Oxidative Enzyme Changes in Sorghum Infested by Shoot Fly

P. G. Padmaja,1,2 B. L. Shwetha,1 G. Swetha,1 and J. V. Patil1

1Directorate of Sorghum Research, Rajendranagar, Hyderabad 500 030, India
2Corresponding author, e-mail: padmaja@sorghum.res.in

ABSTRACT. This research investigated the role of oxidative enzymes in the defense response of sorghum, Sorghum bicolor (L.) Moench (Poales: Poaceae), to the sorghum shoot fly, Atherigona sociata Rondani (Diptera: Muscidae). Changes in polyphenol oxidase and peroxidase activity and total protein content were observed in resistant and susceptible sorghum genotypes in response to A. sociata feeding. Resistant plants exhibited higher levels of peroxidase and polyphenol oxidase activities and total protein content compared with susceptible plants. Peroxidase and polyphenol oxidase activities and total protein content in the infested resistant and susceptible genotypes were higher when compared with their control plants, respectively. These findings suggest that resistant genotypes may be able to tolerate shoot fly feeding by increasing their peroxidase and polyphenol oxidase activities. Among the enzymes examined, differences in isozyme profiles for peroxidase and polyphenol oxidase were detected between control and infested IS 18551, M35-1, 296B, SSV 84, and DJ 6514 plants. Differences in protein profiles were observed between A. sociata infested and their respective uninfested controls of all the genotypes. In conclusion, this study revealed that these defense enzymes and proteins might contribute to the resistance mechanisms in sorghum plants against A. sociata infestation.

Key Words: sorghum, shoot fly, oxidative enzyme, protein, host plant resistance
was carried out in a randomized complete block design with five replications. The plants were thinned at 7 d after seedling emergence (DAE) to maintain a spacing of 10 cm between plants. Overall resistance was recorded as the percentage of dead hearts (DH%) caused by *A. soccata* infestation. Plants with dead hearts were recorded in all the plots at 28 DAE. The DH% (ratio of the number of dead hearts to the total number of plants × 100) recorded at 28 DAE was used for evaluating resistance.

**Sample Collection.** Sorghum seedlings (leaves and stems) (1 g) of each of the five genotypes infested with *A. soccata* were collected from field for protein analysis at 21 DAE. Plants without dead heart symptom served as uninfested control.

**Preparation of Samples.** Samples were prepared for protein assay following the protocol by Hildebrand et al. (1986). Soluble proteins were extracted by grinding plant tissues in a chilled mortar with 3.0 ml of 20 mM 4-2-hydroxy-methyl-1-piperazineethane sulfonic acid buffer (HEPES) buffer (pH 7.2) containing a protease inhibitor cocktail [0.3 g/1 g of tissue contains 4-(2-aminoethyl)benzenesulfonyl fluoride, bestatin, pepstatin A, E-64, leupeptin, and 1,10-phenanthroline] and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was collected and stored (<3 h) at 4°C until protein analyses.

**Protein and Enzyme Assays.** The effect of *A. soccata* feeding on plant protein content and enzyme (peroxidase [POX] and polyphenol oxidase) activities were examined using a spectrophotometer. Total protein content was measured with bovine serum albumin as a standard (Lowry et al. 1951). POX activity was measured by monitoring the increase in absorbance at 470 nm for 1 min by using a protocol by Hildebrand et al. (1986) and Hori et al. (1997). The enzymatic reaction was started by adding 10 μl of 30% hydrogen peroxide to a cuvet containing 300 μl of 18 mM guaiacol, 100 μl of 200 mM HEPES (pH 7.0), 585 μl of distilled water, and 5 μl of sorghum extract. The specific activity of POX was determined using the molar absorptivity of guaiacol at 470 nm (26.6 × 10³/M/cm). Polyphenol oxidase activity was measured following a protocol modified from Hori et al. (1997). Polyphenol oxidase activity was monitored at 470 nm for 1 min after the start of the reaction. The reaction was initiated by adding 20 μl of sorghum extract to a cuvet containing 500 μl of 1.6% catechol in HEPES buffer, 380 μl of distilled water, and 100 μl of 200 mM HEPES buffer (pH 6.0). Polyphenol oxidase activity was calculated as the change in A470 per minute milligram of protein.

**Native Gel Electrophoresis.** Samples were analyzed for isozyme expression by native gel electrophoresis using precast 12-well 12.0% polyacrylamide gels. A continuous buffer system of Tris–glycine (3 g of Tris-base, 14.4 g of glycine, and 1,000 ml of distilled water) (pH 8.3) was used. Equal amounts of protein as determined by the BSA protein assay were loaded in each lane. Samples were diluted 1:1 with a gel loading buffer consisting of 62.5 mM Tris–HCl (pH 6.8), 0.01% bromphenol blue, 25% glycerol, 5% β-mercaptoethanol, and 2% SDS before loading. The protein samples were then heated for 5 min at 95°C before loading on the gel. Equal amounts of protein were loaded in each lane. Electrophoresis was conducted at 120 V for 2 h at room temperature. Proteins were visualized by Coomassie brilliant blue staining according to standardized methods. The molecular weights of the different protein bands in each sample were determined from the standard curve drawn between log molecular weight and relative mobility.

**Statistical Analysis.** The data obtained from field experiments were subjected to analysis of variance using the statistical software Windostat (Indostat services 2004). Genotypes were used as fixed effects and blocks and years as random effects. LSD was used to compare the treatment means. Enzyme activity values were analyzed using one-way Analysis Of Variance (ANOVA) using “PROC GLM.” The means were separated using Tukey’s Honest Significant Difference (HSD) test (SAS Institute Inc. 2009).

**Results**

**Evaluation of Resistance to *A. soccata* Under Field Conditions.** There were significant differences among the genotypes for dead heart formation in both the seasons (rainy and post rainy) during both the years (2011 and 2012) (Table 1). M35-1 was as good as the resistant genotype IS 18551 for *A. soccata* dead hearts, whereas the dead heart formation was greater on the genotypes 296B, DJ 6514, and SSV 84 (Table 1).

**Protein Assays.** Infested plants showed an overall increase in total protein content compared with uninfested plants in all the five genotypes (Fig. 1). *A. soccata*-resistant genotypes IS 18551 and M35-1 had a higher protein content compared with the susceptible genotypes 296B and DJ 6514 (*P* < 0.0001). However, SSV 84 recorded highest protein content (10 mg/g fresh weight).

**Enzyme Assays.** *A. soccata*-infested IS 18551, M35-1, 296B, DJ 6514, and SSV 84 plants had higher levels of POX activity compared with their control plants (*P* = 0.0001) (Fig. 2). *A. soccata*-resistant genotypes IS 18551 and M35-1 had a higher POX activity compared with the susceptible genotypes 296B, SSV 84, and DJ 6514. The greatest difference in POX activity between infested and control 296B and SSV 84 plants was observed. *A. soccata*-infested IS 18551 showed approximately twofold increase in POX activity.

**Polyphenol Oxidase Activity.** *A. soccata*-infested IS 18551, M35-1, 296B, DJ 6514, and SSV 84 plants had higher levels of polyphenol oxidase activity compared with their control plants (*P* = 0.0006) (Fig. 3). *A. soccata*-resistant genotype IS 18551 had

| Genotypes          | Dead hearts (%) |
|--------------------|-----------------|
| Khairf 2011        | 88.1 ± 2.1      |
| Khairf 2012        | 88.5 ± 1.3      |
| Rabi 2011          | 75.3 ± 2.4      |
| Rabi 2012          | 76.7 ± 2.9      |
| IS 18551           | 32.9 ± 1.2      |
| SSV 84             | 33.0 ± 1.3      |
| DJ 6514            | 23.7 ± 1.4      |
| M35-1              | 24.5 ± 1.7      |
| Mean               | 27.4 ± 1.9      |
| CV                 | 7.8 ± 1.9       |
| SEM                | 1.2 ± 0.1       |
| F ratio            | 16.7 ± 6.1      |
| P                  | 0.00000          |

**Table 1. Performance of genotypes resistant to shoot fly *Atherigona soccata* in sorghum**
a higher polyphenol oxidase activity compared with the susceptible genotypes 296B, SSV 84, and DJ 6514.

**Isozyme Profile Studies.** Native gel electrophoretic separation of enzyme extract from different genotypes of sorghum plants showed different Peroxidase (PO) isoform patterns. Native gels stained for POX-specific activity showed four isozymes (PO1, PO2, PO3, and PO4) in 296B plants infested with shoot fly, whereas two isozymes (PO1 and PO2) in control plants (Fig. 4A). Two of them (PO3 and PO4) were induced after infestation with shoot fly compared with their controls. In contrast, resistant genotype IS 18551 expressed two isoforms in both infested and control plants.

Native gels stained for polyphenol oxidase-specific activity showed four isozymes in 296B, SSV 84, DJ 6514, and M35-1 plants infested with *A. soccata*, whereas there were only two isoforms in respective control plants (Fig. 4B). Two of them were induced after infestation with *A. soccata* compared with their controls. In contrast, resistant genotype IS 18551 expressed two isoforms in both infested and control plants.

**Discussion**

An increase in the activities of phenolic-related enzymes and the accumulation of phenolic compounds has been correlated with resistance of cereals to biotic stresses (Mohammadi and Kazemi 2002). Plant resistance to biotic and abiotic stresses is often regulated by the metabolism of phenolic compounds. Sorghum phenolic compounds, or allelochemicals, are involved in plant resistance to all kind of stresses (Lo et al. 1999, Weston et al. 1999, Weir et al. 2004). POXs play an important role in stress-related resistance. One of the important physiological roles of POXs is the synthesis of cell-wall polymers (lignin and suberin), which constitute physical barriers for both biotic and abiotic stresses (Cosgrove 1997). In sorghum, POXs are involved in thermal tolerance (Choudhary et al. 1993) and resistance to fungal infection (Luthra et al. 1988). Polyphenol oxidases play an important role in plant defense via the oxidation of endogenous phenolic compounds into o-quinones, which are toxic to invading pathogens and pests (Mohammadi and Kazemi 2002).

In this study, we examined *A. soccata* feeding-induced damage on sorghum plants and its subsequent effects on the plant biochemical and enzymatic changes. Infestation by *A. soccata* increased the total extractable protein content of five sorghum cultivars. In most of the insect-wounded plants due to herbivory, there has been quantitative increase in the levels of biochemicals such as proteins, phenols, and...
The increase of the Peroxidase (POD) activity in herbivore-damaged plants can be attributed to the fact that these are the key enzymes that participate in several plant cell wall building processes (Chittoor et al. 1999). The final products of such enzymatic activities would be considered antinutritive because they cannot be effectively digested and assimilated by insects (Constabel 1999). From the above evidences, it is assumed that the activity of PO might attribute to the reduced A. soccata damage and their preference to sorghum seedlings.

In our studies, all the five genotypes had higher levels of polyphenoloxidase (PPO)-specific activity as a result of A. soccata infestation. The association of PPO activity with host plant resistance to insects occurs in many plants including tomato, potato, coffee, and poplar (Duffy and Felton 1991, Constabel et al. 1996, Chaman et al. 2001, Wang and Constabel 2004, Thipyapong et al. 2006). Polyphenol oxidase reduces the nutritional quality of infested plants by converting soluble phenolic compounds into quinones that eventually prevent the digestion of proteins in insects. Similarly, considerable evidence from the earlier work implicates that increased accumulation of PPO in plants against tomato fruit borer (Helicoverpa armigera and Helicoverpa zea) has affected the growth and development of these insects (Ismann and Duffey 1982a,b). Saravanakumar et al. (2007) demonstrated that increased activity of PPO reduced the growth and development of Cnaphalocrosis medinalis on rice plants. From the earlier reports, it is suggested that PPO might interrupt with the insect fecundity and digestion which ultimately leads to delay in developmental period. Further research is needed to determine the role of these enzymes in the defense response of sorghum genotypes to A. soccata feeding and to evaluate these differences as potential molecular markers for selecting A. soccata-resistant sorghums.

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**Table 2. Protein profile of different genotypes of sorghum infested with shoot fly**

| Molecular weight (kDa) | 296B | IS 18551 | SSV 84 | DJ 6514 | M35-1 |
|------------------------|------|----------|--------|---------|-------|
| 112.2                  | C    | C        |        | C       | C     |
| 104.7                  |      |          |        |         |       |
| 95.5                   |      |          |        |         |       |
| 63.1                   | +    | +        |        | +       | +     |
| 25.1                   | +    |          |        | +       |       |
| 22.9                   |      |          |        | +       |       |

C = control plants; I = infested plants.

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Figure 4. (A) Native gel stained for peroxidase activity. Lane 1, 296B control; lane 2, 296B infested; lane 3, IS 18551 control; lane 4, IS 18551 infested; lane 5, SSV 84 control; lane 6, SSV 84 infested; lane 7, DJ 6514 control; lane 8, DJ 6514 infested; lane 9, M35-1 control; and lane 10, M35-1 infested. (B) Native gel stained for polyphenol activity. Lane 1, 296B control; lane 2, 296B infested; lane 3, IS 18551 control; lane 4, IS 18551 infested; lane 5, SSV 84 control; lane 6, SSV 84 infested; lane 7, DJ 6514 control; lane 8, DJ 6514 infested; lane 9, M35-1 control; and lane 10, M35-1 infested.

Carbohydrates and an increase in enzymes activities. Ni et al. (2001) reported an increase in total protein in wheat infested with the Russian wheat aphid, Diuraphis noxia (Mordvilko), and the corn leaf aphid, Rhopalosiphum padi (L.). We found that shoot fly-resistant cultivar IS 18551 had nearly twofold increase in POX-specific activity after infestation compared with uninfested plants. Plant oxidative enzymes (e.g., POX, polyphenol oxidase, and catalase) play an important role in the plant’s response to biotic and abiotic stresses (Van Loon 1976; Castillo et al. 1984; Hildebrand et al. 1986; Felton et al. 1994a,b; Zhang and Kirkham 1994; Stout et al. 1999; Chaman et al. 2001; Ni et al. 2001).

Our enzyme activity assays and protein profiles suggest that A. soccata feeding leads to a loss in POX activity in susceptible sorghum genotypes. Tolerant genotypes, however, may be able to tolerate A. soccata feeding by increasing their POX activity. Hydrogen peroxide is thought to be produced in response to plant stress such as insect feeding (Dowd and Lagrimmnini 1997). The level of hydrogen peroxide is mediated by the presence of oxidative enzymes such as POX and catalase (Levine et al. 1994, Mehdy 1994, Allen 1995). Hildebrand et al. (1986) suggested that increased POX activity in resistant plants may allow the plant to detoxify peroxides and therefore sustain less tissue damage than susceptible plants. POX is also involved in lignin synthesis in cell walls. Lignification can be beneficial to the plant because it serves to strengthen and reinforce cell walls (Fincher and Stone 1986). The synthesis of lignin in response to insect feeding may provide cell wall reinforcement and thereby increase the plant’s tolerance to insect feeding.
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