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Communication

Production of Iridoids and Phenolics by Transformed *Harpagophytum procumbens* Root Cultures

Harpagophytum procumbens (Devil's claw) is an important medicinal plant, which tubers are used for the treatment of different inflammatory diseases. In this study, the time courses of growth of Devil's claw hairy roots and accumulation of intracellular total iridoids and phenolics were investigated during cultivation under submerged conditions. After 21 days of growth in liquid hormone-free MS medium, a growth index of 81.3 was achieved and the accumulated biomass reached 0.58 g/flask. It was found that the time courses of total iridoids and phenolics production showed almost the same patterns with a maximum on day 21 from the beginning of cultivation (15.93 mg harpagoside equivalents/L and 261 mg gallic acid equivalents/L, respectively). The methanolic extracts possessed relatively high DPPH-radical scavenging properties (IC_{50} 4.08 mg/mL), while for the culture media no antiradical activity was detected. According to our best knowledge, the present report is the first one dealing with the investigation of transformed root cultures from *Harpagophytum procumbens* plants.

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1 Introduction

Harpagophytum procumbens (Devil's claw) is a plant species, belonging to the Pedaliaceae family, which grows in South Africa and especially in the Kalahari dessert region [1]. The extracts from *H. procumbens* tubers are used as a drug for the treatment of different inflammatory disorders, such as arthritis, lumbago and muscular pain [2]. Recent studies showed that the aqueous extracts from the secondary roots of *Harpagophytum* plants exhibit analgesic and antidiabetic properties, which are due to the iridoid glycosides harpagoside, 8-*p*-coumaroyl-harpagide, harpagide, prucumbide, etc. [1, 3].

Currently the biggest exporter of Devil's claw tubers is Namibia. In 2002, the total export reached 1018 tons and the majority of the dried *Harpagophytum* spp. tubers were exported to Europe (mainly Germany and France). In 2001, *H. procumbens* became the third most frequently used medicinal plant in Germany, with sales of approximately 30 million Euros [4]. However, supplying commercial amounts of Devil's claw mass became very difficult, owing to the climatically features of South Africa. In order to be able to continue the supply of fertile plants, Levieille and Wilson [2] offered a rapid and reproducible two steps protocol for the *in vitro* propagation of *H. procumbens* and *H. zeyheri* plants. However, micropropagation does not exclude field cultivation.

Plant *in vitro* technologies were recognized as an alternative for the production of economically important metabolites because of their independence of seasonal and geographical conditions [5, 6]. Therefore, several technologies have been developed [7]. The genetic transformations with *Agrobacterium rhizogenes* received more attention in the recent years and they are preferable, when the target metabolites are synthesized in the underground plant parts (such as roots, tubers, rhizomes, etc). Previous reports [2, 3], showing that the iridoid glycosides are localized mainly in the tubers of *H. procumbens* plants, as well as the lack of reports for hairy roots induction from this plant species together with the high medicinal value of the Devil's claw iridoids provoked our interest to investigate this area further.

The aim of the present research was to study the growth of *H. procumbens* hairy roots under submerged culture conditions, the production of total iridoids and phenolics, as well as the evaluation of the antiradical activities of extracts from the transformed roots. This work presents the first report dealing with transformed root cultures from Devil's claw plants.

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2 Materials and Methods

2.1 *H. procumbens* Hairy Roots and Culture Conditions

H. procumbens hairy roots were obtained in the SIAB, Leipzig (from A. P. and G. K.) using the method of co-cultivation of sterile leaves from Devil's claw and *A. rhizogenes* ATCC 15834 (agropine-type strain), and following the procedure described earlier [8]. The transformed roots obtained were maintained on solid MS-hormone free media [9] for 21 days. The experiments were performed in 250 mL Erlenmeyer flasks containing 50 mL MS medium on a New Brunswick rotatory shaker (Innova 4340) at 110 rpm, 26 °C, in the dark. For inoculation, 2–3 g fresh roots/L were used from a 14-day-old maintenance culture grown on solid MS medium. Samples were taken every 3 days from the shaker.

2.2 Characterization of *H. procumbens* Growth

The hairy roots (taken from the shaker) were separated from the culture medium through filtration. The liquid culture medium was analyzed using a pH/conductivity meter and then evaporated under vacuum at 40 °C to reduce its volume and freeze-dry (lyophilizer Alpha 1-2, Christ). After washing with water, the roots were harvested (fresh biomass, FB), frozen and freeze-dried for 24 h for the gravimetrical determination of dry biomass (DB). The growth of *H. procumbens* hairy roots was monitored using the dry biomass (ADB) and growth index (GI) [10]:

ADB = Final Dry Biomass – Initial Dry Biomass

GI = (Final Dry Biomass – Initial Dry Biomass)/Initial Dry Biomass

2.3 Extraction and Analyses of Total Iridoids and Phenolics from *H. procumbens* Biomass

The freeze-dried roots were extracted with pure methanol. Extractions were performed at room temperature (about 23 °C), using a magnetic stirrer (650 rpm) and a solid:liquid ratio 1:50, for 2 h. The extracts were centrifuged (6000 × g for 10 min) and filtrated consecutively through filter papers and 0.2 µm filters (VWR).

The total content of iridoids in the extracts was determined according to the methodology described by Levieille and Wilson [2]. The reaction mixture contains 0.1 mL sample and 0.9 mL reagent solution (82 mL methanol, 100 mg vanillin and 8 mL concentrated sulfuric acid). For each assay, a blank sample was prepared with the same content, except vanillin. After 15 min, the obtained colored fulvoiridoid complex was measured spectrophotometrically at 538 nm (Spectrophotometer Helios Beta, TEC, England). Pure harpagoside (5−60 mg/L) (Extrasynthese, France) was used for establishing the standard curve ($r^2 = 0.9891$).

The total phenolic content in the extracts was determined according to the Folin-Ciocalteau method [11] as follows: 2.5 mL of Folin-Ciocalteau reagent was mixed with 0.5 mL extract and 2 mL sodium carbonate (7.5 % v/v aqueous solution). Absorbance at 765 nm was measured after 2 h at room temperature (about 23 °C). The total phenolics content is expressed as gallic acid (5–100 mg/L) (Sigma-Aldrich, USA) equivalents ($r^2 = 0.9982$).

2.4 Radical Scavenging Activities

For the evaluation of radical scavenging activities, 2,2'-diphenyl-1-picrylhydrazyl free radicals ($DPPH^{\cdot}$) (Sigma-Aldrich Chemie) were used [12]. The reaction mixture contains 0.8 mL (0.1 mM) DPPH and 0.2 mL sample solution (5 mg dry extracts or culture medium/mL). The absorbance was measured at 515 nm after 30 min and the percentage of decolorization was calculated according to the formula:

$$\% \text{ of decolorization} = (A_{control} - A_{sample})/A_{control} \cdot 100$$

Further, the IC_{50} values were calculated, which represent the necessary concentration of sample for a 50 % reduction of the $DPPH^{\cdot}$ absorbance.

The presented results are averages of two independent experiments each repeated twice ± standard deviation (SD). For spectroscopic measurements, the results are the averages of at least three measurements.

3 Results and Discussion

The obtained *H. procumbens* transformed roots were maintained for several weeks on solid MS medium with the aim to get hairy roots with stable growth and morphological characteristics. The culture grew on a length without forming many lateral branches, as the growing tips of the hairy roots were colorless or weak yellow colored and the pigmentation at the active-growing stage was mainly in yellow. After this period, the hairy root cultures were adapted to submerged cultivation conditions in consecutively cultivating them on liquid and solid media over a 3-month period. The adapted cultures were then grown under submerged conditions. It was observed that the intensive growth of *H. procumbens* hairy roots and biomass accumulation continued till the end of the process on day 21. ADB was about 0.58 g/flask and the achieved Growth Index was 81.3 (see Fig. 1). This high growth index together with the low amounts of the inoculum used (about 2−3 g fresh roots/L) will make further scaling-up from flasks to bioreactors easier. Surprisingly, the changes in the conductivity of the medium did not follow the time course of growth after the 15^{th} day of cultivation and no linear dependence could be observed (see Fig. 1). The enhancement of the conductivity after day 15 of cultivation is most probably due to the active transport of some compounds to the culture medium.

The time course of iridoids production in the roots of *H. procumbens* follows the growth of the culture being more intensive between day 15 and 21. The achieved volumetric yields at the end of the cultivation process were about 15.93 mg harpagoside equivalents/L (see Fig. 2). These quantities are lower in comparison with the amounts detected in the tubers of micropropagated *H. procumbens* plants and 34 % lower than the amounts detected in the leaves of the same spe-

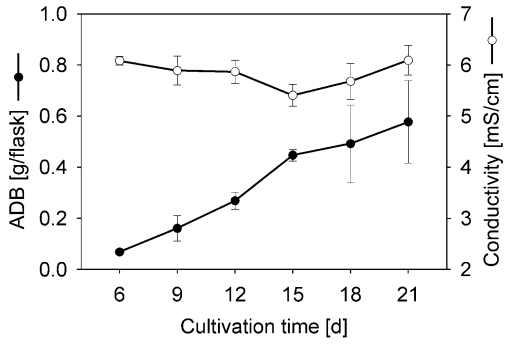

Figure 1. Time courses of growth of *H. procumbens* hairy root culture and conductivity changes during the cultivation under submerged conditions. (The error bars represent the standard deviation [$n = 4$].)

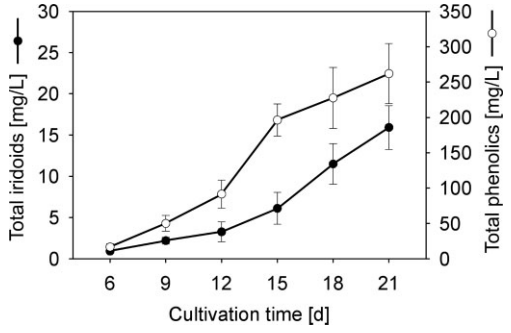

Figure 2. Time courses of the accumulation of total iridoids (expressed as harpagoside equivalents) and phenolics (expressed as gallic acid equivalents) during the cultivation of *H. procumbens* in liquid MS medium. (The error bars represent the standard deviation [$n = 4$].)

cies [2]. However, obtaining total iridoids from micropropagated plants requires a much longer cultivation time (about 18 months), which lead to smaller production rates (about 17 times and 1.7 times lower, respectively, compared to the production rates calculated for the hairy root cultures) (see Tab. 1). Furthermore, this initial yield offers a good possibility for future investigations, dealing with the exploitation of the biosynthetic potential of the transformed roots.

With the aim for a better characterization of the biosynthetic potential of *H. procumbens*, the investigation of the accumulation of total phenolics (which are known to possess a large variety of biological activities) was performed. As it can be seen from Fig. 2, the dynamics of total phenolics production is comparable with the iridoids one, showing a maximum on day 21 (261 mg GA equivalents/L). Using the GC/MS investigations (through derivatization method) it was found that ferulic acid, sinapinic acid, syringic acid, vanillic acid and some other phenolics are present in trace amounts in the extracts from the cultures (Ludwig-Mueller and Georgiev, unpublished results).

The DPPH$^{\cdot}$ is a stable radical, which could be easily used for the detection of antioxidant properties of different compounds

Table 1. Comparison between the yields of total iridoids from *H. procumbens* hairy roots and micropropagated Devil's claw plants.

Plant species/ in vitro culture	Cultivation time	Total iridoids [mg HS equivalents/g dry weight]	Production rate [µg HS equivalents/g dry weight/day]
H. procumbens hairy roots	21 days	0.66	31.43
Propagated	18 months		
H. procumbens plants[a]:		1.00	1.85[b]
– leaves of plants		10.00	18.52[b]
– tubers of plants			

[a] Data from Levieille and Wilson [2]. [b] Calculated for a 540-day period of cultivation.

in term of hydrogen donating ability. The advantages of the method are expressed mainly in its rapidity and selectivity. Because of these reasons DPPH$^{\cdot}$ has been widely used in recent years for the assessment of different antioxidants [12, 13]. The extracts and the culture medium from *H. procumbens* hairy roots were checked for their DPPH-radical scavenging activities. The methanolic extracts showed 61.3 % inhibition of DPPH radicals and the calculated IC_{50} value was 4.08 mg/mL, while the culture medium did not show radical scavenging properties. The latter phenomenon is probably due to the oxidation of the active compounds in the culture medium. In the literature there is no data available for the radical scavenging properties of extracts from this plant species.

4 Conclusions

The present work demonstrates that obtaining transformed root cultures from Devil's claw is possible, and when cultivated in liquid medium these cultures showed stable growth and accumulated high biomass amounts. Also, the hairy roots produced iridoids and phenolics and the extracts from them possess antioxidant activities. Further experiments will be focused on the determination of specific iridoids and phenolics produced as well as the application of different strategies for the exploitation of *H. procumbens* secondary metabolism.

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