

## 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* (AIA). 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 (*Gliocladium 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,  $\beta$ -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  $\beta$ -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  $\beta$ -1,3-glucanase activity. Isozyme patterns revealed the expression of two different  $\beta$ -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<sup>nd</sup> day up to 6<sup>th</sup> 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.