Literature Review

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Plants are exposed to a variety of pathogens but they protect themselves from invasion by pathogens using various defense mechanisms. The defense mechanisms include defense barriers (such as cuticle, cell wall, deposition of lignin, callose, phenolic compounds), formation of papillae, synthesis of pathogenesis related proteins (Aist, 1976; Benhamou *et al.*, 1990). Plant responses to fungal attack lead to resistance or susceptibility following interaction between plant and the pathogen. Any agriculture programme needs fresh healthy seedlings for healthy plants. Seedlings in the seedbeds face several disease problems. Healthy seedlings again when planted to remote areas from its natural habitat become susceptible to many fungi and other disease causing organisms. Pathogenesis and disease resistance are closely related to each other. Pathogenesis is related to compatible interactions while resistance is related to incompatible ones.

At the onset of the present study it was considered to review the reports presented by the earlier workers. The observation of the previous workers in concord with the present line of investigation is being presented, in a selective manner, in the following paragraphs. For convenience, the observations have been grouped in to some aspects. The different aspects of this review are:

- Diseases of tea
- Growth and physiology of the pathogen
- Pathogenicity and serology
- Induction of systemic resistance (systemic acquired resistance & induced systemic resistance)

Diseases

The fungus, *Botryodiplodia theobromae* is presently known as *Lasioodiplodia theobromae* (Pat.) Griffon & Mauble. *Lasiodiplodia theobromae*, the causal agent of diplodia disease of tea is one of the most common fungal pathogen of tea in north-east India. The pathogen can attack any part of tea plant, young or mature. Severe attacks are experienced when the tea plants are debilitated by other causes. The symptoms are of different types, which appear

hairy cushions, given a shooty appearance on leaves and collar regions. The colour of the symptoms varies from grayish-black to coal-black. The infection is believed to expand from infected tea plants to healthy plants by conidia, which are dispersed by air. All woody parts, dying roots and twigs show *Lasiodiplodia theobromae* infection.

Sarmah (1960) suggested for the improvement of general health of tea plants by subsoil irrigation, manorial and cultural practices for protecting the plants from diplodia root disease. Venkata Ram (1960) observed the predisposing factors like low starch reserves, high soil temperature and low soil moisture were essential as a requisite for fungal invasion to cause diplodia disease. Chandramauli (1988) reported that diplodia root disease is a secondary root disease of tea and caused by *Lasiodiplodia theobromae*. He also reported that the disease was very common in north-east India.

Tea rhizosphere/plane harbours diverse population of microorganisms and their mode of nutrition are also different like saprophytic on dead tea plants, symbiotic (mycorhizal association) on root of tea plants and parasitic to tea plants. The parasitic microorganisms cause disease on tea. In 1964, Agnihothrudu reported 385 species of fungal pathogens on tea plants. In 1989, Chen and Chen revised the situation and listed 507 fungi as fungal pathogens. In the same year (1989) Barua reported 190 fungal pathogens from north-east India. However, in the following paragraphs the works on some of the major diseases of tea are being presented.

Other than *Botryodiplodia*, several other diseases were reported to affect the tea plants throughout the world. Saha *et al.* (1980) observed that red rust of tea caused by *Cephaleuros parasiticus* Karst was the most important and lone algal disease of tea. Arpon and Supachi (1980) reported that blister blight of tea caused by *Exobasidium vexans* Massee. It was the most serious disease in many tea plantations on the mountains. Smit and Knox-Devies (1989) reported about crown and root rot disease caused by *Macrophomina phaseolina* (MP) and *Neocosmospora vesinfecta* (NV) which occurred in rooibos tea throughout the main production areas. Tissue infected by MP was gray with numerous black sclerotia when broken, open and the branches were characteristically twisted. Tissue infected by NV was typically maroon to black with superficial orange to red perithecia. Disease developed on plants inoculated during winter. Number of

propagules recovered from the soil varied greatly from field to field with the height levels being 32 viable MP sclerotia and 315 visible ascospores per gram of soil.

Wang *et al.* (1990) studied diseases of tea in 6 zones of Zheijiang province of Peoples Republic of China from 1985 to 1988. Symptoms and biology of pathogens of 20 diseases were described. Among them, 5 of the 20 diseases caused by *Colletotrichum camelliae, Monochaetia camelliae, Pastolotia guepini, Phyllosticta theicola* and *Fusarium ventricosum* were distributed more widely and cause more severe damages and those caused by *Pestalotia algeriensis.*

Barthakur (1994) reported that root diseases caused by *Ustulina zonata, Fomes lamaoensis, Rosellina arcnata* and *Armillaria mellea* and stem diseases caused by *Tunstallia aculeate* and *Poria hypobrunnea* were also common in Darjeeling tea-gardens but they were difficult to control. He also reported that among the common diseases of tea in the hills of Darjeeling, blister blight caused by *Exobasidium vexans* was the most serious.

Park (1995) observed that in May and June, 1992-94 tea plants with white scab symptoms, i.e. numerous small, circular, reddish or yellowish brown spots on young tea leaves was observed in a plantation in Boseung, Chonnum Province, Korea Republic. At the late growth stages the center of the spot became light gray. The causal agent was identified as *Sphaceloma theae.* Similar symptoms occurred on leaves 5-6 days after inoculation with *S.theae.* Park *et al.* (1996) reported the occurrence of gray blight of tea in several tea plantations in Boseung, Chonnam Province, Korea Republic, during 1992-94 after harvesting and pruning of the second crop. Circular to irregularly shaped dark brown spots developed in concentric rings on leaves and black, dot-like acervuli formed randomly on them. The causal fungus of gray blight was identified as *Pestalotiopsis longiseta.* Typical symptoms by *P. longiseta* appeared 11 days after inoculation.

During 1991-1993, Khodaparast and Hedjaroude (1996) surveyed several tea plantations in the north of Iran in order to determine the main fungal diseases of tea. The result of the survey and pathogenicity tests showed that *Botrytis* sp., *Glomerella cingulata, Fusarium solani, Botryodiplodia theobromae, Pestalotiopsis longiseta, P. nattrassii, P. theae, Phyllosticta theacearum* and *Corticium rolfsii* were pathogens of tea. Among the 12 tea growing districts of Kenya, *Armillaria* root rot disease was most severe in the district east of rift valley. Investigations showed that infection of tea bushes were primarily by the mycelial growth from

residual tree roots and from infected tea roots rather than from rhizomorphs. Inoculum from residual tree in debris in the soil was the most important source of infection in plantations of seed origin (Onsando *et al.*, 1997).

In 1999, Chandramouli gave a brief account on abiotic and biotic problems in the nurseries in India, and suggested several remedial measures against the diseases like stalk rot caused by *Pestalotia theae, brown blight by Colletotrichum camelliae*, root rot caused by *Pythium* spp. / *Cylindrocladium* spp. or by *Fusarium* spp., blister blight caused by *Exobasidium vexans* and leaf spot caused by *Cercosporella theae*. Hu-shuXia (1996) found two highly resistant cultivars to *Pestalotiopsis theae* among the 18 cultivars tested in Anhui Province of People Republic of China. The superiority of Fushan variety of green tea over Yabukita (green tea) based on productivity, disease and pest resistance have been reported by Yamaguchi *et al.* (1992).

Lasiodiplodia theobromae has also been reported to attack a number of other plants like *Eucalyptus,* Chashew *etc.* The survey of diseases of *Eucalyptus* plantations (mostly *E. grandis*) were performed in southern Uganda during June, 1999. Roux *et al.* (2001) collected root, stem and leaf samples from the trees ranging in age from a few months to approximately 10 years. The most commonly isolated pathogen was *Lasiodiplodia theobromae*, which was frequently associated with stem cankers and die back. Bacterial wilt caused by *Ralstonia solanacearum*, was the most common cause of death of trees less than two years old in the warmer areas around Kampala. In the eastern part of Uganda, the wilt pathogens *Ceratocystis fimbriata* was isolated from dying *Eucalyptus grandis* and, together with *Lasiodiplodia theobromae*, was considered the greatest threat to *Eucalyptus* plantations in Uganda.

From the feeding injury caused by *Helopeltis antonii* Sign a fungus viz. *Botryodiplodia theobromae* was also isolated consistently from the dead tissues of cashew. The primary cause for entry and establishment of the pathogen seemed to be infestation of the insects. Controlled experiment revealed that die back occurred only when the fungus was inoculated in the lesion caused by feeding of *H. antonii* Sign (Verma and Balasundaran, 1990).

In addition, Pavlic *et al.* (2004) reported different synonyms of *Lasiodiplodia theobromae* and their host range including the country from where the report originated (Table 1).

Table 1: Host range of *Lasiodiplodia theobromae.*

Synonyms of *L. theobromae*	**Host range**	**Origin**
Lasiodiplodia theobromae	*Camellia sinensis*	India
Botryodiplodia theobromae Pat.	*Mangifera indica* L.	India
B. theobromae Pat.	*Papaya* sp.	Brazil, Maxico
B. theobromae Pat.	*Malus sylvestris*	USA
Diplodia gossypina Cooke	*Gossypium* sp.	India
B. theobromae Pat.	*Theobroma cacao*	Ecuador
Macrophoma vestita Prill. & Delacr.	*Theobroma cacao*	Equatorial America
L. tubericola Ellis. & Everh.	*Ipomoea batatas*	Java
Diplodia cacaoicola P.Henn	*Theobroma cacao*	Kamerun
B. gossypii Ellis. & Barthol.	*Gossypium herbaceum*	U.S.A.
L. nigra K. R. Appel & Laubert	*Carica papaya*	Samoa
L.theobromae(Pat.)Griffon & Mauble	*Theobroma cacao*	Equatorial America
Diplodia rapax Massee	*Hevea brasiliensis*	Singapore, Ghana
Diplodia natalensis Pole-Evans.	*Citrus* sp.	South Africa
L. triflorae B.B. Higgins.	*Prunus* sp.	U.S.A.
Diplodia maniothi Sacc.	*Manihot utilissima*	-
Diplodia musae Died.	*Musa sapientium*	-
Diplodia ananassae Sacc.	*Ananas sativus*	Philippines
Diplodia theobromae (Pat.)W.Nowell	*Theobroma cacao*	-

Growth and physiology of the pathogen

The understanding of the pathogen and their activities are of increasing importance in various economic fields like agriculture and industries. A thorough understanding of the physiological processes of pathogens is also of immense

use in understanding the host-parasite relationship and mechanism of pathogenicity. Physiological studies are also helpful in determining various control measures.

Brambl *et al.* (1978) reported that germination of dormant conidia of the fungus *B. theobromae* could be inhibited by the antilipogenic antibiotic cerulenin. The spore remained viable in the presence of the antibiotics, however, after prolonged incubation they were able to overcome the inhibition. Cerulenin inhibition of germination was reversed by Tween 40 and Tween 60 (derivatives of palmitate and stearate) respectively. They also reported that the lipid synthesis is essential for *B. theobromae* spore germination and required for membrane assembly.

The effect of some environmental factors on germination of *Bipolaris carbonum* Nelson, a pathogen of tea was described by Saha and Chakraborty (1990). Under identical humid condition, the optimal concentration of spores, temperature and pH for spore germination were recorded to be 11.2 x 10^5 spores/ml^{-1} at 32^{0}C for pH 6.75 respectively. Temperature pretreatment at 50^{0}C for 20 minutes significantly reduced spore germination, whereas pretreatment at 0^{0}C for even 12 hours had no effect on spore germination and germ tube elongation. Light condition and age of the conidia did not affect the spore germination.

Meah *et al.* (1991) studied the formation of pigment and pycnidia of *B. theobromae* isolated from mango stem-end rot on five different media. They observed that the highest number of complex pycnidia was formed on mango leaf extract agar followed by PDA and found pink coloured pigment.

In 1995, Chakraborty *et al.* showed that factors associated with conidial germination and appressoria formation of *Glomerella cingulata* causing the brown blight disease of tea were studied *in vitro*. Spore germination and appressoria formation were optimum at a temperature of 25^{0}C, pH 5.0, at 7 hours light /day regime and a 24 hours incubation period. At a concentration of conidia of 1200/10 days old culture, *G. cingulata* exhibited a maximum germination and appressoria formation. Maximum production of lesions was also evident on detached tea leaves at this spore concentration and in diffuse light. Diffusates of phenolic nature collected from tea varieties susceptible and resistant to *G. cingulata* inhibited spore germination and appressoria formation. Diffusates from resistant varieties were more fungitoxic than from susceptible varieties.

Ho and Ko (1997) reported that *B. theobromae* produce conidia when they were grown on 10% V-agar (10% V-8 juice, 0.02% $CaCO^3$ and 2% Bacto agar) at 24^0 C for 2 weeks under light. Fatty acid and their solvent (ethanol) had no adverse effect upon spore germination.

The mycelial growth of three isolates of *Stenocarpella maydis* from maize seed increased progressively from 15^0C to a maximum of 30^0C. The maximum numbers of conidia were produced by all three isolates after 8 days of incubation at temperatures ranging from 22^0C to 30^0C (Achar, 2000).

Harden *et al.* (2002) reported that the effect of temperature and pH on the growth and sporangial sporulation of isolates from each of the four known races of *Phytophthora clandestine* Taylor, Pascoe & Greenhalgh were investigated. Mycelial growth occurred at temperatures from 10^0C to 30^0C and pH 3.5 to 9.0 with highest growth rates of all isolates being at 25^0C with a pH of 6.0 to 6.5. Sporangial production was greatest between 20^0C to 25^0C and pH 5.0 to 7.0 with all races. However, sporulation occurred over no consistent differences between the four pathogenic races of *P. clandestina* in their relative growth rate extent of sporangial production over a range of temperatures and pH values.

Miller *et al.* (2003) studied conidial germination *in vitro* and foliar expansion by *Sphaerotheca macularis* f.sp *fragariae*. Detached strawberry (Fragaria x ananassa) leaves were inoculated, then held in controlled environments of constant temperature (4^0C to 36^0C) and relative humidity (RH, 32 to 100%) representing the range of these variables observed under California commercial production conditions. Percent germination and lesion expansion rate were determined by destructive sub sampling over time. Conidia germinated at all temperatures by 6 hours and reached a maximum by 48 hours, with the optimum near 20^0C. Lesions were marked with the aid of a microscope and measured by computer-assisted image-analysis to determine expansion rate. Maximum rates occurred at 25^0C. Several growth models were fit to the expansion rate data with high significance. Predicted optima from these models ranged from 22^0C to 27^0C and/or 17 to 27mm Hg VP (water@ 100% RH). Neither RH, partial vapour pressure of water VP (water) nor vapour pressure deficit (VPD) correlated with lesion expansion rate, adding to studies minimizing the importance of RH and VPD as determinants of asexual phase powdery mildew growth other than specifically at spore germination.

Creelman *et al.* (1992) first reported isolation of jasmonic acid from culture filtrates of the fungus *Lasiodiplodia theobromae*. MeJA, found in the culture filtrate was also reported to be a component of the essential oil of *Jasminum grandifirum* L. and *Rosmanum officinalis* L.

Pathogenicity and serology

The concept of common antigens between a plant and a pathogen is an important feature in determining resistance or susceptibility. It is believed that the degree of compatibility and susceptibility of a plant cultivar to a pathogen is correlated to levels of common antigens present in both host and pathogen (Alba *et al.,* 1983; Purkayastha and Banerjee, 1990; Chakraborty and Saha, 1994; Kratka *et al.*, 2002; Ghosh and Purkayastha, 2003; Musetti *et al.*, 2005; Eibel *et al.*, 2005; Dasgupta *et al.*, 2005).

Ala-El-Dein and El-Kady (1985) used crossed immunoelectrophoresis to show that the tested isolates of *Botrytis cinerea* were serologically different and some antigens were specific for each isolate. Isolate no.1 of *Botrytis cinerea* had four specific antigens. Although these antigens were absent in other isolates. At least sixteen antigens were common in the isolates tested. Some isolates were serologically similar when tested by double gel diffusion test while they were distinguishable when CIE techniques were used. Numbers of precipitin peaks obtained with CIE techniques were more than double the number of precipitin lines detected with double gel diffusion test. Results revealed that CIE techniques could be used as valuable analytical tools in resolving the spectrum of antigens present, in *Botrytis cinerea* isolates. By using CIE techniques antigenic structures of *B. cinerea, B. tulipae, B. paeoniae* and *B. allii* isolates were also compared. Antisera against antigens of these isolates gave 24, 15, 20 and 14 precipitin peaks respectively, when analyzed in homologous reactions. CIE with an intermediate gel and CIE with antibody absorption *in situ* revealed that each isolate was serologically different from the other and has species-specific antigens. *B. cinerea* has eight distinct antigens which distinguished them from the other species of *Botrytis*.

Evaluation of antisera raised against pooled mycelial suspensions from five isolates (Pf-1, Pf-2, Pf-3, Pf-10 and Pf-11) representing five physiologic races of *Phytophthora fragariae* for detecting the red core disease of strawberries by enzyme-linked immunosorbent assay (ELISA) were performed. Cross-reactivity

of antiserum raised against *P. fragariae* with other *Phytophthora* as a genus detecting antiserum has been reported. Antiserum of *P. fragariae* isolates (Anti-PfM) reacted strongly with antigens from several *Phytophthora* species. Some cross-reaction with antigens from *Phythium* species was decreased by fractionating on an affinity column of sepharose 4 B bound to extracts of *Fragaria vesca* roots infected with *P. fragariae.* The affinity purified anti PfM retained its high cross-reactivity with the various *Phytophthora* species. Anti-PfM could not be made specific for *P. fragariae* because it was raised against components shown to be antigenically similar in all *Phytophthora* species tested. However, immunoblotting with the affinity purified anti-PfM produced distinct patterns for *P. fragariae, P. erythroseptica* and *P. cactorum* (Mohan, 1988).

Competitive types of two novel enzyme-linked immunosorbent assays (ELISA) for specific detection of *Fusarium oxysporum* f. sp. *cucumerinum* as well as for general detection of ten strains of common *Fusarium* species has developed, that show specific pathogenicities to different plants. Antiserum against a strain of *Fusarium oxysporum* f. sp. *cucumerinum* (F 504) was elicited in rabbits and a highly specific, sensitive and accurate ELISA for the homologous strain was developed by using the antiserum with β-D-galactosidase-labelled anti-rabbit IgG as a secondary antibody and cell fragments of the strain attached to amino-Dylark balls as the solid-phase antigens. This assay was specific for strain F 504 and showed little cross-reactivity with nine other strains of *Fusarium* species including strain F 501 of *F. oxysporum* f. sp. *cucumerinum* (FO). F 501 possesses pathogenicity against cucumber similar to that of strain F 504, although slight differences have been observed between these two strains regarding their spore formation and pigment production. Cell fragments of strain F 501 absorbed on amino-Dylark balls possessed sufficient immune activity against anti-FO antibody to use in a heterologous ELISA for general detection of ten *Fusarium* species with high sensitivity (Kitagawa *et al.,* 1989).

Common antigenic relationships between soybean and *Colletotrichum dematium* var. *truncata* using immunodiffusion, immunoelectrophoresis and indirect ELISA technique was studied by Purkayastha and Banerjee (1990). Cross-reactive antigens were detected between susceptible soybean cultivars and the virulent strain of *C. dematium* but no cross-reactive antigen was detected between soybean cultivars and avirulent pathogen (*C. dematium*) or non-pathogen *C. corchori.* Results of immunodiffusion and immunoelectrophoresis

showed absence of common antigen between resistant cultivars (UPSM-19) and the pathogen, while the results of indirect ELISA indicated the presence of common antigen between the two at a very low level. They compared antigenic patterns of untreated and cloxacillin treated soybean leaves which induced resistance of soybean against anthracnose disease. The disappearance of one antigen from cloxacillin treated leaves of susceptible soybean cv. "Soymax" was correlated with alteration of disease reaction.

Polyclonal antiserum raised against mycelial extracts of the rot fungus *Phialophora mutabilis,* reacted strongly with its homologous antigen and cross-reacted strongly to moderately with six other *Philosophora* soft rot spp. in ELISA (Daniel and Nilsson, 1991).

With the help of an indirect ELISA technique, Ricker *et al.* (1991) showed that increase in cross-reactivity in late bled antiserum (anti-Bc IgG), raised against water soluble antigens from *Botrytis cinerea* corresponded with an increase in the overall serum titers for anti-Bc IgG to antigens of *B. cinerea.* Polyclonal antiserum of mycelial proteins of *Verticillium dahliae* reacted positively with 11 of 12 isolates of *V. dahliae* from potato, cotton and soil but negatively with one isolate from tomato in indirect ELISA (Sundaram *et al.,* 1991). He also found positive results in detecting, *V. dahliae* and *V. albo-atrum* from infected roots and stems of potato in a double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA).

Lyons and White (1992) compared results of conventional isolation techniques for *Pythium violae* using polyclonal antibodies raised to *P. violae* or *P. sulcatum* in competition ELISA. Priestley and Deway (1993) developed a double antibody sandwich ELISA test for the detection of *Pseudocercosporella herpotrichoides* using a highly specific monoclonal antibody pH 10 as the capture antibody and genus specific polyclonal rabbit antisera as test antibody. The assay recognized extracts from plants both artificially and naturally infected with *P. herpotrichoides,* at least three-fold higher absorbance values with extracts of *P. herpotrichoides* infected tissue than with extracts from healthy tissues. The high molecular weight fraction of immunogen (mycelial extracts) was shown to contain cross-reactive antigens; it induced antiserum in mice that cross-reacted with the other stem base fungi even at high dilution.

Chakraborty and Saha (1994) compared antigens obtained from tea varieties, isolates of *Bipolaris carbonum* and non-pathogens of tea (*Bipolaris*

tetramera and *Bipolaris setariae*) by immunodiffusion, immunoelectrophoresis and enzyme linked immunosorbent assay to detect cross reactive antigens (CRA) shared by the host and the parasite. CRA were found among the susceptible varieties (TV 9, 17 and 18) and isolates of *B. carbonum* (BC-1, 2, 3 and 4). Such antigens were not found between isolates of *B. carbonum* and resistant varieties (TV 16, 25 and 26), non-pathogens and tea varieties, as well as non-pathogen and *B. carbonum*. CRA were also found concentrated mainly around the epidermal cells of leaves of TV-18 in cross section following indirect staining of antibodies using fluorescein isothiocyanate (FITC). They indicated the presence of CRA in the young growing hyphal tips and conidia following treatment with antisera of leaves (TV-28) and indirect staining with FITC.

Polyclonal antibodies (PAbs) were produced against culture filtrates and mycelial extracts immunogen from the soybean (*Glycine max*) and fungal pathogen *Phomopsis longicolla*. Polyclonal antibodies were purified to the immunoglobulin fraction and tested in indirect ELISA and in direct DAS-ELISA, the PAbs raised to culture filtrate were more specific but less active in binding to members of Diaparthe-*Phomopsis* complex than were those to mycelial extract immunogen preparation. DAS-ELISA was more specific and 100-fold more sensitive in detecting members of the complex than was indirect ELISA. Variability in specificity between different PAbs was lower in DAS-ELISA compared to indirect ELISA (Brill *et al.*, 1994).

Extensive cross reactions were found when two monoclonal and three polyclonal antisera, raised against the cell wall/membrane fractions of *Pythium violae* and *P. sulcatum* screened with a collection of 40 isolates of the genus *Pythium* including 20 species and the H-S group. However, when the binding of the antibodies was assessed in an enzyme-linked immunosorbent assay (ELISA) using cytoplasmic fraction antigens, the combined recognition patterns produced profiles unique to each species (White *et al.*, 1994).

Polyclonal antisera against whole (coded: 16/2) and sonicated (coded: 15/2) resting spores of *Plasmodiophora brassicae* were raised as well as soluble components prepared by filtration and ultracentrifugation (coded: SF/2), cross-reactivity of all three antisera with a range of soil fungi, including *Spongospora subterranean* was low (Wakeham and White, 1996). Test formats including western blotting, dipstick, dot blot, indirect ELISA and indirect immunofluorescence were assessed for their potential to detect resting spores of

P. brassicae in soil. Dot blot was least sensitive, with a limit of detection level of $1x10^7$ resting spores/g in soil. With western blotting, the lower limit of detection with antiserum 15/2 was $1x10^5$. This antiserum showed the greatest sensitivity in a dipstick assay, indirect ELISA and indirect immunofluorescence, for all of which there was a limit of detection of $1x10^2$. Of the assays tested, indirect immunoflourescence appears to be the most rapid and amenable assay for the detection in soil low levels of resting spores of *P. brassicae*.

Polyclonal antibodies against prehelminthosporol, a phytotoxin produced by the plant pathogenic fungus *Bipolaris sorokiniana* were raised in rabbits immunized with a prehelminthosporol–hexon conjugate. The IgG was isolated from the serum and the specificity of the purified antibodies was investigated with indirect ELISA. The antibodies bound both to free prehelminthosporol and to a prehelminthosporol-bovine serum albumin conjugate bound to micro titer wells. The antibodies showed less affinity to structurally related compounds from the fungus. No cross-reactivity was shown for proteins extracted from mycelium of *B. sorokiniana*. Low-temperature preparation techniques for electron microscopy were used in combination with immunogold labeling for localization of prehelminthosporol in hyphae and germinated conidia of *B. sorokiniana*. A low level of labeling was obtained throughout the cytoplasm, and the main labeling was seen in membrane-bound organelles identified as Woronin bodies (Akesson *et al.,* 1996).

Kratka *et al.* (2002) prepared four polyclonal and two monoclonal antibodies were prepared and tested to detect *Colletotrichum acutatum,* a quarantine pathogen of strawberry. They observed that only one polyclonal antibody was sensitive enough to recognize the pathogen. The antibody was genus specific that did not cross react with several other fungal pathogens of strawberry. They also detected *C. acutatum* by Plate trap antigen enzyme linked immunosorbent assay (PTA-ELISA), dot blot and immunoprint in roots, crowns, petioles and fruits in the latent age of the disease after artificial infection of strawberry (cvs. Elsanta, Vanda and Kama).

Ghosh and Purkayastha (2003) used polyclonal antibodies and antigens of ginger and *Pythium aphanidermatum*, a causal organism of rhizome rot disease for early diagnosis of rhizome rot disease of ginger. They detected *P. aphanidermatum* in ginger rhizome after eight weeks of inoculation by agar gel

double diffusion and immuoelectrophoretic tests, but only one week after inoculation by indirect ELISA.

Polyclonal antibodies were raised against mycelium from the logarithmic growth phase of a shake culture of *Ustilago nuda*, and developed a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with biotinylated detection antibodies. Other species of *Ustilago* reacted with the antibodies. Cross-reactivity was highest with *U. tritici.* No signal was obtained with the tested isolates of *Tilletia, Rhizoctonia, Pythium* and *Fusarium.* With naturally infected barley seeds, the results of the ELISAs were always in good agreement with those obtained with the routinely used seed embryo test. They suggested that potential fields of application of the ELISA include the early prediction of the efficacy of protection agents, e.g. in screenings for seed treatments, the elucidation of the biology of the fungus and characterization of resistance mechanisms (Eibel *et al.*, 2005).

Besides fungus Indirect ELISA was used to monitor the distribution of *Mycoplasma* like organism (MLO) in the experimental host *Vicia faba.* Post-embedding colloidal gold indirect immunolabelling was developed to identify, without ambiguity, the various forms of MLO cells in the different infected parts of the plant by transmission electron microscopy. Silver enhancement of the gold probe gave accurate histological and cellular localization of MLOs in tissue sections, by light microscopy. Both ELISA and immunolocalization first detected MLO in roots 17 days after inoculation with infectious leafhoppers (Lherminier *et al.*, 1994).

Hema *et al.* (2001) evaluated double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and direct antigen coating (DAC)-ELISA for detection of sugarcane streak mosaic virus (SCSMV-AP). The virus was detected up to 1/3125 and 1/625 dilutions in infected sugarcane leaf, 5µl and 10 µl/well in sugarcane juice, 1/3125 and 1/625 dilutions in infected sorghum leaf and 10 ng and 50 ng/ml of purified virus in DAS-ELISA and DAC-ELISA tests, respectively. Abou-Jawdah *et al.* (2001) in a survey detected potato virus Y (PVY), potato virus A (PVA), potato virus X (PVX), potato virus M (PVM), potato virus S (PVS) and potato leaf roll virus (PVX), potato virus M (PVM) potato virus S (PVS) and potato leaf roll virus (PLRV) by ELISA from potato fields in the two main production areas of Lebanon, the Bekaa and Akkar plains.

Wang *et al.* (2006) observed that an indirect enzyme-linked immunosorbent assay (ID-ELISA) protocol is capable of detecting Rice black-streaked dwarf virus (RBSDV) in very dilute wheat leaf extracts. Based on the results, they concluded that efficient and economic detection of RBSDV can be performed routinely using polyclonal antiserum against outer capsid protein (P10) expressed in prokaryotic cells.

Several other workers also used ELISA for detecting virus (Petrunak *et al.,* 1991; Abou-Jawdah *et al.,* 2001; Hema *et al.,* 2001; Devaraja *et al.*, 2005; Chen *et al.*, 2005) and bacterial (Mazarei and Kerr, 1990) pathogens of plants.

Cellular location of different proteins or antigens can be done by immunolocalization. Location of cross reactive antigens (CRA) was successfully done by several workers. In a study, DeVay *et al.* (1981a) inoculated young cotton (Acala 2) roots with antiserum to *Fusarium oxysporum* f. sp. *vasinfectum* and stained with FITC conjugated, antirabbit globulin-specific goat antiserum. Strong fluorescence was observed at the epidermal and cortical cells, and the endodermis and xylem tissues that indicated a general distribution of the CRA determinants in roots. In a similar fashion, Chakraborty and Saha (1994) labelled polyclonal antiserum with FITC and found that CRA between tea leaves and the pathogen *Bipolaris carbonum* was present mainly around the epidermal cells and mesophyll tissues of leaves of the host and in hyphal tips and in patch like areas on conidia and mycelium of the pathogen. Location of CRA was also studied in tea leaves that were treated with antiserum raised against two pathogens of tea. Indirect labelling of the antibodies with FITC showed that CRA was concentrated mainly in the epidermal cells and also spread throughout the cortical cells (Dasgupta *et al.,* 2005).

Immunolocalization studies were also performed using immunogold labelling which is successfully used for electron microscopy (Lee *et al.*, 2000; Trillus *et al.*, 2000; Nahalkova *et al.*, 2001; Kang and Buchenauer, 2002; Wang *et al.*, 2003). For light microscopy, silver enhancement is done after gold labelling (Santen *et al.*, 2005; Saha *et al.*, 2006). However immunogold labeling has not yet been utilized for location of CRA in compatible host and pathogens. Kuo (1999) used a gold sol which was found to be able to localize the ECM (Extra cellular matrix) of *C. gloeosporioides* very well. In case of *C. gloeosporioide*s, the ECM secreted out from conidium just before germination took place. The area that ECM covered was wide-spread and could reach up to several times the

spore width. With gold sol, the composition and nature of the ECM could be easily identified using cytochemical and biochemical approaches.

Immunogold labelling showed specific labelling of chitinase in the interaction of pepper stems with *Phytophthora capsici.* Chitinase was found on the cell wall of the oomycete in both compatible and incompatible interactions at 24 h after inoculation. In particular, numerous gold particles were deposited on the cell wall of *P. capsici* with a predominant accumulation over areas showing signs of degradation in the incompatible interaction. Chitinase labelling was also detected in the intercellular space and the host cytoplasm. However, healthy pepper stem tissue was merely free of labelling (Lee *et al.*, 2000).

Immunolocalization experiments for locating *Pinus nigra* ARN lectin (PNL) were also performed by Nahalkova *et al.* (2001) who observed that the protein was mainly located on the cytoplasmic membranes and on the primary cell walls. In infected seedlings (infected by *Heterobasidium annosum* and *Fusarium avenaceum*), a strong labelling of hyphal materials with PNL antisera was recorded only at the early stages of infection but not at the later stages of hyphal invasion.

Two antisera raised against acidic β-1,3-glucanase and acidic chitinase from tobacco and used to investigate the subcellular localization of the two enzymes in *Fusarium culmorum*-infected wheat spike by means of the immunogold labelling technique. These studies demonstrated that the accumulation of the enzymes in the infected wheat spikes differed distinctly between resistant and susceptible wheat cultivars (Kang and Buchenauer, 2002). Wang *et al.* (2003) used immunogold labelling technique for localization of PB90 which is a novel protein elicitor secreted by *Phytophthora boehmeriae*. The anti-90 kDa protein antiserum was used for immunocytolocalization studies of PB90 elicitor, on the mycelium and encysting zoospores of *P. boehmeriae* grown *in vitro* in liquid culture and also in solid medium. In liquid culture, immunogold labelling was located mainly in the cell wall. In solid medium, gold particles were observed not only in the cell wall, but also in the solid medium near the hypha.

Induction of Systemic resistance:

Systemic acquired resistance (SAR) & induced systemic resistance (ISR)

Plants have the ability to develop mechanisms in the presence of pathogenic organisms that inhibit the invasion of pathogens. The interactions between plants and pathogens are based on recognition of specific elicitor molecules from avirulent pathogen races (*avr* gene products), which is described in the gene-for-gene resistance theory. Another type of resistance, multigenic (horizontal) resistance, is a less well-studied phenomenon that depends upon multiple genes in the plant host. All plants possess resistance mechanisms, which can be induced upon pre-treatment of plants with a variety of organisms or compounds. This general phenomenon is known as induced systemic resistance (ISR). At least in some plant species, ISR depends on the timely accumulation of multiple gene products, such as hydrolytic enzymes, peroxides or other gene products related to plant defenses. The pre-treatment of plants with an inducing organism or compound appears to incite the plant to mount an effective defense response upon subsequent encounters with pathogens, converting what would have been a compatible interaction to an incompatible one Tuzun (2001).

According to Agrios (1988) resistance is the ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor. Disease resistance in plants is manifested by limited symptoms, reflecting the inability of the pathogen to grow or multiply and spread, and often takes the form of a hypersensitive reaction (HR), in which the pathogen remains confined to necrotic lesions near the site of infection. Induced resistance is the phenomenon that a plant, once appropriately stimulated, exhibits an enhanced resistance upon 'challenge' inoculation with a pathogen.

Although, induced resistance has attracted recently (Ryals *et al.*, 1994; Hammerschmidt and Kuc, 1995), but the first systematic enquiry was done by Ross (1961a,b). He observed that the inducible resistance response to tobacco mosaic virus (TMV) in *N* gene containing, hypersensitively reacting tobacco was not confined to the immediate vicinity of the resulting local necrotic lesions, but extended to other plant parts. A ring of tissue around the developing lesions became fully refractory to subsequent infection (localized acquired resistance; Ross, 1961a), whereas challenge inoculation of distant tissues resulted in much

smaller, and occasionally fewer lesions (systemic acquired resistance (SAR); Ross, 1961b) than in non-induced plants. Even leaves that were mere initials at the time of the primary inoculation became induced, suggesting that as a result of the initial infection, a signal was generated, transported and propagated, that primed the plant to respond more effectively to subsequent infection (Bozarth and Ross, 1964).

Lee *et al.* (2000) reported that plants are exposed to a wide variety of pathogens including viruses, bacteria and fungi. However, plants protect themselves from invasion by pathogens using various defense mechanisms.

Systemic acquired resistance (SAR) is a broad-spectrum resistance that can be induced in plants following a localized infection with a necrotizing pathogen or treatment with elicitors (Sticher *et al.,* 1997; Mauch-Mani and Metraux, 1998). SAR development is mediated by a mobile signal that originates at the primary infection or treatment site and thought to be translocated systemically in the phloem (Rasmussen *et al.,* 1991; Smith-Becker *et al.,* 1998; Kaur and Kolte, 2001; Sharma *et al.,* 2001; Paul and Sharma, 2002).

Systemic acquired resistance (SAR) can be induced in plants following a localised infection with a necrotizing pathogen or treatment with chemical elicitors (Mauch-Mani and Metraux, 1998; Sticher *et al.*, 1997). SAR development is mediated by a mobile signal that originates at the primary infection or treatment site and is thought to be translocated systemically in the phloem (Rasmussen *et al.*, 1991; Smith-Becker *et al.*, 1998). SAR was induced in mature plant part after localized treatment with 0.2 mM salicylic acid (SA) or previous inoculation with the same pathogen. SAR was expressed in adjacent untreated leaves as a reduction in lesion diameter (Reglinski *et al.*, 2001).

Ton *et al.* (2002) observed that three signals salicylic acid, jasmonic acid and ethylene play an important role in inducing defense resistance in plants. Salicylic acid is a key regulator of pathogen-induced systemic acquired resistance (SAR), whereas jasmonic acid and ethylene are required for rhizobacteria-mediated induced systemic resistance (ISR). Both types of induced resistance are effective against a broad spectrum of pathogens (oomycete, fungal, bacterial, and viral pathogen). In non-induced *Arabidopsis* plants, these pathogens are primarily resisted through SA-dependent basal resistance (*Peronospora parasitica* and Turnip crinkle virus [TCV]), JA/ET-dependent basal resistance responses (*Alternaria brassicicola*), or a combination of SA-, JA-, and

ET-dependent defenses (*Xanthomonas campestris* pv. *armoraciae*). They suggested that SAR and ISR constitute a reinforcement of extant SA- or JA/ET-dependent basal defense responses, respectively.

In compatible or incompatible interactions between plant and pathogens, pathogenesis-related proteins (PRs) are often induced in response to infection (Van Loon, 1997) those play an important role in plant defense response. Plant growth promoting rhizobacteria (PGPR) are known to induce systemic resistance to fungal, bacterial and viral diseases (Wei *et al.*, 1991; Maurhofer *et al.*, 1994; Liu *et al.*, 1995a,b; Chen *et al.*, 1998). This phenomenon termed as induced systemic resistance (ISR) (Kloepper *et al.*, 1992) markedly differ from SAR. Many classic SAR proteins are not induced in ISR (Van Loon, 1997). Chen *et al.* (2000) tested PGPR strains (*Pseudomonas* strains 13 and 63-28) significantly induced plant defense enzyme both locally and systemically.

Meera *et al.* (1994) reported that plant growth promoting rhizobacteria (PGPR) were shown to induce systemic resistance (Wei *et al.*, 1991; Van Loon *et al.*, 1998). Some fungal isolates collected from the rhizospheres of zoysiagrass enhanced the growth of a variety of crop plants and thus these isolates were designated as plant growth-promoting fungi (PGPF). The PGPF belonged to the genera *Fusarium*, *Penicillium*, *Phoma*, *Trichoderma* and sterile fungi. It was found that systemic resistance was induced in cucumber using the *Phoma* sp. and the sterile fungus against anthracnose caused by *C. orbiculare*. Cucumber roots treated with culture filtrates (CFs) of PGPF isolates also induced resistance against anthracnose. CF-treated plants expressed resistance to pathogen infection by an alteration of various metabolisms, such as high increases in activities of chitinase, β-1,3-glucanase, peroxidase, polyphenol oxidase and phenylalanine ammonia lyase, indicating that an elicitor substance(s) existed in the CFs.

Plant growth promoting rhizobacteria (PGPR) can suppress the disease caused by foliar pathogen by triggering plant-mediated resistance mechanism called induced systemic resistance, so called ISR (Dube, 2001). Systemic resistance induced by rhizobacteria differs mechanically from SAR, it is designated by a separate term ISR proposed by Kloepper *et al.* (1992) and latter supported by Pieterse *et al.* (1996). SAR and ISR differ in their signaling pathways. SAR is dependent on the synthesis of SA by the plant that acts as an inducer signal and is associated with the accumulation of novel pathogenesis–

related (PR) proteins, POX (peroxidase), PR-1, PR-2 (β -1,3 glucanase), PR-3 (chitinase), PR-4 and PR-5 etc. (Van Loon, 1999). Rhizobacteria-mediated ISR signalling pathway does not involve SA or PR protein, rather it requires Jasmonic acid (JA) and ethylene response and like the SAR, depends on the regulatory protein NPR-1(non-pathogenic rhizobacteria-1) (Dube, 2001).

Van Loon *et al.* (1998) reported that the rhizosphere bacteria are present in large numbers on root surface; few of them stimulate plant growth and are therefore called plant growth promoting-rhizobacteria (PGPR). The strains that were isolated from naturally disease-suppressive soil, mainly fluorescent *Pseudomonas* sp, have been demonstrated to reduce plant disease by suppressing soil-borne pathogens. Some of those biological strains have the ability to reduce disease caused by foliar pathogens by triggering a plant-mediated resistance mechanism called induced systemic resistance.

Ongena *et al.* (2002) demonstrated the ability of *Pseudomonas putida* BTP1 to induce resistance in bean to *Botrytis cinerea. In vivo* assays with samples from successive fractionation steps of the BTP1 supernatant suggested that salicylic acid, pyochelin and pyoverdin, previously identified as *Pseudomonas* determinants for induced systemic resistance (ISR), were not involved in systemic resistance triggered by BTP1 but one main metabolite (not characterized) retained most of the resistance-inducing activity in bean.

Conrath *et al.* (2001) reported that pre-treatment of cultured parsley cells with inducers of systemic resistance, salicylic acid or a benzothiadiazole, leads to the direct activation of a set of defence-related genes and also primes the cells for stronger elicitation of another set of defence genes including those encoding phenylalanine ammonia-lyase. In *Arabidopsis*, pre-treated plants with benzothiadiazole was found to augment the subsequent activation of phenylalanine ammonia-lyase genes by *Pseudomonas* infection, wounding and osmotic stress and also to enhance wound/osmotic stress-induced callose production. From these results, it was concluded that the resistance inducers have at least a dual role in plant defence-gene activation.

Willits and Ryals (1998) reported that Probenazole, which was the first commercialized disease resistance inducer, is an example of such a compound and is widely used for the control of rice blast in Japan. Besides probenazole, other chemical inducers of disease resistance in plants have been described including salicylic acid (SA), 2,6-dichloro isonicotinic acid (INA) and 3-

aminobutyric acid (BABA) (Kessmann *et al.*, 1994a; Cohen, 1994; Sticher *et al.*, 1997). Furthermore, one of the benzothiadiazole compounds (BTHs), acibenzolar-S-methyl (CGA245704: benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester) was developed by Novartis Crop Protection AG and was introduced in 1996 as a 'plant activator' for the control of wheat powdery mildew in Germany and Switzerland (Ruess *et al.*, 1996).

There are many biotic and abiotic inducers used for the establishment of SAR in different plants by several workers. Among the abiotic inducers, Meena *et al.* (2001) used salicylic acid in groundnut, Higa *et al.* (2001) used active oxygen radicals in rice, O'Donnell *et al.* (1996) used ethylene in tomato, Smith-Becker *et al.* (1998) used SA and 4–hydroxybenzoic acid in cucumber, Cohen *et al.* (1993) used jasmonic acid and methyl jasmonate in potato and tomato, Siegrist *et al.* (2000) used β - aminobutyric acid in tobacco, Kaur and Kolte (2001) and Stadnik and Buchenauer (2000) used benzothiadiazole in mustard and wheat plant respectively, Brederode *et al.* (1991) used UV-light in tobacco, Ernst *et al.* (1992) used ozone in tobacco, Klessig *et al.* (2000) used nitric oxide and Kaku *et al.* (1997) applied N-acetylchitooligosaccharide in barley.

Jasmonic acid (JA) plays an important role in plant defense response. Its level is increased under wounding and treatment with pathogen-elicitors that induce genes encoding enzyme involved in flavonoid biosynthesis, chalcone synthase (Creelman *et al.*, 1992) and Phenylalanine ammonia-lyase (Gundlach *et al.*, 1992).

It has been described that SA coordinately induces the full spectrum of SAR genes, encompassing all well-characterized PRs (Ward *et al.*, 1991). Salicylic acid (SA) is an endogenous signal for the development of SAR and it is transported by phloem from the sites of its origin. Leaves inoculated with pathogen exhibits high level of endogenous SA (Malamy *et al.*, 1990). Foliar application of SA at the concentration of 1mM significantly increased the activity of Phenylalanine ammonia-lyase (PAL), Chitinase, β-1,3-glucanase, Peroxidase, Polyphenol Oxidase and Phenolic content in groundnut (Meena *et al.*, 2001). Salicylic acid is a natural phenolic compound present in many plants that play an important role in the signal transduction pathway and involved in local and systemic resistance to pathogens (Delaney *et al.*, 1995).

In tomato, root application or foliage spray with BABA induced the accumulation of the pathogenesis-related (PR) proteins PR-1, chitinase and β-1,

3-glucanase (Cohen and Gisi, 1994; Cohen *et al.*, 1994). In tobacco, foliar spray similarly induced PR-proteins accumulation but, surprisingly, no PR-proteins were detected in plants either stem injected with BABA (Cohen, 1994), although plants were highly protected. Busam *et al.*, 1997 showed that in grape leaves, SA induced basic chitinase III and I whereas INA or BTH induces chitinase III only. The induction by BABA of lignin accumulation in HR-responding mesophyll cells hints at the possible involvement of enzymes like phenylalanine ammonia lyase (PAL) and peroxidase. In fact, BABA was reported (Newton *et al.*, 1997) to increase PAL activity and induce resistance against late blight in potato leaf discs.

Schweizer *et al.* (1999) showed the induction of resistance in rice seedlings by *Pseudomonas syringae*, a biological inducer of resistance, and the chemical inducers benzothiadiazole (BTH) and 2, 6-dichloroisonicotinic acid (INA). Both INA and BTH induced similar patterns of genes, suggesting that these compounds were functional analogues. In contrast, the patterns induced by the chemical inducers and by *P. syringae* were clearly dissimilar.

G¨orlach *et al.* (1996) observed that the benzo (1,2,3) thiodiazole-7-carboxylic acid derivatives have been developed as a novel class of crop protection agent that have no direct anti-microbial properties, but instead increase crop resistance to disease by activating the SAR signal transduction pathway (Lawton *et al.*, 1996). Such plant defense activators are usually applied as a foliar spray treatment, but can also be applied to seed (Jensen *et al.*, 1998; Siegrist *et al.*, 1997).

Dann *et al.* (1998) assessed for severity of white mould disease caused by *Sclerotinia sclerotiorum* following induction of resistance by 2,6 dichloroisonicotinic acid or benzothiadiazole in field or greenhouse grown soybeans. They hypothesized that the decrease in disease severity following treatment with INA or BTH is a result of resistance induction.

Acibenzolar-S-methyl (BTH is the first commercial chemical triggering induced resistance in plants) was recently introduced on the market by Novartis under the tradenames Actigard (USA) and BION (Europe) Buonaurio *et al.* (2002).

Ishii *et al.* (1999) suggested that acibenzolar-S-methyl (CGA 245704) induced resistance to some but not all diseases on cucumber and Japanese

pear. Induction of disease resistance in cucumber was rapidly triggered after treatment with acibenzolar-S-methyl (CGA245704: benzo [1,2,3] thiadiazole-7-carbothioic acid S-methyl ester) which showed no antifungal activity *in vitro.*

Cohen, (1996) reported a new class of resistance-inducing compound belongs to aminobutyric acids. Sticher *et al.* (1997) reported local treatments with DL-3-aminobutyric acid (BABA) can protect tomato, potato, and tobacco, systemically, against *Phytophthora infestans* and *Peronospora tabacina*, respectively.

Acibenzolar-S-methyl (Novartis) induces defense-related compounds in apple seedlings. The protection was associated with the activation of two families of defense-related enzymes, peroxidases and β-1,3-glucanases. Accumulation of both enzymes were induced locally in treated leaves and systemically for β-1,3-glucanases in upper untreated leaves and was sustained for at least 17 days. A pre-flowering foliar spray of the plant activator acibenzolar-S-methyl combined with a fruit dip in guazatine at harvest substantially decreased disease in stored melons caused by *Fusarium* spp., *Alternaria* spp., *Rhizopus* spp. and *Trichothecium* sp. (Huang *et al.*, 2000).

An obligate fungus, *Albugo candida* infects all aerial parts of the mustard (*Brassica juncea*) plants. Plant treated with benzothiadiazole (BTH) exhibited high level of the enzyme PAL, peroxidase and cell wall-bound phenolic compounds (Coumaric and ferulic acid) than in untreated control. It has been shown that transcripts of six typical defense response genes, POX (peroxidase), PR-1. PR-2 (β-1,3-glucanase), PR-3 (chitinase), PR-4 and PR-5 (thaumatin-like protrein) were induced in spray - inoculated heads of the susceptible cv. wheat on and the activation of SAR by 2-6-dichloro isonicotinic acid (INA) or benzo (1,2,3) thiodiazol- 7- carbothioic acid S- methyl ester (BTH) (G¨orlach *et al.*, 1996). Similar activation was reported in barley (Kogel *et al.*, 1994) and maize (Morris *et al.*, 1998). The chemical BTH had no antifungal activity *in vitro* against the pathogen *A. candida*. Under the field conditions, plants treated with BTH at concentrations of 100mgL^{-1}, 250mg $L^{-1}$and 500mgL^{-1} showed protection from staghead development by 59.2%, 61.4% and 82.6% respectively against the challenge inoculation with *A. candida* (Kaur and Kolte, 2001).

Buonaurio *et al.* (2002) used acibenzolar-S-methyl to induce resistance in pepper plants against *Xanthomonas campestris* pv. *vesicatoria* in both growth chamber and open field conditions. In growth chamber experiments of

acibenzolar-S-methyl in pepper plants showed resistance expression systemically and locally that lead to the reduction in the number and diameter of bacterial spots and bacterial growth. Systemic protection was also noticed by the acibenzolar-S-methyl acid derivative, CGA 210007. Under open field conditions both leaves and fruit were protected from the disease perhaps due to SAR activation.

Emmanuel *et al.* (2001) applied Phytogard and BABA to induce systemic resistance in lettuce against downy mildew. Phytogard and BABA completely protected the disease. Pathogenesis related (PR) protein analysis showed that BABA induced weak accumulation of PR-2, but not PR-1, PR-5 and PR-9. Phytogard induced none of these proteins.

Cohen *et al.* (1999) reported the non-protein amino acid BABA (DL-3-aminobutyric acid) to induce local and five other isomers of aminobutyric acid, namely L-2 aminobutyric acid, 2-amino isobutyric acid, DL-2-aminobutyric acid (AABA), DL-3-amino isobutyric acid, and 4-aminobutyric acid (GABA) gave no protection against the downy mildew fungus.

Park *et al.* (2002) observed that the wall glucan elicitor (WGE), mycolaminaran, jasmonic acid (JA), methyl jasmonate and ethelene precursor, 1-amino-cyclopropane carboxylic caid (ACC) are effective in protecting the cells distal to the point of treatment from the site of infection of *Phytpophthora sojae* in soybean.

Penninckx *et al.* (1996) purified a 5-kD plant defensin from *Arabidopsis* leaves after challenged inoculation with the fungus *Alternaria brassicicola* and shown to possess antifungal properties. The corresponding plant defensin gene was induced after treatment of leaves with methyl jasmonate or ethylene but not with salicylic acid *or* 2,6-dichloroisonicotinic acid. When challenged with *A. brassicicola*, the levels of the plant defensin protein and mRNA rose both in inoculated leaves and in nontreated leaves of inoculated plants (systemic leaves). The results indicate that systemic pathogen-induced expression of the plant defensin gene in *Arabidopsis* was independent of salicylic acid but requires ethylene and jasmonic acid to response.

Salicylic acid (SA) is a widely distributed secondary plant product (Raskin *et al.*, 1990). Several plants, especially *Gultheria* species have been reported to be particularly rich in this compound together with 2,3-dihydroxybenzoic acid (2,

3-DHBA) as a minor constituent (Towers *et al.*, 1966). A glucoside of 2,3-DHBA is constitutively present in intact plants of *Catharanthus roseus* (L.) G. Don (Ibrahim and Towers, 1959). Cell suspension cultures of this plant produced 2,3-DHBA after elicitation with fungal cell wall preparations (Frankmann and Kauss, 1994; Moreno *et al.*, 1994a,b).

Foliar application of SA at the concentration of 1 mM significantly reduced late leaf spot and increased the pod yield under greenhouse conditions in groundnut against late leaf spot caused by *Cercosporidium personatum* SA increased the activities of PAL, chitinase, β-1,3-glucanase, peroxidase and polyphenol oxidase in groundnut following challenged inoculation with *Cercosporidium personatum* (Meena *et al.*, 2001).

Treatment of tobacco with a mixture containing reactive oxygen species (ROS) and salicylic acid (SA) provided greater protection of tobacco against infection by *Pseudomonas syringae* pv. *tabaci* than either treatment alone. Synergism in expression from the promoter of the defense gene *PR-1a* was also observed. Although the ROS hydrogen peroxide and peracetic acid were poor inducers alone, they enhanced the level of *β*-glucuronidase (GUS) activity expressed from the *PR-1a* promoter when applied with SA to a transgenic plant bearing a *PR-1a::GUS* fusion. *PR-1a* expression was not correlated with increased cell death as determined by Evans blue staining. There was no effect on the timing at which expression was increased by the mixture compared with the separate treatments Blee (2004).

It has been reported that the oligomers of chitin released by the hydrolytic enzyme were found to be effective elicitors of resistance reactions such as biosynthesis of lignin and phytoalexins in plants (Boller, 1987; Boller, 1991 Mauch-Mani and Metraux, 1998).

Like the abiotic inducers, some biotic inducers have also been used to enhance in plant defense reaction. Some of them are leaf extract of *Azadirachta indica* in barley (Paul and Sharma, 2002), *Acalypha indica* in ginger (Ghosh and Purkayastha, 2003), *Reynoutria sachaliensis* in cucumber (Daayf *et al.*, 1995), plant growth promoting rhizobacteria (PGPR) in cucumber (Wei *et al.*, 1991; Liu *et al.*, 1995a; Chen *et al.*, 2000), *Pseudomonas fluorescens* strain CHAO in tobacco (Maurhofer *et al.*, 1994), *Pseudomonas syringae* in cucumber (Rasmussen *et al.*, 1991), *Pyricularia oryzae* and *Bipolaria sorokiniane* in rice (Manandhar *et al.*, 1999).

Ton *et al.* (2002) observed that in *Arabidopsis thaliana*, non-pathogenic, root-colonizing *Pseudomonas fluorescens* WCS417r bacteria trigger an induced systemic resistance (ISR) that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). In contrast to SAR, WCS417r-mediated ISR is controlled by salicylic acid (SA)-independent signalling pathway that requires an intact response to the plant hormones jasmonic acid (JA) and ethylene (ET). *Arabidopsis accessions* RLD1 and Ws-0 fail to express ISR against *Pseudomonas syringae* pv. tomato and show enhanced disease susceptibility to this pathogen.

The role of PR-proteins in plant defense against pathogens is multi-faceted and induced by biocontrol fungi, *Trichoderma harzianum* (Jangid, *et al.*, 2004). As potential biological agents *Trichoderma harzianum* isolate T39 and *T. virens* isolate DAR 74290 controlled the rot disease in potato and tomato caused by *Phytophthora erythroseptica*. The yield of potatoes from plants treated with *T. virens* DAR 74290 following inoculation of *P. erythroseptica* was significantly greater than in control (inoculated plants) (Etebarian *et al.*, 2000).

De Meyer *et al.* (1998) reported that biocontrol of *Botrytis cinerea* in tomato, lettuce, pepper, bean and tobacco with the treatment of *T. harzianum* T39 reduces the grey mould symptoms inducing systemic resistance that was similar to that of the rhizobacterium *Pseudomonas aeruginosa* KMPCH.

Koike *et al.* (2001) tested five fungal isolates (*Trichoderma*, *Fusarium*, *Penicillium*, *Phoma* and a sterile fungus) for their ability to induce systemic resistance in cucumber plants against *Colletotrichum orbiculare*. Those isolates were collected from zoysiagrass rhizosphere that promotes plant growth.

Pieterse *et al.* (2001) demonstrated that selected strains of rhizosphere bacteria *Pseudomonas fluorescens* WCS417r activating a resistance mechanism in the plant called rhizobacteria-mediated induced systemic resistance (ISR).

Ahmed *et al.* (2000) showed seed and root treatments with *Trichoderma harzianum* in pepper, significantly reduced stem necrosis caused by *Phytophthora capsici*. Accumulation of increased capsidiol in treated inoculated plants was correlated with reduced necrosis. He also showed that *Trichoderma harzianum* induced the accumulation of capsidiol. Reuveni and Reuveni (2000) showed the induction of systemic resistance against powdery mildew by a non-pathogenic isolate of *Alternaria cucumarina*.

De Meyer *et al.* (1999) showed root colonization by rhizobacteria can induce a systemic resistance in plants that is phenotypically similar to systemic acquired resistance induced by a localized pathogen infection but unlike systemic acquired resistance, not associated with PR1a expression at the time of challenge with tobacco mosaic virus.

Landgraf *et al.* (2002) observed that induced systemic resistance by infiltration with *Pseudomonas syringae* pv. *maculicola*, 12-oxo-phytodienoic acid (OPDA) accumulated in infiltrated leaves as well as in non-treated leaves of infected plants. In contrast, jasmonic acid (JA) levels increased only in infiltrated leaves, suggesting that the biosynthetic precursor of JA, OPDA, might play a role in systemic acquired resistance.

Paul and Sharma (2002) observed that the leaves of barley treated with aqueous leaf extract of neem (*Azadirachta indica* Juss.) exhibited significantly high activity of enzymes PAL and tyrosine ammonialyase (TAL) along with rapid and distinct accumulation of fungitoxic phenolic compounds. The population of most of the phylloplane mycoflora species remained unaltered.

Daayf *et al.* (1995) found that the leaf extract of *Reynoutria sachalinensis* induced resistance in long English cucumber against *Sphaerotheca fuliginea* through biochemical changes in the host plant.

Duijff *et al.* (1998) indicated that the suppression of fusarium wilt by *P. fluorescens* WCS417r was ascribed to systemic induced resistance without any detection of the PR-proteins tested (PR-1 and chitinases). In contrast, the suppression achieved by nonpathogenic *F. oxysporum* Fo47 appeared to be mainly ascribed to microbial antagonism but also to a lesser extent to systemic induced resistance. This induced resistance could be related to the accumulation of PR-1 and chitinases.

Many plant enzymes are involved in defense reactions against plant pathogens. These includes oxidative enzymes such as peroxidase (PO) and polyphenol oxidase (PPO), which catalyse the formation of lignin and other oxidative phenols that contribute to the formation of defense barriers for the pathogen to the plant cell structure (Avdiushko *et al.*, 1993). Other enzyme such as tyrosine ammonia lyase (TAL) and phenylalanin ammonia lyase (Bashan *et al.*, 1985; Beaudoin-Eagan *et al.*, 1985) are involved in phytoalexein or phenolic compound biosynthesis.

It is reported that the increased activity of Phenylalanine ammonia lyase (PAL) in the phenylpropanoid pathway lead to the synthesis of defense-related compounds such as lignin, flavonoid, phytoalexin and signaling molecule, salicylic acid (Health, 2002). The defense related enzyme, Phenylalanine ammonia lyase (PAL) frequently increases in plants in respond to pathogen invasion. Maher *et al.* (1994) reported the increased disease susceptibility of tobacco plants to *Cercospora nicotiana* in which PAL activity was suppressed, but over expressed PAL, exhibited reduction of lesion areas caused by two compatible, necrotrophic pathogens in transgenic tobacco plants.

Elicitor treatment and wounding in parsley and sweet potato increased the activity of PAL. About a 3-fold increase in phenolic content was observed 4 days after challenge inoculation with *C. personatum* following pretreatment with SA in groundnut (Meena *et al.*, 2001).

Polyphenol oxidase (PPO) catalyses in the formation of oxidative phenols that contribute to the inhibition of pathogen to the plant cell (Avdiushko *et al.*, 1993).

PRs form a set of pathogen-induced proteins that may be considered as stress proteins. In the past decades it has become evident that plants, when exposed to various environmental stresses, respond by synthesizing sets of specific proteins. Well-known are the heat-shock proteins, that appear to be common to all living organisms, and are transiently induced when ambient temperature exceeds some critical limit (Vierling, 1991). Different sets of proteins are induced by e.g. drought stress or freezing temperatures. For instance, during cold acclimation hardy cultivars of alfalfa synthesize a number of proteins that supposedly function in reducing the deleterious effects of low temperature on plant membranes. The plant hormone abscisic acid (ABA) induces a partly similar set of proteins and increases resistance to freezing stress, indicating that acclimation is hormone-controlled (Mohapatra *et al.*, 1988; Heino *et al.*, 1990). Similar proteins are induced by ABA during the acquisition of desiccation tolerance in developing seeds and upon drought stress of leaves (Skriver and Mundy, 1990). PRs may be considered as stress proteins produced in response to, particularly necrotizing, infections by viruses, viroids, fungi and bacteria, and thought to function in the acquired resistance against further infection (Van Loon, 1989). However, in contrast to most other types of stress proteins, they accumulate in plant tissues to levels that are easily detectable on gels by general

protein stains. Why these inducible PRs may individually reach up to 1% of the total soluble protein in leaves, is unclear.

Gaudet *et al.* (2000) observed that under field conditions of five winter wheat cultivars, all PR-protein (PR-I, chitinase, β-1,3-glucanase, peroxidase) and PAL transcripts exhibited the same basic pattern of expression during the autumn, winter and spring. Transcripts were expressed during the late autumn, reached high levels by mid-winter, than decreased before reaching maximum level during the spring. Conversely PAL expression was low or absent in autumn, reached the highest levels by mid-winter and then gradually decreased during the spring.

Van Loon (1985) reported that the mostly studied plant defense responses are the synthesis of a group of host-encoded proteins, so called pathogenesis-related proteins. One of them known as chitinase has been of particular interest in studies of plant resistance against fungal pathogens. The substrate for chitinase is chitin, the major structural component of the cell wall of many phytopathogenic fungi.

Pelt-Heerschap and Smit-Bakker (1999) reported that chitinase activity was constitutively expressed in the intercellular fluids (IFs) of untreated leaves, stems and roots of carnation cultivar of Pallas. The total chitinase activity in the IFs of stem tissue increased with time after inoculation with 2 near-isogenic races, the avirulent race 1 and the virulent race 8 of *Fusarium oxysporum* f.sp. *dianthi* and four chitinase isoenzymes, three acidic and one basic isoform, were detected. Furthermore, the increases in β-1,3-glucanase activity in IFs of stem tissue were markedly higher in the compatible and incompatible interactions than in the water control. Using an antiserum against β-1,3-glucanase P3 of tomato, 2 bands were detected on immunoblots in the IFs of stem tissue inoculated with races 1 and 8. Total peroxidase activity increased with time in all combinations. One basic and one acidic peroxidase isoform were present in these IFs. Peroxidase activity in a cell wall fraction prepared from stem tissue was clearly higher, and it increased faster, than the activity in the soluble stem fraction.

It is reported that chitinases catalyze the hydrolysis of chitin, a linear polymer of β-1,4-linked N-acetylglucosamine residues that is the predominant constituent of fungal cell walls, nematode eggs, and mid gut layers of insects. Some plant chitinases also exhibit lysozymal activity (Dodson *et al.*, 1993). Three classes of plant chitinases have been proposed based upon protein primary

structure (Shinshi *et al.*, 1990). The highly variable nature of chitinase and the multiplicity of chitinase isozymes in plants suggest that plant chitinase isozymes may carry out specific and differing roles. Some chitinase isozymes, for example, have antifungal activity while others do not, and the activity of antifungal chitinase isozymes isolated from tobacco (Sela-Buurlage *et al.*, 1993) and tomato (Lawrence *et al.*, 1996) have been found to be specific for certain pathogens.

Sela-Buurlage *et al.* (1993) observed that chitinase show marked antifungal properties against several phytopathogenic fungi, inhibiting both spore germination and hyphal growth either alone or acting synergistically with other PR proteins such as β-1,3-glucanase, and ribosome inactivating proteins (Leah *et al.*,1991). The activity of β-1,3-glucanase is higher in resistant cultivars and lower in the susceptible pearl millet cultivars to downy mildew disease. Isoelectric focusing revealed four basic isoforms with pI 9.6, 9.0, 8.9 and 8.2 and two acidic isoforms with pI 4.9 and 6.2 of β-1, 3-glucanase in pearl millet (Kini *et al.*, 2000).

Bodhini *et al.* (2003) found that the tissues of *Zingiber officinale* rhizome contain chitinase of molecular masses of 29 and 34 KDa in case of skin and tissue respectively. Bishop *et al.* (2000) reported that the plant chitinases attack fungal pathogens directly thereby conferring disease resistance by degrading chitin, a major component of the fungal cell wall. Studies at the cellular level have established that chitinases affect the extreme tip of hyphal growth of *Trichoderma longibrachiatum* resulting in thinning of the wall leading to an imbalance of turgour pressure and wall tension which caused the tip to swell and burst (Arlorio *et al.*, 1992).

Kang and Buchenauer (2002) reported similar distribution of β-1,3-glucanase and chitinase in the uninoculated healthy and infected wheat spike. The enzymes were mainly present in the cell walls of different tissues including the lemma, ovary and rachis of the wheat spike, while the cytoplasm and organells of the cells in these tissues have no enzyme. Accumulation of these enzymes differed in resistant and susceptible wheat cultivars. Furthermore, the labelling of β-1,3-glucanase and chitinase also occured over the cell walls of the hyphae in the infected wheat spike, but not over the hyphal cytoplasm.

Exogenous application of SA resulted in increased activity of peroxidase and β-1,3-glucanase enzymes and antifungal PR-proteins in the callus cultures of *Zingiber officinale* against culture filtrate (CF) of *Fusarium oxysporum* f.sp.

zingiberi. Two new protein bands of 97 and 38 kDa molecular weights were obtained (Prachi *et al.*, 2002).

Chakraborty and Sengupta (2001) observed that the activities of PAL and peroxidase in tea plants were decreased with increasing temperatures, but in case of PPO it is increased initially at 40^0C and then declined. In rice suspension culture peroxidase isozyme is secreted under various environmental stress such as heavy metals, salts and temperature (Kiwan and Lee, 2003).

Ghosh and Purkayastha (2003) noticed that systemic protection against *Pythium aphanidermatum* was induced in ginger (Cv. Suprabha) by soaking rhizome seeds separately in selected synthetic chemicals or specific herbal extracts for 1 h prior to sowing. Among 12 plant defense activators tested, jasmonic acid (JA, 5 mM) and 10% leaf extract of *Acalypha indica* (ALE) reduced disease significantly, with concomitant increase of defense-related proteins (DRPs). Growth response of pathogen to both JA and ALE was evaluated *in vitro*. ALE stimulated growth, while JA inhibited growth at high concentration (0.5 mM) and slightly stimulated growth at low dose (0.005 mM). Results suggest their host-mediated role in induced systemic protection against disease (rhizome rot).

It was reported that an incompatible interaction results in an increase in endogenous levels of reactive oxygen intermediates, nitric oxide, salicylic acid (SA), jasmonic acid (JA) and ethylene that trigger the defense responses through different signalling pathways. Signalling pathways activate a series of defense responses that curb or eliminate the pathogen. These responses include the hypersensitive response (HR), up-regulation of phenylalanine ammonia lyase (PAL), a key enzyme in plant defense, deposition of cell wall reinforcing materials, and synthesis of a wide range of antimicrobial compounds including pathogenesis related (PR)-proteins and phytoalexins (Veronese *et al.*, 2003).

Baysal *et al.* (2005) noticed that *Phytophthora capsici* was significantly inhibited by ASM (acibenzolar-S-methylbenzo [1,2,3] thiadiazole-7-carbothioic acid-S-methyl ester) treatment by up to 45% *in planta*. The pepper plants responded to ASM treatments by rapid and transient induction of L-phenylalanine ammonia-lyase (PAL), increase in total phenol content and activities of chitinase and β-1,3-glucanase. No significant increase in enzyme activities were observed in water-treated control plants compared with the ASM-treated plants. Therefore, it may be suggested that ASM induces defense-related enzymes, PAL activity,

PR proteins and phenol accumulation in ASM-treated plants and contribute to enhanced resistance against *P. capsici*.

Changes in phenolic metabolism after elicitation with *Colletotrichum gloeosporioides* (CG) in *Hypericum perforatum* L. (HP) cell suspension cultures was observed by Conceicao *et al.* (2006). HP cultures elicited with the CG elicitor showed a significant increase in xanthone accumulation. Xanthone accumulation increased twelve fold when the cells were primed with methyl-jasmonate (MeJ) or salicylic acid (SA), before elicitation. HP cultures exposed only to MeJ produced a set of flavonoids, the flavones which represent a substantial part (approx. 40%) of the total flavonoids accumulated in these cells. The results indicated that xanthones are important component of defense mechanism of HP against biotic stress.

Guleria and Kumar (2006) observed that aqueous leaf extract of neem (*Azadirachta indica* Juss.) provided the control of *Alternaria leaf* spot pathogen (*Alternaria sesami*) of sesame (*Sesamum indicum* L). Treatment with this extract led to the changes in plant metabolism as leaves of the treated plants exhibited significantly high level of enzymes phenylalanine ammonia-lyase (PAL), peroxidase (PO) and content of phenolic compounds. Furthermore, germination of *A. sesami* spores was not significantly inhibited by neem extract. It is therefore, suggested that, protection of sesame plants against *A. sesami* by neem extract might be due to stimulation of plants natural defense response.