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Differential induction of superoxide dismutase in downy mildew-resistant and -susceptible genotypes of pearl millet

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Differential induction of superoxide dismutase (SOD) in downy mildew-resistant and -susceptible genotypes of pearl millet (*Pennisetum glaucum*) was observed on inoculation with *Sclerospora graminicola*. SOD activity was studied in resistant (IP18292) and susceptible (23B) pearl millet seedlings inoculated with *S. graminicola*. SOD activity increased by 2·3-fold in resistant seedlings upon inoculation. SOD activity was greatest in roots, with a specific activity of 3182 U per mg protein, after inoculation. SOD activity increased in all the resistant genotypes upon inoculation with *S. graminicola*. Native PAGE analysis showed four isozymes of SOD, three of which (SOD-1, -2 and -4) were Cu/Zn-SOD, whereas isozyme SOD-3 was Mn-SOD. This study also revealed increased intensity of all four isozymes of SOD in the resistant genotype upon inoculation. The involvement of SOD in pearl millet (host)–downy mildew pathogen interaction is discussed.

Keywords: downy mildew, host–pathogen interaction, isozymes, pearl millet, *Sclerospora graminicola*, superoxide dismutase

Introduction

Superoxide dismutases (SODs: EC.1·15·1·1) are metal-containing enzymes that catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide. SODs are localized in different subcellular compartments which can scavenge the superoxide radical at the site of formation, thus minimizing the damage to cellular components (Bowler *et al*., 1992). Three types of SOD (Cu/Zn-SOD, Fe-SOD and Mn-SOD) have been identified, and are classified according to the metal at the catalytic site and their sensitivity to cyanide and hydrogen peroxide, as well as their cellular location. Of the three types, the Cu/Zn-SODs are the most abundant in higher plants, and are localized both in chloroplast and cytosol, whilst Mn-SODs are localized in the mitochondrial matrix and Fe-SODs in the chloroplasts (Bowler *et al*., 1992). In plants, exposure to photo-inhibitory light, ozone or other environmental conditions that cause oxidative stress increase superoxide anion ($O_2^{\cdot-}$) levels (Runeckles & Vaartnou, 1997). To date the protective role of SOD in plants has been explored by transgenic approaches, primarily through over-expression or by correlation of SOD over-expression to the degree of oxidative stress resistance (Scandalios, 1997). Some work has been done on the role of SOD during plant infection by pathogenic organisms such as fungi, bacteria and viruses. SOD activity increased in incompatible host–pathogen interactions as observed with potato tuber and the oomycete *Phytophthora infestans* (Doke, 1985), *Phaseolus vulgaris* and *Pseudomonas syringae* (Fridovich, 1986; Croft *et al*., 1990), tobacco and the downy mildew pathogen *Peronospora tabacina* (Edreva *et al*., 1991) and coffee and its rust fungus *Hemileia vastatrix* (Daza *et al*., 1993). Pearl millet (*Pennisetum glaucum*) is an important cereal crop of the arid and semi-arid tropics of the world. *Sclerospora graminicola* is an obligate biotroph that causes downy mildew disease of pearl millet (Jeger *et al*., 1998). Most of the popular F1 hybrids of pearl millet are highly susceptible to this disease. Although several genotypes are known to possess resistance to downy mildew disease (Williams *et al*., 1981), it is not durable. Earlier studies from this department have shown increased activity of lipoxygenase, phenylalanine ammonia lyase, peroxidase, β-1,3-glucanase and RNAase enzymes in downy mildew-resistant pearl millet genotypes on inoculation with *S. graminicola* (Nagarathna *et al*., 1992, 1993; Sreedhara *et al*., 1995; Kini *et al*., 2000; Shivakumar *et al*., 2000). SOD is known to play a role in downy mildew disease resistance of cucumber (Yun *et al*., 1995). The present study was conducted to test the possible involvement of SOD in pearl millet resistance to downy mildew disease.

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Materials and methods

Collection of seed samples

The seeds of downy mildew-resistant gentoypes 7042R, IP18292, IP18293, IP18294, IP18295, IP18296, IP18297, IP18298 and P-310-17 and susceptible genotypes 23B, HB3, 7042S, 700651 and 'Kalukombu' were collected from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India, and from the Project Co-ordinator, All India Co-ordinated Pearl Millet Improvement Programme (AICPMIP), Pune, India.

Seedling inoculation and sampling

Seeds were germinated on discs of moist blotter paper in Petri dishes at 25 ± 2°C for 2 days. *Sclerospora graminicola* was maintained on its susceptible host (HB3 genotype of pearl millet) under glasshouse conditions. A zoospore suspension of 4×10^4 zoospores mL^{-1} was prepared in sterile distilled water and used to dip-inoculate 2-day-old-seedlings (Safeeulla, 1976).

Determination of SOD activity

The seedlings (1 g) were macerated to a fine paste with acid-washed sand in a mortar at 4°C using 3 mL of 50 mM potassium phosphate buffer (pH 7·0). The homogenate was centrifuged at 15 000 × ***g*** for 15 min at 4°C (Himac centrifuge, Hitachi, Japan). The supernatant was used as the enzyme source (Dhindsa *et al*., 1981).

The activity of SOD was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) using the method of Beauchamp & Fridovich (1971). The 3 mL reaction mixture contained 50 mM phosphate buffer, pH 7·8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0·1 mM EDTA, and 0–150 μL enzyme extract. Riboflavin was added at the end and the tubes were shaken and placed 30 cm below a light source consisting of two 15 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 15 min. The reaction was stopped by switching off the light and the tubes were covered with a black cloth. The absorbance of the reaction mixture was read at 560 nm. A nonirradiated reaction mixture did not develop colour and served as control. There was no measurable effect of diffused room light. Preliminary experiments to determine SOD activity were carried out in test tubes, but this was expensive and time-consuming, and only a few samples could be assayed at a time. Microplate assay was therefore adopted. This is a simple, rapid and cost-effective means for screening many samples at a time. Absorbance was read using an ELISA reader (MR 5000 Dynatech, Guernsey, Great Britain) at 540 nm. Percentage inhibition of NBT reduction was plotted as a function of the enzyme extract used in the reaction mixture. From the graph, the volume of enzyme extract corresponding to 50% inhibition of the reaction was read and considered as one enzyme unit (Beauchamp & Fridovich, 1971). Protein content in extracts was estimated by the dye binding method (Bradford, 1976) using bovine serum albumin (Sigma Diagnostics, St Louis, USA) as the standard.

Electrophoretic banding pattern and identification of different isozymes of SOD

Superoxide dismutase activity is most reliably detected using an *in situ* staining technique on native PAGE (Beauchamp & Fridovich, 1971). Essentially, gels that have been dark-incubated in a solution containing NBT and riboflavin are exposed to white light, which catalyses the formation of superoxide from riboflavin. The gel turns uniformly blue except in areas where SOD activity is located, which remain colourless. Isozymes of SOD were separated by anodic electrophoresis on 7·5% polyacrylamide vertical slab gels incorporating a 5% stacking gel (Laemmli, 1970). Enzyme extracts (15 μg protein) were loaded onto the gels. Following electrophoresis (50 A, 4°C), gels were stained for SOD activity. Gels were incubated in the dark for 20 min at room temperature (25°C) in a solution containing 50 mM sodium phosphate buffer (pH 7·8), 0·1 mM EDTA, 3 mM riboflavin and 0·25 mM NBT, and were then rinsed twice in distilled water, placed on a glass sheet and illuminated for 10 min under a 200-W lamp placed 40 cm above the gel.

Different isozymes of SOD were distinguished by their differential sensitivities to inhibitors. The gels were incubated in staining solution supplemented with 2 mM KCN or 3 mM H_2O_2. The activity of Cu/Zn-SOD is inhibited by cyanide and H_2O_2, whilst Mn-SOD is insensitive to these treatments and Fe-SOD is sensitive only to H_2O_2 (Pitcher *et al*., 1992).

Statistical analysis

All the quantitative estimations of SOD were based on three separate experiments, each with three replicates. Values are expressed as mean ± standard error.

Results

Temporal changes in SOD activity in pearl millet seedlings inoculated with *S. graminicola*

The temporal changes of SOD activity during infection of resistant (IP18292) and susceptible (23B) pearl millet seedlings by *S. graminicola* were studied (Fig. 1). The inoculated samples were drawn at hourly intervals up to 24 h. The seedlings treated with distilled water served as control. A differential SOD activity was observed before and after inoculation of seedlings in both the genotypes. In uninoculated control seedlings, SOD activity reached a peak at 6 h and 4 h in resistant and susceptible genotypes, respectively. Thereafter, there was a decline in the activity up to 12 h. From 12 h, the activity remained almost the same up to 24 h in both genotypes. However, upon inoculation, SOD activity increased in the resistant genotype

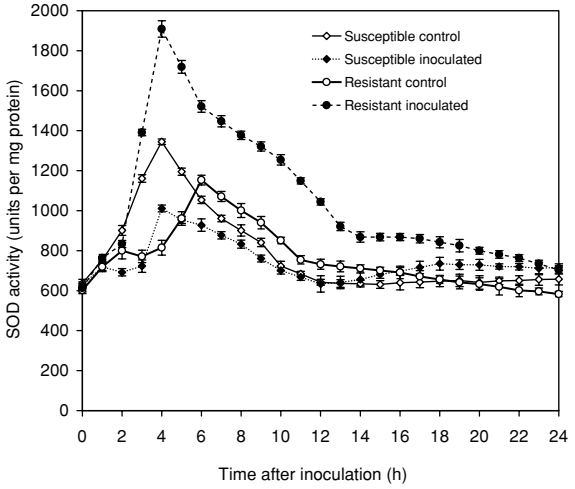

Figure 1 Superoxide dismutase (SOD) activity at different time intervals in seedlings of downy mildew susceptible (23B) and resistant (IP18292) genotypes inoculated with zoospore suspension of *Sclerospora graminicola* with respective uninoculated controls. Values are means of triplicate samples of three separate experiments. Bars indicate standard errors.

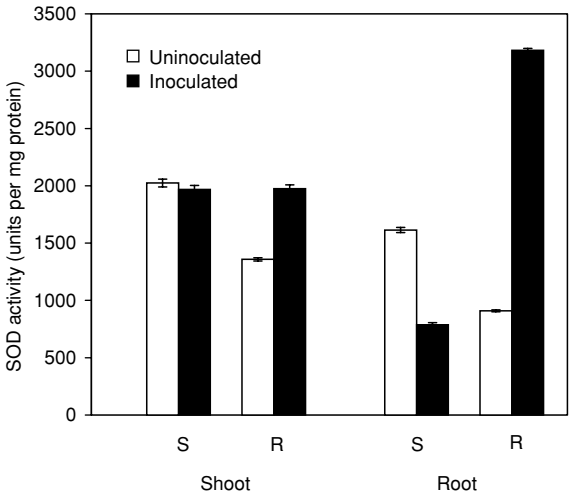

Figure 2 Superoxide dismutase (SOD) activity in different parts of seedlings of susceptible (S) (23B) and resistant (R) (IP18292) pearl millet genotypes inoculated with zoospore suspension of *Sclerospora graminicola* (black bars) with respective uninoculated controls (white bars). At 4 h post-inoculation, shoot and root were excised and enzyme was extracted. Values are means of triplicate samples of three separate experiments. Bars indicate standard errors.

compared with the control. The increase in SOD activity in resistant seedlings was observed within 2 h of inoculation and reached a maximum at 4 h. The increase observed after inoculation was 2·3-fold in the resistant genotype over the uninoculated control 4 h after inoculation. In contrast, there was no remarkable increase in SOD activity in the susceptible genotype after inoculation. SOD activity decreased in the susceptible genotype after inoculation until 12 h, compared with the uninoculated control. However, beyond 12 h, SOD activity increased slightly compared with that of the resistant genotype after inoculation.

SOD activity in different parts of seedlings

Tissue-specific SOD activity was assessed in different parts of pearl millet seedlings after inoculation with *S. graminicola* (Fig. 2). The shoot and root regions of the seedlings were excised from resistant (IP18292) and susceptible (23B) genotypes before and after inoculation with the pathogen, and the SOD activity was determined.

In the uninoculated seedlings of resistant and susceptible genotypes, SOD activity was greater in the shoot compared with the root region. In resistant seedlings a 3·5-fold increase in enzyme activity was noticed in the root region after inoculation. A slight increase in enzyme activity (1·45-fold) was also noticed in the shoot region. In contrast, the enzyme activity decreased in both root and shoot regions of the inoculated susceptible genotype, the decrease being greater in the root region.

SOD activity in different genotypes of pearl millet

Superoxide dismutase activity was analysed in different genotypes of pearl millet after inoculation with *S. graminicola* (Fig. 3). In the resistant genotypes, an increase in enzyme activity over that of the uninoculated control seedlings at 4 h after inoculation was recorded in 7042 R, IP18292, IP18293, IP18294, IP18295, IP18296, IP18297, IP18298 and P-310-17. In susceptible genotypes 23B, HB3, 7042S, 700651 and 'Kalukombu', a decrease in enzyme activity was observed 4 h after inoculation. The percentage increase was greatest in IP18292 (142%) and least in genotype 7042 R (52·2%). The highest percentage decrease (–43%) was observed in 23B, the most susceptible genotype, and the lowest (–5·9%) in 700651.

Electrophoretic banding pattern of SOD

The protein samples of resistant (IP 18292) and susceptible (23B) genotypes before and after inoculation with the downy mildew pathogen were electrophoresed on native polyacrylamide gels. Native gel electrophoresis analysis of SOD showed four SOD isozymes, designated 1–4, in order of increasing migration. These four SOD bands were found in resistant and susceptible genotypes, both before and after inoculation (Fig. 4). This preliminary experiment established that band intensity is proportional to the enzyme activity of the resistant and susceptible uninoculated and inoculated samples. The resistant genotype showed increased intensity of all the bands upon inoculation with the pathogen. Three of the four bands – SOD-1, SOD-3 and SOD-4 – were prominent. Differential staining of SOD for identification of isozymes revealed that the only enzyme that was resistant to cyanide and hydrogen peroxide was isozyme 3. It is likely that SOD-3 is Mn-SOD. The remaining ones were sensitive to both

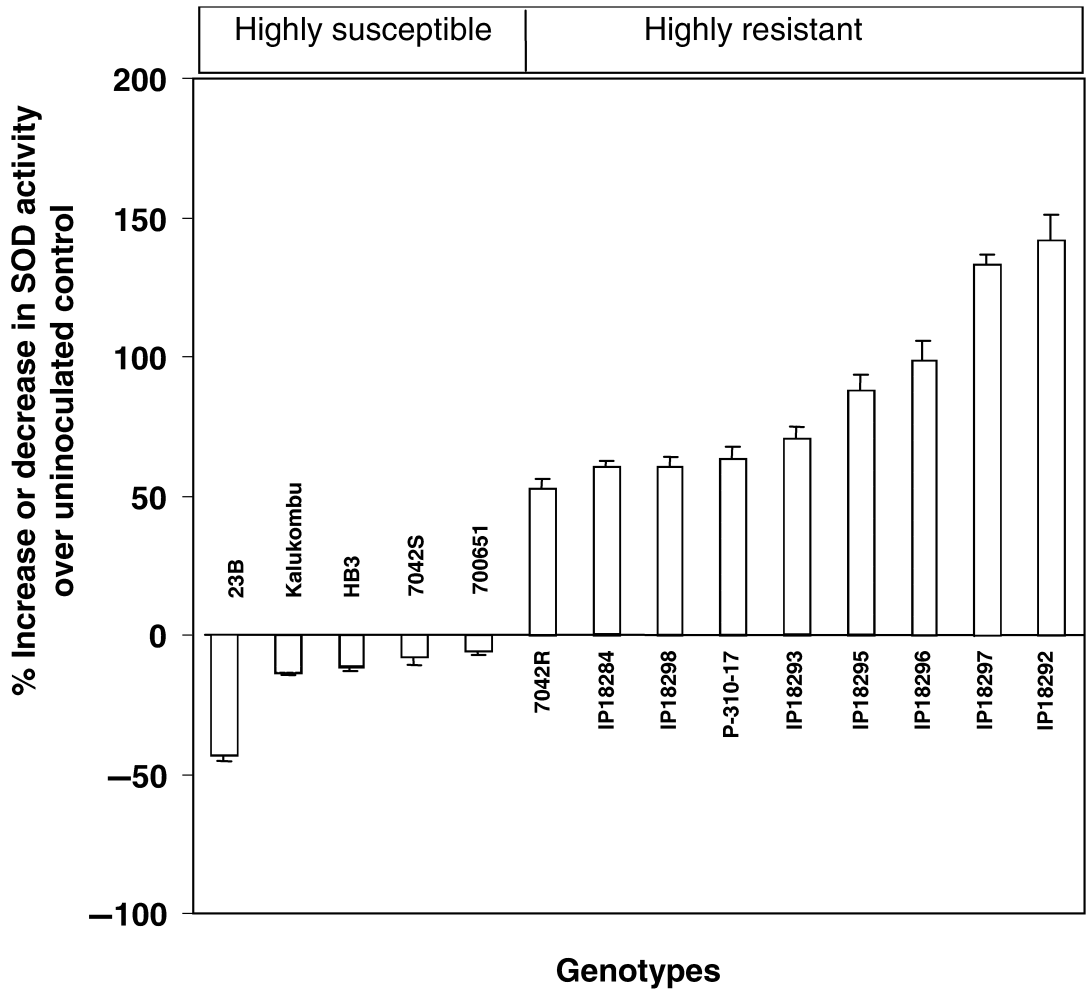

Figure 3 Superoxide dismutase (SOD) activity in seedlings of different genotypes of pearl millet. Pearl millet genotypes susceptible (23B, HB3, 7042S, 700651 and Kalukombu) and resistant (7042R, IP18292, IP18293, IP18294, IP18295, IP18296, IP18297, IP18298 and P-310-17) to downy mildew were analysed for enzyme activity in 2-day-old seedlings 4 h after inoculation with *Sclerospora graminicola*. The values are means of triplicate samples of three separate experiments. Bars indicate standard errors.

inhibitors, indicating that they were isozymes of Cu/Zn-SOD (Fig. 5).

Discussion

Plants are subjected to multiple stresses which generate active oxygen species (AOS). AOS have both beneficial and harmful effects on cells. SOD is known to catalyze the production of hydrogen peroxide (H_2O_2) by scavenging superoxide radicals ($O_2^{\cdot-}$). In this study, the role of SOD in pearl millet against downy mildew disease stress has been investigated.

This is the first report on SOD in relation to the pearl millet-downy mildew host–pathogen interaction. In this system, SOD was studied in resistant and susceptible genotypes with or without *S. graminicola* infection.

SOD activity in uninoculated control seedlings of pearl millet, with different degrees of resistance to downy mildew, depends upon the host genotype used. But on inoculation with *S. graminicola*, a clear-cut differential induction of SOD activity was noticed between resistant and susceptible pearl millet genotypes. This suggests that the enzyme may play a role in downy mildew disease development.

In the resistant genotype IP18292, an increase in SOD activity was observed after inoculating the seedlings with *S. graminicola*. In contrast, in the susceptible inoculated genotype 23B, the enzyme activity was lower than the control value in early time intervals. However, a slight increase was noticed 12 h after inoculation. Earlier reports indicated the induction of SOD activity in plants infected with a pathogen (Doke, 1985; Fridovich, 1986; Croft *et al*., 1990; Edreva *et al*., 1991 Daza *et al*., 1993). The results presented here show an increase in SOD activity in a resistant genotype upon inoculation with the pathogen *S. graminicola*. The enhanced SOD activity might increase oxidative stress due to increased production of H_2O_2 (Tenhaken *et al*., 1995). SOD also catalyses the generation of hydroxyl radicals ($OH^{\cdot}$) from H_2O_2 which

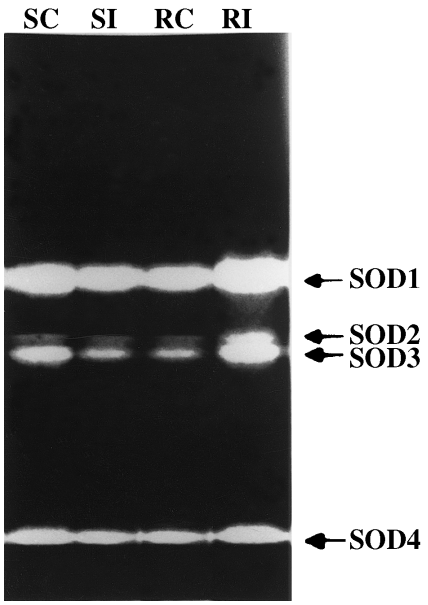

Figure 4 Non-denaturing polyacrylamide gel electrophoresis (7·5%) stained for superoxide dismutase (SOD) of susceptible (23B) and resistant (IP18292) genotypes with and without inoculation with *Sclerospora graminicola*. Fifteen micrograms of crude protein was loaded per lane. The achromatic bands observed on blue background indicate the enzyme activity and are numbered in the order of increasing relative mobility. Four SOD bands were observed: SC, susceptible uninoculated control; SI, susceptible inoculated; RC, resistant uninoculated control; RI, resistant inoculated.

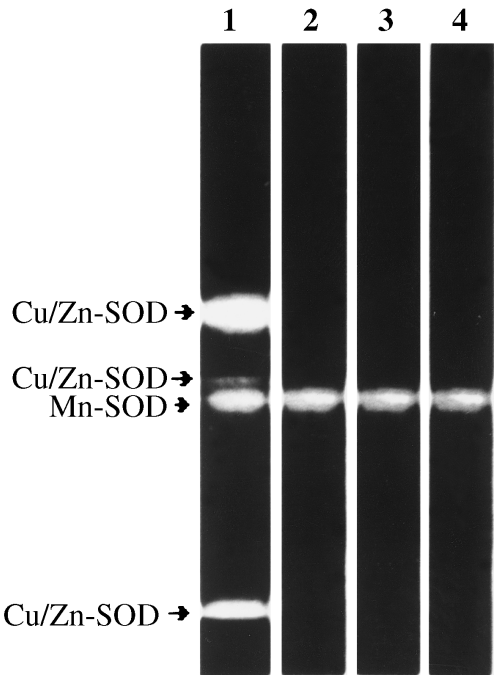

Figure 5 Identification of superoxide dismutase (SOD) isozymes using nondenaturing polyacrylamide gel electrophoresis (7·5%). Fifteen micrograms of crude protein was loaded per lane and the gel was stained for superoxide dismutase. Isozymes were differentiated based on their sensitivity to inhibitors. Lane 1, no inhibitor; lane 2, with KCN; lane 3, with H_2O_2; lane 4, with KCN and H_2O_2.

causes oxidative damage. Increased activity of the enzyme has been reported to induce cell dysfunction and death. Moreover, H_2O_2 has been implicated not only in triggering hypersensitive cell death but also in limiting the spread of cell death by induction of cell protectant genes in surrounding cells. H_2O_2 has also been shown to inhibit the growth and viability of diverse microbial pathogens (Wu *et al*., 1995). The oxidative potential of H_2O_2 also contributes to plant cell wall strengthening during plant pathogen interactions through peroxidase-mediated cross-linking of proline-rich structural proteins and phytoalexin biosynthesis during oxidative burst.

The induction of enzyme activity after inoculation is greater in roots than in shoots in a resistant genotype. It is also evident that in the susceptible genotype the enzyme activity in roots and shoots reduced after infection. Roots are one of the susceptible sites in pearl millet for infection by zoospores of *S. graminicola* (Subramanyna *et al*., 1983). Kong *et al*. (2000) has also reported induced SOD activity in roots of *Pinus massoniana* after infection with *Pislithus tinctorius*. Infection by phytoplasmas in *Paulownia fortunei* also lowered SOD activity in roots compared with those of healthy plants (Zhao *et al*., 1995). Hence, higher SOD activity in the root portion of the resistant seedlings recorded in the present study further suggests their role in defence against the invading pathogen.

In the present study, it has been established that SOD activity increases in all the resistant genotypes after inoculating the seedlings with *S. graminicola*, but in all the susceptible genotypes, a decrease in the enzyme activity was observed. SOD activity was higher in downy mildew-resistant genotypes of cucumber than in susceptible ones (Yun *et al*., 1995). Likewise cotton (*Gossypium hirsutum*) varieties resistant to verticillium wilt (*V. dahliae*) showed increased SOD activity, whilst susceptible ones showed either an insignificant increase or a slight decrease (Zhu *et al*., 1995).

Earlier reports indicated that peroxidase (POD), lipoxygenase (LOX) and phenylalanine ammonia lyase (PAL) activities increase in resistant genotypes after inoculation (Nagarathna *et al*., 1992, 1993; Sreedhara *et al*., 1995). The induction of these enzymes enhances the production of the superoxide anion ($O_2^{\cdot-}$). POD has been shown to be able to oxidize NAD(P)H in the presence of molecular oxygen with subsequent production of $O_2^{\cdot-}$ (Vianello & Macri, 1991). $O_2^{\cdot-}$ may also be generated by LOX action (Roy *et al*., 1994). It is possible that $O_2^{\cdot-}$ produced by POD and LOX is scavenged and elevated levels of H_2O_2 are produced as a consequence of SOD action. The potential accumulation of H_2O_2 by SOD activity may have several important effects on the host–pathogen interaction. Hence, an increase in SOD activity may be observed in resistant genotypes after inoculation.

Native-PAGE studies revealed differential expression of four SOD isozymes upon inoculation with the pathogen. The resistant genotype showed increased intensity of all

four bands. This indicates that all the isoforms are involved in defence against the pathogen. The isozymes were distinguished by differential sensitivities to KCN and H_2O_2. This study revealed two major isozymes and one minor isozyme of SOD identified as Cu/Zn-SOD, and one major isozyme as Mn-SOD.

It is proposed that upon inoculation in resistant genotypes H_2O_2 accumulates due to SOD activity. This may inhibit the growth and viability of the pathogen, directly suppressing attempted invasion. This in turn may impart resistance to pearl millet against downy mildew disease in resistant genotypes. In contrast, a slight increase in enzyme activity occurs too late in the susceptible genotypes. From these studies it appears that SOD is involved in imparting resistance to pearl millet against downy mildew disease.

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