The present study brings out the effect of elicitor (salicylic acid) culture filtrates and formulations of \textit{G. virens} against \textit{C. capsici}, the incitant of anthracnose of \textit{Capsicum annum} (chilli) and the effects of the salicylic acid on the chilli seedlings of \textit{Capsicum annum} under healthy, infected, treated and infection treated conditions.

The optimum inhibitory concentration of the formulations and salicylic acid was found out by their effect on the inhibition of conidial germination and mycelial growth of \textit{C. capsici} in \textit{in vitro} assay and they were given as foliar spray in \textit{in vivo} (field) study. The effect of the treatments on the host plant under healthy, infected, treated and infection treated conditions were found out by studying different physiological and biochemical parameters from the leaf samples collected on the following days.

\textbf{In-vitro STUDY}

\textbf{Conidial germination}

The effect of the (elicitor) salicylic acid, \textit{G. virens} (biocontrol agent) and (chemical fungicide) bavistin was determined by the \textit{in-vitro} conidial germination \textit{C.capsici}. It was found that the above three agents produced higher percentage of inhibition over control. The highest rate of inhibition of conidial germination was recorded in 10mM of salicylic acid (84.5%), 2.5\% \textit{G. virens} (76.7\%).

The inhibition of conidial germination by antagonistic microbes might be attributed to their antibiotic action. The antibiotics produced by the antagonistic
microbes might inhibit the conidial germination, which led to the disintegration of the viable fungal spores. The present finding supports the observation of Umesha et al. (1998) that the inhibition of sporulation and growth process of *Sclerospora graminicola* in the *in vitro* condition was mainly due to the effect of volatile compounds produced from the culture filtrate of *P. fluorescens*. Resca et al. (2001) confirmed that the inhibition of zoospores germination of *P. betae* by *Pseudomonas* treatment was mainly attributed due to the action of antibiotics. Maurhofer et al. (1992), Barea et al. (1998) and Bhatt and Sabalpara (2001) brought out the fact that the antibiotics produced in the *Pseudomonas* culture filtrate was the main factor in the inhibition of the spores of *Pythium ultimum, Glomus mosseae* and *C. falciform*. Sneh et al. (1984) reported that the suppression of chlamydospore germination of *Fusarium oxysporum* was mainly due to the production of antifungal compounds in the *Pseudomonas* culture. Even Swadling and Jeffries (1998) stated that the fungistatic effect of *Pseudomonas* against the conidial germination of *Botrytis cinerea*, the cause of grey mould of strawberries was due to the antifungal compounds present in the culture.

Rajathilagam (1999) observed that the non-volatile antibiotics extracted from the culture filtrate of *Trichoderma* spp. were the main antifungal factors to inhibit the conidial germination of *C. capsici*. Howell (1987) found that the antibiotics of *G. virens* and *Trichoderma* spp. acted as the main factor in the suppression of sclerotial production of *R. solani*. Ciotola (1987) and Carissa et al. (2000) reported that significant inhibition in the ascospore production of *V. inaequalis* was brought out by the antibiotic action of *Trichoderma* spp. Wilhite et al. (1994) concluded that the sporangial germination of *Pythium* sp. was inhibited by the action of virdin antibiotics produced by *G. virens* in the *in
vitro condition. The action of G. virens antibiotics was considered as the main mechanism for the inhibition of sclerotia of *S. rolfsii* (Henis *et al.*, 1983; Papavizas and Collins, 1990); sclerotia of *Pythium ultimum* (Roberts and Lumsden, 1990); *Sclerotinia sclerotium* (Tu, 1980); sclerotia of *T. cucumeris* (Prasad and Rao, 1990) and *C. solani* (Aluko and Hering, 1970). Lorito *et al.* (1993a) Schirmbock *et al.* (1994) found that the synergistic action of hydrolytic enzymes and peptaibol antibiotics of *T. harzianum* inhibited the spore germination of *B. cinerea*. Dutta and Das (2002) confirmed that the spore germination of collar rot fungi of tomato was effectively controlled in *in vitro* mainly due to the secondary metabolites present in the culture filtrates of *Trichoderma* spp. Further report was also made by Roy (1977) against *R. solani* by *Trichoderma viride* and *Trichoderma harzianum* treatment. Dubey (1998) established that the antibiotics of *T. viride* were responsible for the control of spore germination of *Thanatephorus cucumeris* causing web blight of horsegram.

The bavistin (0.1%) treatment recorded 75% inhibition in the conidial germination of *C. capsici*. Similar inhibition of conidial germination due to chemical treatment was supported by Kumari *et al.* (1977) who stated that the chemical fungicide suppressed the spore germination of *Fusarium oxysporum* by decreasing the DNA synthesis and reducing the rate of cell division required for their development.

**Radial mycelial growth study**

The findings of radial mycelial growth reveal that the mycelial growth of *C. capsici* was effectively inhibited by 10mM SA (75%) and 2.5% *G. virens* (70%).

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The radial mycelial growth of *C. capsici* was more effectively inhibited by salicylic acid (85%) which might be due to the chemical present in the SA. The present findings support the work of chemical elicitor Adipic acid monoethyl ester which showed the most effective range of inhibiting activity. It inhibited radial mycelial growth of *A. solani* and *B. cinerea* at 0.5% and also significantly inhibited growth of *Phytophthora* spp. at 0.1% conc. It was also observed that several combinations of FGA (Furfurylamine; 1,2,3,4 tetra-O-acetyl- ß-D glucose) components produced antagonistic effects. In all cases, *in vitro* antimicrobial activity was observed at concentrations 40 times higher than those previously used for plant treatments (Flores *et al.*, 1997).

The suppression of pathogen growth by antagonistic treatment was mainly attributed to the activity of secondary metabolites present in the culture filtrate or by the hyperparasitism. The synergistic action of both antibiotics and mycoparasitism might also lead to effective inhibition in the growth of the pathogen. The action of different antifungal compounds present in the culture filtrate might destroy the germ tube formation or disintegrate the hyphal elongation. The above-mentioned fact finds support from the works of Ramamoorthy and Samiyappan (2001) who concluded that the suppressed mycelial growth of *C. capsici* in the *Pseudomonas* treatment was mainly due to the action of antifungal substances present in the culture filtrate. The effective inhibition of *R. solani* and *S. rolfsii* growth in the dual plate culture by *P. fluorescens* treatment was mainly due to the effect of the antibiotics produced by *P. fluorescens* (Bhowmik *et al.*, 2002). According to Mondal *et al.* (1998, 2000) the *in-vitro* inhibition of *Xanthomonas* growth by *Pseudomonas* treatment was due to the action of HCN antibiotic produced by the *Pseudomonas* spp. Similar report on the HCN which was involved in the inhibition
of mycelial growth of *Thielaviopsis basicola* was made by Piotrowskaseget (1995) and in cotton black rot fungi by Ahl *et al.* (1986). Shekhar Varshney and Chaube (1999) showed that the antibiotic action of *Pseudomonas fluorescens* was responsible for the suppression of mycelial growth of *R. solani, F. oxysporum, S. rolfsii* and *brassica* in dual plate culture. Rosales *et al.* (1995) brought out the efficacy of different types of antibiotics from the *Pseudomonas fluorescens* in the control of mycelial growth of many fungal pathogens (*R. solani, F. moniliforme* and *P. oryzae*) infecting rice plant. *Pseudomonas* antibiotics were found to be the main agents in the inhibition of mycelial growth of *Septoria* sp. (Levy *et al.*, 1992). Gnanamanickam and Mew (1992) brought out that the effective inhibition of rice blast fungal growth by *Pseudomonas* in *in vitro* studies which was mainly due to the action of antibiotics. Weller *et al.* (1988) reported that the inhibition of *Gaeumannomyces graminis* mycelial growth in *in-vitro* culture by *Pseudomonas* treatment was mainly attributed to the action of the antibiotic PCA (phenazine-1-carboxylic acid). Lemanceau *et al.* (1992) found that the growth of *Fusarium* sp. causing wilt of carnation was inhibited by the action of Pseudobactin antibiotics produced in the *Pseudomonas* culture filtrate. Howell and Stipanovic (1979) reported that the lysis of *R. solani* hyphal cell in the *Pseudomonas* treatments was mainly due to the effect of antifungal antibiotic, pyrrolnitrin. Elad and Baker (1985a,b) and Elad and Chet (1987) brought out the fact that the chlamydospore germination and hyphal growth of *Fusarium* spp. was suppressed mainly by the colonization of *P. fluorescens* on the phylloplane. They had reported that the colonization may lead to competition for nutrient and space, which are required for the germination and growth of pathogens and thus result in reduced growth of the pathogen.
Dubey and Patel (2001) brought out that the hyperparasitism of *Trichoderma* spp. acted as the main mechanism in the inhibition of mycelial growth of *T. cucumeris*. The work of Deepak Kumar and Dubey (2001) on the management of collar rot of pea revealed that *in vitro* mycelial growth of *Fusarium solani* was effectively controlled by the mycoparasitic action of *T. viride*, *T. harzianum* and *G. virens*. Similar effect was also evident from the work of Anahosur (2001) on the growth inhibition of *S. rolfsii* by the treatment of *Trichoderma* spp. and *G. virens*. Gupta *et al.* (1999) showed that the hyperparasitism of *Trichoderma* spp. and *G. virens* on *B. theobromae* involved the coiling of the hyphae by forming hooks, haustoria or appressoria like structures which led to wrinkling, bursting and collapse of pathogen’s hyphae. Similar report was available on the hyperparasitic action of *Trichoderma* spp inhibiting the hyphal growth of *R. solani* (Chet *et al.*, 1991; Benhamou and Chet, 1993) and *S. rolfsii* (Chet *et al.*, 1978; Upadhyay and Mukhopadhyay, 1983). Grondona *et al.* (1997) reported that the *in vitro* inhibition of mycelial growth of *R. solani*, *A. cochlioides*, *P. betae*, *A. cucurbitacearum* and *F. oxysporum* was mainly attributed to the action of inhibitory substance present in the culture filtrate of *T. harzianum*.

The antifungal activity of culture filtrate of *Trichoderma* spp. against *C. capsici* mycelial growth was evident from the work of Rajathilagam (1999). Similar report was made by Scarselletti and Faull (1994) on the inhibition of *R. solani* mycelial growth by the culture filtrate treatment of *T. harzianum*. Howell and Stipanovic (1983) confirmed that the *in vitro* mycelial growth of *P. ultimum* was effectively suppressed by the antibiotic gliovirin produced in the culture filtrate of *G. virens*. The gliovirin antibiotics generally coagulate the protoplasm of the pathogen cell and disintegrate the pathogen’s hyphae. The inhibitory
substances (antifungal nature) present in the culture filtrate of *Trichoderma* spp and *G. virens* were found to be responsible for the inhibition of mycelial growth of *F. oxysporum. And R. solani* (Mukhopadhyay, 1994; Mukhopadhyay and Kaur, 1990). Kudryvatseva (1980) brought out the fact that the synergistic action of competition, mycoparasitism and antibiotic activity of *Trichoderma* spp. might be the reason for the successful inhibition of cucumber root rot fungal growth. Similar synergistic action in the inhibition of mycelial growth of *F. Solani* the root rot pathogen of pea was evident from the work of Casterjon and Oyarzun (1995).

Significant inhibition (64%) was recorded in 0.1% bavistin treatment. The present finding supports the findings of Gomathi (2001) and Pugazhenthi (2001) who showed that mancozeb and bavistin treatment brought out the inhibition of conidial germination and mycelial growth of *Colletotrichum* spp. significantly. Fungicidal effect of chemicals on the inhibition of *F. solani* spore germination was reported by Singh *et al.* (2000).

**Determination of OIC of the biocontrol agents**

The present observations reveal that there is no significant difference in the activity of antagonistic microbes 2.5% and salicylic acid 10mM concentrations and bavistin 0.1% used. This might be due to the quantity and quality of inhibitory substances present in the above concentrations of culture filtrate of antagonistic microbes, which might be responsible for the difference in the inhibition activity. Rajathilagam (1999) observed that higher concentration of non-volatile antibiotics of *Trichoderma* spp. did not promote higher antifungal activity against *C. capsici*. Similar dosage response relationship was brought out earlier by Raajimakers *et al.* (1995) in the control of *Fusarium* spp. by *Pseudomonas* treatment; Korsten *et al.*
(1995) in the evaluation of bacterial epiphytes in avocado post harvest diseases. The increased amount/higher concentration of culture filtrate of *Trichoderma viride* did not have any effect on the percentage inhibition of spore germination and mycelial growth of *C. falcatum* (Smitha Jagadish, 1997).

**Cell wall lytic enzyme activity in *C. capsici***

The fungal plant pathogens produce different types of pectic and cellulose lytic enzymes, which are the main agents for disease development. Pectinolytic and cellulolytic enzymes produced by the fungal pathogens act as the major factors for the plant disease development.

The pectinolytic and cellulolytic enzyme activities of *C. capsici* were studied under *in vitro* condition. Maximum pectinolytic activity of *C. capsici* was observed in the control flasks i.e., PME by 33.3 SAU (*in vitro*), PMG by 78% viscosity loss along with 805 SAU of end product released (*in vitro*), and PTE by 58% viscosity loss along with 308 SAU of end product released (*in vitro*); The cellulolytic activity observed was C\_1 by 23 SAU (*in vitro*); C\_x by 69% viscosity loss with 634 SAU *in vitro*; The cellobiase activity recorded 34 SAU *in vitro*.

Significant inhibition in the activity of PME by 90%; PMG by 76; PTE by 96, in *in vitro* conditions and cellulolytic enzyme activity C\_1 by 88; C\_x by 88% and cellobiase by 91% was recorded in the SA treatment. Significant inhibition in the pectinolytic and cellulolytic activity of *C. capsici* was evident from the *G. virens* treatments. The activity of PME was inhibited by 75% in *G. virens* treatment. The percent inhibition of cellulolytic enzymes activity revealed that C\_1 was inhibited by 72% in *G. virens* treatment in *in vitro*. The C\_x activity was inhibited by 63% in *G. virens*. 
treatment and cellobiase activity by 85% in *G. virens* treatment. The lytic enzyme of *C. capsici* may be inhibited by the action of *G. virens*.

The treatment might inhibit the activity of lytic enzymes of the pathogen either by antibiotic action or Induced Systemic Resistance (ISR) in the host cell such as thickening cell wall or it induces the host cell to produce lytic enzymes which are able to inactivate or inhibit the pathogen’s lytic enzymes. The accumulation of phenolic compounds in the treated leaves may be toxic to the enzyme activity of the pathogen. The present result substantiates the observation made by Borowitz *et al.* (1992) who reported that *P. fluorescens* treatment degraded the lytic fungal pathogen’s cellulase, pectinase and xylanase mainly by the action of different antibiotics. Velazhagan *et al.* (1999) found that the extracellular chitinase and β-1,3-glucanase of *Pseudomonas* culture inhibited the chitin and glucan present in the cell wall of *R. solani*. Similar report on the activity of chitinase and β-1,3-glucanase of *Pseudomonas* participating in the inhibition of lytic enzymes of different fungal pathogens was made by Frindlender *et al.* (1991) against soil borne fungal pathogens; Lim *et al.* (1991) against *F. solani* lytic enzymes activity; M’Piga *et al.* (1997) in tomato by *F. oxysporum*; Ramamoorthy and Samiyappan (2001) in *C. capsici* in chilli; Mauch *et al.* (1988) in pea plant; Chen *et al.* (2000) against the lytic enzyme activity of *Pythium* sp. Duijff *et al.* (1997) observed that the *Pseudomonas* treatment in the tomato plant induced higher lignifications in the cell wall of the cortical cell. These thickenings add mechanical strength and resist the lytic enzyme activity of the pathogen. Similar ISR by *Pseudomonas* treatment was observed by Broglie *et al.* (1991) against *R. solani*; Yoshikawa *et al.* (1993) against *R. solani*, *Phytophthora* sp. and *Alternaria* sp; Van Loom *et al.* (1998) and Benhamou *et al.* (2000) against *Pythium* sp. in cucumber plant by *Serratia* sp. treatment.
Rajathilagam (1999) found that the non-volatile antibiotics of *Trichoderma viride* treatment effectively suppressed the lytic enzymes of *C. capsici*. Pugazhenthhi (2001) confirmed that the suppression of lytic enzymes of *Colletotrichum* sp. was mainly due to the antibiotic action of *Trichoderma* spp. Ishikawa *et al.* (1980) and Tokimoto (1982) observed that *Trichoderma harzianum* produced mycolytic enzymes such as ether soluble neutral antifungal compounds which resulted in the hyphal lysis of *L. edodes*. Di Pietro *et al.* (1993) reported that the synergistic action of endochitinases along with gliotoxin antibiotic of *G. virens* controlled the enzyme activity of *B. cinerea*. *Trichoderma* spp. and *G. virens* were reported to produce many antifungal lytic enzymes such as chitinases, proteases, glucanases, lipases, laminarinase and xylanase, which are responsible for the degradation of lytic enzymes of other fungal species. This was observed by Cotes *et al.* (1996) in *Trichoderma* spp.-treated bean plant against *Pythium* sp.; Jones *et al.* (1974) in the degradation of *S. sclerotiorum* by *Trichoderma* treatment; Haran *et al.* (1996) in *Pythium ultimum* by *Trichoderma harzianum* treatment; Lorito *et al.* (1993a,b, 1994) in *B. cinerea* by *Trichoderma harzianum* treatment. Haab *et al.* (1990) reported that the cellulases produced by *Trichoderma* spp were responsible for the inhibition of cellulolytic enzyme activity of *C. capsici*.

The present findings support the studies on the effect of chemical fungicides on pectinolytic enzymes of *S. sclerotiorum* and *B. allii* which revealed that the phaltan or difolatan treatment exhibited minimum pectinolytic enzymes activity (Grover, 1964). The chemical fungicides inhibited the lytic enzymes of *Sclerotinia* sp. (Jones *et al.*, 1974). Bavistin and chitosan were reported to inhibit the production of cellulases in *R. solani* by 58 and 44% (Kannaiyan and Prasad, 1979). The copper oxychloride and mancozeb completely suppressed the cellulases production of *Corynespora* sp. at
different concentrations (Komaraiah and Reddy, 1986). Mancozeb and bavistin were reported to inhibit the pectinolytic and cellulolytic enzyme activity of *Colletotrichum* spp. (Gomathi, 2001; Pugazhenthi, 2001).

**In-vivo STUDY**

**Electrolytic conductivity**

The present study reveals that the *C. capsici*-infected leaves recorded maximum electrolytic leakage (0.242 mS/cm²), which was 6.7 times higher than that of healthy, treated and infected treated plants.

The action of fungal toxin on the host cell resulted in the disintegration of semi-permeable membrane, which in turn led to higher electrolytic leakage. Similar observation was also made by Thoithoi Singh *et al.* (2001) who found that fungal toxin or its enzymes contributed to the higher electrolytic leakage from the infected rice seeds. This higher leakage led to gradual loss of semi-permeability nature of the seed membrane. Padma Singh (2000) showed that the higher electrolytic leakage in the *Alternaria* sp.-infected onion leaves was mainly due to the toxin produced by the pathogen. The damaged cell lost its ability to accumulate the metabolic solutes, which were required for the growth and development of the cell. Similar report was made by Khirbat and Jalali (2000) in *Ascochyta rabiei*-infected chickpea leaves; Cook and Stall (1968) in *Xanthomonas* infected *Capsicum annum* leaves and Burcowicz and Goodman (1969) in *Erwinia* sp infected apple leaves. Wheeler and Black (1962, 1963), Samaddar and Scheffer (1968) and Scheffer and Samaddar (1970) reported that higher electrolytic leakage in the *Helminthosporium*-infected cells was mainly due to the toxic effect of victorin.
The least electrolytic leakage was recorded in the salicylic acid-treated leaves (0.042 mS/cm²) followed by the infected and infected treated leaves did not differ significantly.

The leaves treated with salicylic acid exhibited least amount of electrolytic leakage. This might be either due to the action of antibiotics, which inhibit the establishment and activity of the pathogen or they induce the host cell to develop systemic resistance, which resists the pathogen invasion. Thus the host cell is protected from the damage caused by the pathogen. This finding supports the work of Abad et al. (1996) that minimum electrolytic leakage in the treated tobacco leaves was mainly due to the action of PR protein osmotin. Similar report was made by Liu et al. (1994) in potato plant.

**Photosynthetic pigment content**

The photosynthetic pigments are responsible for absorbing solar energy for the process of photosynthesis in the host plant. Any change in the pigment content would be reflected immediately on the photosynthetic efficacy of the plant and subsequently on its growth and yield. Hence, the analysis of pigment content in the control, infected, healthy treated and infected treated plants becomes essential.

The present study shows that maximum reduction in the total chlorophyll content (78%), carotenoid content (63%) over control and salicylic acid of infected leaves treated plants was more when compared with *C. capsici*-infected plants and infected treated ones.

The toxic effect of the fungal metabolite caused the symptom formation in the host tissue. Similar observation was made by (Asha and Kannabiran 2001b) in the
leaves of chilli infected with *C. capsici*; (Bauer et al., 2000) and (Mayr et al., 2001) in norway spruce infected with *Chrysomyxa rhododendri*; (Pero and Main, 1970) in tobacco infected with *Alternaria* sp. This finding gains support from the work of Pant et al. (2001) where the maize leaf infected with blight disease symptom resulted in the least CO₂ fixation. The reduction in the photosynthetic activity (suppressed ATP formation) was mainly due to the toxic effect of the pathogen. Tu and Ford (1968) observed a heavy reduction in the photosynthetic activity in maize due to virus infection. This might be due to the activity of the pathogen, which initiated the chlorosis and necrosis condition in the phylloplane and reduced the number and size of the chloroplasts. Diener (1963) explained that the reduction of photosynthetic pigment content and activity in the virus-infected leaves was mainly due to the induction of chlorophyllase activity by the pathogen or its toxin. Walters and Ayres (1984) reported decreased pigment content and RuBP case activity in the powdery mildew infected-barley leaves. This fact was due to the activity of toxin produced by the fungal pathogen. Subbaraja (1981) concluded that the decreased chlorophyll content and photosynthetic activity in the *C. capsici*-infected chilli leaves was mainly due the effect of the toxin, colletotin.

Least reduction in total chlorophyll content (6%), and carotenoid content (4%) was noticed in the salicylic acid treatment. The infected and infected treated recorded insignificant reduction in total chlorophyll content (48%), carotenoid (16%) as compared to heavy reduction in the infected leaves.

Healthy treated plants exhibited higher values of pigment concentration than those of control or infected- treated samples. In soybean plants, treatment with salicylic acid increased pigments content as well as the rate of photosynthesis
(Zhao et al., 1995). Sinha et al. (1993) pointed out that chlorophyll and carotenoid contents of maize leaves increased upon treatment with SA. Taking together, the results of the previous authors support our findings.

The treatments might suppress the chlorophyllase activity or they would have produced the needed organic acids for the host growth and development. Another reason was that the disease intensity reduction might be a reason for higher pigment content and enzyme activity in the treated plants as compared to the infected plants. This was confirmed by Lindow (1984) who recorded least reduction of pigment content in the Pseudomonas-treated leaves of pear infected with fireblight disease. The main reason was that the antibiotics produced by the salicylic acid inhibited the activity of the toxin produced by the pathogen. Similar report regarding the least reduction in the salicylic acid-treated leaves due to antibiotic action was made by (Mew and Rosales, 1992) in sheath blight of rice caused by R. solani, (Lindow et al., 1996) in fireblight and frost injury of pear, (Pusey, 1996) and (Zhou and Young, 1996) in scab disease of apple, (Yoshikawa et al., 1993) in fungal disease of tobacco and (Vidhyasekaran et al., 1997) in rice blight disease.

**Total carbohydrates**

The total carbohydrate content was found to be drastically reduced in the leaves of infected plant (total sugar by 65%; reducing sugar by 69%; non-reducing sugar by 55%; sucrose content by 77%; starch content by 69%).

The pathogen normally uses the host carbohydrate metabolites for their growth and survival. This was established Chakrabarty et al. (2002) in cotton plant infected with grey mildew disease, Agarwal et al. (1982) in turmeric leaves infected with leaf
spot disease and Khodhe and Gahukar (1995) in chilli fruit infected with *C. gloeosporioides*. Debnath *et al*. (1998) reported that the decreased starch content in the *Brassica* leaves infected with *Albugo* sp. was mainly due to the fact that these substances were utilized by the pathogen for its growth and development. Padma Singh (2000) found that the loss of carbohydrates from the *Alternaria* sp.-infected onion leaves was due to heavy loss of these contents from the impaired membrane. Then these contents were used by the pathogen for its growth. Dropkin (1972) concluded that the reduced carbohydrate content in *Meloidogyne* galling was mainly due to the activity of the pathogen’s enzymes, which cleaved the host carbohydrate into easily assimilable substances and used it for their growth and development. Similar observation was made by Gomathi (2001) and Asha (1999) in chilli fruit infected with *Colletotrichum* spp. Schiffer and Mirocha (1968) found that the depletion of starch content in the rust-infected bean leaves was mainly due to the activation of starch hydrolyzing enzyme (amylase) by the fungal metabolites.

Healthy sprayed leaves exhibited least reduction in the carbohydrate of about 13% in total sugar, 9% in reducing sugar and 3% in non-reducing sugar content as compared to significant reduction in the infected leaves.

The least reduction in the healthy sprayed leaves might be due to induction of the treated plants systemic resistance (SR) to utilize the carbohydrates for the biosynthesis of phenolic compounds. These phenolic compounds were used for the defense reaction against the pathogen infection. This finding substantiates the works of Neish (1964); Rajavel (2000) in *Pseudomonas* and *Trichoderma*-treated *Capsicum* fruits infected with *C. capsici*. Upadhyay and Mukhopadhyay (1986) in *Trichoderma* spp.-treated rice plants against sheath blight fungus.
Healthy treatment increased the polysaccharide contents as compared with the control plants in this regard; soluble sugar content was also increased in tomato plants in relation to salt stress (Maria et al., 2000). It is suggested that SA application might activate the metabolic consumption of soluble sugars to form new cell constituents as a mechanism to stimulate the growth of maize plants reported in this study. SA treatment might also be assumed to inhibit polysaccharide-hydrolyzing enzyme system on one hand and/or accelerate the incorporation of soluble sugars into polysaccharides. Our assumption could be supported by the result that SA increased polysaccharide level on the sake of soluble sugars. In this connection, Sharma and Lakhvir (1988) postulated that foliar spray of SA to ray plants, resulted in decreasing their soluble sugar level. In summary, it might be concluded that SA treatment of infected plant could stimulate their disease tolerance via accelerating the carbohydrate metabolism.

**Nucleic acid and Protein content**

The DNA and RNA content reduced heavily by 68% in the *C. Capsici*-infected leaves. Maximum reduction in the protein content (72%) and in amino acid content (64%) was noticed in the infected leaves.

Similar observation was made earlier by Asha and Kannabiran (2001a) who found decreased nucleic acid content in the chilli leaves infected with *C. capsici*. This is due to the fact that the pathogen’s enzyme interfered with the cell division process thereby reducing the rate of cell division. Similar report was made by Gomathi (2001) in chilli fruits infected with *Colletotrichum* spp. Chakrabarti and Basuchaudhary (1979) found that maximum reduction in the protein and amino acid content in safflower wilt caused by *Fusarium* sp. was mainly due to their
breakdown by proteolytic enzymes such as protease secreted by the pathogen activity. These enzymes permitted the pathogen to use the host protein as source of nitrogen and amino acid for their growth and development. This was supported by the work of Howell and Krusberg (1966) in alfalfa and pea plant infected with Ditylenclus sp. and Chakrabarty et al. (2002) in cotton plant infected with grey mildew disease.

Least reduction in the nucleic acid content (10%), protein content (13%) and amino acid content (5%) was noticed in the SA-treated leaves.

The reason might be that these treatments induced the host plant to utilize the amino acids of the protein and the sugars of the nucleic acids for the synthesis of phenolic compounds which acted as the defense agents against the fungal infection. The present finding substantiates the work of Veeramohan et al. (1994) that the decreased protein content of chilli infected with Alternaria solani might be due to their participation in the synthesis of phenolic compounds; Dashti et al. (1997) reported increased protein content in the Pseudomonas-treated leaves of soybean. Richmond and Lang (1957) reported that the kinetin production preserved the protein content by the decrease of the proteolytic activity in detached Xanthium leaves. Kloepper (1994) reported that the PGPR treatment recorded higher protein content and total amino acids in rice and wheat plants and high sugar content in sweet potato and sugar beet. This was mainly due to the suppression of the disease and increased the activation of enzymes that were involved in carbohydrate metabolism.
Nitrogen metabolism

The leaves are the major sites of utilization of nitrates. The nitrates that are absorbed by plants are reduced to nitrites and then immediately to ammonia. Finally they are converted to amino acids and proteins. The nitrate reductase is the key enzyme in nitrogen metabolism, which converts the nitrate to nitrite.

Maximum reduction of 79% in total nitrogen, 58% in amino nitrogen and 46% in the activity of NR enzyme over control, SA-treated leaves, and infected with *C. capsici*-infected leaves. Similar observation was made by Prasad (1981) that the heavy reduction in nitrogen content in banana plant under fungal pathogenesis was due to the fact that the pathogen survived at the expense of host nitrogen pool leading to the enhancement of disease development, which brought down the protein and total nitrogen content in the host tissue. Padma Singh (2000) found that decreased nitrogen content in the *Alternaria* sp.-infected onion leaves was due to the disruption of cell structure coupled with enhanced proteolytic enzyme activity which enhanced disease development. Moreover, our finding finds support in the work of Khare and Lakpale (1997) in soybean leaves infected with *Xanthomonas* sp. and Padmanabhan *et al.* (1974) in citrus leaves infected with *Xanthomonas* sp.

The healthy treated leaves recorded higher nitrogen content, amino nitrogen and NR activity over infection and infection treated ones. This might be due to the fact that the treatment may induce the activity of the enzymes involved in the nitrogen metabolism. The least reduction might be due to their participation in defense reaction against the pathogen infection (Krikham, 1954; Murthy and Bagyaraj, 1978). The present finding substantiates the work of a concentration (0.01-1.0mM) of Ca (No₃)₂ in association with SA activated the uptake of nitrogen and the activity of nitrate
reductase (NR) both in the leaves and roots of maize plants, although higher concentration (5mM) proved inhibitory (Jain and Srivastava, 1981). Similarly, SA increased the activity of NR in the presence of NO3 and also favoured protection of the enzymes against proteanase, trypsin (Rane et al., 1995). The plants resulting from wheat grains, soaked in aqueous solution (10^{-5}M) of SA, exhibited high NR activity (Hayet et al., 2005) and also in mustard leaves whose foliage was fed with SA (10^{-5}M) (Fariduddin et al., 2003). Higher NR activity, under the influence of SA, in Glycine max was coupled with protein content (Kumar et al., 1999). Maximal NR activity as herein observed was similar to those of other reports for cassava (Pereira Jose and Splittstoesser Walter 1986) and coffee (Da Matta Fabio et al. 1999).

The increased activity of NR by the dilute concentration (10^{-5} M) of SA could have either been an expression of the interaction of the acid with NR specific inhibitors whose presence is claimed by Srivastava (1980) and/or through the mediation of the other hormone (s). Auxins (IAA and Cl-IAA) are protected by phenols (Schneider and Whitman 1974) and elevate the activity of NR in pea and mustard (Ahmad 1988, 1994, Ahmad and Hayat, 1999; Ahmad et al. 2001). Moreover, the content of any active protein (enzyme) represents a fine balance between its synthesis/activation and degradation/inactivation (Jain and Srivastava, 1981). The concentration of SA might play an active role in such a regulation where the lower concentration favoured an increase in the NR protein and higher quantity of SA decreased it by affecting the above processes.

**Phenol content**

Phenolic compounds are fungitoxic in nature; hence, the accumulation of phenolic compounds increases the physical and mechanical strength of host cell wall
resulting in the inhibition of fungal invasion. The phenol and proline compounds act as adaptive mechanism in the host plant against the fungal infection.

The O.D. phenol, total phenol and proline content exhibited an increase of about 4-time in the infected plants over control and SA-treated ones. The present finding gains support from the work of Asha and Kannabiran (2001b) who recorded higher phenol content in the *C. capsici*-infected chilli leaves. This was due to the hindrance of the glycolysis by the activity of the pathogen, which in turn activated the pentose pathway leading to the formation of 4-carbon compounds for the synthesis of phenols. Farkas and Kiraly (1962) and Jaypal and Mahadevan (1968) found that sharp increased phenol content in the infected plants might be due to the fact that the accumulation of phenols in the infected tissue might come from the surrounding healthy leaves in order to resist the advancement of the pathogen towards the other healthy cells. Increased proline and decreased protein content in the infected plant tissue were also reported by Kumaravadivelu *et al.* (1996).

The healthy treated leaves exhibited an increase of about 1.5 times in the phenol and proline content over control plants.

This might be due to the induction of systemic resistance in the host plant due to treatments. The over-production of phenolic compounds resists the advancement of the pathogen towards the other healthy cells. Similar findings have been reported by Ramamoorthy and Samiyappan (2001) that the higher content of phenol in the *Pseudomonas*-treated chilli plants infected with *C. capsici* was mainly attributed to the fact that the phenols are fungitoxic in nature and their accumulation increased the physical and mechanical strength to the host cell wall resulting in the inhibition of pathogen invasion.
These results are in agreement with those obtained by Shehata et al. (2001), Raskin, (1987), Shakirova et al. (2003) and Rashad, (2003) found that SA and phenol content in maize leaves significantly increased by acetyl salicylic acid application (20 and 40 µM). Abdel and Wahed et al. (2000) found that foliar application of salicylic acid at low dose of 1 µM and 2 µM resulted in significant increase in total sugars, crude protein, oil and total carotenoids content of yellow maize grains, while, the high doses (3 µM) significantly decreased it. The maximum values of biochemical content in onion bulb were obtained by salicylic acid treatment (100 mg/l) on total soluble sugars, free amino acid and total phenols. The combined treatments enhanced the above mentioned biochemical constituents in most cases compared with the individual effect of salicylic acid treatments or untreated plants. 100 or 50 mg/l and the lower and the moderate concentration of salicylic acid (50 or 100 mg / l ) showed higher total soluble sugars, free amino acids, total phenols and total indoles compared with SA- treated plants or untreated plants. Foliar application of salicylic acid at the concentration of 50 and 100 mg/l gave the highest values for chemical constituents of onion bulb compared with the higher concentration (200 mg/l) or untreated plants. This effect might be due to these substances on enzymatic activity and translocation of the metabolites to onion bulb.

**Antioxidant enzymes activity**

The active oxygen species (AOS) such as superoxide anion radical, hydrogen peroxide and hydroxyl radical are generally produced in the plants as the result of the metabolic processes that take place in chloroplast, mitochondria and plasma membrane-linked electron transport system. During the infection process the pathogen/or its activity interfered with the electron transport system thereby resulting
in the leakage of electron. These electrons altered the structure of the molecular oxygen resulting in the production and accumulation of AOS within the cell. The accumulation of AOS damaged the cell and caused lipid peroxidation, protein denaturation, DNA mutation, molecular disfunction and led to cell death. Generally the plants produce many antioxidant and low molecular weight enzymes such as SOD, CAT, PO, ascorbate peroxidase and glutathione reductase etc. The SOD converts the superoxide radicals to hydrogen peroxide. Then the CAT and PO reduced the hydrogen peroxide to water and oxygen molecules.

Significant increase in the antioxidant enzyme activity (2.5% in SOD, CAT and 3.5 times in PO and PPO) was found in the SA-treated leaves.

The enhanced antioxidant enzyme activities in the treated host tissue might be due to induction of systemic resistance in response to the pathogen infection. These enzymes participate in rapid detoxification of reactive oxygen species (superoxide anion (\(O_2^−\)); hydrogen peroxide (\(H_2O_2\)); singlet oxygen (\(·OH\)) into water. The rapid conversion inhibited the toxic effect caused by ROS to the host plant. In addition the host cell under pathogenesis might accelerate the terminal respiratory pathway, which may lead to increase in the CAT activity reported that the higher activity of PO and PPO in the infected tomato leaves might be due to their participation in the oxidation of phenolic residues into cell wall polymers in the pathogen-infected cell. This finding was confirmed in \(C.\ capsici\)-infected turmeric leaves (Agarwal et al., 1982). Asha and Kannabiran (2001a) found out that the higher PO and PPO activity in the chilli leaves infected with \(C.\ capsici\) was mainly due to the enhanced respiratory rate induced by the pathogen activity. Similar finding was made by Gomathi (2001). Nonaka (1959) found that increased PO and PPO in the host plant
infected with fungal pathogen might be due to the fact that the enzymes were triggered to meet the catabolic reaction exerted by the pathogen infection. Deverall (1961) found that higher activity of PO and PPO in chocolate leaf spot disease was due to action of lytic enzyme activity of the pathogen, which involved in the activation of the latent PPO activity of the host plant. Similar finding was made by (Naffaa et al., 1999) in perennial ryegrass infected with endophytic bacteria, Zhang et al. (1996) in anthracnose disease of cucumber, Sreedhara et al. (1995) in pearl millet infected with Sclerospora sp. (Kwon and Anderson, 2001) reported enhanced SOD and CAT isozymes activity in the wheat leaves infected with Fusarium like fungus isolate. The fungi generate hydrogen peroxide as part of its weaponry to enhance the penetration process into the host cell. Baker and Orlandi (1995) explained that the accumulation of active oxygen species in the plant cell during interactions with potential pathogens affect many cellular processes (proteins, lipid, polysaccharides and nucleic acids) that are involved in the plant/pathogen interaction. Yim et al. (1990) reported that the elevated concentration of ROS in the infected plant cell generally induced disfunction of all metabolic activities and led to cell death. Levine et al. (1994) and Jabs et al. (1996) concluded that hydrogen peroxide produced as a result of pathogen activity in the plant-pathogen interaction, led to direct cause for cellular death of the infected tissue. Bowler et al. (1992) explained that the increased SOD activity in a susceptible host infected with a virulent pathogen was mainly responsible for the detoxification of the oxyradicals. Rusterucci et al. (1996) brought out the fact that the elicitin (capsicein and cryptogein)-treated Nicotiana cells exhibited increased activity of active oxygen species. This was mainly due to their participation in the detoxification of the reactive oxygen species.
Keen (1999) explained that the scavengers (SOD, PO, CAT) of active oxygen species act like antibiotic against the invading pathogen. The enhanced activity of SOD, PO, CAT recorded in the treated cells generally restricted the pathogen activity. Schinkel et al. (2001) brought out the fact that the main function of SOD was to scavenge the superoxide anion radicals, generated in various physiological processes and prevent the oxidation of biological molecules.

A mode of action of SA was proposed based on the finding that SA binds to, and inhibits catalase (Chen et al., 1993). Catalase inhibition would lead to an increase in the concentration of hydrogen peroxide (H2O2) or active oxygen species derived from it that arise during respiration, photosynthesis, photorespiration or during the hypersensitive response against pathogens. H2O2 could have a direct antibiotic activity against invading pathogens. H2O2 and its derivatives could also act as intermediates in the signalling cascade for the expression of genes related to defence (Chen et al., 1993).

An increase in enzyme activities associated with antioxidative processes was observed in non-infected leaves of TMV-infected tobacco (Fodor et al., 1997). Increase in ROS such as H2O2 and superoxide anions in infected plants could provide a mechanism for the rapid deployment of a variety of early defense responses (Klessig and Malamy, 1994).

Significant increase in SOD, CAT, PO activity about 1.5% times over healthy plant was evident in the SA-treated plants. The treatments generally induced systemic resistance in the host cell, which in turn enhanced activation of these enzymes in the conversion of reactive oxygen species or radicals to water in order to reduce the infection. Thus the rapid conversion reduced the severity of the infection caused by
the pathogen. The systemic induced resistance by *Pseudomonas* treatment (Van Loom *et al.*, 1998) includes increased cell wall structure modification in response to pathogen attack (Chen *et al.*, 2000; Benhamou *et al.*, 2000); accumulation of phenolic compounds (Ramamoorthy and Samiyappan, 2001); induced biochemical and physiological changes in the treated plants such as enhanced chitinase activity against red rot of sugarcane (Viswanathan and Samiyappan, 1999) and synthesis of phytoalexin and other secondary metabolites (Maurhofer *et al.*, 1994).

**PR-Protein β-1, 3 glucanase**

The activity of β -1, 3 glucanase in the control is (13U/mg), Infection (8U/mg), treatment with SA (25U/mg) and infected treatment (21U/mg).

The two plant 'antifungal hydrolases', chitinase (EC 3.2.1.14) and β-1,3-glucanase (EC 3.2.1.39), have attracted increasing interest over the past few years, particularly due to their putative role in the defence response of higher plants especially against fungal pathogens (Van Loom, 1989). β 1,3-glucanase are induced by several abiotic (e.g. treatment with chemicals, heavy metals or certain gases) as well as biotic (e.g.infection with pathogens) stress situations. In addition, chitinases and β -1, 3-glucanase can be activated not only upon challenge of plant tissues by microbes, but also upon exposure to certain elicitors (Mauch *et al.*, 1988; Hahlbrock, 1986).

β -1, 3 glucanase enzyme is called a PR protein, and has been considered one of the main factors involved in the mechanism of resistance induction in plants (Manandhar *et al.*, 1999).
Plants have several lines of defense against invading pathogens including preformed barriers and induced responses. The latter include a rapid production of reactive oxygen species, enhancement of preformed structural barriers, hypersensitive cell death, synthesis of phytoalexins, and the production of large amounts of pathogenesis related (PR) proteins. The biochemical function of PR proteins has only been established for some groups, but they have all been shown to possess antimicrobial activity.

These proteins are synthesised in response to infection, many of which have β glucanase activity. Some PR-protein disrupts pathogen nutrition. This enzyme exists in multiple forms. These enzyme solubilize elicitor active glucan molecules from the fungal cell walls and also induce plant defense enzymes. In the present study, β 1, 3 glucanase activity was higher on SA treated plants.

The production of extracellular β 1, 3 glucanase has been reported as an important enzyme in bio control of microorganisms.

The PR-proteins were first described as a novel set of abundant proteins accumulating in leaves of resistant tobacco cultivars reacting with hypersensitive response (HR) to infection with tobacco mosaic virus (TMV). Salicylic acid is a known elicitor of PR proteins in many plants.

Resistance induction in plants may be defined as the ability of the plant to prevent or restrict the development and consequent multiplication of a pathogenic agent. This may be achieved by the use of elicitors, substances that induce defense reactions in plants, including the production of phytoalexins, proteins related to pathogenesis, such as PR proteins (β1,3 glucanase). Thus, resistance induction has
been observed and suggested as an alternative control method in different pathosystems.

Generally the content of β 1, 3 glucanase is low in healthy plant. When the plant was infected by the pathogens or induced by the elicitors the activity of the enzyme will promote quickly. It has the function of degrade the cell wall of fungal pathogens, then the growth of fungi ceased. The decomposed product (oligose) can elicit the accumulation of defense-related enzymes such as PAL, also accelerate the synthesis of plant phytoalexin and lignin, which can enhance the plant’s resistance further. Therefore, the results presented here suggest that SA induction may not only enhance the ability of the plant cell physical barriers and prevent the penetration, but also induce the accumulation of antimicrobial products.

**Phenylalanine ammonia-lyase (PAL)**

PAL is the key enzyme in the plant phenyl propanoid pathway catalyzing synthesis of secondary metabolites from L-phenyl alanine including lignin, flavanoid and phytoalexins. It has been suggested that infestation of plants causes an increase in the activity of phenylalanine ammonia-lyase (PAL), a key phenolic biosynthesis enzyme.

The activity of PAL in the leaves of control, healthy treated, infected and infected treated plants as the infected leaves showed minimum activity (0.0003 nmol/mg of protein) compared to that of the control (0.0005 nmol/mg of protein) leaves. All the treatments showed generally higher activity of PAL. It was followed by the infected ones sprayed with SA (0.0014 nmol/mg of protein) and the highest activity was recorded in the leaves of healthy ones sprayed with SA (0.0022nmol/mg
of protein). This finding confirms the fact that the higher rate of synthesis of PAL enhanced the SA content and induced resistance in capsicum fruit (Meena et al., 2000).

The content of salicylic acid (SA) is known to increase in dozens of times when plant tissues are affected by incompatible pathogens and elicitors (Leon et al., 1995 and Raskin et al., 1997) SA immediately participates in activating several plant signal systems, such as SA accumulation due to the pathogen or elicitor effects was shown to result from the enhanced activity of phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), the key enzyme of phenylpropanoid metabolism; such enhancement activates the metabolic chain phenylalanine– cinnamic acid–benzoic acid–SA(Coquoz et al., 1998).

Phenylalanine ammonia-lyase (PAL) is the key enzyme that is involved in phenylpropanoid biosynthesis (Smith and Banks, 1986) and branch pathways lead to biosynthesis of a wide variety of secondary metabolites which has a diverse function in plants. The resulting secondary metabolite involve in defense mechanism such as cell wall strengthening and repair (e.g. lignin and suberin), antimicrobial activity (e.g. furanocoumarin, pterocarpan and isoflavonoid phytoalexins) and as signaling compounds such as salicylic acid (Hammerschmidt, 1999).

The first reaction of this biosynthetic pathway is the deamination of phenylalanine to cinnamic acid by PAL. Then the condensation reaction occurs and it leads to the synthesis of flavonoids (Ryder et al., 1984, 1987; Cramer et al., 1989; Hahlbrock and Scheel, 1989). Increase in level of the PAL enzyme shows the increase in defense mechanism. PAL induction has been linked to defense responses that involve phenylpropanoids in numerous diseases.
The product of PAL activity is trans-cinnamic acid, which is an immediate precursor for the biosynthesis of salicylic acid, a signal molecule in systemic acquired resistance (SAR) (Klessig and Malamy 1994). Increased activity of PAL was observed in *P. fluorescens* treated tomato and pepper plants (during flowering stage) in response to infection by *F. oxysporum fsp lycopersici* and *C. capsici* (Ramamoorthy and Samiyappan 2001).

Phenylalanine ammonia lyase was induced by various biotic (infection by viruses, bacteria, fungi, etc.) and abiotic (low and high temperatures, UV-B light and wounding, etc.) stresses, which resulted in the accumulation of such phenolic compounds as phenolic acids and flavonoids.

Plants inoculated with pathogenic organism showed increase in PAL activity and followed by declined activity rapidly. Similarly, the activity of PAL, PO and PPO declined rapidly in tomato after 4 days of inoculation with *Fusarium oxysporum f.sp. lycopersici* compared to bacterized plants challenged with the pathogen (Ramamoorthy *et al.*, 2002).

The high PAL activity reported in SA treated leaves strengthens the hypothesis of wall-stiffening by lignin synthesis and subsequently reduction in the root growth. These compounds are often induced and play specific roles in plant protection against biotic and abiotic stresses (Hahlbrock and Scheel, 1989; Jones, 1984)

In conclusion, the results presented here indicate that the considerable amount of conjugated SA (Coquoz *et al.*, 1995) is not found in the other healthy plants where
SA biosynthesis occurs. The synthesis of SA induced by AA clearly involves PAL and is unlikely to take place via a PAL-independent pathway.

**Endogenous salicylic acid content**

Salicylic acid (SA, 2-hydroxybenzoic acid) is considered as one of the key endogenous signals involved in the activation of numerous plant defense responses (Shah and Klessig, 1999). Early evidence showed that application of SA induced resistance against several pathogens and the expression of pathogenesis-related proteins in a variety of plants (White, 1979; Ward et al., 1991).

The hypersensitive response (HR) is a common manifestation of plant disease resistance that is characterized by rapid cell death around the point of infection, restricting the systemic spread of pathogens.

Salicylic acid (SA) is a critical signal for the activation of plant defense responses against pathogen infections. SA is thought to be synthesized by conversion of phenylalanine to trans cinnamic acid by phenylalanine ammonia lyase a key enzyme in the phenyl propanoid pathway leading to phytoalexin, lignin and hydroxybenzoic acid synthesis.

The infected leaves showed minimum activity (1.0 µg/g) compared to that of the control (2.1 µg/g) plants. Healthy treated and infected treated plants showed generally higher amount of SA followed by the infected ones sprayed with SA (2.3 µg/g) and the highest activity was recorded in the healthy leaves sprayed with SA (3.5 µg/g). The infected leaves showed minimum SA activity which could be attributed to infection of chilli with *C. capsici* leading to SAR against the pathogen and to an increase in the level of SA in the leaf.
Infection of cucumber cotyledons with TNV leads to SAR against *C. lagenarium* and to an increase in the level of SA in the leaf. This system exhibits the same features as the cucumber plants used previously, from which leaf 1 was used for induction and leaf 2 was used for challenge infection (Madamanchi and Kuc, 1991). Cucumber cotyledons utilize BA as a precursor for the synthesis of SA as was demonstrated for cucumber and tobacco leaves (Yalpani et al., 1993; Meuwly *et al.*, 1995). SA was previously reported in the phloem sap of infected cucumber (Métraux *et al.*, 1990; Rasmussen *et al.*, 1991) as well as in tobacco plants (Malamy *et al.*, 1990; Yalpani *et al.*, 1991), suggesting the transport of SA from the site of infection to the upper, uninfected leaves. This observation led to the hypothesis that SA might be the long-distance systemic signal for SAR (Malamy *et al.*, 1990; Métraux *et al.*, 1990).

The treatment with SA showed maximum activity of endogenous SA the finding is confirmed in induction of SAR by SA is associated with the accumulation of PR proteins. Exogenous application of SA also induces SAR in several plant species (Gaffney T, *et al.*, 1993, Ryals JA, *et al.*, 1996).

At least eight of the ten major PRs induced in tobacco in response to pathogens causing hypersensitive necrosis, were found in the intercellular washing fluid (IWF) of leaves of plants grown in autoclaved soil in the presence of *P. fluorescens* CHA0, which is likely to produce SA in the rhizosphere (Maurhofer *et al.*, 1994). Treatment of radish leaves with avirulent pathogens or millimolar concentrations of SA induced SAR and PR-homologues of the families PR-1, -2 and -5 (Hoffland *et al.*, 1995).

The first indication that SA, a common plant phenolic (Ryals *et al.*, 1994), plays an important regulatory role in plants came from the study on thermogenesis in the inflorescences of *Arum* lilies (Raskin *et al.*, 1987). Subsequently, SA was shown
to be a signal in acquired resistance to pathogens in tobacco (Malamy et al., 1990; Gaffney et al., 1993; Vernooij et al., 1994) and cucumber (Métraux et al., 1990). The development of acquired resistance often follows a localized tissue death at the site of pathogen penetration called the HR. After the inoculation with the inducing pathogen, SAR to subsequent pathogen attack develops in the pathogen-free tissues (Ross, 1961). SA may be a signal responsible for the induction and maintenance of SAR (Ryals et al., 1994). Increased amount of SA is required for the induction of tobacco SAR (Gaffney et al., 1993), and SA likely acts via the local and systemic induction of PR proteins, which possess antipathogen activity (Ward et al., 1991).

SAR is characterized by an accumulation of salicylic acid (SA) and pathogenesis-related proteins (PRs). Accumulation of SA occurs both locally and, at lower levels, systemically, concomitant with the development of SAR. Exogenous application of SA also induces SAR in several plant species. (Gaffney et al., 1993)

**Polygalacturonase inhibiting protein (PGIP) - Purification**

Polygalacturonase- inhibiting proteins (PGIPs) are plant cell wall proteins that are directed against fungal polygalacturonase (PGs). The inhibiting activity of PGIPs directly reduces the aggressive potential of PGs. In addition, it causes PGs to form more long-chain oligogalacturonides that are able to induce defense responses, thereby indirectly contributing to the plant defense. Our evidence showed that PGIPs are efficient defense proteins and limit fungal invasion.

The Previous studies (Barmore and Nguyen, 1985) of PGIP have described the occurrence of a PGIP in ‘Valencia’ oranges and suggested a role for it in the regulation of Diplodia natalensis PG activity in infected tissue.
Since plants express more than one PGIP, it is difficult to investigate the inhibitory activity of a single PGIP without going through a laborious purification protocol.

The results of the present study show the partially purified PGIP from Capsicum annuum. The two activity peaks were obtained by ion exchange chromatography (Fig. 1). Ion exchange of these two peaks using a low salt eluant resulted in a different activity profile for each run. However, similar activity profiles, each with two activity peaks, were seen following the two gel permeation peaks when the sephadex G75 column was eluted with buffer (Figs. 2A and B). Thus far, complete purification has not been achieved, but from the SDS-PAGE results (Fig. 4, lanes 3 and 4) it can be assume that the MW of the active protein is approximately 44 kDa. The 44 kDa MW is consistent with molecular weights reported for PGIPs from several other species, which range from 35 to 45 kDa (Abu-Goukh et al. 1983; Cervone et al. 1987; Favaron et al. 1994). The MW of the partially purified PG-inhibiting protein extracted from ‘Valencia’ orange peel was reported to be near 54 kDa (Barmore and Nguyen, 1985). A polygalacturonase-inhibiting protein (PGIP) was purified from Capsicum annuum leaves. The protein had a molecular mass of about 44 kDa which was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PGIP is a glycoprotein with varying size. The smallest PGIP reported was from peach, with molecular weight 15 kDa (Fielding, 1981), and the largest was isolated from pear with a molecular weight of 91 kDa (Abu-Goukh, et al., 1983). However, most of the identified PGIPs fall in the range of 34 to 54 kDa. The PGIPs are relatively heat-stable up to 50°C and they slowly lose their activity when heated
above 55°C (Albersheim, and Anderson, 1971; Cervone, et al.,1987 and Abu-Goukh et al.,1983) Protease inhibitors such as leupeptin (20 mg/ml), polymethyl sulphonyl fluoroide (2 mM) and pepstatin (50 mM) did not inhibit PGIP of bean (Cervone et al.,1987), while trypsin destroyed PGIP of peach (Fielding, 1981). Kinetic studies have suggested that the inhibition caused by these proteins is competitive (Abu-Goukh, et al., 1983); PG–PGIP complex dissociates at a pH lower than 4.5 and higher than 6 and at a salt concentration above 500 mM Na-acetate (Cervone et al., 1987).

The plant cell wall provides structural stability and protection against invading pathogens. Among the cell wall constituents, the polysaccharide pectin accounts for major part of primary cell walls in dicotyledons (Favaron et al., 1994) and some monocotyledons. Successful colonization and infection of host plants by invading pathogens depend on the enzymatic potential for coping with the pectinaceous barrier. Collmer and Keen (1986) reported pectinases to be the first activated enzymes by phytopathogenic fungi during early stages of penetration. Among the pectinases, polygalacturonase (PG) especially the endo-cleaving forms, play a significant role, while the other enzymes viz., proteases, pectin methyl esterases (PME) and pectate lyases (PL) often work synergistically. The significance of PG for host infection was reported mainly in fungi: Aspergillus flavus, Botrytis cinerea and Alternaria citri (Issiki et al., 2001) and, among bacteria, in Ralstonia solanacearum (Huang and Allen, 2000). These enzymes are exported from the pathogen’s cytoplasm to the host tissue milieu. During this process, they may either encounter inhibitors or protected substrates, or they may cleave structural polymers in the primary cell wall and middle lamella, thus facilitating pathogen penetration and colonization. The importance of pectinases in fungal pathogenesis was demonstrated by the detection of pectinase inhibitors in the cell walls of most plants, which are indicative of an adaptive response
of plants to pectinases produced by pathogens (Favaron, 2001). EndoPGIPs are the major class of pectinase inhibitors so far investigated. They retard the advancement of fungal hyphae, reduce tissue decay, and permit activation of defence responses in the surrounding healthy tissues not yet colonized by pathogens (De Lorenzo et al., 2001). These proteins are soluble glycoprotein molecules with a molecular mass around 40 kDa. They are bound to the extracellular matrix (ECM) of plant cells by ionic interactions (Salvi et al., 1990). They have greater affinity for fungal endoPGs compared with bacterial or endogenous endoPGs (Johnston et al., 1993).

Selective inhibition of pectic enzymes by PGIPs was reported in several experimental systems (Albersheim and Anderson, 1971; Fisher et al., 1973; Fielding, 1981; Hoffman and Turner, 1982; Lafitte et al., 1984; Barmore and Nguyen, 1985; Cervone et al., 1981, 1986, 1987). De Lorenzo et al. (2001) reviewed PGIPs and related proteins identified to date. Hoffman and Turner (1982) purified a 42kDa soluble cell wall protein from pea capable of inhibiting both, endoPG and poly methyl galacturonase (PMG), Ascochyta pisi, the pathogen causing leaf blight. However, the proteinaceous inhibitor isolated from beans inhibited exclusively endoPG, not exoPG. Cell walls containing ionically bound proteins from the chickpea susceptible cultivar PV 24 were more extensively degraded by exoPG and PL forms (PL I and PL II) produced by the wilt pathogen, Fusarium oxysporum f. sp. ciceri, than similar preparations obtained from the resistant cultivar WR 315.

Cell wall bound proteinaceous inhibitors of 91 kDa were isolated from unripened pear fruits and were found to be effective against PGs produced by Aspergillus niger and B. cinerea, but not against PGs from Penicillium expansum. Endogenous plant PGs digest fruit cell wall pectin, while solubilized PGIPs, which
are closely associated with the cell wall components during early stages of ripening, alter the inhibitory efficacy of endogenous PGIPs. Gao and Shain (1995) detected high amounts of a PG inhibitor protein in the bark of Chinese chestnut (*Castanea mollissima* L.), rendering the plant resistant to *Cryphonectria parasitica*. The absence of the protein in American chestnut (*Castanea dentate* L.) seems to render those susceptible to the pathogen. Inhibitors from different bean cultivars are equally able to inhibit endoPGs from different races of *Colletotrichum lindemuthianum* (Anderson and Albersheim, 1972). Essentially isogenic lines of beans resistant to *Colletotrichum lindemuthianum* contain higher levels of PGIPs than the susceptible ones. The inhibitor is bound ionically to hypocotyls cell walls and effectively protects the walls against degradation by PG in vitro (Lafitte *et al.*, 1984). However, the inhibitor does not seem to be involved in race-cultivar specificity, since PGs isolated from race α; β and γ of *Colletotrichum lindemuthianum* and PGIPs isolated from four cultivars of bean showed no differences in PG or PGIP activity, when assayed against one another in vitro, regardless of whether they were isolated from a compatible or an incompatible host/pathogen combination. PGs were detected in culture filtrates of *Cladosporium cucumerinum* and *Colletotrichum lindemuthianum*, while they could not be detected in infected cucumber tissues or bean hypocotyls, from which PGIPs were isolated. PGIPs exist in two different isoforms at a higher level in the pericarp of bean fruits compared with hypocotyl. Both isoforms differ in two and possibly three residues of the deduced Nterminal amino acid sequence for hypocotyl PGIP. Such small alterations in the structure of PGIP may represent a strategy in bean plants for resistance against a variety of pathogens (Pressey, 1996).

Favaron *et al.* (1994) observed close relationship between PGIP activity in vitro and its ability to protect leak tissues from endoPG degradation. More than 20
PGIP isoforms were detected in leak leaves (*Allium porrum* L.), with considerable differences in their inhibitory activity (Favaron, 2001). PGIP purified from raspberry inhibited *Aspergillus niger* PG and two PGs (PGs I and II) from *B. cinerea* (Johnston et al., 1993). Purified apple PGIP showed varying degrees of inhibition against the PGs (PGs I-IV) of *B. cinerea*. The most effective one inhibited PGs I and II, and was ineffective against the fifth PG (PG V) purified from apple fruit infected with *B. cinerea* (Yao et al., 1995). Isolation of PGIPs has also been reported from onion, sweet paprika (*Capsicum annuum* var. *longum*) and beans (Albersheim and Anderson, 1971; Anderson and Albersheim, 1972; Fisher et al., 1973), soybean (Favaron et al., 1994), and several fruit species (Fielding, 1981).

Although several lines of evidence show that the proteinaceous inhibitors may modulate the action of pectic enzymes in host tissues, evidences have also been presented that only small inhibitor amounts are available in the cell wall of host tissues (Hoffman and Turner, 1982, Turner and Hoffman, 1985). The proteinaceous inhibitor in pea leaves inhibits the degradation of PG by *Ascochyta pisi*, but is inactive against degradation of insoluble cell wall fragments *in vitro* (Turner and Hoffman, 1985). Despite the detection of PGIPs in oranges (Barmore and Nguyen, 1985), peach and plums (Fielding, 1981). In *in vitro* it inhibited the PGs of their pathogens (*Diplodia natalensis*, *Glomerella cingulata* and *Monilinia* sp.), the pathogens penetrate and macerate their host tissues *in vivo*. These observations suggest that the inhibitors might not be present in sufficient quantities to prevent infection.
Polygalacturonase (PGs) enzyme inhibition by Polygalacturonase-inhibiting protein (PGIPs) (Inhibition of PGs by PGIP)

Plants possess a polysaccharide-rich cell wall that acts as a barrier to pathogenic fungi (De Lorenzo et al., 2001). Fungal endo polygalacturonases (PGs) are implicated as important enzymes in the early stages of plant pathogenesis (Albersheim and Anderson, 1971), and have been shown to be among the first enzymes to be secreted by fungi growing on plant cell wall material in vitro (English et al., 1971). The action of endo PGs is sometimes a prerequisite for cell wall degradation by other enzymes since it was demonstrated that PGs were necessary before other enzymes such as glycosidases, cellulases, and hemicellulases could degrade cell wall polysaccharides (Karr and Albersheim, 1970). The degradation of the cell wall leads to host tissue maceration, providing nourishment for the invading fungus (Cook et al., 1999).

PGIPs constitute a general mechanism developed by plants to protect against fungal PG attack and to look for new sources of PGIP. Investigations have been undertaken on Capsicum annuum. Evidence was also provided that PGIPs from C. annuum Linn., inhibit fungal Colletotrichum capsici endo-PGs but little.

Plant pathogens produce a number of cell wall-degrading enzymes (Collmer, and Keen, 1986). Polygalacturonase (PG) is the first enzyme secreted by plant fungal pathogens when cultured on isolated cell walls.

PGIP on fungal growth in in-vitro showed a clear effect on C. capsici development. The effects of the PGIP on the pectinolytic enzymes of the fungal species tested, as reported for PGIPs from other species (Cook et al. 1999, Stotz et al. 2000), are somehow responsible for differential PGIP effects on fungal growth is
suggested by the fact that the PGIP effects on growth are only seen when the medium in which these assays were carried out contained polymeric pectinaceous substrates.

The purified PGIP was active since it inhibited PGs from *C. capsici* (Fig.5). The amount of PGIP required to inhibit the PGs from *C. capsici* by 50% was 25 ng (Fig.5), which was calculated to represent 4000 units of PGIP activity mg\(^{-1}\) of PGIP, whereas approximately half this amount (12.5 ng; Fig.5) was sufficient to inhibit the PGs from *C. capsici* by 50%.

PGs of *C. capsici* were used to screen the fractions collected during the purification of PGIP by gel permeation for PGIP1 activity using the agarose diffusion assay in order to identify which peak in the gel permeation elution profile contained the PGIP. A reduction in zone size (well 1 Fig. 5) relative to the activity zone of fungal PGs alone (well 5 and 6 Fig. 5), indicated inhibition of the fungal PGs, and thus presence of the PGIP in the gel permeation elution profile was recorded (fraction 11; Fig. 4).

In *in-vitro* tests revealed that Chilli PGIP showed differential inhibitory activity against *Colletotrichum capsici* polygalacturonase (PG).

**Conclusion**

Based on the results obtained in the present study it is concluded that salicylic acid in the form of foliar spray was found to inhibit the growth of *C. capsici* infecting *C. annuum* by inducing hypersensitive responses. This might be due to the following effects of SA.
• Maximum inhibition of conidial germination and mycelial growth of
  *C. capsici* in-vitro.

• Inhibition of the cell wall degrading enzymes viz., pectinolytic and
cellulolytic produced by *C. capsici* in-vitro.

• Minimum electrolytic leakage in the cells of host plant by SA.

• Higher rate of synthesis of SA by increasing the synthesis of phenyl
alanine ammonia lyase (PAL).

• Inhibition of polygalacturonase (PG) of pathogen by PGIP of host
  plant.

• Induction of SAR by the cumulative action of the above phenomena.

The above observations suggest that SA induces SAR in *C. annuum* by
cauising the host cells to synthesis antioxidant enzymes, phenols, PR-proteins like
ß-1,3-glucanase and PGIP which enable the host plant to resist the spread of
*C. capsici* this in turn showed enhanced production of biochemical constituents such
as chlorophyll pigments, carbohydrate, proteins and nucleic acids in the leaves
leading to better growth and yield of *C. annuum*.

Earlier studies have established the role of SA in inducing hypersensitive
responses (HR) against several pathogens. HR includes the synthesis of lignin and
phenolic compounds and strengthening of the physical barrier (cell wall) and
synthesis of antioxidant enzymes such as SOD, peroxidase, polyphenol oxidase,
PR-protein like ß-1,3-glucanase, PGIP and defense related protein like PAL and
which in turn led to the induction of SAR in the host plant against the pathogen.
The present study brings out that foliar spray of SA at lower concentration (10mM
SA) induces SAR in *Capsicum annuum* by the synthesis of phenols, antioxidant
enzymes (SOD, PPO, PO) PAL and consequent enhancement of various synthetic processes including synthesis of carbohydrate, protein and nitrogenous compounds in the *C. capsici*-infected leaves of *C. annum*. Healthy plants of *C. annum* sprayed with SA showed no adverse physiological and biochemical effects. Rather it showed enhanced values in some parameters.

Hence, it may be concluded that SA at 10mM concentration elicits hypersensitive responses and induces SAR against the invading pathogen *C. capsici* leading to better growth and higher yield in *C. annum*. 
SUMMARY

The present study brings out the Salicylic acid (Elicitor) induced hypersensitive responses in *Capsicum annum* L. infected with *Colletotrichum capsici*. The chemical fungicide bavistin was used for comparision in *in-vitro* condition. The optimum inhibitory concentration (OIC) of the SA was selected based on their efficacy in the control of *C.capsici* sporulation and growth under *in vitro* condition. The OIC of SA was 10mM. This concentration was used for the study of enzymatic and biochemical activity and further *in vivo* experiments.

The chilli seedlings were sprayed in OIC of the elicitor SA (10mM) before *in vivo* studies. *In-vivo* Studies were designed as follows:

**Control:** Control (healthy) Plants of *Capsicum annum* sprayed with distilled water on 45 DAS and left untreated.

**Control (Healthy) plants under treatment:** Healthy plants of *C.annum* (control) sprayed with salicylic acid (10mM) on 52nd DAS

**Infection:** *C.annum* plants inoculated with *C.capsici* on 45 DAS and left untreated.

**Infected plants under treatment:** Inoculated *C.annum* plants sprayed with salicylic acid (10mM) on 52 DAS.

**Harvest for estimation:** 59 DAS

The biochemical and physiological changes in the leaves of *Capsicum annum* were investigated control, healthy, infected and infected treated conditions. Estimations were carried out from the leaf samples collected on 59 DAS.
The following results were obtained in the *in vitro* studies:

- Maximum inhibition of *C. capsici* conidial germination in 10mM SA (84.5%) and *G. virens* 2.5% (76.5%).
- Maximum inhibition of mycelial growth of *C. capsici* in 10mM SA (75%), followed by *G. virens* 2.5% (75.1%) and 0.1% bavistin (64%).
- Maximum inhibition of pectinolytic enzymes produced by *C. capsici* such as pectin methyl esterase (92%), pectin methyl galacturonase (88%) and pectin transeliminase (75%) and cellulolytic enzymes Cx (90%) and C1 (84%) was recorded in the plants sprayed with 10mM SA as compared to other treatments.
- The above concentrations were selected as OIC and used for *in vivo* study.

The following results were obtained in the *in vivo* study:

- The least electrolytic leakage was recorded in the 10mM SA sprayed leaves (0.042mS/cm²) compared to other treatments like control, infection treated and the infected leaves recorded the highest electrolytic leakage (0.242mS/cm²).
- The higher chlorophyll and carotenoid contents were noticed in the SA sprayed leaves (1.97mg/g) compared to other treatments. This was followed by infection and infection treated.
- Higher carbohydrate content was found in the SA sprayed leaves compared to other treatments. The total carbohydrate content was found to be drastically reduced in the infected plant as total sugar by
65%; reducing sugar by 69%; non reducing sugar by 55%; sucrose content by 77%; starch by 69% in the infected leaves.

- Least reduction in the nucleic acid and protein content was recorded in the SA treatment (14%; 12%) as compared with 28% and 25% in other treatments. The nucleic acid and protein content were reduced heavily by 68% and 72% in the C. Capsici-infected leaves.

- Higher NR activity (1048 U), total nitrogen content (2.19 mg/g) was recorded in the SA-sprayed leaves among other treatments. Maximum reduction of 79% in total nitrogen, 58% in amino nitrogen and 46% in the activity of NR enzyme in infected leaves.

- The healthy treated leaves exhibited an increase of about 1.5 times in the phenol and proline content over control plants. This might be due to the induction of systemic resistance in the treated host plant against the infection caused by the pathogen. The O.D. phenol, total phenol and proline content exhibited an increase of about 4-times.

- The healthy treated leaves exhibited significant increase (1.5%) in the antioxidant enzyme metabolism over healthy plants. The C. capsici-infected leaves recorded an increase of about 2.5% in SOD, CAT and 3.5 times in PO and PPO over infected leaves.

- Phenyl alanine ammonium lyase enzyme (PAL) activity was found to increase in the leaves of healthy and infected plants treated with salicylic acid compared to the infected ones. The activity of PAL in the leaves of control, healthy treated, infected and infected treated plants is presented as the infected leaves showed minimum activity (0.0003nmol/mg of protein) compared to that of the control...
(0.0005nmol/mg of protein) leaves. All the treatments showed generally higher activity of PAL. It was followed by the infected ones sprayed with SA (0.0014nmol/mg of protein) and the highest activity was recorded in the leaves of healthy ones sprayed with SA (0.0022nmol/mg of protein).

SA synthesis was higher in the infected leaves than those of control. The SA treatment in healthy plants showed highest value. The infected leaves showed minimum activity (1.0 µg/g) compared to that of the control (2.1 µg/g) plants. Healthy treated and infected treated plants showed generally higher amount of SA followed by the infected ones sprayed with SA (2.3 µg/g) and the highest activity were recorded in the healthy leaves sprayed with SA (3.5 µg/g). The infected leaves showed minimum SA activity which could be attributed to infection of chilli with C. capsici leading to SAR against the pathogen and to an increase in the level of SA in the leaf.

Based on the results obtained in the present study it is concluded that the SA (10mM) in the foliar spray was found to be the best elicitor in controlling C. capsici infecting C. annuum and also in maintaining the growth of seedling under pathogenesis as that of healthy plants. Hence, it may be concluded that SA at 10mM concentration elicits hypersensitive responses and induces SAR against the invading pathogen C. capsici leading to better growth of C. annuum.