

THE EFFECT OF SPIROTETRAMAT APPLIED AGAINST RENIFORM  
NEMATODE, *ROTYLENCHULUS RENIFORMIS*, ON PINEAPPLE, *ANANAS  
COMOSUS*, AND TOMATO, *SOLANUM LYCOPERSICUM*

A THESIS SUBMITTED TO THE  
GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII AT MĀNOA IN  
PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

TROPICAL PLANT PATHOLOGY

MAY 2015

By

Philip Waisen

Thesis Committee:

Brent S. Sipes, Chairperson

Koon-Hui Wang

Zhiqiang Cheng

*Key words: Ananas comosus, chemical control, movento, pineapple, reniform nematode,  
Rotylenchulus reniformis, Solanum lycopersicum, spirotetramat, tomato*

## **ACKNOWLEDGEMENTS**

I thank my adviser, Dr. Brent Sipes, for guiding me throughout my research. I also thank my committee members, Dr. Koon-Hui Wang and Dr. Zhiqiang Cheng for their comments and suggestions during my studies. I thank the nematology laboratory support staff, Donna Meyer, Steve Yoshida, Mike Young and Gareth Nagai for technical support and assisting with my experiments. I also thank the U.S. Department of State for funding my master's program and the East-West Center for administering my fellowship. Many thanks to my parents who have struggled yet been supportive throughout my educational endeavors.

## **DEDICATION**

This thesis is dedicated to my late biological father, Kezorifa Isi, who just passed away seven hours ago at 5:00 am GST on April 30, 2015, as I am writing. Nothing lucrative I will have in my life will worth your commitment and dedication to raise me and educate me to be this person. Your legacy left behind will live through me and my children. May your soul rest in eternal peace. God bless you daddy.

## ABSTRACT

Reniform nematode, *Rotylenchulus reniformis*, is a major pest of pineapple (*Ananas comosus*) and many vegetable crops in Hawaii reducing yields by 26.8-50%. Damage thresholds are low (300-1000 nematodes/250 cm<sup>3</sup> soil) and host-plant resistance is lacking. Therefore management of reniform nematode depends on other tactics. Spirotetramat is a systemic phloem-and-xylem mobility pesticide which acts as a lipid biosynthesis inhibitor. Lipids play a significant physiological role in molting and embryogenesis in plant-parasitic nematodes, hence spirotetramat may provide a viable nematode management tool. The objective of this research was to determine if spirotetramat was active against reniform nematode. An *in vitro* assay was conducted where reniform nematode eggs were subjected to different spirotetramat rates to assess its effect on hatching. A greenhouse experiment pot experiment where 14-day-old tomato (*Solanum lycopersicum*) plants were transplanted, inoculated and treated with spirotetramat to assess nematode penetration. A second greenhouse assays were conducted in which 4-month-old potted-pineapple plants were treated with different rates of spirotetramat 1 month post-inoculation (Pi) with *R. reniformis* and terminated at 4 or 10 months post-treatment (Pt) to assess nematode population and plant growth. A third greenhouse trial was conducted in which tomato plants were treated 14 days Pi and terminated 14 days Pt or 28 days Pi to assess nematode fertility. Spirotetramat treatment 14 days prior to inoculation significantly reduced 92% nematode eggs/g of root. Nematode hatch was similar in all treatments. Penetration was suppressed 100% at 50 g/ha in the tomato greenhouse experiment. In the pineapple trial, 200 g a.i/ha reduced 93% nematode eggs/g of root and increased 34% pineapple growth. Rhizosphere

vermiform nematodes/g of root was reduced 89% at 88 g a.i/ha. The lack of effect on hatching implies that spirotetramat is only active through nematode ingestion. Spirotetramat affected reniform nematode fertility on tomato and pineapple. Spirotetramat holds potential for management of reniform nematode in pineapple and vegetable crops.

## Table of contents

Contents	Pages
CHAPTER 1: INTRODUCTION	
<i>Host range</i> .....	1
<i>Lifecycle</i> .....	2
<i>Economic importance</i> .....	3
<i>Biological control measures</i> .....	4
<i>Cultural control measures</i> .....	5
<i>Chemical control measures</i> .....	7
<i>Spirotetramat</i> .....	8
<i>Objectives</i> .....	10
Literature cited.....	10
CHAPTER 2. EFFECT OF SPIROTETRAMAT ON HATCH, PENETRATION, AND REPRODUCTION OF <i>ROTYLENCHULUS RENIFORMIS</i>	
Introduction.....	23
Materials and methods.....	24
Results and discussion.....	26
Conclusion.....	29
Literature cited.....	29

CHAPTER 3. EFFECT OF SPIROTETRAMAT ON THE FERTILITY OF  
*ROTYLENCHULUS RENIFORMIS* ON *ANANAS COMOSUS* AND *SOLANUM*  
*LYCOPERSICUM*

Introduction.....	31
Materials and method.....	32
Results and discussion.....	34
Conclusion.....	46
Literature cited.....	46

**List of tables**

*Table 1 Root penetration trial: Penetrations per gram of root*.....30

*Table 2 Pineapple trial 1: Shoot weight and nematodes in each subplot*.....40

*Table 3 Pineapple trial 2: Shoot weight and nematodes per gram of root* .....43

*Table 4 Tomato trial 2: Nematode eggs and vermiform per gram of root*.....48

**List of figures**

*Figure 1 Nematode hatch per gram of tomato root*.....29

*Figure 2 Pineapple trial 1: Nematodes per gram of root* .....39

*Figure 3 Pineapple trial 1: Nematode eggs per gram of root*.....42

*Figure 4 Pineapple trial 1: Vermiform nematodes per gram of root* .....42

*Figure 5 Tomato trial 1: Nematode eggs per gram of root*.....44

*Figure 6 Tomato trial 1: Vermiform nematodes per gram of root* .....45

*Figure 7 Tomato trial 2: Nematodes per gram of root* .....46



## CHAPTER 1. INTRODUCTION

Reniform nematode, *Rotylenchulus reniformis* Linford and Oliveira, was first observed in 1938 by Francis Yap, a technician at Hawaii Pineapple Research Institute on roots of cowpea, *Vigna sinensis*, grown in a pineapple field on the Island of Oahu in Hawai'i, USA (Linford and Oliveira, 1940). The genus was subsequently described as a novel species (Linford and Oliveira, 1940). *Rotylenchulus reniformis* is one of ten described species in the genus. *Rotylenchulus reniformis* is well investigated and documented because of its economic impact on agricultural crops (Robinson *et al.*, 1997).

### **Host Range**

*Rotylenchulus reniformis* is polyphagous with a wide host range that reflects its cosmopolitan distribution in tropical, sub-tropical and warm temperate zones in Africa, Asia, Australia, the Caribbean Basin, the Middle East, North America, the Pacific, South America, and Southern Europe (Ayala and Ramirez, 1964). *Rotylenchulus reniformis* parasitizes more than 30 plant families (Varaprasad, 1986) and is known to reproduce on 314 plant species including 50 crop plants (Robinson *et al.*, 1997). In Hawaii, 65 host plants were reported (Linford and Yap, 1940). Since 1950, *R. reniformis* has been the key nematode problem in pineapple (Caswell and Apt, 1989). The nematode has been reported on a wide variety of crops, including cotton, vegetable crops, and several tropical and sub-tropical fruit species, in addition to pineapple (McSorley, 1980; McSorley *et al.*, 1981, 1982; Robinson *et al.*, 1997; Wang and Hooks, 2009). Some common weeds and ornamentals also serve as hosts (Inserra *et al.*, 1989, 1994a, b). Several weeds and ornamentals serve as alternate hosts for *R. reniformis* (Inserra *et al.*, 1989, 1994a, b; Robinson *et al.*, 1997; Ganguly and Art, 1998; Roy and Mukhopadhyay,

2005; Davis *et al.*, 2006; Lawrence *et al.*, 2008) during the off-cropping seasons and non-host crop rotations.

### **Life-cycle**

The reniform nematode undergoes four molts before becoming an adult, requiring 24-29 days to complete its life-cycle under optimum environmental conditions (MacGowan, 1977). Embryogenesis to first-stage juvenile (J1) occurs in 3 days, and a first molt occurs within the egg which gives rise to the second-stage juvenile (J2). After 1-3 days of development, the J2 hatches and undergoes a second molt 6-7 days after the initial egg fertilization. The J2 cuticle remains, enclosing the third-stage juvenile (J3), which continues in the vermiform state while the sexes begin to differentiate (Gaur and Perry, 1991). After 2-3 days, the fourth-stage juvenile (J4) develops with a new stylet and well-established cephalic region (Gaur and Perry, 1991; Nakasono, 2004). The adults develop after a final molt. The adult female penetrates the root cortex of a host plant and establishes a permanent feeding site called a syncytium in the stele (Robinson *et al.*, 1997). The female remains sedentary swelling into a diagnostic kidney-shaped nematode 6-15 days after initial infection. The female attracts the male through chemical signals for mating (Nakasono, 1977; Star, 2007). The male does not parasitize roots but remains in the rhizosphere for amphimictic reproduction (Triantaphyllou and Hirschmann, 1964). In some populations, females can reproduce parthenogenetically (Dasguta, *et al.*, 1986) in the absence of males. The sex ratio is almost equal in *R. reniformis* (Fukuhara, 1966). The male inseminates females prior to egg maturation and sperm is stored in the spermatheca until fertilization. After fertilization the eggs are oviposited in a gelatinous

matrix mainly composed of glycoproteins secreted by the uterine glands (Agudelo *et al.*, 2003).

*Rotylenchulus reniformis* has a semi-endoparasitic behavior of feeding in which the anterior portion of the female body remains embedded in the host root while the posterior portion protrudes externally from the root surface. *Rotylenchulus reniformis* is an obligate semi-endoparasite and sedentary in the mature female stage. *Rotylenchulus reniformis* adopts an anhydrobiotic state under desiccating environmental conditions to survive (Radewald and Takeshita, 1964; Tsai, 1978) and can remain quiescent in this state for up to 1.5 years (Apt, 1976) without losing infectivity.

### **Economic Importance**

*Rotylenchulus reniformis* is one of more than 4,100 species of plant-parasitic nematodes (PPNs) (Decraemer and Hunt, 2006). PPNs collectively are an important threat to global food security with estimated crop loss on a global scale valued at US\$173 billion annually, with at least \$13 billion loss in the USA (Elling, 2013). *Rotylenchulus reniformis* is an economically important PPN of many crop plants including pineapple and cotton. In Hawai'i, *R. reniformis* is the major pest of pineapple (Rohrbach and Apt, 1986; Starr, 1990, 1991; Ko and Schmit, 1996; Robinson *et al.*, 1998; Wang *et al.*, 2001, 2002a; Sipes *et al.*, 2005; Wang and Hooks, 2009) with an economic damage threshold between 300 to 1,000 vermiform stages/250 cm<sup>3</sup> of soil (Sipes and Wang, 2000). Without suitable control, Smooth Cayenne pineapple may suffer 30-40% fruit yield reduction and 80-100% loss in subsequent ratoon crops (Py *et al.*, 1986; Sipes *et al.*, 2005). *Rotylenchulus reniformis* can reduce pineapple marketable yield by up to 26.8% (Sipes, 1994) in a plant crop and by 50% in following ratoon crops (Sipes, 1996). In the southern

states of the USA, *R. reniformis* has replaced *Meloidogyne incognita* as a major pest of cotton (Heald and Robinson 1990; Koenning *et al.*, 2004) and reduced cotton yield potential by 40%, estimated at \$130 million annually (Robinson, 2007). *Rotylenchulus reniformis* was reported to cause an average annual loss of more than 5% of the total cotton production in Louisiana, Mississippi and Alabama (Blasingame *et al.*, 2010). Disease complexes with opportunistic soil-borne pathogens like *Fusarium solani*, *Rhizoctonia solani*, and *Verticillium* spp. exacerbate the nematode problem in cotton (Shurtleff and Averre, 2000).

Control of *R. reniformis* relies upon reducing the population in the soil before a crop is planted or controlling the population after the crop is planted. It is nearly impossible and usually impractical to eliminate the nematode from an infested field, therefore management of *R. reniformis* has depended upon biological, cultural and chemical tactics.

### **Biological Control**

Several promising potential biological control agents that could be used for management of *R. reniformis* exist. The nematode trapping fungi (NTF) *Drechslerella dactyloides* and *D. brochopaga* were found parasitizing juveniles of *R. reniformis* (Kumar and Singh, 2006; Singh *et al.*, 2007). The nematode egg-parasite fungus, *Paecilomyces lilacinus* strain 251, reduced *R. reniformis* populations by 36% in the greenhouse conditions and 59% in the microplot experiments on tomato (Walters and Barker, 1994). A host-root colonizing bacterium, *Bacillus firmus* strain GB-126 has been widely investigated for its antagonistic effect against PPNs including *R. reniformis* (Mendoza *et al.*, 2008; Terefe *et al.*, 2009; Schrimsher *et al.*, 2011; Castillo *et al.*, 2013).

*Bacillus firmus* GB-126 ( $1.4 \times 10^7$  CFU/seed) and *P. lilacinus* strain 251 (0.3% v/v) when treated singly or concomitantly in cotton under greenhouse conditions reduced all life stages of *R. reniformis* and increased populations of free-living nematodes (Castillo, 2013). Cotton yields in *R. reniformis* infested fields treated with *B. firmus* GB-126 or *P. lilacinus* 251 were comparable to yields from plots treated with aldicarb (Castillo, 2013). In India, strains of *Pseudomonas fluorescence* reduced *R. reniformis* populations in cotton roots and soil by 70.4% and 44.8% respectively (Jayakumar *et al.*, 2003). In pot experiments, 3 out of 117 isolates of *Pocconia clamydosporia* suppressed an Arkansas population of *R. reniformis* by 77% (Wang *et al.*, 2005). A single application of isolate 37 of *P. clamydosporia* significantly reduced the numbers of *R. reniformis* on cotton roots and in soil (Wang *et al.*, 2005). Cotton seeds treated with *Pasteuria* strain Pr3 at  $1 \times 10^8$  endospores/seed suppressed *R. reniformis* similar to the seed treatment with Aeris® (imidacloprid + thiodicarb) (Schmidt *et al.*, 2010). Biologically derived pesticides may provide alternatives to the organophosphates and carbamates for management of *R. reniformis*. To date, no biological control agents have been successful in achieving economical control of *R. reniformis* (Sipes, 2000).

### **Cultural Control**

Cultural management tactics for PPNs are traditional and remain environmentally friendly management options. Cultural controls include practices such as crop rotation, weed control, solarization, and the use of cover crops. In a field infested with *R. reniformis*, rotations of corn, peanut, and resistant soybean increased cotton yield by 31%, 27% and 27% respectively (Gilman *et al.*, 1978). Weed management is important in the management of *R. reniformis* because they serve as alternate hosts during off

cropping periods and non-host crop rotation cycles. Tropical cover crops including sunn hemp, *Crotalaria juncea*, have been investigated for management of *R. reniformis* in Hawaii. Compared to weedy fallow, sunn hemp is a promising cover crop against *R. reniformis* (Wang *et al.*, 2002). Sunn hemp-amended pineapple field soil significantly suppressed *R. reniformis* on cowpea in the greenhouse and the suppression was shown to positively correlated with enhancement of nematode trapping fungi (NTF) (Wang *et al.*, 2003). Also the suppressive effect on vermiform stages of *R. reniformis* ( $P < 0.01$ ) in the rhizosphere was correlated with bacterivorous nematode enhancement (Wang *et al.*, 2003). Wang *et al.* (2001) also demonstrated that sunn hemp-induced suppression of *R. reniformis* was related to a delay in female development, allelopathy, and enhancement of NTF. Sunn hemp followed by soil solarization suppressed *R. reniformis* ( $P < 0.05$ ) compared to no sunn hemp and no solarization (Marahatta *et al.*, 2012). Furthermore, sunn hemp extract was shown to have nematostatic effects against *R. reniformis* (Wang *et al.*, 2002b). *Tagetes* spp. may suppress *R. reniformis* either acting as a non-host or a poor host, releasing allelopathic compounds such as *a*-terthienyl, creating an environment that favors nematode antagonistic flora or fauna, or behaving as a trap crop (Pudasaini *et al.*, 2008; Wang *et al.*, 2001). These mechanisms may occur singly or concomitantly in reducing populations of *R. reniformis* (Hooks *et al.*, 2010). French marigold, *T. patula*, has been used as a non-host to limit the penetration and the development of *R. reniformis* (Caswell *et al.*, 1991). In Japan, while *T. erecta* served as a moderate host to *R. reniformis*, *T. patula* was shown to markedly reduce populations of *R. reniformis* compared to fallow (Nakasono, 1973). However, these non-chemical alternatives are

currently not as effective as most chemicals in achieving economical control of *R. reniformis* in commercial production (Sipes, 2000).

### **Chemical Control**

Synthetic nematicides have been the primary means of managing damage from *R. reniformis* in the pineapple and cotton cropping systems (Sipes, 2000). Fumigant nematicides had been the primary nematode management tactics employed in Hawaii pineapple fields for over 60 years (Schneider *et al.*, 1995 and 1997; Sipes *et al.*, 2005). Methyl bromide and 1,3-dichloropropene were the main preplant fumigants (Sipes, 2000). Fallow periods of 6-12 months with all fields receiving 1,3-dichloropropene was the industry standard practice in pineapple production in the 1980s (Caswell and Apt, 1989). Oxamyl and fenamiphos were used as non-fumigant postplant nematicides applied through drip irrigation and as foliar applications. With the intent to eliminate or minimize adverse environmental impacts and health related risks, most of these nematicides have been removed from the market. Methyl bromide has been phased out due to concerns with ozone depletion (USDA NASS, 2000). DBCP and DB were withdrawn due to possible health concerns. Fenamiphos was removed from the market due to its toxicity to non-target organisms. Currently, pre-plant fumigation with 1,3-dichloropropene (Telone®) or metam sodium (Vapam®) and post-plant nematicides application such as oxamyl (Vydate®) are used to manage *R. reniformis* (Wang *et al.*, 2011; Moore and Lawrence, 2012). These limited chemical options may create nematicide resistant population of *R. reniformis* or lead to enhanced degradation by soil microbial population over time. Additional alternatives are needed by growers.

## **Spirotetramat**

Spirotetramat, commercially available as Movento<sup>®</sup> from Bayer CropScience, is a group 23 synthetic insecticide. Spirotetramat is the first member of a new chemical class, cyclic ketoenoles. Spirotetramat is a spirocyclic tetramic acid derivative (Bretschneider *et al.*, 2007) that acts as a lipid biosynthesis inhibitor (Nauen *et al.*, 2006, 2008). Spirotetramat is active only by ingestion and largely effective against juvenile stages of sucking insects. Spirotetramat minimally reduces fecundity (number of eggs oviposited) and fertility (viability of eggs oviposited) in an adult insects that result in overall decline in insect pest pressure. Spirotetramat is registered for control of a broad range of hemipterans, the sucking insect pests, including aphids, mealybugs, psyllids, whiteflies, psylla, phylloxera and specific thrips of horticultural and vegetable crops (Nauen *et al.*, 2008). As long as a plant's vascular system is actively transporting spirotetramat-enol and an insect pest is feeding, it takes 2-10 days post-treatment to cause death in target immature insects (Nauen *et al.*, 2008). Once the spirotetramat penetrates the leaf cuticle, it is quickly metabolized through hydrolysis and forms spirotetramat-enol, the metabolite that enters the xylem and phloem and is translocated throughout the entire plant (Bruck *et al.*, 2009). The weak acid formed with xylem fluid demonstrates a two-way systemic movement in both the phloem and xylem (Nauen *et al.*, 2008; Safferling, 2008; Vermeer and Baur, 2008). This unique ambimobile systemic movement is not common in agricultural insecticides and nematicides. This two-way systemic movement allows spirotetramat to move acropetally and bisepetally, thus any hidden pests in the buds, leaf-rolls and roots can be controlled.



Spirotetramat interferes with acetyl CoA carboxylase, an enzyme crucial for fatty acid biosynthesis. Lipids are critical to the survival of PPNs because lipids play a basal role in numerous physiological processes such as embryogenesis and molting. In Western flower thrips, *Frankliniella occidentalis*, spirotetramat was effective against larval stages but had no effect on adults (Herron and James, 2005; Guillen, *et al.*, 2014). In laboratory assays, mortality ( $P < 0.05$ ) of *F. occidentalis* was significantly higher 2-3 days post-treatment (dpt) when treated at 250-500 mg/L (Zheng *et al.*, 2014). Bioassays on sucking pests showed that spirotetramat affected immature stages, with little effect on adults (Nauen *et al.*, 2008, Brück *et al.*, 2009). Spirotetramat controlled the development of Asian citrus psyllid (*Diaphorina citri*) and the citrus rust mite (*Phyllocoptruta oleivora*) populations for more than 2 months (Edenfield and Morris, 2013). The population of the vine mealybug, *Planococcus ficus*, was reduced by 55-80% 21 days after treatment with spirotetramat (Mansour *et al.*, 2010).

Sedentary PPNs, including *R. reniformis*, and sucking insect pests share four characteristics: embryogenesis, molting, a sedentary life stage, and plant feeding. Embryogenesis and molting both involve lipid metabolism. Thus we hypothesize that spirotetramat activity will be common in both groups of organisms. With the lipid biosynthesis mode of action, spirotetramat could possibly act against biosynthesis of 24-ethylcholesterol and cholesterol, the major lipids (sterols) found in *R. reniformis* (Chitwood, 1999), thus embryogenesis and molting in *R. reniformis* could be interrupted.

Spirotetramat has been evaluated for efficacy against several PPNs. McKenry *et al.* (2010) demonstrated that foliage application of spirotetramat reduced the number of *Pratylenca vulnus* in the rhizosphere of walnut (*Juglans* spp.) by 50% over a 6-month

period. Late fall treatments of spirotetramat at 100 ml/ha reduced population levels of *P. vulnus* by 45% for 4 months but reduced *Tylenchulus semipenetrans* populations on grapes (*Vitis* spp.) over 6 weeks. Spring treatments reduced soil populations of *Meloidogyne* spp., *P. vulnus* and *T. semipenetrans* by 50% over a 3-month period (McKenry *et al.*, 2009). Spirotetramat application on wheat (*Triticum* spp.) reduced *Heterodera avenae* populations by 78% and 35% before and after plants exhibited white females respectively (Smiley *et al.*, 2011). Post-harvest population density of *H. avenae* was reduced by 52-71% compared with control treatments (Smiley *et al.*, 2012). Spirotetramat applied twice per year reduced the number of *T. semipenetrans* females, juveniles and eggs (Edenfield and Morris, 2013). Spirotetramat has not been evaluated for its capacity to suppress *R. reniformis*.

### **Objectives**

The overall objective of this research was to determine if spirotetramat is active against *R. reniformis*. The specific objectives were to determine the effect of spirotetramat on 1) hatching, 2) penetration, and 3) fertility of *R. reniformis*.

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## CHAPTER 2. EFFECT OF SPIROTETRAMAT ON HATCH AND PENETRATION OF *ROTYLENCHULUS RENIFORMIS*

Spirotetramat is effective against a range of insect pests acting as a lipid biosynthesis inhibitor (Nauen *et al.*, 2006, 2008). The compound has reduced Western flower thrip, *Frankliniella occidentalis*, larval populations 80% within 7 days of exposure (Guillen *et al.*, 2014). While effective against larval stages, spirotetramat had no effect on adults of *F. occidentalis* (Herron and James, 2005). Spirotetramat controlled the development of Aisan citrus psyllid and citrus rust mite, *Diaphorina citri* and *Phyllocoptruta oleivora*, for more than 2 months (Edenfield and Morris, 2013). The population of the vine mealybug, *Planococcus ficus* was reduced by 55-80% on 21 days post-treatment (dpt) with spirotetramat (Mansour *et al.*, 2010). Bioassays on sucking pests have shown that spirotetramat affects immature stages, with little effects on adults (Nauen *et al.*, 2008, Brück *et al.*, 2009). Spirotetramat exhibits only limited contact activity against sucking insect pests, thus it is primarily effective only after ingestion by the pest (Nauen *et al.*, 2008).

Spirotetramat breaks down into its active metabolite, spirotetramat-enol (EPA, 2008; APVMA, 2009) within days after application. The parent compound undergoes hydrolysis of the side chain ester bond resulting in the formation of the secondary metabolite, BYI 08330-enol. In sucking insect pests, spirotetramat requires 2-10 days post-treatment to kill the larvae when the plant vascular system is actively translocating the metabolite and the insects are actively feeding (Nauen *et al.*, 2008).

Since plant-parasitic nematodes share traits and behaviors with sucking insect pests such as, embryogenesis and molting – all processes that involve lipid

biosynthesis, spirotetramat may be effective against nematodes as well. Nematodes, such as *Rotylenchulus reniformis*, undergo embryogenesis in the egg, hatch, penetrate hosts, and molt to complete their life cycle. After hatching, plant-parasitic nematodes must penetrate the roots of a host plant and establish a feeding site. The efficacy of a nematicide treatment can be determined by evaluating the nematode along the steps in this pathway. Spirotetramat could affect the hatch and penetration of *R. reniformis* through contact activity. Reproduction of *R. reniformis* could be affected by the ingestion of the active metabolite upon establishment of a feeding site. We hypothesize that spirotetramat will reduce populations of *R. reniformis* as it does to insect population by affecting one or more of these steps in the life cycle of *R. reniformis*. The objective of this experiment was to determine the effect of spirotetramat on the hatch, penetration, and reproduction of *R. reniformis*.

#### **MATERIALS AND METHODS**

Field soil was autoclaved and mixed with sand in a 1:1 ratio. Sixty 15-cm-d biodegradable pots (Mayers Lawn & Garden, Canada) were filled with the soil mix. Tomato cv. Patio F1 Hybrid seeds were germinated in 7-cm-d clay pots filled with vermiculite. Seedlings were individually transplanted 14 days later into the pots. Plants were maintained in the greenhouse, watered and fertilized monthly (0.006 g Miracle.Gro/ml water) in all experiments.

*Rotylenchulus reniformis* inoculum was collected from cultures maintained on tomato in the greenhouse. Nematode eggs were collected from tomato roots using a NaOCl extraction method (Hussey and Barker, 1973). Eggs were separated from fine



soil particles and debris using a density-dependent centrifugal sugar flotation method (Jenkins, 1964; Barker, 1985). Inoculum was standardized at 1,000 eggs/ml.

*Hatching test:* To determine the effects on hatch, an *in vitro* experiment was conducted. Spirotetramat concentrations of 0, 50, 100, and 200 g a.i/ha were formulated by mixing Movento 240 SC with tap water. Each treatment was replicated 7 times. Twenty eggs of *R. reniformis* were placed into individual watch glasses filled with 1 ml of the treatment rate. The experiment was arranged in a completely randomized design. The eggs were incubated at 25°C in the dark. Numbers of nematodes hatched was recorded every 2 days for 6 days. The spirotetramat solution was changed at each assessment time.

*Penetration test:* To assess penetration of *R. reniformis* as affected by spirotetramat, twenty-three 14-day-old tomato seedlings were inoculated with 1,000 eggs of *R. reniformis*. Spirotetramat was applied 14 days post-inoculation at 0, 50, 100, or 200 g a.i spirotetramat/ha delivered in 525  $\mu$ l per pot. The plants were foliar sprayed to runoff. The experiment was arranged in a completely randomized design with 4 treatments each replicated 6 times, and maintained in the greenhouse. Irrigation was delayed for 3 days after the spirotetramat treatment and before termination of the experiment. The experiment was terminated 1 month after planting or 2 weeks post-inoculation. Roots were gently removed from the pots and soil, a 1 g root subsample was randomly collected from each root system for assay. The root subsamples were stained following a NaOCl-acid fuchsin-glycerin method (Byrd *et al.*, 1983) and number of nematodes penetrating the roots was recorded.

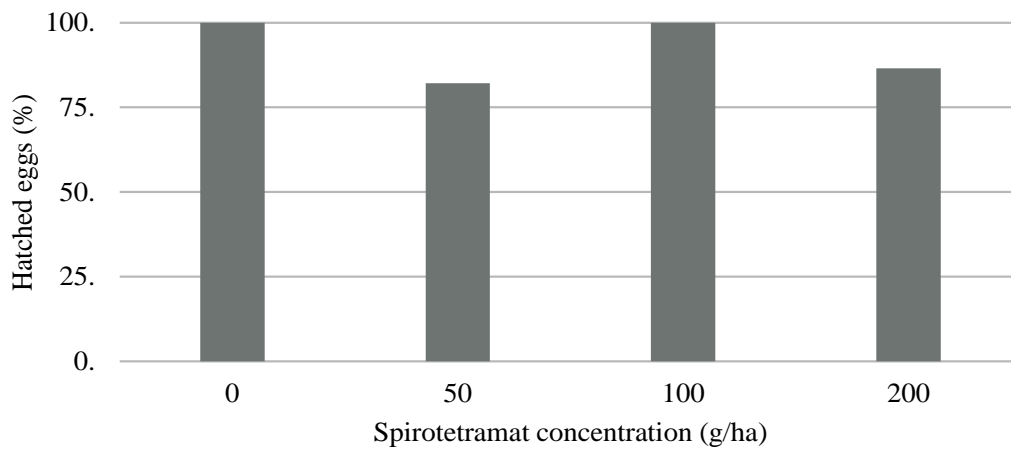
*Statistical analysis:* The hatched egg counts and root penetration counts were tested for normality using  $\log(x + 1)$  before analyses of variance (ANOVA). The data were subjected to ANOVA (SAS Institute Inc., Cary, NC, USA). Where treatment was significant ( $P < 0.05$ ), means were grouped by either Duncan's multiple range test (Duncan, 1955) or Tukey's honest significant difference (HSD) test (Tukey, 1949).

## RESULTS AND DISCUSSION

*Hatching trial:* Spirotetramat did not affect hatch of *R. reniformis*. Hatch was comparable across all the spirotetramat concentrations (Fig.1). No significant difference in hatch ( $P > 0.05$ ) was detected in all the rates tested. This supports the theory that spirotetramat is only active through ingestion (Nauen, *et al.*, 2008). The active secondary metabolite may not have been formed in the water solution or may not have been mobile across the nematode egg shell. Therefore spirotetramat may have been unable to interfere with lipid biosynthesis during embryogenesis or prevent hatch (EPA, 2008; APVMA, 2009).

*Penetration trial:* Spirotetramat affected tomato root penetration by *R. reniformis*. Although not significantly different ( $P > 0.05$ ), spirotetramat tended to suppress the nematode penetration per gram of root. *Rotylenchulus reniformis* failed to penetrate the roots that had been treated at 50 g a.i/ha (Table 1). Conversely, the greatest penetration by *R. reniformis* occurred in plants treated with 100 g spirotetramat/ha (Table 1).

Tomato root growth was not different ( $P > 0.05$ ) among treatments (Table 1). Those plants that were treated with only water (0 g spirotetramat) had the greatest weight and those receiving the highest rate of spirotetramat had the lowest root weight (Table 1). The tomatoes might have experienced some phytotoxicity from the spirotetramat treatments.



**Figure 1.** Hatch of *Rotylenchulus reniformis* as affected by spirotetramat at different rates. Bars represent the mean hatch ( $n=7$ ) and are not different based on Tukey's HSD test ( $\alpha=0.05$ ).

The numbers of rhizosphere vermiform *R. reniformis* per gram of root were reduced 90% ( $P < 0.05$ ) at 50 g a.i/ha compared to all other treatments (Table 1). This reduction agrees with the reduction in the penetration per gram of root (Table 1). Surprisingly, the nematode vermiform stages per gram of root were not supported at the untreated water control (Table 1). This suggests that *R. reniformis* may have been in the roots feeding and reproducing. Generally, the 50 g a.i/ha rate reduced the penetration per gram of root compared to the untreated water control which resulted in lower *R. reniformis* vermiform stages per gram of root.

**Table 1.** *Rotylenchulus reniformis* penetration and numbers of vermiform stages extracted from root rhizosphere. Mean ( $n=6$ ) values within the same row followed by the same letter are not different ( $\alpha=0.05$ ) based on Duncan's multiple range test.

Response variables	Spirotetramat concentration (g/ha)			
	0	50	100	200
Penetrations/g of root	1 <sup>a</sup>	0 <sup>a</sup>	3 <sup>a</sup>	2 <sup>a</sup>
Vermiform nematode/g of root	0 <sup>b</sup>	1 <sup>b</sup>	1 <sup>b</sup>	4 <sup>a</sup>
Root weight (g)	13 <sup>a</sup>	12 <sup>a</sup>	11 <sup>a</sup>	9 <sup>a</sup>

Spirotetramat did not affect the hatch or penetration of *R. reniformis*. This means that spirotetramat in its parent compound form may not act against embryogenesis. Also spirotetramat as a systemic pesticide may not affect hatch through contact but has to be broken down into its active secondary metabolites and ingested to affect embryogenesis or hatch. In the penetration trial, spirotetramat had some effects against *R. reniformis* on root penetration.

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**CHAPTER 3. EFFECT OF SPIROTETRAMAT ON THE FERTILITY OF  
*ROTYLENCHULUS RENIFORMIS* ON *ANANAS COMOSUS* AND *SOLANUM  
LYCOPERSICUM***

Host-plant resistance is often lacking in commercial cultivars of many crops. This is especially so for crops where *Rotylenchulus reniformis* is a major pest (Sipes and Schmitt, 1994; Utery *et al.*, 2005). Consequently, chemical control is the primary tactic to achieve economical control in fields infested with *R. reniformis*. However, many efficacious nematicides have been withdrawn from the market due to environmental impacts and potential health risks (Peoples *et al.*, 1980; Zaki *et al.*, 1982; Thomason, 1987). A larger arsenal of nematicides with a variety of modes of action is desirable for growers. The relatively new insecticide spirotetramat has shown activity against *Meloidogyne*, *Heterodera*, *Tylenchorynchus*, and *Pratylenchus* (McKenry *et al.*, 2009, 2010; Smiley *et al.* 2011, 2012) and could be a potential nematicide for use in integrated pest management of *R. reniformis*.

Spirotetramat acts against sucking insect pests by inhibiting lipid biosynthesis (Nauen *et al.*, 2008; Brück *et al.*, 2009), a macromolecule important in physiological roles (Sakurai, 1974). In insects, spirotetramat affects embryogenesis, flight, metamorphosis and molting. Interestingly, two unrelated groups of organisms, the sucking insect pests (hemipterans) and sedentary plant-parasitic nematodes including *R. reniformis* share several of these characteristics. These characteristics include a sedentary feeding behavior, a plant feeding behavior, embryogenesis and molting. Spirotetramat is expected to affect *R. reniformis* as well. The objective of this research was to determine the effect of spirotetramat on fertility of *R. reniformis* on pineapple and tomato.



## MATERIALS AND METHODS

Greenhouse experiments were conducted in the Magoon research facility (21° 18'24.90"N and 157° 48'33.11"W) at the University of Hawaii at Manoa. *Rotylenchulus reniformis* cultures were maintained on tomato and used for inoculum. The host plants were tomato, *Solanum lycopersicum* cv. 'Patio F Hybrid' and pineapple, *Ananas comosus* cv. 'MG3'. Pineapple crowns were provided by Dole Plantation. Prior to planting, the pineapple crowns were cured for 1 week on a greenhouse bench. Tomato seedlings were prepared by germinating seeds in a pot filled with vermiculite.

Field soil was autoclaved and mixed with commercial sand (Cemex dapis duster sand, CA) in 1:1 ratio. The soil-sand mixture was placed into 20-cm-d (4L) or 15-cm-d (1L) biodegradable pots (Mayers Lawn & Garden, Canada). Pineapple crowns were planted singly into the 4L pots. The experiment was repeated. For the first repeat, pineapples were arranged on a 5 m × 2 m greenhouse bench with 30 cm between plants in a 4 × 4 split plot design. Main plot treatments included a first destructive harvest (FDH), early single application (ESA), mid-double applications (MDA), and late single application (LSA). Subplot treatments were six rates of spirotetramat (0, 50, 88, 100, 175 and 200 g a.i/ha) with 3 replications. The two subplot treatments, 50 and 200 g a.i/ha were missing in all main plots except in LSA. The experiment was repeated once with 5 replications in the second trial.

*Rotylenchulus reniformis* inoculum was prepared from a culture maintained on tomato in the greenhouse. Reniform nematodes were extracted from tomato roots using a NaOCl method (Hussey and Barker, 1973). The nematodes were separated from debris

and fine soil particles following a density-dependent centrifugal sugar flotation method (Jenkins, 1964; Barker, 1985). Inoculum was standardized to 1,000 nematodes (eggs and juveniles) per ml.

*Nematode fertility on pineapple:* Four months post-planting, approximately 3,000 (trial 1) or 5,000 (trial 2) nematodes were inoculated to all pineapple plants. A 100 ml solution of each of the desired spirotetramat (Movento 240 SC) concentrations was prepared using tap water and 5  $\mu$ l Methylated Seed Oil adjuvant. One month post-inoculation, the first spirotetramat application was made using a pressure hand sprayer (Delta Industries, CA, USA). Three months after the first spirotetramat application, the second spirotetramat application was made. Plants were grown in the greenhouse. Fertilizer was applied monthly (0.006 g Miracle.Gro/ml water). Irrigation and insect control were maintained throughout the experiment. Water was withheld for 9 days after each spirotetramat application. The FDH was collected 1 month after the first spirotetramat application. All treatments were terminated 14 months post-planting.

*Nematode fertility on tomato:* Two greenhouse tomato trials were conducted. Fourteen-day-old tomato seedlings were transplanted individually in 1L pots spaced 15 cm apart. In Trial 1, plants were arranged in a completely randomized design (CRD) with 6 treatments (0, 50, 88, 100, 175, and 200 g a.i spirotetramat/ha) and 5 replications. In Trial 2, a 3  $\times$  4 split plot experiment with 3 main plots (application intervals of 7, 14 and 28 days) and 4 subplots (CTR=untreated water control, TIS=treatment and inoculation simultaneously, TBI=treatment before inoculation, and TAI=treatment after inoculation) arranged in CRD was conducted. The spirotetramat application rate was constant at 100 g a.i/ha. Tomato plants in both trials were inoculated with 5,000 nematodes 1 month after

planting. Plants were harvested 1 month after the spirotetramat treatment. Irrigation of the tomato plants was delayed for 3 days after every spirotetramat treatment and before termination of the experiment.

Upon termination of each trial, roots were harvested, a 250 cm<sup>3</sup> soil sub-sample collected, and fresh shoot weight of pineapple were measured. Tomato shoots were not measured because of possible phytotoxicity from the spirotetramat treatment and powdery mildew infection. *R. reniformis* were extracted from soil by elutriation (Byrd *et al.*, 1976) and collected on 25- $\mu$ m pore sieves (Gilson Company, Worthington, OH, USA). Pineapple and tomato roots were shaken in NaOCl to extract eggs (Hussey and Barker, 1973) and the nematodes separated from debris following a density-dependent centrifugal sugar flotation method (Jenkins, 1964; Barker, 1985). Nematodes per ml were counted with the aid of a dissecting microscope. After the nematode extraction, roots were oven-dried at 50°C for 3 days and weighed.

*Statistical analysis:* Root and soil nematode populations were tested for normality using log (x + 1) prior to analysis of variance. All data, including fresh shoot weight, dry root weight, and, root and soil nematode populations were analyzed for variance (SAS Institute Inc., Cary, NC, USA). Where treatment was significant ( $P < 0.05$ ), means were separated by either Duncan's multiple range test (Duncan, 1955) or Tukey's HSD test (Tukey, 1949).

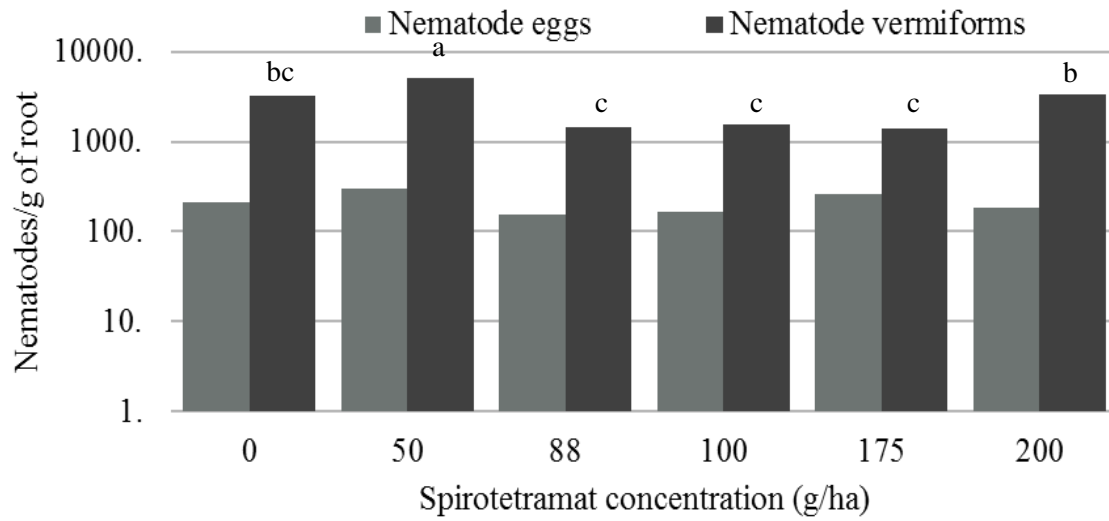
## **RESULTS AND DISCUSSION**

*Nematode fertility on pineapple:* No significant interaction between application time (main plot) and spirotetramat application rate (subplot) was detected. Pineapple shoot weights within the main plots were comparable to untreated water control (Table 2).

However, the pineapple shoot weights between the main plots were significantly different ( $P < 0.05$ ) (Table 2). The shoot weights in FDH were significantly reduced ( $P < 0.05$ ) compared to that of ESA, MDA and LSA (Table 2). This is because the FDH assigned plants were harvested in an early time point prior to fruiting compared to plants that were assigned in other main plots.

Spirotetramat application did not affect the number of reniform nematode eggs per gram of root (Fig.2). Reduction in the number of *R. reniformis* eggs per gram of root was not different ( $P > 0.05$ ) in the main plot treatments (Table 2). However, the rhizosphere vermiform nematodes per gram of root were reduced ( $P < 0.05$ ) in FDH, MDA and ESA (Table 2) compared to LSA. This reduction is probably because these plots received early spirotetramat treatments. Unlike in the FDH, MDA and ESA, the nematode eggs per gram of root in LSA were comparable ( $P > 0.05$ ).

*Rotylenchulus reniformis* vermiform stages per gram of root were affected by the spirotetramat application. The rhizosphere vermiform nematodes per gram of root were significantly reduced ( $P < 0.05$ ) by 56%, 55% and 51% at 175, 88 and 100 g a.i/ha respectively compared to the untreated water control (Fig.2). Surprisingly, the nematode vermiform stages per gram of root were significantly increased ( $P < 0.05$ ) 62% at 50 g a.i/ha rate compared to the untreated water control (Fig.2). This unexpected increase in the nematode vermiform stages per gram of root was because 50 g a.i/ha rate was only applied in an LSA main plot in which spirotetramat was treated in a late time point. This means that the nematode had an adequate time to reproduce and proliferated well before the treatment. Spirotetramat might have some effects but the reduction in the nematode vermiform stages per gram of root remained above the untreated water control (Fig.2).



**Figure 2.** Response of *Rotylenchulus reniformis* eggs and soil vermiform stages per gram of pineapple root. Means ( $n=3$ ) with the same letter are not different ( $\alpha=0.05$ ) based on Duncan's multiple range test.

**Table 2.** Effect of spirotetramat on pineapple shoot growth (g) and eggs and vermiform stages of *Rotylenchulus reniformis*. Means ( $n=3$ ) in the same column between (capital letters) or within (lower case) main plots followed by the same letter are not different ( $\alpha=0.05$ ) based on Duncan's multiple range test. MP=main plot, SP=subplot, FDH=first destructive harvest, ESA=early single application, MDA=mid-double application, LSA=late single application, n.a=not available.

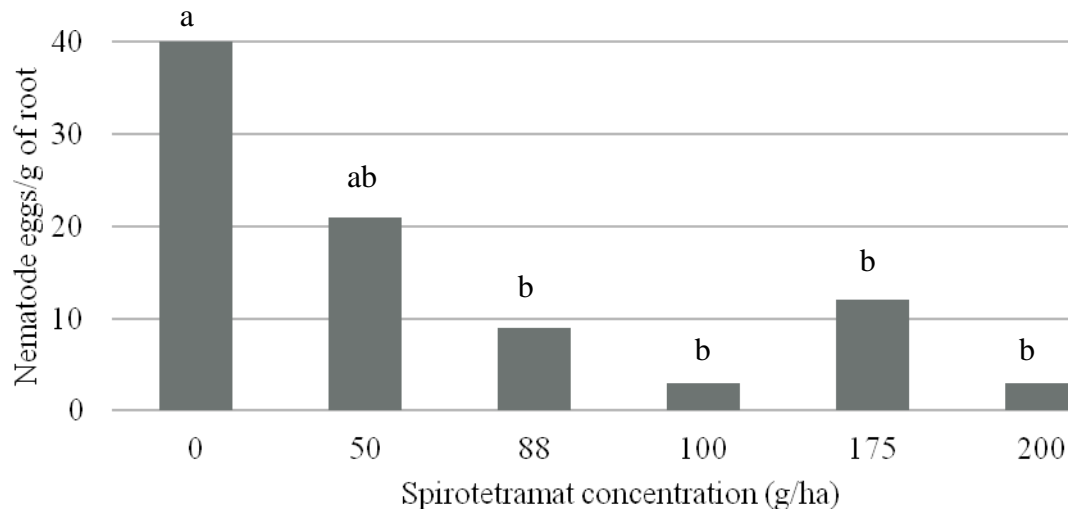
Harvest	Spirotetramat (g a.i/ha)	Shoot (g)	Reniform nematode	
			Eggs (g of root)	Vermiform stages (g of root)
FDH		1240 <sup>B</sup>	n.a	1925 <sup>B</sup>
	0	1249.9 <sup>a</sup>	n.a	3178 <sup>a</sup>
	88	1267.1 <sup>a</sup>	n.a	1275 <sup>a</sup>
	100	1206.7 <sup>a</sup>	n.a	1201 <sup>a</sup>
	175	1237.5 <sup>a</sup>	n.a	1306 <sup>a</sup>
ESA		1596 <sup>A</sup>	218 <sup>A</sup>	1590 <sup>B</sup>
	0	1766.4 <sup>a</sup>	241 <sup>a</sup>	3135 <sup>a</sup>
	88	1394.1 <sup>a</sup>	147 <sup>a</sup>	1364 <sup>a</sup>
	100	1575.3 <sup>a</sup>	161 <sup>a</sup>	575 <sup>a</sup>
	175	1478.6 <sup>a</sup>	219 <sup>a</sup>	1199 <sup>a</sup>
MDA		1835 <sup>A</sup>	181 <sup>A</sup>	2274 <sup>B</sup>
	0	1766.4 <sup>a</sup>	241 <sup>a</sup>	3135 <sup>a</sup>
	88	1879.9 <sup>a</sup>	214 <sup>a</sup>	2149 <sup>a</sup>
	100	2148.3 <sup>a</sup>	129 <sup>a</sup>	3378 <sup>a</sup>
	175	1613.6 <sup>a</sup>	210 <sup>a</sup>	1570 <sup>a</sup>
LSA		1778 <sup>A</sup>	234 <sup>A</sup>	3834 <sup>A</sup>
	0	1766.4 <sup>a</sup>	241 <sup>a</sup>	3135 <sup>a</sup>
	50	1869.9 <sup>a</sup>	328 <sup>a</sup>	5594 <sup>a</sup>
	200	1707.8 <sup>a</sup>	193 <sup>a</sup>	3547 <sup>a</sup>

In the second trial on pineapple, the numbers of *R. reniformis* eggs per gram of root were reduced. The reduction in the reniform nematode eggs per gram of root was not statistically significant ( $P > 0.05$ ) but clearly evident across all treatments compared to the untreated control (Fig.3). The nematode eggs per gram of root were reduced the greatest by 94% and 93% at 200 and 100 g a.i/ha respectively compared to the untreated water control (Fig.3).

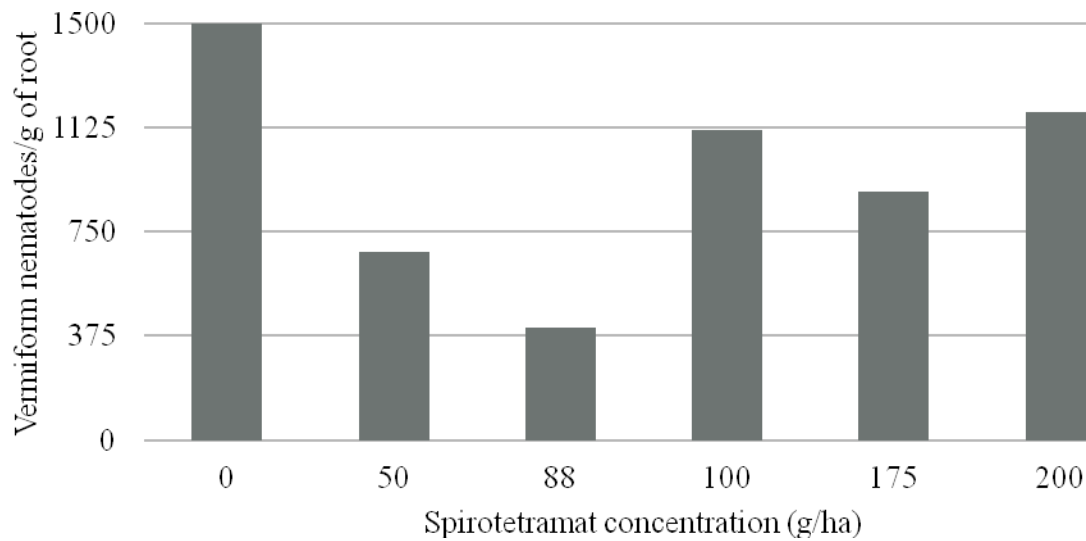
*Rotylenchulus reniformis* vermiform stages per gram of root were affected by spirotetramat application rate. Reductions in vermiform reniform nematode per gram of root were numerically reduced ( $P > 0.05$ ) compared to the untreated control in all the treatments (Fig.4). The greatest reduction (73%) was achieved at 88 g a.i/ha (Fig.4).

Spirotetramat treatment increased pineapple shoot weights. Even though the increase in the shoot weights was not statistically significant, the increase was consistent across all the treatments when compared to the untreated control (Table 3). The greatest increases, 34% and 21%, were achieved with the 200 and 50 g a.i/ha concentrations, respectively (Table 3).

*Nematode fertility on tomato: Rotylenchulus reniformis* eggs per gram of tomato root were affected by spirotetramat. Even though reductions in the reniform nematode eggs per gram of root were not statistically significant ( $P > 0.05$ ), all the treatments exhibited an evident reduction compared to the untreated water control (Fig.5). The greatest reduction (76%) was achieved with the 175 g a.i/ha treatment (Fig.5).



**Figure 3.** Numbers of *Rotylenchulus reniformis* eggs per gram of pineapple root treated with different rates of spirotetramat. Bars represent means ( $n=5$ ) and those with the same letter are not different ( $\alpha=0.05$ ) based on Duncan's multiple range test.

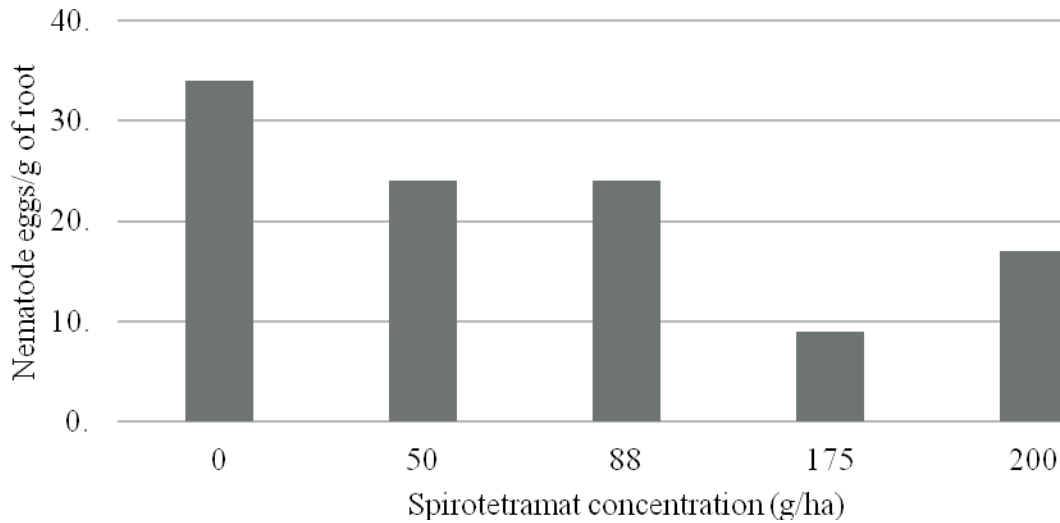


**Figure 4.** Numbers of *Rotylenchulus reniformis* vermiform stages per gram of pineapple root treated with different rates of spirotetramat. Bars represent means ( $n=5$ ) and are not different ( $\alpha=0.05$ ) based on Tukey's HSD test.



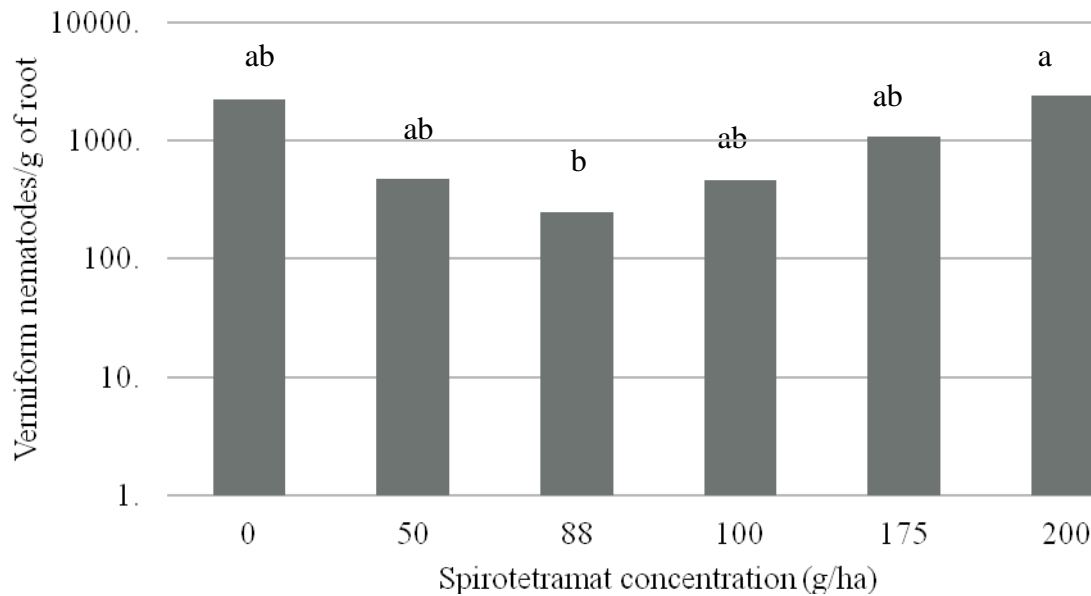
**Table 3.** Pineapple shoot growth (g) and numbers of *Rotylenchulus reniformis* eggs and vermiform stages per gram of root responses to spirotetramat. Means ( $n=5$ ) within the same row followed by the same letter are not significantly different ( $\alpha=0.05$ ) based on Tukey's HSD test.

Response variables	Spirotetramat rate (g a.i/ha)					
	0	50	88	100	175	200
Fresh shoot weight (g)	501.5 <sup>a</sup>	608.2 <sup>a</sup>	579.3 <sup>a</sup>	544.5 <sup>a</sup>	518.9 <sup>a</sup>	673.3 <sup>a</sup>
Eggs/g of root	41 <sup>a</sup>	21 <sup>a</sup>	8 <sup>a</sup>	3 <sup>a</sup>	11 <sup>a</sup>	2 <sup>a</sup>
Vermiform stages/g of root	1526 <sup>a</sup>	678 <sup>a</sup>	406 <sup>a</sup>	1118 <sup>a</sup>	893 <sup>a</sup>	1180 <sup>a</sup>



**Figure 5.** Number of *Rotylenchulus reniformis* eggs per gram of tomato root in response to different concentrations of spirotetramat. Bars represent means ( $n=5$ ) and are not significantly different ( $\alpha=0.05$ ) based on Tukey's HSD test.

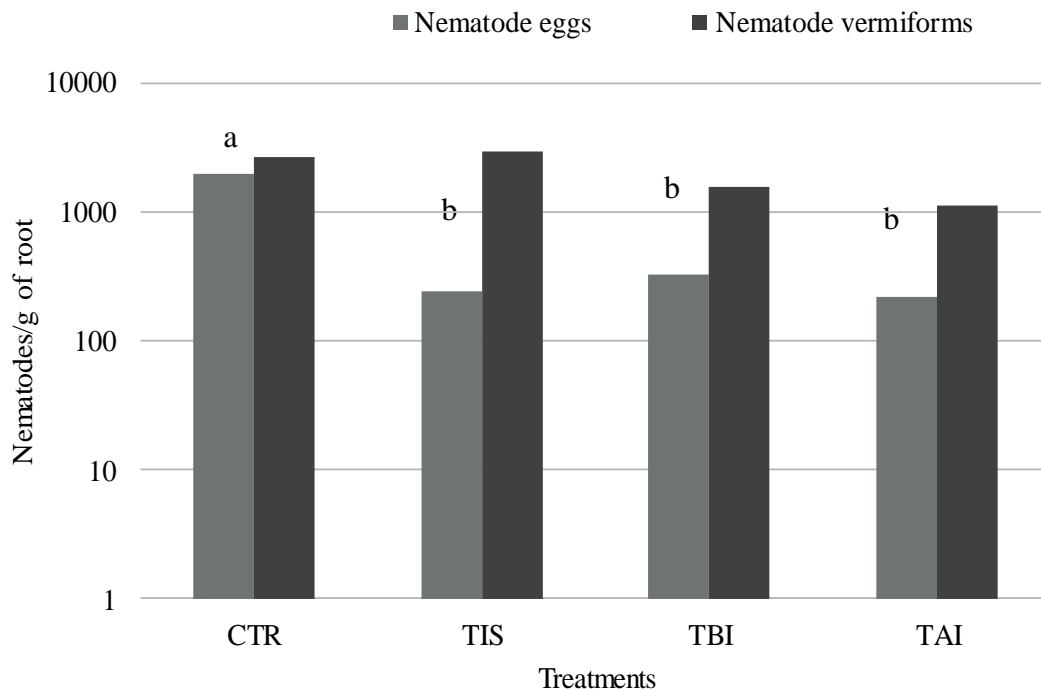
The numbers of vermiform stages of *R. reniformis* were affected. The reniform nematode vermiforms per gram of root were reduced ( $P < 0.05$ ) by 89% at 88 g a.i/ha compared to the untreated water control (Fig.6). Although the nematode vermiforms per gram of root at 50, 100, and 175 g a.i/ha were reduced, no significant difference was detected compared to the untreated water control (Fig.6). The number of vermiform stages per gram of root increased 6% at the 200 g a.i/ha (Fig.6).



**Figure 6.** Response of *R. reniformis* vermiform per gram of root to spirotetramat. Means ( $n=5$ ) are presented. Bars with the same letter are not significantly different ( $\alpha=0.05$ ) based on Tukey's HSD test.

In the second tomato trial, the numbers of eggs of *R. reniformis* per gram of root were reduced. The number of reniform nematode eggs per gram of root was reduced ( $P < 0.01$ ) compared to the untreated control (Fig.7). The greatest reduction (89%) was achieved in TAI compared to the untreated control. However, reduction in the nematode vermiform stages per gram of root were not different ( $P > 0.05$ ) compared to the untreated control (Fig.7). TAI also achieved the greatest reduction (58%) in the nematode vermiform stages per gram of root followed by TBI with a 41% reduction ( $P > 0.05$ ) (Fig.7).

Eggs of *R. reniformis* per gram of root were affected by spirotetramat treatment. While the treatment effects on eggs per gram of root among main plots were similar, that among the sub plots (treatment rates) were different ( $P < 0.05$ ) (Table 4). At the 7-day interval, the nematode eggs per gram of root increased ( $P < 0.05$ ) in all the treatments compared to untreated control. The greatest increase (1.73 fold) was achieved at TIS compared to the untreated control. The 7-day interval supported no nematode reproduction.



**Figure 7.** *Rotylenchulus reniformis* eggs and vermiform stages per gram of tomato root. Bars represent means, and with the same letter are not significantly different ( $\alpha=0.05$ ) based on Duncan's multiple range test. CTR=control, TIS=treat & inoculate simultaneously, TBI=treatment before inoculation, TAI=treatment after inoculation.

The 7-day interval between inoculation and treatment is too short for the nematodes to penetrate and establish feeding sites. Although spirotetramat may have been hydrolyzed into its active secondary metabolite in the plant, it may not have effects on the nematode because *R. reniformis* may not have been penetrated and formed feeding sites. In contrast, the 14- and 28-day intervals reduced ( $P < 0.05$ ) the number of nematode eggs per gram of root (Table 4). These intervals were sufficient for the nematodes to penetrate and formed the feeding sites, thus the nematodes exposed to spirotetramat-enol, the active metabolite, resulted in the reduction of the number of nematode eggs per gram of root.

**Table 4.** Effect of spirotetramat on eggs and vermiform stages of *Rotylenchulus reniformis* infecting tomato. The spirotetramat rate was constant at 100 g a.i/ha with differences in the timing of nematode inoculation and chemical application. Means ( $n=6$ ) within the same column followed by same letter are not different ( $\alpha=0.05$ ) based on Duncan's multiple range test. MP=main plot, SP=subplot, CTR=control, TIS=treat & inoculate simultaneously, TBI=treat before inoculate, TAI=treat after inoculate.

MP/SP	<i>Rotylenchulus reniformis</i>	
	Eggs/g root	Vermiforms/g root
7 days	485 <sup>A</sup>	1109 <sup>AB</sup>
CTR	279 <sup>b</sup>	532 <sup>a</sup>
TIS	762 <sup>a</sup>	1770 <sup>a</sup>
TBI	364 <sup>ab</sup>	979 <sup>a</sup>
TAI	534 <sup>ab</sup>	1155 <sup>a</sup>
14 days	169 <sup>A</sup>	408 <sup>B</sup>
CTR	308 <sup>a</sup>	556 <sup>a</sup>
TIS	215 <sup>a</sup>	456 <sup>a</sup>
TBI	26 <sup>b</sup>	369 <sup>a</sup>
TAI	177 <sup>ab</sup>	315 <sup>a</sup>
28 days	1061 <sup>A</sup>	3524 <sup>A</sup>
CTR	3636 <sup>a</sup>	4733 <sup>a</sup>
TIS	0 <sup>b</sup>	5111 <sup>a</sup>
TBI	509 <sup>b</sup>	2622 <sup>a</sup>
TAI	100 <sup>b</sup>	1630 <sup>a</sup>

## CONCLUSION

Spirotetramat application affected *R. reniformis* fertility. Spirotetramat treatment at 100 g/ha before inoculation significantly suppressed ( $P < 0.05$ ) *R. reniformis* eggs/g of root at 14 days interval. However, at 28 days interval the reniform nematode eggs/g of root were reduced regardless of the spirotetramat treatments. Therefore spirotetramat can be applied 14 days at 100 g/ha before the reniform nematode infection on tomato plants.

Spirotetramat suppressed ( $P < 0.05$ ) *R. reniformis* fertility in pineapple at 88, 100 and 175 g/ha and reduced ( $P > 0.05$ ) the reniform nematode fertility in tomato at 175 g/ha. In pineapple, farmers can apply spirotetramat as low as 88 g/ha to reduce *R. reniformis*. These results suggested that spirotetramat effects on fertility may vary across different crops.

The inhibition of lipid biosynthesis may only manifest an effect on the fecundity of females of *R. reniformis*. The effect of spirotetramat on *R. reniformis* might be slightly different that its effects on nematodes such as *Meloidogyne* or *Pratylenchus*. *Rotylenchulus reniformis*, unlike other nematodes, does not establish a feeding site until reaching the adult stage, completing all molting. Consequently, the inhibition of lipid biosynthesis may only manifest an effect on the fecundity of females of *R. reniformis*. Further study is needed to evaluate the effect of spirotetramat on different PPNs on pineapple and other vegetable crops.

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