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Effects of UV-B and DMSO Treatments on the Production of Artemisinin in Low-Artemisinin Producing *Artemisia annua* Cell Cultures

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ABSTRACT

Artemisinin is a sesquiterpene lactone derived from *Artemisia annua* L., and has a variety of biological properties, such as immunoregulatory and anticancer effects, and therapeutic applications. As a result of its naturally low production and compartmentalised synthesis, the irregular agricultural supply often leads to price fluctuations and reduction of the artemisinin inventory. In this study, elicitation efficiencies of ultraviolet B (UV-B) and dimethyl sulfoxide (DMSO) on a low-artemisinin producing (LAP) chemotype of the species *A. annua* were investigated. Exposure of cell suspension cultures to short-term UV-B radiation and DMSO treatment did not result in significant changes in artemisinin yield. The lack of stimulation could be associated with the growth condition such as the incubation duration after treatment, the physiological state of suspension-cultured cells, and the regulation of cellular metabolic homeostasis. Further molecular analysis with RT-PCR revealed the absence of mRNA transcripts of key genes *ADS*, *DBR2*, and *ALDH1* which might affect artemisinin synthesis. This study demonstrated the complexity of stress-induced responses of *A. annua* cell suspension cultures in relation to metabolic processes which are important for artemisinin formation.

Keywords: Artemisinin; dimethyl sulfoxide; liquid cell suspension; oxidative stress; ultraviolet B.

INTRODUCTION

The highly oxygenated endoperoxide sesquiterpene lactone, artemisinin, is an effective antimalarial compound that is derived from the Chinese medicinal plant, *Artemisia annua*. In view of the rapid development of parasite resistance to antimalarial drugs, the World Health Organization (WHO) has endorsed the artemisinin-based combination therapies as the first-line treatment for uncomplicated malaria caused by *Plasmodium falciparum* (Ye et al., 2016). Apart from its antimalarial activities, artemisinin is also a multi-functional compound that is associated with a variety of novel biological properties, such as immunoregulatory, anticancer, antituberculosis, antidiabetic, antiparasitic and antiviral functions (Li et al., 2017; Saeed-ur-Rahman et al., 2019).

Given the economic importance of artemisinin, the sole dependency on plant-based production periodically fails to meet the global demand due to irregular agricultural supply, which then causes changes in the market value of artemisinin and creates a decrease in artemisinin inventory (Shretta and Yadav, 2012). To mitigate supply uncertainties and price volatility, biotechnological alternatives such as genetic modification, crop breeding and semi-synthetic production were implemented with semi-synthesis method being far more successful (Pulice et al., 2016; Khairul Ikram et al., 2017). The semi-synthesis approach has the production capacity to yield approximately 60 tonnes of artemisinin annually, but it also involves the trade-off between efficiency and profitability. The multiple chemical conversion steps to produce a semi-synthetic variant of artemisinin, compared to the more economical leaf-derived artemisinin extraction, demands high financial and environmental costs, inevitably increasing raw material prices (Amara et al.,

2015). Furthermore, when demand plateaued due to periodic excess of agricultural supply and improving malaria diagnosis, plant-derived artemisinin was sold at a price less than US$250 per kg, while semi-synthetic artemisinin was valued between US$350 to 400 per kg (Peplow, 2016). Although *Artemisia* farmers can occasionally produce enough raw materials during favourable growing seasons, an alternative production strategy which is rapid in establishment should be considered to fill in the agricultural production gap in times of tight supply.

Artemisinin biosynthesis occurs in the glandular secretory trichomes that are present on foliage, stems and inflorescences of the plant (Ferreira and Janick, 1996a). Due to its compartmentalised synthesis and influence by physiological, seasonal, and environmental factors, artemisinin concentration is highly variable with reported values of 0.1 to 10 mg/g dry weight (DW) (Pulice et al., 2016). With the biosynthetic pathway of artemisinin formation almost completely elucidated (Zhou et al., 2020), it was learnt that within *A. annua* two contrasting chemotypes can be distinguished, which are characterised by the differing contents of artemisinin and its precursor (Figure 1). While both possess artemisinin, the high-artemisinin producing (HAP) chemotype contains relatively higher levels of dihydroartemisinic acid (DHAA) and artemisinin, while the low-artemisinin producing (LAP) chemotype has higher contents of artemisinic acid (AA) and arteannuin B (AB, a non-antimalarial product) (Wallaart et al., 2000; Zhang et al., 2016). For example, HAP cultivars 3M and Artemis® have leaf artemisinin concentrations ranging from 0.25 to 1.30% DW and 0.58 to 1.00% DW, respectively. The LAP cultivar Sandeman, on the other hand, has lower artemisinin concentrations of 0.07 to 0.20% DW (Ferreira et al., 2018). Despite the difference in biochemical phenotype which is attributed to the differential expression of one artemisinin biosynthesis specific gene artemisinic aldehyde Δ11(13) reductase (DBR2), both chemotypes undergo similar spontaneous photo-oxidation reactions to produce artemisinin or AB from its direct precursors (Brown and Sy, 2007; Yang et al., 2015).

As a plant secondary metabolite, artemisinin production would be naturally induced and enhanced in response to environmental stresses. Resulting from the exposure to biotic and/or abiotic stress factors, the elevation of secondary oxidative stress in plants has been shown to be in a synergistic relationship with artemisinin production (Domokos et al., 2018; Zehra et al., 2020). On such basis, the biotechnological method of elicitation which mimics the natural induction of plant stress is the method of choice to enhance the production of artemisinin. The positive effects of ultraviolet B (UV-B) and dimethyl sulfoxide (DMSO) on the production of artemisinin had been verified by previous studies on *A. annua* seedlings (Rai et al., 2011; Pan et al., 2014), *in vitro* propagated plantlets (Pandey and Pandey-Rai, 2014), and shoot cultures (Mannan et al., 2010). Former studies had also shown that artemisinin production can be enhanced by different elicitors such as potassium nitrate, methyl jasmonate, cobalt nanoparticles and a combination of coronatine and sorbitol in *A. annua* cell cultures (Chan et al., 2010; Caretto et al., 2011; Ghassemi et al., 2015; Salehi et al., 2019). However, to our knowledge, the effect of UV-B and DMSO elicitation on the content of artemisinin synthesised by a LAP chemotype cell culture has not previously been reported. As LAP chemotype preferentially converts its precursor AA to AB rather than artemisinin under normal circumstances, it seemed possible that stress factors could induce a shift in its biosynthetic mechanism favouring artemisinin formation. This notion was motivated by preliminary studies of the same stress treatments, but on young soil-grown LAP plants (unpublished data). The potential of LAP chemotypes as alternative sources for artemisinin has not been given much attention, as selection and breeding programmes primarily focus on producing commercial germplasm of high artemisinin-yielding hybrids. Given that DHAA is a direct precursor of artemisinin with the implicated role as a reactive oxygen species (ROS) scavenger (Wallaart et al., 1999a, b), our hypothesis was that UV-B and DMSO applications on a LAP plant may trigger an oxidative burst in cells resulting in an increased DHAA production for ROS quenching, which in turn enhances artemisinin production. Therefore, this study investigated the above-mentioned hypothesis by evaluating the effects of UV-B and DMSO elicitation on the content of artemisinin in cell cultures of a LAP chemotype.

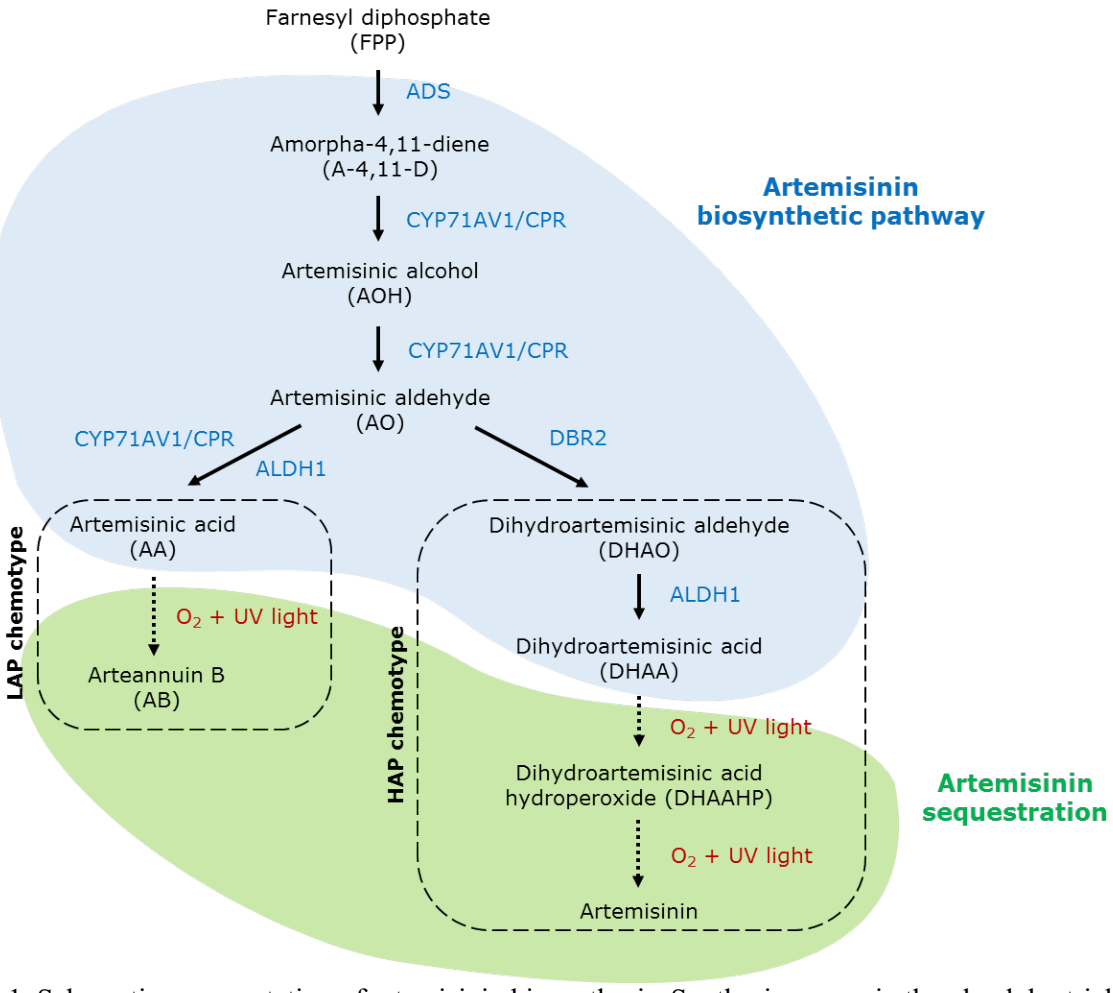

Figure 1. Schematic representation of artemisinin biosynthesis. Synthesis occurs in the glandular trichome secretory cells of *A. annua* and artemisinin sequestration takes place in the subcuticular space. The branching pathways leading to artemisinin and AB formation (dashed line box) are depicted with enzymes shown in blue and the final non-enzymatic photo-oxidation reaction is shown in red. ADS, amorpha-4,11-diene synthase; CYP71AV1, cytochrome P450 monooxygenase; CPR, cytochrome P450 reductase; DBR2, artemisinic aldehyde Δ11(13) reductase; ALDH1, aldehyde dehydrogenase 1.

MATERIALS AND METHODS

Plant materials and culture conditions

A. annua seeds of European origin (Chiltern Seeds Ltd, UK) were germinated and grown in sterile soil under controlled conditions. Seedlings (approximately 15 cm) were transplanted into pots. Plants were cultivated in a growth chamber under a 16 h photoperiod at 25°C to prolong the vegetative stage. The plants were watered every alternate day with direct application to the soil and fertilised (N:P:K 15:15:15) once every 2 weeks. Stock plants were maintained and propagated via stem cuttings.

For *in vitro*-raised stock plants, seeds were surface-sterilised according to Pras et al. (1991) with slight modifications. Seeds were disinfected in 3% (v/v) Clorox® (containing 5.25% sodium hypochlorite) for 20 min with prior soaking in distilled water for 4 h. The seeds were rinsed three times with sterile distilled water and germinated on basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (Duchefa Biochemie B. V., Netherlands) containing 3% (w/v) sucrose and 0.35% (w/v) Phytagel™ (Sigma-

Aldrich, USA). Thereafter, *in vitro*-raised plantlets were subcultured at 4-week intervals into fresh basal MS medium with 3% (w/v) sucrose and 0.25% (w/v) Phytagel™. All media were adjusted to pH 5.8 and autoclaved at 121°C for 15 min. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod (40 μmol/m^2/s) provided by cool-white fluorescent lamps.

Callus induction

Young leaves of *in vitro*-raised plants (1- to 2-month-old) were excised (0.5 to 1.0 cm) and cultured onto MS medium supplemented with 0.5 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L α-naphthaleneacetic acid (NAA) (Duchefa Biochemie B. V.), 3% (w/v) sucrose and 0.35% (w/v) Phytagel™ (Chan et al., 2010). After 3 weeks of callus initiation, friable calli were separated from explants and transferred to the same fresh medium for further proliferation. Subsequent maintenance of calli were performed at 4-week intervals.

Establishment of *A. annua* cell suspension

Fine cell suspension cultures were initiated with the same medium composition used for callus induction. Friable calli (2 g) were inoculated into 100 mL Erlenmeyer flasks containing 25 mL liquid MS medium with 0.5 mg/L BAP and 0.5 mg/L NAA. Flasks were placed on an orbital shaker (120 rpm) and incubated under the same culture conditions as above. After 3 weeks of inoculation, established suspension cultures were filtered using a sterile stainless-steel sieve (aperture of 1.5 to 2.0 mm) to obtain a homogenous cell suspension. The fine cell suspension was then diluted with the same volume of fresh medium (dilution of 1:1 [v/v]), distributed into two 100 mL Erlenmeyer flasks and incubated in the same conditions. Medium volume was increased to 50 mL in the following subcultivation step. Homogenised suspension cultures (25 mL) were transferred into 250 mL Erlenmeyer flasks and refreshed by adding equal volumes of fresh medium (25 mL). Cell cultures were gently swirled and further incubated.

Growth kinetics of suspension cultures

Growth of suspension cultures was evaluated by determining the cell DW during a culture cycle of 35 days with measurements taken at 5-day intervals. Cells were harvested by filtration through filter paper, rinsed with distilled water and blotted to remove excess water. The cells were then oven dried at 60°C for 24 h to determine the DW. Triplicate flasks were used in this experiment.

Cell viability analysis

To evaluate viability of *A. annua* cells over the growth period of 35 days, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay was carried out according to Castro-Concha et al. (2006) with minor modifications. Suspension cells (1 mL) were washed twice with 50 mM phosphate buffer (pH 7.5) and resuspended in 1 mL of the same buffer. MTT (115 μL) was added to a final concentration of 1.25 mM. Samples were then incubated in darkness for 1 h at 37°C. To dissolve the formazan crystals, 1.5 mL solubilisation solution (50% [v/v] MeOH containing 1% [w/v] sodium dodecyl sulfate) was added to the samples and incubated at 60°C for 30 min. Samples were centrifuged at 1880 ×g for 5 min at room temperature and the supernatant was recovered. Absorbance was quantified at 550 nm.

UV-B elicitation and sample collection

The effects of UV-B treatment in enhancing the capacity of *A. annua* cell cultures to produce artemisinin were tested on 10-day-old suspension cultures (after subculture). Flasks were exposed to artificial UV-B radiation for 0, 1, 2, 3, 4 and 5 h, as described by Pandey and Pandey-Rai (2014) with modifications. UV-B radiation was artificially provided by UV-B Narrowband TL lamp (TL 20W/01 RS SLV/25) (Philips

Lighting Holding B. V., Netherlands) at a given dosage of 2.9 W/m^2. Cells were harvested immediately by filtration after every hour of UV-B treatment for biochemical and molecular analyses.

DMSO treatment and sample collection

DMSO concentrations were manipulated to evaluate impact on artemisinin production in 5-day-old suspension cultures (after subculture). Old liquid medium was removed by filtering through a 100 µm nylon cell strainer, the trapped cells were then returned to the flask and 50 mL fresh liquid media supplemented with the same hormone regime was added. Thereafter, filter-sterilised DMSO (Sigma-Aldrich) was added at increasing concentrations (0, 0.1, 0.25, 0.5, 1.0, 2.0% [v/v]). Flasks were cultured under the same growth conditions and cells were harvested one-week post-treatment.

Artemisinin extraction and determination

For crude extract preparation, treated and untreated cells were harvested and dried in the same manner as described above, and ground into fine powder using a pestle and mortar. Artemisinin was extracted as described by Xiang et al. (2015) with minor modifications. Briefly, 500 mg of dry powder were extracted with 50 mL of petroleum ether in an ultrasonic bath for 30 min, the extraction mixture was filtered, followed by a second extraction with 50 mL of petroleum ether and sonicated for 10 min. Filtrates from both extractions were pooled and evaporated to dryness *in vacuo* at 50°C. Residue was re-dissolved in 5 mL methanol and stored in -20°C. Prior to HPLC analysis, 1 mL of the solution was filtered through a 0.45 µm nylon membrane filter.

Artemisinin detection was performed using an Agilent 1260 Infinity LC system equipped with a Hypersil GOLD C18, 250 × 4.6 mm column (pore size 175 Å, particle size 5 µm) (Thermo Scientific, USA) coupled with a guard column. The mobile phase consisted of acetonitrile (Merck) and water (60:40 [v/v]) at a constant flow rate of 1 mL/min. Injection volume and detection wavelength were set at 20 µL and 195 nm, respectively. Artemisinin standard (≥ 98%; LKT Laboratories Inc., USA), dissolved in methanol and properly diluted, was used to prepare standard curves for quantification. Putative artemisinin peaks were confirmed by re-analysing a sample co-injected with standard artemisinin. For each sample, at least three replications ($n \geq 3$) were performed.

RNA isolation, cDNA synthesis and reverse transcription (RT)-PCR

Pre-weighed cell samples (0 to 5 h old cultures after UV-B treatment; 8-day-old cultures after DMSO treatment) and leaf tissues (100 mg from 1-month-old plantlets) were frozen in liquid nitrogen and ground to a fine powder using a sterile pestle and mortar. Total RNA was extracted using innuPREP Plant RNA kit (Analytik Jena). The purity and yield of RNA samples were measured with a NanoDrop™ 1000 (Thermo Scientific) and integrity tested by 1% (w/v) agarose gel at 90V for 55 min. RNA samples were stored in -80°C until further use. First-strand cDNA was synthesised from 1 µg of total RNA using QuantiTect® Reverse Transcription kit (Qiagen, Germany).

RT-PCR was performed using primers ADS_Ex4$_{for}$ and ADS_Ex4$_{rev}$ for the detection of DNA contamination, as the primers were designed to span intron 4 of the amorpha-4,11-diene synthase (*ADS*) gene. The amplification of a 251 bp fragment indicates the authenticity of the cDNA products that are contamination-free, whereas a single fragment of 363 bp indicates genomic DNA contamination (Czechowski et al., 2018). Experiments were replicated at least thrice and only samples that resulted in the amplification of the 251 bp fragment were taken for further analysis. Routine detection of the artemisinin biosynthesis specific genes which were *ADS*, *DBR2*, aldehyde dehydrogenase 1 (*ALDH1*), cytochrome P450 monooxygenase (*CYP71AV1*), cytochrome P450 reductase (*CPR*), and housekeeping genes namely ubiquitin (*UBI*) and actin 2 (*ACT2*), was carried out using Mastercycler® nexus gradient. Reactions were performed in a total volume of 25 µL with 100 ng cDNA, 10 µM forward and reverse gene-specific primers and 5× Green GoTaq® Flexi Buffer. Cycling conditions were 10 min at 95°C, 40 cycles of 30 s at 95°C, 30

s at 60°C, and 30 s at 72°C. Amplified products (10 µL) were electrophoresed on 1% (w/v) agarose gel at 90V for 45 min. All primers are listed in Table 1. HAP plants or cell cultures were not investigated in this study, therefore no control from this chemotype was available.

Table 1. Gene-specific primer sequences

Primer name/gene	Primer sequences (5'–3')	Expected amplicon size (bp)
ADS_Ex4	forward: GGCTGTCTCTGCACCTCCTC	-
	reverse: CAGCCATCAATAACGGCCTTG	
ADS	forward: GAATGGGCTGTCTCTGCACCTCC	165
	reverse: GGTTTGGGCATACTCCTCATTGACA	
DBR2	forward: GGCATAAATGACAGAACGGACGAAT	133
	reverse: CGATTGCAGGGGAGATTCTAAGACC	
ALDH1	forward: CCTTGGAATGGTCCTGCCTTCA	168
	reverse: GTTAACCACATTAATCACGC	
CYP71AV1	forward: GGAATTGAGGAAAGCATTGAACGG	124
	reverse: CAGAACCAAGGGTAGTGGAGGGTG	
CPR	forward: CCATTTCTTCTTCCCCAAGGTTTG	133
	reverse: GTCATAGGCACGGCATTCTTCATC	
UBI	forward: TGATTGGCGTCGTCTTCGA	58
	reverse: CCCATCCTCCATTTCTAGCTCAT	
ACT2	forward: CCATCCTTCGTTTGGACTTGGC	105
	reverse: CAATTTCCCGCTCTGCTGTGGT	

Data analysis

Each of the two treatments (UV-B and DMSO) was replicated six and nine times, respectively, with each flask treated as one replicate. Experiments were set up in a randomised complete block design. Significance of stress treatments was determined by Kruskal-Wallis test. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using the SPSS software (IBM SPSS Statistics 24).

RESULTS AND DISCUSSION

Growth kinetics of *A. annua* cell suspension

The growth dynamics of *A. annua* cell suspension cultures were determined via dry cell weight estimation. It appeared that *A. annua* cells were better able to adapt to the new culture environment as evidenced by the rapid entry of cells into the exponential growth phase within the first 5 days of culture initiation (Figure 2). After day 10 of culture, the cells then entered plateau, followed by a phase of diminishing growth. The addition of growth hormones (0.5 mg/L BAP and 0.5 mg/L NAA) in media for callus induction from leaf explants derived from *in vitro* plants cultured in basal MS might have conditioned cells to adapt and respond to the varying physicochemical conditions of its environment. Such phenomenon is known as priming in which a transient abiotic stress cue leads to modified stress responses upon exposure to a recurring stress (Lämke and Bäurle, 2017). Thus, following its transition into liquid suspension supplemented with the same hormonal regime, cells exhibit enhanced tolerance to recurring stress in terms of rapid growth as lag phase is often associated with minimal growth that is compromised for the exploitation of new environmental conditions (Silveira et al., 2004; Brooks et al., 2011). It is also likely that the lag phase had been limited to

a duration of only 1 to 3 days when subculture was conducted during the exponential or linear phases. The subculture period of 3-week intervals of the present study might justify the shortened lag phase. Furthermore, Lo et al. (2012) reported prolonged lag phase (9 days) when Vietnamese *A. annua* suspension cultures with initial inoculum of 0.25 g were subcultured at 2-week intervals. Based on the growth curve, *A. annua* cells in the exponential phase were selected for elicitation experiments but because of the shorter duration of UV-B treatments which could be completed in a day and cells were harvested immediately, 10-day-old cell cultures were chosen. Conversely, DMSO treatment required an incubation period of a week and for this reason, 5-day-old cells were used so that when cells were harvested, they would not enter the stationary or death phase.

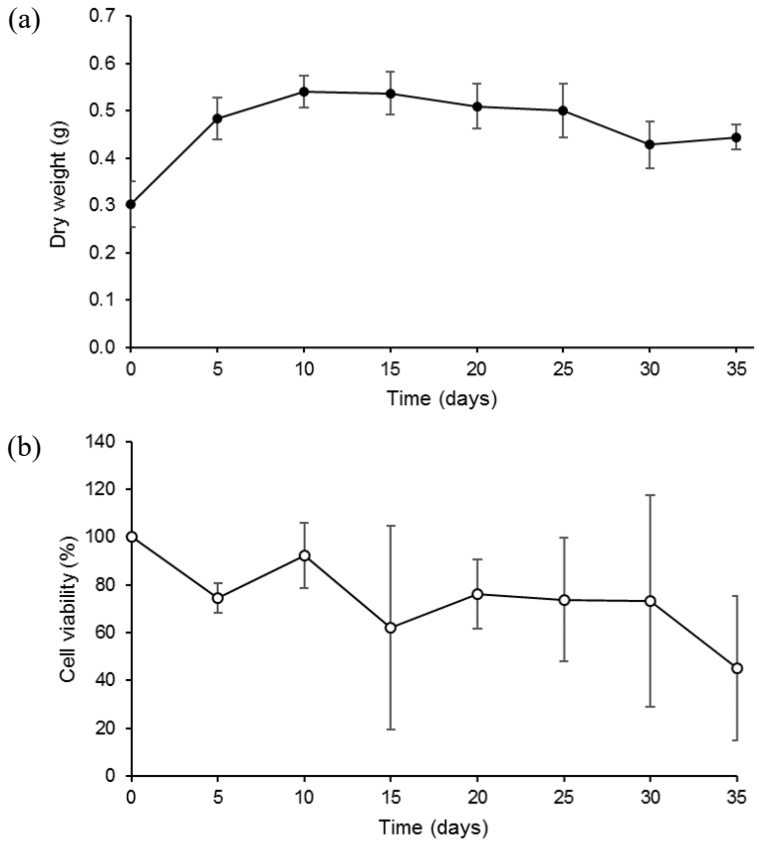

Figure 2. Evaluation of growth kinetics and MTT assay of *A. annua* suspension cultures. (a) Time profile of growth and (b) viability of *A. annua* cell cultures in liquid MS supplemented with 0.5 mg/L BAP and 0.5 mg/L NAA in shake flasks. Values were mean ± standard deviation of triplicate cultures.

Stress effects on the artemisinin content of *A. annua* suspension cells

To test our hypothesis that elicitation with physical and chemical stress may lead to a shift in the biosynthetic mechanism of a LAP favouring artemisinin formation instead of AB, *A. annua* suspension cultures were subjected to UV-B radiation and DMSO application, independently. The effects of these stress treatments were shown in Figures 3 and 4. In this study, short term UV-B radiation showed only limited effect on the production of artemisinin with the 2 h exposure yielding the highest artemisinin content (1.73 mg/g DW) among other exposure hours but was marginally low compared to the untreated cultures (1.80 mg/g DW). The treated cell suspension cultures showed no significant effect on the accumulation of artemisinin (Figure 3). Similarly, suspension cultures treated with varying DMSO concentrations did not

show any enhancement in artemisinin production as well, in which case artemisinin was not detected (Figure 4).

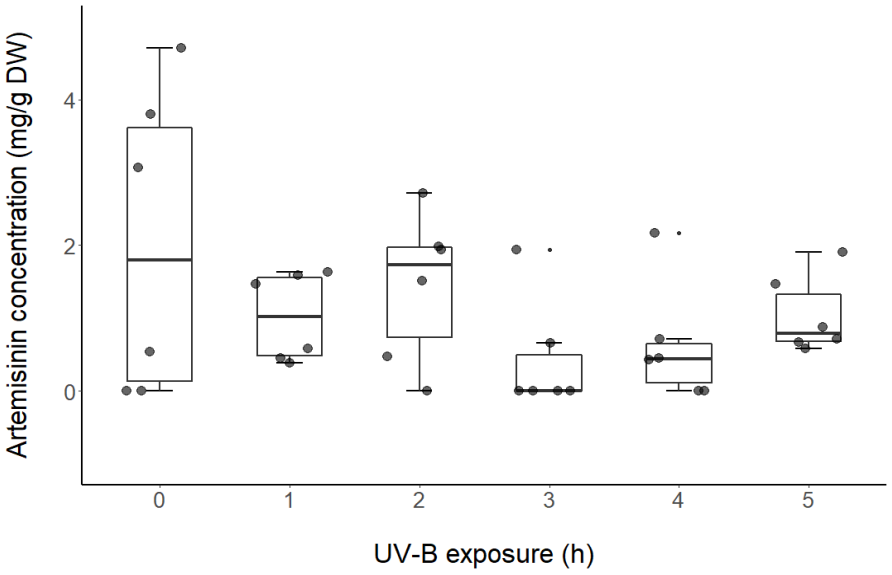

Figure 3. Artemisinin concentration in cell suspension cultures after the application of stress treatment with UV-B radiation at different durations. Data were presented as median values of six independent experiments with one flask each.

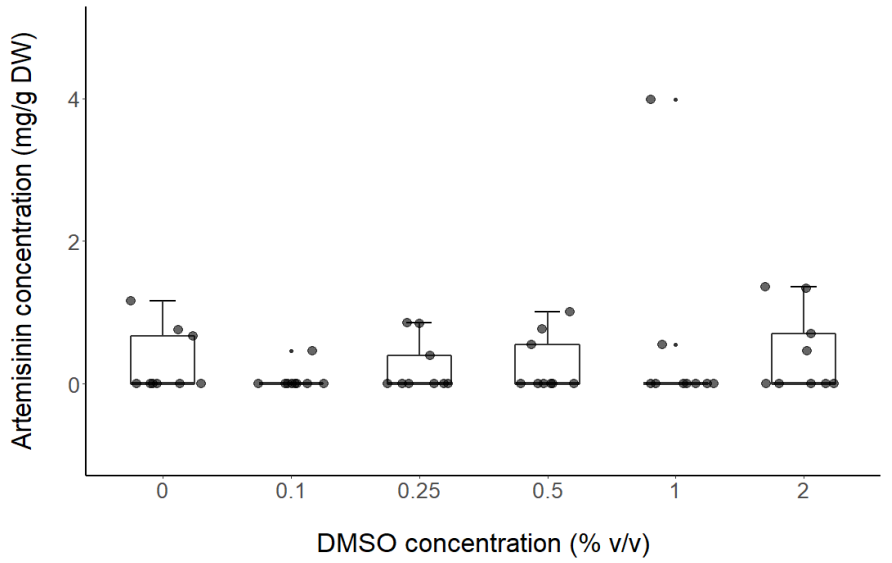

Figure 4. Effect of different concentrations of DMSO elicitation on artemisinin accumulation. Data were presented as median values of nine independent experiments with one flask each.

Based on these results, the hypothesis that artemisinin production in cell cultures of a low-yielding chemotype could be improved by either UV-B or DMSO elicitors was rejected. The fact that artemisinin was detected in suspension cultures subjected to UV-B elicitation confirmed that de-differentiated cells of *A. annua* of European variety could produce artemisinin. Although flavonoid and anthocyanin contents were not determined in the present study, the decrease in artemisinin concentrations following UV-B

irradiation could be a result of resource allocation to produce these sunscreen metabolites to absorb UV-B radiation, inhibit ROS generation and quench ROS (Qi et al., 2018; Thomas and Puthur, 2020). The competition between these antioxidant agents and DHAA for ROS might occur, thus possibly affecting the efficacy of UV-B in enhancing artemisinin production in cell cultures. This could be observed in UV-B-treated *in vitro A. annua* plantlets where the concentrations of artemisinin were lower compared to flavonoids, though they showed similar accumulation patterns and peaked at the same treatment duration of 2 h (Pandey and Pandey-Rai, 2014).

The lack of stimulation in the DMSO elicitation study seemed to be tied to several physiological attributes that possibly explain the roadblock responsible for failure. The physiological state of cultures and the regulation of cellular metabolic homeostasis were identified as important factors that might influence stress responses of *A. annua* that in turn might result in a distortion of elicitation outcome (Kam and Yap, 2020). While Ferreira and Janick (1996b) also reported either trace or no artemisinin in HAP cell cultures, recent elicitation experiments on *A. annua* suspension cultures improved artemisinin production by several folds (Baldi and Dixit, 2008; Chan et al., 2010; Caretto et al., 2011; Durante et al., 2011; Salehi et al., 2019; Zhu et al., 2020). The discrepancy in elicitation successes may lie in the different time point selection as to when cells were harvested post-treatment. In the present study, 5-day-old cell cultures were subjected to DMSO treatments and harvested 7 days later for further analysis. The literature provided evidence that prolonged incubation periods might affect intracellular artemisinin accumulation and in culture medium. Highest artemisinin accumulation was recorded when elicited cell cultures were harvested after incubation periods ranging from 4 h to 5 days (Baldi and Dixit, 2008; Caretto et al., 2011; Durante et al., 2011; Salehi et al., 2019; Zhu et al., 2020). Caretto et al. (2011) showed that cells treated with methyl jasmonate for prolonged periods (24 h, 48 h and 5 days) had artemisinin levels that dropped to values similar to control cells and were accompanied by a decline in *CYP71AV1* expression. Confirming the current results that artemisinin was absent in DMSO-treated cells or if present, might be below the detection limit, Durante et al. (2011) showed that the highest artemisinin content in the culture medium of heptakis(2,6-di-*O*-methyl)-β-cyclodextrin (DIMEB)-treated cells was observed after 3 days (25.19 µmol/g DW) and only 15% of the maximum value (3.78 µmol/g DW) was recorded after 7 days. However, the intracellular artemisinin accumulation of DIMEB-treated cells significantly increased after 7 days (0.19 µmol/g DW) but this content was still lower than the remaining 15% detected in the medium after 7 days of culture (Durante et al., 2011). On the other hand, Chan et al. (2010) also recorded an increased production in *A. annua* cells treated with 2 mg/L of potassium nitrate after 7 days of culture but again the artemisinin content was minute (55.2 µg/g DW). It is interesting to note that the high increase of artemisinin levels in the DIMEB-treated cell cultures has been suggested as a result of β-cyclodextrins forming inclusion complexes with artemisinin, thereby reducing its cytotoxic effects against the cells and a possible negative feedback loop (Durante et al., 2011).

The possibility of artemisinin decomposition in the medium over an extended period of incubation cannot be excluded completely. Woerdenbag et al. (1992) reported that artemisinin rapidly decomposed when incubated with spent culture medium or with a cell homogenate of *A. annua*. After 72 h of incubation, the remaining amount of artemisinin in spent medium was 0 and 14% in cell homogenate. This rapid degradation of artemisinin has been linked to the high peroxidase activity originating from *A. annua* cultures (Woerdenbag et al., 1992). As previously confirmed that artemisinin could be isolated from the culture medium (Caretto et al., 2011; Durante et al., 2011), further extraction studies of artemisinin were performed on culture medium post-treatment in the present study and artemisinin was not detected. Our results agreed with Woerdenbag et al. (1992) in that artemisinin might be degraded in the medium by the hydrolytic and oxidising enzymes present, following the prolonged incubation period after DMSO treatment.

Although artemisinin was detected in UV-B-treated cell extracts, artemisinin was also undetectable in the culture medium. These findings appeared to support the hypothesis that an as-yet-unknown self-resistance mechanism towards self-produced phytotoxic metabolite exists in *A. annua* cell cultures, at least for this European variety. The fluctuating cellular artemisinin contents seemed to parallel the recycling mechanism of sanguinarine detoxification in cell suspension cultures of *Eschscholzia californica*.

Presumably, excreted artemisinin may be rapidly reabsorbed by producing cells and converted into a less toxic product via reduction reactions, in which case the resulting compound may then undergo further biosynthetic reactions (Weiss et al., 2006). Although there is no direct evidence regarding self-resistance in *A. annua* suspension culture, but there remains substantial scope for further research to unravel the mechanism underlying protection against self-intoxication in cells (if any), and provide a deeper insight on the differential control of artemisinin sequestration in relation to developmental stage (cell cultures and intact plants). Of note, we believed that the regulatory mechanism underlying artemisinin production is closely linked to the self-resistance mechanism (Kortbeek et al., 2019). Thus, it is worthwhile to warrant further studies to elucidate the relationship between artemisinin biosynthetic pathway and its detoxification process in cell cultures.

Lastly, we investigated how prolonged incubation of cell cultures might affect artemisinin biosynthesis by determining the presence of mRNA transcripts of artemisinin biosynthetic genes. RT-PCR assays revealed that transcripts of three out of four artemisinin-specific biosynthetic genes namely *ADS*, *DBR2* and *ALDH1* were absent in cultured cells. To ascertain the underlying cause for transcript absence, whether variation in chemotypic characters of this particular *A. annua* variety or the developmental state of cultures could have influenced the regulation of artemisinin biosynthesis, soil-grown plants of the corresponding chemotype were used as comparison and it was found that those genes were expressed in juvenile leaves (Figure 5).

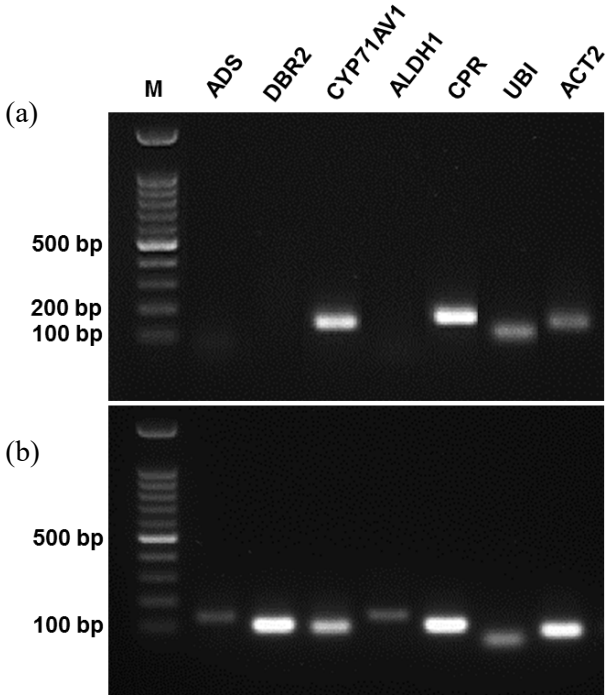

Figure 5. Comparison of PCR profiles of amplified artemisinin biosynthetic genes and housekeeping genes between untreated cultured cells (a), and leaf tissues from soil-grown *A. annua* (b) using gene-specific primers. M, 100 bp DNA ladder.

Thus, these observations suggested the importance of developmental timing on the spatial-temporal regulation of gene expression influencing artemisinin biosynthesis. Similar to previous description of cell suspension cultures of *Lupinus polyphyllus*, *Cytisus scoparius* and *Laburnam alpinum*, quinolizidine alkaloids were produced in orders of magnitude lower (0.01 to 5 µg/g fresh weight) compared to those of differentiated plants (500 to 5000 µg/g fresh weight). Thus, it was inferred that genes responsible for quinolizidine alkaloid formation were expressed at very low levels (Wink, 1989). Likewise, the inability to

detect *ADS* expression in *A. annua* cell cultures has also been reported by Jing et al. (2009) and Caretto et al. (2011), although unfortunately not all genes of the pathway were studied. Our results were consistent with these reports but disagreed with Wallaart et al. (2001), who reported that *ADS* was not detected in young leaves of unstressed *A. annua* plants. Furthermore, as *ADS* is an essential gene for the first committed step of artemisinin formation (Mercke et al., 2000) and the modest effect of yeast expressing only *ADS* on amorpha-4,11-diene (A-4,11-diene) production (Ro et al., 2006), its absence in cultured *A. annua* cells would likely lead to the truncation of artemisinin biosynthesis pathway. Lacking genes encoding both DBR2 and ALDH1 enzymes necessary for the preceding steps in the pathway further disrupt artemisinin biogenesis. It was also noteworthy that our observations were consistent with previous data suggesting the deployment of divergent and distinctive classes of CYP/CPRs in *A. annua* plants to cope with the reductive demand of P450-catalysed reactions (Wu et al., 2011). Since CYP71AV1 is a membrane-bound, multifunctional enzyme that is involved in a wide range of oxidative metabolic reactions, as demonstrated by its efficient *in vivo* conversion of A-4,11-diene to AA by recombinant CYP71AV1 (Ro et al., 2006), it was therefore no surprise that both *CYP71AV1* and *CPR* (a redox partner of the former) genes were present even in cultured cells.

CONCLUSIONS

Taken together, it was reasonable to suggest that the inefficiency of elicitation in the present study was not due to the incompatibility or choice of stress-inducing agents, but one associated with several factors such as the incubation duration post-treatment, and the physiology and biochemistry of cultured cells. Apart from the absence of transcripts of most key genes along the pathway, other processes such as transportation, accumulation and degradation of artemisinin should be considered when determining whether a LAP plant-derived cell culture could produce the antimalarial compound or not. Details of the metabolism of artemisinin in cell cultures, regardless of chemotype, remains a topic that deserves further investigation to evaluate these processes at the molecular level which in addition to biosynthesis are important for artemisinin production.

AUTHORS CONTRIBUTION

MKYY and WYSP conceived and designed the work. MKYY performed the analysis. MKYY and WYSP wrote the paper. WYSP checked and approved the submission.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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