

Foliar Extrafloral Nectar of *Humboldtia brunonis* (Fabaceae), a Paleotropic Ant-plant, is Richer than Phloem Sap and More Attractive than Honeydew

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ABSTRACT

The ant-plant *Humboldtia brunonis* secretes extrafloral nectar (EFN) despite the lack of antiherbivore protection from most ants. EFN was richer in composition than phloem sap and honeydew from untended Hemiptera on the plant, suggesting that EFN could potentially distract ants from honeydew, since ants rarely tended Hemiptera on this plant.

Key words: ant–plant interaction; extrafloral nectar; honeydew; India; phloem sap; trophic mutualism; tropical wet forest; Western Ghats.

THE IMPORTANCE OF EXTRAFLORAL NECTAR (EFN) AS AN INDIRECT DEFENSE STRATEGY (Bentley 1977, González-Teuber *et al.* 2012) against herbivores is widely reported (Koptur 1992, González-Teuber & Heil 2009, Weber & Keeler 2013). Besides chewing and grazing herbivores, plants are also vulnerable to phloem-feeding Hemiptera that are often tended by ants for honeydew (Way 1954, 1963, Del-Claro & Oliveira 1993, Blüthgen *et al.* 2003). Such Hemiptera not only deplete phloem sap but also render plants vulnerable to pathogens (Maramosoch 1963). Becerra and Venable (1989) hypothesized that EFN was richer than honeydew; consequently, EFN may function to distract ants from tending Hemiptera on plants. This hypothesis may apply only to facultative ant–plant interactions (Fiala 1990, Fiala & Maschwitz 1991, Rosumek *et al.* 2009), and when EFN is more attractive to ants than honeydew. Indeed, when EFN forms a major part of the ant's diet, it increases ant colony growth (Byk & Del-Claro 2011).

If EFN is important in a plant's antiherbivore defense strategy, do plants actively modulate EFN composition within the nectary to produce a secretion attractive to ants, or is EFN merely a phloem exudate? Few studies have compared EFN and phloem sap to examine this question (Orona-Tamayo *et al.* 2013). We investigated sugar and amino acid compositions of foliar EFN relative to phloem sap in the myrmecophyte *Humboldtia brunonis* (Fabaceae), which dominates the understory of some low elevation, rain forests in the Indian Western Ghats (Ramesh & Pascal 1997). While all individuals do not produce caulinary domatia (hollow, swollen internodes), all plants bear active nectaries on young expanding leaves (Fig. S1, Fig. S2, Appendix S1) and floral bud bracts. Of 16 domatia-occupying ant species, this plant receives protection from only one ant, *Technomyrmex albipes*, and only in the region where herbivory is highest (Shenoy & Borges 2010, Shenoy *et al.* 2012, Chanam *et al.* 2014a). Domatia-bearing plants are rewarded by nitrogen

absorbed from domatia that are occupied even by non-protective EFN-consuming ants (Chanam *et al.* 2014b). Could all plants receive benefits from EFN? Ant-tending of Hemiptera was almost never observed on *H. brunonis*, although non-ant-tended Hemiptera colonies were sometimes present on petioles and laminas of young leaves, so we expected foliar EFN to divert ants from tending Hemiptera. Therefore, we also compared compositions of EFN and honeydew.

The study was conducted at Agumbe Reserve Forest (13° 31' N, 75° 04' E), Karnataka, India. We chose 15 young green leaves (four leaflets to a leaf) with Hemiptera (Fulgoroidea, unidentified species) colonies on their petioles. We collected all three types of samples (EFN, phloem sap, and honeydew) from these leaf sets to control for high plant or leaf variation (Shenoy *et al.* 2012). These fulgorids were the only Hemiptera, except for one case (see later), found regularly on *H. brunonis*. To sample honeydew, we bagged woody stems with the Hemiptera colonies using sterile plastic bags with ventilation holes. We collected the honeydew squirted by these Hemiptera within 24 h, and noted its volume. We removed the Hemiptera colonies, cleaned the leaves and bagged them for 24 h after which we collected EFN secreted by each leaf, and noted its volume. We sampled phloem sap using the EDTA-exudation technique of King and Zeevaart (1974) described by Douglas (1993).

We immediately added HPLC-grade methanol (50 μ l) to each vial of all three types of samples (EFN, honeydew, and phloem sap) to deactivate enzymes such as invertase (Selisko *et al.* 1990, Rodríguez *et al.* 1997) whose activity might change sugar compositions, and also to inhibit microbial growth (Shenoy *et al.* 2012). Vials were kept at 0°C till their contents were lyophilized at Bangalore. Sugar and free amino acid compositions (final sample size: honeydew: $N = 5$; EFN: $N = 6$; phloem sap: $N = 9$) were analyzed using gas chromatography-mass spectrometry (Appendix S2). Since phloem sap of *H. brunonis* woody stems is not abundant, does not ooze out of excised stems, and because the collection method employed did not allow accurate measurement of volume change post-extraction in EDTA, we could not

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determine the volume of sap collected and thereby absolute values of constituent concentrations. Therefore, relative proportions of constituents were compared across EFN, phloem sap, and honeydew.

To visualize differences between EFN, honeydew and phloem sap, an unsupervised classification based on relative constituent concentration was performed using the MDSplot function of the randomForest package. To obtain a quantitative measure of these differences, we employed the *pvclust* function of the *pvclust* package with 10,000 bootstrapping iterations using Euclidean distance as the distance measure and Ward's minimum variance as the agglomerative method. Clusters with approximately unbiased (AU) values of ≥ 95 were considered stable. We analyzed data using R software (v. 2.14.1; R Development Core Team 2010).

Glucose, sucrose, fructose, galactose, altrose, and the sugar alcohol inositol were detected in EFN (total concentration: 9.17 ± 7.84 g of sugar/100 ml). The concentration of inositol was lowest (0.2%) compared to the other sugars, while concentrations of sucrose (28.7%) and fructose (32.2%) were the highest (Table 1). The eight amino acids present in quantifiable amounts in EFN (Table 1) were the essential amino acids isoleucine, leucine, phenylalanine, and valine (total concentration: 0.22 ± 0.29 g/100 ml), and the non-essential amino acids ala-

nine, glycine, glutamine, and proline (total concentration: 0.13 ± 0.057 g/100 ml).

No amino acids were detected in phloem sap – only sugars, *viz.*, sucrose, glucose, fructose, galactose, the sugar alcohol inositol, and traces of altrose (Table 1). Unlike in EFN, the average percent composition of inositol was the highest, followed by that of fructose and sucrose. Galactose and glucose were present in much lower proportions (Table 1).

In honeydew, most detected sugars were also found in EFN, of which fructose had the highest concentration, followed by galactose. However, honeydew also contained sugars absent from EFN, *viz.*, maltose, turanose, lactose, and melibiose, which were present in lower proportions than the major sugars (Table 1). Total sugar concentration in honeydew (13.12 ± 14.9 g/100 ml) was significantly greater than in EFN (Wilcoxon rank sum test: $W = 2.5$, $N = 11$, $P = 0.03$). Honeydew had higher concentrations of inositol ($W = 3$, $N = 11$, $P = 0.03$), and lower concentrations of sucrose ($W = 27$, $N = 11$, $P = 0.03$) compared to EFN. Fructose, galactose, glucose, and altrose concentrations did not differ between honeydew and EFN. Amino acids, *viz.*, isoleucine, alanine, proline, tyrosine, and lysine, were detected as traces in honeydew samples, and hence were not quantified. Honeydew samples had fewer types of amino acids compared to EFN (Table 1).

TABLE 1. Relative percent compositions and concentrations of sugars and free amino acids detected in the phloem sap, and EFN of *H. brunonis*, and in honeydew obtained from *Hemiptera* foraging on *H. brunonis* petioles. All values are mean \pm SD. ND = not detected.

Components	Phloem sap (N = 9 stems)	EFN (N = 6 leaves)		Honeydew (N = 5 Hemiptera colonies)	
	Percent	Concentration (g/100 ml)	Percent	Concentration (g/100 ml)	Percent
Sugars					
Sucrose	21.59 \pm 11.58	3.08 \pm 3.002	28.69 \pm 6.21	0.63 \pm 0.66	8.17 \pm 9.21
Fructose	23.34 \pm 8.62	2.92 \pm 2.38	32.18 \pm 2.65	3.38 \pm 3.68	26.32 \pm 2.59
Galactose	9.70 \pm 5.33	1.86 \pm 1.63	19.78 \pm 1.58	2.45 \pm 2.30	19.89 \pm 4.71
Glucose	5.68 \pm 2.10	0.53 \pm 0.40	6.06 \pm 0.81	0.86 \pm 0.83	6.87 \pm 1.5
Inositol	37.61 \pm 10.01	0.21 \pm 0.17	2.28 \pm 0.68	0.97 \pm 1.01	11.06 \pm 7.29
Altrose	2.08 \pm 6.24	0.56 \pm 0.35	7.45 \pm 2.33	0.90 \pm 0.77	7.91 \pm 2.54
Lactose	ND	ND	ND	1.72 \pm 1.98	11.04 \pm 6.75
Maltose	ND	ND	ND	2.14 \pm 4.06	8.38 \pm 9.95
Turanose	ND	ND	ND	0.06 \pm 0.07	0.36 \pm 0.12
Melibiose	ND	ND	ND	Trace	Trace
Amino acids					
Isoleucine	ND	0.01 \pm 0.02	0.07 \pm 0.10	Trace	Trace
Leucine	ND	0.06 \pm 0.07	0.53 \pm 0.51	ND	ND
Phenyl-alanine	ND	0.09 \pm 0.16	0.44 \pm 0.72	ND	ND
Valine	ND	0.06 \pm 0.06	0.40 \pm 0.49	ND	ND
Lysine	ND	ND	ND	Trace	Trace
Alanine	ND	0.04 \pm 0.05	0.62 \pm 1.03	Trace	Trace
Glycine	ND	0.02 \pm 0.04	0.44 \pm 0.80	ND	ND
Glutamine	ND	0.01 \pm 0.02	0.27 \pm 0.31	ND	ND
Proline	ND	0.01 \pm 0.02	0.09 \pm 0.15	Trace	Trace
Aspartic acid	ND	0.01 \pm 0.01	0.05 \pm 0.16	ND	ND
Tyrosine	ND	0.03 \pm 0.06	0.65 \pm 1.60	Trace	Trace

The compositions of EFN, honeydew and phloem sap were distinct (Fig. 1A). EFN samples were tightly grouped, while phloem sap and honeydew samples were less tightly clustered. In the cluster analysis (Fig. 1B), honeydew and EFN were closer to each other than to phloem sap. All EFN samples and all, except one sample (outlier), of honeydew formed clusters that were distinct from each other and also from phloem sap ($P < 0.05$) with high AU/BP (approximately unbiased/bootstrap probability) values.

EFN was considered merely secreted phloem sap (Bentley 1977, Heil 2011). Like floral nectar, however, EFN is a complex mixture of various components absent from phloem sap (Orona-Tamayo *et al.* 2013, Lohaus & Schwardtfefer 2014) and whose synthesis likely occurs in secretory cells found in extrafloral nectaries of numerous ant-plants (Díaz-Castelazo *et al.* 2005, Escalante-Pérez & Heil 2012, Villamil *et al.* 2013). In *H. brunonis*, the richer amino acid composition and significantly lower proportion of inositol in EFN compared to phloem sap indicates that EFN

is actively synthesized in the nectaries. The presence of secretory cells in the foliar nectary (Fig. S2), and vascular bundles at the nectary base which could upload carbohydrates from sieve tubes to secretory cells for processing into nectar sugars (Heil 2011, Orona-Tamayo *et al.* 2013, Lin *et al.* 2014), further supports active synthesis of EFN in *H. brunonis*. The nectaries remain functional and do not change in size throughout leaf expansion (Fig. S1; Appendix S1), indicating a merocrine type of EFN secretion (Escalante-Pérez *et al.* 2012). More research on ultrastructure and physiology, such as the photosynthetic ability (Lüttge 2013) of the greenish extrafloral nectaries of *H. brunonis*, would enhance our understanding of EFN secretion in this system and in ant-plants in general.

In honeydew, the relative proportion of sucrose attractive to ants in this system (Shenoy *et al.* 2012) was low; it also contained oligosaccharides (maltose, turanose, lactose, and melibiose) that are less attractive to ants than sucrose (Blüthgen & Fiedler 2004). These oligosaccharides are present in most honeydew compositions reported to date (Völkl *et al.* 1999, Blüthgen *et al.* 2004), and are synthesized in Hemipteran guts to reduce osmotic pressure due to a high sugar diet (Rhodes *et al.* 1997). Further, inositol concentration was significantly higher in honeydew than in EFN. Inositol is considered either unimportant (Rudgers & Gardener 2004) or with an equivocal effect on ants (Shenoy 2008). The absence of asparagine, cysteine, and methionine in all samples (EFN, honeydew, and phloem sap) is supported by observations in other ant-plants where these amino acids were rarely present (Blüthgen *et al.* 2004). Our results therefore suggest that *H. brunonis* EFN is likely a more attractive food resource for ants than honeydew and might distract them from tending Hemiptera (Becerra & Venable 1989, Savage & Rudgers 2013). This may explain why during our study period spanning over five field season years, we only once came across ant-tended Hemiptera colonies (mealybugs, Suborder: Stenorrhyncha, Family: Pseudococcidae) on *H. brunonis*. Taken together, the tighter grouping of EFN compared to honeydew and phloem sap samples reflects lower variation in the composition of EFN, and suggests active synthesis of EFN with a composition attractive to ants as well as greater selection pressure on its constituents.

Most reported ant-Hemiptera trophobioses are with Hemiptera of the suborder Stenorrhyncha (Delabie 2001) where honeydew droplets are directly solicited by ants. A few cases of suborder Auchenorrhyncha (Fagundes *et al.* 2013) also release honeydew as droplets. Very few studies (*e.g.*, Del-Claro & Oliveira 1996, Naskrecki & Nishida 2007) report stable trophobiotic associations between ants and fulgorid Hemiptera of the suborder Auchenorrhyncha in which honeydew is flicked away rather than directly solicited by ants (but see Steiner *et al.* 2004, Holzinger 2009). Such flicked honeydew may not be as reliable a resource as EFN whose availability at nectaries of young leaves is assured. Ant-Hemiptera trophobiotic interactions could be viewed as a gradient in a spectrum ranging from strong (*i.e.*, constantly ant-tended) to facultative, to non-ant-tended honeydew flicking Hemiptera (*e.g.*, the commonly found fulgorid Hemiptera on *H. brunonis*).

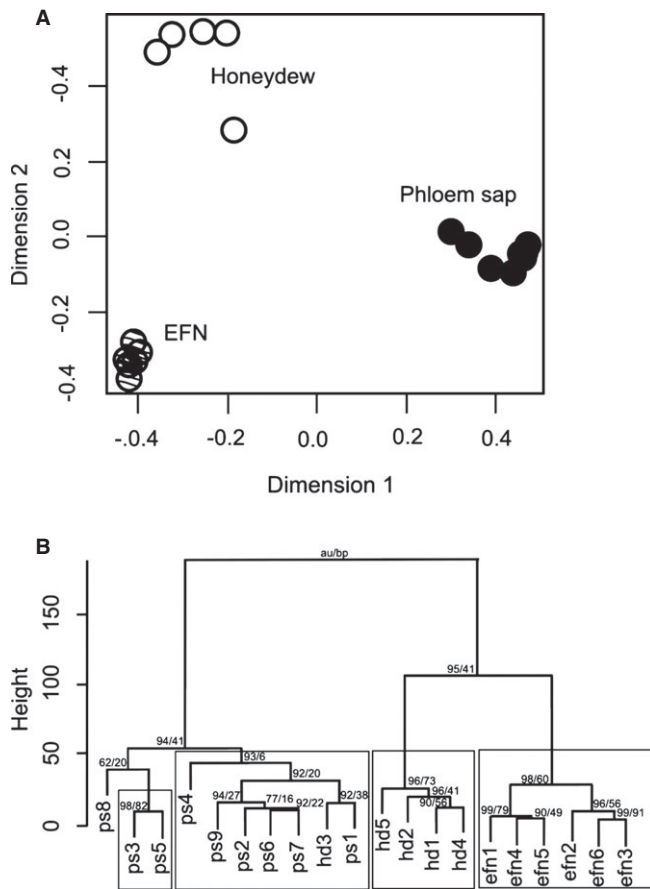


FIGURE 1. Relationship among EFN, honeydew, and phloem sap of *Humboldtia brunonis* based on the relative percent values of their free amino acid and sugar compositions in a multidimensional scaling plot (A), and (B) cluster dendrogram (hd: honeydew and ps: phloem sap). Gray boxes in the cluster dendrogram indicate distinct clusters ($P < 0.05$). Approximately unbiased (AU) and bootstrap probabilities (BP) values are shown.

Although most ant species on *H. brunonis* are not effective for protection, such ants do not tend Hemiptera on the host plant. Instead, they feed on EFN and via domatia occupancy feed the plant nitrogen absorbed from the occupied domatia (Chanam *et al.* 2014b). The high volume of EFN contains not only sugars but also essential amino acids and is constitutively produced even from the very young leaf phase. This indicates that EFN in *H. brunonis* could have evolved not only to attract ants for protection (Shenoy & Borges 2010, Shenoy *et al.* 2012, Chanam *et al.* 2014a) and within the context of a trophic mutualism with ants (Chanam *et al.* 2014b) but also to distract the ants from tending Hemiptera. However, the possibility that EFN production in this species could result from phylogenetic inertia cannot be ignored (Keeler 1985, Nogueira *et al.* 2012). This study is the first to report a comparative analysis of EFN, honeydew, and phloem sap within the same plant species, and is also a rare investigation in an Asian tropical ant-plant.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

FIGURE S1. Expansion of *Humboldtia brunonis* leaf, observed from leaf bud to mature leaf stages.

FIGURE S2. Morphology of the foliar extrafloral nectaries of *Humboldtia brunonis*.

APPENDIX S1. Nectary size and leaf expansion.

APPENDIX S2. Gas chromatography-mass spectrometry (GC-MS) analysis.

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