In vitro studies on *Ambrosia maritima*: I-Morphogenic responses and algal toxins elicitation

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ABSTRACT

The molluscicidal effect of sesquiterpene lactones of Ambrosia maritima on snails (intermediate vectors of Schistosomia) has been proven in Egypt. Here we present our results regarding establishment of tissue culture system for the production of active ingredients in vitro. Calli cultures were proliferated from leaf explants onto MS medium supplemented with 1 mg/l BA + 1 mg/l Kin + 0.1 mg/l NAA. The highest percentage of explants forming callus from leaf explants of Ambrosia (80%) was recorded onto MS medium supplemented with 1 mg/l BA + 1 mg/l Kin + 0.1 mg/l NAA. Shoot proliferation was restricted to MS medium contained 2 mg/l Kin + 0.5 mg/l NAA. The obtained data regarding the effect of increasing levels of toxic algal extracts (Microcystis aeruginosa and Nodularia harveyana) on damsin and ambrosin content of Ambrosia suspension cultures indicated that a maximum damsin and ambrosin yields can be achieved by adding either 10% of Nodularia harveyana extract or 20% of Microcystis aeruginosa extract. These results showed that high levels of damsin and ambrosin can be successfully produced from cell suspension cultures under standardized conditions.

Key words: *Ambrosia maritima, tissue culture, biotic elicitors, algae.*

INTRODUCTION

Ambrosia maritima L., locally known as damesisa, is a wild plant, native to Southern Europe and Africa, growing in the coastal areas near rivers, canals and rice fields and sometime, is reported as a weed in crop fields. Its medicinal interest is due to its molluscicidal activities. The lethal effect of sesquiterpene lactones of the plant on snails (intermediate vectors of *Schistosomia*) has been proven in Egypt (Sherif and El Sawy, 1962, 1977 and Abdel-Salam *et al.*, 1984). damesisa is not toxic to non-target organisms (rats, rabbits, algae and daphnia (Geerts *et al.*, 1992, 1994). Nowadays, its used in some renal tea due to it is proved effect in renal colic and expel renal stones.

Depending on genetic and environmental factors, Egyptian Damesisa shows different morphology, higher efficiency and molluscicidal activity than the Senegalese species (Triest *et al.*, 1989) and many trials have been done to cultivate *A. maritima* of Egypt for the replacement of *A.senegalensis* of Senegal (Vassiliades *et al.*, 1986). Accordingly, a great demand for Egyptian damesisa is raised. Due to insufficient wild plant material needed for local market and unavailability of agricultural lands and water resources for cultivation of damesisa, introduction of plant tissue culture is needed,

for many reasons, including, preservation of Egyptian wild germplasm, as a first step towards *in vitro* production of sesquiterpene lactones and genetic improvement of the existing Egyptian germplasm. However, search in available database revealed that no body paid attention either to tissue culture of damesisa or the employment of recent advances in plant biotechnology for the improvement and/or production of their sesquieterpene lactones *in vitro*.

In a number of suspension cultures, the level of desired secondary product accumulated can be dramatically increased by elicitation. The response may be induced by UV light, salt stress, drought, water stress (abiotic elicitors) and toxins of fungal, bacterial or algal culture filtrate (biotic elicitors). In this context, a stimulating effect of osmotic stress caused by mannitol on alkaloid production of cultured *Hyoscymus muticus*, *Atropa belladonna* and *Datura stramonium* cells is reported (Saker *et al.*, 1997). It was found that the exposure of *Catharanthus roseus* cell suspension cultures to *Aspergillus niger* homogenate caused a marked increase in total alkaloid (Godoy-Hernandez and Loyola-Vargas (1991). Saker and El-Ashal (1995) mentioned that although the salt stress reduces growth of *Hyoscyamus* suspension cultures, the alkaloid content on 0.6% NaCl containing media is 2.4 times greater than that on salt-free medium and the total alkaloid content of cultures (cells + media) is about 5.3 times greater than that of the original whole plant.

Certain blue-green algal species inhabiting fresh water bodies are termed toxic and causing sickness death to livestock, pets, wild animals and humans following ingestion of water containing toxic algal cells, algal blooms or the toxin released by aging cells (Gorham and Carmichael, 1979). Microcystins are hepatotoxins produced by *Microcystis aeruginosa* (unicellular, closely compacted and irregularly arranged in colonies) and Nodularin is another hepatotoxin produced by *Nodularia harveyana* (very short, compressed cells with heterocysts and the filaments have a thin and close sheath).

The present study was conducted to optimize *in vitro* tissue culture system for Egyptian *Ambrosia maritima* and studying the possibility of stimulating the biosysnthesis of sesquiterpene lactones of *Ambrosia maritima* by elicitation using algal toxins.

MATERIALS AND METHODS

Plant material

Seeds of *Ambrosia maritma L.* endemic in Egypt were kindly provided by Horticulture Research Institute, Egyptian Ministry of Agriculture.

Tissue culture

Seeds were surface sterilized in 70% ethanol for 2 min, 0.02% mercuric chloride for 20 min, then rinsed four times in sterilized distilled water. Leaf explants, three weeks old aseptically seedlings, were cultured on MS medium Murashige and Skoog (1962) containing basal MS salts, 0.7% agar, 3% sucrose, B5 vitamins and supplemented with various selected combinations of auxins and cytokinins. Culture media were adjusted to pH 5.8 before autoclaving for 20 min at 121 ^{0}C (1.2 kg/cm^2). All cultures were incubated in a growth chamber at 25± 2^{o}C, 16/8 light dark photoperiod (25 µmole/m^2/s). Cultures were subcultured at one month intervals. After eight weeks of cultivation, percentages of explants forming callus, growth rate, fresh and dry weights of proliferated callus were recorded. Frequencies were calculated as the number of responding explants/ total number of explants x 100. The experiments were repeated three times and each value is the average of ten replicates. Suspension cultures were initiated by transferring one gram fresh callus into 100

ml liquid MS medium contained 1 mg/l BA + 1 mg/l Kin + 0.1 mg/l NAA, incubated in dim light at 25 °C on shaker at 150 rpm. Different concentrations of algal crude extracts were included.

Axenic algal cultures

The cultures of *Microcystis aeruginosa and Nodularia harveyana,* were kindly provided by Pasteur Culture Collections (PCC) and they have given the numbers PCC 7806 and PCC 7804, respectively. Cultures were maintained on Bold's nutritive medium and incubated at 25 ±1°C, under continuos illumination by cool fluorescent lamps, which give a light intensity of $10\mu molm^{-2}s^{-1}$. Subculturing took place regularly at 10 days intervals. Algal cultures, ten days old (at the end of the exponential growth phase, where the maximum toxin production) were centrifuged at 6000 rpm for 20 min at 20°C, the algal cells were washed and recentrifuged to eliminate culture medium. Concentrated algal suspension (20 ml) were sonicated at 90 k cycles for 6 min using Fisher Sonic dismenbrator model 150. To calculate the initial algal concentration used in this experiment, estimation of chlorophyll a content in 5 ml algal suspension using 90% acetone according to Metzner *et al.* (1965), as well as the fresh and dry weight of another 5 ml were used. Chlorophyll a content was calculated from the following equation:

$11.9 \times E_{665} \times V$ acetone.

One ml of *Microcystis aeruginosa* contained 1.73498-mg chl a/mg dry wt. One ml of *Nodularia harveyana* contained 1.93441-mg chl a/mg dry wt.

Extraction and determination of sesquiterpene lactones

Total sesquiterpene lactones were isolated and determined as described by Amin (1990).

RESULTS AND DISCUSSION

Data in Table (1) summarizes the effect of different combinations of growth regulators on the morphogenic responses of *Ambrosia* leaf explants. Data given in this table showed that the four selected combinations of growth regulators induced callus proliferation at different frequencies. The physical appearance of proliferated callus ranging from friable greenish white to compact green, based on the type and level of auxin and cytokinin added. However, sorts of pink callus were formed onto MS supplemented with 1 mg/l BA + 1 mg/l Kin + 0.1 mg/l NAA . The highest percentage of leaf explants forming callus of *Ambrosia* (80%) was recorded on MS medium supplemented with 1 mg/l BA + 1 mg/l Kin + 0.1 mg/l NAA. The highest fresh and dry weight yields (4.4g and 0.21 g) and growth rate (146 mg/day) were also recorded on the same medium. Shoot proliferation was restricted to MS medium contained 2 mg/l Kin + 0.5 mg/l NAA.

Figure (1) shows different callus lines proliferated from leaf explants, including friable greenish callus (Fig.-A), greenish callus with white and pink sorts (Fig.1-B), shoot proliferation onto MS medium + 2 mg/l Kin+0.5 mg/l NAA (Fig1-D) and elongation and rooting of proliferated shoots on basal MS medium (Fig.1-E). The most striking side observation is the strong antimicrobial activity of damesisa callus. It was noticed that contaminated cultures can resist bacterial contaminations and clear inhibition zones were recorded as that illustrated in Figure (1-C).

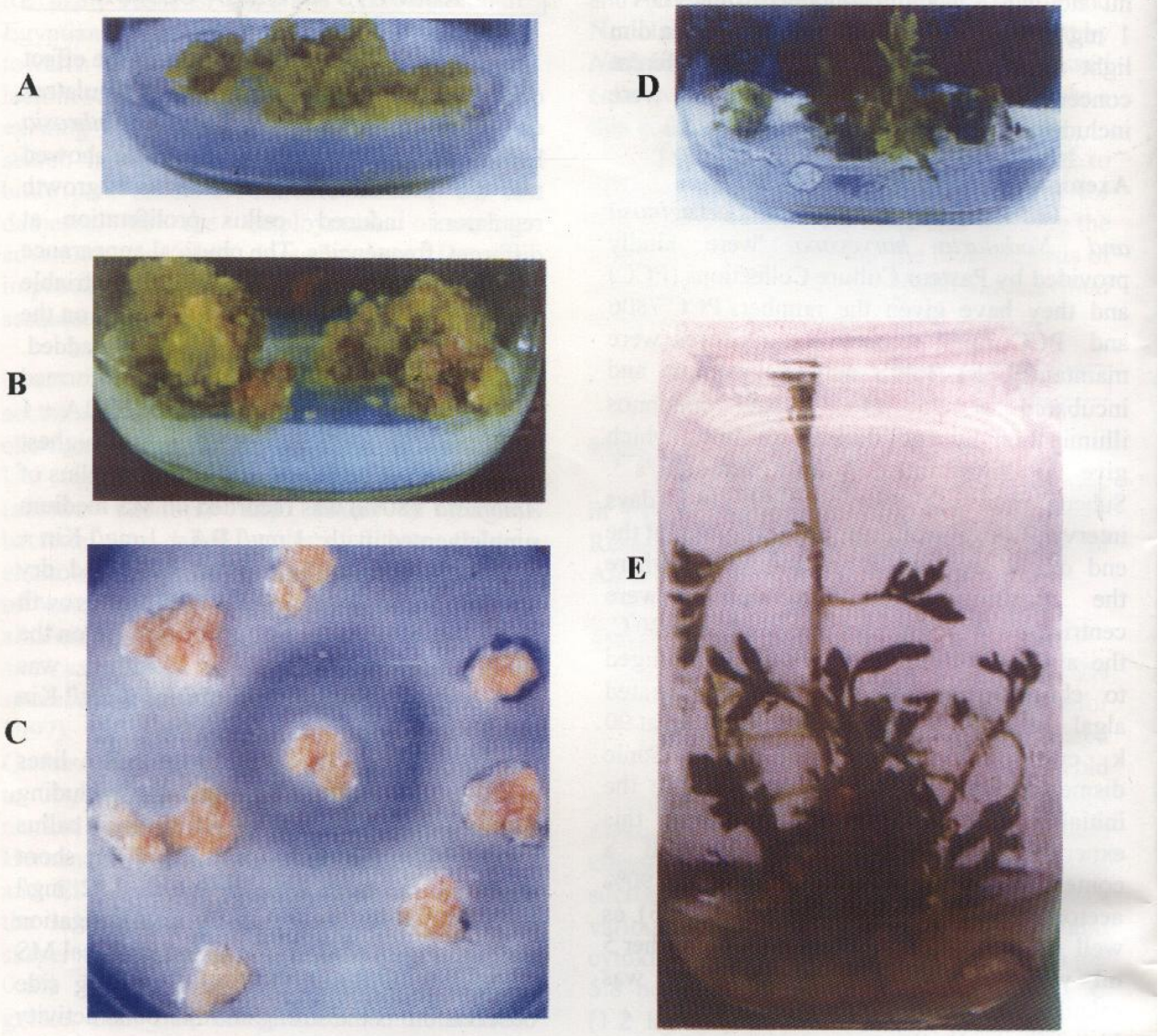

Fig. (1): Different callus lines proliferated from leaf explants of damesisa.
(A): Friable greenish yellow callus on MS+2 mg/12,4-D+0.5 mg/ 1Kin.
(B):Greenish callus with white and pink sorts on MS+1mg/1BA+1mg/1 Kin+0.1mg/1NAA.
(C):Clear inhibition zone indicates the strong antimicrobial activity of damesisa.
(D):Shoot proliferation onto MS medium+2 mg/1Kin+0.5mg/1NAA.
(E):Elongation and rooting of proliferated shoots onto basal MS medium.

Table (1): Morphogenic response of Ambrosia leaf explants, cultured for eight weeks onto MS medium supplemented with selected combinations of auxins and cytokinins.

Culture media	% of explants forming callus	Fresh weight (g/explant)	Dry weight (g/explant)	Growth rate (mg/day)	Remarks
MS+2mg/l 2,4-D+0.5 mg/l Kin	70	4±0.1	0.18	133	Yellowish, white and friable callus
MS+2mg/l BA+0.5 mg/l NAA	60	3±0.6	0.12	120	Greenish, compact callus
MS+1mg/l BA+1 mg/l Kin+0.1mg/l NAA	80	4.4±0.2	0.21	146	Greenish callus with pink and white sorts
MS+2mg/l Kin+0.5 mg/l NAA	65	3.8±0.3	0.18	126	Greenish, friable callus and shoot differentiation

Each value is the average of ten replicates ±SE, 50 explants were used per treatment.

Table (2): Effect of alga toxins on the fresh and dry weight yield of Ambrosia suspension cultures.

Toxin Conc. (%)	Fresh weight (g)		Dry weight (g)	
	Nodularia harveyana	***Microcystis aeruginosa***	***Nodularia harveyana***	***Microcystis aeruginosa***
0.0	2.50	2.50	0.22	0.42
10	3.25	2.15	0.25	0.22
20	2.20	1.80	0.21	0.20
30	2.00	1.70	0.20	0.12
40	1.80	1.50	0.15	0.10
50	1.50	1.40	0.17	0.13

Table (3): Effect of algal toxins on Damsin and Ambrosin content of Ambrosia suspension cultures.

Toxin Conc. (%)	***Nodularia harveyana***		***Microcystis aeruginosa***	
	Damsin (%)	Ambrosin (%)	Damsin (%)	Ambrosin (%)
0.0	1.6	0.0	1.60	0.0
10	5.0	3.5	2.60	1.0
20	3.5	2.3	2.40	2.2
30	3.4	1.9	1.60	2.1
40	3.1	1.2	1.33	0.0
50	2.4	0.0	0.95	0.0

Table (2) illustrates the effect of toxic algal crude extracts on fresh and dry weight yield of *Ambrosia* suspension cultures, low concentration (10%) of both *Microcystis* and *Nodularia* toxic extracts caused an increase in fresh and dry weights of damesisa. Increasing algal extract concentrations (20, 30, 40 and 50%) led to an inhibitory effect on growth, expressed as fresh and dry weight yield. Data extracted from Table (2) indicated that the highest values of fresh and dry weight yields (3.25 g and 0.25 g) were recorded on medium contained 10% of toxic crude extract and the lowest values (1.5 g and 0.17 g) were recorded on 50 %. Generally, crude extracts of *Microcystis* showed more toxicity than that of *Nodularia.*

Data summarized in Table (3) shows the effect of toxic algal crude extract on damsin and ambrosin produced by *Ambrosia maritima* suspension cultures. Results clearly indicate that, the toxic algal crude extracts stimulate damsin production in all the concentrations used in case of *Nodularia* and most concentrations of *Microcystis.* Ambrosin, which is absent in the control, its induction is stimulated by lower concentrations of algal crude extracts, then its content decreases progressively with increasing algal concentration till complete inhibition at concentration 50%. Maximum damsin and ambrosin content were recorded on media contained either 10% *Nodularia* or 20% *Microcystis* crude extract. The high percentages (5 % and 3.5 %) of damsin and ambrosin produced by *Ambrosia* suspension cultures exposed to biotic elicitation by crude toxic algal extracts were about two times greater than the previously known levels of flowers and five times of stem of *in vivo Ambrosia* plants.

The obtained results show clearly that lower concentrations of algal crude extracts (present naturally in the water body by aging cells, releasing their endotoxins to the surrounding environment where damesis was planted) stimulates the growth of damesisa (fresh and dry weight increases) and their effects were mainly on the metabolic activities and stimulated the production of ambrosin and damsin, which control Bilharziasis. It is not surprising that tissue-cultured cells of higher plants typically accumulate large amounts of secondary metabolites only when subjected to specific conditions. In this context, It was found that the exposure of *Catharanthus roseus* cell suspension cultures to *Aspergillus niger* homogenate caused a marked increase in total alkaloid (Godoy-Hernandez and Loyola-vargas (1991). Saker and El-Ashal (1995) mentioned that although the salt stress reduces growth of *Hyoscyamus* suspension cultures, the alkaloid content on 0.6% NaCl containing media is 2.4 times greater than that on salt-free medium. A stimulating effect of osmotic stress caused by mannitol on alkaloid production of cultured *H. muticus, A. belladonna* and *D. stramonium*, cells are also reported (Saker *et al.*, 1997).

The relatively high levels of damsin detected here agreed with the fact that damsin can be regarded as the parent lactone from which all other lactones can be generated by further oxidation (Branchman and Periera 1992). However, most of the recent researches are directed towards the isolation of more and more sesquiterpene lactones other than the two main constituents (damsin and ambrosin). The reasons for this new trend were the fact that some damesisa from different origin show considerable variations in the level of active substances and its molluscicidal activity. In some South African populations, the two main active substances are totally lacking or present in trace amounts (Triest *et al.* 1992) and what about its molluscicidal activity? We think much more

work is needed to test the activity of other lactones on snails. The shortage in studies dealing with biotechnology and even basic research of damesisa may be due to the fact that damesisa is domestic in developing countries (Africa) and it may be impracticable for poor peoples to go deeply through this kind of research. Correlation with different microorganisms (phytoplanktons or zooplankton), or other plants inhabiting the same environment may be present.

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الملخص العربي

دراسات معملية على مزارع الأنسجه لنبات الدمسيسه (أمبروزيا ماريتيما)
أ. تأثير تنوع بيئات النمو و السموم الطلبيه على انتاجية المواد الفعالة المبيدة لقواقع البلهارسيا.

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ثبت علميا في مصر التأثير المبيد لمركبات السسكوتربين لأكتون المستخلصة من نبات أمبروزيا ماريتيما (المعروفة محليا بالدمسيسة) على القواقع التي تحمل العائل الوسيط للبلهارسيا. و لقد كان الغرض من هذا البحث هو التوصل الى ظروف مثلى و قياسية لعمل مزارع انسجة و معلق خلوى معمليا لهذا النبات البرى ثم محاولة زيادة انتاجية المواد الفعالة باستخدام بعض الأجهادات الحيوية.

اظهرت النتائج التي حصلنا عليها امكانية الحصول على أفضل نمو للكالوس من اجزاء مفصولة من أوراق نبات الدمسيسة و ذلك عند زراعتها على بيئة MS مضافا اليها مجم / لترمن كل منKIN و BA بالأضافة الى ٠,١مجم / لترNAA وتكشفت الأفرع الخضرية على الكالوس فقط على بيئة MS محتوية على٢مجم / لترKIN مضافا اليها ٠,٥ مجم/ لتر NAA.

و لقد اظهرت النتائج ايضا ان استخدام تركيزات مختلفة من المستخلصات الطحلبية السامه لبعض الطحالب الخضراء المزرقه, أدت الى الحصول على أعلى انتاجيه من المواد الفعاله المبيدة للقواقع ممثله بالدمسين و الأمبروزين عند استخدام تركيز ١٠% من مستخلص طحلب النوديولأريا هارفيانا و ٢٠% من مستخلص طحلب الميكروسيستس اوريجنوزا.