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Full Length Research Paper

Effect of (2-chloro-4-pyridyl)-N-phenylurea (CPPU) on artemisinin production in hairy root culture of *Artemisia annua* L

Elias Gebremariam Oba[1]*, Phithak Inthima[2] and Kanyaratt Supaibulwatana[2]

[1]Jimma Agricultural Research Center, P. O. Box 192, Jimma, Ethiopia.
[2]Plant Cell Biotechnology Unit, Department of Biotechnology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.

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Enhancement of artemisinin content in hairy root culture was investigated by using plant bioregulator in order to provide other alternative sources of artemisinin production besides the field grown plants. Synthetic cytokinin at different concentration: 1 mg L^{-1} (4 µM), 2.5 mg L^{-1} (10 µM) and 5 mg L^{-1} (20 µM) in the form of (2-chloro-4-pyridyl)-N-phenylurea (CPPU) was applied to culture media after 10 days of subculture. Artemisinin content was measured at different exposure periods (0, 24, 48, and 72 h). Our result shows that the highest artemisinin content was observed from hairy root that was treated with 5 mg L^{-1} (20 µM) of CPPU for 72 h which were about 3 folds higher than that of non-treated culture.

Key words: *Artemisia annua* L., hairy root, artemisinin, bioregulator.

INTRODUCTION

Artemisinin is a sesquiterpene lactone endoperoxide derived from shoot part of Chinese medicinal plant called *Artemisia annua* (sweet or wormwood) (Klayman, 1985). In addition to its antimalaria activity, artemisinin now has been known for its various medicinal properties such as antiviral and anti-cancer activities (Efferth et al., 2001; Romero et al., 2005). Currently, the global health community has ambitious proposal to eliminate deaths caused by malaria through Global Malaria Action plan by 2015 and Artemisinin combination therapies (ACT) is the major component of this proposal (Elspeth, 2010). Moreover, at this time, artemisnin is considered as the only available drug to fight multi-drug resistant *Plasmodium* spp (Enserink, 2010). Therefore, urgent increase in artemisinin production quality as well as quantity is a priority. Nevertheless, one of the major challenges facing artemisinin production nowadays is its low yield (~0.01 to 0.8% dry weight) (Abdin et al., 2003). On the other hand, it is predicted that the demand for artemisinin will increase (double of 2008 level by 2012) for the next decade (Elspeth, 2010). Therefore, increasing artemisinin production is an urgent task that must be undertaken in order to guarantee supply for increasing demand.

To date, tremendous effort has been exerted in different direction to increase artemisinin production with different level of success. For instance, total synthesis of artemisinin is difficult, costly and environmentally damaging (Vorman et al., 1999). The second option is using engineered *Saccharomyces cerevisiae* to produce artemisinin precursor followed by purification and semi-synthetic production. This method was relatively successful by producing high titres of artemisinic acid (up to 100 mg L^{-1}), but no industrial scale-up method is available (Ro et al., 2006). Recently, in *Escherichia coli* system, high levels of amorpha-4, 11-diene, an artemisinin precursor, were also obtained (Tsuruta et al., 2009). On the other hand, field grown *A. annua* is now the major source of artemisinin. Recently, Graham et al. (2010) published the genetic map of *A. annua* loci that directly related to artemisinin yield. The work of Graham and co-workers under fast-track breeding program is a promising source of artemisnin from high yielding *A. annua* verities but field grown *A. annua* is affected by environmental factors which

*Corresponding author. E-mail: elini2050@gmail.com.

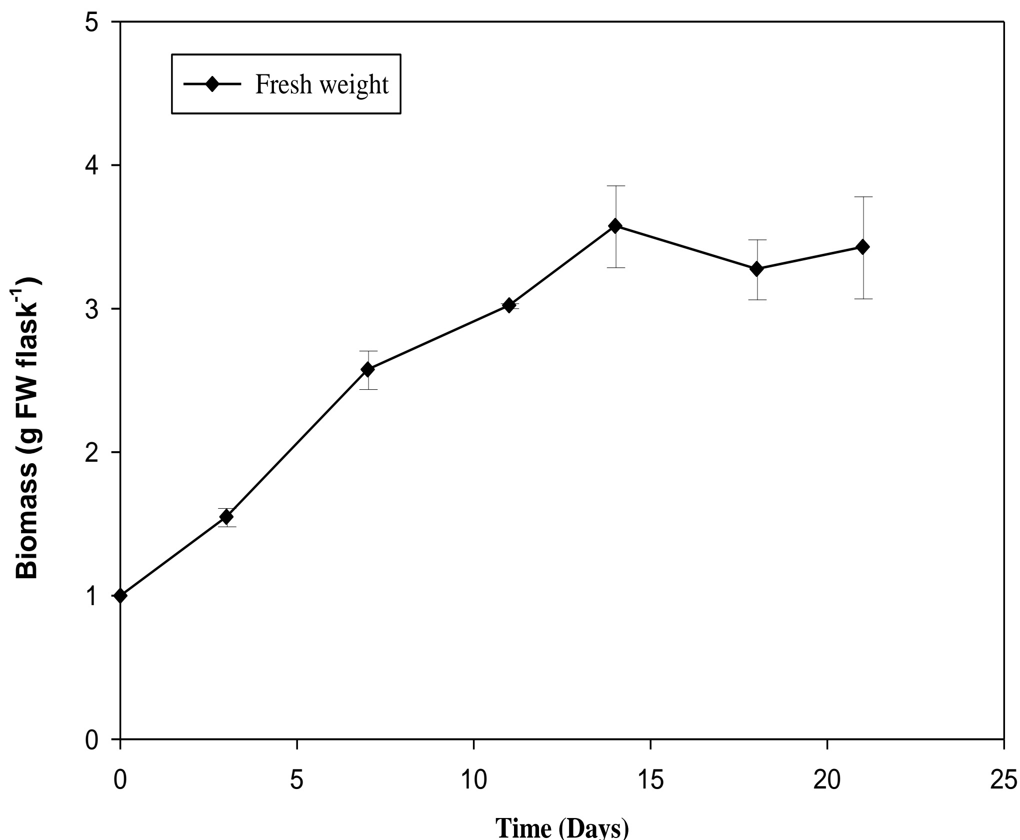

Figure 1. The growth curve of *A. annua* hairy root culture. Values are means ± SE.

directly influence yield. The final option is the use of *in vitro* cultures, which is considered as alternative source of high-valued secondary metabolite than whole plant. This is in part because production is independent of geographical, seasonal and environmental factors and ensures continuous supply of products (Rao and Ravishankar, 2002).

Among *in vitro* culture methods, hairy root provide better advantage over cell suspension such as high growth rate, genetic stability and growth in hormone free media (Guillon et al., 2006) and can be used as an experimental system to study secondary metabolism (Bais et al., 2001). For this reason, in this study, hairy root culture was selected to study the effect of CPPU (N-phenyl-N′-(2-chloro-4-pyridyl) urea) on artemisinin production of *A. annua*.

Bioregulators regulate growth and secondary metabolites accumulation in medicinal plants (Sangwan et al., 2001). In this regard, several studies reported extensively the effect of plant growth regulators on artemisinin level of *A. annua*. For example, high accumulation of artemisinin was observed in intact plant, shoot culture, hairy root and suspension culture of *A. annua* after treating with Gibberellic acid (GA_3) (Woerdenbag et al., 1993; Liu et al., 1997) and abscisic acid (Jing et al., 2009). Likewise, signaling molecules such as jasmonic acid, methyl jasmonate and salicylic acid are known to induce defense gene expression and elicit artemisinin in *A. annua* plant and suspension culture (Guo et al., 2010; Wang et al., 2010). Despite their diversity in structure and function, not all bioregulators were screened for their effect to induce artemisinin. The focus of this study was to study the effect of CPPU derivatives synthetic compound on artemisinin content in the hairy root culture of *A. annua*.

MATERIALS AND METHODS

Transformed hairy roots of *A. annua* L. (clone 241) were obtained from previous studies of hairy root stock cultured on plate (Plant cell lab, Faculty of Science, Mahidol University). Approximately, 1 g of hairy root was transferred to 30 ml hormone-free MS (Murashige and Skoog, 1962) liquid medium containing 3% (w/v) sucrose in 125 ml Erlenmeyer flasks and grown at 25°C on a rotary shaker (120 rpm) under 16 h light per day, and were subsequently subcultured every 14 days to activate hairy root. To investigate the pattern of hairy root growth, 1 g of hairy root was subcultured into 125 ml flasks containing 30 ml MS liquid medium, and hairy roots were then harvested to determine the fresh weight. Filter sterilized (0.2 µm Acrodisc® syringe filters, Supor membrane®) CPPU was added to 10-day-old transgenic hairy root cultures (Figure 1) to a final concentration of 1 mg L^{-1} (4 µM), 2.5 mg L^{-1} (10 µM) and 5 mg L^{-1} (20 µM), whereas the control cultures did not receive CPPU. Artemisinin content and fresh weight was measured at 0, 24, 48,

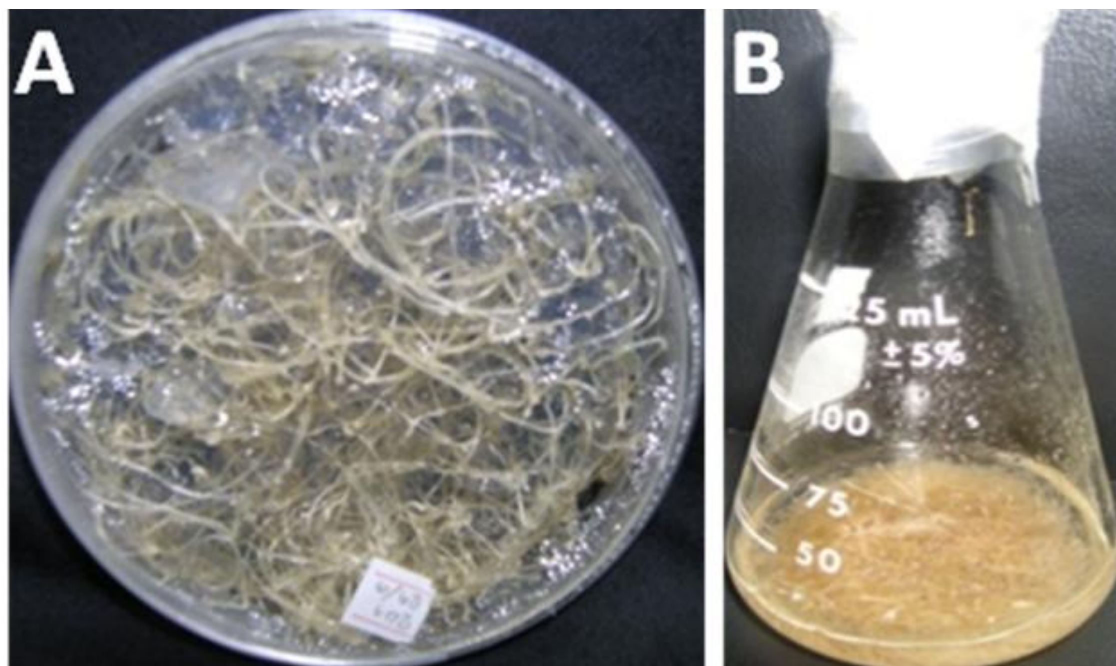

Figure 2. Appearance of hairy root culture of *A. annua* clone 241. (A) Transformed hairy root grown in 0.7% (w/v) agar-solidified MS medium, and (B) well established hairy root culture proliferated in liquid medium after routinely subcultured every 14 days for six weeks.

and 72 h after treatment. All experimental cultures were conducted in triplicate and collected samples were stored at - 80°C after briefly immersing in liquid nitrogen. Artemisinin was extracted and quantified high performance liquid chromatography (HPLC (Waters2695 separation module, Alliance®) by the modified method of Van Nieuwerburgh et al. (2006) and Vandenberghe et al. (1995) respectively. 1 g of hairy root was ground in liquid nitrogen before extracted with 10 ml dichlorome-thane and vortex for 1 min. The supernatant was filtered and allowed to evaporate in fume hood at room temperature until drying. Crude extract was dissolved in 1 ml of methanol and vortex briefly before the samples were converted to the Q260 derivative. Then, 200 µl of crude extract were resuspended in 800 µl of 0.2% (w/v) sodium hydroxide solution. After brief vortex, the mixture was incubated in water bath at 50°C for 30 min. After cooling at room temperature, 200 µl of methanol and 800 µl of 0.05 M acetic acid were added and mixed with vortexing. The mixture was filtered through 0.45 µm Sartorious® membrane and run on Luna C18 reverse phase column (5 µm, 4.6 × 150 mm) C18 guard column (Phenomenex, USA). Isocratic flow was used for separation with 55% (v/v) acetonitrile and 45% (0.1% (v/v) formic acid) at a rate of 1 ml/min and detected at 254 nm by UV-dual λ-absorbance detector (Waters 2487). Finally, artemisinin was quantified against external standard curve with R2 ≥ 0.996 using standard artemisinin (Khunming Pharmaceutical Corporation, China). For statistical com-parisons of the fresh weight and artemisinin content, triplicate flasks were used in all experiments and all data were the mean ± SE.

RESULTS AND DISCUSSION

The growth kinetics of *A. annua* hairy roots is shown in Figure 1. The hairy roots cultured on liquid MS medium supplemented with 30 g L^{-1} sucrose showed rapid growth at up to 10 days, during a 21 day period of the study, and the maximum fresh weight of 3.55 gm $flask^{-1}$ was obtained at day 14 (Figure 2). Based on this result, we determined that application of plant growth regulator at 10 day age of hairy root is appropriate. On the other hand, artemisinin content in hairy roots of *A. annua* generally increased significantly ($p \leq 0.05$) with increase in concentration of CPPU from 4.04 to 20.2 µM (Figure 3). Maximum accumulation (0.0827 ± 0.013 mg g^{-1} FW) of artemisinin occurred after treatment with 20.2 µM CPPU for 72 h. This was three-fold higher than for the non-treated (0.0235 ± 0.0032 mg g^{-1} FW). Hairy root treated with 20.2 µM CPPU for 24 h had relatively higher artemisinin content (0.0684 ± 0.01 mg g^{-1} FW) as compared to untreated one (0.0168 ± 0.0046 mg g^{-1} FW). Likewise, hairy root culture treated with similar concentration of CPPU for 48 h also reveal high artemisinin content (0.0802 ± 0.00736 mg g^{-1} FW). However, there is no statistically significant difference between 48 and 72 h of 20.2 µM CPPU treatment. Control hairy root culture did not show any significant variation in accumulation of artemisinin content at any given time point, moreover, it was observed that hairy root exposed to CPPU for 72 h resulted in culture browning (Figure 4). This is in agreement with previous results in a sense that exogenous cytokinin cause root disorganization in *Cichorium intybus* L (Bais et al., 2001).

Likewise, other synthetic cytokinin such as N^6-benzylaminopurine (BA) and 6-(γ, γ- dimethylallylamino) purine (2iP) inhibited hairy root growth in *A. annua*

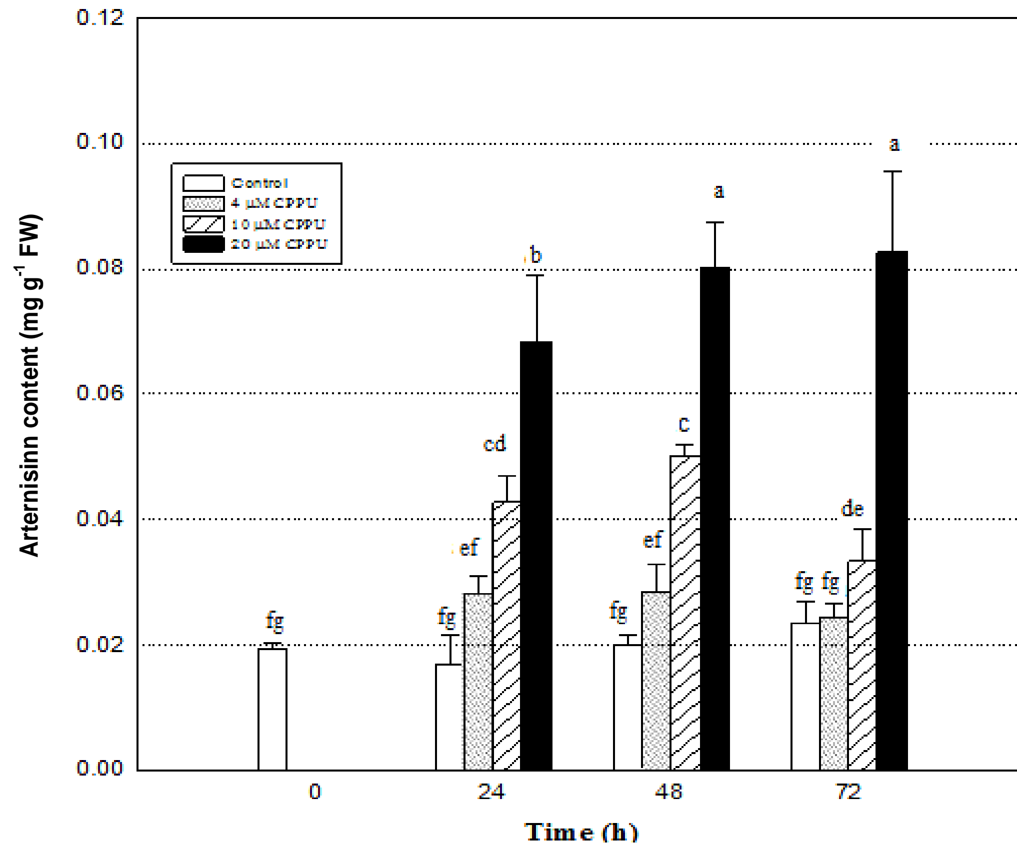

Figure 3. Effect of CPPU on artemisinin content in hairy root culture of *A. annua*. Values represent means of triplicate results and error bars show SD (n = 3). Letters indicate statistical significance at $p \leq 0.01$.

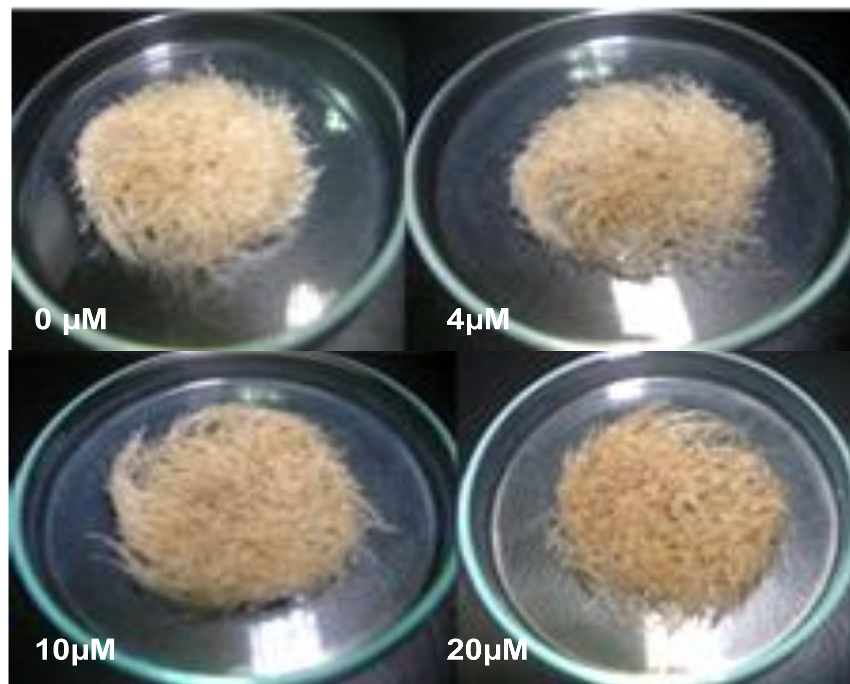

Figure 4. Effect of CPPU on hairy root cultures of *A. annua* after 72 h of treatment.

(Weathers et al., 2005)

Previous research report showed that secondary metabolite started to accumulate during stationary phase when nutrients depleted as culture turned aged (Lindsey et al., 1983; Smith et al., 1997). Therefore, most authors suggested that exponential or late exponential phase is suitable for elicitation experiment (Vasconsuelo et al., 2003). Based on this suggestion, the hairy root of *A. annua* was treated with syenthetic cytokinin (CPPU) during exponential phase (10 days after subculture). Here, it has been demonstrated that CPPU or N-phenyl-N′-(2-chloro-4-pyridyl) urea, synthetic urea derivative with cytokinin like activity, enhanced artemisinin content in hairy root culture on concentration dependent manner. Our result confirmed the potential of CPPU as chemical elicitor to induce artemisinin content in *A. annua.* This increasing artemisinin content could be explained based on the following two research reports. The first one published by Laloue and Fox (1989) revealed that CPPU was a potent inhibitor of cytokinin oxidase, an enzyme which is involved in degradation of endogenous cytokinin. Interestingly, the second report by Geng et al. (2001) showed that overexpression of *ipt*-gene which was key gene in cytokinin biosynthesis could enhance endogenous cytokinin to 3-fold and increased artemisinin content by 70% as compared to control. Therefore, the increase in artemisinin level in hairy root culture exposed to CPPU treatment might be due to accumulation of endogenous cytokinin as a consequence of inhibition of cytokinin oxidase. On the other hand, CPPU by itself could switch on signal transduction pathway by directly activating AHK receptor and this might lead to accumulation of artemisinin.

Conclusion

Addition of CPPU at late exponential phase of growth stage of hairy root culture of *A. annua* clone 241 increased accumulation of artemisinin. Our data suggests that harvesting hairy root at 48 h after treatment with 5 mg L^{-1} (20 µM) CPPU will give the highest result.

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