THE ROLE OF OLFACTORY AND VISUAL CUES IN THE HOST SELECTION BEHAVIOR OF BIOCONTROL CANDIDATE SPECIALIST HERBIVORES FOR THE BIOLOGICAL CONTROL OF AN INVASIVE PLANT

A Thesis

Presented in Partial Fulfillment of the Requirements for the

Degree of Master of Science

with a

Major in Entomology

in the

College of Graduate Studies

University of Idaho

by

Bijay Subedi

Approved by:

Major Professor: Mark Schwarzländer, Ph.D.

Committee Members: Sanford D. Eigenbrode, Ph.D.; Philip Weyl, Ph.D.

Department Administrator: Edwin Lewis, Ph.D.

December 2021

ABSTRACT

Pre-release host range assessment of weed biological control agent (BCA) candidates typically rely on no-choice and choice feeding, oviposition, and development tests. However, these tests may exclude an environmentally safe BCA candidates from consideration if they can develop on nontargets that they would not colonize post-release because of the behavioral barriers. An accurate assessment of the post-release host range should, therefore, consider the host selection behavior. The seedpod weevil *Ceutorhynchus peyerimhoffi* and root-crown weevil *Ceutorhynchus rusticus* are two BCA candidates for the invasive Eurasian mustard *Isatis tinctoria.* Here, to assess the environmental safety of these weevils to native North American Brassicaceae plant species, I examined the behavioral response of *C. peyerimhoffi* to floral olfactory and visual cues, and *C. rusticus* to foliar olfactory and visual cues of *I. tinctoria* and selected native North American and Eurasian Brassicaceae plant species that supported larval development in previous oviposition and developmental tests. Results indicate that *C. peyerimhoffi* distinguishes *I. tinctoria* from the other tested confamilial plant species, including federally listed, threatened and endangered *Boechera hoffmannii*, during host finding using olfactory and visual cues. *Ceutorhynchus rusticus* is also able to distinguish *I. tinctoria* from the other tested nontargets using olfactory cues primarily and visual cues to some degree during the host finding. Based on these data, it appears unlikely that *C. peyerimhoffi* and *C. rusticus* would be drawn to the tested native North American confamilial plant species post-release, illustrating the utility of this approach as a component of environmental safety assessments of weed BCAs.

Acknowledgements

I cannot begin to express my thanks to Dr. Mark Schwarzländer, my major advisor, who has been supportive of my career goals and who worked actively to provide me with the protected academic time to pursue those goals.

Each of my Thesis Committee members has provided me with extensive personal and professional guidance and taught me a great deal about scientific research and life in general. Dr. Sanford D. Eigenbrode has taught me more than I could ever give him credit for here and has shown me, by his example, what a good scientist (and person) should be. I would also like to extend my deepest gratitude to Dr. Philip Weyl for his constructive advice on my research and writings and for providing many weevils to work on with.

Special thanks to Dr. Bill Price for helping with statistical analysis. I appreciate the help I received from Dr. Sanaz Safian, who lend me ASD FieldSpec-instrument to use as a part of my research.

I am grateful to all of the members of Dr. Schwarzländer lab (Sujan Panta, Karuna Nepal, Samikshya Aryal, Anjila Thapa, Jessica Fung, and Bradley L. Harmon) for their insightful suggestions and help in greenhouse and lab work. I would also like to extend my sincere thanks to members of Dr. Eigenbrode lab (Dane Elmquist, Ying Wu) for helping me with benchtop instruments. Financial support was kindly provided through: USDI, Bureau of Land Management Agreement L19AC00080 to MS.

Dedication

To my family!!

Table of Contents

Abstract ii

Acknowledgements iii

Dedication iv

Table of Contents v

List of Tables viii

List of Figures ix

Chapter 1: INTRODUCTION TO THE THESIS 1

Chapter 2: EXAMINING PRE-ALIGHTMENT HOST SELECTION OF THE BIOLOGICAL WEED CONTROL CANDIDATE *CEUTORHYNCHUS PEYERIMHOFFI* USING OLFACTORY AND VISUAL CUES 21

- Abstract 21
- Introduction 22
- Methods and Materials 25
- Results 32
- Discussion 37

Chapter 3: UNDERSTANDING THE PRE-ALIGHTMENT HOST FINDING OF *CEUTORHYNCHUS RUSTICUS* A BIOLOGICAL WEED CONTROL CANDIDATE FOR THE INVASIVE PLANT *ISATIS TINCTORIA* AS CONTRIBUTION TO PRE-RELEASE RISK ASSESSMENTS 52

- Abstract 52
- Introduction 53
- Methods and Materials 56
- Results 62
- Discussion 67

References 86

Appendix A: Summary statistics for initial choice (IC) and final choice (FC) of naïve and experienced peyerimhoffi females in a modified Y-tube choice bioassay with olfactory cues, visual cues and combined olfactory and visual cues of target *I. tinctoria*, and test plant species against control (purified air and/or empty arm), and combined olfactory and visual cues of test plant species against *I. tinctoria*..... 103

Appendix B: Summary statistics for the test of synergy for the naïve and experienced weevil's average response times in individual (olfactory and visual) cue bioassay vs combined cue bioassay for individual tested plant species, and contrast of sum of response times across all bioassays (olfactory cues, visual cues, and combined cues) between *Isatis tinctoria* and test species in a modified Y-tube choice bioassay with olfactory, visual and combined olfactory-visual cues of target *I. tinctoria*, and test species, and control (purified air and/or empty arm)..... 104

Appendix C: Floral reflectance and electroretinography (ERG) 104

Appendix D: Floral volatile organic compounds..... 111

Appendix E: Summary statistics for behavioral responses of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of target *I. tinctoria*, test plant species, and control (purified air and/or empty arm)..... 112

Appendix F: Summary statistics for Initial choice (IC) and final choice (FC) of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of target *I. tinctoria*, test plant species against control (purified air and/or empty arm)..... 113

Appendix G: Summary statistics for response times (RT) of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cue, visual cue, and combined olfactory and visual cues of target *I. tinctoria*, test plant species, and control (purified air and/or empty arm)... 114

Appendix H: Summary statistics for the test of synergy for the *C. rusticus* average response times in individual (olfactory and visual) cue bioassay vs combined cue bioassay for individual tested plant species, and contrast of sum of response times (RT) across all bioassays (olfactory cues, visual cues, and combined cues) between *I. tinctoria* and test

species in a modified Y-tube choice bioassay with headspace volatile organic compounds of target *I. tinctoria*, test plant species, and control (purified air and/or empty arm) 115

Appendix I: Summary statistics for behavioral responses of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of test plant species against respective cues of target *I. tinctoria* 116

Appendix J: Summary statistics for Initial choice (IC) and final choice (FC) of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of test plant species against respective cues of target *I. tinctoria* 117

Appendix K: Summary statistics for response times (RT) of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of test plant species against respective cues of target *I. tinctoria* 118

Appendix L: Foliar volatile organic compounds 119

Appendix M: Foliar reflectance 124

List of Tables

Table 1.1. List of selected North American test species along with their reason for inclusion in the experiment..... 18

Table 2.1. List of selected North American test species along with their reason for inclusion in the experiment..... 44

Table 3.1. List of selected North American test species and reason for inclusion in the experiment. ……..... 72

List of Figures

Figure 2.1: Modified Y-tube olfactometer set-up. In the center is the Y-tube (4 cm Y-stem, 12 cm arms, 2 cm internal diameter) resting on cylindrical plastic ring (diameter: 20 cm; height: 5 cm) with transparent plastic top**.** The cylindrical plastic ring consists of 2 openings (diameter: 3 cm) which serves the purpose of introducing visual cues (flowering sprigs) into the bioassay. The Rena ® 400 pumps were used to push the purified air with eluted VOCs into the Y-tube's arm and pull it out through the stem-end, and flowmeters regulated the amount of airflow into each Y-tube arm. The Y-tube olfactometer arena was illuminated using a full spectrum LED light by placing it directly above Y-tube. 1 µL eluted volatile organic compounds (VOCs) is applied to 2 mm^2 filter paper for each replication. Black arrow on the figure indicates the direction of airflow 45

Figure 2.2: Modified Y-tube device (4 cm Y-stem, 12 cm arms, 2 cm internal diameter) raised 5 cm from floor of the experimental arena using circular plastic ring (diameter: 20 cm; height: 5 cm) with 2 circular openings (diameter: 3 cm) to introduce visual cues. Experiments conducted in behavioral choice assays: **a** Olfactory cues of test plant species vs control (purified air) **b** Visual cues of test plant species vs control (empty arm) **c** Combined cues of test plant species vs control (purified air and empty arm) **d** Combined cues of test plant species vs *I. tinctoria*. Experiments **a**, **b**, and **c** conducted with both naïve and experienced weevils, whereas experiment **d** only with experienced weevils**.** Roman numerals in **a**, **b**, **c**, and **d**: i (test plant olfactory cues); ii (test plant visual cues); iii (*I. tinctoria* olfactory cues); iv (*I. tinctoria* visual cues); v (3 cm decision line); vi (weevil-release point)..... 46

Figure 2.3: Proportion of time spent (mean ± SE) by naïve and experienced female *C. peyerimhoffi* in a modified Y-tube choice bioassay with olfactory cues (**a, d**), visual cues (**b,**

e), simultaneously offered olfactory and visual cues (**c, f**) of test plant species (grey bars) and *I. tinctoria* (black bars) against control (purified air and/or empty arm, white bars), and by experienced female *C. peyerimhoffi* in a modified Y-tube choice bioassay with simultaneously offered olfactory and visual cues of test plant species (grey bars) against *I. tinctoria* cues (black bars) (**g**). Generalized linear mixed model (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s.: not significant). Numbers (N) on the top of each right bars indicates the sample size for that experiment.... 47

Figure 2.4: Proportion of naïve female *C. peyerimhoffi* (mean ± SE) initial (first) arm choice (IC) and final choice (FC) among two arms of the Y-tube in a modified Y-tube choice bioassay with olfactory cues (**a**), visual cues (**b**), simultaneously offered olfactory and visual cues (**c**) of test plant species (grey bars) and *I. tinctoria* (black bars) against control (purified air and/or empty arm, white bars). Generalized linear mixed model (n.s.: not significant). Numbers on the top of each bars indicates the number of naïve female weevils choosing for initial and final choice in the respective panel..... 48

Figure 2.5: Proportion of experienced female *C. peyerimhoffi* (mean ± SE) initial (first) arm choice (IC) and final choice (FC) in a modified Y-tube choice bioassay with olfactory cues (**a**), visual cues (**b**), simultaneously offered olfactory and visual cues (**c**) of test plant species (grey bars) and *I. tinctoria* (black bars) against control (purified air and/or empty arm, white bars), and simultaneously offered olfactory and visual cues of native North American confamilial plant species (grey bars) against *I. tinctoria* cues (black bars) (**d**). Generalized linear mixed model (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s.: not significant). Numbers on the top of each bars indicates the number of experienced female weevils choosing for initial and final choice in respective panel... 49

Figure 2.6: Mean (± SE) response times (RT) of naïve and experienced female *C. peyerimhoffi* in a modified Y-tube choice bioassay with olfactory cues (**a, d**), visual cues (**b, e**), simultaneously offered olfactory and visual cues (**c, f**) of test plant species (grey bars) and *I. tinctoria* (black bars) against control (purified air and/or empty arm, white bars), and of experienced female *C. peyerimhoffi* in a modified Y-tube choice bioassay with simultaneously offered olfactory and visual cues of test plant species (grey bars) against *I. tinctoria* cues (black bars) (**g**). Generalized linear mixed model (* $P<0.05$; n.s.: not significant). 50

Figure 2.7: Mean (±SE) response times of naïve and experienced female *C. peyerimhoffi* choosing the Y-tube cue offered arm in behavioral bioassays with visual cues, olfactory cues, and simultaneously offered olfactory and visual (combined) cues of *I. tinctoria* (black bars) and test plant species (grey bars) against control (purified air and/or empty arm). Generalized linear mixed model (* $P<0.05$, ** $P<0.01$; n.s.: not significant). Small brackets are a single degree of freedom contrast tests between the average of response times of individual cue bioassay (first and second bars from top on each species panel) and combined cue bioassay (third bars from top on each plant species panel, respectively). Big brackets indicate a single degree of freedom contrast tests between the combined response times in all the bioassays with the cues of *I. tinctoria* and test species... 51

Figure 3.1: Modified Y-tube olfactometer set-up. In the center is the Y-tube (4 cm Y-stem, 12 cm arms, 2 cm internal diameter) resting on cylindrical plastic ring (diameter: 20 cm; height: 5 cm) with transparent plastic top**.** The cylindrical plastic ring consists of 2 openings (diameter: 3 cm) which serves the purpose of introducing visual cues (flowering sprigs) into

the bioassay. 1 µL eluted volatile organic compounds (VOCs) is applied to 2 mm^2 filter paper for each replication. Black arrow on the figure indicates the direction of airflow. 75

Figure 3.2: Modified Y-tube device (4 cm Y-stem, 12 cm arms, 2 cm internal diameter). A single Y-tube resting on the cylindrical plastic ring (diameter: 20 cm; height: 5 cm) consisting of 2 openings (diameter: 3 cm) to introduce visual cues. Experiments in behavioral choice assays: Olfactory cues vs control (purified air) (**a**), Visual cues vs control (empty arm) (**b**), Combined cues vs control (purified air and empty arm) (**c**), Olfactory cues of test plant vs *I. tinctoria* (**d**), Visual cues of test plant vs *I. tinctoria* (**e**), Combined cues of test plant vs *I. tinctoria* (**f**). Roman numerals in **a**, **b**, **c**, **d, e, and f**: i (test plant olfactory cues); ii (test plant visual cues); iii (*I. tinctoria* olfactory cues); iv (*I. tinctoria* visual cues); v (3 cm decision-line); vi (weevil-release point)... 76

Figure 3.3: Proportion of time spent (mean ± SE) by female *C. rusticus* in experiment with olfactory cues (**a**), visual cues (**b**), and simultaneous olfactory and visual cues (**c**) of *I. tinctoria*, test plant species, and control treatments (purified air and/or empty arm; white bars). Black bar represent *I. tinctoria*. Grey bars represent confamilial nontarget plant species. Generalized linear mixed model, **P* < 0.05; n.s., indicates not significant. Numbers (N) on top of right bars indicates sample size for that experiment for year 1 and year 2, respectively.... 77

Figure 3.4: Proportion of time spent (mean ± SE) by female *C. rusticus* in experiment with olfactory cues **(a)**, visual cues **(b)**, and simultaneous olfactory and visual cues **(c)** of test plant species (grey bars) and *I. tinctoria* (black bars). Generalized linear mixed model; **P* < 0.05, ***P* < 0.001, ****P* < 0.001; n.s., not significant. Numbers (N) on top of right bars indicate replicate numbers for year 1 and 2, respectively..... 78

Figure 3.5: Proportion of initial (first) arm choice (IC) and final choice (FC) (mean ± SE) of female *C. rusticus* in experiment with olfactory cues **(a)**, visual cues **(b)**, and simultaneous olfactory and visual cues **(c)** of *I. tinctoria* (black bars), test plant species (grey bars), and control treatments (purified air and/or empty arm; white bars). Generalized linear mixed model; *$P < 0.05$; n.s., not significant. Numbers on the top of each bars indicates the number of female *C. rusticus* choosing that arm..... 80

Figure 3.6: Proportion of initial (first) arm choice (IC) and final choice (FC) (mean ± SE) of female *C. rusticus* in experiment with olfactory cues **(a)**, visual cues **(b)**, and simultaneous olfactory and visual cues **(c)** of test plant species (grey bars), and *I. tinctoria* (black bars). Generalized linear mixed model; *$P < 0.05$; n.s., not significant. Numbers on the top of each bars indicates the number of female *C. rusticus* choosing for initial and final choice in respective panel..... 82

Figure 3.7: Mean (± SE) response times (RT) of *C. rusticus* females *rusticus* in experiment with olfactory cues (**a**), visual cues (**b**), and simultaneous olfactory and visual cues (**c**) of test plant species (grey bars) and *I. tinctoria* (black bars) against control (purified air and/or empty arm, white bars). Generalized linear mixed model (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s.: not significant..... 83

Figure 3.8: Mean (± SE) response times (RT) of *C. rusticus* females *rusticus* in experiment with olfactory cues (**a**), visual cues (**b**), and simultaneous olfactory and visual cues (**c**) of test plant species (grey bars) against respective cues of *I. tinctoria* (IT, black bars). Generalized linear mixed model (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s.: not significant..... 84

Figure 3.9: Mean (± SE) for the contrast of sum of response times (RT) across all bioassays (olfactory cue (year 1, year 2), visual cue (year 1, year 2), and combined cues (year 2))

between *Isatis tinctoria* and individual tested test plant species in a modified Y-tube choice bioassay with headspace volatile organic compounds of target *I. tinctoria*, test plant species, and control (purified air and/or empty arm). Black bars represent *I. tinctoria* and grey bars indicate nontarget species. Generalized linear mixed model followed by single degree of freedom contrast between *I. tinctoria* and selected nontargets species (black brackets) (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s.: not significant)..... 85

Chapter 1: INTRODUCTION TO THE THESIS

Alien invasive plants

Alien plant species, which after their introduction to a new natural or semi-natural habitat outside their native range, can form self-sustaining populations without human aid, are referred to as naturalized plant species (Richardson et al. 2000). About 4% of the world's currently known vascular flora (i.e., >13,000 plant species) have become naturalized in at least one region outside their native distribution range (Rejmánek 2015; van Kleunen et al. 2015). Of all naturalized plant species, only a small proportion, i.e., about one percent, have negative impacts in their new natural or semi-natural habitat (Williamson and Fitter 1996). When naturalized alien plants cause impacts, they can be referred to as alien invasive plants (AIP) (Milanović et al. 2020). Negative impacts can include replacement of native biota or depletion of soil and water resource (D'Antonio and Vitousek 1992; DiTomaso 2000; Mack et al. 2000; Pysek et al. 2012; Pysek and Richardson 2010; Vilà et al. 2011), ultimately threatening native biodiversity and ecosystem services such as nutrient cycling or water availability (Pysek et al. 2012; Vilà et al. 2011), native herbivore dynamics (Bezemer et al. 2014), climate regulation, and fire regimes (D'Antonio and Vitousek 1992). AIPs can also cause significant economic losses in agriculture and forestry (Pejchar and Mooney 2009; Pimentel et al. 2005; Rai et al. 2020). In addition, management of AIPs have been proven costly (DiTomaso 2000; Simberloff et al. 2013).

Conventional control strategies such as chemical control (use of herbicides; DiTomaso 2000), mechanical control (hand-pulling, mowing, tilling; Mack et al. 2000) and cultural control methods (prescribed burning, grazing; DiTomaso 2000) are widely used for the management of AIPs (Culliney 2005; Sheley et al. 2011). Their outcomes can range from

complete success to ineffective and often they are considered costly or unfeasible depending upon the scale of the invasions, accessibility of invaded areas, terrain invaded and agricultural or ecological value of invaded areas (Culliney 2005; Mack et al. 2000; Sheley et al. 2011). Classical biological control can be an environmentally more benign and cost-effective alternative to conventional control means for IAPs (Clewley et al. 2012; McFadyen 1998; Schwarzländer et al. 2018).

Classical biological control of weeds

Classical biological control of weeds (BCW) is the reunion of co-evolved host-specific natural enemies with an IAP in the invaded range to reduce the competitiveness, reproductive output or population growth of the targeted invasive plant (Müller-Schärer and Schaffner 2008; Schwarzländer et al. 2018). The ecological basis for BCW is the enemy release hypothesis (ERH), which posits that exotic plant species introduced to a new region experience a decrease in natural enemy pressure, which results in an increase in distribution and abundance (Elton 1958; Keane and Crawley 2002). Because BCW, if successfully implemented, is self-perpetuating, with biocontrol organisms actively dispersing, the control strategy has the potential to cover large, remote and topographically challenging areas, which makes it extremely cost-effective (Clewley et al. 2012; Culliney 2005; McFadyen 1998). As of 2014, 551 biological control agents (hereafter BCA) have been intentionally released against more than 220 invasive weeds in 130 countries and most of these releases occurred in the USA, Canada, Australia, South Africa, and New Zealand (Winston et al. 2014). At least some level of control has been recorded for 66% of all the targeted invasive plants across all the countries and regions (Schwarzländer et al. 2018).

However, the release of BCAs to manage invasive weeds is not without risks (Louda 2000; Louda et al. 2005; Simberloff and Stiling 1996a; Strong 1997). Without extensive pre-release host range that assess and reliably predict a BCA candidate's post-release host range, BCA releases have the potential to cause negative nontarget effects (directly on other plant species and indirectly on the respective ecosystems) (Simberloff 2011). Direct nontarget effects include utilization by BCA of native flora (De Clercq et al. 2011). For example, two frequently cited direct nontarget attack cases are *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) attacking native *Opuntia* species in the USA (Simberloff and Stiling 1996b), and the seed-feeding weevil *Rhinocyllus conicus* (Froel.) (Coleoptera: Curculionidae), attacking native thistle species in the genus *Cirsium* in the USA (Louda 2000). However, in the former case, *C. cactorum* was not intentionally introduced but likely accidentally moved via plant nursery trade from the Dominican Republic to Florida (Hinz et al. 2020). In the latter case, pre-release host range testing included only very few native plant species but more importantly, despite the broader host range of the weevil this was not regarded as a problem in the 1960s when *R. conicus* was investigated and considered for release (Hinz et al. 2020; Suckling and Sforza 2014). In addition, native thistles were socio-economically not valued in the 1960s and potential feeding on native thistles was not regarded problematic (Schaffner et al. 2001).

Indirect nontarget effects of BCAs include ecosystem impacts, for example, apparent competition with native herbivores, trophic cascades, and indirect mutualism, but these effects can be subtle and difficult to predict (Paynter et al. 2020). Based on the BCA release data, both Suckling and Sforza (2014) and Hinz et al. (2019) concluded that less than 1% of weed BCAs released to date have known population-level adverse nontarget effects on nontarget

plant species. Releases of BCAs that did result in population-level nontarget effects occurred mostly in the 1950s and 1960s when host range testing protocols for BCW were at their infancy and when native flora was not considered during host range testing (Hinz et al. 2019; Suckling and Sforza 2014). If *R. conicus* would have been held to present day host specificity testing standards, the insect would clearly not have been approved for release in the USA (Hinz et al. 2020; Murphy and Evans 2009).

Pre-release host specificity testing

Because of the potential for nontarget attack on native plant species, the most important aspect of any weed biocontrol program is to ensure the environmental safety of any BCA candidate (Hinz et al. 2019). The host specificity must be evaluated pre-release, i.e., before the BCA is introduced to a new environment (Briese et al. 2002; Heard 2000). Many different types of pre-release host specificity tests need to be conducted with closely related confamilials to assess their post-release environmental risk of a candidate BCA (Hinz et al. 2020; Schaffner 2001). A test plant species list is typically generated based on the centrifugal phylogenetic method (Wapshere 1974; Schaffner 2001). The centrifugal phylogenetic method states that plant species more closely related to the target species are more likely to support BCA development than distantly related plant species (Wapshere 1974; 1989). Closely related plant species are assumed to have more similar phytochemical and morphological characteristics, which would make it less likely that insect herbivores discriminate against these close relatives compared to more distantly related plant species (Briese and Walker 2002; Wheeler and Schaffner 2013). Typically, plant species representing all taxa of the target plant family are represented in the test plant list and more closely related plant taxa are chosen. Plant species are tested in the order of relatedness to the target plant until the host

range of the BCA candidate is adequately described (Wapshere 1974). In addition to closely related confamilial plant species, plant species with similar plant chemistry traits, plants of economic importance (as crops or ornamentals), and rare, threatened and endangered plant species within the family of the targeted invasive plant are also included in the test plant list (Schaffner 2001; Wapshere 1974).

Pre-release host range assessments of BCA candidates typically rely on feeding and developmental tests to determine their physiological and ecological host ranges (Heard 2000; Schaffner 2001). The physiological host range, determined through no-choice tests, comprises plant species on which the larvae can complete development. The ecological host range, determined through different type of choice tests (in cages, field cages or in the open), comprises plant species used under natural conditions by a species (Schaffner 2001). In no-choice tests only one plant species is offered to a BCA candidate, whereas in choice tests, the BCA candidate is offered a choice between the target plant species and one or more test plant species (Schaffner 2001). When combined, physiological and ecological host range data should be adequate to predict the post-release environmental risk of BCAs (Hinz et al. 2019; Schaffner 2001).

The centrifugal phylogenetic method has been successfully used for more than four decades to generate test plant lists in BCW because the test plant lists are based on phylogenetic relatedness (Simberloff 2011). Recent rearrangements of plant phylogenies (e.g., Bailey et al. (2006), Beilstein et al. (2006), Beilstein et al. (2008), Al-Shehbaz (2012 in the Brassicaceae family)) have reduced previous classification limitations (Al-Shehbaz 2012) and allowing more precise host range predictions for BCA candidates. Refined classification has also shown that no single or few characters adequately delineate plant species to a specific

lineage or tribe (Al-Shehbaz 2012), i.e., common plant traits, such as fruit morphology, secondary metabolites, plant architecture, may not be phylogenetically conserved. Rapo et al. (2019) found strong feeding intensity associated with phenotypic trait similarity more so than genetic similarity. That study suggests that phenotypic traits, e.g., secondary metabolites, can in some instances be better predictors of host preference for specialist herbivores than phylogenetic relatedness (Hinz et al. 2008; Wheeler and Schaffner 2013). Phylogenetically disjunct host ranges — the development of an insect herbivore on a distantly related plant species — has been demonstrated in specialist insect herbivores (Hinz et al. 2008). For example, *Ceutorhynchus cardariae* Korotyaev (Coleoptera: Curculionidae), a gall-forming weevil associated with the Eurasian mustard *Lepidium draba* L (Brassicaceae), developed successfully on the native North American confamilial *Caulanthus anceps* Payson, a plant species only distantly related to the weevil's field host *L. draba* (Hinz et al. 2008) but with four glucosinolates shared with *L. draba* (Rapo et al. 2019). This example demonstrates that additional studies into the host selection behavior and host utilization in relation to plant phenotypic traits could benefit risk evaluations and more accurately predict post-release nontarget risks (Simberloff 2011, Hinz et al. 2019, 2020). For many insect herbivores, host volatile organic compounds (VOC) and floral and/or foliar visual cues play an important role during host finding (Bernays and Chapman 1994). For example, *Ceutorhynchus obstrictus* (Paykull) (Coleoptera: Curculionidae), a crucifer-feeding insect concerning olfactory cues (Bartlet et al. 1997), *Ceutorhynchus cardariae* Korotyaev (Coleoptera: Curculionidae), a BCA candidate for *Lepidium draba* (L.) Desv (Brassicaceae), concerning visual cues (Rendon 2019), *Mogulones borraginis* (F.) (Coleoptera: Curculionidae), a BCA candidate for

Cynoglossum officinale L. (Boraginaceae), concerning visual and olfactory cues (Park et al. 2018).

Discrepancies between physiological and ecological host range data

Discrepancies between physiological and ecological host range data exist (Schaffner et al. 2018). The physiological host range is considered a safe and conservative estimate for potential nontarget attack of BCAs, however, release decisions based solely on the physiological host range can lead to the rejection of otherwise safe BCAs candidates (Hinz et al. 2020; Schaffner 2001). This is because the physiological host range, determined through no-choice tests, often indicates a much broader host range and typically overestimates the risks for nontarget attack (Schaffner 2001). On the other hand, the ecological host range, determined through choice tests, is typically much narrower than the physiological host range and often consists of a subset of the plant species within the physiological host range (Schaffner et al. 2018). The difference between no-choice and choice host specificity tests is that the latter allow a BCA candidate to express its host selection behavior, i.e., the insect herbivore can utilize different plant cues to provide it with information regarding plant identity and quality, which, under no-choice condition, may be distorted or by-passed (Schaffner et al. 2018). Studies on the host selection behavior that mediates host recognition via examination of host plant cues – could potentially explain the differences between conventional physiological and ecological host specificity data (Hinz et al. 2014; Hinz et al. 2019; Wheeler and Schaffner 2013).

Catenary process of host plant selection

Finding, examining, and accepting are the three main stages of insect host selection (Bernays and Chapman 1994; Miller and Strickler 1984). Different plant cues such as visual, olfactory, mechanosensory, and gustatory cues mediate the host selection process by providing information regarding host location (Couty et al. 2006), host defense characteristics (De Moraes et al. 2001), host nutritional quality and identity (Bruce et al. 2011; Tasin et al. 2011), all of which together determine the acceptance or rejection of a plant for feeding and/or oviposition (Bernays and Chapman 1994; Bruce et al. 2005; Miller and Strickler 1984; Reeves 2011). Among all cues available, insect herbivore uses only a few during the pre-alightment host finding stage, i.e., those that provide the insect with the most accurate information and that are least costly to assess during the host selection process (Fawcett and Johnstone 2003). In most cases, olfactory cues are essential in the early host finding stages as many insects can perceive them from both short (Egonyu et al. 2013) and long (Ballhorn et al. 2013) distances. In addition, olfactory cues can help insect herbivores through early signaling of potential predation risks or saving energy costs through avoidance of landings on unsuitable hosts (Fawcett and Johnstone 2003).

Insects can utilize visual cues alone or in combination with olfactory cues during the host finding stage (Bernays and Chapman 1994; Reeves 2011). Few phytophagous insects (Stenberg and Ericson 2007) and many flower visitors (Kinoshita et al. 2017) are known for using visual cues to locate the host. Insects could also benefit from the integration of different plant cues (Silva and Clarke 2019); for example, pollinators (e.g., Raguso and Willis 2005), parasites (e.g., Bradbury and Bennett 1974), parasitoids (e.g., Jang et al. 2000), and

herbivores (e.g., Park et al. 2018) can respond to the cues in synergistic (Park et al. 2018; Raguso and Willis 2005) or additive manner (Park et al. 2018).

The host selection behavior of insects has been studied extensively with herbivorous insects (Knollhof and Heckel 2014). However, few studies have addressed the role of olfactory or visual plant cues in the host selection of weed biological control candidates (Wheeler and Schaffner 2013; but see Andreas et al. 2009; Cosse et al. 2006; Fung et al. 2021; Müller et al. 2011; Park et al. 2018; Reddy et al. 2009). Even fewer studies have been conducted investigating the role of visual and olfactory cues together by BCA candidates (Park et al. 2018; Park et al. 2019). The IAP *Isatis tinctoria* L. (Brassicaceae) and its two BCA candidates *Ceutorhynchus rusticus* Gyllenhal (Coleoptera: Curculionidae) and *Ceutorhynchus peyerimhoffi* Hustache (Coleoptera: Curculionidae) (Weyl et al. 2019), provide a suitable system to study the host selection behavior for these two BCA candidates using visual and olfactory plant cues simultaneously.

Study system

Isatis tinctoria

Isatis tinctoria L. (Brassicaceae), commonly known as dyer's woad, is a biennial or short-lived perennial herbaceous mustard native to Eurasia (Farah et al. 1988). The plant has a characteristic two-layered rooting pattern comprised of a taproot and lateral roots. The combined total root length can vary from about 2.2 m to 2.6 m in North America (Zouhar 2009).

Plants germinate in autumn months or in the spring (Farah et al. 1988) and form rosettes with waxy, slightly pubescent, oblanceolate to elliptic basal leaves. To form

reproductive meristem from vegetative meristem, *I. tinctoria* rosettes require vernalization (i.e., temperature below 4ºC for 4-7 weeks) (Asghari et al. 1992). After snowmelt from mid-April to the end of May, plants develop up to 20 stems with lanceolate to elliptic aerial leaves when bolting (West and Farah 1989). Stems are typically 50 cm to 90 cm tall, (range: 35 cm to 120 cm), usually glabrous throughout and paniculately branched with an inflorescence at the tip (Zouhar 2009).

Flowering occurs between April-June (FNA 2021). Numerous compound racemes bear yellow-colored flowers (individual flower width: 6 mm and length: 3.5 mm), forming a terminal panicle. Plants set seed in May-June (Gibson 2017). Fruits are pods and are hairless or shortly hairy winged indehiscent silicles (length: 8 mm - 18 mm, width: 2.5 mm - 7 mm) hanging freely from a deflexed or recurved pedicel. The color of the pod changes from green to black at maturity. Each pod contains one seed, rarely two, which is oblong or ovate and yellowish to light brown. Seed dispersal takes place from end of July through the first snowfall (Farah et al. 1988) aided by human, wildlife, wind, and rain (Callihan 1990; Pokorny and Krueger-Mangold, 2007). *I. tinctoria* reproduces sexually through seed production and vegetatively through adventitious buds on root crown and upper roots (Zouhar 2009; Callihan et al. 1990 and references therein).

Introduction of Isatis tinctoria into *North America*

The initial introductions of *I. tinctoria* were most likely deliberate for cultivation and dye production. Later introductions were accidental as contaminant of alfalfa seed shipments (McConnel et al. 1999). The plant was first reported from Virginia and adjacent states during the colonial period (Varga and Evans 1978), Brigham City, Utah, in 1910 (McConnel et al. 1999), and Siskiyou County, California, in the early 20th century (Young and Evans 1977). *I.*

tinctoria is only distributed sporadically throughout Illinois, Maryland, Missouri, New Jersey, New York, Virginia, and West Virginia (Callihan et al. 1984; USDA NRCS 2021) but is persistent and invasive in western USA states (USDA NRCS 2021). Dyer's woad is considered particularly problematic in two distinct geographical areas heavily invaded by the weed in southern Oregon and northern California and southern Idaho and northern Utah (Gaskin et al. 2018) because of negative impacts on native flora and losses in crop and rangeland productivity (FWS 2006; Jacobs and Pokorny 2007). It is a declared noxious weed in Arizona, California, Colorado, Idaho, Montana, New Mexico, Nevada, southern Oregon, Utah, Washington, and Wyoming (USDA NRCS 2021).

Negative effects of Isatis tinctoria *invasions*

I. tinctoria exhibits great morphological and physiological plasticity in response to environmental factors and performs well under harsh and nutrient-poor conditions (Monaco et al. 2005; Sonmez et al. 2008). The plant competes successfully with native plant communities and causes decreases in native flora abundance (DiTomaso et al. 2013). For example, habitats of the endangered *Phlox hirsuta* E. E. Nelson (Yreka phlox) Polemoniaceae and *Calochortus persistens* Ownbey (Siskiyou mariposa lily) Liliaceae are threatened by invasions of *I. tinctoria* in northern California (Diggles et al. 2003; FWS 2006) through competition and allelopathic properties (FWS 2011). Infestation of *I. tinctoria* in 75.2% of known habitat of *C. persistens* was determined by surveys conducted in 2003 (FWS 2012).

In addition to ecological effects, *I. tinctoria* also has negative economic impacts in the USA. In Utah, the dispersal rate of *I. tinctoria* doubled within ten years between 1971 and 1981 and reduced crop and rangeland productivity accounting for a loss of $2 million to the state (Evans and Chase 1981; Evans and Dewey 1994). The United States Department of

Interior (USDI) Bureau of Land Management (BLM) reported an annual 14% increase of invaded areas on rangelands in the Pacific northwestern USA (Jacobs and Pokorny 2007). Cattle grazing capacity is reduced by 38% on western USA rangelands due to *I. tinctoria* invasions (Jacobs and Pokorny 2007; Young 1988). Between 1969 and 1985, a 35-fold increase of *I. tinctoria* invaded areas was recorded by the United States Department of Agriculture (USDA) Forest Service for the Intermountain West region (USDA, 1986).

Management of Isatis tinctoria *invasions*

Cultural, mechanical, and chemical control strategies are used to manage *I. tinctoria* invasions. Grazing with sheep is an option for *I. tinctoria* management but is not considered particularly successful as aboveground biomass reduction rates reported for sheep grazing are low (DiTomaso 2000; West and Farah 1989). In some cases, *I. tinctoria* seems to be avoided by livestock (DiTomaso 2000). The plant is considered unpalatable even though there are no reported cases of livestock poisoning or *I. tinctoria* toxicity (DiTomaso et al. 2013; Young 1988). Mechanical control strategies such as tilling, mowing, clipping, hand-pulling, or digging are generally practiced on very small spatial scales (Evans and Chase 1981). While successful at those scales, they are considered ineffective for managing large area infestations (Callihan 1990). Prescribed burning is not recommended for the management of *I. tinctoria* as the outcomes of this management practice are not well understood (DiTomaso et al. 2013). Fire effectively removes aboveground biomass but has little effect on the root system or the soil seedbank, which may facilitate quick reestablishment of *I. tinctoria* populations (Zouhar 2009).

Herbicides are the most common approach to control *I. tinctoria* infestations, using herbicides such as 2,4-D, metsulfuron, chlorsulfuron, and imazapic aminocyclopyrachlor plus

chlorsulfuron (Callihan 1990; DiTomaso et al. 2013; Evans and Dewey 1994; Jacobs and Pokorny 2007; PNW 2016; Varga and Evans 1978). Some herbicides are restricted to be used only in certain habitats because of concerns about adverse nontarget effects, such as chlorsulfuron plus aminocyclopyrachlor in California and some Colorado counties (DiTomaso et al. 2013; PNW 2016). The use of herbicides to manage large scale *I. tinctoria* invasions in rangelands, pastures, and inaccessible terrains is often not considered because of its cost and the low agricultural value of rangeland (DiTomaso 2000; Kropp et al. 1995).

Biological control

The dyer's woad rust, *P. thlaspeos* Ficinus & Schubert, was federally approved in 2002 as a biological control organism for the management of *I. tinctoria* infestation in the USA (EPA 2002). *Puccinia thlaspeos* 'woad strain' infects *I. tinctoria* during spring after the germination of teliospores and production of basidiospores (Flint et al. 1993). Infected rosettes show symptoms of chlorosis, leaf distortion, and stunted growth and produce spermatia and telia on the underside of leaves (Gibson 2017; Kropp et al. 1995). Upon bolting, plants infected with *P. thlaspeos* 'woad strain' produce reproductive stems that remain vegetative with distorted and chlorotic leaves (Flint and Thomson 2000). Infected plants produce flowers and viable seeds but in low numbers (McConnel et al. 1997). In a field survey experiment by Gibson (2017), more than 97% overall impact on seed production of *I. tinctoria* by *P. thlaspeos* 'woad strain' was found. Application of *P. thlaspeos* 'woad strain' inoculum is effective in reducing *I. tinctoria* plant densities (Kropp and Darrow 2006) as the infection often leads to plant sterilization, i.e., the prevention of floral and seed production (Daines 1988). Currently, the rust fungus is not commercially available for several reasons, including natural presence of *P. thlaspeos* 'woad strain' in the infestations of *I. tinctoria* in the

Intermountain West, expiration of a permit for the production of commercial product (i.e., ‘woad warrior’), and lack of a commercial backer (Gibson 2017). However, because *P. thlaspeos* ‘woad strain’ is well established throughout the Intermountain West, it can be collected from infected *I. tinctoria* plants in the field (Gibson 2017; Winston et al. 2014).

Classical biological control agent candidates under consideration for the management of Isatis tinctoria

Classical biological control agents for *I. tinctoria* have been investigated since 2004 at CABI in Switzerland (Hinz et al. 2014). Surveys conducted at 40 field sites across European and western Asian countries resulted in nine BCA candidates (Hinz et al. 2007; Hinz et al. 2005). However, only four BCA candidates were studied in greater detail: the root-mining weevil, *Aulacobaris fallax* Brisout, the root-crown weevil, *Ceutorhynchus rusticus* Gyllenhal, the seedpod weevil, *Ceutorhynchus peyerimhoffi* Hustache (all three, Coleoptera: Curculionidae), and the stem-mining flea beetle, *Psylliodes isatidis* Heikertinger (Coleoptera, Chrysomelidae). Following host specificity testing, *A. fallax* and *P. isatidis* investigations with these BCA candidates were suspended because of insufficient host specificity of these beetles: *A. fallax* attacked most of the native North American species tested (16 of 39 in no-choice tests and 7 of 17 in multiple-choice tests) (Gerber et al. 2009), and *P. isatidis* supported larval development on economically important crops (i.e., *Brassica rapa* L., *B. juncea* (L.) Czern, *B. napus* L.) and a federally threatened and endangered (T&E) listed native North American plant species (*Boechera hoffmannii* (Munz) Al-Shehbaz) (Hinz et al. 2014). Host specificity testing with the root-crown weevil *C. rusticus* and the seed-feeding weevil *C. peyerimhoffi* are underway at CABI and show promising control potential (Weyl et al. 2019).

Experimental host range of Ceutorhynchus rusticus

Host specificity testing with the root-crown weevil, *C. rusticus* was conducted between 2005 and 2021. Host specificity tests included no-choice, choice, open field oviposition and larval development tests. A total of 142 plant species has been tested, including 37 European and 105 native North American plant species. Under no-choice conditions, 14 native North American species in eight genera supported the development of *C. rusticus* to adulthood (Weyl et al. 2019). However, in open-field tests conducted between 2009 and 2018 on those 14 native North American species, only limited oviposition was observed on nine test plant species, often on individual replicates. *C. rusticus* preferred *I. tinctoria* in all host specificity tests conducted (Weyl et al. 2019). Host specificity data suggest a narrow physiological host range and an even narrower ecological host range for *C. rusticus* (Weyl et al. 2019).

Test plant species attacked during no-choice tests included confamilial species in the following six tribes across three Brassicaceae lineages[1]: Descurainieae, Euclidieae, Eutremeae, Isatideae, Sisymbrieae, and Thelypodieae (BrassiBase 2021). The Descurainieae (genus: *Descurainia* Webb & Berthel) belongs to lineage I, the Eutremeae (genus: *Eutrema* R. Br), Isatideae (genus: *Isatis* L.), Sisymbrieae (genus: *Sysimbrium* L.), and Thelypodieae (genera: *Caulanthus* S. Wats., *Stanelya* Nutt.) belong to lineage II, and the Euclidieae (genus: *Braya* Sternb. & Hoppe) belongs to lineage III (Table 1.1; BrassiBase 2021). Development of the weevil on species in the genera *Sysimbrium*, *Caulanthus*, *Stanelya*

[1]Chloroplast gene *ndhF* was used to determine the lineages. Beilstein et al. (2006) reconstructed the phylogeny of the gene using parsimony, likelihood, and Bayesian methods. Genera grouped into monophyletic groups are called lineages.

(lineage II) could be expected according to the centrifugal phylogenetic method, which states that plant closest related to the target AIP are more likely to be attacked by BCA candidates and/or support their development (Wapshere 1974). However, the development of the weevil on *Descurainia* (lineage I) and *Braya* (lineage III) is not in accordance with centrifugal phylogenetic method predictions indicating a phylogenetically disjunct physiological host range of *C. rusticus*.

Experimental host range of Ceutorhynchus peyerimhoffi

Host specificity investigations with *C. peyerimhoffi* have been conducted between 2008 and 2021. In no-choice host range tests with more than 120 test plant species (88 native to North America), 34 test species were accepted by weevils for oviposition (Weyl et al. 2019). In no-choice development tests with 29 out of these 34 test plant species, four species (three native to North America: *Braya alpina* var *americana* Hooker, federally threatened and endangered (T&E) listed *Boechera hoffmannii* (Munz) Al-Shehbaz, *Caulanthus heterophyllus* (Nutt.) Payson, and one Eurasian plant species: *Isatis glauca* L.) supported larval development to some degree (Weyl et al. 2019). Whereas in the multiple-choice cage tests, very few eggs/larvae were recorded on those three native North American confamilial plant species, compared to the target *I. tinctoria* (Weyl et al. 2019). *Isatis glauca* is a congener of the target *I. tinctoria*, and *C. heterophyllus* belongs to the same lineage as *I. tinctoria* (lineage II). Therefore, larval development on these test species was in line with the centrifugal phylogenetic method. But *B. alpina* (lineage III) and *B. hoffmannii* (lineage I) belong to different lineages than *I. tinctoria* (lineage II) (Table 1.1) again indicating a potentially phylogenetically disjunct physiological host range of *C. peyerimhoffi*.

For both these BCA candidates the comprehensive conventional host specificity data suggest a broader physiological host range compared to the respective ecological host ranges (Hinz et al. 2016, Weyl et al. 2017, Weyl et al. 2019). One potential reason for the observed difference between the physiological and ecological host range data may be that under open-field testing conditions, a BCA candidate can freely exercise choice (Schaffner et al. 2018). With the free expression of host selection, the BCA candidates could have utilized visual and/or olfactory plant cues to assess plant quality and plant taxonomic identity. In contrast, no-choice condition could have distorted or bypassed components of host-selection, leading to a distorted assessment of the insect's ecological host range (Schaffner et al. 2018). Studying the host selection behavior of the two BCA candidates could potentially help interpret the differences between physiological and ecological host specificity data and explain why distantly related plant species are considered part of the host range of these specialist insect herbivores (Hinz et al. 2014; Hinz et al. 2019).

Objectives

The objectives of this research are to 1) investigate the host selection behavior *C. peyerimhoffi* and *C. rusticus* in response to olfactory and visual cues of select confamilial plant species to more accurately predict the ecological host range of both BCA candidates, and 2) assess the risk of the nontarget effect of *C. peyerimhoffi* and *C. rusticus* with regard to select native North American plant species. Here, the potential role of visual and olfactory cues during the insect herbivore pre-alightment host selection was studied.

Table 1.1 List of selected North American test species and reason for inclusion in the experiment.

Lineages[1]	Tribe[1]	Species[1]	Flowering time[2]	Elevation (m)[2]	Distribution[2]	Life history trait[2]	Native range	Reason for inclusion
Lineage I	Boecherae	*Boechera hoffmannii* (Munz) Al-Shehbaz	Feb–Mar	0-100	United States (California/Santa Cruz Island in Santa Barbara County)	Annual[5]	North America[5]	T & E; Oviposition and larval development[5,6,7,8,9]
	Descurainieae	*Descurainia nelsonii* (Rydb.) Al-Shehbaz & Goodson	late May-mid Jul	800-3000	B.C., Calif., Idaho, Mont., Nev., Oreg., Wash., Wyo	Annual[4]	North America[4]	Non-host; Limited feeding and oviposition[4,5]
		Descurainia californica (A. Gray) O. E. Schulz	Jun–Aug	1700-3400	Ariz., Calif., Colo., Nev., N.Mex., Oreg., Utah, Wyo	Perennial[4]	North America[4]	Non-host; Limited feeding and oviposition[4,5]
	Lepidae	*Lepidium sativum* L.	Apr–Aug	N/A	Introduced; Alta., B.C., Man., Nfld. and Labr. (Nfld.), N.W.T., N.S., Ont., P.E.I., Que., Sask., Conn., Idaho, Iowa, Maine, Md., Mass., Mich., N.H., N.Y., Ohio, Oreg., Pa., R.I., Tenn., Wash., Wyo., Europe, SW Asia, perhaps NE Africa, introduced also in South America (Argentina), Australia	Annual[8]	South-west Asia (perhaps Iran)[3]	Limited feeding and adult development[8]

[1](BrassiBase 2021), [2](FNA 2021), [3](Sabaghnia et al. 2015), [4](Hinz et al. 2012), [5](Hinz et al. 2014), [6](Hinz et al. 2015), [7](Hinz et al. 2016), [8](Weyl et al. 2017), [9](Weyl et al. 2019); T & E: Threatened and Endangered, Alta.: Alberta, Ariz.: Arizona, B.C: British Columbia, Colo.: Colorado, Conn.: Connecticut, Ill.: Illinois, Kans.: Kansas, Man.: Manitoba, Mass.: Massachusetts, Mich.: Michigan, Mo.: Missouri, Mont.: Montana, N Africa: North Africa, N.Dak.: North Dakota, Nev.: Nevada, N.H.: New Hampshire, Pa.: Pennsylvania, Nfld. And Labr (Nfld.): Newfoundland and Labrador, N.W.T.: Northwest Territories, Ont.: Ontario, Que.: Quebec, Calif.: California, N.Mex.: North Mexico, N.Y.: New York, Oreg.: Oregon, P.E.I.: Prince Edward Island, R.I.: Rhode Island, Sask.: Saskatchewan, Tenn.: Tennessee, Va.: Vancouver, Wash.: Washington, WC California: West Coast California, W.Va.: Washington Vancouver, Wyo.: Wyoming, C, SW Asia: Central, Southwest Asia

Lineage II	Isatideae	*Isatis tinctoria* L.	Apr-Jun	300-2200	B.C, Nfld. and Labr. (Nfld.), Ont., Que., Calif., Idaho, Ill., Mo., Mont., Nev., N.Mex., N.Y., Oreg., Utah, Va., Wash., W.Va., Wyo., Europe, C, SW Asia, N Africa, South America (introduced; Chile, Peru)	Annual, biennial or Perennial[4]	Eurasian[4]	Host plant[8,9]
		Isatis glauca Aucher ex Boiss.	N/A	N/A	N/A	Perennial[5]	Eurasian[5]	Non-host; Limited feeding and oviposition, adult emergence[5,6,8]
	Eutremeae	*Eutrema salsugineum* (Pall.) Al-Shehbaz & Warwick	May–Jun	600-2500	B.C., N.W.T., Sask., Yukon, Colo., Mont., C, E Asia	Annual[5]	North America[5]	Non-host; Limited feeding and oviposition, adult emergence[5,6,8]
	Sisymbrieae	*Sisymbrium linifolium* (Nutt.) Nutt. ex Torr. & A. Gray	Apr–Aug	700-2800	B.C., Ariz., Colo., Idaho, Mont., Nev., N.Mex., Oreg., Utah, Wash., Wyo.	Perennial[6]	North America[6]	Non-host; Limited feeding; supported larval development[6,7,8,9]
	Thelypodieae	*Caulanthus flavescens* (Hook.) Payson	Mar-May	200-700	United States (WC California)	Annual[7]	North America[7]	Non-host; Limited feeding and oviposition, adult emergence[7,8,9]
		Caulanthus heterophyllus (Nutt.) Payson	Mar-May	0-1400	Mexico (Baja Calif.), United States (SW Calif.).	Annual[2]	North America	Non-host; Limited feeding; supported larval development[5,6,7]
		Stanleya pinnata (Pursh) Britton *(Stanleya pinnata* var. *pinnata)*	Apr–Sep	200-2500	Ariz., Calif., Colo., Idaho, Kans., Mont., Nev., N.Mex., N.Dak., Oreg., S.Dak., Utah, Wyo.	Perennial[4]	North America[4]	Non-host; Limited feeding and oviposition, adult emergence[4,5,7,8]
		Stanleya tomentosa Parry	Jun–Aug	1300-2300	Idaho, Wyo.	Biennial, Perennial[5]	North America[5]	Non-host; Limited feeding and oviposition, adult emergence[5,6,7,8,9]

		Stanleya viridiflora Nutt.	May–Jul	1300-2700	Calif., Colo., Idaho, Mont., Nev., Oreg., Utah, Wyo	Perennial[4]	North America[4]	Non-host; Limited feeding and oviposition, adult emergence[4,5,7,8]
Lineage III	Euclidieae	*Braya alpina* var *americana* Hooker *(Braya glabella* subsp. *Glabella)*	Jun–Jul	0-3700	North America	Perennial[8]	North America[8]	Non-host; Limited feeding and oviposition, larval development[8]

Chapter 2: EXAMINING PRE-ALIGHTMENT HOST SELECTION OF THE BIOLOGICAL WEED CONTROL CANDIDATE *CEUTORHYNCHUS PEYERIMHOFFI* USING OLFACTORY AND VISUAL CUES

Abstract

Pre-release host specificity testing can reliably predict the environmental safety of weed biological control agent candidate (BCA) species, but typically does not consider their pre-alightment host discrimination. Incorporating pre-alightment behavior into pre-release testing could improve predictions of post-release host ranges of BCA candidates. We addressed this topic with the seedpod weevil *Ceutorhynchus peyerimhoffi*, a BCA candidate for the Eurasian mustard *Isatis tinctoria* that is invasive in the USA. Behavioral responses of naïve and experienced female *C. peyerimhoffi* weevils to olfactory, visual, or combined plant cues of the native North American confamilial nontarget plants *Braya alpina*, *Caulanthus heterophyllus* and the federally listed *Boechera hoffmannii* were compared to responses to *I. tinctoria* or control (purified air and/or empty arm) treatments using a modified Y-tube olfactometer device. Both naïve and experienced weevils responded with attraction to both olfactory and combined cues of *I. tinctoria*, whereas only experienced weevils were attracted to *I. tinctoria* visual cues. In contrast, there was no attraction by either naïve or experienced weevils to nontarget plant cues, except for attraction to combined visual and olfactory cues of *C. heterophyllus* by experienced weevils. Visual cues of *B. alpina* and *B. hoffmannii* were repellent to experienced weevils, and olfactory cues alone of *B. alpina* repelled naïve weevils. Weevils responded faster to simultaneous cues of *I. tinctoria* compared to single cues, whereas the response time did not differ between individual cues and combined cues for confamilial nontarget species. Weevils also responded faster to the cues of *I. tinctoria* compared to cues of nontargets when presented against control treatments. We conclude that

C. peyerimhoffi uses visual and olfactory cues to discriminate between its host plant *I. tinctoria* and North American nontargets. Based on our data, it appears unlikely that *C. peyerimhoffi* would be drawn to the nontargets *B. alpina* or *B. hoffmannii* post-release, illustrating the utility of this approach as a component of environmental safety assessments in weed biological control.

Introduction

One of the most important aspects of classical biological weed control programs is the assessment and assurance of the environmental safety of biological control agent (BCA) candidate species (Hinz et al. 2020), which must be evaluated prior to release into a new environment (Heard 2000; Hinz et al. 2020). The host range of a BCA is typically assessed through no-choice, choice, field cage and open field experiments (Schaffner 2001). No-choice tests are used to assess the physiological host range, which comprises plant species on which the larvae can complete development; choice tests (in cages, field cages or in the open) are used to assess the ecological host range, which comprises plant species used under natural conditions by a species (Schaffner 2001). However, the determination of a BCA's physiological and ecological host range using no-choice and choice tests provides little information about its pre-alightment host selection behavior (Hinz et al. 2014; Park et al. 2018, 2019), which is the behavior expressed by an insect in the early stages of host selection process when foraging for a host.

The ecological host range often constitutes a subset of the physiological host range. That is potentially the case because it allows an insect to express its host-choice behavior to the degree the testing conditions allow, and these choices may limit the plant species that support development to those that are attractive or otherwise acceptable to the insect

(Schaffner 2001; Schaffner et al. 2018). Understanding the pre-alightment host selection behavior of a BCA candidate could help explain the discrepancies between physiological and ecological host range data (Heard 2000; Park et al. 2018), and more reliably predict potential risks for nontarget plant attack (Hinz et al. 2014; Hinz et al. 2019).

Pre-alightment host selection by an herbivorous insect involves a sequence of steps: searching, finding, and locating (Bernays & Chapman, 1994). In herbivorous insects, volatile organic compounds (VOCs) emanating from plants as olfactory cues and specific wavelengths of light reflected from plant surfaces as visual cues are known to mediate the pre-alightment stage of the host selection process (Bernays and Chapman 1994). An insect herbivore's physiological and behavioral responses to these host plant cues determine early whether a plant is accepted or rejected (Balkenius et al. 2009). Understanding an insect herbivores' preferences to plant cues may help to predict whether a given plant species would be sought out as host under field conditions, and thus may improve our ability to predict whether nontarget plant species are at attack risk of attack should a BCA be released (Heard 2000; Park et al. 2018, 2019; Hinz et al 2019).

The host selection behavior of insects has been studied extensively in the context of interactions between pollinators and flowers (Leonard and Masek 2014) and insect pest management (Szendrei and Rodriguez-Saona 2010), but few studies have addressed the role of host selection in the context of weed biological control host range assessments (Wheeler and Schaffner 2013 but see Andreas et al. 2009; Cosse et al. 2006; Fung et al. 2021 [olfactory cues]; Müller and Nentwig 2011; Reddy et al. 2009 [visual cues]). Since both, olfactory and visual plant cues are important during host finding it would be ideal to consider both plant cue modalities individually and simultaneously (Park et al. 2018; Park et al. 2019). The Eurasian

herbaceous mustard *Isatis tinctoria* L. (Brassicaceae), which is invasive in the USA along with select native North American confamilial nontarget plant species and *Ceutorhynchus peyerimhoffi* Hustache (Coleoptera: Curculionidae), a BCA candidate considered for *I. tinctoria,* provide an interesting system to investigate the host selection behavioral responses to visual and olfactory plant cues.

Isatis tinctoria (dyer's woad) is a Eurasian annual or biennial mustard that is invasive in the western United States (Gaskin et al. 2018) and a declared noxious weed in 11 U.S. states (USDA NRCS 2021). Invasions reduce crop and rangeland productivity (Evans and Chase 1981; Young 1988), and cattle grazing capacity (Jacobs and Pokorny 2007). *I. tinctoria* performs well under harsh and nutrient-poor conditions (Monaco et al. 2005), which enables the invasive plant to compete well with native plants and decrease their abundance (Diggles et al. 2003). Cultural, mechanical, and chemical control measures for the management of *I. tinctoria* invasions on rangelands and natural areas are widely used but can be unfeasible depending on the size, accessibility, and terrain of invasions (DiTomaso et al. 2013). The seedpod weevil *C. peyerimhoffi* is under consideration as a BCA for the control of *I. tinctoria* in the United States (Weyl et al. 2019). Female *C. peyerimhoffi* feed on inflorescences and lay single eggs into the developing pods of *I. tinctoria*, and the hatched larvae feed on the seeds in the pod as it matures (Cortart et al. 2008). *C. peyerimhoffi* attack can reduce seed output of *I. tinctoria* by up to 98.5% through adult feeding and larval mining (Hinz et al. 2016).

In no-choice host range tests with more than 120 test plant species (88 native to North America), 34 test species were accepted by weevils for oviposition. In no-choice development tests with 29 out of these 34 test plant species, four species (three native to North America: *Braya alpina* var *americana* Hooker, the United States Fish and Wildlife Service

federally threatened and endangered (T&E) listed *Boechera hoffmannii* (Munz) Al-Shehbaz, *Caulanthus heterophyllus* (Nutt.) Payson, and one Eurasian congener of the targeted invasive plant species, *Isatis glauca* L.) supported larval development to some degree (Weyl et al. 2019). In multiple-choice cage tests, very low number of eggs/larvae were recorded on those three native North American confamilial plant species, compared to the target *I. tinctoria* (Weyl et al. 2017). There may be behavioral and/or ecological filters, for example host and non-host plant cues, that govern the weevil's host selection and oviposition behavior and as a result restrict its ecological host range.

The aim of this study is to inform pre-release host specificity assessments for *C. peyerimhoffi* through behavioral bioassays using visual and olfactory plant cues. We were especially interested in investigating the host selection behavior of *C. peyerimhoffi* in response to confamilial nontarget plant species that supported development of the weevil to a certain degree in previous host range investigations. We hypothesized that *C. peyerimhoffi* uses both, visual and/or olfactory plant cues to discriminate nontarget plant species from *I. tinctoria* during pre-alightment host selection that were attacked in no-choice larval development tests. We tested whether *C. peyerimhoffi* females respond to visual and olfactory cues of confamilial nontarget plants in the absence of cues of its Eurasian host, *I. tinctoria*, and whether *C. peyerimhoffi* females prefer visual and olfactory cues of *I. tinctoria* over the respective cues of native North American confamilial plant species.

Methods and Materials

Insects

Adult *C. peyerimhoffi* were shipped from CABI Switzerland to the quarantine facility at the University of Idaho, Moscow, ID in March 2019 (n = 300) and April 2020 (n = 241),

respectively. Weevils were maintained in an environmental chamber (I-35 VL, Percival Mfg. Co., Boone, Iowa) at 17 ºC during the day and 8 ºC at night, 60-70% RH, and 16:8 h L:D. Fresh *I. tinctoria* flowers were fed to weevils as needed. Since these *C. peyerimhoffi* were allowed to feed on *I. tinctoria* flowers, they were termed 'experienced' during bioassays. In April 2020, a shipment of neonate *C. peyerimhoffi* weevils in cocoons was received from CABI Switzerland and kept at 5 ºC in a rearing chamber (Percival Scientific Incubator, Model C-30, Percival Scientific, Inc., Perry, Iowa) for emergence. Sixty-seven adult *C. peyerimhoffi* emerged from this rearing and since these weevils had no feeding experience, they were used as 'naïve' in bioassays.

Plants

Three North American nontarget species confamilial to *I. tinctoria* were selected to study the pre-alightment host selection behavior of *C. peyerimhoffi*: *B. alpina, B. hoffmannii*, and *C. heterophyllus* (Table 2.1). These plant species were selected because they supported *C. peyerimhoffi* in no-choice developmental tests (Weyl et al. 2019). In addition, *B. hoffmannii* is restricted to few locales in San Bernardino and San Diego counties, California, with the largest populations occurring on Santa Rosa and Santa Cruz Islands in Santa Barbara County, California (FNA 2021). The plant is a federally listed threatened and endangered species in the USA (ECOS 2021).

All plant species were grown from seeds received from CABI Switzerland. Plants were grown in 3-liter black plastic pots (diameter: 25 cm, height: 17 cm; T-pot Three, Stuewe and Sons, Inc, Tangent, OR, USA), using the following soil mix: 1-part sand and 3-part Sunshine Mix No. 4 (Sun Gro Horticulture Canada Ltd). The following nutrient supplements were added to every 12 kg mixture of soil and sand: trace elements: 2.5 g (FRIT Industries,

Inc., Ozark, Alabama); chelated iron: 1.25 g (Grow More Inc., Garden, California); limestone: 47.5 g (Grow More Inc., Garden, California); triple super phosphate: 47.5 g (Bonide Products Inc., Oriskany, New York); and Osmocote®: 187.5 g (The Scotts Company LLC., Marysville, Ohio). All plants were watered as needed and maintained at 26 ºC day and 15 ºC night, and 16:8 h L:D. Since the flowering phenostage was required to conduct bioassays with *C. peyerimhoffi*, 12-week old plants (except for the annual *C. heterophyllus*) were vernalized in a cooling room at 12:12 h L:D, and a constant temperature of 4º C for 10-12 weeks. *Caulanthus heterophyllus* was germinated once the other test plants were moved out of the cooling room.

Collection of floral headspace volatiles and flowering stems

We used a portable volatile collection system (PVCS) for dynamic headspace VOC collection, which consists of a push pump to push the air into the volatile collection bag and a pull pump to pull out the equal amount of air, and flowmeters to regulate the amount of air flow (Park et al. 2019). For each collection, purified air (300 ml/min) was pushed using a Rena Air 400 pump (RENA, Chalfont, PA, USA) into a sealed sterilized polyvinyl bag (14 cm × 24 cm; Reynolds, Richmond, VA, USA) enclosing a flowering stem of a test plant at one end. The enclosed air along with emitted floral headspace VOCs from the enclosed plastic bag were pulled out at the same rate at the other end, passing through a VOC trap (40 mg Porapak Q, 80-100 mesh; Southern Scientific Inc. Micanopy, FL, USA) to absorb the emitted headspace VOCs (Park et al. 2019). Volatile collections were conducted for 3 hours between (10:00 and 13:00) on sunny days for each six plants with one empty bag as a control. Trapped VOCs were eluted with 200 µL of dichloromethane into screw cap vials (Supelco, Bellefonte, PA, USA) and stored at -80 ºC until further use. Flowering stems of individual test plants

were cut and kept in 10 cm transparent aqua tubes (Syndicate Sales Inc., Kokomo, IN, USA) to use as a visual cue in visual bioassay.

Olfactometer bioassays

A modified glass Y-tube device (4 cm Y-stem, 12 cm arms, 2 cm internal diameter) was used to assess the weevil's behavioral responses to host and non-host visual and olfactory cues (Fig. 2.1, Fig. 2.2; Park et al. 2019). The Y-tube olfactometer was placed on the top of the cylindrical plastic ring (internal diameter: 16 cm, external diameter: 20 cm; height: 5 cm; Fig. 2.1, Fig. 2.2) with a transparent plastic top (Plastic Wrap, WinCo Foods, Boise, ID, USA). The cylindrical ring consisted of two openings (diameter: 3 cm) on the side that aligned with the arms of the Y-tube resting on its top. The two openings in the cylindrical ring introduced visual cues into the bioassay. The Y-tube was cleaned with 70% ethanol after each trial to remove residual olfactory cues and rotated 180º to avoid positional effects (Park et al. 2019). In a sterilized 2 mm^2 filter paper, eluted VOCs (1 µL) was applied using a 10 µL manual syringe (Agilent Technologies, Sydney, Australia). Filter paper with eluted VOC was then placed into a Tygon tube (R-3603, Saint-Gobain Corporation., Valley Forge, PA, USA) connected to the Y-tube arm. The Rena ® 400 (RENA, Chalfont, PA, USA) pumps were used to push the purified air with eluted VOCs into the Y-tube's arm and pull it out through the stem-end. Flowmeters (MR3000, Key Instruments, Hatfield, PA, USA) regulated the amount of airflow @ 300 ml/min into each Y-tube arm. Both olfactory cues (VOCs) and visual cues (flowering stems) collected from six plants each of target and nontargets were replaced every five replicates during the bioassay. The Y-tube olfactometer arena was illuminated using a full spectrum LED light (Jansjö ® LED lamp, Inter Ikea System B. V., Delft, Netherlands) by placing it directly above Y-tube. Each day, after completing trials, the olfactometer (Y-tube

and connecting tubes) was cleaned with 70% ethanol and placed in a heated oven (60 ºC) to dry for 10 minutes. The testing arena was enclosed in a double-layered rectangular box (180 cm x 90 cm x 60 cm) covered with a white cloth on the inside to minimize visual distraction.

All *C. peyerimhoffi* females were starved for 24 hours prior to bioassays to increase the responsiveness to plant cues (Defagó et al. 2016). Single *C. peyerimhoffi* females were introduced in the Y-tube at the weevil-release point (Fig. 2.1, Fig. 2.2) using forceps by briefly disconnecting the outlet hose at the stem end. A camera (Contour Roam 2, Contour Inc., and Seattle, Washington) was used to record the weevil behavior. The bioassay with each replicate lasted 10 minutes. If the *C. peyerimhoffi* female did not cross a decision line (3 cm into arms) (Fig. 2.1, Fig. 2.2), the female was considered unresponsive, and the bioassay was discarded from analyses. For each bioassay, the total time spent (residence time; RET), initial choice (first arm entered; IC), time to reach the decision line in the Y-tube arm (response time; RT) and final choice (arm entered at end of bioassay; FC) were recorded for each arm of the Y-tube. The residence time was used to measure the strength of a female's preference to cues offered. The IC and RT respectively were used to measure the weevil's ability and agility to discriminate between the offered cues. The FC, a females' location in a Y-tube's arm at the end of the recording period, was considered the weevil's ultimate preference to offered cues. There were three possible outcomes: attraction, indifference, and repellence. Attraction is defined as the preference of plant VOCs and/or visual cues over respective control treatments (Vet et al. 1983). Indifference occurs when a plant's VOCs and/or visual cues were not more or less preferred by *C. peyerimhoffi* than control cues (purified air and/or absence of a visual cue) (Martini et al. 2015). And repellence occurs if *C. peyerimhoffi* prefers control cues (purified air or empty arms) over VOCS and/or visual cues

of plant species (Vet et al. 1983). All bioassays were conducted at the University of Idaho's entomological quarantine laboratory at 21 ºC, and 50% RH. At the beginning of experimentation, a blank test with purified air was conducted to test for orientation bias between Y-tube arms. Olfactometer choice tests were conducted with experienced females in summer 2019 and with naïve weevils in summer 2020.

Experiment 1: Olfactory cues versus purified air with naïve females: Eluted VOCs of *I. tinctoria* or confamilial test plants were presented in one arm and purified air in the other arm of the Y-tube to understand the weevil responses to olfactory cues.

Experiment 2: Visual test plant cues versus empty arms with naïve females: Visual cues of *I. tinctoria* and test plants (flowering stems) were presented below one arm of Y-tube in a modified Y-tube device in comparison to an empty arm to assess responses to visual cues. The Y-tube was maintained with no airflow to prevent positive anemotaxis, which is the tendency of insects to move towards air flow (Farkas and Shorey 1972).

Experiment 3: Combined olfactory and visual test plant cues versus control cues (purified air and empty arm) with naïve females: Both plant cues were presented simultaneously to *C. peyerimhoffi* in a modified Y-tube device as described above for individual plant cue modalities to understand the weevil responses to combined olfactory and visual cues.

Experiment 4, 5 and 6 are analogous to experiments 1, 2 and 3, respectively, but were conducted with experienced females.

Experiment 7: Combined olfactory and visual cues of nontarget species versus combined I. tinctoria *cues with experienced females*: Olfactory and visual cues of *I. tinctoria* were offered

to weevils versus olfactory and visual plant cues of test plant species to study the preference or lack thereof of *C. peyerimhoffi* for its host plant.

Experiments 1, 2, and 3 were conducted with all test plant species, except for *B. hoffmannii*, because of the unavailability of the plant in the flowering phenostage.

Statistical analysis

To standardize weevil responses, we used the proportion of time spent in each arm (RET) as the response expressed as percentage relative to the total time spent in the Y-tube minus the time spent in the weevil-release area. A generalized linear mixed model was used to analyze the behavioral responses for each bioassay, assuming a binomial response for the percentages with arm as fixed effect, and replicate and replicate × arm as random effects. For the initial choice (IC) and final choice (FC), a generalized linear mixed model was used assuming a binary response with residuals as random effect to estimate the proportion of the weevils that chose one arm or the other. A generalized linear mixed model was used to test for the weevil response times (RT), assuming a lognormal distribution for the response time. A single degree of freedom contrast was subsequently used to assess whether the RT of female *C. peyerimhoffi* differed between individual (olfactory or visual) plant cues and combined olfactory and visual cues. An additive effect was defined as no detectable difference between the weevil's RT to simultaneously offered cues and the average RT to each cue individually. A synergistic effect (non-additive effect) was defined when the RT to simultaneous offered cues was greater than the average RT to the individual cues. To test whether RTs of the weevil differed between bioassays with *I. tinctoria* cues and those with nontarget plant species cues, a single-degree-of-freedom contrast was used to compare the sum of RTs of *C. peyerimhoffi* across all bioassays (olfactory cues, visual cues, and combined cues bioassay) in

experiments against control (purified air and/or empty arm). SAS version 9.4 (SAS Institute 2021) was used for all analyses.

Results

Experiment 1: Olfactory cues versus purified air with naïve females

For the residence time (RET), naïve *C. peyerimhoffi* females spent more time in arms with *I. tinctoria* volatiles compared to arms containing purified air (t=-5.65, $P<0001$; Fig. 2.3a, first bars from top). In contrast, females spent more time in arms with purified air compared to *B. alpina* olfactory cues (t=-3.54, $P=0.0017$; Fig. 2.3a, second bars from top), or responded with indifference with regard to RET for *C. heterophyllus* volatiles (t=0.37, $P=0.7171$; Fig. 2.3a, third bars from top).

Weevils did not prefer any plant species including *I. tinctoria* over purified air for the initial choice (IC) and final choice (FC), respectively ($P > 0.05$; Fig. 2.4a, Appendix A), though a relatively larger number of females chose the *I. tinctoria* arm for both IC and FC (Fig. 2.4a) when compared to the nontarget plant species.

Similarly, there was no difference for the response time (RT) of naïve females between olfactory cues of the nontarget plant species and *I. tinctoria* and the purified air control (*I. tinctoria*: t=0.48, $P=0.6399$; *B. alpina*: t=0.74, $P=0.4748$; *C. heterophyllus*: t=-0.29, $P=0.777$; Fig. 2.6a, first, second, and third bars form top, respectively).

Experiment 2: Visual test plant cues (flowering stems) versus empty arms with naïve females

There was no difference in the response of naïve *C. peyerimhoffi* females between visual cues of *I. tinctoria* (t=-1.51, $P=0.1459$; Fig. 2.3b, first bars from top), *B. alpina* (t=-

0.68, *P*=0.4999; Fig. 2.3b, second bars from top) and *C. heterophyllus* (t=-1.1, *P*=0.2824; Fig. 2.3b, third bars from top) and empty control arms, respectively.

Similarly, weevils responded with indifference to flowering stems of all tested plant species for IC and FC, respectively ($P > 0.05$; Fig. 2.4b, Appendix A).

The average RT of naïve females to visual cues and empty arms also did not differ for all tested plant species (*I. tinctoria*: t=-0.53, *P*=0.601; *B. alpina*: t=1.9, *P*=0.0818; *C. heterophyllus*: t=-0.2, *P*=0.845; Fig. 2.6b, first, second and third bars from top, respectively).

Experiment 3: Combined olfactory and visual test plant cues versus control with naïve females

Naïve weevils preferred combined olfactory and visual cues of *I. tinctoria* over purified air and empty arms (t=-4.14, *P*=0.0005; Fig. 2.3c, first bars from top), but not those of *B. alpina* (t =-0.21, *P*=0.8365; Fig. 2.3c, second bars from top) and *C. heterophyllus* (t =0.59, *P*=0.5646; Fig. 2.3c, third bars from top), respectively.

Weevils did not prefer any tested plant species based on IC and FC ($P>0.05$; Fig. 2.4c, Appendix A).

The average RT of weevils to arms with olfactory and visual cues did not differ from RT to purified air and empty arms (*I. tinctoria*: t=-1.13, *P*=0.287; *B. alpina*: t=1.25, *P*=0.2895; *C. heterophyllus*: t=-0.83, *P*=0.4238; Fig. 2.6c, first, second, and third bars from the top, respectively). The RT of female weevils to combined olfactory and visual cues of all the tested species was not different when compared to the RT to individual olfactory and visual plant cues (*I. tinctoria*: t=-1.13, *P*=0.287; *B. alpina*: t=1.25, *P*=0.289; *C. heterophyllus*: t=-0.83, *P*=0.4238; Fig. 2.7). The sum of RT of all bioassays conducted (olfactory, visual and

combined) also did not differ between *I. tinctoria* and the nontarget plant species tested (*P*>0.05; Fig. 2.7, Appendix B).

Experiment 4: Olfactory test plant cues versus purified air with experienced females

Experienced *C. peyerimhoffi* females spent more time (RET) in arms with *I. tinctoria* volatiles than those with purified air (t=5.08, *P*<0.0001; Fig. 2.3d, first bars from top). There was no preference for RET in *C. peyerimhoffi* for olfactory cues of nontarget plant species compared to purified air (*B. alpina*: t=-0.1, *P*=0.9226, *B. hoffmannii*: t=-0.23, *P*=0.819, and *C. heterophyllus*: t=-1.57, *P*=0.126; Fig. 2.3d, second, third, and fourth bars from top, respectively).

When VOCs from plants were presented in the Y-tube against purified air, experienced *C. peyerimhoffi* preferred *I. tinctoria* for the initial and final choice (IC: t=-2.54, *P*=0209, Fig. 2.5a; FC: t=-2.57, *P*=0.0212, Fig. 2.5a). Females responded with indifferent to all nontarget plant species for IC and FC (*P* > 0.05; Fig. 2.5a, Appendix A).

The average response time of *C. peyerimhoffi* for olfactory cues of all tested plant species did not differ control arms with purified air (*I. tinctoria*: t=-0.08, *P*=0.9375; *B. alpina*: t=0.51, *P*=0.6203; *B. hoffmannii*: t=1.34, *P*=0.1968; *C. heterophyllus*: t=0.72, *P*=0.4847; Fig. 2.6d).

Experiment 5: Visual plant cues versus empty arms with experienced females

Experienced female *C. peyerimhoffi* spent more time (RET) in arms with *I. tinctoria* flowering sprigs below compared to the empty control arms (t=5.08, *P*<0.0001; Fig. 2.3e, first bars from top). There was no difference in RET between arms with *C. heterophyllus* visual cues and empty control arms (t=0.48, *P*=0.632; Fig. 2.3e, fourth bars

from top). Females were repelled by visual cues of *B. alpina* (t=-2.03, *P*=0.0483; Fig. 2.3e, second bars from top) and *B. hoffmannii* (t=-2.44, *P*=0.0186; Fig. 2.3e, third bars from top), respectively compared to empty control arms.

Experienced females preferred *I. tinctoria* flowering stems over empty arm based on IC (t= 2.6, *P*=0.017, Fig. 2.5b) and FC (t=2.27, *P*=0.0344, Fig. 2.5b) and responded with indifference to flowering stems of the nontarget plant species tested based on IC and FC, respectively (*P* > 0.05; Fig. 2.5b, Appendix A).

Female weevils took longer to respond to visual cues of *C. heterophyllus* (t=-3.31, *P*=0.0033; Fig. 2.6e, first bars from the top) compared to empty arms. For the other tested plant species, the response time did not differ from that of empty arms (*I. tinctoria*: t=0.55, *P*=0.5883; *B. alpina*: t=-0.79, *P*=0.4373; *B. hoffmannii*: t=1.43, *P*=0.1673; Fig. 2.6e, respectively).

Experiment 6: Combined olfactory and visual cues versus control treatments with experienced females

Experienced females preferred olfactory and visual cues of *I. tinctoria* (t=5.08, *P*<0.0001; Fig. 2.3f, first bars from top) and those of *C. heterophyllus* (t =-2.45, *P*=0.0272; Fig. 2.3f, fourth bars from top) over purified air and empty arms for the time spent in arms (RET). They responded with indifferences for the time spent to combined cues of *B. hoffmannii* (t=-0.81, *P*=0.4243; Fig. 2.3f, third bars from top), and were repelled by olfactory and visual cues of *B. alpina* when compared to control arms (t=-2.99, *P*=0.0053; Fig. 2.3f, second bars from top).

For the initial choice and final choice, females preferred *I. tinctoria* (IC: t=-2.47, *P*=0.028, Fig. 2.5c; FC: t=-2.3, *P*=0.0423, Fig. 2.5c) over empty arms and were indifferent towards *B. alpina*, *B. hoffmannii* and *C. heterophyllus* (IC and FC: *P*>0.05; Fig. 2.5c, Appendix A).

Experienced female *C. peyerimhoffi* responded faster (lower RT) to the combined olfactory and visual cues of *I. tinctoria* compared to purified air and empty arms (t=2.67, *P*=0.0146; Fig. 2.6f, first bars from top). There was no difference in RT between the tested nontarget plant species and control treatments (*B. alpina*: t=0.48, *P*=0.6396; *B. hoffmannii*: t=3.97, *P*=0.0663; *C. heterophyllus*: t=0.33, *P*=0.7438; Fig. 2.6f, second, third, and fourth bars from top, respectively). The RT to combined olfactory and visual cues of *I. tinctoria* differed from RT to individual olfactory and visual cues (Fig. 2.7, Appendix B), but no difference was detected for any of the tested nontarget plant species (*P*>0.05; Fig. 2.7, Appendix B). The sum of RT in all the bioassays conducted (olfactory, visual, and combined cues) compared to control treatments (purified air and/or empty arm) differed for experienced females between *I. tinctoria* and the nontarget species tested (Fig. 2.7, Appendix B).

Experiment 7: Combined olfactory and visual cues of nontarget species versus combined cues of I. tinctoria *with experienced females*

In bioassays testing the preference of experienced *C. peyerimhoffi* between olfactory and visual cues of *I. tinctoria* versus those of confamilial nontarget species, females preferred *I. tinctoria* over all nontarget species tested based on time spent in arms (RET) (*B. alpina*: t=-2.58, *P*=0.0189, *B. hoffmannii*: t=-2.64, *P*=0.017, and *C. heterophyllus*: t=3.87, *P*=0.0013; Fig. 2.3g, first, second, and third bars from top, respectively).

For the initial and final choice, weevils preferred *I. tinctoria* over *B. alpina* (IC: t=2.35, *P*=0.035, Fig. 2.5d; FC: 2.15, *P*=0.0497, Fig. 2.5d) for both IC and FC, and over *C. heterophyllus* (t=-2.42, *P*=0.0277; Fig. 2.5d) for IC.

There was no difference in RT between combined cues of *I. tinctoria* and the nontarget plant species (*B. alpina*: t=0.89, *P*=0.3589; *B. hoffmannii*: t=0.7, *P*=0.4144, *C. heterophyllus*: t=-1.98, *P*=0.0668; Fig. 2.6g).

Discussion

We investigated the role of pre-alightment host finding for the biological weed control candidate *C. peyerimhoffi* with regard to its Eurasian field host *I. tinctoria* and three native North American confamilial nontarget plant species, *B. alpina, B. hoffmannii* and *C. heterophyllus* using both visual and olfactory plant cues. We found that *C. peyerimhoffi* females are attracted to *I. tinctoria* plant cues and that they prefer the plant over the three North American nontarget species. Our data show no pattern of preference by *C. peyerimhoffi* for visual and/or olfactory cues for any of the three nontarget species. Most responses to these plant species in bioassays indicated indifference or repellence with one notable exception: experienced *C. peyerimhoffi* females were attracted to combined olfactory and visual cues of *C. heterophyllus.*

When exposed to olfactory cues of *I. tinctoria* with purified air as a control, both naïve and experienced *C. peyerimhoffi* females responded with attraction, suggesting that the weevil perceives olfactory cues and utilizes them for host finding and discrimination (Park et al. 2018). Similarly, visual cues of *I. tinctoria* elicited attraction in experienced females. For naïve females, a relative greater number of females chose the *I. tinctoria* visual cues for the initial choice (n=9) and final choice (n=10) over empty arms (n=2 and n=3, respectively) but

the number of replications (n=12 (naïve) vs n=23 (experienced)) was likely too small, about half of the comparable bioassay with experienced females, because of limited availability of naïve females to compute a statistical inference. Overall, our data show that *C. peyerimhoffi* responds with attraction to both olfactory and visual cues individually and combined when compared to respective controls and independent of the prior feeding experience.

In bioassays between *I. tinctoria* and nontarget species with combined (olfactory and visual) cues, experienced *C. peyerimhoffi* females strongly preferred their Eurasian field host *I. tinctoria* over native North American tested species suggesting that the weevil can discriminate *I. tinctoria* against all three tested native North American plant species. This finding could explain the contrast observed in *C. peyerimhoffi* attack between *I. tinctoria* and each of the test plant species in choice field cage tests (see Weyl et al. 2017). Our findings corroborate findings of conventional host specificity choice tests and combined suggest that visual and olfactory cues might play an important role in the host finding and host acceptance of *C. peyerimhoffi*, leading to a highly selective behavior of and host specialization to *I. tinctoria* (Janike 1990).

We found indifferent and repellant responses of naïve and experienced *C. peyerimhoffi* females to various plant cues of *B. alpina* and *B. hoffmannii,* respectively, that suggest that the weevil is not able during the early stages of host finding to discern these plant species as potential hosts in the field. Olfactory cues are an essential component in host finding (Bernays and Chapman 1994) as they can provide reliable information regarding a host-plant quality (host suitability, nutritional quality; Tasin et al. 2011). The observed indifferent responses to olfactory cues of *B. alpina* and *B. hoffmannii* suggest that these plant species may be

considered low quality or non-hosts by *C. peyerimhoffi* (Tasin et al. 2011) and may be unrecognized or repelled in the field (Byers et al. 2004).

Indifference and repellence to visual cues of *B. alpina* and *B. hoffmannii* in naïve and experienced weevils suggest a similar outcome as described for olfactory cues above. Even if *C. peyerimhoffi* were to encounter the nontarget plant species in the field by chance the repelling effect of visual cues of these nontarget species on the weevil would likely deter *C. peyerimhoffi* females (Deletre et al. 2016). Reeves (2011) suggest that host and non-host visual cues are perceptible and reliably used by herbivores and for specialist herbivores with narrow host ranges such as *C. peyerimhoffi* they may be used to efficiently distinguish suitable hosts from non-hosts (Stenberg and Ericson 2007) in order to maintain their specialized narrow host-fidelity.

The eggs of *C. peyerimhoffi* found on *B. alpina* and *B. hoffmannii* plants in multiple-choice cage tests with *I. tinctoria* (Weyl et al. 2017), could be explained by the perception of VOCs from the host plant *I. tinctoria* in close physical proximity to the nontarget plant species, leading to cases of oviposition 'mistakes' (Heard 2000). In behavioral bioassays the seed-feeding weevil *Mogulones borraginis* F. (Coleoptera: Curculionidae), a biological weed control candidate for *Cynoglossum officinale* L. (Boraginaceae), was repelled by olfactory cues of the confamilial nontarget plant *Adelinia grande* (Douglas ex Lehm.) J.I. Cohen, but the weevil accepted the plant for oviposition when it was given a choice along with its host plant *C. officinale* in narrow sleeved bags (Park et al. 2018). The authors attributed the oviposition to odor plum admixture (OPA), the mixture of nontarget olfactory cues with that of the host plant within the restricted air volume of the sleeves (Park et al. 2018).

In bioassays with individual olfactory and visual cues of *C. heterophyllus*, both naïve and experienced *C. peyerimhoffi* females responded with indifference, suggesting that based on one cue modality at a time, *C. peyerimhoffi* would not be able to discover *C. heterophyllus* as a host plant regardless of its prior experience. However, experienced females preferred combined olfactory and visual cues of *C. heterophyllus* over purified air and an empty arm, indicating that experienced weevils could identify *C. heterophyllus* as a host plant. It is unclear why only weevils with feeding experience on *I. tinctoria* preferred *C. heterophyllus* plant cues.

Both naïve and experienced *C. peyerimhoffi* tended to prefer olfactory cues of *C. heterophyllus* over controls, suggesting that the floral headspace volatile profile of *C. heterophyllus* may include at least one potentially attractive VOCs. For instance, in the case of *Mogulones crucifer* Pallas (Coleoptera: Curculionidae), the preference of one bioactive VOC (i.e., methyl isovalerate) in *C. officinale* triggers the attraction (Kafle 2016). The greater preference of weevils to *I. tinctoria* combined cues over the *C. heterophyllus* cues suggests that *I. tinctoria* headspace volatile profile may include other potentially attractive VOCs that are not in the profile of *C. heterophyllus.* In addition, in our experiments, *C. heterophyllus* produced only yellow-colored flowers (similar to the flower color of *I. tinctoria*), but it has been reported that *C. heterophyllus* has different inflorescence morphotypes with sepals and petals of differing colors (sepals: purple or yellow to creamy white; petals: purple or yellowish, often with darker purple veins) (Brassibase 2021). Because weevils were only presented with one of the inflorescences morphotypes, i.e., the one that resembles *I. tinctoria* the most, the responses of *C. peyerimhoffi* may have been more favorable to visual cues complemented by its headspace VOCs than they would have to entire range of inflorescence

morphotypes constituting *C. heterophyllus*. It also could explain the *C. peyerimhoffi* attraction observed for combined visual and olfactory cues of *C. heterophyllus* in the bioassays.

Ideally, olfactometer bioassays conducted with naïve insects exclude potentially confounding factors like female age, egg load, or gravidity that could potentially affect test results. In the case of *C. peyerimhoffi* this was not possible because the availability and logistics of obtaining weevils prevented use of naïve individuals. We speculate that the female age, egg load, or gravidity (Thompson 1988) might have affected the preference of the experienced weevils.

It has been suggested that metabolic pathways are shared by compounds that determine floral color and volatiles in inflorescences (Zvi et al. 2008), suggesting pleiotropic effects of the genes involved and resulting in a correlated selection of traits, i.e., the selection of specific floral volatile compounds due to direct selection of floral color and vice-versa (Zvi et al. 2008). It could be assumed that the differing inflorescence morphotypes of *C. heterophyllus* not only differ in floral coloring but also exhibit differing volatile profiles. For example, Ascrizzi and Flamini (2020) found that the floral volatile profile of *Iris lutescens* Lam. (Iridaceae) differed between two inflorescence morphotypes growing in serpentine soil. It would be possible that different morphotypes of *C. heterophyllus* could produce different floral reflectance pattern and olfactory floral VOC profiles and consequently elicit different behavioral responses of *C. peyerimhoffi* to those cues than those observed in our study.

Our data on response times for experienced *C. peyerimhoffi* females showed both additive and synergistic effects between confamilial nontarget species cues and *I. tinctoria* cues, respectively, suggesting that females respond differently to cues of their host plant and those of non-hosts depending on the plant species emanating the cues**.** Both, additive and

synergistic effects to plant cues have been reported for other insect herbivores (e.g., Park et al. 2018; Campbell and Borden 2009).

Electrophysiological experiments using insect herbivores' sensory organs associated with plant cue modalities are expected to explain some of the behavioral responses observed in bioassay (Park et al. 2018). We attempted electroretinography (ERG) with the compound eye of *C. peyerimhoffi*, but the results were inconclusive due variability in the data, resulting in three pronounced ERG response groups of weevils whose source of variation could not be identified (Appendix C). The potential source of variability could be from the experimental insect preparations such as electrode placement and depth of electrode penetration in the compound eye of weevils. Conducting ERG with different wavelengths and/or intensity adaptation, varying electrode placement on compound eye, and mapping the photoreceptors could provide insight into the weevil's vision physiology. With olfactory cues, we were not able to obtain the electrophysiological response of female *C. peyerimhoffi*, which could most likely be due to factors such as, insensitive GC-EAD/FID method (Moorhouse et al. 1969), or low signal-to-noise ratio (Myrick and Baker 2018).

In sum, our data show that *C. peyerimhoffi* utilizes visual and olfactory cues to discriminate its host plant *I. tinctoria* from at least two of the three tested native North American confamilial nontarget species. Our data emphasize that both, visual and olfactory plant cues should be included in behavioral bioassays. The findings presented here facilitate to delineate the physiological and ecological host ranges of *C. peyerimhoffi* as assessed in conventional host specificity investigations. While the results of our experiments are encouraging, it should be noted that volatile emissions (Holopainen and Gershenzon 2010) and plant phenotypic attributes (Lacey and Herr 2005) can be dynamic in nature and affecting

olfactory and visual cues, respectively. While some attraction to *C. heterophyllus* was observed, we conclude that *C. peyerimhoffi* would is unlikely to be able to recognize and cause any potential nontarget attack to *B. alpina* or the threatened and endangered listed *B. hoffmannii* post-release.

Table 2.1. List of selected North American test species and reason for inclusion in the experiment.

Lineages[1]	Tribe[1]	Species[1]	Flowering time[2]	Elevation (m)[2]	Distribution[2]	Life history trait	Native range[2]	Reason for inclusion
Lineage I	Boechereae	*Boechera hoffmannii* (Munz) Al-Shehbaz	Feb-Mar	0-100	United States (Calif./Santa Cruz Island in Santa Barbara County)	Perennial[2]	North America	Non-host; T & E; supported larval development[4,5,6,7]
Lineage II	Isatideae	*Isatis tinctoria* L.	Apr-Jun	300-2200	B.C, Nfld. and Labr. (Nfld.), Ont., Que., Calif., Idaho, Ill., Mo., Mont., Nev., N.Mex., N.Y., Oreg., Utah, Va., Wash., W.Va., Wyo., Europe, C, SW Asia, N Africa, South America (introduced; Chile, Peru)	Annual, biennial or perennial[3]	Eurasian	Host plant[7]
	Thelypodieae	*Caulanthus heterophyllus* (Nutt.) Payson	Mar-May	0-1400	Mexico (Baja Calif.), United States (SW Calif.).	Annual[2]	North America	Non-host; Limited feeding; supported larval development[5,6,7]
Lineage III	Euclidieae	*Braya alpina* var *americana* Hooker (*Braya glabella* subsp. *glabella*)	Jun-Jul	0-3700	North America	Perennial[3]	North America	Non-host; Limited feeding; supported larval development[7]

[1](BrassiBase 2021), [2](FNA 2021), [3](Hinz et al. 2014), [4](Hinz et al. 2015), [5](Hinz et al. 2016), [6](Weyl et al. 2017), [7](Weyl et al. 2019); T & E: Threatened and Endangered, B.C: British Columbia, Nfld. And Labr (Nfld.): Newfoundland and Labrador, Ont.: Ontario, Que.: Quebec, Calif.: California, N.Mex.: North Mexico, N.Y.: New York, Oreg.: Oregon, Va.: Vancouver, Wash.: Washington, W.Va.: Washington Vancouver, Wyo.: Wyoming, C, SW Asia: Central, Southwest Asia, N Africa: North Africa

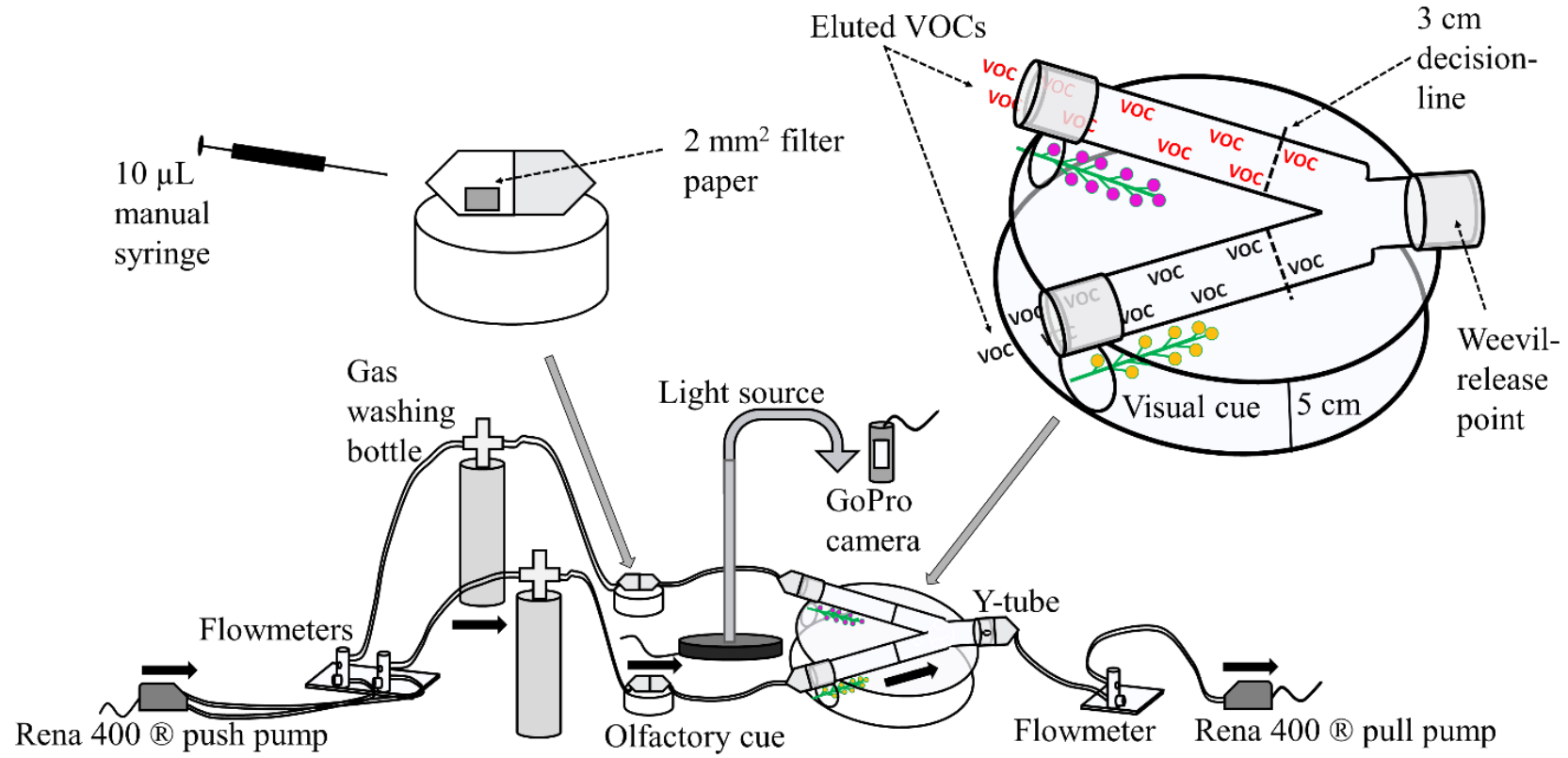


Fig. 2.1: Modified Y-tube olfactometer set-up. In the center is the Y-tube (4 cm Y-stem, 12 cm arms, 2 cm internal diameter) resting on cylindrical plastic ring (diameter: 20 cm; height: 5 cm) with transparent plastic top**.** The cylindrical plastic ring consists of 2 openings (diameter: 3 cm) which serves the purpose of introducing visual cues (flowering sprigs) into the bioassay. The Rena ® 400 pumps were used to push the purified air with eluted VOCs into the Y-tube's arm and pull it out through the stem-end, and flowmeters regulated the amount of airflow into each Y-tube arm. The Y-tube olfactometer arena was illuminated using a full spectrum LED light by placing it directly above Y-tube. 1 µL eluted volatile organic compounds (VOCs) is applied to 2 mm^2 filter paper for each replication. Black arrow on the figure indicates the direction of airflow. Note: Figure not drawn to scale.

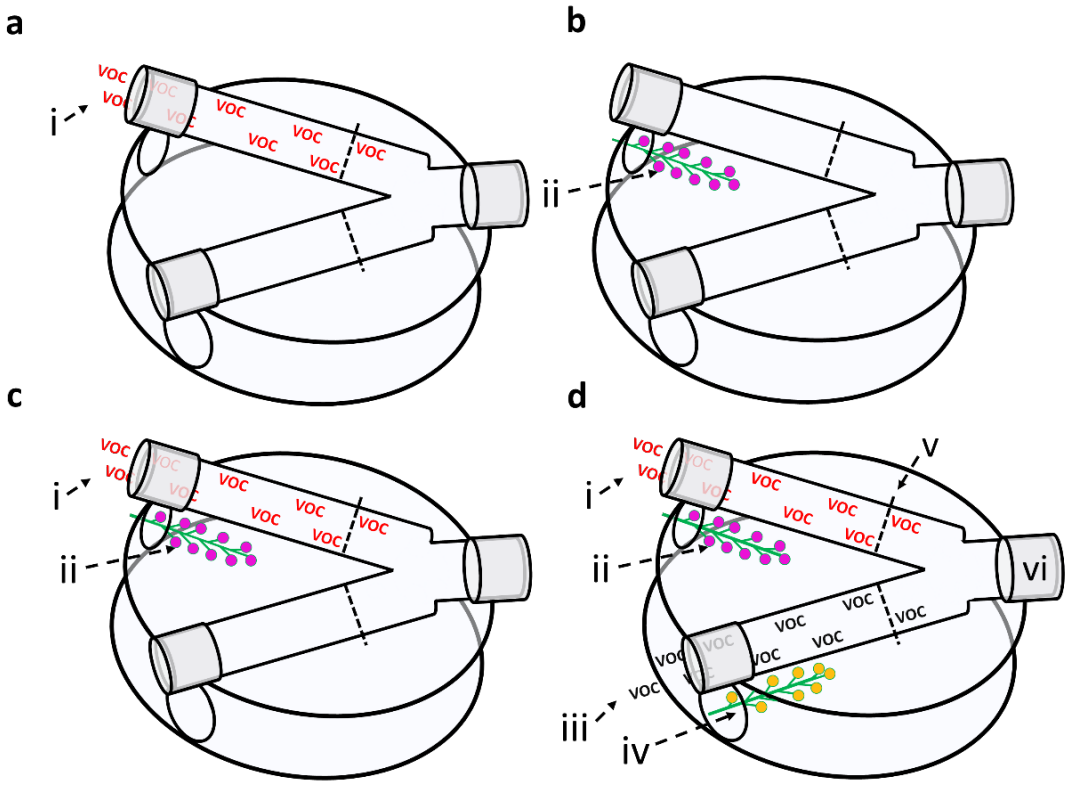


Fig. 2.2: Modified Y-tube device (4 cm Y-stem, 12 cm arms, 2 cm internal diameter) raised 5 cm from floor of the experimental arena using circular plastic ring (diameter: 20 cm; height: 5 cm) with 2 circular openings (diameter: 3 cm) to introduce visual cues. Experiments conducted in behavioral choice assays: **a** Olfactory cues of test plant species vs control (purified air) **b** Visual cues of test plant species vs control (empty arm) **c** Combined cues of test plant species vs control (purified air and empty arm) **d** Combined cues of test plant species vs *I. tinctoria*. Experiments **a**, **b**, and **c** conducted with both naïve and experienced weevils, whereas experiment **d** only with experienced weevils**.** Roman numerals in **a**, **b**, **c**, and **d**: i (test plant olfactory cues); ii (test plant visual cues); iii (*I. tinctoria* olfactory cues); iv (*I. tinctoria* visual cues); v (3 cm decision line); vi (weevil release point).

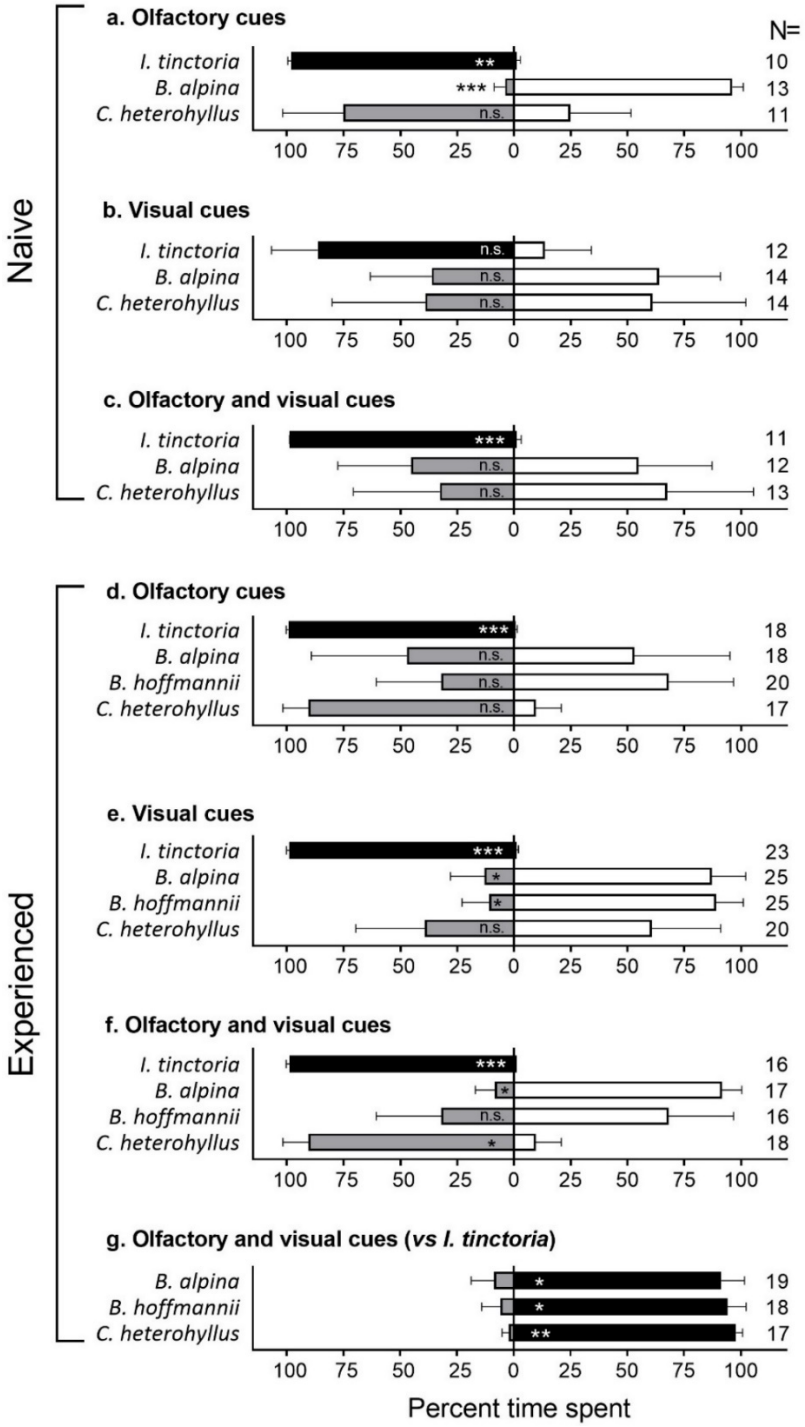


Fig. 2.3: Proportion of time spent (mean ± SE) by naïve female *C. peyerimhoffi* in Y-tube arms with olfactory cues (**a, d**), visual cues (**b, e**), combined olfactory and visual cues (**c, f**) of test plant species (grey bars) and *I. tinctoria* (black bars) against control (purified air and/or empty arm, white bars), and by experienced female *C. peyerimhoffi* in Y-tube arms with combined olfactory and visual cues of test plant species (grey bars) against *I. tinctoria* cues (black bars) (**g**). Generalized linear mixed model (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s.: not significant). Numbers (N) on the top of each right bars indicates the sample size for that experiment.

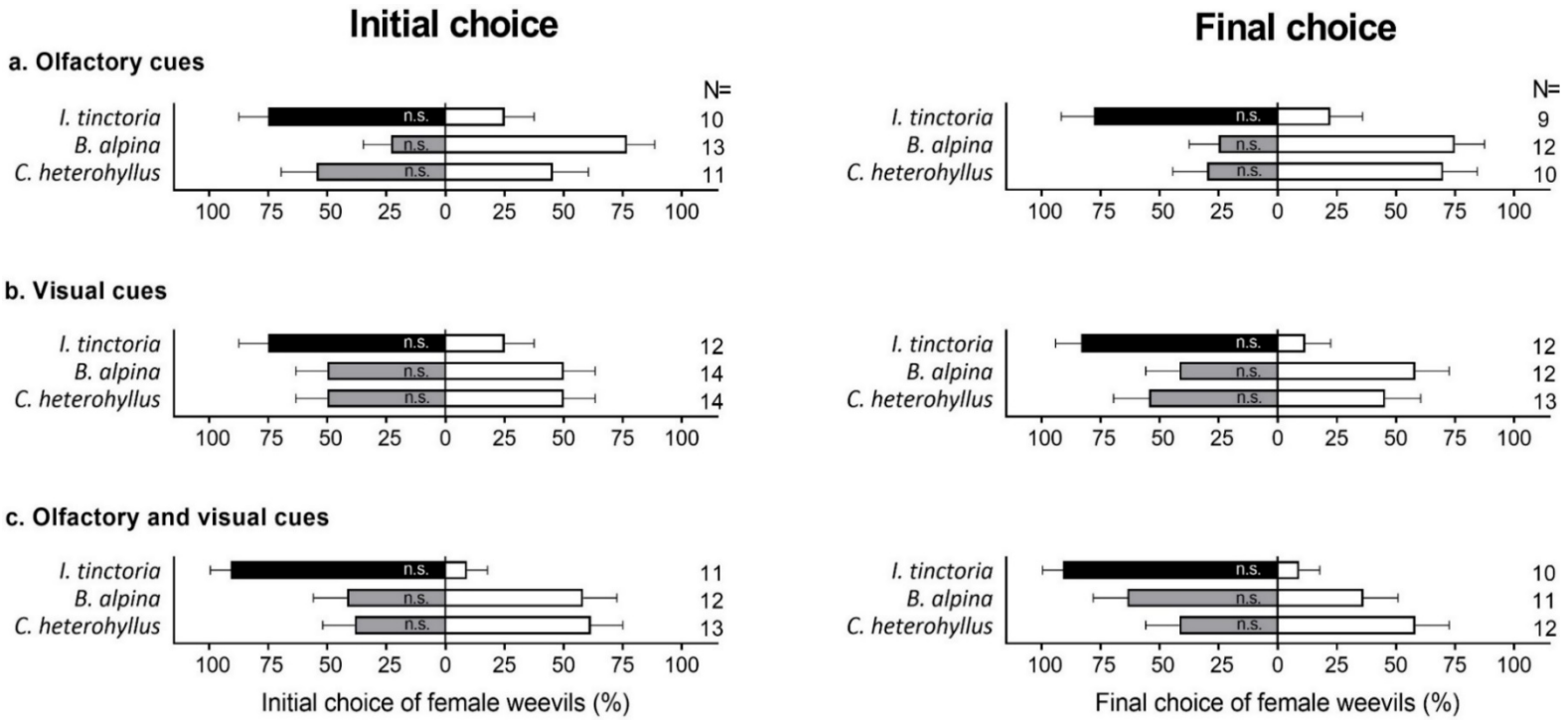


Fig. 2.4: Proportion of naïve female *C. peyerimhoffi* (mean ± SE) initial (first) arm choice (IC) and final choice (FC) among two arms of the Y-tube with olfactory cues (**a**), visual cues (**b**), combined olfactory and visual cues (**c**) of test plant species (grey bars) and *I. tinctoria* (black bars) against control (purified air and/or empty arm, white bars). Generalized linear mixed model (n.s., not significant). Numbers (N) on the top of each right bars indicates the number of naïve female weevils choosing for initial choice and final choice in respective panel.

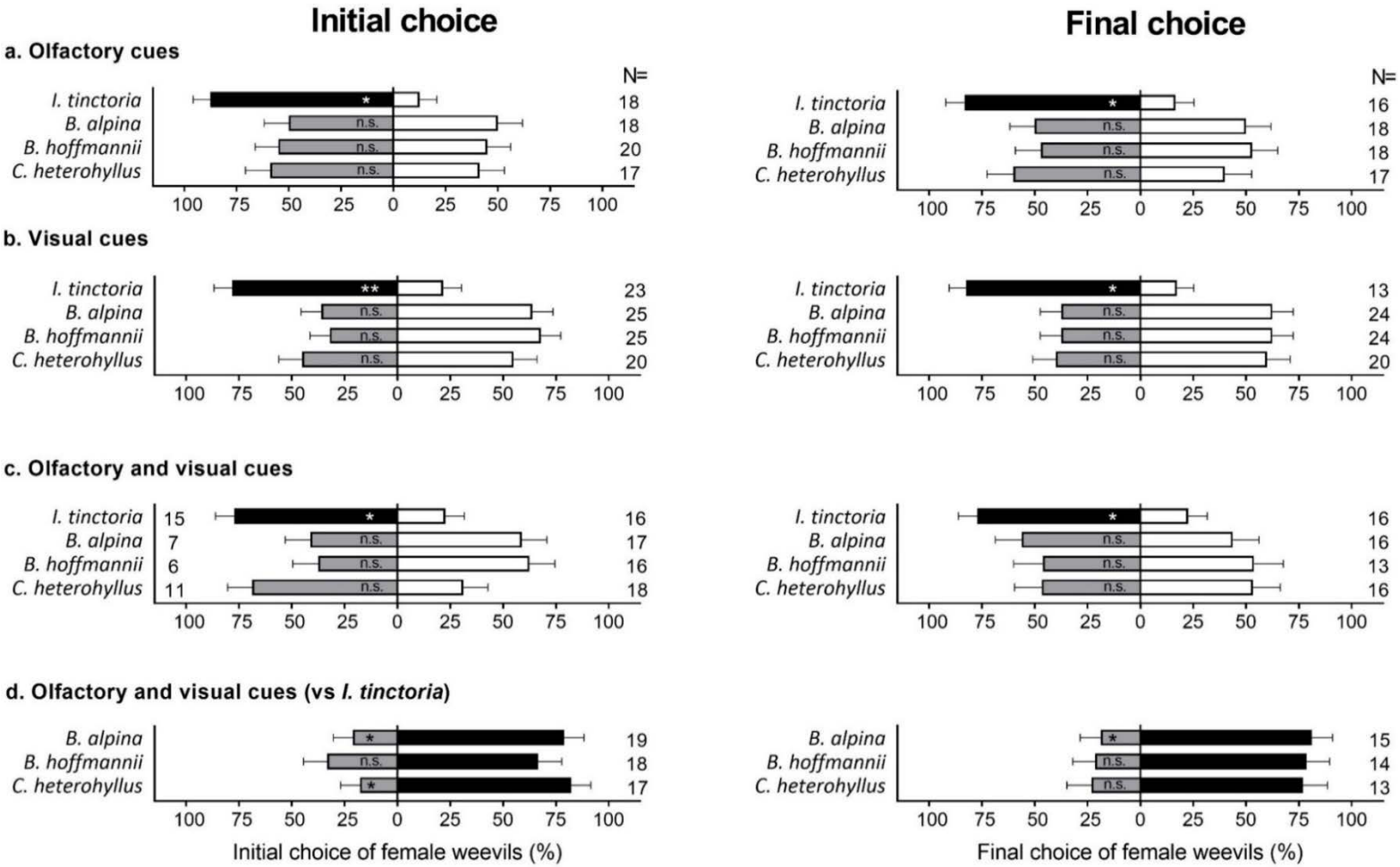


Fig. 2.5: Proportion of experienced female *C. peyerimhoffi* (mean ± SE) initial (first) arm choice (IC) and final choice (FC) among two arms of the Y-tube with olfactory cues (**a**), visual cues (**b**), combined olfactory and visual cues (**c**) of test plant species (grey bars) and *I. tinctoria* (black bars) against control (purified air and/or empty arm, white bars), and combined olfactory and visual cues of test plant species (grey bars) against *I. tinctoria* cues (black bars) (**d**). Generalized linear mixed model (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; n.s.: not significant). Numbers (N) on the top of each right bars indicates the number of naïve female weevils choosing for initial choice and final choice in respective panel.

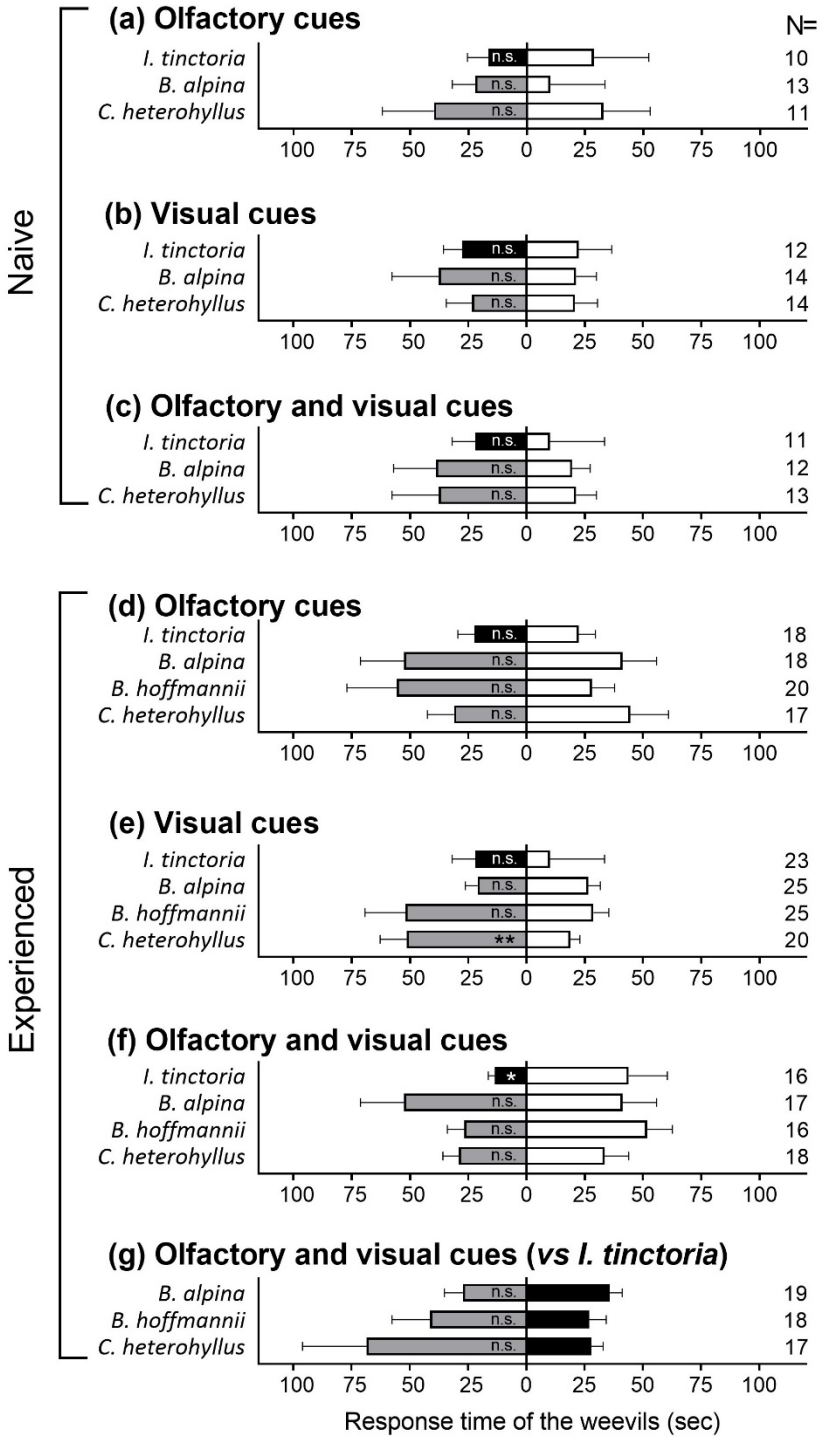


Fig. 2.6: Mean (± SE) response times (RT) of naïve female *C. peyerimhoffi* in a modified Y-tube choice bioassay with olfactory cues (**a, d**), visual cues (**b, e**), combined olfactory and visual cues (**c, f**) of test plant species (grey bars) and *I. tinctoria* (black bars) against control (purified air and/or empty arm, white bars), and of experienced female *C. peyerimhoffi* in a modified Y-tube choice bioassay with combined olfactory and visual cues of test plant species (grey bars) against *I. tinctoria* cues (black bars) (**g**). Generalized linear mixed model (* $P<0.05$; n.s.: not significant).

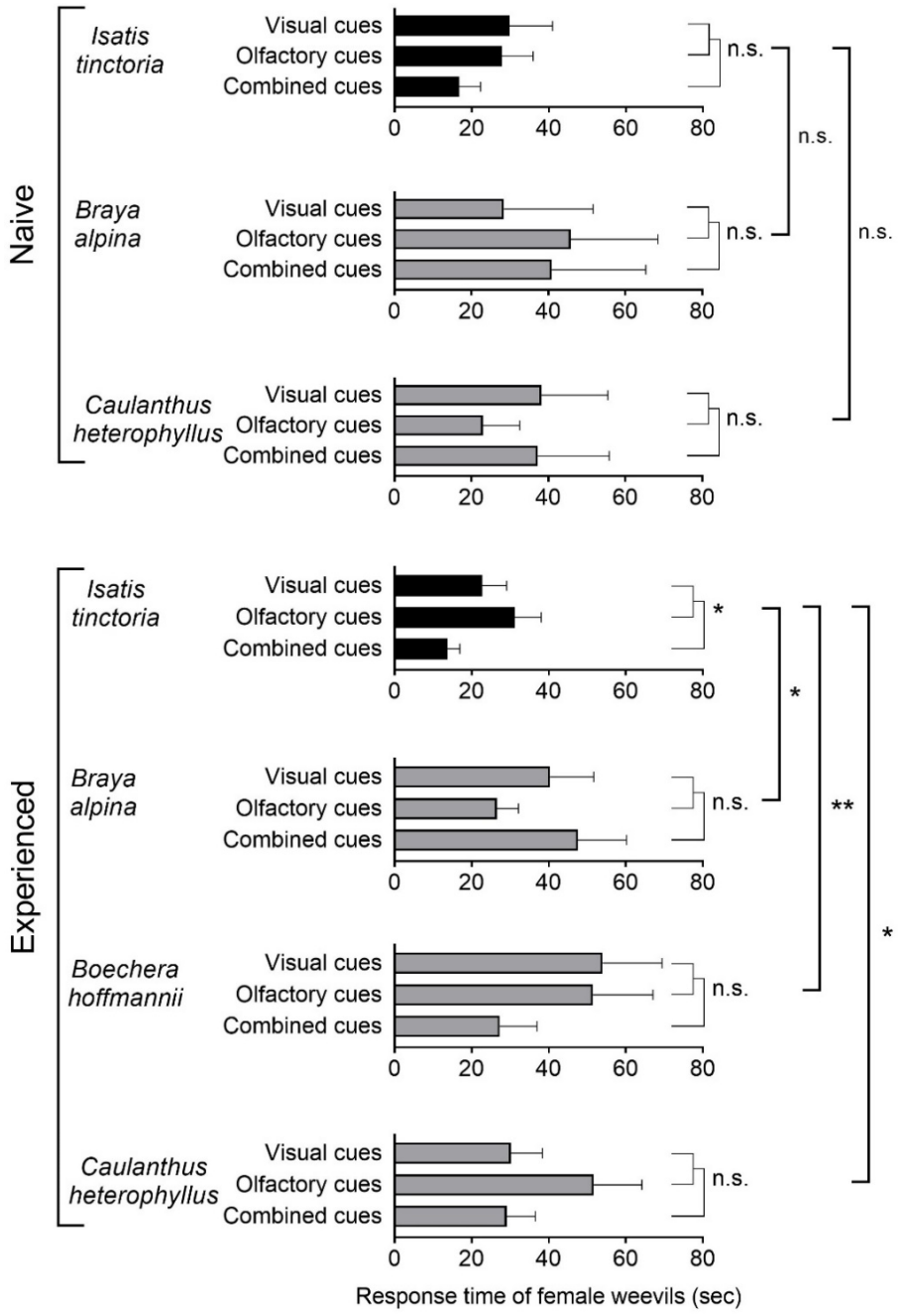


Fig. 2.7: Mean (±SE) response times of naïve and experienced female *C. peyerimhoffi* choosing the Y-tube cue offered arm in behavioral bioassays with visual cues, olfactory cues, and combined olfactory and visual (combined) cues of *I. tinctoria* (black bars) and test plant species (grey bars) against control (purified air and/or empty arm). Generalized linear mixed model (* $P<0.05$, ** $P<0.01$; n.s.: not significant). Small brackets are a single degree of freedom contrast tests between the average of response times of individual cue bioassay (first and second bars from top on each species panel) and combined cue bioassay (third bars from top on each plant species panel, respectively). Big brackets indicate a single degree of freedom contrast tests between the combined response times in all the bioassays with the cues of *I. tinctoria* and test species.

Chapter 3: UNDERSTANDING THE PRE-ALIGHTMENT HOST FINDING OF *CEUTORHYNCHUS RUSTICUS* A BIOLOGICAL WEED CONTROL CANDIDATE FOR THE INVASIVE PLANT *ISATIS TINCTORIA* AS CONTRIBUTION TO PRE-RELEASE RISK ASSESSMENTS

Abstract

Pre-release host range assessments of weed biological control agent (BCA) candidates typically rely on no-choice and choice feeding, oviposition, and development tests. However, these tests may lead to the rejection of environmentally safe BCA candidates from consideration if they can develop on nontarget plant species in no-choice tests that they would not colonize because of potential behavioral barriers. An accurate assessment of the ecological host range should, therefore, consider the host selection behavior. Here, we present data on the pre-alightment host finding of the root-crown weevil *Ceutorhynchus rusticus*, a BCA candidate for the invasive Eurasian mustard *Isatis tinctoria.* We examined the behavioral responses of *C. rusticus* to olfactory and visual plant cues individually and combined for nine native North American and two Eurasian confamilial plant species of *I. tinctoria* that supported larval development in previous oviposition and developmental tests. We compared behavioral responses to nontarget plant cues to control cues (purified air/empty arm) or to corresponding cues of *I. tinctoria* using a modified y-tube device. In behavioral bioassays with olfactory cues of nontargets, weevils showed no preference for any tested plant species when compared to purified air. In contrast, for visual plant cues, *C. rusticus* was attracted to cues of three of the tested plant species when compared to empty arms. As expected, *C. rusticus* preferred individual olfactory and visual cues of *I. tinctoria* over the respective cues of most nontarget species, and preferred combined plant cues of *I. tinctoria* over all nontarget species tested. Weevils also responded faster to *I. tinctoria* plant

cues when compared to confamilial nontarget species. Our data suggest that sensory host recognition studies could provide useful data explaining discrepancies between the physiological and ecological host range of BCA candidates and thus, contribute additional information to environmental safety assessments in biological weed control.

Introduction

Isatis tinctoria L. (Brassicaceae), dyer's woad, is a Eurasian winter annual or biennial mustard that has been repeatedly introduced into North America (hereafter NA) during the early 20th century (Evans and Chase 1981). Since, it has naturalized, become invasive, and is now a declared noxious weed in 11 western USA states (Gaskin et al. 2018; USDA NRCS 2021). Dyer's woad is considered particularly problematic in two distinct geographical areas heavily invaded by the weed in southern Oregon and northern California and southern Idaho and northern Utah (Gaskin et al. 2018) because of negative impacts on native flora and losses in crop and rangeland productivity (FWS 2006; Jacobs and Pokorny 2007). Conventional management strategies including hand pulling, digging, clipping and herbicide applications are all widely used and can be successful on small spatial scales (Jacobs and Pokorny 2007; Pokorny and Krueger-Mangold 2007), but given the widespread invasions in remote areas and/or public lands these management means are considered increasingly economically infeasible (Zouhar 2009, and references therein). In 2004, a grassroots effort of local land managers and ranchers in Idaho supported by state and federal agencies led to a project investigating the feasibility of classical biological control of *I. tinctoria*. Currently, two biological control agent (hereafter BCA) candidates are being evaluated for their potential introduction into the USA (Weyl et al. 2019). One of these insect herbivores is the root-crown mining weevil, *Ceutorhynchus rusticus* Gyllenhal (Coleoptera: Curculionidae). Adult *C.*

rusticus feed on foliage and larvae of the weevil mine during spring in the root crowns of *I. tinctoria* (Weyl et al. 2019). Attack by *C. rusticus* can reduce the seed production of *I. tinctoria* plants up to 72%, plant biomass up to 46%, and heavy larval feeding can kill affected plants outright (Hinz et al. 2008).

One of the most important components of any classical biological weed control program is the determination of the host range of a BCA candidate to determine its environmental safety. The release decisions by respective federal authorities rely on pre-release predictions of any potential post-release environmental risk of nontarget attack (Hinz et al. 2020). Typically, environmental safety assessment procedures include host-specificity testing to assess the BCA candidate's physiological and ecological host range (Schaffner 2001). The physiological host range of a BCA candidate, determined through no-choice tests, comprises all test plant species on which the larvae can complete their development whereas the ecological host range, determined through series of choice tests (in cages, field cages or in the open), comprises the plant species that insect uses under natural conditions (Schaffner 2001).

The physiological host range often comprises more plant species than the ecological host range (Schaffner 2001; Schaffner et al. 2018). The resulting discrepancies between the host ranges can lead to overstating post-release nontarget attack risks (Hinz et al. 2014, 2019). In traditional host-specificity tests, 14 native North American confamilial plant species within eight genera supported *C. rusticus* development to adulthood to some and often low degree in no-choice tests (Weyl et al. 2019). But of those 14 native North American confamilial plant species only nine species and often only one plant replicate per species were accepted for oviposition in open-field tests (Weyl et al. 2019). One explanation for these different test

outcomes could be that under open-field conditions, a BCA candidate can utilize different cues, for instance, olfactory and/or visual plant cues, to provide it with information regarding plant quality and express its host-choice behavior, which, under no-choice condition, may be distorted or by-passed (Schaffner et al. 2018). Studying BCA candidates' behavioral responses to these cues may help interpret observed discrepancies between traditional physiological and ecological host specificity test results (Hinz et al. 2014; Hinz et al. 2019).

Although studying the host selection of weed biological control candidates has been acknowledged as an area of research that could help better understanding conventional host-specificity data and potentially improve predictions of post-release host use (Wheeler and Schaffner 2013), few studies exist. Of these, most studies focused on one cue modality, i.e., olfactory cues (Andreas et al. 2009; Fung et al. 2021) or visual cues (Müller and Nentwig 2011; Reddy et al. 2009). Even less studies exist that investigate both cue modalities important in pre-alightment host selection (but see Park et al. 2018; Park et al. 2019).

In this study, we aimed to investigate whether the root-crown mining weevil *C. rusticus,* a BCA candidate for the control of the Eurasian mustard *I. tinctoria* invasive in the USA uses olfactory and visual cues during pre-alightment host selection and if so whether the weevil will discriminate against native North American confamilial plant species in the presence and absence of its Eurasian host plant *I. tinctoria.* We hypothesized that *C. rusticus* uses foliar volatile and visual cues to distinguish between *I. tinctoria* and confamilial nontarget plant species, and that the combination of these cue modalities provides for the greatest discrimination among plant species.

Methods and Materials

Insects

For our experiments, *Ceutorhynchus rusticus* adults were shipped from CABI Switzerland to the quarantine facility at the University of Idaho, Moscow, Idaho, in September 2018 (*n* = 200) and March 2020 (*n* = 238). Weevils were maintained in an environmental chamber (I-35 VL, Percival Mfg. Co., Boone, Iowa) at 17 ºC day and 8 ºC night, 16:8 h L:D, and 60% RH throughout the experimentation period.

Plants

Nine native North American, one Eurasian and one Iranian originating confamilial nontarget species were selected to study the host selection behavior of *C. rusticus* using both visual and olfactory cues: *Braya alpina* Sternb. & Hoppe, *Caulanthus flavescens* (Hook.) Payson, *Descurainia californica* (A. Gray) O. E. Schulz, *Descurainia nelsonii* (Rydb.) Al-Shehbaz & Goodson, *Eutrema salsugineum* (Pall.) Al-Shehbaz & Warwick, Eurasian *Isatis glauca* Aucher ex Boiss., Iran origin *Lepidium sativum* L., *Sisymbrium linifolium* (Nutt.) Nutt. ex Torr. & A. Gray, *Stanleya pinnata* (Pursh) Britton, *Stanleya tomentosa* Parry, and *Stanleya viridiflora* Nutt. (see Table 3.1). These test plant species were selected because limited feeding and larval development were recorded in no-choice tests (Hinz et al. 2015; Weyl et al. 2017; Weyl et al. 2019) (Table 3.1).

For all test plants, individuals were propagated by seed. CABI Switzerland kindly provided seeds of the nontarget species. Seeds with hard seed coats were soaked in water for 6 hours and the coats peeled off to facilitate the germination process. The bare seeds were sown directly into seedling starter trays (length: 15 cm, breadth: 12 cm, height: 5 cm; Stuewe &

Sons, Inc. Tangent, Oregon) with Sunshine Professional Growing Mix Number 4 (Sun Gro Horticulture Canada Ltd., Vancouver, Canada). Seeds of *I. tinctoria* did not require peeling off the seed coat; however, seeds were separated from the fruit pods before sowing. Two-week-old seedlings were transplanted into the tree-pots (19.68 cm x 31.75 cm; Stuewe & Sons, Inc. Tangent, Oregon) with the soil mix of 1-part sand and 3-part Sunshine Mix No. 4 (Sun Gro Horticulture Canada Ltd., Vancouver, Canada). Nutrient supplements added (for each 12 kg of soil and sand): 2.5 g trace elements (FRIT Industries, Inc., Ozark, Alabama), 1.25 g chelated iron (Grow More Inc., Garden, California), 47.5 g limestone (Grow More Inc., Garden, California), 47.5 g triple superphosphate (Bonide Products Inc., Oriskany, New York), and 187.5 g Osmocote® (The Scotts Company LLC., Marysville, Ohio). Transplanted pots were maintained in a greenhouse at the Parker Research Farm in Moscow, Idaho, at 26ºC day and 15ºC night, 16:8 h L:D, and watered as needed.

Collection of floral headspace volatile for olfactory cues and leaves for visual cues

The method developed by Park et al. (2019) was adopted to collect volatile organic compounds (VOCs) of plants. In summary, purified air is pushed (300 ml/min) into pre-baked polyvinyl acetate bags (15 cm × 15 cm; Reynolds Consumer Products LLC., Richmond, Virginia) containing test plant leaves, from one edge of the plastic bag and pulled out from the other edge at the same flow rate. A volatile collection trap (40 mg, 80-100 mesh Porapak-Q adsorbent, Southern Scientific Inc. Micanopy, FL, USA) was inserted into the plastic bag's pull end. A Rena ® Air 400 pump was modified to pull the air out from the plastic bag. Air was purified using activated charcoal filters (Orbo™, Supelco, Sigma-Aldrich Co. LLC, St. Louis, Missouri). Flowmeters (Model: MR3A14BVBN; Zoro Tools, Inc., Buffalo Grove, IL, USA) were used to regulate the airflow into and out of the plastic bag. Foliar headspace

volatiles were collected from five plants with one control (empty bag) simultaneously for each species during sunny afternoons for six hours (0900 h to 1500 h). Greenhouse collected VOCs were eluted in the lab with 200 µL of dichloromethane and stored at -80ºC. Leaves of individual plants were cut and stored in 10 cm transparent aqua tubes for visual bioassays.

Modified Y-tube olfactometer setup

Behavioral responses of the weevil to host and non-host olfactory cues were assessed using a modified Y-tube device (Fig. 3.1). It consists of a single Y-tube (Y-stem: 4 cm, arms: 12 cm, internal diameter: 2 cm), placed on top of the cylindrical plastic ring (internal diameter: 16 cm, external diameter: 20 cm; height: 5 cm) with two circular holes (diameter: 3 cm) on the side that align with arms of the Y-tube placed above. Circular holes in the plastic ring were used to present visual cues. The Y-tube was maintained identically to the D-SYD's upper Y-tube, as explained by Park et al. (2019): The Y-tube was cleaned with ethanol (70%) after every trial to remove the residual effects of previous cues and was rotated 180º to address positional bias. A 2 mm^2 filter paper was primed with 1 µL aliquot of eluted VOCs using a 10 µL manual syringe and placed into a Tygon^{tm} tube connected to the Y-tube's arm. A Rena ® 400 push pump was used to push the purified air and eluted VOCs into each Y-tube arm through a Tygon tube. Flowmeters (MR3000, Key Instruments, Hatfield, PA, USA) were used to regulate the amount of airflow of 300 ml/min in each Y-tube arm. From the Y-tube's stem end, the air was pulled out at the rate of 600 ml/min using a Rena ® 400 pump, whose diaphragm was modified to make it a pull pump. A full-spectrum LED light (Jansjö ® LED lamp, Inter Ikea System B. V., Delft, The Netherlands) was placed directly above the Y-tube to illuminate the olfactometer set-up. A double-layered rectangular box (180 cm x 90 cm x 60

cm) with an internal layer of white cloth enclosed the entire arena to minimize the effects of surrounding visual and daylight.

Bioassay protocols

Females were starved 24 hours before bioassays to increase the responsiveness of weevils to the plant cues offered (Defagó et al. 2016). The Y-stem outlet hose was removed, and a single female was placed at the weevil-release point (Fig. 3.1, Fig. 3.2). A camera (Contour Roam 2, Contour Ins., Seattle, Washington) was turned on to record the weevil's behavior for 10 minutes. A weevil was marked 'unresponsive' if it failed to cross the 3 cm decision-line (Fig. 3.1) after 3 minutes of recording and was excluded from the analysis. Total time spent (residence time), initial choice (IC), response time (RT), and final choice (FC) parameters were recorded. Total time spent in each arm of the Y-tube was used as a measure of the strength of preference of cues presented. IC was recorded to understand the weevil's choice for the presented cues, and RT, the time taken by a weevil to reach the decision line, was used to assess the weevil's agility for choosing a cue. FC, the position of the weevil at the end of the recording period, was recorded to understand the final preference of the weevil. Possible outcomes of the bioassay were: indifference, repellence and attraction. Indifference was noted when a plant's olfactory and/or visual cues were not more or less preferred by *C. rusticus* than control (purified air and/or empty arm) (Martini et al. 2015). Attraction was noted when a plant's olfactory and/or visual cues were preferred over the control treatment, and repellence was recorded when *C. rusticus* preferred control treatments over test plant cues (Vet et al. 1983). All bioassays were conducted between 0900 hours and 1700 hours at 21ºC room temperature at approximately 50% relative humidity at the University of Idaho's

quarantine facility. A blank test was conducted with purified air in both arms to assess any unintended bias of the setup.

Experiments

Weevils were presented with a choice between olfactory and/or visual cues to evaluate their response in a modified Y-tube olfactometer device. The following experiments were conducted:

Experiment 1 – Olfactory cues versus control (purified air): Olfactory cues (eluted VOCs) from the test plant species were presented in one arm as described above and purified air in the other arm of the Y-tube to assess the weevil responses to olfactory cues.

Experiment 2 – Visual cues versus control (empty arm): Visual cues from test plants (leaves) were presented in a modified Y-tube device below one Y-tube arm keeping the other arm empty to understand the weevil preference for visual cues.

Experiment 3 – Combined olfactory and visual cues of test plant cues versus control (purified air and empty arm): Both plant cues were presented in a modified Y-tube device as described for individual cues to test the response of *C. rusticus* to olfactory and visual cues combined.

Experiment 4 – Olfactory cues of test species versus olfactory cues of I. tinctoria: Olfactory cues from the test plants were presented in one arm, and headspace volatiles collected from *I. tinctoria* in the other arm of the Y-tube to assess the weevil response to olfactory cues of nontargets in the presence of olfactory cues of *I. tinctoria.*

Experiment 5 – Visual cues of test species versus visual cues of I. tinctoria: Nontargets plants visual cues (foliage) were presented below one arm of the Y-tube and *I. tinctoria* foliage

under the other arm of the Y-tube in a modified Y-tube device to understand the weevil response to visual cues of nontargets in the presence of visual cues of *I. tinctoria.*

Experiment 6 – Combined olfactory and visual cues of test species versus combined cues of I. tinctoria: To assess the response of *C. rusticus* to nontargets combined olfactory and visual cues in the presence of combined cues of nontargets, *I. tinctoria* and nontargets headspace VOCs were pushed into each arm of the Y-tube, and visual cues (foliage) from the respective plant species were placed right under each respective arm.

Statistical analysis

All statistical analyses were carried out using the software package SAS version 9.4 (SAS Institute 2021). Experiments 1, 2, 4, 5, and 6 were conducted in two consecutive years. Since there were no detectable differences between the two years, data for these experiments were pooled for analysis. Experiment 3 was conducted only in year two because of logistical reasons. A generalized linear mixed model, assuming a binomial response, was used to analyze the weevils' behavioral response for each bioassay. Under this model, experiment year, treated arm and replicate were treated as fixed treatment effects, whereas each run within year and replicate × treated arm within year were treated as random effects. Proportions of time spent (i.e., residence time (RET)) in both arms were compared to test if they are equal. To estimate the proportion of the weevils that chose one or the other arm (initial choice (IC) and final choice (FC)), a generalized linear mixed model was used assuming a binary response with residuals as a random effect. To test for the weevil response times (RT), a generalized linear mixed model was used, assuming a lognormal distribution for the response time, followed by a single degree of freedom contrast to assess whether the RT of female *C. rusticus* differed between individual (olfactory and visual) cues and combined

olfactory-visual cues. An additive effect was defined when no difference was detectable between the weevil's RT for both cues offered simultaneously and the average of individual cues. In contrast, a synergistic effect (a non-additive effect) was defined as a difference between the estimated response to simultaneously offered cues and the average of the estimated individual cues. To test whether response times of the *C. rusticus* differed between bioassays with *I. tinctoria* cues and nontarget plant species cues, a single degree of freedom contrast was used to compare the sum of response times of the weevils.

Results

Experiment 1 – Olfactory cues versus control (purified air)

Female *C. rusticus* spent more time in *I. tinctoria* arms when tested against purified air (t= -2.79, P=0.0087) but showed no preference for any other tested plant species (P>0.05; Fig. 3.3a, Appendix E).

Weevils preferred *I. tinctoria* but was indifferent to all other plant species for the initial choice (IC) (t=0.0068, P=0.0068; Fig. 3.5a, Appendix F) and final choice (FC) (t=0.0112, P=0.0112*;* Fig. 3.5a, Appendix F).

Weevils required less time to decide on *I. tinctoria* volatiles compared to arms with purified air (F=7.37, P=0.0106*;* Fig. 3.7a, Appendix G), and this difference in response time was not detected for any of the other plant tested species (P>0.05; Fig. 3.7a, Appendix G).

Experiment 2 – Visual cues versus control (empty arm)

Female *C. rusticus* preferred the visual cues of *I. tinctoria* (t=-2.47, P=0.0194), *D. nelsonii* (t=-4.42, P=0.0001), *E. salsugineum* (t=-0.071, P=0.0171), and *S. linifolium* (t=-2.37, P=0.0263) (Fig. 3.3b, Appendix E) over empty arms for residence time (RET). An

indifferent response was recorded for visual cues of *B. alpina, C. flavescens, D. californica, I. glauca, L. sativum, S. pinnata, S. tomentosa* and *S. viridiflora* (all $P>0.05$; Fig. 3.3b, Appendix E) when compared to an empty arm.

Weevils preferred *I. tinctoria* leaves over purified air based on IC (t=2.26, $P=0.0316$; Fig. 3.5b, Appendix F) and FC (t=2.24, $P=0.0335$; Fig. 3.5b, Appendix F). Weevils were attracted to visual cues of *D. nelsonii* (t=2.41, $P=0.0224$; Fig. 3.5b, Appendix E) and *E. salsugineum* (t=2.27, $P=0.0349$; Fig. 3.5, Appendix F) based on IC, and reacted with indifference to leaves of *B. alpina, D. californica, I. glauca, L. sativum, S. linifolium, S. pinnata, S. tomentosa, S. viridiflora,* and *C. flavescens* for both IC (all $P>0.05$; Fig. 3.5b, Appendix F) and FC (all $P>0.05$; Fig. 3.5b, Appendix F).

When presented with visual cues versus empty arms, female weevils took less time to decide for *D. nelsonii* (F=5.63, $P=0.0245$), and *I. glauca* (F=6.08, $P=0.0246$) (Fig. 3.7b, Appendix G), whereas took more time to decide when presented with visual cues of *S. pinnata* (F=-2.46, $P=0.0237$) and *S. tomentosa* (F=-2.66, $P=0.0134$) compared to empty arms (Fig. 3.7b, Appendix G). For the other tested plant species, there was no difference in the response time between visual cues and the empty arm (all $P>0.05$; Fig. 3.7b, Appendix G).

Experiment 3 – Combined olfactory and visual cues of test plant cues versus control (purified air and empty arm)

Weevils were attracted to combined olfactory and visual cues of *I. tinctoria* (t=-2.77, $P=0.0162$; Fig. 3.3c, Appendix E) and *I. glauca* (t=-2.65, $P=0.0138$; Fig. 3.3c, Appendix E). Female weevils responded with indifference to the combined cues of *B. alpina, C. flavescens, D. californica*, D. *nelsonii, E. salsugineum, L. sativum, S. pinnata,* and *S.*

viridiflora (all $P > 0.05$; Fig. 3.3c, Appendix E). *C. rusticus* was repelled by combined cues of *S. tomentosa* (t=2.49, P=0.0303*;* Fig. 3.3c, Appendix E).

Based on the initial (IC) and final choice (FC), *C. rusticus* preferred *I. tinctoria* (t=2.29, P=0.037*;* Fig. 3.5c, Appendix F) and (t=2.29, P=0.037*;* Fig. 3.5c, Appendix F), respectively over purified air and empty arms. In contrast, there was no preference for combined cues for any of the tested plant species based on IC (all P>0.05; Fig. 3.5c, Appendix F) and FC (all P>0.05; Fig. 3.5c, Appendix F).

The response time for *C. rusticus* to decide for the control treatments was shorter compared to olfactory and visual cues of *S. linifolium* (F=15, P=0.0011) and *S. pinnata* (F=5.27, P=0.0347) (Fig. 3.7c, Appendix G). There was no difference in the response time for any other plant species (P>0.05; Fig. 3.7c, Appendix G). The response time of female weevils to combined olfactory and visual cues of the tested species did not differ from that to individual olfactory and visual cues, except for *E. salsugineum* (P>0.05; Appendix H). The sum of response times across all bioassays conducted (olfactory, visual, and combined) between plant cues and the control treatments differed between *I. tinctoria* and the following nontarget species: *D. nelsonii* (F=11.99, P=0.0006), *E. salsugineum* (F=11.55, P=0.0008), *I. glauca* (F=8.97, P=0.003), *L. sativum* (F=12.01, P=0.0006), *S. linifolium* (F=6.79, P=0.0096), *S. pinnata* (F=5.51, P=0.0196), and *S. tomentosa* (F=8.44, P=0.0039) (Fig. 3.9, Appendix H). In contrast, there was no difference in the response time of *C. rusticus* between *I. tinctoria* and *B. alpina*, *C. flavescens*, *D. californica* and *S. viridiflora* (P>0.05*;* Fig. 3.9, Appendix H).

Experiment 4 – Olfactory cues of nontarget species versus olfactory cues of I. tinctoria

Female *C. rusticus* preferred *I. tinctoria* olfactory cues over those of *C. flavescens* (t=5.75, *P*<.0001), *D. californica* (t=-2.31, *P*=0.0309), *E. salsugineum* (t=-3.3, *P*=0.0026), *I. glauca* (*P*=0.011), *S. linifolium* (t=2.39, *P*=0.0234), *S. pinnata* (t=7.84, *P*<.0001), *S. tomentosa* (t=3.61, *P*=0.016), and *S. viridiflora* (t=2.81, *P*=0.0096) (Fig. 3.4a, Appendix H). In contrast, weevils did not prefer *I. tinctoria* over *B. alpina, D. nelsonii* and *L. sativum* (*P*>0.05; Fig. 3.4a, Appendix I).

For IC and FC, weevils preferred *I. tinctoria* olfactory cues only over *C. flavescens* (IC: t=-2.5, *P*=0.02, Fig. 3.6a; FC: t=-2.61, *P*=0.0172, Fig. 3.6a), and *D. californica* (IC: t=2.12, *P*=0.0448, Fig. 3.6a; FC: t=2.21, *P*=0.0333, Fig. 3.6a). In addition, *C. rusticus* preferred *I. tinctoria* olfactory cues for IC over *L. sativum* (t=-2.47, *P*=0.0214; Fig. 3.6a, Appendix F) *S. viridiflora* (t=-2.26, *P*=0.0333; Fig. 3.6a, Appendix J) and for FC *S. pinnata* (t=-2.1, *P*=0.04; Fig. 3.6a, Appendix J).

Weevils took less time to decide for VOCs of *I. tinctoria* when compared to *B. alpina* (F=7.55, *P*=0.0112) and *D. californica* (F=5.16, *P*=0.0332) (Fig. 3.8a, Appendix K). The average response time of the weevil did not differ between the VOCs of the *I. tinctoria* and the tested nontarget plant species regardless of their choice (all *P*>0.05; Fig. 3.8a, Appendix K).

Experiment 5 – Visual cues of nontarget species versus visual cues of I. tinctoria

Female *C. rusticus* preferred *I. tinctoria* leaves only over those of *D. nelsonii* (t=-2.17, *P*=0.0388) and *S. linifolium* (t=3.02, *P*=0.0051), for all other tested species the weevil did not differentiate between visual cues (all *P*>0.05; Fig. 3.4b, Appendix I).

For the initial (IC) and final choice (FC) for visual cues, *C. rusticus* only preferred *I. tinctoria* only over *S. linifolium* for FC (t=-2.54, *P*= 0.0172; Fig. 3.6b, Appendix J). For all other plant species, no preference was observed either for IC or FC (all *P*>0.05; Fig. 3.6b, Appendix J).

Weevils took less time to decide on *I. tinctoria* foliage compared to *B. alpina* (t=18.73, *P*=0.0002) and *D. californica* (t=9.38, *P*=0.0049) (Fig. 3.8b, Appendix K), but the average response time of the weevils did not differ between the *I. tinctoria* and the other nontarget species tested (all *P*>0.05; Fig. 3.8b, Appendix K).

Experiment 6 – Combined olfactory and visual cues of nontarget plant species versus combined cues of I. tinctoria

Female *C. rusticus* preferred combined olfactory and visual cues of *I. tinctoria* over all tested nontarget species for the time spent in arms (all *P*<0.05*;* Fig. 3.4c, Appendix I).

For the initial and final choice, weevils preferred *I. tinctoria* cues over *B. alpina* (IC: t=2.71, *P*=0.0114; FC: t=2.26, *P*=0.0333), *S. linifolium* (IC: t=-2.39, *P*=0.0173; FC: t=-2.68, *P*=0.0278), *S. pinnata* (IC: t=-3.4, *P*=0.0247; FC: t=-2.3, 0.0148), and *C. flavescens* (IC: t=-2.53, *P*=0.002; FC: t=-2.32, *P*=0.0299) (Fig. 3.6c, Appendix J).

Weevils took less time to choose *I. tinctoria* combined cues compared to *D. nelsonii* (F=6.03, *P*=0.0289; Fig. 3.8c), whereas for all other plant species there was no difference in the decision time regardless of choice (all *P*>0.05*;* Fig. 3.8c, Appendix K).

Discussion

Pre-alightment host selection studies are instrumental in explaining discrepancies between physiological and ecological host range data of biological weed control candidates where typically the physiological host range includes more plant species related to the targeted invasive plant than the ecological host range (Fung et al. 2021; Heard 2000; Park et al. 2018). Our results are consistent with those of other studies (e.g., Fung et al. 2021) demonstrating that BCA candidates utilize the pre-alightment phase of host selection to discriminate between their host plant and confamilial non-host plants. The clear preference of *C. rusticus* especially for olfactory and combined (visual and olfactory) pre-alightment cues of *I. tinctoria* both in the presence and absence of other tested confamilial species might explain the weevil's selective host finding behavior that has been documented in host specificity tests assessing the ecological host range (Weyl et al. 2019).

In bioassays with olfactory cues alone against purified air as a control treatment, *C. rusticus* responded with attraction to *I. tinctoria* and indifference to all tested confamilial nontarget species, suggesting that the *C. rusticus* may effectively locate and recognize *I. tinctoria.* In contrast, the weevil may not locate and/or recognize any of the tested confamilial species based on this cue modality alone. Host volatile odors can provide valuable information to insect herbivores, such as food availability and oviposition sites, which are essential for insect herbivores' survival and reproduction (Webster and Cardé 2017). Indifferent responses to olfactory cues of nontarget species imply that *C. rusticus* cannot perceive these plant species and thus, not seeking them out in the field (Bruce and Pickett 2011). In an open-field trial with *I. tinctoria* and select confamilial nontarget species low levels of attack were observed (*S. pinnata* and *S. viridiflora*; average 0.2 – 0.6 eggs/plant; *I.*

tinctoria; average 36 – 59 eggs/plant) (Hinz et al. 2015) but that attack could have occurred because the VOC plum from *I. tinctoria* may have masked confamilial nontarget plants at close proximity, i.e., olfactory plume admixture (OPA) (Park et al. 2018). The potential olfactory resemblance of confamilial nontarget plant species and resulting oviposition 'mistakes' on nontarget plant species in proximity to the host plant (e.g. Catton et al. 2014, 2015) is currently being studied in our lab.

Vision is considered as important as olfaction during host-finding (Reddy et al. 2011, Park et al. 2019). In our study, *C. rusticus* was attracted to visual cues of *I. tinctoria* but also to those of *B. alpina*, *D. nelsonii, E. salsigineum*, and *S. linifolium.* But despite attraction disappeared when olfactory and visual cues were offered combined. For the remaining nontarget species tested, the weevil responded with indifference. Finch and Collier (2000) developed the 'appropriate/inappropriate landings' theory, which posits that insect pests of Brassicaceae are capable of utilizing visual cues to discriminate between green plants and non-green plant parts or materials and that detection of the host VOCs along with relevant visual cues indicates the presence of the host plant. The attraction of *C. rusticus* to visual cues of certain nontarget species observed may suggest that the weevils are able to discern green leaves from empty arms (i.e., non-green material, e.g., Müller and Nentwig 2011) as suggested by Finch and Collier (2000) but potentially not between different reflectance spectra within the green wavelength (550 nm) area. The subsequent loss of attraction to the respective species when offered combined visual and olfactory cues may imply that the visual cues alone might be insufficient for host discrimination (Bell 1990) and emphasizes the importance of olfactory cues in *C. rusticus* for accurate host plant discrimination. It also

underlines that visual cue alone cannot be relied on to provide accurate information on host selection decisions for *C. rusticus* (Fawcett and Johnstone 2003).

When olfactory plant cues are included in pre-alightment host-selection, insect herbivores may have sufficient ecologically relevant information to assess the host plant quality (Bernays and Chapman 1994; Campbell 2004). For instance, Campbell (2004) reported the trapping of curculionid and cerambycid beetles in similar numbers with different colored traps (black (host), white (non-host)) in the absence of the olfactory stimuli. But when volatile cues were added, the weevils shifted their preference to black-colored traps (host) over white-colored traps (non-host) (Campbell 2004). Analogously, data of bioassays reported here for visual cues and combined visual and olfactory cues compared to purified air and empty arms suggest that host acceptance in *C. rusticus* depends to a greater extent on olfactory cues.

It is unclear what led *C. rusticus* to attack confamilial nontargets to some degree in open-field trials (see Hinz et al. 2015; Weyl et al. 2019). One possibility is ‘spillover’ attack, which can occur at high insect densities and when *I. tinctoria* is close to nontarget plant species, i.e., the acceptance of suboptimal host plants due to competitive interactions rather than a purposeful choice (Catton et al. 2014; Hinz et al. 2015, 2019). Alternatively, cues operating locally on the host surface, for instance, defensive plant compounds (Ballhorn et al. 2013), or an herbivores’ physiological status, for example, egg load, or the delayed search time or both (Janike 1990), could have led to the acceptance of the nontargets for feeding/oviposition in the open field tests.

Bioassays with olfactory cues of *I. tinctoria* against olfactory cues of confamilial plant species showed the clear preference of weevils to olfactory cues of *I. tinctoria* over all

confamilial plant species tested. For visual cues, *C. rusticus* only preferred *I. tinctoria* over visual cues of some confamilial plant species tested but not others, suggesting that weevils may use olfactory cues primarily to differentiate between plant species. The results of combined olfactory and visual cues of *I. tinctoria* against combined plant cues of the confamilial nontarget plant species showed a preference of *C. rusticus* for *I. tinctoria* over all the plant species tested, highlighting the importance of the combination of cues in host finding behavior study. The results of our study are consistent with findings of previous conventional host specificity tests with *C. rusticus* (see Hinz et al. 2015, Weyl et al. 2019), where oviposition on *I. tinctoria* was much greater than on confamilial nontarget species included in our study, i.e., in choice tests with the eleven nontarget species included here only nine were attacked and often only single plants had small numbers of eggs laid on them in choice tests (Weyl et al. 2019). The preference of *C. rusticus* for *I. tinctoria* observed in our bioassays and conventional host specificity data for no-choice, field cage and open field choice tests (Weyl et al. 2019) suggest a specialization of the weevil on *I. tinctoria*.

Our data on the host findings behavior of *C. rusticus* underline that the weevil can be considered an environmentally safe BCA candidate for *I. tinctoria.* However, the underlying physiological mechanisms of the host selection would complement the understanding of the environmental safety of *C. rusticus.* We attempted conducting gas chromatographic-electroantennographic detection (GC-EAD) and electroretinography (ERG) experiments with *C. rusticus* but were constrained by the availability of weevil numbers. Conducting electrophysiological experiments with *C. rusticus* could identify bioactive volatile organic compounds from identified VOCs profile (Appendix L) and/or wavelengths from reflectance spectra (Appendix M), i.e., those to which the weevil responds electrophysiologically. These

bioactive VOCs and wavelengths of light could then be further investigated for attractive or repelling responses triggered in *C. rusticus*.

There are still few studies on pre-alightment host selection in insect herbivores considered as biological weed control agents in response to visual and olfactory cues, but the number is slowly increasing (e.g., Andreas et al. 2009; Müller and Nentwig 2011; Park et al. 2018; Park et al. 2019, Fung et al. 2021). Our study aimed to contribute to the increasing body of research investigating the role of visual and olfactory cues in host finding. The results may help rendering some species within the physiological host range of BCA candidates environmentally safe from post-release nontarget attack (Park et al. 2018; Schaffner et al. 2018). The findings are particularly beneficial to the United States Department of Agriculture Animal Plant Health Inspection Service (USDA APHIS) and the Technical Advisory Group (TAG, a federal interagency group of subject experts that reviews petitions and advises USDA APHIS), the regulatory agency responsible for biological control introductions in the USA (Park et al. 2018). The regulatory agencies responded very positive to the inclusion of such data in field release petitions (S. Sing, M. Schwarzländer, personal communication)

In sum, our data demonstrate that *C. rusticus* utilizes pre-alightment cues, especially olfactory cues, to identify its host plant and that it cannot perceive any of the tested confamilial nontarget plant species as host plants. The results presented here illustrate the utility of behavioral bioassays with visual and olfactory cues as part of pre-release environmental safety assessments in weed biological control.

Table 3.1. List of selected North American test species and reason for inclusion.

Lineages[1]	Tribe[1]	Species[1]	Flowering time[2]	Elevation (m)[2]	Distribution[2]	Life history trait	Native range	Reason for inclusion
Lineage I	Descurainieae	*Descurainia nelsonii* (Rydb.) Al-Shehbaz & Goodson	late May-mid Jul	800-3000	B.C., Calif., Idaho, Mont., Nev., Oreg., Wash., Wyo	Annual[4]	North America[4]	Non-host; Limited feeding and oviposition[4,5]
		Descurainia californica (A. Gray) O. E. Schulz	Jun–Aug	1700-3400	Ariz., Calif., Colo., Nev., N.Mex., Oreg., Utah, Wyo	Perennial[4]	North America[4]	Non-host; Limited feeding and oviposition[4,5]
	Lepidae	*Lepidium sativum* L.	Apr–Aug	N/A	Introduced; Alta., B.C., Man., Nfld. and Labr. (Nfld.), N.W.T., N.S., Ont., P.E.I., Que., Sask., Conn., Idaho, Iowa, Maine, Md., Mass., Mich., N.H., N.Y., Ohio, Oreg., Pa., R.I., Tenn., Wash., Wyo., Europe, SW Asia, perhaps NE Africa, introduced also in South America (Argentina), Australia	Annual[8]	South-west Asia (perhaps Iran)[3]	Limited feeding and adult development[8]

[1](BrassiBase 2021), [2](FNA 2021), [3](Sabaghnia et al. 2015), [4](Hinz et al. 2012), [5](Hinz et al. 2014), [6](Hinz et al. 2015), [7](Hinz et al. 2016), [8](Weyl et al. 2017), [9](Weyl et al. 2019); Alta.: Alberta, Ariz.: Arizona, B.C: British Columbia, Colo.: Colorado, Conn.: Connecticut, Ill.: Illinois, Kans.: Kansas, Man.: Manitoba, Mass.: Massachusetts, Mich.: Michigan, Mo.: Missouri, Mont.: Montana, N Africa: North Africa, N.Dak.: North Dakota, Nev.: Nevada, N.H.: New Hampshire, Pa.: Pennsylvania, Nfld. And Labr (Nfld.): Newfoundland and Labrador, N.W.T.: Northwest Territories, Ont.: Ontario, Que.: Quebec, Calif.: California, N.Mex.: North Mexico, N.Y.: New York, Oreg.: Oregon, P.E.I.: Prince Edward Island, R.I.: Rhode Island, Sask.: Saskatchewan, Tenn.: Tennessee, Va.: Vancouver, Wash.: Washington, WC California: West Coast California, W.Va.: Washington Vancouver, Wyo.: Wyoming, C, SW Asia: Central, Southwest Asia

Lineage II	Isatideae	*Isatis tinctoria* L.	Apr-Jun	300-2200	B.C, Nfld. and Labr. (Nfld.), Ont., Que., Calif., Idaho, Ill., Mo., Mont., Nev., N.Mex., N.Y., Oreg., Utah, Va., Wash., W.Va., Wyo., Europe, C, SW Asia, N Africa, South America (introduced; Chile, Peru)	Annual, biennial or Perennial[4]	Eurasian[4]	Host plant[8,9]
		Isatis glauca Aucher ex Boiss.	N/A	N/A	N/A	Perennial[5]	Eurasian[5]	Non-host; Limited feeding and oviposition, adult emergence[5,6,8]
	Eutremeae	*Eutrema salsugineum* (Pall.) Al-Shehbaz & Warwick	May–Jun	600-2500	B.C., N.W.T., Sask., Yukon, Colo., Mont., C, E Asia	Annual[5]	North America[5]	Non-host; Limited feeding and oviposition, adult emergence[5,6,8]
	Sisymbrieae	*Sisymbrium linifolium* (Nutt.) Nutt. ex Torr. & A. Gray	Apr–Aug	700-2800	B.C., Ariz., Colo., Idaho, Mont., Nev., N.Mex., Oreg., Utah, Wash., Wyo.	Perennial[6]	North America[6]	Non-host; Limited feeding; supported larval development[6,7,8,9]
	Thelypodieae	*Caulanthus flavescens* (Hook.) Payson	Mar-May	200-700	United States (WC California)	Annual[7]	North America[7]	Non-host; Limited feeding and oviposition, adult emergence[7,8,9]
		Stanleya pinnata (Pursh) Britton *(Stanleya pinnata* var. *pinnata)*	Apr–Sep	200-2500	Ariz., Calif., Colo., Idaho, Kans., Mont., Nev., N.Mex., N.Dak., Oreg ., S.Dak., Utah, Wyo.	Perennial[4]	North America[4]	Non-host; Limited feeding and oviposition, adult emergence[4,5,7,8]
		Stanleya tomentosa Parry	Jun–Aug	1300-2300	Idaho, Wyo.	Biennial, Perennial[5]	North America[5]	Non-host; Limited feeding and oviposition, adult emergence[5,6,7,8,9]
		Stanleya viridiflora Nutt.	May–Jul	1300-2700	Calif., Colo., Idaho, Mont., Nev., Oreg., Utah, Wyo	Perennial[4]	North America[4]	Non-host; Limited feeding and oviposition, adult emergence[4,5,7,8]

Lineage III	Euclidieae	*Braya alpina* var *americana* Hooker *(Braya glabella* subsp. *Glabella)*	Jun–Jul	0-3700	North America	Perennial[8]	North America[8]	Non-host; Limited feeding and oviposition, larval development[8]

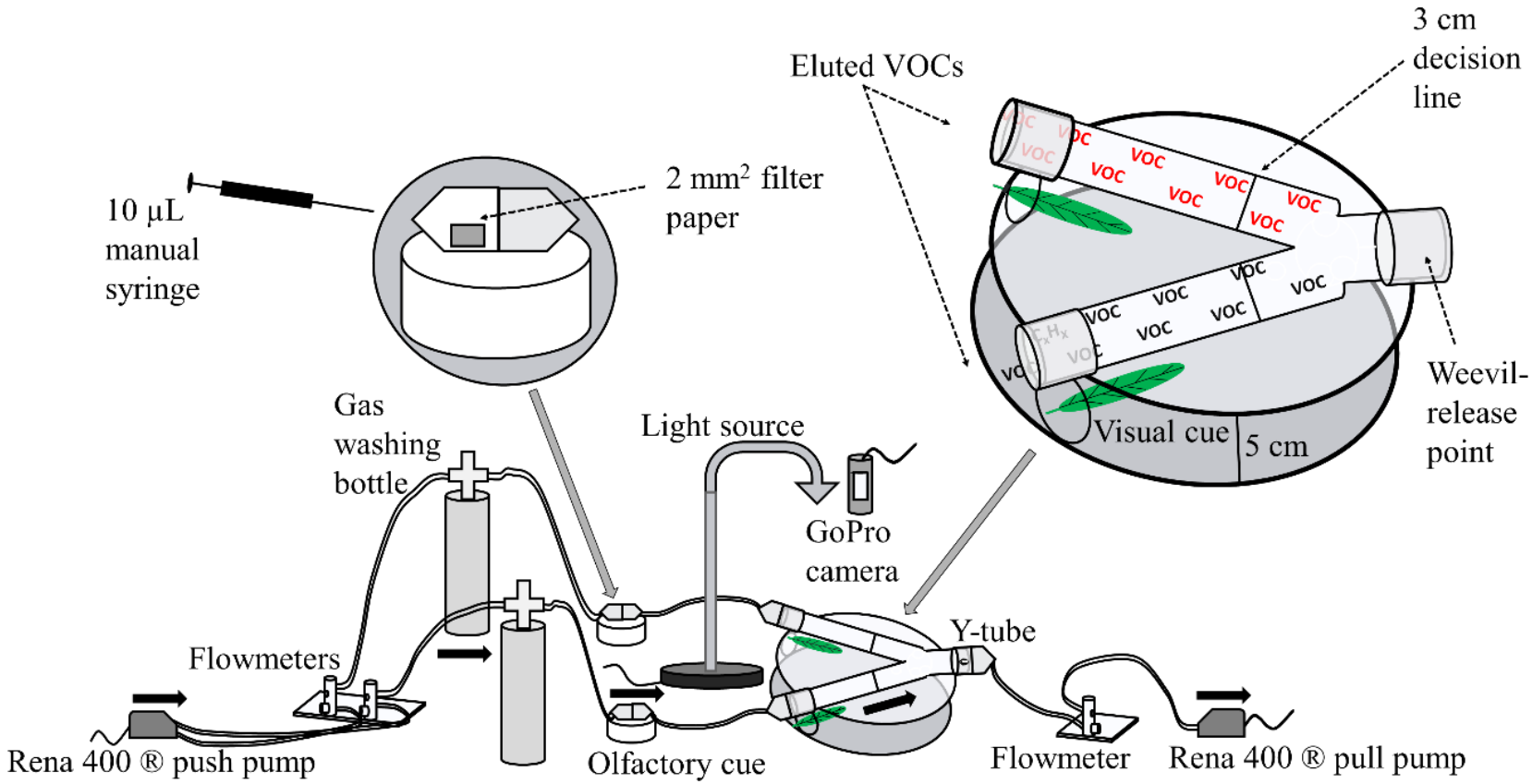


Fig. 3.1: Modified y-tube olfactometer set-up. In the center is the Y-tube (4 cm Y-stem, 12 cm arms, 2 cm internal diameter) resting on the cylindrical plastic ring with transparent plastic top (diameter: 20 cm; height: 5 cm)**.** The plastic ring consisted of 2 openings (diameter: 3 cm) which served the purpose of introducing visual cues into the bioassay. 1 µL eluted volatile organic compound (VOCs) is applied to 2 mm^2 filter paper for each replication. Black arrow on the figure represents the direction of airflow. Note: Figure not drawn to scale.

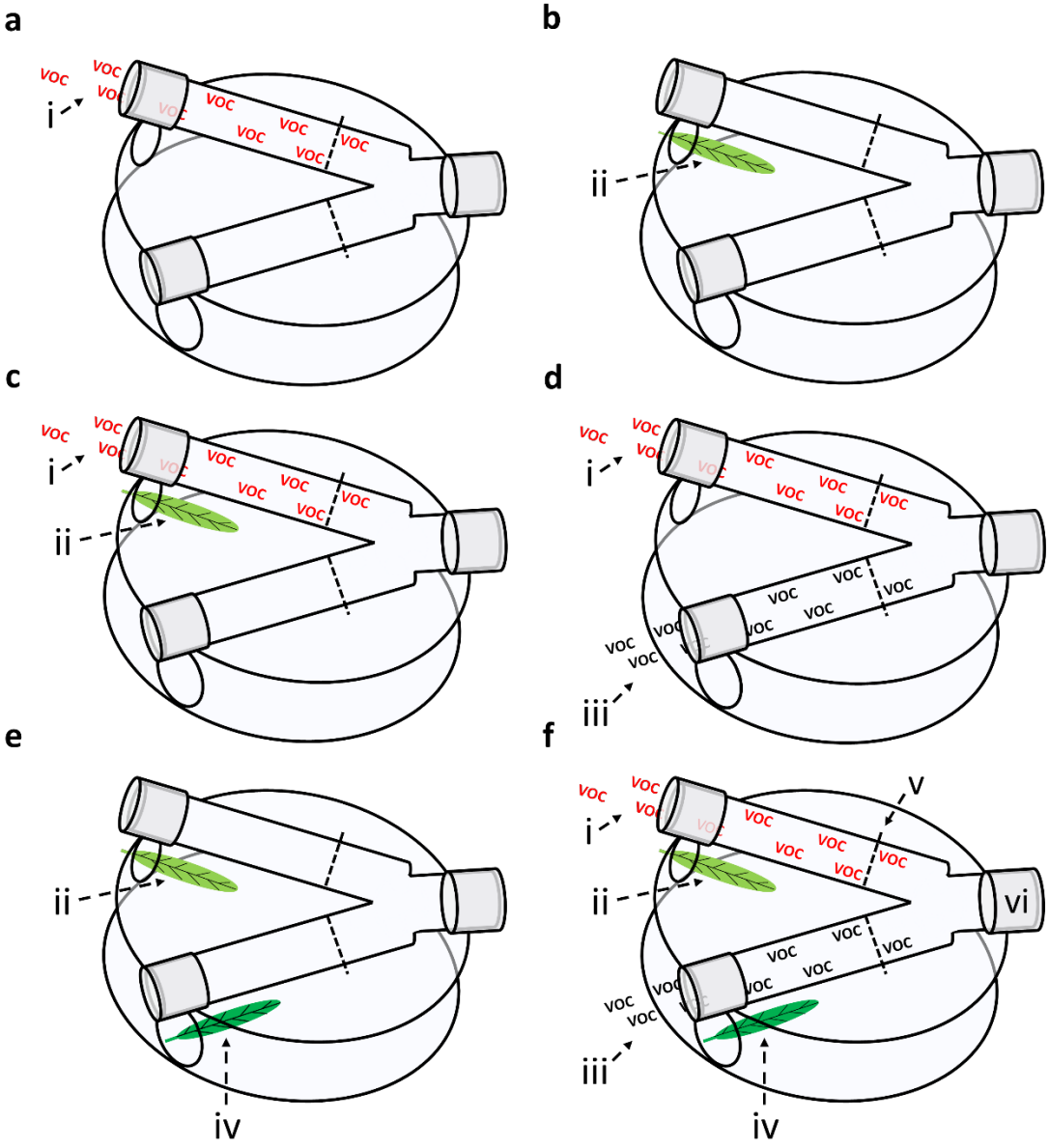


Fig. 3.2: Modified Y-tube device (4 cm Y-stem, 12 cm arms, 2 cm internal diameter). A single Y-tube resting on the cylindrical plastic ring (diameter: 20 cm; height: 5 cm) consisting of 2 openings (diameter: 3 cm) to introduce visual cues. Experiments in behavioral choice assays: Olfactory cues vs control (purified air) (**a**), Visual cues vs control (empty arm) (**b**), Combined cues vs control (purified air and empty arm) (**c**), Olfactory cues of test plant vs olfactory cues of *I. tinctoria* (**d**), Visual cues of test plant vs visual cues of *I. tinctoria* (**e**), Combined cues of test plant vs combined cues of *I. tinctoria* (**f**). Roman numerals in **a**, **b**, **c**, **d, e, and f**: **i** (test plant olfactory cues); **ii** (test plant visual cues); **iii** (*I. tinctoria* olfactory cues); **iv** (*I. tinctoria* visual cues); **v** (3 cm decision-line); **vi** (weevil-release point).

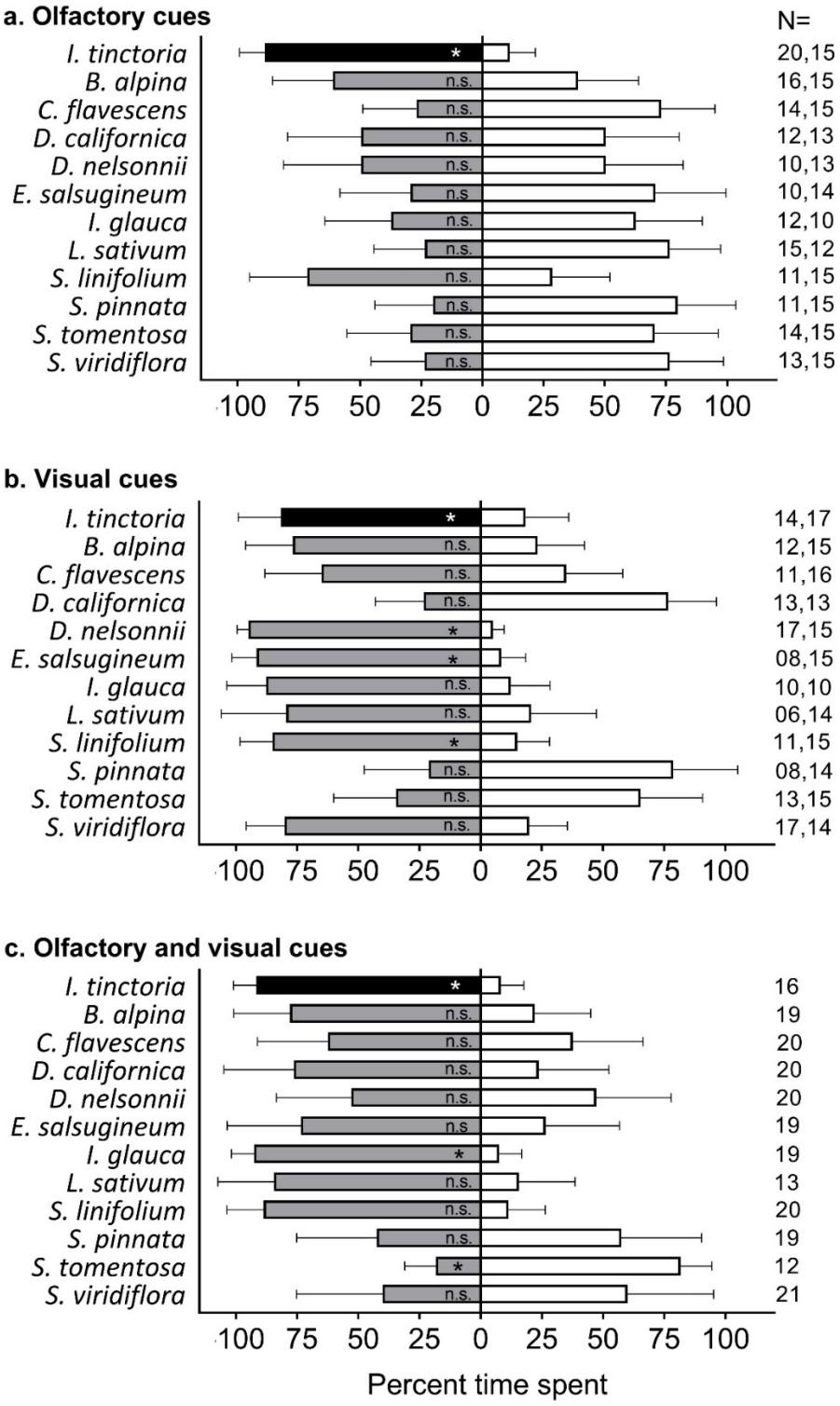


Fig. 3.3: Proportion of time spent (mean ± SE) by female *C. rusticus* in experiment with olfactory cues **(a)**, visual cues **(b)**, and combined olfactory and visual cues **(c)** of *I. tinctoria* (black bars), test plant species (grey bars), and control treatments (purified air and/or empty arm; white bars). Generalized linear mixed model; *$P < 0.05$; n.s., not significant. Numbers (N) on top of right bars indicate replicate numbers for year 1 and 2, respectively.

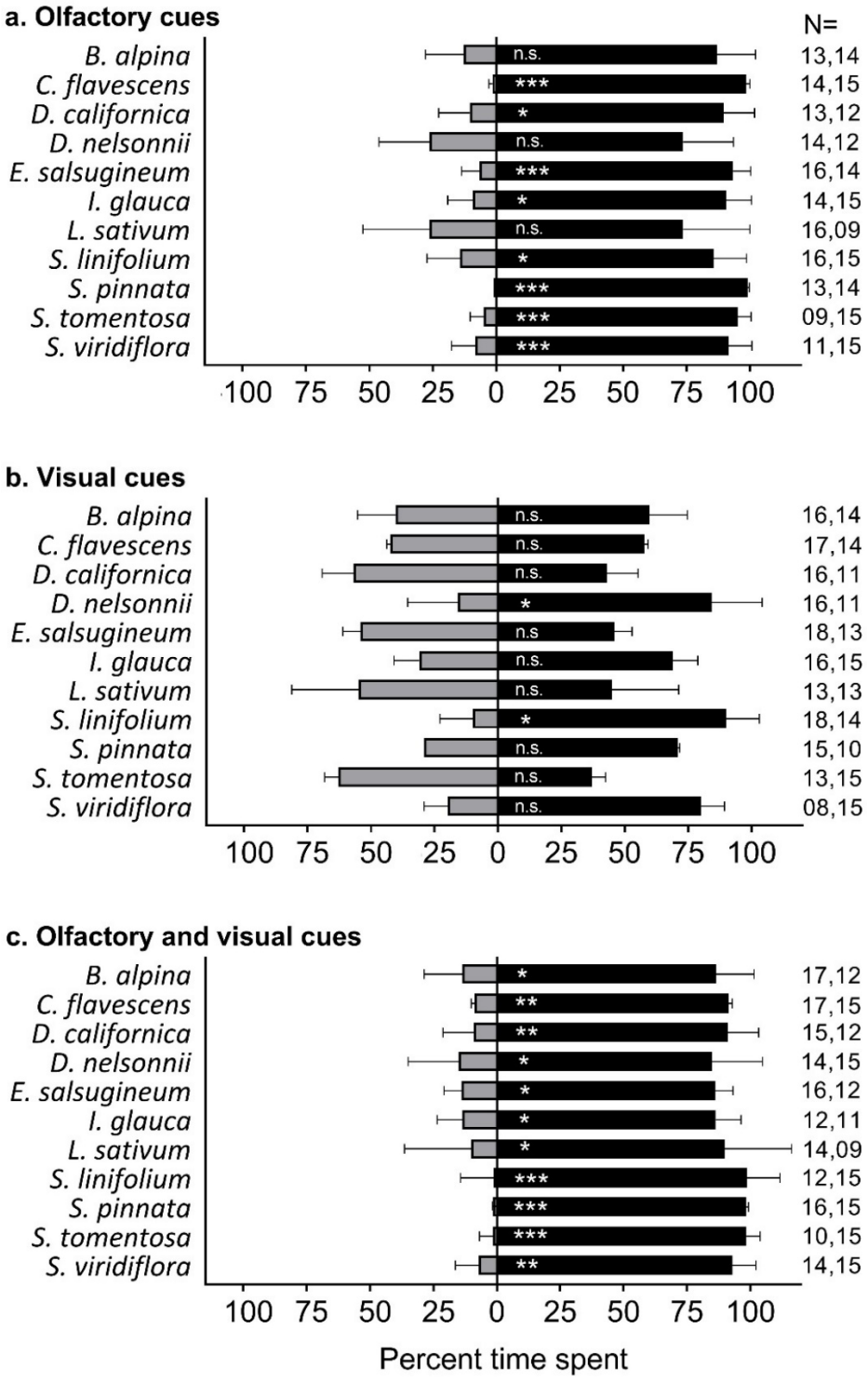
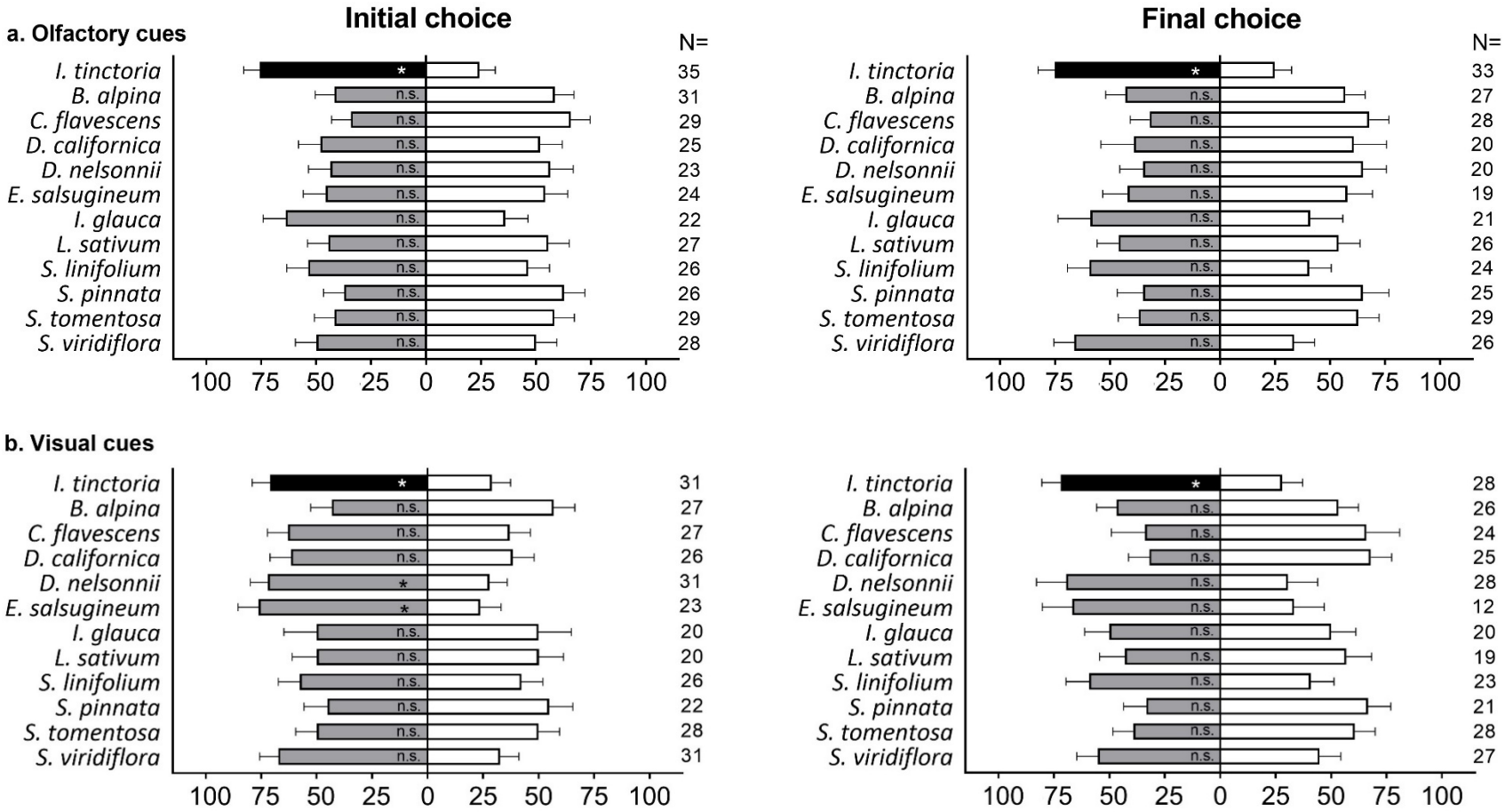


Fig. 3.4: Proportion of time spent (mean ± SE) by female *C. rusticus* in experiment with olfactory cues **(a)**, visual cues **(b)**, and combined olfactory and visual cues **(c)** of test plant species (grey bars) and *I. tinctoria* (black bars). Generalized linear mixed model; **P* < 0.05, ***P* < 0.001, ****P* < 0.001; n.s., not significant. Numbers (N) on top of right bars indicate replicate numbers for year 1 and 2, respectively.


a. Olfactory cues
Initial choice
Final choice
N=
I. tinctoria
B. alpina
C. flavescens
D. californica
D. nelsonnii
E. salsugineum
I. glauca
L. sativum
S. linifolium
S. pinnata
S. tomentosa
S. viridiflora
*
n.s.
35
31
29
25
23
24
22
27
26
26
29
28
33
27
28
20
20
19
21
26
24
25
29
26
100 75 50 25 0 25 50 75 100
b. Visual cues
31
27
27
26
31
23
20
20
26
22
28
31
28
26
24
25
28
12
20
19
23
21
28
27

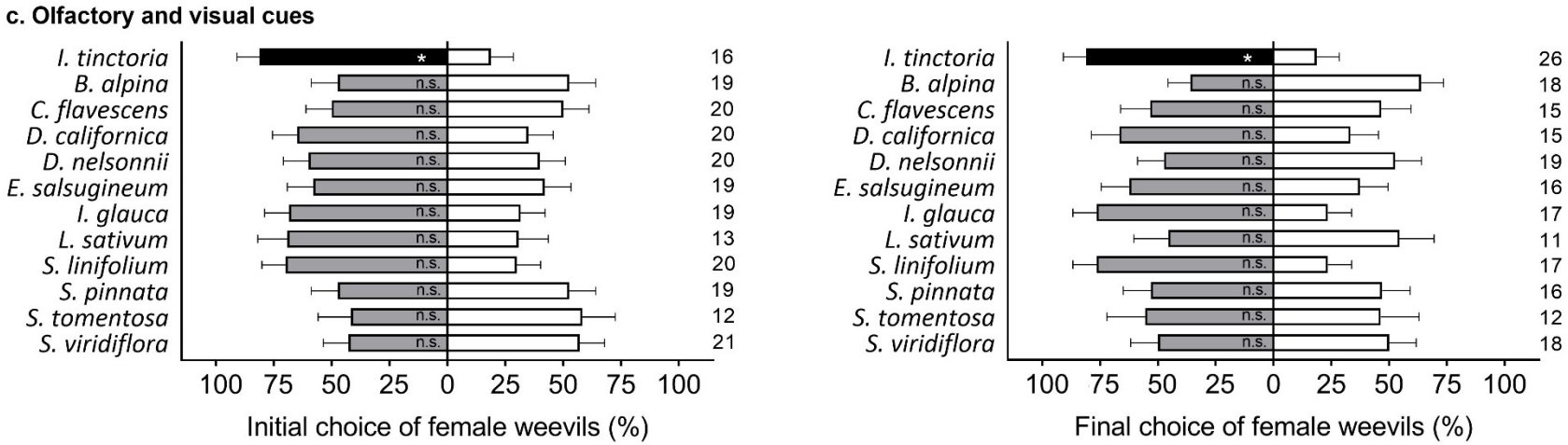
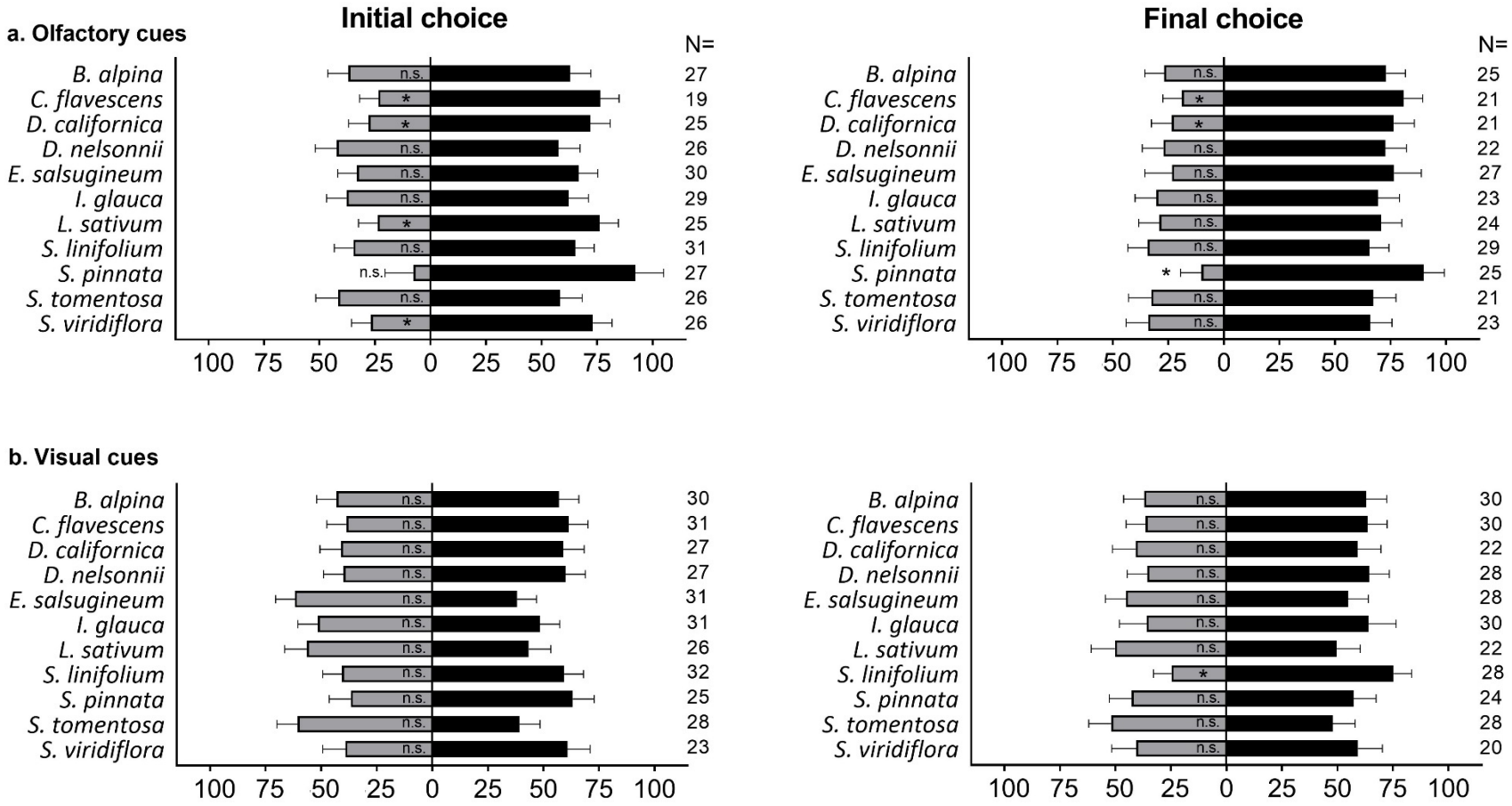


Fig. 3.5: Proportion of initial (first) arm choice (IC) and final choice (FC) (mean ± SE) of female *C. rusticus* in experiment with olfactory cues **(a)**, visual cues **(b)**, and combined olfactory and visual cues **(c)** of *I. tinctoria* (black bars), test plant species (grey bars), and control treatments (purified air and/or empty arm; white bars). Generalized linear mixed model; **P* < 0.05; n.s., not significant. Numbers on the top of each bars indicates the number of female *C. rusticus* choosing for initial choice and final choice in respective panel.


a. Olfactory cues
Initial choice
Final choice
N=
B. alpina
C. flavescens
D. californica
D. nelsonnii
E. salsugineum
I. glauca
L. sativum
S. linifolium
S. pinnata
S. tomentosa
S. viridiflora
n.s.
*
27
19
25
26
30
29
25
31
27
26
26
25
21
21
22
27
23
24
29
25
21
23
100 75 50 25 0 25 50 75 100
b. Visual cues
30
31
27
27
31
31
26
32
25
28
23
30
30
22
28
28
30
22
28
24
28
20

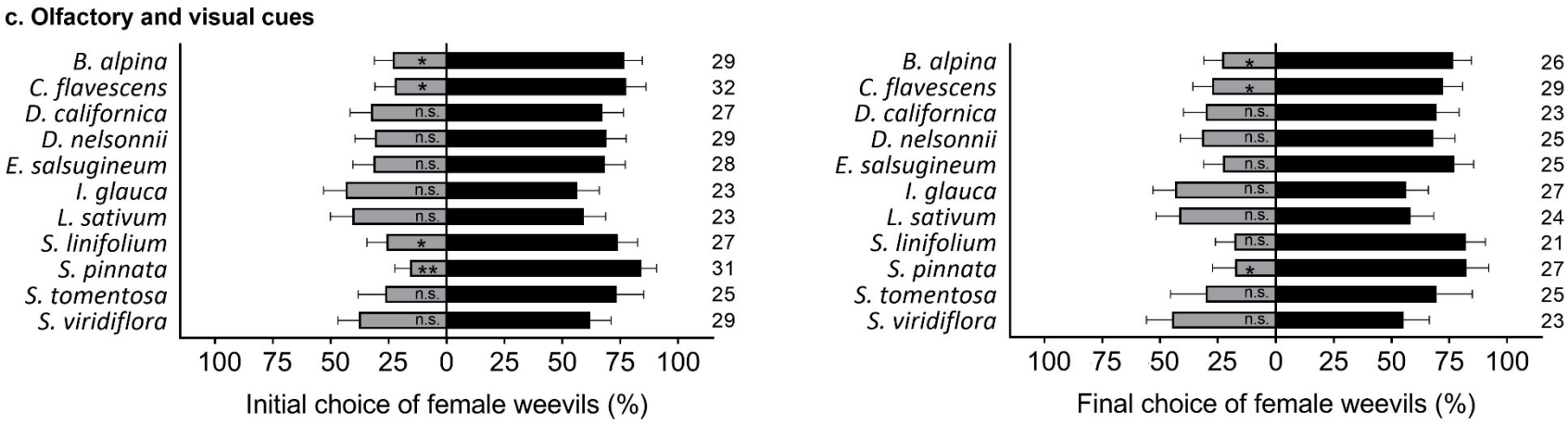


Fig. 3.6: Proportion of initial (first) arm choice (IC) and final choice (FC) (mean ± SE) of female *C. rusticus* in experiment with olfactory cues **(a)**, visual cues **(b)**, and combined olfactory and visual cues **(c)** of test plant species (grey bars), and *I. tinctoria* (black bars). Generalized linear mixed model; *$P < 0.05$; n.s., not significant. Numbers on the top of each bars indicates the number of female *C. rusticus* choosing for initial choice and final choice in respective panel.

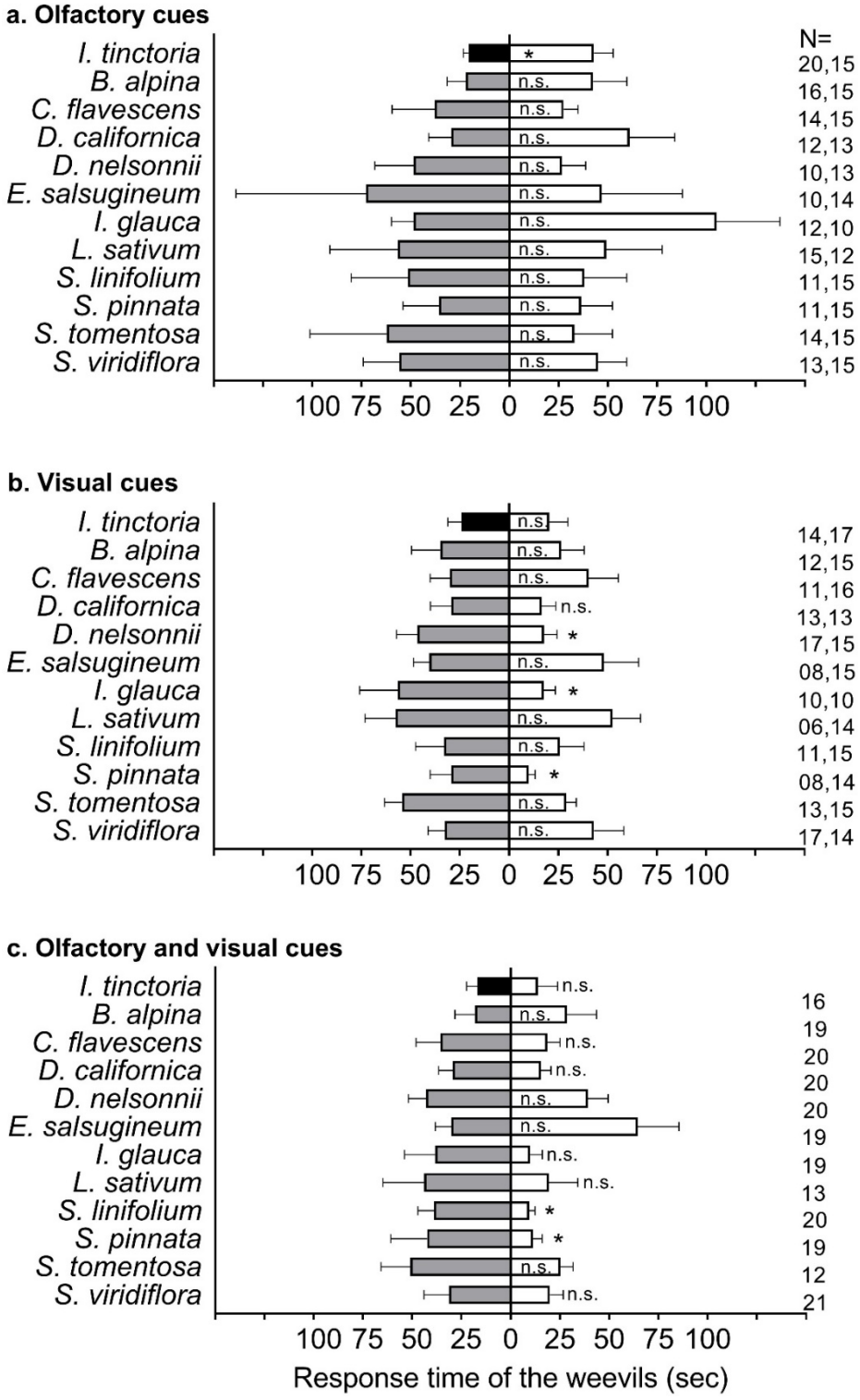


Fig. 3.7: Mean (± SE) response times (RT) of female *C. rusticus* in experiment with olfactory cues (**a**), visual cues (**b**), and combined olfactory and visual cues (**c**) of *I. tinctoria* (black bars), test plant species (grey bars), and control treatments (purified air and/or empty arm; white bars). Generalized linear mixed model; *P < 0.05; n.s., not significant.

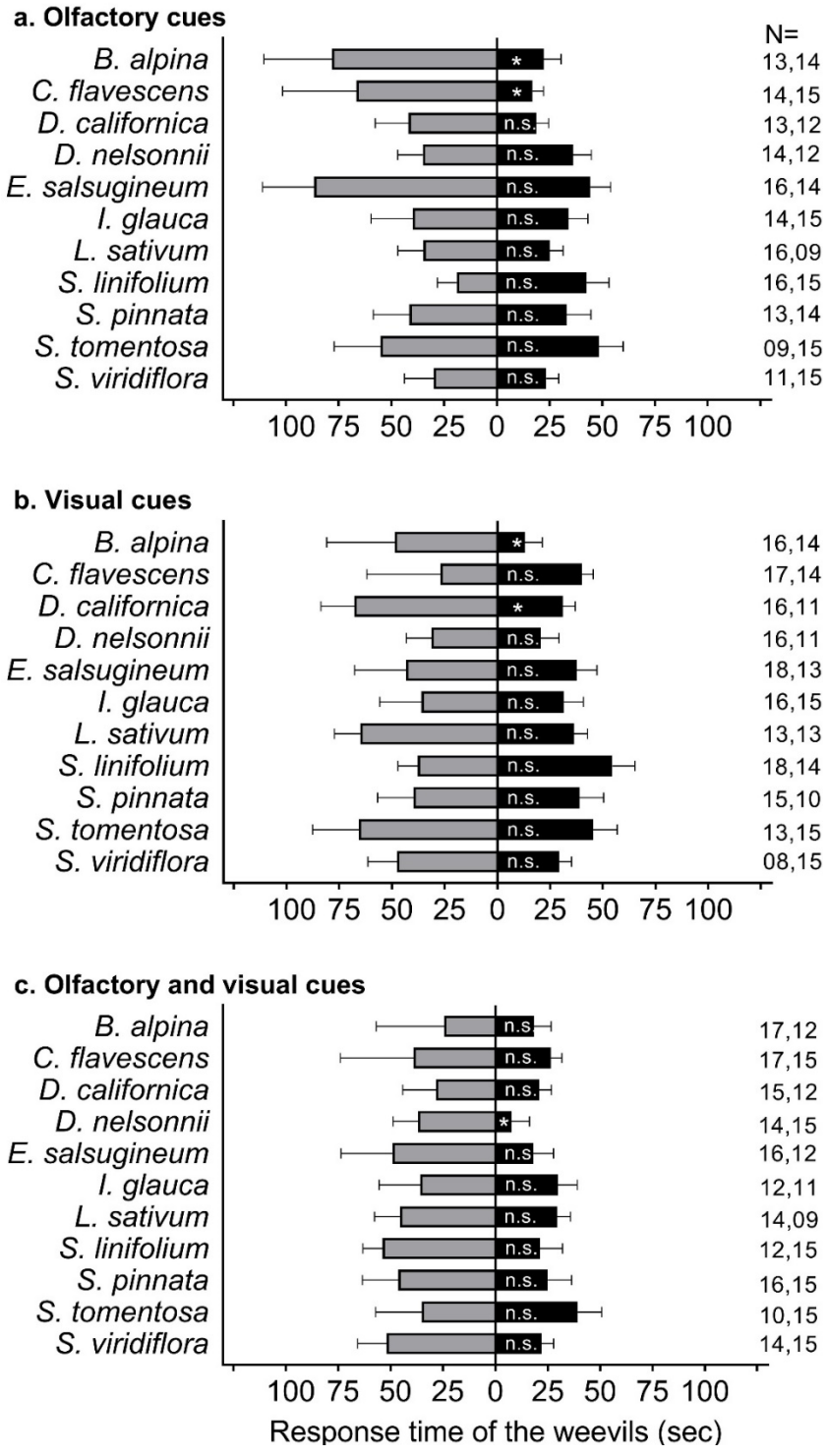


Fig. 3.8: Mean (± SE) response times (RT) of female *C. rusticus* in experiment with olfactory cues **(a)**, visual cues **(b)**, and combined olfactory and visual cues **(c)** of test plant species (grey bars) and *I. tinctoria* (black bars). Generalized linear mixed model; **P* < 0.05; n.s., not significant.

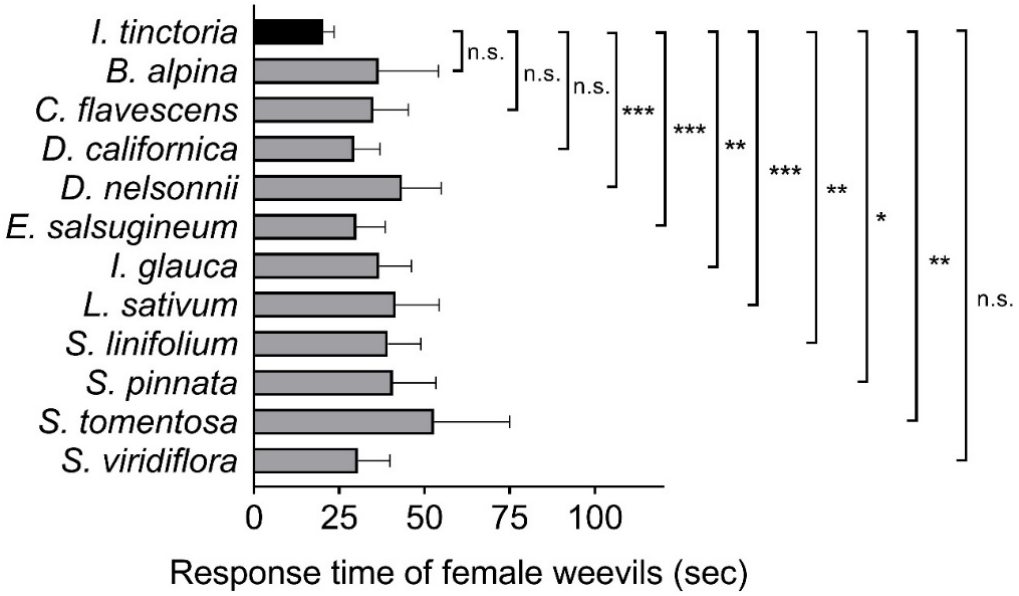


Fig. 3.9: Mean (± SE) for the contrast of total response times (RT) of female *C. rusticus* between *I. tinctoria* and individual test plant species across bioassays with olfactory and/or visual cues of target *I. tinctoria*, test plant species, and control (purified air and/or empty arm). Generalized linear mixed model followed by single degree of freedom contrast between *I. tinctoria* and test plant species (black brackets) (**P*<0.05, ***P*<0.01, ****P*<0.001; n.s., not significant).

References

Al-Shehbaz IA (2012) A generic and tribal synopsis of the Brassicaceae (Cruciferae). Taxon 61:931-954

Andreas JE et al (2009) Post-release non-target monitoring of *Mogulones cruciger*, a biological control agent released to control *Cynoglossum officinale* in Canada. In: Julien MH, Sforza R, Bon MC, Evans HC, Hatcher PE, Hinz HL, Rector BG (eds) Proceedings of the 12th international symposium on biological control of weeds. CAB International, Wallingford, United Kingdom, pp 75–82

Ascrizzi R, Flamini G (2020) *Iris lutescens* on serpentine soil: volatile emission profiles in different organs of its two colour morphs. Plant Biosyst 8:1-9

Bailey CD, Koch MA, Mayer M, Mummenhoff K, O'Kane SL, Warwick SI, Windham MD & Al-Shehbaz IA (2006) Toward a global phylogeny of the Brassicaceae. Mol Biol Evol 23:2142-2160

Balkenius A, Bisch-Knaden S, Hansson B (2009) Interaction of visual and odour cues in the mushroom body of the hawkmoth *Manduca sexta*. J Expt Biol 212:535-541

Ballhorn DJ, Kautz S, Heil M (2013) Distance and sex determine host plant choice by herbivorous beetles. PLoS ONE 8:e55602

Bartlet E, Blight MM, Lane P, Williams IH (1997) The responses of the cabbage seed weevil *Ceutorhynchus assimilis* to volatile compounds from oilseed rape in a linear track olfactometer. Entomol Exp Appl 85:257-262

Beilstein MA, Al-Shehbaz IA, Kellogg EA (2006) Brassicaceae phylogeny and trichome evolution. Am J Bot 93:607-619

Bell WJ (1990) Searching behavior patterns in insects. Ann Rev Entomol 35:447–467

Bernays EA, Chapman RF (1994) Host plant selection by phytophagous insects. Chapman and Hall, New York, NY

Beyaert I, Hilker M (2014) Plant odour plumes as mediators of plant–insect interactions. Biol Rev 89:68–81

Bezemer TM, Harvey JA, Cronin JT (2014) Response of native insect communities to invasive plants. Annu Rev Entomol 59:119-41

Bradbury WC, Bennett GF (1974) Behavior of adult Simuliidae (Diptera). II. Vision and olfaction in near-orientation and landing. Can J Zool 52:1355-1364

BrassiBase (2021) BrassiBase. https://brassibase.cos.uni-heidelberg.de/ Accessed 26 January 2021

Briese DT (2003) The centrifugal phylogenetic method used to select plants for host-specificity testing of weed biological control agents: can and should it be modernised? Proceedings of the CRC for Australian Weed Management Biological Control of Weeds Symposium and Workshop, pp 23-33

Briese DT, Walker A (2002) A new perspective on the selection of test plants for evaluating the host-specificity of weed biological control agents: the case of *Deuterocampta quadrijuga*, a potential insect control agent of *Heliotropium amplexicaule*. Biol Control 25:273-287

Briese DT, Walker A, Pettit WJ, Sagliocco JL (2002) Host-specificity of candidate agents for the biological control of *Onopordum* spp. thistles in Australia: an assessment of testing procedures. Biocontrol Sci Technol 12:149-163

Bruce TJA, Pickett JA (2011) Perception of plant volatile blends by herbivorous insects—Finding the right mix. Phytochemistry 72:1605–1611

Bruce TJA, Wadhams LJ, Woodcock CM (2005) Insect host location: A volatile situation. Trends Plant Sci 10:269–274

Byers J, Zhang Q, Birgersson G (2004) Avoidance of nonhost plants by a bark beetle, *Pityogenes bidentatus*, in a forest of odors. Naturwissenschaften 91:215-219

Calflora (2021) Calflora: Information on California plants for education, research and conservation. https://www.calflora.org/ Accessed March 2021

Callihan RH (1990) Dyers woad: biology, distribution and control. Bulletin - Idaho Agricultural Experiment Station (USA) 20:18-32

Callihan RH, Dewey SA, Patton JE, Thill DC (1984) Distribution, biology, and habitat of Dyer's woad (*Isatis tinctoria*) in Idaho. Journal of the Idaho Academy of Science 20:18-32

Campbell SA (2004) Integration of visual and olfactory cues by host-seeking forest coleoptera (Scolytidae, Cerambycidae). Thesis, Simon Fraser University

Campbell SA, Borden JH (2006) Close-range, in-flight integration of olfactory and visual information by a host-seeking bark beetle. Entomol Exp Appl 120:91-98

Campbell SA, Borden JH (2009) Additive and synergistic integration of multimodal cues of both hosts and non-hosts during host selection by woodboring insects. Oikos, 118(4):553-63

Catton HA, Lalonde RG, De Clerck-Floate RA (2014) Nontarget herbivory by a weed biocontrol insect is limited to spillover, reducing the chance of population-level impacts. Ecol Appl 25:517–530

Clement SL, Cristofaro M (1995) Open-field tests in host-specificity determination of insects for biological control of weeds. Biocontrol Sci Technol 5:395-406

Clewley GD, Eschen R, Shaw RH, Wright DJ (2012) The effectiveness of classical biological control of invasive plants. J Appl Ecol 49:1287-1295

Committee FoNAE (1993) Flora of North America: Magnoliophyta: Salicaceae to Brassicaceae. Oxford University Press

Cosse AA et al (2006) Behaviorally active green leaf volatiles for monitoring the leaf beetle, *Diorhabda elongata*, a biocontrol agent of saltcedar, *Tamarix* spp. J Chem Ecol 32:2695-2708

Couty A, Van Emden H, Perry JN, Hardie J, Pickett JA, Wadhams LJ (2006) The roles of olfaction and vision in host-plant finding by the diamondback moth, *Plutella xylostella*. Physiol Entomol 31:134–145

Crook DJ et al. (2009) Laboratory and field response of the emerald ash borer (Coleoptera: Buprestidae), to selected regions of the electromagnetic spectrum. J Econ Entomol 102:2160-2169

Culliney T (2005) Benefits of classical biological control for managing invasive plants. Crit Rev Plant Sci 24:131-150

D'Antonio CM, Vitousek PM (1992) Biological invasions by exotic grasses, the grass/fire cycle, and global change. Annu Rev Ecol Evol Syst 23:63-87

De Clercq P, Mason PG, Babendreier D (2011) Benefits and risks of exotic biological control agents. BioControl, 56(4):681-98

De Moraes CM, Mescher MC, Tumlinson JH (2001) Caterpillar-induced nocturnal plant volatiles repel conspecific females. Nature 410:577–580

Defagó MT, Videla M, Valladares G (2016) To smell you better: prior food deprivation increases herbivore insect responsiveness to host plant odor cues. J Insect Behav 29:527-534

Deletre E, Schatz B, Bourguet D, Chandre F, Williams L, Ratnadass A, Martin T (2016) Prospects for repellent in pest control: current developments and future challenges. Chemoecology 26:127-42

Diagne C, Leroy B, Vaissière AC, Gozlan RE, Roiz D, Jarić I, Salles JM, Bradshaw CJ, Courchamp F (2021) High and rising economic costs of biological invasions worldwide. Nature, 592(7855):571-6

Diggles MF, Kanim NR, Figura P, Knight M (2003) The Siskiyou Mariposa Lily: an expedition to determine the remaining extent of an endangered species. Flag Report, Flag 161, California: The Explorers Club

DiTomaso J et al. (2013) Weed control in natural areas in the western United States. University of California Weed Research and Information Center, Davis, California, USA, pp 544

DiTomaso JM (2000) Invasive weeds in rangelands: species, impacts, and management. Weed Sci 48:255-265

ECOS (2021) Environmental Conservation Online System. U.S. Fish & Wildlife Service. https://ecos.fws.gov/ecp/species/5909 Accessed 04 March 2021

Egonyu JP, Ekesi S, Kabaru J, Irungu L, Torto B (2013) Cashew volatiles mediate short-range location responses in *Pseudotheraptus wayi* (Heteroptera: Coreidae). Environ Entomol 42:1400–1407

Elton CS (1958) The ecology of invasions by animals and plants. Methuen, London, UK

EPA (2002) Biopesticide registration action document: *Puccinia thlaspeos* 'strain woad' (PC Code 006489). Office of Pesticide Programs, Biopesticides and Pollution Prevention Division

Evans J, Chase R (1981) Dyer's woad control. Utah State University Extension Service Bulletin EL-188, Logan, UT

Evans JO, Dewey SA (1994) Dyer's woad. In: Sheley, RL (eds) The identification, distribution, impacts, biology and management of noxious rangeland weeds. Scientific Contract Report

Farah KO, Tanaka AF, West NE (1988) Autecology and population biology of Dyer's Woad (*Isatis tinctoria*). Weed Sci 36:186-193

Fawcett TW, Johnstone RA (2003) Optimal assessment of multiple cues. Proc R Soc Lond B Biol Sci 270:1637-1643

Finch S, Collier RH (2000) Host-plant selection by insects - a theory based on 'appropriate/inappropriate landings' by pest insects of cruciferous plants. Entomol Exp Appl 96:91–102

FNA (2021) Flora of North America. Volume 17. http://beta.floranorthamerica.org/Volume_17 Accessed 26 January 2021

FWS (2006) Recovery plan for *Phlox hirsuta* (Yreka Phlox). U.S. Fish and Willife Service, Sacramento, California

Gerber E, Cortat G, Hinz HL (2009) Biology and host specificity of *Aulacobaris fallax* (Coleoptera: Curculionidae), a potential biological control agent for dyer's woad, *Isatis tinctoria* (Brassicaceae) in North America. J Appl Entomol 133:345-354

Gibson RD (2017) Biology and interaction of the invasive forb *Isatis tinctoria*, dyer's woad, with the native rust fungus *Puccinia thlaspeos* in populations in the intermountain west, United States. Thesis (M.S., Environmental Science), University of Idaho, Moscow, Idaho

Heard TA (2000) Concepts in insect host-plant selection behavior and their application to host specificity testing. In: Van Driesche RG, Heard T, McClay A, Reardon R (eds) Forest Health Technology Enterprise Team, Proceedings of Session: Host Specificity Testing of Exotic Arthropod Biological Control Agents - the biological basis for improvement in safety. Proceedings of the X. international symposium on biological control of weeds. pp 1-10

Hinz HL et al (2015) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2014. CABI Switzerland, Delemont

Hinz HL et al (2016) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2015. CABI Switzerland, Delemont

Hinz HL, Blair L, Closca C, Gerber E (2012) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2011. CABI Switzerland, Delemont

Hinz HL, Closca C, Castellan, I, Heijs, W, Morelon, S (2016) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2015. CABI Switzerland, Delemont

Hinz HL, Closca C, Pardo P (2013) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2012. CABI Switzerland, Delemont

Hinz HL, Closca C, Pardo P (2014) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2013. CABI Switzerland, Delemont

Hinz HL, Closca C, Stattegger S, Winkler L (2015) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2014. CABI Switzerland, Delemont

Hinz HL, Cortat G, Edelmann L, Gerber E (2008) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2007. CABI Switzerland, Delemont

Hinz HL, Cortat G, Gerber E (2007) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2006. CABI Switzerland, Delemont

Hinz HL, Gerber E, JoyeS, Ye Z (2011) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2010. CABI Switzerland, Delemont

Hinz HL, Gerber E, Krebs C, Cortat G (2005) Biological control of dyer's woad, *Isatis tinctoria*, Annual Report 2004. CABI Switzerland, Delemont

Hinz HL, Schwarzländer M, Gaskin J (2008) Does phylogeny explain the host-choice behaviour of potential biological control agents for Brassicaceae weeds? In: R. Sforza, Bon MC (eds) Proceedings of the XII International Symposium on Biological Control of Weeds. La Grande Motte, France, 2007 2008. CABI, pp 410-417

Hinz HL, Schwarzlander M, Gassmann A, Bourchier RS (2014) Successes we may not have had: A retrospective analysis of selected weed biological control agents in the United States. Invas Plant Sci Mana 7:565-579

Hinz HL, Winston RL, Schwarzländer M (2019) How safe is weed biological control? A global review of direct nontarget attack. Q Rev Biol 94:1–27

Hinz HL, Winston RL, Schwarzländer M (2020) A global review of target impact and direct nontarget effects of classical weed biological control. Curr Opin Insect 38:48-54

Jacobs J, Pokorny M (2007) Ecology and Management of Dyer's Woad (*Isatis tinctoria* L.). US Department of Agriculture, Natural Resources Conservation Service

Jaenike J (1990) Host specialization in phytophagous insects. Ann Rev Ecol Syst 21:243–273

Jang EB, Messing RH, Klungness LM, Carvalho LA (2000) Flight tunnel responses of *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) to olfactory and visual stimuli. J Insect Behav 13:525-538

Janz N, Nylin S (1997) The role of female search behaviour in determining host plant range in plant feeding insects: a test of the information processing hypothesis. Proc R Soc B 264:701–707

Keane RM, Crawley MJ (2002) Exotic plant invasions and the enemy release hypothesis. Trends Ecol Evol 17:164-170

Kerr JL, Kelly D, Bader MK, Brockerhoff EG (2017) Olfactory cues, visual cues, and semiochemical diversity interact during host location by invasive forest beetles. J Chem Ecol 43:17-25

Kevan PG, Baker HG (1983) Insects as flower visitors and pollinators. Annu Rev Entomol 28:407-453

Kinoshita M, Stewart FJ, Ômura H (2017) Multisensory integration in Lepidoptera: insights into flower-visitor interactions. BioEssays 39:1600086

Knollhof LM, Heckel DG (2014) Behavioral assays for studies of host plant choice and adaptation in herbivorous insects. Annu Rev Entomol 59:263-278

Kropp BR, Albee S, Flint KM, Zambino P, Szabo L, Thomson SV (1995) Early detection of systemic rust infections of dyers woad (*Isatis tinctoria*) using the polymerase chain reaction. Weed Sci 43:467-472

Lonsdale WM (1999) Global patterns of plant invasions and the concept of invasibility. Ecology 80:1522–1536

Louda SM (2000) *Rhynocyllus conicus*- Insights to improve predictibility and minimize risk of biological control of weeds. Svata M. Louda Publications, pp 24

Louda SM, Rand TA, Arnett AE, McClay AS, Shea K, McEachern AK (2005) Evaluation of ecological risk to populations of a threatened plant from an invasive biocontrol insect. Ecol Appl 15:234-249

Mack RN, Simberloff D, Lonsdale WM, Evans H, Clout M, Bazzaz FA (2000) Biotic invasions: causes, epidemiology, global consequences, and control. Ecol Appl 10:689-710

Martini X, Hughes MA, Smith JA & Stelinski LL (2015) Attraction of redbay ambrosia beetle, *Xyleborus glabratus*, to leaf volatiles of its host plants in North America. J Chem Ecol 41: 613-621

McConnell EG, Evans EO, Dewey SA (1999) Dyer's Woad. In: Sheley RL, Petroff JK (eds) Biology and management of noxious rangeland weeds. Oregon State University Press, Corvalis, OR, pp 231-237

McFadyen REC (1998) Biological control of weeds. Annu Rev Entomol 43:369-393

Mellor H, Bellingham J, Anderson M (1997) Spectral efficiency of the glasshouse whitefly *Trialeurodes vaporariorum* and *Encarsia formosa* its hymenopteran parasitoid. Entomol Exp Appl 83:11-20

Miller JR, Strickler KL (1984) Finding and accepting host plants. In: Bell WJ, Carde RT (eds) Chemical ecology of insects. Springer US, Boston, MA, pp 135-160

Monaco TA, Johnson DA, Creech JE (2005) Morphological and physiological responses of the invasive weed *Isatis tinctoria* to contrasting light, soil-nitrogen and water. Weed Res 45:460-466

Moorhouse J, Yeadon R, Beevor P, Nesbitt BF (1969) Method for use in studies of insect chemical communication. Nature, 223:1174-1175

Müller E, Nentwig W (2011) How to find a needle in a haystack–host plant finding of the weevil *Ceratapion onopordi*. Entomol Exp Appl 139:68-74

Müller-Schärer H, Schaffner U (2008) Classical biological control: exploiting enemy escape to manage plant invasions. Biol Inv 10:859-874

Murphy ST, Evans HC (2009) Biological control of invasive species. In: Clout MN, Williams P (eds) Invasive species management. A handbook of principles and techniques. Oxford University Press, Oxford, Oxford, pp 77–92

Myrick AJ, Baker TC (2018) Increasing signal-to-noise ratio in gas chromatography - electroantennography using a deans switch effluent chopper. J Chem Ecol, 44:111-126

Park I, Eigenbrode SD, Cook SP, Harmon BL, Hinz HL, Schaffner U, Schwarzlander M (2018) Examining olfactory and visual cues governing host-specificity of a weed biological control candidate species to refine pre-release risk assessment. BioControl 63:377–389

Park I, Schwarzlander M, Hinz H, Schaffner U, Eigenbrode S (2019) 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. Arthropod-Plant Interact 13:139–149

Park I, Thompson DC (2021) Host recognition by *Rhinocyllus conicus* of floral scents from invasive and threatened thistles. Biol Invas 20:1-6.

Paynter Q, Paterson ID, Kwong RM (2020) Predicting non-target impacts. Curr Opin Insect Sci 38:79-83

Pejchar L, Mooney HA (2009) Invasive species, ecosystem services and human well-being. Trends Ecol Evol 24:497-504

Pimentel D, Zuniga R, Monison D (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol Econ 52:273-288

Pysek P, Jarosik V, Hulme PE, Pergl J, Hejda M, Schaffner U, Vila M (2012) A global assessment of invasive plant impacts on resident species, communities and ecosystems: the interaction of impact measures, invading species' traits and environment. Glob Change Biol 18:1725-1737

Pysek P, Richardson DM (2010) Invasive species, environmental change and management, and health. Annu Rev Env Res 35:25-55

Raguso RA, Willis MA (2005) Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta.* Anim Behav 69:407-418

Rai PK, Singh JS (2020) Invasive alien plant species: Their impact on environment, ecosystem services and human health. Ecol Indic, 111:106020

Rapo CB, Schaffner U, Eigenbrode SD, Hinz HL, Price WJ, Morra M, Gaskin J, Schwarzländer M. (2019) Feeding intensity of insect herbivores is associated more closely with key metabolite profiles than phylogenetic relatedness of their potential hosts. PeerJ 17;7:e8203

Reddy GVP, Cruz ZT, Braganza N, Muniappan R (2009) Response of *Melittia oedipus* (Lepidoptera: Sesiidae) to visual cues is increased by the presence of food source. J Econ Entomol 102:127–132

Reeves JL (2011) Vision should not be overlooked as an important sensory modality for finding host plants. Environ Entomol 40:855–863

Reeves RD, Brooks RR, Macfarlane RM (1981) Nickel uptake by Californian *Streptanthus* and *Caulanthus* with particular reference to the hyperaccumulator *S. polygaloides* Gray (Brassicaceae). Am J Bot 68:708-12

Rejmánek M (2015) Global trends in plant naturalization. Nature 525:39-40

Richardson DM, Pysek P, Rejmánek M, Barbour MG, Panetta FD, West CJ (2000) Naturalization and invasion of alien plants: concepts and definitions. Divers Distrib 6:93-107

Sabaghnia N, Ahadnezhad A, Janmohammdi M (2015) Genetic variation in garden cress (*Lepidium sativum* L.) germplasm as assessed by some morphological traits. Genet Resour Crop Evol 2:733-45

SAS Institute (2021) Base SAS 9.4 Procedures Guide. SAS Institute, Cary, North Carolina, United States of America

Schaffner U (2001) Host range testing of insects for biological weed control: how can it be better interpreted? BioScience 51:1–9

Schaffner U, Smith L, Cristofaro M (2018) A review of open-field host range testing to evaluate non-target use by herbivorous biological control candidates. BioControl 63:405–416

Schwarzländer M, Hinz HL, Winston RL, Day MD (2018) Biological control of weeds: an analysis of introductions, rates of establishment and estimates of success, worldwide. BioControl 63:319-331

Sheley RL, James JJ, Rinella MJ, Blumenthal D, DiTomaso JM (2011) Invasive plant management on anticipated conservation benefits: a scientific assessment. Conservation benefits of rangeland practices: assessment, recommendations, and

knowledge gaps. USDA Natural Resources Conservation Service, Lawrence, KS, USA. 2011 Oct, 1:291-336

Sheley RL, Sheley JL, Smith BS (2015) Economic savings from invasive plant prevention. Weed Sci 63:296-301

Silva R, Clarke AR (2019) The “sequential cues hypothesis”: A conceptual model to explain host location and ranking by polyphagous herbivores. Insect Sci pp 1–11

Simberloff D (2011) Risks of biological control for conservation purposes. BioControl 57:263-276

Simberloff D et al. (2013) Impacts of biological invasions: what's what and the way forward. Trends Ecol Evol 28:58-66

Simberloff D, Stiling P (1996a) How risky is biological control? Ecology 77:1965-1974

Simberloff D, Stiling P (1996b) Risks of species introduced for biological control. Biol Conserv 78:185-192

Sonmez O, Bukun B, Kaya C, Aydemir S (2008) The assessment of tolerance to heavy metals (Cd, Pb and Zn) and their accumulation in three weed species. Pak J Bot 40:747-754

Speranza J, Miceli N, Taviano MF, Ragusa S, Kwiecień I, Szopa A, Ekiert H (2020) *Isatis tinctoria* L.(Woad): a review of its botany, ethnobotanical uses, phytochemistry, biological activities, and biotechnological studies. Plants 9:298

Stenberg JA, Ericson L (2007) Visual cues override olfactory cues in the host-finding process of the monophagous leaf beetle *Altica engstroemi*. Entomol Exp Appl 125:81-88

Strong DR (1997) Fear No Weevil? Science 277:1058

Suckling DM, Sforza RFH (2014) What magnitude are observed non-target impacts from weed biocontrol? PloS one 9(1):e84847

Tasin M, Betta E, Carlin S, Gasperi F, Mattivi F, Pertot I (2011) Volatiles that encode host-plant quality in the grapevine moth. Phytochemistry 72:1999–2005

Thompson JN (1988) Evolutionary ecology of the relationship between oviposition preference and performance of offspring in phytophagous insects. Entomol Exp Appl 47:3–14

USDA NRCS (2021) The PLANTS Database [Online]. Greensboro, NC, USA: National Plant Data Team. http://plants.usda.gov/core/profile?symbol=ISTI Accessed 15 February 2021

Van Kleunen M, Dawson W, Essl F, Pergl J, Winter M, Weber E, Kreft H, Weigelt P, Kartesz J, Nishino M, Antonova LA (2015) Global exchange and accumulation of non-native plants. Nature 525(7567):100-3

van Klinken R (2000) The meaning of "host-range" and "host specifity", and implications for host-specificity testing. Proceedings of the X International Symposium on Biological Control of Weeds. pp 688-689

van Klinken RD, Edwards OR (2002) Is host-specificity of weed biological control agents likely to evolve rapidly following establishment? Ecol Lett 5:590-596

van Lenteren JC, Bale J, Bigler E, Hokkanen HMT, Loomans AM (2006) Assessing risks of releasing exotic biological control agents of arthropod pests. Annu Rev Entomol 51:609-634

Varga WA, Evans JO (1978) Dyer woad - from cultivated - to cursed. Utah Science 39:87-89

Vet LE, Lenteren JV, Heymans M, Meelis E (1983) An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects. Physiol Entomol 8:97-106

Vilà M et al. (2011) Ecological impacts of invasive alien plants: a meta-analysis of their effects on species, communities and ecosystems. Ecol Lett 14:702-708

Wagner DL, Van Driesche RG (2010) Threats posed to rare or endangered insects by invasions of nonnative species. Annu Rev Entomol 55:547-68

Wapshere AJ (1974) A strategy for evaluating the safety of organisms for biological weed control. Ann Appl Biol 77:201-211

Wapshere AJ (1989) A testing sequence for reducing rejection of potential biological control agents for weeds. Ann Appl Biol 114:515-526

Webster B, Cardé RT (2017) Use of habitat odour by host-seeking insects. Biol Rev 92:1241–1249

Welsh SL, Atwood ND, Goodrich S, Higgins LC (1987) A Utah flora. The Great Basin Naturalist Memoir No. 9

West NE, Farah KO (1989) Effects of clipping and sheep grazing on dyers woad. J Range Manag 42:5-10

Weyl P, Cloşca C, Hinz HL, Vidović B, Petanović R, Cristofaro M, Dieudonné E, Vasilev L (2017) Biological control of dyer's woad, *Isatis tinctoria,* Annual Report 2016. CABI Switzerland, Delemont

Weyl P, Cloşca C, Hinz HL, Vidović B, Petanović R, Cristofaro M, Marini F, Besomi G (2019) Biological control of dyer's woad, *Isatis tinctoria,* Annual Report 2018. CABI Switzerland, Delemont

Wheeler GS, Schaffner U (2013) Improved understanding of weed biological control safety and impact with chemical ecology: a review. Invas Plant Sci Mana 6:16–29

Williamson M, Fitter A (1996) The Varying Success of Invaders. Ecol 77:1661-1666.

Winston RL, M. Schwarzländer, H.L. Hinz, M.D. Day, and MJWC, M.H. Julien E (2014) Biological control of weeds: a world catalogue of agents and their target weeds, 5th edition. USDA Forest Service, Forest Health Technology Enterprise Team, Morgantown, West Virginia

Woad, dyer's (*Isatis tinctoria*) (2016) http://pnwhandbooks.org/weed/other-items/control-problemweeds/woad-dyers-isatis-tinctoria. Accessed March 2021

Young J (1988) Dyer’s woad wages chemical war in west. Agric. Res.

Young J, Evans R (1977) Dyer's woad. Weeds Today 21:76-78

Zouhar K (2009) *Isatis tinctoria* In: Fire Effects Information System [Online]. U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station, Fire Sciences Laboratory. http://www.fs.fed.us/database/feis/plants/forb/isatin/all.html Accessed 2018 March

Zvi MM et al (2008) Interlinking showy traits: co-engineering of scent and colour biosynthesis in flowers. Plant Biotechnol J 6:403-15

Appendix A. Summary statistics for initial choice (IC) and final choice (FC) of naïve and experienced *peyerimhoffi* females in a modified Y-tube choice bioassay with olfactory cues, visual cues and combined olfactory and visual cues of target *I. tinctoria*, and test plant species against control (purified air and/or empty arm), and combined olfactory and visual cues of test plant species against *I. tinctoria*.

Bioassay	**Test species**	**Naive**				**Experienced**			
		Initial choice		**Final choice**		**Initial choice**		**Final choice**	
		t value	**p-value**	**t value**	**p-value**	**t value**	**p-value**	**t value**	**p-value**
Olfactory cues vs purified air	*Isatis tinctoria*[b]	1.65	0.1276	1.56	0.1568	**-2.54**	**0.0209**	**-2.57**	**0.0212**
	Braya alpina[a]	1.83	0.0924	1.65	0.1276	0	1	0	1
	Boechera hoffmannii[a]	-	-	-	-	0.45	0.6603	-0.24	0.8116
	Caulanthus heterophyllus[a]	0.3	0.7695	-1.23	0.2506	0.72	0.4797	0.77	0.4545
Visual cues vs empty arm	*Isatis tinctoria*	1.65	0.1276	2.08	0.0619	**2.83**	**0.0097**	**2.53**	**0.0189**
	Braya alpina[a]	0	1	0.57	0.5771	1.38	0.18	1.21	0.238
	Boechera hoffmannii[a]	-	-	-	-	1.76	0.0915	1.21	0.238
	Caulanthus heterophyllus[a]	0	1	0.3	0.7695	-0.45	0.6603	-0.89	0.3855
Olfactory & visual cues vs purified air & empty arm	*Isatis tinctoria*[b]	2.2	0.0528	2.08	0.0668	**-2.47**	**0.028**	**-2.3**	**0.0423**
	Braya alpina[a]	0.57	0.5771	-0.89	0.3929	0.72	0.4797	0.5	0.6252
	Boechera hoffmannii[a]	-	-	-	-	0.99	0.3383	0.28	0.7864
	Caulanthus heterophyllus[a]	-0.82	0.4258	-0.57	0.5771	1.46	0.1644	-0.26	0.8002
Olfactory & visual cues of test plant vs *I. tinctoria*	*Braya alpina*[a]	-	-	-	-	**2.35**	**0.0305**	**2.15**	**0.0497**
	Boechera hoffmannii[a]	-	-	-	-	1.39	0.1836	1.99	0.0675
	Caulanthus heterophyllus[a]	-	-	-	-	**-2.42**	**0.0277**	-1.83	0.0924

[a]Native North American confamilial plant species (FNA 2021); [b]Eurasian confamilial plant species (Weyl et al. 2019). Test statistics are generalized linear mixed models, assuming a binary response for the choice in the treated arm. Significant values highlighted in bold.

Appendix B. Summary statistics for the test of synergy for the naïve and experienced weevil's average response times in individual (olfactory and visual) cue bioassay vs combined cue bioassay for individual tested plant species, and contrast of sum of response times across all bioassays (olfactory cues, visual cues, and combined cues) between *Isatis tinctoria* and test plant species in a modified Y-tube choice bioassay with olfactory, visual and combined olfactory-visual cues of target *I. tinctoria*, and test plant species, and control (purified air and/or empty arm).

Test species	**Test of synergy**				**Contrast**			
	Naive		**Experienced**		**Naive**		**Experienced**	
	t value	**p-value**	**t value**	**p-value**	**t value**	**p-value**	**t value**	**p-value**
Isatis tinctoria[b]	1.85	0.1844	**5.22**	**0.0271**	-	-	-	-
Braya alpina[a]	0.05	0.8298	1.38	0.2482	1.93	0.1697	**6.18**	**0.0143**
Boechera hoffmannii[a]	-	-	2.86	0.1062	-	-	**9.14**	**0.0030**
Caulanthus heterophyllus[a]	0.13	0.7206	0.98	0.3304	0.23	0.6348	**5.86**	**0.0169**

[a]Native North American confamilial plant species (FNA 2021); [b]Eurasian confamilial plant species (Weyl et al. 2019). Test statistics are generalized linear mixed models, assuming a lognormal response for the response time for the treated arm. Significant values highlighted in bold.

Appendix C. Floral reflectance and electroretinography (ERG)

Floral reflectance measurement

To measure the floral reflectance method used by Crook et al. (2009) was adopted. Reflectance data were recorded from flowers of each species. Relative reflectance was recorded for each spectral reading using ASD FieldSpec 4 Hi-Res: High-Resolution Spectroradiometer (Malvern Panalytical Ltd, Cambridge, UK). Each spectral reading consisted of 10 scans. White reference readings were measured before recording reflectance from each sample. To record reflectance from flowers, samples from each test species were prepared by placing the flowers from each plant on a black cloth. Measurements were taken by placing the fiber optic cable assisted by the pistol grip directly above the flower at the height of 1 cm on a sunny afternoon for all test species, except for *B. hoffmannii*, whose floral

reflectance was measured in a dark lab setting with ASD Contact Probe. Because *B. hoffmanni* flowers were only available during winter, gloomy weather did not permit floral reflectance measurement in an outside setting.

Electroretinography (ERG)

ERG was conducted to determine whether female *C. peyerimhoffi* can detect and respond to a specific wavelength from the wavelength spectrum of 350 nm to 750 nm. Instrumentation and set-up used to conduct electroretinography are described by Park et al. (2018). In summary, Xenon 75 W short arc lamp (Oriel Instruments, Irvine, CA, USA) was used to generate the full spectrum light beam. A monochromator (aperture: 3.16 mm; Oriel Instruments, Irvine, CA, USA) controlled by Newport 74004 software (Oriel Instruments, Irvine, CA, USA) was used to produce the specific bands of the light spectrum at 10 nm interval, which was delivered to insect preparation using a liquid light guide cable.

Legs and antennae of each female weevil (n = 30) were excised and placed laterally on playdough facing right eye up. The head of the weevil was fixed by pressing the snout of the weevil into playdough. The abdomen and thorax were immobilized using sticky tape. Glass insulated tungsten electrodes (Taper angle: 10º – 12º, impedance: @ 1KHz: MΩ; Tunglass-1, Kation Scientific, LLC, Minneapolis, USA) were used for the recording. The recording electrode was inserted into the center of the eye, and an indifferent electrode was inserted into the thorax (Mellor et al. 1997). The whole insect preparation was dark-adapted for 30 minutes before experimenting.

Weevils were only selected for ERG recordings if the test flash of full-spectrum light generated by LED light (Jansjö ® LED lamp, Inter Ikea System B. V., Delft, Netherlands)

elicited a typical ERG response, i.e., a monophasic waveform with negative potential change (Fig. C2).

At each specific wavelength single pulse was provided by opening a shutter controlled by Newport 74004 software (Oriel Instruments, Irvine, CA, USA), and eyes were allowed to recover for 60 – 90 seconds before presenting the light pulse of another wavelength. ERG responses were amplified 10X by EAG combi electrode (Syntech, Hilversum, Netherlands) and 10X by IDAC-232 box (Syntech, Hilversum, Netherlands). The amplified ERG signals were captured and visualized using GcEad/2014 Version 1.2.5. All the experiments were conducted between 08:30 am and 05:00 pm.

Statistical analysis

For sensitivity response, PCA was conducted to see if there were any groupings in individual weevil's sensitivity response. Then, the correlation between plant reflectance spectra and optical sensitivity responses was estimated using a bootstrap procedure. A replication of a complete set of spectral observations was randomly selected with replacement within each plant species. Correspondingly, a replication of optical observations was also randomly selected and paired with the spectral observations by wavelength. This process was repeated to provide 5000 paired sets of spectral reflectance and sensitivity observations. The Pearson correlation between reflectance and sensitivity was then computed for each of the 5000 bootstrap replications, and the resulting distribution of correlations was summarized and plotted. For all tests, *P*-values <0.05 were considered as significant. SAS version 9.4 (SAS Institute 2021) was used to carry out the analyses.

Results

Floral reflectance measurement and electroretinography (ERG)

In the spectrum range from 350 nm – 750 nm, average floral reflectance of *I. tinctoria, B. hoffmannii*, and *C. heterophyllus* overlaps in three regions (~ 385 nm, ~ 520 nm, and ~ 680 – 730 nm) other regions remaining disjoint (Fig. K1). Average floral reflectance curve of *B. alpina* was found to be greater than the other test species recorded.

Preliminary analysis with the PCA suggested three possible groupings (Group 1, Group 2, and Group 3) of the compound eyes' sensitivity response (Fig. K3). Components 1 and 2 altogether explained 72.06% of variability in the data. Principal component 1 (PC1) (58.8%; loadings: 370 nm, 380 nm, 400 nm – 450 nm, 660 nm – 680 nm) separated Group 1 and Group 3 from Group 2, and principal component 2 (PC2) (13.26%; loadings: 460 nm – 480 nm, 500 nm, 550 nm, 610 nm – 640 nm) separated Group 1 and Group 2 from Group 3.

With bootstrap procedure, the three groups identified previously manifested themselves as pronounced smaller peaks within the overall distributions (Fig. D4). Some sub distributions show significant negative relationships; however, other sub distributions do not and are substantially centered over 0.0 (Fig. K4, Table K1).

Table C1. Confidence intervals of the Pearson correlation between reflectance and sensitivity computed for each of the 5000 bootstrap replications.

Test species	**95 % confidence intervals**	
	Lower	**Upper**
Isatis tinctoria	-0.5021	0.06729
Braya alpina	-0.32547	0.10279
Boechera hoffmannii	-0.46585	-0.24364
Caulanthus heterophyllus	-0.41335	0.03756

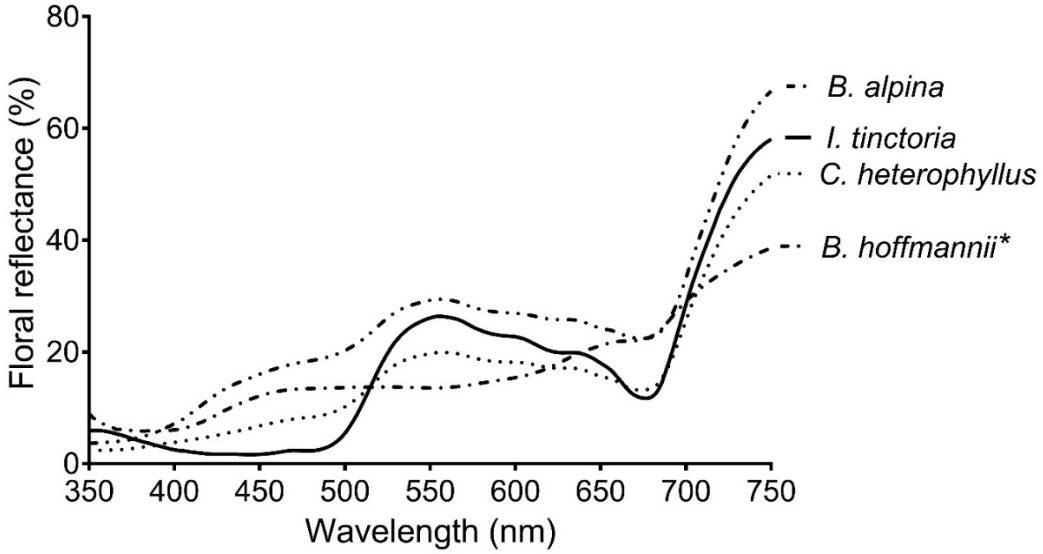


Fig. C1: Average floral reflectance of three Brassicaceae species (i.e., *I. tinctoria*, *B. alpina, B. hoffmannii, and C. heterophyllus*) compared to a white reference plate (*n* = 9 for each plant species, * indicates lab measurement).

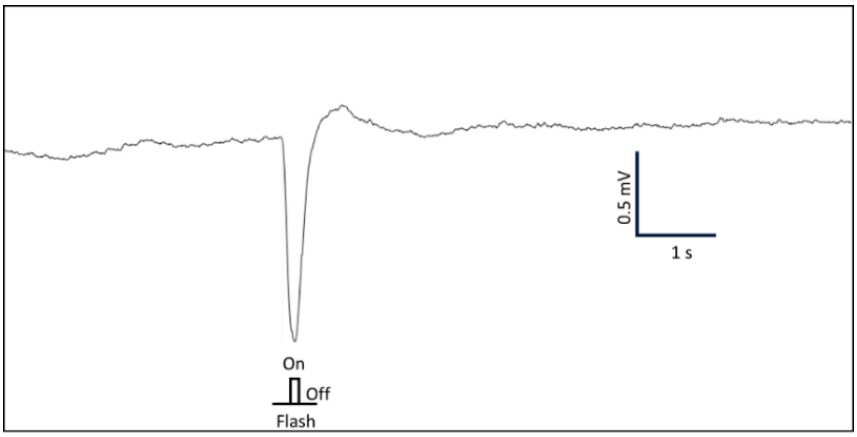


Fig. C2: ERG recording from the compound eye of female *C. peyerimhoffi* in response to a test flash of white light.

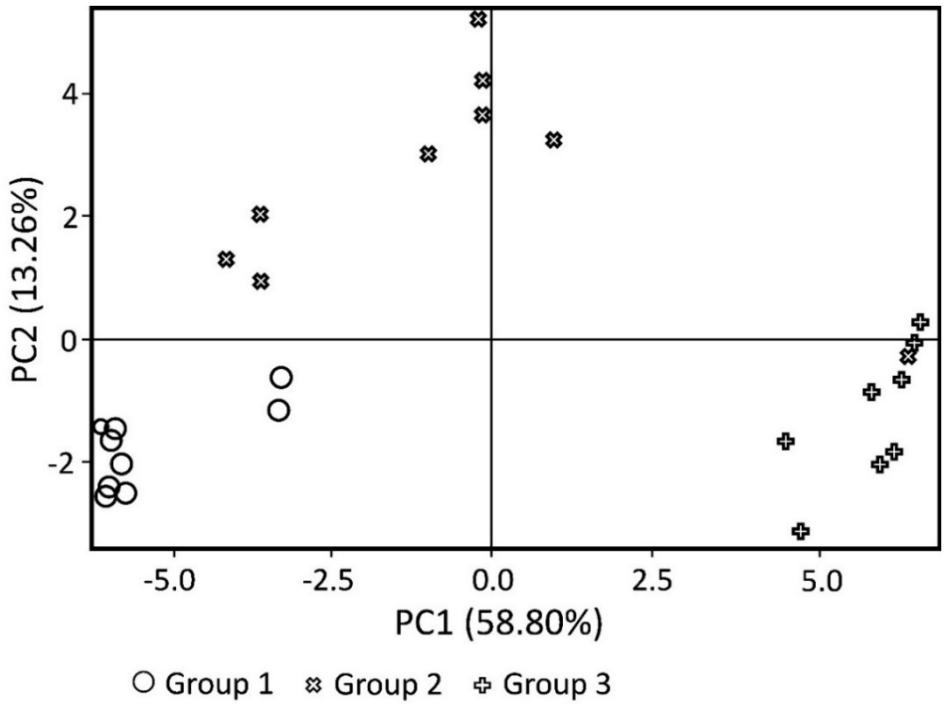


Fig. C3: Principal component analysis (PCA) based on individual weevil's sensitivity response.

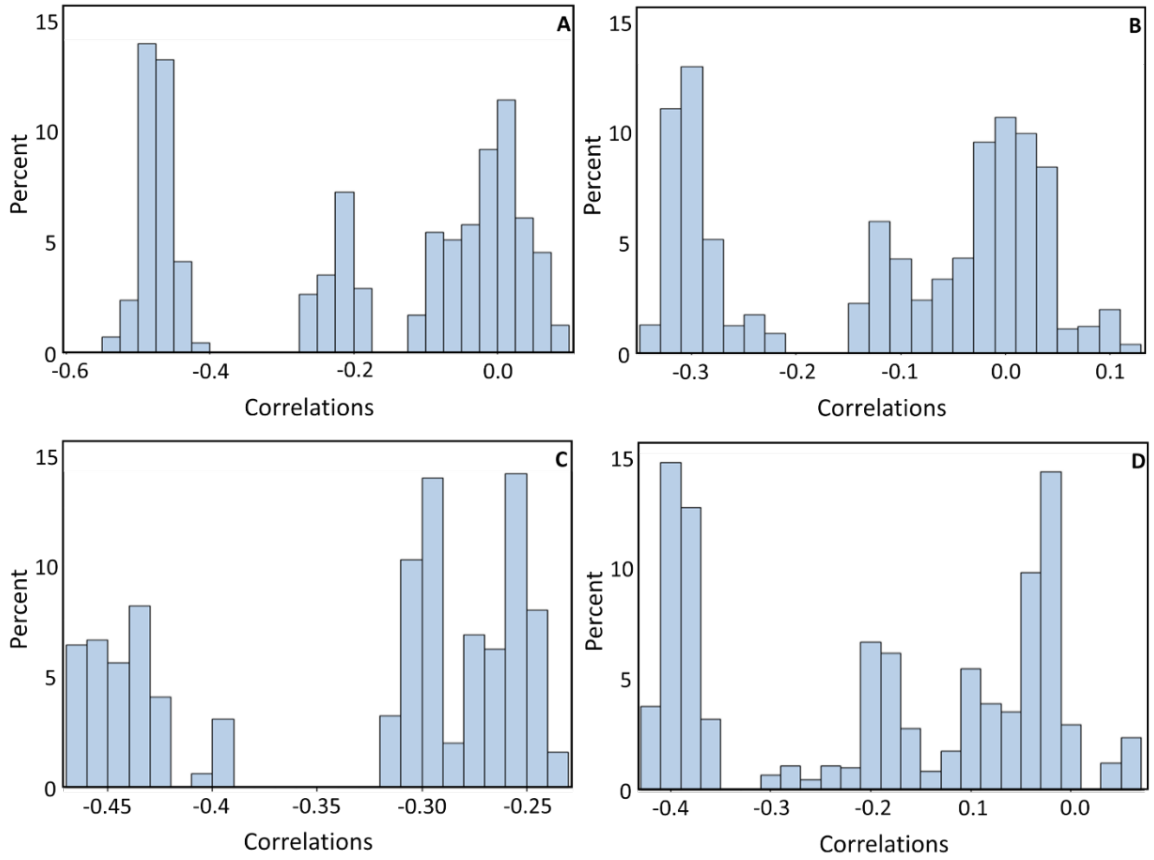


Fig. C4: Histogram of summary of distribution of Pearson correlation between reflectance and sensitivity determined through bootstrap procedure (5000 bootstrap replications). A) *I. tinctoria*, B) *B. alpina*, C) *B. hoffmannii*, D) *C. heterophyllus.*

References

Crook DJ et al. (2009) Laboratory and field response of the emerald ash borer (Coleoptera: Buprestidae), to selected regions of the electromagnetic spectrum. J Econ Entomol 102:2160-2169

Mellor H, Bellingham J, Anderson M (1997) Spectral efficiency of the glasshouse whitefly *Trialeurodes vaporariorum* and *Encarsia formosa* its hymenopteran parasitoid. Entomol Expt et Appl, 83:11-20

Park I, Eigenbrode SD, Cook SP, Harmon BL, Hinz HL, Schaffner U, Schwarzlander M (2018) Examining olfactory and visual cues governing host-specificity of a weed biological control candidate species to refine pre-release risk assessment. BioControl 63:377-389

Appendix D. Relative total ion concentration (TIC) peak area percentage of VOCs collected in the headspace of test plant species.[1]

Compound	RT (min)	IT	BH	BA	CH
Furfural	4.165	-	24.92	-	4.99
3-Hexen-1-ol, formate, (Z)-	4.629	2.62	2.62	-	-
1-Penten-3-one	5.934	-	17.77	-	-
Benzaldehyde	7.052	10.52	-	-	-
3-Hexen-1-ol, acetate, (Z)-	8.413	-	45.29	-	-
4-Hexen-1-ol, acetate	8.422	32.61	-	5.35	-
Acetic acid, hexyl ester	8.591	13.18	-	-	-
Benzeneacetaldehyde	9.354	6.23	-	-	-
Bicyclo [3.1.1] hept-2-ene, 3,6,6-trimethyl-	9.528	11.60	-	-	-
Phenylethyl Alcohol	11.34	2.34	-	-	-
Geranyl nitrile	11.479	6.80	-	-	-
Ethanone, 1-(4-ethylphenyl)-	15.519	-	1.35	-	-
Hexane, 3,3-dimethyl-	16.564	-	-	1.35	-
2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester	17.206	-	-	-	27.81
2,6-Octadienoic acid, 3,7-dimethyl-, methyl ester, (E)-	17.210	-	-	-	9.68
Cyclopentane, 1,1,3-trimethyl-	21.441	2.47	-	-	-
2-Butenoic acid, 2-methyl-, phenylmethyl ester	21.601	-	-	-	5.03
Oxalic acid, cyclobutyl octadecyl ester	31.594	-	-	1.08	-
1,7-Dimethyl-4-(1-methylethyl) cyclodecane	32.431	-	-	1.16	-
Number of volatile compounds (shared with *I. tinctoria*)		9	5(1)	4(1)	4(0)

[1]IT: *Isatis tinctoria*, BA: *Braya alpina*, BH: *Boechera hoffmannii*, CH: *Caulanthus heterophyllus*. Only compounds with library match score of 60 or higher were selected for reporting. Headspace VOCs samples were analyzed using the methods and instruments described by Park et al. (2018). In summary, an Agilent 7890A (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with HP-5MS column (30 m x 250 µm x 0.25 µm, Agilent Technologies Inc., Santa Clara, CA, USA) in conjunction with Hewlett Packard (HP) 5973 mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA) was used to analyze the volatile samples. Helium gas was used as a carrier gas. Ionization was achieved by electron impact at 70 eV. Initially, the oven temperature was maintained at 40ºC for 1 min and then was programmed as 5ºC min^{-1} to 200ºC (first ramp), 10ºC min^{-1} to 300ºC (second ramp) finally held for 2 min at 300ºC. Compound identification was carried out by comparing the obtained spectra and retention times in the NIST spectral library database (National Institute of Standards and Technology, Gaithersburg, MD).

Appendix E. Summary statistics for behavioral responses of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of target *I. tinctoria*, test plant species, and control (purified air and/or empty arm).

Test species	**Olfactory cues**		**Visual cues**		**Combined olfactory and visual cues**	
	t value	**p-value**	**t value**	**p-value**	**t value**	**p-value**
Isatis tinctoria[b]	**-2.79**	**0.0087**	**-2.47**	**0.0194**	**-2.77**	**0.0138**
Braya alpina[a]	0.61	0.5484	1.57	0.1296	1.34	0.1968
Caulanthus flavescens[a]	1.27	0.2159	-2.01	0.0541	0.59	0.564
Descurainia californica[a]	0.02	0.9842	1.53	0.1379	-1.04	0.3128
Descurainia nelsonnii[a]	0.02	0.9856	**-4.42**	**0.0001**	0.02	0.8979
Eutrema salsugineum[a]	0.89	0.3814	**-2.59**	**0.0171**	0.87	0.3643
Isatis glauca[b]	0.64	0.5306	-1.87	0.0776	**-2.65**	**0.0162**
Lepidium sativum[c]	1.44	0.1604	-1.17	0.2573	-1.37	0.1956
Sisymbrium linifolium[b]	-1.12	0.2734	**-2.37**	**0.0263**	-1.94	0.0729
Stanleya pinnata[a]	1.32	0.1987	1.18	0.2516	0.32	0.7532
Stanleya tomentosa[a]	0.99	0.3295	0.8	0.4309	**2.49**	**0.0303**
Stanleya viridiflora[a]	-1.36	0.1844	-2.01	0.0541	0.39	0.7011

[a]Native North American confamilial plant species (FNA 2021); [b]Eurasian confamilial plant species (Weyl et al. 2019); [c]perhaps Iran origin (Sabaghnia et al. 2015). Test statistics are generalized linear mixed models, assuming a binomial response for the percentage of time spent in the treated arm. P-values <0.05 significant (highlighted in bold).

Appendix F. Summary statistics for Initial choice (IC) and final choice (FC) of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of target *I. tinctoria*, test plant species against control (purified air and/or empty arm).

Test species	**Olfactory cues**				**Visual cues**				**Combined olfactory and visual cues**			
	Initial choice		**Final choice**		**Initial choice**		**Final choice**		**Initial choice**		**Final choice**	
	t value	**p-value**	**t value**	**p-value**	**t value**	**p-value**	**t value**	**p-value**	**t value**	**p-value**	**t value**	**p-value**
Isatis tinctoria[b]	**2.89**	**0.0068**	**2.7**	**0.0112**	**2.26**	**0.0316**	**2.24**	**0.0335**	**2.29**	**0.037**	**2.29**	**0.037**
Braya alpina[a]	0.94	0.3571	-0.58	0.5656	-0.7	0.4876	-1.17	0.2548	0.23	0.8212	-1.39	0.1836
Caulanthus flavescens[a]	-1.67	0.1064	-1.85	0.0754	1.33	0.195	-0.99	0.3336	0	1	0.26	0.8002
Descurainia californica[a]	-0.19	0.8515	-0.7	0.4943	1.17	0.2551	-1.76	0.092	1.32	0.2024	1.27	0.2263
Descurainia nelsonnii[a]	0.62	0.5388	-1.32	0.203	**2.41**	**0.0224**	1.29	0.2074	0.89	0.3855	-0.23	0.8212
Eutrema salsugineum[a]	-0.41	0.6824	-0.69	0.4997	**2.27**	**0.0349**	1.13	0.2817	0.69	0.5019	0.99	0.3383
Isatis glauca[b]	1.29	0.2124	0.59	0.5629	0	1	0	1	1.57	0.1346	2.06	0.0559
Lepidium sativum[c]	-0.58	0.5703	-0.39	0.6986	-0.01	0.9947	-0.6	0.5555	1.35	0.2021	-0.3	0.7695
Sisymbrium linifolium[b]	0.37	0.7138	0.92	0.3668	0.78	0.4422	0.85	0.4043	1.74	0.0987	2.06	0.0559
Stanleya pinnata[a]	-1.29	0.2104	-1.19	0.2446	-0.45	0.6562	-1.5	0.1505	-0.23	0.8212	0.24	0.8116
Stanleya tomentosa[a]	-0.89	0.3806	-1.33	0.1943	0	0.9996	-1.12	0.2709	-0.57	0.5771	0.33	0.748
Stanleya viridiflora[a]	0	0.9996	1.63	0.1159	1.89	0.0693	0.54	0.5973	-0.65	0.5216	0	1

[a]Native North American confamilial plant species (FNA 2021); [b]Eurasian confamilial plant species (Weyl et al. 2019); [c]perhaps Iran origin (Sabaghnia et al. 2015). Test statistics are generalized linear mixed models, assuming a binary response for the choice in the treated arm. P-values <0.05 significant (highlighted in bold).

Appendix G. Summary statistics for response times (RT) of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cue, visual cue, and combined olfactory and visual cues of target *I. tinctoria*, test plant species, and control (purified air and/or empty arm).

Test species	**Olfactory cues**		**Visual cues**		**Combined olfactory and visual cues**	
	F value	**p-value**	**F value**	**p-value**	**F value**	**p-value**
Isatis tinctoria[b]	**7.37**	**0.0106**	0.19	0.6666	0.26	0.6197
Braya alpina[a]	3.61	0.0679	0.43	0.5199	0.43	0.5192
Caulanthus flavescens[a]	2.06	0.1628	0.8	0.3786	1.86	0.19
Descurainia californica[a]	2.11	0.1607	1.63	0.2144	2.72	0.1161
Descurainia nelsonnii[a]	1.37	0.2553	**5.63**	**0.0245**	0.09	0.7677
Eutrema salsugineum[a]	1.24	0.2785	0.1	0.7512	3.32	0.0863
Isatis glauca[b]	4.01	0.0598	**6.08**	**0.0246**	4.36	0.0523
Lepidium sativum[c]	0.07	0.7992	0.06	0.8094	1.35	0.2696
Sisymbrium linifolium[b]	0.6	0.4461	0.4	0.5313	**15**	**0.0011**
Stanleya pinnata[a]	0.01	0.9268	-2.46	0.0237	**5.27**	**0.0347**
Stanleya tomentosa[a]	2	0.1687	-2.66	0.0134	3.26	0.1012
Stanleya viridiflora[a]	0.39	0.54	0.69	0.4937	0.73	0.4022

[a]Native North American confamilial plant species (FNA 2021); [b]Eurasian confamilial plant species (Weyl et al. 2019); [c]perhaps Iran origin (Sabaghnia et al. 2015). Test statistics are generalized linear mixed models, assuming a binary response for the choice in the treated arm. P-values <0.05 significant (highlighted in bold).

Appendix H. Summary statistics for the test of synergy for the *C. rusticus* average response times in individual (olfactory and visual) cue bioassay vs combined cue bioassay for individual tested plant species, and contrast of sum of response times (RT) across all bioassays (olfactory cues, visual cues, and combined cues) between *I. tinctoria* and individual tested confamilial species in a modified Y-tube choice bioassay with headspace volatile organic compounds of target *I. tinctoria*, test plant species, and control (purified air and/or empty arm).

Test species	**Test of synergy**		**Contrast of response times**	
	F value	**p-value**	**F value**	**p-value**
Isatis tinctoria[b]	0.02	0.8757	-	-
Braya alpina[a]	2.29	0.1448	1.01	0.3165
Caulanthus flavescens[a]	0.92	0.3484	1.77	0.1846
Descurainia californica[a]	0.24	0.6281	1.4	0.2382
Descurainia nelsonnii[a]	0.06	0.8097	**11.99**	**0.0006**
Eutrema salsugineum[a]	**8.3**	**0.0077**	**11.55**	**0.0008**
Isatis glauca[b]	0.62	0.4402	**8.97**	**0.003**
Lepidium sativum[c]	0.64	0.432	**12.01**	**0.0006**
Sisymbrium linifolium[b]	0.13	0.7208	**6.79**	**0.0096**
Stanleya pinnata[a]	0.1	0.7538	**5.51**	**0.0196**
Stanleya tomentosa[a]	0.12	0.7321	**8.44**	**0.0039**
Stanleya viridiflora[a]	0.11	0.7413	1.21	0.2715

[a]Native North American confamilial plant species (FNA 2021); [b]Eurasian confamilial plant species (Weyl et al. 2019); [c]perhaps Iran origin (Sabaghnia et al. 2015). Test statistics are generalized linear mixed models, assuming a binary response for the choice in the treated arm. P-values <0.05 significant (highlighted in bold).

Appendix I. Summary statistics for behavioral responses of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of test plant species against respective cues of target *I. tinctoria*.

Test species	**Olfactory cues**		**Visual cues**		**Combined olfactory and visual cues**	
	t value	**p-value**	**t value**	**p-value**	**t value**	**p-value**
Braya alpina[a]	-2.01	0.0562	-0.6	0.5526	**-2.53**	**0.0174**
Caulanthus flavescens[a]	**5.75**	**<.0001**	0.42	0.6786	**2.89**	**0.0071**
Descurainia californica[a]	**-2.31**	**0.0309**	0.36	0.7193	**-2.98**	**0.0063**
Descurainia nelsonnii[a]	-1.42	0.168	**-2.17**	**0.0388**	**-2.17**	**0.0388**
Eutrema salsugineum[a]	**-3.3**	**0.0026**	0.22	0.8287	**-2.14**	**0.0415**
Isatis glauca[b]	-2.73	0.011	-1.04	0.3058	**-2.27**	**0.0339**
Lepidium sativum[c]	1.07	0.2927	-0.26	0.8005	**2.38**	**0.0268**
Sisymbrium linifolium[b]	**2.39**	**0.0234**	**3.02**	**0.0051**	**6.12**	**<.0001**
Stanleya pinnata[a]	**7.84**	**<.0001**	1.21	0.2366	**6.69**	**<.0001**
Stanleya tomentosa[a]	**3.61**	**0.0016**	-0.81	0.4259	**5.08**	**<.0001**
Stanleya viridiflora[a]	**2.81**	**0.0096**	1.57	0.1312	**3.53**	**0.0015**

[a]Native North American confamilial plant species (FNA 2021); [b]Eurasian confamilial plant species (Weyl et al. 2019); [c]perhaps Iran origin (Sabaghnia et al. 2015). Test statistics are generalized linear mixed models, assuming a binomial response for the percentage of time spent in the treated arm. P-values <0.05 significant (highlighted in bold).

Appendix J. Summary statistics for Initial choice (IC) and final choice (FC) of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of test plant species against respective cues of target *I. tinctoria*.

Test species	**Olfactory cues**				**Visual cues**				**Combined olfactory and visual cues**			
	Initial choice		**Final choice**		**Initial choice**		**Final choice**		**Initial choice**		**Final choice**	
	t value	**p-value**	**t value**	**p-value**	**t value**	**p-value**	**t value**	**p-value**	**t value**	**p-value**	**t value**	**p-value**
Braya alpina[a]	0.59	0.5618	1.38	0.1807	0.76	0.4537	0.36	0.7179	**2.71**	**0.0114**	**2.26**	**0.0333**
Caulanthus flavescens[a]	**-2.5**	**0.02**	**-2.61**	**0.0172**	-1.25	0.2224	-1.47	0.1526	**-2.53**	**0.0173**	**-2.32**	**0.0278**
Descurainia californica[a]	**2.12**	**0.0448**	**2.29**	**0.0333**	0.92	0.3667	0.85	0.4062	1.77	0.0895	1.83	0.082
Descurainia nelsonnii[a]	0.78	0.4422	2.05	0.0538	1.09	0.2857	1.5	0.1469	1.99	0.0568	1.77	0.0904
Eutrema salsugineum[a]	1.8	0.0826	1.73	0.0961	-1.3	0.2028	0.51	0.6149	1.9	0.0689	0.7716	0.08396
Isatis glauca[b]	1.29	0.2072	1.82	0.0824	-0.18	0.8587	1.08	0.2873	0.66	0.5125	0.66	0.5125
Lepidium sativum[c]	**-2.47**	**0.0214**	-1.98	0.0607	0.65	0.5217	0.03	0.9778	-0.96	0.3458	-0.8	0.4301
Sisymbrium linifolium[b]	-1.66	0.1069	-1.64	0.1116	-1.05	0.3001	-2.54	0.0172	**-2.39**	**0.0247**	**-2.68**	**0.0148**
Stanleya pinnata[a]	-1.39	0.178	**-2.1**	**0.047**	-1.31	0.2036	-0.72	0.4817	**-3.4**	**0.002**	**-2.3**	**0.0299**
Stanleya tomentosa[a]	0.4167	0.1006	-1.55	0.1386	-1.12	0.2709	0.2	0.8433	-1.7	0.1025	-1.15	0.2634
Stanleya viridiflora[a]	**-2.26**	**0.0333**	-1.49	0.1512	-1.03	0.3128	-0.83	0.4176	-1.29	0.209	-0.47	0.6456

[a]Native North American confamilial plant species (FNA 2021); [b]Eurasian confamilial plant species (Weyl et al. 2019); [c]perhaps Iran origin (Sabaghnia et al. 2015). Test statistics are generalized linear mixed models, assuming a binary response for the choice in the treated arm. P-values <0.05 significant (highlighted in bold)

Appendix K. Summary statistics for response times (RT) of *C. rusticus* females in a modified Y-tube choice bioassay with olfactory cues, visual cues, and combined olfactory and visual cues of test plant species against respective cues of target *I. tinctoria*.

Test species	**Olfactory cues**		**Visual cues**		**Combined olfactory and visual cues**	
	F value	**p-value**	**F value**	**p-value**	**F value**	**p-value**
Braya alpina[a]	**7.55**	**0.0112**	**18.73**	**0.0002**	0.18	0.6784
Caulanthus flavescens[a]	0	0.9771	0.1	0.7505	0.49	0.4879
Descurainia californica[a]	**5.16**	**0.0332**	0.12	0.7368	0.49	0.6309
Descurainia nelsonnii[a]	2.87	0.1036	**9.38**	**0.0049**	**6.03**	**0.0289**
Eutrema salsugineum[a]	0.02	0.8829	0.25	0.6199	4.22	0.0502
Isatis glauca[b]	3.49	0.073	0.36	0.554	0.05	0.8316
Lepidium sativum[c]	0.02	0.881	0	0.9606	1.1	0.3056
Sisymbrium linifolium[b]	0.52	0.4756	0.38	0.5436	2.84	0.1046
Stanleya pinnata[a]	0.05	0.8319	0	0.9773	0.53	0.4732
Stanleya tomentosa[a]	0.15	0.7014	0.17	0.6795	0.14	0.7102
Stanleya viridiflora[a]	0.03	0.8538	3.6	0.0722	2.39	0.134

[a]Native North American confamilial plant species (FNA 2021); [b]Eurasian confamilial plant species (Weyl et al. 2019); [c]perhaps Iran origin (Sabaghnia et al. 2015). Test statistics are generalized linear mixed models, assuming a binary response for the choice in the treated arm. P-values <0.05 significant (highlighted in bold).

Appendix L. Relative total ion concentration (TIC) peak area percentage of volatile organic compounds collected in the headspace of plant species.[1]

Compounds	RT (min)	IT	BA	CF	DC	DN	ES	IG	LS	SP	SL	ST	SV
Indolizine, 2-(4-methylphenyl)-	3.875	-	-	-	-	-	-	-	0.282	-	-	-	-
Acetic acid, dichloro-	3.922	2.995	-	1.853	-	-	1.420	-	-	1.452	1.853	0.923	1.053
Benzo[h]quinoline, 2,4-dimethyl-	3.929	1.800	-	-	-	-	-	-	-	-	-	-	-
Benzenesulfinic acid, 4-chloro-	3.930	-	0.419	5.547	4.117	0.046	0.932	1.429	-	-	5.547	-	-
Indole-2-one, 2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl-	3.931	-	-	-	-	-	-	-	-	-	-	-	-
1H-Indole, 5-methyl-2-phenyl-	3.933	-	-	-	-	-	-	-	-	-	-	-	-
3-Hexen-1-ol	4.621	-	-	-	-	-	-	-	-	-	-	2.820	-
beta. -Phellandrene	7.478	-	-	-	-	-	-	-	-	-	-	1.720	65.339
Bicyclo[3.1.0]hex-2-ene, 4-methyl-1-(1-methylethyl)-	7.479	-	-	-	-	-	-	-	-	-	-	-	8.015
Cyclohexene, 4-methylene-1-(1-methylethyl)-	7.486	-	-	-	-	-	-	-	-	-	-	-	10.350
Cyclohexane	7.544	-	-	0.753	-	-	1.042	-	-	-	0.753	-	-

[1]Tentative identification of compounds was based on comparison of their mass-spectra in the NIST library. For most species, values are averaged across five individual plants.; RT: Retention time (in minutes), IT: *Isatis tinctoria*, BA: *Braya alpina*, CF: *Caulanthus flavescens*, DC: *Descurainia californica*, DN: *Descurainia nelsonnii*, ES: *Eutrema salsugineum*, IG: *Isatis glauca*, LS: *Lepidium sativum*, SP: *Stanleya pinnata*, SL: *Sisymbrium linifolium*, ST: *Stanleya tomentosa*, SV: *Stanleya viridiflora*. Only compounds with library match score of 60 or higher were selected for reporting. We followed the protocols for the analysis of headspace volatile organic compounds using an Agilent 7890A (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with HP-5MS column (30 m x 250 µm x 0.25 µm, Agilent Technologies Inc., Santa Clara, CA, USA) in conjunction with Hewlett Packard (HP) 5973 mass selective detector (Agilent Technologies Inc., Santa Clara, CA, USA) as described in detail in Appendix D.

Compounds	RT (min)	IT	BA	CF	DC	DN	ES	IG	LS	SP	SL	ST	SV
N-Chloro-N-fluoro-trifluoromethylamine	8.023	-	-	-	-	-	-	-	-	-	-	-	2.214
2-Propanol, 1-[2-(2-methoxy-1-methylethoxy)-1-methylethoxy]-	8.331	-	-	-	-	-	-	-	-	-	-	7.798	-
1H-1,3-Benzimidazole-1-carboxylic acid, 2-(chloromethyl)-, methyl ester	8.337	-	-	-	-	-	-	-	-	-	-	-	2.693
2,4-Diamino-N, N, 5-trimethyl-6-quinolinesulfonamide	8.345	-	-	-	-	-	-	1.581	-	-	-	-	-
3,6-Bis(N-dimethylamino)-9-ethylcarbazole	8.348	-	-	-	-	-	5.018	-	-	-	-	-	-
2,6-Dimethyl-5-methylphenylaminopyridin-3,4-dicarboxyimide	8.351	-	-	5.560	5.840	-	-	-	-	-	5.560	-	-
6,7-Benzo-phenothiazine-5,5-dioxide	8.351	-	-	-	-	-	-	-	3.235	-	-	-	-
2-Propanol, 1-(2-methoxy-1-methylethoxy)-	8.445	-	-	-	-	-	-	-	-	-	-	13.108	2.904
Acetic acid	8.482	-	-	-	-	-	-	-	11.086	-	-	-	-
3-Hexen-1-ol, acetate, (Z)-	8.488	15.335	11.223	-	-	-	-	24.795	-	11.788	-	-	-
4-Hexen-1-ol, acetate	8.492	16.642	24.257	-	-	-	-	46.656	-	25.107	-	-	-
Butanoic acid, 3-hexenyl ester, (Z)-	8.502	-	-	-	-	-	-	17.824	-	-	-	-	-
3-Hexyne	8.537	-	-	-	-	-	-	-	4.013	-	-	-	-
2-Butynoic acid	8.547	5.407	-	-	-	-	-	-	-	1.444	-	-	-
3-Aminocrotononitrile	8.560	-	-	-	-	-	-	-	-	-	-	-	-

Compounds	RT (min)	IT	BA	CF	DC	DN	ES	IG	LS	SP	SL	ST	SV
Silanediol, dimethyl-	8.627	-	-	-	-	-	-	-	29.564	-	-	-	-
Acetic acid, hexyl ester	8.673	-	-	-	-	-	-	-	-	4.540	-	-	-
2-Propanol, 1-(2-methoxypropoxy)-	8.725	-	-	-	-	-	-	-	-	-	-	9.436	-
1-Silacyclo-3-pentene	8.784	-	-	-	-	-	-	-	-	1.734	-	-	-
Cyclopropanecarboxamidine	8.806	-	-	-	-	-	-	2.242	-	-	-	-	-
D-Limonene	9.012	-	-	-	-	-	-	-	-	4.942	-	1.109	8.925
1-Hexanol, 2-ethyl-	9.085	-	-	-	-	-	-	-	-	-	-	-	11.653
Propanal, 2,2-dimethyl-	9.119	-	-	-	-	-	-	-	-	-	-	-	1.827
trans-. beta. -Ocimene	9.334	-	3.846	-	-	-	-	-	-	-	-	-	-
1,6-Heptadiyne	9.352	-	-	-	-	1.303	-	-	-	-	-	-	-
Oxime-, methoxy-phenyl-	9.603	-	-	-	-	-	-	-	5.102	-	-	-	-
1,3,6-Heptatriene, 5-methyl-	9.633	1.633	-	-	-	-	-	-	-	-	-	-	-
beta. - Ocimene	9.638	-	23.108	-	-	-	-	-	13.553	-	-	6.074	-
3-Butyn-2-ol, 2-methyl-	11.588	-	-	-	-	-	-	-	-	41.859	-	-	-
1,7-Octadien-3-one, 2-methyl-6-methylene-	11.59	-	-	-	56.419	-	-	-	-	-	-	-	-
Acetic acid, 1,3,7-trimethylocta-2,6-dienyl ester	11.590	-	-	-	56.966	-	-	-	-	-	-	-	-
Trifluoroacetyl-lavandulol	11.591	-	-	-	27.999	-	-	-	-	-	-	51.580	-
(E)-2-Butenoic acid, 2-(methylenecyclopropyl) prop-2-yl ester	11.5916	-	-	-	26.345	-	-	-	-	15.179	-	40.920	-

Compounds	RT (min)	IT	BA	CF	DC	DN	ES	IG	LS	SP	SL	ST	SV
2-Pentene, 4-methyl-, (Z)-	11.643	-	-	-	-	-	-	-	-	1.158	-	-	-
3-Penten-2-one, (E)-	11.751	-	-	-	1.171	-	-	-	-	-	-	-	-
Benzaldehyde, 3-ethyl-	12.876	-	-	-	-	-	-	4.765	-	-	-	-	-
Benzaldehyde, 4-ethyl-	12.892	-	-	-	-	-	-	-	-	10.124	-	-	19.232
Benzene, 1-ethyl-2,4-dimethyl-	12.900	-	-	-	2.706	-	-	-	-	-	-	-	-
1H-Benzotriazole, 1-methyl-	12.918	-	-	-	-	-	-	-	-	4.820	-	-	-
Benzene, 1-isocyanato-2-methyl-	13.029	-	-	-	-	-	-	-	-	4.107	-	-	-
2-ethenyl-3-ethylpyrazine	13.038	-	-	-	-	-	-	1.945	-	-	-	-	-
Phthalimidine	13.312	-	-	-	-	-	-	2.558	-	-	-	-	-
o-Methoxybenzonitrile	13.350	-	-	-	-	-	-	-	-	1.644	-	-	2.613
1H-Indol-4-ol	13.414	-	-	-	-	-	-	1.660	-	-	-	-	-
Benzenemethanol, alpha. -ethynyl-	13.48	-	-	-	-	-	-	-	-	-	-	-	1.264
Ethyleniminoacetonitryl	13.707	-	-	-	-	-	-	-	-	-	-	-	-
Methyl salicylate	13.763	-	-	-	-	-	-	-	-	5.405	-	-	-
cis-3-Hexenyl-. alpha. -methylbutyrate	14.882	-	-	-	-	-	-	-	-	2.355	-	-	-
cis-3-Hexenyl isovalerate	14.990	-	-	-	-	-	-	-	-	1.355	-	-	-
N-Allyl-N-ethylformamide	15.390	-	-	-	-	-	1.819	-	-	-	-	-	-
1-Oxaspiro [2.5] oct-5-ene, 8,8-dimethyl-4-methylene-	15.463	3.281	-	-	-	-	-	-	-	-	-	-	-
Benzaldehyde, 4-methyl-, oxime, (Z)-	15.478	-	-	-	-	-	-	1.174	-	-	-	-	-

Compounds	RT (min)	IT	BA	CF	DC	DN	ES	IG	LS	SP	SL	ST	SV
Ethanone, 1-(4-ethylphenyl)-	15.662	3.621	-	-	7.626	-	-	-	-	7.932	-	-	21.769
Cyclopropanamine, 2-phenyl-, trans-	15.687	-	-	-	-	-	2.585	-	-	-	-	-	-
1,5,6,7-Tetramethylbicyclo [3.2.0] hepta-2,6-diene	15.688	-	-	-	-	-	-	6.666	-	-	-	-	-
Benzene, 1-ethyl-4-(1-methylethyl)-	16.192	-	-	-	-	-	-	-	-	12.297	-	-	-
Benzene, 1,4-dimethyl-2-(1-methylethyl)-	16.204	-	-	-	3.998	-	-	-	-	-	-	-	-
[1,1'-Bicyclopentyl]-2-one	16.206	-	-	-	-	3.211	-	-	-	-	-	-	-
(+)-Cyclosativene	18.441	14.291	-	-	-	-	-	-	-	-	-	-	-
alpha. -ylangene	18.550	12.032	-	-	-	-	-	-	-	-	-	-	-
Selina-3,7(11)-diene	20.407	2.892	-	-	-	-	-	-	-	-	-	-	-
Tricyclo [4.1.0.0(2,4)] heptane, 3,3,7,7-tetramethyl-5-(2-methyl-1-propenyl)-	20.407	2.153	-	-	-	-	-	-	-	-	-	-	-
1,2,3,6-Tetrahydropyridine, 4-[4-hydroxy-5-methoxyphenyl]-	22.098	-	-	-	-	-	-	-	-	-	-	-	-
Number of volatile compounds (Shared compounds with *I. tinctoria*)		12	5 (2)	4 (1)	10 (0)	3 (0)	6 (1)	12 (2)	7 (0)	19 (5)	4 (1)	10 (1)	14 (2)

Appendix M. Foliar reflectance

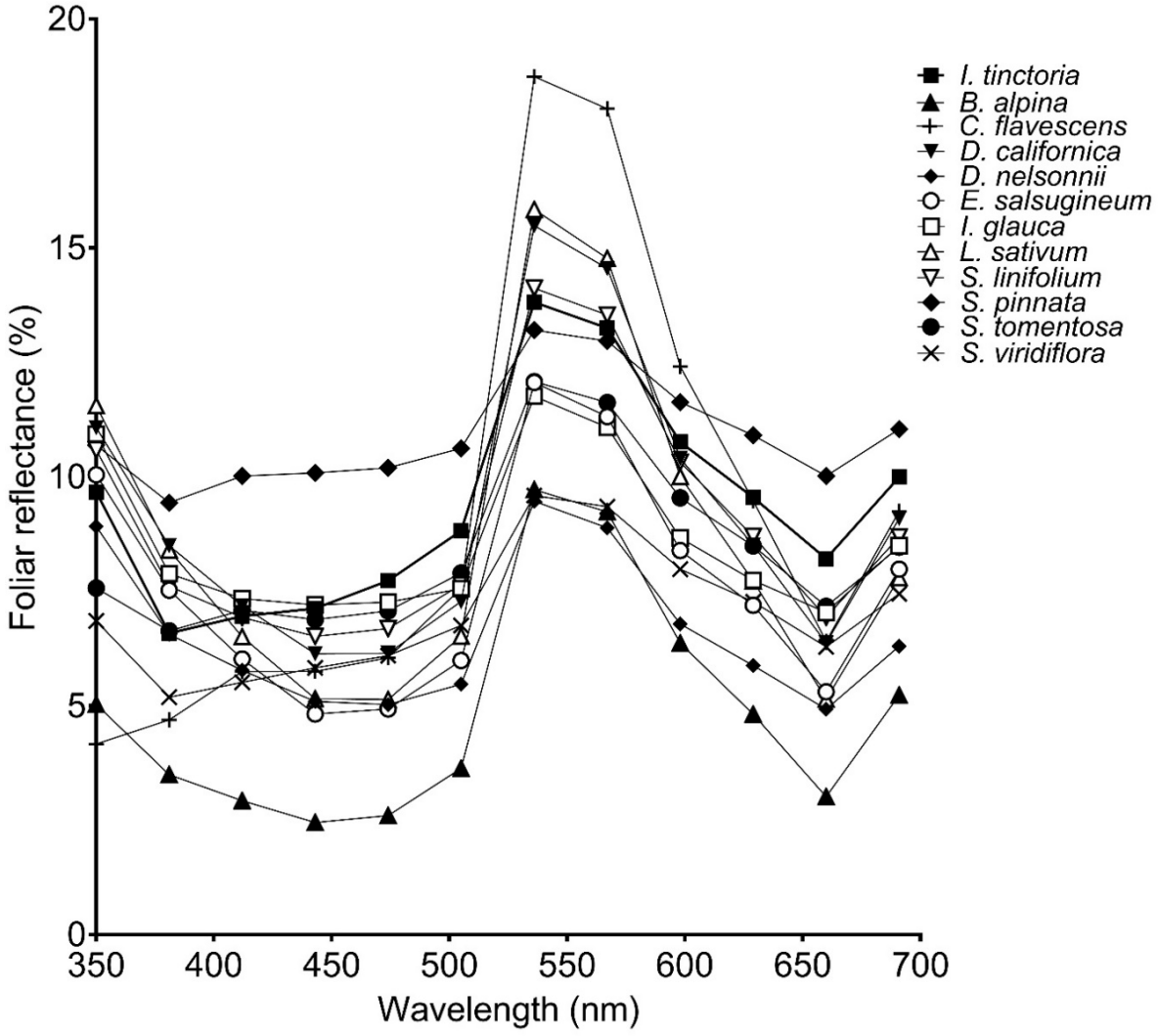


Fig. M1: Average foliar reflectance of twelve Brassicaceae species compared to a white reference plate (n = 9 for each plant species). We followed the protocols for foliar reflectance measurement using ASD FieldSpec 4 Hi-Res: High-Resolution Spectroradiometer (Malvern Panalytical Ltd, Cambridge, United Kingdom) as described in detail in Appendix K.