

## Below-ground herbivory limits induction of extrafloral nectar by above-ground herbivores

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Received: 17 October 2014 Returned for revision: 21 November 2014 Accepted: 23 December 2014

• **Background and Aims** Many plants produce extrafloral nectar (EFN), and increase production following above-ground herbivory, presumably to attract natural enemies of the herbivores. Below-ground herbivores, alone or in combination with those above ground, may also alter EFN production depending on the specificity of this defence response and the interactions among herbivores mediated through plant defences. To date, however, a lack of manipulative experiments investigating EFN production induced by above- and below-ground herbivory has limited our understanding of how below-ground herbivory mediates indirect plant defences to affect above-ground herbivores and their natural enemies.

• **Methods** In a greenhouse experiment, seedlings of tallow tree (*Triadica sebifera*) were subjected to herbivory by a specialist flea beetle (*Bikasha collaris*) that naturally co-occurs as foliage-feeding adults and root-feeding larvae. Seedlings were subjected to above-ground adults and/or below-ground larvae herbivory, and EFN production was monitored.

• **Key Results** Above- and/or below-ground herbivory significantly increased the percentage of leaves with active nectaries, the volume of EFN and the mass of soluble solids within the nectar. Simultaneous above- and below-ground herbivory induced a higher volume of EFN and mass of soluble solids than below-ground herbivory alone, but highest EFN production was induced by above-ground herbivory when below-ground herbivores were absent.

• **Conclusions** The induction of EFN production by below-ground damage suggests that systemic induction underlies some of the EFN response. The strong induction by above-ground herbivory in the absence of below-ground herbivory points to specific induction based on above- and below-ground signals that may be adaptive for this above-ground indirect defence.

**Key words:** Extrafloral nectar, indirect defence, induced defence, above- and below-ground interactions, herbivory, specificity, tallow tree, *Triadica sebifera*, flea beetle, *Bikasha collaris*.

### INTRODUCTION

Plants are frequently attacked by various shoot and root herbivores and have evolved a diverse array of defensive strategies (Agrawal, 2007; Mithöfer and Boland, 2012). Induced defence is thought to be a particularly effective and efficient strategy for plants through creating a specific and targeted defence response (Frost *et al.*, 2008; Karban, 2011). In many cases, induced defence is systemic, crossing above- and below-ground boundaries, such that shoot herbivores can affect root defence level and root herbivore performance, and vice versa (Johnson *et al.*, 2009; Rasmann *et al.*, 2009; Kutyniok and Muller, 2012; Erwin *et al.*, 2014; Huang *et al.*, 2014). Thus, cross-talk of induced defence may determine herbivore population dynamics and ultimately affect community stability in both above- and below-ground compartments (Bezemer and van Dam, 2005; Erb *et al.*, 2008; Soler *et al.*, 2013).

Induced defences function either directly by reducing the impact of herbivores, or indirectly by increasing attraction of herbivore natural enemies (Kessler and Heil, 2011; Mithöfer and Boland, 2012; Hanley *et al.*, 2013). Interactions between induced above- and below-ground direct defences are fairly well

studied (Kaplan *et al.*, 2008a; Rasmann *et al.*, 2009; Huang *et al.*, 2013; Erwin *et al.*, 2014). In general, intra-guild feeding increases induced direct defence responses of plants through shared signalling pathways, while inter-guild feeding, which often activates different signalling pathways, weakens induced direct defences of plants, probably through negative crosstalk (Soler *et al.*, 2013). However, indirect defences are less well studied, and the effects of herbivore interactions on induced indirect defences are not well understood, particularly between shoot and root herbivores feeding simultaneously. Indirect defence can be classified as information-providing traits (e.g. volatile organic compounds) as well as resource-providing traits (e.g. extrafloral nectar) (Arimura *et al.*, 2005). To date, limited studies have focused mainly on the former (Erb *et al.*, 2008; van Dam and Heil, 2011). For example, maize plants attacked by root herbivores produced more (*E*)- $\beta$ -caryophyllene than plants attacked by both shoot and root herbivores (Rasmann and Turlings, 2007). In cotton, plants increased (*Z*)-3-hexenyl acetate upon attacks by shoot herbivores, and the presence of root herbivory strengthened this increase (Olson *et al.*, 2008).

Extrafloral nectar (EFN) is a common indirect defence, being recorded so far in 3941 species and estimated to occur in more than 8000 species (Weber and Keeler, 2013). Many plants benefit by producing EFN to attract arthropod predators and parasitoids of herbivores by providing food as EFN consists mainly of sugars, amino acids and other important nutrients (Arimura *et al.*, 2005; Heil, 2011; Villamil *et al.*, 2013). Many examples have shown that secretion of EFN can be induced via shoot damage caused by artificial clipping, specialist and generalist herbivory, and exogenous hormone application (Heil *et al.*, 2001; Radhika *et al.*, 2010; Wang *et al.*, 2013). By contrast, EFN production is sometimes also significantly increased upon attack by root herbivory and mechanical damage (Wäckers and Bezemer, 2003; Mathur *et al.*, 2013). We predict that interactions between shoot and root herbivory-induced EFN production are likely to occur. However, to date, a lack of manipulative experiments investigating EFN production induced by simultaneous shoot and root herbivory has limited our understanding of how root herbivores affect shoot herbivores and their natural enemies mediated by indirect plant defence.

The flea beetle *Bikasha collaris* (Coleoptera: Chrysomelidae; hereafter *Bikasha*) is a major chewing herbivore on *Triadica sebifera* (Euphorbiaceae; hereafter *Triadica*), a well-known ornamental and medicinal plant in China (Zheng *et al.*, 2005). We have recently shown that *Triadica* seedlings with above-ground adult herbivory increased leaf tannins, and that below-ground larvae herbivory amplified this increase. This led to the presence of larvae inhibiting adult survival, although we did not consider the effects of indirect defence (Huang *et al.*, 2012, 2013). Chewing herbivores can elicit plant expression of jasmonic acid, which activates a wide range of defensive responses including production of phenolics (Ali *et al.*, 2007; Gadzovska *et al.*, 2007) and EFN (Heil *et al.*, 2001; Radhika *et al.*, 2010). If indirect defences respond similarly to direct defences induced by simultaneous shoot and root herbivory in *Triadica*, then we would expect that root herbivory will increase the EFN production elicited by shoot herbivory. Specifically, we ask: (1) Does root herbivory induce EFN production? (2) Does root herbivory increase, limit or have no effect on EFN production induced by shoot herbivory?

## MATERIALS AND METHODS

### Study organisms

*Triadica sebifera* is a rapidly growing, subtropical tree of southern China (Zhang and Lin, 1994). *Triadica* produces EFN both on glands at the bases of petioles and on the undersides of leaf margins in response to leaf damage (Carrillo *et al.*, 2012a). Recently, we have shown that specialist herbivory induced greater EFN production than generalist herbivory (Wang *et al.*, 2013), and that leaf-chewing herbivores induced EFN while phloem-feeders did not, indicating specificity in this defence response (Carrillo *et al.*, 2012b).

*Bikasha collaris* is one of the most abundant chewing insects on *Triadica* in China. Adults feed on leaves producing irregular scars while larvae feed on roots forming tunnels (Huang *et al.*, 2011). Preliminary host range tests indicated that both adults and larvae are monophagous specialists that feed exclusively on *Triadica* (Huang *et al.*, 2011). *Bikasha* pass through more

than five generations per year in Wuhan, China, and adult and larval life stages of different generations can feed on the same plant simultaneously.

### Seeds and seedlings

We collected seeds of *Triadica* from a natural population near Wuhan (31°33'N, 114°07'E). We removed the seeds' waxy coats by soaking them in water with laundry detergent (10 g L<sup>-1</sup>) for 2 d and then stored them in sand at a depth of 5–10 cm in a refrigerator (4°C) for 35 d. We sowed the seeds in growing medium (50 % topsoil and 50 % sphagnum peat moss) in an unheated greenhouse at Wuhan Botanical Garden, Chinese Academy of Sciences (30°32'N, 114°24'E). To avoid potential adverse effects of host-specific soil biota, we used topsoil from fields where no *Triadica* grew (Yang *et al.*, 2013). Seven weeks later, we transplanted individual seedlings into pots (16 cm height, 21 cm diameter) containing the same growing medium and caged them in nylon mesh (100 cm height, 27 cm diameter) to exclude insect herbivores. We watered seedlings three times per week.

### Experimental design

Three weeks after transplanting, we selected 72 similar-sized plants (mean height: 22.4 ± 0.2 cm; stem diameter: 3.36 ± 0.04 mm; number of leaves: 17.6 ± 0.2) and randomly assigned them to above- and/or below-ground herbivory treatments in a factorial design. The density and timing of both above- and below-ground herbivores were similar to those of Huang *et al.* (2013). In brief, plants assigned to below-ground (BG) or below- and above-ground herbivory (Both) treatments received 10 newly laid eggs per plant (day 0). We transferred eggs directly to a hole (3–4 cm deep, 1 cm diameter) in the soil at the base of each plant and covered them with moist soil. Plants assigned to above-ground (AG) or 'Both' herbivory treatments received ten adults per plant nine days later (day 9). We left adults on the plants for 18 d and then removed them (day 27). This procedure ensured that the periods of above- and below-ground herbivory coincided, as the average egg and larval development times are 9 and 18 d, respectively (Huang *et al.*, 2011). Furthermore, ten adults removed approx. 10 % of the leaf area in 18 d when ten larvae attacked the plant simultaneously and such experimental damage levels resemble natural leaf damage levels. We obtained eggs and adults from laboratory colonies originally collected from locally established natural populations. To avoid adults ovipositing in the soil and to make above- and below-ground herbivory independent, we sealed the mesh cage of each pot to the plant stem below all leaves using string.

At the end of the herbivory period (day 27), accumulated EFN production was measured. Previous studies found that EFN production peaked approx. 4 d after herbivory (Carrillo *et al.*, 2012b) but that EFN induction could last from weeks to months after damage (Carrillo *et al.*, 2012a, 2014; Wang *et al.*, 2013). Furthermore, in this study, EFN was collected on plants that were continuously attacked by herbivores over the entire experimental period rather than on plants attacked only at the early stage. Specifically, the numbers of leaves and leaves

producing EFN were recorded for each plant. Then, the EFN was collected from every leaf using microcapillary tubes (5 µL) for each plant to measure EFN volume. When sufficient EFN was obtained, the percentage of soluble solids was estimated using a low-volume hand-held refractometer (45-05, Bellingham + Stanley, Basingstoke, UK). EFN soluble solids was calculated as EFN volume multiplied by the percentage of soluble solids. To examine the relationship between leaf loss and EFN production, the percentage of leaf area damaged was determined by visual estimate (to the nearest 5 %) for each leaf, then averaged for all leaves for each plant.

Data analyses

We used two-way analyses of variance (ANOVAs) to examine the effects of above-ground herbivory (with or without), below-ground herbivory (with or without) and their interaction on the percentage of leaves producing EFN, EFN volume and EFN soluble solids ( $n = 18$ ). When a significant effect was detected for an interaction term, we used adjusted means partial difference tests to test whether treatments were significantly different. We used a one-way ANOVA to test whether above-ground damage (leaf area damaged) depended on below-ground herbivory (with or without) ( $n = 18$ ). We analysed the relationship between EFN volume and leaf area damaged using regressions ( $n = 72$ ). We performed an analysis of covariance (ANCOVA) with below-ground herbivory as the main effect and leaf area damage as the covariate to examine how EFN volume depended on leaf damage and below-ground herbivory for plants that also had above-ground herbivores ( $n = 18$ ). We performed all data analyses with SAS, version 9.1 (SAS Institute, Cary, NC, USA).

RESULTS

EFN production depended on above-ground herbivory and the interaction of above- and below-ground herbivory (Table 1). Above-ground herbivory resulted in a 4.9-fold increase in the percentage of leaves producing EFN relative to control plants, while below-ground herbivory or both types of herbivory together respectively resulted in 2.8- and 3.4-fold increases compared with control (Fig. 1A). Below-ground herbivory induced a greater volume of EFN and mass of soluble solids than controls, but not as much as both types of herbivory, or above-ground herbivory only, which induced the greatest volume and soluble solid mass of EFN (Fig. 1B, C). The percentage of leaf

TABLE 1. Two-way ANOVAs showing the effects of above-ground herbivory (adults, AG) and/or below-ground herbivory (larvae, BG) by *Bikasha collaris* on EFN production of *Triadica sebifera*; significant results are in bold type

Effect	d.f.	% leaves producing EFN		EFN volume		EFN soluble solids		
		F	P	F	P	d.f.	F	P
AG	1, 68	92.72	<b>&lt;0.0001</b>	133.17	<b>&lt;0.0001</b>	1, 60	90.78	<b>&lt;0.0001</b>
BG	1, 68	0.46	0.5015	1.58	0.2127	1, 60	1.06	0.3073
AG × BG	1, 68	49.22	<b>&lt;0.0001</b>	34.25	<b>&lt;0.0001</b>	1, 60	21.46	<b>&lt;0.0001</b>

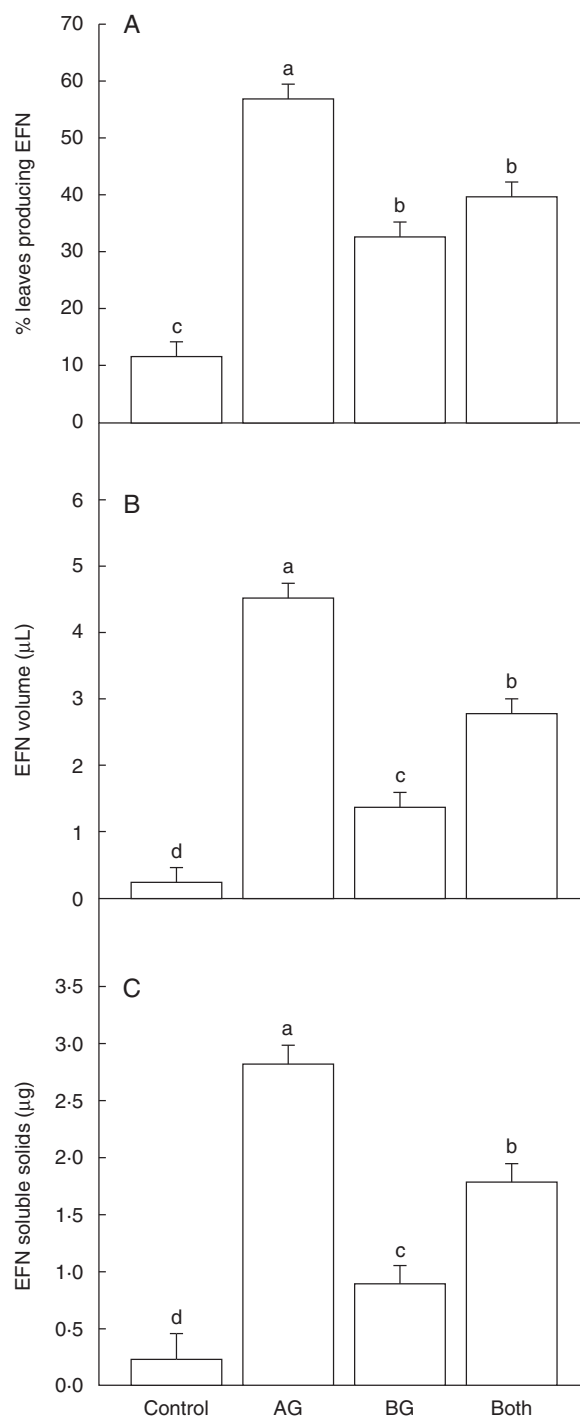


FIG. 1. Impact of above-ground herbivory (adults), below-ground herbivory (larvae) by *Bikasha collaris* and their interaction on (A) the percentage of leaves producing EFN, (B) EFN volume and (C) EFN soluble solids of *Triadica sebifera*. Control, no herbivory; BG, below-ground herbivory; AG, above-ground herbivory; Both, above- and below-ground herbivory. Adjusted means and SEs are from two-way ANOVAs. Means with the same letters were not significantly different ( $P < 0.05$ ) in post-hoc tests.

area damaged was higher for plants exposed to only above-ground herbivory than for those exposed to both types of herbivory (AG:  $15.29 \pm 0.66$  %; Both:  $10.77 \pm 0.63$  %;  $F_{1,34} = 24.67$ ,  $P < 0.0001$ ; Fig. 2). EFN volume increased with

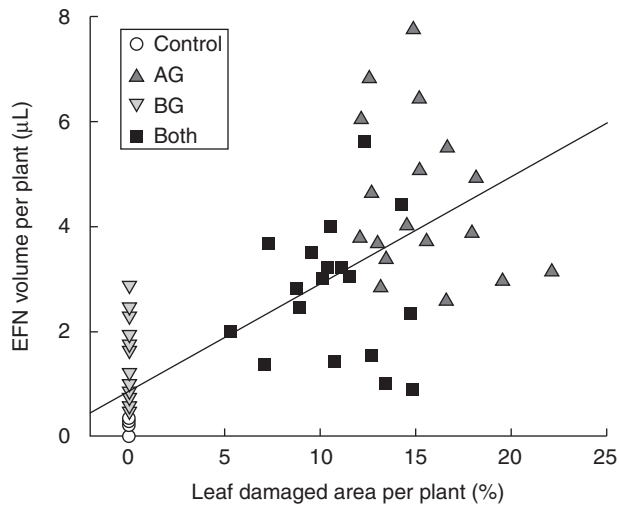


Fig. 2. Regression of EFN volume and percentage of leaf area damage. Circles, no herbivory (Control); downward triangles, below-ground herbivory (BG); upward triangles, above-ground herbivory (AG); squares, above- and below-ground herbivory (Both). EFN volume =  $0.8784 + 0.2049 \times \text{Damage}$ ,  $R^2 = 0.568$ ,  $P < 0.0001$ .

the percentage of leaf area damaged (Fig. 2). For plants with above-ground herbivores, EFN volume depended on the presence of below-ground herbivores ( $F_{1,33} = 12.38$ ,  $P = 0.0013$ ) but not on the percentage of leaf area damaged ( $F_{1,33} = 0.94$ ,  $P = 0.3389$ ).

## DISCUSSION

Our study provides evidence that below-ground herbivores limit rather than increase EFN production induced by above-ground herbivores. Thus far, studies on the effects of the interactions between above- and below-ground herbivores on plant defence have focused mainly on direct defence strategies and found that these interactions can mediate anti-herbivore chemical allocation in shoots and roots, such as secondary compounds, nutrients and latex (Kaplan *et al.*, 2008b; Rasmann *et al.*, 2009; Kutyniok and Muller, 2012; Johnson *et al.*, 2013). However, studies on the effects of such interactions on indirect defence are rare and have considered only herbivore-induced plant volatiles (Rasmann and Turlings, 2007; Olson *et al.*, 2008; Pierre *et al.*, 2011). To the best of our knowledge, this study shows for the first time that above- and below-ground herbivores interact to affect EFN production, adding new insight into our understanding of plant-mediated herbivore–herbivore interactions.

EFN is believed to function by attracting enemies to the site of herbivore attack, as damaged leaves often produced greater EFN than undamaged leaves on the same plant (Wäckers *et al.*, 2001). Therefore, it is somewhat surprising that below-ground larval herbivory induced EFN production. It may be possible that ants recruited by above-ground EFN could attack below-ground herbivores. In fact, ants are known to attack a diverse array of soil organisms (Brian, 1977; Wardle *et al.*, 2010). Furthermore, below-ground herbivory may indicate a greater risk of simultaneous above-ground herbivory especially for species with both above- and below-ground feeding stages

(van Dam and Heil, 2011). *Bikasha* are multivoltine; the adults and larvae of different generations can be feeding on the same plant simultaneously for nearly the whole growing season (Huang *et al.*, 2011). Thus, increased EFN by below-ground herbivory by larvae may provide a predictive defence value to co-occurring above-ground herbivory by adults. To date, however, there are no studies that examine the consequences of above-ground EFN production on below-ground herbivore survival or success, or how EFN induced by below-ground herbivores influences the success and survival of above-ground herbivores.

In this study, above-ground adult herbivory induced EFN production. This result was consistent with previous studies using leaf damage caused by artificial clipping, specialist and generalist herbivory, as well as exogenous hormone application (Heil *et al.*, 2001; Radhika *et al.*, 2010; Wang *et al.*, 2013). However, the presence of below-ground larvae limited this increase. These results suggest that below-ground herbivores are not only able to induce EFN production but also alter EFN production induced by above-ground herbivores. In this case, below-ground herbivores may be able to affect above-ground herbivores and their natural enemies via altering EFN production, and ultimately affect herbivore population dynamics and the plant community (Wäckers and Bezemer, 2003).

It is generally assumed that the amount of herbivore damage and the strength of induced defence responses are positively correlated, as is the case for protease inhibitors in *Solidago altissima* (Bode *et al.*, 2013), volatiles in *Vaccinium corymbosum* (Rodríguez-Saona *et al.*, 2009) and EFN in *Macaranga tanarius* (Heil *et al.*, 2001). Such a positive pattern between leaf damage and EFN production was also observed in *Triadica* when attacked by leaf-chewing caterpillars (Carrillo *et al.*, 2012b). In this study, we found that the plants exposed to both types of herbivores had lower leaf damage than those exposed to above-ground adults alone. The lower levels of leaf damage could explain why the presence of below-ground herbivores decreased EFN production induced by above-ground herbivores. Mainly, the indirect effect of reducing above-ground damage may have limited the EFN production. The significance of below-ground herbivore presence but not leaf area damaged on EFN volume suggests that this effect, if present, is not sufficient to explain the effect of below-ground herbivores in reducing induction by above-ground herbivory. It may be that the combination of reduced leaf and root tissue resulting from simultaneous above- and below-ground herbivory may diminish the ability of the plant to produce costly defences.

In addition to producing EFN, *Triadica* often employs multiple secondary chemicals to defend against herbivores, such as phenolics (Huang *et al.*, 2010; Wang *et al.*, 2012). Our previous study with *Triadica* and *Bikasha* found that plants increased tannin in the leaves with above-ground adult herbivory, and that below-ground larvae herbivory amplified this increase (Huang *et al.*, 2013). Tannin is a costly carbon-based secondary metabolite (Barbehenn and Constabel, 2011). Likewise, EFN mainly comprises carbon-based primary metabolites, such as sucrose, glucose and fructose (Heil, 2011) and its production depends on sugar fluxes in the phloem and on a jasmonic acid-responsive invertase (Millán-Cañongo *et al.*, 2014). In addition, recent studies indicated that EFN is also an expensive plant defence and more costly than previously thought (Heil, 2011;

Ballhorn *et al.*, 2014). Thus, we speculate that decreased EFN may be closely related to increased tannins, as combining above- and below-ground herbivory might simply result in plant-wide patterns of carbohydrate re-allocation that limit the amounts of sucrose available at the site of the nectaries. Further work is required to address the dissimilarity we observed in tannin and EFN responses mediated by above- and below-ground herbivores.

Plants battle against the negative effects of herbivory on two fronts, namely above and below ground (Erb *et al.*, 2008; van Dam and Heil, 2011). This study adds to the growing number of examples that these two arenas are linked through plant-induced responses to herbivore attack, as we demonstrate that below-ground herbivory can limit the defensive response of plants to above-ground attack. Through reducing the EFN production of plants, below-ground herbivores may reduce attraction of above-ground natural enemies, potentially benefiting above-ground herbivores. This is similar to below-ground herbivory reducing the attraction of parasitoids to above-ground herbivores due to changes in herbivore-induced plant volatiles and herbivore quality (Kostenko *et al.*, 2013; Kruidhof *et al.*, 2013). These interactions are complex, involving multiple trophic levels and are potentially important drivers of community dynamics.

#### ACKNOWLEDGEMENTS

We thank Hui Wei, Xuefang Yang, Xue Gu and Lin Zhu for EFN collection. This work was supported by the National Natural Science Foundation of China (31470447 and 31200286 to W.H., 31370404 to J.D.), an NSF Postdoctoral Fellowship (to J.C.), the US National Science Foundation (DEB 0820560 to E.S.) and the Foreign Visiting Professorship of the Chinese Academy of Sciences (to E.S.).

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