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Mass spectral characterization of fatty acid amides from alfalfa trichomes and their deterrence against the potato leafhopper

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Abstract

A homologous series of *N*-(3-methylbutyl)amides of normal saturated C_{14}, C_{15}, C_{16}, C_{17} and C_{18} fatty acids were identified as major components of glandular trichome extracts from *Medicago sativa* G98A, an alfalfa genotype resistant to the potato leafhopper, *Empoasca fabae*. A second homologous series of *N*-(2-methylpropyl)amides of C_{14} through C_{18} normal fatty acids were minor components. Saturated free fatty acids C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17} and C_{18} were present in trace amounts, as was the *N*-(3-methylbutyl)amide of linoleic acid ($C_{18:2}$). *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides of C_{14} through C_{18} fatty acids, along with the *N*-(3-methylbutyl)amide of linoleic acid, were synthesized and bioassayed for leafhopper deterrence by applying the compounds to the surface of a sachet containing an artificial diet. Leafhoppers were then offered a two-way choice between diet surfaces treated with the synthetic amides or an untreated control. *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides of C_{14} through C_{18} fatty acids did not deter leafhopper settling in a dose-dependent fashion. In contrast, when tested singly, *N*-(3-methylbutyl)amide of linoleic acid exhibited dose-dependent deterrence against leafhopper settling. Fatty acid amides localized in alfalfa glandular trichomes likely contribute to leafhopper resistance.

Keywords: Alfalfa; *Medicago sativa*; Leguminosae; Papilionaceae; Insect resistance; Fatty acid amides

1. Introduction

Plant glandular trichomes are known to produce and secrete a wide variety of biologically active secondary metabolites, including terpenoids, phenolics, alkaloids, cardiac glycosides, and sucrose esters (Kelsey et al., 1984; Duke et al., 2000). Such trichome metabolites provide a key line of defense for plants against insect herbivores; *Solanum* spp., *Lycopersicon* spp., and *Nicotiana* spp. have been particularly well studied (Tingey and Sinden, 1982; Lin et al., 1987; Johnson and Severson, 1984).

Glandular trichomes present on *Medicago* spp. are associated with resistance to insect herbivores such as the alfalfa weevil, *Hypera postica* (Gyllenhal), and the potato leafhopper, *Empoasca fabae* (Harris) (Shade

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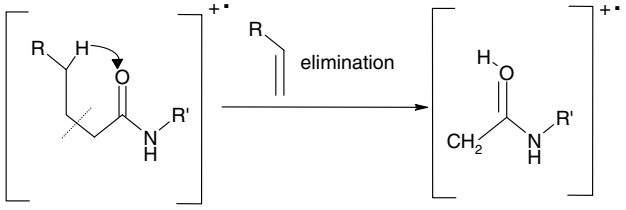
et al., 1975, 1979; Elden and McCaslin, 1997; Ranger and Hower, 2001a, 2002; Shockley et al., 2002). With the recent (1997) commercial release of glandular-haired alfalfa, *Medicago sativa* L., resistant to the potato leafhopper, increased attention is being paid to the role played by the glandular trichomes in conferring resistance. Erect and procumbent glandular trichomes are the two morphologies found on selected *Medicago* spp. and cultivars, and both trichome types actively secrete an exudate (Kreitner and Sorensen, 1979; Ranger and Hower, 2001b).

Potato leafhopper's are deterred from settling on Parafilm®-covered artificial diet sachets after the surfaces are treated with crude glandular trichome extracts from *M. sativa* G98A (Ranger et al., 2004a). Fatty acid amides $C_nH_{2n+1}NO$ ($n = 19$–23) are major components of the G98A extracts (Ranger et al., 2004a), but are absent in trichomes of the less resistant glandular-haired *M. sativa* G98C and the susceptible, nonglandular *M. sativa* Ranger. A mixture of fatty acid amides purified from *M. sativa* G98A glandular trichomes by silica gel or alumina chromatography also deterred potato leafhopper settling (Ranger et al., 2004b). In this paper, we report further on the structural characterization of fatty acid amides from trichomes of *M. sativa* G98A, along with the ability of synthetic fatty acid amides identified from G98A to deter potato leafhopper settling.



R = $C_{10}H_{21}$ through $C_{14}H_{29}$

R' = C_4H_9, m/z = 115
R' = C_5H_{11}, m/z = 129

(1)

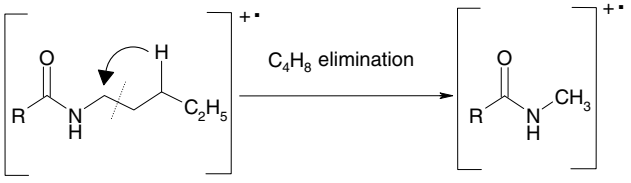
2. Results and discussion

2.1. Characterization of major and minor trichome components

In a previous paper (Ranger et al., 2004a), we described the isolation and mass spectral characterization of a homologous series of fatty acid amides unique to glandular trichome extracts from *M. sativa* G98A. Analysis of EI-MS spectra concluded that these major trichome components were C_5 amides of straight chain fatty acids, C_{14} through $C_{18}[CH_3(CH_2)_{n-7}C(=O)NHC_5H_{11}$, $n = 19$, 20, 21, 22, or 23] (Ranger et al., 2004a). A base peak at *m/z* 129, the result of a McLafferty rearrangement (cleavage of the bond *beta* to the carbonyl carbon with retention of hydrogen from the *gamma* carbon, Eq. (1)), characterized each member of the homologous series (Ranger et al., 2004a). The carboxylic acid moiety was identified by an acyl ion ($R'CO^+$) at M-86 and a carboxamido ion ($R''(C{=}OH)NH_2^+$) at M-69. The straight fatty acid chain was revealed by a series of even electron fragment ions separated by 14 amu's at M-15, M-29, M-43, M-57, M-71,...,*m/z* 114, due to fragmentation of a corresponding chain.

Since each member of the homologous series exhibited the same *m/z* 129 base peak, all of the amides have five-carbon nitrogen substituents. However, interpretation of EI-MS spectra of the natural fatty acid amides isolated from *M. sativa* G98A did not completely reveal the nature of the nitrogen substituent. Nevertheless, odd electron ions at M-56 (cleavage of the bond *beta* to nitrogen with retention of hydrogen from the *gamma* carbon, Eq. (1)), and *m/z* 73 (a combination of Eqs. (1) and (2)), strongly suggested a 2° amide group (i.e., an amide lacking a second alkyl substituent on nitrogen).[2] Thus, several possibilities, such as *n*-pentyl-, 2-methylbutyl-, 3-methylbutyl-, or 2,2-dimethylpropylamine were suspected as the precursor amine. To characterize the structure of the C_5 nitrogen substituent, different amides of hexadecanoic acid were synthesized using several of the aforementioned amines. Only the *N*-(3-methylbutyl)amide exhibited a GC retention time and EI-MS spectrum that matched the natural material. The other amides had different retention times and exhibited significantly different mass spectra. Subsequently, the remaining members in the series (i.e., *N*-(3-methylbutyl)amides of normal C_{14}, C_{15}, C_{17} and C_{18} saturated fatty acids) were also synthesized, furnishing materials that likewise matched the corresponding natural amides isolated from trichomes of *M. sativa* G98A. EI-MS and FAB-MS data for synthetic *N*-(3-methylbutyl)amides of C_{14} through C_{18} fatty acids, as well as *n*-pentyl and 2-methylbutyl amides of hexadecanoic acid, are provided in Table 1. Ratios of the *N*-(3-methylbutyl)amides extracted from G98A trichomes are approximately 1:3:7:1.25:1.5 for the C_{14}:C_{15}:C_{16}:C_{17}:C_{18} acids, respectively.

[2] The mass spectrum of the tertiary amide, *N*-methyl-*N*-butyl hexadecanoamide, shows a peak at *m/z* 87 rather than at *m/z* 73 indicative of the second nitrogen substituent (MS data not included in Table 1).



(2)

Closer examination of EI-MS spectra, in particular, the intensity of the peak at *m/z* 115, revealed another family of amides that was largely obscured by the major *N*-(3-methylbutyl)amide series. By selecting *m/z* 115 and *m/z* 129 ion traces separately, it was determined that 115 amu peaks did not fully overlap with 129 amu peaks (Fig. 1), indicating another series of minor fatty acid amides. Interpretations of EI-MS determined the minor series of fatty acid amides were C_4 amides (a butyl series) with a straight fatty acid chain of C_{14}, C_{15}, C_{16}, C_{17} and C_{18}. The minor series of C_4 amides are all characterized by a base peak of *m/z* 115. As before (Eq. (1)), a McLafferty rearrangement gives $CH_2C(=OH)NHC_4H_9$ at *m/z* 115. Also, the carboxylic acid moiety is identified by an acyl ion (RCO^+) at M-72 and a carboxamido ion ($R(C=OH)NH_2^+$) at M-55. A series of even electron fragment ions separated by 14 amu's at M-15, M-29, M-43, M-57, M-71,…,*m/z* 100 corresponds to fragmentation of a C_{14}, C_{15}, C_{16}, C_{17} and C_{18} carboxylate side chain.

GC retention times and EI-MS spectra of *N*-(2-methylpropyl)amides of C_{14} through C_{18} fatty acids matched the corresponding minor series of $C_nH_{2n+1}NO$ (n = 18–22) fatty acid amides isolated from trichomes of *M. sativa* G98A. The remaining synthetic C_4 amides exhibited considerably different retention times and mass spectra. EI-MS and FAB-MS data for the synthetic *N*-(2-methylpropyl)amides of C_{14} through C_{18} fatty acids, as well as *n*-butyl, 2°-butyl and *t*-butyl amides of hexadecanoic acid are provided in Table 1. Ratios for the *N*-(2-methylpropyl)amides extracted from G98A trichomes are approximately 1:2:5:1:2 for C_{14}:C_{15}:C_{16}:C_{17}:C_{18}, respectively. Representative comparisons of mass spectra of synthetic *N*-(3-methylbutyl)- and *N*-(2-methylpropyl)amides with those extracted from G98A trichomes are shown in Figs. 2–5.

The *N*-(3-methylbutyl)amide of C_{16} fatty acid and the *N*-(2-methylpropyl)amide of C_{17} fatty acid comprised almost one-half of the total fatty acid amides (see Fig. 1(a), peak with retention time of 16.30 min). Fig. 1 also demonstrates that overlapping GC peaks are comprised of compounds with the same total number of carbons, thus *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides with the same fatty acid chain lengths exhibited different retention times. For instance, the *N*-(2-methylpropyl)amide of C_{16} fatty acid exhibited a retention time of 15.42 min, while the *N*-(3-methylbutyl)amide of C_{16} fatty acid exhibited a retention time of 16.30 min (Fig. 1(b) and (c)).

After identifying major and minor components isolated from trichomes of *M. sativa* G98A, attention was shifted to identification of trace compounds. By passing the *M. sativa* G98A trichome extracts through a column of basic Alumina (Al_2O_3), any peaks that disappeared from the extracts after elution through basic Al_2O_3 were suspected as being free fatty acids. Subsequent EI-MS comparisons of the natural free fatty acids with authentic free fatty acids indicated the presence of C_{12} (dodecanoic), C_{13} (tridecanoic), C_{14} (tetradecanoic), C_{15} (pentadecanoic), C_{16} (hexadecanoic), C_{17} (heptadecanoic), and C_{18} (octadecanoic) acids. Hexadecanoic acid was the predominant free fatty acid in *M. sativa* G98A trichome extracts.

Among the additional trace compounds evident in trichome extracts from *M. sativa* G98A was the *N*-(3-methylbutyl)amide of linoleic acid ($C_{18:2}$). Structural identifications were made using the aforementioned procedures, namely by comparing retention times and EI-MS of the natural trace component with those of synthetic *N*-(3-methylbutyl)amide of linoleic acid. EI-MS and FAB-MS data for synthetic *N*-(3-methylbutyl)amide of linoleic acid ($C_{18:2}$) are provided in Table 1.

2.2. Bioactivity of synthetic fatty acid amides

Compared against a solvent control, a mixture of *N*-(3-methylbutyl)amides of C_{14} through C_{18} fatty acids tested at 100, 50, and 12.5 μg/cm^2 did not significantly ($P > 0.05$) deter settling by the potato leafhopper (data not shown). However, at 25 μg/cm^2, the mixture of *N*-(3-methylbutyl)amides was marginally deterrent (Treatment $F = 9.77$, df = 1; $P = 0.052$, Time $P > 0.05$; Treatment × Time $P > 0.05$; data not shown). A mixture of *N*-(2-methylpropyl)amides of C_{14} through C_{18} fatty acids tested at 100, 25, and 12.5 μg/cm^2 did not significantly ($P > 0.05$) deter leafhopper settling (data not shown). In fact, at 50 μg/cm^2, a significant Treatment × Time interaction was detected, whereby the mixture of *N*-(2-methylpropyl) amides was more attractive than the control (Treatment $P > 0.05$; Time $P > 0.05$; Treatment × Time $F = 1.72$, df = 18, $P = 0.046$; data not shown). Both the *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides formed a white powder on the Parafilm® surface of the artificial diet sachet once the solvent evaporated. Refer to Ranger (2004) for bioactivity data not shown.

When tested singly, *N*-(3-methylbutyl)amide of linoleic acid ($C_{18:2}$) exhibited dose-dependent deterrence against settling by the potato leafhopper (Fig. 6(a)–(d)). At 100 μg/cm^2, a significant degree of deterrence

Table 1
Mass spectral and gas chromatographic data for synthetic amides R^1–CO–NH–R^2

Compound		EI MS (*m*/*z*(rel int))[c]	FAB MS		GC retention time (min)
R^1	R^2		[MH^+] theo	[MH^+] found	
n-$C_{13}H_{27}$–	–$CH_2CH_2CH(CH_3)_2$	41(24), 43(43), 44(19), 55(21), 57(15), 69(9), 71(12), 72(8), 73(47), 86(13), 114(14), 129(100), 142(19), 156(3), 170(2), 184(3), 198(5), 211(3), 212(2), 226(1), 228(2), 240(3), 241(5), 254(3), 268(1), 282(3), 297(10)	298.3110	298.3111	14.57
n-$C_{14}H_{29}$–	–$CH_2CH_2CH(CH_3)_2$	41(22), 43(40), 44(18), 55(21), 57(16), 69(9), 71(13), 72(8), 73(48), 86(13), 114(15), 129(100), 142(20), 156(3), 170(2), 184(4), 198(4), 212(3), 225(2), 226(2), 240(1), 242(2), 254(2), 255(6), 268(3), 282(1), 296(3), 311(11)	312.3266	312.3265	15.44
n-$C_{15}H_{31}$–	–$CH_2CH_2CH(CH_3)_2$	41(20), 43(39), 44(16), 55(20), 57(16), 69(9), 71(13), 72(8), 73(47), 86(12), 114(14), 129(100), 142(19), 156(3), 170(2), 184(3), 198(4), 212(1), 226(2), 239(2), 240(2), 254(1), 256(2), 268(2), 269(5), 282(3), 296(1), 310(2), 325(10)	326.3423	326.3419	16.28
n-$C_{15}H_{31}$–	–$CH_2CH_2CH_3CH_2CH_3$	41(23), 43(38), 44(11), 55(19), 57(18), 69(10), 71(17), 72(9), 73(29), 86(30), 87(25), 88(13), 100(12), 114(58), 129(100), 142(43), 156(6), 170(4), 184(8), 198(8), 212(3), 226(4), 239(6), 240(5), 254(3), 256(3), 268(4), 269(3), 282(6), 296(17), 310(2), 325(7)	326.3423	326.3427	16.60
n-$C_{15}H_{31}$–	–$CH_2CH(CH_3)CH_2CH_3$	41(25),43(39),44(5),55(20),57(23),58(21),60(19),69(10),71(17), 72(12), 73(20), 86(13), 114(19), 129(100), 142(24), 156(4), 170(3), 184(5), 198(5), 212(2), 226(3), 239(13), 240(5), 254(2), 256(49), 268(6), 269(3), 282(4), 296(5), 310(3), 325(6)	326.3423	326.3418	16.28
n-$C_{16}H_{33}$–	–$CH_2CH_2CH(CH_3)_2$	41(26), 43(49), 44(19), 55(21), 57(20), 69(10), 71(15), 72(9), 73(56), 86(13), 88(11), 114(16), 129(100), 142(21), 156(2), 170(2), 184(4), 198(3), 212(1), 226(1), 240(2), 253(1), 254(1), 268(1), 270(1), 282(2), 283(4), 296(2), 310(1), 324(2), 339(8)	340.3579	340.3583	17.10
n-$C_{17}H_{35}$–	–$CH_2CH_2CH(CH_3)_2$	41(24), 43(44), 44(19), 55(23),57(22), 69(11), 71(16), 72(10), 73(58), 86(14), 88(13), 114(15), 129(100), 142(20), 156(3), 170(2), 184(3), 198(3), 212(1), 226(1), 240(1), 254(2), 267(1), 268(1), 282(1), 284(1), 296(1), 297(3), 310(1), 324(1), 338(1), 353(6)	354.3736	354.3738	17.89
n-$C_{17}H_{33}$–[a]	–$CH_2CH_2CH(CH_3)_2$	41(25), 43(35), 44(12), 55(39), 57(11), 67(15), 69(22), 71(18), 72(11), 73(44), 81(10), 83(10), 86(13), 88(30), 114(15), 129(65), 142(100), 156(9), 168(2), 182(4), 184(10), 196(12), 198(11), 210(4), 212(3), 224(6), 226(4), 238(4), 252(4), 266(3), 280(2), 294(6), 308(4), 322(1), 336(3), 351(9)	352.3579	352.3580	17.65
n-$C_{15}H_{31}$–[b]	–$CH_2CH_2CH(CH_3)_2$	41(33), 43(42), 44(15), 55(45), 57(9), 67(34), 69(22), 71(23), 72(14), 73(54), 79(20), 81(22), 83(10), 86(23), 87(10), 88(62), 95(15), 114(21), 129(79), 142(100), 156(12), 184(13), 196(19), 198(13), 208(2), 210(6), 212(5), 224(7), 226(4), 236(4), 238(6), 240(4), 250(4), 252(5), 254(3), 264(2), 266(3), 268(2), 278(2), 280(2), 292(4), 294(6), 306(3), 308(4), 334(2), 336(2), 349(13)	350.3423	350.3434	17.57
n-$C_{13}H_{27}$–	–$CH_2CH(CH_3)_2$	41(15), 43(16), 55(15), 57(22), 60(19), 69(6), 71(8), 72(10), 73(5), 74(4), 83(4), 100(6), 115(100), 128(18), 142(3), 156(2), 170(2), 184(2), 198(1), 211(7), 212(2), 226(1), 228(10), 240(3), 254(1), 268(1), 283(2)	284.2953	284.2956	13.61
n-$C_{14}H_{29}$–	–$CH_2CH(CH_3)_2$	41(15), 43(16), 55(13), 57(23), 60(17), 69(6), 71(8), 72(9), 73(5), 74(5), 83(4), 100(6), 115(100), 128(19), 142(3), 156(1), 170(3), 184(2), 198(1), 212(1), 225(7), 226(2), 240(1), 242(10), 254(4), 268(1), 282(1), 297(3)	298.3110	298.3112	14.54
n-$C_{15}H_{31}$–	–$CH_2CH(CH_3)_2$	41(12), 43(14), 55(10), 57(22), 60(14), 69(5), 71(7), 72(8), 73(6), 74(4), 83(4), 100(5), 115(100), 128(17), 142(3), 156(1), 170(2), 184(2), 198(1), 226(1), 239(6), 240(2), 256(8), 268(3), 282(1), 311(2)	312.3266	312.3264	15.42
n-$C_{15}H_{31}$–	–$CH_2CH_2CH_2CH_3$	41(12), 43(14), 55(11), 57(18) ,69(5), 71(5), 72(5), 73(24), 74(8), 86(8), 100(19), 115(100), 128(27), 142(4), 156(2), 170(4), 184(4), 198(1), 212(1), 226(2), 239(3), 240(2), 254(1), 256(1), 268(4), 282(2), 296(2), 311(4)	312.3266	312.3266	15.80
n-$C_{15}H_{31}$–	–$CH(CH_3)CH_2CH_3$	41(19), 43(23), 44(91), 55(17), 57(21), 58(17), 59(16), 60(13), 69(7), 71(7), 72(11), 86(12), 100(12), 115(100), 128(22), 142(3), 156(1), 170(3), 184(3), 198(1), 212(1), 226(1), 239(1), 240(1), 254(1), 256(14), 268(2), 282(7), 296(1), 311(5)	312.3266	312.3264	15.14
n-$C_{15}H_{31}$–	–$C(CH_3)_3$	41(12), 43(23), 55(9), 57(19), 58(50), 59(16), 60(9), 72(10), 100(6), 115(100), 128(13), 142(2), 156(1), 170(2), 184(1), 198(1), 212(1), 226(1), 239(1), 240(1), 254(1), 256(20), 268(1), 282(1), 296(1), 311(3)	312.3266	312.3264	14.33
n-$C_{16}H_{33}$–	–$CH_2CH(CH_3)_2$	41(9), 43(13), 55(11), 57(18), 60(13), 69(5), 71(6), 72(7), 73(3), 74(5), 83(4), 100(5), 115(100), 128(19), 142(3), 156(1), 170(3), 184(2), 198(1), 212(1), 226(1), 240(1), 253(5), 254(1), 268(1), 270(9), 282(4), 296(1), 310(1), 325(3)	326.3423	326.3421	16.27
n-$C_{17}H_{35}$–	–$CH_2CH(CH_3)_2$	41(11), 43(14), 55(12), 57(19), 60(14), 69(5), 71(7), 72(8), 73(4), 74(5), 83(4), 100(4), 115(100), 128(16), 142(2), 156(1), 170(2), 184(2), 198(1), 212(1), 226(1), 240(1), 254(1), 267(3), 268(1), 282(1), 284(7), 296(3), 310(1), 324(1), 339(2)	340.3579	340.3582	17.07

[a] *cis*-$CH_3(CH_2)_7CH{=}CH(CH_2)_7$–
[b] *cis*,*cis*-$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7$– (containing ca. 7% oleic acid).
[c] Only the most significant peaks based on relative intensity and mass are shown.

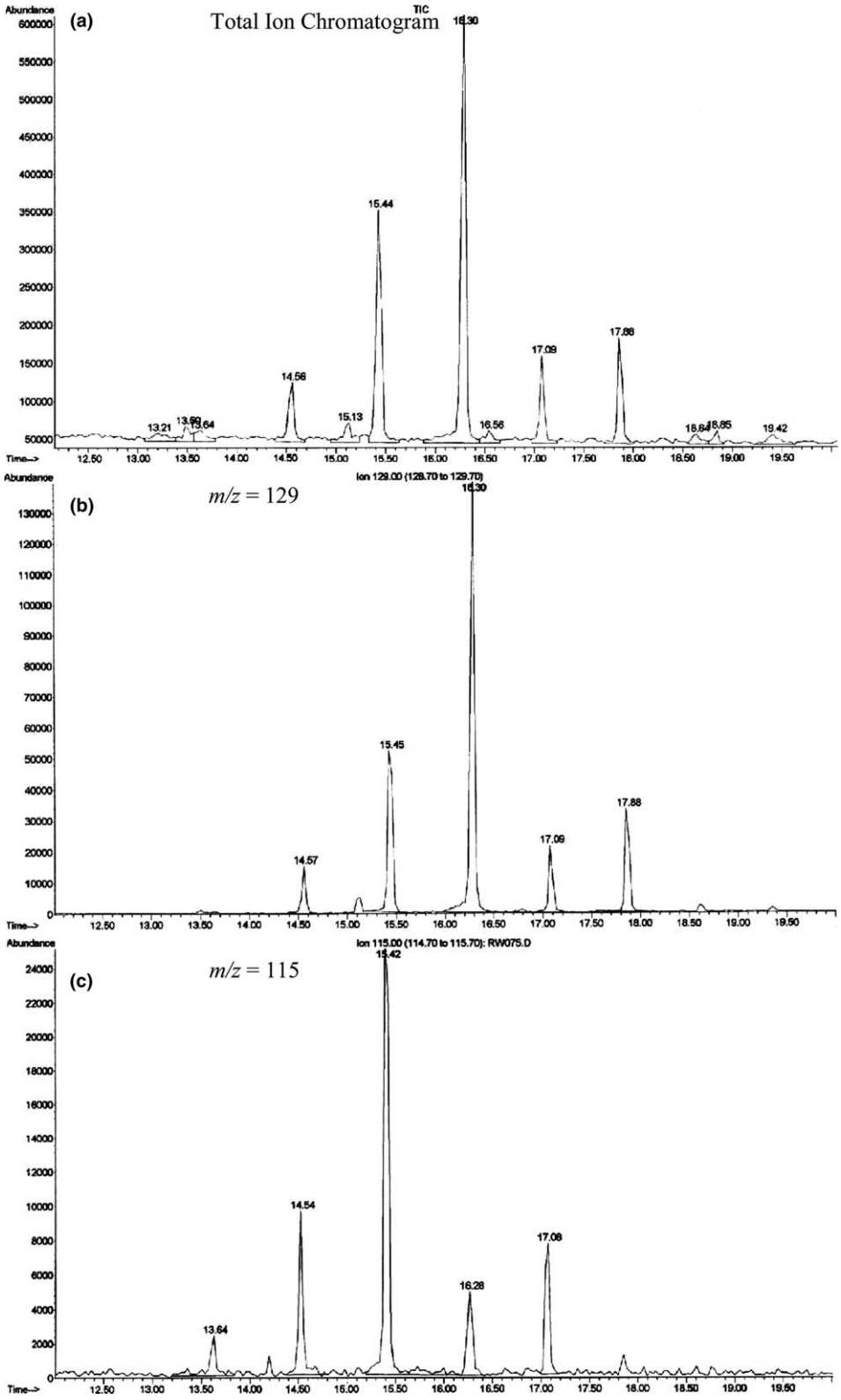


Fig. 1. EI-MS total ion chromatogram (a) of trichome extracts from *Medicago sativa* G98A. Selective ion traces at (b) *m*/*z* 129 and (c) *m*/*z* 115 document the presence of *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides of C_{14} through C_{18} fatty acids, respectively.

was observed (Fig. 6(a); Treatment $F = 118.73$, df = 1, $P < 0.0001$; Time $P > 0.05$; Treatment × Time $P > 0.05$). Leafhoppers were also deterred from settling on artificial diet sachets treated with *N*-(3-methylbutyl)amide of linoleic acid at 50 μg/cm^2 (Fig. 6(b); Treatment $F = 27.87$, df = 1, $P = 0.001$; Time $P > 0.05$;

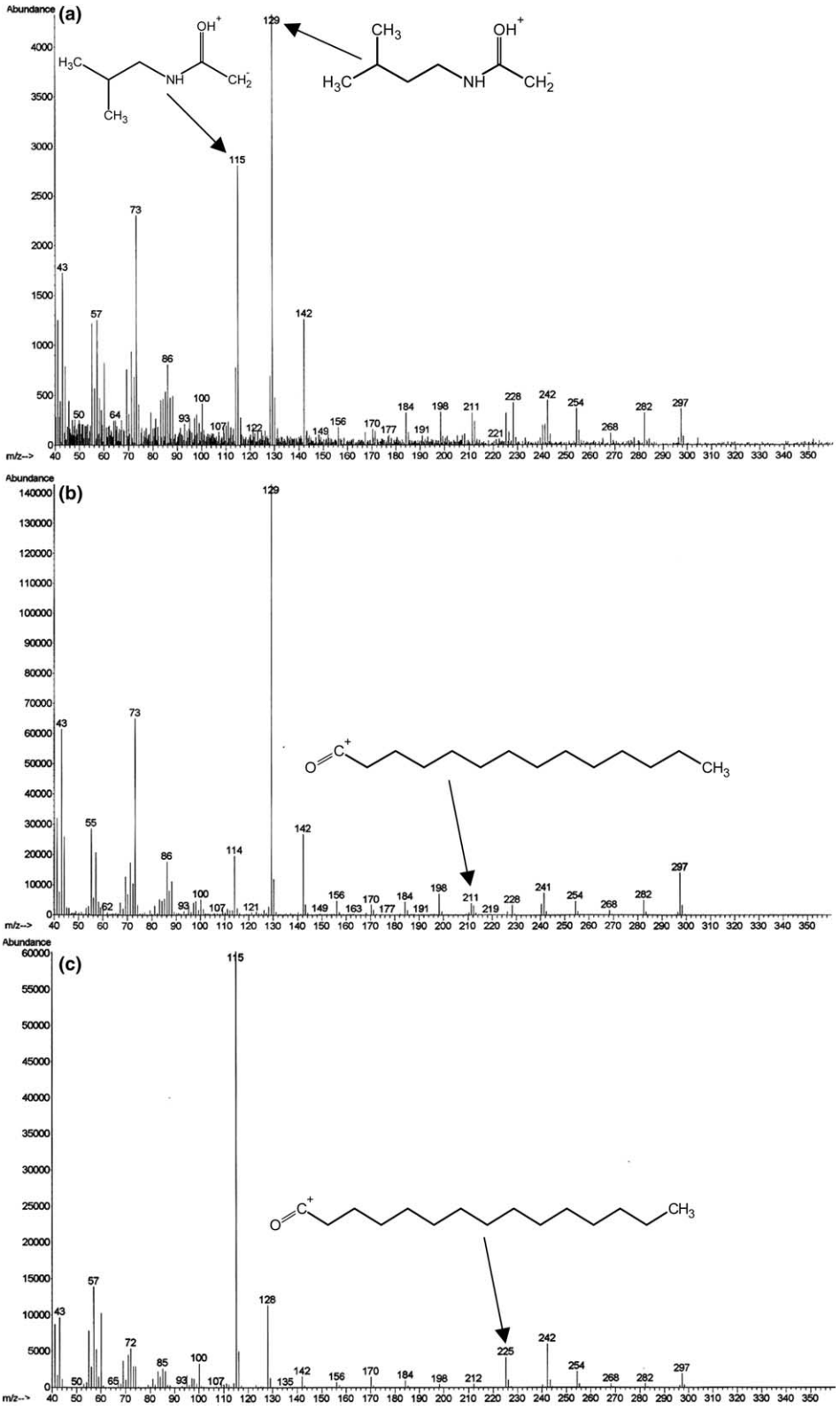


Fig. 2. Identification of two fatty acid amides from trichomes of *Medicago sativa* G98A by comparison of EI-MS from (a) the natural fatty acid amide with (b) synthetic *N*-(3-methylbutyl)amide of tetradecanoic acid (C_{14}) and (c) synthetic *N*-(2-methylpropyl)amide of pentadecanoic acid (C_{15}).

Treatment × Time $P > 0.05$). At 25 μg/cm^2, *N*-(3-methylbutyl)amide of linoleic acid was marginally ($P < 0.01$) deterrent (Fig. 6(c); Treatment $F = 3.96$, df = 1, $P = 0.0868$; Time $P > 0.05$; Treatment × Time $P > 0.05$). Deterrence was not detected at 12.5 μg/cm^2 (Fig. 6(d); Treatment $P > 0.05$; Time $P > 0.05$; Treat-

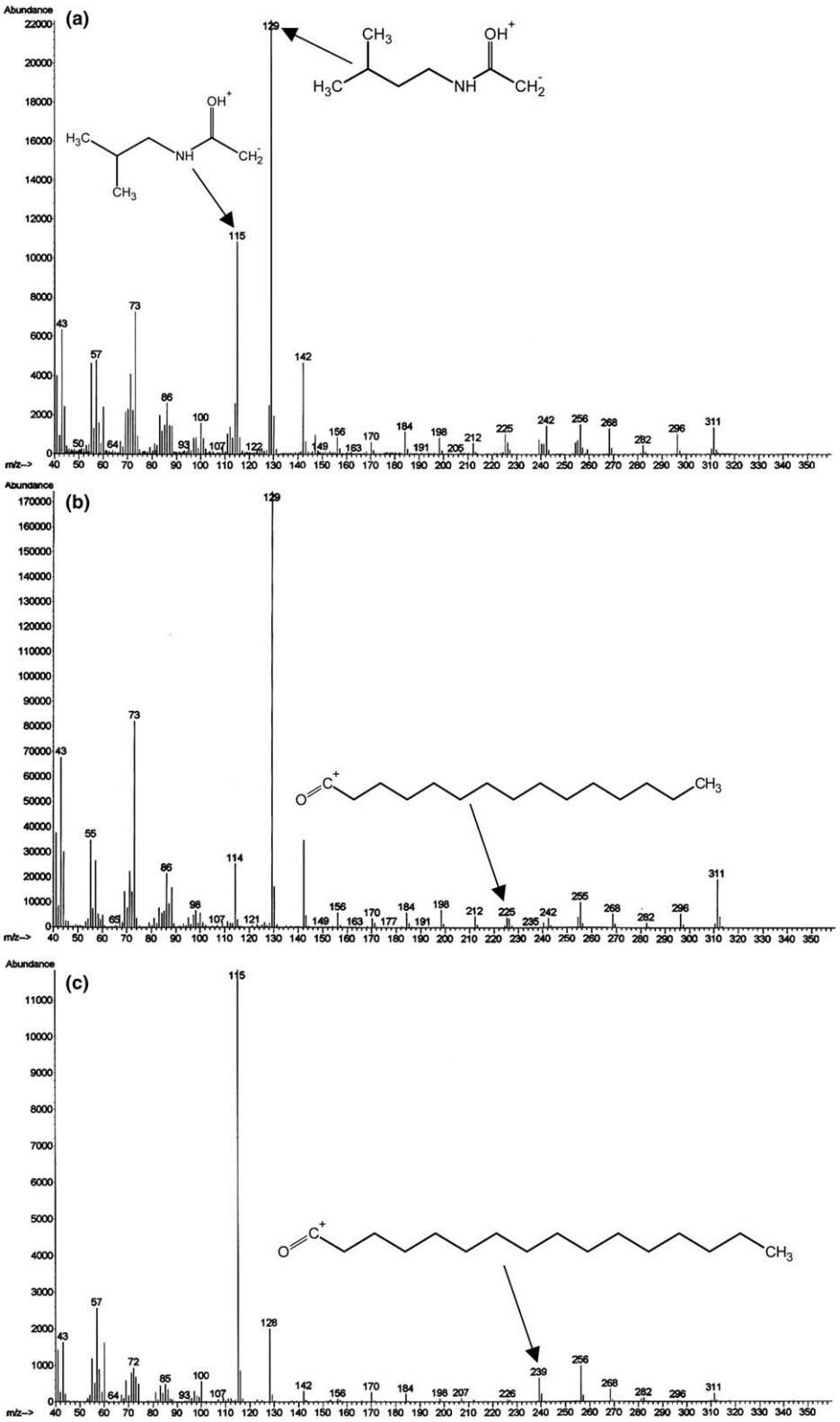


Fig. 3. Identification of two fatty acid amides from trichomes of *Medicago sativa* G98A by comparison of EI-MS from (a) the natural fatty acid amide with (b) synthetic *N*-(3-methylbutyl)amide of pentadecanoic acid (C_{15}) and (c) synthetic *N*-(2-methylpropyl)amide of hexadecanoic acid (C_{16}).

ment × Time $P > 0.05$). In contrast to the solids formed by *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides of C_{14} through C_{18} acids, *N*-(3-methylbutyl)amide of linoleic acid formed an oily residue on the Parafilm® surface of the diet sachet after the solvent evaporated.

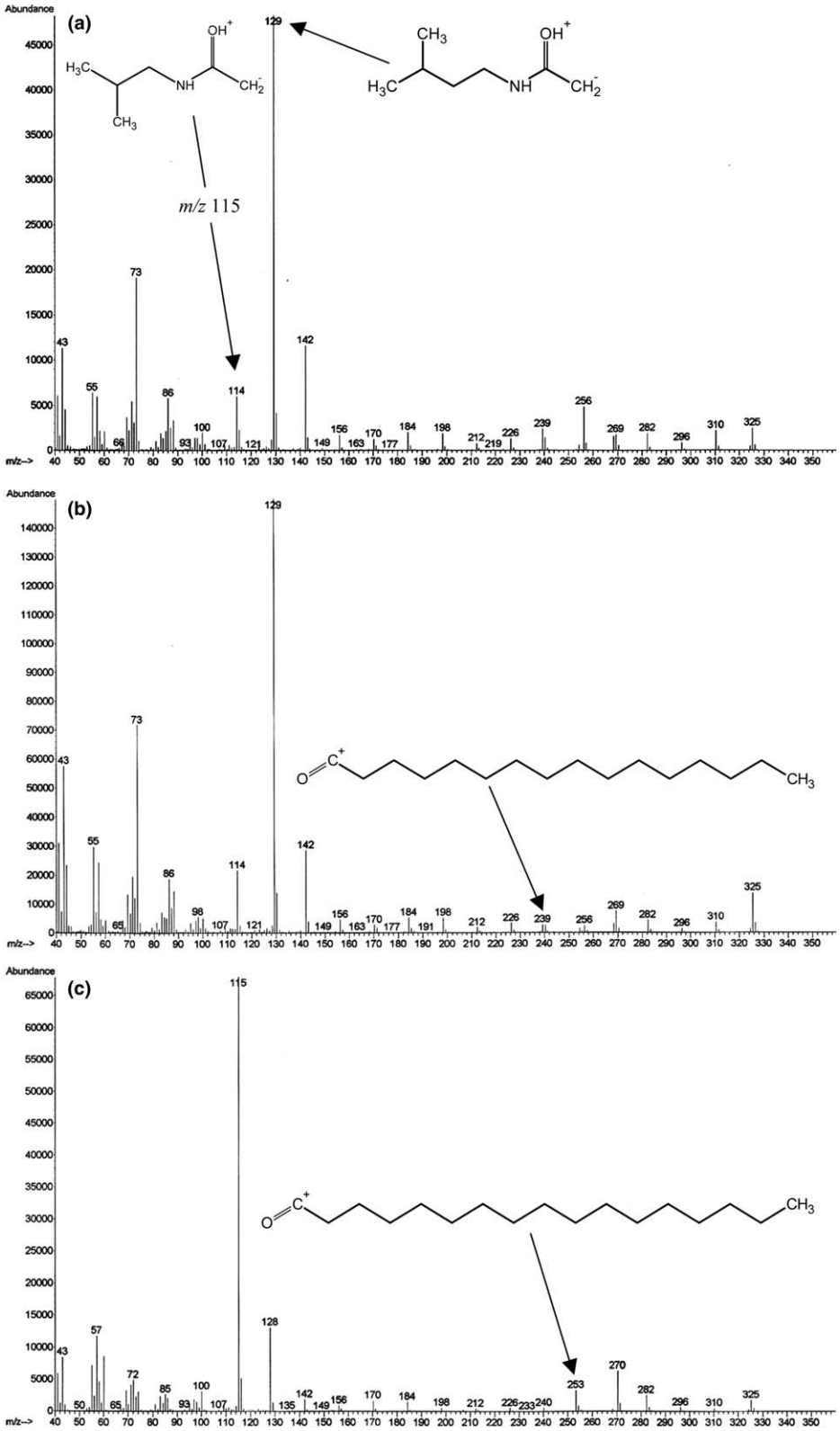


Fig. 4. Identification of two fatty acid amides from trichomes of *Medicago sativa* G98A by comparison of EI-MS from (a) the natural fatty acid amide with (b) synthetic *N*-(3-methylbutyl)amide of hexadecanoic acid (C_{16}) and (c) synthetic *N*-(2-methylpropyl)amide of heptadecanoic acid (C_{17}).

The lack of activity associated with the saturated fatty acid *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides may be due to the formation of solids in their natural state, compared to the oil formed by the *N*-(3-methylbutyl)amide of linoleic acid. *In planta*, the *N*-(3-methylbutyl)amide of linoleic acid may assist

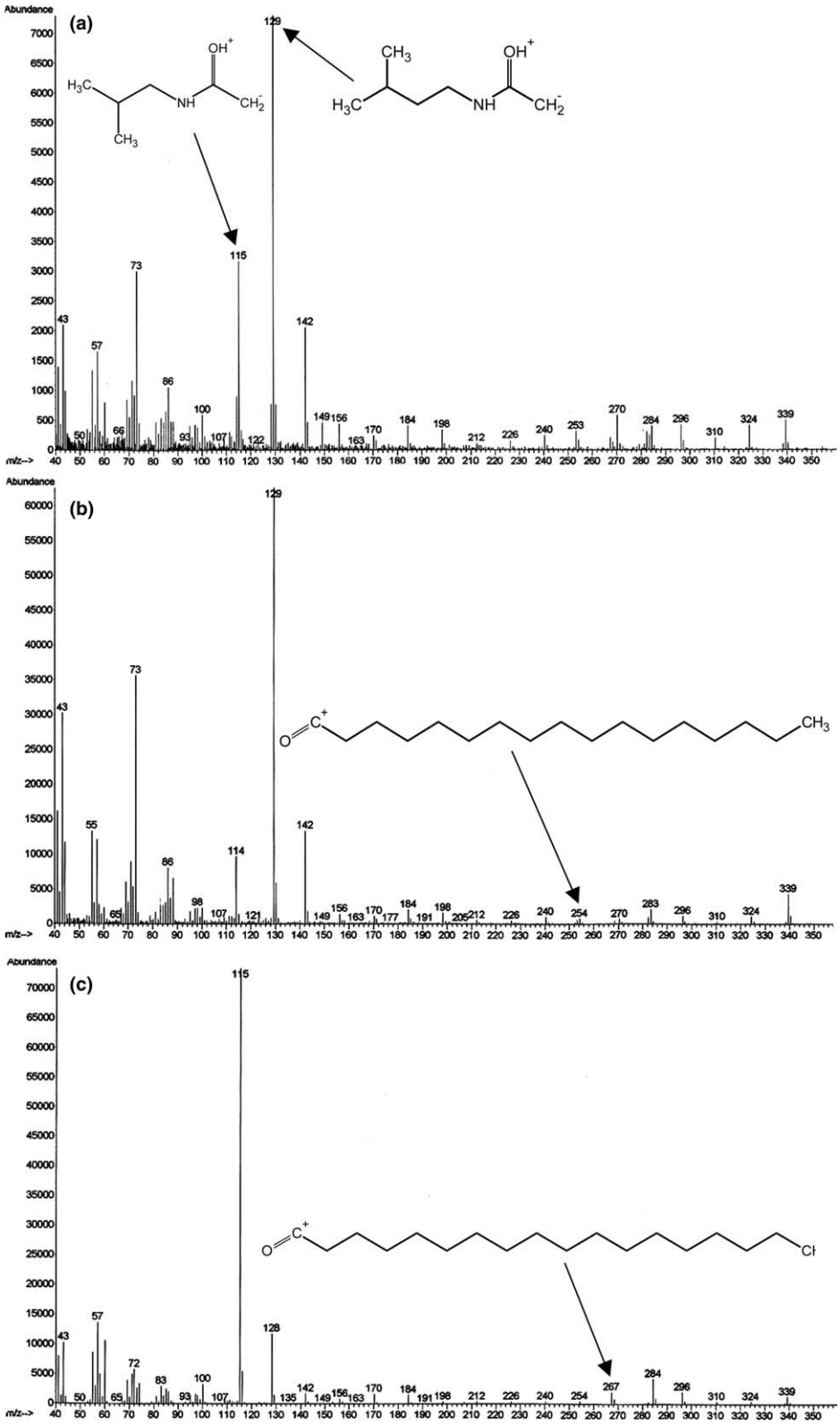


Fig. 5. Identification of two fatty acid amides from trichomes of *Medicago sativa* G98A by comparison of EI-MS from (a) the natural fatty acid amide with (b) synthetic *N*-(3-methylbutyl)amide of heptadecanoic acid (C_{17}) and (c) synthetic *N*-(2-methylpropyl)amide of octadecanoic acid (C_{18}).

in keeping other fatty acid amides in a liquid state. Indeed, erect and procumbent glandular trichomes on alfalfa secrete a viscous exudate that can adhere to the cuticle of the potato leafhopper (Kreitner and Sorensen, 1979; Ranger and Hower, 2001a). Similarly, the normal odd-chained ketone, 2-tridecanone, is found in

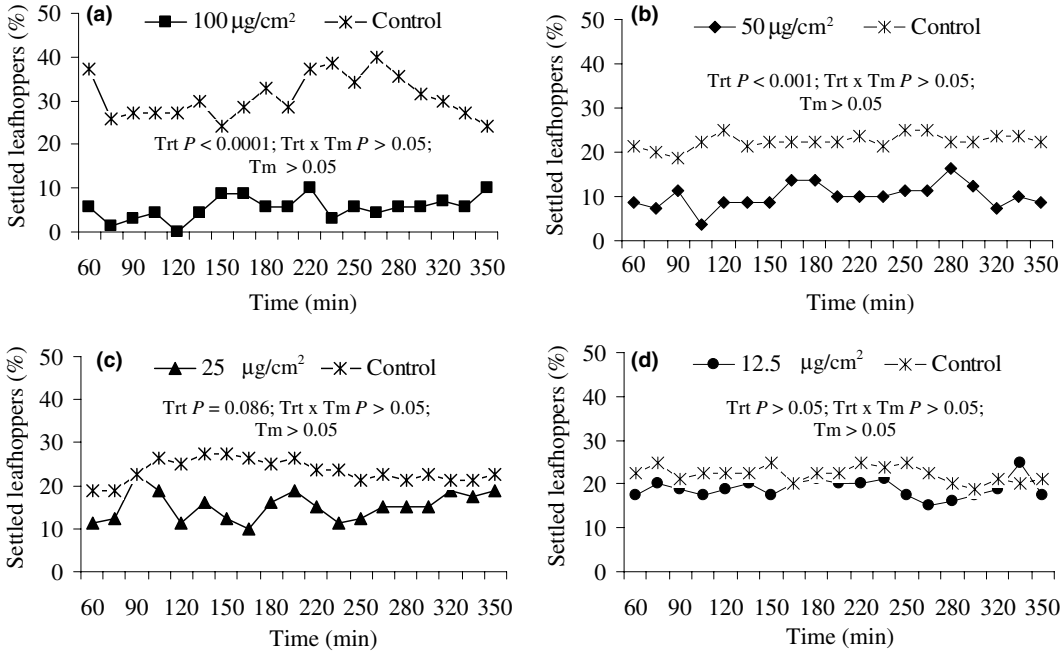


Fig. 6. Two-choice bioassay of the potato leafhopper for settling on artificial diet sachets treated with *N*-(3-methylbutylamide) of linoleic acid ($C_{18:2}$). Extracts were applied to the surface of an artificial diet sachet at a concentration of (a) 100 µg/cm^2, (b) 50 µg/cm^2, (c) 25 µg/cm^2, and (d) 12.5 µg/cm^2. $N = 7$ for (a), and $N = 8$ for (b)–(d).

glandular trichomes of *Lycopersicon* f. *glabratum* and exhibits insecticidal properties (Lin et al., 1987). The authors proposed that since 2-tridecanone forms a solid in its natural state, 2-undecanone (which forms a liquid) may assist in keeping 2-tridecanone in a liquid form and subsequently in a more biologically-active state.

A relatively diverse array of fatty acid amides were identified from glandular trichomes of *M. sativa* G98A. Rather than such compounds being considered as redundant, the Screening Hypothesis (Firn and Jones, 1996) proposes that the production of numerous compounds with no apparent activity ultimately leads to the evolution of a pathway that produces a compound with biological activity. Therefore, plant natural products are not necessarily redundant, but instead are a consequence of the pathways needed to generate chemical diversity (Firn and Jones, 1996). The activity of synthetic *N*-(3-methylbutyl)amide of linoleic acid, but apparent inactivity of synthetic *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides of C_{14} through C_{18} normal fatty acids, supports the Screening Hypothesis (Firn and Jones, 1996).

Plant-derived amides of fatty acids are well-documented to possess insecticidal activities (Elliot et al., 1987), and isobutylamides of unsaturated, aliphatic, straight chain C_{10}–C_{18} acids are toxic to a variety of insects (Jacobson, 1971). In *Chrysanthemum morifolium* (Ramat), concentrations of the unsaturated fatty acid isobutylamide, *N*-isobutyl-2*E*, 4*E*, 10*E*, 12*Z*-tetradecatetraen-8-ynamide, are positively correlated with defense against western flower thrips, *Frankliniella occidentalis* (Pergande) (Tsao et al., 2003).

Previous studies have documented the deterrent properties of crude glandular trichome extracts from *M. sativa* G98A against settling by the potato leafhopper (Ranger et al., 2004a). Furthermore, a purified fraction from trichomes of G98A, containing *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides of C_{14} through C_{18} fatty acids, and *N*-(3-methylbutyl) amide of $C_{18:2}$ fatty acid, also deterred leafhopper settling (Ranger et al., 2004b). By documenting that *N*-(3-methylbutyl)amide of linoleic acid acts as a potato leafhopper settling deterrent, results from this study suggest fatty acid amides localized in alfalfa glandular trichomes play a role in resistance of glandular-haired alfalfa against the potato leafhopper. Resistance levels of glandular-haired alfalfa may therefore be improved by selecting for plants producing higher amounts of the fatty acid amides.

3. Experimental procedures

3.1. Plants and insects

Cuttings (i.e., ramets) of resistant glandular-haired *M. sativa* genotype G98A were provided by Cal/West Seeds (West Salem, WI). Plants were vegetatively propagated and grown under greenhouse conditions. Metal halide lamps (photoperiod: 16:8 h L:D) were used to

supplement the natural lighting. Experimental plants were about 4 months old and harvested 3 times prior to use. A colony of potato leafhoppers was maintained according to Hunter and Backus (1989). Insects were reared on greenhouse-grown fava beans (*Vicia faba* cv. 'Windsor') using an environmental growth chamber (temperature: 25 ± 2 °C; photoperiod: 16:8 h L:D).

3.2. Trichome isolation and extraction

Trichomes were isolated from the stem sections of *M. sativa* G98A according to a modified protocol of Yerger et al. (1992), as described in Ranger et al. (2004a,b). Entire stems were harvested during the hours of 11:00 a.m. to 3:00 p.m. and immediately cut into 2–5 cm sections. These stem sections were then transferred to a test tube in small portions and lowered into a Dewar flask containing liquid N_2. After submersion, the test tube was raised out of the coolant and the liquid N_2 was allowed to evaporate. The test tube was then vortexed for 3–5 s, resulting in the free trichomes adhering to the test tube walls. Trichomes were rinsed from the test tube walls using methylene chloride and pooled samples were soaked for 24 h in methylene chloride with sodium sulfate (as an overnight drying agent). Samples were filtered using glass fiber circles (G6, Fisher Scientific, Pittsburgh, PA) and concentrated to dryness using a Kuderna-Danish Evaporative Concentrator (Kontes Glass Company, Vineland, NJ). Residues were redissolved in methylene chloride for analysis.

3.3. Chemical analysis

Trichome extracts from *M. sativa* G98A stems were analyzed using a Hewlett–Packard GC/MS System Model 5698A operating in electron impact (EI) mode (70 eV). A 15 m Restek Rtx-1 or an equivalent column (internal diameter of 0.25 mm, and a film thickness of 0.3 μm) was used. The injector port was held at 250 °C, and the oven was programmed from 100 to 320 °C at 10 °C/min and held at 320 °C for 5 min. Fast atom bombardment (FAB) mass spectrometry was conducted using a JEOL MStation JMS 700 double focusing mass spectrometer operating in positive mode. The particle beam was xenon gas, and the matrix was *meta*-nitrobenzyl alcohol. Retention times and mass spectral comparisons were made between natural amides isolated from *M. sativa* G98A and synthetic fatty acid amides (see Section 3.4). Free fatty acids from G98A were also compared against authentic samples of commercially available fatty acids.

3.4. Synthesis of fatty acid amides

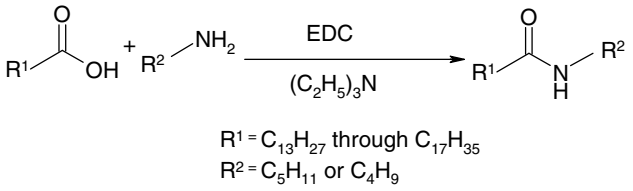
N-(Alkyl)amides of C14:0 through C18:0 fatty acids were prepared according to (Eq. (3)), with the following procedure being employed for all of the amides. A solution of the appropriate amine (1 mmol) in 1 mL of methylene chloride was added to a mixture of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC) slurried in 4 mL of methylene chloride and containing the appropriate fatty acid (1 mmol each). After addition of several drops of triethylamine, the mixture was swirled and the resulting solution was allowed to stand undisturbed at room temperature. After approximately 48 h, the reaction mixture was poured into 10 mL of cold 0.05 M hydrochloric acid (HCl) and the aqueous layer was thoroughly extracted with methylene chloride. The organic layers were combined, washed with 0.05 M HCl (5 mL) and brine (ca. 5 mL), and subsequently percolated through 10 g deactivated basic aluminum oxide using sufficient additional methylene chloride to assure complete elution of all neutral compounds. Solvent was then evaporated under reduced pressure and the products either recrystalized from methanol or purified by flash chromatography, as appropriate. All amides were characterized by GC–MS and high resolution FAB-MS (Table 1).

3.5. Bioassay procedures

A mixture of *N*-(3-methylbutyl)amides and *N*-(2-methylpropyl)amides of normal, saturated C_{14} through C_{18} fatty acids were tested separately for deterring settling by the potato leafhopper. Mixtures of the methylbutyl- and methylpropylamides were prepared using ratios approximating those detected in a particular sample of trichome extracts from *M. sativa* G98A, namely 1:3:7:1.25:1.5 for C_{14}:C_{15}:C_{16}:C_{17}:C_{18}, respectively. *N*-(3-methylbutyl)amide of linoleic acid was also tested singly for deterring leafhopper settling. Synthetic amides were dissolved in acetone and bioassayed according to Ranger et al. (2004a,b). Artificial diet feeding sachets (Habibi et al., 1993) were prepared by pipetting an agarose-based artificial diet into a single plastic gasket (1×1 cm^2) positioned on a microscope cover slip. Parafilm® was then stretched over the gasket and trimmed back to within the gasket edges. A micropipette was used to apply aliquots from stock solutions of the synthetic amides to the exposed Parafilm® surface of the sachet. Since Parafilm® comprised the diet sachet surface, a polar solvent (acetone) was required for applying the synthetic amides to the Parafilm® surface. Solvent was allowed to evaporate from the sachet surface under ambient conditions for about 25 min. Synthetic amides were bioassayed at 100, 50, 25 and 12.5 μg/cm^2.

Comparisons of leafhopper settling on untreated diet sachets or those treated with synthetic amides were made by placing a treated and control sachet at opposing regions within test arenas, which consisted of a clear plastic tube (7.0 cm in diam, and 3.5 cm in height)

positioned upright in a Petri dish lid (Ranger et al., 2004a,b). Once sachets were situated, a second Petri dish lid was



(3)

used to seal the test arena, and 10 acclimated leafhoppers were aspirated into each arena. Test arenas were arranged in a completely randomized design in an aluminum tray under constant fluorescent light, and water was added to the bottom of the tray to prevent leafhopper desiccation. After 60 min of acclimation, the number of insects settling/feeding on a particular diet surface was recorded at 15 min intervals over the next 350 min.

3.6. Statistical analysis

Numbers of leafhoppers settling on each sachet were converted into proportions and arcsine square root-transformed. Data were analyzed with the SAS general linear model (GLM) procedure (SAS Institute, 1985) using a repeated measures split plot analysis of variance (ANOVA). Treatment was used as the main plot effect in the linear statistical model for comparisons of settling. The subplot contained the effects of time and treatment × time. Differences among means for ANOVAs were compared with least significant difference (LSD procedure), Statistical Analysis System (SAS Institute, 1985).

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