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HOST-PLANT CHEMICALS AND DISTRIBUTION OF *Neuquenaphis* ON *Nothofagus*

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Abstract—Extractable metabolites from leaves (EM) and volatiles released from six *Nothofagus* species were analyzed by TLC and GC-MS, respectively. Aphids of the genus *Neuquenaphis*, closely associated to *Nothofagus*, were sampled on each *Nathofagus* species. Cluster analyses of *Nothofagus* species were performed based on the presence or absence of EM and volatiles. Dissimilarity distances, from the cluster analyses of EM and volatiles, were used to evaluate their association with the aphid distribution. A major component identified from EM and volatiles of three species of *Nothofagus*, α-agarofuran, was attractive to alates of the oligophagous *Neuquenaphis sensoriata*, which use them as hosts. These results suggest that chemicals play a significant role in the host-plant associations between *Neuquenaphis* and *Nothofagus*.

Key Words—*Nothofagus*, *Neuquenaphis*, volatiles, olfactometry, α-agarofuran.

INTRODUCTION

The genus *Nothofagus* (Fagaceae), the southern beech, comprises about 35 living species distributed in South America, Australia, New Zealand, New Caledonia, and Papua New Guinea (Poole, 1987). This disjunct distribution constitutes a canonical example of biogeographical patterns explained by the continental drift hypothesis (Poole, 1987). In Chile, 10 species belonging to this genus have been

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described (Ramírez, 1987). They account for nearly half of the area covered by native forests (Carrillo and Cerda, 1987).

The biogeographic relevance of this genus (Schlinger, 1974; McQuillan, 1993; Starý, 1994; Burckhardt, 1997) as well as its economic significance for forestry (Carrillo and Cerda, 1987) has promoted the study of its entomofauna. Among herbivore guilds, sap-suckers show one of the highest species richness (McQuillan, 1993). In particular for South America, aphids, represented by the genus *Neuquenaphis* (Hemiptera: Aphididae), are a major component of this diversity (McQuillan, 1993; Quednau and Remaudière, 1994).

A recent review of the aphid fauna from Chile reported that *Nothofagus glauca* (Phil.) Krasser, *Nothofagus obliqua* (Mirb.) Oerst. var. *obliqua*, and *Nothofagus dombeyi* (Mirb.) Oerst. share five of the 12 extant *Neuquenaphis* species, while the seven remaining aphid species are mainly monophagous on different *Nothofagus* species (Fuentes-Contreras et al., 1997).

During host selection by aphids, visual and olfactory stimuli play an important role in host recognition, while epidermal and internal constituents are important during host acceptance (Niemeyer, 1990). Thus, laboratory and field studies have demonstrated the response of aphids to plant volatiles (Pettersson, 1970; Campbell et al., 1993; Nottingham and Hardie, 1993; Pettersson et al., 1995; Quiroz and Niemeyer, 1997), chemical and physical characteristics of the plant surface (Klingauf, 1987), and extractable chemical constituents (Niemeyer, 1991). Furthermore, volatiles (Jackson *et al.*, 1996; Jackson and Dixon, 1996) and internal secondary metabolites (Zucker, 1982) explain the distribution of aphids on different microhabitats within a tree.

The reported patterns of *Neuquenaphis–Nothofagus* associations led us to initiate research that explores the role of chemical factors, particularly the leaf volatiles of the six main *Nothofagus* species in central Chile, in determining the distribution of *Neuquenaphis*.

METHODS AND MATERIALS

Study Sites. During December 1996, aphid and chemical samplings were performed in two protected areas near Talca, Chile: (a) Altos de Lircay National Reserve (35°37′S; 71°03′W), where five species of *Nothofagus* [*No. glauca, No. obliqua, No. dombeyi, No. pumilio* (P. et E.) Krasser and *No. antarctica* (G. Forster) Oerst.] were present and studied, and (b) Los Ruiles National Reserve (35°49′S: 72°31′W), where *No. alessandrii* Esp. was studied.

Aphid Sampling. Five sampling sites were designated, and the aphid distribution was evaluated in all *Nothofagus* species present at each site. Three branches of each tree were randomly chosen, and the aphids present in the apical 15 cm of each branch were counted. Aphids from every tree sampled were

collected and kept in 70% ethanol for later taxonomic identification, according to the key of Quednau and Remaudière (1994).

Entrainment of Nothofagus Volatiles. The apical 30 cm of a branch 1 m above the ground of each *Nothofagus* species was enclosed in a plastic bag. Air entering the bag was purified by passage through a filter with activated charcoal and exited the bag through a filter containing Porapak Q. The air was drawn with an oil-free pump at 1 liter/min for 1 hr. A blank trapping was carried out in each site, 1 m from the sampled branch. The trapped volatiles were desorbed from the Porapak Q with distilled diethyl ether (2.5 ml) and concentrated (200 μl) under a stream of nitrogen to give an extract containing the leaf "volatiles."

Analysis of Volatiles. Aliquots (1 μl) of the concentrated volatile extracts were analyzed by GC-MS. A capillary Ultra-2 HP GLC column (25 m × 0.2 mm ID) was directly coupled to a mass detector with an integrated data system (GC model HP-5890, MD model HP-5972). Ionization was by electron impact at 70 eV and 280°C. The GC oven was programmed to remain at 40°C for 4 min and then increased at 5°C/min to 300°C. A library was constructed of the retention time and mass spectra of all compounds detected in the volatile extracts of each *Nothofagus* species studied. The presence or absence of a given compound in the profile of each *Nothofagus* species was determined by comparing retention times and mass spectra with this library. The GC peaks were considered to be coincident and the compounds identical if their retention times did not differ by more than ±0.03 min and the similarity index of their mass spectra (Pensyna, 1976) was higher than 95%. The components of the volatile extract were identified by comparing their GLC Kováts indexes and mass spectra with those of commercial standards. Where standards were not available, spectra were either compared with a library database by a reverse search technique, which verifies that the main peaks in a reference spectrum are present in the unknown spectrum (Pensyna, 1976), or by analysis of the mass fragmentation patterns.

Extraction of Nonvolatiles. Young leaves of each *Nothofagus* species were collected, air-dried, ground, and extracted (70 g) with CH_2Cl_2 (0.5 liter) for 20 min. The mixture was filtered, and the residue reextracted with MeOH (0.5 liter) for 20 min. Both extracts were concentrated to dryness in a rotary evaporator. Each extract was dissolved in acetone, and aliquots of these solutions (corresponding to 100 mg of dry plant material) were analyzed by TLC (Silica gel F-254) by development with CH_2Cl_2 (for the CH_2Cl_2 extract) and MeOH/CH_2Cl_2 (1 : 9) (for the MeOH extract). The TLC plates were visualized with Liebermann reagent, vanillin/H_2SO_4, or phosphomolybdic acid. A table of the presence or absence of extractable metabolites was produced in terms of R_f values.

*α-Agarofuran (Compound **30**).* Dry leaves (100 g) of *No. glauca* were steam distilled. The aqueous distillate was extracted with CH_2Cl_2 (×3) and the organic phase concentrated to a pale yellow oil (0.5 g). The oil was subjected to chromatography on silica gel, with a stepwise hexane–CH_2Cl_2 gradient. Pure

α-agarofuran (50 mg) was eluted with hexane–CH_2Cl_2 (55 : 45) as an oil, $[\alpha]_D$ = +36.4° ($CHCl_3$, c = 2.5), lit. +41.5° (Barrett and Buchi, 1967). ^1H NMR (300 MHz, d_6-benzene) δ: 5.55 (1H, br s), 2.06 (1H, ddd), 1.97 (1H, dd), 1.90 (2H, m), 1.71 (3H, s), 1.70 (1H, dd), 1.66 (1H, dd), 1.54 (2H, m), 1.49 (1H, d), 1.29 (3H, s), 1.14 (3H, s), 1.09 (1H, ddd), 0.99 (1H, dd), 0.86 (3H, s). ^{13}C NMR δ: 133.3 (C-4), 126.7 (C-3), 84.7 (C-5), 80.6 (C-11), 44.6 (C-7), 37.3 (C-10), 34.8 (C-9), 33.2 (C-1), 32.7 (C-6), 30.5 (C-12), 24.8 (C-8), 23.0 (C-2), 23.0 (C-13), 22.0 (C-15), 19.3 (C-14). El-MS m/z (rel. int. %): 220 (M^+, $C_{15}H_{24}O$, found, 220.1833, requires, 220.1827, 87), 205 (28), 202 (21), 187 (11), 177 (9), 162 (12), 147 (34), 131 (11), 124 (14), 123 (36), 122 (14), 121 (14), 119 (12), 109 (32), 107 (12), 105 (22), 95 (15), 91 (25), 82 (100), 55 (22), 43 (30), 41 (46).

Olfactometry. Behavioral studies were performed in an olfactometer as described by Pettersson (1970). The olfactometer consisted of a quadratic arena permeated by air (250 ml/min) coming from the stretched-out corners (arms) and drawn on through a hole above its center. Two glass tubes containing the same single chemical stimulus were connected to the end of two consecutive arms; tubes containing control stimulus (hexane) were connected to the other two arms. The observation arena was divided into four arm zones and one indifferent zone in the center. A field-collected test aphic (*Neuquenaphis sensoriata*, alate and apterous, and *Ne. bulbicauda*) was placed in the center of the arena and the time the aphid spent in each arm was recorded during 15 min with the olfactometer being rotated by 90° every minute. Each experiment was replicated 10 times and results analyzed by nonparametric statistics (Wilcoxon one-tailed rank-sum test for two groups); the mean time spent in the treatment arms was compared with the mean time spent in the control arms.

Dissimilarity and Correlation Analyses. For each *Nothofagus* species, the presence or absence of EM, volatiles, and *Neuquenaphis* species was tabulated, and cluster analysis performed to compare the chemical profiles with aphid distribution. Distance measures between species were calculated according to the dissimilarity algorithm 1 – (Pearson's r), which assigns a value of 0 to maximum similarity and a value of 1 to minimum similarity. Clusters of species were estimated by using unweighed pair-group of arithmetic average (UPGMA) as a linkage rule (Manly, 1994). Subsequently, the values of distance obtained from the cluster analysis of volatiles and internal secondary metabolites were separately used to estimate simple Kendall's correlations with aphid distribution (Siegel and Castellan, 1988).

RESULTS AND DISCUSSION

The distribution of aphids observed on the six *Nothofagus* species studied is shown in Table 1. This distribution generally agrees with data reported in

TABLE 1. AVERAGE NUMBER OF APHIDS PER BRANCH OBSERVED ON SIX *Nothofagus* SPECIES IN CENTRAL CHILE

	Nothofagus (mean ± SD)						Previous
Neuquenaphis	*glauca*	*obliqua*	*dombeyi*	*pumilio*	*antarctica*	*alessandrii*	reports[a]
sensoriata	7.8 ± 16.7	7.8 ± 9.7			4.5 ± 7.7		*No. glauca* *No. obliqua*
bulbicauda			2.0 ± 6.9				*No. dombeyi* *No. obliqua*[b]
similis				10.4 ± 12.9			*No. pumilio* *No. obliqua*[c]
staryi						0.5 ± 0.5	*No. alessandrii*

[a]Information from Fuentes-Contreras et al. (1997).
[b]British Museum of Natural History, possible wrong identification of the host plant.
[c]Hille Ris Lambers (1968), possible wrong identification of the host plant.

the literature (Quednau and Remaudière, 1994; Fuentes-Contreras et al., 1997), although this is the first report of *Ne. sensoriata* occurring on *No. antarctica*. The eventual exceptions to the general agreement with previously published data are *Ne. bulbicauda* Hille Ris Lambers and *Neuquenaphis similis* Hille Ris Lambers. In the first instance, a single record at the British Museum of Natural History (BMNH) collection indicates that *Ne. bulbicauda* was collected on *No. obliqua*. However, all other records of this species in the BMNH collection (72 mounted specimens) and information from the literature (Fuentes-Contreras et al., 1997) report this aphid on *No. dombeyi*. In the second instance, *No. obliqua* was originally mentioned as a host plant for *Ne. similis* (Hille Ris Lambers, 1968). Since all the subsequent collections of *Ne. similis*, including the present one, have been obtained form *No. pumilio* (Blackman and Eastop, 1994), it is likely that the six specimens of the type series (one holotype and five paratypes) of *Ne. similis* were labeled incorrectly by their original collectors. Neither *Ne. similis* nor *Ne. bulbicauda* were observed on *No. obliqua* in spite of the coexistence of these three species in a same sampling site.

From the GC and TLC profiles of the volatile and extractable metabolites, respectively, cluster analysis enabled dendrograms of the six *Nothofagus* species to be constructed. A nonsignificant Kendall's correlation ($\tau = 0.31$, $N = 14$, $P = 0.10$) was found between aphid distribution and the TLC profile of extractable leaf metabolites (principally nonvolatiles). Similarly, a nonsignificant Kendall's correlation ($\tau = -0.06$, $N = 15$, $P = 0.74$) was found between aphid distribution and the GC profile of the entrained leaf volatiles. Although the probability values are not significant at the 5% level in either case, comparison of their magnitudes suggests a weak but closer to significant correlation with the extractable metabolites profile and aphid distribution. However, the correlations

of extractable metabolites and volatiles may not be absolutely independent since components in one group may also occur in the other group.

The identity and relative abundance of the constituents of the entrained volatiles collected from the foliage of each *Nothofagus* species are shown in Table 2. The compounds listed can be categorized into three groups. The first group consists of commonly found leaf volatiles (Knudsen et al., 1993), *viz*, monoterpenes (compounds **1**, **2**, **3**, **9**, **10**, and **12** in Table 2), methylheptenone (**6**), hexene esters (**7** and **8**), aldehydes (**4**, **13**, and **18**), phenolics (**5**, **11**, and **18**), and ethyl octanoate (**16**). The second group, corresponding to ca. 46% of all compounds listed in Table 2 are provisionally identified as sesquiterpenes (**19–27**, **29–34**, and **36**). Some of these compounds are common plant constituents, e.g., α-cubebene, copaene, and caryophyllene, while others have restricted occurrence. The third group of volatiles are those compounds (**14**, **15**, **28**, and **35**) that remain to be identified. Both the relative importance of these three groups and the abundance of individual compounds were highly variable between *Nothofagus* species.

The major sesquiterpene occurring in the volatile profile of *No. obliqua*, *No. glauca*, and *No. antarctica* was identified as α-agarofuran (5,11-oxy-5β,10α-eudesm-3-ene, **30**) (Figure 1), a sesquiterpene previously isolated from agarwood (*Aquillaria agallocha*) (Barrett and Buchi, 1967). α-Agarofuran was readily isolated from a steam distillate of dried leaves of *No. glauca* and its identity established from careful analysis of its proton and carbon NMR spectra. A 2D-COSY experiment quickly established carbon–proton connectivities, and the eudesmane skeleton and position of the oxy ring was established from a Heteronuclear Shift Correlation Multiple Bond Connectivities experiment. A Nuclear Overhauser Enhancement of the C-15 angular methyl on irradiation of the C-6 proton ($\triangleq$ 1.46) showed that the ring junction was *trans*, consistent with **30**.

The occurrence of α-agarofuran in *No. obliqua*, *No. glauca* and *No. antarctica*, and its absence in *No. dombeyi*, correlates with the former trees being host-plants and the latter tree a nonhost of the oligophagous *Ne. sensoriata*. Therefore, α-agarofuran is a good candidate for studying the role of host-plant chemicals in the orientation behavior of these aphids. Olfactometric bioassays showed that alate morphs of *Ne. sensoriata* were significantly attracted by α-agarofuran compared to the solvent control (Table 3), but the apterous morph did not show a significant response. Such differential response between alate and apterous morphs has been reported for other aphid species (Quiroz and Niemeyer, 1997). Apterous individuals of the monophagous *Ne. bulbicauda*, which associate with *No. dombeyi*, a species lacking α-agarofuran, was not significantly affected by α-agarofuran in olfactometric tests (Table 3). The alate morph of *Ne. bulbicauda* was not observed in the field and has not been reported in the literature (Hille Ris Lambers, 1968; Quednau and Remaudière, 1994; Blackman and Eastop, 1994). It could not be tested.

TABLE 2. COMPOSITION OF VOLATILES FROM SIX *Nothofagus* SPECIES[a]

Peak and Cmpd No.	Compound	Identification method[b]	GLC Kovats index Ultra-2	Relative areas (%)					
				N. o.	N. g.	N. d.	N. an.	N. al.	N. p.
1	3-Carene	B	920	nd	nd	3.6	nd	nd	nd
2	α-Pinene	A	933	nd	nd	23.2	nd	nd	1.9
3	Camphene	A	949	nd	nd	19	nd	nd	1.1
4	Benzaldehyde	A	960	0.8	0.2	2.6	4.4	8.5	1.1
5	Phenol	A	977	0.3	nd	7.5	8.2	9.9	7.5
6	6-Methyl-5-hepten-2-one	A	984	nd	nd	5.3	4	6.5	nd
7	(*Z*)-3-hexenyl acetate	A	1004	40.1	2.4	nd	9.9	22.4	14.4
8	Hexyl acetate	A	1009	0.7	0.4	nd	4.1	nd	nd
9	Limonene	A	1030	nd	nd	nd	1.4	3.6	12.5
10	(*E*)-3,7-Dimethyl-1,3,6-octatriene	B	1046	0.9	0.1	nd	6.6	nd	nd
11	Acetophenone	A	1068	nd	nd	nd	nd	2.4	nd
12	Linalool	A	1089	3.1	0.5	2.3	nd	nd	nd
13	Nonanal	A	1102	0.7	0.4	5.7	6	8.7	1.5
14	Unknown 1 M_r 150 (*m/z*:41, 53, 69,[c] 81, 107, 121, 135)		1114	0.4	nd	nd	2.8	nd	nd
15	Unknown 2 M_r 115 (*m/z*:29,[c] 44, 56, 72, 86, 100)		1129	nd	nd	nd	nd	nd	5.1
16	Ethyl actanoate	B	1193	1.3	nd	nd	2.5	3.8	2
17	Decanal	A	1203	0.4	0.3	3.2	2.8	4.1	0.7

TABLE 2. CONTINUED

Peak and Cmpd No.	Compound	Identification method[b]	GLC Kovats index Ultra-2	Relative areas (%)					
				N. o.	N. g.	N. d.	N. an.	N. al.	N. p.
18	4-Ethylacetophenone	B	1267	nd	0.4	nd	1.4	nd	nd
19	α-Cubebene	A	1356	nd	0.6	nd	nd	nd	nd
20	α-Amorphene	B	1378	nd	0.8	nd	nd	nd	nd
21	Copaene	A	1386	0.2	10.6	nd	4	nd	nd
22	Sesquiterpene M_r 204 (*m/z*: 41, 55, 57, 77, 91, 105,[c] 119, 133, 147, 161, 175, 189)	B	1421	2.2	nd	nd	nd	nd	nd
23	Caryophyllene	A	1437	1.3	20.4	nd	nd	nd	nd
24	Sesquiterpene M_r 204 (*m/z*: 41,[c] 55, 69, 79, 91, 105, 119, 133, 147, 161, 189)	B	1475	10.7	1.8	nd	nd	nd	2.7
25	Sesquiterpene M_r 204 (*m/z*: 41, 55, 67, 81,[c] 91, 107, 121, 133, 147, 161, 189)	C	1484	1.1	nd	nd	nd	nd	nd
26	Sesquiterpene M_r 204 (*m/z*: 41, 55, 67, 80, 91, 105,[c] 121, 133, 147, 161, 189)	C	1497	0.6	nd	nd	nd	nd	nd
27	Saturated naphthalene M_r 204 (*m/z*: 41, 55, 67, 79, 91, 105, 119, 133, 147, 161,[c] 189)	B	1507	6.7	14.7	nd	4	nd	0.5
28	Unknown 3 M_r (*m/z*: 43,[c] 55, 69, 81, 95, 109, 123, 137, 149, 161, 189)		1519	nd	0.6	nd	nd	nd	nd

29	Sesquiterpene M_r 204 (*m/z*: 41, 55, 67, 79, 91, 105, 119, 133, 147, 161,[c] 189)	C	1536	0.3	1.6	nd	nd	nd	nd
30	α-Agarofuran	A	1567	19.9	38.7	nd	26	nd	nd
31	Sesquiterpene M_r 204 (*m/z*: 43,[c] 55, 69, 81, 91, 107, 121, 133, 147, 161, 189)	C	1603	nd	nd	nd	nd	nd	7.6
32	Sesquiterpene M_r 204 (*m/z*: 43,[c] 55, 67, 80, 91, 105,[c] 121, 133, 147, 161, 189)	C	1612	nd	nd	3.5	nd	nd	13.7
33	Sesquiterpene M_r 204 (*m/z*: 43,[c] 55, 69, 81, 91, 107, 122, 133, 147, 161, 189)	C	1623	1.1	nd	nd	nd	nd	nd
34	Saturated naphthalene M_r 204 (*m/z*: 59, 77, 105, 119, 135, 149, 161,[c] 189)	C	1672	1.5	0.8	nd	nd	nd	nd
35	Unknown 4 M_r 218 (*m/z*: 41, 55, 65, 77, 91, 105, 115, 128, 135,[c] 149, 162, 175, 203)		1785	3.7	1.5	24.2	11.9	30	27.7
36	Saturated naphthalene M_r 204 (*m/z*: 43, 55, 67, 79, 91, 105, 119, 133, 147, 161,[c] 189)	C	1795	2	1.3	nd	nd	nd	nd
Relative total area[d]				3.56	6.11	1.46	1.40	1.39	2.34

[a]As percent of total volatile compounds. N. o. = Nothofagus obliqua var. obliqua, N. g. = No. glauca, N. d. = No. domeyi, N. an. = No. antarctica, N. al. = No. alessandrii, N. p. = No. pumilio, nd = not detected.

[b]Identification codes: A = comparison with authentic compounds; B = GC-MS comparison between the recorded and library mass spectra with a similarity index higher than 95%; C = tentative identification based on fragmentation patterns in the mass spectra.

[c]Main peak.

[d]Calculation based on total area/area of internal standard (docosane).

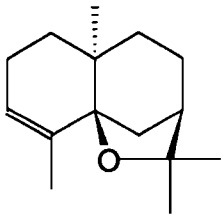

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FIG. 1. 5,11-Oxy-5β,10α-eudesm-3-ene, or α-agarofuran, the major sesquiterpene occurring in the volatile profile of *Nothofagus obliqua*, *Nothofagus glauca*, and *Nothofagus antarctica*.

TABLE 3. RESPONSE OF *Neuquenaphis* INTRODUCED SINGLY INTO AN OLFACTOMETER AND EXPOSED TO 10 μg OF α-AGAROFURAN AND HEXANE

Test aphid	Time spent (min)[a]		P^b
	Hexane	10 μg of α-agarofuran	
N. sensoriata alatae	4.9 ± 1.7	8.0 ± 1.1	0.01
N. sensoriata apterae	5.5 ± 1.5	6.4 ± 1.8	0.20
N. bulbicauda apterae	7.9 ± 3.3	4.6 ± 2.8	0.09

[a]Mean ± standard error.
[b]Wilcoxon one-tailed rank-sum test for two groups.

In summary, the results show that patterns of host-plant volatiles emitted from leaves, and α-agarofuran in particular cases, may play a significant role in host orientation of aphids that feed on *Nothofagus*. Careful identification and testing of the remaining *Nothofagus* volatile compounds with *Neuquenaphis* will be required before a complete picture can emerge. The nonvolatile or extractable metabolites undoubtedly play a role in host acceptance and the correlations described in this work, although weak, will need to be confirmed by detailed chemical and behavioral assays.

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