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Retention index database for identification of general green leaf volatiles in plants by coupled capillary gas chromatography–mass spectrometry

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Abstract

A series of ubiquitously occurring saturated and monounsaturated six-carbon aldehydes, alcohols and esters thereof is summarised as 'green leaf volatiles' (GLVs). The present study gives a comprehensive data collection of retention indices of 35 GLVs on commonly used non-polar DB-5, mid-polar DB-1701, and polar DB-Wax stationary phases. Seventeen commercially not available compounds were synthesised. Thus, the present study allows reliable identification of most known GLV in natural plant volatile samples. Applications revealed the presence of several seldom reported GLVs in headspace samples of mechanically damaged plant leaves of *Carpinus betulus* and *Fagus sylvatica*. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Retention indices; *Fagus sylvatia*; *Carpinus betulus*; Closed loop stripping analysis; Plant materials; Green leaf volatile compounds; Volatile organic compounds

1. Introduction

Following mechanical damage, green plant tissue releases a characteristic odour known as 'green leaf odour'. Investigation of the involved processes revealed that this odour is due to a series of saturated and monounsaturated six-carbon aldehydes, i.e., hexanal, (*E*)-2-hexenal, (*E*)- and (*Z*)-3-hexenal and the corresponding alcohols which are formed by enzymatic degradation of unsaturated fatty acids [1,2]. Esterification of most abundant alcohols (*Z*)-3-hexenol, (*E*)-2-hexenol, and 1-hexanol with common metabolic carboxylic acids additionally leads to the formation of green leaf esters. For some of these so-called 'green leaf volatiles' (GLVs) antimicrobial activity has been shown suggesting that these compounds might serve as wound disinfectants [3]. Several GLVs have been shown to contribute significantly to the flavour of many plant-derived foods [4–7] and, thus, are commonly used by the flavour industry as aroma chemicals in flavourings and fragrances [8]. On the other hand, several studies have investigated the behaviour-modifying properties of GLVs towards phytophagous arthropods. GLVs may, e.g., attract herbivores to their host plant [3,9,10], synergistically enhance the attractiveness of insect pheromones [11], inhibit the response of

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insects to their sex pheromone [12], or may even be directly used by insects to locate sexual mates [13]. Thus, scientists from different disciplines are interested in this class of compounds. Headspace enrichment and coupled capillary gas chromatography–mass spectrometry (GC–MS) is normally used for chemical analysis of GLVs [10,14–19]. However, several GLVs are not commercially available as synthetic reference compounds and, therefore, identification of less common GLVs is sometimes based merely on comparison of mass spectra with library spectra [17,18]. This status of identification is not satisfying because several GLVs occur as configurational isomers with different sensory properties [20] but very similar mass spectra. For identification of monoterpenes, a class of compounds where similar problems have to be faced, it can be helpful to involve retention data from the literature [21], because modern capillary columns are manufactured with a high degree of reproducibility. It has been shown that retention indices of many aroma chemicals on comparable stationary phases can be estimated at different laboratories with standard deviations of far less than 1% [22]. This paper presents a data collection of retention indices of 35 GLVs on non-polar, medium polar, and polar stationary phases and describes easy methods for the synthesis of commercially not available GLVs to allow optimisation of GLVs identification in natural plant samples. Additionally some applications are given to demonstrate practical relevance of the data.

2. Experimental

2.1. Commercially available reference chemicals

Authentic reference samples of hexanal (98%), (*E*)-2-hexenal (95%), 1-hexanol (98%), (*Z*)-2-hexen-1-ol (95%), (*E*)-2-hexen-1-ol (96%), (*Z*)-3-hexen-1-ol (98%), (*E*)-3-hexen-1-ol (98%), (*Z*)-4-hexen-1-ol (97%), (*Z*)-3-hexenyl formate (99%), hexyl acetate (99%), (*Z*)-3-hexenyl acetate (98%), (*E*)-2-hexenyl acetate (98%), hexyl butyrate (98%), (*Z*)-3-hexenyl butyrate (98%), hexyl isobutyrate (98%), (*Z*)-3-hexenyl tiglate (97%), hexyl hexanoate (97%) and (*Z*)-3-hexenyl benzoate (97%) were purchased from Sigma–Aldrich, Deisenhofen, Germany.

2.2. Chemical syntheses

(*Z*)-3-Hexenal, (*E*)-3-hexenal, and (*Z*)-2-hexenal were synthesised by oxidation of the corresponding alcohols using Dess-Martin periodinane (Lancaster, Mülheim/Main, Germany) [23]. Periodinane (0.0015 mol) was suspended in 10 ml of dichloromethane (HPLC-grade, Fisher Scientific, Loughborough, UK). After addition of 0.001 mol of the respective alcohol, the suspension was stirred at room temperature and the reaction was monitored by GC–MS. After 3 h the oxidation was complete and the reaction mixture was cleaned up by adsorption chromatography using silica gel 60 (70–230 mesh, Merck, Darmstadt, Germany) and dichloromethane as eluent. To prevent losses of the highly volatile aldehydes during solvent removal, eluates were not concentrated but were used after dilution directly for GC–MS analysis.

Reference samples of hexyl propanoate, hexyl tiglate, (*Z*)-3-hexenyl propanoate, (*Z*)-3-hexenyl isobutyrate, (*Z*)-3-hexenyl 2-methylbutyrate, (*Z*)-3-hexenyl 3-methylbutyrate, (*Z*)-3-hexenyl tiglate, (*Z*)-3-hexenyl hexanoate, (*E*)-2-hexenyl propanoate, (*E*)-2-hexenyl butyrate, (*E*)-2-hexenyl isobutyrate, (*E*)-2-hexenyl 2-methylbutyrate, (*E*)-2-hexenyl 3-methylbutyrate, (*E*)-2-hexenyl tiglate, and (*E*)-2-hexenyl hexanoate were synthesised from 1-hexanol, (*Z*)-3-hexen-1-ol, or (*E*)-2-hexen-1-ol by esterification with one of the following carboxylic acids: propionic acid (99.5%), butyric acid (99.5%), isobutyric acid (99%), 2-methylbutyric acid, 3-methylbutyric acid (99%), or hexanoic acid (99,5%), all purchased from Sigma–Aldrich. For esterification 4-dimethylaminopyridine (DMAP, Merck) was used as a catalyst and *N*,*N*′-dicyclohexylcarbodiimide (DCC, Merck) as a condensation reactant [24]. Equimolar quantities (0.01 mol) of alcohols and carboxylic acids and 0.001 mol DMAP were dissolved in 50 ml of dichloromethane. After cooling the solution to 0°C, 0.01 mol of DCC was added, and the reaction mixture was stirred for 6 h. After filtration of the precipitated dicyclohexyl urea and evaporation of the solvent, the reaction product was cleaned by adsorption chromatography using silica gel 60 and dichloromethane as eluent. Fractionation was monitored by GC–MS and fractions that were free from educts and by-products were pooled and

finally freed from the solvent using a rotary evaporator.

2.3. Gas chromatography–mass spectrometry

Analytical separations were performed on a Fisons 8060 GC system, coupled with a Fisons MD800 quadrupole mass spectrometer (Thermoquest, Egelsbach, Germany) using J&W 30 m×0.32 mm I.D. fused-silica columns, film thickness 0.25 μm coated either with non-polar 5% phenylmethylpolysiloxane (DB-5ms), mid-polar 14% cyanopropylphenylmethylpolysiloxane (DB-1701) or polar polyethylene glycol (DB-Wax) stationary phases. Columns were equipped with uncoated 1 m×0.32 mm I.D. fused-silica retention gaps. Helium (purity >99.996%, Air Liquide, Berlin, Germany) was used as carrier gas (inlet pressure 10 kPa). The temperature program started at 40°C, held for 3 min and ran with 3°C min^{-1} to the final temperature, i.e., 280°C for DB-5ms, 260°C for DB-1701, and 230°C for DB-Wax. Injector temperature was kept at 240°C. The column effluent was ionised by electron impact ionisation (EI) at 70 eV at a source temperature of 200°C. Mass range was from 35 to 450 m/z at a scan time of 0.9 s and an interscan delay of 0.1 s. The solvent delay was 1.5 min in case of DB-5ms and DB-1701 columns, and 3 min in case of DB-Wax column. Standard solutions (1 μl) containing reference compounds at 100 ng μl^{-1} in dichloromethane were injected at a 1:10 split ratio together with 0.5 μl of a hydrocarbon mixture (C_7–C_{26}) in hexane, containing odd-numbered *n*-alkanes at concentrations of 160 ng μl^{-1} and even-numbered *n*-alkanes at 80 ng μl^{-1}. Retention indices of each compound on the three stationary phases were calculated according to van den Dool and Kratz [25].

2.4. Applications

2.4.1. Closed loop stripping analysis (CLSA) of green leaf volatiles after insect feeding

Volatiles emitted by a twig of *Fagus sylvatica* (about 30 leaves) being fed on by 13 males of the forest cockchafer, *Melolontha hippocastani* F., were collected for 5 h at room temperature by use of a commercially available apparatus for the Grob closed loop stripping analysis (Brechbühler, Schlieren, Switzerland) [26]. The adsorption tube was eluted with 25 μl of dichloromethane, and 1 μl was injected splitless into the GC–MS system using the DB-5ms column. Identification of green leaf volatiles was done by comparison of mass spectra and retention indices with those estimated by analysis of authentic reference compounds.

2.4.2. Analysis of green leaf volatiles by purge and cold trap technique

Volatiles emitted by *Carpinus betulus* L. were collected by purge and cold trapping using a Tekmar AEROTrap 6000 thermal desorption system (Thermoquest, Egelsbach, Germany) modified for on-line analysis of volatile compounds from living samples by GC–MS [27]. Samples were placed into a 500-ml gas wash bottle that was connected to a liquid nitrogen cooled cryotrap. After an equilibration time of 10 min, volatiles were purged for 1 min to the cryotrap that was kept at −100°C, using clean synthetic air as purge gas (Air Liquide) at a flow-rate of 30 ml min^{-1}. After enrichment, volatiles were thermally desorbed by flash-heating the cryotrap for 3 min to 250°C and transferred to the GC–MS system by purging the trap with carrier gas (helium, head pressure 10 kPa). Prior to GC–MS analysis, volatiles were condensed a second time right before the GC column in a liquid nitrogen-cooled focuser (−100°C) and were finally injected splitless by flash-heating the focuser for 1 min to 250°C. This procedure allowed narrow starting bands resulting in sharp peaks during successive GC–MS analysis. For this solventless injection technique no solvent delay had to be adjusted (for more details compare Ref. [27]). The following *C. betulus* samples were analysed: (a) a twig with 20 intact leaves; (b) 20 leaves, 10 min after mechanical damage (fresh damage); (c) 20 leaves, 1 h after mechanical damage (old damage). Mechanical damage was achieved by cutting the leaves into four pieces using a pair of scissors.

3. Results and discussion

The retention indices estimated for 35 GLVs on non-polar DB-5ms, mid-polar DB-1701, and polar DB-Wax stationary phases are listed in Table 1.

Table 1
Retention indices of green leaf volatiles on columns of different polarities[a]

Compound	M_r	BP (m/z)	Retention index		
			DB-5ms[b]	DB-1701[c]	DB-Wax[d]
Aldehydes					
(1) Hexanal	100	41	802	877	1067
(2) (*Z*)-3-Hexenal	98	41	801	885	1132
(3) (*E*)-3-Hexenal	98	41	802	881	1127
(4) (*Z*)-2-Hexenal	98	55	841	946	1187
(5) (*E*)-2-Hexenal	98	41	848	955	1201
Alcohols					
(6) 1-Hexanol	102	56	869	981	1351
(7) (*Z*)-3-Hexen-1-ol	100	67	849	970	1378
(8) (*E*)-3-Hexen-1-ol	100	41	846	960	1356
(9) (*Z*)-2-Hexen-1-ol	100	57	865	991	1410
(10) (*E*)-2-Hexen-1-ol	100	57	861	982	1400
(11) (*Z*)-4-Hexen-1-ol	100	67	879	1001	1422
Esters					
(12) (*Z*)-3-Hexenyl formate	128	67	920	995	1258
(13) Hexyl acetate	144	43	1015	1082	1264
(14) (*Z*)-3-Hexenyl acetate	142	43	1008	1080	1308
(15) (*E*)-2-Hexenyl acetate	142	43	1019	1090	1323
(16) Hexyl propionate	158	57	1108	1171	1332
(17) (*Z*)-3-Hexenyl propionate	156	67	1100	1170	1375
(18) (*E*)-2-Hexenyl propionate	156	57	1111	1180	1392
(19) Hexyl butyrate	172	43	1194	1257	1406
(20) (*Z*)-3-Hexenyl butyrate	170	67	1187	1256	1448
(21) (*E*)-2-Hexenyl butyrate	170	71	1198	1265	1466
(22) Hexyl isobutyrate	172	43	1151	1208	1335
(23) (*Z*)-3-Hexenyl isobutyrate	170	82	1144	1208	1380
(24) (*E*)-2-Hexenyl isobutyrate	170	71	1152	1214	1391
(25) (*Z*)-3-Hexenyl 2-methylbutyrate	184	67	1231	1296	1463
(26) (*E*)-2-Hexenyl 2-methylbutyrate	184	57	1238	1302	1470
(27) (*Z*)-3-Hexenyl 3-methylbutyrate	184	67	1237	1302	1477
(28) (*E*)-2-Hexenyl 3-methylbutyrate	184	57	1245	1311	1490
(29) Hexyl tiglate	184	101	1330	1405	1602
(30) (*Z*)-3-Hexenyl tiglate	182	82	1322	1404	1647
(31) (*E*)-2-Hexenyl tiglate	182	83	1338	1419	1672
(32) Hexyl hexanoate	200	43	1387	1453	1596
(33) (*Z*)-3-Hexenyl hexanoate	198	82	1380	1453	1643
(34) (*E*)-2-Hexenyl hexanoate	198	99	1391	1461	1660
(35) (Z)-3-Hexenyl benzoate	204	105	1564	1681	2093

[a] M_r, relative molecular mass; BP, base peak of the mass spectrum.
[b] 30 m×0.32 mm I.D. DB-5ms, 0.25 μm film thickness. GC oven program: initial temperature, 40°C for 4 min; rate, 3°C min^{-1} to 280°C.
[c] 30 m×0.32 mm I.D. DB-1701, 0.25 μm film thickness. GC oven program: initial temperature, 40°C for 4 min; rate, 3°C min^{-1} to 260°C.
[d] 30 m×0.32 mm I.D. DB-Wax, 0.25 μm film thickness. GC oven program: initial temperature, 40°C for 4 min; rate, 3°C min^{-1} to 230°C. All columns were equipped with an uncoated 1 m×0.32 mm I.D. fused-silica retention gap.

Results show that there are several critical pairs of GLVs on both non-polar DB-5ms and mid-polar DB-1701 stationary phases with differences <2 retention index units. On DB-5ms not all examined aldehydes and alcohols were resolved, whereas on DB-1701 esters of 1-hexanol and (*Z*)-3-hexenol co-eluted. Thus, individual compounds may be obscured by co-eluting components when these or even less

polar stationary phases like pure methylsilicone are used for plant volatile analysis. This is particularly likely when one compound occurs as main component and the other only in traces. Only polar DB-Wax stationary phase was able to resolve all 35 compounds satisfactorily.

Fig. 1 shows the total ion current chromatogram resulting from the injection of a volatile extract won by closed loop stripping of *F. sylvatica* leaves being fed on by *M. melolontha*. Nineteen of the compounds examined in this study were identified in this extract, among them a series of esters of the most common leaf alcohols 1-hexanol, (*Z*)-3-hexenol, and (*E*)-2-hexenol with butyric acid (compounds 19–21), isobutyric acid (compounds 22–24), 2-methylbutyric acid (25,26), and isovaleric acid (27,28). These compounds may be derived from enzymatic esterification, and some of them have been reported in plant-related systems before [16–18,28]. However, often identification of individual compounds is only tentative because position or configuration of the double bonds have not finally been determined [14,17,18]. Interestingly it has been found for some of these esters that, like in the present study, they are only produced by plants after insect feeding [16–18,28]. Methyl-branched carboxylic acids like isobutyric acid, 2-methylbutyric acid, and isovaleric acid are known to derive from amino acid degradation [29], and are common metabolites of microorganisms developing on protein containing substrates [30]. It will be interesting to investigate the possible role of insect- or plant-associated microorganisms in the formation of these GLV esters. The present paper allows exact identification of all isomers of the relevant esters, and may be a good starting point for future studies on this aspect.

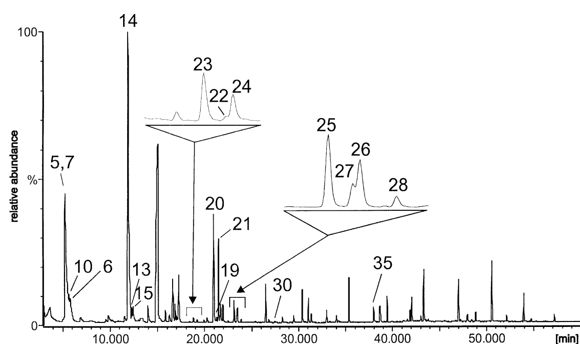

Fig. 1. Total ion current chromatogram of volatiles emitted by *Fagus sylvatica* leaves being fed on by the forest cockchafer, *M. hippocastani*. Volatiles were collected for 4 h by the closed loop stripping technique [26] and eluted with 25 μl of dichloromethane. Separation was performed on a 30-m×0.32-mm I.D. DB-5ms capillary column, 0.25 μm film thickness, equipped with an uncoated 1 m×0.32 mm I.D. retention gap. GC oven program: initial temperature, 40°C for 4 min; rate, 3°C min^{-1} to 280°C. Carrier gas, helium; inlet pressure, 10 kPa. Detection by EI mass spectrometry. Injection volume, 1 μl splitless; injector temperature, 240°C. Peak numbers correspond to Table 1.

The second application using *C. betulus* (Fig. 2) demonstrates very well the fact that GLV emission dramatically increases immediately after artificial damage of plant tissue. Highly volatile aldehydes are the predominating compounds in the headspace of freshly damaged leaves, whereas apart from (*Z*)-3-hexenyl acetate GLV esters are missing. The interesting aspect of this example is the fact that not only common GLV aldehydes hexanal, (*Z*)-3-hexenal, and (*E*)-2-hexenal occurred in the headspace of freshly damaged leaves but also (*E*)-3-hexenal and (*Z*)-2-hexenal, which rarely have been reported among GLVs. One hour after artificial damage of *C. betulus* leaves, aldehydes have nearly vanished from the headspace and (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate are the predominating GLVs.

4. Conclusions

Like monoterpenes, GLVs are common plant constituents and every scientist working on volatile analysis of plant-related systems has to deal with the identification of these classes of compounds. Both monoterpenes and GLVs contain isomeric compounds resulting in similar mass spectra and, thus, mass spectral data alone are not sufficient for reliable identification. For monoterpenes a comprehensive collection of retention data is available [21], which together with mass spectral data has proved to be a helpful tool for identification of those compounds that are not available as synthetic references. The present paper gives a data collection of retention indices of GLVs on three commonly used stationary phases of different polarities and describes convenient methods for the synthesis of commercially not available reference compounds, allowing reliable identification of these compounds. The application

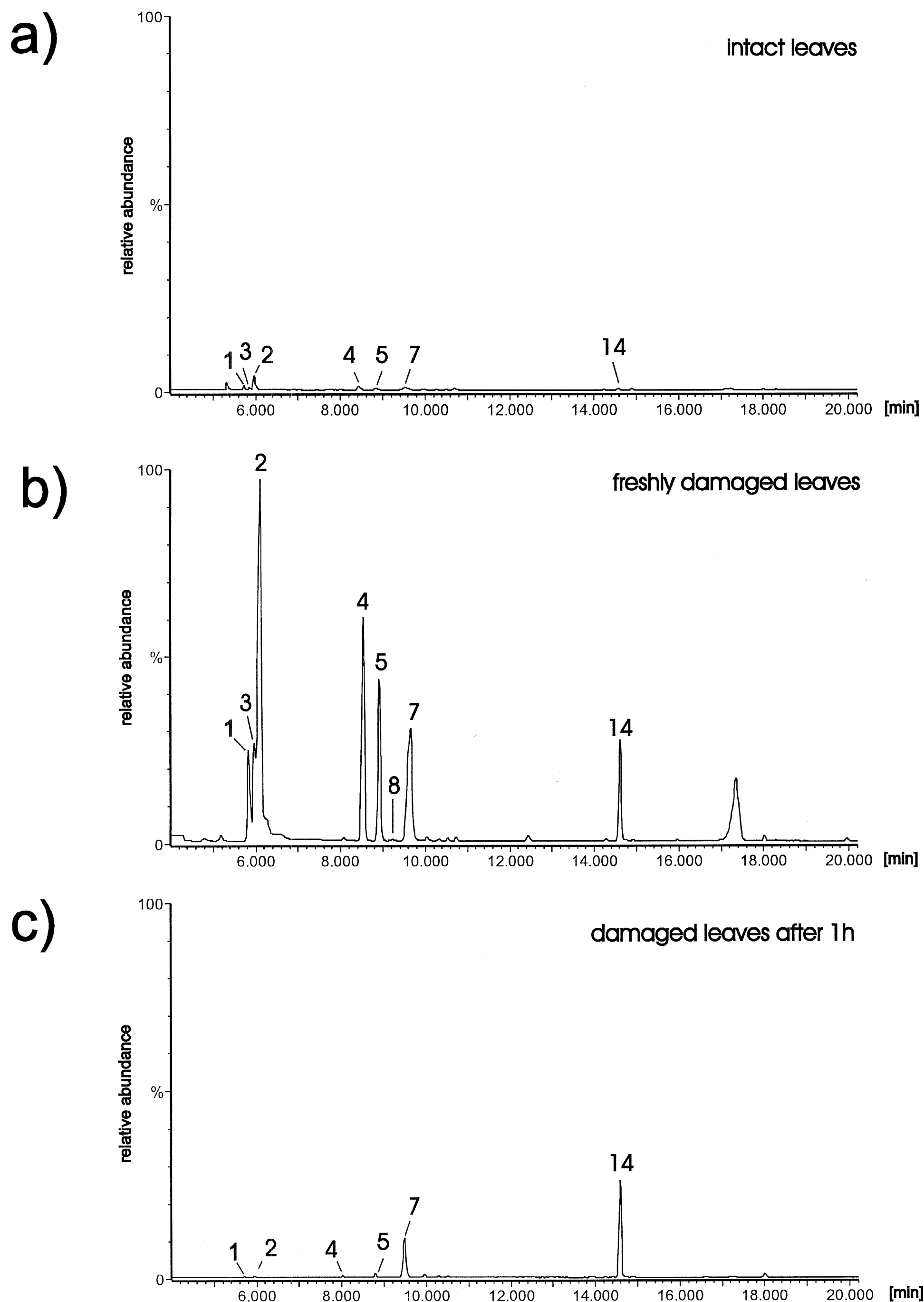

Fig. 2. Total ion chromatograms of volatiles emitted by 20 undamaged *Carpinus betulus* leaves (a), 20 freshly damaged leaves (b) and 20 damaged leaves sampled after 1 h (c). Volatiles were collected by purge (clean air, 1 min at 30 ml min^{-1}) and cold trapping (−100°C) according to Ruther and Hilker [27]. On-line injection (splitless) by thermal desorption at 250°C. Separation was performed on a 30-m×0.32-mm I.D. DB-1701 capillary column, 0.25 μm film thickness, equipped with an uncoated 1 m×0.32 mm I.D. retention gap. GC oven program: initial temperature, 40°C for 4 min; rate, 3°C min^{-1} to 280°C. Carrier gas, helium; inlet pressure, 10 kPa. Detection by EI mass spectrometry. Peak numbers correspond to Table 1.

examples given in this paper revealed that several seldom reported GLVs occurred in headspace samples of mechanically damaged plant leaves. Thus, the present paper will be the base for more detailed studies on GLVs in plants including the evaluation of their physiological and behaviour-modifying properties towards insects.

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