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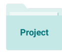

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Interrelationship Among Lipoxygenase Isozymes, Polyunsaturated Fatty Acids and Trypsin Inhibitor During Seed Development in Soybean

VINEET KUMAR[1], ANITA RANI[2] AND G. S. CHAUHAN[3]

National Research Centre for Soybean (ICAR), Khandwa Road, Indore 452 017, M. P., India
(E-mail: vineetksahni@yahoo.com)

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ABSTRACT

Lipoxygenase isozymes, polyunsaturated fatty acids and trypsin inhibitor content were determined in four selected soybean varieties during seed development. A genotypic variation was observed for accumulation pattern of lipoxygenase isozymes and trypsin inhibitor, besides, significant levels of trypsin inhibitor in the early stage of soybean seed development. Linolenic acid was found to be maximum at 30 days after flowering and thereafter decreased continuously at varying rate in all the genotypes till maturity. Lipoxygenase isozymes levels were comparatively low in the early stage of development when linolenic acid was very high. A significant positive correlation of trypsin inhibitor content with lipoxygenase I as observed in present studies suggests a coordinated expression of these biological components during seed development in soybean.

Key words: Soybean, lipoxygenase isozymes, polyunsaturated fatty acids, trypsin inhibitor, seed development

Lipoxygenase (Linoleate: oxygen oxidoreductase, EC 1.13.11.12) and trypsin inhibitor are considered undesirable components in soybean seeds (Rackis *et al.* 1979, Anderson-Hafferman *et al.* 1992). In general, normal soybean seed lipoxygenase (Lox) exists in three isozymic forms namely Lox-I, Lox II and Lox III (Axelrod *et al.* 1981) and constitutes about 1-2 percent of the proteins present in dry seeds (Kitamura 1984). These isozymes catalyse the hydroperoxidation of polyunsaturated fatty acids (PUFA), linoleic and linolenic acid, containing *cis cis 1,4 pentadiene* moiety and have been categorized into two classes. Class I lipoxygenase (Lox-I) is characterized by high pH optima of around 9.0 and formation of large amounts of 13-hydroperoxides while class II lipoxygenase (II+III) show pH optima of around 7.0 and formation of equal amounts of 9 and 13-hydroperoxides. The hydroperoxidation reaction catalysed by the lipoxygenase isozymes lead to the formation of volatile hexanal compounds

[1]*Scientst Sr. Scale (Plant Biochemistry),* [2]*Scientist Sr. Scale (Plant Breeding),* [3]*Prof (Food Technology) and Director*

ɔnsible for the development off-products, the prime deterrent in ptability by Indian populace. Fur-ɛan seed lipoxygenases have also to cause seed deterioration . During storage and transport, ıechanical or bacterial damage, mbrane bound and storage lipids ne favorable substrates for ɔ catalyse oxidation. Free radi-in the process, set the chain re-on of membrane lipids, ultimately ːmbrane integrity (Vick and 87).

hibitor (TI), the protease inhibi-eeds, is responsible for reducing ɔroteins by inhibiting tryptic ac-TI is heat labile, the heat treat-izes the much-valued proteins 2) and cause loss of essential soy proteins (Rios-Iriarte and

genotypic variability for Lox A and TI has been reported in ıs (Marczy *et al.* 1995, Yang *et et al.* 2001). Recently, variabil-ıaracters has been reported in among Indian genotypes (Kumar ʼ2; 2003; Rani *et al.* 2004). The he developmental expression of ınd their substrates, PUFA, and growth is important to view the of immature soybean pods for ption and to understand the in-ımong these biological compo-ed development. However, the ; on the expression and interre-ological components during seed e few and scattered (Yao *et al. t al.* 1986, Liu and Markakis ıe present study was undertaken the interrelationship between ʼUFA and TI in developing seed ndian soybean varieties.

MATERIALS AND METHODS

Four commercial varieties of Indian soybean *viz.* JS 335, Pb 1, NRC 37 and Shilajeet were sown in 3 meters rows with a spacing of 45 cm in the experimental fields of National Research Centre for Soybean (ICAR), Indore on 27th June 2003. Sufficient number of plants was tagged in each variety on the day of flowering. Hand picking of green immature pods commenced from 30 days after flowering (daf) and continued till harvest maturity with an interval of 5 days. The seeds were removed from picked pods for further analyses.

Extraction and estimation of lipoxygenase isozymes

For determination of lipoxygenase isozymes, fresh green seeds were ground using pestle and mortar in liquid nitrogen. The ground freeze-dried samples were defatted with petroleum ether and air dried to evaporate petroleum ether. The enzyme was extracted with soybean extract with 100 volumes of phosphate buffer (0.2 M, pH 6.8) by agitation in a micro tissue homogenizer for 20 minutes at 0-4°C. The homogenized solution so obtained was centrifuged at 10, 000 rpm for 10 minutes at 4°C. The supernatant so obtained was used as the crude extract for the assay of lipoxygense isozymes following the standard method (Axelrod *et al.* 1981). The reaction mixture for lipoxygenase-I consisted of crude extract as enzyme source, 2.8 ml of 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, 0.2 M phosphate buffer (pH 6.8) and 10 mM sodium linoleate as a substrate. The change in absorbance was recorded in 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 absorb-

per minute due to conjugate diene in [...]atic hydroperoxidation at 234 nm.

[...]tion and estimation of trypsin [...]itor (TI)

One gram of fresh green pods was ex[...]ed in 50 ml of 0.01N NaOH for 4 hours constant stirring at 125 rpm in an orbital [...]er so as to keep the samples in suspension. suspension so obtained was appropriately [...]ed so that 2 ml of the sample extract inhib- 40-60 percent of the trypsin used as a stand-[...]n the analysis. TI activity was determined [...]tandard procedure (Kakade *et al.* 1974) as [...]ified by Hammerstrand *et al.* (1981). Of the test tubes taken, 2 ml aliquots of the diluted [...]ple were added to the four test tubes. A test tube was prepared for the trypsin stand-[...]y adding 2 ml of distilled water. To three of [...]our test tubes containing the sample extract, of trypsin solution (prepared by dissolving 4 g of the trypsin in 200 ml of 0.001 N HCl) added and were maintained at a constant [...]erature water bath 37°C for 10 minutes. milliliters of benzoyl DL- arginine para[...]anilide hydrochloride (prepared by dissolv-[...].08 g of benzoyl DL arginine paranitroanilide [...]ochloride in 2 ml of dimethyl sulfoxide and [...]ed to 200 ml with 50 mM Tris buffer (pH containing 20 mM calcium chloride and the [...]ents were warmed to 37°C) was rapidly [...]d into each tube. The contents were stirred [...]ediately on a vortex mixture and the tubes [...] placed in a water bath at 37°C. The reac-[...] was terminated after exactly 10 minutes by [...]apid addition of 1 ml of 30 percent glacial [...]c acid. The fourth tube containing sample [...]act (sample blank) was prepared by the [...] procedure except that the trypsin solution added after the reaction was terminated by [...]addition of 30 percent glacial acetic acid. absorbance of each solution was determined [...]0 nm against the sample blank. Values ob-[...]d from each of the two sample extracts were subtracted from trypsin standard. These values were averaged and the trypsin content was determined as follows:

TI mg /g of defatted sample = Differential absorbance x Dilution factor / 0.019 x 1000

Percent Inhibition = 100 x Differential absorbance / Absorbance of the standard

Estimation of polyunsaturated fatty acids

Oil was extracted from oven dried shelled seeds using petroleum ether (bp 40-60°C). Methyl esters were prepared from the oil by interesterification in methanol using sodium methoxide as the catalyst (Luddy *et al.* 1968). Fatty acid methyl esters (FAMEs) prepared were separated and analyzed in gas liquid chromatograph (GLC), Shimadzu GC 17A, using capillary column with length and diameter of 30 meter and 0.32 millimeter, respectively. Oven temperature of the GLC was programmed at 140°C for 3.6 minutes, and subsequently increased to 170°C at the rate of 13.5°C per minute and maintained for 3.8 minutes and finally to 182°C at the rate of 5°C per minute for obtaining best resolution of methyl esters. The temperatures of flame ionization detector (FID) and injector were maintained at 240°C. Nitrogen, the carrier gas used, was maintained at a flow rate of 15 ml/minute with column pressure at 90 kpa. The peaks for individual FAMEs were identified by comparing the retention times with those of standard methyl esters (procured from Sigma, USA). The analysis was done in triplicate samples and the mean values were reported.

Qualitative analysis of Kunitz inhibitor

Kunitz inhibitor from developing seeds was extracted in Tris-Cl buffer (100 mM, pH 6.8) containing 0.23 M $CaCl_2$ and 5 mM phenyl methyl sulfonyl chloride (PMSF) following Kollipara *et al.* (1991) and was resolved using non denaturing discontinuous polyacrylamide slab gel con-

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