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Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*

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Abstract

Artemisinin, isolated from the shrub-*Artemisia annua*, is a sesquiterpene lactone used to treat multi-drug resistant strains of falciparum malaria. It is also effective against a wide variety of cancers such as leukemia and colon cancer. To counter the present low content in leaves and uneconomical chemical synthesis, alternate ways to produce artemisinin have been sought. But this compound remains elusive in cell cultures of *A. annua* despite the extensive studies undertaken. This work reports the first successful approach for production of artemisinin by cell cultures of Indian variety of *A. annua*.

In the present study, an integrated yield enhancement strategy, developed by addition of selected precursor (mevalonic acid lactone) and elicitor (methyl jasmonate) at optimized concentrations, resulted in 15.2 g/l biomass and 110.2 mg/l artemisinin, which was 5.93 times higher in productivity in comparison to control cultures.

Keywords: Artemisinin; *Artemisia annua*; Yield enhancement; Methyl jasmonate; Mevalonic acid lactone

1. Introduction

Malaria is a serious endemic disease in many parts of the world, affecting 5% of the worlds population (White and Pukrittayakamee, 1993). Approximately 2100 million people are at risk of developing the disease annually, and some 270 million are infected each year (WHO, 1997). *Artemisia annua* L., a Chinese medicinal herb, has evoked wide interest for its artemisinin content, a sesquiterpene lactone, which is effective against both chloroquine-resistant and sensitive strains of *P. falciparum* as well as cerebral malaria with high safety profile. Significant biological activity and novel chemical structure have prompted efforts in developing a series of derivatives which are more potent than the parent compound, such as artesunate, artemether, arteether (Titulaer et al., 1991) and sodium salt of artelinic acid (Lin et al., 1987). Highest artemisinin content has been reported in leaves (0.01–0.5%) in Chinese varieties of plant (Nair et al., 1986). It was introduced in India, cultivated and naturalized in Kashmir and Lucknow (Singh et al., 1988) but showed low artemisinin content. Synthesis of artemisinin has not proved to be commercially feasible (Avery et al., 1992) and low yield from natural source (E1Sohly et al., 1990) along with disappointing results from experiments with undifferentiated cultures of *A. annua* with respect to the artemisinin production (He et al., 1983; Kudakasseril et al., 1987; Jha et al., 1988; Martinez and Staba, 1988; Tawfiq et al., 1989) have triggered search for high yielding biotechnological approaches for improved artemisinin production.

Feeding of precursors and elicitation has proved to be an effective way to enhance secondary metabolites in plant cell cultures. A number of precursors such as sodium acetate (Wu et al., 1999; Yuan et al., 2001; Zeng et al., 2003), mevalonic acid lactone, casein acid hydrolysate (Woerdenbag et al., 1993) and elicitors like jasmonates

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(Walker et al., 2002), salicylates (O'Donnell et al., 1996), chitosan and gibberlic acid (Woerdenbag et al., 1993) have been investigated to enhance yield of plant based secondary metabolites. Effect of single precursor/elicitor on a particular cell line was investigated in most of these studies. But considering the complexicity of artemisinin biosynthesis, it is reasonable to examine the combined effect of these yield enhancement strategies. For this reason, the present study was undertaken on cell suspension cultures of *A. annua*. The aim of this work was to develop a strategy for synergistic improvement of artemisinin production in cell cultures. This appears to be the very first successful tissue culture study on Indian variety of *A. annua*.

2. Methods

2.1. Callus cultures

Seeds of Indian variety of *A. annua*, obtained from Prof. R. Jafar (Jamia Milia, New Delhi), were sterilized by treating first with 90% alcohol for 3 s, and followed by 0.1% (w/v) mercuric chloride for 5 m. Seeds were then rinsed thoroughly three times in sterile distilled water and 10 seeds per Petri plate were allowed to germinate on padding of sterile filter paper and absorbent cotton, moistened with 15 ml of Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) supplemented with 5% (w/v) sucrose. Seeds were then incubated at 25 ± 2°C under 2200 lux intensity of light and 16/8 h light/dark photoperiod in a growth chamber. Germination started within 2–3 d. High yielding callus culture of *A. annua* from aseptically germinated seedlings was developed and maintained on MS medium supplemented with NAA and Kn (0.5 mg/l of each) as growth regulators (Unpublished data).

2.2. Suspension cultures

To establish the suspension cultures in 250 ml Erlenmeyer flasks, 2 g/l cells (dry cell weight basis) were transferred to 50 ml of liquid MS media with 3% (w/v) sucrose and 0.1 mg/l of each of NAA and Kn. The flasks were incubated on a rotary shaker (125 rpm) at 27 ± 2 °C with a photoperiod of 16/8 h light/dark cycle. Cells from 21 d old suspension culture were used for further experiments.

2.3. Kinetics of biomass and artemisinin accumulation

Suspension cultures were initiated by transferring friable fraction of callus equivalent to 2 g/l dry cell weight into 250 ml Erlenmeyer flask containing 50 ml of liquid Murashige and Skoog (MS) medium. The plant hormone and sucrose concentration were the same as in the inoculum preparation media. Cultures were incubated on a gyratory shaker at 125 rpm at 27 ± 2 °C under 16/8 h light/dark regime. To establish growth and production kinetics, individual flask was harvested in duplicate at an interval of 5 d and analyzed for dry cell weight (DW), residual substrate concentrations (sucrose, nitrate and phosphate) and artemisinin content.

2.4. Analysis

For DW estimation, cells were harvested and collected by centrifugation at 3000 rpm for 15 m and washed with distilled water. Cells were allowed to dry at 25 ± 2 °C until constant weight was achieved. The culture supernatant was analysed for residual sucrose (Dubois et al., 1956), nitrate (Cataldo et al., 1975) and phosphate (Murphy and Riley, 1962) by colorimetric methods. All experiments were done in duplicate to check the reproducibility of the results. Sodium derivative of artemisinin was analyzed by HPLC with some modification in the method of Zhao and Zeng (1985). For analysis of artemisinin, 100 mg of dried and powdered cells were allowed to macerate with 2.0 ml diethyl ether for 48 h. The filtrate was then dried under vacuum and the residue was dissolved in 1.0 ml of methanol. This was then treated with 4.0 ml NaOH (0.2%, w/v) and incubated at 50 ± 2 °C with occasional shaking to get a sodium derivative of artemisinin.

2.5. Improvement of artemisinin production

2.5.1. Addition of precursors

To improve artemisinin production in suspension cultures, some known precursors of artemisinin biosynthesis [sodium acetate (SA) and mevalonic acid lactone (MAL), casein acid hydrolysate (CAH)] and cholesterol (CH) were added. Stocks of sodium acetate and casein acid hydrolysate were prepared in double distilled water and the pH was adjusted to 5.7. Cholesterol and mevalonic acid lactone were dissolved in acetone and 95% ethanol respectively. Filter sterilized precursors were added to the culture medium on 15th d at following concentrations: sodium acetate, 10, 25 and 50 mg/l; mevalonic acid lactone, 10, 20 and 50 mg/l; cholesterol, 10, 50 and 100 mg/l; casein acid hydrolysate, 0.1, 0.5 and 1.0 g/l. Cultures were harvested on 30th d, in duplicate, and analyzed for biomass and artemisinin content. In control cultures, volume of sterile distilled water, equal to volume of solvent for precursor, was added aseptically to culture medium.

2.5.2. Addition of elicitors

Some known abiotic elicitors for plant based secondary metabolites such as signaling molecules [methyl jasmonate (MJ), acetyl salicylic acid (ASA)], metal ion [calcium as calcium chloride (CC)] and blooming agent [gibberlic acid (GA)] were also tested for their effect on AN content. Stock solutions of acetyl salicylic acid, and calcium chloride were prepared by dissolving them in double distilled water and adjusting the pH to 5.7. Methyl jasmonate was dissolved in 95% ethanol. Abiotic elicitors were aseptically added to cultivation medium at the following concentrations: methyl jasmonate and acetyl salicylic acid, 10, 20, 30 and