

Chemotaxis of *Phytophthora sojae* Zoospores to Soybean Roots is Altered by  
Isoflavone Silencing

Kara Riggs

Advisor: Dr. Terrence Graham

May 27, 2010

**Table of Contents:**

Introduction	2
Materials and Methods	5
Results	7
Discussion	9
Acknowledgments	11
Figures	12
Literature Cited	16

## Introduction

Using their complex biochemistry, plants have been beating the incredible odds of survival for thousands of years. Because plants cannot uproot and escape danger, they must face pathogens with defense mechanisms that will stop infection. Unfortunately for plants, pathogens have evolved to tolerate the defense tactics of their hosts. As research advances, plant-pathogen interactions continue to reveal high levels of complexity. The interactions between soybean (*Glycine max*) and its major pathogen *Phytophthora sojae* do not make exception to this observation.

Soybean stem and root rot are caused by the pathogen *Phytophthora sojae*. In 2005, *P. sojae* cost soybean farmers \$250 million in yield suppression (Wrather, 2006). As shown by Brett Tyler, *P. sojae* is highly specialized to recognize soybean roots in the soil (Tyler, 1996). Daidzein and genistein, two isoflavones that are prevalent in soybean, are attractive to *P. sojae* in sub-nanomolar amounts (Morris and Ward, 1992). The high sensitivity of *P. sojae* towards daidzein and genistein presents a challenge in controlling diseases caused by the pathogen, since recognition of the host is the first step of pathogenesis.

Zoospores are the infecting bodies of *Phytophthora sojae*. The spores are biflagellate and free-swimming, capable of propelling themselves through soil in wet conditions. Zoospores swim in a helical pattern up to speeds of 100-200 um/sec depending on temperature and species (Carlile, 1983).

Navigation towards soybean roots is controlled by chemotaxis (chemo- “chemical,” – taxis “movement”). Chemotaxis is due to the high chemotactic sensitivity of zoospores towards the isoflavones daidzein and genistein (Morris and Ward, 1992). Nearly 90% of the daidzein and genistein that is found in a soybean root is located immediately behind the root tip (Graham

1991). The high concentration of isoflavones in this section of the root, the zone of elongation, is very chemoattractive to the zoospores. Therefore, the zone of elongation is an ideal area to observe zoospore chemotaxis.

After arriving at the host root, zoospores turn away briefly from the root and rest their flagella against the root surface. The zoospores then encyst, dropping their flagella and attaching to the surface via adhesion proteins (Deacon and Donaldson, 1993). After encystment, the pathogen creates a germ tube to enter the host. *P. sojae* releases enzymes to digest the polymers in soybean cell walls, breaking the cells apart. This provides the pathogen its required room for growth without entering the cells. Unfortunate to the soybean, the plant can identify the pathogen only after infection has begun (Ebel, 1986). Interestingly, the soybean's limited sensitivity to pre-infection events is a sharp contrast to the pathogen's sub-nanomolar recognition of isoflavones present in root exudates (Morris and Ward, 1992).

One proposed method of plant defense is pathogen avoidance. Because zoospores are dependent on chemotaxis to find soybeans in the soil, the opportunity presents itself to hide the roots by silencing the production of their chemoattractive chemicals. If the root no longer exudes the chemicals that zoospores use to navigate, the zoospores should have decreased ability to find the root in the soil. If zoospores cannot find the root, they cannot enter the tissue, and infection will be prevented.

The enzyme isoflavone synthase (IFS) controls the production of the isoflavones, daidzein and genistein. Knocking down this enzyme, by silencing the expression of the genes encoding IFS (Subramanian, et al., 2005), stops the synthesis of daidzein and genistein (See Figure 1). IFS silenced roots should become the unattractive to *P. sojae* zoospores because they will contain neither daidzein nor genistein. Previous research from the Graham lab shows

decreased attraction to IFS silenced roots, although results were highly variable (K. Riggs, T. Graham, unpublished data).

Roots without gene silencing should attract the normal number of zoospores to the most appropriate zone for infection. These control roots will contain the natural levels of isoflavones and portray the chemotaxis that takes place in the field between soybean roots and *Phytophthora sojae* zoospores.

A novel method of defense against *P. sojae* is preventing encystment. Zoospores cannot infect a host if they cannot encyst on the root. Because *P. sojae* zoospores are dependent on chemotaxis, the zoospores should have severe difficulty navigating to a root that does not exude chemoattractants. By altering their chemoattractive nature to pathogens, the disease resistance of soybeans and similar legumes could improve. Although fascinating, the preliminary data mentioned above are the results seen with a single race of *P. sojae*. These findings need to be confirmed through extensive testing. Various races and isolates of *P. sojae* must be studied to confirm the trends in chemotaxis are not race specific.

This project determines the attraction of several races (races 1, 7, 25) of *P. sojae* to soybean roots that contain decreased levels of genistein and daidzein. Roots will be silenced by knocking down the enzymes chalcone reductase and isoflavone synthase, two enzymes that are involved in daidzein and genistein synthesis. The zoospore confusion already achieved by preliminary tests of silencing chalcone reductase and isoflavone synthase proves that the visibility of hosts to their pathogens can be prevented or directed to another root zone. Confirmation that these patterns are not race and isolate specific will extend the application of this research possibly to all root-zoospore interactions in the field.

## **Materials and Methods**

### *Soybean Varieties and Zoospore Races*

The most popular soybean varieties grown today are largely based on the Williams soybean variety as background. The field isolates of several *P. sojae* races (races 1, 3, 7) pathogenic on this line are used in the chemotaxis assay.

### *Growth of Soybean Plants*

Williams soybean (*Glycine max* L.) seedlings are grown at 26°C with 500 uE/m<sup>2</sup>/s of light, receiving 14 hours of light each day. After five days, the drying seed coats are removed to prevent pinching of the cotyledons. After seven days, the cotyledons are harvested for use.

### *Initiation of Transgenic Soybean Roots*

Roots are initiated from soybean cotyledons using the hairy root generating organism, *Agrobacterium rhizogenes*. This same organism is used to transform soybean with an RNAi construct to silence genes for the enzyme isoflavone synthase (IFS) involved in isoflavone biosynthesis.

Ten cotyledons are twisted from seven-day-old soybeans. Only unblemished cotyledons are counted for experimentation. The lower epidermis of the cotyledon is sterilized by gently rubbing it with a white felt square that is soaked in 70% ethanol. A single-edged razor blade is also sterilized using the similar technique.

A thin piece of the lower epidermis is sliced from the cotyledon, beginning at the petiole with a steady, downwards motion. The depth of the cut exposes the large vein running through

the center of the cotyledon. At least half of the vein remains in the cotyledon. The width and length of the area that is removed measures approximately  $5.0 \times 10^{-1}$  cm in diameter.

The cotyledons are placed in a Petri dish that is lined with damp filter paper. 20 ul of *Agrobacterium rhizogenes* (optical density at 600 nm, 0.5) with the RNAi construct for isoflavone synthase (IFS) silencing is applied to the exposed areas on the cotyledons. Additional cotyledons are transformed with *A. rhizogenes* containing a blank vector without a silencing construct. A green fluorescent protein (GFP) marker is used to select successfully transformed roots (Figure 2).

After five to seven days, the cotyledon transformation sites are treated with 100 ug/ml carbenicillin to kill the residual *A. rhizogenes*. Sterilizing the cotyledon surface with carbenicillin prevents *A. rhizogenes* from spreading to the roots and interfering with the interaction between the zoospores and the root surface.

The roots are allowed to grow from the cotyledons for three weeks (Figure 3). The plates are given 14 hours of light each day ( $500 \text{ uE/m}^2/\text{s}$ ). The filter paper on the bottom of the plates is kept damp by applying sterilized water. This creates humidity inside the plate and prevents the cotyledons and roots from dehydrating (Subramanian et al., 2005).

#### *Chemotaxis Assay*

2 cm of the root (including the tip) from a transformed root is sliced off with a sterile razor blade. Only the end closest to the harvest site is handled or touched with tweezers. The tip of the root is placed in the center of a 35 mm petri dish. Water suspensions of zoospores are introduced on top of the root, breaking surface tension and submersing the root. Two concentrations of zoospore suspensions were used: 300 zoospores/ml and 500 zoospores/ml.

Photographs were taken of the elongation zone of the root every ten seconds for a period of five minutes. After each minute, the number of zoospores attached to the root periphery was recorded (Figure 4).

The number of zoospores that encyst on a silenced root are analyzed in comparison to the number of encysted zoospores on a non-silenced root. Zoospores that encyst on a root are also referred to as attracted zoospores. If the number of attracted zoospores is higher on IFS-silenced roots than non-silenced roots, they are more chemoattractive than wild type roots.

The elongation zone of the roots are collected for LC-MS analysis. The analysis confirms the silencing phenotypes and allows a precise correlation of specific metabolites to biological activity.

#### *P. sojae* Zoospore Initiation

Liquid *P. sojae* cultures are started in 500 ml of soybean broth. When the culture is five days old, the suspended mycelial mat is washed and kept in 4°C for 24-36 hours. To produce a suspension of 300 z/ml, the cultures are taken out of 4°C and wrapped in foil for 12 hours at room temperature. One hour before use, the cultures are unwrapped from the foil. To produce a suspension of 500 z/ml, the cultures are taken out of 4°C and left for 24 hours at room temperature before use.

## **Results**

Two concentrations of zoospores are tested for their chemotactic response to transformed roots. The two concentrations that are used, 300 z/ml and 500 z/ml, give unexpected results.



The two concentrations show different zoospore behavior patterns instead of a decreased or amplified response of the same behavior.

#### *500 z/ml Suspension:*

Roots transformed with an IFS-silencing construct are half as attractive to the 500z/ml suspension than non-silenced roots. After five minutes, the average number of zoospores attached to the elongation zone of IFS-silenced roots is 16 zoospores. An average of 28.5 zoospores are attracted to non-silenced roots under the same conditions.

The rate of zoospore attachment is measured by counting the number of zoospores along the root after each minute. Over the span of five minutes, zoospores (500 z/ml) introduced to IFS-silenced roots attach to the root at a linear rate. In contrast, the zoospores introduced to non-silenced roots attach to the root at a polynomial rate. Zoospore (500 z/ml) chemotaxis to the different roots appears to occur with the same success for a period of two minutes after introduction to the root. After this period, the chemotaxis of zoospores to non-silenced roots becomes more efficient, as encystment on non-silenced roots occurs at a much higher rate.

#### *300 z/ml Suspension*

IFS-silenced roots are more attractive to zoospores (300 z/ml) than non-silenced roots. An average of 7.1 zoospores attach to the elongation zone of IFS-silenced roots after 5 minutes. An average of 4.1 zoospores attach to non-silenced roots under the same conditions. On average the zoospores (300 z/ml) favor encysting on roots without isoflavones over roots that contain isoflavones.

The rate of zoospore (300 z/ml) attraction to IFS-silenced roots over five minutes is linear. The rate of zoospore (500 z/ml) attraction to non-silenced roots is polynomial, but the data is also analyzed with a lower  $R^2$  value as a significant linear curve.

## **Discussion**

The number of zoospores attached to IFS-silenced roots after five minutes is similar in both concentrations of zoospores. Regardless of the population density of the zoospores, they chemotax toward IFS-silenced roots at a constant rate. The linear accumulation of zoospore attachment may reflect a non-specific encystment on the root as they simply collide with it. Although zoospores are known to encyst randomly on physical structures upon impact, it remains possible that the root may provide additional factors that favor encystment upon impact, but which are chemotaxis independent. If this is true, the interesting possibility exists that the signals affecting chemotaxis and encystment are different. This could be explored in future experiments.

The polynomial accumulation of zoospores to non-silenced roots demonstrates chemotactic behavior. At high concentrations of zoospores, isoflavones play a role in chemotaxis that is exponential in nature. As zoospores encyst on the root, the rate of attachment and encystment on the root increases among the remaining zoospores. Taken alone, zoospore (500 z/ml) chemotaxis data suggest that chemotaxis is dependent on an isoflavone chemical gradient. As isoflavones diffuse into the suspension, zoospores swim up the gradient toward the root. As time goes on, more and more zoospores interact with the gradient, and the rate of zoospore chemotaxis increases. This explanation is not able to reconcile the decreased rate of chemoattraction of zoospores at lower numbers (300 z/ml) toward isoflavone-rich roots.

*P. sojae* zoospore chemotaxis toward soybean roots is affected by zoospore population density. We hypothesize that signaling between zoospores may play a key role in chemotaxis toward soybean roots. A signal between the zoospores may complement the detection of isoflavones to facilitate chemotaxis. If such a signal were not needed to find the root, chemotaxis of low population density zoospores to isoflavone rich roots would have been higher than chemotaxis to isoflavone-less roots. This proposed signal between zoospores is necessary for chemotaxis in the presence of isoflavones.

It is also possible that isoflavones are deterrents of chemotaxis in low population density zoospore suspensions. Zoospores favor encystment on isoflavone-less roots when their population density is low. This could occur because the isoflavone gradient emanating from the root overwhelms the zoospores. In the presence of genistein alone, zoospores begin encystment in the medium without adhering to surfaces (Riggs and Graham unpublished data). Thus a certain number of zoospores may begin their encystment process when they come into contact with isoflavones, setting a limit to the time remaining before their flagella detach. Zoospores in low population density would be unable to detect a signal from surrounding zoospores and find the root in time before the flagella are dropped.

What is the nature of the signaling between zoospores? It has been shown by Rivera-Vargas et al. that *P. sojae* can readily hydrolyze malonyl glucosyl daidzein (MGD) into free daidzein (Rivera-Vargas 1993). It is possible that zoospores are attracted to free daidzein but not MGD. As free daidzein is released by individual zoospores, surrounding zoospores may be attracted to the area. MGD may act as a repellent or overwhelming sensory chemical, disabling the zoospore from localizing the root in low population densities. If isoflavones conjugates themselves were helpful in chemotaxis, zoospore attraction to isoflavone-rich roots would have

been higher than random encystment on isoflavone-less roots, even without signaling help from neighboring zoospores.

### **Acknowledgments**

This research has been supported by funds appropriated by the Ohio General Assembly to The Ohio State University, Ohio Agricultural Research and Development Center.

All research was conducted in Dr. Terrence Graham's lab, Room 474, in Kottman Hall on the Columbus campus of The Ohio State University. The isoflavone synthase silencing construct was graciously provided by Dr. Oliver Yu of the Donald Danforth Plant Science Center in St. Louis, Missouri. The RNAi strain of *A. rhizogenes* carrying the IFS silencing construct was prepared by Madge Y. Graham.

## Figures

Figure 1. The Phenylpropanoid Pathway (Yu 2005). The enzyme isoflavone synthase (IFS) outlined in blue is involved with the production of daidzein and genistein.

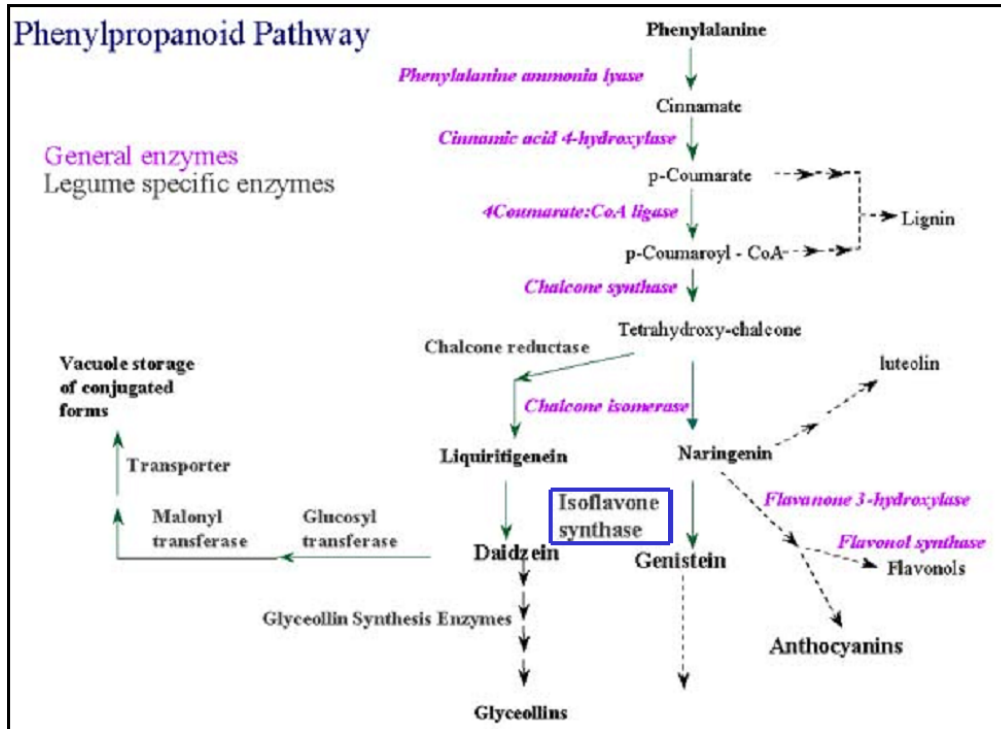


Figure 2. GFP (Green Fluorescent Protein) is inserted along with the RNAi construct to identify successfully transformed roots.

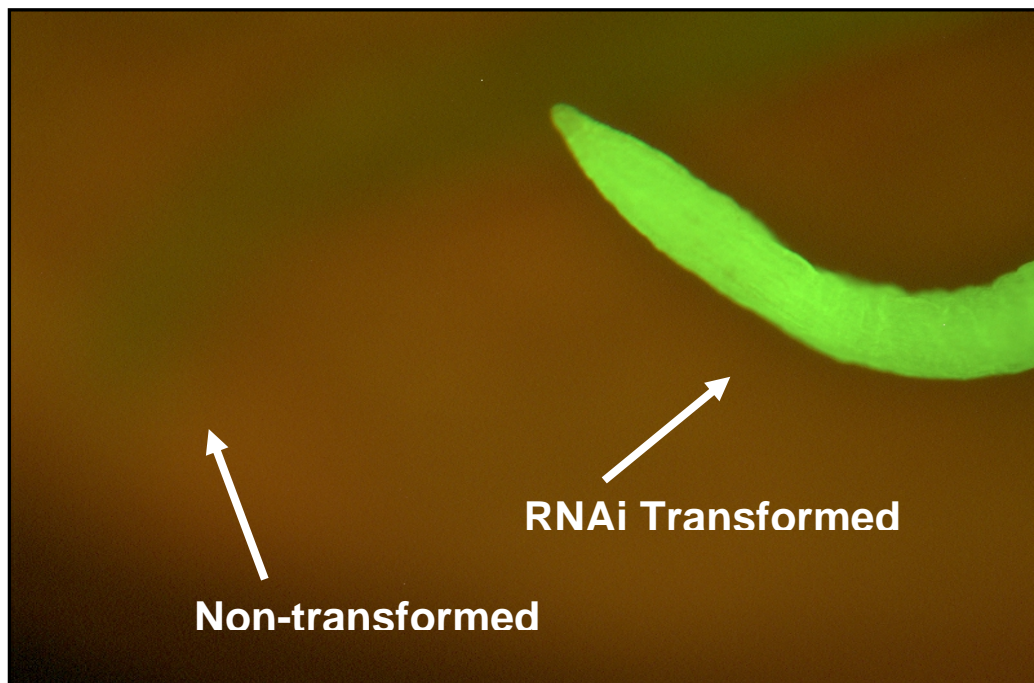


Figure 3. The growth of the initiated roots from a callous ridge (Day 2) to mature roots (Week 3).

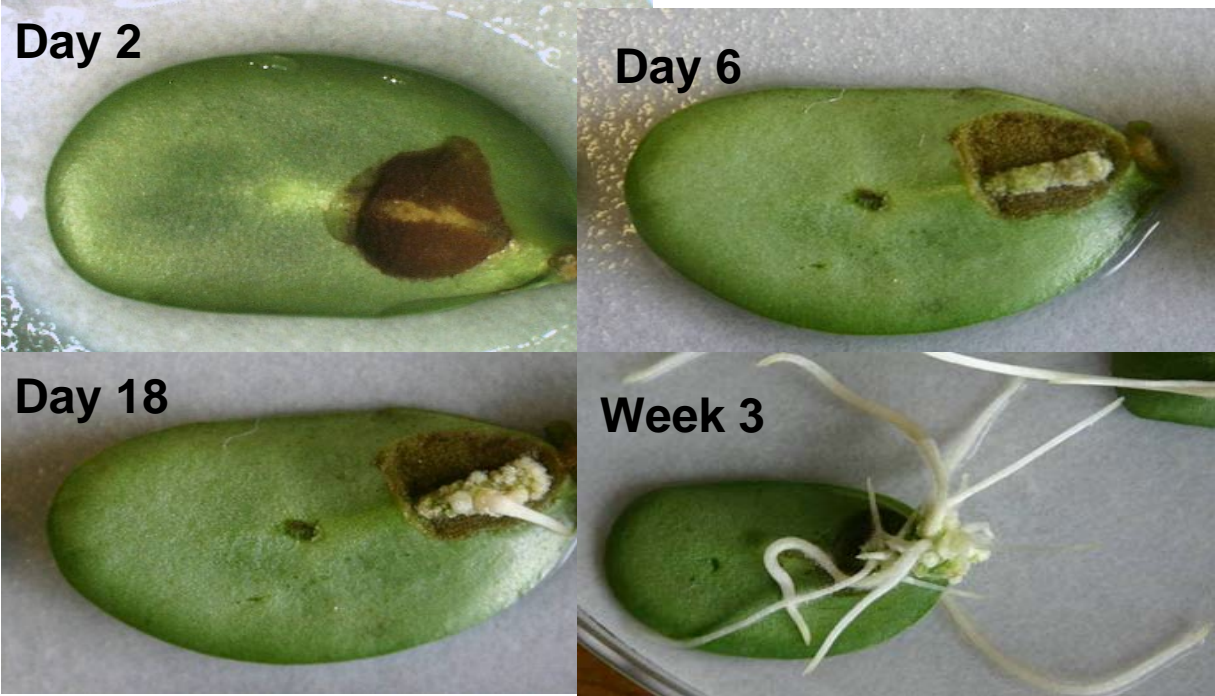


Figure 4. A transformed soybean root with zoospores encysting on the root periphery in the elongation zone (E).

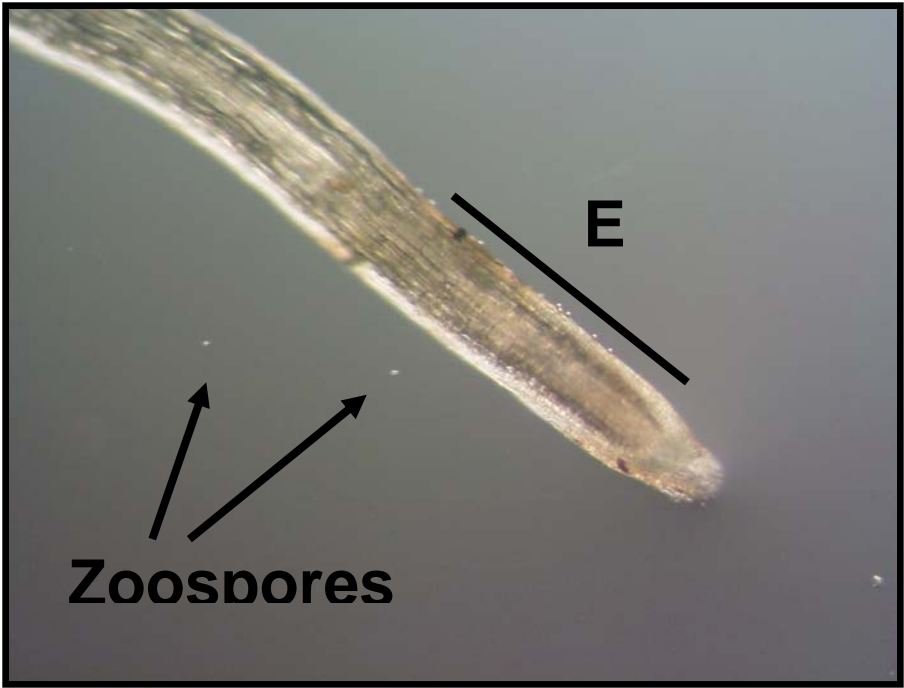


Figure 5. The average number of *P. sojae* zoospores attracted to the roots at the elongation zone, where 90% of the isoflavone population is present in non-silenced roots. Averages represent attachment to the roots five minutes after zoospore introduction.

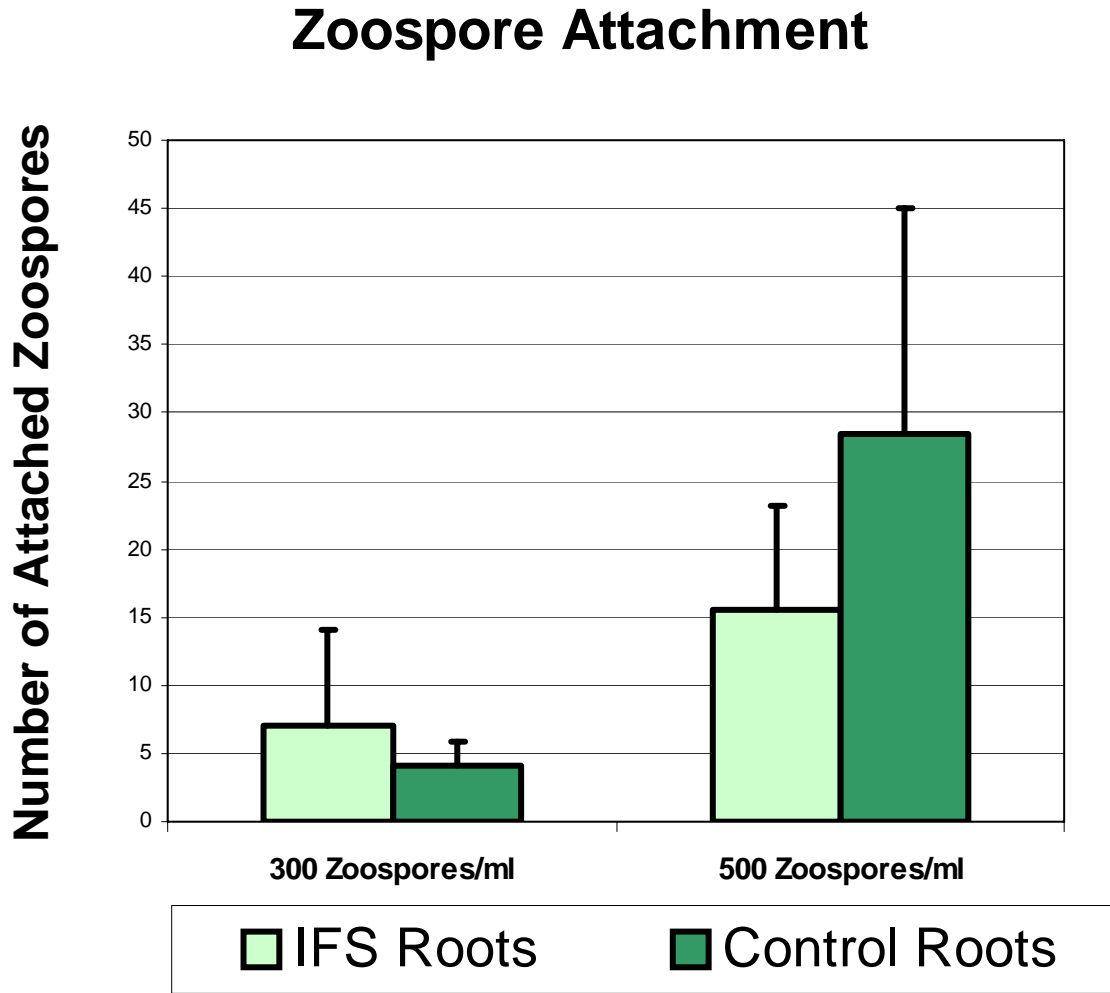
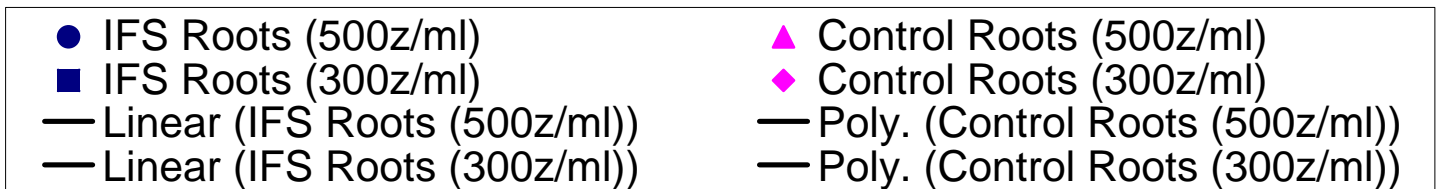
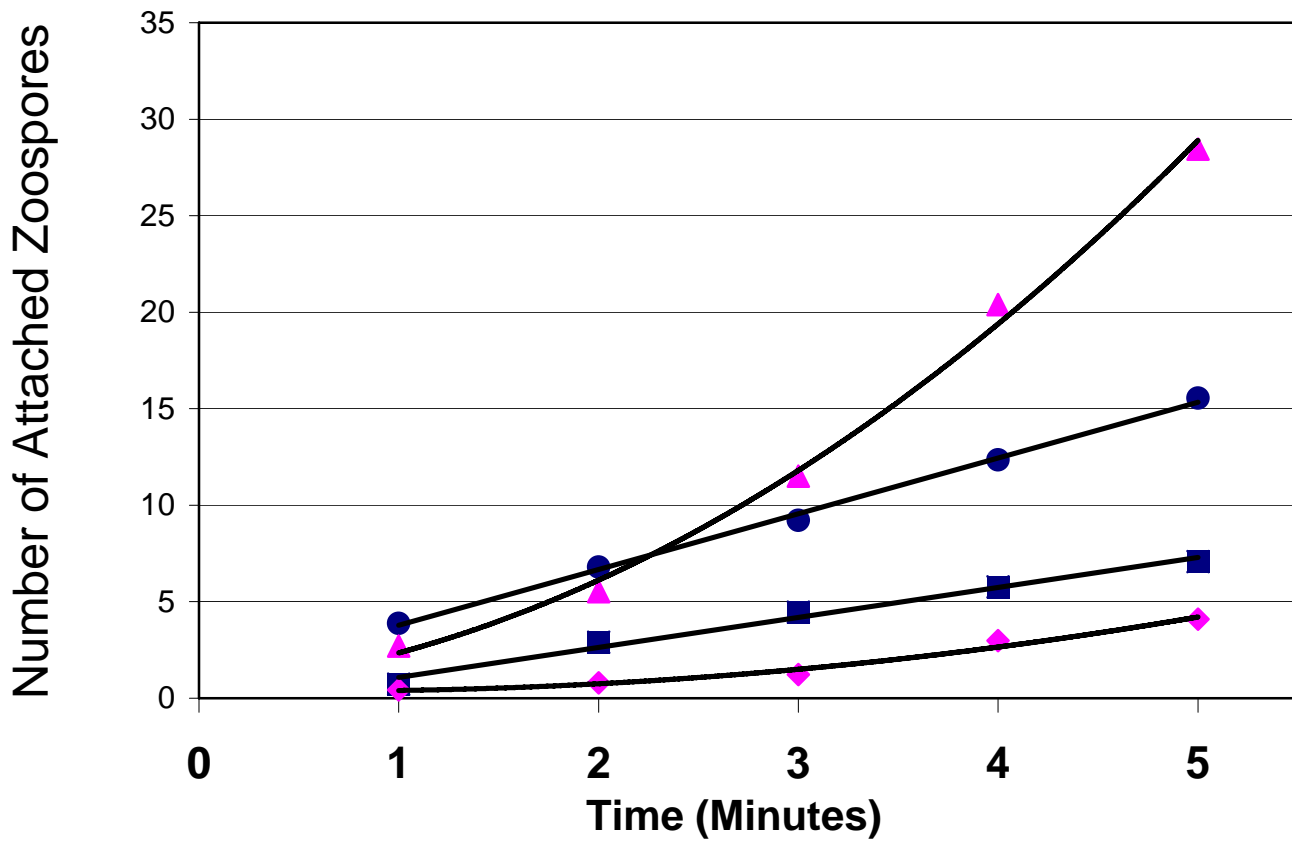


Figure 6. The rate of *P. sojae* zoospore attachment to the roots at the elongation zone using two concentrations of zoospore cultures.

## Zoospore Attachment Rates





## Literature Cited

- Carlile, M. J. (1983). Motility, taxis and tropism in *Phytophthora*. In *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology* (ed. DC Erwin, S Bartnicki-Garcia & PH Tsao) pp. 95-107. American Phytopathological Society, St Paul.
- Deacon, J.W., Donaldson, S.P. (1993). Molecular Recognition in the homing response of zoosporic fungi, with special reference to *Pythium* and *Phytophthora*. *Mycological Research*, 97, 1153-1171.
- Graham, T. L. (1991). Flavonoid and Isoflavonoid Distribution in Developing Soybean Seedling Tissues and in Seed and Root Exudates. *Plant Physiology*, 95, 594-603.
- Morris, P. F., Ward, E. W. B. (1992). Chemoattraction of zoospores of the soybean pathogen, *Phytophthora sojae*, by isoflavones. *Physiological and Molecular Plant Pathology*, 40, 17-22.
- Rivera-Vargas, L.I., Schmitthenner, A.F., Graham, T.L. (1993). Soybean flavonoid effects on and metabolism by *Phytophthora sojae*. *Phytochemistry (Oxford)*, 32, 851-857.
- Subramanian, S., Graham, M. Y., Yu, O., Graham, T. L. (2005). RNA interference of soybean isoflavone synthase genes leads to silencing in tissues distal to the transformation site and to enhanced susceptibility to *Phytophthora sojae*. *Plant Physiology*, 137, 1345-1353.
- Tyler, B. M., et. al. (1996). Chemotactic preferences and strain variation in the response of *Phytophthora sojae* zoospores to host isoflavones. *Applied and Environmental Microbiology*, 62(8), 2811-2817.
- Wrather, J. A., Koenning, S. R. (2006). Estimates of disease effects on soybean yields in the United States 2003 to 2005. *Journal of Nematology*, 38, 173-180.

Yu, O. (2005). *Enzymatic Interactions*. November 12, 2005,

<http://www.danforthcenter.org/yu/researchfiles/enzymfiles/enzym.htm>.