Biochemical characterisation of grain mould resistant and susceptible genotypes and PGPR-induced resistance in the host to Curvularia lunata and Fusarium proliferatum

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Resistance to biotic stresses in plants is either due to the presence of preformed biochemical compounds or induced in response to external stimulus. In this study, 13 grain mould resistant and seven susceptible lines of sorghum were analysed for biochemical defence mechanism. The levels of total phenols and phenylalanine ammonia lyase were almost same in the resistant and susceptible genotypes. However, two additional isoforms of peroxidase were found in the three of the 13 resistant genotypes. The isoform peroxidase corresponding to the R_f value of 0.25 was found in the resistant genotypes IS 13969, ICSB 377 and IS 8219-1, and two genotypes IS 13969 and ICSB 377 had an additional isoform corresponding to the R_f value of 0.32. The results indicated the genotype specific association of peroxidases with grain mould resistance in sorghum. Nine bacterial strains (*Bacillus pumilus* SB 21, *Bacillus megaterium* HiB 9, *Bacillus subtilis* BCB 19, *Pseudomonas plecoglossicida* SRI 156, *Brevibacterium antiquum* SRI 158, *B. pumilus* INR 7, *P. fluorescens* UOM SAR 80, *P. fluorescens* UOM SAR 14, *B. pumilus* SE 34) were tested to induce systemic resistance in sorghum cultivars 296B and Bulk Y against the highly pathogenic grain mould pathogens *Curvularia lunata* and *Fusarium proliferatum*, respectively. The bacterial isolates were effective in inducing resistance in sorghum. Among the strains tested, SRI 158 was found highly effective in reducing grain mould severity in both the genotypes.

Keywords: grain mould; induced resistance; isoforms; peroxidase; phenols; phenylalanine ammonia lyase

Introduction

Grain mould is a major disease of sorghum (*Sorghum bicolor* (L.) Moench) that affects grain production and quality especially in short duration cultivars that mature during the rainy season in the humid, tropical and subtropical climates. Damage due to grain mould has been associated with losses in seed mass, grain density, seed germination, storage quality and market value. Some of the mould fungi produce mycotoxin(s) that are harmful to human and animal health and productivity (Thakur et al. 2006). Fungi belonging to more than 40 genera are reported to be associated with sorghum grain

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mould (Navi et al. 1999). In India, *Fusarium proliferatum, Curvularia lunata* and *Alternaria alternata* are more pathogenic among the fungi associated with grain mould complex (Thakur et al. 2003).

Host plant resistance is the most effective strategy for managing sorghum grain mould (Thakur et al. 2003, 2006). However, it is important to understand mechanism of grain mould resistance in sorghum. Plant resistance mechanism can be broadly divided into two types which relate either to constitutive features of the structure and biochemical composition of the plant cells or to inducible systems, which are only switched on when the plant is challenged by infection, damage or treatment with a chemical elicitor. The constitutive resistance is conferred by the presence of antifungal proteins, peptides and other biochemical compounds either in the apo-plasm or within the cells, whereas the biochemical defence mechanism may consist of the presence or absence of a particular chemical substance or group of substances in a host plant, which inhibits the growth and multiplication of a pathogen. Such a condition may exist constitutively either before the pathogen attacks the plant or as a reaction of the host to infection by the pathogen. Among the biochemicals involved in defence process, phenolics, peroxidases and phenylalanine ammonia lyases (PAL) are significantly important (Dicko et al. 2002, 2005, 2006; Heldt 2005).

Role of phenolic compounds in disease resistance in sorghum has been documented (Nicholson and Hammerschmidt 1992). Studies have shown that the plant resistance to both biotic (pathogens and predators) and abiotic (UV radiation, drought etc.) stresses is related to the presence of phenolic compounds (Dicko et al. 2002, 2005, 2006). PAL is indirectly associated with the synthesis of phenol polymers including lignin and suberin (Parr and Bolwell 2000; Heldt 2005). In sorghum, the infection of the seedlings by the pathogen involves rapid accumulation of PAL mRNA (Cui et al. 1996). Inhibition of PAL and cinnamyl alcohol dehydrogenase has been reported to increase the susceptibility of barley to powdery mildew (Carver et al. 1994).

Plant peroxidases are ubiquitous, haeme containing glycoproteins that catalyse the oxidation of diverse organic and inorganic substances at the expense of hydrogen per-oxide (Castillo 1992). Their roles in defence mechanism include the oxidation of hydroxycinnamyl alcohols into free radical intermediates, phenol oxidation (POX), cross-linking of polysaccharides and of extensin molecules, lignification and suberisa-tion (Chittoor et al. 1997).

In the early twentieth century, evidence began to accumulate that plants could be protected against pathogens by prior infection of the plant with other avirulent strains. This phenomenon is known as induced or acquired resistance to disease (Hammerschmidt and Kuc 1995; Sticher et al. 1997). One of the characteristics of acquired resistance is that it is effective against a broad spectrum of pathogens. A number of plant growth promoting rhizobacteria (PGPR) have been identified as potential inducers of systemic resistance (ISR). Increased PAL activity has been reported in the sorghum when inoculated with *Azospirillum* (Mohan et al. 1988). Bacteria differ in their ability to induce resistance, some bacteria are more active on certain plant species with varying results within the same species, and in some cases they have no effect on other species (Van Loon 1997). The present study was undertaken for the biochemical characterisation of grain mould resistant geno-types and to identify the PGPR effective in reducing the grain mould severity in sorghum.

Table 1.   Grain mould reaction of sorghum lines selected for biochemical characterisation.

Entry no.	Genotype	Grain mould reaction
1	IS 12932-2	Resistant
2	IS 13969	Resistant
3	SGMR 24-5-1-2	Resistant
4	SGMR 11-3-5-1	Resistant
5	IS 14384-1	Resistant
6	ICSB 377	Resistant
7	IS 8219-1	Resistant
8	SGMR 33-5-6	Susceptible
9	PVK 801-4	Susceptible
10	SGMR 23-10-2-1	Susceptible
11	SGMR 40-1-2-3	Resistant
12	IS 41397-3	Resistant
13	ICSV 96094-2	Resistant
14	ISCB 402-3	Resistant
15	ISCB 402-1-2	Resistant
16	SPV 462-3	Resistant
17	IS 36469C 1187-1-2-9-8-2	Susceptible
18	SP 72521-2-6-6-6	Susceptible
19	SPV 104	Susceptible
20	Bulk Y	Susceptible

Notes: Resistant $\leqslant 3.0$ grain mould score and Susceptible $\geqslant 7.0$ score on a 1–9 progressive scale.

Materials and methods

Biochemical characterisation of grain mould resistant sorghum genotypes

Plant materials

Thirteen grain mould resistant and seven susceptible genotypes were used in this study (Thakur et al. 2003, Table 1). Seeds of these 20 genotypes were surface sterilised with 1% chlorox for 3 min and washed three times with sterile distilled water. Sterilised seeds were plated in humidity chambers and incubated at 30 °C for 5−6 days. The plumules of the seedlings were used as a source material for the estimation of phenols, PAL and peroxidase enzymes.

Assay for peroxidase, phenols and PAL

Isoenzyme analysis for peroxidase was carried out using the native polyacrylamide gel electrophoresis (native-PAGE). Proteins were extracted using Tris-HCl buffer. Protein content was estimated as per the method of Lowry et al. (1951). Electrophoresis was performed at 50 v for 7−8 h in a vertical gel electrophoresis using a gradient gel of 10–155 concentration. Isoforms were visualised by incubating the gel in a solution containing *O*-dianisidine. The activity of PAL was determined using the method of Subba Rao and Towers (1970). The absorbance was measured at 290 nm using trans-cinnamic acid at varying concentrations as the standard. Enzyme activity was expressed in μM trans-cinnamic acid/g protein/min. Total phenols were determined as per the method of Malik and Singh (1980). The absorbance was read using a spectrophotometer at 750 nm. Phenol content was expressed as mg phenols/g sample.

Induction of resistance in sorghum with PGPRs

Seed source

Seeds of two susceptible sorghum genotypes 296B and Bulk Y used in the study were obtained from Sorghum Breeding Unit, ICRISAT, Patancheru.

ISR agents and inoculum preparation

Nine bacterial strains (*Bacillus pumilus* SB 21, *Bacillus megaterium* HiB 9, *Bacillus subtilis* BCB 19, *Pseudomonas plecoglossicida* SRI 156, *Brevibacterium antiquum* SRI 158, *B. pumilus* INR 7, *P. fluorescens* UOM SAR 80, *P. fluorescens* UOM SAR 14 and *B. pumilus* SE 34) obtained from the department of Applied Sciences and Biotechnology, University of Mysore, Karnataka and Biocontrol Unit, ICRISAT were used in these studies. The bacterial suspensions were obtained by inoculating in the nutrient broth with bacterial isolates and incubating at 27 °C at 120 rpm in a shaker cum incubator for 48 h. The 48-h-old cultures were used for inoculation of sorghum panicles for the induction of resistance.

The panicles of both 296B and Bulk Y were spray inoculated at pre-flowering stage (three days before anthesis) with the 48-h-old bacterial suspensions in the greenhouse at 25 °C. Proper controls were maintained by spraying sterile water. Each bacterial treatment consisted of eight replications, one panicle/replication in each genotype.

Pathogen inoculation

C. lunata and *F. proliferatum*, the two major grain mould pathogens of sorghum were used in this study. The pathogens were isolated from the moulded sorghum grains collected from the grain mould nursery conducted at ICRISAT, Patancheru during 2010. The conidial suspensions of *C. lunata* and *F. proliferatum* were prepared in the sterile distilled water from 10-day-old cultures grown on potato dextrose agar. Spore concentration was adjusted to 1×10^5 conidia/ml and spray inoculated on the bacteria-treated panicles at 80% anthesis stage. The inoculated panicles were exposed to over head mist for 48 h for facilitating the mould infection. The inoculated plants were maintained in the greenhouse chambers at 25 °C until physiological maturity. At physiological maturity, the plants were exposed to mist for 72 h for the grain mould development. The inoculated panicles were harvested, dried and the grains were collected for further use.

Observations on grain colonisation

The grains were assayed for fungal colonisation using blotter paper method (Thakur et al. 2006). The grains were surface sterilised with clorox (1%) for 3 min and thoroughly washed with sterilised distilled water. The grains were then kept in sterilised moist Petri plates, 25 grains/Petri plate; two Petri plates/treatment. The plates were incubated at 28 ± 1 °C for five days with 12 h photoperiod. The data were recorded on the number of grain colonised by the pathogen inoculated as well as total moulded grains due to natural infection by other fungi. Per cent grain colonisation was estimated as

$$\text{Grain colonisation } (\%) = \frac{\text{Moulded grains}}{\text{Total grains}} \times 100$$

Statistical analysis

The data-sets were subjected to analysis of variance to determine significant differences among treatments using GENSTAT statistical package (Rothamsted Experiment Station, Herpenden, Herts AL52JQ, UK).

Results

Biochemical characterisation of grain mould resistance in sorghum

Isozyme analysis

Zymogram of peroxidase isoforms in resistant and susceptible genotypes of sorghum is presented in Figure 1. Six isoforms were common across resistant and susceptible genotypes, but two additional isoforms corresponding to the R_f value of 0.25 were found in three resistant genotypes (IS 13969, ICSB 377 and IS 8219-1) and two genotypes (IS 13969 and ICSB 377) had an isoform corresponding to R_f value of 0.32.

Estimation of PAL

Comparative PAL activity in the resistant and susceptible genotypes is presented in Figure 2. There were significant differences in the PAL activity among test lines; however, no significant variation between the resistant and susceptible groups was observed. The highest enzyme activity (41.75 μM/min/g protein) was found in resistant genotype
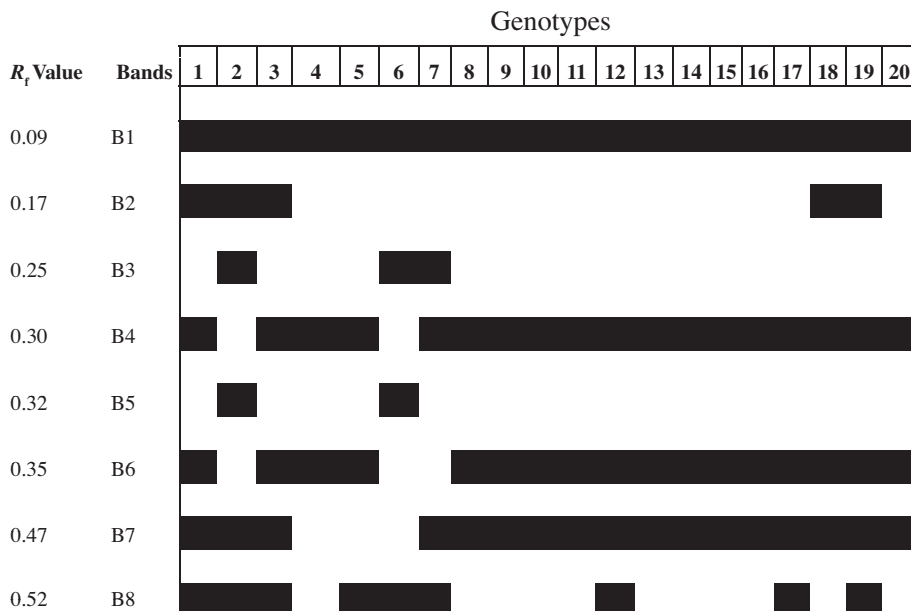

Figure 1. Zymogram showing banding pattern of peroxidase isozyme in grain mould resistant and susceptible sorghum lines.
Notes: Lanes 1−20 represents genotypes IS 12932-2, IS 13969, SGMR 24-5-1-2, SGMR 11-3-5-1, IS 14384-1, ICSB 377, IS 8219-1, SGMR 33-5-6, PVK 801-4, SGMR 23-10-2-1, SGMR 40-1-2-3, IS 41397-3, ICSV 96094-2, ISCB 402-3, ISCB 402-1-2, SPV 462-3, IS 36469C 1187-1-2-9-8-2, SP 72521-2-6-6-6, SPV 104 and Bulk Y.
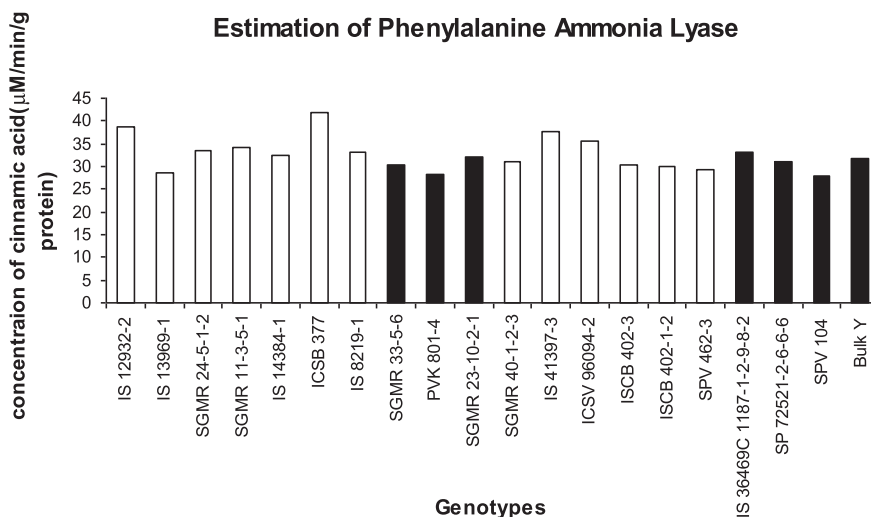
Figure 2.   Concentration of cinnamic acid in the selected resistant and susceptible sorghum lines.
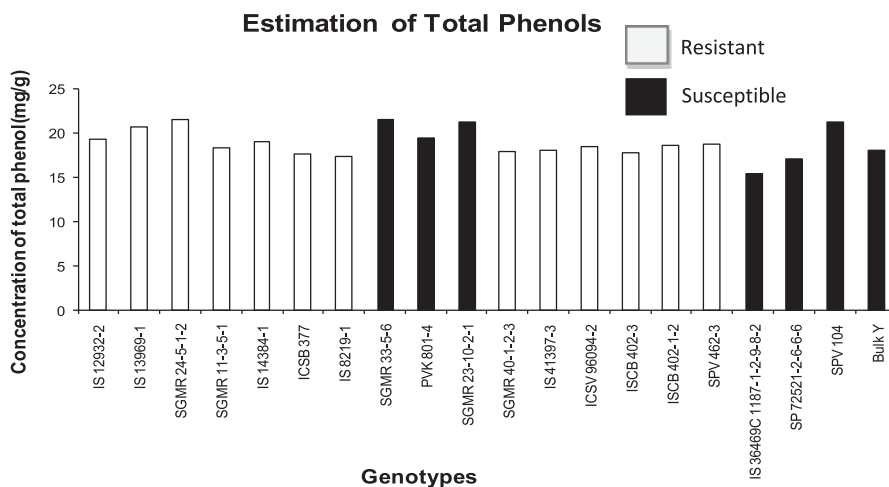

Figure 3.   Total phenol content in the resistant and susceptible sorghum lines.

ICSB 377 while the lowest (27.88 µM/min/g protein) in the susceptible genotype SPV 104.

Estimation of total phenols

There was no significant variation in the phenolic contents of resistant and susceptible genotypes (Figure 3). The highest amount of phenols (21.60 mg/g) was recorded in two genotypes SGMR 3-3-5-6, a susceptible genotype and SGMR 24-5-1-2, a resistant genotype. The lowest amount (15.51 mg/g) was recorded in the susceptible genotype, IS 36469C 1187-1-2-9-8-2.

Table 2. ANOVA for efficacy of ISR agents in reducing grain colonisation (%) by *C. lunata* on 296B and *F. proliferatum* on Bulk Y.

		Mean square	
Source of variation	Degree of freedom	296B	Bulk Y
Replications	7	393.6	69.18
ISR agents	9	3637.5**	333.75**
Residual	143	144.9	53.46
Total	159		

Note: **significant at $p < 0.01$

Induction of disease resistance in sorghum

Efficacy of bacterial strains as inducers of grain mould resistance

Analysis of variance indicated that the bacterial strains were significantly effective in reducing the gain mould severity in 296B and Bulk Y inoculated with *C. lunata* and *F. proliferatum*, respectively (Table 2). In 296B, the grain colonisation by *C. lunata* in the bacteria-treated plants was significantly lower compared to control (Table 3). The lowest grain colonisation by *Curvularia* (4%) was observed in SRI 158 treated plants followed by treatment with *P. fluorescens* UOM SAR 14, that resulted in 5% grain colonisation, whereas 56% grain colonisation was observed in the control. Also per cent total mould infection (by pathogen inoculation as well as due to natural infection by other fungi) was higher in the control (67%) compared to bacteria-treated plants. Treatment with SRI 158 resulted in lowest mould infection (6%) compared to 67% in control.

In Bulk Y, the lowest grain colonisation (2%) by *F. proliferatum* was observed in treatment with SRI 158 against the control (17%) (Table 3). However, treatment with *P. fluorescens* UOM SAR 14 resulted in lowest total mould infection (18%) compared to control (30%).

Table 3. Efficacy of ISR against grain colonisation of sorghum genotypes caused by *Curvularia lunata* and *Fusarium proliferatum* in greenhouse conditions.

		Grain colonisation (%)[a]			
		296B		Bulk Y	
Treatment no.	ISR agent	*C. lunata*	Other fungi	*F. proliferatum*	Other fungi
T 1	*B. pumilus* SB 21	27	34	12	28
T 2	*B. megaterium* HiB 9	23	70	3	47
T 3	*B. subtilis* BCB 19	21	40	4	50
T 4	*P. plecoglossicida* SRI 156	13	30	9	52
T 5	*Brevibacterium antiquum* SRI 158	4	6	2	28
T 6	*B. pumilus* INR 7	25	32	5	34
T 7	*P. fluorescens* UOM SAR 80	12	20	7	44
T 8	*P. fluorescens* UOM SAR 14	5	18	5	18
T 9	*B. pumilus* SE 34	13	20	10	54
T 10	Control (Distilled water)	56	67	17	30
	Mean	20	34	7	38
	LSD ($p < 0.05$)	8.4	13.7	5.1	16.1

Note: [a]Based on the mean of 8 replications.

Discussion

The biochemical basis of disease resistance in plants is a complex phenomenon. Total phenols and phenolics have long been considered as important defence-related compounds whose levels are naturally high in the resistant varieties of many crops (Onyeneho and Heltiarachchy 1992; Saini et al. 1988). According to Nicholson and Hammerschmidt (1992), some antibiotic phenols occur in plants constitutively to function as preformed inhibitors, while some occur in response to ingress of pathogens, exhibiting active pathogen defence.

Many attempts have been made to find and identify toxic compounds, which, by their presence in the resistant varieties and absence or smaller concentrations in the susceptible varieties, could be assigned a role in the host defence against a particular pathogen. However, few cases in which such compounds are correlated with pre-infectional defence against the pathogen have been adequately documented. In the present study, no significant differences in the total phenol content between susceptible and resistant genotypes were found. Leaves of sorghum resistant to fungi have been found to contain a higher content of total phenols than those of susceptible upon pathogen challenge (Luthra et al. 1988). This suggests that total phenols in sorghum grains, which are not challenged by the pathogens, are not good indicator for resistance to biotic stress.

POX is involved in cross-linking extensin molecules to form lignin (Brisson et al. 1994). Increased lignin deposition is believed to play a role in barricading the pathogen from invading the plant through physical exclusion (Milosevic and Slusarenko 1996). According to Yang et al. (1984), isozyme bands of peroxidases are the expression of individual genes. Among 20 sorghum lines tested, the three resistant genotypes (IS 13969, ICSB 377 and IS 8219-1) shared some common loci (Figure 1), indicating genotype specific association of peroxidases with disease resistance. Castor and Frederiksen (1980) observed that sorghum resistant to one genus of fungus or mode of fungal attack was not necessarily resistant to other genera or modes of attack. The sorghum varieties in this study may have different resistance mechanisms to the grain mould fungi. This could explain the variation in peroxidase isozyme banding patterns even among resistant varieties.

PAL activity has been reported to be associated with the biosynthesis of toxic metabolites such as phytoalexins, phenols, lignins and salicylic acid in plant defence pathways (Mauch-Mani and Slusarenko 1996). In the present study, the PAL activity was almost same in both resistant and susceptible genotypes. The PAL expression might increase in response to pathogen attack. Therefore, PAL activity should be compared in resistant and susceptible lines following inoculation of a pathogen to determine the association of the enzyme with mould resistance. The total phenols and related enzymes analysed in this study did not correlate well with grain mould resistance. However, genotype specific association of peroxidase for grain mould resistance was observed in this study. Thus, the ability of sorghum to resist fungal attack does not appear to be due to a single factor, but is most likely the result of interaction and combination of many factors.

The bacterial isolates were effective in inducing resistance in sorghum against the mould fungi. Among strains tested, SRI 158 was most effective in inducing disease resistance in both the genotypes tested. A number of PGPR have been selected for their ability to systemically control various diseases when localised to plant roots, as an soil drench, transplant mix, root dip or seed treatment (Van Loon et al. 1998; Chen et al. 2000). *B. pumilus* INR7 is an exemplary example of a PGPR strain that effectively

protected cucumber plants against angular leaf spot and anthracnose in several field trials (Raupach and Kloepper 1998; Wei et al. 1996). Hence, spray application of SRI 158 at the anthesis stage coupled with reasonable levels of resistance in the host could become an integral component of integrated disease management in sorghum. However, the efficacy of SRI 158 as a potential ISR agent needs to be further confirmed at field level.

Acknowledgements

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