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ORIGINAL ARTICLE

Identification of gene markers to predict proanthocyanidins biosynthesis in cell culture of *Croton lechleri*

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Abstract

Croton lechleri Müll. Arg. is a traditional medicinal tree growing in South America, which produces a deep red sap known as *Dragon's Blood Sap* (DBS) used for its many therapeutic benefits. DBS bioactivity is mainly due to the high content of proanthocyanidins (PAs), important plant secondary metabolites involved in plant defence, but also utilised as antioxidant for human health. Since the process to directly obtain DBS from the *C. lechleri* tree is expensive, time-consuming and strongly dependent on the environmental conditions, the use of plant cell cultures for DBS production seems to represent a suitable alternative. The aptitude of plant cell cultures to synthesize specific secondary metabolites can be elicited by different types of abiotic stresses. In this study, the *C. lechleri* main genes putatively involved in the PAs biosynthetic pathway were identified and their expression was evaluated in both calli in solid medium and cell culture suspensions to identify the most suitable predictive gene markers for PAs biosynthesis. *C. lechleri* cell cultures were grown and elicited by N-starvation to induce the production of PAs and were then monitored by assessing the expression of previously identified gene markers, by quantifying the content of PAs and measuring few phenotypical parameters. The results show a new perspective on how PA gene transcript abundance could be used to predict the biosynthesis of useful secondary metabolites in plant cell cultures and to speed up the screening of putative elicitors.

Key message

This study provides an attempt to obtain a cell culture from a Croton lechleri sample and to early detect the production of proanthocyanidins through gene expression analysis.

Keywords *Croton lechleri* · Cell culture · Proanthocyanidins · Gene expression · Nitrogen deficiency

Abbreviations

PAs Proanthocyanidins
DBS Dragon's Blood Sap

Introduction

Novel plants-derived drugs for the treatment of diseases continue to be developed through research (Hidalgo et al. 2018). Plant derived compounds are getting more and more attention due to their affordability comparable to high-cost synthetic drug agents and to their accessibility and eco-friendly aspects (Yuan et al. 2016).

Croton lechleri Müll. Arg. (fam. Euphorbiaceae) is a traditional medicinal tree growing in South America, which produces a deep red sap, known as "Sangre de Drago" or "Dragon's Blood Sap" (DBS), which is obtained by carving the bark of plants older than two–three years. Amazonia natives use this sap for wound healing and as remedy for several diseases (Jones 2003).

Regarding its therapeutic potential, DBS is used to reduce pain and inflammation, against bacterial, fungal and viral

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infections thanks to its immunomodulatory and antioxidant effects, but it is also employed to hinder bleeding, sepsis, diarrhoea, ulcer, and skin affections, and as antitumoral and wound healer (Gupta et al. 2008). DBS bioactivity is mainly due to the high content of proanthocyanidins (> 90% dry weight of the resin) (Cai et al. 1991), but probably also to its content in taspine, catechin, epigallocatechin, epicatechin, and a small percentage of terpene compounds (De Marino et al. 2008). Proanthocyanidins (PAs), also known as condensed tannins, are secondary plant metabolites involved in plant defence, and have many potential benefits for human health, related to their antioxidant properties (Yang et al. 2018). They are oligomeric and polymeric flavan-3-ols that are produced from the flavonoid pathway (Dixon et al. 2005). Usually, oligomeric proanthocyanidins are mainly composed by catechin and epicatechin, but they could also contain epigallocatechin and/or gallocatechin, depending on the plant species. The first step of the biosynthetic pathway starts with the key enzyme DFR (dihydroflavonol 4-reductase) that catalyses the NADPH dependent reduction of dihydroflavonols to produce leucoanthocyanidins, which in turn are the substrate to produce anthocyanidins through the anthocyanidin synthase (ANS) also known as leucoanthocyanidin dioxygenase (LDOX). Finally, anthocyanidins can be converted into epicatechin, epiafzelechin, and epigallocatechin by ANR (anthocyanidin reductase) or into catechin, afzelechin, and gallocatechin by LAR (leucoanthocyanidin reductase) (Dixon and Sarnala 2020; Lim and Ha 2021). Regarding DBS, the FDA approved the Crofelemer (CF) as a drug for the treatment of non-infectious diarrhea in adult patients with HIV/AIDS undergoing antiretroviral therapies. CF is natural botanical biopolymer obtained from DBS as a mixture of PAs oligomers which are primarily composed of gallocatechin with a small contribution from catechin (Frampton 2013).

The processes to directly obtain DBS from the tree of *C. lechleri* is expensive, time-consuming and strongly dependent on several factors, including environmental, political and labour instabilities in the producing countries (Jones 2003). Furthermore, DBS chemical synthesis is extremely difficult, due to its complex chemical structure, and economically infeasible (Gupta et al. 2008).

The use of plant tissue cultures, such as callus or cell culture suspensions, to produce secondary metabolites, could represent an alternative method to bypass such critical issues (Kolewe et al. 2008; Murthy et al. 2014). Under in vitro conditions, properly induced plant cells form an unorganized mass of cells known as callus, which can then be used to develop single-cell suspension cultures, by employing either batch or continuous fermentation (Xu et al. 2011; Wilson and Roberts 2012). Then, specific growth protocols aimed at forcing or manipulating the secondary metabolites biosynthesis pathways of calli and suspension cell cultures could be set-up to maximize the production of the molecules of interest (Chandran et al. 2020; Mohaddab et al. 2022). Besides, plant cell cultures have many advantages: independency of geographical and seasonal variations and environmental factors, stabilization of a defined production system that ensures the continuous supply of the desired metabolite, uniform quality and yield, potential for selection of cell lines with higher production of secondary metabolites, automatization of cell growth control and regulation of metabolic processes, so that costs can decrease and productivity increase (Smetanska 2008; Niazian and Sabbatini 2021).

Despite this, the plant cell culture technology is challenging, and the problems facing it can be classified as biological (slow growth rate, physiological heterogeneity, genetic instability, low metabolite content, product secretion) and operational (wall adhesion, light requirement, mixing, shear sensitivity, aseptic conditions) (Zhong 2001; Guerriero et al. 2018). Nowadays, some high-value pharmaceutical chemicals (Wu et al. 2021), food additives and cosmetic ingredients (Krasteva et al. 2020) are synthesized via plant cell cultures. Some examples are paclitaxel, an anticancer taxoid compound found in *Taxus brevifolia* partially produced in suspension cultured cells (Wickremesinhe and Arteca 1994; Marupudi et al. 2007), and vincristine, a mitotic inhibitor and anti-cancer obtained from *Catharanthus roseus* (Mitsunaga et al. 2000). However, these examples of successful exploitation of plant cell culture at the industrial level are just a few due to the problems that arise during the industrial scale-up (Knockaert et al. 2015).

The production of specific secondary metabolites can be boosted by treating the plant cell cultures with different type of stresses and growth factors (i.e. the ratio between auxins and cytokinines), or changing the growing conditions (light, temperature, oxygen) (Nielsen et al. 2019; D'Alessandro et al. 2022). For instance, it was shown that MS medium containing 0.2 mg/L IAA, 1.5 mg/L 6-BA, and 50 µM MeJA induced anthocyanins and PAs accumulation in apple callus due to *MdMYB9* or *MdMYB11* up-regulation (An et al. 2015), as well as elicitation with 50 µM MeJA in *Pyrus communis* callus cultures up-regulated the expression of *PcANR2a* and *PcLAR1*, two genes important in the biosynthesis of PAs (Premathilake et al. 2020), while light stress and nitrogen deficiency induced higher levels of PAs in poplar leaves (Gourlay and Constabel 2019).

In this study, the *C. lechleri* sequences of the main genes involved in the proanthocyanidins biosynthetic pathway were identified and their expression was evaluated in both calli in solid medium and in cell culture suspensions, to identify the most suitable predictive markers for proanthocyanidins biosynthesis. *Croton lechleri* cell cultures were also grown and elicited by N-starvation in an attempt to induce the production of PAs. The cell culture was then monitored by assessing the expression of the previously identified gene

markers, the content of proanthocyanidins and other phenotypic parameters.

Materials and methods

Croton lechleri cell culture induction

Tissue portions (young branches and leaves) of *Croton lechleri* were used as starting plant material to generate a cell culture. The plant material was kindly provided by the Montgomery Botanical Center (Florida, USA). The botanical origin of *Croton lechleri* was confirmed by fingerprint DNA analysis made from Parco Tecnologico Padano, Lodi, Italy (Nybom et al. 2014). The young branches of *Croton lechleri* were washed under running water and sterilized by means of a treatment in sequence with 70% (v/v) ethanol (Honeywell, Wunstorfer Straße 40, D-30926 Seelze, Germany), water for about 4 min, 2% (v/v) of sodium hypochlorite solution (6–14% active chlorine, MERCK KGaA, 64271 Darmstadt, Germany) and 0.1% (v/v) Tween 20 (Duchefa, Postbus 809, 2003 RV-Haarlem, The Netherlands) for 4–5 min and, finally, at least 5 washes with sterile distilled water.

The sanitized plant tissue has been cut into minute fragments (explants) which have been deposited in Petri dishes containing solidified nutrient medium Gamborg B5 (Gamborg et al. 1968) with different combinations and concentrations of plant growth regulators (Tables 1 and 2) and incubated at 25 °C in dark condition. Four weeks after from the date of explant, the first repair scar tissue formed. The highest rate of callus growth was observed using the solid medium Gamborg B5 supplemented with 20 g/L sucrose (Sudzucker), 0.8% (w/v) of plant agar (Duchefa), 1 mg/L of (P) picloram (Duchefa), 0.5 mg/L of (K) kinetin (Duchefa) and at pH 6.5 (Cle medium). The callus grown in these media were regularly transferred to new plates every 2 weeks, selecting the most friable and homogeneous portions.

Cell cultures grown on Cle medium had a heterogeneous appearance due to the presence of calluses with different phenotypes: at the beginning they were much darker in colour, then they became lighter. Both callus phenotypes were subjected to subculture for at least 3 months until they became friable and homogeneous, with a constant growth rate.

Cell suspension cultures were generated by transferring 10% (w/v) of selected callus into 250 mL of liquid culture medium Gamborg B5 supplemented with 20 g/L sucrose (Sudzucker), 1 mg/L of P (Duchefa) and 0.5 mg/L of K (Duchefa). The pH was adjusted to 6.5 before autoclaving (Cle liquid medium).

The suspension cultures were maintained in a climatic room, in dark condition, at 25 °C on rotary shaker in constant agitation at 120 rpm and were sub-cultured in a new liquid medium every 14 days of fermentation. The cell suspensions were transferred using 12% v/v of inoculum.

Croton lechleri cell suspension induction to produce proanthocyanidins

To stimulate the biosynthesis of proanthocyanidins, after 14 days of fermentation in Cle liquid medium, the cell suspensions were transferred to Cle final liquid media (12% v/v of inoculum) with 25 mM KNO_3 and 1 mM $(NH_4)_2SO_4$ as control (CTR) or without KNO_3 and without $(NH_4)_2SO_4$ as treatment (No Nitrogen). The proanthocyanidin content, the cell fresh weight, the density, the color by spectrophotometer (colorimeter) and the expression of PAs' related genes, during the cell suspension culture growth were evaluated at two time-points: T7 and T14, at 7 and 14 days of fermentation in the Cle liquid medium, respectively. Accordingly, 7 days represented halfway of the fermentation time required for the *C. lechleri* cell culture optimum growth, which were assessed to 14 days by the analysis previously described. At every time-point, 10 mL of cell suspension culture were sampled to analyze the proanthocyanidin content, while 50 mL, corresponding to 10–12 g of cells, were used to extract RNA and evaluate gene expression of the target genes. Cell fresh weight, density and color were evaluated only at T14. Three biological replicates were set for each time point.

Ultra-performance liquid chromatography method with diode array detection (UPLC-DAD) analysis for the evaluation of the total proanthocyanidins content

10 mL of *Croton lechleri* suspension culture or 10 g of callus from solid medium were transferred into a 15 mL test tube and 2 mL of a solution containing 20 g/L of citric acid monohydrate (ACEF) and 10 g/L of ascorbic acid (Ph.Eur. E300-ACEF) in water were added for the quantification of the total proanthocyanidins expressed as catechin equivalent. The suspension was mixed for 10 s and frozen at − 20 °C until UPLC-DAD analysis. Before the analysis, the suspension was thawed and transferred into a 50 mL test tube and 10 mL ethanol were added. The suspension was mixed for 10 s with a vortex mixer and homogenized for 1 min with ultraturrax (T18 digital IKA) at 20000 rpm in an ice bath; finally, it was centrifuged at 4500 rpm for 5 min at 6 °C. At the end of centrifugation, 2 mL of the supernatant were transferred in an Eppendorf test tube and further centrifuged at 14000 rpm for 3 min at 6 °C. The supernatant was transferred in a new Eppendorf test tube and preserved in ice until

Table 1 Composition of plant growth regulators (2,4 dichlorophenoxyacetic, 6-benzylaminopurine, kinetin and picloram) in solid culture media used for *Croton lechleri* callus induction

Composition	2,4 dichlorophenoxyacetacid (mg/L)	6-benzylaminopurine (mg/L)
1	0.5	1
2	1	0.5
3	1	1
4	1	2
5	2	1
6	2	2
Composition	2,4 dichlorophenoxyacetacid (mg/L)	Kinetin (mg/L)
1	0.5	0
2	1	0
3	0.5	0.3
4	0.5	0.5
5	1	0.3
6	1	1
Composition	Picloram (mg/L)	6-benzylaminopurine (mg/L)
1	0.2	0.4
2	0.4	0.2
3	0.5	1
4	1	0.5
5	1	1
6	1	2
Composition	Picloram (mg/L)	Kinetin (mg/L)
1	0.2	0.4
2	0.4	0.2
3	0.5	1
4	1	0.5
5	1	1
6	1	2

The compositions indicated in the table contain one or two plant growth regulators

Table 2 Composition of plant growth regulators (naphthalene acetic acid, indole 3-acetic acid and 6-benzylaminopurine) in solid culture media used for *Croton lechleri* callus induction

Composition	naphthalene acetic acid (mg/L)	indole 3-acetic acid (mg/L)	6-benzylaminopurine (mg/L)
1	0.5	0.5	0.5
2	0.5	0.5	1
3	1	0.5	0.5
4	0.5	1	0.5
5	1	1	0.5
6	1	1	1

The compositions indicated in the table contain three plant growth regulators

loading into the UPLC system. The sample was diluted in water (from 1:1 to 1:5 depending on the content of proanthocyanidins of the sample) and filtered over 0.22 µm filters before being loaded into the UPLC system.

The chromatography system used for quantification of the total proanthocyanidins consists in an Acquity UPLC BEH C18 1.7 µm column, size 2.1 × 100 mm, coupled to an Acquity UPLC BEH C18 1.7 µm VanGuard Pre-Column 3/Pk, size 2.1 × 5 mm. The platform used for the UPLC-DAD analysis comprises a UPLC system (Waters Corporation, Milford, MA, U.S.A) consisting of an eluent management module, Binary Solvent Manager model I Class, and an auto-sampler, Sample Manager–FTN model I Class, coupled to a PDA eλ diode array detector. The chromatography

method used was the following: solvent A: water, 0.1% formic acid; solvent B: 100% acetonitrile. The initial condition was 99% solvent A; the flow remained constant at 0.350 ml/min throughout the duration of the analysis. The chromatography column was temperature controlled at 30 °C. Elution of the molecules was conducted by alternating gradient and isocratic phases, as indicated in Online Resource 1.

Quantification of total proanthocyanidins in the samples were based on absorbance UV/VIS spectra measured at 277 nm. The content of total proanthocyanidins (expressed as equivalent of catechin) were evaluated through the comparison with calibration curves obtained from serial dilution of the authentic commercial standard of (+)-catechin (purity ≥ 99%; batch 241217/0, Extrasynthese). Three independent biological repetitions for each sample were evaluated and quantified. Empower 3 (Waters Corporation, Milford, MA, U.S.A.) software was used to acquire and analyse the data.

RNA extraction and cDNA synthesis

Cell suspension cultures were sampled at each time-point (T7 or T4, namely 7 or 14 days of fermentation). 50 mg were sampled from each cellular pellet, in three independent biological repetitions, and immediately frozen in liquid nitrogen.

Total RNA was extracted using SpectrumTM Plant Total RNA Kit (Sigma, St Louis, MO, USA). RNA was quantified with a Nanodrop1000 (Thermo Scientific, Nanodrop Products, Wilmington, DE, USA) and RNA quality was assessed via agarose gel electrophoresis. 500 ng of RNA for each sample was then reverse transcribed to cDNA as described by Manoli et al. (2012).

Gene bank screening and primer design

Despite the great pharmacological potential of *Croton lechleri*, there are still no genomic data available for this species. Nevertheless, Canedo-Téxon and colleagues (2019) performed the sequencing and de novo assembly of RNA libraries from various organs of *Croton draco* trees. They also provided an annotation to public databases and categorized the transcripts according to their functions and metabolic pathways. Since both *C. lechleri* and *C. draco* are sources of dragon's blood sap (DBS) (Gupta et al. 2008) and are closely related in the *Croton* sect. *Cyclostigma* taxonomy (Riina et al. 2009), we used the unigenes annotated by Canedo-Téxon et al. (2019) to find genes related to PAs pathways in *C. lechleri* and to design specific primers for quantitative Real-time PCR (qRT-PCR) by using Primer3 v.0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). The list of genes and of the primers used are reported in Online Resource 2.

Quantitative Real-time PCR (qRT-PCR)

qRT-PCR was performed using the StepOne Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) as described by Nonis et al. (2007). SYBR Green reagent (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA) was used in the reaction, according to the manufacturer's instructions. Melting-curve analysis confirmed the absence of multiple products and primer dimers. Target gene relative expression was determined according to the Livak and Schmittgen (2001) method, using *UBIQUITIN CONJUGATING ENZYME 10 (UBC10)* as reference gene (Souček et al. 2017). The results of gene expression were calculated according to the $2^{-\Delta CT}$ method, which was normalized to a reference gene (Schmittgen and Livak 2008).

Three technical replicates were performed on three independent biological repetitions. ANOVA was performed as statistical analysis with significance set to $p < 0.05$ using the web tool SATQPCR (http://satqpcr.sophia.inra.fr/cgi/home.cgi) (Rancurel et al. 2019).

Fresh weight, density and coloring of cell culture

After 14 days of fermentation in Cle final liquid media, the *C. lechleri* cell suspensions were used for phenotypical analysis through the evaluation of cell fresh weight and colour by a portable spectrophotometer colorimeter (CM-2500d, Konica Minolta). Cell fresh weight was evaluated by transferring the cell suspension into a 50 mL falcon tube and letting it settle for 30 min. The volume occupied by the sedimented cells was considered as fresh weight by decantation. After that, the 50 mL falcon tubes were centrifuged for 15 min at 3000 rpm and 4 °C, the supernatant was discarded, while the cellular pellet was weighted, and its color was evaluated through the Hue angle by the colorimeter (Kortei and Akonor 2015).

Results

Proanthocyanidins quantification from both callus and liquid culture

The content of total proanthocyanidins (expressed as equivalent of catechin) was assessed to evaluate whether the callus or the liquid cell culture derived from it were able to produce PAs as the *Croton* plant in vivo (Fig. 1A). Accordingly, the *C. lechleri* explant firstly induced to form callus with a dark appearance (herein called "dark callus") showed the highest rate of PA accumulation as detected by the UPLC-DAD analysis (0.32 g/Kg of total PAs expressed as equivalent of catechin). However, as soon as the callus stabilized, it

A

Sample	Total PAs as equivalent of catechin
Dark callus	0.32 g/Kg (***)
Dark cell suspension	0.093 g(L (**)
Pale callus	0.11 g/Kg (***)
Pale cell suspension	0.067 g/L (**)
Pale cell suspension elicited with N deficiency	0.055 g/L (**)

Abbr: PAs, proanthocyanidins

B

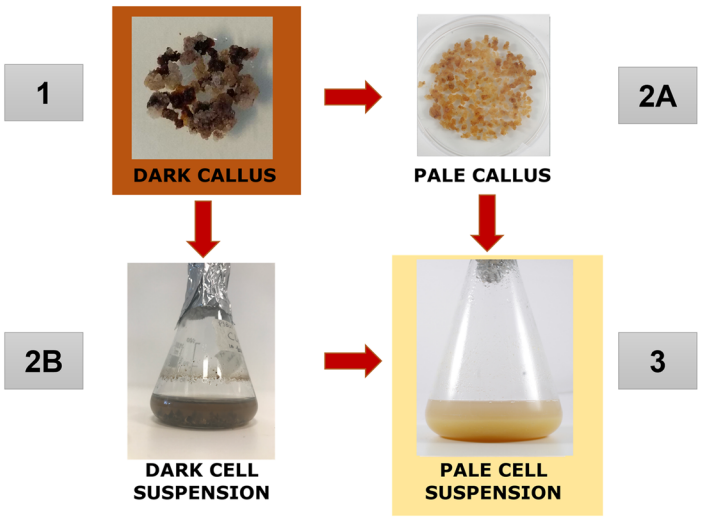

Fig. 1 Panel A: Quantitative UPLC-DAD analysis results on total proanthocyanidins (PAs) contents expressed as equivalent of catechin in calluses (g/Kg) and cell suspensions (g/L). Three biological replicates were analysed for each sample. Asterisks in brackets represents significance codes for ANOVA: *** for $p < 0.001$; ** for $p < 0.01$; * for $p < 0.05$. Panel B: *Croton lechleri* callus and cell suspension phenotypes: just after callus induction (dark callus) (1) and after some months in the solid medium (pale callus) (2**A**); dark phenotype (dark cell suspension) (2**B**) of cell suspensions obtained from the dark callus in the Cle medium, and the pale cell suspension phenotype (3) developed as soon as the cell fresh weight increased

became paler (herein called "pale callus") with a reduction of 65% in the equivalent of catechin value (0.11 g/Kgof total PAs expressed as equivalent of catechin) compared to the dark callus. Moreover, once transferred in the liquid Cle medium, the pale callus produced a pale cell suspension (called "pale cell suspension") with a further drop in the content of PAs (−40% if compared to the pale callus, −80% if compared to the dark callus) (Fig. 1B). If the dark callus was transferred in the liquid Cle medium before its stabilization in pale callus, for the first weeks the deriving cell suspension showed a dark phenotype (herein called "dark cell suspension") with a PA content statistically equal to that present in the pale callus (0.093 g/L of total PAs expresses as equivalent of catechin). As soon as the cell fresh weight increased, the culture became increasingly paler, reaching the "pale cell suspension" phenotype with its very low PA content (Fig. 1A). Finally, PA content in the cell suspension elicited with N deficiency showed very low levels of catechin in the samples (0.055 g/L of total PAs expresses as equivalent of catechin), statistically equal to the one reported for the pale cell suspension non elicited, but significantly higher compared to those detected in those detected in the control cell culture (Fig. 1A).

Identification of gene sequences involved in PAs-related pathways

In-silico study and bioinformatics analysis of the PAs biosynthetic pathway of *C. lechleri* allowed to obtain a list of genes to be tested in Real-time PCR (Online Resource 3). Starting from the annotation of the *C. draco* non-redundant unigenes provided by Canedo-Téxon et al. (2019) and of the corresponding *Arabidopsis thaliana* homologs, the dataset was screened to identify those genes encoding enzymes involved in the PA biosynthesis, transport and regulation (Fig. 2). Genes involved in reactive oxygen species (ROS) production and scavenging and in cell cycle related were also selected. The synthesis of PAs in the plant cell is an adaptive response to oxidative stress, where ROS are major players, so plants with high levels of PAs have excellent antioxidant capabilities.

A list of putative housekeeping genes (Online Resource 3) was also tested based on the most promising candidates from the literature (Souček et al. 2017) by means of Real-time PCR, and *UBC10* (*ubiquitin-conjugating enzyme 10*) was found as the most.

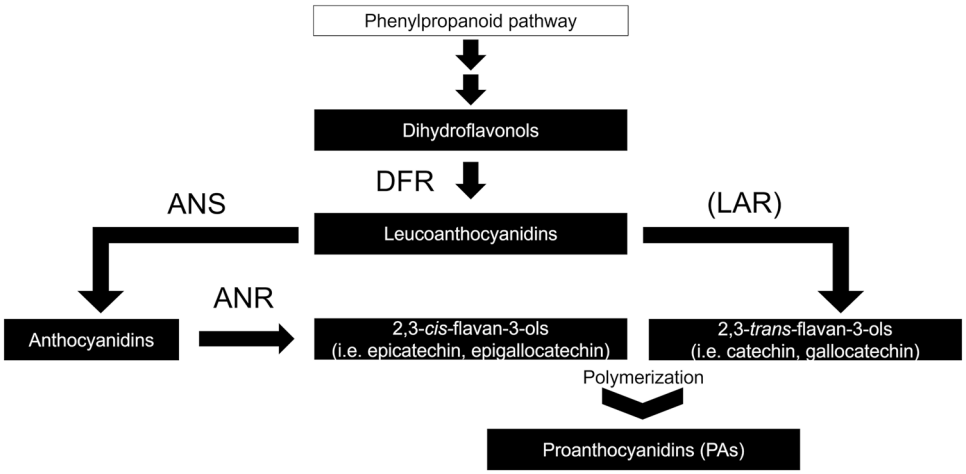

Fig. 2 Simplified biosynthetic pathway of proanthocyanidins (PAs). The enzymes involved in each step are reported with their acronym and their encoding genes were analyzed in this study (Online Resource 3), with the only exception of *LAR* (between bracket), which was not identified in *A. thaliana*. Abbr: ANR: anthocyanidin reductase; ANS: anthocyanidin synthase; DFR: dihydroflavonol 4-reductase; LAR: leucoanthocyanidin reductase. For more detailed information about PAs biosynthesis, we suggest the reviews by Yu et al. (2020) and Lim and Ha (2021)

Gene expression analysis on callus (solid culture) and cell suspension (liquid culture) of genes involved in proanthocyanidins biosynthesis and transport, cell cycle and oxidative stress regulation

Preliminary gene expression analyses were conducted to choose the most reliable candidate gene markers, among those pre-selected (Online Resource 3), that may show correlation with and predict the production of proanthocyanidin (PAs) through quantitative gene expression analysis on *C. lechleri* cell cultures (Fig. 3).

To set up the methodology, gene expression was initially tested on samples obtained from both *C. lechleri* callus and cell suspension. The expression of all the three genes chosen as indicative of PAs biosynthesis (*DFR*, *ANS* and *ANR*) was markedly higher in dark callus then in the pale one and dropped to basal levels in the cell suspension (Fig. 3A). Due to this almost overlapping trend among the three genes, for subsequent analysis, only *ANS* and *ANR*, encoding anthocyanin synthase and anthocyanin reductase respectively, were selected.

Considering those genes involved in the regulation of PAs biosynthesis (*TG1*, *TT2*, *TT8*) and in PAs transport (*TT9*, *TT12*, *TT13*) (Fig. 3B), no significant variations were detected among *TG1* and *TT13* expressions, while *TT2* was up regulated in the pale callus. *TT8, TT9* and *TT12* displayed, instead, a pattern similar to that observed for *ANS*, *ANR* and *DFR*, thus being also good putative markers for PAs accumulation.

Among genes involved in cell cycle regulation (Fig. 3C), only *CYCT1;4* showed an increased expression in the cell suspension with respect to both dark and pale callus. *CYCT1;4* encodes a cyclin T partner involved in the positive regulation of DNA-templated transcription, elongation, regulation of transcription by RNA polymerase II, and appeared promising as marker of an intense cellular transcription in the pale cell suspension. As far as genes involved in oxidative stress response were concerned, all except *GPX* displayed a higher mRNA accumulation on the dark callus compared to the cell suspension, leading us to hypothesize the existence of a correlation between oxidative stress and PAs production (Fig. 3D).

To resume, the following gene markers were chosen to be used in the elicitation tests: *ANS* and *ANR* for PAs biosynthesis, *CYCT1;4* for cell cycle activation, and *CAT2*, *SOD2*, *APX1* and *GPX2* for oxidative stress.

Croton lechleri cell culture elicitation with N-deficient media

Nitrogen shortage in the nutrient media was chosen as elicitor to stimulate PA biosynthesis in *C. lechleri* cell suspension.

To test the effect of N deficiency on *C. lechleri*, the unstable dark callus and the cell suspension derived from it (dark cell suspension) were tested together with stable but pale cell suspension treated for 7–14 days with normal Cle media (CTR, control) or with the Cle media deprived of any nitrogen source (No Nitrogen) for 7–14 days. Gene expression of *ANS*, *ANR*, *CYCT1;4*, *CAT2*, *SOD2*, *APX1* and *GPX2* was evaluated (Fig. 4). As previously described (Fig. 1B), the dark callus represented the original phenotype of the *C. lechleri* cell culture, rich in PA content but with an unstable

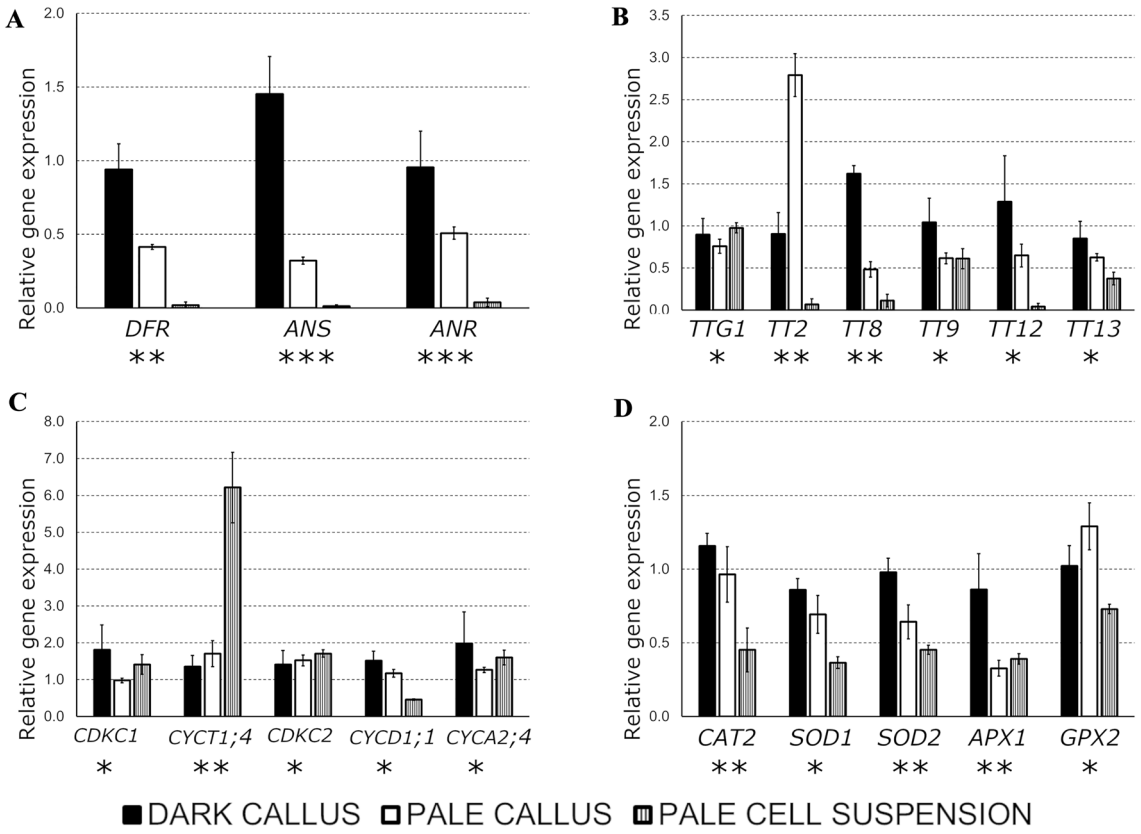

Fig. 3 Gene expression analysis on *Croton lechleri* callus and cell suspension of genes involved in proanthocyanidins biosynthesis (panel **A**) and transport/regulation (panel **B**), cell cycle (panel **C**) and oxidative stress (panel **D**). Data are mean ± SE for three biological replicates. Asterisks below the genes names represent statistical significance codes for ANOVA: *** for $p < 0.001$; ** for $p < 0.01$; * for $p < 0.05$. The expression levels of genes are presented using mRNA levels normalized to *UBC10* (UniGene59055, Canedo-Téxon et al. 2019). For the complete gene description please refer to Online Resource 3

growth rate, that was not able to maintain its characteristics but gradually transformed into a pale callus from which it the pale cell suspension could be derived. At the same time, the dark callus, before its transformation into pale callus, could be transferred to the liquid medium to obtain the dark cell suspension, which again resulted to be unstable and through time reached the final phenotype of pale cell suspension. Dark callus and the derived dark cell suspension were then compared to the stabilized pale cell suspension fermented in normal Cle medium (CTR, control) and pale cell suspension elicited in the N-deprived nutrient medium (No Nitrogen).

Both *ANS* and *ANR* were 10-times up-regulated in No Nitrogen for 14 days samples with respect to their CTR, while 7 days of N deficiency were not enough to stimulate their expression (Fig. 4A). These data suggest that 14 days of N deficiency could induce PAs biosynthesis. Nevertheless, the expression of selected genes in all cell suspensions showed a level significantly lower if compared to that measured for dark callus, suggesting that the step of transferring the cells of the callus to liquid media induced a relevant reduction in PA level.

CYCT1;4 was used as marker of cell growth (Fig. 4B). Its transcription was unchanged with respect to the control (CTR) in conditions of No Nitrogen both for 7 and 14 days, suggesting that N deficiency, even if prolonged, did not have a negative impact of the cell viability.

Among the oxidative stress marker genes, both *CAT2* and *GPX2* were 2-times up-regulated in No Nitrogen for 14 days if compared to CTR 14 days (Fig. 4B), leading us to hypothesize that nitrogen deficiency induced oxidative stress which is in turn possibly correlated to the increased PA production.

Fresh weight, density and coloring of cell culture

The same *C. lechleri* pale cell suspensions fermented for 14 days and used for gene expression analysis were also assessed for phenotypical analysis through the evaluation of cell fresh weight, density, and colour by colorimeter (Fig. 5). The volume occupied by the sedimented cells by decantation was considered as fresh weight (Fig. 5A). Cell suspension of

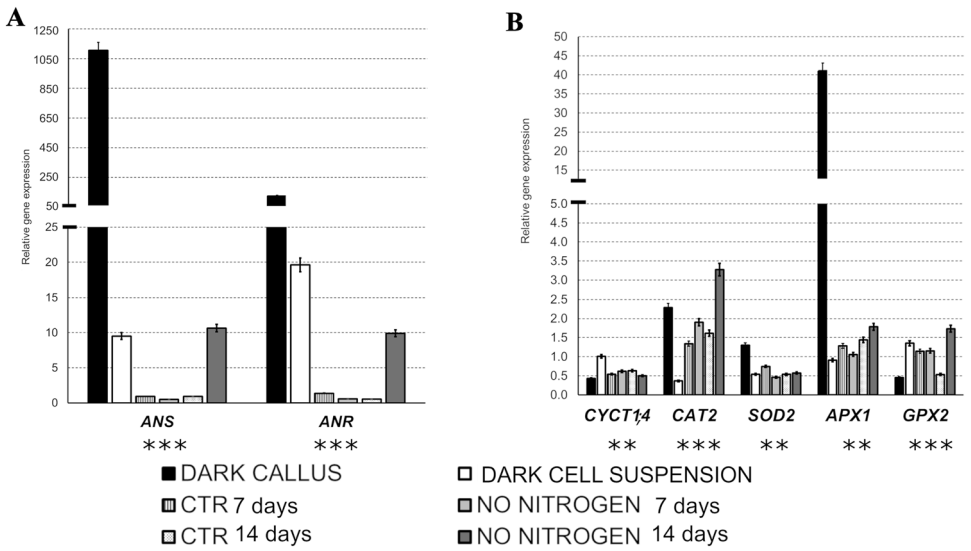

Fig. 4 Gene expression analysis of genes involved in proanthocyanidins biosynthesis (*ANS*, *ANR*) (panel **A**), cell cycle (*CYCT1;4*) and oxidative stress (*CAT2*, *SOD2*, *APX1*, *GPX2*) (panel **B**) on *Croton lechleri* dark callus, dark cell suspension and pale cell suspension fermented in normal Cle medium (CTR, control) or N-deprived nutrient medium (No Nitrogen) for 7 to 14 days. Data are mean ± SE for three biological replicates. Asterisks below the gene names represent statistical significance codes for ANOVA: *** for $p < 0.001$; ** for $p < 0.01$; * for $p < 0.05$. The expression levels of genes are presented using mRNA levels normalized to *UBC10* (UniGene59055, Canedo-Téxon et al. 2019). For the complete gene description please refer to Online Resource 3

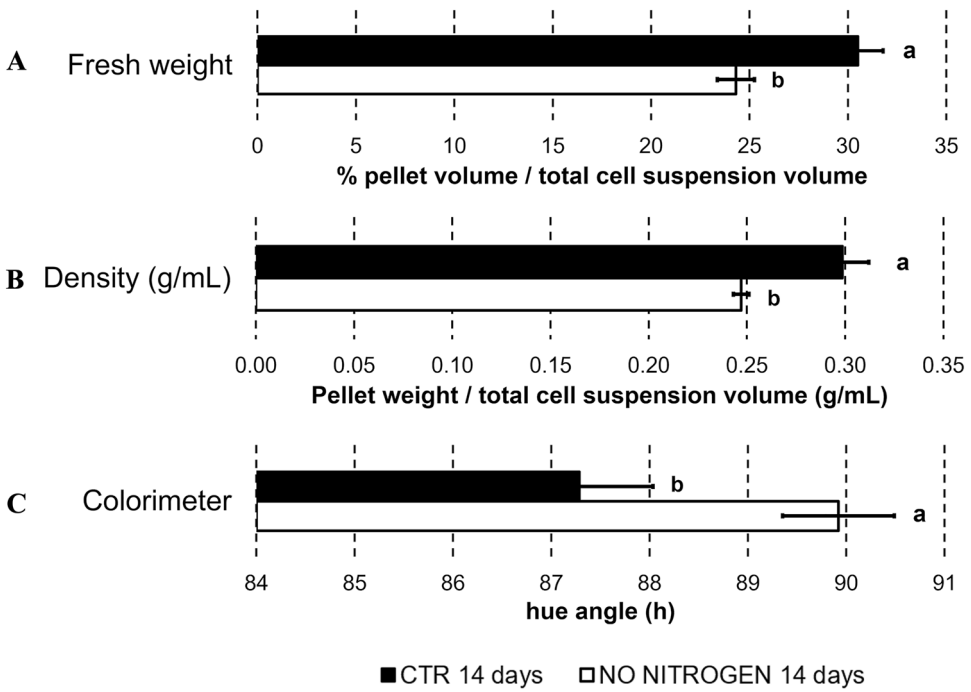

Fig. 5 Phenotypical analysis of *C. lechleri* cell suspensions after 14 days of fermentation in Cle liquid medium, stimulated to produce proanthocyanidins by removal of nitrogen in the medium (No Nitrogen) or grown in the standard nutrient medium supplemented with 25 mM KNO_3 and 1 mM $(NH_4)_2SO_4$ as N source (CTR, control). Cell fresh weight was evaluated by letting the cell suspension settle for 30 min, so the volume occupied by the sedimented cells was considered as fresh weight by decantation. After the centrifuge of the cell suspension, the cellular pellet was weighted and evaluated by colorimetry to assess the density and the hue angle (h), respectively. Different letters above the bars indicate statistically significant difference at $p < 0.05$ for ANOVA

C. lechleri fermented in the control media (CTR) showed a significant increase (+6%) in the fresh weight if compared to liquid culture grew in the absence of nitrogen in the medium (No Nitrogen). After centrifugation, the control samples (CTR) still displayed a significant higher density if compared to the treated cells (No Nitrogen) (Fig. 5B). Regarding the color, the colorimeter used in the analysis allowed to discriminate among the control liquid culture (CTR) and the treated *C. lechleri* culture (No Nitrogen). A higher hue angle represents a lesser yellow character; thus, the hue angle for both samples was close to 90, where 0 represents the red color, and 90 represents the yellow color. Visually, the control cells appeared paler than the *C. lechleri* cells treated with N deficiency, and the colorimeter confirmed the small but significant difference in the Hue angle among the two different liquid cultures (Fig. 5C).

Discussion

Croton lechleri is a South America tree of the Euphorbiaceae family well known for its production of a deep red latex known as "Dragon's blood sap" (DBS). The chemical constituents of *C. lechleri* latex are proanthocyanidins (PAs), polyphenolic components, alkaloids such as taspine, and minor amounts of terpenoid compounds (Gupta et al. 2008). This latex is used as a starting material in the isolation and purification of the botanical drug Crofelemer, a polymeric proanthocyanidin product that is clinically used for treating HIV-associated secretory diarrhea (Kleindl et al. 2017).

In plants, PAs play important roles in increasing the tolerance to several abiotic and biotic stresses (Lim and Ha 2021), such as low temperature (An et al. 2018), drought (Malisch et al. 2016), UV-B radiation (Mellway et al. 2009), herbivory attack (Yu et al. 2020) and infection by biotrophic fungi (Ullah et al. 2017). In human health, PAs provide many benefits, and they are considered important food-derived bioactive compounds in both the pharmacological and cosmetic industries (Santos-Buelga and Scalbert 2000). As many other secondary metabolites, such commercially important metabolites as PAs are generally present at low abundance and only occur in specific plant organs, making difficult their extraction and production in large amounts. Therefore, alternative methods to obtain them such as through plant cell suspension cultures have been proposed for large-scale production. Once settled, plant suspension cell cultures have the advantages of safety, regulatory compliance, scalability, and low cost (Wu et al. 2021). The knowledge of the molecular mechanisms underlying the secondary metabolites biosynthesis is crucial to set up effective plant cell culture growth protocols, but this goal is not always easy to reach due to the limited amount of information available on the selected plant species or on the biosynthetic pathways (Yonekura-Sakakibara and Saito 2009; Guerriero et al. 2018). For instance, a study led to the identification of biosynthetic genes up-regulated during the elicitation with Methyl jasmonate (MeJA) in *Isatis indigotica* hairy root cultures to increase the production of lignans (Chen et al. 2015). In another study, a metabolite and transcript profiling of *Nicotiana* cells elicited by MeJA allowed to discover new genes involved in tobacco secondary metabolism (Goossens et al. 2003), while a similar functional genomic techniques allowed to identify many genes involved in the terpenoid indole alkaloid biosynthesis in *Catharanthus roseus* cell cultures (Rischer et al. 2006). Furthermore, a transcriptome analysis of *Rosmarinus officinalis* Lour. cell suspension allowed to identify several differentially expressed genes involved in the biosynthesis of the active compounds and induced by MeJA (Yao et al. 2021). Similarly, the characterization of genes involved in the biosynthesis of artemisinin (an important drug commonly used in the treatment of malaria) in *Artemisia annua* plants paved the way to engineer the production of this valuable metabolite in cells of both plants and microorganisms (Guerriero et al. 2018). Nevertheless, it is still rare to find studies showing how a marker genes could be used to predict the biosynthesis of a secondary metabolite without needing an expensive validation with quantitative methods (i.e. mass spectrometry), which is the attempt of the present research.

PAs are synthesized as one of the end-products of the flavonoid pathway, sharing the biosynthetic pathway with anthocyanins (Fig. 2; Dixon and Sarnala 2020; Lim and Ha 2021). From a chemical point of view, PAs are oligomers or polymers resulting from the condensation of two or more flavan-3-ol units. The biosynthetic pathway begins with the condensation and subsequent cyclization of three malonyl-CoA molecules with one 4-coumaroyl-CoA molecule to produce a naringenin chalcone (He et al. 2008). The second step of the pathway is the isomerization of the naringenin chalcone to naringenin. Then, naringenin is converted to its two derivatives eriodictyol and pentahydroxy flavanone which serve as substrates to produce dihydroflavanols. The key enzyme DFR (dihydroflavonol 4-reductase) catalyses the NADPH dependent reduction of the keto group in position 4 of dihydroflavonols to produce leucoanthocyanidins. Leucoanthocyanidins are then synthesized into anthocyanidins by ANS (anthocyanidin synthase). Anthocyanidins can then be converted into epicatechin, epiafzelechin, and epigallocatechin by ANR (anthocyanidin reductase). Other conserved proteins involved in PA biosynthesis include the transcription factors TRANSPARENT TESTA 2 (AtTT2), TRANSPARENT TESTA 8 (AtTT8/MtTT8), TRANSPARENT TESTA 9 (AtTT9), TRANSPARENT TESTA GLABROUS 1 (AtTTG1, with ortholog WD40-DOMAIN PROTEIN, MtWD40-1, in *M. truncatula*), and the transporter AtTT12, orthologous to MULTIDRUG AND TOXIC

COMPOUND EXTRUSION FAMILY 1 (MtMATE1) in *M. truncatula* (Lu et al. 2022). Leucoanthocyanidins are also synthesized into (2R, 3S)-flavan-3-ols (i.e. catechin, afzelechin, and gallocatechin) by LAR (leucoanthocyanidin reductase). Nevertheless, the *LAR* gene was not identified in *A. thaliana,* which has been reported to have only epicatechin (Abrahams et al. 2003), so the homologous sequences were not available for *Croton draco* annotation (Canedo-Téxon et al. 2019), nor for the present work. However, the PA production could be determined by the expressional competition and/or the presence/absence of *ANS* and *ANR*, confirming that these two genes could represent valuable markers to predict the PAs biosynthesis among the many genes herein evaluated (Fig. 3).

In our study we identified genes for biosynthesis and transport of PAs in *Croton lechleri* starting from sequence from *Croton draco* (Canedo-Téxon et al. 2019) and among these we have identified some marker genes useful to early detect PA enhanced biosynthesis in *Croton lechleri* cell culture (Fig. 3). Particularly, we showed that *ANS* (*anthocyanin synthase*) could be considered the most effective marker gene for its strong induction in the dark callus if compared to the pale cell suspension (Fig. 3A).

On one hand we showed a correlation between the higher levels of PAs (Fig. 1A) and the up-regulation of *ANS* (Fig. 3A), on the other hand we also showed the existence of a correlation between oxidative stress and PA production (Fig. 3D). Oxidative stress is essentially a state of redox imbalance originating from the overproduction and accumulation of pro-oxidants such as reactive oxygen species (ROS), or from the relative insufficiency of antioxidant molecules such as glutathione (GSH) and enzymes such as CAT, SOD, and GSH-Px (Yang et al. 2018). The synthesis of PAs in the plant cell is an adaptive response to oxidative stress, so plants with high levels of PAs have excellent antioxidant capabilities. In general, suboptimal temperatures, high irradiance, water stress, wounds and nitrogen deficiency stimulate the production of ROS and consequent oxidative stress (Yu et al. 2020; Mannino et al. 2021). Accordingly, it was shown that high light stress and nitrogen (N) deficiency both generate ROS in hybrid poplar (*Populus tremula* x *Populus tremuloides*), while exposure to natural sunlight for 14 days caused a 14-fold increase in foliar PA levels, whereas the N deficiency in the soil led to a 4- to fivefold increase and an induction of genes involved in the regulation of PA biosynthesis (Gourlay and Constabel 2019). In another study, shoot cultures of *V. corymbosum* showed that the reduction of macro-nutrients stimulated anthocyanins production and higher antioxidant power (Lucioli et al. 2018). Since the nutrient deficiency, and in particular the N deficiency, appeared as the cheapest method to stimulate PA production, we applied this abiotic stress to the *C. lechleri* cell suspension (Fig. 4). Overall, the elicitation with N shortage was successful since both the PA biosynthesis marker genes *ANS* and *ANR* showed a significant 10-times up-regulation if compared to the control cell suspension in normal Cle liquid medium (Fig. 4A). Moreover, the treatment with no nitrogen significantly induced a state of oxidative stress, as shown by the up-regulation of *CAT2* and *GPX2* after 14 days in N deficiency (Fig. 4B). Unfortunately, this elicitation was not sufficient to restore the level of PA detected in the dark callus (Fig. 1A), but it was a good indication for further studies in this way.

Despite the promising results of the PA elicitation in N-deprivation, the growth rate of the *C. lechleri* liquid culture resulted critic. Accordingly, when the *C. lechleri* callus was transferred to Cle liquid medium to obtain the *C. lechleri* cell suspension, a remarkable loss of PA productivity and fitness was observed (Fig. 1A), with cells growing slowly (14 days needed for each subculture) and without producing PAs anymore. In future studies, it could be interesting to firstly push the growth rate of the cell culture, then to elicit the PA production with the abiotic stress (N-deprivation), also studying how to combine the N-deprivation to other inducing stress (i.e. osmotic stress, low temperature).

One alternative explanation is that the failure to accumulate the metabolite of interest in cultured cells may be due either to feedback-inhibition of key biosynthetic enzymes or to non-enzymatic degradation of the product in the culture medium (Nielsen et al. 2019). In any case, the herein identification of *ANS* as marker of PA biosynthesis in *C. lechleri* could be used to discriminate among the most promising elicitation conditions tested together with N-deprivation.

Another valuable result achieved in this study is the indication to use a pre-screening to evaluate the cell culture before the gene expression. Accordingly, fresh weight, density and colors of the treated cell cultures could be correlated with PA production (Fig. 5). The easy employment of a colorimeter could be particularly useful. Colorimeters are electronic devices for color measurement that express colors in numerical coordinates and the Hue angle is one of the parameters used to evaluate the color quality (Kortei and Akonor 2015). Hue is the attribute of color perception by which a color is judged to be red, orange, yellow, green, blue, purple, or intermediate between adjacent pairs of these colors, considered in a closed 360° ring or wheel. Hue angles of the four primary colors are: red, 0°; yellow, 90°; green, 180°; and blue, 270°. PAs are brown-pigmented phytochemicals, so a correlation between the dark phenotype of callus and cell suspension and their higher amounts of PAs was supposed and validated by quantitative UPLC-DAD analysis (Fig. 1). On the contrary, the stabilized liquid cell culture appeared paler than the *C. lechleri* cells elicited with N deficiency, a significant difference confirmed by the different Hue angle among the two different liquid cultures (Fig. 5C).

In conclusion, this study provides for the first time an attempt to obtain a cell culture from a *Croton lechleri* sample and to induce the production of proanthocyanidins, thus of oxidative stress, through the imposition of a nutrient deficiency as eliciting abiotic stress. In particular, the results show a new perspective on how gene expression analysis could be used to predict the biosynthesis of useful secondary metabolites in plant cell cultures without the requirement of expensive quantitative analysis. Further studies will be needed to force the elicitation by chemicals or abiotic stress combinations, but the identification of *ANS* as a marker gene for PA biosynthesis in *C. lechleri* could be useful to speed up the screening of putative elicitors.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflicts of interest The authors declare no conflict of interest.

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