

Artemisinin Production from Different Shoot Culture Systems of *Artemisia annua* L.

^{1,3}Mahmoud Sharaf-Eldin and ^{2,3}Shereen Elkholy

¹Medicinal and Aromatic Plants Dept., National Research Centre (NRC), Cairo, Egypt

²Plant Transformation and Biopharmaceuticals Lab, Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Centre (ARC), Giza, Egypt

³Department of Biology and Biotechnology, Worcester Polytechnic Institute (WPI), Worcester, MA, USA

Abstract: Shoot cultures of *Artemisia annua* L. were cultivated in three different micropropagation systems: an ultrasonic nutrient mist bioreactor (UNMB), a modified ultrasonic nutrient mist bioreactor (MUNMB) and solid culture in Magenta boxes. The shoots cultivated in the UNMB and MUNMB showed excellent growth. The dry weight increase (35 times) of shoot cultures in the MUNMB was higher than those (25 times and 19 times) in both the UNMB and the Magenta boxes. Additionally, artemisinin content of shoot cultures in the MUNMB was 1.2- and 2.0-fold higher than those in both the UNMB and the Magenta boxes, respectively. The modified ultrasonic nutrient mist bioreactor was found to be advantageous for *A. annua* L. shoot cultures and artemisinin production.

Key words: Bioreactor, *Artemisia annua* L., shoot culture, growth, artemisinin biosynthesis

INTRODUCTION

Artemisia annua L., belongs to the family Compositae, is known for the drug artemisinin; an effective antimalarial drug against chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum* and against cerebral malaria. Likewise, its effectiveness has been demonstrated in the treatment of skin diseases and it is also a natural herbicide. Presently, the only commercial source of the drug is extracted from field-grown leaves and flowering tops of *Artemisia annua* L., which are subject to seasonal and somatic variation (Klayman, 1985 & Paniego and Giulietti, 1994). Malaria is a serious disease that affects more than 275 million people worldwide and is the cause of at least 1 million deaths every year (Butler, 1997).

Studies on the biochemistry and medicinal efficacy of *Artemisia annua* requires an efficient large-scale system for production of plant tissues free from seasonal and somatic variations, infestations of bacteria, fungi, or insects and environmental pollution that can affect the pharmaceutical value of the harvested tissues (Murch *et al.*, 2000; Saxena, 2001). Bioreactor technologies are considered as a key factor for realization of mass propagation of important and sterile plants in the optimized environment conditions (Honda *et al.*, 2001; Liu *et al.*, 1998). Several experimental bioreactors for shoot cultures have been developed with the aim of reducing the production costs while maximizing the plant growth (Akita and Ohta, 1996; Liu *et al.*, 2000; Simonton *et al.*, 1991; Teisson *et al.*, 1996; Weathers and Giles, 1988; Ziv and Shemesh, 1996). However, the use of bioreactors for large-scale cultivation has been limited because of the high costs and abnormal shoot morphogenesis associated with liquid culture (Aitken-Christie *et al.*, 1995; Liu *et al.*, 1998; Ziv *et al.*, 1998). Most of the shoot cultures are sensitive to shear stress and may diminish after prolonged liquid culture (Liu *et al.*, 2001; Ziv, 1991). Therefore, here we report the effect of various micropropagation systems on the growth and yield of the main secondary metabolite of sweet wormwood; artemisinin. The long term goal of this work is to provide artemisinin producers with an effective production technology that is environmentally safe for producing higher quantity of artemisinin.

MATERIALS AND METHODS

Plant Materials and Culture Maintenance:

Sweet wormwood (*Artemisia annua* L.) seeds (YU strain) were surface sterilized in 10% (v/v) bleach for 10 minutes followed by 70% (v/v) ethanol for 5 minutes. Then seeds were washed with 10ml 0.1% (v/v) sterile PPM (Plant Preservative Mixture, Plant Cell Technology, Inc.), 3 times, 5 minutes each time in a 50ml sterile

Corresponding Author: Mahmoud Sharaf-Eldin, Medicinal and Aromatic Plants Dept., National Research Centre (NRC), 33 Elbehoth St., Dokki, Cairo-12622, Egypt; Tel.: +202/3337/1499
Fax: +202/3337/0931
E-mail: sharafeldin99@yahoo.com

centrifuge tube. The sterilized seeds were incubated in 25ml Gamborg's B5 medium (Gamborg *et al.*, 1968) with 3% (w/v) sucrose at pH5.7 with no light in the refrigerator for 3 days, then transferred to continuous cool-white fluorescent light ($100\mu\text{Em}^{-2}\text{s}^{-1}$) at $25\pm 2^\circ\text{C}$, and at 120rpm (Lab-line Orbit Shaker, Lab-Line Instruments Inc., Melrose Park IL). After 4 days, most seeds developed to the two-cotyledon stage. Four seedlings at the two-cotyledon stage were inoculated into a Magenta box containing 20ml autoclaved B5 medium with 0.23% (w/v) Phytigel to which 3% (w/v) sucrose at pH5.7 were added. After 4-6 weeks, most seedlings developed to the maximum height of the Magenta box (W x L x H; 77x77x97 mm), of which shoot cultures of *A. annua* were used in this study. Shoot cultures with an average shoot length of about 0.5 cm were selected as explants for all experiments.

Micropropagation Systems and Cultivation Condition:

Fig. 1A shows the configuration of the Ultrasonic Nutrient Mist Bioreactor (UNMB) that used in this study along with its three main parts; growth chamber (GC), mist generator (MG), and medium reservoir (R). The GC is made of an autoclavable material (polypropylene) with 3.5 L working volume (210 mm i.d. x 190 mm h) and a stainless mesh with 2 mm pore size topped with a filter paper was served as a plant platform (Fig. 1B), which replaced with clay stones in the Modified Ultrasonic Nutrient Mist Bioreactor (MUNMB) as shown in Fig. 1C.

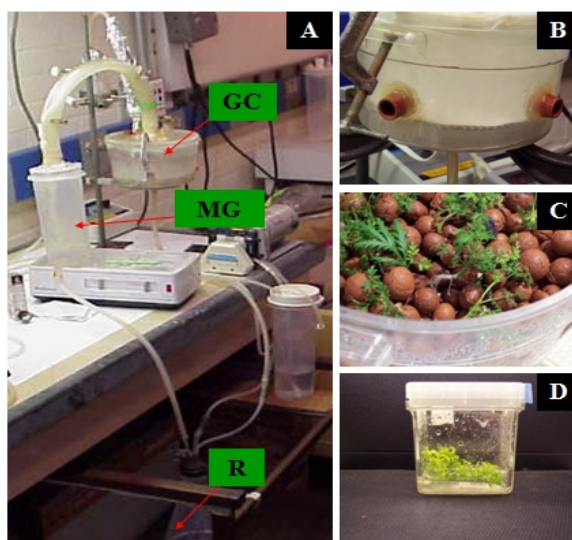


Fig. 1: A; configuration of the Ultrasonic Nutrient Mist Bioreactor (UNMB), growth chamber (GC), mist generator (MG), medium reservoir (R), B; plant platform in the UNMB, C; plant platform in the Modified Ultrasonic Nutrient Mist Bioreactor (MUNMB), D; *A. annua* plantlets grown into Magenta boxes

The mistifier with transducer and mist generator system was controlled by timer to produce nutrient mist periodically; 10 minutes on/hour (10 on/50 off). Air was supplied during the periods with mist through a disc filter. Before starting the cultivation, the bioreactors and the whole system were sterilized by autoclaving at 121°C for 45 min. These bioreactors were inoculated by placing shoot cultures (from Magenta boxes culture) homogeneously on top of each; stainless steel mesh which is covered by a filter paper, top of the clay stones and into Magenta boxes (Fig. 1D). The cultivation conditions in various micropropagation systems are shown in Table 1. Triplicate micropropagation systems were used in all experiments.

Extraction and Analysis of Artemisinin:

To determine the fresh weight; shoot cultures were gently blotted dry on filter papers to remove excess water and weighed, then they were dried in an oven at 60°C for 48 h and the dry weight was recorded. Artemisinin was determined as follows: the dried shoots of all seedlings harvested from the same reactor or Magenta box were pooled together, weighed, and extracted with toluene in a chilled water sonicator for 30 minutes. The supernatant was decanted and the sonication was repeated twice. The 3 supernatants from shoots harvested from the same reactor or Magenta box were pooled and dried under nitrogen at 30°C . Samples were stored in the freezer at -20°C until analysis by high performance liquid chromatography (HPLC). Artemisinin in each sample was analyzed as the Q260 method by HPLC according to Smith *et al.* (1997). For HPLC analysis the artemisinin samples were first converted to their Q260 derivatives Smith *et al.* (1997), and then

applied to a 15 cm Microsorb-MV C-18 column with a 4.6 mm i.d., containing 5 μm silica beads with 10 nm pore size (Varian, Walnut Creek, CA). The mobile phase was 0.01M sodium phosphate buffer: methanol, 55:45 (v/v) pH 7.0, at 1.0 mL/min. A linear calibration curve of artemisinin (Sigma-Aldrich, St. Louis, MO) was measured in the 0.1-0.5 $\mu\text{g}/\text{mL}$ range. The retention time of artemisinin under these conditions was about 12.0 min. Each sample was co-injected a second time with a known amount of authentic artemisinin to validate peak identification.

Table 1: Cultivation conditions of *A. annua* L. shoot cultures in various micropropagation systems

	UNMB	MUNMB	Magenta box
Inoculum weight (g fresh wt L ⁻¹)	5.0	5.0	5.0 (0.2 g M. box ⁻¹)
Light irradiation	cool-white fluorescent light (100 $\mu\text{Em}^{-2}\text{s}^{-1}$) at 25 \pm 2°C, 16 h day ⁻¹		
Medium volume (L)	1.0	1.0	1.0 (20 ml M. box ⁻¹)
Liquid flow rate (ml min ⁻¹)	2.0	2.0	
Misting cycle*	10/50	10/50	

UNMB: ultrasonic nutrient mist bioreactor, MUNMB: modified ultrasonic nutrient mist bioreactor, * minutes of misting ON/minutes of misting OFF

Statistical Analyses:

All the experiments consisted of three replicates, repeated twice, and collected data analyzed using the Student–Newman–Keulls means separation test of CoStat Version 3.03, a computerized interactive statistical program.

RESULTS AND DISCUSSION

The development characteristics of *Artemisia annua* L. shoots in various micropropagation systems (ultrasonic nutrient mist bioreactor; UNMB, modified ultrasonic nutrient mist bioreactor; MUNMB and Magenta box) showed obvious differences. The shoot cultures in both the UNMB and the MUNMB showed excellent growth, but those in the Magenta boxes resulted in deprived growth maybe to the poor ventilation and severe physical stress of the limited space. The biomass results of *A. annua* L. shoot cultures are shown in Fig. 2A & B.

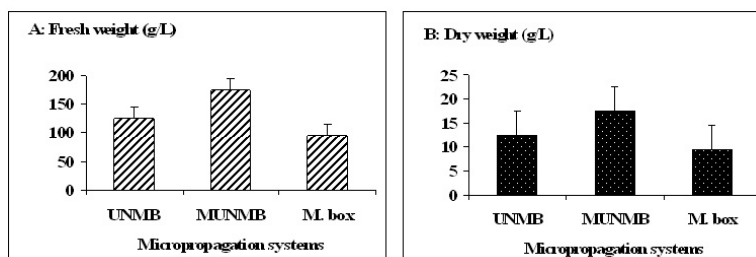


Fig. 2: *A. annua* fresh (A) and dry (B) weight in various micropropagation systems. Values are means of triplicate results and error bars represent standard deviations. UNMB; ultrasonic nutrient mist bioreactor, MUNMB; modified ultrasonic nutrient mist bioreactor and Magenta box

After 28-day batch culture, the dry weight escalating (35 times) of shoot cultures in the MUNMB was higher than those (25 times and 19 times) in both the UNMB and the Magenta boxes, respectively. Compared with that in the UNMB, where the liquid nutrient mist bioreactor was only supplied to the top of the shoot cultures inoculated on top of a filter paper, the growth of shoots was improved significantly in the MUNMB where the liquid nutrient was made into mist form and then delivered efficiently to each part of the shoot cultures cultivated on top of clay stones, which developed best rooting system because of the aeration that filter paper slightly prevented in the case of UNMB. Compared with that in the Magenta box, the effect of limited aeration was avoided because the shoot cultures were exposed directly to air exchange in both UNMB and MUNMB during misting cycle (10 min h⁻¹). The ratio of fresh weight to dry weight under the cultivation conditions using a filter paper as a platform in the UNMB was evidently higher than those in both the MUNMB and the Magenta box. The hyperhydrated shoots in the UNMB had the highest water content. Park and Hu (1989) reported that shoots of *A. annua* L. in a rectangular shape airlift bioreactor containing a single carbon and nitrogen source showed normal growth and root formation, but the fresh weight of *A. annua* shoot cultures increased eight times after 29 days under the completely submerged condition in their culture system. Fulzele *et al.* (1995) also reported the biomass of *A. annua* shoots increased 4- to 5-fold in 1-L capacity bioreactors under submerged conditions after 30 days. Comparing these studies with our findings, we concluded that the Magenta box is not suitable as a rapid micropropagation system for *A. annua* L. shoot culture. In

contrast, a gas-phase nutrient mist bioreactor, which we tested in this study (UNMB & MUNMB), provided an excellent environment conditions for shoot growth of *A. annua* by allowing adequate gas exchange and providing sufficient nutrients in a lower shear stress environment. In the three various micropropagation systems, the artemisinin contents and productions in *A. annua* L. shoot cultures are shown in Fig. 3.

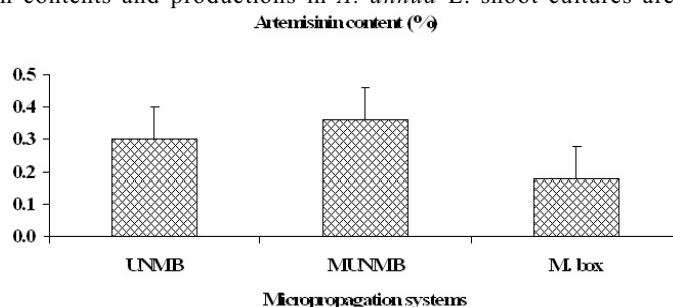


Fig. 3: artemisinin biosynthesis in various micropropagation systems. Values are means of triplicate results and error bars represent standard deviations. UNMB; ultrasonic nutrient mist bioreactor, MUNMB; modified ultrasonic nutrient mist bioreactor and Magenta box

In the mist bioreactor, which provided an excellent gas-phase environment and sufficient nutrient supply for both shoot growth and artemisinin biosynthesis, the artemisinin content of shoot cultures grown into the MUNMB was 1.2- and 2.0-fold higher than those in both the UNMB and the Magenta box, respectively. Artemisinin is a sesquiterpene lactone endoperoxide found mainly in the aerial parts of *A. annua* plants, and contains the therapeutically active endoperoxide bridge (Meshnick *et al.*, 1996). The biosynthesis of artemisinin requires a considerable involvement of oxygen (Wallaart *et al.*, 1999), and the higher content in both the UNMB and the MUNMB might be partly due to the greater availability of oxygen. Kim *et al.* (2001) reported the higher artemisinin content of *A. annua* L. hairy roots in mist reactors than in bubble column reactors because of oxygen availability. Our presented data with *A. annua* shoot cultures might further support that oxygen is a key factor for artemisinin biosynthesis in *A. annua* tissue cultures. In addition, lesser hyperhydration stress might improve artemisinin biosynthesis of the shoot cultures in the UNMB and in contrary; the advantage of using the clay stones is enhancing the development of better rooting system. In conclusion, we have demonstrated that the type of UNMB and/or MUNMB condition can significantly affect growth and artemisinin biosynthesis of *A. annua* shoot cultures. The obtained results are not only providing a potential alternative for artemisinin production, but also permit a prompt mass propagation of individual plant selection in a suitable bioreactor system.

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