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ALFALFA SNOUT BEETLE, *OTIORHYNCHUS LIGUSTICI* L. (COLEOPTERA: CURCULIONIDAE): METHODS FOR EGG COLLECTION AND LARVAL REARING

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ABSTRACT

The alfalfa snout beetle, *Otiorhynchus ligustici* L., is the most serious pest of alfalfa in northern New York State. Recent research efforts focused on the biological control of this insect require the availability of all life stages. With a 2-year lifecycle and a mandatory diapause, the artificial rearing of a laboratory culture appears to be a non-viable option at present, but methods described here can be used to obtain sufficient numbers of eggs and larvae over an extended period of time for research purposes. The crowding of adult beetles in egg production units (cups) had a significant, negative effect on egg production per beetle but the total egg production per cup was still higher with higher number of beetles per cup resulting in a significant saving of labor per egg produced. Larval survival rates in alfalfa-planted cans were surprisingly low given the protected conditions of the greenhouse. The larval survival rates were not significantly different among the dates for the second instar and later instars, suggesting that larval mortality occurs in the first instar in alfalfa-planted cans.

The alfalfa snout beetle, *Otiorhynchus ligustici* L. (Coleoptera: Curculionidae), was introduced into the United States from Europe via wooden sailing ships carrying soil as ballast (Lindroth 1957, York et al. 1971). The beetle was first recorded in New York State in 1896 at the Port of Oswego and was first recorded as a pest of alfalfa when alfalfa was introduced into the area in the 1920s (York et al. 1971). In subsequent years, this flightless and parthenogenetic insect has spread to nine Northern New York counties, infested over 200,000 hectares of cropland and has become the most serious pest of alfalfa in northern New York State (Schroeder et al. 1994, Ferguson et al. 1995, Shields et al. 1999). The larvae feed on the lateral roots and later on the tap roots of the host plants. Alfalfa snout beetle has a 2-year lifecycle. The biology and life history of alfalfa snout beetle has been studied and described by several authors in Eurasia and North America (Vassiliev 1914 in York 1974, Lincoln and Palm 1941, Hanuss 1958, Nyilas 1962, York 1974, Jermy and Balázs 1990) and is very similar throughout Europe and Northern New York. Most larvae mature by late fall and move down in the soil to varying depths depending on soil type, temperature, and other factors. Mature larvae remain quiescent deep in the soil for ca. 8 months before pupation the following summer. After eclosion, adults remain in the pupal cells and only move to the soil surface the following spring after spending the second winter in the soil (Lincoln and Palm 1941). Adults start moving up to the soil surface when spring soil temperatures warm to 3°C and appear on the surface from late April to early May throughout the geographical distribution in the US. After reaching the surface, adults feed on the available host plants after which oviposition commences (Schroeder et al. 1995).

Research efforts focused on this insect require the availability of all life stages for an extended period of time; usually large numbers of individuals are needed. With a 2-year lifecycle and a mandatory diapause, the artificial rearing of a laboratory culture appears to be a non-viable option. However, the adults

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can be collected in the spring in large numbers and placed in cold storage at 4°C for 4 months without significant death, extending the adult availability from 3 weeks to 4 months. Adults will survive in cold storage for 4 months with low mortality and a few individuals will survive in cold storage for up to 9 months (EJS, personal observation). Adults can be removed from cold storage, fed and used to produce eggs. The purpose of this report is to illustrate a series of economically cost effective procedures to produce eggs, collect eggs and rear larvae to predetermined stages using alfalfa plants in containers. These techniques are currently being used in ongoing research projects focused on biological control and developing a snout beetle-resistant alfalfa.

MATERIALS AND METHODS

Egg production. Alfalfa snout beetle adults were field collected in late April 2006 near Great Bend, NY, immediately after emergence. The adult beetles were kept in cold storage (4°C) for 4 weeks on moist filter paper. The beetles were then placed into paper cups (82 mm diameter, 70 mm depth). The cups contained a layer of approximately 1 cm of autoclaved soil to encourage egg laying. The soil was sifted through an 18 mesh screen (1 mm opening) to remove the large particles. The soil was lightly moistened as needed. Fresh alfalfa foliage was provided to the beetles every other day as food.

The impact of adult crowding on egg production was investigated by placing adult females singly, two, four, eight, or 16 beetles/cup. Each treatment had 30 replicates. The cups were monitored daily and eggs were collected 6 and 12 days after the detected onset of oviposition. The total number of eggs was recorded for each cup. The average egg production per beetle in cups was calculated by dividing the total number of eggs by the number of beetles in the cup. A regression analysis was conducted with the number of beetles in a cup being the independent factor and the per beetle egg production being the response. Because of the heteroskedasticity of the data, the weighted least-square method was used.

Egg collection. The soil from the cups was rinsed in a 60-mesh screen (250 micron openings) to separate the eggs and large soil particles from the small soil particles. The remaining soil and eggs were placed into 300 ml of a 40% sugar solution in a 500 ml glass beaker. Eggs were suspended in the solution or floated to the surface, while the majority of the soil sank to the bottom of the beaker. The soil on the bottom of the beaker was stirred gently with a plastic stirring rod to free any eggs trapped in the soil. The egg-sugar solution was then decanted from the beaker through a 60 mesh sieve that retained the eggs. Eggs were then washed off the sieve with water into a 50 ml beaker. In water, the eggs sank to the bottom of the container while the remainder of the debris (frass, leftover plant material) floated to the top and could be simply decanted leaving a small volume of the water on the bottom of the beaker with the eggs. The eggs were then poured onto a filter paper in a filter funnel. The eggs were surface sterilized with a 5% bleach solution by pouring the bleach solution over the eggs. After one minute, the eggs were immediately rinsed with deionized water.

Egg hatch rate. Eggs from adult beetles were collected using the sugar flotation method described above. Three different methods of egg incubation were used: 1) control: 100 eggs were rinsed in deionized water and placed on a moist filter paper in a Petri dish (10 cm diameter); 2) treatment 1: 100 eggs were suspended in a 0.5% agar solution (10 °C temperature) and left undisturbed for one hour; they were then pipetted off onto a filter paper to drain the excess agar and were placed onto a second filter paper moistened with deionized water in a Petri dish; 3) treatment 2: a Petri dish was filled with Cornell mix (consisting of 1:2 peat moss-vermiculite mixture). One hundred eggs were suspended in 0.5% agar solution (10°C temperature) and left undisturbed for one hour. Eggs were

then placed onto a filter paper to absorb the excess moisture. The eggs were then moved from the filter paper using a soft brush onto the surface of potting soil. These Petri dishes were left uncovered and were lightly moistened daily with a water sprayer bottle.

All eggs were incubated at 23°C in the dark. The number of eggs hatching was determined daily for a total of 14 days and the proportion of eggs hatching was recorded. Each treatment was replicated four times. Proportional data was transformed using arcsine square root transformations and then mean hatch rates in the different treatments were compared using ANOVA and Tukey's HSD procedure. Statistical analyses were conducted using SAS™ system for Windows™, release 8.02 (SAS Institute Inc., Cary, N.C.).

Inoculation of alfalfa plants with eggs. Alfalfa plants were grown in plastic waste paper cans (21 cm width, 27 cm length, and 33 cm depth). The waste paper cans had four small holes (approximately 1 cm diameter) drilled in the bottom to allow for water drainage during watering and the bottom of the cans was lined with fine mesh fiberglass window screen to prevent the escape of larvae from the cans. The cans were then filled with Cornell Mix. Alfalfa seeds were planted in the cans and allowed to grow and establish for 6 weeks before being inoculated with snout beetle eggs.

Alfalfa snout beetle eggs were collected as described above and suspended in 0.5% agar solution and cooled to 10°C at a concentration of 5 eggs/ml. The eggs remained in the agar solution for approximately one hour before application into the soil around the plants. The use of the dilute agar solution thickened the liquid so the eggs remained suspended without gravitational settling. Each can was inoculated with 500 eggs by spreading 100 ml of egg suspension on the surface of the potting soil. A subset of the eggs used to inoculate the cans was returned to the laboratory and monitored for hatching. The inoculated cans were incubated in a growth chamber at a constant temperature of 24°C. A total of 22 cans were inoculated.

Alfalfa snout beetle larval survival in the cans. Larvae were recovered from the cans by breaking down the cans and sifting the soil at eight different times after inoculation: 22 days (three cans), 28 days (three cans), 35 days (two cans), 40 days (four cans), 49 days (three cans), 54 days (two cans), 60 days (two cans), and 68 days (three cans). The larval instars were determined by measuring the width of the head capsule of the larvae. The presence of 1st instar larvae was not tabulated due to the small size of the instar and the difficulty of accurately counting the larvae accurately. The proportion of surviving alfalfa snout beetle larvae was calculated by dividing the number of recovered larvae by 500 (the number of eggs inoculated). Proportional data was transformed using arcsine square root transformation and then mean survival rates at the different times were compared using ANOVA and Tukey's HSD procedure.

RESULTS AND DISCUSSION

Egg production. The mean egg production per beetle was the highest, 82 ± 9.5 (mean ± SE) , when only a single beetle was present in a cup and the lowest, 43.1 ± 1.9, when 16 beetles were in the same cup. The mean egg production per beetle was 66.1 ± 5.9 with two beetles in the same cup, 62.3 ± 3.0 with four beetles, and 51.5 ± 2.9 with eight beetles in a cup. There was a negative linear relationship ($F = 35.25$, df = 1, 148, $P < 0.000$, $r^2 = 0.19$) between the number of beetles in a cup and the per beetle egg production (Fig. 1). The mean total egg production per cup was the highest, 689.1 ± 31.1, with 16 beetles in a cup and the lowest, 82.0 ± 9.5, with a single beetle per cup. The mean total egg production per cup was 132.10 ± 11.70 with two beetles in the same cup, 249.1 ± 11.9 with four beetles, and 412.1 ± 23.5 with eight beetles in a cup. The high egg production variability which is the most obvious in the single insect per

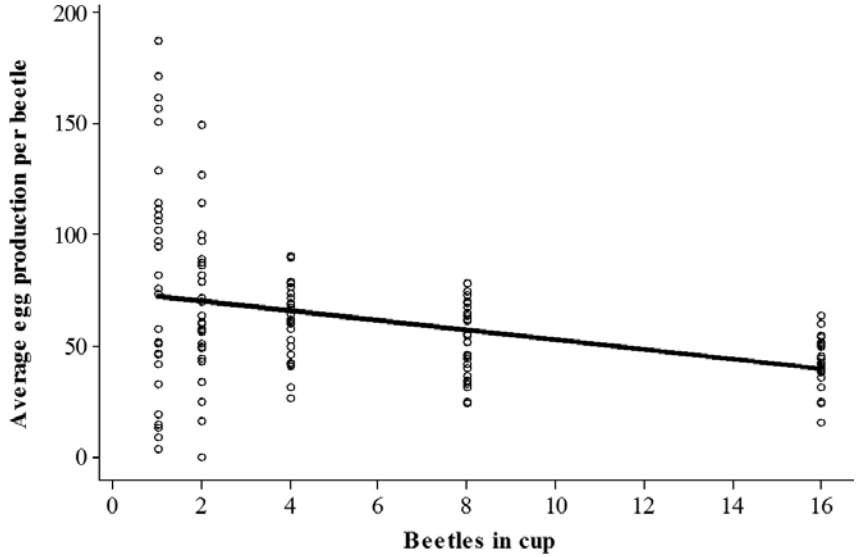

Fig. 1. Alfalfa snout beetle egg production in 10-cm-diameter paper cups. Each data point represents the total egg production in a cup in a 12-day time period divided by the number of beetles in the cup. There is a very apparent decreasing heteroskedasticity in the data. The regression analysis was therefore done using the weighted least square method. The line represents the best fit line of the regression (egg production per beetle= 71.4 - 1.8 number of beetles in cup). High egg production variability between individuals has been reported by numerous authors and is consistant with the data shown. Some individuals lay few eggs while some individuals lay numerous eggs. As more beetles are placed in each cup, the individual variability between insects is masked by the averaging procedure of calculating average eggs per beetle.

cup data is typical of this parthenogenetic species, where egg production from individual insects commonly range from zero to several hundred per individual (Lincoln and Palm 1941, York 1974).

It appears that the crowding of the beetles results in decreased oviposition per beetle but it is not known whether this effect would be observed over the entire lifetime of the beetle. Since we were mostly interested in the efficiency of harvesting the eggs, only the first 12 day period was investigated. It is not clear why adult beetles would decrease their egg production when crowded, but one possible explanation could be that in natural conditions, oviposition under crowded conditions increases the intraspectific competition for larval resources.

Our main objective was to produce a large number of eggs efficiently. Although crowding the beetles (16 per cup) resulted in reduced per beetle egg production compared to the single beetle per cup, the total egg production per cup was still much higher, resulting in a significant saving of labor per egg produced. If the availability of beetles is not the limiting factor, then crowding the beetles is the most labor efficient method. However, if beetle availability is an issue, egg production is the greatest when a single beetle is caged individually.

Egg hatch rate. The percentages of hatching eggs were 47.0 ± 3.2% in the control, 51.0 ± 4.6% in treatment 1, and 46.5 ± 2.5 in treatment 2. Other researchers have reported a similar hatch rate ranging between 50-60%

(e.g., Lincoln and Palm 1941, York 1974). The proportions of hatching eggs among the three different incubation methods were not significantly different ($F = 0.48$, df = 2, 9, $P = 0.631$). Therefore, we conclude that the sucrose-flotation method had no adverse effects on snout beetle egg hatching rate. Suspending insect eggs in agar solution with a specific density is a widespread method for insect egg applications, because the eggs neither sink nor float on the surface of the suspension so the rate of application is even (Fery et al. 1979, McEwen 1996, Abel et al. 2000).

Larval survival. The survival of alfalfa snout beetle on alfalfa plants grown in waste paper cans ranged between 3.13 ± 0.07% and 4.20 ± 0.20%. Survival rates did not change significantly with increasing time since inoculation ($F = 0.75$, df = 7, 14, $P = 0.638$) (Table 1.). The hatching rate we observed did not differ from early research conducted on snout beetle (Lincoln and Palm 1941). The larval survival rates were surprisingly low given the protected conditions of the greenhouse but were higher than reported by Schroeder et al. (1994). The cans had sufficient alfalfa plants to support a higher number of larvae with a large root mass in every container, so food availability was not considered a limiting factor. It would appear that a large amount of larval mortality occurs from the 1st instar larvae failing to find a root as a food source shortly after hatching.

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Table 1. Mean number of surviving alfalfa snout beetle larvae in different instars recovered from cans planted with alfalfa at different times and the mean percent total survival.

Time (days)	2nd instar	3rd instar	4th instar	5th instar	6th instar	% survival
22	15.7 ± 0.3	0	0	0	0	3.1 ± 0.1
28	16.3 ± 3.8	2.7 ± 0.9	0	0	0	3.8 ± 0.6
35	7.0 ± 5.0	11.0 ± 1.0	3.0 ± 3.0	0.5 ± 0.5	0	4.2 ± 0.2
40	2.5 ± 1.0	13.0 ± 1.7	1.3 ± 0.8	0	0	3.2 ± 0.2
49	0.3 ± 0.3	8.0 ± 3.8	9.7 ± 1.7	0.3 ± 0.3	0	3.7 ± 0.7
54	0	3.0 ± 2.0	13.0 ± 3.0	0	0	3.3 ± 0.1
60	0	0	9.5 ± 4.5	9.0 ± 3.0	0	3.7 ± 0.3
68	0	0	0.7 ± 0.7	13.3 ± 1.3	2.00 ± 2.00	3.2 ± 0.4

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