Discussion
V. DISCUSSION

The Finger millet (*Eleusine coracana* L.) is grown extensively in different parts of the world as a staple food crop. The largest area in India under this crop is in Karnataka, followed by different states in India. Recognizing the importance of this crop ICAR (Indian Council of Agricultural Research) has established an AICRP (All India Coordinated Research Project) on millets scheme at UAS, Bangalore, for its improvement. The single major constraint in the production of this crop is the fungal disease mainly caused by *Pyricularia grisea*, which infects at different stages of the crop (seedling stage and grain filling stage). The infection of the leaf causes the necrotic lesions causing reduced photosynthesis and the yield. The infection that found at grain filling stage is more devastating causing the disease in the neck region of the inflorescence leading to production of chaffy grains. So an extent of 28 per cent to 90 per cent loss in the crop production is caused by this disease. Several attempts are being made by the plant breeders to introduce the resistant cultivar from Africa & other places and also through breeding hybrids produced by Indo-African crosses. The result of these studies has helped to a large extent to develop the moderately resistant cultivars for this disease, e.g. GPU-28. However, it is still far from satisfactory in identifying the approaches to develop highly resistant cultivars against this disease.

In view of the above the present investigation was conducted aiming to develop techniques to identify the highly resistant genotypes against the disease and also to identify biodiversity among the available resistant and susceptible genotype groups selected based on morphological and disease incidence studies under field conditions. In addition to this experiments were carried out to identify the biochemical differences with particular reference to phenolics, tannins and sugar content; enzymatic differences which are known to be related to disease resistance namely Peroxidase (POD), Phenylalanine ammonia lyase (PAL) and Polyphenol oxidase (PPO); differences among the isoforms of these enzymes (POD and PPO). Furthermore experiments were also carried out to identify the genotypic difference applying the modern molecular marker techniques RAPD and AFLP to understand the biodiversity among 17 selected cultivars of Finger
millet for their biodiversity in relation to resistance or susceptible to disease infection. An attempt was also made to know the possibilities of using Isotope Ratio Mass Spectrophotometer, to identify the resistant or susceptible cultivars at the shortest period of time using non-destructive methods, which could be adoptable in the lab conditions. The salient findings of the above studies are discussed in this chapter.

A. Reconfirmation of resistant and susceptible cultivars under field conditions for the blast disease infestation

The coordinated project on millets, UAS, Bangalore, has reported some resistant and susceptible cultivars of Finger millet. In order to select the cultivars for the present investigation from these reported cultivars, reconfirmation of the severity of infection was carried out by artificially inoculating the blast fungus under field conditions. Based on the results obtained for the different parameters considered (Plate1 and figure 7) two cultivar namely GPU-28, GPU-45 were confirmed to be resistant and two cultivars namely K-7 & KM-245 were found to be susceptible to the disease. Further studies were carried out with these cultivars.

The parameters selected for assessing the intensity of infection are on par with the studies conducted to assess the disease resistance. The apparent rate of infection is an epidemiological concept put forth by Vander Plank (1963), which has been widely used to identify resistance sources particularly that of horizontal and partial resistance in rice blast (Villareal et al., 1980). The other concept viz. area under disease progress curve (AUDPC) is also very useful in identifying resistance (Wilcoxson et al., 1975). In fact, AUDPC values are being used extensively to identify the behavior of genotypes against the disease (Raju, 1987). There are also similar reports regarding screening for resistance to blast disease in Finger millet (Annon, 1980; Somashekara, 1988; Sanath Kumar, 2002).
5.1 Biochemical differences between resistant and susceptible cultivars

5.1.1 Biochemical changes in the resistant and susceptible cultivars upon germination

The differences among the resistant and susceptible cultivars were studied with respect to total phenolics, tannins, total sugar and protein content in the germinated seeds at different intervals of time (Fig 6). The total Phenolic content was found to be significantly higher in both the resistant cultivars when compared to the susceptible cultivars. The present data also confirms similar biochemical relationship with the fungal diseases. Marginally higher tannin content was observed in resistant cultivars than in susceptible cultivars suggesting the positive relationship of tannin content to disease resistance. The total sugar content was higher in resistant cultivar compared to susceptible cultivars. The present investigation confirms the relationship of high sugar content and disease resistance. There are several reports available to show the relationship between higher phenolic content, higher tannin content, the protein content and sugar content for fungal disease resistance.

5.1.2 Biochemical changes in the resistant and susceptible cultivars after infection of the blast disease

5.1.2.1 Changes in total phenolics between inoculated and uninoculated resistant and susceptible cultivars

5.1.2.1.a Total quantitative phenolic content

Phenolics have been found to play an important role in determining resistance or susceptibility of a host to parasitic infection. Experiments were carried out to know the biochemical changes in the host upon fungal infection, in both resistant and susceptible cultivars at different period of intervals after inoculation. It was observed that large quantity of total phenol accumulation in the resistant plants as compared to the susceptible cultivars after the fungal infection and also over the uninoculated control (fig 8a &8b). Increase in phenolic content upon infection at relatively higher level suggests
the positive correlation for the disease resistance. Similar observations are reported earlier in other crops.

The resistant variety may contain more phenolics than a susceptible one [Sathiyanathan and Vidhyasekaran, (1981); Bhide et al., (1997); Sabitha Rani and Reddy (1998); Mandavia et al., (1997)]. Alteration in phenolic metabolism following infection was observed in many diseases and was more marked in resistant varieties.

A higher level of pre-formed phenolic compounds, particularly ortho-dihydric phenolics was found to occur in resistant cultivars than in susceptible ones due to infection by various pathogens. At early stages of infection, the resistant varieties were shown to contain increased levels of phenolics followed by decreased levels with an increase in DAI, however, in the resistant varieties, the infection was found to cause more pronounced increase in the levels of phenolics, but it was less pronounced in susceptible varieties of many crop species due to infection of various pathogens [Ashoka (1998); Hakulkinen et al (1999); Karunanithi and Usman (1999); Gawande et al., (2002); Menden et al., (2003); Scarpari et al., (2005)]. Geetha et al., (2005) found the higher accumulation of wall-bound phenolics in resistant cultivar seedlings of pearl millet to downy mildew disease than in susceptible cultivars as evidenced by Folin-Ciocalteau reagent staining of thin layer chromatography plates. BalamuraliKrishnan et al., (2005) observed the enhanced induction of total phenolics which might have contributed for the induced systemic resistance in sorghum to sugarcane mosaic virus. These reports support the changes observed in total phenolics in resistant and susceptible varieties and the trend in change in the present study.

5.1.2.1.b Qualitative differences in the phenolics between resistant and susceptible cultivars

In order to identify the differences in the type of phenolics present between the resistant and susceptible cultivars, the qualitative phenolics in the resistant and susceptible plants with or without inoculation of fungus, were studied and compared (fig
One of the bands (band 4), was specifically found to be in large quantity in resistant plant in response of the fungal inoculation at different period of studies. It appears that there is a relationship between disease resistance in these cultivars and the type of phenol. Band 2 was found to be induced with in 24 hours of inoculation in resistant plants whereas it had appeared 3 DAI in susceptible plants. Band 7 phenolic with $R_f$ value 10.75 was specifically induced only in the resistant cultivars after 3 hours of inoculation and persisted throughout the study. It was interesting to note that this particular phenolic was known to be specifically induced after infection only in the resistant cultivars. Therefore, qualitative phenolic has a direct relation with the disease resistant which was induced only after infection. This seems to be fungal specific induced phenol. More studies are needed on such phenolics for the disease management.

There are some phenolics (band 2 & 4), which were found to increase upon inoculation in susceptible cultivars similar to resistant cultivars. However, the phenolic band 3 with the $R_f$ value 7.0 was found to be present only in resistant cultivars irrespective of inoculation suggesting that this is a resistant cultivar specific phenol. From the above studies, it was found that there are qualitative differences in phenolics, which are specific to resistant and susceptible cultivar, induced by pathogen upon infection and the phenolics produced by certain stages of inoculation, which are developmentally regulated.

5.1.2.2 Total tannin content in resistant and susceptible cultivars

Tannins are oligo- and polymeric phenolics that are divided into two classes – hydrolysable and condensed tannins. The term tannin was introduced in 1796 to describe a group of polyphenolic compounds present in some plants, certain dihydric –phenolics are conjugated with each other or with glucose molecules to form polyhydroxy phenolic oligomers and polymers called tannins that contribute to the disease resistance. In general, the resistant cultivar was found to have higher tannin content than susceptible ones. The resistant cultivars had higher tannin irrespective of inoculation compared to the susceptible cultivars (Fig 10a & 10b). The tannin content in resistant plants upon
infection was found to increase relatively at a higher rate than in susceptible cultivar suggesting that higher tannin content upon infection has relationship with the disease resistance.

It has been recognized that endogenic pre-formed levels of tannins have positive correlation with disease resistance (Seetharam and Ravikumar, 1993). A positive association of tannins with disease resistance has been reported in apricot (Misirli et al., 1995). Ashoka (1998) observed that the tannin content was found to decrease up to 13 DAI in the blast resistant rice variety, IET-7191, in HR-12 the susceptible one this was found on all DAI compared to their respective controls when inoculated with *Pyricularia oryzae*.

Karunanithi and Usman (1999) found post-infectional changes of phenolics, tannins, proteins, sugars, lignins in groundnut leaves induced by leaf blight pathogen *Alternaria alternata*. Menden et al., (2003) showed that biochemical changes in the content of phenolics, tannins and proteins, were correlated with the resistance of wheat to stem rust.

Scarpari et al., (2005) investigated biochemical changes in shoots of cocoa during the development of witches' Broom (WBD), which is the most important disease of cocoa in Brazil caused by *Crinipellis perniciosa*. The tannin content increased prior to symptom development and declined with the death of the infected tissues. Thus a perusal of literature supports the findings in the present investigation.

**5.1.2.3 Total sugars in resistant and susceptible cultivars**

The sugars are of fundamental importance in living organisms, as a source of metabolic energy. Carbohydrates are important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. The total sugar content was found to be marginally lower in resistant ones suggesting that the susceptible plants have relationship with the higher sugar content and disease infection (fig 11a
The inoculated susceptible plants had higher sugar content after infection as compared to the resistant cultivar. The increase in sugar content upon infection has direct relationship to higher susceptibility of the cultivar.

Omokolo et al., (1996) studied the changes in carbohydrate, contents in cocoa pods from three clones after infection with Phytophthora megakarya. Variations of the contents in carbohydrates, ketohexoses, were studied in the cortex for 4 d. After pod infection, the amount of carbohydrates decreased more rapidly in the clone SNK10 (highly susceptible) than in the clones SNK413 (low susceptibility) and ICS95 (mildly susceptible). At the same time, the amount of ketohexoses also decreased by 22 % in the clone SNK10, remained almost constant in the clone SNK413, and increased by 53 % in the clone ICS95. Variations of the contents in the biochemicals during Phytophthora black pod development were genotype-dependent, and the patterns of the changes could be related, at least in part, to the susceptibility of the genotype to P. megakarya.

Karunanithi and Usman (1999) found the post-infectional changes of sugars in groundnut leaves induced by leaf blight pathogen Alternaria alternata. They correlated the susceptibility of the disease to increase in sugar content. Menden et al., (2003) showed that biochemical changes in the sugar content were correlated to susceptibility of wheat to stem rust. In cocoa Scarpari et al., (2005) observed high sugar content in shoots of infected cocoa plants. The contents of soluble sugars were analysed in cocoa (Theobroma cacao) shoots during the infection and development of WBD. Among other biochemical changes, there were alterations in the content of soluble sugars (sucrose, glucose, and fructose).

5.2 Changes in the host enzymes due to fungal infection

5.2.1 Total peroxidase activity

Peroxidase (EC 1.11.1.7) occurs in plants and in certain animal cells. Peroxidases are iron-containing enzymes, which catalyze oxidation of an indefinite number of phenols and aromatic amines in the presence of hydrogen peroxide. Peroxidases are ubiquitous
class of oxidoreductase enzymes distributed throughout the plant and animal kingdoms. Peroxidase is a multipurpose enzyme that catalyses the condensation of phenolics into lignins, play a specific role in the hypersensitive containment of the pathogen. Increases in peroxidase activity have been reported to be associated with number of host-parasitic interactions and increase in host plants following pathogen infection (Van Loon, 1997).

Studies were carried out to know the changes in host enzymes in the resistant and susceptible cultivar upon infection of the fungus. Maximum increase in the total peroxidase was found in resistant cultivars (Fig 12a &12b) than in the susceptible cultivars suggesting the relationship with POD activity and disease resistance, which was found to be increased at higher level in the resistant cultivars than in susceptible cultivars under similar conditions. The results are on par with the results obtained by other studies.

The peroxidase enzyme is believed to be contributing to the disease resistance by the oxidizing phenolic compounds to quinines, which are toxic to microorganisms. Increase in total activity of peroxidase has been implicated in the mechanism of resistance due to various fungal diseases (Agarwal et al., 1982). POD is a member of the family of PR9 proteins, which is involved in plant defense by enhancing the formation of lignin (Ray et al., 1998). POD activity is associated with disease resistance in plants. Lin and Kao, (2001) also reported that increased POD activity host plants following pathogen infection, which is also supported by several researches (Borden and Higgins, 2002; Koike et al., 2001; Zeng et al., 2004).

Gawande et al., (2002) did not find any significant difference in the level of peroxidase activity in the resistant and susceptible varieties before infection. However drastic changes in the degree of increase in this activity in resistant genotypes were observed compared to susceptible genotypes. Anguelova et al., (2002) studied the effect of leaf rust (Puccinia triticina) on peroxidase activity in resistant and susceptible wheat lines at seedling, stem elongation and flag leaf stages of plant growth. The levels of activity of peroxidase were low at seedling and stem elongation stage, and high at flag
leaf stage. Induction of POD in wheat head by infection with pathogenic strain of *Fusarium graminearum* has been reported by Mohammadi and Kazemi (2002).

Diby Paul and Sarma (2004) reported that increase in production of peroxidase activity upon infection with *Pseudomonas* infection was higher in the healthy leaves compared to inoculated leaves of black pepper. Zeng Hu-zhe *et al.*, (2005) showed changes in lignification–related enzymes like peroxidase in pepper in response to inoculation of *Phytophthora capsici*. Balamuralikrishnan *et al.*, (2005) found the enhanced induction of peroxidase in sorghum against sugarcane mosaic virus. Boudjeko *et al.*, (2005) showed changes in peroxidase activities from roots of cocoyam upon *Pythium myriotylum* inoculation. Their findings suggest that peroxidase might be involved in the defense mechanism of cocoyam against *P. myriotylum*. From these studies it appears that higher peroxidase activity is related to disease resistance and similar mechanism might be operating in resistant cultivars of Finger millet which recorded higher peroxidase activities.

5.2.1.1 Isoenzymes of anionic peroxidase

The isozymes of anionic POD studies showed five different isozymes in all the cultivars irrespective of their resistant or susceptibility (fig 13). However, a specific isozyme was found to be induced only after inoculation of the fungus after 3DAI in resistant cultivars only and found to be present even after 15DAI (band 2). It appears that this particular isozyme of anioic POD has a direct relationship with disease resistance. Upon infection particularly in resistant cultivars different isozymes bands 1, 3, 6 & 7 were relatively in higher quantity in suggesting that these are induced by the pathogen at higher level in resistant plants. There are isozymes band 3 & 6, which are fungal, specific but not specific to resistant or susceptible cultivars. It was found to be induced in all the cultivars irrespective of whether they are resistant or susceptible. Isozyme band 1 & 7 were found to be induced specifically by the fungus only in the resistant cultivars suggesting a direct relationship of this enzyme with resistant cultivars which are induced.
The anionic peroxidase isozyme has been studied in general plant systems and induction of new isoforms specific to pathogen infection has been studied. The isozymes identified from the present study which have relation to disease resistance needs to be further studied for developing the disease resistant cultivars.

The induction and disappearance of some isozymes due to inoculation of blast fungus *Pyricularia oryzae* in rice seedlings was studied by Ashoka, (1998). Vasquez *et al.*, 2004 detected 2-7 POD isoforms in control and inoculated seedlings but two extracellular forms were enhanced after inoculation. Saravanan *et al.*, 2004 observed two isoforms of peroxidase in banana plants infected with *Fusarium oxysporum*. Similar results were obtained by Zheng *et al.*, (2005) who found the three major isozymes, of which, the third band of 45KDa POD isozyme was induced by the pathogen infection of *Phytophthora capsici* in pepper. Boudjeko *et al.*, 2005 found three isoforms of POD in the resistant lines of cocoyam infected with *Pythium myriotylum* and a new band was found specifically in inoculated fraction.

5.2.2 Polyphenol oxidase (PPO)

Polyphenol oxidase, a copper – containing enzyme from higher plants and fungi oxidizes a great variety of monophenolic and o- diphenolic compounds to brown pigments. The enzyme is extensively studied because of its relative ubiquity in importance in the food and agricultural industry. In plants, it is suggested that the enzyme is involved in resistance to infection, in synthesis of plant constituent and as an oxygen scavenger in photosynthetic tissue. PPO is involved in the terminal oxidation of diseased plant tissue, which was attributed for its role in disease resistance

The total PPO studied between resistant and susceptible cultivars showed that there was a gradual increase in total PPO in all the cultivars from 3 hours to 15 days (fig 14). However, marked increase was observed after third day in all the cultivars. The total PPO content of the resistant cultivar was consistently higher in both the resistant cultivars studied and was almost two fold more than the susceptible cultivar. Therefore it appears
that there is a significant correlation between total PPO content in resistant cultivar for the disease resistance. Several workers have investigated the role of PPO in disease resistance in different systems. In these studies they observed both positive and negative correlations with disease resistance.

The PPO enzyme is also believed to be contributing to disease resistance by the oxidation of phenolics to quinines, which are toxic to microorganisms (Vidyasekaran, 1975). Increase in total activity of PPO had been implicated as a mechanism of resistance to various parasitic infections (Gupta et al., 1995). They determined the specific activities of PPO, in healthy and infected leaves of Brassica species, which were tolerant (B. carinata, B. napus) and susceptible (B. juncea, B. campestris) to A. brassica and A. brassicicola. The results indicated that the specific activity of PPO remained high while that of peroxidase remained low in the tolerant as compared to the susceptible species. In response to infection, the activity of both the PPO and POD increased comparatively at a much faster rate in the susceptible species.

Sharma and Sharma (1997) observed that the resistant lines of wheat against leaf rust pathogen Puccinia recondita showed higher levels of polyphenol oxidase activity in comparison to susceptible cultivars. The specific activity of polyphenol oxidase remained higher in the leaves of resistant genotypes of chickpea in response to inoculation from 6 to 10 days with two isolates of blight pathogen, Ascochyta rebiei. However, the activity of the enzyme had sharply declined after 10 days of inoculation with both the isolates in the susceptible genotype (Khirbat and Jalali, 1998). Jite and Teresa (1999) observed that the polyphenol oxidase activity in Jasminum grandiflorum was enhanced in plants infected with Verticillium hobsoni as compared to the healthy ones. Nagesh et al., (1999) observed for higher activity of polyphenol oxidase in root-knot nematode infected roots of China aster in first two weeks after inoculations, which declined, in third and fourth week of infection compared to that of control.

Borua and Das (2000) found increased activity of polyphenol oxidase in developing chilli varieties, susceptible and resistant to Colletotrichum capsici. There was a significant
increase in polyphenol oxidase activity. Gawande et al., (2002) did not find any significant difference in the level of PPO activity in the resistant and susceptible varieties before infection. However drastic changes in the degree of increase in this activity in resistant genotypes were observed compared to susceptible genotypes.

Diby Paul and Sarma (2005) reported that increase in production of PPO upon 
*Pseudomonas* infection was higher in the healthy leaves compared to inoculated leaves of black pepper. Zeng et al., (2004) showed the changes in lignification – related enzymes like PPO activity in pepper in response to inoculation of *Phytophthora capsici*. Balamuralikrishnan et al., (2005) also found the enhanced induction of PPO in sorghum against sugarcane mosaic virus.

5.2.2.1 Polyphenol oxidase isoenzymes

Different isozymes of PPO were studied between cultivars of resistant and susceptible with or without inoculation of the fungus causing blast disease (fig 15). The data obtained from the above studies clearly showed that there were three isozymes irrespective of the cultivar studied in both resistant and susceptible genotypes. Isozyme 1 & 3 did not differ between the cultivars irrespective of resistance or susceptibility and also different period of growth from 3 hours to 15 days after inoculation. However, very interestingly isozyme 2 was significantly induced in the 3rd and 24 hours after inoculation in all the cultivars suggesting that this isozyme is an inducible PPO by the disease causing fungus, in the early stages of the infection. However, after third day this isozyme was also found in the uninfected plants in all the cultivars. Therefore, the data suggests that this isozyme PPO is present in all the cultivars but its production is influenced by the disease causing organism earlier than the non-infected plants. This appears that there is an interaction between the fungus and isozyme, which needs to be studied in greater detail although there was no relation to resistance or susceptibility. Similar observations have been reported by different workers in specific systems.
Vasquez et al., (2004) detected 2-7 POD isoforms in control and inoculated seedlings but two extracellular forms were enhanced after inoculation. Saravanan et al., (2004) observed five isoforms of PPO in banana plants bacterized with Fusarium oxysporum. Similar results were obtained by Zheng Hu-zhe et al., (2005). Active staining of PPO in pepper revealed the presence of three isozymes (53000, 95000 and 119000). The isozyme with molecular weight of 60000 appeared in pathogen treated plants only, suggesting that this PPO was induced by the pathogen infection.

Boudjeko et al., (2005) found three isoforms of POD in the resistant lines of cocoyam infected with Pythium myriotylum and a new band was found specifically in inoculated fraction. In our study we did not find any isozyme specific to resistant genotypes which suggest that this response may be species specific.

5.2.3 Phenylalanine ammonia lyase

Phenylalanine ammonia lyase is one of the key enzyme in the phenyl propanoid pathway and the flavonoid pathway, which catalyses deamination of phenylalanine to trans-cinnamic acid. PAL is involved in biosynthesis of phytoalexins, lignins and salicylic acid associated with disease resistance expression (Mauch mani and Slusarenko, 1996). In disease resistance mechanism, the enzyme plays an important role in the conversion of phenylalanine to coumaric acids. These provide the phenylpropane carbon skeleton for the synthesis of flavonoids, phenylpropanes and lignin. Resistant varieties are characteristic of rapid conversion of phenylalanin to coumaric acids.

PAL was present in all the cultivars irrespective of resistance or susceptibility both in inoculated and uninoculated plants (Fig 16a). However, large quantity of the enzyme was found in the infected plants than the uninfected plants in both the resistant cultivars significantly after three days of infection. The data suggests that higher quantity of the above enzyme in the plant induced due to fungus in the resistant plant 3 DAI has direct relevance for the resistance to the disease. More studies are required to identify the genotypes responding to inoculation similarly to identify the genotypes, which are
resistant to the disease. Since this enzyme is induced to produce more quantity than in uninoculated resistant plants and inoculated and uninoculated susceptible plants, this seems to be a good result for studying this enzyme in relation to blast disease resistance. The relationship between PAL and disease resistance has been reported by earlier workers, which are as follows.

Peltonen et al., (1995) determined the PAL activity in two barley seedlings after infection with necrotrophic pathogen (*Bipolaris sorokiniana*). PAL activity increased in leaves of both the cultivars after 16 h after fungal inoculation. More activity was recorded in resistant cultivars. Similarly they also found increased activity of PAL in wheat and oats to this pathogen. Thus they linked the PAL activity to their resistance to fungal pathogen. In contrast, Sindhu et al., (1995) found more PAL activity in susceptible seedlings of chick pea than in varieties which are resistant to *Ascochyta* blight pathogen, indicating that this enzyme is not directly involved in disease resistance.

Two lines of China aster (AST-5 and IIHR-55) which was resistant and susceptible to root-knot nematode were analyzed for the activity of PAL. PAL activity was comparatively higher in the infected AST-5 roots compared to its uninoculated control and IIHR-55 roots. The percent increase in PAL activity was higher in infected AST-5 roots from 21 days after infection (Nagesh et al., 1999).

Angela et al., (2003) obtained significant changes in PAL activity in four cultivars of common bean after three days of inoculation with delta races of *Colletotrichum* (inductive fungus) when compared to control. They concluded that, an increase in the activity of PAL could be considered as a biochemical marker for resistance, given that this enzyme is key for the synthesis of phenols associated with resistance.

Geetha et al., (2005) studied the PAL activity in pearl millet cultivars with different levels of resistance to the downy mildew disease caused by *Sclerospora graminicola*. PAL activity was elevated in resistant host cultivar and decreased in susceptible cultivars following downy mildew pathogen infection. The enzyme activity varied between cultivars and was correlated with the degree of resistance to downy
mildew disease. Diby Paul and Sarma (2005) reported that increase in production of PAL activity upon *Pseudomonas* infection was higher in the healthy leaves compared to inoculated leaves of black pepper. Zeng et al., (2004) showed the changes in lignification – related enzymes like PAL activity in pepper in response to inoculation of *Phytophthora capsici*. Balamuralikrishnan et al., (2005) found the enhanced induction of PAL in sorghum against sugarcane mosaic virus.

5.3 Comparative studies of Pathogenesis related proteins (PR-Proteins) studies between blast resistant and susceptible varieties of finger millet upon fungus inoculation

A group of plant-coded proteins induced by different stress stimuli, named “pathogenesis related proteins” (PR- proteins) is assigned an important role in plant defense against pathogenic constraints and in general adaptation to stressful environment. Assumption flowed from initial findings that these proteins are commonly induced in resistant plants, expressing a hypersensitive necrotic response (HR) to pathogens of viral, fungal and bacterial origin. Later, however, it turned out that b-proteins are induced not only in resistant, but also in susceptible plant – pathogen interactions, as well as in plants, subjected to abiotic stress factors (Van Loon, 1985).

Acidic and basic PR proteins were studied in both resistance and susceptible cultivars with inoculation of the blast fungus to know the changes from 3 hours to 15 days after inoculation over the uninoculated.

With respect to acidic PR protein standard, it was observed that there are PR proteins, which are specific to resistant and susceptible genotypes (Fig 17). Around 70 KDa protein (band 13) was found to be accumulating in both inoculated and uninoculated susceptible plants, but not in resistant plants thus indicating its specificity to susceptible cultivar. It appears that this protein has some relevance to the susceptibility of the cultivar, which needs to be studied at the level of expression with time and its relation to the proteomics and to disease susceptibility.
Interestingly, a PR protein of around 50 KDa was found only in the resistant plants which was absent in the susceptible, irrespective of inoculation of the fungus consistently throughout the period of study and it was negligible in the susceptible cultivar during the same period. It appears this protein is a resistant cultivar specific and it may be having a role for disease resistance. It appears to be a constitutive protein not affected by the fungal infection. More studies are required about this protein and its relation to disease resistance to develop improved varieties. Screening of different resistant cultivars for this protein has to be carried out to know whether it is cultivar specific or not.

A PR-protein of around 43 KDa (band 7 & 8) was found to be induced both in resistant and susceptible cultivar with the growth of plant irrespective of fungal infection. However, this protein has appeared in large quantity significantly earlier in the resistant cultivar (3DAI) and was consistently found after 15 DAI, where as in susceptible cultivar the above two proteins appeared relatively late. Therefore, more studies are required to know the appearance of these induced proteins with time, earlier to have resistance to the disease. Similar was the trend with other two proteins band 5 & 6 of low molecular weight of around 35 KDa which also appeared earlier in the resistant plants as compared to susceptible plants. Therefore, the interaction of resistant specific protein (band 9) with this kind of inducible protein (5, 6, 7 and 8) with time needs to be studied with reference to the tolerance to blast disease.

With respect to basic PR-protein studied between resistant and susceptible plants by native PAGE (fig 18), it was observed that two proteins (bands 7 & 11) were found to be resistant cultivar specific which was found to be consistently present irrespective of the fungal infection relatively in large quantity than in the susceptible cultivar suggesting this is definitively a resistant cultivar specific basic PR protein. However, two proteins were found to be inducible by the fungus only in the resistant cultivars after three day after infection and confirmed to be present even after 15 DAI (band 2 &8). Therefore it is interesting to know that these two proteins are resistant cultivar specific fungal inducible
proteins, which are not found in, uninoculated resistant cultivar and also both inoculated and uninoculated susceptible cultivar. This fungal specific inducible protein in resistant cultivar has greater relevance for further studies for developing cultivars resistant with this kind of protein. A protein band (3&10) was found to be present only in the resistant cultivars which were inoculated with the fungus. The same was absent in the uninoculated resistant cultivar. Therefore, this protein is highly specific to produce in the inoculated resistant cultivar and it appears only after 3DAI suggesting that it is a developmentally regulated in resistant cultivar. The above data suggest that there are cultivars specific, resistant and susceptible specific and induced by the fungus in the resistant plants. The relationship of these protein needs to be explored for characterize these protein band to understand the disease resistance mechanism in Finger millet in particular and other crops in general.

Many defense reactions in plants are triggered in response to invasion by pathogen or other stress-inducing agents. Among these production of PR-proteins is well documented (Fluhr et al., 1991). These proteins are known to be induced by a wide range of pathogens including viruses, fungi and bacteria, application of chemicals and treatment with elicitors (Van Loon, 1983). These PR-proteins not only accumulate locally in the infected leaf, but are also induced with the development of systemic acquired resistance (SAR) against further infection by fungi, bacteria and viruses.

Several workers have earlier reported the presence of PR-proteins in plants due to infection: It is essential to underline, that PR-proteins members induced in resistant or SAR-expressing plants and resistant transgenic plants to exhibit PR-proteins with high antimicrobial activity (Rauscher et al., 1999; Tonón et al., 2002; Anand et al., 2004), thus suggesting their direct role in disease resistance.

Tamas and Fric (1995) separated proteins synthesized in intercellular washing fluids from powdery mildew infected barley leaves by PAGE. They detected induction of PR-proteins at the pre-parasitic stage of the host-parasitic interaction (24h after
inoculation). A marked accumulation of PR-protein in incompatible host-parasitic interactions than in compatible interactions was also observed. Liu et al., (1995) found some new soluble proteins in the roots and leaves of the cotton infected with vesicular-arbuscular mycorrhizal fungi and Verticillium dahliae. More than ten types of new proteins were identified as PR-proteins of which one of them exhibited chitinase activity.

Rahimi et al., (1996) found increased activity of PR-proteins in the leaves of potato infected by root parasite of the genus Globodera. Ashoka (1998) observed increase in number of acidic and basic PR-proteins due to inoculation with the fungus, Pyricularia oryzae in both IET 7191 (resistant) and HR-12 (susceptible) rice varieties. The band intensity also varied due to inoculation over the control. Fusarium clmorum infection of germinating wheat seeds caused induction of specific PR-proteins upon infection (Caruso et al., 1999). Fusarium moniliformae in sorghum genotypes also caused induction of PR-proteins (Krishnaveni et al., 1999). Egea et al. (1999) observed an induction of PR-proteins in stem tissue of pepper plants infected with Phytophthora capsici, soon after inoculation.

5.4 Studies on genetic differences between blast resistant and susceptible varieties of Finger millet by employing molecular marker techniques

Molecular marker techniques are being extensively used to identify and characterize disease resistant and susceptible genotypes among the germ-plasm available and also to know the biodiversity in relation to disease resistance. In the present investigation two different molecular marker techniques were employed to identify the differences if any among the resistant and susceptible cultivars.

5.4.1 Randomly amplified polymorphic DNA (RAPD)

Seventeen diverse genotypes of Finger millet comprising of ten number of resistant and seven number of susceptible cultivars were studied. After selecting 40 different random primers from the initial study of 120 primers which were polymorphic
the data obtained from other studies was used to know the possibility of differentiating resistant genotypes from that of susceptible ones and also to know the extent of diversity among them. The dendograms obtained from the above data was clearly clustered into two groups namely resistant and susceptible and the degree of resistance or susceptibility among the above genotypes could be identified from the RAPD data obtained. It was also possible to identify the highly resistant, moderately resistant, moderately susceptible and highly susceptible based on the disease incidence data obtained from the field which could be corroborated with marker data. There is a very good correlation to the disease incidence and the diversity recorded with this 40 random primers. Two and three dimensional PCA constructed from the above data strongly supported the above correlation (fig. 20 and 21). Therefore, it is possible to differentiate resistant and susceptible cultivars by using these selected random primers to identify resistant and susceptible genotypes and the degree of susceptibility. This information should be useful for screening large number of global germplasm collected and maintained in the coordinatory centre, Bangalore.

Liu (1996) studied the genetic diversity and relationships among 40 accessions of *Lablab purpureus* genotypes using RAPD markers, a high level of genetic variation in this species was detected but this was mainly restricted to the difference between cultivated and wild forms. In cultivated genotypes, genetic variation among Asian collections was significantly higher than that among African collections. The three most divergent cultivated genotypes were all from Asia. Similarly, RAPD was used to assess genetic diversity studies in grape cultivars (Qu et al., 1996), cotton varieties (Tatineni et al., 1996), watermelon cultivars (Lee et al., 1996) and strawberry cultivars (Graham et al., 1996).

Levi and Rowland, (1997) identified blue berry cultivars and evaluated their genetic relationship using 15 RAPD and 3 SSR primers. However estimates of relative genetic similarity between genotypes did not agree well with known pedigree data and thus indicated that RAPD and SSR data do not accurately assess the genetic relationships of cultivars within species.
Bartolozzi et al., (1998) studied genetic characterization and relatedness among California almond cultivars and breeding lines were detected by 37 RAPD markers. Genetic diversity within almond was found to be limited despite its need for obligate out crossing. Perry et al., (1998) used RAPD to evaluate their suitability in distinguishing cocoa varieties.

Sandip Das et al., (1999) evaluated the genetic relationship among nine cultivars of *Brassica campestris* by employing RAPD marker. It generated a total of 125 bands using 13 decamer primer of which nearly 80% were polymorphic. Angiolillo et al., (1999) studied the genetic diversity of olive accessions using RAPDs.

Pradeepkumar et al., (2001) used 22 accessions of *Piper nigrum* for molecular characterization using RAPD markers. They found cultivar specific bands for all the released varieties. The range of polymorphic markers per primer was 3 to 21 with a mean of 21.53 polymorphic bands per primer. Analysis of genome differentiation between high toxin and low toxin accessions of *Lathyrus sativus* was also done using RAPD (Qayyum Khan and Abdul Majid, 2001). Irrespective of their geographical diversity, high toxin and low toxin varieties clustered into distinct genetic groups in the phylogenetic tree. Esha and Shirish (2001) studied molecular distinction amongst varieties of mulberry using RAPD profiles.

Chowdhury et al., (2002) studied the genetic relationship among 48 soyabeau germplasm lines, which were genetically and geographically distinct from the existing lines. They found that 31% were polymorphic which indicated that high level of genetic similarity existed in those exotic cultivars.

The biodiversity studies in core germplasm collections of sandalwood with RAPD revealed genetic dissimilarity of 51 genotypes ranging from 15-91% (Shashidhar et al., 2003). RAPD markers were used to study the genetic diversity in nine cultivars of strawberry differing in reaction to photoperiod, 29 % polymorphism in molecular
diversity of strawberry was found and 15% of the markers were specific to cultivars (Jadwiga et al., 2003). Orapi and Pittaya (2003) evaluated 37 accessions of *Alpinia spp.* by RAPD technique. They did not find any relation with their morphological characters. Neeraj Jain et al., (2003) studied molecular diversity in *Phyllanthus amarus* through RAPD profiling.

Badiane et al., (2004) used RAPD marker to screen the cowpea varieties by inducing water deficit by PEG-6000. They found that samples were genetically diverse. Nayak et al., (2004) analysed 12 species of bamboo using 30 tenmer primers to study the diversity. They found RAPD technique has the potential for use in species identification and understanding genetic relationship between taxa and species of bamboo for breeding program.

Debnath (2005) differentiated the 13 cranberry (*Vaccinium spp.*) cultivars using RAPD marker. Out of 134 bands, 114 were polymorphic (85.1%) Cultivars were divided into two clusters by their distribution on the UPGMA dendrogram at a similarity value of around 0.5. Suja George et al., (2005) analyzed the diversity and species relationship in 13 accessions of pearl millet and related species using seven random primers. The RAPD profiles showed variation among accessions from different geographical regions. Prakash Nowbuth et al., (2005) assessed the genetic diversity of some Anthurium cultivars. They used eight decamer primers and found low average genetic distance among various cultivars indicating the less genetic variation. Vibha et al., (2005) did the molecular characterization of 32 Indian rice varieties of different agro-climatic zones, which resulted in mean heterozygosity by RAPD, ISSR and STMS markers.

5.4.2 Amplified fragment length polymorphism analysis of finger millet genotypes (AFLP)

In order to validate the results obtained from RAPD studies, further studies were carried out using another advanced highly reproducible molecular marker technique namely AFLP. The 17 genotypes studied earlier were subjected to AFLP studies and analysis was carried out to know the diversity among these cultivars and their
relationship to the resistance and susceptible cultivar information obtained from the field data (fig 22 and 23). It is interesting to know from the above study that the data was in confirmation with the RAPD. With the further final details among the cultivars, the African world types namely 18IE and IE1012, which were found to be highly resistant to blast disease, clustered separately. Similarly, the blast susceptible, mildly susceptible and highly susceptible genotypes clustered distinctly and away from resistant cultivars. PCA data with two-dimensional scattered diagrams for the above were found to be highly divergent among them. Further analysis with three-dimensional plots showed the dispersion of different cultivars indicating the considerable genetic variation among these groups. The above data will be definitely useful for grouping the germplasm based on the relative resistant and susceptible character for selecting the parents to develop the resistant cultivars. It is possible to use both the marker technique to differentiate resistant and susceptible cultivars, the degree of susceptibility or resistance and the biodiversity among the genotype studied. This information will be very useful and with the techniques standardized here should be possible to use to screen large number of germplasm whose response to disease is not known. Our results are in line with the results obtained by several groups who have successfully used AFLP in studying genetic diversity with respect to particular phenotypic character.

Maughan et al., (1996) assessed the extent of variation in cultivated soybean and wild soybean using AFLP. They found that adapted cultivars were tightly clustered, illustrating the relatively low genetic diversity present in clustered soybean. Thome et al., (1996) used AFLP analysis to evaluate the genetic structure between and within gene pools of a core collection of wild Phaseolus vulgaris.

Paul et al., (1997) employed AFLP markers to detect diversity and genetic differentiation among Indian and Kenyan populations of tea. Kenya type was more dispersed on the PCO plot which is a reflection of wider genetic variation.
Perera et al., (1998) evaluated genetic relationships between indigenous coconut accessions from Sri Lanka by means of AFLP profiling. This study provided insights into the genetic relatedness of coconut accessions.

The genetic diversity of Indian neem was assessed with AFLP markers (Singh et al., 1999). 37 neem accessions from different eco-geographic regions of India and 4 from Thailand were screened. The Indian and Thailand accessions clearly separated indicating that Indian accession revealed broad genetic base, while Thailand type had a very narrow genetic base.

Beebe et al., (2001) used AFLP analysis to determine the genetic structure of a large sample of common bean from Andean landraces, and to establish a relationship between landraces and wild bean. They concluded that there is narrow genetic base in Andean bean and there is need to broaden the genetic base of the Andean gene pool. Aytul et al., (2001) assessed the polymorphism of Triticum species by AFLP markers. Capo-chichi et al., (2001) estimated the diversity and phenetic relationships in a collection of 40 velvet bean accessions from cultivated species and different eco-geographic regions using AFLP markers.

Potokina et al., (2002) compared DNA fingerprinting of 673 accessions of common vetch of Vavilov Institute of Plant Industry (VIR) and compared their genetic variability with that of the 450 accessions obtained world over by AFLP. They found that all the AFLP fragments generated could be detected with varying frequency throughout the entire distribution area. Marcos et al., (2002) established the genetic relationship among Arachis species based on AFLP. Coulibaly et al., (2002) used AFLP analysis to evaluate genetic diversity among 117 cowpea accessions including 47 domesticated and 52 wild and weedy annuals and 18 perennial accessions.

Maass et al. (2003) used AFLP analysis to evaluate the genetic structure between and within gene pools of a core collection of wild Phaseolus vulgaris L. They analyzed 114 genotypes collected from different parts and found greater insights into the genetic structure of wild bean than other methods of analysis. Jasmina et al., (2003) found the genetic diversity within lamb’s lettuce and across related species by AFLP markers.
Nguyen et al., (2004) evaluated the genetic variation among cultivated chickpea and wild *Cicer* relatives using AFLP. They found 98.6 per cent polymorphism across the 95 accessions that represented 17 species of *Cicer*. The genetic variation within a species was highest in *C. pinati* then followed by *C. reticulatum* and lowest in *C. macracantha*. Twenty six landraces of black gram were screened for their genetic diversity using AFLP by Shivaprakash et al., (2004).

Prakash (2005) studied the genetic diversity studies among field bean genotypes using AFLP markers. He found a higher genetic variation within the field bean genotypes. The result obtained was useful in choosing the most dissimilar genotypes in breeding program and for developing mapping population to identify markers, which are linked to desirable qualitative and quantitative traits of this crop. Rosales et al., (2005) revealed the genetic relationships within and among races of Mexican common bean cultivars based on AFLP which would be useful to establish the genetic basis of improved germplasm, to facilitate the use of that diversity and to implement the use of markers in selection.

Singh et al., (2006) used RAPD technique to assess genetic diversity and species relationship among 28 accessions of eggplant representing five species. A total of 144 polymorphic amplified products were obtained from 14 decamer primers, which discriminated all the accessions. Genetically distinct genotypes identified using RAPD markers could be potential sources of germplasm for eggplant improvement.

### 5.5 Isotope discrimination studies between blast resistant and susceptible varieties of finger millet upon fungus inoculation

An attempt was made in the present investigation to examine the pattern of stable carbon isotope discrimination during the process of photosynthesis. The pattern of $\Delta^{13}C$ in relation to mesophyll efficiency, thus Water Use Efficiency of plants was studied between resistant and susceptible cultivars. Any stresses, both biotic and abiotic, are known to alter photosynthesis by varying mesophyll efficiency. In the present
investigation, the pattern of $\Delta^{13}$C in two distinct blast resistant cultivars of Finger millet was compared with two highly susceptible cultivars. The $\Delta^{13}$C values were assessed in the seeds, the 15days old healthy seedling, the seedlings infected, neck infected with blast disease and compared with uninfected plants. There was no significant difference among any of these samples studied in the $\Delta^{13}$C values. The findings indicate that the $\Delta^{13}$C values cannot be correlated with disease resistance.

To conclude, from the above studies, in general the total phenols, tannins and sugars were more in resistant plant and no relation with the total proteins was observed. There were qualitative and quantitative changes in the infected plants with blast disease at different period of plant growth. There were resistant and susceptible cultivars specific, inducible by the fungus and constitutive in resistance and susceptible plants, with respect to isozymes namely anionic peroxidases, PPO, PAL, PR- proteins.

By application of molecular marker techniques it is certainly possible to distinguish resistant and susceptible genotypes and the degree of susceptibility and resistance and their biodiversity in this regard using RAPD and AFLP technique. The marker identified from this study could be used for large scale screening of germplasm and for developing the improved resistant cultivars using marker assisted breeding technique. However, an isotope discrimination study appears to be less revealing with respect to disease resistance or susceptibility character.

**Future line of work**

The host enzymes involved in disease resistance to blast disease can be used for over expression in the susceptible genotypes to tolerate the disease. The PR- protein studies could be used to isolate the blast disease specific proteins responsible for disease resistance. From the molecular marker studies it was found that genetic variation among the finger millet genotypes was high. The genetic distant information can be used to choose the genotypes for crossing purpose to develop mapping population, or to facilitate the identification of diverse parents for use hybridization programme in order to maximize the expression of heterosis.