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Original Research

Influence of abiotic elicitors on improvement production of artemisinin in cell culture *of Artemisia annua* L.

Alireza Zebarjadi[1,2*], Saeideh Dianatkhah[1], Payam Pour Mohammadi[3], Ardeshir Qaderi[4]

[1] Department of Agronomy and Plant Breeding, Razi University, Kermanshah, Iran
[2] Biotechnology for Drought Tolerance Research Department, Razi University, Kermanshah, Iran
[3] Department of Agronomy and Plant Breeding, Faculty of Agriculture, Ramin University of Agriculture and Natural Resources, Ahwaz, Iran
[4] Medicinal Plant Research Center, Institute of Medicinal Plants, ACECR, Karaj, Iran

Correspondence to: zebarjadiali@yahoo.com

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Abstract: A significant sesquiterpene lactone used as a drug is artemisinin. It is definitely an anti-parasitic drug isolated from field-grown *Artemisia annua* L. a plant from Asteraceae family. It is the best treatment for *Plasmodium falciparum* malaria. Unfortunately, artemisinin content in *A. annua* is extremely low (0.01-0.8% dry weight). So, some researchers focused on enhancing artemisinin content either in tissue/cell culture or the whole plant of *A. annua* sp. The aims of the current study were the effect of plant growth regulators on callus production and improvement of artemisinin content in cell suspension culture of *A. annua,* an alternative to the whole plant using abiotic elicitors. For callus induction, an experiment was laid out as a factorial experiment with three factors (explant type, different concentrations of BAP and 2,4-D) based on completely randomized design with three replications. The maximum frequency of callus induction (100%) was found in leaf explant on MS medium with a combination of 2, 4-D (3 mg/l) and BAP (1.5 mg/l). Therefore, the best calli were used for cell suspension culture and the effects of GA3 and ABA as abiotic elicitors were evaluated on the improvement of artemisinin production. The results indicated that both ABA and GA3 increased artemisinin content (2.02 fold and 1.67 fold in comparison to control respectively) in cell suspension culture.

Key words: Artemisinin; Cell suspension culture; Plant growth regulators; *Artemisia annua*.

Introduction

Plants have just been a significant supply of pharmaceutical for thousands of years. Indeed, even today, the World Health Organization (WHO) estimates that around 80 percent of people groups still depend mostly on traditional cures, for example, herbs because of their medicines. It is evaluated that almost one-fourth of recommended drugs contain plant concentrates or active substances acquired from or exhibited on plant substances (1).

Plant secondary metabolites are noted for their significant part in the adjustment of plants with their conditions. They represent an important supply of active pharmaceuticals (2). Among secondary metabolites, sesquiterpene lactones and flavonoids are the most interesting from the pharmacological perspective. These substances are noted for their testified medical value e.g. strong anti-inflammatory, antimalarial, antioxidant, additionally; they increase immunity and reduce the chance of atherosclerosis, arthritis and gastrointestinal disorders (3-6). Recently, anticancer activity of artemisinin has been reported (7).

Among the essential sesquiterpene lactones utilized as drug artemisinin is very important, it is undeniably an anti-parasitic medication isolated from field-grown *A. annua* (8). Artemisinin has been regarded as the last line of protection against malaria for a long time (9). In 2004, the World Health Organization (WHO) proposed that artemisinin-based combination therapy (ACT) is the best treatment for *P. falciparum* malaria, and it has been also accepted in most malaria-endemic countries (10). Furthermore, in 2015, it was valued that 429 000 deaths from malaria happened globally. Nearly all of the death (99%) resulted from *P. falciparum* malaria (11). Artemisinin has been reported to store in aerial parts of the plants and seeds. Artemisinin content was found more in leaves and in florescence, but it was not found in the root, also artemisinin content in *A. annua* is really low (0.01- 0.8 % dry weight) (12). Artemisinin structure is exceptionally complex, in this manner, artemisinin and its derivatives were hard to synthesize and would not be economically competitive with the natural plant-derived artemisinin (13, 14).

Klayman (8) announced that one course of treatment needs 2-3 grams of the medication in clinical trials and that repeated repetition may demand multiple courses of treatment per year. One ton of dry *A. annua* leaves create only 6 kg of artemisinin (15). Therefore, the improved production of artemisinin either in the cell/tissue culture or in the complete plant of A. annua is highly favourable (12).

Biotechnological tools are very important for multiplication and genetic enhancement of the medicinal plants by adopting methods such as in-vitro regeneration and genetic transformations (1). The cell culture procedure is known as an admirable method to the investigation of the biosynthesis of secondary metabo-

lites in plants (16). Plant cell cultures are more profitable in comparison to cultures in a solid form medium for the reason that cells are consistently soaked in the liquid culture medium which results in tissue completely connect with the nutrients in the medium (17, 18). An effective way to improve production of secondary metabolites in field-grown plant, tissue and cell suspension culture is the using of precursors and elicitors (19). Abiotic elicitors and phytohormones like: methyl jasmonate (19-21), salicylic acid (SA) (19, 22), gibberellic acid (GA3) (23, 24), abscisic acid (ABA) (25, 26) or biotic like: chitosan (27), yeast extract (19, 28) significantly improves the assembly of artemisinin and shows the capability of biotechnology based approaches for a large-scale assembly of artemisinin, also several precursors such as sodium acetate (29), artemisinic acid (30), mevalonic acid lactone and casein acid hydrolysate (31) have been examined to enhance artemisinin in *A. annua.* Therefore the aims of current study were optimizing callus production and use of abiotic elicitors to enhance the production of artemisinin in the cell suspension cultures of *A. annua*.

Materials and Methods

Plant materials and growth conditions

Seed's of *A. annua* were collected from the north of Iran. They were sterilized by 70% alcohol treatment for 1 minute and followed soaking in 2.5% sodium hypochlorite for 10 minutes, then the surface-sterilized seeds were washed with sterile distilled water for three times. Then the seeds were cultured on Murashige & Skoog (32) as a basal medium without any plant growth regulators and they were incubated in a growth chamber at 25±2 ˚C with fluorescent tubes with 16 h photoperiod.

Protocol for callus induction

The leaves and stems from 2 weeks old plants of *A. annua* were cut and used as source of explants, then explants were cultured on petri dishes containing solidified MS medium supplemented with different combinations of 6-Benzylaminopurin (BAP) and 2,4-Dichlorophenoxyacetic acid (2-4, D), as follows: (BAP: 0, 0.5, 1.5 mg/l and 2-4, D: 0, 1, 2, 3, 4 mg/l). The pH of the media was adjusted to 5.7- 5.8 before adding agar then sterilized by autoclaving at 121˚C for 20 minutes. All media with sucrose (30 mg/l) and agar (7 mg/l) were incubated in a growth chamber at 25±2 ˚C under dark conditions. The Calli were transferred to fresh media every 2 weeks.

Cell suspension culture

After 35 days, the best calli (fresh and breakable) were used to establish the cell suspension of *A. anuua*. The calli with a weight of 1±0.5 g were transferred into 500 ml sterilized flasks, containing 100 ml liquid medium with sucrose 30 g/l, BAP 1.5 mg/l, and 2,4-D 3 mg/l. The flasks were continuously shaken on the shaker with rate 120 rpm. The cultures were maintained in the culture room with a temperature of 25±2°C and dark conditions.

Induction of artemisinin production

To increase the artemisinin production in the cell suspension of *A. annua*, different concentrations of ABA and GA3 were used as abiotic elicitors. After 45 days, in order to investigate the effect of different concentrations ABA and GA3 treatments on artemisinin content, fresh cells were transferred into liquid MS medium supplemented with ABA (5 and 10 mg/l) and GA3 (5 and 10 mg/l). After 5 days, the content of artemisinin in each concentration and inducer elicitors was measured by high-performance liquid chromatography (HPLC). For analysis of artemisinin content, 100 mg of dried and powdered cells were permitted to drench 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. Then was treated with 4.0 ml NaOH (0.2% w/v) and incubated at 50±2C for one hour with shaking.

Data analysis

All experiments were analyses based on a completely randomized design with three replications and 10 explants in each petri dish. Means were compared using Duncan's multiple range tests at the 1% probability level ($p \leq 0.01$). The computations were made using the SAS statistical analysis package (SAS Inc. Cary, USA). All residuals data were checked for normal distribution using R software before statistical analysis.

Results

The seeds of *A. annua* cultured on MS medium were germinated within 10- 14 days under *in vitro* conditions. After fourteen days the explants began callus development (Fig. 1). The results of analysis of variance (ANOVA) for callus formation were presented in Table 1. These results were indicated that callus induction was variable and depended on applied concentration and combination of plant growth regulators. The results of ANOVA demonstrated that there was the non-signifi-

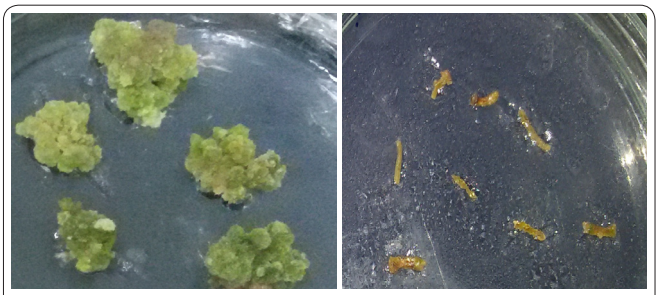

Figure 1. Callus induction on MS medium including 3 mg/l 2, 4-D and 1.5 mg/l BAP (Left) and into control medium (Right).

Table 1. Analysis of variance for effects of explant types and PGRs on callus induction in *A. annua*.

Source of variations	Df	Mean of Squares
2,4-D	4	7486 **
BAP	2	19670 **
Explant	1	1.6 ns
2,4-D * BAP	8	854 **
Explant * 2,4-D	4	1244 **
Explant * BAP	2	1887 **
Explant * BAP * 2,4-D	8	788 **
Error	60	282

Df: Degree of freedom, ns: Non-significant, **: Significant difference at $p \leq 0.1$. 0.

Table 2. Effect of different concentrations of plant growth regulators on percent callus formation from leaf and stem explants of *A. annua* L.

explant	Growth regulator (mg/l)		Callus formation (%)
	BAP	2,4-D	Mean
Leaf	0	0	0^{i}
	0	1	$20^{h\ i}$
	0	2	$16^{h\ i}$
	0	3	$24^{h\ i}$
	0	4	$37^{f\ g\ h}$
	0.5	0	0^{i}
	0.5	1	$24^{h\ i}$
	0.5	2	$37^{f\ g\ h}$
	0.5	3	$41^{e\ f\ g\ h}$
	0.5	4	$62^{c\ d\ e\ f}$
	1.5	0	$12^{h\ i}$
	1.5	1	$83^{a\ b\ cd}$
	1.5	2	$87^{a\ b\ c\ d}$
	1.5	3	100^{a}
	1.5	4	100^{a}
stem	0	0	0^{i}
	0	1	$45^{e\ f\ gh}$
	0	2	20^{hi}
	0	3	$29^{g\text{-}i}$
	0	4	20^{hi}
	0.5	0	25^{hi}
	0.5	1	$66^{b\text{-}f}$
	0.5	2	$41^{e\text{-}h}$
	0.5	3	95^{ab}
	0.5	4	20^{hi}
	1.5	0	24^{hi}
	1.5	1	$58^{d\text{-}g}$
	1.5	2	$69^{a\text{-}e}$
	1.5	3	$91^{a\text{-}c}$
	1.5	4	100^{a}

Means in each column followed by different letter (s) had significant difference at $p \leq 0.1.0$.

cant difference between explants types for callus formation, but there were significant differences among main levels of BAP, 2,4-D concentrations and their interactions ($p<0.01$) (Table 1).

Means comparison for triple interactions (Explant * BAP * 2, 4-D) was done (Table 2). The results were indicated the explants cultured on medium without growth regulators (control) did not show callus formation. According to table 2, the percentage of callus induction ranged from 12 to 100%. The maximum percentage of callus induction (100%) occurred on MS medium with combinations of 2, 4-D (3 and 4 mg/l) and BAP (1.5 mg/l) for leaf explants and for stem explants in combinations of 2, 4-D (4 mg/l) and BAP (1.5 mg/l).

Influence of ABA and GA3 treatments on artemisinin content

The data was analyzed using means comparison and indicated significant differences among the treatments (ABA: 0, 5 and 10 mg/l as an elicitor) at 5% level of

Table 3. The effect of concentration of abiotic elicitor (ABA) on artemisinin content in *A. annua* cell culture.

Artemisinin (mg/g)	Elicitor (mg/l)
Mean (mg/g)	ABA
5.36^{b}	0
6.80^{b}	5
10.86^{a}	10

Means in each column followed by different letter (s) are significantly different at 5% level, analyzed by Duncan's multiple range tests.

Table 4. The effect of concentration of abiotic elicitor (GA3) on artemisinin content in *A. annua* cell culture.

Artemisinin (mg/g)	Elicitor (mg/l)
Mean (mg/g)	GA3
5.36^{b}	0
6.5^{b}	5
8.98^{a}	10

Means in each column followed by different letter (s) are significantly different at 5% level analyzed by Duncan's multiple range tests.

significance by Duncan's multiple range tests (Table 3). This result was showed that the ABA have significant effect on artemisinin content. It was observed maximum increasing (2.02 fold) when ABA used in 10 mg/l as an abiotic elicitor in MS medium.

According to Table 4, it was defined that content of artemisinin as a secondary metabolite in GA3 (Gibberellin) 5 and 10 mg/l was more than control and the amount of this metabolite increased in concentration of 10 mg/l of GA3. The maximum of increasing was about 1.67 fold (Fig. 2 and 3).

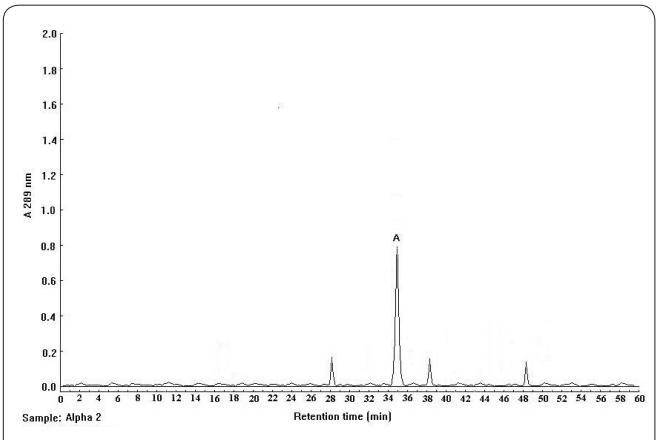

Figure 2. HPLC results of the effect of GA3 on artemisinin content in *A. annua* (check).

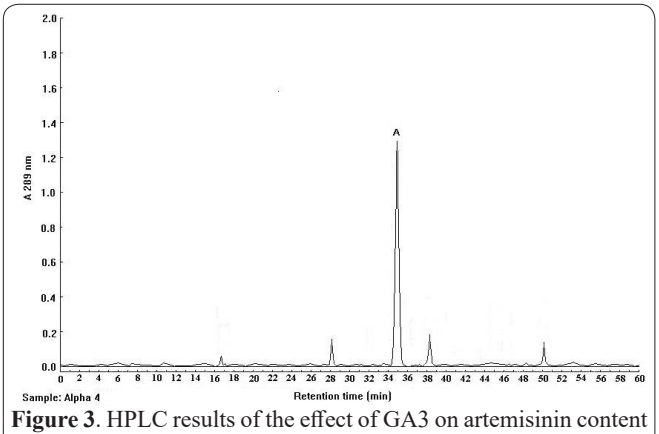

Figure 3. HPLC results of the effect of GA3 on artemisinin content in *A. annua* (in 10 mg/l GA3 treatment).

Discussion

Increasing content of artemisinin either in cell/tissue culture or in the complete plant of *A. annua* is highly favourable (12). *In vitro* culture of *Artemisia* species have been the object of some researches (33-35)**.** Aslam *et al*. (34), announced the maximum frequency of callus formation (80-100) was obtained from leaf explants of *A. annua* were cultured on MS medium supplemented with 2, 4-D and NAA. Gansan and Paulsamy (35), reported that the BAP and 2, 4-D induced callus formation in *A. annua* L. (40-55%), this result was in agreement with our results. Dangash *et al.* (33) reported that the highest percentage of callus induction was obtained from explants cultured on MS+ 0.5 mg/l BAP + 0.5, 1.5 mg/l 2, 4-D in *A. annua*. The ability of 2,4-D along and in combination with cytokinins in callus formation might be for their function in DNA synthesis and mitosis (36). External factors such as nutrient levels, stress factors, light and growth regulators were affected on numerous secondary metabolite pathways. Artemisnin content is known to be seriously affected by both genotype and external factors. Enhancement of artemisinin production by biotic and abiotic elicitors has been reported in *A. annua* plant (19, 25, 37). Abscisic acid (ABA), characterized as a stress hormone, plays a substantial role in responses to biotic and abiotic stresses including drought, salt, osmotic, and cold stress (38). ABA acts through a complex signaling cascade to induce changes in gene expression and in adaptive physiological responses (26). They reported that artemisinin content increased by ABA treatment. Spraying ABA onto *A. annua* plant and transgenic *A. annua* whit ABA receptor also salt stress can increase the production of artemisinin by inducing the expression of key genes in artemisinin biosynthesis pathway. In addition, it was reported that ABA could stimulate the production of some secondary metabolites (39). According to Jing *et al* (40) artemisinin content in plants treated by 10 μM ABA was 65% higher than that in the control plants, and gene expression analysis indicated that in both the ABA-treated plants and cell suspension cultures, expression of the important genes in the artemisinin biosynthetic pathway, were significantly boosted. Gibberellic acid, a plant hormone that can induce blooming, has been reported to improve growth and artemisinin biosynthesis in shoot cultures, root cultures, cell culture and plantlets of *A. annua* (19, 41-45). The current study indicated GA3 also improved artemisinin production compared to control and maximum content (8.96 mg/L) was obtained (1.67 fold) when GA3 (10 mg/l) was added. Since GA is known to induce flowering in some plants (46), and maximum artemisinin accumulation in field-grown crops occurs at or near the onset of flowering in most strains (44, 47-49). Development of artemisinin content and leaf biomass in *A. annua* by exogenous GA3 treatment were reported by Banyai et al. (23). The GA3 increased synthesis of key enzymes in the artemisinin biosynthesis pathway. Remarkably, exogenous GA3 application constantly enhanced the artemisinin content from the vegetative stage to the flower initiation in both plant lines and have a significantly higher leaf biomass than in the control plants. Subsequently, the artemisinin yield in GA3-treated plants was much higher than in the control plants. Therefore, increased artemisinin content from exogenous GA3 treatment was related with increased expression of key enzymes in the artemisinin biosynthesis pathway. The current study indicated that artemisinin production continuously increased until 96 h after application of GA3.

There was the non-significant difference between explant types but there were significant differences among levels of plant growth regulators and interactions for callus formation. Finally, in *A. annua* it was found that the most important secondary metabolite (artemisinin) content of calli derived explants could be enhanced by abiotic elicitors application.

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Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

References

1. Tripathi L, Tripathi JN. Role of biotechnology in medicinal Plants. Trop J Pharm Res 2003; 2(2): 243-253.
2. Grech-Baran M, Pietrosiuk A. Artemisia species *in vitro* cultures for production of biologically active secondary metabolites. Bio Technologia 2012; 93(4): 371-380.
3. Liu CZ, Murch SJ, El-Demerdash M, Saxena PK. *Artemisia judaica* L. micropropagation and antioxidant activity. J Biotechnol 2004; 110: 63-71.
4. Stojanowska A. *In vitro* cultures of some plants from the Asteraceae family as a source of bioactive molecules. International Conference of Perspectives Cytostatic Compounds Production Using Biotechnological Methods, Warsaw 13 April 2010; 152.
5. Kazemi M, Dakhili M, Dadkhah A, Yasrebifar Z, Larijani K. Composition, antimicrobial and antioxidant activities of the essential oil of *Artemisia kermanensis* Podl., an endemic species from Iran. Journal of Medicinal Plants Research 2011; 5(18): 4481-4486.
6. Nakase I, Lai H, Singh NP, Sasaki T. Anticancer properties of artemisinin derivatives and their targeted delivery by transferrin conjugation. Int J Pharm 2008; 342(1-2): 28-33.
7. Slezakova S, Ruda-Kucerova J. Anticancer activity of artemisinin and its derivatives. Anticancer Res 2017; 37: 5995–6003.
8. Klayman DL. Qinghaosu (Artemisinin) an anti-malarial drug from China. Science 1985; 228: 1049-1055.
9. Bryant L, Flatley B, Patole C. Proteomic analysis of *Artemisia annua*—towards elucidating the biosynthetic pathways of the anti-malarial pro-drug artemisinin. BMC Plant Biol 2015; 15: 175.
10. Hommel M. The future of artemisinins: natural, synthetic or recombinant. J Biol Sci 2008; 7: 38-42.
11. WHO. World Malaria Report. http://www.who.int/malaria/publications/world-malaria-report-2016/report/en.
12. Abdin MZ, Israr M, Rehman RU, Jain SK. Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. Planta Medica 2003; 69: 289-229.
13. Xu XX, Zhu J, Huang DZ, Zhou WS. Total synthesis of artemisinin and deoxyartemisinin. Tetrahedron 1986; 42: 819-828.
14. Zhao Y, Hantan WK, Lee KH. Antimalarial agents, artesunate,

an inhibitor of cytochrome oxidase activity in *Plasmodium berghei*. J Nat Prod 1986; 49: 139-142.
15. Van Geldre E, Vergauwe A, Van den E. State of the art of the production of the antimalarial compound artemisinin in plants. Plant Mol Biol 1997; 33: 199–209
16. Ramachandra Rao S, Ravishankar GA. Plant cell cultures: Chemical factories of secondary metabolites. Biotechnol Adv 2002; 20(2): 101-153.
17. Soomro R, Memon RA. Establishment of callus and suspension culture in *Jatropha curcas*. Pakistan Journal of Botany 2007; 39(7): 2431-2441.
18. Hippolyte I, Marin B, Baccou J, Jonard R. Growth and rosmarinic acid production in cell suspension cultures of *Salvia officinalis* L. Plant Cell Rep 1992; 11(3): 109-112.
19. Baldi A, Dixit VK. Yield enhancement strategies for artemisinin production by suspension cultures of *Artemisia annua*. Cur Trends Biotechnol Pharm 2008; 99: 4609-4614.
20. Yu ZX, Lia JX, Yang CQ, Hu WL, Wang LJ, Chen XY. The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua* L. Molecular Plant 2012; 5(2): 353-365.
21. Xiang L, Zhu S, Zhao T, Zhang M, Liao Z. Enhancement of artemisinin content and relative expression of genes of artemisinin biosynthesis in *Artemisia annua* by exogenous MeJA treatment. Plant Growth Regul 2014; 75(2): 435-441.
22. Pu GB, Ma DM, Chen JL et al. Salicylic acid activates artemisinin biosynthesis in *Artemisia annua* L. Plant Cell Rep 2009; 28: 1127–1135.
23. Banyai W, Mii M, Supaibulwatana K. Enhancement of artemisinin content and biomass in *Artemisia annua* by exogenous GA3 treatment. Plant Growth Regul 2011; 63: 45–54.
24. Weathers PJ, Elkholy S, Wobbe KK. Artemisinin: the biosynthetic pathway and its regulation in *Artemisia annua*, a terpenoid-rich species. In Vitro Cell Dev Biol Plant 2006; 42: 309-317.
25. Jing F, Zhang L, Li M et al. Abscisic acid (ABA) treatment increases artemisinin content in *Artemisia annua* by enhancing the expression of genes in artemisinin biosynthetic pathway. Biologia 2009; 64(2): 319-323.
26. Zhang F, Lu X, Lv Z et al. Overexpression of the Artemisia orthologue of ABA receptor, AaPYL9, enhances ABA sensitivity and improves artemisinin content in *Artemisia annua* L. PLoS One 2013; 8(2): e56697.
27. Putalun W, Luealon W, De-Eknamkul W, Tanaka H, Shoyama Y. Improvement of artemisinin production by chitosan in hairy root cultures of *Artemisia annua* L. Biotechnol Lett 2007; 29: 1143–1146.
28. Darwati I, Manohara D, Rohimatun, Nurhayati H. The application of biotic elicitor on *Artemisia annua* L. to increase artemisinin content. IOP Conf. Ser.: Earth Environ Sci 102 012013; 2017.
29. Zeng Y, Yan F, Tang L, Chen F. Increased crocin production and induction frequency of stigma-like structure from floral organs of *Crocus sativus* by precursor feeding. Plant Cell Tiss Org Cult 2003; 72: 185-191.
30. Arsenault PR, Vail DR, Wobbe KK, Erickson K, Weathers PJ. Reproductive development modulates gene expression and metabolite levels with possible feedback inhibition of artemisinin in *Artemisia annua* L. Plant Physiol 2010; 154: 958–968.
31. Woerdenbag HJ, Lfiers JFJ, van Uden W, Pras N, Malingr TM, Alfermann AW. Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L. Plant Cell Tiss Org Cult 1993; 32: 247-257.
32. Murashige T, Skoog FK. Revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 1962; 15(3): 473-497.
33. Dangash A, Ram M, Niranjan R, Bharillya A, Misra H. *In vitro* selection and hormonal regulation in cell culture of *Artemisia annua* L. Plant. JSM Cell Dev Biol 2015; 3(1): 1-7.
34. Aslam N, Zia M, Chaudchary MF. Callogenesis and direct organogenesis of *Artemisia scoparia*. Pak J of Biol Sci 2006; 9(9): 1783-1786.
35. Ganesan CM, Paulsamy S. Standardized protocol for the *in vitro* culture of *Artemisia annua* L. – A medicinal plant at high altitudes of Nilgiris, the Western Ghats. Journal of Research in biology 2011; 1(3): 173-178.
36. Sen MK, Nasrin S, Rahman S, Jamal AHM. *In vitro* callus induction and plantlet regeneration of *Achyranthes aspera* L., a high value medicinal plant. Asian Pac J Trop Biomed 2014; 4(1): 40-46.
37. Zare- Mehrjerdi M, Bihamta M, Omidi M, Naghavi M, Soltanloo H. Effects of exogenous methyl jasmonate and 2 isopentenyladenine on artemisinin production and gene expression in *Artemisia annua*. Tubitak 2013; 37: 499-505.
38. Smet ID, Zhang H, Inze D, Beeckman T. A novel role for abscisic acid emerges from underground. Trends Plant Sci 2006; 11: 434-439.
39. Smith JI, Smart NJ, Kurz WG, Misawa M. Stimulation of indole alkaloid production in cell suspension cultures of *Catharanthus roseus* by abscisic acid. Planta Med 1987; 53: 470-474.
40. Jing F, Zhang L, Li M et al. Abscisic acid (ABA) treatment increases artemisinin content in *Artemisia annua* by enhancing the expression of genes in artemisinin biosynthetic pathway. Biologia 2009; 64(2): 319-323.
41. Fulzele DP, Heble MR, Rao PS. Production of terpenoid from *Artemisia annua* L. plantlet cultures in bioreactor. J Biotechnol 1995; 40: 139-143.
42. Weathers PJ, Bunk G, Mccoy MC. The effect of phytohormones on growth and artemisinin production in *Artemisia annua* hairy roots. In Vitro Cell Dev Biol Plant 2005; 41: 47-53.
43. Zhang YS, Ye HC, Liu BY, Wang H, Li GF, Russian J. Exogenous GA and flowering induce the conversion of artemisinic acid to artemisinin in *Artemisia annua* plants. Plant Physiol 2005; 52: 58-62.
44. Woerdenbag HJ, Pras N, Chan NG et al. Artemisinin, related sesquiterpenes, and essential oil in *Artemisia annua* during a vegetation period in Vietnam. Planta Med 1994; 60: 272-275.
45. Smith TC, Weathers PJ, Cheetham RC. Effect of gibberellic acid on hair root cultures of *Artemisia annua* growth and artemisinin production. In Vitro Cell Dev Biol Plant 1997; 33: 75-73.
46. Crozier A, Kamiya Y, Bishop G, Yokota T. Biosynthesis of Hormones and Elicitor Molecules. In: Biochemistry and molecular biology of plants. Buchanan B., Guissem W., and R Jones. eds. American Society of Plant Physiologist, Rockville, MD 2000: 850-929.
47. Bunk GJ. Hormone influences on growth and secondary metabolite production in *Artemisia annua*. M.Sc. Thesis. Worcester Polytechnic Institute, Worcester, MA USA 1997.
48. Morales MR, Charles DJ, Simon JE. Seasonal accumulation of artemisinin in *Artemisia annua* L. Acta Hortic 1993; 344: 416-420.
49. Ferreira JFS, Simon JE, Janick J. Developmental studies of *Artemisia annua*: Flowering and artemisinin production under greenhouse and field conditions. Planta Med 1995; 61: 167-170.