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Callus Production and Suspension Elicitation of *Impatiens balsamina* L., Plant for Enhancing Accumulation of Phenolics and Flavonoids Content

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ABSTRACT

This research was conducted in the Laboratory of Plant Tissue Culture, Vegetable and Floriculture Department, Mansoura University, Egypt during 2016/2017. The aim was to use tissue culture technique for callus production and enhancing total phenolics (TPC) and flavonoids (TFC) content in suspension culture of garden balsam (*Impatiens balsamina* L.) which belongs to Family Balsaminaceae, which has many important secondary metabolites in its different organs. Callus was initiated on leaf discs cultured on MS medium supplemented with either 2,4-D or NAA at different concentrations (0.5, 1.0 and 1.5 mg/l), besides the control (MS-free hormone medium) with a fixed BAP concentration (0.5 mg/l). Also, suspensions were supplemented with either yeast extract or chitosan (0, 50, 100 and 200 mg/l) for enhancing accumulation of phenolics and flavonoids content for two separated elicitation periods (24 and 48hrs.). Data showed that the highest callus formation percentage (100%) was obtained from leaf explants cultured on MS medium supplemented with either 1.0 mg/l 2,4-D+0.5 mg/l BAP or 1.5 mg/l NAA+0.5 mg/l BAP. Also, these superior treatments produced the biggest callus volume (9.38 and 9.19 mm^3, respectively). The heaviest callus fresh and dry weights (8.25 and 1.15 g/jar) were recorded for MS medium fortified with 1.0 mg/l 2,4-D+0.5 mg/l BAP. In addition, after 24hrs from the suspensions elicitation, the highest TPC value of 8.298 mg/gdw with 4.18 fold higher than the control was recorded for suspensions which received 50 mg/l yeast extract. In the same time, 50 mg/l chitosan increased TFC (3.929 mg/gdw). The matter was partially similar after 48hrs, with a slight increase in all the elicitation treatments, since the superior treatments after 24hrs still giving a higher TPC and TFC.

Abbreviations: TPC; Total phenolics content, TFC; Total flavonoids content, 2,4-D; 2,4-Dichlorophenoxyacetic acid, NAA; 1-Naphthaleneacetic acid, BAP; 6-Benzylaminopurine.

Keywords: *Impatiens balsamina*; Callus induction; Callus suspension; Phenolics and Flavonoids content.

INTRODUCTION

More than 67.5% of individuals on the planet depend on plant for their hygienic and nutrition (Moses and Goossens, 2017), exceptionally the derived secondary metabolites from it. Traditionally, secondary metabolites can be acquired by extracting directly from the different organs of the plant. Nonetheless, this method requests farming on a huge scale in addition to the extraction, isolation and purification procedures which are more costly. Meantime, when it creates synthetically, the cost will be exceptionally expensive because of the complex processes of that way (Smetanska, 2008). One elective strategy that can be used for producing these bioactive substances is plant tissue culture. Different ways in plant tissue culture could be used for production of secondary metabolites such as organ, callus and suspension cultures by using many strategies for example, manipulating the culture conditions, optimizing the macro and micro-nutrients in the culture medium, feeding of elicitors and precursors in the *in vitro* growth medium (Karuppusamy, 2010). In this way, many researchers have concentrated on creating effective procedures for enhancing the accumulation and production of these important metabolites in plant without breeding or gene modification (Kim, 2011). As numerous secondary metabolites biosynthesis in plants is normally a plant protection reaction to different stresses, biotic and abiotic elicitors could enhance secondary metabolites accumulation in plant tissue cultures (Narayani and Srivastava, 2017). The most well-known and viable elicitors utilized in many previous researches mainly incorporate the microbial cells segments particularly oligo and polysaccharides.

Chitosan is a deacetylated subordinate effectively possible from different sources, especially from the exoskeletons of crustaceans. Also, it is found in some algae as well as outer skin of insects and the fungi cell walls (Nwe *et al.*, 2014). Being present in the ineffective micro-organisms wall, it could be perceived such as a microbe linked molecular type by the immune system of the plant. Finally, this activates the plant defense restraint (Choudhary *et al.*, 2017), beside accumulation of the related secondary metabolites. Consequently, chitosan has been generally utilized as a vigorous biotic elicitor in suspension cultures to improve the production of secondary metabolites, like artemisinin (Putalun *et al.*, 2007), phenylethanoid glycosides (Liu and Cheng, 2008) and phenylpropanoid (Chakraborty *et al.*, 2009). In addition, yeast extract as a biotic elicitor derived from fungal sources, modifies the enzyme effectiveness of phenylpropanoid metabolism that finally produces the phenolic compounds (Ramachandra and Ravishankar, 2002).

Impatiens balsamina L. belongs to Family Balsaminaceae and native to southern Asia in India. It is an annual herbaceous plant reach 20-75 cm height, with soften thick stem. The leaves have spirally arrangement with approximately 5.5 cm long and 1.75 cm broad, with a pointedly serrated margin. The flowers include color variations like red, pink, mauve, white or lilac. All the balsam parts have many active constituents which accumulate in the root, fruit, seed and flower. Many compounds have been isolated from I. *balsamina* L. plant parts, including phenolics (Kang *et al.*, 2013). Phenolic compounds contain an extensive variety of substances that show antioxidant characteristics which are used as additions, cosmetics and food preservatives (Capecka *et al.*, 2005). In addition, the plant parts have many naphthoquinone derivatives and flavonoids (Siatka and Kasparova, 2010; Zielinska *et al.*, 2012), which had memorable antioxidant, antitumor, hypocholesterolemic, antidiabetic and antimicrobial activities. Moreover, the plant parts are utilized in conventional therapy for skin

afflictions, snakebite, burns, dandruff and splitting hair ends (Baskar *et al.*, 2012).

To the best of our knowledge, there were no previous reports about the effects of biotic elicitors such as yeast extract or chitosan for enhancing production and accumulation of phenolics and flavonoids contents by the callus suspension culture of *Impatiens balsamina* L. Therefore, the aim of this research was to study the influence of some plant growth regulators concentrations (2,4-D and NAA) on callus production of this important medicinal plant, besides the influence of some biotic elicitors (chitosan and yeast extract with different concentrations and elicitation periods) on accumulation of phenolics and flavonoids content in callus suspension cultures.

MATERIALS AND METHODS

This study was conducted during 2016/2017 in Biotechnology Lab of Plant Cell and Tissue Culture, Vegetable and Floriculture Department, Faculty of Agriculture, Mansoura University, Egypt on *Impatiens balsamina* L., plant.

Seed sterilization and germination

Balsam seeds were obtained from China (pink flowers) and sanitized by washing under a running faucet water for service sterilization from solid particles or the dust followed by immersing in 70% ethyl alcohol for 30 seconds, rinsed with a sterilized distilled water for two times. The main sterilizer was 30% commercial clorox (1.25 % sodium hypochlorite) for 15 min, then washing by sterilized distilled water for four times was repeated. Sterilized seeds were germinated on pre-sterilized medical cotton pads moistened with distilled water in aseptic conditions in 200 ml glass jars (Figure, 1A). After one week from the seeds culturing, the full germinated seedlings were transferred on a solid MS (Murashige and Skoog, 1962) free hormone medium supplemented with 7 g/l agar and 3% (w/v) sucrose. Medium pH was adjusted to 5.8 by using 0.1 N NaOH or 0.1 N HCl solutions and autoclaved for 20 min under pressure of 15 lb and 121°C. Jars were incubated for 16 hrs. photoperiod with light intensity of 3000 lux and 25 ± 2°C heat.

Callus induction

For callus induction, *in vitro* germinated seedlings (21 days old from culturing) were utilized as a source of leaf explants (Figure, 1B). The excised leaves (approximately 1 cm^2) were aseptically cultured in 200 mm jars containing 25 ml of MS salts supplemented with either 2,4-D at different concentrations (0.0, 0.5, 1.0 and 1.5 mg/l) or NAA (0.5, 1.0 and 1.5 mg/l) with a fixed concentration from BAP (0.5 mg/l), 30 g/l sucrose and 7 g/l agar. After 4 weeks from the callus initiation (Oluk *et al.*, 2010), data were recorded for callus formation percentage, callus volume (mm^3), callus fresh and dry weight (g) and callus morphology (texture and color).

Callus elicitation in the suspension cultures

One month old brown friable callus pieces (5 pieces) of approximately 5-10 g fresh weight (Azeez and Ibrahim, 2013) were cultured on 250 ml Erlenmeyer flasks containing 100 ml of MS liquid medium fortified with either yeast extract or chitosan at different concentrations (50, 100 and 200 mg/l) on a rotary shaker (110 rpm). The control treatment was cultured on a liquid MS free elicitor medium. Three replicates were utilized for each treatment. Samples of the treated callus were collected after 24 and 48 hours from the elicitation treatments. Each elicitation treatment was repeated twice within the following 2 months. Data were recorded for total phenolics and flavonoids content.

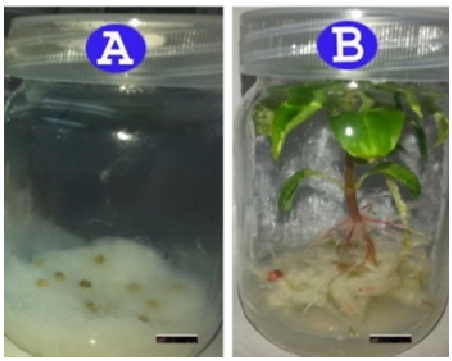

Figure 1. Explants source of *Impatiens balsamina*. A, Seeds cultured on sterilized medical cotton; B, Seedling leaves as a source of callus explants.

Samples preparation

Callus pieces and cells in Erlenmeyer flasks for each elicitation treatment were filtered over Whitmann No.1 filter paper and then dried for 48 hrs. at room temperature followed by, grinding using mortar and pestle, then stored at 5°C until next use. The powder of each dried sample (200 mg) was extracted with 70% methanol (20 ml) at room temperature for one week. The residue was extracted again with 70% methanol (30 ml), at room temperature, for two days. Finally, extracts were filtered over Whitmann No.2 and the extracts were kept under -20°C for further use.

Measurement of total phenolics content

Total phenolics content (TPC) in I. *balsamina* methanolic extracts was colorimetrically evaluated (Singleton and Rossi, 1965). Since, the response of 0.5 ml methanolic extract solution mingled with 0.5 ml of 10% Na_2CO_3 and 0.5 ml of the Folin-Ciocalteau reagent, followed by keeping at room temperature in the dark for one hour. The absorbance was then done by using UV spectrophotometer at 700 nm wavelength. Standard solution of gallic acid was utilized in making the curve of calibration. The total phenolics content was expressed as mg of gallic acid equivalents/g of dry weight. In addition, total phenolics fold increases were calculated by dividing each elicitation value on the control treatment.

Measurement of total flavonoids content

Total flavonoids content (TFC) in the extract was estimated using a methodology of Meda *et al.* (2005) with a minor modification. In brief, 1.5 ml of the methanolic extracts was mixed with 2% (w/v) $AlCl_3$ in a tube for 10 min at room temperature. Solution absorbance was estimated at 415 nm wavelength using UV spectrophotometer. Standard solution of quercetin was utilized to quantify flavonoids content and the obtained results were presented in mg quercetin equivalents/g dry weight. Moreover, total flavonoids fold increases were

calculated by dividing each elicitation value on the control treatment.

Statistical analysis

The results were reported as mean ± standard error (SE). The experiments were designed in a completely randomized design (one way) with twelve replicates for the callus induction experiment. The same design was used for phytochemical analysis of the elicited callus suspension culture, but with three replicates for each elicitation treatment. Means of each experiment represented a twice separated experiments average. COSTAT v.63 statistical software was used for analysis of variance (ANOVA) and subsequently Duncan's new multiple range test (Duncan, 1995) was used for means comparison at $P \leq 0.05$.

RESULTS

Callus induction

Callus formation%

This part of the study aimed to record the various responses for callus formation using the leaf explants of *Impatiens balsamina* cultured four weeks on MS medium supplemented with different concentrations (0.5, 1.0 and 1.5 mg/l) from either 2,4-D or NAA each alone, plus the control medium (free hormone MS medium). The obtained results illustrated in Figure (2) revealed that the callus formation was varied depending on the auxin type and concentration used. In addition, no callus was observed on MS medium free of growth regulators. This result was in agreement with that of Rao *et al.* (2015) who revealed that callogenic response in *Centella asiatica* was observed in all combinations of growth regulators except for on media free of growth regulators. Also, they indicated that media containing a low concentration from either 2,4-D or NAA less than 0.5 mg/l was not supportive for the callus formation and the suitable concentrations were between 1-2 mg/l.

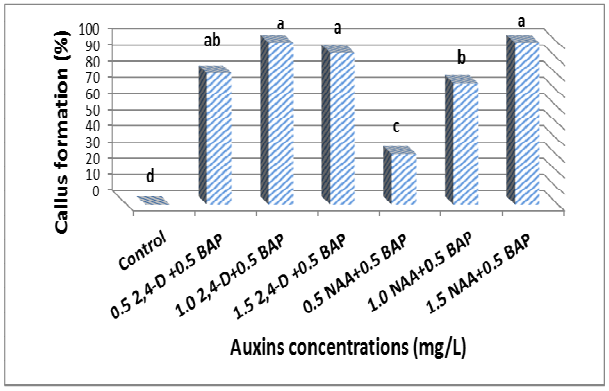

Figure 2. Effect of auxin types and concentrations on callus formation% of *Impatiens balsamina* leaf explants. Columns with different letters indicate statistically significant difference among groups at p < 0.05.

The best results for callus formation (100%) were obtained from leaf explants cultured on MS medium supplemented with either 1.0 mg/l 2,4-D + 0.5 mg/l BAP or 1.5 mg/l NAA + 0.5 mg/l BAP without significant differences between them. These results agreed with Gandi and Giri (2013) who observed that the callus growth in *Centella asiatica* was increased on medium contained 2,4-D + BAP and Patel and Patel (2013) on *Tecomella undulata* who found that callus induction percentage increases with increasing the concentration of NAA and 2,4-D up to 3.0 mg/l and 2,4-D still giving higher callus formation percentage comparing with NAA. In contrast, the lowest significant value of callus formation percentage (31.20%) was recorded for MS medium supplemented with 0.5 mg/l NAA. However, it was clear that 2,4-D concentrations were stronger compared with the NAA concentrations even with using the lowest concentration of 0.5 mg/l., and this was in agreement with Saensouk *et al.* (2007) who indicated that the highest callus formation percentage was obtained from young leaves of *Cornukaempferia larsenii* cultured on the medium supplemented with 0.5 mg/l 2,4-D in the light condition. Also, Sakpere *et al.* (2014) concluded that 2,4-D is better auxin for high callus induction in *Telfaria occidentalis* explants as compared with NAA.

Callus volume (mm^3)

For callus volume, data illustrated in Figure (3) shows that there was a positive relationship between callus formation % and the callus volume. Since the superior treatments in producing a higher callus formation percentage also significantly produced the higher callus volume values. As, MS medium supplemented with either 1.0 mg/l 2,4-D + 0.5 mg/l BAP or 1.5 mg/l NAA + 0.5 mg/l BAP gave 9.38 and 9.19 mm^3, respectively with non-significant differences between them. Also, the least value (3.83 mm^3) for that characteristic was recorded for MS medium supplemented with 0.5 mg/l NAA + 0.5 mg/l BAP.

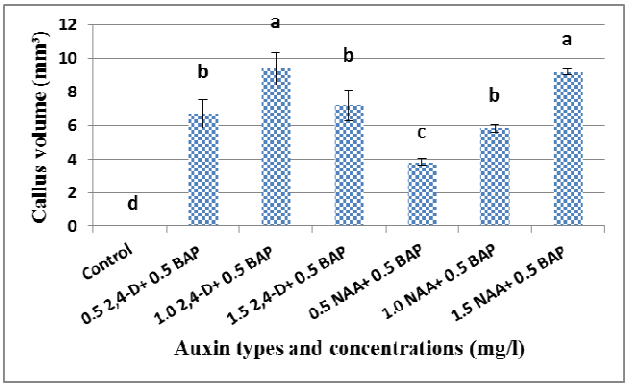

Figure 3. Effect of auxin types and concentrations on callus volume (mm^3) of *Impatiens balsamina* leaf explants. Columns with different letters indicate statistically significant difference among groups at p<0.05. Bars on the columns represent the standard error(n = 12).

Callus fresh and dry weights

The obtained data for callus fresh and dry weights of *Impatiens balsamina* L., tabulated in Table (1) reveal that MS medium supplemented with 1.0 mg/l 2,4-D + 0.5 mg/l BAP gave significantly the heaviest fresh and dry weights of I. *balsamina* callus, as it were 8.25 and 1.15 g callus fw/jar, respectively. Similar records were tabulated by Ameen and Mohammed (2010) on *Salvia officinalis* using the same 2,4-D concentration (1.0 mg/l) plus a lower BAP concentration (0.2 mg/l). The next positive results were recorded for MS media fortified with either 1.5 mg/l 2,4-D + 0.5 mg/l BAP or 1.5 mg/l NAA + 0.5 mg/l BAP and 0.5 mg/l 2,4-D + 0.5 mg/l BAP, since they recorded 6.06, 5.72 and 5.60 g callus fw/jar, respectively. In a

similar trend, corresponding values were recorded for the callus dry weight.

Callus morphology characteristics

Data presented in Table (1) and illustrated in Figure 4 (4A to 4F) clear that adding the two auxins (2,4-D and NAA) to the callus growth media in the low concentration (0.5 mg/l) produced light green calli. However, the higher concentration (1.5 mg/l) turned the calli colors of brown to green and brown to white with using 2,4-D and NAA, respectively. Similar results were obtained by Rahayu *et al.* (2016) on *Centella asiatica*. Moreover, with increasing the two types of auxin concentrations the calli texture was friable. In contrary, with the low auxin concentrations, compact calli were formed. However, callus produced on NAA medium was amorphous, friable, and ranged in colors from green to brown.

Table 1. Influence of 2,4-D and NAA concentrations on callus vegetative parameters initiated from leaf explants of *Impatiens balsamina* after 28 days from culturing.

Auxin type and Conc.(mg/l)	Fresh weight (g)	Dry weight (g)	Morphology	
			Texture	Color
Control	-	-	-	-
0.5 2,4-D+0.5 BAP	5.60±0.35^{bc}	0.53±0.03^{bc}	compact	G
1.0 2,4-D+0.5 BAP	8.25±1.09^{a}	1.15±0.38^{a}	compact	B
1.5 2,4-D+0.5 BAP	6.06±0.57^{b}	0.63±0.11^{bc}	friable	B to G
0.5 NAA+0.5 BAP	4.12±0.17^{d}	0.39±0.02^{c}	compact	G
1.0 NAA+0.5 BAP	5.15±0.19^{c}	0.46±0.01^{bc}	compact	G to Y
1.5 NAA+0.5 BAP	5.72±0.18^{bc}	0.70±0.11^{b}	friable	B to W

Values represent the mean ± SE of two independent experiments. Means followed by different letters in each column are significantly different at the 0.05 probability level using Duncan's multiple range tests; – means that no data had represented; G, green; B, brown; Y, yellow; W, white.

Measurement of total phenolics and flavonoids

Total phenolics and flavonoids content after 24 hours from callus suspension elicitation

Data presented in Table (2) and illustrated in Figure 4 (4G and 4H) clear that after 24 hours from adding the elicitor's concentrations into the callus suspension, yeast extract at the lower concentration had the upper hand in increasing the total phenolics content comparing with the other biotic elicitor (chitosan) concentrations. Moreover, there was a negative relationship between increasing the elicitor concentrations and the total phenolics content. This was in a similar trend with the findings of Sanchez-Sampedro *et al.* (2005) who showed that yeast extract did not work as biotic elicitor in the higher doses. Fortifying the callus suspension by 50 mg/l yeast extract significantly produced the highest phenolics content which was 4.18 fold higher (8.298 mg/gdw) than that of control (1.983 mg/gdw). This result was in agreement with Hamza (2013) who found that 50 mg/l yeast extract act as a promoter for increasing phenolics content of the *in vitro* cultured *Lupinus termis* seedlings. Also, Ishikawa *et al.* (2007) revealed that, adding yeast extract into the growth media enhanced and increased accumulation of the active constituents in *Glehnia littoralis*. In addition, Zhao et al. (2004) indicated that elicitation of *Cupressus lusitanica* with yeast extract enhanced also metabolism and production of polyphosphoinositol. The next positive value in that respect, was recorded when the callus suspension medium was fortified with 100 mg/l yeast extract with 3.79 fold (7.520 mg/gdw) comparing with the control. Besides, only the lowest chitosan concentration (50 mg/l) produced higher phenolics content of 7.037 mg/gdw comparing with the other two higher concentrations of 100 and 200 mg/l, as it recorded 3.854 and 3.373 mg/gdw, respectively. This was in agreement with the findings of Abraham *et al.* (2011) who showed that with increasing the concentration of chitosan into the culture medium up to 150 mg/l, caused reduction of phenolics content in the *Curcuma mangga* plantlets *in vitro*.

Table 2. Influence of callus suspension elicitation on total phenolics and flavonoids contents (mg/gdw) of I. *balsamin*a after 24 hours from elicitation.

Elicitation treatments	Total phenolics content (mg/g GAE)	Phenolics fold increase	Total flavonoids content (mg/g QE)	Flavonoids fold increase
Control	1.983±0.18^{g}	-	0.837±0.19^{e}	-
50 mg/l yeast extract	8.298±0.23^{a}	4.18	2.896±0.59^{b}	3.45
100 mg/l yeast extract	7.520±0.39^{b}	3.79	2.687±0.52^{bc}	3.21
200 mg/l yeast extract	4.851±0.17^{d}	2.44	2.179±0.28^{cd}	2.60
50 mg/l chitosan	7.037±0.10^{c}	3.54	3.929±0.16^{a}	4.69
100 mg/l chitosan	3.854±0.28^{e}	1.94	1.535±0.27^{d}	1.83
200 mg/l chitosan	3.373±0.06^{f}	1.70	1.573±0.24^{d}	1.87

Values represent the mean ± SE of two independent experiments. Means followed by different letters in each column are significantly different at the 0.05 probability level using Duncan's multiple range test.

As for total flavonoids content, the matter was differed comparing with phenolics content, as supplying the callus suspension with chitosan at 50 mg/l, significantly enhanced accumulation of flavonoids with 4.69 fold higher (3.929 mg/gdw) than the control (0.837 mg/gdw). In the same way, Mendhulkar and Vakil (2013) found that feeding the suspension culture of *Andrographis paniculata* by 20 mg/l chitosan for 24 hrs. duration explored the maximum flavonoids elicitation of 3.51 mg/gdw. In addition, adding all the tested concentrations from yeast

extract was in the second order for increasing the flavonoids content to reach 2.896, 2.687 and 2.179 mg/gdw with 3.45, 3.21 and 2.60 fold higher than the control for using 50, 100 and 200 mg/l, respectively. Moreover, the highest concentration of chitosan (100 or 200 mg/l) gave the least flavonoids content (1.535 and 1.573 mg/gdw, respectively) after the control suspension.

Figure 4. Responses of *Impatiens balsamina* leaf explants to different plant growth regulator types and concentrations on callus induction and suspension elicitation with different biotic elicitors. A, Callus on MS medium fortified with 0.5 mg/l 2,4-D+ 0.5 mg/l BAP; B, Callus on MS medium fortified with 1.0 mg/l 2,4-D+ 0.5 mg/l BAP; C, Callus on MS medium fortified with 1.5 mg/l 2,4-D+ 0.5 mg/l BAP; D, Callus on MS medium fortified with 0.5 mg/l NAA+ 0.5 mg/l BAP; E, Callus on MS medium fortified with 1.0 mg/l NAA+ 0.5 mg/l BAP; F, Callus on MS medium fortified with 1.5 mg/l NAA+ 0.5 mg/l BAP; G, Callus suspension elicited by 50 mg/l yeast extract; H, Callus suspension elicited by 50 mg/l chitosan. Scale bars = 1 cm.

Total phenolics and flavonoids content after 48 hours from callus suspension elicitation

Table (3) shows that overall, the lowest concentration of yeast extract and chitosan (50 mg/l) or 100 mg/l yeast extract, gave significantly the maximum amount of phenolics content with 2.88, 2.74 and 2.61 fold higher (8.627, 8.220 and 7.816 mg/gdw) than the control (2.990 mg/gdw), respectively after 48 hours from the elicitation process and non-significant differences were shown between them. In addition, supplying the callus suspension by 100 or 200 mg/l chitosan reduced the phenolics content to 4.036 and 3.930 mg/gdw, respectively. The control suspension significantly produced the lowest phenolics content (2.990 mg/gdw), compared with all of the other suspension treatments.

The total flavonoids content showed significant ($P \leq 0.05$) differences among the different elicitor types and concentrations as shown in Table (3). Dealing with yeast extract and chitosan elicitation treatments, 50 mg/l chitosan in the calli suspension gave the maximum flavonoids content value with 4.09 fold higher (3.665 mg/gdw) than the control. This finding was in agreement with Mendhulkar and Vakil (2013) who cleared that after 48 hrs. from chitosan elicitation, the total flavonoids content of *Andrographis paniculata* was decreased with increasing concentrations of chitosan from 5 to 10 and 20 mg/l with the differ in chitosan concentrations in our research.

This was followed by 2.880, 2.873 and 2.756 mg/gdw when 50, 100 and 200 mg/l yeast extract were added to the callus suspension, respectively with non-significant differences between them. Moreover, the three examined yeast extract concentrations (50, 100 and 200 mg/l) recorded 3.21, 3.20 and 3.07 fold higher than the control. The least flavonoids content was recorded for the suspensions supplemented with either 100 and 200 mg/l chitosan or the control suspension which did not receive any type of elicitors, as they were 2.373, 1.226 and 0.896 mg/gdw, respectively.

Finally, comparing the total phenolics and flavonoids content after 24 and 48 hrs. from the callus suspension elicitation, there was an increment in that respect. This positive variations in phenolics and flavonoids content due to the treatment period might be attributed to the differential mode of elicitor concentrations that were only turned fitly favorable when interacting tardily with the elicitor like yeast extract or chitosan.

Table 3. Influence of callus suspension elicitation on total phenolics and flavonoids content (mg/gdw) of I. *balsamina* after 48 hours from elicitation.

Elicitation treatments	Total phenolics content (mg/g GAE)	Phenolics fold increase	Total flavonoids content (mg/g QE)	Flavonoids fold increase
Control	2.990±0.12^{d}	-	0.896±0.17^{d}	-
50 mg/l yeast extract	8.627±0.28^{a}	2.88	2.880±0.72^{b}	3.21
100 mg/l yeast extract	7.816±0.32^{ab}	2.61	2.873±0.47^{b}	3.20
200 mg/l yeast extract	7.665±0.62^{b}	2.56	2.756±0.40^{b}	3.07
50 mg/l chitosan	8.220±0.86^{ab}	2.74	3.665±0.47^{a}	4.09
100 mg/l chitosan	4.036±0.32^{c}	1.34	2.373±0.16^{b}	2.64
200 mg/l chitosan	3.930±0.41^{c}	1.31	1.226±0.19^{c}	1.36

Values represent the mean ± SD of two independent experiments. Means followed by different letters in each column are significantly different at the 0.05 probability level using Duncan's multiple range test.

DISCUSSION

Plant cell and tissue culture assume a noteworthy part in the preservation of imperiled medicinal and aromatic plants, besides enhance accumulation of their secondary metabolites. *In vitro* callus induction and expanding their secondary metabolites were recorded for *Convolvulus alsinoides* (Kaladhar, 2012), *Citrullus colocynthis* (Tanveer *et al.*, 2012) and *Solanum trilobatum* (Priya and Chellaram, 2014). Gathering treatments of 2,4-D with BAP was more effective for callus induction than the other combinations of NAA with BA especially with the lower concentrations of 2,4-D (0.5 and 1.0 mg/l). This

demonstrates the effectiveness of 2,4-D in cell growth and division. Role of 2,4-D in calli induction was also studied with other medicinal plants like *Ocimum sanctum* (Lim *et al*., 2009), *Ionidium suffruticosum* (Arunkumar and Jayaraj, 2011), *Achyranthes aspera* (Sen *et al.*, 2014) and *Glinus lotoides* (Teshome and Feyissa, 2015). Influence of the interaction between 2,4-D and BAP gained significant noticeable results on calli formation percentages compared with the interaction among NAA and BAP concentrations. These results agreed with many other publications with different plants (Wani *et al.*, 2010; Acemi *et al.*, 2012; Guruchandran and Sasikumar, 2013; Lalabadi *et al.*, 2013; Kumar *et al.*, 2014; Padmavathy, 2014). In contrast, there was a positive interaction of NAA with BAP in forming fast grown callus equal with 2,4-D in combination with BAP. Also, this was observed with some plants like *Solanum nigrum* (Sridhar and Naidu, 2011) and *Orthosiphon aristatus* (Reshi *et al.*, 2013).

Elicitors are abiotic and biotic compounds triggering the accumulation of active constituents. Using such biotic or abiotic elicitors for enhancing production of secondary metabolites has become a very important strategy for that purpose in decreasing the pathway period needed to produce high product concentrations (Cai *et al.*, 2012). Chitosan is a polymer of β-1,4 -glucosamine residues (Chakraborty *et al.*, 2009) and an exogenous biotic elicitor gotten from fungal cell wall (Montesano *et al.*, 2003), which utilized as a part of the plant cell, tissue and organ cultures for induction of imperative secondary metabolites. Also, it has been considered for their roles on accumulation of rosmarinic acid in *Ocimum basilicum* (Kim *et al.*, 2005), phenylpropanoid enzymes (Chakraborty *et al.*, 2009) and oleanolic acid in *Calendula officinalis* (Wiktorowska *et al.*, 2010). The capacity of chitosan to expand the phenolics and flavonoids production by such a large quantity could be because of the imitating of a natural fungal infection, that initiated a characteristic safeguard reaction (Iriti and Faoro, 2009) or it may have upgraded the enzymes involved in phenolics and flavonoids biosynthesis, much the same as the phenylpropanoid pathway of other biosynthetic active constituents (Baque *et al.*, 2012). Nonetheless, various examinations have detailed that chitosan stimulates jasmonic acid, a single particle identified with defense-gene regulation (Doares *et al.*, 1995) and phospholipase C/protein kinase cascades (Vasconsuelo *et al.*, 2004). Generally, the impacts of elicitors on any active constituent production for a plant cell or tissue culture fundamentally rely upon the elicitation dosage and the elicitation period (Murthy *et al.*, 2014). Surpassing the ideal conditions may bring about physiological or metabolic damage. However, higher doses of chitosan (100 and 200 mg/l) resulted in a decrease of total phenolics and flavonoids content when compared to those at 50 mg/l. This might be identified with the harmfulness of chitosan to the living cells (Amborabe *et al.*, 2008).

Yeast extract is an essential elicitor in light of its rich substances from glucan, chitin, ergosterol, vitamin B-complex and glycopeptides (Boller, 1995); these compounds inspire plant protection reactions by activating metabolite synthesis (Putalun *et al.*, 2007; Cai *et al.*, 2012). Likewise, George *et al.* (2008) suggested that yeast extract is used as biotic elicitor in order to increase the plant growth, as a result of its high amino acids content. The enhancer impacts of yeast extract in amassing of phenolics and flavonoids contents may be an outcome that yeast extract upgrade and trigger synthesis and gathering of methyl jasmonate and/or jasmonic acid which promote phenolics acid production (Sanchez-Sampedro *et al.*, 2005; Abraham *et al.*, 2011).

CONCLUSION

In conclusion, both of 2,4-D or NAA have the ability for initiating callus formation with higher percentages and characteristics, but the 2,4-D one was more effective than NAA especially in its lower concentrations (0.5 to 1.0 mg/l). Also, the presence of lower concentrations of yeast extract or chitosan (50 mg/l) promoted the production of total phenolics and flavonoids in the callus suspensions of *Impatiens balsamina*. However, higher yeast extract and chitosan concentrations gradually decreased the total phenolics and flavonoids content.

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إنتاج الكالس وتحفيز المعلق لنبات البلسم بغرض تحسين محتوى الفينولات والفلافونيدات الكلية

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أجرى البحث بمعمل زراعة الانسجة النباتية بقسم الخضر والزينة كلية الزراعة جامعة المنصورة، مصر خلال الفترة ٢٠١٦/٢٠١٧. وكان الهدف يتمثل فى استخدام تقنية زراعة الانسجة النباتية كأداه لإنتاج خلايا الكالس لنبات البلسم والذى يتبع العائلة البلسمية ومحاولة لتحسين محتواه من الفينولات والفلافونيدات الكلية بمعلقات الكالس، حيث يحتوى على العديد من المركبات الثانوية الهامة فى أعضاءه المختلفة. تم البدء بإنتاج الكالس من خلال اجزاء من أوراق البادرات السابق تنبيتها معمليا ثم زرعت على بيئة موراشيج وسكوج (١٩٦٢) كاملة القوى والمزودة بتركيزات مختلفة (٠.٥، ١.٠ ، ١.٥ ملليجرام/اللتر) من 2,4-D و NAA كل على حده مع تركيز ثابت من BAP (٠.٥ ملليجرام/اللتر)، بالإضافة لمعاملة الكنترول (بيئة موراشيج وسكوج الخالية من الهرمونات). أيضا معلقات الكالس قد اضيف اليها اثنان من المحفزات الحيوية (مستخلص الخميرة، الشيتوزان) كل على حده بثلاث تركيزات مختلفة (٥٠، ١٠٠، ٢٠٠ ملليجرام/اللتر) بالإضافة لمعاملة الكنترول (معلق خلوى خالى من المحفزات) وذلك لدراسة قدراتهم التحفيزية لمدتين منفصلتين (٢٤ ، ٤٨ ساعة من بداية التحفيز). وقد أظهرت النتائج ان اعلى نسبة مئوية لتكوين الكالس (١٠٠%) قد تم الحصول عليها من الاجزاء الورقية المنزرعة على بيئة موراشيج وسكوج والمزودة بكل من ١.٠ ملليجرام/اللتر 2,4-D + ٠.٥ ملليجرام/اللتر BAP أو ١.٥ ملليجرام/اللتر NAA + ٠.٥ ملليجرام/اللتر BAP. كذلك فإن هذه المعاملات المتفوقة قد انتجت اكبر حجم للكالس (٩.٣٨ و ٩.١٩ مللم^3، على التوالى). علاوة على ذلك فإن اثقل الاوزان الطازجة والجافة للكالس (٨.٢٥ و ١.١٥ جرام) قد سجلت لبيئة موراشيج وسكوج المضاف اليها ١.٠ ملليجرام/اللتر 2,4-D + ٠.٥ ملليجرام/اللتر BAP. بالإضافة لذلك فإن معلقات خلايا الكالس المحفزة بمستخلص الخميرة بمقدار ٥٠ ملليجرام/اللتر ولمدة ٢٤ ساعة ، قد سجلت اعلى محتوى للفينولات الكلية (٨.٢٩٨ ملليجرام/الجرام وزن جاف) بزيادة بمعدل ٤.١٨ ضعف بالمقارنة بمعاملة الكنترول. فى نفس وقت التحفيز فإن معاملة الشيتوزان بتركيز ٥٠ ملليجرام/اللتر قد اعطت اعلى نسبة للفلافونيدات الكلية (٣.٩٢٩ ملليجرام/الجرام وزن جاف) بمقدار زيادة ٤.٦٩ ضعف بالمقارنة بالكنترول. كما تشابهت النتائج بشكل كبير بعد ٤٨ ساعة من التحفيز لتلك المسجلة بعد ٢٤ ساعة مع زيادة بسيطة لجميع معاملات التحفيز فى نسبتى الفينولات والفلافونيدات الكلية.