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ESTABLISHMENT OF REGENERATION SYSTEM *VIA* ORGANOGENSIS IN SOME EGYPTIAN CULTIVARS OF *VICIA FABA*

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ABSTRACT:

Faba bean (*Vicia faba* L.) is one of the most important legume crops worldwide particularly in Egypt. The present investigation was designed to establish the regeneration system of two cultivars of faba bean (cv. G 461 and G 674). The regeneration system was carried out *via* organogenesis using shoot apices and cotyledon explants obtained from the two cultivars used. The VSI medium (MS + B5 vitamins) containing different concentrations of BAP and NAA for callus as well as shoot induction was used and the cotyledon explants appeared to be more effective than shoot apex explants. After 3 weeks from culturing at 28±2°C and photoperiod 16/8 h light/dark, the VSI medium supplemented with 2 mg/L BAP and 1 mg/L NAA or 3 mg/L BAP and 0.05 mg/L NAA was successful for producing calli for the two cultivars, respectively. The VSI medium combination with 5 mg/l BAP and 0.5 mg/L NAA and 3 mg/L BAP and 0.05 mg/L NAA were the best media for shoot induction using the shoot apex explants from the two cultivars (G 461 and G 674), respectively. Six different media were used for root induction and the M4 medium (B5 medium free hormones) was the most successful one.

INTRODUCTION:

Seed legumes have not been particularly amenable to *in vitro* culture, as plant regeneration from callus proved to be especially difficult and the major problems have been encountered in this connection with the large seeded legumes (Dale, 1983). Faba bean tissue culture often releases a substantial amount of phenolic substances and the callus tissue is often characterized by poor growth rates, with a tendency to become necrotic after some time (Schulze *et al*., 1985).

Glazy and Hamoui (1981) studied the induction of organogenesis on the calluses of *V. faba* minor cultivar Ascot derived from shoot tips. Schulze *et al*. (1985) and Busse (1986) obtained shoot development from explants containing axillary meristems (shoot tips, nodal tissue either from stem or hypocotyl and cotyledonary nodes), but in addition they obtained shoot regeneration from callus derived from these explants. Fakhrai *et al*., (1989) used nodule stems, leaves, roots and cotyledons from *V. faba* as explants for shoot induction. High frequency rooting of these adventitious shoots was obtained on half strength MS medium with 1.5 % sucrose, 0.1 mg NAA and 0.5 mg kinetin/L. Thynn *et al*., (1989) reported that in shoot apex meristems with leaf primordias of *V. faba* cv. Tp667, addition of low concentration (0.01 mg/L) of auxin induced regeneration of whole plants at high frequency (100%). The combination of NAA and kinetin or GA3 also induced a high yield of plant regeneration. Tao-Ym (1991) showed that NAA at 1-5 ppm induced callus from all germinating seeds but inhibited subsequent shoot growth, thus demonstrating an effect similar to that of doctinomycin (20 ppm). Marked inhibition of shoot growth was observed when NAA and doctinomycin were applied together without preventing callus induction. The rooting process of *in vitro*

regenerated shoots is one of the problems indeveloping regeneration systems of *V. faba*. Busse (1986) described a meristems culture of *V. faba*, which is potent for multiple shoot bud propagation and many trials have been performed to root such shoots. Furthermore, it was possible to increase rooting frequency by application of the method described by Schulze *et al*., (1985), who used *V. faba* shoots originated from cultured meristems without efficient propagation phase. The rooting frequencies obtained in this one-step experiment were not higher than 20%. Ahmed *et al*., (1997) found that the optimal medium for root induction on shoots obtained from nodal segments of lens was the Ms medium supplemented with 5.3 µM NAA. This work was designed to establish the regeneration system for *V. faba* cultivars Giza 461 and Giza 674 *via* organogenesis using different explants and different combinations of cytokinin(s) and auxin(s).

MATERIALS AND METHODS:

Source of seeds: Seeds of *Vicia faba* L. cultivars Giza 461 (G 461) and Giza 674 (G 674) were obtained from the Field Crops Research Institute, (ARC), Giza, Egypt.

Seed sterilization: Mature seeds of faba bean cultivars (G 461 and G 674) were surface sterilized by soaking them in 70% ethanol for 30 seconds, then rinsed in sterile water before transferring to 20% commercial household bleach (active ingredient 5.25% sodium hypochlorite) for 10-15 min with 1-2 drops of Tween 20. Seeds were washed 5-6 times with sterile distilled water (d.H_2O).

Seed germination: Seeds were germinated in a pre-autoclaved wet cotton pads, placed in 10 cm glass jars and covered with aluminum foil followed by incubation at 28°C±2 and under 16 h photoperiod provided from cool white fluorescent lamp (3000 lux).

Explants: *In vitro* grown seedlings of the two cultivars (10-13 days old) were used as a source of explants. Mature cotyledon and shoot apexes were excised and scratched using a sharp scalpel.

Callus and shoot induction using shoot apex explants: The mature cotyledon and shoot apexes explants of cvs. G 461 and G 674 faba bean were cultured on Vicia shoot induction (VSI) medium which consist of MS salts with B5 vitamin supplemented with different combinations of BAP (1.0, 2.0, 3.0, 4.0 and 5.0 mg/L) and NAA (0.05, 0.5 and 1.0 mg/L). A VSI medium free growth regulator was used as control. Ten explants were used for each treatment. All cultured plates were incubated at 28± 2°C for 3-4 weeks. Shoot production in faba bean cultivars, G 461 and G 674, were tested on VSI medium. Cultures were routinely maintained on a fresh medium every 3-4 weeks. Brown dark area tissues were dissected regularly using a sharp scalpel every time to minimize death of the tissues. The number of the responded explants; explants produced callus and numbers of shoots per explant were determined.

Callus and shoot induction using cotyledon explants: Mature seeds were surface sterilized as previously mentioned and were soaked overnight in sterile water and dehisced there after. Seeds were longitudinally sectioned into two halves followed by cutting the two mature cotyledons in small sections after separating the embryo, the explants were tested on VSI media for callus and shoot induction. In addition, another cytokinin (Kinitin, 1.0 and 2.0 mg/L) and auxin (2,4-D, 0.5 and 1.0 mg/L and IAA 0.1 and 1.0 mg/L) were used with cotyledon explant to choose the best media for this explant. Cultured were transferred to a freshly prepared VSI medium every 3-4 weeks. Brown and dead tissues were dissected all the time using a sharp scalpel. The data were recorded and statistically analyzed using ANOVA test.

Rooting stage: The excised cultured shoots 4-5 cm long were transferred to different rooting media. M1: B5 medium plus BAP (0.01 mg/L), NAA (0.1 mg/L). M2: B5 medium

contains indolebutric acid (2 mg/L), Kinetin (1 mg/L), ammonium sulfate (100 mg/L), and sucrose (10 gm/L). M3, B5 medium contains NAA (2 mg/L), Kinetin (0.1 mg/L), ammonium sulfate (200 mg/L), and sucrose (30 g/L). M4: B5 free hormones (Gamborg *et al.,* 1968). M5: MS medium free hormones (Senreich and Eisenreich. 1989) and M6: ½ strength MS medium with 1.5 % sucrose, NAA 0.1 mg/l, and kinetin 0.5 mg/L at pH 5.8 solidified with 0.6 % agar (Fakhrai *et al.,* 1989). The cultures were incubated for 12 h photoperiod at 22-27°C for about one month.

RESULTS AND DISCUSSION:

Tissue culture techniques provide a new and valuable approach in crop improvement and genetical studies as it save effort and time to the breeder by providing material that can never be obtained through conventional breeding methods (Angeloni *et al.,* 1992).

Establishment of regeneration system in *V. faba via* organogenesis: The production of transgenic plants requires an efficient shoot regeneration system. The *in vitro* "plant-to-plant" process usually includes the induction of callus from which morphogenesis must be achieved (Fontana *et al.,* 1993).

Callus induction stage: For callus induction, shoot apex and cotyledon explants of *V. faba* cultivars, G 461 and G 674, regeneration medium (MS medium with B5 vitamin) containing different combinations of NAA and BAP were used. The obtained results showed that the highest percentage (70 %) of callus induction in *V. faba* cv. G 461 was obtained using the cotyledon explants with medium composed of 2.0 mg/L BAP and 1.0 mg/L NAA (Table 1). While in *V. faba* cv. G 674, cotyledon explants gave 90% of callus induction with medium composed of 3 mg/L BAP and 0.05 mg/L NAA (Table 2). This proved that the cotyledon explants were the most suitable for callus induction in the two cultivars applied (Figure 1).

When shoot apices were used as explants the percentage of callus induction was decreased to 26.6% in *V. faba* cv. G 461 on medium composed of 5.0 mg /L BAP and 1.0 mg/L NAA (Table 3), and to 80 % in *V. faba* cv. G 674 on medium composed of 4.0 mg/L BAP and 1.0 mg/L NAA (Table 4 and Figure 2). Similar observations were reported by Thynn *et al., (*1989) who found that addition of low concentrations of auxins (0.01 mg/L) using shoot apex meristems with leaf primordias of TP667 faba bean induced 100% regeneration of whole plants. The combination of NAA and kinetin was also induced a high degree of plant regeneration. It is of importance to note that the cultures in this study were incubated in the dark during callus induction phase to reduce the oxidation of phenolic compounds and at the same time to increase callus productivity.

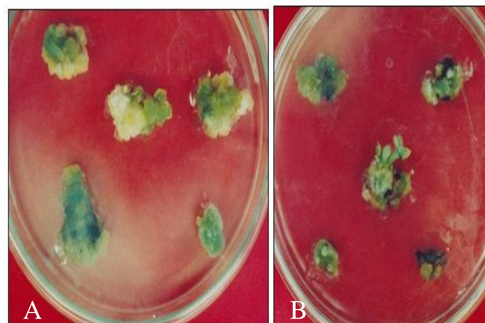

Figure (1): Callus induction using mature cotyledon explants obtained from *V. faba* after 3 weeks from culturing on VSI medium. (A) *V. faba* cv. G 461 on VSI medium composed of MS medium + B5 vitamin, were containing 2.0 mg/L BAP and 1.0 mg/L NAA. (B) *V. faba* cv. G 674 on VSI medium composed of MS medium plus B5 vitamin supplemented with 3.0 mg/L BAP and 0.05 mg/L NAA.

The importance of dark incubation during the callus induction phase in several legume crops was reported by Bhat and Chandel (1991) and Becker *et al*., (1994).

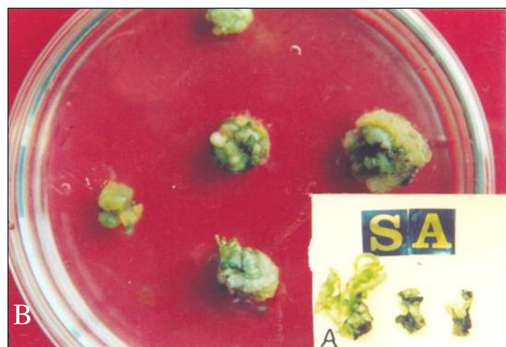

Figure (2): Callus induction using shoot apex explants obtained from *V. faba* after 3 weeks from culturing on VSI medium. (A) *V. faba* cv. G 461 on VSI medium composed of MS medium plus B5 vitamin, were containing 5.0 mg/L BAP and 1.0 mg/L NAA. (B) *V. faba* cv. G 674 on VSI medium composed of MS medium plus B5 vitamin, supplemented with 4.0 mg/L BAP and 1.0 mg/L NAA.

Previous report dealing with legume crops such as *V. faba L.* (Selva *et al.,* 1989; Taha and Francis, 1990; Herscovich *et al*., 1992 and Tegedar *et al.,* 1995) and *Glycine max* (Dhir *et al.,* 1991) also reported that dark incubation was found to be important in reducing polyphenolic compounds oxidation. In Egypt, Saleh (1998) reported that the dark incubation of *V. faba* explants for callus induction affected positively the reduction in phenols oxidation and increasing callus productivity.

Shoot induction stage: The production of shoots from faba bean explants was studied using *Vicia* shoot induction medium (VSI) and two explants (shoot apexis and cotyledons), for the two cultivars (G 461 and G 674). In the case of *V. faba* cv. G 461 results showed that the percentage of shoots produced from shoot apex explants was 20% on VSI medium composed of 5.0 mg/L BAP and 0.5 mg/L NAA (Table 5 and Figure 3A). The obtained results also showed that shoot apices (as explants) of *V. faba* cv. G 674 were more sufficient than cotyledon explants for regeneration. As 40% from the calli were produced shoots on VSI medium composed of 3.0 mg/L BAP and 0.05 mg/L NAA (Table 6 and Figure 3B). While the cotyledon explants were found to be more effective for shoot induction in cv. G 674 faba bean, as 3.3% of the calli were developed shoots, in comparison with cv. G 461, which failed to give shoots from cotyledon explants (Figure 4). In contrast, shoot apices were the most suitable explants for shoot induction and plant regeneration in the two cultivars that have been confirmed statistically. Selva *et al.,* (1989) found that BA at 4 mg/L was the most effective concentration in promoting high rates of shoot development using cotyledonary nods and stem nods on *V. faba* L. Fakhrai *et al.,* (1989) found that shoot organogenesis was only obtained with nodal stem and cotyledonary node explants when cultured on MS medium with 3% sucrose, 2.0 mg/L BA and 0.2 mg/L NAA. Schulze *et al.*. (1985) and Busse (1986) obtained shoot developed from explants containing axillary meristems, but also calli were derived from these explants. Saleh (1998) found that half-strength MS basal (MSB) medium supplemented with 3 mg/L BAP was better for cv. G Blanca, where the highest number of proliferated buds were produced. On the other direction, full strength MSB medium with 5 mg/L BAP was better for cv. G 461. Similarly, Mohamed (1999) used different combinations of BAP and NAA to proliferate multiple shoot formation from nodal cutting explants in *V. faba* cv. G 461 and the optimum concentrations were found to be 0.1 mg/L NAA and 4.0 mg/L BAP.

Figure (3): Shoot apex explants of V. faba 4 weeks post culturing on VSI medium. (A) V. faba cv. G 461 on VSI medium (MS medium supplemented with B5 vitamin, 5.0 mg/ BAP and 0.5 mg/L NAA). (B) V. faba cv. G 674 on VSI medium (MS medium supplemented with B5 vitamin, 3.0 mg/L BAP and 0.05 mg/L NAA).

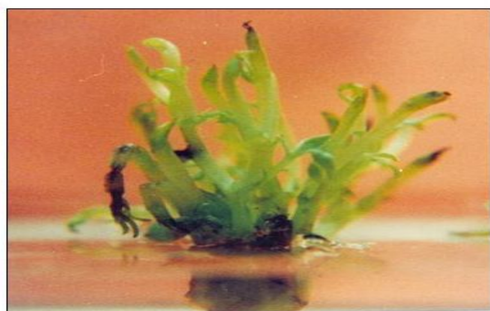

Figure (4): Shoot induction and elongation of mature cotyledon explants obtained from V. faba cv. G 674 four weeks post culturing on VSI medium (MS medium plus B5 vitamin and 1.0 mg/L BAP and 0.05 mg/L NAA).

Rooting stage: Sayegh (1988) described a technique for the culture of explants such as apical tips, nodal segments, epicotyls, hypocotyl, roots, and cotyledons and epicotyls-hypocotyls junction (EHJ) tissue. Shoot were developed after 1 week from apical tips, nodal segments and EHJ tissue, while the other types of explants were failed to develop any shoots. After 4 weeks, 59 shoots of 2 genotypes were transferred into a rooting MS medium, and 55% and 73% of these shoots were rooted within 3 and 5 weeks, respectively.

In this study, the rooting process of *in vitro* regenerated shoots is one of the main problems in developing regeneration system of *V. faba.* In the present investigation, 6 media for the rooting stage were used as illustrated in Figure-5. B5 free hormones medium (M4) was found to be the most suitable medium for roots formation as 6.6 and 10% of rooted shoots were obtained for cv. G 674 and cv. G 461, respectively.

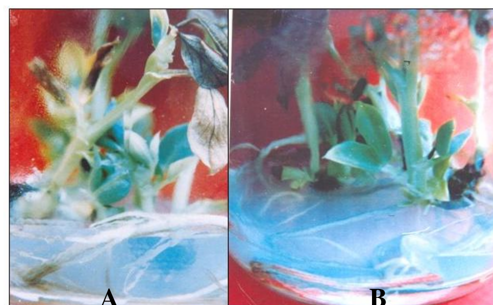

Figure (5): Rooting stage of V. faba cvs. G 461 (A) and G 674 (B). Complete shoot cultured on a rooting medium (B5 major and minor salts without hormone). The data was recorded after 21 days from cultivation on rooting medium.

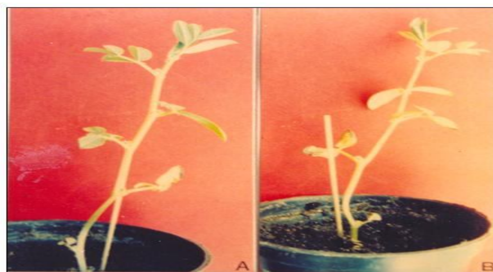

Figure (6): Normal regenerated V. faba plants acclimatized using soil mixture composed of peat-moss: clay (2:1, w:w). (A), cv. G 461 and (B) cv. G 674.

Busse (1986) described a meristems culture of *V. faba*, which is potent for multiple shoot lend propagation. Suman *et al*., (1988) showed that both the number of roots initiated and the length of the longest root were significantly higher on MS medium than on half-strength MS medium. Polisetty *et al*. (1997) showed that a high BAP concentration inhibited subsequent rooting of shoots, while kinetin was stimulated root differentiation and elongation. Thynn *et al.* (1989) found that shoot and root differentiation and regeneration occurred with epicotyl explants cultured in B5 medium supplemented with 0.2 mg/L NAA. While Mohamed (1999) used different concentrations of NAA, IAA and IBA in order to determine the best concentration for rooting efficiency. She also indicated that grafting is the most appropriate method for *V. faba* rooting.

Acclimatization stage: It is a very important step towards the production of normal and mature regenerated plants. In the present work, acclimatization of the regenerated faba bean plantlets was achieved using soil mixture composed of peat-moss and clay in a ratio of 2:1 (v/v) for the two cultivars used as shown in Figure (6).

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Table -1: Survival, callus induction and average weight of callus regenerated on MS medium plus B5 vitamins and different concentrations of NAA and BAP for mature cotyledon explants of *V. faba* cv. G 461.

NAA (mg/L)	BAP (mg/L)	Responded explants		Explants produced callus		Average weight of callus (g)
		No.	%	No.	%	
0.05	1.0	0	0	0	0	0
	2.0	4	13.3	3	10	0.93
	3.0	11	36.6	9	30	3.00
	4.0	0	0	0	0	0
	5.0	0	0	0	0	0
0.5	1.0	0	0	0	0	0
	2.0	0	0	0	0	0
	3.0	7	23.3	5	16.6	1.73
	4.0	4	13.3	2	6.6	1.18
	5.0	16	53.3	12	40	3.10
1.0	1.0	0	0	0	0	0
	2.0	21	70	21	70	3.03
	3.0	4	13.3	2	6.6	0.68
	4.0	9	30	2	6.6	1.05
	5.0	13	43.3	6	20	2.60
SE		1 .7		1.5		0.3
Control		1	1	0	0	0

Fifty explants were used for each treatment

Table -2: Survival, callus induction and average weight of callus regenerated on MS medium plus B5 vitamins and different concentrations of NAA and BAP for cotyledon explants of *V. faba* cv. G 674.

NAA (mg/L)	BAP (mg/L)	Responded explants		Explants produced callus		Average weight of callus (g)
		No.	%	No.	%	
0.05	1.0	24	80	23	76.6	2.75
	2.0	5	83.3	22	73.3	6.85
	3.0	30	100	27	90	3.62
	4.0	17	56.6	17	56.6	3.24
	5.0	6	20	5	16.6	2.75
0.5	1.0	23	76.6	15	50	2.39
	2.0	7	36.6	6	33.3	2.00
	3.0	25	83.3	20	66.6	3.84
	4.0	3	10	1	3.3	1.55
	5.0	15	50	6	20	1.88
1.0	1.0	15	50	9	30	2.47
	2.0	12	40	11	36.6	1.20
	3.0	12	40	9	30	1.65
	4.0	25	83.3	16	53.3	3.36
	5.0	16	53.3	9	30	2.80
SE		2.1		2		0.3
Control		0	0	0	0	0

Fifty explants were used for each treatment.

Table -3: Survival, callus induction and average weight of calli on MS medium plus B5 vitamins and different concentrations of NAA and BAP for shoot apex explants of *V. faba* cv. G 461.

NAA (mg/L)	BAP (mg/L)	Responded explants		Explants produced callus		Average weight of callus (g)
		No.	%	No.	%	
0.05	1.0	2	6.6	2	3.3	0.31
	2.0	2	6.6	1	6.6	0.28
	3.0	6	20	2	3.3	0.39
	4.0	4	13.3	1	6.6	0.49
	5.0	6	20	2	6.6	0.18
0.5	1.0	2	6.6	2	3.3	0.48
	2.0	5	16.6	1	10	0.40
	3.0	4	13.3	3	3.3	0.24
	4.0	2	6.6	1	6.6	0.44
	5.0	4	13.3	2	13.3	0.78
1.0	1.0	9	30	4	16.6	0.47
	2.0	4	13.3	5	13.3	0.75
	3.0	1	3.3	4	3.3	0.08
	4.0	4	13.3	1	10	0.74
	5.0	8	26.6	3	26.6	0.46
SE		0.5		0.5		0.05
Control		1	4	0	0	0

Fifty explants were used for each treatment.

Table -4: Survival, callus induction and average weight of callus regenerated on MS medium plus B5 vitamins and different concentrations of NAA and BAP for shoot apex explants of *V. faba* cv. G 674.

NAA (mg/L)	BAP (mg/L)	Responded explants		Explants produced callus		Average weight of callus (g)
		No.	%	No.	%	
0.05	1.0	12	40	12	40	1.02
	2.0	18	60	9	30	0.57
	3.0	24	80	18	60	1.32
	4.0	24	80	21	70	1.43
	5.0	24	80	18	60	2.54
0.5	1.0	21	70	18	60	0.65
	2.0	6	20	3	10	0.19
	3.0	21	70	18	60	1.29
	4.0	12	40	12	40	0.17
	5.0	12	40	12	40	0.25
1.0	1.0	24	80	18	60	1.49
	2.0	21	70	9	30	0.70
	3.0	18	60	12	40	0.60
	4.0	24	80	24	80	0.64
	5.0	6	20	3	10	0.78
SE		1.7		1.7		0.2
Control			0	0	0	0

Fifty explants were used for each treatment.

Table -5: Shoot induction using MS medium with B5 vitamins and containing different concentrations of NAA and BAP with shoot apex explants obtained from *V. faba* cv. G 461.

NAA (mg/L)	BAP (mg/L)	Explant produced shoot		No. of shoot/explant
		No.	%	
0.05	1.0	0	0	0
	2.0	0	0	0
	3.0	1	10	1
	4.0	1	10	1
	5.0	1	10	1
0.5	1.0	1	10	2
	2.0	1	10	1
	3.0	1	10	1
	4.0	1	10	1
	5.0	3	20	2
1.0	1.0	0	0	0
	2.0	2	13.3	1
	3.0	1	10	1
	4.0	1	10	1
	5.0	2	13.3	1
SE		0.2		0.1
Control		1	10	0

Fifty explants were used.

Table -6: Shoot induction using MS medium with B5 vitamins and containing different concentrations of NAA and BAP with shoot apex explants obtained from *V. faba* cv. G 674.

NAA (mg/L)	BAP (mg/L)	Explant produced shoot		No. of shoot/explant
		No.	%	
0.05	1.0	3	10	1
	2.0	3	10	1
	3.0	12	40	4
	4.0	6	20	1
	5.0	12	40	2
0.5	1.0	6	20	1
	2.0	3	10	1
	3.0	12	40	2
	4.0	3	10	1
	5.0	3	30	1
1.0	1.0	9	30	2
	2.0	3	10	1
	3.0	6	20	1
	4.0	3	10	1
	5.0	0	0	0
SE		1.1		0.3
Control		0	0	0

Fifty explants were used.

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