REPORT

Developing the seed-feeding weevil *Mogulones borraginis* as a biological control agent for houndstongue (*Cynoglossum officinale*)

For Wyoming Weed & Pest Council

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Executive summary

1. During 2011, we conducted oogenesis tests at the University of Idaho with native congeneres of houndstongue in which, similar to tests at CABI, only weevils fed with houndstongue produced viable eggs.

2. We have begun to develop devices and techniques that allow us to analyze the attractiveness or repellence of the headspace (the scent) of test plant species to the candidate species *Mogulones borraginis* as part of Mr. Ik Ju Park’s Ph.D. research project and to boost host-specificity data in case TAG should require additional data on the weevil.

3. We have drafted the first 70 pages of the petition for release of *M. borraginis*, entailing all information regarding the weed, its status in North America and environmental effects of the proposed action. We also summarized the host-specificity data but have not yet written the narrative for that data. The petition will have to be submitted no later than July 2012, in order to be considered during that year by the Technical Advisory Group.

4. The rearing of *M. borraginis* was successfully continued at CABI during 2011. More than 200 adults emerged from rearing containers. On 2 May 2011, Mark Schwarzländer hand-carried 52 females and 48 males on a special hand-carrying permit issued by USDA APHIS PPQ from Switzerland back to the quarantine facility in Pullman, Washington to conduct additional host-specificity tests. The remaining weevils at CABI were used to maintain the rearing colony. More than 2,500 mature *M. borraginis* larvae were reared on 80 houndstongue plants during the summer of 2011. The larvae are currently being overwintered for adult emergence in March 2012. Unexpectedly, many weevils already started to emerge in autumn 2011, probably due to exceptionally warm autumn temperatures. Mark Schwarzländer therefore hand-carried another 250 females and 100 males back to the U.S.

5. We repeated an experiment in which we overwintered weevils at six different temperatures during the 2010/11 winter. Although we took care to lower temperature gradually and we adapted temperatures, again, weevils emerged only from containers with larvae placed at temperatures at or above 0 °C. Since we had enough weevils available from our rearing colony, we set up an additional experiment in fall 2011 with temperatures just below zero, and not at -5 °C as before. Results will be available in spring 2012.

6. Because the native *Cynoglossum grande* again started flowering very early in spring, and no flowering houndstongue plants were available at that point, we established additional “oogenesis” tests, which test whether female weevils can develop viable eggs when exclusively fed with foliage of a given plant species. Results clearly confirmed that *M. borraginis* females are unable to develop mature eggs when only feeding on *C. grande*. 
Background

*Mogulones borraginis* (F.) (Coleoptera, Curculionidae) is a host-specific and effective candidate agent for the biological control of houndstongue in the United States. The weevil is a seed-feeder with the potential to reach outbreak densities, thus capable to severely impair houndstongue plant growth and reproduction. The seeds of houndstongue present the greatest problem for land managers since they are randomly dispersed by livestock, wildlife and by anthropogenic means. A biological control agent that would attack and reduce flower and the fruit production of houndstongue would directly contribute to the management of the noxious weed.

Of 79 plant species in the Boraginaceae family (28 native North American) tested between 1993 and 2010 in single-choice oviposition tests, only seven species in the genera *Cynoglossum* and *Lappula* were accepted (to some extent) as hosts. In no-choice and multiple-choice development tests with 17 test plant species, mature larvae were only retrieved from three European *Cynoglossum* species and from houndstongue. *M. borraginis* females did, however, lay a total of 3 eggs on the native *C. grande* during single-choice oviposition tests. Unfortunately, no larval development tests could be conducted for the two native North American congeneres of houndstongue, *Cynoglossum grande* and *C. occidentale* and the native North American *Hackelia californica*, because of difficulties to procure plants and/or to advance *C. grande*, *C. occidentale* and *H. californica* plants under greenhouse conditions to the flowering and fruiting stage. However, tests with these three species are mandatory in order to submit a petition for field release of *M. borraginis* in North America to USDA APHIS and the Technical Advisory Group (TAG), the two entities charged with the evaluation and approval of these petitions.

During the past years, field populations of all three species have been identified in Washington and Oregon and methods to collect, transfer, and grow plants in greenhouses have been steadily improved to the point that we can produce flowering plants of these species under greenhouse conditions at the University of Idaho and CABI Europe - Switzerland. The native North American relatives of houndstongue are sensitive, clonal, tap-rooted plants that are difficult to transplant. Further, we do not know of any laboratory that has succeeded growing these plants from seed.

We believe that we have found methods to translocate these plants from the field and still allow them to produce fruits in a greenhouse setting and/or controlled quarantine facility at the time *M. borraginis* lays its eggs during the second half of May. However, it is difficult to synchronize the flowering time of the three plant species with the oviposition to that period. While *C. grande* typically flowers in March, both *C. occidentale* and *H. californica* do usually not flower before end of June, while houndstongue typically flowers in May. In order to maximize the probability to successfully conduct the remaining host-specificity tests with the three plant species, the University of Idaho has in the past distributed plant material to CABI Europe - Switzerland. This is to facilitate the successful conduct of the remaining host-specificity tests at least at one of two locations. While we have conducted tests with some of the three aforementioned plant species since 2008, a petition to for field release of the seed feeding weevil has been prepared in a parallel effort and will be submitted to TAG this year. At the same time we are preparing to expand the North American rearing efforts for the seed weevil.
1 Tests conducted at the University of Idaho

1.1 M. borraginis host-specificity tests at the Northwestern Biocontrol Insectary and Quarantine (NWBIQ)

The focus during 2011 was to perform host location behavior bioassays with native confamilials of houndstongue. We were especially interested to determine whether Cynoglossum occidentale is a suitable host plant for M. borraginis and whether the weevil would be attracted to this plant species. We received a total of 100 M. borraginis from CABI-Switzerland (see below). We set up 94 weevils in groups of eight weevils (4 males, 4 females) in 12 plastic cylinders (diameter: 10 cm, height: 26 cm) and fed the weevils with houndstongue rosette leaves, flowers and fruits. We conducted two types of tests during 2011: 1) Dual-choice D-SYD bioassays with C. occidentale between 29 June and 15 July; and 2) Dual-choice D-SYD bioassays with Plagiobothrys hirtus between 29 June and 6 July.

Dual-choice D-SYD bioassays with C. occidentale

Methods On 24 June, a portable volatile collection system (PVCS: see 2.2) was operated to collect headspace volatile organic compounds (VOCs) from five individual C. occidentale for 3 hours in Bend, OR (Plate 3). On 25 June, the same approach was applied to collect VOCs from three individual houndstongue plants in Moscow, ID. The trapped VOCs were eluted with dichlomethane (CH₂Cl₂) to make 200 µl solution and were stored at 4°C. For visual cues, individual flowering stems of C. occidentale and C. officinale were collected in the field and were stored at 4°C. On 29 June, adult M. borraginis were placed into a double stacked y-tube device (D-SYD) to assess host location behaviors (see 2.3 and Plate 3). In the bioassays, we assessed M. borraginis preferences on the apparatus when offered nothing as control, olfactory cues, and visual cues. Also, weevils’ preferences were compared between VOCs from a potted plant and PVCS. For statistical analysis, Chi Square Goodness of Fit test was used.

Results D-SYD itself did not affect the behavioral response of Mogulones borraginis ($\chi^2$ =0.66, n=24) (see Plate 1). However, M. borraginis distinguished between C. officinale and its native North American congener Cynoglossum occidentale when only visual cues were tested ($\chi^2$ =7.00, p<0.001, n=24). Using olfactory cues only, M. borraginis also distinguished C. officinale from its sensitive native congener regardless of whether potted plants or PVCS VOCs were used (location on C. officinale compared to C. occidentale using VOCs from potted plants: $\chi^2$ =15.36, p<0.001, n=44; and eluted VOCs from PVCS: $\chi^2$ =11.56, p<0.001, n=25). Overall, M. borraginis highly prefers to locate on C. officinale compare to C. occidentale (Plate 1).
Plate 1 Results of dual choice D-SYD bioassays with *C. occidentale*. In visual cues, a flowering stem from *C. occidentale* and *C. officinale* was offered in D-SYD and 75% of adult *M. borraginis* preferred to locate on *C. officinale*. In olfactory cues, adult *M. borraginis* preferred to locate on *C. officinale* in all comparisons. *** denotes a significant difference of *M. borraginis* location between two arms in D-SYD at $P < 0.001$ (Chi Square Goodness of Fit test, SAS) and ns denotes not significant. Grey represents negative control (nothing offered).

**Dual-choice D-SYD bioassays with *P. hirtus***

Methods. On 29 June, adult *M. borraginis* were placed into D-SYD to assess host location behaviors (See 2.3). In the bioassays, we assessed *M. borraginis* preference in response to given visual cues only.

Results. D-SYD itself did not affect the behavioral response of *Mogulones borraginis* ($\chi^2 = 0.66^{ns}$, n=24) (see Plate 2). In contrast, all *M. borraginis* distinguished between *C. officinale* and native North American T&E species *Plagiobothrys hirtus* when only visual cues were tested ($\chi^2 = 7.00$, $p<0.001$, n=24). As expected, results were even more pronounced as for *C. occidentale*. These results support the previous single-choice oviposition test data, where no eggs were found on *P. hirtus* (Plate 2).
Plate 2 Results of dual choice D-SYD bioassays with *P. hirtus*. A flowering stem from *Plagiobothrys hirtus* and *C. officinale* was offered in D-SYD and none of adult *M. borraginis* located on *P. hirtus*. *** denotes a significant difference of *M. borraginis* location between two arms in D-SYD at *P* < 0.001 (Chi Square Goodness of Fit test, SAS) and ns denotes not significant. Grey represents negative control (offered nothing).

### 1.2 Development of Portable Volatile Collection System (PVCS)

PVCS consists of three major parts: a polyvinyl acetate bag, a push-pull pump, and two pairs of push-pull flowmeters (Plate 3). We chose a polyvinyl acetate bag for floral VOC collections because it was the most commonly used material (Steward-Jones and Poppy, 2006). Using a vacuum sealer, it is possible to make various sizes of a polyvinyl acetate bag. Also, carrying and using glass can be complicated especially under field conditions. A Rena Air 400 pump (Mars Inc. Hackettstown, NJ) was modified to create a push-pull pump by switching the direction of diaphragms within the pump assemblage. Then, two pairs of flowmeters were connected to the outlets of the pump via a Teflon® Y-splitter. Pure air, filtered by an activated charcoal filter, entered a polyvinyl acetate bag while volatile was trapped in Toyopearl Super-Q adsorbent resin (Tosoh Bioscience LLC, King of Prussia, PA). Floral headspace VOCs with control were collected for 3 hours at 300 ml per minute in the field. The trapped VOCs were eluted with dichlomethane to make 200 µl solution and will be stored at 4°C until further use. Heat and humidity in a glass chamber or a polyvinyl acetate bag would increase in a static headspace volatile system (Tholl et al, 2006). Alternatively, in our dynamic headspace volatile system, the push-pull pump maintains constant air flow between air outlet and inlet in the enclosed polyvinyl acetate bag, and thus sensitive T&E plant will endure less stress during the 3-hour VOC collection time.

### 1.3 Development of Double Stacked Y-tube devices (D-SYD)

D-SYD is a portable apparatus for assessing behavioral responses of a weed biological control candidate agent to given olfactory or visual cues or both cues simultaneously (Plate 3). D-SYD consists of two glass y-tubes. For assessing responses to olfactory cues, the two arms of the top glass Y-tube were connected to plastic caps in which a filter paper (2 mm²) was placed for injection of 2 µl of eluted VOCs from PVCS for behavioral bioassays. Each plastic cap was connected to a push-push pump via Tygon tube (diameter: 3 mm, length: 450 mm, Murdock Industrial Inc. Akron, OH). For assessing visual cues, the identical size bottom glass Y-tube was placed under the top Y-tube. Fresh leaves and/or flowers of test plants were placed in one arm and the control or a standardized shape of leaves/flowers in the other arm. For each test
plant species, *M. borraginis* weevils were placed on the tip of the bottom part of the top glass Y-tubes and were monitored for 5 minutes. For VOCs in the top Y-tube flow rate was kept at 10 ml per minute. The D-SYD was covered with a white plastic dome (40 by 30 by 20 cm) to prevent any potential distraction. A single LED light source was used and was the sole light source in the laboratory during trials. The top glass Y-tube was cleaned using 70% ethanol every two trials and it was rotated 180 degrees every five trials to prevent potential false negative/positive results. If a weevil did not reach the decision line (5 cm from the bifurcation point in each arm within the top glass Y-tube) after 5 minutes, it will be recorded as non-responding individual (NI).

Plate 3 Left: three portable volatile collection systems (PVCS) were directly connected to Duracell DPP-600HD powerpack for collecting floral VOCs from five *C. occidentale* with one control (empty) in Bend, OR as a field demonstration; Right: a double stacked y-tube device (D-SYD) for assessing behavioral responses of *M. borraginis* to olfactory-, visual- or both cues simultaneously. The green arrow indicates the initial release point of adult *M. borraginis* during bioassays.

2 Tests conducted at CABI Europe-Switzerland

2.1 Rearing and shipments

In spring 2011, 231 *M. borraginis* adults emerged from our rearing colony and the overwintering experiment established in 2010. Survival of weevils kept in our underground insectary was 65.1%. Of the 91 weevils that had emerged prematurely in autumn 2010 and that were overwintered in cylinders, only 26 survived until spring 2011. A total of 142 weevils (79 females and 56 males) were placed into cylinders and kept at 5 °C to delay egg laying, while 105 were placed on potted houndstongue rosettes (n=21).

On 2 May, Mark Schwarzländer (University of Idaho) hand-carried 52 females and 48 males (half from plants and half from cylinders) of *M. borraginis* back to the quarantine facility in Pullmann, Washington to conduct additional host-specificity tests.

Since we had problems getting houndstongue to germinate in 2010, and we had no reproducing plants available in spring 2011, plants were collected from the Rhine Valley, Germany, and an additional 100 houndstongue plants were sent to us by Bradley Harmon (University of Idaho). To continue the rearing of *M. borraginis*, between 1-5 females and 1-3 males were released
onto individually potted, gauze-covered houndstongue plants. Once these plants started to reproduce, and eggs were found, weevils were retrieved and placed onto new flowering plants. In total, 80 rearing plants were set up. On 28 June, we started to attach vials to the rearing plants to collect mature larvae leaving the seeds. Between 1 July and 5 August, over 2,500 *M. borraginis* larvae emerged. Larvae (n=10 or 25) were transferred into plastic cups (6.5cm diameter, 8cm height) filled with sifted soil for pupation. Vials were placed in an underground shelter for adult emergence in spring 2012 or were used in an additional overwintering experiment (see below).

### 2.2 Influence of temperature on overwintering survival

In October 2010, we had established an additional overwintering experiment with *M. borraginis*, choosing temperatures that simulated conditions in the winter of 2009-10 in Wyoming. Temperature treatments used were: 1) constant 0°C, 2) constant -5°C, 3) constant -5°C and one week at -10°C, 4) constant -5°C and two weeks at -10°C, 5) constant -5°C and three weeks at -10°C, and 6) constant -5°C and four weeks at -10°C. At the beginning of October 2010, 60 cups with ten *M. borraginis* each, were placed in an incubator that was gradually lowered to 0 °C. Thereafter, ten cups were randomly assigned to each of the six treatments and placed into incubators accordingly. For the -5°C treatment, temperature was again gradually lowered, while for the -10°C treatments, cups were directly placed from -5 to -10°C. Unfortunately, successful emergence was only observed for cups placed at 0 °C (78.6%).

Since many larvae emerged from our rearing colony in summer 2011 (see above), we decided to try overwintering at different temperatures one more time. In order to determine at which permanent overwintering temperatures between 0 and -5 °C, *M. borraginis* is able to survive, three temperatures were chosen: -0.5, -1.0 and -1.5 °C. At the beginning of November 2011, 30 cups with 9-10 *M. borraginis* each, were placed in an incubator at 0 °C for 11 days. Thereafter, ten cups were randomly assigned to each of the three treatments and placed into incubators accordingly. At the beginning of March 2011, i.e. at a time when *M. borraginis* starts to emerge naturally, we will retrieve all cups and record adult emergence.

### 2.3 Host-specificity tests

Previous tests have shown that *M. borraginis* does accept *C. grande* to a certain extent for egg laying under no-choice conditions, and that this non-target plant is able to support larval development. Several single-choice tests, i.e. exposing *C. grande* together with *C. officinale* showed that *M. borraginis* clearly prefers the latter.

Because no reproducing houndstongue plants were available (see above) at the time the native North American *Cynoglossum grande* started flowering, we established another oogenesis test to see whether females would be able to produce mature eggs when only fed with *C. grande*. On 8 and 22 March 2011, newly emerged *M. borraginis* (14 females and 11 males) were placed onto five flowering *C. grande* plants. When no flowers were left, weevils were retrieved and placed onto new flowering plants. In mid April, the first fruits were produced, and weevils were left on these reproducing *C. grande* plants until fruits were mature. Weevils were then retrieved, all fruits were dissected and females were killed to check their ovariole development. No eggs were found in the 53 *C. grande* fruits, and no mature eggs or ingested plant material was found in any of the eight females dissected. In fact, ovariole structures were not visible anymore, and females may have absorbed a good part of their reproductive system
due to the unsuitability of *C. grande* as a food source.

3. **Outlook and conclusions**

The University of Idaho is in possession of larger quantities of potted *C. grande*, *C. occidentale*, and *H. californica* plants. We also just received seeds of a number of USFWS listed T&E Boraginaceae species and will continue to acquire additional plants from native plant nurseries. While the NWBIQ is not a perfect facility to conduct tests, it does provide space to work with non-permitted organisms and there is a sufficiently large number of plant light fixtures to set up host-specificity tests. In addition, CABI Europe – Switzerland has and can continue to provide us with sufficient numbers of *M. borraginis* adults. *M. borraginis* is a sensitive insect that stops laying eggs when fed incorrectly throughout the spring or when exposed to continuous artificial light conditions. Nonetheless, we have slowly overcome these hurdles during the past 3 years and although we are determined to continue tests and expand our data set, we think that our currently existing data set suffices to submit a petition for field release of the weevil in the U.S.

4. **Work planned for 2012**

*Work planned at UI*
- Setup quarantine rearing colony at NWBIQ;
- Increase number of replicates for single-choice oviposition tests with *C. occidentale* and *H. californica*;
- Conduct additional oogenesis tests with *C. occidentale* and *H. californica*;
- Conduct GC-EAD/FID and olfactometer tests with *C. grande*, *C. occidentale* and *Hackelia californica*;
- Optimize methodology to collect headspace volatile from rare conffamilial species in the field;
- Finish host-specificity part of petition for release to TAG and submit petition no later than July;
- Defend petition to TAG during TAG’s annual meeting in October 2012.

*Work planned at CABI*
- Continue rearing at the center;
- Obtain results of overwintering experiment at different temperatures;
- Continue summarizing host-specificity data collected at CABI;
- Assist in preparing the host-specificity section of the petition for field release.

**References**
