plant biology

Plant Biology ISSN 1435-8603

RESEARCH PAPER

Methyl jasmonate and miconazole differently affect arteminisin production and gene expression in *Artemisia annua* suspension cultures

S. Caretto, A. Quarta, M. Durante, R. Nisi, A. De Paolis, F. Blando & G. Mita

Istituto di Scienze delle Produzioni Alimentari, CNR, Lecce, Italy

Keywords
Gene expression; malaria; plant cell cultures.

Correspondence
S. Caretto, Istituto di Scienze delle Produzioni Alimentari – CNR, Via Prov.le Monteroni, 73100 Lecce, Italy.
E-mail: sofia.caretto@ispa.cnr.it

Editor
J. Whelan

Received: 3 August 2009; Accepted: 17 November 2009.

doi:10.1111/j.1438-8677.2009.00306.x

ABSTRACT

***Artemisia annua* L. is a herb traditionally used for treatment of fevers. The glandular trichomes of this plant accumulate, although at low levels, artemisinin, which is highly effective against malaria. Due to the great importance of this compound, many efforts have been made to improve knowledge on artemisinin production both in plants and in cell cultures. In this study, *A. annua* suspension cultures were established in order to investigate the effects of methyl jasmonate (MeJA) and miconazole on artemisinin biosynthesis. Twenty-two micro molar MeJA induced a three-fold increase of artemisinin production in around 30 min; while 200 µm miconazole induced a 2.5-fold increase of artemisinin production after 24 h, but had severe effects on cell viability. The influence of these treatments on expression of biosynthetic genes was also investigated. MeJA induced up-regulation of *CYP71AV1*, while miconazole induced up-regulation of *CPR* and *DBR2*.**

INTRODUCTION

Artemisinin is an endoperoxide sesquiterpene lactone produced by the plant *Artemisia annua* L. (Asteraceae), which is native to Asia and has been used for many centuries in traditional Chinese medicine for treatment of fever (Ferreira *et al.*1997). Artemisinin is effective against a variety of parasites and, most importantly, is highly effective against the malarial agents *Plasmodium falciparum* and *P. vivax* (Ferreira *et al.* 1997; Hsu 2006). Unfortunately, artemisinin is produced by plants at low levels (0.1–1.0% dry weight), is almost exclusively produced in leaves and inflorescences, and accumulates in glandular trichomes (Duke & Paul 1993; Duke *et al.* 1994). Due to the great importance of this compound and to its complex chemical nature, several studies have been conducted to improve knowledge on artemisinin production (for a review see Covello 2008). Many efforts have been made to identify and fully characterize genes and enzymes involved in artemisinin biosynthesis (Fig. 1). Bouwmeester *et al.* (1999) reported that the first committed precursor for artemisinin biosynthesis is amorpha-4,11-diene, synthesised by the amorpha-4,11-diene synthase (ADS) enzyme. The *A. annua ADS* gene has been cloned (Mercke *et al.* 2000; Wallaart *et al.* 2001), as well as some downstream genes in the artemisinin biosynthetic pathway: *CYP71AV1*, a gene encoding a cytochrome P450 monoxygenase (Ro *et al.* 2006; Teoh *et al.* 2006), *CPR*, a cytochrome P450 reductase (accession number DQ984181) and *DBR2* encoding an artemisinic aldehyde Δ11(13) reductase (Zhang *et al.* 2008). Elucidation of the artemisinin biosynthetic pathway is of primary importance for research programmes aiming at either microbial engineering for artemisinin production or increasing the artemisinin content in genetically modified plants (Ro *et al.* 2006; Chang *et al.* 2007; Covello *et al.* 2007; Zeng *et al.* 2008a,b; Tsuruta *et al.* 2009).

Attempts have also been made to produce artemisinin in *A. annua* cell and tissue cultures. However, due to the costs of tissue culture and the low yields obtained, these *in vitro* systems have not been used for the industrial production of artemisinin (Paniego & Giulietti 1994, 1996; Souret *et al.* 2003; Towler *et al.* 2007). However, plant *in vitro* cultures can be a valuable tool to widen knowledge on biochemistry and molecular biology of plant primary and secondary metabolism, since they make it possible to operate in very well-defined experimental conditions and to gain information on the role that specific chemical treatments have on plant metabolite production (Mülbach 1988).

Jasmonic acid and its methyl ester, methyl jasmonate, are well known signalling molecules that induce reprogramming of gene expression and elicit the pathway of secondary metabolism in plant cells (Pauwels *et al.* 2008). Recently, Liu *et al.* (2009) reported that jasmonic acid increases the density of glandular trichomes on *A. annua* leaves and enhances artemisinin production.

Miconazole is an antifungal compound, known to inhibit sterol biosynthesis and to redirect carbon flux towards the terpenoid pathway (Kudakasseril *et al.* 1987; Zarn *et al.* 2003). Recently, Towler & Weathers (2007) reported that miconazole induces a significant increase in artemisinin in *A. annua* seedlings.

Here, we report on the establishment of *A. annua* suspension cultures, which were subjected to methyl jasmonate or

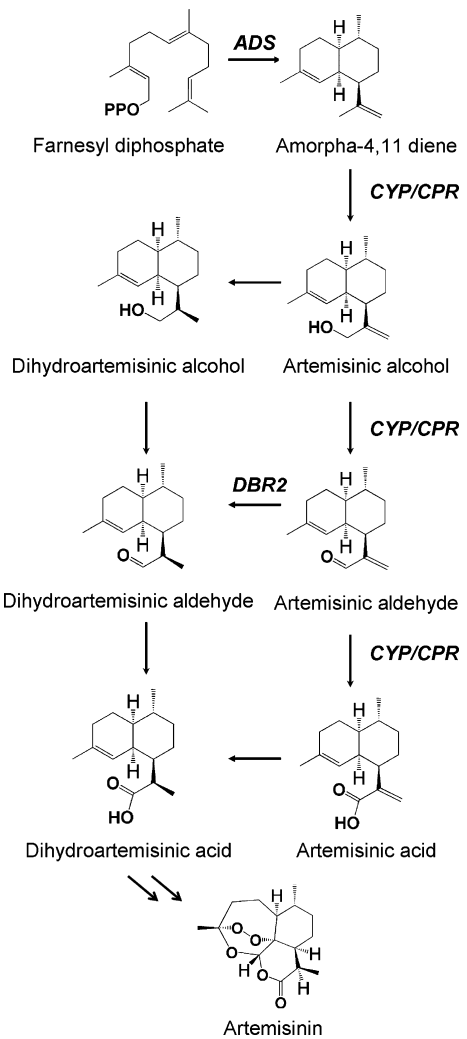

Fig. 1. Artemisinin biosynthetic pathway, adapted from Teoh *et al.* (2006). *ADS*, amorpha-4,11-diene synthase, *CYP*, cytochrome P450 monoxygenase, *CPR*, cytochrome P450 reductase, *DBR2*, artemisinic aldehyde Δ11(13) reductase.

miconazole treatment. The influence of these treatments on artemisinin content and expression of biosynthetic genes was investigated.

MATERIALS AND METHODS

Establishment and treatment of *Artemisia annua* cell cultures

Artemisia annua L. plants were grown in experimental fields at the Consiglio per la Ricerca e Sperimentazione in Agricoltura, Lecce, Italy. Plant leaves were surface sterilized by extensive washes in sterile distilled water containing a few drops of Tween20 (Sigma, St Louis, MO, USA), followed by 20 min immersion in a NaClO solution (1% active chloride) and three further washes in sterile distilled water. Axenic leaf explants were then incubated for callus induction in continuous light conditions (125 $\mu mol \cdot m^{-2} \cdot s^{-1}$) in a growth chamber at 25 °C on agarized (0.8% plant agar Duchefa) Murashige and Skoog (MS) basal medium (Murashige & Skoog 1962), supplemented with 30 $g \cdot l^{-1}$ sucrose and various concentrations of naphthalene acetic acid (NAA, from 0 to 2 $mg \cdot l^{-1}$) or 2,4-dichlorophenoxyacetic acid (2,4-D, from 0 to 2 $mg \cdot l^{-1}$) and 6-benzyl amino purine (6-BAP, from 0 to 0.2 $mg \cdot l^{-1}$).

Suspension cultures were initiated by transferring small pieces of calli into 100-ml Erlenmeyer flasks containing 5 ml of G6 liquid medium (MS medium containing 2 $mg \cdot l^{-1}$ 2,4-D and 0.15 $mg \cdot l^{-1}$ BAP) and incubated on a rotary shaker (120 rpm) in continuous light at 25 °C. The medium volume was gradually increased to 25 ml. Once established, cultures were regularly sub-cultivated every 35 days into 500-ml Erlenmeyer flasks by transferring 15 ml of the 35-day-old suspension into 85 ml of fresh G6 medium. Growth was evaluated by measuring the fresh weight of the cultures at regular intervals. Cell viability was assayed by staining with fluorescein diacetate (Wildholm 1972). For dry weight determination, cultures were filtered through nylon filters (30-μm pore size), then frozen in liquid nitrogen and lyophilized overnight (Labconco, Kansas City, MO, USA).

Methyl jasmonate (Sigma) was added to G6 liquid medium at a final concentration of 22 μM. Fifteen-day-old suspension cultures (25 ml) were incubated in G6 medium containing methyl jasmonate for 30 min, 1 h, 2 h, 4 h, 24 h, 48 h or 5 days. Equivalent volumes of 95% ethanol (used as MeJA solvent) were used as control samples. Miconazole (Sigma) was dissolved in dimethyl sulfoxide (DMSO; Sigma) and added to G6 medium at 5, 10, 20, 50, 100 or 200 μM final concentrations. Miconazole treatment intervals were as reported for MeJA. Control samples were treated with equivalent volumes of DMSO.

Artemisinin determination

After filtration, the cell samples were frozen in liquid nitrogen and lyophilized overnight. Aliquots of 20–50 mg lyophilized powder were then extracted with 4 ml *n*-hexane for 16 h under magnetic stirring, then for 15 min in an ultrasonic water bath (L&R SweepZone Technology, Kearny, NJ, USA). The extract was centrifuged at 4000 *g* for 10 min, and the supernatant was removed and placed in new tubes. The pellet was resuspended in 1 ml *n*-hexane and centrifuged again for 10 min at 4000 *g*. This second supernatant was added to the first, and the pellet discarded. Extracts were then dried under vacuum and stored at −20 °C until HPLC analysis. Culture medium was lyophlilized and the powder extracted with *n*-hexane as described above for cell samples. For artemisinin determination, the Q_{260} derivative was analysed by HPLC according to Smith *et al.* (1997). Samples were resuspended in 100 μl absolute ethanol and derivatised by addition of 200 μl 60 mM NaOH, samples were then briefly vortexed and incubated in an oven at 45 °C for 30 min. After cooling to room temperature, samples were acidified with 200 μl acetic acid (62.5 mM final concentration), briefly vortexed, and centrifuged for 1 min in a microcentrifuge at maximum speed. Artemisinin standard (Sigma), derivatised as described above and properly diluted, was used to prepare standard curves for artemisinin quantification. HPLC analyses were carried out using an Agilent 1100 Series HPLC system equipped with pre-column, guard, Ultrasphere ODS (0.46 × 4.5 cm, 5 μm particle size; Beckmann, Fullerton, CA, USA) and a C18 Ultrasphere ODS column (0.46 × 25 cm, 5 μm particle size; Beckmann). The mobile

phase was methanol:sodium phosphate buffer, pH 7.0 (55:45 v/v) at 1 ml·min^{-1} constant flow rate, 35 °C column temperature and 260 nm wavelength for detection. The injection volume was 20 µl. Putative artemisinin peaks were confirmed by re-analysing a sample co-injected with standard artemisinin and by comparing spectra of the standard and putative artemisinin peaks (Figure S1).

Expression analysis of artemisinin biosynthetic genes

Suspension cultures were first filtered as described above, frozen in liquid nitrogen, lyophilized and ground to a powder. RNA was isolated using the SV Total RNA Isolation System (Promega, srl, Milan, Italy). RNA concentration was determined spectrophotometrically, and the quality was checked by agarose gel electrophoresis. cDNAs were obtained from both control and treated cells, starting from 1 µg total RNA and using random primers and the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's instructions.

For quantitative real-time PCR experiments, primers and dual-labelled probes were designed for *ADS*, *CYP71AV1*, *CPR* and *DBR2* genes, on the basis of public available sequences (accession numbers EF197888, DQ315671, EF104642 and EU704257, respectively). Primers and probes for the *A. annua* EST accession number EY075668, described as similar to *A. thaliana LOX3*, were also designed on the basis of the nucleotide sequence available in the CAZI *A. annua* normalised leaf library (Newman K.L., unpublished data). As a housekeeping gene, ubiquitin (UBIQ) was used (EU258763). The probes were labelled at the 5′ end with 6-carboxy-fluorescein (FAM) and at the 3′ end with tetramethylrhodamine (TAMRA). The primers and probes used are listed in Table 1, and were all purchased from PRIMM srl (Milan, Italy). Amplifications were performed in an Applied Biosystems 7500 apparatus using the sequence-specific primer set (900 nM each primer), specific probe (200 nM), 0.5 µl of the first strand cDNA and 12.5 µl of 2× TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in a total volume of 25 µl. Negative controls were performed by omitting the template in the PCR reaction mix. Experiments were conducted in triplicate and using cDNAs obtained from at least two independent experiments. Real-time PCR cycles were as follows: 50 °C for 2 min (1 hold); 95 °C for 10 min (1 hold); 95 °C for 15 s, 60 °C for 1 min (40 cycles). Quantification of transcripts was carried out using the comparative quantitation module, as described for the ABI 7500 Sequence Detection System, (User Bulletin 2; Applied Biosystems) based on the $2^{-\Delta\Delta C}{}_{T}$ method (Livak & Schmittgen 2001). The relative expression was normalised against ubiquitin, and calculated using the untreated samples as a calibrator, whose expression was given equal to one.

Semi-quantitative RT-PCR experiments were performed using the same cDNA samples used for quantitative real-time PCR. The amplifications were performed in a *One personal* PCR instrument (EuroClone SpA, Milan, Italy). The primer sequences are reported in Table S1. Negative controls were performed by omitting the template in the PCR reaction mix. PCR cycles were as follows: 94 °C for 3 min (1 hold), 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min (30 cycles) for *CYP71AV1*, *DBR2*, *CPR* and *LOX3*; 35 cycles were used for *ADS*. The thermal profile used for the ubiquitin gene was: 94 °C for 3 min (1 hold), 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s (28 cycles). PCR products were analysed on 1% agarose gels containing ethidium bromide and photographed under UV light.

Statistical analysis

Experiments were performed at least in triplicate and data expressed as the mean ± SD. Data were analysed statistically by two-way ANOVA, followed by Tukey HSD *post hoc* tests, using SIGMASTAT software Version 3.1 (SPSS Inc., Chicago, IL, USA). Significance level was set at 5%.

Table 1. Target gene, primer and probe sequences used for quantitative real-time PCR assays in *A. annua* cell cultures.

target gene	primer set	sequences	reference
ADS	RTADSfor	5′-TGGTCGACGCCTAAATGATCT-3′	Mercke *et al.* 2000
	RTADSrev	5′-TGGGCATACTCCTCATTGACAT-3′	
	ADSprobe	5′-TGACCCACAAGGCCGAGCAAGAA-3′	
CYP71AV1	RTCYPfor	5′-GTTTGGAGCTGGGAGAAGGAT-3′	Teoh *et al.* 2006
	RTCYPrev	5′-GGTCATGTCGATCTGGTCATAGC-3′	
	CYPprobe	5′-ACGTGCAGCTCCCGCTCGCTAATAT-3′	
DBR2	RTDBR2for	5′-GGGTTACAAGCTGTGGCTCAAG-3′	Zhang *et al.* 2008
	RTDBR2rev	5′-CAAGGTCAGGATTCGAGACAAAA-3′	
	DBR2probe	5′-TGCTGATTTGGTCGCTTTTGGTCGTT-3′	
CPR	RTCPRfor	5′-GCTCGGAACAGCCATCTTATTCT-3′	GenBank Acc. no. EF104642
	RTCPRrev	5′-CCCCAGTCTCCACGAAATTATT-3′	
	CPRprobe	5′-CGGATGCAGGAATCGCAAAGTGGAT-3′	
UBQ	RTUBIfor	5′-CGGACCAGCAGAGGTTGATATT-3′	Zeng *et al.* 2008b
	RTUBIrev	5′-CAGCCTTAAGACCAAATGGAGAGT-3′	
	UBIprobe	5′-CAGGAAAGCAGCTTGAAGATGGCCG-3′	
LOX3	RTLOX3for	5′-AGCTTGGTATGCGGAGGTGAT-3′	GenBank Acc. no. EY075668
	RTLOX3rev	5′-TAATGTGGGCCACCAGTTCTC-3′	
	LOX3probe	5′-TGTGGGCCATGCAGATTTGCGT-3′	

RESULTS

A. annua suspension cultures

Leaf-derived *A. annua in vitro* cultures were started on MS agarised medium supplemented with several different hormone combinations of NAA or 2,4-D and 6-BAP to identify optimal conditions to obtain actively growing and friable calli. This made it possible to identify G6 (MS basal medium supplemented with 2 $mg \cdot l^{-1}$ 2,4-D and 0.15 $mg \cdot l^{-1}$ 6-BAP) as the optimal medium composition for obtaining rapidly growing green and friable calli (data not shown). G6 callus cultures were then used to obtain suspension cultures, which, once established, were sub-cultivated at 35-day intervals. The growth rate of these suspension cultures was measured at regular intervals, showing a four-fold biomass increase at the end of the subculture cycle (Fig. 2).

Artemisinin production

Artemisia annua cell cultures were analysed for their ability to produce artemisinin during the subculture cycle. The results showed that suspension cultures were indeed able to produce artemisinin continuously during the sub-cultivation cycle, ranging from 4.90 to 5.44 $\mu g \cdot g^{-1}$ DW (Table 2). Artemisinin accumulated in the suspension cultures up to 20 $\mu g \cdot l^{-1}$ after 25 days (Fig. 3). The presence of artemisinin was also evaluated in the culture medium, revealing an increasing content from 5.20 to 15.35 $\mu g \cdot l^{-1}$ by the end of the subculture cycle (Fig. 3).

Exponentially growing *A. annua* cultures were subjected to 22 μM MeJA treatments for different intervals before sample collection. As shown in Fig. 4, cell viability was only slightly affected by MeJA, being about 85% after a 5-day treatment. Artemisinin levels were then analysed, revealing that cells exposed to MeJA for 30 min had a significantly higher artemisinin level (12.44 $\mu g \cdot g^{-1}$ DW) compared to the level in ethanol-treated cells used as a control (5.94 $\mu g \cdot g^{-1}$ DW). The highest artemisinin level (14.40 $\mu g \cdot g^{-1}$ DW) was observed in 4-h treated samples, with a 2.4-fold increase compared to the control. In cells treated for prolonged periods (24 h to 5 days), artemisinin levels dropped to values similar to control cells; no significant differences in artemisinin content were observed between untreated and ethanol-treated cells (Fig. 5).

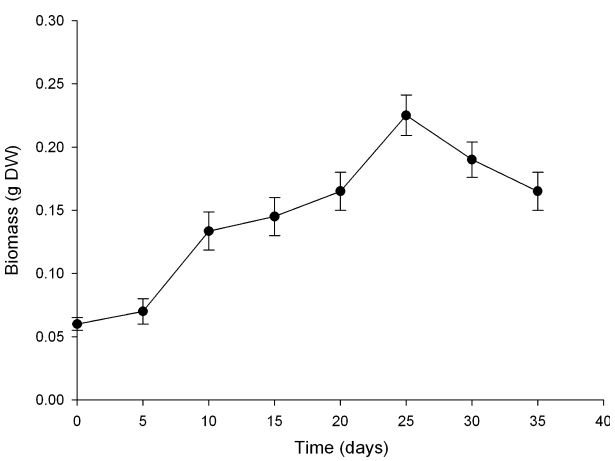

Fig. 2. Time course of cell growth of *A. annua* suspension cultures subcultured in G6 culture medium at 35-day intervals. Values are the means of three independent experiments ±SD.

Table 2. Artemisinin biosynthesis during the sub-cultivation cycle.

Days after sub-cultivation	Artemisinin ($\mu g \cdot g^{-1}$ DW)
0	5.14 ± 0.06
5	5.15 ± 0.07
10	4.96 ± 0.06
15	4.90 ± 0.01
20	5.03 ± 0.16
25	4.98 ± 0.54
30	5.04 ± 0.48
35	5.44 ± 0.79

Values are the means of three independent experiments ±SD.

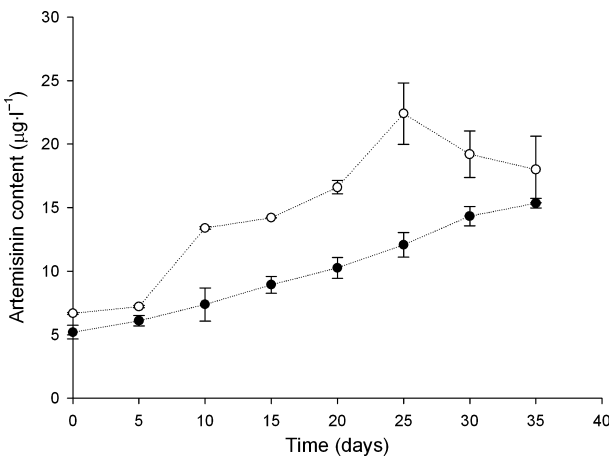

Fig. 3. Intracellular (…○…) and extracellular (…●…) artemisinin determination in *A. annua* suspension cultures. Values are the means of three independent experiments ±SD.

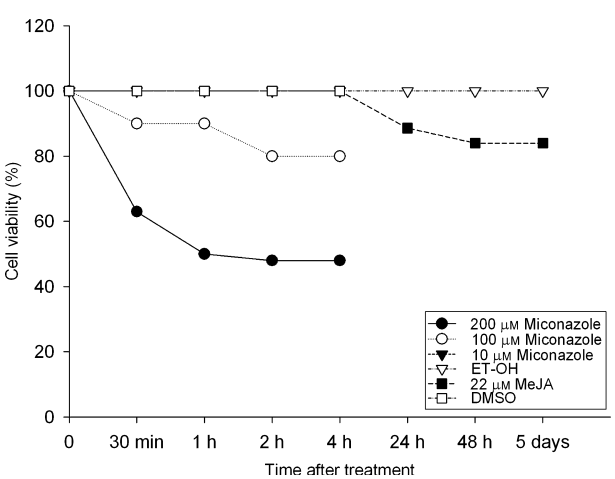

Fig. 4. Cell viability of *A. annua* suspension cultures treated with MeJA or miconazole. Cell viability (% of control) was evaluated by staining with fluorescein diacetate. The SD did not exceed 10%.

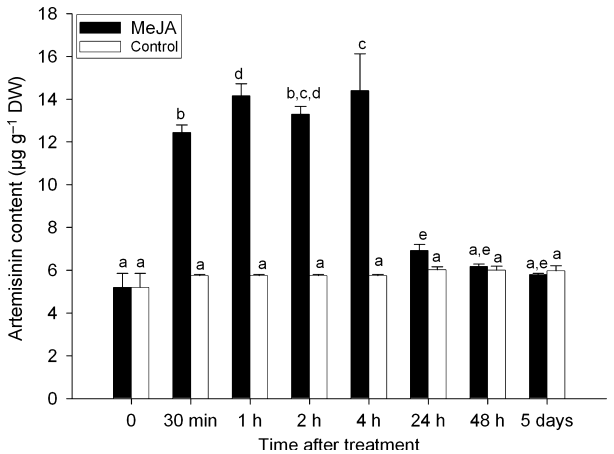

Fig. 5. Effect of MeJA on artemisinin production by *A. annua* suspension cultures. As a control, suspension cultures were treated with equivalent volumes of 95% ethanol. Values are the means of three independent experiments ±SD. Letters indicate statistical differences at P = 0.05.

Artemisia annua cell cultures were also subjected to treatment with different concentrations of miconazole for various intervals. After 30 min in the presence of 200 μM miconazole, suspension cultures showed a 40% reduction in cell viability. Lower miconazole concentrations had less severe effects. Prolonged treatment with 200 or 100 μM miconazole made it impossible to precisely estimate the viability of the cultures. No changes in cell viability were observed in control, DMSO-treated suspension cultures (Fig. 4). Miconazole-treated suspension cultures were analysed for artemisinin production. As reported in Fig. 6, 10 μM miconazole did not induce a significant variation in artemisinin level. Treatments with 100 and 200 μM miconazole induced a significant increase in artemisinin levels after a 24-h treatment. The highest artemisinin level (13.04 $\mu g \cdot g^{-1}$ DW) was observed when cultures were treated with 200 μM miconazole for 5 days. In suspension cultures exposed to DMSO (as a control), a slight but significant increase of artemisinin levels was also observed after prolonged periods (24 h, 48 h and 5 days).

Expression of artemisinin biosynthetic genes

To verify possible effects of MeJA and miconazole on regulation of artemisinin biosynthetic genes (Fig. 1), expression of *ADS*, *CYP71AV1*, *CPR* and *DBR2* genes was analysed in control and treated *A. annua* cell cultures using semi-quantitative RT-PCR (Figure S2) or quantitative real-time PCR. As a positive control for MeJA induction, expression of the *A. annua* EST EY075668 was also analysed. This EST has been described as similar to the *A. thaliana LOX3* (Newman K.L., unpublished data), which is induced by MeJA in *A. thaliana* cell cultures (Pauwels *et al.* 2008). Results obtained show that, after a 30-min MeJA treatment, *CYP71AV1* was seven-fold up-regulated compared to control samples. After prolonged periods, expression of *CYP71AV1* declined, although it was always higher than in control samples (Fig. 7). MeJA did not induce significant changes in the expression of *CPR* and *DBR2*. As far as expression of the *A. annua* putative *LOX3* gene is concerned, clear induction

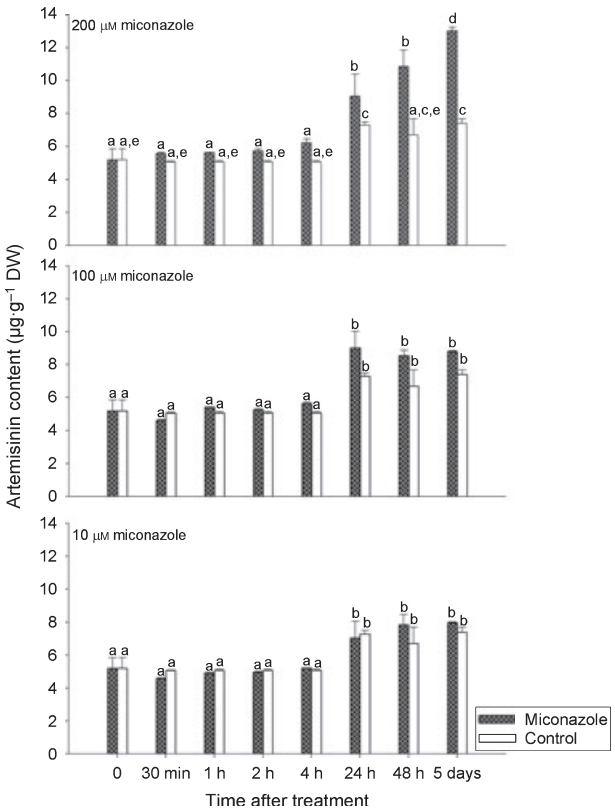

Fig. 6. Effect of 200, 100 or 10 μM miconazole on artemisinin production of *A. annua* suspension cultures. As a control, suspension cultures were treated with equivalent volumes of DMSO. Values are the means of three independent experiments ±SD. Letters indicate statistical differences at P = 0.05.

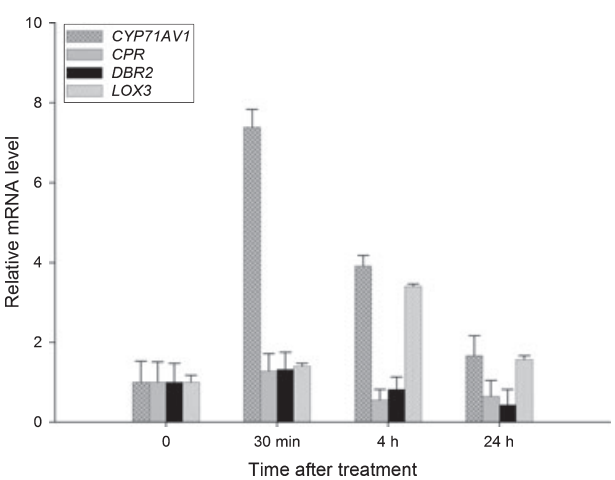

Fig. 7. Estimation of the relative mRNA levels of the artemisinin biosynthetic genes *CYP71AV1*, *CPR* and *DBR2* in *A. annua* suspension cultures treated with 22 μM methyl jasmonate. The expression of an *A. annua* putative lipoxygenase (*LOX3*) was also assayed as a positive control.

was observed in the 4-h MeJA-treated samples. In suspension cultures treated with 200 μM miconazole, expression of *CPR* was more than three-fold increased in 4-h treated samples.

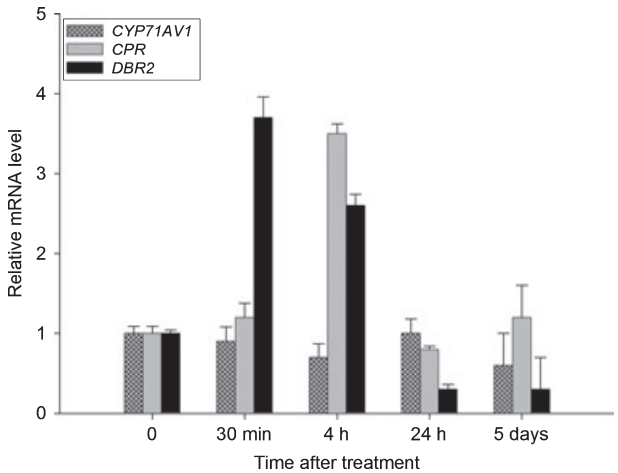

Fig. 8. Estimation of the relative mRNA levels of the artemisinin biosynthetic genes *CYP71AV1*, *CPR* and *DBR2* in *A. annua* suspension cultures treated with 200 μM miconazole.

Treatment for 30 min and 4 h with miconazole induced up-regulation of the *DBR2* gene about four- and three-fold, respectively. In contrast, expression of *CYP71AV1* was always similar to that observed in DMSO-treated control samples (Fig. 8).

In spite of several attempts using semi-quantitative PCR or quantitative RT-PCR, different annealing temperatures, as well as different primers, it was never possible to detect expression of the *ADS* gene in cDNA samples obtained from *A. annua* suspension cultures. In contrast, clear amplification products were obtained using cDNA samples from *A. annua* leaves, as well as with genomic DNA isolated from *A. annua* cell cultures and leaves. These amplification products were cloned and sequenced, and the *ADS* gene identity confirmed (data not shown).

DISCUSSION

The goal of increasing the availability of artemisinin has driven research to explore *A. annua in vitro* cultures as an alternative source of this valuable compound. However, in spite of several attempts, this approach is still far from attractive for commercial production of artemisinin (Covello 2008). However, *in vitro* cell cultures can be a valuable tool to study plant cell metabolism, since it can be assessed in strictly controlled conditions. In this work, actively growing *A. annua* suspension cell cultures were established and used to study the effects of MeJA and miconazole on the production of artemisinin and expression of genes involved in the artemisinin biosynthetic pathway. Among several callus cultures obtained, it was possible to select a cell line able to produce artemisinin, although at low levels, when cultivated in suspension culture. It is interesting that it was possible to observe artemisinin accumulation also in the culture medium. This could be a consequence of some cell lysis in shaken flasks and consequent leakage of metabolites into the culture medium, or to active release of artemisinin by viable cells. This latter explanation can be supported by the observation that extracellular artemisinin levels increased during the subculture cycle. However, this needs to be further investigated. Moreover, intracellular artemisinin production in suspension cultures, expressed as $\mu g \cdot l^{-1}$, gradually increased during the subculture cycle, while artemisinin content, expressed as $\mu g \cdot g^{-1}$ DW, did not show significant variations. These results indicate that the biosynthetic capability of these cell cultures did not change significantly during the subculture cycle; such constant biosynthetic capacity made these suspension cultures a suitable *in vitro* system to investigate the effects of putative elicitors.

MeJA is considered a key compound in the signal transduction pathway, involved in the induction of low-molecular weight compounds that take part in several plant responses (Creelman & Mullet 1997). Several reports have shown that exogenous application of MeJA can induce expression of plant genes for various biosynthetic pathways (Gundlach *et al.* 1992; Pauwels *et al.* 2008). In *A. annua* suspension cell cultures, the observed significant increase of artemisinin levels soon after the beginning of MeJA treatment indicated a rapid response of these cells to MeJA, as well as the ability of MeJA to stimulate artemisinin biosynthesis. Similar stimulatory effects of MeJA were recently reported by Liu *et al.* (2009) in *A. annua* plants treated with jasmonic acid, by Putalun *et al.* (2007) in *A. annua* hairy root cultures, and by Baldi & Dixit (2008) in cell cultures of an Indian variety of *A. annua*. In our cell cultures, along with the artemisinin increase, expression of the *CYP71AV1* gene was also significantly enhanced in response to MeJA treatment. This gene encodes a monooxygenase enzyme that catalyses the three-step oxidation of amorpha-4,11-diene to artemisinic acid (Teoh *et al.* 2006). Furthermore, the time course of artemisinin production clearly resembled the *CYP71AV1* gene expression profile, thus indicating that the stimulatory effect of MeJA on artemisinin production could be due to up-regulation of the *CYP71AV1* gene. However, expression of the *DBR2* and *CPR* genes was not enhanced in the MeJa-treated samples. This could indicate that the cell culture mRNA levels were not rate limiting for artemisinin biosynthesis, but further experiments are needed to draw definitive conclusions. On the basis of these results, *CYP71AV1,* being regulated at the transcriptional level by MeJA, could be a suitable candidate for metabolic engineering strategies to improve artemisinin production.

Miconazole is known to inhibit sterol biosynthesis by inhibiting sterol 14-α-demethylases and thus channelling carbon flux towards the terpenoid pathway (Kudakasseril *et al.* 1987; Zarn *et al.* 2003; Towler & Weathers 2007). Despite the known inhibitory effects of miconazole on specific enzymes, its effects on plant gene expression have not yet been elucidated. In *A. annua* suspension cultures treated with 200 μM miconazole, a late and gradual increase in artemisinin production was observed, indicating that miconazole could be useful to enhance artemisinin production. However, this treatment had severe effects on the viability of the cultures, and lower and less toxic miconazole concentrations induced only a slight increase in artemisinin, which was probably due to the effect of DMSO. An increase in artemisinin level induced by DMSO was recently reported by Towler & Weathers (2007). These authors also reported a positive effect on artemisinin production of miconazole treatment in *A. annua* seedlings, with only slight chlorosis as an adverse effect. Such lower susceptibility of seedlings can be expected in comparison with undifferentiated cell cultures. Analysis of gene expression revealed that miconazole treatment had no

significant effect on *CYP71AV1*, while up-regulation of *DBR2* and *CPR* was observed after 30 min and 4 h, respectively (Fig. 8). Furthermore, the up-regulation of these genes did not appear to be strictly time-correlated to the observed increase in artemisinin, which was detected much later and could involve mechanisms other than activation of the *DBR2* and *CPR* genes. On the basis of these results, the miconazole-induced increase in artemisinin production could be independent from the transcriptional activation of these biosynthetic genes. Further analyses, such as evaluation of the artemisinin biosynthetic precursors, could help clarifying this point.

Recent evidence indicates that the last steps of artemisinin biosynthesis involve transformation of dihydroartemisinic acid (DHAA) to artemisinin through spontaneous autoxidation processes independent of enzymatic catalyses (Brown & Sy 2004). Moreover, there is emerging evidence that stress conditions, involving the generation of reactive oxygen species (ROS), could enhance this spontaneous transformation (Pu *et al.* 2009). This could be the case in the miconazole treatment, since production of increased levels of ROS is thought to be involved in the mechanism of cellular damage induced by miconazole (Kobayashi *et al.* 2002). On the other hand, production of ROS is also known to be induced by the stress hormone MeJA (Zhang & Xing 2008), suggesting that spontaneous transformation of DHAA could also have occurred in MeJA-treated suspension cultures, together with up-regulation of the artemisinin biosynthetic pathway. The role of ROS in *A. annua* cell cultures will be further investigated to verify possible new strategies in the over-production of artemisinin.

It is important to note that we have never been able to observe *ADS* expression in *A. annua* cell cultures, although several experimental conditions have been tested. ADS is involved in cyclisation of farnesyl diphosphate to amorpha-4,11-diene, suggested to be the first committed step in artemisinin biosynthesis (Bouwmeester *et al.* 1999). The inability to detect *ADS* expression could be due to the very low expression level of *ADS* in *A. annua* suspension cultures, consistent with the reported tissue-specific expression of this gene (Kim *et al.* 2008). Moreover, *ADS* was not detectable in unstressed *A. annua* young leaves (Wallaart *et al.* 2001). The regulation of *ADS* is still under investigation, since it has not been completely elucidated whether it occurs at the transcriptional or at the translational level (Zeng *et al.* 2008b).

Taken together, the results presented here indicate that *A. annua* suspension cell cultures are a suitable system to investigate the effects of chemical factors on modulation of artemisinin production and to shed more light on regulation of artemisinin biosynthetic genes in response to specific elicitors such as MeJA and miconazole.

ACKNOWLEDGEMENTS

The authors are grateful to Dr Giovanna Frugis for helpful comments and discussion, Giovanni Colella and Leone D'amico for skilful technical assistance, Dr P. Greco, (CRA, Lecce) for cultivating *A. annua* plants and H. Caffery for English revision of the manuscript. A. Quarta and M. Durante are supported by fellowships funded by the Regione Puglia. This work was supported by the Regione Puglia, Italy, Progetto strategico PS070. The authors wish to acknowledge the memory of Dr Luigi Villanova (Lachifarma) for enthusiastic encouragement in this project.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. HPLC chromatograms obtained from derivatised standard artemisinin (—) and suspension culture extracts (—) (A). Putative artemisinin peaks were confirmed by re-analysing a sample co-injected with standard artemisinin (B), and by comparing spectra of the standard (—) with the putative artemisinin peaks (—) (C).

Figure S2. Semi-quantitative RT-PCR analysis of artemisinin biosynthetic genes, *CYP71AV1*, *CPR* and *DBR2* in *A. annua* suspension cultures. Ubiquitin (*UBQ*) was used as housekeeping gene to show normalisation of the templates in PCR reactions. *A. annua* putative lipoxygenase (*LOX3*) was assayed as a positive control. Panel A: samples treated with 22 μM methyl jasmonate for 0 (lane 1), 30 min (lane 2), 4 h (lane 3) or 24 h (lane 4). Panel B: samples treated with 200 μM miconazole for 0 (lane 1), 30 min (lane 2), 4 h (lane 3), 24 h (lane 4) or 5 days (lane 5).

Table S1. Target genes and primer sequences used for semi-quantitative RT-PCR assays in *A. annua* cell cultures.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

REFERENCES

Baldi A., Dixit V.K. (2008) Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. *Bioresource Technology*, **99**, 4609–4614.

Bouwmeester H.J., Wallaart T.E., Janssen M.H., van Loo B., Jansen B.J., Posthumus M.A., Schmidt C.O., De Kraker J.W., König W.A., Franssen M.C. (1999) Amorpha-4,11-diene synthase catalyses the first probable step in artemisinin biosynthesis. *Phytochemistry*, **52**, 843–854.

Brown G., Sy L.K. (2004) *In vivo* transformations of dihydroartemisinic acid in *Artemisia annua* plants. *Tetrahedron*, **60**, 1139–1159.

Chang M.C.Y., Eachus R.A., Trieu W., Ro D.K., Kiesling J.D. (2007) Engineering *Escherichia coli* for production of functionalized terpenoids using plant P450s. *Nature Chemical Biology*, **3**, 274–277.

Covello P.S. (2008) Making artemisinin. *Phytochemistry*, **69**, 2881–2885.

Covello P.S., Teoh K.H., Polichuk D.R., Nowak G. (2007) Functional genomics and the biosynthesis of artemisinin. *Phytochemistry*, **68**, 1864–1871.

Creelman R.A., Mullet J. (1997) Biosynthesis and action of jasmonates in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, **48**, 355–381.

Duke S.O., Paul R.N. (1993) Development and fine structure of the glandular trichomes of *Artemisia annua* L. *International Journal of Plant Sciences*, **154**, 107–118.

Duke M.V., Paul R.N., Elsohly H.N., Sturtz G., Duke S.O. (1994) Localization of artemisinin and artemisitene in foliar tissues of

glanded and glandless biotypes of *Artemisia annua* L. *International Journal of Plant Sciences*, **155**, 365–372.

Ferreira J.F.S., Simon J.E., Janick J. (1997) *Artemisia annua*: botany, horticulture and pharmacology. *Horticultural Reviews*, **19**, 319–371.

Gundlach H., Müller M.J., Kutchan T.M., Zenk M.H. (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proceedings of the National Academy of Sciences of the USA*, **89**, 2389–2393.

Hsu E. (2006) The history of qing hao in the Chinese *materia medica*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **100**, 505–508.

Kim S.H., Chang Y.J., Kim S.U. (2008) Tissue specificity and developmental pattern of amorpha-4,11-diene synthase (ADS) proved by ADS promoter-driven GUS expression in the heterologous plant, *Arabidopsis thaliana*. *Planta Medica*, **74**, 188–193.

Kobayashi D., Kondo K., Uehara N., Otokozawa S., Tsuji N., Yagihashi A., Watanabe N. (2002) Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. *Antimicrobial Agents and Chemotherapy*, **46**, 3113–3117.

Kudakasseril G.J., Lam L., Staba E.J. (1987) Effect of sterol inhibitors on the incorporation of ^{14}C-isopentenyl pyrophosphate into artemisinin by a cell-free system from *Artemisia annua* tissue cultures and plants. *Planta Medica*, **53**, 280–284.

Liu S., Tian N., Li J., Huang J., Liu Z.. (2009) Isolation and identification of novel genes involved in artemisinin production from flowers of *Artemisia annua* using suppression subtractive hybridization and metabolite analysis. *Planta Medica*, doi: 10.1055/s-0029-1185809.

Livak K.J., Schmittgen T. (2001) Analysis of relative gene expression data using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. *Methods*, **25**, 402–408.

Mercke P., Bengtsson M., Bouwmeester H.J., Posthumus M.A., Brodelius P.E. (2000) Molecular cloning, expression, and characterization of amorpha-4,11-diene synthase, a key enzyme of artemisinin biosynthesis in *Artemisia annua* L. *Archives of Biochemistry and Biophysics*, **381**, 173–180.

Mülbach H.P. (1988) Use of plant cell cultures in biotechnology. *Biotechnology Annual Review*, **4**, 113–176.

Murashige T., Skoog F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **51**, 473–497.

Paniego N.B., Giulietti A.M. (1994) *Artemisia annua* L.: dedifferentiated and differentiated cultures. *Plant Cell Tissue and Organ Culture*, **36**, 163–168.

Paniego N.B., Giulietti A.M. (1996) Artemisinin production by *Artemisia annua* L.-transformed organ cultures. *Enzyme and Microbial Technology*, **18**, 526–530.

Putalun W., Luealon W., De-Eknamkul W., Tanaka H., Shoyama Y. (2007) Improvement of artemisinin production by chitosan in hairy root cultures of *Artemisia annua* L. *Biotechnology Letters*, **29**, 1143–1146.

Pauwels L., Morreel K., De Witte E., Lammertyn F., Van Montagu M., Boerjan W., Inzé D., Goossens A. (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured *Arabidopsis* cells. *Proceedings of the National Academy of the Sciences USA*, **105**, 1380–1385.

Pu Gb., Ma D.M., Chen J.L., Ma L.Q., Wang H., Li G.F., Ye H.C., Liu B.Y. (2009) Salicylic acid activates artemisinin biosynthesis in *Artemsia annua* L. *Plant Cell Reports*, **28**, 1127–1135.

Ro D.K., Paradise E.M., Ouellet M., Fisher K.J., Newman K.L., Ndungu J.M., Ho K.A., Eachus R.A., Ham T.S., Kirby J., Chang M.C., Withers S.T., Shiba Y., Sarpong R., Keasling J.D. (2006) Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature*, **440**, 940–9433.

Smith T.C., Wheathers P.J., Cheetham R.D. (1997) Effects of giberellic acid on hairy root cultures of *Artemisia annua*: growth and artemisinin production. *In Vitro* Cellular and Developmental Biology, **33**, 75–79.

Souret F.F., Kim Y., Wyslouzil B.E., Wobbe K.K., Weathers P.J. (2003) Scale-up of *Artemisia annua* L. hairy root cultures produces complex patterns of terpenoid gene expression. *Biotechnology and Bioengineering*, **83**, 653–667.

Teoh K.H., Polichuk D.R., Reed D.W., Nowak G., Covello P.S. (2006) *Artemisia annua* L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. *FEBS Letters*, **580**, 1411–1416.

Towler M.J., Weathers P.J. (2007) Evidence of artemisinin production from IPP stemming from both the mevalonate and the nonmevalonate pathways. *Plant Cell Reports*, **26**, 2129–2136.

Towler M.J., Wyslouzil B.E., Weathers P.J. (2007) Using an aerosol deposition model to increase hairy root growth in a mist reactor. *Biotechnology and Bioengineering*, **96**, 881–891.

Tsuruta H., Paddon J.C., Eng D., Lenihan J.R., Horning T., Anthony L.C., Regentin R., Keasling J.D., Renninger N.S., Newman J. (2009) High-level production of amorpha-4,11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. *PLoS ONE*, **4**(2): e4489, doi:10.1371/journal.pone.0004489.

Wallaart E.T., Bouwmeester H.J., Hille J., Poppinga L., Maijers N.C. (2001) Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. *Planta*, **212**, 460–465.

Wildholm J.M. (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. *Stain Technology*, **47**, 189–194.

Zarn J.A., Brüschweiler B.J., Schlatter J.R. (2003) Azole fungicides affect mammalian steroidogenesis by inhibiting sterol 14-α-demethylase and aromatase. *Environmental Health Perspectives*, **111**, 255–261.

Zeng Q., Qiu F., Yuan L. (2008a) Production of artemisinin by genetically modified microbes. *Biotechnology Letters*, **30**, 581–592.

Zeng Q., Zhao C., Yin L., Yang R., Zeng X., Huang Y., Feng L., Yang X. (2008b) Cloning of artemisinin biosynthetic cDNAs and novel ESTs and quantification of low temperature-induced gene overexpression. *Science in China Series C Life Sciences*, **51**, 232–244.

Zhang L., Xing D. (2008) Methyl jasmonate induces production of reactive oxygen species and alteration in mitochondrial dynamics that precede photosynthetic dysfunction and subsequent cell death. *Plant and Cell Physiology*, **49**, 1092–1111.

Zhang Y., Teoh K.H., Reed D.W., Maes L., Goossens A., Olson D.J., Ross A.R., Covello P.S. (2008) The molecular cloning of artemisinic aldehyde Delta11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in *Artemisia annua*. *Journal of Biological Chemistry*, **283**, 21501–21508.