In vitro Cultures of *Cyclopia* Plants (Honeybush) as a Source of Bioactive Xanthones and Flavanones

Adam Kokotkiewicz[a], Malgorzata Wnuk[b], Adam Bucinski[b], and Maria Luczkiewicz[a,*]

[a] The Chair and Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Gdansk, al. gen. J. Hallera 107, 80-416 Gdańsk, Poland. Fax: +4 85 83 49 31 60. E-mail: mlucz@amg.gda.pl

[b] The Chair and Department of Biopharmacy, Faculty of Pharmacy, Ludwig Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Torun, ul. M. Sklodowskiej-Curie 9, 85-094 Bydgoszcz, Poland

* Author for correspondence and reprint requests

In vitro shoot and callus cultures of the endemic South-African shrubs: *Cyclopia intermedia* E. Mey., *Cyclopia subternata* Vogel, and *Cyclopia genistoides* (L.) Vent. (Fabaceae) were established and examined for the presence of polyphenolic compounds. The xanthones mangiferin and isomangiferin, as well as the flavanones hesperidin and eriocitrin were identified by LC-ESI-MS and LC-DAD, and analyzed quantitatively by HPLC. The respective intact plants were analyzed for comparison. From all *in vitro* cultures, the highest levels of mangiferin (1.55%) and isomangiferin (0.56%) were recorded in *C. subternata* microshoots, compared to 1.31% and 0.49% found in the intact plant. Callus cultures of all species synthesized only trace amounts of mangiferin and isomangiferin. Hesperidin and eriocitrin contents were significantly lower in all *in vitro* cultures, in comparison to the respective intact plants. Among the obtained *in vitro* biomasses, the highest hesperidin content was recorded in *C. intermedia* (0.9%) and *C. subternata* (0.87%) microshoots, whereas *C. subternata* callus was characterized by the best growth parameters and highest hesperidin content (0.69%) from all examined *Cyclopia* calli.

Key words: Honeybush, Plant *in vitro* Culture, LC-ESI-MS Analysis

Introduction

The genus *Cyclopia* (Fabaceae family, Podalyrieae tribe) comprises over 20 species of shrubs, endemic to the Cape Floristic Region of South Africa (Joubert *et al.*, 2008a; Kokotkiewicz and Luczkiewicz, 2009). *Cyclopia* plants are characterized by trifoliate leaves and yellow, sweet-scented flowers. The distribution of some species, due to their peculiar environmental requirements, is highly limited, for example to hilltops or marshy areas (Kokotkiewicz and Luczkiewicz, 2009).

Since the early 19th century, the herb of a number of *Cyclopia* species has been used to produce honeybush herbal tea, traditionally obtained by the fermentation process, but more recently available also in an unfermented version (the so called "green honeybush") (Joubert *et al.*, 2008a, b; Kokotkiewicz and Luczkiewicz, 2009). The commercially most exploited species include *C. intermedia* E. Mey., *C. subternata* Vogel, *C. genistoides* (L.) Vent., and *C. sessiliflora* Eckl. & Zeyh. Honeybush infusions have a distinctive sweet, honey-like flavour, low tannin content, and are coffeine-free. In recent years, they are gaining popularity as a beverage for everyday use and a substitute for tea. Significant amounts of plant material are also exported (Joubert *et al.*, 2008a; Kokotkiewicz and Luczkiewicz, 2009).

Apart from a characteristic honey-like taste and aroma, honeybush extracts may exert numerous health-promoting activities. Research showed

---

Abbreviations: 4-CPPU, *N*-(2-chloro-4-pyridyl)-*N*'-phenylurea; 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, 6-(γ,γ-dimethylallylamino)purine; DW, dry weight; Gi, growth index; HPLC, high-performance liquid chromatography; IBA, indole-3-butyric acid; KN, kinetin; LC-DAD, liquid chromatography-diode array detection; LC-ESI-MS, liquid chromatography-electrospray ionization-mass spectrometry; MS, Murashige and Skoog; NAA, α-naphthaleneacetic acid; PVP, polyvinylpyrrolidone; SH, Schenk and Hildebrandt; TDZ, thidiazuron; 2,4,5-T, 2,4,5-trichlorophenoxyacetic acid.

that extracts obtained from *Cyclopia* plants have substantial antioxidant and antimutagenic potential demonstrated in various *in vitro*, *ex vivo* and *in vivo* models, and thus may be potentially beneficial in cancer prevention. Moreover, some honeybush extracts exhibit significant phytoestrogenic activity (Joubert *et al.*, 2008a; Kokotkiewicz and Luczkiewicz, 2009).

The main chemical components of *Cyclopia* plants are polyphenols, represented by xanthones, flavanones, flavones, flavonols, isoflavones and coumestans (Joubert *et al.*, 2008a; Kokotkiewicz and Luczkiewicz, 2009). The most important compounds, present in highest amount, include the xanthones mangiferin and isomangiferin, as well as the flavanone hesperidin (Joubert *et al.*, 2003, 2008b).

The aim of the present study was to establish, for the first time, *in vitro* cultures of three *Cyclopia* species (*C. subternata*, *C. intermedia*, and *C. genistoides*) and to evaluate their potential to produce xanthones and flavanones. The reason for this is the endemic character of *Cyclopia* together with its chemical uniqueness, manifested by the presence of the xanthones mangiferin and isomangiferin, which are rarely recorded in the Fabaceae family (De Nysschen *et al.*, 1996). Until now, *in vitro* cultures of legume plants have been extensively studied in terms of isoflavone production (Dixon, 1999), whereas little is known about the xanthone metabolism within the Fabaceae family. At present, most experiments concerning the xanthone accumulation in plant cell cultures are conducted with the use of plants from the genera *Hypericum* (Clusiaceae) and *Centaurium* (Gentianaceae) (Dias *et al.*, 2000, 2001; Pasqua *et al.*, 2003; Mulinacci *et al.*, 2008). Previous *in vitro* experiments concerning *Genista* plants (Fabaceae) showed that the isoflavonoid content in the plant biomass can be substantially elevated by establishing *in vitro* cultures, and further selection of the medium and culture type (Luczkiewicz and Glod, 2003, 2005). Due to positive results (in terms of the bioflavonoid content) observed in the case of *Genista* cell lines, it was decided to examine *in vitro* cultures of legume plants representing a non-standard, xanthone-oriented secondary metabolism, namely those of *Cyclopia*, for the presence of polyphenolic derivatives, and to compare the results with those of the respective intact plants.

Material and Methods

Seeds and intact plant material

Seeds of *C. intermedia*, *C. subternata*, and *C. genistoides* were obtained from Silverhill Seeds, Kenilworth, Cape Town, South Africa. Herbs of the respective intact plants were obtained from Cape Honeybush Tea, Mossel Bay, South Africa.

In vitro cultures

General procedures

All *Cyclopia* cultures were cultivated on Schenk and Hildebrandt (SH) (Schenk and Hildebrandt, 1972) and Murashige and Skoog (MS) (Murashige and Skoog, 1962) media supplemented with growth regulators (Table I) and 3.0% w/v sucrose, and solidified with 0.6% w/v (SH medium) or 0.7% w/v (MS medium) of agar. The pH of the media was adjusted to 5.80 prior to autoclaving (0.1 MPa, 121 °C, 20 min). The cultures were maintained in baby food jars (Sigma-Aldrich, USA) in a growth chamber at (24 ± 1) °C under continuous light [Phillips white fluorescent lamps, 36 W, (88 ± 8) μmol m^{-2} s^{-1} light intensity].

Germination

Prior to sterilization, the seeds were scarified with fine (P120) sand paper. Next, they were surface-sterilized in 70% ethanol for 1 min, followed by 0.5% NaOCl for 30 min. After sterilization, the seeds were rinsed three times with sterile, double distilled water, and put into Petri dishes lined with wet filter paper. The seeds were germinated in the dark at (24 ± 1) °C. After germination, the seeds were moved to a growth chamber for 10 d and the obtained seedlings were then used in further experiments. Germination rates and time of germination were determined by repeating the experiment twice.

Callus cultures

For callus initiation, cotyledone, hypocotyl and root fragments of *Cyclopia* seedlings (0.5–1.0 cm) were placed on solid SH and MS media supplemented with growth regulators alone [0.5 mg/l kinetin (KN) and 5.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D)] or together with antioxidants: 100.0 mg/l L-cysteine or 1000.0 mg/l polyvinylpyrrolidone (PVP) (6 media modifications). The cultures were subcultured 6 times onto fresh media

with the same growth regulators and antioxidants every 4 weeks.

After 6 passages, the initial calli were transferred onto solid SH and MS media supplemented with various growth regulators (Table I) and grown for 6 months (6 subsequent passages), in order to determine the conditions for continuous callus growth. After that period, the *in vitro* biomasses were evaluated for growth, structure and morphological tendencies (Table I). The callus growth indices (Gi) were evaluated after 30 d using the Klein formula: $Gi = [(Fw – Fo)/Fo]·100$, where Fw is the fresh weight after 30 d and Fo is the fresh weight of the inoculum (Zenkteler, 1984). Polyphenolic compounds concentrations were determined by HPLC for the calli representing the best growth parameters.

Microshoot cultures

For microshoot culture initiation, cotyledone and shoot tip fragments (0.5–1.0 cm) of *Cyclopia* seedlings were placed on solid SH medium supplemented with growth regulators alone [2.0 mg/l 6-(γ,γ-dimethylallylamino)purine (2iP) and 0.22 mg/l thidiazuron (TDZ)] or together with antioxidants: 100.0 mg/l L-cysteine or 1000.0 mg/l PVP. The cultures were subcultured 6 times onto fresh media with the same growth regulators and antioxidants every 4 weeks.

After 6 passages, the initial microshoot cultures were transferred onto solid SH medium supplemented with the same growth regulators, but without antioxidants, and cultivated for 6 months (6 subsequent passages), in order to obtain stable microshoot cultures (Table I). After that period, the microshoot growth indices were evaluated in a 30-d experiment using the Klein formula (Zenkteler, 1984), and polyphenolic compounds concentrations were determined by HPLC.

Root cultures

Root fragments (2.0–3.0 cm) of *Cyclopia* seedlings were placed on solid SH and MS media without growth regulators or supplemented with 1.0 mg/l indole-3-butyric acid (IBA) or 5.0 mg/l α-naphthaleneacetic acid (NAA). The cultures were transferred onto fresh media with the same growth regulator concentrations every 4 weeks.

Preparation of extracts

1000.0-mg portions of pulverized, freeze-dried plant materials (herbs of the intact plants and *in vitro* biomasses) were extracted with two portions of methanol (2 × 100 ml, 2 × 3.0 h, 40 °C) and one portion of 90% aqueous methanol (100 ml, 3.0 h, 40 °C) with the use of a hotplate magnetic stirrer. The methanolic extracts were concentrated under reduced pressure (40 °C). The dry residues were diluted in water and partioned into chloroform and aqueous fractions. The aqueous phases were then vacuum-concentrated (40 °C), and the resulting dry residues were dissolved in 10 ml of methanol (HPLC grade) and stored at –20 °C. Prior to HPLC analysis, the extracts were centrifuged (15 min, 4 °C) at $7500 \times g$.

In order to test the recovery of the analyzed compounds, *Cyclopia* plant matrices of known compound content were spiked with known amounts of xanthone and flavanone representatives, that is mangiferin (0.2, 1.0, 3.0% DW) and hesperidin (0.2, 1.0, 3.0% DW), prior to extraction. The determined recoveries were $(93.50 \pm 4.23)\%$ for mangiferin and $(92.06 \pm 3.6)\%$ for hesperidin.

Chemicals

All chemicals used for media preparation were from Sigma-Aldrich (USA). Solvents used for extract preparation were from POCH (Poland). Water used for HPLC analyses was from Baker (USA). Acetonitrile and formic acid were from Merck (Germany). Mangiferin and hesperidin standards were obtained from Sigma-Aldrich. Eriocitrin was from Extrasynthese (France). Isomangiferin was isolated from *C. intermedia* intact plant material and identified by spectroscopic methods (Kokotkiewicz *et al.*, 2006).

HPLC analysis

The qualitative and quantitative HPLC analyses were performed with the use of LC-ESI-MS and LC-DAD systems (Shimadzu, Kyoto, Japan) consisting of two solvent pumps LC-20AD, an autosampler SIL-20AC (15 °C), a diode array detector SPD-M20A, a mass spectrometry detector (ESI) 2010EV, a column oven CTO-20AC (30 °C), and a DGU-20A3 degasser. Chromatography was performed on a Supelcosil LC-18 (150 × 4.6 mm, 3 μm) column (Sigma-Aldrich). The mobile phase

consisted of A: water/formic acid (100:0.1 v/v), and B: water/acetonitrile/formic acid (50:50:0.1 v/v/v). The gradient elution applied was as follows: 0–15 min, 12–25% B; 15–75 min, 25–100% B; 75–77 min, 100% B; 77–80 min, 100–12% B; 82 min, stop. The flow rate was 0.6 ml/min, and the injection volume was 3 μl.

Peaks in chromatograms were identified by comparison of their retention time with those of standards, and additionally by LC-DAD and LC-ESI-MS. Mass spectrometric detection of the polyphenolic compounds was performed in the negative ion mode over the range *m/z* 50–1800. The following parameters of electrospray ionization were applied: desolvation temperature, 230 °C; nitrogen flow rate, 1.5 l/min; capillary voltage, 2000 V.

Quantification of the polyphenolic compounds, performed with the use of external standards, was based on the peak area at 280 nm. Peaks were integrated by LC-MS solution (ver. 3.40, Kyoto, Japan) software. Standard calibration curves

were plotted using dilution series of mangiferin (13–200 μg/ml), hesperidin (15–230 μg/ml) and eriocitrin (17–350 μg/ml). Isomangiferin was quantified using mangiferin as standard.

Results and Discussion

Establishment of Cyclopia in vitro cultures

As a result of biotechnological experiments, *in vitro* cultures of three *Cyclopia* species (*C. intermedia*, *C. subternata* and *C. genistoides*) have been established for the first time, and their productivity in terms of selected xanthones and flavanones has been evaluated.

In general, plants from the *Cyclopia* genus proved to be rather difficult to cultivate *in vitro*. The problem of poor germination rates was effectively overcome by an initial seed scarification, which has been previously reported to break the seed coat-imposed dormancy of *Cyclopia* seeds (Sutcliffe and Whitehead, 1994). The scarified seeds of all examined species germinated after 10

Table I. Influence of the medium type on the growth of *Cyclopia* sp. biomass.

Medium composition	*C. intermedia*		
	Colour	Structure	Gi[a]
SH + 5.0 mg/l (22.72 μM) 2,4-D + 0.5 mg/l (2.32 μM) KN	Green	Cauliflower-like callus	88.4 ± 27.0
SH + 5.0 mg/l (19.57 μM) 2,4,5-T + 0.5 mg/l (2.32 μM) KN	Yellowish-green	Cauliflower-like callus	76.5 ± 17.9
SH + 5.0 mg/l (20.19 μM) 4-CPPU + 0.5 mg/l (2.27 μM) 2,4-D	Yellowish-green	Slimy callus	98.6 ± 23.3
SH + 5.0 mg/l (20.19 μM) 4-CPPU + 0.5 mg/l (1.96 μM) 2,4,5-T	Green	Slimy callus	56.3 ± 14.5
SH + 2.0 mg/l (9.84 μM) 2iP + 0.22 mg/l (0.10 μM) TDZ	Green	Shoot primordia	450.6 ± 35.7
MS + 5.0 mg/l (22.72 μM) 2,4-D + 0.5 mg/l (2.32 μM) KN	Green	Slimy-parenchymatic callus	220.4 ± 12.3
MS + 5.0 mg/l (19.57 μM) 2,4,5-T + 0.5 mg/l (2.32 μM) KN	Bright green	Cauliflower-like callus	191.4 ± 21.6
MS + 5.0 mg/l (20.19 μM) 4-CPPU + 0.5 mg/l (2.27 μM) 2,4-D	Green	Cauliflower-like callus	250.5 ± 43.8
MS + 5.0 mg/l (20.19 μM) 4-CPPU + 0.5 mg/l (1.96 μM) 2,4,5-T	Bright green	Parenchymatic callus	481.0 ± 31.7

[a] Growth indices measured after 30 d of cultivation on solid media. The values represent the mean ± SD of 10 samples.

days with the following rates: 74% for *C. subternata* and *C. genistoides*, and 95% for *C. intermedia*.

In order to initiate callus cultures, *Cyclopia* seedling fragments were placed on the so-called rich media (SH and MS) supplemented with 2,4-D and KN. These media have been previously shown to be effective for *in vitro* cultivation of many legume plants, including shrubs from the *Genista* genus (Luczkiewicz and Glod, 2003). Hypocotyl fragments of all *Cyclopia* seedlings placed on SH and MS media supplemented with 2,4-D and KN formed cauliflower-like initial calli with strong necrotic tendencies, manifested by poor growth and intensive browning of the explants and the surrounding area. This phenomenon was probably caused by the formation of simple phenolic compounds, as a result of the stress-induced defense mechanism (Luczkiewicz, 2008). Cotyledone fragments of the examined species placed on the above media showed a significantly stronger necrotic tendency than hypocotyls, and failed to form calli. Supplementation of the growth media

with antioxidants (L-cysteine or PVP) proved to be ineffective against callus browning, but fortunately, this tendency declined autogenously after 4–6 passages. After 6 passages, the initial calli of all examined *Cyclopia* species were transferred on various SH and MS media and grown for subsequent 6 months. After that time, they were evaluated for consistency and growth parameters (Table I). Although the MS and SH media supplemented with 2,4-D and KN proved to be good for callus initiation, very low Gi values for all initial calli made them unsuitable for continuous cultivation of the biomasses (Table I). Significantly better results, in terms of callus growth and biomass stability, were obtained on MS and SH media with the addition of *N*-(2-chloro-4-pyridyl)-*N*'-phenylurea (4-CPPU) (Table I). In fact, this growth regulator was previously successfully used in the cultivation of friable-parenchymatic *G. tinctoria* callus (Luczkiewicz, 2008). The MS medium supplemented with 5.0 mg/l 4-CPPU and 0.5 mg/l 2,4,5-trichlorophenoxyacetic acid

C. subternata			*C. genistoides*		
Colour	Structure	Gi[a]	Colour	Structure	Gi[a]
Green	Cauliflower-like callus	160.4 ± 34.6	Yellowish-green	Cauliflower-like callus	94.3 ± 25.0
Yellowish-green	Cauliflower-like callus	120.5 ± 21.6	Green	Slimy callus	170.2 ± 32.5
Green	Slimy-parenchymatic callus	100.6 ± 16.4	Green	Cauliflower-like callus	252.0 ± 20.4
Green	Slimy callus	90.4 ± 30.4	Bright green	Cauliflower-like callus	160 ± 25.6
Green	Shoot primordia	520.4 ± 43.7	Green	Shoot primordia	704.6 ± 55.2
Bright green	Parenchymatic callus	120.6 ± 12.4	Greyish-green	Slimy callus	55.6 ± 12.6
Green	Slimy-parenchymatic callus	320.5 ± 45.0	Green	Slimy callus	67.8 ± 19.1
Yellowish-green	Parenchymatic callus	360.1 ± 15.6	Green	Cauliflower-like callus	110.5 ± 31.5
Bright yellow	Friable-parenchymatic callus	1269.9 ± 98.6	Green	Callus with shoot primordia	150.4 ± 42.1

(2,4,5-T) proved to be optimal for the cultivation of *C. intermedia* and *C. subternata* calli, in terms of morphology and growth indices, whereas *C. genistoides* callus was established on SH medium supplemented with 5.0 mg/l 4-CPPU and 0.5 mg/l 2,4-D (Table I). The attempts to obtain one optimal medium for continuous growth of *Cyclopia* biomasses were unsuccessful. *C. genistoides* callus clearly favoured the SH medium whereas *C. intermedia* and *C. subternata* biomasses exhibited the best growth parameters on the growth medium based on MS salts (Table I). These results are surprising, as numerous experiments involving several legume plants from one genus showed that *in vitro* growth requirements of closely related species are mostly similar, enabling the use of one medium (Luczkiewicz, 2008).

Contrarily to *Cyclopia* calli, microshoot cultures of the examined species had similar medium requirements. The SH medium supplemented with 2iP and TDZ (Table I), previously used for the establishment of microshoots of related *Genista* plants (Luczkiewicz and Piotrowski, 2005), was applied for *Cyclopia* shoot cultures initiation. Cotyledone and shoot tip fragments of *Cyclopia* seedlings placed on the above medium formed shoot primordia with substantial necrotic tendencies, manifested by excessive browning. A substantial number of abnormal leaves with the tendency to vitrify was observed too. As in the case of callus cultures, the addition of antioxidants (L-cysteine or PVP) was ineffective, but the tendency to browning vanished autogenously after 6 passages. The initial microshoot cultures were grown on the same medium, but without antioxidants, for subsequent 6 months. After that period, stable microshoot cultures were formed, without tendencies to form vitrified abnomal leaves.

Root explants from all *Cyclopia* seedlings showed necrotic changes, which could not be overcome by the addition of antioxidants, subsequent passages or specific media composition (growth regulators). As a result, all explants decayed after 3–4 passages and attempts to initiate root cultures were unsuccessful. This phenomenon can be explained by the weakened growth of root explants under sterile *in vitro* conditions, resulting from the lack of symbiotic *Rhizobium* strains which are often necessary the for proper development of a legume root system (Luczkiewicz, 2008).

Accumulation of xanthones and flavanones in *Cyclopia in vitro* cultures and intact plants

In vitro cultures of *Cyclopia* sp., which represented the best growth parameters, were analyzed by HPLC for the presence of compounds constituting the majority of the polyphenols found in intact plants, namely the xanthones mangiferin and isomangiferin, as well as the flavanones hesperidin and, to a lesser extent, eriocitrin. The previous research on the *Cyclopia* chemical composition reported the use of a C12 column, as the C18 column was shown to generate strong tailing of the analyzed compounds and failed to separate the xanthone isomers mangiferin and isomangiferin (Joubert *et al.*, 2003). Contrarily, our research proved, that the more popular C18 column, commonly used in polyphenol analysis (De Rijke *et al.*, 2006), together with gradient elution and the addition of an acidic modifier, can be successfully applied for the separation of xanthones and flavonoids found in *Cyclopia* intact plants and *in vitro* cultures. The above compounds were identified by comparison of their retention times with those of the standards (mangiferin, 22.09 min; isomangiferin, 23.01 min; eriocitrin, 29.55 min; hesperidin, 35.00 min), and additionally by the presence of respective pseudomolecular and fragmentary ions in LC-ESI-MS spectra: mangiferin and isomangiferin, m/z 421 [M-H]$^-$; hesperidin, m/z 609 [M-H]$^-$ and m/z 300 (aglycone); eriocitrin, m/z 595 [M-H]$^-$ and m/z 286 (aglycone).

Quantitative analyses of xanthones and flavanones in *Cyclopia* biomasses showed significant differences depending on the species and culture type. Generally, the concentrations of all examined compounds (exept form mangiferin and isomangiferin in the case of *C. subternata* shoots) were lower *in vitro* than in the respective intact plants, indicating the suppressed phenylpropanoid metabolism in most *Cyclopia* cell lines (Table II). Moreover, the xanthone biosynthesis in *Cyclopia* plants seems to be morphogenesis-dependent, as calli of all examined species produced only traces of mangiferin and isomangiferin (< 0.05% DW) in comparison to substantial amounts of these compounds found in microshoot cultures. Among these, *C. subternata in vitro* microshoots produced more mangiferin (1.55% DW) and isomangiferin (0.56% DW), in comparison to the intact plant material (1.31% and 0.49% DW, respectively – Table II). Interestingly, the previous research on

in vitro cultures of plants from the *Hypericum* and *Centaurea* genera proved the ability of undifferentiated cell lines to produce xanthones (Dias *et al.*, 2000; 2001; Pasqua *et al.*, 2003; Mulinacci *et al.*, 2008). In the case of *Hypericum* sp., xanthones were the main group of secondary metabolites found in undifferentiated cells, whereas only small amounts of these compounds were reported in developed shoots, producing mainly hypericins (Pasqua *et al.*, 2003; Mulinacci *et al.*, 2008). Our research on *Cyclopia in vitro* cultures proved the possible relation between xanthone biosynthesis and organogenesis level. The results also suggest, that this dependence is seemingly genus-related. In the case of *Hypericum* sp. intact plants, xanthones constitute only a small part of the secondary metabolite composition, but their content is significantly elevated in unorganized cell lines (Pasqua *et al.*, 2003). On the other hand, *Cyclopia* shrubs contain substantial amounts of xanthones, which are almost completely absent in callus cultures. Moreover, there are no significant differences of mangiferin and isomangiferin accumulation between calli representing various morphological tendencies (firm cauliflower-like for *C. genistoides*, parenchymatic for *C. intermedia* and friable-parenchymatic for *C. subternata*), suggesting that the production of the mentioned metabolites is only possible in fully developed *Cyclopia* shoots (Tables I and II). Additionally, there was no clear correlation between hesperidin content and morphogenesis level. While only traces of this metabolite could be detected in both microshoots and calli of *C. genistoides* (< 0.05% DW), substantial amouns of hesperidin were found in *C. subternata* shoots (0.87% DW), as well as its undifferentiated, parenchymatic calli (0.69% DW). Differently, only microshoots were able to produce hesperidin in the case of *C. intermedia* (0.9% DW) (Table II). Among the examined *in vitro* biomasses, eriocitrin was only present in *C. subternata* microshoots, although in lesser amount than in the respective intact plant (Table II). It seems, that the flavanone biosynthesis in *Cyclopia in vitro* cultures is more species-, than morphogenesis-dependent.

Summing up, the experiments proved that *in vitro* cultures of *Cyclopia* show a certain potential to produce biologically active xanthones and flavanones. As conclusion could be drawn that *Cyclopia* biomasses are particularly appropriate for further *in vitro* experiments for establishing plant systems used for xanthone and flavanone produc-

tion. The obtained cultures can also be used in research the mechanisms of xanthone and flavonoid biosynthesis.

Table II. Quantitative analysis of xanthones and flavanones in intact plants and *in vitro* cultures of *Cyclopia* sp.[a].

Species	Material	Medium composition	Mangiferin	Isomangiferin	Hesperidin	Eriocitrin
C. intermedia	Intact plant	–	2.320 ± 0.190	0.857 ± 0.050	1.638 ± 0.154	0.206 ± 0.017
	Callus	MS + 5.0 mg/l 4-CPPU + 0.5 mg/l 2,4,5-T	0.004 ± 0.000	0.012 ± 0.004	0.012 ± 0.002	–
	Shoot culture	SH + 2.0 mg/l 2iP + 0.22 mg/l TDZ	1.320 ± 0.100	0.355 ± 0.050	0.899 ± 0.075	–
C. subternata	Intact plant	–	1.312 ± 0.050	0.485 ± 0.034	1.949 ± 0.183	0.471 ± 0.032
	Callus	MS + 5.0 mg/l 4-CPPU + 0.5 mg/l 2,4,5-T	0.004 ± 0.000	0.004 ± 0.000	0.694 ± 0.041	–
	Shoot culture	SH + 2.0 mg/l 2iP + 0.22 mg/l TDZ	1.547 ± 0.134	0.562 ± 0.048	0.873 ± 0.095	0.222 ± 0.012
C. genistoides	Intact plant	–	2.895 ± 0.218	1.369 ± 0.134	1.023 ± 0.120	0.046 ± 0.060
	Callus	SH + 5.0 mg/l 4-CPPU + 0.5 mg/l 2,4-D	0.004 ± 0.000	0.004 ± 0.000	0.033 ± 0.009	–
	Shoot culture	SH + 2.0 mg/l 2iP + 0.22 mg/l TDZ	0.968 ± 0.110	0.225 ± 0.010	0.004 ± 0.000	–

[a] All values are expressed as percentage of the DW. Each value represents the mean ± SD of 5 samples.

De Nysschen A.-M., van Wyk B., van Heerden F.-R., and Schutte A.-L. (1996), The major phenolic compounds in the leaves of *Cyclopia* species (honeybush tea). Biochem. Syst. Ecol. **24**, 243–246.

De Rijke E., Out P., Niessen W.-M.-A., Ariese F., Gooijer C., and Brinkman U.-A.-Th. (2006), Analytical separation and detection methods for flavonoids. J. Chromatogr. A **1112**, 31–63.

Dias A.-C.-P., Seabra R.-M., Andrade P.-B., Ferreres F., and Fernandes-Ferreira M. (2000), Xanthone biosynthesis and accumulation in calli and suspended cells of *Hypericum androsaemum*. Plant Sci. **150**, 93–101.

Dias A.-C.-P., Seabra R.-M., Andrade P.-B., Ferreres F., and Ferreira M.-F. (2001), Xanthone production in calli and suspended cells of *Hypericum perforatum*. J. Plant Physiol. **158**, 821–827.

Dixon R.-A. (1999), Isoflavonoids: Biochemistry, molecular biology, and biological functions. In: Comprehensive Natural Products Chemistry, Vol. 1: Polyketides and Other Secondary Metabolites including Fatty Acids and their Derivatives (Barton D., Nakanishi K., Meth-Cohn O., and Sankawa U., eds.). Pergamon, Elsevier Science Imprint, Oxford, pp. 773–822.

Joubert E., Otto F., Grüner S., and Weinreich B. (2003), Reversed-phase HPLC determination of mangiferin, isomangiferin and hesperidin in *Cyclopia* and the effect of harvesting date on the phenolic composition of *C. genistoides*. Eur. Food Res. Technol. **216**, 270–273.

Joubert E., Gelderblom W.-C.-A., Louw A., and de Beer D. (2008a), South African herbal teas: *Aspalathus linearis*, *Cyclopia* spp. and *Athrixia phylicoides* – A review. J. Ethnopharmacol. **119**, 376–412.

Joubert E., Siân Richards E., van der Merwe J.-D., de Beer D., Manley M., and Gelderblom W.-C.-A. (2008b), Effect of species variation and processing on phenolic composition and *in vitro* antioxidant activity of aqueous extracts of *Cyclopia* spp. (honeybush tea). J. Agric. Food Chem. **56**, 954–963.

Kokotkiewicz A. and Luczkiewicz M. (2009), Honeybush (*Cyclopia* sp.) – A rich source of compounds with high antimutagenic properties. Fitoterapia **80**, 3–11.

Kokotkiewicz A., Luczkiewicz M., and Marszałł M. (2006), *In vitro* cultures of several *Cyclopia* plants – growth conditions and phytochemical analysis of phenylpropanoid metabolites. In: XI Ogólnopolska Konferencja Kultur *In vitro* i Biotechnologii Roślin „Kultury *In vitro* Podstawą Biotechnologii Roślin”, Międzyzdroje (Kępczyński J., Rybczyński J.-J., and Kępczyńska E., eds.). Print Group, Szczecin, p. 68.

Luczkiewicz M. (2008), Research into isoflavonoid phyto-oestrogens in plant cell cultures. In: Bioactive Molecules and Medicinal Plants (Ramawat K.-G. and Merillon J.-M., eds.). Springer-Verlag, Berlin, Heidelberg, pp. 55–84.

Luczkiewicz M. and Glod D. (2003), Callus cultures of *Genista* plants – *in vitro* material producing high amounts of isoflavones of phytoestrogenic activity. Plant Sci. **165**, 1101–1108.

Luczkiewicz M. and Glod D. (2005), Morphogenesis-dependent accumulation of phytoestrogenes in *Genista tinctoria in vitro* cultures. Plant Sci. **168**, 967–979.

Luczkiewicz M. and Piotrowski A. (2005), Two-stage system for micropropagation of several *Genista* plants producing large amounts of phytoestrogens. Z. Naturforsch. **60c**, 557–566.

Mulinacci N., Giaccherini C., Santamaria A.R., Caniato R., Ferrari F., Valletta A., Vincieri F.-F., and Pasqua G. (2008), Anthocyanins and xanthones in the calli and regenerated shoots of *Hypericum perforatum* var. *angustifolium* (sin. Fröhlich) Borkh. Plant Physiol. Biochem. **46**, 414–420.

Murashige T. and Skoog F. (1962), A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant **15**, 473–497.

Pasqua G., Avato P., Monacelli B., Santamaria A.-R., and Argentieri M.-P. (2003), Metabolites in cell suspension cultures, calli, and *in vitro* regenerated organs of *Hypericum perforatum* cv. Topas. Plant Sci. **165**, 977–982.

Schenk R.-U. and Hildebrandt A.-C. (1972), Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. **50**, 199–204.

Sutcliffe M.-A. and Whitehead C.-S. (1994), Role of ethylene and short-chain saturated fatty acids in the smoke-stimulated germination of *Cyclopia* seed. J. Plant Physiol. **145**, 271–276.

Zenkteler M. (1984), Hodowle komórek. In: Hodowle komórek i tkanek roślinnych (Zenkteler M., ed.). PWN, Warszawa, p. 285.