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# Lipoxygenase isozymes and trypsin inhibitor activities in soybean as influenced by growing location

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**Abstract**

Lipoxygenase isozymes, responsible for the off-flavour associated with soy-based foods, and trypsin inhibitor, that affects protein digestibility, are two undesirable biological components present in soybean. The information on influence of growing location on lipoxygenase isozymes and trypsin inhibitor is negligible. The dry seeds of seven Indian soybean varieties, grown at four locations widely differing in latitude and therefore climate, were evaluated for isozymes of lipoxygenase and trypsin inhibitor. Variation in different varieties, at different locations, for the activities of lipoxygenase-I and lipoxygenase-II + III ranged from 450 to 2042 and 118 to 600 units per gramme of soy flour, respectively, while, for trypsin inhibitor, it varied from 42 to 113 mg per gramme of soy flour. Averaged over seven genotypes, Palampur, the coolest location, showed maximum mean values for Lox-I as well as Lox-II + III, indicating the influence of minimum mean temperature prevalent from flowering to maturity on lipoxygenase activitity. Genotypic, locational and genotypic×locational variations were found to be significant for Lox-I as well as Lox-II + III activity. Genotypic variation and genotypic×locational interaction were found to be significant for trypsin inhibitor activity.


*Keywords:* Lipoxygenase isozymes; Trypsin inhibitor; Soybean; Growing location

## 1. Introduction

Soybean has emerged as one of the most economical and nutritious foods that can combat diseases ascribed to mal- and under nutrition in developing countries. Lately, it is gaining importance in developed nations as well because of its nutraceutical ingredients, namely isoflavones, that prevent the risk of diseases such as breast cancer, cardiac arrest, osteoporosis, kidney stone and menopausal blues (Messina, 1997). However, barring far eastern countries, such as China and Japan, where soy-based foods have been in use for a long time, the off-flavour associated with soy-based foods is the prime deterrent for soybean, not being relished in many nations, e.g. India, North America. The major culprit of this off-flavour is seed lipoxygenase (EC 1.13.11.12), an iron containing dioxygenase that catalyses the oxidation of unsaturated fatty acids resulting in the formation of aldehyde and ketone compounds (Rackis, Hinig, Seessa, & Steggard, 1979). These volatile compounds lend objectionable off-flavours associated with soy-based foods. Lipoxygenase in soybean seeds is present in the form of three isozymes i.e., Lox-I, Lox-II and Lox-III, which have been categorized into two classes. Class I is characterized by high pH optima of around 9.0 and formation of large amounts of 13-hydroperoxides, e.g. lipoxygenase-I (Lox-I) while class II has pH optima of around 7.0 and formation of equal amounts of 9 and 13-hydroperoxides, e.g. lipoxygenase- II and III (Lox-II + III).

Soybean suffers from another shortfall due to the presence of trypsin inhibitor that affects protein digestibility (Liener & Kakade, 1980). Though trypsin inhibitor is heat-labile, heat-treatment insolubilizes the much-valued proteins (Anderson, 1992) and, more importantly, excessive heat-treatment can cause loss of essential amino acids in soy proteins (Rios-Iriarte & Barnes, 1996). At high temperatures, amino acids, such as lysine, bind with reducing sugar and are rendered unavailable, while cystine, already limiting in soybean seeds, is destroyed.

*Abbreviation:* LOX, lipoxygenase.

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Genotypic variation in lipoxygenase isozymes and trypsin inhibitor have been reported globally (Kumar, Patra, Rani, & Tiwari, 2002; Kumar, Rani, & Prakash, 2001; Marczy, Simon, Mozsik, & Szajani, 1995; Viktorova, 1982; Yang et al., 1999) and soybean lines with null levels of lipoxygenases and trypsin inhibitor have also been developed (Bernard & Hymowitz, 1986; Hajika, Igita, & Kitamura, 1991; Kitamura, Ishimoto, Ktikuchi, & Kaizuma, 1992). However, barring isolated studies (Chapman, Robertson, & Burdick, 1976; Marczy et al., 1995) where the effect of environment on lipoxygenase isozymes has been studied, there is no information available on the influence of growing location on the activities of lipoxygenase isozymes and trypsin inhibitor in soybean. Though Madhaya Pardesh is the epicentre of soybean cultivation in India, soybean has made inroads into new geographical locations widely differing in latitude. This study was undertaken to evaluate lipoxygeanase isozymes and trypsin inhibitor content in the mature seeds of soybean varieties grown at different locations with varying latitude in India with an aim to study the effect of growing location on the activities of lipoxygenase isozymes and trypsin inhibitor.

## 2. Materials and methods

### 2.1. Materials

Seeds of cultivars [Punjab-1, Shilajeet, JS 335, NRC 37, Hardee, KhSb 2, Kalitur (black seeded), Harit Soya from the same lot] were grown in the fields of H. P. Krishi Vishva Vidyalya, Palampur (32°N); G.B. Pantnagar University of Agriculture and Technology, Pantnagar (29°N); National Research Centre for Soybean, Indore (22.2°N) and University of Agricultural Sciences, Bengalore (12.6°N). All the above- mentioned cultivars were sown at Palampur, Pantnagar, Indore and Bengalore on 18 June, 12 June, 27 June and 28 July 2001, respectively, according to the recommended date of sowing at these locations. Freshly harvested seeds of these cultivars from all four locations were analysed for lipoxygenases and trypsin inhibitor content.

### 2.2. Sample preparation

For determination of lipoxygenase isozymes, freshly harvested seeds were ground. The ground soy flour was defatted with *n*-hexane until the soy flour became fat-free to ascertain the absence of fats interfering in the analysis. The air-dried samples were sieved (150 mesh).

### 2.3. Preparation of crude extract and estimation of lipoxygenase isozymes

The soybean extract was prepared by homogenization of 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, Cheesebrough, & Laaskso, 1981). Reaction mixture for lipoxygenase-I consisted of crude extract as enzyme source (25 μl), boric acid borax buffer (0.2 M, pH 9.0) and 10 mM sodium linoleate as a substrate. Lipoxygenase- II and III were analysed collectively with the reaction mixture, consisting of crude extract as enzyme source (50 microlitre), 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.

### 2.4. Extraction and estimation of trypsin inhibitor

The sieved soy flour samples so obtained were extracted with 50 ml NaOH (.01 N) 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 acvtivity was determined by standard procedure (Hammerstrand, Black, & Glover, 1981). Of the five test tubes taken, 2 ml aliquots of the diluted sample were added to the 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 of trypsin solution (prepared by dissolving 0.004g of the trypsin in 200 ml of 0.001 N HCl) were added and then were maintained at a constant temperature water bath 37 °C for 10 min. Five millilitres of benzoyl DL-arginine para nitroanilide hydrochloride (prepared by dissolving 0.08 gm of benzoyl DL-arginine paranitroanilide hydrochloride in 2 ml of dimethyl sulfoxide and diluted to 200 ml with 50 mM tris buffer of pH 8.2 containing 20 mM calcium chloride and the contents were warmed to 37 °C) were rapidly added to each tube. The contents were stirred immediately on a vortex mixture 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 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 30% 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:

Table 1
Lipoxygenase isozymes' activities (units)[a] in various soybean cultivars grown at different locations[b]

| Variety | Lox-I | | | | | Lox-II + III | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | P'pur | P'nagar | Idore | B'lore | Mean | P'pur | P'nagar | I'dore | B'lore | Mean |
| Punjab-1 | 844±32 | 450±17 | 780±22 | 844±25 | 730 | 450±17 | 410±11 | 218±11 | 208±11 | 321 |
| Shilajeet | 1352±45 | 784±25 | 870±21 | 798±19 | 951 | 496±21 | 231±8 | 142±9 | 308±15 | 294 |
| JS 335 | 1540±45 | 1020±25 | 1188±29 | 1225±32 | 1243 | 265±14 | 155±13 | 148±10 | 440±13 | 252 |
| NRC 37 | 1856±38 | 892±17 | 882±23 | 1388±26 | 1255 | 315±16 | 150±12 | 188±7 | 316±15 | 242 |
| KhSb 2 | 1508±35 | 1444±35 | 1421±45 | 1476±29 | 1462 | 476±13 | 230±11 | 118±9 | 184±9 | 252 |
| Kalitur | 1280±35 | 1504±45 | 1283±25 | 1260±35 | 1332 | 360±14 | 373±8 | 526±12 | 517±13 | 444 |
| Harit Soya | 2042±29 | 1430±45 | 1186 25 | 820±18 | 1369 | 600±11 | 155±7 | 160±8 | 372±13 | 322 |
| Mean | 1489 | 1075 | 1087 | 1116 | 1192 | 423 | 286 | 214 | 335 | 315 |

[a] 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; values given are average of three replications + S.D.
[b] P'pur, Palampur; P'nagar, Pantnagar; I'dore, Indore; B'lore, Bengalore.

$$\text{TI mg/g of defatted sample} = \frac{\text{Differential Absorbance} \times \text{dilution factor}}{0.019 \times 1000}$$

$$\text{Percent inhibition} = \frac{100 \times \text{differential absorbance}}{\text{Absorbance of the standard}}$$

## 3. Results and discussion

Table 1 shows that activity of lipoxygenase-I for different varieties at different locations ranged from 450 to 2042 units per gramme of soy flour. The highest and lowest activities for lipoxygenase-I were observed in Harit Soya from Palampur and Punjab-1 from Pantnagar, respectively. Genotypic, locational and genotypic×locational variations were found to be highly significant ($P < 0.01$, ANOVA). All the varieties, except Kalitur, expressed maximum Lox-I activity at Palampur. However, Punjab-1 showed same values of maximum Lox-I activity at Palampur as well as at Bengalore. Averaged over four locations, Punjab-1 showed the least mean value for Lox-1 activity, followed by Shilajeet, JS 335, NRC 37, Kalitur, Harit Soya and KhSb 2. Averaged over eight genotypes, Palampur showed the maximum mean value for Lox-1 activity, followed by Bengalore, Indore and Pantnagar.

Table 1 also shows that activity of lipoxygenase-II + III for different varieties, at different locations, ranged from 118 to 600 units per gramme of soy flour. The highest and lowest activities for Lox-II + III were observed in Harit Soya from Palampur and KhSb 2 from Indore, respectively. Genotypic, locational and genotypic×locational variations were found to be highly significant ($P < 0.01$, ANOVA). All the varieties, except Kalitur and JS 335, expressed maximum Lox-II + III activity at Palampur. However, NRC 37 showed the same values of maximum activity of Lox-II + III at both Palmpur and Bengalore. Averaged over four locations, NRC 37 showed the least values for Lox-II + III activity, followed by KhSb 2, Shilajeet, Punjab-1, Harit Soya. Averaged over eight genotypes, Palampur showed the maximum mean value for Lox-II + III activity, followed by Bengalore, Pantnagar and Indore.

Trypsin inhibitor activity ranged from 42 to 113 mg per gramme of defatted soy flour among all locations and genotypes (Table 2). Minimum activity was observed in Punjab-1 at Indore while Shilajeet and Kalitur expressed maximum activity. Genotypic and genotypic×locational interaction were found to be significant ($P < 0.01$, ANOVA). However, locational effects were found to be non significant. At all locations, except at Bengalore, Punjab-1 showed minimum activity. Averaged over four locations, the least average trypsin inhibitor activity was observed in Punjab-1, followed by KhSb 2, Hara Soya, Kalitur, JS 335, NRC 37 and Shilajeet.

Varieties did not respond similarly to changes in growing conditions. The largest difference for Lox-I value for any cultivar was 149% for Harit Soya between

Table 2
Comparative activity of trypsin inhibitor[a] in various Indian soybean cultivars grown at different locations

| Cultivar | Trypsin inhibitor activity[b] | | | | |
|---|---|---|---|---|---|
| | P'pur | P'nagar | I'dore | B'lore | Mean |
| Punjab-1 | 53±3.2 | 46±3.8 | 42±3.7 | 72±5.1 | 52.6 |
| Shilajeet | 110±4.8 | 97±6.5 | 113±7.4 | 109±7.4 | 107 |
| JS335 | 104±6.3 | 97±5.8 | 75±3.7 | 77±5.8 | 88.3 |
| NRC37 | 93±4.9 | 100±7.0 | 102±8.2 | 85±6.3 | 95.0 |
| KhSb2 | 70±4.6 | 63±5.6 | 66±5.8 | 65±5.9 | 66.0 |
| Kalitur | 95±6.1 | 70±4.7 | 113±6.5 | 72±5.6 | 87.3 |
| Harit Soya | 64±4.2 | 84±4.0 | 63±3.0 | 73±3.8 | 71.5 |
| Mean | 84.1 | 79.6 | 82.0 | 79.0 | 81.2 |

[a] mg per gramme of defatted soy flour; values given are averages of three replications + S.D.
[b] P'pur, Palampur; P'nagar, Pantnagar; I'dore, Indore; B'lore, Bengalore.

Table 3
Different weather parameters at different locations during soybean growth period

| Location | Developmental stages[a] | Total rainfall mm | Temperature (°C) | |
|---|---|---|---|---|
| | | | Max. | Min. |
| Palampur (32°N) | I | 1247.3 | 27.1 | 19.8 |
| | II | 173.6 | 26.8 | 14.1 |
| | III | 1420.9 | 26.9 | 17.4 |
| Pantnagar (29°N) | I | 602.4 | 32.7 | 26.0 |
| | II | 628.0 | 29.0 | 21.4 |
| | III | 1230.0 | 30.4 | 23.2 |
| Indore (22°N) | I | 242.7 | 30.1 | 23.8 |
| | II | 225.2 | 32.5 | 23.8 |
| | III | 467.9 | 31.6 | 23.8 |
| Bengalore (12°N) | I | 209.0 | 26.6 | 19.8 |
| | II | 442.0 | 27.0 | 19.1 |
| | III | 651.0 | 26.8 | 19.4 |

[a] I, sowing to flowering; II, flowering to maturity; III, sowing to maturity.

Bengalore and Palampur while Kalitur showed the least variation (19%). The largest difference for Lox-II + III value for any cultivars was 303% for KhSb 2 between Palampur and Indore, while least variation was observed in Kalitur (44%). Thus black seeded Kalitur was most stable for both classes of lipoxygenase isozymes over different locations. KhSb 2 was the most stable genotype for trypsin inhibitor activity while Punjab-1, which is one of the genotypes with lowest activities, showed maximum variation (71%) for trypsin inhibitor activity. Thus the levels of lipoxygenase isozymes and trypsin inhibitor have been found to be influenced to different degrees by cultivar as well as climatic effect. Correlation between trypsin inhibitor and both classes of lipoxygenase isozymes was also studied and no correlation was present ($P > 0.5$) indicating that both the classes of lipoxygenases and trypsin inhibitor are not genetically linked and the genetic improvement for all these three factors is possible by simple breeding methods.

Table 3 and Fig. 1 detail the weather conditions and rainfall pattern at the four locations, Palampur, Pantnagar, Bengalore, and Indore, during the main developmental stages of soybean growth. The total growth duration, i.e. from flowering to maturity varies from location to location. It was 18, 17, 16 and 15 weeks at Palampur, Pantnagar, Indore and Bengalore, respectively. Rainfall is considered to be the most important factor influencing the ontogeny of soybean. In this regard, Palampur faced very low precipitation during the most crucial soybean growth stage, i.e. from flowering to maturity and, during this stage, with regard to temperature as well, Palampur was the coolest and most northerly location. Changes in the environmental growth conditions have been reported to affect seed composition with respect to protein content, fatty acid composition, carbohydrate and seed yield of soybean (Nielson, 1996). Changes in the activities of both the classes of lipoxygenase isozymes, at different locations, as observed in our studies, may be the result of the varying temperatures (Table 3) and rainfall pattern (Fig. 1) during the soybean growth period at different locations. The same cultivar genotype was subjected to different climatic conditions during the period of biosynthesis of lipoxygenase isozymes at different locations. Thus highest activity for Lox-I and Lox-II + III, averaged over all the varieties at Palampur, than at other locations may be explained on the basis of low

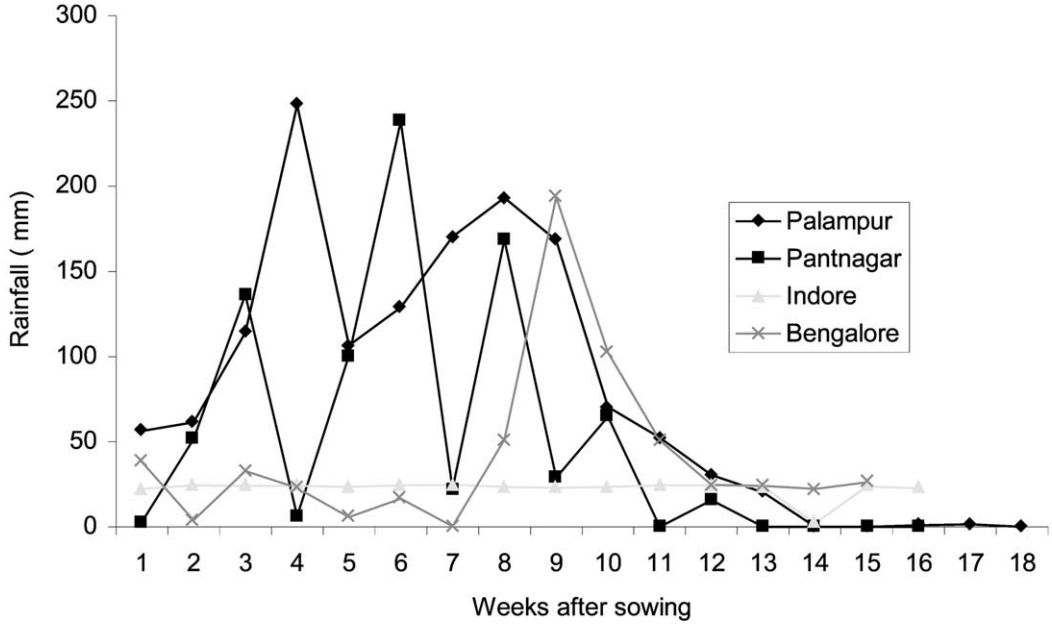


Fig. 1. Distribution of rainfall at different locations during soybean growing season 2001.

temperature, coupled with low rainfall prevailing from the flowering to pod maturity. This is supported by the earlier study (Marczy et al., 1995) where it has been suggested that higher temperatures diminish the biosynthesis of both the classes of lipoxygenase isozymes. However, the results obtained are in contrast with the earlier report (Chapman et al., 1976) where the soybean lipoxygenases have been suggested to be uninfluenced by the environment.

Conclusively, lipoxygenase isozyme activities in soybean seeds are influenced significantly by the growing locations and there exists a genotype×latitude interaction. Trypsin inhibitor activity is not influenced significantly by location itself but there exists a genotype×location interaction. A location specific evaluation of seed lipoxygenase isozymes and trypsin inhibitor is required to evaluate the overall potential of all the released varieties of soybean for food uses.

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