



## Physiology

## Duration of emission of volatile organic compounds from mechanically damaged plant leaves

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## ABSTRACT

Classical biological control of invasive alien weeds depends on the use of arthropod herbivores that are sufficiently host specific to avoid risk of injuring nontarget plants. Host plant specificity is usually evaluated by using a combination of behavioral and developmental experiments under choice, no-choice and field conditions. Secondary plant compounds are likely to have an important influence on host plant specificity. However, relatively little is known about the volatile organic compounds (VOCs) that are emitted by target and nontarget plants, and how environmental conditions may affect their emission. Previous studies have shown that mechanical damage of leaves increases the composition and content of VOCs emitted. In this study we measured the VOC emissions of five species of plants in the subtribe Centaureinae (Asteraceae) – *Carthamus tinctorius*, *Centaurea cineraria*, *Centaurea melitensis*, *Centaurea rothrockii*, and *Centaurea solstitialis* – that have previously been used in host specificity experiments for a prospective biological control agent of yellow starthistle (*C. solstitialis*). Leaves of each plant were punctured with a needle and the VOCs were collected by solid-phase microextraction (SPME) periodically over 48 h and analyzed by GC–MS. A total of 49 compounds were detected. Damage caused an immediate increase of 200–600% in the composition of VOCs emitted from each plant species, and the amounts generally remained high for at least 48 h. The results indicate that a very unspecific mechanical damage can cause a prolonged change in the VOC profile of plants.

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## 1. Introduction

In order to use phytophagous arthropods as classical biological control agents to control invasive alien weeds, it is critical to determine that they are sufficiently host specific in order to avoid the risk of attacking nontarget plants (Horner, 2004; Sheppard et al., 2005). Host plant specificity is usually evaluated by a series of complementary no-choice, choice and field experiments to assess the risk that the prospective agent will damage nontarget plants (Withers et al., 1999; van Klinken, 2000; Schaffner, 2001; Spafford Jacob and Briese, 2003; Briese, 2005). Such experiments provide empirical evidence that they are useful for obtaining regulatory approval to use the agent. Nevertheless, host specificity experiments do not provide an explanation why an agent prefers one plant species over another. There is

growing evidence that secondary plant compounds play an important role in determining which plants are acceptable to oligophagous arthropods (Bernays and Chapman, 1994; Schoonhoven et al., 2006; Wheeler and Schaffner, 2013). Volatile organic compounds (VOCs) are known to attract both phytophagous arthropods and some of their natural enemies, and there have been many reports on the latter phenomenon (e.g., Tumlinson, 1991; Turlings et al., 1990, 1993; Kugimiya et al., 2010; Mumm and Dicke, 2010; van Dam et al., 2010; Hare, 2011). However, relatively little is known about the role of plant secondary metabolites and the specificity of biological control agents of weeds (Wheeler, 2005; Andreas et al., 2008; Park et al., 2012; Rapo et al., 2012; Wheeler, 2012; Wheeler and Schaffner, 2013; Wheeler et al., 2014).

VOCs emitted by plants can act as attractants or repellants to phytophagous insects (e.g., Visser, 1986; Mitchell, 1994; Zhang and McEvoy, 1995; Moyes and Raybould, 2001; van Tol et al., 2002; Heil, 2004; Junker and Blüthgen, 2008; Otalora-Luna et al., 2009; Piesik et al., 2011a,b, 2012). Although undamaged plant leaves typically emit VOCs, physical damage usually causes them to emit additional compounds or greater amounts. Most of the studies of this phenomenon have focused on the multitrophic interactions between host plant, herbivore and natural enemies of the

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herbivore (parasitoids and predators). However, experiments on the host specificity of biological control agents of weeds typically use intact plants or plant cuttings without much concern that this would substantially affect the relative attractiveness of the plants (Palmer, 1999). A previous study showed that mechanical damage of leaves greatly changed the profile (composition and content) of VOCs emitted from three species of test plants (Beck et al., 2008). Multivariate statistical analysis indicated that the plant species could be more easily distinguished when they had been damaged, and that different VOCs were involved for discrimination of damaged versus undamaged plants (Smith and Beck, 2013). Furthermore, the type of mechanical damage (cut, punctured or scraped) had relatively little effect on the VOC profiles, albeit cut leaves generally emitted fewer VOCs than punctured or scraped leaves.

There are few studies regarding the duration of the change in VOC emissions of damaged plants (Turlings et al., 1998; Schaub et al., 2010). VOC emission is thought to occur during one of three time scales, depending on the synthetic pathway utilized and whether the compounds are stored in plant structures (Baldwin, 1994). “Preformed-induced responses” occur immediately after damage and are restricted to the damaged tissue. “Rapidly-induced responses” occur within hours or days of the injury and can be either systemic or restricted to the damaged leaf. “Delayed-induced responses” do not occur until the next growing season of a perennial plant. Herbivore-induced damage is expected to produce responses for long enough to improve plant fitness by helping to protect against herbivore damage (Karban and Baldwin, 1997; Agrawal, 2000). However, simple mechanical damage is expected to release primarily “green leaf volatiles” that evaporate from the contents of damaged cells, resulting in immediate emission that stops within a few hours (Turlings et al., 1998; Dudareva et al., 2006).

There have been few studies on the duration of changes in VOC profiles of plants that have been mechanically damaged. Given that host specificity experiments are usually conducted for periods lasting several days, we wanted to determine if mechanical damage would result in changes that persisted during most of this period. The purpose of this study was to measure changes in VOC emissions during 48 h after mechanically puncturing leaves of five plant species that represent a variety of phylogenetic clades within the tribe Centaureinae. These plant species have previously been used to evaluate the host plant specificity of a weevil (*Ceratapion basicorne* (Illiger), Coleoptera: Apionidae) as a prospective biological control agent of yellow starthistle (*Centaurea solstitialis* L., Asteraceae) (Smith, 2007; 2011; Cristofaro et al., 2013).

## 2. Materials and methods

Plants were grown from seed in flower pots under outdoor conditions and were tested indoors in the rosette stage of development. The species that were evaluated – *Carthamus tinctorius* L. (safflower varieties Seedtec 518 [oleic], CalWest 4440 [linoleic]), *Centaurea cineraria* L. (dusty miller), *Centaurea melitensis* L. (tocalote; Napa thistle), *Centaurea rothrockii* Greenm. (Rothrock's basket flower), and *C. solstitialis* – represent a variety of close relatives that were previously evaluated for host plant preference for a prospective insect biological control agent, the weevil *Ceratapion basicorne*, which feeds on and oviposits in leaves of *C. solstitialis* (Smith, 2007).

### 2.1. Leaf damage

The effect of mechanical damage on production of VOCs by an intact leaf was evaluated by use of six treatments: no damage (control, Ctrl), and puncturing an intact leaf ten times with a sterile

22-gauge needle then starting the collection of VOCs at 0, 4, 8, 24 and 48 h afterward (Beck et al., 2008). Plants were damaged in the early morning so that samples for 0, 24 and 48 h were collected in the morning, 4 h was in the afternoon, and 8 h was after sunset. Volatiles were collected from four replicates of *C. tinctorius* and from two replicates of each of the other plant species.

### 2.2. Collection of volatiles

Volatiles were adsorbed onto solid-phase microextraction (SPME) (Supelco, Bellefonte, PA; 100  $\mu$ m, polydimethylsiloxane) fibers using methods identical to previously published methods (Beck et al., 2008; Smith and Beck, 2013). Briefly, a Teflon<sup>®</sup> bag (SKC West, Fullerton, CA) was placed over a leaf and gently sealed over the stem using a twist tie. Five minutes after leaf enclosure a SPME fiber was inserted and the control (undamaged) headspace volatiles collected for 55 min. Immediately after the control headspace collection the bagged leaf was damaged as described above and 5 min after damage the headspace volatiles collected onto the SPME fiber for 55 min.

### 2.3. Analysis of collected volatiles

For all experiments the adsorbed volatiles were immediately analyzed (less than 1 min after collection) via gas chromatography-mass spectrometry (GC-MS) using identical methods previously published (Beck et al., 2008; Smith and Beck, 2013). NIST and Wiley databases were initially used for fragmentation pattern identification. The retention indices (RIs) were calculated using a homologous series of *n*-alkanes on DB-Wax and DB-1 columns. Volatile identifications were verified by injection of authentic samples for comparison of retention times, retention indices, and fragmentation patterns. Compounds that we could not authenticate with commercial or isolated standards were labeled as either tentative or unknown in Table 1. The labeling of unknown sesquiterpenes coincides with an earlier report (Smith and Beck, 2013) and lists the new unknown sesquiterpenes in alphabetical order and ascending order of RI value.

Data from GC-MS analyses were transferred to Microsoft Excel for comparison of retention times and compound identification for same-column analysis. Calculated RIs were used to assist in compound identification and to perform comparison of DB-1 to DB-Wax column results. Compounds consistent through all replicates are included in Table 1. The GC-MS data were error-checked by plotting the area of each identified peak from DB-Wax versus that from DB-1. Outliers from the regression line were reviewed for errors of interpretation or transcription and were corrected when appropriate. The area of the peak on DB-Wax was used as the response variable because it was generally larger than that on DB-1 (on average by a factor of 2.1). Peaks with areas less than about  $1 \times 10^6$  were not reliably detected, as was our previous experience (Smith and Beck, 2013).

### 2.4. Statistical analysis

The area of GC-MS peaks was transformed by  $\log(\text{area} + 1)$  to improve normality by reducing skewness and kurtosis, which also reduced the coefficient of variation ( $\text{CV} = \text{sd}/\text{mean}$ ). The significance of plant species, treatment and their interaction were analyzed by GLM using plant species and treatment as classified effects followed by 1-way GLM analysis of the effect of treatment on each VOC for each species (SAS Institute, 2003). Contrasts were used to test specific hypotheses: control (undamaged leaves) vs. 0 h to test the immediate effect of puncture damage; 0 h vs. 4 h, 0 h vs. 24 h and 0 h vs. 48 h to test for delayed changes in VOCs after puncturing; (4 h and 24 h) vs. 8 h to test the effect of morning vs. evening; and

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