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Physiological Analysis Reveals the Possible Resistance Mechanisms of *Glycine max* to *Fusarium solani*

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Abstract

Sudden death syndrome (SDS) of soybean is a complex root rot disease caused by the semi-biotrophic fungus *Fusarium solani* (*F. solani*) and a leaf scorch disease; caused by toxins produced by pathogen in the roots. However, the mechanism of soybean resistant to *F. solani* is still poorly understood. Eighteen soybean cultivars were screened for SDS resistance, with one cultivar showing susceptibility and one cultivar showing resistance to *F. solani* infection. Histochemical analysis with diaminobenzidine (DAB) and Trypan blue staining indicated an accumulation of reactive oxygen species (ROS) and cell death in surrounding area of SDS which was higher in susceptible cultivar than in resistant cultivar. Furthermore, exogenous salicylic acid (SA) application also induced some level of resistance to *F. solani* by the susceptible cultivar. A biochemical study revealed that the activities of superoxide dismutase (SOD), peroxidase (POD), and enzymes involved in scavenging ROS, increased in susceptible cultivar after SDS infection. In addition, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) content also increased in the susceptible cultivar than in resistant cultivar. High-performance liquid chromatography (HPLC) analysis indicated that free and total salicylic acid (SA) content increased in the susceptible cultivar than in resistant cultivar. In addition, a real-time quantitative PCR analysis showed an accumulation of pathogen related (*PR*) genes in the resistant cultivar than in susceptible cultivar. Our results show that (i). *F. solani* infection can increase endogenous SA levels, antioxidase activities, ROS and cell death in susceptible soybean cultivar to induce resistance against *Fusarium solani*. (ii). *F. solani* infection induced the expression of SA marker genes in resistant soybean cultivar to enhance resistance.

Keywords: *Fusarium solani*, soybean, disease resistance, salicylic acid, antioxidants

1. Introduction

Soybean production is often affected by many diseases in major cropping areas (Wrather et al., 2010). Sudden death syndrome (SDS) caused by *Fusaruim solani* (*F. solani*) occurs frequently in the top eight soybean producing countries in the world (Wrather et al., 2010). SDS is associated with root rot, vascular discoloration of stems, root chlorosis and necrosis, defoliation and plant death (Roy et al., 1997). Mostly, SDS is more easily detected in soybean fields after flowering, when the leaves show interveinal choroids and necrosis. Meanwhile, SDS can be expressed in severe and mild forms. The occurrence and geographical distribution of pathogens causing the latter is unknown (Scandiani et al., 2011).

Exposure of plants to unfavorable conditions, makes them develop an integrated defense mechanism against fungal diseases which include chemical and physical barriers; and inducible defense (Dixon et al., 1994). So far as resistance responses other than susceptibility and immunity is concerned, the invasion of plant by fungal hyphae is likely to induce and sustain expression of some plant defense-related genes. Induced defenses attempts

to prevent or reduce pathogen access by activating molecules that are antimicrobial, antioxidants, involved in the SA signaling pathways (Lamb & Dixon, 1997). Phytohormones play important roles in regulating developmental process and signaling networks which are involved in plant response to a wide range of abiotic and biotic stresses (Robert-Seilaniantz et al., 2007; Bari & Jones, 2009). Three major signaling molecules, Salicylic acid (SA), Jasmonic acid (JA) and Ethylene (ET) are recognized as major defense hormones against various pathogens (Glazebrook et al., 2003; De et al., 2005; Koornneef & Pieterse, 2008). SA is associated with resistance against biotrophic and hemibiotrophic pathogens, and with triggering systematic acquired resistance (SAR) in many species including *Arabidopsis thaliana* and wheat (*Triticum aestivum*) (Görlach et al. 1996). Induction of SAR is accompanied by accumulation of SA and up-regulation of a set of genes encoding pathogenesis-related (*PR*) proteins in dicot plants such as tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* providing a wide range of protection against pathogens (Ward et al., 1991; Uknes et al., 1992). The elevated expression of defense genes have been assumed as a molecular evidence of induced resistance (Sumayo et al., 2014). Natriuretic Peptide Receptor 1 (*NPR1*) gene is a key regulator of the SA signaling pathways (Yan & Dong, 2014). Enhanced Disease Susceptibility 1 (*EDS1*) gene was important for mediating resistance to a broad range of pathogens (viral, bacterial and fungal pathogens) yet showed specificity to the class of resistant *R* genes that it affected (Hu et al., 2005). The *EDS1* protein has been found to be complex with both the pathogen effectors and their cognate proteins and partitioning of the *EDS1* complex in the cytoplasm nucleus is required for full activation of local resistance (Zheng & Dong, 2013). *EDS1* is required to induce SA biosynthesis (Zheng & Dong, 2013).

Also, Chitosan which is used in agriculture in seed treatment and biopesticide helping plants to fight off against fungal infections, induced a significant increase in the activities of polyphenoloxidase, peroxidase, and enhanced the content of phenolic compounds in tomato fruits, thus providing protection against gray mould and blue mould diseases (Liu et al., 2007). Plants resistance can be induced by application of synthetic compounds such as functional analogs of SA, for example benzothiadiazole-7-carbothioic acid (acibenzolar-S-methyl) or benzothiadiazole (BTH). It has been shown that BTH which is a nontoxic compound, induced systematic resistance by exogenous root-treatment in tomato and controlled crown and root rot caused by *F.oxysporum* radices-lycopersici (Benhamou & Bélanger, 1998). *Fusarium wilt* of tomato was effectively controlled by foliar spray of validamycin A or validoxylamine A, which induced SA accumulation and development of systematic resistance (Ishikawa et al., 2005). Exogenous application of SA induces plant resistance to different kinds of pathogens that are associated with oxidative burst, cell wall enforcement and up-or down-regulation of gene expression (Oostendorp et al., 2001).

Production of ROS such as the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), as one of the earliest response to pathogen attack, can trigger hypersensitive cell death. Abnormally high production of ROS causes damage to biomolecules, whereas ROS at moderate concentrations act as a second messenger in signal cascades that mediate several responses in plant cells including program cell death (Sharma et al., 2012). The hypersensitive response (HR), as an early defense response, restricts pathogen infection to the site of attempted ingress by necrosis and cell death. Plants have an efficient antioxidative, enzymatic and non-enzymatic protective mechanisms to scavenge excess ROS. Several antioxidative enzymes including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) are involved in detoxification of ROS (Zhang et al., 1995; D. H. Lee & C. B. Lee, 2000).

Although much efforts have been made to identify mechanisms of resistance against SDS, much remains to be elucidated about the physiological and molecular capabilities of the soybean plant against *F. solani* infection. Here we performed a comparative study between resistant and susceptible soybean cultivars to distinguish the effects of some antioxidant and biosynthetic enzymes in response to *F. solani* infection. We also investigated the effect of exogenous application of SA, as a key factor in SAR, on soybean resistance to *F. solani* infection. Finally, to identify possible *PR* genes involved in the resistance process between the resistant and susceptible cultivar, RT-qPCR analysis was performed.

2. Materials and Methods

2.1 Plant Materials and Chemicals

Eighteen soybean cultivars used in the study were provided by Key Laboratory for Crop Genetics and Breeding of Sichuan Agricultural University, China. Seeds were surface-sterilized for 20 min in a 20% solution of sodium hypochlorite and then rinsed three times with sterilized water. Seeds were grown in an 11.5 cm diameter paper cups filled with perlite and placed in a climatically grown chamber. Conditions in the growth chamber included day/night at a regulated temperature of 25 ºC. Watering was done as required to promote seed germination.

2.2 Fungal Growth and Inoculation

The fungi *Fusarium solani* isolate 2hao3 was provided by the Key Laboratory for Major Crop Diseases of Sichuan Agricultural University, China. Isolate was obtained from soybean roots and preserved on potato dextrose agar (PDA) prior to sub cultures. 2 mm plug from the stock culture was used for sub cultures for inoculation by placing fungal isolate on PDA medium in petri dishes. The fungus was incubated at a temperature of 28 ºC for 7 days in the dark in a growth chamber. The seedlings were removed from the perlite after 3-day growth period and washed with distilled water prior to inoculation. Inoculation was performed by using the hypocotyl inoculation method described previously by (Haas & Buzzell, 1976). Disease development was observed for 2 days. Control plants were inoculated with 7 days grown PDA on a petri dish without fungus. Fungal inoculated and non-fungal inoculated seedlings were placed in a growth chamber at a temperature range of about 25 ºC without light condition.

2.3 Screening and Disease Assessment

Assessment of disease severity on all the 18 soybean cultivars was done according to (Ishikawa et al., 2005) with some modifications. At 2 days post inoculation (dpi) by *F. solani* the disease index (on 1-5 scale) on each plant was recorded according to vascular browning and the mean value of 10 plants from each cultivar calculated for disease severity. For evaluation of vascular browning, the basal stems were cut and vascular browning was rated on a scale of 1-5; where 1 = no symptoms or vascular browning; 2 = 1-25% vascular; 3 = 6-50% vascular browning; 4 = 51-75% vascular browning; 5 = more than 75% vascular browning. The mean value recorded for Nandou12 was 1 = no symptoms and Juiyuehuang recorded 5 = more than 75% vascular browning (Figure 1). These formed our basis for selecting Nandou12 as resistant and Juiyuehuang as susceptible cultivars.

2.4 Histo-chemical Stainings

Tissue staining with Trypan blue (1.25 mg/ml, sigma) and DAB (1 mg/ml, sigma) was performed as previously described (Thordal-Christensen et al., 1997; Shirasu et al., 1999; Lam, 2004) with suitable modifications. Samples were stained at 48 hours post inoculation (hpi).

2.4.1 Exogenous SA Application

To determine whether exogenous SA application can induce systematic acquired resistance in soybean, we first sprayed (For 48 h: at every 6 h intervals) the seedlings with SA which was dissolved in deionized water at a concentration of 100 µM and 200 µM in order to compare the disease development to seedlings inoculated with fungus without exogenous SA application. This assay was conducted as previously described (Spletzer & Enyedi, 1999).

2.4.2 Reverse Transcription and Quantitative PCR (RT-qPCR) Analysis

Total RNA was extracted using a plant total RNA Miniprep purification Kit (Tiangen, http://www.tiangen.com/) cDNA was reversely transcribed from 2 µg of total RNA using an oligo dT_{20} primer and MLV reverse transcriptase (http://www.invitrogen.com). First strand cDNAs of reversely transcribed 50ng of RNA was used for semi-quatitative RT-PCR analysis *Extaq* DNA polymerase (TaKaRa) and qPCR with Universal SYBR® GREEN qPCR Master Mix (2×) (Gangchi Bio). The parameters of the semi-quantitative PCR were as follows: 95 ºC for 5 min, 95 ºC for 15 s, 50 ºC for 30 s, 72 ºC for 1kb min^{-1}, and another cycle (step 2) was repeated according to the gene expression level of the specific genes. Parameters of the qPCR were as follows 95 ºC for 3 min, 95 ºC for 15 s and 55 ºC for 15 s, and 72 ºC for 20 s, go to step 2 for 39 more cycles. Then increment of 0.5 ºC from 65 ºC to 95 ºC for 5 seconds was used for melt curve analysis. ΔΔCq method was used to normalize the qPCR data according to (Du et al., 2016). *GmACT3* (Glyma09g17040) was amplified as an internal control. Gene-specific primer pairs were designed using Primer 5.0 (Table 1).

2.4.3 Endogenous SA Measurement

SA was extracted and measured according to modification from previous studies (Wang et al., 2011). 200 mg of soybean tissue was ground to fine powder with N_2 and extracted once with 1.5 ml of 90% methanol followed by one extraction with 1.5 ml of 100% methanol. The methanol fraction was equally split into two micro centrifuge tubes (for total and free SA analyses, respectively) and dried in the fume hood overnight. The pellet was dissolved by adding 500 µl of 100 mM sodium acetate (pH 5.5). To half of the duplicated samples, 40 µl of β-glucosidase (Sangon, A662003-0010) were added to digest glucosyl-conjugated SA (total SA) for 1.5 h at 37 °C. (About 80 units/g fresh weight). All the samples were treated with an equal volume of 10% trichloroacetic acid (TCA) and centrifuged at 10,000g for 10 min. The supernatant was extracted twice with 1 ml of extraction solvent (ethylacetate: cyclopentane: 2-propanol 100:99:1, v/v). The top (organic) phase was collected in a micro centrifuge tube and dried in a fume hood overnight. The residual fraction was re-suspended in 0.5 ml of 55%

methanol by vortex and was passed through a 0.2-um nylon spin-prep membrane (Fisher 07-200-389) via centrifugation for 2 min (14,000 g) before being subjected to HPLC analysis. A Dionex AS50 HPLC instrument with an Acclaim 120C18 reverse column (4.6 × 250 mm) and an RF2000 fluorescence detector was used to separate and detect SA. The mobile phase included a gradient of methanol and 0.5% acetic acid. SA was detected at 6.5 min with 301-nm excitation/412-nm emission. The standard curve was made from quantification of SA at concentration of 10, 8, 6, 2, and 1 mg mL^{-1} and used to calculate the final concentration in each sample using Microsoft Excel software.

2.4.4 Determination of Antioxidant Enzymatic Activity

The enzymes activity was carried out by grinding 0.5 g of 2 days infected soybean tissues with 2 mL ice-cold 25 mM HEPES buffer (pH 7.8) containing 0.2 mM, EDTA, 2 mM ascorbate and 2% PVP. Further, the homogenates were centrifuged at 4 ºC for 15 minutes at 13,000 *g* and the supernatant was used for enzyme activities analysis. All the various steps in the preparation of the enzyme extract were carried out at 4 °C. Peroxidase (POD) activity was measured according (Egley et al., 1983), the total volume of 2 mL mixture contained 25 mM (Ph 7.0) sodium phosphate buffer, 0.1 mM EDTA, 5% guaiacol (2-ethoxyphenol), 1.0 mM H_2O_2 and 100 µl enzyme extract. Superoxide dismutase (SOD) activity was also measured as previously described (Giannopolitis and Ries 2010).

2.4.5 H_2O_2 Measurement

Hydrogen peroxide H_2O_2 content from the 2days infected soybean tissues was measured according to (Du et al., 2011). About 1 g of soybean hypocotyl was homogenized in an ice bath with 5 ml 0.1% (w/v) TCA. The homogenate was transferred into a tube and centrifuged at 12,000 g for 20 min at 4 ºC. 0.5ml of the supernatant was added to 0.5 ml 10 mm potassium phosphate buffer (Ph.7.0) and 1 ml potassium iodide (KI). The absorbance of supernatant was read at 390 nm. H_2O_2 content was determined by a standard curve.

2.4.6 Malondialdehyde (MDA) Content Measurement

MDA content of the 2days infected soybean tissues was quantified according to (Sun et al., 2006; Qian et al., 2007). About 1 g of soybean tissues (hypocotyl) was homogenized with 5% trichloroacetic acid (TCA) on ice and centrifuged at 3,000 g for 10 min at 4 ºC. 2 mL of the supernatants was transferred into another tube added with 2 ml 0.67% thiobarbituric and incubated at 100 ºC for 15 min. The cooled mixture was centrifuged at 4,000 g for 10min. The supernatants were subjected to analysis at 450 nm (A_{450}) 532 nm 600 nm (A_{600}) in spectrometer. The amount of MDA was calculated using an extinction coefficient of 155 Nm^{-1} cm^{-1} and according to the formula: MDA (µmol L^{-1}) = $6.45\times (A_{532}- A_{600}) - 0.56\times A_{450}$.

2.5 Statistical Analysis

All experiments were repeated three times, with three replication each. Statistical calculations were performed using SPSS-20 (SPSS, Chicago, IL, USA). For disease severity assessment, a minimum of ten plants were evaluated for each replicate. Tests for significant difference among physiological parameters under different treatment were conducted using analysis of variance (ANOVA) with mean separation using Duncan's multiple range tests (DMRT) at the 0.05 level of confidence.

3. Results

3.1 Phenotypic Expression of Soybean Seedlings to F. solani Infection

In the current study, 18 soybean cultivars were evaluated for SDS resistance to *F. solani* infection. Inoculated seedlings were monitored and disease symptoms were recorded at 2dpi. Jiuyuehuang showed severe disease symptoms under *F. solani* infection whiles Nandou12 showed no disease symptoms (Figure 1; Figure 2: JT and NT). Once the symptoms of the disease appeared, fungal inoculated and non-fungal inoculated plants were collected at 2 dpi. The appearance of the symptoms provided a confirmation that the pathogen had penetrated the host tissues and infection was successful. Visual disease assessment (VDS) was used to evaluate the resistance level between the two soybean cultivars. See Materials and Methods section for details.

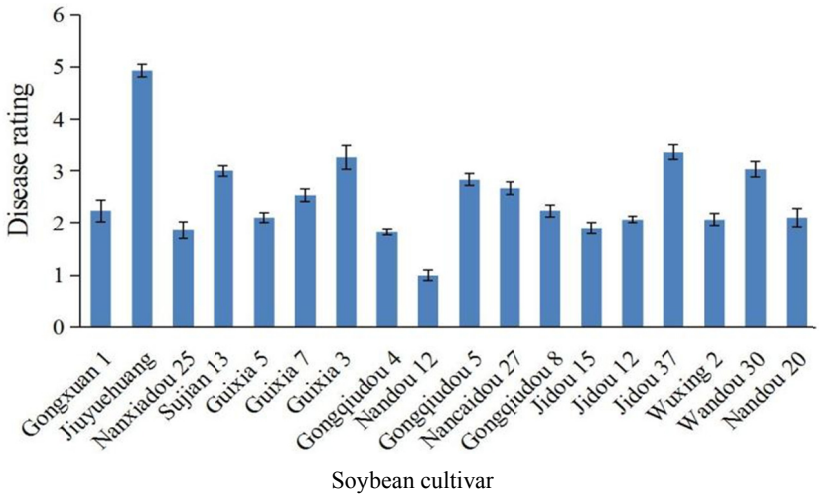

Figure 1. Response of 18 soybean cultivars to sudden death syndrome (SDS). Data on vascular browning of the stems were recorded at 2dpi of soybean plants by *Fuasrium solani*. Columns represent the mean disease ratings on 1-5 scale as described in Materials and methods section. Data bars are the means ±SD of three replicates. The experiments were repeated three times with three replicates

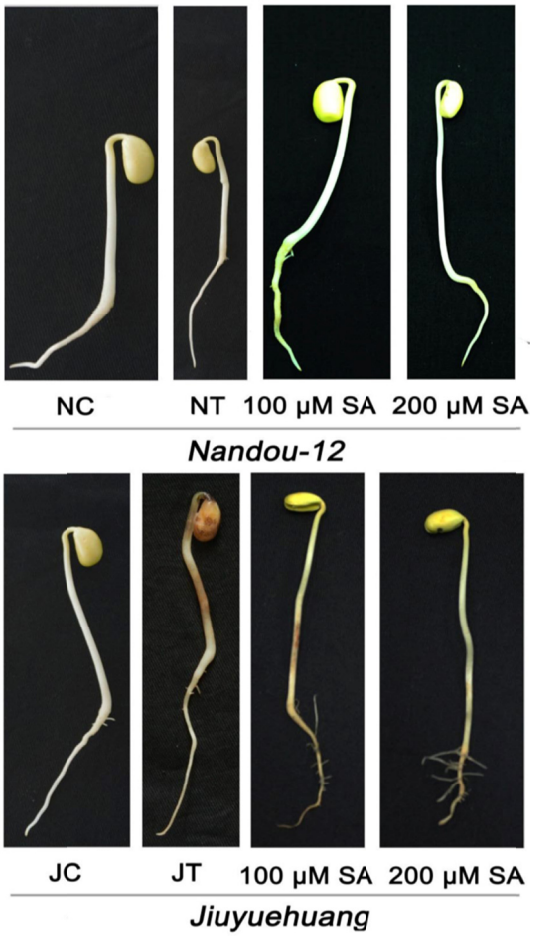

Figure 2. Early symptoms of Soybean sudden death syndrome (SDS) induced artificially by inoculating with Fusarium solani at 2 dpi. NC: Nadou12 control; NT: Nadou12 treatment; JC: Jiuyuehuang control; JT: Jiuyuehuang treatment. And in order to observe the effect of exogenous SA application on inducement of disease resistance, different SA concentrations (100-200 μM) were sprayed (For 48 h: for every 6 h intervals) on different soybean seedlings of the same cultivars prior to fungal inoculation

3.2 Histo-chemical Stainings

Results from the histo-chemical staining showed a higher intensity of diaminobenzidine (DAB) and programmed cell death (PCD) in Jiuyuehuang than in Nadou12 (Figures 3b and 3d) and (Figures 4b and 4d) respectively. Through staining, it was observed that the tissue with DAB stained brownish at the infected or penetrated site. Likewise cells with PCD stained deep blue at the infected site as a result of fungal infection.

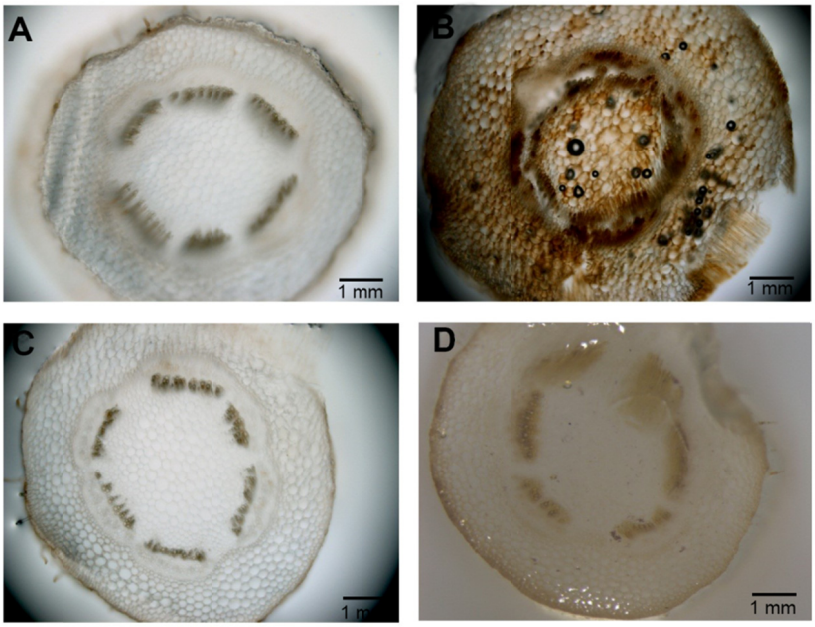

Figure 3. Cross sectional hypocotyl levels of ROS was visualized by DAB-staining at 48hpi with *F. solani*. (A): Jiuyuehuang control; (B): Jiuyuehuang treatment; (C): Nadou12 control; (D): Nadou12 treatment. Scale bars represents 1 mm

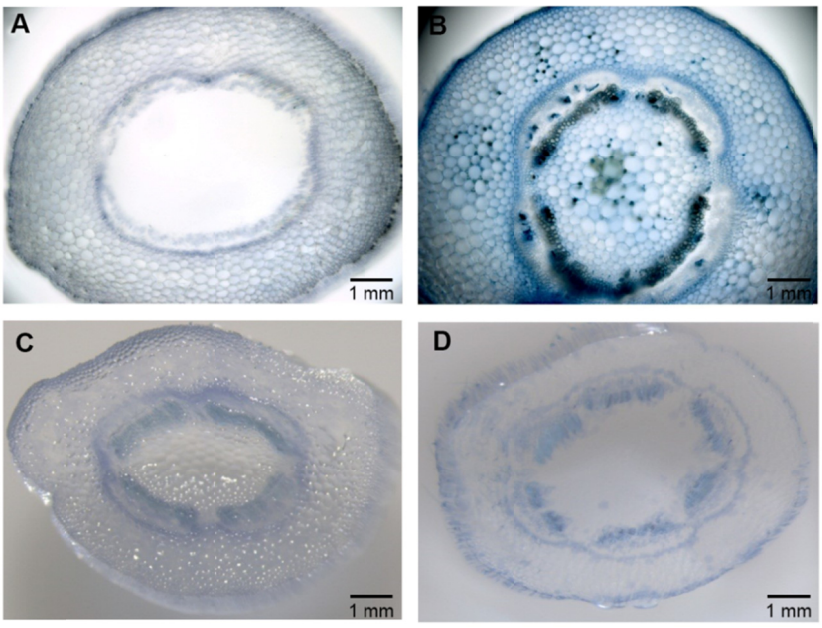

Figure 4. Cross sectional hypocotyl levels of plant cell death was visualized by Trypan blue staining at 48hpi with *F. solani*. (A): Jiuyuehuang control; (B): Jiuyuehuang treatment; (C): Nadou12 control; (D): Nadou12 treatment. Scale bars represents 1 mm

3.3 Exogenous SA Treatment of Soybean Seedlings Induced Resistance Against F. solani

Exogenous SA application results indicated that, exogenous SA application induces systematic acquired resistance in soybean. Vascular brown level in Jiuyuehuang (Figure 2: JT) reduced significantly after exogenous SA feeding of 200 µM prior to inoculation. No difference was observed in Nadou12 after the exogenous SA application.

3.4 Detection of SA From Soybean Seedlings

The effect of SA on plants response to pathogenes in susceptible and resistant cultivars under *F. solani* infection was investigated. Results from the study suggest that endogenous SA content in soybean can be increased under *F. solani* infection. *F. solani* infection induced Free/Conjugated SA content in Jiuyuehuang than in Nadou12 (Figure 5).

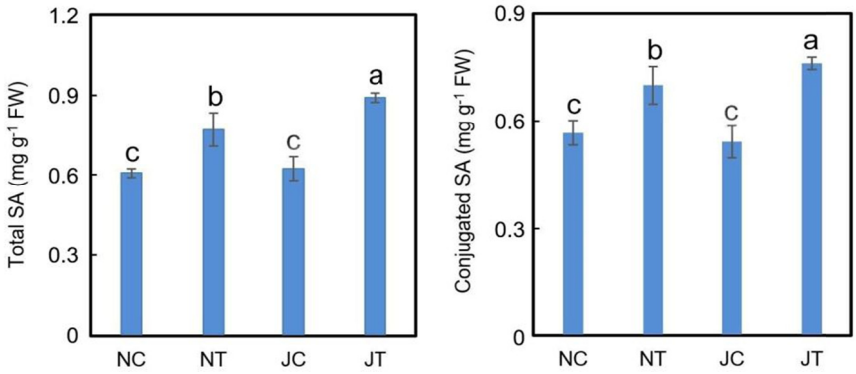

Figure 5. Accumulation of salicylic acid (Total and Conjugated SA) in soybean tissue at 48 hpi with *F. solani*. (NC) Nadou12 control; (NT)-Nadou12 treatment; (JC)-Jiuyuehuang control, (JT) Jiuyuehuang treatment. Data bars are the means ±SD of three replicates. Letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests

3.5 Temporal Expression of Defense-Related Genes

We monitored the differential expression of genes related to biotic- stress responses. From all the treatments there was some advancement in the expression of SA and PR- related genes. Most of the genes were unregulated in the resistant cultivar than in the susceptible cultivar. At 24 hpi the relative expression of *NPR1*, *PR1*, *PAD4*, *EDS1*, *EDS5* and *SID2* was higher in the resistant cultivar (Nandou12) than in the susceptible cultivar (Jiuyuehuang) (Figure 6). This result indicates that *F. solani* inoculation induced higher *PR* genes at 24 hpi in Nadou12 than in Jiuyuehuang. Similarly, at 48 hpi there was a higher expression of *PR1* and *SID2* in the susceptible cultivar than in resistant cultivar. *PAD4* and *EDS5* also expressed a little higher in the resistant cultivar than in susceptible at 24 hpi. In contrast, *EDS1* expression level in Jiuyuehuang than in Nadou12.

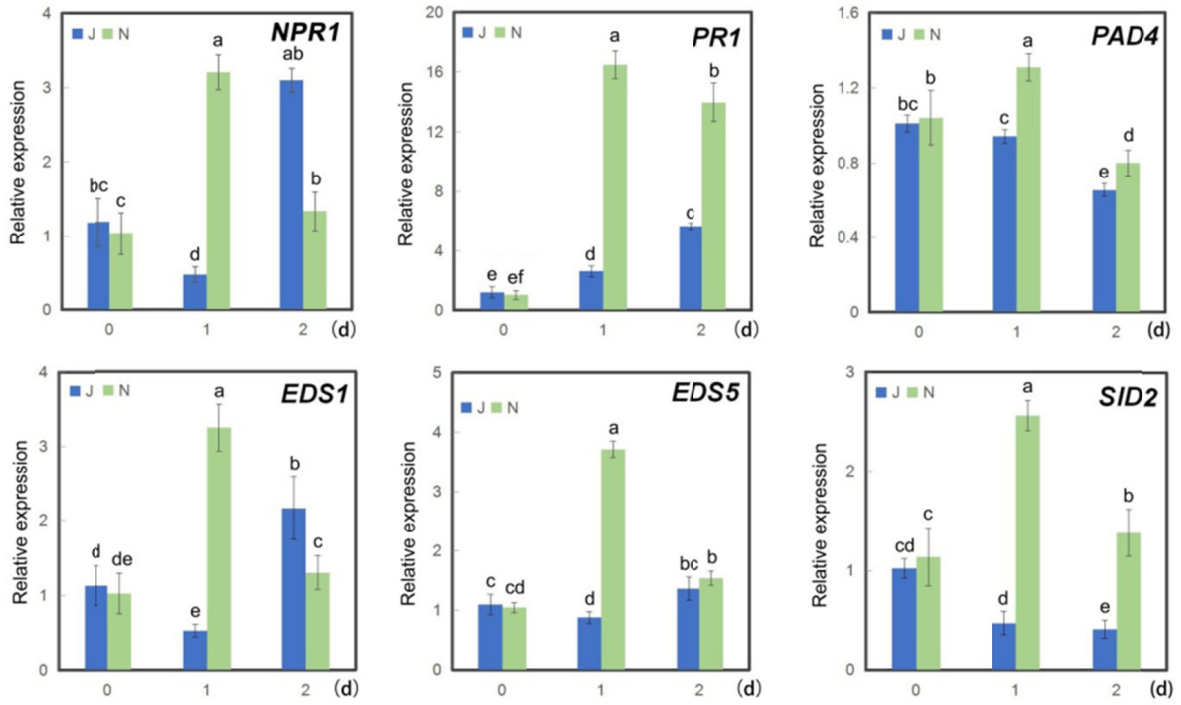

Figure 6. Relative expression of *GmNPR, GmPR1, GmPAD4, GmEDS1, GmEDS5, GmSID2* in two varieties of soybean (*Glycine max L.*), Jiuyuehuang (J) and Nandou12 (N), at 0, 1, 2 days (d) as determined by fluorescent quantitation PCR analysis. Error bars indicate SD (N = 3). Lowercase letters significant difference from Jiuyuehuang at 0 day (Student's *t* test, $p < 0.01$)

Table 1. Primer sequences used in RT-qPCR analysis

GENE ID	GENE NAME		qPCR PRIMER(5' to 3')
Glyma15g06790	*GmPR1*	Forward	CGCTGCTTTTGCACAGAACTA
		Reverse	CTCCCCGTATTTTCCATCAC
Glyma09g02430	*GmNPR1-1*	Forward	CTCTGGGTTCTCTGGCATCA
		Reverse	ACACAGCCACCCCAGAAAAC
Glyma04g34800	*GmEDS1*	Forward	CACTCCTCTGGTGCTGCAAT
		Reverse	TGGCGTGGGTTTTTGGAT
Glyma01g25690	*GmSID2/GmICS1*	Forward	GTCAGCATTGGTGGAAAAGG
		Reverse	TCATCCCACTCCAGGTAAGG
Glyma11g11970	*GmEDS5*	Forward	ATATCGAGGCAGGGTGAGAA
		Reverse	AGTTGCTGGTCCTGTGAACA
Glyma08g00420	*GmPAD4*	Forward	AAGAGAGCCAAGTGGGTGAA
		Reverse	ATGCATCCCCTTCCCATAAT

3.6 Activities of Antioxidant Enzymes

As indicators of antioxidants response in plants, the current study observed an increase in peroxidase (POD) and superoxide dismutase (SOD) activities in Jiuyuehuang than in Nadou12 (Figures 7a and 7b). Increased and decrease in antioxidants activity in plants have frequently been correlated with disease resistance and susceptibility. As indicators of oxidative stress, malondialdehyde (MDA) and hydrogen peroxide H_2O_2 content was determined for both cultivars. From the current study, the content of MDA and H_2O_2 was higher in Jiuyuehuang than in Nadou12 (Figures 7c and 7d).

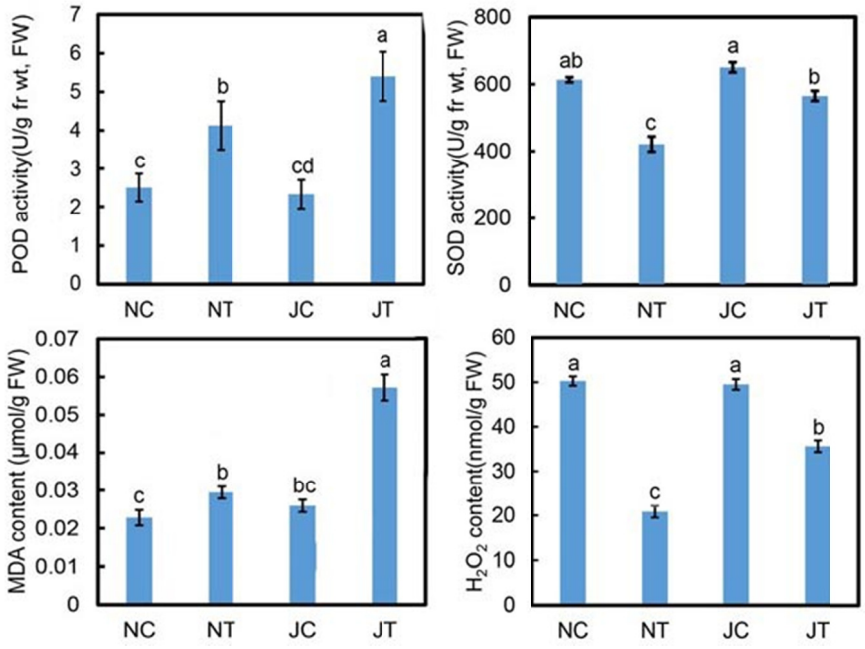

Figure 7. Antioxidants activity on two soybean cultivars at 48hpi after with *F. solani.* (a) Peroxidase (POD) content; (b) Superoxide dismutase (SOD) content; (c) Malondialdehyde (MDA) content; (d) Hydrogen peroxide (H_2O_2) content; (NC)-Nadou12 control; (NT)-Nadou12 treatment; (JC)-Jiuyuehuang control, (JT)-Jiuyuehuang treatment. Letters indicate significant differences ($P < 0.05$) according to Duncan's multiple range tests. Data bars are the means ± SD of three replicates

4. Discussion

The current study investigated a temporal resistance response between two soybean cultivars (Nadou12 and Jiuyuehuang) to *F. solani* infection. Our results provide a novel insight into the *F. solani* soybean interaction by analyzing plant pathogen defense response assays. Our assessment was based on physiological and molecular assays. SA is well known for its endogenous signal molecule playing an important role in development of systematic acquired resistance in plants (Dempsey et al., 1997). SA application induces accumulation of PR proteins (Loake & Grant, 2007). Many of the PR proteins have antimicrobial activity in vitro and they serve as molecular markers for the onset of disease responses (Durrant & Dong, 2004). Application of Exogenous SA and its functional analog potentiated plant tissue to respond rapidly and effectively to a variety of defense mechanisms after pathogen challenge (Katz et al., 1998). Our exogenous SA application showed a decrease in disease levels in Jiuyuehuang which suggested that exogenous SA application induced the endogenous SA level leading to the disease resistance. This observation has some similarities to a recent report that exogenous application of 200 μM SA to tomato plants prior to inoculation provided increased *F. oxysporum* resistance as evidenced by reduced foliar necrosis and plant cell death in Arabidopsis (Edgar et al., 2006). Similar response was also observed in tobacco plants treated with 100 μM SA (Oostendorp et al., 2001). All these suggest that, exogenous SA application plays a significant role in inducing SAR.

In the present study, a significant increase of ROS was observed in Jiuyuehuang than in Nadou12 through DAB staining (Figures 3b and 3d). Accumulation was stronger and more intense in Jiuyuehuang. These results suggest that the induction of ROS in Jiuyuehuang maybe a defense responses against the invading pathogen. In a similar study, the inoculation of *Botritis cinera* induced an oxidative burst in *Arabidopsis thaliana*, and the aggressiveness of this pathogen was directly dependent on the level of ROS accumulation (Levine, 2000). The same was also reported by (Singh & Upadhyay, 2014) where Ferric acid – treated tomato leaves exhibited visible necrotic lesions as a result of cell death which was evident by Evans blue staining, due to enhanced ROS levels and DNA damage. In the current study, increased accumulation of cell death was also observed in Jiuyuehuang at the site of infection than in Nadou12 which indicates a defense response against the attacking pathogen.

Plants infected by different pathogens induce different hormonal signaling pathways (Wege & Siegmund, 2007). Salicylic acid (SA) and jasmonic acid (JA) signaling pathways are two most important pathways for plant

response to fungal infection (Luo et al., 2011). SA pathways is often induced by pathogen infection and is effective in mediating resistance against biotrophic pathogens (Thaler et al., 2012). SA signaling was shown to be important in defense against *F. graminaruim* infection in *A. thaliana* and in wheat (Makandar et al., 2006; Makandar et al., 2010; Makandar et al., 2012). From the current study, endogenous SA content (Total and conjugated SA) increased in Jiuyuehuang than in Nadou12 which suggests the accumulation of SA as a result of the fungal infection.

The result from the gene expression analysis indicated that more defense genes were expressed in Nandou12 than in Jiuyuehuang. The expression of *NPR1* was also up-regulated in Nandou12 than in Jiuyuehuang indicating that the gene expression involved in SA signaling pathways was up-regulated in Nandou12 than in Jiuyuehuang. We suggest from the current result that in the event of fungal stress in soybean, SA mediated disease resistance plays an important role in Nandou12 resistance to *F. solani.*

In addition, results from the current study also showed that there was an increase in Peroxidase (POD), superoxide dismutase (SOD) and malondialdehyde (MDA) as a result of the fungal stress (Figure 7). SOD is one important enzyme in ROS metabolism and catalyzes the dismutation of oxygen (O_2)—and hydrogen peroxide (H_2O_2) (Gill & Tuteja, 2010). Increased and decrease in SOD activity have frequently been correlated with disease resistance and susceptibility (Yang et al., 2003). Vanacker et al. (1998) reported that an increase in SOD activity following pathogen attack might be required to catalyze the synthesis of H_2O_2 during the oxidative burst and to prevent the accumulation of superoxide. The current study recorded an increase in SOD activity in the fungal infected plants of Nandou12 more than the fungal infected plants of Jiuyuehuang. Similar result was recorded in the POD level with increased activity in the fungal infected plants of the resistant cultivar than in fungal infected plants of the susceptible cultivar. This suggests that antioxidant defense mechanism activated under stress remained operative throughout that challenging period, enabling plants to adopt to such conditions (Pérez-Clemente et al., 2012). MDA is one final decomposition product of lipid peroxidation and has been used as an index for the presence of lipid peroxidation (Esim et al., 2012). While more ROS was accumulated, more MDA was also accumulated in Nandou12 (Figures 3d and 7c) respectively. Which we conclude that the fungal stress had more damaging effect on Jiuyuehuang more than the Nandou12. MDA content increased in water stressed olive plants which has resemblance with our results (Sofo et al., 2004). The increased level of activated oxygen species could contribute to the symptoms development and pathogenesis in compatible plant-virus interactions. In the current study, the higher level in ROS is consistent with the increase in H_2O_2 activity (Xi et al., 2007).

5. Conclusion

In conclusion, *F. solani* infection revealed disease symptoms in Jiuyuehuang with no disease symptoms in Nandou12. The plant cells stained by DAB and Trypan blue produced a high amount of ROS and cell death in the area of infection respectively. Enzymatic and non-enzymatic antioxidative pathways which are involved in the production of signaling molecules were increased in Jiuyuehuang. More *PR* genes were induced in Nandou12. The current findings suggest that these differences are associated with resistance. SA treatment of both cultivars rendered them more resistant to *F. solani*. The current results can provide novel insights for better recognition of the responsible mechanisms needed to regulate SDS resistance in soybean. The direct effect of SA should further be examined on the above-mentioned enzyme activities and their gene expression in conferring resistance to pathogens. Exogenous SA application was able to induce SAR against *F. solani* of which we propose that SA played this role through regulation of the plant antioxidative system or through the genes involve in the SA signaling pathways.

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