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Role of Superoxide Dismutase and Peroxidase Isozymes in Pigeonpea during Wilt Disease

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Abstract

Superoxide dismutase (SOD) and peroxidase (POX) are important antioxidant enzymes which scavenge superoxide ion and hydrogen peroxide, respectively. These isozymes were analysed in fusarium wilt resistant (ICPL 87119, ICP 8863 & Vaishali) and susceptible (ICP 2376 & T15-15) genotypes of pigeonpea at 0 day after infection (DAI), 5 DAI and 10 DAI in infected and non-infected tissues. The intensity of SOD isozymes was higher in resistant genotypes than the susceptible ones at pre-infection stage. While, SOD 3 was detected only in resistant genotypes and disappeared in susceptible genotypesat 5 DAI, it may be correlated with decreasing SOD activity in susceptible genotypes. Further, POX 3 was only present in infected and non-infected resistant genotypes at 5 DAI. However, at 10 DAI, two isoforms were observed in all the genotypes. The intensity of POX 1 was increased in infected resistant genotypes compared to susceptible genotypes and non-infected genotypes. These results indicated the specific gene expression of SOD and POX in resistant genotypes, and depression in susceptible genotypes might be the cause for resistance or susceptibility.

Keywords: Fusarium; Isozymes; Resistant; Pigeonpea; Wilt

Abbreviations

DAS: Days After Sowing; DAI: Days After Infection; EDTA: Ethylenediaminetetraacetic Acid; NBT: Nitro Blue Tetrazolium; POX: Peroxidase; PVP: Polyvinylpyrrolidone; SOD: Superoxide Dismutase; TEMED: Tetramethylethylenediamine

Introduction

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is one of the major grain legume crops of tropics and sub tropics. It finds an important place in the farming systems adopted by small and marginal farmers in a large number of developing countries because it is drought resistant, a cheap source of protein in the diet and a source of fuel and foliage for livestock [1,2]. The main constraints in boosting the yield of the crop are its susceptibility to diseases, insects and other physiological stresses. Pigeonpea is known to be affected by more than hundred pathogens. However, Fusarium wilt caused by *Fusarium udum* Butler is the most important disease of pigeonpea worldwide [3]. Host resistance has been the most effective and efficient strategy for the control of plant diseases. Considerable progress has been made over the past few years in understanding the mechanisms of disease resistance or susceptibility [4] and it has been established that resistance to any pathogen depends on plant metabolism [5]. Enzyme coding genes are fairly easy to map because of their superior genetic properties. The existence of multiple forms of isozymes in plants has been recognized with the relationship of individual isozymes to specific plant disease resistance [6-8]. Superoxide dismutase (SOD) and peroxidase (POX) are important antioxidant enzymes which scavenge superoxide ion and hydrogen peroxide, respectively. The peroxidase isozymes which increase in activity during infection function to inhibit pathogen growth, perhaps through participation in biosynthesis of phenolic compounds or by direct inhibition of fungal growth, for example, through reactions involving inorganic ions [9]. Considering these observations, the present study was conducted to determine the role of superoxide dismutase and peroxidase isozymes in wilt resistant and susceptible genotypes of pigeonpea during wilt disease.

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Table 1: Superoxide dismutase (SOD) isozyme banding pattern in roots of pigeonpea genotypes at pre and post infection stage.

Isozyme	Susceptible		Resistant		
	ICP 2376	T_{15-15}	ICPL 87119	Vaishali	ICP 8863
Pre-infection stage (15 DAS or 0 DAI)					
SOD-1	+	+	+	+	+
SOD-2	-	-	-	+	-
SOD-3	+	++	+	+	+
SOD-4	+	+	+	+	+
SOD-5	+	++	+	+	+
Non-infected at post infection (10 DAI)					
SOD-1	-	+	+	+	+
SOD-2	+	++	+	+	+
Infected at post infection (10 DAI)					
SOD-1	++	+	+	-	+
SOD-2	++	++	++	++	++

(+present, -absent)

Table 2: Superoxide dismutase (SOD) isozyme banding pattern in leaves of pigeonpea genotypes at pre and post infection stage.

Isozyme	Susceptible		Resistant		
	ICP 2376	T_{15-15}	ICPL 87119	Vaishali	ICP 8863
Pre-infection stage (15 DAS or 0 DAI)					
SOD-1	+	+	+	+	+
SOD-2	+	++	++	++	+
SOD-3	+	+	++	++	++
Non-infected at post infection (5 DAI)					
SOD-1	+	+	+	+	+
SOD-2	-	-	+	+	-
SOD-3	+	+	+	+	+
SOD-4	+	+	-	-	-
SOD-5	++	+	+	+	+
SOD-6	+	+	-	-	-
SOD-7	++	++	+	+	++
Infected at post infection (5 DAI)					
SOD-1	+	+	+	+	+
SOD-2	++	+	+	+	++
SOD-3	-	-	+	+	+
SOD-4	++	++	+	+	++
SOD-5	+++	++	+	+	++
Non-infected at post infection (10 DAI)					
SOD-1	+	+	+	+	+
SOD-2	+	+	+	+	+
SOD-3	+	+	++	++	++
Infected at post infection (10 DAI)					
SOD-1	+	+	+	+	+
SOD-2	+	+	+	+	+
SOD-3	+	+	++	++	++

(+present, -absent)

Material and Methods

Growing of pigeonpea genotypes and infection with *Fusarium udum*

The seeds of three fusarium wilt resistance genotypes (Vaishali, ICPL87119 and national check ICP 8863) and two susceptiblegenotypes (T15-15 and national check ICP 2376) were procured from the Pulse Research Station, Navsari Agricultural University, Bharuch. Resistant and susceptible genotypes were selected based on screening of genotypes in wilt sick plot. Pigeonpea genotypes were raised in germination papers under glass bottlesat Department of Plant Molecular Biology andBiotechnology, N. M. College of Agriculture, Navsari Agricultural University, Navsari during 2011-12. Plants (15 days after sowing, DAS) of all the five genotypes were infected with *Fusarium udum* by mechanical method as described by Swami et al. [10].

Isozyme study

Isozymes were analyzed at three stages: (i) pre-infection [15 daysafter sowing (DAS)], (ii) post-infection [5 days afterinfection (DAI) i.e. 20 DAS and (iii) 10 days after infection (DAI) i.e. 25 DAS]. Fresh leaf samples for were collected from secondupper leaf while roots were taken at only at 0 and 10 DAI to remain the same plant for all the stages (0, 5 and 10 DAI) of analysis. Infected and non-infected leavesand roots were washed with sterilized Milli Q water and analyzed in duplicate for isozymes.

Five hundred milligram leaves were homogenized with a pre-chilled mortar and pestle under ice cold condition in 2.5ml of extraction buffer, containing 50 mM sodium phosphate buffer (pH 7.4) with 1mM EDTA and 1% (w/v) polyvinylpyrolidone (PVP). The homogenates were centrifuged at 10,000 rpm for 20min and supernatant was used for SOD and POX isozymes [11]. Electrophoresis was carried out on GENEI vertical electrophoresis unit using 1mm gel. Electrophoresis was performed at 30mA until tracking dye moved at bottom. Enzyme extracts (100µg protein) were loaded for each isozyme and mixed with 2µl tracking dye. Protein concentration was estimated by method of Lowry et al. [12].

Superoxide dismutase isozymes

Isozymes of SOD were separated on 10% non-denaturing polyacrylamide gels. After electrophoresis, SOD isoforms were visualized by following the method described by Mahatma et al. [13]. Gels were stained in 50mM sodium phosphate buffer, pH 7.8 containing 0.24mM NBT and 28µM riboflavin for 20min in the dark followed by immersing in 50mM sodium phosphate buffer, pH 7.8 containing 28mM TEMED, which were then exposed to a light source at room temperature until white bands were appeared in blue background.

Peroxidase isozymes

Peroxidase isozymes were separated on 10% non-denaturing polyacrylamide gels and visualized by following the method described by Mahatma et al. [14]. The gel was incubated in 100ml sodium phosphate buffer (0.025M, pH 6.0) containing 100µl of 30% H_2O_2, for min with gentle shaking. Followed by o-dianisidine (50mg dissolved in 1ml methanol) was added and kept in dark with occasional shaking until bands were appeared. Gels were scanned with scanner.

Results and Discussion

Total five SOD isozymes (SOD 1, SOD 2, SOD 3, SOD 4 & SOD 5) were observed in roots at pre-infection (15 DAS or 0 DAI) stage (Table 1). Among all five isoforms SOD 2 was present in resistant genotype Vaishali. The intensity of SOD 3 & SOD 5 was more in

Table 3: Peroxidase (POX) isozyme banding pattern in roots of pigeonpea genotypes at pre and post infection stage.

Isozyme	Susceptible		Resistant		
	ICP 2376	T_{15-15}	ICPL 87119	Vaishali	ICP 8863
Pre-infection stage (15 DAS or 0 DAI)					
POX- 1	+++	+++	+++	+++	+++
POX- 2	+	+	+	+	+
POX- 3	++	+	+	+	++
POX- 4	+	+	+	+	+
POX- 5	+	+	+	+	+
Non-infected at post infection (10 DAI)					
POX- 1	++	++	++	+	++
POX- 2	++	++	++	+	++
POX- 3	-	+	+	+	+
POX- 4	+	+	+	+	+
Infected at post infection (10 DAI)					
POX- 1	++	++	++	++	++
POX- 2	++	++	++	++	++
POX- 3	+	+	+	+	+
POX- 4	+	+	+	+	+

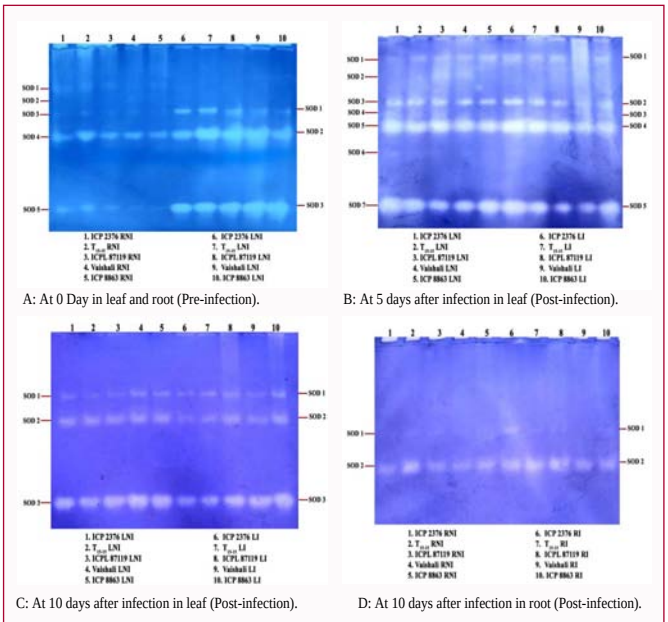
(+present, -absent)

Table 4: Peroxidase (POX) isozyme banding pattern in leaves of pigeonpea genotypes at pre and post infection stage.

Isozyme	Susceptible		Resistant		
	ICP 2376	T_{15-15}	ICPL 87119	Vaishali	ICP 8863
Pre-infection stage (15 DAS or 0 DAI)					
POX- 1	+	++	++	+++	++
POX- 2	+	+	+	++	+
POX- 3	-	-	+	+	+
Non-infected at post infection (5 DAI)					
POX- 1	+	+	+	+	++
POX- 2	+	+	++	+	+
POX- 3	-	-	+	+	-
Infected at post infection (5 DAI)					
POX- 1	++	++	++	+++	+++
POX- 2	+	+	-	-	-
POX- 3	-	-	++	+	+
POX- 4	+	+	-	-	-
Non-infected at post infection (10 DAI)					
POX- 1	+	+	+	+	+
POX- 2	+	+	+	+	+
Infected at post infection (10 DAI)					
POX- 1	+	+	++	++	++
POX- 2	+	+	+	+	+

(+present, -absent)

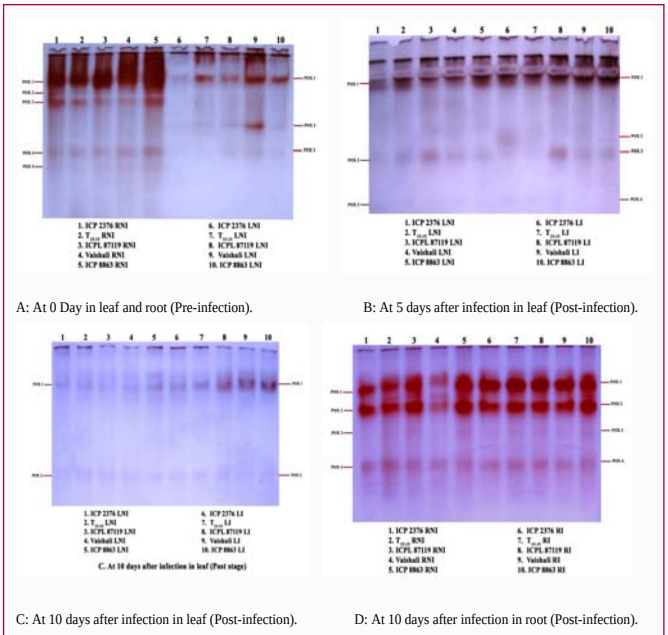
susceptible genotype T_{15-15} than the other genotypes. At 10 DAI, two different isozymes (SOD 1 & SOD 2) were observed in both infected and non-infected genotypes. The SOD 1 was not detected in non-infected susceptible genotype ICP 2376 but it was induced in infected one with high intensity. On the other hand, resistant genotype Vaishali lacks one isozyme (SOD 1) in infected ones. Isozymes from



A: At 0 Day in leaf and root (Pre-infection). B: At 5 days after infection in leaf (Post-infection).

C: At 10 days after infection in leaf (Post-infection). D: At 10 days after infection in root (Post-infection).

Figure 1: Superoxide dismutase isozyme in leaves and roots of wilt resistant and susceptible pigeonpea genotypes at pre and post-infection with *Fusarium udum*. Wilt resistance genotypes (Vaishali, ICPL87119 and national check ICP 8863) and two susceptible genotypes (T15-15 and national check ICP 2376).
NB: RNI: Root Non-Infected; RI: Root Infected; LNI: Leaf Non-Infected; LI: Leaf Infected.



A: At 0 Day in leaf and root (Pre-infection). B: At 5 days after infection in leaf (Post-infection).

C: At 10 days after infection in leaf (Post-infection). D: At 10 days after infection in root (Post-infection).

Figure 2: Peroxidase isozyme in leaves and roots of wilt resistant and susceptible pigeonpea genotypes at pre and post-infection with *Fusarium udum*. Wilt resistance genotypes (Vaishali, ICPL87119 and national check ICP 8863) and two susceptible genotypes (T15-15 and national check ICP 2376).
NB: RNI: Root Non-Infected; RI: Root Infected; LNI: Leaf Non-Infected; LI: Leaf Infected.

infected roots were expressed intensely than the non-infected ones, indicating enhanced levels of these isozymes in roots.

Native PAGE analysis of leaves at 0 DAI showed the presence of three different isozymes (SOD 1, SOD 2 & SOD 3) in all the genotypes, where the intensity of these isozymes was more in resistant genotypes

than the susceptible ones (Table 2 and Figure 1). At 5 DAI, seven different isozymes (SOD 1, SOD 2, SOD 3, SOD 4, SOD 5, SOD 6 and SOD 7) were observed in non-infected genotypes. Among the 7 isoforms, SOD 4 and SOD 6 were absent in resistant genotypes whereas, SOD 2 was absent in susceptible genotypes. In the infected genotypes only five isoforms of SOD were observed. Interestingly, SOD 3 was detected only in resistant genotypes and disappeared in susceptible genotypes; it may be correlated with decreasing SOD activity in susceptible genotypes at 5 DAI.

At 10 DAI, three SOD isozymes were observed in all the genotypes. The intensity of SOD 3 isozyme was more in infected and non-infected leaves of resistant genotypes than the susceptible ones.

Native PAGE analysis of POX at pre-infection (15 DAS or 0 DAI) showed the presence of five isoforms (POX 1- POX 5) in roots of all pigeonpea genotypes (Table 3). The intensity of POX 1 was much higher than the other isoforms. At 10 DAI, four isoforms of POX was observed in non-infected pigeonpea genotypes except ICP 2376 where POX 1 was absent but present in infected genotype. No specific isoform as observed in resistant or susceptible genotypes (Figure 2). Native PAGE analysis of leaves at pre-infection (15 DAS or 0 DAI) showed three isoforms (POX 1- POX 3) in the all pigeonpea genotypes (Table 4). Among all isoforms, POX 3 was absent in ICP 2376 and intensity of other two bands were less.

At 5 DAI, three isoforms were observed in non-infected genotypes whereas four isoforms were observed in infected genotypes. Among all isoforms POX 2 & POX 4 was appeared due to infection in susceptible genotypes (ICP 2376 & $T_{15\text{-}15}$) but absent in resistant genotypes (ICPL 87119, Vaishali, ICP 8863). The intensity of bands was more in infected genotypes. Moreover, POX 3 was only present in infected and non-infected resistant genotypes. At 10 DAI, two isoforms were observed in all the genotypes. The intensity of POX 1 was increased in infected resistant genotypes compared to susceptible genotypes and non-infected genotypes. Appearance of specific isoform (POX 3) suggested its possible involvement in wilt resistance and higher activity of enzyme. The intensity of POX 1 at 10 DAI may be correlated with higher activity of resistant genotypes in present study (data not shown). The absence of this isoform (POX 3) at pre-infection stage and 5 DAI in susceptible genotypes may also support its implication in imparting resistance to wilt disease in pigeonpea. Increased peroxidase activity and induction of new isoenzyme have been observed in cotton bolls inoculated with *R. solani* [15]. Similarly, zymograms of SOD and POX in the resistant genotypes of taro, with *Phytophthora* leaf blight infection, showed increased activity for anodal isoform of SOD and increased expression and/or induction of either POX 1 or POX 2 isoforms of POX. While, in susceptible genotype, expression of the above isoforms was faint for SOD and nearly absent for POX under both blight free and induced blight conditions [8]. These results indicated the specific gene expression of SOD and POX in resistant genotypes, and depression in susceptible genotypes might be the cause for resistance or susceptibility. Induction of particular isoform of SOD and POX may also be important for the synthesis of certain substance(s) which may act as a barrier(s) to the spread of invading pathogens and led to the apparent conclusion of linkage of isozyme expression with wilt resistance in pigeonpea.

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