CHAPTER 5: DISCUSSION

Biological control using plant based biocides are now being extensively used for pest and disease control. They are non-toxic, systemic and easily biodegradable. Plants acquire resistance upon treatment with an inducing agent like biocide leads to its priming.

In the present study, the morphological, physiological and biochemical tests confirmed the pathogen isolated from naturally infected tomato fruits to be *Pseudomonas syringae* pv. *tomato.* The agar well diffusion assay demonstrated that aqueous fruit extracts of neem could not significantly inhibit the *in vitro* growth of *Pseudomonas syringae* pv. *tomato*. Similar observations were made by Mahfuzul Hoque *et al*. (2006) who reported that aqueous leaf extracts of neem did not show any antibacterial activity against *Listeria monocytogenes, Staphylococcus aureus, Vibrio parahaemolyticus, E. coli* 0157:H7, *Salmonella, Bacillus cereus, Pseudomonas spp., Alcaligenes faecalis*, and *Aeromonas hydrophila* but the ethanol extract inhibited the growth of *L. monocytogenes S. aureus* and *V. parahaemolyticus.* Sukanya *et al*. (2009) demonstrated that aqueous neem leaf extracts had no *in vitro* antibacterial activity against *Xanthomonas vesicatoria* and *Rhizoctonia solanacearum*, but its methanol extract was inhibitory. Aqueous leaf extracts could not inhibit *in vitro* growth of *Bacillus subtilis, S. aureus, E. coli, P. vulgaris, P. aeruginosa, S. typhi, Aspergillus niger, Candida albicans,* while the methanol extract of *Azadirachta indica* exhibited pronounced activity against *Bacillus subtilis* [Grover *et al*., 2011]. Neem oil had 92 % *in vitro* antibacterial activity against *P. aeruginosa, S. pyogenes, E. coli, Proteus* group and *K. aerugenes* [Jahan *et al*., 2007]. Acetone extract of neem leaf had higher antibacterial activity as compared to aqueous and methanolic extracts against *E. coli, B. subtilius, S. typhi, Pseudomonas, S. aureus, K. pneumoniae, S. epidermitis* [Irshad *et al*., 2011]. Ijato *et al*. (2010), however, demonstrated the *in vitro* inhibitory effects of both aqueous and methanolic leaf extract of *Azadirachta indica* against *Rhizopus stolonifer* and *Aspergillus niger.* Neem seed kernel extract (NE) could significantly reduce the *in vitro* growth of *Monilinia fructicola, Penicillium expansum, Trichothecium roseum* and *Alternaria alternata,* which could help in preventing postharvest diseases in plum and yali pear [Wang *et al.*, 2010]. Higher concentrations of neem extracts effectively reduced the *in vitro* growth of brinjal wilt pathogen, *Fusarium solani* [Joseph *et al*., 2008]. Methanol and ethanol extracts of neem leaf could effectively control *in vitro* growth of *Bacillus pumillus, Pseudomonas aeruginosa and Staphylococcus aureus* [Maragathavalli *et al*., 2012]. Various concentrations of aqueous neem

leaf extracts could effectively suppress the *in vitro* mycelial growth of *F. oxysporum* and *A. solani* [Hassanein *et al*., 2008]. The germination of *A. sesami* spores were not significantly inhibited by neem extract but the sesame plants had low incidence of spot pathogen [Guleria and Kumar, 2006]. *In vitro* growth of *X. campestris* could be significantly inhibited by methanolic extracts of neem leaf and fruits [Britto and Gracelin, 2011]. Amadioha (2000) reported that neem seed oil and its aqueous and ethanolic extracts could effectively control the *in vitro* radial growth of *Pyricularia oryzae*.

In the present study, neem fruit extract could significantly ($P \leq 0.05$) induce SAR in tomato against *Pseudomonas syringae* pv. *tomato* thereby reducing the incidence of bacterial speck in both the local and F1 varieties. The application of neem extract on a single leaf of the plant could elicit the expression of POX, PPO and LOX both at the site of treatment and in distal untreated leaves of the plants. The isozyme expression pattern of POX and PPO was also significantly altered. However, the extract had no significant effect on lysozyme activity and expression of its isoforms.

Induction of SAR by application of plant extracts and subsequent reduction in the incidence of disease has also been demonstrated in an array of plant-pathogen systems [Sarvamangala *et al*., 1993; Daayf *et al*., 1995; Kagale *et al*., 2004; Enikuomehin, 2005; Latif *et al*., 2006; Mostafa and Gado, 2007; Akinbode and Ikotun, 2008; Balestra *et al*., 2009; Hassan *et al*., 2009; Belabid *et al*., 2010; Stangarlin *et al*., 2011; Ahmed *et al*., 2012; Majeed *et al*. 2012].

Singh and Prithviraj (1997) demonstrated the induction of resistance in pea against *Erysiphe pisi* by Neemazal, a product of neem. Olufolaji (1999) reported the control of wet rot disease of *Amaranthus* sp. caused by *Choanephora cucurbitarum* with extracts of *A. indica.* Water and ethanol leaf extracts, and seed oil of *A. indica* could significantly reduce the spread of blast disease in rice under greenhouse conditions [Amadioha, 2000]. *R. solani*, *F. solani* and *S. rolfsii* were effectively controlled on cucumber plants by weekly sprays of neem fruit extract [Tohamy *et al*., 2002]. Aqueous extract of neem leaves controlled the leaf stripe pathogen, *Drechslera graminea* on barley [Paul and Sharma, 2002] and *A. sesami* on sesame [Guleria and Kumar, 2006] through induction of systemic acquired resistance. Neem seed powder significantly reduced the severity of *Fusarium* and root-knot pathogen on tomato in both greenhouse and field [Agbenin *et al*., 2004]. Aboellil (2007) demonstrated that neem extract could enhace resistance in cucumber against *Podosphaera xanthii*. Early blight and wilt diseases in tomato could be

effectively controlled by neem extract due to suppression of the mycelial growth of the pathogens [Hassanein *et al*., 2008]. Wilt incidence in *Phaseolus vulgaris* was significantly reduced by neem extract [Obongoya *et al*., 2010]. Neem extract substantially reduced the incidence of crinkle virus disease in Urd bean [Binyamin *et al*., 2011]. Amongst several tested plant extracts, neem leaf extract was the most effective in reducing the damping-off disease in tomato, chilli and eggplant [Pattnaik *et al*., 2012; Islam and Faruq, 2012; Nwogbaga and Utobo, 2012]. Subramani *et al*. (2012) reported that *A. indica* extracts inhibited coffee leaf rust upto 61.40%.

The results of the present study indicated that 8 to 10 weeks old tomato plants were most resistant towards the pathogen. Treatment of young plants with neem fruit extract alone or in combination with pathogen reduced disease severity, but in older plants the disease severity was significantly higher. Similar observations in tobacco have also been made by Mandal *et al*. (2007). Among all the treatments, the pathogen only inoculated plants had maximum disease severity irrespective of the age.

The 12 weeks old plants were the most susceptible probably because they inadequately responded to the neem treatment. These plants had significantly reduced activity of POX, PPO, LOX and lysozyme and expression of fewer isozymes which resulted in insignificant induction of SAR. The plants of 6 and 8 weeks age were more susceptible than 10 weeks old plants but less than 12 weeks old plants probably because the SAR induction by neem treatment at these ages was insignificant as compared to the 10 weeks old plants. Therefore, it appears that SAR induction is age-dependent and the molecular events leading to establishment of SAR by plant extracts is not activated prior to a specific age possibly which is species specific. Progressive increase in the defense response was observed between 6 and 10 weeks of age. The local variety was more responsive to neem treatment as compared to the F1. Higher disease severity was observed in the F1 plants. Enhancement in the activity of defence enzymes and induction of novel acidic POX and PPO isoforms were significantly reduced in F1 plants. This resulted into F1 being more susceptible to the pathogen than the local variety. However, neem fruit extract could still significantly reduce disease symptoms in F1 variety. The age dependence of SAR inducibility of F1 plants was observed to be similar to the local plants, indicating that the developmental stage of tomato is crucial in its natural ability to sustain against the pathogens as

well as in its capacity to be induced for systemic expression of defense compounds by the application of certain elicitors such as neem fruit extract.

The role of age in the susceptibility or resistance of plants was critical for both the varieties. The intermediate stages of growth were least susceptible and the mature stages were the most susceptible to the bacterial speck pathogen. Earlier studies also demonstrated that the developmental stage of the plant has a vital role to play in the expression of resistance against the invading pathogens [Develey-Rivière and Galiana, 2007], although there are instances where plant age didn't play any significant role in its susceptibility [Visker *et al*., 2003].

Padmanabhan and Ganguly (1954) observed that susceptibility in rice was least at younger stages and it increased with age of plants. Bateman and Lumsden (1965) hypothesized that during the processes of tissue maturation, pectin is converted to calcium pectate which renders the pectic materials resistant to polygalacturonase and thereby increases resistance of bean hypocotyl tissue to *R. solani.* Griffin and Hunt (1972) documented the negative correlation between increase in age and susceptibility of *Alfalafa* plants against gall disease. The resistance of cotton seedlings to *Rhizoctonia solani* increased between 5 and 12 days after planting [Hunter *et al*., 1977]. Soybean plants displayed increase in resistance against *Phytophthora megasperma* var. *sojae* as the plant matured [Ward *et al.*, 1981]. Decrease in susceptibility of groundnut to *Puccinia arachidis* with increasing plant age has been reported, probably due to the reduced ability of the host plant to recognize the pathogen [Savary, 1986]. Kim *et al*. (1989) have noted the existence of age-related resistance (ARR) in pepper which could be attributed to the physiological changes during transition from vegetative to flowering stage. Increase in leaf age was accompanied by increase in resistance in three incompatible cowpea cultivars inoculated with race 1 of *Uromyces vignae* [Heath, 1994]. Younger Chickpea leaves were more resistant to *Ascochyta rabiei* than the mature leaves [Dolar, 1997]. Dowley *et al*. (1997) reported that onset of fruiting could increase resistance in mature tomato plants against *Phytophthora infestans.* Increase in vulnerability of tomato to *Alternaria solani* due to reduction in the thickness of the epicuticular wax layer with increasing age has been reported by Vloutoglou and Kalogerakis (2000). Dat *et al.* (2000) have reported the differential expression of foliar antioxidants in *Pisum sativum*, known to be critical for plant defense, leading to variation in susceptibility of the host plants towards pathogens at different ages. Zeier (2005) demonstrated that *Pseudomonas syringae* pv. *maculicola*-induced expression of phenylalanine

ammonia lyase (PAL), glutathione-S-transferase (GST) and Salicylic acid was higher and accumulated faster in younger leaves of *Arabidopsis* as compared to the older ones. Ripening in tomato was accompanied by expression of cell wall-modifying polygalacturonase and expansin proteins which increased susceptibility of the fruit towards the necrotrophic pathogen *Botrytis cinerea* [Cantu *et al*., 2009]. Ando *et al*. (2009) demonstrated the increase in resistance of Cucurbit fruit against *Phytophthora capsici* as it grew from young waxy green to mature green stage, probably due to increased expression of "*R*" gene, responsible for imparting resistance to the host. Higher resistance against *Cladosporium fulvum* in mature tomato plants was observed due to expression of an avirulence gene *Cf-9B* specifically in mature plants [Shibata *et al.*, 2010]. Rwegasira & Rey (2012) reported that higher incidence of brown streak virus disease in mature cassava plants was due to increase in the time of exposure of plants to the pathogen.

The identification of similar transduction pathways governing various developmental or defense processes in different plants suggests that these pathways have been conserved through evolution. Plants may therefore have acquired some capacity to resist pathogens by adaptation or exaptation (the acquisition by a protein of a function other than that for which it was originally selected) of physiological or developmental functions. Different components of defense responses may therefore not only defend the plant against stresses, in addition to those exerted by plant pathogens, but also play other roles in plant development, structure and function [Develey-Rivière and Galiana, 2007].

The results of present study demonstrated that application of neem fruit extract significantly increased POX activity both locally and systemically in distal leaves with variable magnitude at different plant ages and was effective in induction of expression of its acidic isoforms in both the cultivars. The appearance of additional isoforms after neem treatment suggested that either the already expressed but inactive POX isoforms were activated or new ones were expressed as a result of neem elicited reactions. POXs have been implicated in several physiological processes during the induction of hypersensitive responses resulting in acquisition of SAR due to pathogen attack or elicitor application. In the present study, neem elicited-POX might have created toxic environment inside the cell cytoplasm by generation of ROS which can directly inhibit the invading pathogens. POX-mediated formation of physical barriers in host cell walls by enhanced lignifications, polymerization of lignin and suberin and cross linking of other wall proteins to

prevent the entry of the pathogen into the cell might also have increased resistance of tomato against *Pseudomonas syringae* pv. *tomato*.

One of the main functions of POX is to ensure the detoxification of activated O_2 forms which is very important for metabolic responses of plants to different stress factors [Bybordi *et al*., 2010]. Enhanced peroxidase activity has been associated with induced systemic resistance of cucumber to *Colletotrichum lagenarium* [Hammerschmidt *et al.,* 1982]. Four different POX isoforms were present in tomato plants attacked by *P. syringae* pv. *tomato* as compared to only one in control plants which was correlated to lesser disease index in them [Bashan *et al*., 1985]. Higher POX activity in resistant muskmelons as compared to the susceptible ones has been considered as a marker of resistance against *Pseudoperonospora cubensis* [Reuveni *et al.,* 1992]. Alcazar *et al*. (1995) reported the *Phytophthora capsici*-induced expression of an acidic isoPOX in the cytoplasm of resistant *Capsicum annum* var. Smith-5 which was confirmed by histochemical analysis. Inoculation of tomato leaves with tobacco necrosis virus resulted into systemic expression of three novel POX isoforms which led to induction of systemic resistance in the host plant [Anfoka and Buchenauer, 1997]. He *et al*. (2001) reported that rapid induction of hypersensitive cell death in *Asparagus densiflorus* was associated with restriction of *Fusarium* growth, and activation of peroxidase. It has been suggested that inoculation of maize dwarf mosaic virus resulted in increased activity of a specific anionic isoform of POX in resistant inbred lines of maize which was involved in the synthesis of barriers in cell walls thus preventing the translocation of the virus into the cytoplasm [De Souza *et al*., 2003]. Yeast-elicited cassava cells demonstrated induced-expression of a peroxidase gene *MecPOD1* which probably led to cross-linking of extensin monomers in the cell walls leading to increased resistance towards the pathogen [Gomez-Vasquez *et al.,* 2004]. Oxidative stress tolerance in pepper was enhanced by active participation of POX involved in the detoxification of ROS and thus reducing damage to the ROS-susceptible chlorophyll [Sarowar *et al*., 2005]. It has been suggested that expression of *Ep5C* (corresponding to POX *CEVI16*) in tomato upon inoculation with *Pseudomonas syringae* pv. *tomato* might be necessary for modification of a cell surface molecular target that could be a component of a basal host defense complex [Coego *et al*., 2005]. Wang *et al*. (2005) reported that a cytosolic POX gene *APX* was overexpressed in transgenic tomato in order to minimize the damage caused by ROS synthesised during seed germination hence conferring tolerance to chilling and salt stress. Upon infection of *A. thaliana* by *Orobanche ramose*, a gene coding for a

class III peroxidase was upregulated which probably resulted in an increase in lignin accumulation and cross-linking of cell wall proteins [Passardi *et al*., 2005]. It has been reported that POX mediated generation of H_2O_2 in *Arabidopsis* was crucial in imparting resistance to a wide range of pathogens [Bindschedler *et al.*, 2006]. Vital involvement of POX in induction of ISR in tomato has been studied by Halfeld-Vieira *et al*. (2006). *Botrytis cinerea* induced increase in peroxidase activity led to oxidation of phenol compounds to oxidized products (quinones) and polymerization of hydroxycinnamyl alcohols to yield lignin and cross-linking isodityrosine bridges in cell wall in Faba bean which resulted in lower disease incidence [Hassan *et al.*, 2007]. Cucumber plants after challenge-inoculation with *P. cubensis* showed induction of different isoforms of POX as compared with control plants indicating its potent role in defense response [Anand *et al*., 2007]. Resistant sunflower cultivar expressed four POX isoforms as compared to only two in the susceptible cultivar which might be critical in imparting resistance against *M. phaseolina* [Aboshosha *et al.*, 2008]. Kavitha and Umesha (2008) demonstrated that the expression of POX was higher in resistant (safal) tomato cultivar than the susceptible (PKM-1) one, thus linking it directly to higher resistance of safal variety towards *Xanthomonas axonopodis* pv. *vesicatoria*. Expression of a novel POX isoform was responsible for lignification of cell walls in cold stressed buckwheat plants [Lucic *et al.*, 2009]. Increased POX activity and affinity for the substrates of lignification, as well as for the formation of H_2O_2 clearly suggested that POX was involved in formation of barrier in pearl millet during *Sclerospora graminicola* attack [Arun *et al.*, 2010]. Ashry *et al*. (2011) demonstrated positive correlation of resistance in flax with the increase in POX activity, accompanied by production of microtoxic free oxygen radicals and H_2O_2 upon infection with Powdery mildew pathogen, *Odium lini* Skoric. The induction of resistance in tomato plants against *Pseudomonas syringae* pv. *tomato* has been correlated to the increased activity of POX and increase in number of its isoforms Bhuvaneshwari and Paul, 2012]. Taheri (2010) and Taheri and Tarighi (2012) reported that in a compatible tomato-*R. solani* interaction, resistant variety of tomato had significantly high expression levels of *CEVI1* POX gene as compared to the susceptible one, which might be involved in the polymerization of lignin or suberin, the crosslinking of wall glycoproteins or polysaccharides, and the dimerization of antimicrobial phenols in response to pathogen invasion.

The present study also demonstrated that the expression of POX is developmentally regulated. The expression of acidic POX isoforms and its overall activity were noted to be variable at

different developmental stages of the plants. The progressive increase in expression of acidic POX isoforms was observed up to 10 weeks of age in the leaves of tomato plants which reduced dramatically at older age. The induction of POX and change in its activity after the foliar application of the elicitor (neem fruit extract) also increased upto 10 weeks of age. This might be possible due to active involvement of POX in auxin metabolism during the younger growth stages of plants. However, in older plants reduced POX expression might have reduced auxin activity which is critical for the onset and promotion of flowering by reducing leaf proliferation.
Kar and Mishra (1976) have reported that POX activity was higher in rice plants during senescence. Expression of new POX isoforms in *Capsicum annum* leaves reduced auxin metabolism, thereby permitting floral development [Bernal *et al*., 1993]. Mohan *et al*. (1993) suggested that the POX *tap1* gene was expressed upon wounding or pathogen attack and played a critical role in normal developmental processes in tomato. A positive correlation between age and number of anionic POX isoforms in tobacco was reported by Klotz *et al*. (1998). Older tomato plants had higher tendency of POX induction by JA application [Cipollini and Redman, 1999]. Scialabba *et al*. (2002) noticed the reduction in the expression of a POX isoform with increasing age in Radish seeds. The expression of POX gene *PR9* was induced under non-pathogenic, developmentally regulated events such as flowering, cytokinin fluctuation or as a consequence of expression of the *LOX* gene [Wielgoss and Kortekamp, 2006]. In *Arabidopsis*, the mRNA of two Ascorbate peroxidase genes *APX4* and *tAPX* showed strong plant-age-dependant reduction in levels at an early stage of leaf senescence [Dabrowska *et al*., 2007]. One of the attributes of increased resistance of tobacco towards *Peronospora tabacina* at higher ages was increased POX activity [Ebrahim *et al*., 2011].

Local and systemic increase in PPO activity with varying magnitude at different stages of plant growth was observed in both cultivars of tomato. Elevated levels of PPO activity and enhanced expression of its acidic isoforms in tomato plants after neem treatment could be related to its critical role in imparting resistance. Neem could induce higher expression of PPOs both at the site of treatment and in leaves away from it leading to induction of SAR in tomato. This could have possibly activated the PPO-mediated phenylpropanoid pathway resulting in the synthesis of quinones from cytoplasmic phenols and production of microtoxic ROS. Enhanced PPO activity could have promoted accelerated cell death of the cells surrounding the infection site thus preventing the spread of pathogen. Also, cross linking of carbohydrates, glycoproteins and lignin

in the cell walls might have occurred, thereby reducing pathogen ingress. Successful SAR establishment can thus be attributed to the multifaceted defensive functions of PPO in tomato. Elevations in PPO activity and enhanced plant resistance have also been reported by few earlier investigators.

Bashan *et al*. (1985) reported the induction of 8 PPO isoforms leading to synthesis of antimicrobial phenolics in response to *Pseudomonas syringae* pv. *tomato* attack in tomato. Plant growth promoting rhizobacteria (PGPR) elicited PPO activity was responsible for induction of resistance in cucumber against *P. aphanidermatum* [Chen *et al*., 2000]. Tyagi *et al*. (2000) suggested that the increase in number of PPO isoforms due to *A. triticana* infection in wheat led to increased contents of oxidized quinone derivatives, thus increasing its resistance towards the pathogen. Li and Steffens (2002) reported that quinones generated in PPO over-expressing tomato plants could hinder the ingress of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* by generating microtoxic ROS to directly inhibit the pathogen growth inside the cells, accelerating cell death of plant cells nearby infection site, alkylation of proteins and reduce protein bioavailability to the pathogen and by synthesis of cross-linked phenolics barrier in the cell wall. A positive correlation of PPO isoforms expression and enhanced resistance against *Alternaria helianthi* due to accumulation of its oxidation products in sunflower has been studied by Madhavi *et al.* (2005). Nakkeeran *et al*. (2006) reported that application of *Bacillus subtilis* strain BSCBE4 and *Pseudomonas chlororaphis* strain PA23 on hot pepper could induce PPO, which resulted in cross-linking of hydroxyproline rich glycoproteins (HRGPs) and lignin barriers in cell walls against *Pythium aphanidermatum* invasion. Induced systemic resistance (ISR) in cucumber against *P. cubensis* and *Erysiphe cichoracearum* was mediated by expression of an additional PPO isoform [Anand *et al*., 2007]. Cavalcanti *et al*. (2007) reported that PPO mediated lignifications in tomato cell walls increased its resistance against *Xanthomonas vesicatoria* by application of aqueous suspension of *Crinipellis perniciosa* mycelium. Benzothiadiazole treatment of onion plants increased lignin biosynthesis, oxidative crosslinking in cell walls and generation of ROS due to enhanced PPO activity leading to resistance against leaf blight pathogen, *Stemphylium vesicarium* [Abo-Elyousr *et al.*, 2008]. Chatterjee and Ghosh (2008) observed hyperactive expression of PPO characterized by rapid accumulation of phenolics at infection site and increased cell death in yellow vein mosaic disease virus infected mesta plants. Bacterial spot in tomato was effectively controlled by foliar application of SA

which led to enhancement of PPO activity [Ibrahim, 2011]. The transcript levels of PPO genes were altered and its activity increased after application of commercial extract from the brown seaweed *Ascophyllum nodosum* to cucumber [Jayaraman *et al*., 2011]. The PPO activity was observed to be higher in all the developmental stages of resistant genotype of maize in comparison to the susceptible ones which was responsible for lignifications of the host cell walls [Purwar *et al*., 2012]. PPO has been observed to be instrumental in imparting resistance to potato against soft rot infection by oxidation of chlorogenic acid which inhibits the cell wall degrading activity of *Pectobacterium* sp. [Ngadze *et al.*, 2012]. Richter *et al*. (2012) demonstrated that a specific, single PPO isoform was significantly responsible for imparting resistance in Dandelion. Bhuvaneshwari and Paul (2012) also demonstrated that application of neem extract could lead to the systemic induction of defense enzymes like POX and PPO, responsible for reduced bacterial speck symptoms in tomato.

The findings of present study also demonstrated that the expression of PPO in tomato is developmentally regulated. Differential expression of the PPO isoforms and variable overall PPO activity was observed at different ages. PPO expression progressively increased with the increase in age of tomato plants, was maximum at 8 to 10 weeks of age and reduced significantly as the plant aged. The induction of PPO isoforms by neem extract was also found to be dependent on the plant/leaf age. Higher expression of PPO in young leaves might be because of their higher susceptibility towards pathogen or more vulnerability towards pathogen attack. Since PPOs are actively involved in cell wall modifications, it can be hypothesized that higher expression of PPO in young leaves might lead to cross linking of proteins in cell walls possibly for prevention of leakage of cytoplasmic proteins during leaf growth as well as to stop the invasion of the pathogens. Reduced PPO expression in mature plants could be attributed to the transition of plants from vegetative to reproductive phase during which the physiological machinery could have undergone several changes required for flowering. The variation in the expression of PPO in concurrence with age has been observed in a variety of plants. Younger leaves of hybrid poplar had more PPO isoforms than the older leaves in response to wounding, which seemed to be an adaptation against herbivory, since young leaves are often preferred by herbivorous insects [Constabel *et al*., 2000]. Li and Steffens (2002) reported that endogenous PPO activity was found to be negatively correlated with age in tomato and its induction by various elicitors such as SA, JA or ethylene was age-dependent, probably because of the involvement of several signaling

mechanisms which are activated at different stages of life cycle. Doan *et al*. (2004) concluded that younger plants of *Physalis angulata* were more responsive in expressing PPO after JA application possibly because of physiological changes related to the shift from vegetative to reproductive phase in older plants. Thipyapong *et al*. (2004) reported that in water stressed tomato plants, PPO expression was higher in younger leaves as compared to older ones probably in order to protect the juvenile tissues from subsequent attack by pathogens and pests. In their further studies on aspen and hybrid poplar, Thipyapong *et al*. (2007) detected leaf-age-dependent local and systemic induction of PPO expression. Higher PPO expression in young *Solanum tuberosum* leaves has been correlated with the increase in crosslinking of proteins in cell wall for prevention of cytoplasmic leakage during leaf expansion [Poiatti *et al*., 2009].

Foliar application of neem fruit extract could significantly enhance LOX activity both at the site of application and systemically in distal parts of the plant in both the tomato cultivars at all the ages, leading to induction of SAR. Lipoxygenases are actively involved in hypersensitive resistance responses leading to signaling of the defense related reactions and ultimately facilitating the host cell in getting immunized to any kind of unfavourable stress conditions. Lipoxygenase could directly inhibit the spread of pathogen by synthesizing oxylipins which induce membrane damage in the infected cells, thus releasing antimicrobial compounds. Another possible role of LOXs could be the conversion of cytoplasmic polyunsaturated fatty acids into hydroperoxides, which act as precursors for JA pathway, ultimately synthesizing Jasmonic acid which is an integral defense compound. Neem elicited LOX might have possibly mediated the synthesis of antimicrobial phytoalexins leading to the synthesis of Salicylic acid, a secondary signaling molecule which is translocated to the untreated parts of the plant and promoting *de novo* synthesis of PR proteins for a successful SAR establishment. The rise in LOX activity as a consequence of neem treatment in tomato may be responsible for initiation of the defense pathways, pointing towards its unique role in the enhancement of resistance in the host plant.

Peever and Higgins (1989) demonstrated that inoculation with pathogens or application of elicitors was followed by the increase in LOX activity. LOX mRNA accumulation during tomato-*Pseudomonas syringae* interaction resulted in membrane damage leading to hypersensitive necrosis of the affected cells [Koch *et al*., 1992]. The *LOX1* gene in *Arabidopsis* was upregulated by the application of virulent as well as avirulent pathogen, which was suggested to be the first step in initiation of a signal transduction mechanism leading to JA

pathway and hence inducing systemic resistance in them [Melan *et al*., 1993]. Several antifungal unsaturated aldehydes were synthesised from polyunsaturated fatty acids via the lipoxygenase pathway in soybean (*Glycine max*) in response to wounding and pathogenic infection [Vaughan and Gardner, 1993]. 13-hydroperoxyoctadecadienoic acid (13HPODE) and 13-hydroperoxyoctadecatrienoic acid (13HPOTrE) were synthesized via lipoxygenase pathway which were not only toxic to the pathogen but also acted as the precursors of JA synthesis in groundnut, pigeonpea and tomato against *A. niger, F. udum* and *Pseudomonas putida* strain BTP1 respectively [Sailaja *et al*., 1997; Devi et al., 2000; Akram *et al*., 2008; Mariutto *et al*., 2011]. Absence of LOX gene in tobacco plants made them susceptible against *Phytophthora parasitica* var. *nicotianae* while inclusion of a LOX gene in transgenic tobacco led to greater accumulation of LOX and its products, thereby enhancing host resistance against the pathogen [Rance *et al*., 1998; Mene-Saffrane *et al*., 2003]. Pathogen-induced hypersensitive reactions involved LOX-mediated synthesis of oxylipins, responsible for accelerated cell death in the infected tissues of *Arabidopsis thaliana* upon infection with *Ralstonia solanacearum* [Montillet *et al*., 2002]. El-Khallal (2007) reported that bio-elicitation of tomato plants by arbuscular myccorhiza induced LOX-mediated synthesis of phytolexins and increased accumulation of salicylic acid which led to increased resistance of the plants against *F. oxysporum*. Inactivation of the lipoxygenase gene *ZmLOX3* increased susceptibility of maize to *Aspergillus* spp. suggesting its critical role in plant defense [Hu *et al*., 2011]. Yang *et al*. (2011) demonstrated that the wound-induced JA synthesis was regulated by LOX at transcriptional levels and its activation was necessary in wound-mediated defense response responsible for increased tolerance of pea seedlings to wounding. In *Arabidopsis*, 9-Lipoxygenases (9-LOXs) initiate fatty acid oxygenation resulting in the formation of oxylipins, thereby activating defense against hemibiotrophic pathogenic bacteria [Vellosillo *et al*., 2012].

The present study also demonstrated that LOX activity was highest in the young plants (6 weeks) in both the varieties. However, at 8, 10 and 12 weeks stage LOX activity didn't vary significantly. At an older age in both the varieties, LOX activity was significantly reduced after pathogen inoculation, indicating the reduced ability of the host plant to initiate LOX expression upon pathogen attack at mature stages. At this stage the pathogen and neem combinations were able to elicit higher levels of LOX activity in the new leaves emerging after 2 weeks of treatments, pointing towards the efficacy of neem extract in inducing LOX expression at all ages

leading to SAR establishment in tomato. Age regulated expression of LOX and difference in the responsiveness of LOX genes at different stages of plant growth has been a characteristic feature of LOX expression. Higher activity of LOX in younger plants could be a strategy of plant protection during comparatively more susceptible phase by modifying the plasma membranes in tomato leaves. However, LOXs might have also served as storage proteins (required for normal growth and development) in early stages of seedling development.

Hertel *et al*. (1987) reported that maize seedlings had highest LOX activity at younger stages which decreased with maturation, indicating its possible role in defense of young susceptible seedlings from potential pathogens. A progressive loss of LOX activity with ripening of the fruits of belrrubi and agridulce varieties of pepper was observed by Mínguez-Mosquera *et al*. (1993). Saravitz and Siedow (1995) suggested that reduction of LOX expression with increase in age of soybean leaves might be necessary to facilitate flowering. LOX activity in olive fruits was high at early stages of development and underwent a steady decrease thereafter, probably playing an important role in physiological response of plants to stress at younger stages [Salas *et al*., 1999]. Porta and Rocha Sosa (2002) have reported the expression of different LOX isoenzymes during germination and in mature seedlings serving as storage and defense proteins respectively in soybean. Yi *et al*. (2005) suggested that the higher LOX activity during early stages of germination in oat could possibly have a role in plasma membrane modifications for protection against pathogens. The *TomloxD* transcript was observed to be higher in younger tomato leaves, playing a critical role in prevention of mechanical injury in them and acting as a precursor of the octadecanoid pathway during induction of SAR in susceptible young plants [Hu *et al*., 2011].

Variable response to neem extract in induction of cytoplasmic lysozyme activity and in-gel-activity-staining was observed. 8 and 10 weeks old plants responded adequately to the neem treatment. The younger and older plants didn't show significant variation in lysozyme expression after foliar treatment with the neem fruit extract either singly or in combination with the pathogen. However, when the plants were inoculated only with the bacterial speck pathogen, they showed marked increase in lysozyme activity at all the ages. The release of pathogen virulence compounds including effector proteins into the host cytoplasm possibly triggered the breakage of vacuoles and the discharge of lysozyme. This could have possibly acted as an effective second line of defense when the pathogen causes tissue damage. Once the vacuole is disrupted, lysosome is released into the cell cytoplasm, thereby attacking the pathogen. This may

occur in the hypersensitive reaction of the plant where a small group of plant cells around an invading pathogen die and release their contents [Boller and Vogelli, 1984; Van Loon *et al*., 2006]. This could be one of the factors responsible for insignificant response of tomato to the neem treatment as neem application might be ineffective in boosting the release of lysozyme from the vacuoles of the host cells. In plants, induction of chitinase/lysozymes and other hydrolytic enzymes is a coordinated, often complex and multifaceted defense mechanism triggered in response to phytopathogen attack. Due to their apparent anti-pathogen activities and their inducibility in plants upon infection by pathogens, lysozymes have been classified as pathogenesis-related (PR) proteins [Mayer *et al.*, 1996].

In the leaves, stems, and roots of *Hevea brasilensis,* chitinase/lysozyme activity rapidly increased following exposure of the plant to elevated levels of the phytohormone ethylene, bacterial, viral or fungal pathogens or a number of other biotic or abiotic stresses, which has been correlated to the defensive role of lysozyme [Martin, 1991]. Introduction of a T4 lysozyme gene in transgenic potato could effectively protect the plant from the attack of *Erwina carotovora* spp. [During *et al*., 1993]. Busam *et al*. (1997) suggested that the expression of *VCH3,* a type III chitinase possessing lysozyme activity, could successfully induce SAR in *V. vinifera* against *Plasmopara viticola*. BTH (elicitor)-treated tomato leaves had higher lysozyme activity as compared to the controls indicating its important role in plant defense [Inbar *et al*., 1998]. Silverleaf whitefly feeding on the host plants significantly induced lysozyme activity both locally and systemically in the hosts leading to their increased resistance against the pest [Mayer *et al*., 2002]. Wang *et al*. (2005) and Wang *et al*. (2011) reported the presence of lysozyme in mung bean seeds and *Momordica charantia L.* respectively which exhibited broad spectrum antifungal and antibacterial properties, suggesting its important role in constitutive host defense mechanisms against microbial pathogens. Sawasdipuksa *et al.* (2011) isolated a lysozyme possessing antifungal properties against *Macrophomina phaseolina* from the seeds of *Pithecellobium dulce.*

In the present study, no additional lysozyme isoforms were observed after treatment with neem extract in both the varieties. The 8 weeks old plants of local variety had significantly higher lysozyme activity as compared to other ages. This indicates that tomato plants have an inherent tendency towards higher expression of lysozyme and it is more active at this stage of growth. The concentration analysis of the in-gel-activity stained bands of lysozyme also supported the

above results. The F1 plants however, didn't show any such behavior. The lysozyme activity in them didn't deviate significantly at different ages. The presence of a highly basic chitinase/lysozyme in cucumber seeds which disappeared completely after 3 days of germination has been noticed by Majeau *et al*. (1990). Pilet *et al*. (1983) reported increase in lysozyme expression in accordance with the growth stages. Two lysozyme isoforms were observed specifically during the early stages of seed germination in Barley, which acted synergistically alongwith ribosome-inactivating protein and β-glucanase for lysis of seed rot fungi, *T. reesei* and *F. sporotrichioides* [Jacobsen *et al*., 1990; Leah *et al*., 1991].

The quantitative real time PCR results demonstrated that *PPOA* gene was significantly upregulated at the site of treatment in plants inoculated with pathogen prior to or after neem treatment. When the pathogen inoculation preceded neem treatment, either the elicitor was able to overcome the inhibitory effects of pathogen or the pathogen was unable to significantly inhibit *PPOA* gene expression. Similarly, when neem treatment preceded pathogen inoculation, the pathogen could not suppress its elicitation effects. Graham *et al*. (2003) observed upregulation of defense-associated *PR-1a*, *PR-10*, *PR-2*, *PR-4* and *PR-6* genes upon treatment with glucan elicitor (WGE) from the cell wall of *Phytophthora sojae* both at the site of treatment as well as in distal parts of soybean. Djonovic *et al*. (2006) reported the upregulation of *GLU* and *CHT* (PR proteins), *CAD1-C* and *HMG* (terpenoid phytoalexin pathway), and *POD6* and *GhLOX1* (related to oxidative burst and hypersensitivity reactions) genes in response to application of Sm1, a proteinaceous bioelicitor secreted by *Trichoderma virens*. Chujo *et al*. (2007) reported that application of cerebroside elicitor isolated from *Rhizoctonia* sp. MAFF3055045 on rice calli could enhance the expression of a transcription factor OsWRKY53 which was responsible for upregulation of pathogenesis-related *PBZ1* gene, thus increasing resistance against *Magnaporthe grisea.* Subramanian *et al*. (2011) could significantly induce the expression of *PR1* and *PDF1.2* genes in *Arabidopsis* against *Pseudomonas syringae* pv. *tomato* DC3000 and *Sclerotinia sclerotiorum* by application of methanolic extracts of *Ascophyllum nodosum* on the roots of host plants.

The lipoxygenase *TomLoxD* gene was downregulated in distal node leaves of plants inoculated with pathogen after neem treatment. The *CEVI1* POX gene was downregulated in both the 3rd and distal node leaves of plants inoculated with pathogen prior to neem treatment. The pathogen seems to interfere and eventually suppress the inductive effect of neem, thereby suppressing the

expression of *CEVI1* gene. The neem treatment could not overcome the inhibitory effects of the pathogen. Moreover, it appears that the pathogen interferes with the elicitation abilities of the neem fruit extract because significantly lower expression of RNA was observed in the 3rd node leaves of plants simultaneously treated with pathogen and neem extract. This could be due to possible modulation of the host genomic machinery by the effector molecules secreted by the pathogen into the host cytoplasm. This appears to be in agreement to the findings of Rico and Preston (2008), who reported that such effector proteins can potentially inactivate plant surveillance mechanisms and signaling pathways, thus allowing the survival of the pathogen on the leaf surface. Hauck *et al*. (2003) demonstrated that AvrPto, an effector molecule of *Pseudomonas syringae* pv. *tomato* strain DC3000 could downregulate the expression of a set of genes in *Arabidopsis* which encoded cell wall and defense proteins, thus increasing the susceptibility of the host towards the pathogen. Coronatine, an effector molecule synthesized by *Pseudomonas syringae* pv. *tomato*, acts as a molecular mimic of methyl jasmonate and hence inhibits the activation of JA signaling pathway responsible for defense response during stress conditions [Nomura *et al*., 2005]. *Pseudomons syringae* released an effector molecule HopAI1 which inactivated by dephosphorylating two mitogen-activated protein kinases MPK3 and MPK6 in *Arabidopsis* resulting into the downregulation of cell wall defense and transcriptional activation of PAMP (pathogen-associated molecular patterns) response genes [Zhang *et al*., 2007]. The *AvrPto* specifically targets and inhibits the functioning of FLS2, a receptor kinase responsible for perception of bacterial pathogens and initiation of defense response in *Arabidopsis* [Xiang *et al*., 2008].

The aim of this study was to evaluate the effect of a bioelicitor (aqueous neem fruit extract) on the expression of defense associated PR proteins peroxidase, polyphenol oxidase, lipoxygenase and lysozyme leading to induction of systemic acquired resistance in tomato, at different plant ages. Application of neem extract could significantly reduce disease severity in leaves emerging two weeks after neem treatment in both local and F1 (Roopsi) varieties of tomato. The reduction in disease severity was accompanied by significant enhancement in activity of POX, PPO and LOX by neem extract at all ages, both at the site of application as well as in the distal untreated leaves away from it, thus proving the successful induction of SAR in them. However, the magnitude of the activity of the enzymes varied as a function of plant age, which might be due to their possible implications in physiological processes of the plant during the life cycle.

Expression of novel POX and PPO isoforms was induced by neem extract both locally and systemically. This indicates the possible mobilization of a signaling molecule such as salicylic acid from the neem elicited cells to the distal untreated leaves which might have induced the *de novo* expression of POX and PPO isoforms and increase in the activity of POX, PPO and LOX. Neem extract could significantly enhance lysozyme activity in 8 and 10 weeks tomato only. However, the plants which were inoculated with *Pseudomonas syringae* pv. *tomato* only demonstrated significant increase in lysozyme activity at all the ages. It was probably because the effector molecules from the pathogen could have induced the release of lysozyme from the vacuole while neem extract was unable to do so. Real time PCR quantification of *PPOA* gene demonstrated that it was upregulated at the site of application of elicitor either prior to or after pathogen inoculation, thus confirming the role of neem extract as a potential bioelicitor. However the *CEVI1* gene was down regulated both at the site of neem treatment and in distal leaves away from it when preceded by pathogen inoculation. *TomloxD* gene was downregulated in the distal untreated leaves of plants which were inoculated with pathogen after neem treatment. This indicates that the pathogen could inhibit neem elicitation effects on POX and LOX genes. Therefore, the increase in the activity of these PR proteins leading to SAR in tomato could be because of neem-elicited-activation of already expressed yet inactive isoforms present in the cytoplasm.

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