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Medicinal Herb Extracts Inhibit Growth of *Rhabditis elongata* (Nematoda: *Rhabditidae*)

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We tested the influence of extracts from three medicinal herbs -- *Salvia miltiorrhiza, Schizandra chinensis,* and *Eugenia caryophyllata* -- on activity of the nematode *Rhabditis elongata*. Treatment with *E. caryophyllata* was most useful, causing the greatest decrease in populations and mobility, but did not have any detrimental effect on the initial growth of the host microorganism, *Escherichia coli*. For example, when 0.5 g/L of the extract was added to an inoculated liquid culture, we counted 710 nematodes/mL, with a multiplication rate 5 times greater than the initial population. This was in contrast to the control sample, which had a count of 1100 nematodes/mL and a growth ratio of 11. For our field test, nematode mobility in the presence of the extract also decreased, to 6.8 mm/day, compared with 20 mm/day for the control. Likewise, when 1.0 g/L of the extract was added to the soil, the total number of nematodes was reduced to only 30- to 40% of the control population.

Keywords: *Eugenia caryophyllata*, medicinal herbs, nematocide, *Rhabditis elongata*

The current practice of disinfestation to control soil-borne plant pathogens and parasitic nematodes depends heavily on the use of chemical pesticides or fumigant nematocides, e.g., 1,3-dichloropropene (Jatala et al., 1980; Gaugler and Kaya, 1990; Bohn and Homnick, 1996). Because of the reduced availability of effective, conventional insecticides, as well as new laws that restrict their use, researchers are working to develop new types of nematocides (George, 1992; Kaya and Gaugler, 1993). However, these endeavors have not been very successful because of their low efficacy and high costs (Hooper, 1986; van der Walt and de Wale, 1989). Moreover, little attention has been paid to the use of plant extracts and/or other derivatives to control nematode growth even though they may potentially reduce production costs from those involved with the manufacture of other biopesticides.

Some saprophytic nematodes have relatively short life cycles, and can be grown in a liquid culture using *Casenorhabditis elegants* Maintenance Medium (CbMM) with *Escherichia coli* or *Xenorhabdus nematophilus* as the host organism (Buecher and Popiel, 1989; Matsuno, 1993; Bessho et al., 1995). For example, *Rhabditis* sp. can be monoxenically cultivated in a simple nutrient broth medium with high agitation speed (Bedding, 1984; Grewal, 1991). This means that it is relatively easy to maintain mass amounts of nematodes in liquid culture (Hooper, 1986), thereby providing a method for comparing the killing efficacy of biological nematocides and biopesticides when their activities are enhanced with biological substances from natural sources. Three medicinal herbs, *Salvia miltiorrhiza*, *Schizandra chinensis*, and *Eugenia caryophyllata* have been widely used as natural preservatives in foods and other products (Sofos et al., 1988; Park et al., 1992), and were selected for our investigation into the effectiveness of plant extracts to inhibit nematode growth.

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MATERIALS AND METHODS

Nematode and Herb Extract Preparation

Our source of *Rhabditis elongata* (Nematoda: *Rhabditidae)* inoculum was perished potatoes from the United States Department of Agriculture. These potatoes were held on solid potato agar at 4°C for long-term maintenance, then cut into 1- to 2-cm pieces and inoculated for one week at 28°C. Afterward, they were placed on a Baermann funnel for 5 d at 28°C to extract the nematodes (Faulkner and Darling, 1961;

Bordie, 1984). These nematodes were then grown in a solid nutrient broth (NB) medium (Gibco, USA), and identified as *R. elongata* by microscope. The nematodes were disinfected with a solution containing 500 mg/L streptomycin sulfate and 50 mg/L mercury chloride for 5 min, then washed with distilled water (Faulkner and Darling, 1961). About 2000 adults and juveniles were collected under a microscope and transferred into the NB medium (Grewal, 1991). Leaves of the three herbs were separately dried and extracted with 80% ethanol at 80°C for 6 h. Each extract was then concentrated by vacuum rotary evaporator and freeze-dried. The powdered samples were stored at 4°C before being added to the nematode cultures.

In-Vitro Cultivation of *R. elongata* with Herb Extracts

About 100 nematodes/mL were inoculated into flasks containing 300 mL of the NB medium (20 g of NB/L). Each flask also contained 1×10^6 cells of *E. coli*/mL that had already been growing for 2 d. The flasks were maintained at 28°C in a shaking incubator at 150 rpm for 5 to 6 d. Nematode activity was monitored by checking the opacity of the culture medium; the broth became clear in the presence of living nematodes. Growth of the host bacteria was also observed by measuring the optical density of the culture broth at 340 nm.

Either 0.1 g/L or 0.5 g/L of the individual extracts was filtered through 0.45 µm filter paper, then added to flasks containing the inoculum and cultivated for 10 d. One-mL samples of the culture were collected daily from each flask so that we could count the nematodes under a microscope to determine their rates of growth and multiplication. The multiplication rate was calculated as the ratio of the number of nematodes from the culture broth to the initial number in the inoculum. To measure their mobility, we dropped approximately 20 nematodes onto the center of a Petri dish containing 1.5% agar medium, with or without extract supplements. All these Petri dishes were placed in the dark at 28°C for 24 h. Afterward, mobility was estimated according to the distance the nematodes had traveled from the center of the dish.

For our field test, we ground the potatoes to powder and added 5% (w/v) of that powder to loamy soil (30% moisture content). This soil had already been mixed with an NB medium containing 10 nematodes/mL and 0.5 g of extract/g soil. Each of the soil treatments was placed in a 30- × 20- × 15- cm tightly sealed container and incubated at 28°C. After one month, the number of nematodes in each container was measured every 5 d via the Baermann funnel.

For our controls in each trial, we monitored the growth and behavior of nematodes that were not treated with any herb extract. All experiments were replicated at least four times. Data were analyzed for means and standard deviation, using the Statistical Analysis System (SAS Institute; Cary, NC, USA) (Norman and Smith, 1981).

RESULTS AND DISCUSSION

In-vitro growth of *R. elongata* was confirmed in the NB liquid culture when *E. coli* was used as the host (Fig. 1). The culture broth became clearer as the nematode population increased (data not shown), which implies that inoculated nematodes can grow in such a culture. The total number of nematodes in the control treatment was greatly increased after 3 d of inoculation, and reached a maximum at 7 d, after which the population was maintained at about 1150 nematodes/ml in the culture broth.

We estimated the maximum specific growth rate at ca. 0.271 (1/day) for the control, with the multiplication rate also gradually increasing up to 11 times greater than at the time of inoculation. However, this multiplication ratio was lower than the 20 to 25 that can be expected when a conventional solid medium is used (Chung, 1975; Jaffee, 1980). This variability may have been due to different and/or unfavorable envi-

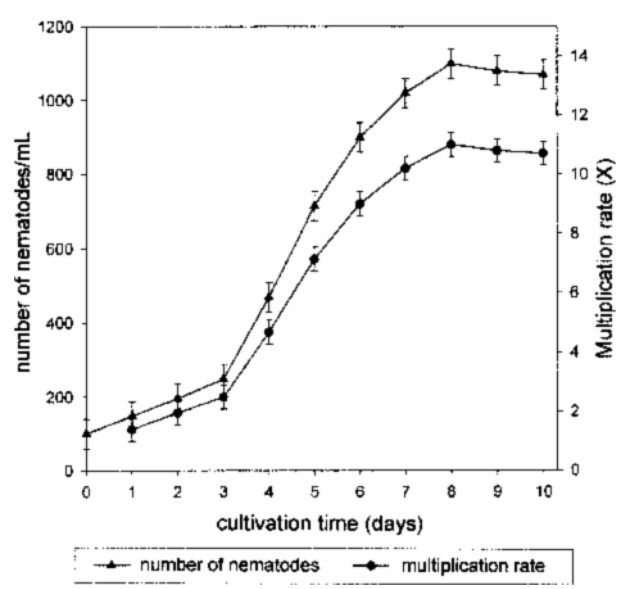

Figure 1. Growth of *R. elongata* in liquid culture with *E. coli*.

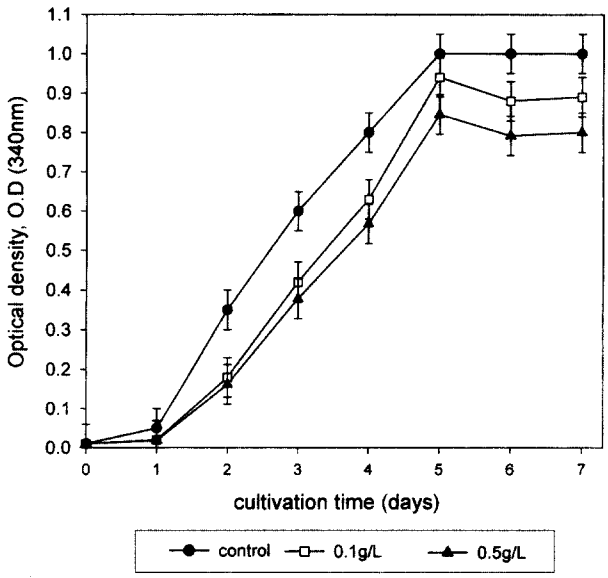

Figure 2. Effect of herb extracts from *E. caryophyllata* on growth of the host organism, *E. coli*.

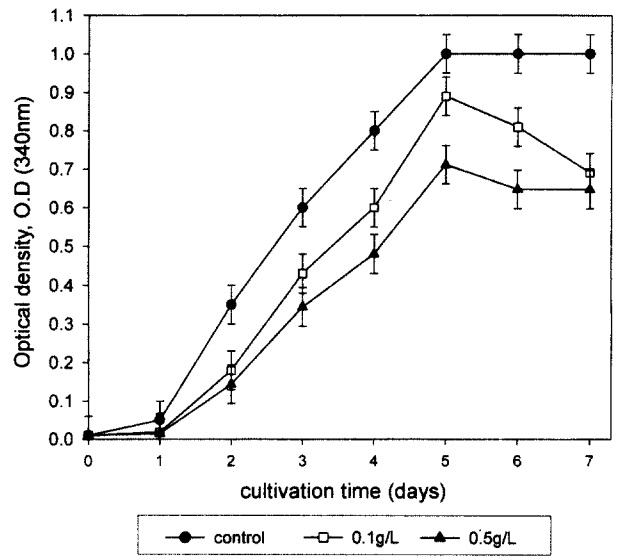

Figure 3. Effect of herb extracts from *S. chinensis* on growth of the host organism, *E. coli*.

ronmental conditions inherent to a liquid culture, such as high shear stress, a limited oxygen transfer rate, etc. Nevertheless, we were able to demonstrate that the liquid culture could maintain a certain population level, which would reduce the substantial amount of labor normally required for nematode production.

Medicinal herb extracts can inhibit the growth of a broad spectrum of microorganisms (Faulkner and Darling, 1961; Bordie, 1984). They may also retard growth of the host bacterium in a liquid culture. This latter effect means that the nematodes would be killed when the extracts were added to the NB culture medium (Grewal, 1991). Therefore, one of our objectives was to examine the effect of specific concentrations of each herb extract on the growth of *E. coli*. In our experiments, all three types of extracts seemed to inhibit initial *E. coli* growth (Figs. 2, 3, and 4). However, the addition of 0.1 g/L of the *E. caryophyllata* extract was least detrimental. In fact, after exponential growth, the maximum cell density with that extract was very close to that found from the control, with only an 8.7% decrease in opacity. In contrast, the addition of 0.1 g/L of *S. miltiorrhiza* or *S. chinensis* greatly reduced the growth of *E. coli* cells by 31.0 and 48.1%, respectively. Of all the extracts, that of *Salvia miltiorrhiza* most severely hampered *E. coli*. Likewise, when 0.5 g/L of any of the extracts was added, overall growth of the *E. coli* host decreased by up to 45% compared with the control, and *S. miltiorrhiza* showed the highest rate of inhibition.

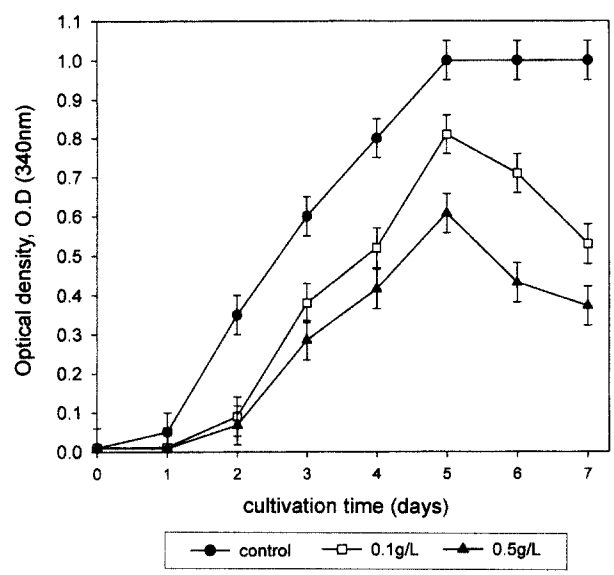

Figure 4. Effect of herb extracts from *S. miltiorrhiza* on growth of the host organism, *E. coli*.

We also noted that the addition of the extracts could affect cell growth at stages later than initial or exponential growth. This was illustrated by a greater decrease in opacity at the stationary phase than during the other growth phases. Based on our results, we propose that *E. caryophyllata* has the best potential for use as a natural nematocide because it least affected the growth of the host organism when a relatively high concentration, i.e., 0.5 g/L, was added (Fig. 2).

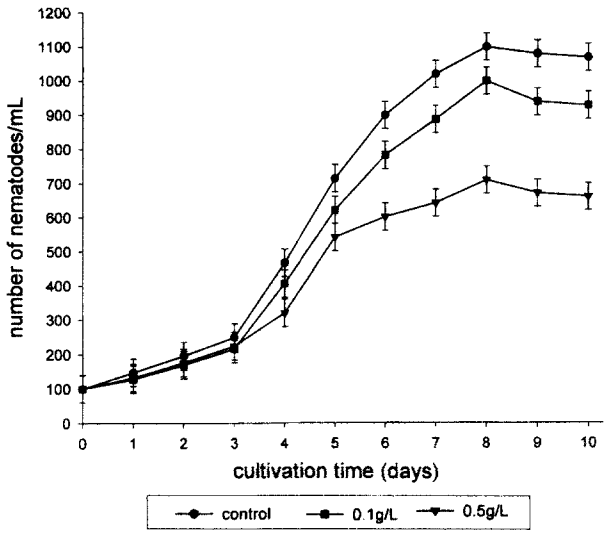

Figure 5. Growth of *R. elongata* as a function of the concentration of the *E. caryophyllata* extract in the liquid culture.

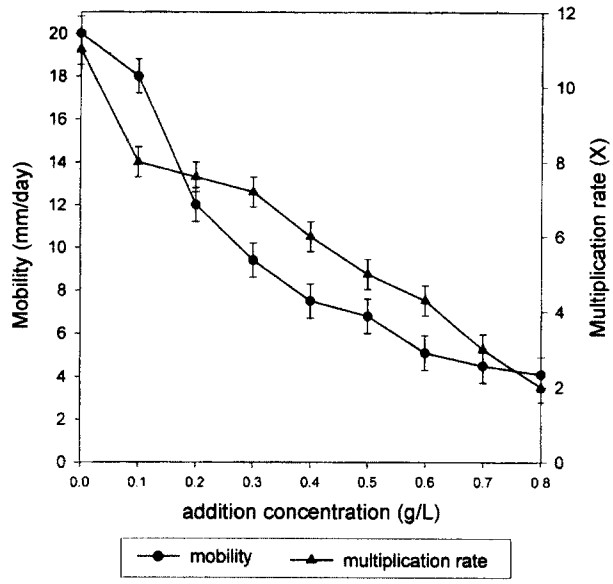

Figure 6. Effect of *E. caryophyllata* extract on mobility and multiplication rate of *R. elongata* on agar medium in 24 h.

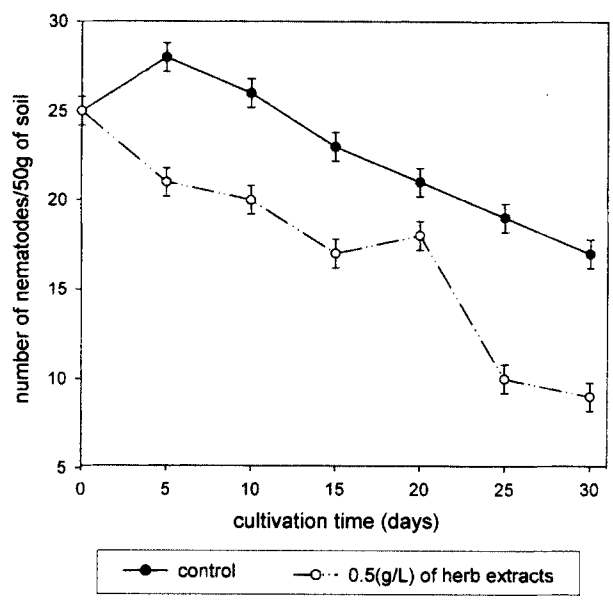

Figure 7. Change in the number of *R. elongata* counted via Baermann funnel from experimental soils with or without added extracts.

Because *S. miltiorrhiza* and *S. chinensis* so strongly inhibited growth of the *E. coli* (Fig. 2), we chose not to screen either of them for their ability to selectively inhibit nematode growth.

We tested two different levels of the ethanol extract from *E. caryophyllata* in liquid culture (Fig. 5). At a concentration of 0.1 g/L, the number of *R. elongata* nematodes slightly decreased to ca. 1000 nematodes/mL, compared with 1100 nematodes/mL in the control. However, when 0.5 g/L of the extract was added, the nematode population was greatly diminished, to 710 nematodes/mL. Therefore, the ethanol extract of *E. caryophyllata* can selectively inhibit ca. 30- to 40% of the growth of *R. elongata*, while not affecting the growth of *E. coli* in liquid culture. However, this particular extract did influence nematode growth at a latter period of cultivation, possibly because of the decreasing numbers of host bacteria at this phase.

We also evaluated the effect of the extract on nematode mobility, because it an important factor when determining their survivability (Hooper, 1986). For example, nematodes in the control dishes moved 20 mm/day away from the center (Fig. 6). When 0.1 g/L of an extract was added, mobility decreased only a bit, to ca. 18 mm/day. Likewise, the multiplication rate was not much decreased, maintaining a ratio of 8 to 11. This demonstrates the ability of the nematodes to remain active under these conditions. When more than 0.3 g/L of the extract was added, mobility was sharply reduced, to 9.4 mm/day. At 0.5 g/L, mobility dropped even more, to 6.8 mm/day, with an accompanying decrease in the multiplication rate. Overall, the addition of extracts seemed to affect mobility much more than the multiplication rate. Nonetheless, applications at concentrations >0.5 g/L did not further decrease nematode movement, although at 0.8 g/L, the multiplication rate for nematode growth was diminished. This also suggests a critical point at which the

addition of herb extracts is effective in killing nematodes while not decreasing the level of the host in the liquid culture. This point was reached at 0.5 g/L for *E. caryophyllata*.

In our field tests, we inoculated the soil with *R. elongata*, with or without extracts. As seen in Figure 7, the number of nematodes increased to 28 in a 50-g sample at 5 d post-inoculation. The gradual reduction later on, to 18 nematodes/sample, was probably caused by a lack of oxygen and/or the presence of toxic products in the soil. When 0.5 g/L of the extract was added to the soil, the nematode population did not show any initial increase, but rather was rapidly reduced compared with the control.

This lack of nematode growth implies that these extracts had a direct influence on nematode activity. Their numbers were greatly decreased during the latter period of the experiment, possibly because the nematodes became weakened by the extracts and/or by other toxic materials in the soil. It may also be evidence of the long-term efficacy of *E. caryophyllata*, both in liquid culture and in field soil. This phenomenon should be further investigated through consecutive purification processes to identify the key compounds and particular nematocidal mechanisms inherent to these herb extracts. Answers to these questions might confirm the usefulness of herb extracts as biological nematocides. Long-term field trials are also necessary to evaluate the effectiveness of extracts in controlling nematode growth.

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