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Production of Benzyl Isothiocyanate from *Moringa peregrina* (Forssk.) Fiori via Suspension Cultures

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Abstract

Benzyl isothiocyanate (BITC) is one of the main constituents of *Moringa peregrina*, an endangered species in the flora of Egypt. The purpose of this study was to establish a procedure to enhance the *in vitro* production of BITC in *Moringa peregrina* suspension cultures. This could then aid in the mass production of this useful pure active compound and avoid the exploitation of natural resources of this valuable plant. Suspension cultures of *Moringa peregrina* was established from callus cells in liquid MS medium supplemented with 10.74 µM naphthalene acetic acid, 2.325 µM kinetin, in addition to salicylic acid as an elicitor and/or L-phenylalanine as a precursor of BITC. The combination of both salicylic acid and L-phenylalanine gave the highest accumulation of BITC after two weeks with 10.42-fold increase, compared to its content in the mother plant. This study is promising for the large-scale production of BITC, which has been identified as an anticancer agent with possible applications in the pharmaceutical industry.

Introduction

Benzyl isothiocyanate (BITC) is one of the products of hydrolysis of glucosinolates in particular plant species. It forms via myrosinases (endogenous thioglucosidases) that catalyze the hydrolysis of benzyl glucosinolate. BITC has been shown to prevent many types of human cancers and more specifically it induces human cell apoptosis in cancer cells of breast (Xiao et al. 2008), pancreas (Sahu et al. 2009) and hepatocellular carcinoma (Zhu et al. 2016; 2017). Moreover, BITC may inhibit and restrain angiogenesis of human glioma cells (Zhu et al. 2013, 2014).

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Moringa peregrina (Forssk.) Fiori. is an endangered tree in the flora of Egypt, from family Moringaceae. Its Arabic name is Moringa or Al-Yassar. It is recorded in Sinai Mountains, Eastern desert, Red Sea coast and Elba massive (Boulos 1999). *Moringa peregrina* is described as a miracle tree because it possesses various medicinal uses, such as anticancer, immune-modulatory hypoglycemic, antimicrobial, antioxidant, anti-inflammatory, analgesic, and hypolipidemic activities (Mansour et al. 2019). Seeds and leaves of *Moringa peregrina* contain several amino acids, vitamins, minerals and protein (Al Rawashdeh et al. 2016). *Moringa peregrina* leaf extract possesses antimicrobial and anticancer properties which make it ideal for isolation of its bioactive compounds that can be used to develop new drugs, especially for liver cancer chemotherapy (Mansour et al. 2019). Also, the plant seeds contain a highly valuable oil, which is used for medicinal, cooking, cosmetics, and lubrication purposes. The seed cake is also used in soil and water treatments (Al-Ghamdi 2018).

Isothiocyanates are known to be present in *Moringa peregrina* (Dehshahri et al. 2012). However, the over-collection of plants for extracting valuable secondary metabolites can dangerously reduce natural populations and narrow the species genetic basis. Therefore, plant cell and tissue culture is increasingly being employed to produce high quality secondary metabolites of medicinal value from plants (Cardoso et al. 2019). These techniques will ensure the sustainable production of bioactive secondary metabolites since it is independent of geographical and climatic conditions for providing a continuous, economical, and viable source of bioactive compounds (Chandran et al. 2020).

Both elicitation and precursor feeding are effective methods for enhancing secondary metabolites production in plant callus and suspension cultures. They are responsible for triggering the synthesis of these bioactive compounds (Narayani and Srivastava 2017). Elicitation stimulates stress induction and alters metabolism favouring pathways for the desired product. Elicitors are either of abiotic or non-biological origin, which could be physical agents, such as temperature, ultraviolet irradiation, mechanical wounding, or chemical substances, such as jasmonic acid, methyl jasmonate and salicylic acid (SA). Biotic elicitors on the other hand have a biological origin (a microbe or plant), such as yeast extract (Narayani and Srivastava 2017; Klimek-Szczykutowicz et al. 2022). The glucosinolate amino acid precursor L-phenylalanine (Phe) is involved in glucosinolate biosynthesis (Tian et al. 2018).

Until now, to our knowledge, there are no publications on the *in vitro* production of BITC from suspension culture of *Moringa peregrina*. Therefore, the aim of this study was to improve the previous work of Mustafa (2017), which was carried out on callus induction from various seedling explants of *Moringa peregrina* and accumulating BITC using SA and the precursor amino acid Phe. The present study aimed to establish a suspension culture protocol to scale up the *in vitro* production of the anticancer compound; BITC from suspension cultures of *Moringa peregrina* using both SA as an elicitor and Phe as a precursor of BITC.

Materials and Methods

This study aimed to establish suspension cultures from callus of *Moringa peregrina* derived from cotyledonary leaves to enhance the *in vitro* accumulation of the anticancer compound; BITC using both SA as an elicitor and Phe as a precursor of BITC.

Plant material: Moringa peregrina seeds were collected from wild trees grown in Wadi Zaghra and Wadi Feiran, South Sinai, Egypt. They were washed under running tap water for one hour with few drops of tween-20. Seeds were aseptically sterilized by 50% of commercial bleach (Clorox containing 5.25% sodium hypochlorite solution) for 30 min, followed by three rinses with sterilized distilled water. Seeds were decoated, then cultured for germination on solid Murashige and Skoog (1962) basal medium (MS medium; Duchefa, Haarlem, the Netherlands) supplemented with 0.01% myo-inositol and 3% sucrose. The pH of the media was adjusted to 5.7–5.8 and solidified by 0.25% phytagel (Duchefa, Haarlem, the Netherlands). Equal volumes of the medium were dispensed into 350 ml glass jars. Jars containing the nutrient medium were sealed with autoclavable polypropylene caps and autoclaved for 20 min at 121°C under 1.1 kg/cm^2 pressure in the autoclave (Harvey Sterilemax autoclave, Thermo Scientific, USA), then left to cool and stored at room temperature till used. Cultured seeds were incubated in the darkness for 10 days at 25±2°C to initiate germination, then were transferred to a 16-hour photoperiod under fluorescent tubes supplied by cool white light of 2500-3000 lux (F140t9d/38, Toshiba) as described by Mustafa (2017).

Callus induction: Cotyledonary leaves were taken from the *in vitro* germinated seedlings of *Moringa peregrina* as explants for callus induction. The explants were cultured on solid MS basal medium supplemented with 0.01% myo-inositol, 3% sucrose, 10.74 µM naphthalene acetic acid (NAA) and 2.325 µM Kinetin (kin; Sigma Cell Culture, min. 90%, St. Louis, USA), which was the best medium for callus induction as reported by Mustafa (2017). Media were adjusted to pH 5.7-5.8 and solidified by 0.25% phytagel, then autoclaved. The cultures were incubated at 25 ± 2°C under a 16-hour photoperiod. Fresh and dry weights of callus were scored weekly, during four weeks from culturing. For further proliferation of callus, explants were subcultured twice on fresh media with the same composition.

Elicitation and precursor feeding: Salicylic acid (SA; Sigma Cell Culture, St. Louis, USA) SA concentration of 0.25 mM was added to the medium as an elicitor for BITC production in *Moringa peregrina* callus and suspension cultures. It was added to the culture medium at a concentration of 0.0250 mM. L-phenylalanine (Phe; Sigma Cell Culture, St. Louis, USA) was used as a precursor of BITC and was added to the medium at a concentration of 1 mM. The concentrations of both SA and Phe were the best treatments for BITC accumulation in callus cultures of *Moringa peregrina* as reported in our previous study of Mustafa (2017). Also, a combination of SA and Phe was tested for enhancing BITC accumulation in the callus and suspension cultures.

Suspension cultures establishment: After callus formation and proliferation, 3 g of fresh friable callus were transferred to 250 ml Erlenmeyer flasks containing 50 ml liquid MS basal medium supplemented with 0.01% myo-inositol, 3% sucrose, 10.74 µM NAA, 2.325 µM Kn without phytagel. The pH of the medium was adjusted to 5.7-5.8 before autoclaving. Suspension cultures were established in a rotary shaking incubator (DAIHAN Scientific, Korea) at a constant agitation of 120 rpm and 24 ± 2°C. SA as an elicitor and/or Phe as a precursor of BITC were added aseptically to the suspension cultures on the 14th day during the stationary phase of cells growth. A control culture without elicitor or precursor was also maintained. After the addition of SA and Phe, BITC content (ppm) in the suspension cultures and the increase in BITC concentration (fold) compared to its content in the mother plant were scored weekly for a total period of four weeks.

Extraction and determination of benzyl isothiocyanate: A weight of 2 g callus from suspension cultures of each treatment as well as fresh actively growing shoots from the mother plant of *Moringa peregrina* were taken for BITC extraction. Six ml of methanol (70%, diluted with deionized water) were added to the samples, homogenized, and incubated in a water bath at 70°C for 30 min. The extracts were centrifuged for 20 min at 8000 rpm three times. The supernatants were filtered using 0.45 mm filters and 20 µl were injected in the high-performance liquid chromatography (HPLC; Dionex Ultimate 3000 equipped) for determination (Kiddle et al. 2001). The HPLC was performed at 246 nm wavelength by UV-VIS detector at 40°C, 250×4.6 mm C18 column, acetonitrile, and deionized water (60: 40 v/v) mobile phase and 1 ml/min flow rate. The calibration curve was plotted between the concentration of BITC (Sigma-Aldrich, Germany) and the peak area. BITC content was calculated in the samples and expressed as ppm.

Experimental design and Statistical analysis: Experiments were designed as completely randomized with at least three replicates for each treatment. Data were tested according to the analysis of variance (ANOVA) using Costat statistical package software. The differences among mean values for treatments were tested for significance at 0.05 level using Duncan's multiple range test (Duncan 1995).

Results and Discussion

Plant cell and tissue culture techniques allow the easy establishment of suspension cultures, which in turn are convenient not only to produce secondary metabolites but also enable tweaking the culture conditions to enhance their production. Most useful among the secondary metabolites are anticancer substances (Gonçalves and Romano 2018). The advantage of *in vitro* techniques for producing plant-derived medicinal compounds is the sterile and controlled environmental conditions that are necessary for successful production. The precise environment also allows better control of the metabolic processes and the efficient use of different elicitors and precursors that are

responsible for triggering the synthesis of a high amounts of medicinal compounds (Cardoso et al. 2019).

In the present study, *Moringa peregrina* cotyledonary leaves had the ability to produce friable yellow callus on MS medium supplemented with 10.74 µM NAA and 2.325 µM kin, either in the presence or absence of the elicitor SA and/or the precursor Phe. It could be observed from data in Table 1 that the medium supplemented with 1 µM Phe gave the highest fresh and dry weight of *Moringa peregrina* callus in the first (Fig. 1a), second (Fig. 1b) and third weeks, however it significantly gave maximal weight after two weeks from culturing (fresh weight of 4.25 g/jar and dry weights of 0.62 g/jar). The control treatment without SA or Phe ranked next in the first three weeks regarding both fresh and dry weight of callus. Concerning SA individually or in combination with Phe, it gave the least response on callus biomass, compared to media with Phe individually and the control medium. In the fourth week, in comparison with the first three weeks, the biomass of cells showed minimum values.

Table 1. Effect of MS medium supplemented with 10.74 µM NAA and 2.325 µM kin in addition to SA and /or Phe on fresh and dry weight of *Moringa peregrina* callus during the four harvest periods.

Duration (week)	Concentration (µM)		Fresh weight (g/jar)	Dry weight (g/jar)
	SA	Phe		
1st	0.000	0.0	2.46^{ef}	0.25^{f-h}
	0.000	1.0	3.63^{b}	0.33^{c}
	0.025	0.0	2.19^{g}	0.29^{i}
	0.025	1.0	2.32^{fg}	0.26^{e-g}
2nd	0.000	0.0	3.19^{c}	0.36^{j}
	0.000	1.0	4.25^{a}	0.62^{a}
	0.025	0.0	2.25^{fe}	0.24^{f-i}
	0.025	1.0	2.19^{g}	0.23^{e-g}
3rd	0.000	0.0	2.77^{c}	0.27^{d}
	0.000	1.0	3.72^{b}	0.38^{b}
	0.025	0.0	2.55^{de}	0.25^{f-h}
	0.025	1.0	2.30^{fg}	0.24^{g-i}
4th	0.000	0.0	2.26^{fg}	0.22^{hi}
	0.000	1.0	2.36^{e-g}	0.25^{f-h}
	0.025	0.0	2.72^{d}	0.26^{ef}
	0.025	1.0	2.36^{e-g}	0.28^{de}

Values with the same letter within a column are not significantly different, at the 0.05 level, according to Duncan's multiple range test.

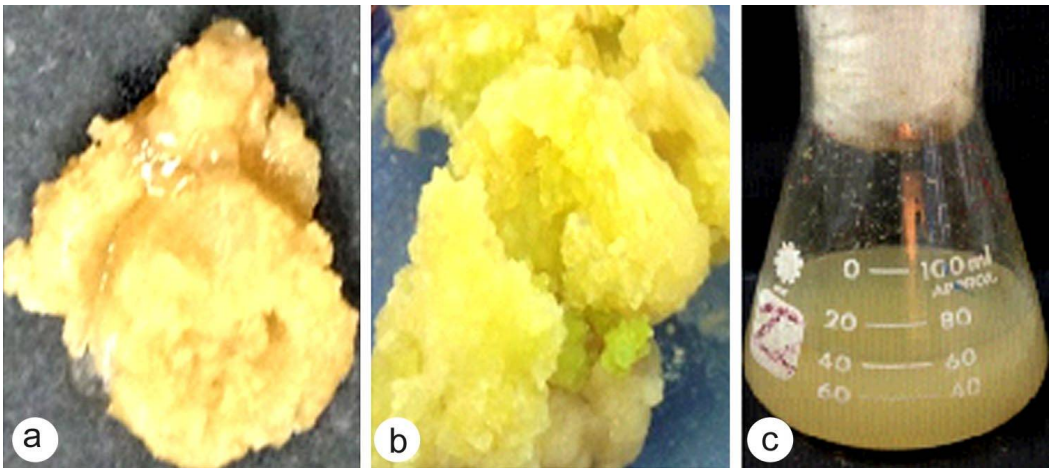

Fig. 1. Callus of *Moringa peregrina* from cotyledonary leaf explants on MS medium supplemented with 10.74 µM NAA, 2.325 µM kin and 1 µM Phe **a.** after one week, **b.** after two weeks, and **c.** suspension culture

The promotive effect of Phe on the fresh and dry weight of *Moringa peregrina* callus in the first three weeks, followed by SA agrees with the studies carried out on *Salvadora persica* for enhancing the production of BITC *in vitro* and showed that the biomass of callus (Hegazi et al. 2016) and suspension cultures (Hegazi 2017) increased by the application of different concentrations of Phe as a precursor of BITC with two biotic elicitors; chitosan and SA, respectively. However, the prolonged culture caused a decrease in the callus biomass as reported by Silja et al. (2014), who found that the prolonged incubation period and high elicitor concentration affect the viability of cultures. High concentrations of elicitors may induce hypersensitivity that leads to the death of cells; however, the optimum level is required (Naik et al. 2016). The negative results of using SA on callus biomass agree with Al-Khayri and Naik (2020), who reported that cell suspension culture of date palm treated with SA showed a decrease in cells biomass. Similarly, Cai et al. (2017) reported that when suspension cells of *Changium smyrnioides* were treated with different elicitors (including SA), all tested elicitors and concentrations showed a negative impact on cells biomass, and high concentrations significantly suppressed cell growth.

Suspension cultures of *Moringa peregrina* were established, by transferring 30-day old yellow friable callus to liquid MS medium supplemented with 10.74 µM NAA and 2.325 µM kin in the rotary shaking incubator. The callus was easily broken apart and dispersed into single cells and cell clumps. On agitation cell clumps rapidly disintegrate into single cells and small cell aggregates. SA as an elicitor and Phe as a precursor were added aseptically to the 14-day old liquid culture medium. Results in Table (2) show the effect of SA and Phe on the accumulation of BITC in the suspension culture of *Moringa peregrina* during the four harvest periods. After two weeks, the combination between SA as an

elicitor and Phe as a precursor was superior in the accumulation of BITC, since this gave its highest concentration compared to all tested treatments and durations (499.93 ppm) with 10.42-fold increase more than the mother plant (Fig. 1c). The treatment of Phe individually ranked next and produced a 9.692-fold increase (465.15 ppm BITC). After the third and fourth weeks, BITC accumulation decreased for all treatments. It is noticed from Table (2) that the combination between SA and Phe gave the highest accumulation of BITC, followed by Phe individually, compared to the other treatments for each period. Moreover, for each treatment, the concentration of BITC increased in the second week which gave the optimum concentrations then decreased gradually with increasing the duration of incubation.

Table 2. Benzyl isothiocyanate content in suspension cultures of *Moringa peregrina* cultured on MS medium supplemented with 10.74 µM NAA and 2.325 µM kin in addition to SA and /or Phe during the four harvest periods.

Duration (week)	Concentration (µM)		BITC content (ppm)	Increase of BITC content compared to the mother plant (fold)
	SA	Phe		
Mother plant	47.999^{k}	-		
1	0.000	0.0	393.238^{c}	8.193
	0.000	1.0	164.415^{i}	3.425
	0.025	0.0	161.383^{i}	3.362
	0.025	1.0	401.294^{c}	8.360
2	0.000	0.0	309.839^{de}	6.455
	0.000	1.0	465.154^{b}	9.691
	0.025	0.0	335.134^{d}	6.982
	0.025	1.0	499.927^{a}	10.415
3	0.000	0.0	267.991^{fg}	5.583
	0.000	1.0	246.810^{gh}	5.142
	0.025	0.0	161.742^{i}	3.370
	0.025	1.0	298.240^{e}	6.213
4	0.000	0.0	183.168^{i}	3.816
	0.000	1.0	225.890^{h}	4.706
	0.025	0.0	96.909^{j}	2.019

Values with the same letter within a column are not significantly different, at the 0.05 level, according to Duncan's multiple range test.

The ease of establishing the suspension culture from callus of *Moringa peregrina* was influenced by the friable nature of the callus tissue. A fine suspension of cells was obtained when the yellow friable callus was cultured in liquid MS medium supplemented with 10.74 µM NAA and 2.325 µM kin. According to previous reports,

there is an increase in the degree of friability of the callus tissue when it was sustained in liquid or semiliquid media (John et al. 2018). The combination between SA as an elicitor and Phe as a precursor was superior in the accumulating BITC in the suspension cultures of *Moringa peregrina*, it gave 10.42-fold increase more than the mother plant after two weeks of incubation. This process of feeding by the elicitor and/or precursor was necessary to improve the accumulation of BITC content in *Moringa peregrina* callus and suspension cultures. These results agree with Naik and Al-Khayri (2016), who reported that elicitation and precursor feeding improve the synthesis and accumulation of novel secondary components in plant *in vitro* cultures.

The stimulatory effect of SA as an elicitor and Phe as a precursor on the accumulation of BITC in the cell suspension culture of *Moringa peregrina* was in harmony with the results of Hegazi et al. (2016), who found that higher BITC content was accumulated in the callus of *Salvadora persica* with Phe at the concentration of 12.5 mg/l that increased the amount of BITC in callus cultures by about 4.5 times that in the stem of the intact plant and 8 times that in the leaves of the intact plant. Also, Hegazi (2017) found that elicitation with the two biotic elicitors: chitosan and SA, increased callus biomass and BITC accumulation in the suspension culture of *Salvadora persica*. Additionally, SA stimulated the accumulation of other bioactive compounds in plants such as phenolic compounds in cell cultures (Dong et al. 2010) and cells (Li et al. 2016) of *Salvia miltiorrhiza*, glycyrrhizin in *in vitro* cultured *Glycyrrhiza glabra* (Shabani et al. 2009), stilbene in the cell suspension of *Vitis vinifera* (Xu et al. 2015) and carotenoids in *Artemisia aucheri* cultures (Abbaspour and Ehsanpour 2016). Golkar et al. (2019) produced the highest amount of rebaudioside A from stevia by the addition of 0.25 mg/l SA to the medium. Also, Sharifi et al. (2019) showed that SA enhanced phenols and flavonoids content in callus culture of *Ruta graveolens*, depending on the dose and type of compound to be elicited.

Elicitors are as a rule, compounds which fortify plant defense reactions and promote the generation of secondary metabolites to ensure plant protection (Baenas et al. 2014). Particularly, SA is a biotic elicitor and plays an important role in regulating physiological, biochemical and respiration processes in plants. SA is an important signaling molecule for modulating plant response to environmental stress of both biotic and abiotic nature. SA has been considered as a phytohormone, mediating several reactions (Rowshan et al. 2010; Muthulakshmi and Lingakumar 2017). Besides, it is recognized and bound by particular cell membrane receptors, causing a signal transduction cascade for biosynthesis of secondary metabolites (Baenas et al. 2014).

Conclusions

Plant suspension cultures are reliable substitutes to whole plant cultivation to produce desired secondary metabolites. In this study, the suspension cultures of *Moringa peregrina* were established for the mass production of BITC. The best medium for callus

induction from cotyledonary leaves was MS medium supplemented with 10.74 µM NAA and 2.325 µM kin in addition to 1 µM Phe for the maximum friable callus biomass after two weeks from culturing. The suspension culture of the obtained callus was established and gave the highest accumulation of BITC after the second week of culture in MS medium supplemented with 10.74 µM NAA and 2.325 µM kin in addition to 0.025 µM SA and 1 µM Phe, with 10.42-fold increase in BITC content compared to its content in the mother plant. This protocol could be applied to scale up the production of this important anticancer compound commercially. A variety of other biotic and abiotic elicitors may further improve the accumulation of BITC from *Moringa peregrina* through both suspension cultures and the bioreactor.

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