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IMPACT OF FUNGAL ELICITOR AND CULTURE CONDITIONS ON INDUCTION OF CALLI AND ALKALOIDS PRODUCTION IN *Narcissus tazetta* var. *italicus* TISSUE CULTURES

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ABSTRACT

The use of plant-derived drugs has been increased throughout the world as a result of health hazards and toxicity associated with the use of synthetic pharmaceutical compounds. Tissue culture techniques are developed as an alternative strategy to produce active secondary metabolites. Fungal elicitation with a combination of tissue cultures optimization is effective strategy to enhance biologically active compounds. Callus cultures of *Narcissus tazetta* var. *italicus* (Ker-Gawler) Baker were prepared from bulb explants. They were cultured on a modified Murashige and Skoog medium, supplemented with 3% sucrose, 3mgl^{-1}naphthalene acetic acid (NAA) and1.5mg l^{-1} benzyl adenine (BA). Five factors included: fungal growth medium, fungal elicitor concentration, fungal age, exposure time, and callus age were optimized to improve alkaloids production. Culture filtrate of *Fusarium sporotrichioides* Sherbakoff isolated from the rhizosphere of *N. tazetta* var. *italicus* was used as a biotic elicitor. Elicitor of *F. sporotricoides*, grown on Potato Dextrose broth, stimulated alkaloids production by calli (23.4 mg. g -1dry cell) more than that grown on other media. Elicitor concentrations above 20% enhanced alkaloid accumulation significantly particularly at 80% (32.3 mg. g -1dry cell). Fungal ageing was accompanied by a progressive increase in alkaloid contents of calli. Elicitors prepared from older fungal cultures were effective in stimulating calli growth and alkaloid production more than younger cultures. Alkaloids were increased by increasing exposure time. After 15 days exposure time, there was an increase in alkaloid contents of calli by 2.52 – fold compared to the control. Alkaloid production was directly proportional to callus age until the eighth

week then declined significantly by ageing. Eight-week-old callus cultures produced maximum amount of alkaloids (39.2 mg. g^{-1} dry cell) after elicitation for 15 d. In this treatment alkaloid contents increased by 1.8-fold, compared to that produced by 2-week-old callus.

Keywords: Amarylidaceae; *Narcissus tazetta var*. *italicus;* alkaloids; fungal elicitor; tissue culture conditions.

INTRODUCTION

Narcissus tazetta var. *italicus* (Ker-Gawler) Baker is a perennial plant of the Amaryllidaceae family. Plants belonging to the Amaryllidaceae family have been shown to be a promising source of biologically active natural compounds [1-3]. Over 500 alkaloids have been isolated from plants of family Amaryllidaceae [4]. Amaryllidaceae alkaloids have antitumor, antibacterial, antifungal, antimalarial, antiviral and analgesic activities [5,6]. Also, some types of alkaloids act as acetylcholin esterase and butyrylcholin esterase inhibitors [5]. Alkaloids have great attention due to their valuable therapeutic uses. Stepharine, an aporphine alkaloid of *Stephania glabra* plants is anti-aging, anti-hypertensive, and anti-viral agent [7].

With increasing health hazards and toxicity associated with the indiscriminate use of synthetic pharmaceutical compounds, the use of plant-derived drugs has been increased throughout the world [8]. The production of medicinal herbal materials in an appropriate manner becomes importantin pharmaceutical industry. Cell and tissue culture techniques are developed as an alternative strategy to produce active secondary metabolites [9,10]. Shoot and root cultures established in culture media supplemented with various combinations of growth regulators have the ability to produce the same secondary metabolites as the intact plant [11]. In certain cases, higher levels of biologically active compounds have been obtained by optimization of culture conditions.

Combination of tissue cultures with elicitation can be very good strategy for increasing production of secondary metabolites in plant which have a viable industrial and medical applications [12]. Fungal elicitation has been an effective tool to improve the yield of secondary metabolites [13,14]. When cell cultures of *Catharanthus roseus* were treated with *Aspergillus niger*, *Fusarium moniliforme*, and *Trichoderma viride*, ajmalicine accumulation increased by about 3-fold [15]. The effect of elicitors depends on several factors such as elicitor concentration and selectivity, duration of elicitor exposure, culture age, cell line, growth regulation and nutrients [16].

Several strategies have been improved to enhance alkaloid production in cell culture of plants, including optimization of nutrient media [17], growth regulators [18,19], chemical treatment [20], and using of fungal elicitors [21,22].The effects of fungal growth medium, elicitor concentration, fungal age, exposure time and callus age on alkaloid production by callus culture are important factors in elicitation process. However, data about the response of *N. tazetta* var. *italicus* calli to these factors are rare or absent.

Therefore, the target of this study was to optimize the previous parameters in an attempt to enhance alkaloid production by callus cultures of *N. tazetta* var. *italicus*.

MATERIALS AND METHODS

Plant Material

The bulbs of the selected plant were taken from the Agriculture Research Center, Giza, Egypt. The plant was identified as *Narcissus tazetta* var. *italicus* (Ker-Gawler) Baker by the Herbarium of Medicinal and Aromatic Plants Department, National Research Centre, Giza, Egypt.

Fungal Elicitor

Fusarium sporotrichioides Sherbakoff was isolated from the rhizosphere of *N. tazetta* var. *italicus* [23]. The fungus was identified by The National Research Center, Chemistry of Natural and Microbial Products Department and Experts in Fungal identification at Botany and

Microbiology department, Faculty of Science, Cairo University. After growing the fungus at 28°C under the different experimental factors, culture filtrate was sterilized by membrane filtration (Millipore filter GS 0.22 pm) and used as elicitor [23].

Establishment and Subculture of Calli Derived from Bulb Explants

Callus cultures were prepared from bulb explants after removing of papery scales and roots, and washing with soap and disinfectants according to the method of Squires and Langton [24] and described by Abu Taleb et al. [23]. After preparing the primary bulb explants, they were cultured on a modified Murashige and Skoog medium [25,26], supplemented with 3% sucrose, 3mgl^{-1}naphthalene acetic acid (NAA) and1.5mg l^{-1} benzyl adenine (BA). The medium was solidified with 0.6% Agar (Sicomol, Portugal) and autoclaved for 20 min at 121°C. This medium was optimum for production of maximum amount of total intracellular alkaloids by calli of *N. tazetta* var. *italicus* [23]. The calli induced from the explants, after 2 weeks of initiation, were used as inocula for subcultures (4 pieces jar^{-1}). The cultures were incubated at 18 ± 2°C, for 16 h photoperiod d^{-1}.

Factors Affecting Calli Growth and Alkaloid Contents

Five factors were used to stimulate calli growth and alkaloid production. Czapek-Dox broth (CDB), Modified Czapek-Dox broth (molasses broth, MCDB), Malt extract glucose broth (MEGB) and Potato Dextrose broth (PDB), were the four types of fungal growth media used in this work. CDB composed of (g l^{-1}): sucrose, 20.0; $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; $FeSO_4.7H_2O$, 0.01. Sucrose was replaced by sugar cane molasses (45 g l^{-1}) as a sole carbon source in MCDB. Sugar cane molasses was obtainedfrom the Egyptian Sugar Company and Integration Industries. MEGB composed of (g l^{-1}): malt extract, 30; glucose, 50.0. PDB composed of (g l^{-1}): potato, 200; dextrose, 20.0. The pH of media was adjusted at 6.5-7.0. The levels of the other factors were elicitor concentrations: 0.0, 10, 30, 50, 80 and 100%; fungal age: 0.0, 3, 5, 7, 10 and 15 d; contact time: 0.0, 3, 7, 10, 15 and 20 d; and callus age: 2, 4, 6, 8, 10 and 12 weeks.

Fungal elicitor (5 ml) was added to *N. tazetta* var. *italicus* callus cultures of different ages and incubated at 18 ± 2 °C for different incubation periods.

Measurement of Calli Growth

Calli were harvested at the end of incubation period and, weighed to measure fresh weight (Fwt), and dried in an oven at 50°C for 3 d to obtain dry weight (Dwt).

Extraction and Determination of Alkaloids

Known weight of callus dry biomass (0.2-0.3 g) was extracted three times with 5 ml methanol for 15 min at 50ºC in an ultrasonic bath. The concentrated extract was dissolved in 2 ml of 3% sulfuric acid. The solution was extracted with diethyl ether, basified with 1 ml of 25% ammonia, then extracted with one ml chloroform for three times. After filtration over anhydrous sodium sulfate, the chloroform extracts were evaporated to dryness and weighed [27].

Statistical Analysis

Analysis of variance (ANOVA) was carried out using SPSS statistical program version 15. All measurements were done in triplicate and the statistical evaluation was achieved using analysis of variance and the significance was determined using LSD at level of 5%. Data were represented as mean ± standard error.

RESULTS AND DISCUSSION

Importance of Elicitors

Elicitors are compounds of biological or non-biological origin, which upon contact with plant cells, could significantly enhance defense responses in plant against pathogens due to the up-regulation of the transcription and expression of resistance-related genes as well as increasing the activity of resistance-related enzymes and antimicrobial compounds [28,29] Fungal elicitor can induce cell synthesis and accumulation of

secondary metabolites in some hypersensitive plants, such as phytoalexin, flavonol, alkaloid and others [30].

Factors Affecting Calli Growth and Alkaloid Contents

Effect of fungal growth media

One month old callus cultures of *N. tazetta* var. *italicus* were exposed for 10 d to culture filtrate of *F. sporotrichioides* (50%) which was grown in CDB, MCDB, MEGB or PDB for 7 d (Table 1). It was found that elicitor prepared from PD culture filtrate was the most effective in enhancing alkaloid biosynthesis. This enhancement was significant when compared with elicitors prepared from the other culture filtrates. At the end of contact time, 2.39, 1.32 and 1.72-fold increases in alkaloid contents, fresh and dry weights, respectively, were observed when compared the results of elicitor prepared from PD culture filtrate with that prepared from CD culture filtrate. MEGB came next to PDB followed by Molasses broth. Callus cultures exposed to elicitor prepared from CD culture filtrate, produced the minimum amount of total intracellular alkaloids.

F. sporotrichioides is considered as one of the phytopathogens, which have the ability to produce mycotoxins and it produces higher amount of trichothecenes when grown on potato dextrose agar [31]. So, elicitor prepared from *F. sporotrichioides* can be used as a stress factor to induce the synthesis of secondary metabolites. This finding may explain our results.

Sharma and Pandey [32] concluded that colony diameter, culture characteristics and sporulation of fungi were greatly influenced by the type of growth medium used. Growth of *Penicillium* sp. and *Acremonium kiliense* was maximum on Potato Dextrose Agar, when compared with Czapek's Dox + Yeast Extract Agar or Lignocellulose Agar. Pradeep et al. [33] observed that growth and pigment production by *F. moniliforme* KUMBF 1201were best on PDA followed by Malt extract Agar and Oat Meal Agar.

Effect of elicitor concentrations

Culture filtrate of *F. sporotrichioides* (after growing on PD broth for 7 d), was used at different concentrations (10, 30, 50, 80 and 100%) to determine the most effective one in enhancing total intracellular alkaloids production by 4 -week old callus culture (Table 2). The Table shows that elicitor above 20% enhanced alkaloid accumulation significantly, whereas below 50% the enhancement was not significant, compared to the control. Elicitor concentrations at 80 and 100% induced a 2.69 and 2.28-fold increases, respectively in alkaloid contents of calli as compared to the control. On the other hand, fungal elicitor concentrations appeared to have no significant effect on calli growth.

Cultivation of leaf explants of *Atropa belladonna* in liquid MS-medium containing 1mg l^{-1} of each of NAA and BA in the presence of 5 mg ml^{-1} of *Aspergillus niger* for 10 d appeared to be the most favorable conditions for stimulating total tropane alkaloids production [34]. On the other hand, Harkes, et al. [35] indicated that only 0.5 $mg.ml^{-1}$ of *A. niger,* increased anthraquinone content to 500 $\mu g.g^{-1}$ fresh weight in culture medium of *Cinchona lederiana*, as compared with the control. Aslam et al. [36] disagreed with our findings and concluded that the higher concentration of fungal elicitor had toxic effects and resulted in the loss of cell viability.

Effect of fungal age

Our results show that alkaloid contents were increased significantly as compared to the control, by the addition of 80% (v/v) culture filtrate of *F. sporotrichioides*, after growing in PDB for different incubation periods (3, 5, 7, 10 or 15 d), to 4- week old callus cultures of *N. tazetta* var. *italicus* for 10 d (Table 3). Total intracellular alkaloid contents of calli recorded maximum value (32.6 mg. g^{-1} dry cell) when calli were treated with elicitor prepared from 10- d old fungal culture. At this treatment, alkaloid increased by 1.80- fold when compared to control (0 time). Alkaloids began to decrease till reached 29.5 mg. g^{-1} dry cell when calli exposed to 15- d old fungal culture. On the other hand, a progressive increase in calli growth was observed with ageing of fungal culture. Elicitors prepared from older fungal cultures were effective in stimulating calli growth and alkaloid production more than that prepared from younger culture filtrates. This finding was confirmed by [36].

Mansonone F (phytoalexin) production in elm callus induced by elicitors of *Ophiostoma ulmi* was affected by cultural and experimental conditions [34]. These factors included fungal isolates, fungal culture age, elicitor concentration and the time of elicitor treatment.

Effect of exposure periods

When 4-week-old callus cultures were exposed to *F. sporotrichioides* elicitor (prepared from 10- d old fungal culture), for different exposure periods (3, 7, 10, 15 or 20 d), fresh weight alkaloid accumulation increased significantly (Table 4). Highest amount of alkaloids (36.5 mg g^{-1} dry cell) was induced by 80% fungal elicitor when incubated with callus cultures for 15 days. In this treatment alkaloid increased by 2.52- fold, compared to the control. However, alkaloid began to decrease (32.7 mg. g^{-1} dry cell) after 15d exposure time. This result was confirmed by Namdeo et al. [15]. Data obtained from this investigation indicated that the period of elicitor contact with callus is important parameter in improving growth and alkaloid production. Yangib et al. [37] confirmed this conclusion. Indole alkaloid production by *Catharanthus roseus* cultures was affected by the elicitor type and concentration as well as exposure time [38]. Elicitor specificity, elicitor dosage and time of harvest after elicitation are important factors in enhancing alkaloid levels [34].

Effect of callus age

Elicitor prepared from culture filtrate of *F. sporotrichioides* (after growing on PD broth for 10 d) was added to callus cultures of different ages (2, 4, 6-, 8-, 10- or 12-week-old) for 15 d to measure growth and alkaloid accumulation (Table 5). It was found that alkaloid production was directly proportional to callus age until the eighth week then declined significantly by ageing. Eight-week-old callus cultures produced maximum amount of alkaloids (39.2 mg. g^{-1} dry cell) after elicitation for 15 d. In this treatment alkaloids increased by 1.8-fold, compared to that produced by 2-week-old callus cultures. On the other hand, there was a progressive significant increase in both fresh and dry weights by increasing age of the elicited callus. The Table shows that, 12-week-old callus cultures recorded maximum growth (11.93 and 1.87 g. jar^{-1} fresh and dry weights, respectively) after elicitation.

A progressive increase of fresh and dry weights was observed during all stages of calli growth [35]. Culture age is very important factor for improving elicitation. Addition of elicitors is preferred when the cells are rapidly dividing [39].

Aslam et al. [36] concluded that optimal induction of the target occurred when the elicitors were added to cultures at late exponential or early stationary phase of plant cell growth. The total alkaloid content in static cultures was maximum (1.8 mg /100 ml) in 6-week-old tissues in the suspension culture of *Papaver rhoeas* Linn callus [40]. However, the highest root growth and alkaloid contents were obtained in 5-week-old cultures of *Vernonia cinerea* [41]. These results proved that callus age producing maximum amount of alkaloids is not constant but differs according to plant type and other factors.

Maximum alkaloid production was elicited by sterilized culture filtrate of *F. sporotrichioide*s at a concentration of 80% v/v (after growing the fungus on potato dextrose broth for 10 days), when incubated for 15 days with 8-week-old calli of *N. tazetta var*. *italicus* on a modified Murashige and Skoog medium. On the other hand, 12-week-old calli recorded maximum growth exposed to fungal elicitor under the previous conditions.

Our results are in agreement with those of Sarin [40] who reported that the effect of any elicitor to induce maximum response depended on the culture age, elicitor concentration and contact time with the elicitor. Wiktorowska et al. [42] suggested that one of the primary actions of the bio industry is improving production of commercially valuable compounds through elicitor application. It is affected by several parameters such as specificity, concentration and exposure time of the elicitor, as well as culture conditions (nutrient composition of the medium, growth regulators and light), in addition to the growth stages of the cell culture.

Table 1. Elicitation of growth and alkaloids production in *N.tazetta* var. *italicus* callus cultures under the influence of growth media of *F. sporotrichioides*

Broth	Fresh weight (g. jar^{-1})	Dry weight (g. jar^{-1})	Alkaloids (mg. g^{-1}dry cell)
CD	5.51 ± 0.086	0.76 ± 0.08	9.8 ± 0.66
MCD	6.14 ± 0.57	0.85 ± 0.04	11.4 ± 0.95
MEG	6.85 ± 0.94	1.18 ± 0.29	15.0 ± 3.0
PD	7.29 ± 0.58	1.31 ± 0.34	23.4 ± 2.0
LSD 5%	1.33	0.37	3

Legend: LSD 5% - Least significant difference at 5% level, CD-Czapek-Dox, MCD-Modified Czapek-Dox, MEG-Malt extract glucose, PD - Potato Dextrose
Values are mean ± standard error

Table 2. Growth and alkaloids production in *N. tazetta* var. *italicus* callus cultures after exposure to different concentrations of fungal elicitor

Fungal elicitor concentration (%)	Fresh weight (g. jar^{-1})	Dry weight (g. jar^{-1})	Alkaloids (mg. g^{-1} dry cell)
Control	6.01 ± 0.64	0.70 ± 0.03	12.0 ± 1.0
10	5.75 ± 0.91	0.66 ± 0.11	12.2 ± 1.0
20	4.21 ± 0.95	0.56 ± 0.05	13.0 ± 1.0
50	7.10 ± 1.64	0.97 ± 0.31	23.1 ± 2.0
80	6.48 ± 1.06	0.79 ± 0.26	32.3 ± 0.56
100	6.40 ± 0.24	0.73 ± 0.04	27.4 ± 0.8
LSD 5%	1.78	0.31	2.0

Legend: LSD 5% - Least significant difference at 5% level,
Values are mean ± standard error

Table 3. Elicitation of growth and alkaloids production in *N. tazetta* var. *italicus* callus cultures under the influence of fungal age

Fungal age (Day)	Fresh weight (g. jar^{-1})	Dry weight (g .jar^{-1})	Alkaloids (mg. g^{-1} dry cell)
Control	6.72 ± 0.33	0.74 ± 0.03	18.1 ± 1.0
3	7.73 ± 0.60	0.90 ± 0.02	21.2 ± 0.8
5	8.67 ± 0.44	0.66 ± 0.46	23.5 ± 1.0
7	8.95 ± 0.38	1.02 ± 0.05	28.6 ± 1.0
10	9.35 ± 0.70	1.16 ± 0.18	32.6 ± 0.76
15	10.34 ± 0.71	1.54 ± 0.24	29.5 ± 2.0
LSD 5%	0.98	0.40	2.0

Legend: LSD 5% - Least significant difference at 5% level
Values are mean ± standard error

Table 4. Induction of growth and alkaloids production in *Narcissus tazetta* var. *italicus* callus cultures exposed to fungal elicitor for different exposure periods

Exposure time (Day)	Fresh weight (g. jar^{-1})	Dry weight (g. jar^{-1})	Alkaloids (mg. g^{-1} dry cell)
Control	4.55 ± 0.85	0.64 ± 0.24	14.5 ± 0.7
3	5.06 ± 1.08	0.56 ± 0.13	19.8 ± 0.76
7	5.51 ± 0.94	0.57 ± 0.20	22.4 ± 3.0
10	5.64 ± 0.44	0.63 ± 0.41	24.6 ± 1.6
15	6.70 ± 0.44	0.66 ± 0.42	36.5 ± 6.0
20	9.03 ± 0.50	1.00 ± 0.07	32.7 ± 6.5
LSD 5%	1.34	0.26	3.0

Legend: LSD 5%, - least significant difference at 5% level
Values are mean ± standard error

Table 5. Effect of callus age of *Narcissus tazetta var. italicus* on elicitation of growth and alkaloids production

Callus age (week)	Fresh weight (g. jar^{-1})	Dry weight (g. jar^{-1})	Alkaloids (mg. g^{-1} dry cell)
2	3.67 ± 0.46	0.37 ± 0.01	21.8 ± 1.0
4	5.73 ± 0.67	0.51 ± 0.33	27.3 ± 1.0
6	7.67 ± 0.82	0.80 ± 0.25	33.6 ± 0.76
8	8.85 ± 0.39	1.08 ± 0.13	39.2 ± 1.0
10	10.50 ± 0.36	1.45 ± 0.14	35.2 ± 0.42
12	11.93 ± 0.39	1.87 ± 0.09	32.3 ± 0.76
LSD 5%	0.97	0.34	2.0

Legend: LSD 5% - least significant difference at 5% level
Values are mean ± standard error

Table 6. Optimization of culture conditions to improve growth and alkaloid production in callus cultures of *N. tazetta* var. *italicus*

Treatment No.	Fungal growth media	Fungal elicitor concen. (%)	Fungal age (Days)	Exposure time (Days)	callus age (Weeks)	Fresh weight (g. jar -1)	Dry weight (g. jar -1)	Alkaloids (mg g-1 dry cell)
1	CD	50	7	10	4	5.51	0.76	9.8
2	MCD	50	7	10	4	6.14	0.85	11.4
3	MEG	50	7	10	4	6.85	1.18	15.0
4	PD	50	7	10	4	7.29	1.31	23.4
5	PD	0	7	10	4	6.01	0.70	12.0
6	PD	10	7	10	4	5.75	0.66	12.2
7	PD	30	7	10	4	4.21	0.56	13.0
8	PD	50	7	10	4	7.10	0.97	23.1
9	PD	80	7	10	4	6.48	0.79	32.3
10	PD	100	7	10	4	6.40	0.73	27.4
11	PD	80	0	10	4	6.72	0.74	18.1
12	PD	80	3	10	4	7.73	0.90	21.2
13	PD	80	5	10	4	8.67	0.66	23.5
14	PD	80	7	10	4	8.95	1.02	28.6
15	PD	80	10	10	4	9.35	1.16	32.6
16	PD	80	15	10	4	10.34	1.54	29.5
17	PD	80	15	0	4	4.55	0.64	14.5
18	PD	80	10	3	4	5.06	0.56	19.8
19	PD	80	10	7	4	5.52	0.57	22.4
20	PD	80	10	10	4	5.64	0.63	24.6
21	PD	80	10	15	4	6.71	0.67	36.5
22	PD	80	10	20	4	9.03	1.01	32.7
23	PD	80	10	15	2	3.67	0.37	21.8
24	PD	80	10	15	4	5.73	0.51	27.3
25	PD	80	10	15	6	7.67	0.80	33.6
26	PD	80	10	15	8	8.85	1.08	39.2
27	PD	80	10	15	10	10.50	1.45	35.2
28	PD	80	10	15	12	11.93	1.87	32.3

Legend: CD - Czapek-Dox, MCD - Modified Czapek-Dox, MEG - Malt extract glucose, PD - Potato Dextrose

CONCLUSION

Finally, 8-week-old calli of *N. tazetta* var. *italicus*, cultivated with 80% (v/v) culture filtrate of *F. sporotrichioides* (after growing on PD broth for 10 d) for 15 d, represented the optimum conditions for induction of alkaloid production (Table 6, treatment no. 26). Also, maximum growth obtained under the previous conditions, but when elicitor was added to12-week old calli (Table 6, treatment no. 28).

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of

research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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