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# Application of Elicitation Process for Achievement of Phenols and α-Tocopherol Accumulation Rates in Suspension Cultures of Brassica *rapa* L.

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**ABSTRACT**

A reproducible protocol for *in vitro* enhancement of phenolic and α- tacopherol accumulation rate in *Brassica rapa* L leaf suspension cultures was established. The maximum frequency, fresh and dry weights of calli and cell suspension cultures were recorded with MS medium supplemented with 0.1mg/l NAA in combination with 0.5 mg/l BAp compared with other supplementations. Moreover, the effects of elicitation cell cultures with chitosan at different concentrations (0, 25, 50, 100 and 150 mg/l) on either cell number, fresh and dry weights or total accumulation rate of phenolic and α tacopherol content compared with those quantified in non-elicited cell cultures were investigated. It could be mentioned that elicitation of *B. rapa* leaf cell culture with chitosan at different concentrations have negative effects on either cell number, fresh or dry weights compared with non elicited treatment. However, it could be recommended that augmentation of modified MS medium with 100 mg/l of chitosan enhanced both accumulation rate of total phenolic (18.35 ng GAE/g DW ) and α tacopherol (45.4 µg/g FW) contents compared other concentrations of chitosan or non elicited cell culture .

**Keywords**: *Brassica rapa* L, callus, suspension, chitosan, phenolic, α- tacopherol

**Abbreviations:** NAA=α-Naphthaleneacetic acid, BAp= 6-benzylaminopurene

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## INTRODUCTION

Plants are a main valuable source of a wide range of secondary metabolites. Despite advancements in synthetic chemistry, biological sources are usually preferred due to lesser side effects and better biodegradability [1-2].

*Brassica rapa* L. subsp. sylvestris, belongs to the family Brassicaceae and cultivated commonly as a vegetable plant for its succulent root [turnip] [3, 4]. The leafy vegetable short cycle has a remarkable commercial importance since consumers tend to associate its local and traditional use to its high quality and health-promoting properties [5]. Brassicaceae family plants conceder as a target source of natural antioxidants due to the high levels of carotenoids, tocopherols and ascorbic acid. Strong evidence shows that these compounds may help to protect the human body against damage by reactive free oxygen species [6-8]. In particular, α-Tocopherol, together with carotenes, are the most abundant lipidsoluble antioxidant highly accumulating in chloroplasts [9]. It prevents lipid peroxidation by removal of singlet oxygen and lipid peroxyl radicals [10]. In addition to its antioxidant activity and ability to scavenge free radicals, this vitamin may reduce the risk of cancer and prevent progression of precancerous lesions [11]. Plant biotechnology applies to three major areas of plants and their uses: [1] control of plant growth and development; [2] protection of plants against biotic and abiotic stresses; and [3] expansion of ways by which specialty foods, biochemicals, and pharmaceuticals are produced [12].

Cell cultures show a higher rate of metabolism than intact differentiated plants due to fast proliferation of cell mass and condensed biosynthetic cycle and secondary metabolite formation can take place within a short cultivation time. In addition plant cells are biosynthetically totipotent and be able to produce the range of chemicals found in the parent plant [13]. Elicitation has been one of the most effective strategies for improving the productivity of bioactive secondary metabolites [14]. Elicitors have been used to stimulate secondary metabolite product formation in plant cell cultures by reducing the process time for increased culture volumes and high product concentrations [15]. Various elicitors such as chitosan, ß-glucan, and yeast extracts, as well as plant hormonal chemicals such as jasmonic acid [JA] and MeJA have been used to induce biotic and abiotic stresses upon plants [16].

Chitosan is considered an important structural component of the cell wall of several plant pathogen fungi. In plants, it can be applied in vivo as antimicrobial agent against fungi, bacteria and viruses, and in vitro can be used as elicitor of defense mechanisms [17]. Moreover, it is a one of the most abundant natural amino polysaccharides and its natural molecule that are applied to crops aiming of reducing or replacing more costly and environmentally damaging chemical bactericides. With reduced input costs and the potential for increased yields, farmers could gain substantial benefits from these applications of chitosan and its oligosaccharides to crops. Moreover it is highly biodegradable [18], non-toxic [19] and non-allergenic features of chitosan especially encourage its potential use as a bioactive material [20]. In addition, the Food and Drug Administration [FDA] agency in U.S. approved chitosan as a feed additive in 1983.

The present investigation intended to study the effect of in *vitro* elicitation of Brassica *rapa* L. using chitosan on enhancement the accumulation rates of phenols and α tacopherol in leaf cell suspension cultures compared with those quantified in non-elicited cell cultures.

## MATERIALS AND METHODS

### Plant material

*Brassica rapa* L. seeds were secured from the Horticulture Division, Faculty of Agriculture, Cairo University, Giza, Egypt.

### Methods

### Establishment of sterilized *Brassica rapa* L. seedlings

Seeds of *Brassica rapa* L. were surface sterilized by immersing in 70% ethanol for 10 sec., followed by three washes with sterile distilled water. Further, the seeds were immersed in 20% of commercial Clorox

(5.25 Cl g/l) solution containing a drop of Tween 20 for 15 min.. Whereas sterilized seeds were germinated aseptically on 50 ml of solidified basal MS-medium [21] in 300 ml glass jars in a few 5 to 8 days. Ten replicates, each of 10 seeds per jar were used. Agar was added prior to autoclaving at 1.2 kg/cm2 for 20 min. The pH of the medium was adjusted to 5.8 by the addition of 0.1 N HCL or 0.1 N KOH. Cultured jars were incubated in a growth chamber at 26 ± 1ºC and exposed to 16 h/day photoperiod at intensity of 3000 Lux from white cool light of fluorescent lamps.

**Establishment of *Brassica rapa* L. calli cultures.**

After 10 of aseptically geminated seeds on basal MS-medium where's leaf explants were used as a sources of calli cultures. Three sections 3-4mm in diameter of leaf were excised and cultured in 150 ml of glass jars containing 25 ml of the following modified MS-medium as follow:

1-MS free growth regulators
2-MS + 0.1 mg/l NAA
3-MS + 0.1 mg/l BAp
4-MS + 0.1 mg/l NAA + 0.2 mg/l BAp
5-MS + 0.1 mg/l NAA + 0.5 mg/l BAp
6-MS + 0.1 mg/l NAA + 1.0 mg/l BAp
7- MS + 0.1 mg/l NAA + 2.0 mg/l BAp

Cultures were incubated in darkness in a growth chamber at a constant temperature of 28°C ± 1 then incubated under light condition 16/8 h (2000 Lux) from cool white fluorescent lamps, and subcultured every 4 weeks on fresh new medium. After three subcultures, yellowish green calli were initiated and observed.

The following parameters were recorded after 4 weeks of cultivation and incubation.

1- Percentage of calli production (%).
2- Fresh and dry weights (g/jar).

**Preparation and establishment of cell cultures**

According to the described method by Taha et al. [22] cell cultures of *Brassica rapa* L. was established and sub-cultured in 100 ml of liquid (MS) culture medium with sucrose (3 %, w/v), myoinositol (100 mg/l) and supplemented with 0.1 mg/l NAA and 0.5 mg/l BAp (The best modified MS medium for callus production ). Suspension cultures were grown in gyratory rotary shakers at 120 rpm, 25 ± 1°C under for 3 days then under light conditions (16/8 h) 200 Lux.

**Elicitation with chitosan**

The obtained leaf suspension cultures were elicited using chitosan (Sigma Aldrich low molecular weight CAS –Number 9012-76-4) Synonym: Deacetylated chitin, Poly(D-glucosamine) at different concentrations 0,25,50,100 and 200 mg/l.

**Measurement of cell growth parameters**

According to **Taha et al.** (23) the cell number ($x10^5$) was counted and estimated during the growth period of cultivation as growth parameters. As well as the fresh &dry weights were recorded after 4 weeks of cultivation and incubation at 26±1 ºC.

**Sample preparation for chemical analysis**

Non-elicited and elicited cell cultures were harvested for the quantification of major secondary metabolites. Subsequently cells were filtered from their media using Whatman's filter paper (No. 1). Then, all samples were lyophilized under completely darkness for completely dryness, further milled into a fine powder.

**Determination of total phenolic content**

The phenolics were extracted and isolated according to described method by Taha et al. [22]. Five grams of fine powder macerated in 25 ml methanol: water (80:20, v/v) for 24 h at room temperature. The crude preparation was filtered, and the residue re-extracted twice with 50 ml of the same hydro-alcoholic solvent for 24 h at room temperature. The extract was filtered. The filtrates of hydro-alcoholic were combined. After removing the alcohol under vacuum at 40 °C, the phenolic compounds were extracted three with ethyl acetate (1:1, v/v) in the presence of an aqueous solution containing 20% ammonium sulphate and 2% of orthophosphoric acid solution. The three organic phases were combined; the residual water in the ethyl acetate was eliminated with anhydrous sodium sulphate, and then evaporated to dryness using a rotary evaporator. The extracted phenolics were dissolved in 20 ml of methanol and then filtered using filter paper. Methanolic solutions of phenolic were kept frozen until analysis.

The amounts of total phenolic contents in lyophilized cell line extracts were determined using the described method by Singleton and Rossi [24]. Briefly, reaction mixture contained one 100 µl of methanolic extracts (three replicates), 500 µl freshly prepared dilute (1:10) Folin–Ciocalteu reagent, and 2 ml of Na2CO3 (20% w/v). Mixtures were shaken and left to stand at room temperature for 30 min before measuring the absorbance at 760 nm using a spectrophotometer (UV–visible spectrophotometer; Shimadzu). The TPC was determined colorimetric through standard gallic acid curve and expressed as milligrams of gallic acid equivalents per 100 g of dry weight cell lines material (GAE/100 g dw).

**Determination of α-Tocopherol content**

The α-Tocopherol content was assayed as described by Taha et al. [25] where five hundred milligrams of fresh tissue were homogenized with 10 ml of a mixture of petroleum ether and ethanol (2:1.6, v/v). Then the extract was centrifuged at 10,000 rpm for 20 min and the supernatant was used for estimating α-Tocopherol. To one ml of the extract, 0.2 ml of 2% 2,2-dipyridyl in ethanol was added and mixed thoroughly and kept in dark for 5 min. Resulting red color was diluted with 4ml of distilled water and mixed well. The resulting color in the aqueous layer was measured at 520 nm. The **α-**Tocopherol content was calculated using a standard graph made with known amount of α-Tocopherol and expressed in µg /g fresh weight (FW).

**Statistics**

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test [(26)]**.** The values are mean ±SE. for five samples in each group. p values ≤0.05 were considered as significant.

**RESULTS**

**Establishment of *Brassica rapa* L. calli cultures.**

In this experiment sterilized segments of leaves (3-4mm) were *in vitro* cultured on MS basal medium free growth regulators or MS medium fortified with 0.1 mg/l of each NAA or Bap as alone treatments. Moreover, MS medium was augmented with 0.1 mg/l of NAA in combination with different concentrations of BAp (0.2, 0.5, 1.0, or 2.0 mg/l). The percentage of calli production , fresh and dry weights (mg/Jar) were recorded after 4 weeks of cultivation . Data in Table (1) and Fig. (1) display that the highest percentage of calli cultures (85 %), highest values and significant responses of fresh and dry weights 3.15 and 0.285 (g/jar) were recorded with augmentation of MS medium with 0.1 mg/l NAA + 0.5 mg/l of BAp .While the lowest value 31(%), 0.28 and 0.032 (g/jar) were recorded with supplementation of MS medium with 0.1 mg/l NAA as individually supplementation. However, modified of MS medium encouraged of primary callus induction. It could be mentioned that MS medium as nutrient medium free supplementation of NAA as auxin or BAp as cytokinin haven't any response on calli production from leaf explants of *Brassica rapa* L. As well as all determined morphological parameters of callus production either frequency ; fresh or dry weights were significant in their responses.

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**Table 1: Effect of supplementation MS-medium with0.1 mg/l of NAA individually or in combination with BAp at different concentrations on frequency, fresh and dry weights (g/jar) of calli production from leaf explants of *Brassica rapa* L. Cultures were incubated under light (16/8 h) conditions at 26 ±1 ºC for 28 days.**

| MS medium supplemented with: (mg/l) | Callus production from leaf explants of *Brassica rapa* L | | |
|---|---|---|---|
| | % of callus formation | F.W of callus (g/jar) | D.W of callus (g/jar) |
| MS free growth regulators | 0.00 | 0.00 | 0.00 |
| MS + 0.1 NAA | 31±4.15d | 0.28±0.01a | 0.032±0.001c |
| MS + 0.1 BA | 52±5.05c | 0.49±0.02d | 0.0467±0.002c |
| MS + 0.1 NAA + 0.2 BA | 67± 8.12c | 1.65±0.14c | 0.175±0.005b |
| MS + 0.1 NAA + 0.5 BA | 85±9.16a | 3.15±0.16d | 0.285±0.007a |
| MS + 0.1 NAA + 1.0 BA | 73±6.22a | 2.45±0.14b | 0.188 ±0.005a |
| MS + 0.1 NAA + 2.0 BA | 64± 6.09b | 1.75±0.13c | 0.12±0.006b |
| Mean | 53.14 | 1.4 | 0.121 |
| P value | *** | *** | *** |

Means in a column with similar letters are not significantly different according to LSD. *** = significant at P < 0.05.

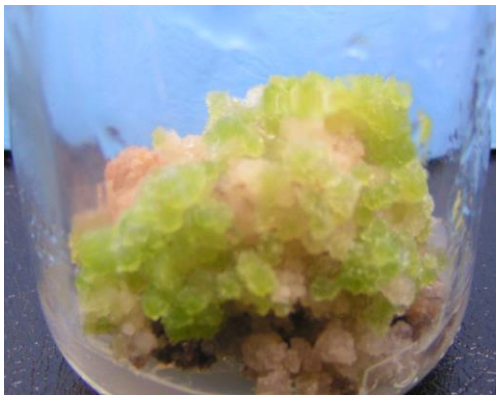

**Figure 1: Callus production from leaf explants of *Brassica rapa* L. cultured on MS medium supplemented with 0.1 mg/l NAA + 0.5 mg/l BAp. Cultures were incubated under light conditions 16/8 h 26 ±1 ºC for 28 days.**

**Establishment of *Brassica rapa* L. cell cultures**

Friable calli obtained from leaf explants of *Brassica rapa* L. were re-cultured and re-suspended in an agitated liquid MS medium augmented with 0.1 mg/l NAA in combination with 0.5 mg/l BAp (the best medium for friable calli production).

**Elicitation with chitosan**

Fresh and viability leaf suspension cell cultures were elicited using chitosan (Fig.2) at different concentrations 0,25,50,100 and 200 mg/l.

As shown in Fig. (3) the maximum cell number ($x10^5$) was recorded with leaf cell cultures free chitosan concentrations which recorded 2.4, followed by 1.2, 1.15, 0.97 and 0.87 ($x10^5$) cell cultures supplemented with 25, 50, 100 and 150 mg/l chitosan. In same respect, the descending order of fresh and dry weights (g/jar) recorded 0.97,0.57,0.37,0.24 and 0.21(g/jar) as fresh weight , and 0.07, 0.047, 0.014, 0.011 and 0.009 (g/jar) as dry weight for 0, 25, 50, 100 and 150 mg/l chitosan, respectively. It could be mentioned that elicitation of ***B.rapa*** cell cultures with chitosan at different concentrations have negative effects on either cell number, fresh or dry weight compared with non elicited treatment.

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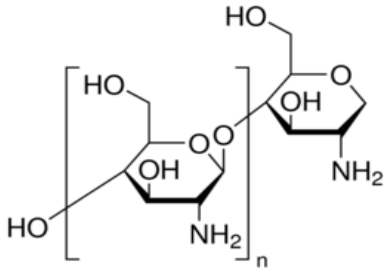


**Figure 2: Chitosan structure**

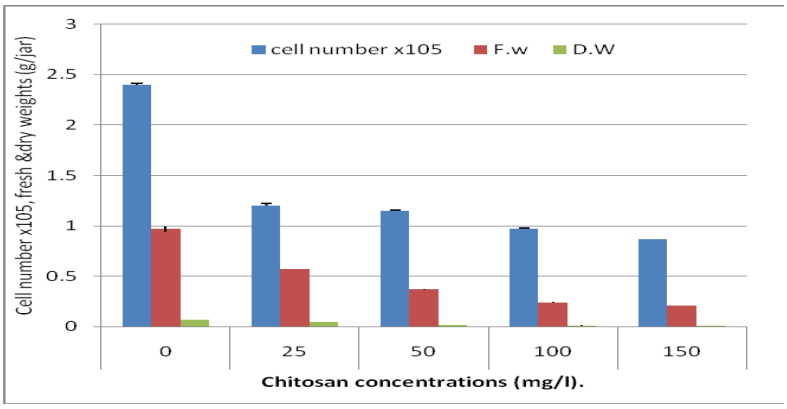


**Figure 3: Effect of elicitation of *B. rapa* leaf cell cultures with chitosan at different concentrations on cell number (x10$^5$), fresh and dry weights (g/jar). Cultures were incubated under light condition 16/8h at 26±1 ºC for 4 weeks.**

**Effect of chitosan at different concentrations on total phenolic and α tacopherol contents**

The total phenolic ( ng GAE/g dw) and α-tacopherol (µg/g FW) contents were determined in lyophilized *Brassica rapa* L. cell cultures which elicited with different concentrations of chitosan. Illustrated data in Fig.(4) clearly stated that elicitation of *B. rapa* cell cultures with 100 mg/l of chitosan gave the highest value of total phenolic content (18.35 ng GAE/g dw ). However, increasing of chitosan concentration up to 200 mg/l reduced the accumulation of total phenolic content to 13.4 ng GAE/g dw. Regarding evaluation and determination of α-tacopherol (µg/g FW) in different elicited cell lines of *B. rapa* with different concentrations of chitosan , it can be mentioned that the descending order of α-Tocopherol highest value contents (45.4, 40.85, 38.7, 35.6 and 27.92 µg/g (F.W) were recorded with augmentation of MS medium with 100, 200, 50, 25, 0.0 mg/l of chitosan, respectively.

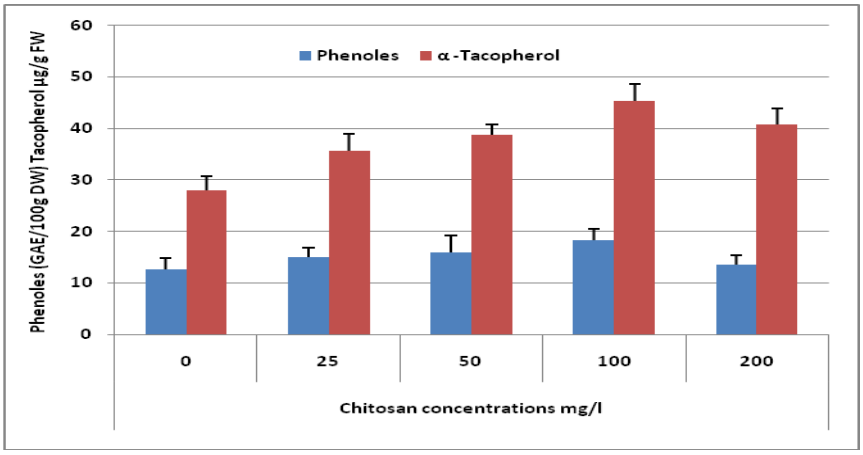


**Figure 4: Effect of elicitation of *B. rapa* leaf cell cultures with chitosan at different concentrations on total phenolic (ng GAE/g dw) and α-tacopherol (µg/g FW) contents.Cultures were incubated under light condition 16/8h at 26±1 ºC for 4 weeks .**

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## DISCUSSION

Plant cell culture offers an alternative way for the production of various photochemical, medicinal compounds, flavors, fragrances, and colorants, which cannot be produced by microbial cells or chemical synthesis. Further, biotechnological applications of plant cell cultures presents the most up-dated reviews on current techniques in plant culture in the field. The principle advantage of this technology is that it may provide continuous, reliable source of plant pharmaceuticals and could be used for the large-scale culture of plant cells from which these metabolites can be extracted. In addition elicitation and metabolic engineering can help to upscale the compounds and produce new compounds [27]. In the present study, it is observed that phenols and α-tacopherol accumulation were highly influenced with the addition of chitosan as biotic elicitor in *B. rapa* cell cultures. Regarding of reported publications on chitosan affecting the production of secondary metabolites in cell suspension cultures of various important plant species, attempts were made at quantification of secondary metabolites in the elicited cell suspension cultures of *B. rapa* with chitosan at different concentrations (0,25,50,100 and 150 mg/l) and were compared with those quantified in non-elicited leaf cell cultures. Regarding total phenols content it was recorded 18.35 ng GAE/g dw with elicited leaf cell cultures with 100 mg/l chitosan. The obtained results were in agreement with those obtained by Chakraborty et al. [28] who reported that the highest content of phenol compound recorded with elicitation of Ocimum cell cultures with 200 mg/l chitosan. In addition, it was found by many investigators that utilization of chitosan has been individually increased the total alkaloid content in cell cultures of *Eurycoma longifolia* [29]. Moreover, those obtained results were recorded in hairy root cultures of *Brugmansia candida* [30,31].

On other hand, it has been recommended that highly accumulation of secondary metabolite content can be obtained by combined elicitor treatment of MeJA and chitosan in few other suspension cultures such as the increase in podophyllotoxin content from elicitation of *Juniperus chinensis* cell cultures with MeJA and chitopentaose [32]. As well as increase in paclitaxol production from *Taxus cuspidata* var. nana, cell cultures elicited with MeJA and chitoheptaose was recorded [33]. Moreover, *Taxus chinensis* cell cultures elicited with MeJA and chitosan was performed [34]. Further, it was stated by Rakwal et al. [35] that application of chitosan to plants through cut stems, led to a rapid increase in jasmonic acid content, confirming the activation of the octadecanoic pathway. Chitosan was reported to increase the endogenous levels of 2-oxo-phytodeionic and jasmonic acids of rice, leading to the activation of the octadecanoic acid pathway. Recently, Mathew and Sankar [36] reported that quantification studies showed total phenols to be the highest in leaves (90 days old) of all three Ocimum species. Total alkaloids and terpenoids were observed as the highest at the combined elictor treatment MeJA 25 µM + chitosan 200 mg/L (8 h) for *O. basilicum*, individual elicitor treatment MeJA 25 µM (48 h) for *O. sanctum* and chitosan 50 mg/L (24 h) for *O. gratissimum*.

Tocopherols (vitamin E) are lipophilic antioxidants, which are important in human health [37, 38]. Concerning achievement the accumulation rate of α-tacopherol in cell cultures of *B. rapa* using chitosan, it was found that elicitation of cell cultures with 100mg/l enriched of accumulation rate of α-tacopherol to 45.4 mg/g FW. In addition, it was reported by Caretto et al. [39] that optimization of culture conditions, precursor feeding and elicitor application are essentially to improve the tocopherol yield in cell cultures. The modulation of alpha-tocopherol levels in plant cell cultures can provide useful hints for a regulatory impact on tocopherol metabolism.

## CONCLUSION

Secondary metabolites have resulted in a high level interest in the possibility of altering their production through cell culture technology. With establishing of cell culture technology, several strategies were pointed forward for the enhanced production of metabolites in less amount of time when compared to the normal cell cultures. One such strategy was the use of elicitors to trigger cell growth and secondary metabolite formation in less time. From this study chitosan was found to trigger the high volumes of the major secondary metabolites such as phenols and α-Tocopherol content in the cell cultures of B. *rapa* because of its effective action on the shikimate pathway, the pathway mainly responsible for the production of secondary metabolites from the primary metabolites. It can be concluded that supplementation of MS medium with 0.1 mg/l NAA + 0.5 mg/l BAp in the presents of 100 mg/l chitosan as elicitor enhanced and

achievement of accumulation rate of total phenolic and α-Tocopherol contents compared with those non-elicited cell cultures of *B. rapa*.

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