Published August 18, 2016

RESEARCH

Variation in Seed Protein Concentration and Seed Size Affects Soybean Crop Growth and Development

Florencia Poeta, Lucas Borrás, and José L. Rotundo*

ABSTRACT

Developing high protein (HP) cultivars is often precluded by the inverse relationship between protein and yield. We hypothesized that attaining HP concentration based on contrasting seed size impacts crop growth and development differently. We screened 97 soybean genotypes and found lines with HP concentration (~450 g kg^{-1}) associated with (i) increased protein content (mg $seed^{-1}$) in large seed genotypes, and (ii) reduced oil and carbohydrate contents in small seed ones. Then, we evaluated different growth traits in a subset of three HP large and three HP small seed genotypes, as well as in three high-yielding genotypes with average seed size and protein concentration. High-yielding genotypes showed higher leaf area duration and harvest index when compared with HP genotypes, regardless of seed size. High protein large seed was associated with more assimilate availability per seed during seed filling, while HP small seed showed higher leaf area at the beginning of seed fill, more canopy biomass production, and very low levels of assimilate per seed. Results show that selecting for seed protein concentration can impact crop growth and development differently, depending on the strategy used for selection in terms of seed size. These findings, if utilized for parental selection, might contribute to eliminating negative correlations between seed protein and yield, since these strategies may be under different genetic control and/or determine different biophysical constraints.

F. Poeta, L. Borrás, and J.L. Rotundo, Dep. de Producción Vegetal, Facultad de Ciencias Agrarias, Univ. Nacional de Rosario, S2125ZAA, Zavalla, Santa Fe, Argentina. Received 14 Jan. 2016. Accepted 23 June 2016. *Corresponding author (jrotundo@unr.edu.ar). Assigned to Associate Editor Zenglu Li.

Abbreviations: HI, harvest index; HP, high protein; LAI, leaf area index; LAI_{R5}, leaf area index at the beginning of seed filling; $LAI_{perSeed}$, LAI_{R5} per unit seed; LAD, leaf area duration; QTL, quantitative trait loci; SenCoef, leaf senescence coefficient; SFD, duration of linear phase of seed filling; SGR, individual seed growth rate; $TotBio_{R7}$, total biomass at maturity.

Primary soybean [*Glycine max* (L.) Merr.] processing involves oil extraction by solvent or pressure and the subsequent production of protein meal (Lusas, 2004). High protein meal, used for animal feeding, can only be achieved with seed protein concentrations above 380 g kg^{-1} (dry weight basis) (Hurburgh, 1994; Brumm and Hurburgh, 2006). However, a problem that international soybean markets currently face is seed protein deficits (Dardanelli et al., 2006; Naeve and Huerd, 2008; Medic et al., 2014; Rotundo et al., 2016). These protein deficits can preclude the production of HP meal required for profitable marketing. Regardless of cultivar selection, factors associated with reduced seed protein include different environmental conditions and management practices (Medic et al., 2014). For example, high yields determined by early planting dates or abundant water availability during seed filling are usually associated with reduced protein concentration (Bastidas et al., 2008; Rotundo and Westgate, 2009; Bellaloui et al., 2011). Also, reduced temperature at higher latitudes is associated with lower protein (Naeve and Huerd, 2008; Rotundo et al., 2016). One possible avenue to offset environmentally- and/or management-induced seed protein deficits is to increase seed protein concentration of commercial cultivars.

Published in Crop Sci. 56:1–13 (2016).
doi: 10.2135/cropsci2016.01.0025

Selection for high seed protein concentration is feasible, but the development of competitive commercial cultivars with superior protein concentration is hampered by the negative correlation between protein and yield (Brim and Burton, 1979; Carter et al., 1982; Wilcox and Zhang, 1997; Wilcox and Shibles, 2001). Different physiological explanations of this negative correlation have been proposed. Sinclair and de Wit (1975, 1976) originally suggested that yield limitations are related to the high seed N requirements of HP genotypes that determine accelerated leaf senescence due to N remobilization, shortened seed filling, and faster assimilate partitioning to the seed. While many studies agree with this self-destructive hypothesis (e.g., Salado-Navarro et al., 1985; Leffel et al., 1992) others propose that higher seed protein does not affect the normal growth and development of the crop. For example, Egli and Bruening (2007b) showed no evidence that selection for HP concentration affected N remobilization, leaf senescence, or seed filling duration (SFD). On the other hand, lower yields of HP cultivars were also associated with increased assimilate supply per seed during seed filling required to fill large seeds with HP concentration (Rotundo et al., 2009, 2011). Increased assimilate availability per seed is commonly a consequence of reductions in seed number, rather than the result of actual increases in assimilate supply during seed filling (Rotundo et al., 2009, 2011). This effect is responsible for yield limitations observed in large seed, HP concentration genotypes.

Seed protein concentration (g kg^{-1}) is a mathematical ratio between protein content (mg $seed^{-1}$) and total seed weight (the sum of protein, oil, and carbohydrates; Ishii et al., 2010). Therefore, high seed protein concentration can be achieved by (i) more-than-proportional increases in seed protein content (mg $seed^{-1}$) relative to increases in oil and carbohydrate content in large seeds, or (ii) more-than-proportional reductions in oil and/or carbohydrate content relative to protein content reductions in small seeds. Studies dealing with soybean protein concentration at the physiological or genetic level have ignored seed size as a factor (e.g., Brim and Burton, 1979; Salado-Navarro et al., 1985; Wilcox and Zhang, 1997; Cober and Voldeng, 2000; Egli and Bruening, 2007b), or showed a positive correlation between seed size and protein (Alt et al., 2002; Panthee et al., 2005; Rotundo et al., 2009). The prospect of attaining HP concentration via small seeds and its possible impact on crop functioning have been ignored. We hypothesize that contradictory results regarding the impact of seed protein concentration on crop growth and development (e.g., Salado-Navarro et al., 1985; Egli and Bruening, 2007b) could be related to this ignored seed size effect.

The objectives of our study were to (i) assess whether equivalent HP concentration can be achieved in genotypes with large and small seed size, and (ii) test how differing seed size in HP concentration genotypes impacts several growth and development traits. Evaluated traits were (i) yield, seed number, canopy biomass at maturity, and harvest index (HI); (ii) green leaf area during grain filling (Salado-Navarro et al., 1985; Leffel et al., 1992); (iii) seed growth rate and duration (Egli and Bruening, 2007b); and (iv) assimilate supply per seed during seed filling (Rotundo et al., 2009).

MATERIALS AND METHODS

Two field experiments were conducted at the Campo Experimental Villarino, Facultad de Ciencias Agrarias, Universidad Nacional de Rosario, in Zavalla, Argentina. Soil type was a silty clay loam (Vertic Argiudoll, Roldán series). Soil water availability at sowing, rainfall during the growing season, and mean temperature are reported as supplemental information (Supplemental Table S1).

Screening for Increased Seed Protein Concentration in Small and Large Seed Soybeans

We selected 97 genotypes from the USDA Germplasm Resources Information Network (GRIN) and other sources (Supplemental Table S2). Selection criteria included that the majority of cultivars belonged to maturity groups III and V (plus two maturity VI cultivars), reported yields were higher than 1500 kg ha^{-1}, and protein concentration was higher than 410 g kg^{-1} (dry weight basis). Selected maturity groups are adapted to the experimental site latitude. We evaluated these 97 selected genotypes in the 2010 to 2011 growing season at two sowing dates (6 Dec. and 27 Dec.). Planting dates were used as environmental replication for testing genotypic differences under conditions that are known to severely affect seed protein concentration (Medic et al., 2014). Plots were three rows, 1 m long, with a row spacing of 0.52 m. Plots were overseeded and thinned to a final stand density of 30 plants m^{-2} at V1. Weeds were chemically controlled at sowing and hand removed afterward. Pests and diseases were controlled by spraying recommended insecticides and fungicides.

Physiological maturity (R7, Fehr and Caviness, 1977) was determined on a plot basis three times per wk on 20 consecutive plants in the center row. At R7, 0.75 m of the central row was hand clipped and threshed with a stationary combine. Protein concentration was estimated by NIRS (Near Infrared Spectroscopy, Infratec 1241, Foss, Denmark) and expressed on a dry weight basis. The NIRS equipment had calibration equations developed by the University of Rosario in cooperation with Foss Argentina. The NIR calibration contained green seed coat genotypes. Seed dry weight (mg $seed^{-1}$) was calculated using 200-seed samples dried at 60°C for 5 d. Protein content (mg $seed^{-1}$) was calculated by multiplying individual seed dry weight by protein concentration.

The experimental design was a split plot with three blocks. Planting dates were the main plots, and genotypes the subplots. Data were analyzed using the general linear model (GLM) procedure of SAS 9.1 (SAS Institute, 1999). The statistical model included block, sowing date, and genotype as main factors. Differences at the 0.05 probability level were considered significant. Multiple comparisons among means were performed using a LSD test.

High Seed Protein Concentration in Large and Small Seed Soybeans and Its Relationship with Crop Growth and Development

Six high seed protein concentration genotypes were selected from the experiments conducted in the 2010 to 2011 growing season—three with large and three with small seed size. These six genotypes were evaluated for yield, yield components, and other relevant crop-level traits. In addition, three modern Argentinean commercial cultivars were included in the evaluation. They have high yield but average seed size and protein concentration, as reported in the National Seed Institute from Argentina. Standard high-yield cultivars were NK34, DM3100 and SPS3x1. Only three genotypes per category were included in this experiment due to the difficulty of doing a detailed ecophysiological characterization in a larger genotype set. The experiment was conducted in 2011 to 2012 and 2012 to 2013, and sowing dates were 1 November and 13 November, respectively. Stand density in both years was 35 plants m^{-2}. Plots were four rows, 5.5 m long and 0.52 m between rows. Seeds were inoculated with *Bradyrhizobium* before planting. Weeds were chemically controlled before planting and hand removed if necessary. Pests and diseases were controlled by spraying recommended fungicides and insecticides.

Phenological stages were recorded three times per wk on 20 consecutive plants per plot. Recorded stages were R1 (beginning bloom), R3 (beginning pod), R5 (beginning seed), R6 (full seed) and R7 (physiological maturity) (Fehr and Caviness, 1977). Days between R5 to R7 determined the effective SFD (Egli et al., 1981).

Aboveground biomass was sampled by hand clipping the two center rows at five different growth stages during the seed filling period: R5, R5 + 10 d, R6, R6 + 10 d, and R7. Sampled area for each sampling date was 0.52 m^2. Consecutive sampling areas had a buffer area of 0.26 m^2 to minimize border effects. Samples were separated into stem plus petioles, green leaflets, nongreen leaflets, and pods. Seed weight to pod weight ratio was determined on a 100-pod subsample on all but R7 samples. This ratio was used to determine seed weight per unit area in the pre-R7 samples. At R7, pods were threshed with a stationary combine. To correctly account for total aboveground biomass production, abscised leaves and petioles were collected with plastic nets placed from R5 until R7 (Salvagiotti et al., 2009). Under common field-growing conditions, leaf senescence before R5 accounts for <15% of leaf biomass (Hanway and Weber, 1971; Hanway et al., 1984). Therefore, placing nets at R5 ensured capturing most senesced leaves. Abscised leaves were recovered twice each wk, and this biomass was added to the harvested biomass. All samples were dried at 60°C for 5 d.

Seed yield (seed weight per unit land area at R7) was expressed on a dry weight basis. Numerical yield components (individual seed weight and seed number per area) were determined using the R7 sample. Two hundred seeds were counted and weighed to determine individual seed dry weight. Total seed number was calculated as the ratio between seed yield and individual seed weight. Harvest index was calculated as the ratio between seed yield and total plant biomass at R7 ($TotBio_{R7}$) (Donald & Hamblin, 1976). Individual seed growth rate was estimated as the ratio between individual seed weight at maturity and duration of seed filling (R5–R7) as in Egli et al. (1981).

Leaves were separated for determining leaf area index (LAI, surface of green leaves per unit ground area) during the seed filling period using a LI-3100 area meter (LI-COR, Nebraska, USA). Samples were maintained in hermetically sealed plastic bags and stored in a cold room (5°C) to avoid leaf wilting until processing. Senescence of green leaf area was estimated as a quadratic LAI decay from R5 to R7 using the following model:

$$y = a + bx^2 \quad [1]$$

where y is LAI, a is the intercept (LAI_{R5}, leaf area index at R5), b is the leaf senescence coefficient (SenCoef), and x is days after R5. The SenCoef (b) takes negative values. A more negative value implies a faster rate of green leaf area loss. The linear term in the quadratic function (Eq. [1]) was considered zero to properly model the decay in leaf area assuming maximum LAI at R5 (days after R5 = 0). Leaf area duration (LAD) was estimated by integrating Eq. [1] from R5 to R7.

Assimilate supply to the seed during seed filling was estimated following Rotundo et al. (2009), as the ratio between the parameter a in Eq. [1] (LAI_{R5}, m^2 m^{-2}) and seed number per unit land area at R7.

Seed protein and oil concentrations (g kg^{-1}) were estimated by NIRS on the R7 seed sample and expressed on a dry weight basis. The calibration contained green seed coat genotypes. Seed residual concentration (carbohydrates and ash) was estimated as the residual fraction of the seed (1000 − protein [g kg^{-1}] − oil [g kg^{-1}]). Protein, oil, and residual content (mg $seed^{-1}$) were calculated as the product between individual seed dry weight and protein, oil, or residual concentration, respectively.

For this experiment, we used a randomized complete block design with four replicates. Data were analyzed using the GLM procedure of SAS (SAS Institute, 1999). The model included year, block nested within year, seed size (standard, HP large seed, and HP small seed), and genotype nested within seed size as main factors. This genotype factor quantifies residual variation after accounting for the seed size effect. Differences were considered significant at the 0.05 probability level. Multiple comparisons between means were performed using a LSD test. A principal component analysis was conducted with all the crop physiological traits that were significantly associated with seed protein concentration strategies.

RESULTS

Screening for Strategies to Increase Seed Protein Concentration

Seed protein concentration (g kg^{-1}) and seed protein content (mg $seed^{-1}$) showed significant variability among the 97 genotypes phenotyped at both sowing dates (Fig. 1, Supplemental Table S2). The genotype × sowing date interaction was not significant ($P > 0.05$) for seed protein concentration and content (data not shown). Average protein concentration was 416 g kg^{-1} for the first sowing date and 430 g kg^{-1} for the latter date. Seed protein content ranged from 20 to 140 mg $seed^{-1}$ at the first sowing date and 30 to 120 mg

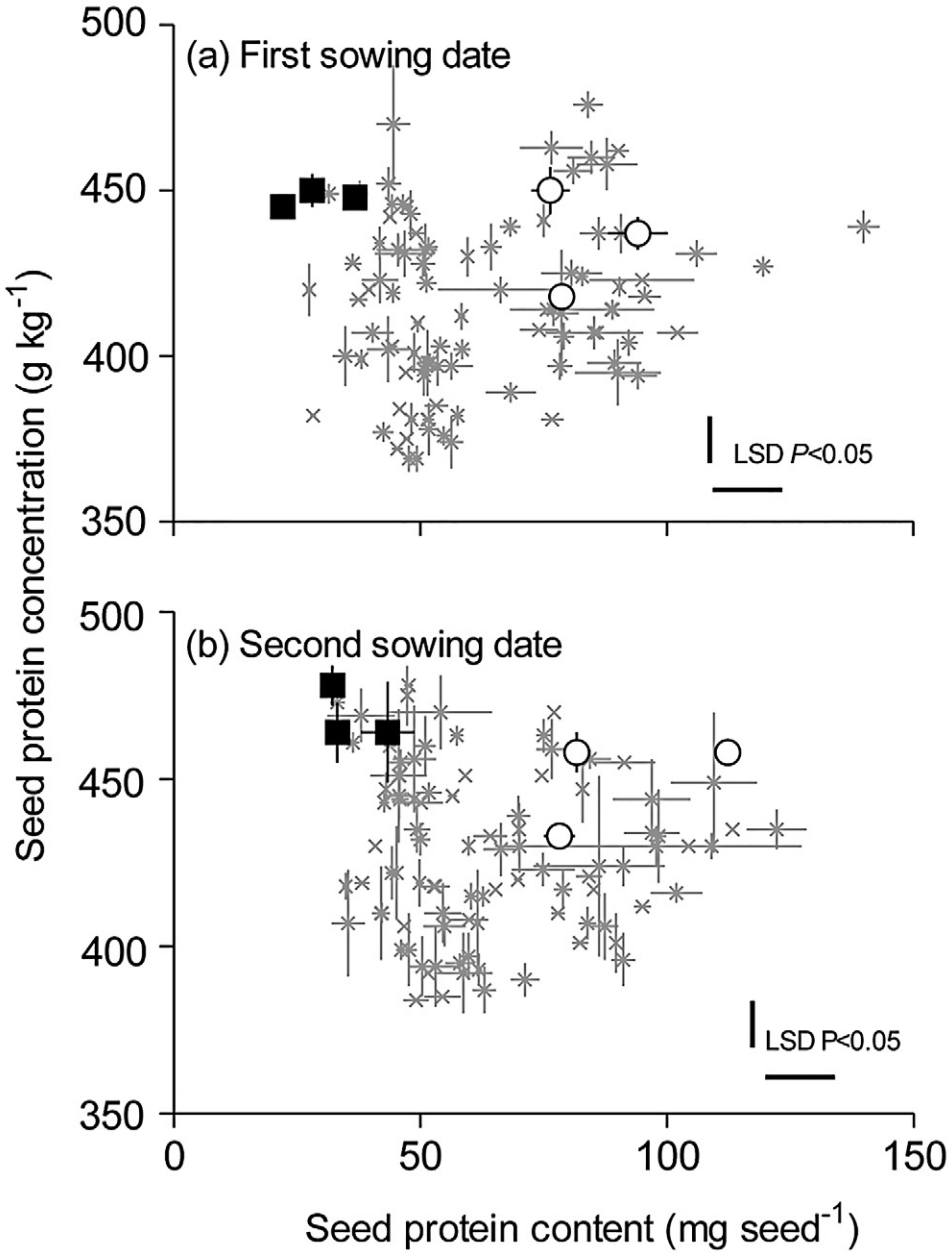

Fig. 1. Scatterplot depicting the variation in seed protein concentration and content for (a) 6 Dec. and (b) 27 Dec. planting dates. Genotypes not selected for further analysis are shown with an (x). Empty circles indicate selected high protein (HP) large seed size genotypes having increased seed protein concentration due to high seed protein content (HP large seed); full squares indicate selected HP small seed size genotypes with increased seed protein concentration based on reduced seed oil and residual contents (HP small seed). Each point is the average of three replicates ± SE. Vertical and horizontal lines show the LSD ($P < 0.05$).

seed^{-1} for the second sowing date (Fig. 1). Seed size varied from 50 to 318 mg seed^{-1} at the first sowing date and 68 to 282 mg seed^{-1} for the second sowing date

From this initial screening, we selected six genotypes with HP concentration (mg mg^{-1}) associated with either (i) HP content and large seed weight (HP large seed size) or (ii) reduced protein, oil, and residual contents and small seed weight (HP small seed size). Table 1 describes the genotypes we selected from the 97 total genotypes, and Fig. 1 displays where they fit relative to the total explored variability. The main criteria for selecting these genotypes were high seed protein concentration with contrasting low or high seed protein content (and therefore small or large seed size). Some restrictions for selection, however, were imposed by agronomic problems that were evident for some genotypes, such as lodging and disease susceptibility. Therefore, genotypes having higher seed protein concentration than the genotypes we actually selected were evident but were not used (Fig. 1). Mean seed protein concentration for the six selected genotypes was 449 g kg^{-1}.

Table 1. Seed protein concentration, seed protein content, individual seed weight, days to physiological maturity, and growth habit for six high protein (HP) genotypes having contrasting seed size selected in the 2010 to 2011 screening of 97 diverse cultivars (Fig. 1). High protein large seed genotypes have increased seed protein concentration due to high seed protein content (IA3011, PI555396, PI538376); HP small seed genotypes have high seed protein concentration based on reduced seed oil and residual content (PI518757, PI398970, PI196177). The value LSD is Fisher's protected least significant difference ($P < 0.05$). The proportion of explained sums of squares accounted for by each source of variation is denoted as %SS. Specific values for the 97 evaluated genotypes at each sowing date are included as supplementary information (Supplemental Table S2).

Source of variation		Protein concentration	Protein content	Seed weight	Days to maturity
		g kg^{-1}	mg seed^{-1}	mg seed^{-1}	d
Sowing date					
First		443	56.8	128.9	109.4
Second		459	63.5	139.4	103.2
Seed size					
HP large seed		444	86.7	195.2	105.8
HP small seed		457	32.6	71.3	108.1
Genotype					
IA3011	(S-D)†	426	78.4	185.2	104.8
PI555396	(I)	453	78.5	173.2	105.4
PI538376	(S-D)	445	101.4	227.3	107.2
PI518757	(S-D)	461	29.8	64.5	113.0
PI398970	(S-D)	454	39.4	86.5	105.6
PI196177	(D)	454	27.7	60.8	105.6

	P value (LSD)	%SS	*P* value (LSD)	%SS	*P* value (LSD)	%SS	*P* value (LSD)	%SS
Statistical significance								
Block	–	2.8	–	0.6	–	0.3	–	0.2
Sowing date (SD)	** (6)	30.0	NS	1.8	NS	0.8	NS	39.8
Seed size	** (11)	26.7	*** (5.4)	86.5	*** (11.2)	89.2	NS	5.7
SD* Seed size	NS	1.0	NS	0.1	NS	0	NS	0.1
Genotype (Seed size)	** (15)	36.0	*** (7.7)	10.1	*** (15.8)	8.7	* (5.6)	38.9
SD*Genotype (Seed size)	NS	3.4	NS	0.9	NS	0.9	NS	15.3

* Significant at 0.05 probability level.

** Significant at 0.01 probability level.

*** Significant at 0.001 probability level.

† Plant growth habit: (I) indeterminate, (D) determinate, (S-D) semideterminate.

Growth and Development of Genotypes with High Seed Protein Concentration and Large versus Small Seed Size

Seed Protein, Oil, and Residual Concentrations, Seed Size, and Component Contents

Selected genotypes showed that seed size categories, including commercial high-yielding genotypes, differed for protein, oil, and residual concentration and contents ($P < 0.01$; Fig. 2). Average seed protein concentration was 364 g kg^{-1} for commercial genotypes, 432 g kg^{-1} for HP large seed size, and 412 g kg^{-1} for HP small seed size (seed size main effect, $P < 0.05$). There were also significant differences among genotypes within each category (Fig. 2). Oil and residual concentrations were larger for the commercial genotypes (219 g kg^{-1} for oil and 417 g kg^{-1} for residual) than for the other two categories (199 and 181 g kg^{-1} for oil and 369 and 407 g kg^{-1} for residual, for HP large and small seed size, respectively). As expected, the higher protein concentration of HP genotypes was associated with increased protein content per seed in the HP large seed size, and with reductions in oil and residual contents per seed in the HP small seed size genotypes, when compared with the commercial ones.

Seed protein, oil, and residual concentrations and contents were affected by year × seed size, and year × genotype interactions ($P < 0.05$). However, these effects explained a very low proportion of variation in seed component concentration and content (<2.5% of the sums of squares). A complete description of these interactions is available in Supplemental Table S3.

Seed Yield, Biomass, and Harvest Index

As expected, commercial genotypes outyielded HP large and small seed genotypes during both years (4300 kg ha^{-1} vs. 2900 kg ha^{-1}, respectively) (Table 2). In the 2011 to 2012 growing season, HP small seed genotypes outyielded HP large seed ones, but yields were similar during the 2012 to 2013 season. Residual genotypic effects after

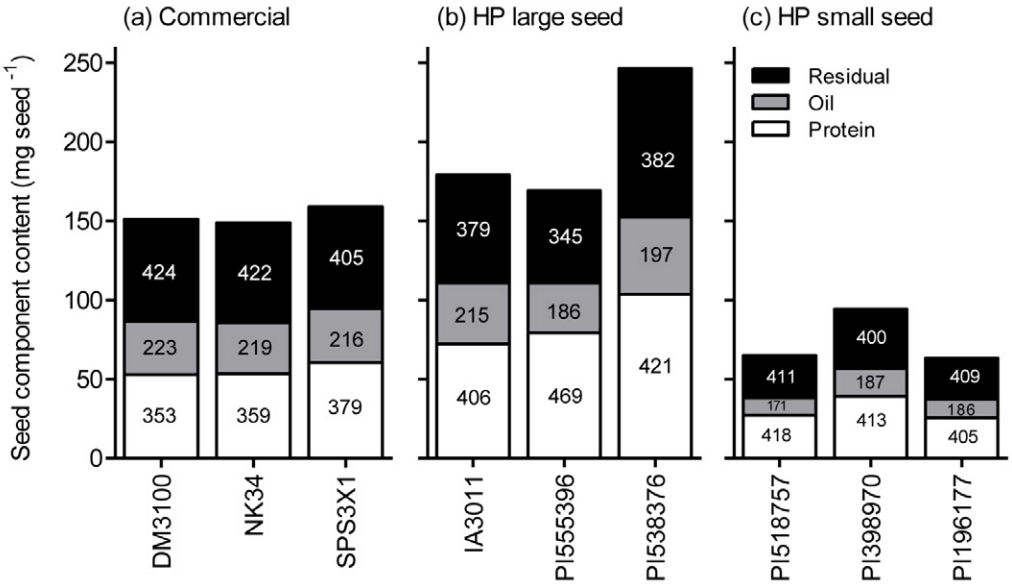

Fig. 2. Genotypic effects on seed protein, oil, and residual content for genotypes differing in seed size and composition. (a) Commercial are elite cultivars with average seed protein and seed size, (b) high protein (HP) large seed are genotypes having increased seed protein concentration due to high seed protein content, and (c) HP small seed are genotypes with high seed protein concentration based on reduced seed oil and residual content. Numbers within each bar are component concentration (g kg^{-1}) on a dry weight basis. LSD ($P <$ 0.05) for content (and concentration) values are: protein 1.6 mg seed^{-1} (4 g kg^{-1}), oil 0.7 mg seed^{-1} (2 g kg^{-1}), and residual 1.2 mg seed^{-1} (3 g kg^{-1}). Each value is the average of four replicates and two growing seasons (2011–2012 and 2012–2013).

accounting for seed size category effects were not significant (Table 2). Mean yields in seasons 2011 to 2012 and 2012 to 2013 were significantly different (2995 vs. 3845 kg ha^{-1}, respectively).

Numerical yield components, seed number, and individual seed weight were strongly associated with seed size category (Table 2). Harvested seed number m^{-2} was 2993, 1460, and 4333 for commercial, HP large seed, and HP small seed cultivars, respectively; significant genotypic differences within each strategy were also evident ($P < 0.001$, Table 2). Seed weight was 153, 196, and 74 mg seed^{-1} for commercial, HP large seed, and HP small seed cultivars, respectively. Within each seed size category, genotypic differences were also evident for HP large and small seed cultivars ($P < 0.001$, Table 2). Interactions between year × seed size and year × genotype within seed size category were statistically significant, but explained a very small proportion of total model variance (Table 2).

Total plant biomass at maturity was larger for commercial and HP small seed categories (~980 g m^{-2}) and smaller for HP large seed genotypes (~760 g m^{-2}). Harvest index was larger for commercial (~0.45) and smaller for HP large and small seed categories (~0.40) (Table 2). A significant year × seed size interaction showed that differences in total biomass between commercial and HP large seed genotypes were higher during 2011 to 2012 (270 g m^{-2} difference) than during 2012 to 2013 (160 g m^{-2} difference, Table 2). There was a significant year effect for HI (0.32 in 2011 to 2012 and 0.42 in 2012 to 2013). Even though there was a significant seed size category effect on HI, differences between HP seed size genotypes were more evident in 2011 to 2012 (0.06 difference) than in 2012 to 2013 (0.02 difference).

Seed Growth and Development

Seed filling duration was longest for the commercial genotypes (48 d), shortest for the HP small seed genotypes (36 d), and intermediate for the HP large seed genotypes (42 d) (Fig. 3). There was a significant year × seed size category interaction ($P < 0.05$); in 2012 to 2013, there were no differences between HP large and small seed size categories (average 40 d), but they were different during 2011 to 2012 (43 vs. 35 d, HP large and small seed, respectively). There were more differences across seed size genotypes for seed growth rate than for SFD. Commercial genotypes had an intermediate seed growth rate (3.6 mg seed^{-1} d$^{\times 1}$), HP large seed the highest seed growth rate (5.3 mg seed^{-1} d^{-1}) and HP small seed the smallest rate (2.2 mg seed^{-1} d^{-1}) (seed size main effect, $P < 0.05$) (Fig. 3).

Leaf Area Traits

Green leaf area at the beginning of seed filling (LAI_{R5}) was largest for HP small seed genotypes (4.3 m^2 m^{-2}), intermediate for the commercials (3.8 m^2 m^{-2}), and smallest for HP large seed (3.3 m^2 m^{-2}) (Table 3). Although there was a significant year × seed size category interaction, the three groups showed a similar ranking during both seasons (Table 3). The mean LAI_{R5} differed by year, with 3.08 and 4.54 m^2 m^{-2} in years 2011 to 2012 and 2012 to 2013, respectively ($P < 0.05$). Some differences among genotypes

Table 2. Seed yield, and numerical and physiological yield components for genotypes differing in seed size and protein concentration evaluated in 2011 to 2012 and 2012 to 2013. Commercial genotypes are elite cultivars with average seed protein and seed size, high protein (HP) large seed are genotypes with increased seed protein concentration due to high seed protein content, and HP small seed are genotypes with high seed protein concentration based on reduced seed oil and residual content. Genotypes 1 to 3 are commercial genotypes, 4 to 6 are HP large seed genotypes, and 7 to 9 are HP small seed genotypes. The value LSD is the Fisher's protected least significant difference ($P < 0.05$). The proportion of explained sums of squares accounted for by each source of variation is denoted as %SS.

Source of variation		Seed yield	Numerical components		Physiological components	
			Seed number	Seed size	Total biomass	Harvest index
		kg ha^{-1}	seed m^{-2}	mg seed^{-1}	g m^{-2}	kg kg^{-1}
Seed size						
	Commercial	4362	2993	152.8	978.9	0.445
	HP large seed	2818	1460	196.2	764.1	0.361
	HP small seed	3093	4333	74.2	973.5	0.320
Year × seed size						
2011–2012	Commercial	4134	2836	146.2	996.2	0.422
	HP large seed	2230	1256	183.5	727.2	0.307
	HP small seed	2622	3738	73.3	1058.4	0.249
2012–2013	Commercial	4613	3149	160.1	961.5	0.468
	HP large seed	3357	1646	207.9	801.1	0.414
	HP small seed	3565	4928	75.1	888.7	0.391
Genotype						
	1. DM3100	4374	2906	151.1	939.3	0.466
	2. NK34	4585	3469	149.0	1077.1	0.427
	3. SPS3X1	4158	2615	159.3	920.3	0.443
	4. IA3011	3039	1668	179.3	772.4	0.392
	5. PI555396	2648	1552	169.2	787.0	0.333
	6. PI538376	2760	1116	246.5	732.9	0.357
	7. PI518757	3073	4713	65.0	1089.9	0.285
	8. PI398970	3043	3307	94.2	851.6	0.351
	9. PI196177	3164	4980	63.3	979.1	0.324

	P value (LSD)	%SS	P value (LSD)	%SS	P value (LSD)	%SS	P value (LSD)	%SS	P value (LSD)	%SS
Statistical significance										
Year	** (186)	23.5	** (125)	5.0	** (4.0)	1.1	NS	2.1	*** (0.008)	38.5
Block (Year)	–	6.0	–	1.2	–	0.2	–	9.2	–	0.6
Seed size	*** (370)	62.5	*** (383)	77.2	*** (7.4)	83.9	*** (66.9)	46.0	*** (0.021)	40.6
Year × Seed size	* (741)	3.7	* (766)	2.4	* (14.8)	0.4	** (133.8)	11.4	*** (0.042)	5.9
Genotype (Seed size)	NS	3.0	*** (1150)	13.2	*** (22.2)	13.7	** (200.6)	23.2	*** (0.063)	9.8
Year × Genotype (Seed size)	NS	1.3	NS	1.1	* (44.5)	0.7	NS	8.1	* (0.125)	4.6

* Significant at 0.05 probability level.

** Significant at 0.01 probability level.

*** Significant at 0.001 probability level.

within the HP small seed category were evident (4.8 vs. 3.9 m^2 m^{-2} for the two most contrasting genotypes).

Faster leaf senescence after R5 was strongly associated with seed size categories, accounting for almost 70% of model sum of squares ($P < 0.05$, Table 3). High protein small seed genotypes had the fastest leaf senescence ($b = -0.0029$) compared with HP large seed ($b = -0.0016$) or commercial ($b = -0.0013$) genotypes; these differences were evident both years. There were genotype differences within each seed size category, especially within HP small seed, where PI518757 showed the fastest leaf senescence ($b = -0.0036$) compared with the other two genotypes ($b = -0.0026$) (Table 3).

Leaf area duration during seed filling was strongly dependent on the growing season (93.7 vs. 141.6 m^2 d, 2011 to 2012 and 2012 to 2013, respectively). Seed size category was the second most important source of variation, as shown by the proportion sum of squares (Table 3). Commercial genotypes had the highest LAD (137.6 m^2 d), while HP large seed had the lowest (102.7 m^2 d), and these differences were similar during both growing seasons. There were significant genotypic differences within each category (Table 3).

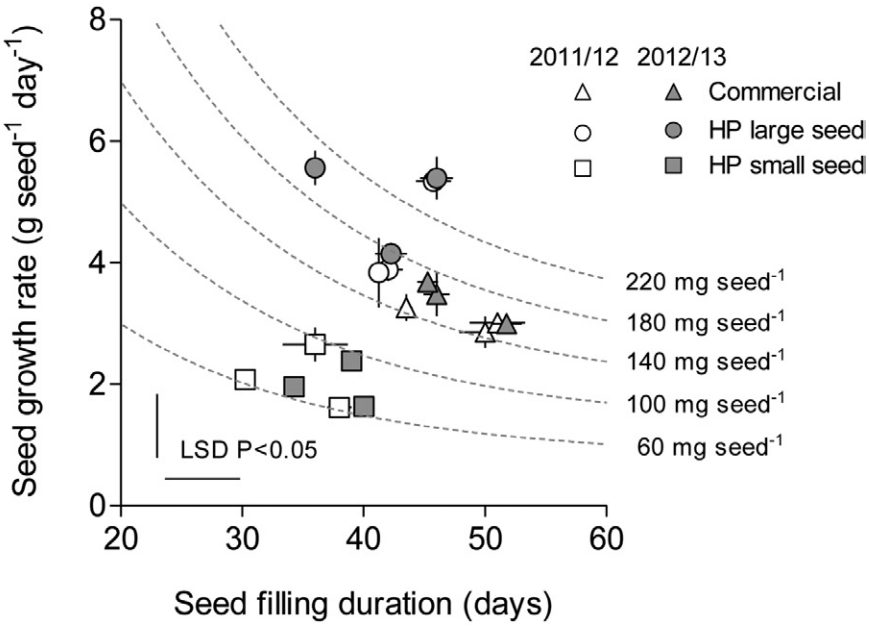

Fig. 3. Seed growth rate and seed filling duration for genotypes differing in seed size and composition. Triangles indicate cultivars with average seed protein and seed size (commercial), circles are genotypes having increased seed protein concentration due to high seed protein content [high protein (HP) large seed], and squares indicate genotypes with high seed protein concentration based on reduced seed oil and residual content (HP small seed). Each value is the average of four replicates. Dashed lines indicate combinations of seed growth rate and seed filling duration for the same seed size.

Assimilate Supply per Seed during Seed Filling

Green leaf area at the beginning of seed filling (R5) was used as a proxy for total assimilate supply for seed filling (Rotundo et al., 2009). Since pod and seed numbers at R5 are mostly set (Board and Tan, 1995; Jiang and Egli, 1995), the ratio between green leaf area at R5 and seed number at R7 can be considered an estimate for assimilate availability per seed.

This trait was strongly affected by the seed size category ($P < 0.05$), explaining more than 60% of total variation. High protein large seed genotypes always displayed the highest assimilate availability per seed during seed filling, with 23.8 cm^2 $seed^{-1}$; commercial genotypes had 13.6 cm^2 $seed^{-1}$, and the HP small seed genotypes only 10.2 cm^2 $seed^{-1}$. There was a significant genotypic effect within seed size category on assimilate availability per seed, accounting for 27% of model sum of squares. Variability in assimilate availability per seed was explained by differences in seed number per unit land area ($R^2 = 0.92$, $P < 0.05$), and not by differences in leaf area per unit land area ($R^2 = 0.18$, $P > 0.05$).

An asymptotic relationship between seed size and assimilate availability per seed was observed (Fig. 4a). A single function accommodated all genotypes and years, indicating a strong relation between those variables. However, no relationship was observed between seed protein concentration and assimilate availability per seed (Fig. 4b).

Seed Size Category Multivariate Comparison

A principal component analysis was conducted for identifying traits associated with each seed size category. Genotypes were used as entries, and the analyzed crop traits were those that showed significant genotypic effects across years.

The first two axes of the principal component analysis accounted for 73% of total variation. The analysis clearly grouped genotypes according to their seed size category. The first axis (47% of the variation) separated HP large seed genotypes from HP small seed ones based on assimilate availability per seed during seed filling, seed growth rate, and leaf area at the beginning of the seed filling period (Fig. 5). The second axis (26% of the variation) helped separate commercial genotypes from HP large and small seed size genotypes, which were associated with higher HI, LAD, SFD, and SenCoef (Fig. 5).

DISCUSSION

Our results support the idea that improvements in seed protein concentration can be achieved via alternative strategies in terms of seed size. High protein concentration can be obtained by more-than-proportional increases in seed protein content (mg $seed^{-1}$) relative to increases in oil and carbohydrate content in large seeds, or by more-than-proportional reductions in oil and/or carbohydrate content relative to protein content reductions in small seeds. Egli (1998) proposed that selection for seed chemical composition may affect the normal growth and development of field crops. Our experiments show that this impact can also depend on the ultimate mechanism

Table 3. Leaf traits for genotypes differing in seed size and protein concentration, evaluated in 2011 to 2012 and 2012 to 2013. Commercial genotypes are elite cultivars with average seed protein and average seed size, high protein (HP) large seed are genotypes with increased seed protein concentration due to high seed protein content, and HP small seed are genotypes with high seed protein concentration based on reduced seed oil and residual content. Genotypes 1 to 3 are commercial genotypes, 4 to 6 are HP large seed size genotypes, and 7 to 9 are HP small seed size genotypes. LSD is the Fisher's protected least significant difference ($P < 0.05$). The proportion of explained sums of squares accounted for by each source of variation is denoted as %SS.

Source of variation		Leaf area at R5	Leaf senescence Coefficient	Leaf area Duration
		$m^2\ m^{-2}$		$m^2\ d$
Seed size				
	Commercial	3.77	−0.0013	137.6
	HP large seed	3.33	−0.0016	102.7
	HP small seed	4.33	−0.0029	112.7
Year × Seed size				
2011–2012	Commercial	3.22	−0.0011	116.9
	HP large seed	2.47	−0.0012	75.5
	HP small seed	3.55	−0.0026	88.8
2012–2013	Commercial	4.32	−0.0015	158.4
	HP large seed	4.18	−0.0020	129.9
	HP small seed	5.11	−0.0032	136.7
Genotype				
	1. DM3100	3.43	−0.0012	124.8
	2. NK34	4.01	−0.0012	154.4
	3. SPS3X1	3.87	−0.0015	133.7
	4. IA3011	3.25	−0.0019	90.7
	5. PI555396	3.03	−0.0013	98.3
	6. PI538376	3.71	−0.0016	119.1
	7. PI518757	4.38	−0.0036	102.1
	8. PI398970	3.86	−0.0023	107.1
	9. PI196177	4.75	−0.0029	129.0

	P value (LSD)	%SS	P value (LSD)	%SS	P value (LSD)	%SS
Statistical significance						
Year	*** (0.59)	59.8	* (0.0001)	11.3	** (6.1)	54.7
Block (Year)	–	5.7	–	2.0	–	5.4
Seed size	*** (0.29)	18.9	*** (0.0003)	68.0	*** (9.9)	20.5
Year × Seed size	* (0.58)	1.9	NS	1.1	NS	0.7
Genotype (Seed size)	*** (0.87)	10.2	*** (0.0010)	16.5	*** (29.7)	13.8
Year × Genotype (Seed size)	NS	3.5	NS	1.1	* (59.5)	4.9

* Significant at 0.05 probability level.

** Significant at 0.01 probability level.

*** Significant at 0.001 probability level.

modifying seed composition associated with contrasting seed sizes. The idea that it is feasible to select for the same trait (i.e., seed protein concentration) through different strategies (i.e., large vs. small seeds) is relevant for finding alternative avenues to eliminate negative correlations frequently associated with biophysical constraints or genetic linkage (Stearns, 1989; Weih, 2003).

Ignoring the role of seed size in determining seed composition may lead to misleading conclusions on the impact of selection for high seed protein concentration on crop function, even for genotypes with similar yield. For example, $TotBio_{R7}$ was higher for commercial high-yielding genotypes than for the HP ones (979 vs. 869 g m^{-2}, respectively), regardless of seed size. However, taking into account the contrasting seed size, the HP small seed cultivars had the potential to produce similar $TotBio_{R7}$ as the commercial cultivars, while the HP large seed ones did not. Results from Egli and Bruening (2007a) suggest that a larger vegetative reservoir at the beginning of seed filling is not associated with high seed protein concentration. Our results showed that this holds true for the HP large seed size, but not for the HP small seed category; the latter showed the largest green leaf area at the beginning of seed filling. Faster leaf senescence rate during seed filling is another trait commonly associated with high seed protein concentration (Salado-Navarro et al., 1985). High protein large seed genotypes had similar leaf senescence rate when compared with the commercial ones, but the

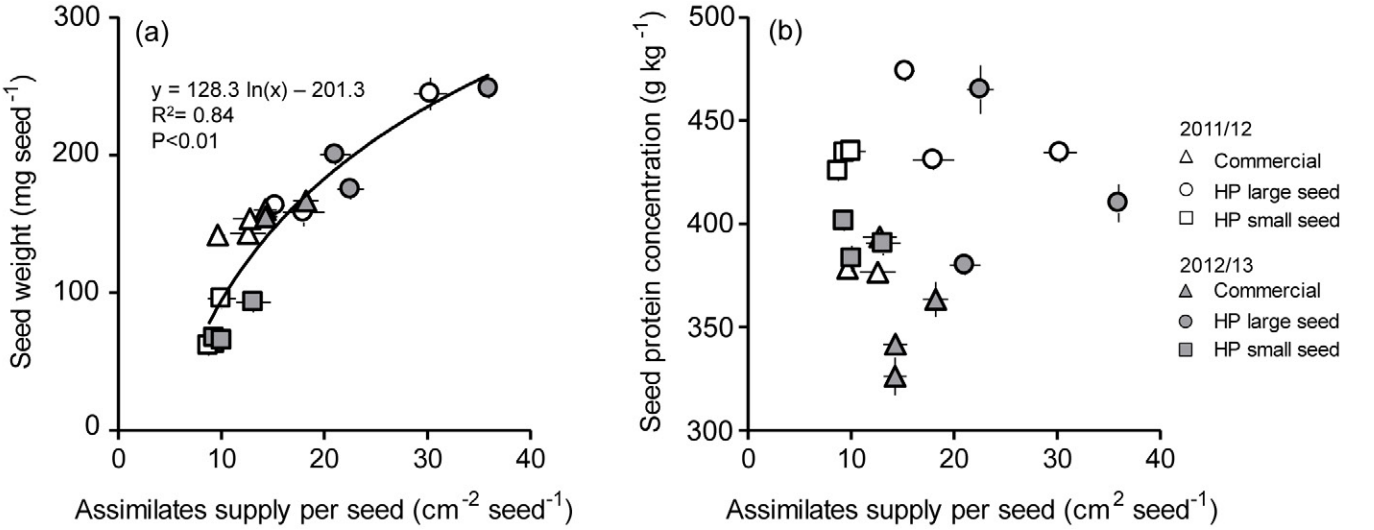

Fig. 4. Relationship between seed size (a) and seed protein concentration (b) versus assimilate supply per seed. Triangles indicate cultivars with average seed protein and seed size (commercial), circles are genotypes having increased seed protein concentration due to high seed protein content [high protein (HP) large seed], and squares indicate genotypes with high seed protein concentration based on reduced seed oil and residual content (HP small seed). Each value is the average of four replicates.

HP small seed genotypes had a faster leaf senescence rate than the commercial ones. The growth habit of the evaluated HP cultivars was mostly semideterminate, but there was one indeterminate in the HP large seed category, and one determinate in the HP small seed category. Therefore, some of the genotypic effects observed for leaf area traits, e.g., the difference in LAD between the indeterminate PI555396 and the determinate PI196177, could be related to the difference in growth habit. However, there is large evidence supporting the concept that growth habit does not affect final seed protein concentration (Bernard, 1972; Escalante and Wilcox, 1993; Wilcox and Zhang, 1997), total leaf area and dry matter accumulation (Beaver and Johnson, 1981), or seed yield (Ouattara and Weaver, 1994). Therefore, to understand the impact of HP on leaf area dynamics, it is critical to take into consideration the strategy in terms of seed size that is associated with the increase in seed protein.

Similar to Egli and Bruening (2007b), we observed a similar seed growth rate between commercial genotypes and the HP genotypes as a group, without considering differences in seed size (3.6 vs. 3.7 g seed^{-1} d^{-1}, respectively). When taking into account the contrasting seed sizes, however, seed growth rate was either higher (5.3 g seed^{-1} d^{-1}, HP large seed) or lower (2.2 g seed^{-1} d^{-1}, HP small seed) compared with the commercial genotypes. Since seed size is determined by changes in both seed growth rate and SFD, a positive association between seed size and seed growth rate implies there was no variation in SFD among the HP concentration genotypes (Egli et al., 1981, 1987; Swank et al., 1987). These examples show that considering the strategy in terms of seed size that determines the high seed protein concentration phenotype is critical for understanding any potential impact on soybean growth and development.

Assimilate availability per seed plays a central role in determining seed protein concentration when this high concentration is based on increased protein content (mg protein seed^{-1}) as related to larger seed size. In previous work, we showed that increasing assimilate supply to the seed, which is required for attaining high seed protein concentration in large-seeded genotypes, was the result of reducing seed number rather than increasing total assimilates (e.g., leaf area at R5; Rotundo et al., 2009, 2011). This reduction in seed number was responsible for the limited yields attained by these high seed protein concentration genotypes. There is substantial evidence of a mechanistic relationship between assimilate supply per seed and seed weight (Jenner et al., 1991; Borrás et al., 2004; Rotundo et al., 2009). However, by understanding that there are different strategies in terms of seed size for attaining high seed protein concentration, we could identify high seed protein concentration genotypes with lower assimilate supply per seed (HP small seed size) when compared with commercial genotypes. This indicates that increased assimilate supply per seed is not a requisite to attain high seed protein concentration, as previous evidence suggested (Rotundo et al., 2009).

Several quantitative trait loci (QTL) or genomic regions were associated with soybean seed protein concentration (Diers et al., 1992; Csanádi et al., 2001; Chung et al., 2003; Panthee et al., 2005; Li et al., 2007; Zhaoming et al., 2011). The ultimate goal of these studies was to identify candidate genes responsible for the trait of interest (Bolon et al., 2010). Recognizing the existence of different seed size strategies to attain higher seed protein concentration is critical to facilitate the identification of molecular mechanisms responsible for this trait. For instance, high protein concentration may be determined

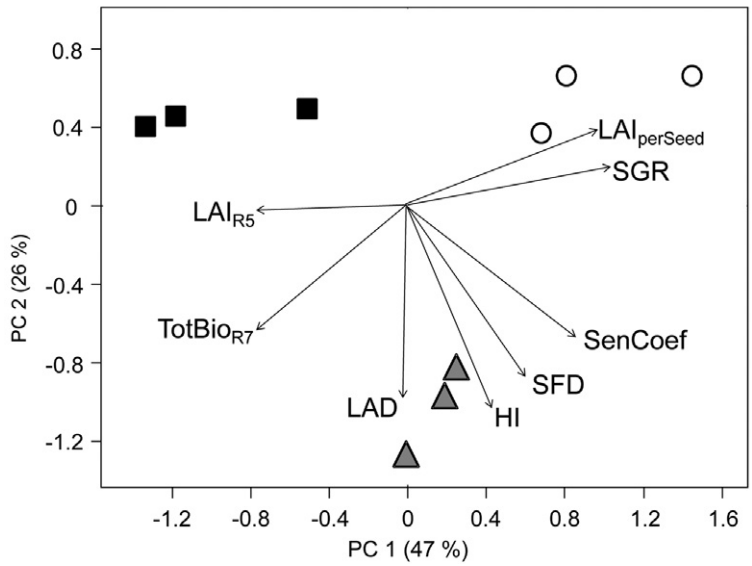

Fig. 5. Biplot of the first and second principal components for physiological traits associated with commercial cultivars (gray triangles), and high protein (HP) genotypes with contrasting physiological strategies to attain HP content: genotypes having increased seed protein concentration due to high seed protein content (empty circle, HP large seed), and genotypes having the alternative strategy to attain high seed protein concentration based on reduced seed oil and residual content (full square, HP small seed). Variables are: TotBio$_{R7}$ (kg ha^{-1}), total biomass at maturity; HI (%), harvest index; LAI$_{R5}$, leaf area at beginning seed filling; LAD (m^2 d), green leaf area duration during the seed filling period; SenCoef, leaf senescence coefficient—smaller (more negative) values imply faster leaf area loss; SFD (d), duration of linear phase of seed filling; LAI$_{perSeed}$ (cm^2 seed^{-1}), LAI$_{R5}$ per unit seed; SGR (mg seed^{-1} d^{-1}), individual seed growth rate.

by a gene related to reduced carbohydrate and/or oil synthesis, rather than one related to increased protein synthesis. Ishii et al. (2010) demonstrated that ignoring these alternatives may result in detecting QTL for seed protein concentration that have no actual function in protein synthesis. The utility of such QTL for marker-assisted breeding to increase the actual synthesis of protein is limited.

Our results have implications for parental selection aimed to simultaneously breed for increased protein concentration and yield (Medic et al., 2014). Parental selection for HP donors should take into account the contrasting influence of seed size on crop function. For example, choosing HP donors based on small seed size would, hypothetically, be beneficial over larger seed sized parents, since the former produce more leaf area at beginning seed fill (larger N reservoir), more total biomass, increased seed set, and require lower assimilate supply per seed. To test this hypothesis, we developed breeding populations using a common commercial high-yielding cultivar crossed with a HP large (population 1) and small (population 2) seed size donor. Our results suggest that there is a higher chance of obtaining a HP high-yielding cultivar in population 2.

CONCLUSIONS

Breeding for increased seed protein concentration in soybean can be attained by contrasting strategies based on seed size. Here, we described two possibilities: (i) seeds with increased seed protein content in large seed genotypes, and (ii) seeds with reduced oil and carbohydrate contents in small seed genotypes. Past research describing crop growth and development effects resulting from selection for high seed protein concentration have almost always tested genotypes with higher seed size, and have not included the (high seed protein) small seed genotype alternative.

We demonstrated that similar seed protein and yield can be obtained through different strategies when seed size differences are taken into account. High protein concentration in large seed size genotypes was associated with longer SFD, more assimilate per seed, and faster seed growth rates compared to the strategy based on reduced seed oil and carbohydrate contents in small seed size genotypes. The latter exhibited higher LAI at the beginning of seed filling and faster leaf senescence compared with the former, and produced the same biological yield as commercial genotypes.

Our results show that selecting for the same trait (i.e., high seed protein concentration) can impact crop growth and development differently depending on the mechanism behind the final trait of interest.

Supplemental Material Available

Supplemental material for this article is available online.

Acknowledgments

This research was supported by Agencia Nacional de Investigaciones (PICT-2011-1292), CONICET (PIP-202), and Fundación Ciencias Agrarias-UNR (PICT 2012). Authors thank valuable comments from Jill Miller-Garvin. F. Poeta held a graduate scholarship from, and L. Borrás and J.L. Rotundo are members of, CONICET, the Scientific Research Council of Argentina.

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