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COMPOSITIONAL TRAITS OF SOYBEAN SEEDS AS INFLUENCED BY PLANTING DATE IN INDIA

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SUMMARY

Information on the influence of date of planting on protein, oil and fatty acid composition of soybean seeds is meagre, whilst similar studies on lipoxygenase isozymes and trypsin inhibitor contents are lacking. A field experiment was conducted with nine Indian genotypes and three planting dates (spread over 23 days) to study the influence of planting dates on these seed traits. Results based upon a one-year study indicated that oil content declined with delays in planting. Oleic acid content increased from the first to third planting, while the reverse trend was observed for linolenic acid. Lipoxygenase-1 activity was reduced by delayed plantings. Protein content was the lowest and trypsin inhibitor content was the highest for the second planting. However, no differences were observed for protein and trypsin inhibitor contents between the first and third planting. Significant interactions, observed between genotype and planting date for most of the seed compositional characters suggest that the influence of late planting is genotype-dependent.

INTRODUCTION

Soybean (*Glycine max*), a relatively new crop in India, has acquired the status of a cash crop in the last few decades, changing the fortunes of soybean-growing farmers. This is attributed largely to the demand for Indian soybean meal in the international market, where it earns substantial foreign exchange annually. Since soybean meal is priced on its protein content, maintaining high levels in soybean seeds is essential. Soybean oil extracted in the process of producing soybean meal contributes significantly to the vegetable oil economy of the country. However, soybean oil extraction industries prefer soybean seeds that produce not only a large oil yield but also oil with high oleic and low linolenic acid contents. This obviates the need for partial hydrogenation during industrial processing to improve oxidative stability, and to produce soybean oil free of *trans*-fatty acids (Fehr and Curtiss, 2004). Lipoxygenases and trypsin inhibitor are two undesirable components in soybean seeds. Lipoxygenases not only develop off-flavours in various soy preparations by catalyzing the oxidation of polyunsaturated fatty acids present in seeds (Rackis *et al.*, 1979), but they also disrupt seed membranes following mechanical or bacterial damage during seed storage and transport (Vick and Zimmerman, 1987). Trypsin inhibitor, the protease inhibitor present in soybean seeds, is responsible for reducing the digestibility of soybean proteins by inhibiting tryptic activity (Anderson, 1992).

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Soybean, as in many regions across the globe, is a rain-fed crop in India. The time of planting of a soybean crop depends upon the onset of rains (monsoons) which is erratic over the years. Farmers have to postpone planting whenever the monsoon is delayed or there is incessant rain during the recommended planting period. There are numerous reports regarding the influence of delayed planting on yield responses (Kane *et al.*, 1996; Peltzer, 2004). However, studies on the influence of date of planting on lipoxygenases and trypsin inhibitor are lacking and information on the influence of delayed sowing on industrially important characters, namely, oil, protein and fatty acids is meagre (Kane *et al.*, 1997). The objective of this study was to characterize the changes in oil, protein, fatty acid composition, lipoxygenase isozymes and trypsin inhibitor contents of a selection of soybean genotypes in relation to the time of planting.

MATERIAL AND METHODS

The experiment was laid out in a randomized complete block design with three replicates under rainfed conditions in the fields of National Research Centre for Soybean (ICAR), Indore (22°N, 76°54'E, 618 masl) which is the epicentre of the soybean cultivation in India. The soil was a deep, montmorillonitic clay, dark greyish brown in colour and slightly alkaline. The treatment combinations were three planting dates, namely, 24 June 2003 (the recommended date of planting), 5 July 2003 (second planting) and 17 July 2003 (third planting) and nine Indian genotypes, namely, Hardee, Hara soya, JS 335, Kalitur, KHSb2, LSb1, NRC 37, Pb1 and Shilajeet, chosen for their different times to maturity. The distances between row to row and plant to plant were maintained at 0.45 m and 50 mm respectively. Before planting, the seeds were treated with *Bradyrhizobium japonicum* culture and the recommended fungicide, thiram (2 g kg⁻¹). NPK fertilizers at the rate of 20:26:17 kg ha⁻¹ were applied at planting. Standard agronomic practices for weed and insect control were followed. Days to 50 % flowering and days to maturity when 90 % of the pods had turned brown were recorded. Harvested mature seeds were subjected to different biochemical analysis, namely, protein, oil, fatty acid composition, lipoxygenase isozymes and trypsin inhibitor content.

Weather data

Total rainfall, daily maximum and minimum temperatures and mean air temperatures were recorded from flowering to maturity for each genotype across all three planting dates (Table 1).

Seed size

Seed size was expressed as the weight of 100 seeds after drying at 40 °C in an oven for six days. The values given in Table 2 are the means of three observations.

Crude protein analysis

The crude nitrogen of the soy flour sample was determined by the standard micro-Kjeldahl method. It was converted to protein content by using the conversion factor 5.71. Values given in Table 3 are the means of three observations.

Table 1. Climatic conditions during the period from flowering to maturity.

Genotype	Planting	Maximum temperature (°C)	Minimum temperature (°C)	Mean temperature (°C)	Rainfall (mm)
Hardee	I	29.6	21.2	25.4	515
	II	29.6	21.2	25.4	515
	III	29.6	20.1	24.8	422
Hara soya	I	29.4	22.4	25.9	472
	II	29.4	22.4	25.9	472
	III	29.6	20.2	24.9	515
JS 335	I	29.3	22.5	25.9	447
	II	29.6	22.4	26.0	472
	III	29.9	20.2	25.0	421
Kalitur	I	29.4	22.4	25.9	472
	II	29.6	21.2	25.4	515
	III	29.9	20.2	25.0	421
KHSb2	I	29.4	22.4	25.9	472
	II	29.6	21.2	25.4	515
	III	29.5	20.9	25.2	474
LSb1	I	29.1	23.9	26.5	498
	II	28.6	23.3	25.9	351
	III	29.0	22.6	25.8	585
NRC 37	I	29.0	20.5	24.5	525
	II	29.4	22.4	25.9	472
	III	29.5	20.9	25.2	474
Pb1	I	29.0	20.5	24.7	525
	II	29.4	22.4	25.9	472
	III	29.1	21.7	25.4	551
Shilajeet	I	28.9	22.8	25.9	491
	II	29.4	22.4	25.9	472
	III	29.6	21.2	25.4	515

Extraction and estimation of oil

Oil was extracted with n-hexane in Soxhlet apparatus for 7 h and after the evaporation of excess solvent, the weight of residual oil was used to obtain the oil content. Values given in Table 2 are the means of three observations

Fatty acids analysis

Oil extracted from the Soxhlet was trans-esterified in methanol with 1N sodium methoxide as the catalyst following Ludy *et al.* (1968). Fatty acid methyl esters (FAMEs) were separated and analysed in a gas chromatograph, Shimadzu GC 17A, using a capillary column of length 30 m and diameter 0.32 mm respectively. The oven

Table 2. Mean and range for days to flowering, days to maturity, seed size and seed compositional traits averaged for all nine soybean genotypes at different dates of planting (DOP).

Character	DOP	Range	Mean	<i>s.e.</i>	Character	DOP	Range	Mean	<i>s.e.</i>
Days to flowering	I	28–58	44.7	0.64	Oleic acid %	I	21.5–40.1	30.1	1.00
	II	27–52	41.6			II	24.6–42.8	32.0	
	III	28–45	39.4			III	24.5–41.6	32.9	
Days to maturity	I	68–117	103.6	0.80	Linoleic acid %	I	40.0–55.8	48.0	1.42
	II	70–108	97.7			II	35.9–52.6	46.0	
	III	72–101	93.0			III	36.9–53.9	45.3	
Seed size (g 100 dried seeds ⁻¹)	I	9.8–14.2	11.9	0.23	Linolenic acid %	I	4.7–7.9	6.0	0.14
	II	7.9–13.7	11.0			II	4.8–7.4	5.2	
	III	6.0–14.8	10.9			III	4.8–7.7	5.6	
Oil %	I	16.7–21.3	19.7	0.12	Lx-1 (units g ⁻ defatted soy flour)	I	624–1800	1214	25.5
	II	16.8–20.2	19.1			II	693–1424	1060	
	III	16.1–20.5	18.0			III	477–1504	1012	
Protein %	I	37.8–41.2	39.4	0.30	Lx-2+3 (units g ⁻ defatted soy flour)	I	210–500	314	20.8
	II	36.2–39.0	38.1			II	240–464	354	
	III	37.8–42.6	39.7			III	224–540	341	
Palmitic acid %	I	8.4–17.3	11.8	0.52	Total Lx (Units g ⁻ defatted soy flour)	I	834–2182	1528	25.7
	II	9.6–12.8	11.5			II	866–1868	1414	
	III	8.9–12.4	10.9			III	729–2024	1353	
Stearic acid %	I	1.9–4.8	3.1	0.42	Trypsin inhibitor (mg g ⁻ defatted soy flour)	I	40.1–87.5	56.6	2.96
	II	2.2–4.4	3.5			II	54.1–81.0	67.8	
	III	3.2–4.9	4.0			III	34.9–69.7	57.0	

Table 3. Correlation between seed compositional traits, seed size, days to maturity, minimum and mean air temperature during seed development (flowering to maturity) ($n = 81$).

Temperature	Protein	Oil	C16:0	C18:0	C18:1	C18:2	C18:3	TI	Lx1	Lx2+3	Total Lx	Days to maturity	Seed size
Minimum	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.404*	0.479**
Mean	n.s.	n.s.	n.s.	-0.414*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.284*	0.431**

*, ** and *** significant at $p = 0.05$, $p = 0.01$ and $p = 0.001$ respectively.

temperature of the gas liquid chromatograph was programmed at 140 °C for 3.6 min, then increased to 170 °C at the rate of 13.5 °C min⁻¹ and maintained at this for 3.8 min and finally increased to 182 °C at the rate of 5 °C min⁻¹. This gives the best resolution of methyl esters. The temperatures of the flame ionization detector and the injector were maintained at 240 °C. Nitrogen, the carrier gas used, was maintained at a flow rate of 15 ml min⁻¹ with a column pressure at 90 kpa. The peaks for individual fatty acid methyl esters were identified by comparing the retention times with those of standard methyl esters (Sigma-Aldrich, India). Data given in Table 2 for the different fatty acids (namely, palmitic acid [C16:0], stearic acid [C18:0], oleic acid [C18:1], linoleic acid [C18:2], linolenic acid [C18:3]) are the means of determinations of three samples.

Extraction and estimation of trypsin inhibitor (TI)

The sieved soy flour samples were extracted with 50 ml NaOH (0.01N) for 4 h with constant stirring at 125 rpm in an orbital shaker so as to keep the samples in suspension. The suspension so obtained was appropriately diluted so that 2 ml of the sample extract inhibited 40–60 % of the trypsin used as a standard in the analysis. Trypsin inhibitor activity was determined by a standard procedure (Hammerstrand *et al.*, 1981). For each sample, 2 ml aliquots of the diluted extract were added to four test tubes. A fifth test tube was prepared for the trypsin standard by adding 2 ml of distilled water. To three of the four test tubes containing the sample extract, 2 ml of trypsin solution (prepared by dissolving 0.004 g of the trypsin in 200 ml of 0.001N HCl) was added and the tubes were maintained in a constant temperature water bath at 37 °C for 10 min. Five millilitres of benzoyl DL-arginine-para-nitroanilide-hydrochloride (prepared by dissolving 0.08 g of crystalline benzoyl DL-arginine-paranitroanilide-hydrochloride in 2 ml of dimethyl sulfoxide, diluting to 200 ml with 50 mM tris buffer of pH 8.2 containing 20 mM calcium chloride and warming to 37 °C) was rapidly added to each tube. The contents were stirred immediately on a vortex mixer and the tubes were placed in a water bath at 37 °C. The reaction was terminated after exactly 10 min by the rapid addition of 1 ml of 30 % acetic acid. The fourth tube containing a sample extract (sample blank) was prepared by the same procedure except that the trypsin solution was added after the reaction was terminated by the addition of glacial acetic acid. The absorbance of each solution was determined at 410 nm against the sample blank. Values obtained from each of the two sample extracts were subtracted from the trypsin standard. These values were averaged and the trypsin content was determined as follows:

$$\text{T1 of defatted sample (mg g}^{-1}\text{)} = \frac{\text{Differential absorbance} \times \text{dilution factor}}{0.019 \times 1000}$$

$$\text{Inhibition (\%)} = \frac{100 \times \text{differential absorbance}}{\text{Absorbance of the standard}}$$

Values given in Table 2 are the means of three independent samples.

Extraction and estimation of lipoxygenase isozymes

For the determination of lipoxygenase isozymes, freshly harvested seeds were ground. To ensure the absence of fats interfering in the analysis, the ground soy flour was defatted with n-hexane until it was fat-free. The air-dried samples were sieved through a 150-mesh.

An extract was prepared by homogenization of the sieved soy flour with 100 volumes of phosphate buffer (0.2 M, pH 6.8) in a microtissue homogenizer for 20 min at 0–4 °C. The homogenized solution so obtained was further centrifuged at 10 000 rpm for 10 min at 4 °C. The supernatant so obtained was used as the crude extract for assaying lipoxygenase isozymes following the standard method (Axelrod *et al.*, 1981). The reaction mixture for lipoxygenase-1 (Lx-1) consisted of the crude extract as the enzyme source (25 µl), boric acid–borax buffer (0.2 M, pH 9.0) and 10 mM sodium linoleate

as a substrate. Lipoxygenase-2 and 3 (Lx-2 + 3) were analysed collectively with the reaction mixture consisting of crude extract as enzyme source (50 μ l), phosphate buffer (0.2 M, pH 6.8) and 10 mM sodium linoleate as a substrate. The change in absorbance was recorded in a Shimadzu UV-160 spectrophotometer at 234 nm. One unit of enzyme was taken as equivalent to the amount of enzyme that generated an increase in absorbance of 1.0 per min at 234 nm. Values given in Table 2 are the means of three samples.

RESULTS

Table 1 indicates the climatic conditions experienced by genotypes between flowering and maturity. Days to flowering, days to maturity and seed size decreased as plantings were delayed (Table 2). Significant variations between genotypes, planting dates and genotype \times planting date interaction ($p < 0.01$) for oil and protein content were observed. Among all the genotypes across all three planting dates, the protein content ranged from 36.2 % for the second planting to 42.6 % for the third planting and the oil content ranged from 16.1 % for the third planting to 21.3 % for the first planting (Table 2). The protein content observed for the second planting was lower than that from the normal planting date; however, for the third planting there was no significant difference. It is also evident from Table 2 that the oil content declined with delay in planting, i.e. the oil content in the second and third planting was less than the first planting. Three genotypes of the nine genotypes had stable oil contents across all three planting dates.

Table 2 also indicates the fatty acid profiles. Significant genotypic variation was observed for palmitic acid ($p < 0.01$) and stearic acid ($p < 0.05$), the two saturated fatty acids. Genotypic variation was found to be more significant ($p < 0.01$) than planting date ($p < 0.05$) for palmitic acid. Among all the genotypes across the three planting dates, the palmitic acid content was observed to range from 8.4 % in the first planting to 17.3 % in the second planting, while stearic acid ranged from 1.9 % in the first planting to 4.9 % in the third planting. Genotypic variation in oleic acid content was found to be more significant ($p < 0.001$) than planting date ($p < 0.05$) and genotypic \times planting date interaction was evident ($p < 0.05$) for oleic acid content. Oleic acid content of different genotypes ranged from 21.5 % for the first planting to 42.8 % for the second planting. Genotypic and genotype \times planting date variation were found to be significant ($p < 0.01$) for linoleic acid, ranged from 35.9 % in the second planting to 55.8 % in the first planting. Genotypic and genotype \times planting date interaction were found to be more significant ($p < 0.001$) than planting date for linolenic acid ($p < 0.01$). Linolenic acid ranged from 4.7 % to 7.9 %. Oleic acid increased from the first to third planting while linolenic acid showed the reverse trend; the differences were not significant for linoleic acid.

Soybean seed lipoxygenase exists in three isozymic forms, i.e. lipoxygenase-1 (Lx-1), lipoxygenase-2 (Lx-2) and lipoxygenase-3 (Lx-3) which have been divided into two classes. Class I is characterized by neutral pH optima such as lipoxygenase-1 while class II designates high pH optima (8.5) such as lipoxgenase-2 and lipoxygenase-3. The

Table 4. Correlation (r) between various seed compositional traits, seed size and days to maturity ($n = 81$).

	Oil	C16:0	C18:0	C18:1	C18:2	C18:3	TI	Lx -1	Lx -2+3	Total Lx	DM	Seed size
Protein	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	-0.337*	n.s.	n.s.	n.s.	n.s.	n.s.
Oil		n.s.	n.s.	-0.354*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.601***	n.s.
C16:0			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
C18:0				n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
C18:1					-0.963***	n.s.	-0.523**	n.s.	n.s.	n.s.	-0.530**	n.s.
C18:2						n.s.	0.480**	n.s.	n.s.	n.s.	-0.562**	n.s.
C18:3							n.s.	n.s.	0.475**	n.s.	n.s.	n.s.
TI								n.s.	0.351**	n.s.	n.s.	n.s.
Lx1									0.543***	0.973***	n.s.	n.s.
Lx-2+3										0.733***	-0.353*	n.s.
Total Lx											n.s.	n.s.
DM												-0.284*

*, ** and *** significant at $p = 0.05$, $p = 0.01$ and $p = 0.001$ respectively.

total lipoxygenase and lipoxygenase-1 activities were observed to be higher in normal than in delayed plantings (Table 2). Genotype, planting date and genotype \times planting date variations were found to be highly significant for lipoxygenase-1 and total lipoxygenase activity ($p < 0.001$). Genotype and genotype \times planting date variation for lipoxygenase-2 + 3 were also found to be highly significant ($p < 0.001$). Among all the genotypes across all three planting dates, lipoxygenase-1 ranged from 477 to 1800 units g^{-1} defatted soy flour in the third and first planting respectively, while lipoxygenase-2 + 3 ranged from 210 units to 540 units g^{-1} in the first planting and third planting respectively. Genotypic, planting date variations were found to be more significant ($p < 0.001$) than genotypic \times planting date interactions ($p < 0.01$) for trypsin inhibitor content. Among all the genotypes across all three planting dates, trypsin inhibitor content ranged from 40.1 to 87.5 mg g^{-1} defatted soy flour. Trypsin inhibitor content was highest in the second planting, i.e. planting delayed by 12 days; however, there was no difference in the trypsin inhibitor content between third planting and normal planting (Table 2).

Tables 3 and 4 indicate the various correlations identified. Daily mean air temperature and minimum temperature showed negative correlations with days to maturity ($p < 0.05$) and positive correlations with seed size ($p < 0.01$), while the minimum temperature showed a negative correlation with stearic acid content ($p < 0.05$) (Table 3). A highly significant positive correlation was observed between oil content and days to maturity ($p < 0.001$), while there was a negative correlation between oleic acid content and days to maturity and ($p < 0.01$) (Table 4). Trypsin inhibitor was observed to be negatively correlated with protein content ($p < 0.05$) and oleic acid ($p < 0.01$) and positively correlated with linoleic acid ($p < 0.01$) (Table 4). Lipoxygenase-2 + 3 activity showed a positive correlation with lipoxygenase-1 ($p < 0.001$), linolenic acid and trypsin inhibitor content ($p < 0.01$), while a negative correlation was observed between lipoxygenase-2 + 3 and days to maturity ($p < 0.05$).

DISCUSSION

As the genotypes selected for the study differed in days to flowering and maturity, the temperatures they experienced at these times also differed between planting dates (Table 1). Maximum temperatures experienced by all the genotypes across the three planting dates from flowering to maturity more or less remained the same while the minimum temperature ranged from 20.1 to 23.9°C. All cultivars experienced lower daily mean temperature in the third planting compared with the first and second planting except for cultivars Pb1 and NRC 37 which experienced lower mean temperatures from flowering to maturity in the first planting. None of the genotypes at any of the dates of planting experienced moisture stress (Table 1). Delays in planting resulted in reductions in the number of days to flowering and to maturity (Table 2). This may be attributed to the photosensitive nature of soybean and the reduction in photoperiod experienced by genotypes from the first to third planting. The growing location (22°N) experiences a reduction in photoperiod from 21 June to 21 December. Genotypes varied in how days to flowering and days to maturity changed for different planting dates. The early maturing cultivar LSb1 was the least sensitive for the time taken to flower and days to maturity under different planting dates while the late maturing genotype Hardee was the most sensitive. This is consistent with a previous report (Byth, 1968) which suggested that earlier maturing soybean genotypes are less affected in terms of days to flowering and to maturity by changes in photoperiod than later maturing types. Table 2 also shows that there was a decrease in seed size due to delayed planting. However, there was no significant difference in the seed size between the second and third plantings.

The variations ($p < 0.01$) observed for genotype, planting date and genotype \times planting date interaction for oil and protein content are consistent with earlier reports (Alvarez *et al.*, 2004; Kane *et al.*, 1997). The decline in oil content in delayed plantings and genotypic variation in the rates of decline also agree with an earlier report (Kane *et al.*, 1997). It has also been reported that soybeans grown in warm conditions produce seeds with higher oil content than those grown in cooler conditions (Kane *et al.*, 1997; Piper and Boote, 1999). Table 1 indicates that, barring NRC 37 and Pb1, daily mean temperatures from flowering to maturity were, in general, higher for the crop planted at normal time than for the third planting. Reduced oil contents in the second and third plantings could not be explained by temperature differences, although there was a positive correlation between oil content and days to maturity.

Kane *et al.* (1997) showed that planting date had little or no influence on palmitic acid, stearic acid and linoleic acid contents. Our results agreed with their findings except in the case of stearic acid, which increased with late planting. Increases in oleic acid and decreases in linolenic acid contents agree with an earlier report by Kane *et al.* (1997). However, they attributed the increase in oleic acid and decrease in linolenic acid contents to elevated temperatures during seed fill. However, no correlation was observed between oleic acid and linolenic acid contents and temperature over the period from flowering to maturity in our study. This may be because temperature differences were small. However, the positive correlation between days to maturity

and linoleic acid content and negative correlation between days to maturity and oleic acid content were consistent with an earlier study in sunflower, which reported oleic acid content to have a positive association with earliness (Fernandez-Martinez *et al.*, 1989).

Total lipoxygenase and lipoxygenase-1 activity declined in delayed planting while trypsin inhibitor increased in the second planting and then decreased in the third planting. These results cannot be compared with any previous work as reports are lacking. However, negative correlations observed between trypsin inhibitor and protein content (Table 4) are in agreement with an earlier report (Fu and Lu, 1992).

Although, the studies were conducted for one year only, significant interactions were observed between genotype and planting date for most of the characters. This suggests that the influence of late planting is genotype-dependent. Furthermore, oil content, lipoxygenases and seed size were considerably reduced with delayed planting. However, no consistent changes were observed for protein and trypsin inhibitor contents. The recommended time of planting (late June) was observed to be the most appropriate planting date for obtaining the maximum oil contents. Changes in fatty acid composition, leading to an increase in oleic acid and a decrease in linolenic acid with delayed planting, suggest that soybean varieties specially bred for high oleic acid and low linolenic acid for oxidative stability of oil may have an advantage if planting is delayed. Reduced total lipoxygenase activity in seeds harvested from delayed plantings suggest that these seeds may have improved keeping quality in storage and yield soy-products with reduced off-flavours.

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