Establishment and Comparative Secondary Metabolite Production of Callus and Cambial Meristematic Cells of Coffea arabica and Coffea canephora

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Research Article

Keywords: Cambial meristematic cells, Cellular agriculture, Coffea arabica, Coffea canephora, Elicitation, Plant cell culture

Posted Date: June 12th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9847662/v1

Additional Declarations: No competing interests reported.

Abstract

Coffee is a globally important agricultural commodity whose production is increasingly challenged by climate change and sustainability constraints, necessitating alternative production strategies independent of traditional cultivation. Plant cell culture represents a promising cellular agriculture approach for producing valuable coffee secondary metabolites, including purine alkaloids (e.g., caffeine and theobromine) and chlorogenic acids, under controlled conditions. This study aimed to establish cambial meristematic cells (CMCs) of *Coffea arabica* and evaluate their biotechnological performance relative to dedifferentiated cells (DDCs) with respect to growth characteristics and metabolite production. For the first time, stable *C. arabica* CMC lines were successfully established and cultivated alongside DDCs in shake-flask systems under heterotrophic and mixotrophic conditions. Cellular morphology, growth kinetics, and secondary metabolite accumulation were systematically analysed. CMCs exhibited characteristic morphology with small vacuoles and significantly smaller cell size (59.51 ± 14.22 µm) compared with DDCs (82.34 ± 20.21 µm). Under heterotrophic cultivation, DDCs achieved higher intracellular caffeine yields (0.285 ± 0.067 mg/g_{CDW}) than CMCs (0.041 ± 0.005 mg/g_{CDW}). In contrast, mixotrophic conditions markedly enhanced CMC metabolic performance, resulting in increased caffeine accumulation (0.447 ± 0.015 mg/g_{CDW}) and substantial chlorogenic acid production (0.314 ± 0.057 mg/g_{CDW}), whereas DDCs showed reduced productivity and increased stress sensitivity. Comparative analysis of CMCs from *C. canephora* revealed species-specific differences, with caffeine concentrations nearly twice those observed in *C. arabica* (0.85 ± 0.15 vs. 0.45 ± 0.02 mg/g_{CDW}). These findings identify CMCs as a promising platform for light-driven coffee cell biotechnology and provide a foundation for developing sustainable, cell culture–derived coffee production systems.

Key message

Cambial meristematic cells outperform dedifferentiated cells under light conditions, achieving superior caffeine and chlorogenic acid production for sustainable coffee biotechnology applications.

Introduction

The plants of the genus *Coffea*, belonging to the family *Rubiaceae*, are commonly known as coffee plants. While 124 coffee species are recognised, global trade is dominated by *Coffea arabica* (approximately 60% of production) and *Coffea canephora* (Robusta, 40%) (About Economics & Statistics | International Coffee Organization; Davis et al. 2019). Annual global coffee production exceeds 9.5 million tons, with an estimated trade value surpassing USD 50 billion in 2024 (The Observatory of Economic Complexity). However, coffee production is associated with a substantial environmental footprint, with life-cycle assessments indicating carbon emissions ranging from 3.7 to 15.8 kg CO_2-equivalents per kilogram of green coffee (Nab and Maslin 2020; Ritchie and Roser 2020). With rising temperatures, changing rainfall patterns and the increasing frequency of extreme weather events, climate change poses an increasingly threat on coffee yields and bean quality. These environmental changes not only threaten the agricultural viability of coffee-growing regions, but also affect the taste, aroma and overall

market value of coffee (Girma 2023). Consequently, coffee prices have increased on a global scale, with climate-induced supply disruptions contributing to increased volatility and less predictable costs across the supply chain (Ahmed et al. 2021; Bilen et al. 2022). Furthermore, changing climatic conditions facilitate the spread of phytopathogens such as *Hemileia vastatrix* (coffee leaf rust), necessitating increased pesticide application and exacerbating environmental concerns (Alfonsi et al. 2019; Tadesse et al. 2021). The ongoing decline in biodiversity of wild coffee species is a matter of particular concern, as it threatens to hinder future advancements in plant breeding (Davis et al. 2019). Concurrently, the demand for coffee is predominantly driven by augmented consumption in Asia and developing countries, underpinned by economic growth that fosters a growing middle class and urban lifestyles (Purnomo et al. 2021; Hanley et al. 2026). Projections indicate that demand will triple by 2050, while global production is expected to halve over the next 30 years due to climate change (Grüter et al. 2022). These projections underscore the coffee industry's imminent challenges.

To address these challenges, future strategies must prioritize both reducing the environmental impact of coffee production and ensuring its sustainability. One possible route to sustainable coffee is the use of coffee substitutes, also known as surrogates (Tahmouzi et al. 2024). Traditionally, these surrogates have been derived from roasted plant materials such as barley, rye, malted grains, etc. They were introduced as affordable, locally sourced alternatives that produced a coffee-like infusion when brewed with hot water, with some consumer segments preferring their low or no caffeine content. In recent years, multiple commercial ventures have emerged to develop coffee alternatives with reduced environmental impact. Companies such as Atomo Coffee, Northern Wonder, Prefer, Minus, Ciao Coffee, and Ryze Superfoods are pursuing strategies to create coffee-like beverages without relying on conventional agricultural production, thereby addressing sustainability concerns associated with climate-intensive coffee cultivation (Khushvakov et al. 2024). In contrast, the present work is directed toward the production of authentic coffee through plant cell culture technology. Rather than developing coffee substitutes, the full biochemical complexity of genuine coffee is targeted by enabling the biosynthesis of native coffee metabolites from cultured plant cells. Biotechnological strategies, particularly cell-based agriculture, have emerged as promising alternatives to conventional crop cultivation. Within this framework, two principal plant cell culture systems are employed, including dedifferentiated cells (DDCs) and cambial meristematic cells (CMCs). DDCs are obtained by subjecting plant tissues to a process of induction, thereby inducing an undifferentiated state. This results in the formation of heterogeneous cell populations, characterised by variable morphology and metabolic performance. These cultures are frequently utilised due to their ease of induction; however, they often exhibit limited stability and inconsistent productivity (Li and Hall-Ponselè 2025). Conversely, CMCs are derived from the vascular cambium and thus possess meristematic characteristics, including uniform cell size, elevated division rates, and diminished vacuolisation (Partap et al. 2023; Mehring et al. 2020). These properties contribute to improved metabolic stability and, in certain cases, increased metabolite accumulation. It is noteworthy that several companies have already set up pilot-scale facilities for the fermentative production of coffee and cocoa cells. These enterprises, including PluriAgtech/Coffeesai (Haifa, Israel), California Cultured (Davis, CA, USA) and Food Brewer (Zurich, Switzerland), have set up pilot-scale

facilities for the fermentative production of coffee and cocoa cells, respectively (Raviv and Ben Eliezer 2023; Turrell 2024). These initiatives highlight the considerable commercial potential of employing cell culture technology to produce plant-derived commodities.

The concept of substituting green coffee beans with cultivated coffee cultures can be traced back to Townsley's seminal work in 1974 (Townsley 1974). The first utilization of *Coffea arabica* cell cultures can be traced back to the 1970s. Buckland established early callus suspension cultures in small Erlenmeyer flasks (Buckland 1972), while Staritsky described the cultivation of callus cells on Murashige-Skoog (MS) solid media (Staritsky 1970). Building on these approaches, Waller et al. (1983) cultured callus cells in small reaction vessels and showed that much of the synthesized caffeine was released into the medium (Waller et al. 1983). Recent advancements, as exemplified by the work of Aisala et al., have expanded these methodologies by increasing the scale in wavebag bioreactors (10–25 L working volume) and determining product concentrations (e.g., caffeine and chlorogenic acid) in callus cells (Aisala et al. 2023). The significance of coffee extends beyond its status as a popular beverage, as it is a significant source of phytochemicals. These include, but are not limited to, caffeine and chlorogenic acids, as well as diterpenes, trigonelline, proanthocyanidins, ferulic acid, caffeic acid, and *p*-coumaric acid (Makiso et al. 2024). A notable aspect of coffee's nutritional profile pertains to its potential applications in cancer, diabetes, cardiovascular diseases, and obesity therapy. This potential benefits from a body of evidence that has emerged from ongoing research (Stoikidou and Koidis 2023). To meet the growing demand for these bioactive compounds and enable sustainable production independent of agricultural constraints, plant cell culture systems offer a promising alternative. While cambial meristematic cells (CMCs) have been established as robust production platforms in several plant species, offering potential advantages such as greater genetic stability, faster growth, and enhanced metabolite production (Ochoa-Villarreal et al. 2015), their application to *Coffea* spp. has not yet been systematically explored. In this study, we report the establishment and characterization of stable CMC lines from *Coffea arabica* and *Coffea canephora*, thereby extending the CMC concept to coffee cell biotechnology.

The present study aimed to establish CMCs from the two most commonly cultivated coffee species (*C. arabica* and *C. canephora*) and to comparatively evaluate their performance against conventional DDCs as platforms for authentic coffee metabolite production. Both cell types were cultivated in suspension systems and investigated with respect to growth behaviour, cellular morphology, and the accumulation of target metabolites relevant to biological activity. Furthermore, a comparative biochemical evaluation was performed against *C. canephora* cell cultures to determine metabolic similarity. Therefore, cell culture biotechnology can be integrated to bridge plant cell cultivation and coffee product development, providing fundamental insights into the feasibility of producing genuine coffee compounds through sustainable, controlled, and agriculture-independent production systems.

Materials and methods

Establishment of CMC and DDC cell cultures

CMC and DDC Cell cultures were established from young shoot and cotyledon leaves, respectively, from young plants of *C. arabica* and *C. canephora*. The plant material was kindly provided by Prof. Dr. Peter Nick (Botanical Institute, Karlsruhe Institute of Technology, Karlsruhe, Germany). Young shoots and cotyledon leaves were cut to 2 cm in size and surface sterilized according to an adapted protocol by (Mehring et al. 2020). The explant material was incubated in 0.56 mM ascorbic acid solution for 90 min, followed by 10 min incubation in 70% (v/v) ethanol and incubation in 1% (v/v) sodium hypochlorite for 30 min. Then, leaves and shoots explant were rinsed in sterile deionized water for 5 min after each sterilization step. Afterwards, shoots were quartered along the shoot axis in approximal one cm long pieces and incubated on agar plates containing 30 g/L sucrose, 4.4 g/L Linsmaier-Skoog (LS) media, 4 g/L plant agar and 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D). Leaves were cut into approximately 2 cm^2 squares and placed with the adaxial side on LS-induction media containing, additionally 1 mg/L 6-furfurylaminopurine. The induction of both cell types was conducted under both dark and illuminated conditions, employing a photoperiod of 16 h of light and 8 h of darkness, with a constant light intensity of 100 ± 10 $\mu mol_{photons}/(m^2 \cdot s)$. The treated shoot explants were examined under a light microscope (Nikon Eclipse NI, Düsseldorf, Germany) at two-week intervals to ascertain the emergence of new cells in the cambial layer during the induction of the CMCs. Following the formation of cells, they were carefully scraped off using a scalpel and transferred to agar plates for strain maintenance. Cell morphology was routinely inspected with the light microscope.

Strain maintenance and pre-culture

CMCs and DDCs of both *Coffea* sp. were maintained on agar plates containing LS medium (30 g/L sucrose, 4.4 g/L LS-media, 1 g/L MES ((2-(*N*-morpholino)ethanesulfonic acid) and 1 mg/L 2,4-D, pH adjusted to 5.7 with sodium hydroxide) with an addition of 5.5 g/L plant agar. LS-medium for DDCs additionally contained 1 mg/L 6-furfurylaminopurine. Subculturing of agar-grown cultures was performed at monthly intervals to maintain cell viability, sustain active growth, and ensure physiological stability throughout the experimental period. All agar cultures were maintained in a controlled incubator (Binder, Tuttlingen, Germany) at 28°C. For the establishment of pre-suspension cultures, DDCs or CMCs were inoculated at an initial density of 1 g/L cell wet weight (CWW) into 300 mL non-baffled shake flasks containing 50 mL LS medium. Cultures were incubated for 14 days in an orbital shaking incubator (Multitron, Infors GmbH, Einsbach, Germany) at 28°C, 120 rpm, and an orbital eccentricity of 2.5 cm to promote homogeneous suspension growth and adequate mass transfer. All experimental procedures were conducted either under complete darkness or under a controlled photoperiod of 16 h light and 8 h darkness, depending on the respective cultivation conditions. Additional mechanical homogenisation of the cultures was not required, as both cell types exhibited a friable morphology that enabled spontaneous dispersion in liquid medium.

Cultivation in shake flasks

To assess growth and productivity in 300 mL shake flasks over time, 1 g/L_{CWW} DDCs or CMCs from precultures were incubated in 50 mL medium in an incubation shaker for up to 35 days at 28°C and 120 rpm in darkness. Flasks were capped with cellulose plugs. All samples of the cultivation media were taken under sterile conditions. Similarly, mixotrophic suspension cultures were established by transferring 1 g/L_{CWW} DDCs or CMCs of light-induced, optically green DDC/CMC cells from agar plates to 250 ml shake flasks containing 50 mL of medium with 15 g/L sucrose. These cultures were maintained at 28°C and 120 rpm in a shaking incubator equipped with broad-spectrum white LEDs lighting, under a photoperiod of 16 h of light and 8 h of darkness at a constant light intensity of 100 ± 10 $\mu mol_{photons}/(m^2 \cdot s)$. At the end of the cultivation, plant cell biomass was harvested by vacuum filtration and frozen at -20°C until further use.

Product extraction and analysis

Frozen biomass was lyophilized (Christ, Osterode, Germany) at − 20°C and 1.03 mbar for 24 h. Alkaloids, specifically caffeine and theobromine, and chlorogenic acid were extracted by grinding 50 mg dry biomass in a mortar with 250 mg sea sand (particle size 0.1–0.315 mm) and 750 µL demineralized water for 2 min. Samples were collected in 15 mL reaction vessels by washing the mortar with another 750 µL of water and mixed with a vortex mixer (Reax Top, Heidolph Instruments, Schwabach, Germany). The products were then extracted for 30 min at 60°C with a nominal output of 160 W in an ultrasonic bath (Sonorex Digiplus, BANDELIN electronic GmbH & Co. KG, Berlin, Germany). Afterwards, the extracts were stored at − 20°C until further use.

Extracts were prepared for chromatographic analysis by filtration through nylon membrane filters (pore size 0.22 µm) to remove particulate material. Reversed-phase high-performance liquid chromatography (RP-HPLC) analyses were performed using a Knauer HPLC system equipped with an AZURA DAD 2.1L diode array detector (Knauer, Berlin, Germany). Separation was carried out under isocratic conditions at a flow rate of 0.5 mL/min with an injection volume of 20 µL and a column temperature maintained at 80°C.

The mobile phase consisted of 30% (v/v) methanol containing 0.1% (v/v) formic acid in water. Chromatographic separation was achieved using a Eurospher II 100-5 C18 column (5 µm particle size, 250 × 3 mm) coupled with a corresponding guard column to protect the stationary phase and ensure reproducible performance. Detection was performed by UV absorbance at 273 nm, and full UV spectra were recorded for peak identification and purity assessment. Quantification was conducted using external calibration with authentic reference standards, applying a five-point calibration curve for each analyte to ensure accurate determination of metabolite concentrations.

Neutral red for vacuole staining

For differentiation between DDC and CMC cells based on their distinct vacuolar morphology, 50 mg cells from agar plates were incubated in 1 mL 0.005% (w/v) neutral red solution prepared in culture medium

for 3 min. Suspensions were then centrifuged twice for 2 min at 14,000 rpm. Supernatant was discarded at each stage, and cells were resuspended in 1 mL phosphate buffer as established by Sörensen (pH 7.2). All staining steps were performed in darkness. Suspended cells were pipetted on a microscopy slide and observed under the light microscope.

Determination of cell size

About 50 mg CMCs or DDCs from suspension cultures were filtered and resuspended in 1 mL of the respective liquid media. Suspended cells were pipetted on a microscopy slide and observed under a light microscope. Across ten slides per cell type, a total of 476 cambial meristematic cells and 384 dedifferentiated cells were evaluated based on the recorded images. Cell size was determined by measuring the cell diameter using ImageJ software (Version 1.54g, National Institutes of Health, USA).

Statistical analysis

Values are presented as means ± standard deviation (n = 384 for cell size measurements) unless otherwise noted. The Shapiro–Wilk test was used to assess data normality. The Mann–Whitney U test was applied to determine significant differences between data sets, with $p < 0.001$ indicated by triple asterisks (***). Statistical analyses were performed using Origin software (Version 2023, OriginLab Corporation, Northampton, MA, USA).

Results and discussion

Establishment of C. arabica CMCs and DDCs and their morphology

Within nine days following explant preparation, light microscopic examination revealed the initiation of cell formation originating from the vascular cambium of *C. arabica* (Fig. 1A, B). These newly formed cells were carefully excised and transferred onto solid culture medium for further propagation. The classification of these cells as CMCs was based on their characteristic morphology, including their unique small, isodiametric shape and the presence of numerous small vacuoles (Fig. 2A, B). In addition, the cells exhibited a darker, brownish colour compared to DDCs (Fig. S1).

In contrast, DDCs originating from the same species were first observed two weeks after explant treatment and displayed significantly larger cell dimensions, a light to white coloration, and a single large vacuole (Fig. 2C, D). These morphological distinctions are consistent with previously reported observations distinguishing CMCs from DDCs (Lee et al. 2010; Ochoa-Villarreal et al. 2015; Kaňuková et al. 2024).

The suspension cultures of CMCs were primarily composed of individual cells and cell pairs, in addition to small clumps consisting of three to four cells. Conversely, DDC suspension cultures exhibited a higher frequency of cell clumps and larger aggregates, reaching sizes of up to 2 cm. Cells within these aggregates were frequently connected end-to-end, forming filamentous, thread-like strands of variable length (Fig. 1C, F). These strands commonly consisted of at least ten interconnected cells exhibiting

active cytoplasmic streaming. Similar filamentous morphologies have previously been reported in *C. arabica* callus suspension cultures (Di Bonaventura et al. 2024; Padua et al. 2014).

Earlier studies have demonstrated that phytohormone composition exerts a substantial influence on cellular morphology, including the induction of filamentous growth patterns (Nishi and Sugano 1970). As filamentous structures are predominantly described in *C. arabica* callus cultures, such growth behavior may represent a characteristic in vitro response of this species. Moreover, callus morphology has been shown to depend strongly on explant origin. For example, nodal explants containing meristematic tissues primarily generate compact, granular callus with embryogenic potential, whereas leaf explants display greater morphological heterogeneity, with callus characteristics varying according to tissue position within the leaf (Padua et al. 2014). These observations indicate that the differentiation status of the source tissue, whether parenchymal, epidermal, or vascular, plays a decisive role in determining both regenerative capacity and structural organization of the resulting callus.

In older callus cultures, numerous small discrete bodies were observed within cellular compartments. Based on the hypothesis proposed by Buckland (1972), these structures were presumed to represent amyloplasts containing starch granules. To verify this assumption, samples were treated with 2% Lugol's iodine solution. Following staining, the structures developed a characteristic dark blue to black coloration (Fig. S2), indicative of starch–iodine complex formation and thereby confirming the presence of starch-containing amyloplasts.

To further differentiate callus-derived cells from CMCs, a neutral red staining was performed, exploiting the selective accumulation of the dye within acidic compartments such as vacuoles. This approach confirmed that CMCs possess numerous small vacuoles, while DDCs exhibit a single large central vacuole that occupies most of the cellular volume (Fig. 2B, C), representing a characteristic morphological distinction. The longstanding reliability of neutral red as a vacuolar marker is attributed to its ion-trap mechanism, whereby the protonated dye accumulates within acidic intracellular compartments (Timmers et al. 1995; Stefano et al. 2018; Schwab and Hülskamp 2010).

Quantitative morphometric analysis further substantiated these qualitative observations. The mean cell diameter of CMCs was determined to be 59.53 µm (Fig. 2D; black box), with an interquartile range of 14.23 µm and values ranging from 34.33 to 138.47 µm. In contrast, DDCs exhibited significantly larger dimensions, with a mean diameter of 82.34 ± 20.22 µm (Fig. 2D; red box), an interquartile range of 20.22 µm, and a size distribution between 40.89 and 150.18 µm. These findings are consistent with previously reported size differences between meristematic and dedifferentiated plant cell systems, including observations in Ocimum basilicum cell lines (Mehring et al. 2020).

Hence, the combined morphological, cytological, and quantitative analyses confirmed the successful establishment of two distinct *C. arabica* cell culture systems, including both CMCs and DDCs, characterised by fundamentally different cellular organisation, vacuolar architecture, and aggregation behaviour. These structural differences are expected to influence growth dynamics and secondary

metabolite biosynthesis, thereby providing a foundation for subsequent comparative metabolic analyses.

Growth characteristics and secondary metabolite production of CMCs compared to DDCs under heterotrophic conditions

The growth characteristics of CMCs and DDCs were monitored over 30 and 36 days, respectively (Fig. 3). CMCs reached 6.15 ± 0.77 g_{CWW}/L after 30 days, while DDCs achieved 5.25 ± 1.47 g_{CWW}/L after 36 days. Maximum specific growth rates were 0.08 ± 0.01 /day for CMCs and 0.07 ± 0.01 /day for DDCs, corresponding to doubling times of 8.84 ± 0.66 and 9.81 ± 0.61 days, respectively. These growth rates are consistent with previously reported values for other plant species (Omar et al. 2006; Yegorova et al. 2024; Lee et al. 2010). Both cultures continued to grow until the end of cultivation.

Initially, the productivity of both cell lines was monitored through the quantification of extracellular metabolites accumulated in the culture medium. The extracellular accumulation of secondary metabolites differed markedly between the two cell culture systems (Fig. 3). CMC cultures exhibited delayed alkaloid production, with theobromine and caffeine concentrations reaching 2.21 ± 1.15 mg/L and 2.21 ± 1.88 mg/L, respectively, by day 28 of cultivation. In contrast, DDCs demonstrated substantially higher extracellular alkaloid accumulation, with theobromine attaining 13.52 ± 4.15 mg/L and caffeine 13.80 ± 6.46 mg/L by day 34. The intermediate metabolite 7-methylxanthine remained detectable only at low concentrations in both culture systems. Chlorogenic acid was not detected in the culture medium of either cell type.

Hence, DDC cultures displayed markedly enhanced extracellular alkaloid production under heterotrophic conditions, characterised by earlier activation of the biosynthetic pathway and higher final metabolite concentrations. These observations suggest that DDCs preferentially channel purine alkaloid metabolism toward secretion into the surrounding medium. The observed accumulation pattern further highlights the regulatory importance of the conversion step from theobromine to caffeine, supporting earlier reports indicating that caffeine secretion is closely associated with specific cellular growth phases (Ogutuga and Northcote 1970). The absence of extracellular chlorogenic acid was consistent with previous findings demonstrating limited secretion of phenolic compounds under heterotrophic cultivation conditions (Baumann and Röhrig 1989).

Comparison with previously published studies remains challenging because most reports describe total purine alkaloid concentrations without distinguishing between intracellular and extracellular fractions or analyse disrupted cultures in which compartmentalisation cannot be resolved. Consequently, direct quantitative comparison is possible only to a limited extent. Frischknecht and Baumann (1980) reported total purine alkaloid concentrations ranging from 5 to 130 mg/L in heterotrophically cultivated *Coffea arabica* suspension cultures, encompassing the combined concentration of 28.73 mg/L observed in the present study. With respect to extracellular caffeine accumulation, Waller et al. (1983) reported

concentrations of 8.52 ± 0.93 mg/L in the culture medium, while Sartor et al. (2000) described maximum extracellular caffeine levels of 4.3 mg/L. Both studies similarly identified the extracellular compartment as the primary location of product accumulation.

Therefore, the metabolite concentrations obtained in the present investigation fall within the range reported in the literature. However, methodological differences, including cultivation strategies, extraction procedures, and analytical quantification approaches, limit strict quantitative comparison across studies. Nevertheless, the results clearly demonstrate distinct secretion behaviours between CMCs and DDCs, indicating fundamental differences in metabolic regulation and transport processes between meristematic and dedifferentiated coffee cell systems.

As outlined in Table 1, the analysis of cell extracts demonstrated that DDCs cultivated under heterotrophic conditions produced higher concentrations of all three purine alkaloids compared to CMCs. These results suggest that heterotrophically cultivated CMCs have a distinct and less efficient profile for the synthesis of purine alkaloids in comparison to DDCs under the same conditions, indicating inherent differences in their biosynthetic capacities. The lower product concentrations observed in the cell extracts compared to the media are consistent with the conclusions of previous studies conducted on coffee suspension cultures, which reported preferential extracellular accumulation of these compounds (Waller et al. 1983; Sartor and Mazzafera 2000; Pech-Kú et al. 2018). The absence of detectable CGAs in heterotrophically cultured cells may be attributed to the downregulation of the phenylpropanoid biosynthetic pathway under non-photosynthetic conditions.

The results indicate that, while CMCs exhibit superior growth kinetics, as evidenced by faster doubling times and enhanced biomass accumulation, the DDCs possess a higher capacity for synthesising the studied metabolites under heterotrophic conditions. According to Ashihara (2015), caffeine levels vary among different plant tissues. The highest concentrations of caffeine are found in cotyledons (2.0% of dry weight), followed by leaves (0.8–1.1%), and very low levels are found in stems (0.02–0.12%) and roots (0.02%) (Ashihara 2015; Ashihara et al. 2017; Ashihara and Suzuki 2004). Given that CMCs are derived from stem tissue, it is reasonable to hypothesise that they possess the metabolic profile of this tissue, which naturally produces lower concentrations of caffeine than the cotyledon/Leaf tissue from which the DDCs are isolated. These tissue-specific differences in caffeine biosynthesis are likely the reason why CMC cultures have lower caffeine concentrations than leaf-derived DDC cultures.

The intracellular concentrations determined in the present study are largely within the range previously reported for *C. arabica* cell cultures. However, notable differences were observed depending on the cultivation regime and cell type. For heterotrophically cultivated cells in a wavebag bioreactor system, Aisala et al. (2023) reported 0.22 mg/g caffeine. Kim et al. (2025) observed caffeine concentrations between 0.118–0.214 mg/g in heterotrophic callus cultures depending on phytohormone treatment. Sartor et al. (2000) reported comparatively low intracellular caffeine levels in suspension cultures of 0.06 mg/g after 42 days of cultivation. In contrast, Pech-Kú et al. (2018) were unable to detect caffeine in heterotrophic suspension cultures but measured 0.090 ± 0.001 mg/g under photoperiodic conditions.

Upon supplementation with 5.55 µM theobromine, caffeine levels increased substantially to 0.942 ± 0.027 mg/g (heterotrophic) and 0.900 ± 0.026 mg/g (photoperiodic), indicating a strong precursor dependency of alkaloid biosynthesis (Pech-Kú et al. 2018). To our knowledge, Buckland and Townsley (1975) are the only authors who have published a paper describing 0.38 mg/g of caffeine and, more importantly, 1.35 mg/g of CGA under heterotrophic conditions.

Table 1
Secondary metabolite production in DDCs and CMCs suspension cultures of *C. arabica* under heterotrophic (H) and mixotrophic (M) conditions. Mixotrophic cultures were maintained under a 16:8 h light:dark photoperiod at 100 ± 10 $\mu mol_{photons}/(m^2 \cdot s)$. Values represent intracellular concentrations ($mg/_{gCDW}$) and extracellular concentrations in culture medium (mg/L) after cell harvest. Data are presented as mean ± standard deviation from three independent experiments (n = 3). ND: not detected.

Secondary Metabolite	**Cultivation condition**	**DDC**		**CMC**	
		Cell extracts [mg/g_{CDW}]	**Media [mg/L]**	**Cell extracts [mg/g_{CDW}]**	**Media [mg/L]**
7-Methylxanthine	H	0.028 ± 0.003	1.408 ± 0.183	0.018 ± 0.002	0.435 ± 0.536
	M	ND	ND	ND	2.706 ± 0.229
Theobromine	H	0.260 ± 0.035	13.516 ± 4.155	0.050 ± 0.004	2.214 ± 1.147
	M	0.456 ± 0.025	0.267 ± 0.062	0.101 ± 0.034	1.751 ± 0.590
Chlorogenic acid	H	ND	ND	ND	ND
	M	0,269 ± 0.029	3.929 ± 2.213	0.314 ± 0.057	ND
Caffeine	H	0.285 ± 0.067	13.798 ± 6.455	0.041 ± 0.005	2.211 ± 1.880
	M	0.151 ± 0.013	3.065 ± 0.180	0.447 ± 0.015	49.983 ± 3.186

Growth characteristics and secondary metabolite production of CMCs compared to DDCs under mixotrophic conditions

Caffeine biosynthesis in *C. arabica* involves a four-step enzymatic pathway comprising three S-adenosyl-L-methionine (SAM) dependent methylation reactions and one nucleosidase-mediated hydrolysis (Ashihara and Suzuki 2004). Although SAM serves as the methyl group donor in three of the four steps of caffeine biosynthesis, previous studies suggest that SAM availability is not the limiting factor for alkaloid accumulation. Kurata et al. (1997) demonstrated that the supplementation of 7-methylxanthine

and theobromine led to their immediate conversion into downstream methylated products, indicating that methylation capacity remains sufficient under culture conditions (Kurata et al. 1997). Conversely, the initial limitation appears to lie in the availability of purine ring precursors, which accumulate only after several days of cultivation. This delayed availability is likely to represent a metabolic bottleneck in the early cultivation phase and may provide a rationale for the lag phase observed in theobromine and caffeine production in both DDC and CMC cultures. The underlying cause of this delay could be attributed to a delayed onset of de novo purine biosynthesis or reduced salvage pathway activity during the early stages of culture development. The application of abiotic elicitors such as aluminium chloride, salt stress, and varying light intensities has been reported to positively influence purine metabolism and purine alkaloid biosynthesis in plant cell cultures of *C. arabica* (Frischknecht and Baumann 1985; Aisala et al. 2023; Pech-Kú et al. 2018; Prenosil et al. 1987). Interestingly, while previous studies have suggested that the biosynthesis of caffeine and theobromine under heterotrophic conditions is limited, we observed substantial accumulation of these purine alkaloids in DDC cultures even under heterotrophic conditions. This unexpected result prompted further investigation of light as a potential elicitor not only of purine alkaloid biosynthesis but also of CGA production. To this end, both cell types were grown under mixotrophic conditions to assess the influence of light as an abiotic stimulus on the accumulation of secondary metabolites (Fig. 4).

In order to evaluate the role of light as an abiotically elicited stimulus, DDC and CMC cultures maintained on solid media were exposed to LED illumination under a photoperiod. After a period of three weeks, a gradual development of green pigmentation was observed, indicating chloroplast differentiation (Fig. 4). This demonstrates that both DDC and CMC lines retain substantial developmental plasticity, allowing metabolic and structural adaptation to altered environmental stimuli. Light-induced greening and chloroplast reactivation in coffee callus cultures have been previously reported (Herman and Haas 1975; Söndahl and Sharp 1977; Teixeira et al. 2004), though photosynthetic capacity typically remains incomplete compared to intact leaves.

Following two successive sub-cultivations under identical light conditions, photo-adapted CMC and DDC cells were successfully established as suspension cultures (Fig. 4). The transition from heterotrophic to mixotrophic conditions is assumed to reactivate plastid biogenesis and light-dependent pathways, including the phenylpropanoid and chlorogenic acid biosynthetic routes (Kurata et al. 1997; Frischknecht and Baumann 1985; Teixeira et al. 2004). To evaluate this effect, CMC and DDC suspension cultures were cultivated under mixotrophic conditions for 30 days (Fig. 5). In mixotrophically cultivated CMC cultures, caffeine was the dominant metabolite, reaching 46.68 ± 1.34 mg/L at day 30. Notably, CGA, which was absent in heterotrophic cultures, was detected under light exposure, reaching a transient maximum of 51.01 ± 6.58 mg/L on day 8 before declining to undetectable levels by day 20, confirming that light acts as a strong inducer of phenolic metabolism. Conversely, DDC cultures exhibited reduced overall productivity with CGA reaching only 6.15 ± 1.71 mg/L on day 6, and caffeine remaining low at 0.27 ± 0.06 mg/L by day 30.

The product formation pattern in both cultures is consistent with earlier observations in *C. arabica* cell cultures under photoperiodic conditions. Waldhauser and Baumann (1996) observed that the induction of purine alkaloid biosynthesis occurred within 24–48 h, coinciding with a rapid onset of CGA synthesis that peaked after 5–7 days before gradually declining (Waldhauser and Baumann 1996). Comparable trends were evident in the present study across the different cell lines and cultivation conditions, supporting the notion that light simultaneously activates phenylpropanoid and purine alkaloid pathways.

Overall, the substantially lower product formation rates observed in DDC cultures indicate reduced metabolic activity, likely caused by increased sensitivity to stress imposed by suspension cultivation and light exposure. CMCs appear to possess higher physiological robustness under such conditions, consistent with their more homogeneous characteristics (Lee et al. 2010; Partap et al. 2023).

The analysis of intracellular extracts from mixotrophically cultivated cultures (Table 1) revealed light-dependent shifts in secondary metabolite allocation. In both cell types, CGA was exclusively detected under mixotrophic conditions, confirming light as a key inducer of phenylpropanoid metabolism. CMC extracts demonstrated the highest caffeine yields among all conditions (0.447 ± 0.015 mg/g_{CDW}), which notably exceeds most previously reported values for heterotrophic cultures and approaches the levels achieved by Pech-Kú et al. (2018) under theobromine supplementation (0.942 mg/g). Additionally, CMCs produced substantial CGA yields (0.314 ± 0.057 mg/g_{CDW}), which, while lower than the 1.35 mg/g reported by Buckland and Townsley (1975), should be interpreted in the context of the cultivation dynamics observed. Given that substantially higher CGA concentrations were detected in the culture medium during early cultivation phases (up to 51.01 ± 6.58 mg/L on day 8), it is reasonable to assume that intracellular CGA levels would have been higher if cells had been harvested before the observed decline. In contrast, DDC extracts exhibited high theobromine accumulation (0.456 ± 0.025 mg/g_{CDW}) and reduced caffeine productivity, indicating potential restrictions in downstream methylation processes. These results demonstrate that mixotrophic cultivation selectively enhances intracellular CGA and caffeine production and identifies CMCs as the more promising platform for light-driven coffee cell cultivation.

Comparison of C. arabica and C. canephora CMC cultures under mixotrophic conditions

Given the pronounced differences in intracellular metabolite allocation observed between CMCs and DDCs of *C. arabica*, it is pertinent to consider whether these trends are conserved across coffee species with distinct phytochemical profiles. Plants of *C. canephora* (Robusta) has been shown to accumulate substantially higher levels of caffeine and related purine alkaloids than *C. arabica* (Table 2). These results align with those previously reported by (Monteiro et al. 2019) who described similar concentrations in the young leaves of both species. The high caffeine concentrations observed in the present study can be explained by the fact that explant material was obtained from very young plants of both species (Fig. S3), which naturally exhibit elevated alkaloid levels during early developmental stages compared to mature plant tissues.

To compare secondary metabolite yields between *C. arabica* and *C. canephora*, CMC cultures were induced from *C. canephora* explants following the established protocol and adapted to mixotrophic conditions before transferred to suspension culture. Comparison of metabolite accumulation profiles revealed distinct differences between the two species (Fig. 6).

Table 2
Mass-specific secondary metabolite yield in mg/g_{CDW} of freeze-dried biomass of leaves, beans and CMCs of *C. arabica* and *C. canephora*. Data represent the mean ± standard deviation from three independent experiments (n = 3).

Secondary Metabolite	***C. arabica***			***C. canephora***		
	Leaves [mg/g_{CDW}]	**Beans [mg/g_{CDW}]**	**CMCs [mg/g_{CDW}]**	**Leaves [mg/g_{CDW}]**	**Beans [mg/g_{CDW}]**	**CMCs [mg/g_{CDW}]**
Theobromine	0.34 ± 0.02	0.29 ± 0.01	0.10 ± 0.03	0.08 ± 0.01	0.23 ± 0.02	0.14 ± 0.07
Chlorogenic acid	4.81 ± 0.42	6.27 ± 0.09	0.32 ± 0.06	1.08 ± 0.03	3.78 ± 0.05	0.39 ± 0.07
Caffeine	12.55 ± 0.01	12.84 ± 0.57	0.45 ± 0.02	21.09 ± 0.18	18.26 ± 1.74	0.85 ± 0.15

CGA was the most rapidly accumulating metabolite in both species, peaking on day 8 (*C. canephora*: 92.29 ± 16.69 mg/L; *C. arabica*: 51.01 ± 6.58 mg/L) before declining to undetectable levels. Purine alkaloids accumulated continuously throughout cultivation, with caffeine following a sigmoidal pattern. *C. canephora* exhibited earlier onset and substantially higher final caffeine concentrations (73.36 ± 11.07 mg/L) compared to *C. arabica* (47.73 ± 1.70 mg/L). Final biomass yields were comparable between species, with *C. canephora* reaching 0.232 ± 0.045 g_{CDW} and *C. arabica* 0.206 ± 0.015 g_{CDW}, indicating that the observed differences in metabolite production are not attributable to variations in growth performance.

Similar trends were observed in cell extracts, with *C. canephora* producing nearly double the intracellular caffeine concentrations (0.85 ± 0.15 mg/g_{CDW}) compared to *C. arabica* (0.32 ± 0.06 mg/g_{CDW}) (Table 2). When these levels are compared to those previously reported for coffee cell cultures, the differences are particularly noteworthy. The *C. canephora* concentrations achieved in this study approach the levels reported by Pech-Kú et al. (2018) under theobromine supplementation (0.942 mg/g) and substantially exceed most reported values for heterotrophic cultivation. These include the levels reported by Aisala et al. (2023) (0.22 mg/g), Kim et al. (2025) (0.118–0.214 mg/g), and Sartor et al. (2000) (0.06 mg/g). Both CGA and caffeine yields in cell extracts were substantially lower than those reported for differentiated leaf tissue, likely reflecting the dedifferentiated state of CMC cultures. Furthermore, caffeine biosynthesis occurs independently of cell division, suggesting that rapidly dividing CMC cultures may be inherently less productive on a per-cell basis than metabolically quiescent leaf tissues. This hypothesis is supported by earlier observations demonstrating higher specific productivity in slow-growing cell

aggregates compared to highly dispersed, rapidly proliferating cells (Dubuis et al. 1995; Frischknecht and Baumann 1985).

Overall, *C. canephora* cultures consistently exhibited higher caffeine concentrations than *C. arabica*, consistent with the known metabolic characteristics of Robusta coffee (Ashihara et al. 2008; Koshiro et al. 2006). The temporal separation between early CGA and later purine alkaloid accumulation suggests a biphasic metabolic pattern that may reflect nutrient-dependent regulation or developmental transitions within the cell population.

Conclusion and outlook

This study provides the first comprehensive characterization of CMCs from *C. arabica* and *C. canephora* and demonstrates their potential as a platform for sustainable coffee production. The observed morphological traits, including small cell size (median: 59.53 µm) and the presence of numerous small vacuoles, are consistent with previously described CMC characteristics. Comparative analyses of DDCs and CMCs under heterotrophic and mixotrophic conditions revealed distinct metabolic behaviours. DDCs exhibited higher purine alkaloid production under heterotrophic conditions, whereas CMCs outperformed DDCs under mixotrophic cultivation. Notably, CMCs achieved the highest intracellular and extracellular caffeine concentrations (0.447 ± 0.015 mg/g_{CDW} and 49.98 ± 3.19 mg/L) and were the only cell type showing substantial chlorogenic acid accumulation (0.314 ± 0.057 mg/g_{CDW} and 51.01 ± 6.58 mg/L), highlighting their superior biosynthetic performance under light exposure. Species-specific differences were preserved in cell culture, with *C. canephora* CMCs producing nearly twice the caffeine levels of *C. arabica*. This demonstrates that intrinsic metabolic traits remain stable in vitro and can be exploited for targeted production of defined metabolite profiles.

Overall, this work demonstrates the feasibility of producing coffee metabolites using CMC-based systems and identifies mixotrophic cultivation as a key factor for enhanced secondary metabolism. Future work should focus on optimizing cultivation parameters, elicitation strategies, scaling up bioreactor processes, and integrating metabolic engineering strategies to further improve yields. In combination with downstream processing advancements, CMC-based cellular agriculture represents a promising, climate-resilient alternative to conventional coffee production.

Declarations

Ethical approval

Not applicable.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This work was supported by State Rhineland-Palatinate in the frame of the research initiative NanoKat.

Author Contribution

JH: Conceptualization, Methodology, Formal analysis and investigation, Writing—original draft preparation; TS: Formal analysis and investigation; PF: Formal analysis and investigation, JS: Writing—review and editing; AZ: Writing—review and editing; RU: Conceptualization, Supervision, Funding acquisition, Writing—review and editing.

Acknowledgement

The authors thank Prof. Dr. Peter Nick (Botanical Institute, Karlsruhe Institute of Technology, Karlsruhe, Germany) for providing the Coffea arabica and Coffea canephora explants.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

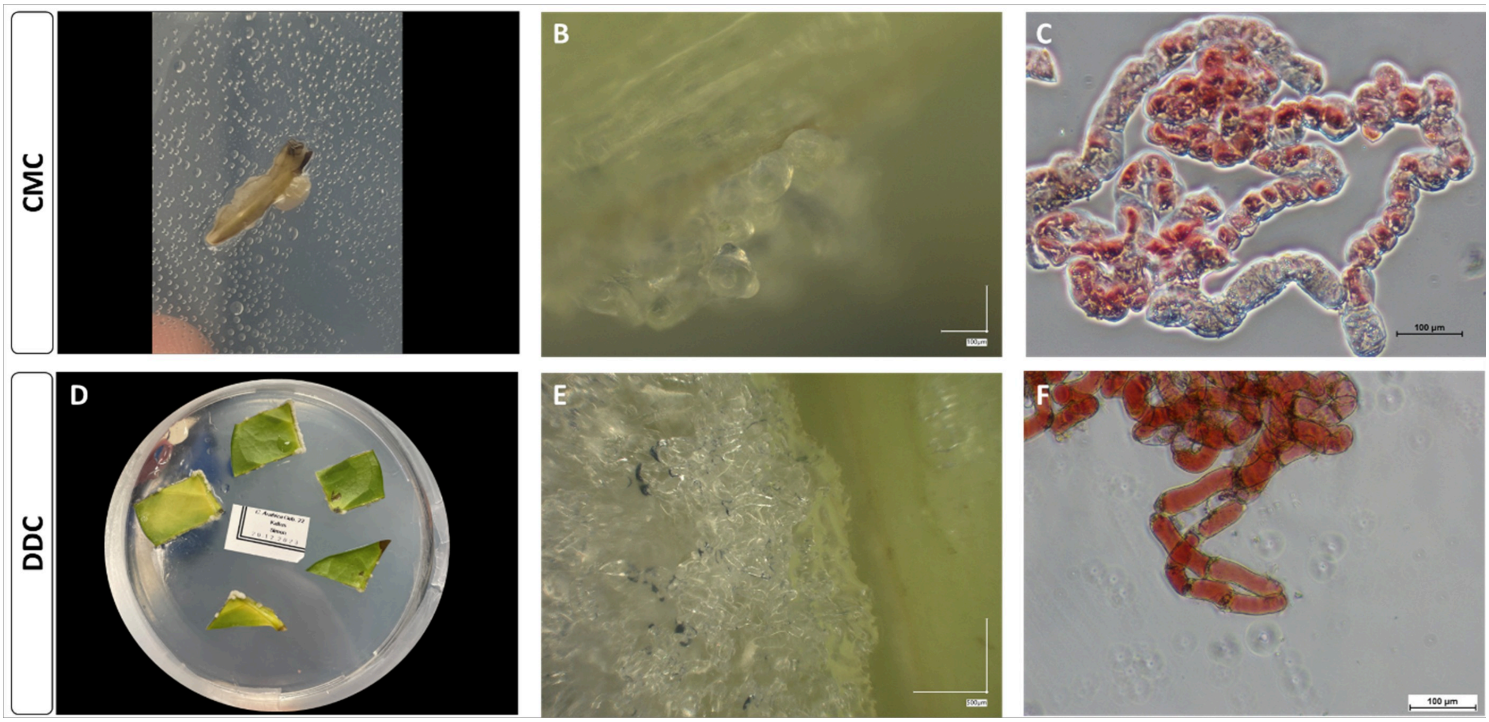

Figure 1

Comparison of Coffea arabica cell morphologies: Cambial meristematic cells (CMCs) and dedifferentiated cells (DDCs). **(A)**CMCs growing on solid media three weeks after induction; arrows indicate nascent cells. **(B)** CMCs growing adherend to steam tissue. Scale bar = 100 µm. **(C)** CMC after staining with 0.005% neutral red. Scale bar = 100 µm. **(D)** DDCs growing on solid media three weeks after induction; arrows indicate freshly formed callus. **(E)** DDCs growing adherend to leaf tissue. Scale bar = 500 µm. CMC and **(F)**DDC after staining with 0.005% neutral red. Scale bar = 100 µm.

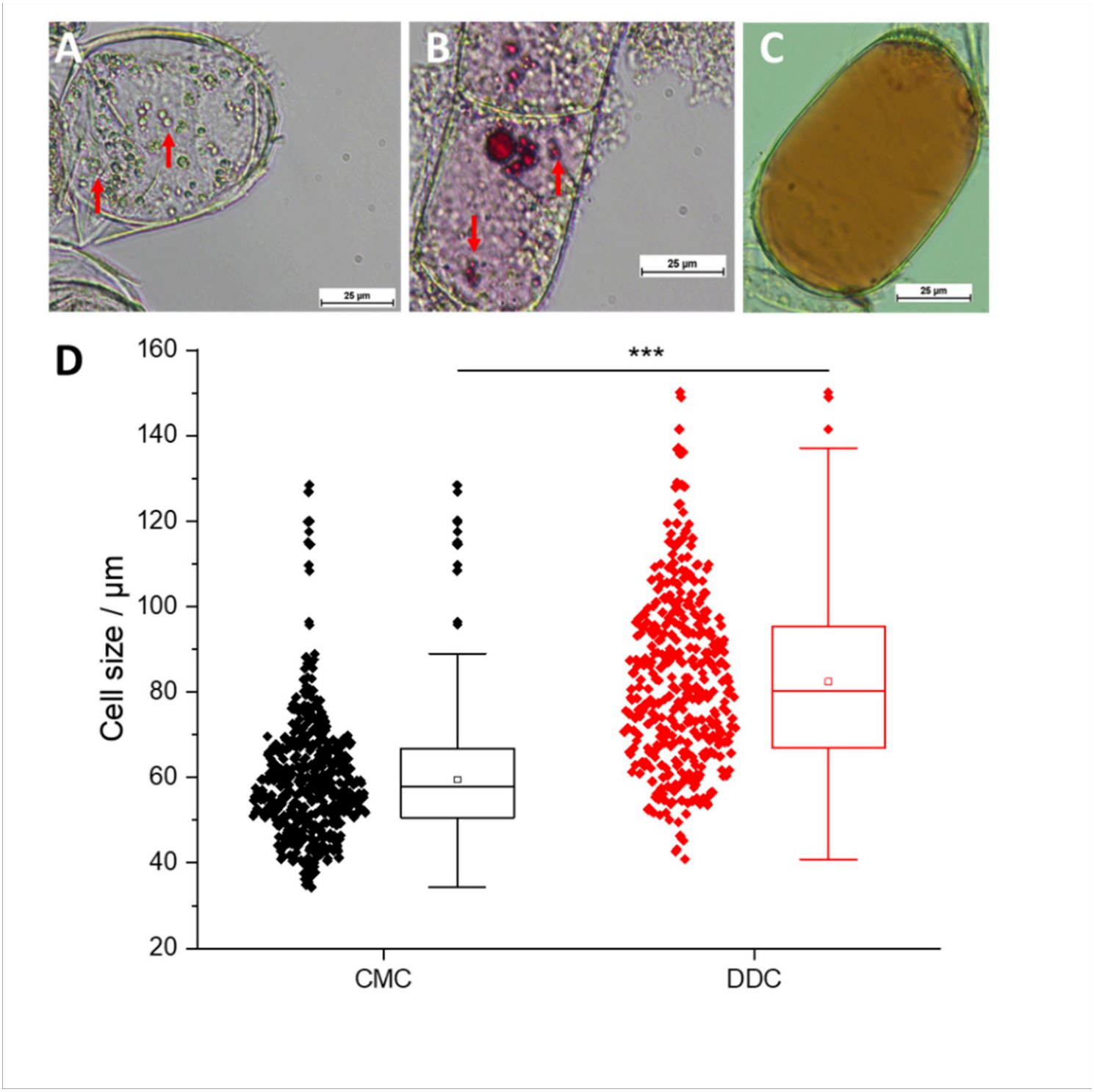

Figure 2

Comparison of Coffea arabica cell morphologies: Cambial meristematic cells (CMCs) versus dedifferentiated cells (DDCs). **(A)** CMCs growing in suspension cultures; arrows indicating numerous small vacuoles. **(B)** CMCs and **(C)** DDCs after staining with 0.005% neutral red. Scale bar = 25 µm. **(D)** Box plot depicting cell sizes for CMCs (left) and DDCs (right). Horizontal lines in boxes depict median values for the populations. Single data points are indicated beside the corresponding boxes (n = 476 for

CMCs, n = 384 for DDCs). Statistical significance was determined using the Mann–Whitney U Test; ***p < 0.001.

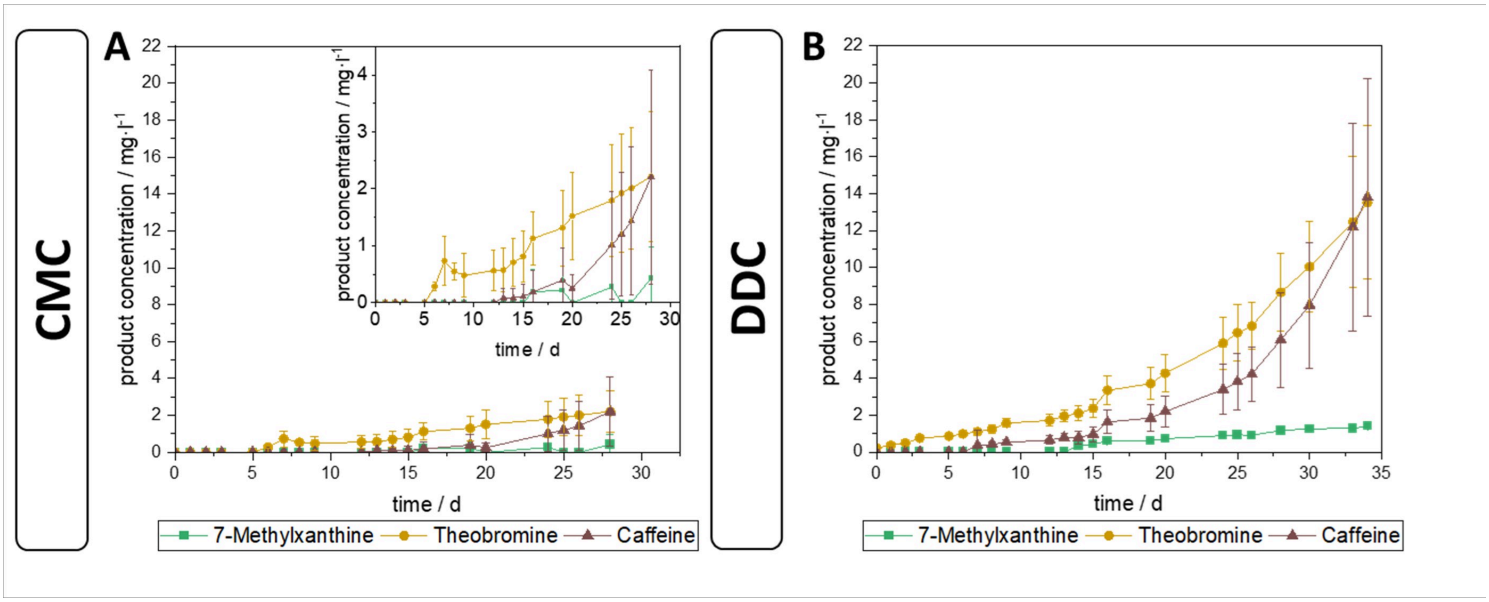

Figure 3

Secondary metabolite concentrations in suspension cultures of C. arabica cambial meristematic cells (CMCs) and dedifferentiated cells (DDCs). Panels **(A)** and **(B)** illustrate extracellular metabolite concentrations in mg/L in CMC and DDC culture media over cultivation periods of 30 and 36 days, respectively. To facilitate comparison between **(A)** and **(B)** the same scale is used. The inset shows in **(A)** the scaled-up details. Data represent the mean ± standard deviation from three independent experiments (n = 3).

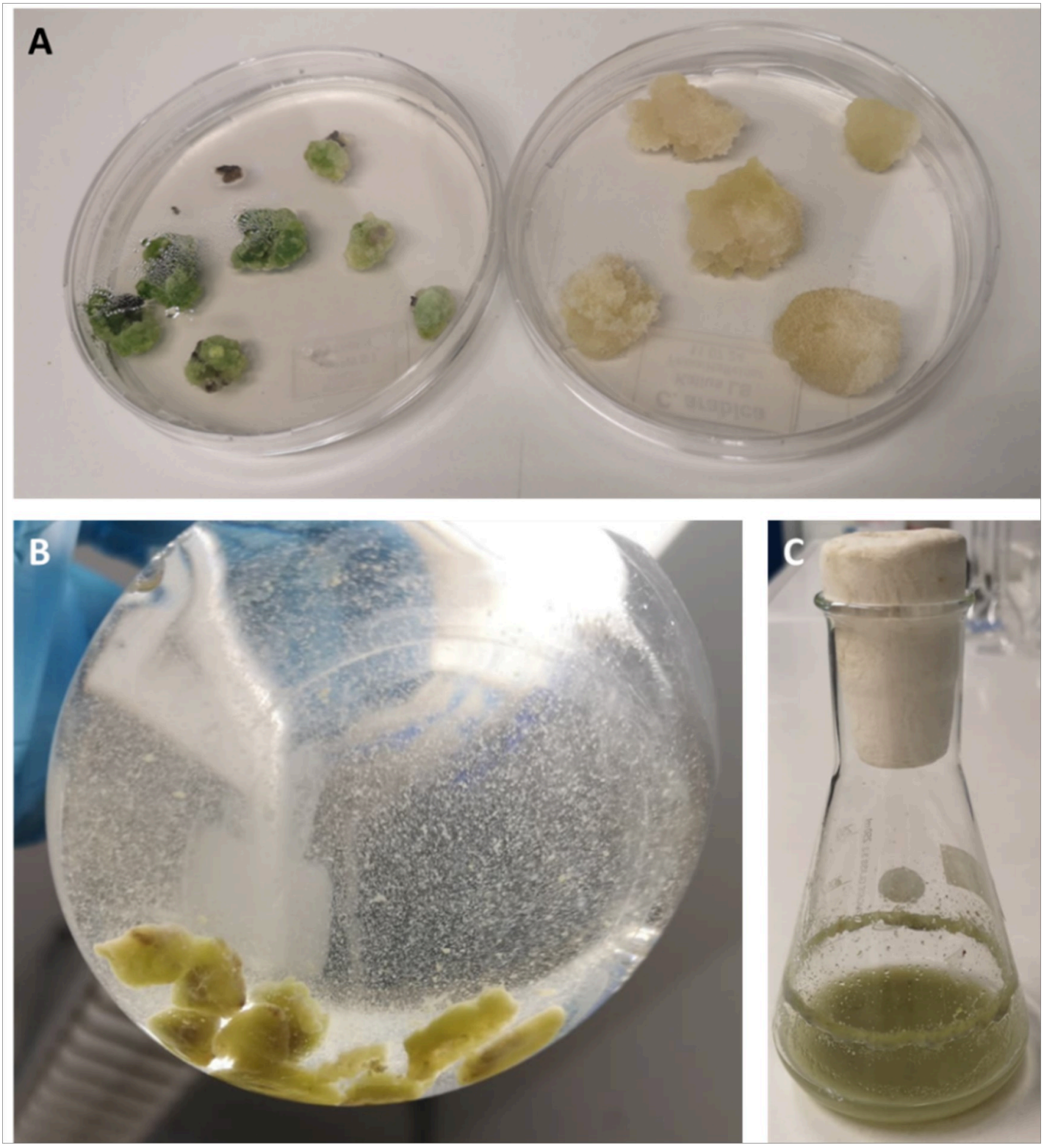

Figure 4

Morphological comparison of Coffea arabica DDC cultures grown on solid LS medium under mixotrophic (left) and heterotrophic (right) conditions **(A)**. Suspension cultures of C. arabica DDCs after 30 days of mixotrophic cultivation **(B)** and CMC suspension cultures after 30 days of mixotrophic cultivation **(C)**. The photoperiod was 16:8 h light:dark at 100 ± 10 $\mu mol_{photons}/(m^2 \cdot s)$.

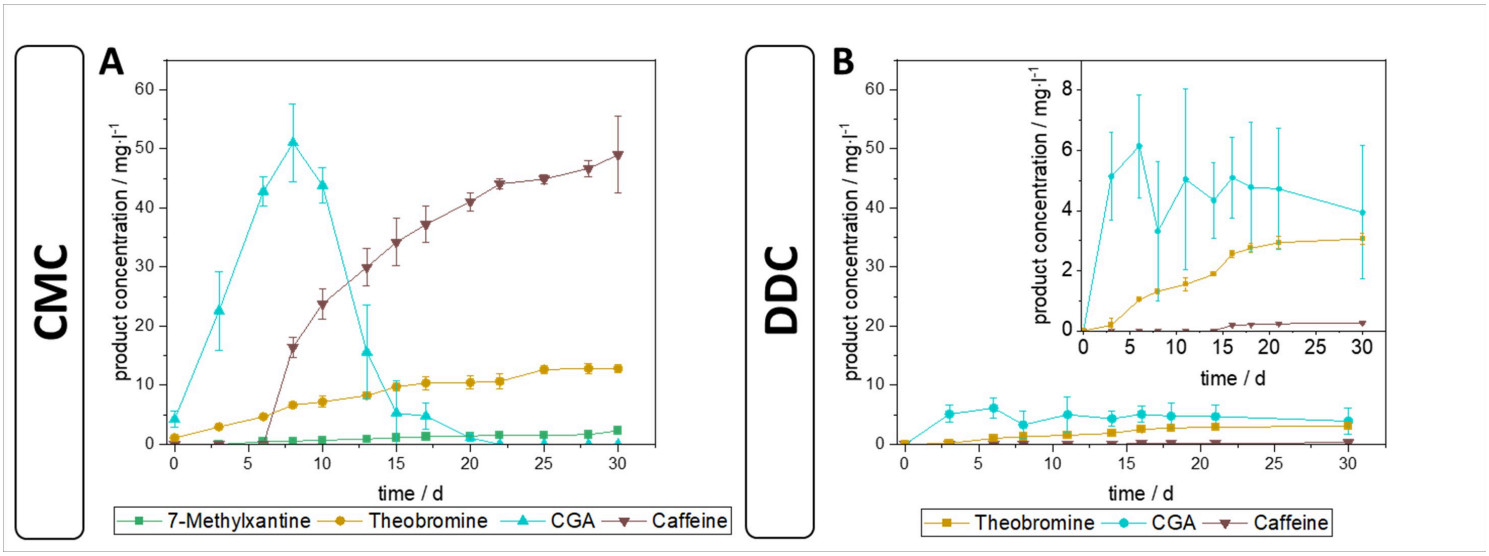

Figure 5

Secondary metabolite concentrations in mixotrophic suspension cultures of C. arabica cambial meristematic cells (CMCs) and dedifferentiated cells (DDCs). Panels **(A)** and **(B)** illustrate extracellular metabolite concentrations in mg/L in CMC and DDC culture media over cultivation periods of 30 days. To facilitate comparison between **(A)** and **(B)** the same scale is used. The inset shows in **(B)** the scaled-up details. Data represent the mean ± standard deviation from three independent experiments (n = 3). The photoperiod was 16:8 h light:dark at 100 ± 10 $\mu mol_{photons}/(m^2 \cdot s)$.

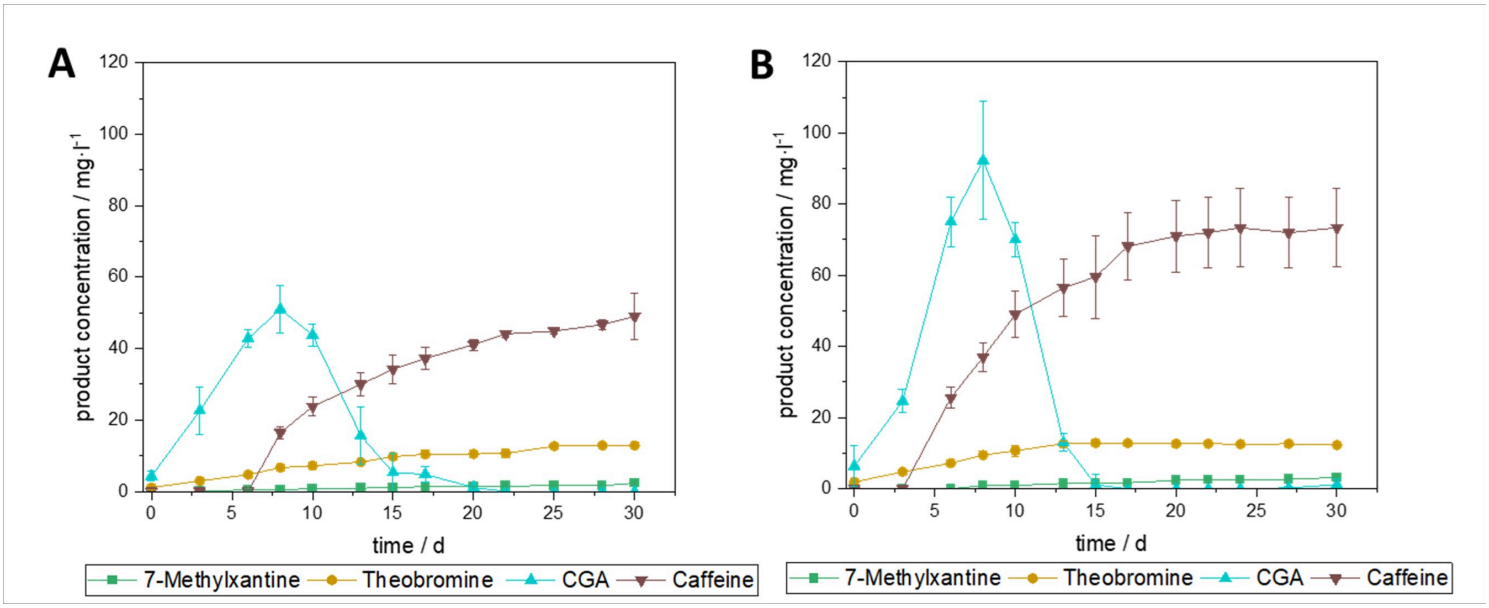

Figure 6

Secondary metabolite concentrations in suspension cultures of cambial meristematic cells (CMCs) from C. arabica **(A)** and C. canephora **(B)** under mixotrophic conditions. The photoperiod was 16 h of light and

8 h of darkness at a constant light intensity of 100 ± 10 $\mu mol_{photons}/(m^2 \cdot s)$. Data represent the mean ± standard deviation from three independent experiments (n = 3).

Supplementary Files

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- SupplementaryMaterialfinal.docx