Chapter 15

Methods for Replicating Leaf Vibrations Induced by Insect Herbivores

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Abstract

Testing plant responses to natural sources of mechanical vibration requires methods that can precisely reproduce complex vibrational stimuli. Here we describe a method for conducting high-fidelity vibrational playbacks using consumer audio equipment and custom-written signal processing software.

Key words Plant mechanosensing, Plant–insect interactions, Vibrational communication, Herbivory

1 Introduction

There is great current interest in the role of acoustic information in plant–environment interactions. Recent trends in the field include a focus on sources of acoustic energy relevant to plants in natural environments [1–8], study of the sensory receptors involved in responses to acoustic stimuli and of downstream responses to activation of these receptors [9–18], the agricultural use of sound to influence plant growth and development [16–19], and adoption of experimental methods from the study of plant-borne vibrational communication in animals [5, 20, 21]. The terminology in this literature is in flux. The term "plant acoustics" has often been used, though "acoustic" typically refers to airborne sound and its detection by sensory structures that have evolved to detect pressure waves. Plants lack specialized sound-detecting structures, and although airborne sound does induce mechanical vibrations in plant tissues [22], we are unaware of any published work on whether natural sources of airborne sound are relevant to plants. Some authors have used the phrase "sound vibration" to draw attention to the function of plant mechanoreceptors in sensing vibrations in plant tissue [13, 14, 18], and the term "biotremology" has been proposed to encompass study of the biological role of any form of mechanical wave [23]. The study of plant responses to acoustic or

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vibrational stimuli is also closely related to the study of plant responses to wind or touch, or "thigmomorphogenesis" [11]. In this chapter we follow recent literature, using "acoustic" as a general reference to information carried by some form of mechanical wave, and "vibration" to refer to the stimuli perceived by plants.

The activity of herbivores, whether feeding, moving, or signaling, is one of the most prevalent biotic sources of mechanical vibrations in plants in nature [20]. The importance of herbivore-induced vibrations in insect–plant interactions is highlighted by the finding that the feeding vibrations of herbivores can cause priming of plant defenses [5]. Other types of ecological interactions may also involve plant responses to mechanical vibrations, and a host of questions remain to be addressed about how plants perceive herbivore vibrations, distinguish them from nonrelevant vibrations, and respond both locally and systemically. To address questions about the influence of vibrations produced by herbivores or other natural sources, it is necessary to be able to measure and experimentally reproduce the mechanical vibrations of plant tissues. This vibration-measurement-and-reproduction step has been rate-limiting for the study of vibration-mediated interactions between plants and their environment, given the need for specialized and expensive instruments. Airborne sound has often been used as a stimulus, but although it does induce vibrations in plants, plant structure greatly influences the properties of those vibrations, yielding an uncertain relationship between the stimulus that is produced by the experimenter and the one that is experienced by the plant (*see* below).

Our laboratories are developing vibrational playback methods based on relatively inexpensive, off-the-shelf consumer equipment. Vibration recording and measurement is a broad topic that is outside the scope of this chapter, and here we only discuss those sensors and procedures that are necessary for properly calibrating vibrational playbacks. Our focus is on experimentally testing plant responses to mechanical vibrations, while ensuring that the stimuli experienced by the test plants have the desired properties. Although many of the issues that arise when conducting vibrational playbacks on plants have been discussed elsewhere [20], the high throughput required for testing plant responses requires carefully calibrated vibrational stimuli on multiple plants at the same time. Here we describe a method that achieves calibrated, multichannel vibrational playbacks using a combination of signal processing software and consumer audio equipment.

2 Materials

Overview: The basic setup described here (Fig. 1a) for playing back herbivore vibrations to plants consists of a computer with signal processing software; an audio interface device; an amplifier; a set of

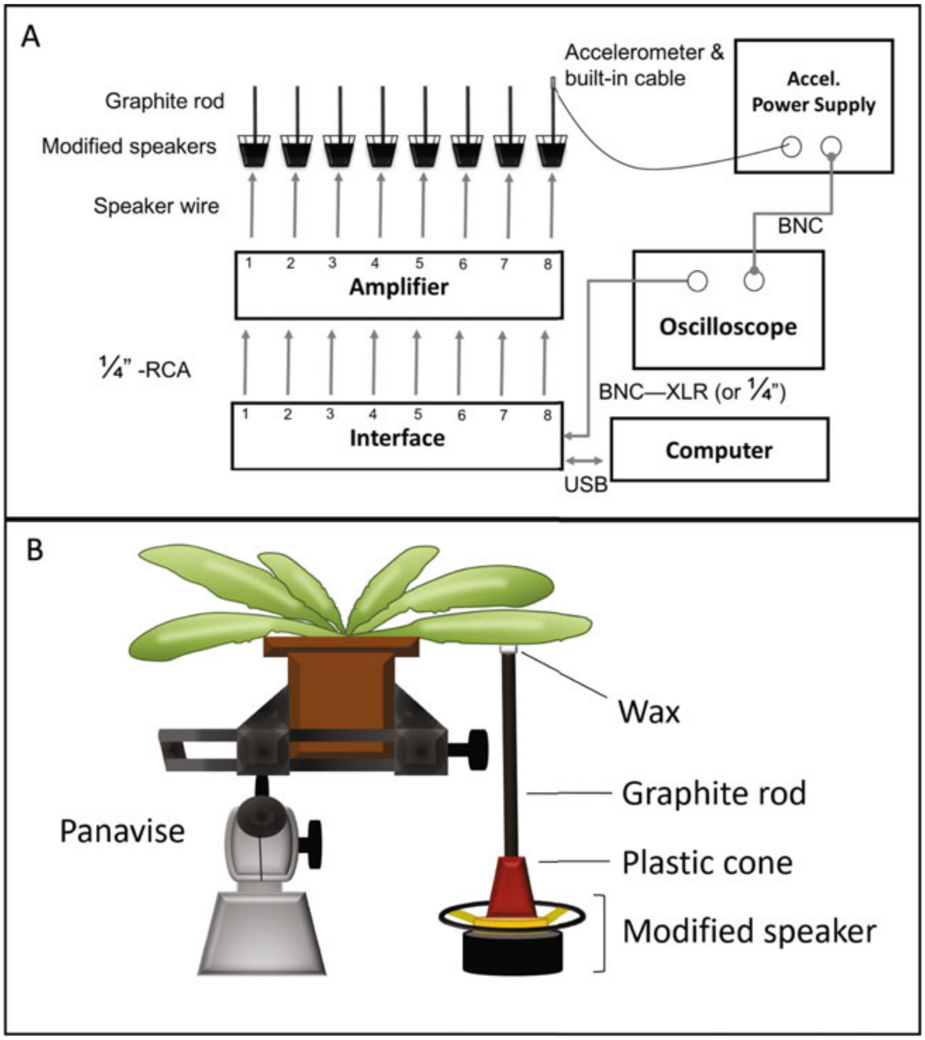


Fig. 1 The experimental setup: (**a**) layout; (**b**) diagram of method of attachment of a plant leaf to the vibration playback device

vibration transducers; a vibration sensor; an oscilloscope; cables for connecting the equipment; vibration isolation; and a playback device and a method for connecting it to the plant (Fig. 1b).

1. Computer with appropriate hardware drivers installed (*see* **Note 1**).
2. Software:
   (a) For signal processing: Matlab (Mathworks, Inc.) including the Signal Processing toolbox (*see* **Note 2**). A custom-written script for prefiltering the playback stimuli and adjusting their amplitude is the centerpiece of high-fidelity vibrational playbacks. We guide the user through a custom-written Matlab script with a graphical user interface to be used for vibrational playbacks. The script can be downloaded at the following web address: https://greenvibes.missouri.edu/vibe-ware/.

   (b) For recording and editing signals: Audacity (https://www.audacityteam.org/), a user-friendly free shareware program.
   (c) For playing back stimuli: a digital audio workstation for playing back stimuli on multiple channels simultaneously (*see* **Note 3**).

3. Multichannel audio interface (*see* **Note 4**).
4. Vibration transducers for playback: a small 4- or 8-ohm speaker (*see* **Note 5**) with suitable modification as described in **Note 6**.
5. Amplifier: a multichannel amplifier for driving the vibration playback devices to deliver vibrational stimuli to several plants at a time (*see* **Note 7**).
6. Small, calibrated vibration sensor such as a miniature accelerometer (*see* **Note 8**).
7. Vibration isolation (*see* **Note 9**).
8. Oscilloscope: a digital or analog oscilloscope (*see* **Note 10**).
9. Cables: for each channel in the setup below, use one ¼″ (6.35 mm) male to RCA male mono cable for connecting the interface outputs to the amplifier input; a length of speaker wire, 5–10′ (1.5–3 m) depending on the configuration of the lab space, for connecting each amplifier output to the corresponding speaker; one BNC female—BNC female cable for connecting the accelerometer power supply output to the oscilloscope input via one BNC T-connector; and one BNC female to ¼″ (6.35 mm) male mono cable for connecting the oscilloscope via the T-connector to the input channel of the interface.
10. Adhesive: accelerometer mounting wax, soft dental wax, or other nonpermanent adhesive to couple the playback device and the vibration sensor to the playback device and/or plant (Fig. 2; *see* **Note 11**).
11. A clamp for holding and positioning the plant pot (*see* **Note 12**).

3 Methods

The two basic challenges in conducting vibrational playbacks are (1) to compensate for the filtering effect of the playback device and substrate and (2) to play back the stimulus at an appropriate amplitude [20]. When these issues are addressed using appropriate hardware and signal processing software, any of a wide variety of vibration playback devices can produce high-fidelity results like those shown in Fig. 3a, b. If these issues are not addressed, then regardless of the vibration playback device used, the stimulus delivered to the plant will depart in unknown ways from the stimulus the

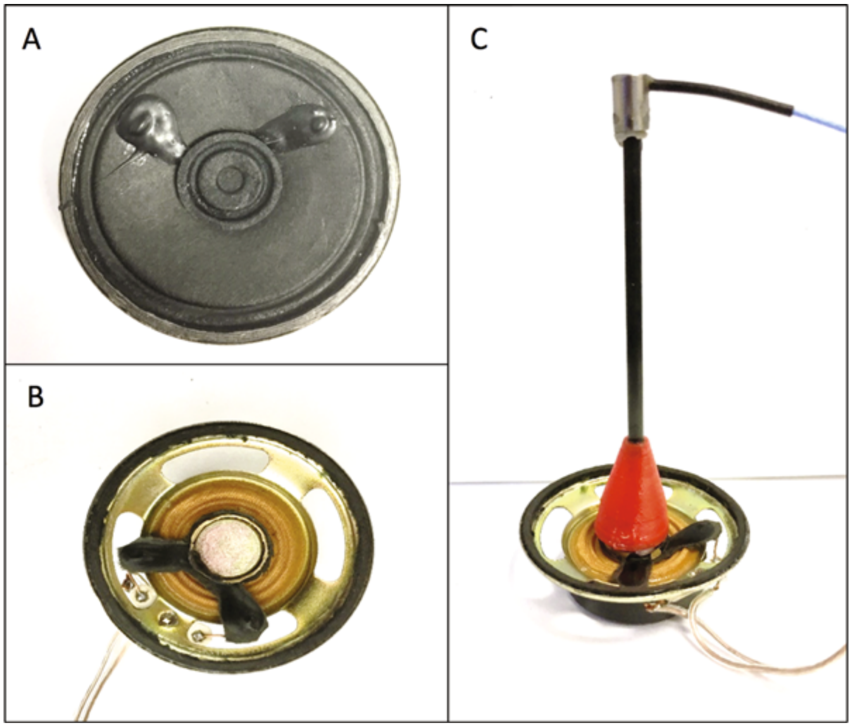


Fig. 2 Modifying a small audio speaker to make it a suitable vibrational playback device. (**a**) The speaker before modification; (**b**) the speaker with the paper membrane removed; (**c**) the speaker with a graphite rod added and an accelerometer attached

experimenter intended to deliver. Likewise, filtering by plant structures will markedly change the spectrum of stimuli played back as airborne sound (Fig. 3c). Here we assume that the investigator plans to use natural stimuli with a broad frequency range, such as excerpts from vibration recordings of herbivore activity on plants. However, the methods described here are equally suitable for other stimuli including those generated using software.

3.1 Input Calibration

1. Before calibrating the input gain, ensure that the computer is communicating properly with the interface. Check all connections and switches; make sure the computer is set to record and play back through the desired device; and check the sound card settings on your computer (*see* **Note 13**).
2. Calibrate the input channel gain on the interface to a level of 1.0 to adjust the amplitude of a playback. Perform this procedure initially; periodically check; and re-do when using a different sensor or if the gain control setting is incidentally changed.
   (a) For the speaker–accelerometer combination, attach the accelerometer to the vertical rod on the modified speaker using wax (Fig. 2c) when calibrating the playback device (*see* **Note 14**).

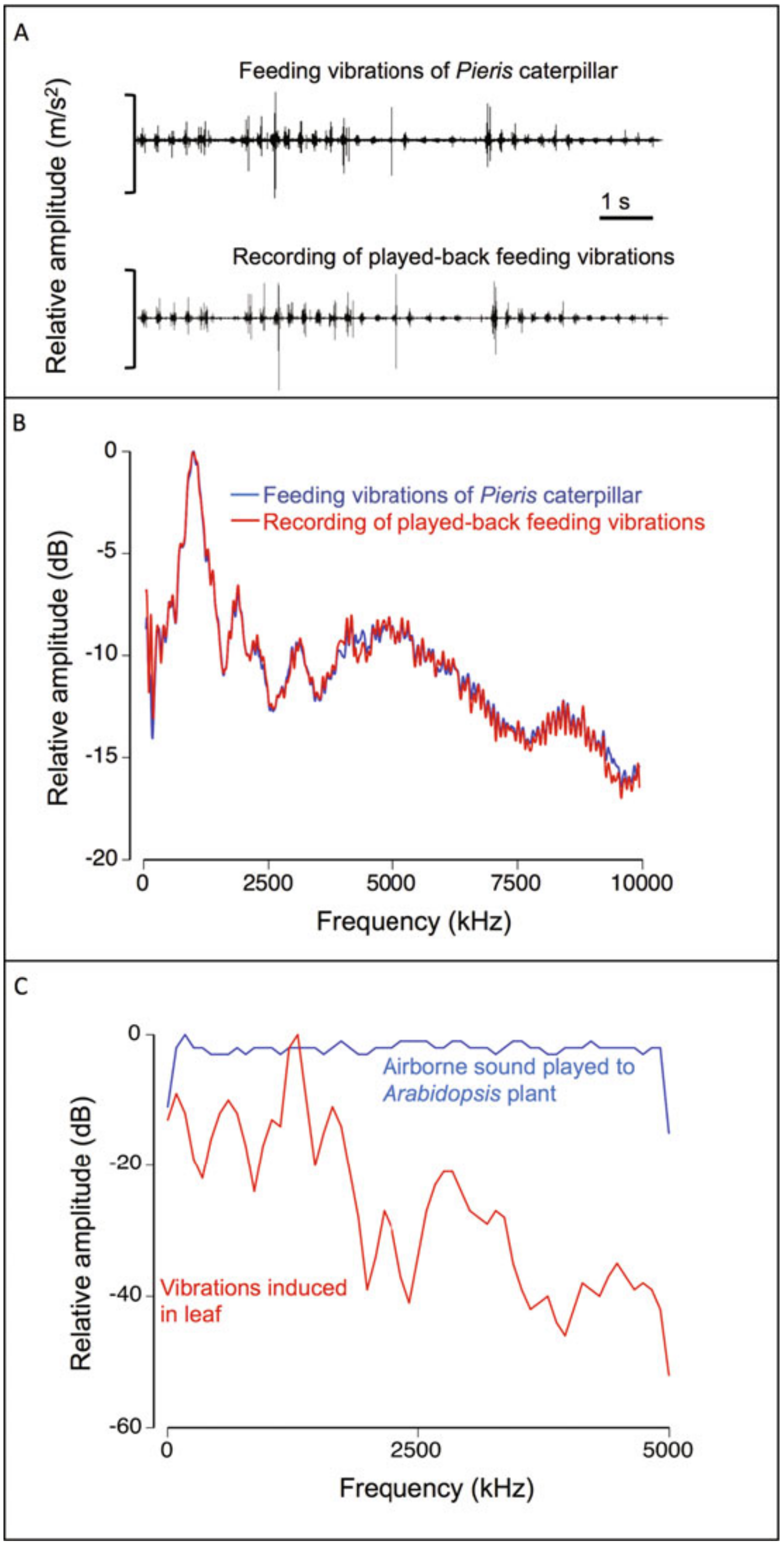
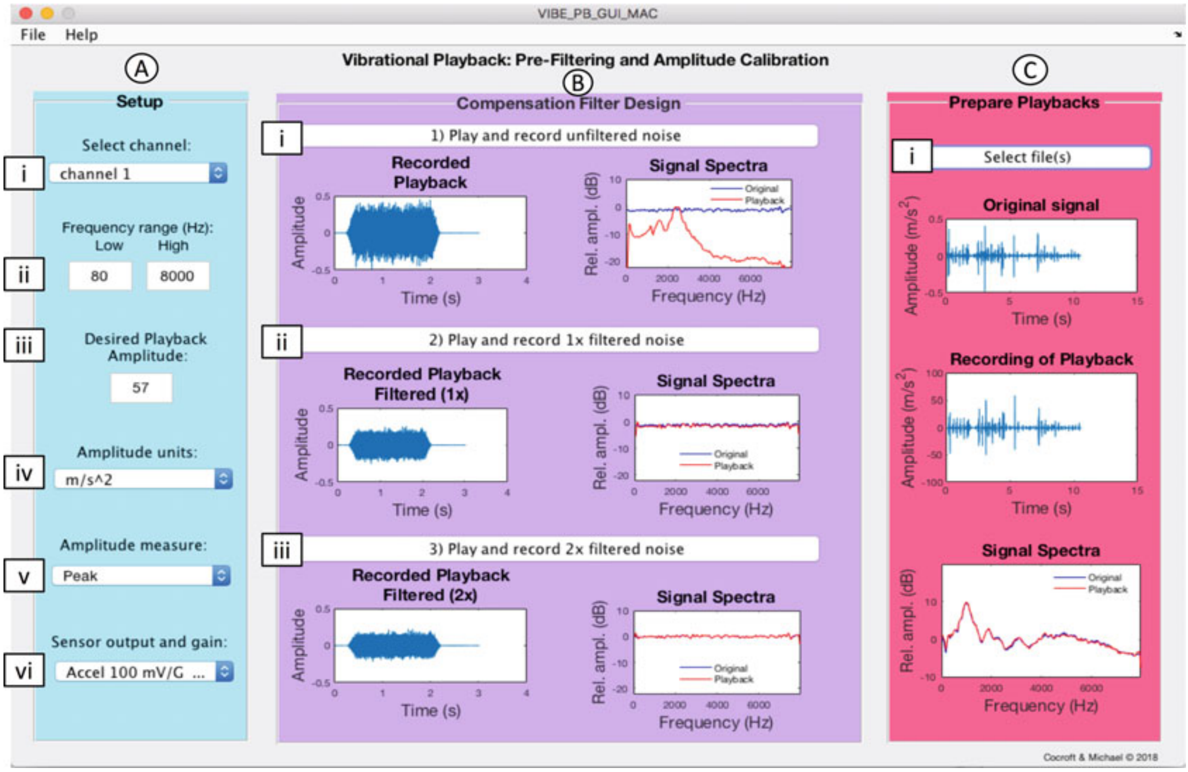


Fig. 3 Comparison of a recording of vibrations produced by a feeding caterpillar, and a recording of the played-back vibrations using the modified speaker shown in Fig. 2b, c and the procedure shown in Fig. 4. (**a**) Waveforms of the original recording (after conversion to acceleration; *see* **Note 17**) and the recorded playback (duration of waveforms = 10 s). (**b**) Frequency-vs.-amplitude spectra



i Select channel: channel 1

ii Frequency range (Hz): Low 80 High 8000

iii Desired Playback Amplitude: 57

iv Amplitude units: m/s^2

v Amplitude measure: Peak

vi Sensor output and gain: Accel 100 mV/G ...

Fig. 4 The graphical user interface (GUI) created in Matlab for prefiltering vibrational playback stimuli and adjusting their amplitude. The initial settings can be adjusted in panel **a**. The system filter is calculated in panel **b**. The calculated system filter is applied to the experimental playback stimuli and the amplitude is also adjusted and applied in panel **c**. *See* Subheading 3.3 for detailed instructions

To calibrate the audio interface input gain for channel 1, generate a pure-tone stimulus of several hundred Hz (this can be done in Audacity), and loop it to play continuously. To play the tone while recording it, in Audacity select Preferences → Recording and check "Overdub: play other tracks while recording new one." Then adjust the gain for input channel 1 until the amplitude of the tone in Audacity matches that on the oscilloscope. Note that the scale in Audacity is +/− 1 V, so that if the oscilloscope shows a peak amplitude of 300 mV, adjust the input gain until the peak amplitude in Audacity is 0.3.

Fig. 3 (continued) of the same two signals, showing the close match of the recorded playback and the original recording. (**c**) Amplitude spectra of airborne sound (a noise burst) played from a speaker 20 cm from an *Arabidopsis thaliana* plant, and of the induced vibrations measured on one of the leaves using a laser vibrometer, showing the marked differences between the airborne sound and the leaf vibrations

3.2 Output Calibration

1. Set the output gain on the amplifier and interface. Start with a low gain setting on the amplifier, about ¼ of the maximum on each amplifier channel that will be used (*see* **Note 15**).
2. Once the input gain is calibrated, use the provided Matlab script (*see* Subheading 2, **step a**) to prefilter the playback stimuli to compensate for the frequency response of the playback device and substrate, and to adjust the amplitude of the playbacks (Fig. 4). Run the procedure for each channel used in the experiment (*see* **Note 16**).

3.3 Playback Stimulus Prefiltering and Amplitude Adjustment

1. Use the custom software (*see* Subheading 2, **step a**) to measure and compensate for the frequency response of the playback device and match the amplitude of the playback stimuli to that of the original stimuli. It is necessary to match the average peak or RMS amplitude of those stimuli, or to match other amplitude levels called for by the experimental design (*see* **Note 17**). In this case, this step was done *before* the leaf is attached (*see* **Note 18**).
2. Ensure that the following files are in the same folder: For example, VIBE_PB_GUI_PC.m, VIBE_PB_GUI_PC.fig, and all stimulus files in .wav format.
3. Choose the channel corresponding to the playback device for which stimuli are currently being prepared, and to which the accelerometer is attached (Fig. 4a(i)).
4. Enter a frequency range that slightly exceeds the relevant frequency range of the stimuli (Fig. 4a(ii)) (*see* **Note 19**).
5. Enter the desired amplitude level for the playbacks (Fig. 4a(iii)) (*see* **Note 17**).
6. Enter the appropriate amplitude units. For an accelerometer, set to m/s^2 (Fig. 4a(iv)).
7. Select the type of amplitude measurement, peak (used for most stimuli) or RMS (Fig. 4a(v)) (*see* **Note 17**).
8. Select the type of sensor used for calibrating the playbacks and the gain used (Fig. 4a(vi)).
9. If desired, save the above settings for future use using the File menu (top left, just below the title).
10. Click on “Play and record original signal”(Fig. 4(i)). The program will generate a short burst of random noise and play it through the playback device, while recording from the accelerometer. After a few seconds, the waveform of the recorded signal should appear, along with the amplitude spectra of the random noise and the recorded signal. If there is clipping of the recorded signal, the program will lower the amplitude and play the signal again until the signal is not clipping. After the waveform and spectra appear, and assuming the recorded signal

looks like the one shown in Fig. 4, proceed to the next step. If the signal does not appear to be correct refer to **Note 20**.

11. Click on "Play and record 1× filtered signal" (Fig. 4b(ii)). The program will play back the original noise signal modified by the calculated filter. The waveform and spectra will appear, and the spectrum of the recorded signal should be closer to that of the original playback. It may not match the original very closely, however; especially for frequencies that are strongly attenuated by the playback system, the calculated system filter will be imprecise.
12. Click on "Play and record 2× filtered signal" (Fig. 4b(iii)). The spectra of the recording and the original playback should now be very similar, because the original noise has been modified to compensate for the system filter (e.g., the amplitude of frequencies attenuated by the playback system will have been correspondingly increased). If the played-back signal does not appear to be correctly filtered, refer to **Note 20.**
13. Click on "select file(s)" (Fig. 4c(i)); a browser window will open and the stimuli to be played from the current channel can be selected. Once a stimulus has been filtered and its amplitude adjusted (the default is to within +/− 1 dB across the desired frequency range), it will be saved and the waveform and spectra displayed; if there are additional stimuli to be filtered, it will move onto the next after a few seconds. If some of the playback stimuli adjusted for the current channel will be played back at different amplitudes, click on "select file(s)" again after changing the amplitude value (*see* Fig. 4a(iii)).
14. Once all of the stimuli for the current channel have been saved, move the accelerometer to the next playback device or plant. Plug the Channel 1 output (from interface device) to the next channel on the amplifier, and select the corresponding channel (*see* Fig. 4a(i)), which will cause a channel identifier to be added to each filtered file (*see* **Note 21**).

3.4 Attaching Playback Device to Plant

1. Once all of the playback stimuli have been filtered and amplitude-adjusted for each channel, attach plants to the playback devices using wax, either accelerometer mounting wax or dental wax, the best method tested thus far for coupling the playback device to the plant. Take care when attaching the leaf to the playback device and (especially) when detaching it at the end of the playback, to avoid injury to the leaf (*see* **Note 22**). Because this method involves physical contact with the plant, include a control treatment in which leaves are likewise attached to silent playback devices or a sham.
2. To position the plant to allow contact with the playback device, use a clamp (Fig. 1b) to hold an empty plant pot and then insert the pot containing the playback plant into the empty pot.

3. Use the clamp to adjust the height and orientation of the plant and to position the target leaf. Position the end of the dowel so that it is lightly touching the target leaf (*see* **Note 23**). If the leaf needs to be bent toward the dowel, the force exerted by the leaf petiole will tend to detach the leaf from the wax.
4. Once the leaf is in position, apply gentle pressure for 2–3 s from the opposite side of the leaf using a cotton swab, so that the leaf is lightly but firmly adhered to the wax to achieve a nondestructive attachment. Test the connection by gently lifting the edge of the leaf 1–2 mm from below.

3.5 Creating Playback Files

1. To deliver the same stimulus at regular intervals:
    - (a) Open wav file containing the filtered and adjusted playback stimulus.
    - (b) Select "Generate" and then "Silence."
    - (c) Choose the desired time of silence needed before and after the playback stimulus.
    - (d) Use loop play in Reaper to generate a continuous playback track (*see* below).
2. To create a playback file in Audacity with multiple filtered and adjusted playback stimuli and various silent gaps:
    - (a) Follow the steps above in Subheading 3.5, **step 1** to add silence before and after stimulus. Use "Loop play" in Reaper to generate a continuous playback track (*see* below).
    - (b) If using multiple stimuli in one playback channel, simply copy one stimulus exemplar from the filtered/adjusted file. To paste, first have the Audacity file to be used for the playback open, and zoom in on the waveform until the individual samples are visible. Paste the stimulus where the individual sample is at zero.
3. Load the adjusted playback stimuli for playing back to the plants into Reaper:
    - (a) Insert a separate track (Select "Track" and then "Insert new track") for each playback channel.
    - (b) Load the playback files one at a time (and only one per track) by selecting "Insert" and then "Insert Media File."
    - (c) Select the "Route" icon on each track and deselect the "Master Send" option, then select the output channel for each track. For example, the first track should be sent to "Output Channel 1"; the second to "Output Channel 2"; etc.

4 Notes

1. Apple computers usually allow trouble-free connection to commercial audio devices. ASUS and other PC computers are also suitable with the appropriate drivers.
2. We use Matlab for methods described in this chapter, but there is a free shareware program, GNU Octave (https://www.gnu.org/software/octave/), which mimics Matlab, including the signal processing toolbox. However, we have not tested this Matlab script in Octave.
3. For playing back stimuli: a digital audio workstation for playing back stimuli on multiple channels simultaneously, such as Reaper (which offers an inexpensive education license and was used in this chapter), ProTools, or Cubase.
4. An audio interface functions as an external sound card for the computer and provides multichannel input and output. In this chapter we used the Tascam US 20×20.
5. Virtually any small 4- or 8-ohm speaker can be used to produce high-fidelity vibrational playbacks, with the proper modifications and signal processing. The procedures described in the chapter are based on an inexpensive 8-ohm speaker.
6. We used small audio speakers, modified to reduce the emission of airborne sound and to allow coupling of the playback signal to the plant (Figs. 1b and 2). When using a speaker as a playback device, first remove the paper membrane to reduce the production of airborne sound. Then attach a graphite rod (or a wooden dowel) to the speaker to allow coupling between the moving speaker coil and the plant (Fig. 2b). Some airborne sound will still be produced by the speaker, but the vibrations transmitted to the plant via the rod will be substantially higher in amplitude than any vibrations induced in the plant by the airborne sound.
7. An ever-changing array of makes and models of amplifiers is available commercially, typically with a flat frequency range of 20–20,000 Hz (and usually the ability to amplify signals with frequencies outside this range). The procedures described in the chapter use a Behringer HA8000 amplifier, which is used primarily for headphones but is also capable of driving 8 ohm speakers.
8. The basic requirements for a vibration sensor to be used in the filtering and amplitude adjustment steps are that the vibration sensor is calibrated (i.e., the relationship between vibration amplitude and sensor output has been measured); and that it is small, to minimize mass loading of the structure it is attached to, because mass loading changes a structure's vibration-

transmitting properties. We suggest using a miniature accelerometer with an output of 100 mV/G and a frequency range from <10 Hz to 15 or 20 kHz, such as the VibraMetrics model 9002A accelerometer and P5000 power supply (or, with a less broad frequency range, the PCB 352A24 accelerometer and 480E09 signal conditioner). If a laser vibrometer is available, it is an ideal sensor for recording and for playback calibration, but laser vibrometers are expensive and are not essential for calibrating playbacks. However, a laser may be required for recording herbivore vibrations from small plant structures such as an *Arabidopsis* leaf, since attaching a contact sensor such as an accelerometer will greatly alter the vibration of the leaf.

9. Some degree of isolation is needed to reduce the influence of the unwanted vibrations present in buildings. The best isolation is achieved using a large vibration table with active damping, but the minimum requirement of a heavy mass resting on a springy material can be met using a steel plate or concrete block resting on a partially inflated bicycle inner tube.
10. An oscilloscope is needed for calibrating the gain on the input channels on the audio interface. For procedures described in this chapter, we used a Tenma 72-2580 digital oscilloscope.
11. Accelerometer mounting wax is useful, but dental wax is universally available and, if it is non-scented and sufficiently soft and tacky, is a suitable alternative. Other methods of coupling the playback device to the plant are currently under development.
12. We used the Panavise 201 PV Jr. (Panavise, Reno, NV, USA) for positioning plants.
13. If using a PC and TASCAM, ensure that "TASCAM 20X20" is selected for the input and output device for use in Audacity and "TASCAM 20X20 Mixer" is selected for input and output device in the sound settings.
14. The mass of the sensor used for calibrating the playback device can potentially change the device's frequency response. This issue is moot for laser vibrometers, but empirical testing is required to determine the effect of attaching an accelerometer on the playback device's frequency response. For the speaker–accelerometer combination we describe below, measurements with a laser vibrometer confirm that the presence of the VibraMetrics accelerometer leaves the frequency response intact to within an average of 2 dB across the relevant frequency range. To calibrate the playback device, the accelerometer should be attached to the vertical rod on the modified speaker using wax (Fig. 2c). Here we assume that the plant structure receiving the playback is sufficiently small and light that attaching it to the playback device will not change the

frequency response of the device, i.e., the relative amplitude of different frequencies in the output signal, compared to those in the input signal. If the frequency response is flat, the relative amplitude of different frequencies in the signal will be the same in the input and output). Our measurements confirm that the output of the modified speakers described below is minimally affected by attaching an *Arabidopsis* leaf. We note that if there are enough vibration sensors available to use one per channel and leave the sensor in place during the experiment, changes in the output of the playback system due to attachment or detachment of a sensor can be avoided entirely. Assessing whether attaching the accelerometer influences the frequency response of the playback system: If a laser vibrometer is available, then a few seconds of noise (which can be generated in Audacity) can be played through the playback device with and without the accelerometer attached, and recorded with the laser from the top of the dowel or the top of the attached accelerometer. The spectra can be visually compared in Audacity or quantitatively compared in Matlab. Note that the accelerometer cable should be supported 10–15 cm from the accelerometer, to reduce the influence of the cable on the playback device.

15. For the device used in the setup here (The Tascam US20x20), there is a gain control for channels 1–2, but not for channels 3–10. The gain control for the first two channels [“LINE OUT 1–2”] reduces the amplitude below that of the other channels. This gain knob should be turned all the way to the right (maximum gain) to make the output gain of channels 1 and 2 equal to that of channels 3–10.

16. Rationale: The Matlab script characterizes the frequency response of the playback system by sending a noise signal through the system, then comparing the recorded output to the input signal. The amplitude-vs.-frequency spectrum reveals which frequencies are over- and under-represented when the stimulus is played through the playback device. The frequency response of the playback system is then inverted and used to design a digital filter that compensates for that frequency response. When the digital filter is applied to the original noise stimulus, the signal recorded by the vibration sensor will now have a flat amplitude spectrum without over- or under-represented frequencies. The resulting digital filter will then be used to filter the playback stimuli, to ensure that each stimulus has the desired amplitude spectrum (Fig. 3b). The result of running this script for each stimulus for each channel will be a set of playback-ready audio files, prefiltered to compensate for the frequency response of the system and adjusted to the desired amplitude. Because inexpensive vibration playback devices (such as modified speakers) often differ from each

other in the details of their frequency response characteristics, delivering a uniform stimulus to a set of plants requires generating calibrated stimuli for each individual playback device. Because we have found that it is not always possible to configure Matlab to communicate with sufficient output channels, the Matlab GUI we provide here simply generates a set of playback-ready files, and is designed to play and record to only one channel at a time; the multichannel playback is done by loading those files into another program.

17. For most stimuli the "Peak" measurement is appropriate; for constant-amplitude stimuli such as pure tones or noise without silent gaps the "RMS" measurement is also appropriate. If both the original recordings from which the playbacks are drawn, and the playback calibrations, are done with the same sensor and the same sensitivity/gain settings, it is straightforward to match the peak of the playback stimulus to that of the recording, or to another desired amplitude. However, if different sensors have been used for recording the original stimulus and for calibrating the playback device, achieving the correct amplitude requires careful accounting of units. For example, if a stimulus is recorded with a laser vibrometer but the calibration process is done with an accelerometer, the output of the two sensors is initially not comparable because the laser measures velocity while the accelerometer measures acceleration. Furthermore, each type of sensor usually has multiple settings that control its input sensitivity and/or output gain. We provide a second Matlab script for converting a velocity recording to acceleration, taking into account the sensors' sensitivity and the output gain (https://greenvibes.missouri.edu/**vibe-ware/**). The result is a proxy for the recording the accelerometer would have made, given the motion recorded by the laser. This procedure will allow one to calibrate playbacks with an accelerometer, when the original recordings were done with a laser.

18. In this case, it is appropriate to measure the frequency response of the device and calibrate the amplitude of the playback stimuli *before* the leaf is attached; this has the advantage that the experimental subjects experience stimuli only during the experiment, and not during calibration. For larger structures such as woody stems or heavier leaves, it will be necessary to do the prefiltering and amplitude calibration *after* the plant is attached. When the need arises to measure the frequency response of playback device and plant structure after they are connected, this creates a nuisance variable that must be controlled for. In particular, the plant will experience a series of brief vibrational stimuli during the filtering and calibrating process, and thus the procedure should be done with both experimental and control plants so that the effect of the

vibrations delivered during the calibration process is constant across plants.

19. The low and high values entered by the user will determine the frequency range over which the stimulus will be matched to the original file. There are disadvantages of entering a wider range than necessary. For example, entering a high frequency of 22k Hz when the highest frequency in the stimulus is 5 kHz may reduce the quality of the end result. For most playback devices, achieving a flat frequency response up to 22 kHz will require dropping the amplitude of the lower-frequency components of the signal, and boosting the higher frequencies; if there is no signal energy in those frequencies, this will simply increase the representation of any high-frequency noise present in the original recording. Entering a low-frequency value of 1 Hz, when the lowest frequency in the signal is 100 Hz, also is problematic; any low-frequency noise will be amplified, and low-frequency building vibrations that are not sufficiently attenuated by the vibration isolation used will interfere with the estimate of the playback signal amplitude at those frequencies. In the example illustrated, the range chosen is 80–10,000 Hz; the low value is above the low-frequency noise but below what appears to be the lowest frequency in the stimulus, and the high value is at the point where the stimulus amplitude at those frequencies is 30 dB or more below the peak amplitude.

20. If the waveform does not resemble the one shown, it is possible that either the computer is not playing back through the interface, not recording from the interface, or both. First, check all connections. If Matlab appears to be recording but not playing back it could be that (1) the playback device is not working correctly (check by playing back a tone through Audacity), (2) the computer is playing back through the computer's output (check sound preferences), or (3) output gain on amplifier is too low. If Matlab appears to be playing back but not recording, it could be that (1) the recording device is not functioning properly; (2) the computer is recording through the microphone input (check sound settings) rather than through the interface; or (3) the input gain is too low or is not correctly calibrated. If the two spectra are not closely matched, one possibility is a transient source of ambient noise that interfered with the recording, in which case running the routine in Fig. 4c once again may resolve the issue. Also, make sure that the accelerometer, or other recording device, is close to the playback device and firmly attached.

21. The custom software will allow you to select a channel in Fig. 4a(i). The channel number will be added as a prefix to the filtered, amplitude-adjusted playback files.

22. Other methods are currently being tested for use on plants for which a wax attachment is unsuitable, such as those with dense glandular trichomes that would likely be damaged when attaching and detaching the leaf from the playback device, or with a waxy cuticle to which the wax does not adhere.
23. In terms of biological relevance, it is reasonable to attach the playback device to the area of the leaf where the herbivore would likely feed.

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