South African Journal of Botany 2005, 71(2): 160–166
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SOUTH AFRICAN JOURNAL OF BOTANY
ISSN 1727–9321

Flavonoids in phylloclades discriminate endemic *Semele androgyna* chemotypes from Madeira

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Received 10 May 2004, accepted in revised form 12 August 2004

Thirty-five randomly-collected *Semele androgyna* Kunth samples were screened by RP-HPLC for their phenolic composition. Fraction analysis allowed the detection of 17 different compounds. According to their retention times and UV spectra obtained by diode array analysis, these phenolics represent three classes: phenolic acids, flavones and flavonols. Co-chromatography with specific standards enabled identification of quercetin, rutin and quercitrin in *Semele* tissues for the first time. Polymorphism based on phenolic composition was evaluated using multivariate analysis and showed four distinct *S. androgyna* clusters. This polymorphism was not associated with morphological diversity or different in ambient light intensities. Biochemical differentiation is thus present in this species. The application of multivariate analysis techniques to RP-HPLC data has allowed the classification of samples into two groups, previously proposed on the basis of morphological and cytotaxonomical information. Therefore, the use of phenolics as chemotaxonomic markers in *Semele* is highly recommended because of its diagnostic value, even at a subspecies level. Discriminant canonical analysis and Mahalanobis distances confirmed these clusters as recognisable chemosystematic units. However, these units do not support the separation of *S. pterygophora*.

Introduction

Semele, an endemic genus from the Macaronesian phytogeographical region, is rare in the wild in Madeira. It occurs mainly in rocky, wooded ravines of the interior and occasionally in damp places on the north coast. It is also widely cultivated in gardens. Species of the genus are perennial with a shrubby or climbing habit, and show great morphological variability (Vale Lucas *et al*. 1998). In *Semele*, the leaves are reduced to tiny scales and broad, flat leaf-like outgrowths of the stem called phylloclades are responsible for most photosynthetic activity. Several taxa which delimit the range of field variation into several species have been proposed (Menezes 1922, Costa 1927, Costa 1949, Costa 1950). However, *Semele androgyna* (L.) Kunth (Ruscaceae) remains the only species presently recognised in Madeira (Vickery 1994). Nevertheless, a recent evaluation of morphological and reproductive characteristics of the plants combined with their ecology has clearly shown that two different *Semele* taxa, *S. androgyna* (L.) Kunth (or *S. androgyna sensu stricto*) and *S. maderensis* (Costa) Pinheiro de Carvalho, should be recognised in Madeira.

The morphological variability and taxonomy within *S. androgyna sensu stricto* (s.s.) remains unclear and requires better evaluation. In addition, the biochemical features of the genus are poorly characterised. One biochemical study demonstrated the presence of sapogenins, such as androgenin and ruscogenin, in the phylloclades (Gonzalez 1976). However, the chemical diversity of secondary metabolites in this endemic species has not been investigated adequately. The phenolic composition of leaf tissue has been used to identify and discriminate different chemotypes of *Quercus rubra* (McDougal and Parks 1986), cultivars of walnut (Jay-Allemand *et al*. 1999) and apple trees (McRae and Lidster 1990) as well as azaleas and roses (Van Sumere *et al*. 1993). Phenolic contents of the flower heads of *Leontondon* spp. have been demonstrated to be the most reliable markers in *Leontondon* chemosystematics (Zidorn and Stuppner 2001) and, in a general review, Crawford (1978) showed that flavonoids are useful taxonomic markers, even at the lowest taxonomic levels. Mirov *et al*. (1996) and Kaundun *et al*. (1997) have distinguished *Pinus*

halepensis from *Pinus brutia* through differences in their terpene compositions. Lebreton *et al*. (1990) have shown that *Pinus halepensis* and *Pinus brutia* can also be separated by the relative contents of quercetin and isorhamnetin in their leaves.

The purpose of this study was to obtain analytical information about the phenolic components of plant samples belonging to *Semele* by RP-HPLC in order to assess their taxonomical significance. We examined the phenolic composition of samples from *S. androgyna* s.s. in relation to plant morphology and ecology in order to assess whether the data support or reject the separation of the species into several taxa, as proposed by Costa (1950).

Material and Methods

Thirty-five *S. androgyna* s.s. samples distributed across Madeira (Table 1) were screened for phenolic composition. Voucher specimens were deposited at the University of Madeira Herbarium (MADU). Plants were selected to represent the greatest morphological variability within *S. androgyna* s.s. in Madeira. To evaluate the influence of light exposure on the phenolic content of phylloclades, samples from plants growing under different light exposures were analysed. Plants growing in wild populations with different light exposures and plants with the same origin, but cultivated in gardens for at least a year under similar light exposure, were included in the study. All phylloclade samples were collected from the lower part of the plant near the site of emergence of the secondary branches during the first three months of 2000. Only phylloclades at comparable stages of development and without any obvious tissue injury were collected. After harvesting, the plant material was frozen in liquid nitrogen, and stored at –85°C.

For the phenolic extraction and analysis, phylloclades were lyophilised and phenols extracted by sonication in 80% acetone containing 10^{-4}M of methoxyflavone (internal standard) and 0.1M of gluconolactone (b-glucosidase

Table 1: *Semele androgyna* samples used for flavonoid analysis, their locations, light intensities at these locations and morphological features

Case number	Sample code	Origin	UTM[1]	Light exposure[2]	Costa classification[3]
1	JBM2	Botanical garden	CB 2114	2	1
2	JBM4	Botanical garden	CB 2114	2	2
3	JBM3	Botanical garden	CB 2114	2	2
4	JBM5	Botanical garden	CB 2114	2	2
5	JBM23	Botanical garden	CB 2114	2	1
6	JBM24	Botanical garden	CB 2114	2	2
7	MAC2	Private garden	CB 2114	3	1
8	MAC3	Private garden	CB 2114	3	2
9	JUMa16	Madeira University	CB 2114	3	2
10	JUMa21	Madeira University	CB 2114	3	2
11	JUMa36	Madeira University	CB 2114	3	2
12	QV-p	Q. Vigia-Funchal	CB 2114	3	2
13	QV-j	Q. Vigia-Funchal	CB 2114	3	2
14	QP-e	Q. Palmeira-Funchal	CB 2114	2	2
15	PMCPC1	Porto Moniz	BB 9533	2	1
16	PMCPC4	Porto Moniz	BB 9533	2	2
17	PMCPC6	Porto Moniz	BB 9533	2	2
18	PMCPC5	Porto Moniz	BB 9533	1	2
19	LRJ2	L. Ribeira da Janela	BB 9636	2	2
20	LRJ37	L. Ribeira da Janela	BB 9636	2	2
21	LRJ39	L. Ribeira da Janela	BB 9636	2	2
22	LRJ42	L. Ribeira da Janela	BB 9636	2	2
23	SVJPI1	São Vicente	CB 0830	3	2
24	SVJPI2	São Vicente	CB 0830	3	2
25	ESVS41	S.Vicente – Seixal	BB 9935	3	1
26	RJ/Sxest	R. da Janela – Seixal	BB 9935	3	1
27	Ilha-Est(Ver)	Ilha	CB 2131	3	2
28	RibF-JPI	Ribeiro Frio	CB 2323	2	2
29	S-PA	Santana	CB 2330	3	1
30	S-EstI	Santana, S. António	CB 2130	3	1
31	LRB1	L. Ribeiro Bonito	CB 1528	1	1
32	LRB49	L. Ribeiro Bonito	CB 1528	3	2
33	LRB50	L. Ribeiro Bonito	CB 1528	2	2
34	LRB51	L. Ribeiro Bonito	CB 1528	2	2
35	LRB52	L. Ribeiro Bonito	CB 1528	3	2

1 UTM = Universal Transverse Mercator geographical units
2 Exposure to direct sunlight: 1) full shade, 2) dappled shade, 3) continuous exposure possible for at least 1–2 hours per day
3 Costa classification (based in morphological characters): 1 = *S. pterygophora* (Costa 1950); 2 = *S. androgyna* (Costa 1950)

inhibitor), according to Jay-Allemand *et al*. (1999). Separation and identification of the phenolic compounds was done by RP-HPLC (Radix *et al*. 1998), using a Lichrospher column (5µm) 100 RP-18 (250mm x 4mm) with detection at 340nm, a flow rate of 1ml min^{-1} and an injection volume of 30µl. Mobile phase A (1% acetic acid in water) and mobile phase B (50% methanol, 50% acetonitrile) were mixed as follows, using linear gradients: (i) 15% to 40% of B in A (20min), (ii) 40% to 60% of B in A (5min), (iii) 60% to 100% of B in A (5min), (iv) 100% B (5min) and (iv) 100% to 15% of B in A (3min). Relative quantitative values were determined from HPLC chromatograms as described previously (Lagrange *et al*. 2001). Compounds were characterised by their retention times and UV spectra in comparison with standards. All analyses were performed at least four times. Quantitative values represent the averages of four chromatograms. Co-chromatography with standards was used to identify phenolics.

Principal Component Analysis (PCA) and Canonical Discriminant Analysis (DA) were performed on the abundances of phenolics. Multivariate analysis was accomplished by SPSS (Statistical Package for the Social Sciences) 10.0 (Kinnear and Gray 1999).

Results and Discussion

Table 1 shows the origins of samples, their light exposure and classification, according to Costa (1949, 1950). Plants with small secondary branches, small simple phylloclades and flowers only in the lower secondary branches and lower phylloclades of the secondary branch were classified as *S. androgyna* sensu Costa (or *S. androgyna* (L.) Kunth). Those with large secondary branches, large and composed phylloclades and flowers in all secondary branches and all phylloclades of the secondary branch were referred as *S. pterygophora* sensu Costa (1950).

The phenolic composition of *Semele* phylloclades was examined. *Semele* phylloclades possess three major phenolic classes. Seventeen phenolic peaks were detected (Table 2). Four flavones, Peaks 8–11, appear as major compounds. Peak 9 has the highest relative content in most samples, and was identified as an apigenin glycoside, based on its retention time and UV spectra obtained by diode array analysis. The basic phenolic metabolism in *Semele* therefore seems to be characterised by high flavone production, in contrast to a low level of production of phenolic acids (Peaks 1–6, Table 2, Figure 1). However, it is among the third phenolic class, the flavonols (Peaks 13–17), where the more dramatic variations between samples are seen (Table 2). Three of these flavonols (Peaks 13, 16 and 17) corresponded to rutin, quercitrin and quercetin respectively. All three compounds are biosynthetically related, as rutin and quercitrin are glycosidic derivatives of quercetin (quercetin-3-O-rutinoside and quercetin-3-O-rhamnoside, respectively). They are not present in all *Semele* samples, and their presence or absence seems to be one of the features strongly associated with the overall phenolic polymorphism.

Principal Components Analysis (PCA) was performed on the relative contents of phenolics. The Kaiser-Meyer-Olkim test (KMO > 0.6) confirms the validity of the statistical analysis performed. The results show that the samples can be grouped into four clusters (Figure 2). The first three regression factors explain 89.5% of observed cumulative variance (F1 and F3 presented in Figure 2 account for 59%) and illustrate the polymorphism found in phenolic composition of *S. androgyna* s.s.; Principal Component Analysis shows that the most important characters that distinguish the clusters (Table 3) are the apigenin-related compound (Peak 9), an unidentified compound (Peak 10), rutin (Peak 13) and quercetin (Peak 17). Apigenin glycoside was the most strongly correlated phenolic with regression factor 1, whereas rutin (Peak 13) is the phenolic most strongly correlated with regression factor 3. Discriminant Analysis (DA) based on Mahalanobis distance confirms the existence of the clusters obtained by PCA analysis.

Table 2: Phenolic compounds relative contents in *Semele androgyna* phylloclades. Values are expressed in equivalents of mg metoxyflavone per gram of dry weight

	Cluster 1 mean (min.–max.)	Cluster 2 mean (min.–max.)	Cluster 3 mean (min.–max.)	Cluster 4 mean (min.–max.)
Peak 6*	0.559 (0.313–0.698)	0.213 (0.000–0.542)	0.056 (0.000–0.138)	0.624 (0.341–1.096)
Peak 7*	1.099 (0.548–1.527)	1.148 (0.308–2.243)	0.346 (0.000–0.985)	0.691 (0.000–1.489)
Peak 8*	1.374 (0.542–1.892)	0.544 (0.000–2.148)	0.782 (0.029–3.148)	2.210 (1.146–4.270)
Apigenin glicoside	10.459 (7.968–15.401)	5.960 (3.341–8.444)	2.603 (1.100–5.286)	9.990 (7.887–13.525)
Peak 10**	2.237 (0.994–6.460)	1.724 (0.609–8.226)	4.295 (0.547–11.708)	2.321 (1.210–5.722)
Peak 11**	3.245 (0.890–4.844)	0.981 (0.205–2.165)	0.593 (0.142–1.143)	2.245 (1.370–3.501)
Peak 12**	0.541 (0.000–3.243)	0.365 (0.000–1.313)	0.085 (0.000–0.466)	0.000
Rutin	0.000	0.068 (0.000–1.015)	0.413 (0.000–2.264)	5.381 (3.800–8.943)
Peak 14**	0.558 (0.315–1.024)	0.350 (0.029–0.899)	0.316 (0.000–0.963)	0.712 (0.597–0.976)
Peak 15***	0.690 (0.041–2.337)	0.119 (0.000–0.378)	0.081 (0.010–0.213)	0.000
Quercitrin	0.000	0.000	0.000	0.446 (0.175–0.693)
Quercetin	3.351 (0.204–5.903)	1.384 (0.031–9.269)	1.560 (0.007–7.666)	0.669 (0.412–1.230)

* Unidentified phenolic acid
** Unidentified flavone
*** Unidentified phenolic compound

Complete discrimination (Wilks statistics, P < 0.0001) of the four PCA clusters was achieved with all the *Semele* genotypes correctly grouped. Obtained F values show that all clusters are well separated with Clusters 1 and 3 the most closely-related groups and Clusters 2 and 3 more distantly related (Table 4). The results show that Peaks 8 and 10 are the best discriminators for separation of Cluster 1 from 3, with Mahalanobis distances of 16.81 and 14.01, respectively. Peak 7 is the best discriminator to separate Clusters 2 from 4, with a Mahalanobis distance of 2.34.

The distribution of the more discriminating phenolics between *Semele* clusters was examined in detail. The average relative contents of major phenolic compounds used for *Semele* cluster discrimination are shown in Figure 4 and Table 2. The clusters are generally characterised by the absence of quercitrin and rutin for Cluster 1, and the practical absence of quercitrin in Clusters 2 and 3. At the same time, these clusters can be distinguished by the increase of rutin in Clusters 3 and 4, and the absence of Peaks 12 and 15 in Cluster 4. The variation in identified flavonols and apigenin derivatives represented by Peaks 7, 8 and 10 appears to provide the clearest discriminators that define *S. androgyna* clusters. In spite of that, the average Peak 9 to Peak 10 ratios shows that Cluster 3 is clearly distinct from the others (Table 5).

The chromatographic analysis of *S. androgyna* s.s. phylloclade samples from 35 plants shows that this species has a diverse phenolic metabolism, with the production of 17 compounds belonging to three phenolic classes. These results seem to agree with existing data showing that the genus *Danae*, a taxon closely related to

Table 3: Contribution of principal phenolic compounds to Regression Factors 1 and 3 (Component Score 1 and 3), Principal Components Analysis and the separation of *Semele androgyna* accessions into clusters

Variables	Component Score 1	Component Score 3
Peak 6	0.004	0.002
Peak 7	0.029	–0.029
Peak 8	–0.005	0.092
Peak 9	0.907	–0.027
Peak 10	–0.320	0.238
Peak 11	0.101	–0.044
Peak 12	0.016	–0.030
Peak 13	–0.137	0.801
Peak 14	0.001	0.003
Peak 15	0.009	–0.020
Peak 16	–0.001	0.005
Peak 17	0.119	–0.501

Table 4: F values of Discriminating Analysis obtained during the pairwise group comparison between clusters

	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Cluster 1	–	24.82	14.19	41.21
Cluster 2	24.82	–	49.44	35.18
Cluster 3	14.19	49.44	–	40.13
Cluster 4	41.21	35.18	40.13	–

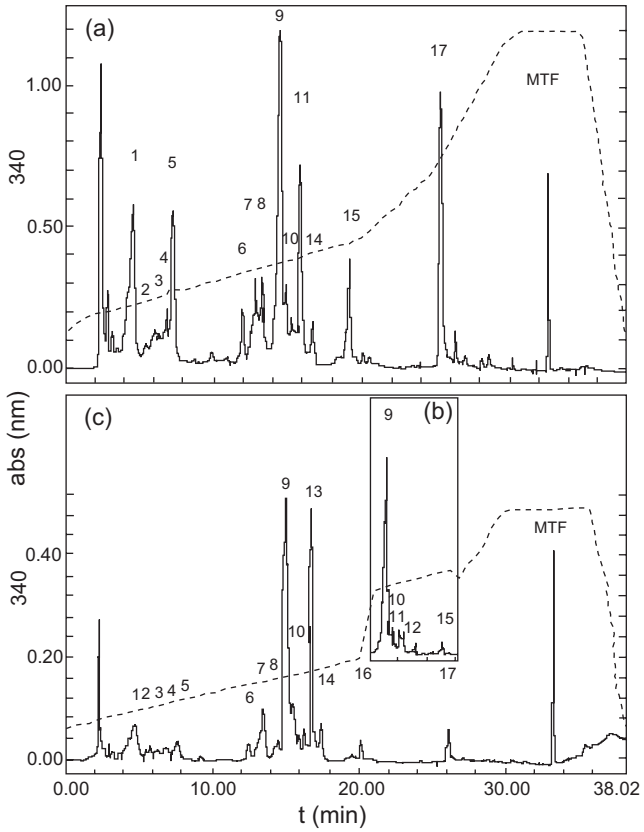

Figure 1: *Semele* phylloclade phenolic profiles. The profiles were obtained from sample QV-p (1a), JBM2 (1b) and MAC2 (1c). Peak 13 corresponds to rutin (1C), Peak 16 was identified as quercitrin (1c) and Peak 17 was identified as quercetin (1a). MTF = methoxyflavone (internal standard)

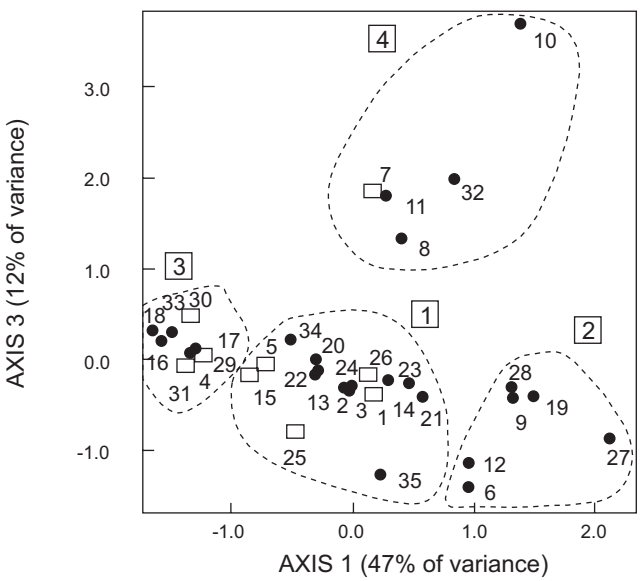

Figure 2: Phenolic polymorphism in *S. androgyna*. Principal Components Analysis of 35 *Semele* samples indicates four clusters. Scores for Regression Factor 1 and Regression Factor 3 are based on relative phenolic contents. Case numbers correspond to those provided in Table 1: • = *S. androgyna* (Costa 1950) and □ = *S. pterygophora* (Costa 1950)

Semele, produces the flavonols quercetin and kaempferol (Watson and Dallwitz 1992). However, according to these authors, flavonols are absent in the remaining genera of *Ruscaceae*, namely *Ruscus* and *Semele* (Watson and Dallwitz 1992). Gonzalez (1976) also reported the absence of flavonols in *Ruscus*. Here we report the widespread occurrence of flavonols in *S. androgyna* s.s. from Madeira and the identification of rutin, quercitrin and quercetin. Among the identified *Semele* phenols, the compound detected in highest relative amounts is an apigenin glycoside (Table 2, Figure 3).

Variation in the occurrence of apigenin glycoside, quercetin and its rutin and quercitrin glycosides, as well as other phenolic compounds, reveals the existence of a biochemical differentiation in *S. androgyna* phenolic metabolism. At the same time, we can exclude the influence of light exposure on production of these phenolics, which was reported previously (Olsson *et al*. 1999). The striking morphological differentiation (large secondary branches and phylloclades, higher floration patterns and composed phylloclades) which, according to Costa (1949, 1950), distinguishes *S. pterygophora* from *S. androgyna sensu lato*, is not supported by any differentiation in phenolic metabolism.

Table 5: Average Peak 9/Peak 10 ratio in the identified *Semele* clusters

Identified *Semele* clusters	Average Peak 9/Peak 10 ratio
Cluster 1	6.27±2.60
Cluster 2	5.56±2.51
Cluster 3	0.54±0.13
Cluster 4	5.61±2.02

The PCA analysis explaining 89.5% of phenolic polymorphism shows the presence of four clusters, differentiated by the presence or absence of rutin and quercitrin, and/or the ratio between Peak 9 and Peak 10 (Tables 2 and 5, Figure 2). The phenols and their glycosides reported here are commonly used for plant chemosystematic purposes (Lebreton *et al*. 1990, Zidorn and Stuppner 2001, Grayer *et al*. 2002, Frison-Norrie and Sporns 2002, Zidorn *et al*. 2002). Discriminant analysis based on Mahalanobis distance co-efficients supports the existence of several clusters within *S. androgyna* s.s. based on PCA analysis. Complete discrimination of the four clusters was achieved with all cases correctly grouped. Values of F or Mahalanobis distance for phenolic chemical distances are enough to consider that PCA clusters also define chemosystematic units. However, an assessment of their taxonomic significance remains to be undertaken. Kaundun *et al*. (1997) used a Mahalanobis distance of 1.94 to separate *Pinus halepensis* and *Pinus brutia*. However, they supported this conclusion with additional genetic data. In the absence of such supporting data, *Pinus eldarica* is not separate from *Pinus brutia* at the species level, even with a Mahalanobis distance of 3.16. For similar reasons, we cannot separate *S. androgyna* chemotypes (Figure 2), even at the sub-specific level. Firstly, there is no evidence that any of them are specifically associated with major morphological traits previously reported (Costa 1949, 1950). Secondly, we do not have additional genetic data. Our data suggest that *Semele* phenolic biochemistry is not related to plant epigenetic modification at the morphological level, at least for the characters currently considered to be the most relevant for taxonomic delimitation within this species (Figure 2). Presently, we can refute the hypothesis that *S. androgyna* chemotypes result from the influence of environmental conditions. No relationship between these and light exposure or growing condi-

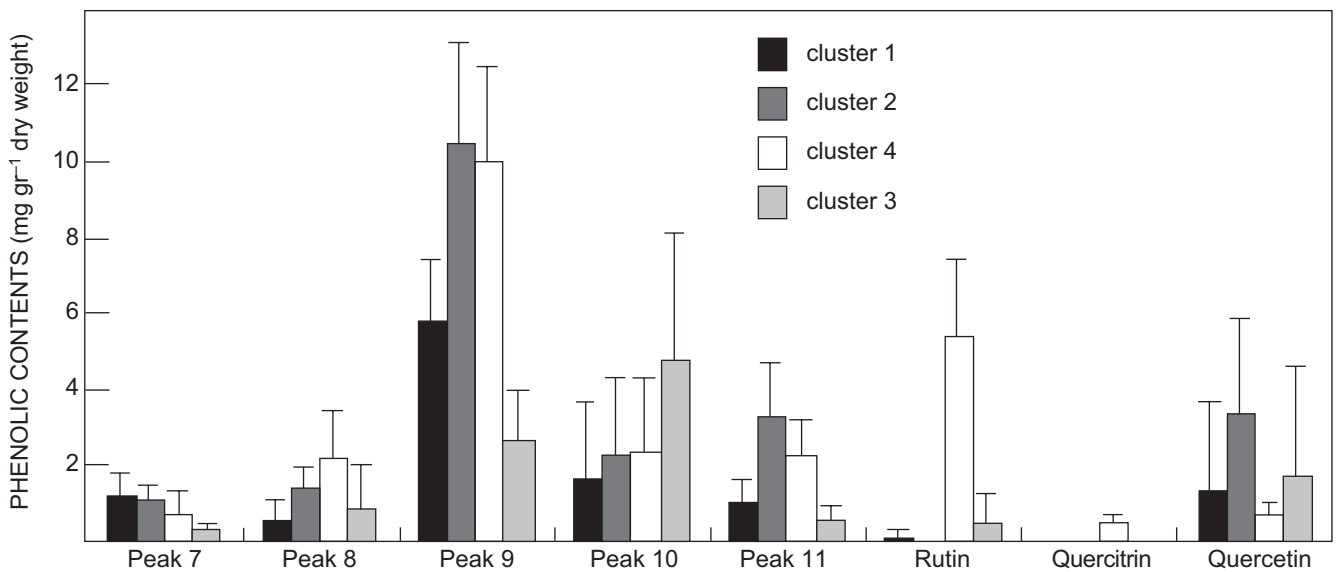

Figure 3: Phenolic composition in *S. androgyna* phylloclades. Means and standard deviations of the eight phenolic compounds in the *S. androgyna* chemotypes are shown (Cluster 1, n = 16; Cluster 2, n = 6; Cluster 3, n = 8; Cluster 4, n = 5). The relationship between phenolic abundances and discrimination of chemotypes by Principal Component Analysis and Discriminant Canonical Analysis is represented

tions is apparent, since the PCA clusters grouped plants with different light exposures or ecology (Figure 2).

During this work, samples of *Semele* were used to evaluate *S. androgyna* chemotype differentiation. Discriminant Analysis of outgroups (*Ruscus streptophyllus and S. maderensis*) and *S. androgyna* samples maintained the differentiation between chemotypes (Figure 4). The F values between *S. androgyna* chemotypes are similar to those previously reported. According to these values, Clusters 2 and 4 represent chemosystematic units significantly displaced from the new *S. maderensis*. Therefore, according to F values for phenolic chemical distances, Clusters 1 and 3 appear to be closer to outgroups. The phenolic compound analysis of new species of *Semele* suggests a similar basic metabolism, which is typical for *S. androgyna* Chemotype 1 reported here. However, they can be differentiated by simpler phenolic metabolism, and the absence of phenolic acids and the flavonols, rutin and quercitrin. In fact, the distinct phenolic profiles of these *S. maderensis* samples appear associated with specific morphological and reproductive traits described within the genus (Vale Lucas *et al*. 1998). Principal Component Analysis and DA both show that the identified *S. androgyna* chemotypes can be assigned at least a variety status. In the case of Chemotype 3, members of which possess a very distinct phenolic profile and Peak 9/Peak 10 ratio, this status may even be higher. However, this conclusion needs to be supported by additional traits, because our data also show that morphological divisions previously proposed for *S. androgyna* (Costa 1949, 1950) are not in agreement with the phenolic chemical distances.

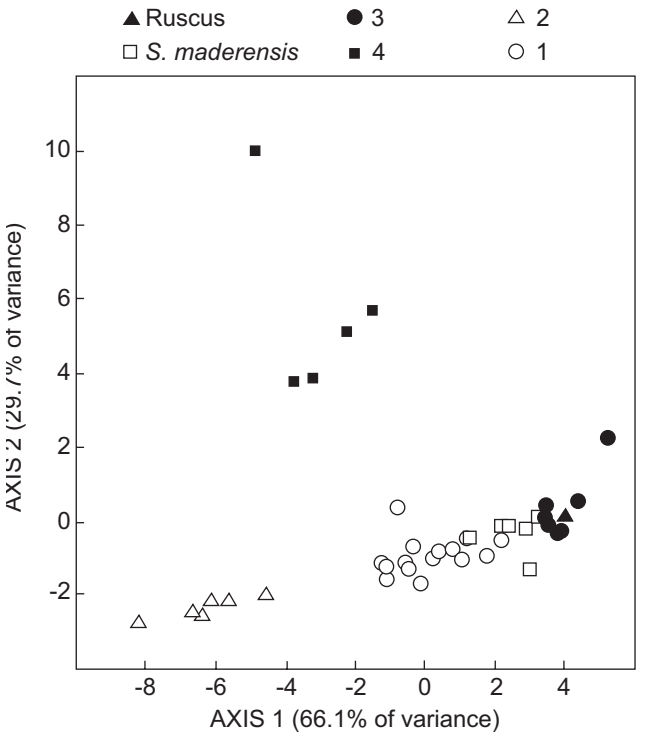

Figure 4: Discriminant Canonical Analysis of *Semele* and *Ruscus* samples. Both discriminant functions are based on the relative contents of phenolic compounds. Case numbers correspond to those provided in Table 1

Acknowledgements — The Portuguese Foundation for Science and Technology (FCT) funded this work through the Centre of Macaronesian Studies (CEM). The authors are grateful to the Madeiran Centre of Science and Technology (CITMA), the Berardo Foundation and European Social Funding for financial assistance given during execution of this work. The assistance rendered by Mr Rogério Correia during field collection is gratefully acknowledged.

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Edited by PD Hare