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Effect of thinning and shade removal on green stem disorder in soybean

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ABSTRACT

Green stem disorder (GSD) in soybean (*Glycine max* (L.) Merrill) retains green stems and leaves as the pods mature, thereby reducing the harvest efficiency and impairing seed quality. In order to elucidate the causes of GSD, the factors that promote GSD need to be identified. In our experiments, we adjusted plant density at the developmental growth stage R1 (the beginning of flowering) or at R5 (the beginning of seed filling), from dense (22.2 plants m^{-2}) to sparse (5.56 plants m^{-2}) by thinning. We found that GSD occurrence was increased when plant density was changed, compared to the treatments that were maintained under either dense or sparse conditions. GSD was promoted more strongly when thinning was conducted at R5 than at R1 stage. Shading equipment surrounding plants, except for their upper-most leaves, was implemented to determine the association of shading and GSD. The results of the shade experiment revealed that GSD occurrence generally increased in treatments subjected to shade removal, compared to those that were shaded until R8 stage (full maturity) or never shaded since the time of sowing. GSD was strongly promoted by shade removal at R5 than at R1 stage. The shading results coincide with the results of the plant density experiment, indicating that an increase in light availability enhances source activity relative to sink at R5 stage, thereby promoting GSD occurrence in soybean. Thinning is expected to be used as an easy experimental method to create GSD for research purpose.

1. Introduction

Typically, as soybean pods mature and reach harvesting stage, the leaves turn yellow and drop, and the green stem turns pale and loses moisture. Green stem disorder (GSD) in soybean is defined as the condition in which the stems and leaves stay green and retain some moisture even when the pods normally mature (Harbach et al., 2016; Hobbs et al., 2006). GSD has also been termed delayed leaf senescence (Phillips et al., 1984) and inharmonious maturation (Furuya et al., 1988). GSD often negatively affects harvesting and seed appearance. Harvest efficiency is greatly reduced compared to normally matured soybean plants because it is difficult to cut the moist stems of GSD soybeans by using combine harvesters (Harbach et al., 2016; Hill et al., 2006). Seed appearance is also impaired because the seed surface is stained with the sap of moist stems and leaves in combine harvesters (Ogiwara, 2002).

In order to develop a solution to prevent the occurrence of GSD in soybean, the underlying mechanism of GSD must be elucidated. Toward this, it will be effective to find factors or experimental treatments that promote GSD, and then to analyze the effects of these factors or treatments on soybean physiology and ecology.

Studies have suggested factors that promote GSD, such as excessively wet soil conditions during the reproductive period (Sato et al., 2007), drought at the flowering and pod set periods after excessively wet conditions during initial growth (Tsujimoto et al., 2006), high temperature during the reproductive period (Mochizuki et al., 2005), pest attacks at the pod set and filling stages (Boethel et al., 2000), and diseases occurrence (Takehara et al., 2016). Depodding at the pod set and filling stages has also been used as an experimental treatment to promote delayed senescence of leaves and stems including GSD (Crafts-Brandner & Egli, 1987; Crafts-Brandner et al., 1984; Egli & Bruening, 2006; Htwe et al., 2011; Leopold et al., 1959; Mondal et al., 1978; Wittenbach, 1982). The common feature behind these factors or treatments is thought to be a decrease in the number of pods due to some type of stress during the reproductive phase. Depodding also leads to the accumulation of vegetative storage proteins

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in the vegetative organs, suggesting a surplus of assimilation products (Ogiwara & Ishikura, 1994; Wittenbach, 1983a, 1983b). These factors have been thought to imply that GSD is related to a relative increase in source levels resulting from sink limitation although the conclusive evidence has not been shown and GSD has sometimes also been observed without reduction in the number of pods (Mochizuki et al., 2005). It was also reported that fungicides application promoted GSD incidence (Hill et al., 2013) and that there were differences in GSD occurrences among cultivars (Fujii et al., 2015; Hill et al., 2006; Isobe et al., 2015; Yamada et al., 2014) although these results were not related to sink limitation.

In addition, the above-mentioned factors or treatments are difficult to repeat in experimental set up with high reproducibility, particularly in research in field conditions. Depodding can not only lead to a decrease in the number of pods but also cause physical injury stresses from cutting. Depodding may also have unintended effects on soybean physiology apart from sink limitation, for instance, by up-regulation of the stress responses (Turner et al., 2012). Therefore, another experimental treatment to promote the occurrence of GSD must be incorporated in order to analyze and elucidate the mechanisms of GSD.

In the current study, we hypothesized that increased light availability to enhance the source–sink ratio would also promote GSD. To examine this hypothesis, we incorporated thinning and shade removal at R1 and R5 stages to alter the light environment and availability. Studies on altered light conditions have been previously conducted with soybean (Hayati et al., 1995; Mathew et al., 2000; Schou et al., 1978). Hayati et al. (1995) reported the effect of shading from R1 to R5 on the leaf chlorophyll content until R7 stage as the indicator of leaf senescence and discussed that increased photosynthesis did not accelerate leaf senescence. However, they did not mention about GSD occurrences, which must be evaluated by conditions of both leaves and stems at R8 stage. Thus, to our knowledge, this study is the first to examine the relationship

between increased light availability and occurrence of GSD in soybean. We also discuss the experimental advantages of thinning and shade removal in field research compared to other treatments used for studying GSD. We believe these can be effectively used to analyze and elucidate the mechanism of GSD.

2. Materials and methods

2.1. Plant materials and experimental site

Two experiments were conducted in the experimental fields of NARO, Western Region Agricultural Research Center, Hiroshima, Japan (lat. 34°30′N, long. 133°23′ E, and 2 m elevation; Typic Fluvaquents soil type) in 2014, 2015, and 2016. The leading soybean cultivar at this site 'Sachiyutaka' was used in all the experiments. Exp. 1 during all years were conducted in a field where the ground water level was maintained at 30 cm below the ground surface by a farm-oriented enhanced aquatic system, FOEAS (Wakasugi & Fujimori, 2009). Exp. 2 was conducted without irrigation.

2.2. Treatments

2.2.1. Exp. 1: thinning at R1 or at R5 stages

Exp. 1 was conducted in 2014, 2015, and 2016. The sowing dates were 25 June 2014, 24 June 2015, and 27 June 2016 (Table 1). There were eight plots each (four treatments and two replications) in 2014 and 2015, and four plots (two treatments and two replications) in 2016. The size of each plot was 3.6 m × 3.0 m in 2014, 3.0 m × 3.3 m in 2015, and 3.0 m × 2.1 m in 2016. Planting density was either sparse (0.6 m row and 0.3 m plant spacing; 5.56 plants m^{-2}) or dense (0.3 m row and 0.15 m plant spacing; 22.2 plants m^{-2}). In addition to the plots in which plant population densities were maintained as sparse or dense from sowing until R8 stage, there were also plots in which plant population density was changed from dense to sparse

Table 1. Dates of growth stage and treatments in Exp. 1.

Year	Treatment[a]	The date of the growth stage or treatment					
		Sowing	R1	Thinning at R1	R5	Thinning at R5	R8
2014	Sparse	25 June	9 August	–	5 September	–	25 October
	R1	25 June	10 August	11 August	4 September	–	25 October
	R5	25 June	10 August	–	6 September	11 September	25 October
	Dense	25 June	10 August	–	5 September	–	24 October
2015	Sparse	24 June	4 August	–	31 August	–	22 October
	R1	24 June	5 August	7 August	31 August	–	21 October
	R5	24 June	6 August	–	31 August	4 September	22 October
	Dense	24 June	5 August	–	1 September	–	24 October
2016	R5	27 June	4 August	–	28 August	5 September	2 November
	Dense	27 June	4 August	–	29August	–	1 November

Notes: [a]Sparse, plant population density was kept at 5.56 plants m^{-2}. R1, thinning was conducted at R1. R5, thinning was conducted at R5. Dense, plant population density was kept at 22.2 plants m^{-2}.

by performing thinning activities. Thinning involved cutting-off all the above-ground parts of the plant in every other row of the plot, and of every other plant in the remaining rows. In 2014, thinning was conducted on either 11 August or on 11 September, corresponding approximately to R1 and R5 stage (Table 1). In 2015, thinning was conducted on 7 August (R1) or 4 September (R5) (Table 1). In 2016, there were two plots, in which either thinning was conducted at R5 (5 September), or the plant population density was maintained as dense (Table 1). All data were recorded by sampling individual plants randomly selected from each plot, and excluding plants on the border of the plot. The number of plants selected was 12 or 11 in 2014, and six in 2015 and 2016. The value for each plot was the average score of the recorded plants, and the mean of the replications was the representative score for each treatment group.

2.2.2. Exp. 2: shade removal at R1 or at R5 stages

Exp. 2 was conducted in 2015. The results of Exp. 1 indicated that thinning at R5 stage promoted the GSD occurrences. Light intensity is one of the environmental factors changed by thinning. To examine the specific effects of altered light conditions on GSD, a shading equipment in which plants were surrounded by a shade sheet to mimic the shading by neighboring plants was designed (Figure 1). The equipment was 2.1 m long, 0.4 m wide, and the height was adjustable to match with plants' height. In each plot, seven plants in a single row (plant spacing, 0.3 m) were surrounded by this shade equipment. As the central portion of 0.1 m width on the upper surface of the equipment was opened, a part of uppermost leaves was not shaded to mimic actual field situations where a part of the uppermost leaves of plants in the canopy is not shaded by neighboring plants. The shade sheets were raised as the plants became taller. When one of the uppermost leaves of the plants in the plots became 3 cm taller than the upper side of the shade equipment, the shade sheet was raised immediately above this uppermost leaf. Three types of black colored polypropylene or polyethylene shade sheets were used. Their shading strength were 16, 82, and 94% (eliminating 16, 82, and 94% of PAR in sunlight on average, respectively). The shading strength of each shade sheet was measured by SunScan Canopy Analysis System – type SS1 (Delta – T Devices Ltd, UK). We covered the PAR sensor put on the ground with the shade sheet and measured PAR. At the same time, total PAR without any shading was measured by the other PAR sensor. Shading strength was calculated by the ratio of PAR covered with shade sheet to total PAR without shading. The scores were the average of 5 measurements for each shade sheet. The sowing date was 24 June 2015 (Table 2). There were ten treatment groups

with two replications, which differed in the type of shade sheet and the timing of shade removal (Table 2). In some plots, the shade sheet was removed on 4 August (R1) or 1 September (R5), corresponding to the date of thinning in Exp. 1. In addition, there were the plots shaded until R8 stage or not shaded for the entire growth period (Table 2). Data were recorded for every five plants, except two plants at the border of the plot. The value for each plot was the average score of all the plants recorded, and the mean of the replications was the representative score for each treatment group.

2.3. Measurements

Following Fehr and Caviness (1977), the dates of growth stages R1, R5, and R8 were recorded for each plant. The severity of GSD was assessed for each plant at R8 stage using the scoring method shown in Table 3, adopting the method of Furuya and Umezaki (1993), in which GSD score was assigned based on stem color and the number of green leaves left on the stem. When more than one leaflet was left on the stem, the trifoliate was counted as one green leaf left on the stem. Completely yellowed leaves left on the stem, which was rarely observed, were not counted as green leaves. A high GSD score represents severe GSD symptom in this study, although the severer GSD is, the lower the GSD score is in the study of Furuya and Umezaki (1993). After R8 stage, seed weight, pod number, and the number of total nodes in all branches and main stem were measured. The dry matter N concentration of the main stem was measured using Vario MAX CN (Elementar Analysensysteme GmbH, Germany), except in Exp. 1 in 2014. The sixth to eighth nodes or the seventh to ninth nodes counted from the cotyledonary node of the main stem were sampled because the three nodes were approximately at the center of the main stem.

2.4. Statistical analysis

All experiments were conducted in a completely randomized design. Analysis of variance (ANOVA) and Tukey's test or t-test were used to test the differences in values and compare the means among the treatment groups ($p < 0.05$ or $p < 0.01$). GSD scores were analyzed after Box-Cox transformation. GSD ratio (ratio of the number of plants with a certain GSD score to that of all plants examined in each plot) were analyzed after angular transformation. Correlation coefficient of GSD score and N concentration of the main stem for each plant sampled was calculated. All the analyses were performed using the statistical software, BellCurve for Excel (Social Survey Research Information Co., Ltd.).

a.
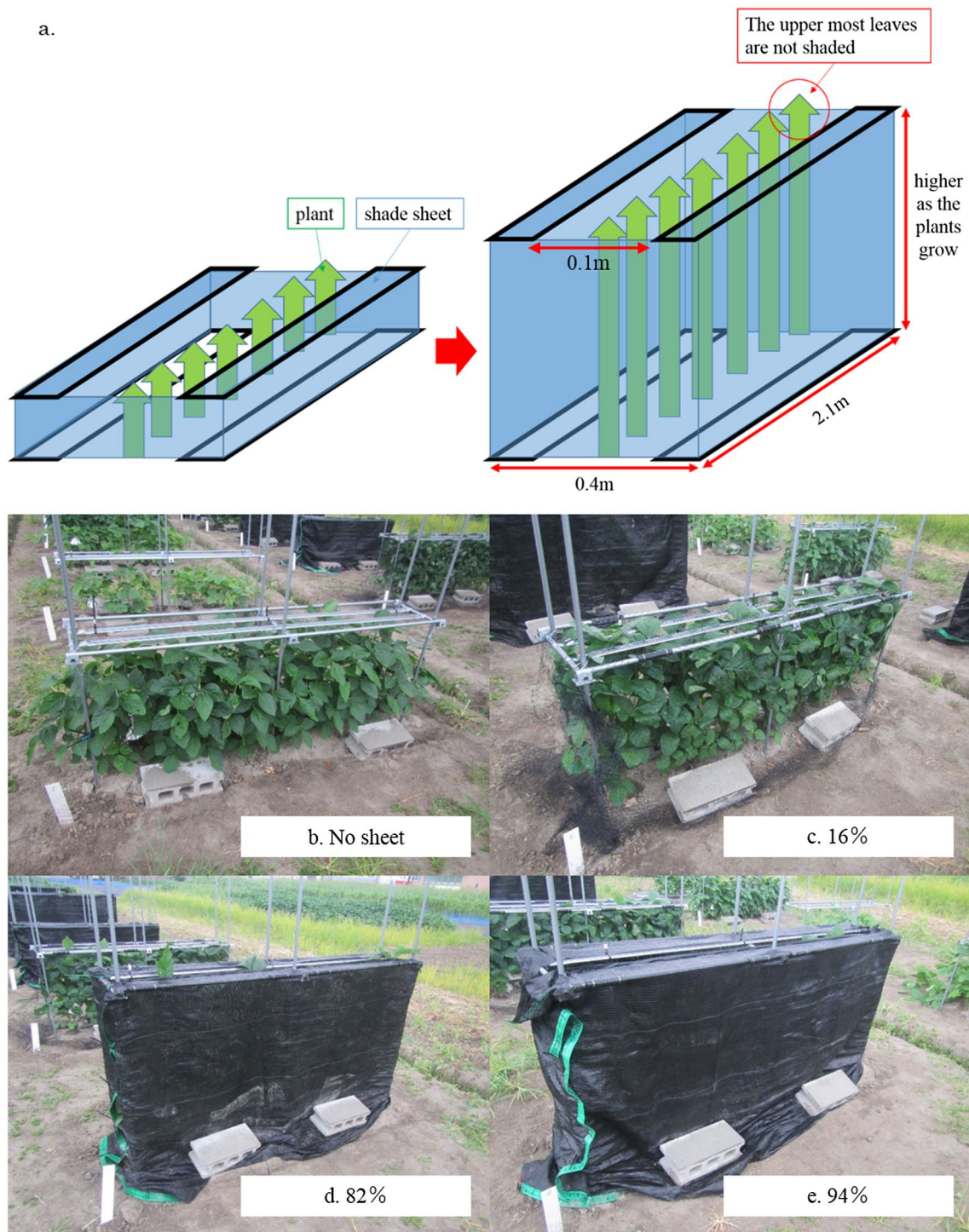
Figure 1. The shade equipment used in Exp. 2. (a) The concept of shading by the shade equipment. The equipment was 2.1 m long and 0.4 m wide. The shade sheet surrounded seven plants planted in a single row, except for their uppermost leaves. The shade sheet can be elongated as the plant grows. (b to e) The appearance of the shade equipment in the experimental field with (b) no sheet, (c) 16% shade sheet (eliminating 16% of PAR from sunlight on average), (d) 82% shade sheet (eliminating 82% of PAR from sunlight on average), and (e) 94% shade sheet (eliminating 94% of PAR from sunlight on average).

3. Results

3.1. Exp. 1: thinning at R1 or at R5 stages

3.1.1. Plant appearance at maturity

The appearance of representative plants from Exp. 1 in 2014 at maturity is shown in Figure 2. The plants from the continuously dense population (22.2 plants m^{-2}) or the continuously sparse population (5.56 plants m^{-2}) showed no GSD symptoms: all leaves had fallen and the stems turned brown and dried, even though the number of pods was less, and the stems were thinner and longer in the dense population than in the sparse population. The plants

Table 2. Dates of growth stage and treatments in Exp. 2.

Treatment[a]							
Shade sheet	Stage of shade removal	Sowing	R1	Shade removal at R1	R5	Shade removal at R5	R8
None	–	24 June	3 August	–	28 August	–	25 October
16%	R1	24 June	2 August	4 August	25 August	–	24 October
	R5	24 June	3 August	–	27 August	1 September	25 October
	R8	24 June	3 August	–	27 August	–	25 October
82%	R1	24 June	1 August	4 August	24 August	–	25 October
	R5	24 June	2 August	–	24 August	1 September	17 October
	R8	24 June	2 August	–	24 August	–	18 October
94%	R1	24 June	1 August	4 August	24 August	–	22 October
	R5	24 June	2 August	–	24 August	1 September	18 October
	R8	24 June	2 August	–	24 August	–	20 October

Notes: [a]None means that plants were not surrounded by a shade sheet. 16, 82, and 94% mean that plants were surrounded by a shade sheet eliminating 16, 82, and 94% of PAR on average, respectively.

Table 3. The scoring method used to evaluate the severity of GSD, following Furuya and Umezaki (1993).

GSD score	The appearance of the plant at R8
5	The stem is green and green leaves remain at more than one-third of all the nodes of the plant.
4	The stem is green and green leaves remain at fewer than one-third of all the nodes of the plant.
3	The stem is green or yellow-green and no green leaves remain.
2	The stem is yellow and retains some moisture, and no green leaves remain.
1	The stem is brown and dry, and no green leaves remain.

Notes: GSD score was judged based on the criteria in this table at R8 for each plant. A high score means the plant shows severe GSD symptoms.

thinned at R1 stage showed GSD symptoms (GSD score 3): yellow-green stems with no leaves. The plants thinned at R5 stage showed severe GSD symptoms (GSD score 4): green stems and some remaining leaves.

3.1.2. Severity of green stem disorder

GSD scores of the treatments thinned at growth stage R5 were significantly higher (3.6 to 4.0) than those of the other treatments in the three years (Table 4). Mean GSD score in 2014 and 2015 of the treatment thinned at growth stage R1 was significantly higher than those of the treatment in which plant population was kept dense or sparse and

Figure 2. Appearance of representative plants after R8 in the four treatment plots in Exp. 1 in 2014.
Notes: Sparse means plant population density was kept at sparse (5.56 plants m^{-2}) from sowing to harvest. Thinning at R1 means plant population density was changed from dense (22.2 plants m^{-2}) to sparse by thinning on 11 August (R1). Thinning at R5 means plant population density was changed from dense to sparse by thinning on 8 September (R5). Dense means plant population density was kept dense from sowing to harvest.

Table 4. The effects of thinning on GSD and plant characteristics in Exp. 1.

Year	Treatment[a]		GSD score[b]	GSD ratio (%)[c]			N (mg g^{-1})[d]	Leaf number left at R8 per node[e]	Seed weight (g plant^{-1})	Pod number per node	Total node number per plant
				≥3	≥4	=5					
2014	Sparse		2.4b	39b	5b	0	nd	nd	73a	1.8ab	62a
	R1		2.8b	71b	13b	0	nd	nd	69a	1.9a	55ab
	R5		4.0a	100a	96a	0	nd	nd	33b	1.4bc	35ab
	Dense		2.4b	50b	13b	4	nd	nd	25b	1.2c	34b
	ANOVA		**	**	**	ns	nd	nd	**	*	*
2015	Sparse		2.3b	50b	0b	0	7.2b	0.000b	50a	1.4	71a
	R1		2.8b	67b	8ab	0	7.3b	0.004b	39a	1.6	44b
	R5		3.7a	100a	67a	0	18.5a	0.119a	19b	1.1	27c
	Dense		2.3b	42b	8ab	0	6.0b	0.004b	13b	0.9	29c
	ANOVA		**	**	*	ns	**	**	*	ns	**
mean of 2014 and 2015	Sparse		2.4c	45b	4b	0	nd	nd	62a	1.6ab	67a
	R1		2.8b	69b	16b	0	nd	nd	54a	1.7a	50b
	R5		3.8a	100a	68a	0	nd	nd	26b	1.2bc	31c
	Dense		2.4c	46b	16b	2	nd	nd	19b	1.0c	31c
	ANOVA	Treatment	**	**	**	ns	nd	nd	**	**	**
		Year	*	ns	*	ns	nd	nd	**	**	ns
		Interaction	ns	ns	ns	ns	nd	nd	ns	ns	ns
2016	R5		3.6	100	58	0	11.4	0.06	34	1.8	31
	Dense		2.3	25	8	0	3.6	0.002	24	1.4	36
	t-test		**	**	ns	ns	ns	ns	ns	ns	ns

Note: Within columns in each year, means followed by the same letter are not significantly different according to Tukey's test (0.05).

**Significant at the 0.01 probability level; *Significant at the 0.05 probability level. ns, nonsignificant at the 0.05 probability level. nd, no data.

[a]Sparse, plant population density was kept at 5.56 plants m^{-2}. R1, thinning was conducted at R1. R5, thinning was conducted at R5. Dense, plant population density was kept at 22.2 plants m^{-2}.

[b]ANOVA, Tukey's test, and t-test were conducted after Box-Cox transformation.

[c]GSD ratio indicates the ratio of the number of plants with GSD score ≥3, ≥4, or =5 to the number of all plants sampled in the plot. ANOVA, Tukey's test, and t-test were conducted after angular transformation.

[d]N (mg g^{-1}) is dry matter N concentration in main stem.

[e]Leaf number left at R8 per node is the number of green leaves left at R8 divided by total node number, which was calculated for each plant.

was significantly lower than that of the treatment thinned at R5.

GSD ratio (≥3) of the treatments thinned at R5 were significantly higher (100.0%) than those of the other treatments in the three years (Table 4). GSD ratio (≥4) of the treatments thinned at R5 was significantly higher than those of the other treatments in 2014 and in the mean of 2014 and 2015. There were no significant differences in GSD ratio (=5) among any treatments in every year (0–4%).

The number of leaves remaining at the R8 stage per node, which is one of the measures used to score GSD, showed similar tendency to GSD scores. In Exp. 1 in 2015 (Table 4), groups thinned at R5 stage (0.119) had significantly higher values than the other groups with a low GSD score (0.000–0.004).

3.1.3. N concentration in the main stem

In Exp. 1 in 2015 (Table 4), N concentration of the main stem at maturity in the group thinned at R5 stage was significantly higher than that in the other groups. In Exp. 1 in 2016 (Table 4), the group thinned at R5 stage also tended to have higher N concentration than that in the dense group ($p = 0.067$). In 2015 and in 2016, N concentration of the main stem showed a significantly positive correlation to GSD scores for each recorded plant (Table 6).

3.1.4. Seed weight, pod number per node, and total node number

In Exp. 1 (Table 4), seed weight tended to increase as the sparse planting period became longer in the three years. Pod number per node and total node number tended to be higher in the group thinned at R1 than the group thinned at R5 in 2014 and in 2015.

3.2. Exp. 2: shade removal at R1 or at R5 stages

3.2.1. Severity of green stem disorder

In Exp. 2 (Table 5), the GSD scores of the treatments of 82% shade removal at R5 and of 94% shade removal at R5 and R8 (4.1–4.5) were significantly higher than that of no shading in treatment (2.6). There were no significant differences in GSD scores among the other treatments (2.7 to 3.3) and the no shading treatment. GSD ratio (≥4) showed the same tendency as those of GSD score. In GSD ratio (≥3), the treatments of 16% shade removal at R5 and of 82% shade removal at R1 also showed significantly higher GSD ratio (100%) than the no shading treatment. There were no significant differences among treatments in GSD ratio (=5).

In Exp. 2 (Table 5), the number of leaves remaining at R8 stage per node of the high GSD score groups (82% shade removal at R5 and 94% shade removal at R5 and 94%

Table 5. The effects of shade removal on GSD and plant characteristics in Exp. 2.

Treatment			GSD ratio (%)[c]							
Shade sheet[a]	Stage of shade removal	GSD score[b]	≥3	≥4	=5	N (mg g^{-1})[d]	Leaf number left at R8 per node[e]	Seed weight (g plant^{-1})	Pod number per node	Total node number per plant
None	–	2.6d	50b	10b	0	4.9cd	0.005c	150a	2.4abc	98a
16%	R1	2.7cd	70b	10b	0	4.6cd	0.001c	134ab	2.4bcd	91a
	R5	3.2bcd	100a	20b	0	9.2cd	0.008c	121ab	2.1cd	82a
	R8	2.7cd	60b	10b	0	6.3cd	0.001c	106bc	2.1cd	82a
82%	R1	3.3abcd	100a	30b	0	8.8cd	0.026bc	108bc	3.1a	56b
	R5	4.2ab	100a	100a	20	22.4b	0.191abc	47de	2.0cd	43bc
	R8	2.8cd	70b	10b	0	4.1d	0.002c	38e	1.8de	45bc
94%	R1	2.7cd	70b	0b	0	7.7cd	0.000c	80cd	2.9ab	42bc
	R5	4.5a	100a	100a	50	29.3a	0.368a	13e	1.3e	24c
	R8	4.1abc	100a	100a	10	10.3c	0.221ab	14e	1.2e	27c
	ANOVA	**	**	**	ns	**	**	**	**	**

Note: Within columns means followed by the same letter are not significantly different according to Tukey's test (0.05).
**Significant at the 0.01 probability level. ns, nonsignificant at the 0.05 probability level.
[a]None means that plants were not surrounded by a shade sheet. 16, 82, and 94% mean that plants were surrounded by a shade sheet eliminating 16, 82, and 94% of PAR from sunlight on average.
[b]ANOVA, Tukey's test, and t-test were conducted after Box-Cox transformation.
[c]GSD ratio indicates the ratio of the number of plants with GSD score ≥3, ≥4, or = 5 to the number of all plants sampled in the plot. ANOVA, Tukey's test, and t-test were conducted after angular transformation.
[d]N (mg g^{-1}) is dry matter N concentration in main stem.
[e]Leaf number left at R8 per node is the number of green leaves left at R8 divided by total node number, which was calculated for each plant.

Table 6. Correlations between GSD score and dry matter N concentration in the main stem for each recorded plant.

	Exp. 1 (2015)	Exp. 1 (2016)	Exp. 2 (2015)
Experiment (Year)	$n = 24$	$n = 72$	$n = 100$
r	0.68	0.73	0.68
Significance test	**	**	**

Note: **Significant at the 0.01 probability level.

continuous shading until R8) tended to be higher than those of all other low GSD score groups.

3.2.2. N concentration in the main stem

In Exp. 2 (Table 5), N concentrations in the main stem at maturity in the two high GSD score groups shaded until R5 stage with the 82 and 94% shade sheet was 22.4 and 29.3 mg g^{-1}, respectively. These were significantly higher than the values obtained for the other groups (4.1–10.3 mg g^{-1}). Although the group shaded until R8 stage with the 94% shade sheet was one of the groups with a high GSD score, this group had lower N concentration (10.3 mg g^{-1}) than the other two high GSD score groups.

N concentration of the main stem showed a significantly positive correlation to GSD scores for each recorded plant (Table 6).

3.2.3. Seed weight, pod number per node, and total node number

In Exp. 2 (Table 5), seed weight with no shade sheet during the whole growth period was the highest (150 g plant^{-1}). The seed weight tended to decrease as the shading period and intensity increased. In any shade sheets, pod number per node and total node number tended to be higher in the group the shade of which was removed at R1 than that at R5 stage.

4. Discussion

4.1. GSD score and delayed leaf and stem senescence characterize GSD

Harbach et al. (2016) reported that in soybean, delayed senescence symptoms, including GSD, are manifested through various combinations of delayed maturation of the stem, leaves, and pods, but these symptoms have not been adequately distinguished from each other. In the current study, GSD was primarily evaluated using the GSD score, which was based on the color of the stem and the ratio of existing leaves at R8 stage (when almost all pods have matured), as in previous studies (Fujii et al., 2015; Furuya & Umezaki 1993; Isobe et al., 2015; Mochizuki et al., 2005; Takehara et al., 2016; Tsujimoto et al., 2006; Yamada et al., 2014). In both the present experiments, the developmental progression of the reproductive growth stage (days from sowing to R1, R5, and R8 stages) were not significantly different (Tables 1 and 2), suggesting that GSD in our experiments was characterized not by the accelerated maturation of the pods, instead by delayed maturation of the leaves and stems. We also found that the GSD score was positively and significantly correlated to the N concentration of the main stem in all experiments (Table 6). Previous study has shown that depodded GSD soybean plants tend to have high dry matter N concentration in the stem (Egli & Bruening, 2006). Taken together the

above-mentioned results suggest that GSD scores properly represent GSD symptom, and that GSD in this study is characterized mainly by delayed leaf and stem senescence.

4.2. Improved light availability at R5 stage promotes GSD

Depodding is the most frequently used treatment in research of leaf and stem senescence, including GSD research (Crafts-Brandner & Egli, 1987; Crafts-Brandner et al., 1984; Egli & Bruening, 2006; Htwe et al., 2011; Leopold et al., 1959; Mondal et al., 1978; Wittenbach, 1982), which decreases sink size and then increases the source–sink ratio. In this study, a novel approach was employed to increase the source–sink ratio.

During the three years of Exp. 1, thinning at R5 stage following dense cultivation showed a significantly higher GSD score and GSD ratio (≥3) than that in a continuously dense cultivation (Table 4). In Exp. 2, removal of the 82% shade sheet at R5 stage that mimicked improved above-ground light conditions resulting from thinning produced a significantly higher GSD score or higher N concentration in the main stem compared to that in continuous shading until R8 stage (Table 5). These results suggest that increased light availability at R5 stage promotes GSD, probably by enhancing the source relative to the sink. This finding just reconfirms the results of previous studies in which the sink was reduced in order to promote GSD (Crafts-Brandner & Egli, 1987; Crafts-Brandner et al., 1984; Egli & Bruening, 2006; Htwe et al., 2011; Leopold et al., 1959; Mondal et al., 1978; Wittenbach, 1982). However, to our knowledge, it has never been reported that the enhanced source also promoted GSD.

4.3. Difference in treatments at R1 and R5 stages

In the context of relative increases in source-to-sink, it is also notable that the effects of thinning and shade removal on GSD were significantly stronger at R5 than at R1 stage as was shown in the GSD score in 2014 and 2015 (Table 4) and of the GSD ratio (≥4) (Table 5). Schou et al. (1978) reported that light enrichment before R5 stage resulted in higher yield compared to that after R5 stage. It was reported that source restriction by defoliation or shading determine pod and seed number between R1 and 10–12 days after R5 (Board & Tan, 1995) or between R1 and 14–21 days after R5 (Egli, 2010). Thus, one important difference between the plants at R1 and R5 stages is their ability to increase the number of pod per plant in response to improved light environment.

Stephenson and Wilson (1977) demonstrated that carbon assimilated in a leaf basically accumulated in pods in the axil of the leaf. Nobuyasu et al. (2003) showed that the involved leaf and pods collectively formed a source–sink unit with the subtending internode. Therefore, it would be important to consider a source–sink balance on the basis of the source–sink unit. The number of pod per node at R8 stage can serve as a good estimate for an averaged sink size per source–sink unit after R5 stage, which was higher in the groups thinned or shade-removed at R1 than at R5 stage (Tables 4 and 5). Meanwhile, an averaged source ability per source–sink unit after R5 stage would be no higher in the groups treated at R1 than at R5 stage because of mutual shading provided by the leaves themselves. The degree of mutual shading could be rationally estimated based on the total node number per plant, which tended to be higher in the groups treated at R1 than at R5 stage (Tables 4 and 5). Therefore, the source–sink ratio based on the source–sink unit after R5 stage could be lower in the group treated at R1 stage than that at R5 stage, leading to differences in GSD between treatments at R1 and R5 stages.

4.4. Continuous strong shading also promotes GSD-like symptom

The groups with no change in the light environment tended to have low GSD scores, except under the 94% shade sheet in Exp. 2 (Table 5). The continuously shaded group with high GSD score also showed a different trend in terms of N concentration in the main stem, with a significantly lower N concentration than that in the other plants with a high GSD score in Exp. 2. The reason for this result remains to be elucidated. Strong shading above certain levels (such as the 94% shade sheet, eliminating 94% of PAR) may also enhance GSD-like symptom without altering the N status, as seen in Exp. 2. This result is in line with those of previous studies in which dark incubation of whole *Arabidopsis* plants delayed senescence, while dark incubation of individual leaves promoted senescence (Rolland et al., 2006; Weaver & Amasino, 2001). Although the underlying mechanism of delayed senescence in strongly shaded soybean plants and dark-incubated *Arabidopsis* plants needs to be elucidated, the mechanism described seems to be different from that in naturally occurring GSD.

4.5. Thinning and shade removal as experimental methods to reproduce GSD symptoms

The treatment used in this study, particularly thinning, is a simpler, less time/labor consuming method than depodding, and is especially valuable for field experiments and elucidating the precise mechanism of GSD. Depodding causes injury stresses due to the cutting of pods, whereas thinning or shade removal does not. Turner et al. (2012) reported that sink limitation by cutting organs in soybean

induces the expression of stress response genes and the metabolic shifts involved in abiotic or biotic stresses in distant leaves. They suggested considering the unintended consequences due to cutting the organs.

Thinning and shade removal may also be useful for breeding GSD insensitive cultivars. In breeding and QTL analysis, many comparisons of the occurrence of GSD in each line or cultivar are needed. However, it is difficult to accurately identify GSD insensitive lines and cultivars, because the occurrence of GSD largely varies by location and year (Fujii et al., 2015; Hill et al., 2006). Given that thinning and shade removal can stably promote the occurrence of GSD, these treatment techniques may be useful as a means to produce GSD symptoms in experimental lines and cultivars, particularly in localities or during years with extremely low occurrence of GSD.

5. Conclusions

The improved light availability at R5 stage by thinning or shade removal promoted the occurrence of GSD. These results suggested that enhanced source relative to sink promotes GSD. Especially, thinning is the easiest experimental technique to reproduce GSD symptoms. Thus, thinning is expected to be used for studying the precise mechanism of GSD and for breeding of GSD insensitive cultivars.

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Disclosure statement

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