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Enhancement of Alkaloids Production in Tissue Culture of *Narcissus Tazetta* Var. *Italicus* I: Effect of Growth Regulators and Fungal Elicitors

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Abstract The objective of this study is to improve total alkaloid contents of *Narcissus tazetta* var. *italicus* tissue cultures by using different types of growth regulators and fungal elicitors. Seven and six species belong to four genera were isolated from both rhizosphere and bulbs of *N. tazetta* var. *italicus*, respectively. *Aspergillus niger*, *A. ochraceus*, *A. oryzae*, *Fusarium oxysporum*, *F. sporotrichioides*, *Penicillium. chrysogenum* and *Trichoderma viride* were isolated from the rhizosphere. On the other hand, *A. flavus*, *A. niger*, *F. oxysporum*, *F. semitectum*, *P. chrysogenum* and *T. viride* were isolated from the bulbs. Explants derived from bulbs and leaves of *N. tazetta* var. *italicus* were cultured on Murashige and Skoog medium (MS) supplemented with different combinations and concentrations of plant growth regulators. Calli derived from both bulb and leaf explants, and bulbs derived from bulb explants, had maximum growth and highest amount of total intracellular alkaloids, when cultured on MS4, MS6 and MS2 media, respectively (without the addition of fungal elicitor). MS4 contained benzyl adenine (BA 1.5 mg/l) and naphthalene acetic acid (NAA 3 mg/l), MS6, contained BA (2 mg/l) and indole acetic acid (IAA 0.5 mg/l), whereas, MS2 contained BA (2 mg/l) and indole butyric acid (IBA 1mg/l). The isolated fungi were screened for their potentiality to elicit alkaloids production. *F. sporotrichioides* culture filtrate was the most effective elicitor in enhancing growth and total intracellular alkaloids production when incubated on MS4 medium for 10 days with 4 week old callus cultures of *N. tazetta* var. *italicus*.

Key words: *Narcissus tazetta* var. *italicus*, tissue cultures, alkaloids, fungal elicitors

Introduction

Narcissus tazetta var. *italicus* (Ker-Gawler) Baker is a perennial ornamental plant, of the Amaryllidaceae family, that grown in Egypt from a

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bulb. Plants belong to family Amaryllidaceae are well known for their ornamental value, but they are also interesting for their alkaloids. Ghosal *et al*. (1985) recorded that the large numbers of structurally diverse Amaryllidaceae alkaloids are classified mainly into nine skeleton types, for which the representative alkaloids are: norbelladine, lycorine, homolycorine, crinine, haemanthamine, narciclasine, tazettine, montanine and Galantamine. The Amaryllidaceae-type alkaloids possess antiviral and antitumor properties (Gabrielsen *et al*., 1992; Weniger *et al*., 1995), as well as an anticholinesterase activity. The extraction of chemical plant products from intact plants has several inherent problems, including seasonal variations, pests, diseases, and inconsistent product quality and yield (Kargi and Potts, 1991). Several strategies have been studied to enhance the alkaloid production in cell culture such as optimization of nutrient media (Van der Heigden *et al.*, 1989), growth regulators (Ganapthi and Kargi, 1990), chemical treatment (Zhao *et al.*, 2000) and employment of fungal elicitors (Karthikeyan *et al.*, 2007). Also, metabolic engineering has focused on increasing production by precursor feeding, and introduction of genes encoding specific metabolic enzymes into the plant (El-sayed and Verpoorte, 2007). *In vitro* shoot and root cultures established in growth media containing various combinations of growth regulators (auxins and cytokinins) are able to produce the same secondary metabolites as the intact plant, and by medium optimization higher levels have been obtained in certain cases. Jacobs *et al.* (2005) suggested that plant cell culture systems are viable alternatives for the production of secondary metabolites that are of commercial importance in food and pharmaceutical industries. Ingram (1977) recorded that tissue culture techniques offer many advantages over the *in vivo* cultivation of whole plant because of the exclusion of contaminating microorganisms, control of the environmental parameters (such as temperature, light and nutrients), ability to inoculate host cells without wounding and ease of application or removal of materials from cultured cells.

Elicitation induces or enhances biosynthesis of metabolites due to addition of biotic and/or abiotic elicitors. Singh (1999) defined elicitation as the induction of secondary metabolites production by biotic or abiotic molecules or treatments. Many components derived from fungi (cell wall fragments, polysaccharides, oligosaccharides, glycoproteins, etc.) have been used as elicitors to enhance secondary metabolites production in plants.

Lee and West (1981) showed that pectinolytic enzymes released into the culture filtrates of *Rhizopus stolonifer* elicited phytoalexin accumulation in castor bean.

The low yield of alkaloids in the plant combined with their high market price made *Narcissus tazetta* important model systems for plant biotechnology and

secondary metabolism studies. It is well known that plant alkaloids exhibit inter alia antitumor, antiviral, antimalarial, antibacterial, antifungal and anticholinergic activities. Some types of alkaloids have been used in the treatment of myasthenia gravis, myopathy and diseases of the nervous system (Martin, 1987).

This research was intended to investigate the production of callus cultures to approach maximum amount of alkaloid production.

Material and methods

Plant materials

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

Fungal elicitors

Isolation of fungi from the rhizosphere and bulbs of N. tazetta var. italicus

Czapek-Dox Agar medium (CDA) was used to isolate fungi from the rhizosphere and bulbs of *N. tazetta* var. *italicus*. The isolated fungi were used for the preparation of elicitors.

Rhizospheric soil was added to sterilized distilled water and put in the shaker for 20 minutes. After making different dilutions, samples of each dilution (one ml) were transferred to Czapek-Dox agar plates, and incubated at 28-30 ℃ for 5 to 7 days.

Pieces of plant bulbs that showed symptoms of diseases were submerged for five minutes in 5% sodium hypochlorite for surface sterilization. Bulb pieces were thoroughly washed with sterile distilled water, blotted between two folds of sterilized filter paper and putted in Petri dishes containing CDA. The plates were incubated at 28-30 ℃ for 5-7 days (Abou-Zeid *et al*., 2008). Fungi isolated from rhizosphere and bulbs were purified, identified and stored on slants containing potato dextrose agar (PDA) at 4 ℃ until use.

CDA medium composed of (g/l): sucrose, 20.0; NaNo3, 3.0; KH2PO4, 1.0; MgSO4.7H2O, 0.5; KCL, 0.5; FeSO4.7H2O, 0.01; agar, 15 and distilled water, 1000 ml. pH was adjusted at 6.5-7.0. CDA medium was amended with streptomycin sulphate (30 mg/l) to suppress bacterial growth. PDA composed of (g/l): potato extract, 250; dextrose, 20.0; agar, 15 and distilled water, 1000 ml. pH was adjusted at 6.5-7.0.

Identification of fungi

The isolated fungi were identified by The National Research Center, Chemistry of Natural and Microbial Products Department according to their morphological characters (Booth, 1971; Gilman, 1975; Raper and Fennell, 1973).

Preparation of fungal elicitors

From the margin of seven day old fungal cultures, two discs (each of 1 cm diameter) were cut and transferred to 250 ml Erlenmeyer flasks each containing 100 ml CD broth. Flasks were incubated in shaking incubator (150 rpm) at 28 ℃ for 7 days. At the end of incubation period Fungal cells were harvested, filtered (Whatman paper #1) culture filtrates of fungi were sterilized by membrane filtration (Millipore filter GS 0.22 pm) and used as elicitors. Fungal cells dried at 65 ℃ for 24 h, and ground up with a mortar. Dry cell powder was dissolved with water to obtain 0.2 gl^{-1}. The solutions were autoclaved and filtered with Whatman paper #1. Mycelial extracts and culture filtrates (5ml) were separately added to one month old calli and bulbs of N. tazetta var. italicus and incubated at 18 ± 2 ℃ for ten days. Pectinolytic activity in fungal culture fjiltrates were measured by the modified Nelson procedure (Somoygi, 1952).

Establishment of calli derived from bulb and leaf explants

To obtain callus cultures and bulb propagation, the bulb explants were cultured following basically the method of Squires and Langton (1990). After removing of papery scales and roots, bulbs were washed with soap, rinsed with tap water and then disinfected according to the following scheme: bulbs were placed in 80% ethanol for 5 min then immersed in 20% (v/v) household bleach for 30 min. Bulbs were vertically cut through two-third of their height (leaving one-third portion of its basal part intact) and placed in 5% bleach containing 0.003% (w/v) of Tween 80 for15 min, then they were washed several times with sterilized distilled water. From each bulb, the two-third of the apical portion, the two external scales of the remaining portion and a thin layer of the basal plate were discarded. The remaining portion of the bulb was longitudinally sectioned to obtain explants formed by segments of twin-scales joined by a thick segment (2-3 mm) of basal plate tissue. Leaves explants were cut into small pieces, placed in 80% ethanol for 20 minutes, and then washed several times with sterilized distilled water.

Primary explants of bulbs and leaves were cultured on a modified Murashige and Skoog medium (Hussey, 1982) with 3% sucrose and supplemented with different types of growth regulators at different concentrations (Table 1). The media were solidified with 0.6% Agar (Sicomol, Portugal) and autoclaved at 121 ℃ for 20 minutes. Glass growth jars each containing 20 ml of Murashige and Skoog medium were used for the initial phases of the cultures at 18 ± 2 ℃ for 16 hrs photoperiod/day (Santos *et al.*, 1998).

Table 1. Different types of modified Murashige and Skoog medium (Hussey, 1982)

Media	Media composition
MS_1	4.4 g/l MS basal medium + 30 g/l sucrose + 6.0 g/l agar + 2 mg/l BA + 10 mg/l NAA
MS_2	4.4 g/l MS basal medium + 30 g/l sucrose + 6.0 g/l agar + 2 mg/l BA + 1mg/l IBA
MS_3	4.4 g/l MS basal medium + 30 g/l sucrose + 6.0 g/l agar + 0.5mg/l BA + 0.5 mg/l NAA
MS_4	4.4 g/l MS basal medium + 30 g/l sucrose + 6.0 g/l agar + 1.5 mg/l BA+ 3 mg/l NAA
MS_5	4.4 g/l MS basal medium + 30 g/l sucrose + 6.0 g l agar + 5 mg/l BA + 1 mg/l 2,4-D
MS_6	4.4 g/l MS basal medium + 30 g/l sucrose + 6.0 g/l agar + 2 mg/l BA + 0.5 mg/l IAA

Growth regulators**:** 2, 4, D, 2, 4- dichlorophenoxy-acetic acetic acid; BA , benzyl adenine; NAA , naphthalene acetic acid; IAA , indole acetic acid; IBA , indole butyric acid.

Method of subculture

After 2 weeks of culture initiation, the obtained cultures were cut into pieces (approximately with similar size, 20 - 30 mm) and used as inocula for subculture (4 pieces / jar). Cultures were incubated at 18 ± 2 ℃, for 16 hours photoperiod/day.

Measurement of callus and bulb growth parameters

Induction Frequency (IF), size, color and nature of the calli and bulbs were recorded 4 weeks after callus initiation.
Callus Induction Frequency (CIF %) was calculated as follow:

$$CIF\% = \frac{\text{No. of induced calli}}{\text{Total No. of explants}} \times 100$$

The regeneration of roots and / or shoots from calli was observed. Calli and bulbs were harvested and weighed to measure fresh biomass (FB), and

dried in an oven at 50◦C for 3 days to obtain dry biomass (DB). Total alkaloids content was measured also.

Extraction and determination of alkaloids

Known weight of dry biomass (0.2-0.3 g) was extracted three times with 5 ml methanol in an ultrasonic bath for 15 min at 50 ℃, combined extracts were concentrated under vacuum and dissolved in 2 ml of 3% sulfuric acid. The neutral compounds were removed by extraction (three times) with diethyl ether. After basification of the extract with 1 ml of 25% ammonia, it was extracted with one ml chloroform for three times. The chloroform extracts were filtered over anhydrous sodium sulfate and evaporated to dryness and weighed (Sellés *et al*., 1999).

Statistical analysis

Analysis of variance (ANOVA) was carried out using SPSS statistical program version 15. All measurements described in this work were the means of three replicates ± standard error. The results obtained were analysed using analysis of variance and the significance was determined using LSD at level of 5%.

Results and discussion

Fungi isolated from rhizosphere and bulbs

Table (2) represents fungal species isolated from both rhizosphere and bulbs of *N. tazetta* var. *italicus.* Seven species were isolated from the rhizosphere *Aspergillus niger, A. ochraceus, A. oryzae, Fusarium oxysporum, F. sporotrichioides, Penicillium chrysogenum* and *Trichoderma viride* were collected from the rhizosphere. On the other hand, and six species were isolated from bulbs, respectively. *A. flavus, A. niger, F. oxysporum*, *F. semitectum*, *P. chrysogenum* and *T. viride* were isolated from the bulbs. Beaumont and Hudson (1929) isolated *Fusarium moniliforme, Gibberella moniliformis, F. moniliforme* var. *maius, F. solani, F. bulbigenum, Cylindrocarpon album, Mamularia macrospora*, and *Fusariom* spp. from *Narcissus* bulbs showing rotting of the scales from the neck downward, the base remaining healthy. Isolates of *Penicillum simplicissimum, P. verrucosum* var. *cyclopium, P. brevicompactum, P. multicolor, P. oxalicum, P. paxilli, Botrytis cinerea*, and *Gliocladium* sp. were obtained from necrotic virus-tested narcissus twin-scales (Lyon, 1978).

Table 2. Fungi isolated from the rhizosphere and bulbs of *Narcissus tazetta* var. *italicus*

Fungal species	Rhizosphere	Bulbs
A. flavus	-	+
A. niger	+	+
A. ochraceus	+	-
A. oryzae	+	-
F. oxysporum	+	+
F. semitectum	-	+
F. sporotrichioides	+	-
Pencillium chrysogenum	+	+
Trichoderma viride	+	+
Total species	7.00	6.00
Total genera	4.00	4.00

+, present ; -, absent

Influence of plant growth regulators on induction and growth of calli and bulbs

For the successful application of the tissue culture technique in crop breeding, callus growth and plant regeneration potential of each crop must be determined. Both callus induction and plant regeneration from explants require the presence of appropriate concentrations and combinations of plant growth regulators in the growth media (Kaya and Akı, 2013). The plant growth regulators used in this work were cytokinin such as benzyl adenine (BA) and auxins such as naphathalene acetic acid (NAA), indole butyric acid (IBA), 2, 4-dicholrophenoxy-acetic acid (2, 4-D) and indole acetic acid (IAA). Explants began to inflate after 7 days incubation and produce a small amount of callus cells or small bulbs at excised surface after 10-12 days incubation, followed by rapid growth. Callus and bulb induction frequency, growth, size and nature were determined 30 days after initiation. The data revealed that high induction frequencies of calli and bulbs were observed in all media. Induction frequency and growth of calli ranged between 88-96% and 5.10-6.66 g/jar fresh biomass and o.47- 0.63 g/jar dry biomass, when bulb explants were cultured on MS medium supplemented with different combinations and concentrations of plant growth regulators (Table 3).

Table 3. Calli induction, growth and nature after development from bulb explants of *Narcissus tazetta* var. *italicus* on MS medium supplemented with different concentrations and combinations of plant growth regulators

Media	CIF (%)	Growth (g/jar)		Size	Color	MAI	MAS
		fresh wt	dry wt				
MS1	88± 1.0	5.10 ± 0.06	0.47 ± 0.01	+	Y	C	H
MS2	91± 1.4	5.13 ± 0.00	0.48 ± 0.00	+	YB	C	F
MS3	95± 0.8	6.23 ± 0.08	0.54 ± 0.02	++	YW	C	S
MS4	96± 2.0	6.66 ± 0.11	0.63 ± 0.02	+++	YW	C	S
MS5	93± 1.1	5.81 ± 0.032	0.53 ± 0.01	++	YW	C	S
MS6	93± 0.6	5.19 ± 0.00	0.47 ± 0.00	++	YG	C	C

CIF, Callus induction frequency; MAI, Morphology after induction; MAS, Morphology after subculturing; Y, yellow; B, brown; W, white; G, green; C. compact; H, hairy; F, friable; S, smooth; G, granular
Data represented as mean ±standard error

However, induction frequency and growth of calli recorded 84-96%, and 1.88- 3.62 g/jar fresh biomass and 0.13-0.24 g/jar dry biomass, when leaf explants were cultured under the previous conditions (Table 4).

Table 4. Calli induction, growth and nature after development from leaf explants of *Narcissus tazetta* var. *italicus* on MS medium supplemented with different concentrations and combinations of plant growth regulators

Media	CIF (%)	Growth(g/jar) fresh wt	dry wt	Size	color	MAI	MAS
MS1	89± 1.4	3.20 ± 0.03	0.20 ± 0.01	++	Y	C	S
MS2	94± 1.8	3.31 ± 0.12	0.22 ± 0.07	++	YB	C	C
MS3	89± 1.6	2.78 ± 0.18	0.19 ± 0.02	++	Y	C	F
MS4	86± 1.4	2.26 ± 0.12	0.17 ± 0.01	++	Y	C	F
MS5	84± 2.3	1.88 ± 0.09	0.13 ± 0.00	+	YW	C	F
MS6	96± 1.1	3.62 ± 0.14	0.24 ± 0.03	+++	YG	C	CG

CIF, Callus induction frequency; MAI, Morphology after induction; MAS, Morphologyafter subculturing; Y, yellow; B, brown; W, white; G, green; C. compact; F, friable; S, smooth; G, granular
Data represented as mean ±standard error

Bulb induction frequency and growth ranged between 70-97%, and 6.16-8.30 g/jar fresh biomass and 0.55- 0.91 g/jar dry biomass, under the same conditions (Table 5).

These results are in agreement with those suggested by Islam *et al*. (2005) who concluded that growth regulators have a major effect and a regulatory role on the growth of callus and root cultures. Wiktorowska *et al*. (2010) revealed that auxins have important role in callus induction and their action was facilitated by lower concentrations of cytokinins. Auxins are involved in cell division, cell elongation, vascular tissue differentiation, rhizogenesis and root formation, embryogenesis and inhibition of axillary shoot growth (Chawla 2002; George *et al*. 2008; Park *et al*., 2010). Cytokinins are derivatives of adenine and seem to be required to regulate the synthesis of proteins.

This study showed that MS4, in which level of cytokinin is lower than auxin, was the most suitable medium for induction and growth of calli derived from bulb explants. However, MS6 and MS2, in which level of cytokinin is higher than auxin, were the most suitable media for induction and growth of leaf derived calli and bulb propagation, respectively. Formation of maximum number of calli derived from bulb explants required higher level of auxin than cytokinin, and vice versa in case of calli and bulb derived from leaf and bulb explants, respectively. These results are in agreement with those of Verma *et al.* (2012) who concluded that low auxin and higher cytokinin concentrations were

found to be better for callus proliferation from leaf explants of *Catharanthus roseus*. Janet *et al*. (2005) concluded that the ovary explants of *Narcissus* required high levels of auxin for the induction of callus.

Table 5. Bulbs induction, growth and nature after development from bulb explants of *Narcissus tazetta* var. *italicus* on MS medium supplemented with different concentrations and combinations of plant growth regulators

Media	BPF (%)	Growth(g/jar)		Size	Color	MAI	MAS
		fresh wt	dry wt				
MS1	79± 1.9	7.83 ± 0.13	0.88 ± 0.00	+	YB	C	S
MS2	97± 1.1	8.30 ± 0.13	0.91 ± 0.01	+++	YG	C	C
MS3	78± 0.7	7.14 ± 0.15	0.69 ± 0.01	+	Y	C	C
MS4	76± 1.7	6.18 ± 0.01	0.63 ± 0.05	+	Y	C	C
Ms5	70± 1.2	6.16 ± 0.00	0.55 ± 0.01	+	YW	C	C
MS6	75± 1.1	6.36 ± 0.04	0.66 ± 0.00	+	YW	C	C

BPF, Bulb propagation frequency; MAI, Morphology after induction; MAS, Morphology after Subculture; Y, yellow; B, brown; W, white; G, green; C. compact; S, smooth
Data represented as mean ±standard error

The presence of BA in lower concentration (2mg/l) than NAA (10mg/l) in MS1inhibited significantly bulbs derived calli induction frequency and growth when compared with the other types of media and stimulated shoot and root regeneration. Kaya and Aki, (2013) reported that minimum callus formation was obtained on MS medium supplemented with 10 and 2 mg/l of NAA and BAP, respectively, which support our results. On the contrary, the highest number of somatic embryos of *Narcissus* L. was noted under the influence of 25 µM 2, 4-D and 5 µM BA in explants cultivated for 8 weeks in liquid medium and then, for 4 weeks, on solid medium (Malik, 2008).

The level of auxin (10mg/l) is higher than that of cytokinin (2mg/l) in MS1. Moreover, the ratio between cytokinin and auxin (1:5) in this medium is higher than that in the other media. The ratio between the concentration of cytokinin and auxins was either equal (1:1) as in MS3, narrow as in each of MS4 (1:2) and MS2 (2:1) or wide as in each of MS5 (5:1) and MS6 (4:1). This may be the reason that induction and growth of calli derived from bulb explants in MS1 were lower than that in the previous media. Dessouky (2000) found that one mg/l of each of NAA and BA was more suitable for cell culture induction

of different explants of *Atropa belladonna.* On the other hand, minimum number and lowest growth of calli derived from leaf explants were attained in MS5 which contained higher concentration of cytokinin than auxin (5:1). Janet *et a*l. (2005) reported that the levels of various growth regulators were found to be important in obtaining shoot apices of *Narcissus* cultivars. They also concluded that the relative ratio of cytokinin and auxin is critical in tissue cultures.

In leaf explants, combinations of BA at 2 mg/l with either IAA at 0.5 mg/l (4:1) in MS6, or IBA at 1mg/l (2:1) in MS2 were associated with best growth and formation of maximum number of calli. Callus induction frequency recorded 94 and 96% when leaf explants were cultured on MS2 and MS6, respectively. In MS2 fresh biomass and dry biomass reached 3.31 and 0.22 g/jar, respectively, whereas, the two growth parameters recorded 3.62 and 0.24 g/jar, respectively in MS6.

In this investigation maximum bulb propagation frequency (97%) and growth (8.30 g/j fresh biomass and 0.91 g/jar dry biomass) were detected on MS2 medium containing BA and IBA at a concentrations of 2 and 1mg/l, respectively. On the other media, bulb propagation frequency was less than 80%, whereas fresh weight and dry weight ranged between 6.16- 7.83 and 0.55-0.88 g/j, respectively.

Data of the current study provide strong evidence that type and concentration of growth regulator requirements for callus induction and growth in *N. tazetta var. italicus* varied depending on the source of explant. Such response has been established previously in other plants (Nikam and Shitole, 1999; Zouine and El hadrami, 2004).

Our results are in agreement with those of Dhar and Joshi (2005) who concluded that responses to organogenesis in *Saussurea obvallata* are influenced by explants type, age and specific hormonal concentrations in the medium. They concluded that this response possibly resulted from the variation in morphological and biochemical characters of the different explant types, which affects cytokinin uptake and competence of cells to initiate callus or shoots. Explant type, and probably its anatomical structure seem to play a significant role in callus initiation.

In this investigation the colour of calli and bulbs was mostly yellow (Tables 3, 4 and 5). The appearance of calli and bulbs were compact after induction in all types of media. However, after first subculture the appearance of calli and bulbs remained compact or changed to hairy, friable, smooth or granular.

Influence of plant growth regulators on total alkaloids biosynthesis

Various strategies have been employed to increase the production of secondary metabolites in cell cultures for commercial exploitation. These include manipulation of culture media (hormonal and nutrient stress) and environmental conditions (temperature, pH and osmotic stress), precursor addition, elicitation and combination of these strategies.

Thirty day old calli and bulbs were used for estimation of total intracellular alkaloid contents after growing on growth media supplemented with different types and concentration of plant growth hormones (Fig. 1). It was evident from the data that the highest production of total intracellular alkaloids (14.2 mg/g dry cell) by bulbs derived calli was obtained on MS4. On the other hand, culturing leaves derived calli on MS6 resulted in maximum production of total intracellular alkaloids (9.8 mg/g dry cell) when compared with the other media. Highest amount of total intracellular alkaloid content of bulbs (12.4mg/g dry cell) was recorded when bulbs derived bulbs were cultured on MS2 medium. The other types of media were also suitable for enhancement of alkaloid production.

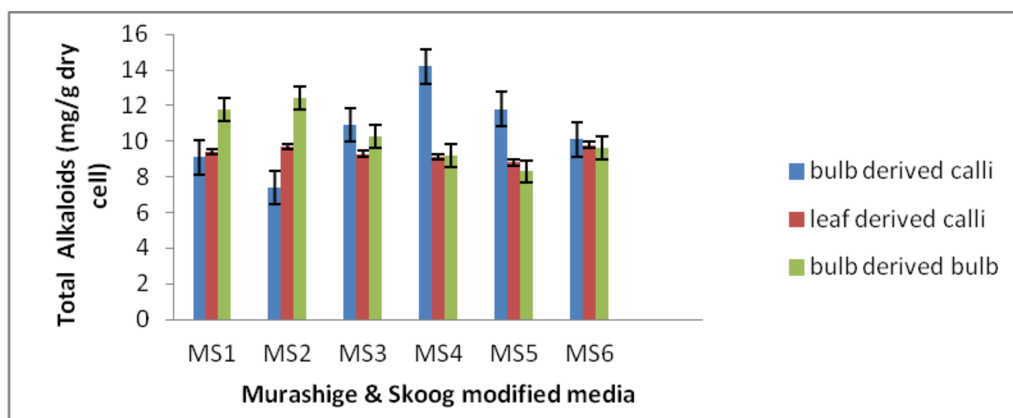

Fig. 1. Alkaloid contents (mg/g dry cell) of *Narcissus tazetta* var. *italicus* calli and bulbs when grown on modified Murashige and Skoog medium supplemented with different combinations and concentrations of growth regulators. Data represented as mean ± standard error

A few workers have attempted enhancement of alkaloid content in callus cultures of *N. tazetta* var. *italicus*. The induction of adventitious meristems of leaf base explants is a particularly promising method for the propagation of virus-free material and for the rapid propagation of valuable horticultural material**s** (Janet *et al.*, 2005). In order to produce galanthamine, an alkaloid

currently being tested in Alzheimer's disease therapy, Montserrat *et al.*(1997) used *in vitro* organ cultures of *Narcissus confuses*. Our results indicate that alkaloid production is enhanced by interacted or combined application of cytokinins and auxins, this is in agreement with results of Kadi *et al.* (2013). Types and concentrations of plant growth regulators and nutritional factors affect the production of secondary metabolites, as well as growth of cell cultures of higher plants (Vida *et al*., 2000). MS media supplement with 1:0.5 and 0.5:1 mg/l of 2,4-D and BA were found to be suitable media to increase callus biomass and total alkaloid contents (Verma *et al*., 2012). In our study it is important to mention that the amount of total intracellular alkaloid produced in bulbs derived calli was higher than that produced in leaves derived calli and bulbs derived bulbs. Also the total intracellular alkaloids production increased by increasing callus induction frequency and growth, the more callus induction frequency and growth the more total intracellular alkaloids production.

Fungal elicitors and biosynthesis of total intracellular alkaloids in callus cultures

Many studies have shown that both biotic and abiotic stresses when become in contact with higher plant cells, enhance secondary metabolite synthesis in plant cell cultures (Singh, 1999). However, no elicitor has been found to have a general effect on many culture systems, and no system has been found to respond to all elicitors. So it is necessary to screen various elicitors for a particular system to produce a desired compound.

In the present study, the isolated fungi from both rhizosphere and bulbs of *N. tazetta* var. *italcus* were screened for elicitation intracellular alkaloids production by callus cultures derived from bulb explants and grown on MS4 medium. Enhancement of secondary metabolites by elicitation is one of the few strategies recently finding commercial application.

In this work elicitors prepared from *A. ochraceus, F. sporotrichioide*s, *P. chrysogenum* and *T. viride* stimulated the biosynthesis of total intracellular alkaloids and increased dry biomass of *N. tazetta* var. *italicus* calli (Table 6). On the contrary, elicitors prepared from *A. flavus*, *A. niger*, *A. oryzae*, *F. oxysporum* and *F. semitectum*, inhibited significantly the total intracellular alkaloids biosynthesis compared to the control, whereas their effect on growth was variable. Response of calli to elicitors depended upon elicitor type, as recorded also by (Rijhwani and Shanks, 1998). Our investigation showed that culture filtrate was more effective than mycelial extract in elicitation alkaloids production. This result indicates that the extracellular fungal metabolites may be more effective than the intracellular in stimulating the total intracellular

Table 6. Growth and alkaloids production in *Narcissus tazetta* var. *italicus* callus cultures, after exposure to different types of fungal elicitors for 10 days

Fungal elicitors	Fresh weight (g/jar)	Dry weight (g/jar)	Intracellular alkaloids (mg/g dry cell)
Control	8.047 ± 0.34	0.80 ± 0.11	25.0 ± 1
A. flavus			
mycelial extract	6.18 ± 1.04	0.59 ± 0.09	18.4 ± 1
culture filtrate	8.32 ± 1.05	0.82 ± 0.06	20.2 ± 0.71
A. niger			
mycelial extract	6.29 ± 0.43	0.94 ± 0.03	14.7 ± 2
culture filtrate	5.22 ± 1.12	0.50± 0.03	22.3 ± 2
A. ochrceus			
mycelial extract	6.11 ± 0.03	1.39 ± 0.05	30.5 ± 1
culture filtrate	5.28 ± 0.44	0.67 ± 0.06	32.2 ± 0.4
A. oryzae			
mycelial extract	5.77 ± 1.58	1.37 ± 0.16	13.3 ± 1
culture filtrate	4.42 ± 0.70	1.29 ± 0.22	21.8 ± 2
F. oxysporum			
mycelial extract	7.73 ± 0.88	0.74 ± 0.07	21.3 ± 0.9
culture filtrate	7.62 ± 0.64	0.72 ± 0.06	24.8 ± 0.25
F. semitectum			
mycelial extract	3.84 ± 0.40	0.83 ± 0.20	18.9 ± 0.72
culture filtrate	5.46 ± 0.67	0.71 ± 0.24	21.7 ± 0.46
F. sporotrichioides			
mycelial extract	5.54 ± 1.04	0.91 ± 0.19	32.1 ± 0.55
culture filtrate	9.50 ± 0.67	1.08 ± 0.18	33.4 ± 0.25
P. chrysogenum			
mycelial extract	6.59 ± 0.64	0.95 ± 0.12	26.9 ± 0.57
culture filtrate	4.88 ± 0.11	0.91 ± 0.01	27.6 ± 0.66
T. viride			
mycelial extract	5.35 ± 0.21	0.91 ± 0.03	25.2 ± 0.56
culture filtrate	5.92 ± 0.60	0.78 ± 0.02	30.5 ± 1
LSD 5%	1.26	0.21	2

LSD 5%, least significant difference at 5% level; Data represented as mean ± standard error

alkaloids production. In addition, elicitor prepared from culture filtrate of *F. sporotrichioide*s was the most efficient one in increasing total intracellular alkaloid contents after incubation for 10 days with one month old callus cultures of *N. tazetta* var. *italicus.* Total intracellular alkaloid contents, fresh and dry biomasses of callus culture reached to 33.4 mg/g dry cell, 9.50 and1.08 g/jar respectively, after exposing for 10 days to the culture filtrate of *F. sporotrichioide.*

F.sporotrichioides is considered as one of the phytopathogens which have the ability to produce mycotoxins in host tissues. So, it can be used as a stress factor to stimulate hosts to produce secondary metabolites. Abu Taleb *et al*. (2012) reported that *F. sporotrichioides* is one of the trichothecenes producers. Karthikeyan *et al.* (2007) stated that the biosynthesis of terpenoid alkaloids can be stimulated by the addition of exogenous elicitors of *F. sporotrichioides*. Bobák *et al*. (1995) concluded that the fungal elicitor prepared from *Botrytis cinerea* affected sanguinarine alkaloid formation and accumulation in callus cells of *Papaver somniferum* L. pectinolytic activity in the culture filtrate of *F. sporotrichioides* was determined Lee & West (I981) showed that pectinolytic enzymes released into the culture filtrates of *Rhizopus stolonifer* elicited phytoalexin accumulation in castor bean.

Pectinolytic activity in the culture filtrate of *F. sporotrichioides* was determined, it was found that *F. sporotrichioides* can produced 12.6 units/ml. Lee & West (I981) showed that pectinolytic enzymes released into the culture filtrates of *Rhizopus stolonifer* elicited phytoalexin accumulation in castor bean.

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