SHST
Horticulture and Forestry Society of Transylvania

Anshu AK *et al.* (2022)
Notulae Scientia Biologicae
Volume 14, Issue 1, Article number 10994
DOI:10.15835/nsb14110994
Research Article

NSB
Notulae Scientia Biologicae

Phosphatidylcholine content in soybean: Genetic variability and the effect of growing year

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Abstract

Information on the genetic variability of phosphatidylcholine (PC) content in soybean (*Glycine max* L.) is scanty. In the present investigation, estimation of PC content in 408 soybean accessions from 15 countries revealed a range of 2.04-8.80 mg/g soy flour, thereby exhibiting about 4.3-fold genetic variation. Both high (> 8.00 mg/g soy flour) and low PC (< 4.50 mg/g soy flour) genotypes identified in 2018 were raised in 2019 to investigate the effect of growing year on PC content in soybean seeds. Effect of growing year and genotype × growing year interaction on PC content was found to be significant ($p < 0.05$). Results suggested that higher average minimum temperature during the seed filling stage may increase PC content. Mapping populations developed from high and low PC genotypes from diverse genetic background may be used for identifying genomic regions underlying biosynthesis of PC in soybean seeds.

Keywords: genetic variability; germplasm; growing year; phosphatidylcholine; soybean

Introduction

Soybean is the major oilseed crop with worldwide production of 358.28 million metric tons in 2018-19. The United States of America is the leading country in soybean production followed by Brazil, Argentina, China and India. China holds the *numero uno* position in soybean oil production (15,232 thousand metric tons) followed by USA, Brazil, Argentina, European Union and India (USDA, 2019). Commercial lecithin is a brown to pale yellow viscous liquid containing a mixture of phospholipids, namely, phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidic acid and a fraction of crude vegetable oil, which is removed during the degumming process (Shurtleff and Aoyagi, 2016). Phosphatidylcholine is the major phospholipid component of lecithin, and hence both these terms are used interchangeably (Kapalka, 2010). Global market for lecithin is poised to reach 1.59 billion USD by 2023 (Markets and Markets Research Private Ltd, 2018). Besides being used in animal feed with a market share of 42.1% (Grand View Research Inc, 2017), lecithin is ubiquitous in wide array of daily food items, cosmetics, pharmaceuticals, coatings, plastic and rubber industry, paper and printing etc. (Hoogevest and Wendel, 2014;

Received: 14 Jun 2021. Received in revised form: 16 Nov 2021. Accepted: 27 Jan 2022. Published online: 10 Feb 2022.
From Volume 13, Issue 1, 2021, Notulae Scientia Biologicae journal uses article numbers in place of the traditional method of continuous pagination through the volume. The journal will continue to appear quarterly, as before, with four annual numbers.

List, 2015). Soybean oil has high total phospholipids compared to other vegetable oils (Shurtleff and Aoyagi, 2016) and commands 79.6% market share (Grand View Research Inc, 2017). Among all the phospholipids present in commercial soybean lecithin, it is the PC (1,2-Diacyl-sn-glycero-3-phosphocholine) component which is desired to be in high concentration in commercial lecithin for the food industry due to its high polarity and emulsifying property (Shurtleff and Aoyagi, 2016). PC may be implicated in the synthesis of choline molecule required for transport of fats (Van der Veen *et al.*, 2017) and in neurotransmitter acetylcholine for cognitive and brain functions (Blusztajn *et al.*, 2017) and staving off cardiovascular diseases by lowering homocysteine concentration in plasma (Olthof *et al.*, 2005).

Therefore, soy industry seeks to enhance the proportion of PC content in the commercial lecithin, which underscores the importance of development of soybean varieties with high level of PC content to be used as raw material. This necessitates investigating the genetic variability for PC content in soybean germplasm. Barring the sole report of Song *et al.* (2018), genetic variability for PC has been scarcely reported. Even the study of Song *et al.* (2018), which reported PC content in narrow range of 7.09-10.27 mg/g soy flour in 269 soybean samples, has its own limitation as soybean samples analysed in the study were grown in 4 soybean-producing regions of China i.e. the narrow variability for PC in the study was inclusive of variation due to altitude/latitude of different growing locations. In the present study, therefore, it was thought worthwhile to analyse large number of soybean genotypes from different country of origin raised at single location, thereby eliminating the effect of growing location, to investigate the genetic variability for PC. Soybean genotypes identified for high and low PC content in first cropping season were also raised in the next cropping season to assess the effect of growing environment on PC content.

Materials and Methods

Four hundred eight soybean accessions comprising exotic accessions, indigenous collections, advanced breeding lines developed in the flagship plant breeding programme on 'Breeding for food grade characters and oil content' and popular cultivars of India were raised in the field of ICAR-Indian Institute of Soybean Research, Indore (N 22.7196° and E 75.8577°), India in cropping season 2018. Planting was done in the last week of June 2018 in single row plot of 3 m length with row-to-row spacing of 45 cm and plant-to-plant distance of 5 cm. In cropping season 2019, planting of only selected genotypes was carried out with the same crop geometry as in cropping season 2018 in random block design in triplicate. Standard agronomic practices recommended for soybean cultivation in Central India were followed from sowing to harvesting. Genotypes were harvested at maturity and threshed manually in both the cropping seasons. Freshly harvested seeds were subjected to lipid extraction and PC analysis through HPLC.

Extraction of phosphatidylcholine from soybean flour

PC was extracted from soybean flour following Lee *et al.* (2010). One gram of oven dried finely ground soybean flour (60 mesh) from the seeds of three randomly selected plants was mixed with 7 ml of chloroform: methanol (1:1, v/v) solution in a 50 ml centrifuge tube and vortexed for 2 min. Suspension so obtained was incubated for 1 hour at room temperature and centrifuged at 4800 g for 10 min. The supernatant obtained was transferred to the other centrifuge tube and 15 ml of 0.5% aqueous sodium chloride was added. Subsequently, vortexing for 2 min separated the mixture into 2 distinct phases. The lower organic phase containing the total lipids was decanted into 10 ml beaker containing 250 mg anhydrous sodium sulphate and the contents after stirring carefully were kept for evaporation at 55 °C for 12 hours. The residue so obtained was re-dissolved in 2 ml of isopropanol: n-hexane (1:1, v/v) and stored at -20 °C for further analysis.

Determination of phosphatidylcholine through HPLC

PC content was determined through HPLC following Jiang *et al.* (2015). For this purpose, total lipids re-dissolved in isopropanol: n-hexane (1:1, v/v) were passed through syringe filter (PVDF membrane, 0.22 µm pore size, 13 mm diameter,). A fixed volume (20 µl) of the syringe-filtered sample was injected into a Shimadzu chromatograph (LC-10AT VP), equipped with a UV detector (SPD 10AT VP) and oven (CTO-10) housing a silica column (Phenomenex; Luna 5 µm Silica (2) 100 Å, LC Column, 250 × 4.6 mm; Catalogue No. - 00G-4274-E0). The column oven was maintained at 40 °C. The separation of PC was achieved isocratically using mobile phase comprising isopropanol: n-hexane: HPLC grade water in the ratio of 70: 16: 14 (V: V: V) at a flow rate of 1.0 ml/min for 30 min. The PC was detected at a wavelength of 205 nm. The retention time of PC was 14.1 min (Figure 1). A standard curve was generated using different concentration of L-α-phosphatidylcholine procured from Sigma Aldrich (catalogue no. P7443). The concentration of PC in the sample was computed by comparing its peak area with that of the known concentration of the standard. PC content was expressed as mg/g soy flour on dry weight basis and values given are mean of triplicate samples from 3 randomly selected plants.

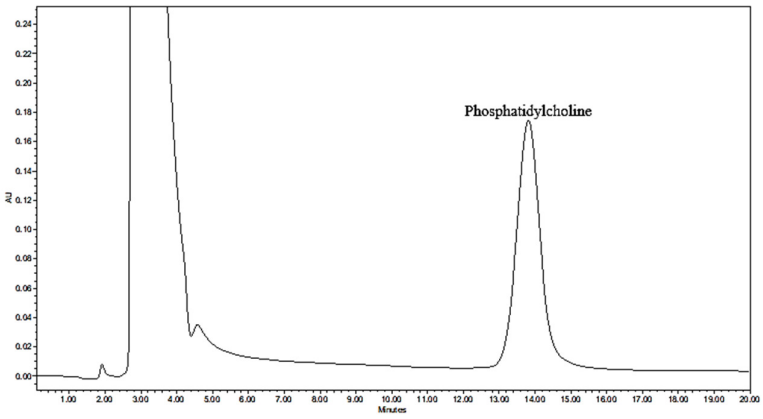

Figure 1. HPLC chromatogram depicting the resolution of phosphatidylcholine standard at 14.1 min

Statistical analysis

All data presented in tables were expressed as the means of triplicate measurements. Multiple comparisons of means were performed using Fisher's Least Significant Difference (LSD) test. Differences were considered significant when the probability value was < 0.05 ($p < 0.05$). All calculations were performed using GraphPad Prism version 9.1.0 for Windows (GraphPad Software, San Diego, California, USA). Effect of genotype, growing year and genotype × growing year was analysed through two-way analysis of variance using IBM SPSS Statistics for Windows, Version 25.0 (IBM Corp, Armonk, NY).

Results and Discussion

As evident from Table 1, which lists high and low PC content soybean genotypes identified across 408 soybean accessions analysed from the cropping season 2018, PC content ranged from 2.04 (IC275) to 8.80 mg/g soy flour (NRC151), thereby exhibiting about 4.3-fold genetic variation. The coefficient of variation of PC was 12.63. IC275 is an indigenous collection, while NRC151 is a lipoxgenase-3 free advanced breeding line developed from JS335 × PI417458 (*lx3lx3*). Figure 2 exhibits the distribution of 408 genotypes among different ranges of PC content, with minimum number of genotypes (4) falling in the range of 2.00-4.00 mg/g soy flour, while maximum number of genotypes (313) were in the range of 6.01-8.00 mg/g soy flour. Seventy-

nine soybean genotypes exhibited PC content in the range of 4.01-6.00 mg/g soy flour, while 12 soybean genotypes exhibited > 8.00 mg/g soy flour. Frequency distribution curve for PC showed skewness -0.87 and kurtosis 2.73, showing symmetrical distribution of the data. Song *et al.* (2018) investigated PC content in 269 soybean genotypes collected from 4 soybean-producing regions of China and reported the concentration in the range of 7.09-10.27 mg/g soy flour with the mean value of 8.16 mg/g soy flour i.e. only 1.45 fold genetic variation was reported by these authors. Though, the authors did not mention the true genetic background of these 269 soybean samples in their study, very low variability for PC content may be because of the narrow genetic base of the soybean samples analysed, and the variation noted may also be contributed by the different growing conditions prevailing at the different soybean-producing regions from where the authors collected these samples. Barring this sole report on assessment of soybean genotypes for PC content with limited information, the authors could not find any other study in the literature to compare the genetic variability of PC content observed in our investigation.

Table 1. Phosphatidylcholine content (mg/g soy flour) in high and low phosphatidylcholine containing soybean genotypes across growing years

Genotype	Growing year	
	2018	2019
NRC151	8.80 ± 0.62	8.91 ± 0.71
AVKS215	8.62 ± 0.53	9.04 ± 0.86
EC456556	8.53 ± 0.45	8.92 ± 0.62
IC243548	8.40 ± 0.35	9.61 ± 0.91
AVKS201	8.34 ± 0.32	9.57 ± 0.88
EC289099	8.26 ± 0.29	9.37 ± 0.75
PI133226	8.16 ± 0.31	8.14 ± 0.23
EC538814	8.11 ± 0.19	8.58 ± 0.43
PI596540	8.03 ± 0.22	8.36 ± 0.26
JS20-98	4.29 ± 0.21	5.47 ± 0.33
JS20-34	4.14 ± 0.30	5.56 ± 0.47
EC606918	3.80 ± 0.15	4.93 ± 0.22
EC606917	3.55 ± 0.18	4.73 ± 0.25
IC574373	3.42 ± 0.12	4.06 ± 0.18
IC275	2.04 ± 0.06	2.48 ± 0.08
Mean	6.43	7.18

Values given are mean of triplicate samples ± standard deviation. Genotype, growing year and genotype × growing year were significant at $p < 0.05$ LSD= 0.49.

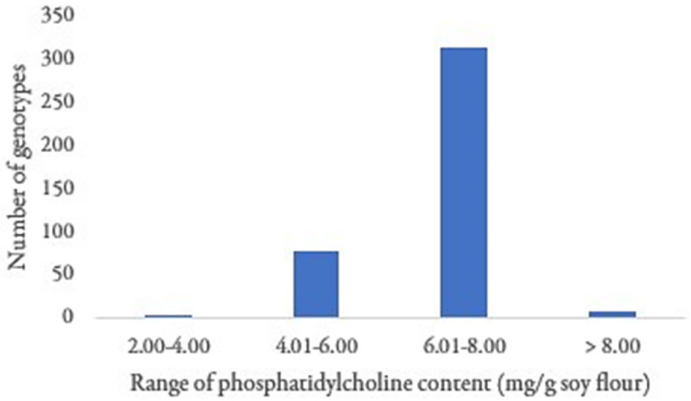

Figure 2. Distribution of 408 soybean genotypes according to phosphatidylcholine content

Table 2 shows the country of origin of 408 soybean genotypes, their range and average PC content investigated in this study. Genetic variability for PC content was the highest in soybean accessions from India (2.04-8.80 mg/g soy flour-4.3 fold) followed by USA (3.55-7.83 mg/g soy flour-2.2 fold). With regard to average PC content of soybean accessions from different country of origin, soybean accessions from Sri Lanka, Indonesia and Japan exhibited average PC content of 7.76, 7.57 and 7.15 mg/g soy flour, respectively. Average PC content of soybean accessions from Australia was 6.88 mg/g soy flour. Soybean accessions from USA and China both showed average PC content of 6.85 mg/g soy flour followed by Ghana (6.82 mg/g flour) and Taiwan (6.67 mg/g soy flour). Maximum number of 240 soybean accessions was from India with average PC content of 6.65 mg/g soy flour. Average PC content in soybean accessions from Brazil, Hungary, Italy, Philippines and Russia was 6.62, 6.54, 6.48, 6.05 and 6.02 mg/g soy flour, respectively. Average PC content of soybean accessions from some of the countries such as China, Ghana, Hungary, Indonesia, Italy, Nigeria, Philippines, Russia and Sri Lanka are based on very limited accessions analysed.

Table 2. Average phosphatidylcholine content (mg/g soy flour) in soybean genotypes from different countries

Country of origin	Number of genotypes	Range of PC content	Average PC content*
Australia	3	6.53-7.45	6.88 ± 0.40
Brazil	4	4.55-7.74	6.62 ± 1.14
China	2	6.22-7.48	6.85 ± 0.63
Ghana	2	5.99-7.65	6.82 ± 0.83
Hungary	2	5.18-7.89	6.54 ± 1.35
India	240	2.04-8.80	6.65 ± 0.84
Indonesia	2	6.98-8.16	7.57 ± 0.41
Italy	1	6.48	6.48 ± 0.00
Japan	6	6.46-7.29	7.15 ± 0.64
Nigeria	1	6.68	6.68 ± 0.00
Philippines	1	6.05	6.05 ± 0.00
Russia	2	5.56-6.48	6.02 ± 0.46
Sri Lanka	2	7.54-7.97	7.76 ± 0.22
Taiwan	24	5.14-8.00	6.67 ± 0.75
USA	33	3.80-8.53	6.85 ± 1.06
Unknown	85	3.55-7.83	6.57 ± 0.75
Total	408	2.04-8.80	6.66 ± 0.84

*Values given are average of phosphatidylcholine content of total soybean genotype from given country ± standard deviation.

Among 408 soybean genotypes assessed for PC content, 7 were the most popular soybean varieties, 42 were special food grade, and 15 were oil traits genotypes developed in India. Variability for PC content in the most popular 7 soybean varieties of India, namely, 'JS335', 'JS20-34', 'JS20-29', 'JS95-60', 'JS93-05', 'RVS 2001-4' and 'JS20-69', which account for 84% of the total soybean breeder seed indent in the country (AICRPS, 2018) and the harvest of which predominantly goes to soybean oil extraction industries, was from 5.14 to 6.76 mg/g soy flour. This underscores the need to enhance the PC content in commercial varieties. P3-19, a high oleic acid line developed from LSb1 × NRC7, showed 5.74 mg/g soy flour PC content, while PC content of LSb1 and NRC7 were 5.66 and 6.83 mg/g soy flour, respectively, indicating low PC trait into P3-19 was inherited from LSb1 (*Glycine max*). NRC127 is Kunitz trypsin inhibitor (KTI) free soybean genotype and is essentially derived variety of 'JS97-52'. PC content of 'NRC127' and 'JS97-52' were statistically same as the former registered a PC value of 6.37 mg/g soy flour while the latter showed 6.67 mg/g soy flour. PC content of 12 Kunitz trypsin inhibitor free lines (BC_3F_4), developed by introgressing null allele of Kunitz trypsin inhibitor, an antinutritional factor in soybean, from PI542044 (*Glycine max*) into 'JS97-52' through marker assisted back crossing (MABC), was also found to be the same as that of 'JS97-52' (6.67 mg/g soy flour). Similarly,

lipoxygenase-2 (the principal contributor to the off-flavour associated with the soy products) free soybean genotypes (BC_3F_4), which have been developed by introgressing null allele of lipoxygenase-2 gene into 'JS97-52' from PI596540 (8.03 mg/g soy flour) (*Glycine max*) through marker assisted backcrossing, were also found to contain the same level of PC content as that of 'JS97-52'.

Fifteen soybean genotypes were grown in two consecutive years, namely, 2018 and 2019 (Table 1). The criterion for selection of 15 soybean genotypes for investigation of PC content for the second year was the data of the first cropping year i.e. 2018, which revealed 9 high (> 8.0 mg/g soy flour) and 6 low PC genotypes (< 4.5 mg/g soy flour). Effect of growing year and genotype × growing year interaction on PC content in soybean was significant ($p < 0.05$). The increase in PC content of high PC genotypes in 2019 was significantly ($p < 0.05$) less compared to the increase in PC content of low PC genotypes. Among high PC containing soybean genotypes, 3 genotypes, namely, 'AVKS201', 'IC243548' and 'EC289099' showed significant ($p < 0.05$) increase in PC content in 2019 cropping season compared to 2018, while remaining 6 high PC content genotypes, namely, 'NRC151', 'AVKS215', 'PI596540', 'PI133226', 'EC456556' and 'EC538814' registered no significant ($p < 0.05$) difference across the two growing years. Among 6 low PC containing soybean genotypes, germplasm accession 'IC574373' showed no significant difference in PC content across two growing years, while the 5 remaining genotypes, namely, 'IC275', 'EC606918', 'EC606917', 'JS20-98' and 'JS20-34' exhibited significant ($p < 0.05$) increase in PC content in cropping year 2019. Temperature of the location of the experiment for the months of June, July, August and September for both the cropping years 2018 and 2019 was accessed (WeatherOnline, 2019).

Table 3. Average minimum, maximum and mean temperature (°C) during crop growth stages in cropping seasons 2018 and 2019 at Indore (N 22.7196° and E 75.8577°), India

Month	Crop growth stage	Temperature (°C)					
		2018			2019		
		Average Min.	Average Max.	Mean Temp.	Average Min.	Average Max.	Mean Temp.
June	Sowing	24.5	36.0	30.2	25.7	38.3	32.1
July	Vegetative	22.7	28.6	25.5	23.6	30.6	27.1
August	Flowering + Seed development (R5)	22.5	27.5	25.0	22.7	27.5	25.1
September	Seed development (R5+R6 +R7)	20.2	29.7	25.0	22.6	28.1	25.4

Data presented in Table 3 depicts average minimum temperature, average maximum temperature and average mean temperature during the vegetative phase and seed development stages i.e. R5, R6 and R7 as described by Fehr *et al.* (1971) during the cropping seasons 2018 and 2019 at the location of the experiment. The data show significantly (1.6 °C) higher mean temperature in July, which corresponds to the vegetative phase, and slightly high mean temperature during the months of August (0.1 °C) and September (0.4 °C), which coincide with the seed development stages of crop growth, in cropping season 2019 than in cropping season 2018. More importantly, average minimum temperature (night temperature) during the month of September 2019, which coincides with the major seed filling duration as the crop passes through part of R5 and whole of R6 and R7 reproductive stages at the location of the experiment, was 2.4 °C higher than in the corresponding month in 2018. But, average maximum temperature (day temperature) was 1.6 °C higher in 2018 than in 2019 during this month. Dornbos *et al.* (1989) showed higher PC content in soybean genotype raised at 33 °C than 27 °C, with the night temperature maintained at 19 °C. Increase in PC content in some genotypes in cropping season 2019 in the present study may be attributed to the increase in average minimum temperature (night temperature) than average maximum temperature (day temperature). Further, soybean oil and nutraceutical industries seek soybean genotypes having high PC content as raw material. To obtain transgressive segregants possessing PC content higher than the maximum limit (8.80 mg/g soy flour-NRC151)

observed in the present study, it is important to cross high PC content genotypes of diverse genetic background i.e. from different country of origin. Of the 9 high PC genotypes, 4 accessions, namely, 'EC289099', 'PI596540', 'EC456556' and 'EC538814' were from USA, 4 genotypes, namely, 'NRC151', 'AVKS215', 'AVKS201' and 'IC243548' from India and the remaining 1 genotype, namely, 'PI133226' was from Indonesia. 'NRC151' is a lipoxygenase-3 free soybean breeding line developed from a cross between Indian soybean variety 'JS335' and PI417458 (a lipoxygenase-3 free accession from Japan), and its higher PC content (8.80 mg/g soy flour) than the diverse parents, namely, 'JS335' (6.50 mg/g soy flour) and 'PI417458' (7.29 mg/g soy flour) exhibited transgressive segregation for the trait. 'AVKS215' is a lipoxygenase-2 free breeding line developed from a cross between JS93-05 (Indian soybean variety) and 'PI596540'. Germplasm accession 'PI596540' (8.03 mg/g soy flour) was identified as a high PC containing soybean genotype in our results, therefore 'AVKS215' might have inherited the genes of high PC from its male parent ('PI596540'). 'AVKS201' is a lipoxygenase-1 free genotype developed by crossing Indian variety 'JS335' with 'PI133226' (lipoxygenase-1 free germplasm accession from Indonesia). Interestingly, since 'PI133226' was also identified as high PC content accession in this study, therefore high PC content in 'AVKS201' may be due to the genes inherited from 'PI133226'. 'IC243548' is an indigenous collection in national germplasm repository of India. Therefore, 4 high PC accessions from USA may be crossed with 4 and 1 high PC genotypes from India and Indonesia, respectively, to develop new genotypes which may yield PC higher than the maximum value observed in the present study. With regard to the biosynthesis of PC, it is synthesized *via* both methylation and nucleotide pathways in animals, the former involves sequential methylation of phosphatidylethanolamine (PE), while in the latter pathway free choline is incorporated into PC *via* enzymes choline kinase (CKase), cholinephosphate cytidylyltransferase (CCTase) and cholinephosphotransferase (CPTase) (Monks *et al.*, 1997). In plants, single pathway which includes the elements of both these pathways has been suggested for the PC biosynthesis, which occurs in conjunction with biosynthesis of other phospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) (Kinney, 1993). Cholinephosphate cytidylyltransferase is the rate limiting step for PC synthesis, though the regulatory roles of choline kinase and cholinephosphotransferase have also been demonstrated (Guschina *et al.*, 2014). Extreme values of PC content (high and low) observed in some genotypes consecutively for 2 years may be because of the difference in the expression of these key enzymes involved in the biosynthesis of PC, which has not been investigated in the present study. Moreover, regulatory and rate limiting role of more than one key enzymes in PC biosynthesis pathway suggests multiple genomic regions, which have not yet been reported in any oilseed crop, underlying it. Mapping population may be developed through biparental crossing between high and low PC content genotypes with different country of origin identified in this study to investigate genomic regions associated with PC biosynthesis.

Conclusions

In brief, assessment of 408 soybean germplasm accessions of different country of origin for PC content exhibited 4.3-fold genetic variability for the trait. Germplasm accessions identified with high PC content were from India, USA and Indonesia, while accessions for low PC content were from India and USA. Change in PC content due to different growing year was genotype dependent. Results also showed that the increase in average minimum temperature (night temperature) during seed filling duration may increase the PC content. The diverse genetic background of these high and low PC genotypes can be exploited by developing mapping population to identify the genomic regions underlying PC biosynthesis, which is known to have multiple rate limiting steps in its pathway, and to develop soybean genotypes with higher PC content value than the maximum value observed for this trait in this investigation.

Authors' Contributions

Conceptualization: VK and AR; Investigation: AKA and TT; Data curation: VK and AKA; Methodology: VK, AR and AKA; Supervision: HSP and VK; Writing original draft: VK and AKA; Writing-review and editing: AKA, VK, AR, TT and HSP. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

Acknowledgements

The authors are grateful to ICAR-Indian Institute of Soybean Research, Indore, Madhya Pradesh, India for providing facilities to carry out the study. Authors acknowledge Agharkar Research Institute, Pune, India for providing some germplasm accessions.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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