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Full Length Article

Biochemical and Molecular Basis of Resistance in Sorghum against *Curvularia* Leaf Spot

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Abstract

Plants employ different strategies to combat infection caused by numerous virulent pathogens. In this study, basis of host defense response was analyzed after *Curvularia lunata* (Wakker) Boedijn infection by biochemical and molecular techniques. Five different varieties of *Sorghum bicolor* were challenged with virulent strain of *C. lunata* for development of leaf spot disease. It was demonstrated that in resistant sorghum variety 'Indian Gold', total phenolics and enzymes involved in phenylpropenoid pathway were in higher amounts as compared to susceptible varieties when calorimetric quantifications were performed at different intervals after pathogen challenge. Isozyme analysis also supported our biochemical analysis by showing extra isozymes of Peroxidase (PO), Polyphenol oxidase (PPO) in resistant variety. Likewise, gene expression analysis showed higher expression of different defense related genes like PR10, Thaumatin, Sormatin, Antifungal proteins (AFPRT) and β-Glucanase in resistant variety. All of these factors may collectively make a front line against *C. lunata* attack in sorghum.

Keywords: *Sorghum bicolor*; *Curvularia lunata*; Leaf spots; Total Phenolics; PO; PPO; PAL; PR-Genes

Introduction

Sorghum bicolor (L.) Moench, diverged from maize, is a major cereal crop in the world after corn and wheat (FAO, 1995). This cereal crop can be cultivated on a wide range of latitudes. This crop suffers from more than 30 fungal diseases (USDA, 1960; Richardson, 1990). A common specie of Curvularia viz: (*C. lunata*) causes leaf spots in different cereal crops including sorghum (Huang *et al*., 2004).

Plant resistance to pathogens is alienated in the constitutive features of the structure of the plant organ or tissue. These features are switched on when plant comes under attack by infection, damage or chemical elicitors (Rooney, 1980; Mansuetus, 1990; Forbes *et al*., 1992). A specific host can be categorized as resistant or susceptible on the basis of interaction with pathogen (Flor, 1971). Resistance interaction leads to activation of resistance gene of host plant against avirulence gene of pathogen that initiates rapid and strong defense reactions in host cells. These involve numerous biochemical changes including lipid peroxidation (Ohta *et al.*, 1991), protein phosphorelation (Ryu *et al*., 2009), production of reactive oxygen entities (Tanaka *et al.*, 2007), production of phytoalexins (Umemura *et al*., 2003) and ultimately expression of Pathogenesis-Related (PR) proteins (Konishi *et al*., 2001; Kim *et al*., 2003; 2004).

Phenolic compounds are toxic secondary metabolites of plants that constitute several antifungal substances in plants (Luthra *et al*., 1988a). Different enzymes convert endogenous biochemicals in quinones that are toxic for invading pathogens (Luthra *et al*., 1988a). Peroxidase (PO) play role in lignification and subrization of cell wall during plant pathogen interactions (Low and Merida, 1996; Wojtaszek, 1997). Polyphenoloxidase (PPO) in plants modify endogenous phenolic compounds into O-quinones (Luthra *et al.*, 1988b). Phenylalanine ammonia-lyase (PAL) also catalyzes biosynthesis of a wide variety of phenolic compounds (Chen *et al*., 2006).

Expression patterns of different genes are considered as determinants of a cell's state. Changes in the gene expression pattern provide us insight into mechanism of differentiation along with signaling pathways between different organisms. Different defense related genes are inducible in plants upon pathogen attack and encode different PR proteins that provide wide array of protection to plants against invading pathogens (Moiseyev *et al.*, 1994). These include Thaumatin-like proteins (Iyengar *et al*., 1979), Chitinase and sormatin proteins (Seetharaman *et al*., 1996), antifungal proteins (AFPs) (Leah *et al*., 1991; Chandrashekar *et al*., 2000) and β -(1,3) glucanase (Cote *et al*., 1991; Nielsen *et al*., 1997). These proteins collectively provide antifungal properties to plants either by strengthening of cell wall or by production of antifungal toxins inside plant body (Watanabe *et al*., 1999; Selitrennikoff, 2001). No work has been done so far on *C.*

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lunata leaf spot of sorghum with respect to characterization of resistance. This manuscript documents biochemical and molecular basis of resistance in sorghum against *C. lunata* leaf spot disease. Previously no such wok has been done to elucidate basis of resistance in sorghum against Curvularia leaf spot disease.

Materials and Methods

Resistance of Sorghum Varieties against *C. lunata* Leaf Spot

Five cultivars of sorghum viz (Local Punjab, Indian Gold, Sadabahar, Sukhar and White Krishna) were used in current investigation. Seeds were surface sterilized by 1.0% sodium hypochlorite. Plastic pots of 30 cm diameter were filled with sterilized soil. Ten seeds per variety were sown in each pot that was thinned to four healthy seedlings after emergence.

Inoculum of *C. lunata* was prepared in sterilized water at the concentration of 10^6 spores/mL with the help of haemocytometer. After 30 days of emergence, plants were sprayed with this inoculum and covered with plastic bags to retain humidity for successful disease development. After four weeks of inoculum application, plants were evaluated for the development of disease. The disease index was calculated by adopting disease-rating scale 0-5, where 0= no symptoms on leaves, 1= <5% infection on leaves, 2=5-25% infection on leaves, 3=25 75% infection on leaves, 4= >75% infection on leaves (Vir and Grewel, 1974). Disease index were calculated by the formula:

$$\text{Disease index} = \frac{\text{Sum of all numerical rating}}{\text{Number of leaves assessed}} \times \frac{100}{4}$$

Then finally varieties were categorized for their susceptibility to *C. lunata* leaf spots for following formulae. 0-10% DI= resistant, 11-25% DI=Moderately resistant, 25-100% DI= Susceptible (Vir and Grewel, 1974).

Determination of Biochemical Basis of Resistance

Biochemical basis of resistance was determined by quantification of total phenolics and defense related enzymes including peroxidase (PO), Polyphenoloxidase (PO) and Phenyl ammonia lyase (PAL). Calorimetric assays were performed from the day of inoculation to final harvest in a time course study. Top two to three leaves were chosen for these assays.

Total phenolics were quantified by Folin ciocaltueau reagent by adopting method as proposed by Zieslin and Ben-zaken (1993). For quantification of defense related enzymes, one gram of fresh leaf samples were ground in a pre-chilled mortar with pestle in 5 mL of ice cold 100 mM phosphate buffer (pH 7.0) followed by centrifugation at 10,000 rpm for 10 min at 4^{o}C and supernatant was used as an enzyme source. PO quantification was performed by using Guaiacol reagent. PPO activity was determined by using catechol as substrate (Mayer *et al.*, 1965). For PAL activity, L-phenylalanine was used as enzyme substrate (Burrell and Rees, 1974).

Isozyme Analysis of Defense Related Enzymes

Native PAGE analysis was performed for isozyme study of defense related enzymes. Samples from diseased plants of each cultivar were taken at final harvest. Enzymes extracts were prepaired by using same method as described in previous section. Polyacrylamide resolving gel of 7.5% was prepared. Gel was run at 180 volts for one hour with Major Sciences Mini vertical electrophoresis unit. The running buffer was of Tris Glycene native page of pH 8.9 (Goldenberg, 1989).

Peroxidase isozymes were localized by incubating gel in Guiacol reagent (0.25%) for 30 min followed by incubation in 0.3% hydrogen peroxide for another 15 min. Isozyme of polyphenol oxidase were visualized by incubating gels in 0.03 M catechol containing 0.05% P-Phenylenediamine in 0.1 M sodium acetate buffer (pH 6.0) for one hour. Isozyme of phenyl ammonia lyase were stained by incubating the gels in 1% ABTS solution in 100 mM sodium citrate buffer (pH 6.0) for five hour. Relative mobility front (Rf) was calculated by using following formula:

$$\text{Rf} = \frac{\text{Distance traveled by the isozyme (cm)}}{\text{Distance traveled by the tracking dye (cm)}}$$

PR Genes Expression Analysis

Semi-Quantitative RT-PCR was used to perform relative gene expression analysis (Leblanc *et al.*, 1991). Actin was used as house-keeping gene. Its expression level was used as internal standard for cDNA template quantity. RNA was extracted using Triazole reagent according to manufacturer instructions. RNA samples were calibrated to a final concentration of 100 ng/µL. Sequences of gene specific primers are given in Table 1. First stranded cDNA was prepared by M-MLV cDNA synthesis kit according to provided instructions by using oligo DT primers. Second strand of cDNA was prepared by using first strand of cDNA as a template DNA. It was carried out in 25-µL reactions. Sequences of gene specific primers are provided in Table 1. PCR reaction mixture provided by enzynomics was used for PCR reactions. Temperature conditions for PCR cycles were: Denaturation 95ºC for 30 s, Annealing 40-55ºC for 30-60 s, Elongation 72ºC for 1 min, Number of cycles= 35, Final elongation= 72ºC for 5 min. When cycles are over, the reaction was kept at 4ºC to prevent DNA degradation.

Table 1: Primer sequences of defense related genes used in RT–PCR analysis

Primer	Sequence 5' to 3'	Melting temp.
PR10rtF	CCG ACG CCT ACA ACT AAA TCT G	60.3 C
PR10rtR	CAT ACA CCA CAC ACC GCA TAG AG	
ThaumRt–rtR	CGC ATC AGG GCA TTT GG	54.0
ThaumRT–rtF	CCG CAG GAT TAC TAC GAC ATC TC	
SormRt–rtF1	GCA CAC GCT TCG TTC TCT AC	59.4
SormRt–rtR1	GTT CAC CAC CGT GAA CAC C	
AFPRT–rtF1	GTC GTC TTC TGC CCA TGA TT	57.3
AFPRT–rtR1	ACG TGG AGC ATG GTG TAT CA	
β –gluc–RtrR1	TTG AAG AGT CCG AAG TGC CTC	59.8
β –gluc–RtrF1	CAG ACC TAC AAC CAG AAC CTC ATC	
ChitRT–rtF1	GCT ATC AAG GGC GTT GGC AAG	25.3
ChitRt–rtR1	GCT GGC TTC GTA TGC TCA TCA GAC	
Actin–F	ACCGAAGCCCCTCTTAACCC	56.9
Actin–R	GTATGGCTGACACCATCACC	

Fig. 1: Symptoms caused by *C. lunata* on different varieties of sorghum

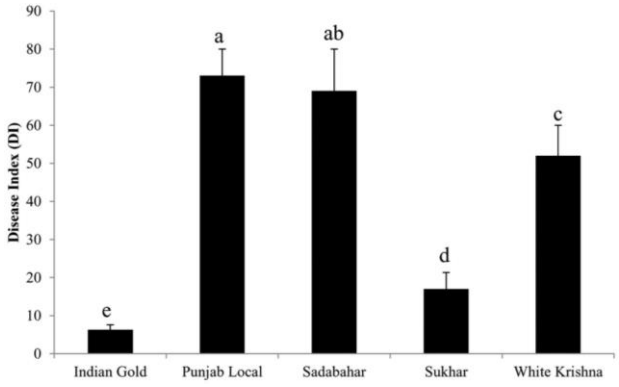

Fig. 2: Disease index of five different sorghum cultivars under attack of *C. lunata.* Vertical bars represents standard error. Small letters shows significance level as governed by ANOVA and DNMRT at $p<0.05\%$

Results

Different cultivars of sorghum represented different susceptibility against *C. lunata* leaf spot (Fig. 1). By keeping in view disease index values (Fig. 2) different cultivars of sorghum were graded under respective susceptibility level. "Indian Gold" was found as the most resistant variety against fungal infection. Moderate resistance was found in "Sukhar" and rests of varieties were susceptible against *C. lunata* leaf spot.

Basis of Biochemical resistance

A significant increase in amounts of total phenolics and enzymes involved in phenylpropanoid pathway were recorded in the resistant variety 'Indian Gold' from 0-12 hours of inoculation (Fig. 3). Second moderate resistant variety 'Sukhar; also showed significant higher levels of phenolics and defense related enzymes as compared to susceptible ones viz: 'Punjab Local', 'Sadabahar' and 'White Krishna'. These had the least quantities of defense related biochemicals (Fig. 3). Indian Gold showed an increase in polyphenol contents of 81, 107.8, 79.68, 6.5 and 81.25% at 6, 12, 24, 48 and 96 h of inoculation. Similarly, more than 100% increase of total phenolics was recorded after 24 h of inoculation in moderately resistant sorghum variety "Sukhar". Overall trend prevailed in this data was of sudden elevation in defense factors just after the inoculation (Fig. 3). But, the quantities of those factors were gradually decreased onwards. Moreover, total phenolic contents were increased in all test varieties, but the percentage of increase was dependent upon level of plant innate resistance against fungal pathogen (Fig. 3).

All three enzymes i.e. peroxidase, phenyl ammonia lyase, polyphenol oxidase showed a significant increase in resistant variety after attack of pathogen (Fig. 3). "Indian Gold" recorded an increase of 30.17% in PO activity after 12 h of inoculation. At same time, Sukhar, Sadabahar, White Krishna and Punjab Local exhibited 27, 13, 17 and 5% elevation in peroxidase activity (Fig. 3). PPO exhibited the trend reminiscent of PO, but PAL showed a different trend which was pattern-less with respect to inoculation period. Its average increase in quantity was highest in "Sukhar" under the influence of pathogen. From data, it was also evident that resistant varieties (Indian Gold, Sukhar) comprised higher amounts of these defenses related biochemicals when quantification were performed at 0 hour interval (Fig. 3). This indicates higher constitutive amounts of these biochemicals in resistant varieties as compared to susceptible ones.

Isozyme Analysis

Isozymes of each enzyme were visible on polyacrylamide gel with variable Rf values. Maximum number of peroxidase isozymes was detected in most resistant variety 'Indian Gold' (Fig. 3). On native gel this variety showed four peroxidase isoforms with Rf values of 0.04, 0.46, 0.813 and 0.850. Moderately resistant variety "Sukhar" had three isozymes with Rf values of 0.04, 0.46, 0.813, and 0.85. While, all the rest of susceptible varieties had two isozymes with Rf values of 0.04 and 0.85 common in each germplasm (Fig. 4).

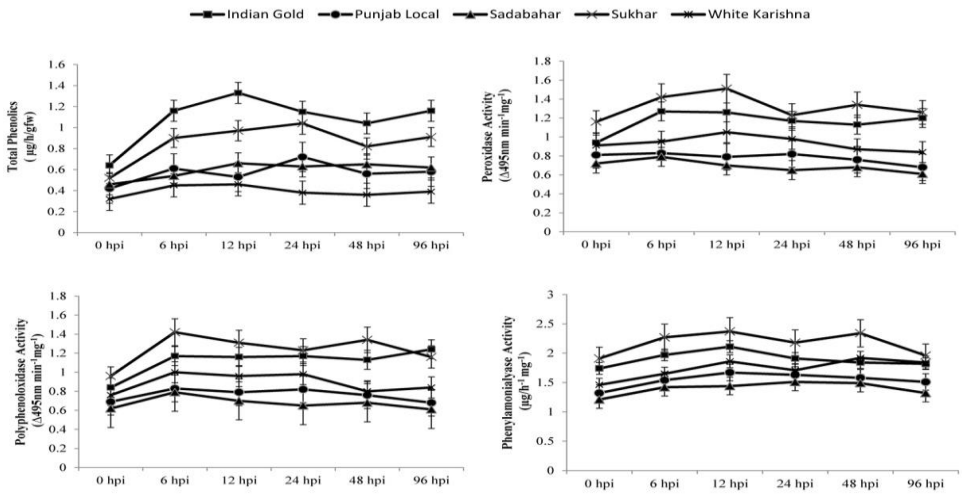

Fig. 3: Changes in total phenolics and enzymes involved in phenylpropenoid pathway in sorghum varieties under influence of *C. lunata* at different intervals. Vertical bars indicate standard error

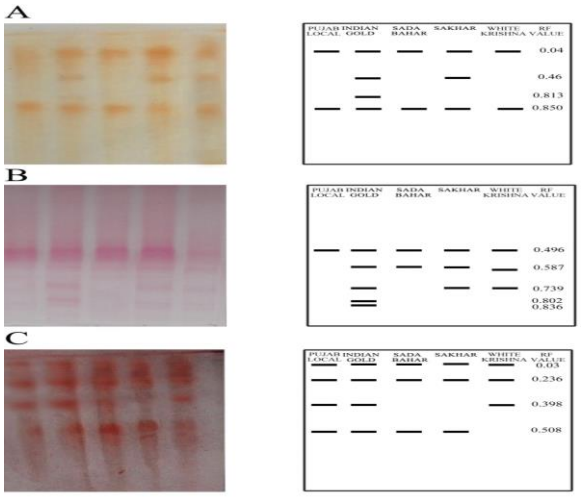

Fig. 4: Zymogram of isozymes pattern of enzymes involved in phenylpropenoid pathway in sorghum verities under influence of C. lunata. A= peroxidase. B=Polyphenoloxidase. C= phenylamonialyase

Similar trend prevailed in case of isozyme study of PPO. Resistant variety not only contained increased number of isozymes of PPO but the amount of an individual isozyme was also increased. Resistant variety (Indian Gold) exhibited five isozymes with Rf values of 0.496, 0.587, 0.739, 0.8.2 and 0.836. Whereas, only three isozymes (with reduced intensity of individual isozyme) were present in "Sukhar" and "White Sorghum". Punjab local had least number of PPO isozymes. In that case only a single isozyme was visible on native gel at Rf=0.496 (Fig. 4).

A great variation of PAL isozymes was observed among different varieties of sorghum. There were only two isozymes (Rf=0.03 and 0.236) commonly present in all sorghum cultivars. While, varieties with enhanced resistance i.e., 'Punjab Local' and 'Indian Gold' reveled two more isozymes of PAL (Rf=0.398 and 0.508) in each sample of them. The rest of the three varieties contained three isozymes each (Fig. 4).

PR Genes Expression Analysis

A significantly higher expression of resistance conferring genes was observed in gene specific, semi-quantitative RT-PCR of sorghum varieties. The highest expression of PR10 was given by the most resistant variety "Indian Gold"; while, expression was gradually decreased towards "Sadabahar" and "Sukhar". Expression of this particular gene was distinctly less in "White Krishna" and "Punjab Local". The general trend can be concluded as "directly related to extent of antifungal resistance" (Fig. 5).

Highest expression of Thaumatin was evident in "Sukhar"; whereas, the most resistant variety stood second in the expression index of this gene. Susceptible varieties (Sadabahar, Punjab Local and White Krishna) showed the least expression providing a sound base for varieties susceptibility. The overall trend for expression of this gene was inclined towards resistant varieties (Fig. 5).

Sormatin gave pattern-less expression levels among sorghum varieties. Punjab Local exhibited the least expression of Sormatin. All the remaining varieties i.e., Sukhar, Indian Gold, White Krishna and Sadabahar had Soratin expression without any significant difference. The only gene which did not show expression in some sorghum varieties (i.e., Punjab Local and Sukhar) was AFP. Its expression was evident in only three varieties i.e., Sadabahar, Indian Gold and White Krishna (Fig. 5). Expression of β-glucanase only differed qualitatively, but not quantatively among varieties. It was observed in all the varieties with only one variety (Indian Gold) having distinctly higher expression than all others.

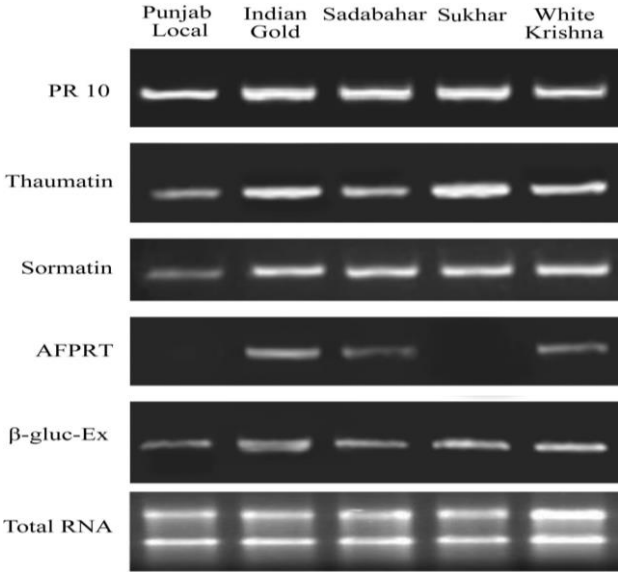

Fig 5: Relative expression of defense related genes in Sorghum varieties under influence of *C. lunata*

Discussion

In the present agricultural and environmental scenario it is necessary to protect economically important crops through some nonhazardous and environment friendly ways. The best and primary measure to control diseases on agricultural crops is to choose the variety having strong resistance against locally prevailing devastating pathogens. Present study gives a convenient approach to explore the possibility to control Curvularia leaf spots on an important agricultural crop (Huang *et al*., 2004). Due to the cultivation of resistant variety in agricultural system, disease would be controlled without any extra efforts and costs of disease controlling practices.

Polyphenols or phenolics are a group of plant metabolites, ubiquitously present throughout the plant kingdom. A deliberately, known phenomenon of polyphenols is antipathogenic responses in plant pathogen relationship (Nemeth *et al*., 2009). A number of studies have been conducted upon plant polyphenols content and level of susceptibility. This study argues upon the pathogen dependent increase in phenolic contents in plant tissues. Such sudden increase of phenolic contents cannot be achieved by only using local machinery of plant cell. So, there is a chance of translocation of phenolic contents from nearby healthy tissues towards diseased portion. This translocation of phenolics is indicating the involvement of an interesting phenomenon of plant signaling which is resulted into inter-cellular transfer of defense materials.

Higher quantities of defense related enzymes including PO and PPO have been previously reported in resistant varieties. These enzymes have being considered as antifungal component in plant body (Bashan *et al*., 1985). Moreover, McLaren *et al.* (1992) observed higher amounts of PPO in resistant plant than the susceptible ones. This study is in agreement with all those previous researches in these directions. It has been proved that higher the quantities of peroxidase and PPO, higher the resistance level would be. So, quantitation of peroxidase and PPO can be used as a parameter for determination of defense levels.

One important enzymes of phenylpropanoid pathway is PAL, which plays a key role in phenolics production and the conversion of phenols in antipathogenic molecules (Cools and Ishii, 2002). But unexpected results recorded in this case were the lesser amounts of PAL in resistant variety than susceptible varieties. There was an immediate increase in PAL activity just after the attack of pathogen up to 12 h, which supported increase in total phenolics. Decrease in PAL activity in further intervals is because when maximum level of total phenolics is reached then its activity declines (Mahadevan and Rangmannar, 1980). Another possible mechanism is the modulation of more than one pathways in which biosynthesis of polyphenols is involved.

Isozyme assays on native PAGE revealed that resistant variety had quantitatively as well qualitatively more enhanced isozyme profile than susceptible variety. This phenomenon was equally applicable to peroxidase and PPO isozymes of sorghum varieties (Sukhwal and Purohit, 2003). While trend of PAL isozymes, was different from the isozymes of above two discussed enzymes.

Pathogenesis related genes encode proteins with antipathogenic properties and restrict fungal invasion (Waniska, 2001). So, the RT-PCR results showed greater expression in resistant cultivars of sorghum. Such types of results were also previously recorded by Chen *et al*. (2006). Thaumatin-like protein cause a loss of electrolyte and cellular contents due to excessive osmotic level disturbance (Sunitha and Chandrashekar, 1994: Shewry and Lucas, 1997). So, the antifungal properties are common in thaumatin-like proteins. A varied trend was present in expression pattern of sormatin gene. This indicates that sormatin does not play an active role in case of plant antifungal defenses.

Hence, present investigation is an important step towards understanding the antifungal factors in sorghum. These results are helpful in designing sorghum antifungal resistance criteria, which will be helpful in plant breeding and variety development program.

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