

Effect of Biotic Stress (*Aspergillus niger*) on the Production and Accumulation of Total Alkaloids in *Atropa belladonna* L. via Tissue Culture

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Abstract

An efficient protocol for enhancement of total alkaloids production from suspension cultures of *A. belladonna* L. was established. The effect of MS-medium supplemented with different concentrations of naphthalene acetic acid (NAA) and benzyl adenine (BA) on growth parameters as well as total alkaloid production was investigated. In the same connection, the effect of biotic stress caused by various concentrations of *Aspergillus niger* on alkaloid accumulation and production was investigated. The optimum values of cell growth parameters and alkaloid production were obtained for leaf, stem and root cell cultures, respectively. The optimum supplementation of liquid MS-medium was 1 mg/l of each of NAA and BA. *A. niger* extract at 10 % (~ 0.5 mg/ mL) gave the highest value for cell growth and alkaloid accumulation in the different types of cell cultures following 10 days of cultivation. Total alkaloid contents of different cell cultures were identified by HPLC.

INTRODUCTION

Plant secondary products are used extensively in commerce, particularly in the food and pharmaceutical industries, and many of these compounds have proved to be target compounds for plant cell culture production. There have been a number of excellent publications on this topic within the last decade. Plant cell cultures have the capability of producing a wide range of secondary compounds (Phillipson, 1990).

Atropa belladonna L. (Solanaceae) is one of the most important medicinal plants and is a source of tropane alkaloids such as hyoscyamine and scopolamine. Medicinally, *A. belladonna* is used for the use of its alkaloids in the treatment of Parkinson's disease for its anti-inflammatory properties, for relief of bronchial asthma and motion sickness and its ability to counteract toxic agents. Belladonna extract is used as an antimuscarinic agent, which accounts for its use as a spasmolytic drug. Also, it is used as a concomitant therapy in the treatment of peptic ulcer and functional digestive disorders, including spastic, mucous, and pancreatitis.

Plant growth regulators and nutritional factors affect the production of secondary metabolites, as well as growth, of cell cultures of higher plants (Sakuta and Komamine, 1987; Szoke et al., 1982, 1992; Vida et al., 2000; Laszlo et al., 2001). Also, types and concentrations of growth regulators affect secondary product formation (Bohm, 1980; Krajewska et al., 1987; Bálványos et al., 2001). The presence of 2,4-dichlorophenoxy acetic acid (2,4-D), indole acetic acid (IAA) and (NAA) increased the production of indole alkaloids in cell cultures of *Cinchona ledgeriana* (Harkes et al., 1985).

Moreover, Nussbaumer et al., (1998) reported that, half strength Gamborg's B5 medium supplemented with 5% sucrose and 1 mg/l of each of NAA and BA gave the best value for root culture growth of *Datura candida* x *D. aurea*. Meanwhile, full strength B5 medium supplemented with the same concentrations of NAA and BA gave the best results for hyoscyamine and scopolamine content.

One of the methods frequently used to increase the productivity of plant cell culture is the use of so-called elicitors (Singh, 1996). Elicitors can be all types of compound that increase the production of phytoalexins (Muller, 1956 and Kuc, 1972). Phytoalexins are antibiotically active compounds and, by that, important factors in the

resistance of plants to microbial attack (Cruickshank, 1980; Darvill and Albersheim, 1984). Many secondary metabolites belong to the group of phytoalexins. So, if the right elicitor can be found, it is possible to enhance the production of the desired secondary metabolite (Eilert, 1987).

The term elicitor can be used for “biotic elicitor” such as plant-derived, endogenous elicitors and for micro-organism-derived compounds (Keen et al., 1972), or “abiotic elicitor” such as physical and chemical stress factors that can also induce product formation, like UV light, extreme temperature, ethane and heavy metals (Tietjen et al., 1983; Davis et al., 1986).

One of the most intensively studied and commonly used biotic elicitors is derived from the fungus *Phytophthora megasperma*, which when used with *Glycine max* cell cultures, increased production of the secondary metabolite glyceollin (Ebel et al., 1989). Other elicitors of microbial origin that are commonly used for elicitation include *Aspergillus niger*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis* and *Penicillium* (Heijden, 1989).

In this respect, Zhao et al. (2000) reported that using combined elicitor treatment of an *A. niger* mycelium and tetraethyl ammonium bromide with *C. roseus* (L.) Don cell cultures enhanced the accumulation of ajmalicine as compared with control medium. Also, Wijnsma et al., (1985) reported that the anthraquinones in *Cinchona ledgeriana* cell cultures were increased when the cells were treated with 0.5 mg/mL of *A. niger* as elicitor.

The main objective of this study was to study the effect of different concentrations of NAA and BA and various concentrations of *A. niger* extract on cell growth and total alkaloid production in different types of cultures of *A. belladonna*.

MATERIALS AND METHODS

This investigation was carried out in the Plant Cell and Tissue Culture, Department, Genetic Engineering Division, National Research Centre., Dooki, Cairo, Egypt.

Plant Material

Seeds of *Atropa belladonna* were secured from the Pharmacognosy Department, Faculty of Pharmacy, Cairo University. Seeds were surface sterilized by immersion in 70 % ethanol for 10 sec., followed by three washes using sterile distilled water, then immersed in 50 % commercial Clorox solution containing a drop of Tween 20, for 15 min. The seeds were subsequently rinsed several times with sterile water. These seeds were then germinated aseptically on solid MS-medium free of hormones (Murashige and Skoog, 1962). Cultures were solidified with 0.7 % agar added prior to autoclaving at 1.2 Kg/cm² for 15 min. The pH of the medium was adjusted to 5.8 by addition of 0.1 N HCL or 0.1 N KOH. Germination of seeds took place within 7-10 days. After one month from germination the different segments i.e., leaf, stem and root were used as a source of callus production.

Callus Production

In this experiment, the following concentrations of NAA and BA were added to MS-basal medium as follow:

- 1-MS-medium, free hormones
- 2-MS + 0.0 mg/l NAA + 0.5 mg/l BA
- 3-MS + 0.5 mg/l NAA + 0.0 mg/l BA
- 4-MS + 0.5 mg/l NAA + 0.5 mg/l BA
- 5-MS + 1.0 mg/l NAA + 1.0 mg/l BA
- 6-MS + 2.0 mg/l NAA + 2.0 mg/l BA

Cultures were incubated under light conditions of 16 h/day photoperiod at an intensity of 3000 Lux from cool light fluorescent lamps for 30 days, and maintained at 26 ±1 °C. 5 replicates of each treatment were used.

Determination of Callus Growth

The following growth parameters were determined 30 days from incubation: fresh weight (g), dry weight (g) and dry matter content (%).

Cell Culture Induction

The obtained callus from different explants, i.e., leaf, stem and root were resuspended, according to Torres (1988), in an agitated liquid MS-medium supplemented with 1mg/l NAA + 1 mg/l BA.

Measurement of Cell Culture Growth

The cell number was counted during the growth period of cultivation as a growth parameter (Neumann, 1966).

Elicitor Preparation

A. niger was obtained from the Department of Plant Pathology of the National Research Centre. *A. niger* was grown in malt extract (20 g/L) in a shake flask (1000 mL) with 200 mL medium on a rotary shaker (120 rpm) at room temperature. After 7 days the cell suspension was autoclaved, and filtered on Whatman no. 1 filter paper. The mycelium was washed several times with sterilized distilled water and suspended in 100 mL water. This mixture was homogenized, autoclaved again and measured through the P.C.V. and used without purification.

In this experiment, concentrations of 0 %, 5%, 10%, 15% and 20% of the 0.1% P.C.V of suspended *A. niger* were added to the culture media.

Chemical Analysis

Total alkaloid content was determined as tropane alkaloid by HPLC according to the method described in the British Pharmacopoeia (1998) and the standard curve for tropane alkaloids (calculated as scopolamine) was estimated according to Milan et al. (1990).

Statistical Analysis

Design of all experiments was completely randomised and the obtained data were statistically analysed using standard error (SE) according to the method described by Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

1. Effect of NAA and BA on Callus Production

Data tabulated in Table 1 show the effect of supplementation of MS medium with different concentrations of NAA (auxin) alone and in combination with BA (cytokinin) on callus production from different explants, i.e., leaf, stem and root of *A. belladonna* (Fig.1). Supplementation of MS-medium with 1 mg/L of each of NAA and BA gave the optimum value for callus production. Leaf explants recorded the maximum value for callus fresh weight (12.10 g/jar followed by 9.25 then 8.45 g/ jar) for stem and root calli cultures, respectively, as compared with the other supplementation. Dry weight was 1.17, 0.81 and 0.75 g for leaf, stem and root callus cultures, respectively. Dry matter content was 9.38, 8.65 and 8.87 % for leaf, stem and root callus cultures of *A. belladonna*, respectively.

The above results clearly showed that supplementation of MS-medium with 1 mg/L of each of NAA and BA was more suitable for callus production from different explants of *A. belladonna* as compared to other concentrations.

In this respect the obtained results were in close agreement with those of Gamborg and Shyluk (1981), who concluded that callus initiation and production were dependant on the presence of auxin and cytokinin, which stimulate cell division and cell elongation, respectively. Moreover, Nussbaumer et al. (1998) reported that

supplementation of B5 medium with 1 mg/l of each of NAA and BA gave the best results for the growth value for *Datura candida* x *D. aurea*. The obtained results are in close agreement with those of Saker and Dessouky (2000), who reported that 1 mg/L of each of NAA and BA were more suitable for cell culture induction of different explants of *A. belladonna*.

2. Cell Culture Induction and Determination of Total Alkaloids

Cell cultures were induced from friable calli cultures of different explants; i.e. leaf, stem and root of *A. belladonna* and recultured on liquid MS-medium supplemented with 1 mg/L of each of NAA and BA. The maximum values for cell number ($\times 10^5$) were counted during 21 days. They were 5.873, 5.012 and 4.875 $\times 10^5$ cells / 1 mL for leaf, stem and root explants, respectively, at the 10th day of cultivation, when maximum cell production was recorded. The optimum values for total alkaloids were determined as 0.032, 0.024 and 0.009% for cell cultures induced from leaf, stem and root explants, respectively (Fig. 2).

The obtained results are in agreement with those of Skoog and Schmitz, (1972), who reported that cytokinins are generally added to a culture medium to promote cell division in calli cultures of plant tissue and this stimulates the rate of protein synthesis in tobacco cell cultures.

3. Effect of addition of different concentrations of *A. niger* as a biotic elicitor on cell number and total alkaloid accumulation.

The effect of different concentrations (0%, 5 %, 10%, 15 % and 20%) of 0.1 P.C.V. of *A. niger* (0, 2.5, 5.0, 7.5 and 10 mg/mL of liquid cell cultures), which were added to the MS medium containing 1 mg/L of each of NAA and BA on cell number (10^5) and total alkaloid production from different types of cell cultures were investigated. Data tabulated in Fig. 2 shows that leaf explants of *A. belladonna* gave the optimum value for cell number (5.92×10^5) with the blank elicitor treatment, as compared with elicitor treatments. The increase in elicitor levels reduced cell growth, but stimulated the accumulation of total alkaloids (calculated as scopolamine %). Stem cell cultures showed a low cell number (1.25×10^5) as well as low total alkaloid production. *A. niger* at 10 % after 10 days from the duration of incubation (21 days) showed the highest value for tropane alkaloid accumulation, in comparison to the other concentrations. The highest values for total tropane alkaloids were 0.048 %, 0.035 % and 0.018% for leaf (Fig. 3), root and stem cell cultures, respectively.

The obtained results are in agreement with those of Wijnsma et al. (1985), who indicated that anthraquinone content can be increased to 500 $\mu\text{g/g}$ fresh weight (as compared with the control) by the addition of 0.5 mg/mL of *A. niger* as a biotic elicitor to the culture medium of *Cinchona ledgeriana* cell cultures. Concerning the time of treatment, our results were in close agreement with those of Eilert (1987), who reported that the optimal moment for elicitor treatment with *Fusarium oxysporum* for thiophene production by *Tagetes* cell cultures is around day 8, when the culture starts to grow more or less exponentially. Most probably, this is due to the correlation between secondary metabolite production and the cell growth cycle. Most plant secondary products are produced in the stationary phase of cell growth.

It may be concluded that cultivation of leaf explants of *A. belladonna* in liquid MS-medium containing 1mg/L of each of NAA + BA in the presence of 5 mg/mL of *A. niger* for 10 days was the most favourable condition for stimulating total tropane alkaloids production.

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Tables

Table 1. Effect of MS-medium supplemented with different concentrations of NAA and BA (mg/L) on fresh weight (g), dry weight (g) and dry matter content (%) of callus cultures produced from leaf, stem and root explants of *A. belladonna*

MS-supplemented with NAA BA	Leaf explants			Stem			Root		
	FW (g)	DW (g)	DMC (%)	FW (g)	DW (g)	DMC (%)	FW (g)	DW (g)	DMC (%)
0.0 0.0	0	0	0	0	0	0	0	0	0
0.0 0.5	3.12 ±0.12	0.12 ±0.054	3.85 ±0.26	2.25 ±0.25	0.08 ±0.002	3.50 ±0.25	1.85 ±0.059	0.05 ±0.008	2.70 ±0.85
0.5 0.0	2.15 ±0.15	0.07 ±0.001	3.22 ±0.14	1.27 ±0.23	0.05 ±0.008	3.95 ±0.26	1.08 ±0.15	0.04 ±0.007	3.69 ±0.75
0.5 0.5	7.58 ±0.25	0.64 ±0.022	9.35 ±1.18	5.42 ±0.59	0.43 ±0.009	7.88 ±0.98	4.13 ±0.29	0.35 ±0.073	8.35 ±1.25
1.0 1.0	12.10 ±0.17	1.17 ±0.053	9.38 ±1.30	9.25 ±1.26	0.81 ±0.058	8.65 ±1.25	8.45 ±1.36	0.75 ±0.006	8.87 ±1.33
2.0 2.0	10.15 ±1.51	0.85 ±0.003	8.56 ±0.65	7.14 ±1.23	0.65 ±0.157	8.26 ±0.75	6.55 ±1.98	0.54 ±0.005	8.73 ±1.25

± SE (Standard error), N=5 replicates

FW = Fresh weight (g), DW = Dry weight (g), DMC Dry matter content (%).

Figures

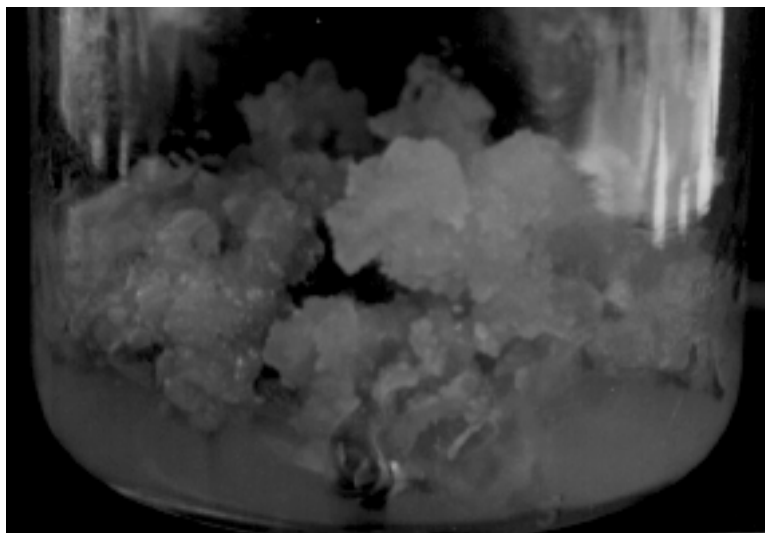


Fig. 1. Callus production from leaf explants of *Atropa belladonna* after 8 weeks of cultivation on MS medium supplemented with 1 mg/l of each of NAA and BA, cultured under light conditions.

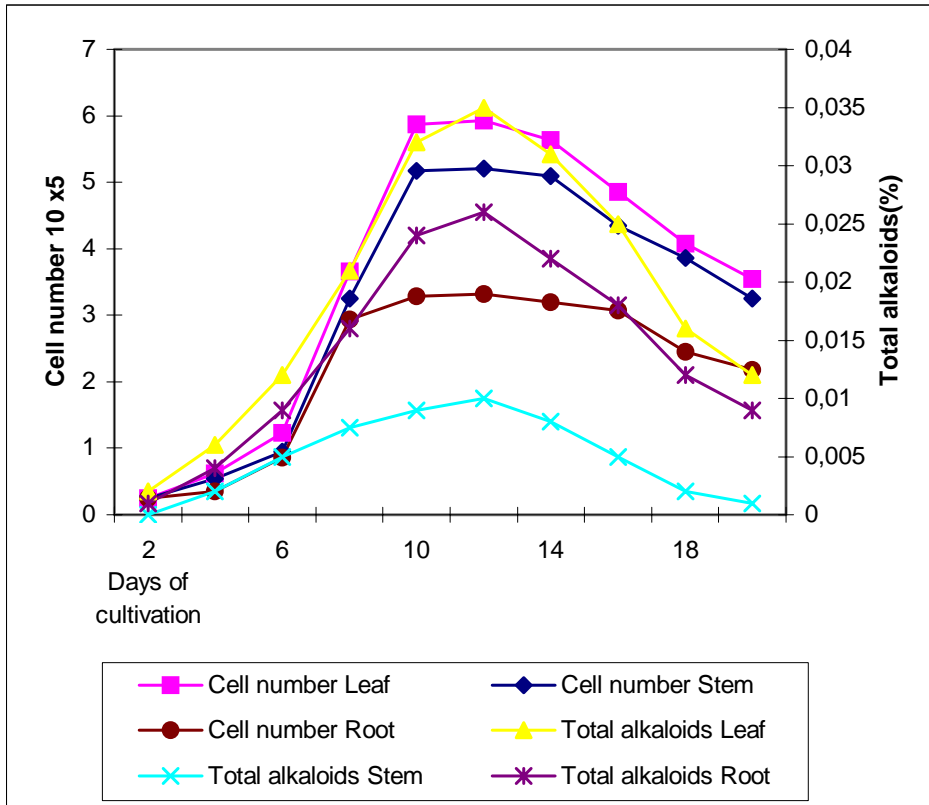


Fig. 2. Cell number (10×5) and percentages of total alkaloids accumulated in the different cell cultures induced from leaf, stem and root explants of *Atropa belladonna* cultured on MS- medium supplemented with 1 mg/l of each of NAA and BA, cultured under light conditions.

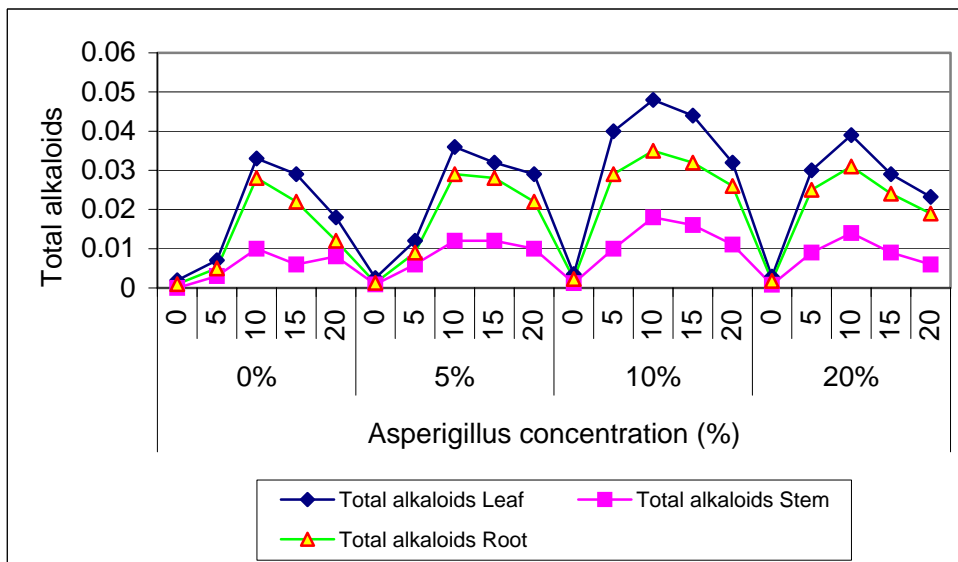


Fig. 3. Effect of different concentrations of *Aspergillus niger* on total alkaloid production from leaf, stem and root cell cultures of *Atropa belladonna* explants during 20 days of cultivation under light conditions.