Seed Science Research (2016) **26**, 84–91
doi:10.1017/S0960258515000367
© *Cambridge University Press 2015*

TECHNICAL UPDATE

Development of a reliable GC-MS method for fatty acid profiling using direct transesterification of minimal quantities of microscopic orchid seeds

Louise Colville[1], Tim R. Marks[1], Hugh W. Pritchard[1], Ceci C. Custódio[2] and Nelson B. Machado-Neto[2*]

[1]Department of Comparative Plant and Fungal Biology, Royal Botanic Gardens, Kew, Wakehurst Place, Ardingly, West Sussex, RH17 6TN, UK; [2]Agronomy College – UNOESTE, Rodovia Raposo Tavares Km 572, Presidente Prudente SP, Brazil19067175

(Received 10 March 2015; accepted after revision 10 November 2015; first published online 22 December 2015)

Abstract

Orchid seeds are among the smallest seeds in nature and they are naturally rich in fatty acids. However, the fatty acid composition of orchid seeds has not been investigated because the sample masses utilized for widely used methods for fatty acid profiling would generally require prohibitively large numbers (i.e. 10,000s) of seeds. The present work aimed to develop a method for fatty acid analysis using gas chromatography–mass spectrometry on small quantities (mg) of seeds. The method was developed using the seeds of two species, *Dactylorhiza fuchsii*, a temperate terrestrial, and *Grammatophyllum speciosum*, a tropical epiphyte. A range of sample masses was tested to determine the minimum mass required to achieve reliable fatty acid composition data. A direct transesterification method was used, which did not require extraction of fatty acids from seeds prior to analysis, and the effects of seed processing (crushed versus intact seeds) and incubation time in toluene on fatty acid yield were tested. Stable fatty acid profiles were obtained using as little as 10 mg of seeds. Neither crushing the seeds nor extending the toluene incubation step had much effect on the fatty acid yield. The simple direct transesterification method presented will enable the fatty acid composition of orchid seeds, and possibly other small seeds, to be determined reliably for studies into seed development, storage and germination.

*Correspondence
Email: nbmneto@unoeste.br

Keywords: ***Dactylorhiza fuchsii*, gas chromatography–mass spectrometry, *Grammatophyllum speciosum*, lipid, Orchidaceae**

Introduction

After the Asteraceae, the Orchidaceae is the largest Angiosperm family, with 736 genera (Chase *et al.*, 2015) and over 26,000 species, with around 500 new species described per year (Chase *et al.*, 2015). There is evidence that orchids arose over 80 million years ago (Ramirez *et al.*, 2007), and possibly up to 100 million years ago (Chase, 2001). Orchids are valued for their ornamental and medicinal uses, and are a prominent focus for plant conservation (Yam *et al.*, 2009). Over the past two centuries the interest in orchid species for their ornamental value has intensified and this, coupled with loss of habitat and changing climate, has increased the pressure on natural populations. Along with habitat preservation, *ex situ* conservation strategies such as seed banking are important for conserving threatened orchid species (Koopowitz, 2001; Machado Neto and Custódio, 2005; Seaton and Pritchard, 2008; Li and Pritchard, 2009; Seaton *et al.*, 2010). Seed conservation offers a simple and fairly inexpensive means of conserving diverse genetic material in a relatively small space. Due to their tiny size many thousands of orchid seeds can fit into a single vial, while the space and resources required to maintain similar numbers of living plants would be prohibitive. Mature orchid seeds are very small and are classified as 'dust seeds' (Eriksson and Kainulainen, 2011). Seed sizes range from 0.28 to 10.09 mm in length, and 0.39 μg to 1.79 mg in weight (Yam *et al.*, 2009).

https://doi.org/10.1017/S0960258515000367 Published online by Cambridge University Press

Orchid seeds consist of rudimentary globular embryos of relatively few cells with no endosperm or cotyledonary storage reserves (Lee *et al.*, 2008). Lipid and protein bodies within the embryonic cells form the main storage reserves, and orchid seeds tend not to accumulate starch. Instead, carbohydrates required during germination are provided by fungal symbionts to support early seedling growth (Peterson *et al.*, 1998; Rasmussen, 2002).

A thorough understanding of seed storage physiology is crucial for successful seed banking, and studies of orthodox seeds of agricultural species have aided understanding of how seed development contributes to seed longevity and germination (Ellis and Pieta Filho, 1992; Sanhewe and Ellis, 1996a, b). Little is known about orchid seed reserve deposition and its consequences for the acquisition of desiccation tolerance, timing of harvest, storage behaviour, germination (Schwallier *et al.*, 2011) and seedling vigour in comparison with well-studied agricultural species (Lee *et al.*, 2008). Some studies have reported that seeds containing lipids as the major storage component are shorter-lived than seeds with mainly protein or carbohydrate reserves (Nagel and Börner, 2010), while others have found no association between seed storage reserves and longevity (Walters *et al.*, 2005; Probert *et al.*, 2009). However, seed storability may be related to lipid composition rather than lipid content, particularly in relation to lipid stability (Ponquett *et al.*, 1992) and thermal properties (Crane *et al.*, 2003, 2006), including that of orchids (Pritchard and Seaton, 1993).

Comparative longevity studies of orchid seeds have shown that seeds of 12 species are desiccation tolerant and have improved lifespan on desiccation (Pritchard *et al.*, 1999; Hay *et al.*, 2010). These studies have shown that orchid seeds are relatively short-lived compared to non-orchid species. While storage at −18°C may be acceptable for some species, seeds of other species might display temperature-specific cold sensitivity, which could be a factor limiting their longevity at some low temperatures (Pritchard *et al.*, 1999; Seaton *et al.*, 2013). Short longevity limits the effectiveness of conventional *ex situ* storage in seed banks as a conservation strategy, but alternative techniques such as cryopreservation show promise (Pritchard *et al.*, 1999; Merritt *et al.*, 2005; Seaton *et al.*, 2013). However, overall data for orchid seed storage are limited, and questions remain concerning the most suitable environmental conditions for storage (Pritchard and Seaton 1993; Shoushtari *et al.* 1994; Pritchard *et al.* 1999; Machado-Neto and Custódio 2005; Hay *et al.*, 2010; Hosomi *et al.*, 2012).

Despite the potential importance of the lipid reserves in relation to seed desiccation tolerance and longevity, no studies to date have attempted to characterize the fatty acid composition of orchid seeds. A search of the Seed Oil Fatty Acid (SOFA; http://sofa.mri.bund.de/) database for records of the Orchidaceae family yielded eight records with fatty acid composition. Six records related to the lipids of leaves, roots and flower spikes, rather than seeds, of *Phalaenopsis* and *Cattleya* hybrids. The remaining two records of fatty acid composition were for an *Oncidium* sp. and *Dendrobium moniliforme*, but it is not clear whether these records are for seeds. While orchids represent around 8% of all angiosperm species, <0.1% of the 7000 plant species records on the SOFA database are for orchids. Similarly, Orchidaceae taxa represent <1% of the 33,346 taxa on the Royal Botanic Gardens, Kew's Seed Information Database and, of the 289 Orchidaceae taxa, oil content data are available for just three species.

Biochemical studies of orchid seeds are limited by the microscopic nature of the seeds, which means that large quantities of seeds are needed for conventional assays. There are numerous methods for fatty acid analysis, most involve initial extraction in organic solvents such as hexane (Younis *et al.*, 2000; Gören *et al.*, 2006; Nedhi *et al.*, 2011), petroleum ether (Matthaus and Özcan, 2011) or, most commonly, chloroform:methanol (2:1, v/v) (Bligh and Dyer, 1959; Colville *et al.*, 2012). This is followed by transesterification to convert saponifiable lipids to fatty acid methyl esters for analysis by gas chromatography. However, these methods tend to use large amounts of tissue. A number of reports in the literature have shown that direct transesterification methods without prior lipid extraction can result in higher yields of fatty acids, are quicker and avoid the use of toxic chlorinated organic solvents (Lepage and Roy, 1984, 1986; Griffiths *et al.*, 2010; Cavonius *et al.*, 2014). Smaller-scale direct transesterification methods have been developed for microalgae (Lee *et al.*, 2010) and for microheterotrophs (Lewis *et al.*, 2000). A sub-microscale assay for fatty acids using gas chromatography–mass spectrometry (GC–MS) has also been described (Bigelow *et al.*, 2011). However, these small-scale assays have yet to be applied to orchid seeds.

The aim of this work was to develop a reliable and simple protocol for fatty acid profiling of orchid seeds using GC–MS. Comparison was made between two species: *Dactylorhiza fuchsii* (common spotted orchid), a temperate terrestrial species belonging to the Orchidoideae subfamily which is widespread across Europe; and *Grammatophyllum speciosum* (common names include giant orchid, tiger orchid and sugar cane orchid), a tropical epiphytic species belonging to the Epidendrioideae subfamily, and the world's largest orchid, native to Burma, Laos, Vietnam, Indonesia and Malaysia.

Material and methods

Seed material

Seeds of *D. fuchsii* were collected in the Wakehurst Place estate in Ardingly, UK in 2013 and *G. speciosum* seeds

https://doi.org/10.1017/S0960258515000367 Published online by Cambridge University Press

were obtained from the Prince of Songkla University, Thailand in 2011 and stored at 15% relative humidity (RH) and 5°C since their arrival in ripe capsules. Seeds of both species were cleaned and stored at 15% RH and 5°C in paper bags. Initial viability was determined using tetrazolium (TZ) staining as described by Hosomi *et al.* (2011). Germination was conducted in three Petri dishes, each with 5 mg seeds. Seeds were disinfected according to Hosomi *et al.* (2012) with 5 g l^{-1} of dichloroisocyanuric acid sodium salt (DCCA; Sigma, Colorado, USA), rinsed three times and sown in Murashige and Skoog medium (Murashige and Skoog, 1962) at half strength.

Fatty acid transesterification

Due to the ease of preparation and high fatty acid recovery (Cavonius *et al.*, 2014) sulphuric acid in methanol was selected as the catalyst for direct transesterification in this study, following a method adapted from Christie (1989) and Colville *et al.* (2012).

Three replicates of each sample mass of 2.5, 5, 10, 15 and 20 mg were used to determine the minimum sample mass required for reliable determination of fatty acid composition. Seeds were placed into 20-ml glass vials with 1 ml of toluene containing 50 mg l^{-1} butylated hydroxytoluene (BHT) as an antioxidant. As an internal standard, 10 μl of 10 mg ml^{-1} heptadecanoic acid was added. The samples were incubated for 4 h at room temperature. Fatty acids were methylated with 2 ml of 1% (v/v) sulphuric acid in methanol overnight at 50°C with constant shaking at 150 rpm. The fatty acid methyl esters (FAMEs) were isolated by partitioning with 5 ml of hexane and 5 ml of NaCl (5%, w/v). The organic phase was transferred to a clean vial and the aqueous phase was washed with another 5 ml of hexane. Removal of the organic phase was undertaken carefully to avoid the aspiration of seeds into the Pasteur pipette. The organic phases were combined and dried at 45°C under a stream of nitrogen gas. The residue was dissolved in 1 ml of hexane and transferred to 2-ml autosampler vials for GC–MS analysis.

The effect of incubation time in toluene was investigated by extending the 4-h incubation to 16 h (overnight) before derivatizing the fatty acids as described above. In addition, comparison was made between the extraction efficiency from intact and crushed seeds using sample masses of 5 mg. Seeds were crushed between glass slides prior to overnight extraction in toluene as described above.

GC–MS analysis of fatty acid methyl esters

FAMEs were separated by GC (Thermo Finnigan Trace GC Ultra) using a FAMEWAX column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness (df); Thames Restek UK Ltd, Saunderton, Buckinghamshire, UK) running a temperature program (initial temperature 70°C; 20°C min^{-1} until 195°C; 5°C min^{-1} until 240°C; 10 min at 240°C) with helium as the carrier gas (constant flow rate of 1 ml min^{-1}). The compounds were detected by MS (Thermo Finnigan Trace DSQ; ionization energy 70 eV, scanning frequency m/z 10–500 at 0.3 s) and identified by comparison with the NIST mass spectral database (National Institute of Standards and Technology, Gaithersburg, Maryland, USA) and analytical standards (FAME Mix C4–C24, Supelco, Bellefonte, Pennsylvania, USA). FAME quantification was performed using quantitative standard curves (FAME Mix GLC-10, -30 and -50, Supelco).

Data analysis

Based on the molar percentage fatty acid composition, the double-bond index (DBI) and peroxidizability index (PI) were calculated according to Pamplona *et al.* (1998). Analysis of variance and a *post-hoc* Tukey test (Genstat v.14; GenStat Committee, 2011) were used to determine statistical significance between sample masses and treatments. All differences quoted in the text were significant at $P < 0.05$.

Results and discussion

Seed viability

Germination and viability of the seed lots were assessed immediately prior to the fatty acid analyses. The total germination of *D. fuchsii* seeds was 89.7% and the viability according to the TZ test was 84.8%. *G. speciosum* seeds failed to germinate, possibly due to the use of inappropriate germination media or induction of dormancy, but TZ staining indicated that 87.6% of seeds were viable. The *G. speciosum* seeds had been stored at 15% RH and 5°C for 2 years since 2011, when the germination and viability of the fresh seeds were 57% and 99%, respectively. This indicates that the *G. speciosum* seeds had experienced some deterioration during storage, but that seed viability was comparable between the two species at the time of analysis.

Fatty acid yield and composition

The fatty acid composition of *D. fuchsii* and *G. speciosum* seeds was fairly similar in terms of the major fatty acid components, which were methyl linoleate, followed by methyl palmitate and methyl oleate (Table 1). These fatty acids are the most common polyunsaturated, saturated and monounsaturated fatty acids, respectively, found in plants.

https://doi.org/10.1017/S0960258515000367 Published online by Cambridge University Press

Table 1. Content of fatty acid methyl ester components in *Dactylorhiza fuchsii* and *Grammatophyllum speciosum* seeds obtained by direct derivatization of different seed masses following incubation in toluene for 4 h or 16 h. Values represent the mean ($n = 3$) fatty acid methyl ester concentration. Lowercase letters indicate significant differences ($P < 0.05$) between the content of individual fatty acids in different sample masses, and uppercase letters indicate significant differences ($P < 0.05$) in the fatty acid content following 4 h or 16 h incubation in toluene

		Seed mass (mg)				
		2.5	5	10	15	20
	Time (h)	FAME [mmol (g FW)$^{-1}$]				
Dactylorhiza fuchsii						
Methyl palmitate (16:0)	4	0.0830 aA	0.0948 aA	0.0909 aA	0.0954 aA	0.1059 aA
	16	0.0799 aA	0.1068 bA	0.0936 abA	0.1051 bA	0.1117 bA
Methyl stearate (18:0)	4	0.0073 aA	0.0090 abA	0.0092 abA	0.0117 bA	0.0119 bA
	16	0.0076 aA	0.0115 bA	0.0096 abA	0.0130 bA	0.0120 bA
Methyl oleate (18:1)	4	0.0847 aA	0.0904 aA	0.0824 aA	0.0918 aA	0.1026 aA
	16	0.0864 aA	0.1003 aA	0.0845 aA	0.0988 aA	0.1030 aA
Methyl octadecenoate* (18:1)	4	0.0008 aA	0.0035 bB	0.0043 bA	0.0071 cA	0.0077 cA
	16	0.0005 aA	0.0051 bA	0.0045 bA	0.0079 cA	0.0076 cA
Methyl linoleate (18:2)	4	0.4656 aA	0.4881 aA	0.4460 aA	0.4323 aA	0.4731 aA
	16	0.4668 aA	0.5522 aB	0.4476 aA	0.4697 aA	0.4505 aA
Methyl linolenate (18:3)	4				0.0002	
	16				0.0002	
Grammatophyllum speciosum						
Methyl pentadecanoate (15:0)	4	0.0000 aA	0.0000 aA	0.0008 abA	0.0016 bA	0.0015 bA
	16	0.0000 aA	0.0000 aA	0.0013 bA	0.0018 cA	0.0021 dB
Methyl palmitate (16:0)	4	0.0107 aA	0.0233 bcA	0.0267 bcA	0.0281 cB	0.0203 bA
	16	0.0171 aA	0.0248 abA	0.0270 bA	0.0267 bA	0.0279 bB
Methyl stearate (18:0)	4	0.0005 aA	0.0067 bA	0.0071 bA	0.0081 b	0.0062 bA
	16	0.0031 aB	0.0063 bA	0.0077 bB	0.0073 b	0.0081 bA
Methyl arachidate (20:0)	4	0.0000 aA	0.0018 bA	0.0021 bcA	0.0033 cA	0.0026 bcA
	16	0.0000 aA	0.0012 bA	0.0026 cA	0.0031 cdA	0.0035 dA
Methyl oleate (18:1)	4	0.0046 aA	0.0136 bcA	0.0143 bcA	0.0160 cB	0.0118 bA
	16	0.0090 aA	0.0138 bA	0.0152 bA	0.0145 bA	0.0152 bA
Methyl octadecenoate* (18:1)	4	0.0000 aA	0.0020 bA	0.0025 bA	0.0037 b	0.0029 bA
	16	0.0000 aA	0.0019 bA	0.0034 cA	0.0033 c	0.0036 cA
Methyl linoleate (18:2)	4	0.1213 a	0.2212 c	0.2025 c	0.2220 bc	0.1586 ab
	16	0.172 ns	0.2273 ns	0.2157 ns	0.1976 ns	0.2039 ns
Methyl linolenate (18:3)	4	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0002 a
	16	0.0000 ns	0.0000 ns	0.0000 ns	0.0000 ns	0.0001 ns

*Methyl octadecenoate – unresolved double-bond position.

Methyl octadecenoate (unresolved double-bond position) and methyl stearate were present at low concentrations (Table 1). *G. speciosum* also contained methyl pentadecanoate, methyl linolenate and methyl arachidate as minor components. The total fatty acid concentration in *D. fuchsii* seeds was more than double that in *G. speciosum* seeds. There was no significant effect of sample mass on the concentration of methyl linoleate or methyl oleate in *D. fuchsii* seeds (Table 1), but for methyl palmitate, methyl stearate and methyl octadecenoate there were increases in concentration with sample mass. For *G. speciosum* seeds, where fatty acid concentrations were lower than in *D. fuchsii* seeds, there was an increase in all fatty acid concentrations as sample mass increased. At the lowest sample mass (2.5 mg) only four fatty acids were detected in *G. speciosum* seeds: methyl palmitate, methyl stearate, methyl oleate and methyl linoleate. This increased to six fatty acids when the sample mass was doubled to 5 mg, with the detection of methyl octadecenoate and methyl arachidate; and seven fatty acids when the sample mass was increased to 10 mg, with the detection of methyl pentadecanoate. Methyl linolenate was not detected at sample masses lower than 20 mg.

The proportion of each fatty acid as a percentage of total fatty acid concentration showed a significant change with sample mass for *D. fuchsii*, and also for *G. speciosum* (Table 2). In both species the percentage of methyl linoleate declined, while the percentages of the fatty acids with lower abundance increased as sample mass increased. Due to their lower abundance, more saturated fatty acids were detected as sample mass was increased, which altered the ratio of unsaturated:saturated fatty acids, and also the double-bond

https://doi.org/10.1017/S0960258515000367 Published online by Cambridge University Press

Table 2. Fatty acid methyl ester composition of the seeds of two orchid species: *Dactylorhiza fuchsii* (a temperate species) and *Grammatophyllum speciosum* (a tropical species). Values represent the mean ± SD ($n = 3$) abundance of each fatty acid as a percentage of the total fatty acid methyl ester (FAME) molar concentration. The unsaturated:saturated fatty acid ratio is shown along with the double-bond index (DBI) and peroxidizability index (PI), which were calculated as described in the text. Letters indicate significant differences ($P < 0.05$) between different sample masses

	Seed mass (mg)				
	2.5	5	10	15	20
	FAME abundance (molar %)				
Dactylorhiza fuchsii					
Methyl palmitate (16:0)	12.918 ± 0.4490 a	13.829 ± 0.1260 ab	14.360 ± 0.0420 ab	14.921 ± 0.8529 b	15.231 ± 1.1514 b
Methyl stearate (18:0)	1.138 ± 0.06870 a	1.312 ± 0.08021 ab	1.452 ± 0.0040 ab	1.840 ± 0.1802 c	1.718 ± 0.1736 bc
Methyl oleate (18:1)	13.201 ± 0.1394 a	13.170 ± 0.1555 a	13.033 ± 0.1347 a	14.378 ± 0.2819 ab	14.850 ± 1.2283 b
Methyl octadecenoate* (18:1)	0.136 ± 0.1178 a	0.511 ± 0.02343 b	0.681 ± 0.0205 b	1.117 ± 0.1255 c	1.113 ± 0.1087 c
Methyl linoleate (18:2)	72.607 ± 0.3641 c	71.179 ± 0.1175 c	70.474 ± 0.1566 bc	67.744 ± 0.7606 ab	67.087 ± 2.1778 a
Unsaturated/Saturated	6.12 ± 0.256 c	5.60 ± 0.038 bc	5.32 ± 0.018 ab	4.97 ± 0.273 ab	4.92 ± 0.382 a
DBI	158.6 ± 0.86 c	156.0 ± 0.16 c	154.7 ± 0.18 bc	151.0 ± 1.47 ab	150.1 ± 3.20 ab
PI	72.9 ± 0.37 c	71.5 ± 0.11 c	70.8 ± 0.15 bc	68.1 ± 0.76 ab	67.5 ± 2.15 a
Grammatophyllum speciosum					
Methyl pentadecanoate (15:0)	0.000 ± 0.0000 a	0.000 ± 0.0000 a	0.337 ± 0.296 ab	0.579 ± 0.0032 bc	0.740 ± 0.0829 c
Methyl palmitate (16:0)	7.785 ± 0.3310 a	8.684 ± 0.6594 ab	10.411 ± 0.6910 c	9.950 ± 0.2415 bc	10.032 ± 0.4492 bc
Methyl stearate (18:0)	0.326 ± 0.5641 a	2.491 ± 0.1992 b	2.764 ± 0.2363 b	2.865 ± 0.1118 b	3.044 ± 0.0159 b
Methyl arachidate (24:0)	0.000 ± 0.0000 a	0.677 ± 0.1687 b	0.841 ± 0.3398 bc	1.158 ± 0.0820 c	1.285 ± 0.0312 c
Methyl oleate (18:1)	3.336 ± 0.2733 a	5.098 ± 0.4131 b	5.572 ± 0.1645 bc	5.651 ± 0.0492 bc	5.749 ± 0.0952 c
Methyl octadecenoate* (18:1)	0.000 ± 0.0000 a	0.745 ± 0.2891 b	1.012 ± 0.5136 b	1.295 ± 0.0468 b	1.454 ± 0.0655 b
Methyl linoleate (18:2)	88.553 ± 1.0632 c	82.305 ± 1.4947 b	79.062 ± 0.8819 a	78.501 ± 0.1136 a	77.609 ± 0.6016 a
Methyl linolenate (18:3)	0.000 ± 0.0000 a	0.000 ± 0 .0000 a	0.000 ± 0.0000 a	0.000 ± 0.0000 a	0.086 ± 0.0772 a
Unsaturated/Saturated	11.41 ± 1.200 b	7.92 ± 0.651 a	6.51 ± 0.222 a	6.67 ± 0.075 a	6.49 ± 0.262 a
DBI	180.4 ± 1.87 c	170.5 ± 2.38 b	164.7 ± 1.12 a	163.9 ± 0.23 a	162.4 ± 1.21 a
PI	88.6 ± 1.06 c	82.5 ± 1.48 b	79.2 ± 0.87 a	78.7 ± 0.11 a	77.8 ± 0.60 a

*Methyl octadecenoate – unresolved double-bond position.

index (DBI) and peroxidizability index (PI) (Table 2). Lewis *et al.* (2000) reported that variation in sample mass (1–20 mg) had no significant effects on the total amount or relative proportion of fatty acids from two microheterotrophs. Likewise, Li *et al.* (2006) observed no difference in fatty acid composition of *Arabidopsis* seeds between direct transesterification of 10 mg of seeds compared to ~1 mg of seeds. It is likely that the minimum sample size for obtaining reproducible fatty acid composition data depends on the oil content of the sample and the limit of detection of the analytical method. In this study there were less significant changes in fatty acid concentration or relative abundance at sample masses ≥10 mg (Tables 1, 2), which indicates that a sample mass of 10 mg is sufficient to obtain reliable fatty acid composition data for *D. fuchsii* and *G. speciosum* seeds. However, optimization of sample mass may be required for other orchid species. Seed weights across the Orchidaceae family are reported to vary from 0.39 μg to 1.79 mg (Yam *et al.*, 2009), which means that the number of seeds required for a sample mass of 10 mg could range between 5 and 26,000. However, given that a single capsule of *G. speciosum* may contain up to 2 million seeds (Seaton *et al.*, 2013), while capsules of *D. fuchsii* may contain ~2000 seeds, each weighing ~1.9 μg (Marks *et al.*, 2014), a sample mass of 10 mg is feasible for many studies.

To determine whether the efficiency of fatty acid extraction could be improved, the solubilization step in toluene prior to derivatization was extended from 4 to 16 h (i.e. overnight). The longer incubation time in toluene had no significant effect on the fatty acid yield from seeds of either species (Fig. 1), indicating that the 4-h solubilization step in toluene is sufficient for obtaining reproducible yields. Other direct transesterification methods, e.g. Lewis *et al.* (2000) and Li *et al.* (2006), do not include a pre-incubation in toluene, but toluene is considered necessary for the solubilization of non-polar lipids such as triacylglycerols (Christie, 1989), which form the bulk of seed-storage lipid reserves. Orchid seeds tend to have a hydrophobic, balloon-like testa, which causes the seeds to clump together and float on the surface of solvents. For this reason a pre-incubation in toluene was included to improve the access for organic solvents in the subsequent derivatization step.

Comparison was also made between the yield of fatty acids from intact seeds and seeds that had been crushed prior to 16 h incubation in toluene.

https://doi.org/10.1017/S0960258515000367 Published online by Cambridge University Press

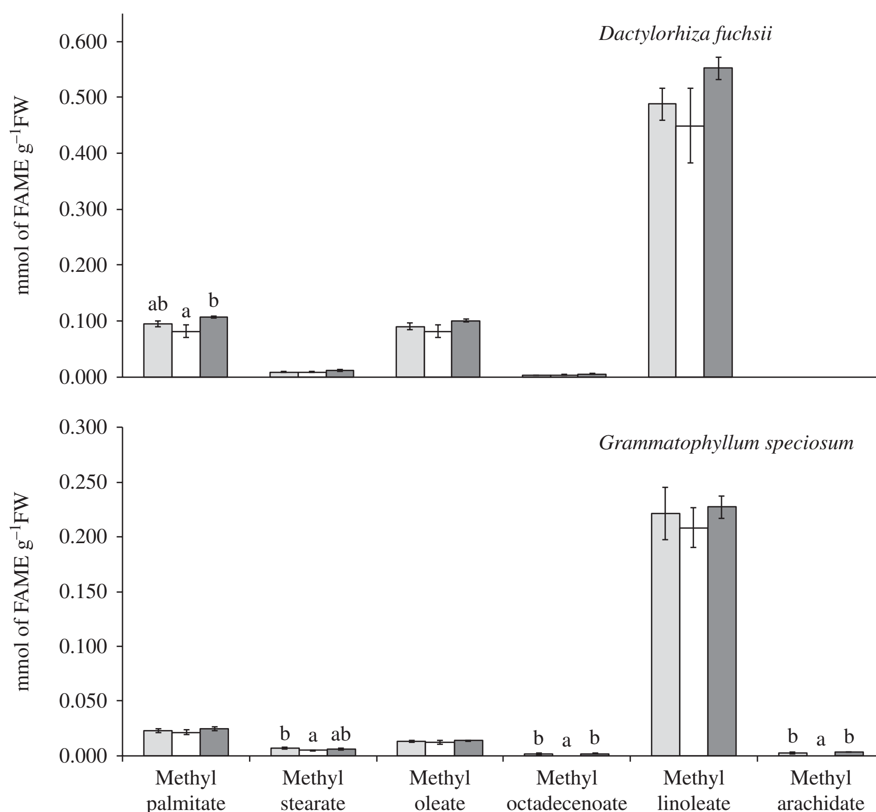

Figure 1. The yield of fatty acid methyl esters from intact *Dactylorhiza fuchsii* and *Grammatophyllum speciosum* seeds (5 mg) following incubation in toluene for 4 h (light grey bars) or 16 h (white bars) compared to the yield from crushed seeds following incubation in toluene for 16 h (dark grey bars). Bars represent mean ± SD ($n = 3$). Letters indicate significant differences ($P < 0.05$) in the yield of individual fatty acids between different extraction protocols.

Crushing the seeds prior to direct transesterification tended to reduce the fatty acid yield, but the reduction was only significant for methyl palmitate ($P = 0.012$) in *D. fuchsii* seeds, and methyl stearate ($P = 0.035$), methyl octadecenoate ($P = 0.007$) and methyl arachidate ($P = 0.007$) in *G. speciosum* seeds. This suggests that crushing the seeds led to loss of a portion of the sample, possibly due to incomplete recovery of material from the surface of the glass slides. Direct transesterification of intact *Arabidopsis* seeds was also reported to result in similar oil yield and fatty acid composition as the derivatization of ground seed samples (Li *et al.*, 2006), indicating that for small seeds with thin seed coats grinding is unnecessary, and in some cases may lead to sample loss.

Fatty acid unsaturation indices and relationships with seed longevity

The data for the 10 mg sample mass was used to compare the fatty acid unsaturation indices between the two species. The ratio of unsaturated to saturated fatty acids was lower in *D. fuchsii* compared to *G. speciosum* ($P = 0.001$; Table 2), indicating a greater degree of lipid saturation. *D. fuchsii* had a DBI of 154.7 ± 0.18 and a peroxidizability index (PI) of 70.8 ± 0.15, which were both significantly lower ($P < 0.001$) than those of *G. speciosum* (DBI = 164.7 ± 1.12; PI = 79.2 ± 0.87). This suggests that *D. fuchsii* seed lipids are less susceptible to oxidative damage than *G. speciosum* seed lipids. This could have implications for seed storage because lipid peroxidation is considered to play a role in seed deterioration during storage (Sung, 1996; Goel and Sheoran, 2003; Ratajczak and Pukacka, 2005). Preliminary storage experiments indicate that *G. speciosum* seeds may be shorter-lived than *D. fuchsii* seeds. Germination of *G. speciosum* seeds declined from 50% to 0% during 24 months of storage at 15% RH and 20°C, compared to germination of *D. fuchsii* which declined from 80% to 62% under the same conditions (T.R. Marks, unpublished data). Both the DBI and PI of the mitochondrial membrane were found to be inversely correlated with maximum life span in mammals (Pamplona *et al.*, 1998). Similarly, a negative relationship between DBI and longevity was reported for desiccated anhydrobiotic plant systems, e.g. seeds, pollen and spores (Hoekstra, 2005), which supports the possibility that fatty acid unsaturation could be a factor contributing to seed longevity. Although Ponquett *et al.*

https://doi.org/10.1017/S0960258515000367 Published online by Cambridge University Press

(1992) did not observe a significant relationship between total lipid unsaturation and longevity for seeds of eight species, relations between seed life span and indices of fatty acid unsaturation require more detailed evaluation.

Conclusions

A straightforward method for profiling the fatty acid composition of orchid seeds using GC–MS is presented. The method can provide reliable composition data using sample masses as low as 10 mg. Direct transesterification of fatty acids requires no pre-processing of seed samples and eliminates time-consuming extraction procedures and reduces the potential for sample loss. The fatty acid profiles of the seeds of two orchid species (*D. fuchsii* and *G. speciosum*) were determined using this method, and are presented for the first time. Characterization of orchid seed lipids will provide a valuable insight into storage-reserve deposition and the role of lipids in seed storability and longevity.

Financial support

This work was supported by Science without Borders fellowships (C.C.C. grant number 245783/2012-1, and N.B.M.N. grant number 245777/2012-1; both from Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil); Royal Botanic Gardens, Kew receives grant-in-aid from Defra.

Conflicts of interest

None.

References

Bigelow, N.W., Hardin, W.R., Barker, J.P., Ryken, S.A., Macrae, A.C. and Cattolico, R.A. (2011) A comprehensive GC–MS sub-microscale assay for fatty acids and its applications. *Journal of the American Oil Chemistry Society* **88**, 1329–1338.

Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* **37**, 911–917.

Cavonius, L.R., Carlsson, N.-G. and Undeland, I. (2014) Quantification of total fatty acids in microalgae: comparison of extraction and transesterification methods. *Analytical and Bioanalytical Chemistry* **406**, 7313–7322.

Chase, M.W. (2001) The origin and biogeography of Orchidaceae. pp. 1–5 *in* Pridgeon, A.M.; Cribb, P.J.; Chase, M.W.; Rasmussen, F. (Eds) *Genera Orchidacearum, Orchidoideae (Part I)*. Vol. 2. Oxford, Oxford University Press.

Chase, M.W., Cameron, K.M., Freudenstein, J.V., Pridgeon, A.M., Salazar, G., van den Berg, C. and Schuiteman, A. (2015) An updated classification of Orchidaceae. *Botanical Journal of the Linnean Society* **177**, 151–174.

Christie, W.W. (1989) Analysis of fatty acids. *Gas chromatography and lipids*. Dundee, P.J. Barnes & Associates (The Oily Press Ltd).

Colville, L., Bradley, E.L., Lloyd, A.S., Pritchard, H.W., Castle, L. and Kranner, I. (2012) Volatile fingerprints of seeds of four species indicate the involvement of alcoholic fermentation, lipid peroxidation, and Maillard reactions in seed deterioration during ageing and desiccation stress. *Journal of Experimental Botany* **63**, 6519–6530.

Crane, J., Miller, A., van Roekel, J.W. and Walters, C. (2003) Triacylglycerols determine the unusual storage physiology of *Cuphea* seed. *Planta* **217**, 699–708.

Crane, J., David Kovach, K., Gardner, C. and Walters, C. (2006) Triacylglycerol phase and 'intermediate' seed storage physiology: a study of *Cuphea carthagenensis*. *Planta* **223**, 1081–1089.

Ellis, R.H. and Pieta Filho, C. (1992) The development of seed quality in spring and winter cultivars of barley and wheat. *Seed Science Research* **2**, 9–15.

Eriksson, O. and Kainulainen, K. (2011) The evolutionary ecology of dust seeds. *Perspectives in Plant Ecology, Evolution and Systematics* **13**, 73–87.

GenStat Committee (2011) *The guide to GenStat release 14 – Parts 1–3*. Oxford, UK, VSN International.

Goel, A. and Sheoran, I.S. (2003) Lipid peroxidation and peroxide-scavenging enzymes in cotton seeds under natural ageing. *Biologia Plantarum* **46**, 429–434.

Gören, A.C., Kiliç, T., Dirmenci, T. and Bilsel, G. (2006) Chemotaxonomic evaluation of Turkish species of *Salvia*: fatty acid composition of seed oils. *Biochemical Systematics and Ecology* **34**, 160–164.

Griffiths, M.J., van Hille, R.P. and Harrison, S.T. (2010) Selection of direct transesterification as the preferred method for the assay of fatty acid content of microalgae. *Lipids* **45**, 1053–1060.

Hay, F.R., Merritt, D.J., Soanes, J.A. and Dixon, K.W. (2010) Comparative longevity of Australian orchid (Orchidaceae) seeds under experimental and low temperature storage conditions. *Botanical Journal of the Linnean Society* **164**, 26–41.

Hoekstra, F.A. (2005) Differential longevities in desiccated anhydrobiotic plant systems. *Integrative and Comparative Biology* **45**, 725–733.

Hosomi, S.T., Santos, R.B., Custodio, C.C., Seaton, P.T., Marks, T.R. and Machado-Neto, N.B. (2011) Preconditioning *Cattleya* seeds to improve the efficacy of the tetrazolium test for viability. *Seed Science and Technology* **39**, 178–189.

Hosomi, S.T., Custódio, C.C., Seaton, P.T., Marks, T.R. and Machado-Neto, N.B. (2012) Improved assessment of viability and germination of *Cattleya* (Orchidaceae) seeds following storage. *In Vitro Cellular & Developmental Biology – Plant* **48**, 127–136.

Koopowitz, H. (2001) *Orchids and their conservation*. Oregon, Timber Press.

Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y. and Oh, H.-M. (2010) Comparison of several methods for effective lipid

https://doi.org/10.1017/S0960258515000367 Published online by Cambridge University Press

extraction of microalgae. *Bioresource Technology* **101**, 575–577.

Lee, Y.I., Yeung, E.C., Lee, N. and Chung, M.C. (2008) Embryology of *Phalaenopsis amabilis* var. *formosa*: embryo development. *Botanical Studies* **49**, 139–146.

Lepage, G. and Roy, C.C. (1984) Improved recovery of fatty acid through direct transesterification without prior extraction or purification. *Journal of Lipid Research* **25**, 1391–1396.

Lepage, G. and Roy, C.C. (1986) Direct transesterification of all lipid classes in a one-step reaction. *Journal of Lipid Research* **27**, 114–120.

Lewis, T., Nichols, P.D. and McMeekin, T.A. (2000) Evaluation of extraction methods for recovery of fatty acids from lipid producing microheterotrophs. *Journal of Microbiological Methods* **43**, 107–116.

Li, D.-Z. and Pritchard, H.W. (2009) The science and economics of *ex situ* plant conservation. *Trends in Plant Science* **14**, 614–621.

Li, Y., Beisson, F., Pollard, M. and Ohlrogge, J. (2006) Oil content of *Arabidopsis* seeds: the influence of seed anatomy, light and plant-to-plant variation. *Phytochemistry* **67**, 904–915.

Machado-Neto, N.B. and Custódio, C.C. (2005) Orchid conservation through seed banking: ins and outs. *Selbyana* **26**, 229–235.

Marks, T.R., Seaton, P.T. and Pritchard, H.W. (2014) Desiccation tolerance, longevity and seed-siring ability of entomophilous pollen from UK native orchid species. *Annals of Botany* **114**, 561–569.

Matthaus, B. and Özcan, M.M. (2011) Lipid evaluation of cultivated and wild carob (*Ceratonia silique* L.) seed oil growing in Turkey. *Scientiae Horticulturae* **130**, 181–184.

Merritt, D.J., Touchell, D.H., Senaratna, T., Dixon, K.W. and Walters, C.W. (2005) Survival of four accessions of *Anigozanthos manglesii* (Haemodoraceae) seeds following exposure to liquid nitrogen. *Cryoletters* **26**, 121–130.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473–497.

Nagel, M. and Börner, A. (2010) The longevity of crop seeds stored under ambient conditions. *Seed Science Research* **20**, 1–12.

Nedhi, I.A., Zarrouk, H. and Al-Resayes, S.I. (2011) Changes in composition of *Phoenix canariensis* Hort. Ex. Chabaud palm seed oil during ripening process. *Scientiae Horticulturae* **129**, 724–729.

Pamplona, R., Portero-Otín, M., Riba, D., Ruiz, C., Prat, J., Bellmunt, M.J. and Barja, G. (1998) Mitochondrial membrane peroxidizability index is inversely related to maximum life span in mammals. *Journal of Lipid Research* **39**, 1989–1994.

Peterson, R.L., Uetake, Y. and Zelmer, C. (1998) Fungal symbioses with orchid protocorms. *Symbiosis* **25**, 29–55.

Ponquett, R.T., Smith, M.T. and Ross, G. (1992) Lipid autoxidation and seed ageing: putative relationships between longevity and lipid stability. *Seed Science Research* **2**, 51–54.

Pritchard, H.W. and Seaton, P.T. (1993) Orchid seed storage: Historical perspective, current status, and future prospects for long-term conservation. *Selbyana* **14**, 89–104.

Pritchard, H.W., Poynter, A.L.C. and Seaton, P.T. (1999) Interspecific variation in orchid seed longevity in relation to ultra-dry storage and cryopreservation. *Lindleyana* **14**, 92–101.

Probert, R.J., Matthew, I.D. and Hay, F.R. (2009) Ecological correlates of ex situ seed longevity: a comparative study on 195 species. *Annals of Botany* **104**, 57–69.

Ramirez, S.R., Gravendeel, B., Singer, R.B., Marshall, C.R. and Pierce, N.E. (2007) Dating the origin of the Orchidaceae from a fossil orchid with its pollinator. *Nature* **448**, 1042–1045.

Rasmussen, H.N. (2002) Recent developments in the study of orchid mycorrhiza. *Plant and Soil* **244**, 149–163.

Ratajczak, E. and Pukacka, S. (2005) Decrease in beech (*Fagus sylvatica*) seed viability caused by temperature and humidity conditions as related to membrane damage and lipid composition. *Acta Physiologiae Plantarum* **27**, 3–12.

Sanhewe, A.J. and Ellis, R.H. (1996a) Seed development and maturation in *Phaseolus vulgaris* I. Ability to germinate and to tolerate desiccation. *Journal of Experimental Botany* **47**, 949–958.

Sanhewe, A.J. and Ellis, R.H. (1996b) Seed development and maturation in *Phaseolus vulgaris* II. Post-harvest longevity in air-dry storage. *Journal of Experimental Botany* **47**, 959–965.

Schwallier, R., Bhoopalan, V. and Blackman, S. (2011) The influence of seed maturation on desiccation tolerance in *Phalaenopsis amabilis* hybrids. *Scientia Horticulturae* **128**, 136–140.

Seaton, P.T. and Pritchard, H.W. (2008) Life in the freezer. *Orchids* **77**, 762–773.

Seaton, P.T., Hu, H., Perner, H. and Pritchard, H.W. (2010) *Ex situ* conservation of orchids in a warming World. *Botanical Review* **76**, 193–203.

Seaton, P., Kendon, J.P., Pritchard, H.W., Puspitaningtyas, D.M. and Marks, T.R. (2013) Orchid conservation: the next ten years. *Lankesteriana* **13**, 93–101.

Shoushtari, B.D., Heydari, R., Johnson, G.L. and Arditti, J. (1994) Germination and viability staining of orchid seeds following prolonged storage. *Lindleyana* **9**, 77–84.

Sung, J.M. (1996) Lipid peroxidation and peroxide-scavenging in soybean seeds during aging. *Physiologia Plantarum* **97**, 85–89.

Walters, C., Wheeler, L.M. and Grotenhuis, J.M. (2005) Longevity of seeds stored in a genebank: species characteristics. *Seed Science Research* **15**, 1–20.

Yam, T.W., Arditti, J. and Cameron, K.M. (2009) 'The Orchids Have Been a Splendid Sport' – an alternative look at Charles Darwin's contribution to orchid biology. *American Journal of Botany* **96**, 2128–2154.

Younis, Y.M.F., Ghirmay, S. and Al-Shirby, S.S. (2000) African *Cucurbita pepo* L.: properties of seed and variability in the fatty acid composition. *Phytochemistry* **54**, 71–75.

https://doi.org/10.1017/S0960258515000367 Published online by Cambridge University Press