Research Journal of Pharmacognosy (RJP) 1(3), 2014: 15-20
Received: Mar 2014
Accepted: Apr 2014

Original article

Tissue culture and regeneration of an antimalarial plant, *Artemisia sieberi* Besser

A. Sharafi[1*], H. Hashemi Sohi[2], A.A. Sharafi[3], P. Azadi[4], A. Mousavi[2]

[1]*Pharmaceutical Biotechnology Department, School of Pharmacy, Zanjan University of Medical Sciences, P. O. Box 45195-1338, Zanjan, Iran.*
[2]*National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran.*
[3]*Novin Giti Gene Biotech. Co. (NGene Biotech. Co.), Biotechnology Incubator Center of NIGEB, P. O. Box 1417863171.Tehran, Iran.*
[4]*Tissue Culture and Genetic Engineering Department, Agricultural Biotechnology Research Institute of Iran (ABRII), P. O. Box 31535-1897, Karaj, Iran.*

Abstract

WHO recommends artemisinin-based combination therapies (ACTs) as the most effective choice to treat malaria. For developing transgenic plants with high accumulation of artemisinin (by introducing genes encoding enzymes which regulate the biosynthetic pathway of artemisinin), an efficient protocol for tissue culture and plant regeneration is necessary. In the present study, leaf explants of *Artemisia sieberi* were cultivated in Murashige & Skoog based medium supplemented by combination of different plant growth regulators including 6-benzyl-aminopurine (BA), α-naphthalene-acetic acid (NAA), indole-3-acetic acid (IAA), picloram (Pic) and 2,4-dichlorophenoxyacetic acid (2,4-D). The highest frequency of shoot induction was obtained on MS medium supplemented with 2 mg/L BA plus 0.05 mg/L NAA (95% regeneration) and MS medium supplemented with 2 mg/L BA plus 0.5 mg/L IAA (85% regeneration). Rooting was obtained on MS medium supplemented with 0.05 mg/L NAA. The present study has revealed a simple, reliable, rapid and high efficient regeneration system for *A. sieberi* Besser as a source of artemisinin in short period via adventitious shoot induction procedure.

Keywords*: Artemisia sieberi* Besser, artemisinin, regeneration, tissue culture

Introduction

627000 malaria deaths have been estimated in 2012 worldwide [1] and world malaria report in 2013 has indicated that 3.3 million malaria deaths were reported between 2001 and 2012. Approximately 90% of the estimated deaths had occurred in the sub-Saharan African region. This has resulted in pressure on economy of African countries. International funds for malaria control has continued to rise, to a peak of 1.97 US$ billion in 2013. The amounts committed by

*Corresponding author: alisharafi@zums.ac.ir, Tel/Fax: +98-21-44580436

WHO to malaria have been estimated to be more than 2.85 US$ billion per year during 2014-2016. WHO has recommended artemisinin-based combination therapies (ACTs) as the most effective choice to treat malaria. In 2012, 79 countries and territories had adopted ACT as the first-line treatment of multi-drug resistance *Plasmodium falciparum* caused malaria. The number of ACT treatment courses have increased greatly from 11.2 million in 2005 to 331 million in 2012 [1]. While artemisinin-based therapy is very effective against malaria, it is too expensive for many people in African countries due to the high demand and low production.

Artemisinin is produced in a few *Artemisia* species (mainly *Artemisia annua* L. and *A. sieberi* Besser). *A. sieberi* is an aromatic plant which has been naturally distributed in the semi-desert regions of Iran [2]. As artemisinin cannot be synthesized chemically in an economically feasible way due to its complex chemical structure, most of artemisinin-related interests have focused on *A. annua* which contains low amounts of artemisinin (less than 1.2% of its dry weight) [3]. However, the presence of artemisinin in *A. sieberi* has been shown to be in comparable levels with *A. annua* (Arab et al. 2006). Nahrevanian *et al.* [4] have demonstrated the first application of *A. sieberi* extracts in treatment of murine malaria in mice. Applying high concentrations of the herbal extract has not shown any toxicity, approving its minimal side effects. Moreover, one of the artemisinin derivatives, dihydroartemisinin has exhibited selective toxicity toward human breast cancer cells [5]. It has been used in treatment of a range of cancer cell lines, including human leukemia, colon and small-cell lung carcinomas [6]. It has also appeared to be effective in treating coccidial infection in broiler chickens [2].

The supply of artemisinin is far from enough in the international markets. The relatively low yield of artemisinin in *A. annua* L. and *A. sieberi* limits the commercialization of the drug. Some efforts focused on enhancing the production of artemisinin have been performed for a long time [7]. In recent years, there has been more progress in the molecular regulation of artemisinin biosynthesis. The genes of the key enzymes involved in the biosynthesis of artemisinin such as farnesyle diphosphate synthase (FPS) and amorpha-4,11-dine synthase (AMS) and the genes of the enzymes relevant to the biosynthesis of artemisinin such as squalen synthase (SQS), have been over-expressed in *A. annua* [7,8]. However, efforts in these cases have been restricted by the lack of efficient protocols for genetic transformation of *A. annua* [9]. Developing transgenic plants with high accumulation of artemisinin can be achieved by introducing genes encoding enzymes regulating the biosynthetic pathway leading to the formation of artemisinin. For successful transformation, an efficient protocol for tissue culture and regeneration is necessary. The composition of plant growth regulators in the culture medium has directed the callus morphotype [10,11] and there have been some successful reports on tissue culture and regeneration of *A. annua* [8,12,13] but there has been no report on tissue culture and plant regeneration of *A. sieberi* Besser.

In the present study, we have described a simple, efficient and reliable regeneration protocol for the medicinal plant *A. sieberi* for the first time, which has a high potential to be used in genetic transformation of this important medicinal plant.

Experimental

Plant material and tissue culture conditions

Seeds of *A. sieberi* were collected from Yazd, Iran. They were sterilized by soaking in 5% sodium hypochlorite for 20 minutes after immersing in 70% ethanol (2 minutes). The seeds were rinsed (at least 30 minutes) with sterile distilled water and then germinated under aseptic condition on agar-solidified MS medium [14]. The pH of the medium was adjusted to 5.8 before adding agar and then sterilized by autoclaving at 121 °C for 20 minutes. The seeds were incubated in a growth chamber under a 16/8 h (light/dark) photoperiod under cool-white light (40-50 µmol m^{-2} s^{-1}) at 25 °C. The leaves from three weeks

old plants were cut and used as the explant.

Tissue culture and regeneration media

The leaves from three weeks old plants were cut and cultured on petri dishes containing solidified MS medium supplemented with different combination of BA, NAA, IAA, 2,4-D and picloram as follows: BA (0, 1, 2 mg/L) plus NAA (0.05, 0.1 mg/L); BA (1, 2, 3 mg/L) plus IAA (0, 0.1, 0.5 mg/L); BA (0, 1, 2 mg/L) plus 2,4-D (0.5, 1, 1.5 mg/L); BA (0, 0.25 mg/L) plus picloram (1.5, 3, 4.5, 6, 7.5 mg/L).

The petri dishes were sealed and incubated in a growth chamber at 25 °C under standard cool white fluorescent tubes with 16 h photoperiod. Explants were transferred to fresh medium every 2 weeks. After callus induction, explants were transferred to MS medium without hormones or supplemented with 0.5 or 1 mg/L BA for shoot induction. Obtained shoots were cut and transferred to MS medium supplemented with 0.05 mg/L NAA for root induction.

Statistical analysis

The experiments were based on a completely randomized design with 4 replications and 12 explants in each petri dish. Means were compared using Duncan's multiple range tests at the 5% probability level ($p \leq 0.05$). The computations were made using the SAS statistical analysis package (SAS Inc. Cary, USA). All percentage data were checked for normal distribution and subjected to arc sine ($\sqrt{x}$) transformation before statistical analysis. The frequency of callus induction or shoot regeneration was defined as the number of explants which induced callus or adventitious shoots per all explants, respectively.

Results and Discussion

Three weeks seedlings leaves were cut and used as explants (figure 1a). They were cultured on callus induction media for regeneration. The explants were transferred to fresh medium every two weeks to avoid browning. After 2-3 sub-cultures, the callus appeared on most of the explants (figure. 1b,c). The explants were transferred to MS medium without hormone or supplemented with 0.5 or 1 mg/L BA for shoot induction. In these media, regeneration was appeared after 3 weeks (figure 1d-f). High frequency of regeneration was observed in MS medium containing 2 mg/L BA and 0.05 mg/L NAA (95% regeneration). The calli originated from combination of 2 mg/L BA and 0.5 mg/L IAA showed the highest value of plant regeneration (85% regeneration), while in combination of BAP and 2, 4-D, a low rate of regeneration was observed.

The characteristics of shoot induction in our study were different from those in Chen *et al.* (2000) on *A. annua*; whereas, leaf segments were used in *A. annua* for shoot induction [15], the whole leaf was used in our study. On the other hand, wounded leaf stalks of *A. annua* produced higher shoot induction [5].

Shoot elongation was obtained in MS medium supplemented with 0.5 mg/L GA_3, while vitrification was reduced. The elongated shoots were transferred to root induction medium containing 0.2 mg/L NAA for 2 weeks. The plantlets were transferred to MS medium without hormone for more growth (figure 1 g, h). Media containing 2 mg/L BA plus 0.05 mg/L NAA and 2 mg/L BA plus 0.5 mg/L IAA showed the highest frequency of regeneration 95% and 85.6%, respectively (figure 2). The plantlets were acclimatized in greenhouse (figure 1i).

A. sieberi biodiversity is endangered because of severe grazing and collection by people to be used in folk medicine. Therefore, this species should be conserved and protected. Sharaf *et al.* [16] have reported two cryopreservation methods to conserve the shoot tips of this plant. Besides, *in vitro* regeneration can be used as a strong tool in conservation for producing high number of genetically alike plants.

The present protocol has introduced a simple and rapid tissue culture system for high frequency regeneration of *A. sieberi* via shoot induction.

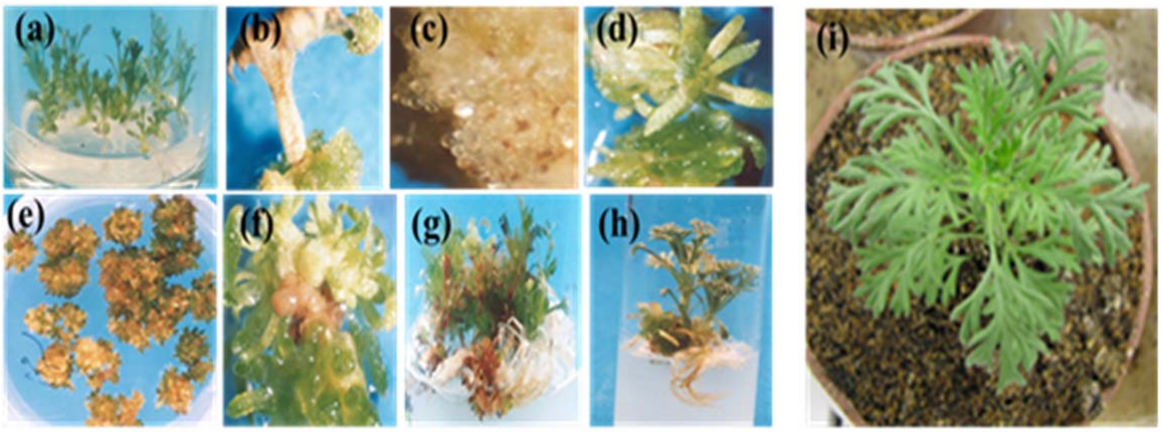

Figure 1. Tissue culture and plant regeneration of *Artemisi sieberi*; (a) three weeks old plants in MS medium; (b) leaf explant; (c) callus induction in MS medium containing 2 mg/L BA and 0.5 mg/L IAA; (d) shoot induction in medium containing 2 mg/L BA and 0.5 mg/L IAA; (e, f) shoot induction in medium containing 2 mg/L BA and 0.05 mg/l NAA; (g, h) root induction; (i) transferring regenerated plant to soil

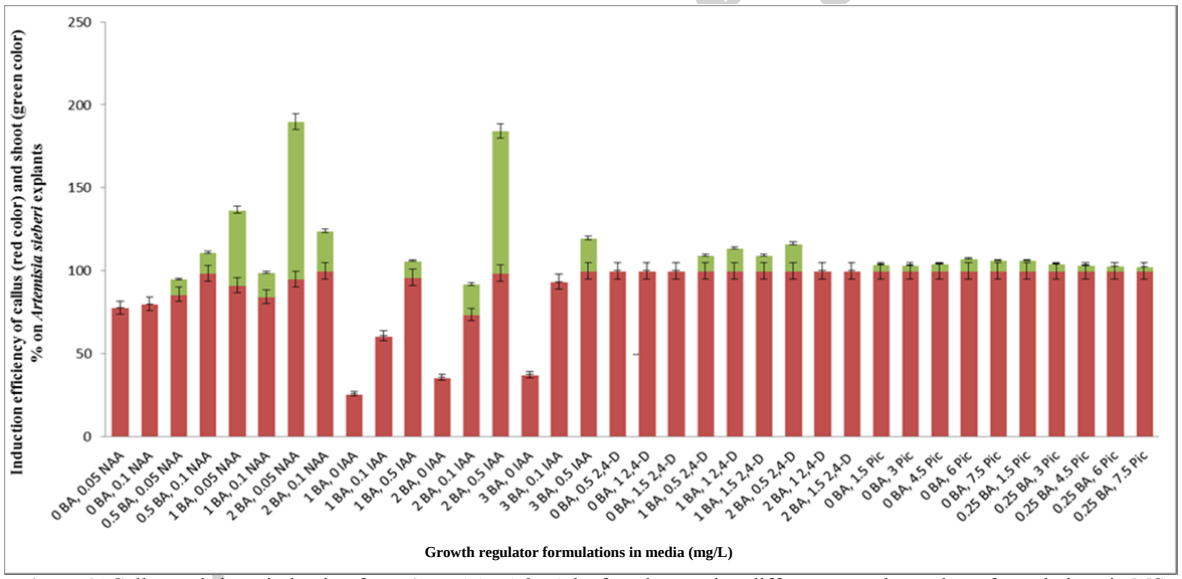

Figure 2. Callus and shoot induction from *Artemisia sieberi* leaf explants using different growth regulator formulations in MS media

Further studies are in progress in our group to engineer *A. sieberi* using genes of the key enzymes involved in the biosynthesis of artemisinin.

Acknowledgments

This work was supported by Novin Giti Gene Biotech. Co. (*NGene Biotech. Co.*), www.ngene.ir, Biotechnology Incubator Center of National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

References

[1] Malaria report (2013), World Health Organization (WHO); Available from: http://www.who.int/malaria/publications.

[2] Arab HA, Rahbari S, Rassouli A, Moslemi MH, Khosravirad F. Determination of artemisinin in *Artemisia sieberi* and anticoccidial effects of the plant extract in broiler chickens. *Trop Anim Health Pro.* 2006; 38: 497-503.

[3] Kindermans JM, Pilloy J, Olliaro P, Gomes M. Ensuring sustained ACT production and reliable artemisinin supply. *Malaria J*. 2007; 6: 125; Doi: 10.1186/1475-2875-6-125.

[4] Nahrevanian H, Milan BS, Kazemi M, Hajhosseini R, Mashhadi SS, Nahrevanian S. Antimalarial effects of Iranian flora *Artemisia sieberi* on *Plasmodium berghei in vitro* in mice and phytochemistry analysis of its herbal extracts. *Malar Res Treat.* 2012; Doi:10.1155/2012/727032.

[5] Han JL, Wang H, Ye HC, Liu Y, Li ZQ, Zhang Y, Zhang YC, Yan F, Li GF. High efficiency of genetic transformation and regeneration of *Artemisia annua* L. via *Agrobacterum tumefaciens* mediated procedure. *Plant Sci*. 2005; 168: 73-80.

[6] Efferth T, Dunstan H, Sauerbrey A, Miyachi H, Chitambar CR. The antimalarial artesunate is also active against cancer. *Int J Oncol*. 2001; 18: 767-773.

[7] Chen DH, Chen H, Guo Y, Li F. Expression of a chimeric farhesyl diphosphate synthase gene in *Artemisia annua* transgenic plants via *Agrobacterium* mediated transformation. *Plant Sci*. 2006; 155: 179-183.

[8] Banyai W, Kirdmanee C, Mii M, Supaibulwatana K. Overexpression of farnesyl pyrophosphate synthase (FPS) gene affected artemisinin content and growth of *Artemisia annua* L. *Plant Cell Tiss Org*. 2010; 103: 255-265.

[9] Covello PS. Making artemisinin. *Phytochemistry*. 2008; 69: 2881-2885.

[10] Ntui VO, Azadi P, Supaporn H, Mii M. Plant regeneration from stem segment-derived friable callus of "Fonio"(*Digitaria exilis* (L.) Stapf.). *Sci Hortic*. 2010; 125: 494-499.

[11] Azadi P, Beirami Zadeh E, Ntui VO A. simple protocol for somatic embryogenesis in *Rosa hybrida* L. cv. Apollo. *J Hortic Sci Biotech*. 2013; 88: 399-402.

[12] Han JL, Liu By, Ye HC, Wang H, Li ZQ, Li GF. Effects of overexpression of the endogenous farnesyl diphosphate synthase on the artemisinin content in *Artemisia annua* L. *J Integr Plant Biol.* 2006; 48: 482-487.

[13] Sharafi A, Hashemi Sohi H, jourabchi E. Tissue culture and regeneration of *Artemisia annua*. *Iran J of Biol*. 2007; 21: 565-573.

[14] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. *J Physiol Plant*. 1962; 15: 473-497.

[15] Chen DH, Ye HC, Li GF. Expression of a chimerical farnesyl diphophate synthase gene in *Artemisia annua* transgenic plant via *Agrobacterium tumefaciens* mediated transformation. *Plant Sci*. 2000; 155: 179-185.

[16] Sharaf SA, Shibli RA, Kasrawi MA, Baghdadi SH. Cryopreservation of wild Shih (*Artemisia herba-alba* Asso.) shoot-tips by encapsulation-dehydration and encapsulation-vitrification. *Plant Cell Tiss Org*. 2012; 108: 437-444.

Abbreviations
2,4-D: 2,4-dichlorophenoxyacetic acid
BA: 6-benzyl-aminopurine
IAA: indole-3-acetic acid
NAA: α-naphthalene-acetic acid
Pic: Picloram
GA_3: Giberlic acid