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Major Isoflavonoid Contents of the 1-Year-Cultivated Phytoestrogen-Rich Herb, *Pueraria mirifica*

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***Pueraria mirifica* is a tuberous plant enriched with active phytoestrogens. There is no established information about the factors influencing isoflavonoid storage in the tubers. We investigated the tuberous storage of the major isoflavonoids of 1-year-old plants. Four cultivars of *P. mirifica* were cultivated in the same field trial during the same period to establish a unique plant age and differentiation under the same environment and soil conditions. The tubers collected from the 1-year-old plants in the summer, rainy season and winter were submitted to an HPLC analysis with a gradient system comprising 0.1% acetic acid and acetonitrile. Five major isoflavonoids, puerarin, daidzin, genistin, daidzein and genistein, were adopted as standards. *P. mirifica* tubers of different cultivars collected in the same season exhibited significant differences in individual and total isoflavonoid contents, showing chemovariety. *P. mirifica* tubers of the same cultivar collected from different seasons also exhibited significant differences in individual and total isoflavonoid contents, showing the influence of season. In conclusion, the tuberous storage of major isoflavonoids in 1-year-cultivated plants was greatly diverse and was strongly influenced by the season and plant genetics.**

Key words: isoflavonoid; phytoestrogen; *Pueraria mirifica*; puerarin

Phytoestrogens are plant compounds with a structure and/or function similar to estrogen. The exposure and bioactivity of phytoestrogens in the human body have been well described.[1)] Soybeans are widely recognized as phytoestrogen-rich food sources. Such plant products have established benefits to humans in terms of cancer protection.[2)] The main phytoestrogens in soybeans are daidzein and genistein, each with potent anti-cancer activities.[3)] There has been an attempt to search for phytoestrogen-rich herbs as supplements or alternatives to soybean consumption. Kudzu, *Pueraria lobata* a tuberous plant found in Japan, Korea and China has been mentioned most. Phytoestrogens in the group of isoflavonoids including puerarin, daidzin, genistin, daidzein and genistein have been isolated from the plant tubers.[4,5)] Nevertheless, the plant crude extract exhibited weak estrogenic activity in a vaginal cornification assay in ovariectomized rats,[6)] and exhibited only antiproliferation, not proliferation, in the test with MCF-7 cells.[7)]

Pueraria mirifica Airy Shaw et Suvatabhandu of the family Leguminosae is an indigenous Thai herb with the domestic name, "White Kwao Krua." The plant's large tubers have been used in traditional remedies for rejuvenation of mature females. The plant has recently been recognized as a strong candidate, together with *P. lobata*, for a phytoestrogen source. *P. mirifica* cultivar Wichai-III crude powder showed strong estrogenic effects, improving the signs and symptoms related to menopause in a human clinical trial[8)] without any significant adverse effect in toxicology tests on animals and human volunteers.[9)] The estrogenic activity tests on the tuberous extracts *in vitro* required metabolic activation *via* specific cellular drug-metabolizing enzymes.[10)] Phytoestrogens from the same plant cultivar exhibited a dose-dependent estrogenic effect on the reproductive system of ovariectomized rats,[11)] cyclic female monkeys,[12)] and aged menopausal monkeys.[13,14)] In a study with male rats, the consumption of a high amount of the same plant cultivar caused disruption to male sex organs, including the epididymis and seminal vesicle, as well as to sperm motility and viability,[15)] although it provided protection against osteoporosis in orchidectomized male rats.[16)] The plant crude extract exhibited both proliferation and anti-proliferation in the test with

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MCF-7 cells.[7] The findings confirmed *P. mirifica* to be a very phytoestrogen-rich plant. It was found that the major isoflavonoid contents in the *P. mirifica* population in Thailand were highly diverse and might influence the estrogenic activity following plant consumption. It was hypothesized that the plant genetics and environment could influence the accumulated amount of tuberous isoflavonoids.[17] It has recently been demonstrated in ovariectomized rats that the estrogenic activity of the same samples of plant tubers was also highly diverse.[6,18] To clarify this conclusion, we established an experiment in field-trial plants to evaluate the influence of season and plant genetics on the tuberous isoflavonoid contents by cultivating four different plant cultivars in the same field trial and over the same time period to help minimize environmental and plant differentiation variance. The results may confirm the previous hypothesis that the chemovariety found in the plant population is highly influenced by environment and genetic differences. The information established in this study should benefit the farmers and manufacturers who have recently recognized *P. mirifica* as a new crop species and cultivated it to serve the emerging global demand for a phytoestrogen-rich raw material.

Materials and Methods

Plant materials. P. mirifica seeds of four cultivars were respectively collected from wild plants in Muang District of Prachuap Khiri Khan Province (N11°48′/E99°48′) in the southern part of Thailand, Phrabuddhabata District of Saraburi Province (N14°45′/E100°45′) in the central part of Thailand, and Chiang Dao District (N19°23′/E98°58′) and Doi Tao District (N17°49′/E98°42′) of Chiang Mai Province in the northern part of Thailand. The plants were identified by Cherdshewasart with the aid of reference[19] in comparison with voucher specimen No. BCU 11045[9] and were assigned as PM-I, PM-II, PM-III and PM-IV, respectively. The leaves (Fig. 1) and pods of the wild plants were submitted to a morphometric analysis, using 50 samples for each cultivar. The four plant cultivars exhibited some distinct botanical characteristics (Table 1). Seedlings were established under greenhouse conditions. The 1-month-old plants, including nine plants per cultivar, were subsequently transferred to a field trial in Banpong District of Ratchaburi Province (N13°37′/E99°52′), in the central part of Thailand, in May 2002. Tuberous roots of the 1-year-old plants were randomly harvested from three plants of each cultivar in April 2003 (summer in Thailand), October 2003 (rainy season in Thailand) and February 2004 (winter in Thailand). These seasons are classified according to the monthly record of daily mean temperature and rainfall in Ratchaburi Province provided by the Meteorological Department, Ministry of Information and Communication Technology, Thailand (Fig. 2). Differentiation among the plants was clearly apparent during the study period. In April 2003, new branches emerged from the old twinning plants, and new leaves rapidly grew from the buds. In October 2003, the twinning plants had become enlarged. The new branches became mature and were covered with plenty of mostly fully-matured leaves. In February 2004, most of the mature leaves turned yellow-brownish and defoliated.

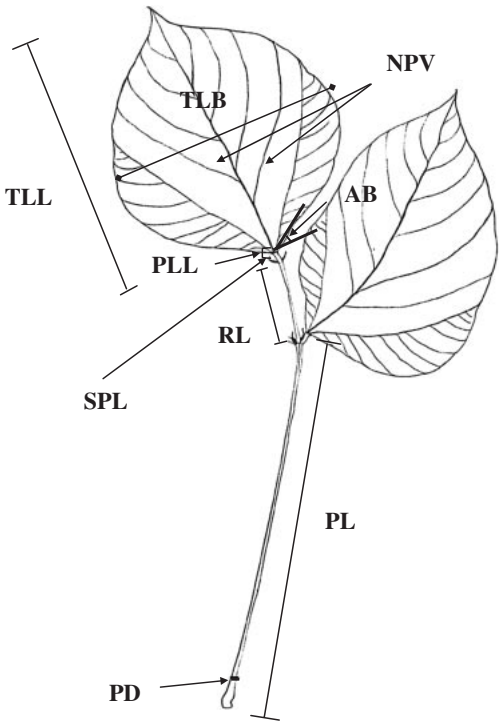

Fig. 1. Leaf Morphometry of Four Cultivars of *P. mirifica.*
PL, petiole length; PD, petiole diameter; RL, rachis length; PLL, petiolet length; TLL, terminal leaflet length; TLB, terminal leaflet breadth; SPL, stipule length; AB, angle of blade leaf; NPV, number of pairs of primary veins.

Chemicals and equipments. The isoflavonoid standards, puerarin, genistin, daidzein and genistein, were purchased from Sigma, (St. Louis, MO, USA) while daidzin was purchased from Fluka Biochemika (Buchs, Switzerland). The organic solvents for chromatography (HPLC grade) were purchased from Merck, Germany. Water with over 16 MΩ/cm for a component of the mobile phase of HPLC was prepared by Maxima Ultrapure Water Systems (ELGA). HPLC system control and data processing were carried out by Waters™ apparatus (incorporating a 717 plus autosampler, 600 controller, and 2996 photodiode array detector). The reversed phase C_{18} column (250 × 4.6 mm) was filled with 5-μm ODS2 (Waters Spherisorb®, Ireland) that had been pre-filtered with a Waters Spherisorb® S5 ODS2 (10 × 4.6 mm) guard cartridge. The filter set was a Millipore membrane of 0.45-μm pore size with a 13-mm diameter for the sample and a 47-mm diameter for the mobile phase, using the HA type for the aqueous solution and HV type for the organic solvent. The chromatography management software Empower™ was operated on a personal computer.

Table 1. Pod and Leaf Morphometry of the Four Cultivars of *Pueraria mirifica*

Plant cultivar	Pod			Leaf							
	Length (cm)	Width (cm)	Seeds/pod	PL (cm)	PD (cm)	RL (cm)	PLL (cm)	TLL (cm)	TLB (cm)	SPL (cm)	AB (degree)	NPV (cm)
PM-I	5.71 ± 0.25^{c}	0.79 ± 0.008^{b}	2.96 ± 0.19^{b}	18.24 ± 0.62^{a}	0.36 ± 0.047^{a}	4.84 ± 0.13^{a}	0.70 ± 0.014^{a}	23.05 ± 0.31^{b}	14.51 ± 0.22^{a}	0.36 ± 0.006^{a}	37.53 ± 0.93^{c}	6.33 ± 0.11^{a}
PM-II	2.97 ± 0.11^{a}	0.57 ± 0.012^{a}	2.32 ± 0.14^{a}	21.18 ± 0.61^{b}	0.338 ± 0.011^{a}	6.24 ± 0.13^{c}	0.93 ± 0.023^{b}	22.54 ± 0.43^{ab}	18.29 ± 0.41^{c}	0.37 ± 0.009^{a}	38.96 ± 0.68^{c}	6.94 ± 0.10^{b}
PM-III	6.10 ± 0.11^{c}	0.88 ± 0.014^{c}	5.90 ± 0.16^{c}	25.75 ± 1.16^{c}	0.345 ± 0.009^{a}	5.45 ± 0.19^{b}	0.99 ± 0.029^{c}	21.53 ± 0.36^{a}	16.00 ± 0.37^{b}	0.37 ± 0.01^{a}	29.83 ± 1.13^{b}	6.81 ± 0.13^{b}
PM-IV	4.31 ± 0.13^{b}	0.85 ± 0.021^{c}	2.82 ± 0.14^{b}	32.44 ± 0.80^{d}	0.44 ± 0.006^{b}	7.68 ± 0.15^{d}	0.93 ± 0.016^{b}	24.34 ± 0.39^{c}	20.59 ± 0.41^{d}	0.42 ± 0.01^{b}	23.76 ± 0.86^{a}	7.84 ± 0.12^{c}

Each value is the mean ± SEM, $n = 50$.
Values with different superscripts within same column are significantly different ($P < 0.05$) by Duncan's multiple-range test.
PL, petiole length; PD, petiole diameter; RL, rachis length; PLL, petiolet length; TLL, terminal leaflet length; TLB, terminal leaflet breadth; SPL, stipule length; AB, angle of blade leaf; NPV, number of pairs of primary veins

HPLC sample preparation and quantitative analysis. The samples were prepared as previously described.[17] The methods used for the isoflavonoid analysis were modified from those previously described[17] by setting the linear gradient system for 50 min from 100:0 to 55:45 with 0.1% acetic acid:acetonitrile at a flow rate of 1 ml/min and analyzed at a wavelength of 254 nm. The standard isoflavonoids were serially diluted with methanol to establish the required concentrations to generate a five-point calibration curve and cover the range of isoflavonoid concentrations in the samples. The analyses of the samples were run in triplicate, identification being made by comparing the retention times and quantified by using standard curves for the peak area of the isoflavonoid standards.

Statistical analysis. The mean ± SEM of the leaf and pod morphometry, tuberous fresh weight and dry weight, and isoflavonoid contents from the samples of *P. mirifica* were analyzed for statistical significance by an unpaired T-test, factorial analysis and Duncan analysis of variance at the significance level of $P < 0.05$.

Results

Tuberous fresh weight and dry weight

The harvested tubers from the four plant cultivars varied in size and mostly appeared in a non-fully developed shape. The tuberous fresh weight and dry weight of the plant samples collected in the three seasons of the study period are presented in Table 2.

Quantitative analysis of the isoflavonoids

The HPLC analysis established for isoflavonoids in plant samples in this study, with a quantification limit of 0.1 mg/100 g, could demonstrate the difference in retention times from the standard isoflavonoids (Fig. 3). Calibration curves for the standard isoflavonoids were obtained with high linearity, $R^2 \geq 0.985$. The isoflavonoids were analyzed from plant samples of different cultivars and collecting seasons. The results showed significant differences in individual and total isoflavonoid contents, with the highest and lowest isoflavonoid contents in PM-I tubers collected during the summer (1007.47 ± 72.22 mg/100 g of powder) and PM-II tubers collected during the rainy season (17.93 ± 1.81 mg/100 g of powder).

Correlation analysis of the isoflavonoid contents with seasonal and genetic variation

Puerarin and the total isoflavonoids were correlated at 99% ($P < 0.01$). Daidzin was also correlated with the total isoflavonoids at 95% ($P < 0.05$) in the three tested seasons, as determined by a factorial analysis (Table 3). No correlation was apparent between the individual and total isoflavonoid contents, and the temperature and amount of rainfall in the cultivars examined.

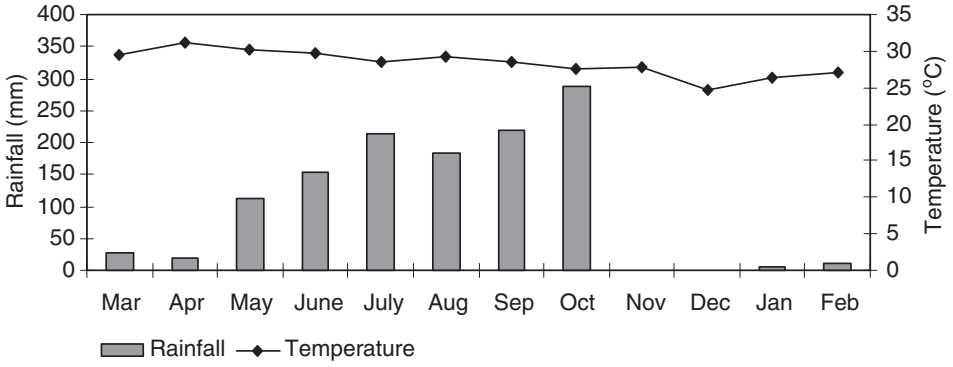

Fig. 2. Climatic Conditions in Ratchaburi Province during the Experiment.
In April 2003, the mean temperature was 31.19 °C, and the amount of rainfall was 20.1 mm. In October 2003, the mean temperature was 27.64 °C, and the amount of rainfall was 287.6 mm. In February 2004; the mean temperature was 27.06 °C, and the amount of rainfall was 10.9 mm.

Table 2. Tuberous Fresh Weight and Dry Weight of *P. mirifica* Collected during Three Seasons of the Experimental Period

Season	Plant cultivar	Fresh weight (g)	Dry weight (g)
Summer	PM-I	72.27 ± 37.26^a	6.84 ± 3.74^a
	PM-II	77.95 ± 40.53^a	7.37 ± 3.92^a
	PM-III	225.51 ± 80.87^a	21.34 ± 7.63^a
	PM-IV	92.88 ± 21.53^a	8.63 ± 1.80^a
	Mean ± SEM	117.15 ± 28.58^a	11.04 ± 2.72^a
Rainy season	PM-I	286.28 ± 146.11^a	27.70 ± 14.51^a
	PM-II	531.31 ± 228.02^a	52.43 ± 22.55^a
	PM-III	107.57 ± 25.59^a	10.10 ± 2.49^a
	PM-IV	131.72 ± 67.56^a	12.63 ± 6.59^a
	Mean ± SEM	264.22 ± 78.49^a	25.72 ± 7.79^a
Winter	PM-I	448.34 ± 233.65^a	44.23 ± 23.29^a
	PM-II	175.13 ± 50.87^a	16.91 ± 5.04^a
	PM-III	105.66 ± 2.68^a	10.07 ± 0.25^a
	PM-IV	237.10 ± 48.07^a	23.07 ± 4.83^a
	Mean ± SEM	241.56 ± 64.78^a	23.57 ± 6.46^a

Each value is the mean ± SEM, $n = 3$.
Values with different superscripts within same column are significantly different ($P < 0.05$) by Duncan's multiple-range test.

Discussion

PM-I exhibited the highest total isoflavonoid contents among the tested plants in April 2003, this being 23.81 and 12.90 times greater than that in October 2003 and February 2004, respectively. A variation in the total isoflavonoid contents was also demonstrated in the analysis of PM-II, PM-III and PM-IV. Some individual isoflavonoids varied with season. The mean value for the tuberous glycosides, puerarin, daidzin and genistin, in the plant population collected in April 2003 was greater than in October 2003 and February 2004. Nevertheless, more daidzein was present in October 2003 than in February 2004 and April 2003, while more genistein was present in February 2004 than in April and October 2003. The ratio of the total isoflavonoids of PM-I, PM-II, PM-III and PM-IV in April 2003 was 2.04:0.58:0.40:1. The individual and total isoflavonoid contents in the plant samples collected in October 2003 and February 2004 also varied to different degrees. In addition, the plants had few or no fully matured leaves in March to act as sites for isoflavonoid synthesis. Temperature also played a significant role in regulating the growth of tubers.[20)]

The isoflavonoid contents in seeds of *P. mirifica* were not analyzed in this study because the amounts of produced seeds were minute as compared with the size of the enlarged tubers. The genistein content was found to be the highest during February. These plants had coped with a period of temperature drop without any heat-shock effect during December, which may have stimulated the tuberous storage of aglycoside genistein. In comparison with the analysis of the mature wild tubers not less than 3 years old collected during March–April from the original location in a previous study,[17)] the 1-year-old tubers of PM-I in this study exhibited total isoflavonoid contents of 18.99 times greater than the mature wild tubers (1,007.47/53.05 mg/100 g). This was derived from the great difference in the amount of puerarin, by 59.52 times (620.22/10.42 mg/100 g), daidzin, by 31.65 times (304.74/9.63 mg/100 g), and genistein, by 9.76 times (5.76/0.59 mg/100 g), but not genistin which increased by 2.53 times (76.74/30.31 mg/100 g) and daidzein which was absent in this plant sample. The great differences of some individual and total isoflavonoid contents between the plants of the same cultivar at different ages may be derived mostly from the influence of differentiation of the plant *per se*. The 1-year-old plants exhibited a small size and fewer differentiated tubers, while the plants that were at least 3 years old exhibited a large size and fully differentiated tubers. It has been found that the isoflavonoid contents in fully differentiated tubers of the same plant population did not exhibit much variation in isoflavonoid contents,[17)] in comparison with those in this study. We can draw the conclusion that the degree of chemo-variation in the same plant cultivar was minimized when the tuber became more differentiated. The different data from the two studies indicate the significant influence of plant age and differentiation (which was highly season-

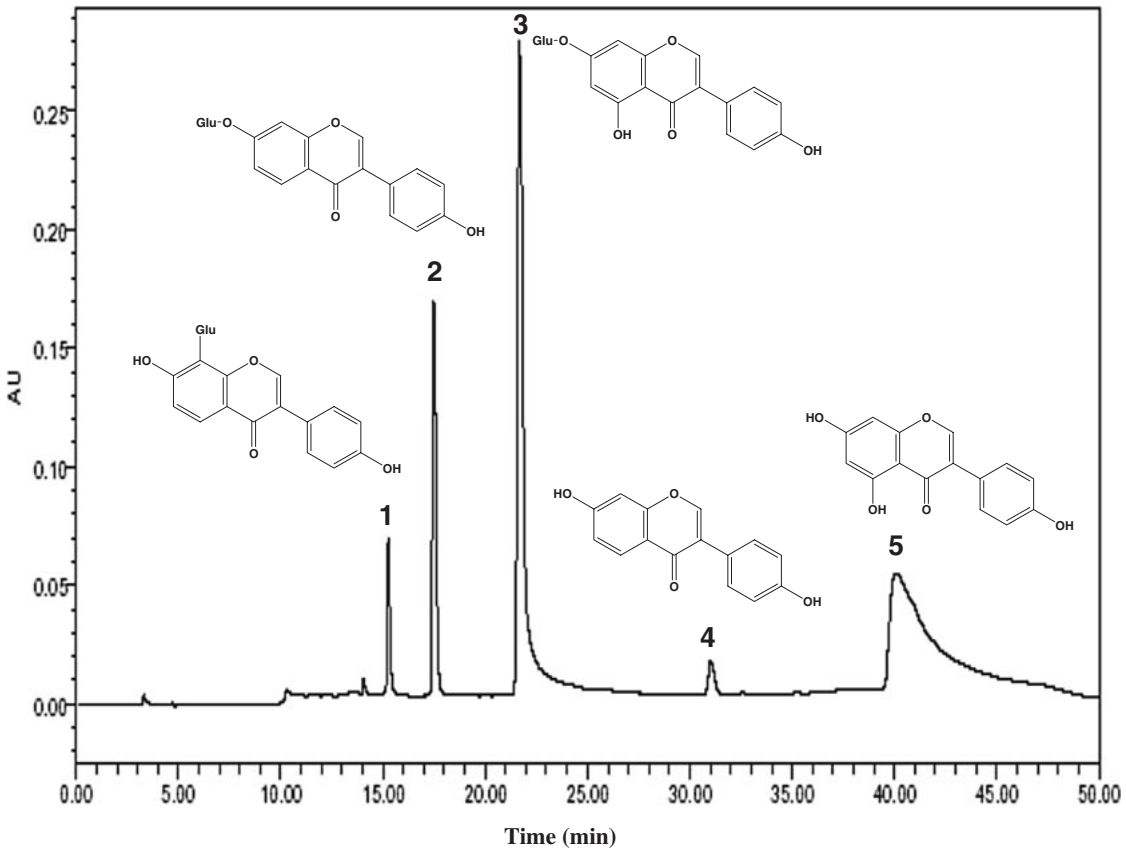

Fig. 3. HPLC Profile of the Standard Isoflavonoids.
The peaks recorded at 254 nm correspond to puerarin (1), daidzin (2), genistin (3), daidzein (4) and genistein (5) at respective the concentrations of 0.025, 0.1, 0.1, 0.05 and 0.25 mg/ml.

Table 3. Individual and Total Isoflavonoid Contents in mg/100 g of Tuberous Powder (DW) of the Four Cultivars of *P. mirifica* Cultivated in Ratchaburi Province, Thailand during the Summer, Rainy Season and Winter

Season	Cultivar	Puerarin††	Daidzin†	Genistin	Daidzein	Genistein	Total
Summer	PM-I	620.22 ± 14.22^{c}	304.74 ± 110.85^{a}	76.74 ± 18.64^{a}	0.00 ± 0.00^{a}	5.76 ± 5.76^{a}	1007.47 ± 72.22^{b}
	PM-II	112.88 ± 0.88^{ab}	90.10 ± 2.10^{a}	53.28 ± 7.02^{a}	29.06 ± 3.94^{ab}	3.88 ± 3.88^{a}	289.19 ± 4.10^{a}
	PM-III	30.10 ± 19.09^{a}	111.34 ± 71.27^{a}	45.93 ± 16.39^{a}	6.94 ± 1.79^{ab}	4.12 ± 4.12^{a}	198.43 ± 74.48^{a}
	PM-IV	150.43 ± 54.80^{ab}	206.61 ± 81.75^{a}	91.56 ± 38.16^{a}	44.95 ± 21.51^{abc}	0.39 ± 0.39^{a}	493.98 ± 196.61^{a}
	Mean ± SEM	228.41 ± 87.80^{b}	178.20 ± 43.55^{a}	66.88 ± 11.10^{a}	20.25 ± 7.92^{ab}	3.54 ± 1.70^{a}	497.27 ± 125.69^{a}
Rainy season	PM-I	8.06 ± 6.45^{a}	9.14 ± 3.32^{a}	4.16 ± 2.98^{a}	7.44 ± 4.83^{a}	13.51 ± 7.73^{ab}	42.32 ± 14.12^{ab}
	PM-II	4.87 ± 0.96^{a}	1.79 ± 0.68^{a}	2.61 ± 0.61^{a}	5.85 ± 0.73^{a}	2.80 ± 0.29^{a}	17.93 ± 1.81^{a}
	PM-III	5.97 ± 1.76^{a}	10.81 ± 1.83^{a}	6.40 ± 2.13^{a}	9.75 ± 2.93^{a}	9.38 ± 3.85^{ab}	42.32 ± 9.62^{ab}
	PM-IV	37.15 ± 19.37^{a}	28.55 ± 7.50^{b}	27.44 ± 2.54^{b}	154.88 ± 119.5^{a}	25.88 ± 6.22^{b}	273.89 ± 131.17^{b}
	Mean ± SEM	14.48 ± 6.03^{a}	13.40 ± 3.25^{a}	10.81 ± 2.90^{ab}	47.21 ± 33.45^{a}	13.29 ± 3.21^{ab}	99.19 ± 42.39^{ab}
Winter	PM-I	10.67 ± 1.76^{a}	20.58 ± 6.29^{ab}	10.32 ± 5.25^{ab}	21.51 ± 7.07^{a}	15.01 ± 5.91^{a}	78.10 ± 13.13^{a}
	PM-II	8.38 ± 3.54^{a}	5.67 ± 4.19^{a}	3.46 ± 3.46^{a}	40.48 ± 9.42^{a}	12.69 ± 0.31^{a}	70.68 ± 1.46^{a}
	PM-III	10.83 ± 1.60^{a}	27.89 ± 10.12^{ab}	28.71 ± 11.03^{b}	39.14 ± 6.54^{a}	24.91 ± 8.45^{a}	131.47 ± 19.68^{ab}
	PM-IV	22.93 ± 7.56^{a}	47.00 ± 13.79^{bc}	29.85 ± 5.20^{b}	41.46 ± 8.62^{a}	44.61 ± 3.24^{b}	185.86 ± 24.25^{b}
	Mean ± SEM	14.63 ± 3.04^{a}	27.91 ± 6.34^{ab}	19.39 ± 3.76^{ab}	33.92 ± 3.60^{a}	25.08 ± 3.92^{a}	126.13 ± 11.71^{ab}

Each value is the mean ± SEM, $n = 3$.
Values with different superscripts within same column are significantly different ($P < 0.05$) by Duncan's multiple-range test.
The plant cultivar samples expressed significant-correlation between individual isoflavonoid and total isoflavonoid contents†† ($P < 0.01$), † ($P < 0.05$) as determined by a factorial analysis.

influenced) on the tuberous isoflavonoid accumulation.

The plants in this study were of the same age and planted in the same type of soil and the same environment in the same field trial. The differences in individual and total isoflavonoid contents during the same collection period would mostly have been derived from the plant genetics *per se*. The plants produced abundant flowers and seeds, resulting in a high degree of genetic

variation. However, the differences in genotypes and gene expression, including genes on the isoflavonoid pathway within the same plant cultivar, should basically be lower than those between different plant cultivars. There was a similar result from the study of isoflavonoid accumulation in the hairy root culture of two different genotypes of soybean, which were different after exposure to pathogenic *Fusarium solani*.[21] This means that different plant cultivars or genetics could produce different amounts of isoflavonoids under the same stress conditions. Our findings are also related to the previous report that different soybean varieties showed different seed isoflavonoid contents. The crop year and climatic differences also influenced their isoflavonoid contents.[22] This would mean that the varied isoflavonoid synthesis and/or storage and/or metabolism in different cultivars of *P. mirifica* depended on the tuberous age and/or differentiation, and on the expression of the plant genes on the isoflavonoid biosynthetic pathway. Our correlation analysis results for seasonal and genetic variations are not related to those of similar studies on soybeans,[23,24] the two factors are not correlated with the phytochemical contents in the tubers. Even though puerarin exhibited the highest content in all the collected tuberous samples, there was a correlation between the amount of both puerarin and daidzin and total isoflavonoids in all tested seasons. These two components should play a more important role in total isoflavonoid contents than the others. A benefit of this finding is that puerarin, daidzin and the total isoflavonoids could be used as chemical markers to evaluate the influence of both climate and genetics on the tuberous storage of isoflavonoids in this plant species. Even though *P. mirifica* contained spinasterol with antitumor activity,[25] miroestrol,[26,27] and deoxymiroestrol[28] with high estrogenic activity, those compounds were found in minute amounts in the plant tubers and might not be practical, especially when determined by HPLC, for use to demonstrate the influence of seasonal and plant genetics on phytochemical storage in the tubers.

P. mirifica is a legume plant with distinct phytoestrogen-rich, large tubers instead of seeds as commonly found in ordinary legume plants. This represents the establishment of the first basic but important information on the physiology of tuberous isoflavonoid storage which could not be achieved in non-tuberous legume plants. We have proof of our recently published hypothesis not only in that there was chemovariety in the *P. mirifica* population but also that plant genetics was one of a major factors influencing the total isoflavonoid contents in this plant species.[17] The novel knowledge established in this study will benefit not only plant breeders for cultivating a selected cultivar/chemovariety with high isoflavonoid contents, but also for tuber harvesters to manipulate this during April, and not October or February, or according to seasonal variations, to obtain the highest concentration of total isoflavonoids.

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