



Growth parameter optimization of hybrid poplar (*Populus tremula* × *Populus alba*) hairy root culture for the production of antioxidant specialized metabolites

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Abstract

Hairy roots cultures resulting from transformation by the soil bacteria *Rhizobium rhizogenes* represent a biotechnological tool to produce specialized bioactive metabolites. We previously developed a protocol allowing the sustainable production of hairy roots from hybrid poplar 717-1B4 (*Populus tremula* × *Populus alba*). In the present study we assessed physicochemical parameters for the optimization of growth of hybrid poplar HP15-3 hairy root line and production of ethyl acetate extracts with antioxidant potential. The use of Woody Plant Medium (WPM) as mineral base significantly improved growth index by 2.1-fold compared to Murashige and Skoog (MS) medium and was selected even if a slight decrease in total phenolic content (TPC) and antioxidant activity could be observed (DPPH and FRAP assays). Among the different concentrations tested (15, 30 and 60 g·L⁻¹), sucrose at 30 g·L⁻¹ made it possible to significantly increase the growth index, without affecting the TPC and the antioxidant potential of the root extracts. Stressful conditions likely to affect the biosynthesis of specialized metabolites were also tested. The cultivation of hairy roots under LED light conditions resulted in a significant 2.45-fold increase in growth index. Antioxidant activity was also increased in relation to increased anthocyanin production. Furthermore, we demonstrated the feasibility of poplar hairy roots biomass production in temporary immersion bioreactors (RITA[®]) under two water and mineral supply conditions. UHPLC/HRMS analysis of the extracts allowed to identify and quantify two phenolic glycosides (tremulacin and nigracin) whose concentrations were increased during cultivation under LED light. Two flavonoids (cyanidin and cyanidin-3-glucoside) were identified in light-grown root extracts. The parameters optimized during this research work could be successfully transposed to produce root biomass in bioreactors and under light to obtain bioactive ingredients for cosmetic formulations.

Key message

LED light and optimized medium composition promoted hybrid poplar hairy root growth while leading to the production of anthocyanins and increased antioxidant activity of the organic extracts.

Keywords *Rhizobium rhizogenes* · Carbohydrate · Lighting · Mineral composition · Temporary immersion bioreactor · *Salicaceae*

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Introduction

Plants are known to produce bioactive specialized metabolites with protective or interacting roles in their environment. Those active molecules can also be used for human well-being as food, cosmetics or pharmaceuticals. In most cases, these metabolites are extracted from the aerial parts of herbaceous plants and trees (bark, buds and leaves) collected in natural habitats, cultivated areas, or produced by biotechnology (D'Amelia et al. 2021). Roots which are highly exposed to biotic and abiotic stress-inducing conditions are also rich in a range of specialized metabolites synthesized for long-term survival that may differ from those accumulated in aerial parts (Bishopp and Lynch 2015). Using roots grown in natural environments requires uprooting, which results in the destruction of the entire plant. This can be impactful for slow-growing herbaceous species or endangered species such as *Podophyllum hexandrum* harvested for its richness in podophyllotoxin, a precursor to anticancer drugs (Farkya et al. 2004). In most cases, low root yield and long growth period also limit the harvest of desirable bioactive ingredients in sufficient quantities. In the context of global climate change leading to the increase of abiotic stresses such as drought, a drastic plant growth decrease is already visible affecting plant root availability as metabolites sourcing. It is then necessary in this context to protect plant resources and to use biotechnological approaches to obtain higher biomass within a short time span and without depending on environmental conditions. To overcome these biomass supply problems, hairy root culture systems can provide an all-year-round accessible source of specialized metabolites from roots (Malik 2017).

Over the past decade, the use of biotechnology in the cosmetics industry has increased due to the sustainable production of large quantities of biomass (Georgiev et al. 2018; Malarz et al. 2023). Indeed, in vitro multiplication of plants is independent of environmental constraints and requests less space and resources (Chen and Hu 2006). Hairy roots are transformed roots obtained after plant cell infection by *Rhizobium rhizogenes*. When in contact with plant wounds, T-DNA is transferred to the plant genome. The generated roots are capable of autonomous growth in vitro without the need of any growth regulator (Georgiev et al. 2018). In this regard, hairy root culture seems one of the most promising strategies to produce root biomass and further valorization of bioactive compounds (Benyammi et al. 2016; Malik 2017; Roy 2021). Studies on the specialized metabolites production revealed that a similar to higher quantity of molecules of interest are produced from hairy roots cultures (Malarz et al. 2023). Indeed, molecules that are naturally present only in the aerial parts of the plants have been found in *Lawsonia inermis* hairy roots (Bakkali

et al. 1997). For *Prunella vulgaris*, the production of rosmarinic acid was found to be 10–15 times higher in hairy roots than in the original plant (Ru et al. 2016). Hairy root cultures are also known for producing higher biomass than traditional cultures in a short time (Chen and Hu 2006; Malarz et al. 2023). Biosynthesis and release of specialized metabolites highly depends on the culture conditions and physical environment (Malik et al. 2011) and they need to be standardized for each plant species. Depending on the species, the needs in mineral and carbohydrate nutrients may vary (Malik 2017). The concentration and nature of carbohydrate source as well as the mineral supplies have to be adjusted to obtain an optimized growth. Light has also been demonstrated to have an impact on growth but also the production of specialized bioactive metabolites in hairy roots (Abbasi et al. 2007; Liu et al. 2002).

Numerous bioactive compounds found in woody plant species offer remarkable potential for use in pharmaceuticals (Abiri et al. 2021; Duh et al. 2012; Malik et al. 2011; Metsämuuronen et al., 2019; Movahedi et al. 2021; Tebbi and Debbache-Benaida 2022). Likewise, poplar (*Populus*, family Salicaceae) is an important tree species distributed worldwide. Apart from its ecological benefits it is cultivated to produce wood used to manufacture, packaging, biofuels and paper pulp (Devappa et al. 2015) and is a source of biologically active compounds (Guleria et al., 2021). Among these biological activities, antioxidant, anti-inflammatory and antibacterial properties are well described in the *Populus* genus (Stanciu et al. 2023; Zhang et al. 2006a, b; Zhong et al. 2012) and are mainly due to the presence of specialized metabolites such as phenolic compounds (gallic acid, cinnamic acid, etc.) including flavonoids (apigenin, kaempferol) (Devappa et al. 2015; Scaysbrook et al. 1992; Guleria et al. 2022; Stanciauskaite et al. 2021). However, up to now, the poplar root compartment has been poorly studied, especially in relation to specialized metabolites contents. Poplars are particularly sensitive to increasing drought in their environment (McDowell et al. 2022) and natural populations may be threatened in the future. Collecting roots in the natural compartment is not possible since it is destructive. Yoshida et al. (2015) demonstrated the presence of anthocyanins in hybrid poplar hairy root lines. Popovic et al. (2016) highlighted the effect of drought on the production of polyphenols in the roots of poplars grown hydroponically. Recently, we have reported the production of phenol glucosides (tremulacin, trichocarpin), flavonoids (catechin, procyanidin) and other phenolic compounds in poplar 717-1B4 hairy roots which are bioactive compounds with antioxidant properties (Laffon et al. 2024).

Under in vitro culture conditions, several parameters such as the composition of the culture medium, can have an impact on plant tissues and organ growth. Employing

optimal culture conditions can enhance the hairy root biomass production. The present study was designed to determine the impact of physicochemical factors including basal media and sucrose concentrations on hybrid poplar hairy root growth and specialized metabolites production. We also tested the effect of several stresses on those roots by growing them under light. We demonstrated the feasibility of scaling up the production of hybrid poplar hairy root biomass in temporary immersion bioreactors. These culture systems could make it possible, by limiting the frequency of immersion, to mimic water stress conditions likely to impact the production of specialized metabolites. The parameters optimized during the present study could be successfully maintained for large-scale commercial use in bioreactors to produce bioactive ingredients for cosmetic and pharmaceutical industries.

Materials and methods

Plant material

Hairy root line HP15-3 was selected as a plant material. This line was obtained by the transformation of hybrid poplar (*Populus tremula* × *Populus alba* 717-1B4 clone) with the *Rhizobium rhizogenes* strain 15,834 as reported earlier by our group and its transgenic state was confirmed by PCR amplification of *rolB* and *rolC* oncogenes (Laffon et al. 2024).

HP15-3 hairy root line was maintained by regular subculturing on MS reference liquid medium (Murashige and Skoog, supplied by DUCHEFA, ref M0255, 4.9 g·L⁻¹) supplemented with sucrose (30 g·L⁻¹), glutamine (0.2 g·L⁻¹), Polyvinylpyrrolidone (PVP, 4.0 g·L⁻¹) and Morpholino ethane sulfonic acid (MES, 0.5 g·L⁻¹). The pH was adjusted to 5.7 before autoclave. Cultures were carried out in a dark culture room at 26 °C on a rotary shaker (Ohaus SHHD6850DG, 51 mm orbital) at 70 rpm (Laffon et al. 2024).

Influence of basal medium composition and sucrose concentration

To study the effect of basal medium composition (macro- and micronutrients, vitamins), MS reference medium and WPM medium (Woody Plant Medium, supplied by DUCHEFA, ref. M0220) were used at full strength, at the concentration indicated by the manufacturer (4.9 g·L⁻¹ and 2.4 g·L⁻¹, respectively), with sucrose (30 g·L⁻¹).

To evaluate the effect of carbohydrate concentration on hairy root growth, 3 concentrations of sucrose were tested: 15, 30, and 60 g·L⁻¹ with WPM as the basal medium.

For each experiment, 2.60 g of hairy roots were used as starting biomass and cultured on liquid medium as mentioned above. After 3 weeks of culture, the medium was discarded and replaced with fresh medium for a period of 2 weeks. Cultures were harvested after a total cultivation time of 35 days. After harvesting, biomass was lyophilized for further extraction and phytochemical analysis. After the lyophilization process, roots were weighted to calculate the dry weight Growth Index (GI) with the following formula: $GI = \text{Final dry weight} / \text{Initial dry weight}$. Each experiment was run in triplicate.

Effect of light

To study the effect of light on growth and phytochemical profile of hairy roots, the roots were inoculated in WPM culture medium with sucrose (30 g·L⁻¹). For technical reasons, the experiment was carried out in a phytotronic chamber at 24 °C equipped with LED lights (Vegeled Bi-phosphorus white 4 K-N1) at 180 μmol·m⁻²·s⁻¹ photosynthetically active radiation on a rotary shaker (KS501 digital, IKA labortechnik, 30 mm orbital) at 70 rpm under a 16 h photoperiod. The control Erlenmeyer flasks were kept in the dark with aluminum foil. The experiment was conducted as before (2.6 g starting biomass, replacement of the medium after 21 days, harvest at day 35) in triplicate.

Effect of immersion frequency in temporary bioreactors

HP15-3 hairy root line was cultivated in temporary immersion bioreactors (RITA[®], Vitropic, France) as an alternative to classic cultivation conditions. Immersion duration was 3 min, and two immersion frequencies were tested: 4/24 h and 8/24 h. Initial fresh biomass varied between 3 and 6 g. Final biomass was collected after 42 days.

Viability assay and microscopic observations

Root viability was assessed using the fluorescein diacetate (FDA) probe. After immersion for 5 min in a FDA solution (0.01 mg·mL⁻¹), the roots were observed under an inverted epifluorescence microscope (Axio observer 3, Zeiss, 500/25 nm bandpass excitation filter; bandpass emission filter 535/30 nm band). The roots were also observed under transmitted light.

Preparation of root extracts

Ethyl acetate was primarily chosen as the solvent to extract moderately polar compounds (Gil-Martin et al., 2022) and prevent too many sugars present in the root tissues from

being extracted at the same time. It was used for all phytochemical analyses. Hairy roots were collected from liquid cultures, rinsed and frozen before freeze-drying. The dry hairy roots were then ground into a fine powder using a mortar. The root powder was then extracted with an ethyl acetate solvent to extract moderate polar compounds, at a ratio of 1:40 with sonication at 80 kHz for 1 h. Acidified ethanol is more appropriate for the extraction of anthocyanins, whose color is preserved at acidic pH. This solvent was chosen for the phytochemical characterization of roots grown under LED lighting conditions. To obtain anthocyanin-enriched extracts the powder was extracted with absolute ethanol acidified with 0.1% acetic acid at a ratio of 1:40 with sonication at 37 kHz for 15 min followed by a 2-h maceration. The liquid extracts were collected and evaporated under nitrogen flux for 24 h. The dried extracts were frozen, lyophilized, weighed and dissolved in absolute ethanol to a final concentration of $10 \text{ mg}\cdot\text{mL}^{-1}$. These samples were filtered using $0.25 \mu\text{m}$ filters before phytochemical analysis.

Determination of total phenolic content

Total phenolics content (TPC) was determined by the Folin-Ciocalteu method. Absolute ethanol was used as a blank and solutions of gallic acid in the same solvent were used as standards. A calibration curve was established from ethanolic solutions ranging from $0.6 \text{ mg}\cdot\text{mL}^{-1}$ to $0.1 \text{ mg}\cdot\text{mL}^{-1}$. Twenty μL of sample at a concentration of $10 \text{ mg}\cdot\text{mL}^{-1}$ was added into a well. Ten μL of Folin solutions were added in all the wells. The plate was incubated at room temperature for 8 min. Then 30 μL of sodium carbonate solution at 20% in water was added and the wells were filled with 140 μL of ultrapure water. The 96 well plate was incubated in the dark at room temperature for 2 h and the absorbance was read at 760 nm using a μQuant microplate spectrophotometer from BioTek. The results were expressed as $\text{mg}\cdot\text{g}^{-1}$ of dry extract gallic acid equivalent (GAE). The assay was run in triplicate.

FRAP and DPPH antioxidant activity assays

FRAP assay

The free radical scavenging activity using FRAP assay was determined based on a method reported by Benzie and Strain (1996). FRAP solution was prepared as follows: a buffer solution at $360 \text{ mg}\cdot\text{L}^{-1}$ sodium acetate was prepared in 1.6% acetic acid aqueous solution. A second solution was prepared by adding iron (III) chloride to the previously prepared solution at $54 \text{ mg}\cdot\text{L}^{-1}$. A third solution was prepared with $2.9 \text{ g}\cdot\text{L}^{-1}$ 2,4,6-Tris(2-pyridyl)-s-triazine in 0.3% hydrochloric acid aqueous solution. The FRAP solution was

obtained from the three solutions following the proportions 80/8/8 (v/v/v). Ten μL samples at the concentration of $10 \text{ mg}\cdot\text{mL}^{-1}$ and 190 μL FRAP solution were added into the wells. Absolute ethanol was used as a blank and solutions of Trolox in absolute ethanol at 1.0, 0.5, 0.3 and $0.1 \text{ mg}\cdot\text{mL}^{-1}$ were used as standards. Trolox $1 \text{ mg}\cdot\text{mL}^{-1}$ was used as a reference for 100% activity. The plate was incubated in the dark at room temperature for 30 min and the absorbance was measured at 590 nm. All the assays were run in triplicate. The results were expressed in % of activity as compared to Trolox.

DPPH radical scavenging assay

A solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) at $788.6 \mu\text{g}\cdot\text{mL}^{-1}$ was prepared. Ten μL of extract were added in the well and completed with a volume of 190 μL of DPPH solution. Absolute ethanol was used as a blank and solutions of Trolox in absolute ethanol at 1.0, 0.5, 0.3 and $0.1 \text{ mg}\cdot\text{mL}^{-1}$ were used as standards. The 96 well plate was incubated in the dark at room temperature for 30 min and the absorbance was read at 516 nm. All the assays were run in triplicate. The results were expressed as percentage inhibition.

HPLC analysis

Hairy root extracts from different biological conditions were analyzed and compared using the HPLC-DAD method, with the target compounds being mainly phenolic compounds. Analysis was performed on the VWR Hitachi Chromaster HPLC. The column used for the analysis was a Phenomenex Luna C18 $3 \mu\text{m}$, $100\text{\AA}$, $150 \times 4.6 \text{ mm}$. HPLC analysis was carried out at 40°C , with a volume of injection of 10 μL , a flow of $1 \text{ mL}\cdot\text{min}^{-1}$, and a mobile phase composition of: A: $\text{H}_2\text{O} + 0.1\%$ formic acid, B: acetonitrile + 0.1% formic acid and C: Isopropanol + 0.1% formic acid, with the following gradient: 0 min (95% A + 5% B); 20 min (100% B); 25 min (50% B + 50% C), 30 min (50% B + 50% C); 31 min (100% B); 32 min (100% B); 33 min (95% A + 5% B); 42 min (95% A + 5% B). Samples were analyzed with DAD at a wavelength from 200 nm to 600 nm and with two selected wavelengths: 280 nm and 366 nm for phenolic compounds and 520 nm for anthocyanins visualization. For quantitative analysis, mixtures of different commercial standards at 4 different concentrations, ranging from 0.1 to $2 \text{ mg}\cdot\text{mL}^{-1}$, were prepared in absolute ethanol. Calibration curves were built, representing the peak areas as a function of standard concentration for each standard. Slopes were used for quantification of identified compounds in each sample obtained from ethyl acetate extracts of hairy roots grown on different conditions with or without stress. Quantitative data

were obtained from biological duplicates of each condition. Commercial standards of tremulacin and nigracin were purchased from CIMIT QUIMICA SL (Barcelona, Spain). Catechin, chlorogenic acid, cyanidin et cyanidin glycoside were purchased from Extrasynthèse (Genay, France).

UHPLC-HRMS analysis

UHPLC-HRMS method was further used to more precisely identify specific specialized metabolites. As anthocyanin compounds produced under light were also targeted for this study, the positive ionization mode was chosen to detect both anthocyanin and phenolic compounds. UHPLC separations were performed using an Ultimate 3000RSLC system (Dionex, Germering, Germany) composed of a binary pump, an automatic sampler, a thermostated column compartment and a DAD detector. Analyses were performed using a Luna Omega C18 column (150 × 2.1 mm; 1.6 µm) (Phenomenex, Le Pecq, France) heated at 40 °C with a flow rate of 0.5 mL.min⁻¹. The mobile phase was composed of A: H₂O + 0.1% formic acid and B: ACN + 0.1% formic acid using the following gradient: 0 min (97% A + 3% B); 0.4 min (97% A + 3% B); 15.1 min (15% A + 85% B); 16.1 min (0% A + 100% B); 18.1 min (0% A + 100% B); 18.3 min (97% A + 3% B); 21.1 min (97% A + 3% B). The injection volume was 2 µL.

HRMS experiments were carried out using a maXis Q-TOF (Bruker, Bremen, Germany) hyphenated with the previous UHPLC system. Data were acquired in ESI positive ion mode in the 50–1650 *m/z* range at a frequency of 1 Hz. Source parameters were as follows: capillary voltage was set to 4.5 kV, nebulizing gas was set to 2 bar and drying gas was heated at 200 °C with a flow rate of 9 L.min⁻¹.

Data were exploited using DataAnalysis 4.4 software (Bruker) and the obtained formulas were searched using the databases PubChem, ChempSpider and Lotus.

Statistical analysis

Data were compared by ANOVA or Welch ANOVA's tests, depending upon the variance homogeneity, and separated by Post-Hoc's Tukey test. All analyses were performed with JASP open software from the University of Amsterdam (JASP team, 2022. Version 0.18.3. Amsterdam, Netherlands).

Results

Effect of culture medium composition

The impact of the modification of culture medium composition, mineral basis and sucrose concentration, on the hairy roots specialized metabolites content and antioxidant activity have also been assessed. All colorimetric assays were performed after extraction with ethyl acetate of the hairy roots grown under different conditions.

Mineral basis composition

To assess mineral basis impact on the hairy root's growth, HP15-3 liquid cultures were grown with two different mineral sources at the concentration given by the supplier. Liquid cultures grown with WPM as a mineral basis showed a significant two-fold higher growth index (GI) than the ones grown with MS (6.69 and 3.13) (Fig. 1a). A higher final biomass was observed for the hairy roots grown with WPM-based medium, but no macroscopic phenotypical differences could be observed (Fig. 1b). The viability of hairy roots was still very high after 35 days of culture for each condition as evidenced by the strong fluorescence after FDA staining (Fig. 1c). The diameter and branching of the hairy roots remained unchanged. Quantities of viable root border cells (RBCs) could be observed, gradually detaching from the root cap (Fig. 1c).

The Folin-Ciocalteu method showed a higher (although not significant) total phenolic content of hairy roots grown with MS compared to WPM as mineral basis (Fig. 2a) (24.9 and 22.0 mg.g⁻¹ GAE, respectively). For FRAP and DPPH antioxidant assays, extracts from cultures grown with MS medium showed significant higher antioxidant activity than those from cultures grown with WPM medium (Fig. 2b) (34.5% vs. 24.1% inhibition for DPPH and 57.8% and 40.3% activity for FRAP, respectively).

The comparison between MS and WPM extracts revealed no major qualitative differences in the phytochemical profile but variations in the concentration of some compounds, the more apolar compounds (retention time between 22.5 and 30 min) being more concentrated in the MS extract. (Fig. 2c and supplemental Fig. S1).

Sucrose concentration

Since a two-fold higher growth index was obtained using WPM as a mineral base, different concentrations of sucrose were tested on hairy root growth in this medium. The sucrose concentration of 30 g.L⁻¹ gave the highest GI (7.70), followed by 15 g.L⁻¹ (4.92) and 60 g.L⁻¹ (3.25) after a culture period of 35 days (Fig. 3a). The sucrose concentration

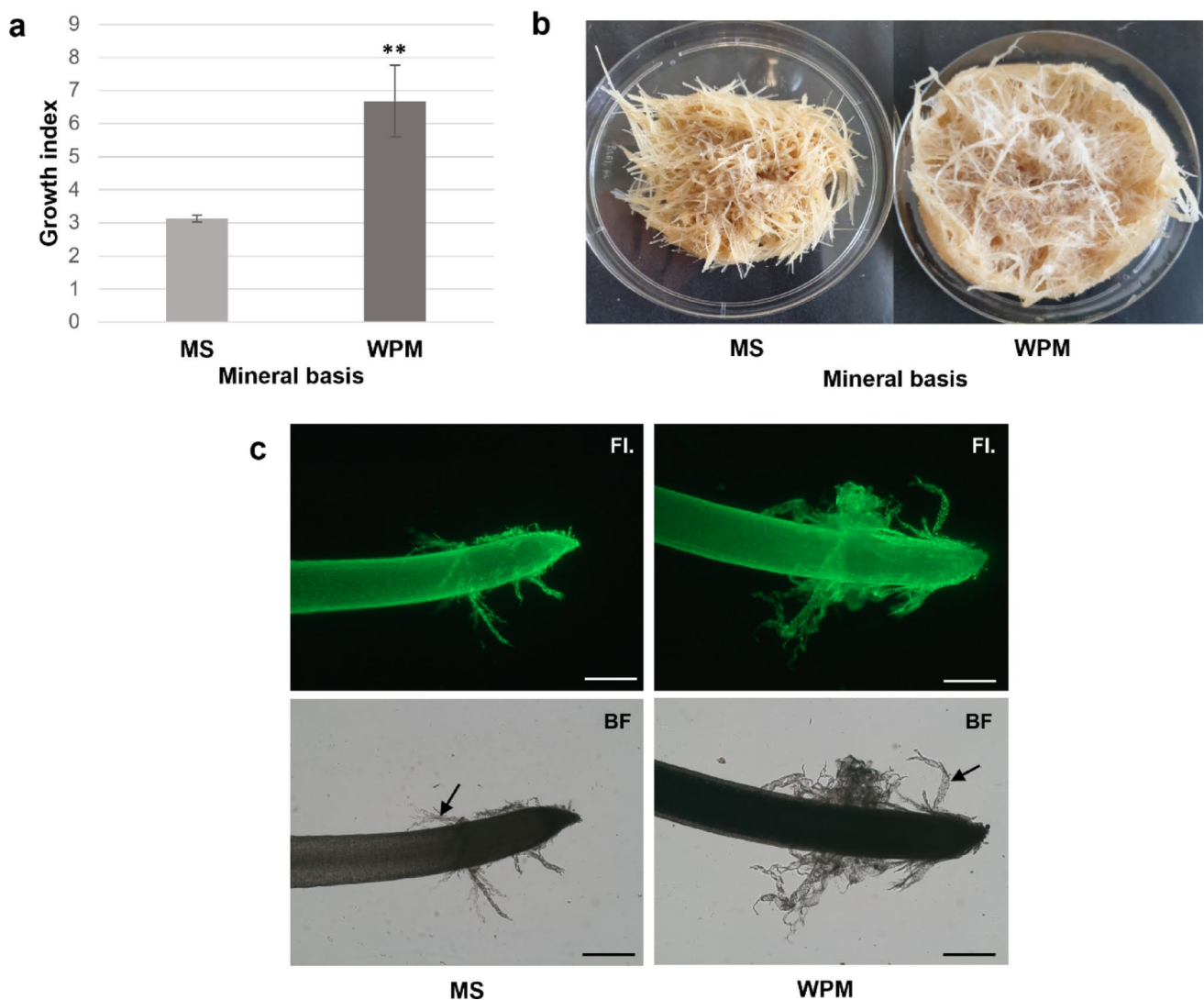


Fig. 1 Effect of macronutrient basis on HP15-3 hairy root growth. Growth index (**a**) and final biomass (**b**) of HP15-3 hairy root line grown 35 days in the dark on MS or WPM macronutrients basis supplemented with 30 g·L⁻¹ sucrose. Represented data are mean and standard deviation from biological triplicates. Root viability of HP15-3

hairy roots line (**c**) was assessed using the fluorescein diacetate (FDA) probe. Roots were observed 5 min after staining with FDA under fluorescence (FL) or bright field (BF) (Bar scale: 500 μ m). The arrows indicate the presence of Root Border Cells. ** indicates significant difference at $p < 0.01$

of the medium did not affect root phenotype (diameter and branching) or viability (Fig. 3b, c) which remained high in the cultures until day 35. RBCs, although not visible on Fig. 3c (the roots had been blotted on filter paper before microscopic observation), were present in quantity around the root tips and in the culture medium.

The total phenolic content of the extract obtained from hairy roots grown in media with 60 g·L⁻¹ sucrose was significantly lower than the ones measured for other sucrose concentrations (Fig. 4a) (16.4 mg·g⁻¹ GAE *versus* 22.7 mg·g⁻¹ GAE and 21.9 mg·g⁻¹ GAE respectively for 15 g·L⁻¹ and 30 g·L⁻¹ sucrose). However, both DPPH and FRAP assays revealed that there was no significant difference

in antioxidant activity for cultures grown with different sucrose concentrations (Fig. 4b).

HPLC analysis showed no qualitative and almost no quantitative differences in the phytochemical profile of hairy root extracts grown in media containing 15 g·L⁻¹ and 30 g·L⁻¹ sucrose (Supplemental Fig. S2). However, the reduction in peak areas revealed a lower quantity of all compounds for the extract from hairy roots grown in a medium containing 60 g·L⁻¹ of sucrose, in accordance with the reduction in TPC (Fig. 4a).

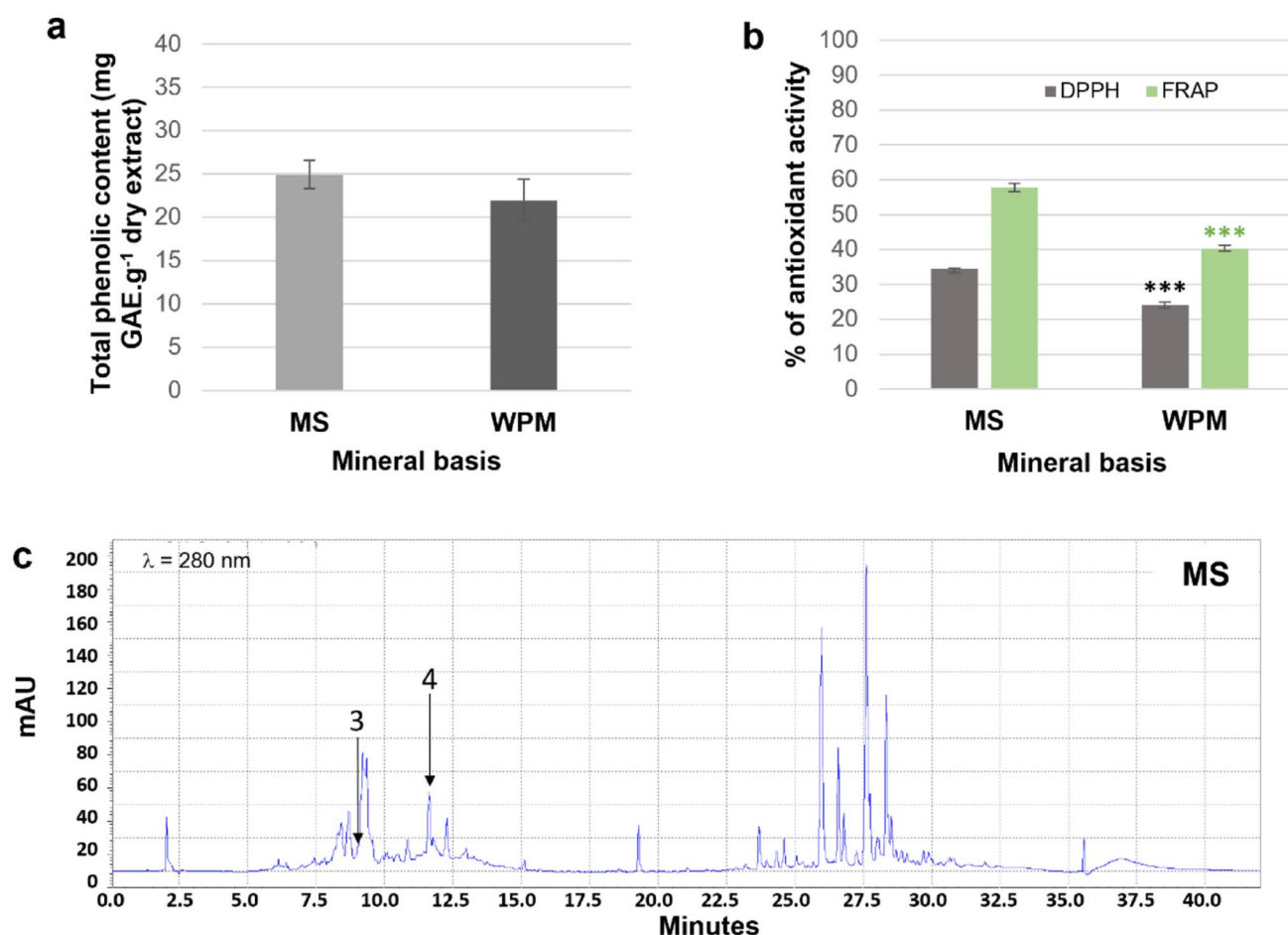


Fig. 2 Effect of macronutrient basis on HP15-3 hairy root specialized metabolites production and antioxidant activity. Total phenolic content (TPC) (**a**) and antioxidant activity (**b**) of extracts of HP15-3 hairy root line grown 35 days in the dark on MS (Murashige and Skoog) or WPM (Woody Plant Medium) macronutrients basis supplemented with 30 g·L⁻¹ sucrose. Results were expressed in mg gallic acid equivalent

(GAE) per gram of dry extract for TPC and in inhibition percentage (%) for both DPPH and FRAP assays. Represented data are means and standard deviation from biological triplicates. *** indicates significant difference at $p < 0.001$. (**c**) HPLC profile of the extract of HP15-3 grown on MS mineral base. Peak 3: nigracin, peak 4: tremulacin

Effect of lighting on root growth and production of specialized metabolites

The presence of light in a plant environment is an important parameter for growth. To assess the impact of lighting conditions on biomass production, liquid cultures were grown under LED light or obscurity (under the previously optimized culture conditions: WPM, sucrose 30 g·L⁻¹). Growth index of hairy roots grown 35 days with light was 2.45-fold higher (although non-significant) than that of roots grown in the dark (5.80 and 2.36 respectively) (Fig. 5a). The roots grown under light were predominantly pink (Fig. 5b). A pink color of cortical cells, root cap cells, as well as separated border cells could be observed under a bright field microscope, presumably due to anthocyanin biosynthesis (Fig. 5c, d). FDA staining revealed high viability of roots grown under light as well as those grown in the dark (Fig. 5c). Cultivation under

LED light did not affect the diameter and branching of hairy roots but it allowed the development of adventitious shoots. This morphogenesis process was not observed on roots grown in the dark. The shoots were then separated from the roots and subcultured on hormone free Agar-gelled half MS medium (30 g·L⁻¹ sucrose, 8 g·L⁻¹ agar). The shoots were a little stunted, they developed leaves and a dense adventitious root system with a characteristic negative geotropism (Fig. 5e).

Two extraction processes were then evaluated on both hairy roots grown under light or dark conditions. Using ethyl acetate solvent (as for previously described experiments), HP15-3 hairy roots grown under light showed a significantly higher amount of total phenolic compounds compared to hairy roots grown in the dark (34.3 mg·g⁻¹ GAE and 28.6 mg·g⁻¹ GAE, respectively) and an associated higher antioxidant activity (37.6% and 28.5% inhibition for DPPH, respectively; 84.6% and 72.2% activity for

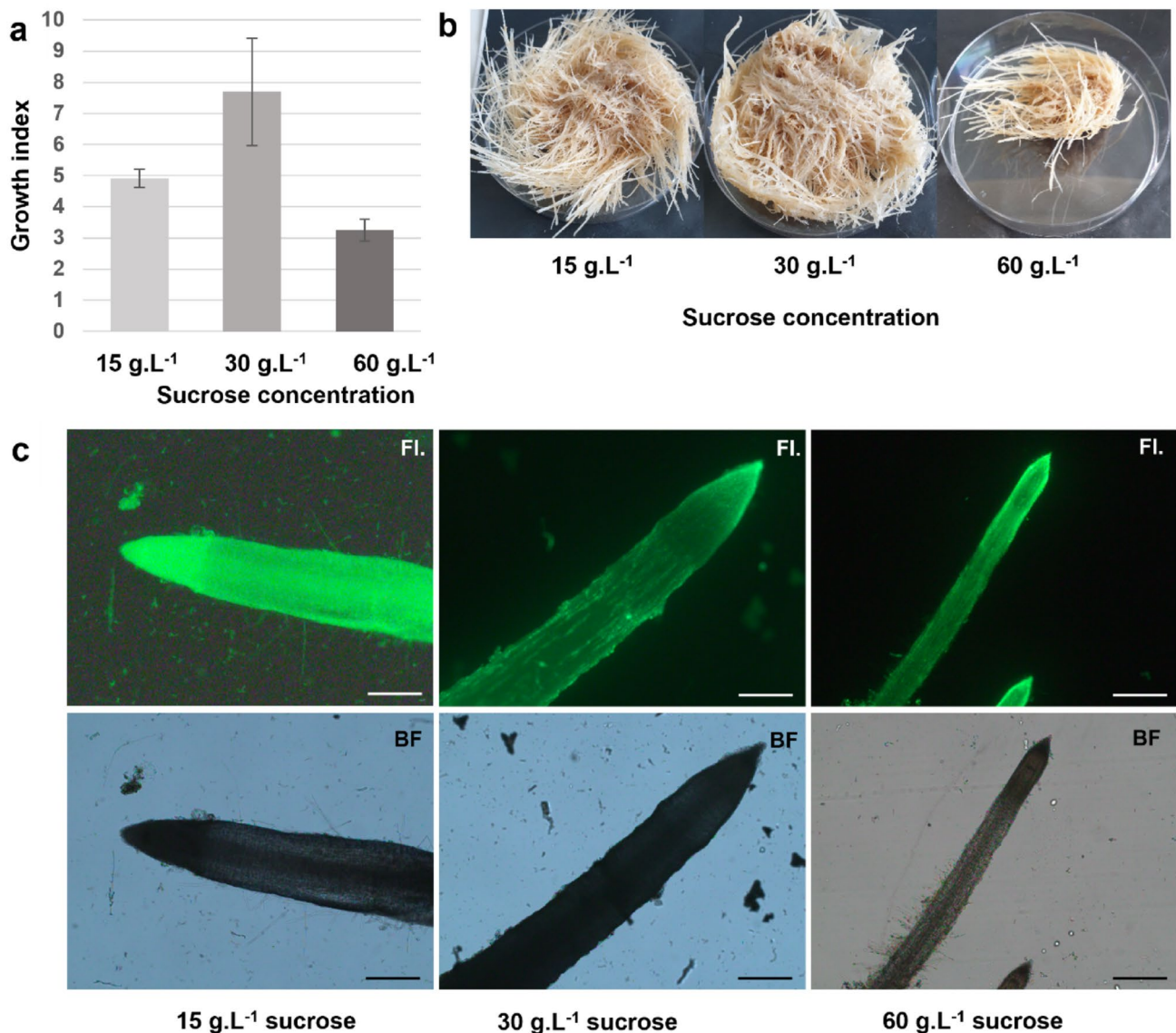


Fig. 3 Effect of sucrose concentration on HP15-3 hairy root growth. Growth index (**a**) and final fresh biomass (**b**) of HP15-3 hairy roots line grown 35 days in the dark on WPM supplemented with 15, 30–60 g.L⁻¹ sucrose. Represented data are mean and standard deviation

FRAP, respectively) (Fig. 6a, b). Comparison of the HPLC chromatograms revealed that all the compounds with a retention time between 5 and 12.5 min and between 24 and 30 min were present in higher quantities in the hairy roots grown with light than the ones grown in dark (Fig. 6c). Two compounds (dotted arrows on Fig. 6c) that have never been found in the previous analysis, possibly phenolic compounds, were found to be present in the ethyl acetate extracts of roots grown under light (retention time 6.50 min and 8.75 min).

To obtain anthocyanin-enriched extracts, the hairy root powder was extracted with absolute ethanol acidified with 0.1% acetic acid as solvent. As previously observed, hairy

roots grown under light revealed significantly higher total phenolic content (Fig. 7a) than those grown in the dark (16.59 mg.g⁻¹ GAE against 12.06 mg.g⁻¹ GAE). Antioxidant activity was also significantly increased when roots were cultivated under light (Fig. 7b). Indeed, FRAP assay showed a more than 1.6 higher antioxidant activity when roots were grown under light (67.8% and 41.2% activity, respectively) and anthocyanin-rich extract had a 1.3-fold higher inhibition percentage of free radicals for DPPH assay (51.4% and 39.2% inhibition, respectively).

The HPLC analysis with DAD revealed two peaks (5) and (6) (retention times of 5.50 and 6.25 min) absorbing at 520 nm for the ethanolic extracts of hairy roots grown

from biological triplicates. Root viability of HP15-3 hairy roots (**c**) was assessed using the fluorescein diacetate (FDA) probe. Roots were observed 5 min after staining with FDA under fluorescence (FL.) or bright field (BF) (Bar scale: 500 µm)

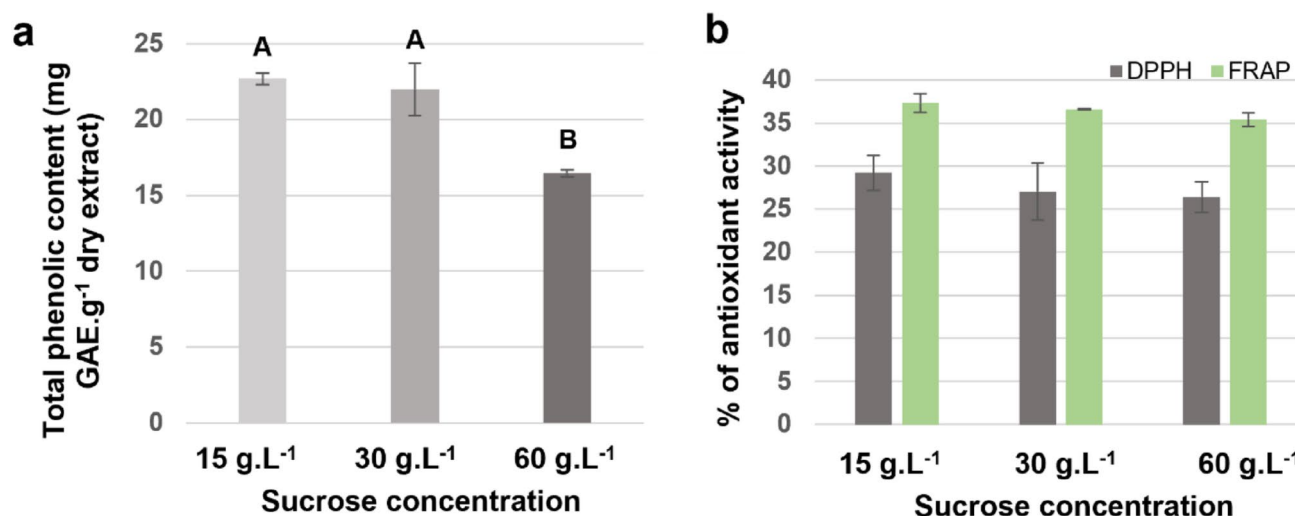


Fig. 4 Effect of sucrose concentration on hairy roots specialized metabolites production and antioxidant activity. Total phenolic content (TPC) (**a**) and antioxidant activity (**b**) of extracts of hairy roots grown 35 days in the dark on WPM supplemented 15, 30–60 g.L⁻¹ sucrose. Results were expressed in mg gallic acid equivalent (GAE) per gram

under light that were absent from the HPLC chromatogram for those grown in the dark. They were further identified as cyanidin-3-glucoside and cyanidin, respectively (Fig. 7c).

Effect of immersion frequency in RITA® bioreactors

An increase in hairy root biomass production was attempted using temporary immersion bioreactors (RITA® systems). The duration of each immersion was set at 3 min. A high immersion frequency of 8/24 h was chosen to maintain good water status of the roots. An immersion frequency of 4/24 hours was tested with the aim of limiting water intake and mimicking water stress. After 42 days, no significant differences were observed in hairy roots growth since the GI respectively reached 2.8 and 3.1 for the immersion frequencies of 4/24 h and 8/24 h (Fig. 8a). At the same time, hairy roots did not grow during the first week of the experiment but began to grow again all along the sides and the bottom of the temporary immersion system (Fig. 8b) showing the same phenotype. Cultivation in a temporary immersion bioreactor had no impact on root diameter and branching, but increased root hair density compared to Erlenmeyer flask culture. The delay in the resumption of growth, linked to the stress produced during subculturing, led us to postpone the biomass harvest by one week, i.e. after 42 days of cultivation. Statistical tests revealed no significant difference in the growth for the two conditions. The root tips remained viable for up to 42 days in both experimental conditions (Fig. 8c). RBCs were also observed in the culture media of RITA® bioreactors (data not shown), probably detached from the root tips during the immersion phases.

of dry extract for TPC and in inhibition percentage (%) for both DPPH and FRAP assays. Represented data are means and standard deviation from biological triplicates. Bars labelled with different letters are significantly different according to Tukey test ($p < 0.05$)

No significant differences in the total phenolic content were observed between the hairy roots grown in RITA systems programmed with 4/24 h and 8/24 h immersions compared to the control condition (Erlenmeyer flasks on a rotary shaker) (respectively 18.0 mg.g⁻¹ GAE, 17.9 mg.g⁻¹ GAE and 18.1 mg.g⁻¹ GAE) (Fig. 9a). DPPH assay showed no significant differences between the antioxidant activity of the hairy roots grown in RITA on 4/24 h and 8/24 h immersion frequencies (30.8% and 30.2% inhibition, respectively) compared to the control (29.1% inhibition). The same trend was observed with the FRAP assay since no significant differences are observed (Fig. 9b). HPLC analysis of the 3 extracts showed identical profiles for each condition (Supplemental Fig. S3).

UHPLC/HRMS identification and quantification of specialized metabolites

By means of UHPLC coupled to HRMS we were able to identify several specialized metabolites within the ethyl acetate extracts. The compounds are listed in Table 1 and their chemical structure is described in Fig. 10. (+) catechin (1), a flavonoid of the flavan-3-ols class, was detected in light conditions, and in trace amounts in the MS condition. Chlorogenic acid (2), an ester of hydroxycinnamic acid, was detected only under light conditions. Two phenolic glycosides, nigracin (3) and tremulacin (4) were detected in all conditions. Finally, cyanidin-3-glucoside (5), an anthocyanin, and cyanidin (6), an anthocyanidin, could be detected in the acidified ethanolic extracts of hairy roots grown under light conditions.

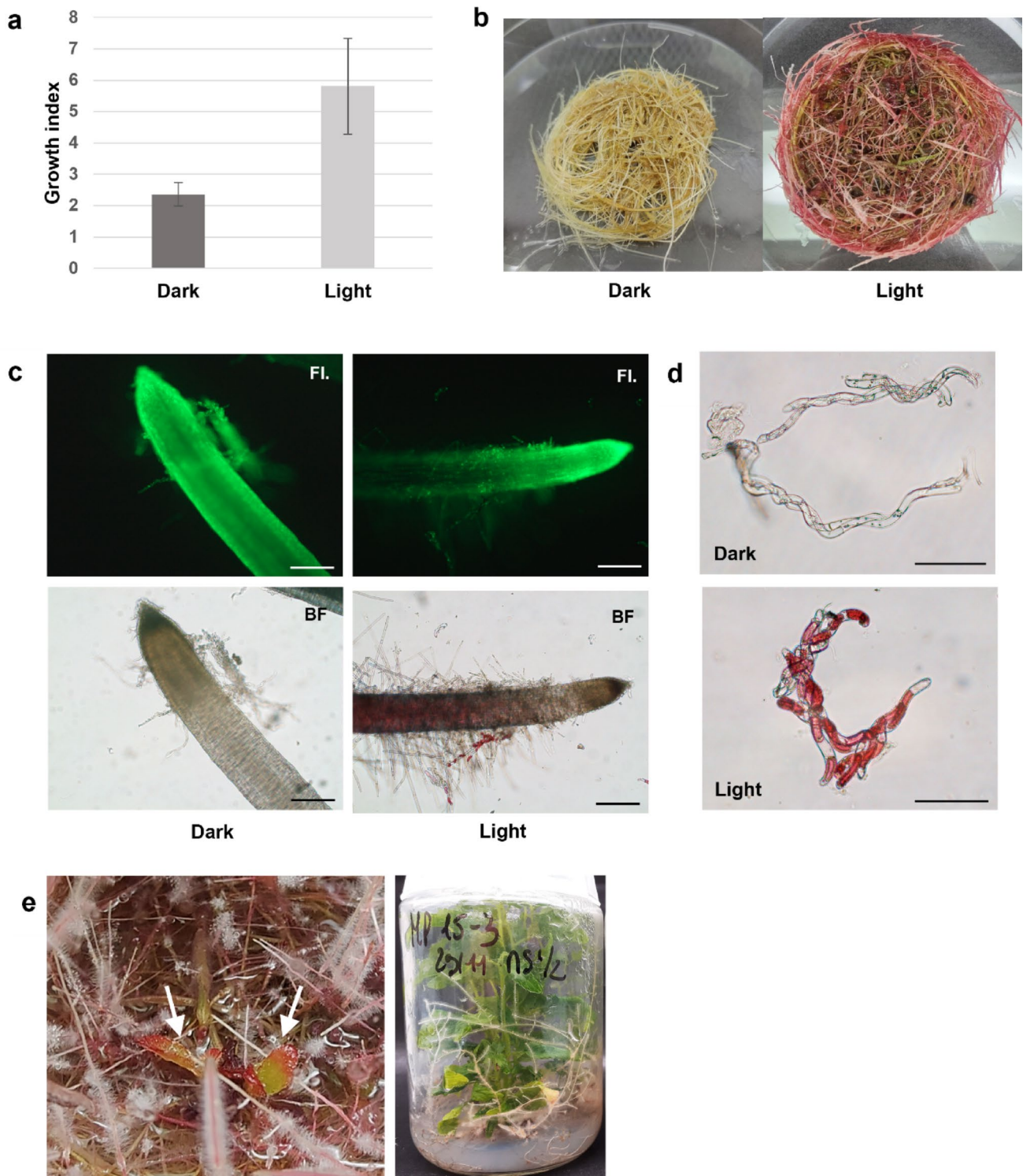


Fig. 5 Effect of light on HP15-3 hairy root growth. **(a)** Growth index and **(b)** final fresh biomass of HP15-3 hairy root line grown 35 days under light or in the dark on WPM supplemented with 30 g·L⁻¹ sucrose. Represented data are mean and standard deviation from biological triplicates. Root viability of HP15-3 hairy roots line **(c)** was assessed using the fluorescein diacetate (FDA) probe. Roots were

observed 5 min after staining with FDA under fluorescence or bright field (Bar scale: 500 µm). **(d)** border cells detached from root caps observed under fluorescence (Fl.) or bright field (BF) (Bar scale: 100 µm). **(e)** development of adventitious shoots (indicated by arrows) under light and subsequent growth in baby food jars

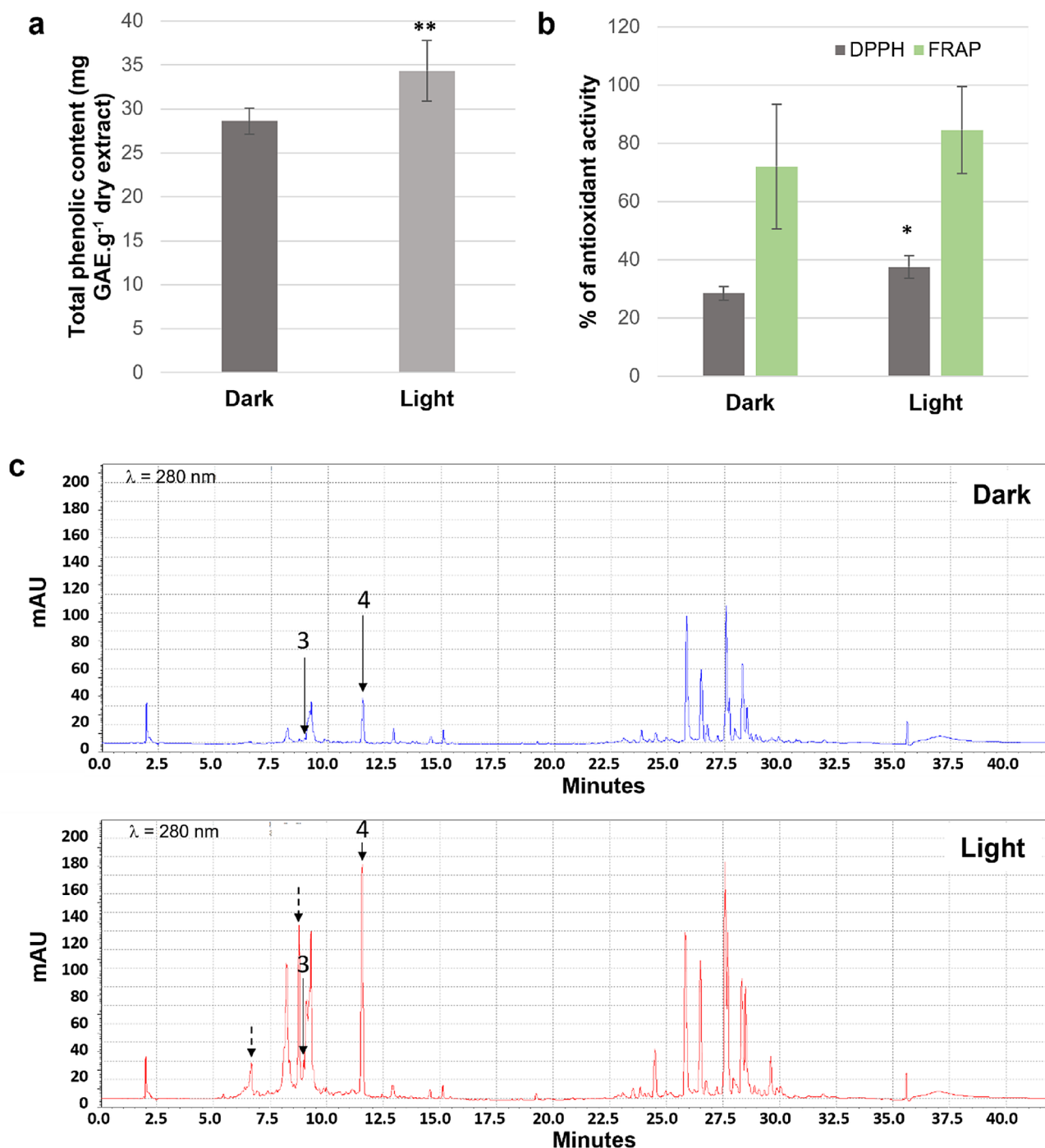


Fig. 6 Effect of light on hairy roots specialized metabolites production and antioxidant activity. Total phenolic content (TPC) (a) antioxidant activity (b) and HPLC profiles (c) of extracts of hairy roots line grown 35 days in the dark or under light on WPM supplemented with 30 g·L⁻¹ sucrose. Results were expressed in mg gallic acid equivalent (GAE) per gram of dry extract for TPC and in inhibition percentage

(%) for both DPPH and FRAP assays. Represented data are means and standard deviation from biological triplicates. * and ** indicate significant differences at $p < 0.05$ and $p < 0.01$, respectively. Peak 3: nigracin, peak 4: tremulacin. Dotted arrows indicate compounds accumulated only under LED light conditions

The HPLC-DAD and the UHPLC-HRMS were performed on two different columns and two different devices, so the retention time of compounds on the HPLC-DAD chromatogram could not be directly transposed from the HRMS

identification. Therefore, commercial standards were also injected into HPLC-DAD conditions. Catechin and chlorogenic acid, although standards were available, could not be quantified because they accumulated in low quantities.

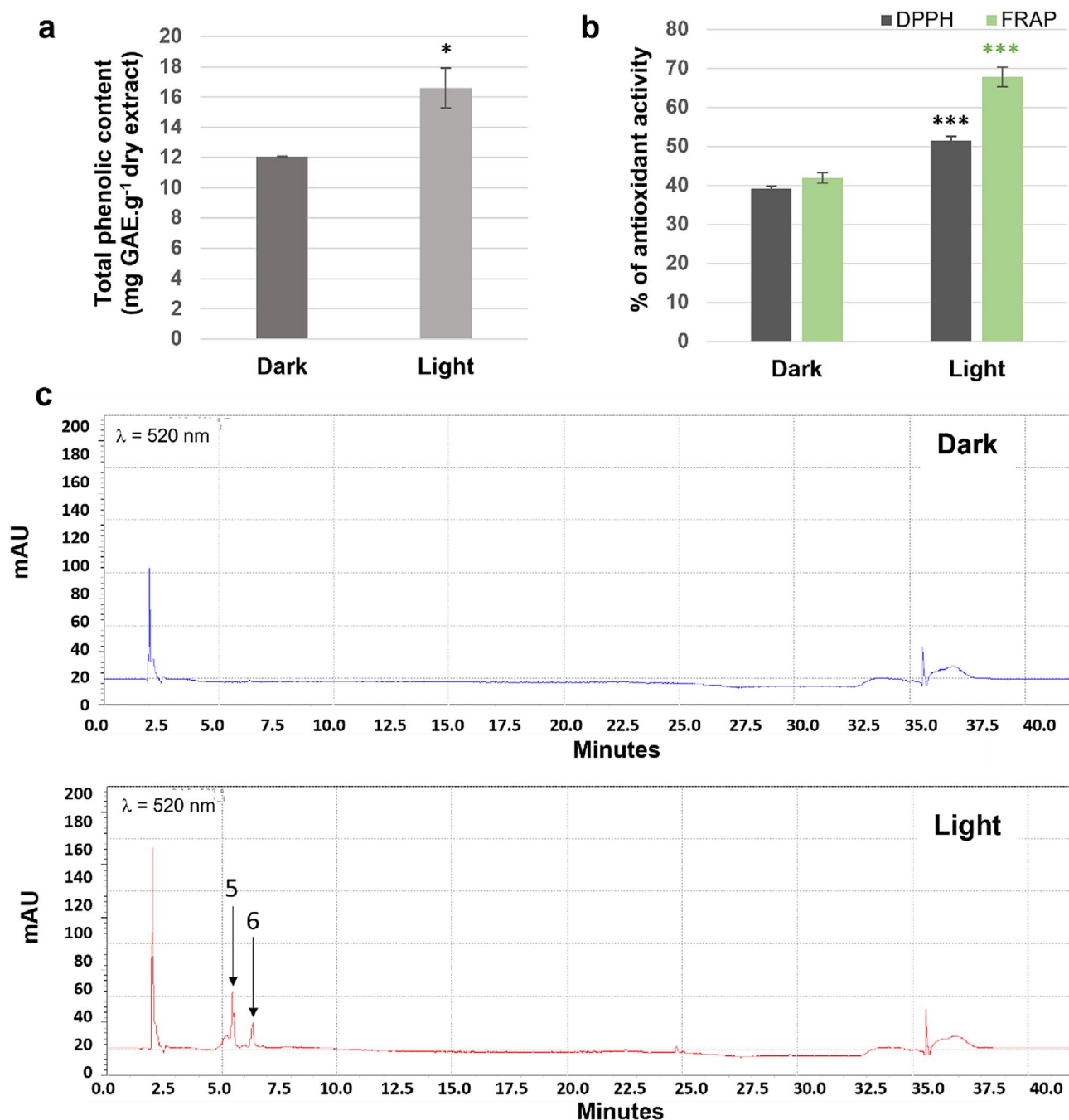


Fig. 7 Specialized metabolites content and antioxidant activity of anthocyanin-rich extracts of HP15-3 hairy roots line grown under light or in the dark. Total phenolic content (**a**) antioxidant activity (**b**) and HPLC profiles (**c**) at 520 nm of acidified ethanolic extracts of hairy root line grown 35 days in the dark or under light on WPM supplemented with 30 g·L⁻¹ sucrose. Results were expressed in mg gallic

acid equivalent (GAE) per gram of dry extract for TPE and in inhibition percentage (%) for both DPPH and FRAP assays. Represented data are means and standard deviation from biological triplicates. * and *** indicate significant differences at $p < 0.05$ and $p < 0.001$, respectively. Peak 5: cyanidin- 3-glucoside, peak 6: cyanidin

The concentrations of the phenolic glycosides nigracin and tremulacin present in all culture conditions could be evaluated (Table 2). Besides, these molecules are quite specific to poplars and salicaceae. The peaks corresponding to these two compounds (nigracin, retention time 9.0 min and

tremulacin, retention time 11.50 min) were identified on the HPLC-DAD chromatograms (Figs. 2c, 6c, 7c, supplemental Fig. S1, S2, S3). Of the two compounds, tremulacin was present at the highest concentration. The latter was a little higher in WPM condition compared to MS. Tremulacin

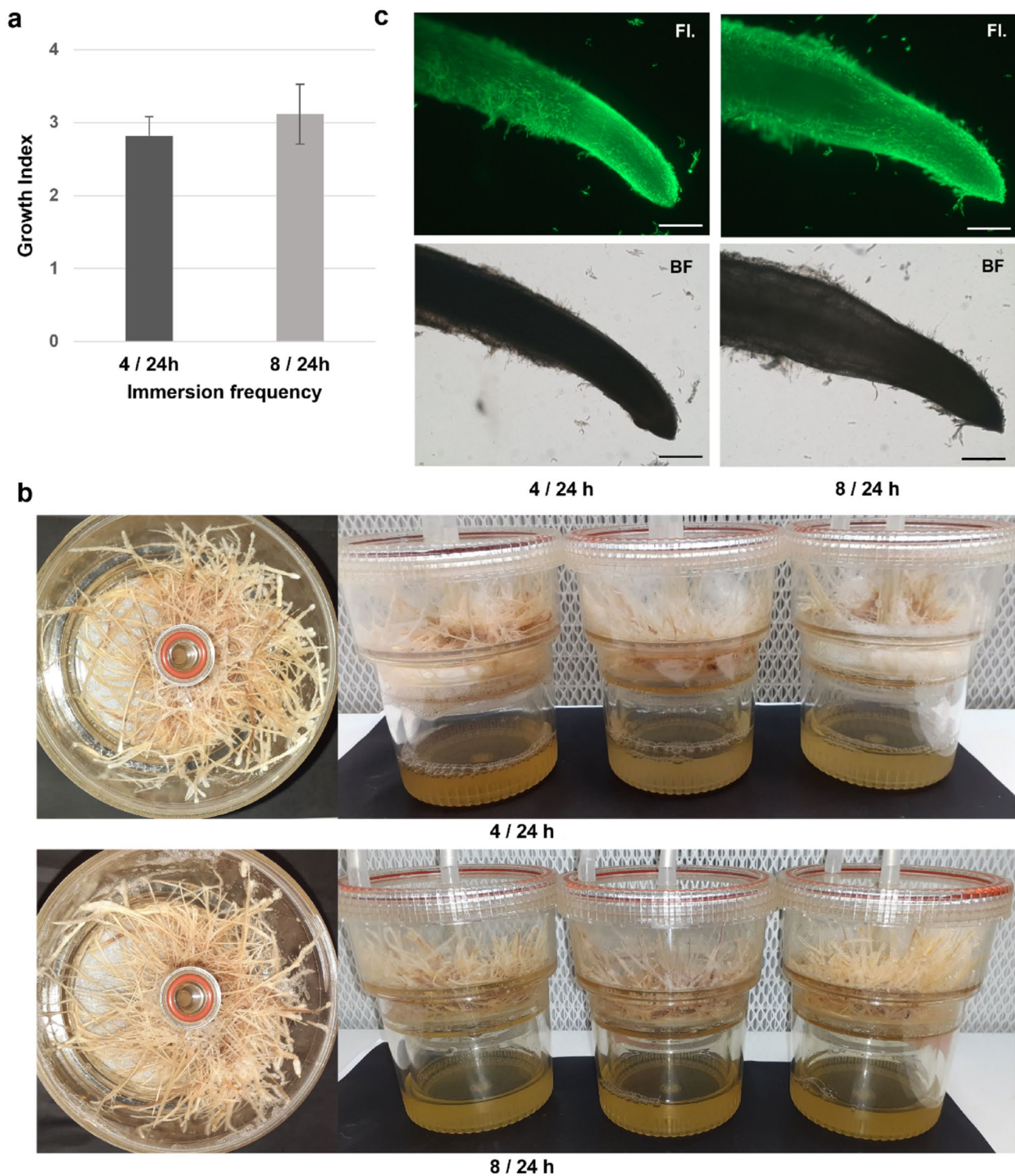


Fig. 8 HP15-3 hairy root line growth in RITA® temporary immersion systems. Growth index (**a**) and final fresh biomass (**b**) of HP15-3 hairy roots line grown 42 days in RITA® systems in WPM with 30 g·L⁻¹ sucrose in the dark. Represented data are mean and standard deviation

from five biological replicates. Root viability of HP15-3 hairy roots line (**c**) was assessed using the fluorescein diacetate (FDA) probe. Roots were observed 5 min after staining with FDA under fluorescence (Fl.) or bright field (BF) (Bar scale: 500 µm)

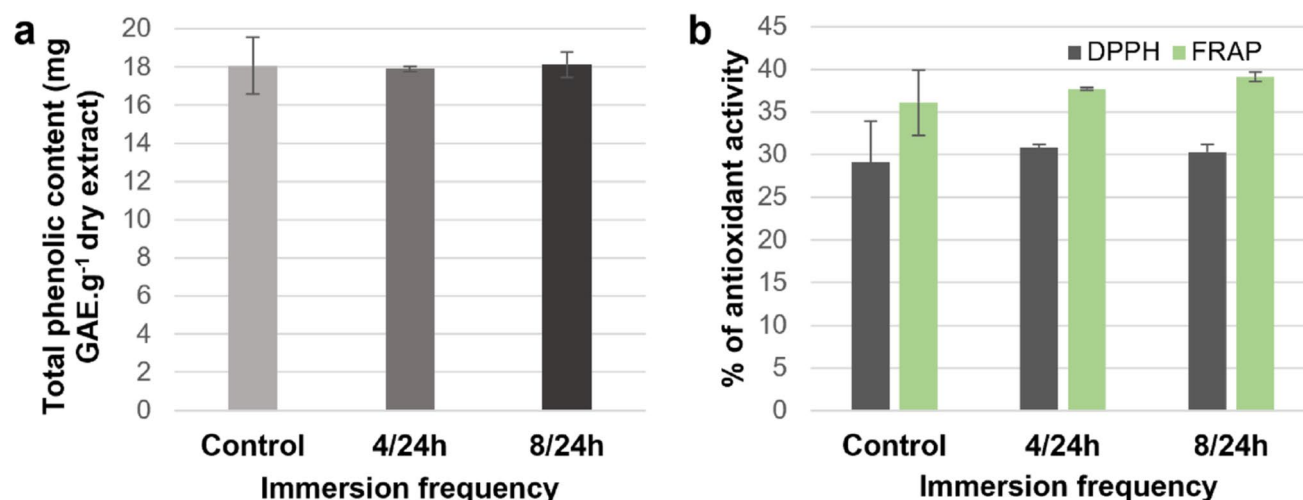


Fig. 9 Total phenolic quantification and antioxidant activity of extracts of HP15-3 hairy roots line grown in RITA® systems with two different immersion frequencies. Total phenolic content (TPC) **(a)** antioxidant activity **(b)** of extracts of liquid cultures grown 35 days in Control Erlenmeyer on rotary shaker or 42 days in RITA systems with 4 or 8 immersions of the hairy roots per 24 h with WPM culture medium (sucrose 30 g·L⁻¹, dark.). A control hairy root extract from a liquid cul-

ture in an Erlenmeyer flask (dark condition, same medium) was also tested. Results were expressed in mg gallic acid equivalent (GAE) per gram of dry extract for TPC and in inhibition percentage (%) for both DPPH and FRAP assays. Represented data are means and standard deviation from biological triplicates. The absence of letters indicates no different according to Tukey test ($p < 0.05$)

Table 1 UHPLC-HRMS detection of specialized metabolites in extracts of HP15-3 hairy roots grown under different experimental conditions

Compound	Number	t_R (min)	$[M+H]^+$ or $[M]^+$	Theoreti- cal mass	Fragments	Formula	Occurrence
(+) - catechin	(1)	5.29	291.0868	291.0863	165.0544/139.0388/123.0440	C ₁₅ H ₁₄ O ₆	Light condition Traces in MS condition
Chloro- genic acid	(2)	5.33	355.1015	355.1023	163.0387/145.0281/135.0440	C ₁₆ H ₁₈ O ₉	Light condition
Nigracin	(3)	7.60	407.1304	407.1336	389.1223/371.1113/353.1012/335.0904/267.0858/249.0753/23 1.0646/225.0754...	C ₂₀ H ₂₂ O ₉	All conditions
Tremulacin	(4)	9.82	529.1692	529.1704	373.1284/267.0888/105.0347	C ₂₇ H ₂₈ O ₁₁	All conditions
Cyanidin- 3- Glucoside	(5)	5.31	449.1075	449.1078	287.0545	C ₂₁ H ₂₁ O ₁₁ ⁺	Light condition
Cyanidin	(6)	6.45	287.0552	287.0550	—	C ₁₅ H ₁₁ O ₆ ⁺	Light condition

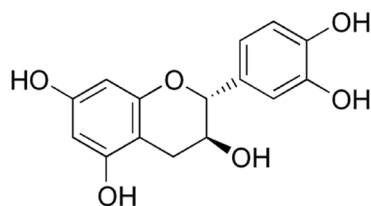
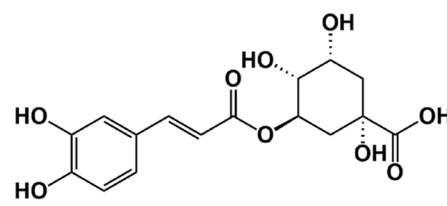
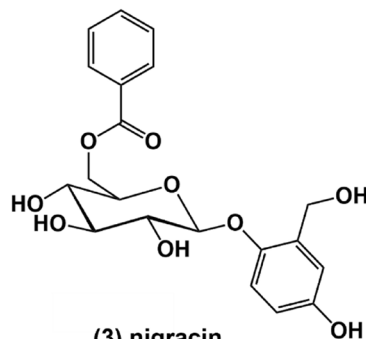
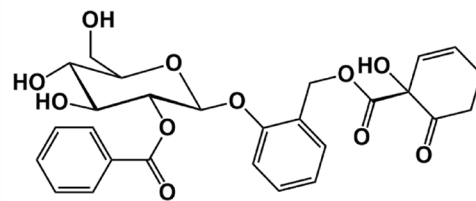
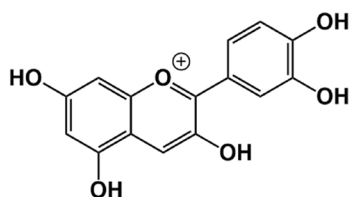
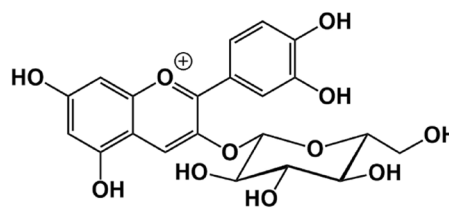
(+) Catechin, chlorogenic acid, nigracin and tremulacin were identified in Ethyl acetate extracts. Cyanidin and cyanidin-3-glucoside were identified in acidified ethanolic extracts. t_R : retention time (min), $[M+H]^+$ or $[M]^+$: experimental m/z

content was the highest at 30 g·L⁻¹ sucrose concentration. It was not affected during cultivation in a temporary immersion bioreactor, whatever the immersion frequency, but increased by 3-fold under light conditions. Nigracin content was decreased 1.8 times in hairy roots grown in WPM compared to MS medium. Increasing sucrose concentration in the medium had a negative impact on nigracin accumulation. The concentration of nigracin was also increased (2.9-fold) under light conditions and particularly high in hairy roots cultured in temporary immersion bioreactors, independently of the immersion frequency.

Discussion

Optimization of media composition

In a previous study, we developed a sustainable hairy root culture medium modified with polyvinylpyrrolidone to circumvent the problem of browning due to phenolics (Lafon et al. 2024). To increase the production of molecules of interest, a balance must be found to obtain a culture medium offering good biomass production as well as a good yield of

Fig. 10 Chemical structures of compounds identified in hybrid poplar hairy root organic extracts**(1) (+)-catechin****(2) chlorogenic acid****(3) nigracin****(4) tremulacin****(5) cyanidin****(6) cyanidin-3-glucoside****Table 2** Quantification of phenolic glycosides nigracin and tremulacin in Ethyl acetate extracts of HP15-3 hairy roots grown under different experimental conditions

Condition	Compound	Concentration (mg.g ⁻¹ dry extract)
MS	Nigracin	0.208 ± 0.002
	Tremulacin	24.90 ± 0.96
WPM	Nigracin	0.113 ± 0.045
	Tremulacin	33.28 ± 4.59
Sucrose 15 g·L ⁻¹	Nigracin	0.147 ± 0.003
	Tremulacin	29.63 ± 0.26
Sucrose 30 g·L ⁻¹	Nigracin	0.142 ± 0.027
	Tremulacin	34.85 ± 2.57
Sucrose 60 g·L ⁻¹	Nigracin	0.064 ± 0.009
	Tremulacin	26.82 ± 0.74
Darkness	Nigracin	0.243 ± 0.078
	Tremulacin	25.01 ± 3.08
Light	Nigracin	0.705 ± 0.126
	Tremulacin	77.59 ± 27.56
RITA 4/24 h	Nigracin	1.451 ± 0.189
	Tremulacin	34.57 ± 7.71
RITA 8/24 h	Nigracin	1.501 ± 0.301
	Tremulacin	33.74 ± 3.18

Data are mean and standard deviation of two independent experiments

specialized metabolites. With this objective, we worked on the optimization of different physicochemical parameters.

Effect of mineral composition

The mineral composition of the environment is an important parameter for plant growth. Here, the use of WPM as a mineral base increased the final biomass of hairy roots of hybrid poplar by more than 2-fold compared to MS medium. The first difference in terms of macronutrients composition is the higher concentration of sulfur in WPM compared to MS medium (7.18 mM and 1.5 mM concentrations given by the supplier, respectively). Indeed, sulfur is an essential macronutrient, component of many amino acids and proteins and involved in diverse plant biological processes. It is also involved in stress management and helps nutrient assimilation. Depending on the species, a different amount of sulfur is required for plant growth, development and survival (Narayan et al. 2023), with no evidence of an increased need for tree species.

Besides, MS medium contains a 4-fold higher concentration of total nitrogen (60 mM *versus* 14.7 mM for WPM).

The two media have a similar $\text{NO}_3^-/\text{NH}_4^+$ ratio of approximately 1.9. Although nitrogen is known for its function in biochemical and physiological mechanisms of plants, leading to the improvement of the yield for all cultures (Leghari et al. 2016) its presence in too high concentrations can lead to the suppression of root differentiation (Singh et al. 2022a). In his study, Cabrera (2003) showed that for two woody plant species nitrate increasing concentrations favored leaf growth but decreased root dry biomass. To enhance plant growth the suitable concentration of nitrogen and $\text{NO}_3^-/\text{NH}_4^+$ ratio must be determined for each species. In this regard, nitrogen excess in MS medium may have a negative impact on hybrid poplar, especially as the organs cultivated in our case are genetically modified roots.

It can also be noted that the two media also differ in their concentration of thiamine (Vitamin B1), the latter being 10-fold higher in WPM than in the MS medium (3 μM vs. 0.3 μM , respectively). Thiamine is involved in plant physiological processes, among which respiration (Fitzpatrick and Chapman 2020) and biotic and abiotic stress resistance. External input of thiamine could therefore lead to increased stress resistance (Subki et al. 2018) and be responsible for increased robustness against stressful conditions for the roots grown with WPM compared to MS medium.

The Folin-Ciocalteu assay revealed that the mineral basis used for hybrid poplar hairy root cultures had a low impact on the total content of phenolics whereas HPLC analysis revealed quantitative differences between the two profiles. Hairy roots grown on MS media seemed to produce more apolar compounds than the ones grown on WPM. Besides, by means of DPPH and FRAP colorimetric assays we measured an antioxidant activity 1.4 times higher in the extracts of hairy roots grown on MS medium compared to those grown on WPM. This could be partly explained by the higher concentration of ammonium nitrate in MS medium which is used for the process of production of many antioxidant compounds (Hazrati et al. 2022). We could hypothesize that in this case, the apolar compounds produced by the hairy roots could be responsible for higher antioxidant activity than polar compounds.

For antioxidant assays we observed an important difference between values for FRAP and DPPH. This could be explained by the decrease of the capacity of antioxidants, such as gallic acid, to reduce DPPH when there are borates in the extracts (Chen et al. 2020). Indeed, boron is part of the composition of the mineral bases of WPM and MS. Hairy roots must have up taken the borates that could further be found in the tested samples after extraction. We can then consider that antioxidant activities measured with the DPPH assay were underestimated for all the experiments, whereas ferric reduction capacities were not affected by the presence of boron (Munteanu and Apetrei 2021). However, although

the antioxidant activity of extracts with the DPPH assay may be underestimated, the results were correlated with those obtained for FRAP.

Integrating all this data, we selected WPM for the following experiments, as it significantly improves hairy root growth with limited impact on the accumulation of specialized metabolites and overall antioxidant activity.

Effect of sucrose concentration

Sucrose concentration in the medium is known to affect both the availability of carbohydrate sources for plant tissues and the water potential of the extracellular compartment. A range of concentrations between 30 and 50 $\text{g}\cdot\text{L}^{-1}$ sucrose has frequently been reported as optimal for biomass accumulation and production of specialized metabolites from adventitious roots and hairy root cultures, whereas higher concentrations of sucrose have an inhibitory effect on plant tissue culture growth (Vazquez-Flota et al., 1994; Baque et al. 2012; Praveen and Murthy 2012). Similarly, the supplementation of cultures with sucrose has also been reported to influence the growth and development of hairy root cultures of *Psoralea corylifolia* (Shinde et al. 2010), *Hypericum perforatum* (Cui et al. 2010), *Azadirachta indica* (Srivastava and Srivastava 2012) and *Daucus carota* (Mukherjee et al. 2019). Very low water potential resulting from a high sugar concentration can impact water and nutrient uptake and limit growth. These stressful conditions can nevertheless lead to the increased production of specialized metabolites. It can therefore be interesting to find a balance that provides a necessary uptake of sucrose for the roots as well as little stress to increase specialized metabolites production. The current study revealed that sucrose concentration affected the production of hairy root biomass and specialized metabolites production. Indeed, 30 $\text{g}\cdot\text{L}^{-1}$ of sucrose favored high biomass production. Similar results have been reported for many hairy root species (Kochan et al. 2014; Praween and Murthy 2012; Yousefian et al. 2021). Kochan and al. (2014) showed that the highest growth rate was obtained for *Panax quinquefolium* with a sucrose concentration between 30 and 50 $\text{g}\cdot\text{L}^{-1}$. They also found that growth was inhibited in cultures when the sucrose was over 70 $\text{g}\cdot\text{L}^{-1}$ and under 20 $\text{g}\cdot\text{L}^{-1}$. Similarly, in our study, cultures grown with 15 $\text{g}\cdot\text{L}^{-1}$ sucrose showed a reduced (although not significant) growth compared to 30 $\text{g}\cdot\text{L}^{-1}$ whereas high concentration of sucrose i.e. 60 $\text{g}\cdot\text{L}^{-1}$ had an inhibitory effect on hairy root growth. This could be related to the osmotic stress or toxicity generated by high concentration of sucrose (Al-Khayri and Al-Bahrany 2002). Osmotic stress elevates the level of reactive oxygen species, which disrupts the proper cell functioning by damaging the macromolecules, DNA and lipid membrane thus leading to cell death (Martinière et al. 2019;

Singh et al. 2022b). In their study, Praween and Murthy (2012) showed that $30 \text{ g} \cdot \text{L}^{-1}$ sucrose gave the highest hairy roots growth for *Withania somnifera*. However, the highest withanolide A production was achieved with a higher concentration of sucrose i.e. $40 \text{ g} \cdot \text{L}^{-1}$. Indeed, together with the lack of environmental constraints, an optimized media leads to the absence of stress for the plant during growth. As stress is known for activating phenolic pathways (Sasaki-Sekimoto et al. 2005), providing to the plant the exact amount of sucrose needed may lead to the decrease of specialized metabolites production. As osmotic stress may affect the survivability of the roots due to reduced water and nutrients uptake we assessed root viability in each of the experimental conditions. Fluorescence revealed that roots were viable whatever the sucrose concentration, leading to the conclusion that the stress occasioned to the roots was moderate.

In our study, the highest total phenolic contents and the highest (although non-significant) antioxidant activities were reached with the lowest $15 \text{ g} \cdot \text{L}^{-1}$ sucrose concentration and decreased with the augmentation of sucrose in the media. Culture on medium with a high concentration of sucrose (i.e. $60 \text{ g} \cdot \text{L}^{-1}$) led to a reduction in growth and antioxidant activity. Total phenolic contents and HPLC profile also showed reduced concentration of extracted metabolites with a $60 \text{ g} \cdot \text{L}^{-1}$ sucrose concentration in the culture media. As the HPLC profiles showed no difference between 15 and $30 \text{ g} \cdot \text{L}^{-1}$ sucrose and the differences in antioxidant activity and total phenolics being negligible, the best compromise to optimize growth and metabolite production was the use of $30 \text{ g} \cdot \text{L}^{-1}$ sucrose in the culture medium.

Effect of lighting on root growth and production of specialized metabolites

Due to growing underground in natural conditions, most roots are rarely exposed to light. Under these conditions, light being stressful for these organs, it is all the more interesting to see their response in terms of growth capabilities and newly synthesized specialized metabolites under artificial lighting. In the case of hybrid poplar hairy roots, light improved the growth index (by 2.45-fold) compared to dark conditions. This experiment was conducted for technical reasons on a different rotary shaker (smaller orbital) and in a different growth chamber (24°C). This could at least partly explain the lower GI (2.36) observed for the standard condition (dark) compared to the previous experiments (6.69 for basal medium experiment, 7.70 for sucrose concentration experiment). In their study, Abbasi et al. (2007) described thicker roots but there was no difference in the growth rate for *Echinacea purpurea* hairy roots. In both studies, light did not damage the hairy root cells of plant species.

Growing hybrid poplar hairy roots under light induced the development of adventitious shoots capable of subsequently regenerating plants. Similarly, Han et al. (1997) were able to regenerate shoots from the hairy root of a hybrid poplar clone 53–246 (*P. trichocarpa* $\times$ *P. deltoides*) but only after supplementing the medium with cytokinin and auxin. The plants showed the same phenotype of dwarfism. In their study on shoot regeneration from horseradish hairy roots, Saitou et al. (1992) described that growth was initiated on light-grown hairy roots but stopped after 8 weeks. As for hybrid poplar, adventitious shoots started to grow after 5 weeks of culture under light but not in the dark. As well as being a good means to improve hairy roots growth, the use of light on hairy root cultures could enable the production of adventitious shoots with original functions and characteristics. Indeed, as suggested by Han et al. (1997), the advantage could be taken of these modifications to select poplars with a more extensive root system and a better capacity to interact with soil microorganisms for better drought tolerance. This could be of interest in the current context of climate change.

In view of cosmetic or healthcare applications, the effect of light on hairy roots production of phenolic and other metabolites has been studied for several plant species. In the case of hybrid poplar, light improved overall phenolic content, as shown by Folin colorimetric assay and HPLC analysis, and antioxidant activity for both extractions tested. Several studies on specialized metabolites reached the same conclusion. A higher baicalein flavonoid accumulation had previously been described for *Scutellaria lateriflora* (Marsh et al. 2014) for hairy roots grown with light than for the ones grown in the dark. Abbasi et al. (2007) also reported a higher production of caffeic acid and derivatives like chicoric acid, caftaric acid and chlorogenic acid concomitantly with increased phenylalanine ammonium lyase (PAL) enzyme activity when *Echinacea purpurea* hairy roots were grown with light.

The pink color of light-grown hybrid poplar hairy roots is associated with the accumulation of anthocyanins in cortical cells, but also presumably in root border cells (RBCs), indicating a strong metabolic activity of the latter. Poplar RBCs have been characterized by their ability to secrete glyco-molecules constituting mucilage (Busont et al. 2023). It has been shown that *Medicago truncatula* seedling border cells are rich in specialized metabolites (Watson et al. 2015) such as flavonoids. As we observed the presence of a significant quantity of RBCs in the medium of both light- and dark-grown cultures, this opens the prospect of using poplar hairy roots as a biotechnological tool to collect RBCs for transcriptomic and metabolomic studies. Further chemical analyses could demonstrate the potential of poplar border cells for anthocyanin biosynthesis.

Total anthocyanin content (measured by spectrophotometer at 520 nm) of acidified ethanolic extracts was 2-times higher in hairy roots grown under light compared to those grown in the dark (data not shown). The overall increase in the concentration of several compounds and the presence of two new compounds for the hairy roots grown under light were correlated with higher antioxidant capacity. Anthocyanins, through their protective activities against UV-mediated damage and melanogenesis (as tyrosinase inhibitors), their antioxidant properties or their effect on cutaneous bacteriostasis, constitute a group of bioactive compounds with many potential applications in cosmetics (Li et al. 2023).

Effect of immersion frequency in RITA® bioreactor

Temporary immersion systems have made a breakthrough in the biotechnology field over the last decades. The many advantages of these bioreactors in terms of culture conditions make it a good system to improve plant growth. Indeed, those systems provide, amongst other assets, better oxygenation of the tissues, a uniform availability of the nutrients and help dilute the toxic compounds produced by the plant (De Carlo et al. 2021). To obtain the highest yield of specialized metabolites as well as good biomass production, it is necessary to determine the frequency and time of immersion of plant tissues to avoid hyperhydricity, known to limit plant growth (Georgiev et al. 2014). Temporary immersion systems have already been tested on tree species for plant micropropagation (Carvalho et al. 2019; Garcia-Ramirez, 2023) but not for hairy roots culture and very little data is available on the cultivation of hairy roots of herbaceous species in these bioreactors (Carlin et al., 2015; Kentsop et al. 2021; Pavlov et al. 2005). Starting from 6 g, a final biomass of 20 g could be obtained after 42 days. To our knowledge, we demonstrated for the first time the feasibility of cultivating hairy roots of tree species in temporary immersion bioreactors. The lower GI obtained in RITA® bioreactors compared to shake flasks could be attributed to the stress caused by temporary immersion. Besides, the culture medium was not discarded and replaced with fresh medium after 3 weeks. This may have led to nutrient depletion. In an aeroponic system, drought conditions simulated by reducing the frequency and duration of misting can lead to alterations in root exudation and production of specialized metabolites (Lin et al. 2023). In our study, reduced frequency of water and nutrient supplies did not have an impact on hairy root growth. Indeed, the high (8/24 h) and low (4/24 h) immersion frequencies tested allowed similar growth and production of specialized metabolites. Under our experimental conditions, the internal atmosphere probably remained too saturated with humidity, thus preventing the generation of water stress conditions. Future experiments deserve to be

conducted by drastically reducing the frequency and duration of immersion to more effectively mimic drought conditions and study their impact on specialized metabolism. Indeed, osmotic stress is known to induce the production of metabolites and improve antioxidant activity (Zahedi et al. 2021). Media compositions should be optimized specifically to improve growth in this culture system. In our study, the production of specialized metabolites and antioxidant activity were comparable in both the temporary immersion system and control Erlenmeyer flasks.

Identification of specialized metabolites

UHPLC-HRMS analysis further allowed the identification of specialized metabolites known to have valuable biological activities. Indeed, the presence of (+)-catechin, a flavan-3-ol known for its antioxidant activity, has been highlighted. Catechins known to be reactive oxygen species (ROS) scavengers are involved in reactions against oxidative stress (Bernatoniene and Kopustinskiene 2018). Chlorogenic acid was first identified in the roots of hybrid poplar grown under light conditions. This caffeic acid derivative has been identified in extracts of poplar buds (*Populus balsamifera* L., *Populus nigra* L.) and in propolis and is believed to contribute to the antimicrobial and antioxidant activities of the latter (Stanciauskaite et al. 2021). Tremulacin and nigracin, two phenolic glycosides, were unequivocally identified for the first time in the hairy roots of hybrid poplar and could be quantified using standards. The concentrations of these two phenolic glycosides were strongly increased in hairy roots grown under LED light. Tremulacin, which is a phenolic glycoside related to salicin, was previously isolated from the leaves of *P. tomentosa* and willow (*Salix* sp.) bark and exerted interesting anti-inflammatory activity (Antoniadou et al. 2021; Cheng et al. 1994). Nigracin was identified in the bark and leaves of black poplar (*P. nigra* L.) by Palo (1984). This compound was also isolated more recently from the bark of *Drypetes klainei* and showed significant wound healing activities (Sferrazza et al. 2020). Nigracin was also previously shown to exert anti-inflammatory activity against COX-2 cyclooxygenase (Chai et al. 2008). Cyanidin (anthocyanidin) and cyanidin-3 glucoside (anthocyanin) were identified in extracts of hairy roots grown under LED light. Anthocyanins (including anthocyanins-3-glucoside), whose antioxidant and anti-UV properties have been described (He et al. 2017), have already been identified in poplar and their biosynthesis is tightly regulated in the presence of light by MYB transcription factors (Ma et al. 2021; Kim et al. 2021). Taken together these results indicate potential to produce valuable bioactive compounds by hairy roots of hybrid poplar, particularly when grown under light conditions.

Conclusions

During this study we optimized the growing conditions of hybrid poplar hairy roots. The choice of the mineral base and the adjustment of the sucrose concentration made it possible to increase the production of biomass and the antioxidant capacity of the extracts. Cultivation under light conditions promoted root growth while leading to the production of anthocyanins helping to increase the antioxidant activity of the extracts. The temporary immersion reactors can be used to maintain hybrid poplar hairy root culture. Using the optimized medium selected in our study, the RITA[®] system could be a promising alternative to produce root biomass with the aim of providing bioactive extracts for cosmetic or pharmaceutical applications.

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Data availability The data that support the findings of this study are available upon request from the corresponding author.

Declarations

Competing interests All the authors have read the manuscript and declare there are no conflicts of interest.

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