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Variation in Diosgenin Levels among 10 Accessions of Fenugreek Seeds Produced in Western Canada

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A collection of 10 accessions of fenugreek (*Trigonella foenum-graecum* L.), an annual legume, was grown during two summers at three plot locations in western Canada to assess whether genetic (accession) and environmental factors (site and year of production) influenced levels of diosgenin, a steroidal sapogenin. The 60 harvested seed samples, each analyzed by single determinations on three subsamples of defatted and dried seed material, were hydrolyzed by a microscale procedure in water containing 2-propanol (70%) and sulfuric acid (1 M). The extracts were analyzed by capillary gas chromatography with 6-methyldiosgenin as internal standard. Diosgenin levels from mature seeds ranged from 0.28 to 0.92% (28−92 μg/10 mg). Analysis of variance on combined diosgenin levels from the three sites and two years revealed that accession, accession × year, and site × year effects were significant for diosgenin content, whereas site, year, and site × accession effects were not. Four accessions, CN 19062, CN 19067, CN 19070, and CN 19071, were identified with high levels of diosgenin on the basis of the 2-year data set. In these accessions, mean levels of diosgenin plus yamogenin from seven site years were estimated at 0.70, 0.98, 0.84, and 0.87%, respectively.

KEYWORDS: Diosgenin; fenugreek; seeds; genotypes; environments; interactions

INTRODUCTION

The occurrence of glycosides of diosgenin [(25*R*)-spirost-5-en-3β-ol] in seeds of fenugreek (*Trigonella foenum-graecum* L.) has been well recognized for over 50 years (*1*). Several authors have estimated levels of this steroidal sapogenin, both with commercial varieties of fenugreek (*2*) and during comparative studies on seeds from related plant species (*3*−*7*). Two publications report on the geographic variability of diosgenin content from fenugreek grown in India (*8*, *9*). The latter study with 39 varieties of fenugreek and a spectrophotometric assay showed diosgenin levels of 0.07−0.75% on a dry weight basis. A detailed agronomic study in Russia on 31 genotypes from genebanks of various countries indicated diosgenin (plus yamogenin, the 25*S* stereoisomer) levels of 1.14−1.64%, determined by thin-layer chromatography (*10*). Although these levels are perhaps higher than usual, some genotypes were reported as significantly elevated in diosgenin plus yamogenin content compared to the standard variety (1.17%).

Identification of genotypes with high levels of diosgenin might help to make the seeds of fenugreek economically competitive with the tubers of *Dioscorea*, the traditional source of diosgenin used for the synthesis of steroid drugs (reviewed in ref *11*). High-yielding genotypes might also be useful as initial material for plant breeders to increase steroid production. Additionally, steroidal saponin fractions of fenugreek have potential value because of their hypocholesterolemic activity (*12*) and other interesting properties (*13*−*15*).

In the present work, we selected 10 genotypes (accessions) of fenugreek from the Canadian seed genebank and have produced seed from these accessions during two consecutive years at three research sites in western Canada. One year of data was additionally obtained from a site at Morden, MB. The seed was analyzed for diosgenin by a microprocedure (*16*), and statistical analyses were performed (excluding the Morden data) on the diosgenin levels to examine the potential sources of variation. Conceptually similar studies have recently been published on the effects of genetics and environment on levels of other phytochemicals, including soyasaponins of Australian sweet lupin (*Lupinus angustifolius*) (*17*), antinutritional factors of Nigerian cowpea [*Vigna unguiculata* (L) Walp] (*18*), kavalactones of Hawaiian kava (*Piper methysticum* Forster) (*19*), and capsaicinoids of *Capsicum annuum* L. (*20*).

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Table 1. Diversity of Fenugreek (*T. foenum-graecum* L.) Examined, Its Origin, and Improvement Status

Canadian (U.S.) accession no.	country of origin	improvement status
CN 19062 (PI 143504)	Iran	wild collected
CN 19063 (PI 195691)	Ethiopia	cultivated, collected
CN 19064 (PI 199264)	Greece	wild collected
CN 19065 (PI 211636)	Afghanistan	wild collected
CN 19066 (PI 269994)	Pakistan	cultivated, collected
CN 19067 (PI 577711)	Morocco	wild collected
CN 19068 (PI 577713)	Spain	wild collected
CN 19069	Canada	Quatro, cultivar released
CN 19070	Canada	ZT-5, experimental line
CN 19071	Canada	X92-23-2, experimental line

MATERIALS AND METHODS

Chemicals. Diosgenin (~98%) was purchased from Sigma Chemical Co. (St. Louis, MO). 6-Methyldiosgenin was obtained from Steraloids Inc. (Wilton, NH). All organic solvents were of OmniSolv glass-distilled grade (Merck, Darmstadt, Germany) and used as received. Sulfuric acid (99.999%) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and freshly diluted with 70% 2-propanol/30% water before use. Water was purified in the laboratory with a Millipore Super-Q system (Bedford, MA).

Seed Selection and Propagation. Ten accessions of fenugreek seed were obtained from Plant Gene Resources of Canada (Saskatoon, SK) with sources of origin and improvement status summarized in **Table 1**. Seeds from each accession were planted in April or May of 1998 and 1999 in small, replicated experimental field plots located at the Saskatoon Research Centre (Saskatoon, SK), Lethbridge Research Centre (Lethbridge, AB), and Pacific Agri-Food Research Centre (Agassiz, BC). A site at the Morden Research Centre (Morden, MB) was utilized in 1998, but crop failure occurred in 1999. The accessions were planted in a completely randomized design. The field plots were manually weeded (no herbicides were used), and all plants were manually harvested at seed maturity (mid-September to early October). Seeds were cleaned and stored at room temperature for ~6 months (18 months for 1998 seeds from Agassiz) before transfer to the laboratory for grinding and analysis. Seed yields were not obtained.

Seed Processing. Seeds (12 g) from each accession were ground for 2 min with a Wiley mill equipped with a 20 mesh (0.8 mm) screen. A 5 g portion of the ground sample was transferred to a Petri dish and dried, initially for 4.5 h at 60 °C (convection oven) and then overnight at room temperature (desiccator). The resulting flours (~4.8 g) were defatted in a Soxhlet apparatus for 6 h with petroleum ether (bp 38−57 °C) as solvent (250 mL) and double-thickness cellulose extraction thimbles (Whatman). The material in the thimble was air-dried, ground with a mortar and pestle, and then oven-dried at 60 °C for 4 h before transfer to a desiccator. The next day, the dried, defatted samples were weighed, transferred to vials, and stored in a desiccator at room temperature until 10 mg subsamples (in triplicate) were removed for analysis.

Extraction and Chromatography. Defatted and dried subsamples of seed material were transferred to a test tube (100 × 30 mm). Following a microscale method (*16*), the saponins were extracted with hot 80% ethanol (5 mL) and hydrolyzed for 2 h with 70% 2-propanol containing 1 M sulfuric acid (2 mL). After addition of water (3 mL) and an internal standard of 6-methyldiosgenin (50 μg), extraction with methyl *tert*-butyl ether (3 × 2 mL), washing with 1 M sodium hydroxide (2 × 1 mL), and evaporating the solvent at 30 °C with a Meyer N-EVAP apparatus (Organomation Associates, Berlin, MA), toluene solutions (1 mL) containing sapogenins were obtained. A portion (2 μL) of these solutions was subjected to GC with a Hewlett-Packard 6890 instrument and HP-5 column (30 m × 0.25 mm or 0.32 mm i.d.) using the conditions previously described (*16*). Calibration curve standards containing various quantities of diosgenin (0−100 μg) and a fixed amount of 6-methyldiosgenin (50 μg) were prepared as before and analyzed along with each set of experimental seed samples. Equations describing the calibration curves were obtained by linear regression analysis of the integrated peak area ratio of diosgenin to 6-methyldiosgenin versus the amount of diosgenin added. The amount of diosgenin found in the experimental seed samples was obtained from the integrated peak area ratio of diosgenin to internal standard and by reference to the equation describing the appropriate calibration curve. Diosgenin levels were calculated as the amount (in micrograms) found in 10 mg samples of defatted seed dried at 60 °C and expressed as percent. Typically, each quantitative experiment consisted of seed material of one accession (three subsamples) from three sites and seven standard curve samples.

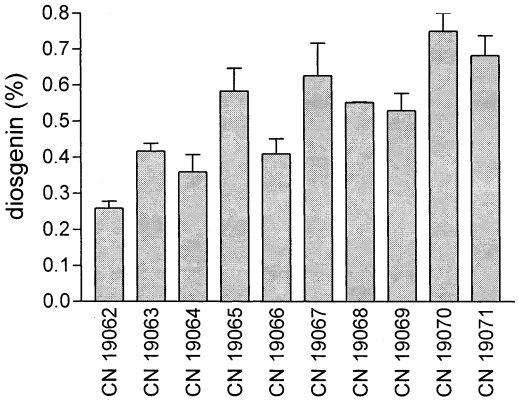

Figure 1. Diosgenin levels found in accessions of fenugreek grown in 1998 at Morden, MB. Error bars illustrate standard deviation from triplicate analyses of each seed sample.

Confirmation of peak identity was obtained by electron-impact mass spectrometry on selected extracts, using a Hewlett-Packard 5989A GC/MS as reported previously (*21*).

Statistics. Precision was obtained from SD/mean × 100. To evaluate the effects of genetic and environmental factors on diosgenin levels (see **Table 3**), the combined data set (10 × 3 × 2) representing numbers of accessions, sites, and years, respectively, was analyzed by the ANOVA and VARCOMP procedures of SAS system release 6.12 (SAS Institute Inc., Cary, NC). A least significant difference (LSD) test was used to evaluate differences among accession means.

RESULTS AND DISCUSSION

Ground fenugreek seed samples subjected to Soxhlet defatting and oven-drying lost an average of 10% (1998) or 11% (1999) of their mass.

The 1998 seeds grown at Morden were analyzed for diosgenin, and the results are plotted in **Figure 1**. Levels ranged from 0.27% (CN 19062) to 0.75% (CN 19070). Diosgenin levels from seeds of CN 19070 (experimental line ZT-5) were known (*16*) to be relatively high (0.76%). Another Canadian experimental line (CN 19071) also produced high levels at Morden (0.68%). The precision, determined from a single analysis of three subsamples of each accession, was <15%. This level of precision was usually observed during subsequent analysis of seeds from the other sites but, inexplicably, samples from Agassiz in 1999 frequently gave higher standard deviations, resulting in precision values that averaged 28% (14% in 1998).

Levels of diosgenin found in 1998 and 1999 seed samples grown at Saskatoon, Lethbridge, and Agassiz are shown in **Table 2**. Using a least significant difference test, CN 19062, CN 19067, CN 19070, and CN 19071 had the significantly highest accession means for diosgenin (0.52−0.64%). Accessions CN 19064 and CN 19066 occupied the low end of the ranking with mean diosgenin values of 0.33−0.34%.

Although site (and year) effects alone were not significant in this study, the diosgenin levels from CN 19062, CN 19070, and CN 19071 were lower at Lethbridge than at Saskatoon and Agassiz, whereas CN 19067 performed relatively well at

Table 2. Diosgenin Levels from 10 Accessions of Fenugreek Seeds Grown for Two Consecutive Years at Three Sites in Western Canada

		diosgenin (%)					
	accession	Saskatoon		Lethbridge		Agassiz	
accession	mean[a]	1998[b]	1999[b]	1998[b]	1999[b]	1998[b]	1999[b]
CN 19062	0.524 abc	0.284	0.727	0.420	0.392	0.398	0.923
CN 19063	0.408 cde	0.411	0.347	0.642	0.246	0.347	0.458
CN 19064	0.329 e	0.431	0.291	0.400	0.361	0.206	0.333
CN 19065	0.444 bcde	0.519	0.402	0.478	0.355	0.528	0.391
CN 19066	0.339 e	0.448	0.243	0.473	0.209	0.286	0.373
CN 19067	0.643 a	0.685	0.663	0.453	0.800	0.354	0.906
CN 19068	0.462 bcd	0.592	0.498	0.522	0.366	0.328	0.464
CN 19069	0.394 de	0.542	0.352	0.539	0.248	0.383	0.302
CN 19070	0.553 ab	0.801	0.506	0.467	0.369	0.633	0.543
CN 19071	0.587 a	0.730	0.555	0.535	0.341	0.747	0.613

[a] $N = 6$, derived from data in the same row without respect to the year of seed production. Means followed with the same letter are not significantly different ($P > 0.05$). The least significant difference was 0.123% ($P = 0.05$). [b] Average values ($N = 3$), determined by single analysis of three subsamples of defatted and dried seed material.

Table 3. Analysis of Variance of Means for Genetic and Environmental Factors Influencing Diosgenin Levels in Fenugreek

factor	degrees of freedom	mean square	*F* value[a]	*P* > *F* (combined analysis)	% variance component[b]
site	2	0.0272	2.65	0.1	0
year	1	0.0187	1.83	0.19	0
site × year	2	0.0800	7.80	0.004	20.3
accession	9	0.0672	6.55	0.0004	6.9
site × accession	18	0.0107	1.05	0.5	1.2
year × accession	9	0.0515	5.03	0.002	40.9
error	18	0.0103			30.7

[a] Calculated *F* values were obtained using as denominator the ANOVA mean square for site × year × accession interaction, the error term. [b] Estimated with the VARCOMP and MIVQUE procedures of SAS. Negative estimates were treated as zero.

Lethbridge (2-year average of 0.63%). The Spanish accession CN 19068 also performed well at Saskatoon, giving a 2-year average of 0.54%.

With regard to 2-year average data at each site, statistically significant differences ($P < 0.05$) were not detected among accessions grown at Lethbridge and were detected only in extreme cases at the other sites. At Saskatoon, CN 19067 was significantly higher than CN 19066, and at Agassiz, CN 19071 was significantly higher than CN 19064. This insensitivity was undoubtedly related to a large year × accession interaction, for example, CN 19062 at Saskatoon and Agassiz, CN 19063 at Lethbridge, and CN 19067 at Lethbridge and Agassiz.

Analysis of variance on the combined data set of **Table 2** revealed a significant effect of accession, site × year, and year × accession on diosgenin levels from fenugreek (**Table 3**). The site or year of production independently did not have a significant effect on this trait. The year × accession interaction accounted for the highest proportion of the variance (41%) followed by site × year (20%) and accession (7%). The error factor, accounting for 31% of the variance, could probably be reduced with additional years of data.

These results showed that the levels of diosgenin in fenugreek seeds from the three sites in western Canada are largely affected by interactions involving environmental (site × year) and environmental plus genetic (year × accession) factors. The relatively small contribution of genetics by itself will present challenges for plant breeders to further increase levels of diosgenin in fenugreek through selection.

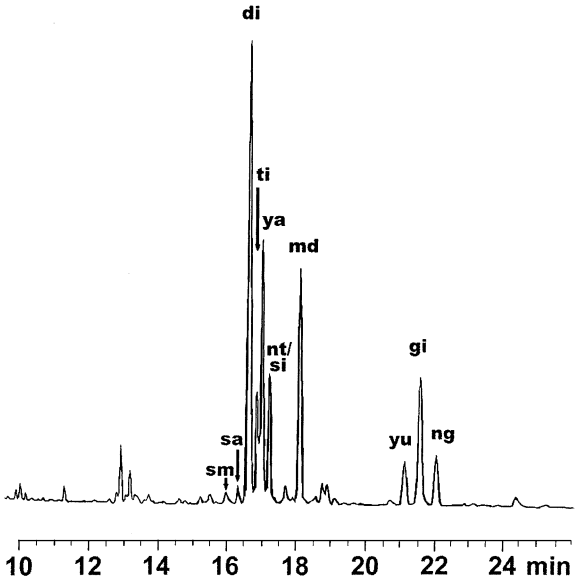

Figure 2. Chromatogram from gas chromatographic analysis with an HP-5 column (30 m × 0.32 mm i.d.) of a fenugreek seed extract obtained from accession CN 19071 by 80% ethanol extraction followed by hydrolysis with 1 M sulfuric acid in 70% 2-propanol/30% water. Labeled peaks refer to identified (or tentatively identified) components: sm, smilagenin; sa, sarsasapogenin; di, diosgenin; ti, tigogenin; ya, yamogenin; nt/si, mixture of neotigogenin and β-sitosterol; md, 6-methyldiosgenin; yu, yuccagenin; gi, gitogenin; ng, neogitogenin. The spirostadienes eluted at 12.8–13.2 min.

Among the 10 accessions studied, the two experimental lines, CN 19070 and CN 19071, would be suitable for producing fenugreek seed of high diosgenin content in western Canada. This recommendation was also true for the single year of data from Morden. Other accessions that may have commercial potential for high diosgenin were identified (particularly from the 1999 data) as CN 19062 from Iran and CN 19067 from Morocco.

Yamogenin, an epimer of diosgenin, is of equal utility to diosgenin as a starting material for steroid drug synthesis (*22*). In fenugreek seeds, an approximate ratio of diosgenin to yamogenin of 2 to 1 has been reported (*5*, *16*). Hence, the potential value of yamogenin is substantial. It was therefore of interest to estimate levels of yamogenin to obtain approximate levels of diosgenin plus yamogenin. A pure sample of yamogenin was unavailable, so it seemed reasonable to calculate the amount in the extracts using the ratios of yamogenin to internal standard and the standard curves obtained with diosgenin, under the assumption that the detector responses were identical. Although it is recognized that this may not be true, we obtained estimates for yamogenin in this manner and report the combined levels in the four highest yielding accessions (**Table 4**). The data showed the same ranking of accessions based on diosgenin levels only (see **Table 2**) and suggested that combined levels from these accessions should be close to 1% on average. On the basis of standard deviations, CN 19071 (**Figure 2**) appeared to represent the most consistent accession of high-saponin fenugreek for western Canada. The ultimate choice for producers, however, would also depend on comparative agronomic characteristics, including intervals from seeding to maturity and long-term seed yields.

Blunden et al. (*11*) discussed the advantages of rapidly growing diosgenin-containing plants such as fenugreek com-

Table 4. Percent Diosgenin plus Yamogenin Levels Estimated from Four Promising Accessions of Fenugreek Seeds Grown in Western Canada

		Saskatoon		Lethbridge		Agassiz		Morden
accession	mean ± SD	1998	1999	1998	1999	1998	1999	1998
CN 19062	0.697 ± 0.335	0.405	1.074	0.581	0.562	0.576	1.310	0.373
CN 19067	0.982 ± 0.295	1.051	1.018	0.720	1.253	0.497	1.354	0.983
CN 19070	0.839 ± 0.266	1.222	0.687	0.663	0.512	0.919	0.722	1.149
CN 19071	0.875 ± 0.208	1.095	0.800	0.823	0.483	1.069	0.864	0.990

pared to the slow-growing tubers of *Dioscorea* species, many of which are cultivated in developing countries (*23*). Although the latter typically have higher concentrations at maturity (3−7% diosgenin plus yamogenin), the tubers can take 3 years or more to mature and are difficult to harvest. In contrast, fenugreek is an annual legume amenable to traditional field crop practices. A detailed analysis of the economics of diosgenin production from our high-saponin fenugreek accessions would be appropriate.

Regarding other steroidal sapogenins in extracts from the 10 accessions, the distribution (from integrated peak areas during gas chromatography) of tigogenin, smilagenin, and sarsasapogenin was similar to those previously reported for Amber fenugreek (*21*), except for CN 19070. Levels of tigogenin were relatively high (10−15%), whereas those of smilagenin and sarsasapogenin were at trace concentrations in this accession. This observation has been noted before (*16*) in other seeds of CN 19070 (ZT-5). Normal levels, however, were found for tigogenin (5.5%) and slighly elevated levels of smilagenin (6.1%) and sarsasapogenin (6.4%) in CN 19070 grown during 1998 at Lethbridge. Gitogenin levels, accounting for ∼2−8% of the total peak areas in the majority of samples, were found to be > 10% in several accessions from Agassiz. The four peaks corresponding to spirostadiene artifacts (*16*) usually represented <10% of the total peak areas of the mixtures.

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