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SELECTION OF ENTOMOPATHOGENIC NEMATODES AGAINST *DALACA PALLENS* (LEPIDOPTERA: HEPIALIDAE)

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Dalaca pallens (Blanchard) is a pest with great impact on grassland production in Chile. The objective of this research was to evaluate the use of entomopathogenic nematodes (EPN) against *D. pallens* larvae. Twenty EPN isolates, collected along Chile, were used for nematode selection. The experimental unit consisted of five 50-mL plastic vials filled with *Nothofagus dombeyi* sawdust and inoculated with 40 dauers per vial. The experimental design was a completely randomized with four replicates per treatment. The results showed that isolate QU N3 (*Steinernema australe*) and QU N13 (*Steinernema unicornum*) produced the highest mortality, with 100 and 95% respectively and no differences ($P < 0.001$) between both. Later, these two isolates were used to calculate the lethal concentration (LC_{50} and LC_{90}). Concentrations of 0, 10, 20, 30, 40, and 50 dauers per 50-mL plastic vials filled with *Nothofagus* sawdust were evaluated. Then, a single larva of *D. pallens* was added to each vial and incubated for 13 d. The results showed that LC_{50} and LC_{90} were equivalent to 14 and 39 dauers mL^{-1} for isolate QU N3, while for isolate QUN13 these figures were 14 and 48 dauers mL^{-1}. Consequently, there are native isolates of EPN with the ability to control *D. pallens* larvae, which require future test under field conditions.

Key words: Biological control, *Steinernema australe*, *Steinernema unicornium*, lethal concentration.

Dalaca pallens (Blanchard) (Lepidoptera: Hepialidae) is an economically important pest, listed as primary pest that justify control measures in the country (Klein and Waterhouse, 2000). The larvae cause significant damage in natural, sowed and regenerated pastures. A density of 50 third stage larvae m^{-2} can consume completely the pastures, by eating leaves and stems during the day and roots at night, leaving the soil completely uncovered (Artigas, 1994). Besides, it also affects blueberries, raspberries, myrtle, and forest species like eucalyptus during establishment. The pest is spread between the Coquimbo (29°57'11'' S, 71°20'36'' W) and General Carlos Ibáñez del Campo Regions (45°34'0'' S, 72°4'0'' W) (Artigas, 1994). Thus, 742537 ha of pastures are threaten annually and 250 000 ha are effectively damaged (Neumann *et al*., 2007). Currently, the control of *D. pallens* larvae is done with chemical insecticides, which are not specific and damage beneficial arthropods, especially predators such as spiders and beetles (Pereira *et al*., 2008), leading to the elimination of natural enemies and eventually the increasing of *D. pallens* population on the pasture.

Entomopathogenic nematodes (EPN) of the families Steinernematidae and Heterorhabditidae are organisms that establish a symbiotic association with specific bacteria, belonging to the genus *Xenorhabdus* and *Photorhabdus*, respectively (Emelianoff *et al*., 2007). These nematodes search actively for the larvae in the soil by following several physical and chemical cues emitted by the host (Ramos-Rodríguez *et al*., 2007). The nematodes enter to the host through its natural openings (mouth, anus, and spiracles), and release a symbiotic bacteria, carried on a ventricular portion of the intestine, into the insect hemocoel. Then, the bacteria multiply and cause a general septicemia, ending in the insect death 24 to 48 h after infection (Kaya and Stock, 1997; Griffin *et al*., 2005).

In Chile, the Insect Pathology Program of Instituto de Investigaciones Agropecuarias (INIA) has been prospecting systematically the country, searching for EPN and demonstrating the presence of *Steinernema* and *Heterorhabditis* in Chilean soils (Edgington *et al*., 2010). Therefore, the objectives of this research were to select native isolates of EPN to control *Dalaca pallens* larvae, to establish lethal concentrations (LC_{50} and LC_{90}), lethal time (TL_{50} and TL_{90}) and to study the foraging strategy of the most effective isolates.

MATERIALS AND METHODS

Nematode rearing

A total of 20 isolates from the EPN collection of INIA, collected throughout Chile by a Darwin project (Edgington *et al*., 2010) were used for isolate selection. The nematodes were reared *in vivo* with *Galleria mellonella*

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larvae (Bedding and Akhurst, 1975). Fifty larvae were placed inside a 15-cm diameter Petri dish lining with Whatman filter paper N° 1, then a concentration of 40 nematode juveniles per larvae were added with a micropipette (Kaya and Stock, 1997). The plates were incubated in darkness and a temperature of 15 ± 2 °C until larvae death (Lacey and Shapiro-Ilan, 2008). The *G. mellonella* cadavers were transferred to 9-cm Petri dishes containing 10 mL of plaster; to allow a solid surface for nematode movement and the absorption of debris as the larvae corpse decompose (Lindegren *et al*., 1993). These dishes were placed inside a 12-cm Petri plates that contain 25 mL of sterile water, where were collected the dauer nematodes that escape from the *G. mellonella* cadaver. The nematodes were collected daily, washed with tap water, counted and stored at 10 ± 2 °C, in plastic containers with tap water. The suspension was bubbling permanently (Realpe *et al*., 2007) with a diaphragm pump for oxygenation, until used in the bioassays.

Collection of *Dalaca pallens* larvae

The larvae of *D. pallens* were obtained by digging a pasture of *Trifolium repens* and *Festuca arundinacea*, located at the INIA experimental station Remehue (40°31'65'' S, 73°04'97'' W), Osorno. Larvae were transported to the lab in plastic containers with *Nothofagus* sawdust as substrate and moistened with distilled water. Then, the larvae were stored individually in conical flasks and observed for 20 d to eliminate sick larvae. The larvae were classified according to their weight and diameter of the head capsule.

Entomopathogenic nematode screening

Twenty isolates of EPN were evaluated on *D. pallens* larvae as screening. Individual larvae of 0.14 ± 0.05 g and 4 mm in diameter of cephalic capsule were placed inside of 50 mL conical plastic vial, filled with 30 g of pasteurized 40:60 *Nothofagus* sawdust and organic soil. Then, 3 mL of sterile water with 40 nematode dauers were added into each vial. The control treatment was supplied only with water. The larvae were fed daily with two leaves of fresh white clover. The experiment was incubated inside chambers at 15 ± 2 °C and darkness (Allan *et al*., 2002), until the first treatment reached 100% of mortality.

Larva mortality was assessed every 24 h, the criteria was to consider a dead larva those that did not move or response after brushing. Dead larvae were transferred to a moist chamber made of Petri dish lined with filter paper Whatman N° 1, moistened with distilled water and incubated at 15 ± 2 ºC until the emergence of the nematodes.

The experimental design was completely randomized, with 21 treatments, each treatment has five replicates and the experimental unit consisted of four conical vials with one larva of *D. pallens* each. The results were expressed as percentage of mortality; data were transformed to arcsine to homogenize the variances previous to the ANOVA. Values of 0 or 100% were corrected according to the formulas (1/4 × N) and (100-1/4 × N), respectively, where N = number of samples used (Campbell and Wraight, 2007). Data were subjected to ANOVA and lately the means were compared using Fisher's protected test with 95% of confidence. The analyses were performed with the statistical program Statistix, version 8.0 (Analytical Software, Tallahassee, Florida, USA).

Study of the CL and TL

Isolates that produced about 90% mortality in the previous trial were used to determine the lethal concentration of 50% (LC_{50}) and 90% (LC_{90}) of the *D. pallens* population, a 3 mL distilled water containing 0, 10, 20, 30, 40, or 50 nematode dauers were used as inoculums and each added to 50 mL conical vials filled with the sawdust soil mixture mentioned above. Then, a single larva of *D. pallens* (0.14 ± 0.05 g and 4 mm of diameter head capsule) was added to each vial. The vials were incubated in a dark chamber at 15 ± 2 °C and the larvae were fed daily with two leaves of fresh white clover. Evaluation consisted in larvae mortality assessed every 24 h; the criteria to consider a dead larvae were the same described above. The dead larvae were incubated in a moist chamber made of Petri dish lined with filter paper Whatman N° 1, distilled water and temperature of 15 ± 2 °C, until the emergence of dauers. The trial was incubated until the first treatment reached 100% mortality. The results were subjected to regression analysis of nematode concentration vs. larvae mortality.

The experimental design was a completely randomized, were each experimental unit consisted of seven conical vials with a single larva of *D. pallens* inside of each vial, and four replicates per each treatment. The TL_{50} and TL_{90} was obtained directly from the linear regressions, while the LC_{50} and LC_{90} were estimated through regression analysis and then interpolated from the probit transformations (Fry, 1993).

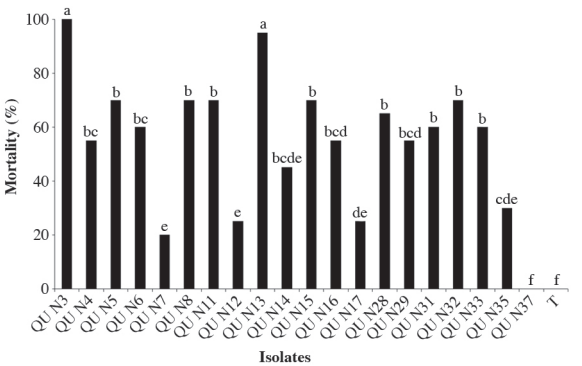
In addition, we calculated chi-square statistic to determine the goodness of fit of the model to the experimental data. Concentrations of the selected isolates were compared through the regression slopes, obtained from each replicate, and using the Student's t-test with a confidence level of 95%. The analyses were performed with the Minitab program version 15 (Minitab, State College, Pennsylvania, USA).

RESULTS AND DISCUSSION

Entomopathogenic nematode screening

Nineteen isolates out of 20 were pathogenic to larvae of *D. pallens*, causing different mortality rates. The dead larvae got a soft consistency and a yellow-brown to black color, characteristic of larvae killed by bacteria of the genus *Xenorhabdus* (Koppenhöfer, 2007). The control

treatment did not show mortality during the course of the bioassay. Isolate QU-N3 was similar to QU-N13 ($P < 0.001$), both reaching 95% of mortality (Figure 1) and were different to the rest of the isolates and the control. Koppenhöfer and Fuzy (2003) noted that differences in the pathogenicity of dauers can be attributed to their foraging strategy, the responsiveness of the host immune system, the pathogenicity of the symbiotic bacteria and the number of bacterial cells transported by the dauers. Furthermore, Koppenhöfer and Kaya (1999) have reported that *Steinernema* dauers can carry different amounts of bacterial cells in their intestines. For example, the species *S. scapterisci* contains a small amount of bacterial cells, instead the species *S. carpocapsae* contains a large number, causing a higher pathogenicity but at the expense of lower survival in the environment (Emelianoff *et al*., 2007). Isolates that had a zero or low value of mortality (Table 1) could be influenced by the immune response mechanisms of the insects, both humoral and cellular, such as encapsulation and melanization of nematodes, preventing the release of bacteria into the insect hemolymph and thereby the larvae septicemia (Alves, 1998). *Heterorhabditis bacteriophora* has a major tendency to be encapsulated within *Acheta domesticus* larvae, while *S. scapterisci* does not have this immune response in the same host (Lewis *et al*., 2006). Thus, some species of *Steinernema* secreted glycoproteins, which inhibit the prophenoloxidase enzyme that acts causing the encapsulation of parasites (Li *et al*., 2009).



Different letters in bars indicate difference in treatments according Fischer protected test ($P \leq 0.05$) (critical value for comparison: 21.093).

Figure 1. Mortality of *Dalaca pallens* larvae inoculated with different isolates of entomopathogenic nematodes. Evaluation after 15 d incubation.

Table 1. Regression slopes of mortality and lethal concentration 50% (LC_{50}) and 90% (LC_{90}) of two isolates of *Steinernema* spp. on *Dalaca pallens* larvae.

Isolate	Slope	LC_{50} dauers mL^{-1}	LC_{90} dauers mL^{-1}
QU N3 (*Steinernema australe*)	4.99 NS	14 (6 ± 20)	39 (35 ± 54)
QU N13 (*Steinernema unicornum*)	4.06	14 (3 ± 20)	48 (38 ± 60)

LC_{50} and LC_{90}: Concentration for killing 50% and 90% of the larvae population treated. NS: non significant according to the Student's t-test.

Isolates QU-N3 and QU-N13 correspond to two new species identified recently in Chile as *Steinernema australe* (Edgington *et al*., 2009a) and *Steinernema unicornum* (Edgington *et al*., 2009b), respectively. QU N3 was obtained from a sample collected in the Isla Magdalena National Park (44°36'42" S, 72°51'11" W), characterized by moist, sandy loam texture and pH 5.3. While the QU-N13 was found in 52 soil samples along Chile, from 33 to 51° S lat, suggesting a better adaptation to different environments. However, the nematode characteristics, both biological and ecological, and their relationships with the symbiotic bacteria need future studies.

Study of the CL and TL

After 72 h incubation the infected larvae die and took the color and consistency characteristic for the EPN infection; described in the first trial. After 4 d dauers start emerging from the dead larvae; this process did not differ from other *Steinernema* species (Koppenhöfer, 2007). At the same time, the control treatment did not show mortality. After 13 d post inoculation the first isolation (QU-N3) reached 100% of larvae mortality, at the concentration of 50 dauers mL^{-1}. The probit analysis (Table 1) showed LC_{50} and LC_{90} values of 14 and 39 dauers mL^{-1}, respectively. While for isolate QU-N13 these lethal concentrations were 14 and 48 dauers mL^{-1} (Table 1). Consequently, both isolates required the same concentration of dauers to eliminate 50% of the larvae population, and a difference of nine dauers to eliminate 90% of the treated population (Table 1). Furthermore, slope analysis indicated no difference between both isolates ($P = 0.932$). The goodness of fit test suggests that both isolates adjust properly to the regression curve ($X^2 = 2.476$, $P = 0.480$ and $X^2 = 0.903$, $P = 0.826$, respectively).

The curve of mortality over time was linear, then TL_{50} and TL_{90} were interpolated directly from the regressions, indicating that dauer concentration was inversely proportional to lethal time (Figure 2). Besides, both isolates have similar time to reach LT_{50} for the different concentrations (Table 2). However, mortality observed in lab trials rarely is coincident with the values obtained under field conditions, where the influence of

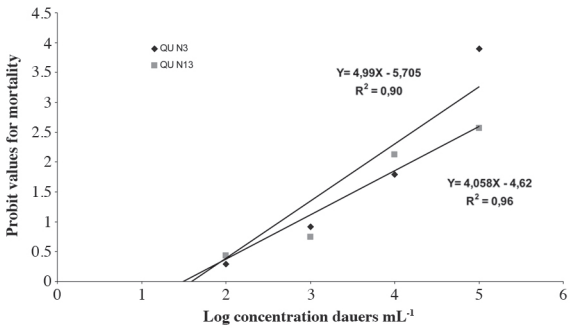


Figure 2. Larvae mortality of *Dalaca pallens* after 13 d of treated with different concentrations of entomopathogenic nematodes.

soil chemistry and physical characteristics, competition with other microorganisms in addition to environmental conditions (Alves, 1998), reduces the amount of dauers reaching an insect.

Table 2. Lethal times (LT) for different concentrations of *Steinernema* spp. dauers (QU-N3 and QU-N13) on *Dalaca pallens* larvae.

Concentration (dauers mL^{-1})	Isolate	LT_{50} (d)	LT_{90} (d)
10	QU N3 (*S. australe*)	11	25
	QU N13 (*S. unicornum*)	13	28
20	QU N3	10	21
	QU N13	10	20
30	QU N3	8	15
	QU N13	8	17
40	QU N3	7	16
	QU N13	6	13
50	QU N3	6	13
	QU N13	5	11

CONCLUSIONS

There are native entomopathogenic nematodes in Chile with the ability to parasitize *Dalaca pallens* larvae. Thus, two isolates of *Steinernema* (*S. austral* and *S. unicornum*) were able to cause above 90% of mortality. Also, the lethal concentrations and lethal time values were calculated and their ranges were appropriate for the control of *Dalaca pallens* larvae, values similar to other *Steinernema* species. Thus, both isolates have the potential for field control of *Dalaca pallens* larvae, facilitated by the food behavior of this species.

Selección de nematodos entomopatógenos para el control de *Dalaca pallens* (Lepidóptera: Hepialidae). *Dalaca pallens* (Blanchard) es considerada una de las plagas de mayor incidencia para la producción de las praderas en Chile. El objetivo de esta investigación fue la selección de aislamientos nativos de nematodos entomopatógenos (EPN) para el control de larvas de *D. pallens*. Se utilizaron 20 aislamientos nativos de EPN, para cada uno se inocularon 40 dauers por frascos cónicos con aserrín de *Nothofagus dombeyi*, y se agregó una larva de *D. pallens* de cuarto instar. Luego, se incubaron a 15 °C y oscuridad y se registró la mortalidad diaria. Se realizaron cuatro repeticiones por tratamiento y la unidad experimental fue de cinco frascos. Los resultados mostraron que el aislamiento QU N3 de *Steinernema australe* alcanzó el 100% de mortalidad a los 15 días de incubación, sin mostrar diferencias ($P < 0{,}001$) con el tratamiento QU N13 (*Steinernema unicornum*), el cual alcanzó un 95%. Para el cálculo de la concentración letal (CL_{50} y CL_{90}) se usaron los dos aislamientos mencionados, y se evaluaron concentraciones de 0, 10, 20, 30, 40 y 50 dauers por frasco que contenían una larva de *D. pallens* de cuarto instar. Para el aislamiento QU N3 la CL_{50} y CL_{90} fue de 14 y 39 dauers mL^{-1}, a los 13 d de incubación, mientras que para QU N13 estos valores fueron de 14 y 48 dauers mL^{-1}. En conclusión, existen aislamientos nativos de EPN con habilidad para parasitar larvas de *D. pallens*, los cuales necesitan ser evaluados en condiciones de campo.

Palabras clave: control biológico, *Steinernema australe*, *Steinernema unicornium*, concentración letal.

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