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Ethyl methane sulfonate (EMS) enhanced the formation of leaf glandular trichomes and the production of artemisinin in *Artemisia annua* L.

E-Shuen Leow[1], Nadali B. Jelodar[2] and Lai-Keng Chan[3,*]

[1]Plant Tissue and Cell Culture Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia
[2]College of Agriculture, Sari Agricultural Sciences & Natural Resources University, Mazandaran, Iran
[3]Sunrich Biotech Sdn. Bhd., Jalan Sungai Daun, Nibong Tebal, Seberang Perai Selatan,Penang, Malaysia
[*]Corresponding author: merrilynchan@gmail.com

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Abstract

Artemisia annua L. can naturally synthesize artemisinin, an anti-malarial compound. Due to the naturally low content of artemisinin available in this plant, many different approaches were used to increase the production of artemisinin. The objective of our study was to determine whether ethyl methane sulfonate (EMS) treated seeds could enhance the formation of leaf glandular trichomes and increase the artemisinin content in the plants. The *in vitro* plantlets were established from EMS treated and untreated seeds on a basic Murashige and Skoog solid medium. The morphology of the leaf glandular trichomes was observed under a fluorescence microscope (Olympus FX53) at the blue wavelength (460-495 nm) while the artemisinin content was determined using High-performance liquid chromatography (HPLC). Results indicated that EMS did not affect the plantlet growth but caused temporary morphological abnormality in < 17% of the *in vitro* plantlets in the form of leaf albinism and chlorosis which disappeared after four subculture cycles. EMS of 0.5-1% (v/v) used for the seed treatment was able to induce the formation of more leaf glandular trichomes for all the three tested *A. annua* L. clones. While EMS ranging between 1.0% (v/v) and 2.0% (v/v) caused an increase in trichome size. A higher artemisinin content was detected in plantlets derived from seeds treated with 1.0% (v/v) EMS. Hence it was concluded that 1% (v/v) EMS could be used to induce the formation of more and bigger leaf trichomes that enhance the production of artemisinin in *A. annua* L.

Keywords: *Artemisia annua* L., Artemisinin, Ethyl methane sulfonate, Glandular trichome, *In vitro* plantlets

1. Introduction

Artemisia annua L. (Asteraceae) is commonly known as *A. annua* L. wormwood or Sweet Annie. It contains artemisinin, an important compound with anti-malarial properties. It is mostly used for the treatment of severe malaria caused by the life-threatening parasite *Plasmodium falciparum* in the form of artemisinin combination therapy (ACT). Due to the rise and spread of chloroquine-resistant *P. Vivax*, it was recommended that ACT should be used for both vivax and falciparum malaria in all co-endemic regions [1]. However, the production of artemisinin in *A. annua* L. is vulnerable to changes in environmental factors, geographical locations, soil nutrients and different genotypes [2-4]. It was reported that *A. annua* L. planted in tropical regions in Vietnam produced only small traces of artemisinin [2]. Thus, *A. annua* L. plants of low artemisinin content are common and would not be economical to cultivate in the tropical regions for the production of artemisinin.

Artemisinin is sequestered in the 10-celled biseriate glandular trichomes mainly on the adaxial and abaxial leaf surfaces and some on the corolla and receptacle of the florets of *A. annua* L. Artemisinin was not detected in the glandless trichome [5-7]. The leaves of *A. annua* L. which have more glandular trichomes, account for 89% of the total artemisinin in the plant [8]. Glandular trichomes are formed from the early development of leaves and inflorescences and the artemisinin content gradually increases and accumulates its maximum amount when the

glands reach the stage of physiological maturity [9]. The leaves of *A. annua* L. are harvested before the flowering stage as this is the time when the plants produce the highest amount of artemisinin. Hence it is reasonable to assume that the artemisinin content would be increased if the number of trichomes is increased and harvested before the flowering stage.

We therefore carried out an *in vitro* study with the aim of inducing the formation of more glandular trichomes on the leaves of *A. annua* L. of three different clones of Vietnam origin using ethyl methane sulfonate (EMS) and to evaluate whether this could influence the production of artemisinin. In addition, we also evaluated the effect of EMS on the external morphology of the *in vitro A. annua* L. plants derived from the EMS treated seeds.

2. Materials and methods

2.1 Establishment of EMS treated in vitro plantlets

The seeds of three selected *A. annua* L. clones (TC1, TC2 and Highland) were obtained from the Institute of Tropical Biology, Ho Chi Minh City, Vietnam. TC1 and TC2 are the high yielding clones while Highland is the wild type. The seeds were surface-sterilized by soaking the seeds in 70% ethanol for 10 min followed by 20% (v/v) Clorox®, a commercial bleach containing 5.3% sodium hypochlorite, for another 10 min. The seeds were then rinsed three times with sterile distilled water. The surface-sterilized seeds were blotted dry with sterile filter paper and treated with filter-sterilized EMS (Sigma M0880) of various concentrations (0.25%, 0.5%, 1.0%, 1.5% and 2.0% [v/v]) in a 0.1 M sodium phosphate buffer solution of neutral pH for two h, agitated on a rotatory shaker (Certomat® Rotory Shaker, B-Braun) at 100rpm and maintained at atemperature of 25 ºC. The seeds immersed for the same duration in 0.1 M sodium phosphate buffer of neutral pH without EMS solution were used as control. EMS treated and untreated seeds (control) were rinsed three times with sterile distilled water before inoculating onto solid basal Murashige and Skoog (MS) medium [10]. They were then incubated under a temperature of 25± 2 ºC and light intensity of 32.5 µmol m^{-2} s^{-1} until the seeds were germinated. The basic MS medium was supplemented with 30 g/l sucrose, 8/l agar (Algas, Chile) and the medium pH was adjusted to 5.7-5.8 before autoclaved (Tommy 325) at 121 ºC for 11 min under 1.05 kg/cm^2 pressure. All EMS-treated seeds and controls were subcultured four times at 8-weeks intervals for each subculture cycle before the morphology of the glandular trichome was recorded and the artemisinin content was determined for each clone. The percentage of seed germination and the height of the seedlings were also recorded after eight weeks of culture. The occurrence of any morphological changes of the plantlets derived from EMS treated seeds was observed and recorded for each clone at each subculture cycle.

2.2 Fluorescence microscopy study on morphology of leaf glandular trichome

A fresh leaf (third from shoot apex) of the control and EMS-induced plantlets was taken from each plant. Sixty samples were taken randomly for each clone. Each leaf piece was mounted directly on the standard glass slide for microscope viewing. A coverslip was placed over the leaf specimen to slow down the dehydration of the leaf while viewing was in progress. Glandular trichome were observed on both sides of the leaf surfaces with the aid of a fluorescence microscope (Olympus FX53) at the blue wavelength (460-495 nm). The number, length, width and surface area of the glandular trichome were recorded and evaluated using the Soft Image System (SIS) program.

2.3 Extraction of samples

The dried leaves of EMS treated and control of *A. annua* L. clones (TC1, TC2 and Highland) were separately pounded to become fine powder using a mortar and pestle. A 0.5±0.002 g of the powdered sample was placed into a test tube and soaked and ultra-sonicated in 3ml n-hexane (Fisher Scientific, UK, AR grade) at 40 ºC for 30 min. The test tube was swirled every 10 min during the 30 min of ultra-sonication to ensure thorough extraction. The supernatant was poured out and fresh 3ml n-hexane was added for the second extraction. The extraction process was repeated three times to obtain the hexane extract which was then evaporated to dryness in a fume chamber.

2.4 Determination of artemisinin content

The extract residue was dissolved with 1.0 ml acetonitrile and filtered via a 0.45 µm nylon micro-filter (Millipore Corporation, USA). The samples were analyzed using ultra high performance liquid chromatography (UHPLC-Agilent Technologies 1260 Infinity) according to the method described by Lapkin et al. [11]. The mobile phase, acetonitrile:water buffer (65:35), was used for the isocratic elution at a flow rate of 0.03 ml/min. The artemisinin was detected at 210 nm with a retention time at 22.2±0.5 min in a chromatogram. The injection

volume was 20 µl for each sample with three replications. An artemisinin standard (Sigma, USA) was used to prepare the calibration curve for the determination of the artemisinin contents in the tested samples. The standard curve was prepared from the artemisinin standard concentration ranging between 62.5-500 mg/L.

2.5 Statistical analysis

Comparison of various studied parameters between the three studied clones of *A. annua* L. were analyzed using one-way Analysis of variance (ANOVA) followed by mean comparison using the Tukey HSD test at $p \leq 0.05$. The percentage data were arch-sine transformed before running the statistical analysis. The comparison of each parameter of glandular trichome morphology between the adaxial and abaxial leaf surfaces for each clone was analyzed using the student t-test at $p \leq 0.05$. While comparison of the artemisinin content for the three clones was analyzed using One-Way ANOVA followed by mean comparison using the Tukey HSD test at $p \leq 0.05$. The statistical analysis was performed using SPSS ver 20.0 software for windows.

3. Results and discussion

3.1 The effects of EMS on seed germination, the growth and morphology of seed-derived plantlets

Ethyl methane sulfonate (EMS) is one of the most popular mutagens being used in creating variability in plants. New varieties of desired traits have been successfully produced in crops, such as barley [12], cucumber [13], cowpea [14], rice [15], wheat [16], sunflower [17] and *Vicia faba* L. [18]. In this study, EMS was found to have different effects on seed germination of the three different clones of *A. annua* L. It has an inconsistent effect on the seed germination of TC1 clone of *A. annua* L. The seed germination percentage was lowest in seeds treated with 0.25% (v/v) EMS but gradually increased as the EMS concentration increased. This phenomenon was contradictory with the normal trend of mutagen effects in which the percentage of seed germination generally reduced when the amount of mutagen used for treatment gradually increased. The trend of seed germination in TC2 seeds treated with EMS was similar but not as apparent to that which occurred in TC1 seed germination. However, the seed germination percentage for Highland clones did follow the normal trend in which the seed germination percentage gradually reduced as the EMS concentration used for treatment increased. The percentage of seed germination was generally higher for the Highland clone as compared to TC1 and TC2 clones (Table 1). The differences in seed germination could be due to the delayed and inhibition of physiological and biological pathways essential for seed germination such as enzyme activity, imbalance of hormonal changes and inhibition of the mitotic process which resulted in a lessening of seed germination [18]. The percentage of seed germination of chick peas was also reported to reduce with increasing doses/concentration of gamma rays and EMS used for treatment [19]. They reported that the reduction of seed germination in chick peas was due to biological damage caused by the mutagens and exerted an inhibitory effect on seed germination.

The effect of EMS on the leaf size was varied and fluctuated between varying EMS concentrations for TC1 and TC2 clones but the leaves of the plantlets derived from EMS treated seeds of the Highland clone was significantly smaller as compared to the control. All of the three clones produced not significantly different leaf size at all EMS concentration levels except the control whereby the Highland clone has a bigger leaf size as compared to TC1 and TC2 clones (Table 1). The changes in leaf shape and size due to various mutagens have been reported by Khursheed and Khan [18] in *Vicia faba* bean. They reported that the altered metabolism due to cellular damage caused by ethyl methane sulphonate (EMS) and gamma rays may be one of the reasons for leaf abnormalities. While Kashid and Salve [20] proposed that the changes in leaf morphology induced by mutagens could be due to changes in physiological and metabolic activities of the developing leaf primordia and the consequent alterations in leaf morphology. Both of the phenomena could have happened to the three tested clones of *A. annua* L. as well.

The height of the eight weeks old plantlets of each tested clone of *A. annua* L. was found to be not affected by the different concentration of EMS used for the seed treatment. However, when comparing among the three clones, the TC1 clone showed ashorter height as compared to the TC2 and Highlands clones (Table 1). The non-effect of EMS on the seedling height of individual clone of *A. annua* L. was contrary to most studies reported in which the seedling height decreased in proportion with the increased concentration of EMS used for treatment such as *Oryza sativa* L. [21], *Withania somnifera* L. Dunal [22] and cowpea [14]. Talebi et al. [21] reported that the effect of EMS on reduction of seedling height was due to the cell cycle arrest at the G2/M phase during somatic cell division or various kinds of damage in the entire genome.

After eight weeks of seed culture, no physical abnormality was observed in plantlets of various clones of *A. annua* L. derived from the untreated seeds or seeds treated with low concentrations of EMS (0.25% and 0.5%). However, 2.4% of the Highlands clone plantlets derived from seeds treated with 0.5% EMS showed abnormalities. The occurrence of morphological changes of the EMS treated plantlets was found to be EMS dose dependence for all the three clones (TC1, TC2 and Highland) of *A. annua* L. The morphological changes could be clearly seen

in the form of abnormal leaf morphology and changes of leaf color. Abnormal leaf morphology includes the formation of irregular leaves, variegation, and non-uniformity of leaf branching. The effect on leaf color was in the form of albinism, chlorosis and discoloration of chlorophyll pigments. Plant vitrification was also observed in some of the plantlets. Murthy et al. [23] also found that EMS has resulted in changes of leaf morphology and occurrence of albinism in mulberry plants. They explained that these changes could be attributed to various factors such as disturbances of phytochromes, chromosome aberrations, disruption of auxin synthesis and mineral deficiencies. Dhulgande et al. [24] reported the occurrence of morphological abnormalities of peas (*Pisum sativum* L.) became more frequent with the increase of EMS concentration used for treatment and this correlated positively with the formation of meiotic abnormalities. Similar findings were reported by Kumar & Yadav [25] on the genotoxicity study of EMS on sesame seeds whereby a wide range of meiotic abnormalities and laggard characteristics of chromosomes occurred in higher frequency. Hence, EMS could also have brought some alterations in the organization of chromosomes which caused the *A. annua* L. clones to have a certain degree of plant abnormalities. However, in our study, the number of plantlets with these morphological changes was found to reduce upon each subculture cycle at 8-weeks intervals and eventually no morphological abnormality was detected after four subculture cycles (32 weeks). This indicated that the morphological changes of the *in vitro* seedlings of *A. annua* L. is a temporary phenomenon.It may also indicate that chimerism occurred in the *A. annua* L. plantlets that were derived from EMS treated seeds at the early stages of growth after seed germination. They are possibly the sectorial chimeras which are unstable and both normal types and mutated types can be produced after mutagen treatment [26]. Upon each subculture cycle at 8 weeks intervals, the chimeras slowly dissolve and the number of abnormal plantlets become reduced and eventually only left with the normal plants. Hence repeated *in vitro* sub culturing was recommended if it is required to dissociate chimeras and discover the real stable plants.

Table 1 The effect of EMS on seed germination and morphology of seed-derived plantlets of three different clones of *A. annua* L. after 8 weeks of culture.

% EMS for seed treatment (v/v)	% Seed germination			Leaf size (cm^2)			Plantlets height (cm)			% of abnormal plants		
	TC1	TC2	Highlands	TC1	TC2	Highlands	TC1	TC2	Highlands	TC1	TC2	Highlands
Control	60.2	42.2	86.7	$0.4\pm0.1^{b}_{a}$	$0.6\pm0.1^{b}_{a}$	$1.2\pm0.3^{a}_{a}$	$0.2\pm0.1^{c}_{a}$	$3.4\pm0.2^{a}_{a}$	$1.7\pm0.2^{b}_{a}$	Nil	Nil	Nil
0.25%	12.5	31.4	83.3	$0.5\pm0.2^{a}_{a}$	$0.5\pm0.1^{a}_{a}$	$0.5\pm0.2^{a}_{b}$	$0.2\pm0.1^{b}_{a}$	$1.7\pm0.2^{a}_{ab}$	$1.6\pm0.1^{a}_{a}$	Nil	Nil	Nil
0.50%	46.3	40.3	78.3	$0.5\pm0.0^{a}_{a}$	$0.5\pm0.0^{a}_{a}$	$0.4\pm0.1^{a}_{b}$	$0.2\pm0.1^{b}_{a}$	$2.4\pm0.5^{a}_{a}$	$1.9\pm0.3^{a}_{a}$	Nil	Nil	2.4
1.00%	34.3	33.2	80.0	$0.4\pm0.1^{b}_{a}$	$0.6\pm0.2^{a}_{a}$	$0.6\pm0.2^{a}_{b}$	$0.3\pm0.1^{b}_{a}$	$1.7\pm0.2^{a}_{ab}$	$1.5\pm0.2^{a}_{a}$	9.5	9.1	10.0
1.50%	73.5	28.4	70.0	$0.2\pm0.2^{b}_{b}$	$0.5\pm0.1^{a}_{a}$	$0.4\pm0.2^{a}_{b}$	$0.2\pm0.1^{b}_{a}$	$1.7\pm0.1^{a}_{ab}$	$2.3\pm0.7^{a}_{a}$	Nil	13.2	11.9
2.00%	79.2	39.5	66.7	$0.5\pm0.1^{a}_{a}$	$0.6\pm0.2^{a}_{a}$	$0.7\pm0.1^{a}_{b}$	$0.2\pm0.1^{c}_{a}$	$1.3\pm0.3^{b}_{b}$	$2.3\pm0.5^{a}_{a}$	14.3	16.3	13.7

Mean values ($\bar{x}$±s.e) of leaf size and plantlet height at each EMS treated level followed by different superscript letters were significantly different between the three different clones of *A. annua* L. (Tukey HSD test, $p \leq 0.05$). Mean values ($\bar{x}$±s.e) of leaf size and plantlet height followed by different subscript letters were significant for different EMS treatment concentrations (Tukey HSD test, $p \leq 0.05$).

3.2 The effect of EMS on leaf glandular trichomes

The density and morphology of the leaf trichomes was evaluated based on the images obtained from the fluorescence microscopy using the control and after the EMS treatment samples as examples (Figure 1). After four subculture cycles (32 weeks), the number of glandular trichomes on the adaxial leaf surface of TC1 clone plantlets derived from seeds treated with 0.25% to 1.5% (v/v) EMS was found to be significantly increased as compared to the control. While the number of glandular trichomes on the abaxial leaf surface of plantlets derived from seeds treated with 0.5% to 1.5% EMS was found to me more than the control. The number of trichomes of both leaf surfaces of plantlets derived from seeds treated with 2.0% (v/v) EMS was drastically reduced within the same culture duration. When comparing the adaxial and abaxial leaf surfaces, the TC1 clone without treatment (control) produced more trichomes on the abaxial leaf surface. It was the reverse for the EMS treated plantlets except the seeds treated with 0.5% (v/v) EMS produced plantlets with a number of leaf grandular trichomes that was not significantly different on both surfaces. The size of the glandular trichomes of the abaxial leaf surface was found to increase for plantlets derived from seeds treated with all tested concentration of EMS except 0.5% (v/v) EMS which had no effect on the size of glandular trichomes. When comparing both the leaf surfaces of the control and those plantlets derived from seeds treated with different concentrations of EMS, the size of the glandular trichomes was smaller at the adaxial surface except those plantlets derived from seeds treated with 1% EMS whereby the size of the trichomes was not significantly different between both surfaces (Table 2).

For the TC2 clone, the number of trichomes on the adaxial leaf surface of the plantlets derived from the EMS treated seeds was not affected by the EMS treatment. Nevertheless, the number of leaf trichomes was significantly increased on the abaxial leaf surface when the seeds were treated with 0.5% to 2.0% (v/v) EMS. There was no significant difference when comparing the number at both leaf surfaces of the controls and those plantlets derived from seeds treated with 0.25%, 1.5% and 2% EMS. While seeds treated with 0.5% and 1% EMS gave rise to more glandular trichomes on the abaxial leaf surface. The size of the glandular trichome was generally bigger for both the adaxial and abaxial leaf surfaces for EMS treated seed-derived plantlets compared with the control except the trichome size on the adaxial surface of plantlets derived from seeds treated with 1.5% (v/v) EMS did not follow the trend. When comparing both the leaf surfaces of the control and at each EMS concentration level, the glandular trichomes of the adaxial leaf surface were bigger except those derived from 1.5% EMS treated seeds (Table 2).

EMS increased the formation of trichomes at different densities on the adaxial and abaxial leaf surface of the Highland clone of *A. annua* L. The number of trichomes on the adaxial leaf surface only increased in plantlets derived from seeds treated with 1.0% and 1.5% (v/v) EMS while the number of trichomes on the abaxial surface increased with all levels of EMS treatment. When comparing the number of trichomes at both leaf surfaces at a fixed concentration of EMS treatment, the trichomes distribution was not significantly different except those derived from seeds treated with 0.5% EMS in which more glandular trichomes were found on the abaxial leaf surface. However, the controls produced more glandular trichomes on the adaxial leaf surface. The effect of EMS generally increased the size of trichomes of both the leaf surfaces except EMS 0.25% (v/v) had reduced the trichome size while EMS 2% (v/v) had no effect on the trichome size of the adaxial leaf surface. When comparing the size of glandular trichomes of both leaf surfaces, the control and plantlets derived from each similar EMS concentration, the glandular trichomes on the adaxial leaf surface were bigger except those plantlets derived from seeds treated with 0.25% EMS in which the reverse was true (Table 2).

The obtained results indicated a general increasing trend in the density and size of leaf glandular trichomes of the TC1, TC2 and Highland clones of *A. annua* L. after seed treatment with various concentrations of EMS. These two features were the permanent effect of EMS on their leaf trichomes (using the TC1 clone as an example) whereby the increased number and size of the trichomes remain the same even after the EMS treated clonal propagated plants were transplanted to soil and placed in plant house conditions (Table 3). As explained by Dalin et al. [27], most plant species can increase their trichomes density in new leaves in response to damage or attack by predators. Some plant species produce glandular trichomes that release secondary metabolites which can be used as a repellent or trap insects or other organisms as a protection mechanism. Several plant species have been proven to produce new leaves with an increased number of trichomes in response to damage caused by herbivores. Baur et al. [28] reported that the *Alnus incara* plant produced new leaves with a 500% increase in trichome density after being attacked by Coleoptera insects. Artificial damage to the leaves also resulted in a 1000% increase in trichome density in birch (*Betula pubescens*) leaves [29]. Hence for *A. annua* L., EMS has induced the plants to produce leaves with higher number as well as bigger trichomes.

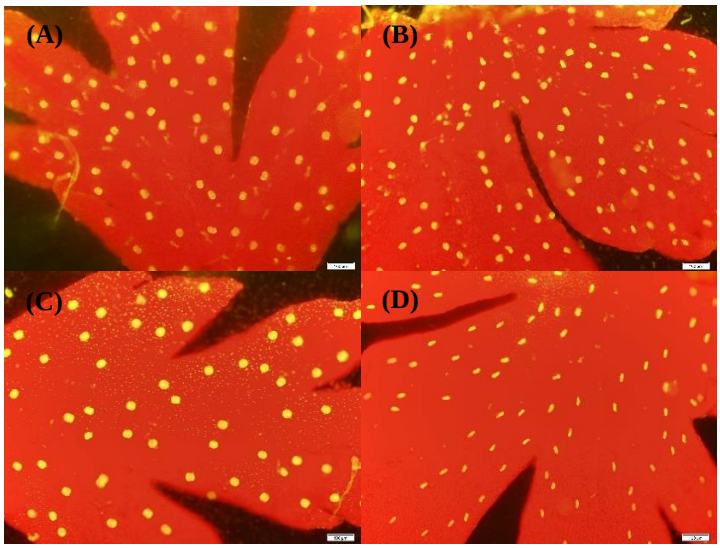

Figure 1 Distribution of *Artemesia annua* L. leaf trichomes as shown in the images from fluorescence microscopy (A): Control; (B), (C), (D): leaf trichomes of seedlings derived from seeds treated with 1% (v/v) EMS of TC1, TC2 and highlands respectively.

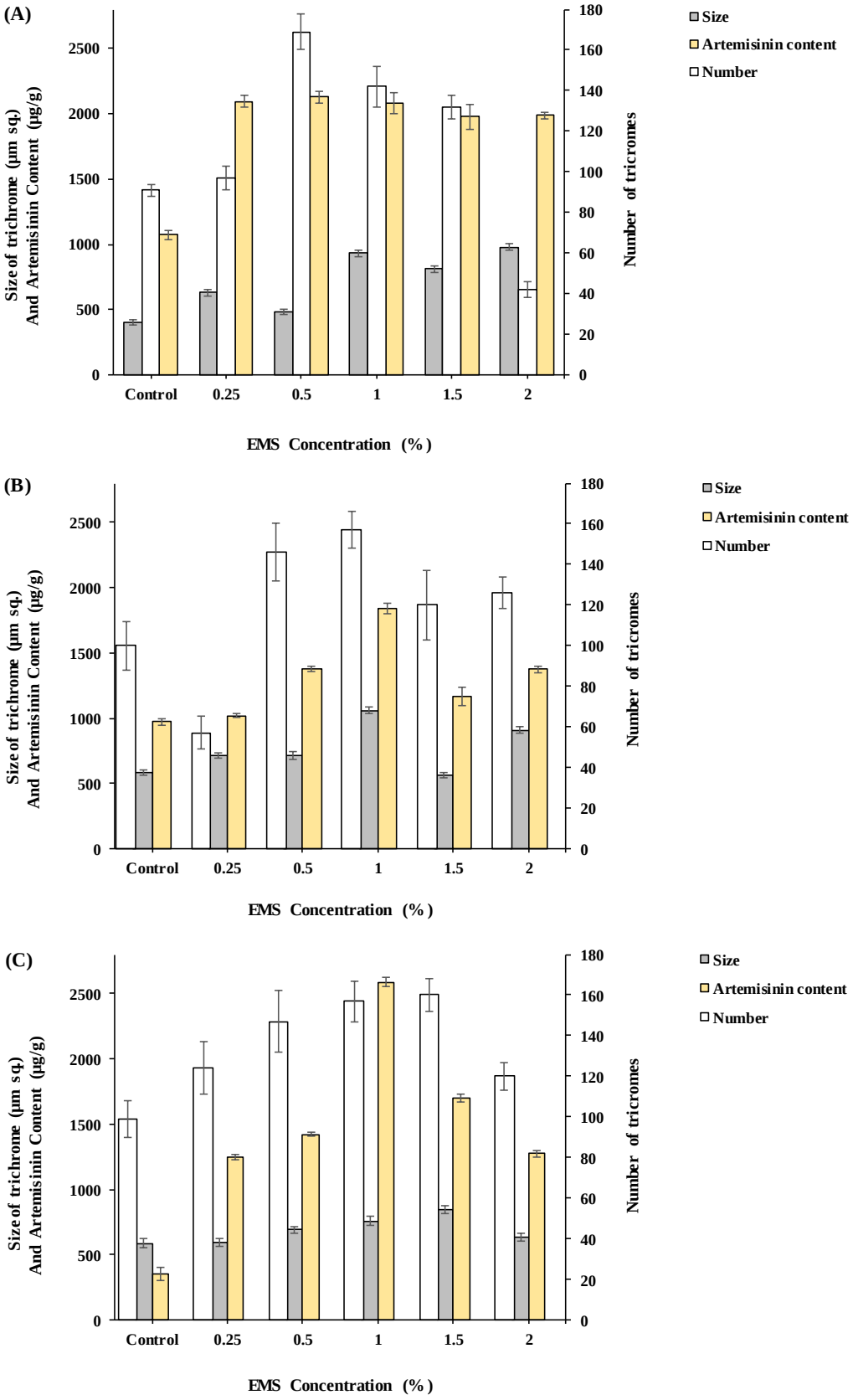

Figure 2 The relationship of density and size of leaf glandular trichomes to the production of artemisinin in (A), (B) and (C) of *Artemisia annua* L.

Table 2 The effect of EMS on distribution and size of leaf glandular trichomes of TC1, TC2 and highlands clones of *A. annua* L.

% EMS for seed treatment	TC1				TC2				Highlands			
	Number (per mm^2)		Size (µm^2)		Number (per mm^2)		Size (µm^2)		Number (per mm^2)		Size (µm^2)	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
Control	$40\pm1^{b}_{d}$	$51\pm2^{a}_{b}$	$308.8\pm18.4^{b}_{e}$	$491.1\pm16.7^{a}_{c}$	$54\pm8^{a}_{ab}$	$46\pm4^{a}_{c}$	$675.5\pm23.5^{a}_{d}$	$498.0\pm18.9^{b}_{c}$	$56\pm5^{a}_{b}$	$43\pm4^{b}_{c}$	$675.5\pm32.8^{a}_{b}$	$498.0\pm34.3^{b}_{c}$
0.25% v/v	$54\pm3^{a}_{c}$	$43\pm3^{b}_{c}$	$591.7\pm23.4^{b}_{c}$	$665.6\pm22.7^{a}_{b}$	$30\pm4^{a}_{b}$	$27\pm4^{a}_{d}$	$809.4\pm25.7^{a}_{c}$	$621.3\pm19.0^{b}_{b}$	$58\pm8^{a}_{b}$	$66\pm5^{a}_{ab}$	$555.2\pm35.6^{b}_{c}$	$628.2\pm25.5^{a}_{b}$
0.50% v/v	$87\pm4^{a}_{a}$	$82\pm5^{a}_{a}$	$473.3\pm25.6^{a}_{d}$	$488.1\pm19.7^{a}_{c}$	$66\pm5^{b}_{a}$	$80\pm9^{a}_{a}$	$780.2\pm27.4^{a}_{c}$	$647.4\pm23.4^{b}_{b}$	$60\pm8^{b}_{b}$	$87\pm7^{a}_{a}$	$838.7\pm23.7^{a}_{a}$	$541.4\pm26.6^{b}_{b}$
1.00% v/v	$81\pm5^{a}_{a}$	$61\pm5^{b}_{a}$	$930.4\pm28.8^{a}_{a}$	$931.9\pm25.5^{a}_{a}$	$71\pm5^{b}_{a}$	$86\pm4^{a}_{a}$	$1281.2\pm21.4^{a}_{a}$	$835.7\pm25.5^{b}_{a}$	$85\pm6^{a}_{a}$	$72\pm4^{a}_{ab}$	$848.1\pm43.2^{a}_{a}$	$665.6\pm31.2^{b}_{b}$
1.50% v/v	$67\pm3^{a}_{b}$	$65\pm3^{a}_{a}$	$690.8\pm27.4^{b}_{b}$	$933.7\pm24.4^{a}_{a}$	$55\pm11^{a}_{ab}$	$65\pm6^{a}_{b}$	$466.2\pm19.9^{b}_{e}$	$656.8\pm22.2^{a}_{b}$	$85\pm5^{a}_{a}$	$75\pm3^{a}_{ab}$	$823.4\pm35.5^{a}_{a}$	$865.1\pm25.5^{a}_{a}$
2.00% v/v	$25\pm2^{a}_{e}$	$17\pm2^{b}_{d}$	$908.4\pm29.5^{b}_{a}$	$1050.6\pm15.7^{a}_{a}$	$62\pm4^{a}_{ab}$	$64\pm4^{a}_{b}$	$1078.9\pm24.4^{a}_{b}$	$735.7\pm21.7^{b}_{ab}$	$58\pm4^{a}_{b}$	$62\pm3^{a}_{b}$	$669.7\pm42.2^{a}_{b}$	$591.7\pm19.6^{a}_{b}$

Mean values ($\bar{x}\pm$s.e) followed by different superscript letters were significantly different for each parameter between the adaxial and abaxial leaf surface of each clone (Student t-test, $p \leq 0.05$). Mean values ($\bar{x}\pm$s.e) followed by different subscript letters for each parameter were significant for different EMS treatment concentrations of each leaf surface (Tukey HSD test, $p \leq 0.05$).

Table 3 The leaf trichome size and density and artemisinin content of *A. annua* L. TC1 clone after being planted in the field and harvested before the flowering stage.

Artemesia annua L. TC1 Clone	Trichome size (µm^2)	No. of trichomes	Days to flowering	Artemisinin content (µg/g)
Control	648.6 ± 30.6^{a}	55 ± 3^{a}	91 ± 4^{a}	689.7 ± 54.1^{a}
EMS treatment (1% v/v)	1109.5 ± 58.6^{b}	86 ± 4^{b}	103 ± 6^{a}	1719.4 ± 88.0^{b}

Mean values for each parameter followed by different were significantly different at $p \leq 0.05$.

3.3 Correlation of leaf trichomes and artemisinin content

Glandular trichomes of *A. annua* L. has been reported as the sole site for artemisinin production [5, 6]. Hence the number and size of trichomes would directly affect the artemisinin content of a particular *A. annua* L. Plant. Our results did indicate that in each tested clone of *A. annua* L., the plantlets derived from seeds treated with EMS produced leaves with an increased number and bigger size of leaf trichomes and both these characteristics enhanced the production of artemisinin and positively correlated (Figure 2). This showed that both trichome density and size together played important roles in influencing the amount of artemisinin produced. Since secondary metabolites within a plant were often produced for defense purposes, the application of EMS mutagen could create biological stress by triggering artemisinin signaling and release pathway, increasing artemisinin production and content. Another possible explanation for the enhanced production thus of artemisinin was that EMS could have mutated the gene coded for artemisinin at the molecular level thus increasing the production of artemisinin. Since mutagenesis is a very random process, it is not uncommon to get inconsistent results even within the same clone and variety. The wild-type Highland clone, which was not a cultivated selected clone, gave a higher variation in the results compared to the selected TC1 and TC2 clones.

4. Conclusions

EMS is useful for inducing *in vitro* plantlets of three different clones of *A. annua* L. to produce higher density and bigger leaf trichomes which directly enhanced the production of artemisinin. The density and size of the leaf trichomes did not change after being transferred to the soil. Therefore, it could be concluded that EMS is potentially a suitable chemical agent for inducing more and bigger sized leaf trichomes in *A. annua* L. for a higher production of artemisinin and this would be beneficial when cultivated in any tropical countries.

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