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Simulated herbivory: the key to disentangling plant defence responses

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January 18, 2019
Dr. Andrea Stephens
Editor
Trends in Ecology & Evolution

Dear Dr. Stephens,

Many thanks for allowing us to submit of a revised version of our manuscript. Please find attached our revised version of ‘Simulated herbivory: the key to disentangling plant defence mechanisms’. We found both yours and the reviewers’ recommendations and comments quite useful, and we feel that we have adequately addressed your concerns and made the appropriate changes.

Once again, all listed co-authors have approved the manuscript’s content and interpretations.

Thank you for your consideration.

Sincerely,

Jamie M. Waterman

Simulated herbivory: the key to disentangling plant defence responses

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Abstract

Plants are subjected to a multitude of stimuli during insect herbivory, resulting in a complex and cumulative defence response. Breaking down the components of herbivory into specific stimuli and identifying the mechanisms of defence associated with them has thus far been challenging. Advances in our understanding of responses to inconspicuous stimuli, such as those induced by microbial symbionts in herbivore secretions and mechanical stimulation caused by insects, have shed light on the intricacies of herbivory. Here we provide a synthesis of the interacting impacts of herbivory on plants and the consequential complexities associated with uncoupling defence responses. We propose that simulated herbivory should be used to complement true herbivory in order decipher the mechanisms of insect herbivore-induced plant defence responses.

Plant Defences Vary Depending on the Nature of Herbivory

Around a quarter of multicellular organisms on the planet are thought to be insect herbivores that have been locked in an evolutionary arms race with plants for over 300 million years [1]. The plant defence mechanisms driving this battle have been the subject of intense study and debate [2]. Insects are typically grouped into two broad categories: chewing insects (e.g. Orthoptera, Coleoptera, and Lepidoptera) and piercing and sucking insects (e.g. Hemiptera) [3]. During **true herbivory** (see Glossary), chewing insects physically lacerate plant tissue as they feed, whereas piercing and sucking insects (e.g. phloem-feeders) typically cause minimal cellular rupture [4]. However, chewing insects such as leafcutter ants can cause relatively less tissue damage due to their razor-like mouthparts (i.e. the surface area of damage might be lower) [5]. It is suggested that defence against phloem-feeders typically involves responses similar to those elicited by microbial pathogens, including programmed cell death, a metabolic process that occurs without wounding recognition [6, 7]. Nevertheless, following penetration and rupture of sieve elements by phloem-feeders, defence responses can be induced [8]. Differences in herbivore feeding habits result in variable perception of attack, which can lead to large differences in defence responses [9, 10].

Multiple Stimuli Trigger Plant Defences During Herbivory

There are multiple stimuli associated with insect herbivores that are each (independently) known to affect responses in plants. Collectively, these stimuli generate the observed responses of plants to insect herbivory (Figure 1). **Wounding** and **mechanical stimulation** induce defence responses in plants [11-13], and plants can recognise self-derived cellular components (e.g. cell wall fragments, glucose, electrolytes, etc.) released in response to tissue damage [14]. Even unwounded plants activate metabolite signalling processes such as employing defensive hormones including jasmonic acid (JA) following repetitive touch or mechanical stimulation [15, 16] (Figure 1.). Similarly, plant defence responses can be altered

by sound vibrations; foliar glucosinolate concentration was shown to increase with higher vibration amplitudes from insect chewing [13]. Further, in rice (*Oryza sativa*), aldolase (a glycolytic enzyme) mRNA expression was significantly upregulated at sound frequencies of 125 and 250 hz, but was downregulated at 50 hz, indicating that responses to sound might be frequency specific [17].

The complexity of defence response becomes greater upon exposure to chemical **elicitors** and **effectors** classified as herbivore-associated molecular patterns (HAMPs) (Figure 1) [18-20]. All else being equal, plant defences can be suppressed [21, 22] or increased in response to said compounds [23-27]. In some instances, responses that weren't previously detectable can be realised in the presence of HAMPs [25, 28]. Considering chewing insects harbour microbes in their **saliva**, digestive tract, and exoskeleton, certain responses may be solely microbe-induced and thus independent of insect-derived compounds, mechanical stimulation, and wounding. It has therefore proven difficult to uncouple whether the observed defence responses are derived from the insect, associated microbes, or both (Figure 1). For example, bacterial symbionts in the **oral secretions (OS)** of both Colorado potato beetle (*Leptinotarsa decemlineata*) and corn earworm (*Helicoverpa zea*) can decrease JA-responsive defences, including polyphenol oxidase activity, relative to OS with lesser amounts of bacteria [27, 29]. Similarly, numerous defence response-associated genes in maize (*Zea mays*) were suppressed to a greater extent by western corn rootworm (*Diabrotica virgifera virgifera*) treated with *Wolbachia sp.* than untreated individuals [30]. It is clear that a multitude of stimuli are responsible for the consequential responses to herbivory, and it is critical to consider each when investigating the underlying mechanisms associated with plant-herbivore interactions.

The Chemical Machinery of Plant Defences

When a plant perceives herbivore attack various complex signal cascades (e.g. electrical and chemical signalling pathways) are activated both locally and systemically, resulting in the activation of defence responses - including the accumulation of reactive oxygen species (ROS), Ca^{+} , defence hormones, and volatile organic compounds (VOCs) - that contribute to the plant's ability to mitigate the effects of the imposed stress [12, 31, 32]. The major plant hormones known to influence the defence response are JA, salicylic acid (SA), and ethylene (ET) [33]. It has been shown that JA and SA can exhibit an antagonistic relationship, that is, JA signalling can suppress the SA pathway and *vice versa* [34]. Many microbes induce SA-responsive defences whereas chewing herbivores often stimulate JA-responsive pathways [34, 35]. In systems in which a plant's JA- and SA-responsive defences interact, microbial symbionts can give herbivores an advantage by inducing the SA pathway and concurrently suppressing JA-dependent defence responses. Although this antagonism has been demonstrated in many plant species, whether or not there is a ubiquitous genetic basis for crosstalk between JA and SA remains contentious [36].

Further, elicitors can trigger a defence response in one species, but have a minimal or differing effect on the same pathway in another [21, 25]. Even within the same plant family, elicitors can have variable effects on the induction of defence responses. For example, inceptin, a short proteolytic fragment of chloroplastic ATP synthase found in the saliva of fall army worm (*Spodoptera frugiperda*), upregulated the production of JA, SA, ET, and total VOCs in cowpea (*Vigna unguiculata*), but had a much lesser influence on the same hormones in soybean (*Glycine max*) [25, 37]. In both lima bean (*Phaseolus lunatus*) and cabbage (*Brassica oleracea*), β -glucosidases found in the OS of the large white (*Pieris brassicae*) triggered the emission of VOCs known to act as **indirect defences** against herbivory by attracting wasps known to parasitise insect herbivores [38-40]. Also in *P. lunatus*, the accumulation of ROS, which affect defence signalling in plants and can result in direct

oxidative injury to insects, was greater in leaves that had been fed on by Egyptian cotton leafworm (*Spodoptera littoralis*) than those simply damaged mechanically [41]. Specifically, the enzyme glucose oxidase and the fatty acid-amino conjugate N-linolenoyl-L-glutamine (both found in Lepidopteran OS) have been shown to promote a significant increase in ROS concentrations within leaf tissue shortly after damage is inflicted [26, 42, 43]. The fatty acid-amino conjugate volicitin (*N*-(17-hydroxylinolenoyl)-L-Glutamine) is found in the OS of lepidopteran larvae and is responsible for the induction of multiple plant VOCs. Additionally, volicitin can stimulate increased activity of both hormone-induced and wound-induced protein kinases [44, 45]. Further, caeliferins (disulphooxy fatty acids named due to their presence in the OS of Orthopteran insects in the suborder Caelifera) induce similar defence responses in multiple plant species [25, 45, 46]. In contrast, glucose oxidase in *H. zea* saliva can inhibit the synthesis and functionality of nicotine in tobacco (*Nicotiana attenuata*) and thus decrease resistance [21, 47]. Insect-derived molecules can also suppress indirect defences, as it has been shown that a silkworm (*Bombyx mori*) specific enzyme (BmFHD) suppressed the production of leaf VOCs in mulberry (*Morus alba*) [48]. In order to realise the nature of the complexities associated with insect feeding, development of techniques that enable the uncoupling of the mechanisms that drive the responses observed in plants is critical.

Simulated Herbivory: A Change in Emphasis

It has been almost 30 years since Baldwin [49] published the seminal review on the value of using mechanical simulations in ecological research. Baldwin's paper identified advantages of **simulated herbivory** (see Glossary), including spatial and temporal precision in the application of damage, the ability to standardise damage without the confounding effects of inherent differences in herbivore feeding behaviour, and control over the

introduction of material from foreign and unidentified organisms (e.g. pathogens). Shortcomings outlined by Baldwin included differences between simulated herbivory as applied by experimentalists and damage caused by true herbivory (e.g. type and age of tissue damaged, inability to accurately mimic certain feeding guilds, and the geometry of feeding patterns). Moreover, simulated herbivory usually failed to replicate environmental changes associated with true herbivory (e.g. enhanced CO₂ microenvironments due to herbivore respiration).

In the past two decades, the differences between simulated and true herbivory have been reviewed in several articles and book chapters [50-52]. The main purpose of these reviews was to describe the fidelity of simulated herbivory as a proxy for herbivory in nature, and how the two differ in terms of their induction of plant defence responses. The rationale for simulating herbivory in experiments has thus far been either for pragmatic reasons (i.e. not having to include herbivorous organisms in experiments) or for standardisation of treatments. Expanding beyond these prior rationales, we suggest that simulated herbivory has an additional and novel benefit: it is an essential tool for separating how plants perceive and distinguish the various factors associated with insect feeding, including mechanical stimulation, wounding, and introduction of foreign compounds.

Plant defences are highly complex, partly due to the fact that both microbes and insects have strongly influenced the evolution of physiological and chemical plant traits [53, 54]. By determining plant responses to specific components of herbivory, it might be possible to identify the evolutionary rationale for a given response; In contrast, live insects are used the exact cause of a response is difficult to determine, as individual stimuli are more difficult to tease apart. Additionally, knowledge of whether a specific response is caused by insect- or microbe-derived compounds can provide insight on how to better manage pests and pathogens. It is clear that identifying novel mechanisms of defence responses to the various

components of herbivory is useful across disciplines, whether by providing coevolutionary insights, or by directing sustainable pest mitigation strategies. As of now, unravelling the individual effects of these interconnected stimuli remains elusive and is thus a subject ripe for synthesis.

Our focus for this synthesis is simulations of chewing-insect herbivory. Although the role of piercing and sucking insects in plant defence induction has been well-documented [22, 55, 56], methods of simulated herbivory aimed at mimicking feeding habits of phloem-feeders are, to our knowledge, absent in the literature. This is presumably due to difficulty replicating proboscis movement, timing of probing, and injection of saliva directly into the phloem [57].

Advantages of Simulated Herbivory

Advantage 1: Specified Elicitors and Stimuli Minimises Bias

The dynamics of defences induced by herbivory are clearly complicated and can be species specific. Using simulated herbivory, it is possible to determine the potential influence of one single stimulus or a customised combination of stimuli on plant defences during insect feeding (Figure 2). Responses found in studies that apply specific herbivore-associated stimuli can be conflated if live insects are used, and therefore studies applying a single stimulus and combinations of stimuli reveal a complexity hidden by true herbivory. Several techniques have been devised in attempts to accurately elicit responses to insect herbivory beyond mechanical wounding, and they typically have two major phases: (1) collection and/or purification of insect-associated compounds and (2) application of herbivore- and pathogen-associated biomolecules (often coupled with wounding) (Box 1).

During bouts of feeding insects secrete variable amounts of OS and saliva. For example, Peiffer and Felton [19] found that insects can secrete anywhere from 0 - 6 nl of OS in 10 min of feeding. Considering this high variability, it is impossible to ensure that all

plants are being treated with the same amount of associated compounds using true herbivory. Chemical, biochemical, and molecular analyses require high-fidelity and consistent treatments, which can be hard to achieve using unpredictable live specimens. Only with artificial herbivory is it possible to run identical treatments and change only one of the variables associated with herbivory. In one method described by Tian et al. [26], plants had holes punched in the same part of the leaf, and phosphate buffer was applied to the resulting wounds. In one treatment, plants were given buffer spiked with a constant volume of *H. zea* saliva. Therefore, any differences in plant response between treatments could be more accurately compared, as the amount of saliva and extent of physical wounds were identical across individuals and treatments respectively. Considering it is well known that herbivores can harbour microbes in their saliva and OS, herbivory simulations using isolated elicitors might be particularly useful in experiments that seek to determine the effects of insect-derived and microbe-derived compounds separately.

Advantage 2: Eliminates the Effects of Tissue Quality

It has been well documented that insects feed differentially based on the physical and chemical attributes of plant material [58-61], and therefore another major challenge associated with the use of true herbivory is the differential feeding patterns likely to be observed between treatments. Ryalls et al. [58] showed that high concentrations of foliar silicon reduced herbivore feeding compared to leaves with lower amounts of silicon. Robin et al. [59] found diamondback moth (*Plutella xylostella*) larvae to feed preferentially on *B. oleracea* plants based on foliar glucosinolate profiles; therefore the size, density, and location of wounding was inconsistent between individual plants and genotypes. Plant phenology also plays a role in determining the extent of herbivory. In *Eucalyptus spp.* the total leaf-area of insect damage was far greater on young leaves compared to mature leaves ($\approx 25\%$ vs. $< 5\%$ respectively) [62]. It is also well known that variation in the intensity of herbivory can alter

plant metabolism [63, 64]. For example, in *Arabidopsis thaliana*, resistance to grey mould (*Botrytis cinerea*) colonisation was increased based on the intensity of damage [65].

Additionally, genetic mutants with particular defence-related genes silenced can be useful in both simulated and true herbivory studies, and have been used with multiple plant species including *A. thaliana*, *O. sativa*, *N. attenuata*, and tomato (*Lycopersicon esculentum*) [66-70]. These genotypes can facilitate the uncoupling of defence mechanisms, as changes in resistance in the absence of possible modes of defence allow for validation or repudiation of hypothesised mechanisms of herbivory-induced defences. Ye et al. [66] showed that the increase in biomass of rice leaf folder (*Cnaphalocrocis medinalis*) was significantly greater in individuals that fed on *O. sativa* with the expression of allene oxide synthase silenced compared to wild-type plants. This in mind, it could be expected that the extent of damage between *O. sativa* genotypes might have varied due to differing feeding preferences. Therefore, variation in response might be influenced by differences in the quality of damage in addition to differing defence capabilities. Simulated herbivory solves this problem; despite genetic variation, the quality of damage is identical between individuals and treatments.

Advantage 3: Timing of Damage and Measurements

Localisation and intensity of damage are also of importance when measuring defence responses at the transcriptome, proteome, and metabolome level. Gene expression can vary in a single plant between the immediate area damaged and areas further away [71, 72]. Furthermore, over time, mechanically damaged *A. thaliana* increased both apoplastic glutamate and cytosolic Ca^{+} concentrations in tissue adjacent to the immediate site of damage [12]. In response to herbivory plants transmit systemic signals to distant tissues in order to upregulate defences in preparation for imminent attack, which can further complicate the decision to measure responses in a given tissue locale [32]; even systemic signalling molecules such as proteins, mRNAs, and large metabolites can be transported at rates of

several hundred $\mu\text{m sec}^{-1}$ [32, 73]. Root herbivory, for example, can influence the quality of above ground tissue and *vice versa* [74, 75], and therefore if one wanted to measure, say, a response in the foliar tissue of a plant to damage undergone in the roots, an understanding of the timing of systemic responses is necessary.

When using live insects, localisation of damage typically requires control over the range in mobility of live insects without interfering with their feeding habits. Mechanisms such as clip cages can confine insects, but these cages have been shown to influence plant growth, which can interfere with the allocation of resources to defence responses [50, 76]. Deciding on the location of the clip cages also presents challenges, as herbivory patterns are often significantly different across, for example, varying leaf phenology [62]. Additionally, the precise timing of feeding can vary considerably between insects over the course of the treatment. Therefore, with true herbivory, measurements of defence responses can differ solely due to inconsistencies in the time at which the wounds were inflicted; although the timing of damage will vary, the timing of harvest will be the same.

Can We Mimic Herbivore Feeding in Time and Space?

A major concern associated with most simulation techniques in ecological studies is that they fail to account for the fact that plants can discriminate between continuous damage and a single wounding event [77]. Herbivores feed on plant material over a period of time, whereas the majority of simulation experiments impose damage in one single application [77, 78], despite the suggestion that the spatial and temporal extent of mechanical damage can alter plant defence responses. Responses can also vary due to differences in the quality of damage and uncontrolled stimuli introduced by the insects but omitted in simulations. Considering the inherent dissimilarity between true and artificial herbivory, experiments that use simulations might fail to elicit a response that would be shown with true herbivory, or elicit an unauthentic response. For example, Massey et al. [79] showed that repeated

wounding events in two grass species increased silicon uptake relative to a single wound application, and that damaging tissues with scissors failed to elicit the same response as tissue damaged by desert locust (*Schistocerca gregaria*). Additionally, stem-boring insects typically prove harmful to plants, however other insects such as leaf defoliators have more variable effects on the intensity of both primary metabolic processes (e.g. photosynthesis) and secondary defence responses depending on the amount of tissue removed [80-84]. It is well known that plant defences and insect feeding patterns can also vary due to circadian rhythm [85], therefore the time of day herbivory simulations occur should be standardised to known circadian patterns of the specific plant-insect system being simulated.

Knowledge of the quality of damage typically inflicted by a given herbivore can yield a more accurate representation of how a plant might respond to herbivory in a ‘natural’ setting; simulations can then be selected accordingly to induce a similar response. Bricchi et al. [86] showed that continuous damage with the MecWorm, a robot designed to spatially and temporally replicate the physical nature of various forms of insect damage (see Table 1), elicited a response in *P. lunatus* VOC emissions more similar to that induced by herbivores than a single entry of damage. Bricchi et al. also showed that only in the presence of OS did ion fluxes closely mimic those induced by true herbivory, regardless if the damage was continuous or not. Similarly, in *B. oleracea*, continuous damage has been shown to induce a response in the production of parasitoid-attracting VOCs more similar to true herbivory damage than final damage or a single-entry and immediate deployment of damage [87].

Technical advancements such as MecWorm simulate herbivory with some success, but there are still knowledge gaps that must be addressed. A better understanding of MecWorm’s effectiveness across multiple systems might help to identify potential modifications that will increase its utility. Refinement of damage to better resemble true herbivory is imperative, especially considering that even gentle touch (e.g. bending leaves

several times without causing wounds) can activate Ca^+ , ROS, and hormone signalling pathways, as well as associated gene expression within minutes of stimulus perception [15, 88]. Defence responses can be sensitive and highly variable, so keeping conditions as similar as possible between individual plants is imperative.

Herbivore Measurements Are Important

Perhaps the biggest issue with herbivory simulations is the most obvious one: they operate in the absence of real insects. Particularly in ecological studies, recording the effects of plant defence on herbivore performance (e.g. biomass, frass production, fecundity, etc.) is required to provide information regarding the nutritive qualities of the plant tissue and the resulting ecological outcomes [45, 58, 89]; measuring defence responses is one thing, knowing if they are of consequence to insects is another. Managing the impacts of herbivory, however, depends on uncoupling the chemical and physiological responses of plants to various types of attack; there are still many gaps in our understanding of the variation in response between herbivores, microbes, and wounding. We propose that many of these knowledge gaps can be best addressed using simulated herbivory, primarily because controlled experiments that clearly distinguish between the effects of each stimulus can be carried out.

Concluding Remarks and Future Directions

Given the impacts of insect herbivory on ecosystem function, agriculture, and the well-being of the global population [90-93], improving our understanding of plant-herbivore interactions is vital across numerous ecological disciplines. This ranges from crop protection against pests (e.g. food security), weed biological control, herbivore invasiveness, plant competition, and even conservation of beneficial herbivores (see Outstanding Questions). Detailed comparisons between various forms of tissue damage, that better characterise

observed variation in responses to plant antagonists, may be best accomplished by herbivore simulations. Consideration of known plant responses to specific herbivores might ensure that simulations most accurately reflect the nature of the interaction, as it has been well established that stimuli that cause change in the metabolism of one plant species can have drastically different effects in another. Development and increased accessibility of technologies such as MecWorm, that facilitate sophisticated mechanical wounding combined with exogenous biomolecules, will enable the uncoupling of elicitor-specific responses from those of wounding alone. Finally, investigations into mimicking sounds associated with herbivory have almost been completely overlooked by ecologists, yet the evidence is now strong to show that specific sound qualities can impart a plant defence response. Incorporating these concepts into artificial techniques will only increase the accuracy of herbivore simulations and make it possible, for the first time, to mechanistically break down the variation in plant defence responses between chemical signals, wounding, and mechanical stimulation during herbivore attack.

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532 insect feeding in *Arabidopsis*. The Plant Cell 12 (5), 707-719.

Glossary:

Effector: A protein derived from an herbivore or microbe that negatively interferes with plant metabolism [7, 10].

Elicitor: A molecule derived from an herbivore, microbe, or the plant itself that stimulates (elicits) a response in the plant [21].

Indirect defence: A volatile organic compound (VOC) emitted by plants that attract predators and parasitoids of herbivores [39].

Mechanical stimulation: Stimulation caused by physical movement or vibrations without wounding tissue [16].

Oral secretions (OS): A combination of bodily fluids derived from both the herbivore gut (regurgitant) and salivary glands (saliva) and secreted from the mouth during feeding [19].

Saliva: Secretions derived solely from salivary glands.

Simulated herbivory: Artificial damage techniques meant to replicate herbivore feeding in the absence of a live herbivore.

True herbivory: Feeding on plant tissue by live insects

Wounding: Mechanical stimulation that causes tissue damage. Encompassing tissue laceration and removal (e.g. defoliation).

559 **Box 1. Simulated herbivory techniques**

560 **Saliva collection:**

561 Saliva is secreted during feeding across feeding guilds, whereas OS is secreted less
562 regularly [19, 94, 95]. After chilling insects on ice, saliva can be collected from the salivary
563 glands using a pipette tip and applied to wounds [26].

564 **Ablation:**

565 To compare insect herbivory both in the presence and absence of insect saliva,
566 ablation of the salivary glands, and thus prevention of salivation, is employed [19, 21, 48,
567 96]. This method is unique in that it uses true herbivory for both treatments and controls. It
568 has been shown that spinneret ablation does not interfere with feeding habits and therefore
569 consistency between treatments should be expected [21]. The ventral eversible gland (VEG)
570 also produces secretions known to elicit a defence response, and can be ablated [97].

571 **Oral secretions and gut contents:**

572 Most OS collection techniques involve agitating the mouthparts of insects after
573 feeding and collecting the regurgitant [98]. The volume of OS able to be collected from a
574 given insect is larger than saliva alone [26]. Contents of the alimentary tracts have also been
575 applied directly to plant tissue [99]. Insects might not secrete all of these extracted
576 compounds when they feed, and even secreted compounds are produced in highly variable
577 volumes [19]. The resulting extract will contain compounds found within the salivary gland,
578 but not necessarily released in saliva, unless appropriate purification techniques are used.

579 **Purified elicitors:**

580 Glucose oxidase (GOX) is a major constituent of the proteome of Lepidopteran saliva,
581 and applying GOX to wounds is often compared against solely mechanical damage in order
582 to elucidate defence responses specific to the introduction of a single HAMP. Results have
583 thus far indicated variability in defence responses [19, 26, 96, 98, 100, 101]. Other known
584 elicitors such as inceptin, fatty acid-amino conjugates, and caeliferins have also been isolated
585 and applied to plant tissue [25, 37, 39, 46, 102, 103].

586 **Mechanical damage of tissue:**

587 Some of the most commonly used mechanical damage techniques are: cutting and/or
588 scratching of the leaf with a razor blade [25, 104], crushing the leaf tissue with apical lamina
589 forceps [105], puncturing the leaf with a tracing wheel [23, 98], punching holes in the leaf
590 [26], puncturing the leaf with a syringe [65], and in few instances the use of a custom-
591 engineered machine designed to simulate the spatial and temporal patterns of insect herbivory
592 as closely as possible [77, 86] (Table 1).

Tables

Table 1. Various types of artificial wounding used in current literature to mimic chewing damage by herbivores

Tissue Damage	Type of Wound	Major Concern(s)	Sources
Razor blade	Clean lesion, number of wounds can be manipulated	Clean lesion, unlike most chewing herbivore damage, low surface area of leaf damaged, often single entry (non-continuous)	[25, 104]
Lamina forceps	Crush desired percentage of leaf	No tissue removed, often single entry (non-continuous)	[105]
Tracing wheel	Run over the surface of tissue and make small puncture wound	No tissue removed, often single entry (non-continuous)	[23, 98]
Hole puncher	Remove disks of tissue from desired location	Often single entry (non-continuous)	[26]
Syringe	Make puncture wounds in leaf tissue	No tissue removed, often single entry (non-continuous)	[65]
MecWorm	Set parameters to remove desired amount of tissue over specified amount of time	Not widely available	[77, 86]

Figure 1. Independent stimuli known to elicit a plant response during chewing insect herbivory. The simplest break down of the various defence-inducing stimuli of herbivory are the physical disturbance and chemical elicitation. Physical disturbance can be further broken down into wounding and mechanical stimulation (i.e. physical movement and/or vibrations), and chemical elicitation can be broken into compounds derived from microbes associated with insects or from the insects themselves.

Figure 2. Possibilities of simulated herbivory not afforded using true herbivory. Plant defence is known to vary between stimuli, and simulated herbivory allows for customised treatments not afforded by true herbivory (see Figure 1). Several studies (numbers shown in brackets correspond to reference number) have used an individual stimulus, two stimuli, or three stimuli to elucidate which components of herbivory were responsible for the observed responses. In all studies listed, regardless of the number of stimuli tested, each was also introduced to the plant independently in order to compare results to the collective response of all the stimuli investigated. Each Venn diagram shows the combination of stimuli (**A**, mechanical stimulation; **B**, wounding; **C**, unseparated elicitors/effectors derived from both insects and microbes; **D**, elicitors/effectors derived from microbes; **E**, elicitors/effectors derived from insects) used by a given study/studies. Each stimuli's respective icon from Figure 1 corresponds to the letter directly above it. The studies referenced are not exhaustive, however to our knowledge no additional combinations directly pertinent to herbivory exist in the literature.

Editor's Comments

1. As you can see from reading the referee reports, both reviewers found the paper well-written and of interest. I would like you to consider Reviewer 2's suggestion for a restructure but I will leave that decision up to you. However, I would like to see their thoughts on Figure 2 implemented -- I strongly agree with them. The Venn diagram structure leads one to expect numbers of studies to be associated with each type of study and you do not provide this (nor do I expect you to).

Response 1: See response to Reviewer 2's comments on Figure 2

2. From an editorial perspective, I thought the paper was well-written and I enjoyed reading it. One aspect I found to be missing was an explanation of *why* one might want to separate out responses to different aspects of herbivore damage -- why does it matter that the plant is responding to, say, herbivore saliva rather than the loss of tissue? Remember that TREE has a very broad audience from 1st year graduate students to professors.

Response 2: We have now addressed this 'why' component (see lines 145-156)

3. I have marked some editorial changes that I would like you to make on the marked up copy of your manuscript.

Response 3: All suggested changes were made with the exception of the comment regarding Table 1. It was suggested that we add a new column to the table to distinguish what type of herbivory (i.e. chewing vs. piercing) each damage technique was meant to replicate. To our knowledge, techniques to mimic piercing insects are absent from the literature, therefore all of these techniques more closely mimic tissue damage induced by chewing insects. We therefore find that the addition of the suggested column might not be necessary, as the values for each cell would be identical. We have however added text to the Figure 1 legend to indicate that all included techniques are meant to emulate chewing insect herbivory (see Table 1 legend).

4. In addition, please also pay particular attention to the following points, not all of which will apply to your manuscript:

- * Please follow our Instructions to Authors for the correct style for the reference list.
- * A solidus (/) can be ambiguous, therefore, please change ALL solidi to 'and', 'or', 'and/or' or '-' where appropriate.
- * Please note that the figures should 'stand alone' and thus figure legends need to be explanatory rather than just descriptive.
- * Please use the accepted nomenclature for all gene and protein symbols, italicising gene names where appropriate.
- * Please include GenBank accession numbers for all genes. Please use bold and underlined text and with uppercase letters (e.g. A12345), stating that they are GenBank accession numbers.
- * For clarity we use 'might' or 'can' rather than 'may' because 'may' implies doubt or permission, please change any use of 'may' to 'might' or 'can' as appropriate.
- * Please note that it is your responsibility to obtain permission to reproduce copyrighted material (i.e. figures, tables or excerpts that have been published online or in print) from the publishers of the original material. This is also relevant to figures that have been altered in any way. You should retain the original permission form on its return from the copyright holder. Please note that it is courteous to inform the author of the original material of your intent to use their published work.

Response 4: We have paid close attention to these guidelines and made all adjustments.

Reviewers' Comments:

Reviewer #1: This is an interesting and thorough review on how we can simulate herbivory for disentangling different insect herbivore-derived factors mediating plant defense regulation. The idea of the review is enticing, and timely.

The paper is very well written (given the excellent writing team!), and most of the relevant literature has been included in my view.

I agree with the authors that mimicking natural herbivory mechanistically remains a challenging task in ecology today, and there is for sure need for novel approaches. I generally appreciate the completeness in presenting the different methods available today. I was just slightly disappointed in not really seeing anything really novel emerging from this summary. In other words, as the authors mention there is still room for improving methods generally, and perhaps a bit naively, I was hoping to extract some more integration from the "long" list of methods that were discussed. For instance, in the introduction, and again in the "outstanding questions" at the end, the authors mention that literature on mimicking phloem feeders is very scarce. That said, something is there and would have been nice to read that too, in addition to perhaps propose novel methods to integrate the different techniques of mimicking herbivory, not just in the lab but also in the field.

I also found some minor imprecisions that should be fixed:

1. lines 106-110: I found this sentence a bit odd. These are not the only those chemicals mentioned here that are activated upon insect feeding (e.g. VOCs). Of course, besides a plethora of secondary and primary metabolites being activated, recently it was shown that plant defense activation is also mediated by electrical signaling.

Response 1: Agreed, the structure of the sentence has been rearranged to highlight more clearly that complex signals (e.g. electrical signals) activate the defence response and lead to the accumulation of a plethora of chemical defence-metabolites (see lines 79-83)

2. line 111-112: while I also generally use this framework, I am actually no more sure about the generality of the JA-SA trade-off. Indeed, evidence for this crosstalk is not universal and several studies have found positive interactions or no interaction between JA and SA pathways. Therefore this might need to be tuned down a bit.

Response 2: We have rephrased this section to explain that the JA-SA antagonism is not universal, and has simply been observed in certain instances (see lines 85-92)

3. line 129: here would be a good place to introduce volicitin (which is mentioned at line 138 but not really explained. The same would apply to caeliferin

Response 3: Briefly introduced volicitin and caeliferins where suggested (see lines 108-114)

4. line 134: "indirect defenses" here come a bit out of nowhere. Please explain them, maybe earlier when VOCs are mentioned.

[Ed note -- perhaps 'indirect defences' should be a Glossary term]

Response 4: Good point. We mentioned indirect defences during earlier discussion about VOCs (lines 99-102) and included the term in the glossary as well.

5. line 172: I must say I was a bit surprised here. I might be a bit biased, but there are some many more papers in the literature about defense gene silenced in Arabidopsis, Nicotiana, Tomato, for instance. Why only mentioning rice?

Response 5: We have now included references that look at defence genes in rice, Arabidopsis, tobacco and tomato (lines 206-209). The rice example was included in the text because we felt the experimental design was particularly relevant to the point we were trying to relay. There are of course many other instances of mRNA silencing used as a tool to decipher plant defences, but the strict word limit meant we could only discuss one example in the text, though the additional references we now include highlight other examples.

6. line 187: I would suggest using "preferentially" if then given specific attributes of the secondary metabolome. otherwise, maybe use "to feed differentially" or something similar.

Response 6: Changed 'preferentially' to 'differentially' (line 192)

Reviewer #2: OVERALL

This manuscript presents a review of the use of simulated-herbivory techniques, with an emphasis on some of the more recently studied subtleties in the effects that herbivores have on their host plants. The main take-home message is that studies employing simulated herbivory can provide precise information that studies using only actual herbivory cannot.

Because this message is certainly not new, my main challenge as a reviewer was to evaluate whether another review article on simulated herbivory is warranted. Indeed, a new article would be useful if it synthesized new information (or old information in a novel way) and provided suggestions that would be of use to researchers in the field of plant-herbivore ecology and evolution. After a few readings of this manuscript, it is my impression that this manuscript does indeed meet these criteria. I did learn some new information, and if I were still an active researcher in this field, it is likely that I would have occasion to cite this article.

Nevertheless, I do think that the article could be a more effective piece of communication with a revision of the overall organization scheme. I think that the formal list of four highlights of the paper (which was presented in a text box) would set up a good organization scheme, but the manuscript does not really follow that scheme. If one just looks at all of the headings in the manuscript, it is hard to discern a logical organizational thread. Moreover, the points made about simulated herbivory are not presented in a fashion that is ideal in terms of appreciation and application by readers of the article. Specifically, the authors' points about simulated herbivory are scattered every few sentences throughout each section of the text. This strategy is not *prima facie* an inferior way to make the points. However, it does affect the tone of the article and the motivation for the take-home message. In a subtle way, it makes the manuscript sound defensive, as though the authors are responding to criticisms of the usage of simulated herbivory in scientific research. Some readers may find this to come across as a flailing counter-attack rather than a sufficiently reasoned plan for inspiring novel research contributions.

My main suggestion is to revise the organization so that the text more effectively leads up to and motivates the need for the creative use of novel simulated-herbivory techniques. Consider starting with a brief review of the different general ways in which herbivores can feed on and affect their host plants. Then delve into highlights of recent research that has elucidated subtle and interesting ways in which herbivores can affect different aspects of their host plants. Then make your points about how these subtle effects can be confounding and hard to tease apart, in terms of how the plants respond specifically to the various stimuli that feeding simultaneously presents. At this point, the readers will be receptive to reading about the benefits of simulated herbivory. You could continue with a brief overview of the history of using different types of manual herbivory, including why they were used, what was gained from using them, and what their limitations were. Then the centerpiece of the article can be presented with maximum impact: how simulated herbivory has a unique potential for elucidating the subtle responses of plants to specific stimuli involved in the feeding of herbivores. The last section could then describe some of the newest innovations in simulating herbivory, as well as suggesting what is on the horizon.

I suspect that the organization scheme I am recommending will generate a better reaction in most of the readers of this article. However, I will acknowledge that the authors might disagree with me completely for reasons that may be justifiable. I do think that the manuscript has some valuable information and suggestions to contribute. I merely request that the authors give this organization scheme genuine consideration, as it is my humble opinion that the novel contributions of the article will then be more effectively communicated.

Response to Reviewer 2 restructure suggestion:

We are thankful for this thorough and diligent review of our manuscript. We agree that the paper will generate a greater impact if changed from its former structure. We have given Reviewer 2's comments serious consideration and feel we have now addressed many of their concerns. The majority of the text remains the same in the revised version, however the arrangement has been changed drastically. The New outline is as follows:

1. Plant Defences Vary Depending on the Nature of Herbivory

2. Multiple Stimuli Trigger Plant Defences During Herbivory
3. The Chemical Machinery of Plant Defences
4. Simulated Herbivory: A Change in Emphasis
5. Advantages of Simulated Herbivory
 - a. Advantage 1: Specified Elicitors and Stimuli Minimises Bias
 - b. Advantage 2: Eliminates the Effects of Tissue Quality
 - c. Advantage 3: Timing of Damage Measurements
6. Can We Mimic Herbivore Feeding in Time and Space?
7. Herbivore Measurements Are Important
8. Concluding Remarks and Future Directions

We consider the revised structure to be better aligned with the order outlined in the 'Highlights Section'. Additionally, the adjustments made to the headings (and now also subheadings) of the manuscript make it easier for the reader to follow, and give it a less defensive tone; it now provides readers with information regarding herbivory and its complexities before discussing our novel rationale for using stimulated herbivory, as well as the overlooked advantages of simulated herbivory. We then conclude the paper by way of addressing critical considerations when deciding whether or not to use simulated herbivory. Additionally, we discuss the importance of realising simulated herbivory's potential, as well as ways in which the fidelity of simulated herbivory might be improved suggestions for the future.

SPECIFIC COMMENTS

1. L99 and L112: I am not providing detailed editorial suggestions. On the whole, I found the text very well written, and what seem like grammatical errors to me are likely just a difference between American and British conventions. However, I suspect that "supress" is a misspelling. [Ed note: TREE is happy with either UK or US English as long as you are consistent]

Response 1: We have checked that all grammar and spelling are UK English (lines 73 and 86)

2. L109: Use an apostrophe for "plant's"

Response 2: Thanks for catching that – now changed (line 83)

3. L233: Do you mean "elicit" rather than "elucidate"?

Response 3: Yes, changed to 'elicit' (line 254)

4. L249: Can you provide any citations to support the claim that "stem boring insects typically prove detrimental to plants"?

Response 4: We have changed 'detrimental' to 'harmful'. The reason here, is that stem-boring damage almost always results in changes in physiological processes (e.g. photosynthetic rate), whereas defoliation events may have less influence on the same physiological processes. Below are the citations that we have included in the text that support the claim that stem-boring insects generally cause substantial damage, whereas defoliators, for example, have much more variable effects: (Refer to lines 255-258)

Stephens, A.E.A. and Westoby, M. (2015) Effects of insect attack to stems on plant survival, growth, reproduction and photosynthesis. Oikos 124 (3), 266-273.

Welter, S.C. (1989) Arthropod impact on plant gas exchange. Insect Plant Interact., 135-150.

Delaney, K.J. and Higley, L.G. (2006) An insect countermeasure impacts plant physiology: midrib vein cutting, defoliation and leaf photosynthesis. Plant Cell Environ. 29 (7), 1245-1258.

5. L520: For consistency with the other terms in the glossary, do not capitalize "secretions" here

Response 5: Changed, thanks.

6. L553: Consider writing out GOX in full

Response 6: Changed, thanks.

7. L559: Consider changing the heading "Application to tissue" to "Mechanical damage of tissue." The former heading seems too general, and it seems that the distinction between this technique and the others is not that this one is the only one that involves an application to tissues, but that it is the only one that involves actually damaging plant tissues.

Response 7: We agree that heading is more appropriate so we have changed it.

Figure 1

Figure 1 is visually effective and provides a useful summary of different ways that chewing can stimulate a plant response. However, it is not clear to me what the category "Elicitors/effectors derived from both insects and associated microbes" adds to the story that the separate categories for insects and for associated microbes do not entail. I note that the icon for "both insects and microbes" is drops of liquid, while those for insects and microbes separately are structural formulas for chemicals. I do not know what I am supposed to infer from this distinction, however.

Figure 1. Response: The logic behind including this category was to suggest that by applying, for example, saliva collected from an insect to plant tissue, researchers encompass both insect and microbial-derived elicitors. It is possible, however, that using techniques outlined in the paper, elicitors specific to either the insect or microbes can be isolated. Therefore, we included the water drops (meant to represent secretions) as a more general, less refined application of the plethora of elicitors found in saliva.

We do agree that the text for this category was a bit vague, so it has been changed to: '*Unseparated elicitors/effectors derived from both insects and associated microbes (e.g. crude saliva or oral secretions)*'

Figure 2

Figure 2 is cute, but I am sceptical regarding whether it effectively communicates essential information better than simple text (or a table) could. In particular, it takes a good bit of effort on a reader's part to translate the letters to stimuli, to figure out what the Venn diagrams are meant to communicate, and to associate the pictures with specific articles.

We appreciate this input, and have modified the figure to be more accessible. We consider the Venn diagram useful for illustrating how multiple stimuli have been investigated and their relative importance separated. We considered including this information in a table, but we found that it was overwhelmed with information and even more challenging to interpret. However, we agreed that the figure would be made more accessible to readers if it was clearer what the letters conveyed, so we have added small icons indicating the different stimuli from Figure 1 next to the relevant letters in Figure 2. We additionally indicated the number of stimuli used in each study to make it more obvious that each circle represents a stimulus.

Essentially, we aim to articulate that there are numerous studies that have taken advantage of the level of refinement associated with simulated herbivory that is not afforded when using live insects. In other words, all of the subtleties in plant response between each of the various combinations of stimuli presented in figure 2 can only be elucidated using simulations, as they would all blend together if live insects were used. This is briefly explained between lines 166-170 in the main text. Including the article references allows for the reader to investigate the precise nature of their experimental conditions and findings.

Text Box: Outstanding Questions

I am not convinced of the effectiveness of this text box. I do think that it is important and useful to include such information in this review. However, I think that these points should be developed thoroughly in the main text. If my suggested organization scheme is followed, I think that these points will be covered near the end of the main text. A separate box to summarize the outstanding questions could still be used. However, it would be more effective if it were more concise. For instance, each bullet point could just contain a question. In the current form, each bullet point contains a question and several lines of explanatory text. If the explanations go into the main text instead, then a box of the questions, summarizing them succinctly, could still be an effective addition.

Outstanding Questions Response: This text box has been included as it is a formal requirement from TREE. Although components of this section are mentioned in the text, the objective of this text box is to provide motivation and probing questions for future research that are beyond the scope of this review.

Text Box: Highlights

In the first bullet point, consider something more descriptive than the phrase "basis of plant-herbivore ecology." (One could argue that there is more to the "basis" of plant-herbivore ecology than chemical signals that underpin plant defences.) Some information in the second and third bullet points is redundant, thus obfuscating the different messages you are trying to communicate with these two separate text blocks. I would suggest simplifying the second bullet point to simply list the different ways signaling pathways are activated by herbivory. The point about how they are challenging to differentiate using conventional feeding is basically the same point that the third bullet makes.

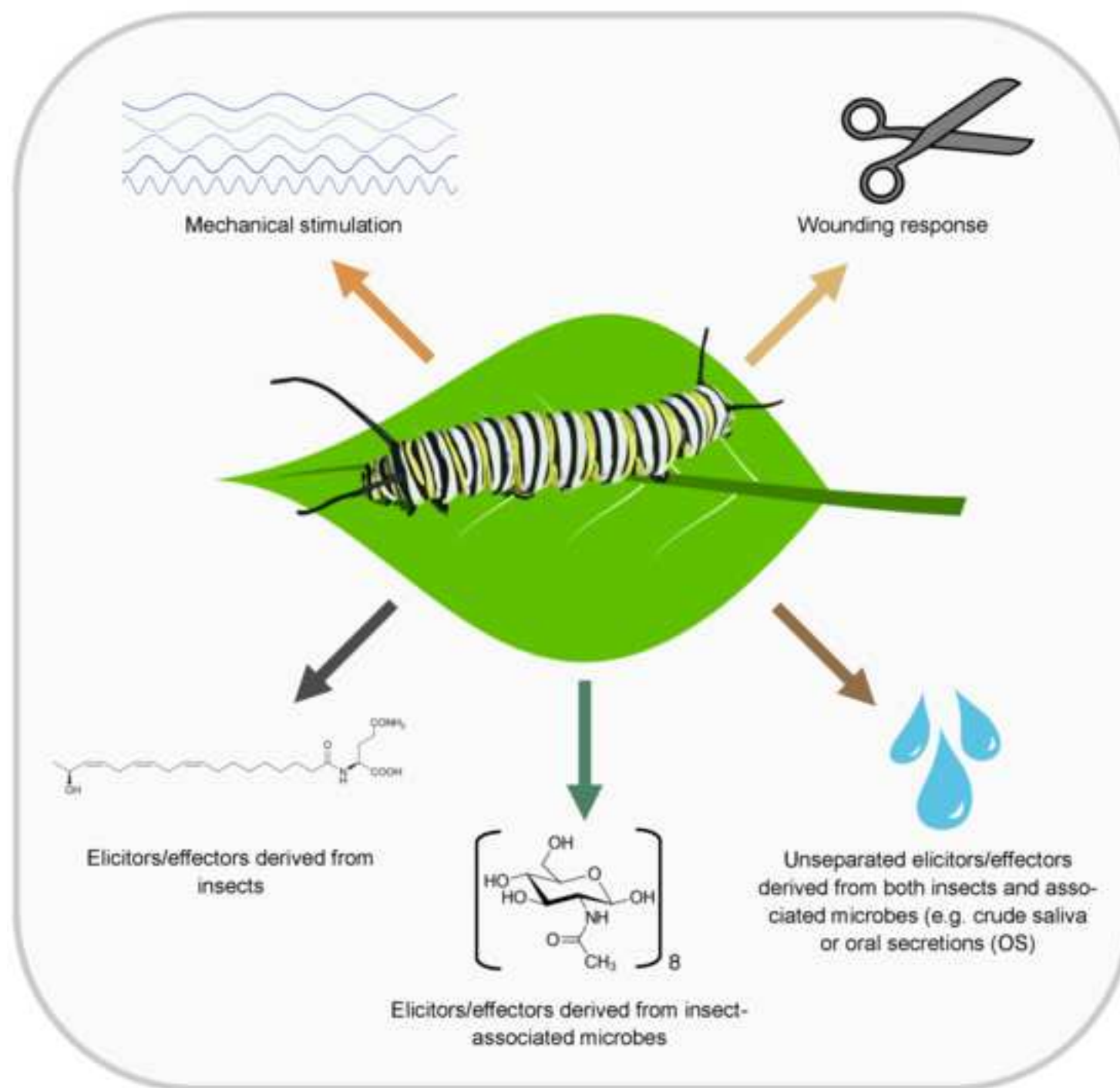
Highlights Response: We agree with all of the suggested changes (see revised highlights attached)

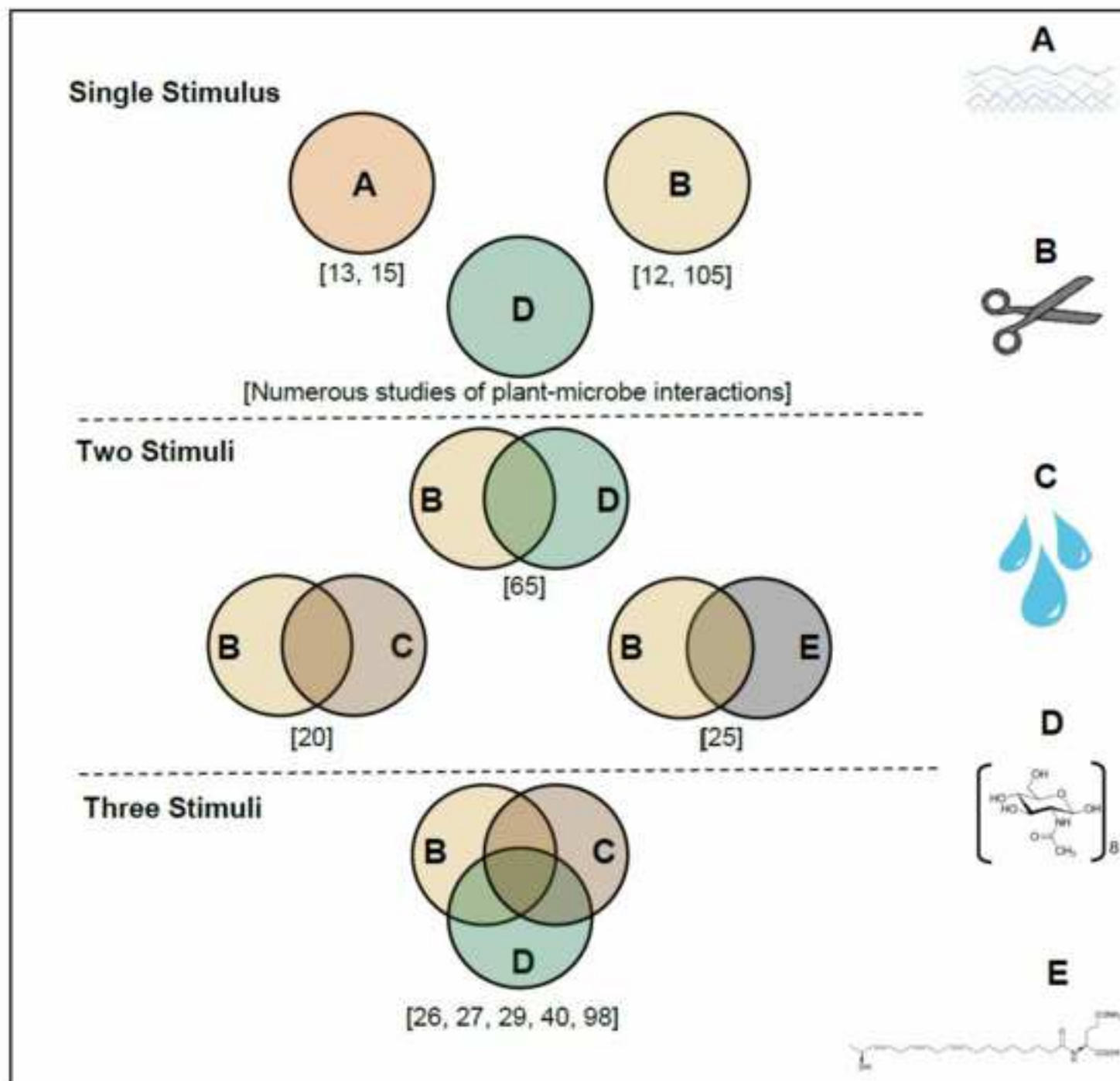
Outstanding Questions

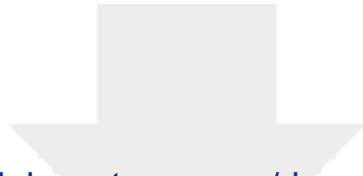
- Researchers usually aim to replicate the total amount of damage inflicted by an herbivore during a bout of feeding. The signalling events that result from this, however, are likely to vary between damage induced suddenly and damage inflicted continuously (i.e. over time). How can we reproducibly optimise the timing of herbivore simulations?
- Can we accurately simulate herbivory for non-chewing herbivores (e.g. phloem feeders)? This is a major knowledge gap given that this feeding guild contains many detrimental global pests and keystone organisms that have mutualisms with other taxa.
- How will environmental change affect insect feeding behaviour? Elevated atmospheric CO₂, for example, often results in metabolic changes within the plant and thus indirectly in compensatory feeding and increased damage. How does this relate to individual and collective defence responses?
- Gene editing techniques (e.g. CRISPR-Cas9) and viral vectors provide cutting edge technologies to control gene expression systemically and untangle plant defence responses. How will the utilisation of these technologies facilitate a greater understanding of the molecular mechanisms associated with plants, microbes, and insects during herbivory?
- Can we breed plants to be more resistant when we have a limited understanding of their defence responses to different components of herbivory? If, for example, the use of simulated herbivory can disentangle the responses to wounding and herbivore-associated microbes, and shows that one contributes a disproportionately larger induction of defence mechanisms or reduction in yield, that information can be used for informing both ecological management and sustainable agriculture.

Highlights

- Ground-breaking research into the chemical and biochemical signals of plant defences has dramatically increased our capacity to understand many of the details that underpin plant-herbivore ecology.
- Herbivore-associated microbes, chemical elicitation, and mechanical stimulation are all known to activate diverse signalling pathways.
- True herbivory can be useful to obtain information on the collective plant response, but it cannot disassociate mechanistic responses of specific defence pathways triggered by the different stimuli associated with herbivore feeding.
- Simulating the chemical and physical factors associated with herbivory in isolation will allow us to disassemble plant defence responses and understand which stimuli are associated with a given defence response.







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Figure360

Waterman_Figure360_2019.mov

