

## TECHNICAL NOTE

# A technique for continuous mass rearing of the black vine weevil, *Otiorhynchus sulcatus*

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### Introduction

The black vine weevil, *Otiorhynchus sulcatus* (F.) (Coleoptera: Curculionidae) is a univoltine, polyphagous insect that is a noxious pest of field and container grown ornamentals as well as small fruit crops world-wide (Moorhouse et al., 1992). In the USA, the environmental horticulture industry (floriculture and nursery crops) is the third largest value crop behind corn and soybeans (USDA fact sheets 2001, <http://www.nass.usda.gov>). *Otiorhynchus sulcatus* is thought to have a northern European origin and was first recorded in North America in 1835 (Smith, 1932). Oviposition occurs at night with eggs being either dropped on the soil surface or inserted into crevices on plants (Smith, 1932). Early instars begin by feeding on small roots while the later instars feed on larger roots, especially on the phloem and cambium tissues near the soil surface (La Lone & Clarke, 1981). Adults are nocturnal and mainly cause aesthetic damage to plants by notching the leaves. Adults are parthenogenic, so a single individual left unchecked can result in the infestation of an entire nursery.

Because of the biology of *O. sulcatus*, the collection of larvae and adults from the field is very tedious and often requires countless hours of digging through field soil, container-grown plants or searching for adults after dark. Indeed, many chemical and biological control companies developing products for *O. sulcatus* larval control still rely on searching through infested plant material in order to gather the needed larvae for efficacy studies. Although an artificial diet initially developed by Shorey & Hale (1965) as modified by Shanks & Finnigan (1973) and Shanks (1980) (hereafter referred to as the 'standard diet') sustains larvae and is commercially available, there has been no documented technique for continuous mass rearing of

*O. sulcatus*. Studies of weevil biology as well as the development of control strategies require a large, uniform, and predictable supply of insects of all life stages throughout the year.

When considering field or potted plants for use in control efficacy or host plant resistant studies, other researchers have often found it useful to store insect eggs for several weeks for subsequent use; many thousands to millions of eggs were needed. The stockpiling of eggs for future use has been necessary with other reared species of Coleoptera (Branson, 1978; Fisher & Edwards, 2002). The egg hatch and viability of the strawberry root weevil, *Otiorhynchus ovatus* L. were not significantly reduced when it was stored for up to 4 weeks at 4 °C (Fisher & Edwards, 2002).

We have developed a technique for continuous mass rearing of *O. sulcatus* in the laboratory using a meridic diet. Since small changes in diet composition can often lead to dramatic changes in larval survival and growth, experiments were also performed to determine if *O. sulcatus* larval survival and development were increased on the improved diet described here, compared to the standard diet. Additionally, we determined if *O. sulcatus* eggs could be stored for up to 4 weeks at 4 °C without significant reductions in larval survival and development.

### Materials and methods

#### Source of insects

Originally, late instar *O. sulcatus* were collected from infested nursery plants obtained from commercial wholesale nurseries in the Willamette Valley of Oregon, USA. Groups of 10–25 larvae were placed in covered plastic deli containers (0.5 l; Reynolds Metal Co., Richmond, VA) containing a 3 cm layer of moist, ground peat moss. Pieces of longitudinally sliced carrots (Masaki & Sugimoto, 1991) were supplied as food and replaced as necessary until pupation. Pupae were transferred to similar containers

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containing only moist peat moss. All larvae were reared in complete darkness at  $21.0 \pm 0.5$  °C. As eclosion proceeded, teneral adults were placed in empty plastic containers containing leaves of strawberry (*Fragaria* × *ananassa* Duchesne) and sprigs of yew (*Taxus* spp.). The strawberry and yew were kept fresh by placing the petioles or the end of the sprig in a floral water-pic (Syndicate Sales Inc., Kokomo, IN). Adults were limited to approximately 10 per cage. They were placed at  $21.0 \pm 0.5$  °C, with a photoperiod of L16:D8. Under these conditions, the adults reached ovipositional maturity within 30 days. Eggs collected from them were then placed on the standard diet (with an additional 120 ml of water for increased flowability) to begin the colony reported here. Periodically, at least once per year, the colony is supplemented with individuals collected from the field.

#### Egg collection

Each week, eggs were collected from adult rearing containers. Live adults were transferred to new containers with fresh plant material and dead adults discarded. As eggs were oviposited on leaf tissue, water pics, and in all other areas of the container, the used adult rearing containers were retained. Eggs oviposited on the foliage and water pics were rinsed into the bottom of the container with a water jet produced by a standard laboratory wash bottle. Aside from the eggs, there were about 1–2 cm depth of water, weevil feces, and leaf debris in the bottom of each container. The contents from 10 to 15 containers were combined in a large beaker (~1000 ml) and allowed to settle. As much fluid as possible (without losing eggs) was decanted. The remaining eggs and debris were minimally rinsed (in as little water as possible) into a 1000 ml separatory funnel fitted with a Teflon® stopcock. The eggs were then separated from the debris by differential flotation.

Previously, 850 ml of a 40% solution of sugar and deionized water (commercially available granulated sugar,

specific gravity ~1.17) were prepared and added to the separatory funnel. The egg and sugar solution suspension was vigorously mixed and allowed to settle. Eggs and very small leaf tissue floated near the top of the solution. The majority of the debris was elutriated into a beaker and discarded. To surface sterilize the eggs, approximately 850 ml of a 1.25% aqueous solution of sodium hypochlorite were added to the separatory funnel. Eggs and sodium hypochlorite solution were then drained from the separatory funnel into a Buchner funnel-suction apparatus fitted with a 90 mm circle of filter paper (Whatman no. 1) and attached to a vacuum line. To remain viable, we found that the maximum amount of time that eggs could be left in the sodium hypochlorite solution was no more than 2 min. The eggs on the filter paper were rinsed several times with deionized water from a wash bottle. Sterilized eggs were kept in 100 mm Petri dishes on the moist filter paper for immediate use, or they were stored at 4 °C on the moist filter paper with the outer edges of the Petri dish wrapped in Parafilm®.

#### Artificial diet

We found that to maximize larval survival and growth, the ingredients in the standard diet (with an additional 120 ml of water for increased flowability) required further modification (Table 1). We achieved superior mold inhibition as well as improved larval survival and growth using sorbic acid potassium salt in place of sorbic acid.

Initially, the agar and water (500 ml) were placed in a beaker and microwaved (1500 W) on a high setting for 15 min. The hot agar solution as well as the lima beans (submerged in 500 ml of water) were each covered with aluminum foil and heated in a Marketforge™ Sterilmatic® bench top autoclave at  $1.1 \text{ k cm}^{-2}$  and 121 °C for 30 min. When the beans and agar were done, they were removed and cooled to 45 and 80 °C, respectively (about 20 min). Temperature was monitored closely with a thermometer. The beans were placed in a cooling bath to speed cooling.

Ingredients	Standard diet <sup>a</sup>	Improved diet
Lima beans	120 g	120 g
Sterile millipore water	500 ml	500 ml
Agar, USP (Moorhead & Co.)	30 g	30 g
Sterile millipore water	500 ml	500 ml
Brewers yeast (Lewis Laboratory International Ltd)	40 g	40 g
L-asorbic acid (Sigma, A-7506)	4 g	4 g
Methyl paraben (Sigma, H-6654)	1.8 g	1 g
Sorbic acid (Sigma, S1626)	0.8 g	0 g
Sorbic acid potassium salt (Sigma, S1751)	0 g	1.25 g
Streptomycin sulfate (Fisher Biotech, BP910-50)	1 g	1 g

**Table 1** Modification of *Otiorynchus sulcatus* diet (1 l)

<sup>a</sup>Shorey & Hale (1965) as modified by Shanks & Finningan (1973) and Shanks (1980) with an additional 120 ml of water.

Once cool, the beans with water were poured into a blender and homogenized. The rest of the dry ingredients were then added and blended thoroughly. The 80 °C agar solution was then added and blended. The diet was then dispensed into 28 g plastic portion cups, filling each to a depth of about 2 cm and set aside to cool. When the diet had gelled, five 2 mm holes were made in the diet and a plastic lid attached. The cups were then placed under ultraviolet light for approximately 1 h. The diet was either used immediately or stored at 4 °C for up to 2 weeks.

#### Rearing procedure

Ten viable eggs (brown to dark brown in color) were placed, using a moistened camel hair brush, in the holes that had been made in the diet (two eggs per hole). Between cups, the brush was decontaminated in a 1.25% sodium hypochlorite solution and rinsed twice in deionized water. After egg inoculation, the lids were replaced on the diet cups. The diet cups with eggs were then maintained in complete darkness at  $21.0 \pm 0.5$  °C.

Six-week-old larvae (3rd–4th instar) (La Lone & Clark, 1981) were removed from the spent diet with a surface sterilized (dipped in 95% ethyl alcohol and flamed) pair of ‘feather-weight’ forceps (BioQuip Products Inc., Gardena, CA) and placed in pairs into cups containing fresh diet (we found that this was the maximum amount of time that the diet was usable at 21 °C). Cups with larvae were capped and maintained in total darkness at  $21.0 \pm 0.5$  °C for another 6 weeks and the larval transfer process repeated. Within the next 4–6 weeks, the larvae began to pupate and eclose in their diet cup. Once newly eclosed adults had melanized, they were placed in adult rearing containers. Total developmental time from egg to adult was 16–18 weeks.

#### Cold storage of eggs

For this experiment, we collected 1250 viable eggs laid within the same week. Eggs were collected and immediately placed on the standard diet (Table 1) or were refrigerated ( $4.0 \pm 0.5$  °C) for 1, 2, 3, or 4 weeks and then placed on the standard diet. For each treatment there were five replications of 10 diet cups, each containing five eggs. Fresh diet (prepared within a week of use) was used for each treatment. The cups were maintained for 6 weeks under standard rearing protocols as described previously. At 6 weeks, each cup was carefully searched and the larvae individually weighed.

This experiment was performed twice and arranged in a completely randomized design. A test of homogeneity of variance was performed to detect variation between the two runs of the experiment (Little & Hills, 1978). Variability was not significant, and the runs were combined. The

mean data from the two runs were analyzed with ANOVA using the General Linear Models Procedure (GLM) and Tukey’s multiple range test was used to separate means (SAS Institute, 1999).

#### Comparison of diets

Experiments were performed to evaluate larval survival and development on the standard vs. our improved diet. Fifty cups of fresh diet of each type (prepared within a week of use) were inoculated with 10 viable eggs using standard rearing protocols. The cups were maintained under standard rearing conditions for 6 weeks. At 6 weeks, each cup was carefully searched and the larvae individually weighed.

The experiment was performed twice and arranged in a completely randomized design with each cup considered a replicate. A test of homogeneity of variance was performed to detect variation between the two runs of the experiment (Little & Hills, 1978). Variability was not significant and the runs were combined. The data were analyzed using GLM, and a t-test was used to separate means (SAS Institute, 1999).

#### Results and discussion

Eggs could be stored for up to 4 weeks at 4 °C without any significant reduction in subsequent larval survival ( $F_{4,5} = 1.05$ ,  $P = 0.47$ ) or average larval weight ( $F_{4,5} = 0.76$ ,  $P = 0.59$ ). The percentage larval survival ( $\pm$  SEM) from eggs placed immediately on diet and those stored for 1, 2, 3, or 4 weeks were  $34 \pm 6.3$ ,  $39 \pm 10$ ,  $37.8 \pm 2.2$ ,  $34.6 \pm 1.4$ , and  $20.4 \pm 4.4\%$ , respectively. The average larval weight ( $\pm$  SEM) from eggs placed immediately on diet and those stored for 1, 2, 3, or 4 weeks were  $12.8 \pm 0.36$ ,  $16.3 \pm 6.2$ ,  $19.6 \pm 0.55$ ,  $16.1 \pm 0.55$ , and  $14.3 \pm 1.9$  mg, respectively. The ability to store eggs for an extended length of time allows for the infestation of large experimental trials with large numbers of eggs. Previously, the infestation of plant material was done by caging adults over plants or by relying on natural infestations. However, egg production by gravid females can vary substantially depending on the plant material on which a particular female has fed (Shanks, 1980; Maier, 1981).

While *O. sulcatus* develops on the standard diet, the improved diet developed here substantially increased larval survival and growth. In our studies directly comparing the two diets, the percentage larval survival ( $F_{1,197} = 116.40$ ,  $P < 0.0001$ ) and the average weight per larvae ( $F_{1,193} = 76.59$ ,  $P < 0.0001$ ) at 6 weeks was significantly higher on the improved diet (Table 2). Larval survival at 6 weeks was 23% higher on the improved diet, and larvae were 10 mg heavier on average. The enhanced larval survival with the

**Table 2** Mean ( $\pm$  SEM) percentage of *Otiorynchus sulcatus* larval survival and weight at 6 weeks on artificial diet

Diet	% survival <sup>a</sup>	Weight <sup>b</sup>
Standard diet <sup>c</sup>	38.7 $\pm$ 1.4a	17.6 $\pm$ 1.0a
Improved diet	62.1 $\pm$ 1.7b	27.7 $\pm$ 0.5b

<sup>a</sup>Means in the same column with different letters are significantly different ( $P = 0.05$ ).

<sup>b</sup>Mean larval weight (mg).

<sup>c</sup>Shorey & Hale (1965) as modified by Shanks & Finnigan (1973) and Shanks (1980) with an additional 120 ml of water.

improved diet was not limited to 6-week-old larvae. In addition to the experimentation performed, there was a period of transition in our colony from the standard to our improved diet. During this time eggs were placed on both types of diet and maintained under standard rearing conditions. To demonstrate that the improvement in larval survival was also observed at the 12 week larval transfer, observational data from our laboratory records of several cohorts of *O. sulcatus* eggs showed increased larval survival at 12 weeks on the improved diet as well (Table 3).

The increase in survivorship and weight, as well as the improvement in uniformity of larvae when reared on the improved diet was remarkable. It is difficult to speculate why this occurred. The diets differed only in a decrease in methyl paraben by 0.8 g and the replacement of sorbic acid with potassium sorbate in the improved diet. The methyl paraben was reduced to offset the negative influence of

**Table 3** Survival of four cohorts of *Otiorynchus sulcatus* on artificial diet at 6 and 12 weeks

Diet	Eggs <sup>a</sup>	6 weeks (%) <sup>b</sup>	12 weeks (%) <sup>c</sup>
Standard diet <sup>d</sup>			
Cohort 1	3000	810 (27)	347 (42, 12)
Cohort 2	2470	454 (18)	207 (46, 8)
Cohort 3	3020	600 (19)	250 (41, 8)
Cohort 4	3040	878 (28)	455 (52, 15)
Improved diet			
Cohort 1	1720	870 (51)	816 (94, 47)
Cohort 2	1100	566 (51)	456 (81, 41)
Cohort 3	2100	1131 (54)	814 (72, 39)
Cohort 4	2160	1685 (78)	1089 (65, 50)

<sup>a</sup>Total number of eggs placed on diet.

<sup>b</sup>Number of 6-week-old *O. sulcatus* larvae and percentage survival from egg to 6 weeks from each cohort of eggs.

<sup>c</sup>Number of 12-week-old *O. sulcatus* larvae and percentage survival from 6 to 12 weeks and from egg to 12 weeks from each cohort.

<sup>d</sup>Shorey & Hale (1965) as modified by Shanks & Finnigan (1973) and Shanks (1980) with an additional 120 ml of water.

preservatives. The change to potassium sorbate increased the mold and bacteria inhibition of the diet. Potassium sorbate is 58% aqueous soluble at 20 °C, whereas sorbic acid is 0.25% aqueous soluble at 30 °C (Budavari et al., 1989). Thus, some of the increased vigor and survivorship may have been the result of a cleaner, more mold- and bacteria-free diet. However, this does not provide a complete answer for the improvement in larval survival and development observed. Reinecke (1985) stated that phytophagous insects need more potassium (K) than other animals that do not have a restricted plant diet. Moreover, Nation (2002) stated that many phytophagous insects need a relatively large amount of K, and only trace amounts of sodium. These ideas and the realization that K (in various compounded forms) is a major component of two of the early and most successful holidic diets (Dadd & Mittler, 1966; Beck et al., 1968) may proffer that the K in the potassium sorbate is responsible for these positive outcomes. The Brewer's yeast contained 844 mg of K and the potassium sorbate added an additional 325 mg (~35% more) over the standard diet.

Because of the biology of *O. sulcatus*, it is difficult to collect large numbers of individuals from the field for experimentation. For many years we and others have spent countless laborious hours obtaining larvae or adults just to conduct limited experiments of weevil biology or control. Presently, we are able to obtain over 1200 6–12-week old larvae/week. Moreover, we have the ability to produce hundreds of adults and thousands of eggs per week. The rearing protocol described here provides a large, uniform, and predictable supply of all stages of *O. sulcatus* year round.

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