

## DEFENSE RESPONSE OF SOYBEAN (*GLYCINE MAX*) TO SOYBEAN CYST NEMATODE (*HETERODERA GLYCINES*) RACE 3 INFECTION

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### ABSTRACT

Soybean plant responses to *Heterodera glycines* (*H. glycines*) race 3 infection were compared between resistant (Peking, LiaoK89102 and Franklin) and susceptible cultivars (Liao dou 10 and Kaiyu 10) at 10, 17, 20, and 23 days after inoculation (DAI). After inoculation with *H. glycines* race 3, the isozymes of peroxidase, polyphenol oxidase, superoxide dismutase, and catalase from the soybean roots were investigated. The time-course analysis of electrophoresis pattern of peroxidase, polyphenol oxidase, and superoxide dismutase isozyme showed increased enzyme activity in both the resistant and susceptible cultivars following inoculation. However, the increase in the resistant cultivars was rapid and greater than that in susceptible cultivars. The newly induced polyphenol oxidase enzyme bands (Rf values 0.80) were recorded for the nematode-infected resistant roots at 10, 13, 17, 20, and 23 DAI and compared to the non-infected ones. A peroxidase isozyme band (Rf value 0.78) was only recorded in the nematode-infected resistant cultivars Peking and Franklin at 13 and 17 DAI, respectively. In the superoxide dismutase and polyphenol oxidase patterns, the remarkable difference between inoculated and control plants was the amount of enzyme. In both resistant and susceptible cultivars infected with nematode, the enzyme was more abundant than in the control roots.

**Key words:** Defense response, Infection, Isozyme, Soybean cyst nematode.

### INTRODUCTION

Soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is one of the most important destructive pests in soybeans. The nematode is widely distributed throughout Northeastern China and the Huang-Huai-Hai Area (Lu *et al.*, 2006), as well as the soybean-producing regions of the United States where different maturity groups with the same sources of SCN resistance are grown (Donald *et al.*, 2006). Soybean yield losses caused by *H. glycines* in the US are greater than those caused by any other disease (Wrather and Koenning, 2006). Up to 30% yield loss has been observed in fields heavily infested with SCN without differences in plant height, susceptible cultivars, resistant cultivars and chlorosis. In China, a country that covers about 267 million ha, the resulting annual yield loss is about 10%–20%, and in some heavily infected fields have no yield. In recent years, agricultural scientists have identified plant resistance as one of the highest research priority for nematode pest management since resistant cultivars are effective against soybean cyst nematode. However, continued planting of the same resistant cultivars will change *H. glycines* populations with increased virulence on those cultivars (Noel and Edwards, 1996; Young, 1998); hence, to accelerate breeding nematode-resistant cultivars, more potential resistant materials should be found in numerous soybean cultivars.

The biochemistry of some plant-nematode

relationships has been fairly well documented in reviews (Kaplan and Keen, 1980; Zinova *et al.*, 2004). These studies have essentially been aimed at unmasking the biochemistry of plant resistance against the invading nematode pathogen. In tissues invaded by *Meloidogyne incognita acrita* and *Heterodera Rostochiensis*, hydrolase, oxidase, and dehydrogenase activity was increased (Giebel *et al.*, 1971; Kathiresan and Mehta Usha, 2005). A series of physiologic and biochemical reactions produced in plants during nematode infection, are similar to many infections by other pathogens. Defense enzymes, phenolic metabolism, the synthesis of phytoalexins, and some suggest superoxide dismutase (SOD) and peroxidase (POD) can be used to assist in selecting nematode-resistant varieties (Kim *et al.*, 1990). These compounds have been studied in response to other plant pathogens, however, little is known about their response to *H. glycines* infection. There are abundant sources of soybean germplasm in China. In this study, information about the characteristics of some soybean cultivars is provided to assist in the breeding of nematode-resistant soybeans.

The objective of this study is to analyze the isozyme patterns of polyphenol oxidase (PPO), POD, catalase (CAT), and SOD in resistant and susceptible soybean cultivars with and without *H. glycines* inoculation at the same time intervals as well as at different time intervals, and to understand the mechanism of disease-resistance in the different resistant cultivars.

## MATERIALS AND METHODS

**Tested cultivars and nematode:** Susceptible cultivars (Liao dou 10 and Kaiyu 10), and resistant cultivars (Peking, LiaoK89102 and Franklin) were tested. The Soybean cultivars were provided by Nematology Laboratory, Shenyang Agricultural University. Soil samples were collected from a field infested with *H. glycines* race 3 at Shenyang Agricultural University Agronomy Research Farm. Mature females were collected from a 180- $\mu$ m-pore sieve by hand-picking under a stereomicroscope (Li *et al.*, 2009). Brown, uniformly sized cysts were selected and crushed to release the eggs. The eggs were then placed into a 500 ml beaker with 300 ml sterilized distilled water and stirred with an automatic stirrer. The number of eggs in the suspension was determined by counting the number of eggs in five random 1 ml aliquots, and a suspension of eggs was prepared (1000 eggs/ml).

**Planting, inoculation, and sampling:** The experiments were conducted in a greenhouse (20–25 °C). Each pot (10 cm diameter) contained sterilized soil (1:1 sand to clay mixture by volume). The seeds were pre-germinated prior to transplantation. One plant was transplanted into each pot and then inoculated. The inoculum consisted of 3000 *H. glycines* eggs in 3ml distilled water applied into three holes located around the seedling. The holes were covered with sterilized soil after inoculation. The soybeans from the same cultivar received the same volume sterilized distilled water as the control. The experiment was completely randomized with three replicates, and water was applied as needed. The tested plants (inoculated and control) were sampled at 10, 13, 17, 20, 23, and 26 days after inoculation (DAI). Three soybean roots were sampled in each cultivar and the control treatment. The roots were washed fully with tap water, rinsed three times with distilled water, and then wiped dry with high-quality tissue paper.

**Extraction of POD and PPO:** POD and PPO were extracted according to the method used by Liu (2002). Briefly, 1 g young root from each of the different cultivars and the control were collected and homogenized in liquid nitrogen to form a fine powder, and extracted with 3 ml 0.05 M PBS buffer (pH 6.5) containing 1% polyvinyl pyrrolidone (w/v). The homogenate was centrifuged for 20 min at 11,000  $\times$  g. The supernatant was transferred to an Eppendorf tubes and stored at -20 °C.

**Isozyme of POD and PPO native PAGE:** The isozymes were analyzed by vertical thin-layer polyacrylamide gel electrophoresis (PAGE) using a 7.5% (w/v) slab gel with a 4.0% (w/v) stacking gel. Aliquots (20  $\mu$ l) of each enzyme extract from the different cultivars (inoculation and the control) were mixed with 5  $\mu$ l loading buffer

[40% (w/v) saccharose and 0.1% Bromophenol blue] and loaded in the wells. Electrophoresis was conducted at a constant 80 V through the stacking gel and a constant 120 V through the separating gel at 4 °C until the tracking dye Bromophenol blue migrated 1 cm from the end of the slab.

The POD isozyme gel was stained using a modified biphenylamine staining method (He and Zhang, 1999). The PPO isozyme gel was stained following the description of Li *et al.* (1991).

**Extraction and native PAGE of SOD and CAT isozymes:** The crude enzymes were extracted with the same method used in PPO extraction. The superoxide dismutase isozymes were analyzed by PAGE using a 10.0% (w/v) slab gel with a 5.0% (w/v) stacking gel under non-denaturing conditions. The enzyme extracts (20  $\mu$ l) from different cultivars (inoculation and the control) were mixed with 5  $\mu$ l loading buffer [40% (w/v) saccharose and 0.1% Bromophenol blue] and loaded into wells. After electrophoresis, the gel was taken carefully and labeled with cut corners, and rinsed with distilled water.

The staining of the SOD isoenzyme followed the methods used by Zhang (2002). The CAT isozyme was stained by the method of He and Zhang (1999) using polyacrylamide gel containing 0.75% soluble starch. All gels were photographed. The Rf values of all isozymes were calculated with Bio-1D analysis soft.

## RESULTS AND DISCUSSION

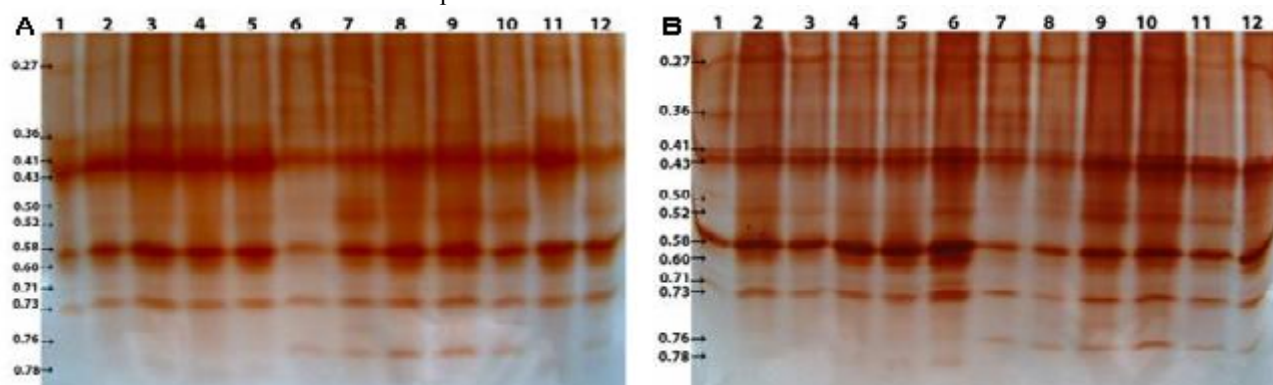
**The peroxidase isozyme activity determined by native PAGE :** The patterns of crude enzyme extracts from the roots of resistant Peking and Franklin, and susceptible Liao dou 10 cultivars inoculated with *H. glycines* were analyzed using native PAGE (Figure 1).

The POD isozyme patterns were characterized by different electrophoretic mobility and Rf values ranging from 0.27 to 0.78. There were 12 bands in the POD isozyme pattern, and Rf values were 0.27, 0.36, 0.41, 0.43, 0.50, 0.52, 0.58, 0.60, 0.71, 0.73, 0.76, and 0.78. The major differences between the inoculated and the control plants of both resistant and susceptible cultivars were in the width of the isozyme band. The isozyme bands among the inoculated cultivars were wider than those of the control roots at 10 DAI.

A higher level of isozyme was found in the nematode-infected roots, especially in resistant cultivars, with Rf values 0.41 and 0.58, in lanes 1 and 2 (Figures. 1A and 1B: Franklin), and lanes 7 and 8 (Liao dou 10). In the roots from susceptible cultivars, no difference was found whether the nematode -infected or and non-infected plants. An isozyme band was found (Rf value 0.76) in Liao dou 10, but not in Peking and Franklin. Meanwhile, the isoenzyme band (Rf value 0.78)

was only recorded in nematode-infected resistant cultivars at 13 (Figure 1A Peking) and 17 (Figure 1B Franklin) DAI. The other bands in the patterns were not different between resistant and susceptible cultivars

except for the width of the bands (Rf values 0.41 and 0.58); the quantity of isozyme increased with the time in both resistant and susceptible cultivars after inoculation.



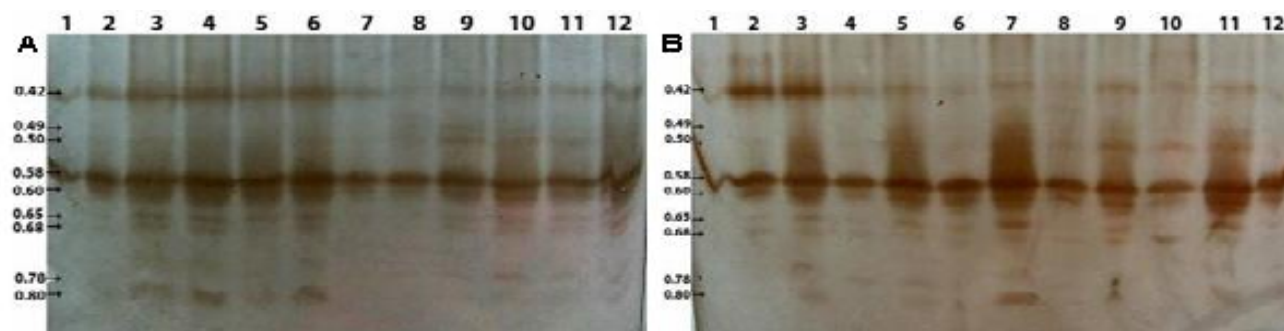
**Figure 1. A and B: The patterns of POD isoenzyme from resistant and susceptible soybean cultivars roots at different time intervals after inoculating *H. glycines* race 3.**

A: Lane 1, 2, 3, 4, 5 and 11. Resistant cultivar Peking, 10 (non-inoculated), 10, 13, 17, 20 and 23 DAI. Lane 6, 7, 8, 9, 10 and 12. Susceptible cultivar Liaodou10, 10 (non-inoculated), 10, 13, 17, 20 and 23 DAI. B: Lane 1, 2, 3, 4, 5 and 6. Resistant cultivar Franklin, 10 (non-inoculated), 10, 13, 17, 20 and 23 DAI. Lane 7, 8, 9, 10, 11 and 12. Susceptible cultivar Liaodou10, 10 (non-inoculated), 10, 13, 17, 20 and 23 DAI.

**The polyphenol oxidase isozyme activity determined by native PAGE:** Figure 2A presents the polyphenol oxidase isozyme pattern. There were nine bands in the patterns, and Rf values 0.42, 0.58, and 0.60 were present in both resistant and susceptible cultivars. No differences in isozyme between resistant and susceptible cultivars in non-infected plants (lanes 1 and 7) were recorded in the electrophoretic mobility ranges. However, a newly induced enzyme band was recorded in the nematode-infected resistant roots at 10, 13, 17, 20, and 23 DAI (Rf value 0.80 in lanes 2, 3, 4, 5, and 6) compared to the non-infected cultivars. The same bands were not found in the nematode-infected susceptible cultivars. From the band width, expression of some isozymes were induced after nematode infection in both resistant and susceptible cultivars (Rf value 0.58 and 0.60); however, there was a greater quantity of polyphenol oxidase isozyme in the resistant cultivars than in the susceptible

cultivars.

Figure 2B presents polyphenol oxidase isozyme patterns of different resistant and susceptible cultivars with or without inoculation at 20 DAI. The major differences between infected and non-infected plants in both resistant and susceptible cultivars are shown. In both resistant and susceptible cultivars, some isozymes were induced (Rf values 0.42, 0.58, and 0.60), and a new polyphenol oxidase isozyme (Rf value 0.80) was recorded in Peking, LiaoK89102, and Franklin, which was not found in Liao dou 10 and Kaiyu 10. However, the isozyme (Rf values 0.58 and 0.60) in Kaiyu 10 was much more intensely stained than that in Liao dou 10. Different mechanisms of susceptibility may exist in the two susceptible cultivars, although they are all susceptible. The two bands (Rf values 0.49 and 0.50) were recorded in susceptible cultivars (Figure 2A lanes 8, 9, 10, 11, and 12; Figure 2B lanes 9, 10, and 11).

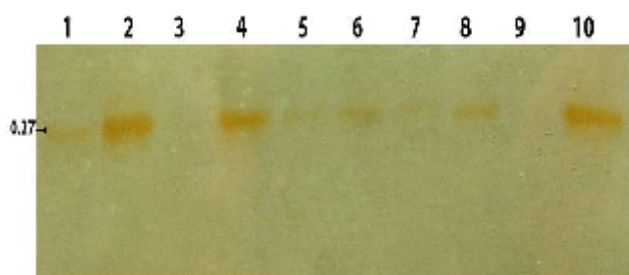


**Figure 2A. Patterns of POD isoenzyme from resistant and susceptible soybean cultivars at different time after inoculating *H. glycines* race 3**

Lane 1, 2, 3, 4, 5 and 6 is resistant cultivar Peking, 10 (non-inoculated), 10, 13, 17, 20 and 23 DAI. Lane 7, 8, 9, 10, 11 and 12 is susceptible cultivar Liaodou10, 10 (non-inoculated), 10, 13, 17, 20 and 23 DAI.

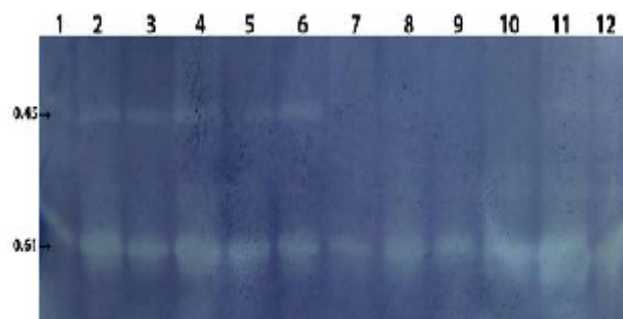
**Figure 2B.** The patterns of POD isoenzyme from roots of resistant and susceptible soybean cultivars at 20 days after inoculating *H. glycines* race 3. Lane1-Peking (non-inoculated 0 DAI), 2-Peking, 3-Peking, 4-LiaoK89102, 5-LiaoK89102, 6-Franklin, 7-Franklin, 8-Liaodou10, 9-Liaodou10, 10-Kaiyu10 (non-inoculated 0 DAI), 11-Kaiyu10, 12-Kaiyu10

**The catalase isozyme activity determined by native PAGE:** Figure 3 is the pattern of catalase isozyme from the roots of different soybean cultivars with *H. glycines* at 20 DAI. Only one enzyme band was observed in both resistant and susceptible cultivars. However, the activity of catalase was greater in nematode-infected Franklin, LiaoK89102, and Kaiyu10 cultivars than those in their controls. In Liao dou 10 and Peking, there were no significant difference between the inoculated treatment and the controls.



**Figure 3.** Patterns of CAT isoenzyme from different soybean cultivars at 20 days after inoculating *H. glycines* race 3. Lane1: Franklin (non-inoculated), 2: Franklin, 3: LiaoK89102 (non-inoculated), 4: LiaoK89102, 5: Liaodou10 (non-inoculated), 6: Liaodou10, 7: Peking (non-inoculated), 8: Peking, 9: Kaiyu10 (non-inoculated), 10-Kaiyu10

**Superoxide dismutase activity analyzed by native PAGE:** The patterns of crude enzyme extract from roots of resistant (Peking, LiaoK89102, and Franklin) and susceptible (Liao dou 10 and Kaiyu 10) soybean cultivars inoculated with *H. glycines* were analyzed by native PAGE (Figure 4). Results showed that two SOD isozyme bands were recorded, and one isozyme (Rf value 0.45) was unique in the Peking cultivar. A small amount of SOD existed in the tissue of soybean plants both inoculated and control. The major difference between inoculated and non-inoculated plant was the lightness and width of enzyme band. The same isozyme band (Rf value 0.61) was present in the resistant and susceptible cultivars, and low activity of superoxide dismutase was expressed in the control plants. The high activity was induced by nematode infection in both resistant and susceptible cultivars. High superoxide dismutase activity was induced earlier in the Peking cultivar at 10 DAI than in the Liao dou 10 cultivar at 17 DAI.



**Figure 4.** Patterns of SOD isoenzyme from different soybean cultivars at different time after inoculating *H. glycines* race 3.

Lane 1, 2, 3, 4, 5 and 6 is resistant cultivar Peking at 10 (non-inoculated), 10, 13, 17, 20 and 23 DAI. Lane 7, 8, 9, 10, 11 and 12 is susceptible cultivar Liaodou10 at 10 (non-inoculated), 10, 13, 17, 20 and 23 DAI.

Meanwhile, SOD isozyme patterns from the *H. glycines*-infected roots of different soybean cultivars at 23 DAI showed that the same two bands were recorded, one (Rf value 0.61) was common to the different soybean cultivars; another (Rf value 0.45) was unique to the Peking cultivar. For the common band, SOD activity was higher in the nematode-infected plants than those in the control both in resistant and susceptible cultivars. We speculate that the unique isozyme band in resistant cultivar Peking is responsible for its nematode resistance.

Information of host defense responses in soybean against *H. glycines* is essential in understanding disease resistance and the development of effective control measures for soybean cyst nematode disease. In this study, electrophoretic analysis revealed the differences in the PPO, POD, SOD, and CAT isozyme patterns of the different resistant and susceptible cultivars. Earlier studies indicate that the peroxidase activity in the roots of resistant cultivars increased more than in susceptible cultivars after nematode infection (Kim *et al.*, 1990). Enhanced SOD activity may also be a result of the non-specific release of free radicals following increased damage to plant tissue during nematode penetration (Rabinowitch and Fridovich, 1983). Enhanced peroxidase and esterase activities may play a role in the lignification of cell walls, which assists in the resistance to penetration by the nematode (Montes *et al.*, 2004). Chitinase isozymes accumulated to a greater extent in the resistant compared with the susceptible cultivars after infestation. The resistant cultivars also had higher chitinase activity and more rapid protein accumulation. The results may be associated with nematode resistance in soybean (Qiu *et al.*, 1997; Wu and Duan, 2004).

Information about enzyme protection against *H. glycines* is not well known. In this study, the results showed that the new peroxidase isozyme band was only recorded in the resistant nematode-infected cultivars after

inoculation, not in the susceptible cultivars. A newly induced polyphenol oxidase enzyme band was also recorded in the nematode-infected resistant roots.

Superoxide dismutase and catalase are important defensive enzymes. Superoxide dismutase can remove superoxide anion and transfer it into  $H_2O_2$ , and catalase can deoxidize  $H_2O_2$  and transfer it into  $H_2O$ .  $H_2O_2$  acts as a signal for gene activation, a trigger for hypersensitive cell death, as well as a powerful antimicrobial agent. Catalase is a very active enzyme that specifically destroys  $H_2O_2$  in cells. The high levels of  $H_2O_2$  required for hypersensitivity response (HR) may be obtained by a strong inhibition of catalase, which must be specific to the tissues involved and limited temporarily. It is currently accepted as plant systemic acquired resistance (Chen *et al.*, 1993). Since spread of  $H_2O_2$  in cells leads ultimately to cell death, cells normally maintain high catalase activity to neutralize this toxic chemical. One of the earliest events considered most important for HR in plants is the generation of active oxygen species (AOS), which produces  $H_2O_2$  (Levine *et al.*, 1994). During the experiment, necrosis was found adjacent to the bodies of juvenile in the roots of resistant soybean cultivars LaioK89102 and Franklin, but not in the susceptible varieties Liao dou 10 and Kaiyu 10 (Wu *et al.*, 2005). However, catalase activity in infected Peking cultivars was not higher than its control (non-inoculated plant) at 20 DAI, which was different from the other resistant cultivars, although it was resistant to soybean cyst nematode as proven in previous research (Concibido *et al.*, 1997; Duan *et al.*, 2008). Therefore, the mechanisms of resistance to *H. glycines* race 3 needs to be examined at the molecular level in future studies.

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