/h (JJ Guiamét, LD Noodén, unpublished data). Thus, differences in dilution by xylem sap are not sufficient to account for the changes in concentrations of CKs in the xylem sap, not even the late rise. Furthermore, a simple dilution mechanism cannot explain the differential changes among the CKs. Therefore, the changes in CK concentrations should reflect proportionate changes in CK metabolism in the roots with an apparent overall decline in production.

Effect of Depodding on Xylem Cytokinins

Flower or fruit removal are known to delay leaf senescence and death in monocarpic plants (19, 20). Defruiting or deflowering appears to increase the levels of CK activity in root bleeding sap or xylem sap from a wide range of species (3, 31, 33). Depodding of soybean at full pod extension greatly increased the xylem sap levels of DZR (5×) and ZR (2×), while Z increased to a lesser degree. Here, DZ clearly differed from the others, for it rose during podfill and this depodding reduced its rise. The level of OGDZR+OGDZ, like that of DZ, was decreased by depodding at full pod extension, whereas OGZR+OGZ and DZMP were unaffected. Depodding at late podfull had much less effect, though it did cause some increase in DZR and a decrease in DZ. Although defruiting increases stomatal resistance (19) and thereby could decrease xylem flux and dilution by the xylem sap, this effect is not great enough to account for the increases in CK concentrations. For example, depodding at full pod extension increased DZR 5×, ZR 2×, and Z up to 2×, while it decreased the transpiration rate for similar plants from about 9.2 mL/h (24 h average) to about 7.5 mL, approximately 20% (JJ Guiamét, LD Noodén, unpublished data). Of course, decreased dilution by xylem sap cannot produce the reduction in DZ levels, an opposite change. Again, the differences in concentrations reflected differences in synthesis by the roots, and the production of each CK showed some independence. In parallel with the effects on the levels of ZR and DZR in xylem sap, depodding at full pod extension prevented leaf yellowing and abscission while depodding in late podfill did not (15). This suggests the resurgence in CK production by the roots may be important in maintaining the depodded plants.

Xylem Flux of CKs

Clearly, a central issue here is what do the concentrations of CK in xylem sap tell about CK flux up into the leaves, but this flux is difficult to determine accurately. Since the CK concentrations represent a sampling of the quantities in transit at a particular time, they can be multiplied by the xylem flow, transpiration rate, to estimate the CK flux up to the leaves. However, this requires the assumption that these CK concentrations represent those throughout the daily cycle. Because the flow rates from soybean rootstocks under pressure (Table I) or suction (11) are much less (0.4–1.9 mL/h) and the root exudation rate without pressure suction is even less than the transpiration rate (7.9–9.2 mL/h, averaged over 24 h), it follows that the former rates cannot be used to calculate flux (3, 11). Since the transpiration does not change greatly during the course of our study, the changes in concentrations of CKs in xylem sap appear to reflect at least proportional changes in the flux of CK into the leaves.

Relation of the Xylem Sap CKs to Their Physiological Activity

Explants, which consist of a leaf, one or more pods, and the stem below, can be used to assess the physiological activity of the various concentrations of CKs in sap by substituting solutions with minerals plus CKs for the roots. The dominant

xylem sap CKs (ZR, DZR, Z, and DZ) are active in delaying leaf yellowing near or above 10 nM (10, 21, 23) in explants (which transpire at a rate equal to or higher than leaves on intact plants [JJ Guiamét, LD Noodén, unpublished data]). The maximum aggregate concentration of 20 nM for these CKs reported by Heindl *et al.* (11) is near the physiological threshold (21) and therefore may have little effect. For this and the reasons discussed above, we suspect their values are underestimated, perhaps 10-fold. How important are the CK O-glucosides and nucleotides? Bioassays using explants (21) also indicate that OGZR is less active than ZR, but still active, and therefore we can expect the O-glucosides to have some effect on leaf yellowing and abscission, albeit small; however, DZMP appears to be much less active and the levels present in xylem sap would probably be inactive. The aggregate concentration of the 229 nM which we observed for the dominant CKs during early reproductive development appears sufficient to override the influence of the developing pods, and therefore the CK flux up through the xylem must decline in order to permit the pods to induce the yellowing and abscission of the leaves (17, 18). The data given here show that the CK flux up through the xylem does decrease to levels which permit the pods to induce leaf senescence. However, studies on explants without pods also indicate that cessation of CK flux does not itself cause senescence (17, 18). The late rise in CK levels may be too late and not great enough to interfere with senescence.

All in all, the decline in CK flux from the roots appears to play an important role in monocarpic senescence of soybean, and this decline appears to be induced by the pods. Since DZR and ZR dominate quantitatively and these are particularly active in delaying senescence of explants (10), they may be the most important. The pods suppress CK production in the roots as they inhibit root growth (19, 20) and this occurs quite early, *i.e.* full extension or before. This signal may or may not be the same as the senescence signal which is exerted on the leaves (18–20).

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