J. King Saud Univ., Vol. 3, *Agric. Sci.* (2), pp. 251–259, Riyadh (1411/1991)

In Vitro Propagation of *Artemisia annua* L.

H. Elhag, F. El-Feraly, J.S. Mossa and M. Hafez
College of Pharmacy, King Saud University,
Riyadh, Saudi Arabia

Key words: *Artemisia annua;* artemisinin, callus; in vitro propagation.

Abstract. Shoot-tips and lateral buds of *Artemisia annua* L. produced numerous shoots of on MS'A' medium and formed 100% roots on half strength Murashige minimal organic medium ($^1/_2$ MMO). In vitro propagated plants were uniform, grew to maturity and formed seeds when transferred to soil. Callus was induced on medium containing Murashige and Skoog inorganic salts and Gamborg B_5 vitamins as a basal medium and was maximal at 1.0 mg/l 2,4-D and 0.1 mg/l kinetin. Preliminary results of the extract of plantlets derived in vitro from shoot-tips or regenerated from callus, indicated the formation of artemisinin in these plants but not in their corresponding callus cultures.

Introduction

Artemisia annua L. (Compositae) an annual medicinal herb native to China, and is one of few species in this genera in which artemisinin, an antimalarial agent, has been detected and isolated [1-4]. The yield of the active compound from wild or cultivated plants (leaves or leaves and flowers) is low and varies from 0.01% to 0.06% but may reach 0.5% in certain Chinese varieties [5]. Enhancement of artemisinin production should consider biomass increase either through cultivation (Leaf biomass) or cell cultures. Populations raised from seeds are characterized by morphological variation in plant height, leaf biomass and flowering dates, thus, resulting in low and variable yield of the active constituent(s).

The objective of this study is to investigate and define an effective protocol for in vitro propagation of *A. annua,* and therefore obtaining of a uniform plant stand and induction of cell cultures for the enhancement of artemisinin production.

Materials and Methods

Seeds of *Artemisia annua* L. were collected from plants grown in the garden of the College of Pharmacy, King Saud Univ. in the 1985/86 season. Seeds were sterilized by soaking in 70% ethanol for 3 min. and then 20% (v/v) sodium hypochlorite solution containing 10-14% chlorine (Kochlight limited, England) for 5 min. and washed 3 times in autoclaved distilled water. Sterilized seeds were then grown on Heller support filter paper dipped in Murashige and Skoog [6] basal liquid medium in 125 mm flasks. Seedlings 2-5 cm long, were removed from the flasks under aseptic conditions. Shoot tips (3-5 mm) and axillary buds were cut from each seedling and cultured separately in culture tubes (16 × 150 mm) containing Murashige's shoot multiplication medium 'A' MS'A' (Gibco Europe, Paisley, Scotland) solidified with 0.8% Bacto Agar.

For callus induction, seedling leaves were cultured on medium containing in (mg/l) Murashige and Skoog [6] inorganic salt mixture (4,330) supplemented with sucrose (20,000), thiamine. HCl (10), nicotinic acid (1.0) pyridoxine. HCl (1.0), glycine (2.0), i-inositol (100) Bacto-agar (8000) and various concentrations of 2,4-D and kinetin as described. Media were sterilized by autoclaving for 15 min. at 121°C after adjusting the pH to 5.7. Warm ungelled medium was distributed into sterile (15 × 60 mm) petridishes 25 ml per plate. Cultures were kept in the dark for callus production and under low intensity illumination (Ca. 1.5 K) from Gro-Lux fluorescent lamps in a walk-in room for shoot multiplication.

To define a suitable rooting medium, four types of media were tested: Murashige's minimal organic medium (MMO) ($^1/_2$ conct)/; Murashige minimal organic medium (4 conct) + NAA (0.3 mg/1), Murashige's shoot multiplication medium 'B' ($MSB/_2$), and Murashige's shoot-tip rooting medium. Shoot explants from three lines (1,8 and 9) were used with 10 explants per treatment.

Extraction and identification of artemisinin

Samples of plants or callus were air dried, weighed, and extracted with n-Hexane for 9-12 hr. They were filtered, evaporated to dryness in a rotating evaporator and dissolved in equal amount of chloroform. Artemisinin and arteannuin B were identified by co-chromatography with authentic samples on silica gel plates. The solvent system used was 5% ETAC in chloroform. The plates were then sprayed with anisaldehyde reagent [7].

Results

Response of shoot tips and axillary buds to MS'A'

Shoot-tip explants from 25 seedlings produced numerous shoots on MS'A' medium after 3 weeks (Fig. 1). The number of shoots per explant was determined after 6 weeks and has indicated clonal variations (Table 1). Shoot proliferation occured from the main shoot apex as well as from axillary buds. Shoot tips from seedlings no. 8 (P_8), produced rosette type branching. Rosette type branching is

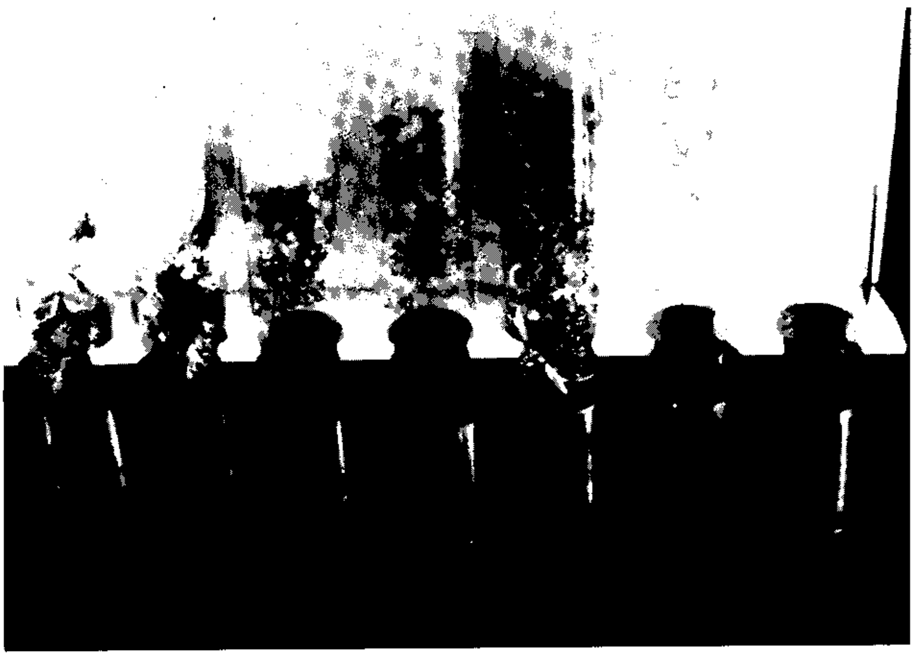

Fig. 1. Shoot multiplication from Shoot-tip explants on MS'A' medium

Table 1. Shoot multiplication in shoot-tip explants of *Artemisia annua* on MSA medium

Clone	No. of shoots/explant	Fresh wt of shoots (g)
P_1	14 ± 3.7[z]	3.77
P_8	8 ± 1.6[y]	1.52
P_9	24 ± 4.3	5.00

z = mean no. of shoots ± SE counting shoots greater than 5 mm length.

y = explants formed rosette branches.

common among plants raised from seeds. Shoots produced in culture were kept separate as clones and recultured every 4-6 weeks by shoot-tip explants on MS'A' or rooting medium.

Rooting of shoot tips

Results shown in Table 2 indicated that Murashige's minimal organic medium, devoid of growth regulators, produced 100% root formation in the three lines after 3 weeks followed by $^1/_2$ MMO + NAA with 100% rooting in two lines 8 and 9. The

Table 2. Effect of culture media composition on root formation, root length and shoot growth in shoots of *Artemisia annua* produced in vitro.

Culture media	% of shoots forming roots after 3 weeks			Average length of root and shoot in clone 1 after 6 weeks[z]	
	Clone 1	Clone 8	Clone 9	Root length (cm)	Shoot length (cm)
1/2 MMO	100	100	100	6.0 ± 1.0	3.5 ± 1.1
1/2 MMO + NAA (0.3 mg/l)	83	100	100	3.5 ± 0.7	3.4 ± 1.7
1/2 MS'B'	14	0	0	2.4 ± 1.2	3.9 ± 0.7
MSTR[y]	0	0	0	0	3.9 ± 0.7

z ± SE

y Murashige shoot-tip rooting medium

other two media did not form roots after 3 weeks but thereafter roots started to form and by the 6th week all explants have formed roots. Roots formed on 4 MMO were vigorous with long tap roots and were formed after one week in culture. Roots formed on 4 MMO + NAA were shorter, spreading and were formed during the 3rd week. This is indicated by measurements of root length taken after 6 weeks (Table 2). Subsequently, shoots that formed roots were transferred to the greenhouse and grown to maturity on peat : sand soil mix (2:1) in pots (Fig. 2).

Callus cultures

Callus was obtained from seedling leaf and stem explants on medium containing 2,4-D and kinetin and was maximal at 1.0 mg/l 2,4-D and 0.1 mg/l kinetin (data not presented). The modification of the vitamin supplement as indicated in materials and methods was found necessary. In fact, a combination of Murashige and Skoog [6] inorganic salts and B_5-vitamins of Gamborg *et al.*, [8] was found effective for callus induction and maintenance, in artemisia (Fig. 3), and other plant species under study in our laboratory.

Identification of artemisinin

Artemisinin production in plants raised in vitro from shoot-tips or their callus cultures have been examined by TLC. As shown in Table 3, artemisinin has been detected in all shoots and plants grown in vitro. The qualitative variation in the intensity of TLC spots, especially in plants transferred to the green house may indicate clonal differences. Clonal difference is expected among clones originally obtained from heterozygote seeds.

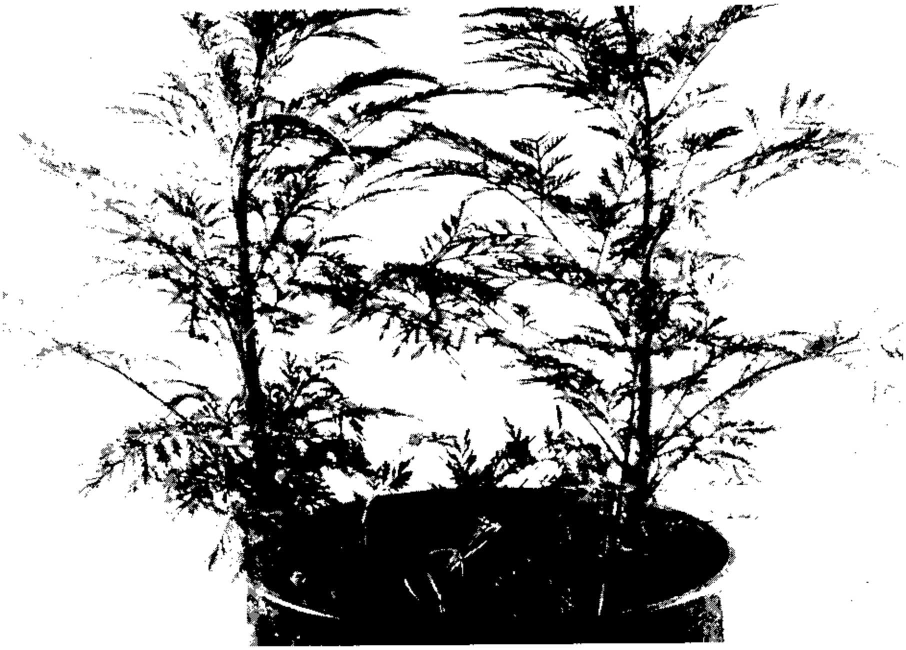

Fig. 2. Artemisia plants produced from shoot tips and transferred to the greenhouse

Fig. 3. Callus induced from artemisia leaf sections on medium containing 2,4-D and kinetin

Table 3. This layer chromatographic (TLC) analysis of the hexane extracts of shoots and plants produced in vitro from *artemisia annua*.

Clone	media	Roots W or W/o[y]	D. wt (g)	Compounds detected* Art.	AB	Others
Shoots from shoot-tips:						
P_8R_2	$^1/_2$MS	W	0.1	±	–	++
P_8R_2	MSB/2	W/o	0.1	±	–	++
P_9R_2	$^1/_2$MS	W	0.1	++	–	++
P_9R_2	$^1/_2$MS	W/o	0.1	±	–	++
$P_9R_{3\text{-}4}$	MSA	W/o	2.0	+	–	++
$P_9R_{3\text{-}4}$	MSA	W	0.87	++	–	++
$P_9R_{3\text{-}4}$	$^1/_2$MS	W	1.81	+++	–	++
Plants from shoot-tips transferred to the G.H.[z] :						
P_8R_1	MSA½MS soil			+	+	+++
P_9R_1	"	W		++	++++	+++
P_1R_1	"	W		+++	++++	+++
P_4R_1	"	W		++	++++	++
$P_{11}R_1$	"	W		+	++++	++

Y_w = with roots; w/o without roots

[z] Greenhouse

* ++++ = strong, + = weak, – = absent; Art = Artemisinin,

AB = Arteannuin B

There was no evidence of hormonal effect on artemisinin production, since artemisinin was detected in shoots grown on $^1/_2$ MS, a medium devoid of growth regulators, and on MSA which is rich in cytokinin and IAA. The extent of artemisinin formation was highest in the presence of roots with clone P_9 on $^1/_2$ MS or MSA medium, (Table 3). Extracts of Artemisia shoots, with or without roots, formed in addition to artemisinin, other compounds that include flavonoids but not arteannuin B. However, plants formed from shoot-tips and transferred to the greenhouse contained artemisinin, and arteannuin B 'AB' (Table 3). Arteannuin B, a precursor of artemisinin [4] was not detected in shoots under culture conditions i.e. before transfer to soil in greenhouse. Artemisinin was also detected in shoots regenerated from callus of one clone (P_5) on a medium containing IAA and Kinetin.

In a single culture, approximately 100 g of callus were obtained from clone P_7R_5 (Dry wt. 13 g). In contrast to shoot-tip cultures, callus cultures from various clones obtained from the same explants as shoot-tips did not form artemisinin or arteannuin B (Table 4).

Table 4. Thin layer chromatographic (TLC) analysis of the hexane extracts from callus cultures of *A. annua*..

Clone	media	Dark/light D or l	D. wt (g)	Compounds detected* Art.	AB	Others
P_6R_2	Callus maintenance medium	D	0.1	–	–	++
P_7R_2	"	D	0.1	–	–	++
P_8R_2	"	D	0.1	–	–	++
P_7R_2	"	D	0.8	–	–	+++
P_7R_5	"	D	13.1	–	–	++++
P_4R_3	MSA	L	1.0	–	–	++
P_3R_3	MSA	L	1.2	–	–	++

* Same as legend in Table 3.

Discussion

Attempts to increase artemisinin content in *A. annua* plants by application of growth regulators such as chlormequat and daminozide [3] or by incorporation of artemisinin precursors in cell cultures [9], have indicated the responsiveness of this plant and its derived tissues to biosynthetic studies, and the possibility of maximizing artemisinin content as well. However the effect of growth regulators would best be studied under in vitro conditions.

In the present investigation, in vitro culture conditions for mass propagation and callus growth of *Artemisia annua* have been determined. Plants or plantlets derived from shoot-tips were uniform and synthesized artemisinin as field grown plants. Artemisinin was also detected in plants regenerated from callus cultures of at least one clone. Results indicated that once a high producing plant is selected, it can be multiplied in vitro and its high qualities maintained.

The qualitative identification of artemisinin, although short in conferring exactly the amount detected, has indicated the presence of artemisinin in in vitro produced plantlets from either shoot-tips or callus. The fact that none of the callus cultures have synthesized artemisinin or its precursor arteannuin B (qinghaosu-II), clearly demonstrated a requirement for cellular differentiation and organogenesis. Such morphogenetic responses have been shown to be associated with secondary product formation [10, pp. 187-197, 11]. The regeneration from callus of plants that accumulated artemisini, suggest that subsequent selection may result in variant plants with enhanced artemisinin content.

References

[1] You-You, T. Mu-Yun N., Yu-Rong, Z., Lan-Na, L., Shu, Lian, C., Mu-Qun, Z. Xian-Zhen, W., Zheng, J. and Xiaotian, L. "Studies on the Constituents of *Artemisia annua* Part II." *Planta Med.*, 44 (1982), 143-145.

[2] Acton, N., Klayman, D.L. "Artemisitene, a new Sesquiterpene Lactone Endoperoxide from *Artemisia annua.*" *Planta Med.*, (1985), 441-442.

[3] Liersch, R., Soicke, H., Stehr, C. and Tullner, H.U. "Formation of Artemisinin in *Artemisia annua* during one Vegetation Period." *Planta Med.*, (1986), 387-390.

[4] El-Feraly, F.S., Al-Meshal, I.A., Al-Yahya, M.A. and Hifnawy, M.S. "On the Possible Role of Qinghao Acid in the Biosynthesis of Artemisinin." *Phytochemistry*, 25 (1986), 2777-2778.

[5] Klayman, D.L. "Qinghaosu (Artemisinin): An Antimalarial Drug from China." *Science*, 228 (1985), 1049-1055.

[6] Murashige, T. and Skoog, F. "A Revised Medium for Rapid Growth and Bioassays with Tobacco Tissue Cultures." *Physiologia Plant.*, 15 (1962), 473-497.

[7] El-Feraly, F.S. and Hufford, C.D. "Synthesis and Carbon-13 Nuclear Magnetic Resonance Assignments of Xenognosin." *J. Org. Chem.*, 47 (1982), 1527.

[8] Gamborg, O.L. Miller, R.A., and Ojima, K. "Nutrient Requirements of Suspension Cultures of Soybean Root Cells." *Exp. Cell. Res.*, 50 (1968), 151-158.

[9] Kudakasseril, F.J., Lam, L. and Staba, E.J. Incorporation of 14C-Isopentenyl pyrophosphate into Artemisinin (Qinghousu) by Cell free system of *Artemisia annua* shoot cultures. Minneapolis, Minn.: VI International Congress of plant tissue and cell culture (Abst.), 1986.

[10] Kurz, W.H.W., and Constabel, F. "Plant cell cultures, a Potential Source of Pharmaceuticals." *Adv. Appl. Microbiol.*, 25 (1979), 209-240.

[11] Luckner, M., and Diettrich, B. "Biosynthesis of Cardenolides in Cell Cultures of *Digitalis lanta* - the Result of a New Stragety. In: Green, C.E.; Somers, D.A., Hackett, W.P., Biesboer, D.D. (eds) *Plant Tissue and Cell Culture*, New York: Alan R. Liss, 1987.

إكثار نبات ارتميسيا أنيوا *(Artemisia annua)* بطريقة زراعة الأنسجة النباتية

حامد الحاج، فاروق الفرلي، جابر سالم موسى و محمد حافظ
قسم العقاقير، كلية الصيدلة، جامعة الملك سعود. الرياض، المملكة العربية السعودية

ملخص البحث. نبات ارتميسيا انيوا من النباتات الطبية المستوطنة لبلاد الصين والتي تحتوي أجزاؤها على مادة «ارتميسنين» التي ثبت أن لها تأثيرًا مضادًا وفعّالاً على مرض الملاريا. في هذه الدراسة استخدمت طريقة زراعة الأنسجة النباتية لإكثار هذا النبات واستخلاص المادة الفعّالة منه.

عند زراعة قمم وبراعم إبطية من هذا النبات على الوسط الغذائي MS'A' انتجت كل واحدة من هذه القمم والبراعم أفرعًا خضرية وكونت جذورًا بنسبة ١٠٠٪ عند نقلها وزراعتها على وسط غذائي آخر بنصف تركيز. وقد لوحظ أن النباتات المنتجة بهذه الطريقة كونت سلالات متجانسة الشكل فيما بينها ومشابهة للنبات الأم واستطاعت إكمال نموها وتكوين بذور عند نقلها للتربة. كما أمكن أيضًا تكوين كدب (Callus) من أوراق هذا النبات على وسط غذائي يحتوي على منظمات النمو 2, 4-D و Kinetin.

وقد أكدت الدراسة أن خلاصة النباتات المنتجة بهذه الطريقة والنباتات المتخلفة من الكدب تحتوي على مادة الارتميسنين المضادة لمرض الملاريا إلا أن الخلايا والأنسجة المستخرجة من النباتات نفسها لا تحتوي على هذه المادة. وتجرى تجارب لتحفيز هذه الخلايا لإنتاج المادة المعنية.