The Role of Olfactory and Visual Cues in the Host Finding Behavior of a Near-Monophagous Specialist Insect Herbivore Considered for Biological Control of Weeds

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AUTHORIZATION TO SUBMIT DISSERTATION

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ABSTRACT

Current pre-release testing procedures in biological weed control primarily rely on choice and no-choice development, feeding, and oviposition tests to evaluate the environmental safety of prospective biocontrol agents. Examining behavioral responses of prospective organisms to olfactory and visual cues can improve pre-release risk assessments because these cues mediate host-plant recognition that necessarily precedes feeding and oviposition in the field. I investigated how the seed-feeding weevil *Mogulones borraginis* distinguishes the rangeland weed, *Cynoglossum officinale* from eight confamilial plant species in North America based on olfactory and visual cues in electrophysiological experiments and behavioral assays. Ten electrophysiologically-active semiochemicals were identified using gas chromatography-electroantennographic detection/flame ionization detection (GC-EAD/FID) with a chiral column and gas chromatography-mass spectrometry (GC-MS). Among them, (-)-α-copaene and (E)-β-farnesene were two sesquiterpenes only found from *C. officinale* among all plant species collected from greenhouse and field conditions. Similarly, four electrophysiologically-active wavelengths of light were identified at 350 nm (ultraviolet), 430 nm (purple), 640 nm (red) and 830 nm (infrared) using electroretinography (ERG) and a photo-radiometer. I designed double stacked y-tube device (D-SYD) and portable volatile collection system (PVCS) for behavioral assays. The results of previous oviposition tests were consistent with the proposed host-finding assays. Weevils clearly distinguished *C. officinale* from each confamilial plant species, notably four federally listed threatened and endangered plant species, by using either floral scents or flowering stems. With the combined cues, *M. borraginis* showed relatively stronger and faster discrimination. However, with

mismatched cues, weevils did not discriminate between two plant species and exhibited the longest searching time in behavioral assays. Further, the relative strength of olfactory and visual cues was equally important in the host selection behavior of *M. borraginis*. Therefore, studies of behavioral responses by biocontrol agents to olfactory and visual cues and underlying electrophysiological mechanisms will advance our understanding of how these agents achieve discrimination among closely related plant species that limit their realized host ranges and reduce potential non-target effects in biological weed control.

DEDICATION

To God and all the people who are surely waiting for me.

"In his heart a man plans his course,

but the LORD determines his steps."

Proverbs 16:9

"사람이 마음으로 자기의 길을 계획할지라도,

그 걸음을 인도하는 자는 여호와시니라."

잠언 16:9

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Introduction to the dissertation

Classical biological control of weeds involves the introduction of biological control organisms deliberately from the native range of invasive plants in order to suppress the population of these plants permanently in the introduced range (McFadyen 1998; Moran & Hoffmann 2015; Seastedt 2015; Van Driesche et al. 2010). Before the decision is made to release prospective biological control organisms into the environment, delineating their host ranges with regard to plant species native to the area of introduction is an essential process in pre-release risk assessments (Smith & Beck 2013). Host specificity tests evaluating the host range of biological control candidate organisms are rigorously conducted as no-choice, choice, field cage or open-field experiments (Clement & Cristofaro 1995; Cullen 1990; Sheppard et al. 2005). However, host specificity solely based on adult feeding, oviposition and larval development as measured in these tests does not explain potential host discrimination of biological control organisms among their hosts (Louda et al. 2003; Marohasy 1998; Rapo 2012; Smith & Beck 2015). Understanding behavioral mechanisms and identifying electrophysiologically-active olfactory and visual cues with regard to the host finding of biological control candidate species may better explain results of conventional host specificity experiments and enhance our ability to better predict realized host ranges of both, prospective and released biological control organisms (Heard 2000; Hinz et al. 2014; Schaffner 2001). Olfactory and visual cues mediate host-plant recognition that necessarily precedes feeding and oviposition in the field (Bernays & Chapman 1994; Miller & Strickler 1984; Raguso 2008) and so should be evaluated as part of host range

tests.

The seed-feeding weevil, *Mogulones borraginis* F. (Coleoptera: Coleoptera) is a prospective biological control organism for *Cynoglossum officinale* L., a Eurasian monocarpic short-lived perennial in the Boraginaceae family, which is an invasive plant and noxious weed in northwestern North America (Schwarzländer et al. 1998). Adult weevils exclusively feed on florescence and developing fruits of *C. officinal*e in their native range. The plant is the only known host plant of *M. borraginis* (Dieckmann 1972; Freude et al. 1983; Koch 1992). To ensure the environmental safety of *M. borraginis* in North America, two types of experiments have been conducted at CABI in Switzerland and the University of Idaho: 1) traditional host specificity tests, including no-choice, choice, and field cage oviposition and larval development tests, and 2) seed-volume measurements in combination with food requirement tests to complete larval development to assess the minimum seed volume for a single *M. borraginis* larva to survive on a plant species (Hinz et al. 2003). Critical North American test plant species that exceeded the threshold seed volume for larval survival were *Adelinia grande* (Douglas ex Lehm.) J. I. Cohen (= *Cynoglossum grande*), *Andersonglossum occidentale* (A. Gray) J. I. Cohen (= *Cynoglossum occidentale*) and *Hackelia californica* (A. Gray) I.M. Johnston. In addition to these nontargets, federally listed threatened and endangered (T&E) plant species were chosen for investigation in order to circumvent problems using so called surrogate species often used for host specificity tests in lieu of T&E species (Colpetzer et al. 2004; Grevstad et al. 2013). The overarching goal of this dissertation is the development of behavioral bioassays and electrophysiological methods that can be incorporated in host range assessments of biological weed control candidates to improve

predictions of and the realized host range and therefore, the potential for non-target impacts during pre-release investigations.

In the first chapter of this dissertation, I describe a simple and cost-effective methodology to evaluate behavioral responses of insects to olfactory and visual plant cues in assays. I designed and tested the functionality of two devices that were constructed based upon a recent review by Knolhoff and Heckel (2014), who emphasized that behavioral bioassays for phytophagous insects should mimic natural conditions as closely as possible. I developed a portable volatile collection system (PVCS) that allows non-destructive collection of greenhouse-propagated or more importantly field-collected volatile compounds. In addition, I tested whether a double-stacked y-tube device (D-SYD) would affect behavioral responses of biological control organisms in the host-finding assays. We used these two devices to test how a rare seed-feeding weevil, *M. borraginis* exploits olfactory and visual cues from its Eurasian field host, *Cynoglossum officinale* L. and the native North American *Andersonglossum occidentale* Cohen during initial host finding.

In the second chapter, I further investigate the host range of *M. borraginis* non-destructively with regard to rare and federally listed threatened and endangered (T&E) confamilial plant species instead of using surrogate confamilial plant species that mimic traits of those T&E species in host-finding bioassays. I chose *M. borraginis* and its host *C. officinale* as a model system to test four T&E plant species using plant cues collected from plants in their natural habitats and from greenhouse propagated plants, including *Amsinckia grandiflora* (Kleeb. ex A. Gray) Kleeb. ex Greene*, Hackelia venusta* (Piper) H. St. John*, Plagiobothrys hirtus* (Greene) I.M. Johnston*,* and *P. strictus* (Greene) I.M.

Johnston. In addition, I included *Dasynotus daubenmirei* I.M. Johnston, a single-population, single location confamilial North American species. I hypothesized that 1) host-finding bioassay results are consistent with results from previous host range tests; 2) female weevils distinguish *C. officinale* from the nontarget plant species based on olfactory and visual cues; 3) in the absence of *C. officinale* plant cues, female weevils are repelled by olfactory and visual cues from the T&E species; and that 4) the behavioral responses of weevils correlate well with electrophysiologically-active volatile profiles of each plant species used in bioassays. Although the species was not tested, I used the findings from these experiments to predict the likelihood of nontarget impact on the remaining confamilial T&E plant species, *Oreocarya crassipes* (I.M. Johnston) Hasenstab & M.G. Simpson.

In the third chapter of this dissertation, I used a similar approach as in the second chapter, but I focused on potential behavioral mechanisms that could explain bioassay responses of female *M. borraginis* to three confamilial native North American plant species that exceed the minimal fruit volume threshold for *M. borraginis* larval development: *A. grande, A. occidentale,* and *H. californica*. Among them, *A. grande* and *A. occidentale* are of particular interest because they are very closely related to C. offiincale, having been previously classified as its North American congeners until a recent phylogenetic revision of the Boraginaceae (Chacón et al. 2016; Cohen 2014, 2015). I hypothesized that 1) inflorescences of *C. officinale* exhibit a unique combination of electrophysiologically-active olfactory and visual cues, and that 2) female *M. borraginis* utilize this plant cue combination cues to distinguish *C. officinale* from phylogenetically closely related plant species.

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Chapter 1. A simple approach to evaluate behavioral responses of insect herbivores to olfactory and visual cues simultaneously: the double stacked y-tube device and portable volatile collection system

Abstract

1. Preceding host feeding and oviposition, herbivorous specialist insects use olfactory and visual cues during host plant finding. Typically, these cues are assessed separately in bioassays, disregarding that in nature an insect herbivore perceives and responds to both cues simultaneously.

2. Here, we demonstrate the functionality of two simple devices that we designed; a double-stacked y-tube device (D-SYD) and a portable volatile collection system (PVCS), to test how a classical biocontrol weed candidate uses visual and olfactory cues to discriminate potential host plants. In dual-modality bioassays, we tested the host finding behavior of the Eurasian specialist seed-feeding weevil, *Mogulones borraginis,* on its Eurasian field host, *Cynoglossum officinale,* which is invasive in North America*,* and a closely related confamilial native North American nontarget, *Andersonglossum occidentale.*

3. Weevils clearly distinguished *C. officinale* from *A. occidentale* based on floral scents or flowering stems. When both cues were offered simultaneously, weevils responded relatively stronger and faster compared to either single cue. When cues were mismatched, weevils were no longer able to discriminate between the two plant species and exhibited longer response times. There were no differences in behavioral responses to floral scents

from greenhouse propagated plants and those collected from the respective plants in the field using the PVCS.

4. The simple bioassay approach proposed here using D-SYD and PVCS can be applied to address theoretical and applied questions concerning the host selection behavior of insect herbivores. It can assess behavioral responses of insects to olfactory and visual plant cues alone or together and measure their relative strengths and potential synergisms.

Introduction

During host selection, herbivorous insects respond to plant cues from multiple sensory modalities (Chittka & Raine 2006; Prokopy 1986; Raguso 2008). Before landing on a potential host (i.e., during the 'finding' phase of host selection) only olfactory and visual cues are accessible (Bernays & Chapman 1994; Miller & Strickler 1984). Much has been learned about how each of these two modalities functions during host selection by considering them individually (Bruce & Pickett 2011; Reeves 2011). Between the two, olfactory cues have been more studied, likely due to practical experimentation reasons (Bruce & Pickett 2011), but visual cues can be at least as important (Jonsson et al. 2007) (Otalora-Luna et al. 2013; Stenberg & Ericson 2007). A fuller understanding of host finding mechanisms, however, requires assessing responses to olfactory and visual cues in combination (bimodal cues) under natural conditions (Leonard & Masek 2014). Despite this, there are few studies that examine responses by insect herbivores to visual and olfactory plant cues simultaneously.

Different approaches have been used to examine the separate roles of olfactory and visual cues in insects. For example, plant parts masked with dark perforated fabric

(Raguso & Willis 2005) or hidden in black perforated glass chambers (Burger et al. 2010) have been used to isolate the effects of olfactory cues from visual cues. Alternatively, visual cues can be isolated by covering plant parts with transparent materials that are impervious to volatiles (Raguso & Willis 2005) or placing them in glass chambers (Milet-Pinheiro et al. 2012). Some studies have examined behavioral responses of insects to olfactory and visual plant cues in a Y-tube olfactometer (Graziosi & Rieske 2013; Mainali & Lim 2011). However, this simple Y-tube olfactometer approach is inadequate for testing responses to olfactory and visual cues of two different plant species, which entails manipulating the four separate types of cues.

In addition to the challenge of evaluating bimodal cues, it is also important to provide cues that resemble natural conditions as closely as possible (Knolhoff & Heckel 2014). For example, in order to release a potential biocontrol agent in the USA, it is also necessary to determine the environmental safety of confamilial federally-listed threatened and endangered (T&E) plant species of the targeted weedy plant species. In our study system this includes five T&E species, including *Hackelia venusta* (Piper) H. St. John, which remains as a single population in the Cascade Mountain Range in Washington State. Collecting visual and olfactory cues directly from the natural habitat aids behavioral bioassays since it is known that plant VOC emissions are affected by abiotic and biotic environmental factors, but these nonetheless are typically not collected *in situ* for logistical and technical reasons such as the weight of collection devices or potential condensation (Kallenbach et al. 2014; Tholl et al. 2006).

Here we describe the design and functionality of two simple devices that were constructed for host finding bioassays to address the challenge of testing bimodal cues

and ensuring that cues used represented natural plant cues as closely as possible. These are a portable volatile collection system (PVCS), which allows simultaneous collection of multiple floral scents from plants growing in their natural habitat in a nondestructive fashion and a double-stacked y-tube device (hereafter D-SYD), which can simultaneously compare olfactory and visual cues from two different plant species (a total of 4 separate cues). Additionally, D-SYD is designed to evaluate two modalities (olfactory and visual cues) simultaneously during host finding of insects. We tested the functionality of both devices in the context of an applied biological weed control system. We conducted bioassays in which we assessed how a Eurasian seed-feeding weevil, *Mogulones borraginis* (F.), exploits olfactory and visual cues from two plant species during its initial host recognition. The two plant species are its Eurasian field host *Cynoglossum officinale* L., which is invasive in North America, and a phylogenetically closely related native North American non-target species, *Andersonglossum occidentale* (A. Gray) J. I. Cohen.

Materials and methods

Study system

Houndstongue, *Cynoglossum officinale,* is a monocarpic, biennial to short-lived, perennial herbaceous plant in the Boraginaceae family that is native to Europe and Asia Minor. It typically forms rosettes in the first year and flowers and sets seeds during the second or third year depending on plant size (de Jong et al. 1990; Upadhyaya et al. 1988b). The plant was accidently introduced into North America in the mid-19th century and has since spread throughout most of the continental United States (NRCS 2017). It is an invasive of rangelands, open woodlands and disturbed areas and a declared noxious

weed in seven northwestern states in the United States and the Canadian provinces of Alberta and British Columbia (NRCS 2017).

A close relative of *C. officinale,* the native North American clonal perennial *Andersonglossum occidentale* (Cohen 2015), was used as an non-target test plant species for behavioral bioassays. Previously considered a congener of *C. officinale, A. occidentale* is now placed in the subtribe Amsinckiinae while *C. officinale* belongs to the subtribe Cynoglossinae (Chacón et al. 2016). The plant, which is sparsely distributed at few location in California and Oregon (NRCS 2017), has fruits sufficiently large to theoretically support larval survival of *M. borraginis* (Hinz et al. 2003).

The seed-feeding weevil, *M. borraginis* is being investigated as a biocontrol candidate for *C. officinale* in the United States. This univoltine weevil is endangered in its native central European range where it is exclusively found on *C. officinale* (Koch 1992). Weevils emerge in spring, begin to feed on *C. officinale* foliage and later on developing buds and flowers (Hinz et al. 2003). Female *M. borraginis* require feeding on pollen and inflorescences of *C. officinale* for successful oogenesis (Hinz et al. 2003). Females lay eggs into maturing seeds of *C. officinale* and larvae feed on the developing seeds for four weeks in early summer before pupating in the soil.

Mogulones borraginis is not permitted for release in the United States, and consequently, all experiments were conducted in a quarantine laboratory at Washington State University, Pullman, WA, USA. Neonate laboratory-reared *M. borraginis* were shipped to the quarantine laboratory from CABI Switzerland in Delémont, Switzerland during the winter of 2011 and early spring of 2012 (n=400 females and 250 males, respectively). Upon receipt, 15 females and five males were placed in a transparent

plastic cylinder (10 cm diameter; 16 cm height) with a mesh-covered lid. Fresh foliage and buds of *C. officinale* were provided to *M. borraginis* twice a week. All cylinders were maintained in an environmental chamber (E-30B, Percival Scientific, Perry, IA, USA) for approximately one month under L18: D6, at 20 °C, and 60 % relative humidity (RH).

Plant material

Rootstocks of *Cynoglossum officinale* were collected from a local population at Idler's Rest Nature Preserve, Moscow, ID (N 46.804160°, W 116.948554°) in March of 2011 and 2012, respectively, just before the time of bolting (*n*=50). The rootstocks were placed in 11.3-L black plastic pots (T-pot Three, Stuewe and Sons, Inc, Tangent, OR, USA) with a standard horticultural soil mix as the propagation medium (Sunshine Mix two, Sun Gro Horticulture, Agawam, MA, USA) and maintained at the University of Idaho's H.C. Manis Entomological Laboratory in Moscow, ID (Manis Lab). Rootstocks of *A. occidentale* were collected in the Deschutes National Forest near Camp Sherman, OR (N 44.47011°, W 121.6282°) in June 2011 (*n*=20). Immediately following the collection, a rooting hormone (10 mg per a rootstock, Schultz TakeRoot, Schultz Company, Bridgeton, MO, USA) was applied to the end of the taproots of rootstocks. The procedure was the same as for *A. occidentale* except field soil was used instead of potting mix because prior work showed potting soil to be an inadequate medium for this *A. occidentale* (Schwarzländer unpubl. data). During the growing season (between March and October), all plants were maintained in an environmentally controlled greenhouse. Photoperiods were ambient and temperatures were maintained near ambient but

excluding extreme heat (> 35 °C) or cold (< 4 °C). Plants were watered as needed. During the remainder of the year (October to March) all plants were vernalized and held in a cold room at the Manis Lab at 4 °C. Boraginaceae species flower sequentially along cymes. Thus, buds, open flowers, and young fruits are present simultaneously on individual plants between April and June. The flowering and young fruit phenostages are used for oogenesis and oviposition by *M. borraginis* (Hinz et al. 2003) and were used for all experiments reported here.

Collection of floral volatile organic compounds (VOCs) and visual cues

As mentioned above, one objective of this research was the development of a nondestructive method to collect olfactory cues of respective plant species for testing because material of T&E species is hard to obtain and plants may be hard to propagate. After receiving permission from USDA Forest Service to collect scents from natural populations of *A. occidentale*, a portable volatile collection system (PVCS) was constructed to collect headspace volatile organic compounds (VOCs) from plants with minimal disturbance to the field site and study plants (Fig. 1.1). Polyvinyl acetate bags (14 cm x 24 cm; Reynolds, Richmond, VA, USA) were purged for volatile contaminants in a drying oven at 140 °C for one hour.

Flowering stems of *C. officinale* and *A. occidentale* were covered with a bag and tied by using purged cotton balls and a cable tie. Opposite sides of the bag had a 1 cm slit cut for an inlet port attached to an activated charcoal filter (ORBO^{TM} 32, Supelco Inc, Bellefonte, PA, USA) and an outlet port attached to a volatile collection trap (30 mg Porapak Q; Southern Scientific Inc., Gainesville, FL, USA in a glass pipette) (Fig. 1.1).

The volatile collection traps were washed with methylene chloride (10ml; EMD Millipore, Billerica, Ma, USA) and wrapped in aluminum foil for an hour at 140 °C.A Rena Air 400 pump (RENA, Chalfont, PA, USA), which was plugged into a Duracell battery Powerpack 600 (Duracell, Bethel, CT, USA), produces an airflow of input and output at 300ml/min, Compared to other volatile collection methods (Tholl et al. 2006), the PVCS allows collecting multiple VOC samples efficiently and simultaneously, reducing time requirements and the impact on plants (Fig. 1.1).

VOCs were collected from five individual plants (0.5 to 1 m apart) for each plant species with the control (an empty bag and charcoal prefilter) for three hours on a sunny day. After trapping for 180 minutes, the volatile collection traps were eluted with 200 µl of methyl chloride. Elutant was placed in a screw cap and stored in a portable cooler during transportation and refrigerated at 4 °C until further use. Following the VOC collection, individual flowering stem (10 cm) were collected with respective agency permission at field sites and placed into 10 cm transparent aqua-tubes (Syndicate Sales Inc., Kokomo, IN, USA), and transported to be used as visual cues during bioassays.

Behavioral bioassays with D-SYD

To precisely control the simultaneous presentation of the two modalities known to function during host finding (Eigenbrode & Bernays 1997), a double stacked y-tube device (D-SYD) was constructed (Fig. 1.2). D-SYD consists of two glass Y-tubes (4 cm y-stem, 12 cm arms, 2 cm internal diameter) placed one on top of the other. The D-SYD was installed in a darkened room and illuminated by a full spectrum light bulb emitting 350 nm to 850 nm wavelength (ES5M827FS, 27 watt, Home Depot,

Atlanta, GA, USA) diffused through a white polyethylene dome (40 cm x 30 cm x 20 cm), 20 cm above the D-SYD. The upper y-tube was used for olfactory cues and was rinsed with 70% ethanol following each bioassay to prevent potential effects of residual olfactory cues. It was also rotated 180°every five trials to exclude potential right or left arm bias. The flow rates in each arm of the upper Y-tube were kept at a rate of 300 ml/min using calibrated flowmeters (MR3000, Key Instruments, Hatfield, PA, USA) one on each inlet arm.

To assess the response of weevils to VOCs collected from plants at field sites, a 2 mm^2 square filter paper was placed in each arm of the olfactory Y-tube of the D-SYD to a plastic cap (Bel-Art Products 5, Bel-Art Products, Wayne, NJ, USA) (Fig. 1.2). A 1-µl aliquot of eluted VOCs was pipetted on the filter paper using a 10 µl manual syringe (Agilent Technologies, Sydney, Australia). The purified air was pushed from the pump to each arm of D-SYD through a 3 mm diameter Tygon tube (R-3603, Saint-Gobain Corp., Valley Forge, PA, USA). To compare the effects of VOCs collected in the field with those of VOCs emitted from greenhouse-propagated (see experiment two below), potted *A. occidentale* (*n*=2) and *C. officinale* (*n*=4) plants on *M. borraginis*, we followed the same method for floral VOC collections as described for the field sites above, except that the outlets of the polyvinyl acetate bags were directly connected to each arm of the D-SYD. All responses were pooled for analysis since there was no evidence of variation in weevil responses among individual plants.

A total of 30 male and 30 female *M. borraginis* were tested with the individual weevil considered a single replicate for experiments testing either visual or olfactory cues alone. For other experiments, limitations on weevils only allowed us to test 20 females in

each bioassay. Combinations of floral VOCs and flowering stems of *A. occidentale* and *C. officinale* were changed every five trials. For every bioassay, one weevil was placed at the weevil release point in the upper glass y-tube (Fig. 1.2). Weevils that moved 3 cm into one arm of the D-SYD were considered to have made a decision (Tooker et al. 2005). If a weevil did not reach the decision line after 5 min, it was recorded as a non-responding individual and excluded from the analysis. The average response time of a weevil was defined as the time in seconds from initiating movement to reaching the decision line and recorded for every weevil and all bioassays. All experiments were conducted between 9:00 am and 16:00 pm at 20-23 °C room temperature and 50 % RH. Tests with purified air were conducted to confirm that the D-SYD was unbiased.

Experiment one: Visual cues

To investigate weevil responses to visual cues, a 10 cm long flowering stem of *C. officinale* was placed into one arm of the visual (lower) Y-tube of the D-SYD while the other arm remained empty (control). In the second set of bioassays, 10 cm flowering stems of *A. occidentale* and *C. officinale* were simultaneously placed in the two arms of the visual D-SYD Y-tube. Weevils were placed in the upper olfactory Y-tube of the D-SYD to prevent the weevils from perceiving olfactory cues from the flowering stems. There was no airflow in the olfactory Y-tube of the D-SYD to prevent positive anemotaxis (i.e., the tendency of insects to move towards air flow) (Farkas & Shorey 1972).

Experiment two: Olfactory cues

To assess responses to olfactory cues, weevils were presented with a choice between VOCs of *C. officinale* in one arm and purified air in the other arm of the D-SYD. In the second set of bioassays, weevils were presented with a choice between VOCs of *A. occidentale* and *C. officinale*. In addition, to test whether VOCs collected from plants at field sites using the PVCS elicit responses differed from those obtained from greenhouse-propagated plants, we duplicated all bioassays using VOCs collected from *A. occidentale* and *C. officinale* field sites and VOCs from greenhouse propagated potted plants of both species.

Experiment three: Combined olfactory and visual cues

To study the response of *M. borraginis* to visual and olfactory cues combined, both plant cues were placed to weevils in the D-SYD as described above for the individual modalities.

Experiment four: Mismatched bimodal cues

To test the effect of mismatching olfactory and visual cues on the host finding ability of female *M. borraginis*, we paired olfactory cues from *C. officinale* with visual cues from *A. occidentale* in the olfactory and visual arms on one side of the Y-tube arms of the D-SYD and vice versa.

Experiment five: Comparing effects of visual and olfactory cues

To test the relative strength of olfactory and visual host plant cues, respectively on behavioral responses of *M. borraginis,* eluted VOCs of *C. officinale* were placed in one olfactory arm of the D-SYD while a flowering stem of *C. officinale* was placed in the opposite arm of the visual Y-tube of the D-SYD.

Statistical Analysis

Behavioral responses of weevils, specifically the numbers of weevils choosing each arm of the D-SYD in each bioassay, were analyzed assuming a completely random design using a generalized linear model with a binomial distribution and a logit link function. The hypothesis within this model was an expected null ratio of 50:50 between the two arms of the D-SYD for every bioassay conducted. Estimated means were compared across individual behavioral bioassays using single degree-of-freedom contrasts based on likelihood ratio chi-square tests to test for the effect of non-host cues from *A. occidentale* on both sexes of *M. borraginis.* Two by four contingency tables (left and right arms of D-SYD among four bioassays in experiment three: olfactory cues) were used to compare the behavioral response of both sexes between the eluted VOCs from plants in natural stands and a greenhouse. To test whether behavioral responses of female *M. borraginis* differed between bimodal cue bioassays (experiment four) and those with single cues (experiments two & three), a single degree-of-freedom contrast was used. To assess whether behavioral responses of female *M. borraginis* differed to olfactory and visual cues offered simultaneously, as compared to their responses to olfactory or visual cues alone, a single degree of freedom contrast was conducted to compare the behavioral

responses of weevils. We defined a synergistic effect as occurring when the least squares mean of responses to the bimodal stimulus significantly exceeded the sum of the behavioral responses (additive effect) from each olfactory and visual cues (Campbell & Borden 2009). The average response time of weevils in each bioassay was analyzed using a generalized linear mixed model with a Poisson distribution and logarithmic link function. The difference of least squares means was calculated to compare the average response time between D-SYD arms for weevils in each bioassay. All analyses were carried out using SAS version 9.4 (SAS Institute Inc, 2013).

Results

Experiment one: Visual cues

Male and female *M. borraginis* preferred flowering stems of *C. officinale* over empty D-SYD arms (Males: Z=-3.49, P=0.0005; Females: Z=-3.04, P=0.0024; Fig. 1.3a and b, second bars from top). The average response time did not differ regardless of choice (Males: Z=0.40, P=0.6914 and Females: Z=0.73, P=0.4662; Fig. 1.4a and b, second bars from top). Both *M. borraginis* sexes preferred *C. officinale* over *A. occidentale* (Males: Z=-3.49, P=0.0005; Females: Z=-2.13, P=0.0334; Fig. 1.3a and b, third bars from top) when flowering stems of both plant species were offered in the two arms. There was no difference in behavioral responses of weevils between empty visual D-SYD arms or *C. occidentale* as visual cue when a *C. officinale* flowering stem was offered as alternative (Males: Z=0, P=1; Females: Z=0.89, P=0.3737; Fig. 1.3a and b, top bracket). The average response time of male *M. borraginis* did not differ between plant species (Z=-1.42, P=0.1542; Fig. 1.4a, third bars from top), but females needed less time

to decide on flowering stems of *C. officinale* when compared to *A. occidentale* (Z=3.02, *P*=0.0025; Fig. 1.4b, third bars from top).

Experiment two: Olfactory cues

When field-site-collected VOCs were used in bioassays, both male and female *M. borraginis* preferred VOCs of *C. officinale* over empty arms in the D-SYD (Males: Z=-3.61, *P*=0.0003; Females: Z=-3.29, *P*=0.001; Fig. 1.3a and b, fourth bars from top). Male *M. borraginis* required less time to decide on empty arms of the D–SYD compared to *C. officinale* VOCs (Z=-2.06, *P*=0.0398; Fig. 4a, fourth bars from top), but there was no difference in the average response time for females (Z=-0.89, *P*=0.3721; Fig. 1.4b, fourth bars from top). Both male and female *M. borraginis* preferred *C. officinale* over *A. occidentale* VOCs (Males: Z=-3.49, *P*=0.0005; Females: Z=-3.29, *P*=0.001; Fig. 1.3a and b, fifth bars from top). As for the visual cues, there was no difference in the behavioral responses of weevils between purified air and VOCs of *A. occidentale* if *C. officinale* VOCs were offered as the alternative (Males: Z=0.85, *P*=0.3980; Females: Z=0, *P*=1; Fig. 1.3a and b, second bracket from top). The average response time between the VOCs of the two plant species did not differ for males (Z=-0.99, *P*=0.3241; Fig. 1.4a, fifth bars from top), but females needed less time to decide on *C. officinale* VOCs when compared to *A. occidentale* (Z=7.97, *P*=0.0011; Fig. 1.4b, fifth bars from top).

When VOCs from greenhouse-propagated plants were used for the same bioassays, male and female *M. borraginis* reacted similarly. Both sexes preferred VOCs of *C. officinale* over empty control arms in the D-SYD (Males: Z=-3.29, *P*=0.001; Females: Z=-3.49, *P*=0.0005; Fig. 1.3a and b, sixth bars from top of graph). The average

response time did not differ for male *M. borraginis* (Z=-1.04, *P*=0.2993; Fig. 1.4a, sixth bars from top), but females needed less time to decide on the empty arms of the D-SYD compared to those with greenhouse propagated VOCs of *C. officinale* (Z=-3.67, *P*=0.0002; Fig. 4b, sixth bars from top). Both sexes preferred *C. officinale* over *A. occidentale* (Males: Z=-2.45, *P*=0.0143; Females: Z=-3.61, *P*=0.0003; Fig. 1.3a and b, seventh bars from top). Again, the behavioral responses of weevils did not differ between empty arms and VOCs of *A. occidentale* if VOCs of *C. officinale* were the alternative (Males: Z=0.93, *P*=0.3507; Females: Z=-0.40, *P*=0.6885; Fig. 1.3a and b, third bracket from top). The average response time did not differ for males (Z=0.41, *P*=0.6819) or females (Z=1.59, *P*=0.1126; Fig. 1.4 a and b, seventh bars from top).

Behavioral responses of males and females to VOCs did not differ between PVCS field collected volatiles and those collected from greenhouse-propagated plants (Males: χ^2_3=4.69, *P*=0.196; Females: χ^2_3 =0.754, *P*=0.860; Fig. 3a and b, bracket between eluted VOCs and whole plants).

Experiment three: Combined olfactory and visual cues

When olfactory and visual cues of *A. occidentale* and *C. officinale* were provided simultaneously in behavioral assays, female *M. borraginis* preferred *C. officinale* to *A. occidentale* (Z=-2.87, *P*=0.0041; Fig. 1.3c, top bar). *M. borraginis* tended to respond much faster to cues of *C. officinale* compared to the one female that chose *A. occidentale* (Fig. 1.4c, top bar). The response of *M. borraginis* females to combined olfactory and visual cues was different compared to the response to olfactory and visual cues alone

(χ^2_1=4.96, *P*=0.026; *n*=30 for olfactory and visual cues, *n*=20 for combined cues; Fig. 1.3b and c, gray brackets).

Experiment four: Mismatched bimodal cues

There was no difference in the behavioral response of *M. borraginis* females in bioassays where olfactory and visual cues from *C. officinale* and *A. occidentale* were mismatched in the D-SYD (Z=0, *P*=1; Fig. 1.3c, second bar from top). The behavioral responses of females between combined bimodal cues and mismatched cues of *A. occidentale* and *C. officinale* differed greatly (Z=-2.63, *P*=0.0085; Fig. 1.3c, bracket between top and center bars). Female *M. borraginis* needed less time to decide for a combination of olfactory cues from *C. officinale* and visual cues from *A. occidentale* when compared to olfactory cues from *A. occidentale* and visual cues from *C. officinale* (Z=5.51, *P*<0.0001; Fig. 1.4c, second bar from top).

Experiment five: Comparing effects of visual and olfactory cues

Female *M. borraginis* responded identically to visual cues of *C. officinale* as they did to olfactory cues when they had to choose between the two cues (Z=0, *P*=1; Fig. 1.3c, third bar from top). However, they responded faster to olfactory cues compared to visual cues (Z=-6.11, *P*<0.0001; Fig. 1.4c, third bar from top).

Discussion

Olfactory and visual cues influencing finding behavior by herbivorous insects have been typically tested separately (Knolhoff & Heckel 2014). More recently, however,

olfactory and visual cues were also tested both singly and in combination. For example, Milet-Pinheiro et al. (2012) offered visual and olfactory cues to oligolectic bees singly or in combination in transparent and opaque bioassay chambers that had a membrane at the bottom and either small holes at the other end to blow air containing floral scent through the cylinder, or no small holes. Lyu et al. (2015) used a method that consisted of a central I-tube arena for the test insect and two storage chambers, connected to both ends of the central I-tube, into which host or non-host plants were placed to provide visual and/or olfactory cues. The D-SYD apparatus is simpler and more flexible than these test designs, as it also easily allows mismatching visual and olfactory cues.

Using the D-SYD method and a model system involving *M. borraginis* and its target and nontarget host plants, we show that both olfactory and visual cues contribute to host selection and that the two modalities together act synergistically. Similar synergistic effects have been demonstrated in species from various insect taxa, e.g. in the hawkmoth *Manduca sexta* (Raguso & Willis 2005), in oligolectic bees (Milet-Pinheiro et al. 2012) and in the longhorned beetle *Anoplophora glabripennis* Motschulsky (Lyu et al. 2015). These findings are consistent with the premise that insects integrate multimodal cues during host selection (Chittka & Raine 2006; Leonard & Masek 2014; Raguso 2008). As an additional corroboration enabled by D-SYD, mismatched visual and olfactory cues from *A. occidentale* and *C. officinale* fail to elicit discrimination by *M. borraginis* between the field host and the non-host plant. Furthermore, when presented with mismatched cues, the weevils spent two- to ten-fold more time searching compared to bioassays with individual modalities or correctly matched ones. This suggests that both cue modalities contribute similarly to host recognition by the weevil.

It has been argued that the inclusion of ecological factors such as cues associated with the host selection behavior of candidate species may increase the accuracy of pre-release predictions of non-target attacks in classical biological control of weeds (Hinz et al. 2014; Louda et al. 2003; Wheeler & Schaffner 2013). The typical reliance on no-choice and choice experiments in environmental safety assessments has on occasion produced false negative or false positive results, and this may in part be due to the limited ability of organisms to express their host selection behaviors in those experimental settings (Heard 2000; Marohasy 1998). Traditional host range tests are descriptive in the sense that false positive or negative results may be documented but may not be well explained with the exiting test designs (Smith & Beck 2015). Thus, interpreting behavioral mechanisms associated with plant cues in classical biological weed control could add data reducing false positive outcomes in host range assessments (Hinz et al. 2014; Sheppard et al. 2005; Wheeler & Schaffner 2013). The experimental greater control of multiple cues afforded by D-SYD can address some of these concerns.

Recently, accounts have been published investigating the role of one plant cue modality on the host selection behavior of biological control organisms such as olfactory cues (Andreas et al. 2009; Müller & Nentwig 2011) or visual cues (Reeves et al. 2009). To our knowledge, ours is the first report to examine combined field-collected olfactory and visual cues in a classical biological weed control system. Since both cue modalities alone resulted in host recognition by *M. borraginis* and their combination produced synergistic effects, we propose that both cue modalities should be studied during initial host selection behavior studies in biological weed control systems whenever possible. As demonstrated here with the D-SYD and the PVCS, simple devices can be used to

improve host range testing procedures and more accurately delineate the realized host range (i.e., the ultimate suite of plant species utilized by a biocontrol organism once released) from the fundamental or forced host range (i.e., the list of all plant species that allows the biocontrol organism to complete its life cycle under confined, no-choice conditions) (Schaffner 2001; Sheppard et al. 2005).

In summary, we demonstrate the use of a novel methodological approach to investigate the host recognition behavior of herbivorous insects in response to olfactory and visual cues from two different plant species in order to better characterize phenotypes of insect herbivores (Knolhoff & Heckel 2014). Using two simple devices, PVCS and D-SYD, we tested whether a Eurasian weevil, *M. borraginis* used olfactory, visual, or a combination of the two plant cue modalities for host recognition and differentiation of a native North American confamilial non-host, *A. occidentale* and its European field host *C. officinale.* While tests using either olfactory or visual cues elicited accurate host recognition behavior by *M. borraginis*, their combination yielded a stronger synergistic response that discriminated host and non-host more reliably and more rapidly. Because it is unlikely that a herbivorous insect will utilize a non-target field host that it does not approach during the early stages of host selection, behavioral bioassays using bimodal cues that function during that stage can complement traditional host range investigations to improve the accuracy pre-release of host range testing. Furthermore, the proposed method can elucidate responses to multiple modalities as part of applied research for integrated pest management or fundamental research on the behavioral and neural mechanisms of host finding by herbivorous insects. Studies of neurophysiological integration during host perception could benefit from the capacity to provide individual

modalities alone or in various combinations.

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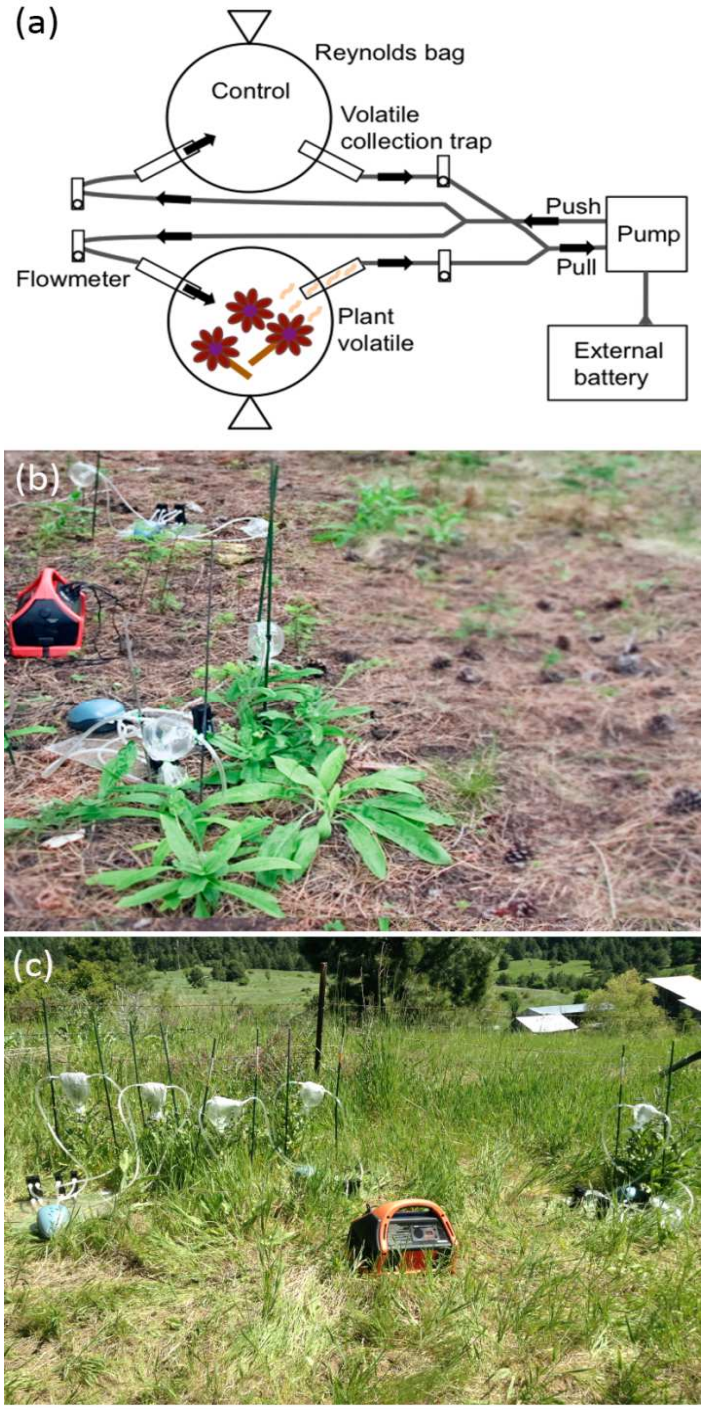

Figure 1.1: Schematic drawing of portable volatile collection system (PVCS) (a); Push-pull floral headspace volatile collection at *Andersonglossum occidentale* field site near Camp Sherman, OR, USA (b) and at *Cynoglossum officinale* field site near Moscow, ID, USA (c).

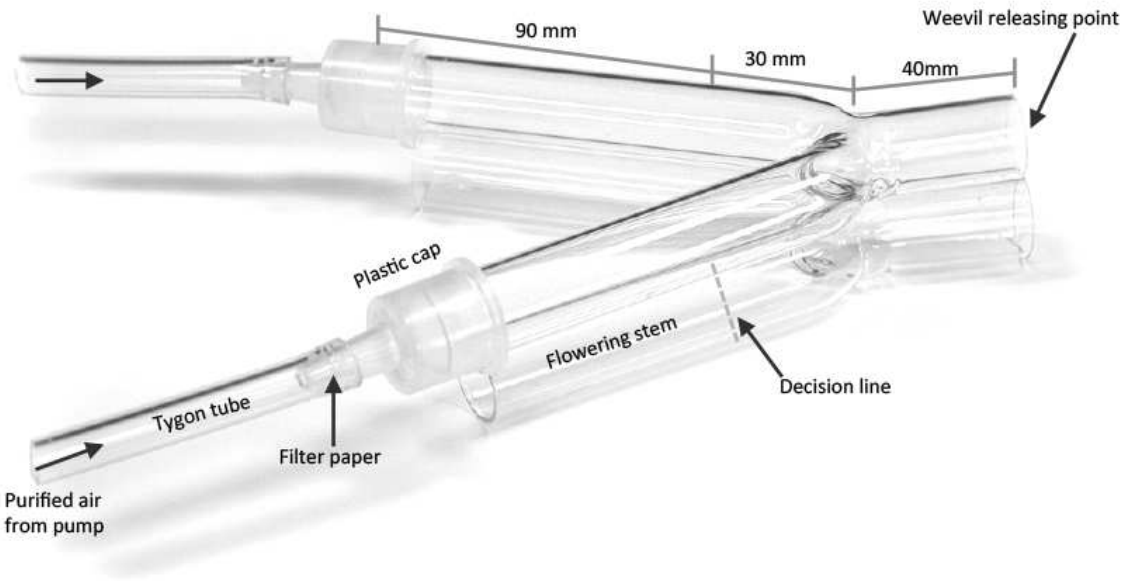

Figure 1.2: Double stacked y-tube device (D-SYD) used to assess effects of olfactory and visual cues individually or combined on the host location of the seed-feeding weevil *Mogulones borraginis*.

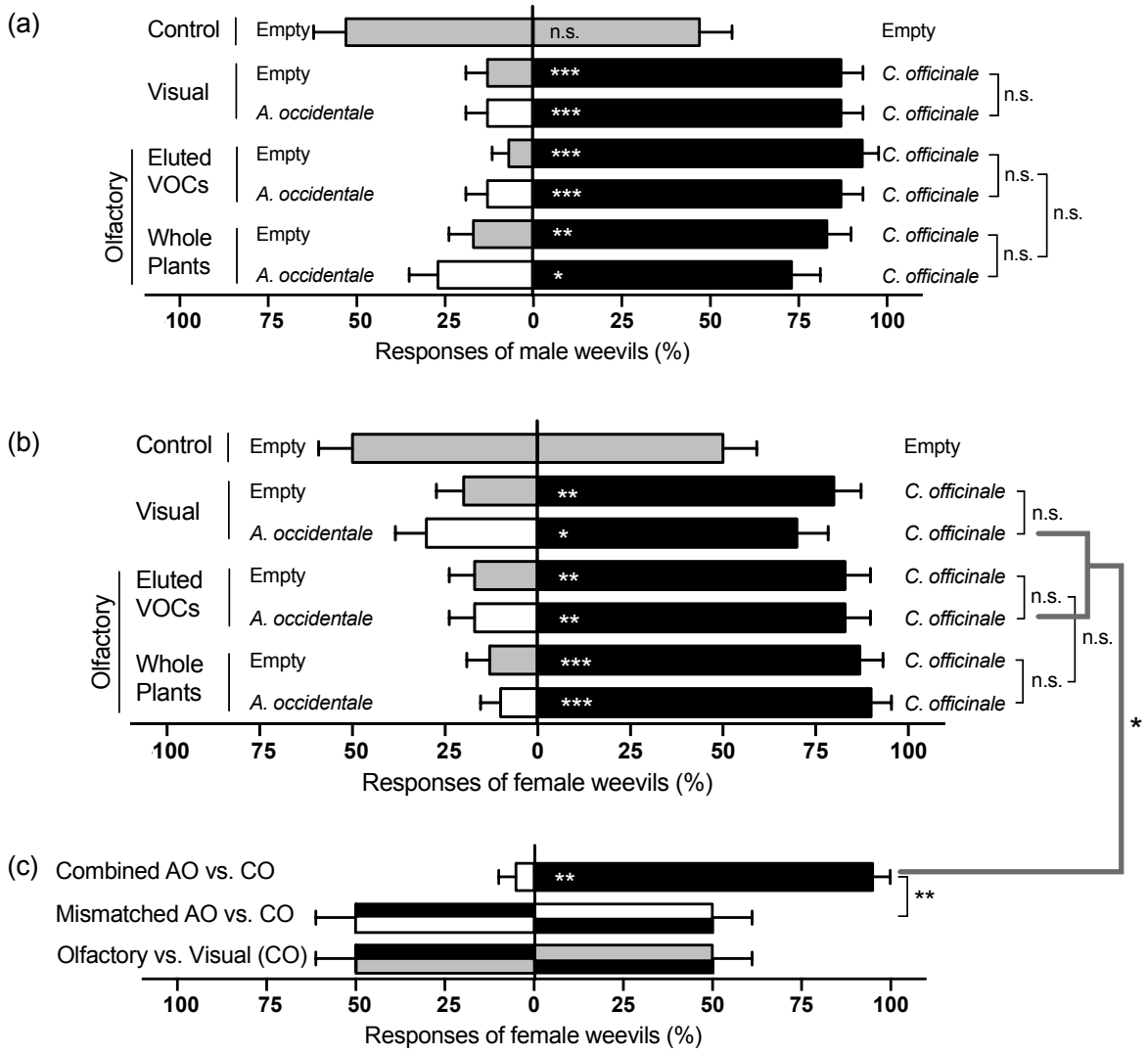

Figure 1.3: Proportional behavioral responses (±SE) of *Mogulones borraginis* in bioassays between *Cynoglossum officinale* and *Andersonglossum occidentale*. Behavioral responses of male (a) and female weevils (b) to olfactory or visual cues. Responses of female *M. borraginis* to combined olfactory and visual cues (c) of *C. officinale* and *A. occidentale* (top bars), mismatched cues of both plant species (center bars), and relative strength between olfactory and visual two cues of *C. officinale* (lower bars). O, olfactory cues; V, visual cues; AO, *A. occidentale*; CO, *C. officinale*. The visual cue was a

flowering stem of each plant species. The olfactory cue was either eluted floral scents collected at field sites or directly from greenhouse-propagated plants (whole plants). Significance levels of generalized linear model of individual bioassays: * $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s., not significant. Black brackets are a single degree of freedom contrast tests between two selected sets of bioassays. Gray brackets indicate a single degree of freedom contrast test between single cue and bimodal cue bioassays.

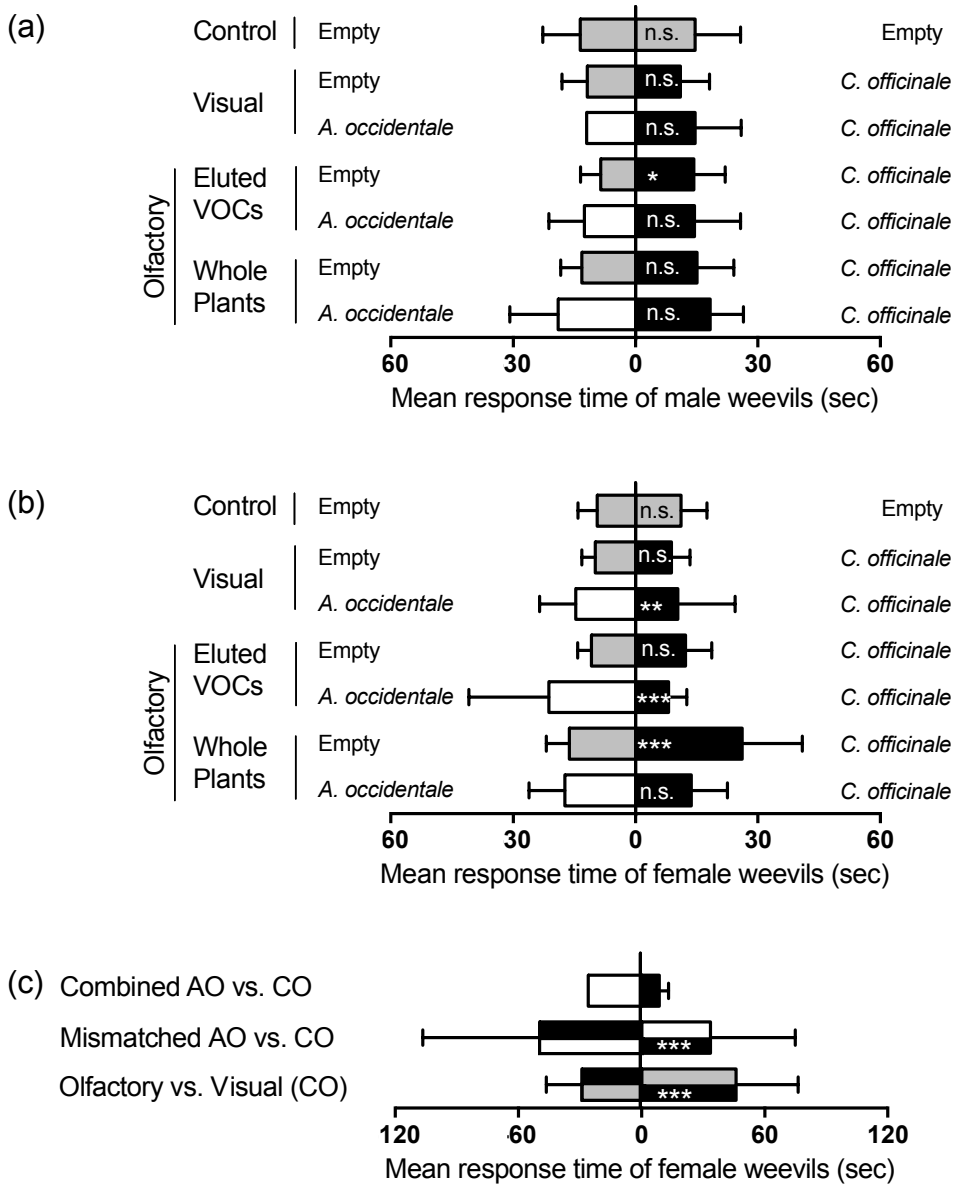

Figure 1.4: Mean (±SE) response times of *Mogulones borraginis* in behavioral bioassays. Response times of male (a) and female (b) *M. borraginis* on single cues and mean response time of females (c) to simultaneously offered olfactory and visual cues of *Cynoglossum officinale* and *Andersonglossum occidentale* (top bars), mismatched cues of both plant species (center bars), and the relative strength of visual and olfactory cues of only *C. officinale* (lower bars). O, olfactory cues; V, visual cues; AO, *A. occidentale*; CO:

C. officinale. The visual cue was a flowering stem of each plant species. The olfactory cue was either eluted floral scents collected at field sites or directly from greenhouse propagated plants. Significance levels of generalized linear model of individual bioassays: * $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s., not significant.

Chapter 2. Non-destructive environmental safety assessment of a biocontrol candidate on rare and threatened or endangered plants in the United States

Abstract

Assessing the likelihood of a non-target attack on rare, endangered or threatened native confamilial plant species is the cardinal objective of host range testing of weed biocontrol candidates, but often it is compromised by logistic difficulties in obtaining propagules, propagating or synchronizing respective plants for pre-release host range tests. To improve testing protocols and increase predictability of the potential non-target attack on rare confamilial plants, we investigated how olfactory and visual cues from five rare, threatened or endangered plant species in Boraginaceae and the invasive weed *Cynoglossum officinale* affect the host finding behavior of the seed-feeding weevil, *Mogulones borraginis*. Female weevils were repelled by olfactory and visual cues from all confamilial plant species in behavioral bioassays whether *C. officinale* was present or not. Further, electrophysiological experiments identified that weevils responded ten volatile compounds; two sesquiterpenes were unique to *C. officinale* among the plant species tested in this study. Since weevils could not identify the confamilial species as host plants, the probability of non-target attack should be infinitely small. Investigating the host finding behavior and underlying electrophysiological mechanisms of biocontrol candidates provide additional data that can greatly enhance the predictability of non-target attack of biocontrol species. The proposed method also made surrogate plants unnecessary because olfactory and visual cues are collected directly from rare, threatened or endangered plant species non-destructively in their natural habitats.

Introduction

Classical weed biocontrol requires predicting the host range of prospective biocontrol organisms to ensure their environmental safety post-release (Winston et al. 2014). Concerns about the predictability and reliability of pre-release host range assessments were raised because of serious non-target attack caused by biocontrol agents on native flora (Louda et al. 2003; Simberloff 2012). However, a recent worldwide review of non-target attack caused by weed biocontrol organisms concluded that less than 1% of released organisms are causing a population level non-target attack (Suckling & Sforza 2014). Among those, there are only two cases of biocontrol organisms severely affecting native plant diversity: *Rhinocyllus conicus* Frölich and *Cactoblastis cactorum* Berg on rare plant species. However, neither of these biocontrol organisms would be petitioned under current pre-release host range testing procedures (Hinz et al. 2014) and they were released before the enactment of the Endangered Species Act (ESA) of 1973 (16 U.S.C. 1531 et. seq.) in the United States (Gassmann & Louda 2001; Moran & Hoffmann 2015).

While current host range testing procedure has been implemented by choice and no-choice feeding and developmental tests and it produced reliable pre-release predictions on the post-release environmental safety of biological weed control agents (Hinz et al. 2014; Suckling & Sforza 2014), it does not apply to rare or threatened or endangered (hereafter T&E) plant species of the target weed if they are unavailable for pre-release host range testing. Investigating olfactory and visual cues collected non-destructively from T&E plant species can improve the predictability of non-target plant use because an insect initially can respond to these cues before landing on a potential host plant (Miller

& Strickler 1984). This is particularly relevant for testing rare or T&E plant species because without an ability to find those plant species non-target attacks are most unlikely occur in the field. However, there has been little focus on the role of both cues underlying the host range of biocontrol candidates on rare or T&E plant species.

Two issues are associated with assessing the likelihood of a non-target attack on rare and federally listed T&E native confamilial plant species in the United States. First, permits to acquire and propagate respective species and to move them across state lines within the United States must be obtained from state and federal wildlife agencies. Second, even permits are granted for plant propagules to be grown into plants in greenhouse settings, it is often unsuitable to successfully propagate these sensitive species to a specific phenostage (e.g. flowering) which needs to be synchronized with the biocontrol candidates before conducting pre-release host range tests. Alternatively, closely-related congeners of rare native species have been commonly used historically as surrogate species for testing purposes of biocontrol candidates (Colpetzer et al. 2004; Grevstad et al. 2013). However, only 50% of federally listed native T&E plant species in the United States (n=213) were grouped with their surrogates based on similar phenotypic traits such as maximum plant height, flower size, life history type (annual/perennial), reproductive mode (vegetative/sexual), or monocarpy/polycarpy (Che-Castaldo & Neel 2012). Further, the volatile profiles are often dissimilar among congeneric plants (Milet-Pinheiro et al. 2015). If the biocontrol candidate utilizes these traits to evaluate host suitability, congeners would be poor surrogates, leading to false positives (i.e., predicting potential non-target attacks when their probability is zero) and false negatives (i.e., not predicting potential non-target attacks when they are possible) on respective rare and

T&E plant species (Marohasy 1996). For these reasons, surrogate species may not provide data that can accurately assess the environmental safety of weed biocontrol candidates on the T&E plant species.

To improve the reliability of pre-release environmental safety assessments of weed biocontrol candidates, we provide an experimental approach that addresses two shortcomings of current host range testing protocols by 1) enabling testing of federally T&E listed plant species non-destructively from their natural habitats without the necessity to propagate those plant species or their surrogates, and 2) improving the predictability of post-release non-target plant use by evaluating olfactory and visual cues on the host recognition behavior of biocontrol candidates (Heard 2000; Wheeler & Schaffner 2013), as a new class of host range data. We used the seed-feeding weevil, *Mogulones borraginis* F., a biocontrol candidate for the management of the invasive *Cynoglossum officinale* L. in the United States and five rare or T&E listed confamilial plant species as a model system. We tested whether *M. borraginis* prefer *C. officinale* over T&E confamilial plant species and whether they do not prefer olfactory and visual cues from T&E plant species to purified air during host-finding bioassays.

Materials and methods

Study system

Cynoglossum officinale L. is a monocarpic, biennial to short-lived perennial plant in the Boraginaceae family native to Europe and Asia Minor (de Jong et al. 1986; Williams 2009). After accidental introductions into North America as cereal contamination, it rapidly spread throughout the continental United States (Upadhyaya et

al. 1988a).

The seed-feeding weevil, *Mogulones borraginis* F. is considered as a classical biocontrol candidate for invasive *C. officinale* in the United States. The extremely rare weevil has a univoltine life cycle and is exclusively found on *C. officinale* in the central European range (Koch 1992). In early spring, weevils emerge and initiate feeding on developing buds and flowers (Hinz et al. 2004). For ovariole development, female weevils have to feed on pollen and inflorescences tissues of *C. officinale.* Females lay eggs into developing fruits for the larvae development (Hinz et al. 2004).

Permits and propagules for four out of five listed T&E species in the Boraginaceae family were acquired (Fig. 2.1). Seeds of *Amsinckia grandiflora* (Kleeb. ex A. Gray) Kleeb. ex Greene (*n*=100) and *Plagiobothrys strictus* (Greene) I.M. Johnst. (*n*=100) were provided by Holly Forbes at Botanical Garden at the University of California at Berkeley and by Cherilyn Burton at Native Plant Program at California Department of Fishery and Wildlife in June 2011 [research and management permit number: 2081(a)-11-08-RP]. Seeds of *Plagiobothrys hirtus* (Greene) I.M. Johnst. (*n*=100) were provided by Kelly Amsberry at Oregon Department of Agriculture in May 2011. Seeds of *Hackelia venusta* (Piper) H. St. John (*n*=50) were provided by Wendy Gibble at Washington Rare Plant Care and Conservation at the University of Washington in March 2011. We were unfortunately not able to receive propagules of the remaining T&E species, *Oreocarya crassipes* (I.M. Johnston) Hasenstab & M.G. Simpson in time for this study, which only occurs in Brewster County, TX. Rootstocks of *Dasynotus daubenmirei* I.M. Johnston (*n*=10) were collected from Walde Lookout, ID, USA (N 46.23528°, W 115.63528°). The permissions were received to collect volatile compounds from the T&E

species *H. venusta* in June 2014 in Leavenworth, WA, USA (N 47.632699°, W 120.725894°; altitude: 357 m) with assistance of Lauri Malmquist at United States Department of Agriculture (USDA) Forest Service, and *P. hirtus* in June 2014 in Wilbur, OR, USA (N 43.330532°, W 123.336050°; altitude: 143 m) with assistance of Kelly Amsberry at the Oregon Department of Agriculture.

While *D. daubenmirei* is not a federally listed T&E species, it is considered a rare plant since it occurs in only one isolated population and is the only species in the genus. Rootstocks of *C. officinale* (*n*=20) were collected from Idler's Rest Nature Preserve, Moscow, ID, USA (N 46.804160°, W 116.948554°). All four were transplanted in 11.3 L black plastic pots filled with Sunshine Mix #2 (Sun Gro Horticulture Canada Ltd., Vancouver, Canada) and placed in an environmentally-controlled greenhouse at the University of Idaho in Moscow, ID in March of the following growing season and were watered as needed. Plants in the Boraginaceae family flower subsequently along cymes, and consequently buds, open inflorescences and young fruits present at the same time. This flowering phenostage is used for oviposition by *M. borraginis* and was used for all experiments described in this study.

Collection of floral headspace volatile and flowering stems for visual cues

Polyvinyl acetate bags (12 cm x 24 cm; Reynolds, Richmond, VA, USA) were purged to prevent volatile contaminants in a drying oven at 140 °C for 60 minutes. Each flowering stem was covered with the polyvinyl bag and sealed with purged cotton balls and a cable tie to minimize potential physical damage on plants. Two sides of each bag was cut for an inlet port connected to an activated charcoal filter and an outlet port

connected to a volatile collection trap (30 mg of Porapak Q; Southern Scientific Inc., Gainesville, FL, USA in a glass pipette). A modified Rena 400 pump (RENA, Chalfont, PA, USA) powered by a Duracell Powerpack 600 (Duracell, Bethel, CT, USA) maintained airflow at 300 ml/min to maintain the ambient pressure inside the bag. Before collecting, the volatile collection traps were washed with methyl chloride (10 ml; EMD Millipore, Billerica, MA, USA) and preconditioned for an hour at 140 °C. Floral scents were collected from three individual plants for each plant species and the control (an empty bag with charcoal filter) for three hours on a sunny day. After collecting for three hours, the volatile collection traps were eluted with 200 µl of methyl chloride. Each elutant was placed in a screw cap vial and stored at 4 °C until further use. Following floral volatile collections as outlined above, flowering stems (5 cm) of the respective three individual plants were collected and placed into 10 cm transparent aqua-tubes and stored in a portable cooler to minimize loss of water potential of flowering stems.

Naïve overwintered *M. borraginis* (*n*=400) were received from CABI Switzerland to the University of Idaho in early spring 2012 to 2014. All weevils fed fresh foliage and buds of C. officinale and kept at the quarantine laboratory at Washington State University in an environmental chamber (E-30B, Percival Scientific, Perry, IA, USA; L18: D6 at 20 °C and 60% relative humidity).

Host-finding bioassays with D-SYD

A double-stacked Y-Tube (D-SYD) was used to assess quantitatively the environmental risk of female *M. borraginis* to feed on or otherwise utilize confamilial rare and T&E plant species (Fig. 2.2), based on olfactory and visual cues either

individually or simultaneously. D-SYD consists of two common laboratory glass Y-tubes (4 cm Y-stem, 12 cm arms, 2 cm internal diameter) stacked together to test behavioral responses to olfactory and visual cues simultaneously (Park 2017). The D-SYD was installed in a darkened room with a full spectrum light bulb (ES5M827FS, 27 Watt, Home Depot, Atlanta, GA, USA; 350 nm to 850 nm wavelength) diffused through a white polyethylene dome (40 cm x 30 cm x 20 cm) placed 20 cm above the D-SYD. The D-SYD was rinsed with 70% ethanol following each bioassay and rotated 180° after each five replicates to minimize left or right-handed bias. A total of 20 female weevils were tested with the individual weevil considered a single replicate in each experiment, except *P. strictus* for which ten females were used due to the limited number of weevils available. One weevil was placed at the release point in the bottom Y-tube in each bioassay (Fig. 2.2). Data from tests on all plants were pooled for analysis because there was no evidence of effects of individual plants on the weevil responses.

Experiment 1: visual cues

To compare the responses to visual cues from two plant species, flowering stems (5 cm) of a rare confamilial plant and *C. officinale* were placed in each arm of the visual Y-tube of the D-SYD. There was no airflow in the olfactory Y-tube when only visual cues were examined due to the possibility of anemotaxis (orientation towards wind) (Farkas & Shorey 1972). Weevils did not differ tests with visual cues and purified air from tests without airflow.

Experiment 2: olfactory cues

To investigate the behavioral responses to olfactory cues, an individual female weevil was presented with floral volatiles of a rare plant species and *C. officinale.* A 1 µl aliquot of the eluted floral scent was deposited on a 2 mm^2 square filter paper on each side of olfactory Y-tube.

Experiment 3: combined visual and olfactory cues

To study the responses to olfactory and visual cues combined, both cues were tested simultaneously with female weevils in the D-SYD, following methods similar to Experiments 1 and 2.

Experiment 4: combined cues vs. purified air

The responses of female *M. borraginis* to combined olfactory and visual cues from each test plant species vs. pure air were measured whether weevils were repelled or indifferent. We used the approach used in Experiment 3, but only using combined cues of test plant species.

Headspace volatile organic compounds (VOC) analysis

Gas chromatography-mass spectrometry (GC-MS) was performed to identify electrophysiologically active chemical compounds and semi-quantifying their concentration in floral scents. We tested floral VOCs collected from greenhouse-propagated plants and at plants at field sites. An Agilent 7890A (Agilent Technologies Inc., Santa Clara, CA, USA) with an HP-5MS column (30 m x 250 µm x 0.25 µm;

Agilent Technologies Inc., Santa Clara, CA, USA) and coupled with Hewlett Packard 5973 mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA). The initial oven temperature was 40 °C for 1 minute. Temperature was increased at 5 °C/minute to 200 °C for the first ramp, at 10 °C/minute to 300°C for the second ramp, then held at 300 °C for 2 minutes. One µl of each trap elutant was injected with 10 ng of nonyl acetate (W278807, Sigma Aldrich) as an internal standard in the splitless mode at 250 °C. Electron ionization (EI) mass spectra of each analyte were produced at an ionization voltage of 70 eV. Identification was based matches of the mass spectra based to those in the NIST database, and on mass spectra and retention times of authentic compounds. To confirm the chirality of an enantiomeric compound, α-copaene, the floral blends were analyzed using an Agilent J&W Cyclodex-B and retention time of the α-copaene was compared with the compound's retention in two essential oils of two different plant species that contain either (+) or (-) enantiomer.

Gas chromatography-electroantennographic detection and flame ionization detection (GC-EAD/FID) was performed to identify electrophysiologically active chemical compounds in the blends of *C. officinale.* The analysis was performed on an Agilent-6890N GC with column specification and other parameters were performed as described above. A 1:1 column splitter delivered effluent to the FID detector and to the EAD. Effluent was delivered, via a Syntech GC effluent conditioner (Syntech, Hilversum, Netherlands), into humidified air flowing at 10ml/sec directed through glass tubing to the antenna of a female *M. borraginis*. Depolarization of the antenna was recorded and combined that with the GC FID signal with Syntech GC-EAD 2000 software (Syntech, Hilversum, Netherlands).

To prepare antennae for recording, female *M. borraginis* were decapitated using a scalpel under a microscope. A decapitated head was placed on a ground probe with Spectra 360 electrode gel (Parker Laboratories, Fairfield, NJ, USA). The distal tip of the antenna was punctured with a minute insect pin (1208SA, Bioquip, Czech Republic) which was placed in contact with the recording probe (Syntech, Hilversum, Netherlands), the undamaged antenna from the head was positioned to receive the entrained effluent from the GC column. MORE INFO NEEDED HERE ABOUT WHAT CONSTITUTED A 'RESPONSE' TO A STIMULUS, ETC. A volatile organic compound was considered electrophysiologically active if all female weevils responded to a chemical compound in the blends of *C. officinale* regardless of greenhouse and field conditions. The performance of the system was checked before each recording using an antenna simulator (Syntech, Hilversum, Netherlands).

Statistical analysis

Behavioral responses of weevils in each assay were analyzed assuming a completely random design using a generalized linear model with an expected null ratio of 50:50 and a binominal distribution. To test whether behavioral responses of *M. borraginis* differed between single cues (Experiments 1 & 2) and combined cues (Experiment 3), the least square means of responses were compared using single degree-of-freedom contrasts based on likelihood ratio chi-square tests. An effect was considered additive if the least square mean of responses from the combined cues equaled the sum of the responses from each individual cues. An effect was considered synergistic (a more-than-additive effect) if the least square mean of responses from the combined cues was

significantly greater than the sum of theresponses from each individual cues. The searching time (i.e., the time delay between the initial movement of a female and its passing the decision line) in each assay was analyzed using a generalized linear model with a Poisson distribution. Least squares mean differences were calculated to compare the searching time of females between arms of the D-SYD in each behavioral assay. To visualize the separation of floral blends among plant species, principal component analyses (PCA) were performed based on the presence of electrophysiologically active volatile compounds from *C. officinale*. All analyses were carried out using the statistical software package SAS version 9.4 (SAS Institute, 2013).

Results

Host-finding bioassays with D-SYD

Experiment 1: visual cues

In bioassays offering visual plant cues, female *M. borraginis* preferred flowering stems of *C. officinale* over each of the five non-target plant species (*A. grandiflora*: $Z=2.87$, $P=0.0041$; *D. daubenmirei*: $Z=2.77$, $P=0.0056$; *H. venusta*: $Z=2.48$, $P=0.0131$; *P. hirtus*: $Z=2.95$, $P=0.0032$; *P. strictus*: $Z=0$, $P=1$; Fig. 2.3, second set of bars from top). The average response time of female weevils did not differ for *P. hirtus* ($Z=0.08$, $P=0.9364$), but was longer for *D. daubenmirei* ($Z=2.00$, $P=0.0452$), and *H. venusta* ($Z=2.24$, $P=0.0250$) compared to *C. officinale* (Fig. 2.4, second bars from top).

Experiment 2: olfactory cues

In bioassays using olfactory plant cues, female *M. borraginis* preferred VOCs of

C. officinale over those of all rare confamilial non-target plant species (*A. grandiflora*: Z=2.48, *P*=0.0131; *D. daubenmirei*: Z=-2.95, *P*=0.0032; *H. venusta*: Z=2.77, *P*=0.0056; *P. hirtus*: Z=2.87, *P*=0.0041; *P. strictus*: Z=0, *P*=1; Fig. 2.3, third bars from top). The average response time of *M. borraginis* females did not differ for *H. venusta* (Z=1.33, *P*=0.1844), shorter for *A. grandiflora* (Z=2.86, *P*=0.0042) and longer for *D. daubenmirei* (Z=3.90, *P*<0.0001) when compared to *C. officinale* (Fig. 2.4, third bars from top).

Experiment 3: combined visual and olfactory cues

In bioassays with both olfactory and visual plant cues offered simultaneously, *M. borraginis* preferred *C. officinale* over all rare non-target species (*A. grandiflora*: Z=0, *P*=1; *D. daubenmirei*: Z=0, *P*=1; *H. venusta*: Z=0, *P*=1; *P. hirtus*: Z=0, *P*=1; *P. strictus*: Z=2.08, *P*=0.0371; Fig. 2.3, fourth bars from top).

Differences of *M. borrgainis* responses in the D-SYD between bioassays with one versus two plant cues showed additive effects for the combined cue in three plant species: *A. grandiflora* (χ^2_1=3.28, *P*<0.0702), *P. hirtus* (χ^2_1=2.34, *P*<0.1261), and *P. strictus* (χ^2_1=2.27, *P*<0.1322) (Fig. 3, brackets to the right). Synergistic effects were observed when combined olfactory and visual cues were offered compared to bioassays with one cue modality: *D. dasynotus* (χ^2_1=4.18, *P*<0.0409) and *H. venusta* (χ^2_1=6.06, *P*<0.0138) (Fig. 2.3, brackets to the right).

Experiment 4: combined cues vs. purified air

There were no differences between the responses of *M. borraginis* to plant cues of non-targets regardless of whether *C. officinale* or only purified air in an empty glass arm

were offered as alternative (comparison of 4th set of bars from top and bottom set of bars in Fig. 2.3) for *A. grandiflora* (*Z*=0, *P*=0.0998) and *H. venusta* (*Z*=0, *P*=0.0998). For *D. daubenmirei, P. hirtus,* and *P. strictus,* female weevils responded identically (Fig. 2.3, brackets to the left).

When visual and olfactory cues of confamilial non-targets were offered to *M. borraginis* with only purified air as alternative, female *M. borraginis* were repelled (preferred purified air) by all plant species tested (*A. grandiflora*: Z=-2.95, *P*=0.0032; *D. daubenmirei*: Z=0, *P*=1; *H. venusta*: Z=2.77, *P*=0.0056; *P. hirtus*: Z=0, *P*=1; *P. strictus*: Z=2.08, *P*=0.0371; Fig. 2.3, bottom set of bars). The average response time of *M. borraginis* females did not differ for *A. grandiflora* (Z=-1.02, *P*=0.3067) and *H. venusta* (Z=-1.65, *P*=0.0981) (Fig. 2.4, bottom bars).

Volatile profiles of plant species

Sixty-one volatile compounds were identified in the floral scents of the six plant species included in this study. Of these, ten that were present in floral scent of *C. officinale* were electrophysiologically active based on EAD responses and two, (-)-α-copaene and (E)-β-farnesene, were unique to *C. officinale* (Table 2.1). PCA calculated based on the concentrations of these ten compounds separated the floral scent of *C. officinale* from those of *D. daubenmirei* and the four tested T&E species (Fig. 2.5).

Discussion

The findings of this study demonstrate that behavioral responses to the combination of olfactory and visual cues can aid current host range testing procedures, as

a new class of host range data, particularly on rare and T&E plant species. We found that based on either olfactory and visual cues alone, female weevils preferred *C. officinale* over all five non-target plant species. For *D. daubenmirei* and *H. venusta,* the effect of combined cues on the host discrimination over a single cue was statistically synergistic while for *A. grandiflora, P. hirtus* and *P. strictus,* the effect was additive. Further, in host-finding bioassays with combined cues, all non-target T&E plant species were repelled by *M. borraginis* compare to purified air. These findings are consistent with current host range testing results with *D. daubenmirei* and *P. hirtus*; female *M. borraginis* laid eggs exclusively into *C. officinale* seeds while none into *D. daubenmirei* (Hinz et al. 2004) and *P. hirtus* (Hinz et al. 2005).

Our finding of the attractancy to *C. officinale* and the repellency on rare and T&E plant species supports the two-phase model to evaluate the host range of biocontrol candidates (Briese 2005). First, the candidate is exposed to a native non-target plant with the target weed to assess a preference ranking (Heard & van Klinken 1998). In the second phase, the candidate is exposed to the non-target plant without the target weed to test whether the lower ranked non-target plant was not attacked by the biocontrol candidate due to the presence of the target weed in the first phase to prevent potential false positive effects (Marohasy 1998). Even the higher ranked target weed, *C. officinale,* is absent, it is difficult to envision a scenario where the weevils would be able to identify any lower ranked T&E plant species as potential host plants due to the repellency.

Although the host finding bioassays may elucidate host recognition cues between *C. officinale* and five non-target plant species, *M. borraginis* may utilize other information during host finding. For example, other visual cues are present in the field

including the floral size (Weiss 1991), floral colors (Milet-Pinheiro et al. 2015), floral patterns (Hansen et al. 2012) and the overall shape of a plant (Degen & Städler 1997). Host recognition based on olfactory cues is perceived by either specific ratio of volatile blends or the presence of specific compounds (Bruce & Pickett 2011; Visser 1986). Among the ten electrophysiologically active VOCs, two sesquiterpenes, (-)-α-copaene and (E)-β-farnesene, were unique to *C. officinale* with little variation regardless of collection locations, suggesting that they may be host specific olfactory cues for *M. borraginis*. Because both sesquiterpenes are reported as attractants for other insects (Flath et al. 1994; Francis et al. 2004), further tests on *M. borraginis* as attractants will be merited. It is also possible that *M. borraginis* may use gustatory or tactile cues to increase the host finding efficiency. However, since the host selection behavior is a catenary process (Miller & Strickler 1984), if a biocontrol candidate is indifferent or even repelled by olfactory and visual cues from non-target plant species, examining gustatory cues is highly unlikely to occur on a rare or T&E plant species.

A particular advantage of the proposed method is that it allows collecting data on the host recognition that uses plant cues, which can be collected almost entirely non-destructively in natural habitats of respective plant species, typically a single population. This becomes particularly relevant in weed biocontrol where it is often difficult or impossible to evaluate native rare or endangered plant species such as those that are listed and protected in the United States under the Endangered Species Act. It is not only difficult to obtain plant material for propagation or to propagate protected species, but also the Endangered Species Act requires confirmation that prospective biocontrol organisms do not harm these species. Historically, these plant species are being

substituted with surrogate species, closely related congeners or confamilials with similar sets of traits, similar habitat requirement and geographic overlap for the purpose of host range testing of potential organisms for use in biological control programs (Colpetzer et al. 2004; Grevstad et al. 2013). The methodological approach proposed here makes such substitutions unnecessary because it is possible to test the T&E plant species in question directly by collecting floral scents and flowering stems from their natural habitats.

In summary, we attempted to incorporate ecological determinants such as plant cues associated with the host selection behavior of biocontrol candidates (Louda et al. 2003; Wheeler & Schaffner 2013) with the current pre-release host range testing (Sheppard et al. 2005) as an approach to improve the prediction on pre-release risk assessment in classical weed biological control (Hinz et al. 2014). The likelihood that female *M. borraginis* encounters one of rare confamilial plant species in their natural habitats is highly unlikely since female weevils were attracted *C. officinale* and repelled by all native non-target plant species tested in this study. Based on host-finding bioassays and electrophysiological applications, this study not only advances our understanding of the role of olfactory and visual cues in the theory, but also serves as a novel approach to improve current host range testing procedure, particularly on T&E plant species instead of using surrogate plant species.

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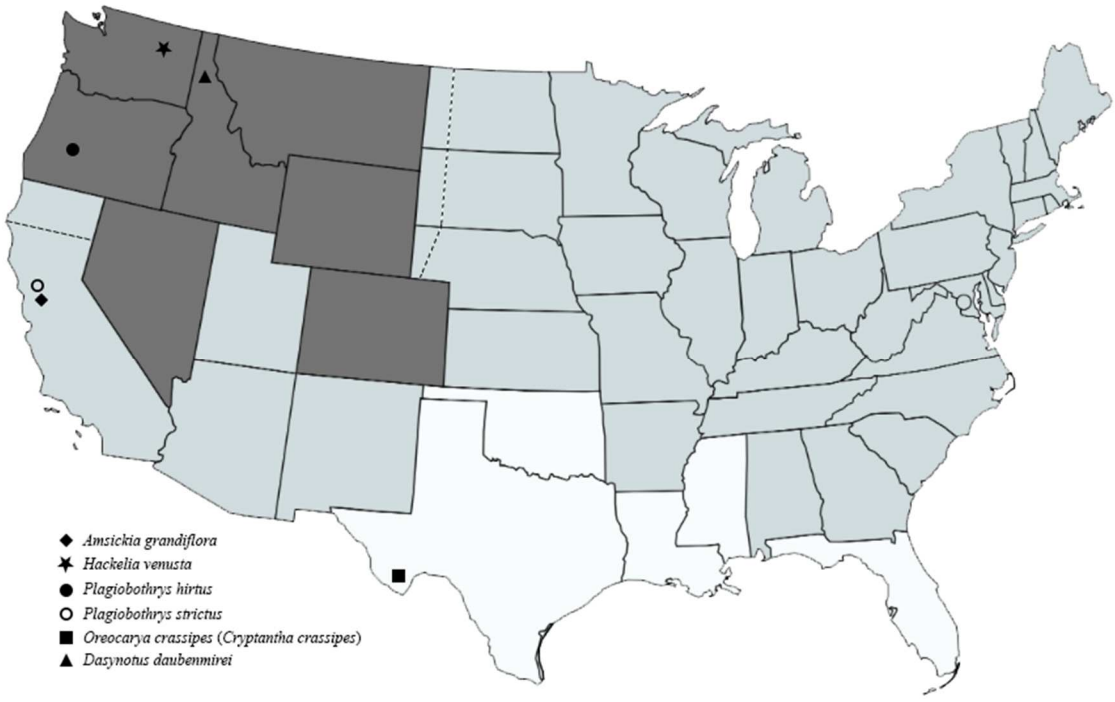

Figure 2.1: Locations of remaining field populations of the five federally listed threatened and endangered (T&E) plant species and one rare single-population species, *Dasynotus daubemirei* in the Boraginaceae family in the United States. Dotted line: the distribution of *C. officinale,* White: absent, Grey: *C. officinale* declared as a noxious weed, Light grey: *C. officinale* infestations.

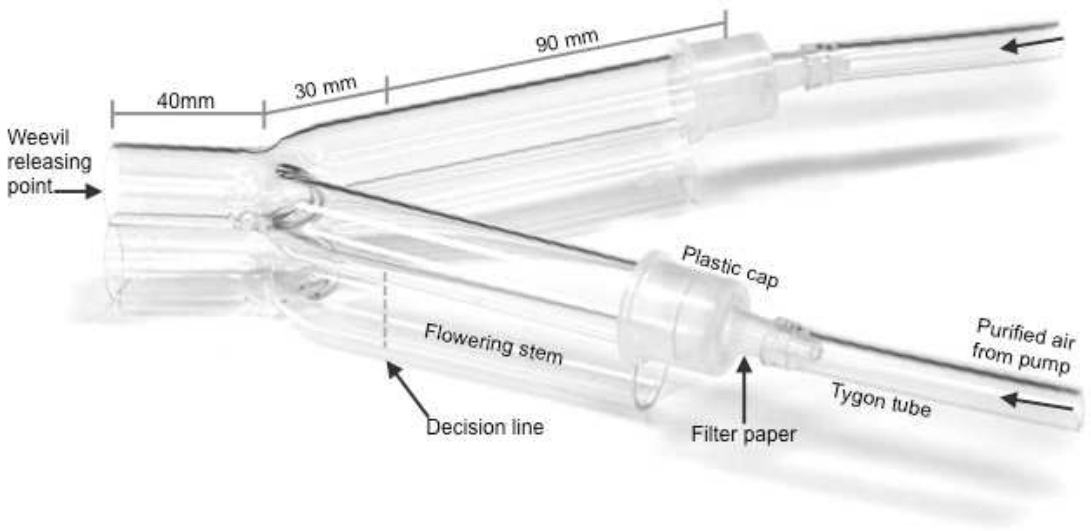

Figure 2.2: Double stacked y-tube device (D-SYD) that evaluate effects of olfactory and visual cues on the host selection behavior of the seed-feeding weevil, *Mogulones borraginis*.

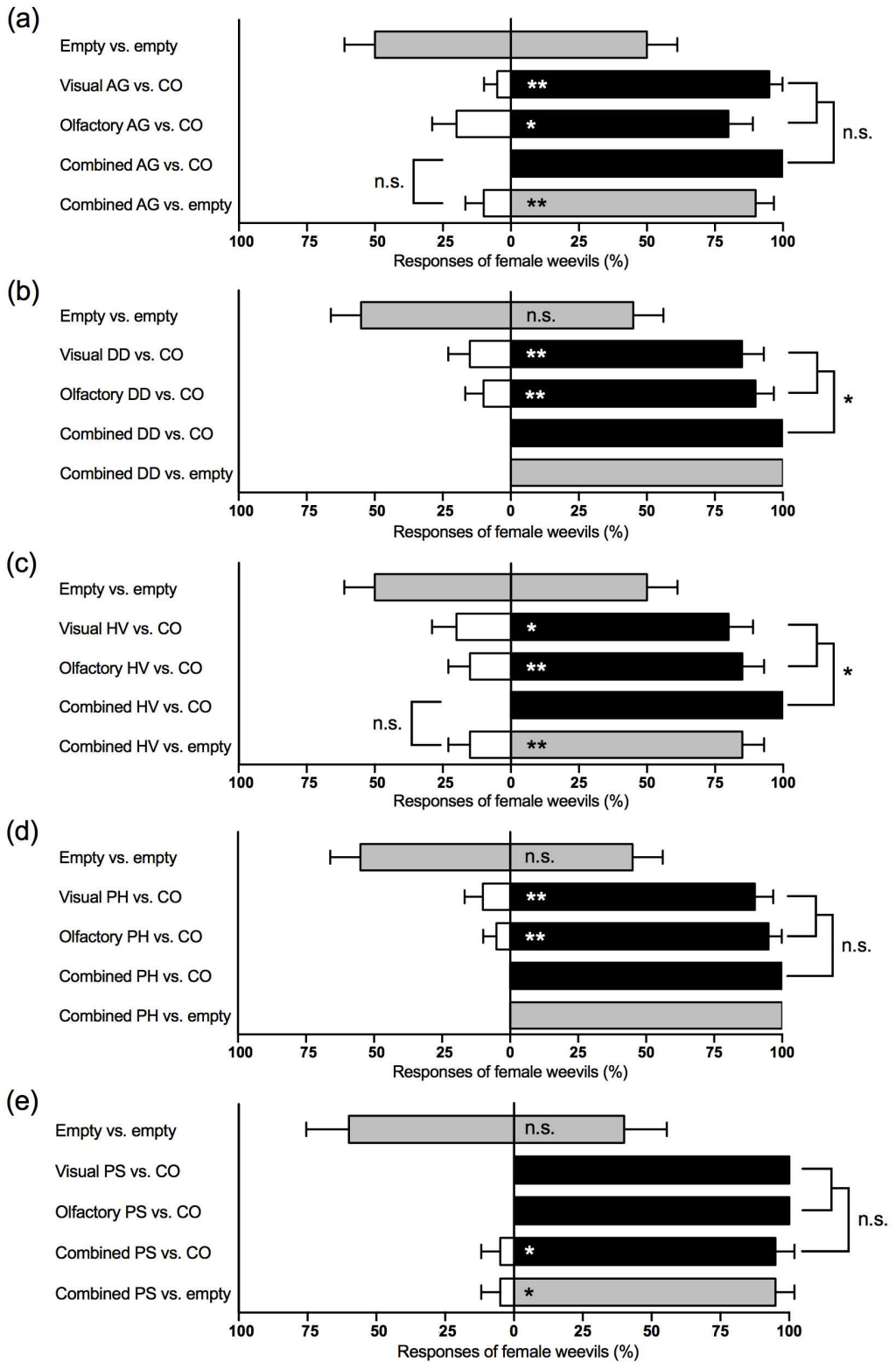

Figure 2.3: Mean (± SE) behavioral responses of *Mogulones borraginis* on olfactory and visual cues in behavioral bioassays. The visual cue was a flowering stem of each plant

species. The olfactory cue was eluted floral VOCs either the natural habitats or greenhouse-propagated plants. AG, *Amsinckia grandiflora*; CO, *Cynoglossum officinale*; DD, *Dasynotus daubenmirei*; HV, *Hackelia venusta*; PH, *Plagiobothrys hirtus*; PS, *Plagiobothrys strictus*. Significance levels of the generalized linear model of individual assays: * $P<0.05$, ** $P<0.01$; n.s., not significant. Left brackets are a single degree of freedom contrast tests between two selected sets of bioassays. Right brackets indicate a single degree of freedom contrast tests between single cues and bimodal cue bioassays.

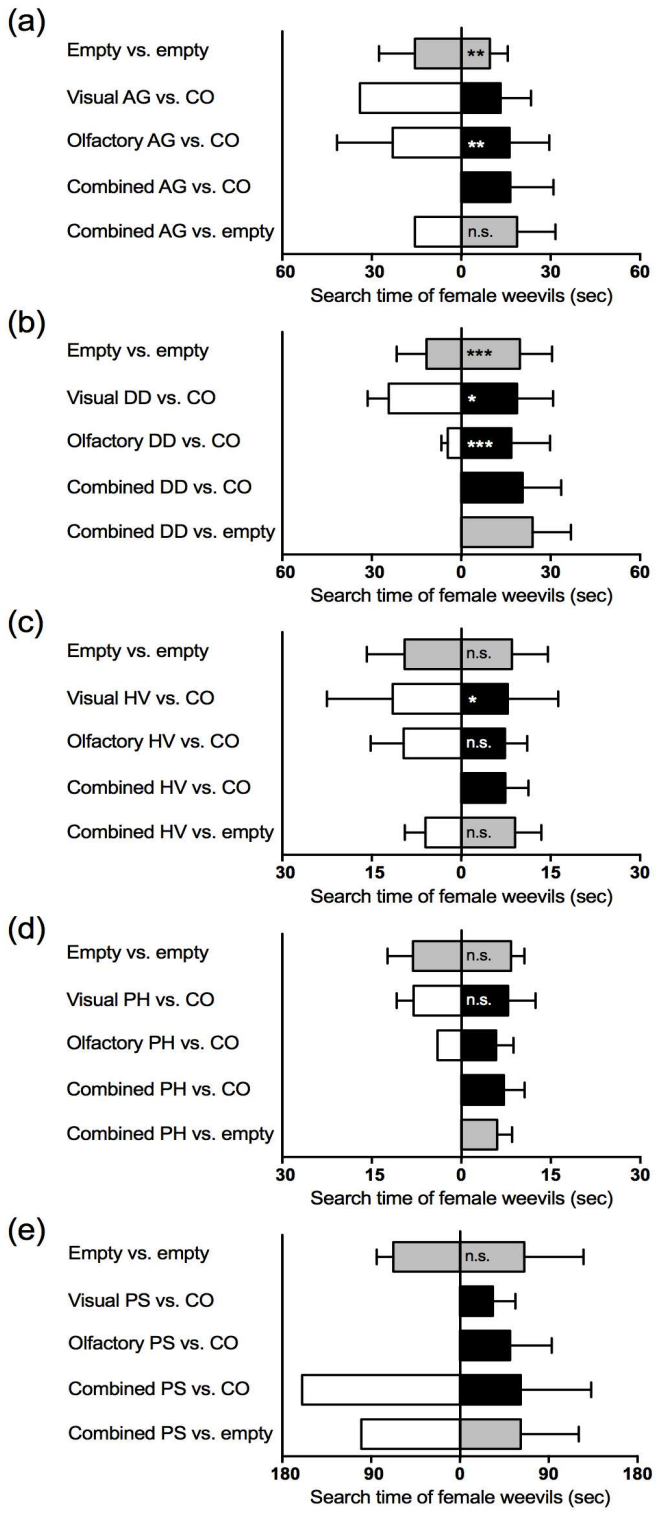

Figure 2.4: Mean (± SE) response time of *Mogulones borraginis* in behavioral bioassays. The visual cue was a flowering stem of each plant species. The olfactory cue was eluted

floral VOCs either the natural habitats or greenhouse-propagated plants. AG, *Amsinckia grandiflora*; CO, *Cynoglossum officinale*; DD, *Dasynotus daubenmirei*; HV, *Hackelia venusta*; PH, *Plagiobothrys hirtus*; PS, *Plagiobothrys strictus*. Significance levels of the generalized linear model of individual assays: $P<0.05$, ** $P<0.01$, n.s.; not significant.

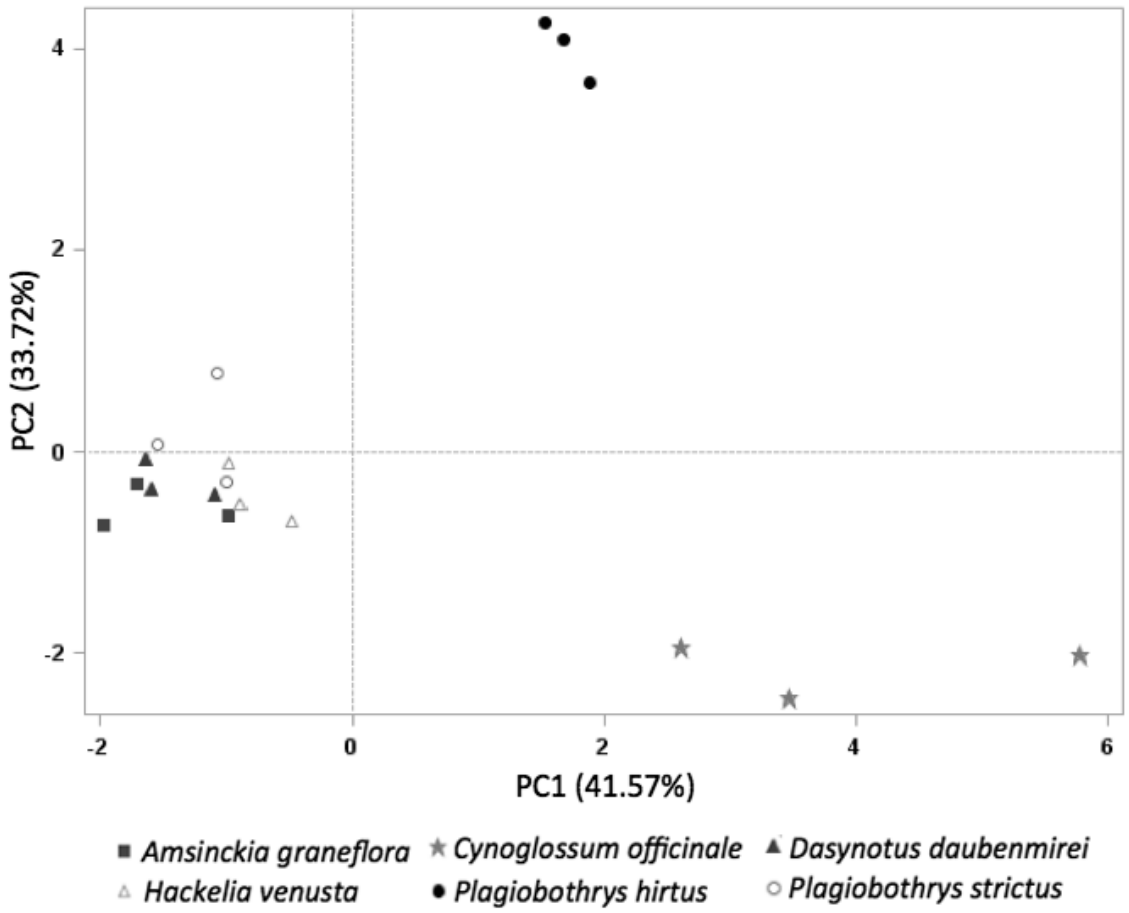

Figure 2.5: Principal component analysis based on the relative proportion of ten electrophysiologically active chemical compounds in volatile headspace blends of plant species used in the study.

Table 2.1: Quantity (µg, mean ± SD) of bioactive compounds emitted from flowering stems from plant species tested in this study.

CO: *C. officinale*; AG: *A. grandiflora*; DD: *D. daubenmirei*; HV: *H. venusta*; PH: *P. hirtus*; PS: *P. strictus*; F: field; G: greenhouse.

Bioactive chemical compounds	RT	CO (F)	CO (G)	AG (G)	DD (G)	HV (F)	PH (F)	PH (G)	PS (G)
2-Heptanone	5.543	1.38 ± 1.99	2.62 ± 2.07	3.31 ± 4.68	0.13 ± 0.18	0.98 ± 1.70	0	0	0
Acetic acid, Pentyl ester	6.142	1.99 ± 2.77	0.52 ± 0.31	0	0	0	1.28 ± 0.76	0	0
Benzaldehyde	7.265	0.66 ± 1.01	0.85 ± 0.63	0.84 ± 0.73	0.13 ± 0.15	0.87 ± 0.89	26.10 ± 5.56	1.73 ± 0.02	2.42 ± 3.76
4-Hexen-1-ol, acetate	8.6	5.16 ± 6.59	1.89 ± 1.65	0	0.89 ± 0.59	0	7.16 ± 4.12	0.07 ± 0.002	0.27 ± 0.19
Hexyl-acetate	8.786	4.38 ± 6.25	0.73 ± 0.57	0	0.38 ± 0.32	0	4.75 ± 2.49	0.04 ± 0.01	0
Linalool oxide	10.867	1.96 ± 2.67	0.83 ± 0.56	0.71 ± 1.07	0.45 ± 0.47	0	0.11 ± 0.10	0	0
Phenylethyl alcohol	11.557	0.57 ± 0.85	0.18 ± 0.12	0	0.02 ± 0.04	0	0	0	0
(-)-α-Copaene	18.71	1.70 ± 2.54	2.33 ± 1.78	0	0	0	0	0	0
(E)-β-Farnesene	20.799	1.69 ± 2.42	4.02 ± 3.43	0	0	0	0	0	0
α-Muurolene	21.849	1.86 ± 2.51	3.24 ± 2.40	0	0.11 ± 0.19	0	0.82 ± 0.88	0.05 ± 0.07	0

Chapter 3. Bimodal host-finding studies can improve environmental safety assessments of weed biological control candidate species and add novel host-specificity data

Abstract

In weed biological control programs, pre-release host-specificity testing relies traditionally on no-choice and choice feeding, oviposition, and development tests. Rarely have they included detailed examination of behavioral responses to olfactory and visual cues of biological control candidates, although host recognition is a pre-requisite for feeding and oviposition. We investigated how the seed-feeding weevil, *Mogulones borraginis*, distinguishes its host plant *Cynoglossum officinale*, from three native confamilial non-target species in North America. In behavioral bioassays, *M. borraginis* responded to olfactory and visual cues individually and, to an even greater extent, to both plant cue modalities when offered simultaneously. In tests with the combined cues, *M. borraginis* was attracted to *C. officinale* but responded with indifference or was repelled by non-target plants. In electrophysiological experiments, we identified that *M. borraginis* responded to ten volatile compounds and four wavelengths of lights from inflorescences of *C. officinale*. We propose that studies of responses to multimodal plant cues can advance our understanding of how biocontrol candidate species discriminate among host plants and closely related non-target species, thereby increasing the accuracy of environmental safety assessments pre-release, ultimately reducing unpredicted non-target attack.

Introduction

Before a decision can be made to release a prospective classical biological weed control organism into a new environment, its environmental safety must be assessed (Heard 2000; Sheppard et al. 2005) in order to prevent or minimize the risk of direct and indirect non-target effects (Louda et al. 2003). To achieve this, the host-specificity of candidate biological control organisms is typically investigated through a series of no-choice and choice laboratory and common garden experiments to assess feeding, oviposition and development on selected non-target species, and where feasible validated with open field tests (Briese 2005; Schaffner 2001). Despite an estimated 99% accuracy of environmental safety predictions for biological weed control organisms, non-target attack by biological weed control agents still occurs (Suckling & Sforza 2014). This raises the question whether traditional host-specificity testing methods could be improved by including assessments of the mechanisms of host selection behavior by candidate biological control organisms (Heard 2000; Marohasy 1998; Miller & Strickler 1984; Visser 1986).

Herbivorous insects typically use olfactory and visual cues during the pre-alightment stage of host selection (Schoonhoven et al. 2005). A better understanding of how these cues are perceived and the resulting behavioral responses by candidate biological control organisms could provide additional data to include in pre-release assessments. For instance, plants within the so-called fundamental host range of a candidate biological control species, as assessed through performance assays, may not be attractive to that species in the field and therefore are rarely or never encountered (Hinz et al. 2014).

Close to 500 weed biological control agents have been intentionally introduced into 130 countries against 175 target plants (Winston et al. 2014) but very few assessments of the host finding behavior of potential agents have been conducted. Most of those assessments focused on responses to olfactory cues (Andreas et al. 2009; Beck et al. 2008; Kafle 2016; Müller & Nentwig 2011), and only four examined responses to visual cues (Müller & Nentwig 2011; Reeves et al. 2009). There are no published studies of the behavioral responses of biological control agents to olfactory and visual cues in combination. Furthermore, there are very few studies that examine the mechanisms of weed biocontrol agent responses to target and non-target plant species (Wheeler & Schaffner 2013) including identification of electrophysiologically active kairomones (Cosse et al. 2006; Kafle 2016) or characterization of the bioactive reflectance spectra from these plants.

Here we employ behavioral bioassays followed by analyses of olfactory and visual cues of the plant species to explain observed host recognition and discrimination behavior of the seed-feeding weevil, *Mogulones borraginis* F., a candidate biological control agent for houndstongue, *Cynoglossum officinale* L. We tested the behavioral responses of *M. borraginis* in dual choice bioassays to olfactory cues, visual cues, or both presented simultaneously, from *C. officinale* and three closely related, endemic North American non-target plant species. We hypothesized that floral olfactory and visual cues contribute to host recognition by *M. borraginis*, and that the two cue modalities in combination allow it to successfully distinguish between *C. officinale* and confamilial, non-target plant species. We coupled these bioassays with tests of electrophysiological responsiveness of *M. borraginis* females to specific olfactory and visual cues. We sought

to determine whether identification of bio-active olfactory and visual cues and comparison of these among target and non-target plants species can help explain observed host-specificity of *M. borraginis* to support environmental risk assessments for this candidate biological control agent.

Materials and methods

Study system

Cynoglossum officinale is a monocarpic, biennial to short-lived perennial herbaceous plant in the Boraginaceae, native to Europe and Asia Minor. The plant forms rosettes in the first year and flowering stems in the second or third year (de Jong et al. 1986; Williams 2009). It was accidentally introduced into North America in the mid 1900s and spread throughout southern Canada and most of the continental United States (Upadhyaya et al. 1988a).

The seed-feeding weevil, *Mogulones borraginis* F., is a candidate for the biological control of *C. officinale* (Koch 1992). Overwintered weevils emerge from the soil in early spring and feed on *C. officinale* foliage, bolting stems, and later on buds and inflorescences (Hinz et al. 2004). Feeding on *C. officinale* inflorescences appears to be a prerequisite for ovariole developments in female *M. borraginis* (Hinz et al. 2004). We selected three North American confamilial non-target plant species to study host discrimination by *M. borraginis* using olfactory and visual cues: *Adelinia grande* (Douglas ex Lehm.) J. I. Cohen (=*Cynoglossum grande*), *Andersonglossum occidentale* (A. Gray) J. I. Cohen (=*Cynoglossum occidentale*), and *Hackelia californica* (A. Gray) I.M. Johnston. The two former congeners were considered to be the closest relatives of *C.*

officinale in North America until a recent phylogenetic revision placed them in their new respective genera (Cohen 2015). *Hackelia californica* was selected because, similar to *A. grande* and *A. occidentale,* it is among the few Boraginaceae that produce fruits large enough to potentially support larval development of *M. borraginis* (Hinz et al. 2003). All are clonal species that have not been extensively tested but, in preliminary tests, *A. grande* was accepted to a limited degree for oviposition by *M. borraginis,* while *A. occidentale* and *H. californica* were not (unpubl. data).

Test plants for greenhouse studies were grown from rootstocks that were collected with surrounding soil from various field sites. Rootstocks of *C. officinale* (n = 20) were collected in Idler's Rest Nature Preserve in Moscow, ID (N 46.804160°, W 116.948554°) in March of 2013 and 2014. Rootstocks of *A. occidentale* (*n*=15; N 44.47011°, W 121.6282°) and *H. californica* (*n*=10; N 44.48194°, W 121.63917°) were collected in the Deschutes National Forest, OR in May 2013. Rootstocks of *A. grande* (*n*=20) were collected in White Salmon, WA (N 45.756892°, W 121.490535°) in March 2013. Rootstocks were potted 11.3 L black plastic pots filled with soil from the collection site. Pots were kept in an environmentally controlled greenhouse at the University of Idaho set to mimic outdoor conditions. The flowering plant phenostage was used for all experimentation described here. Since flowering in Boraginaceae occurs sequentially along cymes, there are typically buds, open flowers and young fruits present at the same time between April and June.

Collecting floral headspace volatiles and flowering stems for visual cues

Except for *H. californica*, floral scents were collected for both greenhouse grown

plants as well as *in situ,* at the same sites where whole rooted plants were collected, using a portable volatile collection system (PVCS) described elsewhere (Park 2017). In summary, polyvinyl acetate bags (12 cm x 24 cm; Reynolds, Richmond, VA, USA) were purged of volatile contaminants in a drying oven at 140 °C for one hour prior to use. Each flowering stem was covered with a bag and tied using a cable tie and previously purged cotton balls. Opposite sides of each bag received a small cut for an inlet port attached to an activated charcoal filter and an outlet port attached to a volatile collection trap (30 mg of Porapak Q; Southern Scientific Inc., Gainesville, FL, USA in a glass pipette). A Rena 400 pump (RENA, Chalfont, PA, USA) powered by a Duracell Powerpack 600 (Duracell, Bethel, CT, USA) provided an airflow of 300 ml/min. Input and output flows were measured and balanced to maintain ambient pressure within the collecting bag. Prior to collecting, volatile collection traps were washed with methylene chloride (10 ml; EMD Millipore, Billerica, MA, USA), wrapped in aluminum foil and preconditioned for one hour at 140 °C. Floral scents were collected from five individual plants for each plant species and the control (an empty bag with charcoal prefilter) for three hours on a sunny day. After trapping for 180 minutes, the traps were removed and eluted with 200 µl of methylene chloride. Elutant was collected in a screw cap vial and stored at 4 °C until further use. Following the headspace volatile collection, individual flowering stems were placed into 10 cm transparent aqua-tubes in a portable cooler for later use as visual cues in bioassays.

Naïve overwintered *M. borraginis* (*n*=400) were shipped from CABI Switzerland to the University of Idaho in winter of 2013 and spring 2014. All weevils were kept at the quarantine laboratory at Washington State University in Pullman, WA, USA, in an

environmental chamber (E-30B, Percival Scientific, Perry, IA, USA; L18: D6 at 20 °C, and 60 % relative humidity (RH)) and fed fresh foliage and buds of *C. officinale.*

Behavioral bioassays with double stacked Y-tube device (D-SYD)

A double-stacked Y-tube (D-SYD) was used to test responses of the weevils to olfactory and visual cues from plants presented either individually or in combination. In brief, the D-SYD consists of two glass Y-tubes (4 cm Y-stem, 12 cm arms, 2 cm internal diameter) stacked on top of each other. One Y-tube, in which a weevil is released is used to test olfactory cues and the second Y-tube, immediately below it contains visual cues (for a detailed description, see (Park 2017)). For bioassays, the D-SYD was installed in an otherwise darkened room and illuminated by a single full spectrum light bulb (350 nm to 850 nm wavelength) (ES5M827FS, 27 Watt, Home Depot, Atlanta, GA, USA) diffused through a white polyethylene dome (40 cm x 30 cm x 20 cm) placed 20 cm above the D-SYD. Following each bioassay, the upper olfactory Y-tube was rinsed with 70 % ethanol to prevent potential effects of residual olfactory cues. The D-SYD was rotated 180° after every five trials to minimize potential left or right arm bias by female *M. borraginis*. The air flow rates in the two arms of the upper Y-tube were maintained at 300 ml/min using calibrated flowmeters (MR3000, Key Instruments, Hatfield, PA, USA), one on each inlet arm.

To examine the behavioral response of weevils to plant volatile organic compounds (VOCs), a 2 mm^2 square filter paper was placed in a plastic cap (Bel-Art Products 5, Bel-Art Products, Wayne, NJ, USA) in each arm of the olfactory Y-tube of the D-SYD. A 1-µl aliquot of eluted VOCs from a test plant (0.015 inflorescence-hours

provided over 5 min approximately equivalent to 0.3 inflorescences) or pure solvent (control) was pipetted onto each filter paper using a 10 µl manual syringe (Agilent Technologies, Sydney, Australia). The purified air was pushed from the Rena 400 pump into the D-SYD to plastic caps in the D-SYD through a 3 mm diameter Tygon tube (R-3603, Saint-Gobain Corp., Valley Forge, PA, USA). There was no evidence of effects of individual plants on weevil responses, so all responses were pooled for analysis.

For all bioassays, 20 female *M. borraginis* were tested with the individual female considered a single replicate. To address intraspecific plant variability of olfactory and visual cues, combinations of floral VOCs and flowering stems from *C. officinale* and the non-target species were changed every five trials. For each bioassay replicate, a single female weevil was placed on the initial release point in the upper Y-tube. When a weevil passed 3 cm into one arm of the D-SYD within 5 minutes it was considered to have made a decision (Tooker et al. 2005). All bioassays were conducted between 9:00 am and 4:00 pm at 20 to 23 °C and 50 % RH. Tests with purified air were used to confirm that the D-SYD was unbiased.

Experiment 1: Effect of visual cues

To evaluate weevil responses to visual cues from plant species, a small flowering stem (9 cm) of one of the non-target species and a flowering stem of *C. officinale* were placed in each arm of the lower Y-tube in the D-SYD. An individual weevil was released at the base of the Y in the upper Y-tube allowing them to perceive the flowering stems visually, through the glass tubing while preventing the perception of floral scents emitted from the flowering stems. To avoid potential anemotaxis (insect movement towards

headwind) (Farkas & Shorey 1972), there was no purified air flowing in the upper olfactory Y-tube during the behavioral bioassays. Tests with purified air and visual cues were conducted to ensure that the behavioral response of weevils did not differ from bioassays conducted without air flow.

Experiment 2: Effect of olfactory cues

To evaluate responses to olfactory cues, individual female weevils were presented with a choice between eluted headspace volatiles of each of the non-target species and headspace of *C. officina*le presented as described above in opposite arms of the olfactory level of the D-SYD.

Experiment 3: Combined visual and olfactory cues (=bimodal cues)

To test the effects of olfactory and visual cues combined, both plant cues were offered to weevils in the D-SYD, following methods as described above for the individual modalities.

Experiment 4: Mismatched bimodal cues

To test whether *M. borraginis* females can distinguish *C. officinale* from a native plant species with incorrect combinations of plant cues, we mismatched olfactory and visual cues from non-target plants and *C. officinale* in the D-SYD. For the purpose of this study, mismatched cues were defined as the combination of olfactory cues from *C. officinale* and visual cues from a non-target plant species placed in one side of the D-SYD, and the combination of olfactory cues from the non-target plant and visual cues

from *C. officinale* in the other side of the D-SYD.

Experiment 5: Combined cues vs. purified air (Control)

Responses of female *M. borraginis* to combined olfactory and visual cues from each plant species vs. pure air and no visual cue were measured to determine if weevils were attracted, were indifferent or were repelled. Methods were otherwise similar to those described above (*n*=20).

Electroretinography (ERG) recording

To investigate electrophysiologically active wavelengths of light to which female *M. borraginis* react, we conducted ERG. We assembled an instrument that consisted of a Xenon 75 watt short arc lamp (Oriel Instruments, Irvine, CA, USA) that can produce the full light spectrum, as the light source and a monochromator (Oriel Instruments, Irvine, CA, USA) that can transmit specific wavelengths of light from 330 nm to 850 nm at 10 nm intervals via a liquid light guide cable. The monochromator was controlled by Newport 74004 software (Oriel Instruments, Irvine, CA, USA). The aperture of the monochromator was set to 3.16 mm. The light beam from the monochromator entered the liquid light guide cable, which terminated 40 mm from the both compound eyes of decapitated female *M. borraginis*. The spectral intervals were presented for 1 second each, with an interval of darkness between them of 0.5 seconds. Each female weevil (*n*=20) was placed onto Spectra 360 electrode gel (Parker Laboratories, Fairfield, NJ, USA) in a sealed Petri-dish. The Petri-dish was kept in a dark box (5 cm x 5cm) for 20 minutes for dark adaptation of compound eyes. Female *M. borraginis* (*n*=20) were

decapitated using a sterilized scalpel under a dissecting microscope. A recording electrode was inserted underneath a compound eye while a ground electrode was inserted in the center between the two compound eyes (Crook et al. 2009). Electrical signals were amplified by an IDAC-232 box (Syntech, Hilversum, Netherlands) and Syntech EAG 2000 software (Syntech, Hilversum, Netherlands).

Floral reflectance spectra

To compare relative reflectance spectra of *C. officinale* and the three non-target plant species and correlate it to potential visual bioactive wavelengths in *M. borraginis,* flowers of each plant species were evaluated using a GER 2600 photo-radiometer (GER Corp., Millbrook, NY, USA). All measurements were taken on a sunny day for one hour around noon. Reflectance data were recorded between 330 nm (ultraviolet) and 900 nm (infrared) wavelengths of light. The reflectance of inflorescences was measured following the methods described by (Crook et al. 2009).

Headspace volatile organic compounds (VOC) analysis

We previously identified ten chemical compounds in the floral headspace of *C. officinale* that elicited electrophysiological signals from *M. borraginis* antennae based on gas chromatography-with electroantennographic detection (GC- EAD) (Table 1) (Park 2017). Gas Chromatography-Mass Spectrometry (GC-MS) analyses were conducted to examine floral volatile blends of the three non-target plant species investigated in this study for the presence of these ten bioactive compounds. We tested volatiles collected at field sites of respective non-target species and from greenhouse propagated plants. An

Agilent 7890A (Agilent Technologies Inc., Santa Clara, CA, USA) coupled with a Hewlett Packard (HP) 5973 mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA) and an HP-5MS column (30 m x 250 μm x 0.25 μm; Agilent Technologies Inc., Santa Clara, CA, USA) with helium gas were used. The initial oven temperature was 40 °C for 1 min. Temperature was increased at 5 °C/minute to 200 °C during the first ramp, at 10 °C/minute to 300 °C during the second ramp, and subsequently held at 300 °C for 2 minutes. A 1 μl aliquot was injected with 10 ng of nonyl acetate (W278807, Sigma-Aldrich) as an internal standard in splitless mode at 250 °C. Electron ionization (EI) mass spectra of each analyte were taken at 70 eV. Identification was based on matches with the NIST database and spectra and retention times of authentic compounds. One of the compounds detected, α-copaene, is enantiomeric. To determine the chirality of the isomer in floral headspace, the blend was analyzed using an Agilent J&W Cyclodex-B and retention time of the α-copaene in the blend compared with that of essential oils of two plant species that have predominantly one or the other enantiomer.

Statistical analysis

Behavioral responses of female weevils for each bioassay were analyzed assuming a completely random design using a generalized linear model based on a binomial distribution and a logit link function. Within this model we tested the hypothesis of an expected null ratio of 50: 50 between the test cue and its corresponding control. The hypothesis was assessed in all treatments through normalized scores using a Z test. This test assesses whether the logit link average of a given treatment equals zero, a condition

that occurs only when the proportion of cue and control responses are approximately equal. To examine whether there are differences in the behavioral responses of female *M. borraginis* to single plant cues (Experiments 1 & 2), and bimodal plant cues (Experiment 3) we compared the least square means using a single degree of freedom contrasts based on likelihood ratio chi-square tests. An additive effect is defined here as the least square mean of behavioral responses from the bimodal cue being equal to the sum of the behavioral responses from each olfactory and visual cues.

To examine the composition of electrophysiologically active olfactory and visual cues in *C. officinale* and the three non-target plant species, principal component analyses (PCA) were performed based upon 1) the ten electrophysiologically active compounds in *C. officinale* (Park 2017) and 2) the four electrophysiologically active wavelengths of lights identified in this study. Multivariate analysis of variance (MANOVA) was conducted to test whether relative floral reflectance differed among the four plant species for the four electrophysiologically active wavelengths of light. All analyses were conducted by using SAS version 9.4 (SAS Institute Inc, 2013).

Results

Host finding bioassays

Experiment 1: Effects of visual cues

Based on visual cues in the form of pieces of flowering stems, *M. borraginis* females preferred *C. officinale* over *A. occidentale* ($Z=-2.95$, $P=0.0032$) and *H. californica* ($Z=-2.77$, $P=0.0056$), but not *A. grande* ($Z=1.32$, $P=0.1867$; Fig. 1a-c, second bars from top).

Experiment 2: Effects of olfactory cues

Based on olfactory cues, female *M. borraginis* preferred *C. officinale* over each of the three non-target plants species: *A. grande* (Z=-2.13, *P*=0.0334), *A. occidentale* (Z=-2.48, *P*=0.0131), and *H. californica* (Z=-2.95, *P*=0.0032; Fig. 1a-c, third bars from top).

Experiment 3: Combined visual and olfactory cues (=bimodal cues)

When olfactory and visual cues were offered simultaneously, *M. borraginis* females preferred *C. officinale* over each of the three non-target plant species: *A. grande* (Z=-2.87, *P*=0.0041), *A. occidentale* (Z=-2.87, *P*=0.0041), and *H. californica* (Z=0, *P*=1; Fig. 1a-c, fourth bars from top). When comparing the strength of *M. borraginis* host discrimination between the bimodal cues and single plant cue modalities, there was an additive effect for *A. occidentale* (χ^2_1=1.28, *P*=0.2576) and synergistic effects for *A. grande* (χ^2_1=10.86, *P*=0.0010) and *H. californica* (χ^2_1=4.18, *P*=0.0409; Fig. 3a-c, right brackets).

Experiment 4: Mismatched bimodal cues

When olfactory and visual cues were mismatched in the D-SYD, *M. borraginis* females were no longer able to distinguish between *C. officinale* and the three non-target plant species: *A. grande* (Z=0, *P*=1), *A. occidentale* (Z=0, *P*=1) and *H. californica* (Z=0.45, *P*=0.6553; Fig. 1a-c, fifth bars from top).

Experiment 5: Combined cues vs. purified air (Control)

Mogulones borraginis females were attracted by the combined *C. officinale* plant cues when compared to purified air (Z=2.77, *P*=0.0056; Fig. 2, a top bar). In contrast, weevils responded with indifference to *A. occidentale* (Z=1.32, *P*=0.1867; Fig. 2, a third bar from top) and were repelled by the bimodal plant cues of *A. grande* (Z=2.77, *P*=0.0056; Fig. 2, a second bar from top) and *H. californica* (Z=2.13, *P*=0.0334; Fig. 2, a bottom bar).

Electroretinography and spectral reflectance of inflorescence

Four bioactive wavelengths of light were found that triggered an electrophysiological response from the compound eyes of female *M. borraginis* during ERG: 350 nm, 430 nm, 640 nm, and 830 nm. The mean floral reflectance curves and the relative reflectance differed between *C. officinale* and the three non-target plant species at each of the four bioactive wavelengths (MANOVA, Pilai's Trace=2.349, $F_{3,32}$=52.44, *P*<0.0001; Fig. 3). The principal component analysis for the four electrophysiologically active wavelengths separated the relative floral reflectance spectra of the four plant species (Fig. 4a). The first two principal components accounted for 98.84 % of the variation. The PC1, which signifies 82.45 % of the variability, consisted of 350 nm, 430 nm and 640 nm. The PC2 explains 16.39 % of the variation and is constructed mainly from 830 nm.

Headspace volatile organic compounds (VOCs) analysis

A total of 60 volatile organic compounds were identified from the floral

headspace of the four plant species using GC-MS (Table S1, Supplemental material). While a number of the ten electrophysiologically active chemical compounds in *C. officinale* were shared by the non-target plant species (Table 1), four chemical compounds were unique to *C. officinale,* (-)-α-copaene, (E)-β-farnesene, hexyl-acetate, and acetic acid, pentyl ester. Floral volatiles of *C. officinale* differed from those of the three non-target plant species based on the proportional composition and presence of the ten bioactive VOCs in field sites and greenhouse (Fig. 4b). The first two principal components (PC1 and PC2) explained 69.50 % of the variation based on the ten electrophysiologically active VOCs (Fig. 4b). PC1 accounted for 57.10% of the variability due to 2-heptanone, acetic acid, pentyl ester, hexyl-acetate, 4-hexen-1-ol, acetate, linalool oxide, (-)-α-copaene, (E)-β-farnesene and α-muurolene. The PC2 corresponds to 12.40% of the variation and correlated with benzaldehyde and phenylethyl alcohol.

Discussion

Potential benefits of incorporating host finding behavioral studies of biological control candidates into pre-release environmental safety assessments have been discussed frequently (e.g. Briese 2005; Heard 2000; Hinz et al. 2014; Knolhoff & Heckel 2014; Louda et al. 2003; Marohasy 1998; Schaffner 2001; Sheppard et al. 2005; Wheeler & Schaffner 2013), but rarely attempted. The few studies examining host finding behavior by potential biological control organisms have focused on volatile cues (Andreas et al. 2009; Cosse et al. 2006; Kafle 2016; Müller & Nentwig 2011) or visual cues (Müller & Nentwig 2011; Reeves et al. 2009; Reeves & Lorch 2009, 2011), but there are none that

consider these modalities together, despite their known importance for host finding (Miller & Strickler 1984), and their potential to act additively or synergistically (Harris & Miller 1982; Harris & Foster 1995).

We addressed this gap using *M. borraginis,* its target *C. officinale,* and three confamilial non-target plant species as a model system. We found that based on visual cues alone female *M. borraginis* preferred *C. officinale* over *A. occidentale* and *H. californica,* but did not distinguish between *C. officinale* and *A. grande*. In bioassays based on olfactory cues alone, *M. borraginis* females preferred *C. officinale* over all three non-target plant species. When both visual and olfactory cues were available to the weevils simultaneously, the preference by female *M. borraginis* for *C. officinale* over all three non-target plant species was stronger than to either plant cue modality presented alone. For two of these non-target species, *A. grande* and *H. californica,* the effect of combining cues in visual and olfactory modalities was statistically synergistic, while for *A. occidentale* the effect was additive. Furthermore, with combined cues, two of the non-target hosts, *A. grande* and *H. californica* became repellent to the weevil relative to pure air. This result confirms the importance of combinations of cues to allow greatest discrimination during host finding by *M. borraginis*, an effect that may be typical of other highly specialized herbivores. The repellency of *A. grande* cues is consistent with a field cage experiment in which *M. borraginis* were placed directly on a *A. grande* in a large field cage (2 x 2 x 1.6 m^3), and all had abandoned the plant within one hour, and were found instead on *C. officinale*, placed in the same cage (unpubl. data).

As an additional corroboration of the importance of multiple modalities for host selection, when visual and olfactory cues from the respective host plants were

mismatched in bioassays, a procedure enabled by our D-SYD bioassay system, the discriminating ability of *M. borraginis* was eliminated. This effect also showed that neither modality was sufficiently strong to override the other, and that they contribute to similar extents to host selection by *M. borraginis.* For example, both suppression and enhancement of odor and color were demonstrated based on different sets of olfactory and visual cues in the mushroom body of *Manduca sexta* (Balkenius et al. 2009).

This study also examined the specific visual and olfactory cues that contribute to discrimination, employing electrophysiological assessments to help explain behavioral responses by *M. borraginis*. Based on the four bands of sensitivity of the weevil's eye, as measured with electroretinography, the reflectance spectrum of the plants included in this study provides sufficient information to distinguish them. Similarly, a set of electrophysiologically active floral scent components potentially allows their separation based on a multivariate analysis and can explain discrimination by the weevils. Knowledge of the specific cues can help understand the basis of discrimination by extreme specialists like *M. borraginis*, contributing to theory, and has potential utility in assessing risks of attack to other non-target species based on their chemical and reflectance phenotypes, especially for test species which are hard to propagate and use in traditional host-specificity tests.

The bioassays employed in this study isolated specific aspects of host discrimination cues within visual and olfactory modalities. The weevil likely uses other information during host finding. For example, although our focus was on reflectance spectra, visual cues available in the field include the overall visual structure of the plant, which can be important during foraging (Degen & Städler 1997), the size of inflorescences (Weiss

1991) and the color and form of specific floral structures (Milet-Pinheiro et al. 2015; Peterson et al. 2015; Reeves & Lorch 2009). Ongoing studies are examining the use of these sorts of cues by *M. borraginis* during host finding.

Olfactory discrimination can be mediated by the presence of specific volatile compounds or the absolute and relative composition of volatile blends released by plants (Bruce et al. 2005; McCormick et al. 2014; Visser 1986). Based on the current study, specific compounds may be the primary mechanism used by *M. borraginis*. Four of the ten electrophysiologically active volatile compounds were unique to *C. officinale* among the plant species tested here. An assessment of five additional confamilial non-target species (unpubl. data) demonstrated that only (-)-α-copaene and (E)-β-farnesene, are unique to *C. officinale.* They are present in *C. officinale* headspace samples regardless of collection locations (field site or greenhouse) with little variation compared to most other compounds in the volatile profile (Table S1), which makes them excellent candidates as host specific cues. Both (-)-α-copaene (Dekker et al. 2011; Flath et al. 1994) and (E)-β-farnesene (Francis et al. 2004) are behaviorally active for other insect species. Females of a sibling species of *M. borraginis, M. crucifer* Pallas, which is also a specialist on *C. officinale,* were attracted to a single volatile compound in foliar headspace, methyl isovalerate, which is unique to *C. officinale* among 11 confamilial plant species tested (Kafle 2016), suggesting that *M. borraginis* may also use single compounds during host selection. Tests of these (E)-β-farnesene and (-)-α-copaene for activity as attractants for *M. borraginis* are merited.

The findings of this study are consistent with other evidence that responses of herbivorous insects to bimodal plant cues differ substantively from responses to cues of

single modalities (Graziosi & Rieske 2013), likely contributing to host recognition accuracy (Milet-Pinheiro et al. 2015) and efficiency at host location in environmentally complex systems. It seems likely that additional modalities, such as gustation would further increase host selection fidelity of *M. borraginis.* On the other hand, if a biocontrol agent is not responsive to olfactory and visual cues before landing on non-hosts, access to gustatory or tactile cues should be rare (Heard 2000; Miller & Strickler 1984).

Overall, our study demonstrates that female *M. borraginis* exploit olfactory and visual cues as bimodal cues to recognize and discriminate *C. officinale* from three North American non-target plant species, which are difficult to propagate to the appropriate phenotypic stage for conventional host-specificity testing. More importantly, to our knowledge, this is the first report illustrating how behavioral bioassays with visual and olfactory plant cues and associated electrophysiological studies can be effectively used in host-specificity assessments of biological weed control candidates. We propose that appropriate host finding bioassays and studies explaining results of such bioassays could constitute a valuable layer of host-specificity data, further improving the accuracy of environmental safety predictions of biological control candidates.

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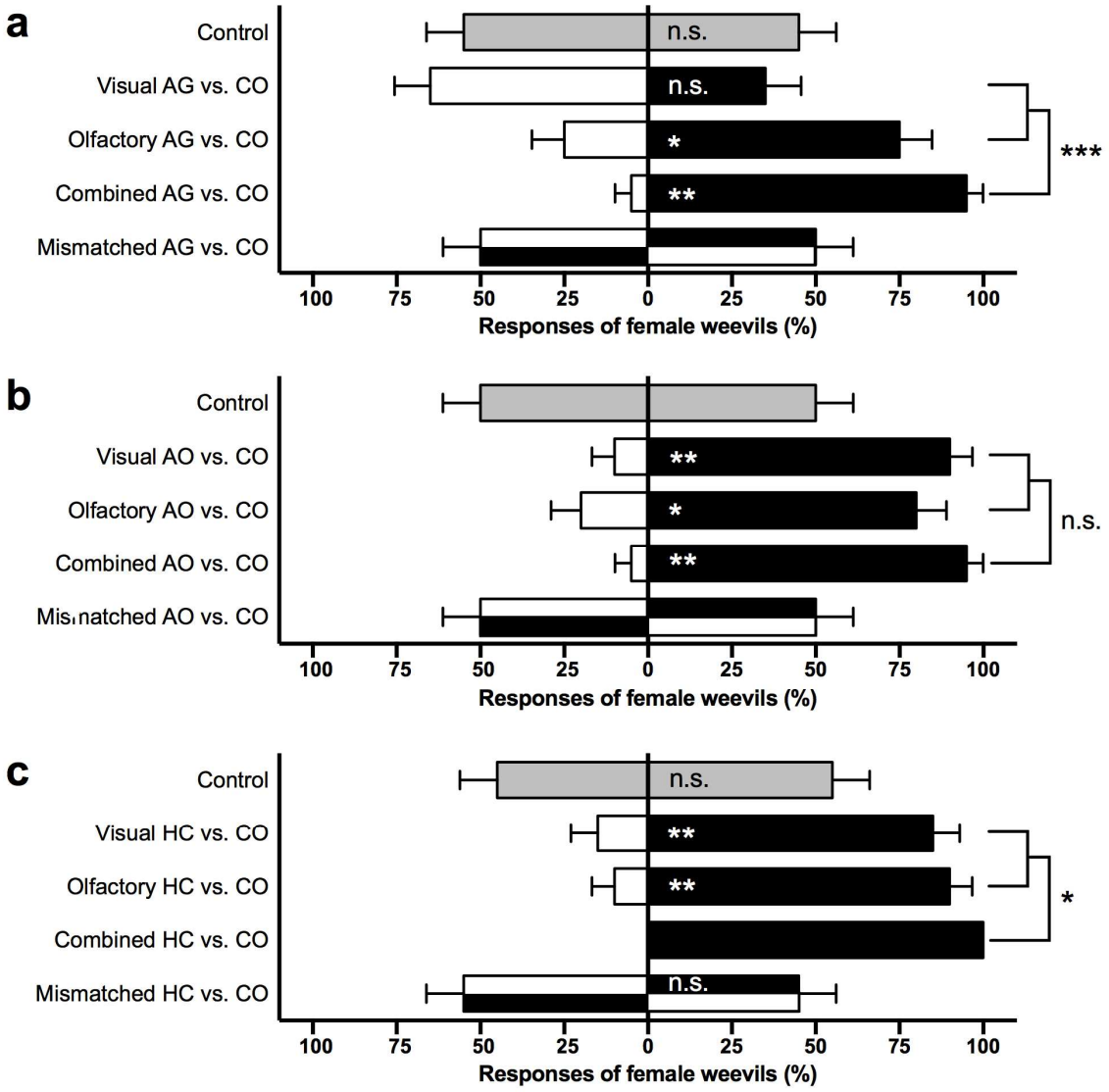

Figure 3.1: Proportional behavioral responses and SE of *Mogulones borraginis* in behavioral bioassays between *C. officinale* (CO)(black bars) and three native plant species (grey bars) (*n*=20 per bioassay): (a), *A. grande* (AG), (b), *A. occidentale* (AO), and (c), *H. californica* (HC). Proportion of females responding to unimodal, combined and mismatched plant cues. Control (white bars): both arms of the D-SYD contained purified air. Mismatched cues: olfactory and visual plant cues of *C. officinale* and one of the three plant species were mismatched in the double stacked y-tubes. Significance

levels of generalized linear model for individual bioassays: * $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s., not significant. Black brackets on the right side denote results of single degree of freedom contrasts test between the average of two single cue bioassays (second and third bars from top) and bimodal cue bioassay (fourth bars from top).

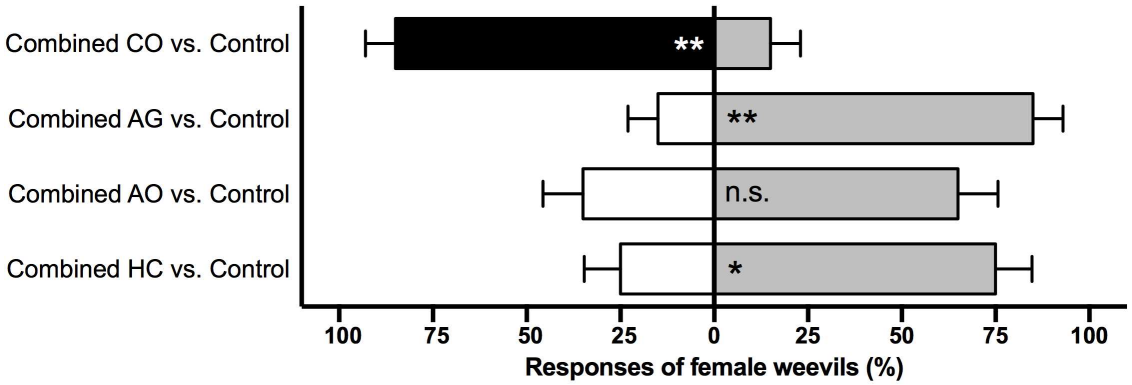

Figure 3.2: Behavioral responses and SE of female *Mogulones borraginis* in bioassays between bimodal (combination of olfactory and visual) plant cues and a control (purified air, white bars) for *C. officinale* (black bar) and three non-target plant species (grey bars) (*n*=20 per bioassay). CO: *C. officinale,* AG: *A. grande,* AO: *A. occidentale*, HC: *H. californica.* Significance levels of the generalized linear model for individual bioassays: * $P<0.05$, ** $P<0.01$; n.s., not significant.

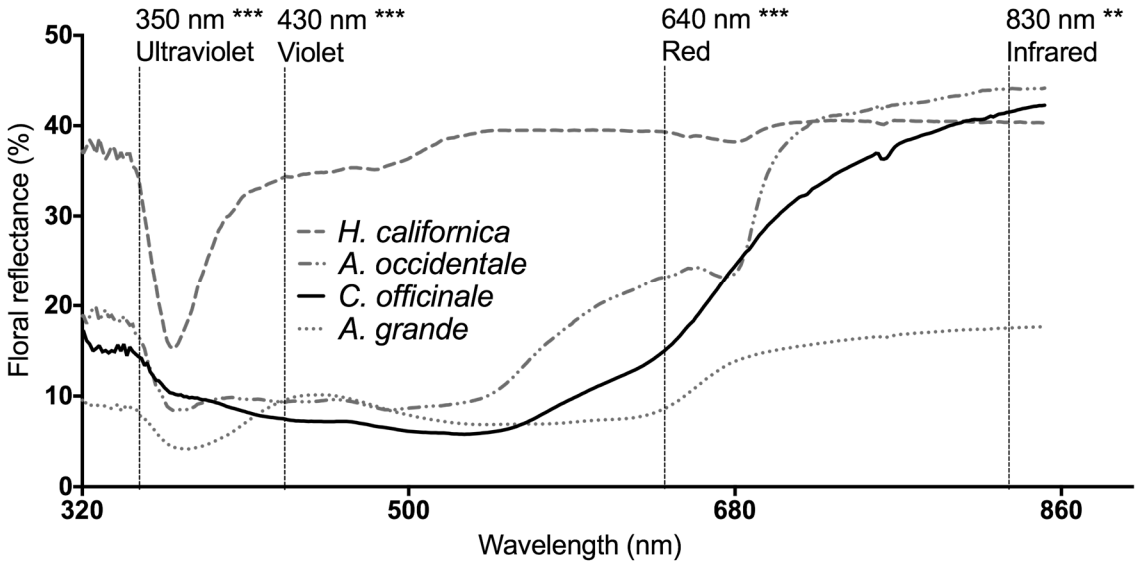

Figure 3.3: Average floral reflectance of four plant species compared to a white reference plate (n = 9 per plant species). Significance levels of MANOVA on differences between relative reflectance of electrophysiologically active wavelengths are denoted to the right of each wavelength: ** P<0.01, *** P<0.001.

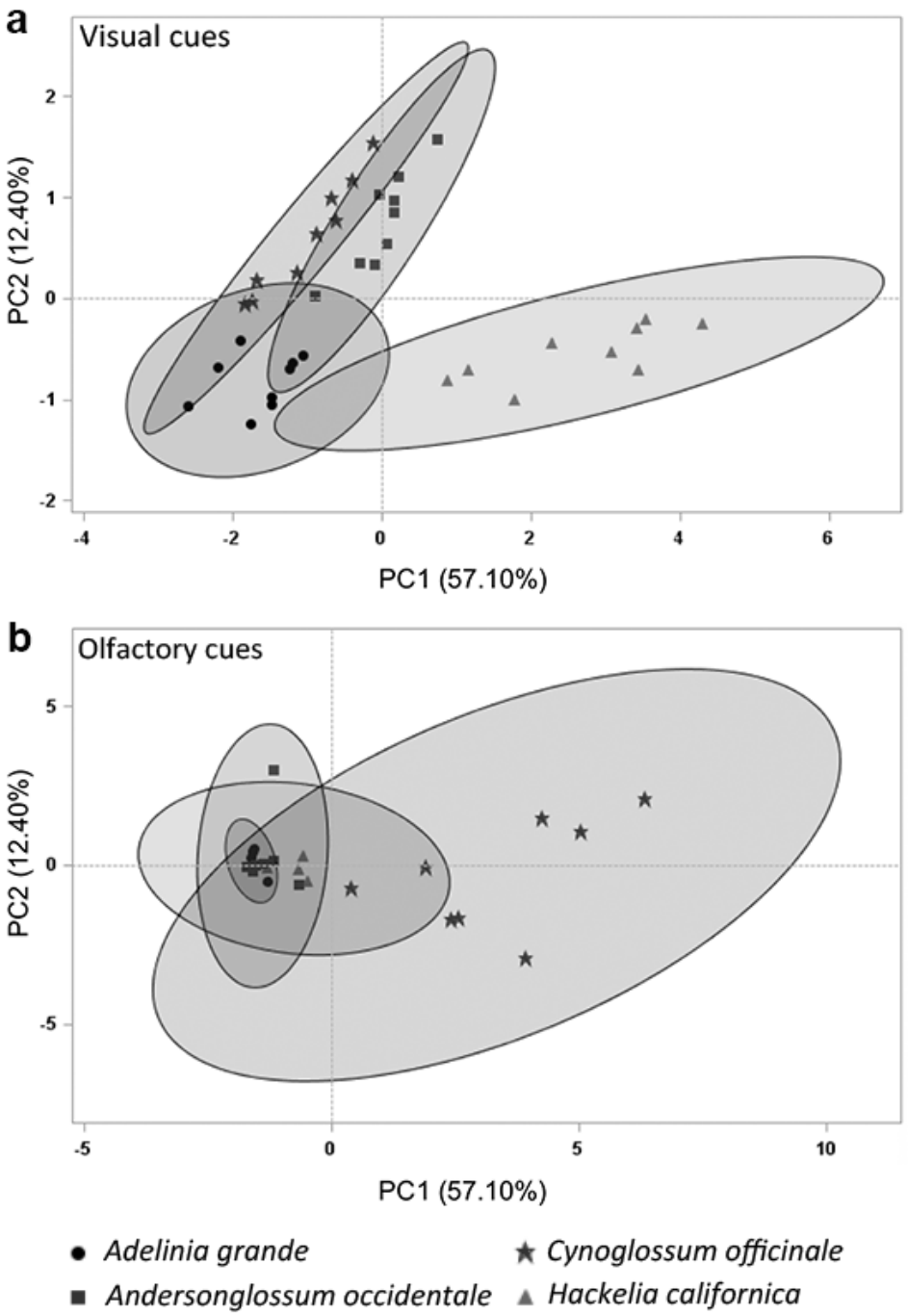

Figure 3.4: Principal component analysis (PCA) based on the floral relative reflectance from four electrophysiologically active wavelengths of light (**a**) and the quantity of ten electrophysiologically active floral volatile organic compounds (**b**). Ellipses represent 95% Confidence Intervals.

Table 3.1: Quantity (µg, mean ± SD) of electrophysiologically active floral volatiles emitted from four plant species. CO, *C. officinale*; AG, *A. grande*; AO, *A. occidentale*; HC, *H. californica*; F, field site; G, greenhouse; RT, retention time (min). Floral volatiles were isolated from Poropak Q and identified using GC-MS analysis with an internal standard.

Bioactive chemical compounds	RT	CO (F)	CO (G)	AG (F)	AG (G)	AO (F)	AO (G)	HC (G)
2-Heptanone	5.543	1.17 ± 1.43	1.97 ± 2.14	-	-	-	-	0.19 ± 0.39
Acetic acid, Pentyl ester	6.142	1.52 ± 2.07	0.47 ± 0.27	-	-	-	-	-
Benzaldehyde	7.265	0.54 ± 0.74	0.69 ± 0.60	0.36 ± 0.26	-	0.36 ± 0.43	-	0.64 ± 0.25
4-Hexen-1-ol, acetate	8.600	3.84 ± 5.02	1.60 ± 1.47	-	-	-	-	1.14 ± 0.54
Hexyl-acetate	8.786	3.57 ± 4.62	0.72 ± 0.47	-	-	-	-	-
Linalool oxide	10.867	2.11 ± 1.96	0.69 ± 0.54	-	2.72 ± 6.08	0.02 ± 0.03	1.21 ± 2.43	1.47 ± 1.01
Phenylethyl alcohol	11.557	0.55 ± 0.63	0.16 ± 0.10	-	-	-	-	0.04 ± 0.07
(-)-α-Copaene	18.710	1.69 ± 1.83	2.13 ± 1.51	-	-	-	-	-
(E)-β-Farnesene	20.799	1.33 ± 1.79	3.43 ± 3.03	-	-	-	-	-
α-Muurolene	21.849	3.13 ± 3.08	3.08 ± 1.99	0.51 ± 0.34	0.87 ± 0.26	2.20 ± 4.40	73.97 ± 45.92	0.20 ± 0.08

Summary and Conclusion

The studies included in this dissertation were conducted with the aim to evaluate the host fidelity of a potential biological control organism based on olfactory and visual cues in behavioral bioassays and electrophysiological experiments. The rationale for this rarely used approach in classical biological weed control is that in herbivorous insects host-plant finding, which precedes feeding and oviposition is mediated by olfactory and visual cues in nature. Thus, recognition or behavioral responses of herbivorous insects to these cues should help to predict the likelihood of nontarget attacks in the area of introduction of biological weed control agents. All three chapters of this dissertation present novel approaches and data on a model system on the applicability of this chemical ecological approach to host fidelity testing in biological control systems.

In the first chapter, weevils clearly distinguished between its field host *C. officinale* and a confamilial nontarget, *A. occidentale* using either floral scents (olfactory cue) or flowering stems (visual cue). *M. borraginis* responded synergistically when both cue were offered simultaneously compared to one cue modality alone. The relative strength of olfactory and visual cues was similar. When visual and olfactory cues were mismatched, weevils were no longer able to discriminate between the two plant species, and searching time in behavioral assays increased significantly.

In the second chapter, behavioral mechanisms and electrophysiologically-active olfactory cues were studied for *C. officinale* and four federally listed T&E confamilial plant species and the single-population species *D. daubenmirei.* Weevils were repelled by olfactory and visual cues from all T&E species tested when *C. officinale* was excluded

form bioassays. Similar to Chapter 1, *M. borraginis* distinguished *C. officinale* from all five plant species based on olfactory and visual cues in behavioral assays. Using GC-EAD and GC-MS, weevils elicited electrophysiological responses to ten semiochemicals in *C. officinale* of which two sesquiterpenes were only found in the floral scents of *C. officinale* regardless of volatile collection locations: (-)-α-copaene and (E)-β-farnesene. Behavioral bioassays were consistent with results of previous host range experiments for *D. daubenmirei* and *P. hirtus*.

In the third chapter, the behavioral responses of female weevils to olfactory and visual cues were investigated for *C. officianale* and three confamilial North American plant species that are not rare or threatened but exceeded the minimum seed volume for larval development, i.e.: *A. grande, A. occidentale,* and *H. californica*. The first two former congeners were considered to be the closest relatives of *C. officinale* in North America*. M. borraginis* responded indifferently to *A. occidentale* while weevils were repelled by *A. grande* and *H. californica* based on the combination of olfactory and visual cues. Using electroretinography, four electrophysiologically-active wavelengths of light were identified at 350 nm (ultraviolet), 430 nm (purple), 640 nm (red) and 830 nm (infrared). The relative reflectance spectra of the plant species differed significantly in these four bands. Likewise, among ten electrophysiologically bio-active floral scent components, (-)-α-copaene and (E)-β-farnesene were unique in *C. officinale* and may contribute to the discrimination of host plants by *M. borraginis.*

The results presented in this dissertation inform biological weed control and allow for improved predictions of the realized host range of biological control candidate species. Experiments presented here may provide interesting opportunities for additional

studies to test 1) bioassays identifying the basis for observed repellency based on olfactory and visual cues, 2) tactile and gustatory cues associated with host selection behavior using electrophysiological methodologies, and 3) behavioral plasticity and genetic variation among biological control candidate organisms. Therefore, the development of behavioral bioassays based on electrophysiologically active olfactory and visual cues proposed in this dissertation does complement our understanding on how biological control organisms discriminate between a targeted weed and closely related nontarget species.

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