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Effects of tannin-rich host plants on the infection and establishment of the entomopathogenic nematode *Heterorhabditis bacteriophora*

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ABSTRACT

Parasitized animals can self-medicate. As ingested plant phenolics, mainly tannins, reduce strongyle nematode infections in mammalian herbivores. We investigated the effect of plant extracts known to be anthelmintic in vertebrate herbivores on the recovery of the parasitic entomopathogenic nematode *Heterorhabditis bacteriophora* infecting African cotton leafworm (*Spodoptera littoralis*). Nematode infective juveniles (IJs) were exposed to 0, 300, 900, 1200, 2400 ppm of *Pistacia lentiscus* L. (lentisk), *Inula viscosa* L. (strong-smelling inula), *Quercus calliprinos* Decne. (common oak) and *Ceratonia siliqua* L. (carob) extracts on growth medium (*in vitro* assay). In control treatments, 50–80% of IJs resumed development to J_4, young and developed adult hermaphrodites, whereas all extracts, except for *C. siliqua* at 300 ppm, impaired IJ exsheathment and development. The highest concentration of *I. viscosa* extract (2400 ppm) had the strongest effect, killing 95% of exposed nematodes. Surviving nematodes did not recover, remaining at the IJ stage. Over the whole cycle, *I. viscosa* extract inhibited recovery to 25% or less, and did not allow full development to adulthood, whereas 65% of IJs in the control treatment recovered and resumed development, 12% reaching complete maturation within 72 h of incubation. When herbivorous *S. littoralis* larvae were fed with different plant extracts *in vivo*, *I. viscosa* had the strongest effect at concentrations above 300 ppm, with 90% of insect-invading IJs not developing to parasitic stages, whereas in the control treatment, 85% of IJs resumed development. Exposure to *C. siliqua* extract also inhibited exsheathment and development of 75% of the IJs. Half of those that resumed development reached full maturation. *P. lentiscus* and *Q. calliprinos* extracts also inhibited development of 50% IJs. Our results suggest that *H. bacteriophora* can be used to study herbal medication against parasites in animals.

1. Introduction

Parasitic nematodes of invertebrates, animals, humans and plants thrive and reproduce in their hosts, but they also spend a substantial portion of their life cycle outside the host. To cope with different environmental conditions, these parasitic nematodes have a developmentally arrested stage, the infective juvenile (IJ), which is adapted to withstand the unfavorable conditions outside the host (Sommerville and Davey, 2002). This stage is further equipped with mechanisms for allocating, and establishing in, new hosts (Glazer, 2002). A similar life cycle exists in the gastrointestinal trichostrongylid parasites of ruminants (Chiejina, 1986). The eggs of these parasites are excreted in the animal's feces and hatch under suitable environmental conditions, producing two nonparasitic larval stages followed by an infective L_3 that is ensheathed, i.e., retains the shed cuticle from the previous molt, for protection. When infective trichostrongylid larvae are ingested by the host, they exsheath and, depending on the species, enter either the gastric glands of the abomasum or the crypts of the small intestine, where they molt, return to the lumen, and after a fourth molting, become egg-producing adults.

The ability of parasitized animals to self-medicate seems to be ubiquitous in the animal kingdom (Villalba and Landau, 2012): for example, chimpanzees suffering from parasite-related diseases consume the bitter pith of the plant *Vernonia amygdalina* (Huffman

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and Seifu, 1989) which, at the ingested doses, contains sesquiterpene lactones and steroid glucosides with antiparasitic activity (Koshimizu et al., 1994); caterpillars of the tiger moths (Lepidoptera: Arctiidae) *Grammia geneura* and *Estigmene acrea* are dietary generalists that defend themselves from infection by insect parasitoids by sequestering pyrrolizidine alkaloids from *Senecio longilobus*, which are toxic to a range of predators and parasitoids (Bernays and Singer, 2005).

Results acquired over the last decade indicate that dietary phenolics, and in particular tannins, ingested from plants might reduce nematode infections in small ruminants (see reviews by Hoste et al., 2006; Mueller-Harvey, 2006). Two main effects have been associated with the consumption of tannin-rich forages by herbivores. First, reduced establishment of the infective larvae (Paolini et al., 2003; Brunet et al., 2008) and second, significant reductions in egg output (Paolini et al., 2003; Landau et al., 2010; Manolaraki et al., 2010). In recent studies, larval exsheathment-inhibition assay (LEIA) (Brunet and Hoste, 2006; Brunet et al., 2007; Azaizeh et al., 2013)—a standardized method that allows comparisons of the effects of plant extracts on larval exsheathment have revealed that not only condensed tannins, but also their constitutive monomers (flavan-3-ols) and some other flavonoids, are responsible for the functional and structural changes induced in L_3 (Brunet and Hoste, 2006; Brunet et al., 2007, 2008).

Insects can be parasitized by rhabditid nematodes that are phylogenetically close to strongyles. One of these parasites is *Heterorhabditis bacteriophora*, which is used as a biological control agent of various soil-inhabiting insect pests (Grewal et al., 2005). The IJ stage of *H. bacteriophora* is J_3, which is developmentally arrested and well adapted for long-term survival in the soil. This stage exhibits host-finding and infection capabilities (Glazer, 2002). The IJs carry a bacterial symbiont, *Photorhabdus luminescens*, in their intestine. After they find a suitable host, they enter into the insect larvae through natural body openings or by penetrating the cuticle, release the bacteria into the insect hemocoel, and resume development. The bacteria replicate rapidly, killing the host within 24–48 h (Ciche and Ensign, 2003). As soon as the IJ releases the bacteria, it resumes development and feeds on the symbiotic bacteria. The process of exiting the IJ stage and resuming development in the insect cadaver is called "recovery" (Golden and Riddle, 1982). The term "recovery", commonly used among entomonematologists, is equivalent to the term "exsheathment" used by animal parasitologists. Subsequently, the IJ grows into a reproductively mature adult hermaphrodite, which then lays eggs and completes two to three life cycles in the host cadaver (Strauch and Ehlers, 1998). As the food resources become depleted, new IJs are produced and leave the insect cadaver in search of a new host. The duration of each life cycle is 5–6 days under suitable conditions. *H. bacteriophora* nematodes can be grown easily *in vitro* and the recovery process, as well as their development, can be observed under a microscope. For these reasons Hallem et al. (2007) suggested to use this nematode-bacterium complex as model system for parasitism research.

Barbercheck (1993), Barbercheck et al. (1995) and Barbercheck and Wang (1996) reported that the food plant of an insect can affect its susceptibility to infection by entomopathogenic nematodes. Hence, *H. bacteriophora* provide an excellent model for determining the effect of plant extracts on IJ recovery/exsheathment processes, and we hypothesize that information on tannin's effects on parasitic nematodes can be interpolated from higher herbivores to insects and vice versa.

Alkaloids are sequestered by insects to combat parasites, but we are not aware of similar reports for dietary tannins. The LEIA is the gold standard for establishing dietary effects on strongyle nematode infection but to our knowledge, there is no standard method for evaluating possible interactions between diet and infection outcome in parasitized insects. Therefore, the aims of the present study were to: (i) investigate the potential contribution of tannins to combating parasitic nematode infection; (ii) develop a method to monitor diet × rhabditid infection interactions in insects *in vitro* and *in vivo*, and (iii) assess the effects of extracts known to be anthelmintic in vertebrate herbivores on the recovery of parasitic rhabditids in insects.

2. Materials and methods

2.1. Plant material

The following plant species were studied: *Pistacia lentiscus* L. (lentisk), *Inula viscosa* L. (strong-smelling inula), *Quercus calliprinos* Decne. (common oak) and *Ceratonia siliqua* L. (carob). These plants grow naturally in Israel and were collected in the spring of each year. Samples of three plants from both the southern and northern slopes of Carmel Mountain in Haifa, Israel, were mixed into one composite sample for each species and used throughout the experiment.

2.2. Preparation of plant extracts and determination of total polyphenol contents

The plant extracts were prepared according to Azaizeh et al., 2013: Briefly, leaves were dried at 50 °C for 24 h. Leaves (10 g) were ground and incubated with 100 ml of 70% ethanol:water (7:3, v:v) at 35 °C for 24 h. The mixture was filtered and evaporated under vacuum in a Hie-VAP rotary evaporator (Hiedolph, Germany) at 45 °C to remove the ethanol and water to dryness. Extraction yield was calculated as gram extract per gram dry matter of plant leaves. Total polyphenols in plant extracts were determined according to the Folin–Ciocalteu method (Zhishen et al., 1999). The extract was kept at −20 °C until analysis. Results of tannin (polyphenol) content were calculated equivalent to quebracho tannin, tannic and gallic acids (Sigma, Israel) as standards.

2.3. Nematode culture

The EN01 strain of the nematode *H. bacteriophora* was used. It was obtained two years earlier from E-nema Company, Kiel, Germany and nematode stocks were maintained by passage through larvae of the greater wax moth, *Galleria mellonella* (Kaya and Stock, 1997). Last-instar were used for nematode maintenance and to produce insect hemolymph.

2.4. In-vitro exsheathment bioassay

The assay was conducted according to Moshayov et al. (2013) with some modifications, as described below. The IJs were exposed to insect hemolymph which was added onto a water agar (Moshayov et al., 2013) mixed with different concentrations of the various plant extracts as described below.

Insect hemolymph was obtained by bleeding fourth-instar of *G. mellonella* via a lesion in the anterior forelimb. Before bleeding the instars were surface sterilized using 70% ethyl alcohol solution. The hemolymph was collected directly into Eppendorf tubes and immediately diluted twofold with sterile PBS (137.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 0.25 μg/ml phenylthiourea, an inhibitor of phenoloxidase (Moshayov et al., 2013). The hemolymph samples were kept frozen at −20 °C until use.

The recovery bioassay was performed in multi-well (12) plates on water–agar medium (1.5% agar in double-distilled water) at 25 °C. The agar (2.5 ml) was added to each well after autoclaving. A stock solution of 12,000 ppm extract was prepared by mixing