BIOSYNTHESIS OF PROTEIN CROSS-LINKING AGENTS BY PLANT CELL CULTURES

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Introduction

Plants can be used as alternative source of potential protein cross-linking metabolites, so-called tanning agents[1]. These metabolites are relevant compounds for producers of collagen based biomaterials in the field of medicine technology and cosmetics as well as food and leather production. Cross-linking of collagen is performed almost exclusively by using substances which bear a toxic potential and are produced on the basis of fossil fuels such as glutaraldehyde, isothiocyanates or chromium salts. Iridoids and Secoiridoids are secondary plant metabolites showing a less toxic behavior but similar cross-linking abilities compared to the common tanning agents[2]. *Centaurium erytraeae* is a well known source of the secoiridoid glycosides swertiamarin, sweroside, gentiopicroside and centauroside (Fig. 1). These compounds belong to the group of mono-terpenoids produced by plants as a defense against herbivores and infection by microorganisms. Plant in vitro cultures growing in closed bioreactors allow a year-round production with constant quality and quantity[3].

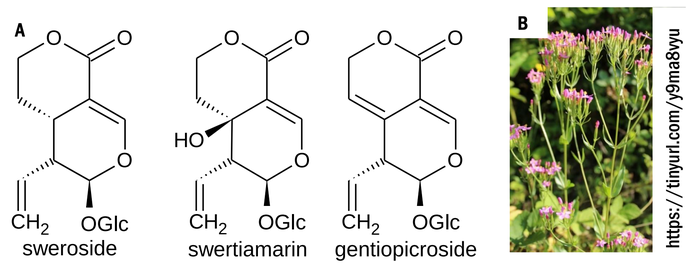
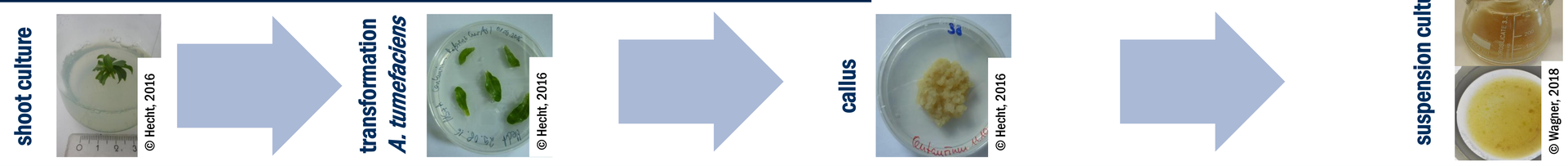


Fig. 1: (A) chemical structure of selected secoiridoid glycosides; (B) *Centaurium erythraeae*

Our work flow for initiation of plant cell suspension cultures



Comparison of the secoiridoid production by (in vitro) plants

- Secoiridoids were extracted from different plant material (Tab. 1).

Tab. 1: Secoiridoid production of *Centaurium erythraeae* material extracted with ethanol/ water 1:1 (v/v), percentage refers to dry biomass used (n = min. 3)

sample	secoiridoid	content [mg/g_{dw}]	
plant leaves	swertiamarin	118	± 23
	gentiopicroside	90	± 1
	sweroside	13	± 7.3
shoot cultures	swertiamarin	46.98	± 0.97
	gentiopicroside	16.90	± 0.74
	sweroside	13.17	± 0.46
callus, hormone based	swertiamarin	0.47	± 0.10
	gentiopicroside	1.83	± 0.42
	sweroside	0.20	± 0.02

- Content of secoiridoids differs among the tested plant material.
- The time required for cultivation should be also taken into account (productivity).

Callus initiation via transformation with *A. tumefaciens*

- Shoot cultures were used as explants for induction of callus cultures via transformation with *Agrobacterium tumefaciens* C58 WT following[4].
- 7 cell lines were induced and cultivated on hormone free MS-Medium (incl. vitamins).
- 2 candidates were selected in terms of growth and morphology for suspension establishment.
- Verification of transformation via virC primers for exclusion of bacterial contamination & tms primer was performed to prove positive transformation results (Fig. 2).

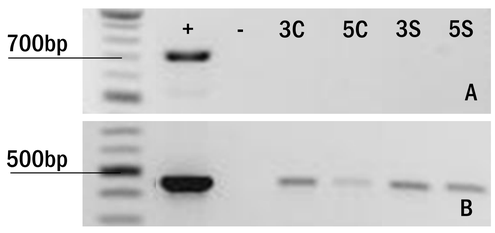


Fig. 2: Stable insertion of *A. tumefaciens* genes in the plant genome was demonstrated by transformation screening: (A) virC primers (730 bp) and (B) tms primer (442 bp). Both callus cell lines (3C, 5C) showed a positive result that was verified by screening the suspension cell lines (3S, 5S). *A. tumefaciens* gDNA was used to validate PCR performance (+) and a water sample (-) to exclude contamination.

Characterization of suspension culture growth and crosslinking properties

- Cell line 5S showed auspicious results in terms of growth and morphology.
- Growth Monitoring was performed by RAMOS® (Tab. 2) using different media.

Tab. 2: Characteristic parameters of growth behavior for *C. erythraeae* cell suspension; conditions: Murashige (MS) or Linsmaier (LS) & Skoog medium hormone free, 30 g/L Sucrose (Suc), 26°C, 110 rpm, dark (n = min. 2)

Parameter[1]	MS	LS
Cultivation duration [d]	ca. 13	ca. 11
OTR_{max} [mmol/(L* h)]	3.34±0.21 (9.8 d)	2.83±0.14 (9.2 d)
CTR_{max} [mmol/(L* h)]	3.63±0.25 (9.8 d)	3.44±0.07 (9.2 d)
cDW_{max} [g/L]	12.78±0.42 (9.2 d)	11.27±0.60 (7.8 d)
otr_{max} [mmol/(g*h)]	0.27 (9.8 d)	0.25 (7.8 d)
μ_{max} (DW) [1/d]	0.33	0.43
μ_{max} (OTR) [1/d]	0.41	0.21
Biomass yield Y [g_{DW}/g_{Suc}]	0.38	0.33
Biomass productivity P_{DW} [g /(L* d)]	0.87	1.04

[1]oxygen (OTR) and carbon dioxide (CTR) transfer rate, dry biomass (DW)

- For both media similar growth was observed.
- Sucrose and glucose were exhausted on day 9, total sugar ca. day 11.
- Maximum of respiration activity was reached after ca. 9-10 d.
- Both *C. erythraeae* cell suspensions showed gentiopicroside production.

- Cross-linking properties were evaluated with extracts from in vitro plants using skin powder: activation of secoiridoids via β-glucosidase, incubation 24 h, 30°C, determination of denaturation temperature T_s (Fig. 3)
- Increase of T_s proved crosslinking properties of in vitro plant extracts containing gentiopicroside and other secoiridoids.

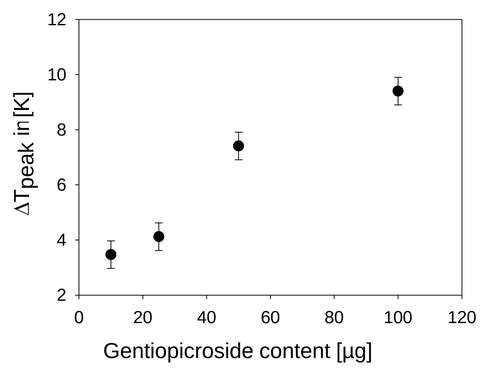


Fig. 3: Increase of denaturation temperature of skin powder after incubation with different amounts of gentiopicroside

Further steps towards scale up

Comparison of growth of transformed *C. erytraeae* cell suspension vs. secoiridoid content and crosslinking properties of extract → Optimization of cell productivity via e.g. elicitation, cultivation strategies → Process transfer to bioreactor → Scale-up

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