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Impact of chitosan on shoot regeneration from faba bean embryo axes through its effect on phenolic compounds and endogenous hormones

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Abstract

Legume crops have been the primary targets for improvement by genetic transformation due to their importance for human and animal consumption worldwide. Many of these important legume crops were difficult to genetically engineer especially faba bean crop, mainly due to high phenolics content and their recalcitrance to *in vitro* regeneration. Therefore, a series of experiments were performed in order to evaluate the growth , morphological changes and production of phenolics in the *in vitro* plantlets of five Egyptian faba bean cultivars (Giza 843, Sakha 1, Sakha 3, Nubaria 2 and Nubaria 3). The obtained results revealed that cultivars Nubaria 2 and Skha 3 had the highest regeneration frequency (85.3% and 78.6%), respectively. Also, both cultivars showed high growth parameters and low in total phenols concentration. Therefore, these cultivars considered as promising candidates to *Agrobacterium* mediated genetic transformation experiments. The effect of different levels of chitosan (0, 2, 4, 8, 15, 30, 60, and 120 mg chitosan/l) on shoot regeneration from mature embryo axes of cv. Nubaria 2 were studied. The obtained results indicated that high levels of chitosan have lethal effect on the development of embryo axes tissues and the plantlets showed morphological abnormalities. However, low levels of chitosan 2 mg /l and 4mg /l in combination with 4.5 mg/l BAP increased the shoot regeneration. Moreover, the total soluble phenols were increased by increasing the age of the faba bean plantlets (six weeks old) cultured *in vitro* on a medium containing low level of chitosan (2, 4 and 8 mg chitosan/l) as compared with plantlets did not expose to chitosan. HPLC analysis showed changes in the polyphenols concentrations and the concentration of Gibberellic acid (GA_3) and Abscisic acid (ABA) in faba bean plantlets that exposed to low levels of chitosan (2, 4 and 8 mg chitosan /l) were increased as compared with control plantlets. The obtained results indicated that the concentration of phenolic compounds, GA3 and ABA in the extracts were increased in the faba bean plantlets that exposed to low levels of chitosan.

Keywords: Embryo axes, regeneration frequency, phenols, GA_3, ABA.

Introduction

Faba bean (*Vicia faba* L.) is an important food legume crop, grown for human and animal consumption globally especially in China, North African countries, parts of Europe as well as North and South Americas (Ray and Georges, 2010) Plant genetic transformation has become important tool for cultivar improvement as well as to study gene function in plants. Successful transformation of plants requires certain criteria. Among the requirements for transformation are target tissues competent for propagation or regeneration, an efficient DNA delivery method, agents to select for transgenic tissues, the ability to recover fertile transgenic plants at a reasonable frequency, in addition to, a simple, efficient, reproducible, genotype-independent and cost-effective process (Hansen and Wright, 1999; Khalafalla and Hattori , 2000 and Babaoglu *et al* .,2000).

Genetic improvement of faba bean using genetic engineering approaches has been hampered due to high phenolic content, low regeneration ability and the difference between cultivars in their responses to *in vitro* techniques (Böttinger *et al.*, 2001, Hanafy *et al.*, 2005 and Hanafy *et al.*, 2013). Despite the effort made by several research groups, improvement of faba bean by genetic transformation remains difficult and need more effort to develop reliable transformation system to economically important faba bean cultivars (Bottinger *et al.*, 2001;Hanafy *et al.*, 2005 and Hanafy *et al.*, 2008).

Moreover, pollination incompatibilities and the limited genetic pool imposed limitation towards faba bean varietal improvement (Bond,1987; Bond *et al.*, 1985 and Selva *et al.*, 1989). So far, transgenic faba bean plants were recovered only by *Agrobacterium*-mediated transformation of either embryo axes (Hanafy *et al.*, 2005) or stem segments from aseptically germinated seedlings (Böttinger *et al*.,2001). As a result, studies on improvement of faba bean regeneration and transformation are of great importance. Direct regeneration of faba bean and establishment of plantlets has been reported from different explants (Taha and Francis,1990; Tegeder *et al.*, 1995; Böttinger *et al.*, 2001and Hanafy *et al.*, 2005). However, the majority of the reported faba bean regeneration protocols are either varietal dependent, not repeatable or successful only in certain research laboratories Anwar *et al*.,(2011). Surveying the literature revealed that faba bean regeneration protocols are quite rare repeatable and this is ascribed to the fact that scientists did not address the problem of secretion of polyphenolic compounds into the culture medium by most of faba bean cultivars (Busse, 1986; Khalafalla and Hattori, 1999; Tegeder *et al.*, 1995 and Selva *et al.*, 1989). The secretion of the polyphynolic compounds is the main reason of explants mortality and negatively affect the regeneration of faba bean explants (Bieri *et al.*, 1984 and Selva *et al.*, 1989). Abdelwahd *et al*.,(2008) pretreated faba bean seeds using polyvinyl pyrrolidone (PVP) and supplemented tissue culture medium by adsorbent (activated

charcoal) and antioxidants to minimize the effect of phenolic compound which causing browning which killing faba bean explants and causing difficulties for *in vitro* regeneration. his treatment improved shoot regeneration by reducing the lethal browning in faba bean explants.

Chitosan is natural polymers composed of randomly distributed *β*-(1-4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit) as described by Islam *et al* .(2017) . Chitosans of defined molecular weight (Mw 10–213 kDa) as mentioned by Huang *et al* .(2005). Chitosan is a biodegradable natural product extracted from the shells of shrimps, crabs and insects which enhancing plant physiological properties. In addition, the chitosan has many applications such as the treatment with chitosan modulate many genes related to plant defense signaling pathways (Köhle *et al.*,1985 and Sharif *et al*., 2018). Moreover, chitosan is used as cell stimulating additive in plant tissue culture (Kowalski *et al.,* 2006 and Nge *et al.*, 2006). Hanafy Ahmed *et al.*,(2016) mentioned that chitosan has a role in increasing the concentration of endogenous plant hormones such as (GA_3 and ABA) as a result of terpenoid formation. This study was undertaken to investigate the effect of chitosan on faba bean regeneration and to evaluate the content of the polyphenolic compounds and endogenous plant hormones (GA_3 and ABA) in different economically important faba bean cultivars in order to improve faba bean regeneration protocol as a first step to establish reliable transformation system for faba bean crop

Material and Method

Evaluation of regeneration capacity and phenolic compounds concentrations in five Egyptian faba bean cultivars

Seeds of five faba bean cultivars (Giza 843, Sakha 1, Sakha 3, Nubaria 2 and Nubaria 3) were obtained from Egyptian Agriculture Research Center (Field Crop Institute - Legume Department). Mature seeds were surface sterilized by immersion for 1 minute in 70 % ethanol and for 6 minutes in 50% Clorox® solution (5.4% sodium hypochlorite) followed by washing four times with sterilized tap water, and soaked overnight in sterile tap water with shaking at 90 to 95 rpm. The embryo axes explants were prepared as described by Hanafy *et al.* (2005) by isolation of the embryo axes and longitudinally slicing them into 2 to 3 slices. Then, a total of 30 embryo axes explants were cultured on SIM medium(Shoot Induction Medium) MS basal salt medium, (Murashige and Skoog ,1962) supplemented with B5 vitamins (Gamborg *et al.*, 1968), 3% sucrose, 4.5 mg/l BAP and 0.1mg/l NAA solidified with 0.2% gelrite). The pH of the medium was adjusted to 5.7 before autoclaving at 121°C for 15 min. The petri dishes (100x15mm) were sealed with parafilm and incubated at 22° in light of 16h duration. Two weeks later, the numbers of regenerated shoots were counted to calculate the regeneration percentage for each cultivar. After 8 weeks from regeneration on Shoot Induction Medium (SIM medium) Shoot height, shoot no per explants, node no per shoot, fresh weight and dry weight were recorded. On the other hand, total phenols (total soluble phenols and conjugated phenols) were determined in the tested faba bean cultivars using Folin–Ciocalteu reagent and spectrophotometric method according to Singleton and Rossi ,(1965). There were at least three replications of each treatment and the experiment was performed twice.

Effect of different chitosan levels on shoot regeneration

Different level (0, 2, 4, 8, 15, 30, 60 and 120 mg chitosan/l) of chitosan were added separately to SIM medium to study the effect of chitosan on the regeneration frequency of faba bean embryo axes. The experiment was performed with 30 mature embryo axes explants from Nubaria 2 cultivar for each treatment with 3 replicates. The number was counted after 2 week from culturing the faba bean embryo axes on SIM supplemented with different chitosan level. After 6 weeks , shoot height, shoot number per explants, leaves number, fresh weight and dry weight were recorded.

Estimation of total soluble phenols

The concentration of total soluble phenols was measured in the regenerated plantlets with the age of 6 weeks from culturing the embryo axes using a modified Folin and Ciocalteu ,(1927) method, employing the reduction of a phosphowolframate–phosphomolybdate complex to blue products by phenolic compounds. Briefly, 2g fresh tissues were extracted with 40 ml ethanol 80%. The extract was soaked in brown bottle for 24 hr at room temperature, sonicated for 10min and the volume was adjusted to 50 ml by ethanol 80%. Finally, the extracts were filtered through whatman filter paper. Total soluble phenols were determined calorimetery by Folin – Ciocalteu reagent and were expressed as µg gallic acid equivalent /g sample as described by Singleton and Rossi, (1965). This analysis carried out at Faculty of Agriculture (Cairo University Research Park) .

HPLC-Analysis of polyphenolics Compounds

Instrument Condition: Agilent1260 infinity HPLC Series (Agilent, USA), equipped with Quaternary pump, a Zorbax Eclipse plus C18 column 100 mm x 4.6 mm i.d., (Agilent technologies, USA), operated at 25°C. The separation is achieved using a ternary linear elution gradient with (A) HPLC grade water 0.2 % H_3PO_4 (v/v), (B) methanol and (C) acetonitrile. The injected volume was 20 µl. Detection using VWD detector set at 284 nm. Instrument condition: temperature 27 °C, Humidity 40% (Schneider, 2014). This HPLC analysis carried out at Faculty of Agriculture (Cairo University Research Park).

Extraction, purification and estimation of plant hormones (GA_3andABA)

Plant hormones (GA_3andABA) were extracted from regenerated shoots exposed to different chitosan level (0, 2, 4, and 8 mg/l) according to Horemans *et al*. (1984). Briefly, faba bean fresh tissues (5 g) were homogenized in ice cold absolute methanol 25ml for 5min at 4ºC the mixture leaved for 3h at 4ºC and followed by another extraction step with methanol 80% for 1 hr at 4C°. The extracts were Filtrated through 0.45µm MF Millipore filters.

After filtration the aqueous methanol phases were collected and cold petroleum ether at 3 times were added to 1/2 volume of filtrate. Take aqueous phase and evaporate it to enrich with rotary evaporator at 45-48 ºC enrich elute is acidify with 0.1 mol/l HCl to pH 2.8-3.0 . the extract partition with ethyl acetate, 3 times at 1/2 volume of aqueous phase. Use Ethyl acetate phase evaluate to dryness with rotary evaporator at 40-42 ºC and Dissolve in 8ml 100% redistilled MeOH. The extract filtered through 0.45µm MF Millipore filters. Then purified samples was used for measuring plant hormones (GA_3 andABA) using HPLC method as described by Jones *et al*. (1980), HPLC (Hewlett packard1050) column phenomenex hypersil 5µ C18(OD5) was used . All standards were dissolved in methanol (l.0mg ml^{-1}). This analysis carried out at Horticulture Institute Central Lab –Agriculture Research Center .

Statistical analysis

All data were subjected to appropriate statistical and conventional method of analysis for variance, Standard deviation and Least significant difference according to Snedecor and Cochran (1989). Computer designed (Microsoft Excel 2007) was used.

Results and Discussion

With the aim of improving faba bean *Agrobacterium*-mediated transformation system, carried several experiments were carried out with 5 elite Egyptian faba bean cultivars e.g., Giza 843, Sakha 1, Sakha 3, Nubaria 2 and Nubaria 3 (Obtained from Agriculture Research Centre (ARC), Egypt) in order to select cultivars with highest regeneration capacity with relatively low concentrations of phenolic compounds. Data presented in Table (1) indicated the regeneration capacity differences between five faba bean local cultivars (recommended by the breeders) from embryo axes. Cultivars Nubaria 2 and Sakha 3 have the highest regeneration capacity from embryo axes. The mean shoot number per explants, shoot length, node number per shoot, fresh weight (FW) and dry weight (DW) were recorded after 6 weeks of cultivation on SIM (Shoot Induction Medium) Table (2). Cultivars Sakha 3, Nubaria 2 and Giza 834 have the highest regeneration capacity from embryo axes, the mean shoot number per explant was around 4 from both cultivars. However, the shoot length for the Sakha 3 and Nubaria 2 was ranged from 6.67 cm and 6.17 cm, respectively(Table 2). On the other hand, we determined the total, free and conjugated phenolic compound in the tested faba bean cultivars using spectrophotometric method (Singleton *et al*., 1965). Sakha 3 and Nubaria 2 have low concentrations of total phenol 2.71 mg/g FW. and 2.19 mg/g FW., respectively. The data presented in Tables 1, 2 and 3 concluded that cultivars Sakha 3 and Nubaria 2 have high growth parameters and relatively low phenolic compounds concentrations. As mentioned above, grain legumes as a general are recalcitrant for *in vitro* culture and regeneration. There are several reports indicated that plant regeneration from preexcited meristematic cells on embryo axes, shoot tip and cotyledonary nodes were used as an efficient shoot regeneration system in grain legumes (Hanafy *et al.,* 2008).

The regeneration efficiency depend on several factor such as type of explant, type of growth regulator and their The regeneration efficiency depend on several factor such as type of explant, type of growth regulator and their combinations and plant species. In this regard, many reports indicated that the regeneration capacity of embryo axes is higher than the regeneration capacity of shoot tips in faba bean plants (Khalafalla and Hattori ,2000 ; Babaoglu *et al* ., 2000 and Metry *et al*., 2007). Moreover, Metry *et al*. (2007) . As mentioned that the regeneration frequency was increased with incubation of the culture in dark for one week and using of charcoal in medium for eliminating the browning problem which result from phenolic compounds. . However, the addition of activated

Table 1: Shoot regeneration percentage from embryonic axes from five faba bean cultivars.

Cultivars	Regeneration %
G843	65.4%
Sakha1	66.3%
Sakha3	78.6%
Nubaria2	85.3%
Nubaria3	73%

charcoal had a negative impact in the regeneration capacity of the explants which reduced the number of shoots per explant to 0-1 shoot/ explant as compared to 2-5 shoots /explant in the absence of charcoal. This ascribed to the fact that activated charcoal absorbed the plant growth regulators. In the same lineMohamed *et al*.,(1992) developed *in vitro* regeneration protocol using embryo axes explants in common bean (*Phaseolus vulgaris* L.). The obtained results are in a harmony with those previously reported by Hanafy *et al*.(2005); Metry *et al*.(2007) ; Khalafalla and Hattori ,(2000) and Babaoglu *et al*.(2000). Fresh weight and dry weight of the regenerated plantlets were recorded after 8 weeks of culture. The fresh and dry weight of the regenerated plantlet of cv. Nubaria 2 were the highest with 2.02g and 0.14g, respectively as compared with other four cultivars Table (2). The obtained results are in agreement with those obtained by Anwar *et al*.,(2011) who used cotyledon explants with half embryonic axis in faba bean cultivars as rapid, efficient regeneration system and regenerated shoot was 5 cm long in average. The total phenolics, free phenolic compounds and conjugated phenolic of the regenerated plantlets faba bean cultivars are summarized in Table (3). Among the different faba bean cultivars analyzed, total phenolics ranged from 2.19 to 8.78 mg/g FW., while free phenolic compounds ranged from 0.05 to 2.83 mg /g FW. and the conjugated phenolic compounds ranged from 2.05 to 5.95 mg/g FW. The cultivar G843 had the highest values for total phenolics, free phenolic compounds and conjugated phenolic compounds (8.78, 2.83 and 5.95 mg/g FW. , respectively) as compared with all other cultivars studied. However, the obtained results indicated that Sakha 3 was the cultivar with the lowest

concentration of total phenolic compound and free phenolic compounds (2.19 and 0.05 mg/g FW, respectively) with moderate level of conjugated phenolic compounds (2.14 mg/g FW). Based on the data presented in Tables 1, 2 and 3, Nubaria2 and Sakha3 cultivars can be recommended for further *in vitro* regeneration and genetic transformation work as these cultivars had higher values for regeneration frequency, shoot numbers per explant, shoot height, node number per shoot, fresh weight than the other tested cultivars with moderate levels of total phenolic compounds, free phenolic compounds and conjugated phenolic compounds.

Table 2 : Shoot height, shoots number per explants, node number per shoot, fresh weight and dry weight of faba bean after 8 weeks of culturing the embryo axes explants on SIM (Shoot Induction Medium).

Cultivars	Plant growth parameters				
	Shoot Length cm ± S.E	Shoots no. per explants ± S.E	Node no. per shoot± S.E	FW.± S.E	DW.± S.E
Nubaria 2	6.67 ± 0.88^{a}	4.00±0.577 ^{a}	3.67±0.333^{a}	2.02±0.221^{a}	0.14±0.012^{a}
Nubaria3	4.33 ±0.60^{a}	3.33±0.882 ^{ab}	3.00 ±0.00^{b}	1.96 ±0.257^{ab}	0.11±.0.0177^{b}
Sakha1	3.67 ± 0.33^{b}	2.33±0.333^{b}	3.00 ±0.00^{b}	1.58±0.198^{ab}	0.11±0.020^{b}
Sakha3	6.17 ± 1.69^{a}	4.00±0.577^{a}	3.33 ±0.333^{b}	1.51±0.124^{ab}	0.11±0.008^{b}
G843	3.67 ±0.60^{b}	4.00±0.000^{a}	3.67±0.333^{a}	1.40±0.133^{b}	0.12±0.003^{ab}
L.S.D. 0.05	2.4	1.4	0.7	0.5	0.03

FW: Fresh weight , DW: Dry weight

Table 3: Determination of total, free and conjugated phenolic compounds in the regenerated plantlets of five faba bean cultivars.

Cultivars	Phenols		
	Total phenolic compound (mg/g FW.)	Free phenolic compound (mg/g FW.)	Conj. Phenolic compound (mg/g FW.)
Nubaria 2	2.71±0.014^{b}	0.54±0.772^{b}	2.17±0.011^{c}
Nubaria 3	2.64±0.032^{c}	0.30±0.011^{d}	2.33±0.015^{b}
Sakha1	2.76±0.019^{b}	0.69±0.02^{c}	2.05±0.017^{d}
Sakha3	2.19±0.011^{d}	0.05±0.003^{e}	2.14±0.011^{e}
G843	8.74±0.021^{a}	2.83±0.014^{a}	5.95±0.014^{a}
L.S.D. 0.05	0.04	0.03	0.03

FW: Fresh weight

Faba bean *in vitro* plant regeneration is limited by phenolic oxidation. These compounds have negative effect on plant tissues such as browning and cell death which cause difficulties in plant regeneration (Selva *et al*.,1989 and Böttinger *et al*., 2001). In order to minimize the lethal effect of the phenolic compound, Abdelwahd *et al* .,(2008) pretreated faba bean seeds with polyvinyl pyrrolidone and supplemented the tissue culture medium with activated charcoal and antioxidants. Klenotičová *et al.* (2013) used shoot apices and cotyledonary nodes of low tannin faba bean cultivars for shoot regeneration system.

Effect of chitosan on faba bean regeneration.

Standard shoot induction medium (SIM) containing MS basal salt medium supplemented with B5 vitamins, 4.5 mg/l BAP, 0.1 mg/l NAA supplemented with different concentration from chitosan at low level (0, 2, 4 and 8mg chitosan /l) and high level (15, 30, 60 and 120 mg chitosan/l). The influence of chitosan levels on the regeneration efficiency and growth after four weeks and six weeks on SIM is shown in Fig (2) and Table (4). High level of chitosan had a lethal effect on the embryo axes as shown in Fig(1). However, low level of chitosan at 2 and 4 mg chitosan/l in combination with 4.5 mg/l BAP enhanced the regeneration frequency to 91.51%, 94.54%, respectively as compared with 83.52% in untreated explants of cv. Nubaria 2. Moreover, with increasing chitosan level to 8mg chitosan/l, the regeneration frequencies decreased to 78.79%. The results obtained are in accordance with Wiszniewska and Piwowarczyk (2014) who reported that the increment of chitosan level resulted in complete destruction of soybean cells. Therefore, it can be concluded that high level of chitosan may has a toxic effect on faba bean explants.

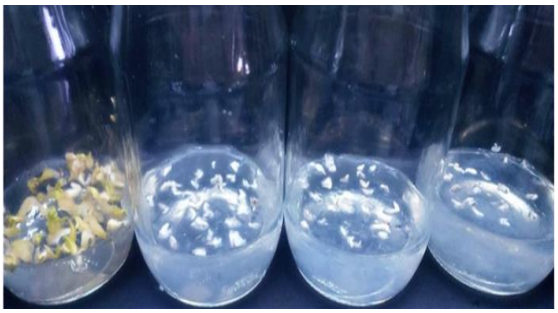

Fig .1: Effect of high level of chitosan (0, 15, 30 and 60 mg chitosan /l, from left to right) on faba bean regeneration from embryo axes of cultivar Nubaria 2 after 2 weeks.

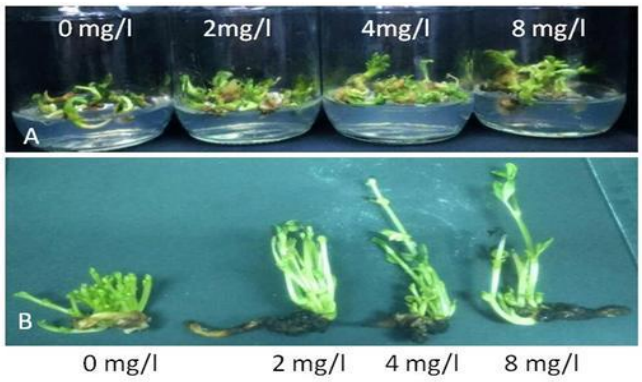

Fig.2 : Shoot development of faba bean in SIM medium (Shoot Induction Medium) containing different chitosan levels (0, 2, 4 and 8 mg chitosan /l) after 4 weeks (A) and 6 weeks (B) of regeneration period.

On the other hand, the obtained results are in contrary to those obtained by Zakaria *et al*.(2009) ; Sopalun *et al*.(2010) and Mondal *et al*.(2013) in minituber yield of *Solanum tuberosum* (L.), *Grammatophyllum speciosum* (L.) and in mung bean, respectively who reported that chitosan at high levels posses variation responses among plant species. Data presented in Table (4) indicate that in all cases, addition of chitosan at low levels increased shoot height as well as fresh and dry weight, , while shoot number per explants was not significantly changed. On the other hand, high levels of chitosan (15, 30, 60 and120 mg chitosan /l) have depressed the growth and regeneration of faba bean plants (Fig. 1) . Chitosan also tended to promote the root system (Fig . 2). Govindaraju and Arulselvi (2016) suggested that the best combination for *Coleus aromaticus* Benth (L) shoot multiplication and elongation was 1.0 mg/l BAP and 40 mg/l chitosan. They ascribed the induced plant growth and development to increased nutrients uptake *via* adjusting the osmotic pressure and key enzyme activities of nitrogen metabolism (Mondal *et al.*, 2012). There are many reports documented the positive impact of chitosan on the improvements of shoot regeneration and growth in different plant species such as in Orchids (Nge *et al*., 2006) andGerbera (Sukwattanasinitt *et al.*, 2001).

Recently, Malerba and Cerana (2016) and Rabêlo *et al.* (2019) suggested that chitosan stimulate photosynthesis process, plant growth in maize and triggered abiotic stress tolerance through induction of antioxidant enzymes as superoxide dismutase, glutathione reductase, catalase, ascorbate peroxidase and guaiacol peroxidase activities to scavenge reactive oxygen species (Mondal *et al.*, 2013), in addition to increasing the total soluble sugars, starch, total amino acids as well as affect on gene expression. Sopalun *et al*. (2010) and Govindaraju and Arulselvi (2016) reported the increment of shoot number per explant in the presence of chitosan in the culture media of *Grammatophyllum speciosum* (L.) and *Coleus aromaticus* (L.**)**, respectively. The obtained findings are in accordance with those of Acemi *et al*.,(2018) who noticed that addition of chitosan at 5 mg/l in the tissue culture media had positive effect on shoot regeneration and gave higher shoot number, shoot length and leaf number in *Ipomoea purpurea* (L.). From the obtained results and the previous investigations (Barka *et al.*,2004; Serivastava *et al*., 2009; Salam *et al*., 2012; Salachna and Zawadzinka, 2014 and El-Tantawy 2017)

Table 4: Effect of different concentrations of chitosan on the regeneration potential of embryo axes of faba bean cv. Nubaria2, Fresh weight (FW), dry weight (DW), shoot length, shoots number per explants and leaves number after 6 weeks on SIM (Shoot Induction Medium) containing different chitosan levels (0, 2, 4 and 8mg chitosan /l).

Chitosan level mg/l	Growth parameters					Regeneration %
	Shoot Length cm ± S.E.	Shoots no. per explants ±S.E.	Leaves no. ±S.E.	FW± S.E.	DW± S.E.	
0	3.66±0.44^{c}	5.33±0.88$^{\mathbf{a}}$	**4.00** ± 0.57^{b}	0.77±0.06^{b}	0.07±0.01^{b}	83.52
2	5.83±0.60^{b}	7.33±1.45$^{\mathbf{a}}$	7.33±0.33^{a}	1.84±0.39^{a}	0.14±0.04^{a}	91.51
4	7.33±0.88^{a}	6.33±0.66^{a}	8.66±1.76^{a}	1.66±0.34^{a}	0.15±0.01^{a}	94.54
8	8.83±0.60^{a}	7.00±0.57^{a}	9.66 ± 1.20^{a}	1.71±0.17^{a}	0.11±0.03^{a}	78.79
LSD (0.05)	1.71	n.s.	2.94	0.73	0.06	

FW: Fresh weight , DW: Dry weight

in the same trend, Esyanti *et al*.,(2019) observed increment in some growth parameters as plant height, leaves number and chlorophyll content in *Capsicum annuum* exposed to chitosan with improved plant resistance to *Phytophthora* infection.

Effect of chitosan on total soluble phenols

Soluble phenols and the phenolic concentration in regenerated plantlets of faba bean on SIM medium(Shoot Induction Medium) containing different levels of chitosan were determined (Tables 4 and 5). Ahmed *et al* .,(2019) mentioned that total phenolic contents is working as a powerful antioxidant and playing a beneficial role in various disorders of inflammatory, cancer and diabetes. However, the high phenolic concentration in faba bean has a negative effect on the shoot regeneration from different explants of faba bean. Variations were observed between control and *in vitro* regenerated plants exposed to different level of chitosan. Data presented in Table (5) illustrate that total soluble phenols was increased significantly with increment in chitosan level. The results obtained were in agreements with those of Chakraborty *et al*.,(2009) in *Cocos nucifera* (L.), Ghasemnezhad *et al*.,(2010) in apricot (*Prunus armeniaca* L.), Mathew and Sankar.(2014) in *Ocimum* spp, Ahmed Hanafy *et al*.,(2016) in washington navel orange tree, Govindaraju and Arulselvi (2016) in *Coleus aromaticus* (L.) and Rahman *et al*. (2018) in strawberry fruit who found increasing in biochemical content by 2.6 fold (as flavonoids, phenolics, anthocyanins and carotenoids) and increasing osmoprotectants as (total soluble phenol, total sugar and total free amino acids) in comparison with control untreated plants; all of these organic molecules have role in improving plant growth and plant osmosis modulation.

Table 5: Determination of total soluble phenols (µg/g) in the regenerated faba bean shoots on SIM medium (Shoot Induction Medium) containing different levels of chitosan.

Chitosan level mg/l	Total phenols (µg/g FW.)
0	49.22^{d}
2	81.92^{c}
4	120.01^{b}
8	170.00^{a}
LSD (0.05)	9.79

FW: Fresh weight

Moreover, Park *et al* .(2019) recorded increasing in phenylpropanoids accumulation after using chitosan at different concentration in Buckwheat (*Fagopyrum esculentum* Moench L.). Our results indicating that increasing in total soluble phenol had no clear negative effect on the growth of faba bean shoots (Table 4). However, high phenolic concentrations slightly decreased the shoot regeneration percentage on SIM medium supplemented with 8mg chitosan/l as compared with other tested chitosan levels (Table 4). Moreover, high phenolic concentrations caused browning in the base of the shoots and the lower part of explants exposed to different level of chitosan (Fig 2).

Precise data for the effect of three levels of chitosan on shoot regeneration frequencies and development in faba bean cv. Nubaria 2 after 6 weeks of culturing the explants are presented in Table (4).

The best shoot regeneration, as determined by shoot number per explants, was found with the inclusion of 2 mg chitosan /l in combination with 4.5 mg BAP /l (Table 4). Ahmed *et al*., (2016) found that there is important character for chitosan biopolymer which make adsorption for phenol and adsorption efficiency was 78.4%.

This adsorption character of chitosan may had role in decreasing necrotic and browning effect of phenolic compound releasing in medium during faba bean shoot growth under chitosan treatment. To obtain precise data on the phenolic composition of the regenerated faba bean shoots from embryo axes exposed to different concentration of chitosan, samples were analyzed by HPLC (Table 6). The obtained data reveale that chitosan treatments make induction for biosynthesis of different polyphenols.

Table(6): Determination of phenolic concentrations in regenerated faba bean shoots on SIM medium (Shoot Induction Medium) containing different level of chitosan using HPLC.

Phenolic compound Conc.(µg/g FW)	Chitosan level (mg/l)			
	0 mg/l	2mg/l	4mg/l	8 mg/l
	Conc. (µg /g FW.)			
Gallic acid	1.10	ND	ND	0.37
Catechol	1.74	2.45	ND	2.70
p- Hydroxy benzoic acid	ND	12.37	17.38	0.48
Caffeine	4.07	0.60	0.36	ND
Vanillic acid	ND	0.31	ND	ND
Caffeic acid	3.53	ND	ND	0.30
Syringic acid	1.76	1.02	0.47	0.21
Vanillin	0.89	ND	1.55	0.53
p- Coumaric acid	0.16	0.25	0.26	0.18
Ferulic acid	4.94	2.21	0.67	2.40
Rutin	2.92	6.86	1.71	0.85
Ellagic acid	1.09	1.52	0.45	ND
Benzoic acid	16.35	16.12	25.49	30.04
o- Coumaric acid	0.78	0.59	0.49	0.49
Salicylic acid	2.17	2.88	0.69	0.84
Cinnamic acid	ND	0.05	ND	0.01
Total	41.5	47.23	49.52	39.4

ND: not detected

These results concluded that polyphenols was increased when using chitosan at level 2 and 4 mg chitosan /l in the medium which resulted in 47.23 µg/g FW and 49.52 µg/g FW , respectively. Whilst these polyphenols was decreased with increasing chitosan level to 8 mg chitosan /l with the value of 39.4 µg/g FW (Table 6). Our results in accordance with Park *et al* .,(2019) who studied the effect of 3 different chitosan levels (0.01 % , 0.1% and 0.5%) on polyphenol

accumulation of buckwheat. They found that phenolic compound accumulation increased significantly when used chitosan at level 0.1% than 0.5% chitosan. Khan *et al* .,(2003) mentioned that chitosan have a role in increasing the activity of enzymes (phenyl–alanine ammonia lyase PAL and tyrosine ammonia lyase TAL. These enzymes are important enzymes in phenylpropanoid pathway in soybean leaves. The derivatives of this pathway used as precursor for many secondary metabolites including flavonoids and polyphenols (Kim *et al*. ,2005 and Chen *et al.*, 2009). They proved that chitosan treatment induced phenylpropanoid biosynthesis genes after soybean sprout treated with either chitosan or salicylic acid which has role in shoot development by Galal(2012); Agami and Mohamed (2013). Their results indicated that salicylic acid was increased to 2.88 µg salicylic acid /g FW when using concentration of chitosan at 2 mg chitosan /l . Whilst with chitosan at level 4 and 8 mg chitosan /l resulted in decreasing the concentration of salicylic acid to 0.69 and 0.84 µg salicylic acid /g FW , respectively. Vlot *et al*. (2009) described that salicylic acid is considered one of phenolic compound which synthesized by plant. Salicylic acid as a plant hormone have role in regulating plant growth as well as regulating plant response to biotic and abiotic stress. Vicente and Plasencia (2011) suggested that salicylic acid induced seed germination, vegetative growth, respiration and photosynthesis process as well as keep cellular redox state through modulation of plant antioxidant enzymes activity. The results of the present study is in line with Vasconsuelo and Boland (2007) who mentioned that the response of plant to elicitor depend on its concentration which varies by plant species. Current result indicated that syringic acid concentration was decreased with increasing chitosan levels 2, 4 and 8 mg chitosan /l to the records of 1.02 ,0.47 and 0.21 µg syringic acid/g FW , respectively. Similar observations was recorded with ferulic acid, caffeine, rutin, gallic, vanillin, p-coumaric acid, ellagic acid and o-coumaric acid. HPLC analysis result of these phenolic acid compounds indicated the reduction in the concentrations of these phenolic acids with increasing chitosan concentrations. Current results are in a agreement with those reported by Kuiter (1989) who reported that high concentrations of salicylic acid, hydroxybenzoic acid, syringenic acid, caffeic acid, vanillic, p-coumaric and ferrullic acid delayed the seeds germination and growth in different plant species. However, low concentration of these phenolic acids had stimulatory effect. Present result is harmony with Conrath *et al*. ,(1989) who investigated that coumarine was decreased with chitosan treatment in suspension culture in orchid and low concentration of coumarine stimulated shoot growth. With regard to the other phenolic acids such as catechol and benzoic acid, it could be noticed that there are increasing in these polyphenols with increment the chitosan concentrations. These results are in agreement with the findings of Reddy *et al.*,(1999) who mentioned that after treating wheat seeds with chitosan, they found increment in hydroxycinammic acid, p-coumaric acid and caffeic. Increasing the concentration of benzoic acid to 30.04 µg /g FW maybe have an negative effect on shoot regeneration percentage specially when using 8 mg chitosan /l as a result of that shoot regeneration percentage was decreased to 78.79%. Our results in the present study are consistent with findings f Ozpinar *et al*.,(2017) who noticed inhibtion in root and stem growth when treating seeds of wheat, corn and chickpea with benzoic acid at high concentration of 0.1g benzoic acid/ 70ml purified water. Whilst plant growth was enhanced when using benzoic acid at low concentration of 0.01g benzoic acid / 70ml purified water .

The effect of low levels of chitosan (0, 2, 4 and 8 mg chitosan /l) on GA_3 and ABA accumulation was studied. Data presented in Table .(7) indicated that there are increasing in GA_3 concentration by percentage of 90.6%, 71.07% and 273.0% with increment in chitosan levels to 2, 4 and 8mg chitosan /l, respectively as compared with the control. Morover, it was noticed increment in ABA concentration by percentage of 8.3%, 16.6% and 50.0% with increment in chitosan levels to 2, 4 and 8mg chitosan /l, respectively.

Table (7). Determination of plant hormones (GA3 and ABA) concentration in regenerated faba bean shoots on SIM medium (Shoot Induction Medium) containing different concentrations of chitosan using HPLC after culturing the embryo axes for 6 weeks.

Chitosan level (mg/l)	Plant hormones (µg/100 g FW)	
	GA_3	ABA
0	363	1.200
2	692	1.300
4	621	1.400
8	1354	1.800

FW: Fresh weight

The obtained results are in agreement with those of Uthairatanakij *et al*.,(2007) and Hanafy Ahmed *et al.*,(2016) in Orchid and in orange tree, respectively who approved that chitosan play a role in increasing the concentration of endogenous plant hormones such as GA_3 and ABA. They ascribed their results to the fact of chitosan might make induction for terpenoid formation. Zhou *et al*.,(2002) reported the elevation of GA_3 and indole acetic acid concentration associated with enhancement of seed germination of peanut seeds after soaking in chitosan. In the same treat, Coqueiro *et al*.,(2015) proved that chitosan treatment lead to change in the metabolism of some hormones in sweet orange. From these results it can be suggested that the treatment with chitosan at level 2 and 4mg chitosan /l were effective for shoot regeneration from embryo axes in combination with BAP at a concentration of 4.5mg BAP /l. In this respect, Ahmed *et al.,* (2017) found that the application of irradiated chitosan to *Mentha piperita* (L.) increased plant active constituents such as menthol. Also ,Gupta and Chakrabatry (2013) mentioned that GA_3 have essential role in the induction of seed germination and transition of meristematic tissue to shoot growth. These findings are in accordance with obtained results from the present study.

Conclusion

In conclusion, the appropriate chitosan level for faba bean regeneration from embryo axes was elucidated. Faba bean regeneration frequency from mature embryo axes was increased when exposed to chitosan at low level . Whilst high level of chitosan completely depressed the plant regeneration. Moreover, chitosan application increased growth parameters and total soluble phenols. Also chitosan treatment at low level lead to changes in polyphenole biosynthesis as salicylic acid which have stimulation effect on shoot regeneration. As well as chitosan induced GA_3 and ABA synthesis.

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