5. Discussion

Niger [*Guizotia abyssinica* (L.f.) Cass.] belongs to the tribe Heliantheae in the family Asteraceae and is grown in Ethiopia, India, Pakistan and Nepal. Ethiopia is also the crop's center of distribution and origin (Seegeler, 1983). The crop was introduced to India from Ethiopia during the third millennium BC (Dogget, 1987). It is also cultivated in several other countries such as Sudan, Uganda, Zaire, Tanzania, Malawi and Zimbabwe, Nepal, Bangladesh and Bhutan, USA and West Indies (Weiss, 1983; Murthy, Hiremath & Salimath, 1993; Getinet & Sharma, 1996; Kandel & Porter, 2002). In India it is cultivated in Madhya Pradesh, Andhra Pradesh, Orissa, Maharastra, Bihar, Karnataka, Nagar Haveli and West Bengal. Seed lipids usually contain over 95% neutral storage lipids in the form of triacylglycerol (TAG) (Ohlrogge & Jaworski, 1997). The seed contains about 400g oil kg^{-1} (Getinet and Sharma, 1996) but it varies (27-50%) with seed quality of niger (Seegeler, 1983; Dutta *et al.*, 1994; Getinet & Teklewold, 1995; Dagne & Jonsson, 1997; Marini *et al.*, 2003; Ramadan & Mörsel, 2003; Asilbekova *et al.*, 2005). Niger seed typically contains fatty acids like Linoleic acid(75%), palmitic acid (7-8%), stearic acids (7-8%), and oleic acid (5-8%) (Getinet and Teklewold,1995). The predominant fatty acid in niger oil is linoleic acid (LA), regardless of the differences among reports in terms of its proportions within the range of 54-85% (Weiss, 1983; Getinet & Teklewold, 1995; Dagne & Jonsson, 1997; Marini *et al.*, 2003; Ramadan & Mörsel, 2003). Niger seed oil has a higher proportion of LA and a lower proportion of oleic acid (OA) as compared to that of wild and/or weedy guizotias (Dagne & Jonsson, 1997), sunflower and safflower (Dutta *et al.*, 1994). Generally, LA, OA and the two major saturated fatty acids in niger seed oil are palmitic acid and stearic acid (Dutta *et al.*, 1994; Dagne & Jonsson, 1997; Ramadan & Morsel, 2003). The high content of LA (an essential fatty acid) in niger seed oil is nutritionally highly valuable, as it is known to prevent cardiovascular disorders such as coronary heart diseases, arteriosclerosis and high blood pressure (Vles & Gottelbos, 1989). About 94-96 percent of α-tocopherol is present in oil and is a good source of vitamin E (Dutta *et al.*, 1994; Ramadan & Mörsel, 2004). The relatively lower level of phenolics, polar lipids and high level of tocopherols are responsible for anti-oxidant activity of niger oil (Ramadan & Mörsel, 2004). The press-cake left after oil extraction is

an excellent poultry and livestock feed, as it contains 33-37% protein and is rich in inorganic constituents and crude fibers (Seegeler, 1983; Kandel & Porter, 2002). The whole plant is also used as fodder (Weiss, 1983).

Presently, niger plants are grown from seeds. In sub-Himalayan West Bengal, several pathogens attack niger plants. Some varieties are well known for their quality and production but they are also susceptible towards some pathogens. A main factor limiting the yield and quality of horticultural crops is their susceptibility to diseases (Rizza *et al.*, 2002). Sometimes, pathogen attack results in failure of the crop production. In the present study, several seed varieties, recognized and certified by different agencies have been collected. In addition some varieties, cultivated locally over the years, have also been collected from the farmers.

Although, several fungi have been reported to attack niger plants but not all are of much importance in the present study area (Sub-Himalayan West Bengal). There are reports that two different fungi (*Alternaria sp.* and *Alternaria porri*) were found to produce diseases in both the young and old plants (Yirgu, 1964; Yitbarek, 1992). But the fungus, *Alternaria alternata* was consistently found to be associated with the niger plants in the present study area (Sub-Himalayan West Bengal). Among the isolated pathogens *A. alternata* was found to cause severe damage to the plants in comparison to the others. Hence, *A. alternata* was used throughout the present study. After verification of Koch's postulates, the pathogen was identified from Indian Type culture Collection, IARI, PUSA, New Delhi.

After the identification of the pathogen, a thorough understanding on the morphological and physiological features becomes necessary. It also forms the basis of further studies on understanding disease development, host-pathogen interaction and control of the disease caused by the pathogen. Hence it was considered worthwhile to know about the basic morphological and physiological aspects of the fungal pathogen. Therefore, a through microscopic observation of the morphological characters of mycelia and spores along with studies on growth conditions and nutritional requirements of the pathogen were performed.

Microscopic study of the fungus revealed that mycelia and conidia of the fungus were shiny black to brown coloured. The length and breadth of the conidia of the fungal isolate ranged between 10-40 µm and 6-12 µm respectively. Mature

conidia were several celled with longitudinal and transverse septa. The diameter of the mature hypha were 3-5 µm. Maiti *et al.* (2006), also reported similar results.

Plant pathogens exhibit considerable variation in cultural as well as in pathogenic characters mostly due to genetic recombination during sexual reproduction (Shaner *et. al.*, 1992). Obtaining fungal cultures in artificial media and inoculum production in the form of spore is important for different laboratory studies. Several workers have recognized the importance of spores as inoculum and studies have been conducted on the effects of various media components along with important physiological parameters that lead to maximum sporulation (Kim *et al.*, 2005; Saxena *et al.*, 2001). During our studies with the pathogen, it was found that the fungus sporulates very poorly in media like potato dextrose agar (PDA) and Czapek dox agar (CDA). Excellent sporulation was found in PCA (potato carrot agar). Mycelial growth was best in MEA (Malt extract agar).Three different liquid media (PDB, PCB, and RM) were used to study the growth of the *A. alternata*. From the results it was evident that PDB was best for both growth of *A. alternata* after 25 days.

Our observations also related with that reported by Karlatti and Hiremath (1989), who observed that the best mycelial growth of *Alternaria zinniae* was on leaf extract dextrose agar and potato dextrose agar media. Prasad *et al.* (2008) reported that growth and sporulation of *Alternaria helianthi*, a pathogen causes leaf blight disease in sunflower were maximum in sunflower leaf extract followed by carrot agar medium whereas *A. helianthi* showed less growth and sporulation on potato dextrose agar (Allen *et al.* 1983; Mukewar *et al.* 1995). Subculturing of *A. helianthi* continuously up to 60 days after isolation reduced the germination capacity of conidia and for successful infection, inoculum concentration was suggested to be 1×10^6 spores ml^{-1} and 20-30 days old plants was considered ideal. Older plants (60 days old) failed to show disease symptom. Our study showed that MEA was better than PDA in mycelia growth but sporulation was excellent in PCA. This was also reported by Allen *et al.* 1983, and Mukewar *et al.* 1995 in case of growth and sporulation of *A. helianthi*.

A. alternata was capable of growing at temperatures that range between 10^0-40^0 C. Best growth was recorded at 28^0C while no growth was observed at temperatures 45^0C and above. These results were in agreement to those

reported by Alam *et al.* (2001), who observed that *Lasiodiplodia theobromae* grew and sporulated at 10^0-40^0C, the optimum being 25-30^0C. In another study, Eng *et al.* (2003) reported similar observations when he studied the effect of temperature on growth characteristics of *Botryodiplodia theobromae*. Amborabé *et al.* (2005) observed that *Eutypa lata*, a vineyard pathogen grow in a large temperature range (2-30^0C) but a higher temperature (35^0C) presented inhibitory effects on mycelial growth. Gock *et al.* (2003) studied the influence of temperature, water activity and pH on growth of some xerophilic fungi and observed that the optimum growth occurred at 25^0C for *P. roqueforti* and *W. Sebi*; at 30°C for *Eurotium* species, *A. penicillioides* and *X. Bisporus* and at 37^0C for *C. xerophilum*. Similar results were also obtained by Lin and Sung, 2006 and Winder, 2006).Thompson-Eagle (1989) reported optimum temperature for growth of one *Alternaria alternata* isolate. Similar results were also found by Slavov *et al.* 2004.

A. alternata was able to grow in a wide range of pH, from 5.0 to 8.0. The fungus however, failed to grow in alkaline environment, beyond pH 8.0. The optimum pH for growth was recorded at pH 6.5. The results indicated that slightly acidic to neutral pH was optimum for the growth of the organism. Thompson-Eagle (1989) also reported similar results in case of a *A. alternata* isolate. Similar results were also shown in case of *Bipolaris carbonum* by Saha and Chakraborty (1990). They showed germ tube growth of the fungus was optimum at pH 7.2. Thakare and Patil (1995) observed that the optimum pH for growth of *Colletotrichum gloeosporioides* was 4.1 to 6.8. Kang *et al.* (2003) also observed that optimum growth of *Colletotrichum gloeosporioides* was around pH 6.0. Harden *et al.* (2002) reported that 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 (of *Phytophthora clandestine*) being at 25^0C with a pH of 6.0 to 6.5.

Nutritional requirement of the pathogen was studied and it was concluded that mannitol was the best carbon source for optimum growth and sporulation of *A. alternata*. Next to mannitol good growth was observed in fructose and sucrose respectively. When nitrogen sources were tested, peptone produced best growth, while potassium nitrate showed best growth among the tested inorganic nitrogen sources. Several workers (Yadav *et al.*, 2002; Jash *et al.*, 2003) studied the influence of various carbon and nitrogen sources on fungal metabolism. Yadav *et al.* 2002 observed that highest mycelia growth and sporulation was recorded

when manittol was used as carbon source and peptone was used as nitrogen source. Wu and Wu (2003) observed that *Alternaria protenta,* a pathogen of sunflower showed abundant sporulation on glucose peptone agar and leonine agar but not on dextrose nitrate agar.

Host-pathogen interaction largely depends on the early stages of disease initiation, which includes spore germination, penetration and early colonization. Several studies have been performed during the present work to evaluate the influence of environmental factors like incubation periods, pH, temperature etc. on spore germination, germ tube elongation appressorium formation. In the present study germination and germ tube elongation of the fungus have been studied at different incubation periods and temperatures. Germination of spores started before 2 hours of incubation and within 12 hours cent percent spores germinated. Germ tube elongation started at a rapid rate and very long germ tubes (340.14µm) were found after 12 hours of incubation. Saha and Chakraborty (1990) also observed that spore germination of *Bipolaris carbonum* begun within 2-4 hours of incubation *in vitro*. Similar influence of environmental factors on spore morphology of *A. alternata* was reported by Misaghi *et al.* (1978).

When *A. alternata* were allowed to germinate at different temperatures, only 2.45% germination occurred even after 12 hours of incubation at 10^0C and there was no germination beyond 40^0C. Spore germination was optimum at 28^0C. The prevailing temperature in the areas covered during the present study and also the adjoining areas does not reach above 40^0C. Hence, spores get their optimum temperature throughout the year.

Degree of susceptibility or resistance of a particular variety to a pathogenic fungus is determined through its differential pathogenicity to different varieties. However, pathogenicity of different fungi to a particular plant variety gives us information about different infecting ability of different pathogens. Pathogenicity of the isolated fungi, *A. alternata* was tested following whole plant inoculation technique. From the results it was found that pathogenicity of *A. alternata* clearly showed LV as the most susceptible and NRS-96-1 as the most resistant among the six different niger varieties tested. The results also indicated differential resistance and susceptibility of the other four varieties. Raja and Reddy (2007) showed disease index of some solanaceus plants by conventional pathogenicity test of *Alternaria* spp, a pathogen of brinjal. Dickens and Cook

(1989) used leaf inoculation technique to detect resistance and susceptibility of *Camellia* plants against *Glomerella cingulata.* Brennan *et al.* (2003) reported pathogenicity of five different fungal pathogens viz. *Fusarium areenaceum*, *F. culmorum*, *F. graminearum*, *F. poae* and *Macrodochium nivale* on coleoptile growth rate of wheat seedlings (cv. Falstaff) *in vitro.* The results of the present study are in conformity with that of the studies of earlier workers. Therefore, the identities of different niger varieties allow us to know about their resistance or susceptibility towards foliar fungal pathogens. The knowledge of resistance or susceptibility might be helpful in integrated disease management practice in niger especially in cases of multiple pathogen attack.

Many workers have reported about the presence of some unique antigenic determinants, so called cross reactive antigens (CRA) between pathogen and compatible host. Thus the concept of CRAs between host and pathogen is a notable feature in determining resistance or susceptibility. The degree of susceptibility of plant cultivars to a pathogen is correlated to levels of CRAs present in both the organisms (Bom and Boland, 2000; Kratka *et al.*, 2002; Ghosh and Purkayastha, 2003; Musetti *et al.*, 2005; Eibel *et al.,* 2005; Dasgupta *et al.*, 2005; Babitha *et al.* 2006). In the present investigation antigens of resistant and susceptible varieties and pathogenic isolate of *A. alternata* were cross reacted separately with antisera of resistant and susceptible host. Reciprocal cross reaction was also carried out with antisera of the pathogen and antigens of both resistant and susceptible varieties. One non-pathogen of niger viz. *Gliocladium virens* was also included in the immunological comparison. The basic immunological techniques like radial immunodiffusion, immunoelectrophoresis and agar gel double diffusion were successfully utilized by several workers while demonstrating cross reactive antigens. Some of the techniques have been used in the present study.

In agar gel double diffusion test no antigenic substance was found to be common in between *A. alternata* and resistant niger varieties NRS-69-1 and RCR-18 but susceptible niger varieties (LV, JNC-6, GA-5 and GA-10) shared CRA with the isolated pathogen *A. alternata.* Antigens of non-pathogen *Gliocladium virens* did not show any precipitation band.

Several authors obtained similar results in different host parasite combinations viz. jute and *Colletotrichum corchori* (Bhattacharya and

Purkayastha, 1985), soybean and *Myrothecium roridum* (Ghosh and Purkayastha, 1990) and tea and *Bipolaris carbonum* (Chakraborty and Saha, 1994). In soybean cultivars similar results were obtained by Purkayastha and Banerjee (1990) when they conducted immunodiffusion between antigen of host and antisera of the pathogen causing anthracnose (*Colletotrichum dematium* var. *truncata*). They were able to detect precipitin bands in cross reaction between the pathogen's antisera and the antigen of susceptible host and *vice versa*, which indicated presence of CRA between susceptible host and pathogen combinations only and not between resistant host and pathogen combinations. Dasgupta *et al.* (2005) were also able to detect CRA only between susceptible varieties of tea and the pathogen *Curvularia eragrostidis*. Therefore, the results of the present study are in conformity with those obtained by previous workers.

In immunoelectrophoretic, studies it was found that antigen of susceptible niger varieties (LV, JNC-6, GA-10 and GA-5) shared one precipitin band with antiserum of *A. alternata* (AIA). Antigen of *A. alternata* showed one precipitin band when cross reacted with antiserum of LV (susceptible variety). No precipitin band was found between antiserum of *A. alternata* and antigens of resistant niger varieties (NRS-69-1 and RCR-18).

The results of Immunoelectrophoretic studies confirmed the results of immunodiffusion. The advantage of immunoelectrophoresis over immunodiffusion is that complex antigenic mixtures are separated from each other due to the additional resolving power of the electrophoretic step. Ala-El-Dein and El-Kady (1985) used crossed immunoelectrophoresis techniques to resolve similarities and dissimilarities between the antigens present in *Botrytis cinerea* isolates and between antigens present in different species of *Botrytis*. They observed that each isolate was serologically different from the other and had species-specific antigens. Purkayastha and Banerjee (1990) observed that the antibiotic cloxacillin when used as an elicitor of the host defense altered the antigenic patterns of soybean cultivars such that one specific precipitin band was found to be absent in immunoelectrophoretic studies between antigen of the treated leaves and untreated leaf antisera when compared with homologous reaction between antigen and antisera of untreated control. Our results were in good agreement with that of several earlier workers.

To quantify the common antigens and to make a gradient of common antigenic similarity indirect enzyme-linked immunosorbent assay (indirect ELISA) was performed. On the basis of certain distinct values a clear picture of compatibility and incompatibility among the hosts and pathogens can be ascertained. For detecting CRA at a very low concentration indirect-ELISA is one of the most specific and rapid methods for identifying fungal diseases (Sundaram *et al.*, 1991; Chakraborty and Saha, 1994b; Kratka *et al.*, 2002; Ghosh and Purkayastha, 2003; Musetti *et al.*, 2005). Dasgupta *et al.* (2005) performed ELISA between tea varieties and *Curvularia eragrostidis*, which revealed the presence of a certain minimum level of antigens for compatible host-pathogen interaction. Eibel *et al.* (2005) developed a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) by raising polyclonal antibodies against *Ustilago nuda* and barley plant. Several other workers have used ELISA for early detection of pathogens (Chakraborty *et al.*, 1996; Ghosh and Purkayastha, 2003).

Higher indirect-ELISA values in cross reactions between antigens and antisera of host and pathogen revealed the presence of more CRA, which indicated the susceptibility of the variety. Similarly, lower indirect-ELISA values revealed lower amount of CRA that indicated resistance. The results obtained by indirect ELISA indicated that the degree susceptibility and resistance was in conformity with the results of pathogenicity tests. In indirect ELISA antigens of *A. alternata* when cross reacted with antiserum of LV (susceptible variety) higher absorbance values were observed but the same antigen when cross reacted with antiserum of NRS-69-1(resistant variety) showed lower absorbance values. In reciprocal cross reactions also higher ELISA values were experienced when antigen LV (susceptible variety) was cross reacted with antisera of *A. alternata* but lower ELISA values was found when antigen of NRS-69-1(resistant variety) cross reacted with antiseara of the pathogen.

CRA was also detected in other host pathogen combinations like *Phytophthora fragariae* and strawberries (Mohan, 1988) and *Phytophthora infestans* and potato (Alba and DeVay, 1985) by indirect-ELISA. Purkayastha and Banerjee (1990) detected cross reactive antigens at a very low concentration using indirect-ELISA technique between susceptible soybean cultivars and the virulent strain of *C. dematium* var. *truncata.* Dasgupta *et al.* (2005) also detected CRA while studying the pathogenicity of *Curvularia eragrostidis* against tea

varieties by analyzing the antigenic patterns of host and pathogen. They used indirect ELISA which revealed the presence of low level of common antigens between all combinations. They observed that a certain level of CRAs was present in compatible host-pathogen interactions than in incompatible interactions.

In the present study, CRA could not be detected between resistant host and pathogen by immunodiffusion and immunoelectrophoresis. But indirect-ELISA showed presence of common antigens in cross reactions between antiserum of the pathogen and antigens prepared from six different niger varieties, both susceptible and resistant. The levels of common antigens between resistant varieties and the pathogen were, however, significantly lower. Indirect-ELISA values, in cross reactions, revealed a direct correlation with results of pathogenicity test and established the degree of susceptibility or resistance of a particular variety. Thus ELISA may be used to determine the pathogenicity of a strain in different cultivars accurately. As the gradient of similarity or disparity of CRA is the indicator of susceptibility and resistance respectively, it would help in selecting resistant varieties for cultivation and contribute towards long term disease control.

Immunocytolocalization techniques are powerful tools to detect and locate specific CRA with great accuracy by utilizing antisera as probe. In the present work 'Immunogold labelling-silver enhancement' technique have been used in susceptible and resistant niger varieties for determining cellular location of CRA in leaf and stem sections. Polyclonal antibodies (raised against the pathogen and the susceptible and resistant host variety) were used as antisera probes. For visualization, these were indirectly labelled with colloidal gold and subsequently enhanced with metallic silver. CRAs were located in the leaf sections of niger and mycelial cells of the fungal pathogens. Although, several authors have used immunofluorescence technique for cellular location of CRA (Chakraborty and Saha, 1994b; Wakeham and White, 1996; Chakraborty *et al.*, 1997; Kratka *et al.*, 2002; Dasgupta *et al.*, 2005) but some others (Kuo, 1999; Lee *et al.,* 2000; Trillus *et al.*, 2000; Nahalkova *et al.*, 2001; Kang and Buchenauer, 2002; Wang *et al.*, 2003) have used immunogold labelling for such studies. Most authors have used electron microscopy to observe immunogold labelled sections for studying cellular locations. But, in the present study, silver enhancement was done with immunogold sections specifically to study in the light microscope (as suggested

in manufacturer's kit Sigma, USA) which is comparatively a new approach for studying cellular location of CRA.

When leaf section of susceptible variety (LV) was treated with antisera of *A. alternnata* and labelled with immunogold particles followed by silver enhancement, CRA was observed mainly in the epidermal regions as strong precipitations. Mesophyll tissues and vascular bundle elements also showed marginal darkening which indicate presence of CRA in those areas also. When leaf section of resistant variety (NRS-69-1) was treated with the antisera of pathogen, no such strong precipitations were observed. Maximum precipitation of silver upon immunogold labels were found in leaf sections treated with homologous antisera.

Now-a-days, environment-friendly disease control measures are being given more importance than chemical fungicides. Disease management is possible if basic knowledge on pathogenesis of disease agents in the host is known. In the present study the level of different defense related enzymes were studied following their induction by different inducers. Subsequently, isozyme analyses of some of the enzymes were also performed.

Studies on defense related enzymes were performed for proper understanding of the mechanism of defense in niger plants. Some abiotic inducers [2-amino butyric acid(2-ABA), 2,1,3-Benzothiodiazole (BTH), 2,3-Dihydroxybenzoic acid (DHB) and Salicylic acid (SA)] and plant extracts (*Acalypha indica* and *Catharanthus roseus*) were used for the treatment of the niger seedlings. Five different defense related enzymes [phenylalanine ammonia-lyase(PAL), polyphenol oxidase(PPO), chitinase, β-1,3-glucanase and peroxidase(PO)] were studied. The induction and accumulation of Pathogenesis related (PR) proteins against pathogen attack and chemical treatments are well documented (Van Loon and Kammen, 1970; Van Loon and Van Strien, 1999). Tea plants of several susceptible varieties have been induced by abiotic inducers including salicylic acid, a very common abiotic inducer of PR proteins (Chakraborty *et al.*, 2005). Major interest have been devoted to plant hydrolases such as β-1,3-glucanases (PR-2) and chitinases (PR-3), as they are capable of cleaving fungal cell walls resulting in pathogen's growth inhibition (Wessels and Sietsma, 1981; Mauch *et al.*, 1988; Neuhans, 1999; Arlorio *et al.*, 1992; Bishop *et al.*, 2000). Several authors (Paul and Sharma, 2002; Ghosh and Purkayastha,

2003; Saha *et al.*, 2007) have used phyto-extracts for the induction of PR-proteins in a variety of crops. Ghosh and Purkayastha (2003) tested 12 elicitors for inducing systemic protection against rhizome rot of ginger caused by *Pythium aphanidermatum*. They reported that leaf extract of *Acalypha indica* showed maximum reduction in disease with associated increase of defense-related proteins. Meena e*t al.* (2001) applied SA at the concentration of 1mM as foliar spray and observed significant increase of the activity of PAL, chitinase, β-1,3-glucanase, PO, and PPO.

Differential expressions of PAL in pre-treated and challenge-inoculated niger plants were studied. Six different inducers were used to treat plants. From the results it was found that when plants treated with *A. indica* leaf extract and 10^{-2} M BTH were challenge inoculated, PAL activity was increased to a very high level. Level of the enzyme was found to be maximum after 4 days following it declined. SA, DHB and *C. roseus* treated plants (both uninoculated and inoculated) showed a significantly high level of PAL activity after 4 days. Similar results were observed by several authors. Cao *et al.* (2006) reported enhanced accumulation of PAL when SA was applied to the trees of *Pyrus bretschneideri* around 30 days after full flowering. Akinwunmi and John (2001) reported transient increase of PAL in cowpea after pre-treatment with BTH following challenge-inoculation by *Colletotrichum destructivum*. Trotel-Aziz *et al.* (2006) reported that grapevine leaves when treated with chitosan led to marked induction of PAL and reduction of *Botrytis cinerea* infection. Qian *et al.* (2005) reported the use of novel synthesized pentafluoropropyle jasmonate (PFPJA) to induce plant defense response, leading to an oxidative burst and activation of PAL. Basha and Chatterjee (2007) used two non-conventional chemicals like zinc sulphate and oxalic acid for the induction of PAL in wheat against *Sclerotinia sclerotiorum.* Paul and Sharma (2002) reported higher PAL activity in barley plants pre-treated with aqueous leaf extract of *Azadirachta indica* and challenge-inoculated by *Drechslera graminea.* Thus, our observation of a higher PAL level after treatment by abiotic and biotic inducers is in conformity with that of earlier workers.

Enzyme PPO is involved in the synthesis of defense chemical like tannin which is toxic to pathogenic microorganisms (Mahadevan and Sridhar, 1996; Chen *et al.*, 2000). In the present work, results revealsd that niger plants pre-treated with leaf extract of *C. roseus* or DHB and inoculated by *A. alternata* showed higher level of PPO in comparison to treated-uninoculated and

untreated-uninoculated controls. Plants pre-treated with other inducers showed increase in enzyme activity but it was much less in comparison to *C. roseus* and DHB induced plants. Meena *et al.* (2001) reported increase of PPO activity in groundnut after pre-treatment with salicylic acid against late leaf spot caused by *Cercosporium personatum*. Thus our results are comparable to that of Meena *et al.* (2001). Baysal *et al.* (2002) reported an effect of induced resistance in the ornamental *Cotoneaster salicifolius* root stock M26, against fire blight caused by *Erwinia amylovara* by using plant extract of *Hedera helix*. Hence, our results are in agreement with several previous workers.

Isozyme analysis showed the expression of three different types of PPO isozyme with R_f values of 0.75, 0.78 and 0.80. Among three PPO isozymes, two isoforms of R_f 0.75 and 0.80 were expressed constitutively in all treatments except control. But plants pre-treated with *C. roseus* aqueous leaf extract and inoculated (with *A. alternata*) showed one additional isozyme whose R_f was 0.78. Several workers have reported similar results in different plants. A unique PPO isozyme was found in tomato after pre-treatment with *Pseudomonas fluorescens* isolate Pf1 against *Fusarium* wilt (Ramamoorthy *et al.*, 2002). Zheng *et al.* (2005) reported PPO isozyme in pepper plants, pre-treated with mycorrhizal fungus of *Glomus intraradices* and challenge-inoculated by *Phytophthora capsici*. Several other workers also indicated that PPO isozymes were induced by various inducer treatments in cucumber and tobacco (Piyada *et al.*, 1995; Ray *et al.*, 1998; Chen *et al.*, 2000).

The defense related enzyme, β-1-3-glucanase is an important plant enzyme having capability of hydrolysing β-glucan. Fungal cell wall is made up of chitin polymers which contains β-glucan in the matrix (Sivam and Chet, 1989). In the present study, the levels of enzyme β-1-3-glucanase were estimated in niger plants after induction by different elicitors. From the results it was evident that SA pre-treated plants showed highest level of β-1-3-glucanase activity in niger plants. Next to SA, plants pre-treated with *C. roseus* aqueous leaf extract and inoculated with *A. alternata* showed high β-1-3-glucanase activity. Bargabus *et al.* (2002) reported increased activity of β-1,3-glucanase and reduction of *Cercospora* leaf spot of sugar beet after pre-treatment of acibenzolar-S-methyl following challenge-inoculation of *Cercospora beticola*. Emmanuel *et al.* (2001) reported rapid induction of defense resistance in susceptible lettuce plants after treatments of DL-β-amino butyric acid (BABA) and Phytoguard against downy

mildew. Paul and Sharma (2002) reported *Azadirachta indica* leaf extract to induce defense in barley. Ghosh and Purkayastha (2003) used six different plant extracts viz. *Catharanthus roseus*, *Acalypha indica*, *Spinacea oleracea*, *Andrographis paniculata*, *Centella asiatica* and *Curcuma longa* for systemic protection against rhizome rot of ginger and found higher systemic protection by using plant extract of *Acalypha indica* in ginger.

Isozyme patterns revealed the expression of two different β-1,3-glucanase isozyme bands with R_f values of 0.08 and 0.13. The band (of R_f 0.13) was found highly induced when treated with *C. roseus* and inoculated with *A. alternata.* In case of untreated-inoculated plants two bands were present but intensity of the bands were very low. Bargabus *et al.* (2002) showed two unique isoforms in sugar beet after pre-treatment with a non-pathogenic microorganism, *Bacillus mycoides,* following challenge-inoculation by *Cercospora beticola.* He also reported that one isoform was available when sugar beet was pre-treated by acibezolar-S-methyl and challenge-inoculated. Lawrence *et al.* (1996) reported two isozymes of β-1,3-glucanase (33 and 35kDa) in all genotypes of tomato.

Chitinase belongs to the category of PR protein which plays a distinct role in plant defense by degrading chitin, a β-1,4-linked polymer of N- acetyl D-glucosamine, a major fungal cell wall component (Lawrence, *et al.*, 1996). In the present study results showed that *C. roseus* leaf extract treated and pathogen inoculated plants showed maximum increase in chitinase activity. Next to *C. roseus* leaf extract BTH showed good induction of the enzyme. Challenge inoculated plants showed higher activity than uninoculated plants. 2-ABA also induced chitinase activity. Our results are in agreement to that of Kozlowski *et al.* (1999), who reported increased activity of chitinase after pre-treatment by methyl jasmonate (MeJA) in *Picea abies* seedlings against *Pythium ultimum.* Kagale *et al.* (2004) induced systemic resistance in rice plants following application of leaf extract of *Datura metel* and challenge-inoculation with *Rhizoctonia solani.* They showed increased accumulation of pathogenesis-related proteins (PRs) including chitinase and other defense related compounds in the pre-treated and inoculated rice plants.

Chitinase isozymes were separated in PAGE gels. The separated isozymes on gels were observed with differential fluorescent intensity developed with treatment of glycol chitin and fluorescent brightner. Alternatively the

isozymes were stained and shown as dark black bands. One chitinase band (R_f 0.62 was visible in both the techniques but intensity of the bands were different in different treatments. *C. roseus* treated and challenge inoculated plants showed maximum activity. From the study it can be concluded that *C. roseus* leaf extract is a potent inducer of defense response in niger plants. Chitinase have been reported to express as multiple isozymes in several plants. Three classes of plant chitinases have been reported based upon primary protein structure (Shinshi *et al.*, 1990). Some of the chitinases have antifungal activity. Chitinase was isolated from tobacco (Sela-Buurlage *et al.*, 1993) and tomato (Lawrence *et al.*, 1996) and have been found to be specific for certain pathogens.

Peroxidase is a well known defense enzyme in plants which is changed under various environmental stresses such as heavy metals, salts, temperature (Kiwan and Lee, 2003) and air pollution (Lee e*t al.*, 2000). It is related with the defense reaction in plants that lead to the detoxification of the reactive oxygen species (Higa e*t al.*, 2001). Results of the present study revealed that among abiotic inducers, 2-ABA pre-treated niger plants showed highest level of peroxidase activity. SA and BTH treated plants also showed increased enzyme activity. *Acalypha indica* leaf extract treated plants showed maximum increase in peroxidase activity. *C. roseus* leaf extract treated plants showed less peroxidase activity than that of *A. indica* leaf extract treated plants. Our results agree with those of some previous workers. Stadnik and Buchenaur (2000) reported higher activity of peroxidase in wheat to *Blumeria graminis* after pre-treatment of BTH. Prachi *et al.* (2002) reported that exogenous application of SA resulted in increased activity of peroxidase in the callus culture of *Zingiber officinale*. Shi *et al.* (2007) reported that the compound osthol (a natural compound extracted from dried fruit of *Cnidii* in *monnieri*), induced pumpkin plants for accumulation of peroxidase and PAL against powdery mildew caused by *Sphaerotheca fuliginea*. Baysal *et al.* (2002) reported plant extract of *Hedera helix* to induce peroxidase in *Cotoneaster salicifolius* root stock M26 against fire blight.

Studies on peroxidase isoform patterns in susceptible niger plants were performed. Niger plants pre-treated with 2-ABA and inoculated with the pathogen showed prominent peroxidase isoforms with three bands of R_f 0.70, 0.72 and 0.75. The expression of the bands were less intense in control (untreated-uninoculated) and treated-uninoculated plants. The peroxidase isozymes induced by pathogen infection appeared to be different from 2-ABA induced-inoculated

plants. The results indicated the possibility of induction of peroxidase isozymes in susceptible niger plants, which in turn, shows resistance to the pathogen. Several authors reported multiple forms of peroxidase isozymes in many higher plants including Korean radish, *Arabidopsis* and rice (Lee and Kim, 1994; Lee *et al.*, 1994; Tognolli *et al.*, 2000; Lee *et al.*, 2001).

Considering the emergence of several alternative ways benign to environment, biological control and control by botanicals were tested against *A. alternata*. Among fungal antagonists, *Trichoderma* spp. are most commonly used, mainly due to their high efficacy in controlling several several diseases. Several authors have reported the successful use of different isolates of Trichoderma for controlling many plant diseases (Maity and Sen,1985; Latunda Dada,1993; Prasad *et al.* ,1999; Biswas, 1999; Jadeja, 2003; Saravanan *et al.*, 2003; Roberts *et al.*,2005). *Bacillus* spp also have been used by several workers for control of plant pathogens. Meena *et al.*(2000) controlled *Phomopsis vexans* by using *Bacillus* sp. There are also reports of use of *Aspergillus* spp to control phytopathogens (Shanmugam and Sukunara Verma, 1999).

Although biological control of many pathogens is reported in literature, no such work has been done to control blight of niger caused by *A. alternata*. In the present study, four *Trichoderma*, one *Aspergillus* and two *Bacillus subtilis* isolates were used for their efficacy against *Alternaria alternata*. From the results it was evident that *Aspergillus flavus* produced maximum inhibition of growth of the pathogen whereas *Bacillus* showed minimum among the seven biocontrol agents tested. Cent percent inhibition of growth of the pathogen was observed when crude culture filtrate of all the *Trichoderma* and *Aspergillus* species were tested in culture filtrate supplemented PDA plates. Additionally, niger plants were challenge inoculated after spraying of culture filtrates on them and disease index was computed. Results revealed a significant control of the disease was done by culture filtrates of all the *Trichoderma* spp and *Aspergillus flavus*. Best control was shown by the culture filtrate of *A. flavus*. Cell free culture filtrates have been used to demonstrate antibiosis, a mechanism of biological control (Khara and Hardwan,1990; Tu, 1992;). Shanmugam and Sukunara Verma, (1999) clearly demonstrated the efficacy of the antagonists *Aspergillus niger, A. fumigates, A.flavus and Trichoderma viride* in inhibiting the rhizome rot pathogen. *Trichoderma viride* was reported to control *C. gleosporioides*, a pathogen of

French bean (Gupta *et al.*2005) and *Sclerotium rolfsi*, a pathogen of brinjal (Jadon *et al.*2005). Thus our results are in agreement with that of earlier workers.

Biological control is essentially a natural phenomenon that safeguards the plant kingdom from diseases. But in cultivations where higher production is all that matters, diseases are often catastrophic and require intense management planning to control them. Leaf diseases are controlled easily by spraying exogenous fungicides but due to awareness of harmful effects of fungicides on environment and human being, it is essential to use eco-friendly measures. In the present study the culture filtrates of *A. flavus* and of the *Trichoderma* spp was used *in vivo* and the results were encouraging like the previous workers (Elad, 1995, 2000; Porello *et al*, 2003, 2006).

Twenty different plant extracts (both aqueous and ethanol) were tested against *A. alternata.* Among the plant extracts *Allium sativum* bulb extract completely inhibited spore germination of *A. alternata*. Leaf extracts of five plants (*Datura stramonium, Hibiscus rosa-sinensis, Plumeria rubra,* and *Xanthium strumarium*) significantly inhibited (above 90%) spore germination of the pathogen. Ethanol and aqueous leaf extract of three plants (*Datura stramonium, Plumeria rubra, Borreria alata and Xanthium strumarium*) were also tested for their efficacy by poisoned food technique. *Plumeria rubra* and *Xanthium strumarium* aqueous leaf extract showed more than 80% inhibition of growth of the fungus in comparison to control. Finally, three plant extracts were sprayed on the susceptible niger plants and then the plants were challenge inoculated. From the results it was evident that the plant extracts significantly controlled the disease in niger plants. Several authors have used plant extracts with antifungal activity to control plant diseases (Singh *et al.* 1995; Bhandary *et al.* 2000; Deena and Thopil, 2000; Ali *et al.* 2001; Chauksey and Srivastava,2001; Digrak *et al.* 1999; Mittal *et al.* 2002; Sharma *et al.* 2002; Saxena *et al.,* 2003; Al-Howiriny *et al.* 2005, Saha *et al.* 2005a,b).

A.sativum has been reported to possess antifungal activity by several workers(Jadeja, 2003; Curtis *et al.*, 2004 and Saha *et al.*, 2005a). The activity of *A.sativum* has been attributed to several compounds like allicin, E-ajoene, Z-ajoene, allin, allitridine etc. (Ankri and Mirelman,1999; Yoshida *et al.* 1999a,b; Miron *et al.*,2002; Liu *et al.*,2004; Hughes *et al.* 2005 and Baghalian *et al.*, 2006).

Both the ethanol and aqueous extracts of *Datura metel* and *A. sativum* were used by Saha *et al.* (2005a) to control some pathogens of tea. Kagale *et al.* (2004) showed that leaf extracts of *Datura metel* significantly reduced the growth of *Rhizoctonia solani* and *Xanthomonas oryzae*. Thus, *it can be concluded* that our findings are in good agreement with that of some earlier workers.

In the present study an approach was made towards environment friendly management of *Alternaria* blight disease in niger through induction of plant defense enzymes using various inducers (abiotic and phyto-extracts). Degree of susceptibility or resistance of a particular variety to a pathogenic fungus is determined through its pathogenicity. Pathogenicity is determined, ordinarily, by studying the level of disease incidence. Disease incidence was assessed and compared in the differentially induced susceptible variety, LV. Four different abiotic inducers (2-ABA, 2,1,3 benzothiodiazole, 2,3 dihydroxybenzoic acid and Salicylic acid) and two leaf extracts of (*Acalypha indica* and *Catharanthus roseus*) were used for induction of resistance in the susceptible variety LV. Assessment of disease was performed from 2nd day up to 6th day at 2-days intervals. All the six inducers effectively reduced disease incidence (mean foliar disease index/plant) in tested niger plants as evidenced by the results. Several scientists have reported similar results in different plants. Cao *et al.* (2006) reported that the Ya Li pear trees pre-treated with SA induced the activities of various defense related enzymes (PAL, β-1,3-glucanase, chitinase and PO) in addition to reduction of disease incident and lesion diameter. Premkumar (1998) has reported systemic action of triazole compound in clonal tea plants (TES-34) which are highly susceptible to *Exobasidium vexans* causing blister blight in tea. Thus our results are in conformity with that of earlier workers. The results were encouraging since several inducers showed significant resistance inducing capacity. Further these may be integrated with other biocontrol agents and may be used in fields as part of integrated disease management system.

All the investigations presented here have confirmed and also extended some of the findings of the earlier workers. During this study, certain new facts of fundamental importance have also been revealed. Pathogenicity of *A. alternata* has been tested in several niger plant varieties in different ways. The significance of antigenic relationship with regard to compatible interaction between *A. alternata* and niger varieties has been demonstrated by various serological

techniques. Correlation between pathogenicity test and different serological experiments was observed and was confirmed with indirect ELISA. Major cross-reactive antigens between the niger plants and the pathogen were detected in niger leaves and mycelia of *A. alternata* through a study by immunogold labelling followed by silver enhancement using light microscope. Resistance was induced in susceptible LV variety using some chemicals and plant extracts. Hence, this study has provided an insight to formulate a definite defense inducer against *Alternaria* blight disease in niger caused by *A. alternata.* The present study would help to design suitable control measures of *Alternaria* blight disease in niger using resistance inducers of different natures: abiotic and botanicals. In addition some plant extracts and biocontrol agents also could control the pathogen both *in vitro* and *in vivo*. The results of *in vivo* studies would definitely help in designing some bioformulations and applicable phytoextracts for control of the blight disease in niger caused by *A. alternata*.