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ORIGINAL ARTICLE

Effect of assimilate competition during early seed development on the pod and seed growth traits in soybean

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Abstract

Although the seed remains small in size during the initial stage of seed development (the lag phase), several studies indicate that environment and assimilate supply level manipulations during the lag phase affect the final seed size. However, the manipulations were not only at the lag phase, making it difficult to understand the specific role of the lag phase in final seed size determination. It also remained unclear whether environmental cues are sensed by plants and regulate seed development or if it is simply the assimilate supply level, changed by the environment, that affects the subsequent seed development. We investigated soybean (*Glycine max* L. Merr.) seed phenotypes grown in a greenhouse using different source-sink manipulations (shading and removal of flowers and pods) during the lag phase. We show that assimilate supply is the key factor controlling flower and pod abortion and that the assimilate supply during the lag phase affects the subsequent potential seed growth rate during the seed filling phase. In response to low assimilate supply, plants adjust flower/pod abortion and lag phase duration to supply the minimum assimilate per pod/seed. Our results provide insight into the mechanisms whereby the lag phase is crucial for seed development and final seed size potential, essential parameters that determine yield.

Keywords Assimilate supply · Lag phase · Embryo and endosperm development · Seed growth traits

Introduction

Seed development is a vital process for plant reproduction as well as for the determination of yield in grain crops. Soybean (*Glycine max* L. Merr.) seed development can be divided into three phases: the early (lag) phase, rapid growth (seed filling) phase, and maturation phase (Fig. 1a). During the lag phase, the embryo develops all the structures (meristem, cotyledons, and hypocotyl) (Chamberlin et al. 1994). Cell division in the cotyledons occurs rapidly, and the cell number reaches its maximum by the end of the lag phase (Egli et al. 1981; Egli 2017). The endosperm undergoes rapid nuclear divisions to generate the multinuclear endosperm and then cellularizes (Dute and Peterson 1992). The seed remains very small throughout the lag phase (< 5% of the final weight). During the seed filling phase, the expansion of cells in the cotyledons takes place, and this is the time when seeds become physically enlarged and significant dry matter accumulation occurs (Egli 2004). Finally, seeds undergo the maturation phase when they start to desiccate and become dormant. A similar pattern of seed development also occurs in other grain crops such as rice (*Oryza sativa* L.), wheat *(Triticum aestivum* L.), and corn *(Zea mays* L.), in which the accumulation of storage materials occurs in the endosperm instead of the embryo (Gao et al. 1992; Brown et al. 1996; Olsen et al. 1999; Sabelli and Larkins 2009).

Although most of the dry weight accumulation that determines final yield occurs during the seed filling phase, several studies in cereal crops have shown that environmental conditions and assimilate supply during flowering and the lag phase also play a role in the determination of maximum seed weight (Chowdhury 1978; Jones et al. 1985; Calderini et al. 1999; Gambín et al. 2006). In soybean, source (production

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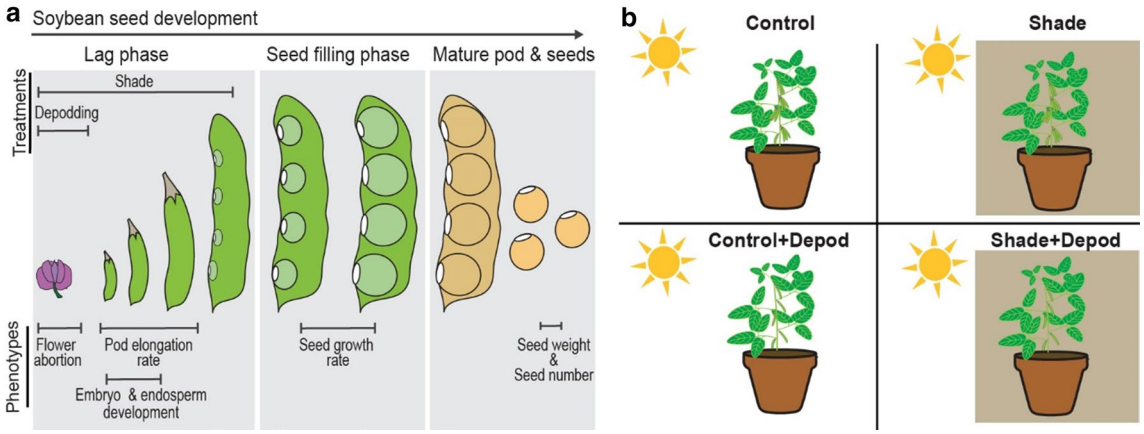

Fig. 1 Schematic diagram showing the reproductive development of soybean (**a**) and treatments imposed (**b**). **a** Development phase durations are not drawn to scale. Horizontal bars represent treatment and data collection periods. **b** Different source-sink manipulation treatments (control, 75% shade during lag phase, control + depodding to one pod per node, 75% shade + depodding to one pod per node) were applied during lag phase seed development. To collect data, a total of 15–40 marked pods from five plants in experiment 1, and seven plants in experiment 2, per treatment per phenotypes, was monitored

of photoassimilates in leaf) and sink (number and size of reproductive organs) manipulations imposed from the beginning of flowering affect cotyledon cell number (Egli et al. 1989), and the number of cells in cotyledons from a diverse group of soybean genotypes showed a close correlation with the potential seed growth rate measured both in vitro and *in planta* (Egli et al. 1981; Guldan and Brun 1985). However, there was still a large gap in knowledge about the effects of environment and assimilate supply specifically during the lag phase on final seed size.

The duration of the lag phase before the start of rapid seed growth influences the temporal distribution of sink strength (or assimilate requirement by growing sinks). For example, longer lag duration delays the start of sink-intensive seed filling phase of each pod and may allow plants to flower more, thus setting more pods to grow. An artificially imposed extended photoperiod, starting at flowering without an increase in daily total photosynthetically active radiation, extends the lag phase duration, increases pod number and final yield (Nico et al. 2015, 2016). Extended photoperiod thus influences the source/sink ratio at the same time; therefore, this made it difficult to distinguish whether the increase in lag phase duration was a result from environmental changes sensed directly by plants, or indirectly through changes in assimilate supply per developing sink.

To further investigate the specific effect of assimilate supply during the lag phase for seed development, we aim to quantify the effect of different source/sink ratios during early seed development on embryo and endosperm development, the lag phase duration, pod elongation rate, seed growth rate, and final seed weight. We hypothesized that changes in source-sink ratios to developing flowers and pods affect seed development not only at the lag phase, but also at the subsequent phases. To address our hypotheses, we conducted two independent experiments and grew soybean plants with different planting dates in the greenhouse under natural light or with 75% shading during the lag-phase with/without depodding treatments. Source-sink manipulation had a significant effect on the rate of flower and pod abortion. Our results provide the evidence that in response to assimilate supply during the lag phase, plants adjusted sink size and lag phase duration to maintain a minimum amount of assimilate supply per pod. From both experiments, we also observed the critical role of assimilate supply during the lag phase on potential seed growth rate and final seed weight determination.

Materials and methods

Culture and experimental design

Two greenhouse experiments were carried out at the University of Kentucky greenhouse facilities in Lexington, KY (38.02° N, 84.50° W). Soybean plants (cultivar PI1A95X) of MG I were grown in 3.8 L pots filled with a 6:1 ratio of pro-mix growing medium and sterilized silt loam soil. Two to three seeds per pot were seeded on April 9, 2018, and May 18, 2018. The difference in the planting dates generated different photoperiods (Table 1). Seeds were inoculated with *Bradyrhizobium japonicum* using a commercial dry peat-based formula (Advanced Biological Marketing, Van Wert, OH). Before seeding, twelve grams of a slow-release fertilizer Osmocote (14:14:14, N:P:K) were mixed with the growing medium in each pot. After emergence, seedlings were thinned to one per

Table 1 Average daily temperature (°C) and day length (hours) during soybean developmental phases in greenhouse experiments 1 and 2 (planted on 04-08-2018 and 05-18-2018, respectively)

	Average daily temperature (°C)			Average day length (hours)		
	VE to R1	R1 to R5	R5 to R7	VE to R1	R1 to R5	R5 to R7
Experiment 1	25.4	26.6	26.5	13.5	14.2	14.7
Experiment 2	26.4	25.8	27.0	14.6	14.8	14.3

pot. In experiment 1, there were five replicated plants for each phenotype observation, a total of 20 plants in each treatment, and in experiment 2, seven replicated plants for each phenotype observation, a total of 35 plants in each treatment. The experimental design in experiments 1 and 2 consisted of a completely randomized design with two main treatments: a control and a shade (plastic mesh with 75% shade) treatment imposed 7 and 8 days after beginning flowering (R1, Fehr and Caviness 1977) in experiment 1 and 2, respectively (Fig. 1b). Opened flowers on all plants at 9 and 19 days after R1 (DAR1) (experiment 1), and 10, 12, and 21 DAR1 (experiment 2) were marked by acrylic paint at the base of the peduncle using a fine brush. Pods developed from the marked flowers were also marked by acrylic paint (Fig. 2a). From the first markings (9 and 10 DAR1 in experiment 1 and 2, respectively), 7–8 developed pods per plant were monitored. For the later markings (19 DAR1 in experiment 1 and 12, 21 DAR1 in experiment 2), 3–4 pods per plant were monitored. For flowers marked 19 DAR1 (experiment 1), and 21 DAR1 (experiment 2), a secondary source-sink manipulation treatment was imposed, where plants were depodded to one marked flower per node (80–85% reduction in pods per plant), or were left un-manipulated (Fig. 1b). All flowers and pods developing after first pod removal were removed continuously throughout the experiment. Plants remained under the shade until all the marked pods were past the end of lag phase (seeds were greater than 3 mm in diameter; 27 DAR1 in experiment 1 and 38 DAR1 in experiment 2). After that, the plastic mesh was removed, and plants returned to the same conditions as the control. In experiment 1, greenhouse air temperature was recorded with a temperature sensor (Argus control, British Columbia, Canada). In the second, air temperature sensors were placed in both shade and control conditions, and the temperature was recorded in Decagon Em50 devices (Decagon Devices, Pullman, WA). The day length data for Lexington, Kentucky, was obtained from Astronomical Applications Dept., US Naval Observatory.

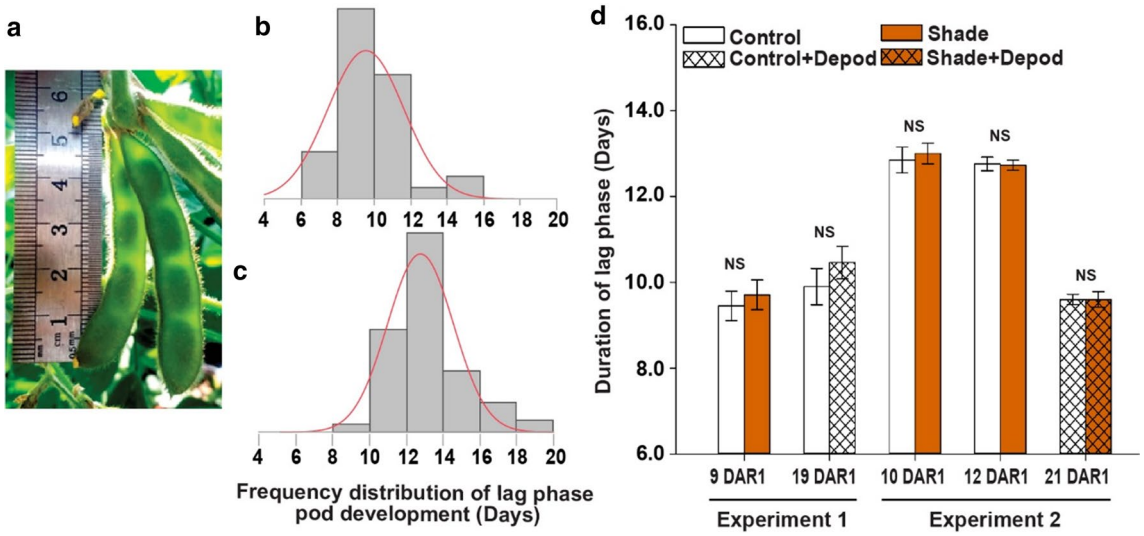

Fig. 2 Duration of soybean lag phase under different source-sink manipulation treatments (control, 75% shade during lag phase, control + depodding to one flower per node, 75% shade + depodding to one flower per node). **a** Pods developed from open flowers marked on the same day show variable lag phase duration. **b**, **c** Frequency distribution of the duration of lag phase in control and shade treatments in experiment 1 (**b**) and experiment 2 (**c**). **d** Lag phase duration measured in flower cohorts marked 9 to 21 days after beginning flowering (DAR1). NS denotes no significant effect of shade and depodding treatments at $p < 0.05$ as determined by t test. Error bars represent standard error

Data collection

The date of the pod developmental stages was recorded for all marked pods, by monitoring plants three times a week: pod of 5-mm length, pod of 20-mm length, seed of 3 mm in diameter, seeds filled the pod locule, pods with mature pod color (Fig. 1a). The duration of the lag phase at the individual pod level was defined as the number of days from opened flower, to a pod with seeds of 3 mm in diameter following the approach of Nico et al. (2016). When pods reached 5 mm, pod length was recorded every other day with an electronic caliper until pods had seeds of 3 mm in diameter. Flower and pod abortion per plant at each developmental stage was determined by the initial number of marked flowers minus the number of developing pods from the marked flowers was divided by the initial number of marked flowers and expressed as a percentage. Pods were sampled to quantify pod and seed growth traits, and to do in vitro cotyledon culture as described in the sections below. At full plant maturity (R8 as defined by Fehr and Caviness 1977), marked and non-marked pods on five plants in experiment 1 and seven plants on experiment 2 per treatment were harvested separately, counted, and dried at 65 °C for 48 h. The number of seeds and dry seed weight were used to calculate dry individual seed weight.

Microscopic assays

For the microscopic assay, seeds from marked pods were collected at three different developmental stages. When the majority of the marked pods reached 10 mm in length, approximately one third pods were collected. The second and third samples were collected once the remaining pods reached 15 mm and 20 mm in length, respectively (Sup. Table 1). A total of four to six marked pods each from five plants were collected for each treatment. The pod was opened by cutting the edge of the pod, and the seeds were gently removed, placed in fixing solution (EtOH/Acetic acid; 3:1), and kept in the 4 °C until further processing. The seeds were processed by following the Feulgen staining method (Braselton et al. 1996). After mounting in LR resin, embryo and endosperm development was observed in the confocal microscope. An FV1200 laser scanning confocal system (Olympus) with fluorescein isothiocyanate (FITC) settings (argon ion laser at 488 nm, emission at 515 nm) was used. Snapshot images were acquired using FV10-ASW 4.2 software. Laser 8–10%, HV 550–600, gain 1.25 and Kalman 3–4 options were applied to capture images. All confocal images were analyzed and processed using Fiji (ImageJ) software.

In vitro seed growth rate assays

For in vitro seed growth rate analysis, all marked pods (5–8 pods per plant) from five plants in experiment 1, and seven plants in experiment 2, per treatment were sampled during the seed filing phase. Pods were harvested 37 DAR1 in experiment 1, and 48, 50, and 59 DAR1 in experiment 2. A random subsample of ten seeds from harvested pods on each plant was cultured in vitro following the protocol of Egli and Wardlaw (1980). First, the harvested pods were washed with liquinox soap and water. Seeds were extracted from clean pods under sterile conditions, the seed coat was removed, and the embryonic axis was excised. One cotyledon per seed was placed in 50-ml Erlenmeyer flasks with 7 ml of culture solution for 7 days, and the other cotyledon from the same seed was dried to determine initial dry weight. The components of the culture solution and the concentration of each components were: K_2SO_4 5.9 mM, $MgSO_4$ 1.5 mM, KH_2PO_4 1.25 mM, H_3BO_4 100 µM, $MnSO_4$ 100 µM, $ZnSO_4$ 30 µM, KI 5 µM, $NaMoO_4$ 0.1 µM, $CuSO_4$ 0.1 µM, $CoCL_2$ 0.1 µM, $CaCl_2$ 3.0 mM, Na EDTA 200 µM, $FeSO_4$ 100 µM, Thiamine 0.3 µM, Nicotinic acid 4 µM, Pyridoxine 2.4 µM, Myoinositol 500 µM, Glycine 26.6 µM, Methionine 10 mM, Sucrose 200 mM, L-Asparagine 7.5 mM and pH was to 6.5. During incubation, flasks were shaken on a rotary shaker at room temperature (approximately 24 °C) in light. After incubation, the cotyledon was taken from the flask and dried to determine the final dry weight. Any contaminated flasks were discarded. The in vitro seed growth rate (mg $seed^{-1}$ day^{-1}) was calculated from the difference of initial and final dry weight for each pair of the cotyledon.

Statistics

Data from lag phase duration, pod elongation rate, in vitro seed growth rate, and final seed weight were analyzed by a *t* test to quantify differences between treatment (control, shade), and depodding manipulation (depodding and no-depodding) within an experiment, and pod cohort. In vitro seed growth rate and final seed weight data collected at 21 DAR1 in experiment 2 that included depodding treatment was analyzed with an ANOVA using PROC GLIMMIX in SAS (SAS v.9.4, SAS Institute, Inc., Cary, N.C.) with treatment (control, shade), depodding manipulation (depodding and no-depodding), and their interaction as fixed effects (Sup. Table 2). We did not have data from all treatment combinations (control, shade, control + depodding, shade + depodding) for all variables measured on 19 and 21 DAR1 due to high flower abortion when imposing shade or no-depodding treatments. In addition, to compare the data between experiments, data from the lag phase duration, pod elongation rate, in vitro seed growth rate, and final seed weight were also analyzed with an ANOVA with

experiment, treatment (control, shade), their interaction, and the date of flower marking nested within experiment and treatment as fixed effects (Sup. Table 3). Data from flower and pod abortion was analyzed by a *t* test to quantify differences between control and shade treatments within an experiment, pod cohort, and seed developmental stage.

Results

Plant growth conditions

The average daily temperature and daylight duration during soybean developmental phases in each experiment are given in Table 1. The mean daily temperature during R1–R5 (Fehr and Caviness 1977) of experiments 1 and 2 was 26.6 and 25.8 °C, respectively. Ten days after R1, when the shade treatment was imposed in experiment 2, the average daily temperature in shade was 0.79 °C lower on average compared to control. In experiment 1, the temperature under shade treatment was not recorded. In experiment 2 during VE-R1 and R1–R5, plants experienced 60 and 45 min, respectively, prolonged photoperiod compared to experiment 1 due to different planting dates (Table 1).

Effect of assimilate supply on the lag phase duration

The shade treatment (75% shade) was applied during the lag phase and the effect on the lag phase duration was monitored. The frequency distribution of the lag phase duration of individual pods under control treatment ranged from 6 to 20 days across both experiments (Fig. 2b, c). In both experiments, the lag phase duration under the shade treatment (no-depodding) was similar to the control (Fig. 2d). The lag phase durations in experiment 2 were approximately 3 days longer compared to those in experiment 1 (Fig. 2d), which is consistent with Nico et al. (2016) where prolonged lag phase duration under extended photoperiod treatment was reported. These results show that the shade treatment itself did not affect the duration of lag phase; however, lag phase duration varied among experiments.

In plants, photoassimilate is transported from the source to sink, and due to high photoassimilate competition among sinks, soybean seed weight cannot achieve the maximum possible (Borrás et al. 2004). Reducing the sink number or depodding is a far less manipulated non-surgical method (Egli et al. 1985, 1989; Chiluwal et al. 2021) compared to the "sugar feeding to the petiole" method (Mason et al. 2014), and depodding during the seed filling phase increased the photoassimilate concentration to the remaining seeds and increased seed size in soybean (Egli and Bruening 2001). To quantify the lag phase duration under conditions of no assimilate competition, we also performed a depodding treatment where one flower was kept in each node and all other flowers and pods were removed continuously throughout experiments 1 and 2 (Fig. 1b). The lag phase duration was similar between the control and the depodding treatments in experiment 1 and between the depodding treatments of control and shade in experiment 2 (Fig. 2d). Due to many flower abortions, we were not able to obtain data from depodding shade treatment in experiment 1 and no-depodding treatment in experiment 2. The lag phase in depodding treatments in experiment 2 was shorter than the no-depodding treatments among treatments in experiment 2 and similar to those in experiment 1 (Fig. 2d and Sup. Table 4). The shortest lag phase duration among experiments including depodding treatments is approximately 9–10 days, suggesting that 9–10 days is the shortest lag phase this particular cultivar can achieve.

Effect of assimilate supply on flower and pod abortion

To quantify the flower and pod abortion rates, open flowers were marked, and we monitored flower and pod abortion of the marked flowers over time. Control plants had a 21 to 28% total flower and pod abortion, whereas abortion increased to 42 to 49% under shade treatments in both experiments (Fig. 3a, b). Only 8 to 12% of marked flowers aborted before developing 5-mm pods in length, and most of the abortions occurred in developing pods before they reached 20-mm length (Fig. 3a, b). In experiment 2, the percentage of flower and pod abortions were also monitored in the depodding treatment (Fig. 3b). Consistent with the previous report (Heitholt et al. 1986), depodding significantly decreased the rate of flower abortion. Interestingly, shade treatment did not increase the rate of flower abortion when plants were depodded, indicating that shade itself is not a direct signal for flower abortion. Our data show a clear effect of assimilate supply on the rate of flower and pod abortion, and indicate abortion provides a mechanism for plants to adapt assimilate supply and maintain assimilate supply per developing flower and pod.

Effect of shade treatment on embryo and endosperm development

Embryo and endosperm development was observed in seeds collected from pods 10 to 15 mm in length (Sup. Table 1). Based on the major developmental phenotypes of the early-stage soybean seeds, we categorized them into four stages: an early globular-shape embryo with multinuclear endosperm (stage 1), a globular-shape embryo with multinuclear endosperm (stage 2), a globular-shape embryo with cellularized endosperm (stage 3), and a late globular embryo with cellularized endosperm (stage 4) (Fig. 4a, b). In the

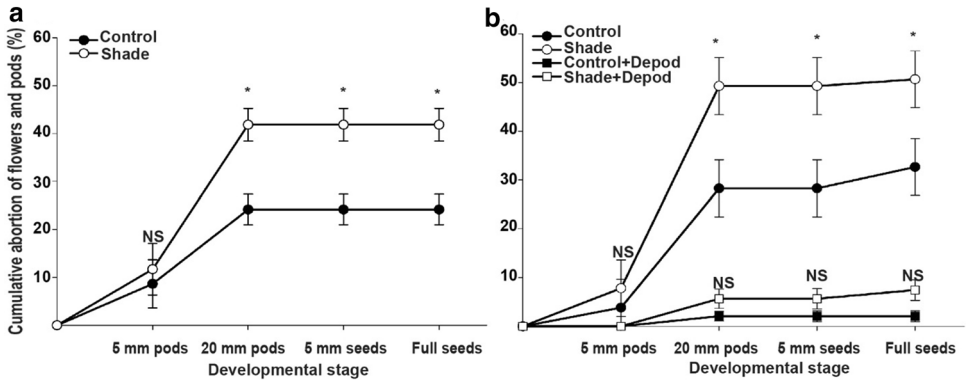

Fig. 3 Cumulative abortion of flowers and pods in experiment 1 (**a**) and experiment 2 (**b**). Flowers and pod abortion were measured in flower cohorts marked 9 days after beginning flowering (DAR1) (**a**), and 10, 21 DAR1 (**b**). Asterisk (*) denote significantly different means between the control and shade treatment at $p < 0.05$ within an experiment and pod cohort (*t* test), NS denote non-significantly different means. The bars represent standard error (SE). "Full" seeds indicate the time when growing seeds had filled the pod locule

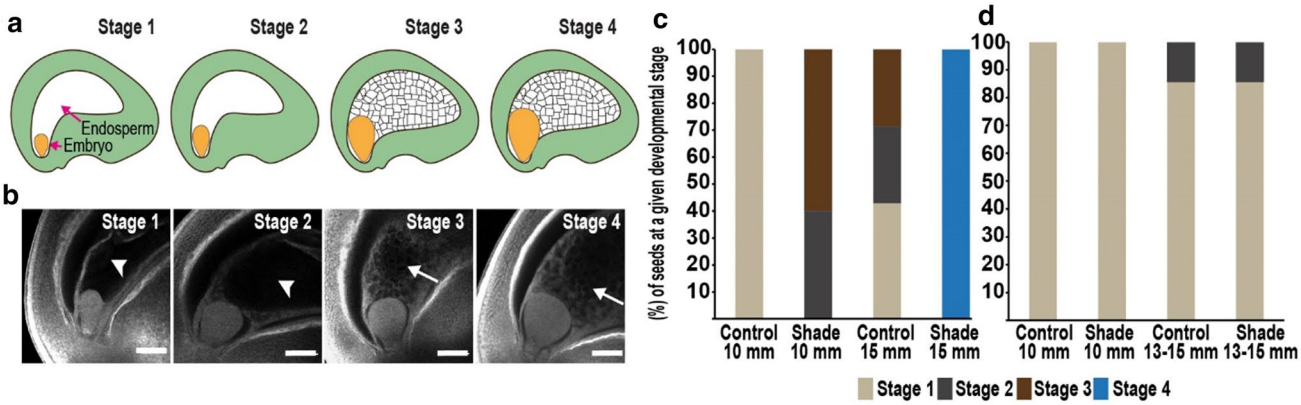

Fig. 4 Embryo and endosperm development of soybean seeds observed in experiments 1 & 2. **a** Cartoon images of embryo and endosperm development stages; early globular embryo with nuclear endosperm (Stage 1), globular embryo with nuclear endosperm (Stage 2), globular embryo with cellularized endosperm (Stage 3), late globular embryo with cellularized endosperm (Stage 4). **b** Confocal representative images of stages 1–4. Arrowhead indicates no cellularization, arrow indicates cellularized endosperm. Scale bar = 100 µm. **c**, **d** Status of embryo and endosperm development of soybean seeds collected from 10- to 15-mm pods. Seeds collected from experiment 1 (**c**), experiment 2 (**d**). The X-axis denotes the length of pods at the time of seed collection and Y-axis denotes the percentage of different stages seeds observed

control treatment of experiment 1, all seeds sampled from 10-mm pods were at stage 1 (Fig. 4c). Under shade treatment, 40% of seeds from 10-mm pods had reached already stage 2 and the remaining 60% had reached stage 3 (Fig. 4c). In seeds from 15-mm pods under the control treatment, 40% of seeds remained at stage 1, 30% of seeds reached stage 2, and the remaining 30% reached stage 3. Furthermore, all seeds from 15-mm pods under the shade treatment had reached stage 4 (Fig. 4c). In experiment 2, both control and shade treatments delayed the embryo and endosperm development compared to experiment 1 (Fig. 4d). 85% of seeds from 13- to 15-mm pods reached stage 1 and the rest of the seeds reached stage 2 condition (Fig. 4d). These results show faster growth of embryo and endosperm development in shade treatment in the experiment 1, whereas in the experiment 2, the embryo and endosperm development are delayed in both treatments compared to the experiment 1.

Effect of assimilate supply on pod elongation rate

The pod elongation rate was not affected by the shade or depodding treatments (Fig. 5a), suggesting that the assimilate supply during the lag phase does not play a major role in the pod elongation rate. Although we did not find any effect of shade on pod elongation, we observed differences in the average pod elongation rate by experiment (Fig. 5a). Experiment 2 no-depodding pods had the lowest pod elongation rate across treatments and experiments (Fig. 5a). The longest lag

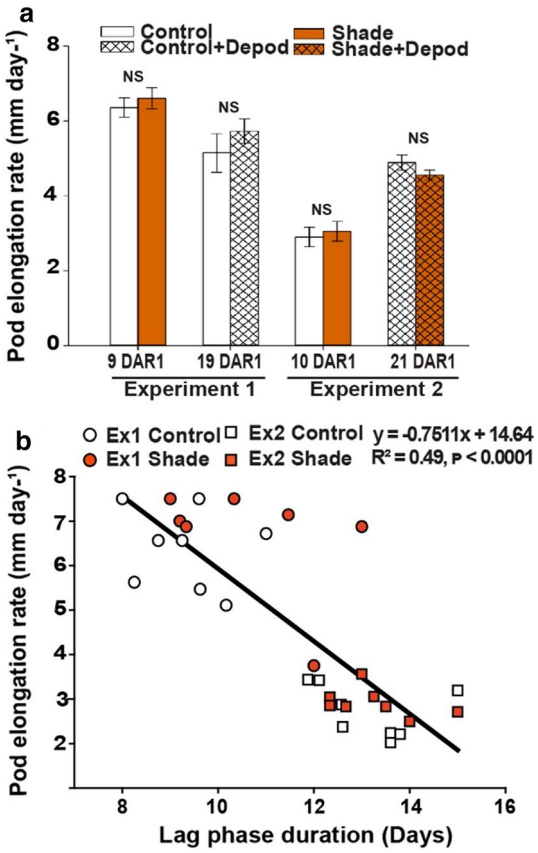

Fig. 5 Pod elongation rate measured in different flower cohorts marked 9 to 21 days after beginning flowering (DAR1) in experiment 1 and 2 and under different source-sink manipulation treatments (control, 75% shade during lag phase, control + depodding to one flower per node, 75% shade + depodding to one flower per node) (**a**), and linear regression of pod elongation rate versus lag phase duration (**b**). NS denotes no statistically significant effect of shade and depodding treatments as determined by *t* test ($p < 0.05$). Error bar represents standard error. **b** The circle and square symbols represent experiment 1 and 2, respectively. Each data point corresponds to the average pod elongation rate and average lag phase duration per plant. Parameters of the fitted linear regression (line) and coefficient of determination (r^2) are also shown

phase duration on average was also observed from these pods (Fig. 2d), and the relationship between the pod elongation rate and the lag phase duration showed a negative correlation coefficient ($r^2 = 0.49$, $p < 0.001$) across the two experiments (Fig. 5b). Furthermore, we observed a negative correlation coefficient ($r^2 = 0.83$, $p < 0.001$) for pod elongation rate and photoperiod, and a positive correlation coefficient ($r^2 = 0.66$, $p < 0.001$) of lag phase duration and photoperiod across the two experiments (Sup. Figure 1).

Effect of assimilate supply during the lag phase on the subsequent seed growth rate and final seed weight determination

The in vitro seed growth rate is a technique that reflects potential seed growth rate without the confounding effect of pod competition or without sucrose and nitrogen limitation during the seed filling phase. Overall, the potential seed growth rate measured from seeds sampled during the seed filling phase was reduced by shade treatment (Fig. 6a). In experiment 2, 10 DAR1 samples show differences similar to those found in experiment 1; however, the effect of shade treatment became not significant over time, in 12 and 21 DAR1 (Fig. 6a). When depodding was applied, in vitro seed growth rates from both control and shade treatments were significantly increased compared to the no-depodding treatments (Fig. 6a). These results indicate that the assimilate supply, but not shade treatment per se, during the lag phase changes the potential seed growth rate of the subsequent seed filling phase. The plant lifecycle in experiment 2, including seed development, was prolonged compared to experiment 1 (Figs. 2, 4), and we also observed an increased number of seeds set per plant in experiment 2 compared to experiment 1 (Sup. Table 5). These conditions caused more assimilate supply competition per pod during development in experiment 2, possibly explaining smaller differences between control and shade treatments at the later stages.

The shade treatment imposed during the lag phase also reduced final seed weight (Fig. 6b and Sup. Table 4). The apparent seed filling phase duration was reduced by shade in experiment 1 9 DAR1, but not in experiment 2 10 DAR1 (Sup. Figure 2). The final seed weight of the control treatment in experiment 2–10 DAR1 was higher than that of the shade treatment (Fig. 6b), suggesting that the actual *in planta* seed growth rate is lower in shade treatment compared to that in the control. Under the depodding treatment in both experiments, seed weight increased significantly compared to the no-depodding treatment (Fig. 6b and Sup. Table 2), and we did not observe any significant change of seed weight between control + depodding and shade + depodding treatments (Fig. 6b). Seed weight was always higher under the depodding treatment than control in all experiments (Fig. 6b). This increase in final seed weight in the depodding treatment is expected since final seed weight in soybean is source limited during the seed filling phase (Borrás et al. 2004; Chiluwal et al. 2021). Overall, our results suggest that shade itself is not the environmental cue, but the change in assimilate supply during the lag phase, is the key to determine the potential final seed weight.

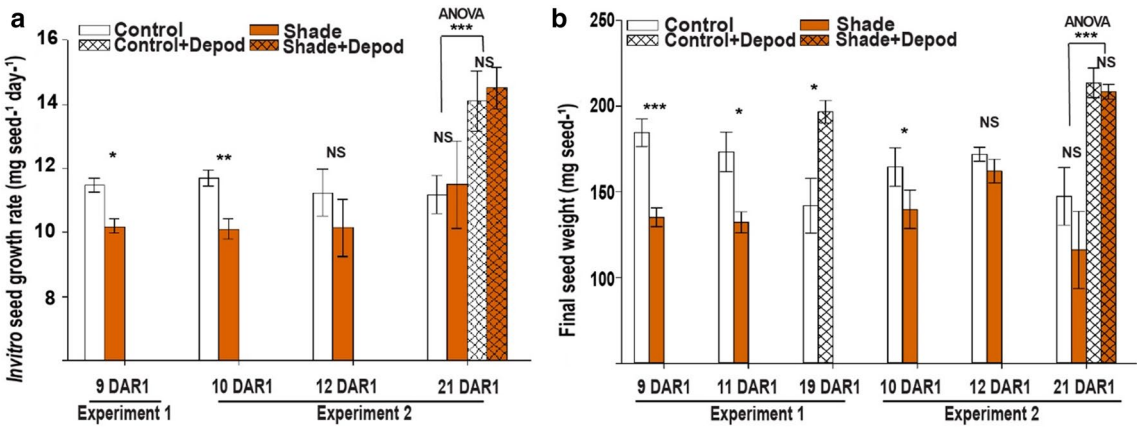

Fig. 6 In vitro seed growth rate **a** final seed weight **b** measured and under different source-sink manipulation treatments (control, 75% shade during lag phase, control + depodding to one flower per node, 75% shade + depodding to one flower per node). **a**, **b** The in vitro seed growth rate and final seed weight were measured in different flower cohorts marked 9 to 21 days after beginning flowering (DAR1). Asterisk denotes the significant effect of shade and depodding determined by *t* test and ANOVA (21 DAR1) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). NS denotes no statistically significant effect. Error bar represents standard error (SE)

Discussion

The seed filling phase is the time when seeds become physically enlarged; therefore, this phase has been the focus of research in understanding physiological processes that determine final seed weight (Egli 2004). There are a large number of studies on the effect of source-sink manipulations during flowering and pod setting on the determination of seed number (Jiang and Egli 1995; Egli 2010; Nico et al. 2016). However, there is a lack of information on the assimilate supply effect specifically during the lag phase on seed development as well as on the determination of the final seed size. We found that the lag phase duration remained unchanged within an experiment in response to the shade treatment (Fig. 2d). Instead, shading increased the percentage of aborted flowers and pods (Fig. 3), indicating that soybean plants abort flowers as an early mechanism to adapt sink number in response to assimilate supply. Overall, the lag phase duration measured at the pod level showed a normal distribution with the peak at 10 and 13 days in experiment 1 and 2, respectively (Fig. 2b, c), and in depodding treatments, the lag phase duration averages 9–10 days (Fig. 2d). These data suggest that this soybean cultivar requires at least 9–10 days of the lag phase if there is no disruption of assimilate supply. It is likely that plants extended or shortened the lag phase duration to maintain a minimum and similar amount of assimilate supply per developing pod (Fig. 2d). Thus, the lag phase duration is an additional trait after flower abortion to adapt sink number to assimilate supply over a window of time.

Overall, comparing the results of both experiments, we observed delayed development of the embryo and endosperm (Fig. 4), lag phase duration (Fig. 2) and pod elongation rate (Fig. 5), and increased number of seeds in experiment 2 (Sub. Table 5). Due to the planting date difference, we observed variations of 0.79 °C and 1 h photoperiod between the two experiments (Table 1). In soybean, 1 °C temperature variation does not show any effects on plant growth and yield (Tacarindua et al. 2013). On the other hand, long photoperiod prolongs the duration of flowering and the lag phase, and increases flowers and pods per node (Kantolic et al. 2013; Nico et al. 2016). Kantolic and Slafer (2005) also reported that prolonged photoperiod (1.5–3 h) increased the duration from the beginning pod (R3) to full seed (R6) developmental stages measured at the plant level. Thus, the longer lag phase duration and slower pod elongation rate in experiment 2 compared to 1 is likely a response to a longer photoperiod. Consistently, we observed a correlation between pod elongation rate and lag phase duration versus photoperiod across the two experiments (Sup. Figure 1). Another possibility is a lower assimilate supply per pod/seed due to the increased number of flowers and pods caused by a longer photoperiod (Table 1 and Sup. Table 5) which indirectly prolonged the lag phase duration. Although small, there is a difference in the temperature between experiments (0.79 °C). The mechanism of how photoperiod and other environmental factors affect lag phase seed/pod development is unknown, and further analyses are awaited.

Precocious endosperm cellularization results in relatively small seeds, while delayed endosperm cellularization is associated with enlarged seeds in *Arabidopsis thaliana* and rice (Scott et al. 1998; Berger et al. 2006; Orozco-Arroyo et al. 2015; Lafon-Placette et al. 2017). In experiment 1, we observed that pods of similar length had faster embryo development and endosperm cellularization with reduced

final seed weight under shade treatment compared to the control treatment (Figs. 4c and 6b). However, we did not see such differences in experiment 2 (Fig. 4d). In experiment 2, plants experienced prolonged lag phase duration compared to experiment 1 (Fig. 2d). Prolonged lag phase duration and delayed embryo development were observed under extended photoperiod (Nico et al. 2016). Therefore, the prolonged lag phase duration and delayed embryo development compared to experiment 1 in both shaded and non-shaded conditions of experiment 2 (Figs. 2d and 4d) may be attributed to the photoperiod difference (Table 1) and/or the difference in assimilate supply per pod. Depodding treatments in diverse photoperiod conditions will determine whether the rate of embryo development and the timing of endosperm cellularization are controlled by assimilate supply and/or photoperiod.

We hypothesized that the assimilate supply during lag phase may affect potential seed growth rate estimated from in vitro culture. We found that the shade treatment reduced this in both experiments and depodding treatment increased the in vitro seed growth rate relative to the control in experiment 2, supporting our initial hypothesis (Fig. 6a). Reducing assimilate supply during the lag phase through shading decreases cotyledon cell number, whereas increasing assimilate supply per developing pod through depodding increased cell number (Egli et al. 1989). Thus, it is likely that assimilate supply during the lag phase regulates the total cell number in the cotyledons, affecting the potential seed growth rate in the subsequent seed filling phase. The final seed size from plants with lag phase shade treatment also has a tendency to reduce the final seed weight compared to the control (Fig. 6b). The shade-treated plants were transferred back to normal light conditions in the subsequent seed filling phase and set a smaller number of pods per plant (Sup. Table 5). Therefore, more assimilates per developing pod should be supplied in these plants compared to the control, yet smaller seeds were produced (Fig. 6b). Taken together, our results suggest that the lag phase effect cannot be rescued by more optimal conditions in the subsequent seed filling phase for the final seed size unless the excess amount of nutrients is supplied per pod like in the depodding treatments (Fig. 6b).

In summary, our study demonstrates that the lag phase is sensitive to assimilate supply and responds by controlling flower/pod abortion, lag phase duration, and potential seed growth rate. Nico et al. (2016) proposed the post-flowering period as a strong candidate for yield improvement. An extended lag phase duration can distribute sink requirement over longer time period and this may allow plants to set and support more pods under an artificially extended photoperiod with the same total photosynthetically active radiation. Further studies evaluating the effect of environmental conditions on the lag phase duration and associated yield components should take into account this potentially confounding effect of assimilate competition that we found in this work. Thus, investigating the physiological and molecular mechanisms regulating pod and seed development during the lag phase under the controlled environment (e.g., growth chambers) with depodding will be crucial to understand the precise role of lag phase in determining final seed weight and yield potential.

Author contribution statement MFA, MS, and TK designed the research; MFA, PB, and JT performed the research; MFA, MS, and TK analyzed data and wrote the manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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