Acta Chromatographica 23(2011)4, 621–628
DOI: 10.1556/AChrom.23.2011.4.8

GC–MS Analysis of Volatile Secondary Metabolites in "Mediterranean" and "Continental" *Festuca arundinacea* (Poaceae) Infected with the Fungal Endophyte *Neotyphodium coenophialum* Strain AR542

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Summary. Profiles of volatile secondary metabolites (VSM) in Mediterranean and Continental *Festuca arundinacea,* either endophyte free or infected with the fungal endophyte *Neotyphodium coenophialum* strain AR542, were determined using gas chromatography–mass spectrometry (GC–MS). The profile of VSM in the endophyte-free Mediterranean *F. arundinacea* germplasm was similar to that of endophyte-free Continental *F. arundinacea* germplasm. However, the VSM profile in AR542-infected Mediterranean *F. arundinacea* was different to that in AR542-infected Continental *F. arundinacea*. Compound 1, identified as N-acetylnorloline, was detected in AR542-infected Mediterranean *F. arundinacea* as being sevenfold greater compared with its level in AR542-infected Continental *F. arundinacea.* Levels of compounds 2, 4, and 5 detected in AR542-infected Mediterranean *F. arundinacea* were significantly lower when compared with their levels in the AR542-infected Continental *F. arundinacea*. Levels of compound 3 were similar in both germplasms infected with endophyte strain AR542. The levels of compounds 2, 4, and 5 but not compound 3 were different between AR542 infected and endophyte free depending on germplasm. On the basis of the mass spectra obtained, compounds 2, 3, 4, and 5 were identified as tridecanoic acid methyl ester, *n*-capric acid, 11, 14, 17-eicosatrienoic acid, and linoleic acid ethyl ester, respectively. Our results highlight key differences between the Mediterranean and Continental germplasms. Comparison of the VSM of AR542-infected Mediterranean *F. arundinacea* with AR542-infected Continental *F. arundinacea* showed that there are quantitative differences between the two germplasms. These differences, which may impact on grazing systems involving horses, most probably arose as a result of intrinsic genetic differences between the two germplasms and are yet to be indentified.

Key Words: *Festuca arundinacea*, *Neotyphodium coenophialum*, endophyte, *N*-acetylnorloline

Introduction

Festuca arundinacea Schreb. (tall fescue) is a perennial grass grown extensively in parts of Europe, North America, South America, Australia, and New Zealand [1–5]. Summer-active "Continental" and winter-active "Mediterranean" are the two commercial germplasms of *F. arundinacea* sown in Australian pasturelands. Virtually all *F. arundinacea* germplasms sown in south-eastern Australian pastures are either endophyte free or infected with the mutualistic fungal endophyte *Neotyphodium coenophialum* strain AR542. The presence of *F. arundinacea* infected with *N. coenophialum* "wild type" strain in Australia is extremely rare [6, 7].

F. arundinacea seed lines infected with AR542 strain do not produce the ergot alkaloids, which are usually produced by seed lines infected with "wild type" endophyte. Ergot alkaloids often induce health problems in grazing animals [8]. Instead, *F. arundinacea* seed lines infected with AR542 produce only the volatile loline alkaloids, which are involved in protecting the grass host from herbivorous insects [9, 10].

Since 2001, AR542-infected seed lines of *F. arundinacea* in Australia have been commercially released in three cultivars of Continental, viz., Jesup, Grasslands Advance, and Quantum, marketed as "Jesup Max P," "Grasslands Advance Max P," and "Quantum Max P," respectively. However, AR542-infected seed lines of Mediterranean have been commercially released after 2003 in cultivars Resolute, Resolute 2, and Flecha, marketed as "Resolute Max P," "Resolute-2-Max P," and "Flecha Max P" [11, 12].

In Australia, horses grazing pastures sown with Mediterranean *F. arundinacea* infected with *N. coenophialum* strain AR542 have been affected by a new toxicosis known as equine fescue edema (EFO) [12]. This toxicosis has not occurred in horses grazing pastures containing Continental *F. arundinacea* infected with *N. coenophialum* strain AR542, endophyte-free Mediterranean *F. arundinacea*, or endophyte-free Continental *F. arundinacea*. This suggests an unusual association between Mediterranean *F. arundinacea*, *N. coenophialum* strain AR542, and horses [12].

Several differences between the Mediterranean and the Continental germplasms have been reported [13, 14]. Growth in Mediterranean cultivars is slower than that in Continental cultivars [13]. Mediterranean cultivars responding to drought periods show higher root–shoot ratios and become smaller in size compared with Continental cultivars raised under identical environmental conditions [14]. The effect of AR542 infection on volatile secondary metabolites (VSM), including loline alkaloids, produced by the Mediterranean and Continental germplasms has not been examined. Therefore, in this study, we have assessed and compared the profiles of VSM, with a particular interest on the volatile loline alkaloids, produced by Medi-

terranean and Continental *F. arundinacea* infected with *N. coenophialum* strain AR542.

Experimental

Collection, Planting and Processing of *Festuca arundinacea*

Mediterranean *F. arundinacea* plants infected with AR542 endophyte (ME^{AR542}) were collected from a pasture at Scone, NSW ($n = 5$). Continental *F. arundinacea* plants infected with AR542 endophyte (CE^{AR542}), Mediterranean *F. arundinacea* endophyte free (ME-), and Continental *F. arundinacea* endophyte free (CE-) were obtained from Glen Innes, NSW ($n = 5$). Endophyte status of *F. arundinacea* material used in this study was assigned after the original seed source and the certification that accompanied each seed line were verified by the seed supplier. Plant populations of ME^{AR542} and ME- were cv. Flecha, and populations of CE^{AR542} and CE- were cv. Jesup.

Collected plants were potted and allowed to acclimatize in the Charles Sturt University (Orange, NSW) glass house for 45 days to nullify possible environmental influences that may have had an impact on the grasses' VSM. Shoots were clipped with a pair of scissors on 26 September 2008. Shoots from each plant population were pooled and air-dried at room temperature (20–23°C) until a constant mass was achieved in each sample. Air-dried samples were ground in a motorized micro hammer-cutting mill (Glen Creston Ltd, Stanmore, UK), so as to pass through a 1-mm sieve and stored at −20°C for extraction and determination of VSM.

Extraction of Volatile Secondary Metabolites

VSM including loline alkaloids were extracted on the basis of the method described by Shelby et al. [15] and Petroski et al. [16] with modifications described below. To 200 mg of the ground plant material from each plant, we added 5 mL of extraction solvent (70% methanol/water (*v/v*)). The pH of the extraction solvent was adjusted to 8.5 with 35% NH_4OH to achieve optimal detection for the VSM. Plant suspensions were maintained on a mechanical shaker (Thermolyine, Barnstead, Waltham, USA) overnight. Suspensions were filtered using 0.45-μM PVDF syringe filters, and the filtrate was extracted by partitioning with 4 mL of chloroform. The chloroform layer was separated from the aqueous layer and evaporated under vacuum (40°C; 19.99 kPa). The residue after evaporating the chloroform was resuspended

with 1 mL of the extraction solvent (70% methanol/water (*v/v*); pH 8.5) and injected into gas chromatography–mass spectrometry (GC–MS).

GC–MS Analysis

Samples were analyzed using a GC–MS (QP5050A, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector, as described by Blankenship et al. [17] with minor modifications. Injection volume was 1 µL. Oven temperature was maintained at 70°C for 2 min and was programmed to rise to 300°C at the rate of 6°C min^{-1}. The temperatures of the injector and the detector were maintained at 250°C and 260°C, respectively. A DB-5 ms column (30 m, 0.25-µm film thickness; 0.25-µm bore diameter) was used. Helium was used as the carrier gas; the total-gas flow and velocity were maintained at 134.3 mL min^{-1} and 43.1 cm s^{-1}, respectively. MS scan speed was 1000 amu s^{-1} and the molecular masses (*m/z*) of the compounds between 70 and 700 *m/z* were acquired. The analysis for each sample was repeated 3-4 times.

VSM including volatile loline alkaloids were identified using NIST05 mass spectral library, and when applicable, their mass spectra were compared with those published in the literature.

Data Analysis

Peaks detected in GC–MS were integrated using GC–MS-solution software version 1.2 (Shimadzu, Kyoto, Japan). The percentage of the area under the curve was determined. For each plant material, an average of four replicates (mean ± SEM) was calculated. Differences between the samples were determined using ANOVA. Data were considered statistically significant when $p < 0.05$.

Results and Discussion

GC–MS was used to examine profiles of VSM, with an interest in loline alkaloids, in Mediterranean and Continental *F. arundinacea*, either endophyte free or infected with *N. coenophialum* strain AR542. Five peaks representing five VSM (compounds **1**, **2**, **3**, **4**, and **5**) occurred with retention times (Rt) 14.7, 20.1, 20.7, 23.1, and 23.6 min, respectively (*Table I* and *Fig. 1*). Either the presence or the absence of these compounds and their levels (measured as

the percentage of the area under the curve) varied among the sampled plant materials *(Table I)*.

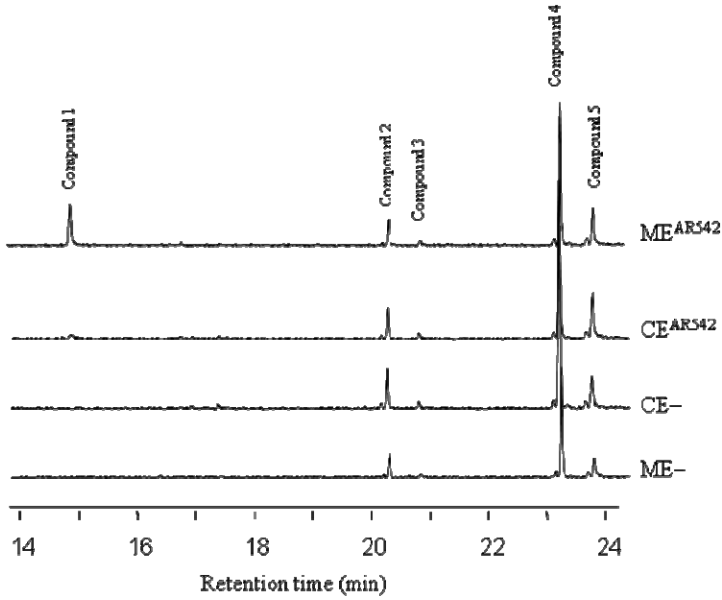

Fig. 1. Total-ion mass chromatograms of AR542-infected Mediterranean *Festuca arundinacea* (ME^{AR542}), AR542-infected Continental *F. arundinacea* (CE^{AR542}), endophyte-free Continental *F. arundinacea* (CE-), and endophyte-free Mediterranean *F. arundinacea* (ME-). Compounds **1**, **2**, **3**, **4** and **5** were identified as *N*-acetylnorloline, tridecanoic acid, *n*-capric acid, eicosatrienoic acid, and linoleic acid, respectively

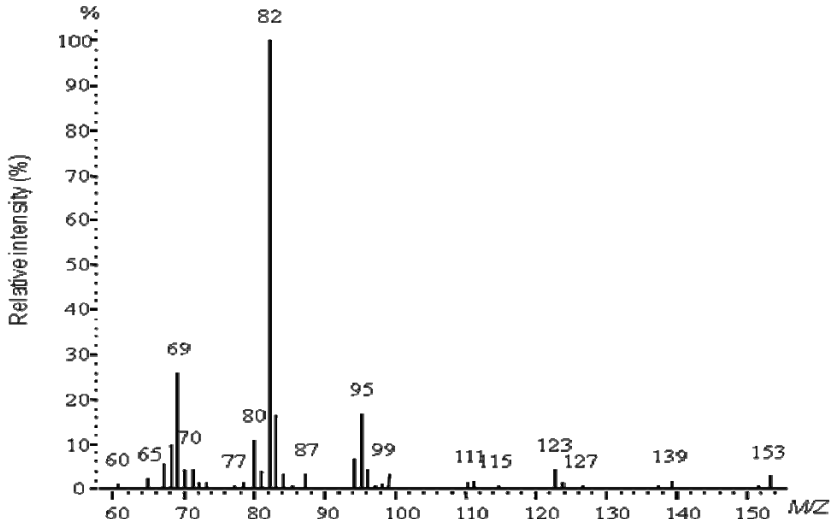

Fig. 2. Representative molecular mass spectrum for compound 1 (*N*-acetylnorloline; Rt 14.7 min) detected in ME^{AR542} and CE^{AR542} using GC–MS

Compound **1** (Rt 14.7 min) was detected only in AR542-infected samples of *F. arundinacea* regardless of it being in Mediterranean or Continental germplasm *(Table I, Fig. 1)*. Compound **1** was identified on the basis of the major *m/z* recorded and their relative intensity: 153 (2.8%), 139 (1.9%), 123 (4.4%), 95 (16.9%), 82 (100%), and 69 (9.8%). The mass specrum *(Fig. 2)* indicated a loline alkaloid. This spectrum was similar to the previously published spectrum for the loline alkaloids [16, 17]. Moreover, the occurrence of the *m/z* 139 and *m/z* 153 is specific to *N*-acetylnorloline [16, 18], confirming that compound **1** is *N*-acetylnorloline. *F. arundinacea* infected with AR542 endophyte is known to produce a single loline alkaloid: *N*-acetylnorloline [19, 20]. The presence of *N*-acetylnorloline confirms the incidence of *N. coenophialum* strain AR542 in the sampled grass materials.

The level of *N*-acetylnorloline in ME^{AR542} was sevenfold greater when compared with its level in CE^{AR542}. *N*-acetylnorloline, an insect deterrent alkaloid, has been implicated with health problems in horses grazing ME^{AR542} pastures but not CE^{AR542} pastures [12]. The large difference in *N*-acetylnorloline levels between ME^{AR543} and CE^{AR542} in this study supports the need for further investigations into any possible link between *N*-acetylnorloline and EFO.

Compounds **2**, **3**, **4**, and **5** were detected in all *F. arundinacea* samples tested, irrespective of their germplasm or their endophyte status. Levels of compounds **2**, **3**, **4**, and **5** in ME- samples were similar ($p > 0.05$) to those found in CE- samples *(Table I)*.

In Mediterranean germplasm, levels of compounds **2** and **4** were significantly lower in ME^{AR542} compared with those in ME- ($p < 0.05$). In contrast, levels of compound **5** were significantly ($p < 0.05$) higher in ME^{AR542} compared with those in ME-. Levels of compound **3** were similar *(Table I)*.

In Continental germplasm, levels of compound **4** were significantly ($p < 0.05$) higher in CE^{AR542} compared with those in CE-. In contrast, levels of compound **5** were significantly ($p < 0.05$) lower. Levels of compounds **2** and **3** were similar *(Table I)*.

AR542 infection variably altered the levels of compounds **2**, **4**, and **5** but not that of compound **3** in Mediterranean and Continental *F. arundinacea*. Levels of compounds **2**, **4**, and **5** in ME^{AR542} were significantly ($p < 0.05$) lower compared with their corresponding levels in CE^{AR542} *(Table I)*.

The major *m/z* recorded and their relative intensity for compound **2** were 69 (15.9%), 74 (100%), 87 (59.3%), 143 (11.9%), and 227 (10.8%); those for compound **3** were 60 (62.8%), 70 (29.5%), 73 (100%), 78 (20.6), 83 (38.3%), 85 (24.5%), and 97 (24.0%); those for compound **4** were 67 (79.3%), 79 (100%), 95 (45.4%), 108 (23.3%), 121 (15.9%), 135 (12.0%), and 149 (5.0%); and those for compound **5** were 65 (15.2%), 67 (100%), 71 (25.2%), 79 (75.5%), 95 (32.8%), 108 (21.5%), 126 (11.7%), 138 (9.3%), 165 (6.6%), and 170 (5.9%).

On the basis of the above *m/z*, compounds **2**, **3**, **4**, and **5** were identified as tridecanoic acid methyl ester, *n*-capric acid, 11, 14, 17-eicosatrienoic acid methyl ester, and linoleic acid ethyl ester, respectively *(Table I)*. Further studies are required to determine the precise biological roles of these four compounds in *F. arundinacea*.

Table I. Compounds detected in AR542-infected Mediterranean *F. arundinacea* (ME^{AR542}; $n = 4$), AR542-infected Continental *F. arundinacea* (CE^{AR542}; $n = 4$), endophyte-free Continental *F. arundinacea* (CE-; $n = 4$), and endophyte-free Mediterranean *F. arundinacea* (ME-; $n = 4$)

Compound No	MW	Molecular formula	Identification	Rt (min)	Base peak (*m/z*)	Area under the curve (%)			
						ME^{AR542}	CE^{AR542}	CE-	ME-
1	182	$C_9H_{14}N_2O_2$	*N*-Acetylnorloline	14.7	82	22 ± 1.5	3 ± 0.5	nd	nd
2	228	$C_{14}H_{28}O_2$	Tridecanoic acid methyl ester	20.1	74	10 ± 0.5	12 ± 0.1	14 ± 0.9	16 ± 0.9
3	172	$C_{10}H_{20}O_2$	*n*-Capric acid	20.7	60	2 ± 0.1	2.1 ± 0.2	3 ± 0.5	3 ± 1.0
4	320	$C_{21}H_{36}O_2$	11,14,17-eicosatrienoic acid methyl ester	23.1	79	46 ± 3.1	65.7 ± 1.8	73 ± 4.5	64 ± 1.3
5	308	$C_{20}H_{36}O_2$	Linoleic acid ethyl ester	23.6	67	15 ± 1.0	21.4 ± 4	15 ± 1.3	12 ± 1.1

MW, molecular weight; nd, not detected; Rt, retention time

Our findings indicate that Mediterranean *F. arundinacea* responds to AR542 infection in a manner which is different to Continental *F. arundinacea*. This study showed that there are differences in the quantities of VSM between ME^{AR542} and CE^{AR542} and in the quantities and quality of VSM between AR542 infected and endophyte free within the same germplasm. These differences, which may have an impact on grazing systems involving horses, most probably arose as a result of intrinsic genetic differences between the two germplasms and are yet to be identified.

Acknowledgments

We thank Suresh Govindaraghavan (Lipa Pharmaceuticals, Sydney, Australia) and S. Raghu (Arid Zone Research Institute, Alice Springs, Australia)

for critical comments on the draft manuscript, and Helen Nicol (Charles Sturt University, Orange, Australia) for statistical advice. A. Raman and W. Wheatley thank the Trustees of the Orange Campus Foundation and the Rural Management Research Institute (Orange, NSW) for financial support.

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Accepted by MWH