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Differences in the induction of the oxidative burst in compatible and incompatible interactions of soybean and *Phytophthora sojae*

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ABSTRACT

The effect of infection by *Phytophthora sojae* on the oxidative state in the soybean cultivars Nannong 493-1 (medium-resistant) and Hefeng 35 (susceptible) was studied. Pro-oxidant activity (H_2O_2 production and lipid peroxidation) and antioxidant systems (enzymatic activities of catalase and glutathione reductase, and low-molecular-weight antioxidants such as reduced ascorbate and glutathione) along with cell death and pathogenesis-related (PR) protein analyses were performed to examine their roles in establishing resistance or susceptibility. During pathogen infection, H_2O_2 accumulation was higher in the soybean cultivar Nannong 493-1 than in Hefeng 35. Cell death was more severe in Nannong 493-1 during the early infection period. However, the malondialdehyde levels were higher in Hefeng 35 than in Nannong 493-1 in the late experiment period. The inherent levels of the antioxidant systems were not consistently higher or lower in Nannong 493-1 than in Hefeng 35 under different pathogen infection duration or antioxidation parameters. The expression of the *PR* genes induced by pathogen infection varied with the cultivar and the pathogen-infection process. Exogenous treatment of soybean with one of three reductants (dithiothreitol, reduced ascorbate, or glutathione) facilitated the disease caused by *P. sojae* in both cultivars. In general, these results show that reactive oxygen species play an important role in the interactions of soybean and *P. sojae*.

1. Introduction

The production of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$), and the hydroxyl radical ($OH^{\cdot}$), via the consumption of oxygen in the oxidative burst is one of the earliest cellular responses following successful pathogen recognition. ROS are thought to be involved in the plant defense response [1–3]. Because hydrogen peroxide is long-lived and able to cross plant cell membranes, it acts as a diffusible and lasting signal in plant–pathogen interactions [4]. Avirulent pathogens, which are successfully recognized via the action of disease-resistance (R) gene products of the plant immune system, elicit a biphasic ROS accumulation that correlates with disease resistance [1]. In contrast, virulent pathogens, which avoid host recognition, induce only the transient, low-amplitude first phase of this response.

To minimize the damaging effects of ROS, plants have evolved various enzymatic and nonenzymatic mechanisms that can reduce oxidative stress by detoxifying harmful oxygen species. Hydrogen peroxide is broken down into water by catalase (CAT) (EC 1.11.1.6) and peroxidase (EC 1.11.1.7) [5,6]. An alternative and more effective detoxification mechanism against H_2O_2 operates in both chloroplasts and the cytosol [5,7]: the ascorbate–glutathione cycle, also referred to as the Halliwell–Asada pathway [7–9]. The levels of reduced ascorbate (AsA) [10] and reduced glutathione (GSH) [11] were found to be elevated in plants after pathogen attack or elicitor treatment. However, decreased AsA levels were reported in infected leaves of Xanthi-nc tobacco (NN genotype) following infection by tobacco mosaic virus [12].

Phytophthora root rot caused by *Phytophthora sojae* Kaufmann & Gerdemann, currently the most devastating disease of soybean (*Glycine max* [L.] Merr), causes severe losses in the soybean harvest worldwide [13,14]. 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 is the oxidative burst. The oxidative burst that has received the broadest scrutiny in plants is the release of H_2O_2 or $O_2^{\cdot-}$ during stimulation by pathogens. Studying the relationship between the

Abbreviations: AsA, reduced ascorbate; CAT, catalase; DHA, oxidized ascorbate; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HR, hypersensitive reaction; MDA, malondialdehyde; SAR, systemic acquired resistance.

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oxidative burst and the resistance of soybean to *Phytophthora* root rot can provide important information for plant breeders and plant pathologists to improve disease management strategies.

Associations of the oxidative burst with disease resistance or susceptibility to pathogens have been reported for many crops. Soybean cultivar Williams 82 suspension cultures exhibit an oxidative burst approximately 3 h after challenge with *Pseudomonas syringe* pv. *glycinea* [15]. Superoxide production was reported in potato tubers [16] and potato leaves [17] 1–4 h after inoculation with an incompatible race of *Phytophthora infestans*, an observation later supported by Jordan and DeVay [18]. Legendre and co-workers investigated the rapid release of H_2O_2 in suspension-cultured soybean cells stimulated with purified polygalacturonic acid, an elicitor [19]. However, very little is known about oxidative metabolism in soybean plants undergoing resistant or susceptible reactions to pathogens, in particular the hemibiotrophic pathogen *P. sojae*.

To investigate the possible role of the oxidative burst in the soybean–*P. sojae* interaction, particularly in soybean resistance to *Phytophthora* root rot, we examined the plant oxidative burst, cell death, changes in the enzymatic activities of CAT and glutathione reductase (GR), and transcript levels of *PR* genes in two host–parasite combinations (soybean cultivars Hefeng 35 and Nannong 493-1 interacting with *P. sojae*). Lipid peroxide was also measured to evaluate possible oxidative stress. The contents of glutathione (GSH and oxidized glutathione, GSSG) and ascorbate (AsA and oxidized ascorbate, DHA) were determined because they are water-soluble antioxidant molecules involved in the ascorbate–glutathione cycle. In addition, the effects of exogenous reductants on the interactions between soybean and *P. sojae* were analyzed.

2. Materials and methods

2.1. Biological material

Seeds of the soybean (*Glycine max* (L.) Merr.) cultivars Hefeng 35 and Nannong 493-1, gifts of Dr. G. Xing (National Center for Soybean Improvement, Nanjing, Jiangsu Province, China), were grown axenically in a greenhouse. The seedlings were maintained as previously described [20], and the first leaves from 12-day-old soybean seedlings were used in the following assays.

P. sojae isolate PS2 (M.J. Kaufmann & J.W. Gerdemann), which is virulent on Hefeng 35 and medium-virulent on Nannong 493-1, was isolated in China from soybean tissues showing root rot, and maintained on 10% V8 agar [13] at 25 °C in darkness. The conditions used for propagation of axenic-grown mycelia and zoospores were as previously described [21].

2.2. Inoculation technique

In this study, the leaf-drop inoculation method was performed as described [22] with the slight modifications noted below. First leaves of uniform size were excised from 12-day-old plants and placed on moist filter paper in Petri dishes with the adaxial surface upward. Fifty microliters of zoospore suspension (approximately 2×10^4 zoospores ml^{-1}) in 0.05% (v/v) Tween 20 were placed on the detached leaves. The Petri dishes were sealed with Parafilm and incubated at 25 °C in darkness for varying times. Control leaves were treated with sterile distilled water. The solutions on the leaves were carefully removed with a pipette, and the leaves were flash-frozen in liquid N_2 for biological analyses.

2.3. H_2O_2 assay

Soybean leaves were inoculated with zoospores and incubated for 8 h. Histochemical *in situ* detection of H_2O_2 in the leaves was performed using 3,3′-diaminobenzidine (DAB) staining following the protocols of Orozco-Cardenas and Ryan [23] and Thordal-Christensen et al. [24] with minor modifications. Briefly, the leaves were harvested and immediately vacuum-infiltrated for 20 min with phosphate-buffered saline, pH 7.4, containing 0.5% (w/v) DAB. The sample leaves were subsequently placed under light (2.8×10^3 lux) for 12 h and then boiled for 30 min in 80% ethanol. The intensity and patterns of DAB staining were assessed visually.

The H_2O_2 concentrations in soybean leaves were determined as described [25]. Briefly, intercellular wash fluid was obtained, and the H_2O_2 concentration in the fluid was measured based on peroxidase-mediated oxidation of Fe^{2+} followed by the reaction of Fe^{3+} with xylenol orange *o*-cresolsulfonephthalein 3′,3′-bis[methylimino]diacetic acid, sodium salt. This method is extremely sensitive and can be used to measure low levels of water-soluble hydroperoxide present in the aqueous phase. The data were normalized and expressed as micromoles per gram fresh weight.

2.4. Detection of cell death in leaves

Cell viability in soybean leaves was measured by (a) kinetics analysis using the Evans Blue method developed by Baker and Mock [26]; and (b) *in situ* detection using trypan blue staining [25]. The cell death in leaves of both cultivars was monitored at 2, 4, 6, 8, 12, and 24 h post-inoculation (hpi) using the Evans Blue method. The Evans Blue approach allows the rapid, reproducible quantification of the stain retained by dead cells. Briefly, for each measurement, five leaf discs (2 cm in diameter) were harvested from the inoculation site and placed in 0.25% Evans Blue (Sigma–Aldrich, St. Louis, MO, USA) for 20 min. Unbound dye was removed by extensive washing, and the leaf discs were finely ground with a mortar and pestle containing 100 μl of 1% SDS and 1 ml of sterile distilled water. The homogenate was centrifuged at $7200 \times g$ for 10 min, and the dye bound to dead cells was quantified by measuring the absorbance at 600 nm.

For the trypan blue assay, sample leaves were submerged in a trypan blue mixture (30 ml ethanol, 10 g phenol, 10 ml water, 10 ml glycerol, 10 ml lactic acid, and 10 mg trypan blue (Gibco-BRL, Cleveland, OH, USA)), placed in a boiling water bath for 3 min, left at room temperature for 1 h, transferred into chloral hydrate solution (2.5 g ml^{-1}), and boiled for 20 min to destain. Then the background was reduced through multiple changes of chloral hydrate solution. After equilibration with 50% (v/v) glycerol, the sample leaves were observed with a stereomicroscope.

2.5. Lipid peroxidation

At 1, 3, 6, 12, 24, and 48 hpi, the level of lipid peroxidation in the crude leaf extracts was spectrophotometrically assessed through the malondialdehyde (MDA) content, based on the MDA reaction with thiobarbituric acid described by Heath and Packer [27].

2.6. Analysis of ascorbate and glutathione

Soybean leaf samples were prepared for AsA, DHA, GSH, and GSSG analyses by homogenizing 1.5 g leaf material (fresh weight) in 10 ml of pre-chilled mega-phosphoric acid [28]. Then the supernatant was collected by centrifuging the homogenate at $22{,}000 \times g$ for 15 min at 4 °C and spectrophotometrically analyzed for ascorbate and glutathione according to the method of Zhang and Kirkham [28].

2.7. Assays of enzyme activities

For assays of CAT and GR, 0.5 g (fresh weight) of zoospore-inoculating leaf samples at 1, 3, 6, 12, 24, or 48 hpi were homogenized

and assayed according to Zhang and Kirkham [28]. For CAT, the decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm ($\varepsilon = 39.4\ M^{-1}\ cm^{-1}$) for 1 min. The 3-ml reaction mixture contained 50 mM phosphate buffer (pH 7.0), 15 mM H_2O_2, and 0.1 ml enzyme extract. The reaction was initiated with the addition of enzyme extract. GR activity was assessed by following the decrease in absorbance at 340 nm due to NADPH oxidation ($\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$) for 1 min. The reaction mixture consisted of 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, 0.1 M Na-phosphate buffer (pH 7.8), and 150 μl enzyme extract. The reaction was triggered by the addition of GSSG.

All spectrophotometric analyses were conducted in a DU-640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). All assays were performed in duplicate, and independent experiments were repeated at least three times with similar results. Error bars indicate mean ± SE.

2.8. Determination of transcript levels of pathogenesis-related genes

Semi-quantitative reverse transcriptase–polymerase chain reactions (RT–PCR) were performed for the *PR-4* and *PR-10* genes [29]. Zoospore suspension (30 μl) containing 1×10^3 zoospores was infiltrated into the mesophyll of soybean plant leaves with a 2.5-ml blunt-end syringe without a needle. Control seedlings were treated with sterile distilled water. At designated time points, the inoculated leaves were excised and snap-frozen in liquid N_2 for total RNA extraction using the TRIzol Reagent kit (Invitrogen, Carlsbad, CA, USA) as directed by the manufacturer. The integrity of the RNA was confirmed using agarose gel electrophoresis. Prior to cDNA synthesis, contaminating DNA was removed from all RNA samples used for RT–PCR by treatment with DNase I (TaKaRa Biotechnology, Dalian, Liaoning province, China) following the manufacturer's protocol. cDNA synthesis was performed using M-MLV reverse transcriptase (RNase H Minus) (Shanghai Promega, Shanghai, China). First-strand cDNA was synthesized from each sample using 1 μg of total RNA and oligo$(dT)_{15}$ primer (Promega). One microliter of each first-strand cDNA sample was subjected to PCR in a 25-μl reaction using the LA PCR kit (TaKaRa).

The *PR-4* and *PR-10* primers were from Graham et al. [29]. The primer sequences were: *PR-4* forward primer, 5′-CTC GTG GCC GTG ATT CTT GT-3′; *PR-4* reverse primer, 5′-GAG CAT CGA GGA TGG AGA GT-3′; *PR-10* forward primer, 5′-AGT TAC AGA TGC CGA CAA CG-3′; and *PR-10* reverse primer, 5′-CCT CAA TGG CCT TGA AGA GA-3′. The *β-tubulin* internal control gene was generated from 20 ng of soybean genomic DNA using the primers 5′-AAC CTC CTC CTC ATC GTA CT-3′ and 5′-GAC AGC ATC AGC CAT GTT CA-3′. PCR reactions were incubated in an MJ PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA) with the following program: 95 °C for 1 min, followed by 28–30 cycles of 95 °C for 15 s, 53 °C for 30 s, and 72 °C for 30 s, and a final extension of 72 °C for 2 min. The amplicon sizes of *PR-4*, *PR-10*, and *β-tubulin* were 246, 377, and 388 bp, respectively. The PCR fragments were visualized using Gel Doc 2000 software (Bio-Rad, Milan, Italy). All RT–PCRs were performed three times.

2.9. Effect of exogenous reductants on the interaction of soybean and P. sojae

The reductants dithiothreitol (DTT), AsA, and GSH (Sigma-Aldrich) were diluted to 1 mM with sterile distilled water. The zoospore concentration used here was approximately 6.7×10^4 zoospores ml^{-1}. Treatment A consisted of 30 μl of a 1:1 mixture of reductant solution and zoospore suspension. Treatment B comprised pre-treatment with 15 μl of zoospore suspension at 30 min, followed by treatment with an equal volume of reductant solution at the same site on the leaf. Control seedlings were treated with 30 μl of zoospore suspension diluted two-fold with sterile distilled water. All solutions were infiltrated using a blunt-end syringe into the mesophyll of soybean leaves. Each treatment was performed on at least ten seedlings, and each leaf received a treatment and a control infiltration on opposite sides. All treated seedlings were incubated at 25 °C. Biological replicates were performed at different times with independent sets of soybean plants and *P. sojae* zoospores.

3. Results

3.1. H_2O_2 burst in soybean leaves during interaction

The accumulation of ROS is characteristic of plant tissues undergoing the hypersensitive reaction (HR) [1]. The H_2O_2 levels in the leaf tissues were monitored at various times after inoculation with zoospores. DAB staining showed that infection with zoospores provoked rapid H_2O_2 accumulation in both susceptible and medium-resistant host plant leaves at 8 hpi (Fig. 1).

The total H_2O_2 content was determined in these leaves and non-inoculated leaves over time (Fig. 2). Three independent experiments were performed with similar results. In Nannong 493-1 leaves, an increase was observed by 1 hpi, with peaks at 1.5 and 8 hpi. In contrast, in Hefeng 35 leaves, the first peak appeared only at around 3 hpi. In both susceptible and medium-resistant host plant leaves, H_2O_2 accumulated in a biphasic manner within 10 h. However, the H_2O_2 content in Nannong 493-1 leaves was greater than in Hefeng 35 leaves within 10 h. These data clearly showed that pathogen infection can trigger H_2O_2 accumulation in both susceptible and medium-resistant host plants, and the induction of H_2O_2 in medium-resistant host plants was quicker and stronger than in susceptible host plants.

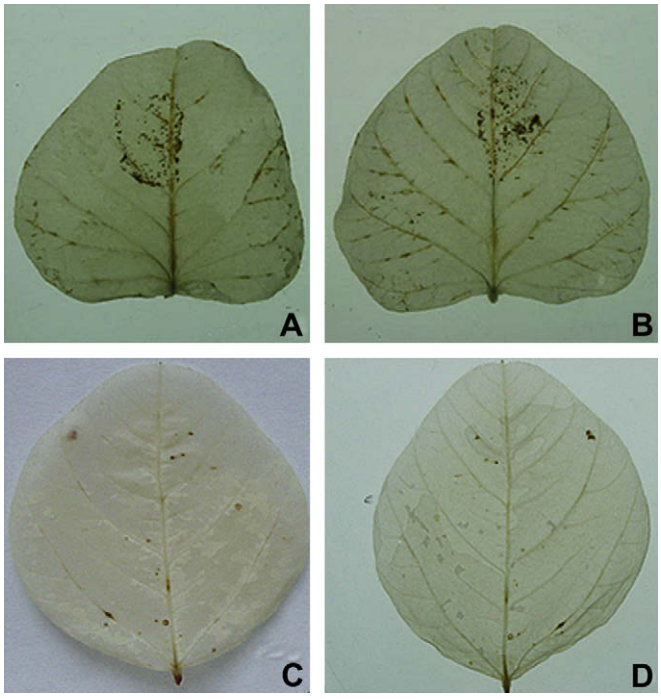

Fig. 1. Histochemical identification of H_2O_2 by DAB staining in soybean leaves. (A, B) Leaves at 8 h after inoculation with zoospores were stained by DAB. (C, D) Control leaves treated with sterile distilled water. (A, C) The leaves of susceptible cultivar Hefeng 35. (B, D) The leaves of medium-resistant cultivar Nannong 493-1.

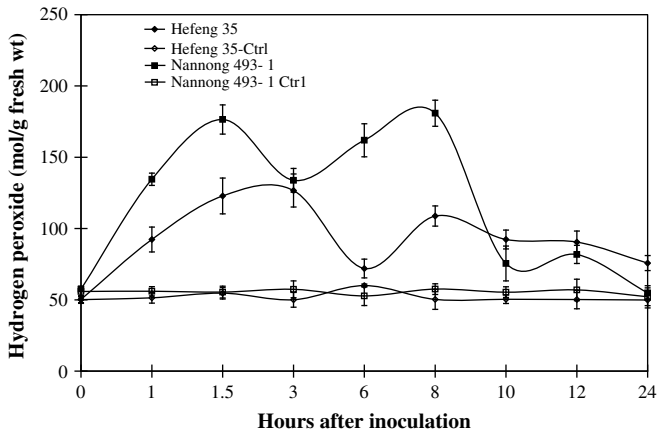

Fig. 2. Kinetics of hydrogen peroxide accumulation induced by the pathogen *P. sojae*. The concentration of H_2O_2 in the leaves of Hefeng 35 or Nannong 493-1 was measured at different intervals: 0 (uninfected), 1, 1.5, 3, 6, 8, 10, 12, 24 h after inoculation with zoospores. Ctrl, control leaves of either Hefeng 35 or Nannong 493-1 treated with sterile distilled water at each designated time point instead of zoospore suspension. Bars represent ±SE. Three replicates yielded the same result.

3.2. Induction of cell death in soybean leaves inoculated with pathogen zoospores

Since one of the direct results of ROS accumulation is the induction of cell death and the restriction of pathogen proliferation [30], cell death was monitored in soybean leaves inoculated with pathogen. Inoculation of 1×10^3 zoospores on either susceptible or medium-resistant host plant leaves reproducibly induced visible cell death, observed at 6, 8, and 10 hpi using trypan blue staining (Fig. 3). The cell death in medium-resistant host plant leaves was more severe than that in susceptible host plant leaves at 6 hpi. However, with increasing time, the only significant increase in the dead cell numbers resulting from infection by the pathogen occurred in Hefeng 35 at 8 and 10 hpi. In inoculated Nannong 493-1, there was little to no significant increase in the dead cell numbers at 8 or 10 hpi.

The kinetics of cell death in leaves of both cultivars was further investigated by staining with Evans Blue, which accumulated in dead cells over time. The inoculation of zoospores provoked cell death in Hefeng 35 and Nannong 493-1 leaves (Fig. 4). In the first 8 hpi, the cell death in medium-resistant host plant leaves (Nannong 493-1) was more severe than in susceptible host plant leaves (Hefeng 35). After 8 h, the extent of cell death in both plants was inverted, with the cell death in medium-resistant host plant leaves remaining steady, but with susceptible host plant leaves showing increasing cell death.

3.3. Lipid peroxidation

Lipid peroxidation, estimated by the MDA content, is an indicator of the prevalence of free-radical reactions in tissue. The lipid peroxidation in leaves of both soybean cultivars changed significantly after pathogen infection. The MDA content was much higher in Hefeng 35 than in Nannong 493-1 over time (Fig. 5). Pathogen infection caused increased MDA content in Hefeng 35, but decreased MDA content in Nannong 493-1 at the late stage of infection. These results fit with the hypothesis that pathogen infection can induce membrane–lipid peroxidation by means of activated oxygen species.

3.4. Concentrations of ascorbate and glutathione

In plant cells, the most effective detoxification mechanism against H_2O_2 is the ascorbate–glutathione cycle [7–9]. AsA and GSH are two very important ROS-scavenging antioxidants. The

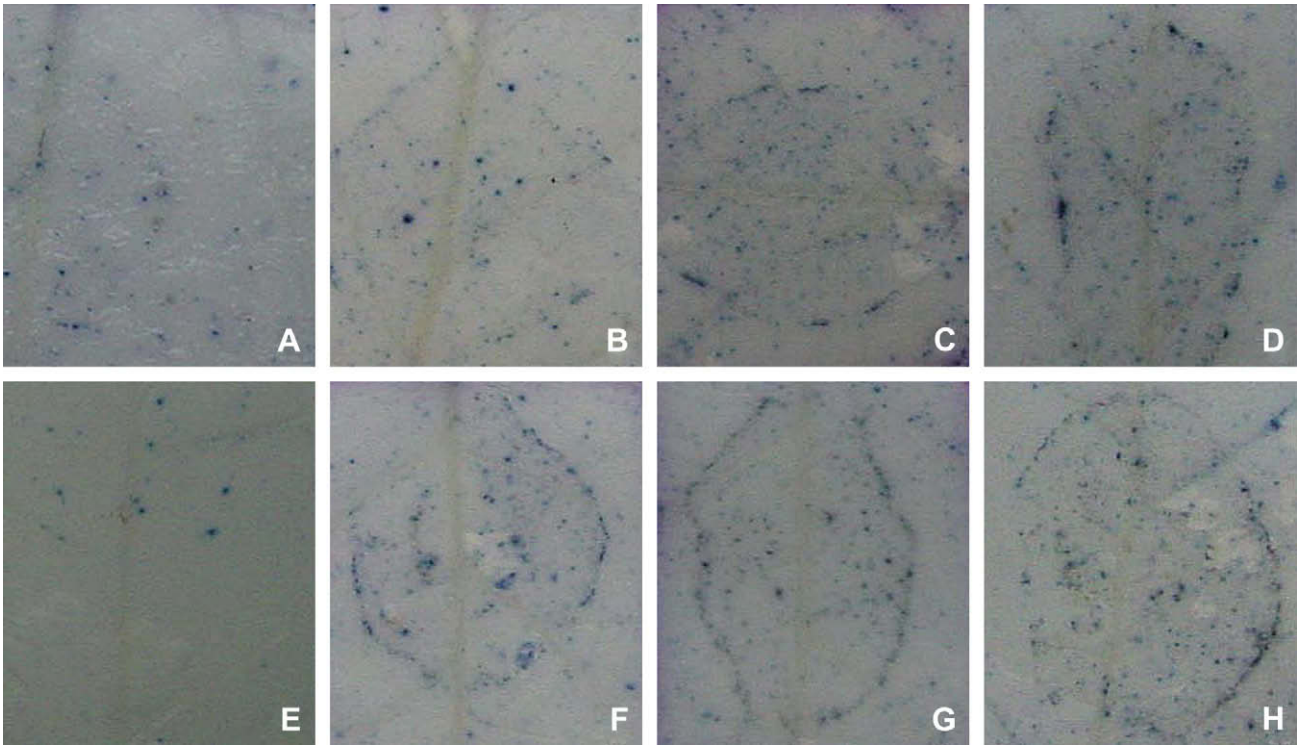

Fig. 3. Cell death detected *in situ* in soybean leaves infected by *P. sojae* zoospores. The top row (A–D) shows the leaves of susceptible cultivar Hefeng 35. The bottom row (E–H) shows the leaves of medium-resistant cultivar Nannong 493-1. (A, E) Control leaves treated with sterile distilled water. (B, F) Treated leaves stained with trypan blue at 6 h post-inoculation (hpi) with zoospore suspension. (C, G) Leaves stained with trypan blue at 8 hpi. (D, H) Leaves stained with trypan blue at 10 hpi.

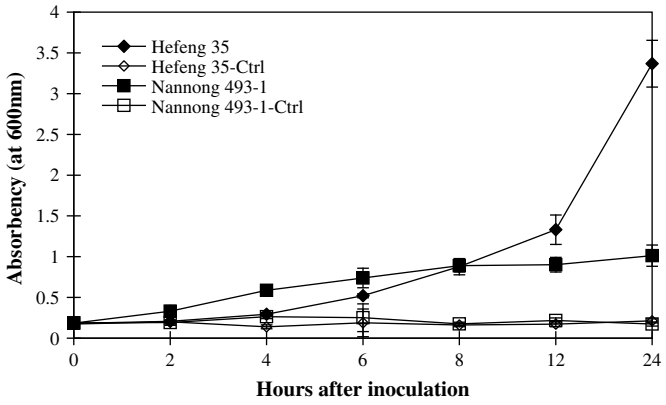

Fig. 4. Kinetics of cell death of soybean caused by *P. sojae*. Cell viability kinetics was measured by Evans Blue method and indicated at designated time points as absorbency at 600 nm for two cultivars. Ctrl, control leaves of either Hefeng 35 or Nannong 493-1 treated with sterile distilled water at each designated time point instead of zoospore suspension. Data presented are the means of three independent experiments and error bars represent the standard error (±SE).

antioxidant responses of the cultivars to the pathogen were examined by measuring the contents of both compounds. Compared to healthy plants, AsA content increased in both cultivars challenged with the pathogen over all post-inoculation period. At 1 hpi, this index was significantly higher in Nannong 493-1 than in Hefeng 35. However, at the other time points, the difference between them was insignificant (Fig. 6). Therefore, under infection conditions, Nannong 493-1 did not have consistently higher AsA contents than Hefeng 35. It is unclear why changes in DHA levels were not accompanied by changes in the AsA contents (Fig. 6).

A major function of GSH in the protection of cells against the toxic effects of free radicals is to keep the free radical-scavenging ascorbate in its reduced, and hence active form via the ascorbate–glutathione cycle. The GSH contents of Hefeng 35 and Nannong 493-1 under pathogen-infected conditions were not similar (Fig. 7). Pathogen infection increased the GSH level in Hefeng 35 by 1 hpi. The first transient peak of GSSG in Hefeng 35 appeared at 6 hpi, and a higher peak (maximum concentration 57.7 μM) appeared at 24 h after inoculation. In Nannong 493-1, the GSH contents changed in a distinctly biphasic manner within the experimental period, but the GSSG contents varied in a monophasic manner at 1–48 hpi, with a peak at 6 hpi.

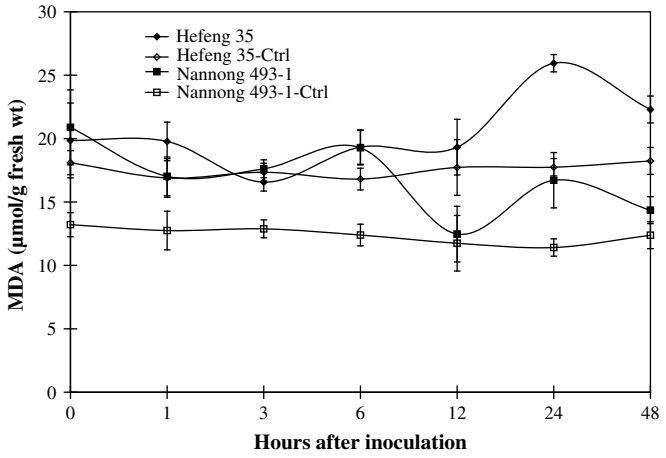

Fig. 5. Kinetics of MDA accumulation in soybean seedling leaves infected by *P. sojae*. The level of lipid peroxidation of soybean leaves at designated time points in both cultivars was measured in terms of MDA content. Ctrl, control leaves of either Hefeng 35 or Nannong 493-1 treated with sterile distilled water at each designated time point instead of zoospore suspension. Each value represents the mean of three replicates and bars represent ±SE.

3.5. Activities of ROS-related enzymes

Various ROS-scavenging systems, including CAT and GR, maintain ROS homeostasis in different compartments of plant cells [31].The oxidative stress measured by means of enzymatic activities may represent the severity of the interplay of plants with pathogens. These enzyme activities in soybean were influenced by PS2 infection, but there were quantitative or temporal differences in some of the observed changes between the compatible and incompatible interactions. CAT, the enzyme responsible for eliminating H_2O_2, was generally affected by pathogen infection. Hefeng 35 and Nannong 493-1 showed various relative CAT activities (the content in treated plants divided by the content in the corresponding control) during the same intervals, with the relative activities in Nannong 493-1 higher than in Hefeng 35 except at 6 hpi when the ratio between cultivars was opposite (Fig. 8). The relative CAT activities in Nannong 493-1 leaves increased rapidly after inoculation, reaching a first peak at 3 hpi and a second at 24 hpi. The observed changes in Hefeng 35 leaves were more moderate than those in Nannong 493-1 leaves, although the relative CAT activities peaked within the first 6 h (Fig. 8).

GR plays a crucial role in the protection of plants against oxidative stress by maintaining the levels of GSH. At the beginning of the experiment, Hefeng 35 had higher relative GR activities than Nannong 493-1 (Fig. 9), with a peak at 3 hpi. However, after 6 hpi, the relative GR activities in Nannong 493-1 increased rapidly, more than in Hefeng 35, peaking at 24 hpi, and then decreasing.

3.6. RT–PCR analysis of transcript levels of pathogenesis-related genes

RT–PCR was used to characterize the expression patterns of *PR* genes during plant infection. The *PR-4* and *PR-10* genes were chosen for analysis based on a previous report [29].

Following zoospore infection, the *PR* genes were activated in the leaves of both plant cultivars (Figs. 10 and 11). The expression of *PR-4* was downregulated as early as 1 h after inoculation in Hefeng 35 leaves, and its transcript levels continued to decrease for 3 h. However, strong upregulation was seen over the 6–12 hpi time frame, peaking at 12 hpi and falling off rapidly after 24 hpi. Similarly, the expression of *PR-10* was as low as in the control in the first 3 hpi, peaked at 6 hpi, and then began to decrease.

In comparison, in Nannong 493-1 leaves, the accumulation of transcripts of the two genes began as early as 1 h after inoculation, and the transcript levels continued to increase for at least 24 h. Although RT–PCR is not quantitative, the use of housekeeping genes such as *β-tubulin* as controls allows a general comparison of the transcript abundance at different infection time points.

3.7. Effect of exogenous reductants on the plant–pathogen interaction

After inoculation of leaves by infiltration, the leaves of Hefeng 35 exhibited large, water-soaked, necrotic lesions at 24 hpi, whereas Nannong 493-1 leaves showed such lesions at 48 hpi. The leaf lesion area of both plants was scored at 48 hpi for Hefeng 35 and at 96 hpi for Nannong 493-1. Statistical analysis of the lesion areas suggested that exogenous treatment of soybeans with reductants (DTT, AsA, and GSH) facilitated the infection of the pathogen in both cultivars (Figs. 12–14).

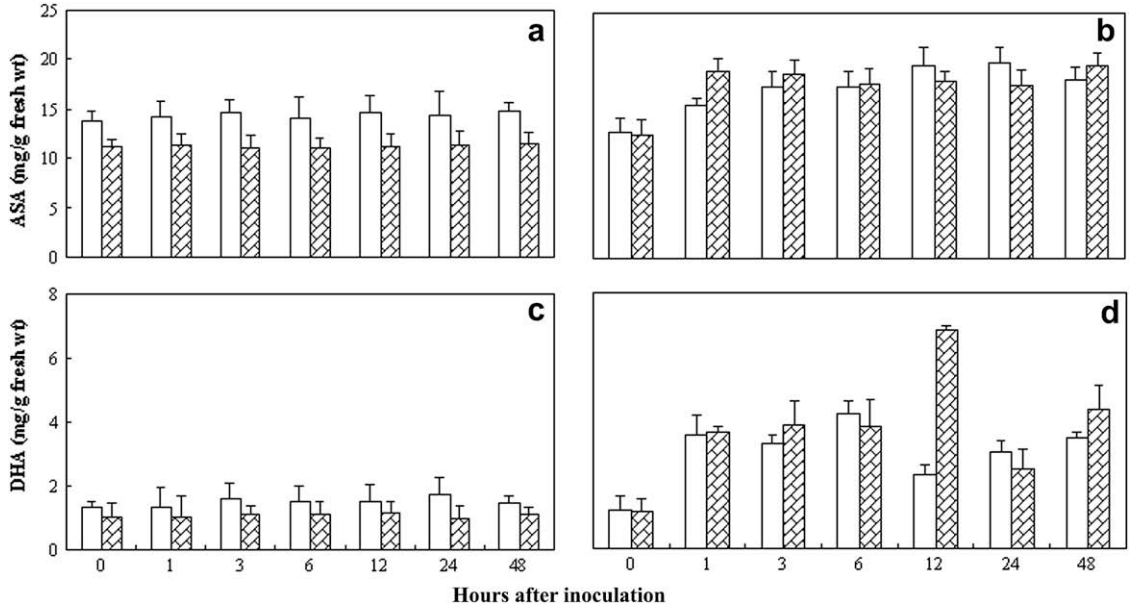

Fig. 6. Changes in reduced ascorbate (AsA) and the oxidized ascorbate (DHA) in leaves of Hefeng 35 (white columns) and Nannong 493-1 (gridded columns) infected by *P. sojae*. (a) and (b) show AsA content whereas (c) and (d) represent DHA content. (a, c) Control leaves treated with sterile distilled water. (b, d) Leaves treated with *P. sojae* zoospore suspension. Each value represents the mean of three replicates and bars represent the standard error (±SE).

4. Discussion

The rapid production of ROS in the apoplast in response to pathogens has been proposed to orchestrate the establishment of different defensive barriers against the pathogens. The production of ROS, including O_2^- and H_2O_2, has been widely observed in plant tissues [24,32–35] and suspension-cultured cell systems [4,19,36,37] associated with the expression of HR and systemic acquired resistance (SAR). Wang and co-workers [38] demonstrated that PB90, a novel protein elicitor from *Phytophthora boehmeriae*, can trigger HR and SAR in tobacco and non-heading Chinese cabbage. Subsequently, PB90-induced hypersensitive cell death of tobacco suspension cells was confirmed through the observation of apoptosis [39]. Melgar et al. [40] determined the effect of race-specific and non-race-specific resistance on the accumulation of soluble peroxidases in soybean seedlings after inoculation with *P. sojae*. However, little is known about the relationship of the oxidative burst with the resistance of soybeans to *Phytophthora* root rot.

The aim of the present work was to investigate the possible role of ROS production in *Phytophthora* root rot. We studied various biochemical parameters of oxidative metabolism during the

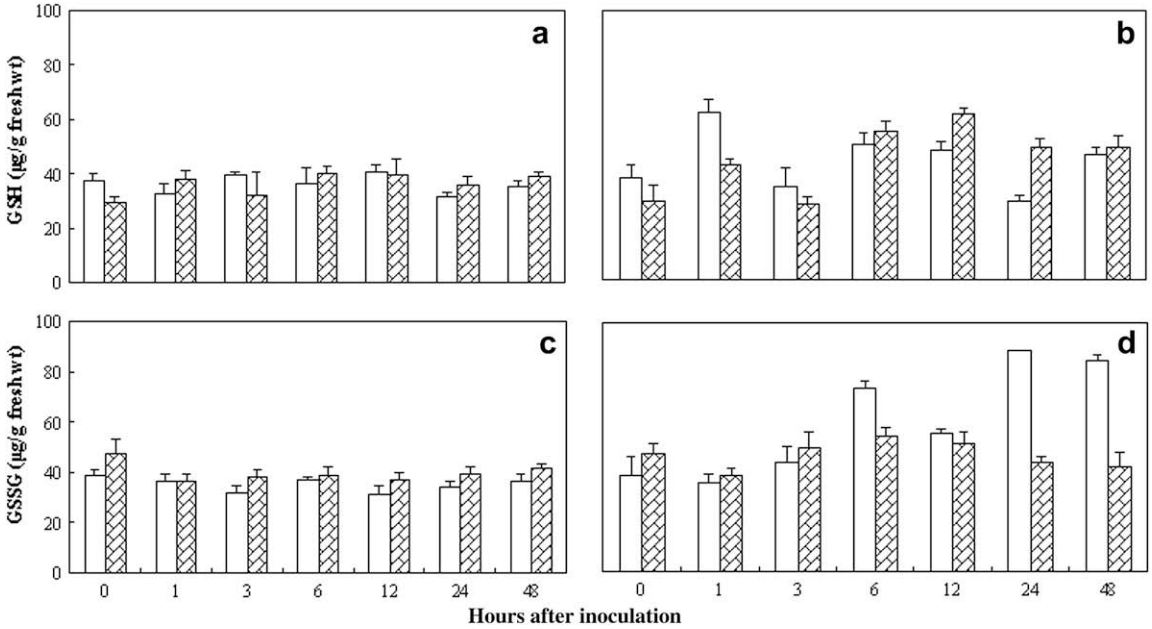

Fig. 7. Modifications in reduced glutathione (GSH) and oxidized glutathione (GSSG) in leaves of Hefeng 35 (white columns) and Nannong 493-1 (gridded columns) infected by *P. sojae*. (a) and (b) show GSH content whereas (c) and (d) represent GSSG content. (a, c) Control leaves treated with sterile distilled water. (b, d) Leaves treated with *P. sojae* zoospore suspension. Each value represents the mean of three replicates and bars represent the standard error (±SE).

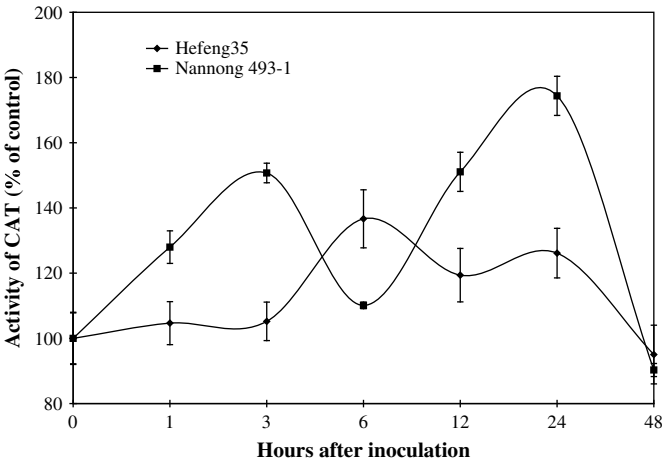

Fig. 8. Changes of the activity of CAT in soybean seedling leaves infected by *P. sojae*. CAT activities are represented as relative percentage values, which are the content values of treatment sets divided by the content values of the corresponding controls at the same intervals. Each value represents the mean of three replicates and bars represent ±SE. Three replicates yielded the same result.

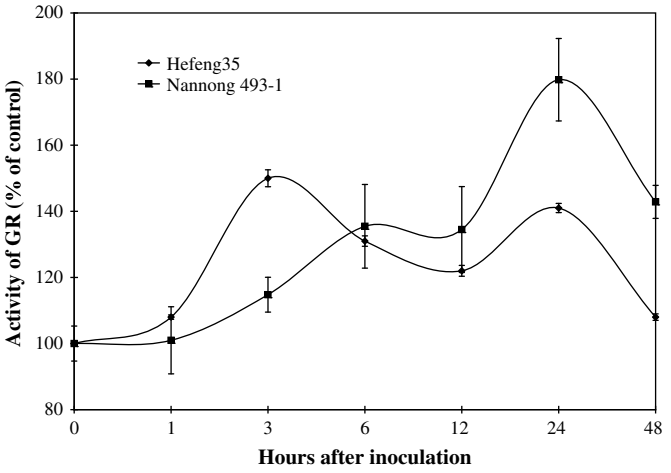

Fig. 9. GR activity in soybean seedling leaves infected by *P. sojae*. GR activities are represented as relative percentage values, which are the content values from treatment sets divided by the content values of the corresponding controls at the same intervals. Each value represents the mean of three replicates and bars represent ±SE. Three replicates yielded the same result.

interaction of the *P. sojae* strain PS2 with the soybean cultivars Hefeng 35 (susceptible) and Nannong 493-1 (medium-resistant) using leaf inoculation as a model of root rot. Although similar studies have been performed with a limited number of pathosystems, all of these studies targeted infections of foliar tissues or suspension-cultured cells by obligate biotrophic or necrotrophic pathogens or bacterial pathogens [10,12,41–44]. In contrast, little is known about plant–hemibiotrophic pathogen interactions, which are characterized by a transition from biotrophic to necrotrophic growth as the infection progresses, partially because of practical difficulties in working with this type of pathosystem. Similar to most *Phytophthora* species, *P. sojae* can infect all parts of the soybean plant [13]. Furthermore, we previously showed that soybean leaf experiments closely mirror the infection process that occurs in the field [22]. Therefore, we used the mimic leaf inoculation method in this study.

Here, we investigated the oxidative burst, cell death, *PR* gene expression, the effect of reductants on infection, and oxidative-stress parameters such as the degree of lipid peroxidation, the activity levels of two antioxidant enzymes (CAT and GR), and the contents of the nonenzymatic antioxidants ascorbate and glutathione. The results showed that both soybean cultivars undergo oxidative burst and cell death when inoculated with *P. sojae*. The oxidative burst is the earliest typical event of plant–pathogen interactions [1,4,45,46]. Besides acting as a signal molecule that induces plant defense [30,47,48], H_2O_2 can directly kill pathogens. Peng and Kuc determined that hydrogen peroxide could be a source of antifungal activity *in vitro* and on tobacco leaf discs [49], a result supported by Baker and Orlandi [50] and Missall et al. [51]. To keep redox equilibrium inside cells, the plants produce the endogenous antioxidants such as AsA or GSH to crack down on the excess ROS, to some extent which facilitates the infection of the pathogens. In our work, treating both soybean cultivars with one of three reductants (DTT, AsA, and GSH) might have facilitated the infection by the pathogen (Figs. 12 and 13), in accordance with these reports. The results showed that these substances, i.e. the endogenous antioxidants and exogenous reductants, may favor compatibility, at least in the inoculation points, with the potential to clear up the ROS. The effects of the exogenous reductants AsA and GSH on the infection of Hefeng 35 by the pathogen were great, whereas those of DTT and AsA on the infection of Nannong 493-1 by the pathogen were predominant. Furthermore, the amplitude of the effects of the exogenous reductants on pathogen infection was slightly higher in Hefeng 35 than in Nannong 493-1. The facilitation of pathogen infection by the application of exogenous reductants was more obvious with treatment B than with treatment A (Figs. 12 and 13). Perhaps the redox state of the pathogen itself was affected by the exogenous reductants when they were mixed together for treatment of the host with treatment A, therefore influencing the infection capacity of the pathogen.

To our knowledge, this is the first report of a novel analysis method for the interaction of soybean and the hemibiotrophic pathogen *P. sojae*, including analysis of the expression of *PR* genes induced by *P. sojae* infection. This system provides a model for the investigation of oxidative stress-related processes and molecular mechanisms involved in *Phytophthora* root rot resistance.

The results of the spectrophotometric assays indicated that infection by *P. sojae* led to substantial changes in the antioxidant status of soybean, and there were clear differences between compatible and incompatible interactions. It was possible to distinguish between responses associated with both interaction

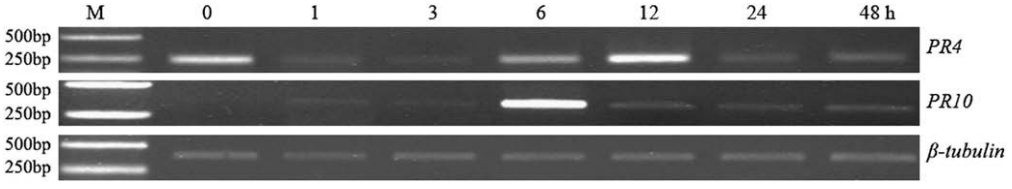

Fig. 10. Response of pathogenesis-related (*PR*) genes in leaves of susceptible soybean cultivar Hefeng 35 to *P. sojae*. Semi-quantitative reverse transcription–polymerase chain reaction (RT–PCR) was employed to explore the *PR* genes' expression. RT and PCR were performed with RNA isolated from zoospore-infected and non-infected leaves with primers specific for *PR-4* and *PR-10* of soybean. *β-tubulin* gene of soybean was used as the internal control. M, 2000-bp DNA marker (TaKaRa).

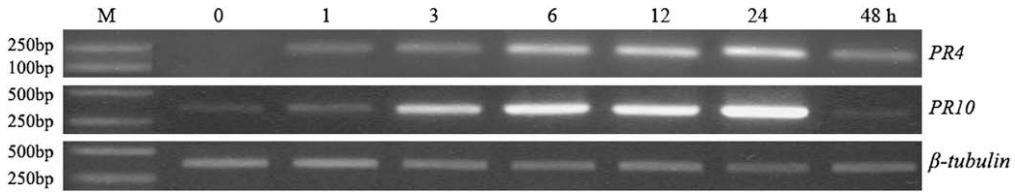

Fig. 11. Expression of defense-related genes in leaves of medium-resistant soybean cultivar Nannong 493-1 to *P. sojae*. For details, see legend of Fig. 10.

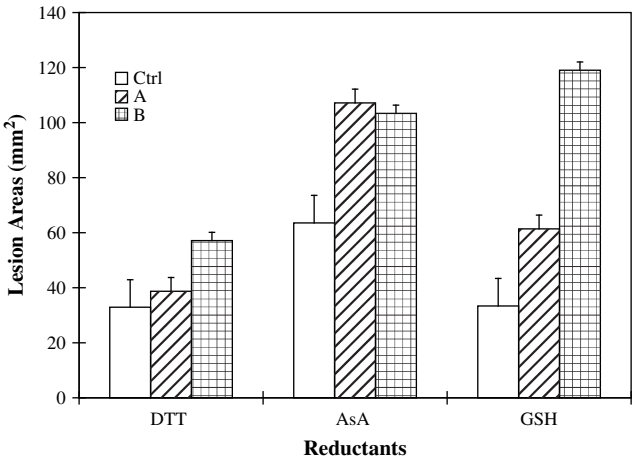

Fig. 12. The effect of three exoreductants (DTT, AsA and GSH) on the interaction between *P. sojae* and soybean cultivar Hefeng35. (A) Leaves were inoculated with 30 μl of 1:1 mixture of reductant solutions (1 mM each) and zoospore suspension (approximately 6.7×10^4 zoospores ml^{-1}). (B) Leaves were treated with 15 μl of zoospore suspension in 30 min, followed by treatment with equal volume of reductant solutions at the same sites on the leaf surface. Ctrl, control seedlings were treated with 30 μl of two-fold diluted zoospore suspension.

types and those specific to either the compatible or the incompatible interaction. The common responses were the changes in the degree of lipid peroxidation, the contents of reductants (ascorbate and glutathione), and the CAT and GR antioxidant enzyme activities that resulted from infection by the pathogen. The induction of CAT suggests that O_2^- and H_2O_2 production are enhanced. However, responses common to both interactions appeared earlier in the incompatible interaction than in the compatible interaction; in the latter, the responses may have occurred too late to afford protection if they are important for resistance.

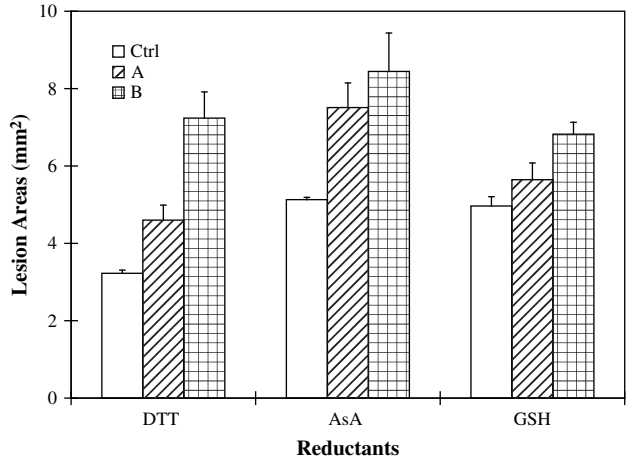

Fig. 13. The effect of three exoreductants (DTT, AsA and GSH) on pathogenicity of *P. sojae* on soybean cultivar Nannong 493-1. For details, see legend of Fig. 12.

Responses specifically linked to the compatible interaction were the induction of MDA and GR. The MDA content was much higher in Hefeng 35 than in Nannong 493-1 (Fig. 5), indicating that the degree of lipid peroxidation in Hefeng 35 was greater than in Nannong 493-1. This may indicate a positive correlation between the degree of lipid peroxidation and disease resistance. The relative GR activity was clearly related to the ascorbate–glutathione cycle [5], the most general mechanism of H_2O_2 detoxification in plants. Since the pathogen enters the cortical cells of susceptible soybeans but not those of resistant plants [52], one could speculate that the activation of the ascorbate–glutathione cycle is a response to H_2O_2 production that differs greatly between compatible and incompatible interactions.

As a substrate of ascorbate peroxidase, endogenous AsA participates in the removal of H_2O_2. AsA might also directly reduce O_2^-, quench $^1O_2$, or regenerate reduced α-tocopherol [7]. Any of these routes of AsA oxidation, as well as a slow synthesis rate of AsA or a decreased reduction rate of either oxidation product (monodehydroascorbate or DHA), could lead to a decrease in AsA in infected Nannong 493-1 from 3 to 6 hpi (Fig. 6).

Endogenous GSH plays an important role in the protection of cells against the toxic effects of free radicals by keeping the free

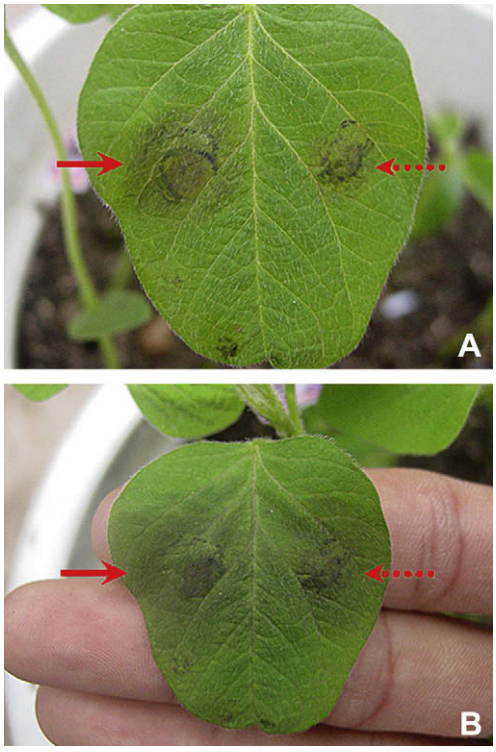

Fig. 14. The effect of exogenous reductants on pathogenicity of *P. sojae* on soybean cultivar Hefeng 35. (A, B) Photographs showing necrotic lesions 48 and 72 h after infiltration, respectively. Serial arrows indicate the inoculation with 30 μl of 1:1 mixture of reduced glutathione solution (GSH) (1 mM) and zoospore suspension (approximately 6.7×10^4 zoospores ml^{-1}). Aserial arrows indicate the inoculation spots with 30 μl of two-fold diluted zoospore suspension.

radical-scavenging ascorbate in its reduced, active form by its involvement in the ascorbate–glutathione cycle. Under conditions of oxidative stress, such as O_3, herbicides, or drought, the GSH level increases above control levels [53], in accordance with our research results.

In summary, we have demonstrated the induction of the oxidative burst during the mimic infection of the soybean leaves by *Phytophthora* root rot pathogen, suggesting that the changes in oxidative metabolism may be a general plant defense response, not restricted to biotrophic and necrotrophic pathogens but also appearing in hemibiotrophic pathogens, such as *P. sojae*. Our results also suggest that increased levels of ROS, built up by either enhanced production or decreased scavenging potential, contribute to the resistance of soybean to *Phytophthora* root rot.

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