



### Short Communication

## Effects on Trypsin Inhibitor in Roots of Resistant Soybeans after *Heterodera glycines* Invasion

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### Abstract

The relationship of Soybean Trypsin Inhibitor (STI) and soybean resistance to Soybean cyst nematode (*Heterodera glycines* Ichinohe, SCN) were studied by two soybean resistant varieties and two susceptible soybean cultivars. The assay results showed that the activity of STI with inoculation was changed extensively in the soybean roots comparing with the no inoculation. A conclusion that STI had attended the resistance of soybean to SCN was drawn; but the trend curve of four varieties were not consistent, it would open out that there was some difference among four varieties, which were defended against the invading of SCN, that meant the different resistant mechanism in different soybeans. © 2016 Friends Science Publishers

**Keywords:** Soybean trypsin inhibitor; Soybean cyst nematode; Resistance

### Introduction

Plant parasitic nematodes are obligate parasites causing serious reduction in crop yields (Ali *et al.*, 2015). Soybean cyst nematode, *Heterodera glycines* Ichinohe, is the most destructive pest of soybean (*Glycines max*) worldwide, which causes extensive damage to soybean and accounts for over one billion dollars loss annually in the US (Allen and Koenning, 2009). Current nematode control strategies include resistant cultivars, crop rotation, nematicides and biocontrol, but each has serious limitations (Chitwood, 2002). Soybean seeds contain two kinds of proteinase inhibitors, the Kunitz trypsin inhibitor and the Bowman-Birk proteinase inhibitor. These inhibitors are localized within protein bodies and are specific for serine proteases. The Kunitz trypsin inhibitor is the most prevalent soybean protease inhibitor, is represented by a 21-kD protein, and is specific for trypsin (Cecilia *et al.*, 1995). By contrast, the Bowman-Birk consists of several related 8-kD proteins, and inhibits trypsin, chymotrypsin and elastase (Cecilia *et al.*, 1995). High levels of enzyme inhibitors found in the seeds of many plant species serve as storage or reserve proteins, as regulators of endogenous enzymes, and as defensive agents against attacks by animal predators and nematodes or microbial pests (Pernas *et al.*, 1999). Trypsin inhibitor (TI) is the most extensively studied enzyme inhibitor. Direct evidence of TI were involved in plant defense. TI gene in tobacco increased host resistance against herbivorous insects (Hilder *et al.*, 1987). Antifungal

activities had also been reported for TI proteins from several crops, including TI from barley (Thevissen *et al.*, 1993), trypsin and chymotrypsin inhibitors from cabbage as well as TI from corn (Quang *et al.*, 1992) and acysteine protease inhibitor from pearl millet (Joshi *et al.*, 1998). However, most were described to be active only against a very limited group of pathogens. Therefore, the objectives of the present study were to express this protein in soybean roots, obtain the TI activity and test for inhibition of SCN. We investigate its mechanism of inhibition through the efforts to test its efficacy against SCN.

### Materials and Methods

#### Collecting of Cyst and Eggs

Soil was collected from a field at the Shenyang Agricultural University Farm in May or June. This particular field, used for several years for SCN screening, was known to have high populations of SCN races. Cysts were extracted by elutriation, placed in a grid dish, and counted with the aid of a stereoscopic microscope. Selected a fine cyst without epiphyte, laid on a sheet of glass, dropped a glob of water, crushed the cyst gently with a anatomical acus, wiped off the crust. The eggs was counted and collected by 2mL tube. Liaodou 18 was planted in no disease soil and inoculated with these eggs. Thirty-five days after inoculation, soil was gently removed from the roots, cysts were removed from the roots and were counted. Then reproduction of cyst was

performed until enough cysts for inoculation were obtained. Cysts were kept in icebox at 4°C (Liu, 1995).

### Plant Material and Inoculation

Two resistant and two susceptible cultivars from maturity groups were grown at the field of Shenyang Agricultural University in 2006. The soybean varieties used were Huipizhi, Harbin, Zhonghuang 28, Liaodou 18. Soybean seeds were placed on gauze saturated with distilled water, germinated in glass trays in the dark at 25°C, and harvested after 3 days (Liu, 1995).

The soil was treated at 165°C for 3 h to killed microorganism (Liu, 1995). Then the soil (30% sand, 60% soil, 10% vegetable charcoal) was encased in 20-cm-d pots. The four soybean varieties were transplanted by planting in noninoculated soil or inoculated soil. Six thousand eggs were added to a pot. When seedlings reached the first trifoliolate stage, timing sampled, individual plant sampled. Samplings were conserved at -80°C.

### Detecting of Inoculation

After the soybean germinated at 15 days, we sampled the susceptible varieties in a randomized design. We detected with the method of Coloration of nematode tissues (Liu, 1995) and calculated the quantity of nematodes. At 35d, Cysts were investigated from roots and counted with the aid of a stereoscopic microscope.

### Activity Assays

Soybean trypsin inhibitor activity was measured according to the method of American Association of Cereal Chemists and determination of trypsin inhibitor activity of soyabean and soya products (BS EN ISO 14902-2001) except for minor modifications. Trypsin was bought from Amresco Chem. Co. (U.S.A.). N $\alpha$ -Benzoyl-DL-Arginine-P-Nitroannilide Hydrochloride (BANPA) was product of Sigma Chem. Co. (USA). Other chemicals were of analytical or pure grade.

### Results

We are successful to inoculate SCN in the soybean roots through Coloration of nematode tissues and collecting cysts of root. We found that the second-stage juveniles (J<sub>2</sub>), the third-stage juveniles (J<sub>3</sub>) of SCN had invaded the varieties of soybean (Fig. 1). We collected the number of cysts of varieties (Table 1). Liaodou 18 and Zhonghuang 28 were susceptible cultivars; Harbin was a resistant variety and Huipizhi proved to be immune variety to race 3 of SCN. Fig. 2 indicates the curve of the STI activity of four non-inoculated soybean varieties after 35 d timing sampling and assay. We could reach a conclusion that Huipizhi had a high activity (Max: 0.81 mg/ g, Min: 0.4 mg/ g), but Zhonghuang 28 had a low activity in the roots (Max: 0.16 mg/ g, Min: 0.05 mg/ g). Zhonghuang 28, Liaodou 18,

Harbin had the similar change curve. Their STI activity was raised when they developed before 20 days and as the seedlings grew, theirs STI activity began weak. We just considered that STI attended the metabolism firstly as the storage protein and its effect became subdued later.

Fig. 3 is the curve of the STI activity of four inoculated soybean varieties after 35d timing sampling and assay. We also could find that Huipizhi STI activity had fallen all the time and so did Zhonghuang 28. The STI activity of the susceptible Check(CK) Liaodou 18 and resistant Harbin were quite high, later fell and reached the lowest at 25 d, had come back at last. For the inoculation was at the first trifoliolate stage of seeding, SCN had invaded the roots heavily at 15 days. It is confirmed that SCN had stimulative effect to soybean roots, resulted in producing of STI and also indicated that the inoculation was successful.

As SCN invaded, the STI activity of every variety changed. The resistant varieties had high expression. The susceptible varieties STI expression was also high but was lower expression distinctly than the resistant. The STI activity of inoculated roots was higher than the STI activity of inoculated roots at 15 days. The expression of Zhonghuang 28, Liaodou 18 and Harbin were outstanding, but Huipizhi was slight. The STI activity of all varieties was falling after SCN inoculated. Liaodou 18, Harbin became rising at 25 days and became stable when SCN had achieved one life cycle at 35 days. Zhonghuang 28 that did not contain STI in seeds had a sharp rising when SCN invaded, arrived at 0.33 mg/ g, was almost equal with Liaodou 18. Then, the STI activity decreased all the time. At 35 days, the inoculated STI activity was 0.13 mg/ g, but the non-inoculated STI activity was only 0.05 mg/ g. It meant to increase 260% after inoculation comparing with CK. So we could clear about that whether soybean is resistant or not, STI would increase after inoculation and STI was correlated with soybean resistance.

### Analyzing of the Single Variety

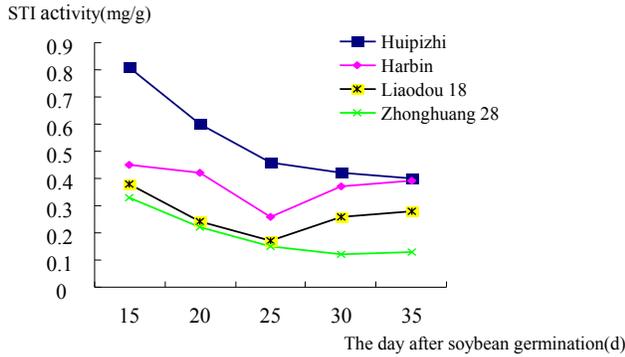
As shown in Fig. 4, the STI activity of Huipizhi was falling all through even that SCN had invaded. However, it was a climax time of the invading of J<sub>2</sub> at 15d, the STI activity of inoculation (0.81 mg/g) was higher than the STI activity of non-inoculation (0.77 mg/g). The STI activity of inoculated roots was smooth after 25 days. This is shown that SCN had stimulation effect and caused the STI activity changed, not distinctly.

From Fig. 5, the enormous effect to Zhonghuang 28 with inoculation can be visualized. Zhonghuang 28 is a special cultivar; its seed had no STI expression, but in the growth, it had STI expression. Although we can see that the STI activity of non-inoculation was low at the beginning, the STI activity of inoculation was very high. Zhonghuang 28 had the most cysts quantity at the detection that predicated the resistance to 3 race of it was quite bad. STI may attend the course of resistance, but could not

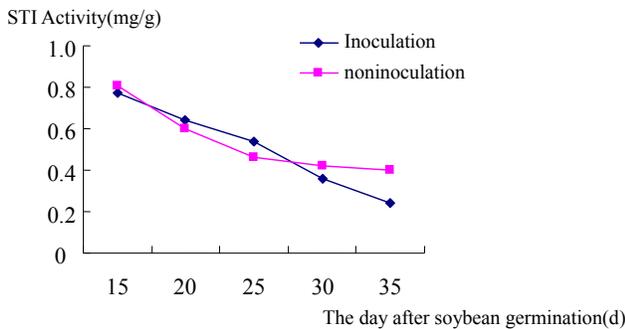
**Table 1:** The number of cysts in the roots of soybeans

Soybean varieties	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean	R or S
Zhonghuang 28	32	17	25	30	28	26.4	S
Liaodou 18	16	24	15	20	21	19.2	S
Harbin	2	3	2	3	3	2.6	R
Huipizhi	0	0	0	0	0	0	R

\*The number of cysts that is over 10 confirmed "S", on the contrary is "R"  
 "R" is short for resistant, "S" is short for susceptible



**Fig. 3:** The STI activity of inoculated soybean varieties



**Fig. 4:** Analyzing with STI activity of Huipizhi

stop SCN invading or spreading in the roots for lower activity.

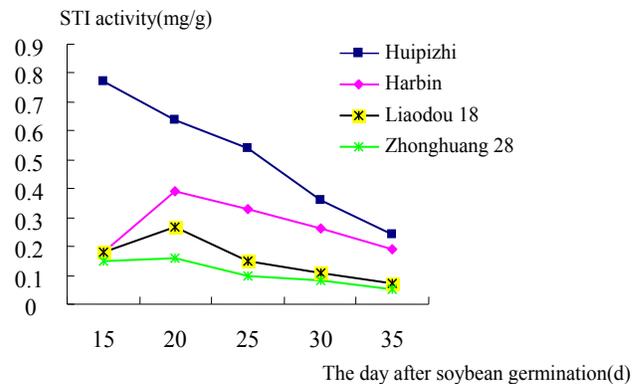
From Fig. 6, it is clear that STI took part in normal metabolism in non-inoculation soybean. The activity of STI rised in inoculated soybean at first, then went down. The activity of Liaodou 18 and Harbin went up together. The only difference was the content of STI. Harbin was higher than Liaodou 18 all the time; maybe this was why Harbin is more resistant than Liaodou 18.

**Discussion**

Every plant would start a series of itself defense system when they suffered threat of pathogen. At the period, they spontaneously adjust the content of enzyme system to protect themselves and avoid, prevent or stop the pathogen invading and spreading in the tissue. So, the resistance to SCN takes on preventing, stopping SCN reproduction and so on. From our test, the activity of SCN is distinct higher

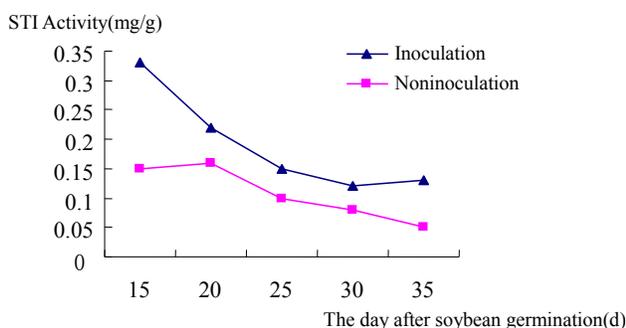


**Fig. 1:** J<sub>2</sub>, J<sub>3</sub> and the female in the soybean roots  
 A: J<sub>2</sub> invaded in the soybean roots. B: the middle stage between J<sub>2</sub> and J<sub>3</sub> invaded in the soybean roots  
 C: J<sub>3</sub> invaded in the soybean roots. D: the female invaded in the soybean roots

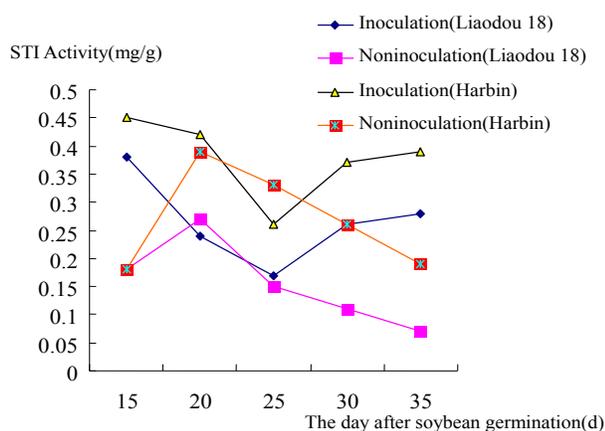


**Fig. 2:** The STI activity of non-inoculated soybean varieties

than CK. It means that STI had assisted the resistance to SCN. All varieties were invaded by large quantities of J<sub>2</sub> from our coloration of nematode tissues, which means that STI had little contribution to prevent SCN for invading. But in the later cysts detecting, the resistant varieties had no cyst or few cysts that indicated STI had a active effect on stopping reproduction of SCN. For SCN has the activity of trypsin itself, it will excrete trypsin when it takes food (Lilley *et al.*, 1997). Soybean releases STI to restrain SCN and stop spreading when stimulating by SCN. STI could result that the body of *Globodera pallida* became



**Fig. 5:** Analyzing with STI activity of Zhonghuang 28



**Fig. 6:** Analyzing with STI activity of Liaodou 18 and Harbin

smaller and the ratio between male and female was changed (Arora, 1983). It might be the resistant mechanism of STI, but STI could not restrain SCN directly (unpublished data).

The activity of STI will be different because SCN invading are in all kinds of environment such as soil, humidity, light. If environment condition is equal, the different varieties will be the only reason of the different activity of STI. The resistant variety has not a good strategy to prevent SCN invading. Mass of  $J_2$  will stab roots, but they could not develop cysts. They are buried because of the defense system of the resistant variety. The susceptible cultivars are in a hole. Lots of  $J_2$  invade their roots and procreate their generation. Although they also excrete STI or something else to prevent or stop SCN, they could not resist the SCN for the lower content of STI or something else. To get to the bottom of the heterogeneity of different varieties is an important question of this study. Huipizhi, this immune variety, could not defend  $J_2$  invading, had few  $J_3$ , had no female later and had no cyst at last. Its STI activity was very high and went down all along even SCN had invaded. Something else resist SCN and the effect was more powerful. The trait of STI, this single defense protein, was not expressing better for other defense system. Zhonghuang 28, this special cultivar short of STI, was powerless to defend SCN. Even its STI activity went up

rapidly, it acquired the last place for the most number of cysts. Liaodou 18, the susceptible check, had a similar place with Zhonghuang 28. Harbin, the other resistant variety, had a different place. Its STI activity was in a low state at no inoculation and a high state at inoculation.

## Conclusion

The contribution of STI was revealable in its roots. This is a inducing system resistance. Certainly, it also had other defense system but not expressed better than Huipizhi. We know that STI has two sorts, Kunitz and Bowman-Birk; but which one is working in chief or they work together? They have different activity for different varieties. Kunitz is researched in resistant pests deeply, but BBI is about animal medicine mostly. We could not make sure which is useful. So the STI mechanism need thorough search.

## Acknowledgements

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