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Authors

Karban, Richard
Shiojiri, Kaori
Ishizaki, Satomi

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Natural History Note

An Air Transfer Experiment Confirms the Role of Volatile Cues in Communication between Plants

Richard Karban,^{1,*} Kaori Shiojiri,² and Satomi Ishizaki³

1. Department of Entomology, University of California, Davis, California 95616; 2. Center of Ecological Research, Kyoto University, Otsu 520-2113, Japan; 3. Course in Ecological Genetics, Graduate School of Environmental Science, Hokkaido University, Sapporo 060-0810, Japan

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ABSTRACT: Previous studies reported that sagebrush plants near experimentally clipped neighbors experienced less herbivory than did plants near unclipped neighbors. Blocking air flow with plastic bags made this effect undetectable. However, some scientists remained skeptical about the possibility of volatile communication between plants since the existence and identity of a cue that operates in nature have never been demonstrated. We conducted an air transfer experiment that collected air from the headspace of an experimentally clipped donor plant and delivered it to the headspace of an unclipped assay plant. We found that assay plants treated with air from clipped donors were less likely to be damaged by naturally occurring herbivores in a field experiment. This simple air transfer experiment fulfills the most critical of Koch's postulates and provides more definitive evidence for volatile communication between plants. It also provides an inexpensive experimental protocol that can be used to screen plants for interplant communication in the field.

Keywords: cue, eavesdropping, herbivory, Koch's postulates, plant communication, volatile.

Introduction

The notion that plants communicate via airborne signals to adjust their defenses against herbivory has been with us for approximately 30 years (Baldwin and Schultz 1983; Rhoades 1983). Recently, this idea has gained greater acceptance as ecologists have accumulated several convincing examples of communication between plants that affects herbivory under both lab and field conditions (reviewed in Dicke and Bruin 2001; Karban 2008; Heil and Karban 2010). These studies, both classic and recent, assume that the cues that coordinate communication are volatiles emitted by damaged plants. Indeed, the first widely accepted demonstration of this phenomenon involved volatile cues

emitted by clipped sagebrush branches incubated in vacuum jars with potted tomato plants (Farmer and Ryan 1990). These plants were not in direct physical contact, implicating airborne chemicals; subsequent experiments involving volatile methyl jasmonate released from cotton wicks further supported the hypothesis that volatile chemicals were acting as cues under more natural conditions (Farmer and Ryan 1990). Since this initial influential contribution, the role of methyl jasmonate as a signaling molecule has remained controversial (Preston et al. 2001, 2004), and the precise nature of the cues remains unresolved for any system (Arimura et al. 2010).

Sagebrush (*Artemisia tridentata*) has been a model system for the study of plant-plant communication (Farmer and Ryan 1990; Baldwin et al. 2006; Heil and Karban 2010). Sagebrush is the most common and the defining plant of the Great Basin biome of western North America. It is a long-lived native shrub that is often found in nearly monospecific stands. Sagebrush individuals experienced reduced levels of herbivory when neighbors were experimentally clipped (Karbon et al. 2004). This result was found when neighbors were up to 60 cm away from the experimental clipping (Karbon et al. 2006). Young actively growing plants were most effective as both emitters and responders to cues (Shiojiri and Karban 2006). When the experimental clipping was conducted inside a plastic bag that was subsequently sealed with a wire twist tie, neighboring plants demonstrated no response (Karbon et al. 2006). This result suggested that volatiles were serving as the cues that mediated plant-plant communication, although the possibility exists that the plastic bags not only blocked emission of cues but also caused other unknown effects that ultimately produced the observed reductions in damage experienced by neighbors.

This problem of determining the causal agent of communication is analogous to one faced by bacteriologists investigating the causes of diseases. To establish causation,

* Corresponding author; e-mail: rkarban@ucdavis.edu.

Robert Koch developed a set of postulates that must be fulfilled if we are to conclude that an infectious agent is the actual source of the symptoms (Brock 1999). These rules, originally designed with a living microorganism as a model, have been modified to apply to infectious agents that are nonliving (Walker et al. 2006). Several of the postulates have proven to be problematic in general, although the expectation that the introduction of the agent must be sufficient to cause the disease in a healthy organism remains the critical requirement (Cohen 1994; Walker et al. 2006).

Here we wish to apply this requirement that introducing the suspected causal agent does in fact cause the predicted symptoms to our study of the putative cues of induced resistance in sagebrush. We hypothesize that volatile chemicals emitted by experimentally clipped plants are the agents responsible for increased resistance to herbivory in neighboring plants. To evaluate this hypothesis, we captured the volatile emissions released by experimentally clipped sagebrush plants enclosed in plastic bags and introduced these headspace volatiles to other healthy plants. The accumulation of damage over the growing season caused by herbivores was compared for sagebrush plants inoculated with headspace volatiles from clipped plants versus sagebrush plants inoculated with headspace volatiles from unclipped controls. If the volatiles are responsible for changes in resistance, then we expected that introducing them to otherwise healthy plants should reduce the herbivory that these plants experience.

Methods

We selected and marked 60 young sagebrush plants on the north side of Sagehen Creek (39°26'67"N, 120°12'90"W) in Tahoe National Forest, north of Truckee, California. These assay plants were randomly assigned to two treatments: those receiving air from the headspace of experimentally clipped air donor plants and those receiving air from the headspace of unclipped control plants. One branch of each assay plant was selected, and the branch tip, including the distal 100 leaves, was marked with flagging tape. A young neighboring plant was selected haphazardly near each of the 60 marked assay plants and designated as the air donor. Air donor plants were approximately 2 m from their assay plant but more than 60 cm away since this is the distance over which communication has been observed to occur naturally (Karban et al. 2006). One branch of each air donor was enclosed in a new plastic bag. We clipped the distal half of 25% of the leaves on the enclosed branch for air donors. Following clipping, the plastic bag surrounding each branch was sealed with a wire twist tie around the stem. Volatiles emit-

ted by the clipped branch were collected in the plastic bag for 24 h.

After 24 h, one branch on each of the assay plants was enclosed in a new plastic bag with a twist tie around its stem. A small hole was made in the side of the plastic bag surrounding each air donor and was kept pinched off such that the air inside the plastic bag did not escape. An entomological aspirator (pooter) made of flexible Tygon tubing with rigid plastic tubing at both ends was used to remove the air from the headspace of each donor plant. The end of the aspirator was inserted into the hole in the bag surrounding the donor, and one of us (R. Karban) inhaled the air (approximately 1 L) from the bag surrounding each donor plant until the walls of the bag had completely collapsed. He then moved to the corresponding assay plant, inserted the end of the aspirator into a small hole in the plastic bag surrounding the assay branch, and exhaled until the bag was inflated. The hole in the bag surrounding the assay plant was then closed off with a wire twist tie. A similar procedure was implemented when moving air from the headspace of unclipped control donors to their corresponding bagged assay plants. We were able to confirm that the plastic bags and twist ties were effective by feeling pressure when the air had been completely pulled out of the bag surrounding the donor and when the bag surrounding the assay remained inflated.

One branch on each assay plant was enclosed in a plastic bag that contained air from the headspace of either a clipped air donor plant or an unclipped control on May 17, 2009. After 24 h, the bags were removed on May 18. Assay plants accumulated natural damage primarily from grasshoppers, caterpillars, and deer over the summer season. On September 20, the assay branches (100 leaves) were collected. One of us (K. Shiojiri) counted the number of leaves with herbivore damage on each assay branch, with no knowledge of which treatment that assay branch represented (air from clipped donor or control).

We performed an ANOVA on the number of leaves of the initial set of 100 that were damaged by herbivores in the two treatments (JMP, ver. 8.0). The data were normalized using a log transformation before analysis, although the untransformed data are presented in figure 1.

Results

Four of the replicates were lost during the experiment because the plastic bags were not airtight or because the assay branch died during the summer. Leaves of those assay branches that received air from experimentally clipped donors were approximately 40% less likely to be damaged by herbivores than were leaves of assay branches that received air from the headspace of unclipped donors (fig. 1; $F_{1,54} = 4.89$, $P = .032$).

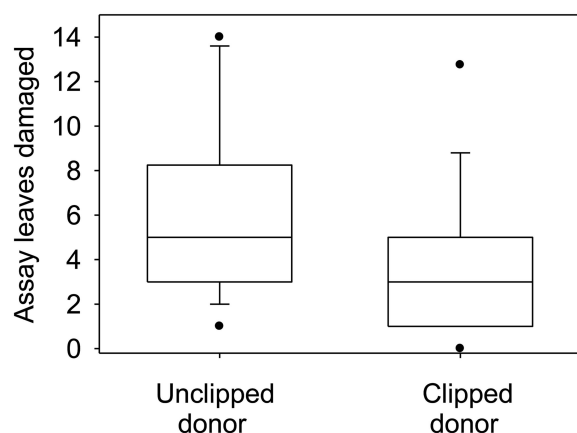


Figure 1: The number of assay leaves damaged by herbivores on branches of 100 leaves (percentage with damage) that received air from either unclipped control donor plants or clipped donor plants. The lower edge of the box indicates the twenty-fifth percentile, the line within the box marks the median, and the top edge of the box indicates the seventy-fifth percentile. Whiskers above and below the box indicate the ninety-fifth and tenth percentiles, and dots indicate the ninety-fifth and fifth percentiles.

Discussion

These results provide more definitive support for the hypothesis that a volatile cue is involved in communication between sagebrush individuals that alters their defenses against herbivores. Since air from the headspace of experimentally clipped donor plants was transferred to healthy assay plants and those healthy assay plants developed the phenotype of induced plants, we conclude that the transferred volatiles are causing the observed reduction in damage by herbivory. This is not a new inference. Experiments in this system, starting with Farmer and Ryan's (1990) initial laboratory demonstration in vacuum jars and continuing with our field demonstrations using plastic bags to block the signal (Karban et al. 2006; Shiojiri and Karban 2006), have suggested that a volatile cue is required to produce the effects we observed. Nonetheless, skeptics have challenged this inference because our results could potentially be explained by alternate mechanisms and because we lack knowledge about the chemical identity of the volatile cue. Since sagebrush is a model system for communication between plants, it is valuable to elucidate clearly the causes behind the reductions in damage for plants near experimentally clipped neighbors.

Workers in this field hold strong opinions about how best to proceed, and several aspects of our design seem worthy of explanation. First, we used artificial clipping with scissors, and this may potentially produce results different from those obtained by actual herbivores. Past ex-

periments have demonstrated that the artificial clipping that was used here produced reductions in damage similar to those of natural levels and modes of damage inflicted by beetle larvae (Shiojiri and Karban 2008). Assay plants of both treatments accumulated natural herbivore damage throughout the season that could potentially cause induction. However, we have found that sagebrush are far more inducible early in the growing season compared to other times during the year (Shiojiri and Karban 2008), such that our early-season treatment effects persisted.

Second, our experimental unit for clipping and then measuring natural herbivore damage was a branch rather than an entire plant. This is probably the appropriate scale since branches of sagebrush are highly sectored and vascular integration among branches is limited (Cook and Stoddart 1960; Karban et al. 2006).

Third, using a living system (R. Karban) to transfer air may modify the chemical nature of the cue. Human respiratory tissues harbor enzymes that detoxify a wide variety of inhaled chemicals (Su et al. 2000; Zhang et al. 2002; Hecht 2008), and these enzymes may have reacted with the volatiles during the transfer process. Air from the headspace of clipped and unclipped donor plants was subjected to similar exposure to respiratory enzymes so that this factor should not bias our results. If anything, respiratory enzymes might be expected to reduce the activity of the volatiles, reducing the likelihood of seeing treatment effects and making these experiments potentially conservative.

Fourth, we enclosed plants of all treatments in plastic bags, and several workers have argued that this technique may increase the responsiveness of plants to volatiles (Baldwin et al. 2006; Paschold et al. 2006). Plants in sealed chambers have access to a limited volume of air that may accumulate unnaturally high concentrations of volatile cues and low concentrations of CO₂, although this has not been documented. These workers advocate instead for using genetic variants of model plant systems (e.g., *Nicotiana*) that are "mute" or "deaf" and therefore limited in their potential to emit or receive cues. While this is a potentially powerful and elegant experimental tool, it is limited because no species that is well characterized genetically has also been found to exhibit interplant communication that affects defenses against herbivores. For example, *Nicotiana attenuata* has mute and deaf genotypes (Baldwin et al. 2006; Paschold et al. 2006) but has not shown evidence of interplant communication between conspecific individuals that affects herbivory of neighbors (Karban et al. 2003).

The air transfer experiment reported here was very simple and used only materials that are readily available. As such, this experiment can be easily repeated in this and other plant systems and may provide an easy and inexpensive method to screen for volatile communication be-

tween plants. This represents an advance because it allows researchers and students without access to specialized equipment or genetic materials to study interplant communication.

In conclusion, our finding that transferring air from an experimentally clipped donor plant to an unclipped assay plant whereon the assay plant received less damage satisfies the most critical of Koch's postulates. This information provides more definitive support for the hypothesis that volatiles act as cues in plant-plant communication.

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