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Medicago spp. as potential sources of bioactive isoflavones: Characterization according to phylogenetic and phenologic factors

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ABSTRACT

A high variety of plant species are often proposed as potential natural sources of specific bioactive components, with emphasis in phenolic compounds. However, the ability to produce a determined phytochemical might be variable, even among species with close phylogeny. Furthermore, the metabolic dynamics vary greatly according to phenologic factors. Herein, it was verified whether isoflavone production in *Medicago* spp. is more associated with phylogenetic or phenologic determinants, to define the optimal productive conditions. Isoflavone profiles were characterized in field-grown *Medicago* species in three phenologic stages. Isoflavones were extracted by matrix solid-phase dispersion method and analyzed using high-performance liquid chromatography coupled with a diode-array detector. The obtained data were evaluated by a generalized linear model (GLM) and linear discriminant analysis (LDA). Formononetin, genistein and irilone were the most abundant isoflavones, reaching values higher than those present in acknowledged plant sources like soy or red clover. Outputs from GLM and LDA indicate that the phylogenetic factors are the most defining criteria. This study promotes *Medicago* spp. as potential isoflavone sources, particularly because the effects of these compounds are highly dependent on their type and concentration, with potential application as foodstuff, feedstuff, or in the nutraceutical and pharmaceutical industry.

1. Introduction

The genus *Medicago* is part of the botanical family of *Leguminosae* and includes about 56 different species mainly distributed in Mediterranean climatic conditions areas (Farag et al., 2007). Besides alfalfa (*Medicago sativa*), which is the main *Medicago* species grown throughout the world, inclusively as source of phytochemicals (Nunes et al., 2008; Silva et al., 2013), not much attention has been given to other *Medicago* species. *Medicago truncatula*, which is often chosen as a model for genomic studies of *Fabaceae* due to its small diploid genome ($\approx 5 \times 10^8$ bp), self-fertilization, easy genetic transformation and prolific nature, is the sole exception (Farag et al., 2007; Huhman and Sumner, 2002; Schliemann et al., 2008; Stochmal et al., 2009). *Medicago* species are important sources of phytochemicals, including carotenoids, saponins or phytoestrogens (Yildiz, 2005), which are known to act as antimicrobial agents, phytoanticipins, phytoalexins, structural barriers, modulators of pathogenicity, plant defense genes activators, or fungitoxic agents (Von Baer et al., 2006). Isoflavones are synthesized, accumulated and constitutively expressed as phytoalexins in response to pathogen attacks (Dakora and Phillips, 1996), contributing to the global strategies of plant defense mechanisms (Aloui et al., 2012) and modulation of the interaction of *Fabaceae* species with nitrogen-fixing bacteria in rhizobium-legume symbiosis (Antunes et al., 2006). Isoflavones are also known as having a wide range of beneficial biological activities in the human body (Mortensen et al., 2009), but their overconsumption have been suggested as potentially causing adverse effects (Setchell and Cassidy, 1999). Hence, the intake of isoflavones has been limited by International Organisation (such as Food Safety Commission of Japanese Government or The Nutrient Data Laboratory of the Agricultural Research Service of the United States Department of Agriculture) to very restricted

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values (Sakamoto et al., 2015). Nevertheless, the intake amounts of isoflavones are usually evaluated on the basis of aglycone forms because the isoflavone glycosides are converted into aglycones by intestinal flora *in vivo* and *in vitro* (Sakamoto et al., 2015).

The three main classes of phytoestrogens are isoflavones, lignans and coumestans (Jacobs et al., 2009), but their biosynthesis varies greatly with environmental and genotypic factors (Hoeck et al., 2000). Soybean, for instance, present mainly isoflavone aglycones (daidzein, glycitein and genistein) and glycoside, acetylglycoside and malonylglycoside forms. In contrast to soybean, red clover contains biochanin A and formononetin (aglycones), and their glycosides and malonyl derivatives as the major components, while in *Medicago* species, the most abundant isoflavones are formononetin, irilone and genistein (Visnevschi-Necrasov et al., 2014a). The isoflavones profile also varied greatly with the phenological stage, varying along the plant maturity (D'Agostina et al., 2008). In lupin plants, the content of isoflavones was reported as negligible in seeds when compared to the amounts detected in leaves and roots (Bednarek et al., 2001). In a different study with *M. truncatula*, constitutive root isoflavonoids, like formononetin malonyl glycoside or medicarpin malonyl glycoside, accumulate increasingly with the development of mycorrhizal symbiosis (Aloui et al., 2012). Furthermore, isoflavones profile is highly affected by genotype × environment interactions (Morrison et al., 2010).

Herein, the profiles in free and conjugated isoflavones were compared among open-field grown *Medicago* spp. and the changes along vegetative cycle were monitored by evaluating three different phenologic stages: vegetative elongation, late bud and late flower. With this approach, it was intended to evaluate the effect of the plant species and the phenologic stage (as well as the interaction of both factors) in the potential yield of individual and total isoflavones.

2. Results and discussion

2.1. Phylogenetic and phenologic influence on isoflavone profiles

In this study eleven isoflavones were quantified, eluting in the following order: (1) puerarin (7,4′-dihydroxy-8-*C*-glucosylisoflavone), (2) daidzin (daidzein-7-*O*-β-D-glucoside), (3) genistin (genistein-7-*O*-β-D-glucoside), (4) daidzein (4′,7-dihydroxyisoflavone), (5) glycitein (4′,7-dihydroxy-6-methoxyisoflavone), (6) genistein (4′,5,7-trihydroxyisoflavone), (7) pratensein (4′-methoxy-3′,5,7-trihydroxyisoflavone), (8) formononetin (7-hydroxy-4′-methoxyisoflavone), (9) irilone (9-hydroxy-7-(4-hydroxyphenyl)-[1,3]dioxolo[4,5-g]chromen-8-one), (10) prunetin (4′,5-dihydroxy-7-methoxyisoflavone) and (11) biochanin A (5,7-dihydroxy-4′-methoxyisoflavone). Besides the chromatographic data, the proposed identification was also supported by UV spectra as described previously (Rodrigues et al., 2014). Chromatographic parameters, namely limit of detection (LOD), limit of quantification (LOQ), linearity, recovery and repeatability were accepted as previously assessed (Visnevschi-Necrasov et al., 2014a).

The effects of plant species (PSp) and phenologic stage (PhS), as well as the interaction of both factors, were assessed simultaneously by evaluating changes in isoflavones composition of *Medicago* spp. grown in open-field conditions. Studying the combined effect of both factors (PSp and PhS), allows understanding the influence of each single one, without having biased results; *i.e.*, it is possible to identify the PSp with highest potential to produce a determined set of isoflavones independently of the PhS (which could be useful for monoculture crops) and also defining the most suitable PhS independently of the PSp (which would be particularly valuable in polyculture crops). Table 1 shows the

Table 1
Isoflavone contents (mg/kg of dry matter) in the studied *Medicago* species.

		Puerarin	Daidzin	Genistin	Daidzein	Glycitein	Genistein	Pratensein	Formononetin	Irilone	Prunetin	Biochanin A	Total quantified isoflavones
Plant species (PSp)	*M. arabica*	1 ± 1	nd	1 ± 1	19 ± 7	nd	104 ± 38	19 ± 5	2010 ± 490	52 ± 22	96 ± 37	40 ± 34	2342 ± 538
	M. doliata	7 ± 10	nd	3 ± 3	7 ± 3	7 ± 5	664 ± 118	nd	1546 ± 395	225 ± 73	7 ± 11	37 ± 10	2502 ± 572
	M. minima	nd	nd	5 ± 2	nd	21 ± 9	42 ± 11	25 ± 6	1158 ± 435	141 ± 30	18 ± 26	28 ± 25	1437 ± 448
	M. murex	22 ± 32	6 ± 8	4 ± 1	9 ± 2	63 ± 10	115 ± 18	4 ± 3	1215 ± 298	64 ± 14	8 ± 3	18 ± 4	1527 ± 279
	M. orbicularis	nd	nd	4 ± 1	1 ± 1	9 ± 13	206 ± 23	21 ± 16	2806 ± 192	103 ± 16	nd	16 ± 13	3166 ± 231
	M. polymorpha	nd	nd	5 ± 4	nd	4 ± 5	390 ± 227	47 ± 5	11 ± 16	1432 ± 272	nd	nd	1889 ± 455
	M. rigidula	nd	nd	38 ± 15	nd	nd	104 ± 51	nd	1185 ± 353	26 ± 18	nd	25 ± 6	1378 ± 378
	M. tornata	26 ± 37	1 ± 2	169 ± 78	3 ± 4	7 ± 4	103 ± 51	3 ± 4	620 ± 219	217 ± 113	38 ± 18	63 ± 36	1250 ± 382
	M. truncatula	nd	nd	15 ± 13	1 ± 1	1 ± 1	440 ± 119	45 ± 23	824 ± 243	696 ± 249	nd	nd	2021 ± 535
	p-Value (n = 36)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Phenologic stage (PhS)[a]	1 – VE	10 ± 21	2 ± 5	26 ± 43	4 ± 8	11 ± 15	208 ± 172	17 ± 18	1104 ± 779	242 ± 317	16 ± 23	42 ± 36	1682 ± 707
	2 – LB	nd	nd	39 ± 84	5 ± 7	11 ± 19	269 ± 252	22 ± 24	1478 ± 751	390 ± 473	15 ± 34	22 ± 14	2252 ± 596
	3 – LF	9 ± 24	1 ± 1	15 ± 30	4 ± 5	15 ± 25	245 ± 229	16 ± 17	1208 ± 903	354 ± 536	25 ± 41	13 ± 16	1904 ± 790
	p-Value (n = 108)	<0.001	<0.001	0.009	0.277	0.234	0.122	0.053	<0.001	0.005	0.058	<0.001	<0.001
PSp × PhS	*p*-Value (n = 324)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[a] VE: vegetative elongation (stem length <30 cm, no visible buds or flowers); LB: late bud (three or more nodes with visible buds, no flowers or seed pods); LF: late flowering (one or more nodes with 50% open flowers, no seed pods).

isoflavone composition reported as the mean value of each PSp considering the three PhS, as well as mean value of each PhS, individually containing the values for all nine PSp. Accordingly, the presented standard deviations should not be understood in *sensu strictu*, since the mean values obtained in each case were calculated using the results given by different PSp or PhS.

As it can be concluded from Table 1, the interaction PSp × PhS was significant for all isoflavones, meaning that the variation in isoflavones contents resulted from the conjunct action of both factors simultaneously. Accordingly, it was not possible to classify the results through multiple comparison tests (such as Tukey's HSD or Tamhane's T2). Nevertheless, some conclusions could be drawn from the corresponding estimated marginal mean (EMM) plots (data shown only in specific cases).

For instance, late bud (LB) tended to present greater isoflavones levels (except for puerarin and daidzin) independently of PSp. This trend is verified either for individual isoflavones as well as for their total amounts (Fig. 1A–D). The crossed lines are also evidence of the strong interaction between the two factors. These difference are likely to be due to the greater impact of biotic (like pathogen attack) and environmental stresses on plant survival and reproduction in younger plants and the consequent need to maximize plant defense (Bednarek et al., 2001). Actually, the impact of these stress conditions varies within the vegetative cycle, producing different needs in the plant defense system and causing differences in the expression of isoflavones (Posmyk et al., 2005). In comparison with previous results obtained with the same species in the late flower (LF) PhS, a general trend to higher contents in vegetative elongation (VE) and LB was verified for most isoflavones. Besides these quantitative differences, some qualitative dissimilarities were also observed in the obtained profiles. The following isoflavones were not detected in the LF of the indicated species: puerarin in *Medicago doliata* and *Medicago murex*; daidzin in *M. murex*; genistin in *Medicago polymorpha*; daidzein in *Medicago orbicularis* and *Medicago tornata*; glycitein in *M. doliata*, *M. polymorpha* and *M. truncatula*; pratensein in *M. orbicularis*; formononetin in *M. orbicularis*; prunetin in *M. doliata*; bioachanin A in *Medicago arabica*, *Medicago minima* and *M. orbicularis*. The highlighted differences are good indicators of the relevance of the PhS in which plants should be harvested to benefit from a determined isoflavones' profile. In similar studies (Guo et al., 2011), soybean plants released greater amounts of daidzein and genistein at the pod and seed maturity stages than at the seedling period. A variation in the total isoflavones along the PhS was also reported in *Lupinus albus* leaves sowing in different seasons, alfalfa, red clover and soybean (D'Agostina et al., 2008; Seguin et al., 2004). The influence of plant age on the concentration of various isoflavonoids, and specifically the increase in biochanin A and formononetin sugar conjugates,

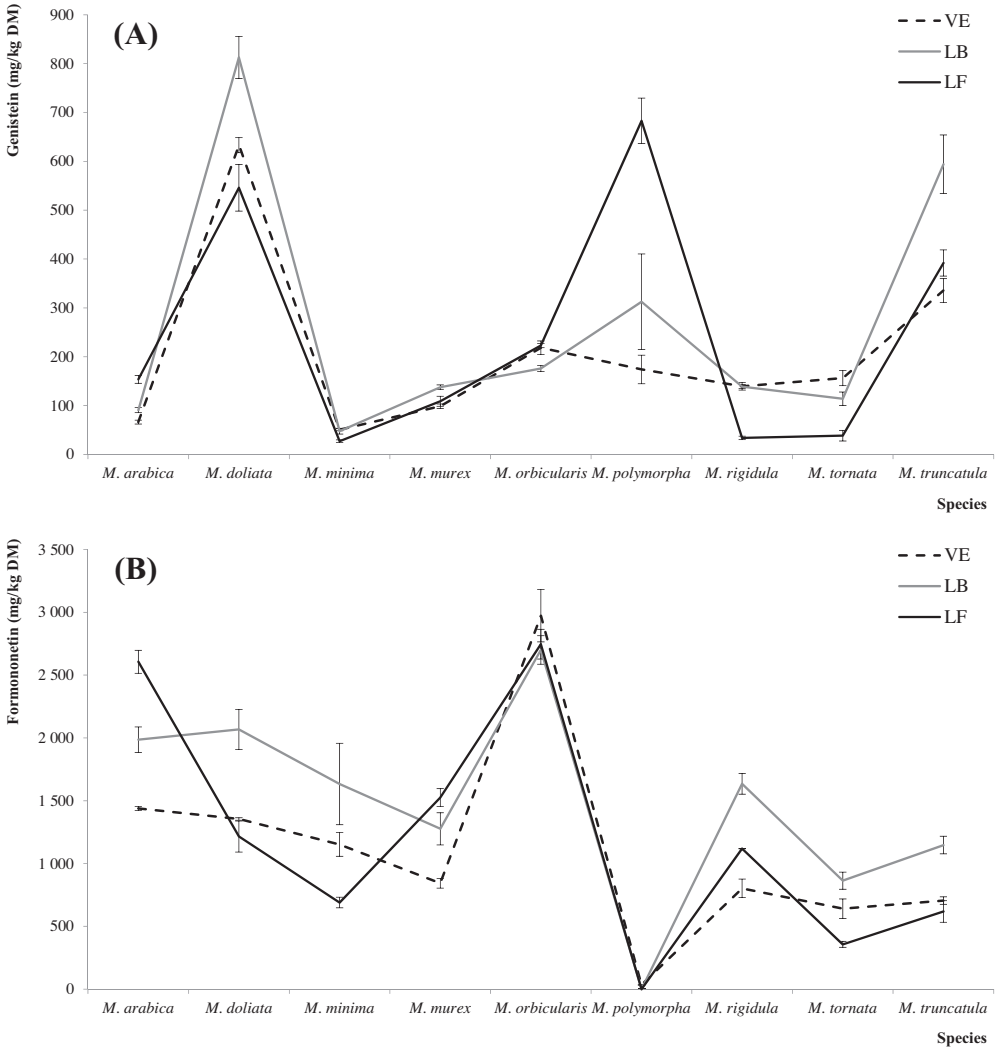

Fig. 1. Estimated marginal mean plots representing the effect of PhS on the isoflavone contents of the assayed *Medicago* species. (A) genistein; (B) formononetin; (C) irilone; (D) total isoflavones.

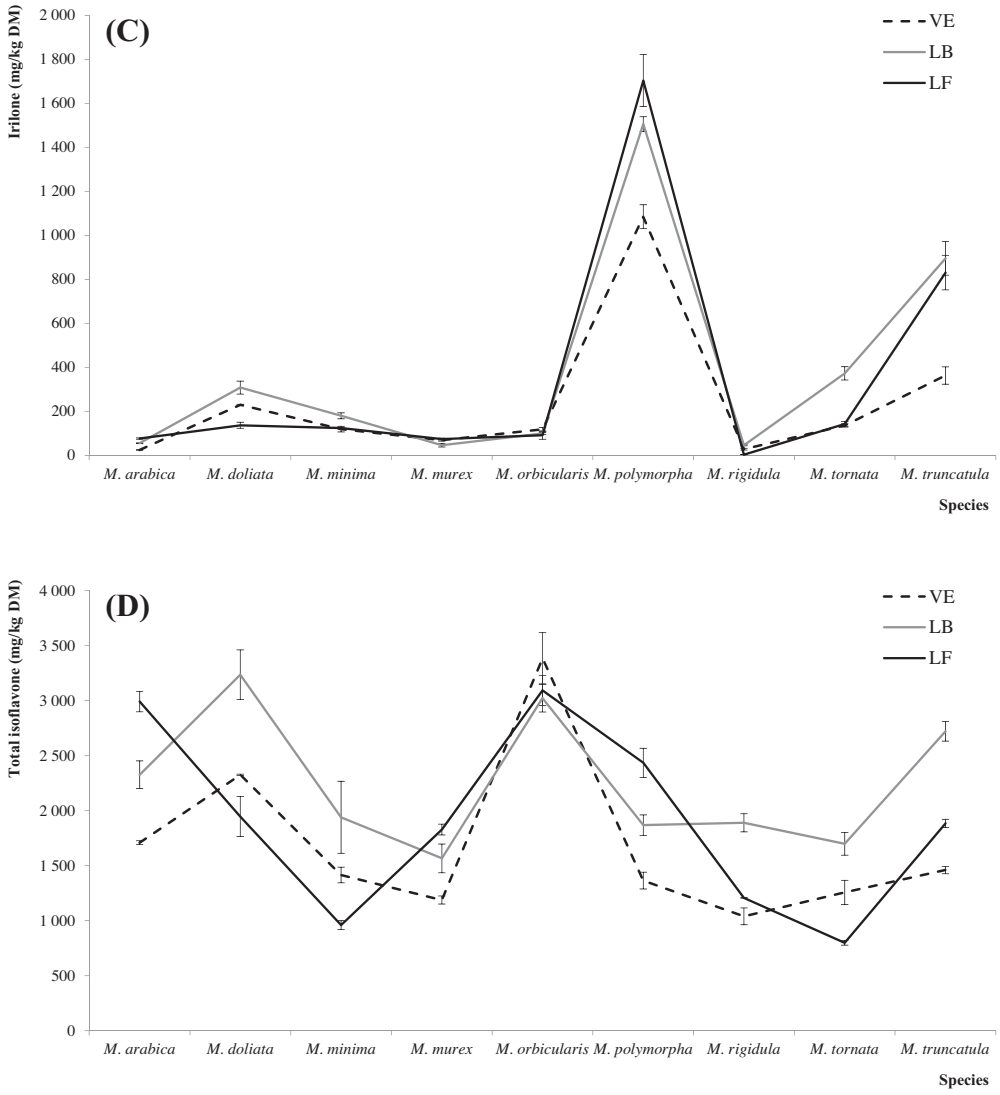

Fig. 1 (*continued*)

was reported in red clover foliage, where these isoflavone derivatives increased markedly during plant growth (Edwards et al., 1997).

The phylogeny seemed to exert the most marked effects on the production of isoflavones in *Medicago* leaves. Considering once again the EMM plots (show only for genistein, formononetin and irilone in Fig. 1), and independently of the PhS, genistin and biochanin A tended to be present in higher quantity in *M. tornata*, daidzein and prunetin in *M. arabica*, glycitein in *M. murex*, genistein in *M. doliata*, formononetin in *M. orbicularis* and irilone in *M. truncatula*. Regarding the isoflavone amounts in a global perspective, *M. arabica*, *M. doliata* and *M. orbicularis* presented the highest overall content of isoflavones (Table 1), while *M. tornata* presented the lowest.

It was also possible to identify the binomial combination of PSp × PhS that maximizes the production of each isoflavone: puerarin, *M. tornata* × late flowering (LF); daidzin, *M. murex* × vegetative elongation (VE); genistin, *M. tornata* × late bud (LB); daidzein, *M. arabica* × VE; glycitein, *M. murex* × LF; genistein, *M. doliata* × LB (detailed in the next paragraph); pratensein, *M. truncatula* × LB; formononetin, *M. orbicularis* × VE; irilone, *M. polymorpha* × LF; prunetin, *M. arabica* × LF; biochanin A, *M. tornata* × VE; total isoflavones, *M. orbicularis* × VE.

These different combinations are also good indicators of the strong interaction among PhS and PSp. In addition, the evolution of the three major isoflavones (genistein, formononetin and irilone) along the PhS may be observed in Fig. 1A–C. As it can be seen, the variation of these three isofavones along the PhS for each PSp is a striking example of their dissimilar behavior.

Regarding the isoflavone with more reports describing its effects on human health, genistein contents ranged from 27 (*M. minima* × LF) to 813 mg/kg DM (*M. doliata* × LB), a very high value, even when compared with soybean, in which genistein was reported as the major isoflavone, ranging from 84 mg/kg to 583 mg/kg DM along the reproductive stages (Kumar et al., 2009).

In what concerns the total amounts of quantified isoflavones, the obtained values were also higher than those presented by other vegetable species like green bean, carrot, white cabbage, cauliflower, iceberg lettuce or artichoke (Konar et al., 2012). Even regarding soybean, that typically yields 1.4–6.9 kg (considering 0.6–3.0 g of isoflavones/kg of soybean and 2.3 tons of soybean/ha) of isoflavones (sum of daidzein, genistein, and glycitein in aglycone equivalents)/ha (Mortensen et al., 2009), the productive potential of *Medicago* spp. might be considered high. These species might reach isoflavone productivities near 50 kg/ha, considering the average value of total isoflavones in the species assayed herein, and a

Table 2
Physiological parameters of the evaluated *Medicago* species grown in the Agrarian Station of Vairão at the experimental field of University of Porto, Portugal.

Species	Phenologic parameters[a]									
	Accession ID	H	ST	TE	EpF	pFF	LC	E	pF	F
Medicago arabica	31, 32	45 ± 7 a	35 ± 1 c	25 ± 1 c	118 ± 14 a	13 ± 1 a	191 ± 16 ab	25 ± 1 b	143 ± 14	156 ± 9 ab
Medicago doliata	39, 41	17 ± 2 b	41 ± 2 bc	36 ± 3 bc	124 ± 2 a	8 ± 1 b	210 ± 2 ab	36 ± 3 ab	160 ± 9	168 ± 7 a
Medicago minima	67, 68	28 ± 2 ab	59 ± 5 b	39 ± 12 bc	109 ± 8 ab	9 ± 1 ab	216 ± 9 a	39 ± 5 ab	148 ± 4	157 ± 7 ab
Medicago murex	62, 69	20 ± 2 b	57 ± 6 bc	48 ± 5 ab	95 ± 5 ab	9 ± 1 ab	208 ± 6 ab	48 ± 5 a	143 ± 7	152 ± 4 ab
Medicago orbicularis	66, 73	42 ± 7 a	39 ± 5 bc	24 ± 2 c	126 ± 3 a	10 ± 1 ab	199 ± 1 ab	24 ± 4 b	149 ± 6	160 ± 6 ab
Medicago polymorpha	82, 83	40 ± 1 a	37 ± 3 bc	23 ± 4 c	111 ± 6 a	11 ± 1 ab	182 ± 5 b	23 ± 4 b	134 ± 3	145 ± 2 b
Medicago rigidula	96, 97	19 ± 1 b	86 ± 2 a	18 ± 2 c	95 ± 1 ab	7 ± 1 b	206 ± 6 ab	28 ± 3 b	136 ± 5	146 ± 3 ab
Medicago tornata	111, 116	33 ± 3 ab	40 ± 10 bc	28 ± 5 bc	113 ± 10 a	10 ± 1 ab	192 ± 4 ab	28 ± 5 b	141 ± 2	151 ± 4 ab
Medicago truncatula	123, 127	30 ± 7 ab	49 ± 8 bc	66 ± 4 a	79 ± 8 b	8 ± 1 b	202 ± 10 ab	49 ± 8 a	137 ± 7	146 ± 5 ab
p-Value (Tukey's test)	–	0.001	<0.001	<0.001	0.003	0.015	0.034	0.002	0.101	0.040

[a] ID: identification number of the accession of each species of *Medicago*; H: plant height (cm); ST: period since sowing in greenhouse and transplantation to the experimental field (days); TE: period among transplantation and elongation (days); EpF: period among elongation and pre-flowering (days); pFF: period among pre-flowering and 50% flowering (days); LC: life cycle (days); E: elongation (days in first phenologic stage); pF: pre-flowering (days in second phenologic stage); F: flowering (days in third phenologic stage).

productivity of 23 tons of dry matter/ha (Rassini et al., 2000). In addition, all of the most studied isoflavones (genistin, daidzein, glycitein, genistein, formononetin and bioachanin A), thereby with the most well-known activity, were detected in these *Medicago* species, with special relevance to formononetin. Furthermore, the applied extraction procedure proved to be much more effective than the maceration with polar extracts (Rodrigues et al., 2014).

The differences verified for the phenologic indicators may also explain some of the observed variability. As it can depicted from Table 2, the assayed specimens presented significant differences ($p < 0.050$) for at least one of the assayed species, except for the pre-flowering (days in the second phenologic stage) period ($p = 0.101$). The species with highest isoflavone contents (*M. orbicularis*, *M. doliata* and *M. arabica*) presented some common features in their phenology. All their accessions presented low ST (period since sowing in greenhouse and transplantation to the experimental field) and TE (period among transplantation and elongation) and high EpF (period among elongation and pre-flowering) and F (flowering period). However, it was not possible to completely correlate these differences with the quantified isoflavone amounts. Either way, the qualitative and quantitative differences found among PSp or PhS may result from the variation induced by the type or age of the plants, besides the effect of environmental conditions. In this particular, the stress induced by temperature, light exposure and drought may explain some variation among *Medicago* species, since plants faced different temperatures during their vegetative development, as it is indicated by the periods of sowing, elongation, transplantation or flowering (Table 2) (Bednarek et al., 2001; Von Baer et al., 2006).

2.2. Linear discriminant analysis

The lack of well identified correlations and the high standard deviations resulting from mixing different PSp or PhS hindered obtaining accurate conclusions. Accordingly, it was necessary to apply a statistical classification technique to understand the real variations of isoflavone profiles according to the *Medicago* species or the phenologic stage in which the plants were obtained. Hence, to obtain a better comprehension about the influence of phylogenetic and phenologic effects over isoflavone profiles, the results were evaluated through LDA, verifying the differentiation power of the selected variables. The significant independent variables (isoflavones) were selected using the stepwise procedure of the LDA, according to the Wilks' λ test. Only those with a statistical significant classification performance ($p < 0.050$) were kept for analysis. As it could be expected for the mean values presented in Table 1, the differences in isoflavone profiles gave a good classification performance, allowing classifying correctly 100.0% of the assayed species for the originally grouped cases as well as for the cross-validated cases. In this discriminant model, six functions were defined as being significant, from which the first three were plotted, (Fig. 2A) integrating 82.4% of the observed variance (first: 46.0%; second: 23.6%; third: 12.8%), with all variables being selected by the model. Function 1, mainly correlated with irilone and formononetin, as deduced from the canonical discriminant functions standardized coefficients (Table 3) separated primarily *M. arabica* (high contents in formononetin and low in irilone) and *M. polymorpha* (low contents in formononetin and high in irilone) from the remaining species. Function 2, more strongly correlated with genistin, projected *M. tornata* (in which genistin was quantified in the highest values) far from any other species. Finally, function 3, more powerfully correlated with glycitein, was particularly effective in separating *M. murex* (supporting the relevance of its high contents in glycitein). Nevertheless, all species were individualized, indicating that the isoflavone profile of each *Medicago* species is particularly affected by phylogenetic factors.

Regarding the effect of the PhS, the discriminant model defined two significant functions (Fig. 2B) integrating 100.0% of the observed variance (first: 70.2%; second: 29.8%). The classification performance was, as it could also been expected from the *p*-values in Table 1, quite lower than the obtained for the effect of PSp, classifying correctly only 61.7% (18 VE as LB, 42 VE as LF; 5 LB as VE, 29 LB as LF; 22 LF as VE, 8 LF as LB) of the assayed species for the originally grouped cases and 60.2% (18 VE as LB, 42 VE as LF; 5 LB as VE, 29 LB as LF; 22 LF as VE, 13 LF as LB) for the cross-validated cases, with genistein as the only variable being excluded by the model. This result indicates that the PhS, *per se*, should not be the main concern when electing the optimal conditions for harvesting plants with a desirable isoflavone profile, highlighting that using monoculture crops would be a more reliable option.

Overall, this study evaluated the influence of phenologic and phylogenetic effects on the isoflavone content of *Medicago* spp leaves grown on open-field conditions, with particular relevance for PSp. Considering the detected isoflavone amounts, *Medicago* species might be considered as interesting alternative source of these phytoestrogens. The most important criterion to be considered when using these plants as isoflavone sources resulted to be the selected species, since the variation along the vegetative cycle seemed to be less pronounced.

The influence of genotypic factors was also found to be the most relevant source of variation in our previous study with different *Fabaceae* genera, namely *Biserrula*, *Lotus*, *Ornithopus* and *Scorpiurus* (Visnevschi-Necrasov et al., 2014b). With the present results, it becomes clear that the genetic modulation of isoflavones' biosynthetic pathway goes beyond the genus, being associated with the lowest taxonomical rank.

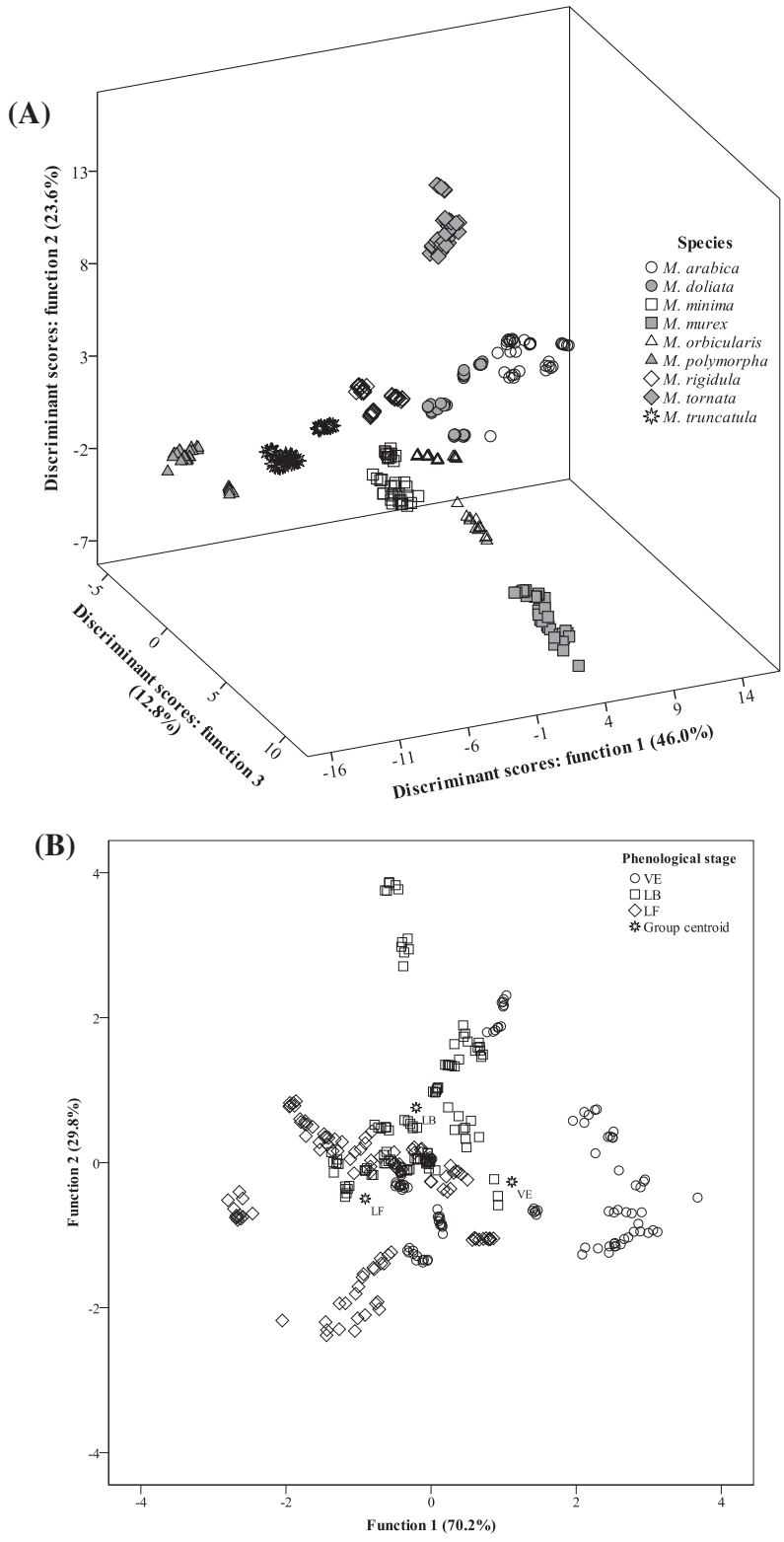

Fig. 2. Mean scores of different of *Medicago* species projected for the three first discriminant functions (A) and phenologic stages projected for the two first discriminant functions (B) defined from the isoflavone profiles.

In addition the best combination of both factors (PSp and PhS) that maximize the yield in a determined isoflavone were found. This provides a more natural way to obtain suitable isoflavone concentrations, in comparison to the metabolic engineering of isoflavonoid biosynthesis. The results obtained in this work might be considered as a step forward in the process of using *Medicago* spp. as isoflavone sources, particularly because the effects of these compounds are highly dependent on their type and concentration.

Besides their application as foodstuff, leaves of *Medicago* spp. (especially *M. truncatula*, *M. orbicularis* and *Medicago rigidula*) can be useful to the nutraceutical and pharmacological industries as a potential source of isoflavones, particularly genistein and

Table 3
Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions.

Isoflavone	Discriminant functions							
	PSp effects						PhS effects	
	1	2	3	4	5	6	1	2
Glycitein	0.042	−0.226	0.681	0.065	0.152	−0.147	−0.085	−0.115
Prunetin	0.166	0.123	−0.089	0.364	−0.029	−0.266	−0.110	−0.176
Genistein[a]	−0.056	−0.077	−0.249	−0.209	0.618	0.498	−0.126	0.108
Formononetin	0.261	−0.155	−0.084	−0.153	−0.383	0.685	−0.100	0.320
Irilone	−0.392	−0.042	−0.261	0.341	0.428	0.644	−0.139	0.142
Pratensein	−0.161	−0.099	−0.182	0.217	−0.074	0.157	−0.006	0.248
Genistin	−0.016	0.368	0.143	−0.051	−0.032	0.091	0.062	0.299
Biochanin A	0.083	0.149	0.043	−0.010	0.0110	−0.053	0.590	−0.045
Daidzin	0.013	−0.029	0.156	0.029	0.066	−0.021	0.264	−0.240
Daidzein	0.213	−0.022	−0.024	0.323	0.228	−0.151	0.044	0.149
Puerarin	0.015	0.056	0.125	0.004	0.085	0.054	0.069	−0.418

[a] Genistein was excluded by the linear discriminant model when evaluating the PhS effects.

formononetin. These novel applications might boon the increase of agronomical exploitations, either in number, as well as in surface, boosting the local development of economically constrained areas.

3. Material and methods

3.1. General experimental procedures

Acetonitrile (HPLC grade) and formic acid (analytical grade) were from Merck (Darmstadt, Germany). Purified demineralized water used was from a "Seradest LFM 20" system (Seral, Ransbach-Baumbach, Germany). The eluents were filtered through 0.45 μm filters and degassed under reduced pressure and ultrasonic bath. Disposable syringe filter PTFE 0.45 μm was from Macherey–Nagel (Düren, Germany). The C_{18}-bonded silica (particle size 55–105 μm) used as sorbent for MSPD was from Waters (Milford, MA, USA).

3.2. Plant material and field experimental site

Nine species of *Medicago* (*M. arabica*, *M. doliata*, *M. minima*, *M. murex*, *M. orbicularis*, *M. polymorpha*, *M. rigidula*, *M. tornata* and *M. truncatula*) were sown in November 2008 at the Experimental Field of the University of Porto (Agrarian Station of Vairão). The vegetal germplasm was obtained from the Portuguese collection of *Leguminosae* provided by the National Institute of Biological Resources (*Instituto Nacional dos Recursos Biológicos*, *I.P.*). Voucher specimens of each *Medicago* species were numbered and deposited in the local herbarium. Samples were collected from February to July in three phenologic stages: (1) vegetative elongation (stem length <30 cm, no visible buds or flowers); (2) late bud (three or more nodes with visible buds, no flowers or seed pods); and (3) late flower (one or more nodes with 50% open flowers, no seed pods). For each species and phenologic stage, three independent samples were selected (in different locations within the limits of the indicated Experimental Field) consisting in fresh leaves from randomly selected plants (5 plants for each sample) belonging to 2 different accessions; samples were dried at 65 °C during 72 h and milled, at particle size of 0.1 mm, using an A11 analysis mill (IKA Werke, Staufen, Germany).

3.3. Physiological parameters

The periods required to fulfil each of the considered physiological stages were accurately measured to obtain a more complete characterization of the physiological response of each species to the edaphoclimatic conditions of the experimental site. The assayed parameters (in days) included: (i) period since sowing in greenhouse and transplantation to the experimental field (ST); (ii) period among transplantation and elongation (TE); period among elongation and pre-flowering (EpF); period among pre-flowering and 50% flowering (pFF); life cycle (LC); elongation – days in first phenologic stage (E); pre-flowering – days in second phenologic stage (pF); flowering – days in third phenologic stage (F).

3.4. Extraction procedure

MSPD extraction of isoflavones was performed following a previous method (Visnevschi-Necrasov et al., 2014a). The same amounts of sample, C_{18} and internal standard (2-methoxyflavone) were used. The extraction mixture was transferred to an empty column, connected to a vacuum system, washed with distilled water and the isoflavones were eluted with methanol:H_2O (9:1, v/v). Before HPLC analysis, the extracts collected in amber vials were filtered through a 0.45 μm PTFE membrane. Different samples of two distinct accessions of all species were extracted.

3.5. HPLC determination of isoflavones

Purity-corrected individual isoflavones stock solutions (1 g/L) were prepared in methanol:H_2O (75:25, v/v). A composite stock standard solution of multiple isoflavones containing 40 mg/L of each standard: biochanin A (⩾97%), puerarin (⩾99%), glycitein (⩾97%), daidzein (⩾98%), daidzin (⩾95%), prunetin (⩾98%), genistein (⩾98%), genistin (⩾95%) and formononetin (⩾99%) (Sigma–Aldrich, St. Louis, MO, USA); pratensein and irilone (both ⩾ 98%) (Chromadex Inc., Barcelona, Spain) was prepared. The internal standard (IS) 2-methoxyflavone was obtained from Sigma. A working 2-methoxyflavone solution was prepared in methanol at 1 g/L. All the solutions were stored at −20 °C in amber glass vials when not in use.

Chromatographic analyses were performed following the previously optimized methodology (Visnevschi-Necrasov et al., 2014a). A high-performance liquid chromatograph (Jasco, Tokyo, Japan) equipped with a PU-2080 quaternary pump and a Jasco AS-950 automatic sampler were used. Detection was performed with a multi-wavelength diode-array detector Jasco, MD-2010 and the chromatographic separation of the compounds was achieved under the conditions described previously (Visnevschi-Necrasov et al., 2014a). Data were analyzed using the Borwin-PDA Controller Software (JMBS, Le Fontanil, France). Compounds were identified by chromatographic comparisons with authentic standards and UV spectra. Quantification was made using the calibration curves obtained for each identified isoflavone (DAD at 254 nm) based on the internal standard (2-methoxyflavone) method.

The limits of detection (0.011–0.171 mg/L) and quantification (0.037–0.569 mg/L), as well as the linearity (correlation coefficients higher than 0.99 in all cases), accuracy (recoveries in the range of 82–104%) and repeatability (relative standard deviation values ranged from 4% to 9%) of the method were accepted as previously obtained (Visnevschi-Necrasov et al., 2014a).

3.6. Statistical analysis

All extractions were performed in triplicate and each replicate was quantified in duplicate. Data were expressed as means ± standard deviations. All statistical tests were performed at a 5% significance level using the SPSS software, version 22.0 (SPSS Inc.).

3.6.1. General linear model

An analysis of variance (ANOVA) with type III sums of squares was performed using the GLM (General Linear Model) procedure of the SPSS software. The dependent variables were analyzed using 2-way ANOVA, with the factors "plant species" (PSp) and "phenologic stage" (PhS), fixed to evaluate properly the effects of phylogeny and phenology. With this approach, it was expected to identify the most suitable PhS, independently of the PSp, as well as verifying if a determined PSp would provide optimal isoflavone amounts, regardeless of the PhS. In this analysis, when a statistically significant interaction (PSp × PhS) is detected, the two factors are evaluated simultaneously by the estimated marginal means plots for all levels of each single factor. Alternatively, if no statistical significant interaction is verified, means obtained for each of the assayed factors (PhS or PSp) are compared using multiple comparison tests adequate for homoscedastic (e.g., Tukey's HSD) or heteroscedastic (e.g., Tamhane's T2) distributions.

3.6.2. Stepwise linear discriminant analysis (LDA)

In addition, a linear discriminant analysis (LDA) was used to assess the global influence of each factor on isoflavone profile. The basic purpose of these discriminant analyses was estimating the connection between a single categorical dependent variable (*Medicago* species in the first analysis and phonological stage in the second) and a set of quantitative independent variables (the amounts quantified for each isoflavone). The significant independent variables were selected following the stepwise method of the LDA, according to the Wilks' λ test, with the usual probabilities of F (3.84 to enter and 2.71 to remove). Only variables with a statistically significant classification performance ($p < 0.05$) were kept in the analysis. This procedure uses a combination of forward selection and backward elimination procedures, where before selecting a new variable to be included, it is verified whether all variables previously selected remain significant (Palacios-Morillo et al., 2013). With this approach, it is possible to identify the significant variables that contribute in higher extent to the discrimination of a determined set of samples. To verify which canonical discriminant functions were significant, the Wilks' λ test was applied. A leaving-one-out cross-validation procedure was carried out to assess the model performance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2015.04.011.

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