Mycopathologia (2006) 161: 37–42
DOI: 10.1007/s11046-005-0721-y

Peroxidase activity in soybeans following inoculation with *Phytophthora sojae*

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Received 7 December 2004; accepted in revised form 16 June 2005

Abstract

The effects of race-specific resistance as conditioned by *Rps* genes (*rps*, *Rps*1-k, *Rps*2, *Rps*3, *Rps*6) in two genetic backgrounds (Williams & Harosoy) on accumulation of soluble peroxidases were determined by a soybean peroxidase capture assay (SPCA) after inoculation with *P. sojae* races 2, 7, or 25. Peroxidase activity increased in all isolines during the 72 h after inoculation, but reactions varied depending on time after inoculation, genetic background, *Rps* gene and *P. sojae* race. Peroxidase activity was higher in race-specific resistant than in susceptible reactions at 72 h. after inoculation, except for plants with the *Rps*2 gene which confers a unique form of root resistance in addition to the whole plant race-specific resistance. Williams isolines had larger increases in peroxidase activity than Harosoy isolines when data were averaged across *Rps* genes, and was most evident when plants were inoculated with race 2. When soybeans were inoculated with race 7 *Rps*1-k resistant plants had the highest increase in peroxidase activity, but *Rps*2 susceptible plants had a significantly higher peroxidase activity than plants with *rps*, *Rps*3, and *Rps*6 that were also susceptible. Results from inoculations with race 25 were somewhat different, *Rps*2 resistant plants had the highest increase in peroxidase activity; however, plants with the *Rps*3 or *Rps*6 gene that were also resistant did not have a significantly higher peroxidase activity than susceptible plants with the *rps* or *Rps*1-k gene.

Key words: Peroxidase, soybean, Phytophthora, susceptible, Rps gene, resistance, race-specific resistance, Phytophthora root rot

Introduction

In the United States and Canada Phytophthora root rot is one of the most devastating diseases of soybeans [1]. Understanding the nature of soybean resistance to Phytophthora root rot is important for soybean production. One of the factors associated with disease resistance in many crops has been peroxidase activity [2–6]. Peroxidases are enzymes whose main function is to oxidize molecules using hydrogen peroxide (H_2O_2) or elemental oxygen as a donor. The study of the relation of peroxidases with disease resistance of soybeans to Phytophthora root rot can provide important information for plant breeders and plant pathologists.

The association of peroxidases with disease resistance or susceptibility to pathogens has been reported for many crops. Many of these have compared peroxidase activity in resistant and susceptible reaction but very few have used near isogenic lines to make these comparisons. High peroxidase activity has been associated with resistance in tomato against *Fusarium oxysporum* f. sp. *lycopercisi* [7] and in pepper to *Phytophthora capsici* [2]. On the other hand, Dalisay and Kuc [8] found that treating cucumber leaves with *Colletotrichum lagenarium* induced an increase in peroxidase

activity, however a positive correlation was not evident between increased peroxidase activity and disease resistance. None of these studies involved near isogenic lines. In wheat near isogenic lines for resistance and susceptibility to *Puccinia graminis* f. sp. *tritici*, resistant plants had higher peroxidase activity than susceptible plants 9 days after inoculation [5, 6]. Graham and Graham [3], treated Williams and Williams 79 soybean seedlings with *Phytophthora sojae* race 1 cell wall glucan and found that peroxidase induction occurred in a non-race-specific manner. The use of cotyledon tissue for inoculation in only one genetic background did not allow the determination of the role of peroxidases in race-specific and non-race-specific resistance.

The purpose of this study was to determine the effect of race-specific and non-race-specific resistance on accumulation of soluble peroxidases in soybean seedlings after inoculation with *Phytophthora sojae*. Race-specific resistance conditioned by *Rps* (Resistance to *Phytophthora sojae*) genes in near-isogenic lines of two different genetic backgrounds was studied. The two genetic backgrounds were Williams and Harosoy that are regularly used by soybean *Phytophthora* researchers. Near isogenic lines (isolines) of both cultivars were selected since the Williams cultivar also has non-race-specific resistance whereas the Harosoy cultivar does not [9]. Isolines with the race-specific *rps*, *Rps*1-k, *Rps*2, *Rps*3, and *Rps*6 genes were used in each genetic background. *Phytophthora sojae* races 2, 7, or 25 were inoculated to each isoline.

Materials and methods

Soybean (*Glycine max* (L.) Merr.) near-isogenic lines with Williams and Harosoy genetic backgrounds were grown in 'rolled-towel-ragdolls' as described by Ashton [10] in growth chambers at 25° C with alternate periods of 13 h of light and 11 h of darkness. Five near-isogenic lines with the *rps*, *Rps*1-k, *Rps*2, *Rps*3, and *Rps*6 race-specific genes for resistance to *P. sojae* from each genetic background were used. *Phytophthora sojae* Kaufmann and Gerdemann (Syn. *Phytophthora megasperma* f. sp. *glycinea* Kuan and Erwin) races 2, 7, and 25 were inoculated to eight seedlings of each isoline as described by Abney et al. [11]. These 3 races were selected because their interaction with the *Rps* genes used gave a diverse combination of susceptible and resistant interactions (Table 1). To determine the peroxidase activity due to inoculation, the *P. sojae* inoculum, wounded and non-wounded check plants also were tested for peroxidase activity. Peroxidase activity due to wounding was subtracted from the increase in peroxidase activity in inoculated plants. After inoculation, plants were maintained in the growth chamber under the conditions previously described.

In this study, peroxidase activity was measured at 1, 24, 48, and 72 h after inoculation. Because of the effectiveness of the inoculation technique used, susceptible plants start dying at about 72 h after inoculation; therefore, all the physiological activities of these plants are considerably reduced or stopped completely. Thus comparisons of peroxidase activity of susceptible versus resistant plants after 72 h could be misleading. Tissue samples were collected from each control, wounded and inoculated plant by cutting a five mm section of hypocotyl that included the point of treatment. Extraction was done by grinding each sample using a Teflon pestle on a Wheaton overhead stirrer. Prior to the extraction, each tube was filled with 0.5 ml of double distilled water. Sections of mycelial mats of *P. sojae* were ground and treated similarly to the tissue samples.

Peroxidase activity of tissue extracts was determined using a soybean peroxidase capture assay (SPCA) as described by Vierling and Wilcox [12]. This technique utilizes a monoclonal antibody that binds peroxidases and therefore only captured peroxidases can oxidize the substrate used. Even though the antibody is a monoclonal antibody, it recognizes all forms of soybean peroxidase as well as recognizes peroxidases in other plant species (R.A. Vierling, unpublished).

Results

Peroxidase activity increased in all soybean isolines as a result of wounding and inoculation with *P. sojae*. Peroxidase activity was significantly higher in inoculated than in non-inoculated plants. No peroxidase activity was detected from samples of *P. sojae*; therefore, it is unlikely that *P. sojae* was the source of the increased enzyme activity. The increase in peroxidase activity in inoculated plants was affected by time, genetic background,

isoline, and *Phytophthora* race as demonstrated by the analysis of variance. The increase in peroxidase activity was greater with time, reaching a maximum 72 h after inoculation. Increased peroxidase activity was generally larger in Williams (cultivar with non-race-specific resistance) isolines than in Harosoy when data were averaged across the five *Rps* genes. Peroxidase activity in resistant interactions (race-specific resistance) was generally higher than in susceptible host pathogen interactions, especially at 72 h after inoculation. Peroxidase activity in non-treated seedlings was similar in all isolines; this is in contrast with results reported for other crops [4] where resistant plants had higher peroxidase activity than susceptible plants. When plants were wounded but not inoculated, there were no significant increases in peroxidase activity; however, at 72 h, plants with the *Rps*6 gene started to show greater increases in peroxidase activity than plants with the other genes. The levels of increase in peroxidase activity reported here are lower than the ones reported in other studies. This is because the majority of the studies to determine peroxidase activity have used guaiacol as substrate without purifying the peroxidase or removing the free radical species; therefore, the high readings they report probably is because non-enzymatic oxidation is also being measured. With the technique used for this study all the oxidation measured is the result of oxidation by peroxidases.

When isolines of both genetic backgrounds were inoculated with race 2, Williams isolines, in general, had larger increases in peroxidase activity than Harosoy isolines when data were averaged across the five *Rps* genes, especially 72 h after inoculation (Table 2). Soybean isolines with the *Rps*1-k, 2, 3, and 6 were resistant to race 2 (Table 1) and the increases in peroxidase activity of these isolines after inoculation with race 2 were larger than the susceptible plants. Because the highly significant differences between Williams and Harosoy isolines the data were not averaged across cultivars and the results are separated for each cultivar. All resistant plants (*Rps*1-k, *Rps*2, *Rps*3, *Rps*6) with Williams background had similar increase in peroxidase activity and only plants with the *Rps*6 gene did not have a significantly larger increase in peroxidase activity than the susceptible (*rps*). Increase in peroxidase activity of plants with Harosoy background was somewhat different, only *Rps*3 had a significantly larger increase in peroxidase activity than the susceptible plants (*rps*). They also were higher than plants with the *Rps*1-k (resistant). These results indicate that the genetic background significantly affected the increase in peroxidase activity when plants were inoculated with race 2.

When soybean plants were inoculated with race 7, Williams isolines generally had larger increases in peroxidase activity than Harosoy isolines when data were averaged across the five *Rps* genes, however the differences were not significant. The levels of increased peroxidase activity of all soybean isolines when inoculated with race 7 were very similar to the levels obtained when they were inoculated with race 2. Increases in peroxidase activity during the first 48 h after inoculation were similar for all isolines. At 72 h after inoculation isolines with the *Rps*1-k gene (resistant to race 7) had higher increase in peroxidase activity than all other isolines (susceptible to race 7). Plants with the *Rps*2 (susceptible) had significantly higher increase in peroxidase activity than plants with *rps*, *Rps*3, and *Rps*6 (also susceptible). The difference

Table 1. Interactions of the soybean *Rps* genes and *Phytophthora sojae* races used for determining peroxidase activity in soybean isolines inoculated with three races of *Phytophthora sojae*

Rps Genes	*P. sojae* races		
	PR-2	PR-7	PR-25
Rps	S	S	S
*Rps*1-k	R	R	S
*Rps*2	R	S	R
*Rps*3	R	S	R
*Rps*6	R	S	R

Table 2. Peroxidase activity (net increase) in soybean plants 72 h after inoculation with *Phytophthora sojae* race 2[x]

Rps genes	Peroxidase activity	
	Williams	Harosoy
Rps	0.432 a	0.334[y] a
*Rps*1-k	1.019 b	0.304 a
*Rps*2	1.048 b	0.504 ab
*Rps*3	0.785 b	0.797 b
*Rps*6	0.759 ab	0.623 ab
Average	0.809[z]b	0.512 a

[x] Average of 3 replications.
[y] Main effect means followed by the same letter are not significantly different (P 0.05) according to the LSD test.
[z] Main effect means followed by the same letter are not significantly different (P 0.05) according to the LSD test.

of plants with the *Rps*2 gene is consistent with observations of unique field reactions associated with this gene. These results support previous reports about the involvement of peroxidases in disease resistance [2–6].

When plants were inoculated with race 25, on the average Williams isolines had slightly larger increase in peroxidase activity than Harosoy isolines at all times, however these differences were not significant. Increase in peroxidase activity 1, 24, and 48 h after inoculation were similar in all isolines. Maximum values were obtained when peroxidase activity was determined 72 h after inoculation. There were significant differences between *Rps* genes when averaged across cultivars. *Rps*2 (resistant) was significantly higher than *rps* (susceptible) and *Rps*3 and 6 (resistant) but not higher than *Rps*1-k (susceptible). There was no significant difference between *rps*, *Rps*1-k, *Rps*3, and *Rps*6, however *Rps*1-k was higher than *Rps*3 (resistant) and *Rps*6, (also resistant). The uniqueness of the reactions involving race 25 especially with plants with the *Rps*1-k gene deserves further consideration.

The regression analysis of increased peroxidase activity on time after inoculation (h) with races 2, 7, or 25 verified the involvement of peroxidases on disease resistance. No significant differences were found during the first 48 h after inoculation. At 72 h after inoculation resistant interactions generally had higher peroxidase activity. Figure 1 shows the regression curves of peroxidase activity in soybean plants when inoculated with race 7. Plants with the *Rps*1-k (the only gene used that confers resistance to race 7) had a significantly larger increase in peroxidase activity than all other plants at 72 h after inoculation. *Rps*2, which is susceptible to race 7, was also significantly higher than all other genes. The regression curves of peroxidase activity in soybean plants inoculated with races 2 and 25 (not shown) were similar to inoculations with race 7, the only difference was a relatively high peroxidase activity of plants with the *Rps*1-k when inoculated with race 25 (susceptible reaction).

The regression curves of peroxidase activity in plants with each of the genes for resistance to *P. sojae*, following inoculation with each of the 3 races further confirmed the role of peroxidases in disease resistance. No significant differences were found in any of the interactions during the first 48 h after inoculation. Plants with the *rps* gene

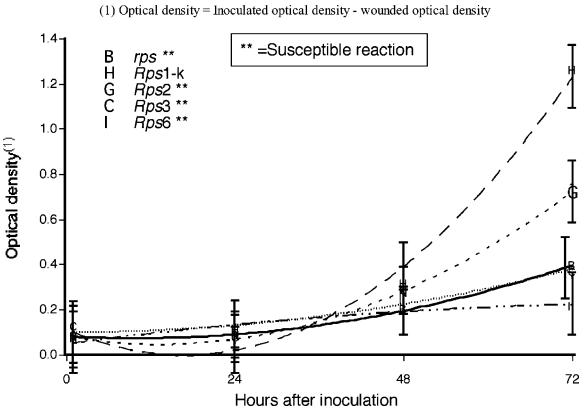

Figure 1. Regression curves of peroxidase activity in susceptible and resistant soybean plants after inoculation with *Phytophthora sojae* race 7.

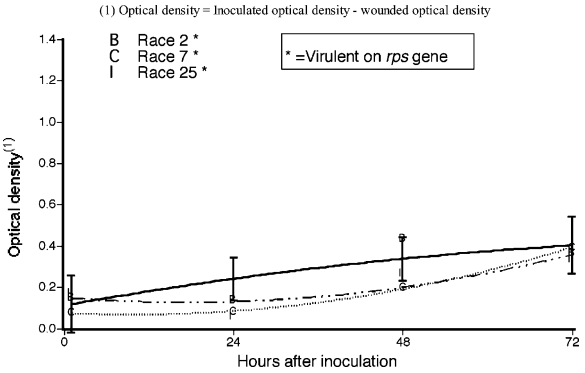

Figure 2. Regression curves of peroxidase activity in soybeans with the *rps* gene after inoculation with *Phytophthora sojae* races 2, 7, or 25.

(susceptible to all races) had the lowest increase in peroxidase activity (Figure 2). Plants with the *Rps*1-k gene had a significantly larger increase in peroxidase activity when inoculated with race 7, a resistant interaction, than when inoculated with races 2 and 25 (Figure 3). Inoculations with race 2, also a resistant interaction, induced larger increase in peroxidase activity than race 25, (susceptible interaction), however the difference was not significant. Peroxidase activity of soybeans with the *Rps*3 and *Rps*6 genes was similar to peroxidase activity of plants with the *Rps*1-k. The reaction of plants with the *Rps*2 gene was unique because the increase in peroxidase activity was identical in susceptible and resistant reactions at all times (Figure 4). The increase in peroxidase activity when plants were inoculated with race 7

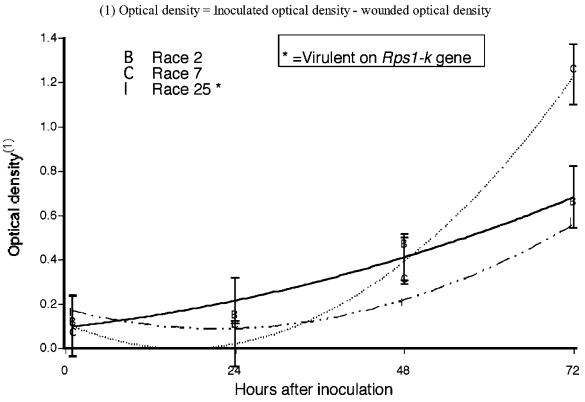

Figure 3. Regression curves of peroxidase activity in soybeans with the *Rps*1-k gene after inoculation with *Phytophthora sojae* races 2, 7, or 25.

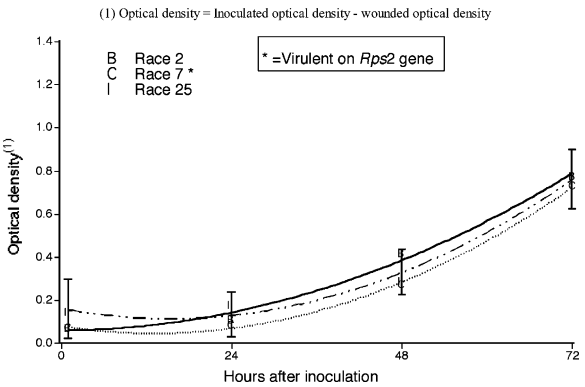

Figure 4. Regression curves of peroxidase activity in soybeans with the *Rps*2 gene after inoculation with *Phytophthora sojae* races 2, 7, or 25.

(Susceptible reaction) was as high as when plants were inoculated with races 2 and 25 (resistant reactions).

Generally, resistant interactions had larger increase in peroxidase activity than susceptible interactions. In some cases the differences were significant. These results confirm the involvement of peroxidases in disease resistance, but because the increase is not observed until resistance is evident peroxidases may not be the determinant factor for disease resistance.

Discussion

The main objective of this study was to determine the effect of race-specific and non-race-specific resistance on activity of soluble peroxidases in soybean seedlings inoculated with *P. sojae*. The inoculation technique used and the controlled conditions under which the experiment was conducted provided an excellent opportunity to study disease reactions that under field conditions can be variable. The use of the SPCA, a very sensitive and reliable technique, also allowed for the accurate measurement of peroxidase activity as it relates to disease resistance. This is the first study that includes near isogenic lines in two genetic backgrounds inoculated with different races of *P. sojae*. Despite all the research that has been done with peroxidases, very little information about peroxidase activity and disease resistance in soybeans is available. The results of this study provide a considerable amount of data that can be used by plant breeders, plant pathologists, and other researchers in breeding programs and understanding host–pathogen interactions.

Inoculation of soybean seedlings with *P. sojae* races 2, 7, and 25 induced an increase in peroxidase activity in all soybean isolines tested. It is unlikely that *P. sojae* is the source of the enzyme activity increase because in tests conducted using mycelia, no activity was detected. Wounding alone induced an increase in peroxidase activity as compared to plants that receive no wounding, however the increases in peroxidase activity due to wounding were very similar in all *Rps* genes tested. When plants were inoculated with *P. sojae*, the increase in peroxidase activity varied depending on genetic background, *Rps* gene tested, *P. sojae* race and time after inoculation.

In this study soybean isolines resistant and susceptible to *P. sojae* races 2, 7, and 25 were used to test the effect of time after inoculation, genetic background, *Rps* gene, and *P. sojae* race on increases in peroxidase activity of soybean seedlings. The highest peroxidase activity was obtained 72 h after inoculation. This time coincides with the time it takes under greenhouse conditions for the start of symptom development in susceptible plants. Also larger increases were generally observed in resistant plants, this is consistent with previous reports for other crops [2, 5, 7]. The fact that during the first 48 h after inoculation the increases in peroxidase activity were small and there were no significant differences between isolines is consistent with greenhouse reactions that show no differences between susceptible and resistant plants during the first hours after inoculation.

When averaged across the five *Rps* genes increase in peroxidase activity was larger in Williams isolines than in Harosoy. This was more evident when plants were inoculated with race 2, Williams isolines had a significantly larger increase in peroxidase activity than Harosoy especially 72 h after inoculation. The higher increase in peroxidase activity in Williams isolines may be related to non-race-specific resistance. Also the term tolerance has been used to describe some susceptible plants that have the ability to perform acceptably in the presence of *P. sojae* [9, 13] as is the case with isolines with Williams background. The increase in peroxidase activity in plants with the *Rps*2 gene was different than in plants with other genes. Plants with the *Rps*2 had similar increases in peroxidase activity in both susceptible and resistant reactions. These results verified observations about unique field reactions (root resistance) of plants with the *Rps*2 gene. These results indicate that peroxidases may be involved in root resistance in soybean plants with the *Rps*2 gene.

The emphasis of this study was to determine if there are differences in induced peroxidase activity at the site of infection. The results presented here demonstrate that genetic background, *Rps* gene and *P. sojae* race are factors that affect peroxidase activity. The largest increases in peroxidase activity were generally obtained in resistant reactions, at the same time that resistant and susceptible reactions were evident in greenhouse inoculations. This is an indication that peroxidases are important for disease resistance, however they are not the determinant factors. Similar results were reported by Seevers et. al. [6] in wheat inoculated with *Puccinia graminis* f. sp. *tritici*. They concluded that increase in peroxidase activity was not determinative of resistance but an end result of resistance. Both of these studies give addition reason to look at the role of induced peroxidase activity and resistance.

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