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**EFFECT OF SOYBEAN PRODUCTS AND OTHER HOST
PRODUCTS ON HATCH OF THE SOYBEAN CYST
NEMATODE (*HETERODERA GLYCINES* ICHINOHE)**

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

MARY ELIZABETH MOBERG

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

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Mary Elizabeth Moberg
346 words

Abstract

The soybean cyst nematode, *Heterodera glycines*, is an economically devastating pathogen of soybeans. The eggs of this nematode can survive within the cyst for eleven years. Current control measures consist primarily of careful use of resistance and crop rotation. New means to control this pathogen are critical.

In 1982, Japanese researchers isolated a hatching factor for *Heterodera glycines*, glycinoeclepin A, from kidney beans. This factor induced hatching apart from host plants. The extraction method yielded 1.25 mg of glycineoclepin A from one metric ton of kidney beans roots. The isolation technique is not practical for commercial purposes. Others have attempted to synthesize the chemical. The synthesis of glycinoeclepin A was achieved, but required 32 steps – also not a commercially viable option. In this thesis, an egg hatch bioassay was developed to conveniently monitor hatch. Treatment solution (80 µl) combined with approximately 100 *Heterodera glycines* eggs, concentrated by centrifugation, were pipetted into each well of 96 well microtiter plates. This technique allowed the testing of many plant products on hatch, a high number of replications and did not rely on estimations. Soybean soapstock, a waste product following soybean oil refinement, was chemically extracted using varying methods. The resulting

extracts were analyzed for effect on hatch of *Heterodera glycines* eggs. Soybean soapstock (25%) at pH 5.0 resulted in significantly increased hatch ($P=0.05$) compared to the control, di/RO (distilled/reverse-osmosis) water. Diluted soybean soapstock (1.0 and 10%) resulted in hatch that was similar to that in di/RO water. Extracts following preparative TLC and chloroform-methanol extraction of soybean soapstock resulted in increased hatch compared to the control, di/RO water. The hatching component was determined to consist of a triglyceride. The isolation technique developed by Masamune *et al.* in 1982 was simplified in an attempt to recover hatching factor(s) from roots of soybeans and kidney beans. Following the final 18 hour liquid to liquid extraction using chloroform, the resulting extract from soybean caused hatch that was significantly greater ($P=0.05$) than that from di/RO water and 3 mM ZnSO₄. An apparent hatching factor from soybean roots was successfully isolated using this modified technique.

Chapter 1: Literature Review

Effect of Soybean Cyst Nematode on Soybean Production

The soybean (*Glycine max* (L.) Merrill) originated in northeastern China and was domesticated around 1100 BC. Upon introduction into the United States in the early 1800's, soybeans were primarily used for forage. However, in the second half of the 1900's, the popularity of soybeans grew to the extent that they became a major grain crop in this country (Poehlman, 1979).

Soybeans are a primary source of vegetable oil and protein. The oil is used in margarine, salad oils, cooking oil, shortening, inks and herbicide adjuvants. The soybean meal that remains following oil extraction is used in swine, poultry, fish and pet food. Soybean products are also becoming important ingredients in high-protein foods and beverages for human consumption (Riggs and Schmitt, 1989). Currently, investigators are exploring possible uses in plastics, fibers, edible films and coatings. Researchers in health and nutrition are also investigating potential cancer prevention properties in soybean products (United Soybean Board, 1993).

During 1987-1988, the United States produced 51.8 million tons of soybean oilseed that represented over 51% of world output on approximately 58 million acres (23 million hectares) (Schmidt, 1989). Soybeans are grown on over 116 million acres (or over 46 million hectares) worldwide. In 1991, Minnesota ranked third nationally in soybean production, and soybeans accounted for 14.3 percent (over \$1 billion) of the cash receipts from farm marketings. Soybeans and soybean

products annually account for approximately \$430 million of Minnesota's \$1.8 billion in agricultural exports (Brungardt, 1991).

An increase in number and severity of soybean diseases has coincided with the expansion of soybean acreage throughout the world. In 1994, total yield losses caused by *Heterodera glycines* Ichinohe, the soybean cyst nematode (SCN), in the ten top soybean producing countries including the United States, Brazil, China, Argentina, India, Canada, Paraguay, Indonesia, Italy and Bolivia were greater than those caused by any other disease (Wrather *et al.*, 1997). In the United States, soybean disease loss estimates from 1994 to 1996 in sixteen southern states showed that the SCN caused the greatest economic loss (\$275.38 million), followed by charcoal rot (\$159.22 million), root and stem rots (\$84.43 million) and root-knot and ectoparasitic nematodes (\$79.50 million) (Pratt and Wrather, 1998).

Disease losses for twelve north central states (these states account for 80% of the total soybean production in the United States) from 1989 to 1991 were estimated at an average of 13.17% and 6.442 million metric tons (Mg) for production loss. This is equivalent to an estimated loss of \$1.3 billion/year due to soybean diseases (Doupnik, 1993). The primary yield-suppressing disease for soybeans in Illinois, Indiana, Iowa, Kansas, Michigan, Minnesota, Missouri, Nebraska, North Dakota, Ohio, South Dakota and Wisconsin was found to be SCN, causing a loss of 1,322,693 Mg (this equals approximately \$267.4 million),

followed by *Phytophthora* root rot at 929,877 Mg (Table 1). In Minnesota, SCN has been identified in 44 counties and is considered the state's number one soybean disease problem (Sperbeck, 1994; S. Chen, personal communication).

Table 1. Summary of north central region soybean disease loss estimates by disease (1989-91). Values are the three-year average production loss for each disease for the north central region in thousands of metric tons (Mg). Equivalent bushels (in millions) are also shown (adapted from Doupenik, 1993).

Three-Year Average Production Loss (Mg) of soybean from diseases.

	Mg x 10 ³	Bu x 10 ⁶
Soybean cyst nematode	1,322.693	48.609
Phytophthora root rot	929.877	34.173
Other seedling diseases	751.455	27.616
Charcoal rot	686.993	25.247
Brown stem rot	553.333	20.335
Rhizoctonia/Pythium root rot	493.932	18.152
Septoria brown spot	266.557	9.796
Pod and stem blight	253.904	9.331
Sudden death syndrome	175.619	6.454
Stem canker	135.673	4.986
Diaporthe/Phomopsis complex	117.142	4.305
Sclerotinia stem rot	72.353	2.659
Anthracnose	64.898	2.385
Bacterial diseases	56.299	2.069
Virus diseases	55.700	2.047
Downy mildew	55.401	2.036
Seed diseases	52.952	1.946
Fusarium disease	50.530	1.857
Other nematodes	44.952	1.652
Cercospora leaf spot	25.251	0.928

The above figures help explain why the SCN is considered the most serious nematode pest of soybean in the United States (Riggs and Kim, 1992). These loss estimates are particularly striking considering that it is difficult to get a true disease loss estimate for the SCN for numerous reasons. The SCN can cause losses consisting from a slight depression in yield to total crop failure. The severity of damage is influenced by soil type, weather, cultivar and nematode population levels in the soil. This nematode affects the root functions of the soybean including uptake and transport of water and minerals. Infected plants may show symptoms of poor root development, fewer root nodules, stunted top growth, chlorosis and reduced pod and seed production. These are all symptoms of general root damage and, hence, not specifically diagnostic for SCN (Dropkin, 1984). Frequently, growers are unaware they have a nematode problem and may attribute yield decline to other numerous biotic and abiotic factors that cause stunting and chlorosis (Noel, 1992). Also, significant yield loss may occur in the absence of any obvious field symptoms (Young, 1996).

The soybean cyst nematode was originally identified as a soybean root parasite in Japan in 1915 from plants with symptoms of the disease referred to as "soybean yellow dwarf disease" (Noel, 1986). The SCN has been currently identified in eleven countries including Japan, People's Republic of China, the

Amur region in eastern Asia, Korea, Taiwan, Indonesia, Columbia, Canada, United States, Brazil and possibly Argentina. In the United States, SCN was first discovered infecting soybeans in North Carolina in 1954 and has since been found in a total of 27 states (Table 2) (Noel, 1992; Mendes and Dickson, 1992). SCN was identified in South Dakota in 1996 (Smolik, 1996).

Table 2. Year of recognized infestation and percentage crop loss due to *Heterodera glycines* in North America during 1987.

State or Province	Year of Recognized Infestation	Loss (%)
<u>United States</u>		
Alabama	1973	4.0
Arkansas	1957	4.0
Delaware	1978	3.0
Florida	1967	1.0
Georgia	1976	1.0
Illinois	1959	4.0 ^a
Indiana	1968	4.0 ^a
Iowa	1978	— ^a
Kansas	1985	— ^a
Kentucky	1957	4.0
Louisiana	1967	2.5
Maryland	1978	3.0
Michigan	1987	— ^a
Minnesota	1979	— ^a
Mississippi	1957	0.3
Missouri	1956	4.0
Nebraska	1986	— ^a
New Jersey	1982	— ^a
North Carolina	1954	5.0
Ohio	1987	— ^a
Oklahoma	1975	0.5
South Carolina	1971	4.5
Tennessee	1956	3.0
Texas	1983	<0.1
Virginia	1958	2.0
Wisconsin	1981	— ^a
<u>Canada</u>		
Ontario	1987	—

^a 1989 data; — = crop loss either ≤ 1.0% or data unavailable.

Adapted from Noel (1992).

The question as to whether *Heterodera glycines* is indigenous to the United States or introduced has been examined critically, but not resolved. Findings from two-dimensional electrophoresis studies showed that certain US populations of the nematode are more closely related to each other than to a Japanese population, suggesting that the US populations may be indigenous. Another consideration, however, was the possible inadvertent import of SCN with soil brought in from China and Japan in the 19th century for *Rhizobium* studies. Its effects could have gone unnoticed until intensive soybean production occurred following World War II (Noel, 1986).

The primary economic hosts of SCN include the following legumes: *Glycine max* (soybean), *Phaseolus vulgaris* (green and kidney bean), *Phaseolus aureus* (mung bean), *Pisum sativum* (green pea) and *Lespedeza striata* (common lespedeza). Table 3 lists hosts for reproduction for *Heterodera glycines* (listed by order, family and species) (Riggs, 1992).

Table 3. Hosts of the Soybean Cyst Nematode.

Centrospermae	
Caryophyllaceae	
	<i>Cerastium vulgatum</i> (mouse-ear chickweed)
	<i>Dianthus chinensis</i> (rainbow pink)
	<i>Gypsophila rosea</i> (gypsophila)
	<i>Stellaria media</i> (common chickweed)
Chenopodiaceae	
	<i>Cycloloma atriplicifolia</i> (winged pigweed)
Geriales	
Geraniaceae	
	<i>Pelargonium</i> sp. (geranium)
Tropaeolaceae	
	<i>Tropaeolum peregrinum</i> (canary-bird-flower)
Rhoeadales	
Capparidaceae	
	<i>Cleome spinosa</i> (giant spider-flower)
Cruciferae	
	<i>Cardamine parviflora</i> var. <i>arenicola</i> (bitter-cress)
	<i>Lunaria annua</i> (honesty)
Rosales	
Leguminosae (Fabaceae)	
	<i>Aeschynomene virginica</i> (sicklepod)
	<i>Amphicarpa bracteata</i> (hog-peanut)
	<i>Astragalus canadensis</i> (Canadian milkvetch)
	<i>Astragalus corrugatus</i>
	<i>Astragalus falcata</i>
	<i>Astragalus glaucophyllus</i>
	<i>Astragalus inflexus</i>
	<i>Astragalus mexicanus</i>
	<i>Astragalus podocarpus</i>
	<i>Astragalus sinicus</i>
	<i>Cajanus cajan</i> (pigeon pea)
	<i>Caragana arborescens</i> (Siberian peashrub)
	<i>Cassia</i> sp. (senna)
	<i>Cassia tora</i> (sickle senna)
	<i>Cytisus canariensis</i> (canary island broom)
	<i>Clianthus puniceus</i> (parrot beak)
	<i>Colutea arborescens</i> (bladder senna)
	<i>Coronilla emerus</i> (scorpion senna)
	<i>Crotalaria intermedia</i>
	<i>Crotalaria incana</i> (fuzzy crotalaria)
	<i>Crotalaria lanceolata</i> (lanceleaf crotalaria)
	<i>Crotalaria leiolooba</i>
	<i>Crotalaria ochroleuca</i>
	<i>Desmodium ovalifolium</i> (kaimi clover)

Desmodium salicifolium
Genista aethnensis (broom)
Genista sp. (broom)
Glycine gracilis
Glycine max (soybean)
Glycine ussuriensis
Hardenbergia violacea (lilac vine)
Indigofera dosua
Indigofera hochstetteri
Indigofera leptosepala
Indigofera parodiana
Indigofera sumatrana (common indigo)
Laburnum alpinum (scotch laburnum)
Laburnum anagyroides (common goldenchain)
Laburnum sp. (goldenchain tree)
Lathyrus aphaca (yellow pea)
Lathyrus inconspicuus
Lathyrus ochrus
Lathyrus sativa (grass pea)
Lathyrus tuberosus (tuberous pea)
Lespedeza bicolor (bicolor lespedeza)
Lespedeza buerger
Lespedeza capitata
Lespedeza cuneata (sericea lespedeza)
Lespedeza japonica
Lespedeza latissima
Lespedeza stipulacea (korean lespedeza)
Lespedeza striata (common lespedeza)
Lespedeza virgata
Lessertia stricta
Lotus angustissimus (narrowleaf trefoil)
Lotus sp. (trefoil)
Lupinus albus (white lupine)
Lupinus hartwegii (annual lupine)
Lupinus luteus (yellow lupine)
Lupinus mutabilis (tarwi)
Lupinus polyphyllus (perennial lupine)
Lupinus pubescens
Lupinus rivularis (stream lupine)
Medicago arabica (spotted burclover)
Medicago hispida (toothed burclover)
Medicago minima (little burclover)
Melilotus elegans (sweet clover)
Melilotus taurica (sweet clover)
Phaseolus vulgaris (green and kidney bean)
Pisum elatius (wild pea)
Pisum sativum (garden pea)
Podalyria sericea

- Psoralea bituminosa* (Arabian scurfpea)
Psoralea physodes
Sesbania aegyptiaca
Sesbania arabica
Sesbania bispinosa (canicha)
Sesbania exaltata (coffeebean)
Sesbania macrocarpa (Colorado River hemp)
Sesbania paulensis
Sesbania speciosa
Spartium junceum (Spanish broom)
Sutherlandia frutescens (cancer bush)
Trifolium rupelianum = *T. semipilosum*
Trifolium semipilosum (Kenya clover)
Trigonella foenum-graecum (fenugreek)
Vicia caroliniana
Vicia disperma
Vicia hirsuta (tiny vetch)
Vicia lutea (yellow vetch)
Vicia micrantha
Vicia villosa villosa (winter vetch)
Vigna angularis (adzuki bean)
Vigna marina
Vigna wilmsii
- Saxifragaceae
Heuchera sanguinea (coral bells)
- Tubiflorae
- Boraginaceae
Borago officinalis (borage)
- Labiatae
Marrubium vulgare (horehound)
Molucella laevis (bells-of-ireland)
- Scrophulariaceae
Linaria bipartita (toadflax)
Linaria canadensis (oldfield toadflax)
Penstemon digitalis (digitalis penstemmon)
Penstemon albertinus
Penstemon angustifolius
Penstemon glaber
Penstemon grandiflorus
Penstemon ovatus
Penstemon unilateralis
Penstemon wilcoxii
- Solanaceae
Lycopersicon esculentum (tomato)
Physalis franchetti (chinese lantern plant)

Biology and Control of Soybean Cyst Nematode

The life cycle of the SCN consists of six stages including an egg stage, four juvenile stages and an adult stage. First-stage juveniles develop within the egg, then molt to become second-stage juveniles that hatch from the egg and serve as the infective stage of the nematode. This stage of the nematode penetrates the root approximately 1 cm or more behind the root tip, migrates to the vascular tissue where it inserts its stylet, induces syncytium and feeds. Once feeding begins, the nematode enlarges and becomes sedentary. The nematode molts three more times and becomes a male or female adult. The males, which are required for reproduction, mature faster than the females. Following insemination, the female nematodes become filled with approximately 200-600 eggs. Their outer body wall hardens and turns into a cyst upon death and serves to protect the eggs. During the growing season, the female produces a gelatinous matrix that contains some eggs that can probably begin to hatch immediately. Development takes place within the temperature range of 18-32 °C, the optimal being 24-28 °C. The entire life cycle takes three to four weeks to complete. The cysts of *Heterodera glycines* are approximately 560-580 µm x 350-590 µm. Second-stage juveniles are approximately 450 µm long and the eggs approximately 100 µm long. The eggs within the cyst may survive for eleven years or more which is due, in part, to the

protection of the cyst wall and may also be due to a genetically controlled diapause (Riggs and Schmitt, 1989; Young, 1992; Sortland and MacDonald, 1986).

The SCN can be disseminated by wind, water, soil peds, machinery and animals (Edwards, 1988). Basically, any means of moving soil can result in the dissemination of the nematode.

Management options for the SCN consist primarily of sanitation, crop rotation and careful use of resistance. Sanitation is key in preventing the inadvertent spread of the nematode. This includes keeping farm machinery clean to prevent field to field spread. Crop rotation is a highly recommended means of control because there are few economic hosts of SCN. Most states recommend rotation with a nonhost crop (such as corn, sugar beet, sorghum, alfalfa and small grains, along with effective weed control) for two to three years following discovery of a SCN infestation, then a resistant variety. Periodic use of a susceptible variety in the crop rotation scheme should prevent a race shift in the nematode. The decision for each year's choice is based on soil analysis for population levels (Wrather *et al.*, 1992). The goal of control is to lower the nematode population below the economic threshold.

Careful consideration is required when selecting resistant soybean varieties for use in an infested field. In 1988, Riggs and Schmitt identified sixteen possible races of SCN based on cyst development on four differential soybean lines (Riggs

and Schmitt, 1988). Only eight of these races, however, are of concern in the United States (Anand *et al.*, 1994). The inheritance of resistance to *H. glycines* in soybeans is highly complex. Anand and Sharma (1996) in an attempt to elucidate the relationship between SCN resistance against races three and five found that resistance to *H. glycines* consists of a genetic system controlling resistance to more than one race. *H. glycines* populations may be reduced to below detectable levels by rotating resistance genes. However, if some populations of *H. glycines* have higher allelic frequencies for parasitism, this approach may not be as effective. When the allelic frequencies for parasitism of soybean by *H. glycines* in a specific field is not known, it is particularly imperative that resistance sources be rotated following the effective use of nonhost crops (Noel and Edwards, 1996).

Nematicides are generally too expensive to consider as an option and not as many are on the market due to environmental considerations. However, they can serve a role in soybean production by improving yields of tolerant or resistant cultivars in those fields harboring heavy infestations of the SCN (Rodriguez-Kabana, 1992).

Trap cropping is another potential means of nematode control that effectively reduces the nematode population by allowing the nematode to hatch and infect the crop. The crop is then destroyed before the nematode population matures. Trap crops were found to significantly reduce *Globodera tabacum*

(tobacco cyst nematode) populations (LaMondia, 1996). In this study, *G. tabacum* populations were reduced up to 96% by destroying tomato or tobacco grown from three to six weeks. This means of control for other nematode populations, particularly *Heterodera* spp. should be investigated further (Sortland and MacDonald, 1987).

One area of current research focuses on various biological control methods for the SCN. Researchers in Arkansas are investigating the effectiveness of a fungus found parasitizing SCN eggs in the field (Kim and Riggs, 1995; Kim and Riggs, 1998). Tylka *et al.* (1997) found that glucosinolates, secondary plant metabolites, effectively and irreversibly inhibited the hatch of SCN eggs. *Pasteuria* spp. have been found to have potential as biological control agents for nematodes. Atibalentja *et al.* (1998) examined an undescribed species of *Pasteuria* that was found to infect *Heterodera glycines* juveniles. They determined that this species could have potential in an integrated pest management program for SCN control. The fungus *Hirsutella rhossiliensis* was observed to parasitize second-stage juveniles of SCN in soybean fields in Minnesota (Chen, 1997). Others are using pheromones to disorient the male nematodes and prevent fertilization. The pheromone is used in conjunction with the fungus *Verticillium lecanii* to destroy eggs resulting from matings by males that escape the pheromone treatment (Huettel and Meyer, 1992; Meyer and Huettel, 1996). The range of

potential biological control agents is quite diverse, yet none are currently used at the commercial level.

The incorporation of a hatching factor into infested soil in the absence of a host to induce hatching of juveniles and consequently their starvation has been a recently suggested control measure for SCN by many researchers. The isolation, identification, structural determination and chemical synthesis of glycinoeclepin A, a natural hatching factor for the SCN, has been accomplished by researchers in Japan. Because host plants of the SCN presumably synthesize the hatching factor as part of their normal growth processes, it is conceivable that products of these host plants may provide a significant source of the hatching factor. The research proposed for this study involves testing products from soybean and other host plants to determine their effect on hatching.

Schmitt (1991), speculating on the future of control for SCN, reported that research has returned to emphasizing cropping and cultural practices. Improvement on current cropping systems, he suggests, will require further research on the biology and ecology, in particular survival mechanisms, of *Heterodera glycines*. If the dormancy of the nematode could be detrimentally affected during periods with nonhosts, the length of crop rotation would be reduced. Masood *et al.* (1985) consider the process of hatch to be of singular import since it marks the beginning of the life cycle of the nematode and the

resulting host infection process. Morton (1986), when discussing the future of the pesticide industry, inquired as to whether there was any chance that glycinoeclepin A could be used in conjunction with a nematicide. These researchers are clearly searching for a new approach for control of the SCN. The process of hatch along with the effects of a hatching factor, perhaps glycinoeclepin A, certainly merits attention.

Role of Hatching Factors in the Life Cycle of Cyst Nematodes

There are several excellent review articles on hatching in nematodes (Perry and Clarke, 1981; Noel, 1986; Perry, 1986; Perry, 1987; Perry, 1989a; Perry 1989b). Host root diffusates have been shown to stimulate egg hatch of cyst nematodes, although the mechanisms leading to egg hatch are still mostly to be determined.

In their work on the pea cyst nematode, *Heterodera goettingiana*, Perry *et al.* (1983) found that root diffusate appeared to elicit a change in permeability (involving trehalose) of the eggshell and, in effect, removed osmotic stress on the juvenile. The juvenile then takes up water, becomes active and begins behavior that leads to hatching from the egg. These authors proposed a hatching mechanism for this nematode that assumes the permeability of the eggshell is changed by hatching agents, and the permeability characteristics of the eggshell were determined by the lipid layer.

Perry *et al.* (1992) studied enzymatic activity during the hatching of *Meloidogyne incognita* and *Globodera rostochiensis*. These researchers found that lipases appeared to be involved in the structural changes of the egg and subsequent hatching of *M. incognita* and discussed whether the inhibition of lipase activity could prevent the hatch of *M. incognita*. Lipases and other enzymes did not appear to be involved in the hatch of *G. rostochiensis*.

Research on the sugar beet cyst nematode, *Heterodera schachtii*, led Zheng and Ferris (1991) to believe that the host root diffusate is a true stimulant of hatch rather than a basic requirement. Their thorough research suggested that several forms of dormancy exist and provide an enhanced means of survival for this nematode species.

Investigations with corn rhizosphere leachates by Hashmi and Krusberg (1992) showed significant hatch of the corn cyst nematode, *Heterodera zaeae*, after treatment with leachates. Leachate obtained from 25-day-old corn plants that was subjected to extremes in temperature continued to demonstrate hatch-inducing activity. These researchers are currently attempting to isolate, purify and identify this hatching factor(s).

Ibrahim *et al.* (1993) studied the hatching behavior of the rice cyst nematodes, *Heterodera sacchari* and *Heterodera oryzicola*, in relation to age of host plants. They found *H. oryzicola* to be very dependent on root diffusates to induce substantial hatch, but host age was not a factor. Surprisingly, host age altered the hatching characteristics of *H. sacchari*. The dependency of *H. oryzicola* on host diffusate for hatch and consequent dormancy when host diffusate was lacking was thought to be similar to the response of *H. glycines*.

Interestingly, root diffusates may not be critical for egg hatch of root-knot nematodes. Research by Krishnan *et al.* (1986) on the root-knot nematode

Meloidogyne incognita suggested that chemical constituents in root exudate along with soil pH play an important role in the hatching of this nematode. However, Idowu and Fawole (1990), studied the effects of cowpea and maize root leachates on hatch of *Meloidogyne incognita*, speculated that root diffusates may not be important for egg hatch in this genus. They surmised that cowpea and maize likely influence the life cycle of the root-knot nematode in some other way.

While studying the golden nematode, *Globodera rostochiensis*, Brodie (Kaniuka, 1985) considered the value of identifying the hatching factor of this nematode that would allow the inducement of "suicidal hatching" when the host isn't present. He speculated on the value of plant species that have a molecularly similar root exudate that could be used as decoy crops to attract the nematode prior to planting the host crop. Another consideration proposed was the development of compounds that could neutralize the hatching factor and therefore prevent nematode hatch and infection of the potato crop.

Stimulating Egg Hatch of *Heterodera glycines*

Research on egg hatch in *Heterodera glycines* has included studies on the effects of inorganic ions, pH, temperature and root diffusates and the interaction of host age and season on hatching factors in root diffusates. Clarke and Shepherd (1966), pioneers in research on hatching of *Heterodera glycines*, tested the effect of various solutions of inorganic ions on egg hatch of nine different cyst nematodes including the SCN. Zinc ions effectively stimulated hatch of SCN, but these scientists noted that adding zinc ions to the soil would be impractical since the ion is readily retained by clays and other minerals.

Calcium was ruled out from being involved in the hatch of *Heterodera glycines* as it is in other cyst nematodes by Tefft and Bone (1984). The authors speculated on the role of zinc in hatch of this nematode and considered the possibility of a role within a metalloenzyme. The effects of zinc *in vitro* are dependent on concentration, time of exposure, temperature and pH, all indicating a physiological function of the metal ion. They found maximal stimulation of hatching at pH 5.3 for zinc that suggested to them a possible kairomone (similar to a pheromone, but benefits one species) influence and considered that the combination of a plant stimulant and zinc may result in hatching.

Tefft and Bone (1985b) subsequently discovered the occurrence of leucine aminopeptidase (LAP) activity in the egg supernatant of the nematode. LAP is a

metalloenzyme that requires zinc as a cofactor for activity. This enzyme was thought to preferentially hydrolyze peptide bonds in the eggshell of *Heterodera glycines* eggs.

Studying the effects of zinc fertilizers on SCN egg hatch, Behm and Tylka (1994) reported no increase in hatch even at phytotoxic rates. Further research was pursued to determine the effect of zinc fertilizers (zinc chelate and sulfate fertilizers) on soil population densities of SCN. These soil fertilizers were found to be ineffective at stimulating the hatch of SCN likely due to the unavailability of free zinc cations in the soil environment (Behm *et al.*, 1995).

Lehman *et al.* (1971) reported the influence of pH on juvenile emergence and found that maximum hatching occurred at pH 3.5, but was depressed at pH 5.5 for cysts. Eggs in egg masses appeared to be insensitive to pH.

Tefft *et al.* (1982) examined numerous factors for their influence on SCN egg hatch. They found the optimal hatching temperature for free eggs to be 24 °C; egg hatching was maximized at a pH of 6.0 and increasing the concentration of root exudates increased hatch. As others had reported, they too experienced 5-10% egg hatch (a majority of the viable eggs) during the first 2-week period of treatments.

In further research, Tefft and Bone (1985a) found the highest level of hatching activity in root diffusate to be from early reproductive plants at the R1

(flowering) stage. The hatching activity from this stage was in fact two times greater than that which occurred in root diffusate from younger or older plants. Hatching activity from root diffusates was lowest from senescing plants. Tefft and Bone found no increase in activity in diffusate from severed roots and stems nor from leafless plants. Nor did they find an increase in hatch from plants treated with the growth regulators gibberellin or kinetin.

Hill and Schmitt (1989) found that air temperature has a greater influence on egg hatch of *Heterodera glycines* than does plant phenology and plays a significant role in dormancy induction. Temperature was postulated to act directly on the nematode by affecting the permeability of the eggshell or perhaps the phase transition of membrane lipids by which it would limit metabolic functions. It was also speculated, however, that temperature may have an effect on nematode activity indirectly by its effect on host growth.

Ten plant species were tested for their influence on hatching of *Heterodera glycines* eggs by Schmitt and Riggs (1991). Plants tested included four soybean cultivars, red clover, alfalfa, hairy vetch, field corn, sweet corn, cabbage, tobacco, cotton and wheat. Soybeans stimulated the most egg hatching. Soybeans from varieties resistant to *Heterodera glycines* were found to stimulate greater hatch than those from susceptible varieties. Hairy vetch was also found to increase hatch, though few nematodes developed to maturity on this plant. The researchers

commented on some of the conditions that could affect the results of hatching studies: the developmental and physiological state of the eggs, daylength and temperature effects on host stimulant concentration, and possible varying stimulatory effects of exudates at different phenological stages.

Tefft and Bone (1984) observed the influence of seasonality (greenhouse grown soybeans infested with SCN) on hatch in hatching experiments. The highest hatching (percent) occurred during May (approximately a two-fold increase compared to other months).

The effect of many herbicides on egg hatch was reported by Wong and Tylka (1992). The herbicide, acifluorfen (a postemergent soybean herbicide), appeared to significantly inhibit egg hatch and is being further investigated for SCN management purposes. In further studies, this inhibition was found to be reversible once the eggs were removed from the herbicide (Wainwright and Tylka, 1994).

Okada (1971) found the peak in egg hatch occurred when eggs were pre-soaked in water at 25 °C or 30 °C for 1-4 weeks then soaked in the root diffusate at 25 °C. Okada (1972) was the first to prove the existence of a hatching stimulant of the SCN. The stimulant was located in aqueous extract of ultrasonically broken cysts of *Heterodera glycines*. Okada suggested that both a hatching stimulant and

an inhibitory factor reside in the cyst and could serve critical roles in hatch and dormancy.

In a breakthrough in hatching factor studies, Masamune *et al.* (1982) successfully isolated a hatching agent from kidney bean roots that was identified as glycinoeclepin A. Glycinoeclepin A (GEA) is a triterpene carboxylic acid with a molecular weight of 446 and a molecular formula of C₂₅H₃₄O₇. The isolation was accomplished by a tedious procedure that began with the preparation of aqueous extracts from kidney bean roots. Aqueous extracts of kidney bean roots were selected rather than root diffusates due to instability of root diffusates at this low level of purification. The roots of kidney bean plants were harvested just prior to flowering from a one hectare field plot, air dried for three months, then crushed. The compound was extracted from 135 kg of powdered kidney bean roots, fractionated through a variety of chromatographic columns such as adsorption, reversed-phase partition, ion exchange, as well as preparative HPLC. The procedure is outlined in Table 4.

Table 4. Isolation of glycinoeclepin A as its bis(*p*-bromophenacyl) esters (Masamune *et al.*).

Dried and powdered roots of kidney bean “Beni-kintoki” [135 kg (1 ha)]
 ↓Extraction with water (2 kl) (<10 °C)
 ↓Evaporation (<30 °C) under reduced pressure
 Concentrates
 ↓Continuous extraction with chloroform at pH 2-3
 Chloroform extracts A (115 g, active at 10⁻⁵ gml⁻¹)
 ↓EtOAc-aq NaHCO₃
 Acidic extracts B (66.5 g, active at 10⁻⁵⁻⁶ gml⁻¹)
 ↓Charcoal-Celite column (aq acetone)
 Fraction C (5.2 g, active at 10⁻⁵⁻⁶ gml⁻¹)
 ↓Silica-gel column (EtOAc-CHCl₃-AcOH, 15:5:1)
 Fraction D (360 mg, active at 10⁻⁸ gml⁻¹)
 ↓Silica-gel column (Ether-AcOH, 99:1)
 Fraction E (117 mg, active at 10⁻⁸ gml⁻¹)
 ↓Siliconized Hyflo Super Cel column
 (MeOH-H₂O-AcOH-CHCl₃-C₈H₁₇OH, 150:150:2:15:15)
 Fraction F (25.5 mg, active at 10⁻⁸⁻⁹ gml⁻¹)
 ↓Amberlyst-15 (Ag⁺ form) column (EtOH)
 Fraction G (14.4 mg, active at 10⁻⁸⁻⁹ gml⁻¹)
 ↓Amberlyst XN-1005 (Ag⁺ form) column (MeOH)
 Fraction H (1.53 mg, active at 10⁻⁹⁻¹⁰ gml⁻¹)
 ↓*p*-Bromophenacylation
 (*p*-BrC₆H₄COCH₂Br, KHCO₃, crown ether in MeCN, 80 °C)
 ↓Silica-gel column
 Fraction I (p-BPE) (0.91 mg, active at 10⁻⁹⁻¹⁰ gml⁻¹ after hydrolysis)
 ↓Preparative HPLC, MicroPak CN-10 (C₆H₁₄-ether, 45:55)
 Fraction J (p-BPE) (0.19 mg, active at 10⁻¹⁰ gml⁻¹ after hydrolysis)
 ↓Preparative HPLC, MicroPak NH₂-10 (C₆H₁₄-CH₂Cl₂-MeCN, 70:18:12)
 Fraction K (p-BPE) (≈50 µg, active at 10⁻¹⁰⁻¹¹ gml⁻¹ after hydrolysis)
 ↓Preparative HPLC, Hitachi Gel #3011 (MeOH-CH₂Cl₂, 9:1)
 Fraction L (p-BPE) (≈5 µg, active at 10⁻¹¹⁻¹² gml⁻¹ after hydrolysis)
 (=GEA p-BPE)

The researchers (Masamune *et al.*, 1982) found that 5 µg of GEA existed as its bis(*p*-bromophenacyl) ester form and was shown to have active hatching ability at 10^{-11} - 10^{-12} g/ml (parts per trillion range). The analysis of the bis(*p*-bromophenacyl) esters indicated that this active compound was probably a dibasic acid. This finding allowed them to revise the purification procedure and achieve much higher recoveries. Also, at one point the quantity of the compound was deemed too small for any useful structural analysis, so approximately 1 metric ton of kidney bean roots were harvested from a 10 hectare field and extracted once again. This process, outlined in Table 5, yielded 1.25 mg of active compound to be used for structural elucidation.

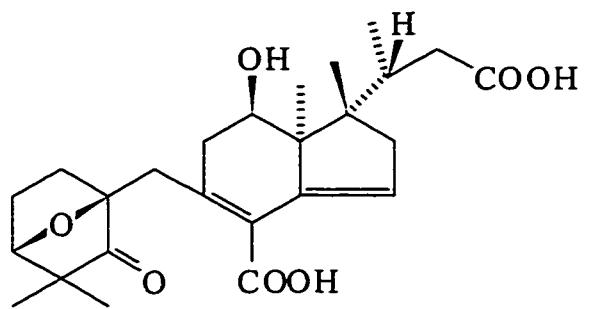
Table 5. Isolation of glycinoeclepin A, glycinoeclepin B and glycinoeclepin C as their bis(*p*-bromophenacyl) esters (Masamune *et al.*).

Isolation of GEA, GEB and GEC as their p-BPEs :

Dried and powdered roots of kidney bean [1058 kg (10 ha)]
 ↓Extraction with water (<10 °C, 15 kl)
 ↓Evaporation (<30 °C) under reduced pressure
 Concentrates
 ↓Continuous extraction with chloroform at pH 2-3
 Chloroform extracts A (1011 g, active at 10⁻⁵ gml⁻¹)
 ↓EtOAc-aq NaHCO₃
 Acidic extracts B (636 g, active at 10⁻⁵⁻⁶ gml⁻¹)
 ↓Charcoal-Celite column (aq acetone)
 Fraction C (197.3 g, active at 10⁻⁶ gml⁻¹)
 ↓Sephadex LH-20 column (MeOH)
 Fraction D (88.9 g, active at 10⁻⁶⁻⁷ gml⁻¹)
 ↓DEAP-Sephadex LH-20 column (72% EtOH-AcOH-NH₄OH)
 Fraction E (18.15 g, active at 10⁻⁷ gml⁻¹)
 ↓Sephadex LH-20 column (CH₂Cl₂-MeOH, 95:5)
 Fraction F (3.59 g, active at 10⁻⁷⁻⁸ gml⁻¹)
 ↓Sephadex LH-20 column (CH₂Cl₂-MeOH, 97:3)
 Fraction G (951 mg, active at 10⁻⁸ gml⁻¹)
 ↓p-Bromophenacylation (*p*-BrC₆H₄COCH₂Br and *i*-Pr₂NEt in MeCN, 20 °C)
 Silica-gel column
 Fraction H (p-BPE) (2057 mg, active at 10⁻⁸ gml⁻¹ after hydrolysis)
 ↓Hitachi Gel #3019 column (MeOH-CH₂Cl₂, 8:2)
 Fraction I (p-BPE) (336 mg, active at 10⁻⁹ gml⁻¹ after hydrolysis)
 ↓Preparative HPLC, μBondapak C18 (MeOH-H₂O, 8:2)
 Fraction J (p-BPE) (79 mg, active at 10⁻¹⁰ gml⁻¹ after hydrolysis)
 ↓Preparative HPLC, μBondapak NH₂ (C₆H₁₄-CH₂Cl₂-MeCN, 76:14:10)
 →Fraction K' (p-BPE) (4.9 mg, inactive ? after hydrolysis)
 ↓μBondapak NH₂ (C₆H₁₄-CH₂Cl₂-MeCN, 60:36:4)
 Fraction K'' (p-BPE) (1.8 mg, inactive at 10⁻⁸ gml⁻¹ after hydrolysis)
 (=GEC p-BPE)
 Fraction K (p-BPE) (8.8 mg, inactive at 10⁻¹¹ gml⁻¹)
 ↓Preparative HPLC, uBondapak NH₂ (C₆H₁₄-CH₂Cl₂-MeCN, 63:33:4)
 →Fraction L' (p-BPE) (2.6 mg, inactive ? after hydrolysis)
 ↓Preparative HPLC, μBondapak NH₂ (C₆H₁₄-CH₂Cl₂-MeCN,
 68:13.2:8.8)
 Fraction L'' (p-BPE) (1.8 mg, inactive at 10⁻⁸ gml⁻¹ after hydrolysis)
 (=GEB p-BPE)
 Fraction L (p-BPE) (1.25 mg, active at 10⁻¹¹⁻¹² gml⁻¹ after hydrolysis)
 (=GEA p-BPE)

These procedures are described in detail by Masamune *et al.* (1987). The structure of glycinoeclepin A was determined by Fukuzawa *et al.* (1985) and is depicted in Figure 1.

Figure 1. The structure of Glycinoeclepin A.



Following structural elucidation, great efforts were undertaken to synthesize glycinoeclepin A (Okawara *et al.*, 1987) and the first total synthesis was published in 1988 (Murai *et al.*, 1988; Murai, 1989). The synthesis involves 32 steps and the overall yield is 0.4%. Others reported varying yields and degrees of difficulty (Watanabe and Mori, 1991; Corey and Houpis, 1990). This chemical synthesis simply does not provide enough of the compound to be economically viable in a SCN management program.

Tylka *et al.* (1992) tested analogs and precursors of the hatching compound, glycinoeclepin A, on egg hatch. They found only one compound, GK1-1991, that stimulated hatch, but not significantly so from the control, zinc sulfate.

Kraus *et al.* (1994) synthesized and evaluated analogs of glycinoeclepin A on hatch of SCN. They found that one analog actually caused inhibition of egg hatch and plan to continue their studies of primarily three analogs.

Due to the difficulties in purifying or synthesizing adequate amounts of GEA, I undertook to find other sources for hatching factors and other methods of partial purification of hatching factors. The purpose of this study was to find a natural product that will induce hatch of *Heterodera glycines* Ichinohe second-stage juveniles from the egg. The objectives included:

1. Develop an effective bioassay system to monitor SCN egg hatch.
2. Determine whether soybean soapstock is a hatching agent for SCN eggs.
3. Partially purify a hatching factor from soybeans and kidney beans using a simplified technique.

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**Chapter 2: A Convenient Bioassay Technique for Counting Nematode Eggs
and Hatched Juveniles of *Heterodera glycines***

Abstract

A new bioassay for *Heterodera glycines* was developed for testing the effect of plant products on egg hatch. The assay utilizes 96 well microtiter plates to quantify egg hatch with multiple replications of each treatment. Treatment solutions (80 µl) combined with approximately 100 *Heterodera glycines* eggs, concentrated by centrifugation, were pipetted into each well. In these studies, up to 12 treatments, including control solutions, were tested simultaneously with 24-32 replications of each treatment. The eggs were observed directly using a stereo microscope. Most egg hatch took place within the first 14 days of the test. This bioassay technique will be a valuable method for testing plant products and other test substances on their ability to stimulate or inhibit *Heterodera glycines* egg hatch.

A Convenient Bioassay Technique for Counting Eggs and Juveniles of *Heterodera glycines*

The soybean cyst nematode, *Heterodera glycines* Ichinohe (SCN), is a serious pest of soybeans around the world. Researchers hope to find an effective means of control of SCN by inducing the hatch and subsequent starvation of juveniles in the absence of a host (Schmitt, 1991; Tylka, 1995). The life cycle of the SCN consists of six stages including an egg stage, four juvenile stages and an adult stage. First-stage juveniles develop within the egg, then molt to become second-stage juveniles that hatch from the egg and are the infective stage of the nematode (Riggs and Schmitt, 1989).

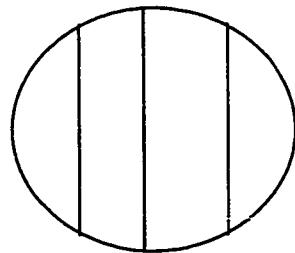
Hatch studies on SCN have found that egg hatch is stimulated by host plant roots, most likely by root diffusates (Tefft and Bone, 1985). In the absence of a host, eggs were found to hatch when incubated in zinc solutions (Clarke and Shepherd, 1966). Hatch from root diffusates is not as great as hatch from zinc solutions. However, it is not practical to apply zinc solutions to the soil so the search for an effective hatch stimulant continues. Because eggs of SCN can remain viable under field conditions for many years, egg hatch stimulants are needed that will result in hatch of the majority of eggs treated. A convenient, accurate and reproducible assay for egg hatch is needed for testing potential egg hatching stimulants.

A few bioassays have been reported for monitoring egg hatch. Tefft and Bone (1984) combined eggs with 200 µl of test or control substances and placed the solutions in the wells of microtiter plates. They used at least 12 wells for each treatment, each containing 10-20 eggs/well. Counts of hatched eggs took place during a 2-week period. In hatch tests using plant leachates, Schmitt and Riggs (1991) constructed hatching chambers consisting of 0.25 µm-pore plastic mesh placed in petri dishes. Root leachate (collected from the bottom of each pot containing soybean plants after pouring water into the pot) was added to the petri dishes to the point where the liquid contacted the screen. After 12 days, juveniles and unhatched eggs were counted. Levene *et al.* (1998), made their egg hatch bioassays by placing 8,000 eggs on a 2 cm circular nylon screen (38 µm pores) that nested in a 3 x 8 x 1 cm plastic tray containing 10 ml of a treatment or control solution. Every three days the hatching screens were transferred to trays containing identical fresh solutions. Following each transfer, the juveniles that passed through the nylon screen into the solution were counted. The remaining unhatched eggs and juveniles in each sieve were counted at the end of the 21-day period. Some of the above techniques require multiple manipulations of hatching chambers, the counting of a subsample of hatched nematodes and the estimation of hatch rather than direct observation. The number of replications in some assays was limited and in some cases only a limited number of eggs could be tested.

A technique using microtiter plates with 96 wells is described that allows for the efficient testing of various plant products on SCN egg hatch. This assay is an accurate, reproducible and convenient means of counting the eggs and juveniles for bioassay purposes.

Materials and Methods

Eggs of the soybean cyst nematode (*Heterodera glycines* Ichinohe race 3) were obtained from Dr. Greg Tylka of Iowa State University, who produced them in greenhouse cultures. Eggs were collected, separated from extraneous debris, surface disinfected with hibitane (chlorhexidine diacetate) and an approximate number per volume was estimated and were then mailed in a vial by Dr. Tylka's lab staff. Untreated flat-bottom 96 well microtiter plates (Dynatech Laboratories) were modified as follows for the bioassay. Three parallel lines approximately 1.5 mm apart were etched with a knife on the outside bottom of each well to aid in counting of nematodes (see below, not to scale).



The eggs were initially kept in suspension in water using a magnetic stirrer and were then concentrated in 10 ml conical Pyrex tubes by centrifugation for 5-7 minutes at 2,750 rpm (IEC Model HN-SII). The supernatant was carefully drawn off using a bulb pipet, replaced with the treatment solution, and the eggs resuspended using a Vortex vibrator. Each treatment consisted of approximately 100 eggs/well plus 80 µl treatment solution/well. Each treatment was repeated in

24 wells. Pipetting was carried out using a Rainin P200 pipette tip trimmed so that eggs did not lodge in the opening.

To limit evaporation, the microtiter plates containing the treatment solutions and eggs were covered with an empty microtiter plate and placed on paper toweling sprayed with water within closed transparent plastic storage containers and stored in darkness in a lab bench drawer at room temperature (approximately 25 °C).

The eggs and any already hatched second-stage juveniles in each well were counted on day "0," to establish a base level; only juveniles were counted on days 7 and 14. Counting was accomplished using an Olympus SZH-10 Research Stereo Microscope (25X) using bottom lighting.

The known hatch stimulants used in these hatch bioassays included: deionized water that had been subjected to reverse osmosis (di/RO), 3 mM ZnSO₄ and soybean root diffusate. The soybean root diffusate was prepared by germinating seeds (cv. Corsoy 79) for one week on dampened paper towels in a covered stainless steel tray (30 cm x 24 cm x 6 cm) at room temperature (25 °C). The seedlings were then inserted into holes (typically 48) drilled through a Styrofoam sheet made to fit an stainless steel tray (30 cm x 24 cm x 6 cm) and support the aerial plant parts. The soybeans were grown hydroponically in Hoagland's nutrient solution (Hoagland and Arnon, 1938) and replenished with

water, as needed. The tray was placed in a growth chamber that provided 14 hours of artificial lighting (24 °C). When soybeans reached the R1 growth stage (initial flowering), approximately 20 plants were removed and the intact roots were placed in a 2-liter glass beaker containing di/RO water (200 – 700 ml, pH 7.0) for four to six days in the growth chamber. The root diffusate was micro-filtered prior to testing.

The treatments were randomized on each microtiter plate. The replications of the treatments in a given microtiter plate, however, could not be randomized. The data for each replication from all plates were combined and the means were separated by Duncan's least significant difference. The results were analyzed by analysis of variance on the raw data and on ranked data with similar results (SAS Institute, Cary, NC).

A number of variables were tested during the development of this hatch bioassay including the number of eggs/well, volume of liquid/well, number of lanes/well, length (days) of hatch test and type of microtiter plate.

Results and Discussion

In order to test plant products on egg hatch of *Heterodera glycines*, a bioassay technique was required that would allow for the convenient testing of many products simultaneously and the monitoring of all eggs in the test solution instead of estimating egg hatch from subsamples. These requirements were met using 96 well microtiter plates. The target of 100 eggs/well was based on the need to have egg numbers high enough to accurately monitor hatch yet also have a manageable number for counting purposes.

In preliminary hatch bioassays, the microtiter plates were irradiated, and the transfer of eggs and treatment solutions into the wells took place under a laminar flow hood. Later in the hatch test studies, this equipment was not used, yet contamination was not a problem. New plates, washed with soap and water, were used for each subsequent test that took place on a laboratory bench. Root diffusate was micro-filtered and the water and ZnSO₄ solution were autoclaved. Microtiter plates from four different manufacturers were tested. The flat-bottom 96 well microtiter plates from Dynatech Laboratories were found to work quite effectively. There was no interference with light reflecting off the sides of the wells that affected the viewing of eggs with the plates from other manufacturers. Lanes were etched on the bottom of each well to aid in counting. Initially, four lines to equal five lanes were attempted, but three lines (1.5 mm apart) to equal four lanes were

found to be sufficient. Varying levels (80 – 200 µl) of the test solutions were pipetted into each well. Higher volumes obscured the viewing of eggs, but with 80 µl of solution the viewing was clear.

Initial studies lasted 21 days (Figure 1) and one study lasted 28 days, but it became obvious that 14 days were sufficient for monitoring the hatch response. Typically, hatch at day 14 would be 1.2 – 1.8 times that at day 7 (Figure 2), and hatch did not increase significantly after 14 days. In initial studies, the numbers of eggs and replications carried out were quite high (approximately 150-300 eggs/well and 32 replications). Using more than 100 eggs/well resulted in clumping of eggs and juveniles, leading to inaccurate counting of hatched eggs. Analysis of data showed that significant separation of means was achieved with 24 replications. It is possible that 16 replications would have sufficed, but this was not tested. This research also confirmed what others including Tefft *et al.* (1982) and Tylka (personal communication) have observed, that a majority of the viable eggs hatch during a two-week period. The lack of further egg hatch after 14 days may have been caused by anoxia. Because fresh solution was not added during the assay, the oxygen supply for the nematodes may have been depleted. In addition, carrying the assay past 14 days increases the risk of contamination to test solutions.

It would be highly advantageous if non-viable eggs could be discerned from the viable ones when determining the final percent egg hatch. The viability stain, Nile Blue A (0.1%, in 10% DMSO) (Meyer *et al.*, 1988), was used without success during this study. The stain was applied to designated wells following counts on day 14, but it reacted with treatments and did not clearly differentiate between viable and non-viable eggs.

The microtiter plates provided a means of monitoring and counting nematode eggs and hatched juveniles using a limited amount of equipment and a limited amount of laboratory space yet also allowed testing of a large number of samples with sufficient replications for an accurate analysis of test solutions. The centrifugation of eggs, consequent suspension and final pipetting of eggs into the wells of the microtiter plates provided a means of even distribution among treatments. This bioassay technique constitutes another method of testing plant products or other test substances on their ability to stimulate SCN egg hatch.

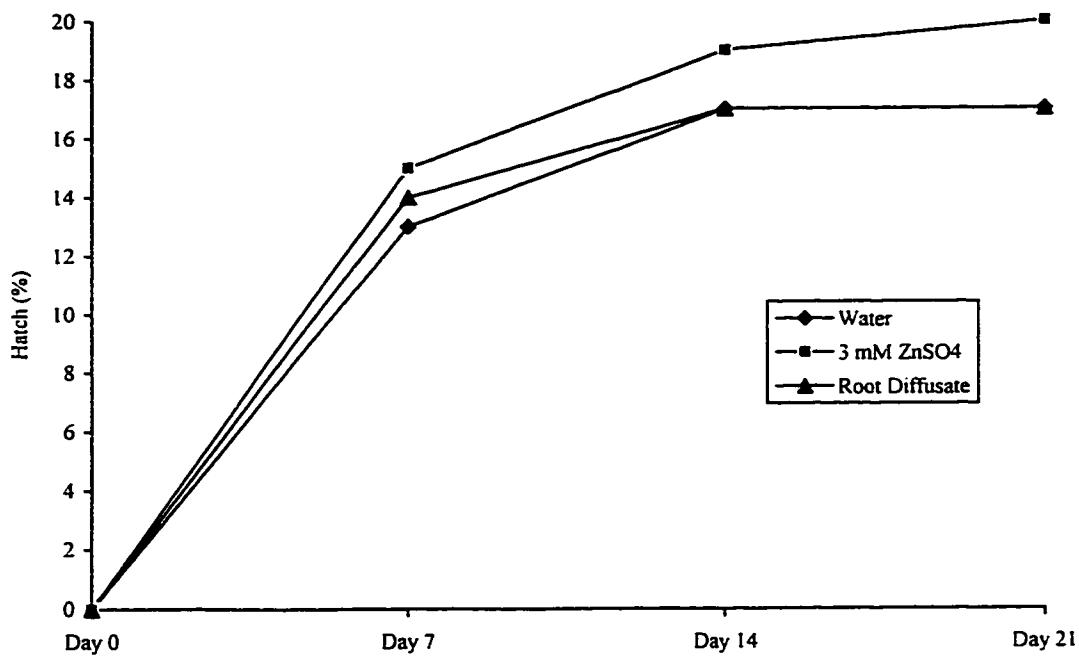


Figure 1. Percentage egg hatch of *Heterodera glycines* exposed to di/RO water, 3.0 mM ZnSO₄ and soybean root diffusate over a period of 21 days. Points are means of 32 replicates.

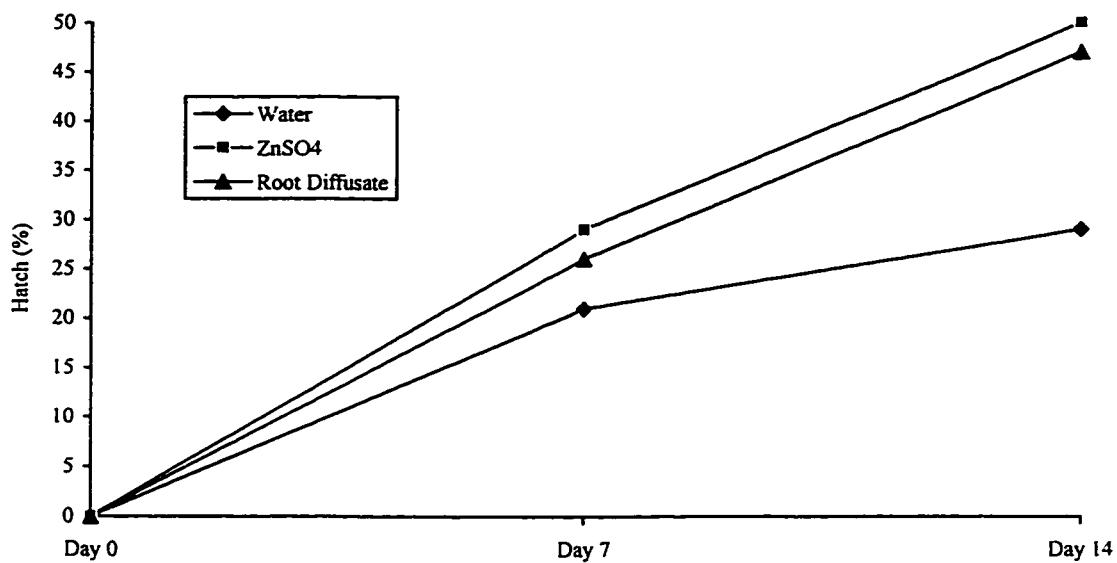


Figure 2. Percentage egg hatch of *Heterodera glycines* exposed to di/RO water, 3.0 mM ZnSO₄ and soybean root diffusate over a period of 14 days. Points are means of 24 replicates.

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Chapter 3: The Effect of Soybean Oil Soapstock on the Hatch of *Heterodera glycines* Ichinohe

Abstract

Soybean soapstock, a waste product of soybean oil refinement, was tested for its effect on hatch of the soybean cyst nematode (SCN), *Heterodera glycines*. Soybean soapstock diluted to 25% at pH 5.0 resulted in significantly increased hatch ($P=0.05$) compared to the control, di/RO water. Diluted soybean soapstock (1.0 and 10%) resulted in hatch that was similar to that in di/RO water. Soybean soapstock was chemically extracted using varying methods, and the resulting components were tested for their effect on SCN using an egg-hatch bioassay. Fractions obtained following preparative TLC and chloroform-methanol extraction of the soapstock significantly increased hatch. The hatching component co-migrated with triglycerides and is most likely a triglyceride or has a triglyceride component. A number of factors affected the assay, primarily the difficulty of working with a nearly opaque substance with an extremely high lipid content. Solvents used to extract test substances also may have affected the assay. A seasonal effect on hatch was noted using greenhouse cultured eggs.

The Effect of Soybean Oil Soapstock on the Hatch of *Heterodera glycines* Ichinohe

In the United States, soybean disease loss estimates from both the southern and northern soybean-producing states showed that *H. glycines*, the soybean cyst nematode (SCN), caused the greatest economic loss compared to any other pathogen (Pratt and Wrather, 1998; Doupenik, 1993). In Minnesota, the SCN has been identified in 44 counties (S. Chen, personal communication).

The life cycle of the SCN consists of six stages including an egg stage, four juvenile stages and an adult stage. First-stage juveniles develop within the egg, then molt to become second-stage juveniles that hatch from the egg and serve as the infective stage of the nematode (Riggs and Schmitt, 1989).

Current measures most widely used to control or primarily manage the SCN include: sanitation (which is really a preventative measure), crop rotation, and careful use of resistance. Nematicides generally are too expensive to be considered as an option, and fewer than before are on the market due to environmental concerns. Trap cropping is another potential means of nematode control that effectively reduces the nematode population by allowing the nematode to hatch and infect the crop. The crop is then destroyed before the nematode population matures. Trap crops were found to significantly reduce *Globodera tabacum* populations (LaMondia, 1996).

Many researchers are searching to find a means of control by better understanding the hatch process in plant parasitic nematodes. In *Heterodera glycines*, zinc ions have been known for some time to effectively stimulate hatch. However, applying zinc to the soil would be impractical because clays and other minerals readily retain the ion (Clarke and Shepherd, 1966). In 1982, Masamune *et al.* successfully isolated a hatching agent for *H. glycines* from kidney bean roots and identified it as glycinoeclepin A. Glycinoeclepin A has been found to stimulate the hatch of SCN eggs at concentrations as low as 10^{-12} g/ml (Masamune *et al.*, 1987).

Morton (1986), when discussing the future of the pesticide industry, inquired as to whether there was any chance that glycinoeclepin A could be used in conjunction with a nematicide. The incorporation of a hatching factor into infested soil in the absence of a host should induce hatching of juveniles and with no host present would result in their starvation. Although glycinoeclepin A, a terpenoid, has been isolated and the structure determined, the synthesis of glycinoeclepin A is long and tedious, requiring 32 steps. Extraction of sufficient glycinoeclepin A for field testing is also impractical. The initial isolation from kidney beans required roots harvested from a 100-hectare field in order to obtain 1.25 mg (Masamune *et al.*, 1987).

Host plants of the SCN may be presumed to synthesize a hatching factor as a part of their normal growth processes. It is conceivable that products of these host plants may provide a significant source of the hatching factor. Soybean seeds may contain hatching factors at low levels or may contain triterpene precursors of glycineoclepin A.

Soybean oil soapstock is an abundant "waste" product of soybean seed oil refinement. Soybean soapstock consists primarily of fatty acids, plant triglycerides and plant steroids. The soapstock, sometimes referred to as acidulated soybean soapstock, has a pH of 5.0 and is a very thick, dark, oily (94-96% fatty acid content) material. See Appendix A for a complete chemical analysis of soybean soapstock.

If the hatching factor(s) could be obtained from soybean soapstock directly, this would be of significant economic importance. Using an egg-hatch bioassay, soybean soapstock and components separated by thin-layer chromatography were tested for effect on SCN egg hatch as were lipid extracts from soybean root, leaf and seed material.

Materials and Methods

Bioassay Technique

Eggs of soybean cyst nematode (*Heterodera glycines*, race 3) were obtained from Dr. Greg Tylka, Iowa State University, who produced them in greenhouse cultures. Eggs were collected in water, separated from extraneous debris, surface disinfected with hibitane (chlorhexidine diacetate), and the approximate number per volume was estimated. Flat-bottom 96 well microtiter plates (Dynatech Laboratories, Chantilly, VA) were modified as follows for the bioassay. To aid in counting of nematodes, three parallel lines approximately 1.5 mm apart were etched on the outside bottom of each well using a knife. The eggs were initially kept in suspension using a magnetic stirrer and were then concentrated in 10 ml conical Pyrex tubes by centrifugation for 5-7 minutes at 2,750 rpm (IEC Model HN-SII). The supernatant was carefully drawn off using a bulb pipet, replaced with the treatment solution (2 ml) and eggs resuspended using a Vortex vibrator. Each treatment consisted of 100 eggs/well using 80 µl treatment solution/well. Each treatment was repeated in 24 wells. Pipetting of eggs was carried out using a Rainin P200 pipette tip trimmed so that eggs did not lodge in the opening.

To limit evaporation, the microtiter plates containing the treatment solutions and eggs were covered with an empty microtiter plate and placed on top of paper

toweling sprayed with water to limit evaporation within closed transparent plastic storage containers and stored in darkness at room temperature (approximately 25° C) in a laboratory bench drawer.

The eggs and any hatched juveniles in each well were counted on day "0" to establish a base level; only juveniles were counted on days 7 and 14. Counting was accomplished using an Olympus SZH-10 Research Stereo Microscope (25X).

The known hatch stimulants included: deionized water following reverse osmosis (di/RO), 3 mM ZnSO₄ and soybean root diffusate. The soybean root diffusate was prepared by germinating seeds (cv. Corsoy 79) for one week on dampened paper towels in a covered stainless steel tray at room temperature (25° C). The seedlings were then inserted into holes (typically 48) drilled through a Styrofoam sheet made to fit a stainless steel tray (30 cm x 24 cm x 6 cm) and support the aerial plant parts. The soybeans were grown hydroponically in Hoagland's nutrient solution (Hoagland and Arnon, 1938) and replenished with water, as needed. The tray was placed in a growth chamber (24° C) that provided 14 hours of artificial lighting. When soybeans reached the R1 growth stage (initial flowering), approximately 20 plants were removed and the intact roots were placed in a 2-liter glass beaker containing di/RO water (200 - 700 ml) for four to six days.

Soybean Soapstock

Soybean soapstock was obtained from Cenex/Land O'Lakes, Inver Grove Heights, MN. Unaltered soapstock was used in hatching bioassays and after adjusting to pH 3.0 with 3% acetic acid or to pH 7.0 with 1% sodium bicarbonate. Soapstock fractions following distillation/extraction, thin-layer chromatography/solvent extraction and liquid chromatography were also tested for effect on SCN hatch. See Table 1 for a general outline of extraction techniques and Table 2 for a schematic on the four extraction procedures used.

Table 1. General outline of the four extraction techniques used on soybean soapstock and other plant material.

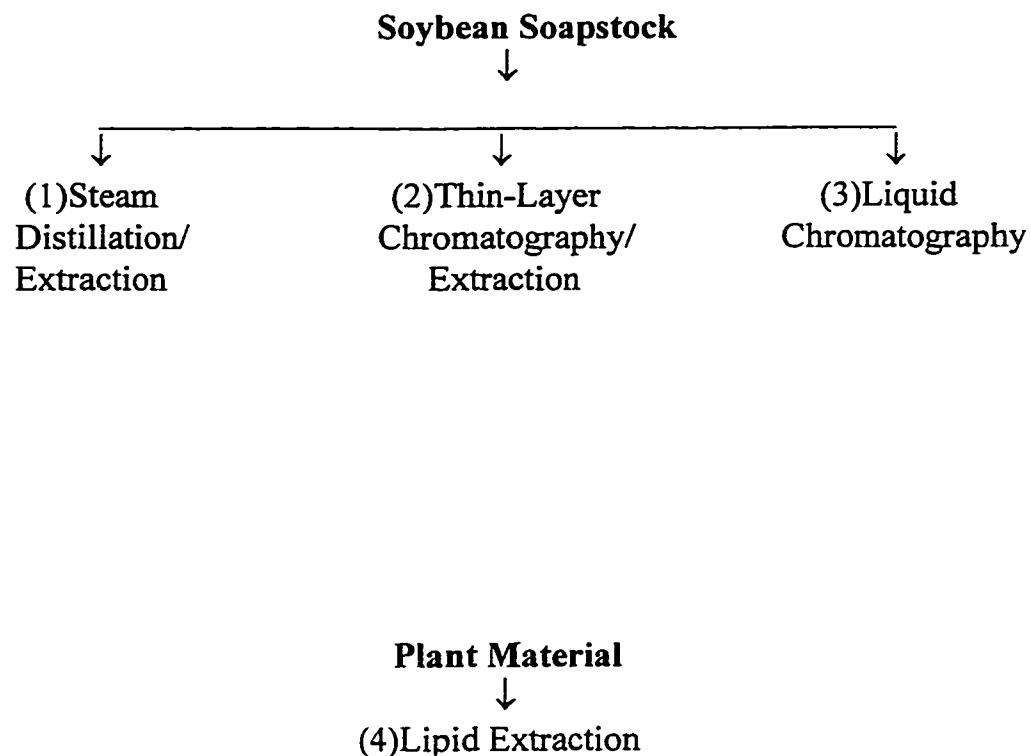
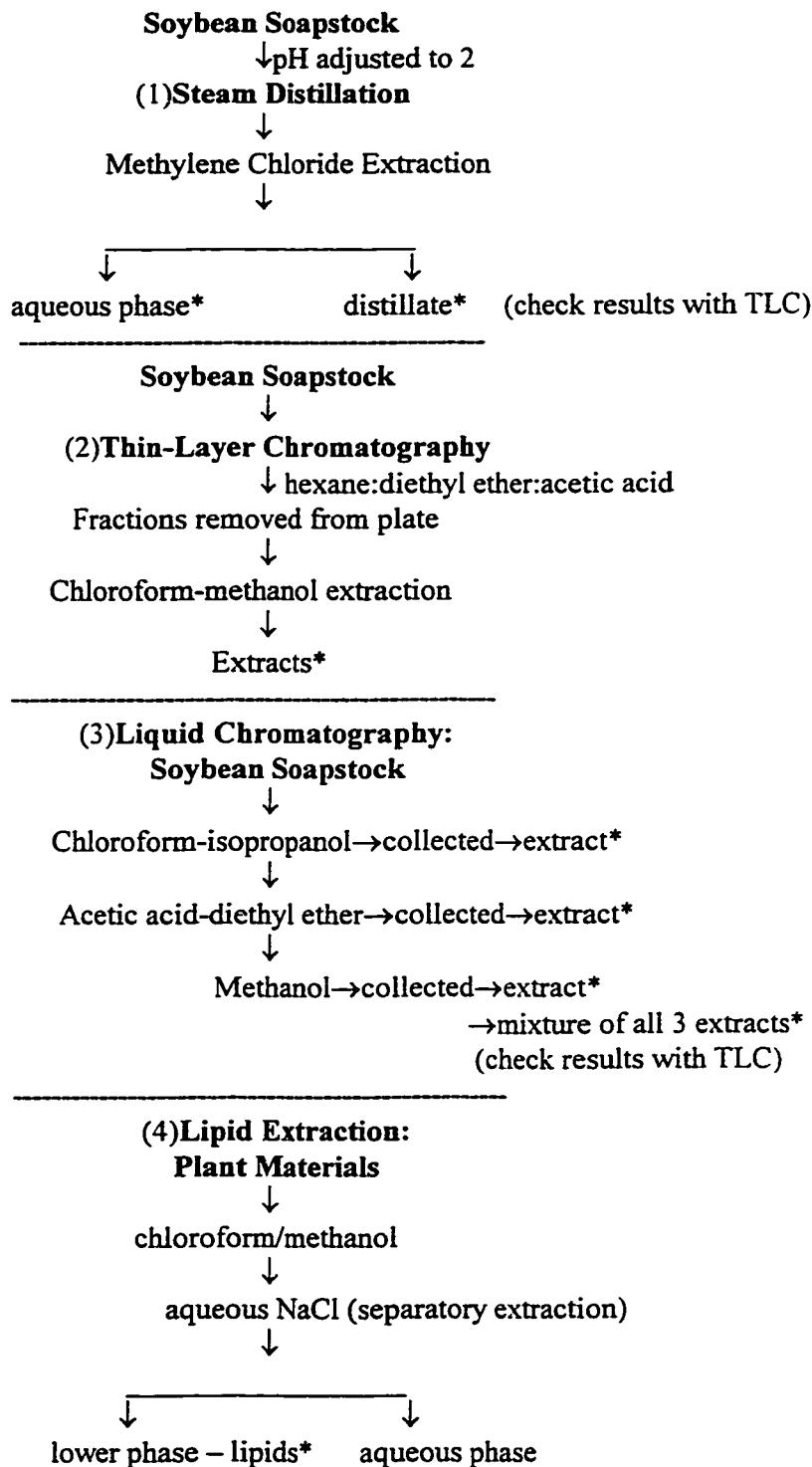


Table 2. Abbreviated schematic on the four extraction procedures used on soybean soapstock and other plant material. * = Samples of the extracts were tested for effect on hatch following these extraction methods.



Distillation-Extraction Technique

In attempts to determine if hatching activity could be found in the steam distillate of the soapstock solution, the following distillation-extraction technique was carried out using a Nickson-Liken Distillation Extraction Apparatus (N-L). Soapstock was diluted to a 5% (v/v) solution using water (di/RO) and 10 ml of this solution was placed in a 250 ml round bottom flask. (A 50% solution caused too much foaming and was not continued). The flask was placed on a cork ring and the solution swirled and allowed to separate. The soapstock solution (originally pH 5.0) was adjusted for basic and acidic distillations. For the acidic distillations, the diluted solution was adjusted to pH 2.0-3.0 using 1 mM phosphoric acid. The flask was placed in a Glas-col heating mantle and attached to the N-L apparatus. The flask was then stirred and heated. Methylene chloride (50 ml) was placed in a round bottom flask and attached to the right side of the apparatus to collect the distillate (lipid compounds). The sample was distilled for two hours. The distillate was concentrated using a thin-film evaporator. The aqueous phase (not dissolved in the solvent) was also saved and tested. For the basic distillation, the pH of the sample for the basic distillation was adjusted to 7.0 using 1% sodium hydroxide. This distillation was foamy and consequently not continued.

Thin-Layer Chromatography/Solvent Extraction

The lipid component of the soapstock was fractionated using 1,000 μm thick silica gel TLC plates (Whatman, 20 cm x 20 cm) and a mobile phase consisting of hexane:diethyl ether:acetic acid at 70:30:2 (v/v). The pure soapstock sample was applied in a 2.5 cm wide band across the entire lower edge of the plate, 3.5 cm from the lower edge. Using a pencil, one lane was drawn approximately 1 cm from the left edge. After separation this lane was not removed, but developed and used for purposes of visualization. The plates were removed from the chamber when the solvent front was approximately 1 cm from the top edge. The front was marked with a pencil and allowed to dry. The plate was divided horizontally into ten equal sections (approximately 1.3 cm wide) and numbered beginning at the top. A spatula was used to cut and scrape each section into a scintillation vial.

Lipids in each soapstock fraction were extracted with enough chloroform:methanol (2:1, v/v) to fill a scintillation vial. Each vial was shaken vigorously, and the end of a spatula was used to further break up the silica gel pieces. After each of three extractions, the solvent was poured through 1PS filters (Whatman) into 50 ml Erlenmeyer flasks. Solvent was evaporated at room temperature in a fume hood. Any solvent left in the flasks was evaporated using a stream of nitrogen gas.

The following standards (Nu Chek Prep, Inc.; Elysian, MN) were selected to compare to soybean soapstock. The standards (3% canola oil, 2% cholesterol, 10% stearin, 10% monostearin, 10% linolenic acid, 10% diolein, 5% triolein, and 10% tristearin) were applied to a 1,000 μm silica gel TLC plate using a 10 μl micropipette. Standards were separated in the same manner as soapstock samples. To visualize standards and separated soapstock, the plates were placed in an iodine chamber or alternatively sprayed with a 50% sulfuric acid and potassium chlorate solution in a fume hood and dried in an oven.

Liquid Chromatography Technique

Columns (Amino - solid phase extraction, 6 ml, 500 mg, Accuband, J&W Scientific) were used in an attempt to separate the lipid components in the soybean soapstock. The columns were washed with 10 ml heptane to remove any contaminants. Two milliliters of 5% soapstock solution in chloroform-isopropanol (2:1, v/v) was applied to each column. Columns were then eluted with four to five milliliters of 2% acetic acid-diethyl ether (1:50, v/v), then with 5 ml methanol. Each eluate was collected and pooled. The procedure was repeated ten times and fractions from each extraction were pooled.

Samples of each fraction were loaded on a TLC plate, along with the original soapstock sample and selected standards (3% canola oil, 2% cholesterol,

10% monostearin, 10% diolein and 10% linolenic acid) to determine the effectiveness of the separation.

The fractions were evaporated at room temperature in a fume hood, then covered with aluminum foil and stored at 6° C until tested. The hatching bioassay included: fraction 1 (following chloroform-isopropanol), fraction 2 (following acetic acid-diethyl ether), fraction 3 (following methanol) and a mixture of all three fractions. These fractions were mixed with a buffer solution (.086 M NaHCO₃, pH 6.6) prior to testing. The buffer solution without any treatment was also tested.

Lipid Extraction Technique

Plant material (roots, leaves or seeds) was pulverized using either a blender (for more than one gram plant material) or mortar and pestle (for less than one gram plant material). Chloroform/methanol (C-M; 2:1 v/v) (100 ml) was added to the plant material after it was pulverized and the suspension was allowed to soak for at least two days at room temperature (25° C) (Folch *et al.*, 1956).

The extracted material was then poured through fluted filter paper (Whatman #2, 110 mm) into an Erlenmeyer flask (250 ml). More solvent (C-M) was added to the material (25 ml x 2) after which it was swirled, filtered and added to the original filtrate. Thirty milliliters of 0.5% aqueous NaCl was added to a separatory funnel containing 150 ml of the extracted material. The funnels were

shaken (x 10), vented and time allowed for phases to separate. After separation, the lower (lipid phase) was collected in a round-bottom flask. An additional 30 ml of an aqueous 0.5% NaCl (30 ml):C-M (150 ml) solution was added to each funnel, mixed and pooled with the previously recovered phase.

The flasks were placed in a warm water bath (54° C) and attached to a thin-film evaporator and the residue collected. If a thin-film evaporator was not available, the extracts were poured into Erlenmeyer flasks (250 ml) and the solvent was evaporated with a stream of nitrogen gas.

All samples were covered with aluminum foil and stored in either a freezer (-12° C) or cooler (6° C) until tested.

Prior to testing, unless stated otherwise, di/RO water was added to the soapstock test samples that were then briefly placed in a sonicator. All solutions containing or derived from plant material (excluding the soapstock samples) were micro-filtered (Nalgene sterile microfilters) prior to use to limit potential contamination.

Statistical Analysis

Data from all of the above tests were analyzed by analysis of variance on the raw data and also on ranked data with similar results (SAS Institute, Cary, NC). Means were separated by Duncan's least significant difference.

Results and Discussion

The bioassay technique used in this study allowed the testing of multiple test substances at the same time. This technique also proved to be a unique means of testing soybean plant products on hatch of the soybean cyst nematode. Each hatch bioassay lasted for fourteen days. In previous unpublished studies (Moberg *et al.*), hatch at day 14 would be typically 1.5 times that at day 7. However, in some of the hatch tests reported here, this tendency was not observed probably due to variables, including the developmental or physiological age of the eggs, that resulted in low hatch overall. Counting juveniles following seven and fourteen days should reflect intermediate and final hatch numbers. This research also confirmed what others including Tefft *et al.* (1982) have observed, namely that 5-10% of the viable eggs hatch during a two-week period. The target of 100 eggs/well was based on the desire to have egg numbers high enough to adequately monitor hatch yet also have a manageable number for counting purposes. Those tests where hatch (designated as a percentage, based on the average of 24 replications) was the same as or greater than the control, di/RO water, were designated as positive hatch treatments.

Soybean soapstock was tested for promoting SCN egg hatch at various concentrations and pH levels. Following sonication, the 5% soapstock samples were emulsified, resulting in unreadable samples. The 10% and 25% soapstock

solutions at or near pH 5.0 proved most conducive to hatch (Figures 1 and 2). The eggs in the 10% soapstock solution at pH 5.0 had the same (Figure 1) and the eggs in the 25% soapstock solution at pH 5.0 had a significantly higher (Figure 2) percent hatch ($P=0.05$) over the control, di/RO water. In their studies, Tefft *et al.* (1982) found egg hatching was maximized at a pH of 6.0 compared to pH 5.4, 6.7, 7.2 and 8.0 using distilled water. In this study, egg hatch in diluted soapstock was found to be optimal at a pH of 5.0. The greatly stimulated hatch by 25% soybean soapstock, pH 5 concentration should have been pursued further.

The effect of soybean soapstock (pH 5.0) on SCN hatch was also tested at three lower concentrations (0.01, 0.1 and 1.0%) (Figures 3 and 4). If overall hatch, including that from the known hatch stimulants (di/RO water, $ZnSO_4$ and root diffusate) was less than 10%, the hatch results were reported as a hatch effect (Figure 3, the original hatch percent data is located in Appendix B, Chapter 3, Figure 3b). The hatch effect was calculated from the percent hatch of eggs in the water control subtracted from the percent hatch off eggs in the treatment divided by the percent hatch of eggs in the water control. The hatch effect of water equals zero, so any increase in egg hatch from the treatments has a positive hatch effect greater than zero. The results from the first study with 0.01 – 1% diluted soapstock are shown in Figure 3. On day 14, diluted soybean soapstock at 0.1 and 1.0% affected hatch that was similar to that in di/RO water. For the second study

(Figure 4), the diluted soybean soapstock samples were dried within the microtiter wells. The nematode eggs, suspended in water, were added directly to the wells. The higher concentrations of soapstock, 0.1% and 1.0%, resulted in a similar level of hatch than control treatments (Figures 3 and 4).

In some hatch tests (Figures 4 and 9), the treatments – while still dissolved in the methylene chloride – were applied to the wells and the solvent was evaporated overnight. Water containing the eggs was then added to the well prior to counting. This technique was used as a means to prevent emulsification that would sometimes occur when the sample was mixed with water and then sonicated. This technique had a limited positive effect on viewing; however, it improved distribution of the eggs within the wells.

A diluted (5%, v/v), acidified (pH 2.0-3.0) soapstock solution was distilled in hopes that a hatching factor could be located in either the solvent or aqueous phases following distillation. Treating eggs with the aqueous phase of the distillate resulted in a positive hatch effect, though not significantly different from the water control (Figure 5, the original hatch percent data is located in Appendix B, Chapter 3, Figure 5b). The samples, however, were difficult to read and overall percent hatch was low. There was no hatch of eggs in the treatment containing the solvent phase distillate. The aqueous phase distillate was tested again a month

later, but this time the percent hatch was zero. The aqueous phase distillate might have had some hatching factor, but the same result was not observed.

Undiluted soybean soapstock (pH 5.0) was subjected to thin-layer chromatography to physically separate out its various lipid components into distinct bands and to attempt to find one with a hatching factor. Soapstock fractions from ten sections of a preparative TLC plate, each 1-2 cm wide, were tested for promoting hatch (Figure 6, the original hatch percent data is located in Appendix B, chapter 3, Figure 6b). The plate sections that contained components promoting the greatest hatch, #5 and #6, were comparable in position to the chromatogram with triolein or the general triglyceride range. This is the general range where one would expect to find a hatching factor to occur if it is comparable to glycinoeclepin A, the known hatching factor of kidney beans. This test was repeated with new TLC fractions (Figure 7, the original hatch percent data is located in Appendix B, Chapter 3, Figure 7b). In this test, compounds in section #4 promoted the greatest hatch of eggs (9.6%) at 14 days, though this was not significantly greater than hatch in di/RO water. This section was comparable to the chromatogram of canola oil, also in the triglyceride range. Based on the results from the preparative TLC, there exists the possibility of a hatching factor in the triglyceride compounds of the soapstock solution.

To determine the effect of dilution on isolation of the putative hatching factor, soybean soapstock at 0.01%, 0.1% and 1% (pH 5.0) were separated by thin-layer chromatography. The hatch effect of extracts are depicted in Figure 8 (the original hatch percent data is located in Appendix B, Chapter 3, Figure 8b). Each TLC plate was divided into the four sections following the pattern in which soapstock separates. Comparing the chromatogram of soybean soapstock to the chromatogram of a set of standards applied to the TLC plate, section #1 appeared to represent the hydrocarbon region; section #2 the upper range of triglycerides; section #3 free fatty acids and section #4 the plant sterols. Extracts from all plate fractions, except from section #2, resulted in positive hatch, although at very low levels. The eggs used for this study were apparently not physiologically ready to hatch and there was very little hatch in any treatments. Also, even with diluted soapstock, emulsification of the test solutions following sonication continued to be a problem.

Because egg hatch was quite low in the previous study, a similar hatching assay was repeated using new TLC fractions and the results are shown in Figure 9. A low level of hatch again occurred in all treatments. A positive effect on egg hatch, though not significantly greater than that in di/RO water, occurred most notably in section #1 at 1.0% concentration and section #3, 0.01% and 0.1% concentrations and section 2, 0.01% concentration. Section #1 contains the

hydrocarbons, section #3 the fatty acids and section #2 the triglycerides. Perhaps this is indicative that a complex compound is involved in promoting hatch of SCN eggs, or that the method is not separating the hatching factor from other components in soapstock.

Liquid chromatography was used to remove the fatty acids in the soybean soapstock that interfered with distributing and viewing eggs (Figure 10). Fraction #1 was the result of the chloroform-isopropanol extraction that is used to extract non-polar lipids. Fraction #2 was the result of the acetic acid-diethyl ether extraction used to extract fatty acids. Fraction #3 resulted from the methanol extraction used to extract polar lipids. The TLC plate of the samples following liquid chromatography revealed a good separation of the fatty acids and triglycerides. Nevertheless, the resultant samples were very cloudy and difficult to read. Fraction #2 and a mixture of all three fractions were impossible to read. Hatch from fractions #1 and #3 was very low. The buffer, which was used to keep fatty acids in solution, was also tested and was found to not have an effect on hatch. There did not appear to be anything close to a positive effect on hatch to warrant a repetition of this method.

The lipid extraction technique was applied to numerous plant materials (freeze-dried soybean leaves and roots, seed extracts from soybean, pea, kidney bean, lima bean and green bean). Only the results from the freeze-dried soybean

leaves and roots will be presented in the text (a majority of the tests were unreadable). The extracts from dried leaves and roots apparently had a significant inhibitory effect on hatch. The percent hatch of eggs in treatments with leaf lipids remained 0% at days 0, 7 and 21, and hatching in treatments containing root extracts ranged from 1.4 to 1.7% over the same time period. The percent hatch for the control treatments included in the same hatch test ranged from 13.4 to 30.1%. Seed extracts from the lipid extraction technique were too opaque to permit viewing of eggs in the hatch test. If a means of overcoming the difficulty in viewing were found, this extraction method could be of merit. Perhaps careful filtering and diluting of the extracted plant material prior to the hatch test would alleviate some of these difficulties.

Summaries of results with root diffusates on egg hatch and corresponding critical values for di/RO water (control) and 3 mM ZnSO₄ are depicted in Figure 11. The percentage hatch that took place in water throughout the reported hatching tests ranged from 0.1 to 29.2%; the range for hatch in 3 mM ZnSO₄ was 0.3 to 50.0% and for soybean root diffusate, the range in which hatch took place was 0.3 to 47.2%. The wide range in values for these three solutions clearly shows that egg hatch in the water control and in the hatch stimulants was also affected by some of the factors (e.g. seasonal effects) that affected hatching in the treatment solutions.

Although root diffusate was considered a hatch stimulant for this study, it actually had a variable effect on hatch (Figure 11). Variable results are expected to occur with root diffusate because so many factors can affect its activity. Most notable are age of the plant and the dilution of the diffusate. There was a wide range in age of plants from which the diffusate was obtained (22-87 days old), the number of plants involved varied (12-40) and the number of days over which the diffusate was obtained (4-6 days). Perry (1989) found that the dilution of PRD (potato root diffusate) usually enhanced hatch. He associated this with the possible reduction in hatch inhibitors. It could also be that the soybean root diffusate varied in its effect on hatch due to the existence of unknown hatch inhibitors.

A number of problems were encountered in using soapstock in the SCN bioassay. One of the problems included the emulsification of the solution upon sonication (which can make for some very difficult viewing through the microscope). Fatty materials in the solution had a tendency to adhere to the pipette tip, which can affect the volume of liquid dispelled. Also, the eggs would sometimes bind to the lipid material in the treatment solution and not sink to the bottom of the well. In addition, many other factors can influence bioassay results. These include: physiological state or “readiness to hatch” of the eggs and sensitivity to treatment solutions (e.g., volatility and toxicity of solvents). The

above factors sufficiently interfered with the hatch tests such that the results were somewhat inconsistent.

Researchers investigating nematode hatch frequently observe dramatic variation of hatch over the various months of the year (Tylka, personal communication). This phenomenon is noted even though using greenhouse grown soybeans to culture nematodes. Tefft and Bone (1984) also observed the influence of seasonality on hatch when using greenhouse-grown soybeans to culture SCN in hatching experiments. They found highest hatching occurred during May, when they observed an almost two-fold increase in hatch compared to other months. The months during which each of the hatch tests in this study were done are shown in Figure 11. The obvious drop in overall hatch during the months of October through January for the control and hatch stimulants further supports this widespread observation. This natural phenomenon also apparently affected the hatch of eggs in test solutions, and low hatch is not necessarily a reflection of an overly sensitive hatch bioassay.

The cause of lower egg hatch during winter months in greenhouse-grown plants is unknown. Previous research by Yen *et al.* (1995) found that dormancy of SCN eggs within cysts, when monitored by decreasing hatching rates, was unrelated to soil temperature. Yen *et al.* (1996) examined the changes in carbohydrate (glucose, trehalose and glycogen) and total protein content of

Heterodera glycines eggs on a monthly basis. Trehalose has been found to function as a cryoprotectant and plays a role in the hatching of some nematode eggs. Their research showed an increase in the amount of trehalose present in the egg with a corresponding decrease in soil temperatures. However, their studies did not reveal any seasonal fluctuation in the trehalose levels of greenhouse-grown soybeans. They suggested that trehalose in SCN eggs may serve as a source of energy reserves during cold weather conditions.

Sikora and Noel (1996), studying the hatch and emergence of SCN in root leachate, noted that hatch and emergence were greater in cysts harvested in October or April compared to January. They suggested that dormancy had been induced in eggs harvested in January that inhibited hatch and emergence of the nematode.

Following their research on hatch of SCN, Schmitt and Riggs (1991) described some of the conditions that could affect the results of hatching studies. They include: the developmental and physiological state of the eggs, daylength and temperature effects on host stimulant concentration and possible varying stimulatory effects of exudates at different phenological stages. The hatch results from this study apparently were likewise affected by the above mentioned conditions.

In summary of the results of this research, a significant positive effect on hatch ($P=0.05$) did occur in some hatch studies using soybean soapstock treatments. When using a 25% soybean soapstock solution at a pH of 5.0, hatch was significantly greater than hatch in di/RO water. Diluted soybean soapstock (1.0 and 10%) resulted in hatch that was similar to that in di/RO water. Following preparative TLC and chloroform-methanol extraction, a significant positive effect on hatch was associated with compounds that co-purified with triglycerides. Also, some positive hatching activity also occurred in treatments containing hydrocarbon and fatty acid compounds. This suggests that a complex compound or multiple compounds may be involved as (a) potential hatching factor(s). When the percent hatch is quite low (< 5%), one hesitates to place too much confidence in the results. However, some of the results suggest a likely hatching factor in the soybean soapstock. One also must be particularly careful when subjecting a small, somewhat delicate organism to substances that have been extracted using toxic solvents. It is imperative that all traces of any solvent are removed prior to testing. Ideally, the test substances should be tested as soon as possible following extraction in order to reduce the risk of oxidation of potential hatching factors. A seasonal effect on hatch was observed using the greenhouse cultured eggs. The bioassay described constitutes a different method of testing soybean plant products for ability to stimulate SCN egg hatch.

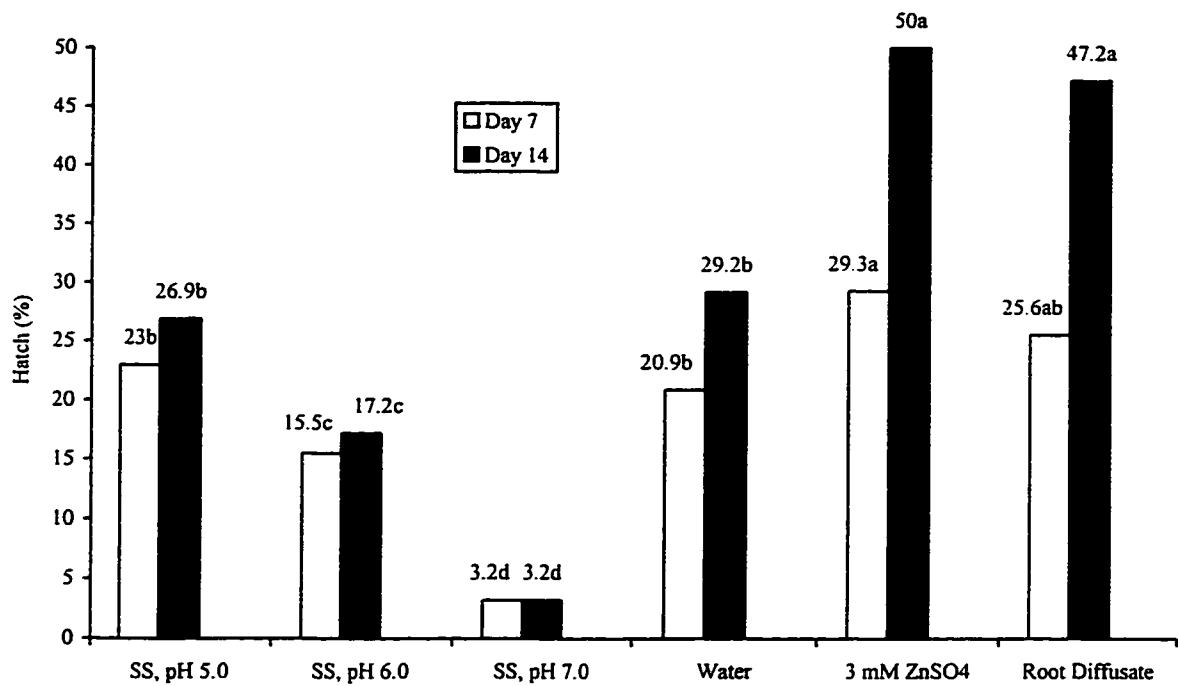


Figure 1. Percentage egg hatch of *Heterodera glycines* exposed to 10% soybean soapstock (SS) at varying pH levels, di/RO water, 3.0 mM ZnSO₄ and root diffusate. Within time points, columns with the same letter are not significantly different ($P=0.05$).

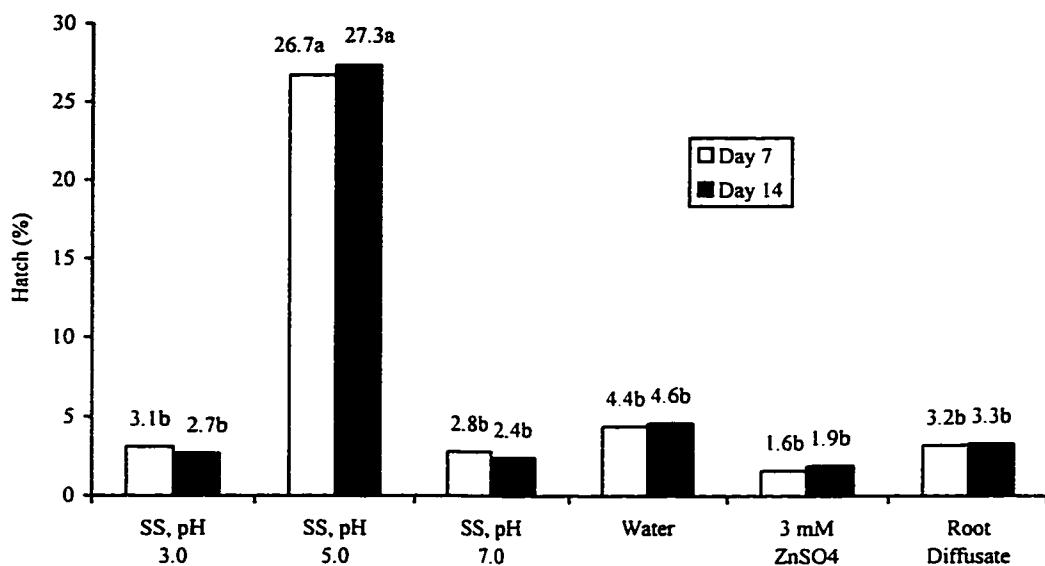


Figure 2. Percentage egg hatch of *Heterodera glycines* exposed to 25% soybean soapstock (SS) at varying pH levels, di/RO water, 3.0 mM ZnSO₄ and root diffusate. Within time points, columns with the same letter are not significantly different ($P=0.05$).

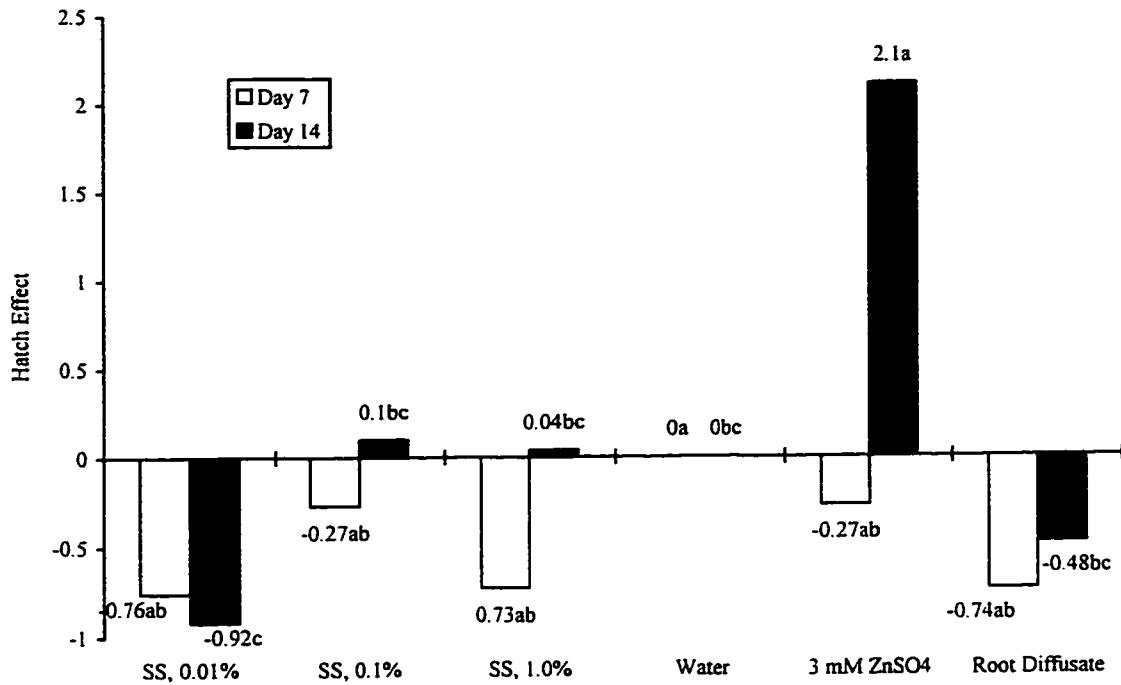


Figure 3. Hatch effect of soybean soapstock (SS) diluted to 0.01, 0.1 and 1.0% (1st study), di/RO water, 3.0 mM ZnSO₄ and root diffusate on eggs of *Heterodera glycines*. Hatch effect = % hatch (treatment) - % hatch (water) / % hatch (water). Within time points, columns with the same letter are not significantly different (P=0.05).

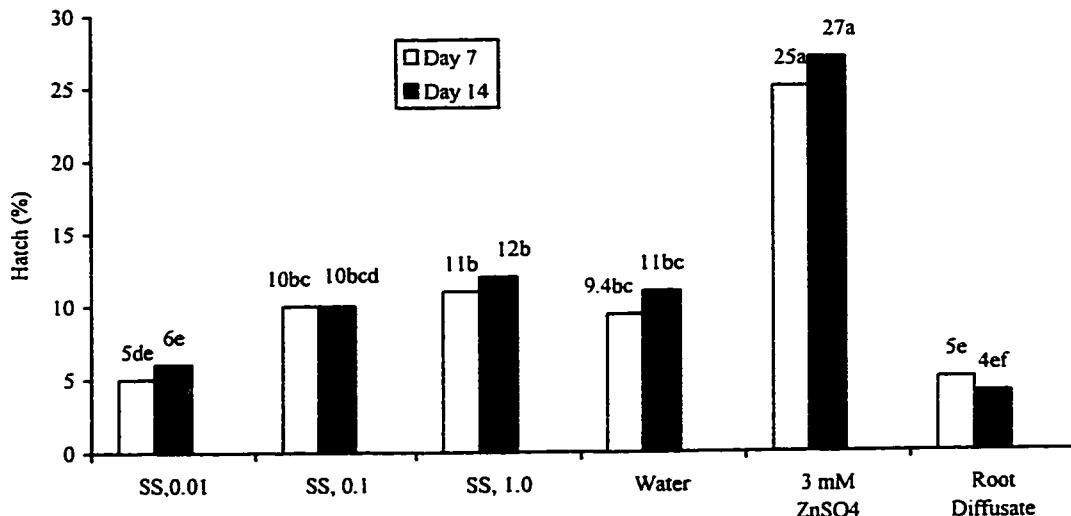


Figure 4. Percentage egg hatch of *Heterodera glycines* exposed to a diluted soybean soapstock (SS) 0.01, 0.1 and 1.0% (2nd study), di/RO water, 3.0 mM ZnSO₄ and root diffusate. The soybean soapstock solutions were evaporated within the microtiter wells. Within time points, columns with the same letter are not significantly different ($P=0.05$).

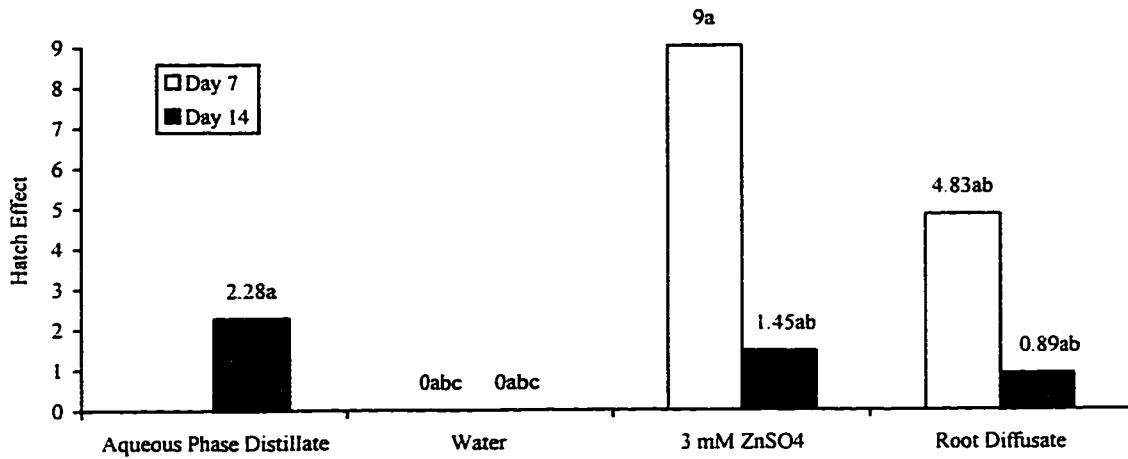


Figure 5. Hatch effect of the aqueous phase following soybean soapstock distillation, di/RO water, 3.0 mM ZnSO₄ and root diffusate on eggs of *Heterodera glycines*. Within time points, columns with the same letter are not significantly different ($P=0.05$). There was no hatch from the aqueous phase distillate on day 7.

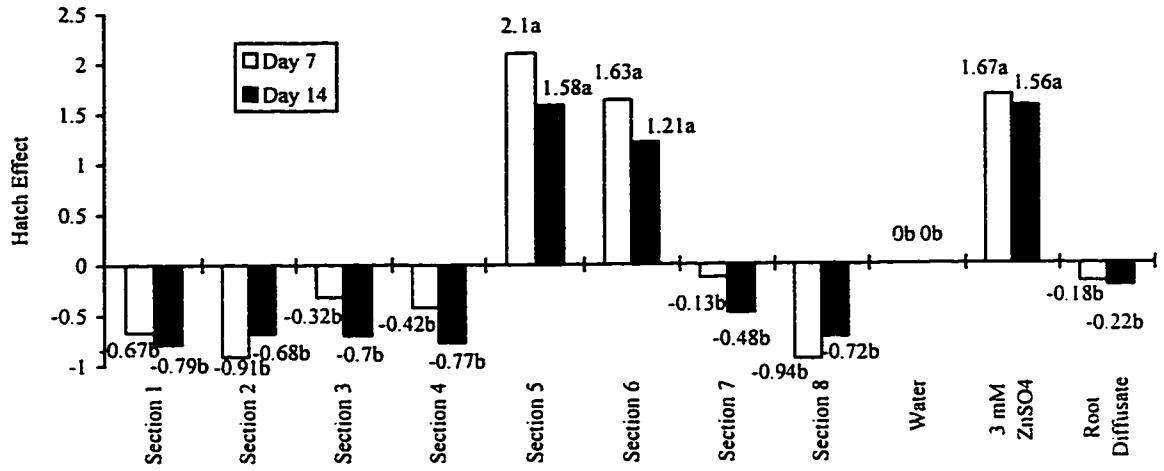


Figure 6. Hatch effect of soybean soapstock following thin-layer chromatography (sections 1-10; 1st study), di/RO water, 3.0 mM ZnSO₄ and root diffusate on eggs of *Heterodera glycines*. Within time points, columns with the same letter are not significantly different ($P=0.05$). There was no hatch from sections 9 and 10.

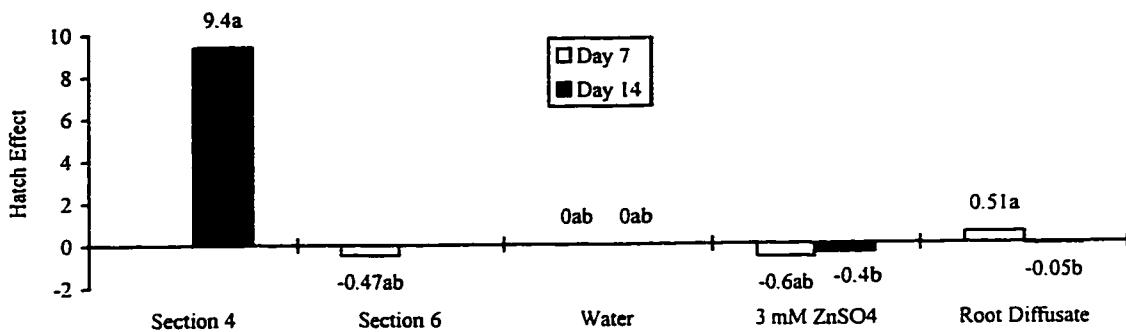


Figure 7. Hatch effect of soybean soapstock following thin-layer chromatography (sections 2-10; 2nd study), di/RO water, 3.0 mM ZnSO₄ and root diffusate on eggs of *Heterodera glycines*. Within time points, columns with the same letter are not significantly different ($P=0.05$). If no hatch occurred, a hatch effect was not recorded.

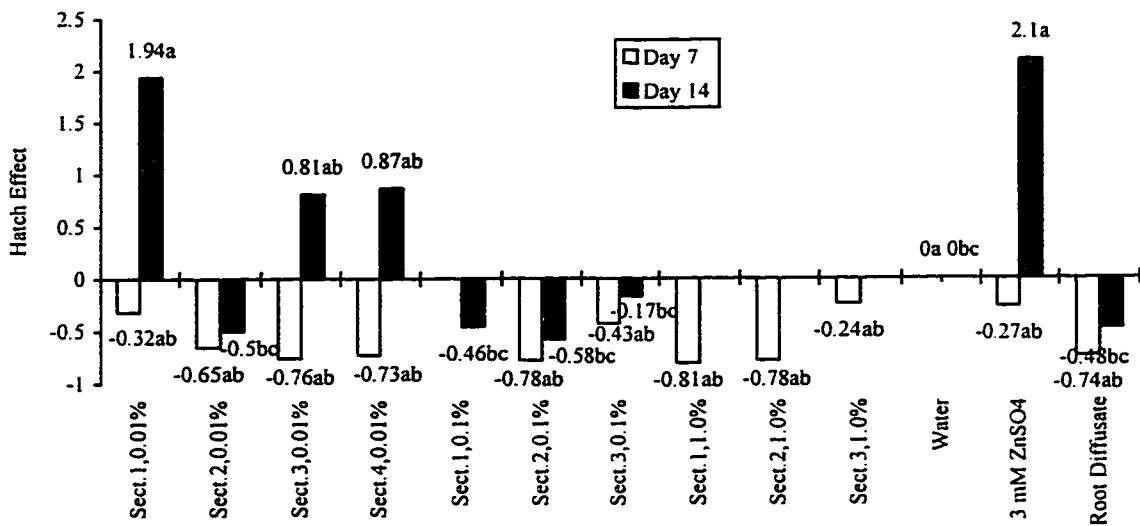


Figure 8. Hatch effect of soybean soapstock, diluted to 0.01, 0.1 and 1%, following thin-layer chromatography (sections 1-4; 1st study), di/RO water, 3.0 mM ZnSO₄ and root diffusate on eggs of *Heterodera glycines*. Within time points, columns with the same letters are not significantly different ($P=0.05$). If no hatch occurred, a hatch effect was not recorded.

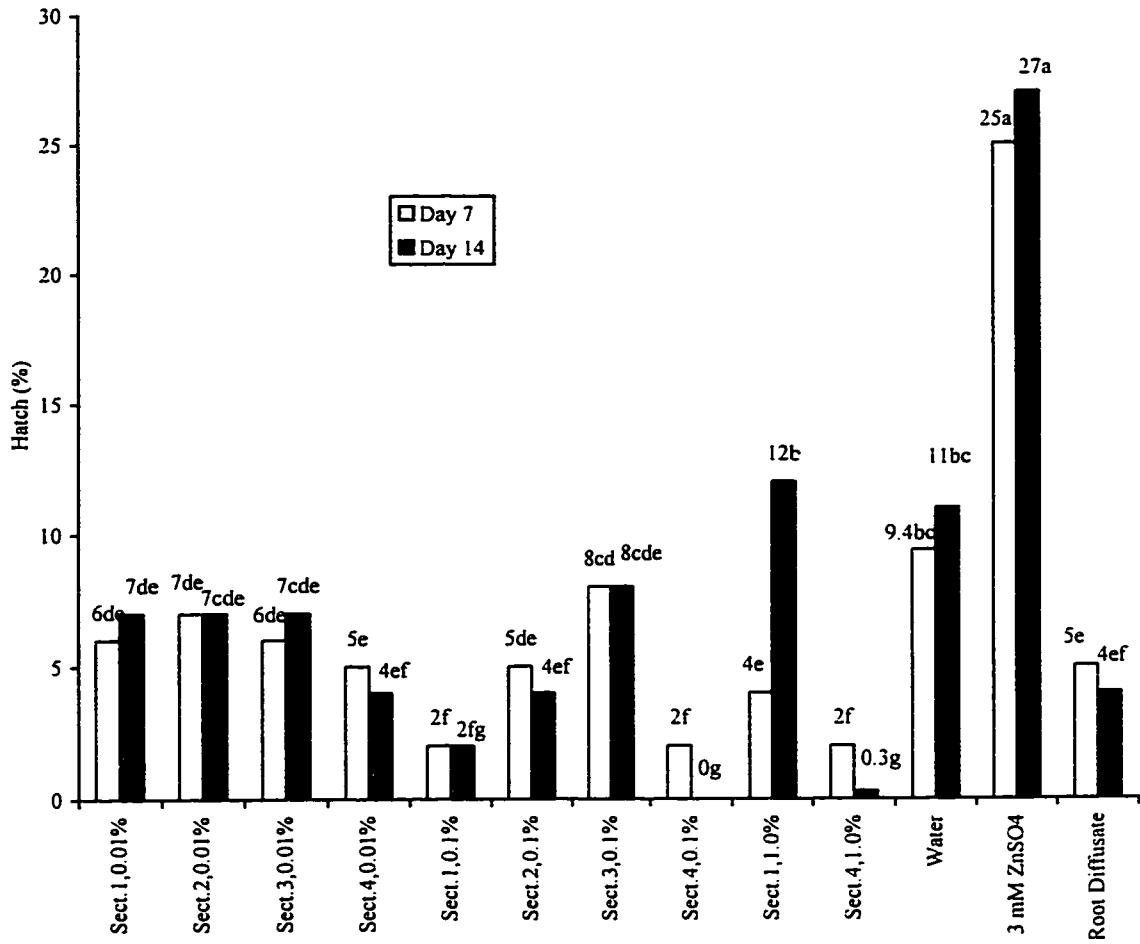


Figure 9. Percentage egg hatch of *Heterodera glycines* exposed to soybean soapstock, diluted to 0.01, 0.1 and 1.0%, following thin-layer chromatography (sections 1-4; 2nd study), di/RO water, 3.0 mM ZnSO₄ and root diffusate. The TLC treatments were evaporated within the microtiter wells. Within time points, columns with the same letter are not significantly different ($P=0.05$).

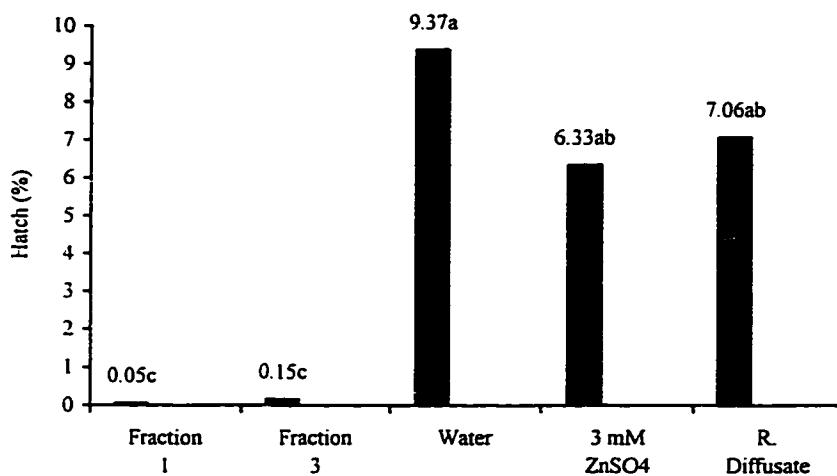


Figure 10. Percentage egg hatch of *Heterodera glycines* exposed to soybean soapstock following column chromatography, di/RO water, 3.0 mM ZnSO₄ and root diffusate. Within time points, columns with the same letter are not significantly different ($P=0.05$).

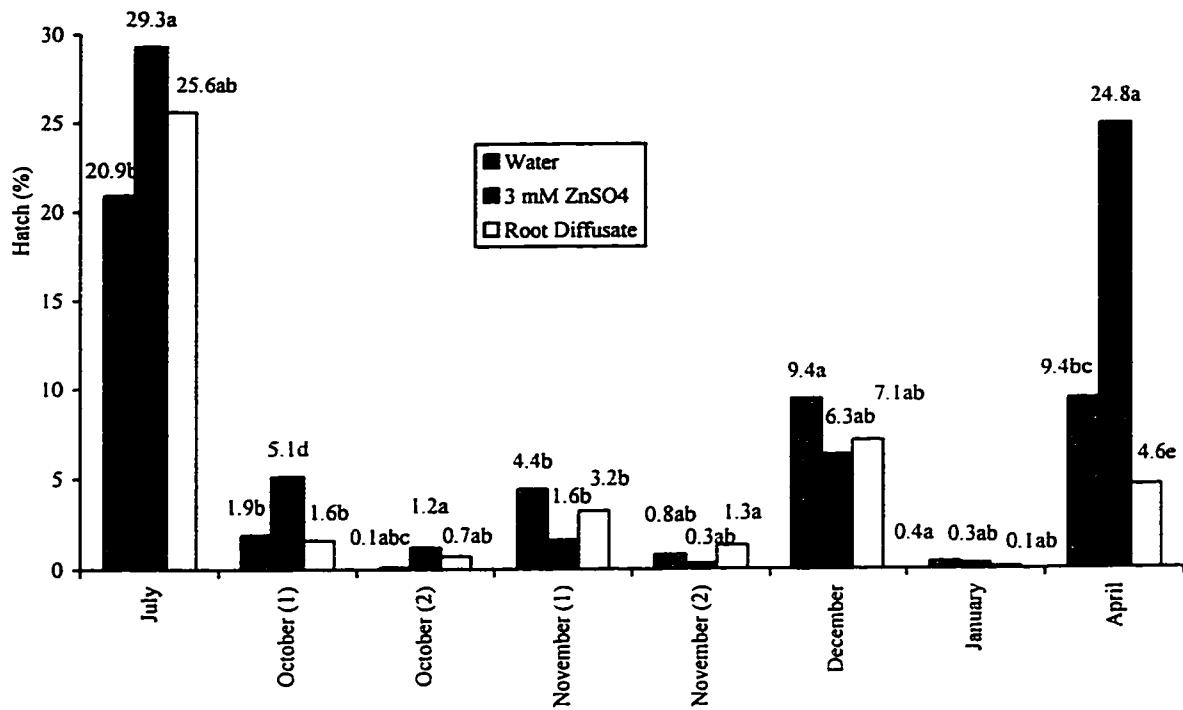


Figure 11. Percentage egg hatch of *Heterodera glycines* exposed to di/RO water, 3.0 mM ZnSO₄ and root diffusate at day 7 during different months of the year (two hatch tests took place during the months of October and November). Within the test periods, columns with the same letter are not significantly different ($P=0.05$).

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Chapter 4: A Technique for Partial Purification of a Hatching Factor from Roots of Soybeans and Kidney Beans

Abstract

The eggs of *Heterodera glycines* have been found to survive in the field without a host for up to eleven years. The current control options for this soybean pest are limited, and broad based, sustainable methods for limiting damage from this pest are needed. Possibly, the incorporation of a hatching factor into infested soil in the absence of a host will induce hatching of juveniles and consequently their starvation. The isolation, identification, structural determination and chemical synthesis of glycinoeclepin A (GEA), the hatching factor for the SCN, have been reported. Yet, the synthesis of GEA is too tedious and the yield too low to be used commercially. In this study, the isolation techniques for GEA were modified and used to extract hatching factors from soybean and kidney bean roots. The egg hatch bioassay revealed a high percentage hatch of eggs treated with the soybean extract following the initial extraction prepared by a 50 hour liquid to liquid extraction using chloroform. This hatch was not as high as that in water, but was significantly ($P = 0.05$) greater than eggs treated with 3 mM ZnSO₄. The final extraction, which consisted of an 18 hour liquid to liquid extraction with chloroform, resulted in hatch from the soybean extract that was significantly greater ($P = 0.05$) than that from di/RO water and 3 mM ZnSO₄. This modified technique resulted in successful isolation of an apparent hatching factor from soybean roots.

A Technique for Partial Purification of a Hatching Factor from Roots of Soybeans and Kidney Beans

The soybean cyst nematode, *Heterodera glycines* Ichinohe, was originally identified as a soybean root parasite in Japan in 1915 and is currently found in eleven countries. The SCN is the primary yield-suppression pathogen for soybeans in both southern and northern soybean-growing areas in the United States (Pratt and Wrather, 1998 and Doupinik, 1993).

Management options for the SCN consist primarily of sanitation, crop rotation and careful use of resistance. Nematicides are generally too expensive to consider as an option and fewer are on the market due to environmental considerations. Trap cropping is another potential means of nematode control by effectively reducing the nematode population by allowing the nematode to hatch and infect the crop. The crop is then destroyed before the nematode population matures. Eggs of the SCN may survive within the cyst – the hardened, body wall of the female nematode – for eleven years or more (Riggs and Schmitt, 1989). If the nematode could be stimulated to hatch with a no host present, infection would not occur and the second-stage juveniles would die. Many researchers have tried to find a means of control by searching for a hatching factor for the nematode.

In a breakthrough in hatching factor studies, Masamune *et al.* (1982)

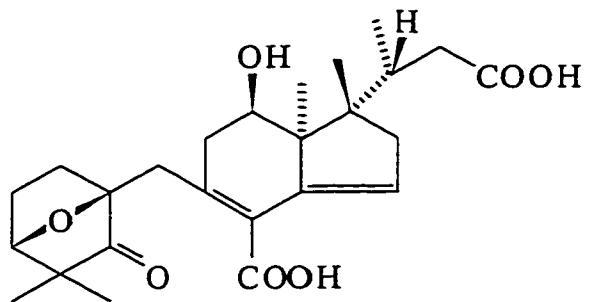
successfully isolated a hatching agent from kidney bean roots that was identified as glycinoeclepin A. Glycinoeclepin A (GEA) is characterized as a triterpene carboxylic acid with a molecular weight of 446 and a molecular formula of C₂₅H₃₄O₇. The isolation was accomplished by a tedious procedure that began with the preparation of aqueous extracts from kidney bean roots. Aqueous extracts of kidney bean roots were selected rather than root diffusates due to instability of root diffusates at this low level of purification. The roots of kidney bean plants were harvested just prior to flowering from a one hectare field plot, air dried for three months, then crushed. The compound was extracted from 135 kg of powdered kidney bean roots, fractionated through a variety of chromatographic columns such as adsorption, reversed-phase partition, ionic exchange chromatography, as well as preparative HPLC.

These researchers found that 5 µg of GEA existed as its bis(*p*-bromophenacyl) ester form and it was shown to have active hatching ability at 10⁻¹¹-10⁻¹² g/ml. The analysis of the bis(*p*-bromophenacyl) esters indicated that the active compound was probably a dibasic acid. This finding led them to revise the purification procedure and resulted in much higher recoveries. However, to obtain sufficient material for structural analysis, approximately 1 metric ton of kidney bean roots was harvested and extracted for GEA. This process (Masamune *et al.*

1987) is outlined in Chapter 1, Table 5, yielded 1.25 mg of active compound to be used for structural elucidation.

The structure of glycinoeclepin A was determined by Fukuzawa *et al.* (1985) and is depicted in Figure 1.

Figure 1. The chemical structure of glycinoeclepin A.



Following structural elucidation, great efforts were undertaken to synthesize glycinoeclepin A (Okawara *et al.*, 1987) and the first total synthesis was published in 1988 (Murai *et al.*, 1988; Murai, 1989). The synthesis involves 32 steps and the overall yield is 0.4%. Others reported varying yields and degrees of difficulty (Watanabe and Mori, 1991; Corey and Houpis, 1990), however, the chemical synthesis of GEA does not provide enough of the compound to be economically viable in a SCN management program.

In this study, the partial purification of a hatching factor from soybean and kidney bean roots was attempted using a simplified technique based on the method of Masamune *et al.* (1987). This is the first report of isolation of a hatching factor from soybean roots, the economic host of the SCN.

Materials and Methods

Root Material

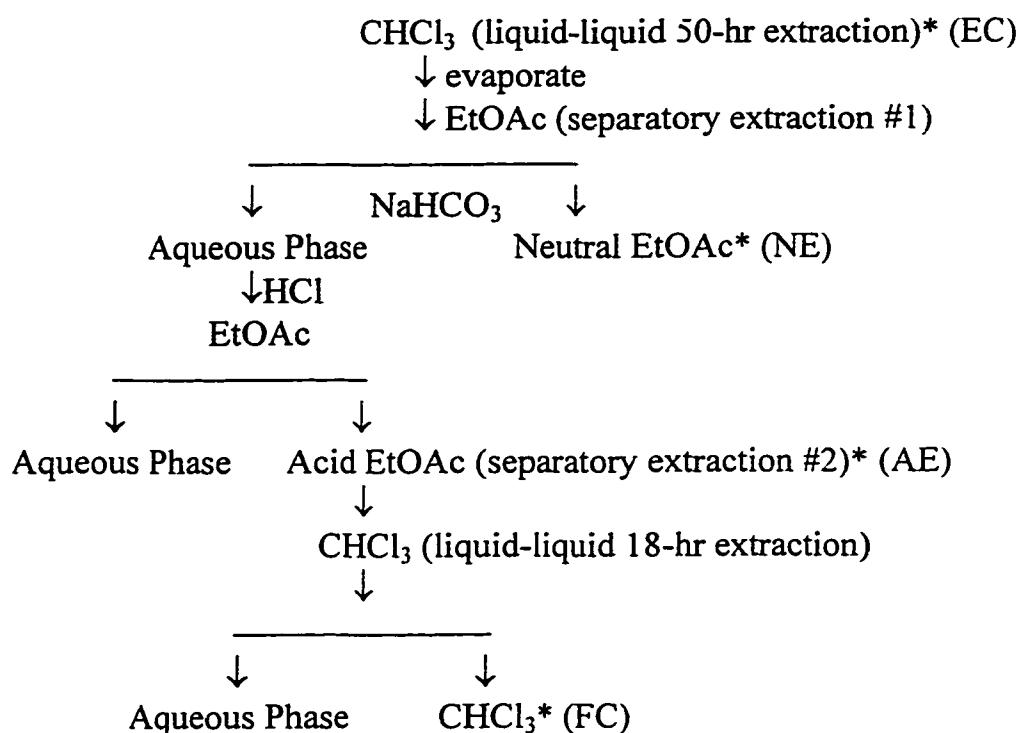
The soybean seeds (Agassi 2, a variety susceptible to race 3 of the SCN) used for this study were obtained from Dr. James Orf, Department of Agronomy and Plant Genetics, University of Minnesota. Soybeans and kidney beans (a dark red field variety obtained from Jordan Seed Ranch, Woodbury, Minnesota) were planted in 15½ x 21½ x 4 inch flats containing vermiculite at a rate of 77 seeds/flat. The flats (24 for each species) were kept in a greenhouse at 30 °C with fourteen hours of supplemental light. The plants were watered as needed (almost daily) and fertilized once/week using Peter's Plant Food (20-20-20, 1 tablespoon/gallon). Two crops of soybeans (SB1;SB2) and two crops of kidney beans (KB1;KB2) were planted at different times, harvested and extracted separately. Plants were harvested when initial flowering had begun, typically four weeks after planting. The plants were harvested by gently pulling up a group of plants and carefully shaking off the vermiculite. The roots were rinsed somewhat vigorously in a bucket of water to dislodge the vermiculite. The excess water was squeezed from the roots and the roots were then cut at the juncture of the hypocotyl and epicotyl. All root material was placed in paper bags. In the lab, the roots were transferred from the paper bags to stainless steel trays (30 cm x 24 cm x

6 cm) with lids. The trays were stacked in plastic bags and stored in the freezer (-18 °C) prior to placing them in a freeze drier.

Extraction and Purification of Hatching Factors

An outline of the procedures followed in the extraction of hatching factor(s) from kidney bean and soybean roots is depicted in Table 1.

Table 1. Outline of the procedure for extracting a hatching factor for *Heterodera glycines* from kidney bean and soybean roots. * = Samples of the extracts were tested for effect on hatch following these extraction methods. Sample codes are shown in parentheses.



The plant roots were placed in a freeze drier (Virtis 10-324, upper unit: Unitop 600L, lower unit: Freezemobile 12) for three to five days. The dried roots were then pulverized using a blender, weighed (SB1 = 185.3 g; SB2 = 328.6 g; KB1 = 204.4 g; KB2 = 125.2 g) and placed in 2,000 ml glass beakers. Di/RO water (16 ml/gram of root material) was poured over the powdered root material. Aluminum foil was used to cover the beakers that were stored in a cooler (10 °C). Following approximately seven days, the liquid root extract was carefully poured into a 2,000 ml Erlenmeyer flask, and additional di/RO water (7 ml/g) was added to the root material. Following approximately seven more days, the remaining liquid was poured into the flask. To ensure the recovery of the maximum amount of root extracts, the root material was pressed hard with a gloved hand using cheesecloth. The root extracts were filtered using cheesecloth to remove any remaining debris prior to pouring into a 2,000 ml round-bottom flask for thin film evaporation. A dry ice trap filled with isopropanol and dry ice was used with a vacuum pump to achieve maximum vacuum to evaporate the large amount of water present.

The temperature of the water bath for thin-film evaporation was always kept below 30 °C. The thin-film evaporator consisted of the following: Buchi

Rotavapor R-124; Buchi water bath B-481; Endocal water bath connected to condenser, 0 °C; 107 rpm.

Following concentration, the root extract concentrates were poured into beakers and acidified with 6M HCl using a stirring bar and pH probe until the pH reached 3.0 (original pH = 6.0). The acidified concentrates were stored in the cooler prior to the liquid-liquid extraction. The following extractions were done in sequence for each root extract.

Liquid-Liquid Extraction – 50 hours

The concentrated root extracts were further extracted using chloroform continuously for 50 hours. The root extracts were poured into a liquid-liquid extractor placed on top of a stir plate, set to achieve a slow stirring motion. Chloroform was added to the root extract until equilibrium was achieved. A cold water condenser unit was attached to the top of the apparatus to keep the chloroform from evaporating. A round bottom flask was placed on a heat mantel and attached to a unit on top of the apparatus using an elbow glass tube wrapped with a towel to retain warmth. The round bottom flask collected the chloroform with the lipid components from the root extracts. A thermometer, attached to a monitor, was placed within the top of the condensing unit and set to prevent the temperature from exceeding 24 °C. A timer was attached to the system and set for 50 hours. Following the liquid-liquid extraction, the chloroform-extract solution

was transferred to a 500 ml round bottom flask and stored in the cooler. Prior to the next step, a sample (1 ml) was taken from each extract, transferred to a vial and stored prior to testing. The chloroform was evaporated from each extract sample using the thin film evaporator.

Separatory Extraction #1

Ethyl acetate was added to each dried extract in the round bottom flask. A total of 250 ml was used, added in three increments (100, 100, 50). The samples were swirled after each addition of ethyl acetate and the solution poured into 600 ml beakers. The ethyl acetate-extract combination was poured into a 500 ml separatory funnel to which a saturated aqueous NaHCO₃ solution was added. This was repeated five more times and each time the aqueous phase (bottom) was collected and more saturated aq NaHCO₃ was added to the top phase. The beakers containing the aqueous phase-extract combination were placed on a stirring plate and acidified using 6M HCl until the pH reached 2-3 (initial pH = 8.4). A 1 ml sample was transferred from each extract into a vial and stored until tested.

Separatory Extraction #2

The extract in the aqueous suspension (approximately 1,000 ml) was poured into a 2,000 ml separatory funnel. Ethyl acetate (100 ml) was added to this suspension five times. Following each addition of the ethyl acetate the lower phase was saved and the upper phase further extracted. A 1 ml sample for each

extract was transferred from the ethyl acetate phase into a vial and stored until tested.

Liquid-Liquid Extraction – 18 hours

Following the second series of ethyl acetate extractions, the aqueous extract was again separated by liquid-liquid extraction. The set-up was the same as described above except the extraction was limited to 18 hours. After this extraction process, each chloroform-extract was poured into 500 ml round bottom flasks, closed with a stopper, wrapped with parafilm around the neck then placed temporarily in the cooler.

Drying and Concentration

After the 18 hour liquid to liquid extraction, 5-20 g (depending on how much liquid was present) of anhydrous sodium sulfate was added to each sample extract to eliminate any water left in the samples. Following four hours, the anhydrous sodium sulfate was filtered out (1PS filter paper) and the samples stored in 500 ml round-bottom flasks.

All solvents were evaporated from each sample extract using the thin-film evaporator. Following evaporation, the samples were left under the hood to ensure any solvent left would evaporate. For the egg hatch bioassay on the samples following the 50 hr chloroform extraction and the samples from the neutral ethyl acetate extraction, each sample was resuspended in 4 ml di/RO water and carefully

sonicated to enhance mixing of the extract with the water. When setting up the second hatch bioassay, a total of 12 ml of di/R-O water was added to the EtOAc samples (which were otherwise too opaque) and 8 ml di/R-O water was added to the final chloroform samples.

Bioassay Technique

The extracts dissolved in di/RO water were used to resuspend eggs of SCN. For each treatment approximately 100 eggs/well were assayed, with 24 replications as described previously (Chapter 2, Materials and Methods).

Statistical Analysis

Results were analyzed by analysis of variance on the raw data and also on ranked data with similar results (SAS Institute, Cary, NC). Means were separated by Duncan's least significant difference.

RESULTS AND DISCUSSION

Hatch Bioassay Results

The purpose of this study was to attempt to extract hatching factor(s) from kidney bean and soybean roots using a modification of the initial extraction steps developed by Masamune *et al.* (1987). A fraction that stimulated egg hatch was isolated from the second crop of soybeans. The egg hatch bioassay was carried out on extracts following the 50 hr liquid to liquid extraction, neutral ethyl acetate extraction, acidic ethyl acetate extraction and final chloroform extraction. Eggs treated with the extract following 50 hr liquid to liquid extraction (EC) from the soybean crop 2 (SB2-EC) had a high percentage hatch, though not higher than hatch from water, but significantly ($P=0.05$) greater than egg hatch in 3mM ZnSO₄ (Figure 2). The percent hatch of eggs treated with the EC extract from soybeans/crop 1 was quite low, as was hatch of eggs treated with the EC extracts from both crops of kidney beans. Greater percentage hatch from samples treated with SB2-EC, KB1-EC and KB2-EC was expected at such an early point in the isolation process. Possibly, these roots contained an inhibitor that suppressed hatching or interfered with the recovery of a hatching factor from these samples at this point. Residual solvent may also have suppressed hatching. Alternatively, the hatching factor in these roots may have been very low and too dilute to be detected by the assay.

The samples, following the 50-hr liquid-liquid extraction, were subsequently extracted in ethyl acetate and treated with saturated aq NaHCO₃ (separatory extraction #1) and samples removed for the hatch bioassay. Unfortunately, the treatment containing SB1-NE, KB1-NE and KB2-NE were too cloudy to be able to count hatched nematodes. Only those samples from SB2-NE were analyzed and the resulting hatch was significantly lower than control treatments and SB2-EC (Figure 2). This result is in agreement with that found by Masamune *et al.* (1987) who also detected no hatching activity from the neutral ethyl acetate extracts.

The aqueous phase following the ethyl acetate and aq NaHCO₃ extraction was acidified with 6 M HCl to pH 2-3 (separatory extraction #2). The acidified ethyl acetate phase was tested for affect on hatch. Hatch from eggs treated with these samples (SB1-AE; SB2-AE; KB1-AE and KB2-AE) was lower than that from water and 3 mM ZnSO₄ (Figure 3; the original hatch percent data is located in Appendix B, Chapter 4, Figure 3b). If overall hatch, including that from the known hatch stimulants (di/RO water, ZnSO₄ and root diffusate) was less than 10%, the hatch results were depicted as a hatch effect. The hatch effect was calculated from the percent hatch of eggs in the water control subtracted from the percent hatch of eggs in the treatment divided by the percent hatch of eggs in the

water control. Masamune *et al.* found these extracts to be active in stimulating egg hatch.

The final extraction step consisted of an 18-hr liquid-liquid extraction using chloroform on the acidic phase following separatory extraction #2. Hatch from treatments with most of the extract samples (SB1-FC;KB2-FC) was lower than treatments with di/RO water and 3 mM ZnSO₄ yet higher than those treatments tested following separatory extraction #2. Hatch of eggs treated with KB1-FC was similar to that from the water treatment whereas hatch from the SB2-FC treatment was significantly greater ($P=0.05$) than that from water and 3 mM ZnSO₄ on day 14 (Figure 3). These extracts were also found to be active by Masamune *et al.* (1987).

Apparently, a hatching factor from the second crop of soybeans was recovered following the 50-hr extraction with chloroform. This hatching factor was still present following the 18-hr extraction with chloroform. It appears that using this modified protocol, a hatching factor was recovered from soybeans (crop 2). This demonstrates that a hatching factor, that may be glycinoeclepin A, exists in soybeans. However, because other factors apparently prevented the recovery of a hatching factor from the other soybean crop and both crops of kidney beans, the protocol should be repeated. The recovery of a hatching factor from soybeans also shows that this modified technique based on those of Masamune *et al.* can be used

to recover and demonstrate the presence of hatching factor(s) without lengthy purification steps.

For this study, it was decided to test all samples at the end of the entire study to limit variability that can occur when conducting hatch bioassays. This variability is due to batch-to-batch variation in the proportion of eggs that are developmentally or physiologically not ready to hatch, or the variability in hatch from eggs harvested during the summer months compared to winter months. However, before undertaking lengthy, exacting and tedious extractions it is imperative that initial samples demonstrate some hatch. It would be recommended, in spite of the variability issues, that a hatch bioassay take place immediately following the recovery of each extract sample. This would also prevent potential detrimental effects that may occur to samples during storage. These effects include possible overexposure to oxygen and harmful effects due to extremes in temperature.

Comparison of Methods

Masamune *et al.* (1987) developed a very refined, detailed extraction process that allowed the isolation and eventual identification of the hatching factor, glycinoeclepin A (GEA). This required the initial acquisition of a large volume of kidney bean root material (10 hectares = 1058 kg) in order to finally recover 1.25 mg of GEA active at $10^{-11}\text{--}10^{-12}$ g ml⁻¹. In this study, the initial extraction

procedures were modified in order to demonstrate the recovery of a hatching factor on a smaller scale in order to test extracts for egg hatch under field conditions.

Both studies required root material that was dried and powdered (Table 2). The root material used by Masamune *et al.* was air dried for one to three months. In this study, the roots were dried using a freeze-drier for three to five days. This dried root material was extracted with water in a cool environment, then eventually concentrated.

One of the major differences between the two studies consisted of how the liquid to liquid extractions using chloroform were set-up. In the study by Masamune *et al.*, they used a specially designed "Soxhlet" apparatus to achieve this extraction. In this study, a modified liquid-liquid extraction technique was developed using a liquid-liquid extractor that resulted in a successful extraction.

The egg hatch from treatments receiving the extracts derived from the first crop of soybeans and both kidney bean crops was disappointing. The root extracts are subjected to many conditions and chemicals that if not handled carefully and precisely may have an effect on hatch. In fact, possibly the months the soybeans and kidney beans were harvested may actually have an effect on the levels of hatching factor recovered. The two crops of kidney beans and the first crop of soybeans were all grown during the months of October through January. The second crop of soybeans was grown during February through March. Perhaps

environmental factors during this later time stimulate hatching compounds in the plant to develop. Schmitt and Riggs (1991) noted that day length and temperature may influence the level of hatching factor derived from the host. It's also possible that during the freeze-drying of the roots the temperature of the freeze-drier may have been too high for some of samples and detrimentally affected any hatching factors. During storage in the cooler, although the samples were sealed, oxidation of hatching factors is always a concern. The extract samples are subjected to numerous solvents, if these are not adequately removed following each extraction and especially prior to the hatch bioassay, these would certainly detrimentally affect the hatch bioassay. In future studies – prior to the egg hatch bioassay – all extract samples should be placed under a nitrogen stream to definitely remove any remaining solvent residue.

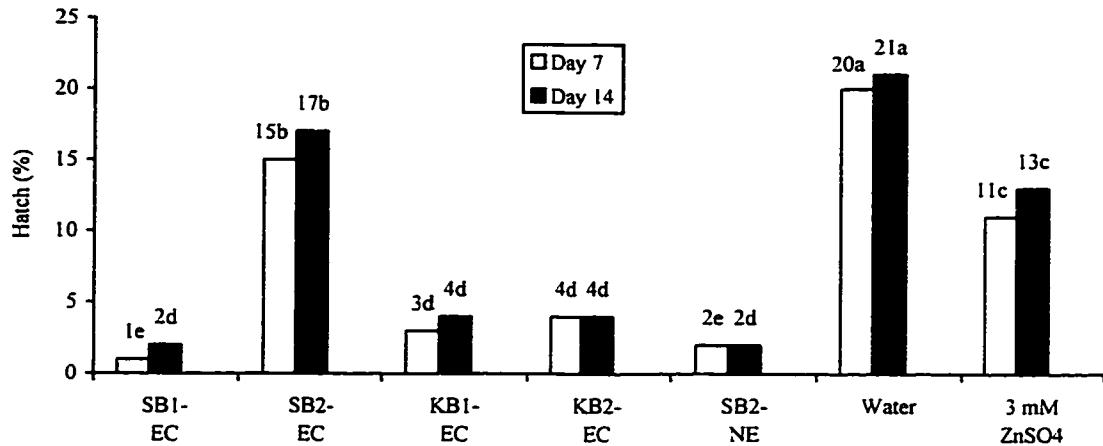


Figure 2. Percentage egg hatch of *Heterodera glycines* exposed to extracts derived from soybean (SB) and kidney bean (KB) roots, di/RO water and 3.0 mM ZnSO₄. Number following abbreviation refers to first (1) and second (2) crops. EC = extract following 50 hr chloroform extraction; NE = extract following neutral ethyl acetate extraction. Within time points, columns with the same letter are not significantly different ($P=0.05$).

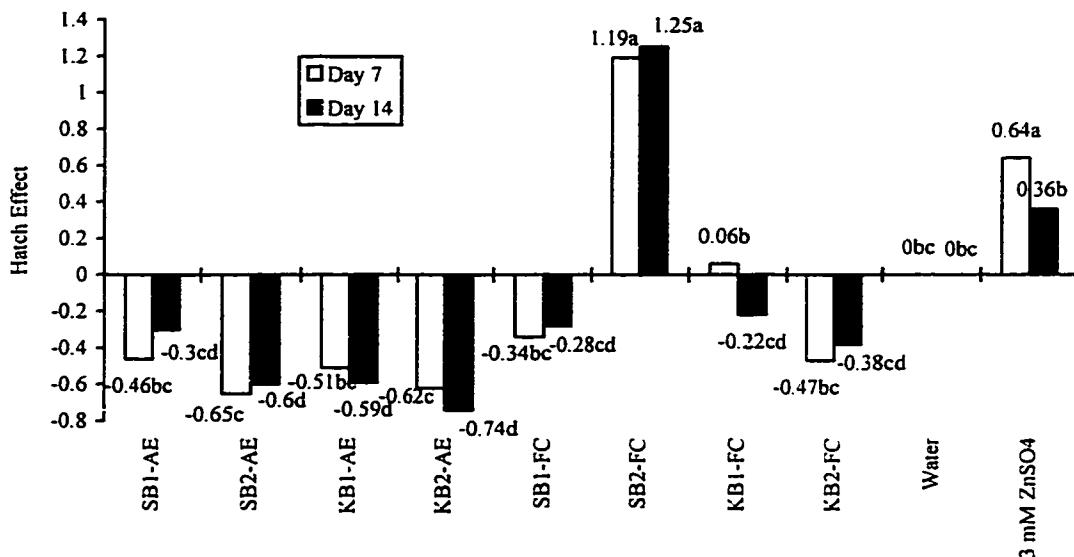


Figure 3. Effect of extracts derived from soybean (SB) and kidney bean (KB) roots, di/RO water and 3.0 mM ZnSO₄ on egg hatch of *Heterodera glycines*. Number following abbreviation refers to first (1) or second (2) crop of plants. AE = extract after acidified ethyl acetate; FC = extract after 18 hr chloroform extraction. Hatch effect = % hatch (treatment) - % hatch (water) / % hatch (water). Within time points, columns with the same letter are not significantly different (P=0.05).

Table 2. An outline of the abbreviated procedures developed by Masamune, *et al.* (1987) to isolate glycinoeclepin A, a hatching factor for *Heterodera glycines* and those techniques followed by Moberg *et al.* in this study. Those techniques modified by Moberg *et al.* appear in boldface.

<u>Masamune et al.:</u>	<u>Moberg et al.:</u>
Kidney bean roots air dried for 1-3 months	Kidney and soybean roots dried in a freeze drier 3-5 days
Grind Roots	Grind Roots
Root material extracted in water	Root material extracted in water
Water evaporated under reduced pressure	Water evaporated using thin-film evaporator and dry-ice vacuum
Extracts acidified with 6 M HCl to pH 2-3	Extracts acidified with 6 M HCl to pH 2-3
"Soxhlet" extraction using chloroform; 50-hrs	Liquid-liquid extraction using chloroform; 50-hrs
Dissolve in ethyl acetate with sat. aq. NaHCO ₃ (Separatory extraction #1)	Dissolve in ethyl acetate with sat. aq. NaHCO ₃ (Separatory extraction #1)
Acidify using 6 M HCl	Acidify using 6 M HCl
Dissolve in ethyl acetate (Separatory extraction #2)	Dissolve in ethyl acetate (Separatory extraction #2)
"Soxhlet" extraction using chloroform; 18-hrs	Liquid-liquid extraction using chloroform; 18-hrs
Anhydrous Na ₂ SO ₄ to absorb water; filter	Anhydrous Na ₂ SO ₄ to absorb water; filter
Evaporate using thin-film evaporator	Evaporate using thin-film evaporator

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Appendix A

Soybean Soapstock Analysis

A typical analysis of acidulated soybean soapstock consists of the following:

Acid Value	80-130
Total Fatty Acids	94-96%
Color	Dark
Iodine Value	118-130
Moisture (Karl-Fischer)	5% max.

A typical analysis of a fatty acid profile for acidulated soybean soapstock consists of the following:

<u>Fatty Acid Profile</u>	<u>% of Total Fatty Acids</u>
14:0 myristic acid	0.1
16:0 palmitic acid	14.1
18:0 stearic acid	4.8
18:1 oleic acid	21.0
18:2 linoleic acid	52.2
18:3 linolenic acid	6.9
20:0 arachidic acid	0.3
22:0 behenic acid	0.4

[The above information was gathered from a patent application for a nonionic soybean oil surfactant].

Appendix B

Chapter 3:

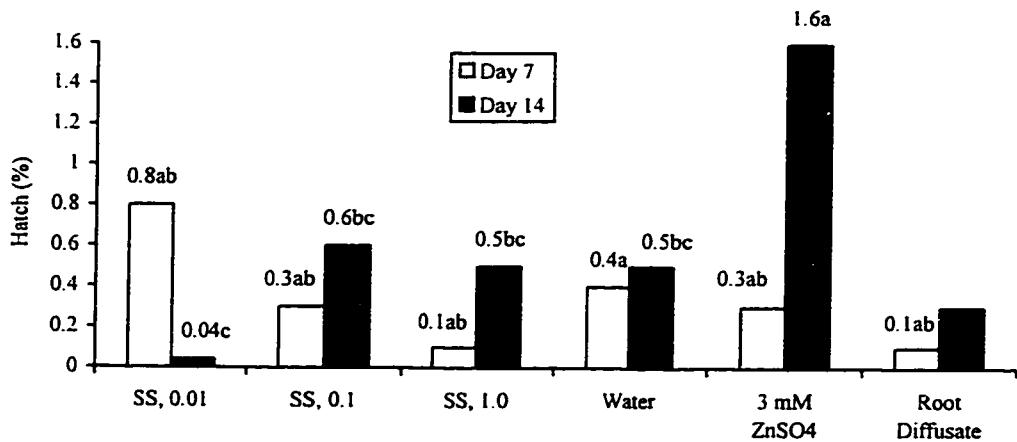


Figure 3b. Percentage egg hatch of *Heterodera glycines* exposed to soybean soapstock (SS), diluted to 0.01, 1.0 and 1.0%, at varying pH levels, di/RO water, 3.0 mM ZnSO₄ and root diffusate. Within time points, columns with the same letter are not significantly different ($P=0.05$).

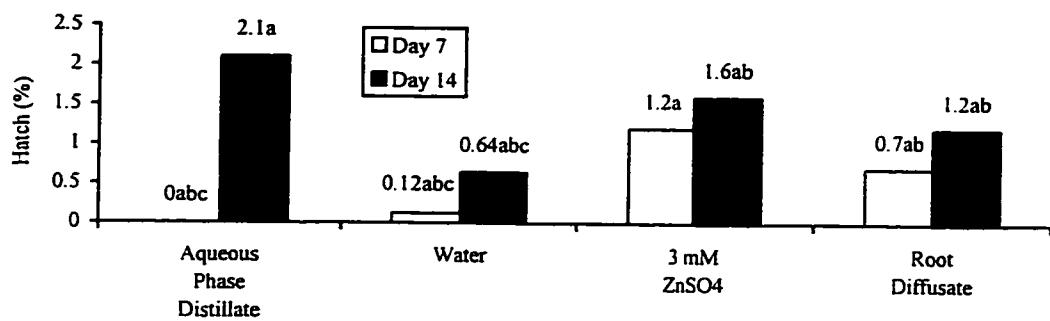


Figure 5b. Percentage egg hatch of *Heterodera glycines* exposed to the aqueous phase following soybean soapstock distillation, di/RO water, 3.0 mM ZnSO₄ and root diffusate. Within time points, columns with the same letter are not significantly different ($P=0.05$).

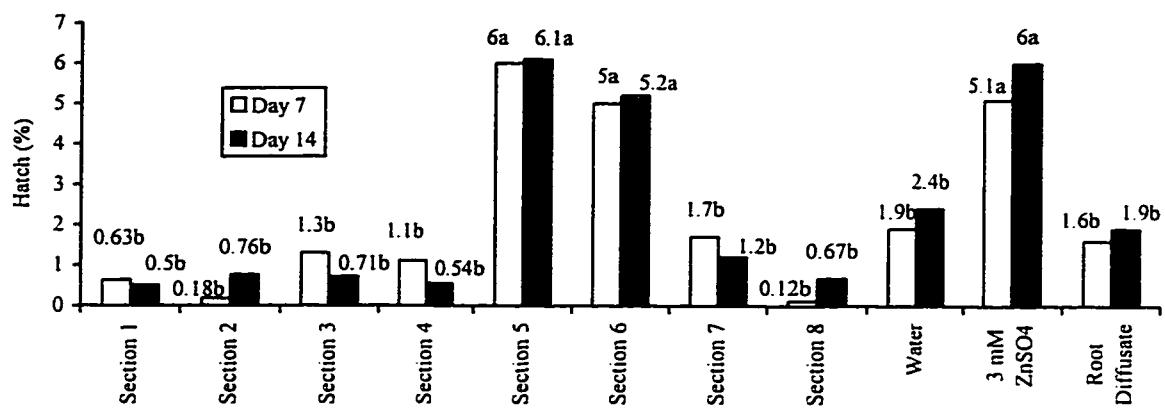


Figure 6b. Percentage egg hatch of *Heterodera glycines* exposed to soybean soapstock following thin-layer chromatography (section 1-10; Group 1), di/RO water, 3.0 mM ZnSO₄ and root diffusate. Within time points, columns with the same letter are not significantly different ($P=0.05$). There was no hatch from sections 9 and 10.

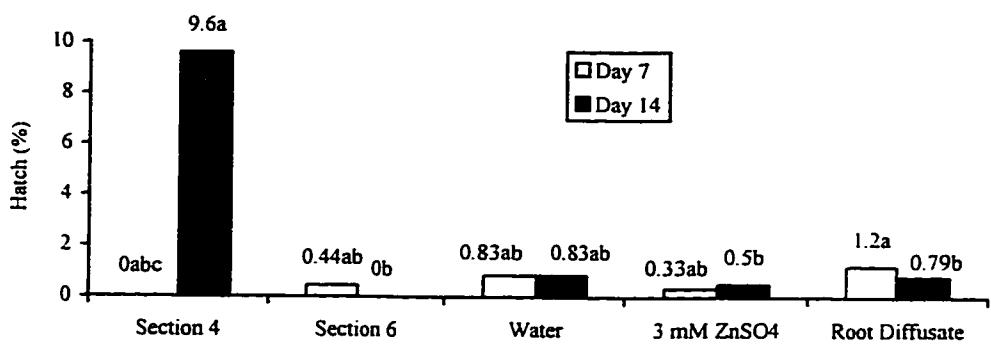


Figure 7b. Percentage egg hatch of *Heterodera glycines* exposed to soybean soapstock following thin-layer chromatography (sections 2-10; 2nd study), di/RO water, 3.0 mM ZnSO₄ and root diffusate. Within time points, columns with the same letters are not significantly different according to Duncan's least significant difference ($P=0.05$).

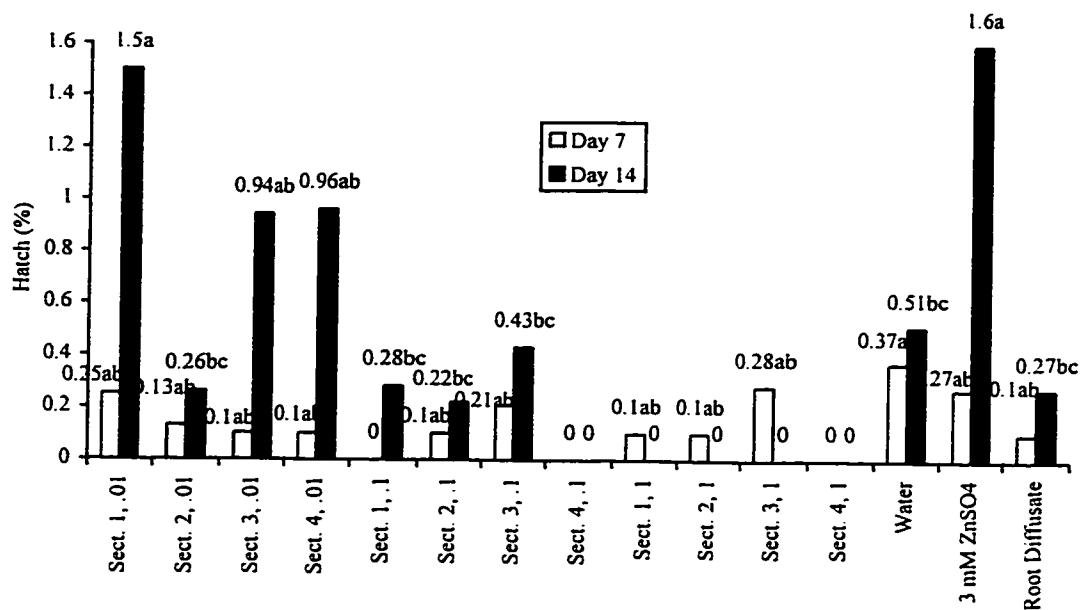


Figure 8b. Percentage egg hatch of *Heterodera glycines* exposed to soybean soapstock diluted to 0.01, 0.1 and 1% following thin-layer chromatography (sections 1-4; 1st study), di/RO water, 3.0 mM ZnSO₄ and root diffusate. Within time points, columns with the same letters are not significantly different according to Duncan's least significant difference (P=0.05).

Chapter 4:

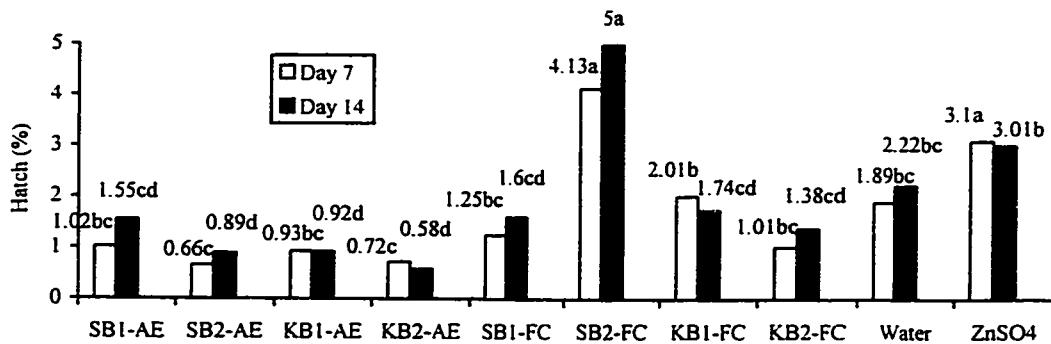
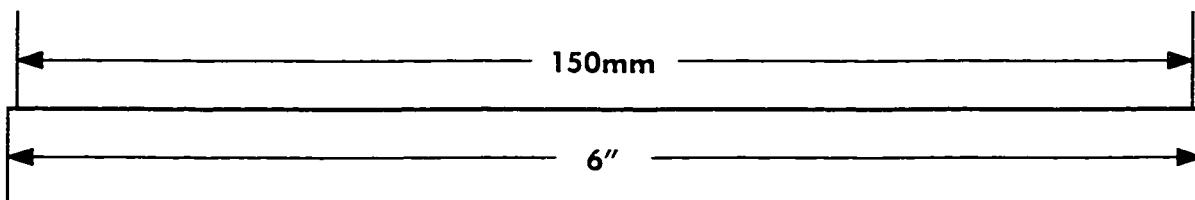
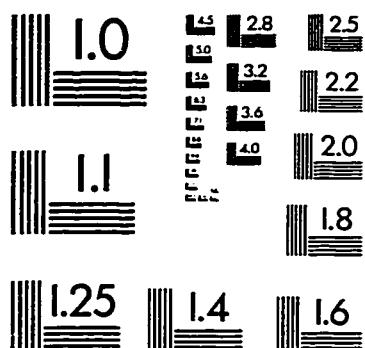
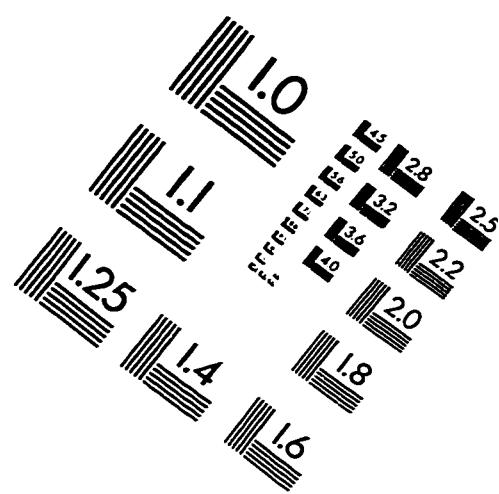
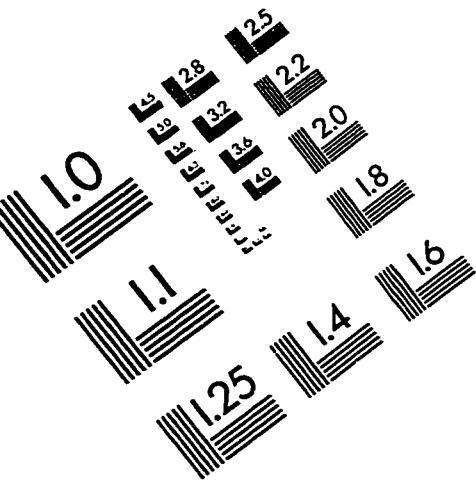


Figure 3b. Percentage egg hatch of *Heterodera glycines* exposed to extracts derived from soybean (SB) and kidney bean (KB) roots, di/RO water and 3.0 mM ZnSO₄. Number following abbreviation refers to first (1) or second (2) crop of plants. AE = extract after acidified ethyl acetate; FC = extract after 18 hr chloroform extraction. Within time points, columns with the same letter are not significantly different ($P=0.05$).

IMAGE EVALUATION TEST TARGET (QA-3)



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