

Pupation by Viburnum Leaf Beetle (Coleoptera: Chrysomelidae): Behavioral Description and Impact of Environmental Variables and Entomopathogenic Nematodes

PAUL A. WESTON¹ AND GAYLORD A. DESURMONT

Department of Entomology, Cornell University, Ithaca, NY

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ABSTRACT *Pyrrhalta viburni* (Paykull), a new landscape pest in the United States, feeds in both the larval and adult stages on foliage of plants in the genus *Viburnum*. The insect is univoltine, with larvae active in spring and adults throughout the summer months. Experiments were conducted to determine the depth of pupation in the soil; the impact of substrate texture, moisture content, and temperature on pupation success; and ability of entomopathogenic nematodes to kill larvae when they enter the substrate to pupate. Larvae burrowed only a short distance into the substrate when pupating; 97–100% were found within the top 3 cm of a column of soil or sand and soil mixture in the laboratory. Larval mortality before pupation was low at 22°C but considerably higher at 30°C; at both temperatures, pupation success was lowest on a mixed substrate and higher (and equivalent) on sand or soil alone. Survivorship to adult was influenced by both temperature and substrate moisture content; at 22°C, 56% percent of pupating larvae emerged as adults at 75% moisture content compared with only 25 at 25% moisture content. Emergence of adults was negligible at 30°C, regardless of moisture content. *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* were very effective biocontrol agents in laboratory bioassays, reducing adult emergence by 76–100%, with nematode applications made before pupation being more effective than those made after pupation, and *H. bacteriophora* consistently (but not significantly) more effective than *S. carpocapsae*. Management methods that take advantage of pupation behaviors are discussed.

KEY WORDS pupation, soil texture, soil moisture, entomopathogenic nematodes

Viburnum leaf beetle, *Pyrrhalta viburni* (Paykull), is a landscape pest with a fairly short history in the United States. Native to Europe, this destructive pest first appeared in the United States in Maine in 1994 and has spread fairly quickly through the northeast (see Weston and Hoebeke 2003 for a detailed invasion history). More recently, the insect was discovered in the state of Washington, likely the result of spread from an outlying population in British Columbia. The insect poses a serious threat to viburnums in both landscapes and natural areas, where some species of *Viburnum* can be dominant members of understory plant communities. Not all species are susceptible to the pest, but the more susceptible species (which are also common landscape choices) are destroyed after repeated defoliation by larvae in the spring and adults in the summer over a period of 2–4 yr (see <http://www.hort.cornell.edu/vlb/suscept.html> for the most recent listing of susceptibility ratings of various *Viburnum* spp.).

Unlike many other gallerucine beetles, *P. viburni* pupates within the soil. Despite the fact the *P. viburni* has been a longstanding pest in Europe, surprisingly little has been published on its natural history, includ-

ing its pupation behavior. Balachowsky (1963) and Zorin (1931) provided the most comprehensive reports to date, but many details are lacking. The reports of Weston and Diaz (2005) and Weston et al. (2007) are filling in some holes in our understanding of the basic biology of the pest, but much work needs to be done.

This study was conducted to increase our understanding of the process of pupation by *P. viburni* and to determine whether nematodes might be effective biocontrols against the pest. The specific objectives were to (1) describe the behavior of larvae as they prepare to pupate, (2) determine how deeply larvae penetrate the substrate when pupating, (3) measure the impact of temperature, moisture content, and substrate texture on ability of larvae to pupate, and (4) determine whether entomopathogenic nematodes might successfully prevent immature *P. viburni* from completing development to adult.

Materials and Methods

Rearing Conditions. Larvae were from colonies started from egg-infested twigs of *Viburnum dentatum* and *V. trilobum* collected from the field in late winter. Twigs were kept in a refrigerator until insects were

¹ Corresponding author, e-mail: paw23@cornell.edu.

needed, transferred to a reach-in chamber at 17°C for 3 d, and transferred to a chamber to 22°C until eggs hatched. Newly eclosed larvae were transferred to a plastic box (30 by 22 by 10 cm) covered with a screened lid and containing shoots of *V. trilobum* or *V. dentatum* (with cut ends in water-filled floral tubes) in a reach-in chamber at 22°C and a 15:9 L:D light regimen.

Pupation Substrates. A variety of substrates were provided for pupation in the various experiments. Sand was commercial white silica play sand obtained locally (Old Castle Retail, Charlotte, NC). Soil was a sandy silt loam collected from nearby research plots.

Observation of Behaviors Leading up to Pupation. Time-lapse video recordings were made of late third-instar larvae. Larvae within a day or two of pupating, based on previous observations of duration of the third instar stage, were placed individually on foliage of *V. trilobum* shoots (\approx 12 cm long and having two leaves) inserted into the substrate (a 50:50 mixture of sand and vermiculite) in a plastic cylinder (21 by 10.5 cm diameter) topped with a vented lid. Video recordings were made with a video camera equipped with an infrared light source on a laboratory bench; the arena was illuminated with daylight coming through a window until sunset, but behavior of the larvae could be observed after sunset because of the infrared illumination. The signal from the video camera went to a time-lapse video recorder, which recorded 2 s of activity every 10 s. Five larvae were recorded.

Pupation Depth. This experiment was conducted in plastic cylinders (21 by 11 cm diameter) covered with a screened lid. The cylinders held one of two substrate types: sand or a 50:50 mixture of sand and soil screened through no. 20 mesh (opening size 850 nm). Twenty third-instar larvae were placed in each container along with leaves of *V. dentatum* or *V. trilobum* and held at our standard rearing conditions. Foliage was replaced until all larvae had pupated. Seven days after pupation of the last larva, the substrate from each container was carefully removed in depth profiles (0–3, 3–6, 6–9, and 9–12 cm deep), and the substrate from each profile was examined for pupae. Six replicates were conducted for each substrate type, and data (percent of pupae found at each depth) were analyzed with analysis of variance (ANOVA; Statistix 8; Analytical Software, Talahassee, FL).

Substrate Texture, Moisture, and Temperature. This experiment was designed to measure the pupation success of larvae under various substrate conditions. Three substrate compositions were evaluated: sand, sifted soil, and a 50:50 mixture of sand and soil. To control the substrate moisture content, the sand and soil were first dried at 130°C, and the soil was screened through a number 20 mesh (opening size 850 nm) after cooling. Substrate (300 ml) was placed in each of 60 plastic cylinders (9 by 9 cm diameter), forming a layer 3.5–4 cm deep. Then, the appropriate amount of water was added to each container to yield a soil moisture content of 25 or 75% saturation. The amount of water to be added was determined previously by measuring how much water could be held by each substrate type before water started to leach un-

der gravity and multiplying this value by 0.25 or 0.75 for the two moisture regimens (the amount of water at saturation varied for the three substrate types). Ten third-instar *P. viburni* and several leaves of *V. trilobum* were added to each container. Finally, the containers were placed in an incubator at 22 or 30°C. The three factors (substrate texture, moisture content, and temperature) formed a 3 by 2 by 2 factorial arrangement, and five replicates were conducted for each combination. Containers were weighed before and after addition of leaves and larvae and every day thereafter for the duration of the experiment. The status of larvae was recorded daily, and the appropriate amount of water was added to return the substrate moisture to the target level. After all larvae had either died or pupated, adult emergence was recorded daily. Pupation success was calculated as the percentage of larvae in each container that burrowed into the substrate to pupate; this variable, therefore, incorporates both mortality of larvae before pupation and inability of larvae to burrow into the substrate. Rate of adult eclosion was calculated as the percentage of insects pupating that reached the adult stage (in all but two cases, this value was identical to the percentage of starting larvae to reach the adult stage). Data (pupation success and adult eclosion) were analyzed with factorial ANOVA (Statistix 8; Analytical Software) after arcsine transformation. Mean comparisons (needed only when comparing statistical differences among substrate type because the other factors had only two levels each) were performed with least square difference (LSD).

Impact of Nematodes. This experiment was conducted in plastic cylinders (9 by cm diameter) filled to a depth of 5 cm with sand. Two species of nematodes were evaluated: *H. bacteriophora* and *Steinernema carpocapsae*. Nematodes were obtained from Rincon-Vitova Insectaries (Ventura, CA) and suspended in water as per instructions that accompanied the shipment. Nematodes were applied at a rate equivalent to the labeled rate of one million per 50 ft², which translated into 1,361 nematodes per container; the appropriate volume of suspension to yield this number of nematodes was pipetted into each container, and additional water was added to nearly saturate the sand. Five late third-instar *P. viburni* on a leaf of *V. trilobum* were placed in each container after adding the nematode suspension. In addition, the impact of timing of nematode application was measured by inoculating an additional set of containers with nematodes 7 d after larvae had burrowed into the sand for pupation. Containers were covered with a vented lid and placed in an incubator at 22°C. Containers were monitored daily for larval mortality, pupation, or adult emergence; this continued until all larvae were accounted for. Five replicates were conducted for each application timing and nematode species. Percent mortality was analyzed with ANOVA after arcsine-transformation; factorial ANOVA testing the effect of both nematode species and timing of application was performed (Statistix 8; Analytical Software).

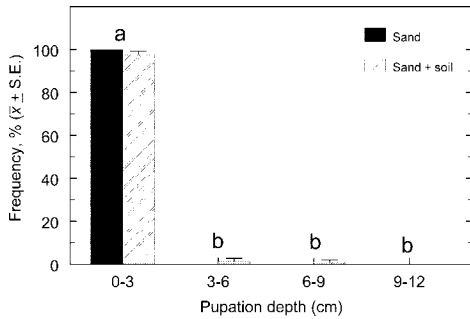


Fig. 1. Pupation depth of larvae of *P. viburni* in the laboratory in two substrate types. Pairs of bars accompanied by the same letter are not significantly different as determined by ANOVA followed by least significant difference (LSD) test (mean comparison test was performed on depth alone, averaging observations over substrate types because substrate type had no impact on pupation depth).

Results and Discussion

Observations of Behavior Leading up to Pupation.

Initially, several of the larvae fed on foliage of the test plant and then rested. Just before pupation, larvae crawled down the stem of the shoot in the bioassay arena. Most larvae that descended the stem crawled onto the surface of the substrate, spending only a few minutes moving about before burrowing. This is consistent with field observations of migration down the stems of host plants by late third-instar larvae (personal observations). One larva ascended the stem after reaching the substrate and spent >30 min exploring the leaves of the test plant before re-descending and burrowing into the substrate.

Pupation occurs within a cell constructed by the larva, apparently made of soil particles glued together by larval secretions. The cavity of the cell is slightly

larger than the exarate pupa and is smooth on the interior. Earlier studies (Weston and Diaz 2005) showed that the pupal stage ranges from 25 d at 27°C to 60 d at 17°C.

Pupation Depth. Nearly all larvae pupated within the top 3 cm of the soil column (Fig. 1). The effect of depth on occurrence of pupae was highly significant ($F = 7104.5$; $df = 3,40$; $P < 0.0001$), but there was no effect of substrate on pupation depth ($F = 0.0$; $df = 1,40$; $P = 1.0$). When sand was used as the substrate, no pupae were found deeper than 3 cm, and when the substrate was a mixture of sand and soil, only one pupa was found between 3 and 6 cm and one between 6 and 9 cm. Pupae were never found deeper than 9 cm. Larvae seem to burrow to a similar depth in the field; casual observations of soil dug up from the base of *V. dentatum* in the field several days after the last larvae were found on the foliage revealed abundant quiescent larvae within several centimeters of the surface. Interestingly, most larvae had already formed pupal chambers, and many became active and crawled away when the pupal chamber was opened. A significant number of larvae were infected with a fungus identified as *Beauveria* spp. (A. Hajek, personal communication). Botanigard, a commercial formulation of *B. bassiana*, had been applied to plants in the same field plots the previous year, so it is not clear if the fungus infecting the larvae was from this source or from an endemic population of *Beauveria*.

Substrate Texture, Moisture Content, and Temperature. Pupation success was 100% on all substrates at 22°C and 75% moisture (Fig. 2a). Pupation success in the other treatment combinations varied with substrate type ($F = 5.39$; $df = 2,48$; $P = 0.0077$) and temperature ($F = 25.0$; $df = 1,48$; $P < 0.0001$); no other main effects or interactions influenced pupation success. Mean comparisons showed that pupation success

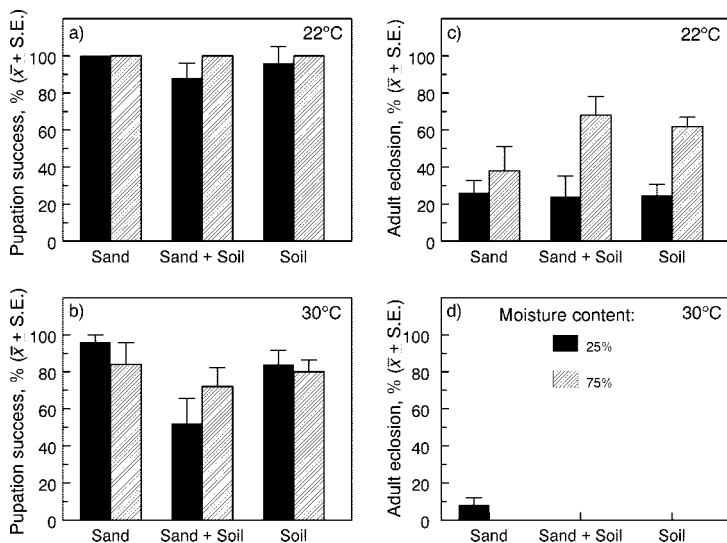


Fig. 2. Pupation success of larval of *P. viburni* (a and b) and eclosion rate of adults (c and d) in the substrate texture experiment.

was significantly lower on sand plus soil than either sand or soil alone (which were equivalent) and that pupation success was higher at 22 (Fig. 2a) than at 30°C (Fig. 2b).

Ecdysis of adults from pupating larvae was moderately low at 22°C (Fig. 2c) and at or near zero for all substrate and moisture content combinations at 30°C (Fig. 2d). The only main effects and interaction that were significant were temperature ($F = 75.3$; $df = 1, 48$; $P < 0.0001$), moisture ($F = 12.7$; $df = 1, 48$; $P = 0.0008$), and their interaction ($F = 16.9$; $df = 1, 48$; $P = 0.0002$); substrate had no significant impact on adult ecdysis. The effect of temperature can be readily comprehended by observing the near total lack of ecdysis at 30°C and the moderate levels at 22°C. Similarly, the impact of moisture can be appreciated by noting the uniformly higher rate of ecdysis at 75% moisture for all substrates at 22°C. The interaction term can be explained by the fact that this difference in response to moisture content was absent at 30°C because ecdysis was virtually equal (and near zero) for all treatment combinations at this temperature.

The fact that pupation success was lower on the mixed substrate suggests that this substrate is more difficult for larvae to penetrate. This might be explained by comparison of the arrangement and consistency of substrate particles between the mixed substrate and the sand or soil treatments. Compared with sand alone, the sand plus soil treatment should have a greater bulk density because spaces between sand particles would be occupied by smaller particles of clay and other fine particulates from the soil, likely making it more difficult for larvae to move particles aside as they attempt to burrow into the substrate. Compared with soil alone, the presence of sand particles in the mixed substrate might interfere with the ability of larvae to push substrate particles aside, making the substrate more difficult to penetrate. The decrease in pupation success with temperature is more difficult to explain but might be related to the fact that 30°C is above the thermal optimum for *P. viburni* and results in developmental abnormalities (Weston and Diaz 2005). Because our measure of pupation success includes both inability to penetrate the substrate and mortality from other causes, it seems likely that the reduced rate of pupation at 30°C is caused by adverse effects of elevated temperature on larval physiology and not on burrowing/pupation behavior per se.

The lack of adult ecdysis at 30°C no doubt resulted from the adverse impact of high temperature on completion of development of immature *P. viburni* (Weston and Diaz 2005). Thus, understanding the impact of substrate texture and moisture on burrowing ability of *P. viburni* should focus on the results obtained at 22°C. The decreased rate of adult ecdysis at lower substrate moisture content might have resulted from desiccation of pupating larvae or decreased ability of newly eclosed adults to work their way through the drier substrate. Turpin and Peters (1971) found that larvae of another chrysomelid, western corn rootworm (*Diabrotica virgifera virgifera* LeConte), were more likely to desiccate after exposure to sand than to

clay, presumably because of damage to the cuticle by sharp edges of the sand grains. Rickelmann and Bach (1991) found that adult ecdysis of another chrysomelid, *Altica subplicata* LeConte, was higher on moist or wet sand than on dry sand. Although it is not possible to equate the moisture conditions of their experiment with ours, the trend is similar.

Our measurements of ability of *P. viburni* to penetrate soil are no doubt conservative because soil structure was totally disrupted by the process of sieving the soil and hydrating it in the process of setting up the pupation bioassay. Soils in the field have many more openings to facilitate insect movement; these include cracks in the soil surface and pores in the soil made by plant roots and soil invertebrates (Gustin and Schumacher 1989). Gustin and Schumacher (1989) found that first-instar larvae of *D. virgifera virgifera* were not able to penetrate compacted soils unless artificial pores at least as large as the width of the head capsule were created in the soil. Third-instar larvae of *P. viburni* are likely more capable of burrowing through the substrate than first-instar *D. virgifera virgifera* because of their larger size.

Heterorhabditis bacteriophora was a very effective mortality agent against pupating larvae. Mortality, as reflected in the number of adults emerging from the bioassay containers relative to the number of starting larvae, was 100% when *H. bacteriophora* was applied before larvae pupated and averaged $92.0 \pm 8.4\%$ (SD) when applied after larvae had burrowed into the substrate to pupate (Fig. 3). *S. carpocapsae* was numerically slightly less effective than *H. bacteriophora* at both application timings, but these differences were not significant. Overall, both the treatment and timing were significant ($F = 48.9$; $df = 2, 24$; $P < 0.0001$ and $F = 6.04$; $df = 1, 24$; $P = 0.022$, respectively), but the interaction was not ($F = 0.75$; $df = 2, 24$; $P = 0.48$). Control mortality averaged $37.0 \pm 18.3\%$. The higher mortality resulting from the application of nematodes before larval burrowing/pupation likely resulted from more intimate contact of nematodes with the insects (larvae that had already pupariated when nematodes were applied were apparently protected to some extent from contact with the nematodes by the puparium). To ensure maximum efficacy of nematodes against *P. viburni*, application should be made no later than the time larvae begin to leave shrubs and seek pupation sites in the soil.

Although pupae are protected to a large extent by their location in the soil profile, the larval/adult transition is a potential control point in the life cycle of *P. viburni*. Biocontrol agents that require elevated relative humidity (e.g., fungi such as *B. bassiana*) or free moisture (e.g., nematodes such as *H. bacteriophora*) might be effective in suppressing populations of *P. viburni* if applied to the soil before movement of fully grown larvae into the soil, provided that the soil remains fairly moist. We have observed great efficacy of *B. bassiana* against larvae of *P. viburni* in laboratory bioassays, but virtually none in field trials when applied to foliage (unpublished data). This difference in efficacy can likely be explained by the difference in relative humidity in the two environments; the labo-

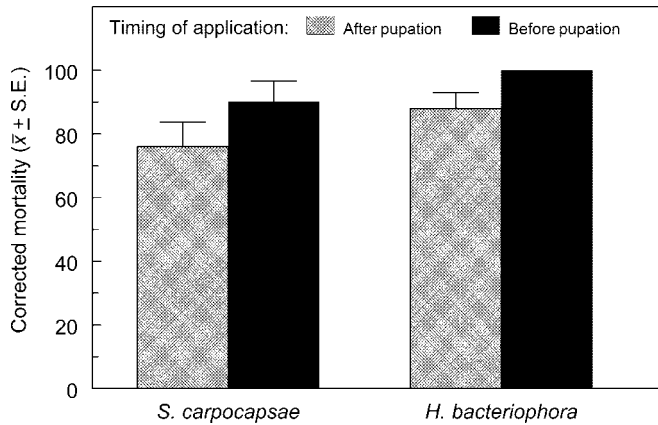


Fig. 3. Efficacy of *H. bacteriophora* and *S. carpocapsae* against pupating larvae of *P. viburni* in laboratory bioassay. Mortality values shown were corrected for control mortality with Abbott's formula.

ratory trial was conducted in closed petri dishes, whereas the field applications were made to foliage on plants, where relative humidity was much lower. The viability of the fungal propagules would be expected to be much higher in soil. Nematodes might also be effective biocontrol agents against *P. viburni* in the field unless soil moisture becomes very low.

Manipulating soil moisture is not likely to serve as a management tactic for *P. viburni*. Although allowing the soil surface to dry out under viburnums in advance of larval migration to the soil might decrease pupation success of *P. viburni*, doing so is also likely to decrease the efficacy of nematodes or soil-inhabiting entomopathogens that might inhabit the soil. These soil-borne mortality agents seem to offer greater potential for controlling *P. viburni* populations than manipulating soil moisture; the decrease in adult eclosion was achieved by decreasing soil moisture from 75 to 25% of holding capacity decreased rates of adult eclosion by roughly 50%, whereas soil-borne agents apparently resulted in very high rates of mortality between the larval and adult stages, judging from the results of the nematode trials reported here and the near absence of adults in some summers after much wetter-than-average springs with large larval populations in both New York State and in the native range of *P. viburni* (personal observation). *H. bacteriophora* may be a more suitable biological control candidate because it moves more deeply in the soil than *S. carpocapsae* (Kaya and Gaugler 1993), which would likely buffer it from hot, dry conditions typical near the soil surface at the time that larvae of *P. viburni* are starting to pupate.

For control methods aimed at the larval-to-adult transition to be effective, they would have to be applied to a rather large area because of the mobility of adults; the efficacy of such tactics would be diminished if large numbers of adults simply immigrate from infested plants on nearby properties.

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