Banat's Journal of Biotechnology
Contact: web: http://www.bjbabe.ro, e–mail: bjb@usab_tm.ro

IMPROVEMENT OF ARTEMISININ PRODUCTION BY DIFFERENT BIOTIC ELICITORS IN *Artemisia annua* BY ELICITATION–INFILTRATION METHOD

DOI: 10.7904/2068–4738–VII(13)–82

Sona Ayadi HASSAN[1*], Tayebeh SOLEIMANI[2]
[1]*Department of biotechnology, Faculty of advanced sciences and technologies, University of Isfahan, Isfahan, **IRAN**,*
[2]*Department of biology, Faculty of biological sciences, University of Nourdanesh institute of higher education, Meymeh, **IRAN**,*
**Corresponding author: sonaayadi@rocketmail.com*

Abstract. Artemisinin, a sesquiterpene lactone; is one of the most well–known secondary metabolites with multi–functional therapeutic effects which is isolated from *Artemisia annua*. Due to low levels of artemisinin in this plant, efforts are being made to optimize its production by various strategies such as elicitation–infiltration. This is a procedure in which, elicitors are injected to the plants by fine–needles directly. In this study, we identified the effect of bacterial and fungal elicitors on artemisinin level and biomass accumulation in *A. annua*. Artemisinin content was measured by high performance thin layer chromatography. The maximum level of artemisinin was accomplished by injection of 0.4 mg total sugar/ml *F. oxysporum* and *B. cereus* decreased artemisinin content in comparison to the controls. 1 McFarland *S. aureus* was the most successful elicitor, increasing the fresh weight 1.34 fold over the controls.
***Keyword**:* artemisinin, elicitor, elicitation–infiltration, secondary metabolite.

Introduction

Since ancient times, the plant–derived chemicals were a significant source for pharmaceuticals [SHAKERAN *et al.*, 2015]. Many active constituents of medicinal, commercial and poisonous of plants belong to secondary metabolites.

Terpenes or terepnoids are one of the classes of secondary metabolites which are built of isoprene units.

Sesquiterpenoid lactones are biologically active secondary metabolites of plants of Compositae family [HANDON *et al.*, 2003]. Artemisinin, a sesquiterpene lactone containing an endoperoxide bridge is isolated from *Artemisia anuua* L. plants.

This compound is effective against both drug–resistant and cerebral malaria causing strains of *Plasmodium falciparum*, different cancers and viruses [NAKASE *et al.*, 2008, EFFERTH *et al.*, 2008, ABDIN *et al.*, 2003]. The low production (0.01–0.8%) of artemisinin in *A. annua* is the main obstacle to the commercialization of the drug.

Therefore, various studies have been conducted to enhance the yield of this invaluable compound either in cell/tissue culture or in the whole plant of *A. annua* [ABDIN *et al.*, 2003].

Since the discovery of this drug, different studies have been carried out to overcome to the low yield of artemisinin, including: hairy root induction, semi–biosynthetic pathway, cell suspension culture and genetic engineering [WEATHERS *et al.*, 1994, DIETRICH *et al.*, 2009, VARMA *et al.*, 2009, CHEN *et al.*, 1999].

Elicitor is a scientific term which is applied for stress factors that triggers the defense mechanisms by direct or indirect routs. Elicitation is described as the most feasible method for enhancing the production of desirable secondary metabolites from cell, organ and plant systems [GEOL *et al.*, 2009].

Furthermore, elicitors improve the release of metabolites in the medium [SHAKERAN *et al.*, 2015]. They are classified in two categories:

Abiotic and biotic.

Abiotic do not have biological origins and are comprised of two groups: physical factors (thermal stress, wounding, *etc*.) and chemical substances (heavy metal salts, vanadyl sulphate $VOSO_4$, *etc*.) [VASCONSUELO *et al.*, 2007].

Biotic elicitors are originated from organisms that include polysaccharides from plant cell walls (pectin, cellulose,

etc.), micro–organisms (chitin, glucans, *etc*.) and G–proteins [GEOL *et al.*, 2009].

For example, *Aspergillus niger* and *Fusarium oxysporum* had increased the production of thiophene in *Tagetes patula* and anthocyanin in *Daucus carota* respectively [NAMDEO *et al.*, 2002, RAJENDRAN *et al.*, 1994].

Furthermore, *Bacillus cereus* and *Staphylocccus aureus* have revealed great impacts on the production of scopolamine in *Scopolia parviflora* [GUILLON *et al.*, 2006]. Injection–infiltration method has been introduced to test pathogenicity of microorganisms which invade the plants.

Cell suspension of bacteria is injected to the leaves of the plants by fine needles.

This method has this advantage over other procedures in that, the investigations can be done on the intact plants [GUILLON *et al.*, 2006].

The aim of this study is to investigate the effect of fungal (*F. oxysporum* and *A. fumigatus*) and bacterial (*S. aureus* and *B. cereus*) elicitors on the biomass accumulation and artemisinin production in the intact plants of *A. annua*.

Material and methods

Seed sterilization and germination

Seeds of *A. annua* were sterilized by immersing in NaClO 2% (v/v) and EtOH 7% (v/v) for 10 and 1 min, respectively [KLEMENT *et al.*, 1963]. Then they were washed for 3 times with sterile distilled water. Sterile seeds were cultured on MS medium for germination. The cultures were placed in the seed germinator, applying this condition: 16 h/8 h light/dark cycle and temperature 25±2°C [KLEMENT *et al.*, 1963]. The samples were 2 month old with similar height and weight.

Preparation of elicitors

Preparation of fungal elicitors

At first, some cells of the fungi (*F. oxysporum* and *A. fumigatus*) were transferred to a 250 mL Erlenemeyer flask containing 50 mL of PDB (Potato Dextrose Broth) media by a sterile spatula. Flasks were incubated into an incubator shaker; rotating at 200 rpm at 25±2 °C 16h/8h light/dark cycle and they were grown for 6 days. The culture broths were past throw Whatman paper No. 1 and the remaining were centrifuged at 5000 rpm for 15 min.

The following procedure was suggested to prepare fungal elicitors:

The culture broth was divided into two portions. One portion was consumed after passing it through 0.22m filter and it was designated as 'filtered culture broth'.

The other portion of culture broth was autoclaved at 15 psig and 121°C for 20 minutes and it was designated as 'autoclaved culture broth'.

The fungal mat was washed for several times with sterile doubled distilled water.

Later it was allowed to dry at 40 ± 2 in a hot air oven to the constant weight.

The dried cells were crushed in a mortar pestle. 10 g of dry cell powder was suspended in 100 mL of doubled distilled water (pH 5.7). Subsequently, it was autoclaved at 15 psig and 121°C for 20 minutes for hydrolysis. The hydrolysate was centrifuged at 5000 rpm for 10 min.

The supernatant was collected and stored at 4°C [VARMA *et al.*, 2009].

To determine the concentration of fungal elicitors, phenol–sulfuric acid method was exploited. In this method, 0 to 1 mg/mL glucose solutions were prepared.

To do so, firstly, 100 mg glucose was dissolved in 100 mL double distilled water. Then, these solutions were diluted to prepare 0.6 and 0.8 mg/mL glucose solutions. Secondly, 4 % phenol solution was prepared by adding 4 g phenol in 100 mL double distilled water.

Thirdly, 1 mL doubled distilled water and 1 mL of 4 % phenol solution was poured in penicillin bottle. Then 5 mL 98 % sulfuric acid was added to it.

This solution was applied as blank after cooling. Fourthly, to prepare standard samples, 1 mL of glucose solutions were added to penicillin bottles and 1 mL 4 % phenol solution was blended to each of them.

Then, 5 mL 98% sulfuric acid was added to all of the bottles. These solutions were designated as standard

after cooling. The wavelength was set on 490 nm by spectrophotometer.

Then the blank samples were adjusted on zero and the calibration curves were depicted by standards. Later fungal elicitors were diluted 100 times by adding double distilled water. 1 mL of fungal elicitor and 1 mL of 4 % phenol solution were added to penicillin bottle.

Then, 5 mL of 98 % sulfuric acid was mixed with them. The concentration of this solution was measured after cooling by spectrophotometer.

This concentration was multiplied by 100. If the concentration was less than the desired one, it can be more concentrated by rotary evaporator and if it was more than the desired concentration, it can be diluted by adding double distilled water. In this study, two fungal concentration were chosen: 0.2 and 0.4 mg total/mL [DUBOIS *et al.*, 1956].

Preparation of bacterial elicitors

B. cereus was cultured in Nutrient broth (NB) in incubator shaker rotating at 120 rpm at 27°C for 24 h. In addition, *S. aureus* was cultured in tryptic soy agar (TSB) with the same condition [SHAKERAN *et al.*, 2015]. Similarly, 0.5 and 1 McFarland bacterial concentrations were applied for elicitation. These concentrations were prepared by a method known as McFarland turbidity standard. In this procedure, at first two solutions of 1 % sulfuric acid (H_2SO_4) and 1.175 % barium chloride ($BaCl_2$) were prepared.

To prepare 1 % sulfuric acid solution: 90 mL deionized water was poured into 100 mL volumetric flask. 1 mL concentrated sulfuric acid was added to the volumetric flask by 1 mL volumetric pipette. Then the volumetric flask was filled with deionized water to 100 mL.

Furthermore, 1.175 g barium chloride was poured in 100 mL volumetric flask. Then, the flask was filled with distilled water to 100 mL. To prepare 0.5 McFarland bacterial concentrations, about 85 mL of 1 % sulfuric acid was added to 100 mL volumetric flask. 0.5 mL barium chloride was added to sulfuric acid solution drop wise by 0.5 mL volumetric pipette while the flask was constantly swirled. Then the solution was brought to 100 mL by adding sulfuric acid.

To have 1 McFarland, 1 mL barium chloride and 99 mL of 1% sulfuric acid were applied. Before using, McFarland standards were thoroughly mixed. In presence of intense light, the turbidity of the samples and McFarland were evaluated by naked eyes.

There are 1.5×10^8 and 3×10^8 bacteria in 0.5 and 1 McFarland respectively. The absorbance was between 0.08–0.1 in 625 nm for 0.5 McFarland and for 1 McFarland was 0.257 [SUTTON *et al.*, 2011].

Elicitation procedure

As mentioned previously, elicitation–infiltration method has been developed as a rapid test for detection of pathogenicity of a microorganism. This procedure is carried out as follows:

Older leaves of the 2–month old–plants were chosen from to inject the elicitors. The injection site was closed to the lateral veins of the leaf, where the tissues were thick enough. 3 mL of each elicitor was injected to intracellular spaces of the *A. annua*'s leaves by a fine and sterile needle. 3 mL sterile distilled water was injected to leaves of control samples [GUILLON *et al.*, 2006].

Measurement of fresh and dry weight of intact plants

The fresh weight of the samples was measured and compared with the control samples after 48 h. The samples were then dried in the dark at room temperature for 5 days before the measurement of dry weight of samples.

Extraction and assessment of artemisinin

1.0 g of dried samples was ground in 2 mL of toluene in mortar. The mortar was cleaned with 1 mL toluene which was transferred to the test tubes.

The extracts were then centrifuged at 5900 rpm for 10 min.

The supernatant was removed and saved. These steps were repeated for several times. The supernatants were dried under nitrogen gas and store –20°C [SMITH *et al.*, 1997].

HPTLC (high performance thin layer chromatography) was used, which is a

specific, accurate, precise and reproducible method.

This method was applied to measure amounts of artemisinin in the samples.

All chemicals were HPLC grade.

The instrument used in the present study was HPTLC system comprising LINOMAT V automatic sample applicator, TLC SCANNER III with WINCATS software; twin through chamber (all from CAMAG, Switzerland) and UV–1601, UV/Vis spectrophotometer, Shimadzu (Kyoto, Japan).

Following chromatographic conditions were set: stationary phase, silica gel F_{254} HPTLC pre–coated plates 60 (20×10 cm with 0.2 mm thickness.; E. Merck, Darmstadt, Germany), mobile phase: acetone: hexane (3:7).; chamber saturation time: 30 min.; sample application: 5 mm.; separation technique: ascending.; temperature: 20 ± 5°C.; migration distance: 75 mm.; scanning mode: absorbance.; detection wavelength: 520 nm and source of radiation utilized: combination of D_2 and tungsten lamps.

A stock solution of artemisinin (ng/mL) was prepared in methanol.

Various amounts of stock solution were spotted in duplicate on TLC plates manually to measure concentrations of 200, 400, 600, 800 and 1000 ng/spot of artemisinin.

The plates were developed in a presaturated twin trough chamber and densitometrically scanned at 520 nm using optomechanical scanning technique. The data of peak area versus drug concentration were treated by linear least–square regression analysis.

A standard calibration curve in the range of 200 to 1000 ng/spot for quantitative analysis was depicted using different concentrations of artemisinin (Sigma Aldrich, USA) as standard substance.

Minimum square method (R^2 value) was used to measure relationship between the concentration and peak height.

Validation of the HPTLC method was calculated as the percent recovery of spiked extract sample with standard artemisinin at 100 ng/mL concentration.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated by using the formula based on the signal to noise ratio. LOD and LOQ were measured by using equations,

LOD = 3×S/N´ and

LOQ = 10× S/N´,

where S = signal height, N´= noise height [SHAHTALEBI *et al.*, 2013].

Experimental design and statistical analysis

In this experiment, 324 plants of *A. annua* were chosen for injection. Two different concentrations were selected for each bacterial (0.5 and 1 McFarland) and fungal (0.2 and 0.4 carbohydrate) elicitors.

The fresh weight, dry weight and artemisinin content were measured in all of the samples.

For each treatment, 3 plants of *A. annua* were prepared for the injection.

All experiments were performed in triplicate.

The variance of the results was analyzed by SPSS software version 20 (SPSS Inc., Chicago, IL). In this study, univariate procedure at $P < 0.05$ was chosen [SHAKERAN *et al.*, 2015].

Results and discussion

The artemisinin content of intact plants of *A. annua* was assayed through HPTLC after elicitation with bacterial and fungal factors.

The level of artemisinin was calculated as the percentage of the dry weight of the intact plants for each sample.

The standard samples were analyzed in 200, 400, 600, 800 and 1000 ng/spot concentrations.

Since artemisinin is a non–UV–absorbing drug, it was converted into a violet colored UV–absorbing compound by applying vanillin (1% w/v) sulphuric acid (5% v/v) in ethanol as derivatizing compound.

The correlation between the concentration and the peak area of the standard was calculated through the minimum square method (R^2 value).

The obtained equation was y = 0.1955x + 17.108 (figure 1).

LOD and LOQ were determined 63 and 210 ng/mL by using the formula which is based on the signal to the noise ratio.

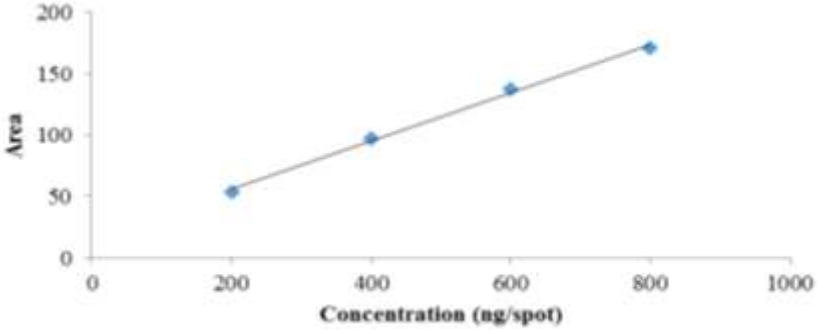

Figure 1. Calibration curve of artemisinin using HPTLC method and mobile phase: acetone: hexane (3:7) was set at 520 nm. Using win CATS software, the calibration curve was calculated by linear regression in the range of 200–1000 ng/spot. The regression equation was y =0.1955x + 17.108 with correlation cofactor R^2 = 0.9972, where x was the concentration of the standard (ng/spot).

The effect of biotic elicitors on artemisinin yield

Figure 2 shows the effect of bacterial elicitors on artemisinin accumulation in the plants of *A. annua*.

No changes in artemisinin content were observed when *S.aureus* was used as a biotic elicitor. In addition, both concentrations of *B. cereus* did not support the enhancement in artemisinin production and led to a dramatic decrease in its production too (150 ng/mL) (table 1).

Table 1.

The ANOVA test's table showing the effect of each bacterial treatment on artemisinin yield. C, B and S stand for control, *B. cereus* and *S. aureus* respectively.

Elicitor	Mean (ng)	Std. Deviation
C0.5	250.0	20
C1	230.0	45
B0.5	163.3	15.28
B1	150.0	20
S0.5	233	15.28
S1	220.0	26.4

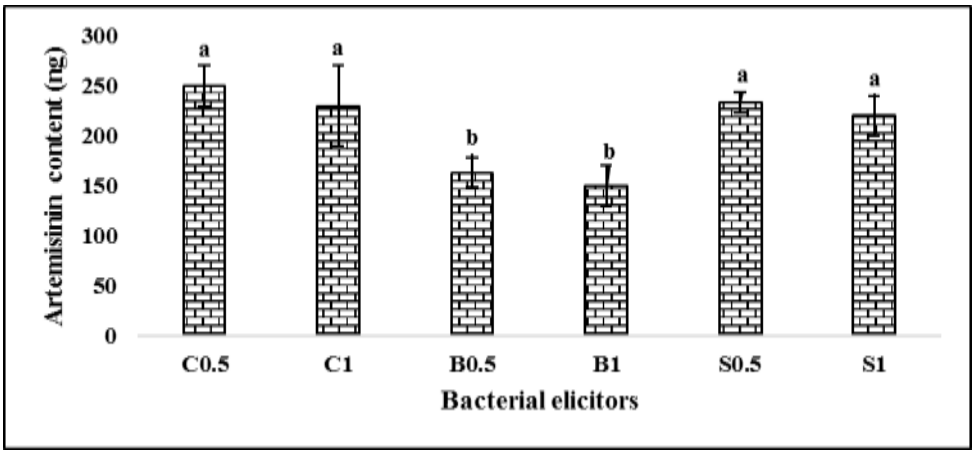

Figure 2. Effect of bacterial (*B. cereus* and *S. aureus*) elicitors on artemisinin content in plants of *A. annua*. Different letters show significant differences in mean values for each parameter using Duncan's test (P < 0.05). C, B and S stand for control, *B. cereus* and *S. aureus* respectively. 0.5 and 1 represent two different concentration for bacterial elicitors according to McFarland.

Available on–line at http://www.bjbabe.ro
Banat's Journal of Biotechnology
2016, VII(13)

Furthermore, the effect of fungal elicitors were assayed and subsequently they were analyzed (table 2). The data revealed that the injection of 0.4 mg total/mL *F. oxysporum* can increase the artemisinin content from (286.7 ng to 140.3 ng) explosively whereas other fungal elicitors could not affect the artemisinin production in comparison to the controls (figure 3).

Table 2.

The ANOVA test's table showing the effect of each fungal treatment on artemisinin yield. C, F and A stand for control, *F. oxysporum* and *A. fumigatus* respectively.

Elicitor	Mean (ng)	Std. Deviation
C0.2	280	15
C0.4	210	30
F0.2	220	28.8
F0.4	1400	10.2
A0.2	180	20.8
A0.4	350	15.2

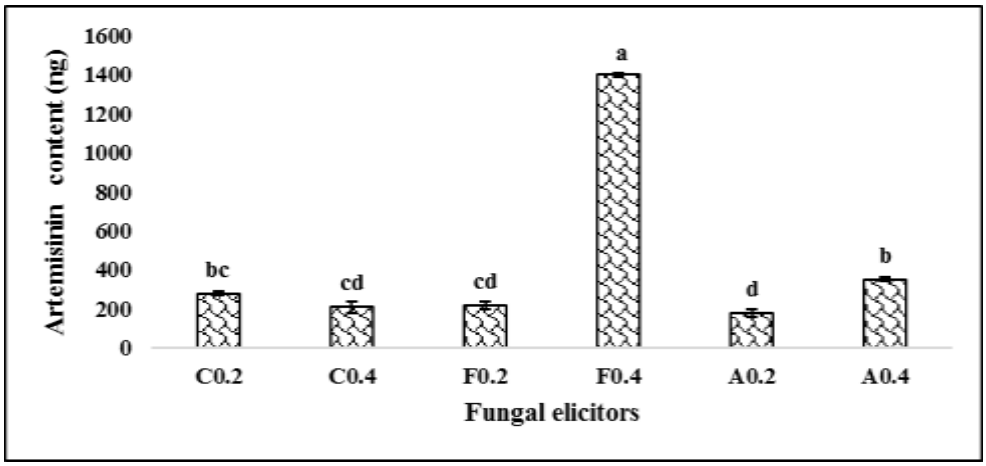

Figure 3. Effect of fungal (*F. oxysporum* and *A. fumigatus*) elicitors on artemisinin content in plants of *A. annua*. Different letters show significant differences in mean values for each parameter using Duncan's test ($P < 0.05$). C, F and A stand for control, *F. oxysporum* and *A. fumigatus* respectively. 0.2 and 0.4 represent two different concentrations for fungal elicitors according to mg total/mL.

The effect of biotic elicitors on fresh weight

The effect of bacterial elicitors' injection (*S. aureus* and *B. cereus*) on the fresh weight of *A. annua* samples are depicted as diagram in figure 4. According to the data analysis (table 3), all elicitors were capable to enhance the fresh weight of the samples.

But in comparison to other elicitors 1 McFarland *S. aureus* showed a remarkable impact on fresh weight of the samples (567.33 mg to 763 mg).

Table 3.

The ANOVA test's table showing the effect of each bacterial treatment on artemisinin yield. C, B and S stand for control, *B. cereus* and *S. aureus* respectively.

Elicitor	Mean	Std. Deviation
C0.5	567.333	7.5055535
C1	556.000	11.5325626
B0.5	635.333	8.3864971
B1	650.000	5.0000000
S0.5	725.333	6.4291005
S1	763.000	7.5498344

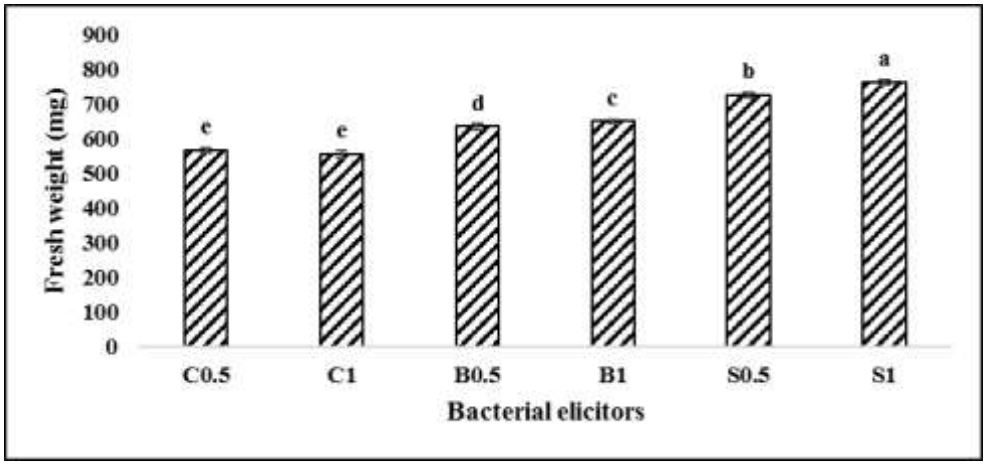

Figure 4. Effect of bacterial (*B. cereus and S. aureus*) elicitors on fresh weight in plants of *A. annua*. Different letters show significant differences in mean values for each parameter using Duncan's test (P < 0.05). C, B and S stand for control, *B. cereus* and *S. aureus* respectively. 0.5 and 1 represent two different concentration for bacterial elicitors according to McFarland.

In addition, the fresh weight of the samples was monitored after injection of fungal elicitors (*F. oxysporum* and *A. fumigatus*). 0.2 mg total/mL *F. oxysporum* and 0.4 mg total/mL *A. fumigatus* revealed no negative change while 0.4 mg total/mL carbohydrate *F. oxysporum* and 0.2 mg total/mL *A. fumigatus* decreased the fresh weight of the samples (table 4 and figure 5).

Table 4.

The ANOVA test's table showing the effect of each fungal treatment on dry weight. C, F and A stand for control, *F. oxysporum* and *A. fumigatus* respectively.

Elicitor	Mean	Std. Deviation
C0.2	561.666667	15.0443788
C0.4	554.000000	14.5258390
F0.2	537.666667	6.8068593
F0.4	517.000000	6.5574385
A0.2	525.000000	6.2449980
A0.4	548.333333	1.5275252

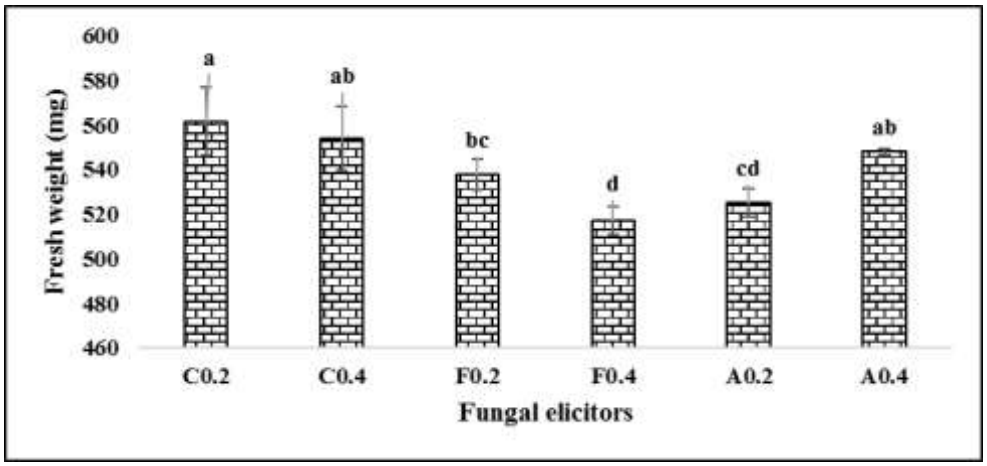

Figure 5. Effect of fungal (*F. oxysporum* and *A. fumigatus*) elicitors on fresh weight in plants of *A.annua*. Different letters show significant differences in mean values for each parameter using Duncan's test (P < 0.05). C, F and A stand for control, *F. oxysporum* and *A. fumigatus* respectively. 0.2 and 0.4 represent two different concentrations for fungal elicitors according to mg total sugar/mL.

Available on–line at http://www.bjbabe.ro
Banat's Journal of Biotechnology
2016, VII(13)

The effect of biotic elicitors on dry weight

By comparing the presented data in figure 6 and table 5, this can be considered that all of the elicitors declared promising results in dry weight increase which led to the maximum amount with 1 McFaeland *S. aureus* (193.66 mg) and the minimum amount with 1 McFarland *B. cereus* (147.33 mg).

Table 5.

The ANOVA test's table showing the effect of each bacterial treatment on dry weight. C, B and S stand for control, *B. cereus* and *S. aureus* respectively.

Elicitor	Mean	Std. Deviation
C0.5	113.000000	2.6457513
C1	114.666667	4.0414519
B0.5	152.666667	9.4516313
B1	147.333333	4.5092498
S0.5	149.666667	2.5166115
S1	193.666667	4.1633320

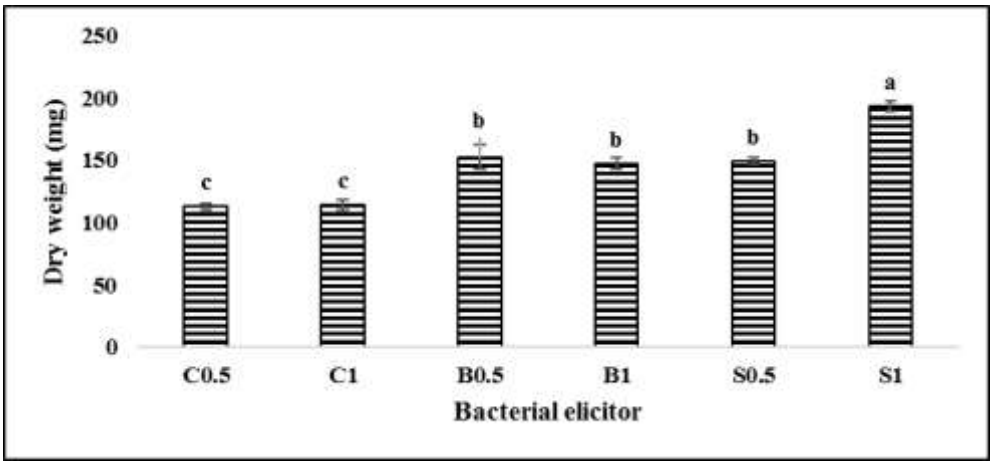

Figure 6. Effect of bacterial (B. cereus and S. aureus) elicitors on dry weight in plants of A. annua. Different letters show significant differences in mean values for each parameter using Duncan's test ($P < 0.05$). C, B and S stand for control, B. cereus and S. aureus respectively. 0.5 and 1 represent two different concentration for bacterial elicitors according to McFarland.

Among fungal elicitors, 0.4 mg total sugar/mL *A. fumigatus* enhanced the dry weight to 1.5 more than the controls (table 6 and figure 7). Other elicitors were slightly significant different in comparison to the controls.

Table 6.

The ANOVA test's table showing the effect of each fungal treatment on dry weight. C, F and A stand for control, *F. oxysporum* and *A. fumigatus* respectively.

Elicitor	Mean	Std. Deviation
C0.2	106.666667	3.0550505
C0.4	108.333333	3.0550505
F0.2	154.500000	3.5355339
F0.4	140.666667	5.8594653
A0.2	132.666667	4.5092498
A0.4	164.333333	3.0550505

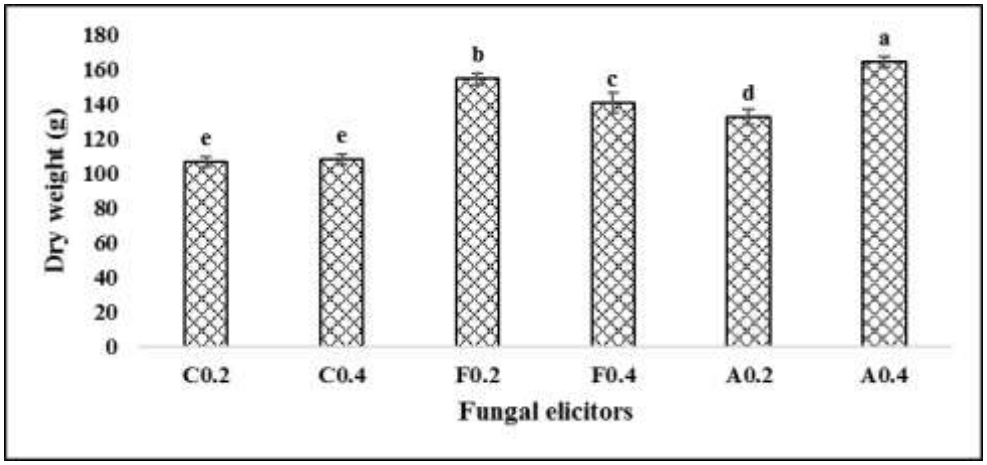

Figure 7. Effect of fungal (*F. oxysporum* and *A. fumigatus*) elicitors on dry weight in plants of *A. annua*. Different letters show significant differences in mean values for each parameter using Duncan's test ($P < 0.05$). C, F and A stand for control, *F. oxysporum* and *A. fumigatus* respectively. 0.2 and 0.4 represent two different concentrations for fungal elicitors according to mg total sugar/mL.

The effect of biotic elicitors on artemisinin yield:

Since the biosynthesis of the secondary metabolites in plants is under control during development.

So these compounds are produced in response to various stresses. In this regard, bacterial and fungal elicitors have been introduced as two outstanding elicitor types to mine new novel strategies for enhancement of secondary metabolite's yield.

For example, bacterial elicitors have been applied to promote the production of ginseng saponin and tropane alkaloids [JUNG *et al.*, 2003; BUTNARIU 2015b].

According to our findings, *B. cereus* could not introduce itself as a promising elicitor for optimizing artemisinin content (figure 2).

This finding is in a total disagreement with presented data by Gomes Silva and collab. [SILVA *et al.*, 2014; BUTNARIU, 2015e; CIOPEC *et al.*, 2015; BUTNARIU, 2015f] in that, *B. cereus* increased tanshinones production to 12 fold (up to 2.7 mgg^{-1} DW) in *Salvia miltiorrhiza*. In addition, *B. cereus* could reveal promising results in enhancement of skopolamin content in *Scopolia parviflora* [DEMIRCI *et al.*, 2015].

While the findings of Shakeran and collab. [SHAKERAN *et al.*, 2015] indicated that this bacteria did not present outstanding signs about atropine production, announcing it as a weak biotic elicitor.

The bacteria perhaps have various impacts on the plants like cell–cell, gene–gene and protein–protein interactions.

These activities surely will affect artemisinin biosynthesis pathways.

On the other hand, in our study the effect of *S. aureus* was investigated on the artemisinin accumulation as well (figure 2 and table 1).

In our results, this bacterial elicitor could not reveal brilliant and meaningful changes for artemisinin content.

But our finding result is in contrast with the findings of Bandeh Ali and collab. [BANDEH ALI *et al.*, 2013; PENTEA, *et al.*, 2015; PETRACHE *et al.*, 2014; RASHED *et al.*, 2014a, RODINO *et al.*, 2014, SAMFIRA *et al.*, 2014] in which, *S. aureus* was introduced as a powerful elicitor in artemisinin enhancement in hairy roots of *A. annua* (0.133 mg/mL DW).

While this elicitor did not show positive changes in scopolamine accumulation in hairy root cultures of *Datura metel* [SHAKERAN *et al.*, 2015].

This paradox can be justified by the phenomena of elicitor specificity.

According to it, accumulation of each secondary metabolite is dependent on plant's species and the nature of elicitors [VASCONSUELO *et al.*, 2007; IANCULOV *et al.*, 2004; NEGREA *et al.*, 2015].

By reviewing articles, it can be concluded that up to now, fungal elicitors were investigated as a promoting factor in artemisinin yield.

For example, *Colletotrichum* spp were used to enhance artemisinin production in hairy roots of *A. annua*, leading to a significant increase (51.63 % increase over the control) in its level [WANG *et al.*, 2001]. Based on an investigation done by Zheng and collab. [ZHENG *et al.*, 2008; BUTNARIU *et al.*, 2015d; CAUNII *et al.*, 2015b], *F. oxysporum* could be a helpful factor to increase the mentioned substance by secreting Nitric Oxide (0.7 mg/g DW to 1.3 mg/g DW).

The findings of this study is similar to the outputs of the previous surveys. The data showed that *F, oxysporum* is the best elicitor among others in enhancing artemisinin level in comparison to the controls (6.5 fold more than the control according to figure 3 and table 2).

To the author's knowledge, there is not any case study about the effect of *A. fumigatus* on artemisinin production in *A. annua*. But its effect on anthocyanins of *Catharanthus roseus* tumor suspension cultures was reported as a significant one [HERNANDEZ *et al.*, 1997; BUTNARIU *et al.*, 2015a; BUTU *et al.*, 2015; RASHED *et al.*, 2014b].

This result is not coordinated with our findings which introduces it as an incapable elicitor according to figure 3. The reason is not yet uncovered.

The effect of biotic elicitors on the biomass accumulation:

In our study, the effect of bacterial (*S. aureus* and *B. cereus*) and fungal (*F. oxysporum* and *A. fumigatus*) elicitors was analyzed on biomass accumulation as a sign of growth.

The results introduced *B. cereus* as a powerful elicitor with considerable impacts on biomass accumulation, increasing fresh weight from 567.33 mg to 650 mg (figure 4).

According to the findings of Niu and collab. [NIU *et al.*, 2011; BUTNARIU *et al.*, 2015c; CAUNII *et al.*, 2015a; SAMFIRA *et al.*, 2013], this microorganism can be named as plant growth–promoting rhizobacterium which is able to induce systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate–and jasmonate/ethylene–dependent signaling pathways.

As mentioned previously, the effect of *S. aureus* was observed on growth. *S. aureus* could enhance biomass accumulation in comparison to the controls vigorously (table 3).

Our findings are in agreement with the results of investigations done by Bandeh Ali and collab. [DEMIRCI *et al.*, 2015] and Shakeran and collab. [SHAKERAN *et al.*, 2015; BUTNARIU *et al.*, 2013; BUTNARIU *et al.*, 2014] in which *S. aureus* is an effective tool for improving growth rate in hairy roots cultures of *A. annua* and *D. metel* respectively.

The reason of this great potential lies in the fact that these kinds of bacteria can lead to a significant modifications in the metabolisms of the plant cells.

In this investigation, 0.2 mg total sugar/ml *F. oxysporum* kept the fresh weight of *A. annua* samples steady and unchanged during 48 h while 0.4 mg total sugar/mL of this elicitor decreased the samples fresh weight exclusively (figure 5). There is relative similarity between our findings and some reported results by Kishor [KISHOR, 1999; BUTNARIU and BUTU, 2015; BUTNARIU and SAMFIRA 2014; SAMFIRA *et al.*, 2015].

In this document, it was reported that *F. oxysporum* can promote defense mechanisms which is predominantly dependent on elicitor concentration.

Furthermore, the elicitation of the samples by *A. fumigatus* was very effective and impressive in dry weight increase (1.5 fold increase over the controls) that, these information are supported by given data in by Kishor [KISHOR, 1999; ANDREEA, *et al.*, 2012, BARBAT, *et al.*, 2013].

He introduced this elicitor as a progressive one in biomass accumulation in the plant cell cultures and presented that carbon supply by *A. fumigatus* is the main factor.

Conclusions

In conclusion, fungal elicitors are powerful elicitors for optimizing artemisinin biosynthesis in *A. annua* by elicitation–infiltration method.

So these kinds of elicitors can be applied in fileds of *Artemisia annua* as a biotic fertilizer to promote its production.

Acknowledgment

This research was funded by the Department of Biotechnology, Faculty of

Advanced Sciences and Technologies, University of Isfahan, Iran.

References

1. Abdin, M.Z.; Israr, M.; Rehman, R.U.; Jain, S.K., Artemisinin, A Novel Antimalarial Drug: Biochemical and Molecular Approaches for Enhanced Production, *Planta Medica,* **2003**, 69, p.289–299.
2. Andreea, G. Samfira, I. Butnariu, M. Corneanu M. The morphological features influence on *Phalaris arundinacea* green mass and dry matter yield, Journal of Biotechnology, **2012**, 161, Supplement, 35, DOI: 10.1016/j.jbiotec.2012.07.102.
3. Bandeh Ali, E.; Keyhanfar, M.; Asghari, GH., Optimizing Artemisinin Production In Hairy Roots of *Artemisia annua* By Using *Staphylococcus aureus*, *Medicinal Plants,* **2013**, 12, p. 82–90.
4. Barbat, C.; Rodino, S.; Petrache, P.; Butu, M.; Butnariu M. Microencapsulation of the allelochemical compounds and study of their release from different products. *Digest Journal of Nanomaterials and Biostructures*, **2013**, 8 (3), p. 945–953.
5. Butnariu, M. Collateral Metabolites of the Aminosulphuric Acids in the *Allium Plants*, *J Bioequiv Availab*, **2015**, 6, e61c.
6. Butnariu, M. Markers, Indicators of Soil Pollution, in book *Environmental Indicators*, **2015**, p. 343–364d.
7. Butnariu, M. Plants, Functions and Processes Adapt to Different Environments, *Journal of Bioequivalence & Bioavailability* **2015**b.
8. Butnariu, M.; Samfira, I.; Sarac, I.; Negrea A.; Negrea P. Allelopathic effects of *Pteridium aquilinum* alcoholic extract on seed germination and seedling growth of *Poa pratensis*, *Allelopathy Journal*, **2015**, 35 (2), p. 227–236a.
9. Butnariu, M. Secondary Metabolites (Pterosin F and B) From *Pteridium aquilinum*, *Journal of Ecosystem & Ecography*, **2015**, 5 (2), 1a.
10. Butnariu, M.; Butu A. Chemical composition of vegetables and their products, in book: *Handbook of Food Chemistry*, **2015**, p. 627–692a.
11. Butnariu, M.; Buțu, A. Functions of collateral metabolites produced by some actinomycetes. In book *Microbial pathogens and strategies for combating them: science, technology and education* (A. Méndez–Vilas, Ed. http://www.formatex.info/microbiology4/vol2/1419–1425.pdf), **2013**, p. 1419–1425.
12. Butnariu, M.; Butu, A. The Effects on Plants by the Insecticides Obtained from *Petroleum By–Products, Petroleum Engineering Vols. I & II, Petrochemical*, Petroleum Engg./Chemical, **2015**a.
13. Butnariu, M.; Rodino, S.; Petrache, P.; Negoescu, C.; Butu M. Determination and quantification of maize zeaxanthin stability. *Digest Journal of Nanomaterials and Biostructures*. **2014**. 9 (2), p. 745–755.
14. Butnariu, M.; Samfira I. Consequences of Acid Rain, *Journal of Ecosystem & Ecography* **2014**.
15. Butu, A.; Rodino, S.; Golea, D.; Butu, M.; Butnariu, M.; Negoescu, C; Dinu–Pirvu, C.E. Liposomal nanodelivery system for proteasome inhibitor anticancer drug bortezomib, *Farmacia*. **2015**, 63(2), p. 224–229.
16. Caunii, A.; Butu, M.; Rodino, S.; Motoc, M.; Negrea, A.; Samfira, I.; Butnariu M. Isolation and Separation of Inulin from *Phalaris arundinacea* Roots, *Revista de Chimie*, **2015**, 66 (4), p. 472–476
17. Caunii, A.; Negrea, A.; Pentea, M.; Samfira, I.; Motoc, M.; Butnariu M. Mobility of Heavy Metals from Soil in the Two Species of the Aromatic Plants, *Revista de Chimie*, **2015**, 66, p. 382–368.
18. Chen, D.; Liu, C.; Ye, H.; Li, G.; Liu, B.; Meng, Y.; Chen, X., Ri–mediated Transformation of *Artemisia annua* with a Recombinant Farnesyl Diphosphate Synthase Gene for Artemisinin Production, *Plant Cell Tissue and Organ Culture,* **1999**, 57, p. 157–162.
19. Ciopec, M.; Negrea, A.; Pentea, M.; Samfira, I.; Motoc, M.; Butnariu M. Studies concerning the immobilisation and stabilization of the mining landfills, *Revista de Chimie*, **2015**, 66 (5), p. 645–653.
20. Demirci, T.; Ozdamar, P.; Baydar, N.G., In Vitro Applications For The Increasing of Root–related Secondary Metabolites Production In Medical Plants And Vegetables, *Turkish Journal of Agriculture and Forestry,* **2015**, 3, p. 261–270.
21. Dietrich, J.; Yoshikuni, Y.; Fisher, K.; Woolard, F.; Ockey, D.; McPhee, D.;

Renninger, N.; Chang, M.; Baker, D.; Keasling, J., A Novel Semi–biosynthetic Route for Artemisinin Production Using Engineered Substrate–Promiscuous P450$_{BM3}$, *Chemistry Biology,* **2009**, 4, p. 261–267.
22. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, PA.; Smith, F., Colorimetric Method for Determination of Sugars and Related Substances, *Analytical Chemistry,* **1956**, 28, p. 350–356.
23. Efferth, T.; Romero, M.; Wolf, D.; Stamminger, T.; Marin, J.; Marschall, M., The Antiviral Activities of Artemisinin and Artesunate, *Clinical Infectious Diseases,* **2008**, 47, p.804–811.
24. Geol, M.; Mehrotra, S.; Kukreja, A., Elicitor–induced Cellular and Molecular Events Are Responsible for Productivity Enhancement in Hairy Root Cultures: An Insight Study, *Applied Biochemistry Biotechnology,* **2009**, 165, p. 1342–1355.
25. Guillon, S.; Tre´ mouillaux–Guiller, J.; Pati, P.; Rideau, M.; Gantet, P., Hairy Root Research: Recent Scenario and Exciting Prospects, *Current Opinion Plant Biology,* **2006**, 9, p. 341–346.
26. Handon, J.R., Natural Products: The Secondary Metabolites, *The Royal Society of Chemistry Publication*, **2003**, p. 7–8.
27. Hernandez, G.; Vargas, V.M., Effect of Acetylsalicylic Acid On Secondary Metabolism of *Catharanthus roseus* Tumor Suspension Cultures, *Plant Cell Reports,* **1997**,16, p. 287–290.
28. Ianculov, I.; Gergen, I.; Palicica, R.; Butnariu M. The determination of total alkaloids from Atropa belladona and Lupinus sp using various spectrophotometrical and gravimetrical methods, *Revista de Chimie*, **2004**, p. 55 (11).
29. Jung, H.; Kang, S.; Kang, Y.; Kang, M.; Yun, D.; Bahk, J.; Yang, J.; Choi, M., Enhanced Production of Scopolamine by Bacterial Elicitors in Adventitious Hairy Root Cultures of *Scopolia parviflora*, *Enzyme and Microbial Technology,* **2003**, 33, p. 987–990.
30. Kishor B.P.K., *Plant Tissue Culture and Technology*. Universities publication, **1999**.
31. Klement, Z., Rapid Detection of the Pathogenicity of Phytopathogenic *Pseudomonas*, *Nature* **1963**, 4890, p. 299–300.
32. Nakase, I.; Lai, H.; Singh, N.; Sasaki, T., Anticancer Properties of Artemisinin Derivatives and Their Targeted Delivery by Transferrin Conjugation, *International Journal of Pharmacy,* **2008**, 354, p.28–33.
33. Namdeo, A.G., Plant Cell Elicitation for Production of Secondary Metabolites: A Review, *Pharmacognosy Review* **2002**, 1, 69–79.
34. Negrea, P.; Caunii, A.; Sarac, I.; Butnariu, M. The study of infrared spectrum of chitin and chitosan extract as potential sources of biomass, *Digest journal of nanomaterials and biostructures*, **2015**, 10(4), p. 1129–1138.
35. Niu, D.; Liu, H.; Jiang, C.; Wang, Y.; Wang, Q.; Jin, H.; Guo, J., The Plant Growth–promoting Rhizobacterium *Bacillus cereus* AR156 Induces Systemic Resistance In *Arabidopsis thaliana* By Simultaneously Activating Salicylate And Jasmonate/Ethylene–dependent Signaling Pathways, *Molecular Plant Microbe Interactions,* **2011**, 5, p. 533–542.
36. Pentea, M.; Butu, M.; Samfira, I.; Cristina, R.T.; Butnariu M. Extraction and analytical study of salvinorin a from leaves of *Salvia divinorum*, *Digest Journal of Nanomaterials and Biostructures*, **2015**, 10 (1), p. 291–297.
37. Petrache, P.; Rodino, S.; Butu, M.; Pribac, G.; Pentea, M.; Butnariu M.; Polyacetylene and carotenes from *Petroselinum sativum* root, *Digest Journal of Nanomaterials and Biostructures*, **2014**, 9(4), p. 1523–1527.
38. Rajendran, L.; Suvarnalatha, G.; Rawishankar, G.A.; Venkataraman, L.V., Enhancement of Anthocyanin Production in Callus Cultures of *Daucus canota* L. Under The Influence of Fungal Elicitors, *Applied Microbiology Biotechnology,* **1994**, 42, p. 227–231.
39. Rashed, K.; Butnariu, M. Antimicrobial and Antioxidant Activities of *Bauhinia racemosa* Lam. and Chemical Content. *Iranian journal of pharmaceutical research*. **2014**. 13 (3), p. 1073–1080.
40. Rashed, K.N.; Butnariu M. Isolation and antimicrobial and antioxidant evaluation of bio–active compounds

from *Eriobotrya japonica* stems. *Advanced Pharmaceutical Bulletin*. **2014**. 4 (1), p. 75–81.
41. Rodino, S.; Butu, M.; Negoescu, C.; Caunii, A.; Cristina, R.T.; Butnariu M. Spectrophotometric method for quantitative determination of nystatin antifungal agent in pharmaceutical formulations. *Digest Journal of Nanomaterials and Biostructures*, **2014**, 9 (3), p. 1215–1222.
42. Samfira I.; Gaitin, D.; Butnariu M. The microelements dynamics in a collection of varieties of Alfalfa in the Romanian Banat plain conditions. *Current Opinion in Biotechnology*, **2013**, 24, Supplement 1, S70, DOI: 10.1016/j.copbio.2013.05.190
43. Samfira, I.; Butnariu, M.; Rodino, S.; Butu, M. Structural investigation of mistletoe plants from various hosts exhibiting diverse lignin phenotypes, *Digest Journal of Nanomaterials and Biostructures*, **2014**, 8(4), p. 1679–1686.
44. Samfira, I.; Rodino, S.; Petrache, P.; Cristina, R.T.; Butu, M.; Butnariu, M. Characterization and identity confirmation of essential oils by mid infrared absorption spectrophotometry, *Digest Journal of Nanomaterials and Biostructures*, **2015**, 10(2), p. 557–565.
45. Shahtalebi, M.; Ghanadian, M.; Farzan, A.; Shiri, N.; Shokri, D.; Fatemi, S.A., Deodorant Effects of A Sage Extract Stick: Antibacterial Activity And Aensory Evaluation of Axillary Deodorancy, *Journal of Research in Medical Sciences,* **2013**,18, p. 833–839.
46. Shakeran, Z..; Keyhanfar, M.; Asghari, G.; Ghanadian M., Improvement of Atropine Production by Different Biotic and Abiotic Elicitors in Hairy Root Cultures of *Datura metel*, *Turkish Journal of Biology,* **2015**, 39, p. 111–118.
47. Silva, F.G.; Horta, L.; De Oliveira Faria, R.; Stehmann, J.; Modolo, L., Stressing Conditions As Tools To Boost The Biosynthesis of Valuable Plant Natural Products, *Recent Patents Biotechnology,* **2014**, p. 8, 89–101.
48. Smith, T.; Weathers, P.; Cheetham, R., Effect of Gibberllic Acid on Hairy Root Cultures of *Artemisia annua*: Growth and Artemisinin Production, *In Vitro Cellular and Developmental Biology,* **1997**, 33, p. 75–79.
49. Sutton, S., Microbiology Topics: Determination of Inoculum for Microbiology Testing, *Journal of GXP Compliance,* **2011**, 15, p. 49–53.
50. Varma, A.; Kharkwal, A.C., *Symbiotic fungi: Principles and Practices*, Springer Publication, Berlin Heidelberg, **2009**, p. 24–25.
51. Vasconsuelo, A.; Boland, R., Molecular Aspects of the Early Stages of Elicitation of Secondary Metabolites in Plants. *Plant Science* **2007**, 172, p. 861–875.
52. Wang, J.W.; Zhang, Z.; Tan, R.X., Stimulation of Artemisinin Production In *Artemisia annua* Hairy Roots By The Elicitor From The Endophytic *Colletotrichum* sp, *Biotechnology Letters*, **2001**, 23, p. 857–860.
53. Weathers, P.J.; Cheetham, R.D.; Follansbee, E.; Teoh, K., Artemisinin Production by Transformed Roots of *Artemisia annua*, *Biotechnology Letters,* **1994**,16, p. 1281–1286.
54. Zanousi, M.; Soleimani, T.; Keyhanfar, M.; Shirali, S.; Raeesi, M., Thin Layer Chromatography (TLC) Technique in The Investigation of Artemisinin Production in *Artemisia annua* L. Medicinal Plant Hairy Roots, *Journal of Medicinal Plants,* **2012**, 6, p. 1842–1845.
55. Zheng, L.; Guo, Y.; Wang, J.; Tan, R.X., Nitric Oxide Potentiates Oligosaccharide–induced Artemisinin Production In *Artemisia annua* Hairy Roots, *Journal of Integrative Plant Biology,* **2008**, 50, p. 49–55.

Received: February 1, 2016
Article in Press: April 23, 2016
Accepted: Last modified on: May 20, 2016