Phytochemical Profiles of *Targionia lorbeeriana*: Grown *in vitro* and in the Open

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The terpenoid constituents of *Targionia lorbeeriana* grown *in vivo* and *in vitro* were compared. The analysis of the dichloromethane extract was performed by HPLC-UV and by HPLC-MS. The obtained results revealed that the sesquiterpene lactones isolated from the dichloromethane extract of the wild *Targionia lorbeeriana* were also produced by the liverwort in *in vitro* cultures, in the same relative amounts.

The composition of essential oils was evaluated by GC and GC-MS. Both, the yield and diversity of the essential oil obtained from wild growing *T. lorbeeriana* gametophytes were higher than those growing *in vitro*. Although, a significant number of compounds produced *in vivo* were maintained *in vitro*, a considerable number of other ones were not detected. Instead, under *in vitro* conditions, some new compounds were found which do not accumulate under wild conditions.

Introduction

The genus *Targionia* (*Targionaceae*) contains three species (Smith, 1991). Asakawa *et al.* (1986) reported the isolation of two monoterpene acetates from *Targionia hypophylla* which comprise the characteristic fragrance of this plant. Recently we reported the isolation of new antifungal sesquiterpene lactones from the dichloromethane extract of wild *Targionia lorbeeriana* (Neves *et al.*, 1999). To our knowledge, no other reports on essential oils of species of this genus have been made.

As typical for liverworts, *Targionia lorbeeriana* grow mixed with other species and is strongly attached by their rhizoids to the soil. Therefore, it is a difficult and time-consuming procedure to obtain pure plant material for phytochemical studies. *In vitro* cultures of liverworts can be a good alternative to obtain biomass for this purpose since several studies with different species suggested that the ability of liverworts *in vitro* cultures to produce secondary metabolites, is similar to those found in field grown plants (Takeda and Katoh, 1981; Becker, 1990, 1994).

In order to compare the terpenoid production by *Targionia lorbeeriana* grown *in vitro* and in nature, the composition of dichloromethane extracts was compared by HPLC-UV and HPLC-MS. Moreover, volatile constituents were compared by analysis of liverwort hydrodistillates by GC and GC-MS.

Results and Discussion

In vitro cultures

The *in vitro* grown plantlets of *Targionia lorbeeriana* showed a quite different morphology relatively to those growing wild. They presented an agglomerate of little greenish thalloids that became dark with time, and developed a considerable amount of white rhizoids. However, its aroma is quite alike to that of wild gametophytes, suggesting a similarity in the composition of volatile constituents. Its *in vitro* growth was low, since the biomass doubling time was only about two months.

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Comparison of dichoromethane extracts of wild and in vitro cultures of *Targionia lorbeeriana*

The use of HPLC associated to mass spectroscopy allowed the identification of the sesquiterpene lactones **TL1-TL5** in the dichloromethane extract of *in vitro* cultures. The respective single ion traces [M+NH4]+ are shown in the Fig. 1. These lactones had already been isolated from the respective wild liverwort (Neves *et al.*, 1999). HPLC-UV chromatograms (Fig. 2) confirmed the similarity in the composition of the dichloromethane extracts of wild and *in vitro* growing *Targionia lorbeeriana,* notwithstanding some additional not identified compounds (**C, D, E, F**) were produced under *in vitro* conditions. The UV spectra of all corresponding compounds were similar (data not shown), confirming the presence of the same kind of compounds in the two extracts which do not absorb in the UV. Moreover, these analyses showed that dehydrocostus lactone (**TL1**) was the major constituent in both extracts, and that the other sesquiterpene lactones (**TL2-TL5**) are produced by the liverwort in similar amounts *in vitro* as in nature (Fig. 3).

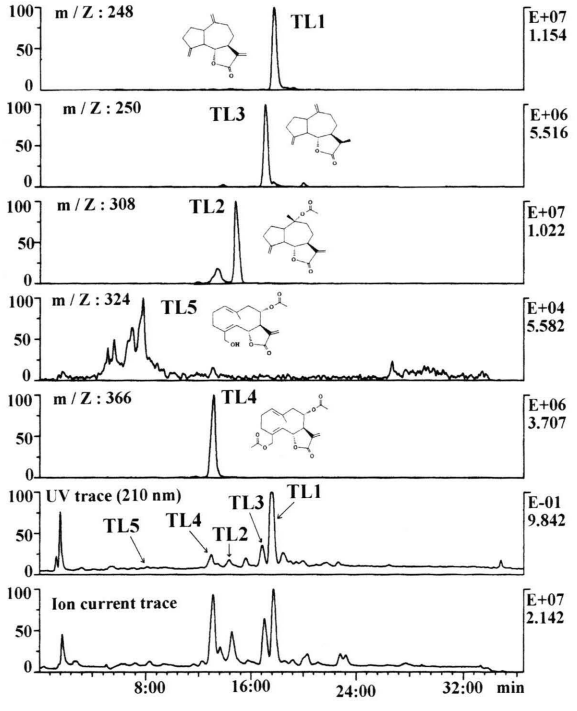

Fig. 1. TPS LC-MS analysis of the dichloromethane extract of *Targionia lorbeeriana* grown *in vitro*. Sample: 20 µl of a 10 mg/ml solution. TL1: Dehydrocostus lactone; TL2: Acetyltriflocuside; TL3: 11αH-Dihydrodehydrocostus lactone; TL4: 8,15-Acetylsalonitenolide and TL5: 8-Acethylsalonitenolide.

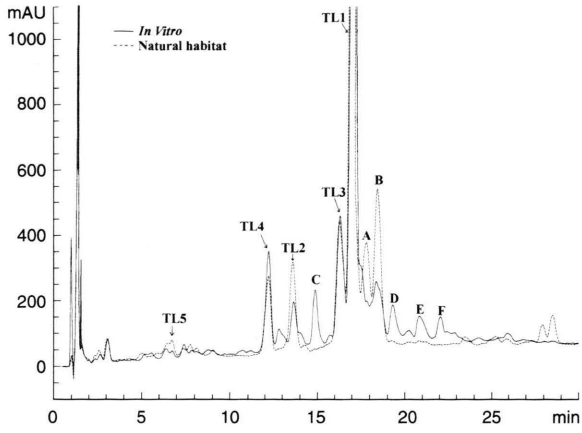

Fig. 2. HPLC/UV chromatograms comparison of the dichloromethane extracts of *Targionia lorbeeriana* grown *in vitro* and in nature. Samples: 10 µl of 10 mg/ml solutions.

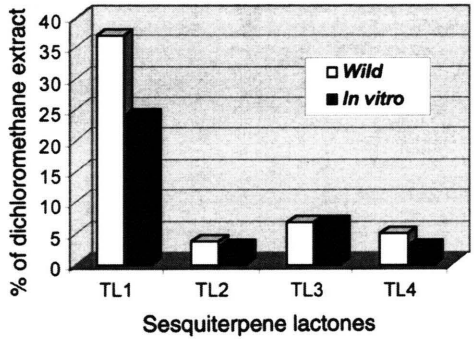

Fig. 3. Percentage composition of the sesquiterpene lactones **TL1-TL4** in the dichloromethane extracts of the *Targionia lorbeeriana* grown *in vitro* and in natural habitat. Compound TL5 was detected in too low amounts to be measured.

Essential oils from wild and in vitro growing Targionia lorbeeriana

The essential oils isolated by hydrodistillation from wild *T. lorbeeriana* consisted of a complex mixture of compounds, which include mainly monoterpene hydrocarbons, oxygen-containing monoterpenes, monoterpene acetates, sesquiterpene hydrocarbons, oxygen-containing sesquiterpenes, sesquiterpene acetates and diterpenes. From more

than sixty detected compounds, fifty-six were identified representing about 82% of the total oil. The remainder, ca. 18%, correspond to the sum of integrated peaks which include four unidentified compounds, detected in over – threshold percentages (> 0.05%), identified compounds present in trace amounts (< 0.05%), and a high number of under – threshold peaks taken by GC as signals independently of being unidentified compounds, artefacts or baseline noise.

Both the content and the composition diversity of the hydrodistilled fraction from the *in vitro* grown *T. lorbeeriana* cultures were lower than those of the wild-growing ones. From the thirty-six detected compounds in the *in vitro* liverwort, only twenty-seven were common to the wild ones (Table I). The monoterpenyl ester, identified as being *cis*-2-methylene-3-(1-methylethenyl)-cyclohexanylacetate (**20**), was the major constituent in both cases. The mass spectrum of this compound is similar to those of *cis*- and *trans*-pinocarveyl acetates (see Experimental), which were isolated from the ether-soluble fraction of a methanol extract of *T. hypophylla* by Asakawa *et al.* (1986). In order to search the presence of these compounds in *T. lorbeeriana* hydrodestillates, we synthesised and analysed *cis*- and *trans*-pinocarveyl acetates as described by Asakawa *et al.* (1986). We confirmed the presence of *cis*-pinocarveyl acetate (**18**) and the absence of the respective *trans*-isomer either in the wild or *in vitro* growing *T. lorbeeriana.*

In addition to **20**, major constituents of the essential oils produced by the wild growing gametophytes of *T. lorbeeriana* were by decreasing order, a phyllocladene isomer (**67**), β-selinene

Table I. Identified essential oil constituents of *Targionia lorbeeriana* growing wild and growing *in vitro.*

Number	Component	Retention index	Wild	*In vitro*
1	n-hexanal	808	–	1.2
2	**α-pinene**	936	0.3	tr
3	**sabinene**	974	2.8	tr
4	**β-Pinene**	979	4.0	2.2
5	myrcene	993	0.1	–
6	α-terpinene	1018	tr	–
7	***p*-Cymene**	1025	tr	tr
8	**limonene**	1029	tr	tr
9	**β-phellandrene**	1030	0.2	tr
10	**γ-terpinene**	1058	0.5	1.0
11	n-nonanal	1104	tr	–
12	**octen-1-ol acetate (a)**	1111	2.1	0.4
13	**4-terpineol**	1177	0.2	1.0
14	n-decanal	1202	0.1	–
15	2-decanol	1260	–	1.1
16	**bornyl or isobornyl acetate**	1285	0.1	0.4
17	***trans*-sabinyl acetate**	1300	0.1	0.4
18	***cis*-pinocarveyl acetate**	1331	0.9	0.9
19	δ-elemene	1338	0.3	–
20	***cis*-2-methylene-3-(1-methylethenyl)-cyclohexanyl acetate (a)**	1376	23.2	16.0
21	β-bourbonene	1385	0.5	–
22	**β-elemene**	1392	2.1	0.5
23	**n-tetradecane**	1400	0.6	1.3
24	β-cedrene	1418	0.4	–
25	β-gurjunene	1428	0.5	–
26	*trans*-α-bergamotene	1435	tr	–
27	**β-humulene**	1441	3.4	1.5
28	*cis*-β-farnesene	1452	0.2	–
29	germacrene D isomer # 1	1460	0.4	–
30	*allo*-aromadendrene	1463	0.4	–
31	α-acoradiene	1466	0.6	–
32	β-chamigrene	1474	0.8	–
33	germacrene D	1480	2.1	–
34	β-selinene	1485	4.1	–
35	valencene	1494	??	–
36	viridiflorene	1496	??	–
37	**α-selinene**	1497	??	??
38	α-longipinene	1499	??	–
39 + 40	**α-muurolene + pentadecane**	1500	??	0.
41	β-bisabolene	1508	0.1	–
42	*cis*-γ-bisabolene	1514	0.5	–
43	*trans*-β-farnesene and/or β-sesquiphellandrene	1523	0.6	–
44	*trans*-γ-bisabolene	1532	0.3	–
45	unknown	1550	–	0.9
46	spathulenol (?)	1568	2.0	–
47	**n-hexadecane**	1600	0.8	1.5
48	unknown	1613	–	1.3
49	cubenol (?)	1620	0.5	–
50	**β-eudesmol**	1639	1.9	2.5
51	unknown	1646	2.4	–
52	**α-cadinol**	1655	1.0	2.4
53	unknown	1671	–	1.0
54	$C_{15}H_{22}O$	1680	–	1.7
55	8-cedrane-13-ol	1687	0.4	–
56	*trans*-α-bergamotol	1690	??	–
57	**$C_{15}H_{22}O$**	1720	1.0	4.0
58	unknown	1740	0.9	–
59	*cis*-lanceol	1762	0.6	–
60	*trans*-α-bergamatol acetate	1793	0.8	–
61	unknown	1831	–	1.6
62	*trans-trans*-farnesyl acetate	1842	??	–
63	unknown	1848	–	4.5
64	*cis*-lanceol acetate	1864	0.4	–
65	**isophyllocladene (?)**	1952	0.4	1.1
66	**$C_{20}H_{32}O$ (manoyloxide ?)**	1967	tr	10.2
67	**phyllocladene isomer # 1 (?)**	1990	14.6	6.3
68	phyllocladene	2002	1.1	tr
69	phyllocladene isomer # 2 (?)	2049	0.3	–
70	unknown	2070	0.2	1.1

* Relative to *n*-alkanes on DB-5 column; tr = trace (< 0.05%); (a) Identification based in mass spectra only; (?) doubtful. Compounds typed in bold were identified in both extracts.

(**34**), β-pinene (**4**), sabinene (**3**), β-elemene (**22**), octen-1-ol acetate (**12**), germacrene D (**33**), spathulenol (**46**) and β-eudesmol (**50**).

Referring to essential oils produced in *in vitro*, in addition to compound **20**, major constituents were the diterpene compounds (**66**) and (**67**). Contrary to the compounds (**20**) and (**67**), which were also the major compounds in the essential oils from wild *T. lorbeeriana*, the oxygen-containing diterpene (**66**) was detected only in *in vitro* cultures. Other compounds detected in greater amounts were, the sesquiterpenes β-eudesmol (**50**) and α-cadinol (**52**), the monoterpene β-pinene (**4**) and the alkanes n-hexadecane (**47**), n-tetradecane (**23**) and the aldehyde n-hexanal (**1**), respectively.

The percentage contents of the compound groups identified in essential oils produced by wild and by *in vitro T. lorbeeriana* gametophytes is documented in Fig. 4.

Both wild and *in vitro* growing liverwort accumulated mostly monoterpenyl acetates, whereas other oxygen-containing monoterpenes and monoterpenes hydrocarbons were produced in minor amounts. Wild *T. lorbeeriana* accumulated monoterpene acetates as well as monoterpene hydrocarbons in percentages higher than those growing *in vitro*. However, differences between the two cultures, were higher in the production of sesquiterpene and diterpene compounds. *In vitro* gametophytes accumulated sesquiterpene hydrocarbons and sesquiterpenyl acetates in percentages 9 times lower and 5 times higher, respectively, when compared with those growing wild. The percentage of other total oxygen-containing sesquiterpenes were similar in both cases. The percentage of total diterpene hydrocarbons accumulated by wild gametophytes more than doubled than found in the *in-vitro* ones. In compensation, *in vitro* cultures produced an oxygen-containing diterpene, tentatively identified as manoyloxide, which accounts for more than 10% of the total hydrodistilled fraction. The percentages of other non-terpenic compounds, namely n-alkanes, in *in vitro* growing gametophytes was about 3 times higher than that of wild ones (Fig. 4).

Table II. Relative contents (%) of grouped compounds isolated from essential oils of *T. lorbeeriana*.

Compound groups		Percentage of grouped compounds	
		Wild gametophytes	*In vitro* gametophytes
Monoterpene hydrocarbons	MH*	7.9	3.2
Oxygen-containing monoterpene	MO	0.2	1.0
Monoterpenyl acetates	MA	24.3	17.7
Sesquiterpene hydrocarbons	SH	> 17.3	> 2.0
Oxygen-containing sesquiterpenes	SO	> 10.7	10.8
Sesquiterpenyl acetates	SA	> 1.2	6.1
Diterpene hydrocarbons	DH	16.4	7.4
Oxygen-containing diterpenes	DO	*tr*	10.2
Other	O	3.9	11.6

* For abbreviations see legend of Fig. 4.

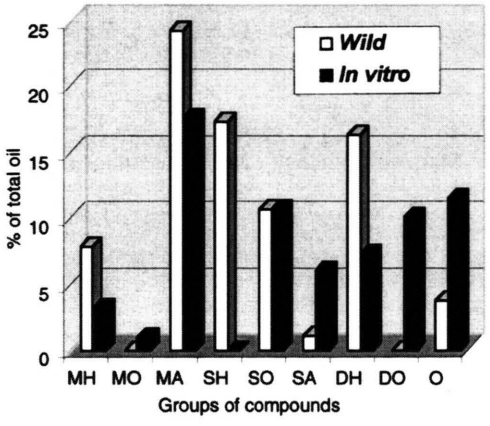

Fig. 4. Percentage composition of identified compounds from essential oils produced by *Targionia lorbeeriana* grown *in vitro* and in natural habitat.
MH- Monoterpene hydrocarbon
MA- Monoterpenyl acetates
SO- Oxygen-containing sesquiterpenes
DH- Diterpene hydrocarbons
O- Others
MO- Oxygen- containing monoterpenes
SH- Sesquiterpene hydrocarbons
SA- Sesquiterpenyl acetates
DO- Oxygen-containing diterpenes

α-Pinene, β-pinene, limonene and β-phellandrene are monoterpenes detected in both *Targionia lorbeeriana* and *Targionia hypophylla*, since Asakawa *et al.* (1986) reported the presence of these compounds in the ether-soluble portion of a methanol extract of *Targionia hypophylla*. However, although *cis*-pinocarveyl acetate (**18**) is present in *T. lorbeeriana* and *T. hypophylla trans*-pinocarveyl acetate and the sesquiterpene alcohol drimenol, were not identified in the essential oils of *Targionia lorbeeriana*, which, according to Asakawa *et al.* (1986) the latter ones, comprise the characteristic fragrance of *Targionia hypophylla*. This finding can explain the differences between the

characteristic fragances of *T. lorbeeriana* and *T. hypophylla* reported by Sérgio and Lopes (1972).

Experimental

Plant material

Targionia lorbeeriana was collected in February 1994 near Águeda, Portugal and identified by Professor Dr. R. Mues as was described by Neves *et al.* (1999).

In vitro cultures of *Targionia lorbeeriana* were initiated on solid modified B5 growth medium with 20 g.l-1 (Gamborg *et al.*, 1968) using gametophytes sterilised with Na-hypochlorite 2% during 5 min., as primary explants. The cultures were then maintained in the same growth medium in a photoperiod of 16 h light (30 μmol.m-2.s-1) at 22 °C.

Essential oil isolation

Essential oils were isolated by hydrodistillation during 3 hours from 5.2 g of fresh plant material in 200 ml of ultra pure water using a Clevenger-apparatus and recovered in 1 ml of *n*-hexane.

Synthesis of *cis*- and *trans*-pinocarveyl acetates

These compounds were prepared from commercially *trans*-pinocarveol as described by Asakawa *et al.* (1986).

Spectral data: MS *m/z* (rel.int.) *cis*-pinocarveyl acetate: 134 (27), 119 (63), 108 (20), 93 (81), 92 (44), 91 (78), 79 (47), 43 (100); *trans*-pinocarveyl acetate: 134 (10), 119 (20), 105 (6), 93 (43), 92 (21), 91 (478), 79 (17), 43 (100).

Gas chromatography- mass spectrometry

The GC unit consisted of a Perkin Elmer 8500 Gas Chromatograph connected to a MS ITD Finnigan Mat operating in EI mode. The chromatograph was fitted with a 30 m × 0.25 mm i.d., film thickness 0.25 μm, DB-5 fused-silica column; carrier gas: He; Transfer line temperature: 260oC; ion trap temperature: 220 °C; ionization energy: 70 eV; temperature programme: 60 °C to 240 °C at 3 °C/min.

HPLC/UV and HPLC/MS

Analytical HPLC/UV Novapak RP-18 column (5 μm, 150 × 3.9 mm i.d.); MeCN-H2O (+ 0.05% TFA) (25:75 → 75:25 in 30 min; 75 to 100 in 1 min; 1 ml/min).

For the HPLC/UV-MS analyses, a Waters 600 MS delivery system was used. The pump was connected to a Hewlett-Packard 1050 DAD detector and to the TSQ 700 mass spectrometer (Finnigan MAT), equipped with a TPS-2 thermospray interface. An aqueous solution of ammonium acetate was added post column. A frequency of 30 scans/min was used.

Asakawa Y., Toyota M. and Cheminat A. (1986), Terpenoids from the French liverwort *Targionia hypophylla*. Phytochemistry **25**, 2555.

Becker H. (1990), Secondary metabolites from *in vitro* cultures of bryophytes. Chapter 22. In: Bryophytes: Their Chemistry and Chemical Taxonomy, H. D. Zinsmeister and R. Mues (Ed.), Oxford Science Publ., New York, p. 339.

Becker H. (1994) Secondary metabolites from bryophytes *in vitro* cultures. J. Hattori Botan. Lab. **76**, 283.

Gamborg O. L., Miller R. A. and Ojima K. (1968), Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. **50**, 151.

Neves M., Morais R., Gafner S., Stoeckli-Evans H., and Hostettmann K. (1999), New sesquiterpene lactones from the Portuguese liverwort *Targionia lorbeeriana*. Phytochemistry **50**, 967.

Sérgio C. and Lopes A. Q. (1972), O género *Targionia* Mich. em Portugal. Boletim da Sociedade Portuguesa de Ciências Naturais, Lisbon, **14**, 87.

Smith, A. J. E. (1991), The Liverworts of Britain and Ireland. Cambridge University Press, Cambridge, p. 312.

Takeda R. and Katoh K. (1981), Growth and sesquiterpenoid production by *Calypogeia granulata* Inoue cells in suspension culture. Planta **151**, 525.