Chapter 2

REVIEW OF LITERATURE
2.1 The need for studies on lipoxygenases in groundnut:

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

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

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

Groundnut is a valuable cash crop for millions of small-scale farmers in the semi-arid tropics. It generates employment on the farm and in marketing, transportation and processing. The largest producers of groundnut are China and India, followed by Sub-Saharan African countries and Central and South America.
China leads in production of peanut having 37.5% share of overall world’s production followed by India (19%) and Nigeria (11%). In India peanut occupies a prominent position in the national edible oil economy.

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

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

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

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

2.2 LOX enzyme classification:

Plant LOXs are classified with respect to their positional specificity of linoleic acid (LA) oxygenation because arachidonic acid is only a minor polyunsaturated fatty acid (PUFA) in the plant kingdom. The generation of oxylipins is initiated by LOXs, which form hydroperoxides from ALA (18: 3) or LA (18: 2) (Feussner and Kuhn, 2000; Feussner and Wasternack, 2002). LA is oxygenated either at carbon atom 9 (9-
LOX) or at C-13 (13-LOX) of the hydrocarbon backbone of the fatty acid leading to two groups of compounds, the (9S)-hydroperoxo- and the (13S)-hydroperoxy derivatives of PUFAs (Fig. 1). With ALA as the substrate, (13S)-hydroperoxyoctadecatrienoic acid (13-HPOTrE) or (9S)-hydroperoxyoctadecatrienoic acid (9-HPOTrE) are formed (Fig. 2).

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

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

2.3 Substrate specificity of LOXs:

The majority of plant LOXs strongly prefer free fatty acids as substrates (Siedow, 1991). However, certain plant and animal LOXs are capable of oxygenating also ester lipid substrates, such as phospholipids (Brash et al., 1987; Murray and Brash, 1988), triacyl glycerols (Holtman et al., 1997; Feussner et al., 1998) and
cholesterol esters (Belkner et al., 1991). Even more complex lipid protein assemblies such as biomembranes (Kuhn et al., 1990a; Maccarrone et al., 1994) were metabolized by LOXs. LOX1 from soybean seeds and a LOX from cucumber roots exhibited activity with PUFAs esterified to phospholipids (Brash et al., 1987, Matsui et al., 1999) in accordance with the suggested involvement of LOXs in membrane permeabilization. As a consequence of altering the physico-chemical properties of membranes via a modification of their fatty acid residues, fluxes of assimilates or ions are facilitated (Hildebrand, 1989; Serhan et al., 1981).

2.4 LOX structure, function and occurrence:

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

LOX proteins contain highly conserved domain and sequence motifs which are important for the distinct structure and the binding of the catalytic iron (LOX motif: His-X_4-His-X_7-His-X_8-His). Plant and mammalian lipoxygenases contain a small N-terminal β-barrel. The function of the β-barrel is unknown, but it is identical
in connectivity to the C-terminal domain of certain lipases and might be related to lipid binding (Gillmor et al., 1997) and to membrane translocation (Chen and Funk, 2001).

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

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

A survey of the literature indicates that LOXs are present in most, if not all, plant organs, depending on developmental stage and environment. Earlier studies reported that most plant LOXs are soluble enzymes located predominantly in the cytosol (Siedow, 1991). Charge modifications of the soluble LOXs permit their association with membranes (Droillard et al., 1993), but non-specific adsorption to membrane fractions has also been observed (Siedow and Girvin, 1980; Mack et al.,
1987). Froehlich et al. (2001) demonstrated that allene oxidase synthase (AOS) and hydroperoxide lyase (HPL) are localized in different membranes of the chloroplast envelope. Thus LOXs might associate with different membranes in the chloroplast, and therefore with enzymes of different pathways. This would lead to the compartmentalization of oxylipin synthesis in the chloroplast (Fig. 3).

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

2.5 The LOX pathway:

It has been suggested that majority of hydro-(pero)xy fatty acids arise from action of LOXs (Feussner et al., 1997, Rusterucci et al., 1999). A minority of PUFAs may be converted by the α-DOX (α-dioxygenase) into α-hydro(pero)xy PUFAs (Hamberg et al., 1999; Saffert et al., 2000) or may give auto-oxidative products such as dinor isoprostanes (Parchmann and Muller, 1998). Therefore, plants predominantly contain as lipid peroxide-derived substances, the (9S)-hydroperoxy and
the (13S)-hydroperoxy derivatives of PUFAs. The LOX immediate products formed from LA and ALA (Fig. 4 & 5), fatty acid hydroperoxides, then are used by at least six different multienzyme branches to form a large number of oxygenated fatty acids or so-called oxylipins (Blee, 2002).

LOX pathways have been implicated in several physiological processes, including plant growth and development, senescence, resistance to insects and pathogens, and environmental stresses (Howe and Schilmiller, 2002; Porta and Rocha-Sosa, 2002). One branch of the lipoxygenase pathway produces traumatic acid, compounds that are known to be involved in plant cell wound responses (Zimmerman and Coudron, 1979), and volatile C6-aldehydes and C6-alcohols. These volatile compounds are the major contributors to the characteristic fresh ‘green’ odour emitted by leaves (Hatanaka, 1996), triggers the production of phytoalexins (Zeringue, 1992), have antimicrobial activity (Croft et al., 1993) and play a role in pathogen defense (Croft et al., 1993). The second branch produces jasmonic acid, a molecule likely to serve a regulatory role in plant cells (Staswick, 1992; Sembdner and Parthier, 1993). A precise physiological role for LOX in plants has not been defined so far, but the diversity of isoymes and the subcellular distribution suggest multiple functions (Siedow, 1991; Rosahl, 1996).
Fig. 4. Various metabolites produced by LOXs from linoleic acid in plant tissues.
Fig. 5: Various metabolites produced by LOXs from α-linolenic acid in plant tissues. (Source: Plant lipoxygenases. Physiological and molecular features. Porta and Rocha-Sosa, 2002)
2.6 Physiological roles of LOX:

2.6.1 Role of LOXs in seedling growth and development:

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

Seeds of chickpea, lentil, broad bean and kidney bean contain two major LOXs, one synthesizing mainly 13-hydroperoxide from linoleic acid, whereas the other produces 9- and the 13-hydroperoxides and 9- and 13-ketodienes (Sanz et al., 1993; Hilbers et al., 1995; Clemente et al., 2000). Expression of 9-LOX is induced to high levels during seed development in maize (Jensen et al., 1997) and almond (Santino et al., 2005). Peanut seeds contain three lipoxygenase isoenzymes with biochemical properties similar to the three soybean isoforms (Burow et al., 2000). Several putative roles for LOXs in seeds were reported such as fatty acid peroxidation in membranes or storage lipids, production of growth regulators (jasmonates, abscisic acid), responses to pathogens and nitrogen storage (Hildebrand, 1989; Siedow, 1991; Rosahl, 1996).
LOXs play a physiological role during seed maturation and seedling growth. New LOXs are induced during the early stages of seedling growth in different plant species such as pea (Anstis and Friend, 1974; Chateigner et al., 1999; Mo and Koster, 2006), watermelon (Vick and Zimmerman, 1976), French bean (Eiben and Slusarenko, 1994), lupin (Beneytout et al., 1988), lentil (Hilbers et al., 1995), barley (Yang et al., 1993; Holtman et al., 1996), cucumber (Matsui et al., 1992), Arabidopsis thaliana (Melan et al., 1994), Papaver somniferum (Bezakova et al., 1994), rice (Suzuki and Matsukura, 1997) and Brassica napus (Terp et al., 2006).

Three LOXs are present in the mature seed of soybean which disappeared during the first few days of germination. Seed or seedling soybean LOXs are not associated with lipid bodies suggesting that soybean LOXs are not used for lipid mobilization during germination (Wang et al., 1999). Prakash et al. (1990) examined the senescence related changes in chlorophyll and protein content and lipoxygenase activity in peanut cotyledons. Protein content of peanut cotyledons decreased continuously during senescence. On the other hand, lipoxygenase activity increased in early stages of germination followed by a decrease in the later course of senescing peanut cotyledons. Mo and Koster (2006) investigated the changes in lipoxygenase isoforms during germination and early seedling growth of Pisum sativum L. During germination, there was a shift of LOX activity from radicles to shoots that accompanied the transition from seed LOXs to vegetative LOXs. Terp et al. (2006) identified two different LOX cDNAs from germinating seeds of Brassica napus. The expression of the corresponding genes and proteins and the accumulation of their metabolites were analyzed during germination. The 13-lipoxygenase derivatives were 6-8-fold more abundant than the 9-derivatives in etiolated seedlings of Brassica.
In *Phaseolus vulgaris* nodules, LOX mRNAs and proteins are detected mainly in nodules in the growing stage, and their levels decreased in nodules that have reached their full size (Porta *et al*., 1999). Transgenic potato plants expressing an antisense, tuber-specific LOX (*POTLOX-1*) gene displayed reduced LOX activity and a several fold reduction in tuber yield. It was suggested that LOXs are involved in the control of tuber growth and development, probably by initiating the synthesis of oxylipins that regulate cell growth during tuber formation (Kolomiets *et al*., 2001).

### 2.6.2 Biosynthesis of regulatory molecules:

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

Bell *et al*. (1995) used a transgenic approach to modify chloroplast lipoxygenase (LOX2) in *Arabidopsis* to observe the consequences of reduced LOX2 expression on plant growth and the synthesis of JA and ABA. The reduction in LOX2 levels caused no obvious changes in plant growth or in the accumulation of abscisic
acid. However, the wound-induced accumulation of JA observed in control plants was absent in leaves of transgenic plants that lacked LOX2.

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

2.6.3 Role of LOX in disease resistance:

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

Increase in lipoxygenase activity was induced by addition of either the pathogen or an elicitor (Fournier et al., 1986; Ocampo et al., 1986; Peever and Higgins, 1989) suggesting that lipoxygenase induction represents a standard component of the plant’s defense response mechanism (Dixon and Lamb, 1990) and does not simply result from mechanical wounding of the tissue during the infection process. In resistant oat lines, the induction of two new lipoxygenase isozymes accounted for an increase in activity observed upon infection with crown rust,
*Puccinia coronata*, while no change in lipoxygenase activity was seen following infection of susceptible lines (Yamamoto and Tani, 1986).

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

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

Largely circumstantial evidence suggests that 9-LOX pathway plays an important role in defense response to pathogen attack (Weber *et al*., 1999; Hamberg *et al*., 2003). 9-hydroxyoctadecadienoic acid (9-HODE) and colneleic acid were significantly stimulated by *Phytophthora infestans* (Gobel *et al*., 2001), and
expression of a 9-LOX gene precedes appearance of visible HR lesions (Kolomiets et al., 2000). Srinivas Reddy et al. (2000) showed that 9-HPOTrE is the major LOX product formed in response to injury/infection in potato tubers, which is neither a precursor of jasmonic acid or traumatic acid, indicating the operation of a new pathway of LOX mediated defense responses in potato tubers. From these results, it was suggested that LOX metabolites of octadecadienoic acid may be involved in mediating the physiological responses, while octadecatrienoic acid metabolites may be mediating defense responses under stress conditions.

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

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

Expression of a maize 9-LOX gene ZmLOX3 (formerly cssap92) was induced by Fusarium verticillioides and Aspergillus flavus in lines accumulating high levels of
mycotoxins (Wilson et al., 2001), suggesting that mycotoxin biosynthesis may be positively regulated by the 9-LOX products. Collectively, these data prompted the hypothesis that the 9-LOX pathway is utilized by mycotoxigenic fungi to induce biosynthesis of mycotoxins and, hence, that some 9-LOX genes are susceptibility factors in maize plants (Sagaram et al., 2006; Tsitsigiannis and Keller, 2006).

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

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

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

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

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

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

2.6.4 Role of LOX in tolerance to abiotic stresses:

Relatively few studies have been carried out to determine the role of LOX in abiotic stresses in plants. In mature Arabidopsis plants, a higher expression of LOX1 was induced by abscisic acid and methyl jasmonate (Melan et al., 1993) whereas the expression of LOX2 was high in the leaves and inflorescence, but low in seeds, roots and stems. Somashekaraiia et al. (1992) observed an increase in LOX activity in seedlings of Phaseolus vulgaris under Cd²⁺ stress. Gallego et al. (1996) found higher LOX activity in sunflower leaf segments treated with Cu²⁺ than in those treated with Cd²⁺. It has been shown that salt stress caused a high level of lipid peroxidation in the cultivated tomato (Lycopersicon esculentum), while the level of peroxidation in the wild relative, salt-tolerant, tomato (L. pennellii) was only marginal (Shalata and Tal, 1998).

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

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

Porta et al. (2008) showed that in Phaseolus vulgaris a novel chloroplast targeted PvLOX6 was highly induced after wounding, non-host pathogen infection, and by signaling molecules as H$_2$O$_2$, SA, ethylene and MJ. These results implied that common bean uses the same LOX to synthesize oxylipins in response to different stresses.

### 2.7 Isolation, purification and characterization of LOXs:

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

In the pea, different lipoxygenases occur in different organs of the plant and in varying amounts (Domoney et al., 1990). Three lipoxygenases have been purified from soybean seeds which differ in pH optimum. Soybean leaves contain other lipoxygenases which are different from the seed enzymes (Grayburn et al., 1991).
Two lipoxygenase isoenzymes present in the embryo of germinating barley seed have been purified to homogeneity and characterized (Doderer et al., 1992). Both isozymes are monomeric proteins with a molecular mass of approx. 90 kDa. The isozymes differed in the products formed upon incubation with linoleic acid. One of the isozymes (lipoxygenase 1) solely formed the 9-HPODE as a product whereas the 13-HPODE was the major product formed by the other isoenzyme (lipoxygenase-2). Rangel et al. (2002) purified lipoxygenase induced in leaves of passion fruit in response to MJ to homogeneity. In vitro analysis of LOX activity using linoleic acid as substrate showed that it possessed C-13 specificity.

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

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

Ohta et al. (1991) observed a marked increase in LOX and lipid hydroperoxide-decomposing activity in the leaves of rice (Oryza sativa cv Aichiasahi)
after inoculation with the rice blast fungus, *Magnaporthe grisea*. Three LOX isozymes (leaf LOX-1, -2, -3) were isolated from both uninoculated and infected leaves using ion-exchange chromatography. Substrate specificity, positional specificity of oxygenation, and pH optimum of the enzyme were quite different from other rice LOXs reported (Ida *et al.*, 1983; Ohta *et al.*, 1986). Fournier *et al.* (1993) purified and characterized the elicitor-induced LOX enzyme from cultured tobacco cells. The purified LOX enzyme was a soluble type-2 lipoxygenase whose physicochemical parameters, notably molecular weight (96kDa) and pI (5.1, 5.3) were very close to those of other lipoxygenases purified from solanaceous plant organs such as potato tubers (Mulliez *et al.*, 1987), tomato and egg-plant fruits (Grossman *et al.*, 1972; Zamora *et al.*, 1987). Despite these similarities and the reported homologies between tobacco genomic DNA sequences and soybean seed cDNA (Bookjans *et al.*, 1988), the elicitor-induced tobacco lipoxygenase did not cross-react with antibodies against soybean seed lipoxygenases.

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

Roopashree *et al.* (2006) purified a protein from *Dolichos biflorus* seed, which exhibited both LOX and haemagglutination activity. The evidence for the lectin protein with LOX activity came from (i) MALDI-TOF (matrix-assisted laser-
desorption ionization-time-of-flight) MS, (ii) N-terminal sequencing, (iii) partial sequencing of the tryptic fragments of the protein, (iv) amino acid composition, and (v) the presence of an Mn$^{2+}$ ion. A hydrophobic binding site of the tetrameric lectin, along with the presence of a Mn$^{2+}$ ion, accounted for the LOX like activity.

2.8 Antifungal activity of LOX products:

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