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Chemical Responses of *Nicotiana tabacum* (Solanaceae) Induced by Vibrational Signals of a Generalist Herbivore

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Abstract
Plants are able to sense their environment and respond appropriately to different stimuli. Vibrational signals (VS) are one of the most widespread yet understudied ways of communication between organisms. Recent research into the perception of VS by plants showed that they are ecologically meaningful signals involved in different interactions of plants with biotic and abiotic agents. We studied changes in the concentration of alkaloids in tobacco plants induced by VS produced by *Phthorimaea operculella* (Lepidoptera: Gelechiidae), a generalist caterpillar that naturally feeds on the plant. We measured the concentration of nicotine, nornicotine, anabasine and anatabine in four treatments applied to 11-weeks old tobacco plant: a) Co = undamaged plants, b) Eq = Playback equipment attached to the plant without VS, c) Ca = Plants attacked by *P. operculella* herbivory and d) Pl = playback of VS of *P. operculella* feeding on tobacco. We found that nicotine, the most abundant alkaloid, increased more than 2.6 times in the Ca and Pl treatments as compared with the Co and Eq treatments, which were similar between them. Nornicotine, anabasine and anatabine were mutually correlated and showed similar concentration patterns, being higher in the Eq treatment. Results are discussed in terms of the adaptive significance of plant responses to ecologically important VS stimuli.

Keywords Nicotine · Plant-insect interactions · Tobacco · Plant response · Plant perception · Plant signaling · Herbivore

Introduction

Studies on behavior and memory have traditionally been performed on animals since it has been assumed that such traits are absent in plants (Applewhite 1975; Trewavas 2017; Vertosick 2002). Nevertheless, recent research has shown that plants can sense their biotic and abiotic environments and selectively respond to many types of stimuli. A number of functions have been described in plants which are analogous to those in animals but differ in mechanisms and capabilities (Karban 2015). For example, plants can assess the external environment, deal with predators and diseases, exhibit flexible reproductive strategies, all in the absence of a central nervous system (Trewavas 2017). Legg and Hutter (2007) propose that intelligence in organisms depends on the ability of individuals to adapt to different objectives and environments and allows them to interact with the environment and succeed when exposed to different challenges. Plants can fit with the previous description, since they exhibit phenotypic and molecular responses to environmental signals, and they are able to improve their fitness through behavioral changes which eventually become adaptive strategies during their life cycle (Karban 2015; McNamara and Houston 1996; Trewavas 2017).

Plants may be exposed to a variety of stimuli (e.g. light, chemicals, wind, temperature, sounds) (Karban 2015; Telewsky 2006) and they are able to scan and sense the environment and respond to prevailing conditions (Karban 2015; Mischra et al. 2016). Light sensing involves phytochromes, cryptochromes and phototropins; chemical sensing involves chemoreceptors; mechanical sensing is performed by mechanoreceptors; and it is not yet clear which specific receptors are responsible for temperature sensing (Karban 2015; Wagner et al. 2008). Although mechanisms of sound or vibrational sensing are just starting to be disentangled, a body of knowledge about their perception and the related feedback by plants has been produced (Appel and Cocroft 2014; Braam 2005; Helms et al. 2013; Karban 2015; Smith 2000; Wagner et al. 2008).

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Vibrational signals (VS) constitute one of the oldest ways of communication between organisms, with an estimated number of 230,000 species of invertebrates and many vertebrates using them (Cocroft et al. 2014; Hill and Wessel 2016). In spite of their widespread occurrence, however, VS remain one of the most understudied communication modes, far behind other modalities such as chemical and visual signals (Cocroft et al. 2014). Recent research has made important advances in the perception of VS by plants and their ecological consequences (Appel and Cocroft 2014; Body et al. 2019; De Luca and Vallejo-Marin 2013; Gagliano et al. 2012; Gagliano 2013, Gagliano et al. 2018). When plants perceive VS they can modify or adjust processes such as root growth, seed germination, defense responses, and responses to water scarcity (Appel and Cocroft 2014; Body et al. 2019; Chowdhury et al. 2014; Gagliano 2013; Jeong et al. 2014). Hence, VS have been recognized as meaningful signals involved in the interaction of plants with their environment (Mischra et al. 2016).

In the realm of plant–insect interactions, the chemical arsenal of plants against herbivory has been studied at different levels and scales (Baluška and Ninkovic 2010), including constitutive internal defensive secondary metabolites, chemicals induced by herbivore attack, and organic volatile compounds that repel herbivores, attract natural enemies of herbivores or alert other plant parts and individuals (Kessler and Baldwin 2002; Meiners 2015; Meinwald and Eisner 2008). Several species of the genus *Nicotiana* (Solanaceae) have been extensively studied from all these approaches, and nicotine and related alkaloids have been described as constitutive and induced defensive metabolites (Baldwin 1988, 1989; Halitschke et al. 2000).

The ecological importance of VS for plants is highlighted by work on the defensive responses of *Arabidopsis thaliana* (Brassicaceae) to feeding vibrations of a specialist predator, *Pieris rapae* (Pieridae). Caterpillar feeding vibrations, alone or in concert with simulated herbivory, can activate hormonal signaling pathways (Body et al. 2019), prime glucosinolate and phenolic based leaf defenses (Appel and Cocroft 2014), and modulate the release of volatile organic compounds from leaves (Body et al. 2019). However, the role of herbivore feeding vibrations in plant defense responses has not been documented in other plant species. Here, we investigated changes in alkaloid concentration in *Nicotiana tabacum* L. induced by VS produced by *Phthorimaea operculella* (Lepidoptera: Gelechiidae), a generalist caterpillar which naturally feeds in the plant leaves.

Materials and Methods

Plants *Nicotiana tabacum* (Solanaceae) plants were grown in a greenhouse at the Universidad de Chile. Lighting was provided by metal halide lamps (36 W) and the room was kept at 25 ± 2 °C with a 16:8 h L:D photoperiod. Seeds were germinated in peat (Kekkilä; Vantaa, Finland) and after six weeks of development seedlings were transplanted into pots (14-cm tall × 17-cm diameter) containing a mix of potting soil (Armony; Pudahuel, Chile), sand (Armony; Pudahuel, Chile) and peat in a 4:1:1 proportion. Plants were watered twice weekly, 1 day with water and the other with an aqueous fertilizer solution (Phostrogen Plant Food, Santiago, Chile; http://www.bayergarden.co.uk/Products/p/Phostrogen-All-Purpose-Plant-Food).

Insect Rearing and Caterpillar Herbivory Patterns *Phthorimaea operculella*, commonly named tobacco splitworm or potato tuberworm, is a global pest of solanaceous crops and weeds (Rondon 2010). Individuals were collected near Curacaví (Metropolitan Region, Chile: 33.49° S, 71.02° W, 185 m above sea level) and reared on *N. tabacum* under the same laboratory conditions as the plants. *P. operculella* caterpillars are leaf-miners, feeding mostly from the mesoderm of leaves (Rondon 2010). Adults of *P. operculella* were maintained in cages containing 3-month old tobacco plants. Adults were fed with 10% aqueous honey solution every 2 days (Golizadeh et al. 2014), while caterpillars were allowed to feed *at libitum* on the tobacco plants until the fourth instar, which was the stage used for bioassays.

Natural patterns of caterpillar feeding were characterized using 20 4th-instar caterpillars. Individuals were placed on the fifth leaf of a tobacco plant (counted from the apex of the plant) and observed for 8 h to characterize: a) temporal patterns of feeding and b) leaf area attacked over time. Photographs of individuals and leaves were taken every 15 min and were analyzed using the software MorphoJ. The amount of leaf area attacked over time constituted the baseline for designing Herbivory and Playback bioassays.

Feeding Vibration Recordings and Playback Setup Vibrational signals produced by a feeding 4th-instar *P. operculella* caterpillar were recorded using the same conditions as in insect rearing. The caterpillar was placed inside a clip cage on the fifth leaf of an 11-week old tobacco plant ($N = 20$), once the caterpillar started to feed, the clip cage was removed. Vibrations were recorded for 10 min per individual with a laser Doppler vibrometer (Polytec CLV-2534) positioned at less than 1 cm from where the caterpillar was feeding.

To reproduce the vibrations produced by the feeding caterpillar, a 9 mm Samsung/Mplus Linear Resonant Actuator (LRA) vibrator was used, driven by a Behringer HA8000 V2 headphone amplifier. An accelerometer (Vibrametrics 9002A with P5000 power supply) was attached with wax to the LRA to calibrate the playback. The LRA and accelerometer were attached to the leaf using a modified hair clip covered with EVA rubber, held in position with a helping hands tool (Quad Hands). The clip held the actuator lightly but securely against the leaf surface; our previous observations

indicate that this procedure avoided injury to the leaf. Before each playback bioassay, the frequency response and amplitude of the actuator were characterized after attaching it to the leaf. Because an accelerometer was used to calibrate playback of laser-recorded signals, the playback recordings were converted from velocity to acceleration using Matlab. Digital filters were applied using the signal processing toolbox of MATLAB R2017a to compensate for the frequency response of the playback setup, and produce playbacks that closely matched the temporal and spectral properties of the original recordings (Appel and Cocroft 2014; Cocroft 2010; Cocroft et al. 2014).

The playback design was based on the natural pattern of feeding behavior of *P. operculella* caterpillars (see Results). Caterpillar vibrations were played back to the fifth leaf of naive tobacco plants ($n = 18$ replicates). Three feeding vibration exemplars with a high signal-to-noise ratio were used, each from a different individual of *P. operculella* feeding on a different tobacco plant.

Plant Chemical Defenses Alkaloids were extracted based on a modification of the protocol by Saitoh et al. (1985). The fifth leaf of each plant used during bioassays was cut, immediately flash frozen with liquid nitrogen and pulverized in a porcelain mortar. The fifth leaf was selected because it exhibits greater induced alkaloidal responses on the fifth day after stimulation (Baldwin 1989, 1999). The powdered leaf was transferred to a glass vial and weighed. Five mL of methanol were added and the vial was shaken at 600 rpm for 30 min. After that, the methanolic extract was filtered through filter paper (Whatman No. 1) and transferred into a Florence flask where it was evaporated to dryness using a rotary evaporator at 40 °C. After adding 5 mL of chloroform to the Florence flask, it was immersed in a 25 °C ultrasound bath for 10 min. The extract was then transferred into a separatory funnel and 5 mL of 5% hydrochloric acid were added, thoroughly mixed and the organic phase collected and discarded; the acidic extract was washed twice with 5 mL of chloroform. After that, 5 mL of 29% aqueous ammonia solution was added and the basic solution was extracted twice with 5 mL of chloroform. The organic phases were collected in a vial and then evaporated to dryness under a nitrogen flow.

The alkaloidal extract was redissolved in a mixture of 70 μL of chloroform and 30 μL of internal standard solution (0.25 mM docosane in chloroform). Then, 1 μL of the redissolved extracts was injected in a Shimadzu model GCMS-QP 2010 Ultra gas chromatograph (Shimadzu, Kyoto, Japan). The mass spectrometer used electron impact (EI) ionization mode (70 eV) with an emission current of 250 μA. The temperatures of the injection port, transfer line and ion source were 250 °C, 280 °C and 250 °C, respectively. An Rtx-5MS Crossbond 5% diphenyl 95% dimethyl polysiloxane capillary GC column (30 m length, 0.25 mm I.D., 0.25 μm film thickness) (Restek, Bellefonte, PA, USA) was used in the splitless mode, with helium as the carrier gas at 50 ml/min. The GC oven temperature was programmed to remain at 30 °C for 3 min, then to increase to 230 °C at a rate of 18 °C/min and finally to remain at 230 °C for 5 min. Retention indexes were determined based on chromatograms obtained from the periodic injection of a standard alkane mixture (Sigma-Aldrich). Compounds were identified based on comparisons of their retention index and mass spectrum with those in the NIST14 database and with authentic standards.

To quantify the four main alkaloids (nicotine, nornicotine, anabasine and anatabine) present in the extracts, calibration curves were constructed for each alkaloid with five concentrations of commercial standards of each one (Sigma-Aldrich) ranging from 0.0224 to 2 mg/mL and containing the same concentration of internal standard described above (Yuan et al. 2018). The concentration of each alkaloid in the extracts was extrapolated from the corresponding calibration curve, and was expressed as μg/g of fresh sample.

Bioassays In all bioassays, 11-week old plants were used. Four types of bioassays were performed. In all of them, alkaloids were quantified in the fifth leaf of the tobacco plants which had been subjected to the following treatments: a) Constitutive, corresponding to the concentration in the undamaged leaf ($N = 18$) (Kaplan et al. 2008). b) Caterpillar, characterizing the chemical response of tobacco to herbivory by *P. operculella* caterpillars. One 4th instar caterpillar was placed on the fifth leaf of an undamaged plant, and enclosed within a clip cage ($N = 18$) in order to limit its movements before it started to feed. The individual was observed every 10 min to determine the start of feeding. Once the caterpillar started to feed, the clip cage was removed, and the caterpillar allowed to eat ad-libitum for 208 min. After this time, it was carefully removed from the plant and the plant was left in the rearing environment described above. Five days after caterpillar removal, the damaged leaf was removed, and alkaloids were extracted and quantified. c) Playback. The same protocol was used as in the Caterpillar treatment except that the caterpillar was replaced by the recording of VS produced by an individual caterpillar chewing the leaf, as described above ($N = 18$). d) Equipment, characterizing the potential effect of the setup used for playback on the induction of secondary metabolites. The complete playback setup was turned on and attached to the fifth leaf of the plant without any sound being reproduced for the same time as the playback replicates ($N = 18$) in order to evaluate the effect of the equipment by itself (e.g. weight, electrical fields). After 5 days the leaf was collected and chemically analyzed. The experimental treatments were conducted during the same time period, with 3–4 plants treated per day, each with a different treatment.

Statistical Analyses A Principal Component Analysis (PCA) was applied to all replicates of alkaloid concentrations in all treatments on R 3.4.4 (R Core Team 2014). Thereafter,

treatments were compared within each alkaloid using General Linear Models (GLM) also on R 3.4.4 (R Core Team 2014). Linear regression was used to generate calibration curves for the alkaloid standards. Pearson correlations were used to evaluate potential associations between the concentrations of different alkaloids.

Results

Caterpillars fed in bouts of 9.70 ± 1.35 min (mean ± sd) with a frequency of 2.48 ± 0.26 bouts per hour and a pause (no feeding) lasting 15.98 ± 1.85 min between feeding events. The waveform of chewing vibrations reflected the repetitive nature of the chewing process (Fig. 1). These data were used in the design of the playback stimulus, which consisted of 1 min of the original feeding vibrations recordings from one individual repeated 10 times (i.e. 10-min stimulus) followed by a 16-min silent pause. This basic 26-min pattern was repeated 8 times, so, the complete stimulus lasted for 208 min. Based on the caterpillar observations described above, during this time period an average of 14.4 ± 1.5 (mean ± sd) % of the leaf area was attacked by a single caterpillar.

The coefficients of determination (R^2) for calibration curves were 0.94 for nicotine, 0.96 for nornicotine, 0.98 for anabasine and 0.95 for anatabine. The most abundant alkaloid in all treatments was nicotine, followed by anatabine, nornicotine and anabasine (mean concentrations over all treatments: nicotine = 873 μg/g, anatabine = 216.4 μg/g, nornicotine = 77.3 μg/g, anabasine = 33.7 μg/g) (Fig. 2).

The PCA with all the alkaloid concentration data showed that most of the variability (94%) was explained with the first two components, the first one involving nornicotine, anabasine and anatabine (74% of variability) and the second one involving only nicotine (21% of variability). The corresponding biplot showed two subgroups, one where Playback and Caterpillar treatments overlapped and another with Equipment and Constitutive treatments overlapped (Fig. 2). Nicotine levels differed between treatments (Table 1), being 1.7 times higher in plants of the Equipment treatment, 2.9 times higher in plants of the Caterpillar treatment and 2.8 times higher in plants of the Playback treatment, with respect to the Constitutive treatment.

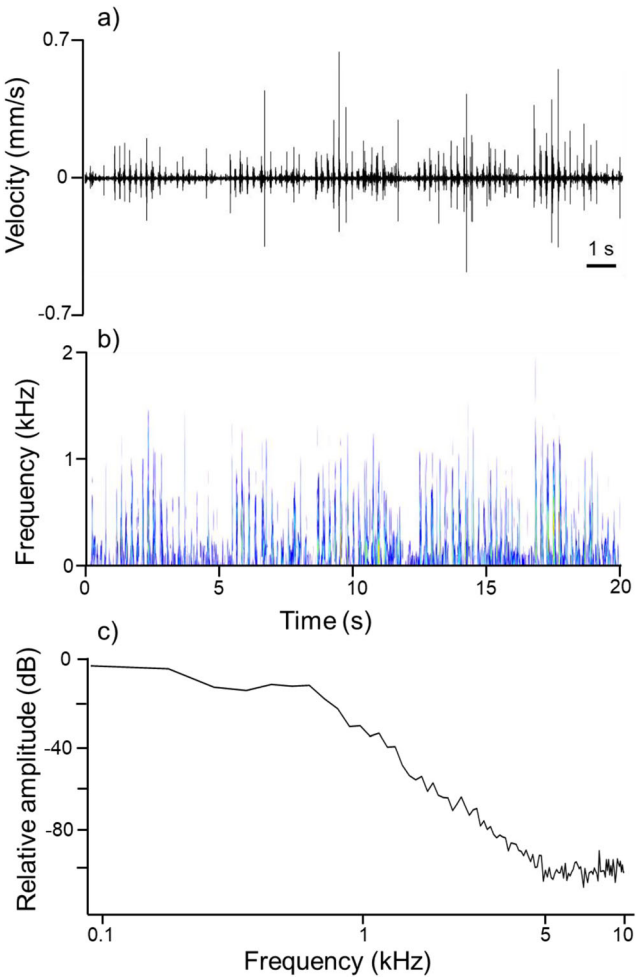

Fig. 1 Chewing vibrations of *P. operculella* on *N. tabacum*. **a** Waveform; (**b**) Spectrogram; (**c**) Amplitude spectrum

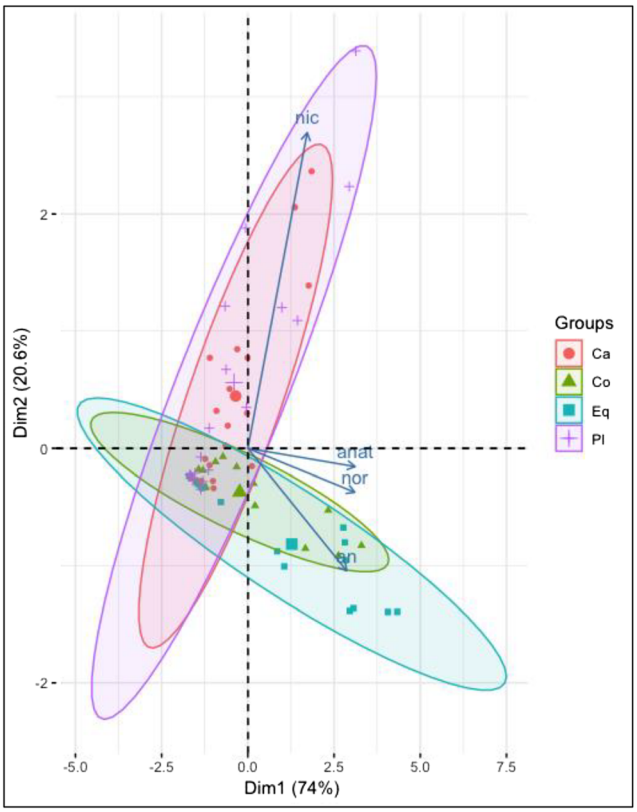

Fig. 2 Biplot of the PCA applied to alkaloid concentraions (*Nic* Nicotine, *Nor* Nornicotine, *An* Anabasine and *Anat* Anatabine) of the four treatments: *Co* Constitutive, *Eq* Equipment, *Ca* Caterpillar, *Pl* Playback

Table 1 Results for the Generalized Linear Model for the concentrations of alkaloids expressed as μg/g of fresh sample. Treatments were: *Co* Constitutive, *Eq* Equipment, *Ca* Caterpillar and *Pl* Playback (see text for description of treatments)

	Estimate	Standard error	t value	Pr(>|z|)
a) Nicotine: ANOVA: F = 3.8326 p = 0.01367* AIC: 1145.8				
Co vs Eq	94.4	329.6	0.286	0.7755
Co *vs* Ca	814.1	307.8	2.645	0.0102
Co *vs* Pl	789.6	307.8	2.565	0.0126
Pl *vs* Eq	−695.2	333.5	−2.085	0.0410
Pl *vs* Ca	24.6	311.9	0.079	0.9375
Eq *vs* Ca	719.7	333.5	2.158	0.0346
b) Nornicotine: ANOVA: F = 6.4086 p = < 0.001 AIC: 786.27				
Co *vs* Eq	85.99	26.77	3.212	0.0021
Co *vs* Ca	−21.95	25.04	−0.876	0.3841
Co *vs* Pl	−10.50	25.04	−0.419	0.6765
Pl *vs* Eq	96.49	26.77	3.604	0.0006
Pl *vs* Ca	−11.45	25.04	−0.457	0.6490
Eq *vs* Ca	−85.99	26.77	−3.212	0.0021
c) Anabasine: ANOVA: F = 8.1874 p = < 0.001 AIC: 615.5				
Co *vs* Eq	24.149	7.627	3.166	0.0024
Co *vs* Ca	−1.640	7.135	−0.230	0.8189
Co *vs* Pl	−13.156	7.135	−1.844	0.0698
Pl *vs* Eq	37.305	7.627	4.891	7.08e-06
Pl *vs* Ca	11.516	7.135	1.614	0.1114
Eq *vs* Ca	−25.789	7.627	−3.381	0.00123
d) Anatabine: ANOVA: F = 3.4086 p = 0.02261 * AIC: 946.06				
Co *vs* Eq	174.34	77.51	2.249	0.0279
Co *vs* Ca	−66.48	72.38	−0.918	0.3618
Co *vs* Pl	−14.88	72.38	−0.206	0.8377
Pl *vs* Eq	189.22	78.42	2.413	0.0186
Pl *vs* Ca	−51.60	73.36	−0.703	0.4843
Eq *vs* Ca	−240.82	78.42	−3.071	0.0031

The GLM analysis showed that the concentration of nicotine in the Playback and Caterpillar treatments did not differ significantly and both differed from those of the Constitutive and Equipment treatments, which had similar nicotine levels (Table 1, Fig. 3). Nornicotine, anabasine and anatabine concentrations differed significantly between bioassays, being higher and significantly different in the Equipment treatment compared with the other three treatments (Table 1, Fig. 3). Concentrations of nornicotine were highly and positively correlated with concentrations of anabasine (R = 0.85, $p < 0.001$) and of anatabine (R = 0.95, $p < 0.001$). Furthermore, concentrations of anabasine were positively correlated with concentrations of anatabine (R = 0.83, $p < 0.001$).

Discussion

Plants of *N. tabacum* chemically responded to natural herbivory by *P. operculella* caterpillars and to VS produced by feeding activity of *P. operculella*. Increased concentration of nicotine was observed under two situations: when leaves were damaged by a caterpillar, and when leaves were stimulated with playbacks of caterpillar chewing vibrations. On the other hand, nicotine concentration did not increase when the equipment for playback was attached to the plant and turned on.

Nicotine constitutes the first effective defensive barrier in the genus *Nicotiana* because it is the most abundant alkaloid in the plant and also because it can be further induced in response to herbivore attack and physical damage (Baldwin 1996, 1999; Baldwin et al. 1997; Halitschke et al. 2000; Stepphun et al. 2004). In fact, plants can sense even small scratches caused by the walking of an insect on the leaf surface and as a consequence activate nicotine induction (Peiffer et al. 2009; Karban 2015). The present study demonstrates for the first time in *N. tabacum* that isolated vibrations produced by a feeding caterpillar can induce a chemical response by the plant. This result shows that tobacco plants can perceive this stimulus, recognize it as a potential threat and choose to invest energy in the induction of defenses. On the other hand, plants do not respond chemically in the same way to different types of damage (e.g. different herbivore species, diseases). Herbivores can alter the ability to affect chemical defenses and even suppress their induction following damage (Tooker et al. 2008; Alba et al. 2011; Witzany and Baluška 2012). For example, in the genus *Nicotiana*, the specialist moth *Manduca sexta* can disrupt the biosynthesis of nicotine in the roots through their oral regurgitant (McCloud and Baldwin 1997; Kahl et al. 2000; Halitschke et al. 2000, 2004). The present

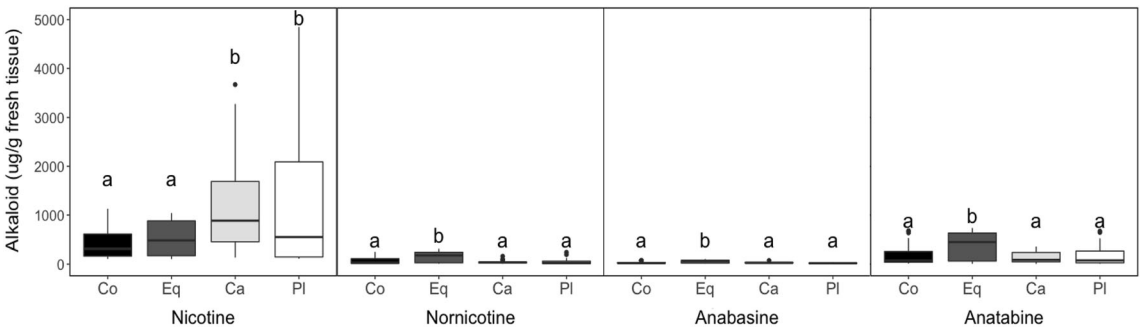

Fig. 3 Concentration of alkaloids expressed as μg/g of fresh sample for each treatment. Different letters (**a**, **b**) indicate significant differences between treatments at $P < 0.05$. Treatments: *Co* Constitutive, *Eq* Equipment, *Ca* Caterpillar, *Pl* Playback

study leaves open the question of which features of the played-back feeding vibrations were important for triggering the defensive responses, and of what responses may be evoked by vibrations produced by other herbivores or other biotic or abiotic sources. Phenolic defenses of *Arabidopsis* plants were induced by playback of *Pieris* feeding vibrations, but not by playback of wind vibrations or leafhopper song (Appel and Cocroft 2014), revealing some specificity in the vibration-induced defensive response. However, the key features of herbivore vibrations necessary for inducing plant responses, and the range of variation in effective vibrational stimuli, have so far not been identified.

The minor alkaloids, nornicotine, anabasine and anatabine were found at significantly higher levels in the Equipment treatment as compared with all other treatments, consistent with the correlations found between these variables. However, the mechanisms leading to these concentration increases are not clear. We lack an explanation for the observation that the Playback treatment did not lead to an increase in the concentration of any of the minor alkaloids. Attaching the equipment should have had the same positive effect in both the 'Equipment' and 'Playback' treatments, and it is possible that the vibrational stimulus downregulated the production of the minor alkaloids.

Plant response to herbivory can be expressed in a variety of traits (e.g. morphology, chemistry, development) (Karban 2015; Karban and Baldwin 1997) and induced chemical responses by the plant may cause herbivores to be less attracted to the defended tissue or may reduce the performance of herbivores that have consumed such tissue (Karban 2015; Karban and Myers 1989). Our experimental approach has assessed plant induced chemical responses to VS isolated from the other effects of insect feeding, such as the mechanical damage to the plant and the insect's saliva, thereby providing a deeper understanding of the role of VS in plant responses to herbivory. We suggest that VS of herbivory constitute an ecological stimulus to *N. tabacum* and is the main reason for nicotine induction as a behavioral response of the plant interprets such signals as a threat. These results, taken together with similar observations made on *A. thaliana* (Appel and Cocroft 2014) suggest that plant perception and responses to VS might be widespread in plants. The adaptive value of the chemical response to VS remains unclear: the inclusion of fitness traits into the experimental design will be necessary to assess these responses as adaptive behaviors expressed by plants.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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