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Retardation of Soybean Leaf Senescence and Associated Effects on Seed Composition

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Abstract. Soybean leaf senescence, leaf abscission, and pod yellowing were markedly delayed by sprays of 10^{-4} M 6-benzylamino-9-(tetrahydropyran-2-yl)purine plus 5×10^{-5} M α-naphthalene acetic acid. The pods on the sprayed plants turned yellow 5–7 days later than those on the control plants, and the treated leaves remained dark green even when the pods had already desiccated. The antisenescence spray did not change pod numbers, seed numbers, seed size, or the yield. By retarding senescence, seed nitrogen content was increased in both TCA-soluble and TCA-insoluble fractions. Seed total protein, buffer-extractable total protein, and globulin were increased by 26, 28, and 33 mg/g of seed flour, respectively, and albumin was decreased by 6 mg/g. The overall increase in seed protein caused by spray treatment is confined to the globulin portion.

Monocarpic leaf senescence of soybean plants can be markedly retarded or even prevented genetically (Abu-Shakra et al. 1978) or chemically (Noodén et al. 1979). Neither approach altered seed yield appreciably while nitrogen and starch contents of leaves were maintained or increased. Thus when senescence is delayed, pods are supplied with current assimilate rather than by nutrient withdrawal from leaves. Protein synthesis in soybean seed lasts until a very late stage of seed development (Rubel et al. 1972, Sale and Campbell 1980, Yazdi-Samadi et al. 1977). Hence it is likely that protein content of seed would be increased if seed development could be prolonged while the assimilate supply from leaves and roots is maintained. This communication reports the assessment of soybean seed composition (particularly protein) after leaf senescence had been markedly delayed and seed development prolonged by spraying with the synthetic cytokinin 6-benzylamino-9-(tetrahydropyran)2-yl)-

purine (9THP-BAP) plus α-naphthalene acetic acid (NAA). In retarding soybean leaf senescence, 9THP-BAP was found previously (Zhang and Letham, unpublished observations) to be more effective than 6-benzylaminopurine (BAP), which was inactivated by formation of the 9-alanine conjugate (Zhang et al. 1986). BAP plus NAA has been used previously to retard leaf senescence of soybeans (Noodén et al. 1979), and NAA appears to act mainly by inhibiting leaf abscission (Noodén 1980).

Materials and Methods

9THP-BAP was synthesized as follows. BAP (6.9 g), dry dioxan (75 ml), 2,3-dihydropyran (13.5 ml), and formic acid (7.5 ml) were refluxed together under anhydrous conditions for 6 h. Thin-layer chromatography indicated almost complete reaction. The mixture was evaporated to dryness *in vacuo*, and a benzene solution (200 ml) of the residue was shaken with 2% Na_2CO_3 (5 300-ml volumes), dried with anhydrous Na_2SO_4, and then passed through an Al_2O_3 column (8 ml, Merck Al_2O_3 90, basic, activity I) to decolorize it. Crystallization from benzene–petroleum ether yielded 9THP-BAP (6.34 g), m.p. 115–117°C. UV spectrum (in ethanol): λ_{max} (nm) 267.0 (ϵ = 19,185), 270.0 (ϵ = 19,310); λ_{min} (nm) 232.0 (ϵ = 2,440). Electron impact mass spectrum (m/z values and relative intensity in parentheses): 309 (M^+, 13), 281 (2), 252 (0.7), 225 (100), 224 (32), 209 (3), 198 (1.5), 197 (2), 148 (7), 121 (5), 120 (19), 119 (5), 106 (87), 91 (38), 85 (18).

Soybean (*Glycine max* (L.) Merr., cv. Anoka) plants inoculated with Nodulaid group H (Agricultural Laboratories, New South Wales, Australia) were grown in 6-in pots filled with potting mixture in a glasshouse at 25/20°C day/night temperature. Uniform plants were selected 30 days after flowering had commenced and were divided into treatment and control groups (usually 6 plants in each group). They were then moved into a growth cabinet (28/22°C day/night temperature, 10 h daylight and 300 $\mu E\ m^{-2}/s$ light intensity). Sprays were applied twice a week for the first 3 weeks and once weekly thereafter until most of the pods had turned yellow. The treated group was sprayed with 10^{-4} M 9THP-BAP plus 5×10^{-5} M NAA in 0.05% Tween 80 solution, and the control group was sprayed with only 0.05% Tween 80 solution until it just began to drip from the leaves. Seeds were harvested when the pods were dry and were then air-dried to about 7.5% moisture.

Seeds (10 per plant) were dried at 75°C to constant weight and used for oil content determination in a Newport Quantity MKII NMR Analyzer (Tonnet and Snudden 1974).

The rest of the seeds of each plant were dehulled, milled to a fine flour, and passed through a 0.2-mm screen. The flour was defatted by sonicating with n-hexane (10 ml/g) for 5 min and then stirring the suspension for 1.5 h at 23°C. After centrifugation the pellet was reextracted by the same procedure and air-dried. The defatted flour was extracted with 10% trichloroacetic acid (TCA) at 0°C for 2 h and then washed once with 5% TCA to yield a soluble and an insoluble fraction from which TCA was removed by repeated extraction with diethyl ether. The nitrogen content of the two dried residues was then deter-

mined using a gas chromatographic elemental analyzer (Carlo Erba model 1106). Protein extraction from the defatted flour with buffer and fractionation into globulin and albumin were performed by the methods of Schroeder (1982). Total buffer-extractable protein (determined after TCA precipitation) and albumin contents were assayed by a microbiuret method (Goa 1953), and globulin content was usually taken as the difference between the two values. To determine directly the protein not extracted by buffer, the pellet was extracted with 0.2 N NaOH (5 ml/g flour) at 38°C for 16 h and then at 23°C for 2 days. The protein content of this fraction was also determined by the microbiuret method.

To analyze seed protein components further, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) were employed. The SDS-PAGE method has been described in detail by Schroeder (1982). HPLC equipment was the same as mentioned before (Entsch et al. 1979), and the system used was as follows: column, Protein PAK DEAE-5PW (7.5 cm × 7.5 mm, Waters Associates); buffers, 0.02 M Tris-acetate pH 8.65 (A), buffer A plus 0.2 M NaCl (B); sequential elution sequence, buffer A (2 min), linear gradient transition from A to B (over 27 min), buffer B (36 min); flow rate 1.0 ml min^{-1}; detection wavelength 280 nm.

Amino acid composition of the globulin and albumin fractions was analyzed on a Beckman 199 CL amino acid analyzer after the two fractions had been hydrolyzed with 6 N HCl in sealed tubes at 110°C for 24 h. Sulfur content of the two protein fractions was determined using the elemental analyzer (Carlo Erba model 1106).

Results

Soybean (cv. Anoka) leaf senescence, leaf abscission, and pod yellowing were markedly retarded by sprays of 9THP-BAP plus NAA (Fig. 1). When evaluated for leaf and pod yellowing by the method of Lindoo and Noodén (1976), the pods on the treated Anoka plants turned yellow 5–7 days later than those on the control plants, whereas most of the treated attached leaves remained dark green at pod harvest (Fig. 1).

In the present experiment, antisenescence sprays did not change seed yield, the pod number per plant, seed number per plant, or seed size (Table 1). The spray treatment increased seed N in both TCA-soluble and TCA-insoluble fractions (Table 2). The seed total protein was increased by 26 mg/g dry seed flour, buffer-extractable total protein by 28 mg/g, and globulin by 33 mg/g, but albumin was decreased by 6 mg/g flour (Table 2). Hence the increase in seed protein caused by the spray treatment is confined to the globulin portion, the major storage protein fraction of soybean seed. The globulin/albumin ratios for treated and control plants were 4.79 and 3.79, respectively.

The effect of the spray treatment on the relative proportions of seed protein polypeptides was also studied. However SDS-PAGE of total protein, globulin, and albumin preparations and also HPLC of the albumin fraction did not reveal any significant treatment-induced differences in the proportions of protein or polypeptide components. Furthermore, the amino acid composition and sulfur

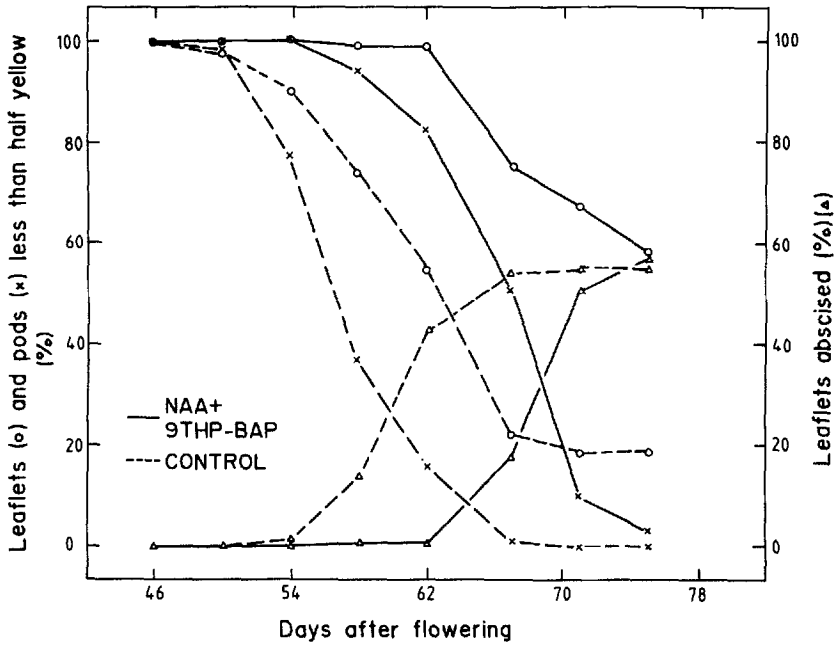

Fig. 1. Retardation of soybean leaf yellowing, leaf abscission, and pod yellowing caused by spraying plants with a mixture of 9THP-BA (10^{-4} M and NAA (5×10^{-5} M). Spraying commenced 30 days after flowering, and the plants were sprayed twice weekly for 3 weeks and thereafter once weekly until pod yellowing was nearly complete.

Table 1. Seed size and yield from control soybean (cv. Anoka) plants and plants showing delayed leaf senescence.

	Pods plant^{-1}	Seeds plant^{-1}	Mean seed dry weight (mg)	Seed yield (g plant^{-1})
Senescence delayed	22.8 ± 1.4	42.3 ± 1.8	239 ± 1.8	10.1 ± 0.3
Control	22.3 ± 2.1	42.3 ± 1.1	241 ± 11.9	10.1 ± 0.3

Values are mean ± SE.

content of the globulin and albumin fractions were not altered significantly by the spray treatment. The mean sulfur content (mg/g protein) for the albumin and globulin fractions of control seed were 12.2 and 6.9, respectively. NMR analysis indicated that the spray caused a significant decrease in seed oil content (Table 3).

In another experiment using cv. Fiskeby V soybean plants, seed total protein, buffer-extractable total protein, and globulin contents were increased by 11, 20, and 20 mg/g flour, respectively, owing to the spray treatment (Table 4). The spray delayed leaf senescence markedly and prolonged pod development, but it did not alter seed yield per plant or mean seed weight.

Table 2. Changes in seed nitrogen and seed protein contents associated with delayed leaf senescence of soybean (cv. Anoka).

	Seed nitrogen content		
	TCA-soluble	TCA-insoluble	Total
Senescence delayed	28.5 (2%)	71.1 (5%)	99.6 (2%)
Control	18.2	66.8	85.0

	Seed protein content				
	Total protein[a]	Protein not extracted by buffer	Buffer-extractable protein		
			Total	Globulin[b]	Albumin
Senescence delayed	444 (5%)	116	323 (2.5%)	267 (2.0%)	56 (5%)
Control	418	123	295	234	62

All values are expressed as mg/g dry flour. When a mean is significantly different from the control mean, the level of probability is given in parentheses.
[a] TCA-insoluble N × 6.25.
[b] The values listed are total buffer-extractable protein content minus albumin content. Direct determination of globulin protein yielded similar values which were significantly different at the 1% level.

Table 3. Change in seed oil content associated with delayed leaf senescence.

	Oil content (mg/g)
Senescence delayed	205[a]
Control	224

[a] Difference from control is significant at the 0.5% level of probability.

Discussion

In the present study, leaf senescence of soybean was retarded markedly by a spray containing 9THP-BAP. As in studies of certain other responses to cytokinin (see references in Letham 1978), this 9-substituted derivative was more effective than BAP itself. Although 9THP-BAP is available commercially, purchase of the amounts required for spray trials with entire large plants is costly. A simple synthesis of this useful growth-regulating compound directly from the inexpensive BAP is reported in this paper.

The increase in seed protein content induced by the growth regulator spray (Tables 2, 4) is probably a consequence of prolonged seed development maintained by a continued supply of assimilate from the nonsenescent leaves and roots. However, the possibility that the supplied growth regulators were translocated into the seed and then directly modified seed development cannot be excluded. Two previous studies are relevant in this connection. By directly applying BAP and NAA to pedicels of pea plants, Schroeder (1984) found an

Table 4. Changes in seed protein contents associated with delayed leaf senescence of soybean (cv. Fiskeby V).

	Total protein[a]	Buffer-extractable protein		
		Total	Globulin	Albumin
Senescence delayed	424	360 (5%)	288 (2.0%)	72
Control	412	340	268	73

Spray applications commenced 3 days after flowering was completed; plants were kept in the glasshouse throughout the experiment.
All values are expressed as mg/g dry flour. When a mean is significantly different from the control mean, the level of probability is given in parentheses.
[a] TCA-insoluble N × 6.25.

increase in, and modification of, pea seed protein. However, addition of kinetin to media for the culture of soybean seed cotyledons in vitro did not change seed protein content, but 2,4-D induced a decrease in protein level (Thompson et al. 1977). It would be worthwhile to determine whether radioactive 9THP-BAP applied to leaves and pod walls is translocated into soybean seed and especially into the developing cotyledons.

A negative correlation between oil and protein contents of soybean seed has frequently been observed (Weiss et al. 1952, Hymowitz et al. 1972). These seed attributes were also found to be negatively correlated in the present study. Oil content of soybean seed is known to decline during the terminal phase of seed development (Sale and Campbell 1982). Hence if seed development is prolonged, as in the present investigation, and protein synthesis maintained, it is likely that seed oil content would decline.

In the present study, retardation of soybean leaf senescence by a spray treatment did not increase seed yield. Similarly, when senescence was delayed genetically (Abu-Shakra et al. 1978) and by hormone application in an earlier study (Noodén et al. 1979), no enhancement of yield resulted. Indeed, in the latter study, seed number was reduced by a spray containing BAP and NAA. However, this reduction is probably simply a consequence of commencing treatment at a very early stage (at early flowering) when auxin is known to induce flower and immature pod abortion (Boize 1982, Noodén and Noodén 1985). Other studies with cv. Anoka and Fiskeby V not detailed in this paper substantiate this view. Yield limitation is probably a multifactor problem, and monocarpic senescence may well be one such factor in soybean, since it commences before seed development is completed. Another limiting factor appears to be pod abortion (see review by Peat and Jeffcoat 1982), which may be as great as 80%. Hormonal retardation of leaf senescence when pod abortion is minimized could well enhance yield, and this possibility merits assessment. However, because of the maintenance of carbon and nitrogen assimilate production when senescence is delayed, deferral of senescence may be one way of enhancing the potential productivity of the soybean.

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