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Characterization of Leaf Senescence and Pod Development in Soybean Explants[1]

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ABSTRACT

Excised soybean (*Glycine max* [L.] Merrill) cv Anoka leaf discs tend to remain green even after the corresponding intact leaves have turned yellow on fruiting plants. We have found that explants which include a leaf along with a stem segment (below the node) and one or more pods (maintained on distilled H_2O) show similar but accelerated leaf yellowing and abscission compared with intact plants. In podded explants excised at pre-podfill, the leaves begin to yellow after 16 days, whereas those excised at late podfill begin to yellow after only 6 days. Although stomatal resistances remain low during the first light period after excision, they subsequently increase to levels above those in leaves of intact plants. Explants taken at mid to late podfill with one or more pods per node behave like intact plants in that pod load does not affect the time lag to leaf yellowing. Explant leaf yellowing and abscission are delayed by removal of the pods or seeds or by incubation in complete mineral nutrient solution or in 4.6 micromolar zeatin. Like chorophyll breakdown, protein loss is accelerated in the explants, but minerals or especially zeatin can retard the loss. Pods on explants show rates and patterns of color change (green to yellow to brown) similar to those of pods on intact plants. These changes start earlier in explants on water than in intact plants, but they can be delayed by adding zeatin. Seed dry weight increased in explants, almost as much as in intact plants. Explants appear to be good analogs of the corresponding parts of the intact plant, and they should prove useful for analyzing pod development and mechanisms of foliar senescence. Moreover, our data suggest that the flux of minerals and cytokinin from the roots could influence foliar senescence in soybeans, but increased stomatal resistance does not seem to cause foliar senescence.

The yellowing and abscission of the leaves during monocarpic senescence of soybean plants generally occur during the completion of seed growth (2, 11, 17, 19, 21). When the seeds or pods are removed before seed maturity, the leaves do not senesce (10, 12). Similarily, leaves excised from soybean plants show abnormal and delayed senescence. We have observed recently that when single leaves are excised along with one or more pods on a subtending stem section and maintained in distilled H_2O, they show a pattern of yellowing and abscission which is accelerated but similar to that observed in intact plants. These explants appear to offer a convenient new experimental system for investigating the role of mineral nutrient and hormone supply in the regulation of pod development and monocarpic senescence in soybeans. As a start in the study of these problems, we report here on some characteristics of the explant system and the effects of cytokinin or mineral nutrients supplied via the xylem on pod development and foliar senescence.

MATERIALS AND METHODS

Plant Material. Soybeans (*Glycine max* [L.] Merrill) cv Anoka were grown in pots as described previously (11). Following 3 to 4 weeks in a greenhouse under continuous illumination, the plants (about 30 cm high, second trifoliate leaf expanded) were transferred to environmental control chambers under 10-h SD with 27°C days and 21°C nights. SD numbers refer to number of SD cycles given to the plants. Note that the number of SD required to reach mid podfill varied a bit between groups of plants probably due to conditions on the greenhouse bench.

Preparation of Explants and Leaf Discs. To maximize uniformity, explants were excised above the third node from the bottom and below the third node from the top. Except where indicated, explants were cut at about mid podfill, when the carpels were still green and the seeds had elongated to fill the seed cavity (candled), SD 45 ± 5 d. Unless otherwise stated, the number of pods was reduced to one (with three seeds) per node 24 h before excision. Explants with 10-cm-stem sections were placed in 125-ml Erlenmeyer flasks filled with distilled H_2O or some other test solution.

Drawing on procedures for maintaining cut flowers (4), quinoline hydroxide (20 μM in 60% v/v ethanol) was added to all solutions to give a final concentration of 0.2 μM (30 μg/ml); this suppressed microbial growth in the treatment solutions. As a further precautionary measure, solutions were replaced every 48 h, and the stem bases were trimmed weekly.

Mite infestations can also accelerate yellowing of leaves on explants or intact plants, thus care must be taken to detect and exclude infested material.

For comparison with the explants, leaf discs (2.2 cm) were excised from the leaf blades with a sharp cork borer and placed on moistened filter paper discs in Petri dishes. Submergence of the discs or the cut edges must be avoided since this induced necrosis.

Explants and leaf discs were cultured in environmental control chambers with the SD light and temperature regime described above for whole plants.

Mineral Nutrient and Cytokinin Solutions. A mineral nutrient solution was formulated to supply N, P, K, S, Ca, and Mg in approximately the ratio found in soybean seeds (3, 5). The N concentration chosen falls in the range found in the xylem exudate of soybean during podfill (15, 22). Allantoin has recently been shown to be the primary form in which nitrogen is transported to the shoots in nodulated soybeans (14, 15, 22) and was therefore used as the major nitrogenous constituent. Micronutrients were formulated in the ratios described by Harper (5); however, the actual levels were determined empirically to avoid toxic damage. The concentrations and types of all mineral nutrients used are shown in Table I.

Cytokinin (zeatin, trans isomer, from Sigma Chemical Co.) was

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Table I. *Nutrient Solution for Explants*

Macronutients[a]	*mmol/l*
Allantoin	2.134
$Ca(NO_3)_2$	0.150
KNO_3	0.160
K_2HPO_4	0.180
KH_2PO_4	0.200
KCl	0.200
$MgSO_4$	0.246
Micronutients[a]	***μmol/l***
H_3BO_3	21.226
$MnSO_4 \cdot H_2O$	1.698
$CuSO_4 \cdot 5H_2O$	4.246
$ZnSO_4 \cdot 7H_2O$	1.698
$(NH_4)_6Mo_7O_{24}$	0.012
FeEDTA	15.280

[a] Containing 0.2 μM quinoline hydroxide.

supplied at 1 μg/ml (4.6 μM), because this is in the range found in bean xylem sap (7).

In more recent studies (Mauk and Noodén, unpublished data), the quinoline hydroxide has been eliminated, and the allantoin has been replaced with 4.2 mM NH_4NO_3.

Assay of Leaf Yellowing, Abscission, and Pod Development. Leaf yellowing (% of leaf area ≤ ½ yellow) was assayed visually as described elsewhere (11) except that data were collected for the individual leaflets (three) on each of the four explants used per treatment. Abscission of leaflets usually occurred a few days after initiation of yellowing and is expressed as a percentage of the initial number of leaflets in the treatment group. Pod development was measured as the FMI[3] (11). FMI is a numerical average of fruit development stages on a scale of 1 (youngest) to 5 (mature) based on pod size and color plus seed size. Seed dry weights were obtained by drying at 75°C to constant weight.

Protein Determination. Levels of soluble proteins in the leaves of explants were measured in green leaves at the time of excision and at initiation of visible yellowing to determine whether yellowing was accompanied by declines in protein levels. Leaves, frozen by direct contact with dry ice, were stored at −13°C and later were ground in ice-cold 0.1 M phosphate buffer (pH 7.1). The protein in the supernatant (soluble protein) obtained by centrifugation at 2,000*g* for 3 min was assayed by dye binding (1).

Stomatal Resistance. A Lambda L1 60 diffusive resistance meter was used to estimate R_s of the underside of the middle leaflets (8).

Variability. All data are the means of assays with at least four explants; all experiments were repeated one or more times. Due to careful selection for uniformity in the explants, the variability (SE) of all measurements (except R_s) was very low within each treatment group.

RESULTS

Color Changes in Leaf Discs and in Foliage on Explants *versus* Intact Plants. Figure 1 shows that the onset of yellowing and abscission in leaves of podded explants maintained in distilled H_2O was accelerated in comparison with intact plants. When pods were removed from explants, the leaves stayed green (Fig. 2) and did not abscise (data not shown); however, they did not turn dark green as in depodded plants. Leaf discs excised at mid podfill and maintained on filter paper moistened with water remained green

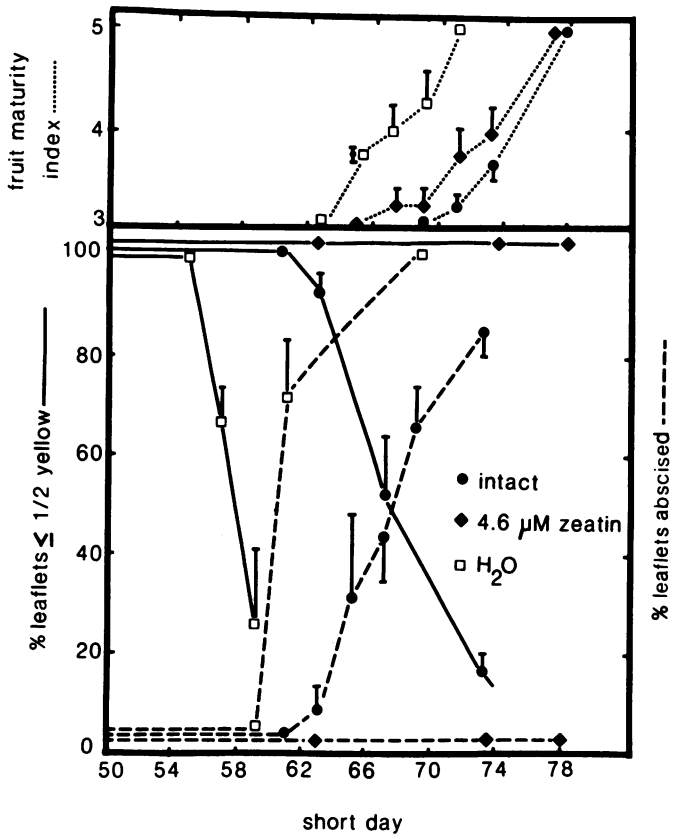

FIG. 1. Kinetics of leaflet yellowing, leaflet abscission and pod maturation in single-podded explants (taken at mid podfill, FMI 3, SD 50) and in corresponding parts of intact plants. Leaflet yellowing is measured visually as percentage of leaflets less than or equal to ½ yellow (11, 19). Fruit maturity index (FMI) is described in detail elsewhere (11). FMI 3 represents mid podfill (both the pods and leaves are fully green), while FMI 5 designates pods greater than ¼ brown (by this time, the leaves usually have yellowed completely and abscised). Each sample represents an average of determinations for 12 leaflets on four explants. SE bars are shown.

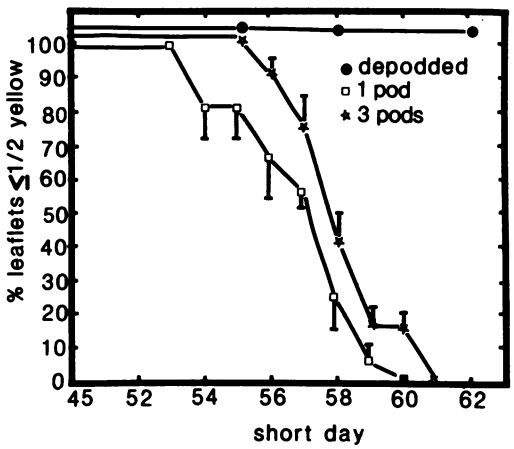

FIG. 2. Effect of pod load on kinetics of leaflet yellowing in explants taken at mid podfill (SD 45). Each sample based on assay of 12 leaflets on four explants.

at least 3 weeks longer than comparable leaves on intact plants (data not shown). Any yellowing which did occur was delayed and abnormal in appearance, and this may be due to stressful conditions. As with intact plants, leaf yellowing in explants usually started immediately over the main veins followed by yellowing in

[3] Abbreviations: FMI, fruit maturity index; R_s, stomatal resistance.

the intervenal areas, and the yellow leaflets abscised before the petiole. During podfill and before visible yellowing, the levels of soluble protein decreased in leaves of explants (Table II) as with intact plants (19).

Effect of Pod Number on the Start of Leaf Yellowing. Mid-podfill explants with either one or three pods showed similar lag periods between excision and initiation of yellowing (Fig. 2). Furthermore, the rate of yellowing (shown by the slope of the descending lines) did not differ for explants with one or three pods.

Seed Growth. Seeds on explants maintained in water continued to grow (increase in dry weight), albeit not as much as seeds in intact plants (Table III).

Effect of Mineral Nutrients on Kinetics of Leaf Yellowing in Explants. Figure 3 shows that explants excised at mid podfill (here SD 50) and maintained on complete mineral nutrient solutions began to yellow 4 d later than those on H_2O. However, leaflet yellowing (Fig. 3) and the pod maturation (data not shown) in explants supplied with minerals were still accelerated in comparison with similar parts of intact plants. Supplying minerals did not prevent the loss of soluble protein (Table II).

Effect of Pod Developmental Stage at Excision. Table IV shows that the lag time from excision to initiation of leaf yellowing varied with stage of pod development at excision. Explants cut during early seed growth (SD 32 and 40) have a longer lag period to initiation of leaflet yellowing, whereas those excised at late podfill (SD 66), when pods were turning yellow, start to yellow after only 2 d. The lag to initiation of yellowing was fairly constant (10–13 d) for explants excised between SD 46 and 57 (early-mid to late-mid podfill).

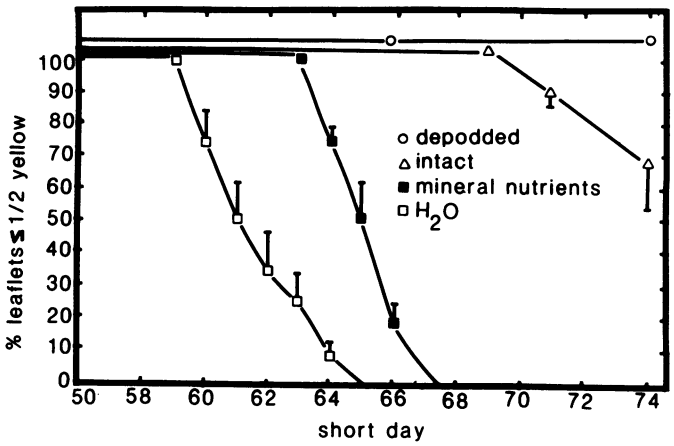

FIG. 3. Effect of mineral nutrients or depodding on kinetics of leaflet yellowing in explants with single pods (excised at mid podfill, SD 50) compared with intact plants. Each sample based on assay of 12 leaflets on four explants.

Table II. *Comparison of Changes in Foliar Soluble Protein in Intact Plants and in Explants Supplied with Water or Mineral Solution or Zeatin*

Treatment	Days from Time Explants Were Cut to Initiation of Leaf Yellowing	Soluble Protein
		mg/g fresh wt[a]
Intact plants		
Initial[a]		16 ± 0.5
Final[a]	27	5 ± 1
Explants		
H_2O	9–13	6 ± 1
Mineral nutrients	13–14	8 ± 1
Zeatin[b]	27	11 ± 1.5

[a] Initial determination of soluble protein levels was made on green leaves from intact plants at the time (SD 44, mid podfill) when the explants were excised. The final determination for intact plants and all determinations for explants were made at the time when yellowing (patches) was first visible. Each sample represents an average of separate determinations for four whole leaves. SE are shown.

[b] Explants maintained on zeatin did not show the characteristic clear-cut, rapid yellowing but gradually faded to a pale green-yellow and finally yellow.

Table III. *Growth of Seeds (Dry Weight) in Intact Plants and Explants Maintained on Water*

	Initial[a]	Final[b]
	g dry wt/seed	
Intact	0.12 ± 0.003	0.23 ± 0.006
Explant (1 pod, 3 seeds)		0.18 ± 0.003

[a] Means ± SE for 12 seeds from four separate plants with green pods at mid podfill.

[b] Seeds harvested when pods turned brown.

Table IV. *Effect of Developmental Stage at Excision on Days to Initiation of Leaf Yellowing in Explants (One Pod, Three Seeds) Held on H_2O or Nutrient Solution*

Excised (SD)	Initial FMI	H_2O	Nutrient Solution	Nutrient-Induced Extension
		days from excision to initial yellowing[a]		
32	1 early seed growth	16 ± 0.6		
40	2 early podfill	15 ± 0.6		
46	2–3 early-mid podfill	13 ± 1.1	17 ± 0.6	+4
50	3 mid podfill	10 ± 0.3	13 ± 0.3	+4
55	3 mid to late podfill	10 ± 0.9	13 ± 0.6	+3
57	3 mid to late podfill	11 ± 0.6	14 ± 0.9	+3
66	3–4 late podfill	6 ± 0.9	7 ± 1.1	+1

[a] Each treatment is based on assay of 12 leaflets distributed over four explants.

Supplying complete mineral solution instead of H_2O generally extended the lag to initiation of leaf yellowing by 4 d, but this extension decreased to about zero by the end of podfill.

Effects of Cytokinins. Zeatin, 4.6 μM (1 μg/ml) delayed leaf yellowing (Fig. 1) and abscission in podded explants. Unlike normal, intact plants or explants on solutions without cytokinins, leaves of explants in media with cytokinin undergo gradual paling of the leaves, and this is not shown by our present visual scoring system. By SD 78, these leaves were a very pale yellow green. The carpels of explants held on zeatin changed from green to yellow and then to brown (FMI stages 3 through 5) in the same way as the pods on intact plants; however, the zeatin treatment delayed the initiation of the color change. Zeatin supplied via the xylem substantially retarded the breakdown of foliar protein (Table II).

Changes in Stomatal Resistance. The kinetics of stomatal closure following excision of explants are shown in Figure 4. Although not shown here, the R_s usually remained at around 4 s cm^{-1} during the first light period following excision and then started to increase. Explants with pods removed behaved essentially the same way as those with intact pods (data not shown). After 2 d on water, the R_s increased from 4 to 20 s cm^{-1}. Explants placed in nutrient solution showed a further 2-d delay before the rise in R_s, and those on zeatin maintained relatively low R_s similar to those found in leaves of intact plants for 7 d.

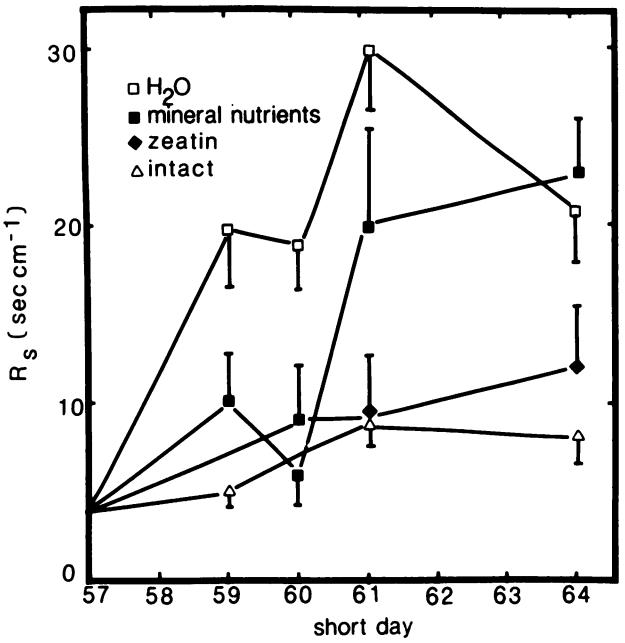

FIG. 4. Leaf stomatal resistances for groups of four intact plants or explants maintained in mineral nutrients, zeatin (4.6 μM), or water.

DISCUSSION

Comparison of Explants and Intact Plants. The patterns of leaf yellowing in explants and intact plants are similar, and leaflet abscission precedes petiole abscission in both. Similarly, the soluble protein in leaves of both the explants and intact plants is largely broken down before yellowing is visible. Alterations in pod load (one *versus* three pods) do not substantially change the time to commencement of yellowing or the rate of yellowing in either explants or intact plants (12, 18). Removal of pods (or surgical excision of seeds; unpublished data) inhibits leaf yellowing and abscission in explants, as it does in intact plants (12).

Foliar yellowing and abscission in explants maintained on water are always accelerated compared with intact plants; visible maturation of the carpels is similarly altered. Addition of mineral nutrients or zeatin delays both leaf yellowing and pod maturation. Zeatin, however, has a greater delaying effect on foliar yellowing than pod development in explants just as it does when applied to the foliage of intact plants (13). Thus, pod development and leaf yellowing show the same sort of interconnection in explants that they do in intact plants. Not surprisingly, the acceleration of leaf yellowing and pod development reduces seed growth (Table III).

Mechanism(s) of Senescence Induction. The effect of zeatin (supplied via the xylem) in delaying leaf yellowing and preventing abscission in soybean explants is similar to the effects of cytokinins applied as foliar sprays to intact plants (13, 17). It has been suggested that a decline in cytokinin flux from the roots may influence shoot senescence (17). In support of this idea, cytokinin activity in the leaves of soybean plants declines progressively during fruit growth (13, 17).

R_s often increases in parallel with leaf senescence (17, 23) and Naim and Neumann (16) showed that accelerated senescence induced in attached bean leaves by sprays of silicone oils correlates with a decrease in leaf diffusivity to CO_2 and lower rates of photosynthesis. R_s increases rapidly (stomata close) in explants placed in water only and less rapidly in mineral solutions, while a supply of cytokinin keeps the stomata open (Fig. 4). Thus, the senescence-retarding effect of cytokinins on podded soybean explants may be related to their ability to maintain stomatal opening and associated photosynthetic or transpirational activities. However, R_s in leaves of depodded plants increases above control values (9, 20), and the R_s of explants maintained on water increases rapidly both with and without pods. Inasmuch as pod removal prevents normal yellowing and abscission of leaves in whole plants and explants, increases in R_s cannot be a primary cause of senescence in these systems. Similarly, the fact that depodded explants maintained on water alone do not show leaf yellowing and abscission suggests that these processes are not initiated simply by decreases in the flux of either essential nutrients or cytokinins into the leaves.

The kinetics of yellowing in leaves of both explants and intact plants (12, 18) appear to be independent of pod load; thus, yellowing is unlikely to be initiated by any accelerated withdrawal of essential nutrients (6) in response to excessive pod demands.

The accelerated foliar yellowing and abscission of explants on water relative to corresponding parts left *in situ* clearly indicates that the loss of the mineral nutrient and cytokinin supply resulting from removal of the roots can hasten foliar senescence but this may be an indirect result of accelerated pod development.

Soybean explants appear to provide a useful model system for investigating the mechanisms which control monocarpic senescence, the nutrient requirements for seed growth and the role of the leaves in feeding the seeds.

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