Soybean (*Glycine max*) seed growth characteristics in response to light enrichment and shading

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ABSTRACT

Seeds are the primary sinks for photosynthates during reproductive growth. Variation in light intercepted during and after seed initiation has been found a major environmental determinant of soybean [*Glycine max* (L.) Merrill] seed size. We investigated the influence of light enrichment and shading on seed growth rate, effective filling, cotyledon cell number, cell volume and endogenous ABA concentrations of cotyledons/testas during seed filling of soybean. Evans, an indeterminate Group 0 soybean, was subjected to light reduction and enrichment treatments from the beginning of pod formation until final harvest for two years in Massachusetts. Higher rates of seed growth, greater seed dry weight, and higher cotyledon cell number were all observed with light enrichment. There was a reduction in seed growth rate and cotyledon cell number, along with a significant lowering of endogenous ABA levels in testa and cotyledon with shade. The level of ABA in cotyledon during seed development was significantly correlated with seed growth rates only under shade treatments. Both the growth rates and seed filling duration were influenced by variation in light interception by the soybean canopy. The effects of varying light treatment on seed size, within one genotype, were most likely due to the differences in seed growth rate and cotyledon cell number.

Keywords: soybean; seed size; growth rate; cotyledon cell number; ABA; light enrichment

Intensity and quality of solar radiation intercepted by the crop canopy, are important determinants of yield components and hence the yield of soybean [*Glycine max* (L.) Merrill] (Willcott et al. 1984, Myers et al. 1987, Board and Harville 1992). Duncan (1986) suggested that light intercepted during and after seed initiation is a major determinant of yield. Mathew et al. (2000) indicated that light enrichment initiated at early flowering can modify seed size with some internal control moderating the final size of most seeds.

Genetic differences in the seed growth rate of soybean were related to the number of cells in the cotyledons (Egli et al. 1980). There was a positive correlation between soybean cotyledon cell number and the ability of the seed to accumulate dry matter (Guldan and Brun 1985). However, within the same soybean genotype seed size may be influenced more by cotyledon cell size than by cell number (Hirshfield et al. 1992). There is evidence that plant hormones are involved in determining both sink size and capacity (Liu 1993, Liu and Herbert 2000). Abscisic acid (ABA), earlier considered an inhibitory substance, is now recognized as a naturally occurring plant hormone of major importance in the coordination of plant growth and development in response to the environment and the regulation of transport and storage of assimilates during grain development (Guerrero and Mullet 1986, Suzuki et al. 2000, Xiong and Zhu 2003). Raschke and Hedrich (1985) reported that ABA reduced photosynthate production directly by a reduction in ribulose-1,5-bisphosphate carboxylase activity in soybean. Increased ABA in leaves caused stomatal closure, which resulted in a decline in photosynthesis due to low intracellular CO_2 levels (Fisher et al. 1986). ABA levels have been found to rise sharply and then fall during seed development of soybean (Quebedeaux et al. 1976, Liu et al. 2000). Soybean embryos require ABA for their continued development and for the accumulation of storage proteins (Eisenberg

and Mascarenhas 1985, Tian and Brown 2000). Schussler et al. (1984) found a high concentration of ABA in large-seeded soybean genotypes compared to small and medium-sized seeds. He proposed that ABA may be involved in the stimulation of rapid unloading of sucrose into the testa of soybean and the ABA in cotyledons may enhance sucrose uptake by the cotyledons. A rapid increase in the fresh and dry weight of soybean seed was found to be correlated with a peak in the rate of ABA accumulation in the developing seed (Quebedeaux et al. 1976). Similarly, a decrease in the rate of dry weight accumulation was associated with a sharp decline in ABA concentration.

The main objectives of this study were: (1) to investigate the response of seed growth rate, effective filling duration to different light treatments; (2) to determine if cotyledon cell number or cell volume were responsible for differences in seed size when plants were subjected to variable light; and (3) to examine a possible role of endogenous ABA concentration in cotyledons and testas on seed filling.

MATERIAL AND METHODS

Cultural practices

A 2-year field study was conducted in 1997 and 1998 in the Connecticut River Valley at the University of Massachusetts Agricultural Experimental Station Farm in South Deerfield, Massachusetts. The soil type was Hadley fine sandy loam (Typic Udifluvent, coarse-silty, mixed, non-acid, mesic). In 1997, the experimental site was tested high for P, thus it only received an application of muriate of potash at a rate of 306 kg K_2O/ha prior to cultivation. No fertilizer was applied for 1998. Evans, an indeterminate, Group 0 soybean, with approximately 92% seed germination was inoculated with commercial powdered-peat inoculant before sowing. Seeds were machine sown on 24 May 1997 and 23 May 1998. The experimental design was a complete randomized block design with four replications and each plot consisted of eight rows 0.25 m apart and 12 m long. Around 48 and 50 plants/m^2 were achieved in 1997 and 1998, respectively. Weed control in 1997 was a post emergence application of 2.06 l/ha poast [2-(1-(ethoxyimino) butyl)-5(2-(ethylthio) propyl)-3-hydroxy-2-cyclohexene] and 2.75 l/ha crop oil. In 1998 weed control was a pre-emergence application of 1.7 kg/ha active ingredient (a.i) alachlor [2-chloro-2',6'-diethyl-N-(methoxymethyl)acetanilide] and 0.85 kg/ha a.i. linuron [3-(3,4-dichlorophenyl)-1-methoxy-1 methylurea] plus hand weeding during early vegetative growth of the soybean. Total rainfall varied from 370 mm (1997) to 522 mm (1998) from May to September. Since the soil was near field capacity at sowing, water availability was judged as being adequate for growth.

Light enrichment and shading treatments

Light treatments consisted of light enrichment (LE) and shading (S) initiated at the early pod formation [R3 stage (Fehr and Caviness 1977)]. Light enrichment was achieved by installing a 90 cm tall wire mesh fencing (mesh hole size 4–5 cm) adjacent to the rows bordering the centre sample row, sloping at approximately a 45° angle away from the centre row of each plot. The fences were inspected periodically, and plants held back by the fence were rearranged to avoid undue crowding to allow near normal growth of fenced border row plants. Plants from neighbouring rows were prevented from encroaching into the centre sample row thus allowing a greater light penetration into the canopy of the centre sample row. Once in place, fences and shade cloth remained in position until soybean maturity. This method was chosen to allow light penetration deeper into the canopy without disturbing the root system of the plants growing in border rows. A control treatment (CK) received no light enrichment or shade. Light intensity at the top canopy, using a Licor line quantum sensor (LI-188B) placed parallel to and beside the centre row plants was 13.06 and 7.34 μmol/m^2 for the CK, LE and S treatments, respectively. At the base of canopy it was 0.19, 3.37 and 0.03 μmol/m^2 for CK, LE and S, respectively (averaged over 1997 and 1998). Thus, the leaves at the base of canopy in LE were receiving more than 25% of the available light. Shading was provided by black polypropylene fabric installed 0.5 m above the soybean canopy. Shade cloth was attached to a vertically movable wire trellis attached to wooden posts, which resulted in 52% light reduction compared to the control.

Tissue sampling for ABA determination

Pods were sampled weekly from 17 d after anthesis at middle nodes (beginning of the linear

seed filling period) through to 38 d after anthesis just before the seed reached physiological maturity. Pods at middle node positions on the main stem were sampled. Node 1 was the unifoliate node, being the first node above the cotyledons. Four middle positioned seeds from three seeded pods were pooled from different plants for ABA analysis. Selected seeds were dissected into testa and cotyledons. Fresh weight was recorded and the samples were immediately frozen on dry ice and stored at approximately –18°C.

ABA extraction and quantification

The technique used for ABA extraction was similar to that of Montero et al. (1994). Plant tissue was homogenized in liquid nitrogen for 5 minutes. It was then extracted overnight, at 4°C in 80% aqueous methanol containing 1 mg/l of butylated hydroxytoluene to avoid oxidation. To remove any impurities, the methanolic extract was passed through a Sep Pak® C18 cartridge prewashed with 1 ml of 80% methanol. This methanolic phase was then evaporated in a vacuum oven at room temperature. The resulting aqueous portion was partitioned 3 times against ethyl acetate at pH 3.0. The ethyl acetate fractions were combined and evaporated to dryness under low pressure. The residue was dissolved in saline tris buffer (TBS) of pH 7.8 and then quantified by enzyme-linked immunosorbent assay (ELISA). A stock solution of 1mM (±) ABA standard was prepared in absolute methanol and diluted in TBS at pH 7.5. Concentrations ranging from 0–2 pmole/µl and 100 pmole/µl were used to give a standard curve. Tissue sample eluates were serially diluted in TBS at pH 7.5.

Strips of flat-bottomed reaction wells coated with ABA McAb were placed in a strip holder plate. To each reaction well, 100 µl of either diluted tissue sample or ABA standard were added. Then, 100 µl ABA-alkaline phosphate conjugate in TBS was added to each well. The holder plates were sealed and placed in the dark at 4°C for three hours to incubate. The solutions were decanted, and the wells were washed with a tween-saline solution. The enzyme tracer activity was assayed by the addition of p-nitrophenyl phosphate substrate to each well. The holder plates were incubated at 37°C in an oven for 60 minutes and removed. One drop of 1N NaOH was added to each well to stop the reaction. Colour absorbance was read at 405 nm by spectrophotometer. To determine if there was any interference in the assay from sample extracts, samples of plant extracts were added to increasing concentrations of ABA standards (Walker-Simmons 1987). ABA standards alone and ABA standards plus extracts were assayed for ABA content and plotted as ABA added versus ABA found. The degree of parallelism between the lines was evaluated. An internal standard was added to account for recovery during extraction and purification.

Seed growth rate and effective seed filling period

Pods and seed samples were collected from the same plants used for ABA quantification every 5–6 d during the linear growth phase. Three seeded pods from middle node positions were collected to minimize bias from position of the seed in the pod. After the sample collection, seed fresh mass was determined and seed volume was estimated by a water displacement method. Seeds were dried at approximately 70°C for 24 hours and seed moisture content was calculated. Linear regression was used to estimate seed growth rates (SGR) for each treatment, after eliminating non-linear points from the initial and final stages of seed development. At maturity, plants were sampled to determine final seed size. The effective filling period (EFP) was estimated by dividing final seed size by the rate of dry matter accumulation in seeds during the linear filling period (Daynard et al. 1971).

Cotyledon cell number and size

The number of cotyledon cells was estimated using the method of Swank et al. (1987). Seeds were dried for 24 hours (70°C) and were then allowed to imbibe water for 8–10 hours, after which the testa and embryo axis were carefully removed. One cotyledon/seed was placed in a fixative formalin acetic acid solution. After 24 hours in formalin acetic acid, each cotyledon was cut finely and digested in 40 ml of chromic acid solution (80 g chromic acid/l water) (Reinert and Yeoman 1982). The chromic acid was removed after 5 d and the cotyledons were macerated before diluting with water. An aliquot of the cell suspension was placed on a hemacytometer, and the cells were counted under × 100 magnification. For not completely digested material, the suspension was filtered, and residues were dried and weighed. The number of cells counted was adjusted by the proportion of

the total cotyledon mass not digested to give the total number of cells in the cotyledon. Cell growth rate was calculated by dividing the seed growth rate by cell number. Cell volume was obtained by dividing maximum fresh seed volume by number of cotyledon cells. The estimated number of cells/cotyledon pair was calculated using the known volumes. Statistical analysis of the data was performed using the SAS ANOVA procedure (SAS Institute 1996). Mean separation was done using the Duncan multiple range test (DMRT).

RESULTS AND DISCUSSION

Rate and duration of seed filling period

In both years there were significant differences among treatments in both seed growth rates and effective filling period (Table 1). Compared to the CK, the rate of seed growth was higher under LE. Shading significantly ($P < 0.05$) lowered seed growth rate (Table 1). Differences in seed growth rate were significantly correlated with final seed size at maturity ($r = 0.99^{***}$, 0.83^{**} in 1997 and 1998, respectively) (Figure 1). There was a negative correlation between seed weight and the duration of seed filling. In both years the time of seed fill was significantly ($P < 0.05$) increased under shade (Table 1). These results indicate that the impact of light enrichment on seed size was probably due to seed growth rate. Previous research also showed that seed growth rate in soybean, rather than filling duration, was positively correlated with seed size (Egli et al. 1981, Guldan and Brun 1985). In contrast to the above results, Swank et al. (1987) reported that a close relationship between final seed size and seed growth rate does not hold true for all soybean genotypes. They found that a variation in the duration of seed fill also made a significant contribution to differences in seed size. As we only used one cultivar in our study, the close relationship between seed growth rate and seed size may not hold true for all cultivars. The significant positive effect of light enrichment on the seed growth and final seed weight at maturity (Figure 1) indicate that a variation in light interception by a soybean crop canopy can strongly influence seed growth rates as well as seed filling duration, which ultimately affects seed size. Egli and Bruening (2001) suggested that soybean seed growth rate was generally sink limited if photosynthesis increased during seed filling, but was source limited if photosynthesis was reduced. Since light treatments affected the number and seeds per plant (Mathew et al. 2000), these potential influences on assimilate supply to the seed and seed growth rate should not be neglected.

Cotyledon cell characteristics

Cotyledon cell number was sensitive to light condition and differed significantly ($P < 0.05$) under the different light treatments (Table 2). The highest cell number was in the light enrichment treatment. Compared to the control, the final cell number in soybean plant cotyledons under shade was reduced significantly ($P < 0.05$) by 30 and 19% in 1997 and 1998, respectively. Shading soybean plants during seed development could have resulted in a reduced assimilate availability, thus affecting cell division in the cotyledons. Shading or defoliation during the initial stages of seed development reduced cell division and cell number in cotyledons and reduced seed growth rates (Egli et al. 1989, Egli and Bruening 2001). Increasing the source to sink ratio by fruit removal increased cotyledon cell number. As was in seed growth rate,

Table 1. Seed growth characteristics of soybean in response to light treatments across years

Treatment	Seed growth rate (mg/seed/day)		Effective filling period (days)	
	1997	1998	1997	1998
CK	10.86b	13.82b	25.8b	23.5b
LE	13.04a	17.25a	25.5b	21.6b
S	8.34c	8.35c	28.9a	26.3a

Data points are means of 4 replicates; CK, LE and S are control, light enrichment and shade treatments respectively; means within each column followed by the same letter are not significantly different ($P < 0.05$) using Duncan's Multiple Range Test

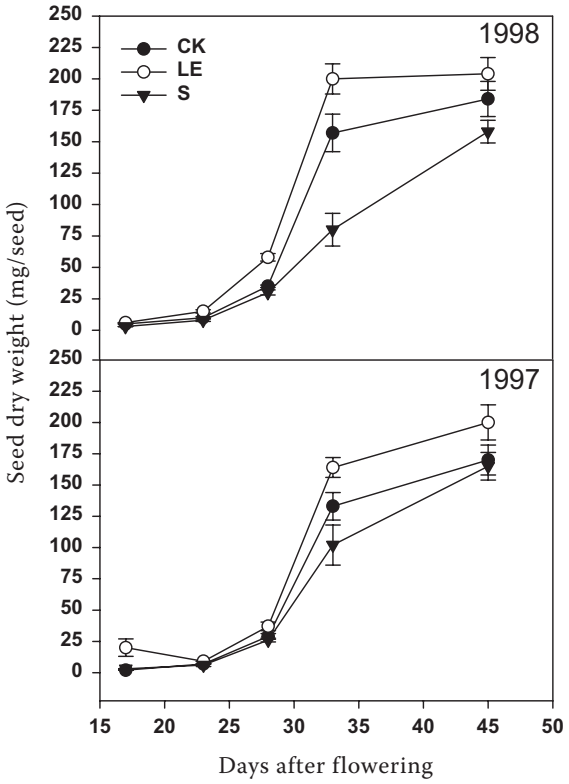

Figure 1. Effects of light enrichment and shade on seed dry weight during seed development of soybean in 1997 and 1998; CK, LE and S are control, light enrichment and shade treatments, respectively; data points are average of 4 replicates; vertical lines are standard error of the mean

in both years differences in cell number among treatments were significantly correlated ($r = 0.925$, $P < 0.05$) with seed dry weight at maturity. Thus, the increase in seed growth rate from increased light was primarily due to increased cell numbers. The growth rate of soybean seed was a function of the number of cells in the cotyledons and the supply of assimilates to the developing cotyledons (Egli et al. 1989). However, Hirshfield et al. (1992) reported that seed size may be influenced more by cotyledon cell size than by cell number.

The growth rate of cells in the cotyledons ranged from 1.02 to 1.86 ng/day across years and treatments. This crop was significantly correlated with seed dry weight at maturity (0.93***) and the rate of seed growth (0.99***) in 1998 but not in 1997 (Table 2). Variations in the growth rate per cell probably reflect the variation in assimilate supply to the seed since this affects seed growth rate in soybean (Egli et al. 1985). Swank et al. (1987) reported significant differences in cotyledon cell number in one soybean genotype, across years, and suggested that the environment may have influenced the number of cells in developing seed cotyledons. So changes in light intensity may be a reason for yearly differences in cotyledon cell number as found here. Other studies showed that low irradiance reduced the number of cells in the endosperm of wheat (*Triticum aestivum* L.) kernels (Wardlaw 1970).

Since there were no significant differences in cell weight or cell volume in seed cotyledons in the three treatments (Table 2), we propose that only the difference in cotyledon cell number, caused by the varying light treatments, was responsible for the observed variations in seed size within the genotype tested. It has to be admitted that the effects of cell number on seed growth rate cannot be separated from possible effects of assimilate supply during seed filling.

Effect of light treatment on endogenous ABA level in testas and cotyledons

The ABA concentration in the seed components under the varying light treatments at 17 d

Table 2. Cotyledon cell characteristics of soybean in response to light treatments across years

Treatment	Cotyledon cell number ($\times 10^6$)		Cotyledon cell volume ($\mu l \times 10^5$)		Growth rate/cell (ng/day)		Cotyledon cell weight (ng)	
	1997	1998	1997	1998	1997	1998	1997	1998
CK	8.32a	8.51a	14.21a	16.01a	1.42a	1.60b	17.61a	21.52a
LE	9.99a	10.12a	13.46a	16.82a	1.43a	1.86a	17.14a	21.64a
S	5.82b	6.93b	15.91a	17.54a	1.56a	1.02c	19.47a	23.28a

Data points are means of 4 replicates; CK, LE and S are control, light enrichment and shade treatments respectively; means within each column followed by the same letter are not significantly different ($P < 0.05$) using Duncan's Multiple Range Test

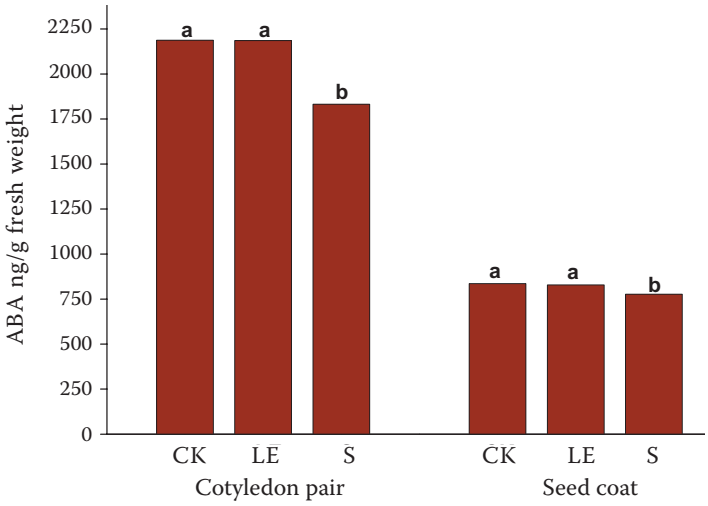

Figure 2. Distribution of ABA in developing soybean seed components sampled in 1998 at 17 days after flowering; data points are the means of seeds averaged over 4 replicates; CK, LE and S are control, light enrichment and shade treatments, respectively; means followed by the same letter are not significantly different at the 5% level of probability using Duncan's Multiple Range Test

after flowering is presented in Figure 2. Figure 2 shows that there were no differences in the ABA concentration in testa and cotyledon of plants grown under CK and LE. However, light reduction significantly ($P < 0.05$) lowered ABA levels in the cotyledons and testa (16% and 7%, respectively at 17 d after flowering compared to the control). Although a significant reduction in ABA levels was found in the cotyledons and testa by shading at 17 d after flowering, no differences existed among treatments at later stages (data were not shown). The lowering of the ABA level in cotyledons and testa, through the seed development, by shade in our study indicates that most of the ABA in plants originates in leaf tissue and that any interference with the photosynthesizing leaves will reduce ABA level in developing soybean seeds. In soybean ABA is reported to be synthesized in the leaves and is exported in substantial quantities via the phloem (Setter et al. 1981, Hein et al. 1984). Quebedeaux et al. (1976) associated rapid increases in the rate of fresh and dry weight accumulation of the soybean seed with a high level of endogenous ABA in the seed. High concentration of ABA in the cotyledons of soybean genotypes was found to coincide with rapid uptake of sucrose, while lower rates of sucrose uptake, later during seed filling, were paired with lower ABA concentrations in the cotyledons (Schussler et al. 1984). Our results also showed that seed growth rate of soybean was significantly correlated with the ABA level in the cotyledons at 17 d after anthesis (0.89**) and testa (0.76*). This indicates that seed tissue ABA concentration is involved in the control of the rate of dry matter accumulation. Morandi et al. (1990) suggested that ABA may increase the assimilate availability during the critical cell division period, and thus regulate cotyledon cell number and seed growth rates. Ackerson (1984) also found that ABA could regulate cell division during early embryo development, thus influencing potential cell storage capacity. However, the involvement of ABA in cell division may only hold true for shaded plants, not under light enriched conditions. Moreover, this is only a preliminary result, and more data is needed to build a solid case for a regulatory role of ABA in seed growth. There is evidence that an increased cytokinin concentration, in certain environments, may also increase seed size and thus total soybean seed yield (Nagel et al. 2001). The progression through cell division and cell enlargement to seed maturity is coordinated by the interactions of stage-specific developmental regulators such as ABA, GAs, and ethylene (Brocard-Gifford et al. 2003). Finally, complex mechanisms are involved in the process of assimilate partitioning during seed filling due to different sources, like leaves, green stems and pod walls, which can all contribute to photosynthates to develop seed.

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