



Production of phenolic compounds, flavonoids, and lupeol by *Lycium schweinfurthii* suspension cultures in shake flasks and a rocking-motion bioreactor

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Abstract

Lycium schweinfurthii, a wild shrub of the Solanaceae family, has received increasing attention in the last decade for its therapeutic potential in traditional medicine due to its diverse array of secondary metabolites, including phenolic substances and terpenoids. The aim of this study was to investigate the accumulation of phenolics, flavonoids, and the terpenoid lupeol in *L. schweinfurthii* cell suspension shake flask cultures and a single-use 2-dimensional rocking motion bioreactor. Three different media formulations were compared for in vitro cell cultures. Various parameters, such as biomass accumulation, settled cell volume, cell viability (assessed via a 2,3,5-triphenyl tetrazolium chloride assay), and sucrose consumption were determined as indicators of cell activity and growth. Total phenolic and flavonoid contents were estimated spectrophotometrically, lupeol was quantified via High-Performance Thin Layer Chromatography (HPTLC). Although a higher fresh biomass concentration of 464 g L⁻¹ was obtained in MS medium supplemented with a combination of each, 1 mg L⁻¹ of 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1-Naphthaleneacetic acid (NAA), the rocking-motion bioreactor cultivation was performed with 2 mg L⁻¹ NAA due to its superior reproducibility in viability, productivity, and content of bioactive compounds (e.g., phenolics, flavonoids, lupeol). A final fresh biomass concentration of 185 g L⁻¹ was achieved in a 16 L cultivation scale with a notable increase in the concentration of phenolics (1.4-fold) and flavonoids (1.7-fold). Most importantly, the concentration of lupeol, a pentacyclic triterpenoid known for its anti-inflammatory, antibacterial, and anti-atherogenic properties, exhibited a remarkable 5.5-fold increase in the bioreactor cultivation (585 µg g⁻¹) compared to shake flask cultivations (106 µg g⁻¹). The current study demonstrated the profound impact of media composition and non-limited fed-batch conditions in a rocking-motion bioreactor on the accumulation of bioactive compounds. The findings are also relevant to other plant cell cultures.

Key message

Scaling-up of *Lycium schweinfurthii* suspension culture is possible in a rocking motion bioreactor for production of phenolics and lupeol.

Keywords Phenolics · Lupeol · Plants suspension culture · Shake flasks · Bioreactor

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Introduction

The genus *Lycium* (family Solanaceae) comprises more than 90 species distributed all over the world. *Lycium barbarum* has received the most attention. Its berry, known as the goji berry, is widely used as a “superfood” with medicinal properties. In contrast, *L. schweinfurthii*, although used by various ethnic groups in Algeria, Morocco, Sudan, and Egypt (Yao et al. 2018) has been studied only rarely so far (Miguel 2022).

L. schweinfurthii leaves are traditionally used for their anti-ulcerogenic activity against gastrointestinal diseases (Bazine et al. 2023). In 2016, Ewais et al. (2016) screened the phytochemical content in different plant parts and showed that the concentration of flavonoids, tannins, saponins, and alkaloids was higher in leaves compared to flowers, stems, and roots. Elbermawi et al. (2019) showed that 4 out of 6 glucosides from the plant suppressed hyperglycemia in diabetic patients. In addition, all 28 secondary metabolites from the plant's methanolic extract are hardly toxic to normal skin and colon cells. However, interestingly, high cytotoxic activity was observed for certain extracted flavonoids against skin and colon cancer cell lines (Elbermawi et al. 2022). Moreover, *L. schweinfurthii* extracts showed sedative, analgesic, antidepressant, and anticonvulsant activity in mice (Bazine et al. 2023; Sherif et al. 2022). In a recent study, 1-heptatriacotanol, which is also known to have anti-hypercholesterolemic effects (Junwei et al. 2018), and lupeol, which has a wide range of proven biological activities to prevent cancer, coronary, and hepatic diseases (Wal et al. 2015), were also found in *L. schweinfurthii* plants (Ragab et al. 2023). Lupeol is a pentacyclic triterpenoid known from lupin seeds, fig trees, and rubber plants. It is also found in some edible fruits and vegetables (Bañares et al. 2023; Muktar et al. 2018) and was investigated for the treatment of acne based on its anti-inflammatory and antibacterial activity (Wal et al. 2015). It can provide considerable protection against the early stage of hypercholesterolemic atherogenesis (Sudhakar et al. 2006).

However, *L. schweinfurthii* is currently not exploited as a potential source of phenolics, flavonoids, and triterpenoids, because its habitat is limited to a warm and dry climate. There are no standard agricultural cultivation techniques, and the content of bioactive compounds is sensitive to fluctuating environmental conditions (Bazine et al. 2023; Ewais et al. 2016; Qian et al. 2017).

For decades, plant cell cultures have been regarded as valuable sources of secondary metabolites with significant applications as food additives, nutraceuticals, and pharmaceuticals (Sytar et al. 2023). Plant cell cultures, as opposed to conventional planting, enable the production of compounds for the food and cosmetics industries with lower

consumption of energy, a potentially lower environmental impact, and seasonal and geographical independence (Eibl et al. 2018). Despite their crucial importance, the production of these metabolites by plant cell cultures encounters various challenges. Overcoming these challenges is fundamental for their mass production (Smetanska 2018). The careful selection of appropriate explants, medium, and plant growth regulators (PGRs) plays a critical role in the initiation of cell cultures (Das et al. 2018). Also, any in vitro culture protocol needs to be customized for each plant species (Das et al. 2018; Kim et al. 2023). Commonly used artificial media for callus and cell cultures include Murashige and Skoog (MS) medium, Gamborg B5 medium, and Linsmaier and Skoog (LS) medium (Binte Mostafiz et al. 2018; Phillips et al. 2019; Rao et al. 2002). It is important to mention, that cell cultures of different plants respond individually to the different cultivation media. Thus, changing the concentration, proportion, and form of medium components is a powerful strategy to influence the growth of in vitro plant cultures and the synthesis of metabolites. Ananga et al. (2013) reported that the transfer of anthocyanin-producing cell suspension of *Vitis vinifera* from the original B5 medium to LS medium completely changed the color pattern from red to yellow, and the cell growth pattern from small colored aggregates to single colorless cells. Zenk (1978) compared the influence of different basal media such as MS, Gamborg, Heller, Nitsch and Nitsch, White, and LS on biomass growth and serpentine production by *Catharanthus roseus* suspension cell cultures. The results indicated that LS medium was optimal for cell biomass growth, while MS medium increased the synthesis of serpentine. Interestingly, cell cultures growing on LS, Gamborg, Nitsch and Nitsch, Heller, and White medium lost their ability to synthesize serpentine, as well as cell cultures cultivated on Gamborg, Nitsch and Nitsch, Heller, and White medium. Therefore, it is necessary to compare different nutrient media in order to select the optimal one for the growth and production of certain secondary metabolites.

The cultivation of plant cells in bioreactors to produce valuable secondary metabolites such as isoprenoids (Nosov et al. 2014), flavonoids (Kokotkiewicz et al. 2013), and pharmaceutical proteins (Tekoah et al. 2015) is not new. Plant cell cultures are used to produce various metabolites and recombinant proteins for human therapy in various types of stirred or shaken bioreactors (Eibl et al. 2008b; T. K. Huang et al. 2009; Kowalczyk et al. 2022). However, the selection of the appropriate bioreactor plays a key role in the successful cultivation of the respective plant cell cultures and, in particular, in the portfolio and content of their active constituents (Werner et al. 2018). The most frequently used bioreactors for plant cell and tissue cultures are made of glass or stainless steel. Various types like stirred tank reactors

(STRs), rotating drums, airlift reactors and bubble columns, fluidized beds, packed beds, and trickle bed bioreactors are applied (Eibl et al. 2008a).

The STR is the most common bioreactor design for plant cell cultures to enhance the mass production of cells and their active constituents. For example, anti-inflammatory compounds such as scopoletin, tomentin, and sphaeralcic acid were produced from *Sphaeralcea angustifolia* cell suspension cultures in a 2 L STR (Pérez-Hernández et al. 2019). An STR was used in combination with the airlift column to produce β -elemene from plant suspension cultures of *Rhizoma zedoariae* (Wang et al. 2019). β -elemene is a sesquiterpene used in the treatment of different cancers, particularly, ovarian, breast, and lung cancers. Another example of the application of an STR is the production of crocin, an anti-oxidant and anti-inflammatory carotenoid, from saffron (stigmas of *Crocus sativus* L.) cell cultures (Amini et al. 2022).

Among the bioreactors that have been applied with plant cell cultures were also single-use bioreactors with various mixing technologies (Werner et al. 2019). Within the commercialized variants of single-use bioreactors, rocking-motion based mixing has been always important. These reactors expose a lower shear force regime, which was also one reason for their first application in mammalian cell culture in the late 1990s (Eibl et al. 2010). They usually consist of a tray and a single-use bag and are applied with a comparably low rocking rate to avoid cell damage and subsequent foam formation (Werner et al. 2018). The bag is commonly pre-sterilized by irradiation (Terrier et al. 2007). Several design variants have been developed, e.g. with a one-dimensional or a two-dimensional movement, as summarized, for example, by Junne et al. (2018). Typically, bags are equipped with sensors for pH and dissolved oxygen tension (DOT) (Junné et al. 2013). Among the commercial bioreactors is the two-dimensionally rocking Cell-tainer[®] bioreactor, which exhibits comparably low mixing times and high oxygen mass transfer rates of up to 600 h⁻¹ (Junné et al. 2013). It turned out to be advantageous compared to traditional stirred tank bioreactors in producing docosahexaenoic acid with the heterotrophically grown microalgae *Cryptocodinium cohnii* as fewer cells were damaged by shear forces (Hillig et al. 2014). The similarity of the shear regime between shake flasks and the rocking-motion bioreactor was also reported for the filamentous fungus *Aspergillus niger*: a similar morphology was obtainable in both, the two-dimensional rocking-motion bioreactor and in shake flasks (Kheirkhah et al. 2023; Kurt et al. 2018). Due to the low to moderate shear regimes and a wide working volume from 100 mL to more than 15 L within a single bag due to the absence of any internal part that needs to be covered with liquid for operation like a stirrer (Junné et al.

2018), this reactor was chosen for controlled cultivation at a larger working volume in the present study.

The main objectives of this study were to examine the effects of culture media components in 0.5 L shake flasks and the scale up to an actively aerated, non-limited fed-batch culture of a maximum working volume of 16 L in a 2-dimensional rocking-motion bioreactor. The focus was on growth and synthesis of secondary metabolites in *L. schweinfurthii* cells.

Materials and methods

Plant material

The plant material of *L. schweinfurthii* callus cultures was obtained from leaf explants according to the method of Mamdouh and Smetanska (2022). The obtained callus was sub-cultured on MS (Murashige and Skoog) medium (Duchefa, Haarlem, The Netherlands) supplemented with 2 mg L⁻¹ NAA (Mallinckrodt, Hazelwood, MO), 30 g L⁻¹ sucrose, and 8 g L⁻¹ solidifying agar (Duchefa). The cultures were incubated in 90 mm Petri dishes at room temperature in the dark. The cultures were sub-cultured into fresh medium every 6 weeks. The friable creamy callus cultures were selected as a starting material for initializing suspension cultures.

Culture conditions and cultivation parameters

To determine the optimal composition of growth regulators for biomass accumulation and secondary metabolite production, a randomized block design experiment was performed by sub-culturing 25 g of callus in 1 L unbaffled shake flasks containing 0.5 L of liquid MS medium supplemented with 1 mg L⁻¹ NAA (N1), 2 mg L⁻¹ NAA (N2), or 1 mg L⁻¹ 2,4-D + 1 mg L⁻¹ NAA (DN1). These growth regulators were selected based on our previous optimization of *L. schweinfurthii* callus production (Mamdouh et al. 2022). The batch cultures were incubated at 23 ± 1 °C at a shaking rate of 120 rpm for 31 days (Edmund Bühler, Bodelshausen, Germany). Sampling was performed randomly and weekly starting from day 3 to day 30 under sterilized conditions from 6 biological replicates of each treatment by taking 10 mL of cell suspension. Afterwards, samples were filtered, weighted, and the viability was determined. The pH and electric conductivity were measured *offline*. Samples were stored at -20 °C prior to further analysis.

The more stable, consistent, and reliable N2 medium was selected to study the accumulation of secondary metabolites by cell cultures cultivated in the rocking-motion single-use bioreactor CELL-tainer[®] CT20 with

2-dimensional movement (Celltainer Biotech, Winterswijk, The Netherlands).

An aerated non-limited fed-batch culture was started by inoculation of 283.5 g fresh friable callus in a 9 L medium corresponding to 31.5 g L⁻¹. The cultivation volume was increased gradually to 16 L on days 16 and 21 of cultivation. Cells were cultivated at a temperature of 25 ± 1 °C, a rocking rate of 15 rpm, and a continuous aeration rate of 0.2 L min⁻¹. Sampling of 3 technical replicates of 50 mL each was conducted weekly for the bioreactor cultures with some additional sampling times between the main sampling dates. Sampling was performed through a tube that lasted up to the bottom of the bag. Rocking was continued during sampling to ensure a constant power input. The bioreactor samples were prepared and stored in the same way as the samples from the shake flask cultures.

Growth parameters of cell cultures

The settled cell volume (SCV) was determined by transferring the collected samples to a graduated cylinder. The volume of settled cells was measured after 20 min at room temperature without centrifugation and is expressed as a percentage (v/v) of the volume of settled cells to the total volume of the sample. The fresh biomass of samples was determined after vacuum filtration using a PC 3001 VARIO® vacuum pump (Vacuubrand, Wertheim, Germany) to observe the biomass accumulation in shake flasks and the bioreactor. Cell viability was immediately assessed. The remaining samples were stored at -20 °C for further analysis. An enzymatic sucrose assay kit (SCA20, Sigma-Aldrich®, St. Louis, MO) was used to analyze the sucrose consumption in the filtered media samples of the bioreactor cultivation. The volumetric and specific consumption rates of sucrose, r_s (g L⁻¹ h⁻¹) and q_s (g g⁻¹ h⁻¹), were calculated from the concentration difference between two sampling points. For q_s , r_s was divided by average fresh biomass in the sampling interval. Sucrose was added by pulse feeding, which was considered in calculations whenever relevant.

The viability test was performed with 2,3,5-triphenyl tetrazolium chloride (TTC) according to CABRI Guidelines (1998) considering the modifications of Mamdouh and Smetanska (2022). Therefore, 50 mg of fresh samples were mixed with 500 µL of 1% TTC solution. After incubation in the dark for 6 h, the reaction mixture was removed after centrifugation at 3,500 rpm for 5 min. The formazan's red color was extracted from cells by adding 3 mL of ethanol before incubation at room temperature overnight. The absorbance of the supernatant was measured at 500 nm using a Specord® 250 Plus UV-Vis spectrophotometer (Analytic Jena, Jena, Germany). The viability of cells was compared with the initial viability at the onset of each cultivation process.

The specific growth rate μ (d⁻¹) was calculated from the fresh biomass analyses between two sampling points by the following equation

$$\mu = \frac{\Delta c_X}{\Delta t \cdot \bar{c}_X}$$

with c_X being the fresh biomass (g L⁻¹).

Sample preparation

The extracts of plant cell cultures were prepared from fresh samples during the cultivation process and from freeze-dried samples harvested at the end of the cultivation.

For phenolic and flavonoid concentration measurement, a 200 mg mL⁻¹ extract of fresh samples and a 50 mg mL⁻¹ extract of lyophilized samples were prepared by mixing 1 g fresh sample with 5 mL 80% aqueous methanol and 500 mg dry samples with 10 mL 80% aqueous methanol, respectively. The samples were sonicated at a frequency of 37 kHz (80% power) and 65 °C for 60 min with an Elmasonic P 30 H water bath (Elma, Singen, Germany). Samples were centrifuged at 3,500 rpm for 5 min. Then, the extracts were stored at -20 °C prior to spectrophotometric measurements of the total phenolic and flavonoid contents.

For lupeol concentration measurements, 500 mg of lyophilized cell samples were extracted with 10 mL of petroleum ether in an Elmasonic P 30 H ultrasonic water bath for 30 min at 75 °C and 80 kHz (80% power). The extracts were filtered and dried at 50 °C in the oven until complete dryness. The extracts were then dissolved in chloroform to a final concentration of 50 mg mL⁻¹ and were stored at -20 °C prior to HPTLC analysis.

Analysis of total phenolics and flavonoids

The total phenolic and flavonoid contents of the suspension cultures were determined by the Folin-Ciocalteu and aluminum chloride assay, respectively, as described by Marinova et al. (2005) and modified as described by Mamdouh et al. (2021). For phenolic compound measurements, gallic acid (Sigma-Aldrich) was used for generating a standard curve using 9 standard solutions of different concentrations between 10 and 300 µg mL⁻¹. The absorbance against the reagent blank was determined at 750 nm with a Specord® 250 Plus UV-Vis spectrophotometer (Analytic Jena). For flavonoid concentration measurements, catechin (Sigma-Aldrich) was used for generating a standard curve using 6 standard solutions of different concentrations ranging between 20 and 120 µg mL⁻¹. The absorbance was measured against the reagent blank at 510 nm. The total phenolic content was expressed as µg g⁻¹ in equivalence to gallic

acid, while the total flavonoid content was expressed as $\mu\text{g g}^{-1}$ in equivalence to catechin.

Analysis of lupeol

High-performance thin-layer chromatography (HPTLC) fingerprinting was used for the estimation of lupeol in shake flask and bioreactor suspension cultures of *L. schweinfurthii* (Fig. 1). A Linomat 5 automatic spraying unit, attached with compressed air, and supplied with a 100 μL syringe (Camag, Muttenz, Switzerland) was used for spotting extracts on TLC silica gel 60 F₂₅₄ aluminum plates (10×20 cm, Merck). The TLC plates were activated at 100 °C for 30 min after submerging them in methanol. The extracts were applied as 8 mm bands from the bottom edge of the plate. Lupeol standard of 50 $\mu\text{g mL}^{-1}$ was applied in volumes of 1–5 μL to deduce a calibration curve against the extracts of the suspension cultures. The plates were developed in a CAMAG Automatic Developing Chamber 2 (ADC 2) connected with a humidity chamber containing magnesium chloride solution to maintain and control the humidity during the development at 25 °C and 40% humidity. A mixture of petroleum ether: ethyl acetate: toluene (7:2:1, v/v/v) was used as the mobile phase. After the development, the plates were derivatized by submersion in anisaldehyde sulfuric acid (0.5 mL anisaldehyde, 10 mL glacial acetic acid, 85 mL methanol, and 5 mL concentrated sulfuric acid) for 1 s and dried at 100 °C for 10 min. Images of plates were taken by a CAMAG TLC Visualizer at 366 nm, captures were analyzed with the VisionCATS software (Camag).

Statistical analysis

Data was derived from 6 biological replicates in shake flask experiments and 3 technical replicates of the bioreactor cultivation. Statistical analysis of variance (ANOVA) was performed using SigmaPlot ver. 12.5 (Systat Software, San Jose, CA). The Shapiro-Wilk normality test was performed to verify the assumptions of ANOVA. The Holm-Sidak

method at $P \leq 0.05$ was used for pairwise comparison between data.

Results and discussion

In this study, we compared the effect of growth regulators as NAA and 2,4-D on biomass growth and synthesis of phenolics, flavonoids, and terpenoid lupeol of *L. schweinfurthii* cell cultures. We compared cultivations in two scales, namely in shake flask and an aerated non-limited fed-batch culture in a 2-dimensional rocking-motion bioreactor.

While plant cell cultures are rich sources of dietary supplements for healthy human nutrition (Ochoa-Villarreal et al. 2016) or for medical applications, understanding the relationship between media composition, growth conditions, and productivity at the different stages of cultivation is essential for identifying sensitive factors for growth and product accumulation. The typical in vitro culture for the accumulation of secondary metabolites (e.g., lupeol and phenolic compounds) requires active living cells with a high division rate under some stressful cultivation conditions (Babich et al. 2021; Kikowska et al. 2020; Zia et al. 2023). In order to identify good conditions for *L. schweinfurthii* secondary metabolite accumulation, the influence of growth regulators was first examined in shake flask cultures.

Influence of growth regulators on biomass formation

Numerous studies investigated the effect of growth regulators such as NAA and 2,4-D on the growth and synthetic potential of callus cultures, but no investigations have been published on the influence of these substances on biomass growth and the synthesis of phenolics and terpenoids in *L. schweinfurthii*. In our experiments, *L. schweinfurthii* cell cultures were grown in 1 L shake flasks with 500 mL MS media supplemented with 1 mg L^{-1} NAA (N1 medium), with 2 mg L^{-1} NAA (N2 medium), and with the mixture of 1 mg L^{-1} 2,4-D and 1 mg L^{-1} NAA (DN1 medium). Although auxins are commonly used to stimulate cell division, callus formation, and biomass growth, a high concentration of auxins can suppress the synthesis of secondary metabolites (Tabata 2006).

As shown in Fig. 2a, the fresh biomass concentration decreased during the first 3 days of cultivation from 50 g L^{-1} to 10.8 g L^{-1} in N2 medium and even to 6.5 g L^{-1} in DN1 medium. Afterwards, the fresh biomass concentration increased gradually in all media, reaching more than 120 g L^{-1} in N2 and DN1 media, but 40% less biomass concentration was achieved in N1 medium. A further almost 4-fold increase in the cells' fresh weight was observed during the

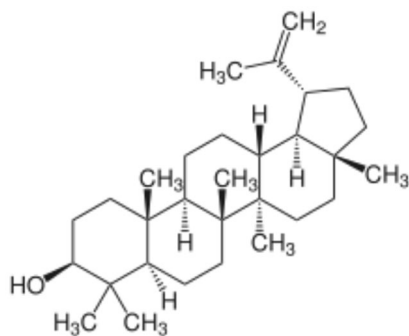


Fig. 1 The chemical structure of lupeol, a plant triterpenoid

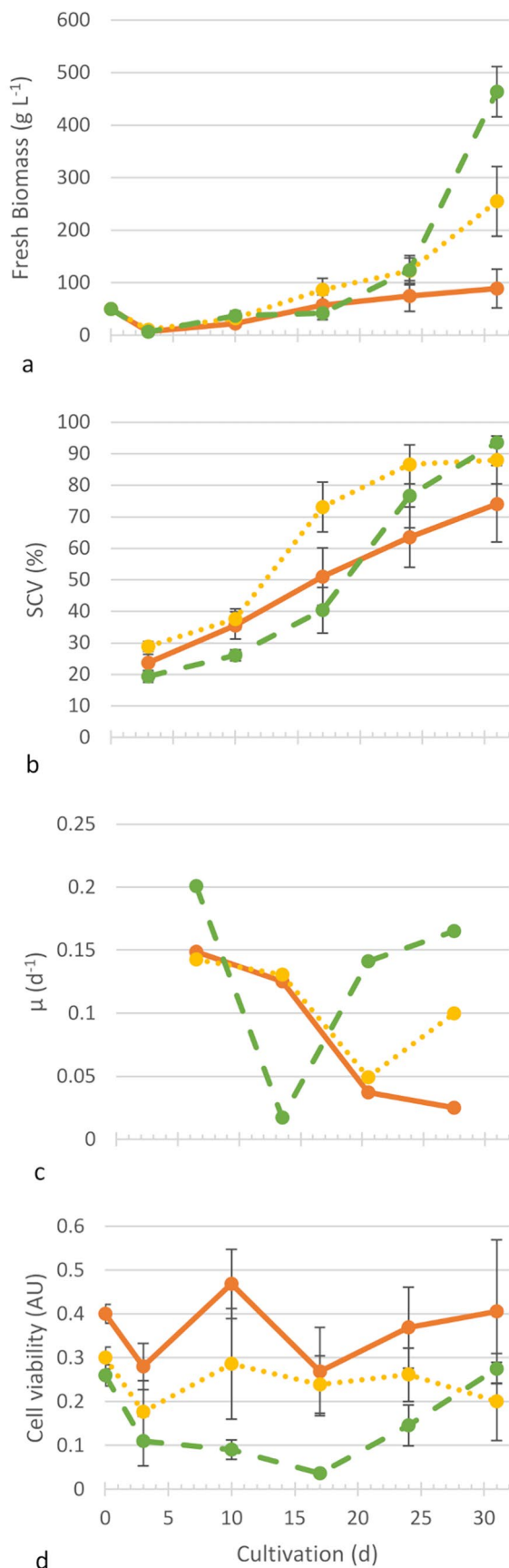


Fig. 2 Growth performance of *L. schweinfurthii* shake flask cultures in N1 (orange solid line), N2 (yellow dot line), and DN1 (green dash line) media: **(a)** biomass accumulation, **(b)** SCV, **(c)** specific growth rate, and **(d)** cell viability

following week in DN1 medium, but not in N1 medium. In a previous study, we cultivated suspension cultures of *L. schweinfurthii* in N2 medium, but in 100 mL flasks containing 50 mL medium with the same sucrose concentration. A final biomass concentration of 240 g L⁻¹ (7.33 g L⁻¹d⁻¹) was achieved after 30 days (Mamdouh et al., 2022) which is consistent with the biomass accumulated in shake flasks in the present study with 500 mL of N2 medium (6.8 g L⁻¹d⁻¹).

The results are in line with other studies. For instance, the addition of plant growth regulators, namely NAA, benzyl adenine, and kinetin, into the culture media of *Panax vietnamensis* cell cultures enhanced the biomass accumulation in shake flasks (Trong et al. 2017). The slightly higher concentration of NAA in N2 medium (2 mg L⁻¹) was more effective in cell culture biomass productivity than the lower concentration in N1 medium (1 mg L⁻¹). However, the combination of NAA and 2,4-D (1 mg L⁻¹ each) enhanced the biomass growth of *L. schweinfurthii* cell cultures regardless of the decline in cell viability and fluctuations in the growth rate during the first 17 cultivation days (Fig. 2). The effect of NAA and 2,4-D for the induction and maintenance of callus and cell cultures has been proven in many plant species such as rice (Binte Mostafiz et al. 2018), *Brucea mollis* (Das et al. 2018) and *Sageretia thea* (Kim et al. 2023). The highest biomass of rice cells was obtained in a medium containing 3 mg L⁻¹ 2,4-D with more than 80% induction frequency of cells while NAA had a higher effect (100% induction frequency) compared to 2,4-D (93% induction frequency) for the production of *Brucea mollis* cells (Binte Mostafiz et al. 2018; Das et al. 2018). Otherwise, an equal concentration of NAA and 2,4-D (0.5 mg L⁻¹ each) was optimal for the initiation and production of *Sageretia thea* cell cultures (Kim et al. 2023). Moreover, the optimization of the PGRs concentration of 2,4-D, NAA, and kinetin to 1.33, 1.76, and 0.15 mg L⁻¹, respectively, resulted in higher cell biomass of 70.62 g L⁻¹ DW of soybean cell cultures (Rani et al. 2022). Consequently, the PGRs concentration as well as the mineral content ensure the reliability, efficiency, and regeneration of the cell suspension initiation protocol (Rani et al. 2022; Schmitz et al. 2016). Based on the results obtained in our experiment and supported by the results obtained by other researchers (Binte Mostafiz et al. 2018; Das et al. 2018; Kim et al. 2023), it is possible to conclude that the optimal concentration of NAA and 2,4-D for biomass growth of *L. schweinfurthii* is 1 mg L⁻¹ for each.

The obtained results showed that SCV of different cultures was approximately the same during the first 10 days of cultivation (19–38%). The highest increase in SCV was in

N2 cultures on day 17 at 73% which was significantly different to DN1 cultures (40%). The SCV steadily increased in all cultures until the harvesting on day 30 (74–94%) in which no significant differences were observable (Fig. 2). The research results were unable to provide a feasible or apparent relationship between the SCV of cultures with either their growth activity or viability. In agreement with the current study, Nomura et al. (2021) considered the SCV of a maximum of 20% as a starting cell density in Bamboo suspension cultures. Furthermore, the SCV of embryogenic cell suspension cultures of *Pinus pinea* reached between 50 and 90% by the end of cultivation in 100 and 200 mL shake flasks (González-Cabrero et al. 2018). The SCV of cell cultures was investigated as a growth indication in two varieties of *Sorghum bicolor* L., where the stationary phase of the ICSB 338 variety and the white variety comprised 65% and 40% SCV, respectively (Ramulifho et al. 2019).

The viability of *L. schweinfurthii* cells which were cultivated in 1 L shake flasks was tested using the tetrazolium test to investigate the activity and stability of cells in the cultivation media (N1, N2, and DN1) during the cultivation period. The white TTC (2,3,5-triphenyl tetrazolium chloride) compound is enzymatically reduced to TPF (1,3,5-triphenyl formazan) in living cells due to the activity of the dehydrogenase enzymes where TTC accepts electrons from the mitochondrial transport chain and is converted to insoluble formazan (Castro-Concha et al. 2012).

The viability of cells was tested prior to inoculation. Results of the cultivations are presented relative to the initial viability. After 3 days of cultivation in shake flasks, the observed viability was 30–58% lower than the initial viability for all media. For DN1 medium the cell viability decreased further until day 17 but then increased strongly

during the last two cultivation weeks. In contrast, for N1 and N2 medium, the cell viability attained its maximum viability on day 10. Thereafter, the N2 culture further declined by one-third until the end of the cultivation, while the N1 culture cell viability increased to the same value as initially on day 31 (Fig. 2).

All in all, a decreased viability during the onset of cultivation is not uncommon, as reported, for example, for a suspension culture in semi-solid matrix of transgenic rice cells (*Oryza sativa*) in the first 2 weeks of cultivation (Varma et al. 2021). Based on our results, we conclude that the applied growth regulators' concentrations are sensitive to cell viability in the later stage of the cultivation of suspension cultures of *L. schweinfurthii*.

The pH value was adjusted to a value of 5.7 before autoclaving and measured throughout the cultivation period (Fig. 3). The culture in N2 medium showed a slight pH increase during the first 10 days of cultivation to pH 6.0 and later decreased gradually to 5.5. Similarly, the pH of N1 medium showed values between 5.55 (the lowest value on day 3) and 6 (the highest value on day 31). The pH value of cultures with DN1 medium showed a bigger variation between 5 and 6.5 with a minimum on day 10 and a maximum on day 31. In all cases, the pH value remained in a range that is suitable for the growth of *L. schweinfurthii* cells. The pH of N2 medium showed a significant difference with DN1 medium on days 10 and 31. Otherwise, no significant differences in pH were observed between the different cultivation media. It was noticed that a low pH in DN1 medium on day 10 was accompanied by a cell viability below 0.1 AU. It recovered after day 17 in parallel with an increasing pH to more than 5.5 (Fig. 2d). This indicates a possible physiological relation between the medium pH and

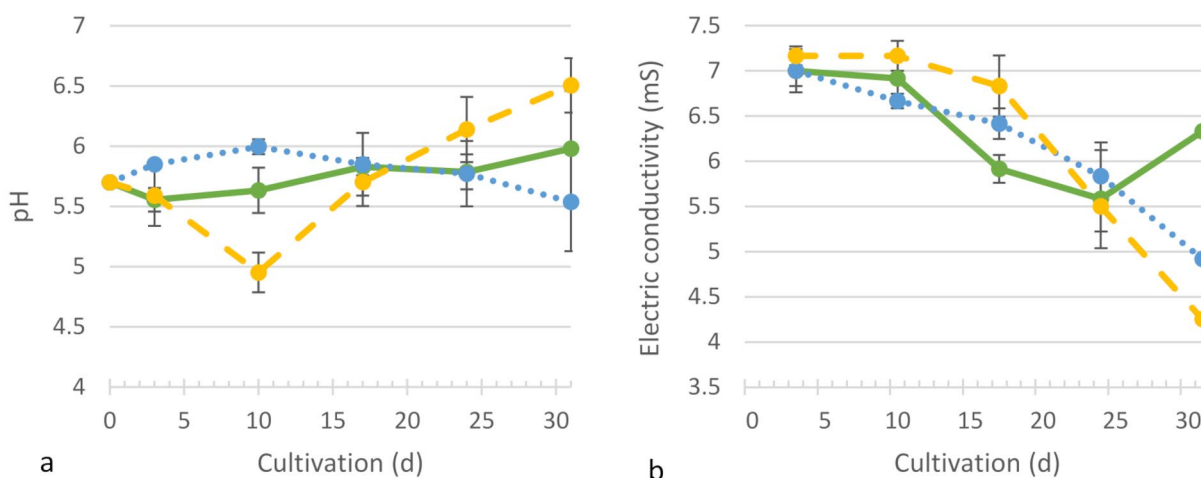


Fig. 3 Changes in pH and electric conductivity of *L. schweinfurthii* shake flask cultures in N1 (green solid line), N2 (blue dot line), and DN1 (yellow dash line) media: (a) pH, and (b) EC

the cell viability. In our previous study, *L. schweinfurthii* cells were grown well in shake flasks in a medium pH range between 5.3 and 6.0 (Mamdouh et al. 2022).

The conductivity decreased in all cultures from 7.0 mS on day 3 to a range of 5.5–5.8 mS on day 24 (Fig. 3). After that, the conductivity continued to decrease in N2 and DN1 media to 4.9 and 4.25 mS, respectively. The conductivity of the N1 cultures increased to 6.25 mS between days 24 and 31. The electric conductivity reflects the cells' consumption or release of salt ions into the media. Any net uptake of ions results in lower conductivity.

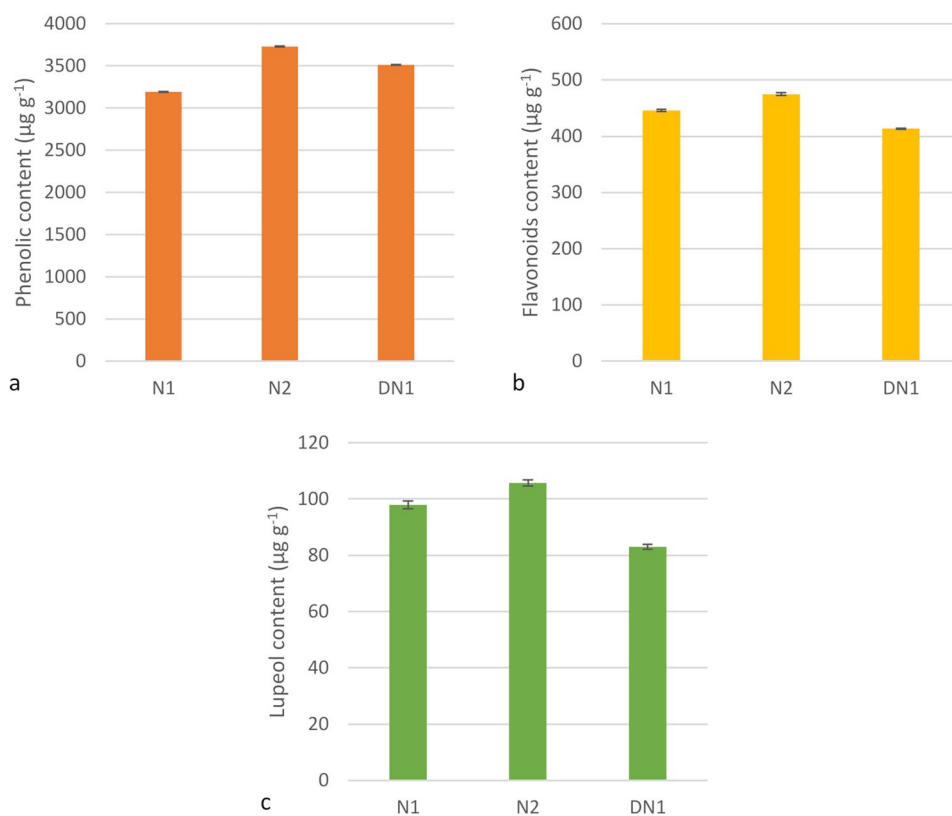
A linear, however negative relation between the conductivity and growth was reported for *Harpagophytum procumbens* cell cultures in shake flasks by Georgiev et al. (2009): the conductivity decreased from 6.5 to 1.0 mS during 30 days of cultivation. The increase or stability of the conductivity at earlier or later stages of suspension cultures could be due to the release of potassium ions from cells through cell membranes (Zhang et al. 2004). Our results have shown that the decrease in conductivity starting from the third week of cultivation occurs in parallel to a higher cell activity when media was supplemented with 2,4-D and NAA (1 mg L⁻¹ each).

Influence of growth regulators on metabolite accumulation

Contents of phenolics and flavonoids were analyzed spectrophotometrically in freeze-dried cell samples from shake flask cultures conducted with different media. Therefore, the suspension cell cultures were harvested after 31 days of cultivation, frozen, dried, extracted, and prepared for phenolics and flavonoids estimation using the Folin-Ciocalteu assay, and an aluminum chloride assay, respectively (Marinova et al. 2005). Cells that were cultivated in N2 media had the highest content of phenolics although a similar concentration was found also in cultures with DN1 media (3.73 versus 3.51 mg g⁻¹). The cultures grown on N1 media had the lowest content of phenolics (3.19 mg g⁻¹). Interestingly, also the flavonoid content was the highest in cultures grown on N2 media (475 µg g⁻¹), followed by cultures grown on N1 (446 µg g⁻¹) and DN1 media (413 µg g⁻¹, Fig. 4). The data showed that no statistically significant differences appeared between the different PGRs on the production of phenolics and flavonoids.

As an auxin, 2,4-D or NAA is frequently used. However, the growth regulator 2,4-D has been shown to inhibit the production of secondary metabolites in a large number of cases (Smetanska 2018). The elimination of 2,4-D or its replacement by NAA enhanced the production of anthocyanins in the suspension of *Daucus carota* and anthraquinones

Fig. 4 Secondary metabolite contents in dried samples of *L. schweinfurthii* suspension cultures grown in shake flasks: (a) phenolics, (b) flavonoids, and (c) lupeol. No significant difference at $P \leq 0.05$



in *Morinda citrifolia* (Zenk 1978). However, 2,4-D stimulated anthocyanin production in *Oxalis linearis* (Meyer et al. 1995). 2,4-D had been used to induce rosmarinic acid accumulation in *Salvia chamelaeagnea* (Huang et al. 2009). Xu et al. (2008) found that supplementation of suspension culture of *Agastache rugosa* (Kuntze) with 2 mg L^{-1} 2,4-D was the best combination for suspension cell growth and producing rosmarinic acid in 100 mL shake flask cultures.

In our experiments, we found that *L. schweinfurthii* cell cultures produce lupeol and that the growth regulators influence this process. The highest content of lupeol was measured in cell cultures grown in N2 medium ($106 \mu\text{g g}^{-1}$ —Fig. 4), followed by cultures grown in N1 medium. The lowest amount ($83 \mu\text{g g}^{-1}$) was found when cultures grew on DN1 media. The lupeol content in the studied media did not show any significant differences.

Pérez-González (2019) demonstrated production of lupeol in plant cell cultures of *Cnidioscolus chayamansa*. In callus cultures, lupeol was induced and identified in the cultures of *Vernonanthura patens* (Kunth) after 20 days of cultivation (Chóez-Guaranda et al. 2021). Also, interestingly, the plant growth regulator NAA ($0.01\text{--}0.1 \text{ mg L}^{-1}$) stimulated lupeol production in in vitro shoot cultures of *Clinacanthus nutans* to $950\text{--}3,930 \mu\text{g g}^{-1}$ from $40 \mu\text{g g}^{-1}$ in pot-grown plants (Wongmaneeeroj et al. 2023). However, due to the unknown regulatory mechanisms, it remains unclear whether results with other cell lines can be compared with results from our study.

Our results show that the plant growth regulators are sensitive to and enhance the productivity of phenolics, flavonoids and lupeol in cell suspensions of *L. schweinfurthii* in shake flasks. The results gave us confidence in selecting a media composition that was further applied in the scale-up to the rocking-motion bioreactor cultivation.

Scale up in a rocking-motion bioreactor cultivation

Biomass accumulation

For the scale-up in an aerated non-limited fed-batch culture in a 2D rocking-motion single-use bioreactor, N2 medium was selected as the shake flask cultivations showed the highest content of phenolics, flavonoids and lupeol and also a good growth performance (i.e. cell viability).

The bioreactor was inoculated with a fresh, viable and friable *L. schweinfurthii* callus culture of 283.5 g into a manufacturer's single-use bag containing 9 L of N2 medium. The cultures were incubated at room temperature of $25 \pm 1 \text{ }^{\circ}\text{C}$ at a rocking rate of 15 rpm and continuous headspace aeration of 0.2 L min^{-1} . All samples were analyzed in 3 technical replicates. During the first week of cultivation, the biomass

fresh weight as determined with cells separated from media by filtration was low ($26.2\text{--}31.9 \text{ g L}^{-1}$). This was likely due to a major part of larger aggregates, which settled down in the middle of the bag when rocking was stopped during sampling. Afterwards, this problem was less prevalent as the agglomerates detached over time. As a consequence, the cell number increased as well as the total mass from day 8 until day 30 (at culture harvest). The highest volumetric increase in fresh biomass concentration of 42.1 g L^{-1} with a rate of $14 \text{ g L}^{-1} \text{ d}^{-1}$ was observed between days 10 and 13. The daily concentration of fresh biomass accumulation decreased to approx. 9 g L^{-1} between the 13th and 15th day of cultivation. A final fresh biomass concentration of 185.1 g L^{-1} was reached before harvest. The specific growth rate (μ) showed a peak increase between days 7 and 10 (0.26 d^{-1}) while a more significant increase was seen between days 15 and 23 (0.91 d^{-1}) of cultivation when fresh medium had been added on days 16 and 21 (Fig. 5). These results are in consistence with the findings of Trong et al. (2017) where the addition of PGRs (e.g. NAA) and other medium contents during the cultivation enhanced the biomass accumulation of *Panax vietnamensis* suspension cultures approximately twofold. In our culture, the addition of fresh medium to the bioreactor (i.e. by replacing one-third of the medium with fresh medium) maintained the growth and activity of *L. schweinfurthii* cells.

As an indication of the cells' activity, the consumption of sucrose was measured during the course of the cultivation. In *L. schweinfurthii* cell cultures, half of the sucrose (approx. 15 g L^{-1}) was consumed within 23 days while approx. 12 g L^{-1} of sucrose was consumed between days 23 and 30. In total, 90% of the sucrose was consumed during the cultivation, while 3 g L^{-1} sucrose was left at the end (Fig. 5). In case of the volumetric growth, similar values were obtained between scales. The period during which fresh media was added (fed-batch mode) in the rocking-motion bioreactor has to be excluded from the comparison between flask cultures in batch mode. Transferability as seen here has been shown before, e.g. the biomass of *N. tabacum* suspension cultures had similar rates of growth and productivity in a single-use stirred tank bioreactor in comparison to shake flask cultivations (Girard et al. 2006).

Other studies described a rather different growth of plant cell cultures in shake flasks and bioreactors. A 12-fold increase in the biomass of transgenic barley suspension cultures was observed in a 1D rocking-motion bioreactor cultivation in comparison to shake flask cultures with 125 mL of media (Ritala et al. 2008). It might be that a certain power input is required to ensure transferability, however, the data in the literature is not sufficient to underline this hypothesis.

The SCV of *L. schweinfurthii* cells in the rocking-motion bioreactor was low in the first week of cultivation,

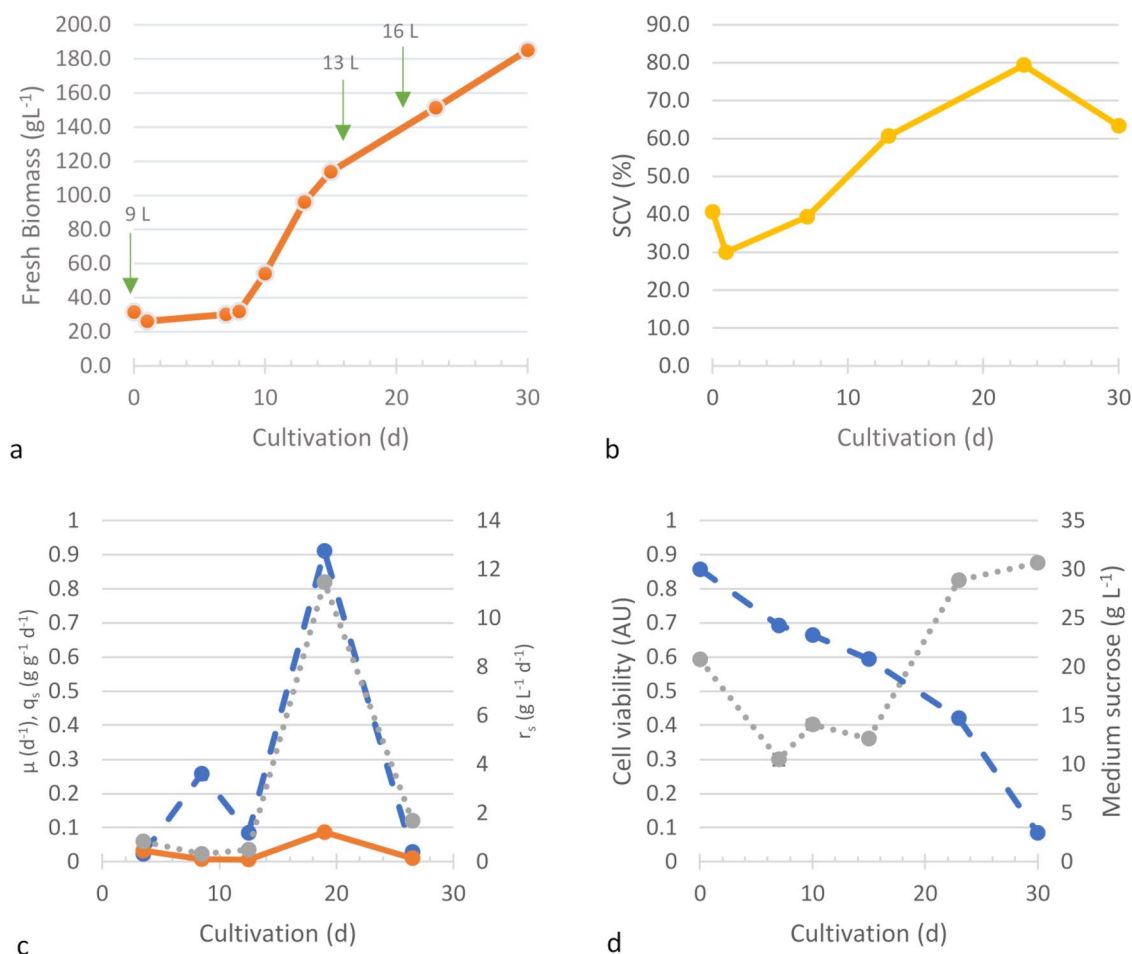


Fig. 5 Growth performance of *L. schweinfurthii* cells cultivated in a rocking-motion bioreactor: **(a)** biomass accumulation and the working volume at the selected points (green arrows), **(b)** SCV, **(c)** specific growth rate (μ , blue dash line), specific substrate consumption rate (q_s ,

orange solid line), and volumetric consumption rate (r_s , grey dot line), and **(d)** cell viability (grey dot line) and sucrose concentration (blue dash line)

comprising 30–40%. Then, the SCV increased steadily to 80% on day 23 before decreasing to 60% when the culture was terminated (Fig. 5). The cell viability of bioreactor cultures decreased by half on the first day of cultivation and then increased to 65% in the next two weeks. The cells showed a 1.5-fold higher viability compared to the initial viability. The results revealed a relation between a high specific growth rate and viability, and the addition of fresh medium on days 16 and 21 (Fig. 5).

Significant differences in plant cell activity and development between the flask and the bioreactor scale have been observed by different research groups. The obtained *Rubia tinctorum* cell biomass obtained from a 1.5 L STR operating at 400 rpm was 29% lower than that obtained in 250 mL shaking flasks at a 100 rpm shaking rate. The growth, activity, and biomass of cell cultures were negatively affected in

the STR bioreactor in comparison to shake flasks (Busto et al. 2008). Similarly, with cell cultures of *Azadirachta indica*, the cell growth and viability were lower in STR cultivations compared to shake flask cultures. It seems that the higher shaking rate (120 rpm) reduced the viability and growth (Villegas-Velázquez et al. 2017). At a stirring of 800 rpm, limonoid production was reduced in the STR compared to an operation of 400 rpm. In contrast to those conditions, the shear rate in the 2D rocking-motion bioreactor as applied in our study is in a similar order of magnitude to that in shake flasks: Zhan et al. (2019) calculated the maximum shear force in a 1D rocking-motion bioreactor of 5 L working volume to be between up to 0.06 Pa in an aqueous solution at 30 rpm and an angle of 7°, similar to this study. It might be that the values are a bit higher in 2D rocking-motion though. The average shear stress in cultivations in unbaffled flasks

is approx. 0.1 Pa or higher, and hence in a similar order of magnitude as in rocking-motion bioreactors (Liu et al. 2016a). It was shown that the maximum shear stress that cells are exposed to in a lab-scale stirred tank reactor can be beyond 10 Pa at the impeller tips (Liu et al. 2016b). So remarkable differences, most likely relevant for many plant cell cultures, do exist with respect to maximum shear rates between a rocking-motion and a STR.

Accumulation of bioactive compounds

In order to investigate the changes of phenolics and flavonoids during the cultivation process between the 1 L flask and bioreactor scale, the phenolic and flavonoid contents were estimated during the cultivation period of 30 days. Due to the low weight of the samples taken during the cultivation, the phenolic and flavonoid contents were estimated in fresh-weighted vacuum-filtered cells. The phenolic and flavonoid content in shake flask and bioreactor cultures showed significantly different values during the whole cultivation time. The highest content of phenolics was estimated in the flask scale at the beginning of the cultivation on day 2 (757.4 $\mu\text{g g}^{-1}$). The total phenolic content decreased gradually until it reached its minimum of 307.2 $\mu\text{g g}^{-1}$ on day 30. Similarly, the highest content of flavonoids of 149 $\mu\text{g g}^{-1}$ was also obtained on day 2. The flavonoid content was declining to its minimum of 78.2 $\mu\text{g g}^{-1}$ on day 24 and then increased non-significantly to 104.3 $\mu\text{g g}^{-1}$ on day 30. The estimated phenolics and flavonoids content of cell cultures in the bioreactor at the culture's onset were 781.2 $\mu\text{g g}^{-1}$ (almost equal to that in flask cultures), and 42.8 $\mu\text{g g}^{-1}$ (one-third lower than that in flask cultures), respectively. The phenolic content changed infrequently by increasing to 1,044 $\mu\text{g g}^{-1}$ on day 7 and decreasing steadily to 748.3 $\mu\text{g g}^{-1}$ on day 17. The content of phenolics increased significantly on days 21, and 30 where 1,277.9 $\mu\text{g g}^{-1}$ and 1,243.8 $\mu\text{g g}^{-1}$

were measured in the extracts, respectively (Fig. 6). Otherwise, the flavonoid content increased steadily in the first 21 days during which 74.3 $\mu\text{g g}^{-1}$ was obtained. Then, the flavonoid content increased significantly to 347.1 $\mu\text{g g}^{-1}$ on day 30. Despite the convergence in the phenolic content at the beginning of the incubation period in the bioreactor and shake flasks (781.2 $\mu\text{g g}^{-1}$, and 757.4 $\mu\text{g g}^{-1}$, respectively), the final content of phenolics on day 30 in the bioreactor cultivation was four times greater than in shake flask cultures (1,243.8 and 307.2 $\mu\text{g g}^{-1}$, respectively). Furthermore, the flavonoid content of the harvested samples on day 30 was 3-fold higher in the bioreactor cultivation (347.1 $\mu\text{g g}^{-1}$) than in flasks (104.3 $\mu\text{g g}^{-1}$). The production of plant secondary metabolites is affected mainly by biotic and abiotic stress factors. The drop in phenolics, flavonoids and lupeol during the first week in shake flask cultures was expected due to the enrichment of nutrients or the absence of stress factors (Narayani et al. 2017). Otherwise, the increase of metabolites in the bioreactor may be related to other factors (e.g., aeration, rocking rate, etc.).

The content of lupeol increased up to 0.58 mg g^{-1} in the bioreactor cultivation, which was over 5 times higher than those in shake flask cultures (Fig. 7). The increase of lupeol and phenolics contents from shake flask to bioreactor cultivations was similarly observed in studies of Busto et al. (2008). The observed differences are significant at $P \leq 0.05$ (Table 1).

One issue that is often discussed when plant cell lines are applied in STRs is that the shear rate not only affects cell growth as discussed before, but also might affect the presence of secondary metabolites. This time, an increase might be due to a higher rate of cell lysis. Hooker et al. (1989) showed that a significantly higher concentration of phenolics was measured in *N. tabacum* cell cultures that had been exposed to shear rates of over 600 s^{-1} for 2 h. Shear rates for rocking motion bioreactors have been reported as being in the order of about 60 s^{-1} , so low enough to prevent significant cell lysis (Zhan et al. 2019). In a similar 2D rocking-motion reactor setup, but with a much higher power input than in our study, Hillig et al. (2014) still measured a substantially lower portion of lysed cells of the heterotrophically grown algae *Cryptocodinium cohnii* compared to a lab-scale STR cultivation as revealed by flow cytometry. In the case of shake flask cultures, approximate values below 100 s^{-1} were present under the conditions that have been applied in our experiments, in particular through the high filling level of 50%, if correlations are assumed, as published by Giese et al. (2014). It is then unlikely that any elevated production and/or release of secondary metabolites in the bioreactor cultivation is caused by shear rates, that either would have led to damage or even lysis. A reason for the difference in secondary metabolites' concentrations lies, most

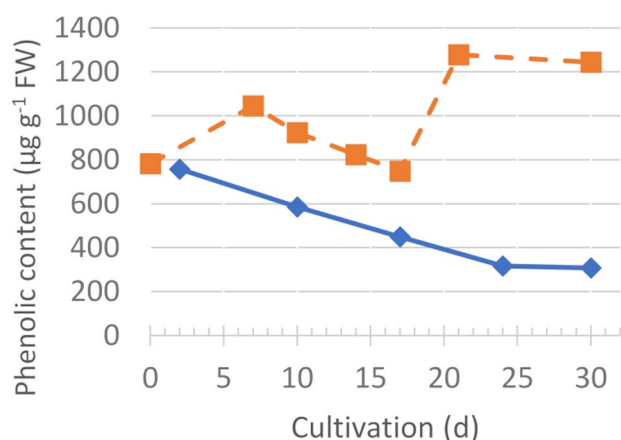
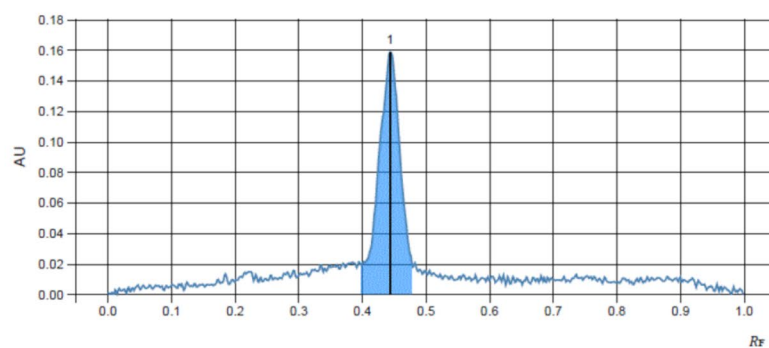
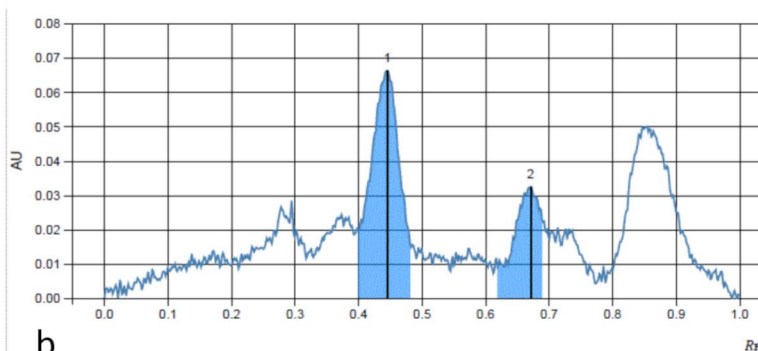


Fig. 6 Total phenolic content of fresh samples in shake flask (blue solid line) and bioreactor cultures (orange dash line) on N2 medium

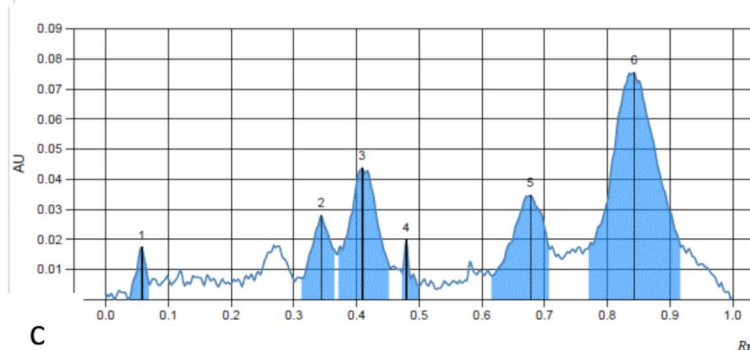
Fig. 7 HPTLC chromatograms of (a) lupeol (500 ng per band; R_f 0.444), (b) *L. schweinfurthii* bioreactor cell culture (30 days cultivation) extract (400 μ g per band), (c) 1 L shake flask cultures (30 days cultivation) extract (800 μ g per band), (d) chromatogram of bioreactor cell culture extract, and (e) chromatogram of DN1 flask cell culture extracts against lupeol after derivatization with anisaldehyde- H_2SO_4 reagent at 366 nm; mobile phase: petroleum ether-ethyl acetate-toluene (7:2:1, v/v/v)



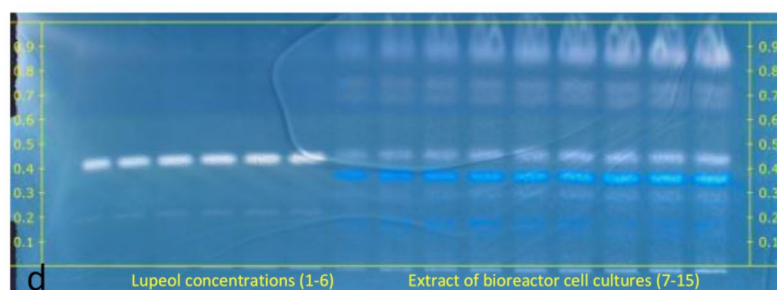
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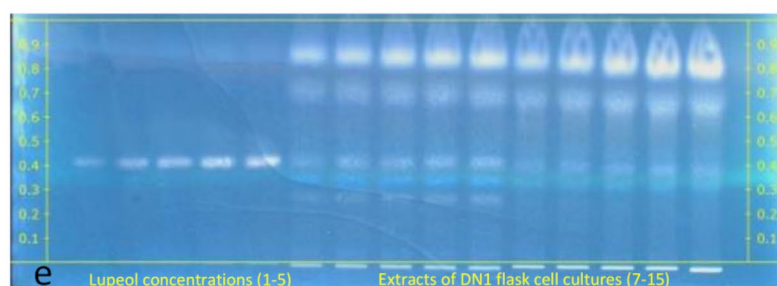
b



c



d



e

Table 1 Phenolics, flavonoids and lupeol content in dried cells cultivated in a rocking-motion bioreactor after 30 days of cultivation

Bioactive compounds	Amount (mg g ⁻¹)	Folds of flask-scale	Total amount (mg harvest ⁻¹)
Total phenolics	5.03 ± 0.15	1.4	1,500
Total flavonoids	0.80 ± 0.13	1.7	237
Lupeol	0.58 ± 0.12	5.5	173

likely, in the different air supply. It can be assumed that the dissolved oxygen concentration in the bioreactor cultivation was higher than in the shake flask cultures, which were not actively aerated. The cells' response to these elevated oxygen concentrations might have led to a comparably stronger synthesis of secondary metabolites. Increased synthesis of phenols and flavonoids at higher oxidative reactivity has been described for several plant cell lines (Saini et al. 2024). Hence, the fed-batch cultivation of *L. schweinfurthii* cells in the rocking-motion bioreactor was suitable to increase the phenolics, flavonoids and lupeol concentration remarkably.

Conclusion

The present study describes the media choice for and comparison of the mass propagation of *L. schweinfurthii* cells in shake flasks and a rocking-motion bioreactor suspension culture. Among the studied media, the N2 culture medium, based on MS formulation and containing 2 mg L⁻¹ NAA, was selected as the optimal one for fast growth, high cell viability, and intensive synthesis of phenolics, flavonoids, and triterpenoid lupeol of *L. schweinfurthii* cell cultures. The viability of suspension cells was negatively affected by the application of NAA during the first two weeks of cultivation. Therefore, any short-term cultivation of cell cultures of *L. schweinfurthii* is not recommended. The flask and bioreactor cultivations of *L. schweinfurthii* have proven to be effective for obtaining biomass and producing bioactive compounds (e.g., phenolics, flavonoids, lupeol). However, bioreactor cultures have an advantage over flask cultures in producing identical cells with higher levels of phenolics, flavonoids, and lupeol due to continuous aeration, type of power input, and the supply of fresh media in a fed-batch mode, while other parameters were maintained similarly across the two scales. Continuous feeding under non-limiting carbon conditions might be beneficial to achieve substantially higher biomass and product yields, as indicated already in this study when fresh media was added to the bioreactor cultivation. This might be explored further in future work.

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Smetanska: Writing– review & editing, Conceptualization, Methodology, Supervision. All authors have read and agreed to the published version of the manuscript.

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Data availability No new data were created or analysed during this study. Data sharing is not applicable to this article.

Declarations

Competing interests Peter Neubauer and Stefan Junne are shareholders of Celltainer Biotech. The authors declare no further conflict of interest.

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