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'Total fluorine' analysis of seed of Australian *Gastrolobium* spp. showing temporal, spatial and morphological variation

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Abstract

Alkali-fusion in conjunction with a fluoride selective electrode were used to quantify 'total fluorine', being organic fluorine + inorganic fluoride, for the seed of 13 species of *Gastrolobium* from south–west Western Australia. Intact seed covering spatial and temporal distributions, as well as *G. bilobum*, *G. calycinum* and *G. parviflorum* seed dissected into cotyledons and testa + aril, were analysed. Analysis found significant intra-and inter-species variation, both temporally and spatially, with intact seed concentrations ranging from 1.6 ± 0.3 mg kg^{-1} in *G. spinosum* from Mundaring to 1063.9 ± 77.8 mg kg^{-1} for *G. cuneatum* from Torbay. Approximately 87% of the 'total fluorine' was found to be in the seed cotyledons. Additional analysis detected little inorganic fluoride, indicating the majority of fluorine in the seed is organically bound. Parent compound(s) of the fluorine, seed toxicity and the implications of the results for seed chemical defense are discussed.

Keywords: *Gastrolobium*; 'Total fluorine' analysis; Seed; Fluoroacetate

1. Introduction

The presence of organo-fluorine compounds in nature is rare, with only a few natural products described [1]. The most common is fluoroacetate, first described by Marais in 1944 in the African plant *Dichapetalum cymosum* Engl. [2] and then subsequently in the Australian plants *Acacia georginae* F.M.Bailey [3], *Gastrolobium grandiflorum* F.Muell. [4] and additional *Gastrolobium* [5–7], *Dichapetalum* [8] and South American species [9]. Additional fluorinated natural products are the ω-fluorinated fatty acids from the seeds of *Dichapetalum toxicarium* [10,11] and fluoroacetone in the leaves of *Acacia georginae* [12,13]. Hall [12] also suggests the likely presence of additional organo-fluorine compounds in *Gastrolobium bilobum* seed (specifically, a fluorinated carbohydrate or amino acid) and in *Acacia georginae* leaf exudate. The most recent fluorinated natural product discovery was 4-fluorothreonine in 1986 [14], although trifluoroacetate can now be considered a natural product [15].

With the strong possibility that *Gastrolobium* seeds could contain fluorinated compounds in addition to the reported fluoroacetate, a comprehensive survey of fluorine levels in seed from an extensive range of *Gastrolobium* spp., and the morphological distribution of seed fluorine, has been carried out. The procedure followed the approach of Hall [12], utilising alkali-fusion in conjunction with a fluoride selective electrode to quantify seed 'total fluorine'. This will facilitate selection of seeds with relatively high fluorine levels for further analysis.

2. Results and discussion

2.1. 'Total fluorine' content of intact Gastrolobium seed

The 'total fluorine' content of intact *Gastrolobium* seed is presented in Table 1. Data indicate that the seeds of *Gastrolobium* species such as *G. spinosum* can contain almost no fluoride or organo-fluorine compounds (although Hall [12] recorded 65 mg kg^{-1} organic fluorine), while species such as *G. cuneatum* recorded a substantial mean 'total fluorine' of 1063.9 ± 77.8 mg kg^{-1} ($\bar{X} \pm$ S.E.; $n = 3$). Significant intra-

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Table 1
Source and 'total fluorine' content of intact *Gastrolobium* seed

Species	Seed source (nearest town, date)	'Total fluorine', $\bar{X} \pm$ S.E. (mg kg^{-1})
G. bilobum	Manjimup, 2000	420.6 ± 44.3 (n = 3)
G. bilobum	Quindanning, January 2000	10.4 ± 3.7 (n = 6)
G. bilobum	Harvey, December 2001	22.8 ± 0.7 (n = 3)
G. bilobum	Tambellup, January 2002	145.6 ± 19.7 (n = 3)
G. bilobum	Albany, January 2002	504.3 ± 25.9 (n = 3)
G. bilobum	Araluen, February 2003	421.0 ± 12.3 (n = 4)
G. calycinum	Boddington, December 1999	816.0 ± 32.5 (n = 6)
G. calycinum	Mundaring, 2001	250.0 ± 27.1 (n = 3)
G. calycinum	Chittering, December 2001	256.3 ± 38.0 (n = 3)
G. calycinum	Boddington, December 2002	619.9 ± 14.4 (n = 3)
G. crassifolium	Tambellup, December 2000	668.2 ± 35.9 (n = 3)
G. cuneatum	Torbay, January 1996	391.8 ± 12.1 (n = 3)
G. cuneatum	Torbay, January 1997	310.0 ± 6.8 (n = 3)
G. cuneatum	Torbay, January 1998	442.2 ± 14.8 (n = 3)
G. cuneatum	Torbay, January 2001	511.6 ± 17.3 (n = 3)
G. cuneatum	Torbay, January 2003	1063.9 ± 77.8 (n = 3)
G. laytonii	Paynes Find, January 1993	46.3 ± 1.4 (n = 3)
G. parviflorum	Cuballing, December 1995	548.7 ± 32.2 (n = 7)
G. parviflorum	Jacup, February 2000	975.5 ± 77.7 (n = 3)
G. parviflorum	Borden, December 2000	118.3 ± 28.4 (n = 3)
G. parviflorum	Tambellup, January 2002	433.3 ± 14.3 (n = 3)
G. parvifolium	Kukerin, December 2000	90.8 ± 6.1 (n = 3)
G. racemosum	'Unknown', 1989	713.5 ± 38.7 (n = 3)
G. racemosum	Jacup, February 2000	904.4 ± 55.8 (n = 3)
G. spathulatum	Dwellingup, 2000	2.8 ± 0.1 (n = 3)
G. spinosum	Mundaring, December 1996	1.6 ± 0.3 (n = 3)
G. spinosum	Hyden, January 1998	3.2 ± 0.9 (n = 3)
G. spinosum	Woogenilup, January 2000	2.3 ± 1.0 (n = 3)
G. stenophyllum	'Unknown', 1989	673.5 ± 18.6 (n = 3)
G. tetragonophyllum	'Unknown', 1989	848.7 ± 46.9 (n = 3)
G. villosum	Jarrahdale, January 1996	430.0 ± 33.2 (n = 3)

species spatial variation was found with seed sourced from different provenances (see Fig. 1). Significant temporal variation in *G. cuneatum* seed sourced from the same site (see Fig. 2) was also found. A lack of data from three intervening years prevents establishing a more conclusive trend, however the data indicates 'total fluorine' for the *G. cuneaum* seed increasing with age. This may simply reflect an increasing capacity of the maturing plants to hyper-accumulate soil fluoride and synthesise organo-fluorine compounds (fluoroacetate).

The results from *G. parviflorum* (Jacup) and *G. racemosum* (Jacup) collected from the same patch of vegetation in February 2000 indicate inter-species variation can be minimal at a given place and time. This suggests that factors such as rainfall, soil type and perhaps resident mycorrhizal fungal associations could have a more significant role in seed 'total fluorine' concentrations than a species' genetic predisposition. The *G. parviflorum* and *G. bilobum* collected from Tambellup in January 2002 were from different patches of vegetation.

Analysis of distilled water blanks by this procedure gave only 2.9 ± 1.0 mg kg^{-1} ($\bar{X} \pm$ S.E.; n = 3), representing background fluorine levels.

2.2. Analysis for inorganic fluoride in Gastrolobium seed

The concentration of inorganic fluoride in *G. bilobum* (Quindanning), *G. calycinum* (Boddington) and *G. parviflorum* (Cuballing) seed is presented in Table 2. Little inorganic fluoride was detected in the seed (max. 9.0 ± 4.0 mg kg^{-1}; $\bar{X} \pm$ S.E.; n = 3), indicating therefore that substantial amounts of organically bound fluorine occur in the seed. Results for the *Camellia japonica* leaves (known to contain fluoride and not known to contain fluoroacetate) indicate that hot NaOH successfully extracts inorganic fluoride. In addition, it was

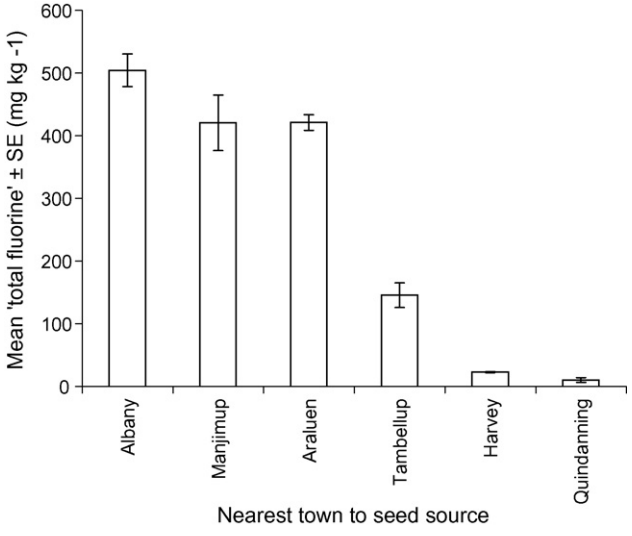

Fig. 1. Spatial variation in *G. bilobum* seed 'total fluorine'.

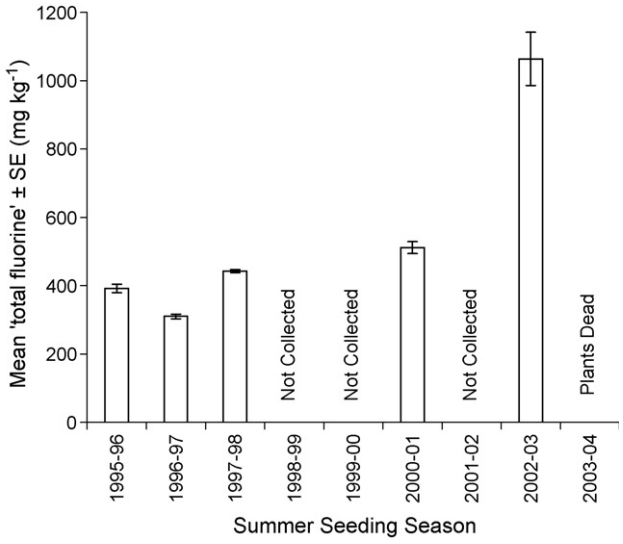

Fig. 2. Temporal variations in *G. cunatum* seed 'total fluorine' from 'Southern Comfort', Torbay, Western Australia.

found that sodium fluoroacetate is not hydrolysed under these conditions and the fluoride results from the seeds do not arise from fluoroacetate hydrolysis.

2.3. Morphological distribution of 'total fluorine' in Gastrolobium seed

Gastrolobium seed was divided into the cotyledons, defined as the seed tissue inside the testa, and the testa + aril, being the seed coat and when present, the attached aril. The large majority of 'total fluorine' is found in the cotyledons (Table 3).

The seeds of *Gastrolobium* were analysed for their 'total fluorine' utilising a process of fusion with sodium hydroxide, with subsequent analysis using a fluoride ion specific electrode. Extreme variation exists between and within species, with species such as *G. spinosum* containing trace levels and *G. cuneatum* (collected from Torbay; January 2003), containing an extremely high concentration of 'total fluorine'. These levels were also found to increase rapidly over time, with the record concentration in *G. cuneatum* collected from Torbay in 2003 double that recorded in 2001. Almost all of this 'total fluorine' is present as organic fluorine rather than inorganic fluoride. For example, *G. calycinum* from Boddington, Western Australia had a 'total fluorine' concentration of 816.0 ± 79.5 mg kg^{-1} ($n = 3$), with only 8.3 ± 7.2 mg kg^{-1} ($n = 3$) free fluoride. Additionally, this study determined that most of this fluorine is stored within the seed's cotyledons rather than the testa and aril. For the *G. calycinum* seed discussed above, this apportioning was 89% in the cotyledons and 11% in the testa + aril. This suggests that species of *Gastrolobium* such as *G. calycinum* have developed the strategy of protecting the developing seedlings with fluorinated toxicants and is supportive of the reports which describe the seedlings as one of the most toxic growth stages [16]. As fluoroacetate is the only currently identified organo-fluorine compound detected in *Gastrolobium* seed [5–7,17], total fluorine results presented here may largely represent concentrations of fluoroacetate. Analysis [18] of milled *G bilobum* (Quindanning) seed, with 10.4 ± 3.7 mg kg^{-1} ($\bar{X}$ ± S.E.; $n = 6$) 'total fluorine', recorded 6.7 ± 0.14 mg kg^{-1} ($\bar{X}$ ± S.E.; $n = 2$) fluoroacetate. Additionally, *G. bilobum* (Tambellup) and the highly fluorinated *G. parviflorum* (Jacup), were analysed using ^{19}F NMR and a triplet coincident with a sodium monofluoroacetate spike was

Table 2
Analysis for inorganic fluoride in *Gastrolobium* seed

Sample	Extraction with hot sodium hydroxide		Extraction with hot water	
	Sample weight, $\bar{X}$ ± S.E. (mg)	Fluoride, $\bar{X}$ ± S.E. (mg kg^{-1})	Sample weight, $\bar{X}$ ± S.E. (mg)	Fluoride, $\bar{X}$ ± S.E. (mg kg^{-1})
G. bilobum (*Quindanning*) seed	500.3 ± 0.5 ($n = 3$)	3.3 ± 0.3 ($n = 3$)	502.4 ± 2.8 ($n = 3$)	1.8 ± 0.5 ($n = 3$)
G. calycinum (*Boddington*) seed	506.3 ± 1.7 ($n = 3$)	8.3 ± 4.2 ($n = 3$)	507.0 ± 0.6 ($n = 3$)	6.0 ± 0.7 ($n = 3$)
G. parviflorum (*Cuballing*) seed	504.5 ± 1.6 ($n = 3$)	9.0 ± 4.0 ($n = 3$)	504.7 ± 1.3 ($n = 3$)	7.7 ± 1.6 ($n = 3$)
Camellia japonica leaves	502.7 ± 1.1 ($n = 5$)	485.5 ± 55.5 ($n = 5$)	501.3 ± 1.1 ($n = 5$)	151.4 ± 1.9 ($n = 5$)
Distilled water	501.1 ± 1.2 ($n = 3$)	0.0 ± 0.0 ($n = 3$)	201.6 ± 0.9 ($n = 3$)	0.0 ± 0.0 ($n = 3$)
Sodium fluoroacetate	0.49 mg fluoride in 0.2 ml ($n = 5$)	7.1 ± 0.2[a] ($n = 5$)	0.49mg fluoride in 0.2 ml ($n = 5$)	5.4 ± 0.1[a] ($n = 5$)

[a] Approximately 2550 ppm fluoride would arise from a complete hydrolysis of this weight of pure sodium monofluoroacetate. These results are believed due to contamination by sodium fluoride, see method section.

Table 3
Partitioning of 'total fluorine' in dissected *Gastrolobium* seed

Gastrolobium species	% of 0.5 g seed[a]		'Total fluorine'[a]		'Total fluorine' distribution[a]	
	Testa + aril, $\bar{X}$ ± S.E.	Cotyledons, $\bar{X}$ ± SE	Testa + aril, $\bar{X}$ ± S.E. (mg kg^{-1})	Cotyledons $\bar{X}$ ± S.E. (mg kg^{-1})	Testa + aril (%)	Cotyledons (%)
G. bilobum (Manjimup)	44.6 ± 0.4	55.4 ± 0.4	105.6 ± 11.4	543.1 ± 92.1	16	84
G. calycinum (Boddington)	46.3 ± 0.06	53.7 ± 0.06	188.5 ± 13.5	1558.4 ± 58.9	11	89
G. parviflorum (Cuballing)	44.6 ± 1.04	55.4 ± 1.04	129.7 ± 8.7	948.8 ± 20.9	12	88

[a] n, number of samples, is 3.

observed in each case, however quantitation of MFA levels was not undertaken in this study. Elemental analysis by ICP-AES of seeds (11 provenance samples from 5 species) found potassium to be, by an order of magnitude, the most dominant cation present at 12–14,000 mg kg^{-1} (unpublished data). Therefore potassium fluoroacetate concentrations in the seed could range from 9.8 ± 1.8 mg kg^{-1} ($n = 3$) in *G. spinosum* (Mundaring) to 6489.8 ± 474.4 mg kg^{-1} ($n = 3$) in *G. cuneatum* (Torbay). At least 6500 mg kg^{-1} fluoroacetate were found in *G bilobum* (Araluen) seed using the thioindigo method [19] (B. Mead, Murdoch University, pers comm. 2001). Future research will be undertaken to establish the presence of any organo-fluorine compounds additional to fluoroacetate, such as the fluorinated carbohydrate or amino acid hypothesised by Hall [12].

3. Conclusions

Analysis of total fluorine levels in the seeds from an extensive range of *Gastrolobium* species showed significant variation in 'total fluorine' ranging from 1.6 mg kg^{-1} to 1063.9 mg kg^{-1}. Seeds of the same species from different provenances showed spatial variation in 'total fluorine' level. Temporal variation in 'total fluorine' level was found in seeds collected from the same patch of vegetation over successive years and indicates these levels to have increased with age.

Seed morphological analysis found the cotyledons to contain approximately 87% of seed 'total fluorine'. If this is largely present as the toxicant fluoroacetate, this suggests a chemical defense strategy of protecting the newly germinated seedling, rather than a strategy of loading up the seed testa to protect the seed itself.

Analysis showed that levels of inorganic fluoride were relatively low indicating that the fluorine was associated with organo-fluorine compounds. This is likely to be principally fluoroacetate, as previously reported, however future research will utilise this baseline data in the search for novel organo-fluorine compounds as proposed by Hall [12].

4. Experimental

4.1. Source of Gastrolobium seed

Gastrolobium seed was purchased from Nindethana Seed Service Pty. Ltd. (Albany, Australia) except *G. bilobum* (Araluen), collected by Robert Davis from the Western Australian Herbarium. All seed was collected in south–west Western Australia. The samples of *Gastrolobium* seed, their source localities with collection dates and results of 'total fluorine' analysis are detailed in Table 1. (Provenance numbers given by Nindethana to each seed sample, or seed locality coordinates, are available from D.E.P.).

Seed identity was supplied by Nindethana Seed Service Pty. Ltd. In addition, the seed samples *G. bilobum* (Araluen, Quindanning and Tambellup), *G. calycinum* (Mundaring), *G. parviflorum* (Jacup and Tambellup) and *G. racemosum* (Jacup) had the identification of their source plants verified by, and lodged with, the Western Australian herbarium (details available from D.E.P.). Nomenclature was as per [20], using *G. cuneatum* rather than *G. forrestii*.

4.2. Analysis for 'total fluorine'

Where possible 0.5 g samples were analysed in triplicate. Where only limited seed was available, triplicate samples of at least 200 mg (e.g. *G. bilobum* ~25 seeds) were used. To examine the morphological distribution of 'total fluorine', 0.5 g samples of *G. bilobum* (Manjimup), *G. calycinum* (Boddington) and *G. parviflorum* (Cuballing) seed were separated using a scalpel and forceps into the cotyledons and the testa + aril. Separated seed was stored at 4 °C prior to being analysed for their 'total fluorine'.

'Total fluorine' content of the seed and seed components was determined using the 'alkali fusion method for total fluorine analysis' [21] in which all organic fluorine is hydrolysed to release the fluoride ion which is then measured by the electrode. The alkali fusion method used in this study was from Shuqi Ma [22] and utilised 2 M NaOH. The Emf (mV) was measured using a Radiometer 'ISE25F-9 Fluoride Selective Electrode' and an Orion '90–02 Double Junction Reference Electrode' and compared with a standard curve. The electrical potential was proportional to the log of the fluoride ion concentration.

Fluoride standard solutions were prepared by diluting a 1000 mg kg^{-1} fluoride standard from AdelLab Scientific (Norwood, Australia) with distilled water. For establishment of the standard curve, standards were prepared by adding fluoride standard solution (4 ml) to TISAB (Total Ionic Strength Adjustment Buffer (0.75 M sodium acetate, 0.25 M acetic acid, 1 M sodium chloride); 4 ml).

All samples and standards were measured while being stirred with a small magnetic stirrer bar. The standard curves of Emf (mV) against log[F^-] were prepared daily using 1, 5, 10, 20, 50 and 100 mg kg^{-1} standards and had an r^2 of ≥ 0.99. Additional 0.1 and 1000 mg kg^{-1} standards were used to assess the sensitivity of the electrode and linearity of the curve. The electrode was found to be sensitive to 0.1 mg kg^{-1} fluoride and linear to 1000 mg kg^{-1}, the highest concentration tested. Fluoride standards were replaced when the r^2 of the regression fell below 0.98.

4.3. Analysis for inorganic fluoride in Gastrolobium seed

In order to determine the proportion of seed 'total fluorine' which is organic or inorganic, the concentration of inorganic fluoride in the seed was investigated using *G. bilobum* (Quindanning), *G. calycinum* (Boddington) and *G. parviflorum* (Cuballing) seed.

A glass test tube was tared, the seed sample added and the seed weight recorded. Solvent (2 M NaOH or distilled water; 6 ml) was added and the total weight recorded (W_0). The mixture was homogenised using a Heidolph Diax 900 homogeniser, then warmed in a water bath @ 50 ± 1 °C for 2 h. The mixture was allowed to cool and settle, then it was reweighed and the appropriate solvent added to adjust the weight to W_0. Supernatant (2 ml) was pipetted into a 25 ml plastic beaker and TISAB

buffer (2 ml) added. The pH was checked and AR grade glacial acetic acid added to mixtures with the NaOH solvent, to adjust pH to 5–7. The Emf (mV) was measured and compared to the standard curve.

As the *Camellia* plant is recognised for its propensity to accumulate free fluoride in its leaves [22], these leaves were used as a control. *Camellia japonica* leaves were collected from a suburban garden in Thorngate, South Australia (Lat. −34° 53.8′; Long. 138° 35.82′-GDA). Leaves of similar appearance were selected, the central vein excised and the leaves cut into minute pieces with scissors. Leaf pieces were then mixed together for replicated sampling. Leaf samples were analysed as per the seed samples, except the mixtures were stirred using a Chiltern MT19 Auto Vortex Mixer rather than homogenised using a Heidolph Diax 900. Additional controls were sodium monofluoroacetate (SMFA), to determine its stability under analysis conditions, and distilled water. A stock solution of SMFA (BDH Chemicals Ltd, Poole England: 86% purity) in 2 M NaOH was prepared by adding 150 mg SMFA to a 10 ml volumetric flask and making it up to the mark with 2 M NaOH or distilled water. Two hundred microliter SMFA stock solution and 6 ml solvent (2 M NaOH or distilled water) were added to a pre-weighed test-tube and the solutions mixed using a Chiltern MT19 Auto Vortex Mixer. Samples were then treated and analysed as per the *Camellia* leaf control.

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