STUDIES ON THE RESISTANCE OF NIGER [*Guizotia abyssinica* (L.f.) Cass.] AGAINST *Alternaria alternata* CAUSING LEAF BLIGHT AND CONTROL OF THE DISEASE USING BOTANICALS AND ANTAGONISTS

THESIS SUBMITED FOR THE DEGREE OF DOCTOR OF PHILOSOPPHY (SCIENCE) OF THE UNIVERSITY OF NORTH BENGAL

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TO WHOM IT MAY CONCERN

This is to certify that Mrs. Chandrani Choudhuri, M.Sc. has worked under my supervision at the Department of Botany, University of North Bengal, for Ph.D. thesis entitled "**Studies on the resistance of niger [*Guizotia abyssinica* (L.f.) Cass.] against *Alternaria alternata* causing leaf blight and control of the disease using botanicals and antagonists.**" I am forwarding her thesis for the Ph.D. degree (Science) of the University of North Bengal. I recommend that she has fulfilled all requirements according to the rules of the University of North Bengal regarding the works embodied in her thesis.

Aniruddha Saha

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Contents

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Chandrani Choudhuri

(Chandrani Choudhuri)

1. Introduction

Guizotia abyssinica commonly known as 'Noug' in Amharic, 'Ramtil' in Hindi and 'niger' in English is cultivated as an oil seed crop (Plate 1.1). India is one of the important niger producing countries in the world. The seeds of niger contain clear, excellent, vegetable edible oil. Niger oil is slow drying and it contributes about 3% of Indian's total oil seed production (Getinet and Sharma, 1996). In India, niger oil is frequently used as a substitute for sesame oil (Weiss, 1983). Niger oil is sweetish in taste, bluish-white in colour and has a faint pleasant odor. It is used for culinary purpose, for anointing the body, as an illuminant and for the preparation of soaps, paints, lubricant and perfumes (Panday and dhakal, 2004; Dutta *et al.*, 1994; Kandel and Potter, 2002). Niger seed oil can also be used as biodiesel through *trans* esterification of its long chain fatty acids with methanol that can partially substitute diesel oil and perform better with lower emission levels (Devi *et al.*, 2006).The seeds are used as bird seed in USA and Europe which earn foreign currency (Kandel and Potter, 2002).

The seeds contain 17-20% protein (Abebe *et al.*, 1978; Kandel & Porter, 2002) that offers an important source of protein and significantly contributes to the human dietary protein intake. It also contains 34-40% carbohydrate and 13.5% fiber and is an important source of thiamine, riboflavin, and niacin (Kandel & Porter, 2002). Niger oil is also used as substitute for olive oil. Whole plants are used as green manure in pre-flowering stage. Oil of the seed is used in rheumatism and asterosclerosis. Niger can grow on a wide range of soils, but it appears to thrive best on clayey loams or sandy clays (weiss, 1983). Average yield of recommended Indian cultivars was recorded by Getinet and Sharma(1996) as 467 kg ha^{-1} which is much higher than that of Purseglove (1979) 398-448^{-1} kg ha. Niger plants grow well in optimum annual precipitation of 6.6 to 17.9 dm, annual temperature of 13.6 to 27.5^{0}C and at pH range of 5.5to 7.5. It can grow on water logged, marginal and poor soils where most other crops fail to grow, as it is able to withstand salinity and low oxygen levels (Abebe *et al.*, 1978). Niger seeds are sown in tropical areas during September to mid-November as a winter season crop. It is a short day, self-sterile plant and requires bee for cross-pollination (Pandey and Dhakal,2004).

Crude oil and their fractions were investigated for their radical scavenging activity (SAR) toward the stable galvinoxyl radical by electron spin resonance (ESR) and by spectrometric method and showed its oxidative stability (Ramadan and Moersel, 2003). A Niger based agar medium can be used to distinguish *Cryoptococcous neoformans* (sant) Vaill, a fungus that causes serious brain aliment, from other fungus (Paliwal and Randhawa 1978). There are reports that Niger oil is used for the treatment of syphilis (Belayneh, 1991).

Due to its wide adaptability niger is often grown over vast region under vatient soil and climatic conditions which make it susceptible to attack by various fungal pathogens. Some of the diseases like Leaf spot by *Cercospora guizoticola* (Yirgu, 1964), Stem and leaf blight by *Alternaria* sp., Seed rot by *Rhizoctonia bataticola* (Yitbarek, 1992) etc. have been recorded but leaf blight of niger caused by *Alternaria* sp. is the most serious disease of niger (Gebre-Medhin & Mulatu, 1992; Getinet & Sharma, 1996). The pathogen attack the leaves of niger plants. The symptoms appear as dark necrotic spots on the leaves later leaves of whole plant is blighted and become dark brown in colour.

Higher plants have the ability to initiate various defense reactions such as the production of phytoalexines, antimicrobial proteins, reactive oxygen species etc. when they are infected by pathogens. If the defense reaction occur too late or are suppressed, the infection process proceed successfully (Somssich and Hahlbrock, 1998). Management of disease is possible by inducing plant defense response by exogenous application of certain biotic and abiotic inducers in order to provide protection against pathogens. Endogenous defensive system of a plant can be enhanced through Immunization. Plant Immunization is the process of activating natural defense system present in plants induced by biotic and abiotic factors. Plants pre-treated with inducing agents stimulate plant defense responses that form chemical or physical barriers that are used against the pathogen invasion. Inducers used usually give the signals to raise the plant defense genes which in turn resulting to induced systemic resistance. In many plant-pathogen interactions, resistance-avirulence gene interactions results in localized acquired resistance or hypersensitive response and at distal end of plant, a broad-spectrum resistance is induced known as systemic acquired resistance (SAR) (Kothari & Patel 2004). During the last decade extensive research work has been performed for the

establishment of SAR by the application of a variety of biotic and abiotic inducers (Meena *et al.,* 2001; Ryals *et al.,*1996). SAR is a broad-spectrum resistance that can be induced in plants following a localized infection with a necrotizing pathogen or treatment with elicitors (Mauch-Mani and Metraux, 1998; Sticher *et al.,* 1997). Salicylic acid (SA) is an endogenous signal for the development of SAR and it is transported by phloem from the sites of its origin. Leaves inoculated with pathogen exhibits high level of endogenous SA (Malamy *et al.,* 1990). Foliar application of SA at the concentration of 1mM significantly increased the activity of several defense-related enzymes like phenylalanine ammonia-lyase (PAL), chitinase, β -1,3 glucanase, peroxidase, polyphenol oxidase and phenolic content in groundnut (Meena *et al.,* 2001).

Many biotic and abiotic inducers have been used for the establishment of SAR in different plants by several workers. Among the abiotic inducers, Meena *et al.* (2001) used salicylic acid in groundnut, O'Donnell *et al.* (1996) used ethylene in tomato, Smith-Beaker *et al.* (1998) used SA and 4–hydroxybenzoic acid in cucumber, Cohen *et al.* (1993) used jasmonic acid and methyl jasmonate in potato and tomato, Brederode *et al.* (1991) used UV-light in tobacco, Ernst *et al.* (1992) used ozone in tobacco, Klessig *et al.* (2000) used nitric oxide and Kaku *et al.* (1997) applied N-acetylchitooligosaccharide in barley.

Similarly, some biotic inducers have also been used to enhance in plant defense reaction. Some of them are leaf extract of *Azadirachta indica* in barley (Paul and Sharma, 2002), *Acalypha indica* in ginger (Ghosh and Purkayastha, 2003), *Allium sativum* bulb extract against many fungi (Singh *et al.*2001), plant growth promoting rhizobacteria (PGPR) in cucumber (Chen *et al.,* 2000; Liu *et al.,* 1995; Wei *et al.,* 1991*), Pseudomonas fluorescens* strain CHAO in tobacco (Maurhofer *et al.,* 1994*), Pseudomonas syringae* in cucumber (Rasmussen *et al.,* 1991*), Pyricularia oryzae* and *Bipolaria sorokiniane* in rice (Manandhar *et al.,* 1999). It has also been reported that 3 potato associated ecto- and endophytically living bacterial strains *Psuedomonas fluorescens* B1 & B2 and *Serratia plymuthica* B4 can effectively control *Rhizoctonia solani* in potato and lettuce (Groseh *et al.* 2005). *Bacillus* species as a group offer several advantages over other bacteria for protection against root pathogens because of their ability to form endospores, and because of the broad-spectrum activity of their antibiotics. The strain *B. subtilis* CE1 have been reported to

be a potential biological control agent against *Fusarium verticillioides* at the root level (Cavaglieri *et. al.* 2005).

Plant growth promoting rhizobacteria (PGPR) can suppress the disease caused by foliar pathogen by triggering plant-mediated resistance mechanism called induced systemic resistance, so called ISR (Dube, 2001). Systemic resistance induced by rhizobacteria differs mechanically from SAR and it is designated by a separate term ISR proposed by Kloepper *et al.* (1992). SAR is dependent on the synthesis of SA by the plant that acts as an inducer signal and is associated with the accumulation of novel pathogenesis–related (PR) proteins, POX (peroxidase), PR-1, PR-2 (β -1,3 glucanase), PR-3 (chitinase), PR-4 and PR-5 etc. (Van Loon, 1999).

Among PR-proteins, two plant hydrolases, β-1,3 glucanase and chitinase are given special importance by the workers because many pathogenic fungi contain β-1,3 glucans and chitin as major structural cell wall component (Wessels and Sietsma, 1981). Several authors (Bishop *et al.*, 2000; Arlorio *et al.*, 1992; Mauch *et al.*, 1988) have demonstrated the activity of β-1,3 glucanase and chitinase to degrade fungal wall components *in vitro*, resulting in growth inhibition of fungi.

Search for effective biocontrol agents for the management of plant diseases have been intensified in recent years to reduce the dependence on ecologically hazardous chemicals (Sharma *et al.*, 2001). Out of 2,50,000 known higher plant species which exist on earth, only relatively few have been thoroughly studied for their therapeutic potential (Deans and Svoboda, 1990). Currently, Secondary metabolites of plants are being tapped for use as pesticides. Numerous defensive chemicals, such as terpenoids, alkaloids, phenols, tannins are very effective in the control of phytopathogenic fungi. Even though many antifungal and antibacterial compounds are reported in literature, plant products have not been used to any significant extent for the control of diseases. In India no such product has been registered till 2002 for the control of phytopathogenic fungi (Narasimhan and Masilamani, 2002). Although several inducer chemicals, antagonistic microorganisms and botanicals are known from different plants through literature but no such information is available for controlling the *Alternaria* leaf blight of niger caused by *Alternaria alternata.* Hence, the present work has been undertaken to find out an environment-friendly alternative strategy for controlling alternaria-leaf blight disease of niger.

A host and a pathogen have been reported to share common antigens, which play an important role in determining compatible/incompatible interactions. Thus, absence of the common antigen leads to incompatible interaction and presence of common antigens lead to compatible interaction. Even within the compatible interactions the degree of compatibility might be determined by the sharing of the common antigens (Dasgupta *et al.*, 2005). At the onset of the present study it was considered to select at least one resistant and one susceptible variety cultivated in India by conventional pathogenicity test as well as by levels of common antigens using serological techniques (Dasgupta *et al.*, 2005).

Sub-Himalayan West Bengal, the present study area (Fig.1.1) is prone to disease caused by *Alternaria* sp. Although several inducer chemicals, antagonistic microorganisms and botanicals are known from different plants through literature but little information is available for controlling the *Alternaria* blight of niger. Hence, in the present study environment-friendly strategies have been taken into consideration for control of the *Alternaria* blight disease of niger. Therefore, the basic objectives of this study are:

Objectives:

1. To determine the pathogenicity of *Alternaria alternata* in different varieties of niger and to select susceptible and resistant varieties of niger.
2. To study morphological and physiological characteristics of the fungus.
3. To determine the common antigenic relationship between *Alternaria alternata* and different varieties of niger by serological techniques.
4. To study whether the disease reactions could be altered in susceptible niger varieties by some SAR inducers.
5. To control the leaf blight disease of niger by SAR inducers, eco-friendly botanicals and biocontrol agents.
6. To select potential SAR inducers, botanicals and biocontrol agents, if any, for preparation of field applicable formulations and field assessment of the formulations.

PLATE 1.1

fig. a : Healthy niger plants in flowering condition at farmer's field at Jalpaiguri

fig. b : Young niger plant

fig. c : Niger seed of local variety (LV)

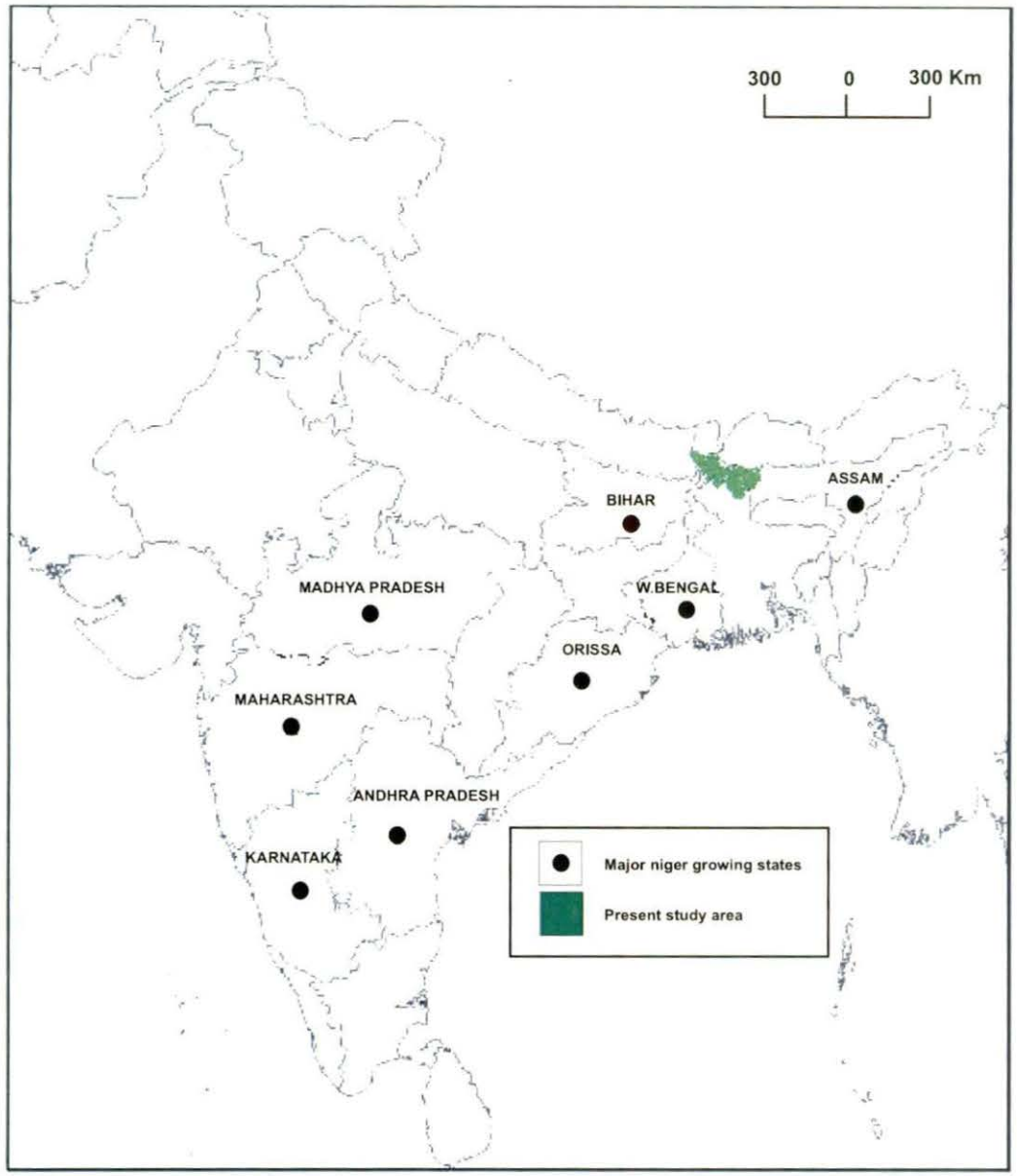

Fig. 1.1 : Major niger growing regions in India

2. Literature review

Plants have evolved sophisticated defense mechanisms including preformed barriers, constitutively expressed antimicrobials and inducible defense mechanisms against potentially pathogenic fungi, bacteria and viruses. Pathogenic microorganisms, non-pathogenic microorganisms and synthetic chemicals can induce resistance when it come in contact with the host plant and provide protection against a broad spectrum of pathogens. It has been shown that plants can recognize general structures associated with microorganisms. The pathogen associated molecular patterns (PAMPs) including some proteins from bacteria and chemicals from fungi (viz. chitin, ergosterol, several cell wall glucans, and proteins) after binding to specific receptor of plant elicits or trigger a signalling cascade which finally resulting to biochemical defense mechanisms such as production of phytoalexins and proteins with antimicrobial activities and mechanical strengthening of the cell walls (Thuerig *et al.* 2006). Like many other plants economically important plants are also attacked by several pathogens and cause substantial yield loss. To control diseases of plants effectively, it is necessary to understand different aspects of host parasite interactions. In case of plant disease management, biological control and botanicals also play important role as they are environment-friendly.

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

- Diseases of niger.
- Diseases caused by *Alternaria alternata.*
- Studies on growth and physiology of the pathogens.
- Antigenic relationship in host and pathogen.
- Induction of systemic resistance (SAR and ISR).
- Disease control by antagonistic organisms.
- Disease control by botanicals.

Diseases of niger

Diseases have been reported on niger plants. Getinet and Sharma, 1996, reported approximately 16 diseases of niger caused by fungi and bacteria. They reported that some *Alternaria* sp. cause stem and leaf blight of niger. They also mentioned that niger blight (*Alternaria* sp.) and leaf spot are most serious diseases among the diseases listed by them (Table 2.1).

Table 2.1 : Diseases of niger

Pathogen	Disease	distribution	References
Alternaria dauci	On seeds and leaf	Ethiopia	Stewart and Yirgu (1967)
Alternaria porri sp. *dauci*	Leaf spot	Ethiopia	Yirgu (1964)
Alternaria sp.	Stem and leaf blight	Ethiopia	Yitbarek (1992)
Aspergillus sp.	-	Ethiopia, India	Kolte (1985)
Bremia lactucae	Downy mildew	Ethiopia	Stewart and Yirgu (1967)
Cercospora guizoticola	Leaf spot	Ethiopia, India	Yirgu (1964)
Cladosporium sp.	-	Ethiopia, India	Yirgu (1964)
Emericella sp.	-	Ethiopia India	Kolte (1985)
Fusarium sp.	-	Ethiopia, India	Kolte (1985)
Ozonium taxanum var. *parasiticum*	Ozonium wilt	India	Kolte (1985)
Macrophomina phaseolina	-	Ethiopia, India	Chaven (1961)
Phoma sp.	Stem lesion, wilting	Ethiopia	Yitbarek (1992)
Phyllosticta spp.	Tar spot	Ethiopia, India	Yirgu (1964)
Plasmopara halstedii	Downy mildew	Ethiopia	Yitbarek (1992)
Puccinia guizotiae	Rust	Ethiopia	Yirgu (1964)
Rhizoctonia solani	Root rot	Ethiopia	Yirgu (1964)
Rhizoctonia bataticola	Seed rot	India	Yitbarek (1992)
Sclerotium rolfsii	Seed rot	India	Kolte (1985)
Sphaerotheca sp.	Powdery mildew	India	Yirgu (1964)
Xanthomonas campestris pv. *guizotiae*	Leaf spot	Ethiopia	Yirgu (1964)
Anguina amsinckia	Leaf gall	Ethiopia	Stewart & Yirgu (1967)
Epicoccum nigrum	-	Ethiopia	Yirgu (1964)
Erysiphe cichoraceurum	-	Ethiopia	Yirgu (1964)
Coniothyrium sp.	-	Ethiopia, India	Kolte (1985)
Penicillium spp.	-	Ethiopia, India	Yirgu (1964)
Xanthomonas campestris pv. guizota var. *indicus*	-	India	Kolte (1985)
Septoria sp.	-	Ethiopia	Stewart & Yirgu (1967)

Disease caused by *Alternaria alternata*

Genus *Alternaria* is one of the most common fungal pathogen of plants. Different species of the fungi attack a large number of vegetables, ornamental plants, orchard plants and cause substantial yield losses (Stranberg, 1992; Farrar *et al.*, 2004). They are polyphagus in nature and have ability to produce mycotoxins and other toxic metabolites that are potentially dangerous food spoilage agents (Repeckiene *et al.*, 2005; Solfrizzo *et al.*, 2005). Alternaria disease decreases nutritive value of vegetables, their storability and resistance to rot (Azevedo *et al.*, 2000; Sidlauskiene & Surviliene, 2002).

Maiti *et al.* (2007) reported *Alternaria alternata* causing leaf spot and leaf blight diseases of some cultivated medicinal plants. They reported leaf spot of *Aloe vera* L.Burm, leaf blight of *Rauvolfia serpentina* L. Benth.ex Kurz., leaf blight of *Mentha arvensis* L., leaf blight of *Ocimum gratissium* L., leaf blight of *Plantago ovata* Forsk., leaf blight of *Catharanthus roseus* L.G. Don., leaf spot of *Cassia angustifolia* Vahl. and leaf blight of *Datura metel* L. of lower gangetic plain of West Bengal.

Chakraborty *et al.* (2006) reported *Alternaria alternata* as a new foliar fungal pathogen of tea in North Bengal, India. They described that disease symptoms first appeared as grayish brown patches around tips and margins of young tea leaves. These lesions extended towards the midrib, resulting in leaf curl, death and defoliation. Microscopic observations showed brown and septate hyphae and conidiophores (17-28 X 3-6 µm). Muriform conidia (23-34 X 7-10 µm) were usually solitary but occasionally in short chains.

Akbari and Parakhia (2007) reported the huge yield loss of sesame, an important oil seed crop due to *Alternaria* blight caused by *Alternaria alternata* (Fr.) Keissler in Saurashtra region of Gujrat, India.

Verma *et al.* (2007) reported leaf spot of Safed Musli (*Chlorophytum borivilianum*), an important medicinal plant, caused by *Alternaria alternata*. The fungus attack only leaves of the plant. The lesions are minute with light brown punctuation in the centre. In later stages the central portions commonly become serious and in a few cases this portion fall-off giving a sort hole like appearance.

Alternaria alternata has been reported in Pakistan as a saprophytic pathogen of tomato causing post harvest losses in high frequency (Akhtar *et al.*, 1994). Among 35 *A. alternata* isolates collected from rotted fruits from fields and markets but only

one isolate from the field was able to produce leaf blight symptoms. Thus they could separate one pathotype of *A. alternata* causing leaf blight. Akhtar *et al.* (2004) also reported that *A. alternata* causing leaf blight in tomato plants in Pakistan. Symptoms on affected plants started with yellowing and browning of the lower leaves, progressing upwards under high humidity conditions. Symptoms often developed from the leaf tips and along the margins of the leaf petiole. Under severe infection, lesions enlarged and coalesced causing blighting of the leaves. Concentric circles with dark layers of spores were observed under moist conditions on blighted leaf portions. Infection under favorable conditions was found to cause severe defoliation, with considerable yield losses when it occurred prior to flowering. In microscopic study showed that conidia formed in long chains and were obclavate and muriform, often with a short conical or cylindrical, pale beak, less than one third of the length of the conidium. Conidia had 3-7 transverse septa and usually several longitudinal or oblique septa.

Maiti *et al.* (2006) first reported that *Alternaria alternata* causing leaf spot on *Stevia rebaudiana.* Symptoms initially appeared as small circular spots, light brown in colour. Later, many became irregular and dark brown to grey, while others remained circular with concentric rings or zones. On severely infected leaves several spots coalesced to form large necrotic areas. On older leaves concentric spots were more common at the tips. Leaf spots varied from 2-18 mm in diameter. Conidial dimensions varied from 10-40 × 6-12 µm, mid to dark brown or olive-brown in colour, short beaked, borne in long chains, oval and bean shaped with 3-5 transverse septa.

Bashan *et al.* (1991) reported that wind dispersal of *Alternaria alternata* spore is the cause of leaf blight of cotton plants. The number of air-borne spores of *A. alternata* was significantly increased by the presence of diseased cotton plants, being highest close to the diseased plants. *Alternaria* blight epidemics occurring in the fields twice a year. The two peaks recorded for the number of spores present in the air above cotton crops correlate with the annual two outbreaks of *Alternaria* blight epidemics. In addition, both wind and plant row direction affect disease development in the fields.

A severe leaf spot disease of cucumber caused by a pathtype of *Alternaria alternata* (Fr.) Keissler was reported by Vakalounakis and Malathrakis (1988), in plastic houses in Crete, Greece. Lesions ranged in size of a pin point to over 5 cm in

diameter, with necrotic tissue on most of their area and a surrounding yellow zone. The pathogen grew satisfactorily on PDA at temperatures between 5°C–40°C and spore germination occurred in the range less than 10°C to over 37°C. Optimum temperature in both cases was near 26°C.

Roy (1976) reported leaf blight spot of *Adhatoda* vasica caused by *Alternaria alternata* at several location of Rajasthan. Singh *et al.* (2006) reported *Altenaria* blight of *Adhatoda vasica* Nees caused *Alternaria alternata* (Fr.) Keissler. They confirmed pathogenicity of the fungus both on leaves and inflorescence of *A. vasica.*

Sreekantiah *et al.* (1973) reported *Alternaria* leaf and fruit spot of Chilli (*Capsicum annuum* L.) caused by a virulent strain of *Alternaria alternata.* Pandey and Vishwakarma (1999) reported leaf blight of brinjal caused by *A. alternata* from Uttar Pradesh. Mangala *et al.* (2006) reported that *Alternaria alternata* is one of the important pathogen causing chilli leaf blight. They investigated the pathogenicity of *Alternaria alternata* on chilli cultivars. They also showed host range of one *Alternaria alternata* pathotype *causing* leaf blight disease in different plants. The host range has been presented in the Table 2.2.

2.2: Table Host range of *Alternaria alternata*

Host plant	Symptoms
Tomato (*Lycopersicon esculentum* Mill.)	Necrotic spots
Redgram(*Cajanus cajan* Millsp)	Necrotic spots
Blackgram(*Vigna mungo* L. Hepper	Necrotic spots
Greengram (*Vigna radiata* L. Hepper)	Necrotic spots
Groundnut (*Arachis hypogea* L.)	Necrotic spots
Cabbage (*Brassica oleracea capitata* L.	Necrotic spots
Mustard (*Brassica nigra* L.)	Necrotic spots
Brinjal (*Solanum melongena* L.)	blighted Symptoms
Tobacco (*Nicotiana tabacum* L.)	blighted Symptoms
Soybean (*Glycine max* L.)	blighted Symptoms
Cluster bean (*Cymposis tetragonaloba* L. Tank)	blighted Symptoms
Potato (*Solanum tuberosum* L.)	blighted Symptoms
Cauliflower (*Brassica oleracea campestris* L.)	blighted Symptoms

Studies on growth and physiology of the pathogens

Knowledge about fungal growth and physiology has immense importance for control of fungal diseases. Use of resistant varieties is one of the important alternatives to overcome the disease problems. A variety which exhibits resistance in one area may show susceptibility in another area due to variation in weather (Dubey, 2005) and variation in pathogen (Paulkar and Raut, 2004). Infection rate and disease development were significantely related with the temperature, wind velocity, relative humidity, soil pH and other physiological factors of nature. Therefore, studies of growth and sporulation of fungi in different conditions is very much helpful in determining various control measures.

Alternaria species are well-adapted to natural conditions with daily fluctuations in temperature and light, but there is considerable variability in the requirements for sporulation in culture (Rotem, 1994). Photosporogenesis in many *Alternaria* spp. consists of two distinct phases: the inductive phase, leading to the formulation of conidiophores; and the terminal phase, leading to the formation of conidia (Aragaki *et al.*, 1973).

It has been shown that nutrition, temperature, light conditions and moisture affected conidiation of *A. alternata* on various agar media (Shabana *et al.*, 1996; Sidky *et al.*, 1999). Maximum production of virulent *A. alternata* conidia was obtained on PDA at 20°C under constant NUV (near ultra violate light), incubated for 4 weeks. Conidiophore induction occurred on nutrient rich media and was stimulated by NUV. Formation of conidia proceeds best in darkness when nutrients are depleted under warm/dry conditions or cool moist conditions (Masangkay *et al.*, 2000).

Babu *et al.*, (2004) reported that conidia production and virulence of *A. alternata* were affected by temperature, light and incubation period. The highest number of conidia were produced on rice seed (120.6×10^5 g^{-1} substrate) followed by wheat (66.2×10^5 g^{-1} substrate), sorghum (60.3×10^5 g^{-1} substrate), maize seeds and cornmeal at 20°C when exposed to near-ultraviolet than on the other substrates, while least conidia (23.2×10^5 g^{-1} to 12.5×10^5 g^{-1} substrate) were observed on these substrates under light conditions. At 20°C, large numbers of virulent conidia (26.8×10^5 g^{-1} substrate) were produced on rice seeds after 4 weeks of incubation under constant dark conditions.

Shahin and Shepard (1979) reported that CaCO3 was required for sporulation to occur, but 10 to 50 g l^{-1} of CaCO3 gave comparable conidial production of *A. solani*. Similarly, when CaCO3 was added to standard PDA medium, *A. solani* mycelium growth was significantly inhibited and sporulation enhanced (Moretto and Barreto, 1995).

Response to light conditions using the S-medium (water agar amended with calcium carbonate [$CaCO_3$] and sucrose) was similar to that on standard agar media. Dark conditions were required to produce large numbers of conidia. Incubation in the light completely inhibited conidial production and exposure to 12 h of alternating light and dark periods dramatically reduced conidial production on all the primary agar media. The effect of temperature on conidiation on S-medium was the reverse of that on standard agar media. Conidiation was inhibited by high temperatures (24 and 28°C) and stimulated by a lower temperature (18°C). Once conidiophores were formed, removal of blue light and lowering of temperature below 20° C was required for conidia production to continue (Aragaki *et al.*, 1973). The wetting of the conidiophores at warmer temperatures stimulated vegetative growth of conidiophores to sterile hyphae (Vakalounakis, 1986).

Consistency in production of inoculum, germination capacity and spore concentration, age of plant and environmental conditions are responsible for disease development. Selection of suitable medium is necessary for the production of inoculums with a high degree of sporulation, minimal aerial mycelia growth and prossessing ability of inoculum to cause a high level of infection (Kong *et al.* 1995).

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* shows less growth and sporulation on potato dextrose agar (Allen *et al.* 1983; Mukewar *et al.* 1974). Subculturing of *A.helianthi* continuously up to 60 days after isolation reduced the germination capacity of conidia and for successful infection inoculam concentration suggested tobe 1×10^6 spores/ ml and 20-30 days old plants were ideal. Older plants (60 days old) failed to show disease symptom.

Normally, *Alternaria* blight was high under high humidity of 80-90%. Temperature of 25°C could stimulate the disease in glass house conditions. The optimum condition determined for lesion development in case of *Alternaria* blight of

Paulownia trees were 25-30°C temperature and RH of 98-100% (Pleysier *et al.* 2006).

The effect of temperature and pH on the growth and sporangial sporulation of isolates from each of the four known races of *Phytophthora clandestine* Taylor, Pascoe & Greenhalgh were investigated by Harden *et al.* (2002). Mycelial growth occurred at temperatures from 10°C to 30°C and pH 3.5 to 9.0 with highest growth rates of all isolates being at 25°C with a pH of 6.0 to 6.5. Sporangial production was greatest between 20°C to 25°C and pH 5.0 to 7.0 with all races.

Umamaheswari *et al.* (2008) reported growth and sporulation of six isolates of two *Alternaria* species (*A.alternata* and *A.cucumerina*) isolated from leaf blight disease of curcarbits. They used four different media e.g. Potato dextrose agar (PDA), Oats agar, watermelon agar and sucrose-calcium carbonate agar to determine the growth and sporulation. Growth of all the six isolates of *Alternaria* spp was slow on PDA and it influenced the sporulation of only one isolate of *A.alternata.*The oats agar hastened the growth of all the *Alternaria* spp. but it influenced sporulation of only two isolates. The fungus took 27-31 days to attain 90 mm growth in special medium amended with calcium carbonate. Watermelon leaf extract was better to induce sporulation of different isolates of *A. alternata* and *A. cucumerina. Pleospora infectoria* (perfect stage of *A.alternata*) with ascoscarps and ascospores were observed in *A.alternata* isolates from pumpkin (*Cucumis pepo*) on oats agar.

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

Rani and Kumar (2007) tested five culture media for variability in culture and morphological characters of six geographical isolates of *Pythium aphanidermatum* (Edson) Fitz. causing damping off of tomato. The isolates varied in mycelia growth, shape, position of sporangia and oospore in thickness. Among different solid media tested, potato dextrose agar supported maximum growth, while the growth was poor in carrot agar. Variations in mycelia, sporangial and oospore characters among six isolates of *P. aphanidermatum* revealed that three isolates produced fluffy aerial

mycelium with inflated sporangia, while two others produced moderately abundant surface mycelium with lobulate sporangia. Another isolate produced sparse aerial mycelium with lobulate sporangia.

Jash *et al.* (2003) observed the effect of different culture media, pH and carbon sources on growth and sporulation of *Alternaria zinniae* Pape causing leaf and flower blight of marigold. They reported that among the different culture media, maximum growth and sporulation of the fungus was obtained in both solid and liquid form on leaf extract dextrose followed by potato dextrose medium. The optimum pH for growth of the pathogenic fungus was found in the range of pH 6.0-6.5. Maximum growth and sporulation of this fungus were obtained with sucrose as carbon source followed by starch and maltose.

Nirmalkar and Lakpale (2007) reported the optimum conditions of some physical factors (*i.e.* temperature, duration of incubation, colours of light and relative humidity) for uredospore germination and subsequent germ tube elongation. The results obtained revealed that uredospore germination and germ tube length were maximum at 20°C followed by 25 and 15°C. Germinatin of uredospore started 8h after incubation, increased up to 20 h and then remained constant up to 24 h. Diffused light, complete dark condition and 75% relative humidity were found ideal for maximum spore germination and germ tube elongation of uredospores of *Uromyces achori.*

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

Kuo (1999) studied the germination and appressorium formation in *C. gloeosporioides* and observed that the size of the conidia of the fungus ranged between 10.7-24.1 µm x 4.0-6.7 µm (15.4 µm x 4.8 µm). They studied the conidial germination and appressorium development by a two step method during a nine

hour period. At first mango decoction was added as supplemental nutrients into the spore suspension in order to trigger germination and this was followed by depletion of the mango decoction to induce the formation of appressorium. It was noticed that in mango decoction, the germlings formed long germ tubes and abundant hyphal branches without forming appressorium during 9 hour period. Appressorium formed mostly at the end of the long germlings or at the end of the hyphal branches if the incubation time was extended. When the spore suspension was first incubated in sterilized mango decoction for two hours and the decoction then removed and replaced with ddH_2O, the percentage of appressorium formation was enhanced dramatically.

Antigenic relationship in host and pathogen

The early and accurate diagnosis of plant disease is a crucial component of any crop managmant system. Plant disease can be managed effectively if control measures can be started at an early stage of disease development. The presence of common antigens among closely related organisms or even among more distantly related organisms is surprising. Studies on both animal and plant hosts and their parasites or pathogens suggest that whenever an intimate continuing association of cells of host and pathogen occurs, partners of this association have a unique serological resemblance to one another involving one or more antigenic determinants. In plants, several studies have shown that the possibility of susceptibility is greater when antigenic similarity is greater. Thus the concept of common antigens between a plant and a pathogen is a notable feature in determining resistance or susceptibility. It is believed that the degree of compatibility and susceptibility of a plant cultivar to a pathogen is correlated to levels of common antigens present in both host and pathogen (Alba *et al.*, 1983; Purkayastha and Banerjee, 1990; Chakraborty and Saha, 1994; Ghosh and Purkayastha, 2003; Kratka *et al.*, 2002; Musetti *et al.*, 2005; Eibel *et al.*, 2005; Dasgupta *et al.*, 2005, Chakraborty and Sharma, 2007). Recent advances in molecular biology and biotechenology are being applied to the development of rapid specific and sensitive tools for the detection of plant pathogen. The novel evolutionary steps in plant pathological research is the development of antibody based immunodiagnosis.

Dazzo and Hubbell (1975) reported cross-reactive antigens and lectin was determinants of symbiotic specificity in the Rhizobium-clover association. Cross-

reactive antigens of clover roots and *Rhizobium trifolii* were detected on their cell surfaces by tube-agglutination, immunofluorescent, and radioimmunoassay techniques. Anti-clover root antiserum had a higher agglutinating titer with infective strains of *R. trifolii* than with noninfective strains. The root antiserum previously adsorbed with noninfective *R. trifolii* cells remained reactive only with infective cells, including infective revertants. Radioimmunoassay indicated twice as much antigenic cross-reactivity of clover roots and *R. trifolii* 403 (infective) than *R. trifolii* Bart A (noninfective). Immunofluorescence with anti-R. trifolii (infective) antiserum was detected on the exposed surface of the root epidermal cells and diminished at the root meristem.

Crossed immunoelectrohoresis (CIE) techniques were used by Ala-El-Dein and El-Kady (1985) to show that the tested isolates of *Botrytis cinerea* were serologically different; some antigens were specific for each isolate. Isolate no.1 of *Botrytis cinerea* had four specific antigens; although these antigens were absent in other isolates. At least sixteen antigens were common in the isolates tested. Some isolates were serologically similar when tested by double gel diffusion test while they were distinguishable when CIE techniques were used. Numbers of precipitin peaks obtained with CIE techniques were more than double the number of precipitin lines detected with double gel diffusion test. Results revealed that CIE techniques could be used as valuable analytical tools in resolving the spectrum of antigens present, in *Botrytis cinerea* isolates.

Evaluation of antisera raised against pooled mycelial suspensions from five isolates (Pf-1, Pf-2, Pf-3, Pf-10 and Pf-11) representing five physiologic races of *Phytophthora fragariae* for detecting the red core disease of strawberries by enzyme-linked immunosorbent assay (ELISA) were performed. Cross-reactivity of antiserum raised against *P. fragariae* with other *Phytophthora* as a genus detecting antiserum has been reported. Antiserum of *P. fragariae* isolates (Anti-PfM) reacted strongly with antigens from several *Phytophthora* species. Some cross-reaction with antigens from *Phythium* species was decreased by fractionating on an affinity column of sepharose 4 B bound to extracts of *Fragaria vesca* roots infected with *P. fragariae*. The affinity purified anti PfM retained its high cross-reactivity with the various *Phytophthora* species. Anti-PfM could not be made specific for *P. fragariae* because it was raised against components shown to be antigenically similar in all *Phytophthora* species tested. However, immunoblotting with the affinity purified anti-

PfM produced distinct patterns for *P. fragariae*, *P. erythroseptica* and *P. cactorum* (Mohan, 1988).

Agar gel double diffusion between cotton seed globulins and the antisera specific to each of the tested *Fusarium oxysporum* f. sp. *vasinfectum* isolates were determined by Abd-El-Rehim *et al.* 1988. The antiserum of *F. moniliforme* revealed that all the tested antisera of *F. oxysporum* f. sp. *vasinfectum* reacted with seed globulins except one cultivar (Menoufi cultivar) globulins. No precipitin lines were detected in the reaction between the antigen of the cotton cultivar Acala SJ2 versus the antiserum of P10 isolate. Five cultivars reacted differently with each fungal antiserum to the extent that they could be distinguished accordingly. When the seed globulins of the susceptible cultivars (Giza 74, and Bahtim 110) reacted with antiserum of the tested *F. oxysporum* f. sp. *vasinfectum* isolates, more precipitin lines were found than the resistant cultivars.

Antigenic relationships between soybean and *Colletotrichum dematium* var. *truncata* using immunodiffusion, immunoelectrophoresis and indirect ELISA technique was studied by Purkayastha and Banerjee (1990). Cross-reactive antigens were detected between susceptible soybean cultivars and the virulent strain of *C. dematium* but no cross-reactive antigen was detected between soybean cultivars and avirulent pathogen (*C. dematium*) or non-pathogen *C. corchori*. Results of immunodiffusion and immunoelectrophoresis showed absence of common antigen between resistant cultivars (UPSM-19) and the pathogen, while the results of indirect ELISA indicated the presence of common antigen between the two at a very low level. They compared antigenic patterns of untreated and cloxacillin treated soybean leaves which induced resistance of soybean against anthracnose disease. The disappearance of one antigen from cloxacillin treated leaves of susceptible soybean cv. "Soymax" was correlated with alteration of disease reaction.

Pathogenicity test of *Fusarium oxysporum* on ten cultivars of soybean revealed Soymax and Punjab-1 to be most resistant while JS-2 and UPSM-19 were most susceptible. Antigens were prepared from the roots of all the ten varieties of soybean and the mycelium of *F. oxysporum*. Polyclonal antisera were raised against the mycelial suspension of *F. oxysporum* and the root antigen of the susceptible cultivar UPSM-19. Cross reactive antigens shared by the host and the pathogen

were detected first by immunodiffusion. The immunoglobulin fraction of the antiserum was purified by ammonium sulfate precipitation and DEAE-Sephadex column chromatography. The immunoglobulin fractions were used for detection of cross-reactive antigens by enzyme-linked immunosorbent assay. In enzyme-linked immunosorbent assay, antigens of susceptible cultivars showed higher absorbance values when tested against the purified anti-*F. oxysporum* antiserum. Antiserum produced against UPSM-19 showed cross-reactivity with the antigens of other cultivars. Indirect staining of antibodies using fluorescein isothiocyanate indicated that in cross-sections of roots of susceptible cultivar (UPSM-19) cross-reactive antigens were concentrated around xylem elements, endodermis and epidermal cells, while in the resistant variety, fluorescence was concentrated mainly around epidermal cells and distributed in the cortical tissues. CRAs were also present in microconidia, macroconidia and chlamydospores of the fungus (Chakraborty *et al.* 1997).

Scala *et al.* (1994) analyzed the possible involvement of cross-reactive antigens in host-parasite interactions between pea and some fungal plant pathogens. Antiserum to pea was used to analyse cross-reactive antigens (CRA) between pea and some fungal plant pathogens with different levels of specificity towards this host by using both double diffusion and immunoblotting techniques. Non pathogens of pea were also included in the study. The three *f. sp. of Nectria haematococca* MPVI (Viz. *dianthi, lycopersici* and *pisi*) of *Fusarium oxysporum* and *Ascochyta pisi* produced strong reactions in both techniques. No CRA was observed in the non-specific pathogens *Rhizoctonia solani, Sclerotium rolfsii* and *Sclerotinia sclerotiorum*, as well as in the non-pathogen *Phytophthora capsici.* The immunoblotting patterns of the most reactive fungi showed common bands with molecular weights of 84, 75 and 62 kDa. Some bands were present only in the specific pathogens *N. haematococca* MPVI and *F. oxysporum* f.sp. *pisi.*

Chakraborty *et al.* 1995, discussed the detection of grey blight of tea caused by *Pestalotiopsis theae* through cross reactive antigen between *P. theae* antigen of tea leaves. Among the 12 varieties of tea tested against three isolates of *Pestalotiopsis theae*, causal agent of grey blight disease, Teen Ali-17/1/54 and TV-23 were found to be highly susceptible while CP-1 and TV-26 were resistant under

identical conditions. Leaf antigens were prepared from all the tea varieties, three isolates of P. theae and a non-pathogen of tea (*Bipolaris tetramera*). Polyclonal antisera were raised against mycelial suspensions of *P. theae* (isolate Pt-2) and leaf antigens of Teen Ali-17/1/54 and CP-1. These were compared in immunodiffusion test and enzyme-linked immunosorbent assay to detect cross reactive antigens (CRA) shared btween the host and the parasite. CRA were found among the susceptible varieties and isolates of P. theae (Pt-1, 2 and 3). Such antigens were not detected between isolates of *P. theae* and resistant varieties, B. tetramera and tea varieties or isolates of *P. theae*. Indirect staining of antibodies using fluorescein isothiocyanate (FITC) indicated that in cross sections of tea leaves, the CRA was concentrated in the epidermal cells and mesophyll tissues. CRA was present in the young hyphal tips of the mycelia and on the setulae and appendages of the conidia of *P. theae*.

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

Polyclonal antibodies against pre helminthosporol, a phytotoxin produced by the plant pathogenic fungus *Bipolaris sorokiniana* were raised in rabbits immunized with a prehelminthosporol–hexon conjugate. The IgG was isolated from the serum and the specificity of the purified antibodies was investigated with indirect ELISA. The antibodies bound both to free prehelminthosporol and to a prehelminthosporol-bovine serum albumin conjugate bound to micro titer wells. The antibodies showed less affinity to structurally related compounds from the fungus. No cross-reactivity was shown for proteins extracted from mycelium of *B. sorokiniana*. Low-temperature preparation techniques for electron microscopy were used in combination with

immunogold labeling for localization of prehelminthosporol in hyphae and germinated conidia of *B. sorokiniana*. A low level of labeling was obtained throughout the cytoplasm, and the main labeling was seen in membrane-bound organelles identified as Woronin bodies (Akesson *et al.*, 1996).

Polyclonal antisera against whole (coded: 16/2) and sonicated (coded: 15/2) resting spores of *Plasmodiophora brassicae were* raised as well as soluble components prepared by filtration and ultracentrifugation (coded:SF/2), cross-reactivity of all three antisera with a range of soil fungi, including *Spongospora subterranean* was low (Wakeham and White, 1996). Test formats including western blotting, dipstick, dot blot, indirect ELISA and indirect immunofluorescence were assessed for their potential to detect resting spores of *P. brassicae* in soil. Dot blot was least sensitive, with a limit of detection level of $1x10^7$ resting spores/ g in soil. With western blotting, the lower limit of detection with antiserum 15/2 was $1x10^5$. This antiserum showed the greatest sensitivity in a dipstick assay, indirect ELISA and indirect immunofluorescence, for all of which there was a limit of detection of $1x10^2$. Of the assays performed, indirect immunoflourescens appeared to be the most rapid and amenable assay for the detection of resting spores of *P. brassicae* in soil.

Polyclonal antibodies were raised against mycelium from the logarithmic growth phase of a shake culturen of *Ustilago nuda,* and a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) with biotinylated detection antibodies was developed. The detection limit of the assay was 15 ng total protein ml^{-1} for the homologous antigen and 50 ng ml^{-1} for a spore extract, Other species of *Ustilago* reacted with the antibodies. Cross-reactivity was highest with *U. tritici*. No signal was obtained with the tested isolates of *Tilletia, Rhizoctonia, Pythium and Fusarium*. With naturally infected barley seeds, the results of the ELISAs were always in good agreement with those obtained with the routinely used seed embryo test. They suggested that ELISA has potential for field application including the early prediction of the efficacy of protection agents, e.g. in screenings for seed treatments, the elucidation of the biology of the fungus and characterisation of resistance mechanisms (Eibel *et al.*, 2005).

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

Abou-Jawdah *et al.* (2001) in a survey detected some potato viruses by ELISA from potato fields in the two main production areas of Lebanon, the Bekaa and Akkar plains. Hema *et al.* (2001) evaluated double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and direct antigen coating (DAC)-ELISA for detection of sugarcane streak mosaic virus (SCSMV-AP). The virus was detected up to 1/3125 and 1/625 dilutions in infected sugarcane leaf.

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

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

Ghosh and Purkayastha (2003) used polyclonal antibodies and antigens of ginger and *Pythium aphanidermatum*, a causal organism of rhizome rot disease for early diagnosis of rhizome rot disease of ginger. They detected *P. aphanidermatum* in ginger rhizome after eight weeks of inoculation by agar gel double diffusion and immuoelectrophoretic tests, but only one week after inoculation by indirect ELISA.

Cellular location of different proteins or antigens can be done by immunolocalization. Location of cross reactive antigens (CRA) was successfully done by several workers. In a study, DeVay *et al.* (1981) inoculated young cotton (Acala 2) roots with antiserum to *Fusarium oxysporum* f. sp. *vasinfectum* and stained with FITC conjugated, antirabbit globulin-specific goat antiserum. Strong

fluorescence was observed at the epidermal and cortical cells, and the endodermis and xylem tissues that indicated a general distribution of the CRA determinants in roots.

In a similar fashion, Chakraborty and Saha (1994) labelled polyclonal antiserum with FITC and found CRA between tea leaves and the pathogen *Bipolaris carbonum*. CRA was present mainly around the epidermal cells and mesophyll tissues of leaves of the host and in hyphal tips and in patch like areas on conidia and mycelium of the pathogen. Location of CRA was also studied in tea leaves that were treated with antiserum raised against two pathogens of tea. Indirect labelling of the antibodies with FITC showed that CRA was concentrated mainly in the epidermal cells and also spread throughout the cortical cells.

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

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

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

Kang and Buchenauer, (2002) raised two antisera against acidic β-1,3-glucanase and acidic chitinase from tobacco and used to investigate the subcellular localization of the two enzymes in *Fusarium culmorum*-infected wheat spike by means of the immunogold labelling technique. These studies demonstrated that the accumulation of the enzymes in the infected wheat spikes differed distinctly between resistant and susceptible wheat cultivars.

Wang *et al.* (2003) used immunogold labelling technique for localization of PB90 which is a novel protein elicitor secreted by *Phytophthora boehmeriae*. The anti-90 kDa protein antiserum was used for immunocytolocalization studies of PB90 elicitor, on the mycelium and encysting zoospores of *P. boehmeriae* grown *in vitro* in liquid culture and also in solid medium. In liquid culture, immunogold labelling was located mainly in the cell wall. In solid medium, gold particles were observed not only in the cell wall, but also in the solid medium near the hypha.

Chakraborty and Sharma (2007) studied the location of CRA in tea (*Camellia sinensis*) leaves treated with antiserum raised against *Exobasidium vexans*, causal agent of blister blight of tea. Indirect staining of antibodies using FITC indicated cross reactive antigens (CRA) were concentrated mainly around epidermal and mesophyll cells in susceptible tea variety (T-78). This finding was substantiated by ultrastructural studies using gold labelled antibodies through transmission electron microscopy (TEM) which shows specific localisation in the chloroplast and host cytoplasm.

Induction of systemic resistance:

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

Plant pathogen intractions are governed by specific interactions between avr (avirulence) gene loci and alleles of the corresponding plant disease resistance (R) locus. When corresponding R and avr genes are present in both host and pathogen,

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

The plant immune response is much more like the innate immune response of animals than the adaptive. Plant does not develop specific resistance to the challenging pathogen but developed a broad spectrum resistance to several (Sticher *et al.* 1997). Plant cannot move to escape enviormantal challenges so they have evolved sophisticated mechanisms to perceive such attacks and to translate that perception into an adaptive response through signal molecules either synthesized by the invading organisms or released from plant cell walls. These signal molecules are collectively termed as 'elicitors', due to the presence of elicitors plant only receive general information that the plant is under attack (Wojtaszek 1997). Plants have several challenged-inducible resistance mechanisms, broadly divisible into local and systemic defences. Local defences include structural changes, such as the formation of papillae, tyloses and abscission zones, necrotic changes etc. Accumulation of phytoalexins, the synthesis of phenolic compounds and their subsequent oxidation of quinines by polyphenol oxidase and peroxidase are also important in plant defence (Agrios, 1997). Systemic defences involve the accumulation of antimicrobial compounds in parts of the plants distinct from the site of infection. Four main classes of compounds can accumulate: hydrolases, particularly the pathogen related proteins (PR), defensins (Broekaert *et al.,* 1995), proteinase inhibitors (Schaller and Ryan, 1996), cell wall componants, particularly hydroxyprolin rich glycoproteins (HRGP) and lignin and its precursor (Sticher *et al.* 1997).

Mainly five types of systemic induced resistance are known: (a) Local acquired resistance (LAR), express in immediate vicinity of the hypersensitive zone caused by attempted pathogen invation. (b) Sytemic acquired resistance (SAR),

expressed in the plant as a whole in response to pathogen attack. (c) Systemic gene silencing (SGS), a putative explanation for the recovery phenomenon by which systemic parts of the plants can exhibit resistance to the virus in infected parts. (d) Induced systemic resistance (ISR), which is induced by plant growth promoting rhizobacteria (PGPR) and expressed systemically. (e) Systemic wounding response (SWR), caused by the wounds inflicted on the plant by chewing insects and leading to the induction of proteinase inhibitors in systemic parts of the plant.

SAR development is mediated by a mobile signal that originates at the primary infection or treatment site and thought to be translocated systemically in the phloem (Rasmussen *et al.,* 1991; Smith-Becker *et al.,* 1998; Kaur and Kolte, 2001; Sharma *et al.,* 2001; Paul and Sharma, 2002). Systemic acquired resistance (SAR) can be induced in plants following a localised infection with a necrotizing pathogen or treatment with chemical elicitors (Mauch-Mani and Metraux, 1998; Sticher *et al.*, 1997). SAR was induced in mature plant part after localized treatment with 0.2 mM salicylic acid (SA) or previous inoculation with the same pathogen. SAR was expressed in adjacent untreated leaves as a reduction in lesion diameter (Reglinski *et al.*, 2001).

Willits and Ryals (1998) reported that Probenazole was the first commercialized disease resistance inducer and was widely used for the control of rice blast in Japan. Besides probenazole, some other chemical inducers of disease resistance in different plants have been described whice include salicylic acid (SA), 2,6-dichloro isonicotinic acid (INA) and 3-aminobutyric acid (BABA) (Kessmann *et al.*, 1994; Cohen, 1996; Sticher *et al.*, 1997). One of the benzothiadiazole compounds (BTHs), acibenzolar-S-methyl (CGA245704: benzo[1,2,3]thiadiazole-7-carbothioic acid S-methyl ester) was developed by Novartis Crop Protection AG and was introduced in 1996 as a 'plant activator' for the control of wheat powdery mildew in Germany and Switzerland (Ruess *et al.*, 1996 ;Buonaurio *et al.* 2002).

In the last two decades many biotic and abiotic inducers have been used for establishment of SAR in different plants. Meena *et al.* (2001) used salicylic acid in groundnut, Higa *et al.* (2001) used active oxygen radicals in rice, O'Donnell *et al.* (1996) used ethylene in tomato, Smith-Becker *et al.* (1998) used SA and 4–hydroxybenzoic acid in cucumber, Cohen *et al.* (1993) used jasmonic acid and

methyl jasmonate in potato and tomato, Siegrist *et al.* (2000) used β - aminobutyric acid in tobacco, Kaur and Kolte (2001) and Stadnik and Buchenauer (2000) used benzothiadiazole in mustard and wheat plant respectively, Brederode *et al.* (1991) used UV-light in tobacco, Ernst *et al.* (1992) used ozone in tobacco, Klessig *et al.* (2000) used nitric oxide and Kaku *et al.* (1997) applied N-acetylchitooligosaccharide in barley.

Salicylic acid is a natural phenolic compound present in many plants that play an important role in the signal transduction pathway and involved in local and systemic resistance to pathogens (Delaney *et al.*, 1995). It has been described that SA coordinately induces the full spectrum of SAR genes, encompassing all well-characterized PRs (Ward *et al.*, 1991). Salicylic acid (SA) is an endogenous signal for the development of SAR and it is transported by phloem from the sites of its origin. Leaves inoculated with pathogen exhibits high level of endogenous SA (Malamy *et al.,* 1990). Foliar application of SAsignificantly increased the activity of Phenylalanine ammonia-lyase (PAL), Chitinase, β-1,3-glucanase, Peroxidase, Polyphenol Oxidase and Phenolic content in groundnut (Meena *et al.*, 2001). Jasmonic acid (JA) plays an important role in plant defense response. Its level is increased under wounding and treatment with pathogen-elicitors that induce genes encoding enzyme involved in flavonoid biosynthesis, chalcone synthase (Creelman *et al.*, 1992) and Phenylalanine ammonia-lyase (Gundlach *et al.*, 1992).

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

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

Ishii *et al.* (1999) suggested that acibenzolar-S-methyl (CGA 245704) induced resistance to some but not all diseases on cucumber and Japanese pear. Induction of disease resistance in cucumber was rapidly triggered after treatment with acibenzolar-S-methyl (CGA245704: benzo [1,2,3] thiadiazole-7- carbothioic acid S-methyl ester) which showed no antifungal activity *in vitro*.

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

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

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

61.4% and 82.6% respectively when challenge inoculated with *A. candida* (Kaur and Kolte, 2001).

Buonaurio *et al.* (2002) used acibenzolar-S-methyl to induce resistance in pepper plants against *Xanthomonas campestris* pv. *vesicatoria* in both growth chamber and open field conditions. In growth chamber experiments of acibenzolar-S-methyl treatment in pepper plants showed resistance expression systemically and locally that lead to the reduction in the number and diameter of bacterial spots and bacterial growth. Systemic protection was also noticed by the acibenzolar-S-methyl acid derivative, CGA 210007. Under open field conditions both leaves and fruits were protected from the disease perhaps due to SAR activation.

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

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

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

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

that systemic pathogen-induced expression of the plant defensin gene in *Arabidopsis* was independent of salicylic acid but requires ethylene and jasmonic acid to response.

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

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

Plant growth promoting rhizobacteria (PGPR) can suppress the disease caused by foliar pathogen by triggering plant-mediated resistance mechanism called induced systemic resistance, so called ISR (Dube, 2001). Van Loon *et al.* (1998) reported that the rhizosphere bacteria were present in large numbers on root

surface; few of them stimulated plant growth. The strains that were isolated from naturally disease-suppressive soil were mainly fluorescent *Pseudomonas* sp. It was demonstrated to reduce plant disease by suppressing soil-borne pathogens. Some of those biological strains had the ability to reduce disease caused by foliar pathogens by triggering a plant-mediated resistance mechanism called induced systemic resistance.

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

Conrath *et al.* (2001) reported that pre-treatment of cultured parsley cells with inducers of systemic resistance, salicylic acid or a benzothiadiazole, leads to the direct activation of a set of defence-related genes and also primes the cells for stronger elicitation of another set of defence genes including those encoding phenylalanine ammonia-lyase. In *Arabidopsis*, pre-treated plants with benzothiadiazole was found to augment the subsequent activation of phenylalanine ammonia-lyase genes by *Pseudomonas* infection, wounding and osmotic stress and also to enhance wound/osmotic stress-induced callose production. From these results, it was concluded that the resistance inducers have at least a dual role in plant defence-gene activation. Chowdhury *et al.* (2003) evaluated the effect of biotic and abiotic elicitors for the managnent of sheath blight of rice caused by *Rhizoctonia solani*. Thre fungal bioagents e.g, *Trichoderma viride*, *T. harzianum* , *T.virens* and nine antagonistic wheat rhizopheric bacteria (WRb) were evaluated *in vitro* by dual culture method for their antagonistic activities against *Rhizoctonia solani*. A maximum inhibition (62.96%) in growth of the pathogen was observed by wheat rhizosphere fluorescent pseudomonas bacteria (WRPf) followed by WRb8 (56.67%) and *T. virens*. Out of 4 plant extracts (*Polyalthia longifolia*, *Polygonum* sp. *Allium sativum* and *Gingiber officinale*), extracts of *Allium sativum* (at 10% concentration) inhibited maximum mycelial growth (100%) and sclerotial production *in vitro*. Among

abiotic elicitors seeds and seedlings treated with K_2HPO_4 (20mM) followed by $FeCl_3$ (10mM), reduced maximum disease incidence.

Abd-El-Kreem, (2007) reported that root rot caused by *Fusarium solani* or *Rhizoctonia solani* and *Alternaria* leaf spot of bean plants (*Phaseolus vulgaris* L.) were controlled with integrated treatment of *Trichoderma harzianum* and humic acid. It was observed that intregated treatments with seed coating with *Trichoderma harzianum* followed by seedling spray with humic acid at the concentration of 6 or 8 ml/L reduced the root rot incidence maximum (more than 80.5%) as compared with plant treated singly with *T.harzianum* or humic acid at concentration of 6 or 8 ml/L (more than 65.7%). *Alternaria* leaf spots were also appeared less when the plants weresubjected to the same treatment. In addition, more increase in chitinase activity was observed.

Role of certain elicitors like jasmonic acid (JA), salicylic acid (SA) and PGPR (*Pseudomonas*) were evaluated on the chemical induction of resistanse in tomato (*Lycopersicum esculantum* Mill.) against the leaf caterpillar *Spodoptera litura* Fab. by Malvin and Muthukumaran (2008). Elicitor was used to manipulate the activities of four putative defense related proteins like protein inhibitors, polyphenol oxidase, peroxidase and lypoxigenase in the leaves of tomato plants. When activities of protinase inhibitors and polyphenol oxidase in leaf tissue were high, growth rates of *S.litura* were low and *vice versa*. In contrast, high activities of peroxidase and lypoxigenase have no effect of growth and development of *S. litura*.

Plants face many biotic and abiotic challenges in the enviorment including drought and pathogen attack and combination of such stresses. Local and systemic induced defense responses were investigated by Fossdal *et al.* (2007) in the Norway spruce-*Rhizoctonia* sp. pathosystem and compared with drought alone or in combination of the two stresses using real-time reverse transcriptase (RT- PCR). They showed that compatible pathogen stress resulted in a transient systemic induction of selected defense related gene transcripts in the shoot. They also showed a persistant local induction in the roots. Drought lead to similar but delayed host response, while the combine stress gave, large and earlier changes in the transcripts than the two stresses separately.

Cellodextrins (CD), water soluable derivatives of cellulose composed of β-1,4 glucosidas residues, have been shown to induce a variety of defense responses in grapevine (*Vitis vinifera* L.) when challenged with *Botrytis cinerea.* The treatment has also resulted in significant reduction of the disease. The large oligomers of CD rapidly induced transient generation of H_2O_2, followed by a differential expression of genes encoding key enzymes of the phenyl propanoid pathway and pathogenesis related proteins. It also stimulated chitinase and β-1,3 glucanase activities(Aziz *et al.* 2007).

Lui *et al.* (2008) investigated the antagonism between acibenzolar-S-methyl (ASM) induced systemic acquired resistance and Jasmonic acid (JA) induced syatemic acquired susceptibility (SAS) to *Colletotrichum orbiculare* infection in cucumber plants. ASM treatment of cucumber plants resulted in much higher accumulation of class III chitinase (*CHI2*) gene, and lesion suppression, than in plants treated with distilled water (DW). In contrast, JA treatment suppressed expression of the *CHI2* gene and caused plant to be more susceptible to *C. orbiculare* infection. In the ASM +JA treatment, the number of lesions and the hybrid signal intensity fell midway between the ASM- and JA-only treatments. There was clear intimation about the antagonism or negative crosstalk between ASM-induced SAR and JA induced SAS.

Role of ethylene in plant development has been well estrablished but role of ethylene in plant defence is contradictory. Ortuno *et al.*, (2008) proposed ethylene as a possible marker of susceptibility of citrus fruits against *A. alternata* pv. *Citri.* They established a positive correlation between susceptibility to *A. alternata* pv. *Citri. and* the different citrus fruits with that of ethylene levels(produced *in vitro*).

Dutsadee and Nunta (2008) purified a novel 75 kDa protein-elicitor from the culture filtrate of *Phytophthora pulmivora*, a pathogen of *Hevea braziliensis.* The protein-elicitor was compared with another renound elicitor 'elicitin'. The new protein elicitor (75 kDa protein) activated defence at a concentration lower than those required for elicitin. The 75 kDa protein induced peroxidase enzyme, scopoletin, phenolic compounds and local resistance of rubber plants against *Phytopthora palmivora*, at about a 2-fold lower concentration than 'elicitin'.

Induction of resistance to downy mildew in sunflower caused by *Plasmopara halstedii* was studied by Nandeeshkumar *et al.* (2008) after treatment with chitosan. Treatment of sunflower with 5% chitosan resulted in decreased (46-52%) disease in field conditions. There was enhanced activation of defense related enzymes like catalase (CAT), phenylealanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxydase (PPO) and chitinase (CHI) in chitosan-pretreated and *P. halstedii* inoculated plants. Northern hybridization analysis revealed increased levels of transcripts for five known defense response genes viz.,pr- 1a, β-1,3 glucanase, chitinase, peroxidase and chalcone synthase.This enhanced and early activation of defense-related responses (due to pretreatment with chitosan) in the susceptible cultivars were comparable to that of resistant cultivars.

Disease control by antagonistic organisms

Plant diseases occur regularly in plants and cause severe economic loss due to low harvest or production of oilseeds/foodgrains from diseased plants. Due to variation in cultivars and climate the resistance towards pathogens also vary. Application of chemical fungicides leads to destroy beneficial microbes on the crop milieu and thus alters the crop scenario and also causes toxicity to human and natural biota (Patro *et al.* 2008). Biological control of plant diseases involves the use of one nonpathogenic organism to control or eliminate a pathogenic organism. Hence, biological control has attracted a great interest in plant pathology (Goto, 1990) and it becomes importent to develop cheaper management practices to control disease and obtain higher yield. To develop biological control strategies for controlling any disease, a thorough knowledge of life cycle of the pathogen(s), their mode of survival, the plant-pathogen interaction processes, the physical relationship of the pathogen to its host during pathogenesis, the time of infection, factors leading to infection and disease development are needed. Several authors have reported antagonistic activity of microorganisms in different crops (Droby *et al.*, 1992; Prasad *et al.*, 1999; Meena *et al.*, 2000; Dwivedi and Johri, 2003; Jadeja, 2003; Kohli and Diwan, 2003; Vestberg *et al.*, 2004; Brewer and Larkin, 2005; Sudha *et al.*, 2005; Singh and Sinha, 2005).

Plant growth promoting rhizobacteria (PGPR) can suppress pathogen and reduce disease incidence by several ways like competition for nutrient and space, production of antibiotics, production of HCN, production of siderophores, increase in

salicylic acids, excreation of lytic enzymes, enhancement of plant defense through Induced systemic resistance (ISR), plant growth promotion by production of auxins and gibberalins etc. In *Trichoderma*, the production of secondary volatile and non-volatile metabolites is one of the criterion to assess its potential as biological agent (Umamaheswari et al. 2008)

Fungal population in the rhizosphere of eggplant was studied by Hundoo and Dwivedi (1993) showing that rhizosphere microorganisms such as *Trichoderma* spp. was found to be antagonistic against *Fusarium solani,* the causal agent of root disease of eggplant. Bucki *et al.* (1998) observed the presence of some biocontrol microorganisms viz., isolates of actinomycetes, fluorescent *Pseudomonads* and *Trichoderma* sp. in the soil which prevent the damping off of egg plant caused by *Fusarium* sp., *Pythium* sp. and *Rhizoctonia* sp.

Trichoderma harzianum has antagonistic effect against four fungal pathogens (viz. *Phytophthora parasitica, Colletotrichum capsici, Sclerotium rolfsii* and *Rhizoctonia solani*) of betel vine (D'souza *et al.* 2001). Ramamoorthy and Samiyappan (2001) suggested that *Pseudomonas flourescens* isolates were effective bacterial antagonist for the management of fruit rot of chilli caused by *Colletotrichum capsici.* Jadeja (2003) observed that fungal antagonists like *Trichoderma* spp. were highly effective for inhibiting mycelial growth and retarding pycnidial formation of *Phomopsis vexans* causing disease in brinjal. *T. koningii* exhibited the maximum antagonistic activity. Bacterial antagonists, e.g. *Bacillus* spp. and *Pseudomonas fluorescens* were also highly effective against the pathogen (Meena *et al.,* 2000).

Baruah and Kumar (2002) isolated an antibiotic and siderophore producing *Pseudomonas* strain from virgin soils (with forest trees) which displayed *in vitro* antibiosis against many plant pathogenic fungi. They noticed that seed bacterization improved germination, shoot height, root length, fresh and dry mass, enhanced yield and chlorophyll content of leaves in the five test crop plants under field conditions. Seed bacterization also reduced the number of infected brinjal plants grown in soil infested with *Rhizoctonia solani.*

Gupta *et al.* (2005) studied on management of anthracnose in french bean caused by *C. gloeosporioides.* On the basis of *in vitro* studies they found *Trichoderma viride* isolate (Tv2), neem extract, carboxin and carbendazim as best treatments in inhibiting the growth of the pathogen. They were then tested in field at

different combinations. The most effective combinations comprised of seed treatement with carboxin and *T. viride* followed by foliar spray of neem extract and carbendazim. This combination treatment resulted in the least disease incidence (1.45%) and severity (0.50%) and maximized yield (126 q/ha).

Jadon *et al.* (2005) carried out experiment with antagonistic microbes and extracts of botanicals on *Sclerotium rolfsii,* incitant of collar rot of brinjal. They tested the efficacy of isolates of *Trichoderma* spp., *Pseudomonas fluorescens,* and *Gliocladium virens* in suppressing the growth of the pathogen by dual culture technique. They observed that *T. viride* isolate was superior than other isolates in reducing colony diameter and sclerotial production of the pathogen.

Some other pathogens of other crops were also controlled by several workers using biological control strategies. For instance, mycostop was a biofungicide that has been effectively used to control a number of soil and seed-borne pathogens like *Botrytis cinerea, Rhizoctonia solani* etc. and seed borne foot rot disease of wheat and barley (Tahvonen and Lahdenpera, 1988; Tahvonen and Avikainen, 1990). The active component of mycostop was the spores and mycelium of *Streptomyces griseoviridis.* The product has been used successfully in seed treatment, soil drench, drip irrigation and as a transplant dip to control various disease causing fungi (Lahdenpera, 1987; Lahdenpera *et al.,* 1990 and Mohammadi, 1992). Mycostop when used at the rate of 0.35 g/l or greater reduced spore germination, plasmolysed germlings and reduced sporulation of *C. radicicola.* In essence, it reduced the inoculum potential of *C. radicicola* (Suleman *et al.*, 2002).

The antagonistic effect of *Trichoderma viride* was well established as reported by several workers. The hyphal coiling and production of inhibitory substances by different species of *Trichoderma,* resulting in dieback and disintegration of *Pythium* spp. were reported by Raju (1991) and Vinod *et al.* (1991). Several other works have shown considerable potential of *Trichoderma* and *Gliocladium* in controlling disease caused by *Sclerotium rolfsii* in snap bean, sugar beet, tomato, chickpea and cotton in greenhouse and field studies (Elad *et al.,* 1983; Upadhyay and Mukhopadhyay, 1983; Punja, 1985; Wokocha, 1990; Ciccarese *et al.*, 1992 and Latunda Dada, 1993). Efficient control of chickpea wilt complex was found when seeds were treated with *Gliocladium virens* (10^7conidia/ml) and carboxin 0.1% (Mukhopadhyay *et al.*, 1992).

Maity and Sen (1985) and Biswas (1999) reported that different isolates of *Trichoderma harzianum* showed differential antagonistic potential as biocontrol agent against *Sclerotium rolfsii*. Filonow (1998) observed that three antagonistic yeasts competed successfully for sugars since their uptake was faster and higher than that of *Botrytis cinerea.* He concluded from this that high competitiveness plays a central role in antagonism.

A comparative study of chemical, biological and integrated control of wilt of pigeon pea caused by *Fusarium udum* was done by Pandey and Upadhyay (1999). In chemical control, bavistin was found highly effective, while *Trichoderma viride* and *T. harzianum*-C isolates were found best among biocontrol agents. Integration of biocontrol agents with bavistin was not beneficial. However, integration of the bioagents with thiram reduced wilt incidence significantly. Thus, seed coating with bioagents proved better and safe for the management of wilt of pigeon pea.

Fourteen isolates of *Trichoderma* and *Gliocladium* speices were tested *in vitro* against *Sclerotium rolfsii,* the causal organism of root/ collar rot of sunflower by Prasad *et al.* (1999). Two isolates of *T. viride,* four isolates of *T. harzianum,* one each of *T. hamatum, T. koningii, T. polysporum, G. virens, G. deliquescens* and *G. roseum* inhibited mycelial growth of the pathogen significantly. Complete inhibition of sclerotial germination was obtained with the culture filtrates of *T. harzianum* (PDBCTH 2, 7 and 8), *T. pseudokoningii* and *G. deliquescens.* The three *T. harzianum* isolates and the *T. viride* isolate (PDBCTV4) were superior under greenhouse conditions with PDBCTH 8 showing maximum disease control (66.8%) followed by PDBCTH 7 (66.0%) PDBCTV 4(65.4%), PDBCTH 2 (61.6%) and were even superior to fungicide captan. *G. deliquescens* gave maximum (55.7%) disease control among *Gliocladium* spp.

Prasad and Rangeshwaran (1999) evaluated a modified granular formulation containing powdered wheat bran, kaolin, acacia powder and biomass of isolates of *Trichoderma harzianum* (PDBCTH 10 and PDBCTH 8), *T. virens* (PDBCTV$_S$ 3 and ITCC 4177) and *Gliocladium deliquescens* (ITCC 3450) for their effect on the reduction of chickpea damping off caused by *Rhizoctonia solani.* Granules with all isolates of bioagents significantly reduced damping off. The above two *T. harzianum* isolates were more effective in reducing saprophytic growth of the pathogen compared to other bioagents.

Ahmed *et al.* (2000) studied the effect of pepper seed and root treatments with *Trichoderma harzianum* spores on necrosis caused in stems by *Phytophthora capsici* inoculation and on the course of capsidiol accumulation in the inoculated sites. They suggested that the treatments significantly reduced stem necrosis, which fell by nearly a half compared with the values observed in plants grown from non-treated seeds. Necrosis was also reduced in plants whose roots were drenched with various doses of *T. harzianum* spores. As potential biological agents *T. harzianum* isolate T39 and *T. virens* isolate DAR 74290 controlled the rot disease in potato and tomato caused by *Phytophthora erythroseptica* (Etebarian *et al.*, 2000).

Ramamoorthy *et al.* (2002) characterized twenty isolates of fluorescent pseudomonads and evaluated their ability to control damping-off in tomato (*Lycopersicon esculentum*) and hot pepper (*Capsicum annuum*). Among these isolates, *P. fluorescens* isolate Pf1 showed the maximum inhibition of mycelial growth of *Pythium aphanidermatum* and increased plant growth promotion in tomato and hot pepper. *P. fluorescens* isolate Pf1 was effective in reducing the damping-off incidence in tomato and hot pepper in greenhouse and field conditions. Moreover, the isolate Pf1 induced the production of defense related enzymes and chemicals in plants.

Weller *et al.* (2002) reported the microbial basis of specific suppression to four diseases, *Fusarium* wilts, potato scab, apple replant diseases and take-all disease. One of the best-described examples occurs in take-all decline soils. In Washington State, take-all decline results from the buildup of fluorescent *Pseudomonas* spp. that produce the antifungal metabolite 2,4-diacetylphloroglucinol. The authors suggested that producers of this metabolite may have a broader role in disease-suppressive soils worldwide.

Perelló *et al.* (2003) evaluated the potential of *Trichoderma harzianum, Trichoderma aureoviride* and *Trichoderma koningii* as biocontrol agents of *D. tritici-repentis* under *in vitro* and greenhouse conditions. Dual cultures in petridishes containing potato dextrose agar showed that the isolates of *Trichoderma* spp. tested inhibited significantly the mycelial growth of *D. tritici-repentis* between 50% and 74%. The results of the greenhouse tests indicated that seven strains of *Trichoderma* spp. significantly reduced the disease severity on wheat plants compared with untreated plants.

Perello *et al.* (2006) also evaluated six isolates of *Trichoderma harzianum* and one isolate of *T. koningii* on the incidence and severity of tan spot (*Pyrenophora tritici-repentis*) and leaf blotch of wheat (*Mycosphaerella graminicola*) under field conditions and noticed significant differences between wheat cultivars, inoculum types and growth stages. Three of the isolates tested showed the best performance in controlling leaf blotch and tan spot when coated onto seed or sprayed onto wheat leaves at different growth stages, with significant severity reduction up to 56%. In some experiments, the biocontrol preparation (T2 and T5) gave a level of disease control similar to that obtained with Tebuconazole (70 and 48%, respectively).

Roy *et al.* (2007) isolated a novel indigenous *Pseudomonous aeruginousa* strain from industrial waste water following dilution plate technique in nutrient agar (pH 7) medium. They used the *Pseudononas* strain as biocontrol agent against several species of *Phytophthora* (viz. *P. nicotianea*, *P. capsici*, *P. colocasiea* and *P. melonis*) and effectively controlled their growth.

Patro *et al.* (2008) reported that *Pseudomonous fluorescence* (*Pf* -1@ 0.6%) can be effectively used as a seed treatment and foliar spray for the management of blat in finger millet in addition to the edifenphos (0.1 %).

Jadeja and Bhatt (2008) isolated four *Bacillus* spp from mango fruit surface and tested against *Lasiodiplodia theobromae*, causing stem end rot disease of mango fruit. The application of any of the four Bacillus species on fruits resulted in reductions by more than 50% of the natural incidence of stem end rot.

Umamaheswai *et al.* (2008) evaluated some biocontrol agents like the isolates of *Pseudomonous fluorescence* (PfC6 and PfCIAH- 196), *Bacillus subtilis* (BSW1and BST1), Trichoderma isolates-CIAH 175 and *Trichoderma harzianum* against *Alternaria alternata* in watermelon. Their result showed that the antibiotics produced by B. subtilis caused swelling of the germ tube while *P. fluorescence* modified hypha into a chain of knotted cells. Volatile metabolites of Trichoderma isolate (CIAH-175) caused a maximum reduction in growth of *A. alternata* (92.2%).

Diseases control by botanicals

Plants naturally synthesize several carbon compounds, basically for their physiological functions or for use as chemical weapons against pathogens, insects and predators (Fatope, 1995). It has been estimated that 70-80% of total world population largely depends on traditional herbal medicine to meet their primary

health care need (Hamayun *et al.* 2006). Plants have been proved as useful source of several antifungal molecules that are harmless and benign to the environment. There are certain advantages in the deployment of botanical pesticides. These are biodegradable, safe to non-target organisms, renewable and suit to sustainability of local ecology and environment. Moreover, repeated application of fungicides to attain desirable level of disease control has been discouraged by some of the farmers (Singh and Sinha, 2005).

Terras *et al.* (1993) noticed synergistic enhancement of antifungal activity of wheat thionins by 2- to 72- folds when combined with albumins of radish or rape and being effective against filamentous fungi and some gram-positive bacteria. Permeabilization of the hyphal plasmalemma of thionins has been shown to be the mode of action. Soil amendments with crop residues lead to build up of allelochemicals and plant nutrients. In a comparative study, it was shown that incorporation of straw was found more effective than burning of straw in containing the symptoms of eye spot disease (*Pseudocercosporella herpotrichiodes*) and sharp eye spot disease (*Rhizoctonia cerealis*) of wheat (Prew *et al.*, 1995).

Kirkegaard *et al.* (1996) while evaluating rape and Indian mustard as companion crop showed that the latter was more effective in minimizing the incidence not only of take-all disease of wheat but also *Rhizoctonia solani, Pythium* and *Cochliobolus sorokiniana.* The tissue extract of Indian mustard was equally effective and hence the role of volatile isothiocyanates was implied. Certain phytochemicals like gallic acid and absisic acid have been shown to be antifungal. For instance, absisic acid was shown to inhibit mycelial growth and sporidial formation and also germination of teliopores (Singh *et al.*, 1997)

Bianchi *et al.* (1997) tested *Fusarium solani, Colletotrichum lindemuthianum, Pythium ultimum* and *Rhizoctonia solani* and found that garlic extracts inhibited mycelial development *in vitro.* They also used aqueous extract of powdered oven-dried (35 °C) garlic bulbs incorporated into the growth medium and reported that the hyphae of *R. solani* and *C. lindemuthianum* showed collapse and for *F. solani* hyphae appeared thinner than in controls.

Ali *et al.* (1999) screened hexane and methanol extracts of sixteen plants of the family Caesalpiniaceae, collected around Karachi, Pakistan and were tested for their antibacterial and antimicrobial activity. As compared to hexane extracts, the methanol extracts of all the examined plants showed stronger growth inhibition

against bacteria and fungi, *Cassia* species being the biologically more active plant. Ethanol extract of *Melia azadirachta* fruit showed fungistatic (MIC 50-300 mg/ml) and fungicidal (MFC60-500 mg/ml) activity against *Aspergillus flavus, Fusarium moniliforme, Microsporum canis* and *Candida albicans* (Carpinella *et al.*, 1999).

Digrak *et al.* (1999) studied the antimicrobial activities of *Valex* (the extract of Valonia), the extracts of mimosa bark, gallnut powders, *Salvia ancheri* Benthum. Var. *ancheri* and *Phlomis bourgei* Boiss. The results of the study indicated that mimosa bark extracts had the greatest antibacterial activity, followed by the *Valex,* gullnut powders, *Salvia ancheri* var. *ancheri* and *Phlomis bourgeie* extracts, respectively. Furthermore, it was found that gullnut powders and the extracts of mimosa bark contained high amounts of tannins and showed antifungal activity.

Ke *et al.* (1999) collected two hundred and four species of traditional Chinese herbal medicines belonging to 80 families from Yunnan Province in People's Republic of China and tested for antifungal activities using a *Pyricularia oryzae* bioassay. Twenty-six herbal medicines form 23 families were active against *P. oryzae* and the ethanol extract of *Dioscorea camposita* (dioscoreaceae) exhibited the most bioactivity among the entire tested sample.

Yoshida *et al.* (1999a) isolated three thiosulfinates with antimicrobial activity from oil-macerated garlic extract and their structures were identified by them as 2-propene-1-sulfinothioic acid S-(Z,E)-1-propenyl ester [AllS(O)SPn-(Z,E)], 2-propene sulfinothioic acid S-methyl ester [AllS (O)SMe] and methane sulfinothioic acid S-(Z,E)-1-propenyl ester [MeS(O)SPn-(Z,E)]. Antimicrobial activities of AllS (O) SPn-(Z, E) and AllS (O) SMe against gram positive and gram negative bacteria and yeasts were compared with 2-propene-1-sulfinothioicacids-2-propenylester [AllS(O)SAll, allicin]. Antimicrobial activity of AllS(O) S Me and All S(O)S Pn-(Z,E) were comparable and inferior to that of allicin, respectively. In another study, Yoshida *et al.* (1999b) isolated and identified an organosulfur compound from oil-macerated garlic extract by silica gel column chromatography and preparative TLC. The antimicrobial activity of isoE-10-DA was inferior to those of similar oil-macerated garlic extract compounds such as E-ajoene, Z-ajoene and Z-10-DA.

Demirci *et al.* (2000) collected the leaves of five *Betula* species, *B. pendula, B. browicziana, B. medwediewii, B. litwinowii* and *B. recurvata* from different parts of Turkey. The leaves were hydro distilled to yield the consequent essential oils. The essential oils showed antifungal activity against various phytopathogenic fungi like

Cephalosporium aphidicola, Drechslera sorokiniana, Fusarium solani and *Rhizoctonia cereals.*

Limonene is the major constituent of essential oil of exocarpic part of *Citrus sinensis* which possessed strong and broad-spectrum antifungal activity against important fungal pathogens of sugarcane (Rao *et al.*, 2000). The mycelial growth of *Ceratocystis paradoxa* at 2000 ppm and that of *Fusarium moliniforme* and *Curvularia lunata* at 3000 ppm concentration of limonene were completely inhibited. It proved fungistatic at minimum inhibitory concentration and exhibited non-phytotoxic toon sugarcane germination and growth.

Ogunwande *et al.* (2001) analysed methanol extracts from leaves, stem bark, root bark, fruits and seed kernels of *Butyrospomum pradoxum* (*Vitellaria paradoxa*) and revealed the presence of alkaloids (in leaves and stem barks), flavones (in stem and root bark), saponins (in root bark), steroids (in stem bark, fruits and seed kernels) and tannins (in leaves and root bark) which have antimicrobial activity against bacteria (*Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, Staphylococcus aureus, Ralstonia solanacearum and Bacillus cereus*) and fungi (*Fusarium oxysporum* and *Candida albicans*).

Jaspers *et al.* (2002) studied the control of *Botrytis cinerea* Pers. leaf colonization and bunch rot in grapes with oils in laboratory and field tests. In detached lateral experiments, the essential oils from thyme (*Thymus vulgaris* L.) and clove (*Syzygium aromaticum* L.), as well as massoialactone (derived from the bark of the tree *Cryptocarya massoia* R.Br.) were not phytotoxic on leaves at concentrations of 0.33% or less. *B. cinerea* sporulation on artificially induced necrotic leaf lesions was significantly reduced by thyme (Thyme R) and masoialactone oils at 0.33%. A single application of either compound at concentrations of 0.33% controlled bunch rot and necrotic leaf lesion colonization by *B. cinerea.* Spray applications of Thyme R oil (0.33%) at 8-10 day intervals from flowering to harvest controlled *B. cinerea* bunch rot but also made senesce to floral tissues.

Bautista-Banos *et al.* (2003) also evaluated the *in vitro* fungicidal effect of chitosan and aqueous extracts of custard apple leaves, papaya leaves and papaya seeds, and the combination of chitosan and plant extracts on the development of *Colletitrichum gloeosporioides,* causative agent of anthracnose on papaya. They found that chitosan had a fungicidal effect on *C. gloeosporioides.* Extracts alone did not show any fungicidal effect while the combination of 2.5% chitosan with all the

tested extracts had a fungistatic rather than fungicidal effect. Changes in the conidial morphology of *C. gloeosporioides* were observed with 1.5% chitosan concentration after 7 h incubation. For *in situ* studies, control of anthracnose disease was obtained with 1.5% chitosan applied before *C. gloeosporioides* inoculation

Almada-Ruiz *et al.* (2003) evaluated antifungal activities of four polymmethoxylated flavons, isolated from cold-pressed orange oil against *Colletotrichum gloeosporioides,* a major plant pathogen of fruits that causes significant damage to crops in tropical, sub-tropical and temperate regions. They noticed that methoxylated flavones were effective in inhibiting mycelial growth of the fungus. Complete inhibition of the growth of the pathogenic fungus *C. gloeosporioides* was observed at a concentration of 100 μg ml^{-1}.

Curtis *et al.* (2004) reported that garlic extract showed activity against the plant pathogenic bacteria *Agrobacterium tumefaciens, Erwinia carotovora, Pseudomonas syringae* pv. *maculicola*, *P.s.* pv. *phaseolicola*, *P.s.* pv. tomato, *Xanthomonas campestris* pv. *campestris*, the fungi *Alternaria brassisicola, Botrytis cinerea, Plectosphaerella cucumerina*, *Magnaporthe grisea,* and the oomycete *Phytophthora infestans.*

Peraza-Sánchez *et al.* (2005) screened seven Yucatecan plant extracts to look for fungicidal activity for the control of *C. gloeosporioides.* Bioassay-directed purification of the root extract of one of the most active plants, *Acacia pennatula*, resulted in the isolation of the new compound 15,16-dihydroxypimar-8(14)-en-3-one (1). The isolated compound showed inhibitory activity on growth, sporulation, and germination of the fungus in "agar dilution" bioassay *in vitro.*

Deepak *et al.* (2005) used methanolic extracts of forty plant species commonly growing across India and screened for antisporulant activity against *Sclerospora graminicola*, the causative organism of pearl millet downy mildew. The methanolic extracts of nine species did not show any effect, whereas the activity of the extracts of *Clematis gouriana, Evolvulus alsinoides, Mimusops elengi, Allium sativum* and *Piper nigrum* were commensurable to that of the marketed botanical fungicides. The extracts of 11 species (*Agave americana, Artemisia pallens, Citrus sinensis, Dalbergia latifolia, Helianthus annus, Murraya koenigii, Ocimum basilicum, Parthenium hysterophorus, Tagetes erecta, Thuja occidentalis* and *Zingiber officianale*) exhibited remarkable antisporulant effect even after 10-fold dilution of the crude extracts. But in the case of remaining 15 plants the crude extracts lost activity

after 10-fold dilution. The antisporulant activity of commercialised *Azadirachta* preparation (Nutri-Neem) was more pronounced than that of Reynutria based on (Milsana) and Sabadilla (veratrin).

Guleria and Kumar (2006) searched for bioactive compounds from lipophilic leaf extracts of medicinal plants used by Himalayan people. They screened antifungal properties by direct bioautography. *Alternaria alternata* and *Curvularia lunata* were used as test organism in bioautography. The results were evaluated by the diameter of the of fungal growth. They showed five effective plant species with antifungal activity among the 12 investigated. They used $CHCl_3$: CH_3OH (1:9, v/v) as a solvent to develop silica gel TLC plates. Clear inhibition zones were observed for lipophilic extracts of *Vitex negundo* (RF value 0.85), *Zantoxylum alatum* (RF value 0.86), *Ipomea carnea* (RF value 0.86), *Thuja orientalis* (RF value 0.80) and *Cinnamomum camphora* (RF value 0.89). The best antifungal activity was shown by lipophilic leaf extract of *T. orientalis*.

Kiran, *et al.* (2006) screened thirty plant extracts (aquous extract) against the pathogen *Sclerotium rolfsii in vitro* to examin the inhibitory effect on mycelial growth and sclerotial production. Maximum inhibition (74%) of mycelial growth was recorded at 10% concentration of plant extract (*Prosopis juliflora)*. Other two antifungal plant extracts were from *Agave Americana* (showed 68% overall inhibition) and *Nerium indicum* (showed 54% overall inhibition). The inhibition(94%) of sclerotial production was exhibited by *Agave americana* and almost similar inhibition was shown by *Clerodendron inerme leaf extracts*. Leaf extract of *Riccinus communis* and fruit extract of *Riccinus communis* also gave good results (showed 72%) inhibition.

Reddy, *et al.* (2007) reported the antifungal component of cloves. They isolated, characterized and tested the efficacy of cloves against *Aspergillus* spp. The major component, eugenol was identified on TLC plate as dark coloured spot with R_f 0.5 along with standard. In TLC plate bioautography test, TLC plates were spray inoculated with four species of *Aspergillus* (*A. flavus*, *A. paraciticus*, *A.niger*, *A. ochraceus*) and eugenol on TLC plates inhibited mycelia growth of all four species of *Aspergillus*.

Meena et al.(2007) evaluated antibacterial activity of seven semi purified plant extracts made from flowers, leaves, fruits, stems, pods and seeds of some

plants and four antimicrobial chamicals. The bacterial plant pathogens were *Pseudomonas solanacearum*, *Xanthomonas campestris* pv. *Campestris*, *Xaxonopodis* and *Xanthomonas* pv. *Citri. Evaluation were done* by disc diffusion technique. Product componantes from mahua flowers and Satyanashi leaves were found effective against *Pseudomonas solanacearum* at 1000 ppm.

Mewari *et al.* (2007) screened two mosses viz. *Entodon plicatus* C. Muell and *Rhynchostegium vagans* jaeg for their antimicrobial activity against *Bipolaris sorokiniana* (Sacc. and Sorok), *Fusarium solani* (Mart.) Sacc.(fungi) and *Pseudomonas sclanacearum*, *Xanthomonas oryzae* pv.*oryzae* (bacteria). Aquous extracts of the two mosses were found to be ineffective. Ethanolic extracts of E. plicatus showed maximum inhibition (42%) of *B.Sporokiniana* and petroleum ether extract of *R.vagans* exhibited max. inhibition (45%) of *B.Sporokiniana.* Extract of *R. vagans* were found to be more effective inhibitors of *F. solani* than those of *E. plicatus.* Ethanolic extract of *R.vagans* showed maximum inhibition (44%) of *F. solani* whereas alcoholic extracts of both the mosses showed more effective antimicrobial activity.

Phyton-T, an extract of seaweed (*Sargassum wightii*) reduced disease incidence, induces defense enzymes against late blight of potato caused by *Phytophthora infestants* and enhances quality of potato. Siddagangaiah *et al.* (2008) reorted that tuber soaking and foliar spray in combination with Phyton-T (0.4%) and mancozeb (0.3%) for thrice at 15 days interval reduced the disease incidence up to 80%.

Phytochemical compound and antimicrobial properties of methanolic extracts of *Aspilia mossambicensis* (Compositae) were evaluated by Musyimi *et al.* (2008) against clinical strain of *Streptococcus pyogenes* (gram positive) and *Salmonella typhi* (gram negative) bacteria and one strain of fungi *Aspergillus niger.* Methanolic plant extract of leaves was found to be more active against the three microorganisms than the root extract.

Malabadi and vijay kumar (2007) evaluated the antifungal activities of aceton, hexane, dichloromethen and methanol extracts of leaves of four plant species (*Acacia pennata*, *Anaphylis wightiana*, *Capparis pepiaria* and *Catunaregum spinosa*) against pathogen viz. *Candida albicans*, *Kluyeromyces polysporus*, *Aspergillus niger*,

Aspergillus fumigantus. High antifungal activity was observed with methanolic extract of *Anaphylis wightiana* against all the test pathogens with the MIC values ranging from 0.02 to 0.06. Methanolic extract of *Acacia pennata*, *Anaphylis wightiana*, *Capparis pepiaria* have very stroung antifungal activity against tested pathogens particularly *C. albicans* and *K. polysporus*.

3. Materials and Methods

3.1. Plant materials

3.1.1. Host plants

3.1.1.1. Collection and selection of niger plants

Seeds of different niger varieties (GA-5, GA-10, RCR-18, BN-1, BN-5, Otcmond, NRS 69-1, JNC-6) were collected from All India Coordinated Research project on sesame and niger (Indian Council of Agricultural Research) Jawaharal Nehru Agricultural University campus, Jabalpur-482004, India. One local seed variety was collected from Jalpaiguri, India. Five different niger varieties (GA-5, GA-10, RCR-18, JNC-6, NRS 69-1) and one local variety widely growing in North Bengal (sub-Himalayan West Bengal) were selected for present study. Selection was done on the basis of the seed germination and growing suitability in the environmental and field condition of North Bengal.

3.1.1.2. Cultivation and maintenance of niger seed varieties

Selected niger varieties were cultivated in pots (Plate 3.1). Some of the plants were also cultivated in fields. Earthen pots of 30 cm in diameter were filled with 4 kg of soil mixture. Soil mixture was prepared by mixing 3.5 kg of fine dry soil and 0.5 kg of green manure. In field also same soil mixture was used for growth of the plants. Seeds were surface sterilised with 0.1 % sodium hypochlorite solution and then washed thrice with sterile distilled water. Sterilised seeds were kept overnight in pre-soaked cotton wool for soaking of water. Water soaked seeds were then broadcasted on prepared soil of pots or field. Finally the seeds were covered with 1 cm thick fine moist soil layer. Watering was done as and when required to maintain the moist condition of the soil. Weeds were removed after 15 days. To provide adequate spacing among the test plants, some niger plants were also removed from the pots or fields.

3.1.2. Collection of plants for extraction of botanicals

On the basis of easy availability in the growing areas of sub-Himalayan West Bengal several plant materials were collected from forests and adjoining areas of the region. Different parts of these plants (leaf, bark, root, rhizome as applicable) were extracted and screened for their fungi toxic properties against

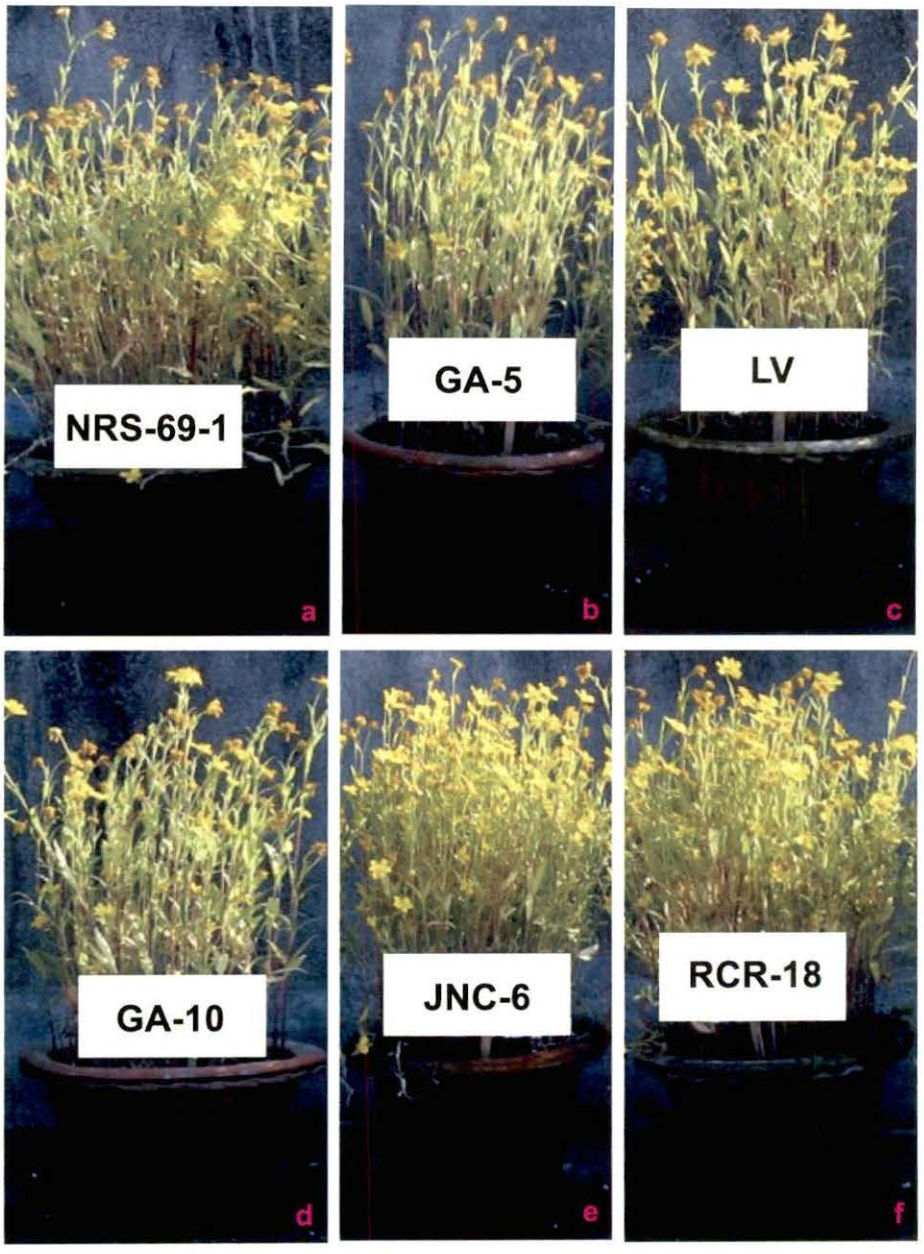

PLATE 3.1

Different varieties of niger in flowering condition

fig. a : NRS-69-1

fig. b : GA-5

fig. c : LV

ig. d : GA-10

fig. e : JNC-6

fig. f : RCR-18

fungal pathogen *Alternaria alternata*. Collected Plants were identified and voucher specimens were deposited in the NBU herbarium, Department of Botany, University of North Bengal.

3.2. Fungal and bacterial cultures used

3.2.1. Source of fungal culture

Three fungal cultures were isolated from the naturally infected leaves of niger plant grown in cultivated fields of North Bengal. Following isolation, the three isolates were subjected to Koch's postulates and have been listed in table 3.1.

Table 3.1: List of fungal cultures isolated from infected niger leaves in North Bengal

Fungal culture	Source	Identified by	Identification no
Alternaria alternata (Fr.) Keissier	Naturally infected niger plant	IARI, New Delhi.	6250.05
Alternaria porri	Naturally infected niger plant	Dr. A. Saha, Dept. of Botany, NBU	-
Aspergillus sp.	Naturally infected niger plant	Dr. A. Saha, Dept. of Botany, NBU	-

**Alternaria alternata* (Fr.) Keissier was used as test pathogen throughout the present study.

3.2.2. Source of microorganisms tested for antagonistic activity

For the study of antagonistic activity two different bacteria and one fungus were isolated from different soil. Three different fungal cultures were procured from Indian type culture collection, IARI, Pusa, New Delhi. One culture of fungal antagonist was collected from Dr. Apurba Choudhury, Uttar Banga Krishi Viswavidyalaya, West Bengal, India. The details of the source of the strains used in the present study are given in Table 3.2.

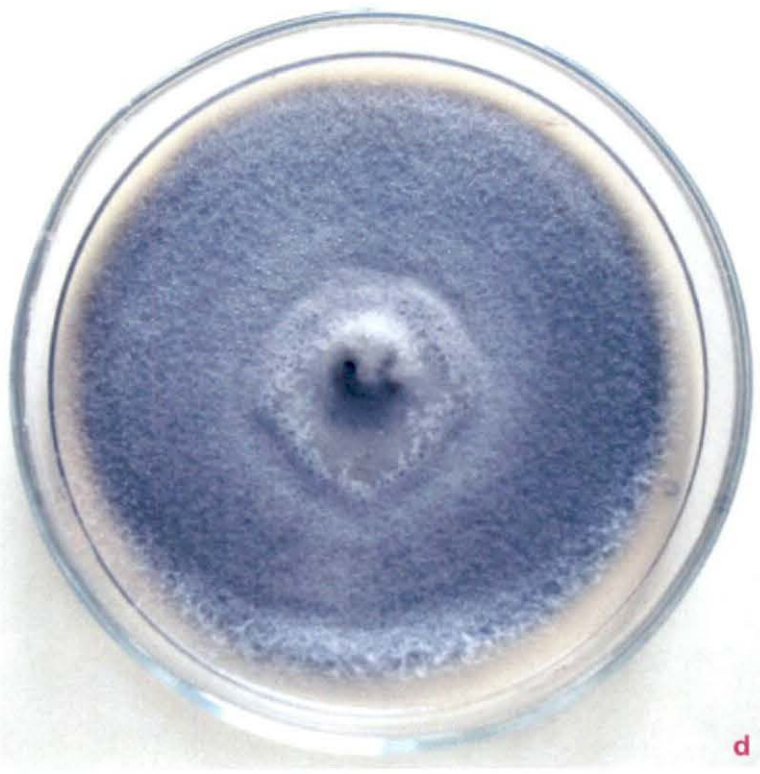

PLATE 3.2

fig. a : Healthy plant.

fig. b : Infected plant.

fig. c : *A. alternata* culture in test tube .

fig. d : *A. alternata* culture in PDA plate.

PDA slants. The inoculated slants were incubated at 28°C and were observed till sporulation. Sporulated cultures were used for microscopic studies. The organisms were confirmed after comparing them with the respective stock cultures. If an organism was consistently re-isolated then it was treated as a pathogen and was identified in the laboratory or elsewhere as mentioned in table 3.1 & 3.2.

3.2.4. Maintenance of stock cultures

Freshly prepared sterile PDA slants were used for the maintenance of the fungal cultures. The bacterial cultures were maintained in nutrient agar as well as in nutrient broth. Pathogens grown on sterile PDA media were stored in two different conditions, viz. at low temperature in refrigerator (at 5°C) and at room temperature. At the interval of two weeks subculture was done for preparation of inoculum for different experiments. Cultures of other fungal and bacterial antagonists were maintained at 5°C and also at room temperature.

3.3. Major chemicals used

In addition to the common laboratory reagents, several chemicals were used during the work. Some of the major chemicals have been enlisted (Table 3.3)

3.4. Composition of media and solutions used

A number of culture media and solutions were used during the present study. The name and compositions of these media and solutions are given below.

POTATO DEXTROSE BROTH (PDB)

Peeled potato	:	40 g
Dextrose	:	2 g
Distilled water	:	100 ml

Peeled potato in required amount was boiled in distilled water. The potato broth was collected by straining through cheese cloth and then required amount of dextrose was added. Finally, the medium was sterilized at 15 lb p.s.i. for 15 minutes.

POTATO DEXTROSE AGAR (PDA)

2% agar powder was added to the final potato dextrose broth solution to prepare potato dextrose agar. The agar was melted by heating the media before sterilization.

Table 3.3: Major chemicals used.

Chemical	Company
3,5- Dinitrosalicylic acid	Hi Media, India
2,1,3-Benzothiodiazole	Fluca,Switzerland.
4- Hydroxybenzoic acid	SRL, India
Acetic acid glacial	E. Merck, India
Acrylamide	SRL, India
Freund's Incomplete Adjuvant	Bangalore Genie
Agarose	SRL, India
Antirabbit IgG	Bangalore Genei
Bromophenol blue	Hi Media, India
Congo red	Hi Media, India
Coomassie brilliant blue	Hi Media, India
di-Sodium tetraborate (Borax)	Merck, India
DL-DOPA	Hi Media, India
Folin and Ciocalteu's phenol reagent	SRL, India
Freund's Incomplete Adjuvant	Difco Lab., USA
Goat anti-rabbit IgG horseradish peroxidase conjugate	Bangalore Genei
2,3-Dihydroxybenzoic acid	Fluca,Switzerland
Glutaraldehyde	Sigma, USA
Glycerol	Merck, India
Glycine GR	Merck, India
Guaiacol	SRL, India
Hydrogen peroxide	Merck, India
L-Alanine	Hi Media, India
Laminarin	Sigma, USA
L-Phenylalanine	SRL, India

Contd...Table 3.3

Table 3.3: (Contd...) Major chemicals used.

Chemical	Company
TEMED	SRL, India
N-Acetyl-D-glucosamine	Hi Media, India
Bisacrylamide	Hi Media, India
O-Dianisidine dihydrochloride	Hi Media, India
p-dimethyl amino benzaldehyde (DMAB)	Hi Media, India
Phenol reagent	Qualigens, India
Polyethelene glycol (PEG)	SRL, India
Polyvinyl pyrrolidone (PVP)	Hi Media, India
Potassium tetraborate	Hi Media, India
Riboflavin	Hi Media, India
Salicylic acid	Qualigens, India
Hydrogen peroxide	Hi Media, India
Sodium tetraborate	SRL, India
Sorbitol powder	Hi Media, India
TMB/H_2O_2	Bangalore Genei,
Triton-X-100	Hi Media, India
Tween 20	Hi Media, India
Immunogold reagent [Affinity isolated aqueous glycerol suspension of antirabbit IgG (Whole molecule)-gold (5nm) from goat]	Sigma, USA
Fluorescent brightener	Sigma, USA
Glycol chitosan	Sigma, USA
Bovine serum albumin	Sigma, USA
2- Aminobutyric acid	Fluca,Switzerland

OAT MEAL AGAR (OMA)

Oat meal	:	40 g
Agar agar	:	15 g
Distilled water	:	1000 ml

Required amount of powdered oat was boiled in distilled water in a water bath, stirred occasionally and strained through cheese cloth. Then agar powder was added to it and melted by heating before the medium was sterilized at 15 lb p.s.i. for 15 minutes.

MALT EXTRACT AGAR (MEA)

Malt extract	:	20 g
Agar	:	20 g
Distilled water	:	1000 ml

Malt extract was dissolved in distilled water by boiling. Then, required amount of agar powder was added. Finally the solution was boiled with constant shaking till the agar was dissolved. Sterilization was done at 15 lb p.s.i. for 15 minutes.

POTATO CARROT AGAR (PCA)

Grated Potato	:	20 g
Grated Carrot	:	20 g
Agar agar	:	20 g
Distilled water	:	1000 ml

Required amount of grated potato and grated carrot were mixed and boiled with distilled water. The broth was strained through cheese cloth and agar powder was added to the filtered broth. Finally, the medium was boiled to dissolve agar before sterilization at 15 lb p.s.i. for 15 minutes.

RICHARD'S SOLUTION / MEDIUM (RM)

Potassium nitrate (KNO_3)	:	10 g
Potassium Dihydrogen Phosphate (KH_2PO_4)	:	5 g
Magnesium sulfate ($MgSO_4$, $7H_2O$)	:	2.5 g
Ferric chloride ($FeCl_3$)	:	0.02 g
Sucrose	:	50 g
Distilled water	:	1000 ml

All the constituents were mixed with required amount of distilled water. Constituents were then dissolved by stirring and sterilized at 15 lb p.s.i. for 15 minutes.

RICHARD'S AGAR (RMA)

2% agar powder was added to the final Richard's solution to prepare Richard's agar. The agar was melted by heating the media before sterilization at 15 lb p.s.i. for 15 minutes.

CZAPEK DOX AGAR (CDA)

Sodium Nitrate ($NaNO_3$)	:	3 g
Potassium hydrogen phosphate (K_2HPO_4)	:	1 g
Potassium Chloride (KCl)	:	0.5 g
Magnesium sulfate ($MgSO_4$, $7H_2O$)	:	0.5 g
Ferrous Sulphate ($FeSO_4$)	:	0.01 g
Sucrose	:	30 g
Agar agar	:	15 g
Distilled water	:	1000 ml

All the ingredients except agar and K_2HPO_4 were dissolved. Then agar was added and dissolved by boiling. Finally K_2HPO_4 was added to the molten solution, mixed thoroughly and sterilized at 15 lb p.s.i. for 15 minutes.

YEAST EXTRACT MANNITOL AGAR (YEMA)

Yeast extract	:	2 g
Mannitol	:	10 g
Potassium Dihydrogen Phosphate (KH_2PO_4)	:	0.5 g
Magnesium sulfate ($MgSO_4$, $7H_2O$)	:	0.2 g
Sodium Chloride (NaCl)	:	0.1 g
Agar agar	:	20 g

All the ingredients except agar were dissolved in distilled water. Finally, agar was added and dissolved by boiling before the medium was sterilized at 15 lb p.s.i. for 15 minutes.

NUTRIENT BROTH

Beef extract	: 3g
Peptone	:10g
NaCl	: 5 g
Distilled water	1000ml

Required amount of beef extract and peptone were dissolved in distilled water by heating. Then NaCl was added. The pH of the solution was adjusted to 7.2 with 10 (N) NaOH. Finally the medium was sterilized at 15 lb p.s.i. for 15 minutes.

NUTRIENT AGAR (NA)

2% agar powder was added to the final nutrient broth solution to prepare nutrient agar. The agar was melted by heating the media before sterilization.

3.5. Inoculation technique

Pathogenecity test was done by artificial inoculation of different varieties of niger plants with the fungal pathogen following the inoculation technique of Dhingra and Sinclair (1995). Inoculation was done by spraying spore suspension bearing $1x10^5$ conidia / ml prepared from 10 d old culture of the pathogen grown on sterile potato carrot agar (PCA) slants. Conidial suspension was sprayed in experimental plants but sterile distilled water was sprayed in control plants by separate automizers. For each treatment ten plants of each variety were taken in experimental pots and were kept for 48 hours in transparent polythene chamber (to maintain high humidity) under normal conditions of light and temperature. The entire experiment was repeated thrice.

3.6. Disease assessment

Numbers of lesions produced on the leaves were counted and diameter of each lesion was measured after 2, 4, 6, 8, and 10 days of inoculation. The results were assessed following Sinha and Das (1972). Lesions were grouped into four categories and value was assigned to each category on the basis of visual observations. The categories are as follows:

- Minute restricted lesions (1-2 mm dia..... 0.10
- Small lesions with sharply defined margins (2-4 mm diameter) 0.25
- Medium slow spreading lesions (4-6 mm diameter) 0.50
- Large spreading lesions of various size with diffused margin (Beyond 6 mm diameter) 1.00

Number of lesions in each category was multiplied by the value assigned to it and the disease index for a plant was calculated as the sum total of such values for all the eaves. Results were computed as the mean of observations of three replications (10 plants in each treatment).

3.7. Morphology and physiology of the pathogen *Alternaria alternata*

3.7.1. Microscopy

Alternaria alternata was sub-cultured in PCA and PDA for ten days. A bit of fungal mycelia was taken from PDA slants, placed on a clean grease free slide and stained with lacto phenol and cotton blue. For study of spores, brown/gray masses of spores distinctly produced on the surface of the PCA slants were carefully taken out with the inoculating needle, placed on a slide and stained with lacto phenol and cotton blue. The slides were observed under light microscope (Olympus, India). Length and breadth of spores, breadth of mycelia etc. were measured by ocular micrometer standardized by stage micrometer. The details of the morphology of the fungus were noted.

3.7.2. Assessment of mycelial growth

Initially, Petri plates with sterile PDA or other media were inoculated with the test fungus (*Alternaria alternata*) and incubated for 7 days at 28 ± 1^{0}C for growth of fungus in the form of mycelia mat. The mycelia mats thus grown in Petri plates were used as inoculum. Mycelia block (4 mm) was cut from the advancing zone of hyphae and was placed one in each Petri plate of 90 mm diameter. The inoculated Petri plates were incubated for 10 days at 28 ± 1^{0}C. Radial growths of mycelia were measured at 2 days intervals to assess the mycelial growth in different solid media.

To assess the mycelial growth in liquid media, mycelia blocks (4 mm diameter) were obtained similarly as mentioned above and were transferred to conical flasks of 250 ml, each containing 50 ml of sterilized liquid medium and incubated at 28 ± 1^{0}C or stated otherwise. The growing fungal mycelia were strained through double-layered cheese cloth after 5, 10, 15, 20 and 25 days of incubation, then blotted on a blotting paper and dried in a hot-air oven at 60^{0}C. The dried mycelia mats were allowed to cool and then dry weight was taken in a precision balance.

3.7.3. Assessment of spore germination

3.7.3.1. Preparation of spore suspension

The fungus was initially cultured in PCA for 14 days at 28±2^{0}C for adequate sporulation. For preparation of spore suspension 4-6 ml (approx.) sterile distilled water was added aseptically and the surface of the fungal culture was gently scrapped using a sterile inoculation needle to loosen the spores only. The tubes were gently shaken for appropriate mixing of the spores with distilled water. The resultant mixture was strained through a muslin cloth and the filtrate was used as spore suspension. Sterile distilled water was added to the suspension to adjust the concentration of the spores following haemocytometer count.

3.7.3.2. Spore germination assay

Assessment of spore germination of the test pathogen was done under various conditions but the basic methodology is essentially the same and is described here. Spore suspension (30 µl) of fungus was placed on slides and the slides were incubated in a humid chamber (in Petri plates) at 28±1^{0}C. The slides were stained with cotton blue-lacto phenol after the desired period of incubation and observed under light microscope. Finally, the percent spore germination [(No. of germinated spores / no. of spores counted) x 100], average germ tube length in each case were calculated for each slide. Percent spore germination was calculated on the basis of 300 spores and average germ tube length was calculated based on 60 germ tubes.

3.8. Collection and maintenance of rabbits for raising antisera

Three New Zealand male white rabbits were used for raising polyclonal antibody in the study (Plate 3.3, fig a). The rabbits were purchased from the laboratory animal supplier and brought to the laboratory at least a month before starting any immunization programme for proper acclimatization. The body weights of rabbits were approximately 1.5 kg and their age was around 9 months. The rabbits were kept in separate cages (75 cm x 75 cm x 90 cm) attached with plastic trays at the bottom and placed in a well-ventilated room. The room was cleaned regularly with permitted room freshening solutions. Rabbits were fed with sufficient quantities of vegetables like soaked gram (*Cicer arietinum* L.), carrot (*Daucus carrota* L.), common grass [*Cynodon dactylon* (L.) Pers.], cabbage

leaves (*Brassica oleraceae* L. var. *capitata*), cauliflower leaves (*B. oleraceae* L. var. *botrytis*), lettuce leaves (*Lactuca sativa* L.) etc. and clean water. The quantity of food was adjusted after a consultation with local veterinary doctor. Routine healths checking of rabbits were also done. The immunization program was started after one month after ascertaining complete body fitness of rabbits.

3.9. Antigen preparation

3.9.1. Antigen preparation from niger leaf

For preparing leaf antigens from young niger leaves, the protein extraction procedure of Alba and DeVay (1985) and Chakraborty and Saha (1994) was followed. For this, fresh young leaves of the each niger varieties were collected from the experimental garden, washed thoroughly with cold water and kept for 2 hours at -20^{0}C. The Frozen leaves (20 g fresh weight in each case) were grounded in prechilled mortar at 4^{0}C with 10 g insoluble polyvinyl pyrrollidone (PVP). The leaf paste was suspended in cold 0.05 M sodium phosphate buffer (pH 7.0) containing 0.85% sodium chloride and 0.02 M ascorbic acid. Then the leaf slurry was strained through cheese cloth and centrifuged at 4^{0}C for 30 min at 12,000 g. The supernatants were collected and ammonium sulphate was added at 4^{0}C to 100% saturation under constant stirring. It was allowed to stand overnight at 4^{0}C and finally centrifuged at 4^{0}C for 15 min at 12, 000 g. The precipitate obtained was dissolved in cold 0.05 sodium phosphate buffer (pH 7.0) and was dialyzed against 0.005 M sodium phosphate buffer (pH 7.0) for 24 hours at 4^{0}C with 16 changes. Finally, the preparation was centrifuged at 4^{0}C for 15 minutes at 12,000 g and supernatant was stored at -20^{0}C until required.

3.9.2. Antigen preparation from fungal mycelia

Antigens from fungal mycelia were prepared following the method as described by Chakraborty and Saha (1994) with some modifications. Mycelial discs (4 mm diameter) from 7 d old PDA culture plates were transferred to 10 conical flasks of 250 ml capacity, each containing 50 ml of sterilized PDB medium and incubated at 28$\pm$1^{0}C. The fungal mycelia were harvested after 15 days, washed with 0.2% NaCl and rewashed with sterile distilled water. Mycelia (50 g fresh weight) were homogenized in 0.05 M sodium phosphate buffer (pH 7.4) containing 0.85% NaCl in a mortar and pestle with sea sand and kept overnight at 4^{0}C. Centrifugation of the homogenates was done at 4^{0}C for 30 minutes at

12,000 g. The supernatants were collected and equilibrated to 100% saturated $(NH_4)_2SO_4$ under constant stirring and again kept overnight at 4^0C. Then, the mixtures were centrifuged at 4^0C for 30 minutes at 12,000 g. Precipitates were dissolved in 5 ml cold sodium phosphate buffer (0.05 M, pH 7.4) after discarding the supernatants. The preparations were dialyzed for 24 hours at 4^0C against 0.005 M sodium phosphate buffer (pH 7.4) with 8 to 10 changes. Following dialysis, the preparations were centrifuged at 4^0C for 15 minutes at 12, 000 g and resultant supernatant were stored at -20^0C until further use. Protein content of both the plant and fungal antigens were determined by the methods of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

3.10. Raising of polyclonal antisera

3.10.1. Immunization of rabbits

Normal sera were collected from each rabbit before immunization. Antisera against antigens of resistant and susceptible host varieties of niger and pathogen (*A. alternata*) were raised in separate rabbits by giving intramuscular injections (Plate 3.3, fig b) 1 ml of antigens emulsified with equal volume of Freund's complete adjuvant. The doses were repeated at 7 days intervals with Freund's incomplete adjuvant for 6 consecutive weeks. The final protein concentration was 5 mg/ml in the emulsion. Blood samples were collected on the 4^{th} day after the last injection and kept at 37^0C for one hour. After incubation antisera were separated from the blood coagulates by the process as mentioned in the following paragraph.

3.10.2. Collection of normal sera and antisera

Blood from rabbits was taken by marginal ear vein puncture and blood was collected in tubes (Plate 3.3, fig c & d). For this, the rabbits were first taken out from the cage, placed on a table and the hairs from the margin of an ear were removed with a sterilized blade. The marginal ear vein was rubbed with xylene-soaked cotton wool to excite blood flow in the veins. The excited ear vein then disinfected with rectified sprit. An incision was made with a sharp sterilized blade on the vein of the ear and about 10 ml blood was collected in a sterile centrifuge tube. Precautions (by using surgical tapes etc.) were taken to stop the flow of the blood from the punctured area of the ear after taking blood. The blood samples were kept undisturbed for an hour at 37^0C for clotting. In order to avoid the loss of

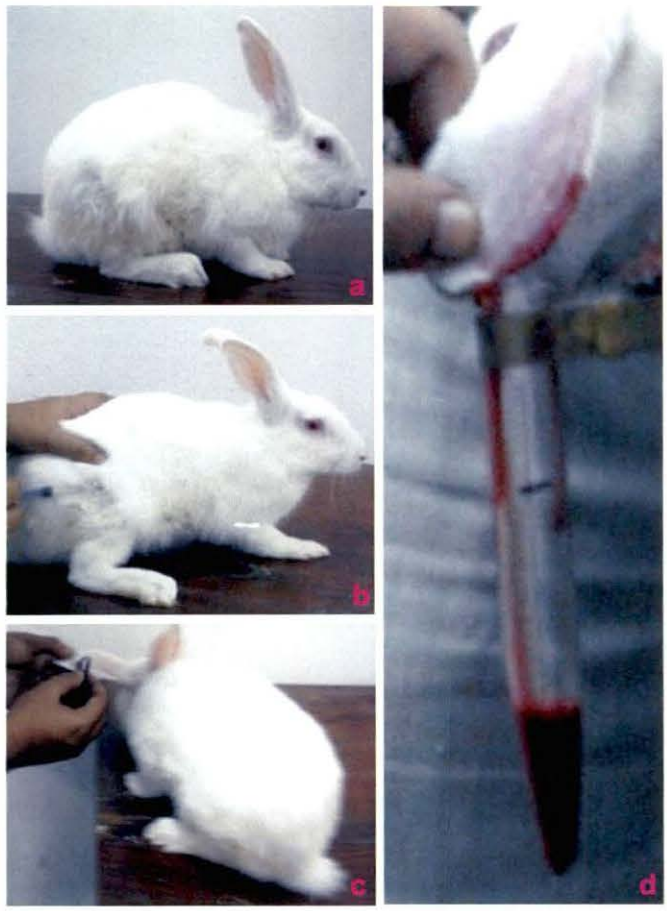

PLATE 3.3

fig. a : Newzealand white male rabbit (approximately 9 month old and 1.2 kg body weight).

fig. b : Intramuscular injection of rabbit

fig. c : Marginal ear vein puncture

fig. d : Collection of blood

serum included within the clot, it was carefully loosened from the glass surface by turning a sterile wooden stick around the clot. Normal sera as well as antisera were clarified by centrifugation at 4^0C for 10 minutes at 3,000 g and were distributed in sterile cryo-vials and stored at -20^0C until required.

3.11. Determination of titre value

Titres of antisera against the homologous antigens and titres of antigens against homologous antisera were determined following immunodiffusion technique as described by Ouchterlony (1967) and Clausen (1969). A constant amount (5 µl) of undiluted antiserum or antigen was placed in the central well, while diluted antigens or antisera (diluted with normal saline with ratios 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128 respectively) were pipetted into the outer well. Diffusion was allowed for 48-72 h at 25^0C in a humid chamber. The highest dilution of antiserum or antigen that reacted with antigen or antiserum giving precipitin lines was determined as titre value.

3.12. Immunotechniques

3.12.1. Immunodiffusion (ID)

Agar gel double diffusion test was performed following the method of Ouchterlony (1958).

3.12.1.1. Agarose coated slide preparation

For preparation of agarose coated slide, barbital buffer (50 ml, 0.05 M, pH 8.6) was taken in a 100 ml Erlenmeyer flask and was placed in a boiling water bath. Agarose (0.4 g) was mixed with hot barbital buffer. The buffer-agarose mixture was carefully placed on water bath. Finally a clear molten agarose solution was prepared. To this, 0.1% (w/v) sodium azide (a bacteriostatic agent) was mixed and the agarose solution was dispensed carefully in clean, dry square glass plates of 6 cm x 6 cm so that no air bubble remained trapped in the agarose medium to avoid asymmetrical diffusion. Before dispensing the molten agarose solution, the glass plates were washed with extran solution and water and then the glass plates were serially dipped in 90% ethanol, ethanol: ethyl ether (1:1, v/v) and ether for removal of grease.

3.12.1.2. Diffusion

In order to perform the experiment, glass plates were placed inside sterile Petri dishes. Four to six wells of 6 mm diameter were cut by a sterile cork borer. The distances of the peripheral wells from the central wells were approximately 5 mm. The antigens, normal sera and undiluted antisera were placed with a micropipette directly into the appropriate wells and diffusion was allowed to continue in humid chamber at 25^{0}C for 48-72 hours. After proper staining of the slides, the precipitation reaction was observed only in cases where common antigens were present.

3.12.2. Immunoelectrophoresis (EI)

3.12.2.1. Slide preparation

Rectangular glass pieces (8 cm x 3.0 cm) were made grease free to perform immunoelectrophoresis. The slides were dried and placed on a clean surface. Thin and uniform layers (2 mm thick) of molten agarose medium (0.8%), containing 0.1% sodium azide in 0.05 M barbital buffer (pH 8.6) were dispensed on each slide, taking care so that no air bubbles were trapped in the agarose medium. This was necessary in order to avoid irregularity that may cause asymmetrical migration and diffusion during later stages. The slides were stored at 4^{0}C in Petri dishes until use.

3.12.2.2. Electrophoresis

Two central wells of 4 mm diameter were dug out from each agarose-coated slide before they were placed in the middle compartment of the electrophoretic platform. The anode and cathode chambers were filled with 0.05 M barbital buffer (pH 8.6). Different antigens were placed into separate wells. To trace the electrophoretic movement of the antigens, bromophenol blue was used as marker. Filter paper (Whatman no-1) strips were soaked in buffer and placed on both ends of the slides, which connected the buffer solution of anode and cathode compartments with the agarose surface of the slides. An electric current of 2.5 mAmp / slide; 10 V/cm was passed through the slides for approximately two hours at 4^{0}C. The current was discontinued and the glass slides were taken out when the bromophenol blue marker reached near the short edge of the glass slides.

3.12.2.3. Diffusion

After electrophoresis, a longitudinal trough parallel to the long edge of the slides was cut between the two central wells of the agarose surface and undiluted antiserum was placed into the trough. Diffusion was allowed to continue up to 72 h in a moist chamber at 25^{0}C. Precipitation arcs if formed were recorded.

3.12.2.4. Staining of slides

After immunodiffusion and immunoelectrophoresis the glass slides were washed with 0.9% aqueous NaCl carefully for 48 hours to remove all the unreacted antigens and antisera widely dispersed in the agarose surface. Next, the slides were washed with distilled water for three hours to remove the NaCl and dried at 40^{0}C for 30 min. Then, the slides were stained either with 0.5% coomassie blue or 0.5% amido black (0.5 g coomassie blue/amido black, 5 g $HgCl_2$ and 5 ml glacial acetic acid dissolved in 95 ml distilled water) for 30 minutes at room temperature. Following that, the slides were washed thrice with 2% v/v acetic acid for 3 h (one hour each time) to remove the excess stain. Finally the slides were washed with distilled water and dried at 40^{0}C for 30 min. The plates were then photographed using a cannon digital camera (Canon, A310).

3.13. Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA)

Indirect ELISA was performed after combining the methods of Koenig and Paul (1982) and Dasgupta *et al* (2005). At first, antigens were diluted with coating buffer [carbonate buffer (0.1 M), pH 9.6] and 100 µl of each diluted antigens was placed on the wells of a flat bottomed micro titre ELISA plate (Tarsons), except one well which was considered as blank. The plate was incubated for 6 hours at 4^{0}C in refrigerator. After incubation, the plate was taken out and each well of the plate was flooded with phosphate buffer saline (PBS) -Tween (0.15 M PBS + 0.8% NaCl + 0.02% KCl + 0.05% Tween 20) and washed thoroughly four to five times. The plate was dried in air after washing. Following this, 100 µl of PBS-BSA (0.15 M PBS containing 1% BSA) was added to each well to coat all the unbound sites and incubated for 2 h at room temperature. The plate was again washed with PBS-Tween, air-dired and 100 µl of diluted antisera (diluted with PBS-Tween) was added to each well except the blank and the control wells where normal sera was added (serially diluted with PBS-Tween containing 0.5% BSA).

The plate was incubated overnight at 4^0C. Next day, thorough washing of the plate was done with PBS-Tween. After washing and drying, 100 µl (1:10000) goat-anti rabbit IgG-Horse radish peroxidase conjugate was added to each well except the blank and the plate was incubated for 2 h at 30 ± 1^0C. After incubation, the plate was again washed with PBS-Tween and shaken to dryness. Then 100 µl (1:20) tetramethyl benzidine / hydrogen peroxide (TMB/H_2O_2), a chromogenic substrate was added to each well except the blank. A blue colour was produced due to the reaction between the enzyme and the substrate. Finally, the reaction was terminated after 30 min by adding 100 µl of 1(N) H_2SO_4 to each well except blank. Absorbance values were recorded in an ELISA reader (Mios Junior, Merck) at 492 nm.

3.14. Immunogold labelling followed by silver enhancement

Fresh healthy leaves of niger varieties were collected and washed thoroughly. The plant parts were kept at 4^0C before use. Thin cross sections of leaves were cut and placed on clean grease free slides. In case of fungus, mycelia as well as spores were taken by a needle and placed on slides containing Mayer's albumin for proper fixing. Water drops (100 µl) were mounted on each section. The slides were incubated at 2-5^0C for 30 min and excess water surrounding the sections was blotted off. Thirty micro litre of blocking buffer (0.15 M PBS pH 7.2 containing 5% normal sera of goat) was placed on the cross sections and incubated for 10 min. Excess solution was wiped off and primary polyclonal antibody (1:50 dilution) raised in rabbit against target antigens were applied on the sections and incubated overnight at 25^0C. After incubation, the sections were carefully rinsed in 0.15 M PBS (pH 7.2) for 4 min. Excess buffer was poured off by tilting the slide slightly. Next, 100 µl of diluted (1:50) immunogold reagent containing 0.5 nm gold particle (Sigma, USA) was applied on the sections. Following incubation for 1 h the sections were again rinsed for 4 minutes with PBS. Excess buffer was poured off and the sections were fixed in 200 µl of PBS-glutaraldehyde (2.5% glutaraldehyde solution in PBS) for 15 min. The sections were rinsed in distilled water and placed on slides for silver enhancement. For this, silver enhancement kit of Sigma (Product No. SE-100) were used. Initially solution A (silver salt) and solution B (an initiator) were mixed (1:1) according to the manufacturer's instruction. Mixed solution (100 µl) was used to flood each section. After 5 min of incubation, the cross sections were washed with distilled water. Distilled water was poured off and 100 µl of sodium

thiosulphate solution (2.5 % aqueous) was placed on the sections and allowed to incubate for 3 minutes. The sections were again washed in distilled water and mounted on slides with a drop of distilled water. Immediately after the staining, photographs were taken in a binocular light microscope (Leica, Germany) using digital camera (Canon, A310) with appropriate attachment system.

3.15. Preparation and application of chemical and botanical inducers

3. 15.1. Preparation of plant extracts as inducers

Several plant materials were collected from the nearby forests and hills. Freshly harvested leaves (50g) were crushed in 100 ml distilled water using mortar and pestle at 4^0C. The crushed slurry was strained through muslin cloth and then centrifuged at 3,000 g at room temperature. Finally the supernatant was stored at 4^0C until used.

3.15.2. Application of chemicals and botanicals as elicitors on niger plants

Niger plants were taken in pots of 30 cm diameter. Aqueous leaf extracts were applied on lower leaves of niger plants of 2 months old using hand sprayer leaving 3-4 topmost leaves. The treated plants were labelled accordingly. In addition, Four different chemicals (salicylic acid, 2, 3-dihydroxy benzoic acid, 2, 1, 3-benzothiadiazole, and 2-amino butyric acid) at a concentration of 10^{-3}M, were also used in the similar way as mentioned for leaf extracts. To ensure adhering both the chemicals and botanical elicitors were supplemented with Tween-20 before spraying. After treatment, treated lower leaves were inoculated with conidial suspension ($1x10^5$conidia/ml) of *Alternaria alternata*. Two control sets (treated-uninoculated and untreated-uninoculated) were also maintained for each treatment to compare. The whole experiment was performed in a sterile environment to avoid contamination. Untreated and uninoculated upper leaves were harvested (for experimental purpose) after 0d, 2d, 4d, 6d and 8d following inoculation by *Alternaria alternata*.

3.16. Extraction and estimation of defence related enzymes

3.16.1. Peroxidase (PO): Peroxidase activity was determined according to the procedure given by Hammerschmidt *et al.* (1982). Freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 0.1M sodium phosphate buffer (pH 6.5) at 4^0C. The homogenate

was then filtered through four-layered muslin cloth and the filtrate was centrifuged at 6,000 g at 4^0 C for 15 min. Supernatant obtained was considered as crude enzyme. For estimation of the enzyme activity, 1.5 ml of 0.05M guaiacol and 200 µl of extracted crude enzyme was mixed in a cuvette. The cuvette was then placed in a UV-VIS Spectrophotometer (Model no.118, Systronics, India) and the initial reading adjusted to zero at 420 nm. Then 100 µl H_2O_2 (1% v/v) was added to the cuvette and the changes in absorbance values were recorded for 5 min at 1min intervals. The enzyme activity was expressed in unit enzyme activity. Change in absorbance [Δ A_{420} $min^{-1}g^{-1}$ fresh weight tissue] of 0.001 were considered as unit of enzyme activity.

3.16.2. Polyphenol oxidase (PPO): The method of Sadasivam and Manickam (1996) was followed for determination of Polyphenol oxidase activity. Freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 5 ml of 50 mM Tris-HCl buffer (pH 7.2) containing 0.4 M sorbitol and 1.0 mM NaCl. The homogenate was centrifuged at 12,000 g at 4^0 C for 10 min and supernatant was considered as crude enzyme. For estimation, 2.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 0.2 ml crude enzyme was mixed in a cuvette. Then the cuvette was placed in a UV-VIS Spectrophotometer (Model no.118, Systronics, India) and the initial reading was adjusted to zero at 495 nm. Following that, 0.3 ml of 0.01M catechol was added to the reaction mixture cuvette and the changes in absorbance were recorded at 1 min intervals up to 5 min. The enzyme activity was expressed as change in absorbance [Enzyme activity = K X (ΔA min^{-1}) µMol min^{-1} g^{-1} fresh weight tissue (K= 0.272 for polyphenol oxidase)].

3.16.3. β-1, 3-glucanase: Colorimetric method of Pan et al., 1991 was followed for the determination of the activity of β-1,3-glucanase. The method is also called as laminarin-dinitrosalicylate method. Freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 5 ml of 0.05 M Sodium acetate buffer (pH 5.0) at 4^0C and filtered through four-layered muslin cloth. The filtrate was then centrifuged at 10,000 g at 4^0 C for 15 min. The supernatant was used as crude enzyme. To estimate the enzyme activity, 15.6 µl crude enzyme extract was added to 15.6 µl of 4% Laminarin (Sigma, USA) and was incubated at 40^0C for 10 min. The reaction was stopped by addition of 94 µl of dinitrosalicylic acid reagent followed by heating for 5 minutes on a boiling water bath. The final colour of the solution was diluted with

1 ml distilled water and absorbance values were recorded at 500 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed on fresh weight basis (nmol min^{-1} mg^{-1}) using D-Glucose as standard.

3.16.4. Phenylalanine ammonialyase (PAL): PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Sadasivan and Manickam (1996). For that, freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 5 ml of 0.25 M borate buffer (pH 8.7) at 4^0C. The homogenate was then filtered through four-layered muslin cloth and centrifuged at 12,000 g at 4^0C for 15 min. The yellowish green supernatant was used as crude enzyme extract. For estimation of the enzyme activity, 0.5 ml borate buffer, 0.2 ml crude enzyme, 1.5 ml distilled water and 1 ml of 0.1 M L-phenylalanine were mixed and was then incubated for 30 min at 30^0 C. After incubation, the reaction was stopped by adding 0.5 ml of 1M Trichloroacetic acid. The absorbance values were recorded at 290 nm in a UV-VIS Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed as µMol $min^{-1}g^{-1}$ fresh weight tissue using trans-cinnamic acid as standard.

3.16.5. Chitinase: The colorimetric assay of chitinase was carried out according to the procedure developed by Mahadevan and Sridhar (1982) with some modifications. Freshly harvested niger leaves (1 g) were instantly dipped in liquid nitrogen and after 10 min the frozen leaves were crushed in 5 ml of 0.5 M sodium acetate buffer (pH 5.2) containing 700 mg of PVP using mortar and pestle at 4^0C. The homogenate was then filtered through four-layered muslin cloth, centrifuged at 10,000 g at 4^0C for 15 min and the supernatant was used as crude enzyme source. For estimation, the assay mixture consisted of 0.5 ml crude enzyme, 0.25 ml of 0.1 M Sodium acetate buffer (pH 5.2) and 1ml colloidal chitin (1.8 mg/ml) was incubated at 37^0 C for 2 h. One ml of reaction mixture was taken and 1ml of distilled water was added to it. The mixture was boiled for 10 min in a boiling water bath and centrifuged at 5,000 g for 3 min. from the mixture, 0.5 ml of the supernatant was taken and 0.1ml of 0.8 M Potassium tetraborate was added to it. This was then boiled exactly for 3 min on a water bath and cooled. After cooling, 3 ml of p-dimethyl amino benzaldehyde (DMAB) reagent was added and incubated at 37^0 C for 20 min. Immediately after incubation the mixture was cooled and absorbances were recorded at 585 nm in a UV-VIS

Spectrophotometer (Systronics, Model no.118, India). Enzyme activity was expressed as mg GlcNAc g^{-1} fresh weight tissue h^{-1}.

3.17. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was done following the method as described by Davis (1964) with some modifications. The compositions of the solutions used for preparing resolving and stacking gels were as follows:-

Solution A

Acrylamide = 30 g, Bis acrylamide = 0.8 g and Distilled water = 100 ml

Solution B

Tris = 18.15 g, 1(N) HCl = 24 ml. Temed = 0.4 ml. and Distilled water = 100 ml. (pH to be adjusted to 8.0)

Solution C

Ammonium persulphate = 60 mg and Distilled water = 100 ml. (to be prepared freshly every time)

Solution D

Acrylamide = 5 g, Bis acrylamide = 1.25 g and Distilled water = 100 ml

Solution E

Tris = 2.10 g, 1(N) HCl = 13 ml. Temed = 0.2 ml. and Distilled water = 86.8 ml. (pH to be adjusted to 6.7)

Solution F

Riboflavin = 2 mg and 2 (M) Sucrose solution = 100 ml.

Preparation of gel: A mini slab gel (8X5 cm) was prepared for isozyme analysis by PAGE. For preparation, two glass plates ware thoroughly cleaned with dehydrated alcohol to remove any traces of grease and air-dried. Spacers (1.5 mm) were placed between the two glass plates on three sides and sealed with 1% agar solution. The slabs were tightly clipped to prevent any leakage of the gel solution while casting the gel. A 10% resolving gel was prepared by mixing solutions A, B and C in the ratio 1:1:1 by a pipette leaving sufficient space (for

comb + 1 cm) for the stacking gel. After pouring the resolving gel solution, it was immediately over layered with water and allowed to polymerize for 1.5 - 2 hours. The stacking gel (4%) solution was prepared by mixing solutions D, E and F in the ratio 2:1:1. The over layered water from the resolving gel was decanted off and the stacking gel was poured on the resolving gel followed by immediate insertion of the comb. Again the gel was over layered with water. The gel was kept for 30 min in strong sunlight. After polymerization of the stacking gel, the comb was removed. The gel was then finally mounted into the electrophoresis apparatus. Chilled tris-glycine running buffer (pH 8.3) was added sufficiently in both upper and lower reservoirs. Any bubbles, trapped at the bottom of the gel, were carefully removed with a bent syringe.

Sample preparation: During sample preparation, ice-cold conditions were maintained throughout the entire process. The samples were prepared by mixing the enzyme extracts (40 µl) and gel loading dye (25 µl). The gel loading dye consisted of 40% sucrose and 1% bromophenol blue in double distilled water. The samples (30 µl) were immediately loaded in a pre-determined order in to the wells with a microlitre syringe.

Electrophoresis: The gel was incubated at 4^0C supplying a constant current of 2.5 m Amp per well continuously for 3-4 hours until the dye front reached the bottom of the gel.

3.18. Analysis of isozyme pattern

3.18.1. Peroxidase: Peroxidase activity was determined following peroxidase specific staining method as described by Patel and Anahosur (2001). After electrophoresis, gels were incubated for 30 min in 0.25% guiaicol solution. After incubation gels were transferred to 0.3% hydrogen peroxide solution and kept for 15 min. Radish brown bands appeared gradually on the gel.

3.18.2. Polyphenol oxidase: Polyphenol oxidase specific staining method as described by Mahadevan and Sridhar (1996) was followed to determine the enzyme activity on PAGE gel. After electrophoresis, the gels were incubated in 0.1M Phosphate-citrate buffer (pH 7.0) for 10 min. Then the gels were transferred in 0.01M DL-3,4-Dihydroxyphenylalanine (DOPA) in the same buffer and incubated under aeration until the bands developed.

3.18.3. β-1,3-glucanase: β-1,3-glucanase specific staining procedure described by Bargabus *et al.* (2002) was followed to detect the enzyme activity on PAGE gel. Following electrophoresis, gels were washed thoroughly with distilled water. After washing, gels were incubated in 0.1M citrate buffer (pH 4.8) containing 0.25% Laminarin at room temperature for 20 minutes. Then the gels were transferred to 0.1% Congo red solution and incubated overnight with constant shaking at room temperature. After proper staining, the gels were destained with 1M NaCl solution. β 1,3 glucanase activity was observed by the formation of yellow orange bands on a reddish purple background.

3.18.4. Chitinase:

Method 1: Chitinase activity was detected on the PAGE gel following the method of Trudel and Asselin (1989) with some modifications. After electrophoresis, gels were incubated in 150 mM sodium acetate buffer (pH 5.0) for 5 min and then in 100 mM sodium acetate buffer (pH 5.0), containing 0.01% glycol chitin for 30 min at 37^0C. The gels were finally transferred into 500 mM tris-HCl (pH 8.9) solution containing 0.01% (w/v) fluorescent brightener 28 (Sigma) and incubated for 5 min. After 5 min the brightener solution was removed and gels were rinsed with distilled water for 1 hour. Finally, lytic zones were observed under UV transilluminator. Results were recorded photographically and R_f of different bands were determined.

Method 2: Alternatively, 'Agar plate polyacrylamide gel assay' was also done to visualize the chitinase bands more prominently as suggested by Gohel *et al.* (2005). Chitin agar plates were prepared by 1% agar, 0.5% glycol chitin and 0.001% (w/v) Fluorescent brightener 28. After electrophoresis of the native PAGE gel slab was directly transferred onto a chitin agar plate. Thin layer of 0.2 M acetate buffer (pH 5.0) was put on each gel for diffusion of chitinase into the chitin agar plate. The plate was then incubated at 37^0C. Activity bands were visible by the formation of dark bands/zones against fluorescent background on chitin agar plate with Fluorescent brightener 28 after7 hours. The plate was observed under UV-transilluminator.

3.19. Evaluation of biocontrol agents

3.19.1. Dual culture test

Microbial antagonists were screened following the dual culture method of Johnson and Curl (1972) for their biocontrol potential. In this technique, both the

test pathogen and biocontrol agents were grown simultaneously in the same plate. Discs (4 mm) cut from 7 days old cultures of the pathogen and of a biocontrol agent were placed aseptically into the peripheral region of a sterile PDA Petri plate (90 mm) in a straight line but opposite to each other and incubated at 28±1 ^{0}C. Radial growth of the pathogen and the biocontrol agent were measured after the desired incubation period and percent inhibition (in comparison to control where no biocontrol agent was placed) was determined for each experiment.

3.19.2. Evaluation of crude culture filtrates of the antagonists

Culture filtrate of fungal antagonists were collected from 15 days old cultures grown in PDB in 100 ml Erlenmeyer flasks. Culture fluid was filtered through cheese cloth and finally centrifuged at 3,000 g and the supernatant was subjected to filter sterilization. One ml of filter sterilized culture supernatant was mixed with 9 ml of sterile molten PDA in a Petridish and allowed to solidify. One mycelial disc of 4 mm in diameter was cut from the advancing zone of a culture (maintained in petridishes) was placed on solidified PDA medium at the centre. In control set 1 ml of sterile distilled water and 9 ml of molten PDA media was poured. The control plates were also inoculated with mycelial discs as mentioned in case of culture fluid supplemented petridishes. All the petridishes were incubated at 28±1^{0}C.

3.19.3. *In vivo* evaluation on whole plants

Selected niger plants in pots (30 cm diameter) were sprayed with the crude culture filtrate supplemented with 0.05% tween 20 and kept in a transparent polyhouse under normal light and temperature conditions. Crude culture filtrates of the biocontrol agents were used for studying their potential to control the disease *in vivo*. After 24 h of spraying, plants were inoculated with the pathogen *A. alternata* following the method of whole plant inoculation technique as described by Dhingra and Sinclair (1995). In control sets, plants were inoculated with the pathogen after spraying with sterile distilled water. Mean disease index/plant was calculated following the method of Sinha and Das (1972) after 2, 4, 6, and 8 days of inoculation.

3.20. Evaluation of botanicals

3.20.1. Extraction

Extracts of the plant parts were done following the method of Mahadevan and Sridhar (1982) with some modifications. Fresh plant materials were collected and washed thoroughly with sterile distilled water and dried at room temperature. Plant materials were weighed, ground and extracted separately with sterile distilled water and 50% ethanol (0.5 g/ml) after drying. The extracts were filtered through double-layered muslin cloth and centrifuged at 5,000 g for 15 minutes. The supernatants of the extracts were sterilized by passing through a Millipore filter (0.2 µm). All extracts were stored at 4^0C. The extracts were screened for their antifungal activity through bioassays.

3.20.2. Spore germination bioassay

Inhibitory effects of botanicals against *A. alternata* were tested. The spores of the pathogen *A. alternata* were allowed to germinate in sterile distilled water drops mounted on sterile grease free slides kept in an humid chamber in case of control. In experimental sets plant extract (30 µl) was placed on the centre of a grease free microscopic slide and allowed to evaporate. After evaporation of the solvent (ethanol), spore suspension was mounted on the slides in the same place where the extract was applied. In solvent control set fresh solvent (50% ethanol) was placed and subsequently evaporated before application of spore suspension. The slides were then incubated at 28±1^{0}C in a humid chamber. Two small glass rods (60 mm in length) were placed in a 90 mm petridish and a slide was placed on the rods in a uniformly balanced position. Sterile distilled water was carefully poured in the petridish so that the bottom of the slide remained just above the water surface. The petridish was then covered and incubated at 28±1^{0}C. Following 12 h of incubation, the slides were stained with lacto phenol-cotton blue and observed under the microscope. Approximately, 200 spores were observed in each slide for germination. The entire experiment was repeated thrice.

3.20.3. Bioassay by poisoned food technique

One millilitre of plant extract was added to 9 ml of the molten PDA medium, mixed well and poured in sterile petridish (70 mm diameter) under aseptic condition and was allowed to solidify. In control sets 1 ml of sterile

distilled ware was added instead of plant extracts. The both experimental and control plates were inoculated with the pathogen and incubated for required period. Radial growth of the pathogen was measured.

3.20.4. *In vivo* bioassay on whole plants

To estimate the antifungal activity of different plant materials, fresh aquous extract (2 g in 10 ml distilled water) was prepared. Niger plants grown in pots (30 cm diameter) were sprayed with the crude extract and were kept for 24 hours in a transparent polyhouse under normal conditions of light and temperature. The plants were inoculated with the pathogen *A. alternata* following the method of whole plant inoculation technique as described by (Section 3.5). Control plants were sprayed with sterile distilled water and maintained under similar conditions of light and temperature. One set of control plants were also sprayed with the pathogen as mentioned. Mean disease index / plant were calculated following Sinha and Das (1972) after 2, 4, 6, and 8 days of inoculation.

3.21. Statistical analysis

Some of the statistical analysis was done using Smith's statistical package (version 2.5). The package was developed by Dr. Gray Smith, Pomona College, Claremont-91711, USA. In some other cases statistical Package for the Social Sciences (SPSS), version 11.0, SPSS Inc., Chicago, Illinois were also used.

4. Experimental

4.1. Studies on morphology and physiology of *Alternaria alternata*

Alternaria alternata affects niger plants of all ages. Sometimes the spread of the disease is so severe that hardly any seeds can be harvested (as experienced in the experimental field as well as in farmer's fields in Jalpaiguri district). It has been experienced in the present study that the fungi attack generally by spores. Germinated spores produced branched mycelia and the mycelia entered through the epidermal layer of the leaves of the host. In isolated culture, white mycelia soon became black and expanded rapidly with formation of septa. Mycelia were either immersed or superficial. Within a week shiny black to brown conidial mass were produced on the surface. For conducting studies on host-parasite interaction it is important to know about the morphology and physiology of the fungus. Hence, it was necessary to culture the fungus in different artificial media. In addition, production of inocula, in the form of spores, and vegetative mycelia are also important for experimental purpose. Hence, it was felt to standardize a medium with optimum growth conditions of the fungus. The present work was therefore undertaken to study the environmental and nutritional requirements of culture media, temperature, pH and light on growth of mycelia and sporulation of *Alternaria alternata*.

4.1.1. Studies on morphology of *Alternaria alternata*

Alternaria alternata, the isolated pathogen of niger, was grown in three different media (PDA, PCA and PDB) and morphology of the pathogen along with mycelial growth was observed. Profuse sporulation of the fungi was observed in PCA (Plate 4.1, fig b).

For microscopic observations, mycelia were taken in microscopic slides from pure culture and stained with cotton-blue in lacto-phenol. The slides were mounted with cover glass, sealed and observed under microscope. Immature mycelia were hyaline in colour but on maturity it became gray in colour. Conidia of the fungus were obclavate to beaked and brownish in colour having transverse and longitudinal septa (Plate 4.1 fig e). Conidia were produced from simple septate conidiophores in simple or branched acropelal chains (Plate 4.1, fig f). The length and breadth of mature conidia were 10-40 µm and 6-12 µm

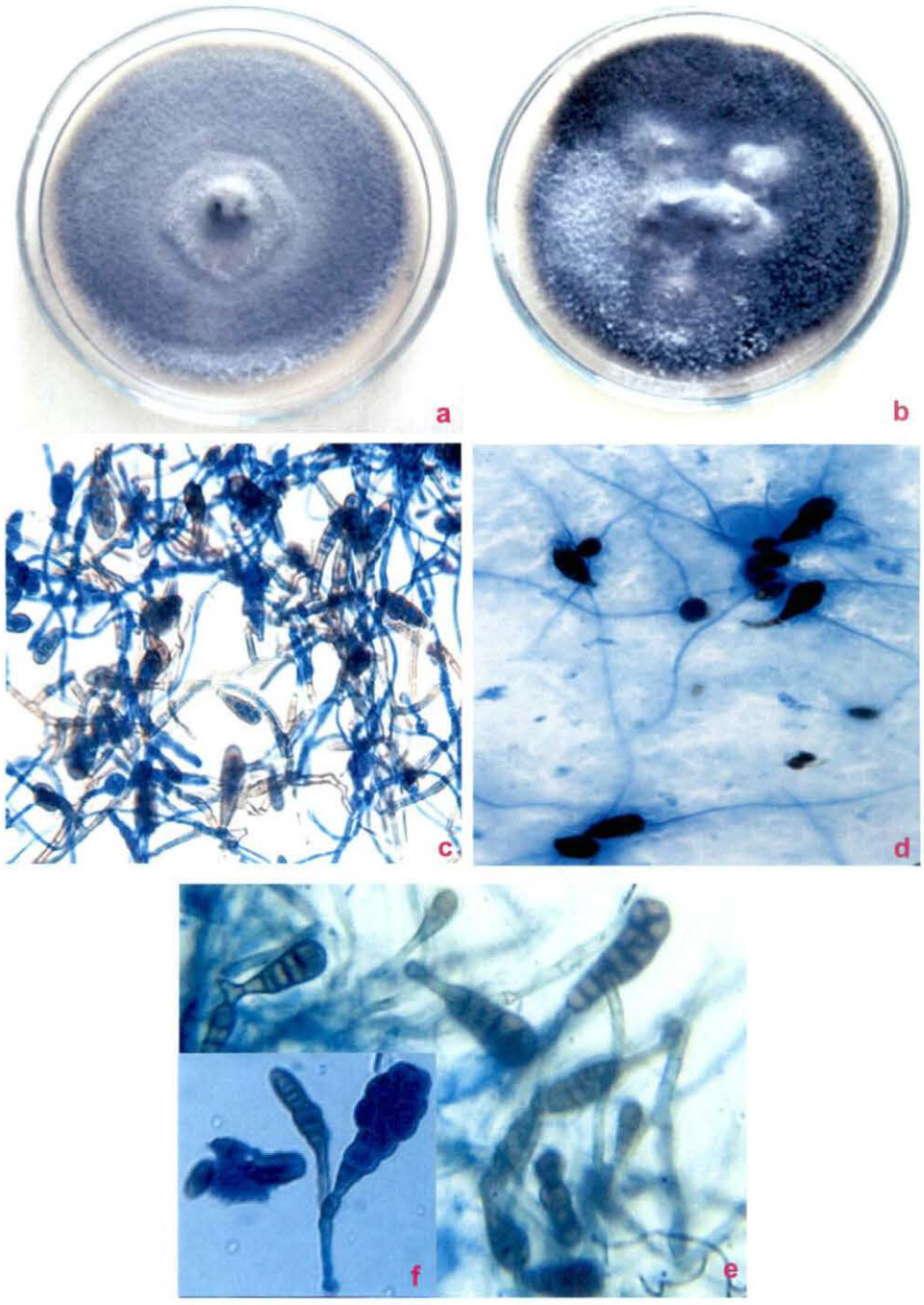

PLATE 4.1

fig. a : *A.alternata* in PDA plate

fig. b : *A.alternata* in PCA plate with profuse spores

fig. c : Mycelia with spores

fig. d : Germinated spores of *A.alternata* with germ tube

fig. e : *A.alternata* spores clearly showing transverse and longitudinal septa

fig. f : Arrangement of spores on conidiophore

respectively. The diameter of the mature hyphae ranged between 3-5 µm (Fig 4.1)

4.2. Culture conditions affecting growth and sporulation of *Alternaria alternata*

In vitro study of growth and sporulation of *A. alternata* was done under different culture conditions and in some solid and liquid media. The details of the study are being discussed in the following sections.

4.2.1. Mycelial growth and sporulation of *A. alternata* in different solid media

In order to evaluate the vegetative growth and sporulation of *A. alternata* in solid media, eight different media viz. potato dextrose agar (PDA), oat meal agar (OMA), Czapek Dox agar (CDA), Richards's agar (RMA), yeast extract mannitol agar (YEMA), malt extract agar (MEA), potato carrot agar (PCA) and corn-meal agar (CMA) were used. Experiments were performed with three replications. Detailed procedure has been presented under materials and methods (Section 3.7.2).

From the results (Table 4.1 & Fig 4.2), it was evident that RMA (Richard's agar) was best for both growth and sporulation of *Alternaria alternata.* After 10 days of incubation on RMA, radial growth of mycelia was 90 mm in diameter and sporulation was also good. Radial growth of mycelia was 84.16 mm in CDA, after 10 days of incubation. Poor sporulation was observed in CDA. In PDA sporulation was comperatively less, although good growth of mycelia was evident. Excellent sporulation(++++) was observed in PCA with moderate growth of mycelia (69.33mm in diameter). Good mycelia growth were also observed in CMA, MEA, OMA. YEMA showed good sporulation but comperatively poor mycelia growth.

4.2.2. Mycelial growth of *Alternaria alternata* in different liquid media

The growth of *A. alternata* was assessed after different periods of incubation at 28±1^{0}C. The test fungus was inoculated in sterile PDB, PCB and RM media. Each medium (50 ml) was taken in a 250 ml conical flask following the methods as described in section 3.7.2. After 5, 10, 15, 20 and 25 days of incubation, fungal mycelia were harvested and strained through muslin cloth. Strained mycelia mat were blotted with blotting paper and then dried at 60^{0}C in a hot air oven. Finally the dried mycelia were allowed to cool and weights of the

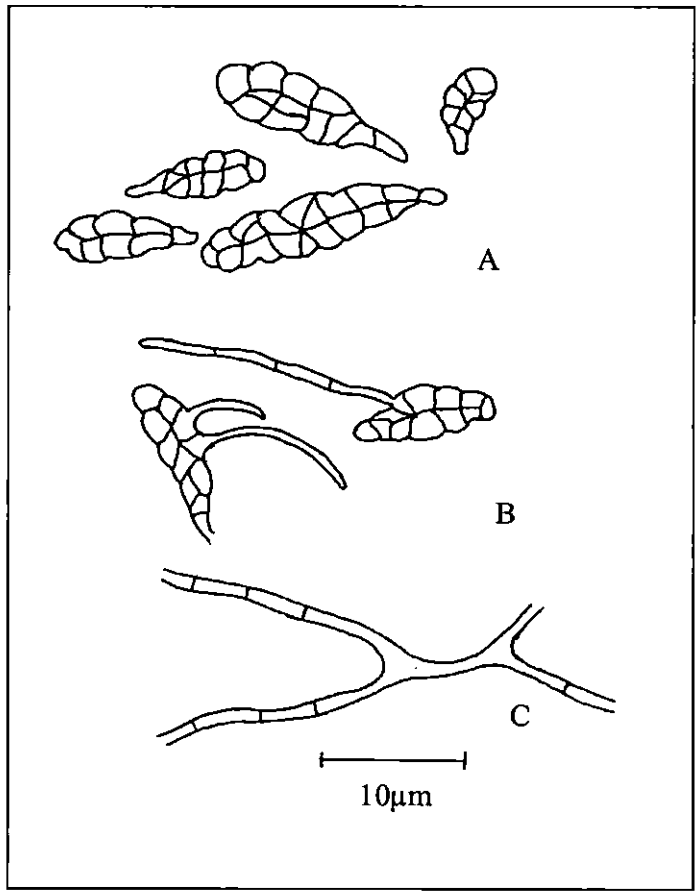

Fig. 4.1
A : Spores
B : Germinating spores
C : Mature hyphae with Septa

dried mycelia (grown in different media) were taken in a precision single pan balance. The results were summarized in table 4.2 and shown in Fig 4.3.

Table: 4.1. Mycelia growth and sporulation of *Alternaria alternata* in different solid media.

Medium of growth	Radial growth (mm)* and sporulation									
	2		4		6		8		10	
	Growth	Sp**	Growth	Sp	Growth	Sp	Growth	Sp	Growth	Sp
RMA	16.66 ±0.34	–	36.66 ±0.92	–	57.50 ± 0.76	+	76.83 ±0.55	++	90.00 ±0.58	+++
CMA	28.16 ±0.57	–	14.83 ±0.17	–	56.16 ±0.74	–	70.16 ±0.45	++	84.16 ±0.45	++
PDA	13.83 ±0.50	–	32.40 ±0.95	–	50.66 ±0.61	–	65.83 ±0.44	–	82.16 ±0.84	+++
MEA	12.00 ±0.29	–	27.66 ±0.88	–	48.16 ±0.93	–	64.33 ±0.67	++	80.33 ±0.60	++
OMA	11.50 ±0.50	–	26.33 ±0.67	–	42.83 ±0.44	–	60.00 ±0.85	++	76.16 ±0.72	++
PCA	11.83 ±0.44	–	26.16 ±0.72	–	41.66 ±0.73	+	55.50 ±0.36	++	69.33 ±0.12	+++ +
YEMA	12.33 ±0.34	–	26.33 ±0.67	–	40.33 ±0.57	+	52.00 ±0.87	++	69.83 ±1.83	+++
CDA	10.33 ±0.17	–	15.33 ±0.67	–	32.16 ±1.09	–	31.00 ±1.00	+	83.33 ±0.88	++
CD (5%)	0.73		1.38		1.89		1.62		2.43	

*Mean of three replications.
**Sp = Sporulation, – = Nil, + = poor, ++ = fair, +++ = good, ++++ = excellent.
Data after ± represent standard error values.
Incubation temperature = 28 ± 1°C,
CD= critical difference.
PDA = Potato dextrose agar
OMA= Oat meal agar
CDA = Czapeak Dox Agar
RMA = Richard's agar medium
YEMA = Yeast extract namitol agar
MEA = Malt extract agar
PCA = Potato carrot agar
CMA = Corn meal agar

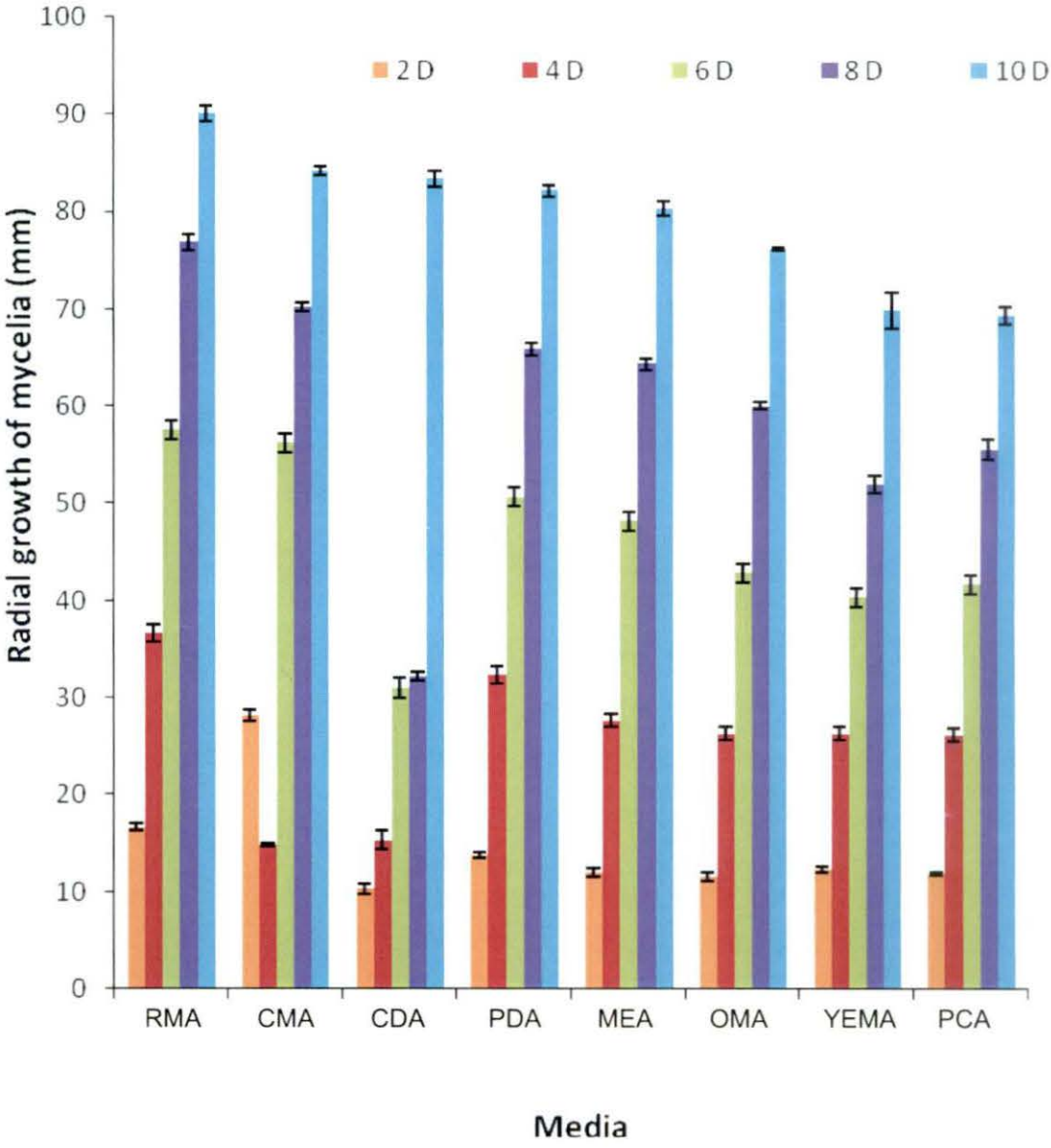

Fig: 4.2 Mycelial growth of *A. alternata* in different solid media
*Mwt = Mycelial dry weight

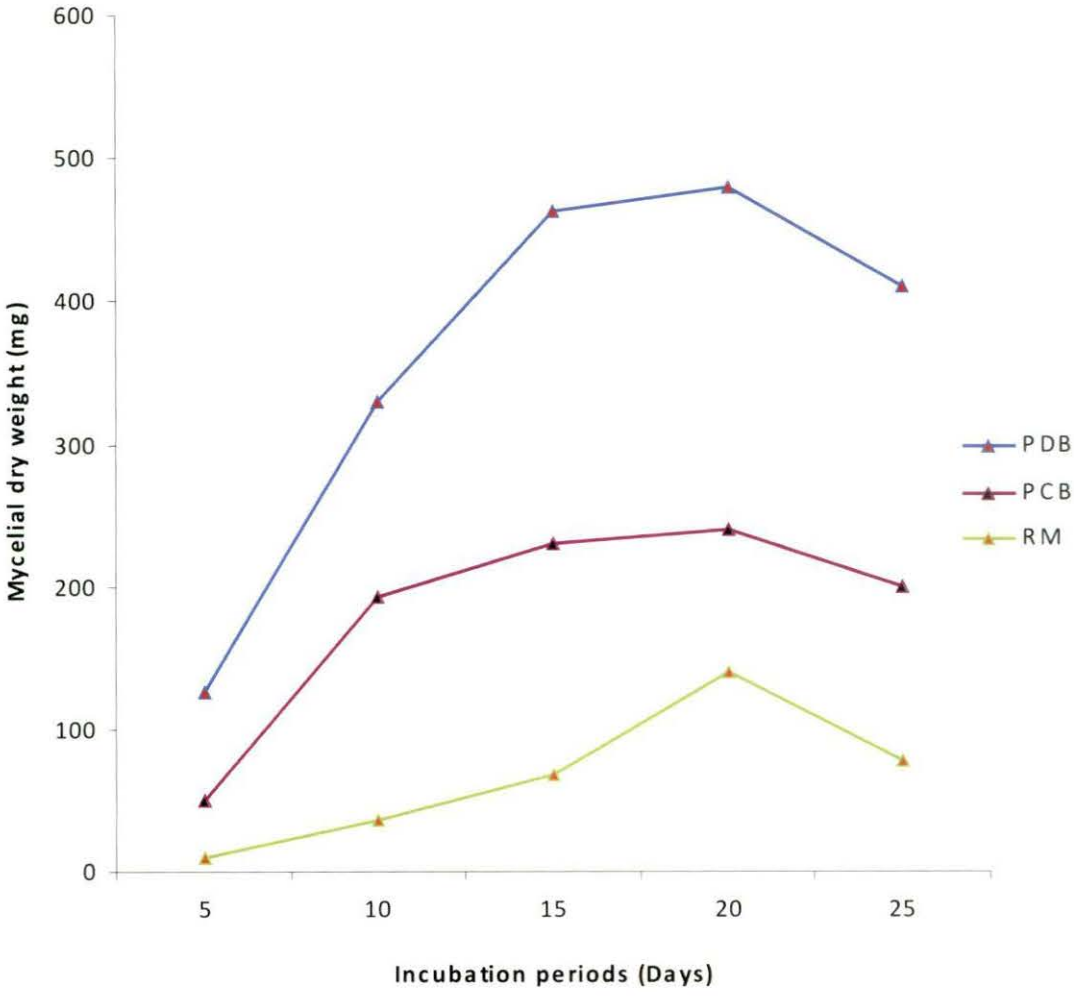

Fig: 4.3. Growth of *A. alternata* in three different liquid media.

Abbreviations:

PDB = Potato dextrose broth
PCB = Potato carrot broth
RM = Richard's medium

Table: 4.2. Growth of *Alternaria alternata* in different liquid media.

Medium of growth	Mycelial dry weight (mg)*				
	5 days	10days	15days	20days	25day
PDB	126.66±0.73	330±1.00	463 ±1.15	480±1.15	410±1.07
PCB	50.00±0.58	193±1.04	230±1.15	240±1.00	200±0.84
RM	10.00±0.58	36 ±0.58	68±1.04	140±1.25	78±0.70
CD (5%)	2.32	3.25	3.72	1.28	2.88

* Mean of three replications. Data after ± represent standard error values.
Dry weight of inoculum was 10 mg.
[PDB=Potao dextrose broth; PCB=Potato carrot broth; RM=Rechard's medium]

From the results (Table 4.2), it was evident that PDB was best for growth of *A. alternata*. Mycelial dry weight was recorded as 480 mg after 20 days of inoculation in PDB medium. In PCB, mycelial dry weight was found 240 mg after 20 days of incubation but in RM mycelial growth was poor. Increasing trend of growth was observed from 5 days after inoculation and it continued till 20 days. Growth was found to decline after 20 days in all the three media tested.

4.2.3. Effect of different pH on mycelia growth of *A. alternata*

Physiology of any organism is controlled by several factors. Among the different factors pH plays an important role on growth and sporulation of a fungus. Potato dextrose broth (PDB) was adjusted to pH 5, pH 5.5, pH 6, pH 6.5 pH 7 and pH 8 by adding 1(N) NaOH or 1(N) HCL drop-wise into the medium before sterilization. After adjusting the pH in PDB the media was sterilized. Each 250 ml Erlenmeyer flask contained 50 ml sterilized medium. Media of different pH were inoculated separately *by A. alternata* following the method as mentioned in section 3.7.2. and incubated at 28±1^{0}C. Mycelial dry weight were taken after 5, 10, 15, 20, and 25 days of inoculation and were tabulated in Table 4.3 and shown in Fig 4.4. It was found that *A. alternata* was able to grow within a wide range of pH, from 5.0 to 8.0 (table 4.3). The fungus however, failed to grow in alkaline environment, beyond pH 8.0. The optimum pH for growth was recorded at the pH 6.5. Highest growth (473.4 mg) was recorded at pH 6.5. Moderate growth was observed at pH 5.5, 6.0 and 7.0. Poor growth was observed at pH 5.0 and at pH

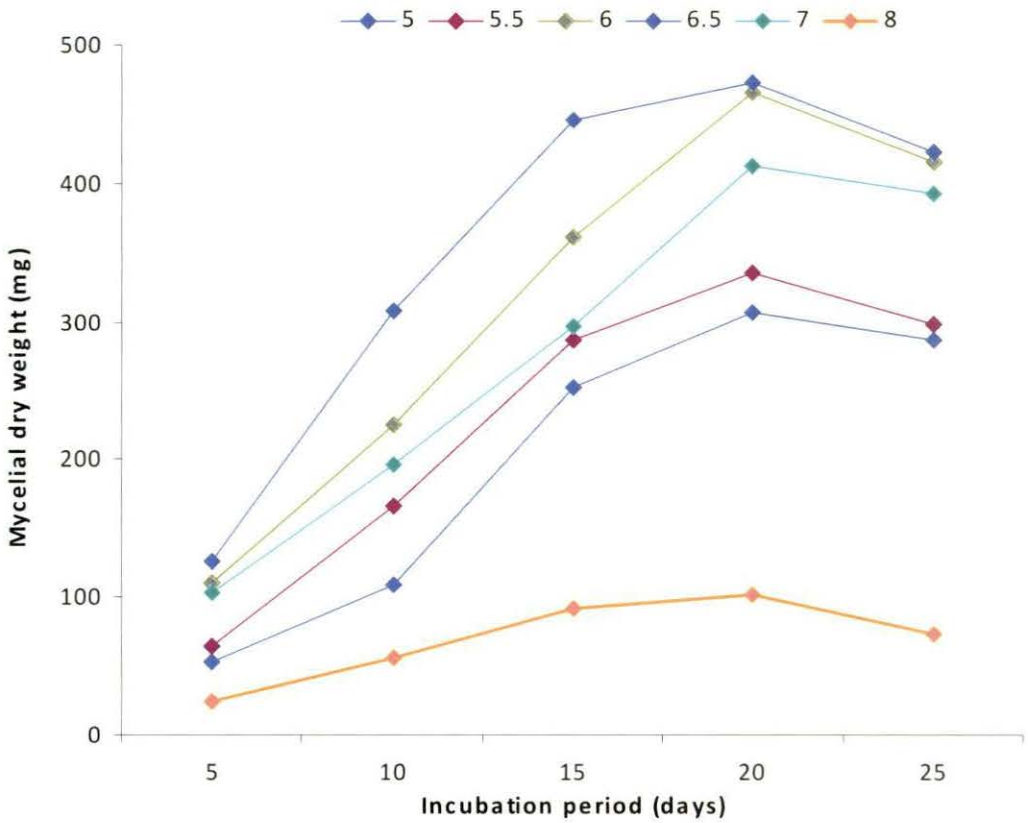

Fig: 4.4 Effect of different pH on growth of *Alternaria alternata*

8.0. The results indicated that slightly acidic pH to neutral pH was optimum for the growth of *A. alternata*.

Table: 4.3. Effect of different pH on growth of *Alternaria alternata*

pH	Mycelia dry weight (mg) *				
	5 days	10 days	15 days	20 days	25 days
5.0	52.6 ±4.32	109.3 ±4.08	252.6 ±3.06	306.4 ±4.94	287.1 ±3.52
5.5	64.5 ±2.06	165.8 ±2.83	286.3 ±2.00	335.6 ±2.40	298.4 ±3.66
6.0	111.0 ±3.55	225.3 ±2.64	361.1 ±4.50	466.3 ±2.04	415.7 ±2.86
6.5	125.7 ±2.80	308.6 ±3.43	445.5 ±2.74	473.4 ±3.22	422.6 ±2.00
7.0	103.2 ±4.09	195.7 ±4.72	296.8 ±1.90	413.3 ±4.56	392.2 ±2.49
8.0	24.4 ±4.08	55.2 ±2.40	92.4 ±1.01	102.1 ±3.13	73.3 ±2.54
CD (5%)	7.12	8.53	7.63	9.50	2.60

*Mean of 3 replications ; Data after ± represent standard error values.
Dry weight of inoculating mycelia block was 10 mg

4.2.4. Effect of different incubation temperatures on mycelial growth of *A. alternata*

The growth of *A. alternata* was assessed at different temperatures. Sterile media (50ml PDB) was taken in each flask of 250 ml. The sterile media in Erlenmeyer flasks were inoculated by the fungus (*A. alternata*). Inoculation was done following the method as described in section 3.7.2. The inoculated flasks were incubated at different temperatures viz.10^{0}C, 150C, 20^{0}C, 25^{0}C, 28^{0}C, 30^{0}C, 35^{0}C and 40^{0}C. After 5, 10, 15, 20 and 25 days of incubation, mycelia were harvested, strained through muslin cloth, blotted and finally dried at 60°C. Mycelial dry weights were taken in a precision balance (Sartorious) and were recorded in table 4.4. The results have also been presented graphically (Fig 4.5).

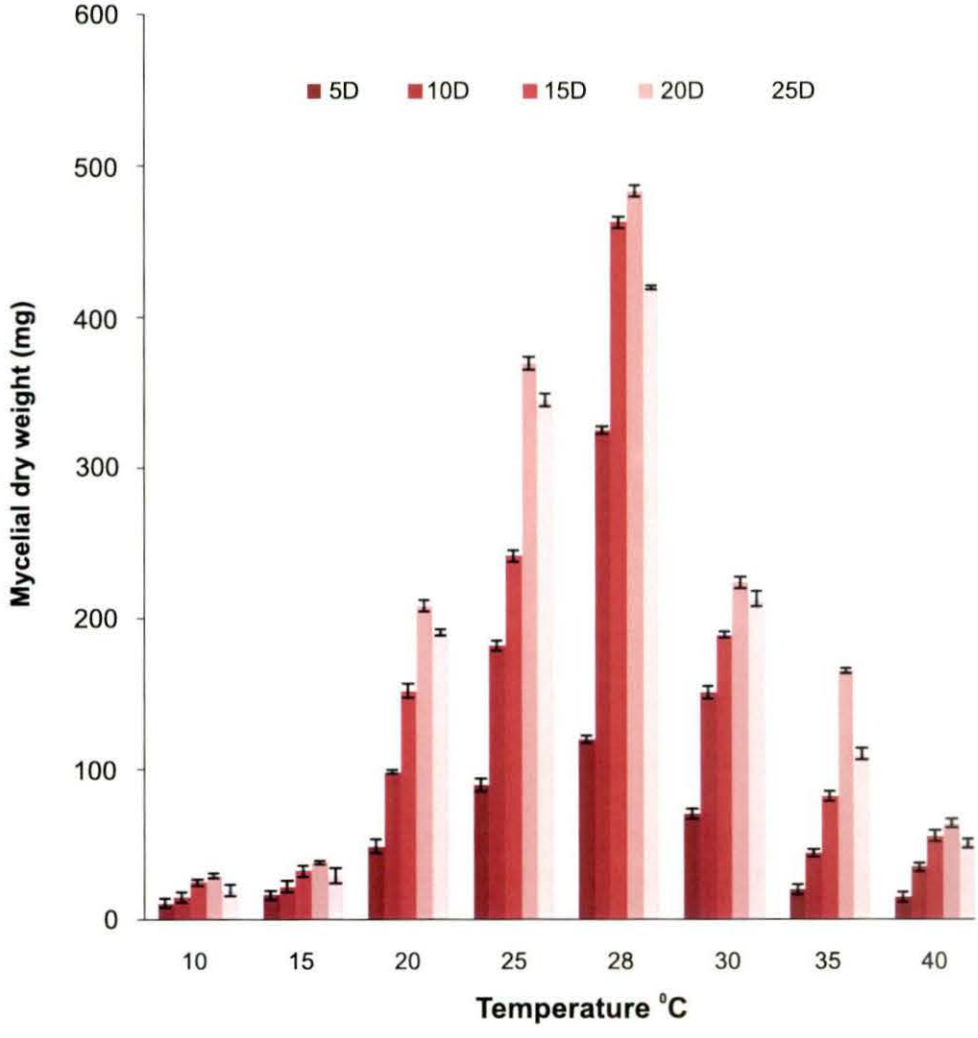

Fig: 4.5 Effect of different temperature on mycelia growth of *A.alternata*

Results revealed that maximum growth was possible at 28^0C at which the mycelia dry weight was recorded as 120.3 mg, 325.1 mg, 462.7 mg, 483.4 mg and 419.5 mg respectively after 5, 10, 15, 20 and 25 days after inoculation. Very poor growth was recorded at incubation temperature of 10 °C. However, results presented in Fig 4.5 indicate that *A. alternata* was capable of growing at temperatures that ranges between 10^0C to 40^0C.

Table: 4.4. Effect of different temperature on mycelial growth of *Alternaria alternata*

Temperature (^{0}C)	Mycelial dry weight (mg)*				
	5 Days	10 Days	15 Days	20 Days	25 Days
10	11.2 ±3.24	15.1 ±3.33	24.8 ±2.36	29.5 ±1.53	19.7 ±3.47
15	16.6 ±2.77	22.1 ±3.86	32.4 ±3.95	38.3 ±1.27	29.2 ±4.87
20	49.0 ±4.60	98.7 ±1.17	152.3 ±4.41	208.4 ±3.84	191.3 ±2.27
25	89.8 ±4.03	182.3 ±3.19	241.7 ±3.92	369.5 ±4.46	345.2 ±4.38
28	120.3 ±2.51	325.1 ±2.36	462.7 ±3.79	483.4 ±3.88	419.5 ±1.40
30	70.6 ±3.48	151.0 ±4.35	189.4 ±2.04	224.2 ±3.80	213.0 ±4.97
35	20.2 ±3.56	44.7 ±2.55	82.4 ±3.39	165.6 ±1.54	110.5 ±3.48
40	15.3 ±3.55	34.9 ±2.77	55.8 ±3.65	64.2 ±2.95	51.0 ±3.14
CD (5%)	1.74	4.11	3.97	4.64	4.26

*Mean of three replicates; Data after ± represent standard error values.

Dry weight of inoculating mycelia block was10.0 mg

4.2.5. Assessment of mycelia growth and sporulation of *A. alternata* and on different carbon sources:

To study different carbon sources for the optimum growth and sporulation of the *A .alternata* a basal medium (Glucose 1%; Asparagine 0.2%; KH_2PO_4

0.1%; $MgSO_4$, $7H_2O$ 0.05%; Zn^{++}, $Mn^{++}$and Fe^{+++} 2 µg/ml) was used for the purpose. The different carbon sources tested were glucose, sorbitol, sucrose, fructose and mannitol. The equivalent amount of carbon present in 1% glucose was used as standard and added separately to the basal medium. The medium (50 ml) was taken in 250 ml Erlenmeyer flasks and sterilized by autoclaving at 15 lb. p.s.i. for 15 minutes. Three flasks were taken for each carbon source. After cooling, the media was inoculated by the pathogen using 4 mm mycelial discs in PDA and incubation was allowed for 5, 10, 15, 20, and 25 days. In control sets, no carbon sources were used in the basal medium. After incubation for the specified time periods, the mycelia were harvested, dried at 60 °C and weighed. The results were tabulated in Table 4.5. After each incubation period but before harvest of mycelia, sporulation of the fungus were also recorded in five different grades on the basis of visual observations.

From the results (Table 4.5 and Fig.4.6) it was evident that *A. alternata* showed a gradual increase in growth until 20 days in all cases. Mycelial dry weight was maximum (500 mg) after 20 days of incubation when mannitol was used as carbon source. Next to mannitol, when fructose and sucrose were supplemented separately mycelial growth were 420.4 mg and 410.6 mg respectively after 20 days of incubation. When glucose was used as carbon source, the growth was minimum among the carbon sources tested and it was only 50 mg after 20 days of incubation. Mannitol was found as the best carbon source among the different carbon sources tested when overall growth pattern of *A. alternata* was observed. Sporulation was excellent in sucrose and mannitol after 20 days of incubation. In all other cases, sporulation was graded as good, fair, poor and nil. Generally good sporulation was found in glucose after 25 days of incubation but in fructose good sporulation was observed after 20 days of incubation. In case of sucrose and mannitol sporulation started after 10 days of incubation but in the other cases sporulation started after 15 days of incubation. In control set, insignificant growth was observed without any sporulation.

4.2.6. Assessment of mycelia growth and sporulation *of A. alternata* and on different nitrogen sources:

To assess the mycelia growth and sporulation of *A. alternata* on different nitrogen sources (both organic and inorganic), modified Asthana and Hawker's medium 'A' (Glucose 10g; KNO_3 3.5; $KH2PO_4$ 1.75g; $MgSO_4$,7H2O 0.75; Agar agar 20g and distilled water 1L) without agar agar was used as basal medium.

The quantity of various nitrogen sources was so adjusted to give the same amount of nitrogen as furnished by 3.5 g KNO_3 in the basal medium. The quantity of various nitrogen sources was prepared and dispersed separately in 50 ml medium and was taken in 250 ml Erlenmeyer flask. The flasks were sterilized by autoclaving at 15lb.p.i. for 15 minutes. After cooling the flasks were inoculated by test fungi as described in section 3.7.2. All the medium (supplemented with different carbon sources) inoculated aseptically were incubated at 28±2° C for 5, 10, 15, 20 and 25 days. In control set no nitrogen source were provided in the basal medium. After specified incubation periods, sporulations were checked and were recorded in five different grades based on visual observation. After that mycelia were harvested, dried at 60°C and weighed. The results were tabulated in the Table 4.6.

Table: 4.5. Effect of different carbon sources on the growth and sporulation of *A. alternata*

Carbon source	Incubation period (Days)									
	5		10		15		20		25	
	Mwt. (mg)	Sp**	Mwt. (mg)	Sp	Mwt. (mg)	Sp	Mwt. (mg)	Sp	Mwt. (mg)	Sp
Glucose	50.00 ±0.89	-	100 ±1.17	-	110 ±0.80	+	125 ±0.90	++	100.00 ±1.29	+++
Sucrose	110.6 ±0.99	-	200.0 ±1.15	+	340.4 ±1.14	++	410.60 ±0.66	+++	385.00 ±0.68	++++
Fructose	100.0 ±1.04	-	190.1 ±0.95	-	310.2 ±1.25	+	420.40 ±0.76	++	390.00 ±1.46	+++
Sorbitol	80.00 ±0.81	-	170.6 ±0.59	-	280.3 ±0.85	+	300.40 ±0.83	++	310.00 ±0.87	++
Mannitol	110.50 ±0.45	-	240.8 ±0.85	+	380.90 ±0.87	++	500.00 ±1.29	++++	495.80 ±0.31	+++
Control***	10.30 ±1.08	-	12.6 ±0.95	-	13.90 ±0.30	-	14.10 ±0.80	-	13.11 ±0.49	-
CD 5%	2.03		2.01		12.55		1.66		2.58	

*Mean of three replicates. **Sp = Sporulation, – = Nil, + = poor, ++ = fair, +++ = good, ++++ = excellent. ***Control Basal medium without any carbon source. Mwt(mg) = Mycelia dry weight in mg; Data after ± represent standard error values

From the results (Table 4.6 and Fig. 4.7) it was evident that *A. alternata* showed highest growth (360.00 mg) in peptone after 20 days of incubation. The other two organic nitrogen sources, yeast extract and beef extract also showed satisfactory

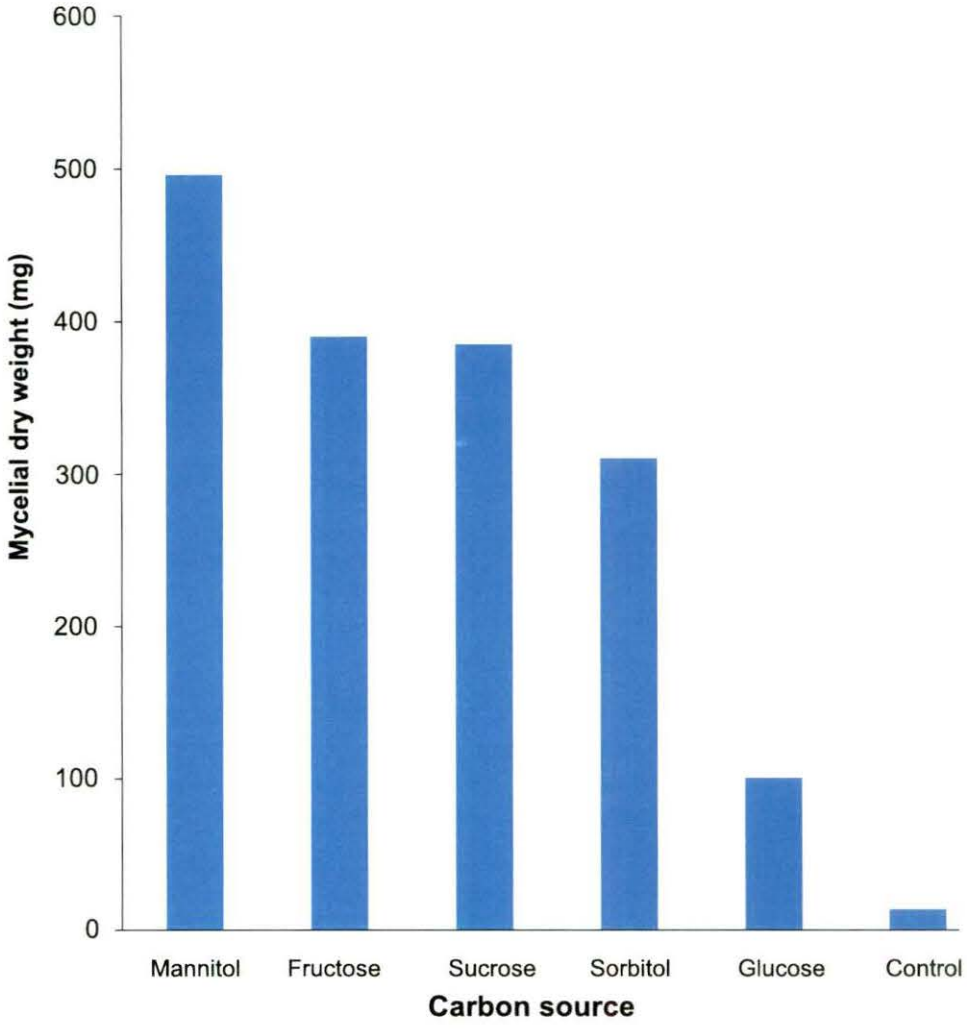

Fig: 4.6 Effect of different carbon source on the growth of *A.alternaria* after 25 days of inocubation

growth (340.00 mg and 235.00 mg respectively) after 20 days of incubation. Among the inorganic nitrogen sources tested, potassium nitrate and Sodium nitrate showed best results (286.00 mg and 275.00 mg respectively) after 20 days of incubation. Media containing Ammonium sulphate as nitrogen source showed minimum mycelial growth (245.00 mg after 20 days) among all the nitrogen sources tested. In all cases, maximum increase in growth was observed within first 15 days of incubation and it declined after 20 days. Sporulation was found good after 15 days of incubation and continued until 25 days. In case of potassium nitrate and yeast extract, sporulation was found Good after 20 days of incubation. Insignificant growth without any sporulation was observed in control set.

Table: 4.6. Effect of different nitrogen source on the growth and Sporulation of *A. alternata*

Nitrogen source	Incubation periods (Days)									
	5D		10D		15D		20D		25D	
	Mwt. (mg)	Sp**	Mwt. (mg)	Sp	Mwt. (mg)	Sp	Mwt. (mg)	Sp	Mwt. (mg)	Sp
Inorganic										
Potassium nitrate	150.0 ±1.00	-	220.5 ±0.66	+	265.2 ±0.70	+++	286.0 ±0.98	+++	250.0± 1.32	+++
Sodium nitrate	130.6 ±0.74	-	186.2 ±0.76	+	210.4 ±0.90	++	275.0 ±0.92	++	225.0± 0.76	++
Ammonium sulphate	120.8 ±0.53	-	163.4± 0.83	+	198.0 ±0.81	++	245.1 ±0.72	++	215.0± 0.72	++
Organic										
Peptone	230.1 ±1.27	-	298.6 ±0.61	+	340.0 ±1.53	++	360.0 ±1.25	++	310.0± 1.15	++
Yeast extract	210.4 ±1.00	-	250.0± 1.26	+	290.3 ±1.12	++	340.0 ±1.44	+++	300.0± 1.15	+++
Beef extract	115.0 ±0.81	-	165.0 ±1.63	-	200.6 ±0.70	++	235.4 ±0.72	++	215.0± 0.72	++
Control ***	5.90 ±0.32	-	9.1 ±0.92	-	10.6 ±0.31	-	11.5 ±0.55	-	14.80 ±0.67	-
CD at 5%	1.58		2.05		1.32		0.99		0.77	

Mwt (mg) = Mycelial dry weight in mg; *Mean of three replicates. Data after ± represent standard error values.**Spn = Sporulation, – = Nil, + = poor, ++ = fair, +++ = good, ++++ = excellent.***Control basal medium without nitrogen source.

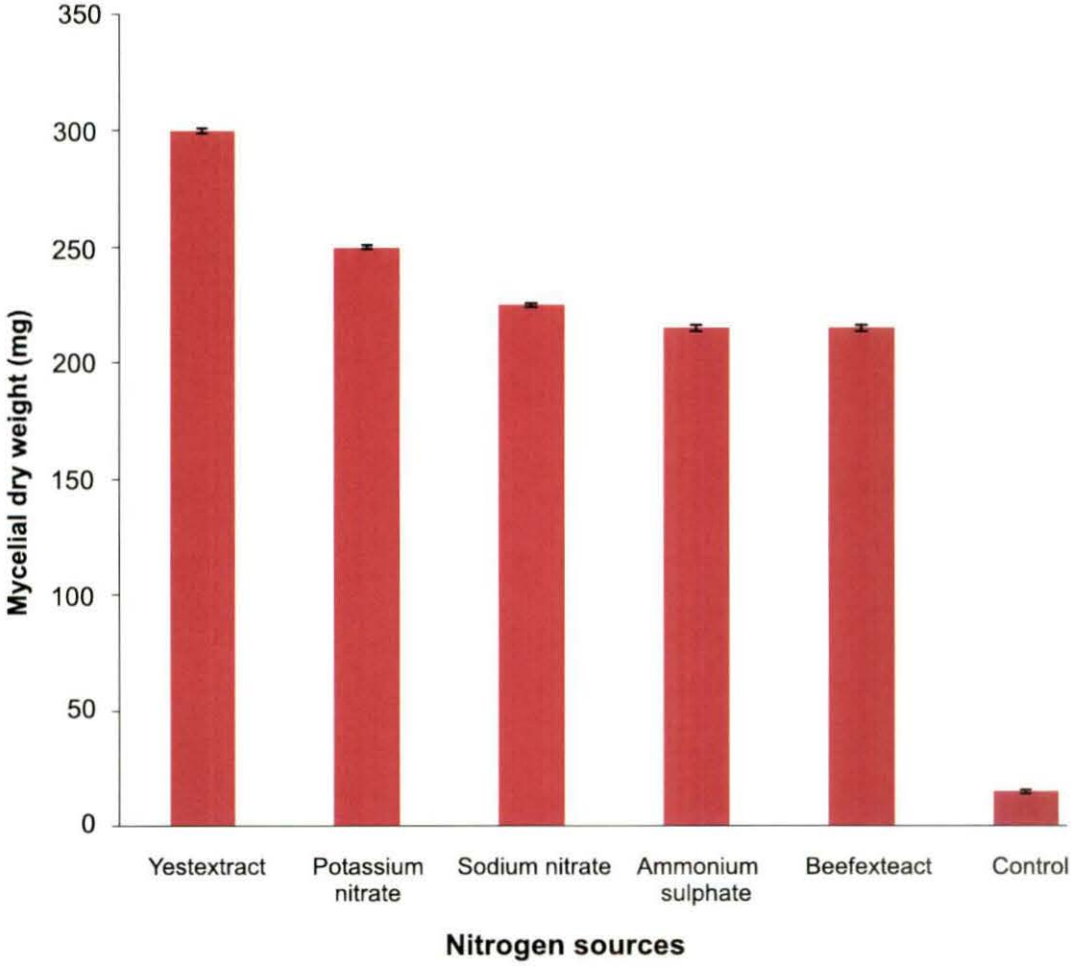

Fig: 4.7 Effect of different nitrogen sources on the growth of *A.alternata* after 25 days of incubation.

4.2.7. Conditions affecting sporulation of *Alternaria alternata*

Fungal spore germination is very important for understanding the host-pathogen interaction. For this, different incubation period, different pH and different temperatures are very important in determining the germination and germ tube elongation.

4.2.7.1. Spore germination and germ tube elongation of *A.alternata* after different periods of incubation

Spore germination, and germ tube elongation of *A. alternata* was studied after different periods of incubation in two different ways. Spore suspensions ($1x10^5$ ml^{-1}) prepared with sterile distilled water was used in one set. Spore suspensions (30µl) was placed on microscopic slides in triplicates and allowed to incubate for 2, 4, 6, 8, 10, and 12 hours at 28±1°C, in a humid chamber as described under materials and methods (Section 3.7.3). The results were tabulated in Table 4.7 and presented graphically in Fig. 4.8.

Germination of spores started after 2 hours of incubation when sterile distilled water was used in spore suspension. An increase in the percent germination of spores and germ tube length were observed with increasing time interval. After 12 hour of incubation germination percentage and germ tube length was recorded as 100% and 340.10 µm respectively (Table 4.7and Fig. 4.8).

Table: 4.7. Percent spore germination at different incubation period

Incubation period (hour)	% of spore germination[1]	Germ tube length[2] (µm)
2H	38.11±1.19	50.26±2.09
4H	60.26±2.33	91.34±1.80
6H	74.70±1.24	137.00±1.87
8H	86.00±1.27	220.39±2.09
10H	97.91±0.85	310.29±2.67
12H	100.00±0.00	340.10±1.19
CD at **5%**	2.66	5.61

[1]Mean of 3 replications. Calculated on the basis of 300 spores per slide.

[2] Mean of 3 replications and average of 60 germ tubes per slide.

Data after ± represent standard error values.

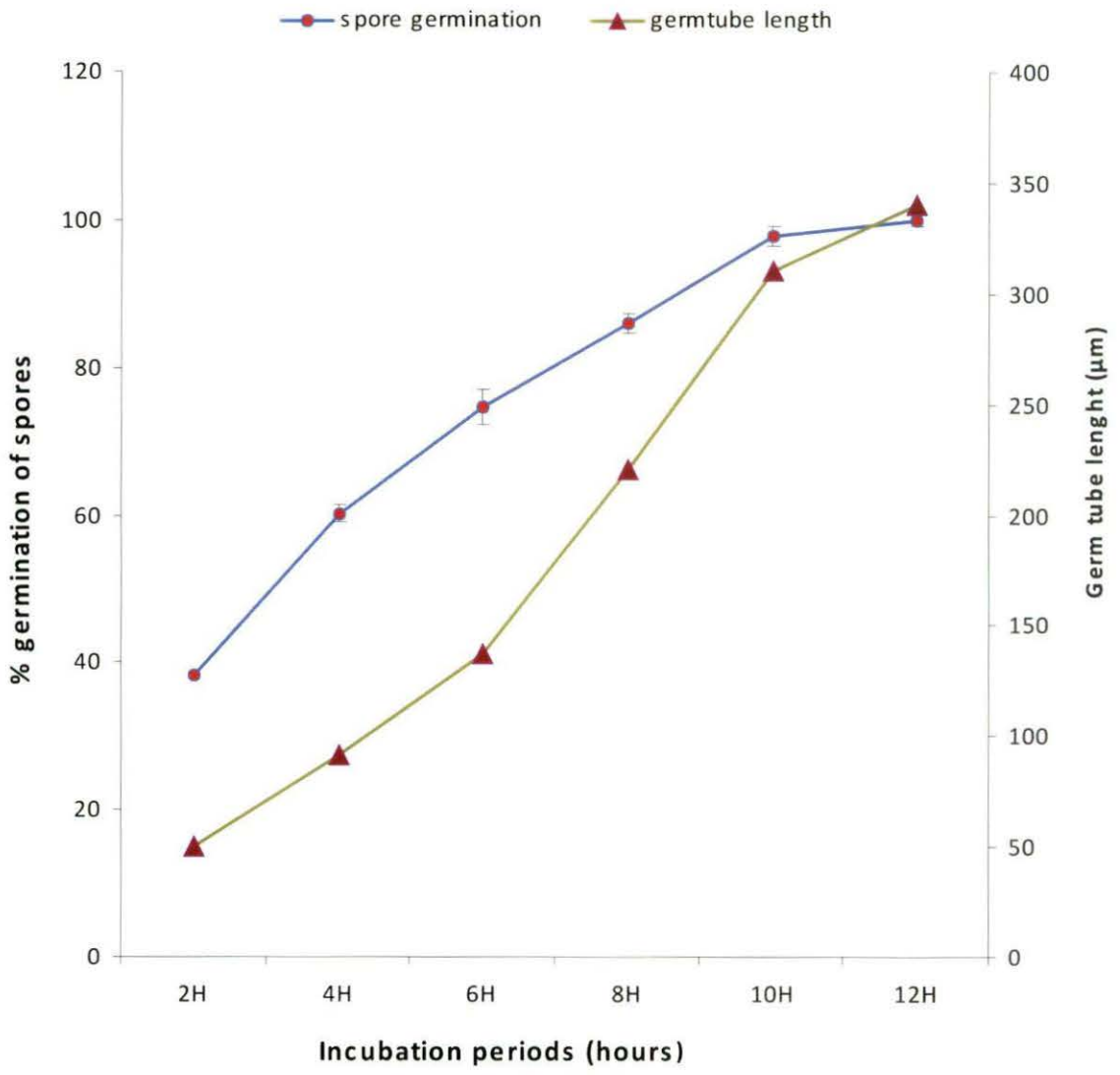

Fig: 4.8 Effect of different incubation periods (hours) on the germination of spores of *A.alternata*

4.2.7.2. Spore germination of *A. alternata* at different temperatures

Spore suspension of *A. alternata* was prepared as mentioned in the materials and methods (Section 3.7.3.1). Sterile distilled water was added to attain optimum concentration ($1x10^5$ ml^{-1}) of the spores. Spore suspension drops (30µl) were placed in different slides in triplicates and incubated at different temperatures (10^0C, 15^0C, 20^0C, 25^0C, 28^0C, 30^0C, 35^0C, and 40^0C). The results were noted in Table. 4.8.

Table: 4.8. Effect of different temperatures on spore germination of *A. alternata* at different incubation period.

Temperature	Germination Percentage * Incubation period (days)					
	2	**4**	**6**	**8**	**10**	**12**
10^0C	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	2.45 ±1.15
15^0C	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	5.11 ±0.62	20.36 ±0.40
20^0C	0.00 ±0.00	18.74 ±0.93	27.15 ±0.70	34.12 ±0.78	40.43 ±0.87	50.30 ±0.51
25^0C	27.26 ±0.62	50.28 ±0.96	69.14 ±0.93	79.56 ±0.64	85.50 ± 0.78	96.00 ±0.64
28^0C	38.13 ±1.07	60.29 ±0.98	71.26 ±0.65	86.26 ±0.96	97.32 ±1.08	100.00 ±1.22
30^0C	0.00 ±0.00	28.54 ±0.98	36.32 ±1.27	50.34 ±1.08	55.16 ±0.64	60.11 ±0.95
35^0C	0.00 ±0.00	11.36 ±0.72	21.22 ±0.53	25.65 ±0.55	30.17 ±0.99	35.36 ±0.35
40^0C	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
CD (5%)	1.02	1.26	1.48	1.15	1.07	1.09

*Mean of 3 replications. Percentage calculated on the basis of 300 spores. Data after ± represent standard error values.

Results revealed that only 2.45% germination took place after 12 hours of incubation at 10°C and no germination took place at 40 °C. Spore germination was optimum at 28 °C (100% after 12 h). Thus optimum temperature of spore germination was found to be 28 °C (Table 4.8 and Fig.4.9)

4.3. Pathogenicity of *Alternaria alternata* in different niger varieties

Pathogenicity of a fungus varies in different cultivated varieties. Differential pathogenicity of a fungus to different varieties gives us information about the degree of susceptibility or resistance of a particular variety to a particular pathogen. Hence, in the present study, pathogenicity of *Alternaria alternata* was tested on 6 different niger varieties viz. LV, JNC 6, GA-5, GA-10, RCR-18 and NRS-96-1.

4.3.1. Pathogenicity test following whole plant inoculation technique

The whole plant inoculation method as described by Dhingra and Sinclair (1995) was followed. Pathogenicity of *Alternaria alternata* was tested on whole niger plants of six different varieties as mentioned in section 3.1.1.1. Ten plants of each variety were taken for each treatment. The inoculation method and procedure of disease assessment has been described in materials and methods (Section 3.5 and 3.6 respectively) and results have been summarized in Table 4.9 and Fig.4.10.

From the data represented in Table 4.9 and Fig.4.10, it was quite clear that LV showed maximum disease development (Mean disease index/ plant was 32.5) after 10 days of inoculation. Therefore, niger plants of LV could be considered as most susceptible variety against *Alternaria alternata* among the vaieties tested. Niger plants belonging to JNC-6 variety were also highly susceptible (MDI/plant=31.6 after 10 days of inoculation). Varieties like NRS-96-1 and RCR-18 were most resistant (MDI/plant=5.3 after 10 days of inoculation) and resistant (MDI/plant=10.4 after 10 days of inoculation) respectively. The other two varieties (GA-5 and GA-10) were less susceptible than most susceptible varieties (LV and JNC-6).

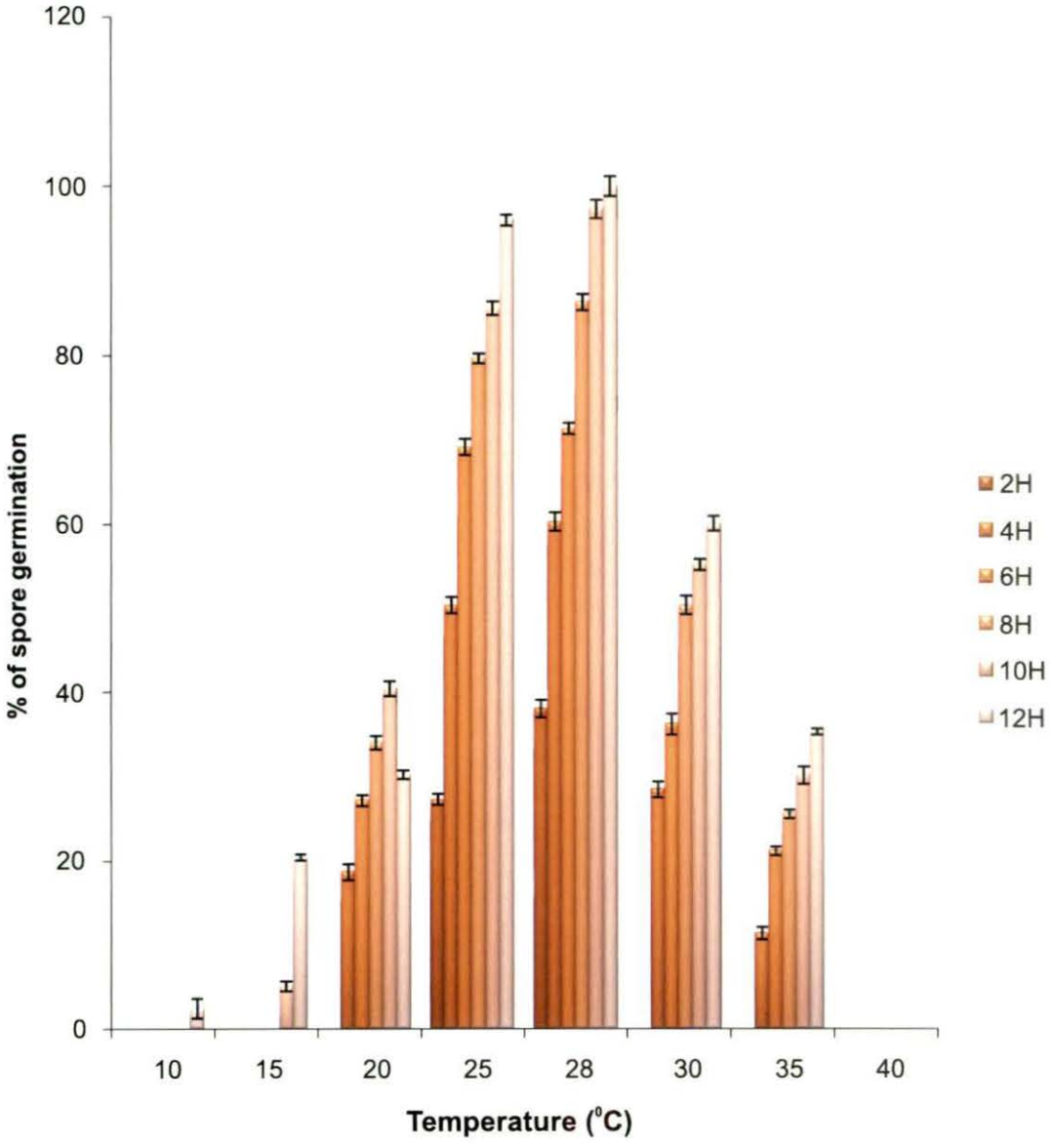

Fig: 4.9 Effect of temperature on spore germination of *A. Alternate*

Table: 4.9. Pathogenicity of *Alternaria alternata* on niger varieties.

Niger varieties	Incubation period (Days)									
	2		4		6		8		10	
	MDI/ plant*	MNL/ Plant	MDI/ Plant	MNL/ Plant	MDI/ plant	MNL/ plant	MDI/ plant	MNL/ plant	MDI/ plant	MNL/ plant
LV	2.8± 0.21	14.3± 1.01	4.6± 1.18	20.2± 1.00	8.4± 0.42	24.3± 1.02	10.1± 0.25	28.4± 1.01	14.3± 0.96	32.5± 0.87
JNC 6	2.8± 0.53	13.2± 1.27	3.7± 0.55	16.6± 0.91	7.5± 1.1	22.5± 1.36	8.5± 1.04	24.3± 1.27	9.3± 0.57	31.6± 0.78
GA-5	2.6± 0.17	12.5± 1.10	3.2± 0.96	14.4± 0.76	4.6± 1.1	16.2± 1.07	5.0± 0.85	18.4± 0.93	8.4± 0.83	22.0± 0.95
GA-10	2.5± 0.20	11.8± 1.14	2.9± 0.10	12.2± 1.14	3.2± 0.4	12.6± 1.46	3.4± 0.57	13.3± 0.79	3.6± 0.81	14.6± 0.64
RCR-18	1.7± 0.35	9.3± 1.24	1.8± 0.26	10.3± 1.34	2.0± 0.25	10.4± 0.67	2.2± 0.59	10.4± 0.40	2.3± 0.74	10.4± 0.76
NRS - 96-1	1.1± 0.30	5.2± 1.12	1.3± 0.35	5.3± 1.19	1.4± 0.20	5.3± 0.45	1.6± 0.12	5.3± 0.71	1.9± 0.21	5.3± 0.20
CD at 5%	0.51	0.41	1.39	2.62	1.6	1.09	1.81	2.10	2.01	2.06

MDI= Mean disease index; MNL =Mean number of lesions

*Mean of 3 replications.

Data after ± represent standard error values.
** Average of 50 lesions.

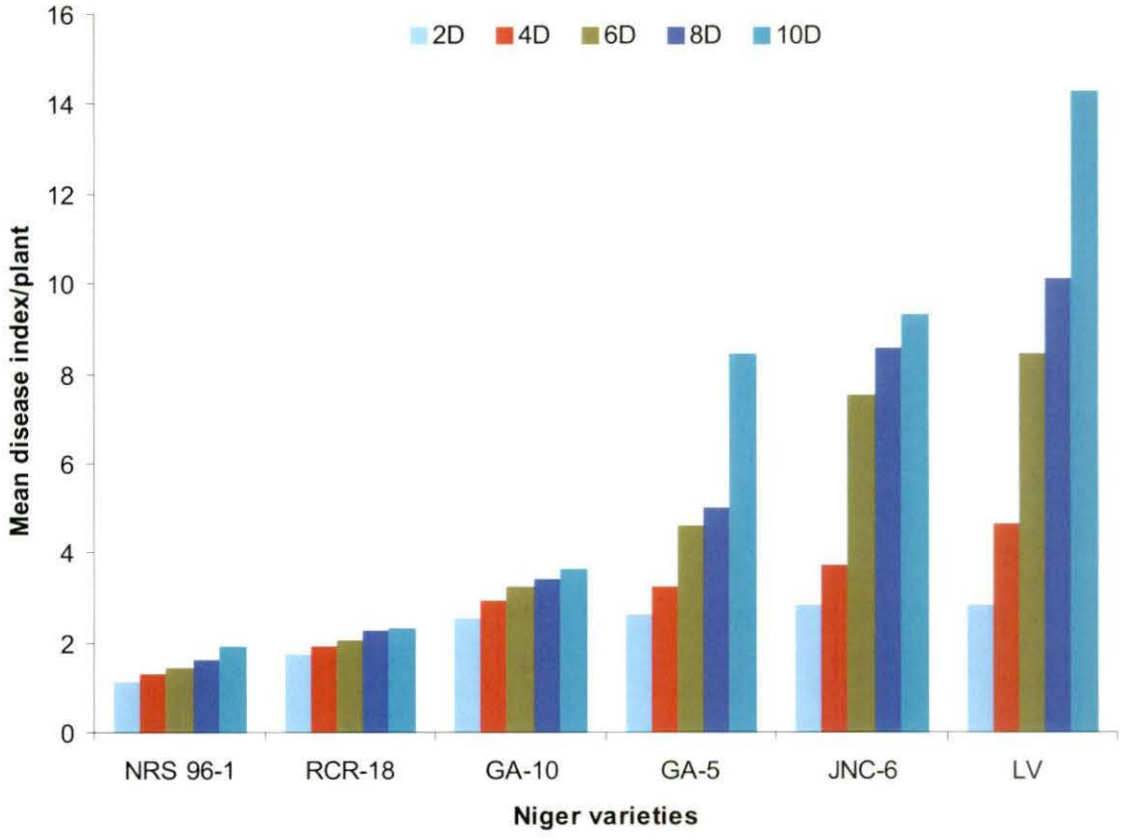

Fig: 4.10. Pathogenicity of *Alternaria alternata* of six different varieties of niger plants.

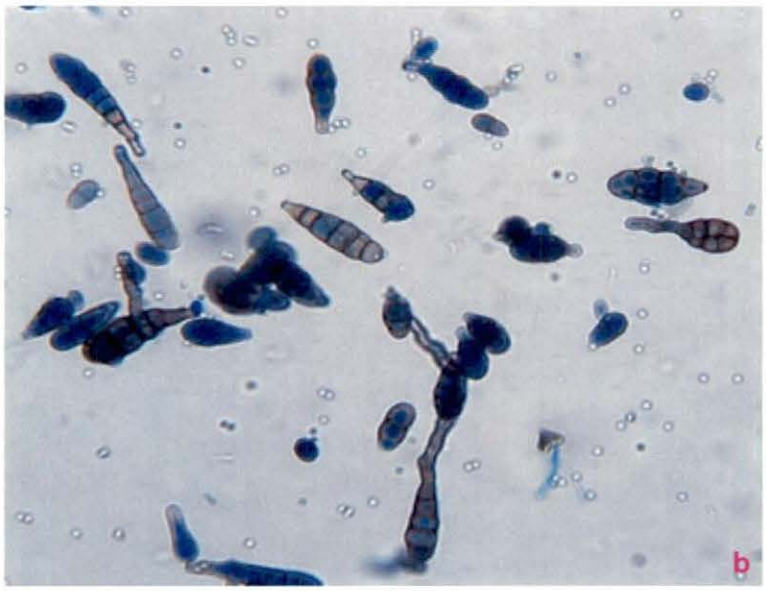

PLATE 4.2

fig. a : Susceptible variety artificially infected with *A.alternata*

fig. b : Spores in spore suspension

4.4. Experiments on Serological studies

Serological techniques are powerful tools in virulence studies. It play a key role in early detection of plant pathogenic microorganisms. The serological techniques determine, on one hand, the pathogenic properties of isolates, and on the other hand, the susceptibility or resistance of host cultivars. In the present investigation, attempt has been taken to determine the presence of common antigens, if any, among the niger varieties and *A. alternata*. Several workers have shown that the possibility of susceptibility is greater when cross-reactive antigens are more (Alba et al., 1983; Purkayastha and Banerjee, 1990; Chakraborty and Saha, 1994. Plant antigens were prepared from healthy leaves of six niger varieties (including susceptible and resistant cultivated varieties). Similarly, fungal antigen was prepared from a virulent isolate of *A. alternata*. Polyclonal antisera were raised in separate male white rabbits against antigens of selected representative type i.e. one resistant (NRS-96-1), one susceptible (LV) niger variety and pathogen (*A. alternata*). Normal sera were collected from rabbits by marginal ear vein puncture before immunization. The details of the antigen preparation procedures have been described in section 3.8 & 3.9 of materials and methods. The antigens of respective varieties were designated by lowercase letter 'a' suffixed with first 2-4 letters of the variety codes of niger plants (i.e. LVa, JNC6a, GA5a, GA10a, RCRa and NRSa). Codes of fungi were 'Al' for *Alternaria alternata* (pathogen) and 'Gv' for *Gliocladium virens* (nonpathogen). Antisera of different niger varieties and fungi were designated by upper case letter 'A' suffixed with respective codes of the niger varieties or fungi. Three different antisera (LVA, NRSA, AlA) and normal sera (NS) were used in the present study. Procedures of preparation of the antisera and normal sera have been discussed in the materials and methods (Section 3.10).

4.4.1. Serological relationship between different niger varieties and *Alternaria alternata* by immunodiffusion (ID) technique

The standard method as described by Ouchterlony (1958) was followed to determine the serological relationship between host and pathogens by agar gel double diffusion. First, semi quantitative estimation of antibody activity of the three different antisera against their homologous antigens as well as titre values of antigens of LVa, NRSa and Ala against their homologous antisera were determined (Table 4.10). Following determination of titre values the antigen of the pathogen *A. alternata,* antigen of non-pathogen *Gliocladium virense* and leaf

antigens of six different niger varieties (LV, JNC-6, GA-5, GA-10, RCR-18 and NRS 69-1) were used in the experiment. Preparation of the antigens, antisera and details of the procedure has already been discussed in the materials and methods (Section 3.9, 3.10 & 3.12). The results have been presented in Table. 4.11.

Table: 4.10. Semi-quantitative estimation of antigens and antisera of niger varieties varieties and *A. alternate*

Host and pathogen	Titre of antigen against homologous antiserum	Titre of antiserum against homologous antigen
Host variety		
NRS-69-1	8	16
Local variety	8	16
Pathogen		
Alternaria alternata	16	32

Incubation time- 72h
Temperature- 25±1° C.

From the results noted in Table 4.11, it was observed that common antigenic relationship were present not only in cases of homologous reactions i.e. between antisera and antigens of *A. alternata* (Plate 4.3 fig. b), LV (Plate 4.3 fig. a & f) and NRS 96-1 (Plate 4.3 fig.e) but also in cross reactions between antisera of *A.alternata* (AlA) and antigens of four niger leaves (LVa, JNCa, GA5a, and GA10a). Very low intensity precipitin band was observed between antisera (AlA) and antigen (RCRa) (Plate 4.3, fig. c). No precipitation band was observed when antigen NRSa was used against antisera of *A.alternata* (AlA) (Plate 4.3, fig. c). Common precipitation bands were also found in reactions between antisera LVA and antigen Ala (Plate 4.3, fig. d). Where the antisera (NRSA) of NRS 96-1 variety was used, common precipitation bands were visible with the antigens LVa, JNCa, GA5a, GA10a, RCRa, and NRSa (Plate 4.3 fig. e). No precipitation bands were observed in any reaction involving antigen of *Gliocladium virens* (Gva) or normal sera (NS) (Plate 4.3, fig. d & f).

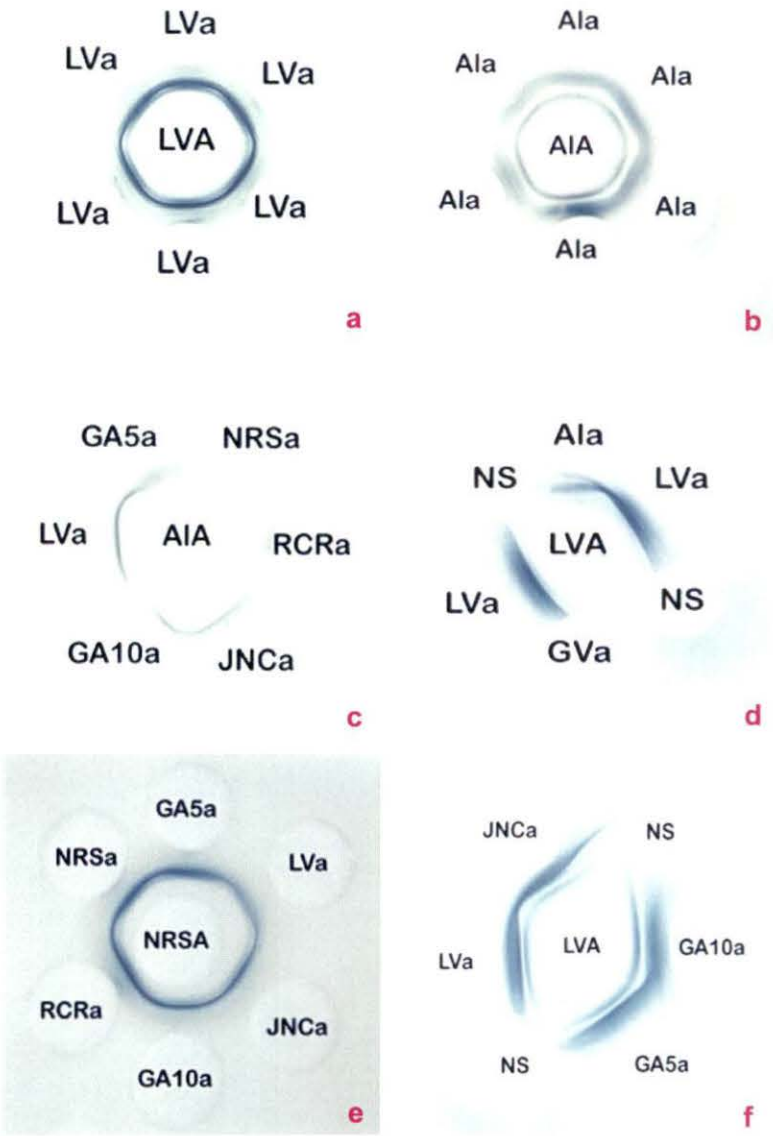

PLATE 4.3

Agar gel double diffusion test using different antigens and antisera

fig. a: Leaf homologous reaction where peripheral wells contained antigen of LV (LVa) and central well contained antisera of LV (LVA).

fig.b: Fungus homologous reaction where peripheral well contained antigen of *A.alternata* (Ala) and central well contained antisera of fungus (AlA).

fig. c: Peripheral wells contained antigens of NRS 96-1 (NRSa), RCR-18 (RCRa), JNC-6 (JNCa), GA10 (AG10a), LV (LVa), GA5 (GA5a) and central well contained antisera of fungus *A.alternata* (AlA).

fig. d : Peripheral wells contained antigen of LV (LVa), Normal sera (NS), *Gliocladium virense (Gva), A.alternata* (Ala) and central well contained antisera of LV (LVA).

fig. e : Peripheral wells contained antigens of NRS 96-1 (NRSa), RCR-18 (RCRa), GA10 (AG10a), JNC-6 (JNCa),LV (LVa), GA5 (GA5a) and central well contained antisera of resistant variety NRS 96-1 (NRSA).

fig. f : Peripheral wells contained antigens of GA10 (AG10a), GA5 (GA5a), Normal sera (NS), LV (LVa), JNC-6 (JNCa), and central well contained antisera of LV variety (LVA).

Table: 4.11. Common antigenic relationship between niger varieties and *Altrenaria altrenata* (Based on agar gel double diffusion)

Antigen of pathogen, host and non-pathogen	Antisera of pathogen and host		
	Alternaria alternata* (AlA)	**Local variety (LVA)**	**NRS-69-1 (NRSA)**
Pathogen *Alternaria alternata* (Ala)	+	+	–
Susceptible varieties			
Local variety (LVa)	+	+	+
JNC-6 (JNCa)	+	+	+
GA-5 (GA5a)	+	+	+
GA-10 (GA10a)	+	+	+
Resistant varieties			
NRS-69-1 (NRSa)	–	+	+
RCR-18 (RCRa)	–	+	+
Non pathogen			
Gliocladium virens (Gva)	–	–	–

Common precipitation band present = +;Common precipitation band absent = –
*Codes of different antigens and antisera are in parenthesis.

Immunodiffusion tests clearly showed the presence or absence of common antigens between hosts and pathogen. Many of the antigen antiserum reactions (precipitin bands) could not be clearly distinguished. Hence, for better resolution it was decided to separate the antigens by electrophoresis before exposing them to antisera.

4.4.2. Serological relationship between different niger varieties and *Alternaria alternata* by immunoelectrophoresis (IE).

A combination of electrophoresis and radial immunodiffusion in agar gel is immunoelectrophoresis. In agar gel, movement of molecules in an electric field is similar to that in liquid medium, with the advantage that free diffusion during and after electrophoresis is lessened (Clausen, 1969). Immunoelectrophoresis was done using the antisera AlA (of *A.alternata),* LVA (of Local Variety) and NRSA (of NRS 96-1 variety) and different niger plant antigens (LVa, JNCa, GA5a, GA10a, RCRa, NRSa), pathogen antigen(Ala), and antigens (Gva) of non-pathogen *Gliocladium virens.* The detail of the procedure has already been

discussed in the materials and method (Section 3.12.2). The results of the experiments were noted in Table 4.12 and Table 4.13.

Table: 4.12. Comparison of precipitation arcs found in immuno-electrophoresis of Niger varieties (Susceptible and resistant), pathogen (*Alternaria aternata*) and non-pathogen (*G. virens*).

Antigen of pathogen, host and non-pathogen	Total No of precipitation arcs		
	Antisera of pathogen and host		
	Alternaria alternata* (AlA)	**Local variety (LVA)**	**NRS-69-1 (NRSA)**
Pathogen			
Alternaria alternata (Ala)	4	1	0
Susceptible varieties			
Local variety (LVa)	1	5	3
JNC-6 (JNCa)	1	5	3
GA-5 (GA5a)	1	4	2
GA-10 (GA10a)	1	4	2
Resistant varieties			
NRS-69-1 (NRSa)	0	3	4
RCR-18 (RCRa)	0	4	4
Non pathogen			
Gliocladium virens (Gva)	0	0	0

*The codes of different antigen and antisera are in the parenthesis.

From Table 4.12, 4.13 and Fig. 4.11 it was evident that the antigen of *A. alternata* shared one precipitin arc or band when treated with the antisera of LV variety (LVA). Antigen of NRS 96-1 variety and RCR-18 shared respectively 3 and 4 precipitin arcs when treated with the antisera of LV variety (LVA). Antigens each of LV, JNC-6, shared five precipitin bands and GA-5, and GA-10 shared four precipitin bands when reacted with antisera of LV (LVA).

Antigens of LV, JNC-6, GA-10 and GA-5, shared precipitation arcs each in all the cases when reacted with antisera of *A.alternata* (AlA) while antigens of NRS 69-1 variety (NRSa), and RCR-18 (RCRa) variety showed no precipitation arcs. Antigen (Ala) of *A. alternata* shared four precipitin bands with antisera (AlA) of *A. alternata*. Antigen (Ala) of *A.alternata* shared one precipitin band with antisera LVA although it shared no precipitin band with antisera (NRSA) of NRS 69-1 variety (Plate 4.4, fig. e & c).

Table: 4.13. Immunoelectrophoretic test of antigens and antisera of niger varieties and *Alternaria alternata*

Antigen of pathogen, host and non-pathogen	**Precipitation arcs**													
	Antisera of *A. alternata* (AlaA)				**Antisera of Local Variety (LVA)**					**Antisera of NRS-69-1 (NRSA)**				
	1	2	3	4	1	2	3	4	5	1	2	3	4	5
Pathogen														
A. alternata (Ala)	+	+	+	+	-	+	-	-	-	-	-	-	-	-
Susceptible varieties														
Local Variety (LVa)	+	-	-	-	+	+	+	+	+	+	+	+	-	-
JNC-6 (JNCa)	+	-	-	+	+	+	+	+	+	+	-	+	+	-
GA-5 (GA-5a)	+	-	-	-	+	+	+	-	+	-	+	+	-	-
GA-10 (GA-10a)	-	+	-	-	+	+	+	-	+	+	+	-	-	-
Resistant varieties														
NRS-69-1(NRSa)	-	-	-	-	+	+	+	-	-	+	+	+	+	-
RCR-18 (RCRa)	-	-	-	-	-	+	+	+	+	+	+	+	+	-
Non pathogen														
Gliocladium virens (Gva)	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Common precipitation band present (+)

Common precipitation band absent (–)

* The codes of different antigens and antisera are in the parenthesis.

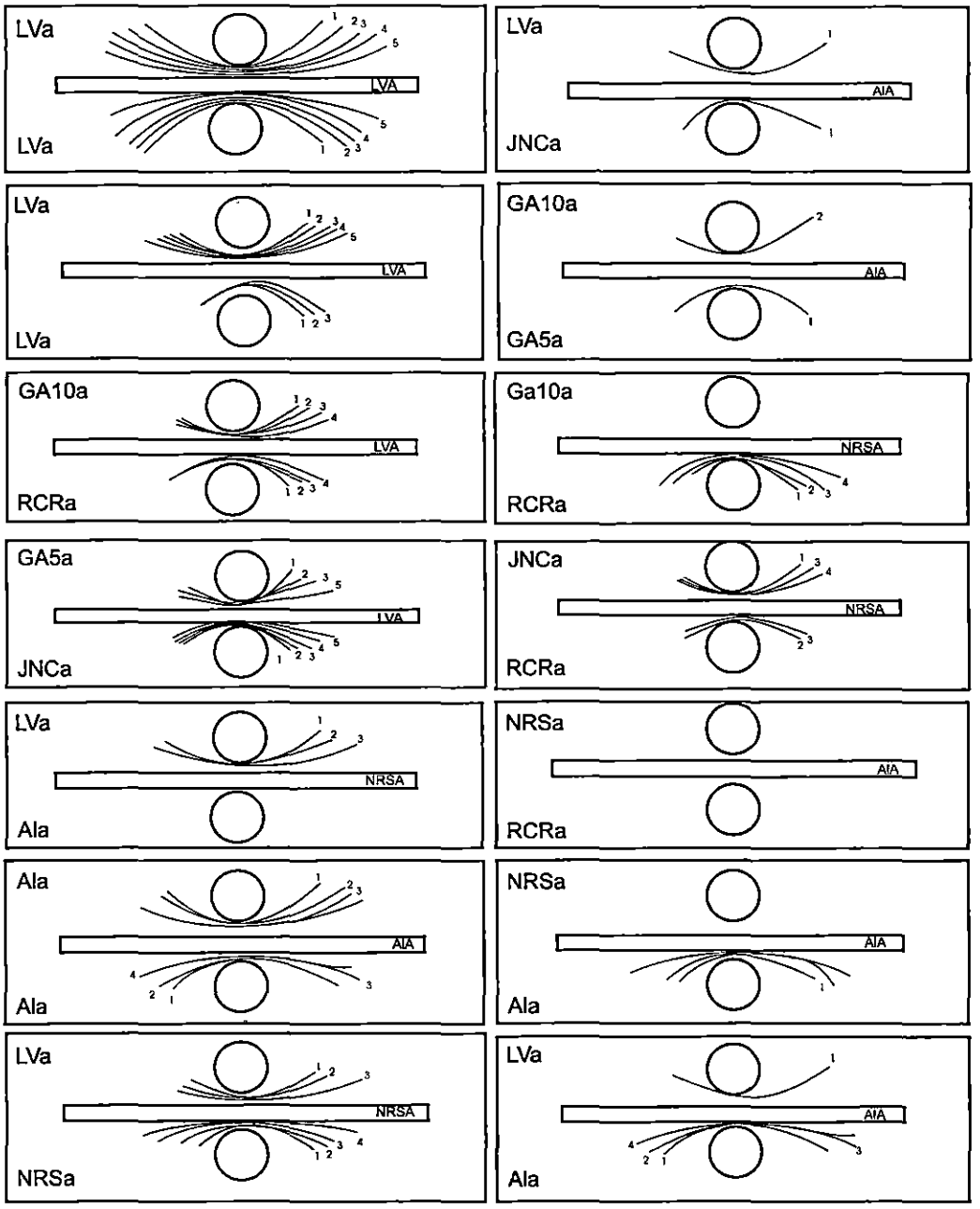

Fig. 4.11 : Immunogram showing immunoelectrophoretic patterns of antigens and antisera of *A. alternata* and niger varieties. Different antisera used in the central rectangular wells are of *Alternaria alternata* (AIA), LV (LVA), and NRS 69-1(NRSA) while different antigens were used in the peripheral wells are LV (LVa), NRS69-1 (NRSa), JNC 6 (JNCa), GA-5(GA5a), GA-10 (GA10a),RCR 18 (RCRa)

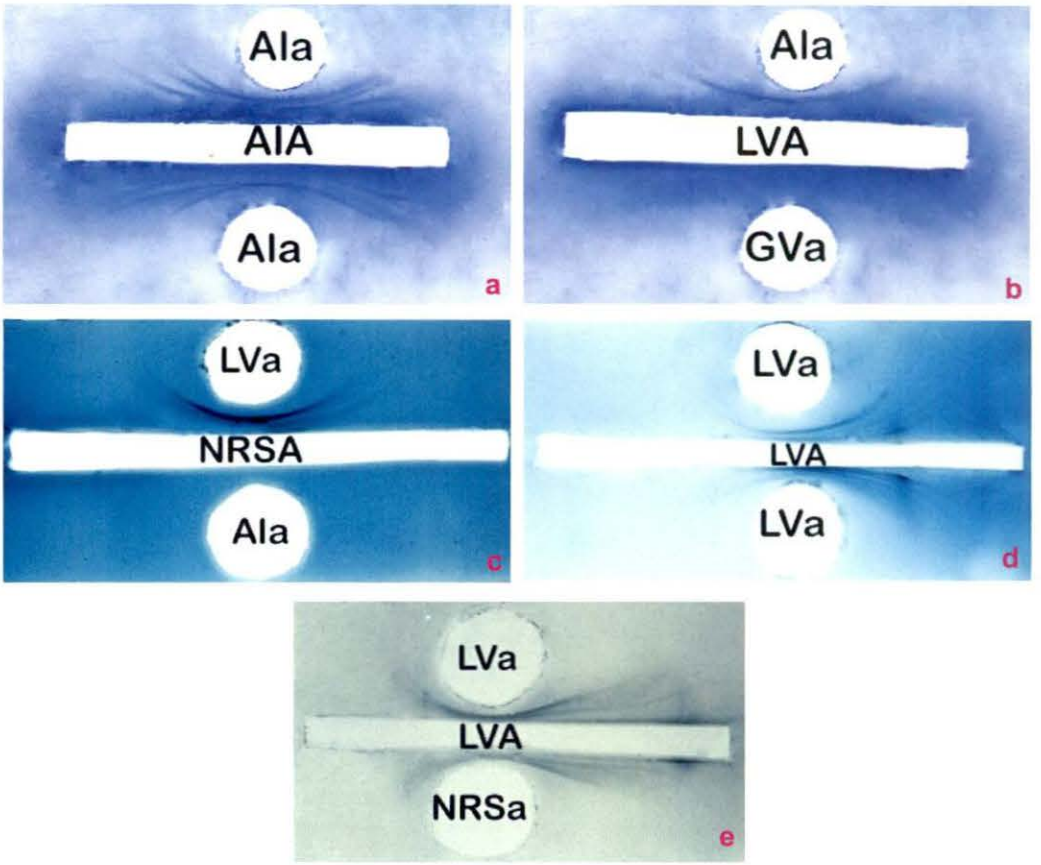

PLATE 4.4

Immunoelectrophoresis showing arc patterns of different antigens and antisera of *A.alternata* and niger varieties

fig. a : Central well contained antisera of *A.alternata* (AIA) and peripheral wells contained antigen of *A.alternata*(Ala).

fig. b : Central well contained antisera of LV (LVA) and peripheral wells contained antigen of *A. alternata* (Ala) and nonpathogen *Gliocladium virens (Gva)*

fig. c : Central well contained antisera of NRS 69-1 (NRSA) and Peripheral wells contained antigen of LV (LVa), and antigen of fungus *A.alternata* (Ala).

fig. d : Central well contained antisera of LV (LVA) and peripheral wells contained antigen of LV (LVa)

fig. e : Peripheral well contained antigen of LV (LVa), NRS 69-1 (NRSa) and central well contained antisera of LV (LVA).

4.4.3. Detection of cross-reactive antigens between leaf antigens of different niger varieties and antisera of *Alternaria alternata* by indirect ELISA

Several formats of enzyme linked immunosorbent assays have become popular as a diagnostic tool (Bom and Boland, 2000; Kennedy *et al.* 2000, Sumarah and Miller, 2005; Babitha *et al.*, 2006). Among the different formats indirect-ELISA technique has been used by several authors (Chakraborty and Saha, 1994; Olsson, 1995; Kratka *et al.*, 2002; Priou *et al.*, 2006). Cross reactive antigens have been successfully detected between different host parasite interactions by indirect-ELISA (Dasgupta *et al.*, 2005; Chakraborty and Sharma, 2007). Cross reactive antigens determine the susceptibility or resistance of a host in a host parasite combination. Therefore, it was considered worthwhile to study cross-reactive antigens between *A. alternata* and niger varieties by using indirect ELISA format, since this technique is one of the most sensitive serological technique to detect and quantify low concentration of antigens. In the present study, leaf antigens of the six niger varieties have been included. In addition, mycelial antigen of the pathogen *A. alternata* and a non pathogen *Gliocladium virens* have been used as antigen while antisera of two niger varieties (LV and NRS 96-1 variety) and the fungal pathogen *A. alternata* were used to perform indirect-ELISA. All the antisera as well as normal sera were diluted to 1/125 and/or 1/250 dilution and were tested against three different concentrations (5 µg ml $^{-1}$,10 µg ml $^{-1}$ and 20 µg ml $^{-1}$) of each antigen separately. The detailed procedures of indirect ELISA as well as the preparation of antigens and antisera have already been discussed in the materials and methods (Section 3.13, 3.9 & 3.10). An ELISA reader (Mios Junior, Merck) determined the absorbance of all the combinations at 492 nm and the results have been presented in Table 4.14 and Fig.4.12.

From Table 4.14, it was clear that all the three concentrations of the antigen *A. alternata* showed higher absorbance values when tested with the antisera of the susceptible variety, LV (0.998 at antigen concentration 20 µg/ml at the antisera dilution of 1/125) than when tested with antisera of the resistant NRS 69-1 (0.236 at antigen concentration 20 µg/ml at the antisera dilution of 1/125). The reciprocal cross of these combinations also showed higher absorbance values produced by antigens of LV (0.522 at antigen concentration 20 µg/ml at the antisera dilution of 1/125) than produced by antigens of NRS 69-1 (0.164 at antigen concentration 20 µg/ml at the antisera dilution of 1/125) when tested with

antisera of *A.alternata*. This clearly indicates that cross-reactivity was higher between pathogen and susceptible variety than between pathogen and resistant variety. Results obtained from all the combinations showed that the absorbance values of normal serum control were lower than the corresponding test values.

Table: 4.14. Indirect ELISA (A_{492}) values of different antigens (antigens of 6 Niger varieties and *Alternaria alternata*) and three different antisera.

Antigen of host varieties, pathogen and nonpathogen	µg protein /ml	**Normal sera (NS) and antisera (AS) of susceptible and resistant niger varieties and pathogen**								
		Local variety (LV)			**NRS-69-1**(NRSA)			**A.alternata (AIA)**		
		NS 1/125	AS 1/125	AS 1/250	NS 1/125	AS 1/ 125	AS 1/ 250	NS 1/125	AS 1/125	AS 1/ 250
Local variety (LVa)	20	0.040	1.118	1.063	0.040	0.750	0.695	0.040	0.522	0.462
	10	0.035	0.972	0.916	0.035	0.742	0.673	0.035	0.400	0.345
	5	0.020	0.905	0.905	0.020	0.720	0.660	0.020	0.365	0.305
JNC-6 (JNCa)	20	0.026	1.110	1.057	0.026	0.785	0.682	0.026	0.356	0.480
	10	0.022	0.965	0.910	0.022	0.761	0.654	0.022	0.386	0.331
	5	0.018	0.945	0.893	0.018	0.733	0.644	0.018	0.327	0.275
GA-5 (GA5a)	20	0.031	0.997	0.942	0.031	0.843	0.785	0.031	0.385	0.335
	10	0.026	0.984	0.931	0.026	0.832	0.776	0.026	0.363	0.311
	5	0.021	0.965	0.915	0.021	0.822	0.769	0.021	0.342	0.292
GA-10 (GA10a)	20	0.034	0.865	0.810	0.034	1.011	0.993	0.034	0.265	0.205
	10	0.030	0.832	0.812	0.030	0.994	0.890	0.030	0.249	0.199
	5	0.026	0.823	0.722	0.026	0.963	0.883	0.026	0.231	0.181
RCR-18 (RCRa)	20	0.028	0.730	0.670	0.028	1.116	1.061	0.028	0.198	0.138
	10	0.022	0.718	0.663	0.022	1.103	1.044	0.022	0.177	0.125
	5	0.020	0.695	0.643	0.020	1.083	1.023	0.020	0.164	0.114
NRS-69-1 (NRSa)	20	0.025	0.716	0.660	0.025	1.134	1.078	0.025	0.193	0.140
	10	0.025	0.688	0.636	0.025	1.110	1.055	0.025	0.173	0.123
	5	0.018	0.662	0.616	0.018	1.086	1.025	0.018	0.153	0.103
A.alternata (Ala)	20	0.024	0.998	0.938	0.024	0.236	0.172	0.024	1.029	0.969
	10	0.020	0.980	0.924	0.020	0.193	0.140	0.020	0.993	0.940
	5	0.017	0.963	0.913	0.017	0.186	0.135	0.017	0.987	0.937
G. virens (Gva)	9	0.027	0.140	0.085	0.027	0.146	0.095	0.027	0.165	0.110
	10	0.025	0.126	0.073	0.025	0.132	0.051	0.025	0.149	0.097
	5	0.023	0.110	0.059	0.023	0.118	0.063	0.023	0.133	0.083

*Codes of antigens and antisera in the parenthesis. NS 1/125=normal sera dialuted to 1:125, AS 1/125=antisera diluted to 1:125, AS 1/250 =antisera diluted to 1:250.

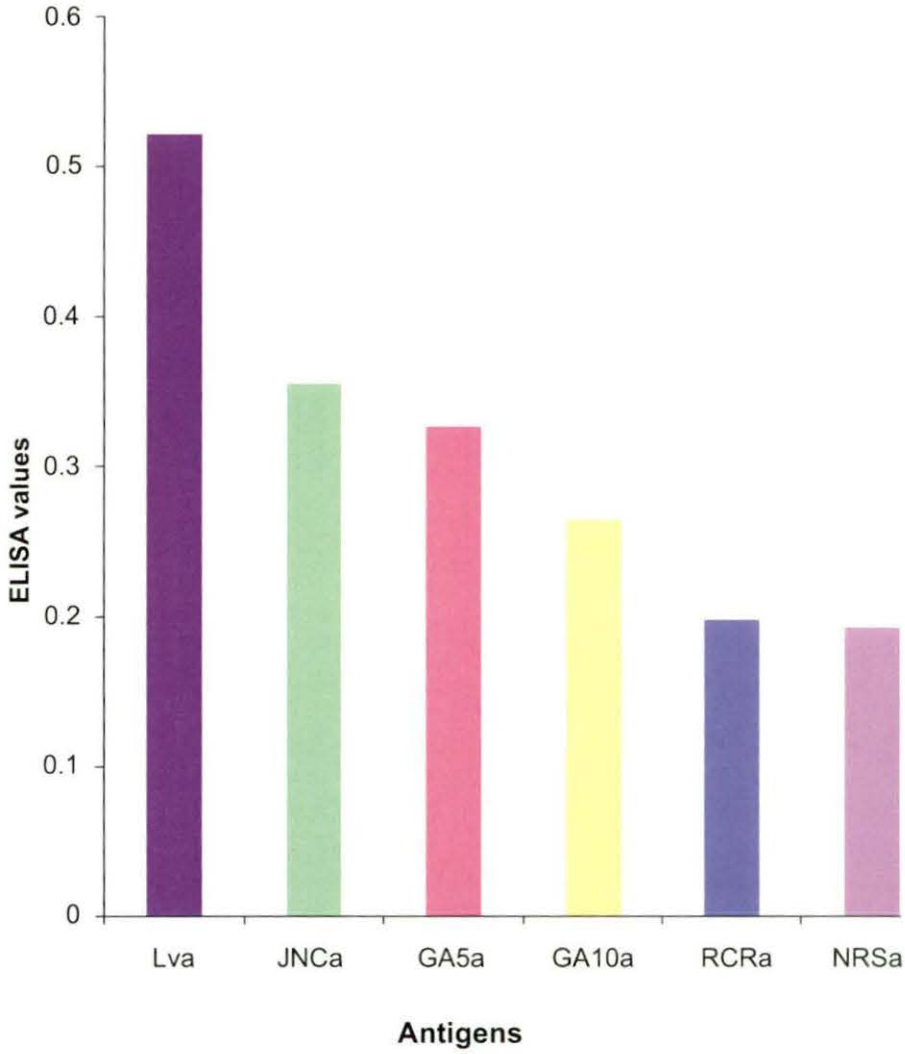

Fig 4.12: Indirect ELISA (A_{492}) results of different combinations of leaf antigens (20µl) against three different antisera.

[Antigens of different niger varieties with their codes in parentheses are : Local variety (LVa), JNC-6 (JNCa), GA-5 (GA5a), GA- 10 (GA10a), RCR 18 (RCRa), NRS 69-1 (NRSa). Three different antisera were of the Local Variety (LVA), NRS 69-1 (NRSA) and *A. Alternata*.]

4.4.4. Immunogold labelling followed by silver enhancement for cellular localization of cross-reactive antigens

For cellular location of different proteins or antigens immunogold labeling followed by electron microscopy is a powerful tool (Lee *et al.*, 2000; Trillas *et al.*, 2000 and Nahalkova *et al.*, 2001). To visualize immunogold labels in light microscope, silver enhancement is essential. Colloidal gold labels are normally visible only at electron microscope level. Silver enhancer enhances the colloidal gold label by precipitation of metallic silver to give a high contrast signal visible under light microscope. Fluorescent antibody labelling with fluorescein isothiocyanate (FITC) is also known to be one of the powerful techniques to determine the cell or tissue location of antigens or proteins. However, autofluorescence present in the plant tissues may mislead the proper understanding of the actual cellular location of CRA or any proteins. Moreover, a fluorescence microscope is more expensive than light microscope. Hence it was considered worthwhile to perform immunogold labeling followed by silver enhancement for tissue location of CRA under light microscope. Earlier serological experiments like immunodiffusion, immunoelectrophoresis and indirect-enzyme linked immunosorbent assay (indirect ELISA) clearly indicated the presence of cross reactive antigens (CRA) between niger varieties and *A. alternata.* To find out tissue and cellular location of CRA shared by the pathogen and niger leaves, immunogold labeling studies followed by silver enhancement were performed. Leaf sections (cut through midrib) of susceptible (LV) and resistant (NRS 96-1) niger varieties and mycelia and spores of *A.alternata* were used as antigens. The antisera of LV (susceptible variety), NRS-96-1 variety (resistant variety) and the pathogen *A.alternata* were used in the experiment. To determine the exact location of CRA, both leaf section and fungal mycelia were treated with antisera and subsequently immunogold labeling and silver enhancement of the host and pathogen was done. The procedure has been discussed in the materials and methods section in details (Section 3.14).

Normal untreated section of niger leaf when observed under light microscope, showed no precipitation and the cells were green in colour. When immunogold labeling and silver enhancement were performed on leaf sections treated with normal sera, the natural greenish colour (Plate 4.5, fig a) disappeared but no precipitation was observed on the cells (Plate 4.5 fig.f). Leaf sections of susceptible variety (LV) when treated with antisera LVA (i.e.

with homologous antisera) and subsequent immunogold labeling followed by silver enhancement, showed maximum precipitation in the epidermal regions, mesophyll tissues and vascular bundle elements of the leaves (Plate 4.5 fig. b). Similar result was observed when leaf sections of NRS 96-1 were treated with homologous antisera (Plate 4.5 fig.d). Heavy precipitation was also observed when a leaf section of LV was treated with antisera of NRS 96-1 variety and vice versa.

In heterologous reaction, when the leaf section of susceptible variety (LV) was treated with antisera of *A. alternata* and labeled with immunogold particles enhanced by silver precipitation, darkening was observed mainly in the epidermal regions. Some precipitation was also found distributed in mesophyll tissues and vascular bundle elements but these were comparatively less dark than observed for homologous reaction (Plate 4.5 fig.c) indicating the presence of CRA. When leaf section of resistant variety (NRS 96-1) was treated with the antisera of pathogen (*A. alternata*) faint precipitation was observed after immunogold labeling and silver enhancement (Plate 4.5 fig.e).

Immunogold labeling and silver enhancement of mycelia and spores of the pathogen *A. alternata* showed that these were grayish in normal condition. When treated with antisera of *A.alternata* (i.e. homologous treatment) mycelia and spores took dark color after immunogold labeling and silver enhancement. When treated with antisera (LVA) of susceptible variety, mycelia showed darkening mainly in the hyphal tips and patches in mycelium and spores but not as much as homologous reaction. Spores and mycelia when treated with antisera of resistant variety (NRS 96-1) showed almost no precipitation and retained normal grayish colour.

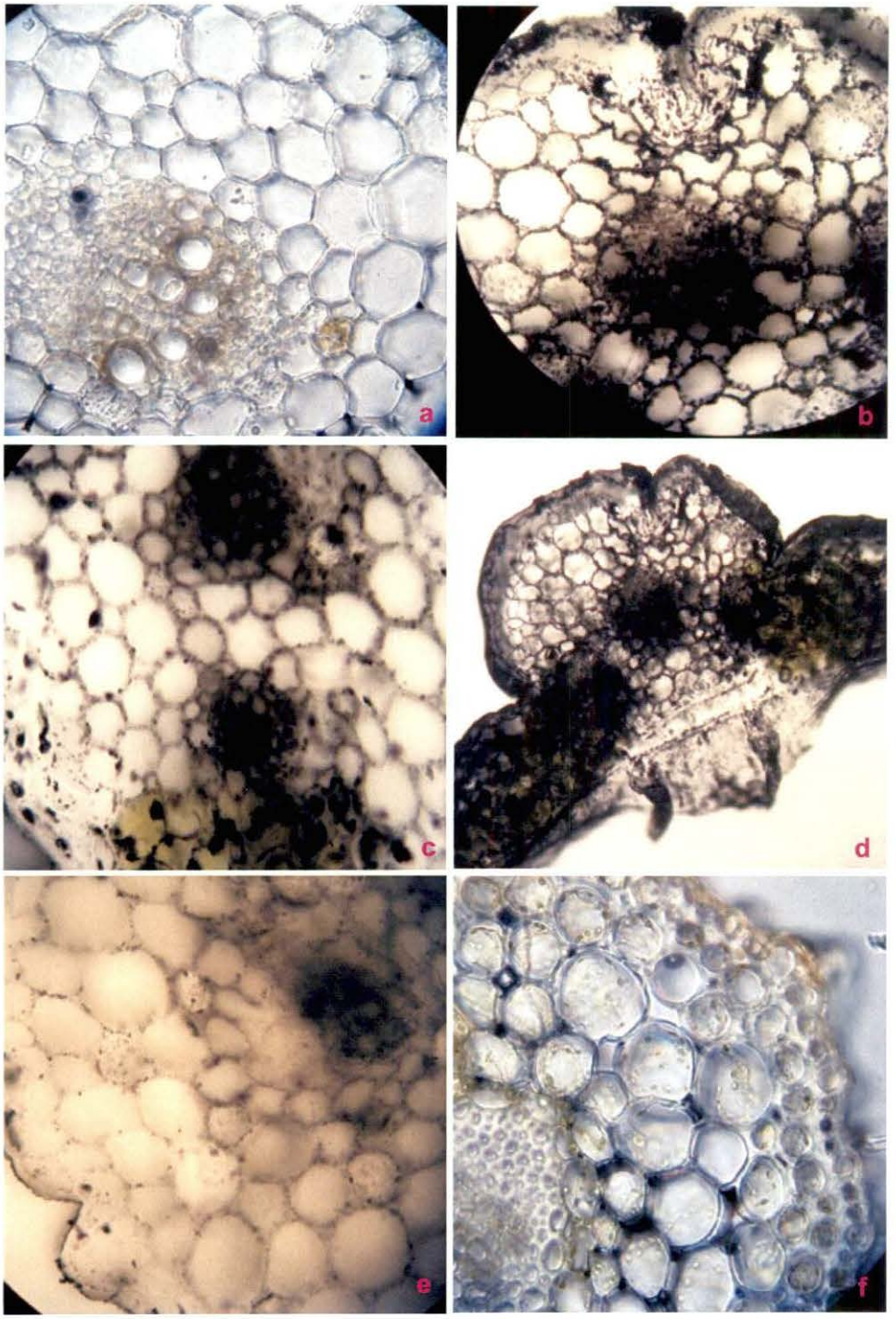

PLATE 4.5

Immuno-gold labeling and siver enhansment of niger leaf tissues for detection of cross- reactive antigens and homologous antigen.

fig. a : Untreated leaf section (LV)

fig. b : Leaf section of LV treated with antisera of LV (LVA)

fig. c : Leaf section of LV treated with antisera of *Alteraria alternata* (AlA)

fig. d : Leaf section of NRS 69-1 treated with antisera of NRS 69-1 (NRSA)

fig. e : Leaf section NRS 69-1 treated with the antisera of *A. alternata* (AlA)

fig. f : Leaf section treated with normal sera

4.5. Studies on defense related enzymes

When plants are infected by pathogens, they initiate various defense reactions such as the production of phytoalexines, antimicrobial proteins, reactive oxygen species etc. These reactions do not allow the infection to proceed, if the reaction occurs in a timely manner. However, if the defense reaction occur too late or are suppressed, the infection process will proceed successfully (Somssich and Hahlbrock, 1998). Hence, it is important for plants to detect infecting pathogens effectively and deliver such information intracellularly / intercellularly to activate their defense machinery (Shibuya and Minami, 2001). To provide protection against pathogens certain biotic and abiotic inducers have been applied exogenously by several workers. In the last two decades extensive research work has been performed for management of diseases in plants by application of a variety of biotic and abiotic inducers.

During the course of the present study, most susceptible niger variety (LV) among the six varieties of niger, was induced for resistance against *A. alternata* by the application of different chemical inducers and botanicals. Details of the application procedures of different inducers have been discussed in the materials and methods (Section 3.15). Salicylic acid (SA) acts as an endogenous signal in the induction of systemic acquired resistance (SAR) (Gaffney *et al.*, 1993) and produces pathogenesis-related proteins (PRs) like peroxidase (PR-9) along with chitinase (PR-3) and β-1,3-glucanase (PR-2). Other than these the enzymes mentioned above polyphenol oxidase (PPO) and phenyl alanine ammonia lyase (PAL) were also studied following inducer treatments.

Niger plants were grouped into four different categories on the basis of treatment by inducers and inoculation by the pathogen. Four groups of plants were 'treated-inoculated', 'treated-uninoculated', 'untreated-inoculated' and 'untreated-uninoculated'.

Most of the defense related enzymes have several isozymes. Some isozymes are present constitutively while some other isozymes are known to be inducible. Inducible isozymes are of great importance in the study of induced resistance in plants as they are indicators of resistance induction. In the present study, it was considered worthwhile to find inducible isozymes, if any, of three different enzymes such as polyphenol oxidase, β-1, 3-glucanase and peroxidase. The detailed procedures of total soluble protein separation by polyacrylamide gel electrophoresis and respective enzyme specific staining techniques have been discussed in materials and methods (Section 3.17, 3.18 respectively).

4.5.1. Peroxidase (PO)

The role of the defense enzyme (peroxidase) was studied in niger plants following application of some chemical and botanical inducers. Peroxidase is a stress related defense enzyme, induced in plants under various environmental changes such as heavy metals, salts, temperature (Kiwan and Lee, 2003), air pollution, (Lee *et al.*, 2000) and pathogen infection. In the present study, role of peroxidases in defense reaction have been studied to find out a suitable inducer, if any, for control of the disease caused by *A. Alternata* in niger plants. The detailed procedures of enzyme extraction and assay have been discussed in materials and methods (Sections 3.16.1).

4.5.1.1. Peroxidase activity with chemical and botanical inducers

2-Amino butyric acid (2-ABA), 2,1,3-Benzothiodiazole (BTH), 2,3-Dihydroxybenzoic acid (DHB) and Salicylic acid (SA) were used as chemical inducers and aquous leaf extract of *Acalypha indica*, *Catharanthus roseus* were used as botanical inducers to induce resistance in susceptible niger plants (LV). Peroxidase activity was studied after induction and/or inoculation of niger plants.

From the results (Table 4.15 Fig. 4.13) it was observed that 2-Amino butyric acid is the best inducer of peroxidase activity which was increased approximately 9 fold on the second day after inoculation with *A. alternata.* Untreated plants that were inoculated with *A. alternata* showed only marginal increase in enzyme activity. SA, BTH treated plants showed almost similar increase in enzyme levels which increased further after inoculation. DHB induced lowest peroxidase activity in case of both treated-uninoculated and treated-inoculated plants. Untreated-uninoculated control plants showed no change in peroxidase level.

From the results (Table 4.15 Fig. 4.14) it was also found that plants treated with *Acalypha indica* leaf extract and challenge-inoculated by *A. alternata* showed maximum increase in peroxidase level. *Catharanthus roseus* leaf extract induced plants showed less peroxidase activity than *Acalypha in*dica leaf extract. Both *A. indica* and *C. roseus* showed very good PO activity. Activity gradually increased and reached a peak on the 4th day with approximately 8 fold increase in comparison to control. Challenge inoculated plants showed higher enzyme activity than uninoculated but treated plants.

Table: 4.15. Peroxidase activity of Niger plants (LV) pre-treated with chemical and botanical inducers followed by challenged inoculation of *Alternaria alternata*

Treatment	Peroxidase activity [Δ_{240} min^{-1} g^{-1} fresh weight tissue] 1 unit = 0.001absorbence			
	Incubation periods [days]			
	0d	2d	4d	6d
Control	50±1.26	50±1.22	50±1.15	50±1.24
Alternaria alternata	50±1.32	100±2.61	150±2.33	50±2.23
2-Amino butyric acid (2-ABA)	50±1.12	450±2.22	200±3.24	150±2.25
2-Amino butyric acid+ *A.alternata*	50±1.17	500±2.53	300±2.21	250±1.13
2,1,3-Benzothiodiazole (BTH)	50±1.24	150±3.61	200±2.26	100±1.26
2,1,3-Benzothiodiazole + *A.alternata*	50±1.36	200±3.26	300±1.15	100±1.65
2,3-Dihydroxybenzoic acid (DHB)	50±1.15	150±3.22	200±1.26	150±2.65
2,3-Dihydroxybenzoic acid + *A.alternata*	50±1.25	200±1.17	250±3.33	150±2.85
Salicylic acid (SA)	50±1.13	200±2.45	250±3.65	200±2.71
Salicylic acid + *A.alternata*	50±1.33	250±2.36	300±3.22	200±2.65
Acalypha indica	50±1.26	150±3.26	200±2.21	150±2.43
Acalypha indica + *A.alternata*	50±1.38	200±4.12	450±2.32	300±2.21
Catharanthus roseus	50±1.22	150±2.10	200±2.56	100±3.10
Catharanthus roseus + *A.alternata*	50±1.34	200±2.44	400±3.18	250±2.61

Data is the mean of three replicates; Data after ± indicates standard error values.

4.5.1.2. Study of peroxidase isoform patterns

The peroxidase isozyme analysis was performed following procedures as described in materials and methods (Section 3.18.1). Among the tested abiotic inducers, 2-ABA was found to be the most effective than others as evident from Fig. 4.13. Hence, peroxidase isoform patterns were studied in susceptible niger plants (LV) treated with 2-ABA. After 2 days, in case of 2-ABA treated plants three major bands (approximate Rf 0.70, 0.72 and 0.75) were observed (Plate 4.6, lane 3 & 4). Isozymes were less intense in control (Untreated-Uninoculated)

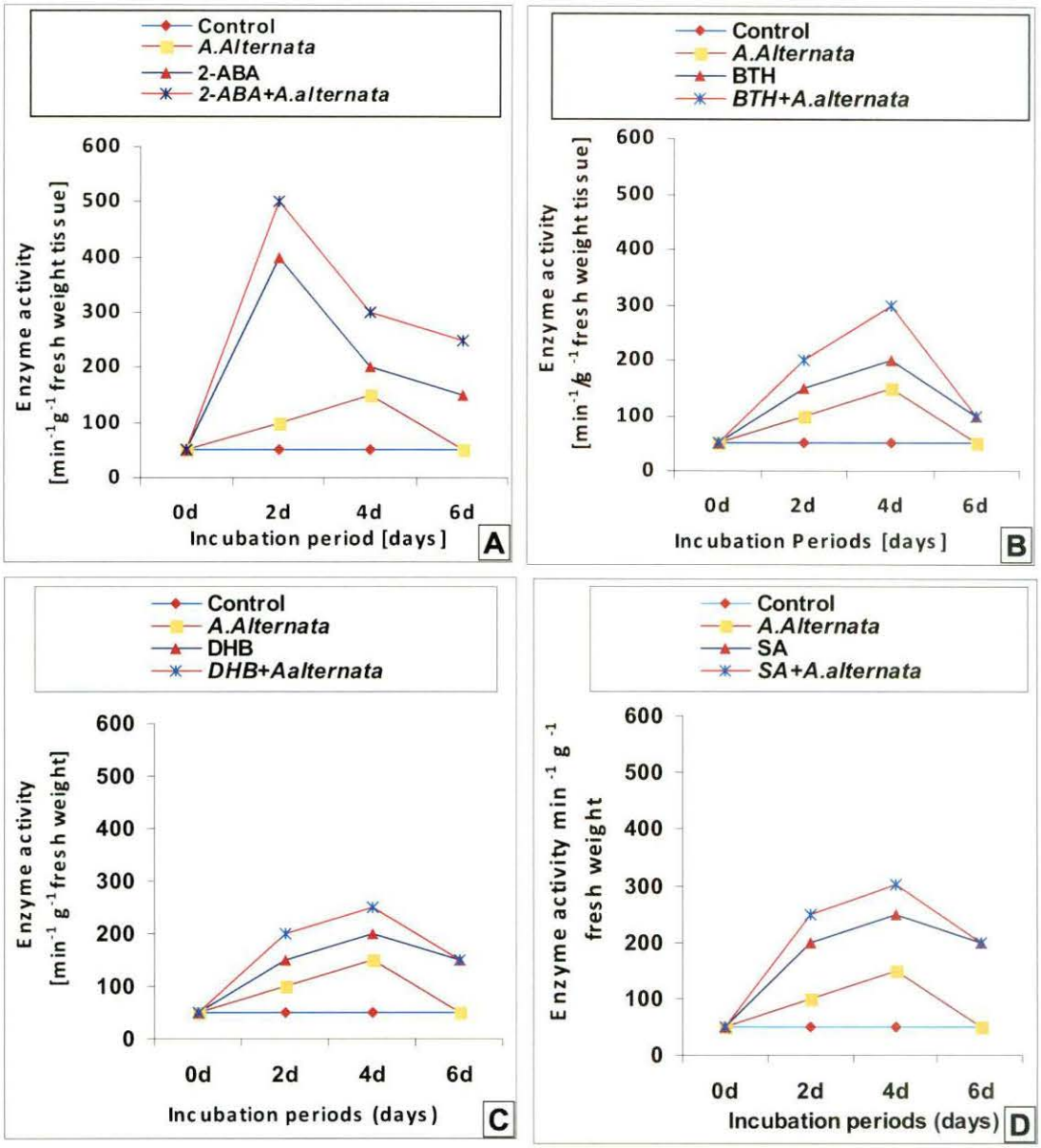

Fig. 4.13: Peroxidase activity in inoculated and treated inoculated niger plant (LV).

A: Treated with 2-Amino butyric acid (2-ABA)

B: Treated with 2,1,3-Benzothiodiazole (BTH)

C: Treated with 2,3-Dihydroxybenzoic acid (DHB)

D: Treated with Salicylic acid (SA)

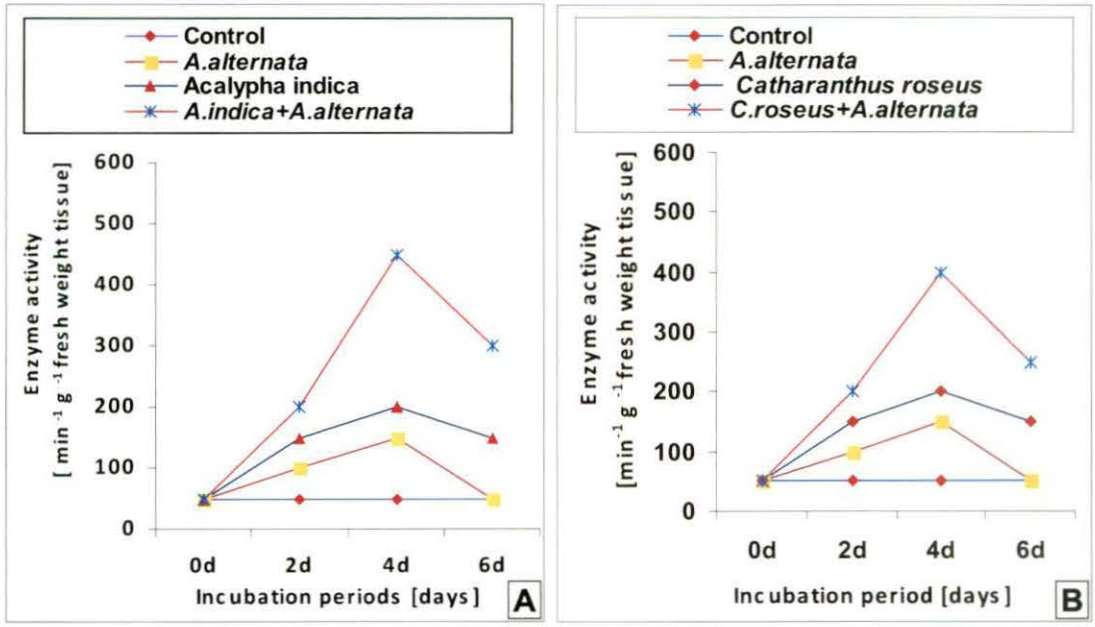

Fig. 4.14: Peroxidase activity in inoculated and treated inoculated niger plant (LV).

A: Treated with *Acalipha indica*

B: Treated with *Catharanthus roseus*

and treated-uninoculated set. Two particular bands with approximately Rf 0.70 and Rf 0.75 showed maximum intensity in the treated-inoculated leaf samples (Plate 4.6: Lane 4). The peroxidase isozymes induced by pathogen infection appeared to be different from that induced following treatment with 2-ABA and then challenge-inoculated with pathogen.

4.5.2. Polyphenoloxidase (PPO)

Polyphenol oxidase (PPO) is a copper-containing defense related enzyme and it is related to phenyl-propanoid pathway. PPO uses molecular oxygen to oxidize o-diphenols to o-quinones (diphenolase activity), which has inhibitory activity against phytopathogenic microorganisms (wang and Peter, 2004 Mahadevan and Sridhar, 1996). It has been reported that PPO activity can be significantly increased by exogenous application of SA (Meena *et al.*, 2001) and by application of different elicitors (Nandeeshkumar *et al.* 2008).

4 .5.2.1. Polyphenol oxidase activity with chemical and botanical inducers

Four different chemical inducers (2-ABA, BTH, DHB, and SA) have been used for induction of PPO in susceptible niger plants (LV). The detailed procedure of application has been discussed in materials and methods (Section 3.15). Enzyme activity was measured after 2, 4, 6, days after treatment and results were recorded. Methods of measuring the enzyme activity have been discussed in materials and methods (Section 3.16.2).

It was evident from the results presented in Table 4.16, Fig. 4.15 & Fig. 4.16 that activity of PPO increased to high lavel when treated with 2,3-Dihydroxybenzoic acid (DHB) and was challenge inoculated by *A.alternata.* The uninoculated but treated plants showed a low increase in enzyme activity which was less than the activity showed by inoculated but untreated plants. Control set showed no significant change in PPO activity. Two different aqueous leaf extracts (*Acalypha indica*, and *Catharanthus roseus*) were used to induce PPO activity in niger plants. The detailed procedures of application of phyto-extracts in order to induce PPO in niger plants have been discussed in materials and methods (Section 3.15).

Results (Table 4.16 & Fig 4.15 & 4.16) revealed that when plants treated with the phytoextract of *C. roseus* and were challenged inoculated with *A. alternata,* showed a significant increase in PPO activity than the treated-uninoculated and untreated-inoculated plants. It was observed that the enzyme

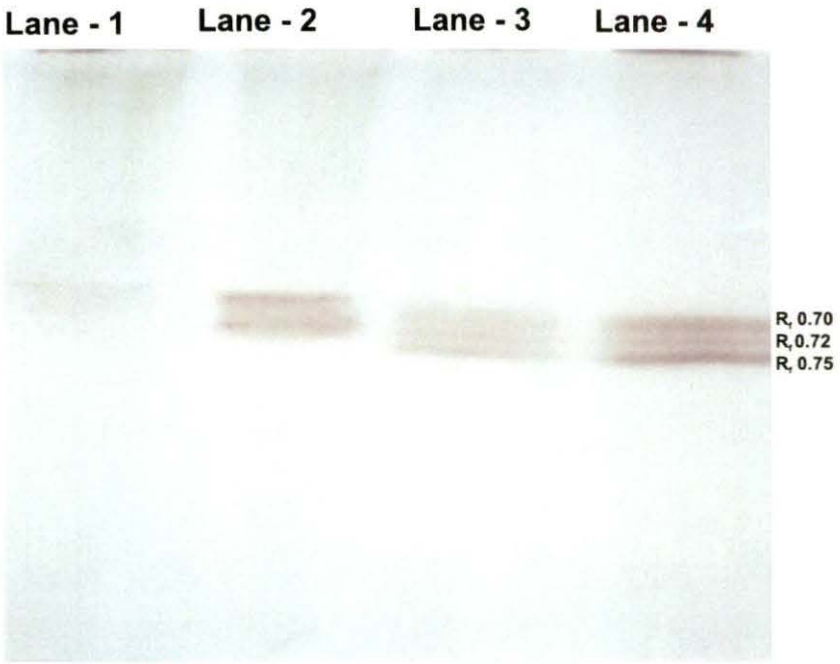

PLATE 4.6

Peroxidase isoform patterns studied on susceptible niger variety (LV) treated with chemical inducer 2-ABA followed by inoculation with *A. Alternata.*

Lane-1: Untreated- uninoculated (Control)

Lane-2: Untreated and inoculated (with *A.alternata*)

Lane-3: Treated (with 2-ABA) uninoculated

Lane-4: Treated (with 2-ABA) and inoculated (with *A. alternata*)

activity in this case was higer then the 2,3-Dihydroxybenzoic acid (DHB) treated inoculated plants.

Table: 4.16. Polyphenol oxidaseactivity of Niger plants (LV) pre-treated with chemical and botanical inducers followed by challenge-inoculation of *Alternaria alternata*

Treatment	PPO activity = K x ($\Delta A\ min^{-1}$) $\mu mol\ min^{-1}\ g^{-1}$ fresh weight tissue (K= 0.272 for polyphenol oxidase) Incubation periods [days]			
	0d	2d	4d	6d
Control	0.03±0.01	0. 03±0.02	0.03±0.02	0.03±0.00
Alternaria alternata	0.03±0.02	0.06±0.03	0.09±0.03	0.04±0.04
2-Amino butyric acid (2-ABA)	0.03±0.02	0.12±0.03	0.34±0.04	0.26±0.03
2-Amino butyric acid+ *A.alternata*	0.03±0.01	0.28±0.04	0.44±0.01	0.29±0.02
2,1,3-Benzothiodiazole (BTH)	0.03±0.01	0.24±0.02	0.41±0.02	0.26±0.02
2,1,3-Benzothiodiazole + *A.alternata*	0.03±0.00	0.29±0.05	0.60±0.03	0.50±0.01
2,3-Dihydroxybenzoic acid (DHB)	0.03±0.03	0.32±0.02	0.46±0.05	0.42±0.03
2,3-Dihydroxybenzoic acid + *A.alternata*	0.03±0.02	0.06±0.03	1.10±0.02	0.95±0.03
Salicylic acid (SA)	0.03±0.01	0.26±0.01	0.50±0.03	0.42±0.04
Salicylic acid + *A.alternata*	0.03±0.02	0.37±0.05	0.65±0.03	0.54±0.05
Acalypha indica	0.03±0.02	0.25±0.04	0.58±0.05	0.45±0.03
Acalypha indica + *A.alternata*	0.03±0.01	0.37±0.02	0.80±0.04	0.75±0.03
Catharanthus roseus	0.03±0.01	0.27±0.06	0.58±0.03	0.46±0.02
Catharanthus roseus + *A.alternata*	0.03±0.03	0.59±0.02	1.18±0.04	1.76±0.02

Data is the mean of three replicates.
Data after ± indicates standard error values.

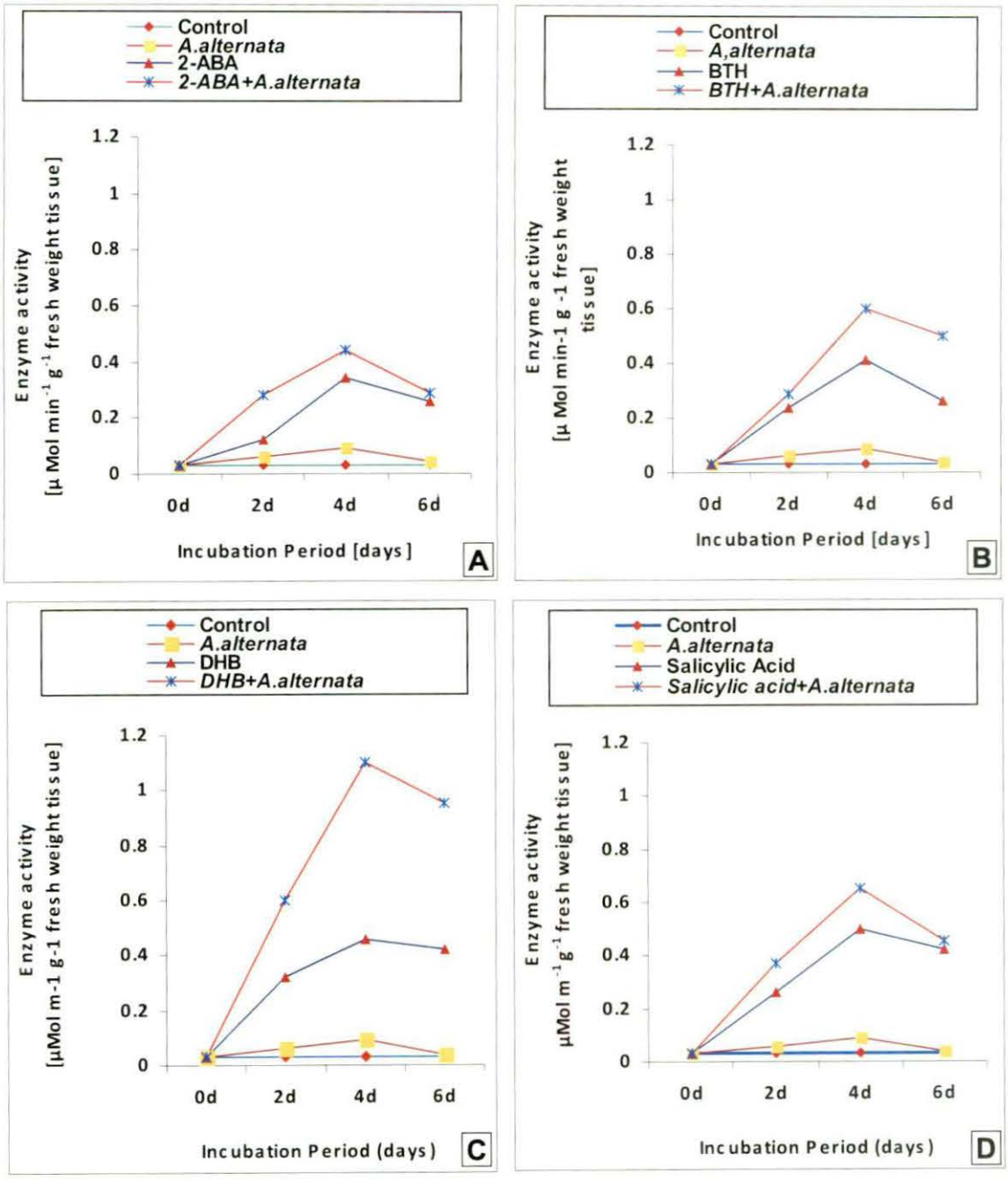

Fig. 4.15: Polyphenol oxidase activity in inoculated and treated inoculated niger plant (LV).

A: Treated with 2-Amino butyric acid (2-ABA)

B: Treated with 2,1,3-Benzothiodiazole (BTH)

C: Treated with 2,3-Dihydroxybenzoic acid (DHB)

D: Treated with Salicylic acid (SA)

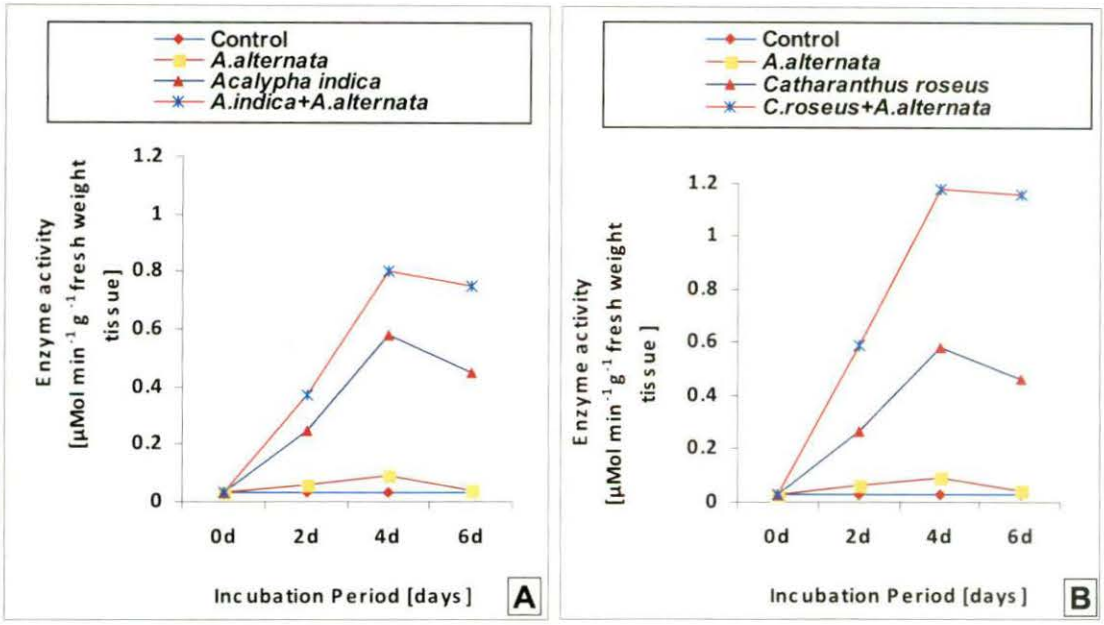

Fig. 4.16: Polyphenol oxidase activity in inoculated and treated inoculated niger pant (LV).

A: Treated with *Acalipha indica*

B: Treated with *Catharanthus roseus*

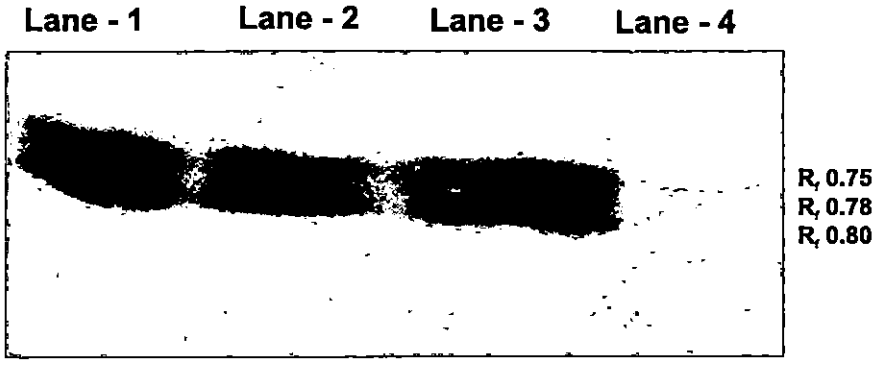

PLATE 4.7

Polyphenoloxidase isoform pattern studied on susceptible niger variety (LV) after different treatments.

Lane-1: Untreated - inoculated

Lane-2: Treated (with leaf extract of *C. roseus*)-uninoculated

Lane-3: Treated (with *C. roesus*)-inoculated (with *A. Alternata)*

Lane-4: Untreated-uninoculated (Control)

the increase was much less in comparison to treated-inoculated sets. In control sets no significant increase in enzyme activity was found.

Induction of β-1,3-glucanase activity was also studied following exogenous application of botanical inducers (leaf extracts of *Acalypha indica*, and *C. roseus*) in niger plants (LV) following methods as described in materials and methods (Section 3.15.2).

From the results (Table 4.17 & Fig.4.18) it was found that plants treated with *C. roseus* leaf extract and challenge-inoculated by *A.alternata* showed maximum increase in β-1,3-glucanase activity after second day of treatment and thereafter it declined. Leaf extracts of *Acalypha indica* showed increase in enzyme activity like SA treated plants but it reached the peak much earlier than the SA trea treated plants. Control plants did not show any significant change in enzyme levels during the experiment.

4.5.3.2. Study of β-1, 3-glucanase isoform patterns

Study of β-1,3-glucanase isozymes were carried out in susceptible niger plants (variety LV) following pre-treatment (separately with *Catharanthus roseus* leaf extract or SA) and then inoculated (by *A. Alternata*). Procedure of total soluble protein separation by PAGE and β-1,3-glucanase enzyme specific staining technique have been discussed in materials and methods (Section 3.17 & 3.18.3 respectively). Separation of β-1, 3-glucanase isozymes in polyacrylamide gels are shown in Plate 4.8. Expression of β-1,3-glucanase isozymes were observed with different colour intensity of the bands that varied with the treatments (Plate 4.8, lane 1-3). Two isozyme bands were visible with the R_f value of 0.08 and 0.13 in each treatment except control. The R_f - 0.13 band was found highly induced when treated with *C. roseus* and inoculated with *A. alternata*. In untreated-uninoculated (control) the band (of R_f value of 0.13) was very faint (Plate 4.8,Lane 4). In case of untreated-inoculated plants, the two bands were present but with much less intensity (Photograph not shown).

Table: 4.17. β-1,3 glucanase activity of Niger plants (LV) pre-treated with chemical and botanical inducers followed by challenge-inoculation by *Alternaria alternata*

Treatment	β-1,3-glucanase activity (nmol $min^{-1}mg^{-1}$ fresh weight tissue)			
	Incubation period [days]			
	0	2	4	6
Control	0.9±0.11	0.9±0.12	0.9±0.15	0.9±0.13
Alternaria alternata	0.9±0.15	1.4±0.14	1.9±0.23	1.0±0.10
2-Amino butyric acid (2-ABA)	0.9±0.12	2.6±0.24	2.9±0.22	2.2±0.26
2-Amino butyric acid+ *A.alternata*	0.9±0.16	2.91±0.22	4.81±0.36	4.1±0.21
2,1,3-Benzothiodiazole (BTH)	0.9±0.12	3.2±0.15	6.9±0.32	5.0±0.31
2,1,3-Benzothiodiazole + *A.alternata*	0.9±0.10	4.2±0.12	9.1±0.24	5.8±0.36
2,3-Dihydroxybenzoic acid (DHB)	0.9±0.14	2.1±0.21	5.8±0.16	4.5±0.15
2,3-Dihydroxybenzoic acid + *A.alternata*	0.9±0.16	3.0±0.26	7.1±0.20	5.8±0.10
Salicylic acid (SA)	0.9±0.15	6.1±0.36	11.7±0.21	7.4±0.25
Salicylic acid + *A.alternata*	0.9±0.13	8.1±0.45	14.4±0.12	13.1±0.21
Acalypha indica	0.9±0.12	11.5±0.22	8.7±0.33	8.1±0.32
Acalypha indica + *A.alternata*	0.9±0.10	15.4±0.24	10.3±0.25	8.7±0.36
Catharanthus roseus	0.9±0.14	12.8±0.17	8.0±0.31	8.0±0.16
Catharanthus roseus + *A.alternata*	0.9±0.15	24.4±-0.25	10.3±0.45	8.7±0.36

Data is the mean of three replicates
Data after ± indicates standard error values.

4.5.4. Chitinase

Chitinase is one of the most important enzyme in plant defense system because it is a glycosyl hydrolase which catalyzes the degradation of chitin, the major component of the cell wall of plant pathogens. This enzyme is released in plant during pathogen infection so it also belongs to Pathogenesis-related protein family. In the present study, chitinase activity and isozyme pattern of chitinase were performed.

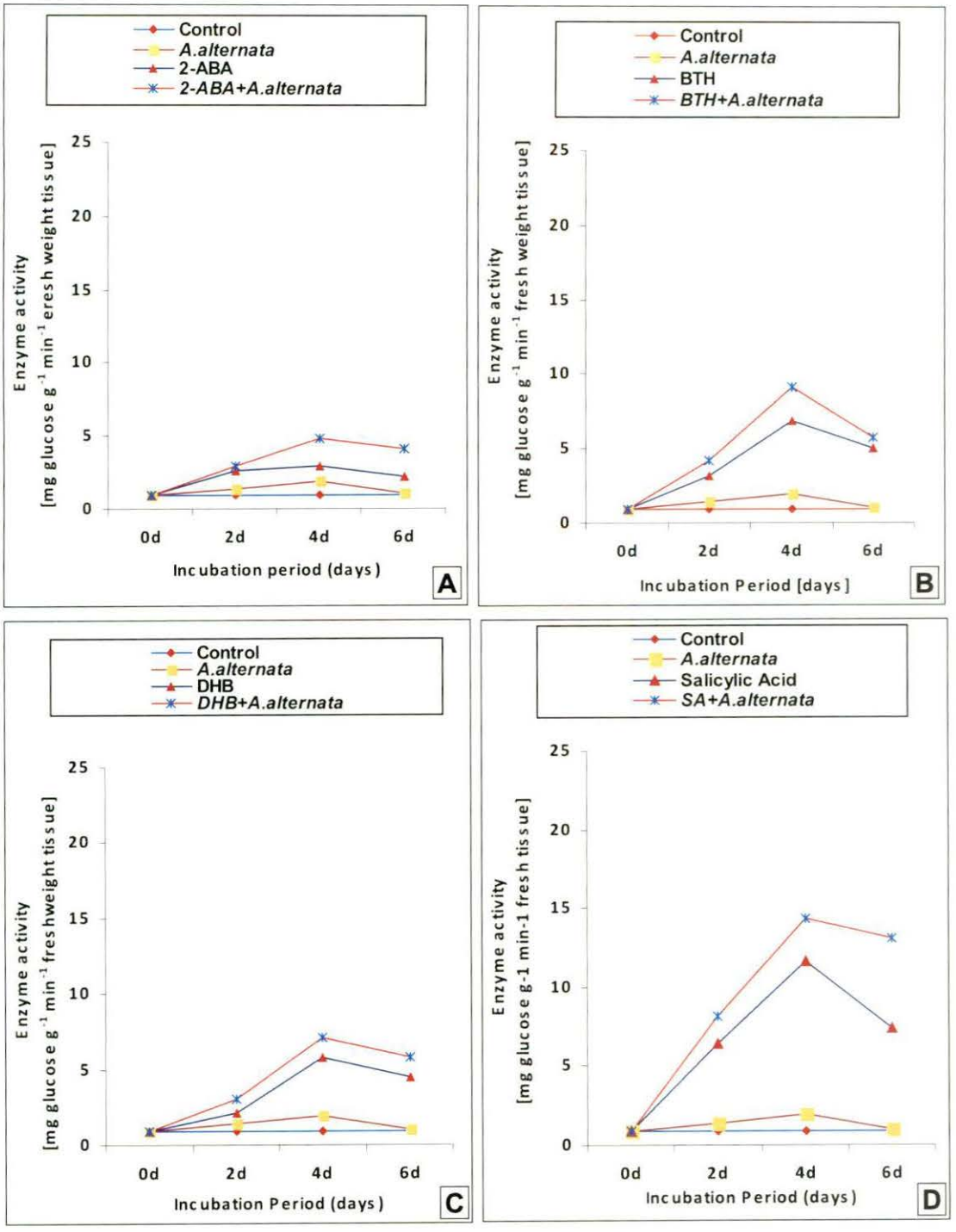

Fig. 4.17: β-1,3-Glucanase activity in inoculated and treated inoculated niger plant (LV).

A: Treated with 2-Amino butyric acid (2-ABA)

B: Treated with 2,1,3-Benzothiodiazole (BTH)

C: Treated with 2,3-Dihydroxybenzoic acid (DHB)

D: Treated with Salicylic acid (SA)

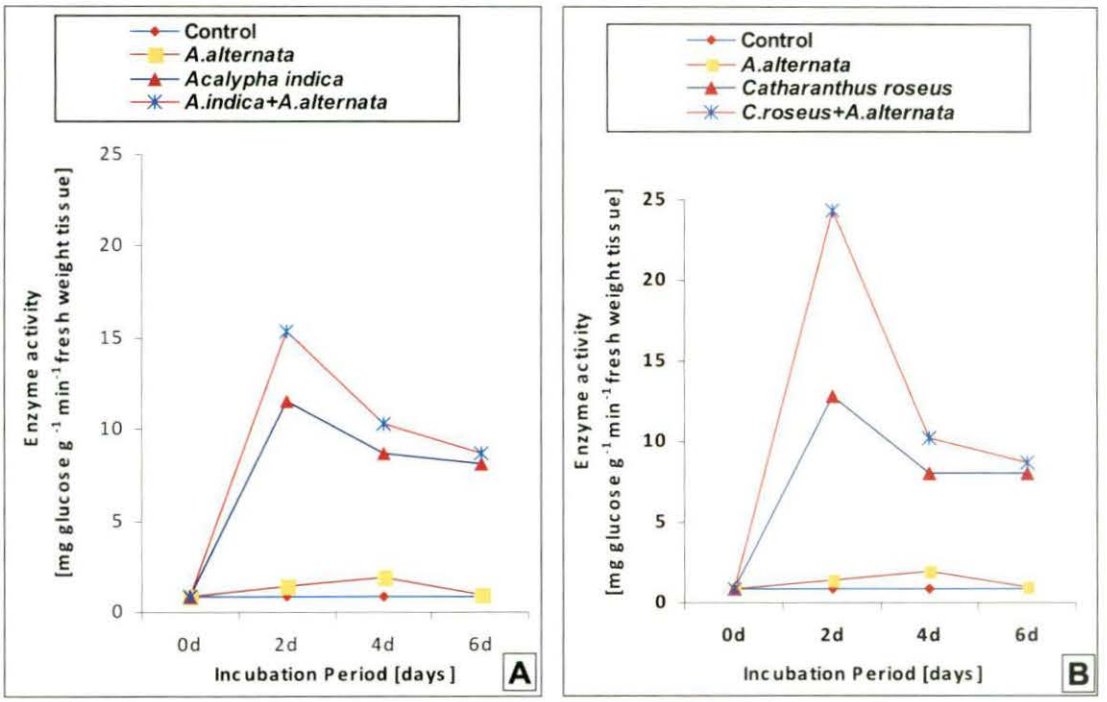

Fig. 4.18: β-1,3-Glucanase activity in inoculated and treated inoculated niger plant (LV).

A: Treated with *Acalipha indica*

B: Treated with 2,1,3-Benzothiodiazole (BTH)

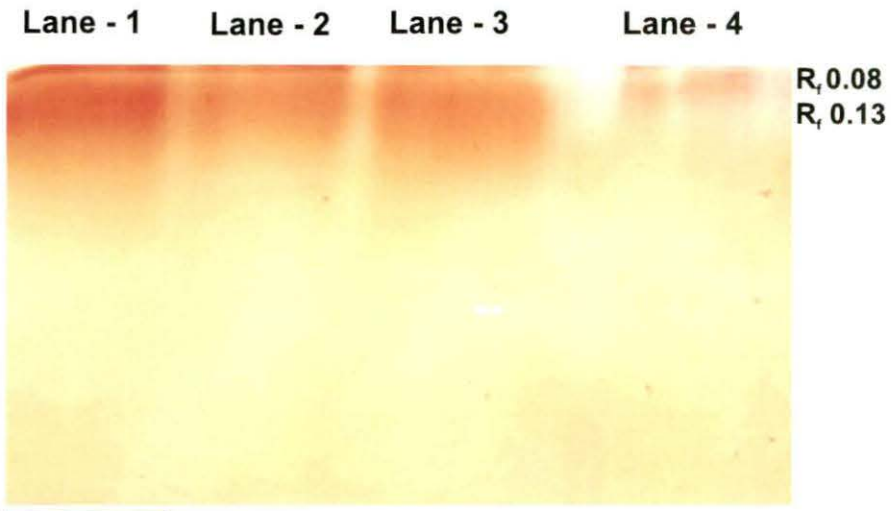

PLATE 4.8

β - 1,3 glucanase isoform pattern studied on susceptible niger variety (LV) treated with leaf extract of *Catharanthus roseus* following inoculation with *A.alternata.*

Lane- 1: Treated with *C.roesus* and inoculated with *A.alternata*

Lane- 2: Treated with SA and inoculated with *A.alternata*

Lane-3: Treated with Botanical inducer *Catharanthus roseus* and inoculated with *A.alternata*

Lane- 4: Untreated- uninoculated (Control)

4.5.4.1. Chitinase activity with chemical and botanical inducers

Four different chemical inducers (2-ABA, BTH, DHB and SA) were used to induce defense response in niger plants (LV). Different level of chitinase activity was observed in different treatments. The detailed procedure of inducer application has been discussed in materials and methods (Section 3.15.2) and the procedure of determining enzyme activity has been discussed in materials and methods (Section 3.16.5).

Results (Table 4.18 & Fig.4.19) indicated that among chemical inducers BTH treated plants showed maximum chitinase activity on fourth day following challenge inoculation with *A.alternata.* Challenge inoculated plants showed higher activity than uninoculated plants. 2-ABA also induced enzyme activity in niger plants. DHB and SA induced more or less similar chitinase activity. In all cases enzyme activity was found to be highest on the fourth day in the treated plants, however, after fourth day it declined. In untreated-inoculated plants chitinase activity increased marginally.

Like chemical inducers plants were also treated with two botanical inducers (*Achalypha indica* and *Catharanthus roseus*). From the results (Table 4.18 & Fig 4.20) it was clear that plants treated (with leaf extract of *C. roseus)* and challenge-inoculated (with *A.alternata)* showed maximum increase in chitinase activity. Leaf extract of *A.indica* also induced enzyme activitybut but much less than *C. roseus.* In control (Untreated-uninoculated) no significant changes in enzyme activity was observed.

4.5.4.2. Study of Chitinase isoform patterns

Study of chitinase isozymes were carried out in two different methods (method 1 & method 2 as mentioned in section 3.18.4). Procedure of protein separation by PAGE has been discussed in materials and methods (Section 3.17). Susceptible niger plants (variety-LV) were pre-treated, separately, with three different inducers (BTH, DHB and SA) and two leaf extracts (*C. roseus* and *A. indica*) and then challenge-inoculated by *A.alternata.*

Separation of Chitinase isozymes in polyacrylamide gels treated with glycol chitin and fluorescent brightner shown in Plate 4.9. Expression of chitinase isozymes were observed with the fluorescent intensity of the bands that varied with the treatments. Alternatively, in Plate-4.10 expression of the enzyme activity have been shown as dark black bands.

Table: 4.18. Chitinase activity of Niger plants (LV) pre-treated with chemical and botanical inducers followed by challenge-inoculation by *Alternaria alternata*

Treatment	Chitinase activity (µmol GlcNAc min^{-1} g^{-1} fresh tissues)			
	Incubation period [days]			
	0	2	4	6
Control	0.8±0.15	0.8±0.12	0.8±0.14	0.8±0.14
Alternaria alternata	0.8±0.12	1.4±0.15	1.9±0.26	1.6±0.25
2-Amino butyric acid (2-ABA)	0.8±0.15	2.8±0.15	4.9±0.34	3.1±0.16
2-Amino butyric acid+ *A.alternata*	0.8±0.14	4.8±0.21	6.4±0.22	5.2±0.54
2,1,3-Benzothiodiazole (BTH)	0.8±0.12	2.7±0.36	5.2±0.45	5.0±0.39
2,1,3-Benzothiodiazole + *A.alternata*	0.8±0.12	3.6±0.0.21	7.0±0.36	7.0±0.32
2,3-Dihydroxybenzoic acid (DHB)	0.8±0.15	2.0±0.31	3.1±0.32	2.9±0.37
2,3-Dihydroxybenzoic acid + *A.alternata*	0.8±0.13	2.2±0.21	5.0±0.62	5.0±0.26
Salicylic acid (SA)	0.8±0.14	3.1±0.10	4.2±0.51	3.6±0.22
Salicylic acid + *A.alternata*	0.8±0.15	3.6±0.15	6.0±0.12	5.2±0.44
Acalypha indica	0.8±0.12	2.8±0.23	3.6±0.16	3.1±0.56
Acalypha indica + *A.alternata*	0.8±0.14	3.9±0.45	4.2±0.26	4.0±0.42
Catharanthus roseus	0.8±0.14	4.1±0.32	6.0±0.39	5.8±0.17
Catharanthus roseus + *A.alternata*	0.8±0.12	5.5±0.62	9.9±0.65	8.4±0.23

Data are mean of three replications

Data after ± indicate standard error values.

One chitinase isozyme band was visible at R_f of 0.62 in each treatment but the intensity of the fluorescent bands were different in different treatments (Plate 4.9). Different treatments were treated with SA & inoculated (lane 3), treated with BTH & inoculated (lane 4) and treated with leaf extract of *C. roseus* & inoculated (lane 5). *Catharanthus roseus* treated and challenge-inoculated (with *A.alternata*) niger plants showed maximum lytic activity of chitinase as compared to other treatments (Plate 4.9). Similar results were also observed in alternative

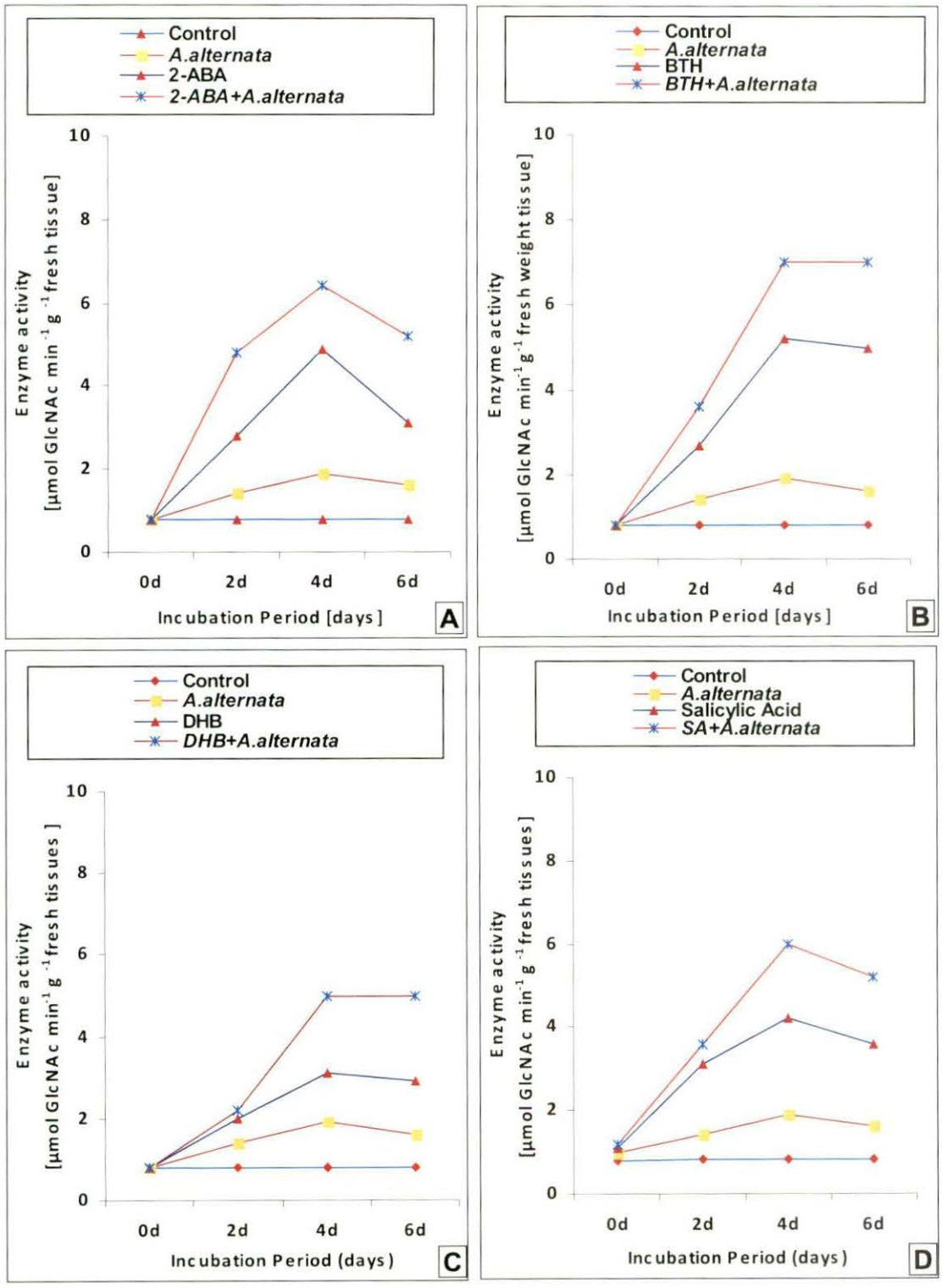

Fig. 4.19: Chitinase activity in inoculated and treated inoculated niger plant (LV).

A: Treated with 2-Amino butyric acid (2-ABA)

B: Treated with 2,1,3-Benzothiodiazole (BTH)

C: Treated with 2,3-Dihydroxybenzoic acid (DHB)

D: Treated with Salicylic acid (SA)

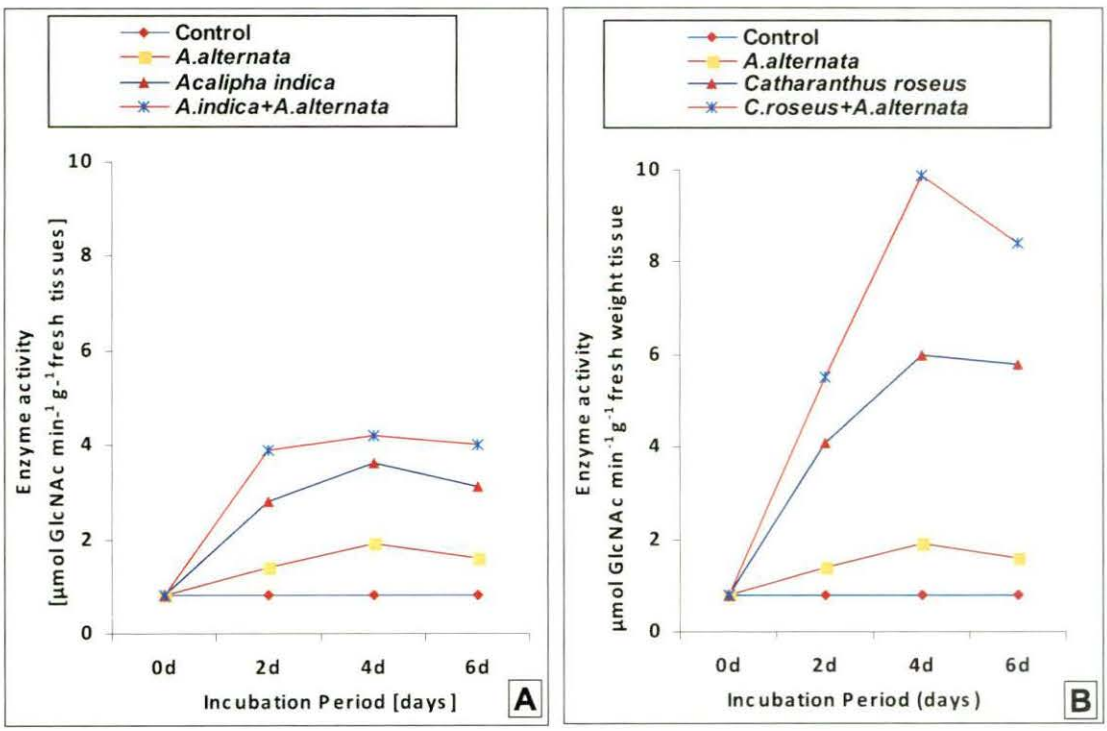

Fig. 4.20: Chitinase activity in inoculated and treated inoculated niger plant (LV).

A: Treated with *Acalipha indica*

B: Treated with *Catharanthus roseus*

Lane - 1 Lane - 2 Lane - 3 Lane - 4 Lane - 5

R_f 0.62

PLATE 4.9

Chitinase isoform pattern studied on polyacrylamide gel (PAGE) and treated with glycol chitin as a substrate and fluorescent brightener used for the resolution of lytic zones (Flurescent bands).

Lane- 1: Untreated- uninoculated (Control)

Lane- 2: Untreated- inoculated with *A.alternata*

Lane- 3: Treated with SA and inoculated with *A.alternata*

Lane- 4: Treated with BTH and inoculated with *A.alternata*

Lane-5: Treated with Botanical inducer *Catharanthus roseus* and inoculated with *A.alternata*

method (method 2) where bands were black coloured and intense bands indicated more activity than the less intense bands (Plate 4.10 & 4.11). In plate-4.11 untreated-uninoculated (control) in lane 1 showed faint band but in the inducer treated-inoculated plants, enzyme activity were found more as observed in the form of intense bands (Lane 2, Lane 3 and Lane 4).

4.5.5. Phenylalanin ammonia layase (PAL)

Phenyl alanine ammonia layase (PAL) is responsible for the conversion of L- phenylealaline to *trans*-cinnamic acid through phenyl propanoid pathway. Cinnamic acid serves as a precursor for the biosynthesis of coumarins, isoflavonoids and liglin. These compounds play an important role in disease resistance mechanism. Changes in PAL activity in fungal, viral and bacterial infections of plants was reported by Mahadevan and Manickam, 1996). PAL activity washas also been reported to increase by exogenous application of Benzothiadiazole (Gorlach *et al.*, 1996), BABA (Newton *et al.*, 1997) and aqueous leaf extract of *Azadirachta indica* (Paul and Sharma, 2002).

4.5.5.1. PAL activity with chemical and botanical inducers

In the present study, different chemical inducers (2-ABA, BTH, DHB and Salicylic acid) and two leaf extracts (*Acalypha indica* and *Catharanthus roseus*) were used to study PAL activity in susceptible niger variety (LV). Fresh young plants were used for induction of PAL. A corresponding control set was also maintained for each treatment. The detailed procedure of application of chemical and botanical inducers has been discussed in materials and methods (Section 3.15.2) and the procedure of enzyme assay has been discussed in Section 3.16.4.

About seven fold increase in PAL activity was observed after 4th day in BTH treated plants that were challenge-inoculated with *A.alternata.* Maximum induction of PAL was observed in *Acalypha indica* treated plants followed by inoculation with *A.alternata.* While 2-ABA treated plants showed only marginal increase (Table 4.19, Fig 4.21& 4.22). Treated plants recorded higher enzyme activity than treated-uninoculated plants. SA, DHB and *Catharanthus roseus* treated plant followed by challenged-inoculated with *A.alternata* showed significant increase in enzyme activity in comparison to 2-ABA treated plants.

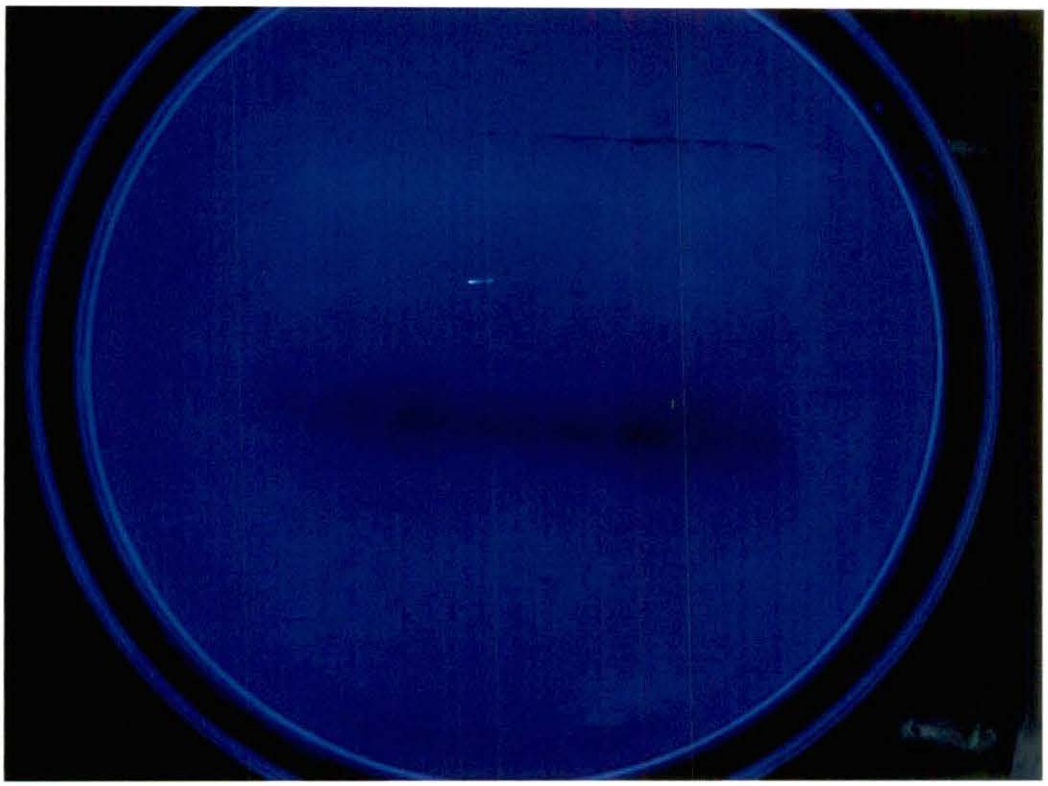

PLATE 4.10

Chitinase isoform pattern studied on agar plate polyacrylamide gel (PAGE) assay where agar plate were treated with glycol chitin as a substrate and fluorescent brightener used for the resolution of lytic zones (black bands)

Lane - 1 Lane - 2 Lane - 3 Lane - 4

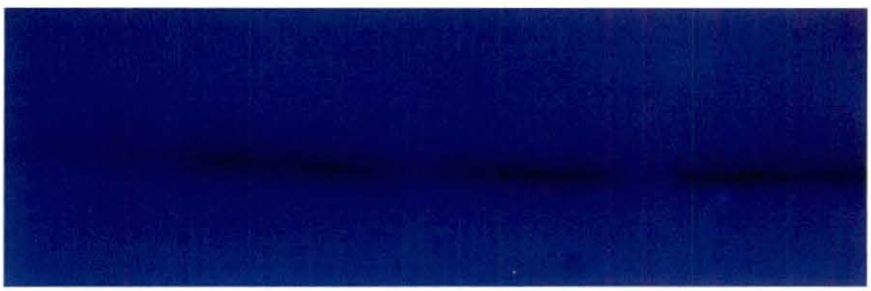

PLATE 4.11

Chitinase isoform pattern studied on agar plate polyacrylamide gel (PAGE) where agar plate were treated with glycol chitin as a substrate and fluorescent brightener used for the resolution of lytic zones (black bands).

Lane- 1: Untreated- uninoculated (Control)

Lane- 2 : Treated with SA and inoculated with *A.alternata*

Lane- 3 : Treated with BTH and inoculated with *A.alternata*

Lane-4: Treated with Botanical inducer *Catharanthus roseus* and inoculated with *A.alternata*

PAL activity increased gradually until 4th day in all treatments following challenge-inoculation by *A.alternata* and then declined. No change in activity was recorded in the untreated-uninoculated control plants. PAL activity was also observed in case of untreated-inoculated plants.

Table: 4.19. PAL activitiy of Niger plants (LV) pre-treated with chemical and botanical inducers followed by challenge-inoculation by *Alternaria alternata*

Treatment	PAL activity (µmol min^{-1} g^{-1} fresh tissues)			
	Incubation period [days]			
	0	2	4	6
Control	0.37±0.01	0.37±0.01	0.37±0.02	0.37±0.01
Alternaria alternata	0.37±0.01	0.65±0.02	0.92±0.01	0.60±0.03
2-Amino butyric acid (2-ABA)	0.37±0.00	1.00±0.02	1.43±0.03	1.22±0.02
2-Amino butyric acid+ *A.alternata*	0.37±0.02	1.27±0.03	1.64±0.03	1.42±0.02
2,1,3-Benzothiodiazole (BTH)	0.37±0.00	1.20±0.01	1.50±0.02	1.32±0.01
2,1,3-Benzothiodiazole + *A.alternata*	0.37±0.00	1.69±0.01	2.8±0.02	2.40±0.03
2,3-Dihydroxybenzoic acid (DHB)	0.37±0.01	1.30±0.04	1.58±0.04	1.40±0.04
2,3-Dihydroxybenzoic acid + *A.alternata*	0.37±0.01	1.67±0.03	1.80±0.03	1.60±0.02
Salicylic acid (SA)	0.37±0.02	1.00±0.03	1.55±0.05	1.23±0.02
Salicylic acid + *A.alternata*	0.37±0.00	1.72±0.01	1.8±0.04	1.70±0.04
Acalypha indica	0.37±0.01	1.69±0.04	1.7±0.01	1.65±0.05
Acalypha indica + *A.alternata*	0.37±0.01	1.78±0.02	4.85±0.02	4.10±0.03
Catharanthus roseus	0.37±0.01	1.12±0.02	1.46±0.01	1.40±0.02
Catharanthus roseus + *A.alternata*	0.37±0.02	1.70±0.01	1.90±0.03	1.80±0.03

Data is the mean of three replicates.
Data after ± indicates standard error values.

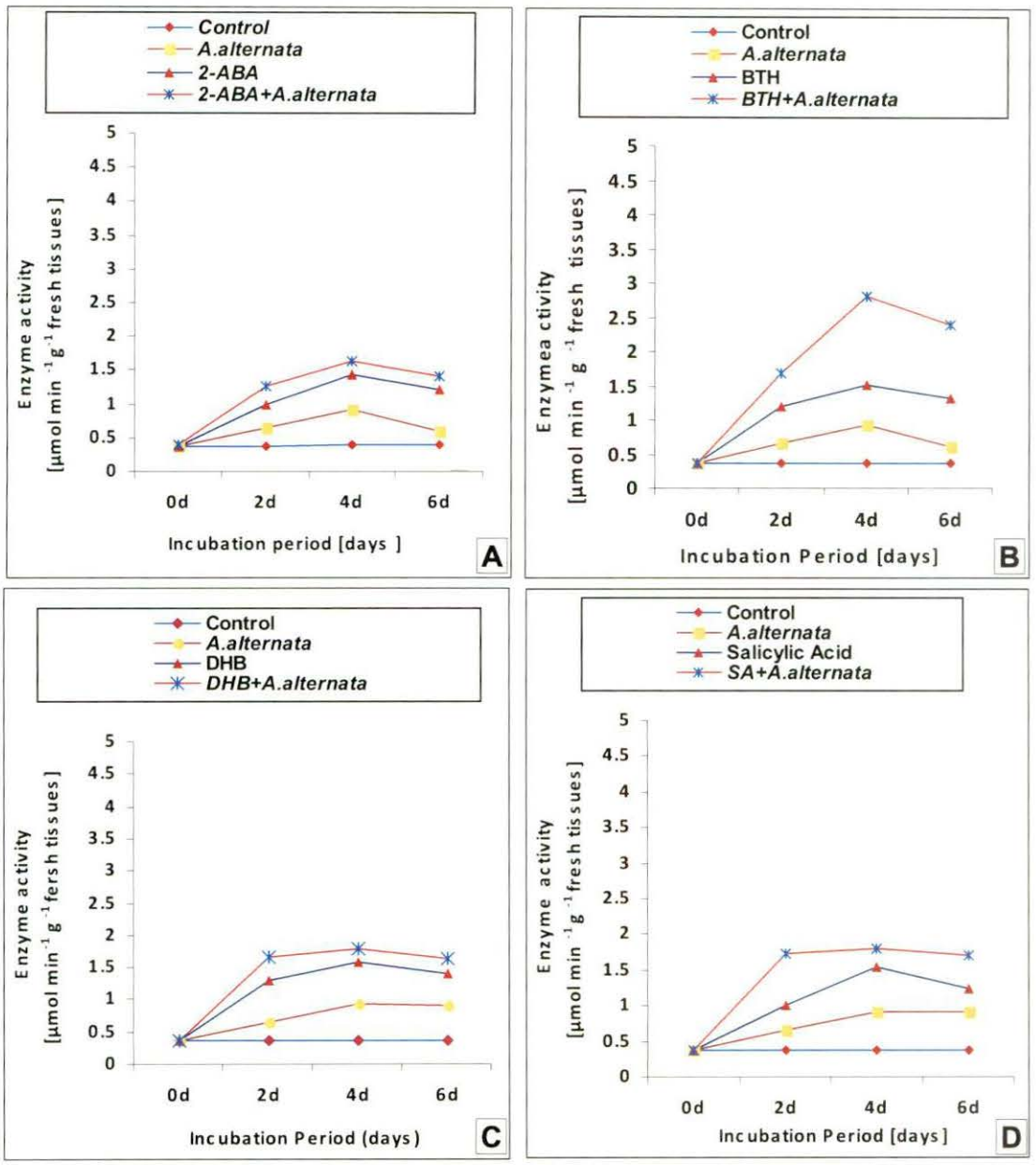

Fig. 4.21: Phenylalanine ammonia lyase activity in inoculated and treated inoculated niger plant (LV).

A: Treated with 2-Amino butyric acid (2-ABA)
B: Treated with 2,1,3-Benzothiodiazole (BTH)
C: Treated with 2,3-Dihydroxybenzoic acid (DHB)
D: Treated with Salicylic acid (SA)

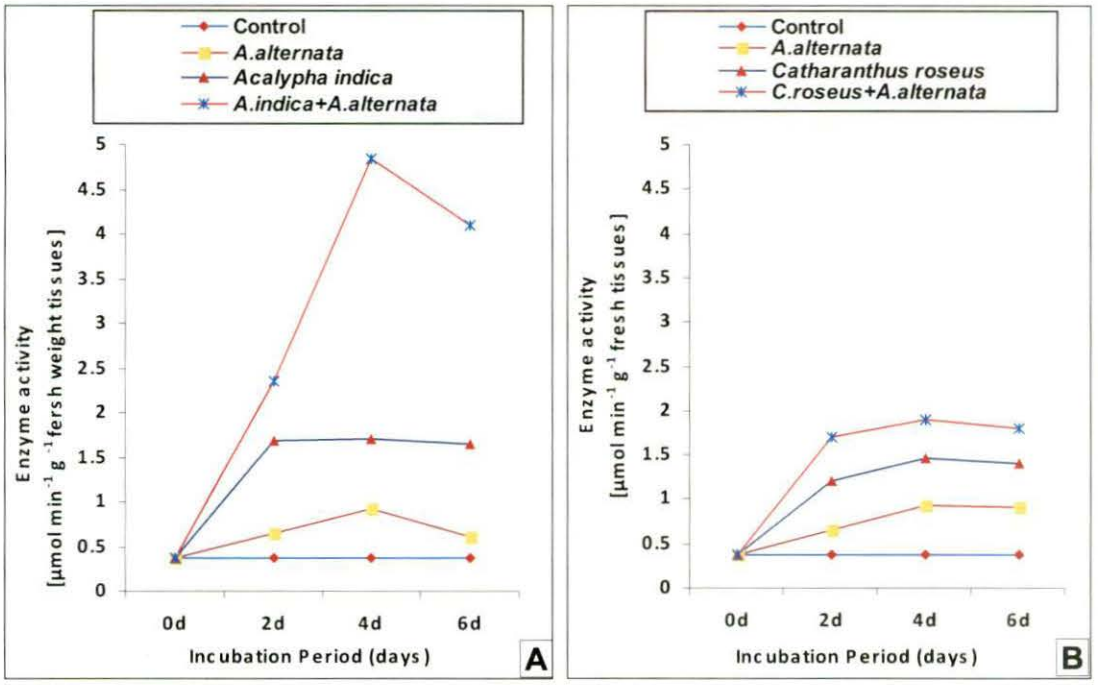

Fig. 4.22: Phenylalanine ammonia lyase activity in inoculated and treated inoculated niger plant (LV).

A: Treated with *Acalipha indica*
B: Treated with *Catharanthus roseus.*

4.6 Foliar application of chemical and botanical inducers, for controlling leaf blight disease caused by *A.alternata* in niger plants

Susceptible niger plants (variety LV) were treated with several chemical and botanical inducers separately and challenge-inoculated by *A.alternata*. In control set, sterile distilled water was sprayed instead of inducer. Mean foliar disease index / plant were calculated after 2, 4, 6 and 8 days of inoculation following the method as described by Sinha and Das (1972). Procedures of inducer application, foliar inoculation technique and assessment of disease have been discussed in materials and methods (Section 3.15.2, 3.5 & 3.6 respectively). The results were noted in Table 4.20.

Table: 4.20. Disease incidence following application of chemical and botanical inducers in susceptible niger variety (LV) against *A. alternata*.

Treatments	Mean foliar disease index/plant*		
	2d	4d	6d
Alternaria alternata	2.81±0.10	4.66±0.08	8.49±05
Catharanthus roseus + *A.alternata*	0.94±0.04	1.10±0.50	1.13±0.08
2-Amino butyric acid *(2-ABA)* + A.alternata	0.98±0.06	1.22±0.16	1.24±0.09
2,1,3-Benzothiodiazole(BTH) + *A.alternata*	1.06±0.07	1.36±0.10	1.38±0.14
Acalypha indica + *A.alternata*	1.12±0.09	1.37±0.06	1.40±0.16
Salicylic acid (SA) + *A.alternata*	1.13±0.08	1.39±0.09	1.40±0.10
2,3-Dihydroxybenzoic acid (DHB) + *A.alternata*	1.25±0.06	1.43±0.11	1.46±0.06
CD at 5%	1.16	0.41	0.12

*Mean of three replicates; Data after ± indicates standard error values

From the results (Table 4.20) it was evident that all the six inducers (both chemical and botanical) when applied, showed significant reduction in mean foliar disease index/plant. It may be noted here that none of the tested leaf extracts showed antifungal activity towards *A.alternata* when tested *in vitro*. Niger plants treated with *Catharanthus roseus* aqueous leaf extracts showed maximum control of the disease with mean foliar disease index of only 1.13 followed by 2-ABA with mean foliar disease index of 1.24 after 16 days of inoculation. *BTH* and *A.indica* treated plants also showed significant reduction in disease index in

comparison to untreated-inoculated (control), where mean foliar disease index was found to be 8.49 after 16 days.

4.7. Evaluation of some biocontrol agents for controlling *A. alternata*

Microbial biocontrol agents are eco-friendly alternative to hazardous fungicides used in agriculture (Bucki *et al.*,1998; Meena *et al.*, 2000; Ramamoorthy and Samiyappan, 2001; Jadeja, 2003 and Perello *et al.*, 2006). Several microorganisms have been identified that can be utilized as antagonists to fungal pathogens. Two genera (one fungus viz. *Trichoderma* and one bacteria viz. *Bacillus)* have been used in the present study for evaluation of their efficacy against the niger leaf blight disease caused by *A.alternata.*

Four *Trichoderma* strains [*Trichoderma viride, T. harzianum, T. virens* (Isolate-I) and *T. virens* (Isolate-II)], one fungus isolated from rubber rhizopher (*Aspergillus flavus*) and two different isolates of bacteria *Bacillus subtilis* (Isolate-I) and *Bacillus subtilis* (Isolate-II) were used in the present study. In dual culture technique (as mentioned in Section 3.19.1) radial growth and percent inhibition over control were calculated after 4 days of inoculation. The results (Table 4.21 and Fig.-4.23 and Plate-4.12) clearly indicated that *Aspergillus flavus* was the best biocontrol agent and showed maximum (95%) inhibition but *Bacillus subtilis* (isolates I) showed minimum (45.2%) inhibition among the fungal antagonists tested.

Table: 4.21. Effect of antagonist on the growth of *A.alternata* tested *in vitro* by dual culture technique.

Antagonists	**Colony diameter (mm) of *A. alternata in PDA**** after 4 days of incubation***	**Percent inhibition over control****
T*richoderma viride*	23.4±1.15	70.9±1.31
T. harzianum	20.0±1.59	75.1±1.18
T. virens (Isolate I)	22.5±1.14	72.0±1.49
T. virens (Isolate II)	27.5±0.47	65.8±1.10
Aspergillus flavus (Af)	6.8±0.89	95.0±0.85
Bacillus subtilis (Isolate I)	44.1±1.36	45.2±1.65
Bacillus subtilis (Isolate II)	15.6±0.86	80.6±1.34
CD at 5%	2.61	3.16

*Control diameter = 80.5 mm; ** Mean of three replications; ***PDA : extract = 9:1.

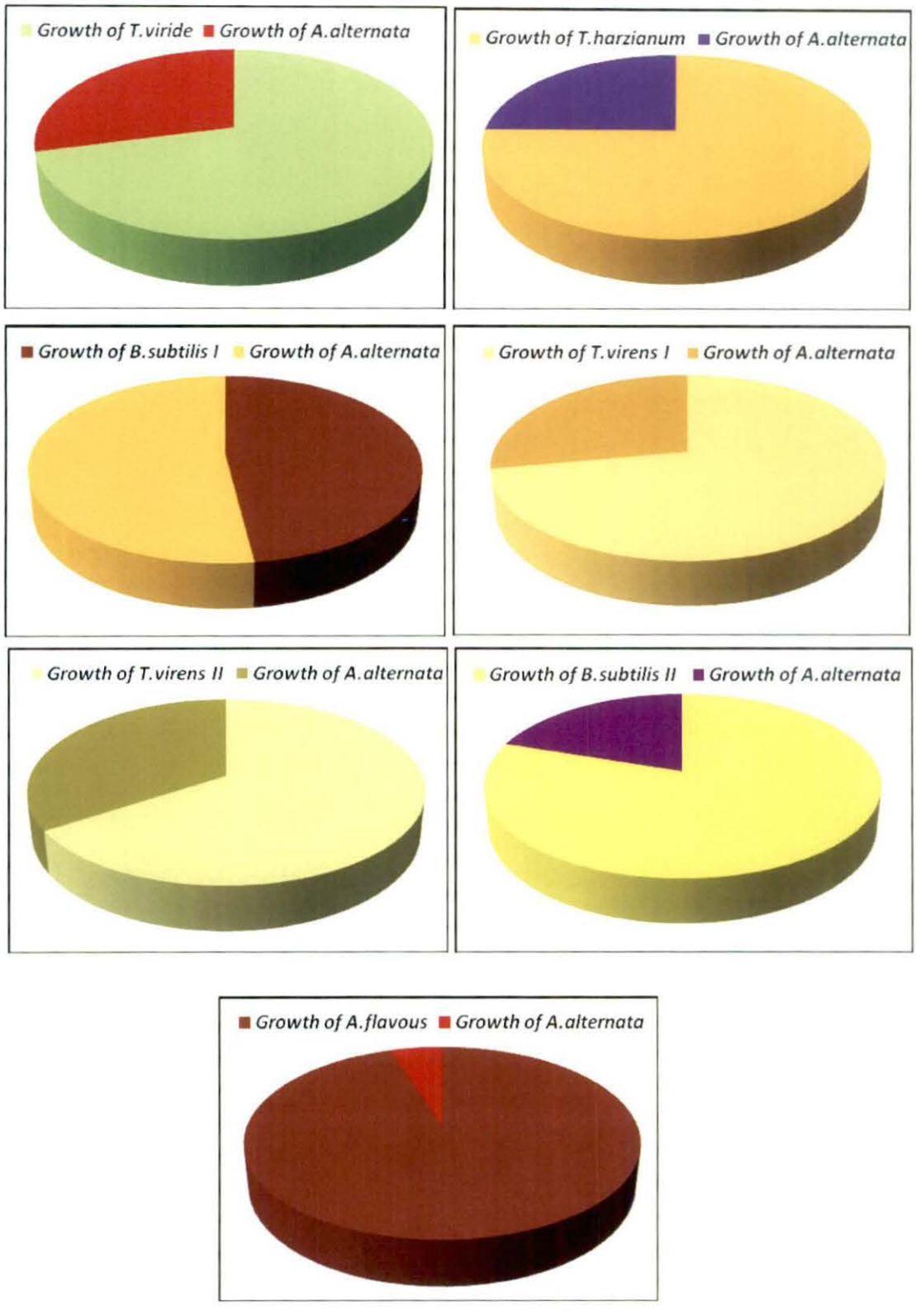

Fig. 4.23 Effect of antagonist on the growth of *Alternaria alternata*

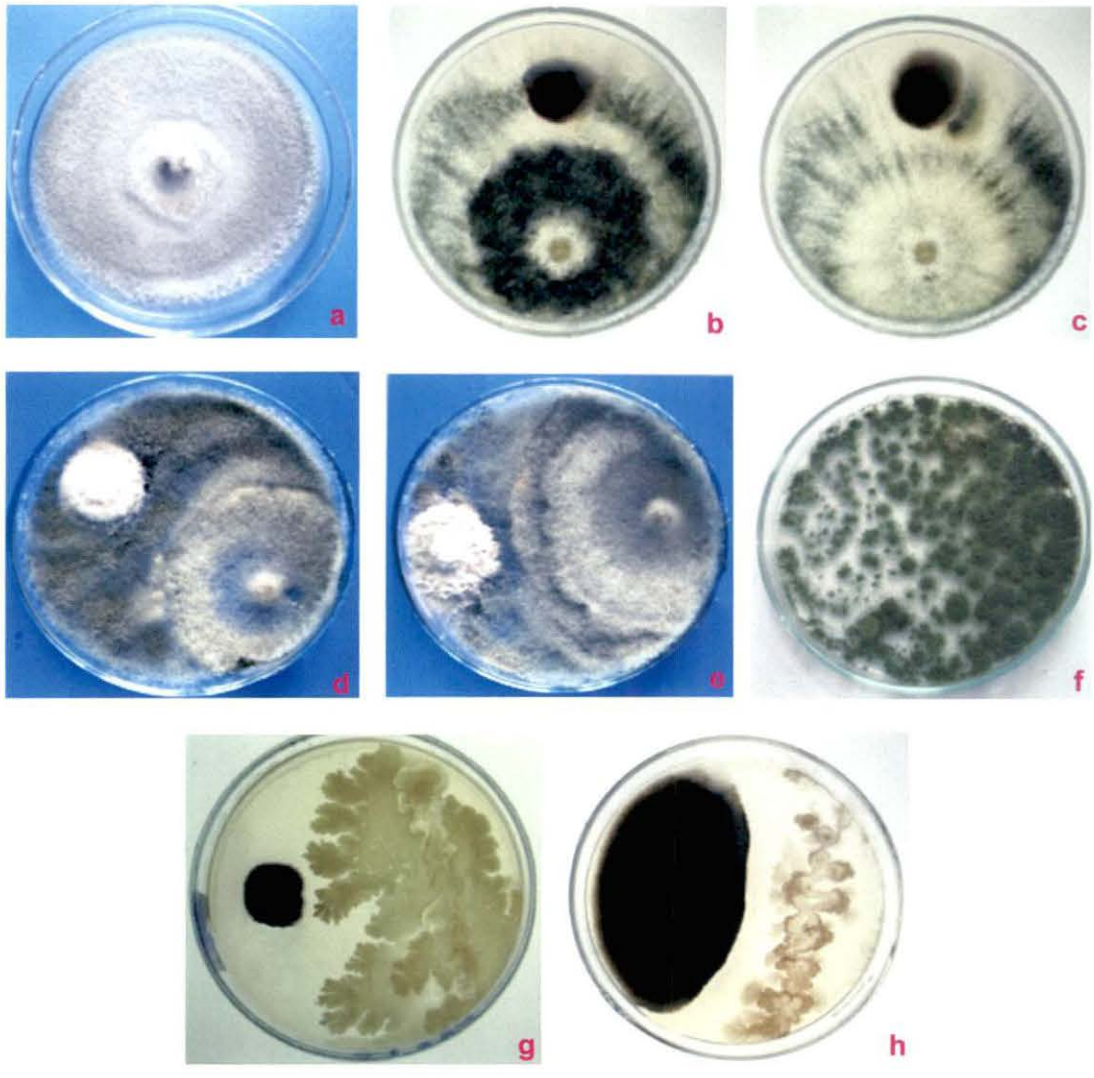

PLATE 4.12

Control of *Alternaria alternata* by fungal antagonists

fig. a : Culture of Control plate (*Alternaria alternata*)

fig. b : Dual culture of *Trichoderma harzianum* and *Alternaria alternata*

fig. c : Dual culture of *Trichoderma Viride* and *Alternaria alternata*

fig. d : Dual culture of *Trichoderma virens* (Isolate-I)and *Alternari alternata*

fig. e : Dual culture of *Trichoderma virens* (Isolate-II) and *Alternaria alternata*

fig. f : Dual culture of *Aspergillus flavous* and *Alternaria alternata*

fig. g : Dual culture of *Bacillus subtilis* (Isolate-I) and *Alternaria alternata*

fig. h : Dual culture of *Bacillus subtilis* (Isolate-II) and *Alternaria alternata*

For this, the pathogen was allowed to grow in PDA mixed with culture filtrates (9:1) as described under materials and methods (Section 3.19.2). Radial growth of the test fungi were measured after 4 days of inoculation. Percent inhibitions with respect to control were calculated in each case and the results were tabulated in Table 4.22. From results it was observed that culture filtrate of five antagonists, (viz.*T. viride, T. harzianum, T. virens* (Isolate-I) *T. virens* (Isolate-II) and *A.flavus*) completely inhibited the growth of the pathogen *A.alternata.*

Table: 4.22. *In vitro* effect of crude culture filtrates of fungal antagonists on the growth of *A. alternata.*

Antagonists	**Radial growth (mm)* of *A. alternata* in culture filtrate supplemented PDA*** after 4 days of inoculation**	**Percent inhibition over control****
Trichoderma viride	0	100
Aspergillus flavus (Af)	0	100
T. harzianum	0	100
T. virens (Isolate I)	0	100
T. virens (Isolate II)	0	100

* Mean of three replications. Data after ± represent standard error values.
**Control diameter = 9.0 cm after 10 days of inoculation.
*** PDA : Culture filtrate = 9:1.

4.7.2. *In vivo* effect of crude culture filtrate of fungal antagonists

Antagonistic activity of culture filtrates of *T. viride, T. harzianum, T. virens* (Isolate-I) and *T. virens* (Isolate-II) against *A. alternata* were tested in susceptible variety of niger plants (LV). The plants were sprayed with crude culture filtrates containing spores of the antagonists. In each experimental set ten plants were kept. The plants in each set were inoculated with *A. alternata* following the technique as described in materials and methods (Section 3.5.). The whole experiment was performed in triplicate. Mean disease index / plant was calculated after 2, 4, and 6 days of inoculation following the procedure as described by Sinha and Das (1972) and was tabulated in Table 4.23 and graphically represented in Fig.4.24. From the results it was clear that crude culture filtrate of *Aspergillus flavus* showed

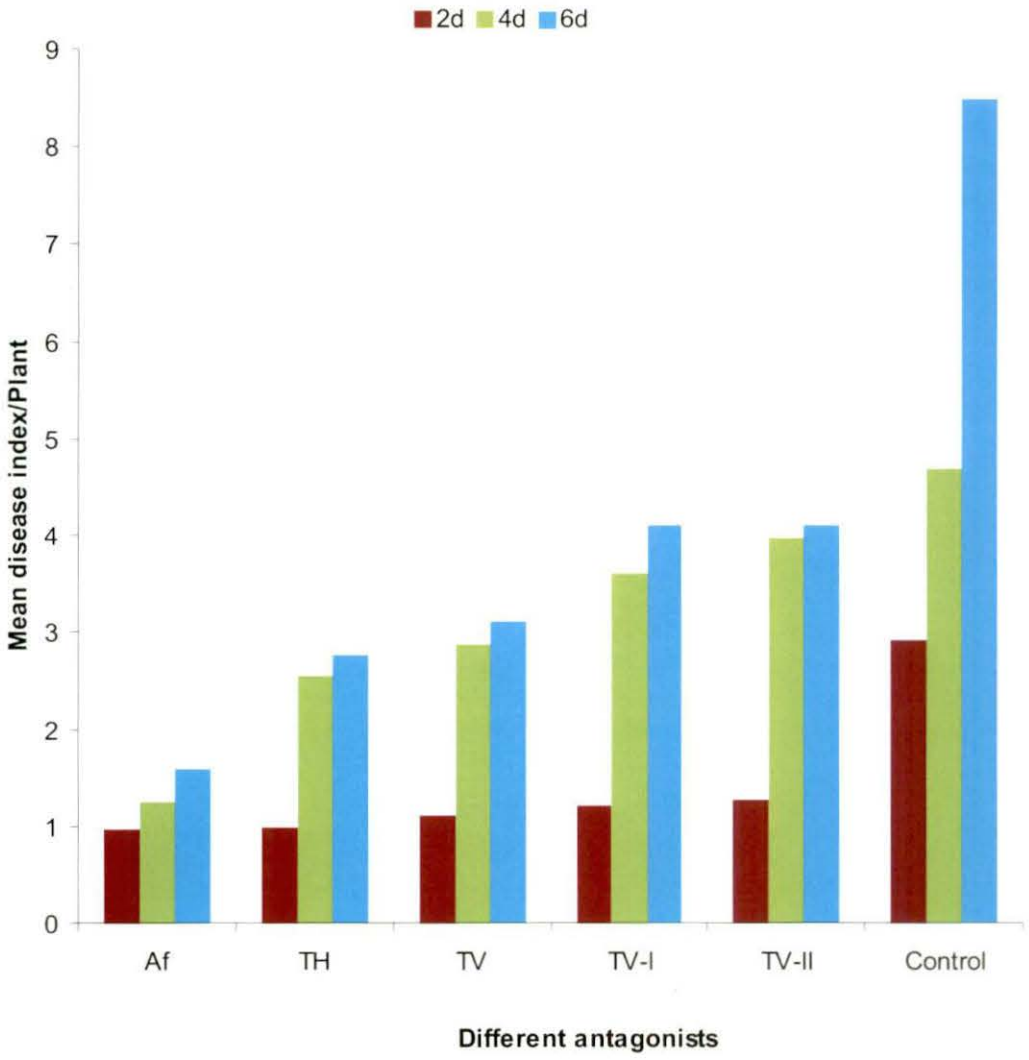

Fig. 4.24 Graphical representation of control of Leaf blight disease of niger (LV variety) caused by *A. alternaria* by foliar treatment of crude culture filtrate of some fungal antagonists.

maximum reduction of disease with mean disease index of 1.59 followed by *T. harzianum* with mean disease index of 2.76. Other two antagonists *T. viride* and *T. virens* (Isolate-I) also showed control of the disease after 6 days of inoculation.

Table: 4.23. *In vivo* control of leaf blight of niger caused by *A. alternata* by foliar treatment of crude culture filtrate of some fungal antagonists

Antagonists	Mean disease index/plant* Incubation periods (Days)		
	2	4	6
Aspergillus flavus (Af)	0.96±0.29	1.23±0.59	1.59±0.40
Trichoderma harzianum	0.98±0.23	2.54±0.19	2.76±0.21
T. viride	1.10±0.59	2.87±0.44	3.10±0.42
T. virens (Isolate I)	1.20±0.42	3.59±0.39	4.10±0.60
T. virens (Isolate II)	1.25±0.16	3.96±0.32	4.62±0.12
Control	2.90±0.15	4.68±0.47	8.50±0.15
CD at 5%	0.56	0.39	0.72

*Mean of three replications. Data after± represent standard error values.

4.8. Screening of potential antifungal phytoextracts against *A. alternata*

Twenty botanical extracts (both aqueous and ethanol) were tested for their antifungal properties *in vitro* following slide germinatiom bioassay. The procedure of preparation of both aqueous and ethanolic extracts of the plants have been discussed in meterials and methods section 3.20.1

4.8.1. Slide germination bioassay to screen potential Botanicals for controlling *A. alternata*

The slide germination bioassay has been discussed in meterials and methods (section 3.20.1.2). The percent germination and percent inhibition of spores against respective plant extracts were calculated and results of some effective plant extracts have been presented in the Table 4.24.

It was evident from the results (Table 4.24) that both the aqueous and ethanolic bulb extracts of *Allium sativum* completely inhibited spore germination of *A.alternata*. Other plant extracts showed significant effects were of *Datura*

stramonium, Hibiscus rosa-sinensis, Pleuromia rubra, Leucus indica, Borreria alata and Xanthium strumarium.

Table: 4.24. Effect of plant extracts on spore germination of *A. alternata* after 48 hours of incubation.

Plant species tested (Family of the plant)	Aqueous extract		50% ethanol extract	
	Percent* germination	Percent* inhibition	Percent germination	Percent inhibition
Allium sativum	0.0±0.00	100.0±0.00	0.0±0.00	100.0±0.00
Datura stramonium	11.3±3.04	88.7±2.66	4.9±0.10	95.1±2.54
Hibiscus rosa-sinensis	0.0±0.00	100±0.00	5±0.53	95.0±0.00
Plumeria rubra	0±0.00	100±0.00	9.9±0.40	90.1±1.08
Leucus indica	12.1±3.30	87.9±1.27	19.6±0.25	80.4±2.27
Borreria alata	9.8±0.22	90.2±2.35	8.2±0.42	91.8±2.21
Xanthium strumarium	0±0.00	100±0.00	5.8±0.20	94.2±1.86
Control	100.0±0.00	0±0.00	100.0±0.00	0±0.00
CD(5%)	3.10	3.16	0.74	4.38

Data are mean of 3 replications. Data after ± represent standard error values.

4.8.2. Antifungal effect of selected plant extracts on growth of *A.alternata* following poisend food technique

Aquous and ethanolic leaf extracts of three plants (*Plumeria rubra*, *Datura stramonium* and *Xanthium strumarium*) were tested following poisoned food technique (as described in Section 3.20.3) to test their antifungal activity.

Radial measurement of inhibition zones of different plant extracts were noted in Table 4.25. From the results it was clear that both aqueous and ethanolic extracts of *Plumeria rubra* showed most inhibitory effect against *A. alternata* with 83% and 72% inhibition respectively. Aqueous and ethanolic extracts of *Xanthium strumarium* showed 77% and 69% percent inhibition of radial growth respectively. Leaf extracts of *Datura stramonium* showed least inhibition among the plant extracts tested(Plate 4.13).

4.9. Foliar application of selected botanicals, for controlling leaf blight disease caused by *A. alternata*

The young niger plants of susceptible variety (LV) were sprayed (in separate sets) with the crude aqueous extracts (2 g in 10 ml distilled water) of *Allium sativum*, *Plumeria rubra* and *Xanthium strumarium.* After 24 hours of spraying,

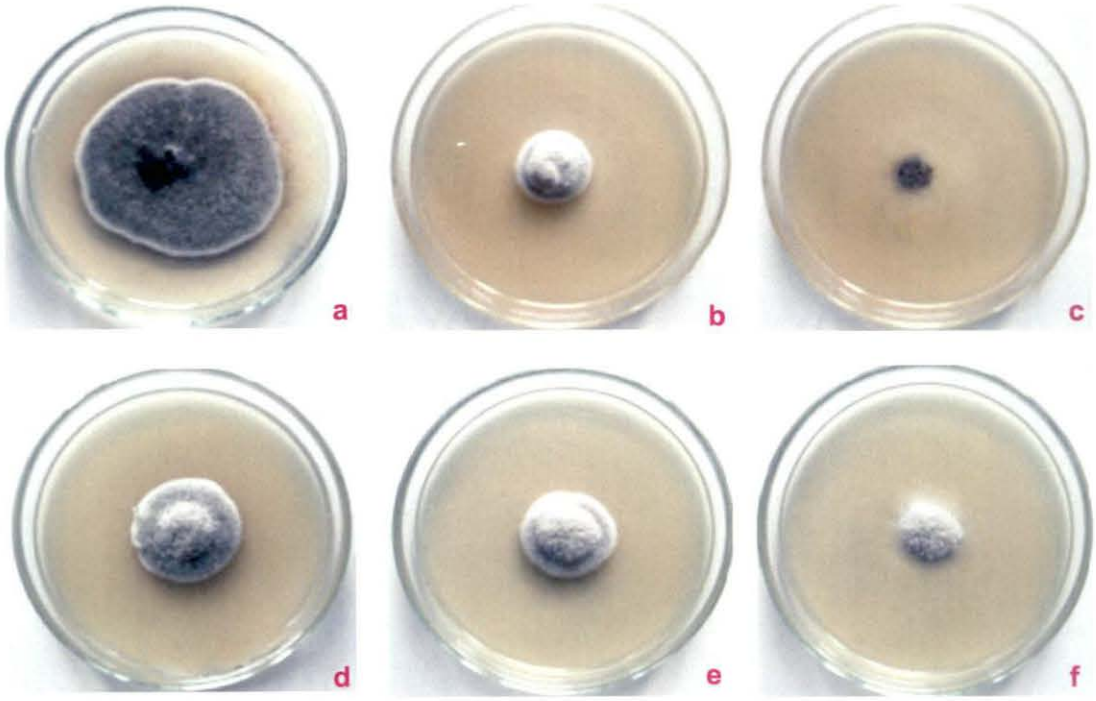

PLATE 4.13

Poisoned food technique done by different plant extracts.

fig. a : Control plate (without extract)

fig. b : Aqueous extract of *Pleuromia rubra*

fig. c : Ethanolic extract of *Pleuromia rubra*

fig. d : Ethanolic extract of *Datura stramoneum*

fig. e : Ethanolic extract of *Xanthium strumarium*

fig. f : Aueous extract of *Xanthium strumarium*

the plants were inoculated with the spore suspention of *A.alternata*. In control set, sterile distilled water was sprayed. After 24 hours, the plants in each treatment were inoculated with *A. alternata* following the whole plant inoculation method as described in section in 3.5. Mean disease index/plant was calculated after 2, 4, 6 and 8 days of inoculation and compared with the control set following the procedure of Sinha and Das (1972). The results were presented in the Table 4.26 & Fig. 4.25.

Table: 4.25. Effect of antifungal activity of selected plant extracts on the growth of *A.alternata* tested by poisoned food technique

Plant extracts		**Radial measure of *A. alternata* in PDA*** after 5 days of inoculation (mm)***	**Percent Inhibition over control****
Plumeria rubra	Aqueous extract	6±1.53	88±1.15
	50% ethanol extract	10±1.15	80±1.53
Datura stramonium	Aqueous extract	26±1.00	48±2.08
	50% ethanol extract	13±0.85	74±1.15
Xanthium strumarium	Aqueous extract	8±1.15	84±0.58
	50% ethanol extract	11±1.13	78±2.00
CD at 5%		2.79	2.37

*Control diameter = 50 mm; ** Mean of three replications; ***PDA : extract = 9:1.

From the result it was evident that all the three aqueous extracts showed significant reduction in mean disease index. *A. sativum* bulb extracts showed maximum control of the disease with mean disease index of only 1.30 followed by *P. rubra* leaf extract with mean disease index of 1.31 after 8 days of inoculation. In control set, disease occurrence was always found to be higher than the experimental sets.

Table: 4.26. *In vivo* control of Leaf blight of niger (LV) caused by *A. alternata* by foliar treatment with crude aqueous plant extracts.

Plant species tested	Mean disease index / plant*			
	Incubation period (Days)			
	2	4	6	8
Allium sativum	0.0±0.00	0.95±0.08	1.28±0.04	1.30±0.41
Plumeria rubra	0.24±0.03	1.16±0.9	1.29±0.10	1.31±0.05
Xanthium strumarium	0.93±0.04	1.64±0.20	2.10±0.18	2.25±0.28
Control	2.8±0.19	4.65±0.21	8.43±0.41	10.4±0.5
CD at 5%	0.31	0.48	0.41	0.57

* Mean of three replications. Data after ± represent standard error values.

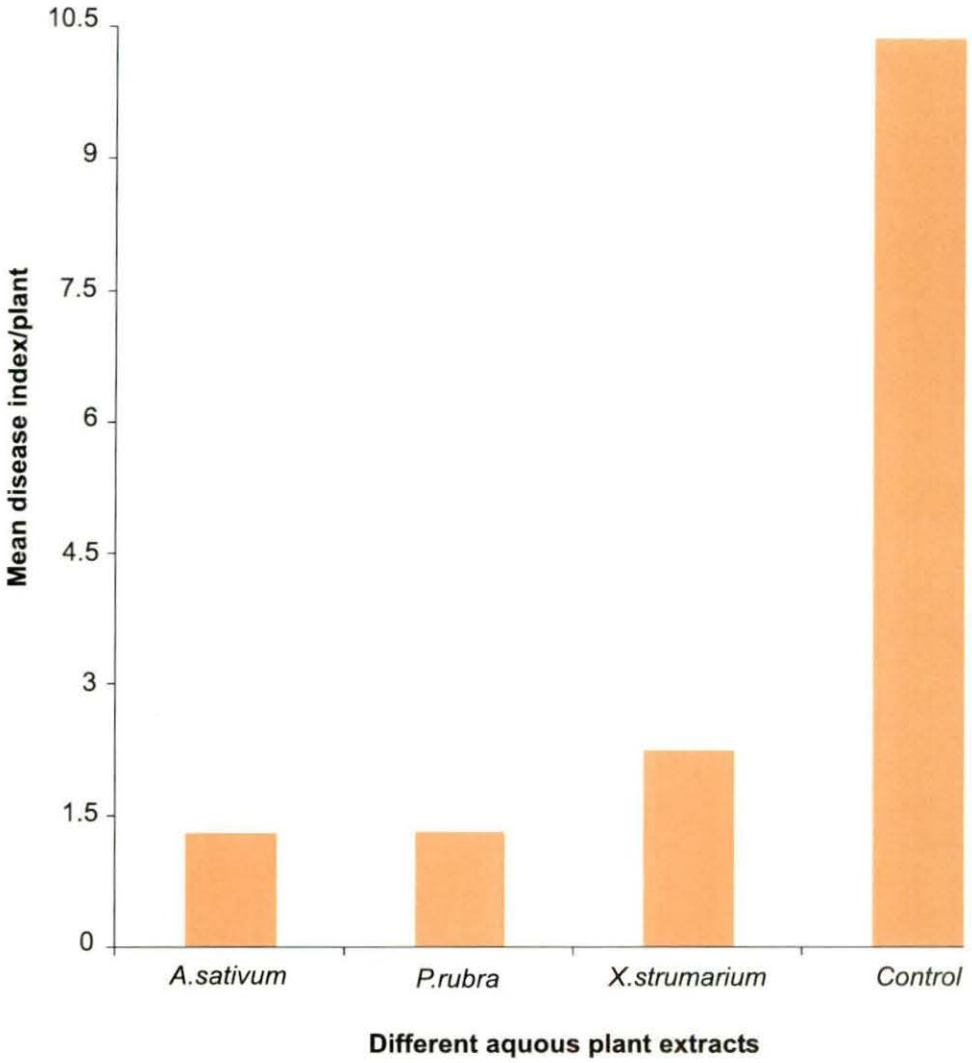

Fig 4.25 Graphical presentation of leaf blight disease of niger (LV) caused by *A. alternata* by foliar treatment with crude plant extract.

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*.

6. Summary

The present study deals with "Studies on the resistance of niger [*Guizotia abyssinica* (L.f.) Cass.] against *Alternaria alternata* causing leaf blight and control of the disease using botanicals and antagonists". The study consists of: i) Pathogenicity of *Alternaria alternata* in different varieties of niger and selection of susceptible and resistant varieties. ii) Morphological and physiological characteristics of the fungus. iii) Common antigenic relationship between *Alternaria alternata* and different varieties of niger by serological techniques. iv) Alteration of disease reactions in susceptible niger varieties by some SAR inducers. v) Control of the leaf blight disease of niger by SAR inducers, eco-friendly botanicals and biocontrol agents. vi) Selection of potential SAR inducers, botanicals and biocontrol agents, if any, for preparation of field applicable formulations and field assessment of the formulations.

At the onset of the work, brief review of literature in the present line of investigation have been presented. The main areas of the review are a) Diseases of niger. b) Diseases caused by *Alternaria alternata*. c) Studies on growth and physiology of the pathogens. d) Antigenic relationship in host and pathogen. e) Induction of systemic resistance (SAR and ISR). f) Disease control by antagonistic organisms. and g) Disease control by botanicals.

Details of different experimental procedures and techniques have been described in details in the materials and methods section.

The present work was carried out after thorough survey of different fungal diseases present in the different niger fields of the present study area. During field survey two fungal pathogens were found to cause diseases in niger plants. Those were isolated and identified as *Alternaria alternata* and *A. porri* following Koch's postulates. *A. alternata* was found more consistently to cause disease in niger plants.

Pathogenicity of *A. alternata* was performed following whole plant inoculation techniques in six different niger varieties. Local variety (LV) was found most susceptible and NRS-69-1 was found most resistant among the six niger varieties tested.

Growth and sporulation of *A. alternata* have been studied on a variety of media. Important physiological parameters have also been studied. From the

results it was found that Richard's agar (RMA) was best for both growth and sporulation of the fungus. Optimum temperature and pH of growth of the fungus were 28° C and pH 6.5 respectively.

To detect cross reactive antigens (CRA) in susceptible and resistant niger varieties against *A. alternata*, serological techniques viz. immunodiffusion and immunoelectrophoresis were performed. Polyclonal antisera were raised against antigens of the susceptible (LV) and resistant (NRS-69-1) niger varieties as well as of *A. alternata*. In immunodiffusion test, antisera, were subjected to react with proteins of susceptible and resistant niger varieties and also with mycelial protein of *A. alternata*. The results showed the presence of CRA in homologous reactions as well as in cross reactions between susceptible niger variety and *A. alternata*. No CRA was found in cross reaction between resistant niger variety (NRS-69-1) and *A.alternata* and *vice-versa*. Immunoelectrophoresis revealed that susceptible niger varieties (LV and JNC-6) shared one precipitin band with antisera of *A. alternata* (AlA). Antisera of susceptible variety (LvA) shared one precipitin band against antigen of *L. theobromae*. No precipitin band was found between antisera of *A. alternata* and antigen of resistant niger variety (NRS-69-1) and *vice-versa*.

The leaf antigens of six niger varieties, mycelial antigen of *A. alternata*, and one non-pathogen proteins (*Gliocladiun virens*) and antisera of two niger varieties (susceptible and resistant) and of pathogen *A. alternata* were used to perform indirect-ELISA. Indirect ELISA was performed to detect the level of cross reactive antigens present in different niger varieties and *A. alternata*. Higher ELISA values in heterologous reactions indicated the presence of CRA in higher level that lead to compatible reactions or more susceptibility. In the present study, higher ELISA values were found in cross reactions between antisera of *A. alternata* and antigen of susceptible niger variety (LV). Low ELISA values (0.193, and 0.198) were found between antisera of *A. alternata* and antigen of resistant varieties (NRS-69-1 and RCR-18). The results of conventional pathogenicity tests were compared with the results of indirect ELISA to establish a guideline for the degree of susceptibility of different niger varieties against *A. alternata*.

To find out the cellular location of CRA in host tissues (niger plants) 'immunogold-silver enhancement' studies were performed using antisera of pathogen (*A. alternata*). In immunogold labelling when stem and root sections of susceptible niger variety (LV) was treated with antisera of *A. alternata* and

labelled with immunogold-silver enhancement, CRA was observed mainly in the epidermal regions and xylem elements as strong precipitations. In leaves of susceptible niger variety, mesophyll tissue and vascular bundle elements also showed marginal darkening which indicated the presence of CRA in those areas. When leaf sections of resistant variety (NRS-69-1) were treated with the antisera of pathogen, no such strong precipitations were observed.

After thorough observation of virulence studies of the pathogen, *A. alternata* on six different niger varieties, further works were carried out to devise environment friendly disease control measures. In this regard, susceptible (LV) niger plants were induced by four abiotic inducers (2-ABA, BTH, DHB and salicylic acid), and two leaf extracts (*Acalypha indica,* and *Catharanthus roseus*) separately for induction of defense related enzymes (chitinase, β-1,3-glucanase, polyphenol oxidase, phenylalanine ammonia-lyase and peroxidase) and disease management.

Susceptible niger plants pre-treated separately with two different inducers (*A. indica* leaf extract and 10^{-2}M BTH) showed maximum phenylalanine ammonia-lyase (PAL) activity after 4 days following challenge-inoculation by *A. alternata*.

From the present study it was also evident that salicylic acid (SA) pre-treated plants showed higher level of β-1-3-glucanase expression in susceptible niger plants. Next to SA, plants pre-treated with *C. roseus* aqueous leaf extract and inoculated (with *A. alternata*) showed higher β-1-3-glucanase activity. 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.

Six different inducers were used for the induction of chitinases in niger plants. *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 when inoculated after treatment. 2-ABA also induced chitinase activity.

One chitinase band (R_f 0.62) was visible in PAGE gels stained with chitinase specific stain. *C. roseus* treated and challenge inoculated niger plants

showed maximum activity as evidenced by intense band. From the study it can be concluded that *C. roseus* leaf extract is a potent inducer of defense response in niger plants.

In the present study, six different types of inducers were used for induction of peroxidase following challenge-inoculation in susceptible niger plants. 2-ABA pre-treated niger plants showed higher 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.

Niger plants pre-treated with 2-ABA and inoculated with the pathogen showed prominent peroxidase isoform patterns 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.

Differential expression of PPO was determined in susceptible niger variety (LV) following pre-treatment (with six different inducers) and challenge-inoculation (by *A. alternata*). Niger plants pre-treated with leaf extract of *C. roseus* and DHB and inoculated by *A. alternata* showed higher level of PPO expression 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.

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.

Pathogenicity is determined, ordinarily, by disease incidence. Disease incidence was assessed and compared in the differentially induced susceptible variety, LV. Four Abiotic inducers and four leaf extracts were used for induction of resistance in the susceptible variety LV. Assessment of disease was performed from 2^{nd} day up to 6^{th} day at 2-days intervals. *Catharanthus roseus* leaf extract

and 2-ABA (abiotic inducer) effectively reduced disease incidence (mean foliar disease index/plant) in tested tea plants. Disease incidence was also found to reduce in niger plants induced by leaf extracts of *Acalypha indica*, Salicylic acid, BTH and *DHB*.

In the present study four *Trichoderma*, one *Aspergillus* and two *Bacillus subtilis* isolates were used for their efficacy against *Alternaria alternata*. *Aspergillus flavus* showed maximum inhibition of growth of the pathogen 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. Significant reduction of disease was also observed when culture filtrates were applied on the plants and disease index was compared with that of control plants.

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, Pleuromia rubra,* and *Xanthium stramonium*) significantly inhibited spore germination of the pathogen. Pleuromia rubra and *Xanthium stramonium* aqueous leaf extract showed more than 80% inhibition of growth of the fungus in comparison to control. Three plant extracts were sprayed on the susceptible niger plants and then the plants were challenge inoculated. Significant control of the disease was observed by the application of the said botanicals.

Implications of the results have also been discussed in the discussion section. The results were encouraging since several inducers showed significant resistance inducing capacity. Further these may be integrated with other bio-control agents and may be used in fields as part of integrated disease management system.

The present study 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 some 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 the cells of niger and the pathogen through 'immunogold-silver enhancement' studies. Resistance was induced in susceptible tea varieties using some chemicals and plant extracts. Hence, this study has provided an insight to formulate a definite defence inducer against *Alternaria*-blight disease. Present study designs the suitable control measures of the disease using resistance inducers of different nature. Additionally, the study also provides some biocontrol agents and botanicals for control of the disease.

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