Biochemical studies on lipoxygenases induced in response to abiotic and biotic stresses in groundnut (*Arachis hypogaea* L.)

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

Haritha Maramreddi
Enrolment No. 02LPPH13

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500 046, INDIA

August, 2009

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
(A Central University established in 1974 by Act of Parliament)
Hyderabad-500 046, INDIA

DECLARATION

I hereby declare that the work embodied in this thesis entitled **"Biochemical studies on lipoxygenases induced in response to abiotic and biotic stresses in groundnut (*Arachis hypogaea* L.)"** has been carried out by me under the supervision of **Dr. G. Padmaja** and this has not been submitted for any degree or diploma of any other university earlier.

Dr. G. Padmaja
(Research Supervisor)

Haritha Maramreddi
(Research Scholar)

Prof. A. S. Raghavendra
(Research Co-Supervisor)

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
(A Central University established in 1974 by Act of Parliament)
Hyderabad-500 046, INDIA

CERTIFICATE

This is to certify that **Ms. Haritha Maramreddi** has carried out the research work embodied in the present thesis under my supervision and guidance for a full period prescribed under the Ph.D. ordinance of this University. We recommend her thesis entitled **"Biochemical studies on lipoxygenases induced in response to abiotic and biotic stresses in groundnut (*Arachis hypogaea* L.)"** for submission for the degree of **Doctor of Philosophy** of this University.

Dr. G. Padmaja
(Research Supervisor)

Prof. A. S. Raghavendra
(Research Co-Supervisor)

Head
Dept. of Plant Sciences

Dean
School of Life Sciences

DEDICATED TO

MY MOTHER AND FATHER

ACKNOWLEDGEMENTS

At the outset I would like to express my gratitude to my mentor ***Dr. G. Padmaja*** *for providing me with an opportunity to work in her lab. I also thank for all her unwavering support, encouragement and guidance throughout my doctoral discourse. I thank* ***Prof. A. S. Raghavendra,*** *my co-supervisor for his constant help and support.*

Words fail to express my gratitude and heartfelt indebtness to ***Prof. P. Reddanna,*** *for providing the lab facilities, his encouragement and moral support he gave me throughout my research work. He is persistent source of encouragement and valuable guidance throughout the course of the present investigation.*

I would especially thank ***Prof. Appa Rao podile, Prof. P. B. Kirti*** *former Heads, Department of Plant Sciences,* ***Prof. A.R. Reddy,*** *Head, Department of Plant Sciences,* ***Prof. A. S. Raghavendra,*** *Dean, School of Life Sciences and the former deans* ***Prof. T. Suryanarayana and Prof. R. P. Sharma*** *for allowing me to use the general facilities of the school.*

My heartfelt thanks to ***Prof. Aparna Dutta Gupta*** *for her timely help, kind support and encouragement.*

I am also indebted to ***Prof. AppaRao Podile,*** *for his support and suggestions given to me throughout my research.*

I would like to thank ***Prof. S. Sokka Reddy,*** *ANGRAU for his suggestions and support.*

My special thanks to ***H. D. Upadyaya,*** *Senior Scientist, ICRISAT and* ***S. Vijaya Kumar,*** *Principal Scientist, Regional Agricultural Station, Raichur, Karnataka for providing me the groundnut seed throughout my experiments.*

I acknowledge ***CSIR*** *for providing me the financial assistance as JRF and SRF.*

Special thanks to Prof. P.Reddanna's research team for their timely help and support.

I take this opportunity to convey my deepest sense of gratitude to all my teachers, since my childhood whose guidance and encouragement at each step was instrumental in shaping my career.

I would also like to express my thankfulness to ***Gangadhar, Prasad Dr. Anil Kumar, Anil*** *and* ***Praveen*** *for their suggestions and help during my research.*

I fall short of words to thank my best friends ***Madhavi, Priya, Chandi*** *and* ***Sony*** *for being there with me all through and for their help and encouragement.*

I wish to extend my thanks to all my friends ***Salomi, Riaz, Hema, Saritha*** *and* ***Neeraja*** *who have helped me in many ways and made my stay at the University of Hyderabad a pleasant and memorable one.*

*I also thank my co-operative and friendly lab mates **Madhumita, Samuel, Sujatha, Dr. Shukla** and **Latha**. Special thanks to Kantha Rao for his help in lab and field experiments.*

Assistance from the non teaching staff of the Department of Plant Sciences is greatly acknowledged.

My deepest sense of gratitude to my mother-in law and father-in-law for their support, and encouragement.

*I would like to express my heartfelt thanks to my parents for their relentless support and abundant patience shown throughout the years. I wouldn't have accomplished all this without their unconditional love and encouragement. My brother's **Ravi** and **Dinesh** have been very cooperative throughout and always there to help whenever and whereever.*

*It's my pleasure to thank my husband **Bharat** for his support, patience and guidance given to me throughout my work. I thank my daughter **Tushara** for her love and affection.*

I thank almighty if not for the one above nothing in this world would be possible.

Haritha

CONTENTS

LIST OF FIGURES

Fig. 18. Native PAGE showing LOX isozyme profiles in roots of 20-day-old seedlings of K-1375 variety after different hours of treatment with 100 μM ABA.

Fig. 19. Native PAGE showing LOX isozyme profiles in roots of 20-day-old seedlings of K-1375 variety after different hours of treatment with 100 μM MJ.

Fig. 20. LOX activity in seedlings of ICG-4747 after different hours of inoculation with *R. solani.*

Fig. 21. Activity staining of LOX isozymes after inoculation of seedlings of ICG-4747 variety with *R. solani.*

Fig. 22. Ten-day-old seedlings of TMV-2 variety after inoculation with *S. rolfsii* along with controls.

Fig. 23. Ten-day-old seedlings of R-2001-1 variety after inoculation with *S. rolfsii* along with controls.

Fig. 24. LOX activity in shoots of resistant and susceptible varieties after 48 h of inoculation with *Sclerotium rolfsii* along with controls.

Fig. 25. LOX activity in shoots and roots of TMV-2 and R-2001-1 varieties after inoculation with *S. rolfsii.*

Fig. 26. LOX activity in shoots of R-2001-1 variety after different intervals (6-96 h) of inoculation with *S. rolfsii.*

Fig. 27. Activity staining of LOX isozymes in shoots of R-2001-1 variety after 6- 24 h of inoculation with *S. rolfsii.*

Fig. 28. Activity staining of LOX isozymes in shoots of R-2001-1 variety after 30-48 h of inoculation with *S. rolfsii*.

Fig. 29. Activity staining of LOX isozymes in shoots of R-2001-1 variety after 54-72 h of inoculation with *S. rolfsii*.

Fig. 30. Activity staining of LOX isozymes in shoots of R-2001-1 variety after 78-96 h of inoculation with *S. rolfsii*.

Fig. 31. LOX activity in shoots of TMV-2 variety after different intervals (6-96 h) of inoculation with *S. rolfsii.*

Fig. 32. Activity staining of LOX isozymes in shoots of TMV-2 variety after 6-24 h of inoculation with *S. rolfsii*.

Fig. 33. Activity staining of LOX isozymes in shoots of TMV-2 variety after 30-48 h of inoculation with *S. rolfsii*.

Fig. 34. Activity staining of LOX isozymes in shoots of TMV-2 variety after 54-72 h of inoculation with *S. rolfsii*.

Fig. 35. Reverse Phase-High Performance Liquid Chromatographic separation of standard 13-HODE.

Fig. 36. RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 18 h interval in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety in comparison to control.

Fig. 37. RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 48 h interval in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety in comparison to control.

Fig. 38. RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 72 h interval in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety in comparison to control.

Fig. 39. RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 30 h interval in shoots of *S. rolfsii* inoculated seedlings of TMV-2 variety in comparison to control.

Fig. 40. RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 48 h interval in shoots of *S. rolfsii* inoculated seedlings of TMV-2 variety in comparison to control.

Fig. 41. RT-PCR analysis of the transcript levels of *PnLOX-1* gene in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety along with controls.

Fig. 42. RT-PCR analysis of the transcript levels of *PnLOX-2* gene in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety along with controls.

Fig. 43. RT-PCR analysis of the transcript levels of *PnLOX-3* gene in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety along with controls.

Fig. 44. Purification of LOX from *S. rolfsii* inoculated seedlings of R-2001-1 variety by DEAE–cellulose column chromatography.

Fig. 45. HPLC chromatogram showing the peaks after passing the purified lipoxygenase obtained from DEAE–cellulose column through the anion exchange HPLC.

Fig. 46a. Native-PAGE of LOX purified from *S. rolfsii* inoculated seedlings of R-2001-1 variety.

Fig. 46b. SDS-PAGE of LOX purified from *S. rolfsii* inoculated seedlings of R-2001-1 variety.

Fig. 47. Effect of pH on activity of purified LOX from *S. rolfsii* inoculated seedlings.

Fig. 48. Effect of temperature on activity of purified LOX from *S. rolfsii* inoculated seedlings.

Fig. 49a & b. SP-HPLC chromatograms of 13-hydroperoxides and hydroxides produced from purified groundnut LOX obtained through anion exchange chromatography.

Fig. 50a & b. SP-HPLC chromatograms showing separation of LOX products produced from commercially available soybean LOX.

Fig. 51. Photomicrographs showing the zone of inhibition on the growth of *S. rolfsii* in the presence of 13-hydroperoxides and 13-hydroxides by using filter disc assay method.

Fig. 52. Photomicrographs showing the zone of inhibition on the growth of *R. solani* in the presence of 13-hydroperoxides and 13-hydroxides by using filter disc assay method.

Fig. 53. Photomicrographs showing the zone of inhibition on the growth of *F. oxysporum* in the presence of 13-hydroperoxide and 13-hydroxides by using filter disc assay method.

Fig. 54. Photomicrographs showing the effects of 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE on mycelial weight in *S. rolfsii*.

Fig. 55a & b. Effects of hydroperoxides and hydroxides on mycelial weight in *S. rolfsii*.

Fig. 56. Photomicrographs showing the effects of 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE on mycelia weight in *R. solani*.

Fig. 57a & b. Effects of hydroperoxides and hydroxides on mycelia weight in *R. solani*.

Fig. 58. Photomicrographs showing the effects of 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE on mycelia weight in *F.oxysporum*.

Fig. 59a & b. Effects of hydroperoxides and hydroxides on mycelia weight in *F. oxysporum*.

Fig. 60. Photomicrographs showing the effects of 13-HPOTrE, 13-HPODE, 13-HOTrE and 13-HODE on formation of sclerotia in *S. rolfsii* after 7 days.

Fig. 61. Photomicrographs showing the effects of 13-HPOTrE, 13-HPODE, 13-HOTrE and 13-HODE on formation of sclerotia in *S. rolfsii* after 14 days.

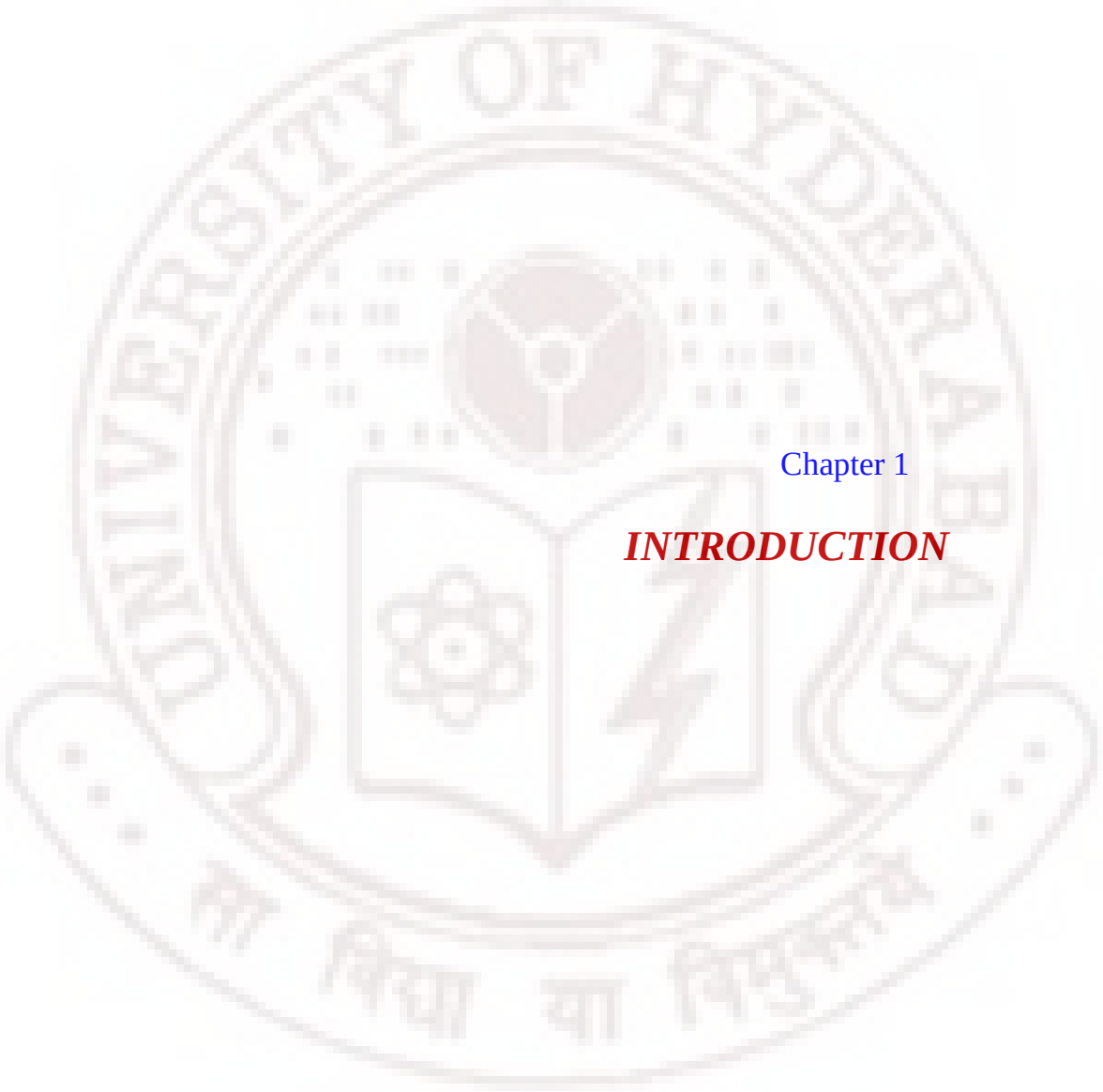
Fig. 62. Photomicrographs showing the effects of 13-HPOTrE, 13-HPODE, 13-HOTrE and 13-HODE on formation of sclerotia in *R. solani* after 7 days.

Fig. 63. Photomicrographs showing the effects of 13-HPOTrE, 13-HPODE, 13-HOTrE and 13-HODE on formation of sclerotia in *R. solani* after 14 days.

TABLES

ABBREVIATIONS

°C	:	Degree centigrade/degree Celsius
µM	:	Micromolar
ABA	:	Abscisic acid
ALA	:	α-linolenic acid
$CaCl_2$	:	Calcium chloride
cDNA	:	complementary Deoxyribonucleic acid
DEAE	:	Diethylaminoethyl cellulose
EDTA	:	Ethylene diamine tetra acetic acid
F. oxysporum	:	*Fusarium oxysporum*
g	:	Gram
h	:	Hour(s)
HCl	:	Hydrochloric acid
13-HODE	:	13-hydroxyoctadecadienoic acid
13-HOTrE	:	13-hydroxyoctadecatrienoic acid
13-HPODE	:	13-hydroperoxyoctadecadienoic acid
13-HPOTrE	:	13-hydroperoxyoctadecatrienoic acid
HPLC	:	High performance liquid chromotography
HR	:	Hypersensitive response
kDa	:	Kilodalton
KN	:	Kinetin
l	:	Litre
LA	:	Linoleic acid
LOX	:	Lipoxygenase
mg	:	Milligram
MIC	:	Minimum inhibitory concentration
min	:	Minute(s)
MJ	:	Methyl jasmonate
ml	:	Millilitre
mM	:	Millimolar
NaCl	:	Sodium chloride
NaCN	:	Sodium cyanide
NDGA	:	Nordihydroguaiaretic acid
nm	:	Nanometer
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
PEG	:	Polyethylene glycol
PGPR	:	Plant growth promoting rhizobacteria
PMSF	:	Phenylmethanesulphonyl fluoride
R. solani	:	*Rhizoctonia solani*
RP-HPLC	:	Reverse phase-High performance liquid chromatography
rpm	:	Revolutions per minute
RT-PCR	:	Reverse transcriptase-polymerase chain reaction
S. rolfsii	:	*Sclerotium rolfsii*
SDS	:	Sodium dodecyl sulphate
SP-HPLC	:	Straight phase-High performance liquid chromatography
TEMED	:	N,N,N',N'-tetra methylenediamine
Tris	:	Tris-(hydroxymethyl) aminoethane
UV	:	Ultraviolet



Chapter 1

INTRODUCTION

INTRODUCTION

1.1 Groundnut - Need for studies on lipoxygenases:

Groundnut (*Arachis hypogaea* L.) is an important legume cash crop, which provides a rich source of high-quality, edible oil (45-50%), easily digestible protein (23-25%), minerals and vitamins. In India, groundnut is an important oil, food and forage crop and millions of farmers depend on its cultivation for their livelihoods.

The yields of groundnut are reduced by diseases including early leaf spots (*Cercospora arachidicola*), late leaf spots (*Phaeoisariopsis personata*), rust (*Puccinia arachidis*), collar rot (*Aspergillus* spp.), root rot (*Macrophomina phaseolina*), stem rot (*Sclerotium rolfsii*) and rhizoctonia damping off caused by *Rhizoctonia solani*. High salinity and drought are also important reducers of yield in many parts of the world. Although the sources with varying levels of resistance to different diseases have been identified in the groundnut germplasm, the mechanisms of resistance are still obscure. Knowledge of the complexity of mechanisms responsible for resistance to different diseases is necessary for strategies to be defined which will improve resistance in highly susceptible crop species (Figueiredo *et al*., 2008). Research on lipoxygenases (LOX) has received attention due to its potentially significant role in plant microbe interactions. LOXs are involved in various physiological processes in plants such as growth and development, senescence, wound and defense responses.

1.2 Substrates for LOXs:

LOXs (LOXs; linoleate-oxygen oxidoreductase; EC 1.13.11.12) constitute a large gene family of non-heme iron containing dioxygenases widely distributed in plants and animals. These enzymes play a key role in lipid metabolism and catalyze the addition of molecular oxygen to polyunsaturated fatty acids (PUFAs) with a 1,4-

cis, cis-pentadiene structure to form conjugated diene hydroperoxide (Hiledbrand *et al*., 1988; Siedow, 1991; Holtman *et al*., 1996). The principal substrates for LOXs in higher plants are linoleic acid (9,12-octadecadienoic acid) and α-linolenic acid (9,12,15-octadecatrienoic acid). Linoleic acid (LA) and α-linolenic acid (ALA) belong to n-6 and n-3 fatty acid families, respectively. These are the terminal fatty acids synthesized in most plants but not by most other higher eukaryotes. Linoleic acid upon the action of lipoxygenase is converted either to 13-hydroperoxyoctadecadienoic acid (13-HPODE) or 9-hydroperoxyoctadecadienoic acid (9-HPODE). Lipoxygenases mediate the conversion of α-linolenic acid into 9- or 13-hydroperoxyoctadecatrienoic acids (HPOTrEs). The conversion of ALA into either 9- or 13-HPOTrE depends on the source of lipoxygenase and specificity of the enzyme (Gardner, 1991).

1.3 Role of LOX metabolites:

The hydroperoxy polyunsaturated fatty acids, synthesized by the action of various highly specialized forms of lipoxygenases, are substrates of at least seven different enzyme families. Signaling compounds such as jasmonates, antimicrobial and antifungal compounds such as leaf aldehydes or divinyl ethers, and a plant-specific blend of volatiles including leaf alcohols are among numerous products (Anderson, 1989; Siedow, 1991; Gardner, 1995; Gardner *et al*., 1996). Due to their reactive nature, fatty acid hydroperoxides can be quite active by themselves and are capable of producing membrane damage and promoting cell death (Hildebrand *et al*., 1988; Siedow, 1991; Ricker and Bostock, 1993). The LOX product, 13S-hydroperoxides are intermediates in the pathway for the production of traumatin, traumatic acid and methyl jasmonate (Siedow, 1991; Gardner, 1995 & 1998).

Traumatic acid is formed from autooxidation of the aldehydes moiety in traumatin. Both traumatic acid and traumatin are involved in wound healing of plant tissues (Zimmerman and Coudron, 1979), which can reduce the entry of pathogens into plant tissues through wounds. Jasmonates (jasmonic acid and/or its methyl ester) have been shown to affect a variety of physiological processes (Koda, 1992; Staswick, 1992). Jasmonates and their octadecanoid precursors were the first oxylipins with an assigned messenger function. Jasmonate products of the LOX pathway can serve as signals that act to induce expression of genes for defense response in plants (Farmer and Ryan, 1992). It has been suggested that plant LOXs may play a role in membrane degradation observed during senescence, wounding, and the hypersensitive response to pathogen attack (Hildebrand *et al*., 1988; Siedow, 1991).

1.4 Involvement of LOX in plant growth and development:

LOX isozymes show large developmental changes and the activity of LOX is generally high in young, rapidly growing tissues (Altschuler *et al*., 1989; Park and Polacco, 1989). Studies looking for possible roles of lipoxygenase in plant growth and development have been limited primarily to correlations between the appearance of lipoxygenase activity and the temporal course of a specific developmental sequence. It has been suggested that LOX may be involved in regulating or triggering various processes in the cell during the early stages of germination rather than being primarily involved in respiration or in mobilization of unsaturated fatty acids (Siedow, 1991). Terp *et al*. (2006) showed that etiolated seedlings of oilseed rape contained more lipoxygenase derived hydroperoxides in non-esterified fatty acids than green seedlings. The 13-lipoxygenase derivates were 6-8-fold more abundant than the 9-derivatives.

LOX and its hydroperoxide products might also directly participate in senescence. Fatty acid hydroperoxides reportedly can cause senescence by several different mechanisms including inactivation of protein synthesis, inhibition of photochemical activity in chloroplasts and deterioration of cellular membranes (Thomas and Stoddart, 1980). Oxidation of polyunsaturated fatty acids in the membranes could lead to increased membrane permeability which could result in increased calcium levels in cells, stimulation of phospholipases and release of free fatty acids. The free fatty acids are then peroxidised by LOX, accelerating the cycle and increasing the deterioration of membranes and metabolic function (Leibowitz and Jhonson, 1971; Mack *et al*., 1987; Hildebrand *et al*., 1988).

1.5 Involvement of lipoxygenases in resistance/tolerance to diseases and abiotic stresses:

LOX pathway has been shown to be activated by abiotic and biotic stresses in different plant species. Products derived from PUFAs *via* lipoxygenase pathway play a significant role in plant resistance against pathogens. The products of LOX pathway contribute to defense reactions by inhibition of pathogen growth and development (Namai *et al*., 1990; Ohta *et al*., 1990), induction of phytoalexin accumulation (Li *et al*. 1991) and/or in signal transduction (Choi *et al.,* 1994).

Increases in LOX activity have been reported for a number of plant/pathogen systems (Koch *et al*., 1992). In those cases in which both virulent and avirulent pathogens were used, LOX activity was found to increase only upon infection by the avirulent pathogen (Keppler and Novacky, 1987; Ohta *et al*., 1991) or to be induced rapidly with the avirulent pathogen and more slowly, if at all, with the virulent strain (Ocampo *et al*., 1986; Croft *et al*., 1990). LOX activity is also induced by treatment

of plants (Peever and Higgins, 1989) or cell cultures (Rickauer *et al*., 1990) or elicitors.

The induction of lipoxygenase activity up to 7-fold was reported in tobacco leaves over a period of 11 days following infection with a powdery mildew, *Erysiphe cichracearum* (Lupu *et al*., 1980). Similarly, induction of LOX activity was recorded in tobacco leaves treated with cryptogein, a protein of the fungus *Phytophthora cryptogea* (Rusterucci *et al*. 1999). These results suggest that the rapid induction of LOX is involved in the defense response to pathogen attack.

Farmer and Ryan (1992) have proposed a model for an octadecanoid signal transduction pathway in plants that is activated by wounding or pathogen attack. According to the model, perception of an extracellular signal results in the activation of a plasma membrane-bound lipase that releases linolenic acid from membrane phospholipids. The linolenic acid is then converted by constitutive enzymes (LOX and others) into the octadecanoid signal molecule, jasmonic acid, which results in gene activation.

LOX have a role in both direct and indirect forms of pest resistance in plants (Hildebrand *et al*., 1988; Shibata and Axelrod, 1995). This was suggested by the immediate massive burst of LOX product formation upon wounding of plant tissues. The production of volatile compounds increases dramatically upon wounding of plant leaves and a major portion of volatile compounds are apparently the products of LOX activity. In direct resistance reactions the lipid hydroperoxy radicals and some of the secondary oxidation products resulting from LOX action on linolenic acid are toxic to plant pathogens. LOX products such as hexenals have an adverse effect on pathogenic organism. The activation of defense responses after mechanical wounding and insect

attack appears to be mediated also by jasmonic acid and its ester methyl jasmonate (MJ) collectively termed jasmonates.

The accumulation of LOX metabolites has been considered as a marker of hypersensitive cell death (HR) in plant pathogen interactions (Montillet *et al*. 2002). Signal transduction pathways are activated during HR, leading to biosynthesis or release of potential anti-microbial effector molecules, which are thought to contribute to both host and pathogen cell death (Wang *et al*. 1996). The lipoxygenase products produced in *Phaseolus vulgaris* were able to inhibit growth of *Aspergillus flavus* grown in liquid cultures. It was observed that trans-2-hexenal possesses anti-protozoan, antifungal and insecticidal activities (Lyr and Basaniak, 1983).

In rice, LOX activity has been described to correlate positively with resistance to blast disease, since the octadecanoid pathway is activated after infection by the fungus (Ohta *et al*., 1991). The LOX activity in the seeds of different genotypes of pearl millet with different susceptibility to downy mildew revealed a good correlation between enzyme activity and their downy mildew reaction in the field (Nagarathna *et al*., 1992). Similarly, LOX enzyme activity showed a positive relationship with resistance to leaf blast disease in Brazilian rice cultivars (Sandhu *et al*., 2007). It has also been reported that maize genotypes, exhibiting field resistance to *Aspergillus flavus*, accumulated higher amounts of volatile aldehydes, produced by the LOX pathway, compared with susceptible varieties (Zeringue *et al*. 1996).

In pearl millet, three LOX isozymes designated LOX-1, -3 and -6 induced in downy mildew resistant seedlings in response to inoculation with *S. graminicola* pathogen have been purified and characterized (Babitha *et al*., 2004). The results

indicated that the LOX isozymes were dimers composed of two unequal subunits of 43 and 40 for LOX-1, 40 and 37 for LOX-3, 38 and 35 for LOX-6.

LOX gene expression has been shown to be regulated by different effectors such as the source/sink status (Fischer *et al*., 1999), jasmonic acid (Creelman and Mullet, 1997), abscisic acid (Melan *et al*., 1993), and also by different forms of stress, such as wounding (Porta *et al*., 1999), water deficiency (Porta *et al*., 1999), or pathogen attack (Melan *et al*., 1993). The *Arabidopsis LOX1* gene was differentially regulated in plant organs and was induced in response to the hormones ABA and MJ, and attack by *Pseudomonas syringae* suggesting a role in plant defense (Melan *et al*., 1993).

Cheng *et al*. (2006) found that the expression levels of *PdLOX1* and *PdLOX2* varied in *Populus deltoides*, when exposed to different stresses with the pathogen *M. brunnea* f. sp. Multigermtubi, exerting a relatively stronger influence on *PdLOX1* expression. It was proposed that the two lipoxygenases play an important role in Poplar resistance to biotic and abiotic stresses.

LOX may also have a direct role in the protection of plant tissues. LOX metabolites might be acting directly on the fungal pathogen leading to the development of resistance. The inhibitory effect of 13-HPOTrE and 9-HOTrE on germination of conidia of rice blast, *Pyricularia oryzae* has been reported (Shimura *et al.* 1983). Similarly, cytospore germination of *Phytophthora capsici* was inhibited by 9- and 13-HPOTrE as well as by the hydroxyl derivatives of arachidonic acid (Ricker and Bostock, 1994). Uma Maheswari *et al*. (2000) showed that HPOTrE was more effective in inhibiting the growth and multiplication of pigeonpea wilt fungus, *Fusarium udum* than HPODEs. It was suggested that ALA metabolites of LOX

pathway might be involved in mediating the defense responses of the plant against wilt fungus.

The involvement of LOX in response to abiotic and biotic stresses has been demonstrated in different species. However, very limited information is available on its role in disease resistance and abiotic stresses in groundnut. Initial biochemical studies had led to the identification of at least three different peanut seed LOX isozymes (Sanders *et al*., 1975; Pattee and Singleton, 1977), two had pH optima of 6.2, and the other an optimum of 8.3 (Sander *et al*., 1975). Three peanut LOX isozymes showed similar pH activity profiles to that of three major soybean LOXs suggesting that one produced primarily 13S-hydroperoxy fatty acids, one primarily 9S-hydroperoxy fatty acids and the other produced significant amounts of both products (Sanders *et al*., 1975; Pattee and Singleton, 1977).

Prakash *et al*. (1990) observed a correlation between the total chlorophyll content and the activity of lipoxygenase of groundnut cotyledons during growth and senescence. Both total chlorophyll content and lipoxygenase activity increased during early stages of germination upto 8th day followed by a sharp decline at later stages. Sailaja *et al*. (1997) reported that the lipoxygenase activities in groundnut increased on treatment with a PGPR strain *Bacillus subtilis* AF1, and with crown rot pathogen, *Aspergillus niger*. Calvo *et al*. (1999) established the sporogenic effects of 9S- and 13S-hydroperoxy fatty acids on *Aspergillus* spp. In peanut seed, *Aspergillus* infections induced expression of *PnLOX1* encoding a mixed-function LOX producing approximately 21% 9S-HPODE and 59% 13S-HPODE (Burow *et al*., 2000). Tsitsigiannis *et al*. (2005) characterized two peanut seed LOX alleles (*PnLOX2* and *PnLOX3*) that were strongly repressed after *Aspergillus flavus* infection. *PnLOX2* and

PnLOX3 were both 13-HPODE producers (13-LOX) and were specifically expressed in seeds. These studies further supported a case for 9-LOX as susceptibility factors and 13-LOX as resistance factors in mycotoxin contamination in seed crops.

Keeping in view the importance of LOXs in several physiological processes, the present study is aimed at investigating the role of LOXs in resistance/tolerance to abiotic and biotic stresses in groundnut.

The specific objectives of the study are:

- To study the induction of LOX in groundnut seedlings subjected to NaCl, PEG, ABA and MJ treatments.
- To study the involvement of LOX in groundnut seedlings after inoculation with *Sclerotium rolfsii and Rhizoctonia solani.*
- Purification and partial characterization of LOX isozymes induced in groundnut seedlings upon inoculation with *Sclerotium rolfsii*.
- To analyze the expression pattern of *PnLOX-1*, *PnLOX-2* and *PnLOX-3* genes using RT-PCR in groundnut seedlings inoculated with *Sclerotium rolfsii*.
- To analyze the antifungal activity of LOX products against *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum* pathogens of groundnut.

Chapter 2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 The need for studies on lipoxygenases in groundnut:

The cultivated peanut or groundnut (*Arachis hypogaea* L.) is one of the major oilseed crops of the tropics and subtropics, although it is also cultivated in the warm areas of the temperate regions (Hammons, 1994). It is a valuable source of edible oil (43-55%) and protein (25-28%) for human beings, and of fodder for livestock. About two thirds of world production is crushed for oil and the remaining one third is consumed as food.

Groundnut is an allotetraploid with 2n = 4X (2A + 2B) = 40 (Husted, 1936; Stebbins, 1957; Seijo *et al*., 2004). It belongs to the subfamily Papilionoideae, family Fabaceae (formerly Leguminosae). Groundnut is a self-pollinating, indeterminate, annual, herbaceous legume. Natural cross pollination occurs at rates of less than 1% to greater than 6% due to atypical flowers or action of bees (Coffelt, 1989). The fruit is a pod with one to five seeds that develops underground within a needle like structure called a peg, an elongated ovarian structure.

Groundnut originated in the southern Bolivia/north west Argentina region in South America and is presently cultivated in 108 countries of the world. It is cultivated on 26.5 million ha in the world, with an average annual production of 35.7 million tons in the year 2003 (FAO, 2003). The average yield world over is 1348 kg/ha.

Groundnut is a valuable cash crop for millions of small-scale farmers in the semi-arid tropics. It generates employment on the farm and in marketing, transportation and processing. The largest producers of groundnut are China and India, followed by Sub-Saharan African countries and Central and South America.

China leads in production of peanut having 37.5% share of overall world's production followed by India (19%) and Nigeria (11%). In India peanut occupies a prominent position in the national edible oil economy.

The major abiotic factors affecting groundnut production include drought, high temperature, low soil fertility, low soil pH, and iron chlorosis. Groundnut is prone to several diseases, the fungi and viruses being the major pathogens compromising its cultivation and economic profit around the world. The major fungal diseases are early leaf spots (*Cercospora arachidicola*), late leaf spots (*Phaeoisariopsis personata*), rust (*Puccinia arachidis*), collar rot (*Aspergillus* spp.), root rot (*Macrophomina phaseolina*), stem rot (*Sclerotium rolfsii*) and rhizoctonia damping off caused by *Rhizoctonia solani*. These diseases cause yield losses of 40 to 60% either singly or in combination (Nigam and Lenne, 1996).

Wild *Arachis* species are a reservoir of high levels of resistances to several stress factors. Because of the low genetic diversity in the peanut crop, wild relatives are an important source of novel genes. Differences in ploidy rendered peanut sexually isolated, giving this species a very narrow genetic base (Stalker *et al*., 1995; Raina *et al*., 2001). Thus, introgression of wild genes into groundnut is only possible through complex crosses or genetic transformation (Proite *et al*., 2007). Breeding efforts have been successful to some extent in the development of groundnut varieties with varying levels of resistance to different diseases. However, the mechanism of resistance is poorly understood. Conventional strategies for disease resistance and management have met with less success due to lack of proper understanding of the mechanism of resistance (Anjana *et al*., 2007). A better understanding of the mechanisms of plant defense against pathogens might lead to improved strategies for

enhancement of disease resistance in groundnut. Efforts are needed to gain insights into the early biochemical defense responses for identification of markers for resistance which accelerates the groundnut improvement.

Plants possess inducible defense system to withstand the attack of the pathogens. Early recognition of the pathogen and activation of resistance responses is often responsible for determining the compatibility or incompatibility of host-pathogen interaction (Lee and Hwang, 2005). Host–pathogen interactions are often accompanied by elevated activities of oxidative enzymes (Lupu *et al*., 1980). The enzyme lipoxygenase (LOX) is known to play a role in disease resistance in many host-pathosystems.

Lipoxygenases (LOXs; EC 1.13.11.12) are a class of enzymes that are widely distributed in eukaryotes (Siedow, 1991; Yamamoto, 1992). These enzymes play a key role in lipid metabolism and catalyze the first step in the dioxygenation of polyunsaturated fatty acids forming hydroperoxy fatty acids. LOXs and the metabolic and signal transduction pathways initiated by them have distinct functions in several physiological processes such as reproductive development, seed germination, senescence, programmed cell death, tolerance to cold, drought and salt stresses and resistance to diverse pathogens and mycotoxins (Rosahl, 1996).

2.2 LOX enzyme classification:

Plant LOXs are classified with respect to their positional specificity of linoleic acid (LA) oxygenation because arachidonic acid is only a minor polyunsaturated fatty acid (PUFA) in the plant kingdom. The generation of oxylipins is initiated by LOXs, which form hydroperoxides from ALA (18: 3) or LA (18: 2) (Feussner and Kuhn, 2000; Feussner and Wasternack, 2002). LA is oxygenated either at carbon atom 9 (9-

LOX) or at C-13 (13-LOX) of the hydrocarbon backbone of the fatty acid leading to two groups of compounds, the (9*S*)-hydroperoxy- and the (13*S*)-hydroperoxy derivatives of PUFAs (Fig. 1). With ALA as the substrate, (13S)-hydroperoxyoctadecatrienoic acid (13-HPOTrE) or (9S)-hydroperoxyoctadecatrienoic acid (9-HPOTrE) are formed (Fig. 2).

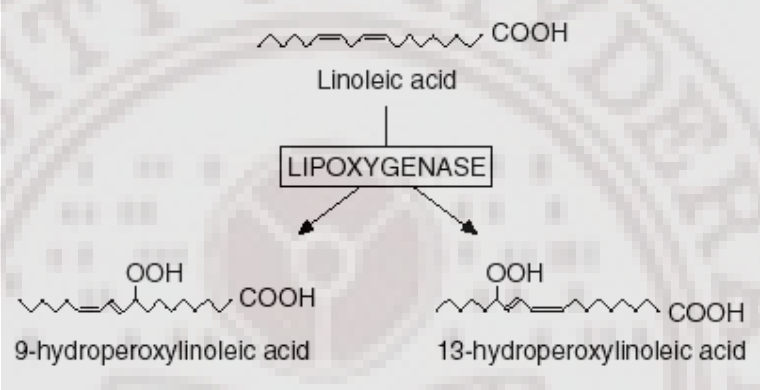


Fig. 1. The formation of 9- and 13-hydroperoxides from linoleic acid by LOXs. (Source: Seed lipoxygenases: Occurrence and functions. Loiseau *et al*. 2001)

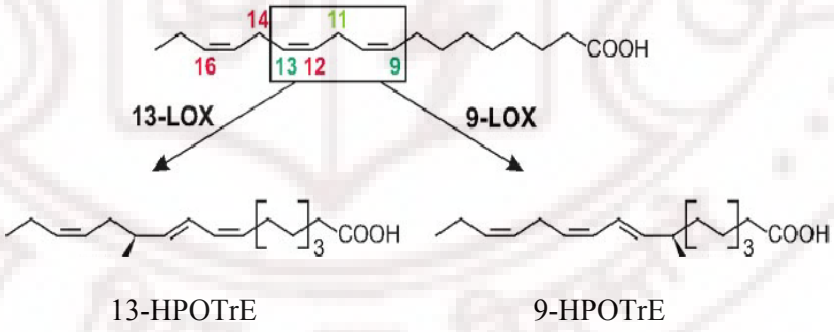


Fig. 2. The formation of 9- and 13-hydroperoxides from α-linolenic acid by LOXs (Source: The lipoxygenase pathway. Feussner and Wasternack, 2002)

Several lipoxygenase isozymes have been identified in different plant species. Their biochemical properties, gene structure and expression, developmental

regulation, tissue distribution and physiological roles have been studied mainly in soybean (Hildebrand, 1989; Siedow, 1991; Gardner, 1995; Rosahl, 1996; Shibata, 1996; Casey, 1999). The various isoforms have been classified as two types according to two criteria. The first, and older criterion, relies on catalytic behaviour, such as the pH for optimum activity and the positional specificity for the hydroperoxide substrates (Siedow, 1991). Type-1 lipoxygenase, later designated lipoxygenase-1 (Christopher *et al*., 1970) has optimum activity at pH 9-10. Type-2 lipoxygenase generally have pH optima of 6-7. Some type-2 lipoxygenases also catalyse secondary reactions leading to pigment bleaching and production of oxodienoic acids (Klein *et al*., 1985; Siedow, 1991).

A more comprehensive classification of plant LOXs, based on comparison of their primary structure, has been proposed (Shibata *et al.,* 1994). According to their overall sequence similarity, plant LOXs are grouped into two gene sub families. Those enzymes harboring no transit peptide have a high sequence similarity (>75%) to one another and are designated type 1-LOXs. However, another subset of LOXs carries a putative chloroplast transit peptide sequence. Based on this N-terminal extension and the fact that these enzymes show only a moderate overall sequence similarity (~35%) to one another, they have been classified as type 2-LOXs. To date, all these LOX forms belong to the subfamily of 13-LOXs.

2.3 Substrate specificity of LOXs:

The majority of plant LOXs strongly prefer free fatty acids as substrates (Siedow, 1991). However, certain plant and animal LOXs are capable of oxygenating also ester lipid substrates, such as phospholipids (Brash *et al.,* 1987; Murray and Brash, 1988), triacyl glycerols (Holtman *et al.,* 1997; Feussner *et al,*. 1998) and

cholesterol esters (Belkner *et al.*, 1991). Even more complex lipid protein assemblies such as biomembranes (Kuhn *et al.,* 1990a; Maccarrone *et al.,* 1994) were metabolized by LOXs. LOX1 from soybean seeds and a LOX from cucumber roots exhibited activity with PUFAs esterified to phospholipids (Brash *et al.,* 1987, Matsui *et al.,* 1999) in accordance with the suggested involvement of LOXs in membrane permeabilization. As a consequence of altering the physico-chemical properties of membranes *via* a modification of their fatty acid residues, fluxes of assimilates or ions are facilitated (Hildebrand, 1989; Serhan *et al.,* 1981).

2.4 LOX structure, function and occurrence:

LOX proteins have a single polypeptide chain with a molecular mass of 75-81 kDa (≈662-711 amino acids) in mammals and 94-103 kDa (≈838-923 amino acids) in plants (Shibata and Axelrod, 1995; Brash, 1999). Exceptions have been reported, molecular weights ranging from 72-108 kDa have been published for lipoxygenases isolated from pea (Galliard and Chan, 1980). Plant LOXs have been characterized to some extent in widely divergent plants as rice, soybean, cotton and sunflower (Eskin *et al.,* 1977, Vick and Zimmerman, 1987). LOX has been extensively studied in soybean (Hildebrand *et al.,* 1988). Soybean seed isoenzymes are 94–97 kDa monomeric proteins with distinct isoelectric points ranging from about 5.7 to 6.4, and could be distinguished by pH optimum, substrate specificity, product formation and stability (Mack *et al.* 1987; Siedow, 1991).

LOX proteins contain highly conserved domain and sequence motifs which are important for the distinct structure and the binding of the catalytic iron (LOX motif: His-X_4-His-X_7-His-X_8-His). Plant and mammalian lipoxygenases contain a small N-terminal β-barrel. The function of the β-barrel is unknown, but it is identical

in connectivity to the C-terminal domain of certain lipases and might be related to lipid binding (Gillmor *et al.,* 1997) and to membrane translocation (Chen and Funk, 2001).

The redox state of iron (Fe^{3+} state) is necessary for the LOX activity (Nelson and Seitz, 1994). The space within the active site and the orientation of the substrate are both important determinants for the positional specificity of plant LOXs and are modified by additional factors such as substrate concentration (Kuhn *et al.,* 1990b), the physico-chemical state of the substrate (Began *et al.,* 1999), pH (Gardner, 1989), or temperature (Georgalaki *et al.,* 1998). Nunez-Delicado *et al.* (1996) reported that the hydroperoxidase activity of LOX was slow, but it was enhanced when a suitable electron donor was included in the reaction mixture as a co-substrate.

LOXs are versatile catalysts because they are multifunctional enzymes, catalyzing at least three different types of reactions: (i) dioxygenation of lipid substrates (dioxygenase reaction), (ii) secondary conversion of hydroperoxy lipids (hydroperoxidase reaction) (Kuhn *et al.,* 1991), and (iii) formation of epoxy leukotrienes (leukotriene synthase reaction) (Shimizu *et al.,* 1984). However, under physiological conditions the first reaction is most prevalent in plants and their major substrates are C18-PUFAs.

A survey of the literature indicates that LOXs are present in most, if not all, plant organs, depending on developmental stage and environment. Earlier studies reported that most plant LOXs are soluble enzymes located predominantly in the cytosol (Siedow, 1991). Charge modifications of the soluble LOXs permit their association with membranes (Droillard *et al*., 1993), but non-specific adsorption to membrane fractions has also been observed (Siedow and Girvin, 1980; Mack *et al*.,

1987). Froehlich *et al*. (2001) demonstrated that allene oxidase synthase (AOS) and hydroperoxide lyase (HPL) are localized in different membranes of the chloroplast envelope. Thus LOXs might associate with different membranes in the chloroplast, and therefore with enzymes of different pathways. This would lead to the compartmentalization of oxylipin synthesis in the chloroplast (Fig. 3).

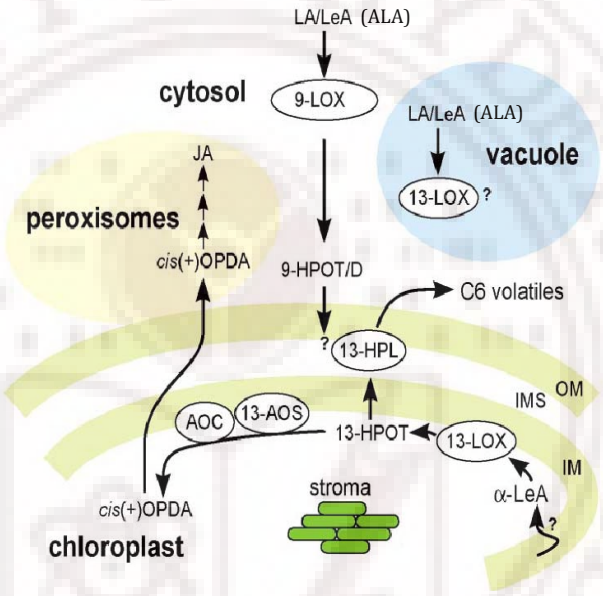


Fig. 3. Intracellular location of LOX pathway reactions. OM/IM, outer and inner membrane of the chloroplast envelope; IMS, inter membrane space. (Source: The lipoxygenase pathway. Feussner and Wasternack, 2002)

2.5 The LOX pathway:

It has been suggested that majority of hydro-(pero)xy fatty acids arise from action of LOXs (Feussner *et al*., 1997, Rusterucci *et al.,* 1999). A minority of PUFAs may be converted by the α-DOX (α-dioxygenase) into a-hydro(pero)xy PUFAs (Hamberg *et al*., 1999; Saffert *et al*., 2000) or may give auto-oxidative products such as dinor isoprostanes (Parchmann and Muller, 1998). Therefore, plants predominantly contain as lipid peroxide-derived substances, the (9*S*)-hydroperoxy and

the (13*S*)-hydroperoxy derivatives of PUFAs. The LOX immediate products formed from LA and ALA (Fig. 4 & 5), fatty acid hydroperoxides, then are used by at least six different multienzyme branches to form a large number of oxygenated fatty acids or so-called oxylipins (Blee, 2002).

LOX pathways have been implicated in several physiological processes, including plant growth and development, senescence, resistance to insects and pathogens, and environmental stresses (Howe and Schilmiller, 2002; Porta and Rocha-Sosa, 2002). One branch of the lipoxygenase pathway produces traumatic acid, compounds that are known to be involved in plant cell wound responses (Zimmerman and Coudron, 1979), and volatile C6-aldehydes and C6-alcohols. These volatile compounds are the major contributors to the characteristic fresh 'green' odour emitted by leaves (Hatanaka, 1996), triggers the production of phytoalexins (Zeringue, 1992), have antimicrobial activity (Croft *et al*., 1993) and play a role in pathogen defense (Croft *et al*., 1993). The second branch produces jasmonic acid, a molecule likely to serve a regulatory role in plant cells (Staswick, 1992; Sembdner and Parthier, 1993). A precise physiological role for LOX in plants has not been defined so far, but the diversity of isozymes and the subcellular distribution suggest multiple functions (Siedow, 1991; Rosahl, 1996).

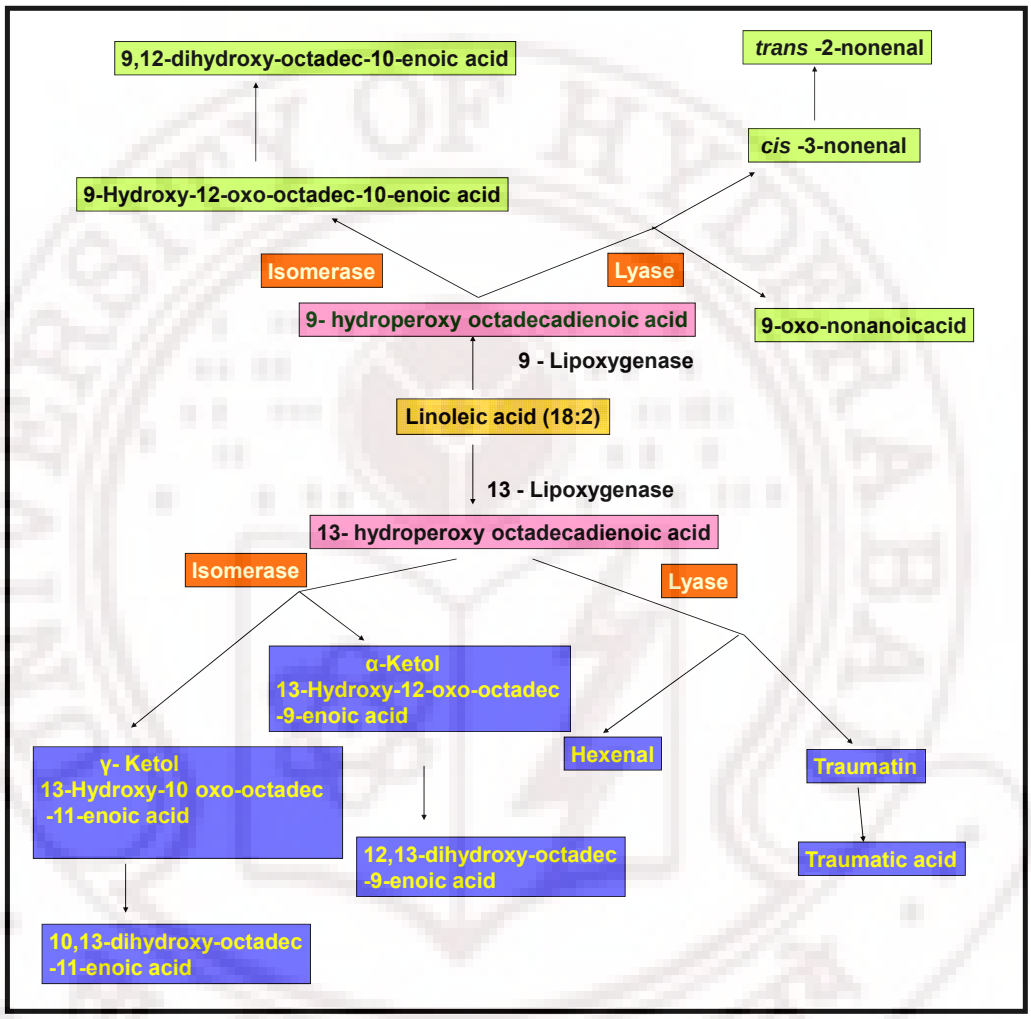


Fig. 4. Various metabolites produced by LOXs from linoleic acid in plant tissues.

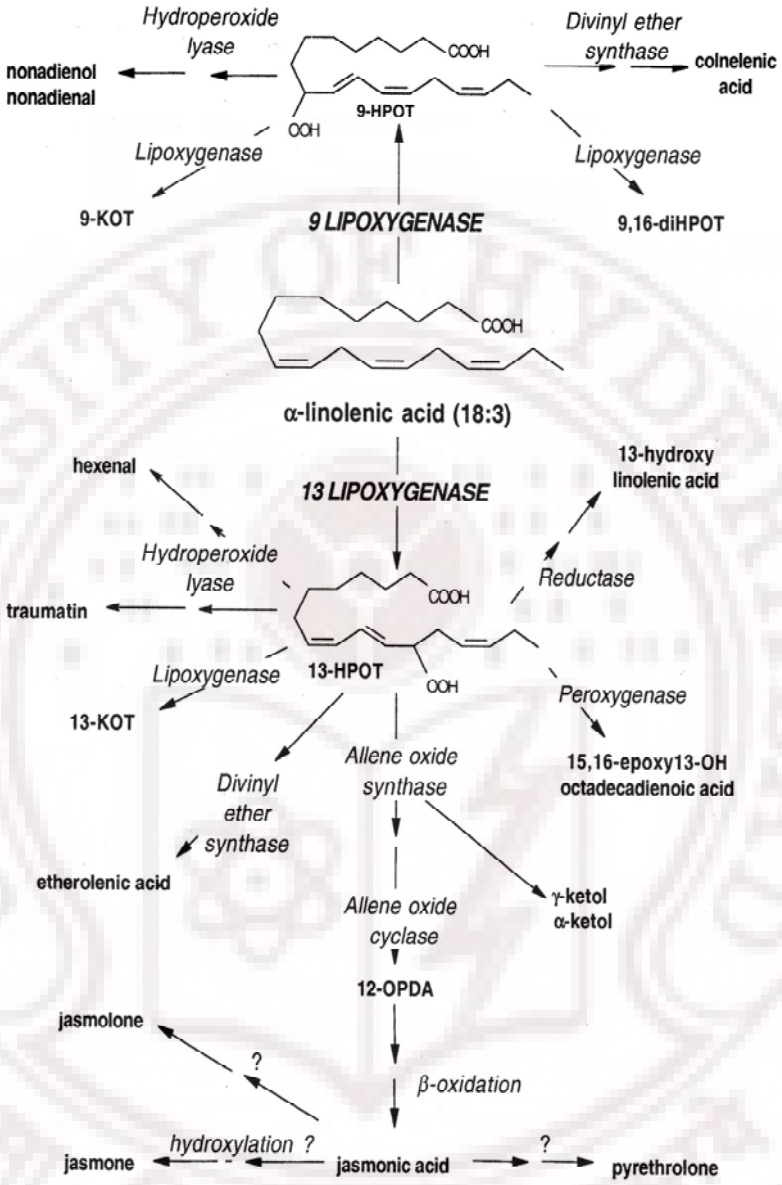


Fig. 5: Various metabolites produced by LOXs from α-linolenic acid in plant tissues. (Source: Plant lipoxygenases. Physiological and molecular features. Porta and Rocha-Sosa, 2002)

2.6 Physiological roles of LOX:

2.6.1 Role of LOXs in seedling growth and development:

LOXs are present in a wide range of biological organs and tissues, but they are particularly abundant in legume and cereal seeds, and potato tubers (Casey, 1999). Chang and McCurdy (1985) grouped 14 legumes into three classes based on their lipoxygenase specific activity *in vitro*. Legumes with a high level of activity were *Glycine max*, *Vigna unguiculata* and *Lens culinaris*; legumes that possessed a medium level of activity were *Phaseolus angularis*, *Vicia faba*, *Pisum sativum* and five biotypes of *Phaseolus vulgaris*. Legumes having a low level of activity were *Cicer arietinum*, *Phaseolus lunatus* and *Phaseolus aureus*.

Seeds of chickpea, lentil, broad bean and kidney bean contain two major LOXs, one synthesizing mainly 13-hydroperoxide from linoleic acid, whereas the other produces 9- and the 13-hydroperoxides and 9- and 13-ketodienes (Sanz *et al.,* 1993; Hilbers *et al.,* 1995; Clemente *et al.,* 2000). Expression of 9-LOX is induced to high levels during seed development in maize (Jensen *et al.,* 1997) and almond (Santino *et al.,* 2005). Peanut seeds contain three lipoxygenase isoenzymes with biochemical properties similar to the three soybean isoforms (Burow *et al.,* 2000). Several putative roles for LOXs in seeds were reported such as fatty acid peroxidation in membranes or storage lipids, production of growth regulators (jasmonates, abscisic acid), responses to pathogens and nitrogen storage (Hildebrand, 1989; Siedow, 1991; Rosahl, 1996).

LOXs play a physiological role during seed maturation and seedling growth. New LOXs are induced during the early stages of seedling growth in different plant species such as pea (Anstis and Friend, 1974; Chateigner *et al.,* 1999; Mo and Koster., 2006), watermelon (Vick and Zimmerman, 1976), French bean (Eiben and Slusarenko, 1994), lupin (Beneytout *et al.,* 1988), lentil (Hilbers *et al.,* 1995), barley (Yang *et al.,* 1993; Holtman *et al.,* 1996), cucumber (Matsui *et al.,* 1992), *Arabidopsis thaliana* (Melan *et al.,* 1994), *Papaver somniferum* (Bezakova *et al.,* 1994), rice (Suzuki and Matsukura, 1997) and *Brassica napus* (Terp *et al*. 2006).

Three LOXs are present in the mature seed of soybean which disappeared during the first few days of germination. Seed or seedling soybean LOXs are not associated with lipid bodies suggesting that soybean LOXs are not used for lipid mobilization during germination (Wang *et al*., 1999). Prakash *et al*. (1990) examined the senescense related changes in chlorophyll and protein content and lipoxygenase activity in peanut cotyledons. Protein content of peanut cotyledons decreased continuously during senescence. On the other hand, lipoxygenase activity increased in early stages of germination followed by a decrease in the later course of senescing peanut cotyledons. Mo and Koster (2006) investigated the changes in lipoxygenase isoforms during germination and early seedling growth of *Pisum sativum* L. During germination, there was a shift of LOX activity from radicles to shoots that accompanied the transition from seed LOXs to vegetative LOXs. Terp *et al*. (2006) identified two different LOX cDNAs from germinating seeds of *Brassica napus*. The expression of the corresponding genes and proteins and the accumulation of their metabolites were analyzed during germination. The 13-lipoxygenase derivatives were 6-8-fold more abundant than the 9-derivatives in etiolated seedlings of *Brassica.*

In *Phaseolus vulgaris* nodules, LOX mRNAs and proteins are detected mainly in nodules in the growing stage, and their levels decreased in nodules that have reached their full size (Porta *et al*., 1999). Transgenic potato plants expressing an antisense, tuber-specific LOX (*POTLOX-1*) gene displayed reduced LOX activity and a several fold reduction in tuber yield. It was suggested that LOXs are involved in the control of tuber growth and development, probably by initiating the synthesis of oxylipins that regulate cell growth during tuber formation (Kolomiets *et al*., 2001).

2.6.2 Biosynthesis of regulatory molecules:

Several lines of evidence suggested that seed LOXs might participate in growth hormone synthesis that mediates seed development. LOXs mediate an essential step in jasmonate synthesis by converting linolenic acid to 13-hydroperoxylinolenic acid (Crozier *et al*., 2000). Conversely, jasmonates have been found to stimulate LOX gene expression, protein, and activity in plants (Saravitz and Siedow, 1996). Jasmonates exert various effects on growth and development and act as signal factors in the plant response to wounding (Hamberg and Gardner, 1992; Sembdner and Parthier, 1993; Tizio, 1996). Jasmonates mediate water stress reactions that occur during physiological dehydration and seed maturation. Some jasmonate-induced proteins in cotton cotyledons exhibit homologies with late embryogenesis abundant proteins (Reinbothe *et al*., 1992).

Bell *et al*. (1995) used a transgenic approach to modify chloroplast lipoxygenase (LOX2) in *Arabidopsis* to observe the consequences of reduced LOX2 expression on plant growth and the synthesis of JA and ABA. The reduction in LOX2 levels caused no obvious changes in plant growth or in the accumulation of abscisic

acid. However, the wound-induced accumulation of JA observed in control plants was absent in leaves of transgenic plants that lacked LOX2.

In potato leaves, the type 2 13-LOXs, LOX-H3 and LOX-H1, were differentially induced upon wounding (Royo *et al.,* 1996). Depletion of LOX-H3 by antisense expression strongly reduced mRNA accumulation of the JA-responsive proteinase inhibitor II (pin2), suggesting a specific role of this LOX form in early JA formation. However, the basal level of JA was increased in these antisense plants, suggesting that some LOX forms can compensate, at least partially, for one another (Royo *et al*., 1999).

2.6.3 Role of LOX in disease resistance:

LOXs have been hypothesized to play a role in the response to plant pathogens (Slusarenko, 1996; Crozier *et al.,* 2000). An increase in LOX activity in response to infection has been reported for several plant-pathogen systems, and LOX activity has been correlated with plant resistance against pathogens. The production of several antimicrobial substances proceeds *via* the lipoxygenase pathway (Doehlert *et al.,* 1993; Burow *et al.,* 1997).

Increase in lipoxygenase activity was induced by addition of either the pathogen or an elicitor (Foumier *et al*., 1986; Ocampo *et al.,* 1986; Peever and Higgins, 1989) suggesting that lipoxygenase induction represents a standard component of the plant's defense response mechanism (Dixon and Lamb, 1990) and does not simply result from mechanical wounding of the tissue during the infection process. In resistant oat lines, the induction of two new lipoxygenase isozymes accounted for an increase in activity observed upon infection with crown rust,

Puccinia coronata, while no change in lipoxygenase activity was seen following infection of susceptible lines (Yamamoto and Tani, 1986).

Potato cDNA clones that encode distinct LOX isoforms have been identified, and their expression was found to be organ-specific and differentially regulated during tuber development and in response to wounding, pathogen infection, and MJ treatments (Geerts *et al.,* 1994; Casey, 1995; Kolomiets *et al.,* 1996; Royo *et al.,* 1996; Fidantsef and Bostock, 1998).

It has been suggested that LOX is involved in the development of an active resistance mechanism known as the hypersensitive response (HR), a form of programmed cell death (Keppler and Novacky, 1987; Croft *et al.,* 1990; Koch *et al.,* 1992; Rusterucci *et al.,* 1999). In the HR, an infection event is followed by rapid death of plant cells localized around the infection site, and this leads to necrotic lesion formation. This reaction limits pathogen spread and prevents further damage to the remainder of the plant organ. In several plant-pathogen systems, HR occurrence is linked tightly to increased activity, protein, or mRNA levels of LOXs (Vaughn and Lulai, 1992; Slusarenko, 1996; Rusterucci *et al.,* 1999). LOX isozyme profiles in the wheat-rust fungus pathosystem revealed that several LOX species were induced differentially during the HR evoked by the pathogen, its specific glycopeptide elicitor, other elicitors like chitosan and chitin oligosaccharides, and MJ (Bohland *et al.,* 1997).

Largely circumstantial evidence suggests that 9-LOX pathway plays an important role in defense response to pathogen attack (Weber *et al*., 1999; Hamberg *et al*., 2003). 9-hydroxyoctadecadienoic acid (9-HODE) and colneleic acid were significantly stimulated by *Phytophthora infestans* (Gobel *et al.,* 2001), and

expression of a 9-LOX gene precedes appearance of visible HR lesions (Kolomiets *et al.,* 2000). Srinivas Reddy *et al.* (2000) showed that 9-HPOTrE is the major LOX product formed in response to injury/infection in potato tubers, which is neither a precursor of jasmonic acid or traumatic acid, indicating the operation of a new pathway of LOX mediated defense responses in potato tubers. From these results, it was suggested that LOX metabolites of octadecadienoic acid may be involved in mediating the physiological responses, while octadecatrienoic acid metabolites may be mediating defense responses under stress conditions.

Komaraiah *et al*. (2003) observed that the relative ratio of 9-HODE and 9-HOTrE in control unelicited roots was approximately 4:1, while it was reversed to 1:3 in elicitor treated hairy root cultures of potato. 9-LOX activity and its hydroperoxide metabolites were found to be sufficient to initiate programmed cell death (PCD) and hypersensitive response (HR) induced by the bacterial pathogen *Ralstonia solanacearum* in tobacco (Cacas *et al.,* 2005).

Purified linoleic acid and hydroperoxy linoleic acids derived from peanut seed exhibited sporogenic activities toward several *Aspergillus* spp. (Calvo *et al*., 1999). In peanut (*Arachis hypogaea*), the gene coding for *PnLOX1* was induced in mature seeds infected with *Aspergillus* spp. (Burow *et al*., 2000). The products of reactions catalyzed by *PnLOX1*, namely (13S)-hydroperoxy-(9Z,11E)-octadecadienoic (13-HPODE) and (9S)-hydroperoxy-(10E, 12Z)-octadecadienoic acid (9-HPODE), are inhibitor and inducer, respectively, of mycotoxin synthesis, conferring a role in plant fungus interaction to this particular LOX (Burow *et al*., 2000).

Expression of a maize 9-LOX gene *ZmLOX3* (formerly *cssap92*) was induced by *Fusarium verticillioides* and *Aspergillus flavus* in lines accumulating high levels of

mycotoxins (Wilson *et al*., 2001), suggesting that mycotoxin biosynthesis may be positively regulated by the 9-LOX products. Collectively, these data prompted the hypothesis that the 9-LOX pathway is utilized by mycotoxigenic fungi to induce biosynthesis of mycotoxins and, hence, that some 9-LOX genes are susceptibility factors in maize plants (Sagaram *et al*., 2006; Tsitsigiannis and Keller, 2006).

Transgenic tobacco plants for an antisense LOX1 construct, infected with an incompatible race of *P. parasitica*, developed disease symptoms similar to those observed in a compatible interaction (Rance *et al.* 1998). Mene-Saffane *et al.* (2003) reported that the over-expression of this LOX in a susceptible tobacco genotype resulted in an increased resistance to this pathogen.

Yamamoto and Tani (1986) investigated the role of lipoxygenase in the mechanism of resistance of oats to *Puccinia coronata avenae*. Based on the experiments using RNA and protein synthesis inhibitors, it was demonstrated that the two LOX isozymes characteristic of the incompatible combination are *de novo* synthesized and their activity was casually linked to the resistance expression.

Gao *et al*. (2009) tested the interactions of a mutant maize line (lox3-4, in which *ZmLOX3* is disrupted) with the mycotoxigenic seed-infecting fungi *Aspergillus flavus* and *Aspergillus nidulans*. The lox3-4 mutant was more susceptible than wild-type maize to both *Aspergillus* species. However, the lox3-4 mutant exhibited resistance to other fungal pathogens (*Fusarium*, *Colletotrichum*, *Cochliobolus*, and *Exserohilum* spp.) in sharp contrast to resistance to *Aspergillus* spp suggesting that outcomes of LOX-governed host-pathogen interactions are pathogen-specific.

LOX have a role in both direct and indirect forms of resistance in plants (Hildebrand *et al*., 1988; Shibata and Axelrod, 1995). This was suggested by the

immediate massive burst of LOX product formation upon wounding of plant tissues. The production of volatile compounds increases dramatically upon wounding of plant leaves and a major portion of volatile compounds are apparently the products of LOX activity. In direct resistance reactions the lipid hydroperoxy radicals and some of the secondary oxidation products resulting from LOX action on linolenic acid are toxic to plant pathogens. LOX products such as hexenals have an adverse effect on pathogenic organism. The activation of defense responses after mechanical wounding and insect attack appears to be mediated also by jasmonic acid and its ester methyl jasmonate (MJ) collectively termed jasmonates.

The activation of systemic resistance by nonpathogenic rhizobacteria has also been associated with the induction of lipoxygenase activity in bean, tomato and peanut (Sailaja *et al*. 1997; Silva *et al*. 2004; Ongena *et al*. 2004; Ongena *et al*. 2007). Sailaja *et al*. (1997) showed that LOX acitivty in groundnut seedlings was rapidly activated by seed treatment with a biocontrol PGPR (Plant Growth Promoting Rhizobacteria) strain. The increase in activity with *B. subtilis* seed treatment was ahead by at least 24 h compared to the increase in *Aspergillus niger*-treated groundnut.

Evidence for a stimulation of the complete LOX pathway correlating with disease reduction was reported in bean protected by *Pseudomonas putida* BTP1 against *B. cinerea* infection (Akram *et al*. 2008). Based on the results it was suggested that a given rhizobacterium can induce the LOX pathway in various plants but with different outcomes regarding the type of oxylipin that will ultimately accumulate in infected tissues and putatively restrict pathogen ingress. This species specificity lies in the hydroperoxide degradation to different end products in function

of the relative activities of peroxidase, divinyl ether synthase, allene oxide synthase, hydroperoxide lyase and lipoxygenase (Akram *et al*., 2008).

2.6.4 Role of LOX in tolerance to abiotic stresses:

Relatively few studies have been carried out to determine the role of LOX in abiotic stresses in plants. In mature *Arabidopsis* plants, a higher expression of LOX1 was induced by abscisic acid and methyl jasmonate (Melan *et al*., 1993) whereas the expression of LOX2 was high in the leaves and inflorescence, but low in seeds, roots and stems. Somashekaraiah *et al*. (1992) observed an increase in LOX activity in seedlings of *Phaseolus vulgaris* under Cd^{2+} stress. Gallego *et al*. (1996) found higher LOX activity in sunflower leaf segments treated with Cu^{2+} than in those treated with Cd^{2+}. It has been shown that salt stress caused a high level of lipid peroxidation in the cultivated tomato (*Lycopersicon esculentum*), while the level of peroxidation in the wild relative, salt-tolerant, tomato (*L. pennellii*) was only marginal (Shalata and Tal, 1998).

Ben-Hayyim *et al*. (2001) analyzed the expression of LOX protein under stress conditions in cells of *Citrus sinensis* L. differing in sensitivity to salt. Lipoxygenase expression was induced very rapidly only in the salt-tolerant cells and in a transient manner. The induction was specific to salt stress and did not occur with other osmotic-stress-inducing agents, such as polyethylene glycol or mannitol, or under hot or cold conditions, or in the presence of abscisic acid. Bachmann *et al*. (2002) reported that the three 13-lipoxygenases were differentially expressed in barley leaves during treatment with jasmonate, salicylate, glucose or sorbitol. Metabolite profiling of free linolenic acid and free linoleic acid, the substrates of lipoxygenases, in water

floated or jasmonate-treated leaves revealed preferential accumulation of linolenic acid.

Skorzynska-Polit and Krupa (2003) conducted studies to determine the involvement of LOX in lipid peroxidation under heavy metal stress in *Arabidopsis thaliana*. LOX activity was dependent on the kind of metal, its concentration and time of plant exposure to the metal.

Porta *et al*. (2008) showed that in *Phaseolus vulgaris* a novel chloroplast targeted *PvLOX6* was highly induced after wounding, non-host pathogen infection, and by signaling molecules as H_20_2, SA, ethylene and MJ. These results implied that common bean uses the same LOX to synthesize oxylipins in response to different stresses.

2.7 Isolation, purification and characterization of LOXs:

Lipoxygenases have been isolated and purified from different sources such as seeds (Rabinovitch-Chable *et al*., 1992), leaves (Jen-Min *et al*., 2006), fruits (Nakayama *et al*., 1995) and bulbs (Reddanna *et al*., 1988). Singleton *et al*. (1978) determined the product specificity of peanut lipoxygenase with linoleic acid as the substrate under different conditions. They observed that oxygen tension, pH value and temperature markedly affected changes in the hydroperoxide isomeric ratio and the total amount of hydroperoxides produced.

In the pea, different lipoxygenases occur in different organs of the plant and in varying amounts (Domoney *et al*., 1990). Three lipoxygenases have been purified from soybean seeds which differ in pH optimum. Soybean leaves contain other lipoxygenases which are different from the seed enzymes (Grayburn *et al*., 1991).

Two lipoxygenase isoenzymes present in the embryo of germinating barley seed have been purified to homogeneity and characterized (Doderer *et al*., 1992). Both isozymes are monomeric proteins with a molecular mass of approx. 90 kDa. The isozymes differed in the products formed upon incubation with linoleic acid. One of the isozymes (lipoxygenase 1) solely formed the 9-HPODE as a product whereas the 13-HPODE was the major product formed by the other isoenzyme (lipoxygenase-2). Rangel *et al*. (2002) purified lipoxygenase induced in leaves of passion fruit in response to MJ to homogeneity. *In vitro* analysis of LOX activity using linoleic acid as substrate showed that it possessed C-13 specificity.

Jen-Min *et al*. (2006) purified and characterized the lipoxygenase (LOX) from banana leaf. The optimal pH of the purified LOX from banana leaf was 6.2, and optimal temperature was 40^{o}C. The products of 18:2 or 18:3 catalyzed by purified LOX were hydroperoxyoctadecadienoic acid or hydroperoxyoctadecatrienoic acid indicating that 9-LOX is the predominant enzyme in banana leaf.

There are several reports showing the involvement of lipoxygenase in defense response in plants. However, in only few cases the respective lipoxygenases have been purified to homogeneity. Babitha *et al*. (2004) described the purification of three isozymes of lipoxygenase from downy mildew resistant seedlings after inoculation with *S. graminicola*. The results indicated that the LOX isozymes were dimers composed of two unequal subunits of 43 and 40 for LOX-1, 40 and 37 for LOX-3, 38 and 35 for LOX-6. The alkaline LOX-1 and -3 purified from pearl millet exhibited maximal acitivty at pH 9.0 while LOX-6 has peak activity at pH 6.5.

Ohta *et al*. (1991) observed a marked increase in LOX and lipid hydroperoxide-decomposing activity in the leaves of rice (*Oryza sativa* cv Aichiasahi)

after inoculation with the rice blast fungus, *Magnaporthe grisea*. Three LOX isozymes (leaf LOX-1, -2, -3) were isolated from both uninoculated and infected leaves using ion-exchange chromatography. Substrate specificity, positional specificity of oxygenation, and pH optimum of the enzyme were quite different from other rice LOXs reported (Ida *et al*., 1983; Ohta *et al*., 1986). Fournier *et al*. (1993) purified and characterized the elicitor-induced LOX enzyme from cultured tobacco cells. The purified LOX enzyme was a soluble type-2 lipoxygenase whose physicochemical parameters, notably molecular weight (96kDa) and pI (5.1, 5.3) were very close to those of other lipoxygenases purified from solanaceous plant organs such as potato tubers (Mulliez *et al*., 1987), tomato and egg-plant fruits (Grossman *et al*., 1972; Zamora *et al*., 1987). Despite these similarities and the reported homologies between tobacco genomic DNA sequences and soybean seed cDNA (Bookjans *et al*., 1988), the elicitor-induced tobacco lipoxygenase did not cross-react with antibodies against soybean seed lipoxygenases.

Tsitsigiannis *et al*. (2005) described the characterization of two peanut seed lipoxygenase alleles (*PnLOX2* and *PnLOX3*) highly expressed in mature seed. *PnLOX2* and *PnLOX3* both are 13S-HPODE producers (linoleate 13-LOX) and, in contrast to 9-LOX or mixed function LOX genes, are repressed between 5-fold and 250-fold over the course of *A. flavus* infection. It was suggested that 9S-HPODE and 13S-HPODE molecules act as putative susceptibility and resistant factors respectively, in *Aspergillus* seed-aflatoxin interactions.

Roopashree *et al*. (2006) purified a protein from *Dolichos biflorus* seed, which exhibited both LOX and haemagglutination activity. The evidence for the lectin protein with LOX activity came from (i) MALDI-TOF (matrix-assisted laser-

desorption ionization-time-of-flight) MS, (ii) N-terminal sequencing, (iii) partial sequencing of the tryptic fragments of the protein, (iv) amino acid composition, and (v) the presence of an Mn^{2+} ion. A hydrophobic binding site of the tetrameric lectin, along with the presence of a Mn^{2+} ion, accounted for the LOX like activity.

2.8 Antifungal activity of LOX products:

Sailaja *et al*. (1997) reported that the products of groundnut LOX (13-HPODE and 13- HPOTrE) significantly inhibited the growth of *Aspergillus niger*. Prost *et al*. (2005) investigated the direct antimicrobial activities of 43 natural oxylipins against a set of 13 plant pathogenic microorganisms including bacteria, oomycetes and fungi. The study showed that most oxylipins are able to impair growth of some plant microbial pathogens, with only two out of 43 oxylipins being compeletely inactive against all the tested organisms, and 26 oxylipins showed inhibitory activity towards at least three different microbes. Mita *et al*. (2007) evaluated the fungicidal properties of C9-aldehydes of (2E)-nonenal and (2E-6Z)-nonadienal, the major products of the almost LOX/HPL metabolism in comparison to that of hexenal and (2E)-hexenal, major compounds produced by 13-HPL which have been reported to possess antifungal and antimicrobial activities (Prost *et al*., 2005). Both (2E)-nonenal and (2E,6Z)-nonadienal showed a marked inhibitory effect on the growth of *Aspergillus carbonarius* with a minimum inhibitory concentration lower than those for hexenal and 2E-hexenal.

Chapter 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Chemicals:

Linoleic acid (LA), α-linolenic acid (ALA), soybean lipoxygenase, ethylene diaminetetraaceticacid (EDTA), nordihydroguaiaretic acid (NDGA), esculetin, sodium cyanide (NaCN) and agarose were procured from Sigma Chemical Co. (St. Louis, MO). O-dianisidine dihydrochloride was obtained from National Chemicals, India. Abscisic acid (ABA) and polyethylene glycol (PEG-6000) were procured from Himedia, India. Methyl jasmonate (MJ) was obtained from Duchefa Chemicals, India. Diethylaminoethyl cellulose (DE-52) was obtained from Whatman Scientific Ltd., UK. HPLC solvents like n-hexane, propane-2-ol, acetic acid and methanol were of high quality HPLC grade chemicals obtained from Qualigens Fine Chemicals, India. RNeasy Plant Mini Kit was obtained from Qiagen, USA. First-strand cDNA synthesis kit was obtained from Invitrogen, USA. Protein Molecular Weight Marker was obtained from MBI Fermentas, Germany.

All other chemicals used in this study were high quality analytical/reagent grades procured from Qualigens, India and Himedia, India.

3.2 Instrumentation: High Performance Liquid Chromatography – Shimadzu (SPD6AV detector and CR4A Chromatopac), PCR machine – MJ Research PTC-200, Spectrophotometer – Hitachi-U-2001, Cooling Centrifuge – Kubota 7700, Microfuge– Eppendorf -5415R, Incubator – Cassia Siamia, Incubator Shaker – Scigenics Orbitek, Nanodrop Spectrophotometer – Nanodrop-ND-1000.

3.3 Plant material: Groundnut varieties *viz*., K-1375, JL-24 and TAG-24 were procured from Agricultural Research Station, Kadiri, Anantapur, Andhra Pradesh. Variety ICG-4747 used for studies on *Rhizoctonia* damping off disease was procured

from International Crops Research Institute for Semi Arid Tropics (ICRISAT), Patancheru, Hyderabad, Andhra Pradesh. Stem rot resistant varieties *viz*., R-9214, R-8808, R-9217, R-9227, R-2001-1, R-2001-2, R-2001-3, K-134 that are resistant and susceptible varieties TMV-2 and S-206 varieties were procured from Regional Agricultural Station, Raichur, Karnataka.

3.4 Fungal cultures: The sclerotia of *Rhizoctonia solani* were obtained from by Prof. Narayan Reddy, Department of Plant Pathology, Acharya N. G. Ranga Agricultural University, Rajendranagar, Hyderabad and the cultures of *Sclerotium rolfsii* and *Fusarium oxysporum* were procured from the laboratory of Prof. Appa Rao Podile, Department of Plant Sciences, University of Hyderabad, Hyderabad.

3.5 Germination experiments:

The seeds of groundnut varieties *viz*., K-1375, TAG-24 and JL-24 were surface sterilized with 70% ethanol for 4 min and rinsed three times with sterilized double distilled water. This was followed by 0.1% $HgCl_2$ for 7 min and rinsed 3 times with sterile double distilled water. The seeds were sown in pots (12 X 13 cms) containing sterilized red soil which were kept in glasshouse (temperature 25^{o}C to 30^{o}C, photoperiod 12 h, light intensity 500-1700 $\mu molm^{-2}s^{-1}$) and watered daily with tap water. The seedlings were harvested at 24 h intervals for 7 days and homogenized with 100 mM potassium phosphate buffer (pH 6.5). The homogenates were centrifuged for 30 min at 10,000 rpm and the supernatant was assayed immediately for lipoxygenase activity. The LOX isozymes were analyzed in the mature seeds and 3-6 day-old seedlings on native-PAGE.

3.6 pH optimal studies:

The pH optima for lipoxygenase induced in 4-day-old seedlings of K-1375 was studied. The crude enzyme extract prepared from 4-day-old groundnut seedlings was added to the reaction buffers containing citrate phosphate buffer (pH range from 5.0 to 5.5), potassium phosphate buffer (pH range from 6.0 to 7.5) and Tris buffer (pH range from 8.0 to 9.0) with linoleic acid as a substrate in 1 ml of final volume and assayed for lipoxygenase activity.

3.7 NaCl and PEG treatments to groundnut seedlings:

Drought tolerant variety, K-1375 and drought sensitive variety, JL-24 were used for NaCl and PEG experiments. The surface sterilization of the seeds was same as described earlier. The seeds were sown in pots (12 X 13 cms) containing sterilized red soil which were kept in glasshouse and watered daily with tap water. The plants were removed from soil after 15 days of germination and the roots were cleaned in tap water. The plants were placed in 250 ml conical flasks containing Hoagland's nutrient solution. After 5 days, they were transferred to 250 ml conical flasks containing 200 mM NaCl or 23% PEG in Hoagland's nutrient solution. The seedlings were removed from the conical flasks at 24 h intervals up to 4 days. The roots and shoots were homogenized separately with 100 mM potassium phosphate buffer (pH 6.5). The homogenates were centrifuged for 30 min at 10,000 g and the supernatant was assayed immediately for lipoxygenase activity by spectrophotometric method. Protein was estimated for all the samples by Lowry's method (1951). LOX isozymes in the roots of the treated plants were analyzed on native-PAGE with equal amount of proteins (20 µg) for all the samples.

3.8 Treatment of ABA and MJ to groundnut seedlings:

The drought tolerant groundnut variety, K-1375 was used in the study. The surface sterilization of the seeds was same as described above. The seeds were sown in pots containing sterilized soil which were kept in glasshouse and watered daily with tap water. Fifteen day-old seedlings were removed from the soil and the roots were cleaned in tap water. They were placed in 250 ml conical flasks containing Hoagland's nutrient solution for 5 days and then transferred to conical flasks containing 100 μM ABA in Hoagland's nutrient solution. For treatment with MJ, the plants were placed in conical flasks containing 100 μM MJ in Hoagland's nutrient solution and covered with plastic sheets. The seedlings were harvested at 24 h intervals for 4 days. LOX activity was estimated in the roots of the treated plants by the spectrophotometric method and the LOX isozymes were analyzed on native-PAGE.

3.9 Infection of groundnut seedlings with *Rhizoctonia solani*:

The seeds of ICG-4747 varity were sown in plastic pots containing autoclaved red soil and placed in glasshouse for germination. The sclerotia of *R. solani* were inoculated on potato dextrose agar medium in petriplates and incubated at 30^{o}C for mycelial growth. The mycelial discs prepared from 3-day-old cultures were placed near the cotyledons of two-day-old seedlings for infection. The seedlings that were not given any inoculum were used as controls. The samples of control and inoculated seedlings were taken at 6 h intervals for 24 h for analyzing the LOX activity through spectrophotometric method and LOX isozymes on native-PAGE.

3.10 Infection of seedlings with *Sclerotium rolfsii*:

Groundnut varieties *viz*., R-9214, R-8808, R-9217, R-9227, R-2001-1, R-2001-2, R-2001-3 and K-134 that are resistant and TMV-2 and S-206 varieties that are susceptible to stem rot disease caused by *S. rolfsii* were used in the study. The seeds were sown in the pots (12 X 13 cms) containing red soil which were kept in glasshouse and watered daily with tap water. Ten-day-old seedlings of resistant and susceptible varieties were inoculated with mycelia of *S. rolfsii*. For the inoculum, the sclerotia were inoculated on petriplates containing potato dextrose agar medium and incubated at 30˚C for fungal growth. The mycelia were completely developed in petriplates after 4 days. The mycelial discs prepared from 4-day-old cultures were placed at the collar region of the seedlings for infection. The seedlings that were not inoculated with the mycelium were used as controls. The samples of control and *S. rolfsii* inoculated seedlings of resistant and susceptible varieties were collected at 6 h intervals from the day of inoculation until 96 h and were assayed for LOX activity by spectrophotometric method. LOX isozymes were analyzed in the control and inoculated seedlings of R-2001-1 and TMV-2 varieties at the same intervals as described above through native-PAGE. 13-HODE formation was determined in the control and inoculated seedlings through HPLC by incubating the crude protein extract of the samples with LA as the substrate. Equal amount of crude protein (20 µg) was used for activity staining through native-PAGE and for analyzing 13-HODE formation in all the experimental samples.

3.11 Preparation of crude extract for lipoxygenase assay and activity staining:

A 20% crude extract was prepared by homogenizing the plant material in 100 mM potassium phosphate buffer, pH 6.5 containing 2 mM sodium metabisulphite, 1

mM EDTA, 1 mM ascorbic acid, 2 mM sucrose and 1 mM PMSF (phenylmethylsulfonyl fluoride). The homogenate was passed through four layers of cheese cloth and centrifuged at 10,000 g for 30 min at 4°C. The resulting supernatant was used for assay of LOX activity and purification of LOX enzyme.

3.12 Estimation of total protein:

Protein content in the crude enzyme preparation of different treatments was estimated by the method of Lowry *et al*. (1951).

3.13 Lipoxygenase assay by spectrophotometric method:

The LOX activity in different samples was measured spectrophotometrically (Shimizu *et al*. 1990). Typical reaction mixture contained 980 µl of potassium phosphate buffer, pH 6.5 and 20 µl of enzyme. The samples were adjusted to zero absorbance by keeping the above mixture as reference. The reaction was initiated by the addition of 12 µl of 20 mM linoleic acid or α-linolenic acid. The reaction was followed for 1 min by monitoring the formation of conjugated dienes at 234 nm. Enzyme activity was calculated by using the formula:

$$\text{LOX activity} = \frac{\text{(Vol. of the reaction mixture) x (absorbance difference per minute)}}{(\varepsilon) \text{ X (Vol. of the enzyme in ml)}}$$

Where ε = (Molar extinction coefficient of the hydroperoxide) =25,000

One unit of enzyme activity was defined as one µmole of hydroperoxide formed per minute. Specific activity was expressed as units per mg protein.

3.14 Analysis of LOX isozymes by Native gel electrophoresis:

LOX isozymes in different samples were analyzed on native-PAGE on vertical slabs. The acrylamide and bisacrylamide (29.2: 0.8) were polymerized in Tris HCl, pH 8.8. The samples consisting of equal amount of protein were dissolved in sample buffer consisting of 10% sucrose and 0.003% bromophenol blue in Tris-HCl buffer, pH 6.8 and loaded onto 10% native-PAGE. The proteins were separated using 80V at 4˚C.

Active lipoxygenase was stained according to the procedure of Heydeck and Schewe (1985). On completion of electrophoresis, the gels were immediately washed with 0.1 M potassium phosphate buffer, pH 6.5 for 15 min at 4˚C. The gels were then incubated with LA with equal volumes of methanol and 0.53 M potassium hydroxide to give potassium linoleate. The potassium linoleate was then mixed with fresh 0.1M potassium phosphate buffer, pH 6.5 to give a final concentration of 2.5 mM substrate in the solution along with 0.1% sodium cholate. The gels were incubated exactly for 5 min at 25˚C. The gels were subsequently washed with 0.1M potassium phosphate buffer of pH 6.5 or with double distilled water to remove the excess of the substrate attached to the gel. The gels were then stained with o-diansidine hydrochloride in 0.1M potassium phosphate buffer.

3.15 Analysis of 13-HODE formation *in vitro* through Reverse Phase-High Performance Liquid Chromatography (RP-HPLC):

Ten day-old seedlings of R-2001-1 and TMV-2 varieties were inoculated with *S. rolfsii* and analyzed for 13-HPODE formation *in vitro* through RP-HPLC at different intervals along with uninoculated controls as per the method of Sunitha *et al.*

(2005). Activity assays were performed by addition of protein supernatant to 0.5 ml of phosphate buffer containing 250 µM linoleic acid as substrate. The mixture was incubated for 15 min at 37°C, and the 13-HPODEs formed were reduced to the more stable hydroxy derivatives (13-HODEs) by addition of 0.1 ml of saturated solution of sodium borohydride in dry ethanol. After acidification to pH 3.0 (acetic acid), 0.5 ml of methanol was added, and the samples were kept on ice for 10 min. The protein precipitate was spun down, and aliquots of the clear supernatant were directly analyzed by RP-HPLC for quantification of the 13-HODE formation in experimental samples using commercial 13-HODE as a standard with mobile phase consisting of methanol, water and acetic acid (850:150:1).

3.16 Analysis of expression of *PnLOX-1, PnLOX-2* and *PnLOX-3* genes in groundnut seedlings inoculated with *S. rolfsii* by RT-PCR analysis:

The seedlings of resistant variety, R-2001-1 variety were inoculated with *S. rolfsii* as described above. The inoculated plants were collected at 6 h intervals upto 96 h along with controls to determine the transcript levels of *PnLOX-1*, *PnLOX-2* and *PnLOX-3* by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's protocol.

The SuperscriptTM III First-strand synthesis system for RT-PCR was used to synthesize first-strand cDNA from total RNA following the manufacturer's recommendations (Invitrogen, USA) as follows. cDNA synthesis was performed in the first step using total RNA (2 µg) primed with 50 uM oligo (dT) 20. Two µg of total RNA, 1 µl of 50 µM oligo (dT) 20 primer, 1 µl of 10 mM dNTP mix were added and made upto 10 µl with DEPC-treated water in 0.2 ml tube. The mixture was

incubated at 65°C for 5 min and then placed on ice for at least 1 min. The cDNA synthesis mix was prepared by taking 2 µl of 10X RT buffer, 4 µl of 25 mM $MgCl_2$, 2 µl of 0.1 M DTT, 1 µl of RNase OUT (40U/µl) and 1 µl of SuperscriptTM III RT (200U/µl). The cDNA synthesis mix was added to each RNA/primer mixture, mixed gently and collected by brief centrifugation. The mixture was incubated at 50°C for 50 min. The reactions were terminated at 85°C for 5 min and chilled on ice. The reactions were collected by brief centrifugation followed by addition of 1 µl of RNaseH to each tube and incubated for 20 min at 37°C. cDNA synthesis reaction was stored at -20°C or used for PCR immediately. First strand cDNA was used as template for PCR amplifications with gene specific primers. The PCR reactions were carried out in a volume of 25 µl containing (final concentration) 1 U of Taq DNA polymerase, 0.2 mM dNTP, 10X reaction buffer and 100 pmol of 5′ and 3′ primers for *PnLOX-1, PnLOX-2* and *PnLOX-3*. The amplification conditions were: LOX-1: 94°C for 1 min for one cycle and 94°C for 30 sec (denaturation) and 58°C for 45 sec (primer annealing) and 72° for 1 min (elongation) for 30 cycles. LOX-2: 95°C for 1 min for one cycle and 95°C for 30 sec and 58°C for 30 sec and 72°C for 1 min for 30 cycles. LOX-3: 95°C for 1 min for one cycle and 95°C for 30 sec and 59°C for 30 sec and 72°C for 1 min for 30 cycles. As an internal control, 18S rRNA gene was used for analyzing the gene expression. The amplification conditions for 18S rRNA was 94°C for 3 min for one cycle, 94°C for 1 min, 55°C for 45 sec, 72°C for 45 sec for 30 cycles. The PCR amplified products were subjected to electrophoresis on 1.2% agarose gel and visualized by ethidium bromide staining and UV irradiation.

Primers were designed for LOX genes of peanut *viz*., *PnLOX-1*, *PnLOX-2* and *PnLOX-3* genes using the gene sequence present in the database with the accession numbers AF231454 for *PnLOX-1*, DQ068249 for *PnLOX-2* and DQ068250 for *PnLOX-3* genes.

Primers used in this study:

PnLOX -1 Forward: 5'- GCG AAC AAG TCC TCA AGT TTC C -3'
Reverse: 5'- ATT GCC TCT TCT AGT GAG CAC C - 3'

PnLOX -2 Forward: 5'- TTT CCA CCA CCT CAC ATC ATT C - 3'
Reverse: 5'- AGC ACC CTT CAA GAT CAA GAA C -3'

PnLOX -3 Forward: 5'- TTG AAT GGG ACGATA GCA TGG G - 3'
Reverse: 5'- TTG TTG GAG AAG AAA ATG CGG G -3'

18S rRNA Forward: 5'- CCA GGT CCA GAC ATA GTA AG - 3'
Reverse: 5'- GTA CAA AGG GCA GGG ACG TA - 3'

3.17 Purification of lipoxygenase isozymes from shoots of groundnut seedlings inoculated with *S. rolfsii*:

The shoots of seedlings of resistant variety, R-2001-1 were harvested after 48 h of inoculation with *S. rolfsii* and used for purification of lipoxygenase.

3.17.1 Ammonium sulphate fractionation:

The crude extract (20%) prepared from the shoots of seedlings inoculated with *S. rolfsii* was subjected to ammonium sulphate precipitation initially to 15% saturation by adding solid ammonium sulphate. The crystals of ammonium sulphate were added slowly to the crude extract at 4°C with constant gentle stirring. The

preparation was centrifuged at 10,000 g for 30 min at 4°C. The pellet with no activity was discarded and the supernatant was again subjected to 20-60% ammonium sulphate saturation. The samples were again centrifuged at 10,000 g for 30 min at 4°C. The supernatant with no substantial activity was discarded and the pellet was dissolved and dialyzed against 40 mM potassium phosphate buffer, pH 6.5 for 12 h at 4°C. The dialysate was centrifuged at 10,000 g for 10 min and the clear supernatant was subjected to further purification.

3.17.2 Anion-exchange chromatography:

Activation of DEAE (DE-52): Twenty grams of DEAE (DE-52) was suspended in 200 ml of equilibration buffer (40 mM potassium phosphate buffer, pH 7.0), washed and degassed thoroughly for 30 min and finally the gel was suspended in 50 ml of equilibration buffer and poured into a glass column (15 mm X 15 cm). The gels were allowed to settle and a reservoir of the same buffer was connected. The gels were equilibrated with potassium phosphate buffer and poured into a glass column (15 mm X 15 cm) for about 12 h at a flow rate of 0.5 ml per min. The 20-60% ammonium sulphate fractionation which showed maximum lipoxygenase activity was applied to DE-52 column, equilibrated with 40 mM phosphate buffer. After reloading the samples thrice, the column was washed with above buffer until all the unbound proteins were washed away by observing the OD at 280 nm. The bound proteins to the column were eluted with a linear gradient of 0 to 0.4 M KCl in the above buffer at a flow rate of 30 ml per hour. The fractions of 1.0 ml were collected and analyzed for total protein by monitoring the absorbance at 280 nm. The fractions were analyzed for lipoxygenase activity spectrophotometrically at 234 nm and those showing

lipoxygenase activity were pooled, dialyzed against 40 mM potassium phosphate buffer and concentrated using Amicon filters.

3.17.3 Anion Exchange HPLC:

Shimpack PA-DEAE column (8 mm X 100 mm) was equilibrated with 40 mM potassium phosphate buffer at a flow rate of 1 ml per min. The concentrated enzyme protein was loaded onto the column through a Rheodyne injector and the proteins were eluted with 0 to 0.4 M KCl gradient in 40 mM potassium phosphate buffer at the same flow rate using Shimadzu L6-6AD pumps. The eluant was continuously monitored at 280 nm on Shimadzu SPD-6AV UV/VIS detectors. The peaks were checked for lipoxygenase activity spectrophotometrically at 234 nm and those showing the activity were collected and the LOX isozymes were analyzed on native-PAGE as described above. The purity of the lipoxygenase was checked on SDS-PAGE following Laemmli's method (1951) with minor modifications as described below.

3.18 Analysis of purified LOX protein by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE):

The purified LOX protein was analyzed on SDS-PAGE according to the method of Laemmli (1970) with minor modifications. The separation of proteins was performed in 12% resolving gel and 4% stacking gel. Both the resolving and stacking gel contained 2.4% bisacrylamide as a cross linker and 0.1% SDS. The final resolving buffer concentration was 0.375 M Tris-HCl (pH 8.8) in resolving gel and stacking buffer final concentration was 0.125 M Tris-HCl (pH 6.8) in stacking gel. Ammonium persulphate and N, N, N′, N′-tetramethyl ethylenediamine (TEMED) was used as polymerizing agents in final concentrations of 0.05% and 0.1%, respectively.

The electrode buffer consisted of 0.025 M Tris and 0.192 M glycine (pH 8.3). The sample buffer consisted of 0.062 M Tris-HCl, 10% glycerol, 2% SDS, 5% β-mercaptoethanol and 0.001% bromophenol blue. The samples were incubated for 3 min in boiling water bath with the sample buffer and centrifuged for 5 min at 3,000 rpm. The clear sample solutions were loaded into wells on the gels of 8 x 10 cm dimension, which were polymerized in glass plates fixed to mini-vertical slab gel apparatus. The gels were run at room temperature at 75 and 100 V (direct current) for stacking and resolving gels, respectively. Electrophoresis was carried out until the bromophenol blue dye front reached about 4–5 mm from bottom. Then the gels were removed, and stained with Commassie Brilliant Blue (CBB) R250 staining. The staining solution was prepared by dissolving 0.1% CBB in methanol: acetic acid: water (40:10:50) v/v for overnight at room temperature. The staining solution was filtered using Whatman No. 1 filter paper and added to the gel and kept under gentle shaking for 1 h. The staining solution was removed and the gels were destained with methanol: acetic acid: water (40:10:50) v/v under constant shaking until the clear bands were detected.

3.19 Characterization of the LOX isozymes purified from *S. rolfsii* inoculated seedlings of groundnut:

3.19.1 Effect of temperature on purified lipoxygenase:

The effect of temperature on purified LOX isozymes was tested by incubating at different temperatures of 20°C, 25°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C for 10 min. The purified LOX incubated at temperatures of 40°C, 50°C, 60°C, 70°C, 80°C and 90°C were cooled to room temperature and the activity was measured spectrophotometrically at 234 nm as described above.

3.19.2 Effect of pH on purified lipoxygenase:

The effect of pH on the purified LOX isozymes was studied by measuring the LOX activity with reaction buffers containing citrate phosphate buffer (pH range from 5.0 to 5.5), potassium phosphate buffer (pH range from 6.0 to 7.5) and Tris buffer (pH range from 8.0 to 11.0) with linoleic acid as a substrate in 1 ml of final volume and assayed for lipoxygenase activity.

LOX activity was determined through spectrophotometrically at 234 nm as described above.

3.19.3 Effect of inhibitors on purified lipoxygenase:

The effect of different inhibitors *viz*., EDTA, NDGA, Esculetin, NaCN and $CaCl_2$ at 1 and 10 mM on the activity of purified LOX was tested. The purified LOX was pre-incubated with inhibitors for 10 min in 50 mM potassium phosphate buffer and the reaction was initiated by addition of substrate and then assayed for LOX activity spectrophotometrically at 234 nm as described above.

3.19.4 HPLC analysis of LOX products from the purified groundnut lipoxygenase:

The hydroperoxides, 13-hydroperoxyoctadecadienoic acid (13-HPODE) and 13-hydroperoxyoctadecatrienoic acid (13-HPOTrE) were prepared with purified LOX enzyme by incubating with linoleic acid (LA) or α-linolenic acid (ALA), respectively as per the method described by Reddanna *et al*. (1990). The reaction mixture contained 100 ml of 100 mM phosphate buffer, pH 6.5, enzyme solution (approximately 100 mg of protein) and 200 µM of LA or ALA at final concentration. The reaction initiated with LA or ALA was stopped by adding 6 N HCl after 2 min. The products were extracted into an equal volume of hexane and ether (1:1) twice in a

separating funnel. The organic solvent, separated from the aqueous layer in a separating flask was passed through the anhydrous granular sodium sulphate and subjected to evaporation in a rotatory evaporator to total dryness. The dried products were redissolved in HPLC solvent in n-hexane and 2-propanol and acetic acid (1000:15:1 v/v/v). A portion of it was kept aside for the preparation of hydroxyl compounds while the rest was loaded onto the Straight Phase HPLC system through a Rheodyne injector.

3.19.4.1 Straight Phase HPLC analysis:

The above reaction products were separated on Shimadzu HPLC using straight phase silica column (CLC-SIL) under isocratic conditions employing HPLC mobile phase consisting of hexane: propanol: acetic acid (1000:15:1) at a flow rate of 2 ml per min. The eluted products were continuously monitored on Shimadzu SPD-6AV UV/VIS detector at 234 nm and the peaks were collected separately. Individual peaks collected from SP-HPLC were analyzed for their absorption spectra on Hitachi spectrophotometer.

3.20 Preparation of HPODEs, HPOTrEs, HODEs and HOTrEs from soybean LOX:

Commercial soybean lipoxygenase was used to generate the standard 13-HPODE and 13-HPOTrE. Hydroperoxy products were generated on a large scale as described above.

3.20.1 Reduction of hydroperoxides:

The hydroperoxides (13-HPODE and 13-HPOTrE) generated by employing purified groundnut lipoxygenases and soybean lipoxygenase were reduced with sodium borohydride as follows:

The hydroperoxides extracted in equal volumes of hexane and ether (50:50) were evaporated to dryness and re-dissolved in 100% methanol. The products were reduced with sodium borohydride ($NaBH_4$) for 2 min. After 30 sec, 6 N HCl was added to remove excess sodium borohydride. The products formed were extracted with equal volumes of hexane and ether (1:1) twice. The organic solvent, separated from aqueous layer in a separating flask, was passed through anhydrous sodium sulphite and subjected to evaporation in a rotary evaporator to dryness. The dried products were redissolved in HPLC mobile phase consisting of n-hexane, propane-2-ol and acetic acid (1000:15:1) and quantified by HPLC.

3.21 Analysis of anti-fungal activity of hydroperoxides and hydroxides using filter disc assay:

***Sclerotium rolfsii* and *Rhizoctonia solani*:** The sclerotia of *S. rolfsii* and *R. solani* were inoculated on potato dextrose agar medium in petriplates and incubated at 30°C. The mycelia were completely developed on the petriplates after 3 days. The mycelial discs (10 mm) of *S. rolfsii* and *R. solani* prepared from 3-day-old cultures were inoculated in the centre of sterile petriplates containing potato dextrose agar medium. Sterile 5 mm filter discs were kept around the fungal mycelial disc with 3.0 cm distance. Different concentrations of hydroperoxides (1-5 µg) (13-HPODE and 13-HPOTrE) and hydroxides (13-HODE and 13-HOTrE) dissolved in 10 µl of 10% ethanol were pipetted on to filter discs in petriplates. As a control, 10 µl of 10% ethanol was pipetted out onto each filter disc in each petriplate. Petriplates were then sealed with parafilm and incubated at 30°C for 24 h and examined for zones of growth inhibition around each disc. The lowest concentration of hydroperoxide and hydroxides that produced detectable zones of inhibition was considered as minimum

inhibitory concentration (MIC) and is expressed as micrograms of hydroperoxide or hydroxide per disc.

***Fusarium oxysporum*:** The cultures of *F. oxysporum* were maintained on potato dextrose agar medium at 30°C. The mycelial discs prepared from 7-day-old cultures were inoculated in conical flasks containing potato dextrose broth and placed in orbital shaker for 3 days for initiating the suspension. One ml of spore suspension was pipetted on to a fresh and dry potato dextrose agar medium previously seeded in 3-partitioned petriplates. Sterile 5 mm filter discs were kept at the centre of each partition in the petriplates. Different concentrations of hydroperoxides (1-5 µg) (13-HPODE and 13-HPOTrE) and hydroxides (13-HODE and 13-HOTrE) dissolved in 10 µl of 10% ethanol were pipetted on to filter discs in petriplates. As a control, 10 µl of 10% ethanol were pipetted out on each filter disc in each petriplate. The petriplates were then sealed with parafilm and incubated at 30°C for 24 h and examined for zones of growth inhibition around each disc. The lowest concentration of hydroperoxide and hydroxides that produced detectable zones of inhibition was considered as minimum inhibitory concentration (MIC) and is expressed as micrograms of hydroperoxide or hydroxide per disc.

3.22 Effects of 13-HPOTrE, 13-HOTrE, 13-HPODE and 13-HODE on the mycelial weight in *S. rolfsii, R. solani* and *F. oxysporum*:

The mycelial discs (10 mm) were prepared separately from 7-day-old cultures of *S. rolfsii*, *R. solani* and *F. oxysporum* inoculated into 150 ml conical flasks, each containing 100 ml of potato dextrose broth and incubated at 30°C on orbital shaker at 120 rpm for fungal growth. The effect of hydroperoxides and hydroxides at 10 and 100 µM on growth of fungi was tested by adding into the

conical flasks containing the respective fungus. After incubation, the entire fungal mycelium was harvested by filtering through previously dried and weighed Whatman filter paper No. 1. The filter papers along with the mycelium were then dried to constant weight at 65^{o} C. Before weighing, the filter papers were allowed to cool and subsequently weighed in a balance. The weight of the mycelium was calculated by deducting the weight of filter paper from the final weight.

3.23 Effects of 13-HPOTrE, 13-HOTrE, 13-HPODE and 13-HODE on sclerotia formation in *S. rolfsii* and *R. solani*:

The effect of different concentrations of 13-HPODE, 13-HPOTrE, 13-HODE and 13-HOTrE on sclerotia formation in *S. rolfsii* and *R. solani* was tested. The fungal mycelial discs were placed in the centre of the petriplates containing potato dextrose agar medium. Sterile Whatman filter paper discs were placed in the petriplates. Different concentrations (5 µg and 10 µg) of hydroperoxides (13-HPODE and 13-HPOTrE) and hydroxides (13-HODE and 13-HOTrE) dissolved in 10 µl of 10% ethanol were pipetted on to filter discs in petriplates. As a control, 10 µl of 10% ethanol were pipetted out on filter disc in control petriplate. The petriplates were then sealed with parafilm and incubated at 30^{o}C for the growth of fungus. The average number of sclerotia formed per petriplate for different treatments was determined after 14 days.

All the experiments consisted of three replicates per treatment and the experiments were repeated thrice at different times.

Chapter 4

ROLE OF LIPOXYGENASES DURING GERMINATION AND ABIOTIC STRESS TOLERANCE IN GROUNDNUT

ROLE OF LIPOXYGENASES DURING GERMINATION AND ABIOTIC STRESS TOLERANCE IN GROUNDNUT

Lipoxygenases are non-heme iron containing dioxygenases widely distributed in the plant kingdom. They are involved in several plant metabolic processes, such as seed development, germination, vegetative growth, wounding, stress responses, senescence and cell signaling (Porta and Rocha-Sosa, 2002). The multiple functions ascribed to this enzyme are consistent with heterogeneity in plant LOX isozymes, which vary in isoelectric point, pH optima, heat stability, response to Ca^{2+}, and primary product formed (Vick and Zimmerman, 1987). In many plant species, germination is accompanied by elevated levels of LOX activities. Maximal accumulation of LOX protein and the corresponding mRNAs lasts from a few hours to a few days after germination (Porta and Rocha-Sosa, 2002). In the soybean, the best-studied plant with respect to lipoxygenase, two classes of lipoxygenase isoenzymes have been described (Kato *et al*., 1992a). They have been inferred to play a role in the development of germination capability or in plant defence (Kato *et al*., 1992b). Analysis of LOX functions and their precise physiological role is complicated by the presence of multiple LOX isozymes in a given plant system.

There is very limited information on the response of LOXs to salt or drought stresses. Bell and Mullet (1991) demonstrated that LOX gene expression in soybean is modulated in plants by water deficit, wounding, and methyl jasmonate. Maccarrone *et al*. (1995) showed that osmotic stress enhanced LOX-1 and LOX-2 expression without the intermediacy of ABA in soybean. Wounding, MJ or ABA treatment of mature leaves of common bean (*Phaseolus vulgaris* L.) induced LOX

mRNA accumulation suggesting that LOX is required during development and stress conditions (Porta *et al*., 1999).

Three LOX isozymes have been identified from peanut seed which exhibited differences in pH optima (Sanders *et al*., 1975; Pattee and Singleton, 1977). Prakash *et al*. (1990) reported that the LOX activity increased in early stages of germination followed by a decrease in the later course of senescing peanut cotyledons. The LOX activity decreased in peanut cotyledons upon treatment with KN and increased by ABA treatment, chemicals which delay or promote senescence. However, the association of LOX with respect to abiotic stresses has not been studied so far. The present study was aimed at analyzing the LOX activity during germination and in response to NaCl, PEG, ABA and MJ treatments.

RESULTS

4.1 pH optima of lipoxygenases in groundnut seedlings:

As a first step, the optimum pH for groundnut lipoxygenase was determined in 4-day-old seedlings of K-1375 variety. The maximum LOX activity (110 units/mg protein) was recorded at pH 6.5 whereas it was low (12 units/mg protein) at pH 9.0 with linoleic acid as the substrate (Fig. 6).

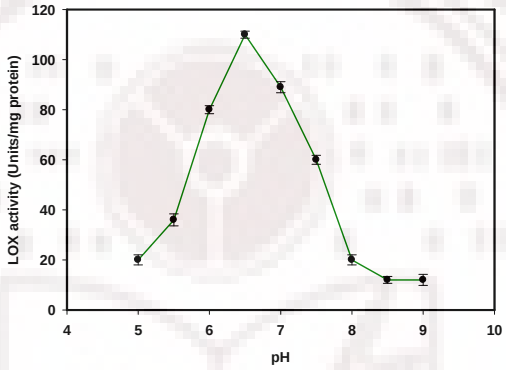


Fig. 6: Determination of the optimum pH for 4-day-old germinating seedlings of K- 1375 variety of groundnut. The LOX activity in the crude extracts prepared from groundnut seedlings was determined spectrophotometrically at 234 nm by incubating in reaction buffers of different pH (5.0-9.0) with linoleic acid as substrate.

4.2 LOX activity in germinating seedlings:

LOX activity was estimated during seed germination at 24 h intervals up to 7 days in three varieties of groundnut. LOX activity increased with the onset of germination followed by a marked increase on 4th day in K-1375 and TAG-24 varieties and decreased during subsequent stages of germination. Maximum LOX activity was observed on 5th day in JL-24 variety and decreased thereafter. The levels of LOX activity in 4-day-old seedlings of K-1375 variety were 7 times higher (128 units/mg protein) as compared to one-day-old seedlings (18 units/mg protein). The

LOX activity in 4-day-old seedlings was 6 times higher (90 units/mg protein) as compared to one-day-old seedlings (15 units/mg protein) in TAG-24 variety. The highest LOX activity was observed in 5-day-old seedlings of JL-24 variety which was 5 times higher (75 units/mg protein) when compared to one-day-old seedlings (15 units/mg protein). The highest LOX activities was found in 4-5-day-old seedlings coinciding with the highest growth rate (Fig. 7).

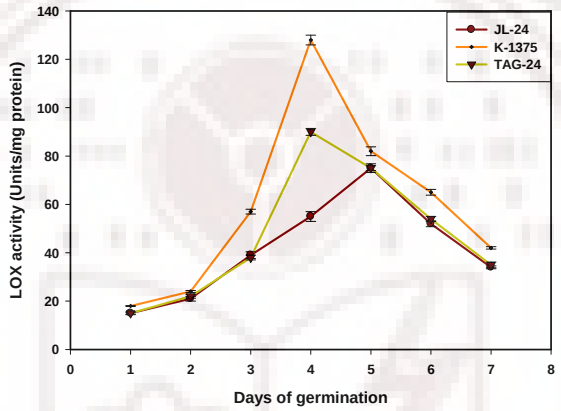


Fig. 7: LOX activity in germinating seedlings of groundnut. The samples were collected at 24 h intervals up to 7 days during seed germination in K-1375, TAG-24 and JL-24 varieties and analyzed for LOX activity spectrophotometrically at 234 nm using linoleic acid as substrate.

The LOX isozyme in mature seeds and 3-6-day-old groundnut seedlings was analyzed using native-PAGE. A single band corresponding to L-3 isozyme was observed in the mature seeds of groundnut. The intensity of this isozyme varied in 3-6-day-old germinating seedlings. Two isozymes *viz*., L-1 and L-2 were induced in 3-day-old seedlings of K-1375, TAG-24 and JL-24 varieties. In addition to L-1, L-2 and L-3 isozymes, three isozymes (L-4, L-5 and L-6) were induced in 4-day-old seedlings of K-1375 and TAG-24 whereas two isozymes (L-4 and L-5) were induced

in JL-24 variety. The induction of new isozymes in 4-5-day-old seedlings was associated with a decrease in the intensity of L-3 isozyme (Fig. 8).

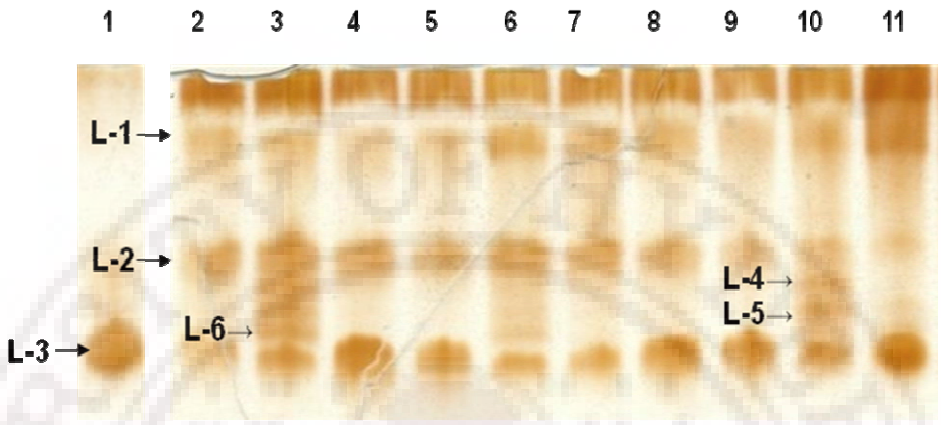


Fig. 8: Native PAGE showing LOX isozyme profiles of 3-6-day-old seedlings along with mature seed of groundnut. The crude protein extracts of different samples (20 µg each) were analyzed on native-PAGE and stained with o-dianisidine. Lane 1: Mature seed of K-1375 variety. Lanes 2-4: 3, 4 and 5-day-old germinating seedlings of K-1375 variety. Lanes 5-7: 3, 4 and 5-day-old germinating seedlings of TAG-24 variety. Lanes 8-11: 3, 4, 5 and 6-day-old germinating seedlings of JL-24 variety.

4.3.1 LOX activity in shoots and roots of groundnut seedlings in response to NaCl and PEG treatments:

LOX activity was estimated in roots and shoots of 20-day-old seedlings of K-1375 variety after 24 h of treatment with 200 mM NaCl or 23% PEG. LOX activity increased in roots and shoots in response to NaCl and PEG treatments as compared to controls. However, the increase in LOX activity was more pronounced in roots (68 units/mg protein) than shoots (50 units/mg protein) in response to NaCl treatment as compared to controls. Similarly, a higher LOX activity was observed in roots (77 units/mg protein) compared to shoots (58 units/mg protein) in PEG treated seedlings. It was observed that PEG treatment resulted in higher levels of LOX

activity compared to NaCl treatment. Since the roots exhibited higher LOX activity compared to shoots upon treatment with NaCl and PEG, they were used in subsequent experiments involving different durations of treatment in JL-24 (drought-susceptible) and K-1375 (drought-tolerant) varieties of groundnut (Fig. 9).

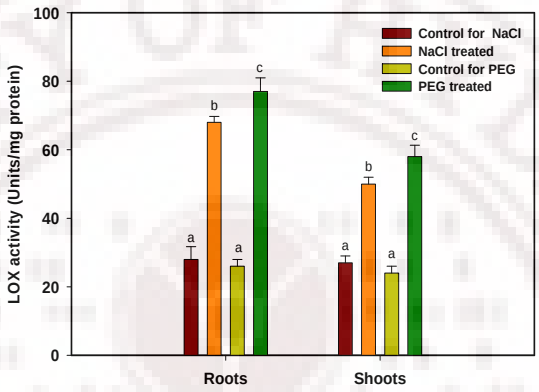


Fig. 9: Effect of NaCl and PEG on lipoxygenase activity in roots and shoots of seedlings treated with 200 mM NaCl or 23% PEG. LOX activity was estimated pectrophotometrically in the protein extracts of roots and shoots of 20-day-old seedlings after 48 h of treatment with NaCl or PEG. Values for each treatment are means ± SE of three replicates. Within each group, bars with the same letter are not significantly different ($P \leq 0.05$) according to Newman-Keul's multiples comparisons test.

4.3.2 Time course alteration of lipoxygenase activity in groundnut seedlings in response to NaCl and PEG treatments:

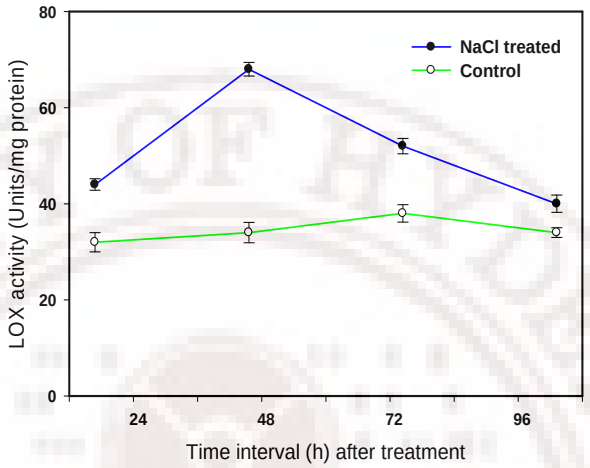
The response of 20-day-old seedlings of JL-24 (drought susceptible variety) and K-1375 variety (drought tolerant variety) after treatment with 200 mM NaCl and 23% PEG was examined. Treatment with NaCl resulted in leaf chlorosis after 24 h in JL-24 variety whereas it occurred after 48 h in K-1375 variety. Leaf chlorosis in PEG treated seedlings was observed after 12 h and 24 h in JL-24 and K-1375 varieties, respectively. Thus the concentrations of NaCl and PEG used in the study caused leaf

chlorosis in both the varieties although the leaf chlorosis was delayed in K-1375 variety compared to JL-24 variety.

LOX activity was determined in roots of 20-day-old seedlings at different time intervals after treatment with 200 mM NaCl in K-1375 and JL-24 varieties of groundnut. Analysis of LOX activity revealed an increase (44 units/mg protein) at 24 h which reached maximum (68 units/mg protein) at 48 h and declined during 72-96 h of treatment in K-1375 variety. In case of JL-24 variety, the maximum activity (30 units/mg protein) was recorded at 24 h and declined thereafter (28-14 units/mg protein). A 2-fold increase in LOX activity was observed at 48 h of treatment in K-1375 variety as compared to controls (Fig. 10a). The LOX activity at 24 h of treatment was found to be 1.6-fold higher than controls in JL-24 variety (Fig. 10b).

Activity staining analysis revealed the presence of three LOX isozymes *viz*., L-1, L-2 and L-3 in roots of control seedlings of K-1375 and JL-24 varieties. A marked increase in the intensity of L-3 isozyme was observed at 48 h of NaCl treatment followed by a decrease during later intervals in K-1375 variety (Fig. 11). In contrast, the intensity of L-3 isozyme was higher at 24 h and decreased gradually in JL-24 variety (Fig. 12). Thus the increase in LOX activity observed in response to NaCl treatment in K-1375 and JL-24 varieties at specific intervals coincided with the increase in the intensity of L-3 isozyme.

a.



b.

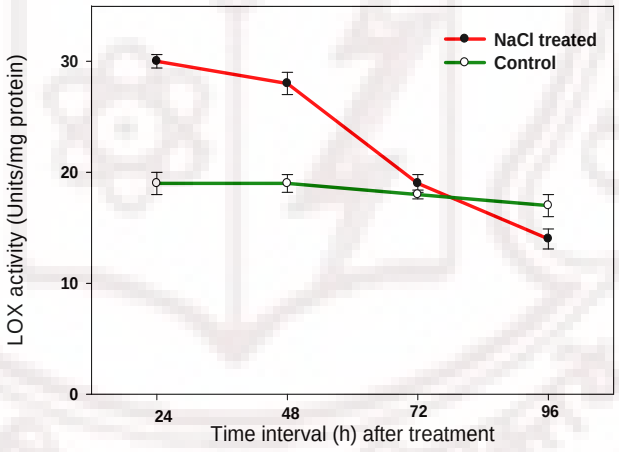


Fig. 10 a & b: LOX activity in roots of groundnut seedlings after treatment with 200 mM NaCl along with controls. The crude protein extracts prepared from roots of 20-day-old groundnut seedlings of K-1375 and JL-24 varieties after 24-96 h of NaCl treatment was assayed for LOX activity spectrophotometrically using linoleic acid as substrate. a. K-1375 variety b.JL-24 variety.

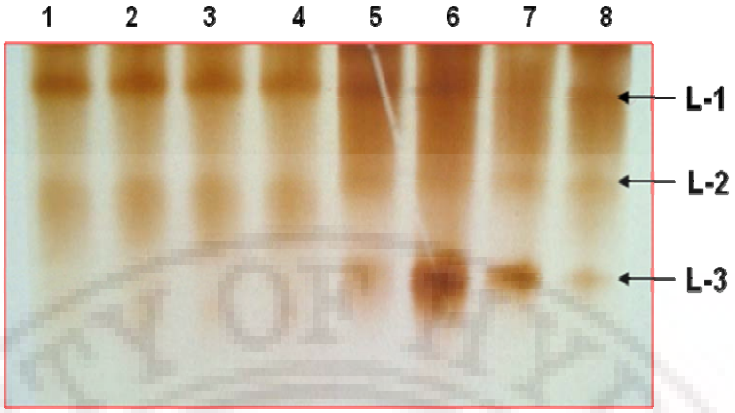


Fig. 11: Native PAGE showing LOX isozyme profiles in roots of 20-day-old seedlings of K-1375 variety after treatment with 200 mM NaCl. The crude protein extracts (20 µg each) of roots of 20-day-old seedlings after 24-96 h of treatment with NaCl were analyzed on native-PAGE. Lanes 1-4, Roots of control seedlings placed in Hoagland's solution for 24 h, 48 h, 72 h and 96 h, respectively. Lanes 5-8, Roots of seedlings treated with NaCl for different durations of 24 h, 48 h, 72 h and 96 h, respectively.

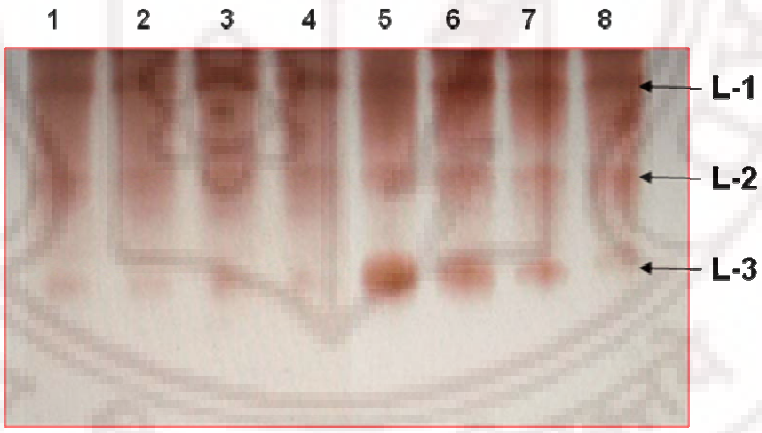


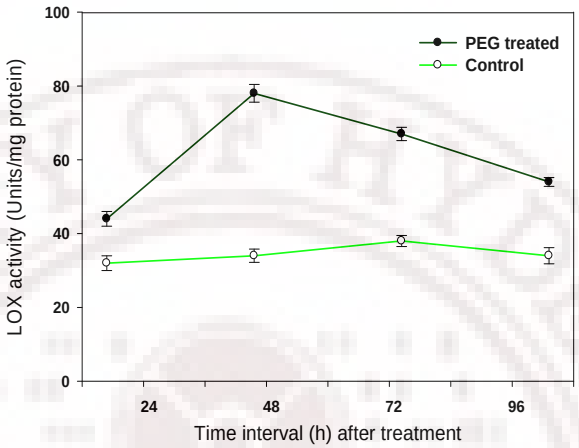
Fig. 12: Native PAGE ahowing LOX isozyme profiles in roots of 20-day-old seedlings of JL-24 variety after treatment with 200 mM NaCl. The crude protein extracts (20 µg each) of roots of 20-day-old seedlings after 24-96 h of treatment with NaCl were analyzed on native-PAGE. Lanes 1-4, Roots of control seedlings placed in Hoagland's solution for 24 h, 48 h, 72 h and 96 h, respectively. Lane 5-8, Roots of seedlings treated with NaCl for different durations of 24 h, 48 h, 72 h and 96 h, respectively.

Treatment of 20-day-old seedlings with PEG resulted in an increase in LOX activity (44 units/mg protein) at 24 h which reached maximum (78 units/mg protein) at 48 h and decreased gradually in K-1375 variety (Fig. 13a). The LOX activity observed at 48 h of treatment with PEG was found to be 2.3-fold higher than controls in K-1375 variety. The LOX activity at 24 h of treatment with PEG was found to be 1.6-fold higher than controls in JL-24 variety (Fig. 13b).

The LOX isozyme profiles in roots of 20-day-old seedlings of K-1375 and JL-24 varieties were analyzed after treatment with PEG. The intensity of L-3 isozyme increased immediately after treatment with PEG followed by a marked increase at 48 h and then decreased subsequently in K-1375 variety (Fig. 14). In case of JL-24 variety, the intensity of L-3 isozyme was higher at 24 h of treatment and thereafter declined (Fig. 15). The increase in LOX activity at 24 h and 48 h of treatment in JL-24 and K-1375 varieties, respectively coincided with the increase in intensity of L-3 isozyme.

In general, the LOX activity induced in response to NaCl and PEG treatments was greater in K-1375 variety as compared to JL-24 variety.

a.



b.

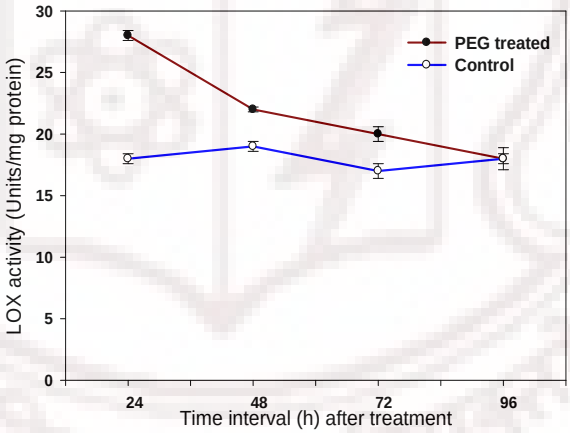


Fig. 13 a & b: LOX activity in groundnut seedlings after treatment with 23% PEG.

The crude protein extracts of roots of 20-day-old seedlings of K-1375 and JL-24 varieties after 24-96 h of treatment were analyzed for LOX activity spectrophotometrically using linoleic acid as substrate. a. K-1375 variety b.JL-24 variety.

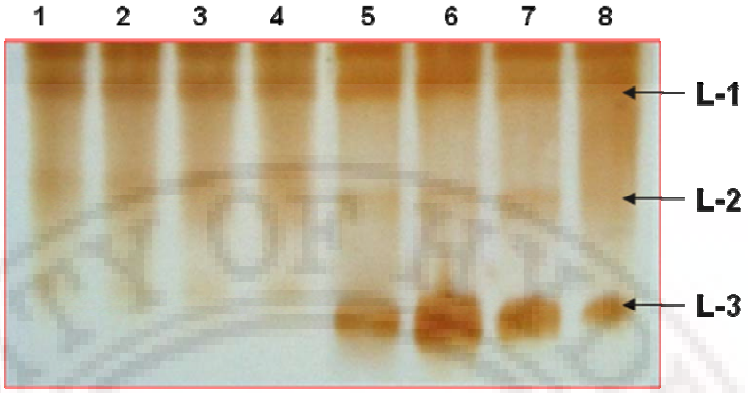


Fig. 14: Native PAGE showing LOX isozyme profiles in roots of 20-day-old seedlings of K-1375 variety after treatment with 23% PEG. The crude protein extracts (20 µg each) of roots of 20-day-old seedlings after 24-96 h of treatment with PEG were analyzed on native-PAGE. Lanes 1-4, Roots of control seedlings placed in Hoagland's solution for 24 h, 48 h, 72 h and 96 h, respectively. Lanes 5-8, Roots of seedlings treated with PEG for different durations of 24 h, 48 h, 72 h and 96 h, respectively.

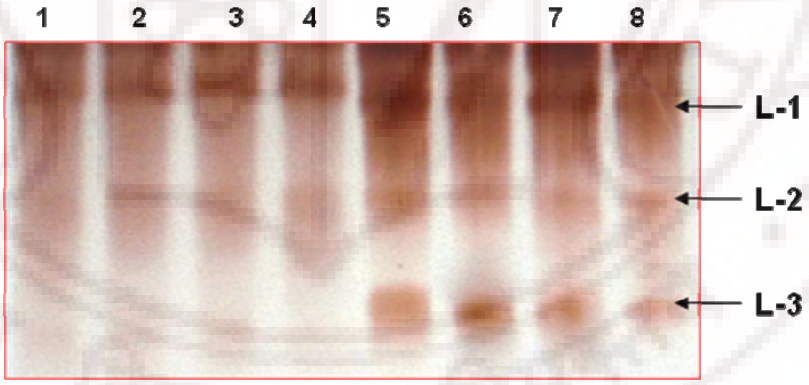


Fig. 15: Native PAGE showing LOX isozyme profiles in roots of 20-day-old seedlings of JL-24 variety after treatment with 23% PEG. The crude protein extracts (20 µg each) of roots of 20-day-old seedlings after 24-96 h of treatment with PEG were analyzed on native-PAGE. Lanes 1-4, Roots of control seedlings placed in Hoagland's solution for 24 h, 48 h, 72 h and 96 h, respectively. Lanes 5-8, Roots of seedlings treated with PEG for different durations of 24 h, 48 h, 72 h and 96 h, respectively.

4.4 Time course alteration of lipoxygenase activity in groundnut seedlings in response to ABA and MJ treatments:

The LOX activity was analyzed at different time intervals in roots of 20-day-old control and treated seedlings of K-1375 variety. A rapid increase in LOX activity was observed at 24 h of treatment with ABA with maximum activity being at 48 h which was 2.5-fold higher than controls (Fig. 16). The LOX activity decreased following 72 h of treatment with ABA although it was higher than controls. A 2-fold increase in LOX activity was observed at 24 h of treatment with MJ compared to control seedlings. The LOX activity decreased with an increase in duration of treatment from 48-96 h (Fig. 17).

Activity staining analysis of LOX isozymes of the roots of 20-day-old seedlings after treatment with ABA and MJ revealed variation in the intensity of L-3 isozyme. The increase in LOX activity observed after treatment with ABA coincided with the increase in intensity of L-3 isozyme as compared to controls. The intensity of L-3 isozyme was higher during 24-72 h of treatment whereas it decreased at 96 h of treatment with ABA (Fig. 18). The marked induction of LOX activity observed at 24 h of treatment with MJ was associated with an increase in the intensity of L-3 isozyme. This isozyme could not be detected at later intervals of treatment (48-96 h) with MJ (Fig. 19).

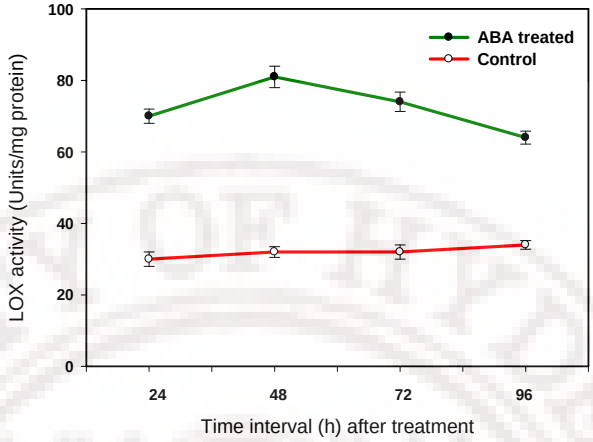


Fig. 16: LOX activity in groundnut seedlings after different hours of treatment with 100 µM ABA. The crude protein extracts of roots of 20-day-old seedlings of K-1375 variety after 24-96 h of treatment was analyzed for LOX activity spectrophotometrically using linoleic acid as substrate.

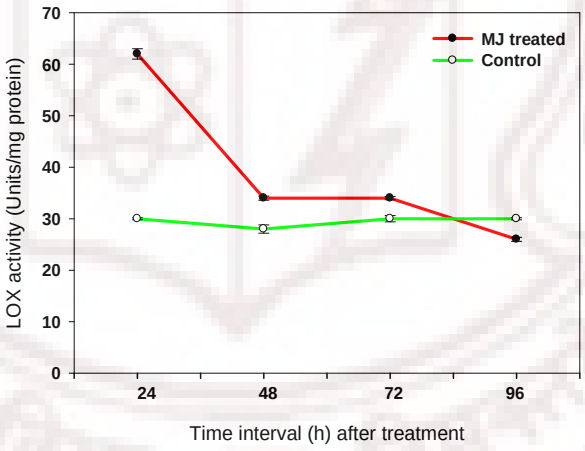


Fig. 17: LOX activity in groundnut seedlings after different hours of treatment with 100 µM MJ. The crude protein extracts of roots of 20-day-old seedlings of K-1375 variety after 24-96 h of treatment was analyzed for LOX activity spectrophotometrically using linoleic acid as substrate.

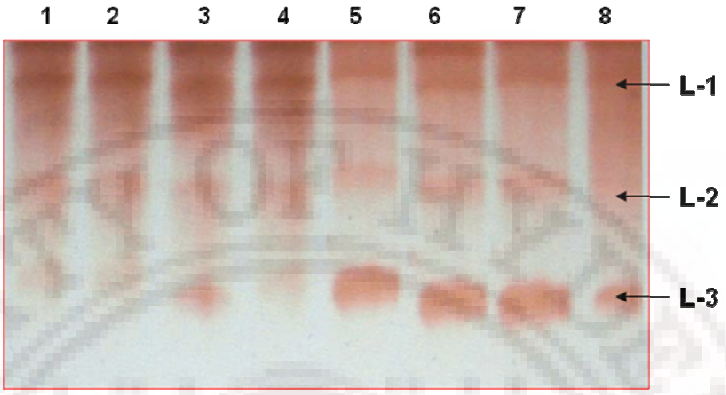


Fig. 18: Native PAGE showing LOX isozyme profiles in roots of 20-day-old seedlings of K-1375 variety after different hours of treatment with 100 µM ABA. The crude protein extracts (20 µg each) of roots of 20-day-old seedlings after 24-96 h of treatment with ABA were analyzed on native-PAGE. Lanes 1-4, Roots of control seedlings placed in Hoagland's solution for 24 h, 48 h, 72 h and 96 h, respectively. Lanes 5-8, Roots of seedlings treated with ABA for different durations of 24 h, 48 h, 72 h and 96 h, respectively.

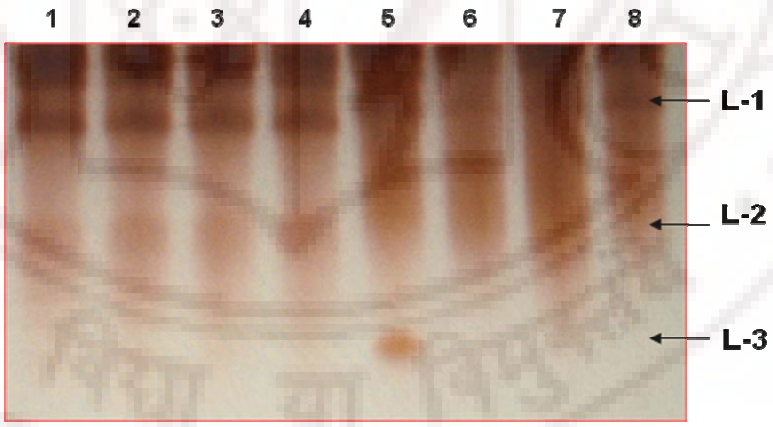


Fig. 19: Native PAGE showing LOX isozyme profiles in roots of 20-day-old seedlings of K-1375 variety after different hours of treatment with 100 µM MJ. The crude protein extracts (20 µg each) of roots of 20-day-old seedlings after 24-96 h of treatment with MJ were analyzed on native-PAGE. Lanes 1-4, Roots of control seedlings placed in Hoagland's solution for 24 h, 48 h, 72 h and 96 h, respectively. Lanes 5-8, Roots of seedlings treated with MJ for different durations of 24 h, 48 h, 72 h and 96 h, respectively.

DISCUSSION

4.5 LOX is induced during germination in groundnut:

The involvement of LOX or the LOX pathway in plant growth and development has been demonstrated in different plant species (Vick and Zimmerman, 1987; Grayburn *et al*., 1991; Hildebrand *et al*., 1991). Increase in lipoxygenase activity soon after germination and during early stages of seedling growth has been reported for a number of plant species. In the present study, analysis of LOX activity during seed germination showed a significant increase in 4-5-day-old germinating seedlings coinciding with the maximum growth rate of the tissue. Variation was observed with respect to LOX activity and isozymes in K-1375, TAG-24 and JL-24 varieties of groundnut. The LOX activity increased to 6-7-fold in 4-day-old seedlings of TAG-24 and K-1375 varieties of groundnut whereas a 5-fold increase in LOX activity was observed in 5-day-old seedlings of JL-24 variety. The high LOX activity observed during the early stages of seedling growth suggests that it might be involved in mobilization of storage lipids or has a protective role as the young seedlings are vulnerable to infection by fungal and bacterial pathogens. In rice, total seedling lipoxygenase activity increased by 20-fold, three days after the germination and most of the activity appeared in the developing shoot (Ohta *et al*., 1986). In corn and sunflower, lipoxygenase activity also increased following germination, but the maximum is not reached until four days after germination (Vick and Zimmerman, 1982). There is also data indicating that LOX is used as temporary storage of nitrogen (Vegetative Storage Protein-94 *i.e* VSP-94) during vegetative growth

(Tranbarger *et al*., 1991). Weichert *et al*. (2002) suggested that LOX induced in germinating cucumber seedlings could be involved in biosynthetic activities during germination producing e.g. C6-aldehydes, jasmonic acid or secondary products.

LOXs are multifunctional enzymes as different isozymes perform specific and peculiar functions during various developmental stages. In the present study, analysis of LOX isozymes by activity staining showed the presence of a single lipoxygenase isozyme (L-1) in the mature seeds which was also detected in germinating seedlings. The increase in LOX activity observed in 4-5-day-old seedlings was associated with induction of new isozymes. Three new isozymes, L-4, L-5 and L-6 appeared in seedlings of K-1375 and TAG-24 varieties whereas in JL-24 variety, two new isozymes, L-4 and L-5 were observed. The induction of new isozymes was associated with the decrease in intensity of L-1 isozyme present in mature seeds although their roles in seed germination remain to be investigated. The appearance of new isoforms during seedling growth indicates that seed isozymes and newly synthesized isozymes have different roles. Similarly, new isozymes were induced during germination and seedling growth in pea (Anstis and Friend, 1974; Chateigner *et al*., 1999), rice (Suzuki and Matsukura, 1997), *Arabidopsis thaliana* (Melan *et al*., 1994) and soybean (Kato *et al*., 1992). In mature soybean seeds, three to four lipoxygenase isozymes were present and during seed germination three additional isozymes appeared in soybean cotyledons (Christopher *et al*., 1970, 1972; Kato *et al*., 1992). Mo and Koster (2006) reported that there was a shift of LOX activity during germination from radicles to shoots that accompanies the transition from seed LOXs to vegetative LOXs. Two new vegetative isoforms appeared in the shoots and gradually replaced

the pea seed lipoxygenases as the principal contributors to LOX enzyme activity during early seedling growth.

The involvement of LOX in growth processes is supported by the observation that during germination the new LOX isoforms appeared just before the growth resumption suggesting that these new isoforms have a role in the remodeling of membrane composition during growth (Chateigner *et al*., 1999). Loiseau *et al*. (2001) suggested several putative roles for lipoxygenases in seeds such as fatty acid peroxidation in membranes or storage lipids, production of growth regulators (jamonates, abscisic acid), storage proteins and protection against pathogen attack during germination and early seedling growth. On the contrary, Wang *et al*. (1999) noted that there is no substantial oxygenation of polyunsaturated fatty acids suggesting that LOX is not used for lipid mobilization during germination. Further studies are required for elucidating the role of lipoxygenases induced in germinating seedlings of groundnut.

4.6 LOX induced by NaCl and PEG treatments exhibit a different pattern in drought tolerant and susceptible varieties:

The activation of LOX pathway in plants in response to pathogens, insects or abiotic stresses and at distinct stages of development has been demonstrated (Feussner and Wasternack, 2002). The role of LOX in abiotic stress and induction of vegetative lipoxygenase by water stress has been reported earlier (Mason and Mullet, 1990; Maccarrone *et al*., 1992; Todd *et al*., 1992). In the present study, LOX activity in the drought tolerant, K-1375 variety was much higher than that in the drought susceptible JL-24 variety at all the periods of germination. An increase in LOX activity at specific time points was observed in groundnut seedlings subjected to NaCl and PEG

treatments, which was more pronounced in the roots compared to shoots. The pattern of LOX induction varied in susceptible and tolerant varieties. A greater increase in LOX activity was observed in roots of drought tolerant variety in response to NaCl and PEG treatments as compared to drought susceptible variety. LOX activity was found to be 2 times higher in roots at 48 h of treatment with NaCl as compared to controls in K-1375 variety. Similarly, treatment with PEG resulted in an increase in LOX activity by 2.3 times as compared to controls in K-1375 variety. In contrast, maximum LOX activity was detected in roots of JL-24 variety at 24 h of treatment with NaCl and PEG and thereafter decreased. However, the level of LOX activity recorded at 24 h of treatment with NaCl and PEG in JL-24 variety was lower than that of K-1375 variety. It was further noted that the LOX activity induced in response to NaCl and PEG treatments was higher than that of controls for all the intervals examined in K-1375 variety. On the contrary, LOX activity at 96 h after treatment with NaCl and PEG in JL-24 variety was lower or equivalent to controls.

LOX isozyme profiles of roots of K-1375 and JL-24 varieties after treatment with NaCl and PEG showed a variation in intensity of isozyme L-3. The high LOX activity observed during 48-96 h of treatment with NaCl and PEG was associated with an increase in the intensity of L-3 isozymes in K-1375 variety. Thus the pattern of LOX induction varied with the genotype and the treatment. Ben-Hayyim *et al*. (2001) reported that LOX was induced very rapidly only in the salt-tolerant cells of *Citrus sinensis* in a transient manner. This increase was not observed in the salt-sensitive cells, suggesting that it does not simply reflect a salt-induced damage caused by the stress, but rather a response that might be related to signaling in the defense mechanism. Furthermore, the induction was specific to salt stress and did not occur

with other osmotic-stress inducing agents such as PEG or mannitol, or under hot or cold conditions or in the presence of abscisic acid. These results are in contrast to present observations where LOX was induced in response to NaCl and PEG treatments although differential pattern of induction was observed in K-1375 and |JL-24 varieties. The increase in LOX activity observed over time in drought tolerant variety indicates that this enzyme could play an important role during stress tolerance.

4.7 LOX is regulated by ABA and MJ stresses:

The similarities between jasmonic acid and abscisic acid in chemical structure and many physiological effects including plant stress responses are well documented in literature (Weidhase *et al*., 1987; Sembdner and Parthier, 1993; Melan *et al*., 1993). Abscisic acid (ABA) and methyl jasmonate (MJ) treatment have been shown to induce LOX expression in different plant species. It was therefore of interest to know whether LOX activity is induced in response to treatment with growth regulators such as MJ and ABA, that have been involved in stress responses. In the present study, treatment of 20-day-old seedlings with ABA resulted in an increase in LOX activity in roots at 24 h which reached maximum levels at 48 h and subsequently decreased. The LOX activity induced in response to ABA treatment remained higher than controls for all the intervals examined. The increase in LOX activity was associated with an increase in the intensity of L-3 isozymes although the intensity of the band varied. These results support the findings of Prakash *et al*. (1990) who reported that KN treatment lowered the lipoxygenase activity where as ABA treatment increased the LOX activity when compared to untreated seedlings in groundnut. It has been suggested that ABA enhanced lipoxygenase activity could be due to the breakdown of cell membranes in senescing tissues, which releases large quantities of free fatty

acids, especially linoleic acid (Draper, 1969). Porta *et al.* (1999) showed that LOX mRNA levels were higher in the mature region than in the growing region of bean seedlings subjected to drought or treated with ABA. The contrasting expression pattern was suggested to be the result of a decrease in the seedling growth rate provoked by drought or ABA treatments (Creelman *et al*., 1990; Colmenoero-Flores *et al*., 1999).

The present study showed a 2-fold increase in LOX activity at 24 h in groundnut seedlings upon treatment with MJ as compared to controls. The LOX activity drastically reduced at 48 h and reached values lower than control at 96 h of treatment with MJ. The increase in LOX activity at 24 h of treatment with MJ was associated with an increase in intensity of L-3 isozyme. These results suggest that LOX activity is induced transiently by MJ in groundnut. This, however, is different from the sustained induction of LOX observed in groundnut seedlings treated with ABA. The rapid and transient expression of *PnLOX1* gene in mature seeds to methyl jasmonate has been reported for peanut (Burow *et al*., 2000). The expression of wound-inducible maize LOX with dual positional specificity was unusual in that it was expressed in both early and late stages of the stress response in exogenously supplied MJ (Kim *et al*., 2003). Melan *et al*. (1993) reported that LOX1 mRNA levels were elevated in *Arabidopsis* roots treated with MJ within 24 h and remained above control levels for at least 96 h.

The present experiments involving NaCl, PEG, ABA, and MJ showed a clear increase in the intensity of L-3 isozyme although variation was noticed with respect to the intensity of isozyme and timing of induction. Since the pattern of LOX induction after treatment with PEG was more similar with that of ABA, it is possible that ABA

acts as a mediator in this response. Tamas *et al*. (2009) hypothesized that the role of LOX in plant metabolic processes in the roots may depend on the level of reactive oxygen species: at physiological concentrations of ROS, LOX may be involved in the processes of root growth, while at the elevated concentrations of ROS induced by different stress conditions, it may be involved in root growth inhibition through ecotopic differentiation.

In summary, the studies showed a maximum induction of LOX activity in 4-5-day-old seedling along with induction of additional isozymes in groundnut. The pattern of LOX induction varied in JL-24 (drought susceptible) and K-1375 (drought tolerant) varieties in response to treatments with NaCl and PEG with the maximum increase being observed in K-1375 variety compared to JL-24 variety. Similarly, treatment with ABA and MJ resulted in an increase in LOX activity in K-1375 variety, but the pattern of LOX induction was sustained with ABA compared to transient induction with MJ. These experiments provided preliminary evidence of the involvement of LOX in tolerance to abiotic stresses in groundnut.

Chapter 5

ROLE OF LOX IN DISEASE RESISTANCE IN GROUNDNUT

ROLE OF LOX IN DISEASE RESISTANCE IN GROUNDNUT

LOXs are widely known to vitally contribute to plant defenses against pathogenic microorganisms. A major difference between resistance and susceptibility of plants to pathogens is in the timing of induction of host-defense genes. Typically, defense-response genes are induced rapidly during a hypersensitive response and more slowly, if at all, in a disease reaction (Dixon and Harrison, 1990). LOX enzyme activity has also been shown to be induced rapidly during a disease-resistance response and more slowly in a susceptible interaction. This association of increased LOX activity and an effective defense response has been observed for several plant pathosystems (Keppler and Novacky, 1987; Croft *et al*., 1990; Ohta *et al*., 1991; Koch *et al*., 1992; Melan *et al*., 1993; Sailaja *et al*., 1997; Bohland *et al*., 1997; Buonaurio and Servili., 1999; Uma Maheswari *et al*., 2000; Burow *et al*., 2000; Gobel *et al*., 2001; Babitha *et al*., 2004).

In plants, LOXs contribute to disease resistance mechanisms through different pathways. According to findings from previous studies, the LOX type 2 13-LOX group mediates disease resistance responses in plants primarily through JA signaling cascades. On the other hand, 9-LOX may mediate antimicrobial activity *via* oxylipin synthesis. LOXs can directly participate in pathogen-induced hypersensitivity reactions in plants (Jean-Luc *et al*., 2002). Compounds formed through both the 13-LOX and 9-LOX pathways participate in plant defense against pests and pathogens. Illustrative of this, the few reported mutant or transgenic plants affected in synthesis, signaling, or perception of selected oxylipins show altered responses to pathogens (Shah, 2005).

The *Arabidopsis LOX1* gene was dramatically induced upon infiltration with pathogenic *Pseudomonas syringae* (Melan *et al*., 1993). Differential kinetics of accumulation of LOX mRNA in compatible *versus* incompatible interactions was also reported for *P. syringae* infection of tomato (Koch *et al*., 1992). Expression levels of *Populus deltoides*, *PdLOX1* and *PdLOX2* increased following exposure to fungal pathogen and JA and also following injury (Cheng *et al*., 2006). Moreover, using antisense approaches, it was demonstrated that expression of *NtLOX1* gene, a pathogen-and elicitor-induced 9-LOX gene was essential for resistance in tobacco (Rance *et al*., 1998; Mene-Saffrane *et al*., 2003). These studies have demonstrated that LOX plays an important role in disease resistance in different plant species.

There is limited information on the role of LOX in disease resistance in groundnut. Earlier reports have showed that *PnLOX1* gene, a mixed function LOX was highly induced by methyl jasmonate, wounding and *Aspergillus* infections in mature cotyledons of peanut (Burow *et al*., 2000). On the other hand, the expression of *PnLOX2* and *PnLOX3* was significantly repressed by the *A. flavus* infections in mature seed (Tsitsigiannis *et al*., 2005).

Stem rot disease caused by the fungus *Sclerotium rolfsii* and *Rhizoctonia* damping off disease caused by *Rhizoctonia solani* are the two important diseases that cause considerable losses at the early stage of crop growth. The role of LOX in resistance to *Sclerotium rolfsii* and *Rhizoctonia solani* has not investigated so far. The present investigation deals with the LOX induction in seedlings in response to inoculation with *S. rolfsii* and *R. solani*. In addition, the LOX isozymes from *S. rolfsii* inoculated seedlings were purified and characterized. The studies were extended to

determine the expression pattern of *PnLOX1*, *PnLOX2* and *PnLOX3* genes in groundnut seedlings inoculated with *S. rolfsii* using RT-PCR.

RESULTS

5.1 LOX activity in groundnut seedlings after inoculation with *Rhizoctonia solani*:

The LOX activity was estimated in two-day-old seedlings of ICG-4747 variety of groundnut after inoculation with *Rhizoctonia solani* pathogen along with the controls. LOX activity increased (18-84 units/mg protein) in the control seedlings as germination progressed. Inoculation of the seedlings with the pathogen resulted in an increase in LOX activity at 6 h (24 units/mg protein) followed by a marked increase (120 units/mg protein) at 18 h and decreased subsequently. A 2.2-fold increase in LOX activity was observed at 18 h of inoculation as compared to controls (Fig. 20).

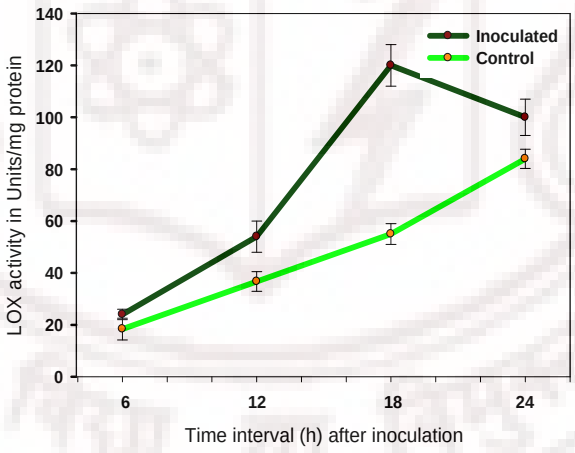


Fig. 20: LOX activity in seedlings of ICG-4747 variety after different hours of inoculation with *Rhizoctonia solani*. The crude protein extracts prepared from two-day old seedlings after 6-24 h of inoculation with *R. solani* along with the controls was assayed for LOX activity spectrophotometrically using linoleic acid as substrate.

LOX isozymes were analyzed on native PAGE in seedlings inoculated with *R. solani* pathogen along with controls. Two isozymes *viz*., L-1 and L-2 were detected in control and inoculated seedlings with an increase in the intensity of L-2 isozyme in inoculated seedlings compared to controls for all the intervals (6 – 24 h) examined (Fig. 21).

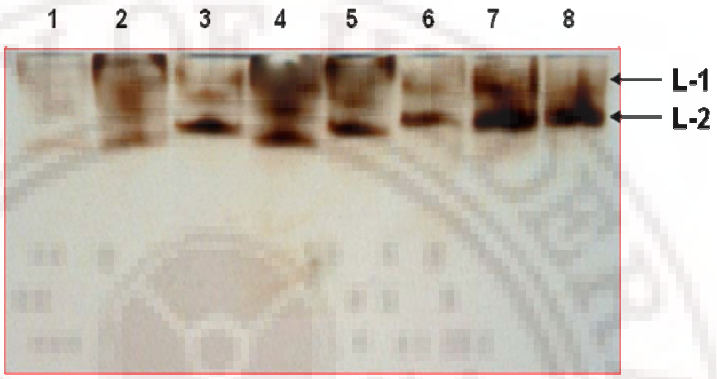


Fig. 21: Activity staining of LOX isozymes after inoculation of seedlings of ICG-4747 variety with *R. solani*. The crude proteins (20 µg each) of two-day-old seedlings after 6 - 24 h of inoculation with *R. solani* were analyzed for LOX isozymes on native PAGE using linoleic acid as substrate and stained with o-dianisidine. Lanes 1-4: Control seedlings harvested at 6 h, 12 h, 18 h and 24 h, respectively. Lanes 5-8: Seedlings harvested at 6 h, 12 h, 18 h and 24 h after inoculation with *R. solani*.

5.2.1 LOX activity in shoots of resistant and susceptible varieties of groundnut after inoculation with *Sclerotium rolfsii*:

The response of the seedlings of resistant and susceptible varieties after inoculation with *S. rolfsii* was determined. The disease symptoms appeared within 36 h in susceptible varieties whereas no disease symptoms appeared up to 36 h in resistant varieties. The disease severity increased in susceptible variety after 72 h of inoculation as reflected by damage in collar region of the shoot whereas the extent of damage was less in resistant variety (Fig. 22b and 23b). The susceptible variety, TMV-2 died after 7 days of inoculation with the stem rot pathogen whereas the

resistant variety, R-2001-1 appeared normal although the growth was lesser than that of uninoculated controls (Fig. 22a and 23a).

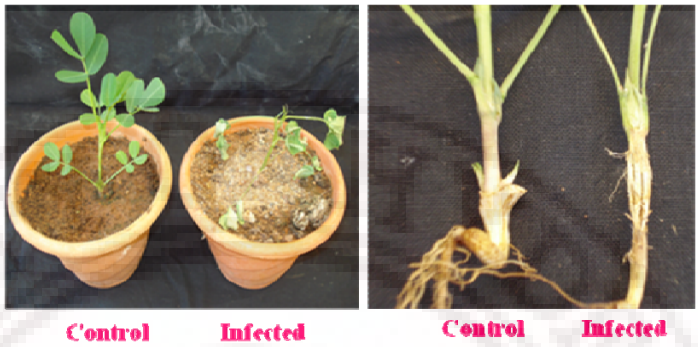


Fig. 22: Ten-day-old seedlings of TMV-2 variety after inoculation with *S. rolfsii* along with controls. a. Left: Control seedlings. Right: Mortality of the seedlings after seven days of inoculation. b. Left: Collar region of control seedling. Right: Damaged collar region of seedlings after inoculation with *S. rolfsii*.

Fig. 23: Ten-day-old seedlings of R-2001-1 variety after inoculation with *S. rolfsii* along with controls. a. Left: Control seedlings. Right: Seedlings of R-2001-1 variety after seven days of inoculation. b. Left: Collar region of control seedling. Right: Less damaged collar region of inoculated seedlings.

The LOX activity was estimated in shoots of 10-day-old seedlings of resistant varieties *viz.*, R-9214, R-8808, R-9217, R-9227, R-2001-1, R-2001-2, R-2001-3, K-134 and susceptible varieties *viz*., TMV-2 and S-206 after 48 h of inoculation with *S. rolfsii* pathogen along with controls. LOX activity in shoots of 10-day-old control seedlings of resistant varieties was significantly higher (21.7-21.3 units/mg protein) than that of susceptible varieties (17.7-18.7 units/mg protein). Inoculation of the seedlings with the stem rot pathogen resulted in a marked increase (2.6-3.6-fold) in LOX activity in disease resistant varieties as compared to uninoculated controls. Only a 1.47-fold and-1.5 fold increase in LOX activity was observed in inoculated seedlings of S-206 and TMV-2 varieties, respectively as compared to controls (Fig. 24).

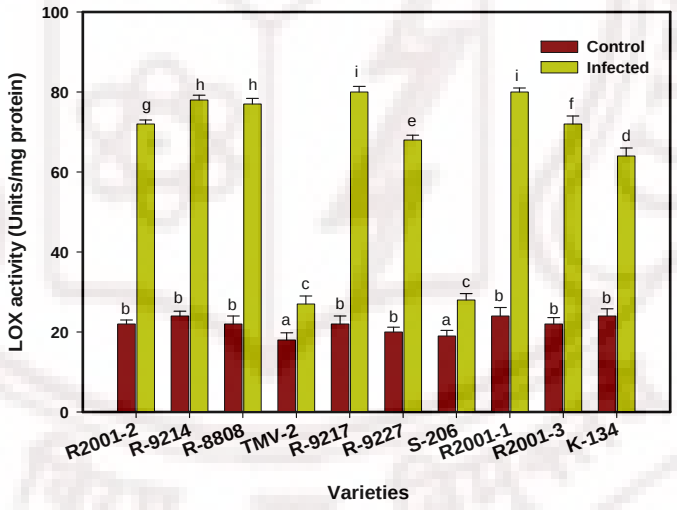


Fig. 24: LOX activity in shoots of resistant and susceptible varieties after 48 h of inoculation with *S. rolfsii* along with controls. The crude protein extracts of different samples were analyzed for LOX activity spectrophotometrically using linoleic acid as substrate. Values for each treatment are means ± SE of three replicates. Bars with the same letter are not significantly different ($P \leq 0.05$) according to Newman-Keul's multiple comparisons test.

LOX activity was estimated in shoots and roots of TMV-2 and R-2001-1 varieties after 48 h of inoculation with stem rot pathogen along with controls. A marked increase (3.2-fold) in LOX activity was observed in shoots of inoculated seedlings of resistant variety after inoculation with the pathogen compared to controls. A 1.78-fold increase in LOX activity was observed in roots of resistant variety after inoculation with the pathogen. In case of susceptible variety, the LOX activity increased by 1.5-fold and 1.37-fold in shoots and roots respectively, after inoculation with the stem rot pathogen (Fig. 25).

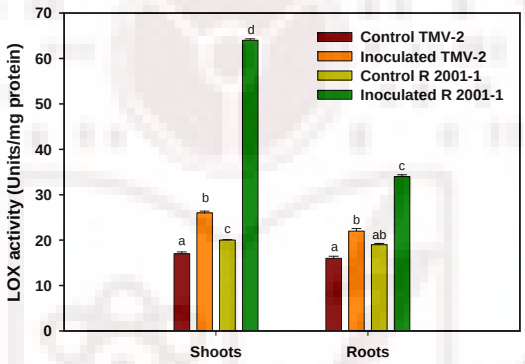


Fig. 25: LOX activity in shoots and roots of TMV-2 and R-2001-1 varieties after 48 h of inoculation with *S. rolfsii*. The crude protein extracts of different samples were analyzed for LOX activity spectrophotometrically using linoleic acid as substrate. Values for each treatment are means ± SE of three replicates. Within each group, bars with the same letter are not significantly different ($P \leq 0.05$) according to Newman-Keul's multiple comparisons test.

5.2.2 LOX activity in shoots of *S. rolfsii* inoculated seedlings of disease resistant (R-2001-1) variety of groundnut:

LOX activity was estimated in roots of R-2001-1 variety after different time intervals of inoculation with the stem rot pathogen. LOX activity increased (24 units mg/protein) from 12 h of inoculation and reached the highest (72-66 units/mg protein) during 42-60 h of inoculation and then decreased (65-40 units/mg protein) during the

subsequent intervals (66-96 h). The maximum LOX activity was observed at 48 h of inoculation which was 3.3 times higher when compared to the controls. Even at 96 h of inoculation, LOX activity remained higher (40 units/mg protein) than that of controls (24 units/mg protein) (Fig. 26).

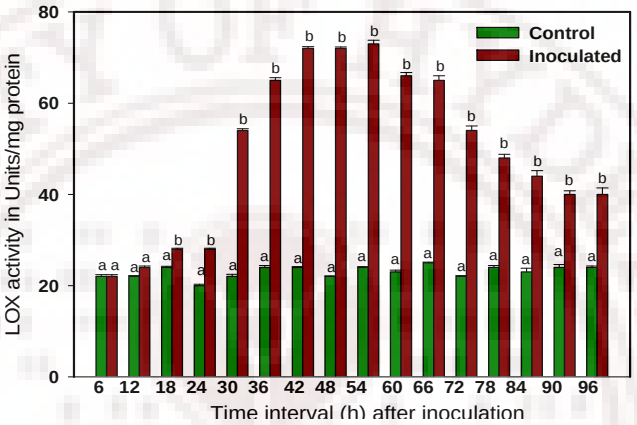


Fig. 26: LOX activity in shoots of R-2001-1 variety after different intervals (6 -96 h) of inoculation with *S. rolfsii*. The crude protein extracts of different samples were analyzed for LOX activity spectrophotometrically using linoleic acid as substrate. Values for each treatment are means ± SE of three replicates. Within each group, bars with the same letter are not significantly different ($P \leq 0.05$) according to Newman-Keul's multiple comparisons test.

LOX isozymes were analyzed on native PAGE in shoots of resistant variety after different intervals of inoculation with *S. rolfsii* along with controls. LOX isozymes *viz*., L-1, L-2 and L-3 were observed in control and inoculated seedlings. There was no difference observed in the intensity of L-1, L-2 and L-3 isozymes from 6-24 h in inoculated seedlings compared to controls. The intensity of L-1 isozyme increased after 30 h of inoculation compared to controls. The intensity of L-3 isozyme was variable in inoculated seedlings during 30-96 h of inoculation compared to controls. The most noticeable difference was the induction of L-4 and L-5 isozymes in inoculated seedlings at 30 h and continued to be expressed upto 96 h

whereas these isozymes were not induced in control seedlings (Fig. 27-30). Thus induction of new isozymes along with the increase in the intensity of L-1 isozyme could have accounted for increased LOX activity in inoculated seedlings.

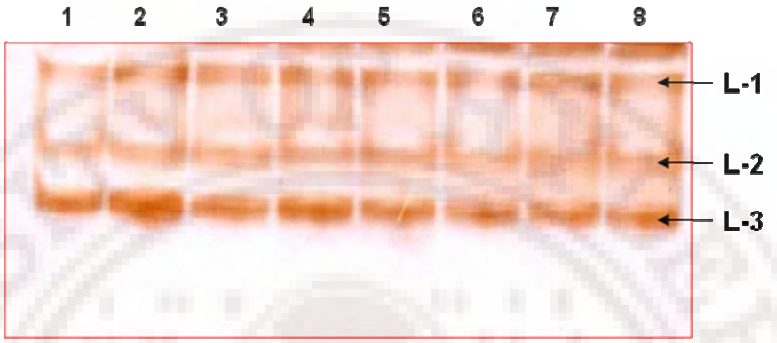


Fig. 27: Activity staining of LOX isozymes in shoots of R-2001-1 variety after 6-24 h of inoculation with *S. rolfsii*. The crude protein extracts of shoots of 10-day-old inoculated seedlings along with controls were analyzed on native PAGE and stained with o-dianisidine. Lanes 1-4: Shoots of control seedlings harvested at 6 h, 12 h, 18 h and 24 h, respectively. Lanes 5-8: Shoots of inoculated seedlings harvested at 6 h, 12 h, 18 h and 24 h, respectively.

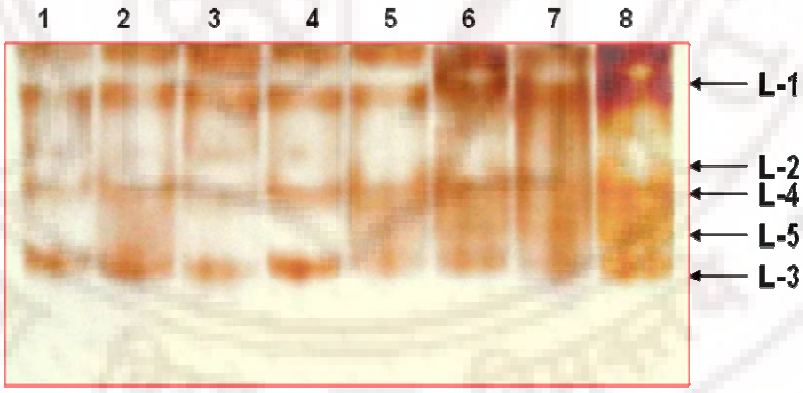


Fig. 28: Activity staining of LOX isozymes in shoots of R-2001-1 variety after 30-48 h of inoculation with *S. rolfsii*. The crude protein extracts of shoots of 10-day-old inoculated seedlings along with controls were analyzed on native PAGE and stained with o-dianisidine. Lanes 1-4: Shoots of control seedlings harvested at 30 h, 36 h, 42 h and 48 h, respectively. Lanes 5-8: Shoots of inoculated seedlings harvested at 30 h, 36 h, 42 h and 48 h, respectively.

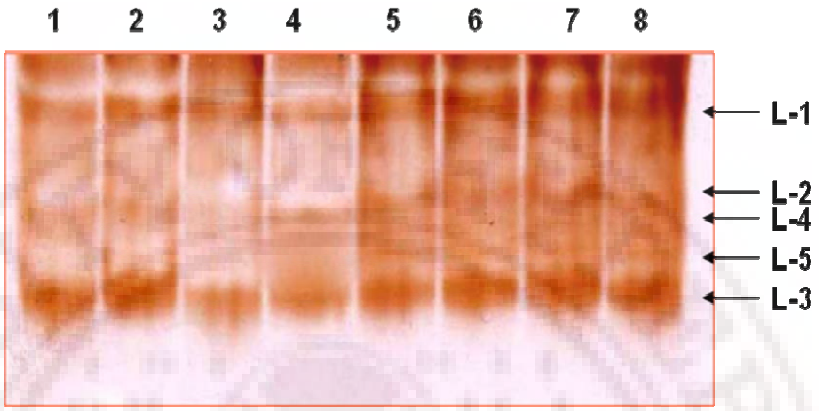


Fig. 29: Activity staining of LOX isozymes in shoots of R-2001-1 variety after 54-72 h of inoculation with *S. rolfsii*. The crude protein extracts of shoots of 10-day-old inoculated seedlings along with controls were analyzed on nativePAGE and stained with o-dianisidine. Lanes 1-4: Shoots of control seedlings harvested at 54 h, 60 h, 66 h and 72 h, respectively, Lanes 5-8: Shoots of inoculated seedlings harvested at 54 h, 60 h, 66 h and 72 h, respectively.

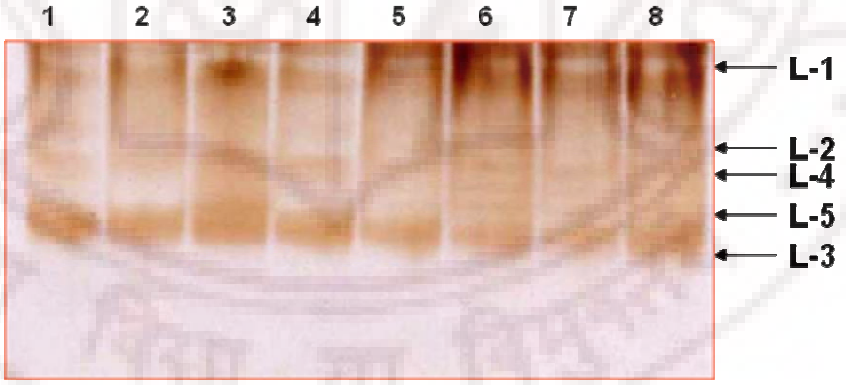


Fig. 30: Activity staining of LOX isozymes in shoots of R-2001-1 variety after 78-96 h of inoculation with *S. rolfsii*. The crude protein extracts of shoots of 10-day-old inoculated seedlings along with controls were analyzed on nativePAGE and stained with o-dianisidine. Lanes 1-4: Shoots of control seedlings harvested at 78 h, 84 h, 90 h and 96 h, respectively, Lanes 5-8: Shoots of inoculated seedlings harvested at 78 h, 84 h, 90 h and 96 h, respectively.

5.2.3 LOX activity in shoots of susceptible variety (TMV-2) after inoculation with *S. rolfsii*:

The LOX activity was estimated in shoots of TMV-2 (susceptible) variety after different time intervals (6-96 h) of inoculation with *S. rolfsii* along with controls. The maximum LOX activity was observed (26 units/mg protein) during 42-48 h of inoculation which was 1.5-1.6 fold higher than controls. It was observed that LOX activity decreased with the increase in time which was lower than controls (Fig. 31).

Analysis of LOX isozymes using native-PAGE in the shoots of TMV-2 variety showed the presence of three isozymes in the control and inoculated seedlings. An increase in the intensity of L-3 isozyme was observed during 42-48 h of inoculation compared to controls (Fig. 32 to 34).

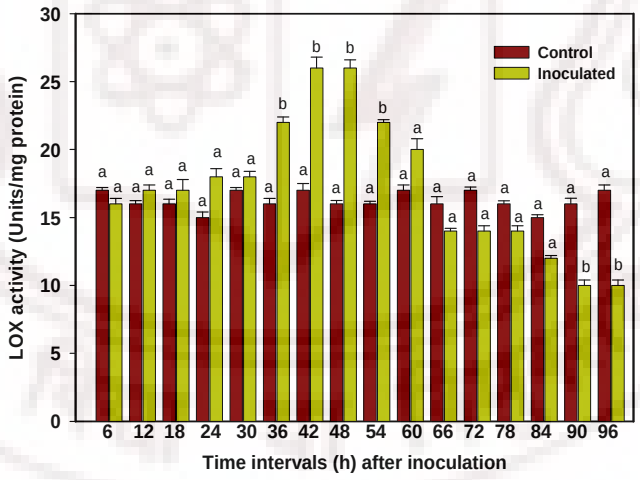


Fig. 31: LOX activity in shoots of TMV-2 variety after different intervals (6-96 h) of inoculation with *S. rolfsii.* The crude protein extracts of different samples were analyzed for LOX activity spectrophotometrically using linoleic acid as substrate. Values for each treatment are means ± SE of three replicates. Within each group, bars with the same letter are not significantly different ($P \leq 0.05$) according to Newman-Keul's multiple comparisons test.

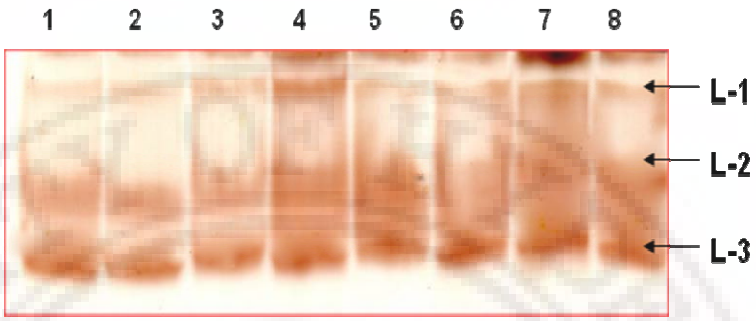


Fig. 32: Activity staining of LOX isozymes in shoots of TMV-2 variety after 6-24 h of inoculation with *S. rolfsii*. The crude protein extracts of shoots of 10-day-old inoculated seedlings along with controls were analyzed on native PAGE and stained with o-dianisidine. Lanes 1-4: Shoots of control seedlings harvested at 6 h, 12 h, 18 h and 24 h, respectively, Lanes 5-8: Shoots of inoculated seedlings harvested at 6 h, 12 h, 18 h and 24 h, respectively.

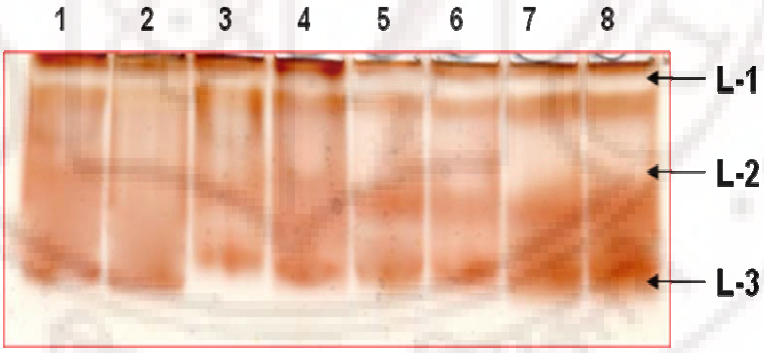


Fig. 33: Activity staining of LOX isozymes in shoots of TMV-2 variety after 30-48 h of inoculation with *S. rolfsii*. The crude protein extracts of shoots of 10-day-old inoculated seedlings along with controls were analyzed on native PAGE and stained with o-dianisidine. Lanes 1-4: Shoots of control seedlings harvested at 30 h, 36 h, 42 h and 48 h, respectively. Lanes 5-8: Shoots of inoculated seedlings harvested at 30 h, 36 h, 42 h and 48 h, respectively.

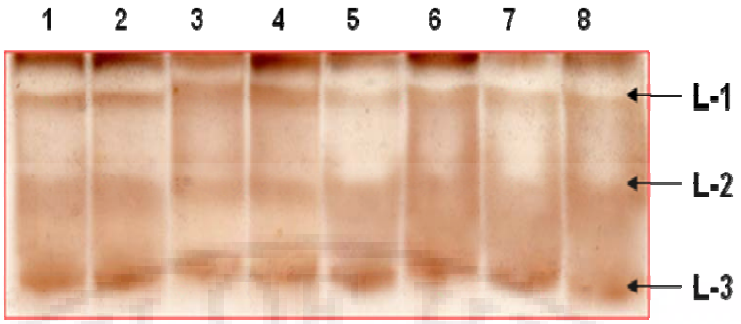


Fig. 34: Activity staining of LOX isozymes in shoots of TMV-2 variety after 54-72 h of inoculation with *S. rolfsii*. The crude protein extracts of shoots of 10-day-old inoculated seedlings along with controls were analyzed on native PAGE and stained with o-dianisidine. Lanes 1-4: Shoots of control seedlings harvested at 54 h, 60 h, 66 h and 72 h, respectively. Lanes 5-8: Shoots of inoculated seedlings harvested at 54 h, 60 h, 66 h and 72 h, respectively.

In general, a greater increase in LOX activity was observed in shoots of resistant variety during 42-54 h of inoculation with the stem rot pathogen as compared to susceptible variety. Further, the LOX activity after 96 h of inoculation remained higher than controls in resistant variety whereas it was lower than controls in susceptible variety.

5.3 HPLC analysis of LOX product formation in *S. rolfsii* inoculated seedlings:

The LOX product formation was analyzed by RP-HPLC in the samples of R-2001-1 and TMV-2 varieties after 6-96 h of inoculation with *S. rolfsii* along with controls using linoleic acid as the substrate. The LOX product formed was identified as 13-HODE in the experimental samples by comparing with the 13-HODE standard which showed a major peak with the retention time of 7.7 min (Fig. 35). The 13-HODE formation was higher in samples of resistant varieties compared to controls for all the intervals examined.

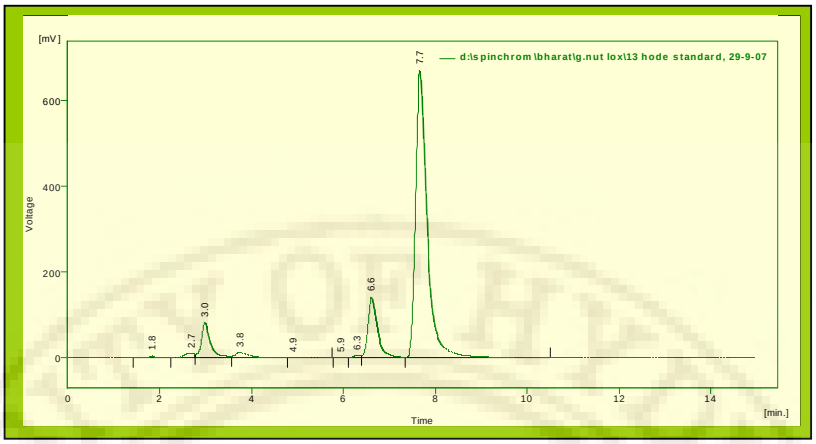


Fig. 35: Reverse Phase-High Performance Liquid Chromatographic separation of standard 13-HODE. The spectrum showing the retention time (RT: 7.7 min) of the 13-HODE obtained from commercially available soybean LOX as enzyme source with LA as substrate when separated on Reverse Phase HPLC.

A marked increase in the 13-HODE formation was observed during 48-54 h of inoculation which was 3.1 times higher than controls. 13-HODE formation decreased with the increase in time after inoculation in R-2001-1 variety (Fig. 36, 37 & 38) but, the 13-HODE formation remained high at all intervals till 96 h compared to controls. Analysis of 13-HODE formation in samples of inoculated seedlings of TMV-2 variety showed a gradual increase at 30 h and reached a maximum at 42-48 h and then decreased. 13-HODE formation was 1.52-fold higher during 42-48 h of inoculation in TMV-2 variety (Fig. 39 & 40).

Thus a rapid increase in 13-HODE formation was observed in samples of R-2001-1 variety which was markedly higher than the susceptible variety for all the intervals examined (Table 1).

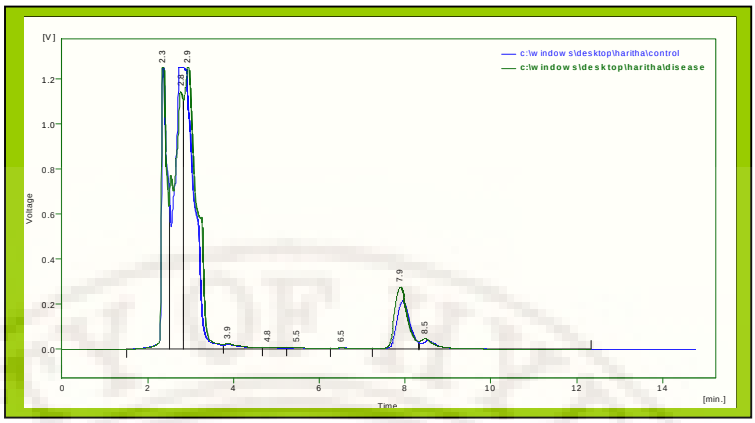


Fig. 36: RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 18 h interval in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety in comparison to control. The protein supernatant from shoots of control and inoculated seedlings were incubated for 15 min with linoleic acid (250 µM final concentration) in potassium phosphate buffer. After addition of sodium borohydride, the products were extracted, and were analyzed by RP-HPLC using the solvent system consisting of methanol: water: acetic acid (850:150:1).

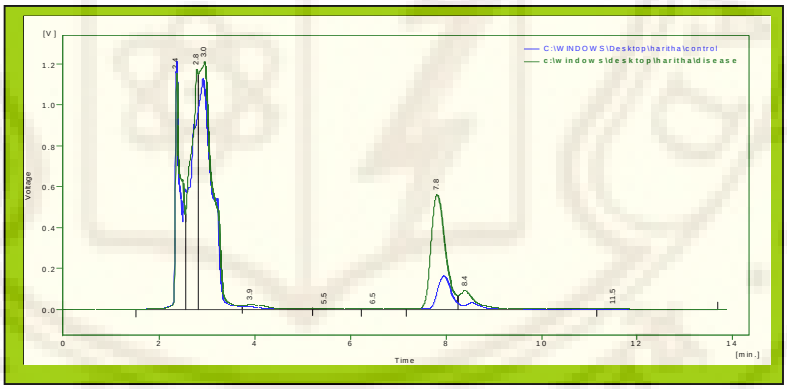


Fig. 37: RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 48 h interval in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety in comparison to control. The protein supernatant from shoots of control and inoculated seedlings were incubated for 15 min with linoleic acid (250 µM final concentration) in potassium phosphate buffer. After addition of sodium borohydride, the products were extracted, and were analyzed by RP-HPLC using the solvent system consisting of methanol: water: acetic acid (850:150:1).

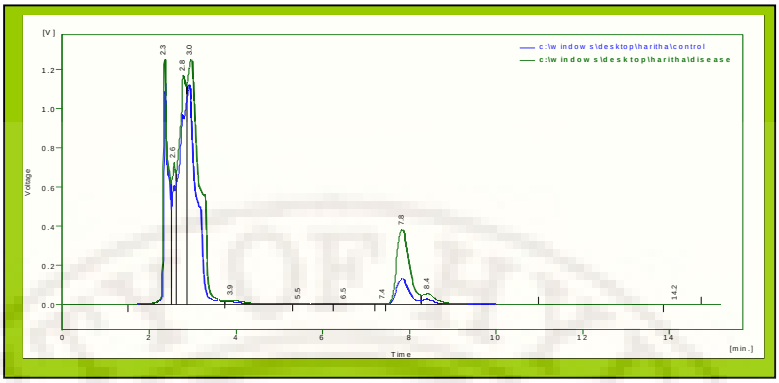


Fig. 38: RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 72 h interval in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety in comparison to control. The protein supernatant from shoots of control and inoculated seedlings were incubated for 15 min with linoleic acid (250 µM final concentration) in potassium phosphate buffer. After addition of sodium borohydride, the products were extracted, and were analyzed by RP-HPLC using the solvent system consisting of methanol: water: acetic acid (850:150:1).

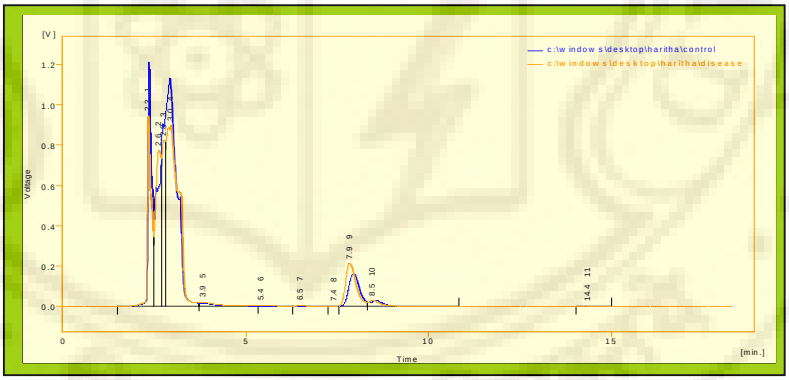


Fig. 39: RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 30 h interval in shoots of *S. rolfsii* inoculated seedlings of TMV-2 variety in comparison to control. The protein supernatant from shoots of control and inoculated seedlings were incubated for 15 min with linoleic acid (250 µM final concentration) in potassium phosphate buffer. After addition of sodium borohydride, the products were extracted, and were analyzed by RP-HPLC using the solvent system consisting of methanol: water: acetic acid (850:150:1).

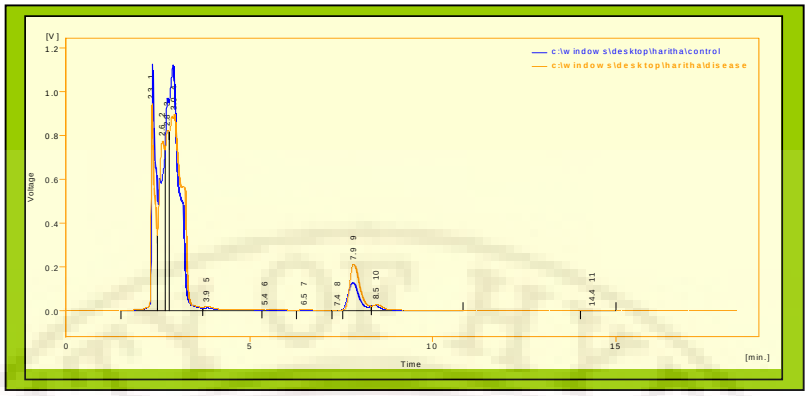


Fig. 40: RP-HPLC analysis of the 13-HODE formed *in vitro* from LA at 48 h interval in shoots of *S. rolfsii* inoculated seedlings of TMV-2 variety in comparison to control. The protein supernatant from shoots of control and inoculated seedlings were incubated for 15 min with linoleic acid (250 µM final concentration) in potassium phosphate buffer. After addition of sodium borohydride, the products were extracted, and were analyzed by RP-HPLC using the solvent system consisting of methanol: water: acetic acid (850:150:1).

Table 1: HPLC analysis of 13-HODE formed *in vitro* using LA in shoots of R-2001-1 and TMV-2 varieties after inoculation with *S. rolfsii* along with controls. The protein supernatant of control and inoculated samples were incubated with linoleic acid in potassium phosphate buffer and analyzed for 13-HODE formation on RP-HPLC using the solvent system consisting of methanol:water:acetic acid (850:150:1).

Time interval in hours	R-2001-1 variety		TMV-2 variety	
	% increase in the area	Increase (fold)	% increase in the area	Increase (fold)
6	0	0	0	0
12	20.0	1.20	0	0
18	22.0	1.22	0	0
24	26.0	1.26	0	0
30	89.0	1.89	40.0	1.40
36	145.0	2.45	40.0	1.40
42	196.0	2.96	52.0	1.52
48	210.0	3.10	52.0	1.52
54	210.0	3.10	30.0	1.30
60	201.0	3.01	10.0	1.1
66	198.0	2.98	0	0
72	196.0	2.96	0	0
78	127.0	2.27	0	0
84	115.0	2.15	0	0
90	98.0	1.98	0	0
96	98.0	1.98	0	0

5.4 RT-PCR analysis of transcripts of *PnLOX-1*, *PnLOX-2* and *PnLOX-3* genes in *S. rolfsii* inoculated seedlings:

The expression pattern of *PnLOX-1*, *PnLOX-2* and *PnLOX-3* genes was examined in ten-day-old seedlings of groundnut after inoculation with *S. rolfsii* using RT-PCR. The transcripts of *PnLOX-1*, *PnLOX-2* and *PnLOX-3* were increased upon inoculation compared to controls although differences were observed with respect to time of induction and intensity of expression. The transcript level of *PnLOX*-1 remained very low in the control seedlings whereas marked increase was observed in inoculated seedlings (Fig. 41).

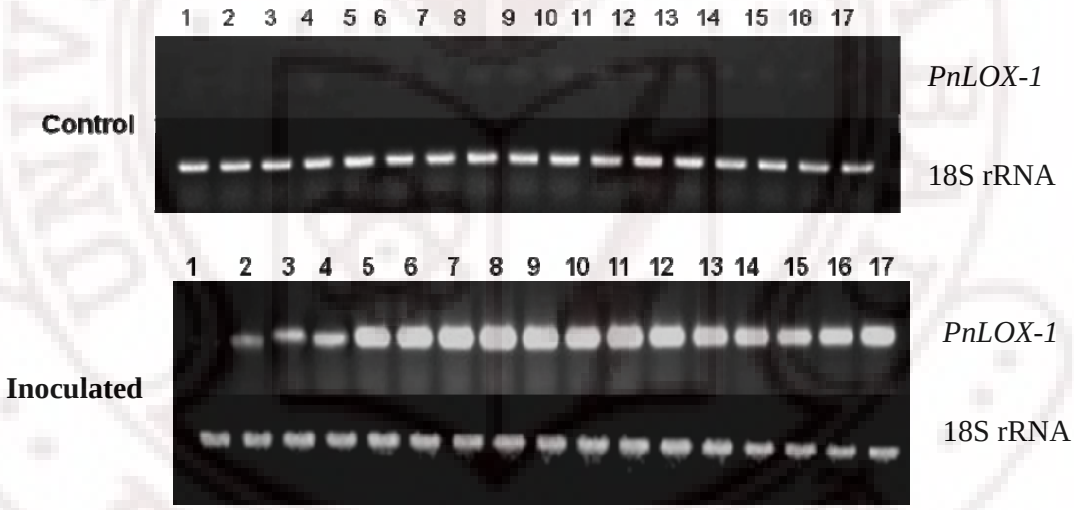


Fig. 41: RT-PCR analysis of the transcript levels of Pn*LOX-1* gene in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety along with controls. The reverse transcribed products of total RNA isolated from control and inoculated seedlings were amplified using *PnLOX-1* or 18S rRNA specific primers and fractionated into 1.2% agarose gel. Lanes 1-17: 0 h, 6 h, 12 h, 18 h, 24 h, 30 h, 36 h, 42 h, 48 h, 54 h, 60 h, 66 h, 72 h, 78 h, 84 h, 90 h and 96 h, respectively.

A gradual increase in the transcript level of *PnLOX-1* was observed from 6 h of inoculation with the highest levels observed during 24-72 h of inoculation. The transcript levels of *PnLOX-2* and *PnLOX-3* were higher than that of *PnLOX-1* in control seedlings. The same expression pattern was observed for *PnLOX-2* and *PnLOX-3* genes with an increase in the transcript level observed during 30-72 h in inoculated seedlings and thereafter declined (Fig. 42 & 43). Thus, *PnLOX-1* gene was found to be highly expressed as compared to *PnLOX-2* and *PnLOX-3* genes in inoculated seedlings compared to controls.

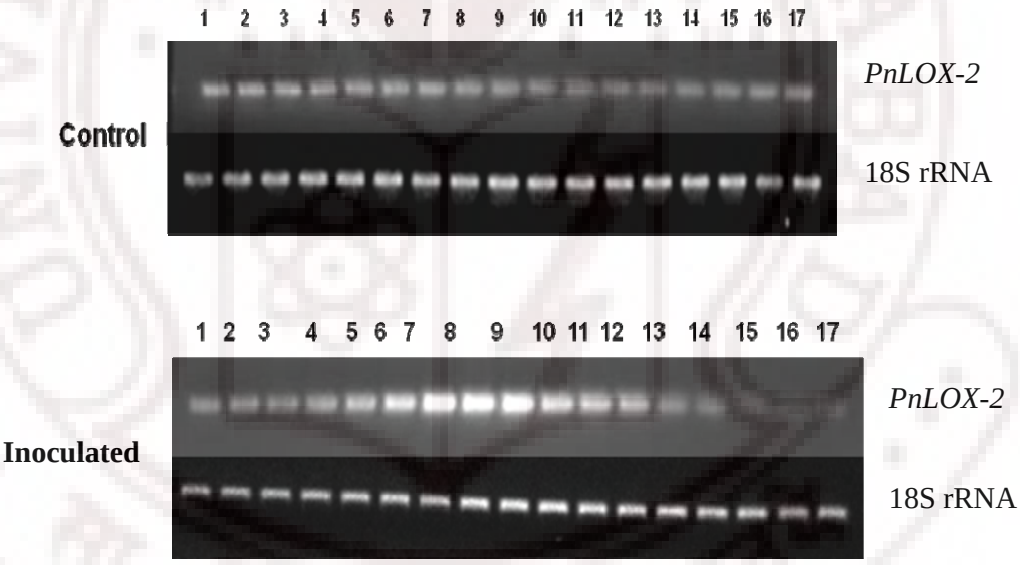


Fig. 42: RT-PCR analysis of the transcript levels of *PnLOX-2* gene in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety along with controls. The reverse transcribed products of total RNA isolated from control and inoculated seedlings were amplified using *PnLOX-2* or 18S rRNA specific primers and fractionated into 1.2% agarose gel. Lanes 1-17: 0 h, 6 h, 12 h, 18 h, 24 h, 30 h, 36 h, 42 h, 48 h, 54 h, 60 h, 66 h, 72 h, 78 h, 84 h, 90 h and 96 h, respectively.

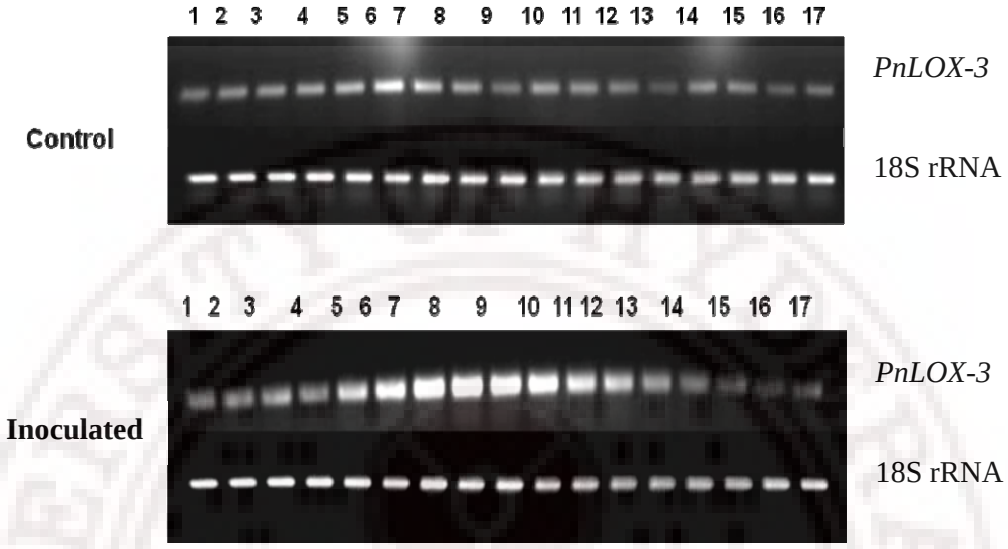


Fig. 43: RT-PCR analysis of the transcript levels of *PnLOX-3* gene in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety along with controls. The reverse transcribed products of total RNA isolated from control and inoculated seedlings were amplified using *PnLOX-3* or 18S rRNA specific primers and fractionated into 1.2% agarose gel. Lanes 1-17: 0 h, 6 h, 12 h, 18 h, 24 h, 30 h, 36 h, 42 h, 48 h, 54 h, 60 h, 66 h, 72 h, 78 h, 84 h, 90 h and 96 h, respectively.

5.5.1 Purification of lipoxygenase induced in shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety:

The crude protein from shoots of R-2001-1 variety after 48 h of inoculation with *S. rolfsii* was purified by ammonium sulphate precipitation followed by anion exchange chromatography and HPLC. Ammonium sulphate saturation removed most of the contaminating proteins and the LOX enzyme was precipitated at 20-60% saturation. A purification fold of 1.9 with a recovery of 76.9% was achieved during ammonium sulphate precipitation. Ammonium sulphate precipitated protein, after dialysis, was loaded on to DEAE-cellulose (ion-exchange) column and washed with

phosphate buffer until all the unbound proteins are washed away by observing the OD at 280 nm. LOX protein was eluted with a linear gradient of 0 to 0.4 M KCl in 40 mM potassium phosphate buffer (pH 6.5). Two peaks, designated aI and aII were obtained based on the absorbance at 280 nm (Fig. 44). However, the peak aI did not show any LOX activity whereas the peak aII exhibited activity with a purification fold of 41.2 and recovery of 64.6% (Table 2). The aII peak corresponding to active LOX fraction was further purified through anion exchange HPLC.

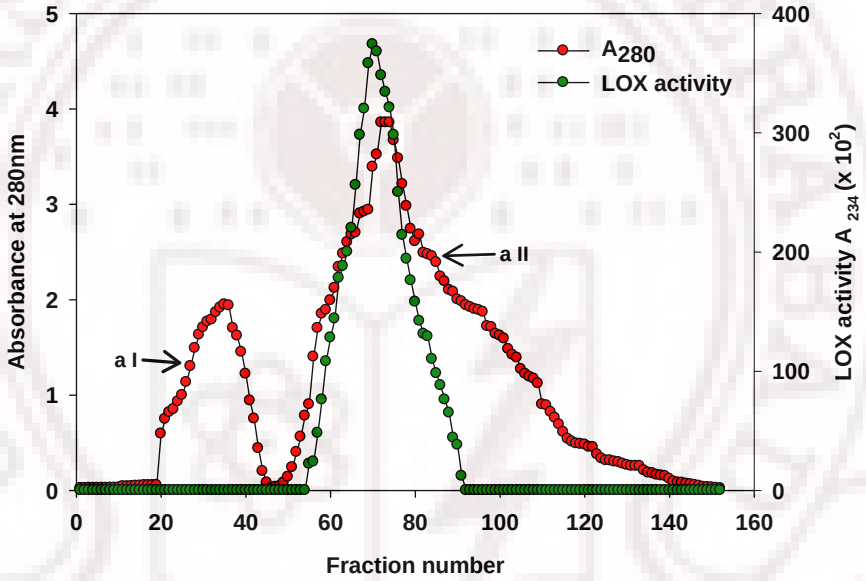


Fig. 44: Purification of LOX from *S. rolfsii* inoculated seedlings of R-2001-1 variety by DEAE–cellulose column chromatography. LOX was purified from shoots of 10-day old seedlings after 48 h after inoculation with *S. rolfsii* by ammonium sulphate precipitation and applied onto DEAE-cellulose column (15 mm × 15 cm), previously equilibrated with 40mM potassium phosphate buffer, pH 6.5. Flow rate was adjusted to 0.5 ml/min with 1 ml per fraction. Protein was separated using a linear gradient of KCl in the buffer and different fractions were collected and analyzed for LOX activity at pH 6.5. (●) Absorbance at 234 nm (●) Absorbance at 280 nm.

The active LOX fractions purified through DEAE-cellulose column were pooled and further purified on HPLC using anion exchange chromatography (Shimpack PA-DEAE -8 mm X 100 mm) column. The column was equilibrated with

40 mM potassium phosphate buffer (pH 6.5). The active LOX fraction was injected into the column and the LOX enzyme was eluted with a linear gradient starting from 0.0 M KCl to 0.4 M KCl in equilibration buffer over a period of 40 min. The peaks when observed at 280 nm on a Shimadzu SPD-6 AV UV/VIS detector were collected and the LOX activity was determined based on the absorbance at 234 nm in UV/VIS spectrophotometer with LA as the substrate. Three peaks designated A, B and C with LOX activity were identified with a retention time of 8.1, 8.9 and 10.0, respectively (Fig. 45). The three peaks with LOX activity were pooled separately and lyophilized. The purification procedure employed in the study resulted in 116.9-fold purification and 13.4% yield for peak A. The fraction corresponding to peak B was purified with a purification fold of 88.7 and yield of 4.6% whereas the purification fold of peak C was 92-fold with a yield of 1.8% (Table 2).

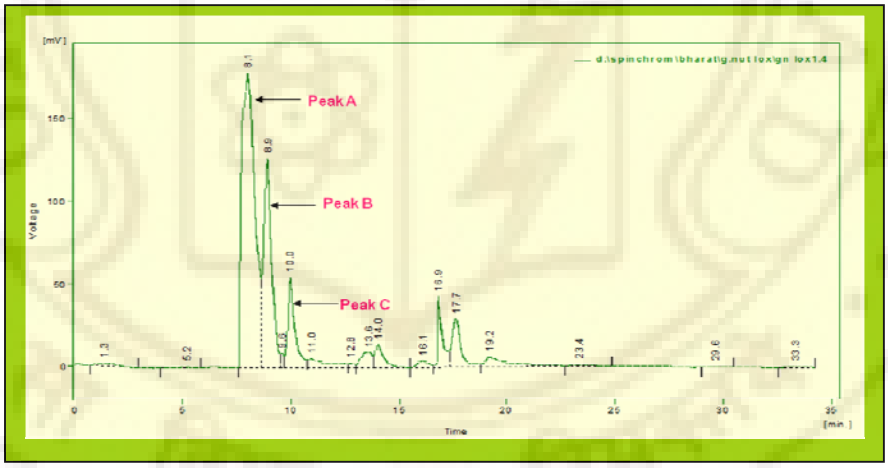


Fig. 45: HPLC chromatogram showing the peaks after passing the purified lipoxygenase obtained from DEAE–cellulose column through the anion exchange HPLC. Shimpack PA-DEAE (8 mm X 100 mm) column was used for the purification and proteins were eluted with 0 to 0.4 M KCl gradient in 40 mM potassium phosphate buffer at the flow rate of 2 ml/min using Shimadzu LC-6AD pumps. The eluant was continuously monitored at 280 nm on a Shimadzu SPD-6 AV UV/VIS detectors.

Table 2: Purification of groundnut lipoxygenase from shoots of *S. rolfsii* inoculated seedlings of R-2001-1 variety. LOX was purified from *S. rolfsii* inoculated seedlings by ammonium sulphate precipitation, anion exchange chromatography and anion exchange HPLC. The peak aII represents the fraction obtained through anion exchange chromatography showing LOX activity. The peaks A, B and C represent the fractions obtained through anion exchange HPLC showing LOX activity.

Fraction		Total protein (mg)	Total activity (No. of units X 10^3)	Yield (%)	Specific activity (No. of units X 10^2)	Purification fold
Crude extract		523.5	325.6	100	6.2	1.0
Ammonium sulphate precipitation		210.4	250.2	76.9	11.9	1.9
Anion exchange chromatography Peak aII		8.2	210.5	64.6	256.2	41.2
Anion exchange HPLC	A	0.6	43.5	13.4	725.4	116.9
	B	0.4	22.3	4.6	550.0	88.7
	C	0.4	24.2	1.8	571.0	92.0

5.5.2 Activity staining of LOX isozymes on native PAGE and SDS-PAGE analysis of lipoxygenase purified from *S. rolfsii* inoculated seedlings of groundnut:

LOX isozymes in the fractions of the three peaks A, B and C were analyzed on the native PAGE. Peak A fraction showed three bands on native gel coinciding with L-3, L-4 and L-5 isozymes. A single band each was observed for peak B and C coinciding with L-2 and L-1 isozymes, respectively (Fig. 46a). The molecular weight of the LOX isozymes in fractions of each of the peaks was determined using SDS-PAGE. SDS-PAGE analysis of purified LOX corresponding to peaks A, B and C fractions showed that they are monomeric proteins with molecular weights of 98 kDa, 95 kDa and 84 kDa, respectively (Fig. 46b).

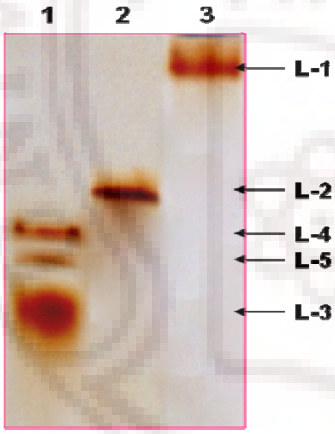


Fig.46a

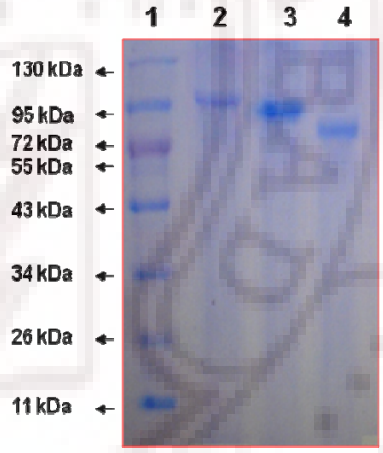


Fig. 46b

Fig. 46 a: Native-PAGE of LOX purified from *S. rolfsii* inoculated seedlings of R-2001-1 variety. Twenty microgram of purified LOX protein of Peak A, B and C fractions obtained through anion exchange HPLC was loaded separately in each well. Gel was stained for LOX activity with o-dianisidine using linoleic acid as the substrate: Lane-1: Peak A; Lane-2: Peak B and Lane 3: Peak C.

Fig. 46 b: SDS-PAGE of LOX purified from *S. rolfsii* inoculated seedlings of R-2001-1 variety. Twenty microgram of purified LOX protein of Peak A, B and C fractions obtained through anion exchange HPLC were loaded separately in each well of 12% acrylamide gel. The proteins were stained with Comassie blue. Lane-1: Protein Molecular weight marker, Lane-2: Peak A, Lane-3: Peak B and Lane-4: Peak C.

5.5.3 Effect of temperature and pH on purified LOX isozymes:

The optimal pH for the purified LOX isozymes was determined. The peak A corresponding with L-3, L-4 and L-5 isozymes exhibited high activity (65-70 units/mg protein) over a pH range of 6.5-8.5. The optimal pH was 6.5 for L-2 and L-1 isozymes with an activity of 52.0 units/mg protein and 42.0 units/mg protein, respectively (Fig. 47).

The effect of temperature on the purified LOX isozymes was tested. The highest activity for all three peaks, A, B and C fractions corresponding to five isozymes was observed at 20°C. The activity progressively decreased with the increase of temperature to 40°C for all the isozymes. The activity of peak A fraction with LOX isozymes L-3, L-4 and L-5, and peak C fraction with isozyme L-1 was completely lost at 50°C. On the other hand, the peak B fraction with LOX isozyme L-2 exhibited activity upto 70°C with complete loss of activity at 80°C. Thus the LOX isozyme L-2 was found to be relatively thermostable than other LOX isozymes (Fig. 48).

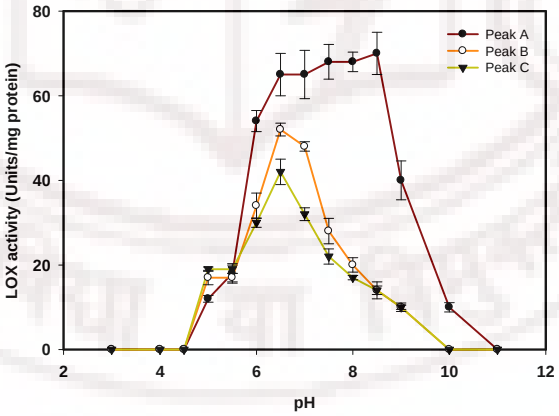


Fig. 47: Effect of pH on activity of purified LOX from *S. rolfsii* inoculated seedlings.
The reaction mixture consisted of the purified LOX in peak A, B and C fractions obtained through anion exchange HPLC, linoleic acid and buffers of different pH values.

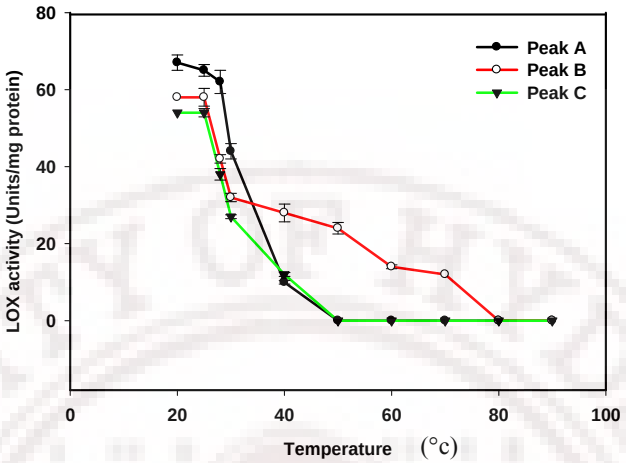


Fig. 48: Effect of temperature on activity of purified LOX from *S. rolfsii* inoculated seedlings. The reaction mixture consisting of the purified LOX obtained through anion exchange HPLC, linoleic acid and potassium phosphate buffer was incubated at different temperatures for 10 min for determination of the LOX activity.

5.5.4 Effects of inhibitors on purified lipoxygenase isozymes:

The effects of five inhibitors on the activity of the purified LOX isozymes was tested. The inhibitors tested had a variable effect on the activity of purified LOX isozymes. NDGA and esculetin at a very low concentration of 1 mM caused 100% inhibition of activity of all the LOX isozymes. EDTA at 1 and 10 mM did not cause any inhibition on LOX activity of peak A fraction with LOX isozymes L-3, L-4 and L-5. Higher concentration (10 mM) of EDTA inhibited the LOX activity by 12-15% for L-2 and L-1 isozymes whereas the extent of inhibition was low in the presence of 1 mM EDTA. NaCN at 10 mM inhibited the LOX activity by 72% for peak A fraction whereas the extent of inhibition remained low (13-17%) for peak B and peak C fractions. $CaCl_2$ at 10 mM was less effective in inhibiting the activity for peak B and C fractions whereas it inhibited the activity by 14% for peak A fraction (Table 3).

Table 3: Effect of inhibitors on activity of purified LOX from shoots of *S. roflsii* inoculated seedlings of R-2001-1 variety. Purified LOX isozymes in peaks A, B and C fractions obtained through anion exchange HPLC were incubated with inhibitors at different concentrations for 10 min and assayed for LOX activity.

Inhibitors	Concentration (mM)	% Inhibition		
		Peak A	Peak B	Peak C
EDTA	1.0	0	1	1
	10.0	0	12	15
NDGA	1.0	100.0	100.0	100.0
Esculetin	1.0	100.0	100.0	100.0
NaCN	1.0	8	1	2
	10.0	72	13	17
$CaCl_2$	1.0	2	0	0
	10.0	14	1	2

5.5.5 Product profile of purified groundnut lipoxygenase:

The activity of the purified lipoxygenase obtained through anion exchange chromatography was confirmed by incubating with LA and ALA as substrates and separating the hydroperoxy metabolites by straight phase HPLC. The wave length spectrum of the hydroperoxy products was monitored on spectrophotometer. The products were separated on straight phase HPLC and monitored at 234 nm. The products with maximum absorption spectra were collected and characterized by recording UV/VIS wave length scanning and co-chromatography with standard. HPLC analysis of the products formed from purified groundnut lipoxygenase with LA and ALA showed major peaks corresponding to standard 13-HPODE, 13-HODE, 13-HOTrE and 13-HPOTrE generated from soybean LOX, respectively, and eluted as

single peaks with corresponding hydroperoxide on co-chromatography under identical conditions. The absorption spectra of the peak fractions had the characteristic spectrum of conjugated diene, with absorption maximum at 234 nm indicating that the products are fatty acid hydroperoxides.

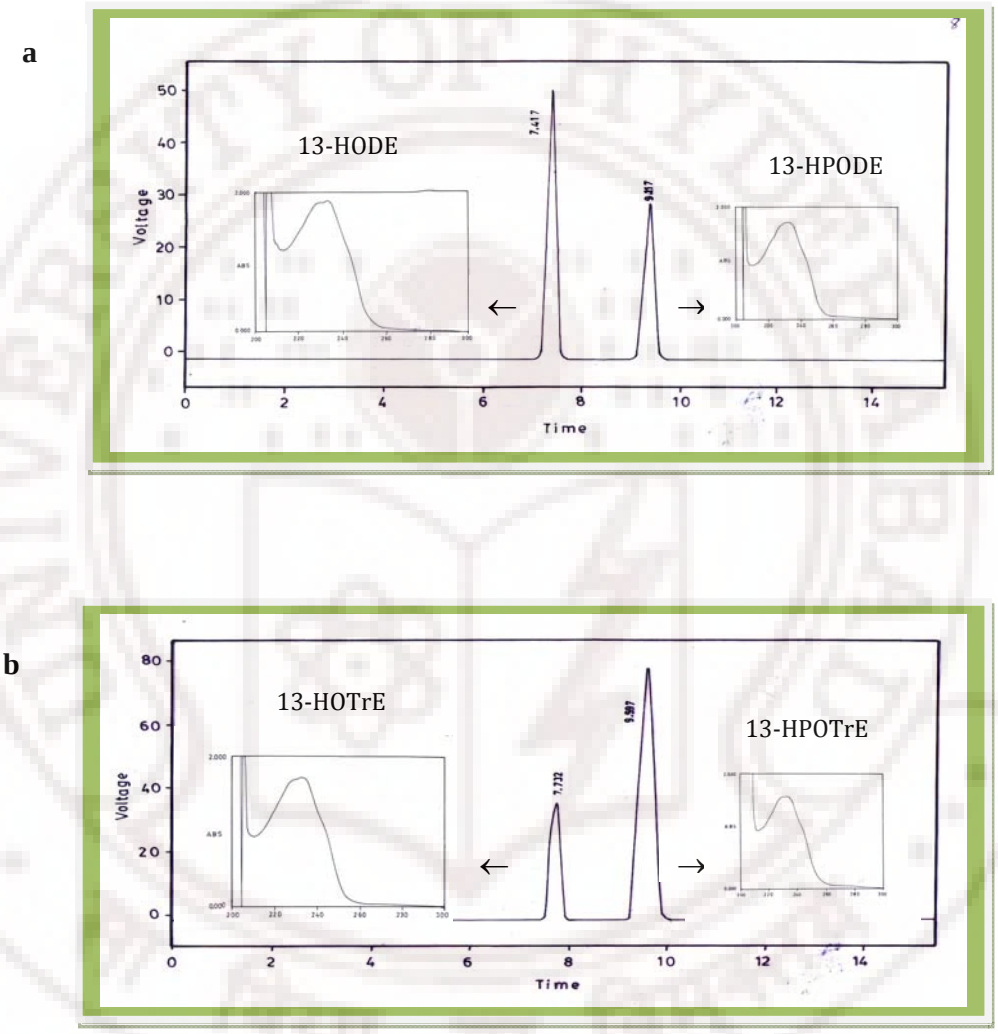


Fig. 49 a & b: SP-HPLC chromatograms of 13-hydroperoxides and hydroxides produced from purified groundnut LOX obtained through anion exchange chromatography. The products were synthesized using linoleic and α-linolenic acid as substrates and purified groundnut LOX as enzyme source. The products were separated on straight phase analytical HPLC with mobile phase of hexane: isopropanol: acetic acid (1000:15:1) with a flow rate of 1 ml/min. a. 13-HPODE + 13-HODE b. 13-HPOTrE + 13-HOTrE.

As shown in Figs 49a and 49b, 13-HPODE and 13-HODE were eluted as major peaks with retention time of 9.3 and 7.4 min. Similarly, 13-HPOTrE and 13-HOTrE were eluted with retention time of 9.5 and 7.7 min, respectively.

DISCUSSION

5.6.1 *S. rolfsii* inoculation induces LOX activity in groundnut seedlings:

High LOX activity may be constitutive in plants resistant to pathogens but with an additional increase upon infection (Uma Maheswari *et al*., 2000). Uma Maheswari *et al*. (2000) reported that LOX activity was higher in the resistant variety than the susceptible varieties of pigeonpea. Similarly, Nagarathna *et al*. (1992) observed maximum LOX activity in seeds of highly resistant genotypes of pearl millet and minimum activity in the highly susceptible genotypes of pearl millet. In the present study also significantly higher basal LOX activity was observed in seedlings of resistant varieties compared to the susceptible varieties of groundnut. Sandhu *et al*. (2007) reported that the lipoxygenase activity was always higher in Brazilian rice cultivars resistant to leaf blast disease than in susceptible cultivars. It was suggested that LOX might be a screening factor for blast disease resistance in rice. Our results also favour the possibility of using LOX as a biochemical marker for screening stem rot disease resistant varieties of groundnut.

In the present study, inoculation of the seedlings with the stem rot pathogen resulted in an increase in LOX activity in shoots compared to controls in both susceptible and resistant varieties of groundnut. A greater increase in LOX activity was observed in shoots of resistant variety as compared to roots in response to inoculation with *S. rolfsii*. These results are in contrast to the observations of NaCl, PEG, ABA and MJ treatments which resulted in a higher increase in LOX activity in

roots than shoots. From these results, it is suggested that a defense mechanism is activated in the tissues or organs that are directly exposed to stresses leading to increased LOX activity which further might trigger the defense responses throughout the plant. This enhanced induction of LOX activity in the resistant varieties of groundnut might confer resistance against pathogen.

Our results are in agreement with that of Babitha *et al*. (2006) where a higher LOX activity was observed in shoot region of the downy mildew resistant pearl millet genotypes which further increased in response to inoculation with *S. graminicola* zoospores. Uma Maheshwari *et al*. (2000) reported that LOX activity enhanced in the resistant cultivars of pigeonpea in response to infection with *Fusarium udum*. They showed that LOX activity decreased immediately after inoculation in susceptible cultivar and remained at lower levels compared to control seedlings. Burow *et al*. (2000) demonstrated that LOX activity increased during early hours and the activity decreased with time in peanut seeds infected with *Aspergillus*. It was suggested that decrease in LOX activity in infected seeds could be due to the metabolization of the *PnLOX1* gene product (and other LOX enzymes) and/or proteolytic activity by *Aspergillus*.

LOX enzyme activity has been shown to be induced rapidly during a disease resistant response and more slowly in a susceptible interaction (Melan *et al*., 1993). Lipoxygenase activity is induced in several incompatible interactions in plants and it seems always to be after a lag phase of several hours (Peever and Higgins, 1989; Slusarenko, 1996; Hornung *et al*., 1999). During this lag phase other defense reactions are in operation, such as signaling pathways, which are important for the selective induction of LOX activity in resistant plants. The present experiments

revealed an increase in LOX activity in *S. rolfsii* inoculated resistant seedlings at 24 h followed by a marked increase during 42-54 h. LOX activity at 48 h of inoculation was found to be 3.3-fold higher than controls in R-2001-1 variety. In the susceptible variety, a higher LOX activity was observed in *S. rolfsii* inoculated seedlings during 42-48 h followed by a decrease during the later intervals. The LOX activity induced in susceptible variety in response to *S. rolfsii* inoculation was lower than that of resistant variety for all the intervals examined. These results are in agreement with the findings of Nagarathna *et al*. (1992) where a significant increase in LOX activity was observed on the second and third day after inoculation of resistant pearl millet seedlings with *S. graminicola* zoospores, while in susceptible seedlings, the activity decreased over respective healthy controls. The decrease in LOX activity could be possibly due to the inactivation of the enzymes by the pathogen and/or reduction in host protein synthesis due to colonization. Differences in LOX activity observed in resistant and susceptible varieties of groundnut after inoculation with *S. rolfsii* strengthen the hypothesis of a relationship between LOX and resistance. Thus a higher LOX activity in resistant variety could be the result of increased expression of LOX gene due to activated signaling pathways leading to the stimulation of hypersensitivity responses. During pathogenesis in wheat it was found that a 92 kDa LOX protein was predominantly formed, which is believed to be responsible for the resistance response of wheat against pathogens (Berner, 2006).

5.6.2 LOX isozymes are induced in response to *S. rolfsii* inoculation in disease resistant variety:

The functional diversity of LOX isozymes enable the plant to respond appropriately to environmental challenges (Feussner and Wasternack, 2002). The

type of LOX isozyme induced will determine the kind of downstream products being formed as a result of LOX activity. Increases in host lipoxygenase activity and individual isozymes after infection with bacterial and fungal pathogens have been observed for several plant species (Yamamoto and Tani, 1986; Keppler and Novacky, 1987; Croft *et al*., 1990; Kato *et al*., 1992b; Koch *et al*., 1992). Our results showed that the intervals at which high LOX activity was observed in *S. rolfsii* inoculated seedlings was associated with the induction of two isozymes, L-4 and L-5 in R-2001-1 variety, whereas these isozymes were not induced in seedlings of TMV-2 variety after inoculation with the *S. rolfsii*. Thus a higher LOX activity along with the induction of new isozymes in resistant variety could be a part of defense strategy for resistance to stem rot pathogen. Similarly, Uma Maheshwari *et al.* (2000) observed an increase in LOX activity in the resistant cultivars of pigeonpea which correlated with the expression of new LOX isozymes (L-5 and L-6), in addition to induction of other LOX isozymes. Yamamoto and Tani (1986) reported the induction of two new lipoxygenase isozymes in resistant oat lines upon infection with crown rust, *Puccinia coronata* that accounted for high LOX activity while no change in lipoxygenase activity was observed following infection of susceptible lines. Babitha *et al*. (2006) described the induction of LOX isozyme, LOX-6 in downy mildew resistant pearl millet seedlings upon inoculation with *Sclerospora graminicola* which was linked to resistance expression. In the tomato plant at least 5 different LOX isoenzymes were found to be responsible for the synthesis of various forms of LOX products (Chen *et al*., 2004). The increase in LOX activity along with the induction of LOX isozymes in inoculated seedlings of resistant variety strengthens the hypothesis of the role of lipoxygenase in defense responses.

The success of defence strategies, lies in the ability of the plant to produce oxylipins *via* LOX (Rayapuran and Baldwin, 2006). The initial products of LOX, the hydroperoxides, are rapidly metabolized by the down-stream enzymes of the LOX pathway. Several of these metabolites act as potent antimicrobial compounds. Being non-toxic for the plants, they might enhance the defence capacity of the plant in addition to the initial hypersensitive cell death (Gobel *et al*., 2003). The present experiments revealed an increase in the formation of 13-HODE in seedlings of resistant variety as compared to controls. The resistant variety displayed a marked increase (1.89-3.1) in 13-HODE formation during 30-54 h of inoculation as compared to controls. In the susceptible variety, 13-HODE formation increased by 1.4-1.52 times during 30-48 h of inoculation as compared to respective controls. Thus the marked increase in the production of hydroperoxides in the inoculated seedlings of resistant variety correlated with the increased lipoxygenase activity which might contribute to defense reactions by inhibition of pathogen growth and development (Namai *et al*., 1990; Ohta *et al*., 1990), induction of phytoalexin accumulation (Li *et al*., 1991) and/or a signal transduction (Choi and Bostock, 1994). Thus a marked increase in fatty acid hyderoperoxides can be considered as a defense mechanism against inoculation by *S. rolfsii*. Rodriguez-Rosales *et al*. (1999) have stated that hydroperoxides formed by the action of lipoxygenase on linoleic acid and α–linolenic acid lead to changes in membrane fluidity and permeability, ultimately leading to dysfunction in the lipid layer. Knight *et al*. (2001) provided cytological and biochemical evidence that tomato cells treated with LOX-products of polyunsaturated fatty acids displayed the classic features of an apoptotic programmed cell death.

In the present study, LOX induction showed a specific pattern depending on the genotype and the pathogen. Inoculation of the seedlings with *Rhizoctonia solani*, which causes damping off disease showed an increase in LOX activity at 12 h with a 2.2-fold increase observed at 18 h of inoculation. Activity staining analysis revealed an increase in the intensity of LOX isozyme L-2 at all the intervals as compared to controls. From these observations, it is suggested that LOX plays an important role in defense response to infection by the *R. solani* pathogen. An increase in the LOX activity can occur because of the activation of pre-existing enzymes and also expression of new LOX isozymes, induced by various stress factors and elicitors produced by pathogenic microorganisms (Babitha *et al*., 2004). It would be interesting to study the isolation, purification and characterization of LOX isozymes, induced in response to pathogen infection, and analyze their involvement in disease resistance.

5.7 LOX isozymes purified from *S. rolfsii* inoculated seedlings exhibited differences in biochemical characteristics:

Numerous reports confirmed the differential induction of LOX protein during incompatible biotic and abiotic stresses. However, only in few cases the lipoxygenases induced in response to pathogens have been isolated and characterized (Wallace and Wheeler, 1979; Noehringer and Blee., 2000). In the present study, LOX induced in groundnut seedlings upon inoculation with *S. rolfsii* have been purified and characterized. The purified LOX fractions when separated in anion exchange HPLC resolved into 3 peaks, A, B and C with an yield of 13.4%, 4.6% and 1.8%, respectively. It was observed that peak A fraction resolved into three isozymes, L-3, L-4 and L-5 on native gels but with a molecular weight of 98 kDa on SDS-PAGE.

The peak B fraction could be separated into L-2 isozyme with a molecular weight of 95 kDa while peak C fraction consisted of L-1 isozyme with a molecular weight of 84 kDa. The purified LOX isozymes were found to be monomeric proteins. Earlier workers have reported that purified lipoxygenase had a molecular weight of 95 kDa for *Solanum melongena* (Nakayama *et al*., 1995), 99 kDa for hazelnut (Santino *et al*., 2003) and 97 kDa for broad bean (Clemente *et al*., 2000). However, the molecular weights of 74 kDa for avocado lipoxygenase (Marcus *et al*. 1988) and 78 kDa for English pea seeds (Eriksson and Svensson, 1970) have been reported. Thus the purified groundnut LOX showed a molecular weight within the range observed by these investigators. Babitha *et al*. (2004) purified the LOX isozymes, LOX-1, -3 and -6 from *Sclerospora graminicola* inoculated resistant seedlings of pearl millet. The molecular weight estimated from SDS-PAGE electrophoresis was 83, 77 and 73 kDa with a subunit molecular weight of 43 and 40, 40 and 37, and 38 and 35 kDa for LOX-1, -3 and -6, respectively. The dimeric nature of LOXs, however, is not confirmed by other investigators.

Many researchers have classified plant lipoxygenases as one of the two types. Type 1 lipoxygenases are characterized by high pH optima at 9 or above while Type II lipoxygenases have an optimum pH of 7.0 (Hildebrand *et al*., 1991). The present experiments showed that the optimum pH of the purified LOX isozymes from *S. rolfsii* inoculated seedlings varied. The peak A fraction obtained through anion-exchange HPLC exhibited maximum LOX activity (70 units/mg protein) at pH 8.5. The optimum pH for peaks B (L-2) and C (L-1) was found to be 6.5 with an activity of 52.0 units/mg protein and 42.0 units/mg protein, respectively. The acidic LOX isozymes L-1 and L-2 purified from *S. rolfsii* inoculated seedlings can be grouped

with Type 2 lipoxygenase with respect to their pH optima of 6.5 (Hildebrand *et al*., 1991; Nicolas *et al*., 1982). Reynolds and Klein (1982) showed that dry English peas seeds (var. Little Marvel) contain a Type-1 lipoxygenase with an optimum pH of 9-10. In the present study, the broad pH range of 6.5-8.5 observed for peak A fraction could be due to the fact that it consisted of 3 isozymes and the pH optima of individual isozymes could be different. Previous experiments have demonstrated that peanut LOX isozymes, *PnLOX2* and *PnLOX3* were active at neutral pH values above 7.7 (Tsitsigiannis *et al*., 2005). In contrast, *PnLOX1* enzyme was active at pH 6.0, 6.5 and 7.0 (Burow *et al*., 2000).

The present experiments showed that the purified LOX isozymes are highly sensitive to temperature. The highest LOX activity was observed at 20^{o}C for all isozymes. The LOX isozyme, L-2 was found to be relatively thermostable and exhibited LOX activity upto 70^{o}C. On the other hand, the L-1, L-3, L-4 and L-5 LOX isozymes exhibited activity upto 40^{o}C and no activity was observed at 50^{o}C. Similarly, Babitha *et al*. (2004) reported that the LOX isozymes, L-1, -3 and -6 purified from downy mildew resistant pearl millet seedlings differed in their temperature stability. LOX-6 was fully active up to 28^{o}C, inactivated up to 50% till 45^{o}C. But, it was completely inactivated above 70^{o}C. Similar results have been reported in *Oscillatoria* sp. (Andrianarison *et al*., 1989) and in tomato (Jadhav *et al*., 1972).

Burow *et al*. (2000) identified a mixed function LOX, *PnLOX1* (21% 9S- and 59% 13S-HPODE) that was activated by wounding and *Aspergillus* infections. Tsitsigiannis *et al*. (2005) purified and characterized two peanut seed lipoxygenase alleles (*PnLOX2* and *PnLOX3*) that were highly expressed in mature seed. HPLC

analysis showed that both are predominantly 13-HPODE producers. In the present study, the LOX purified through anion exchange chromatography from *S. rolfsii* inoculated seedlings catalyzed the formation of 13-HPODE and 13-HODE when incubated with LA whereas 13-HPOTrE and 13-HOTrE were the products formed when incubated with ALA. Although the present experiments indicated that the purified LOX isozymes are 13-LOX, it is possible that they could be mixed function LOXs producing 9-HPODE and 13-HPODE. Further research is required for separating the individual LOX isozymes of peak A fraction to characterize the nature the products produced by each of the isozymes. Metabolites generated from the 13-LOX pathway, particularly methyl jasmonate, were found to function as signals to induce expression of genes for defense response in plants (Farmer and Ryan, 1992) and a series of studies have shown that these metabolites are directly or indirectly involved in the response of plants to pathogen attack (Farmer and Ryan, 1992; Melan *et al*., 1993; Peng *et al*., 1994; Rance *et al*., 1998).

Earlier reports have indicated esculetin to be the best inhibitor for soybean and oscillatoria lipoxygenase (Beneytout *et al*., 1989). NDGA was also a potent inhibitor of potato tubers and soybean LOX (Matsui *et al*., 1991). In the present study, the effect of known lipoxygenase inhibitors on activity of purified LOX was investigated. The inhibition potential of the LOX inhibitors varied for the different LOX isozymes. Among the various inhibitors tested, NDGA and esculetin were found to be potent inhibitors for all the LOX isozymes even at 1 mM. Differential results were obtained for NaCN which inhibited the LOX by 72% for peak A fraction at 10 mM with LOX isozymes, L-1, L2 and L-3 isozymes whereas it was relatively less efficient in inhibiting L-4 and L-5 LOX isozymes. The other inhibitors either had no effect or

inhibited the LOX activity partially depending on the LOX isozymes and concentration of compound used. Similarly, Babitha *et al*. (2004) reported that esculetin and NDGA were the potent inhibitors of the lipoxygenase purified from downy mildew resistant seedlings of pearl millet whereas EDTA, NaCN and calcium chloride inhibited the LOX activity partially. The inhibition of LOX activity of tobacco lipoxygenase and soybean lipoxygenase in the presence of esculetin has been reported earlier (Wheeler and Berry, 1986). Lee and Lillard (1997) demonstrated the inhibitory effect of esculetin on lipoxygenase activity in soybean. It was proposed that esculetin acts as a reducing agent to reduce the catalytic active ferric lipoxygenase to the inactive ferrous form. There have been reports that lipoxygenase can be inhibited by chelator through chelation of nonheme iron which existed in lipoxygenase (Nelson, 1988). Sekiya *et al*. (1982) proposed that lipoxygenase could be inhibited by the radical scavenging property of esculetin.

5.8 *S. rolfsii* inoculation induces the expression of *PnLOX-1*, *PnLOX-2* and *PnLOX-3* genes:

Burow *et al*. (2000) have proposed different roles for three peanut genes, *PnLOX-1*, *PnLOX-2* and *PnLOX-3* genes in plant development. *PnLOX1* gene was highly induced in mature seed of peanut in response to *Aspergillus* infection. In contrast, *PnLOX-2* and *PnLOX-3* genes were repressed in mature seed of peanut over the course of *A. flavus* infection (Tsitsigiannis *et al*., 2005). Given the proposed role of three peanut genes in seed-*Aspergillus* interaction, it was of interest to determine the expression pattern in groundnut seedlings after inoculation with *S. rolfsii*. In the present study, RT-PCR analysis with gene-specific primers revealed an increase in the transcript levels of *PnLOX-1*, *PnLOX-2* and *PnLOX-3* genes at specific points in

shoots of groundnut seedlings following exposure to *S. rolfsii*. The transcript levels of *PnLOX-1* gene were very low in controls whereas a maximum increase in the transcript levels were observed during 24 h to 72 h of inoculation with *S. rolfsii*. The pattern of expression of *PnLOX-2* and *PnLOX-3* was more or less similar with an increase in the transcript levels during 30-72 h of inoculation followed by a decrease in the transcript levels during subsequent intervals. It was observed that *PnLOX-1* was highly expressed in *S. rolfsii* inoculated seedlings compared to *PnLOX-2* and *PnLOX-3* genes. Thus the level of LOX expression could be possibly related to the efficiency of resistance to stem rot disease with higher LOX expression leading to a more effective defence mechanism than a lower expression of LOX. Thus these genes could offer potential targets for manipulating resistance for stem rot disease in groundnut. Similarly, Kolomiets *et al*. (2000a) provided evidence of increases of potato LOX mRNA after pathogen inoculation.

In summary, significantly higher level of LOX activity was observed in resistant varieties of groundnut, which showed a marked increase in response to inoculation in response to inoculation with *S. rolfsii* as compared to susceptible varieties. The purified LOX isozymes from *S. rolfsii* inoculated seedlings showed differences in thermal stability and pH optima. The enhanced LOX activity observed in resistant variety was associated with the increased production of 13-HODE suggesting the involvement of LOX in resistance to stem rot disease in groundnut. Furthermore, the enhanced levels of *Pn LOX-1*, *Pn LOX-2* and *Pn LOX-3* observed in shoots of seedlings inoculated with *S. rolfsii* indicates that they could be used as potential targets for manipulating disease resistance in groundnut. It would be

interesting to evaluate the anti-fungal activities of the LOX products so as to analyze their involvement in disease resistance.

Chapter 6

ANTIFUNGAL ACTIVITIES OF LOX PRODUCTS ON SELECTED FUNGAL PATHOGENS OF GROUNDNUT

ANTIFUNGAL ACTIVITIES OF LOX PRODUCTS ON SELECTED FUNGAL PATHOGENS OF GROUNDNUT

LOX pathway can be activated under different abiotic stress conditions, in response to treatment with chemicals or biotic elicitors but also following interactions with pathogens (Blee, 2002). This metabolic route leads to the synthesis of various compounds displaying antimicrobial (Shah, 2005) or signaling activities or both (Nickstadt *et al*., 2004; Kishimoto *et al*., 2006). The oxylipin pathway could also contribute to plant defense by regulating localized cell death, a powerful plant defense mechanism against pathogens known as the hypersensitive response (La Camera *et al*., 2004). Based on the high chemical reactivity of some oxylipins, Apel and Hirt (2004) suggested that they might act as a plant signal at low concentrations and, at higher doses, are toxic to plants and other cells. It has been well illustrated by the study of Prost and collaborators showing the inhibitory activity of a number of oxylipins toward various bacterial, oomycete and fungal plant pathogens (Prost *et al*., 2005). Shimura *et al*. (1983) reported the inhibitory effects of 13-HPOTrE and 9-HOTrE on the germination of conidia of rice blast, *Pyricularia oryzae*. Ricker and Bostock (1994) observed that several hydroperoxyeicosatetraenoic acids (HPETEs), produced by reaction of lipoxygenase with arachidonic acid proved to be fungitoxic to *Phytophthora infestans* and *Phytophthora capsici* although none of these possessed elicitor activity.

The fungal pathogen, *Botrytis cinerea* of tomato was found to be highly sensitive to some of the LOX-derived products especially polyunsaturated fatty acid hydroperoxides and their reduced forms (Akram *et al*., 2008). The study of Graner *et al*. (2003) demonstrated the inhibitory effects of certain oxylipins on growth and

germination of *Brassica* pathogens. Uma Maheswari *et al*. (2000) reported that 13-HPOTrE, the major product formed in infected pigeon pea seedlings, was more effective in inhibiting the growth of pigeon pea with fungus *Fusarium udum* than 13-HPODE, which is the major product formed in uninfected control seedlings. Calvo *et al*. (1999) found that 13S-HPODE but not linoleic acid or 9S-HPODE had a statistically significant effect on sclerotial production in *Aspergillus flavus* when it was grown in the light.

Sailaja *et al*. (1998) demonstrated the inhibitory effects of groundnut LOX products (13-HPODE and 13-HPOTrE) on the growth of *Aspergillus niger*. The effects of LOX products on other fungal pathogens *viz*., *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum* which cause stem rot, *Rhizoctonia* damping off and *Fusarium* wilt diseases, respectively, have not been studied so far. These diseases are responsible for significant yield losses in groundnut. In the previous chapter, the seedlings of resistant variety when inoculated with *S. rolfsii* displayed higher LOX activity along with increased LOX product formation. The studies were, therefore, extended to examine the inhibitory potential of LOX products against the groundnut fungal pathogens *viz*., *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum*.

RESULTS

6.1 Generation of 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE from soybean lipoxygenase:

13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE were generated using soybean lipoxygenase and linoleic acid and α-linolenic acid as substrates. The products generated were checked for absorption maximum at 234 nm and then

separated on straight phase HPLC by monitoring at 234 nm. Each peak was collected separately and checked for the maximum absorption spectra at 234 nm by using UV/VIS spectrophotometer. The compounds were identified based on the order of elution and retention time of soybean LOX products as per the earlier reports (Kiran Kumar *et al*. 1993). Hydroperoxy compound was eluted as a major peak with retention time of 18.6 min.

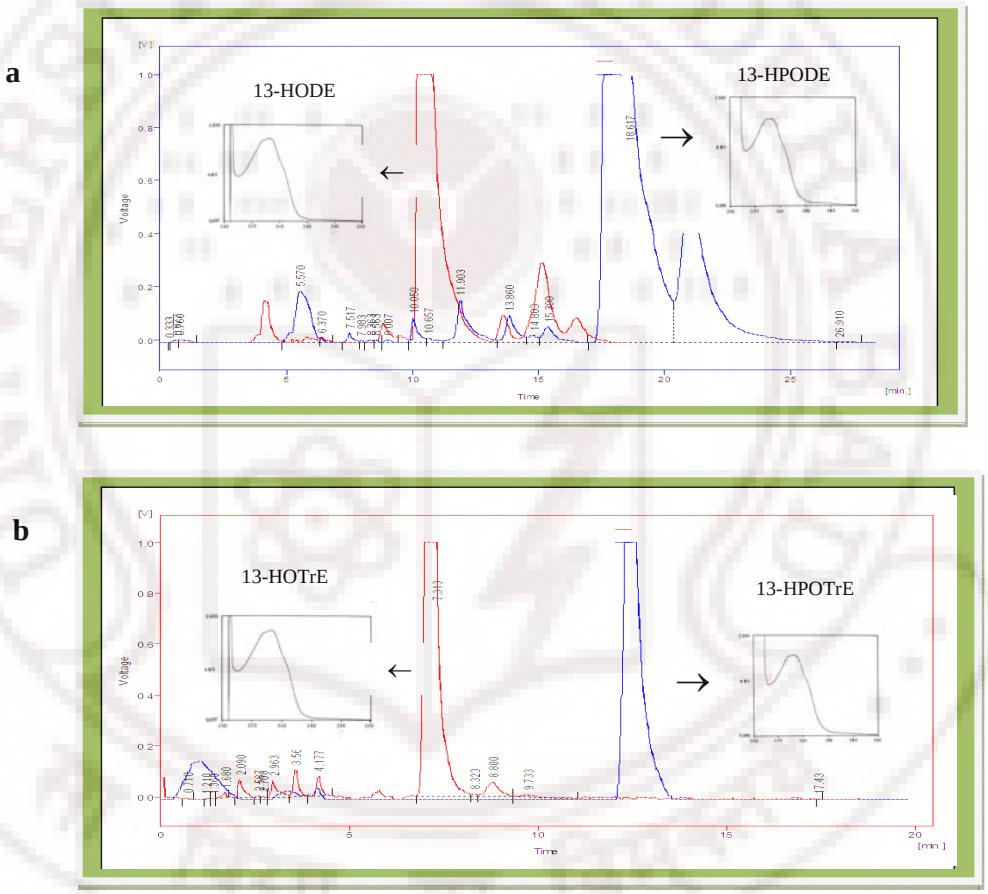


Fig. 50a & b: SP-HPLC chromatograms showing separation of LOX products produced from commercially available soybean LOX. The products were synthesized using linoleic acid and α-linolenic acid as substrates and commercially available soybean LOX as enzyme source. The products were separated on straight phase preparatory HPLC with mobile phase of hexane: isopropanol: acetic acid (1000:15:1) with a flow rate of 10 ml/min. a. 13-HPODE and 13-HODE, b. 13-HPOTrE and 13-HOTrE.

Since the soybean LOX with LA as the substrate is known to generate majorily 13-HPODE, the major peak was considered as 13-HPODE and used for antifungal assays (Fig. 50 a). Similarly, 13-HPOTrE was generated by employing soybean lipoxygenase with ALA as substrate. The major peak was eluted with retention time of 12.8 min (Fig. 50 b).

The hydroperoxy products obtained in the reaction of soybean lipoxygenase with LA and ALA were reduced by using sodium borohydride and separated on straight phase HPLC. The peaks were collected separately and checked for the maximum absorption spectra at 234 nm by using UV/VIS spectrophotometer. The compounds were identified based on the retention time reported earlier for 13-HODE and 13-HOTrE produced from soybean LOX (Kiran Kumar *et al*., 1993). The major peak eluted with LA as the substrate with retention time of 10.8 min was considered as 13-HODE (Fig. 50 a). The major peak eluted with ALA as the substrate with retention time of 7.3 min was considered as 13-HOTrE. The 13-HODE and 13-HOTrE generated were used in antifungal assays (Fig. 50 b).

6.2 Analysis of antifungal activity of hydroperoxides and hydroxides using filter disc assay:

The effects of hydroperoxides and hydroxides on growth of *Sclerotium rolfsii* and *Rhizoctonia solani* were tested using filter disc assay method. The hydroperoxides and hydroxides tested produced a zone of inhibition towards *S. rolfsii*, *R. solani* and *F. oxysporum* although the minimum inhibitory concentration varied with the fungi (Table 4). The minimum inhibitory concentration for the growth of *S. rolfsii* was found to be 1.5 µg for 13-HPOTrE whereas 1.8 µg was required for 13-

HOTrE and 13-HPODE followed by 2.1 µg for 13-HODE (Fig. 51). The order of potency for the inhibition of *R. solani* was found to be 13-HPOTrE (1.4 µg) followed by 13-HOTrE (1.6 µg) whereas 13-HPODE and 13-HODE had similar effects at 2 µg (Fig. 52). 13-HPOTrE inhibited the growth of *F. oxysporum* at a low concentration of 1.0 µg followed by 1.2 µg for 13-HOTrE, 1.5 µg for 13-HPODE and 1.8 µg for 13-HODE (Fig. 53).

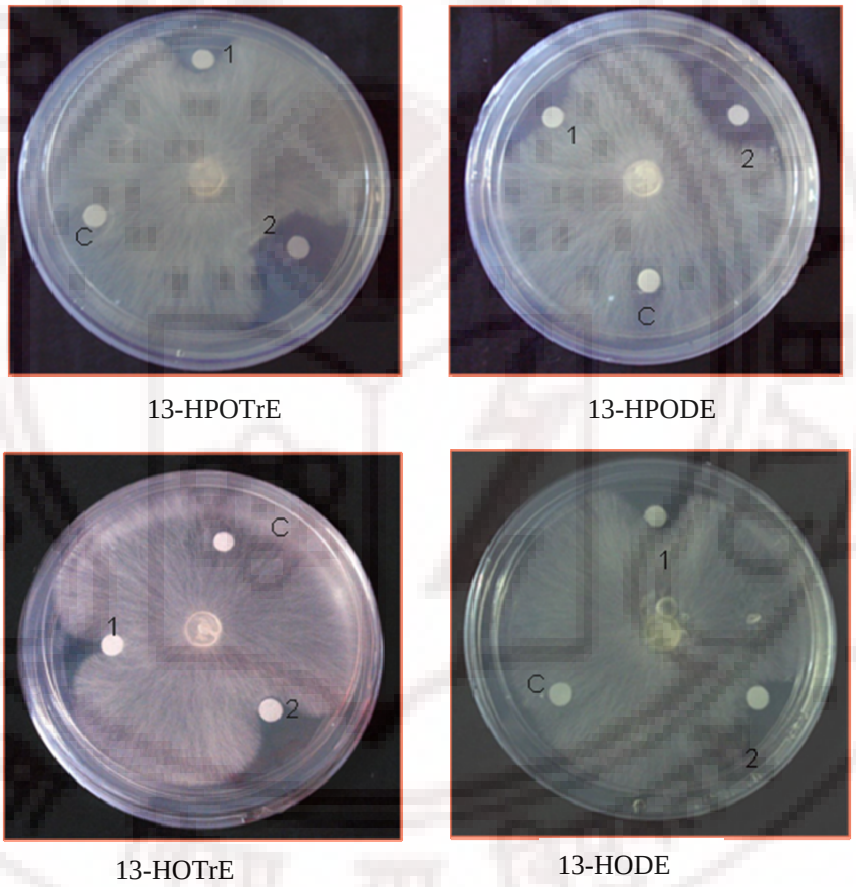


Fig. 51: Photomicrographs showing the zone of inhibition on the growth of *S. rolfsii* in the presence of 13-hydroperoxides and 13-hydroxides by using filter disc assay method. Filter discs containing different concentrations of LOX products or solvent control (C) placed on potato dextrose agar medium seeded with *S. rolfsii*. The plates were incubated at 30°C for 24 h and examined for the zone of inhibition. C=Control, 1=2.5 µg/disc and 2=4 µg/disc.

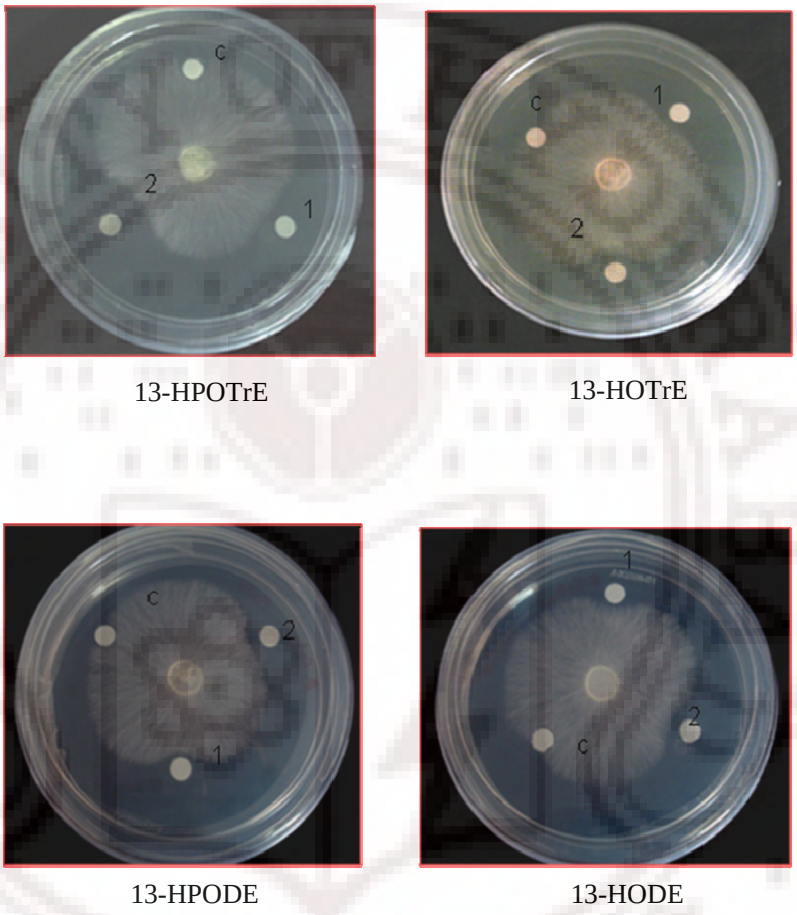


Fig. 52: Photomicrographs showing the zone of inhibition on the growth of *R. solani* in the presence of 13-hydroperoxides and 13-hydroxides by using filter disc assay method. Filter discs containing different concentrations of LOX products or solvent control (C) placed on potato dextrose agar medium seeded with *R. solani*. The plates were incubated at 30°C for 24 h and examined for the zone of inhibition. C=Control, 1=2.5 µg/disc and 2=3 µg/disc.

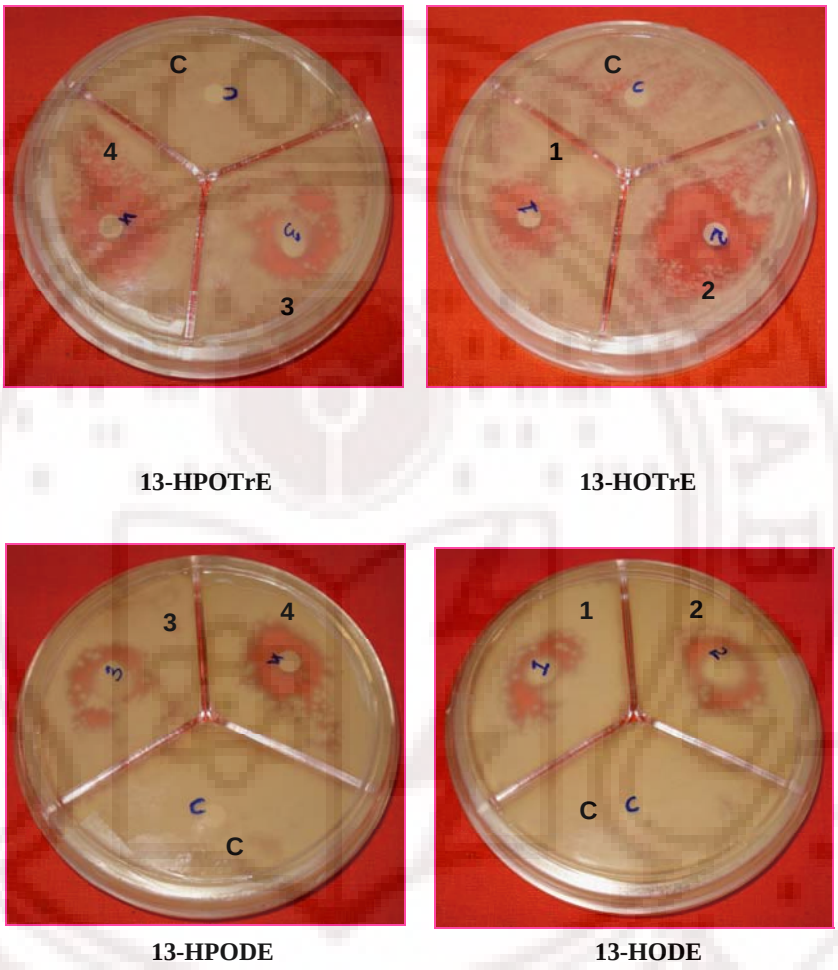


Fig. 53: Photomicrographs showing the zone of inhibition on the growth of *F. oxysporum* in the presence of 13-hydroperoxides and 13-hydroxides by using filter disc assay method. Filter discs containing different concentrations of LOX products or solvent control (C) placed on potato dextrose agar medium seeded with *F. oxysporum*. The plates were incubated at 30°C for 24 h and examined for the zone of inhibition. C=Control, 1, 3=2.5 µg/disc and 2, 4=4 µg/disc.

Table 4: Determination of minimum inhibitory concentration of hydroperoxides and hydroxides against fungal pathogens of groundnut using filter disc assay. LOX products were added in different concentrations on sterile filter paper discs placed in petriplates containing potato dextrose medium with mycelial discs. The minimum inhibitory concentration for the LOX products that produced a detectable zone of inhibition was determined after incubation of cultures at 30°C for 24 h.

Treatment	Minimum inhibitory concentration (µg/disc)		
	S. roflsii	*R. solani*	*F. oxysporµm*
13-HPOTrE	1.5	1.4	1.0
13-HOTrE	1.8	1.6	1.2
13-HPODE	1.8	2.0	1.5
13-HODE	2.1	2.0	1.8

6.3 Analysis of effects of hydroperoxides and hydroxides on the mycelial weight of fungi:

The effects of hydroperoxides and hydroxides on the dry weight of mycelium of *S. rolfsii* were tested. Variable results were obtained depending on the concentration of hydroperoxide or hydroxide and the time of incubation. Low concentration of 13-HPOTrE, 13-HOTrE and 13-HPODE decreased the mycelial weight at 24 h whereas 13-HODE did not have any effect on mycelial weight at the same interval ((Fig. 54: Fig. 55a & b). Both 13-HOTrE and 13-HODE were effective in decreasing the mycelial weight at 48 h but did not have any significant effects with the increase in incubation time (72-96 h). It was interesting to note that all the LOX products tested had no significant effects on mycelial weight of *S. rolfsii* when the

time interval was extended to 120 h. High concentration (100 µM) of LOX products decreased the mycelial weight compared to controls for all the intervals examined. In general, 13-HPOTrE was more effective followed by 13-HOTrE, 13-HPODE and 13-HODE.

When the effects of LOX products on mycelial weight of *R. solani* were examined, it was observed that low concentration of 10 µM did not have any significant effects whereas high concentration (100 µM) decreased the mycelial weight at 24 h (Fig. 56; Fig. 57 a & b). 13-HPOTrE at 10 µM proved to be effective in decreasing the mycelial weight at 48 h whereas no significant effects were observed in the presence of 10 µM of 13-HOTrE, 13-HPODE and 13-HODE at the same interval. The mycelial weight decreased in the presence of 10 µM of LOX products with the increase in time interval (72-120 h). High concentration of LOX products (100 µM) caused a significant reduction in mycelial weight for all the intervals examined.

The antifungal activity of LOX products against *Fusarium oxysporum* was tested. LOX products did not have any effects on mycelial weight at 24 h even when used at 100 µM. 13-HPOTrE at 10 µM proved to be effective in decreasing the mycelial weight at 48 h whereas 13-HOTrE, 13-HPODE and 13-HODE did not have any significant effects at the same interval. However, the mycelial weight decreased in the presence of 10 µM of 13-HOTrE, 13-HPODE and 13-HODE with an increase in time interval (72-96 h). The inhibitory effect of 13-HODE on mycelial weight were lost when incubated upto 120 h (Fig. 58; Fig. 59 a & b). When the concentration of LOX products was increased (100 µM), the inhibitory effects on mycelial weight were observed from 48 h onwards and continued upto 120 h.

Control 13-HPODE 13-HODE

Control 13-HPOTrE 13-HOTrE

Fig. 54: Photomicrographs showing the effects of 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE on mycelial weight in *S. rolfsii*. LOX products (100 μM) were added to the potato dextrose broth containing the fungal inoculam. Conical flasks containing fungal inoculam without addition of products were used as control.

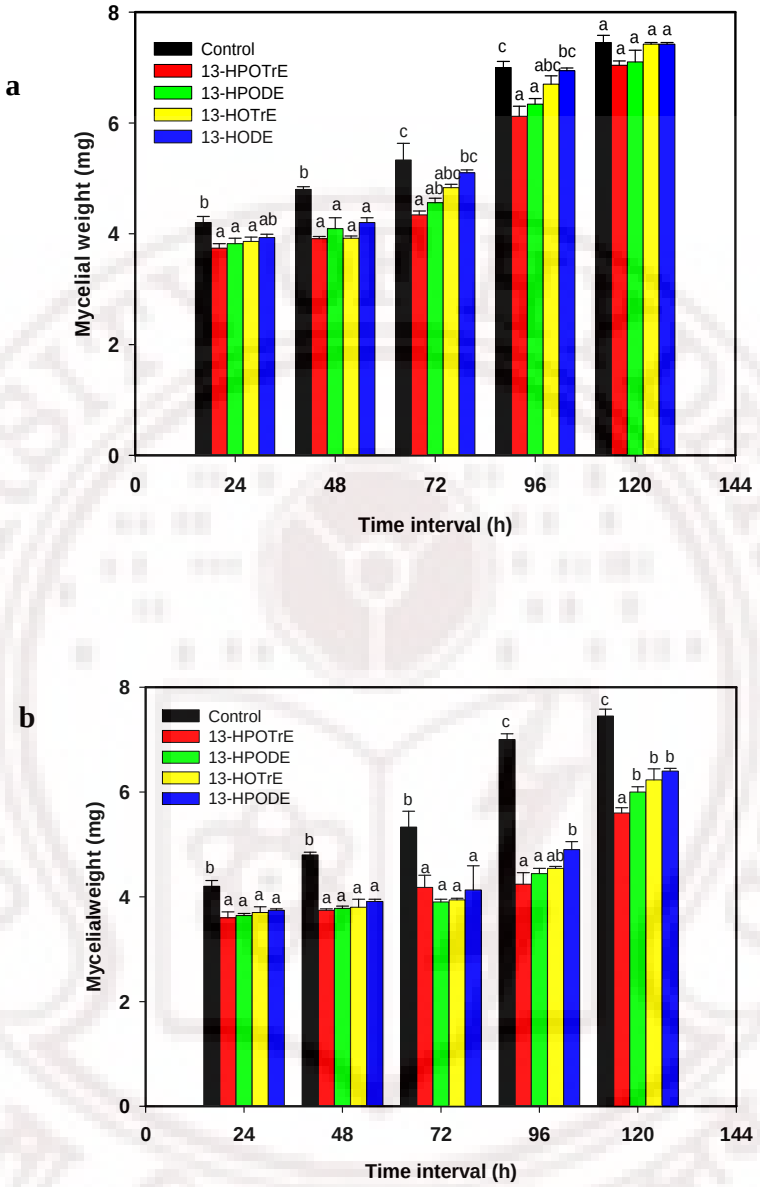
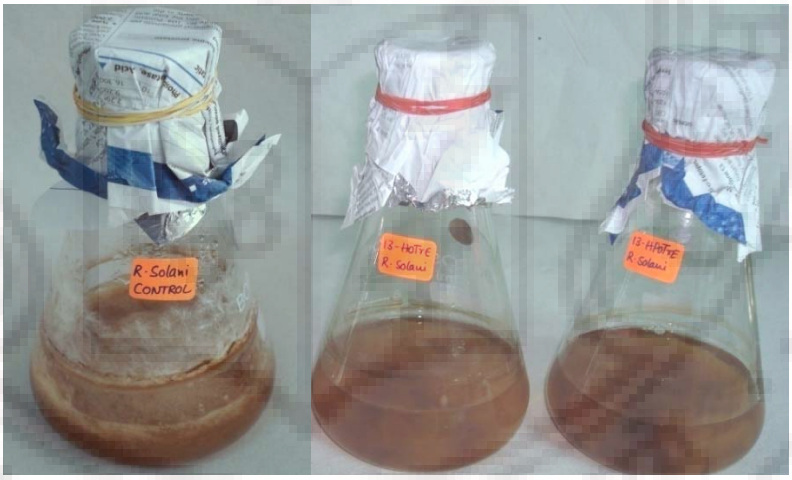


Fig. 55a & b: Effects of hydroperoxides and hydroxides on mycelial weight in *S. rolfsii*. The weight of the mycelium in each flask was recorded at 24 h interval up to 120 h. a. 10 μM; b. 100 μM. Values for each treatment are means ± SE of three replicates. Within each group, bars with the same letter are not significantly different ($P \leq 0.05$) according to Newman-Keul's multiples comparisons test.

Control 13-HPODE 13-HODE



Control 13-HPOTrE 13-HOTrE

Fig. 56: Photomicrographs showing the effects of 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE on mycelia weight in *R. solani*. LOX products (100 μM) were added to the potato dextrose broth containing the fungal inoculam. Conical flasks containing fungal inoculam without addition of products were used as control.

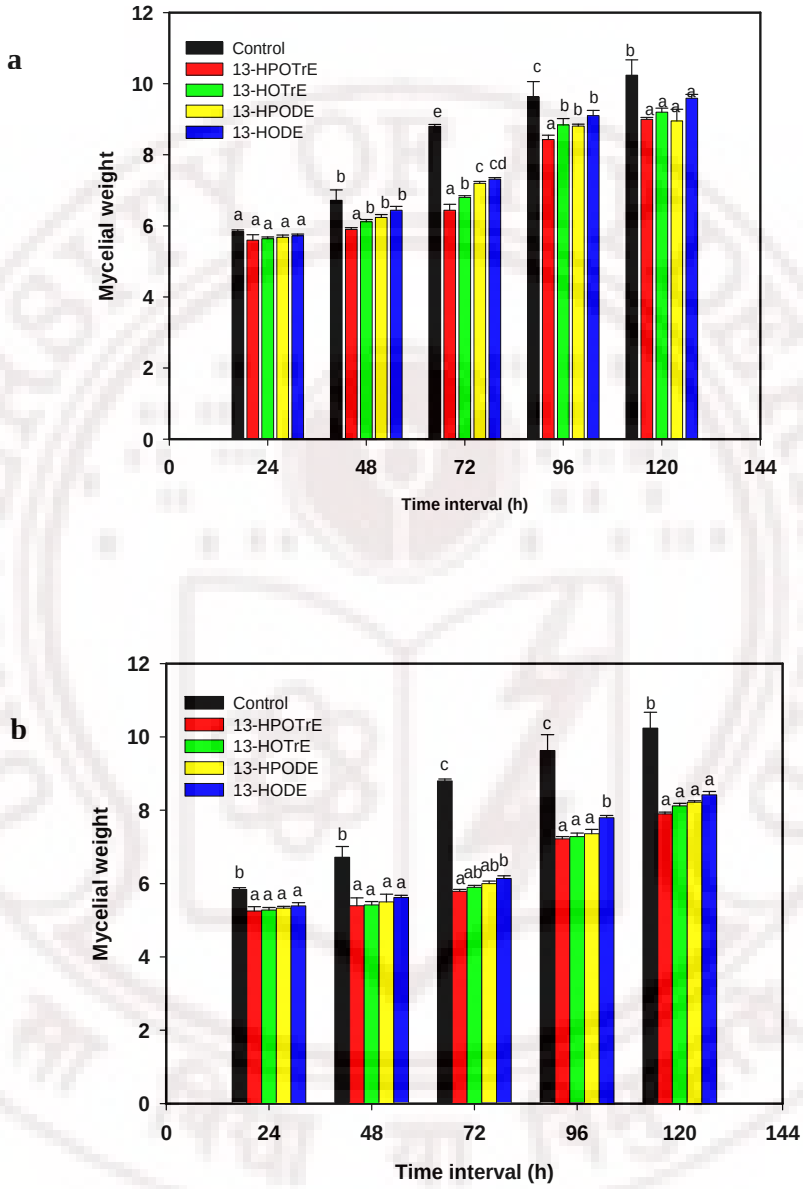
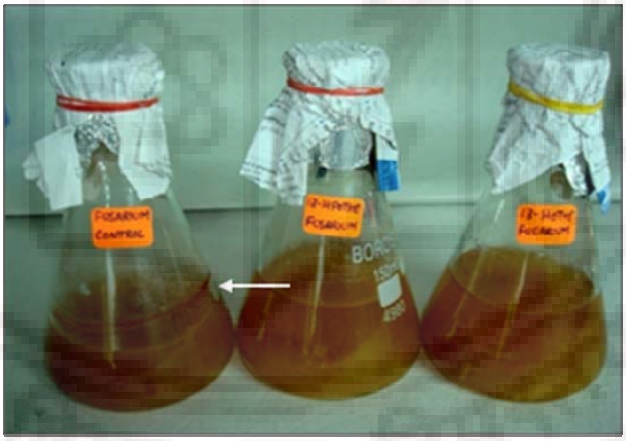


Fig. 57a & b: Effects of hydroperoxides and hydroxides on mycelia weight in *R. solani*. The weight of the mycelium in each flask was recorded at 24 h interval up to 120 h. a. 10 µM b. 100 µM. Values for each treatment are means ± SE of three replicates. Within each group, bars with the same letter are not significantly different ($P \leq 0.05$). according to Newman-Keul's multiples comparisons test.

Control 13-HPODE 13-HODE



Control 13-HPOTrE 13-HOTrE

Fig. 58: Photomicrographs showing the effects of 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE on mycelia weight in *F. oxysporum*. LOX products (100 µM) were added to the potato dextrose broth containing the fungal inoculam. Conical flasks containing fungal inoculam without addition of products were used as control.

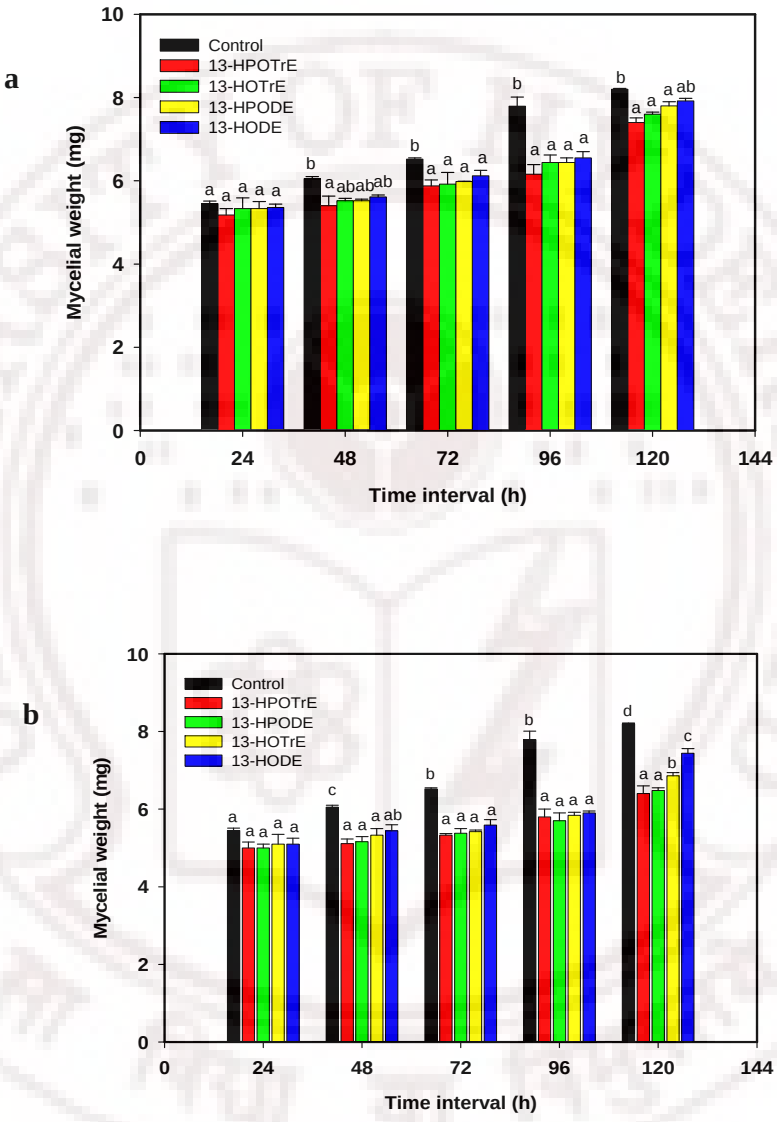
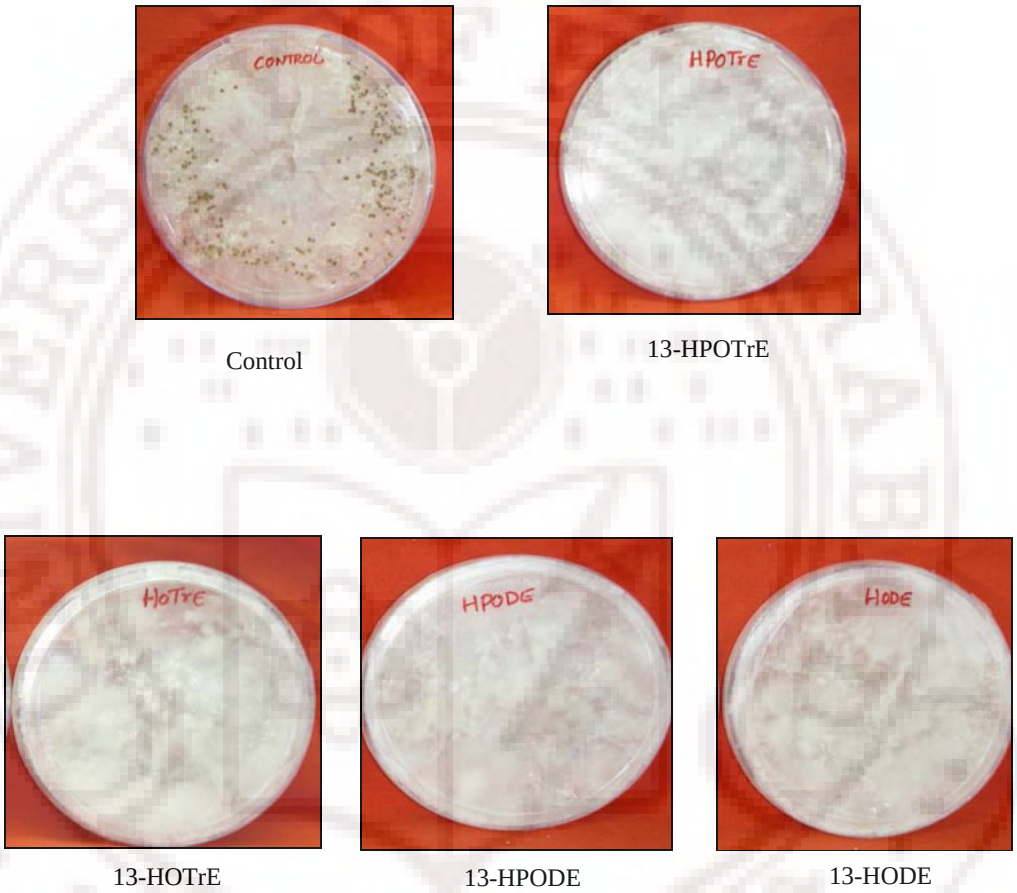


Fig. 59a & b: Effects of hydroperoxides and hydroxides on mycelia weight in *F. oxysporum*. The weight of the mycelium in each flask was recorded at 24 h interval up to 120 h. a. 10 µM b. 100 µM. Values for each treatment are means ± SE of three replicates. Within each group, bars with the same letter are not significantly different ($P \leq 0.05$) according to Newman-Keul's multiples comparisons test.

6.4 Effects of HPOTrE, HPODE, HOTrE and HODE on formation of sclerotia in *S. rolfsii* and *R. solani*:

The effects of 13-HPOTrE, 13-HPODE, 13-HOTrE and 13-HODE on sclerotia formation in *S. rolfsii* and *R. solani* were examined. The hydroperoxides and hydroxides spotted on the sterile filter paper discs caused retardation in the mycelial growth. Complete development of mycelium was observed after 7 days in the presence of LOX products in contrast to 4 days in control petriplates. The sclerotia were formed after 14 days in treated plates as compared to 7 days in control petriplates (Figs. 60 and 62). The number of sclerotia decreased in the presence of all LOX products although the effectiveness varied with the compound (Table 5). Of the four LOX products, 13-HPOTrE proved to be more effective in decreasing the number of sclerotia followed by 13-HPODE, 13-HOTrE and 13-HPODE in *S. rolfsii* (Fig. 60 & 61). The number of sclerotia drastically reduced to 22.8 in the presence of 10 µg of 13-HPOTrE as compared to 343.0 observed for controls. Similarly, 13-HPOTrE was found to be more effective in decreasing the number of sclerotia in *R. solani* followed by HOTrE. The number of sclerotia formed in the presence of 10 µg of 13-HPOTrE was found to be 5.0 in comparison to 51.0 observed in the controls (Fig. 62 & 63). Similar effects were observed in the presence of 13-HOTrE, 13-HPODE and 13-HODE with a significant decrease in the number of sclerotia (10.6-23) as compared to controls (51).



Control 13-HPOTrE

13-HOTrE 13-HPODE 13-HODE

Fig. 60: Photomicrographs showing the effects of 13-HPOTrE, 13-HPODE, 13-HOTrE and 13-HODE on formation of sclerotia in *S. rolfsii* after 7 days. Fungal mycelial disc was kept at the centre of the petriplate and 10 µg/10 µl of LOX products were added to the sterile filter discs placed around the fungal disc. The sclerotia formation in the petriplates was examined after 7 days of incubation at 30°C.

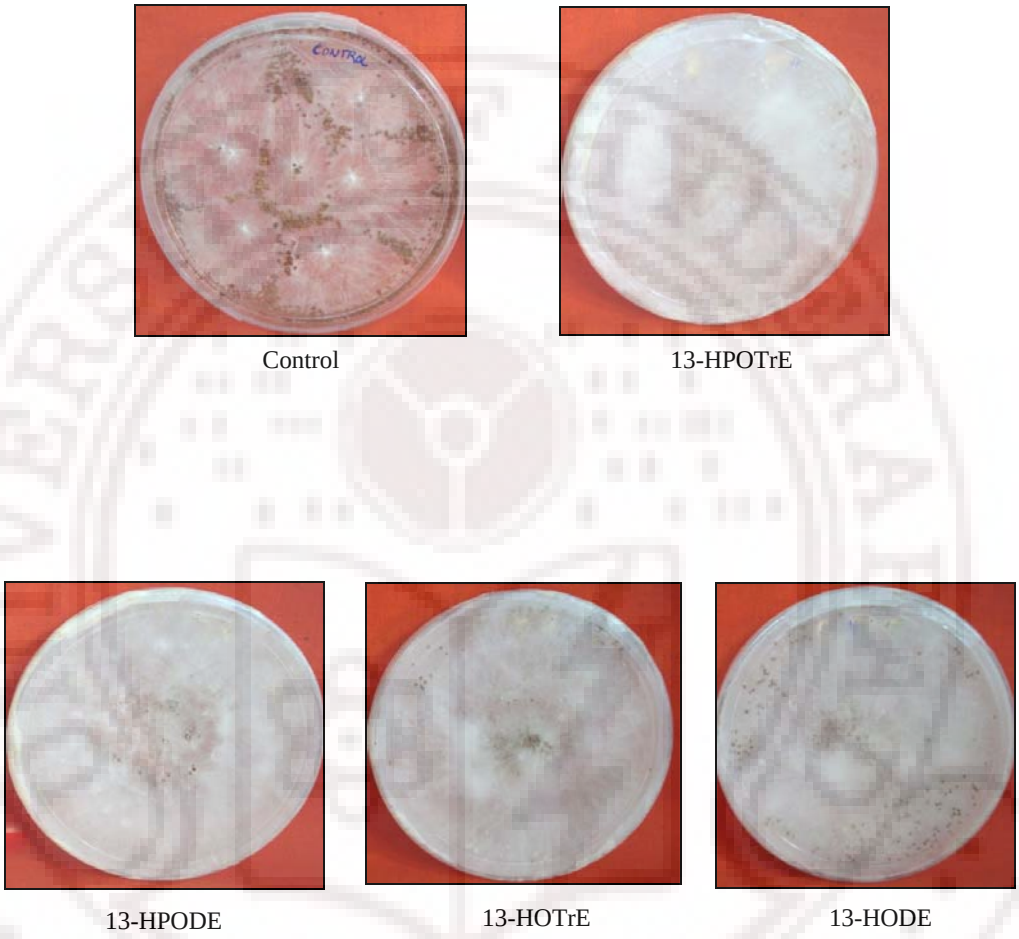


Fig. 61: Photomicrographs showing the effects of 13-HPOTrE, 13-HPODE, 13-HOTrE and 13-HODE on formation of sclerotia in *S. rolfsii* after 14 days. Fungal mycelial disc was kept at the centre of the petriplate and 10 µg/10 µl of LOX products were added to the sterile filter discs placed around the fungal disc. The sclerotia formation in the petriplates was examined after 14 days of incubation at 30°C.

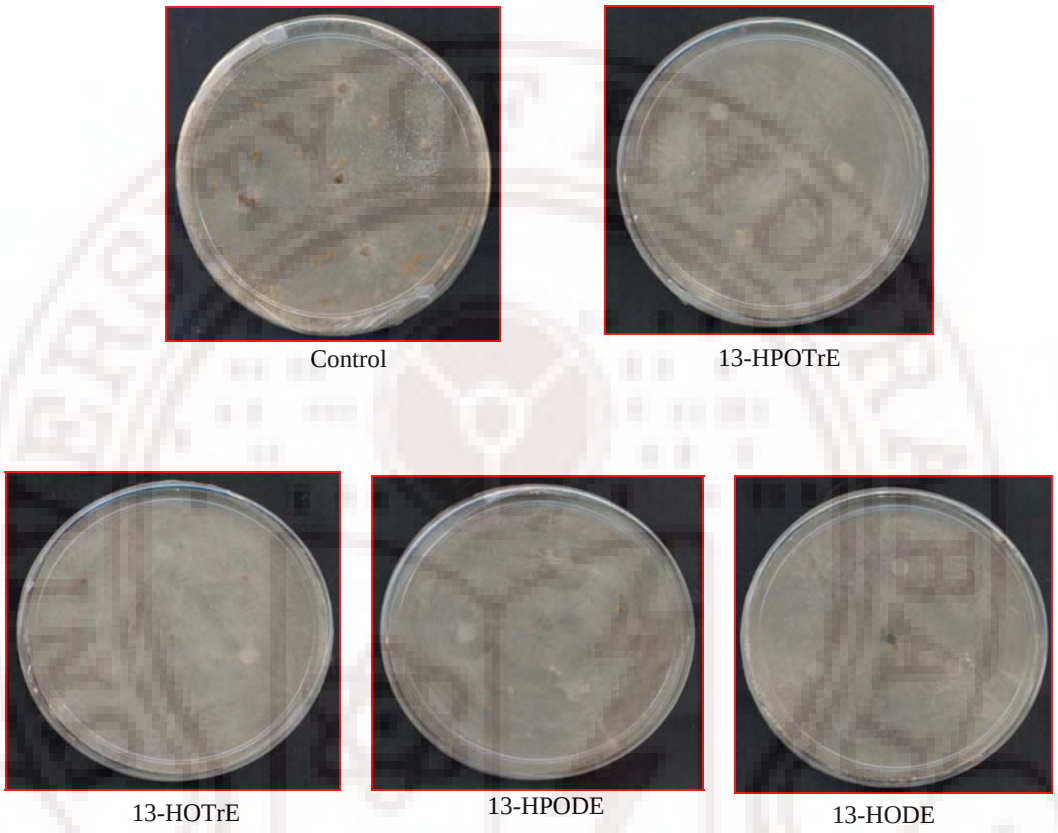


Fig. 62: Photomicrographs showing the effects of 13-HPOTrE, 13-HPODE, 13-HOTrE and 13-HODE on formation of sclerotia in *R. solani* after 7 days. Fungal mycelial disc was kept at the centre of the petriplate and 10 µg/10 µl of LOX products were added to the sterile filter discs placed around the fungal disc. The sclerotia formation in the petriplates was examined after 7 days of incubation at 30°C.

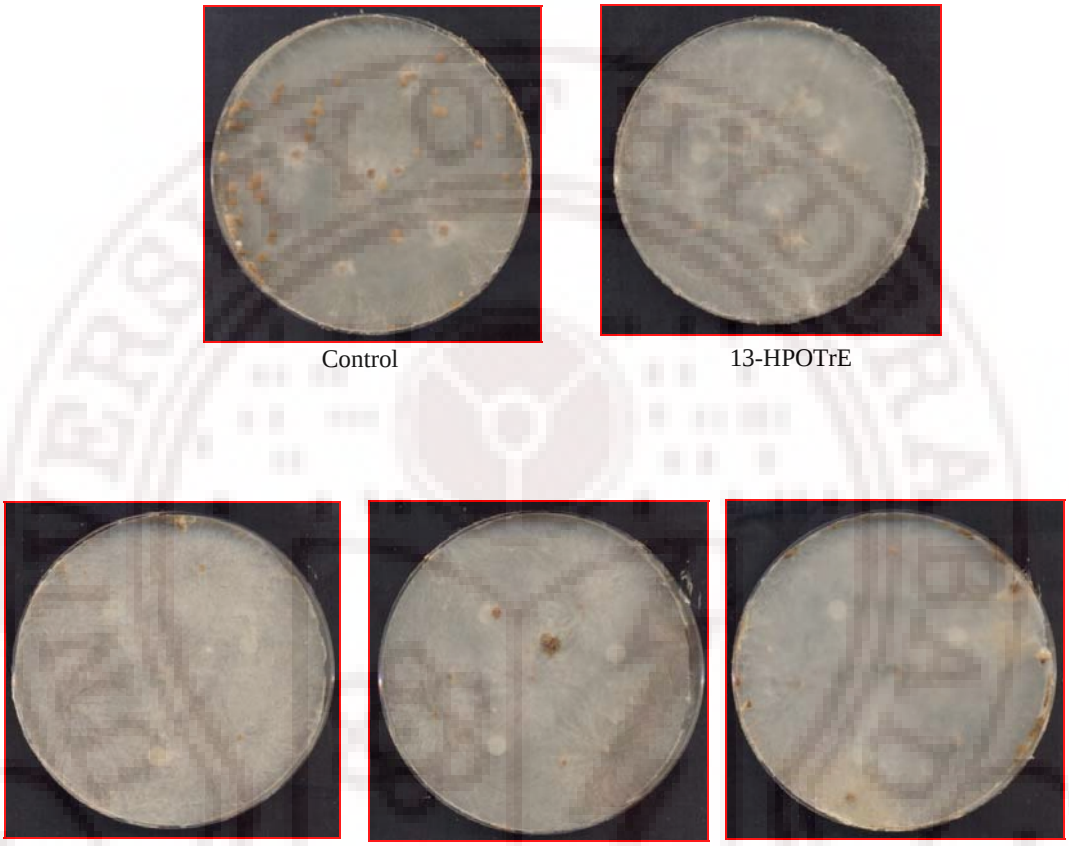


Fig. 63: Photomicrographs showing the effects of 13-HPOTrE, 13-HPODE, 13-HOTrE and 13-HODE on formation of sclerotia in *R. solani* after 14 days. Fungal mycelial disc was kept at the centre of the petriplate and 10 µg/10 µl of LOX products were added to the sterile filter discs placed around the fungal disc. The sclerotia formation in the petriplates was examined after 14 days of incubation at 30°C.

Table 5: Effect of hydroperoxides and hydroxides on sclerotia formation in *S. rolfsii* and *R. solani*. LOX products at 5 µg/10 µl and 10 µg/10 µl concentrations were added to filter discs in petriplate containing potato dextrose agar medium with fungal mycelia discs. The average number of sclerotia formed per petriplate was determined after 14 days of incubation at 30°C. Values are means ± SE of three experiments with each experiment consisting of three replicates/treatment. Means followed by the same letter in a column are not significantly different ($p<0.05$) by Newman-Keul's multiple comparisons test.

Treatment	Average number of sclerotia per petriplate	
	S. rolfsi	*R. solani*
Control	343.0 ± 7.7 h	51.0 ± 1.5 g
13-HPOTrE		
5 µg	42.2 ± 1.0 c	14.6 ± 0.5 d
10 µg	22.8 ± 1.0 a	5.0 ± 0.3 a
13-HPODE		
5 µg	53.4 ± 1.2 d	22.4 ± 0.5 b
10 µg	33.6 ± 1.1 b	10.6 ± 0.4 c
13-HOTrE		
5 µg	80.2 ± 1.3 f	16.6 ± 0.2 e
10 µg	49.8 ± 1.5 cd	7.2 ± 0.4 b
13-HODE		
5 µg	103.2 ± 1.0 g	23.0 ± 1.0 f
10 µg	68.8 ± 2.8 e	12.4 ± 0.2 d

DISCUSSION

6.5 LOX products are inhibitory for the growth of fungal pathogens *in vitro*:

LOX metabolites might be acting directly on the fungal pathogens leading to the development of disease resistance. The present experiments revealed that the four LOX products, 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE, tested had broad spectrum inhibitory effects on the *in vitro* growth of *S. rolfsii*, *R. solani and F.*

oxysporum. Of the four products tested, 13-HPOTrE exhibited high degree of antifungal activity as compared to 13-HPODE, 13-HOTrE and 13-HODE. A relatively low concentration of 13-HPOTrE could inhibit the growth of *S. rolfsii*. Similarly, Graner *et al*. (2003) showed that 13-HPOTrE and 13-HOTrE had antimicrobial effects on fungal pathogens of oil seed rape.

The present studies clearly demonstrated the decrease in mycelial weight of all the three fungi tested in the presence of LOX products. However, the inhibitory potential of the LOX products varied for the fungi depending on the concentration used and the time of incubation. Comparison of the effects of LOX products showed that 13-HPOTrE was more effective in decreasing the mycelia weight of all the three fungi. The data presented here also supports such a possibility on the involvement of LOX products of ALA in anti-microbial defenses. The anti-microbial activity of LOX products of ALA may be mediated by the formation of downstream products such as jasmonic acid, which plays an important role in defense responses (Xu *et al*., 1994; Farmer and Ryan., 1992). This explains why the LOX products of LA, which are not the substrates for jasmonic acid, are less effective in antimicrobial activities. Similar differential effects of LOX products of LA (13-HPODE) and ALA (13-HPOTrE) was reported in the pigeonpea seedlings infected with *Fusarium udam* (Uma Maheswari *et al*., 2000). These differential effects were shown to coincide with the preferential formation of LOX products under physiological (products of LA) and pathological (products of ALA) conditions in the pigeonpeas seedlings (Uma Maheswari *et al*., 2000), potato tubers subjected to injury/infection (Srinivasa Reddy *et al*., 2000) and elicitor treated hairy root cultures of *Solanum tuberosum* (Komaraiah *et al*., 2003). Farmer *et al*. (2003) proposed that several bioactive oxylipins might induce plant cell

damage and defense gene expression through a mechanism based on their electrophilic nature. Avis and Belanger (2001) proposed that cis-9-heptadecenoic acid, an antifungal fatty acid, might insert into the hydrophobic layer of fungal membranes, thereby disrupting membrane properties and possibly causing cell collapse. The work of Ohta *et al*. (1991) reported that 13-HPODE and 13-HODE, the major products of LOX pathway in leaves of rice infected with an incompatible race 131 of the blast *Magnaporthe grisea* had strongly inhibited the conidial germination of the fungus. The results reported here provided preliminary evidence on the inhibitory effects of LOX products on *in vitro* growth of fungi. Additional research is required to determine the exact mechanisms underlying the antimicrobial activity of LOX products *in vitro*, as well as their precise contribution to defense responses in plants.

The work of Prost *et al*. (2005) showed that plant pathogens display greatly varying levels of sensitivity, ranging from highly sensitive to a large number of oxylipins to mostly unaffected by these compounds. These differences might arise either because the pathogens might differ greatly in either the uptake of oxylipins from the aqueous culture broth, features of potential targets such as their membrane composition, or their eicosanoid/oxylipin metabolism. Evidence is accumulating that fungi and oomycetes produce eicosanoids and/or oxylipins that are involved in the regulation of growth, development, and life cycle (Kock *et al*., 2003; Noverr *et al*., 2003; Tsitsigiannis *et al*., 2004). However, it is not clear whether plant oxylipins interfere with microbial metabolism or signaling by competing with structurally related endogenous lipids which remains to be investigated (Prost *et al*., 2005). In the present study, the inhibitory effects of 10 µM 13-HPOTrE and 13-HPODE on

mycelial weight of *S. rolfsii* were observed upto 96 h and were lost when the incubation time was extended to 120 h. The inhibitory effects of 13-HOTrE and 13-HODE on mycelial weight were overcome even when incubated for 72 h. Our results are in conformity with that of Graner *et al*. (2003) who reported that inhibitory effects observed in some fungi by oxilipins are time depedent. Since several of the oxylipins tested were found to be stable in the absence of the fungus this effect was explained by induction of the degrading capacity of the fungus or increased tolerance (Graner *et al*., 2003).

6.6 LOX products affect the sclerotia formation in *S. rolfsii* and *R. solani*:

In the present study, the four LOX products tested affected the mycelial growth and sclerotia formation in *S. rolfsii* and *R. solani*. Addition of LOX products delayed the sclerotia formation along with reduction in the number of sclerotia per petriplate. Of the four products tested, 13-HPOTrE had a considerable effect on sclerotia formation. Even the lowest concentration tested, decreased the number of sclerotia to 42.2 and 14.6 as compared to 343.0 and 51.0 observed in control plates of *S. rolfsii* and *R. solani*, respectively. The biochemical factors responsible for the reduction in sclerotia formation following the incorporation in the medium remain to be determined. Thus the drastic reduction in sclerotia formation in the presence of LOX products could help in sustaining the fungus in an undifferentiated hyphal stage which is vulnerable to degradation by soil microorganisms. The LOX products could be employed in understanding the mechanisms of formation of sclerotia in *S. rolfsii* and *R. solani*. Inhibition of sclerotia formation strongly favours the possible use of LOX products as a control measure against *S. rolfsii* and *R. solani*. Calvo *et al*. (1999) found that 13S-HPODE but not linoleic acid or 9S-HPODE had a statistically

significant effect on sclerotial production in *Aspergillus flavus* when it was grown in the light. It was suggested that the location of the side groups on linoleic acid may determine functional specificities in the genus *Aspergillus*.

In summary, the LOX product, 13-HPOTrE proved to be more effective in inhibiting the growth of *S. rolfsii*, *R. solani* and *F. oxysporum* than 13-HOTrE, 13-HPODE and 13-HODE. Further, the reduction in mycelial weight and sclerotia formation in *S. rolfsii* and *R. solani* by LOX products suggests their involvement in the disease resistance. Thus the LOX products could possibly constitute an important part of the plant's defense system through their antifungal effects or through expression of defense genes in the early stages of interaction. Also studies are required to understand the mechanism of action of LOX products in the inhibition of fungal growth.

The most significant findings of the present study are the identification of specific LOX isozymes associated with drought tolerance (LOX-3) and anti-microbial defenses (LOX-4 and LOX-5) in groundnut seedlings. Further studies, however, are required to validate the hypothesis by isolating specific LOX genes and developing transgenics to over express and evaluate their performances against salt, drought and fungal infection.

Chapter 7

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Plant lipoxygenases (LOXs) are a class of dioxygenases involved in various important processes such as growth and development, senescence, wound and defense responses. The principle substrates for LOXs in higher plants are linoleic acid and α-linolenic acid. The lipoxygenase products produced in plant systems are hydroperoxides, jasmonic acid, traumatic acid, hexenals, nonenals etc which have wide array of functions. The role of lipoxygenase in disease resistance and tolerance to abiotic stresses has been demonstrated in different plant species. However, there is very little information on the role of LOXs in abiotic and biotic stress responses in groundnut. The study was initiated with an objective to determine the role of LOX in resistance/tolerance to biotic and abiotic stresses in groundnut. The first chapter was designed to determine the LOX activity during the process of seed germination and in seedlings subjected to NaCl, PEG, ABA and MJ treatments. The second chapter dealt with the changes in LOX activity and isozymes in groundnut seedlings in response to inoculation with *Sclerotium rolfsii* and *Rhizoctonia solani*. The LOX isozymes from *S. rolfsii* inoculated seedlings of resistant variety were purified and characterized. The expression pattern of *PnLOX-1*, *PnLOX-2* and *PnLOX-3* genes was analyzed in seedlings inoculated with *S. rolfsii* by RT-PCR. The third chapter focused on the preparation of LOX products and analysis of the antifungal activities of 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE on *Sclerotium rolfsii*, *Rhizoctonia solani* and *Fusarium oxysporum*, the specific pathogens of groundnut.

The main findings that emerged from the study are as follows:

- High LOX activity was observed in 4-5-day-old germinating seedlings of drought tolerant (K-1375), TAG-24 and drought sensitive (JL-24) varieties coinciding with high growth rate of tissue. The high LOX activity observed in 4-5-day-old seedlings was associated with the induction of three isozymes in K-1375 and TAG-24 varieties whereas two isozymes were observed in JL-24 variety. The LOX induced during seed germination could have a role in mobilization of stored lipids or might afford protection against fungal and bacterial pathogens.
- LOX activity increased in the roots in response to treatment with 200 mM NaCl and 23% PEG in K-1375 (drought tolerant) and JL-24 (drought susceptible) varieties. However, LOX induced in response to NaCl and PEG treatments was higher in drought tolerant variety than susceptible variety compared to respective controls. The increase in LOX activity at specific intervals was associated with an increase in the intensity of L-3 isozyme in JL-24 and K-1375 varieties. Thus LOX might play a role in salt and drought tolerance in groundnut.
- ABA treatment resulted in a marked increase in LOX activity at 48 h and then decreased in seedlings of K-1375 variety. A rapid increase in LOX activity was observed at 24 h and then decreased following treatment of the seedlings with MJ. Thus LOX was found to be regulated by ABA and MJ treatments in groundnut.
- A significantly higher LOX activity was observed in seedlings of stem rot resistant varieties than susceptible varieties indicating the possibility of using

LOX as biochemical marker for screening stem rot disease resistant varieties of groundnut.

- LOX activity increased in seedlings of resistant and susceptible varieties in response to inoculation with *Sclerotium rolfsii* although the increase was more pronounced in shoots of resistant variety compared to susceptible variety. The increase in LOX activity may be the result of activation of defense responses in the plants due to exposure to pathogen.
- A maximum increase in LOX activity was observed in shoots of R-2001-1 variety during 42-54 h, which was associated with induction of new isozymes, L-4 and L-5 in disease resistant variety whereas these isozymes were not detected in susceptible variety. The induction of new isozymes in resistant variety could be a part of defense strategy for resistance to stem rot pathogen although their role remains to be investigated.
- Inoculation of the seedlings of ICG-4747 variety with *Rhizoctonia solani* resulted in an increase in LOX activity for all the intervals examined compared to respective controls. The increase in LOX activity observed in response to inoculation with *R. solani* could be due to activation of constitutive enzyme or induction of LOX genes.
- RP-HPLC analysis revealed an increase in 13-HODE formation upon incubation with LA in *S. rolfsii* inoculated seedlings of R-2001-1 variety during 30-96 h, which was higher than that of susceptible variety as compared to respective controls. The higher levels of LOX activity observed in resistant variety during 30-96 h of inoculation with *S. rolfsii* correlated with increased 13-HODE formation which might contribute to defense reactions by inhibition

of pathogen growth and development, induction of phytoalexin accumulation and/or signal transduction.

- The LOX isozymes purified from *S. rolfsii* inoculated seedlings showed three peaks, A, B and C on anion exchange HPLC. The peak A fraction resolved into three isozymes, L-3, L-4 and L-5 whereas peak B and peak C fraction separated into L-2 and L-1 isozymes, respectively on native PAGE. SDS-PAGE analysis of purified LOXs revealed a molecular weight of 84 kDa for isozyme L-1, 95 kDa for isozyme L-2 and 98 kDa for isozymes L-3, L-4 and L-5.
- The optimum pH for LOX isozymes, L-3, L-4 and L-5 was found to be 8.5 whereas LOX isozymes, L-1 and L-2 exhibited maximal activity at pH 6.5. The temperature stability of LOX isozymes varied with LOX isozyme, L-2 exhibiting activity up to 70^{o}C whereas LOX isozymes, L-1, L-3, L-4 and L-5 exhibiting activity up to 40^{o}C. Thus LOX isozyme, L-2 with an optimum pH of 6.5 was found to be more thermostable.
- Of the different inhibitors tested, NDGA and esculetin were the potent inhibitors and inhibited the LOX activity of all the purified LOX isozymes at 1 mM concentration.
- RT-PCR analysis revealed an increase in the transcript levels of *PnLOX-1*, *PnLOX-2* and *PnLOX-3* genes at specific time intervals in seedlings of R-2001-1 variety upon inoculation with *S. rolfsii*. The transcript levels of *PnLOX-1* gene were found to be higher than that of *PnLOX-2* and *PnLOX-3* genes in *S. rolfsii* inoculated seedlings. These genes could offer potential targets for manipulation of disease resistance in groundnut.

- The antifungal activity of 13-HPODE, 13-HODE, 13-HPOTrE and 13-HOTrE was demonstrated against *S. rolfsii*, *F. oxysporum* and *R.* solani, specific pathogens of groundnut. Among the four LOX products, 13-HPOTrE proved more effective in inhibiting the mycelial growth of all three fungi and sclerotia formation in *S. rolfsii* and *R. solani*.

In summary, the results showed a marked induction of LOX in seedlings of drought tolerant variety as compared to susceptible variety in response to NaCl and PEG treatments, suggesting a possible role for LOXs in salt and drought tolerance. The mechanism how LOXs are able to confer drought resistance, however, needs to be understood/evaluated. The high LOX activity along with induction of new isozymes observed in the seedlings of resistant variety in response to *S. rolfsii* inoculation as compared to susceptible variety emphasizes their role in defense responses in groundnut. The study demonstrated the antifungal activity of the LOX products and thus substantiating the role of LOXs in disease resistance in groundnut. Further studies on cloning of LOX genes followed by their over expression or down regulation would help in establishing the role of LOX in disease resistance in groundnut.

Chapter 8

REFERENCES

REFERENCES

- Akram, A., Ongena, M., Duby, F., Dommes, J. and Thonart, P. (2008) Systemic resistance and lipoxygenase-related defence response induced in tomato by *Pseudomonas putida* strain BTP1. BMC Plant Biol. 2008: 113.

- Altschuler, M., Collins, G. B. and Hildebrand, D. F. (1989) Developmental expression of lipoxygenases in soybeans. Plant Sci. 63:151-158

- Anderson, J. M. (1989) Membrane derived fatty acids as precursors to second messengers. In: (Eds.) Boss, W. F. and Morre, D. J. Second messengers in plant growth and development. Vol. 6, Alan R. Liss, New York, pp. 181-212.

- Andrianarison, R. H., Beneytout, J. L. and Tixier, M. (1989) An enzymatic conversion of lipoxygenase products by a hydroperoxide lyase in blue-green algae (*Oscillatoria sp*.). Plant Physiol. 91: 1280-1287.

- Anjana, G., Kini, K. R., Shetty, H. S. and Prakash, H. S. (2007) Differential expression of sunflower peroxidase isoforms and transcripts during necrotrophic interaction with *Alternaria helianthi*. Russian J. Plant Physiol. 54: 513-517.

- Anstis, P. J. P. and Friend, J. (1974) The isozyme distribution of etiolated pea seedling lipoxygenase. Planta. 115: 329-335.

- Apel, K., and Hirt. H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55:373–399

- Avis, T. J. and Belanger, R. R. (2001) Specificity and mode of action of the antifungal fatty acid cis-9-heptadecenoic acid produced by *Pseudozyma flocculosa*. Appl Environ Microbiol. 67: 956–960.

- Babitha, M. P., Prakash, H. S. and Shetty, H. S. (2004) Purification and properties of lipoxygenase induced in downy mildew resistant pearl millet seedlings due to infection with *Sclerospora graminicola*. Plant Sci. 166: 31-39.

- Babitha, M. P., Prakash, H. S. and Shetty, H. S. (2006) Induction of lipoxygenase in downy mildew resistant seedlings of Pearl millet in response to inoculation with *Sclerospora graminicola*. Int. J. Agri and Biol. 8: 560-564.

- Bachmann, A., Hause, B., Maucher, H., Garbe, E., Voros, K., Weichert, H., Wasternack, C. and Feussner, I. (2002) Jasmonate induced lipid peroxidation in arley leaves initiated by distinct 13-LOX forms of chloroplasts. Biol. Chem. 383: 1645-1657

- Bachmann, A., Hause, B., Maucher, H., Garbe, E., Voros, K., Weichert, H., Wasternack, C., Began, G., Sudharshan, E. and Rao, A. G. A. (1999) Change in the positional specificity of lipoxygenase-1 due to insertion of fatty acids into phosphatidylcholine deoxycholate mixed micelles. Biochem. 38: 13920-13927.

- Began, G., Sudharshan, E. and Rao, A. G. A. (1999) Change in the positional specificity of lipoxygenase 1 due to insertion of fatty acids into phosphatidylcholine deoxycholate mixed micelles. Biochem. 38: 13920–7.

- Belkner, J., Wiesner, R., Kuhn, H. and Lankin, V. Z. (1991) The oxygenation of cholesterol esters by the reticulocyte lipoxygenase. FEBS Lett. 279: 110-114.

- Bell, E. and Mullet, J. E. (1991) Lipoxygenase gene expression is modulated in plants by water deficit, wounding, and methyl jasmonate. Mol Gen Genet. 230: 456–462

- Bell, E., Creelman, R. A. and Mullet, J. E. (1995) A chloroplast lipoxygenase is required for wound induced jasmonic acid accumulation in *Arabidopsis*. Proc. Natl. Acad. Sci., USA. 92: 8675-8679.

- Beneytout, J. L., Andrianarison, R. H., Rakotoarisoa, Z. and Tixier. M. (1989) Properties of a lipoxygenase in green algae (*Oscillatoria sp*.). Plant Physiol. 91, 367-372.

- Beneytout, J. L., Najid, A. and Tixier, M. (1988) Changes in lipoxygenase activity during seedling development of *Lupinus albus*. Plant Sci. 58: 35-41.

- Ben-Hayyim, G., Gueta-Dahan, Y., Avsian-Kretchmer, O., Weichert, H. and Feussner, I. (2001) Preferential induction of a 9-lipoxygenase by salt in salt-tolerant cells of *Citrus sinensis* L. Osbeck. Planta. 212: 367-375.

- Berner, J. (2006) Biochemistry of Russian wheat aphid resistance in wheat: Involvement of lipid like products. Ph.D thesis. University of the Froe State, Bloemfontein, South Africa.

- Bezakova, L., Mistrick, I., Kovacs, P. and Psenak, M. (1994) Lipids, fatty acids and lipoxygenase activity in developing poppy seedling, *Papaver somniferum* L. Biologia. 49: 339-345.

- Blee, E. (2002) Impact of phytooxylipins in plant defense. Trends Plant Sci. 7: 315-321.

- Bohland, C., Balkenhohl, T., Loers, G., Feussner, I. and Grambow, H. J. (1997) Differential induction of lipoxygenase isoforms in wheat upon treatment with rust fungus elicitor, chitin oligosaccharides, chitosan, and methyl jasmonate. Plant Physiol. 114: 699-685.

- Bookjans, G., Altschuler, M., Brockman, J., Yenofsky, R. and Polacco, J. (1988) Molecular biological studies of plant lipoxygenases. In: (Ed.) Applewhite, T. H. Proc. of the World conference on Biotoch. For the fat and oil industry. Am. Oil. Chem. Soc. Press, Urbana. pp. 301-304.

- Brash, A. R. (1999) Lipoxygenases: Occurrence, functions, catalysis and acquisition of substrate. J. of Biol. Chem. 274: 23679-23682.

- Brash, A. R., Ingram, C. D. and Harris, T. M. (1987) Analysis of a specific oxygenation reaction of soybean lipoxygenase-1 with fatty acids esterified in phospholipids. Biochem. 26: 5465-5471.

- Buonaurio, R. and Servili, M. (1999) Involvement of lipoxygenase, lipoxygenase pathway volatiles, and lipid peroxidation during the hypersensitive reaction of pepper leaves to *Xanthomonas campestris pv. vesicatoria*. Physiol. Mol. Plant Pathol. 54: 155–169.

- Burow, G. B., Gardner, H. W. and Keller, N. P. (2000) A peanut seed lipoxygenase responsive to *Aspergillus* colonization. Plant Mol. Biol. 42: 689-701.

- Burow, G. B., Nesbitt, T. C., Dunlap, J. and Keller, N. P. (1997) Seed lipoxygenase products modulate *Aspergillus* mycotoxin biosynthesis. Mol. Plant-Microbe Inter. 10: 380-387.

- Cacas, J. L., Vailleau, F., Devoine, C., Ennar, N., Agnel, J. P., Tronchet, M., Ponchet, M., Blein, J. P., Roby, D., Triantaphylides, C. and Montillet, J. L. (2005) The combined action of 9-lipoxygenase and galactolipase is sufficient to bring about programmed cell death during tobacco hypersensitive response. Plant Cell Environ. 28: 1367-1378.

- Calvo, A. M., Hinze, L. L., Gardner, H. W. and Keller, N. P. (1999) Sporogenic effect of polyunsaturated fatty acids in development of *Aspergillus* spp. Appl. Environ. Microbiol. 65: 3668-3673.

- Casey, R. (1995) Sequence of a cDNA clone encoding a potato (*Solanum tuberosum*) tuber lipoxygenase. Plant Physiol. 107: 265-266.

- Casey, R. (1999) Lipoxygenases, In: (Eds.) Shewry, P. R. and Casey, R.. Seed proteins, Dordrecht, Kluwer Academic, pp. 685-708.

- Chang, P. R. Q. and McCurdy, A. R. (1985) Lipoxygenase activity in fourteen legumes. Can. Inst of Food Sci and Tech. J. 18: 94-96.

- Chateigner, A. L., Le Deunff, Y. and Jalouzot, R. (1999) Germination-associated changes in the transcript content of pea seedling lipoxygenases. Lipoxygenase-g: a new marker of axis growth resumption. Planta 208: 606-613.

- Chen, G., Hackett, R., Walker, D., Taylor, A., Lin, Z. and Grierson, D. (2004) Identification of a specific isoform of tomato lipoxygenase (TomloxC) involved in the generation of fatty acid-derived flavor compounds. Plant Physiology. 136:2641-2651.

- Chen, X. S. and Funk, C. D. (2001) The N-terminal "β-barrel" domain of 5-lipoxygenase is essential for nuclear membrane translocation. J. Biol. Chem. 276: 811-818.

- Cheng, Q., Zhang, B., Zhuge, Q., Zeng, Y., Wang, M. and Huang, M. (2006) Expression profiles of two novel lipoxygenase genes in *Populus deltoides*. Plant Sci. 170: 1027-1035.

- Choi, D. and Bostock, R. M. (1994) Involvement of *de novo* protein synthesis, protein kinase, extracellular Ca^{2+} and lipoxygenase in arachidonic acid induction of 3-hydroxy-3-methyl glutaryl coenzyme A reductase genes and isoprenoid accumulation in potato. Plant Physiol. 104: 1237-1244.

- Choi, D., Bostock, R. M., Avdiushko, S. and Hildebrand, D. F. (1994) Lipid derived signals that discriminate wound- and pathogen-responsive isoprenoid pathways in plants – methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. Proc. Natl. Acad. Sci. 91: 2329-2333.

- Christopher, J. P., Pistoriu, E. K. and Axeelrod, B. (1970) Isolation of a third isoenzyme of soybean lipoxygenase. Biochimica and Biophysica Acta. 12: 54-62.

- Christopher, J. P., Pistorius, E. K. and Axelrod, B. (1972) Isolation of a third isoenzyme of soybean lipoxygenase. Biochim. Biophys. Acta. 284 : 54-62

- Clemente, A., Olias, R. and Olias, J. M. (2000) Purification and characterization of broad bean lipoxygenase isoenzymes. J. of Agril. and Food Chem. 48: 1070-1075.

- Coffelt, T. A. (1989) Peanut. In: (Eds.) Robbelen, G., Downey, R. K. and Ashri, A. Oil crops of the world. McGaw-Hill Publishing Company, pp. 319-338.

- Colmenero-Flores, J. M., Moreno, L. P., Smith, C. E. and Covarrubias, A. A. (1999) Pvlea-lS, a member of a new late-embryogenesis-abundant protein family that accumulates during water stress and in the growing regions of well-irrigated bean seedlings. Gene expression, protein accumulation, and immunolocalization during stress and development. Plant Physiol. 120: 93-103.

- Creelman, R. A. and Mullet, J. E. (1997) Biosynthesis and action of jasmonates in plants. Ann. Rev. of Plant Physiol. and Plant Mol. Biol. 48: 355-381.

- Creelman, R. A., Mason, H. S., Bensen, R. J., Boyer, J. S. and Mullet, J. E. (1990) Water deficit and abscisic acid cause differential inhibition of shoot *versus* root growth in soybean seedlings. Plant Physiol. 92: 205- 214.

- Croft, K. P. C., Juttner, F. and Slusarenko, A. J. (1993) Volatile products of the lipoxygenase pathway evolved from *Phaaseolus vulgaris* (L.) leaves inoculated with *Pseudomonas syringae* pv *phaseolicola*. Plant Physiol. 101: 13-24.

- Croft, K. P. C., Voisey, C. R. and Slusarenko, A. J. (1990) Mechanism of hypersensitive cell collapse: Correlation of increased lipoxygenase activity with membrane damage in leaves of *Phaseolus vulgaris* cv Red Mexican inoculated with avirulent race/cells of *Pseudomonas syringae* pv. *Phaseolicola*. Physiol. Mol. Plant Pathol. 36: 49-62.

- Crozier, A., Kamiya, Y., Bishop, G. and Yokota, T. (2000) Biosynthesis of hormones and elicitor molecules. In: (Eds.) Buchanan, B., Gruissem, W. and Jones, R. L. Biochem. Mol. Biol. of plants. Rockville, MD, American Soc. of Plant Physiol. pp. 850-929.

- Dixon, R. A. and Harrison, M. J. (1990) Activation, structure, and organization of genes involved in microbial defense in plants. Adv Genet. 28 : 165–234.

- Dixon, R. A. and Lamb, C. J. (1990) Molecular communication in interactions between plants and microbial pathogens. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41: 339-367.

- Doderer, A., Kokkelink, I., van der Veen, S., Valk, B. E., Schram, A. W. and Douma, A. C. (1992) Purification and characterization of two lipoxygenase isoenzymes from germinating barley. Biochimica et Biophysica Acta. 1120: 97-104.

- Doehlert, D. C., Wicklow, D. T. and Gardner, H. W. (1993) Evidence implicating the lipoxygenase pathway in providing resistance to soybeans against *Aspergillus flavus*. Phytopathol. 83: 1473-1477.

- Domoney, C., Firmin, J. L., Sidebottom, C., Ealing, P. M., Slabas, A. and Casey, R. (1990) Lipoxygenase heterogeneity in *Pisum sativum*. Planta. 181: 35-43.

- Draper, S. R (1969) Lipid changes in senescing cucumber cotyledons. Phytochem. 8: 1641-1647.

- Droillard, M. J., Rouet-Mayer, M. A., Bureau, J. M. and Lauriere, C. (1993) Membrane-associated and soluble lipoxygenase isoforms in tomato pericarp – Characterization and involvement in membrane alterations. Plant Physiol. 103: 1211–1219.

- Eiben, H. G. and Slusarenko, A. J. (1994) Complex spatial and temporal expression of lipoxygenase genes during *Phaseolus vulgaris* (L.) development. The Plant J. 5: 123-135.

- Eriksson, C. E. and Svensson, S. G. (1970) Lipoxygenase from peas, purification and properties of the enzyme. Biochim Biophys Acta. 198: 449–459.

- Eskin, N. A. M., Grossman, S. and Pinsky, A. (1977) Biochemistry of lipoxygenase in relation to food quality. Crit. Rev. Food Sci. Nutr. 9: 1-41.

- FAO. (2003) World agriculture towards 2015/2030. An FAO perspective, edited by J. Bruinsma. Rome, FAO and London, Earthscan.

- Farmer, E. E. and Ryan, C. A. (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell. 4: 129-134.

- Farmer, E. E., Almeras, E. and Krishnamurthy, V. (2003) Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Curr. Opin. Plant Biol. 6: 372–378.

- Feussner, I. and Kuhn, H. (2000) Application of lipoxygenases and related enzymes for the preparation of oxygenated lipids. In: (Ed.) Bornscheuer, U. T. Enzymes in lipid modification. Wiley-VCH, Weinheim, Germany, pp. 309-336.

- Feussner, I. and Wasternack, C. (2002) The lipoxygenase pathway. Annu Rev Plant Biol. 53: 275-297.

- Feussner, I., Balkenhohl, T. J., Porzel, A., Kuhn, H. and Wasternack, C. (1997) Structural elucidation of oxygenated storage lipids in cucumber cotyledons – Implications of lipid body lipoxygenase in lipid mobilization during germination. J. Biol. Chem. 272: 21635-21641.

- Feussner, I., Blee, E., Weichert, H., Rousset, C. and Wasternack, C. (1998) Fatty acid catabolism at the lipid body membrane of germinating cucumber cotyledons. In: (Eds.). Sanchez, J., Cerda-Olmedo, E. and Martinez-Force, E. Secr. Publ. Univ, Sevilla, Spain. pp. 311-333.

- Fidantsef, A. L. and Bostock, R. M. (1998) Characterization of potato tuber lipoxygenase cDNAs and lipoxygenase expression in potato tubers and leaves. Physiol. Plant. 102: 257-271.

- Figueiredo, A., Fortes, A. M., Ferreira, S., Sebastiana, M., Choi, Y. H., Sousa, L., Acioli-Santos, B., Pessoa, F., Verpoorte, R. and Pais, M.S. (2008) Transcriptional and metabolic profiling of grape (*Vitis vinifera* L.) leaves unravel possible innate resistance against pathogenic fungi. J.Exp.Bot. 59: 3371-3381.

- Fischer, A. M., Dubbs, W. E., Baker, R. A., Fuller, M. A., Stephenson, L. C. and Grimes, H. D. (1999) Protein dynamics, activity and cellular localization of soybean lipoxygenases indicate distinct functional roles for individual isoforms. Plant J. 19: 543–554

- Foumier, J., Pelissier, B., Esquerre-Tugaye, M. T. (1986) Induction d'une activite lipoxygenased ans les cellules de tabac (*Nicotiana tabacum*) en culture, par des eliciteurs d'ethylene de *Phytophthora parasitica* var. nicotianae. C. R. Acad. Sci. Paris. 303: 651-654.

- Fournier, J., Pouenat, K. L., Rickauer, M., Robinovitch-Chable, H., Rigaud, M. and Esquerre-Tugaye, M. R. (1993) Purification and characterization of elicitor-induced lipoxygenase in tobacco cells. Plant J. 3: 63-70.

- Froehlich, J. E., Itoh, A. and Howe, G. A. (2001) Tomato allene oxide synthase and fatty acid hydroperoxide lyase, two cytochrome P450s involved in oxylipin metabolism, are targeted to different membranes of chloroplast envelope. Plant Physiol. 125: 306-317.

- Gallego, S. M., Benavides, M. P. and Tomaro, M. L. (1996) Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. Plant Sci. 121: 151-159.

- Galliard, T, and Chan, H. W. S. (1980) Lipoxygenases. In: (Ed.) Stumpf, P. K. The biochemistry of plants: A comprehensive treatise. Vol. 4, Lipids: Structure and function, New York, Academic Press, pp. 131-161.

- Gao, X., Brodhagen, M., Isakeit, T., Horowitz-Brown, S., Gobel, C., Betran, J., Feussner, I., Keller, N. P. and Kolomietes, M. V. (2009) Inactivation of the lipoxygenase *ZmLOX3* increases susceptibility of maize to *Aspergillus* spp. 22: 222-231.

- Gardner, C. D., Sherrier, D. J., Kardailsky, I. V. and Brewin, N. J. (1996) Localization of lipoxygenase proteins and mRNA in pea nodules: Identification of lipoxygenase in the lumen of infections threads. Mol. Plant Microbe Interacti. 9: 282-289.

- Gardner, H. W. (1989) Soybean lipoxygenase-1 enzymatically forms both 9(S)- and 13(S)-hydroperoxides from linoleic acid by a pH-dependent mechanism. Biochim. Biophys. Acta. 1001: 274-281.

- Gardner, H. W. (1991) Recent investigations into the lipoxygenase pathway in plants. Biochim. Biophys. Acta. 1084: 221-239.

- Gardner, H. W. (1995) Biological roles and biochemistry of the lipoxygenase pathway. Hort. Sci. 30: 197-205.
- Gardner, H. W. (1998) 9-hydroxy-traumatin, a new metabolite of the lipoxygenase pathway. Lipids. 33: 745-749.
- Geerts, A., Feltkamp, D. and Rosahl, S. (1994) Expression of lipoxygenase in wounded tubers of *Solanum tuberosum* L. Plant Physiol. 105: 269-277.
- Georgalaki, M. D., Bachmann, A., Sotiroudis, T. G., Xenakis, A., Porzel, A. and Feussner, I. (1998) Characterization of a 13-lipoxygenase from virgin olive oil and oil bodies of olive endosperm. Fett/Lipid. 100: 554-560.
- Gillmor, S. A., Villasenor, A., Fletterick, R., Sigal, E. and Browner, M. F. (1997) The structure of mammalian 15-lipoxygenase reveals similarity to the lipases and the determinants of substrate specificity. Nat. Struct. Biol. 4: 1003-1009.
- Gobel, C., Feussner, I. and Rosahl, S. (2003) Lipid peroxidation during the hypersensitive response in potato in the absence of 9-lipoxygenases. J. Biol. Chem. 278: 52834-52840.
- Gobel, C., Feussner, I., Schmidt, A., Scheel, D., Sanchez- Serrano, J., Hamberg, M. and Rosahl, S. (2001) Oxylipin profiling reveals the preferential stimulation of the 9-lipoxygenase pathway in elicitor-treated potato cells. J. of Biolo. Chem. 276: 6267–6273.
- Graner, G., Hamberg, M. and Meijer, J. (2003) Screening of oxylipins for control of oilseed rape (*Brassica napus*) fungal pathogens. Phytochem. 63: 89-95.
- Grayburn, W. S., Schneider, G. R., Hamltonkemp, T. R., Bookjans, G., Ali, K. and Hildebrand, D. F. (1991) Soybean leaves contain multiple lipoxygenases. Plant Physiol. 95: 1214-1218.
- Grossman, S., Trop, M., Avtalion and Pinsky, A. (1972) Eggplant lipoxygenase: Isolation and partial characterization. Lipids 7: 467-473.
- Hamberg, M., Sanz, A., Rodriguez, M. J., Calvo, A. P. and Castresana, C. (2003) Activation of the fatty acid alpha-dioxygenase pathway during bacterial infection of tobacco leaves. Formation of oxylipins protecting against cell death. J. Biol. Chem. 278: 51796-51805.
- Hamberg, M. and Gardner, H. W. (1992) Oxylipin pathway to jasmonates: biochemistry and biological significance. Biochim. Biophys. Acta. 1165: 1-18.
- Hamberg, M., Sanz, A. and Castresana, C. (1999) α–oxidation of fatty acids in higher plants- identification of a pathogen-inducible oxygenase (PIOX) as

an – dioxygenase and biosynthesis of 2-hydroperoxylinolenic acid. J. Biol. Chem. 274: 24503-24513.

- Hammons, R. O. (1994) The origin and history of the groundnut. In: (Ed.) Smartt, J. The groundnut crop. A scientific basis for improvement. Chapman and Hall, New York.

- Hatanaka, A. (1996) The fresh green odor emitted by plants. Food Rev. Int. 12: 303-350.

- Heydeck, D. and Schewe, T. (1985) Improved procedure for the detection of activity of lipoxygenases on electrophoregrams. Biomed Biochem Acta 44: 1261–1263.

- Hilbers, M. P., Kerkhoff, B., Finazzi-Agro, A., Veldink, G. A. and Vliegenthart, J. F. G. (1995) Heterogeneity and developmental changes of lipoxygenase in etiolated lentil seedlings. Plant Sci. 111: 169-180.

- Hildebrand, D. F. (1989) Lipoxygenases. Physiol. Plant. 76: 249-253.

- Hildebrand, D. F., Hamilton-Kemp, T. R., Legg, C. C. and Bookjans, G. (1988) Plant lipoxygenases: occurrence, properties and possible functions. Curr. Plant Biochem. Physiol. 17: 201-219.

- Hildebrand, D. F., Versluys, R. T. and Collins, G. B. (1991) Changes in lipoxygenase isozyme levels during soybean embryo development. Plant Sci. 75: 1–8.

- Holtman, W. L., Duijn, G. V., Sedee, N. J. A. and Douma, A. C. (1996) Differential expression of lipoxygenase isoenzymes in embryos of germinating barley. Plant Physiol. 111: 569-576.

- Holtman, W. L., Vredenbregt-Heistek, J. C., Schmitt, N. F. and Feussner, I. (1997) Lipoxygenase-2 oxygenates storage lipids in embryos of germinating barley. Eur. J. Biochem. 248: 452-458.

- Hornung, E., Walther, M., Kuhn, H and Feussner, I. (1999) Conversion of cucumber linoleate 13-lipoxygenase to a 9-lipoxygenating species by site-directed mutagenesis. Proc. Natl. Acad. Sci., USA. 96:4192–4197.

- Howe, G. A. and Schilmiller, A. L. (2002) Oxylipin metabolism in response to stress. Curr. Opin. Plant Biol. 5: 230-236.

- Husted, L. (1936) Cytological studies of the peanut *Arachis*. Chromosome number, morphology and behaviour and their application to the origin of the cultivated forms. Cytologia. 7: 396-423.

- Ida, S., Masaki, Y. and Morita, Y. (1983) The isolation of multiple forms and product specification of rice lipoxygenase. Agric. and Biol. Chem. 47: 637-641.

- Jadhav, S., Singh, B. and Salunkle, D. K. (1972) Metabolism of unsaturated fatty acids in tomato fruit: linoleic and linolenic acids as precursors of hexanal. Plant Cell Physiol. 13: 449-459

- Jean-Luc, M., Jean-Pierre, A., Michel, P. and Fabienne, V. (2002) Lipoxygenase-mediated production of fatty acid hydroperoxides is a specific signature of the hypersensitive reaction in plants, Plant Physiol. Biochem. 40: 633–639.

- Jen-Min, K., Ann, H., Dong, B. Y., Min, H, P., Mei, L. T. and Bonnie, S. P. (2006) Lipoxygenase from banana leaf: purification and characterization of an enzyme that catalyzes linoleic acid oxygenation at the 9-position, J Agric Food Chem. 54: 3151–3156

- Jensen, A. B., Poca, E., Rigaud, M., Freyssinet, G. and Pages, M. (1997) Molecular characterization of L2 lipoxygenase from maize embryos. Plant Mol. Biol. 33: 605-614.

- Kato, T., Maeda, Y., Kirukawa, T., Namai, T. and Yoshioka, N. (1992b) Lipoxygenase activity increment in infected tomato leaves and oxidation product of α-linolenic acid and its *in vitro* enzyme reaction. Biosci Biotech Biochem. 56: 373–375.

- Kato, T., Ohta, H., Tanaka, K. and Shibata,D. (1992a) Appearance of New Lipoxygenases in Soybean Cotyledons after Germination and Evidence for Expression of a major new lipoxygenase gene. Plant Physiol. 98: 324–330.

- Keppler, L. D. and Novacky, A. (1987) The initiation of membrane lipid peroxidation during bacteria-induced hypersensitive reaction. Physiol. Mol. Plant Pathol. 30: 233-245.

- Kim, E. S., Choi, E., Kim, Y., Cho, K., Lee, A., Shim, J., Rakwal, R., Agrawal, G.K. and Han, O. (2003) Dual positional specificity and expression of non-traditional lipoxygenase induced by wounding and methyl jasmonate in maize seedlings. Plant Mol. Biol. 52: 1203–1213.

- Kiran Kumar, Y. V., Raghunathan, A., Sailesh, S., Prasad, M., Vemuri, M. C., and Reddanna, P. (1993) Differential effects of 15-HPETE and 15- HETE on BHK-21 cell proliferation and macromolecular composition. Biochim. Biophys. Acta. 1167: 102-108.

- Kishimoto, K., Matsui, K., Ozawa, R. and Takabayashi, J. (2006) ETR1-, JAR1- and PAD2-dependent signaling pathways are involved in C6-aldehyde-induced defense responses of *Arabidopsis*. Plant Sci. 171:415–423.

- Klein, B. P., King, D. and Grossman, S. (1985) Cooxidation reactions of lipoxygenase in plant systems. Adva. in Free Radical Biol and Medi. 1: 309-343.

- Knight, V. I., Wang, H., Lincoln, J. E., Lulai, E. C., Gilchrist, D. G. and Bostock R. M. (2001) Hydroperoxides of fatty acids induce programmed cell death in tomato protoplasts. Physiol. Mol. Plant Pathol. 59: 277–286.

- Koch, E., Meier, B. M., Eiben, H. G. and Slusarenko, A. (1992) A lipoxygenase from leaves of tomato (*Lycopersicon esculentum* Mill.) is induced in response to plant pathogenic pseudomonas. Plant Physiol. 99: 571–576.

- Kock, J. L. F., Strauss, C. J., Pohl, C. H. and Nigam, S. (2003) The distribution of 3-hydroxy oxylipins in fungi. Prostaglandins Other Lipid Mediat 71: 85–96.

- Koda, Y. (1992) The role of jasmonic acid and related compounds in the regulation of plant development. Int. Rev. of Cytol. 135: 155-199.

- Kolomiets, M. V., Hannapel, D. J. and Gladon, R. J. (1996) Potato lipoxygenase genes expressed during the early stages of tuberization (Accession Nos. U60200 and U60201) (PGR 96-065). Plant Physiol. 112:445.

- Kolomiets, M. V., Chen, H., Gladon, R. J., Braun, E. J. and Hannapel, D. J. (2000a) A leaf lipoxygenase of potato induced specifically by pathogen infection. Plant Physiol. 124: 1121-1130.

- Kolomiets, M. V., Hannapel, Y., Bishop, G. and Yokota, T. (2000b) Biosynthesis of hormones and elicitor molecules. In: (Eds.) Buchanan, B., Gruissem, W. and Jones, R. L. Biochem. and Mol. Biol. of Plants. American Soc. of Plant Physiol. Rockville, M. D., pp. 85-929.

- Kolomiets, M.V., Hannapel, D. J., Chen, H., Tymeson, M. and Gladon, R. J. (2001) Lipoxygenase is involved in the control of potato tuber development. Plant Cell 13: 613–626

- Komaraiah, P., Reddy, G.V., Srinivas Reddy, P., Raghavendhra, A.S., Ramakrishna, S.V. and Reddanna, P. (2003) Enhanced production of antimicrobial sequiterpenes and lipoxygenase metabolites in elicitor-treated hairy root cultures of *Solanum tuberosum*. Biotechnol. Lett. 25:593-597.

- Kuhn, H., Belkner, J., Wiesner, R. and Brash, A. R. (1990) Oxygenation of biological membranes by the pure reticulocyte lipoxygenase. J. Biol. Chem. 265: 18351-18361.

- Kuhn, H., Sprecher, H. and Brash, A. R. (1990b) On singular or dual positional specificity of lipoxygenases. The number of chiral products varies

with alignment of methylene groups at the active site of the enzyme. J. Biol. Chem. 265: 16300-16305.

- Kuhn, H., Wiesner, R., Rathmann, J. and Schewe, T. (1991) Formation of ketodienoic fatty acids by the pure pea lipoxygenase-1. Eicosanoids. 4: 9-14.

- La Camera, S., Gouzerh, G., Dhondi, S., Hoffmann, L., Fritig, B., Legrand, M. and Heitz, T. (2004) Metabolic reprogramming in plant innate immunity: the contributions of phenylpropanoid and oxylipin pathways. Immunol Reviews: 198: 267-284.

- Laemmli, U. K. (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

- Lee, K. T. and Lillard, D. A. (1997) Effects of esculetin as a lipoxygenase inhibitor in soybean extracts. J of Food Lipids. 4: 119-127.

- Lee, S. C. and Hwang, B. K. (2005) Induction of some defense related genes and oxidative burst is required for the establishment of systemic acquired resistance in *Capsicum annum*. Planta 221: 790-800.

- Leibowitz, M. E. and Johnson, M. C. (1971) Relation of lipid peroxidation to loss of cations trapped in liposomes. J Lipid Res. 12: 662–670.

- Li, W. X., Kodama, O. and Akatsuka, T. (1991) Role of oxygenated fatty acids in rice phytoalexin production. Agric. Biol. Chem. 55: 1041-1047.

- Loiseau, J., Vu, B. L., Macherel M. H. and Deunff Y. L. (2001) Seed lipoxygenases: occurrence and functions. Seed Science Research. 11 : 199–211.

- Lowry, O. H., Rosenbourgh, M. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with Folin-phenol reagent. J. Biochem. Chem. 193: 265-275.

- Lupu, R., Grossman, S. and Cohen, Y. (1980) The involvement of lipoxygenase and antioxidants in pathogenesis of powdery mildew on tobacco plants. Physiol. Plant Pathol. 16: 241-248.

- Lyr, H. and Basaniak, H. (1983) Alkenals, volatile defense substances in plants, their properties and activities. Acta Phytopathol. Acad. Sci. Hung. 18: 3–12.

- Maccarrone, M., Veldink, G. A., Agro, A. F. and Vliegenthart J. F. G. (1995) Modulation of soybean lipoxygenase expression and membrane oxidation by water deficit. FEBS letters. 371: 223-226.

- Maccarrone, M., Van Aarle, P. G. M., Veldink, G. A. and Vliegenthart, J. F. G. (1994) *In vitro* oxygenation of soybean biomembranes by lipoxygenase-2. Biochim. Biophys. Acta. 1190: 164-169.

- Maccarrone, M., Veldink, G. A. and Vliegenthart, J. F. G. (1992) Thermal injury and ozone stress affect soybean lipoxygenase expression. FEBS Letters. 309 : 225–230.

- Mack, A. J., Peterman, T. K. and Siedow, J. N. (1987) Lipoxygenase isozymes in higher plants: biochemical properties and physiological role. Isozymes Curr. Top Biol. Med. Res. 13: 127-154.

- Marcus, L., Prusky D. and Jacoby, B. (1988) Purification and characterization of avocado lipoxygenase. Phytochem. 27: 323–327.

- Mason, H. S., and Mullet, J. E. (1990) Expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding, and jasmonic acid. Plant Cell. 2: 569-579.

- Matsui, K. (1998) Properties and structures of fatty acid hydroperoxide lyase. Belg. J. Bot. 131: 50-62.

- Matsui, K., Hijiya, K., Tabuchi, Y. and Tadahiko, K. (1999) Cucumber cotyledon lipoxygenase during Postgerminative growth. Its expression and action on lipid bodies. Plant Physiol. 119: 1279–1288.

- Matsui, K., Irie, M., Kajiwara, T. and Hatanaka, A. (1992) Developmental changes in lipoxygenase activity in cotyledons of cucumber seedlings. Plant Sci. 85: 23-32.

- Matsui, K., Toyota, H., Kajiwara, T., Kakuno, T. and Hatanaka, A. (1991) Fatty acid hydroperoxide cleaving enzyme: hydroperoxide lyase from tea leaves. Phytochem. 30: 2109- 2113.

- Melan, M. A., Dong, X., Endara, M. E., Davis, K. R., Ausubel, G. M. and Peterman, T. K. (1993) An *Arabidopsis thaliana* lipoxygenase gene can be induced by pathogens, abscisic acid and methyl jasmonate. Plant Physiol. 101: 441-450.

- Melan, M. A., Enriquez, A. L. D. and Peterman, T. K. (1994) The *LOX1* gene of *Arabidopsis* is temporally and spatially regulated in germinating seedlings. Plant Physiol. 105: 385-393.

- Mene-Saffane, L., Esquerre-Tugaye, M. T. and Fournier, J. (2003) Constitutive expression of an inducible lipoxygenase in transgenic tobacco decreases susceptibility to *Phytophthora parasitica* var. *nicotianae*. Mol. Breed. 12: 271-282.

- Mita, G., Fasano, S., De Domenico, G., Perrone F., Epifani, R., Iannacone, R., Casey, R. and Santino, A. (2007) 9-lipoxygenase metabolism is involved in the almond/*Aspergillus carbonarius* interaction. J. Exp. Bot. 58: 1803-1811.

- Mo, B. and Koster, K. L. (2006) Changes in lipoxygenase isoforms during germination and early seedling growth of *Pisum sativum* L. Seed Sci. Res. 16: 97-106.

- Montillet, J. L., Agnel, J. P., Ponchet, M., Vailleau, F., Roby, D. and Triantaphylides, C. (2002) Lipoxygenase-mediated production of fatty acid hydroperoxides is a specific signature of the hypersensitive reaction in plants. Plant Physiol. Biochem. 40: 633-639.

- Mulliez, E., Leblane, J. P., Girerd, J. J., Rigaud, M. and Chottard, J. C. (1987) 5-lipoxygenase from potato tubers. Improved purification and physicochemical characteristics. Biochem. Biophys. Acta. 916: 12-23.

- Murray, J. J. and Brash, A. R. (1988) Rabbit reticulocyte lipoxygenase catalyzes specific 12(S) and 15(S) oxygenation of arachidonoyl-phosphatidylcholine. Arch. Biochem. Biophys. 265: 514-532.

- Nagarathna, K. C., Shetty, S. A., Bhat, S. G. and Shetty, H. S. (1992) The possible involvement of lipoxygenase in downy mildew resistance in pearl millet. J. Exp. Bot. 43: 1283-1287.

- Nakayama, T., Takeura, Y. and Ueda, T. (1995) Visible spectrophotometric assay, purification and molecular properties of a lipoxygenase from eggplant (*Solanum melongena* Linne) fruits. Biochem. Biophys. Res. Commun. 214: 1067-1072.

- Namai, T., Kato, T., Yamaguchi, Y. and Togashi, J. (1990) Time course alteration of lipoxygenase activity in blast infected rice leaves. Ann Phytopath Soc. Japan. 56: 26-32.

- Nelson M. J. (1988) Evidence for water coordinated to the active site iron in soybean lipoxygenase L-1. J. Am. Chem. Soc. 110: 2985–2986.

- Nelson, M. J. and. Seitz, S. P. (1994) The structure and function of lipoxygenase. Curr. Opin. Struc. Biol. 4 : 878–884

- Nickstadt, A., Thomma, B. P. H. J., Feussner, I., Kangasjarvi, J., Zeier, J., Loeffler, C., Scheel, D. and Berger, S. (2004) The jasmonate-insensitive mutant jin1 shows increased resistance to biotrophic as well as necrotrophic pathogens. Mol Plant Pathol. 5: 425–434.

- Nicolas, J., Autran, M. and Drapron, R. (1982) Purification and some properties of wheat germ lipoxygenase. J of Sci and Food Agri. 33: 365–372.

- Nigam and Lenne (1996) Groundnut in ICRISAT programmes. Around the world.
- Noehringer, D. S. and Blee, E. (2000) Lipoxygenase isoforms in elicitor-treated parsley cell suspension cultures. Biochem. Soc. Trans. 28: 827–829.
- Noverr, M. C., Erb-Downward, J. R. and Huffnagle, G. B. (2003) Production of eicosanoids and other oxylipins by pathogenic eukaryotic microbes. Clin. Microbiol. 16: 517–533.
- Nunez-Delicado, E., M. Perez-Gilabert, A., Sanchez, F and Garcia- Carmona, F. (1996) Hydroperoxidase activity of lipoxygenase : A kinetic study of isoproterenol oxidation. Biochim. Biophys. Acta 1293:17-22.
- Ocampo, C. A., Moerschbacher, B. and Grambow, H. J. (1986) Increased lipoxygenase activity is involved in the hypersensitive response of wheat leaf cells infected with avirulent rust fungi or treated with fungal elicitor. Z. Nazurforsch. 41: 559-563.
- Ohta, H., Ida, S., Mikami, B. and Morita, Y. (1986) Changes in lipoxygenase components of rice seedlings during germination. Plant Cell Physiol. 27: 911-918.
- Ohta, H., Shida, K., Morita, Y., Peng, Y. L., Furusawa, I., Shishiyama, J., Aibara, S. and Morita, Y. (1990) The occurrence of lipid hydroperoxide decomposing enzyme activities in rice and the relationship of such activities to the formation of antifungal substances. Plant Cell Physiol. 31: 1117-1122.
- Ohta, H., Shida, K., Peng, Y. L., Furusawa, I., Shishiyama, J., Aibara, S. and Morita, Y. (1991) A lipoxygenase pathway is activated in rice after infection with the rice fungus *Magnaporthe grisea*. Plant Physiol. 97: 94-98.
- Ongena, M., Duby, F., Rossignol, F., Fauconnier, M. L., Dommes, J. and Thonart, P. (2004) Stimulation of the lipoxygenase pathway is associated with systemic resistance induced in bean by a nonpathogenic *Pseudomonas strain.* Mol. Plant Microbe Interact. 17: 1009-1018.
- Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., Arpigny, J. L. and Thonart, P. (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ. Microbiol. 9: 1084-1090.
- Parchmann, S. and Muller, M. J. (1998) Evidence for the formation of dinor isoprostanes EI from α- linolenic acid in plants. J. Biol. Chem. 273: 32650-32655.
- Park, T. K. and Polacco, J. C. (1989) Distinct lipoxygenase species appear in the hypocotyl/radicle of germinating soybean. Plant Physiol. 90, 285–290.

- Pattee, H. E. and Singleton, J. A. (1977) Isolation of isomeric hydroperoxides from the peanut lipoxygenase-linoleic acid reaction. J. Am. Oil. Chem. 54: 183–185.

- Peever, T. L. and Higgins, V. J. (1989) Electrolyte leakage, lipoxygenase, and lipid peroxidation induced in tomato leaf tissue by specific and nonspecific elicitors from *Cladosporium fulvum*. Plant Physiol. 90: 867-875.

- Peng, Y. L., Shirano, Y., Ohta, H., Hibino, T., Tanaka, K. and Shibata, D. (1994) A novel lipoxygenase from rice: primary structure and specific expression upon incompatible infection with rice blast fungus. J. Biol. Chem. 269: 3755–3761.

- Porta, H. and Rocha-Sosa, M. (2002) Plant lipoxygenases. Physiological and molecular features. Plant Physiol. 130: 15-21.

- Porta, H., Elia, R., Balderas, F. and Rocha-Sosa, M. (2008) Wounding and pathogen infection induce a chloroplast-targeted lipoxygenase in the common bean (*Phaseolus vulgaris* L.). Planta 227: 363-373.

- Porta, H., Rueda-Bentez, P., Campos, F., Colmenero-Flores, J. M., Colorado, J. M., Carmona, M. J. Covarrubia, A. A. and Rocha-Sosa, M. (1999) Analysis of lipoxygenase mRNA accumulation in the common bean (*Phaseolus vulgaris* L.) during development and under stress conditions. Plant Cell Physiol. 40: 850-858.

- Prakash, T. R., Swamy, P. M., Suguna, P. and Reddanna.P. (1990) Characterization and behavior of 15-lipoxygenase during peanut cotyledonary senescence. Biochem and Biophy Res.Comm. 172: 462-470.

- Proite, K., Leal-Bertioli, S. C., Bertioli, D. J., Moretzsohn, M. C., da Silva, F. R., Martins, N. F. and Guimaraes, P. M. (2007) ESTs from a wild *Arachis* species for gene discovery and marker development. BMC Plant Biol. 7: 1-10.

- Prost, I., Dhondt, S., Rothe, G., Vicante, J., Rodriguez, M. J., Kift, N., Carbonne, F., Griffiths, G., Esquerre-Tugaye, M. T., Rosahl, S., Castresana, C., Hamberg, M. and Fournier, J. (2005) Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiol. 139: 1902-1913.

- Rabinovitch-Chable, H., Cook-Moreau, J., Breton, J. C. and Rigaud, M. (1992) Purification of lipoxygenase and hydroperoxide dehydrase in flax seeds: interaction between these enzymatic activities. Biochem. Biophys. Res. Commun. 188: 858-864.

- Raina, S. N., Kojima, T., Ogihara, Y., Singh, K. P. and Devarumuth, R. M. (2001) RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification and phylogenetic relationships in

peanut (*Arachis hypogaea* L.) cultivars and wild species. Genome. 44: 763-772.

- Rance, I., Fournier, J. and Esquerre-Tugaye, M. T. (1998) The incompatible interaction between *Phytophthora parasitica* var. *nicotianae* race O and tobacco is suppressed in transgenic plants expressing antisense lipoxygenase sequences. Proc. Natl. Acad. Sci. USA. 95: 6554-6559.

- Rangel, M., Machado, O. L., de Cunha, M. and Jacinto, T. (2002) Accumulation of chloroplast-targeted lipoxygenase in passion fruit leaves in response to methyl jasmonate. Phytochem. 60: 619-625.

- Rayapuram, C. and Baldwin, I. T. (2006) Using nutritional indices to study LOX3-dependent insect resistance. Plant Cell Environ. 29: 1585-1594.

- Reddanna, P., Whelan, J., Maddipati, K. R. and Reddy, C.C. (1990) Purification of arachidonate 5lipoxygenase from potato tubers. Methods Enzymol. 187: 268–277

- Reddanna, P., Whelan, J., Reddy, P. S. and Reddy, C. C. (1988) Isolation and characterization of 5-lipoxygenase from tulip bulbs. Biochem. Biophy. Res. Commun. 157: 1348-1351.

- Reinbothe, S., Machmudova, A., Wasternack, C., Reinbothe, C. and Parthier, B. (1992) Jasmonate induced proteins in cotton: Immunological relationship to the respective barley proteins and homology of transcripts to late embryogenesis abundant (Lea) mRNAs. J. Plant Growth Regul. 11: 7-14.

- Reynolds, P. A. and Klein, B. P. (1982) Purification and characterization of type-1 lipoxygenase from pea seeds. J. of Agricult and Food Chem. 30: 1157–1163.

- Rickauer, M., Fournier, J., Pouenat, M. L., Berthalon, E., Bottin A., Esquerre-Tugaye, M. T. (1990) Early changes in ethylene synthesis and lipoxygenase activity during defense induction in tobacco cells. Plant Physiol Biochem. 28: 647-653.

- Ricker, K. E. and Bostock, R. M. (1994) Eicosanoids in the *Phytophthora infestans* – interaction: lipoxygenase metabolism of arachidonic acid and biological activities of selected lipoxygenase products. Physiol. Mol. Plant Pathol. 44: 65-80.

- Rodriguez-Rosales, M. P., Kerkeb, L., Bueno, P. and Donaire, J. P. (1999) Changes induced by NaCI in lipid content and composition, lipoxygenase, plasma membrane H +-ATPase and antioxidant enzyme activities of tomato (*Lycopersicon esculentum Mill*.) calli. Plant Science.1 43: 143-150.

- Roopashree, S., Sridevi, A. S., Lalitha, R. G. and Appu Rao, A.G. (2006) Dual-function protein in plant defence: seed lectin from *Dolichos biflorus* (horse gram) exhibits lipoxygenase activity. Biochem. J. 395: 629–639

- Rosahl, S. (1996) Lipoxygenases in plants: their role in development and stress response. Z. Naturforsch. 51: 123-138.

- Royo, J., Leon, J., Vancanneyt, G., Albar, J. P., Rosahl, S. (1999) Antisense-mediated depletion of a potato lipoxygenase reduces wound induction of proteinase inhibitors and increases weight grain of insect pests. Proc. Natl. Acad. Sci. 96: 1146-1151.

- Royo, J., Vancanneyt, G., Perez, A. G., Sanz, C., Stormann, K., Rosahl, S. and Sanchez-Serrano, J. J. (1996) Characterization of three potato lipoxygenases with distinct enzymatic activities and different organ-specific and wound-regulated expression patterns. J. Biol. Chem. 271: 21012-21019.

- Rusterucci, C., Montillet, J. L., Agnel, J. P., Battesti, C., Alonso, B., Knoll, A., Bessoule, J. J., Etienne, P., Suty, L., Blein, J. P. and Triantaphylides, C. (1999) Involvement of lipoxygenase dependent production of fatty acid hydroperoxides in the development of the hypersensitive cell death induced by cryptogein on tobacco leaves. J. Biol. Chem. 272: 36446-36455.

- Saffert, A., Hartmann-Schreier, J., Schon, A. and Schreier, P. (2000) A dual function dioxygenase-peroxidase and NaDC oxidoreductase oxidoreductase active enzyme from germinating pea rationalizing –oxidation of fatty acids in plants. Plant Physiol. 123: 1545-1552.

- Sagaram, U. S., Komomiets, M. and Shim, W. B. (2006) Regulation of fumonisin biosynthesis in *Fusarium verticillioides*-Maize system. Plant Pathol. J. 22: 203-210.

- Sailaja, P. R., Podile, A. R. and Reddanna, P. (1997) Biocontrol strain of *Bacillus subtilis* AF1 rapidly induces lipoxygenase in groundnut (*Arachis hypogaea* L.) compared to crown rot pathogen *Aspergillus niger*. Euro J. Plant Pathol. 104: 125-132.

- Sanders, T. H., Pattee, H. E. and Singleton, J. A. (1975) Lipoxygenase isozymes of peanut. Lipids. 11: 681-685.

- Sandhu, S. S., Mazzafer, P., Azini, L. E., Bastos, C. R., Colombo, C. A. (2007) Lipoxygenase activity in Brazilian rice cultivars with variable resistance to leaf blast disease. Bragantia Campinas. 66: 27-30.

- Santino, A., Gallo, A.D.P., Quarta, A., Casey, R. and Mita, G. (2003) Biochemical and molecular characterization of hazelnut (*Corylus avellana*) seed lipoxygenases. Euro. J. Biochem. 270: 4365-4375.

- Santino, A., Iannacone, R., Hughes, R., Casey, R. and Mita, G. (2005) Cloning and characterization of an almond 9-lipoxygenase expressed early during seed development. Plant Sci. 168: 699-705.
- Sanz, L. C., Perez, A. G., Rios, J. J. and Olias, J. M. (1993) Positional specificity of ketodienes from linoleic acid aerobically formed by lipoxygenase isozymes from kidney bean and pea. J. of Agril. and Food Chem. 41: 696-699.
- Saravitz, D. M. and Siedow, J. N. (1992) The differential expression of wound inducible lipoxygenase genes in soybean leaves. Plant Physiol. 110: 287-299.
- Saravitz, D.M. and Siedow, J.N. (1996) The differential expression of 830-838 wound-inducible lipoxygenase genes in soybean leaves. Plant Physiol. 110: 287-299
- Seijo, J. G., Lavia, G. I., Fernandex, A., Krapovickas, A., Ducasse, D. and Moscone, E. A. (2004) Physical mapping of the 5S and 18S-25S rRNA genes by FISH as evidence that *Arachis durannensis* and *A. ipaensis* are the wild diploid progenitors of *A. hypogaea* (Leguminosae). Am. J. Bot. 91: 1294-1303.
- Sekiya, K., Okuda, H. and Arichi, S. (1982) Selective inhibition of platelet lipoxygenase by esculetin. Biochim Biophys Acta. 713: 68-72.
- Sembdner, G. and Parthier, B. (1993) The biochemistry and physiological and molecular actions of jasmonates. Ann. Rev. Plant Physiol. Plant Mol. Biol. 44: 569-589.
- Serhan, C., Anderson, P., Goodman, E., Dunham, P. and Weissmann, G. (1981) Phosphatidate and oxidized fatty acids are calcium ionophores. J. Biol. Chem. 256: 2736-2741.
- Shah, J. (2005) Lipids, lipases and lipid-modifying enzymes in plant disease resistance. Annu. Rev. Phytopathol. 43: 229-260.
- Shalata, A. and Tal, M. (1998) The effect of salt stress on lipid peroxidation and antioxidants in the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. Physiol. Plant. 104: 169-174.
- Shibata, D. (1996) Plant lipoxygenase genes. In: Piazza, G. J. (Ed.) Lipoxygenase and lipoxygenase pathway enzymes. AOCS Press, Champaign, II, pp. 39-55.
- Shibata, D. and Axelrod, B. (1995) Plant lipoxygenases. J. Lipid Mediat. Cell Signal. 12: 213-228.

- Shibata, D., Slusarenko, A., Casey, R., Hildebrand, D. and Bell, E. (1994) Lipoxygenases. Plant Mol. Biol. Rep. 12: 41-42.

- Shimizu T., Honda Z. I., Miki I., Seyama Y., Izumi T., Radmark O., Samuelsson B. Potato arachidonte 5-lipoxygenase: purification, characterization, and preparation of 5(*S*)-hydroperoxyeicosatetranoic acid. Methods Enzymol. 1990;187:296–307.
- Shimizu, T., Honda, Z. I., Miki, I., Seyama, Y., Izumi, T., Radmark, O. and Samuelsson, B. (1990) Potato arachidonte 5-lipoxygenase: purification, characterization, and preparation of 5(*S*)-hydroperoxyeicosatetranoic acid. Methods Enzymol. 187: 296–307.

- Shimizu, T., Radmarmk, O. and Samuelsson, B. (1984) Enzyme with dual lipoxygenase activities catalyses leukotriene A4 synthesis from arachidonic acid. Proc. Natl. Acad. Sci. 81: 689-693.

- Shimura, M., Mose, S. and Iwata, M. (1983) Anti-conidial germination factors induced in the presence of probenazole in infected host leaves: structural elucidation of substances A and C. Agricul Biologi Chem. 47: 1983-1989.

- Siedow, J. N. (1991) Plant lipoxygenases: structure and function. Ann. Rev. Plant Physiol. Plant Mol. Biol. 42: 145-188.

- Siedow, J. N. and Girvin, M. E. (1980) Alternative respiratory pathway. Its role in seed respiration and its inhibition by propyl gallate. Plant Physiol. 65: 669-674.

- Silva, H. S. A., Romeiro, R. D. S., Macagnan, D.N., Halfeld-Vieira, B. D. A., Pereira, M. C. B. and Mounteer, A. (2004) Rhizobacterial induction of systemic resistance in tomato plants: non-specific protection and increase in enzyme activities. Biol. Control. 29: 288-295.

- Singleton, J. A., Pattee, H. E. and Nelson, M. S. (1978) Factors affecting product specificity of peanut lipoxygenase. JAOCS. 55: 387-390.

- Skorzynska-Polt, E. and Krupa, Z. (2003) The activity of lipoxygenase in *Arabidopsis thaliana* (L.) Heynh – a preliminary study. Cell Mol. Biol. Lett. 8: 279-284.

- Slusarenko, A. J. (1996) The role of lipoxygenase in plant resistance to infection. In: (Ed.) Piazza, G. Lipoxygenase and lipoxygenase pathway enzymes. AOCS Press, Champaign, IL, pp. 176-197.

- Somashekaraiah, B. V., Padmaja, K. and Prasad, A. R. K. (1992) Phytotoxicity of cadmium ions on germinated seedlings of mung bean (*Phaseolus vulgaris*): involvement of lipid peroxide in chlorophyll degradation. Physiol. Plant. 85: 85-89.

- Srinivas Reddy, P., Charles Kumar, T., Narsa Reddy, M., Sarada, C. and Reddanna, P. (2000) Differential formation of octadecadienoic acid and octadecatrienoic acid products in control and injured/infected potato tubers. BBA Mol and Cell Biol of Lipids. 1483: 294-300.

- Stalker, H. T., Shew, B. B., Garcia, G. M., Beute, M. K., Barker, K. R., Holbrook, C. C., Noe, J. P. and Kochert, G. A. (1995) Meloidogyne arenaria resistance in advanced-generation *Arachis hypogaea* X *A. cardenasii* hybrids. Proc. of the Amer. Peanut Res. And Edu. Soc. 27:24

- Staswick, P. E. (1992) Jasmonate, genes and fragrant signals. Plant Physiol. 99: 804-807.

- Stebbins, G. L. (1957) Variation and evolution in plants. Univ. Press. Columbia, New York.

- Sunitha, M., Mattihias, W., Ivanov, I., Hammarstro, S., Furstenberger, G., Krieg, P., Reddanna, P. and Kuhn, H. (2005) Sequence determinants for the reaction specificity of murine (12R)-Lipoxygenase targeted substrate modification and site-directed mutagenesis. J. Biol. Chem. 280: 36633-36641.

- Suzuki, Y. and Matsukura, U. (1997) Lipoxygenase activity in maturing and germinating rice seeds with and without lipoxygenase-3 in mature seeds. Plant Sci. 125: 119-126.

- Tamas, L., Dudikova, J., Durcekova, K., Haluskova, L., Huttova, J. and Mistrik, I. (2009) Effect of cadmium and temperature on the lipoxygenase activity in barley root tip. Protoplasma. 235: 17-25.

- Terp, N., Gobel, C., Brandt, A. and Feussner, I. (2006) Lipoxygenases during *Brassica napus* seed germination. Phytochem. 67: 2030-2040.

- Thomas, H. and Stoddart, J. L. (1980) Leaf senescence. Annu. Rev. Plant Physiol. 31: 83-111.

- Tizio, R. (1996) Jasmonic acid and their derivatives as plant growth regulators. Biocell. 20: 1-10.

- Todd, J. F., Paliyath, G. and Thompson, J. E. (1992) Effect of chilling on the activities of lipid degrading enzymes in tomato fruit microsomal membranes. Plant Physiology and Biochemistry. 30: 517–522.

- Tranbarger, T. J., Franceschi, V. R., Hildebrand, D. F. and Grimes, H. D. (1991) The soybean 94-kilodalton vegetative storage protein is a lipoxygenase that is localized in paraveinal mesophyll cell vacuoles. Plant Cell. 3: 973–987

- Tsitsigiannis, D. I., Kowieski, T. M., Zarnowski, R. and Keller, N. P (2004) Endogenous lipogenic regulators of spore balance in *Aspergillus nidulans*. Eukaryot Cell. 3: 1398–1411.

- Tsitsigiannis, D. I., Kunze, S., Willis, D. K., Feussner, I. and Keller, N. P. (2005) *Aspergillus* infection inhibits the expression of peanut 13S-HPODE-forming seed lipoxygenases. Mol. Plant Microbe Interact. 10: 1081-1089.

- Tsitsigiannis, D. L. and Keller, N. P. (2006) Oxylipins act as determinants of natural product biosynthesis and seed colonization in *Aspergillus nidulans*. Mol. Microbiol. 59: 882-892.

- Uma Maheswari Devi, P., Srinivas Reddy, P., Usha Rani, N. R., Reddy, K. J., Narsa Reddy, M. and Reddanna, P. (2000) Lipoxygenase metabolites of α-linolenic acid in the development of resistance in pigeonpea, *Cajanus cajan* (L.) Millsp. seedlings against *Fusarium udum* infection. Euro. J. of Plant Pathol. 106: 857-865.

- Vaughn, S. F. and Lulai, E. C. (1992) Further evidence that lipoxygenase activity is required for arachidonic acid-elicited hypersensitivity in potato callus cultures. Plant Sci. 84: 91-98.

- Vick, B. A. and Zimmerman, D. C. (1976) Lipoxygenase and hydroperoxide lyase in germinating watermelon seedlings. Plant Physiol. 57: 780-788.

- Vick, B. A. and Zimmerman, D. C. (1982). Levels of oxygenated fatty acids in young corn and sunflower plants. Plant Physiol. 69:1103-8

- Vick, B. A. and Zimmerman, D. C. (1987) Oxidative systems for the modification of fatty acids: the lipoxygenase pathway. In: (Eds.) Stumpf. P. K. and Conn, E. E. The Biochemistry of Plants, Vol. 9, Academic Press, New York, pp. 53-90.

- Wallace, J. M. and Wheeler, E. L. (1979) Two lipoxygenase isoenzymes and an activator in wheat germ. Phytochem. 18: 389- 393.

- Wang, C., Croft, K. P. C., Jarlfors, U. and Hildebrand, D. F. (1999) Subcellular localization studies indicate that lipoxygenases 1 to 6 are not involved in lipid mobilization during soybean germination. Plant Physiol. 120: 227-235.

- Wang, H., Li, J., Bostock, R.M. and Gilchrist, D.G. (1996) Apoptosis: A Functional Paradigm for Programmed Plant Cell Death Induced by a Host-Selective Phytotoxin and Invoked during Development. Plant Cell. 8: 375–391.

- Weber, H., Chetelat, A., Caldelari, D. and Farmer, E. E. (1999) Divinyl ether fatty acid synthesis in late blight-diseased potato leaves. Plant Cell. 11: 485-493.

- Weichert , H., Kolbe, A., Kraus, A., Wasternack, C. and Feussner, I. (2002) Metabolic profiling of oxylipins in germinating cucumber seedlings –

lipoxygenase-dependent degradation of triacylglycerols and biosynthesis of volatile aldehydes. Planta. 215: 612–619.

- Weidhase, R. A., Kramell, H., Lehmann, J., Liebisch, W., Lerbs, W. and Parthier, B. (1987) Methyl jasmonate- induced changes in the polypeptide pattern of senescing barley leaf segments. Plant Sci. 51 : 177–186.

- Wheeler, E. L. and Berry, D. L. (1986) *In vitro* inhibition of mouse epidermal cell lipoxygenase by flavonoids: structure-activity relationships. Carcinogenesis, 7, 33-36.

- Wilson, R. A., Gardner, H. W. and Keller, N. P. (2001) Cultivar-dependent expression of a maize lipoxygenase responsive to seed infesting fungi. Mol. Plant Microbe Interact. 14: 980-987.

- Wynne, J. C., Bente, M. K. and Nigam, S. N. (1991) Breeding for disease resistance in peanut (*Arachis hypogaea* L.). Annu Rev. Phytopathol. 29: 279-303.

- Xu, Y., Chang, P. L., Liu, D., Narasimhan, M.l., Roghothama, K.G., Hasegawa, P.M. and Bessan, R.A. (1994) Plant defence genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell. 6: 1077-1085.

- Yamamoto, H. and Tani, T. (1986) Possible involvement of lipoxygenase in the mechanism of resistance of oats *Avena sativa* to *Puccinia coronata* pv. *Avenae*. J. Phytopathol. 116: 329-337.

- Yamamoto, S. (1992) Mammalian lipoxygenases: Molecular structures and functions. Biochimica. Biophy. Acta. 1128: 117-131.

- Yamarnoto, H. and Tani, I. (1986) Possible involvement of lipoxygenase in the mechanism of resistance to oats to *Puccinia coronata* avenae. J. Phytopathol. 116: 329-337.

- Yang, G. S., Schwarz, P. B. and Vick, B. A. (1993) Purification and characterization of lipoxygenase isoenzymes in germinating barley. Cereal Chem. 70: 589-595.

- Zamora, R., Olias, J. M. and Mesias, J. L. (1987) Purification and characterization of tomato lipoxygenase. Phytochem. 26: 345-347.

- Zeringue, H. J., Brown, R. L., Neucere, J. N. and Cleveland, T. E. (1996) Relationships between C_6–C_{12} alkanal and alkenal volatile contents and resistance of maize genotypes to *Aspergillus flavus* and aflatoxin production. J. Agric. Food Chem. 44 : 403–407.

- Zeringue, H. J. (1992) Effects of C6–C10 alkenals and alkanals on eliciting a defence response in the developing cotton boll. Phytochem. 31: 2305 2308.

- Zimmerman, D. C. and Coudron, C. A. (1979) Identification of traumatin, a wound hormone, as 12-oxo-trans-10-dodecenoic acid. Plant Physiol. 63: 536–541.