CHARACTERIZATION OF LOX GENE AND POLYAMINES IN CASTOR (*Ricinus communis* L.) DURING WILT PATHOGEN INTERACTION

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ABSTRACT

CHARACTERIZATION OF LOX GENE AND POLYAMINES IN CASTOR (*Ricinus communis* L.) DURING WILT PATHOGEN INTERACTION

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ABSTRACT

India is the largest producer of castor, contributing to almost 65% of the world share. However, wilt caused by *Fusarium oxysporum* f. sp *ricini* is the major constrain for higher production. The molecular bases for the recognition of pathogens by plants outside the purview of gene-for-gene systems are still elusive. Therefore, in the present investigation the role of lipoxygenase gene and polyamines was studied at molecular and biochemical level in the resistant and susceptible genotypes of castor at 0 days after infection (DAI), 5 DAI and 10 DAI (30 days after sowing) in wilt pathogen infected and non infected tissues. The constitutive level of lipid peroxidation product (MDA content) was higher in susceptible genotypes (VP-1 and VI-9), while induced level was higher in resistant genotypes (48-1 and SKP-84) at both the stages of analysis. Polyamine profiling using HPTLC showed higher spermidine and spermine content in resistant genotypes at 10 DAI. Furthermore, spermidine was detected only in the roots of resistant genotypes at 10 DAI. The lipoxygenase (LOX) and polyamine oxidase (PAO) activities were higher in the incompatible interaction at all the stages of analysis. Sequencing of 6 LOX genes (LOX1, 2, 3, 4, 5 and DOX) were performed and results showed that except LOX1, all the genes had more than 90 % identity with LOX genes on NCBI-BLAST. Only LOX5 protein contains conserved His (Histidine) residues (positions 547, 556, and 715) that are also observed in other plant LOXs. Expression analysis was carried out using RT-PCR

with LOX2, 3, 4, 5 and DOX gene specific primers. Resistant genotypes (48-1 and SKP-84) exhibited appreciably higher expression of LOX5 at 5 DAI, which is responsible for defense mechanism. These results suggest the role of high titers of polyamines, LOX and PAO in disease resistance possibly through HR induction.

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C E R T I F I C A T E

This is to certify that the thesis entitled **Characterization of LOX gene and Polyamines in Castor (*Ricinus communis* L.) During wilt pathogen interaction** submitted by Mr. Mhaske Somnath Dadaso in partial fulfillment of the requirement for the award of the degree of MASTER OF SCIENCE (Agriculture) in PLANT BIOTECHNOLOGY of the Navsari Agricultural University is a record of bona fide research work carried by his under my guidance and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Place: Navsari

Date: 1 Dec. 2011

Major Advisor

DECLARATION

This is to declare that the whole of the research work reported in the thesis for partial fulfillment of the requirements for the degree of **Master of Science (Agriculture)** in **Plant Biotechnology** by the undersigned is the results of investigation done by his under direct guidance and supervision of **Dr. Mahesh Kumar Mahatma**, Assistant professor, Department of Biotechnology, N.M. College of Agriculture, Navsari Agricultural University, Navsari and that no part of the work has been submitted for any other so far.

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Dedicated to

My Beloved Parents

&

Major Advisor

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(S. D. Mhaske)

List of Abbreviations:

µl	Microlitre
µM	Micromolar
µm	Micrometer
Bp	base pair(s)
BSA	Bovine serum albumin
BLAST	Basic local alignment search tool
Cm	Centimeter
DAI	Days after infection
DAS	Days after sowing
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribo nucleic acid
dNTP	2'-deoxynucleotide-5'triphosphates
EC	Enzyme classification
EDTA	Ethylene diamine tetra acetic acid
GABA	γ- aminobutyric acid
G	Gram
H	hour(s)
hai	hour after inoculation
HPTLC	High performance Thin layer chromatography
M	Molar
mcg or µg	Microgram
min	minute(s)
ml	Milliliter
mM	Millimole
NCBI	National centre for biotechnology information
ng	Nanogram
nm	Nanometer
OD	Optical density
PCR	Polymerase chain reaction
ROS	Reactive oxygen species
rpm	revolutions per minute
rRNA	Ribosomal Ribonucleic acid
RT	Room temperature
RT PCR	Real Time Polymerase Chain Reaction
S	second(s)
TBE	Tris Boric acid
TBARS	Yhiobarbituric acid reactive substances
TCA	Tricarboxylic acid
TMV	Tobacco mosaic virus
Tris	Tris (hydroxymethyl) aminomethane buffer
U/g	Units/gram
UV	Ultra violet
FAGB	Formaldehyde agarose gel buffer
DEPC	Diethyl pyrocarbonate
ADC	Arginine decarboxylase

SAMDC	S- adenosylmethionine decarboxylase
PEG	Polyethylene glycol
HCA	Hydroxycinnamic acid
PAMPs	pathogen-associated molecular patterns
PRR	pathogen recognition receptor
Put	Putrescine
Cad	Cadaverine
Spd	Spermidine
Spm	Spermine
MJ	Methyl jasmonate
ISR	Induced systemic resistance
SA	Salicylic Acid
NOX	NADPH oxidase
AOs	Amine oxidases
PAs	Polyamines
ODC	Ornithine decarboxylase
W	Watt
w/v	weight/volume
ε	Enzyme coefficient

Note: The full forms of several rarely used abbreviations have been described within the text.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX

CHAPTER 1

INTRODUCTION

I. INTRODUCTION

Castor (*Ricinus communis* L.) is the industrially important non edible oilseed crop belongs to family *Ephorbiaceae* with chromosome number 2n=20. The major castor growing countries are India, China and Brazil and Russia. India ranks first with respect to area and second with respect to productivity. Total area under castor crop in India for the year 2009-10 is 7.40 lakh hectares with estimated total production of 9.34 lakh tonnes (Nielsen India estimates). In India Gujarat is leading castor growing state, which produces 82 percent of total production in India. The major castor growing states in India are Gujarat, Rajasthan, Andhra Pradesh, Karnataka, Orissa and Tamil Nadu. Castor crop is affected by several biotic and a biotic stresses, which affect successful and profitable cultivation of the crop. Among them, diseases play an important role to cause severe yield losses. Castor wilt (*Fusarium oxysporum* f. sp. *ricini*), is a serious problem in Gujarat state and causes heavy losses. Up to 80 per cent wilt incidence was reported in North Gujarat (Patel *et al.*, 2003). Monocropping has been followed due to its high economical return, which resulted to the endemic development of wilt and wilt has become a limiting factor for castor cultivation in the state (Dange *et al.*, 1997).

Cultivars resistant to this fungus are the most practical way to control this disease. Both compatible and incompatible responses induce alterations in plant metabolism; only in the latter the plant is able to efficiently block pathogen penetration without suffering excessive damage.

The molecular bases for the recognition of pathogens by plants outside the purview of gene-for-gene systems are still elusive. Plants usually recognize pathogen-associated molecular patterns (PAMPs) in the form of chitin, glucan fragments or pathogen recognition receptor (PRR) proteins. After pathogen recognition, a multitude of plant resistance-associated reactions are initiated, such as ion fluxes across plant membranes, the generation of reactive oxygen species (ROS), phosphorylation of specific proteins, activation of cell wall strengthening enzymes, transcriptional activation of several defense related genes, induction of phytoalexins, localized cell death at infection sites (HR response), and induction of systemic acquired resistance in distal

plant organs (Gupta *et al.*, 2010). Hypersensitive cell death appears to not be the result of the direct action of released pathogenic factors but is rather under the genetic control of the host.

One ultimate characteristic of HR is the loss of membrane integrity, and thus HR is often characterized by an associated electrolyte leakage and lipid peroxidation accompanied by the enzyme lipoxygenase (LOX). Initiation of HR membrane damage by LOXs has been suggested as an alternative hypothesis to free radical action, and the process might be propagated by autoxidation (Rusterucci *et al.*, 1999). Oxylipins and other products of lipoxygenase play diverse roles in plant biology as signal molecules for expression of defense related genes or as antimicrobial compounds (Marmey *et al.*, 2007). LOX gene expression was investigated in several plant-pathogen interactions and LOX transcripts were shown to accumulate in rice plants upon inoculation with the fungus M. grisea (Peng *et al.*, 1994), and in the tobacco- *Phytophthora parasitica nicotianae* interaction (Veronesi *et. al.*, 1996).

In addition, polyamine catabolism also contribute to defense responses through two reaction products : γ- aminobutyric acid (GABA), an important metabolite, largely and rapidly produced in response to biotic and abiotic stresses (Cona *et al.*, 2006) and the reactive oxygen speciës, H_2O_2, which has a long been recognized to play a key role in defense (Gechev *et al.*, 2006). H_2O_2 is a mediator of several phys iological events such as programmed cell death, lignifications and wall stiffening. It has been suggested that PAO is important in producing H_2O_2 in vivo in the above mentioned events, during cell growth and differentiation and host-pathogen interaction (Cervelli *et al.*, 2001). To cope with various stress conditions, the expression of stress- associated genes leading to the formation of a wide variety of low molecular weight metabolites like mannitol, proline, glycine betaine and polyamines are also implicated. In recent past polyamines considered as antioxidant molecule and inhibit lipid peroxidation in rat liver microsomes and in phospholipid vesicles. Polyamines have also been reported as direct free radical scavengers or to function as scavengers by interacting with other molecules (Benavides *et al.*, 2000). In general, polyamine metabolism has long been known to distort in plant cells responding to insightful changes in plants interacting with fungal and viral pathogens (Walters, 2003). The correlation between lipoxygenase and polyamines at

molecular and biochemical level is still unclear in Castor plants during disease conditions. Therefore, present investigation is planned to understand the role of lipoxygenase and polyamines with following objectives.

1. To study the expression of lipoxygenase gene during host- pathogen interaction.
2. To access lipoxygenase enzymes and polyamines during host- pathogen interaction.
3. To establish correlation between lipoxygenase and polyamines during host- pathogen interaction.

CHAPTER 2

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

2. The castor plant:

Although commonly referred to as a "bean," castor is not a legume. It belongs to family *Ephorbiaceae* and the chromosome number of castor is 2n=20. The plant has also been called the "castor oil plant." Castor oil, one of the oldest commercial products, was used in lamps by the Egyptians more than 4,000 years ago, and seeds have been found in their ancient tombs. Castor is considered by most authorities to be native to tropical Africa, and may have originated in Abyssinia (Weiss, 1971). Castor was in production as early as the mid-1850s in the central part of the United States, and over twenty three crushing mills reportedly were operational at that time (Zimmerman, 1958).

2.1 Uses:

Production of castor (*Ricinus communis* L., *Euphorbiaceae*) is needed in the United States to supply castor oil for the hundreds of products using this versatile chemurgic raw material. Forty to forty five thousand tonnes of castor oil and derivatives are imported each year (Roetheli *et al.,* 1991) to supply the entire needs of our domestic industries. The United States is the largest importer and consumer of castor oil in the world.

In the United States, castor oil has been used by the military in aircraft lubricants, hydraulic fluids, and in the manufacture of explosives. It has also been used in the synthesis of soaps, linoleum, printer's ink, nylon, varnishes, enamels, paints, and electrical insulations. Textile scientists have used sulphonated castor oil in the dyeing and finishing of fabrics and leather. The most infamous application of castor oil may have been as a purgative popular for the treatment or prevention of many ailments in the first half of the twentieth century (Oplinger *et al.,* 1990).

2.2 Floral biology:

Flowers occur most of the year in dense terminal clusters (inflorescences), with female flowers just above the male flowers. The upper spiny balls (ovaries) with red,

star-shaped stigmas are the female flowers. The lower male buds open into whitish-yellow clusters of stamens. The wind-pollinated flowers have no petals.

The flowers of castor bean are borne near the top of the plant in panicles. But lacking petals they are not especially noteworthy. As the seed mature, the three-celled, spiny capsules turn bright red on foot long panicles and make an interesting distraction to the bold foliage (Gerald, 2002).

Table 2.1: Proximate composition of Castor Seed.

The proximate physico-chemical composition of castor seed is as follows (Lakshminarayana *et al.,* 1984).

Sr.No.	Characters	Content
1	100-seed weight	15.2–30.2 g
2	100-seed volume	15.0–32.5 ml
3	Kernel	64–75%
4	Oil	46.0–51.8%
5	Protein	17.1–24.4%
6	Crude fiber	18.2–26.5%,
7	Ash	2.1–3.4%
8	Oil characteristics, acid value	1.0–2.9
9	Saponification value	176.2–183.7
10	Iodine value	81.4–88.1
11	Hydroxyl value	159.2–167.1
12	Ricinoleic acid content varied from	87.4% to 90.4%

2.3 WILT:

In India, castor wilt was recorded for the first time in 1974 from Udaipur (Rajasthan) by Nanda and Prasad (1974) and causal organism was established as *Fusarium oxysporum* f. sp. *ricini*. The pathogenicity was proved by soil inoculation around the roots of 4 to 6 weeks old castor seedlings. They observed wilt symptoms after 10 days of inoculation and plants died seven days later. They also described the symptoms on leaves as yellowing, sickly appearance and marginal necrosis, which later

advance to interveinal areas and cover them completely. Leaves shrivel and first lower leaves drop down leaving few top leaves, which is followed by irreversible wilting of the plant leading to sudden death. Transverse and longitudinal section of the affected roots revealed presence of fungus in vascular tissue and in xylem parenchyma. Formation of tyloses was also observed in infected roots. Plants were susceptible at all growth stages. However, disease generally appears in the months of October to November, when the crop is about three to four months old and become more prominent during February to March when the crop is in seed formation stage. Initially the disease generally appears in patches in the field.

Andreeva (1979) from Krasnodar, U.S.S.R., reported occurrence of wilt of castor and causal organism was identified as *Fusarium oxysporum* f. sp. *ricini* based on the morphological characters and narrow host specialization. The disease was later recorded from Gujarat state during 1980-81 from Sardarkrushinagar (DOR, 1981).

2.4 BIOCHEMICAL BASIS OF DISEASE RESISTANCE:

Plants have developed elaborate mechanisms to defend themselves against attack by pathogens. Only a small number of pathogens are able to provoke disease in a certain species or cultivar (compatible response), whereas most potential aggressors are recognized and blocked in their penetration by plant defenses (incompatible response). Plants possess physical barriers, such as the cuticle and cell wall and a number of biochemical and molecular mechanisms to counteract pathogen attacks.

2.4.1 ENZYME ACTIVITY:

2.4.1.1 Enzymes related to reactive oxygen species (ROS) generation

The production of reactive oxygen species (ROS) is a key event in HR. Several enzymatic systems have been proposed to be responsible for the oxidative burst characterizing HR (Gara *et al.*, 2003). Reactive oxygen species (ROS) are versatile molecules mediating a variety of cellular responses in plant cells, including programmed cell death (PCD), development, gravitropism, and hormone signaling (Kwak *et al.*, 2006). Upon recognition of pathogens, plants activate a battery of defense responses, including the oxidative burst, the hypersensitive response (HR), cell wall fortification,

and defense-related protein synthesis (Hammond-Kosack and Jones, 1996). One of the most rapid defense reactions to pathogen attack is the oxidative burst, which leads to the transient production of large amounts of reactive oxygen species (ROS), including superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (•OH). The rapid generation of O_2^- or its dismutation product H_2O_2 at the onset of the HR has been documented in many plant-pathogen interactions (Grant and Loake, 2000). ROS produced in the oxidative burst not only protect against invading pathogens but also function as signaling molecules to activate plant defense responses (Tenhaken *et al.*, 1995). During the defense response in plant cells, ROS can inhibit pathogens by strengthening host cell walls via the oxidative cross-linking of glycoproteins, such as the Pro-rich protein (Bradley *et al.*, 1992), or by directly killing pathogens (Levine *et al.*, 1994).

2.4.1.2 Lipoxygenase Enzyme (LOX 1inoleate: oxygen oxidoreductase, EC 1.13.11.12):

Lipoxygenase plays a role in the membrane degradation observed during senescence, wounding and the hypersensitive response to pathogen attack or upon treatment of plant and cell cultures with elicitors (Skorzynska-polit and Krupa, 2003). Some product of LOX metabolism is required to induce the HR, which is a pathogen-induced cell death process at the site of infection in an incompatible interaction that limits pathogen growth. The HR is characterized by the loss of membrane integrity and closely related to the generation of lipid peroxides and active oxygen species. It has been postulated that LOX mediated lipid oxidation is important in causing membrane damage during the HR (Porta and Rocha-Sosa, 2002). The increase in H_2O_2 level may cause pathogen destruction or is involved as a second messenger in the systemic signal network of plant cells (Alvarez *et al.*, 1998).

The hydroperoxy polyunsaturated fatty acids, synthesized by the action of various highly specialized forms of lipoxygenases, are substrate 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 (Feussner and Wasternack, 2002). They are involved in diverse processes of plant growth and development.

Increased lipoxygenase activity has been reported in dicots and monocots after infection with pathogenic microorganisms and viruses e.g., in tobacco infected with tobacco mosaic virus (Ruzicska *et al.*, 1983), in potato infected with *Rhizoctonia soloni* (Reddy *et al.*, 1992), in tomato infected with powdery mildew (Kato *et al.*, 1992), in *Pseudomonas syringae* – inoculated bean (Croft *et al.*,1990), tomato (Koch *et al.*, 1992) and *Arabidopsis thaliana* (Melan *et al.*, 1993), in wheat infected with *Puccinia graminis* (Ocampo *et al.*, 1986) and in rice infected with *Manaporthe griesea* (Ohta *et al.*, 1991). In most cases, a correlation between induction of LOX activity and resistance of the plant has been shown (Devi *et al.*, 2000). Rusterucci *et al.*, (1999) investigated lipid peroxidation in relation with the hypersensitive reaction in cryptogein-elicited tobacco leaves. A massive production of free polyunsaturated fatty acid (PUFA) hydroperoxides dependent on a 9-lipoxygenase (LOX) activity was observed during the development of leaf necrosis. The process occurred after a lag phase of 12 h, was accompanied by the concomitant increase of 9-LOX activity, and preceded by a transient accumulation of LOX transcripts. Lipoxygenase (LOX) activity was measured in germinating pigeon pea *Cajanus cajan* seedlings, resistant (ICP-8863) and susceptible (ICP-2376) to wilt fungus, before and after infection with *Fusarium udum*. LOX activity was significantly higher in the resistant than in the susceptible cultivars of pigeon pea and was enhanced further in response to infection with *Fusarium udum* (Devi *et al.*, 2000).

Lipoxygenase (LOX) activity in seedlings of pearl millet genotypes resistant and susceptible to downy mildew pathogen *Sclerospora graminicola* was recorded. An increase in LOX activity was observed during the incompatible host-pathogen interaction whereas the activity decreased in compatible ones. Resistant pearl millet seedlings exhibited a 2.4-fold increase in LOX activity after inoculation with the pathogen. The enzyme activity was maximum at 18 h after inoculation. The enzyme activity was maximum in shoot portion of resistant genotype after inoculation (Babitha *et al.*, 2006). The lipoxygenase activity was characterize in pigeonpea for response to *Fusarium udum* infection with four known resistant genotypes, viz. ICP 87119, ICP 8863, BDN 2 and BSMR 736 have shown consistently higher lipoxygenase (LOX) activity in comparison

to susceptible genotypes viz. Bahar and TTB 7 (Mandal and Sinha, 2009). Mariutto *et al.,* (2011) analyzed the activities of phenylalanine ammonia-lyase (PAL) and lipoxygenase (LOX), key enzymes of the phenylpropanoid and oxylipin pathways respectively, in tomato treated or not with *Pseudomonas putida* BTP1.The bacterial treatment did not stimulate PAL activity and linoleate-consuming LOX activities. Linolenate-consuming LOX activity, on the contrary, was significantly stimulated in *P. putida* BTP1-inoculated plants before and two days after infection by the fungal pathogen *Botrytis cinereaon* causing gray mold disease of leaves. This stimulation is due to the increase of transcription level of two isoforms of LOX: TomLoxD and TomLoxF, a newly identified LOX gene. They showed that recombinant TomLOXF preferentially consumes linolenic acid and produces 13-derivative of fatty acids. After challenging with *B. cinerea*, the increase of transcription of these two LOX genes and higher linolenic acid-consuming LOX activity were associated with a more rapid accumulation of free 13-hydroperoxy-octadecatrienoic and 13-hydroxy-octadecatrienoic acids, two antifungal oxylipins, in bacterized plants.

2.4.1.3 Polyamine oxidase (PAO) EC 1.5.3.3

The best-known enzyme that catabolizes higher polyamine (Pas), generating hydrogen peroxide (H_2O_2) and reducing intracellular PA titers, is polyamine oxidase. PAOs are localized to peroxisomes and in the apoplast (Moschou *et al.,* 2008). Resulting H_2O_2 plays a role in mediating a complex array of defense responses to microbial pathogens. Furthermore, polyamine derived H_2O_2 has been implicated in cell-wall maturation and lignification during development as well as in wound-healing and cell-wall reinforcement during pathogen invasion (Takahashi and Jun-Ichi, 2010).

Increased activities of copper-containing amine oxidases or polyamine oxidases were reported in barley (*Hordeum vulgare*) seedlings during HR in response to powdery mildew (Cowley and Walters, 2002). Using Tobacco (*Nicotiana tabacum*) mosaic virus (TMV) and intact tobacco cultivars carrying the resistant (N) gene, Yoda *et al.,* (2003) demonstrated that polyamines are indeed one of the sources of hydrogen peroxide during HR. Upon HR induction, polyamines accumulate in apoplasts of tobacco cell and are degraded by polyamines oxidase. Mosohou *et al.,* (2009) reported increased PAO gene

and corresponding PAO enzyme activities in wild-type tobacco (*Nicotiana tabacum 'Xanthi'*) plants, infected by the compatible pathogen *Pseudomonas syringae* pv *tabaci*. Polyamine homeostasis was maintained by induction of the arginine decarboxylase pathway and spermine was excreted into the apoplast, where it was oxidized by the enhanced apoplastic PAO, resulting in higher hydrogen peroxide accumulation. Moreover, plants overexpressing PAO showed pre-induced disease tolerance against the biotrophic bacterium *P. syringae* pv *tabaci* and the hemibiotrophic oomycete *Phytophthora parasitica* var *nicotianae*.

2.5 METABOLITES CONSTITUENT:

2.5.1 Malondialdehyde (MDA), a lipid peroxidation product:

Toxic active oxygen species determine metabolic alterations in cellular membrane systems as a consequence of the peroxidation of the lipid layer of the membrane. Lipid peroxidation is the symptom most easily ascribed to oxidative damage. It occurs via initiation, propagation and termination reactions, in which activated oxygen transitional metals, and lipid hydroperoxides are involved. MDA, a breakdown product of lipid peroxidation, is the most widely measured indicator of oxidative stress (Zhang and Kirkham, 1996). Oxidative stress induces the degradation of a variety of biologically important molecules such as amino acids, proteins and carbohydrates, with the consequent release of malondialdehyde (Costa *et al*., 2002).

Malondialdehyde in rice leaves increased after inoculation by *Magnaporthe grisea*. The rate of lipid peroxidation in the incompatible interaction increased progressively during the first 5 days after inoculation. While, lipid peroxidation in the compatible interaction increased at lower rate (Ge Xiu Chan *et al*., 1998).

Antioxidant enzymes and lipid peroxidation were determined in roots and stems of Fusarium wilt resistant (WR 315) and susceptible (JG 62) genotypes of chickpea. In roots, infection by the pathogen increased lipid peroxidation and catalase and superoxide dismutase activities, although such responses occurred in the incompatible compared with the compatible interactions. In stems, infection by the pathogen increased lipid peroxidation in the compatible interaction (Garcia-Limones *et al*., 2002).

Polkowska-Kowalczyk *et al.*, (2004) observed increased lipid peroxidation only in the *Phytophthora infestans* resistant *Solanum nigrumi* genotype. The increase in lipid peroxidation in *S. nigrum* leaves was coincided with enhanced LOX activity.

Mandal *et al.*, (2008) performed an experiment to study the responses of tomato (*Solanum lycopersicum* L.) against the invading necrotrophic pathogen *Fusarium oxysporum* f. sp. *lycopersici*. They found 2.6 times higher concentration of hydrogen peroxide (H_2O_2) at 24 h post-inoculation (hpi) and 4.4 times higher content of lipid peroxidation at 72 hpi in the extracts of inoculated roots than in the control.

Dubey *et al.*, (2010) reported higher content of GSH in a leaves of downy mildew resistant genotype (Pps-1) of opium poppy at 12 h after inoculation (hai) but a transient and highly significant decrease in content of GSH and increase in content of MDA was observed at 24 hai in comparison to control plants of same genotype and also in comparison to inoculated plants of susceptible genotype (Jawahar-16).

Lipid peroxidation product (malondialdehyde content) was recorded higher in compatible interaction with downy mildew pathogen at pre-infection stage while it was increased in incompatible interaction at post-infection stage in pearl millet leaves (Mahatma *et al.*, 2011).

2.5.2 Polyamines Profiling

Polyamines such as Put, Cad, Spd, and Spm are synthesized in almost all biological systems, including higher plants and their possible role in various growth and physiological processes in plants, microbial, and animal systems has been deduced from exogenous application of polyamines as well as from changes in their endogenous biosynthesis and content. Polyamines have been implicated in a variety of plant growth and developmental processes involving cell proliferation and differentiation, morphogenesis, development and stress tolerance. Additionally, there is accumulating evidence that infections of various pathogenic fungi and viruses bring about dynamic changes in polyamine metabolism not only in the infected cells but also in other regions of the host plants (Terakado-Tonooka and Fujihara, 2008).

Greenland and Lewis, (1984) were the first to show that polyamine levels are altered by pathogen infection. Using barley infected with the biotrophic fungal pathogen *Puccinia hordei*, they found that rust infection resulted in an increase in the concentrations of spermidine to levels six to seven times that in healthy leaf tissue.

Walters *et al.*, (1985) showed that the concentrations of putrescine, spermidine and spermine increased in barley leaves infected by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei.* In work on the interaction between sugarcane and the smut fungus *Ustilago scitaminea*, Legaz *et al.*, (1998) showed that in infected leaves, there was a decrease in the concentrations of free and conjugated forms of putrescine and spermidine, and an increase in free and conjugated forms of spermine.

The concentrations of the free polyamines putrescine/agmatine (Put/Agm), spermidine (Spd), and spermine (Spm)were determined in three near-isogenic lines of the wheat cultivar Prelude differing in their resistance to the wheat stem rust fungus, *Puccinia graminis* f. sp. *tritici* (Pgt) race 32, over entire infection cycles. In highly resistant wheat plants, no changes in polyamine contents were detected. In moderately resistant and fully susceptible plants, Spm concentrations remained unchanged, while Put/Agm contents were increased over control plants between 3 and 7 days after inoculation. Spd concentrations in both interactions started to rise 3 days after inoculation, reaching a maximum at day 6 then declining in susceptible plants (Machatschke *et al.*, 1990).

Kuznetsov and shevyakova, (2007) reported that the level of free put, spd and spm and their conjugates increased markedly on the 4th day after treatment with methyl jasmonate in the barley leaves, which was accompanied by increased resistance to powdery mildew and also Polyamines increase survival of various plants under salt stress, chilling stress, osmotic, acidic stresses, radiation-induced oxidative stress and other stresses. A key polyamine biosynthetic gene arginine decarboxylase, *adc* under the control of a constitutive promoter of cauliflower mosaic virus, CaMV35S was transfer eggplant through *Agrobacterium*-mediated transformation. Polyamine-accumulating transgenic plants exhibited an increased tolerance levels to multiple abiotic stresses such

as salinity, drought, low and high temperature, and heavy-metal and resistance against fungal wilt disease caused by *Fusarium oxysporium*. (Prabhavathi and Rajam, 2007).

The barley leaves were infected with fungal pathogen *Puccinia hordei,* which surround the infection sites of rust and powdery mildew fungi. Spd level in this tissue were enhanced 6-7 fold compared to healthy control. The barley was infected by powdery mildew fungus *Blumeria graminis* f. sp. *Hordei* and observed change of polyamines content was concomitant with increased activities of ADC, ODC and SAMDC, also in wheat leaves infected with black stem rust and observed increased free and conjugated Put and Spd levels, as well as enhanced ADC and ODC activities (Xiao-Ming Pang *et al.*, 2007).

2.6 MOLECULAR BASIS OF DISEASE RESISTANCE:

A cDNA clone of tobacco *(Nicotiana tabacum* L.) lipoxygenase (LOX) was used to study LOX gene expression in tobacco cell-suspension cultures and intact plants in response to infection with *phytophthora parasitica nicotianae (Ppn).* Southern blot analysis of tobacco DNA indicated that only a small number of LOX genes hybridize to this probe. These genes were not constitutively expressed to a detectable level in control cells and healthy plants. In contrast, a rapid and transient accumulation of transcripts occurred in cells and plants after treatment with elicitor and inoculation with zoospores of *Ppn,* respectively. In the infection assays, LOX gene expression and enzyme activity were observed earlier when the plants carried a resistance gene against the race of *Ppn* used for inoculation. (Veronesi *et al.,* 1996). Induction of genes coding for 9-LOX were also shown on other plant-pathogen interactions. The POTLX-3 was induced during the HR of *Solanum tuberosum* with *Phytophthora infestans* (Kolomiets *et al.,* 2000). Marmey *et al.,* (2007) reported that hypersensitive reaction (HR) cell death of cotton to the incompatible race 18 from *Xanthomonas campestris* pv *malvacearum* (Xcm) is associated with 9S-lipoxygenase activity (LOX) responsible for lipid peroxidation. The cloning and the sequencing of of cotton (*Gossypium hirsutum* L.) LOX gene (GhLOX1) revealed that GhLOX1 was found to be highly expressed during *Xcm* induced HR. Sequence analysis showed that GhLOX1 is a putative 9-LOX, and GhLOX1 promoter contains SA and JA responsive elements patterns in the corresponding immune and

susceptible reactions. The study of differentially expressed defense-related that no single gene was up regulated at every time point of post-inoculation, classes of genes encoding oxidizing enzymes, such as lipoxygenases and peroxidases, were up regulated at all time points i.e.1, 6,12,24,48 h post-inoculation (Choi *et al.*, 2008). Oliver *et al.*, (2009) reported that *Pythium irregulare* and *Pythium debaryanum* infection trigger the expression of *CHS*, *PAL*and *LOX* genes, and a slight induction of *PR-1* expression in the moss (*Physcomitrella patens*) tissues. *LOX* transcript accumulation starts at 2 h increasing up to 6–8 h after inoculation. *P. patens* activated multiple responses against *Pythium irregulare* and *Pythium debaryanum*, including the reinforcement of the cell wall.

Lipoxygenases (LOXs) are crucial for lipid peroxidation processes during plant defense responses to pathogen infection. A pepper (*Capsicum annuum*) 9-LOX gene, CaLOX1, which encodes a 9-specific lipoxygenase, was isolated from pepper leaves.

Recombinant CaLOX1 protein expressed in *Escherichia coli* catalyzed the hydroperoxidation of linoleic acid. Expression of CaLOX1 was differentially induced in pepper leaves not only during *Xanthomonas campestris* pv *vesicatoria* (Xcv) infection but also after exposure to abiotic elicitors. Transient expression of CaLOX1 in pepper leaves induced the cell death phenotype and defense responses. CaLOX1-silenced pepper plants were more susceptible to *Xcv* and *Colletotrichum coccodes* infection, which was accompanied by reduced expression of defense-related genes, lowered lipid peroxidation, as well as decreased reactive oxygen species and lowered salicylic acid accumulation. Infection with *Xcv*, especially in an incompatible interaction, rapidly stimulated LOX activity in unsilenced, but not CaLOX1-silenced, pepper leaves (Hwang and Hwang, 2010).

CHAPTER 3

MATERIALS & METHODS

III. MATERIALS AND METHODS

The present investigation on "Characterization of LOX gene and polyamines in Castor (*Ricinus communis* L.) during wilt pathogen interaction" was conducted at the Department of Biotechnology, Navsari Agricultural University, Navsari.

3.1 EXPERIMENTAL MATERIAL

The investigation was carried out using the castor genotypes. The seeds of two resistance genotypes (SKP-84, 48-1) and two susceptible genotypes (VP-1, VI-9) to castor wilt were procured from the Main Castor and Mustard Research Station, Sardar Patel Dantiwada Agricultural University, Sardar Krushinagar, Dantiwada.

3.1.1 LABORATORY WARE

All the glasswares required were obtained from Corning, Borosil and Schott Duran, the disposable plastic wares (centrifuge tubes, PCR tubes, micro tips, etc.) from Tarsons Ltd. Axygen and Eppendorf.

3.1.2 CHEMICALS

All the chemicals used were of analytical reagent grade/molecular grade and were obtained from Sisco Research Lab. (SRL), LOBA Chemicals, Sigma, Hi-Media, E-Merck, Qualigens, Bangalore Genei, Fermentas and QIAGEN.

3.2 PREPRATION OF FUNGAL SUSPENTION AND RAISING OF CASTOR CROP

3.2.1 Isolation of *Fusarium oxysporium* f. sp. *ricini* and preparation of suspension:

The *Fusarium oxysporium* f. sp. *Ricini* was isolated from the root of castor susceptible genotype (VP-1). Few spores from infected root were mixed in sterilized Milli-Q water to prepare spore suspension. The prepared homogenous spore suspension (6 drops) was then transferred with a sterilized pipette, onto the centre of the agar plate and carefully spreaded on the plate. The unsealed plate was incubated at 25 °C

for 12-24 hours. The unsealed plate allows some of the surface water to dry out. The spores were checked within 12 hours and then every 24 hours to establish germination. Once the spores have germinated a sterilized needle was used to pick up a small piece of agar containing a spore. In order to establish that the spore is the one desired, and maintain quality control, a slide was prepared and examined under the compound microscope. Ten germinated spores were transferred and distributed evenly onto two PDA plates and incubated at 25 ° C until their colony diameter were about 1 to 2 cm. A small piece of mycelium with agar was then transferred to another PDA plate and the culture was checked after few days, if there is no contamination, a pure culture has been obtained. This pure culture was again used to infect susceptible genotype (VP-1 and VI-9) to prove Koch's postulates. After that culture was stored on the potato dextrose broth at 4° C (Choi *et al.*, 1999).

The spore suspension of *Fusarium oxysporium* f. sp. *Ricini* was prepared from 7-day-old culture grown on potato dextrose broth and diluted to concentration approximately 6.5 X 10^5 spores/ml.

3.2.2 Tray sowing

Castor genotypes were raised in tray at Department of Biotechnology, N. M. College of Agriculture, N. A. U, Navsari. Only fine sterilized sand was used for sowing of seeds of castor genotypes. Seeds were sown on the surface of the sand and then covered with a 2 cm layer of sand. Twenty plants in each tray (90 x 60 x15 cm) were kept 20 days after germination. The crop was well irrigated regularly to avoid any physiological stress and to maintain high relative humidity condition. Highly susceptible genotypes VP-1, VI-9 and resistant genotypes SKP-84, 48-1 were sown in individual trays.

3.2.3 Pot transplanting:

To collect leaf samples for biochemical and molecular analysis. Plants (20 days old) of all the four genotypes were infected with *Fusarium oxysporium* f. sp. *ricini* by mechanical method. Plants were pulled out gently from the sand and roots were washed with distilled water and roots from terminal sites about 1-2 cm were slotted out and injured roots of castor seedlings were dipped in $1x10^{-6}$ fungal suspension for 10-15

min, while for control treatment the injured roots were dipped in distilled water and transplant in fresh sterilized pot containing sterilized soil and sand in 1:1 ratio.

3.3 BIOCHEMICAL CHARACTERS

All biochemical parameters were analyzed at three stages: (i) pre-infection [20 days after sowing (DAS)] and (ii) post-infection [5 and 10 days after infection (DAI) i.e. 30 DAS].

Fresh leaf samples for biochemical and molecular analysis at all stages were collected from second upper leaf. The lateral roots were collected only at two stage (0 and 10 DAI) to remain same plant for sampling at all three stage (0,5 and 10 DAI). Fresh leaves and roots were washed twice with tap water and then with Milli-Q water. Infected and uninfected leaves without mid rib were analyzed in triplicate for following biochemical estimation:

3.3.1 Determination of enzymes

3.3.1.1 Enzyme related to reactive oxygen species (ROS) generation:

(i) Lipoxygenase (LOX)

(ii) Polyamine oxidase (PAO)

3.3.1.2 Metabolites constituents:

(i) MDA (Malondialdehyde) content

(ii) Polyamines profiling

3.4 MOLECULAR CHARACTERIZATION:

(i) RNA extraction from leaves at regular intervals with and without infection with fusarium wilts pathogen.

(ii) cDNA synthesis

(iii) Isolation of key genes of LOX

(iv) Sequencing of genes

(v) Expression analysis of gene(s) using Real Time PCR

The detail methods applied for various biochemical and molecular parameters are given below with appropriate headings:

3.5 Determination of enzymes:

3.5.1 Enzyme related to ROS generation

(1) Determination of Lipoxygenase (LOX) activity: (EC 1.13.11.12)

Enzyme extraction: The leaves (0.2 g) were homogenized with 2 ml of 0.2 M sodium phosphate buffer (pH 6.5) using a pre-chilled mortar and pestle. The homogenate was centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was used as the enzyme source (Mahatma *et al.*, 2011).

Preparation of Substarte: Stock solutions of 10 mM MBTH and 5 mg/ml hemoglobin (100X their final concentrations) were made by dissolving the reagents in water. A solution containing 20 mM DMAB and 100 mM phosphate buffer was prepared by dissolving 330 mg DMAB in 5 ml of 1 N HCl. This was then diluted to about 80 ml with water and 1.42 g of Na_2HPO_4 was added. The pH was adjusted to 6.0 with HCl and the volume brought to 100 ml with water. A 25 mM stock solution was prepared by adding 155 µl (140 mg) of linoleic acid and 257 µl (280 mg) of tween-20 to 5 ml of water. The mixture was emulsified by drawing back and forth in a Pasteur pipette and then clarified by adding 0.6 ml of 1 N NaOH then after dilution to the final volume of 20 ml.

Assay: This allowed for a simplified standard assay procedure using two working solutions prepared from the stock solutions described above. Solution A was prepared by mixing 10 ml of the 20 mM DMAB, 100 mM phosphate buffer solution (pH 6), 0.4 ml of the 25 mM linoleic acid stock, and 9.6 mL of water. Solution B was prepared by mixing 0.4 ml of 10 mM MBTH, 0.4 ml of 5 mg/ml hemoglobin, and 19.2 ml water. For the standard two-step assay, the sample, in a volume of 80 to 100 µl was incubated with 4.0 ml of solution A. After incubation for the specified amount of time (generally 7 min), 4.0 ml of solution B was added. After an additional 7 min, 1.9 ml of 1% (w/v) sodium lauryl sulfate was added to terminate the reaction. Absorbance at 598 nm was then determined (Anthon *et al.*, 2001).

(2) Polyamine oxidase:

Enzyme extraction: The leaves (0.1 g) were homogenized with 1 ml of 0.1 M sodium phosphate buffer (pH 6.5) using a pre-chilled mortar and pestle. The homogenate was centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was used as the enzyme source (Kaur-Sawhney *et al.*, 1981).

Enzyme Assay: PAO activity in leaves was determined by Peroxidase/guaiacol method. The rate of peroxidative oxidation of guaiacol by H_2O_2 released in enzyme extracts from leaf homogenates was assessed with spermidine or spermine as substrate. The reaction mixture consisted of 1 ml of 0.1 M sodium phosphate buffer (pH 6.5), 0.05 ml each of 25 mM guaiacol and 1 mg/ml of peroxidase (Horseradish Type II, Sigma) and 0.1 ml of crude enzyme. After preincubation of mixture at room temperature for 2 min, 0.02 ml of 10 mM spermine or spermidine was added and at 470 nm was measured (Smith, 1972).

3.6 Estimation of protein:

Protein concentration of each enzyme extract was estimated by the method of Lowry *et al.*, (1951).

Reagents:

(i) Solution A: 2% Na_2CO_3 in 0.1 N NaOH

(ii) Solution B: (a) 1% $CuSO_4.5H_2O$ solution

(b) 2% sodium potassium tartarate solution

Working solution of B: Prepared fresh before use by mixing equal volume of solution B (a) and B (b).

(iii) Solution C: Prepared fresh before use by mixing 50 ml of solution A and 1 ml of working solution of B.

(iv) Solution D: Folin & Ciocalteu reagent (1N) reagent.

Procedure:

Enzymes extract/protein extract (25 µl) were taken in test tube and volume was made up to 1 ml with Mill-Q. A tube with 1 ml of water served as blank. Five ml of solution C was mixed by vortexing and kept for 10 min. Then 0.5 ml of solution D (Folin & Ciocalteu reagent) was mixed with vortex and kept it room temperature for 30 min. Absorbance was recorded at 660 nm. A standard curve was prepared with bovine serum albumin in the range of 10-80 µg.

3.7 Metabolites constituents

3.7.1 Thiobarbituric acid reactive substances (TBARS) / Malondialdehyde (MDA) determination

Lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS) determined by the thiobarbituric acid (TBA) reaction (Heath and Packer, 1968). Leaves of both stages (0.2 g) were homogenized in 1.5 ml of 5% thrichloroacetic acid (TCA). The homogenates were centrifuged at 13,000 x g for 20 minutes. The aliquot of 1.0 ml supernatant was added with 2 ml of 20% TCA containing 0.5% (w/v) TBA. The mixture was heated at 95°C for 25 minutes and then quickly cooled on ice. The contents were centrifuged at 10000 x g for 10 minutes and the absorbance of the supernatant was measured at 532 nm on Spectrophotometer. The value for non-specific absorption at 600 nm was subtracted from the 532 nm reading. The concentration of TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

$$\text{MDA } \mu\text{ mol/g} = \frac{\text{OD } 532\text{ nm} - 600\text{ nm}}{155 \times L} \times 72 \times \text{Dilution Factor} \times 1000$$

Where,

155 = Extinction coefficient

72 = Molecular weight of MDA

L = Path length of the cell in cm

3.8. Polyamines profiling through HPTLC:

3.8.1 Extraction of Polyamines

Tissues were extracted in 5% cold (Perchloric acid) $HCLO_4$ at a ratio of about 100 mg/ml $HCLO_4$ and place the extract in an ice bath for 1 hr. Samples were pelleted at 48,000g X 20 min, and the supernatant phase containing the free polyamines fraction, was stored frozen at -20°C in plastic vials. $HClO_4$ extracts were stable for polyamine analysis by HPTLC for more than 6 months under these conditions.

3.8.2 Sample preparation

The method of Seiler and Wiechmann (1967) was adapted for plant tissues with some modifications. The quantity of 0.2 ml of $HClO_4$ extract were mixed with 400 µl of dansyl chloride (5 mg/ml in acetone, prepared fresh), and 200 µl of saturated sodium carbonate. After brief vortexing, the mixture was incubated in darkness at room temperature for overnight. Excess dansyl reagent was removed by reaction with 100 µl (100 mg/ml) of added proline, and incubated for 30 min. Dansyl polyamines were extracted in 0.5 ml benzene and vortexes for 30 seconds. The organic phase was collected and stored in glass vials at -20°C. Dansylated extracts were stable for up to 1 month. Standards were processed in the same way, and 20 nMol were dansylated for each, alone or in combinations. TLC was performed on high resolution silica gel 60 F 254.

3.8.3 HPTLC profiling of polyamines:

(i) Sample application:

Extracted samples (15.0 µl) were sprayed as 1x9 mm bands along with standards (15.0 µl) on TLC Silica gel 60 F 254 plates 20x10 cm (silica gel plates, Merck) by CAMAG Linomat 5 applicator using nitrogen gas. The plates were activated at 100°C for 10 min before sample application.

(ii) Separation of polyamines:

Solvent system was used as described by Seiler and Wiechmann (1967). Polyamines were separated by the solvent system chloroform: triethylamine (25:2, v/v). Solvent mixture was filled in the CAMAG Twin trough chamber (20x10 cm) and left overnight for saturation. The plates were placed in tank to separate polyamines until solvent reached 8 cm from origin.

(iii) Qualitative and Quantitative analysis:

After separation of polyamines, HPTLC plates were photographed using CAMAG Reprostar 3 system at 366 nm in fluorescent light. Quantitative determination was carried out using CAMAG TLC Scanner 3 at 350 nm.

3.9 MOLECULAR CHARACTERIZATION

1. RNA extraction at regular intervals with and without infection with fusarium wilt.

2. cDNA synthesis

3. Isolation of key genes of LOX

4. Sequencing

5. Expression analysis of gene(s) using Real Time PCR

3.9.1 Total RNA Isolation:

Total RNA was extracted from the leaves by a modified Trizol method. Fresh 21 days old leaves (0.1 gm) from each genotype were powdered in liquid nitrogen using a pestle and mortar. The resulting powder was transferred to a 1.5 ml tight capped eppendorf tube with 1 ml TRIzol reagent and incubated at room temperature for 5 minutes. Two volume of chloroform (0.2 ml) was added with vigorous shaking for 15 seconds and incubated at room temperature for 2-3 min. Tubes were centrifuged at 12000 X g at 2-8°C for 15 min. Aqueous phase was transferred in another eppendorf tube and 0.5 ml of isopropanol was mixed properly. Then eppendorf tubes were kept at room temp

for 10 min and centrifuged. Supernatant was removed and RNA pellet was washed in 1 ml 75% ethanol at 7500 X g for 5 min at 4° C. Pellets were dried about 1-2 hour then dissolved in 25-30 µl DEPC treated water and kept at 55-60°C on dry bath for 10 min. The extracted RNA was loaded on 1.5% agarose gel.

3.9.2 Quantification and quality check of RNA

The quality of RNA was checked by agarose gel electrophoresis and quantification was carried out by Nano Spectrophotometer. The RNA absorbs UV light very efficiently making it possible to detect and quantify either at concentrations as low as 2.5ng/µl. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. The RNA samples with the ratio of 1.7-2.0 at OD 260/280 were retained for RNA fingerprinting.

RNA was separated by denaturing agarose gel electrophoresis. The 1.5% denaturing gel were prepared in 1.0 X formaldehyde agarose gel buffer (Sambrook and Russell, 2001). Agarose (1.5 gm) was dissolved in 10 ml 10x Formaldehyde Agarose gel buffer and volume was made up to 100 ml with RNase-free water. Agarose was boiled in microwave oven and cooled to 65°C in a water bath. Then 1.8 ml of 37% (12.3 M) formaldehyde and 2 µl of a 10 mg/ml ethidium bromide form stock solution were added. Gel solution was mixed thoroughly and poured onto gel support. Prior to running the gel, gel was equilibrated in 1x Formaldehyde Agarose gel running buffer for 30 min. one volume of 5x loading buffer was added per 4 volumes of RNA sample and incubated for 3-5 min at 65°C, chill on ice, and loaded onto the equilibrated Formaldehyde Agarose gel. Gel was run in 1x Formaldehyde Agarose gel running buffer at 5-6V/cm till the dye reaches three fourth of gel. RNA bands were visualized on UV Transilluminator (GeNei^{TM}) and photographed under Gene Genius Imaging System from Syngene.

When resolved by electrophoresis the 28S and 18S RNA should exhibit at near 2:1 ratio on ethidium bromide staining indicating that no significant degradation of RNA has occurred.

3.9.2.1 10x Formaldehyde Agarose Gel buffer

200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)

50 mM sodium acetate

10 mM EDTA

pH to 7.0 with NaOH

3.9.2.2 1x Formaldehyde Agarose Gel Running Buffer

100 ml 10x Formaldehyde Agarose gel buffer

20 ml 37% (12.3 M) formaldehyde

880 ml RNase-free water

3.9.2.3 5x RNA Loading Buffer

16 µl saturated aqueous bromophenol blue solution

80 µl 500 mM EDTA, pH 8.0

720 µl 37% (12.3 M) formaldehyde

2 ml 100% glycerol

3084 µl formamide

4 ml 10 x Formaldehyde Agarose gel buffer

RNase-free water to 10 ml

Stability: Approximately 3 months at 4°C

3.9.3 cDNA synthesis:-

The cDNA was synthesized by using high capacity cDNA reverse transcription kit uses the random primer scheme for initiating cDNA synthesis. Random primers ensure that the first strand synthesis occurs efficiently with all species of RNA molecules present, including mRNA and rRNA. A master mix for cDNA synthesis was prepared by following method

Table 3.1: Preparation of First Strand cDNA Synthesis:

Component	Volume (µl)
Template RNA	2.0
Oligo (dt)18 primer	01
Water, nuclease free	09
Total volume	11
Reaction mixture mixes gently, centrifuge briefly and incubate at 650c for 5 min. Chill on ice, spin down and place the vial. Add the following component in the indication order.	
5X reaction Buffer	04
RibolockTM RNase Inhibitor (20u/µl)	01
10 mM dNTP mix	02
M-MuLV Reverse Transcriptase (20u/µl)	02
Total Volume	**20**

- Mix gently and centrifuge.

- Incubate for 5 min at 25°C followed by 60 min at 37°C.

- Terminate the reaction by heating at 70°C for 5 min.

The reverse transcription reaction product directly used in PCR application or stored at -20°C for less than one week. For longer storage, -70°C was used.

3.9.4 Amplification of lipoxygenase gene(s):-

Different sets (6 primers) were designed to amplify each cDNA by RT-PCR. LOX gene specific sequences of Arabidopsis were obtained at the NCBI data base and gene specific primers were designed using primer 3 software (Table 3.3).

The PCR amplification of lipoxygenase genes were performed using different reverse and forward primers i.e. LOX1, LOX2, LOX3, LOX4, LOX5 and DOX (Table 3.3). The reaction mixtures composed of 5µl 10xPCR buffer, 0.2mM dNTPs, 1µM each primer, 200ng cDNA template, 2.5 units *Taq* DNA polymerase and sterile deionized water to a final volume of 50µl. PCR programme was as follows: initial

denaturation at 94°C for 5min, followed by secondary denaturation (94°C, 30 sec), annealing (65°C, 45 sec), initial extension (72°C, 1 min), and final extension at 72°C for 5 min with 31 cycles. PCR product was stored at 4°C for future use.

All the PCR amplified products were run on 0.7% agarose gel containing 5 µl ethidium bromide, in 0.5X TBE (5X TBE stock, 54g Tris, 27.5g Boric acid, 20ml of 0.5M EDTA, pH8.0). Amplified product (5 µl) was mixed with 1 µl of 5x gel loading dye and loaded in the well. The electrophoresis was carried out at 60 mA (constant) to separate the amplified bands. The standard DNA marker (DNA logic ladder, 100 bp) was also run along with the samples. The single band were seen under UV light and photographed by Gel documentation system and analyzed by Gene tool.

Table-3.2: Sequence of Primers used in present study.

Primer	Sequence
Lox1 F	5'-GGATTACTACATTCACTCCTGCAAC-3'
Lox1 R	5'-GGCAGATATGCCTTGTTAGTAAAGA-3'
Lox2 F	5'-CAAGATGCAACACAGATACACTTTC-3'
Lox2 R	5'-TCTTTCCAACCTTGTTCTTTAAGTG-3'
Lox3 F	5'-GGAATTAGAAAATTGTGCGATAGAA-3'
Lox3 R	5'-CAGAGACTTCTATTCTTCTGCCATC-3'
Lox4 F	5'-ACAGATGACACTAAGGAGTCTCCAG-3'
Lox4 R	5'-TGGAACATTCTTTCTTTGACTTTTC-3'
Lox5 F	5'-AAGTATTACTCCAATAACCGCTGTG-3'
Lox5 R	5'-CATGGAAGGTAGGTCTTGTTAGAGA-3'
Dox F	5'-GATCTAACGGATGACAAAGAAGCTA-3'
Dox R	5'-ACTTTCTGTGGTATTCACCCATTTA-3'

3.9.5 DNA sequencing :

Cycle sequencing of the PCR product was done using Big Dye sequencing kit 3.1v, provided by Applied Biosystems. A 250ng of the PCR product was used for cycle sequencing. To purify the product of cycle sequencing it was first

transferred to a 1.5ml tube. Then a master mix I consisting 10µl Milli-Q H_2O and 2µl of 125mM EDTA per reaction was prepared. 12µl of master mix I was added to 10µl of the reaction and the contents are mixed well. Master mix II consisting 2µl of 3M NaOAc (pH4.6) and 50µl of ethanol per reaction was prepared. 52µl of master mix II was added to 10µl of the reaction and the contents are mixed well. After the incubation of this product at RT for 15min. it was centrifuged at a speed of 12000xg for 20min. at RT. Supernatant was decanted and 250µl of 70% ethanol was added to it. Then it was again centrifuged at RT for 10 min.

After drying the pellet 15µl of Hi-Di Formamide was added to it for denaturation of DNA. Samples were snap chilled and then 10µl of sample was transferred to 96-well sequencing plate. Sequencing was done using AB prism Sequencer, 3130 Genetic Analyzer (Applied Biosystems) with 4 capillaries. Results of sequencing were analyzed by performing online BLAST sequence homology test.

3.9.6 Expression Analysis of Lipoxygenase Gene(S) By Real-Time (RT) PCR:

Real-time PCR is one of the most sensitive and reliably quantitative methods for gene expression analysis. It has been broadly applied to microarray verification, pathogen quantification, cancer quantification, transgenic copy number determination and drug therapy studies. A PCR has three phases, exponential phase, linear phase and plateau phase. The exponential phase is the earliest segment in the PCR, in which product increases exponentially since the reagents are not limited. The linear phase is characterized by a linear increase in product as PCR reagents become limited. The PCR will eventually reach the plateau phase during later cycles and the amount of product will not change because some reagents become depleted. Real-time PCR exploits the fact that the quantity of PCR products in exponential phase is in proportion to the quantity of initial template under ideal conditions. During the exponential phase PCR product will ideally double during each cycle if efficiency is perfect, i.e. 100%. It is possible to make the PCR amplification efficiency close to 100% in the exponential phases if the PCR conditions, primer characteristics, template purity, and amplicon lengths are optimal. Both genomic DNA and reverse transcribed cDNA can be used as

templates for real-time PCR. The dynamics of PCR are typically observed through DNA binding dyes like SYBR green or DNA hybridization probes such as molecular beacons (Strategene) or Taqman probes (Applied Biosystems). The basis of real-time PCR is a direct positive association between a dye with the number of amplicons.

Real-time PCR data are quantified absolutely and relatively. Absolute quantification employs an internal or external calibration curve to derive the input template copy number. Absolute quantification is important in case that the exact transcript copy number needs to be determined, however, relative quantification is sufficient for most physiological and pathological studies. Relative quantification relies on the comparison between expression of a target gene versus a reference gene and the expression of same gene in target sample versus reference samples.

3.9.7 Quantitative RT-PCR analysis:

PCR was then performed using a (≤100 ng/reaction) 2.5 µl aliquot of the first strand cDNA in a final volume of 10 µl containing 10 pmol of specific primers (forward and reverse) for coding region of LOX genes (LOX-2, LOX-3, LOX4, LOX-5 and Dox). As a control, the primer specific to 18S rDNA gene (NCBI accession No.) was used control to normalize each sample for variations in the amounts of RNA used. PCR was carried out using 5µl 2x QuantiFast SYBR Green PCR Master Mix (Qiagen, Valencia, CA, USA) , Primer F and R both are 0.5 µl, Template cDNA 2.5 µl and RNase free water 1.5 µl to final volume 10 µl in a thermal cycler (ABI-7300) programmed as follows: an initial denaturation for 5 min at 95°C, 35 amplification cycles [5 min 95°C (initial denaturation), 10 s at 95°C (denaturation), 30 s at 60°C (annealing)].

Each sample was tested in triplicate for all primers. Melting curve analysis was performed on all samples to ensure amplification of a single product with the expected melting temperature and the absence of primer-dimers. The products of each primer set were tested by agarose gel electrophoresis to verify that a single product of the expected size was produced. Relative RNA quantities were determined with the

delta-delta (ΔΔ)Ct, according to the following formula (Dussault and Pouliot, 2006) comparing the data for each gene of interest with the data for mock-inoculated control samples at each time point. The data was normalized by comparison to gene.

$\Delta\Delta Ct$= [(Ct G.O.I Ctr- Ct Ref. Ctr)- (G.O.I infected- Ct Ref. infected)

Where: G.O.I= Gene of Interest

Ref: reference gene (18s)

Ctr= Contro (non-infected)

Fold increase = $2^{-\Delta\Delta Ct}$

3.10 STATISTICAL ANALYSIS

3.10.1 Biochemical parameters

All the biochemical parameters were analyzed in three replications (one pot/replication/cultivar). The data obtained by biochemical constituents and enzymes determination were subjected to simple completely randomized design for the significance of various data using 'F' test (Gomez and Gomez, 1984).

3.10.2 Sequence Analysis:

Sequences obtained by different gene specific primers were BLAST (Basic Local Alignment Search Too) online on NCBI (National Center for Biotechnology Information) to find the similarity between sequences available on NCBI data base. Multiple sequences of nucleotides and proteins were align using online CLUSTLW programme and phylogenic tree of sequences were constructed.

CHAPTER 4

RESULTS AND DISCUSSION

IV. RESULTS AND DISCUSSION

The results of the present investigation on "Characterization of LOX gene and polyamines in Castor (*Ricinus communis* L.) during wilt pathogen interaction" are presented and discussed in this chapter.

4.1 BIOCHEMICAL CHARACTERIZATION:

All biochemical parameters were analyzed in infected and non infected plants at three stages: (i) pre-infection [20 days after sowing (DAS), only non infected plants] and (ii) post-infection [5 and 10 days after infection (DAI) i.e. 25 & 30 DAS]. Fresh leaf samples for biochemical and molecular analysis were collected from second upper leaf at all stages. The lateral roots were collected only at two stage (pre-infection or 0 and 10 DAI) to remain same plant for sampling at all three stage (0, 5 and 10 DAI).

4.1.1 Metabolites constituent:

(1) Malondialdehyde (MDA) content:

Membrane lipid peroxidation was estimated as the content of MDA to observed membrane damage (Table 4.1). The MDA content was higher in susceptible genotypes (367-375 µmol g-1 FW in leaf and 148-152 µmol g-1 FW in root) (Fig. 4.1) compared to resistant genotypes (308-311 µmol g-1 FW in leaf and 107-128 µmol g-1 FW in root) (Fig. 4.2) at pre-infection (20 DAS) stage. While, after infection (5 and 10 DAI) the MDA content was increased in infected leaves of resistant and susceptible genotypes as compared to their non-infected leaves. However, leaves of infected resistant genotypes had about 1.5-1.7 times more MDA content than infected susceptible genotypes and non-infected resistant genotypes. The MDA content was decline significantly in infected and non-infected tissues of both resistant and susceptible genotypes at 10 DAI compared to 5 DAI. However, the MDA content was higher in both leaf and root tissues of resistant genotypes at 10 DAI as compared to (0 DAI) pre infection.

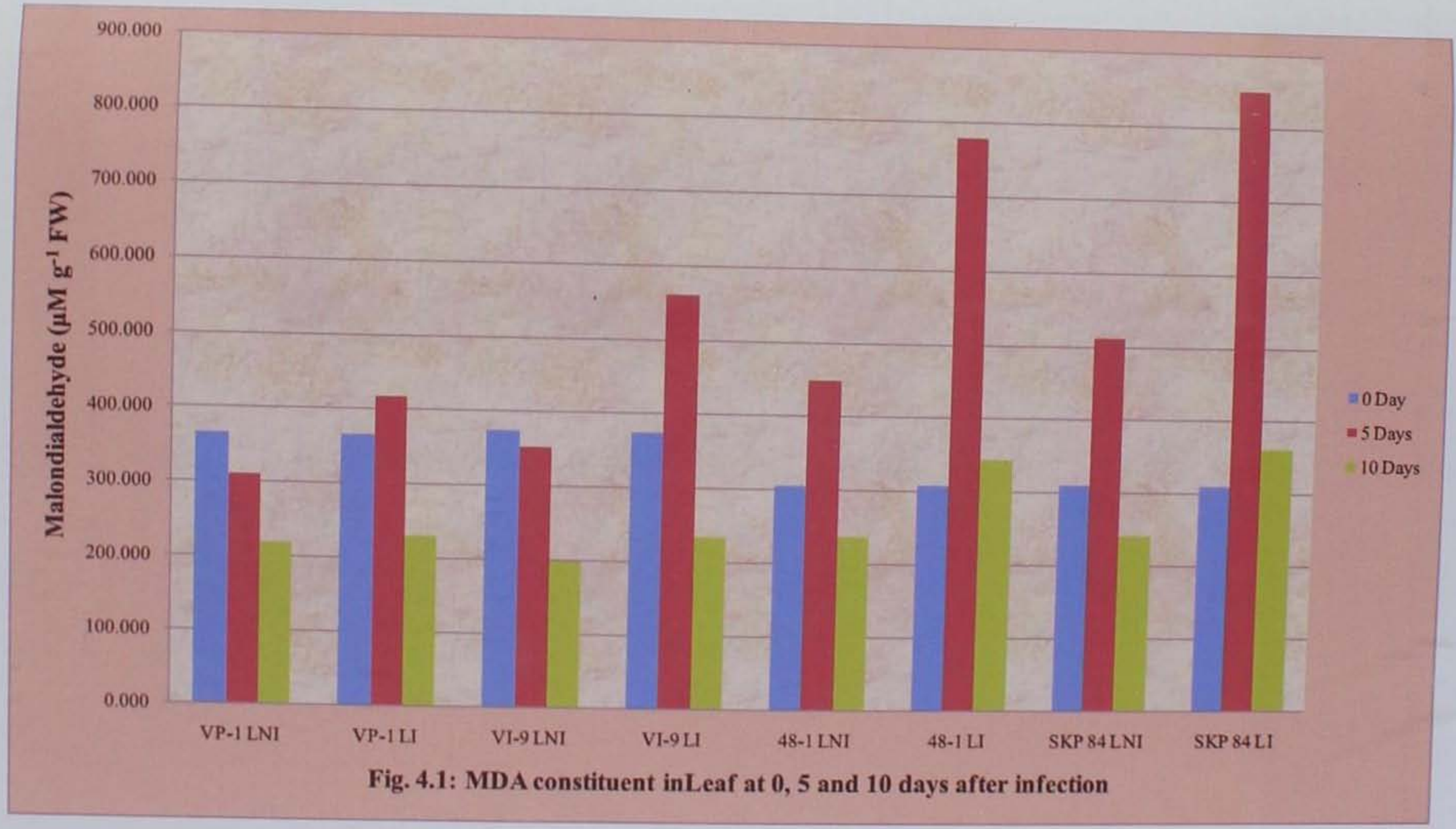


Fig. 4.1: MDA constituent inLeaf at 0, 5 and 10 days after infection

VP-1, VI-9, 48-1, SKP-84 – Genotypes

Note: L= Leaf, R= Root, NI= Non infected, I= Infected

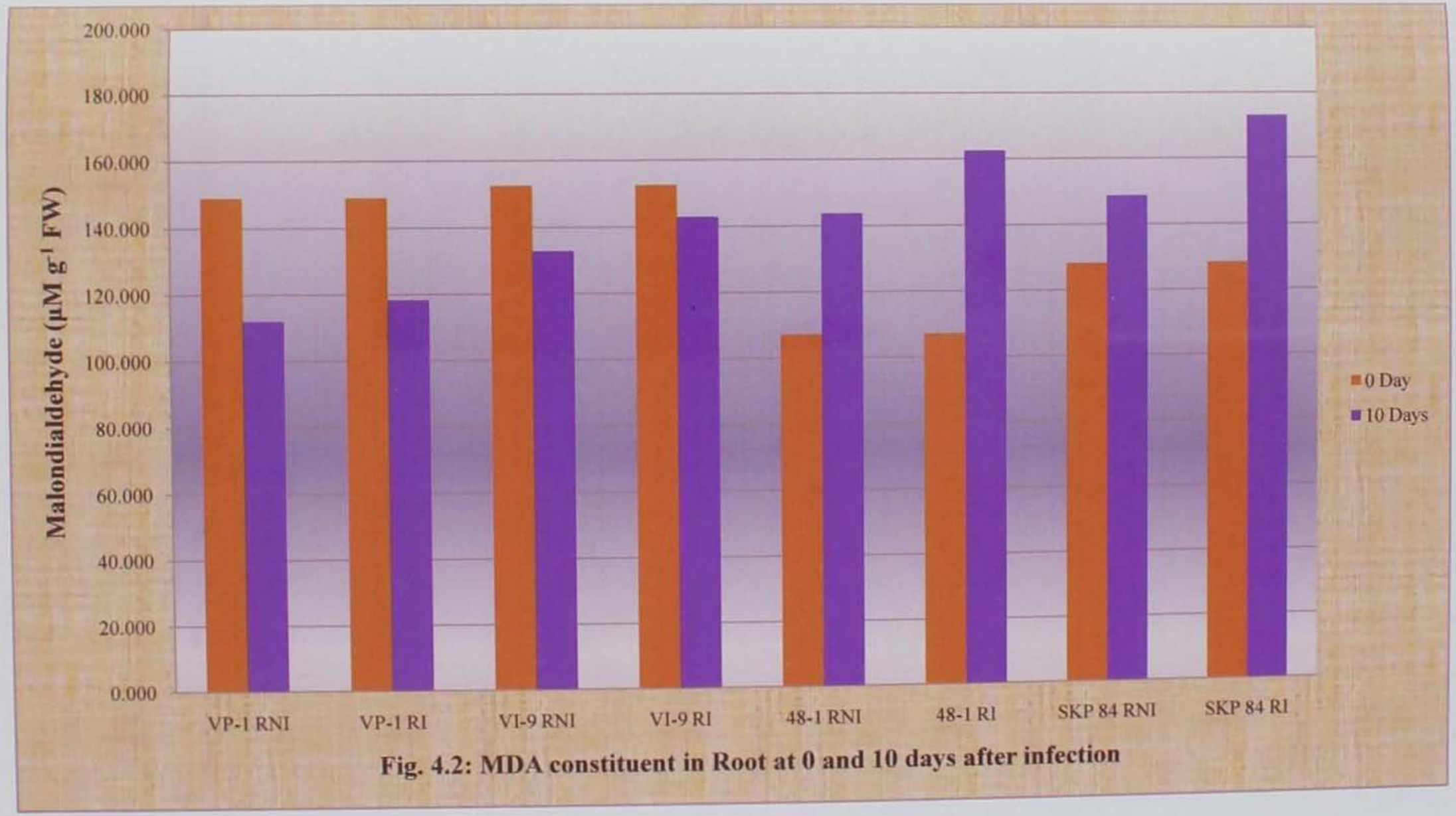


Fig. 4.2: MDA constituent in Root at 0 and 10 days after infection

VP-1, VI-9, 48-1, SKP-84 – Genotypes

Note: L= Leaf, R= Root, NI= Non infected, I= Infected

Table 4.1: Pre- and post-infectional changes in lipid peroxidation product (MDA content) of wilt resistant and susceptible Castor genotypes

No. and name of genotypes	MDA constituents (µmol g^{-1} FW)							
	Pre infection (0 day)		Post infection(5 days)		Post infection (10 days)			
	Leaf NI	Root NI	Leaf NI	Leaf I	Leaf NI	Leaf I	Root NI	Root I
VP-1	367.65	148.99	312.35	418.15	221.23	232.74	111.99	118.29
VI-9	375.84	152.48	355.08	560.53	201.56	235.44	132.76	142.91
48-1	308.23	107.09	450.55	779.92	238.23	345.25	143.73	162.42
SKP-84	311.55	128.18	512.58	850.25	243.56	362.22	148.70	172.93
S.Em.	0.74	0.39	1.88	1.88	1.04	0.94	0.69	0.69
C.D. at 5%	2.22	1.17	5.63	2.63	3.12	2.83	2.08	2.08
C.V.%	0.38	0.50	0.80	0.50	0.80	0.56	0.89	0.80

Pre-infection at 20 DAS, Post-infection at 25 and 30 DAS (5 and 10 days after infection DAI) FW = Fresh weight I= Infected, NI= Non Infected

The rate of lipid peroxidation product increased with *Fusarium oxysporum* f. sp. *ricini* infection in incompatible interaction whereas the rate of lipid peroxidation product decreased in compatible interaction. The rate of increase was more pronounced than rate of decrease. These findings are in accordance with results observed by Ge Xiu Chan *et al.*, (1998) in rice leaves after inoculation by *Magnaporthe grisea* and in grey leaf spot of maize (Guo *et al.*, 2003). Infection by *Fusarium oxysporum* significantly increased levels of H_2O_2 and O_2 production in tomato leaves and its levels increased with increasing the time of infection (El-Khallal, 2007). Our results can be correlated with higher lipoxygenase activity in resistant genotypes in present study and is in good agreement with lipid peroxidation accompanied by the enzyme lipoxygenase.

(2) Polyamines Profiling through HPTLC:

The perusal of data (Table 4.2) revealed the RF of 4 standard polyamines as detected by HPTLC CAMAG SCANNER 3. Total 3 polyamines (Fig. 4.3) putrescine, spermidine and spermine were detected in infected and non-infected tissues of all four castor genotypes VP-1, VI-9, 48-1 and SKP-84.

Table 4.2: R_F value of standard polyamines detected by CAMAG SCANNER 3 at 350 nm

Sr. No.	Name of Polyamines	Start R_F	Max R_F	End R_F	% Area
1	Cadaverine (Cad)	0.63	0.75	0.81	100
2	Putrescine (Put)	0.49	0.56	0.67	99.13
3	Spermidine (Spd)	0.77	0.87	0.92	96.69
4	Spermine (Spm)	0.88	0.92	0.95	64.72

Results of polyamines profiling showed the cadaverine was not detected in any genotype of castor in all the stage of analysis. However, primary diamine putrescine was not detected in non-infected leaf tissues of susceptible genotypes at 10 DAI (Table: 4.3) while it was present in root tissues of same genotypes. On the other hand, putrescine percent area was increased in non-infected leaf tissues of resistant genotypes at 5 DAI and almost maintains its level at 10 DAI. In the infected leaf tissues of susceptible genotypes putrescine, spermidine and spermine was increased at 5 DAI while these polyamines were decreased in resistant genotypes compared to their non-infected leaves. Interestingly, the levels of spermidine and spermine was increased in infected leaves of resistant genotypes at 10 DAI whereas reverse trend was observed in susceptible genotypes. The resistant genotypes had 2-3 times higher content of spermidine and spermine. Similar results were observed in an incompatible interaction between barley and powdery mildew, levels of free spermidine and of conjugated forms of putrescine and spermidine were found to increase after inoculation (Cowley and Walters, 2002a).

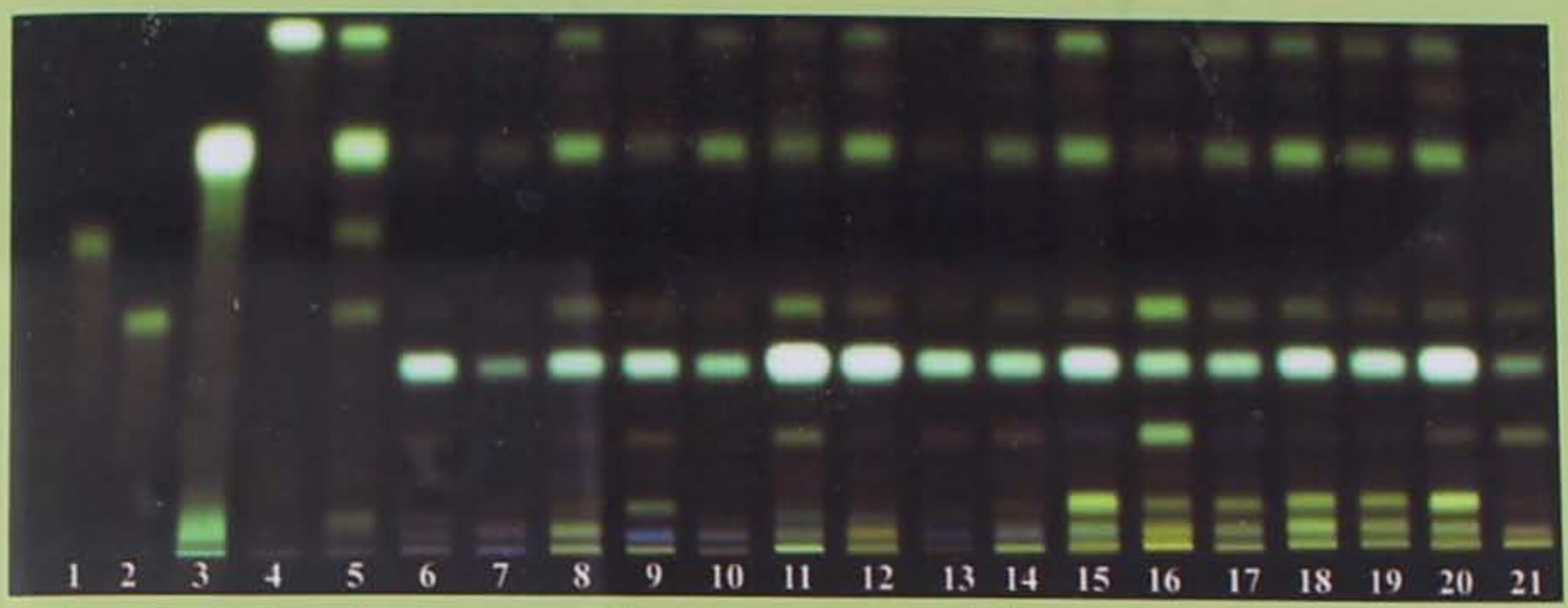


Figure 4.3: Polyamine profiling of susceptible and resistant castor genotypes using HPTLC. Polyamines were detected at 350 nm in fluorescent wavelength.

Furthermore, these results are in agreement with a previous report where the over expression of a key polyamine biosynthetic gene arginine decarboxylase (*adc*) in transgenic eggplant exhibited increased resistance against Fusarium wilt as compared to wild type seedlings. Similarly, the introduction of *samdc* gene in tobacco conferred resistance against Fusarium and Verticillium wilts (Waie and Rajam 2003). Higher content of spermine plays a role as a mediator in defence signalling against plant pathogens (Yamakawa *et al.*, 1998; Takahashi *et al.*, 2003).The levels of PAs, particularly PA conjugates are known to increase during fungal infections and are implicated in providing resistance against various plant pathogens. Therefore, high titers of PAs, in the incompatible interaction suggesting their involvement in fungal resistance. Remarkably, putrescine was only present in infected roots of resistant genotypes of castor and these genotypes also had higher percent area of spermidine. The 'spermine signaling pathway' involves accumulation of spermine in the apoplast, upregulation of a subset of defence-related genes such as those encoding pathogenesis-related (PR) proteins, PR-1, PR-2, PR-3 and PR-5 (Yamakawa *et al.*, 1998) and mitogen-activated protein kinases, and a type of programmed cell death known as the hypersensitive response. This response is triggered by spermine-derived H_2O_2, produced through the action of polyamine oxidase (PAO) localized in the apoplast (Kusano *et al.*, 2008; Moschou *et al.*, 2008). Taken together, these data indicate double-edged roles of spermine in cell survival: as a free radical scavenger in the nucleus and as a source of free radicals in the apoplast, the interaction of spermine with other molecules is involved in the cell death.

The observed results of PAs are accompanied with the polyamine oxidase (PAO) activity in susceptible and resistant genotypes in present study. This might be due to higher PAO activity of resistant genotypes utilized the higher content of polyamines as substrate at 5 DAI. The levels of polyamines in infected resistant genotypes at 10 DAI were almost similar to the level of polyamines in the susceptible genotypes at 5 DAI. Resistant genotypes maintain the PAs and PAO homeostasis in both infected and non-infected tissues.

Table 4.3: Polyamines profiling of Castor genotypes at pre-infection (I 0 hours) and post-infection (II 5 days) and post-infection (III 10 days) stage (Individual polyamines and % Area was detected by CAMAG SCANNER 3 at 350 nm)

Sr. No.	Genotypes	Stage	% Area of Known Polyamines				
			Polyamines	Control Leaf	Control Root	Treated Leaf	Treated Root
1	VP-1	I 0 Day	Cad	--	--	--	--
			Put	3.56	--	--	--
			Spd	7.44	19.77	--	--
			Spm	--	--	--	--
		II 5 Day	Cad	--	--	--	--
			Put	5.95	--	8.96	--
			Spd	16.82	--	23.80	--
			Spm	5.77	--	10.56	--
		III 10 Day	Cad	--	--	--	--
			Put	--	3.52		--
			Spd	9.59	10.81	6.62	3.42
			Spm	7.45	--	6.65	--
2	VI-9	I 0 Day	Cad	--	--	--	--
			Put	9.24	3.07	--	--
			Spd	23.92	5.21	--	--
			Spm	12.22	--	--	--
		II 5 Day	Cad	--	--	--	--
			Put	19.38	--	8.59	--
			Spd	10.46	--	18.70	--
			Spm	10.25	--	17.51	--
		III 10 Day	Cad	--	--	--	--
			Put	--	2.21	--	--
			Spd	17.99	9.58	7.75	--
			Spm	13.54	--	8.38	11.55

3	48-1	I 0 Day	Cad	--	--	--	--
			Put	8.83	19.31	--	--
			Spd	22.34	18.10	--	--
			Spm	12.78	6.81	--	--
		II 5 Day	Cad	--	--	--	--
			Put	10.44	--	6.44	--
			Spd	30.51	--	21.55	--
			Spm	15.75	--	12.40	--
		III 10 Day	Cad	--	--	--	--
			Put	3.25	2.86	2.89	2.66
			Spd	13.07	15.71	17.00	18.76
			Spm	13.83	--	16.55	--
4	SKP-84	I 0 Day	Cad	--	--	--	
			Put	4.97	--	--	--
			Spd	16.52	6.25	--	--
			Spm	7.75	2.75	--	--
		II 5 Day	Cad	--	--	--	--
			Put	9.05	--	7.65	--
			Spd	15.12	--	2.13	--
			Spm	12.30	--	3.56	--
		III 10 Day	Cad	--	--	--	--
			Put	3.70	6.11	4.10	5.63
			Spd	10.43	14.09	25.71	17.15
			Spm	15.66	11.34	20.88	--

4.2. ENZYMES ACTIVITY

The results of different enzymes activity are interpreted and discussed in this section.

4.2.1 Enzymes related to reactive oxygen species generation and metabolism

4.2.1.1 Lipoxygenase (LOX) activity:

At pre-infection (0 day), LOX activity was higher in the resistant genotypes (18.32-19.47 Units min-1 g-1 protein) compared to susceptible genotypes (15.46-15.58 Units min-1 g-1 protein) in leaf (Fig. 4.4) and as same in root (Fig. 4.5) LOX activity was higher in the resistant genotypes (92.27-93.72 Units min-1 g-1 protein) compared to susceptible genotypes (81-82 Units min-1 g-1 protein). At post-infection (5 DAI), LOX activity (Table 4.4) was significantly higher in the infected resistant genotypes (246.14-284.73 Units min-1 g-1 protein) but LOX activity lower in the non-infected resistant genotypes up to 0.5-0.7 fold. Infected susceptible genotypes contain (179-185 Units min-1 g-1 protein) and in non-infected susceptible genotypes (131-139 Units min-1 g-1 protein) such that LOX activity lowers in the non-infected susceptible genotypes than infected genotypes up to 0.4-0.6 fold. We observed that after infection increase LOX activity in resistant genotypes than susceptible at post infection 5 day stage. The LOX activity was decline significantly in resistant and susceptible genotypes at 10 DAI. Furthermore, LOX activity began to rise at the 5^{th} day of stress and thereafter LOX activity began to decline significantly.

Increased LOX activity in plant pathogen interaction is well established (Keppler and Novacky, 1986; Nagarathana *et al.*, 1992; Jain *et al.*, 2002 and Babitha *et al.*, 2004). The increase in lipoxygenase activity suggests its possible involvement in imparting resistance following *Fusarium oxysporum* f. sp. *ricini* infection. Increase in LOX enzyme activities is frequently found to be associated with R-Avr gene mediated incompatibility (Slusarenko, 1996). The increased LOX may generate signal molecules such as jasmonic acid, methyl jasmonic acid or lipid peroxides, which co-ordinately amplify specific responses. Increased LOX activity may also cause irreversible membrane damage, which would lead to the leakage of cellular contents (Mac Carrone *et al.*, 2000).

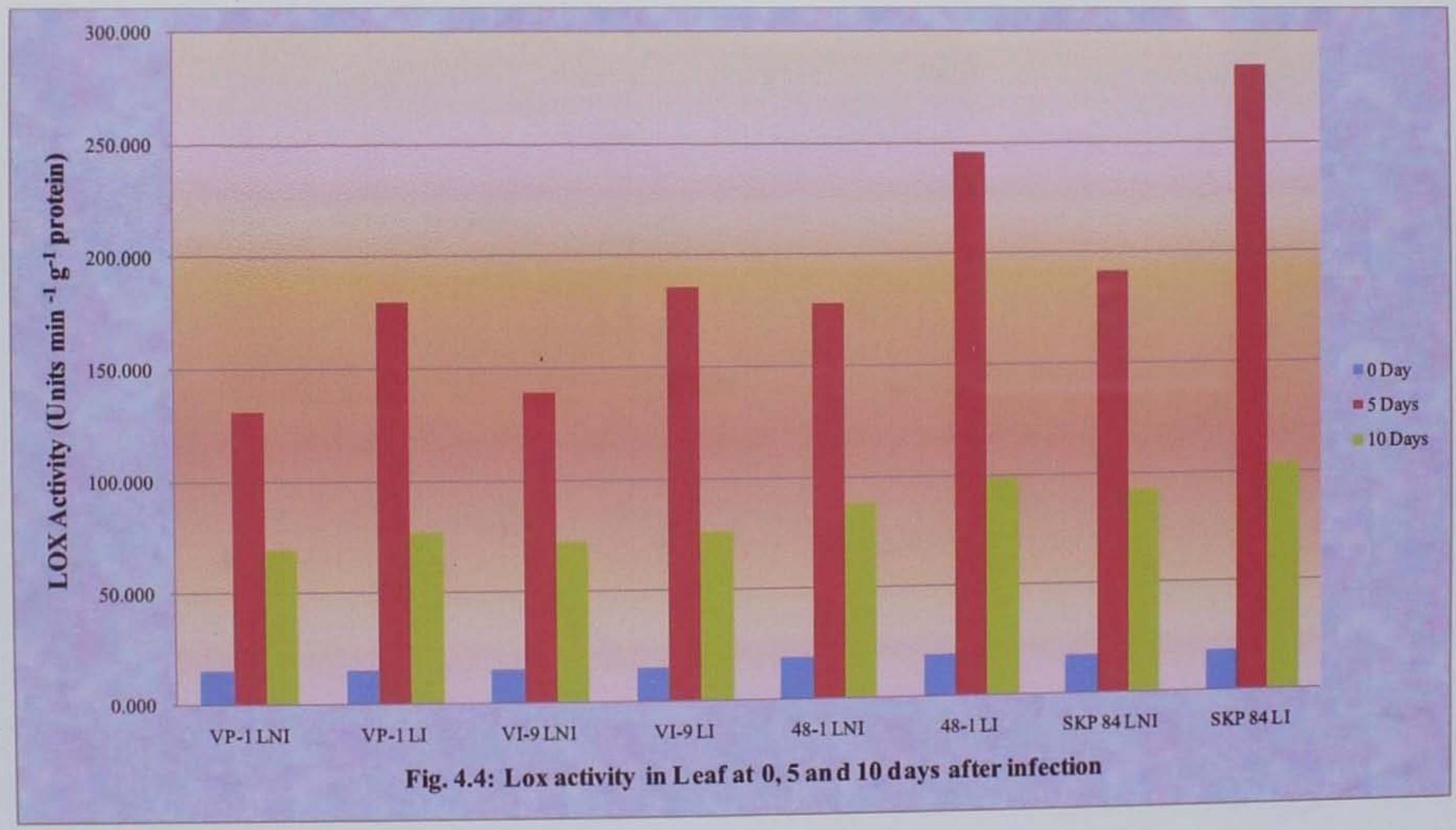


Fig. 4.4: Lox activity in Leaf at 0, 5 and 10 days after infection

VP-1, VI-9, 48-1, SKP-84 – Genotypes

Note: L= Leaf, R= Root, NI= Non infected, I= Infected

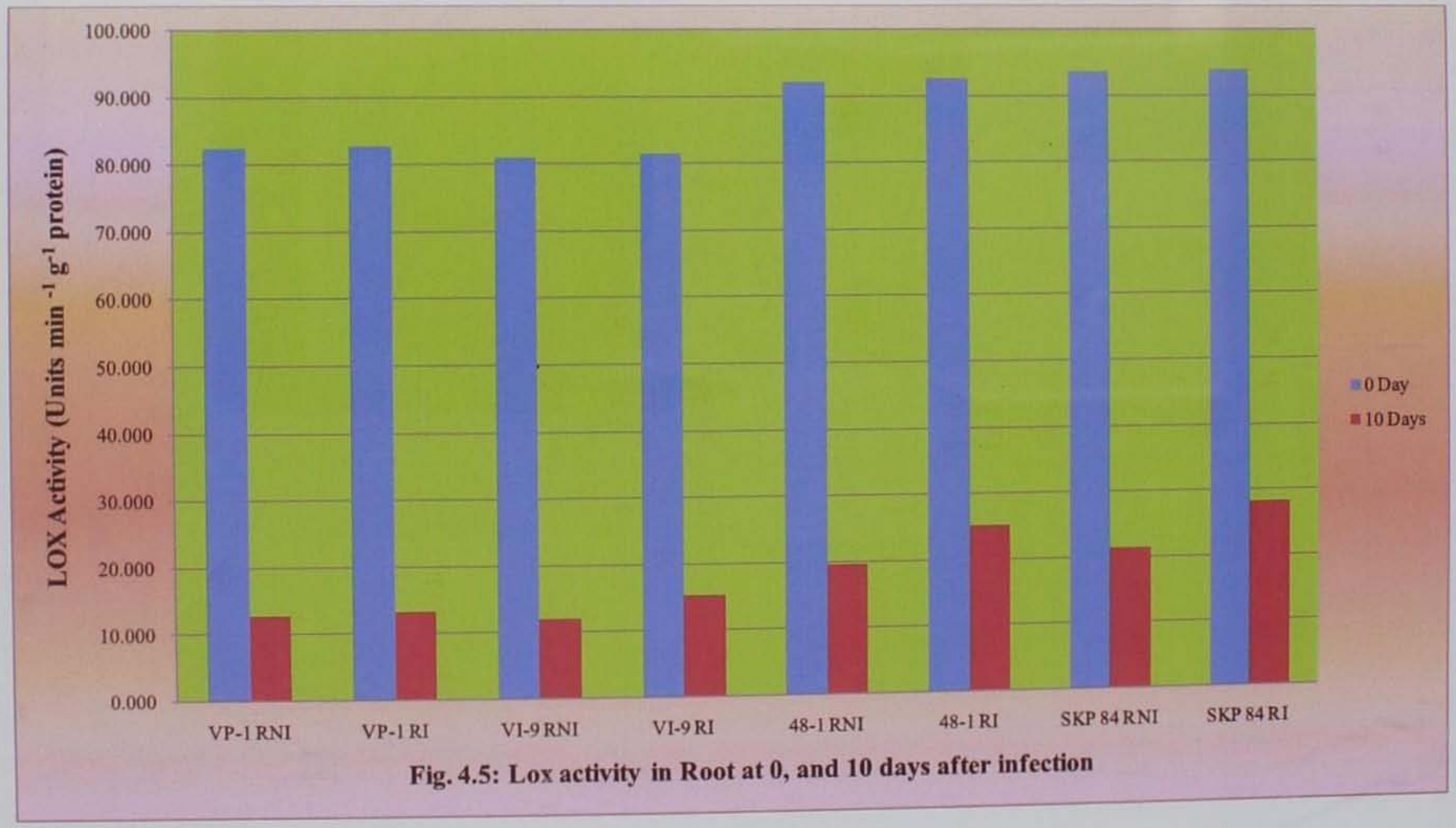


Fig. 4.5: Lox activity in Root at 0, and 10 days after infection

VP-1, VI-9, 48-1, SKP-84 – Genotypes

Note: L= Leaf, R= Root, NI= Non infected, I= Infected

Table 4.4: Pre- and post-infectional changes of enzyme activity in wilt resistant and susceptible Castor genotypes:

No. and name of genotypes	LOX activity (Units min^{-1} g^{-1} protein)							
	Pre infection (0 day)		Post infection (5 days)		Post infection (10days)			
	Leaf NI	Root NI	Leaf NI	Leaf I	Leaf NI	Leaf I	Root NI	Root I
Susceptible VP-1	15.46	82.66	131.27	179.70	69.43	77.54	12.81	13.30
VI-9	15.58	81.18	139.45	185.70	72.46	76.43	12.16	15.44
Resistant 48-1	19.47	92.27	178.48	246.14	88.35	98.78	19.69	25.23
SKP-84	18.32	93.72	191.80	284.73	92.86	104.48	21.44	28.22
S. Em.	0.01	0.43	1.20	1.18	0.85	0.87	0.34	0.24
C.D. at 5%	0.04	1.29	3.60	3.53	2.55	2.60	1.01	0.73
C.V.%	0.13	0.85	1.30	0.91	1.82	1.68	3.53	2.05

Pre-infection at 20 DAS, Post-infection at 25 and 30 DAS (5 and 10 days after infection DAI) FW = Fresh weight I= Infected, NI= Non Infected

Alternatively, LOX catalyzed reactions can result in the production of toxic volatile and non-volatile fatty acid derived secondary metabolites that can directly attack invading pathogens (Croft *et al.*, 1993). ROS (H_2O_2 and O_2) activity is frequently to cause membrane damage through peroxidation of fatty acids, which conceded with a high activity of lipoxygenase in tomato leaves as the results of *Fusarium oxysporum* infection. Levels of MDA and LOX activity gradually increased in leaves of infected tomato plants with increasing the time of infection (El-Khallal, 2007). Resistant genotypes of pigeon pea inoculated with *Fusarium udum* and increased LOX activity at 2-8 day after inoculation as compared to the un-inoculated ones. Increased enzyme activity was prominent at 4-days and at 6-days increasing trend was maintained although

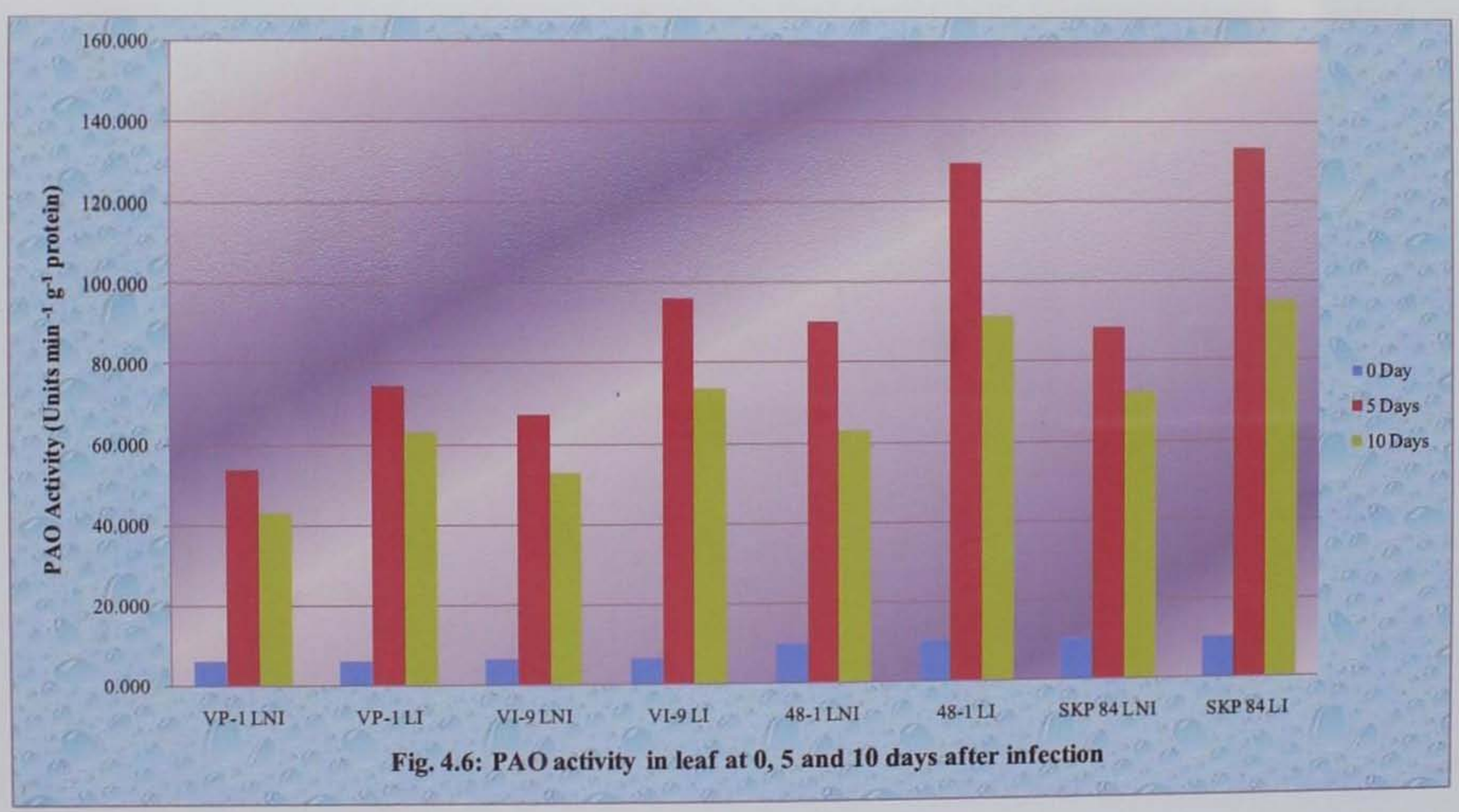


Fig. 4.6: PAO activity in leaf at 0, 5 and 10 days after infection

VP-1, VI-9, 48-1, SKP-84 – Genotypes

Note: L= Leaf, R= Root, NI= Non infected, I= Infected

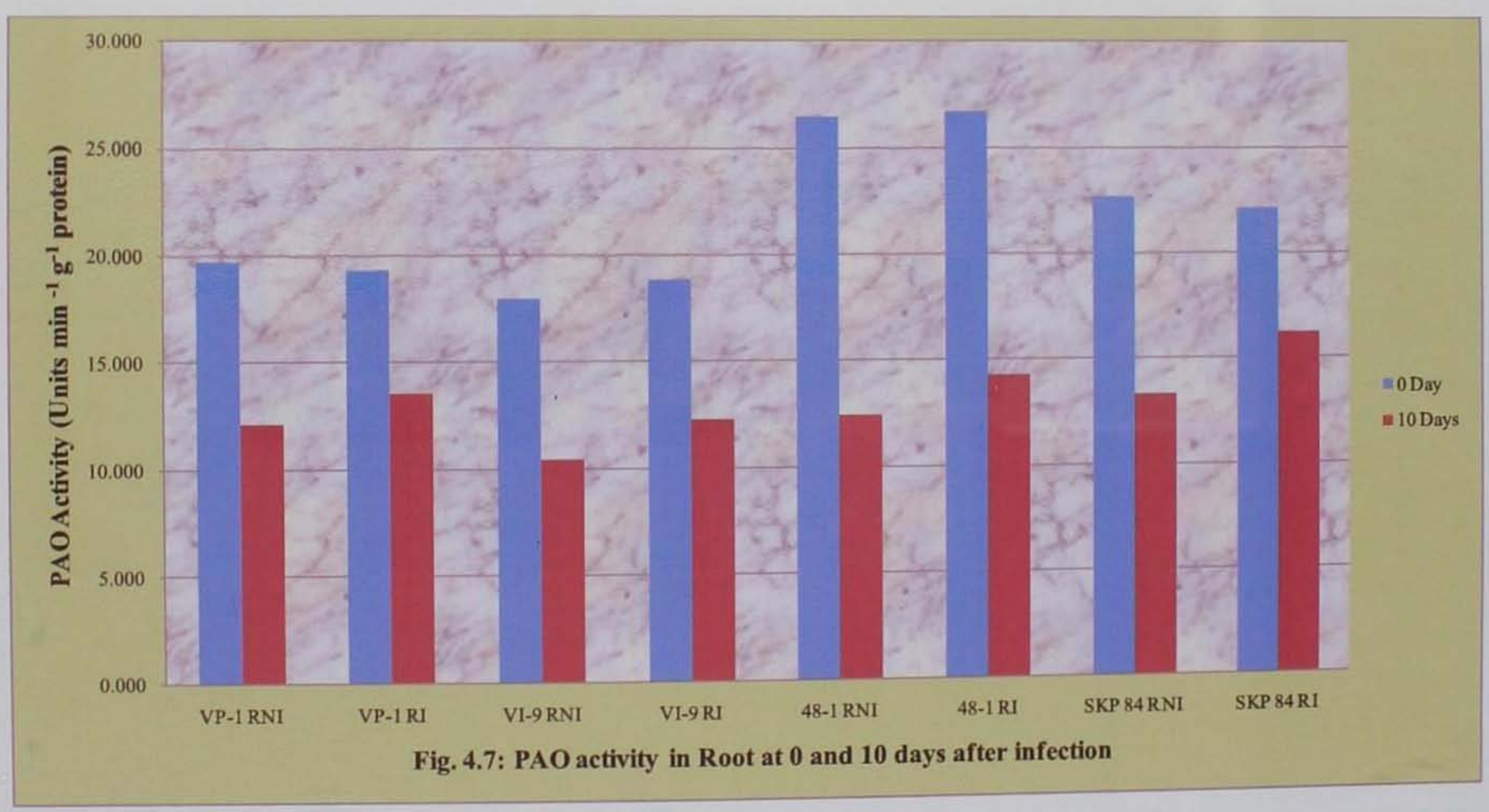


Fig. 4.7: PAO activity in Root at 0 and 10 days after infection

VP-1, VI-9, 48-1, SKP-84 – Genotypes

Note: L= Leaf, R= Root, NI= Non infected, I= Infected

there was slight decline in activity at 8 days (Mandal and Sinha, 2009). A higher level of LOX activity was observed in resistant pigeon pea cultivar than in susceptible cultivar LOX activity, in the *Fusarium udum* inoculated resistant cultivar (ICP-8863), increased progressively starting from 1st day of infection, reached maximum by 4th day and declined by gradually thereafter. (Devi *et al.*, 2000).

4.2.1.2 Polyamine Oxidase (PAO) Activity:

At pre-infection, PAO activity was higher in the resistant genotypes (10.30-10.48 Units min-1 g-1 protein) compared to susceptible genotypes (6.42-6.94 Units min-1 g-1 protein) in leaf and same way PAO activity was higher in the in root of resistant genotypes (22.68-26.44 Units min-1 g-1 protein) compared to susceptible genotypes (18.01-19.77 Units min-1 g-1 protein). PAO activity (Table 4.5) was drastically increased in the infected leaves of both resistant and susceptible genotypes as compared to their non-infected leaves at 5 DAI (Fig.4.6). But at this stage, constitutive levels of PAO in resistant genotypes were about 24-69 % higher than the susceptible genotypes. PAO activity was also higher in infected roots of all genotypes than non-infected roots (Fig.4.7). However, roots of resistant genotypes had significantly higher activity than susceptible genotypes. Similarly increased activities of the catabolic enzymes DAO and PAO were observed during HR induction in powdery mildew resistant varieties of barley at 1-3 days after inoculation (Cowley and Walters 2002b). PAO activity was decline significantly in resistant and susceptible genotypes at 10 DAI but still it was higher in infected leaves as compared to non-infected leaves of 5 DAI. This decline in PAO activity might be attributed due to age of plant.

Table 4.5: Pre- and post-infectional changes of enzyme activity in wilt resistant and susceptible Castor genotypes:

No. and name of genotypes	PAO activity (Units min^{-1} g^{-1} protein)							
	Pre infection (0 day)		Post infection (5 days)		Post infection (10days)			
	Leaf NI	Root NI	Leaf NI	Leaf I	Leaf NI	Leaf I	Root NI	Root I
Susceptible VP-1	6.42	19.77	53.77	74.66	43.18	63.05	12.18	13.63
VI-9	6.94	18.01	67.69	96.53	53.22	74.00	10.49	12.31
Resistant 48-1	10.30	26.44	90.29	129.98	63.15	91.65	12.50	14.32
SKP-84	10.48	22.68	88.89	133.62	72.42	95.30	13.37	16.22
S. Em.	0.06	0.24	0.81	1.02	0.87	0.87	0.28	0.15
C.D. at 5%	0.18	0.72	2.42	3.05	2.61	2.61	0.84	0.44
C.V.%	1.23	1.92	1.86	1.62	2.60	1.86	4.00	1.79

Pre-infection at 20 DAS, Post-infection at 25 and 30 DAS (5 and 10 days after infection DAI) FW = Fresh weight I= Infected, NI= Non Infected

In present study higher activity of PAO in resistant genotypes reflects lignifications of tissues, and is spatially and temporally associated with cell wall strengthening reactions involving cell wall peroxidases (Rodriguez *et al.*, 2008). Resistant genotypes showed the first line of defense against the pathogenic challenge which is associated with the plant cell wall, in which certain modifications take place during attack, respecting the rule "the harder the better." Similarly, Yoda *et al.*, 2003 and 2006 reported that in *Nicotiana tabacum* plants resistant to TMV, *PAO* expression and PA tiers increased in tissues exhibiting TMV-induced HR. Cell death caused by TMV infection or cryptogein, an oomycete-originated elicitor, was partially mediated by H_2O_2 generated through PA catabolism. Interestingly, Kusano *et al.*, 2008 suggested that H_2O_2 produced by PAO could trigger activation of Mitogen Activated Protein Kinases

(MAPKs). *PAO* genes in Arabidopsis are upregulated by elicitors, such as flagellin, thus promoting probably activation of MAPKs involved in pathogen signal perception. Plants employ polyamine catabolism-derived H_2O_2 as a defensive tool upon exposure to biotic stresses has been reviewed by other groups (Walters, 2003.; Cona *et al* 2006)

The chickpea and soybean genotypes were exposed to water stress, Furthermore, Put, Spd and Spm began to rise at the 2nd day of stress and peaked at 4th day in soybean and on 5th day in chickpea. Thereafter, polyamines level began to decline significantly which was accompanied by the accentuated stress injury (Xiao-Ming Pang *et al.*, 2007). Polyamines were found to accumulate in apoplast of infected tobacco leaves and to be degraded by polyamine oxidase, releasing hydrogen peroxide that causes hypersensitive cell death (Yoda, *et al.*, 2006).

4.3 Molecular Characterization:

4.3.1 Total RNA Extraction:

Intact total RNA were run on a 1.5% denaturing gel which showed sharp 28S and 18S rRNA bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band and the 2:1 ratio (28S:18S) is a good indication that the RNA is intact (Fig. 4.8).

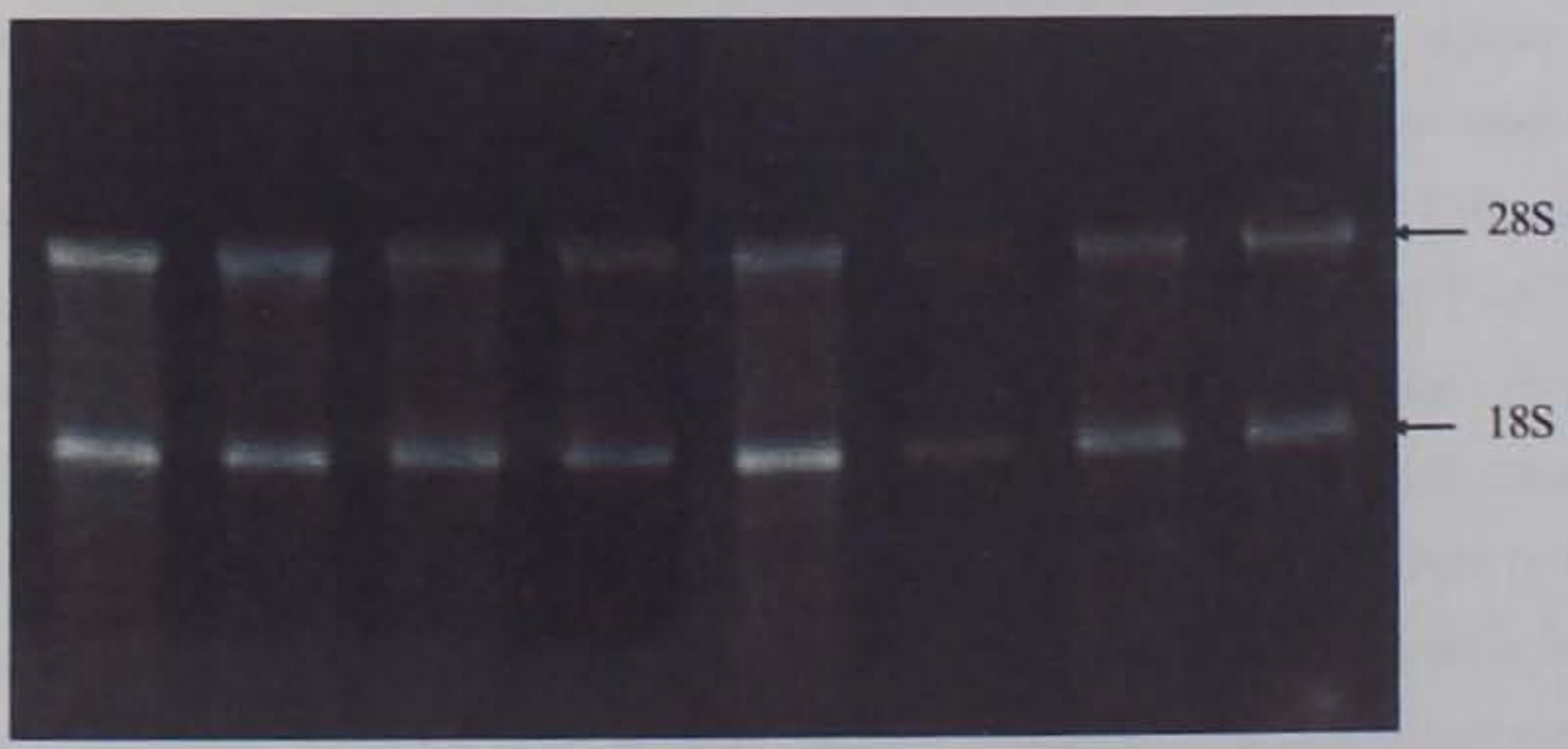
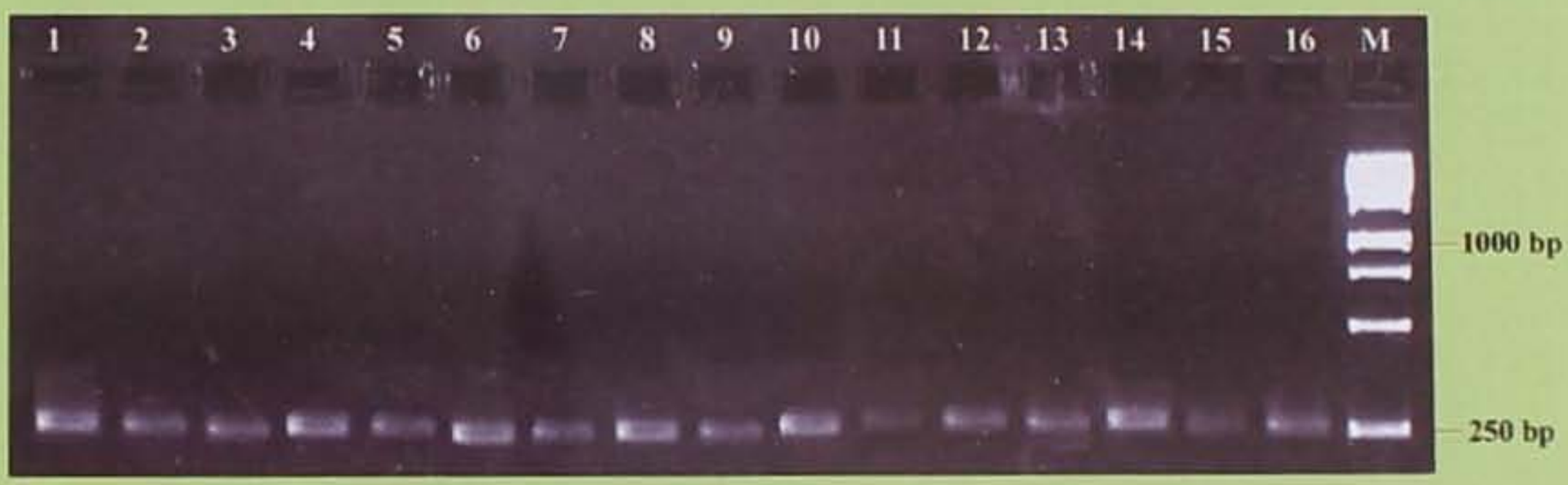


Figure 4.8: Intact total RNA were run on a 1.5% denaturing gel.

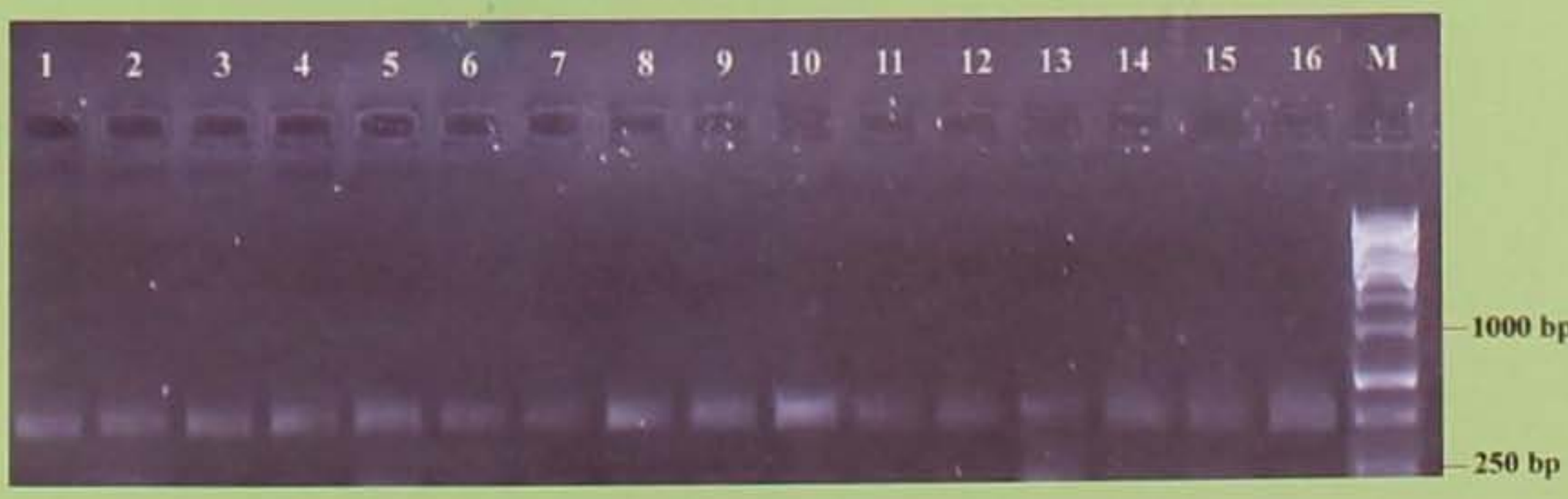
4.3.2 Amplification of LOX genes and its analysis:

LOX gene specific primers were designed by homology based search at NCBI. Total twelve lox genes were available on the data base out of which 6 Lox gene specific primers were designed to amplify castor LOX genes i.e. LOX1, LOX2, LOX3, LOX4, LOX5 and Dox. All these primers were successfully amplified with the cDNA of castor resistant and susceptible varieties for wilt pathogen interaction (Fig. 4.9 and 4.10). Amplified products of resistant genotype 48-1 were purified and then sequenced on genetic analyzer (Applied Biosystem). The obtained sequences of 6 LOX genes were run on BLAST search tool available at NCBI. Except the LOX1, all other genes ie. LOX3, LOX5, and LOX4 were showed more than 90 % identity with castor LOX genes and gene Dox gave 97 % identity with unknown gene of *Oxidoreductase* class. (Appendix I). Translation of the unique open reading frame present in LOX2, LOX3, LOX4 and LOX5 indicated that they codes for an 900, 837, 868 and 786 amino acid protein with an estimated molecular mass of 101.90, 95.03, 98.94 and 89.24 kD, respectively. Analysis of the deduced amino acid sequences of LOX2, LOX3, LOX4 identified conserved LOX domains for plant LOX-related proteins: the PLAT (for polycystein-1, LOX, α-toxin) or LH2 (for lipoxygenase homology 2) domain. It consists of an eight stranded beta-barrel. The domain can be found in various domain architectures, in case of lipoxygenases, alpha toxin, lipases and polycystin; but also as a single domain or as repeats. The putative function of this domain is to facilitate access to sequestered membrane or micelle bound substrates. Analysis of sequences of Lox 5 identified PLAT/ LH2 domain of plant lipoxygenase related proteins. The generally proposed function of PLAT/LH2 domains is to mediate interaction with lipids or membrane bound proteins. Lipoxygenases are nonheme, nonsulfur iron dioxygenases that act on lipid substrates containing one or more (Z,Z)-1,4-pentadiene moieties. In plants, the immediate products are involved in defense mechanisms against pathogens and may be precursors of metabolic regulators. Only LOX5 protein contains conserved His (Histidine) residues (positions 547, 556, and 715) that are also observed in other plant LOXs and that have been implicated in iron binding and enzyme catalytic activity. The other genes (LOX 3 and LOX 4) did not contain His residue at conserved position. However, LOX 2 contained His residue at 548, 553 and 717 position. The His residues that act as ligands

Amplified product of 16 controls (RNA) with gene specific primer LOX 2



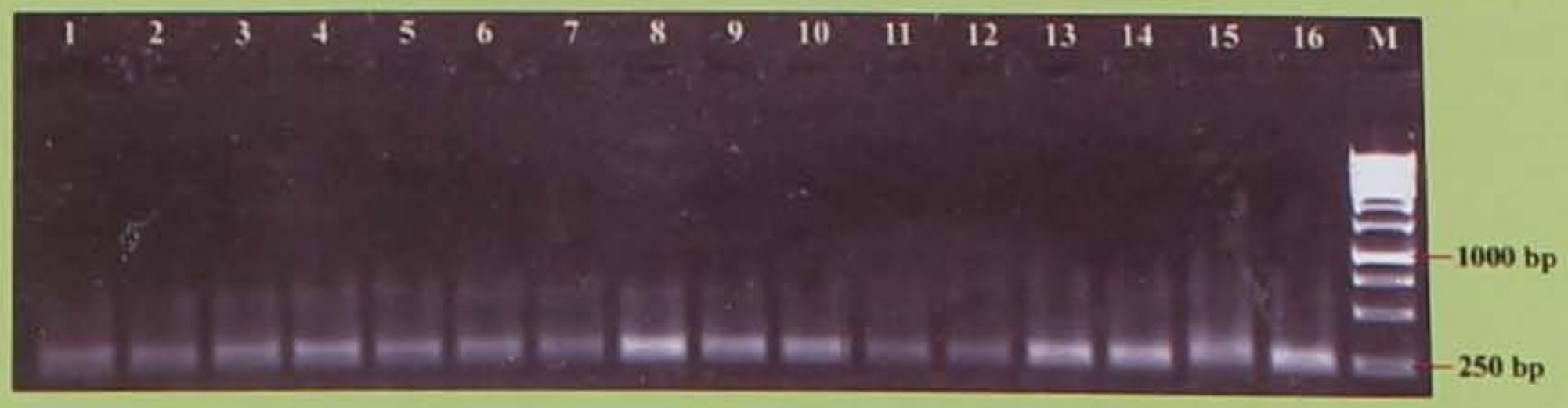
Amplified product of 16 cDNAs with gene specific primer LOX 2



Amplified product of 16 cDNAs with gene specific primer LOX 3

Figure 4.9 : Amplification products of cDNA with LOX gene specific primer

1, 3, 5, 7 - Non infected leaf cDNA of VP-1, VI-9, 48-1 and SKP-84 at 5 DAI
2, 4, 6, 8 - Infected leaf cDNA of VP-1, VI-9, 48-1 and SKP-84 at 5 DAI
9, 11, 13, 15 - Non infected leaf cDNA of VP-1, VI-9, 48-1 and SKP-84 at 10 DAI
10, 12, 14, 16 - Infected leaf cDNA of VP-1, VI-9, 48-1 and SKP-84 at 10 DAI
M - Molecular weight marker (Fermentas # SM0313), DAI - Days after infection



Amplified product of 16 cDNAs with gene specific primer LOX 4

Amplified product of 16 cDNAs with gene specific primer LOX 5

Amplified product of 16 cDNAs with gene specific primer DOX

Amplified product of 16 cDNAs with primer 18S

Figure 4.10 : Amplification products of cDNA with LOX gene specific primer

1, 3, 5, 7 - Non infected leaf cDNA of VP-1, VI-9, 48-1 and SKP-84 at 5 DAI
2, 4, 6, 8 - Infected leaf cDNA of VP-1, VI-9, 48-1 and SKP-84 at 5 DAI
9, 11, 13, 15 - Non infected leaf cDNA of VP-1, VI-9, 48-1 and SKP-84 at 10 DAI
10, 12, 14, 16 - Infected leaf cDNA of VP-1, VI-9, 48-1 and SKP-84 at 10 DAI
M - Molecular weight marker (Fermentas # SM0313), DAI - Days after infection

to the active site iron are highly conserved in LOXs (Prigge *et al.*, 1996). The Castor LOX protein contains conserved His residues involved in catalysis, as observed in other LOXs (Hwang.and Hwang, 2010).

Dendrogram of nucleotide (Fig.4.11) and protein (Fig. 4.12) sequences of all LOX genes were constructed online using CLUSTALW programme on the basis of sequence alignment (Appendix-II and III) of genes, which showed LOX3 and LOX4 was on same sub-cluster of Cluster-I while and LOX2 was found on other sub-cluster. LOX5 was totally different and laid on separate cluster. These results imply the distinct function of LOX5 in disease resistance.

4.3.3 Expression analysis of lipoxygenase gene(s):

Expression analysis of lipoxygenase genes in response to wilt pathogen interaction was examined in infected and non-infected leaves using 4 LOX gene specific primers ie. LOX2, LOX3, LOX4 and LOX5 and one unknown gene of Oxidoreductase (Table 4.6). Expression of LOX2, 3, 4, 5 and DOX were induced 5 and 10 days after infection. Highest expression of LOX genes were observed (Fig.4.13) in resistant genotypes at 5 DAI. Resistant genotypes also showed higher gene expression at 10 DAI. Both resistant genotypes (48-1 and SKP-84) exhibited appreciably higher expression of LOX5, which is responsible for defense mechanism. These results of expression analysis were further confirmed by sequence analysis of LOX5. The expression of LOX5 was maintained in 48-1 genotyped at 10 DAI. Expression of LOX2 was also higher in both resistant genotypes at 5 DAI. LOX2 was laid on separate sub cluster of same cluster in dendrogram of genes on the basis of sequence alignment and shared maximum higher similarity with LOX5.

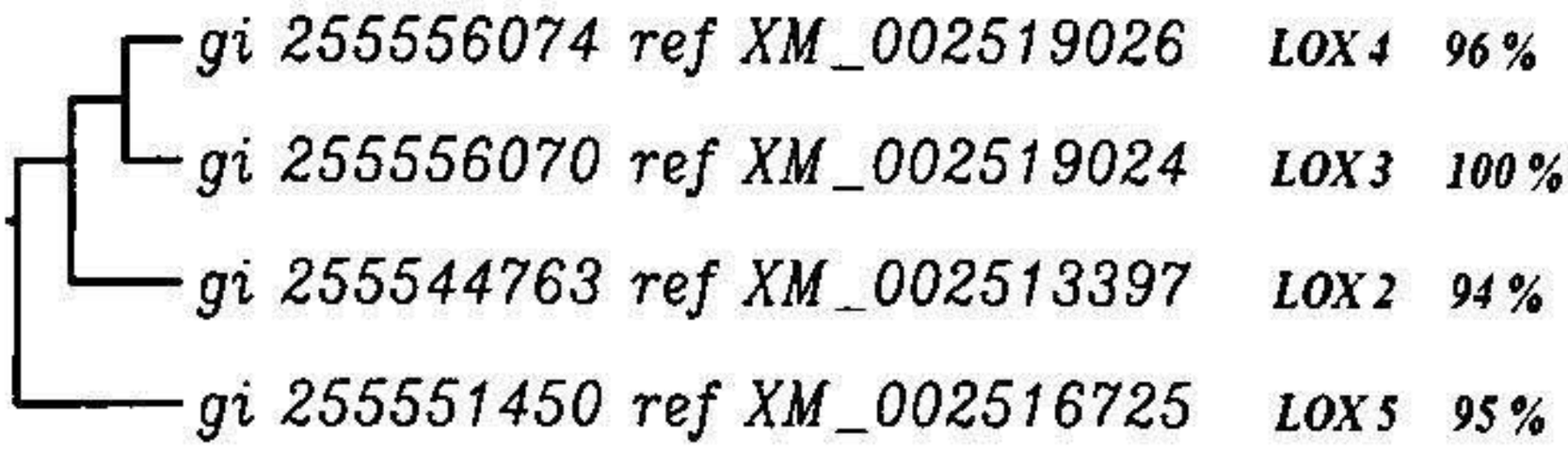


Figure 4.11: Dendrogram of LOX genes on the basis of alignment of nucleotide sequence on CLUSTALW programme

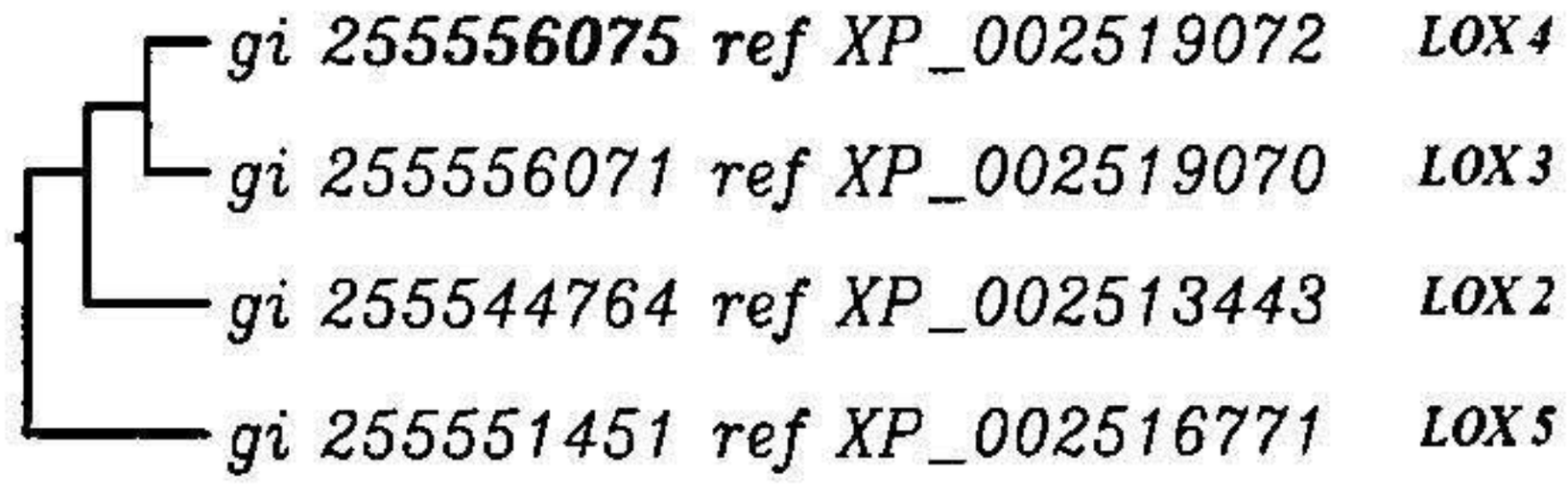


Figure 4.12: Dendrogram of LOX genes on the basis of alignment of protein sequence on CLUSTALW programme

Fig: 4.13 Comparative real time PCR results expressed in fold increase of lipoxygenase genes (LOX2, 3, 4, 5 and DOX) in susceptible and resistant Castor genotypes in response to wilt pathogen interaction.

VP-1, VI-9, 48-1, SKP-84 – Genotypes

Note: L= Leaf, R= Root, NI= Non infected, I= Infected

Table 4.6: mRNA expression relative to infected and non-infected leaves (Fold variation of LOX gene)

Sr. No.	Days	Genotypes	Primers	Sample Mean C	Sample Mean T	Sample Mean C/18s Mean C	Sample Mean T/18s Mean T	ΔΔCT	Fold expression $2^{-\Delta\Delta CT}$
1	5 DAI	VP-1 S	Lox2	21.76	23.425	1.97	2.02	-1.155	2.23
2			Lox3	26.34	28.55	2.38	2.47	-1.7	3.25
3			Lox4	21.19	22.375	1.29	1.52	-0.675	6.84
4			Lox5	14.25	17.535	2.48	2.45	-2.775	1.36
5			Dox	27.425	28.38	1.92	1.93	-0.445	1.60
6	5 DAI	VI-9 S	Lox2	20.255	23.075	1.86	1.94	-1.82	3.53
7			Lox3	27.505	29.005	2.53	2.44	-0.5	1.41
8			Lox4	21.06	22.86	1.32	1.31	-0.8	1.15
9			Lox5	14.405	15.605	2.38	2.36	-0.2	2.17
10			Dox	25.89	28.01	1.94	1.92	-1.12	1.74
11	5 DAI	48-1 R	Lox2	21.895	24.34	1.67	1.72	-1.455	2.74
12			Lox3	27.43	33.07	2.09	2.34	-4.65	25.11
13			Lox4	21.485	24.77	1.14	1.52	-2.295	44.17
14			Lox5	14.985	21.44	2.00	1.96	-5.465	1.42
15			Dox	26.275	27.77	1.63	1.75	-0.505	4.91
16	5 DAI	SKP-84 R	Lox2	22.925	26.05	2.05	1.70	-1.755	3.38
17			Lox3	29.15	33.58	2.61	2.20	-3.06	8.34
18			Lox4	21.501	25.385	1.33	1.19	-2.5	4.06
19			Lox5	15.85	17.04	2.40	1.88	-0.185	1.44
20			Dox	26.845	28.745	1.93	1.66	-0.53	5.66
21	10 DAI	VP-1 S	Lox2	24.46	25.695	1.95	2.09	-1.48	2.79

22			Lox3	28.565	30.845	2.27	2.50	-2.525	5.76
23			Lox4	17.35	18.34	1.31	1.50	-1.235	4.87
24			Lox5	16.5	18.54	2.10	2.34	-2.285	6.73
25			Dox	26.34	28.845	1.38	1.49	-2.75	2.35
26	10 DAI	VI-9 S	Lox2	15.265	19.11	1.36	1.54	-2.74	6.68
27			Lox3	21.28	25.29	1.89	2.04	-2.905	7.49
28			Lox4	23.105	27.915	1.35	1.58	-3.72-05	9.88
29			Lox5	15.19	19.6	2.38	2.36	-3.305	2.54
30			Dox	26.8	29.25	2.05	2.26	-1.345	13.04
31	10 DAI	48-1 R	Lox2	15.645	20.215	1.41	1.79	-4.405	21.19
32			Lox3	24.165	27.695	2.17	2.45	-3.365	10.30
33			Lox4	23.715	24.79	1.52	1.98	-0.91	37.66
34			Lox5	16.935	22.335	2.49	2.64	-5.235	4.10
35			Dox	27.665	29.865	2.13	2.19	-2.035	1.88
36	10 DAI	SKP-84 R	Lox2	21.06	28.08	1.88	2.30	-6.025	65.12
37			Lox3	27.245	29.24	2.43	2.39	-1	2.00
38			Lox4	21.355	23.05	1.30	1.74	-0.7	52.71
39			Lox5	14.6	21.315	2.01	2.24	-5.72	14.98
40			Dox	22.52	27.42	1.90	1.89	-3.905	1.62

Note: DAI- Days after infection, S- Susceptible, R- Resistant.

These results are consistent with LOX enzyme activity in present study and suggesting that LOX is critically involved in the defense response. Similarly, 9-LOX activity and Lox1 mRNA expression are induced upon infection by *Phytophtora parasitica* var *nicotianae* in tobacco. Interestingly, both 9-LOX activity and Lox1 mRNA expression appear earlier in an incompatible plant-pathogen interaction than in a compatible one, thus supporting a role for this 9-LOX in plant defense against fungal infection (Rance *et al.*, 1998).

Plant LOXs have been proposed to play a role in responses to wounding and JA, which triggers gene activation during wound response and necrotrophic fungal pathogen infection (Blee, 2002). One such pathogen, *A.brassicicola,* kills host cells at early stages of infection causing tissue damage (Glazebrook, 2005). Infection with *A. brassicicola* resulted in a resistance response in wild-type and CaLOX1-OX transgenic leaves. These resistance symptoms are consistent with the HR in Arabidopsis Col-0 plants infected by *A. brassicicola* (Narusaka *et al.*, 2003). In contrast, lox1 mutants were highly susceptible to *A. brassicicola*. The remarkable involvement of CaLOX1 and AtLOX1 in mediating resistance to pathogen attack suggests that these LOX genes are highly conserved for disease resistance in plants. In the present investigation higher activity of PAO, lipid peroxidation, and higher expression of LOX5 in resistant genotypes of castor plants may be involved in the H_2O_2 mediated execution of cell death and defense response to wilt pathogen.

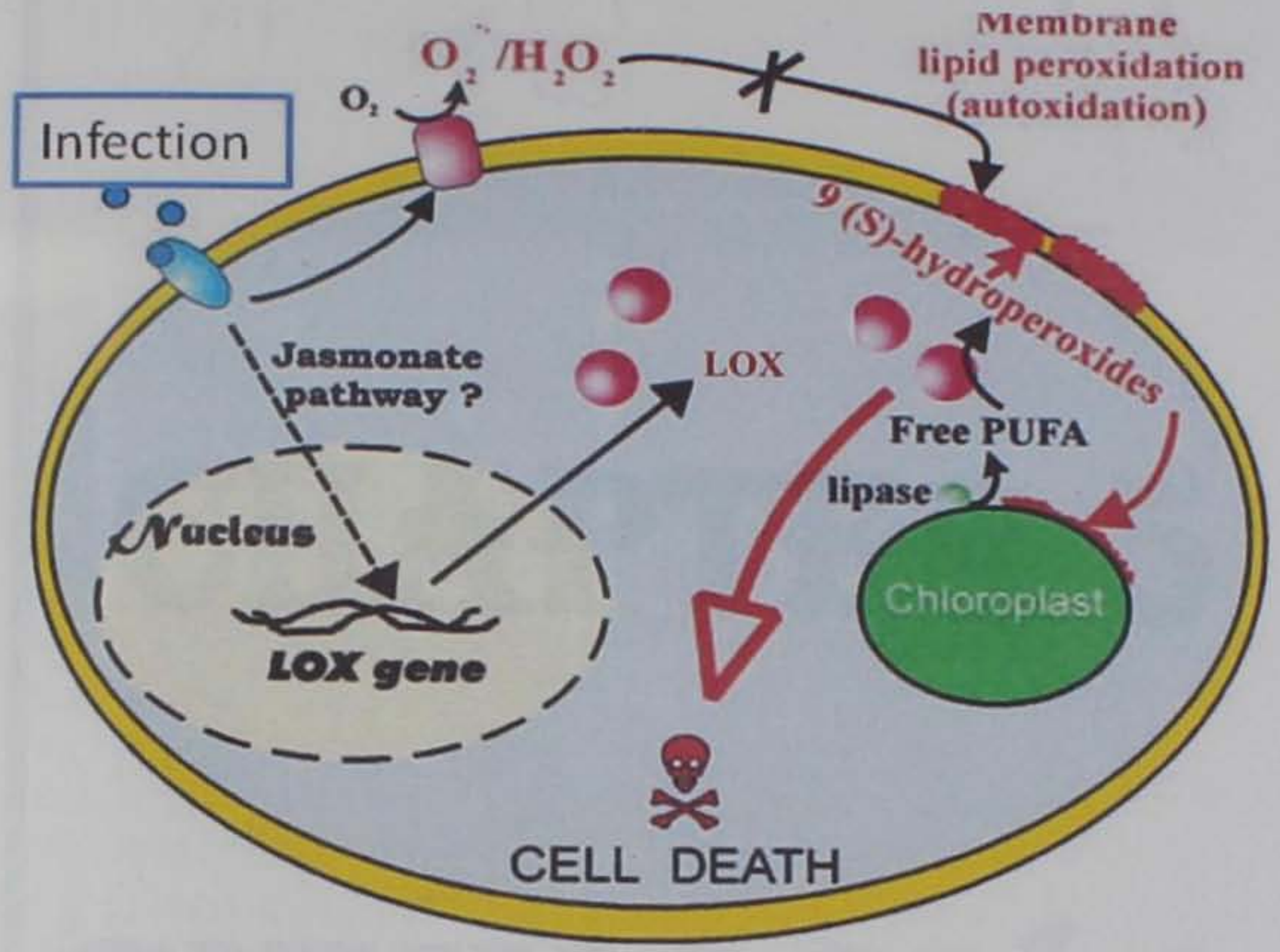


Fig. 4.14: Schematic overviews of the hypothetical mechanism for free fatty acid hydroperoxide production induced by *Fusarium oxysporium* f. sp. *ricini* on Castor and leading to cell death.

Massive free PUFA hydroperoxide formation from membrane PUFAs was shown to induce HR necrotic symptoms. The production was attributed to the conjunction of an increase in LOX and putative lipase activities. Transient accumulation of LOX mRNA transcripts was described, and it was proposed that JA is likely involved in the regulation of this process. Membrane autoxidation, being a minor and late process, was likely attributed to the effects of PUFA hydroperoxides, rather than to AOS, produced early during the oxidative burst.

CHAPTER 5

SUMMARY & CONCLUSION

V. SUMMARY AND CONCLUSION

The present investigation on 'Characterization of LOX gene and polyamines in Castor (*Ricinus communis* L.) during wilt pathogen interaction" is summarized and concluded in this chapter.

Castor (*Ricinus communis* L.) is the industrially important non edible oilseed crop and this crop is affected by several biotic and a biotic stresses, which affect successful and profitable cultivation of the crop. Among them, diseases are major constrain for higher yield. Castor wilt (*Fusarium oxysporum* f. sp. *ricini*), is a serious problem in Gujarat state and causes heavy losses up to 80 per cent wilt incidence in North Gujarat.

All biochemical parameters were analyzed in infected and non infected plants at three stages: (i) pre-infection [20 days after sowing (DAS), only non infected plants (0 DAI)] and (ii) post-infection [5 and 10 days after infection (DAI) i.e. 25 & 30 DAS].

The constituent of two metabolites were analyzed at above mentioned different stages. The results showed that MDA content was higher in susceptible genotypes compared to resistant genotypes at pre-infection stage. While after infection, the MDA content was increased in infected leaves of resistant and susceptible genotypes as compared to their non-infected leaves. However, leaves of infected resistant genotypes had about 1.5-1.7 times more MDA content than infected susceptible genotypes and non-infected resistant genotypes. The MDA content was decline significantly in infected and non-infected tissues of both resistant and susceptible genotypes at 10 DAI compared to 5 DAI. However, the MDA content was higher in both leaf and root tissues of resistant genotypes at 10 DAI as compared to pre infection. The results of polyamines profiling showed that cadaverine was absent in any genotype of castor in all the stage of analysis. In the infected leaf tissues of susceptible genotypes putrescine, spermidine and spermine was increased at 5 DAI while these polyamines were decreased in resistant genotypes compared to their non-infected leaves. The levels of spermidine and spermine was increased in infected leaves of resistant genotypes at 10 DAI whereas reverse trend was observed in susceptible genotypes. The resistant genotypes had 2-3 times higher content

of spermidine and spermine. Remarkably, putrescine was only present in infected roots of resistant genotypes of castor and these genotypes also had higher percent area of spermidine. Therefore, high titers of polyamines in the incompatible interaction suggesting their involvement in fungal resistance. Resistant genotypes maintain the PAs and PAO homeostasis in both infected and non-infected tissues.

The enzymes lipoxygenase (LOX) and polyamine oxidase (PAO) which are related to reactive oxygen species generation were studied. At both pre-infection (0 day) and post-infection (5 DAI), LOX activity was higher in the infected and non-infected resistant genotypes compared to susceptible genotypes in leaf as well as roots. The LOX activity was decline significantly in resistant and susceptible genotypes at 10 DAI. Increased LOX activity may also cause irreversible membrane damage, which would lead to the leakage of cellular contents and conceded with a higher MDA content. Similarly, at pre-infection, PAO activity was higher in the resistant genotypes compared to susceptible genotypes in leaf and also in root. At 5 DAI stage, constitutive levels of PAO in resistant genotypes were about 24-69 % higher than the susceptible genotypes. PAO activity was decline significantly in resistant and susceptible genotypes at 10 DAI but still it was higher in infected leaves as compared to non-infected leaves of 5 DAI. The observed results of the polyamine oxidase (PAO) activity are accompanied with PAs in susceptible and resistant genotypes in present study.

Molecular characterization of castor for wilt pathogen interaction was done using Lox gene sequencing and expression analysis. To amplify the cDNA of castor LOX genes 6 primers were designed i.e. LOX1, LOX2, LOX3, LOX4, LOX5 and Dox. Amplified products of resistant genotype (48-1) were purified and then sequenced. Sequences of all these genes were BLAST on NCBI. Results of these sequences showed, except the LOX1, all other genes i.e. LOX3, LOX4, and LOX5 were showed more than 90 % identity with castor LOX genes and gene Dox gave 97 % identity with unknown gene of *Oxidoreductase* class. Analysis of the deduced amino acid sequences of LOX2, LOX3 and LOX4 identified conserved LOX domains for plant LOX-related proteins: the PLAT (for polycystein-1, LOX, α-toxin) or LH2 (for lipoxygenase homology 2) domain. The putative function of this domain is to facilitate access to sequestered membrane or micelle bound substrates. Similarly, sequence of LOX 5 also shows the presence of

PLAT/LH2 domain of plant lipoxygenase protein. The generally proposed function of PLAT/LH2 domains is to mediate interaction with lipids or membrane bound proteins. In plants, the immediate products are involved in defense mechanisms against pathogens and may be precursors of metabolic regulators. Only LOX5 protein contains conserved His (Histidine) residues (positions 547, 556, and 715) that are also observed in other plant LOXs. The dendrogram of nucleotide and protein sequences of all LOX genes showed LOX3 and LOX4 on same sub-cluster of cluster –I while LOX2 was on another sub-cluster. LOX5 was altogether different and was laid on cluster-II.

Expression analysis of lipoxygenase genes in response to wilt pathogen interaction was examined in infected and non-infected leaves using 4 LOX gene specific primers i.e. LOX2, LOX3, LOX4 and LOX5 and one unknown gene of *Oxidoreductase*. Highest expression of LOX2, 3, 4, 5 and DOX were induced at 5 days after infection. Both resistant genotypes (48-1 and SKP-84) exhibited appreciably higher expression of LOX5, which is responsible for defense mechanism; followed by LOX2. The expression of LOX5 was maintained in 48-1 genotyped at 10 DAI. These results are consistent with LOX enzyme activity in present study and suggesting that LOX is critically involved in the defense response.

In conclusion, resistant genotypes had higher content of lipid peroxidation product and polyamine content after infection. Constitutive levels of LOX and PAO were also higher in resistant genotypes and maintained after infection. These results showed the levels of MDA and polyamines accompanied with the activities of LOX and PAO, respectively. Both LOX and PAO plays important function for HR induction during incompatible interaction. Moreover, the expression level of LOX5 was also higher in the resistant genotypes which was further confirmed by sequence analysis and showed that LOX5 contained all conserved regions of Histidine residues.

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APPENDICES

Appendix I

Sequence details

a. Lox2 gene sequence of Castor (*Ricinus communis* L.) Genotype 48-1

TAGGAACAAAGTCCAATCCTTTCATTGTTCCTGGATCATATAGAATTCTCAGA
AGTGAAGGCGTACACGGTTTCGGAAGCGTCTCCTCAGTATTGCATGCATTGG
TCCCGCAGTAGAGACTGCACTGGTAGACCCTGACCTTGCATTTCCATACTTCA
CAGCCATAGATTCTCTATTCAATGAAGGATTCAACTTGCCCTCCACTTAAAG
AAAAAGGTTGGAAAGAA

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
XM_002513397.1	Ricinus communis lipoxygenase, putative, mRNA	298	298	51%	2e-77	94%

>ref|XM_002513397.1| Ricinus communis lipoxygenase, putative, mRNA
Length=2703

GENE ID: 8274394 RCOM_1576890 | lipoxygenase, putative [Ricinus communis]

Score = 298 bits (161), Expect = 2e-77
Identities = 188/200 (94%), Gaps = 6/200 (3%)
Strand=Plus/Minus

```
Quer33 TCTATGAGCTGTGTAGTATGGGAAATGCAAGGTCAGGG-CGAGAAATGCAAG-CGTCTAA 90
       |||||| |||||| ||||| ||||||||||||||||||| | |  | ||| || | |||||
Sb1040 TCTATG-GCTGTGAAGTAT-GGAAATGCAAGGTCAGGGTCTACCAGTGC-AGTC-TCTAA 985

Quer91 CTGCGGGACCAATGCATGCAATACTGAGTAGACTGTCTTCGCCGAAAACGTGTACTCCTT 150
       ||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
Sbj984 CTGCGGGACCAATGCATGCAATACTGAGTAGACTGTCTTCGCCGAAAACGTGTGCTCCTT 925

Que151 CACTTCTGAGAATTCTTCATCACGAGGAACATAGACGCTGCTGCTTATTGATTCAGAAAG 210
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbj924 CACTTCTGAGAATTCTTCATCACGAGGAACATAGACGCTGCTGCTTATTGATTCAGAAAG 865

Que211 TGTATCTGTGTTGCATCTTG 230
       ||||||||||||||||||||
Sbj864 TGTATCTGTGTTGCATCTTG 845
```

b. Lox3 gene sequence of Castor (*Ricinus communis* L.) Genotype 48-1

CTTTGGGACCCTCTGGAGCCAATACATCGAGCATGGATCATTTGCTGGAGAC
TCCTTGGCGACATCAGCAAAGAATGCTTTATATTTTGACGCGTGCATTCTGTT
TGTCCTGTTGTTCTACAGGCATATGCGATGCTTGTCTCTGAGAATCTTCGAAC
CGTAATACTGAAAAACAAAAGTAAACTCCATGCACGACTGAAAATGTTTTTT
TGATGTACGCCGCCGAAAATACTAAGGTGTCTTCAGCAAATTTTTCATGCCG
ATGTATGTGCCGTGAGACGAAGCACTTTATAGATGGCCAAGAATGTAGTCTC
TGAA

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
XM_002519024.1	Ricinus communis lipoxygenase, putative, mRNA	291	291	48%	3e-75	100%
XM_002519026.1	Ricinus communis lipoxygenase, putative, mRNA	279	279	48%	6e-72	98%

>ref|XM_002519024.1| Ricinus communis lipoxygenase, putative, mRNA
Length=2514

GENE ID: 8279511 RCOM_0937080 | lipoxygenase, putative [Ricinus communis]

Score = 291 bits (157), Expect = 3e-75
Identities = 157/157 (100%), Gaps = 0/157 (0%)
Strand=Plus/Minus

```
Que170 CTGTAGAACTTGGAGGACGTCCAGTTCGACAACGCCTTGGATATGGAAGCATTTCACCTC 229
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbj664 CTGTAGAACTTGGAGGACGTCCAGTTCGACAACGCCTTGGATATGGAAGCATTTCACCTC 605

Que230 CTAAAATTGGCCTATTATATTCCATCCCTTTGTCAGGATTTCCCAAGTCTTTATACGTAT 289
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbj604 CTAAAATTGGCCTATTATATTCCATCCCTTTGTCAGGATTTCCCAAGTCTTTATACGTAT 545

Que290 CATAGTCATAAATTCTATCGCACAATTTTCTAATTCC 326
       |||||||||||||||||||||||||||||||||||||
Sbj544 CATAGTCATAAATTCTATCGCACAATTTTCTAATTCC 508
```

>ref|XM_002519026.1| Ricinus communis lipoxygenase, putative, mRNA
Length=2361

GENE ID: 8279513 RCOM_0937100 | lipoxygenase, putative [Ricinus communis]

Score = 279 bits (151), Expect = 6e-72
Identities = 155/157 (99%), Gaps = 0/157 (0%)
Strand=Plus/Minus

```
Que170 CTGTAGAACTTGGAGGACGTCCAGTTCGACAACGCCTTGGATATGGAAGCATTTCACCTC 229
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbj505 CTGTAGAACTTGGAGGACGTCCAGTTCGACAACGCCTTGGATATGGAAGCATTTCACCTC 446

Que230 CTAAAATTGGCCTATTATATTCCATCCCTTTGTCAGGATTTCCCAAGTCTTTATACGTAT 289
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbj445 CTAAAATTGGCCTATTATATTCCATCCCTTTGTCAGGATTTCCCAAGTCTTTATACGTAT 386

Que290 CATAGTCATAAATTCTATCGCACAATTTTCTAATTCC 326
       |||||||||| ||||| ||||||||||||||||||||
Sbj385 CATAGTCATATATTCTGTCGCACAATTTTCTAATTCC 349
```

c. Lox4 gene sequence of Castor (*Ricinus communis* L.) Genotype 48-1

GCCCTCCATCCCCCCCGCAGTTATGTGCCGCGCAAGTATGCAATATGAAGATT
GCAAAAGAGCAGATATATCTGTTGGGAAGTTGAAGCGGTGGCTAAAAATCT
AGTTCCTGGTCTAAGAAATGGCTCTATTGAAAGCGATTATATCAAGGAATTC
TCTGAAATTAATCATCTCTATAAAAAGAGGAGTAGTGTTGGAGAAAAGTCAA
AGAAAAGAATGTTCCAAAAGCCTCCTTTTTTTAAAAAAAATGGGGTGGGAGG
TCGGTGAAAACCCTTTAAAAAACTTTTTTAAACGGAAAAAATTTTAGCCCCC
TTCCCCCCCCCAAAAATTTTTCTCTTTAAACTCGAACCCCCCCCCGAAAAAGTTT
AGGGTTTTTGGAAAAAAAAATAGGAGGTGAAAATGTGTGTTTGTTGTTAAAA
TTCTCCCACCCAGTG

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
XM_002519026.1	Ricinus communis lipoxygenase, putative, mRNA	326	326	43%	1e-85	97%

>ref|XM_002519026.1| Ricinus communis lipoxygenase, putative, mRNA
Length=2361

Score = 326 bits (176), Expect = 1e-85
Identities = 186/190 (98%), Gaps = 3/190 (2%)
Strand=Plus/Plus

```
Quer42 ATATGAAGATTGCAAAAGAGCAGATATATCTGTTGGGAAGTTG-AAGCGGTGGCTAAAAA 100
       |||||||||||||||||||| |||||||||||||||||||||| |||||||||||||||||
Sbj567 ATATGAAGATTGCAAAAGAGAAGATATATCTGTTGGGAAGTTGAAAGCGGTGGCTAAAAA 626

Que101 TCTAGTTCCTGGTCTAAGAAATGGCTCTATTGAAAGCGATTATATCAAGGAATTCTCTGA 160
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbj627 TCTAGTTCCTGGTCTAAGAAATGGCTCTATTGAAAGCGATTATATCAAGGAATTCTCTGA 686

Que161 AATTAATCATCTCTATAAAAAGAGGAGTAGTGTTGGAGAAAAGTCAAAGAAAAGAATGTT 220
       |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
Sbj687 AATTAATCATCTCTATAAAAAGAGGAGTAGTGTTGGAGAAAAGTCAAAGAAA-GAATGTT 745
```

```
Que221 CCAAAAGCCT 230
       |||||| |||
Sbj746 CCAAAA-CCT 754
```

d. Lox5 gene sequence of Castor (*Ricinus communis* L.) Genotype 48-1

GCCTATATTTTTCAAAATCAACTTTATGACTGAAGCTGTGTAGCATGGGAGTT
CCTGGCGCTTTTATAATTAGAATACCAATATACCGTTATACCTCAAACAGTA
ACTTTAGAGAGTTCCTGGGCATGGTAGGGTACATTTTGTCTGCAATTCTTGGG
TGTATCCAGCTCATTGTTACAATTATGATCGAGTCTTCTTCTCTAAAAGACCT
ACCTTCCATGAA

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
XM_002516725.1	Ricinus communis lipoxygenase, putative, mRNA	276	276	76%	5e-71	95%

>ref|XM_002516725.1| Ricinus communis lipoxygenase, putative, mRNA
Length=2607

GENE ID: 8261460 RCOM_0677750 | lipoxygenase, putative [Ricinus communis]

Score = 276 bits (149), Expect = 5e-71
Identities = 167/175 (95%), Gaps = 3/175 (2%)
Strand=Plus/Plus

```
Quer53 CTGGCGCTATTATAATTAGAAATC-TCATCATAGACAGTTATACCTCAGAACAGTAACTT 111
       |||| ||| ||||||||||||||| |||||||| |||||||||||| ||||||||||
Sbj335 CTGGAGCTTTTATAATTAGAAATCATCATCATAGCCAGTTATACCTCAAAACAGTAACTT 394

Que112 TAGCTGATGTTCCTGGGCATGGTAGGGTACATTTTGTCTGCAATTCTTGGGTGTATCCAG 171
       ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbj395 TAGATGATGTTCCTGGGCATGGTAGGGTACATTTTGTCTGCAATTCTTGGGTGTATCCAG 454

Que172 CTCATTGTTACAATTATGATCGAGTCTTCTTCTCTA-CAAGAC-TACCTTCCATG 224
       |||||||||||||||||||||||||||||||||| |||||| ||||||||||
Sbj455 CTCATTGTTACAATTATGATCGAGTCTTCTTCTCTAACAAGACCTACCTTCCATG 509
```

Appendix II

LOX genes alignment of nucleotide sequence on CLUSTALW programme

CLUSTALW Result

[clustalw.aln][clustalw.dnd][readme]

```
 CLUSTAL 2.1 Multiple Sequence Alignments


Sequence type explicitly set to DNA
Sequence format is Pearson
Sequence 1: gi|255544763|ref|XM_002513397.1|  2703 bp
Sequence 2: gi|255556074|ref|XM_002519026.1|  2361 bp
Sequence 3: gi|255551450|ref|XM_002516725.1|  2607 bp
Sequence 4: gi|255556070|ref|XM_002519024.1|  2514 bp
Start of Pairwise alignments
Aligning...



Sequences (1:2) Aligned. Score: 43.7103
Sequences (1:3) Aligned. Score: 39.7775
Sequences (1:4) Aligned. Score: 42.6014
Sequences (2:3) Aligned. Score: 40.7878
Sequences (2:4) Aligned. Score: 90.4278
Sequences (3:4) Aligned. Score: 39.7772
Guide tree file created:   [clustalw.dnd]

There are 3 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences:   2      Score:42252
Group 2: Sequences:   3      Score:28765
Group 3: Sequences:   4      Score:28499
Alignment Score 51872

CLUSTAL-Alignment file created  [clustalw.aln]
```

```
clustalw.aln

CLUSTAL 2.1 multiple sequence alignment


gi|255556074|ref|XM_002519026.      ----------------------------------------------
gi|255556070|ref|XM_002519024.      ----------------------------------------------
gi|255544763|ref|XM_002513397.      ATGTTGAAGCCACAGGTCTACCAATCTCGGTCTCCAAAAACCCTGTTCTT
gi|255551450|ref|XM_002516725.      ----------------------------------------------

gi|255556074|ref|XM_002519026.      ----------------------------------------------
gi|255556070|ref|XM_002519024.      ----------------------------------------------
gi|255544763|ref|XM_002513397.      ATTGCCTAAACCCTTCATCCATGAAAATGGAGGCCAAACCCATCTTTCTG
gi|255551450|ref|XM_002516725.      ----------------------------------------------

gi|255556074|ref|XM_002519026.      ----------------------------------------------
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gi|255556070|ref|XM_002519024.  ----------------------------------------
gi|255544763|ref|XM_002513397.  TCCTTTCAAGGCCCTCATTACTTAAAACGCAGAGAAAGATTAGAGTCGGG
gi|255551450|ref|XM_002516725.  ----------------------------------------

gi|255556074|ref|XM_002519026.  ----------------------------------------
gi|255556070|ref|XM_002519024.  ----------------------ATGGAAGACGTATTGGAAATGCCTTT
gi|255544763|ref|XM_002513397.  TATAAACCAGGGAACGTACAAGCCATATCAGAAATTACAGAGAAGCAGCT
gi|255551450|ref|XM_002516725.  TGTGCGCAGCCTAGAAATCAGCCAAACGGCGGCACGGTGACAGAGAGATT

gi|255556074|ref|XM_002519026.  ----------------------------------------
gi|255556070|ref|XM_002519024.  CAAGCTCA---AGATGACAGCTCTAGTCAGTGTCAGGAA----CACTATG
gi|255544763|ref|XM_002513397.  CAAGTCTACAAAAGTGAAAGCTATAGTGACTGTGAACAG----AACTGTT
gi|255551450|ref|XM_002516725.  TAAGATCAAAGGAACAGTGGTTTTGATGAAGAAAAATGTTCTCGACTTCA

gi|255556074|ref|XM_002519026.  ----------------------------------------
gi|255556070|ref|XM_002519024.  TTTCATTGGGTGGAATGTTTCAGTAACCTTGGCCAGAAGCACAAGGGTAC
gi|255544763|ref|XM_002513397.  GGAGGGTTCTTGTCAAACCTAGGGATAAATAGAGGACTTGATGATGTTAC
gi|255551450|ref|XM_002516725.  GTGACATTAAAGCTTCATTTCTTGATCGAGTTCATGAGTTGCTGGGGAAA

gi|255556074|ref|XM_002519026.  ----------------------------------------ATG
gi|255556070|ref|XM_002519024.  TG--TTGTTTTGCAACTTGTTAGCAACGACATTGATC-----CAGGGATG
gi|255544763|ref|XM_002513397.  TGATTTGCTTGGCAAAACTCTCCTACTCGAGCTTGTCAGTGCTGAGCTTG
gi|255551450|ref|XM_002516725.  GGTGTTTCTATGCAGCTTATTAGTGCCGTACATCATGACCCAGCGAACAA

gi|255556074|ref|XM_002519026.  AAACCAAAGCTAAG-CAGCGAAACTA--CATTAAAATGGTCTGATCA-GA
gi|255556070|ref|XM_002519024.  AAACCAAAGCTAAG-CAGCGAAACTA--CATTAAAATGGTCTGATCA-GA
gi|255544763|ref|XM_002513397.  ATTCCAGGACTGGGTCGGAGAAGCCAA-CGATTAAAGGGTATGCGCACAA
gi|255551450|ref|XM_002516725.  GCTGCGAGGGAAGCTTGGAAAAGTGGCTTACTTGGAGAAATGGGTGAGAA

gi|255556074|ref|XM_002519026.  GCTTTAA---AATTGAGGATGAGATGACCATTTACAAAGTCGAATTTATG
gi|255556070|ref|XM_002519024.  GCTTTAA---AATTGAGGATGAGATGACCACTTACAAAGTCGAATTTATG
gi|255544763|ref|XM_002513397.  GAAGGAT---GAAAAAGGATCAGA-AATCATATATGAAGCAGACTTGGAG
gi|255551450|ref|XM_002516725.  GTATTACTCCAATAACCGCTGTGGATACAGTTTTCAACATCACTTTTGAC

gi|255556074|ref|XM_002519026.  GTGGACCCTAACTTCGGAGTACCAGGAGCGATCACAGTGATTAGCAAACA
gi|255556070|ref|XM_002519024.  GTGGACCCTAACTTCGGAGTACCAGGAGCGATCACAGTGATTAGCAAACA
gi|255544763|ref|XM_002513397.  GTTGAAGGTAGTTTCGGACAAGTGGGTGCCATTCTTGTTGAAAATGAGCA
gi|255551450|ref|XM_002516725.  TGGGATGAGAGCATGGGAGTTCCTGGAGCTTTTATAATTAGAAATCATCA

gi|255556074|ref|XM_002519026.  TCTAAAGGAGTTCTATTTGGAAAGCGTCACTGTTGAAGATGTCATTC---
gi|255556070|ref|XM_002519024.  TCTAAAGGAGTTCTATTTGGAAAGCGTCACTGTTGAAGATGTCATTC---
gi|255544763|ref|XM_002513397.  TCACAAGGAGATGTTTGTCAAGGATATTGCCCTCGAAGGTTTCATTACA-
gi|255551450|ref|XM_002516725.  TCATAGCCAGTTATACCTCAAAACAGTAACTTTAGATGATGTTCCTGGGC

gi|255556074|ref|XM_002519026.  ------------ATTTTTCCTGCGATTCTTGGGTCCAATCATCACAAAAT
gi|255556070|ref|XM_002519024.  ------------ATTTTTCCTGCAATTCTTGGATCCAATCGTCGCAAAAT
gi|255544763|ref|XM_002513397.  --GGCACTGTCAATATTTCTTGTAATTCGTGGGTTCA--TGCCA-AGAAT
gi|255551450|ref|XM_002516725.  ATGGTAGGGTACATTTTGTCTGCAATTCTTGGGTGTA---TCCAGCTCAT

gi|255556074|ref|XM_002519026.  GATCATGCAGGAAAAAGGATCTTTTTTGCTAATAAGGCCTATTTACCTTG
gi|255556070|ref|XM_002519024.  GATCATGCTGAAAAGCGTATCTTTTTTGCTAACAAGGCCTATTTACCTTG
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gi|255544763|ref|XM_002513397.      GACAACAAGCGTAAAAGAGTCTTCTTCGCTAATAAGTCATACTTACCATC
gi|255551450|ref|XM_002516725.      TGTTACAATTATGATCGAGTCTTCTTCTCTAACAAGACCTACCTTCCATG
                                        *       *  *  **** **  **** *** * **  * ** *

gi|255556074|ref|XM_002519026.      TCAAACGCCGCTAGGACTGAAAGAGCTTAGAGAAATGGATCTTAAGCAAT
gi|255556070|ref|XM_002519024.      TCAAACGCCGCTAGGACTGAAAGAGCTTAGAGAAATGGATCTTAAGCAAT
gi|255544763|ref|XM_002513397.      AGAGACGCCGAATGGATTGAGGAGGTTGAGAGAGGCAGAGCTTGAACTTC
gi|255551450|ref|XM_002516725.      CCAGACACCTAAGCCTTTACGCAAATATAGAGAAGAAGAGCTAATCAATT
                                      * ** **       *          *****    ** **

gi|255556074|ref|XM_002519026.      TGAGGGCAACGGAAGGGAATTAGAAAATTGTGCGACAGAATATATGAC
gi|255556070|ref|XM_002519024.      TGAGGGCAACGGAAAGGGAATTAGAAAATTGTGCGATAGAATTTATGAC
gi|255544763|ref|XM_002513397.      TACGAGGCAATGGCAAAGGGGAGCGCAAGAAAGGCGAGAGGATATATGAA
gi|255551450|ref|XM_002516725.      TCCGTGGAAATGGAAAAGGCAAGCTAGAGGAGTGGGATAGAGTGTATGAT
                                    *  * ** ** ** *  **       *     * ** **  * *****

gi|255556074|ref|XM_002519026.      TATGATACGTATAAAGACTTGGGAAATCCTGACAAAGGGATGGAATATAA
gi|255556070|ref|XM_002519024.      TATGATACGTATAAAGACTTGGGAAATCCTGACAAAGGGATGGAATATAA
gi|255544763|ref|XM_002513397.      TACGATTTCTATAATGATCTCGGCAATCCAGACAGTGACCCAGACCTCAA
gi|255551450|ref|XM_002516725.      TATGCTTACTACAATGATTTGGGAAGTCCAGATAAAGGTAAAGAGTATGC
                                    ** * *   ** ** **  * ** * *** ** *  *     **

gi|255556074|ref|XM_002519026.      TAGGCCAATTTTAGGAGG---TGAAATGCTTCCATATCCAAGGCGTTGTC
gi|255556070|ref|XM_002519024.      TAGGCCAATTTTAGGAGG---TGAAATGCTTCCATATCCAAGGCGTTGTC
gi|255544763|ref|XM_002513397.      AAGACCCGTGCTCGGCGG---CAAAGAGAACCCTTACCCTAGACGTTGCA
gi|255551450|ref|XM_002516725.      ACGTCCAGTTCTTGGCGGAAGTGAACAGTATCCATATCCTCGAAGAGGAA
                                      * **  *  * ** **    **  *  ** ** **  *  *  *

gi|255556074|ref|XM_002519026.      GAACTGGACGTCCTCCAAGTTCTACAGATGACACTA-----AGGAGTCTC
gi|255556070|ref|XM_002519024.      GAACTGGACGTCCTCCAAGTTCTACAGATGACACTA-----AGGAGTCTC
gi|255544763|ref|XM_002513397.      GAACAGGAAGGGCAAGATGCAACACAGAT-ACACT----------TTCT
gi|255551450|ref|XM_002516725.      GAACTGGTCGGAAACCGACAAAAACAGACCCCAATTCCGAGAGCAGATTG
                                    **** **  *              *****   ** *

gi|255556074|ref|XM_002519026.      CAGCAAATGAATCCATGCCGATGTATGTGCCGAGAGACGAAGCATATGAA
gi|255556070|ref|XM_002519024.      CAGCAAATGAATCCATGCCGATGTATGTGCCGAGAGACGAAGCACTTAAA
gi|255544763|ref|XM_002513397.      GAATCAATAAGCAGCAGC--GTCTATGTTCCTCGTGATGAAGAATTCTCA
gi|255551450|ref|XM_002516725.      CCGCTTCTAAATCTAGAT--ATATATGTTCCAAGAGATGAACGATTTGGT
                                        * *          * ***** **  * ** ***  *

gi|255556074|ref|XM_002519026.      GATTGCAAAAGAGAAGATATATCTGTTGGGAA-GTTGAAAGCGGTGGCTA
gi|255556070|ref|XM_002519024.      GATGGCAGAAGAATAGAAGTCTCTGTCGGAAA-ATTGAAAGCAGCCGTCC
gi|255544763|ref|XM_002513397.      GA-AGTGAAGGAGCACACGTTTTCGGCGAAGACAGTCTACTCAGTATTGC
gi|255551450|ref|XM_002516725.      CACATAAAATTTTCAGATTTCCTAGCCTATGC-TCTGAAATCCGTGGTTC
                                     *     *    * *  *   *          *  *  * *

gi|255556074|ref|XM_002519026.      AAAATCTAGTTCCTGGTCTAAGAAATGGCTCTATTGAAAGCGATTATATC
gi|255556070|ref|XM_002519024.      GGAATTTAATTCCTGTTCTAAGAAATGGCTTTGTTAAAAGTGATTGTATT
gi|255544763|ref|XM_002513397.      ATGCATTGGTCCCGCAGTTAGAGACTGCACTGGTAGACCCTGACCTTGCA
gi|255551450|ref|XM_002516725.      AGGTATTAGTTCCAGAGATCAAATCTCTATGTGACAAGACTATTAACGAG
                                        * * **    *     *          *

gi|255556074|ref|XM_002519026.      AAGGAATTCTCTGAAATTA-ATCATCTCTATAAAAAGAGGAGTAGTGTTG
gi|255556070|ref|XM_002519024.      AAGGAATTTTCTGAAATTA-ATGGTCTCTATAAGAAGCGAGGAGGCATTG
gi|255544763|ref|XM_002513397.      TTTCCATACTTCACAGCCATAGATTCTCTATTCAATGAAGGAGTCAACTT
gi|255551450|ref|XM_002516725.      TTTGACAGCTTTGAAGATGTTCTTAAGCTCTATGAAGGGGGTATTAAATT
                                          *    *           **  *   * *          *

gi|255556074|ref|XM_002519026.      G---AGAAAAGTCAAAGAAAGAATGTTCCAAAACCTTGTCTCTACCATGT
gi|255556070|ref|XM_002519024.      G---AGAAAAGTCAAAAACAGAATGTTCGAAAACCTTTCCTCTGCCATGT
gi|255544763|ref|XM_002513397.      G---CCTCCACTTAAAGA-ACAAGGTTGGAAAGATAT---TCTGCCTAAC
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gi|255551450|ref|XM_002516725.      ACCCAGTGGGACCAAAGCCACTAAATTAAGGAATCGCAT-CCCATGGGAG
                                               ***   *  *  **    *         *

gi|255556074|ref|XM_002519026.      ATGCTTAGCAA---GATTTCGGAGTCCTTCTCACAGATTTACAAATTTGA
gi|255556070|ref|XM_002519024.      ATTCCTAATAA---TATTTCAGAGTCCTTATCACAAATTCACAAATTTAA
gi|255544763|ref|XM_002513397.      CTTATCAAGAC---TATTACAGATGAAGCTAAAGAAGTTTTGGCATTTGA
gi|255551450|ref|XM_002516725.      ATGCTCAAGGAACTTGTTCGTAATGATGGCGAGCGATTCCTCAAATTCCC
                                     *   *         **    *                 *     ***

gi|255556074|ref|XM_002519026.      TCCTCCTAAAGCTATCCCCGTTTGGGGGGAAACACCTTGTCTAAGAGATG
gi|255556070|ref|XM_002519024.      TCGTCCTAAAGGCATCTCCT------GGAATGTATTATATTTAAGAGACG
gi|255544763|ref|XM_002513397.      CACCCCGGACACAATGGAAA---GGGACAGATTTTTTTGGTTTAGGGATG
gi|255551450|ref|XM_002516725.      AATGCCTGACGTGATCAAAG---AGGACAAGTCTGCTTGGAGGACAGATG
                                        **  *    **                    *    *  ** *

gi|255556074|ref|XM_002519026.      ATGAATTTGGTCGATTAACACTAAGAGGCATGAACCCTTTAAGCATTGAA
gi|255556070|ref|XM_002519024.      ATGAATTTGGTCGATTAACACTAAGAGGCATGAACCCTTTAAGTATTGAA
gi|255544763|ref|XM_002513397.      AGGAATTTGGTCGCCAAACTCTAGCTGGTCTAAACCCGATGAGCTTACAA
gi|255551450|ref|XM_002516725.      AAGAGTTTGCACGAGAGATGCTTGCTGGAGTCAACCCAGTTATTATCAGT
                                    * ** ****  **    *  **    **  * *****  * *   *

gi|255556074|ref|XM_002519026.      AGGCTTAAGGTGTTTCCACCGGTGAGCAAGCTGGATCCGTCCATCAATGG
gi|255556070|ref|XM_002519024.      AGGCTTAAGGTATTTCCGCCGGTGAGCAAGCTGGATCCTGCCATATGCGG
gi|255544763|ref|XM_002513397.      TTGGTCACGGAATGGCCTTTAAAGAGTGAATTGGATCCTGAAATCTATGG
gi|255551450|ref|XM_002516725.      CGCCTCCAAGAGTTTCCCCCGCCAAGCAAGCTAGATCCTAAAGAATATGG
                                        *   *  *  **       **  *  * *****          **

gi|255556074|ref|XM_002519026.      ATCACATGAGTCGGCACTCAAAGAAGAACACATCATAGGGCATCTTAATG
gi|255556070|ref|XM_002519024.      TTCACATGAATCAGCACTCAAGGAAGAACACATCATAGGGCATCTTAATG
gi|255544763|ref|XM_002513397.      GCCGCCGGAATCAGCAATCACCACAGAAATGATTGAGCAGCAAATCCGAG
gi|255551450|ref|XM_002516725.      AAACCAGAAAAGTTCAATCACAAAGAGCACGTTGAAAAGAGCATGAACG
                                        *   *    ** ***   ***    *      *   *   *

gi|255556074|ref|XM_002519026.      G---AATATCCATACAGCAGGCACTCGAAGAACACAAATTGTTCATACTA
gi|255556070|ref|XM_002519024.      G---CATGTCCGTACAGCAGGCACTTGAAGAAAACAGATTGTTCATACTA
gi|255544763|ref|XM_002513397.      GGTTTATGACAGTTGAAGAGGCTATAAAACAAAAGAAGCTGTTCATGCTT
gi|255551450|ref|XM_002516725.      G---TCTCACAGTAGATCAAGCAATCAGAAATAACAAGTTGTTTATATTA
                                    *    *  *  *  *  * **  *  * *  * *  **** **  *

gi|255556074|ref|XM_002519026.      GACTATCATGACATTTATCTTCCACATCTGAAACGGATAAATGCTCTTGA
gi|255556070|ref|XM_002519024.      GACTATCATGACATCTATCTTCCATATCTGGAACGGATAAATGCTCTTGA
gi|255544763|ref|XM_002513397.      GACTACCACGATTTGTTCTTACCATTTGTGAGCAAGGTAAGACAGCTTGA
gi|255551450|ref|XM_002516725.      GATCATCATGATGCATTGATGCCATACCTGACGAAAATAAA----CTCAA
                                    **  * ** **    *   * ***    **       ***     **  *

gi|255556074|ref|XM_002519026.      TGATCGAAAAG-CTCATGCAACTCGTACAGTATTCTTCTTAACTCCAGCA
gi|255556070|ref|XM_002519024.      TAACCGAAAAG-CCCATGCCACTCGTACAATATTCTACTTGACCCCATTA
gi|255544763|ref|XM_002513397.      AAACACCACAT-TATACGGGTCTCGGACAATTTTCTTCTTAACCCCTGAT
gi|255551450|ref|XM_002516725.      CAACAACAAGGACTTATGCTACAAGAACAATCCTTTTACTGCAAGATGAT
                                      *   *       * *  *  * *** * * *  *

gi|255556074|ref|XM_002519026.      GGAACATTAAACCCCATTGCTATTGAGCTCACTACTATTCCATCTAAGGA
gi|255556070|ref|XM_002519024.      GGAACATTAAAACCCATTGCTATTGAGGTCGCTACTGTTCCATCTACGGA
gi|255544763|ref|XM_002513397.      GGCACATTGCGGCCACTAGCTATTGAGCTC---ACTCGCCCGCCGATGGA
gi|255551450|ref|XM_002516725.      GGGACATTGAAGCCATTGGCAATTGAGCTAAG-TCTTCCGCATCCACAAG
                                    ** *****    **  * ** ****** *     **    *  * *

gi|255556074|ref|XM_002519026.      TTCAAACTCGCCAA----TAAGGCAAGTTCTCACCCCTCCTGTGGATGCC
gi|255556070|ref|XM_002519024.      TTCAAGCTTTCCAG----TTAAGCAAGTTCTCACCTCTCCTGTGGATGCC
gi|255544763|ref|XM_002513397.      CGGAAAGCCACAGT----GGAAGGATGCTTACGTACCAACTTGGCATTCT
gi|255551450|ref|XM_002516725.      GGGAACGTCATGGTGCTGTCAGCAAAGTTTTCACTCCGGCAGAAGATGGT
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gi|255556074|ref|XM_002519026.  TCCACCTATTGGCTATGGCAACTAGCCAAAGCCCATGTTTGCTCCAATGA
gi|255556070|ref|XM_002519024.  ACCACCTATTGGTTATGGCAATTAGCCAAAGCCCATGTTTGCTCGAATGA
gi|255544763|ref|XM_002513397.  ACGGGTGTTTGGCTTTGGAGGCTTGCCAAAGCTCATGTCCTTGCACATGA
gi|255551450|ref|XM_002516725.  GTTGAGGGCTCAGTGTGGCAGCTGGCAAAAGCTTATGCTGCCGTAAATGA

gi|255556074|ref|XM_002519026.  TGCTGGTGCTCATCAATTAATTCATCACTGGTTGCGCGCCCATGCTTGCA
gi|255556070|ref|XM_002519024.  TTCTGGTGCTCATCAACTGATCCATCACTGGTTGCGCGTCCATGCTTGCA
gi|255544763|ref|XM_002513397.  ATCTGGCTATCACCAACTTATTAGCCATTGGTTGAAAACACATGCTTGCA
gi|255551450|ref|XM_002516725.  TTCTGGATACCATCAGCTTATTAGCCACTGGTTGAACACACATGCTGCTA

gi|255556074|ref|XM_002519026.  TGGAGCCGTTTATTATAGCAGCAAACAGGCAACTAAGCGCAATGCATCCA
gi|255556070|ref|XM_002519024.  CGGAACCGTTAATCATAGCAGCAAACAGGCAATTAAGCGTAATGCATCCA
gi|255544763|ref|XM_002513397.  CAGAACCATACATAATTGCAGCCAACCGTCAACTTAGTGTAATGCATCCG
gi|255551450|ref|XM_002516725.  TAGAGCCGTTTATTATTGCCACAAACAGACAGTTAAGTGTACTTCACCCA

gi|255556074|ref|XM_002519026.  ATTTACAAGCTCCTCAAGCCTCACATGAGATACACATTGGCTATAAATGC
gi|255556070|ref|XM_002519024.  ATTTACAAGCTCCTCAAGCCTCACATGAGATACACATTGGCTATAAATGC
gi|255544763|ref|XM_002513397.  ATTTATAGGCTCTTGCACCCTCATTTCCGATATACAATGGAGATCAATGC
gi|255551450|ref|XM_002516725.  ATCTATAAGCTTCTGCATCCACACTTCCGTGATACAATGAACATAAATGC

gi|255556074|ref|XM_002519026.  ACAGGCTCGTGAAGTTTTAACCAATGCCAATGGAATCGTGGAGTCGTGTT
gi|255556070|ref|XM_002519024.  ACAGGCTCGTGAAGTTTTAACCAATGCCAATGGAATCGTGGAGTCGTATT
gi|255544763|ref|XM_002513397.  TTTGGCTCGACAAGCACTAATCAATGCAGGCGGGATTATTGAGTCTTCAT
gi|255551450|ref|XM_002516725.  CTTGGCTAGGCAGATTCTCATCAATGCTGGGGGGATACTTGAGATAACTG

gi|255556074|ref|XM_002519026.  TCGCTCCTGAAAAGTACTGCATGGAGATTACTTCATCTGCCTATAAAGAT
gi|255556070|ref|XM_002519024.  TCGCTCCTGAAAAGTACTGCATGGAGATTACTTCATCTGCCTATAAAGAT
gi|255544763|ref|XM_002513397.  TTTCTCCTGGAAAGTATTGTCTGGAGATGAGCTCTGTTATTTACGACAAG
gi|255551450|ref|XM_002516725.  TTTTCCCAGCCAAATATGCCATGGAATTATCTTCTGTGGTTTACAAGAGC

gi|255556074|ref|XM_002519026.  TGGTGGCGTTTTGACATGGAGGGACTCCCCGCTGATCTTATCAGAAGAGG
gi|255556070|ref|XM_002519024.  TGGTGGCGTTTTGACATGGAGGGACTCCCCGCTGATCTTATCAGAAGAGG
gi|255544763|ref|XM_002513397.  CTATGGAGGTTTGACCAACAGGCAATGCCTCAGGAACTTATCAGCAGGGG
gi|255551450|ref|XM_002516725.  ---TGGGTTTTCACTGAGCATGCACTCCCTGCTGATCTGCTCAAGAGAGG

gi|255556074|ref|XM_002519026.  ATTGGCAATTCCTGACCCGAAACAGCCTCATGGGCTAAGATTACTCATTG
gi|255556070|ref|XM_002519024.  ATTGGCAATTCCTGACGCGAAACAGCCTCATGGGCTAAGATTACTCATTG
gi|255544763|ref|XM_002513397.  AATGGCTGTTCAAGATCCATCGGCTCCACATGGTGTAAAACTAAGAATTG
gi|255551450|ref|XM_002516725.  AGTAGCAGTACCAGATTCAAGCCAACGACATGGTCTGAGACTTCTGATAG

gi|255556074|ref|XM_002519026.  AAGACTATCCTTTTGCAAATGATGGGCTTCTTATATGGTCTGCAATTCAA
gi|255556070|ref|XM_002519024.  AAGACTATCCTTTTGCAAACGATGGGCTTCTTATATGGTCTGCAATTCAA
gi|255544763|ref|XM_002513397.  AAGACTACCCATTTGCCAGTGATGGACTACTTCTATGGGATGCGATTAAA
gi|255551450|ref|XM_002516725.  AAGATTACCCTTATGCTGTTGATGGTTTAGAAGTCTGGTCAGCAATTGAA

gi|255556074|ref|XM_002519026.  GATTTGGTTAAGACATTTGTTAACTACTACTATCCTGAACCAAGTCTAGT
gi|255556070|ref|XM_002519024.  GATTTGGTTAAGACATATGTTAACTACTACTATCCTGAAGCAAGTCTAGT
gi|255544763|ref|XM_002513397.  GCTTGGGTAAGTGACTATGTCAATCACTACTATCCAGACCCAAGCCTGAT
gi|255551450|ref|XM_002516725.  ACTTGGGTGATGGAATATTGTGCTTTCTACTATCCAACAGATGACTTGGT
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gi|255556074|ref|XM_002519026.    CCAATTTGACACTGAGCTTCAGTCCTGGTATAAGGAGTCCATCAATGTAG
gi|255556070|ref|XM_002519024.    CCAATTTGACACTGAGCTTCAGTCCTGGTATAAGGAGTCCATCAATGCAG
gi|255544763|ref|XM_002513397.    ATTATCCGATAAGGAACTCCAGGCATTTTGGACCGAAGTTCGAACAGTTG
gi|255551450|ref|XM_002516725.    TCGGGATGACACTGAACTTCAATCCTGGTGGGCAGAGATCCGTAATGAGG
                                        ** *  ** ** **  * *  *    **       * * *

gi|255556074|ref|XM_002519026.    GGCATGCCGATGTTTCAAATGCTAATTGGTGGCCAAGACTCTCAACTCCA
gi|255556070|ref|XM_002519024.    GGCATGCCGATGTTTCAAATGCTAATTGGTGGCCAAGACTCTCAACTCCA
gi|255544763|ref|XM_002513397.    GTCATGGTGATAAAAAGGATGAACCCTGGTGGCCTGAGTTGAAAACACCT
gi|255551450|ref|XM_002516725.    GACATGGTGACAAAAAAGATGAACCATGGTGGCCTGAGATGCAGACACGA
                                  * **** **       ***     ********     *    ** *

gi|255556074|ref|XM_002519026.    GAGGATCTTATCTCTATACTTAGTACGATCATCTGGATTGCATCAGCACA
gi|255556070|ref|XM_002519024.    GACGATCTTATCTCTATACTTAGTACGATCATCTGGATTGCATCAGCACA
gi|255544763|ref|XM_002513397.    AAAGACCTGATAGAAATCGTCTCAACAATTGCATGGGTAACCTCAGGTCA
gi|255551450|ref|XM_002516725.    GCTGATCTTACGCAAACATGCACAATCATCATTTGGATAGCTTCAGCGCT
                                     ** ** *     *         *  **    *** *  * ****  *

gi|255556074|ref|XM_002519026.    ACATGCAGCAGTCAATTTCGGGCAATATGACTATGGTGGGTACGTCCCAG
gi|255556070|ref|XM_002519024.    ACATGCAGCACTCAATTTTGGGCAATATGACTATGGTGGGTACGTCCCAA
gi|255544763|ref|XM_002513397.    CCATGCTGCAGTGAACTTTGGACAATATGCGTATGCGGGCTATTTCCCTA
gi|255551450|ref|XM_002516725.    CCATGCAGCTGTCAATTTTGGGCAATACCCTTATGCTGGCTATCTCCCTA
                                   ***** **  * ** ** ** *****    ****  ** **  ****

gi|255556074|ref|XM_002519026.    TTAGGCCACCCAAAATGCGAAGATTAGTGCCAATGGAAGGT--GAT--GT
gi|255556070|ref|XM_002519024.    TTAGGCCACCAAACATGCGAAGATTAGTGCCAATGCGAGGT--GAT--GT
gi|255544763|ref|XM_002513397.    ATAGACCTACAACTGCTAGATTGAAAATGCCAAGTGAAGATCCGACTGAT
gi|255551450|ref|XM_002516725.    ACCGTCCAACCGTAAGTCGCCGATTTATGCCTGAGCCAGGA---ACC-CC
                                     * **  *         *         ****      **     *

gi|255556074|ref|XM_002519026.    GGAGTATGCAAATTTCCT-TGCAGACCCGCAAGGATAT-TTCTTATCATC
gi|255556070|ref|XM_002519024.    GGAGTATGCAAAGTTTCT-TTCAGACCCGCAAGGATAT-TTCTTATCATC
gi|255544763|ref|XM_002513397.    GAAGGTTGGAAAATGTTTGCGGAAAAACCTGAAGTTGTACTTTTGACAAC
gi|255551450|ref|XM_002516725.    CGAGTATACTGAACTTGA--GAAAGATCCGAACTTGGCCTTCTTAAAAAC
                                     **  *    *          *    *    *          * **   * *

gi|255556074|ref|XM_002519026.    ATTGCCAAGTTTGAGTCAGACTACTTATTTCATGTCTGTTCTTGATATAC
gi|255556070|ref|XM_002519024.    ATTGCCAAATTTGACTAAAATTACTTCTTTCATGTCTATTCTTGATTTAC
gi|255544763|ref|XM_002513397.    GTTCCCTTCACAGGTCCAGGCAACAAAAGTGATGGCTGTTTTAGATGTGT
gi|255551450|ref|XM_002516725.    AATCACAGCCCAACTACAAACTCTCCTCGGTGTTTCCCTAATCGAGATAT
                                     *  *          *                 * * * * * **  *

gi|255556074|ref|XM_002519026.    TTTCGACTCATTCAGTGGATGAGGAGTACATAGGTGCCAGAAAAGACCTT
gi|255556070|ref|XM_002519024.    TTTCGATGCATTCAGTGGACGAGGAGTACATAGGTGCTAGAAACGACCTT
gi|255544763|ref|XM_002513397.    TATCAAACCATTCTCCTGATGAAGAATATATAGGAGAGAAAATAGAGCC-
gi|255551450|ref|XM_002516725.    TATCTAGGCATCCAACTGATGAGGTATACCTTGGCCAGAGAGATACAGC-
                                  * ** *  *** *    ** ** *  **  * **    * *

gi|255556074|ref|XM_002519026.    TTAAAATGGTCAGGAGAAAACGAAATCATTGAGGCATTTTACAGATTCTC
gi|255556070|ref|XM_002519024.    TTGACATGGTCAGGAGATACTGTAATTACTGAGGCATTTTACAGATTCTC
gi|255544763|ref|XM_002513397.    --AGCCTGGGCTGAGGATCCTAACATAAAAGCAGCATTTGAAAAGTTCGC
gi|255551450|ref|XM_002516725.    --AGAATGGACATCAGATCGTGAACCACTAGCAGCATTTGAAAGATTTTC
                                        *** *    **                * ****** * *  **  *

gi|255556074|ref|XM_002519026.    AATGGAAATAATGAAGATAGAGAAAGAGATTGAGAAGAGAAATGTTGACC
gi|255556070|ref|XM_002519024.    AATGGAAATAATGAAGATAGAGAAAGAGATTGAGAAAAGAAATGTTGACC
gi|255544763|ref|XM_002513397.    TGGAAGGTTGAAGGAACTGGAAGGGATTATCGATGAAAGGAATGCTAATC
gi|255551450|ref|XM_002516725.    AGAAAGGCTGAAAGAAATTGAAAACAAAATCATGGACATGAACAGTGACA
                                        * *   *  * **       **      * *  **   * *
```

```
gi|255556074|ref|XM_002519026.      CAAAACTTAAGAATCGTTGTGGTGCTGGAA-------TTGCGCCTTATGA
gi|255556070|ref|XM_002519024.      CAAAACTTAGGAATCGTTGTGGAGCTGGAA-------TTGCACCTTATGA
gi|255544763|ref|XM_002513397.      CAAGTTTGAAGAATAGGAATGGAGCTGGAA-------TTGTGCCTTATGA
gi|255551450|ref|XM_002516725.      ATAAATACAAGAACAGAATTGGGCCAGTCAAGGTGCCTTACACCTTACTT
                                      *     * ***  *   ***  * *  *         **   *****

gi|255556074|ref|XM_002519026.      ACTGCTATTACC-------AAGCTC-ACATCCAGGTGTTACTGGTAGAGG
gi|255556070|ref|XM_002519024.      ACTGCTATTACC-------AAGCTC-ACATCCTGGTGTTACTGGTAGAGG
gi|255544763|ref|XM_002513397.      GCTTTTGAAGCC-------ATTCTC-TGAGCCTGGAGTTACAGCTCGAGG
gi|255551450|ref|XM_002516725.      TTTCCTAACACCTCAGATGAATCTAGACAGGGTGGACTCACTGGCAAGGC
                                      *  *    **        *  **    *    **  * ** *    **

gi|255556074|ref|XM_002519026.      GGTGCCTAATAGCATATCAATGTAA
gi|255556070|ref|XM_002519024.      GGTGCCTAATAGCATATCAATGTAA
gi|255544763|ref|XM_002513397.      AGTTCCATACAGTATCTCAATCTGA
gi|255551450|ref|XM_002516725.      GATTCCCAACAGTATATCAATATAA
                                      * **  * ** ** ***** * *
```

```
clustalw.dnd
(
(
gi|255544763|ref|XM_002513397.1|:0.20675,
gi|255551450|ref|XM_002516725.1|:0.31540)
:0.23383,
gi|255556074|ref|XM_002519026.1|:0.04256,
gi|255556070|ref|XM_002519024.1|:0.05316);
```

Select tree menu Exec

Appendix III

LOX genes alignment of nucleotide sequence on CLUSTALW programme

CLUSTALW Result

[clustalw.aln][clustalw.dnd][readme]

```
CLUSTAL 2.1 Multiple Sequence Alignments

Sequence type explicitly set to Protein
Sequence format is Pearson
Sequence 1: gi|255544764|ref|XP_002513443.1|    900 aa
Sequence 2: gi|255556075|ref|XP_002519072.1|    786 aa
Sequence 3: gi|255551451|ref|XP_002516771.1|    868 aa
Sequence 4: gi|255556071|ref|XP_002519070.1|    837 aa
Start of Pairwise alignments
Aligning...


Sequences (1:2) Aligned. Score: 42.8753
Sequences (1:3) Aligned. Score: 37.9032
Sequences (1:4) Aligned. Score: 40.6213
Sequences (2:3) Aligned. Score: 40.9669
Sequences (2:4) Aligned. Score: 86.8957
Sequences (3:4) Aligned. Score: 39.0681
Guide tree file created:   [clustalw.dnd]

There are 3 groups
Start of Multiple Alignment

Aligning...
Group 1: Sequences:   2      Score:12180
Group 2: Sequences:   3      Score:6066
Group 3: Sequences:   4      Score:6027
Alignment Score 14942

CLUSTAL-Alignment file created  [clustalw.aln]
```

```
clustalw.aln

CLUSTAL 2.1 multiple sequence alignment


gi|255556075|ref|XP_002519072.      ----------------------------------------------
gi|255556071|ref|XP_002519070.      ----------------------------MEDVLEMPFKLKMTALVSVR
gi|255544764|ref|XP_002513443.      MLKPQVYQSRSPKTLFLLPKPFIHENGGQTHLSVLSRPSLLKTQRKTRVG
gi|255551451|ref|XP_002516771.      -------------MEVFCAQPRNQPNGGTVTERFKIKGTVVLMKKNVLD


gi|255556075|ref|XP_002519072.      ----------------------------------------------
gi|255556071|ref|XP_002519070.      NTMFKWVECFSNLGQKHKGTVVLQLVSNDIDPG---------------
gi|255544764|ref|XP_002513443.      YKPGNVQAISEITEKQLKSTKVKAIVTVNRTVGGFLSNLGINRGLDDVTD
gi|255551451|ref|XP_002516771.      FSDIKASFLDRVHELLGKGVSMQLISAVHHDPAN---------------


gi|255556075|ref|XP_002519072.      --------------MKPKLSSETTLKWSDQSFKIEDENTIYKVEFMVDP
```

```
gi|255556071|ref|XP_002519070.          ----------------MKPKLSSETTLKWSDQSFKIEDEMTTYKVEFMVDP
gi|255544764|ref|XP_002513443.          LLGKTLLLLELVSAELDSRTGSEKPTIKGYAHKKDEKGSEIIYEADLEVEG
gi|255551451|ref|XP_002516771.          ----------------KLRGKLGKVAYLEKWVPSITPITAVDTVFNITFDWDE
                                                        .     *                    ::  :  :

gi|255556075|ref|XP_002519072.          NFGVPGAITVISKHLKEFYLESVTVEDVIH-----FSCDSWVQSSQNDHA
gi|255556071|ref|XP_002519070.          NFGVPGAITVISKHLKEFYLESVTVEDVIH-----FSCNSWIQSSQNDHA
gi|255544764|ref|XP_002513443.          SFGQVGAILVENEHMKEMFVKDIALEGFITG-TVNISCNSWVHAK-NDNK
gi|255551451|ref|XP_002516771.          SMGVPGAFIIRNHHHSQLYLKTVTLDDVPGHGRVHFVCNSWVYPAHCYNY
                                        .:*  **: : ..* .::::: :::::..       : *:**: .    :

gi|255556075|ref|XP_002519072.          GKRIFFANKAYLPCQTPLGLKELREKDLKQLRGNGRGIPKLCDRIYDYDT
gi|255556071|ref|XP_002519070.          EKRIFFANKAYLPCQTPLGLKELREKDLKQLRGNGKGIPKLCDRIYDYDT
gi|255544764|ref|XP_002513443.          RKRVFFANKSYLPSETPNGLRRLREAELELLPGNGKGERKKGERIYEYDF
gi|255551451|ref|XP_002516771.          DR-VFFSNKTYLPCQTPKPLRKYPEEELINLPGNGKGKLEEWDFVYDYAY
                                         : :**:**:***.:**  *:. ** :*  *****:*  :  :*:*:*

gi|255556075|ref|XP_002519072.          YKDLGNPDKGMEYNRPILGG-EMLPYPRRCRTGRPPSSTDDTKES-PANE
gi|255556071|ref|XP_002519070.          YKDLGNPDKGMEYNRPILGG-EMLPYPRRCRTGRPPSSTDDTKES-PANE
gi|255544764|ref|XP_002513443.          YNDLGNPDSQPDLKRPVLGG-KENPYPRRCRTGRARCNTDTLSES-ISSS
gi|255551451|ref|XP_002516771.          YNDLGSPDKGKEYARPVLGGSEQYPYPRRGRTGRKPTKTDPNSESRLPLL
                                        *:***.**.. :  **:*** :  ***** ****   .**  .**  .

gi|255556075|ref|XP_002519072.          SMPMYVPRDEAYEDCKREDISVGKLKAVAKNLVPGLRNGSIESDYIKEFS
gi|255556071|ref|XP_002519070.          SMPMYVPRDEALKDGRRIEVSVGKLKAAVRNLIPVLRNGFVKSDCIKEFS
gi|255544764|ref|XP_002513443.          ---VYVPRDEEFSEVKEHTFSAKTVYSVLHALVPQLETALVDPDLAFPYP
gi|255551451|ref|XP_002516771.          NLDIYVPRDERFGHIKFSDFLAYALKSVVQVLVPEIKSLCDKTINEFDSF
                                           :******   . :   . .  : :. : *:* :..   ..

gi|255556075|ref|XP_002519072.          EINHLYKKRSSVGEKSKKECSKTLSLP--CMLSKISESFSQIYKFDPPKA
gi|255556071|ref|XP_002519070.          EINGLYKKRGGIGEKSKTECSKTFPLP--CIPNNISESLSQIHKFNRPKG
gi|255544764|ref|XP_002513443.          TAIDSLFNEG-VNLPPLKEQGMKDILP--NLIKTITDEAKEVLAPDTPDT
gi|255551451|ref|XP_002516771.          EDVLKLYEGGIKLPSGTKATKLRNRIPWEMLKELVRNDGERFLKFPMPDV
                                              : .      .      :*   : . : :. ...  *  *.

gi|255556075|ref|XP_002519072.          IPVWGETPCLRDDEFGRLTLRGMNPLSIERLKVFPPVSKLDPSINGSHES
gi|255556071|ref|XP_002519070.          ISWN--VLYLRDDEFGRLTLRGMNPLSIERLKVFPPVSKLDPAICGSHES
gi|255544764|ref|XP_002513443.          MERD-RFFWFRDEEFGRQTLAGLNPMSLQLVTEWPLKSELDPEIYGPPES
gi|255551451|ref|XP_002516771.          IKED-KSAWRTDEEFAREMLAGVNPVIISRLQEFPPPSKLDPKEYGNQKS
                                        :        *:**.*  * *:**: :. :  :*  *:***   *  :*

gi|255556075|ref|XP_002519072.          ALKEEHIIGHLN-GISIQQALEENKLFILDYHDIYLPHLKRINALDDRKA
gi|255556071|ref|XP_002519070.          ALKEEHIIGHLN-GMSVQQALEENRLFILDYHDIYLPYLERINALDNRKA
gi|255544764|ref|XP_002513443.          AITTEMIEQQIRGFMTVEEAIKQKKCLFMLDYHDLFLPFVSKVRQLENTTL
gi|255551451|ref|XP_002516771.          SITKEHVEKSMN-GLTVDQAIRNNKLFILDHHDALMPYLTKINSTTTR-T
                                        ::. * :   :.  :::::*:.:::**:**:**  :*.: ::.

gi|255556075|ref|XP_002519072.          HATRTVFFLTPAGTLNPLAIELTTIPSKDSNS-PIRQVLTPPVDASTYWL
gi|255556071|ref|XP_002519070.          HATRTIFYLTPLGTLKPLAIEVATVPSTDSSF-PVKQVLTSPVDATTYWL
gi|255544764|ref|XP_002513443.          YGSRTIPFLTPDGTLRPLAIELTRPP-MDGKP-QWKDAYVPTWHSTGVWL
gi|255551451|ref|XP_002516771.          YATRTILLLQDDGTLKPLAIELSLPHPQGERHGAVSKVFTPAEDGVEGSV
                                        :.:**:: *   ***.*:***::     .       .. ... ..    :

gi|255556075|ref|XP_002519072.          WQLAKAHVCSNDAGAHQLIHHWLRAHACMEPPIIAANRQLSAMHPIYKLL
gi|255556071|ref|XP_002519070.          WQLAKAHVCSNDSGAHQLIHHWLRVHACTEPLIIAANRQLSVMHPIYKLL
gi|255544764|ref|XP_002513443.          WRLAKAHVLAHESGYHQLISHWLKTHACTEPYIIAANRQLSVMHPIYRLL
gi|255551451|ref|XP_002516771.          WQLAKAYAAVNDSGYHQLISHWLNTHAAIEPPIIATNRQLSVLHPIYKLL
                                        *:****:.  :::* **** *** ..**. ** ***:*****.:****:**

gi|255556075|ref|XP_002519072.          KPHMRYTLAINAQAREVLTNANGIVESCPAPEKYCMEITSSAYKDWWRPD
gi|255556071|ref|XP_002519070.          KPHMRYTLAINAQAREVLTNANGIVESYPAPEKYCMEITSSAYKDWWRPD
```

```
gi|255544764|ref|XP_002513443.      HPHPRYTHEINALARQALINAGGIIESSPSPGKYCLEMSSVIYDKLWRPD
gi|255551451|ref|XP_002516771.      HPHPRDTMNINALARQILINAGGILEITVFPAKYAMELSSVVYKS-WVPT
                                    :**:* *: *** **: * **.**:*  . * **.:*::*  *.. * *

gi|255556075|ref|XP_002519072.      MEGLPADLIRRGLAIPDPKQPHGLRLLIEDYPFANDGLLIWSAIQDLVKT
gi|255556071|ref|XP_002519070.      MEGLPADLIRRGLAIPDAKQPHGLRLLIEDYPFANDGLLIWSAIQDLVKT
gi|255544764|ref|XP_002513443.      QQAMPQELISRGMAVQDPSAPHGVKLRIEDYPFASDGLLLWDAIKAWVSD
gi|255551451|ref|XP_002516771.      EHALPADLLKRGVAVPDSSQRHGLRLLIEDYPYAVDGLEVWSAIETWVME
                                     ..:* :*: **:*: *..  **::* *****:* *** :*.**:  *

gi|255556075|ref|XP_002519072.      FVNYYYPEPSLVQFDTELQSWYKESINVGHADVSNAMWPRLSTPEDLIS
gi|255556071|ref|XP_002519070.      YVNYYYPEASLVQFDTELQSWYKESINAGHADVSNAMWPRLSTPDDLIS
gi|255544764|ref|XP_002513443.      YVNHYYPDPSLILSDKELQAFWTEVRTVGHGDKKDEPWWPELKTPKDLIE
gi|255551451|ref|XP_002516771.      YCAFYYPTDDLVRDDTELQSWWAEIRNEGHGDKKDEPWWPEMQTPADLTQ
                                    :  .*** .*:  *.***::: *  . **.* .:  ***.:.*  ** .

gi|255556075|ref|XP_002519072.      ILSTIIWIASAQHAAVNFGQYDYGGYVPVRPPKMRRLVPMEG--DVEYAN
gi|255556071|ref|XP_002519070.      ILSTIIWIASAQHAALNFGQYDYGGYVPIRPPNMRRLVPMRG--DVEYAK
gi|255544764|ref|XP_002513443.      IVSTIAWVTSGHHAAVNFGQYAYAGYFPNRPTTARLKMPSEDPTDEGWKM
gi|255551451|ref|XP_002516771.      TCTIIIWIASALHAAVNFGQYPYAGYLPNRPTVSRRFMPEPG--TPEYTE
                                      : * *::*. ***:**** *.**.* **.  *  :*  .    :

gi|255556075|ref|XP_002519072.      FLADPQGYFLSSLPSLSQTTYFKSVLDILSTHSVDEEYIGARKDLLKWSG
gi|255556071|ref|XP_002519070.      FLSDPQGYFLSSLPNLTKITSFMSILDLLSMHSVDEEYIGARNDLLTWSG
gi|255544764|ref|XP_002513443.      FAEKPEVVLLTTFPSQVQATKVMAVLDVLSNHSPDEEYIG-EKIEPAWAE
gi|255551451|ref|XP_002516771.      LEKDPNLAFLKTITAQLQTLLGVSLIEILSRHPTDEVYLG-QRDTAEWTS
                                    :  .*:  :*.::.   :    ::::::** *. ** *:* ..    *:

gi|255556075|ref|XP_002519072.      ENEIIEAFYRFSMEIMKIEKEIEKRNVDPKLKNRCGAGIAPYELLLPSS-
gi|255556071|ref|XP_002519070.      DTVITEAFYRFSMEIMKIEKEIEKRNVDPKLRNRCGAGIAPYELLLPSS-
gi|255544764|ref|XP_002513443.      DPNIKGAFEKFAGRLKELEGIIDERNANPSLKNRNGAGIVPYELLKPFS-
gi|255551451|ref|XP_002516771.      DREPLAAFERFSERLKEIENKIMDMNSDNKYKNRIGPVKVPYTLLFPNTS
                                    :     ** :*: .: ::*  * . * : . :** *.  .** ** * :

gi|255556075|ref|XP_002519072.      ----HPGVTGRGVPNSISM
gi|255556071|ref|XP_002519070.      ----HPGVTGRGVPNSISM
gi|255544764|ref|XP_002513443.      ----EPGVTARGVPYSISI
gi|255551451|ref|XP_002516771.      DESRQGGLTGKGIPNSISI
                                        . *:*.:*:* ***:
```

```
clustalw.dnd

(
(
gi|255544764|ref|XP_002513443.1|:0.30183,
gi|255551451|ref|XP_002516771.1|:0.31914)
:0.21517,
gi|255556075|ref|XP_002519072.1|:0.05514,
gi|255556071|ref|XP_002519070.1|:0.07590);
```

Select tree menu Exec

CERTIFICATE

This is to certify that I have no objection to supply one copy of any part of this thesis at a time to any scientist through reprographic process for rendering reference services in a library or documentation centre.

Place: Navsari

Date : 1 Dec. 2011

(Mhaske S. D.)