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SALINITY EFFECTS AND TRANSFORMATION OF *Vicia faba* L. CALLUS TISSUES

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ABSTRACT

Faba bean (*V. faba* L.) cultivars Giza 5 and Giza 461 immature embryos were used to obtain callus which was used to study the effect of salinity and transformation by the *Agrobacterium tumefaciens* bacteria carrying the plasmid pBI121. As the salinity level increased, both fresh and dry weights of callus tissue decreased. However, NaCl + $CaCl_2$ had a less adverse effect on both fresh and dry weights of callus tissue as compared to NaCl alone. Generally, Giza 5 cultivar produced higher fresh and dry weights compared to Giza 461 cultivar. Sugars, soluble phenols and proline concentrations increased as the salinity level increased. Proline showed higher concentration in Giza 461 cultivar compared to Giza 5 cultivar. *Vicia. faba* L. callus tissue proved to be sensitive to kanamycin, whereas the survival rate was only about 2% at a concentration of 75 mg/l kanamycin. Giza 5 cultivar recorded higher transformation percentage than Giza 461 cultivar. Thus, the established callus tissue transformation in the present work can offer the introduction of new desirable genes to improve *V. faba* L. nutritional value and increasing resistance to disease and some herbicides and consequently improving the economical plant productivity.

INTRODUCTION

Faba bean (*Vicia faba* L.) is an economically important legume crop as a popular daily diet for most of the Egyptian public as a protein source. It is also used in animal feed beside its role in increasing soil fertility through the process of nitrogen fixation. *Vicia. faba* dry seeds contain 25-30% protein, 50-60% carbohydrates, 10-15% fibers and 5% minerals, especially phosphorus and potassium. Unfortunately, susceptibility to environmental fluctuations and diseases infection, resulted in *V. faba* seed yield reduction due to viral infection, especially in Middle Egypt region. Another obstacle to the expansion of the cultivated area of *V. faba* is the limited resources of agricultural land. Although *V. faba* plants are sensitive to salinity, newly cultivated saline soil may offer a chance for such expansion through conducting salinity research on *V. faba* plant. Plant tissue culture can offer solutions to counteract such problems through the introduction of new genes coding for resistance to disease or herbicides and improving nutritional value by adding genes coding for some essential amino acids. Plant regeneration from somatic cells has been difficult to achieve among large seeded leguminous species. Among such species, *V. faba* L. has received a little attention. Thus, the aim of the present work is to: a) Establish a reliable tissue culture and regeneration system in *V. faba* L. b) Establish and

evaluate a transformation protocol to produce *V. faba* L. transgenic plants through *Agrobacterium* mediated transformation. c) Evaluate the produced callus response to different salinity levels through studying their growth characters and chemical constituents.

MATERIALS AND METHODS

All experiments were conducted at Plant Physiology Section, Faculty of Agriculture, Cairo University and The Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Center (ARC), Ministry of Agriculture, Giza, Egypt.

I. Tissue culture and salinity:

Vicia. faba immature seeds at 15 days after pollination, were used as a source for the immature embryos explants. The immature pods of *V. faba* plants Giza 5 and Giza 461 cultivars were collected from fields in Sakha, Kafr Elsheikh. Immature seeds were washed with commercial soap & water and sterilized in 1% $HgCl_2$ solution for 5 min. then rinsed in sterilized water 6 times. *Vicia faba* immature embryos were excised and cultured on MS medium (Murashige and Skoog, !962) containing 100 mg/l myo-inositol, 4.5 mg/l benzyl amino purine (BAP) and 0.05 mg/l NAA. Explants were incubated at 21^{o}C in complete darkness for 9 weeks and sub-cultured every 2 weeks during that period by transferring explants into the same freshly prepared media. For callus maintenance, the produced callus from the cultivar Giza 5 was kept on the same previously mentioned media plus 3 mg/l BAP, while the produced callus for the cultivar Giza 461 was kept on the same media plus 5 mg/l BAP. Callus tissue was incubated at 21^{o}C in darkness and sub-cultured every 3 weeks.

Callus growth:

The obtained calluses were subjected to different levels of salinity as NaCl alone or in combination with $CaCl_2$ in the ratio of 3:1. Salinity levels; zero (control), 500, 1000, 2000 and 4000 ppm were added to the previously mentioned MS media containing 3 and 5 mg/l BAP for the cultivars Giza 5 and Giza 461, respectively. Sub-culturing was carried out twice, every 21 days. Each treatment consisted of 8 jars (300 ml) containing 40 ml of the proper media with 5 callus pieces (0.5x0.5x0.5 cm for each piece). The obtained callus was harvested and fresh weight (g) was recorded. Sub-samples of the fresh callus matter were dried in an electric oven at 60 ^{o}C for 48 hrs. and the dry weight (g) was recorded.

Chemical analysis:

Reducing (R.S.), non-reducing (N.R.S.) and total sugars (T.S.) as well as total soluble phenols were determined in the hot ethanol extract of the fresh callus material. Reducing, non-reducing and total sugars were determined using the phosphomolybdic acid method (A.O.A.C., 1975) and data were calculated on dry matter bases (mg/g dry weight). Total soluble phenols were estimated using spectrophotometer (Beckman Du40) at wavelength of 660 nm according to Forrest and Bendall (1969). Acetosyringone was used as a standard. Amino acids were determined

according to Anderson *et al.* (1977). Briefly, 0.1 g of the dried *V. faba* callus material was hydrolyzed using 10 ml of 6 N HCl at 110 °C for 22 hrs. in a sealed tube. The hydrolysate was filtered using Whatman No. 1 filter paper. Filtrate sample of 5 ml was evaporated at 50 °C under vacuum. Residue was dissolved in 5 ml of Beckman Na-S buffer and 50 µl of each sample was injected into Beckman Amino Acid Analyzer model 7300.

II. Callus transformation:

Bacterial strains and plasmids:

Vicia faba L. callus tissue derived from immature embryos were genetically transformed by the bacteria *Agrobacterium tumefaciens* strains; LBA4404 (nopaline strain; carrying the necessary trans-acting virulence functions to facilitate the transfer of T-DNA region of binary vectors to plant cells), EHA105, EHA101 and the wild type strain C58. A. *tumefaciens* strains were cultured on 2 ml of LB media containing the following antibiotics:

Strain	Antibiotic name	Concentration
LBA4404	Streptomycin	25 mg/ml
EHA105	Kanamycin	25 mg/ml
EHA101	Kanamycin + Rifampicin	25 + 25 mg/ml
C58	--	--

***Agrobacterium* transformation:**

Agrobacterium *tumefaciens* bacteria were incubated overnight at 28 °C in a mechanical shaker at 150 rpm. *E. coli* (Strain DH5ά) containing the pBI121 construct and the helper strain pRK2013 (Ditta *et al.,* 1980) were incubated overnight in 2 ml LB liquid medium containing 50 mg/l kanamycin at 37 °C. The plasmid pBI121 was introduced to the *Agrobacterium* strains by the triparental crosses. The transconjugant *Agrobacterium* strains which contain the plasmid were selected on a solidified LB media containing 15 g/l Bactoagar supplemented with 50 mg/l kanamycin + 25 mg/l streptomycin + 25 mg/l rifampicin for the A. *tumefaciens* strains LBA4404 and EHA105, respectively. As for strain EHA101, the selection was carried out *on* LB solidified medium containing 100 mg/l kanamycin + 25 mg/l rifampicin. The bacterial cultures were incubated for 2 days at 28 °C.

***Agrobacterium* plasmid DNA isolation:**

Transformed *A. tumefaciens* were detected by using 3-keto-lactose test according to Bernaerts and De Ley (1960 and 1963). Transformed colonies with the plasmid pBI121 showed yellow ring of Cu_2O as 3-keto-lactose positive. The obtained colonies were used for isolation of plasmid and for the 3-keto-lactose test. According to Sambrook *et al.* (1989), a single colony from each strain was transferred and incubated into 20 ml LB media plus 100 mg/l kanamycin in a loosely capped 100-ml flask overnight at 28 °C on a mechanical shaker at 150 rpm. The obtained cultures were placed in a centrifuge tube and kept on ice for 10 min, then centrifuged at 8000 rpm for 10 min. The pellet was resuspended in 14 ml STET solution (0.1 M NaCl, 10 mM Tris.HCl pH 8.0, 1 mM EDTA, 5% Triton-X100) by vortexing briefly. Lysozyme solution (10 mg/ml Lysozyme in 10 mM Tris. HCl, pH 8.0) was added (300 µl). Tubes were gently inverted several times and then transferred into a boiling water bath for exactly 45 seconds. Tubes were

centrifuged at 12000 rpm for 15 min. at room temperature (R.T.) and bacterial debris pellet was removed with a sterilized toothpick. An equal volume of isopropanol and 1.4 ml of 2.5 M sodium acetate (pH 5.2) was added to the supernatant. Tubes were inverted several times followed by incubation for 5-10 min. at R.T. The plasmid DNA pellets were then recovered by centrifugation at 12000 rpm for 20 min. The pellet was resuspended 100 µl TE buffer (10 mM Tris and 1 mM Na_2EDTA, pH 8.0). The obtained samples of plasmid DNA were mixed with the loading TAE buffer (5-10% glycerol, 7% sucrose and tracking dyes such as 0.025% bromophenol blue or xylem cyanol) and loaded on the agarose gel according to Sambrook *et al.* (1989).

Callus transformation:

Callus tissue of *V. Faba* L. derived from the immature embryos was transformed with *A. tumefaciens* strain EHA101 carrying the plasmid pBI121. Strain EHA101 (30 ml) were pelleted at 6000 rpm at 4 ^oC, then suspended in an equal volume of MS hormone-free medium and left for 2 hrs. Calluses pieces (about 0.5 cm diameter) were dipped in the MS media containing the plasmid and mixed with liquid bacterial media and kept for 10-15 min. Explants were blotted on sterile filter paper to dryness, cultured on the MS media for 2-3 days at 22^oC, and placed on bacterial elimination selection medium (500 mg/l carbencillin + 50 mg/l kanamycin. Representative samples of *Agrobacterium* treated callus which grown on kanamycin-containing MS media for 3 subcultures were used in the GUS assay. Samples were transferred in Eppendorff tubes covered with the GUS solution (1 mg X-Glu, i.e. 5-bromo-4-chloro-3-indoyl glucuronide, and 0.3 $MnaH_2PO_4$ at pH 7.0) according to Jefferson *et al.,* (1987) and covered with aluminum foil to prevent light effect. Tubes were incubated overnight in a shaker at 30 ^OC. Explants were placed in a plate and covered with 70% ethanol for 10 min., replaced by 20% bleach for 5-10 min. to remove the green and brown colors, which might interfere with the blue color due to the GUS assay.

Data analysis:

Complete randomized block design was adopted. Data were subjected to the analysis of variance and treatments mean were compared to the control treatment using the least significant difference (L.S.D. 0.05) according to Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

Fresh and dry weight of callus tissues derived from immature embryos of *V. faba* decreased as the salinity level increased in both Giza 5 and Giza 461 cultivars (Table 1). In general, Giza 5 cultivar excelled Giza 461 cultivar in callus tissue growth for both fresh and dry weights. When salinity was used as a mix of sodium and calcium salts, fresh and dry weights of *V. faba* callus tissues were higher compared to salinity applied as sodium salt alone in both cultivars Giza 5 and Giza 461. The percent decreases in fresh weight in Giza 5 cultivar were 11.84, 24.44, 31.58 and 59.96% when

treated with NaCl + $CaCl_2$ at 500, 1000, 2000 and 4000 ppm, respectively when compared to their control treatment. The same treatment resulted in more drastic percent decreases in the fresh weight of *V. faba* callus tissue cultivar Giza 461 where it recorded 33.31, 38.88, 43.88 and 67.89%, in the same respective order as compared to their control treatment.

Table 1: Effect of different salinity levels on fresh (F.W.) and dry (D.W.) weight (g) of *Vicia faba* L. calli tissues derived from immature embryos.

Cultivar	Salt	Ppm	F.W.	D.W.
Giza 5	NaCl	Control	5.32	0.43
		500	4.25	0.29
		1000	3.83	0.18
		2000	2.45	0.07
		4000	1.86	0.05
	NaCl + $CaCl_2$	500	4.69	0.35
		1000	4.02	0.23
		2000	3.64	0.12
		4000	2.15	0.09
Mean			3.58	0.20
Giza 461	NaCl	Control	4.89	0.39
		500	3.01	0.31
		1000	2.55	0.16
		2000	2.34	0.08
		4000	1.61	0.03
	NaCl + $CaCl_2$	500	3.27	0.32
		1000	2.99	0.20
		2000	2.78	0.11
		4000	1.57	0.08
Mean			2.77	0.19

L.S.D. (0.05):

Fresh weight, Cultivars= N.S. Treatments= 0.45
Dry weight, Cultivars= N.S. Treatments=0.02

In the present work, regeneration of callus from *V. faba* L. was accomplished through the use of immature embryos at 15 days after pollination. In this respect, Khalafalla and Hattori (2000) found that *in vitro* rooting of faba bean (*Vicia faba*) shoots regenerated on medium containing thidiazuron (TDZ) was promoted by the ethylene inhibitors; silver nitrate ($AgNO_3$), acetyl salicylic acid (ASA) and cobalt chloride ($CoCl_2$) at 3-10 mg/l. In this connection, faba bean plants were regenerated via somatic embryogenesis of the Egyptian grown cultivars Giza 461 (Aly and Saker, 1998). Also, Saker *et al.* (1999) reported multiple shoot regeneration from immature cotyledon explants in Egyptian *Vicia faba* cv. Giza 3. Proliferation of embryonic callus, followed by the recovery of shoots, were obtained using MS medium containing 2 mg 2,4-D and 1.5 mg BA.

Faba bean (*V. faba* L.) is considered as one of the highly sensitive legume crops to salinity, with a survival percentage of less than 1% when irrigated with 10% sea water (Tal, 1984). The decrease in both fresh and dry weights as the salinity level was increased as observed in tobacco (Sumaryati *et al.,* 1992), tomato (Bressan *et al.,* 1982) and rice (Caplan *et al.,* 1990). In this respect, Abd Alla *et al.* (2001) *reported that s*alinity levels up to 120 mM NaCl significantly suppressed nodulation, nitrogenase activity and growth of hydroponics grown *Vicia faba* plant. Also, Emam *et al*. (2000) reported that mungbean callus growth increased with increasing no. of subculture for the cultivars used in that study, the negative correlation between salt (sea water or NaCl) concentration (up to 12000 ppm) and callus fresh weight and the less damaging effect of sea water compared with NaCl treatments are in agreement with our with results and with those obtained by Gulati and Jaiwal (1992 and 1994) where *Vigna radiata* callus responded differently with stress, salt type, and single salts (Na_2SO_4 and NaCl) were more toxic than a salt mixture (NaCl: KCl: Na_2SO_4).

Concerning sugars accumulation, generally, data revealed that salinity treatments caused higher accumulation of non-reducing and total sugars in the callus tissue of *V. faba* (Table 2).

Table 2: Effect of different salinity levels on reducing (R.S.), non-reducing (N.R.S) and total (T.S.) sugars concentrations (mg/g F.W.) of *Vicia faba* L. calli tissues derived from immature embryos.

Cultivar	Salt	Ppm	R.S.	N.R.S.	T.S.
Giza 5	NaCl	Control	10.1	17.9	28.0
		500	9.9	18.3	28.2
		1000	8.8	22.6	31.4
		2000	7.4	25.6	33.0
		4000	7.8	28.6	36.4
	$NaCl + CaCl_2$	500	10.1	18.7	28.8
		1000	9.6	19.8	29.4
		2000	8.5	24.1	32.6
		4000	8.0	25.4	33.4
Mean			8.9	22.3	31.2
Giza 461	NaCl	Control	10.1	16.9	27.0
		500	8.3	17.4	25.7
		1000	8.1	18.4	26.5
		2000	7.4	19.7	27.1
		4000	7.3	20.0	27.3
	$NaCl + CaCl_2$	500	9.7	18.3	28.0
		1000	9.5	19.1	28.6
		2000	9.0	20.5	29.5
		4000	8.7	21.4	30.1
Mean			8.7	19.1	27.8

In this respect, El-Shafey *et al.* (1994) found that non-reducing sugars was positively correlated with increasing salinity level in the culture medium of wheat calli. Strognov (1970) showed that the accumulation of non toxic substances such as sucrose, proline, organic acid, pigments and protein which have protective properties, are considered to be a protective adaptation and that survival of plant under salinity conditions depends upon the regulation of metabolic processes and the quantitative ratios between the protective and the toxic intermediates of metabolism. On the other hand, it has been suggested that the high concentrations of organic solutes in the cytoplasm can have the following roles; a contribution to the osmotic balance when electrolytes are lower in the cytoplasm than in the vacuole (Steward and Lee, 1974). Also the organic solutes can have a protective effect on enzymes in presence of high electrolytes in the cytoplasm (Pollard and Jones, 1979). The sugars as osmolytes enable plants to keep better water relation under stress condition. It has been found that sucrose can protect isolated chloroplasts against injury during desiccation (Santarius, 1973). Similar results were obtained by El shihy *et al.* (1994 a & b) working on *V. faba*, alfalfa and Egyptian clover.

Soluble phenols concentration in *V. faba* was rapidly increased as the salinity level increased in the growing media (Table 3). It was found that soluble phenols concentration increased almost 290 and 270% when the cultivar Giza 5 was treated with 4000 ppm NaCl and 4000 ppm NaCl + $CaCl_2$, respectively. The same treatment increased soluble phenols concentration in the cultivar Giza 461 by 240 and 180%.

Table 3: Effect of different salinity levels on soluble phenols (mg/g F.W.) of *Vicia faba* L. calli tissues derived from immature embryos.

Cultivar	Salt	Ppm	Phenols
Giza 5	NaCl	Control	1.12
		500	1.53
		1000	1.84
		2000	2.55
		4000	3.27
	$NaCl + CaCl_2$	500	1.38
		1000	1.68
		2000	2.00
		4000	3.06
Mean			2.05
Giza 461	NaCl	Control	1.90
		500	2.16
		1000	2.82
		2000	3.17
		4000	4.49
	$NaCl + CaCl_2$	500	2.05
		1000	2.43
		2000	2.51
		4000	3.48
Mean			2.78

Total amino acids analysis of *V. faba* L.. callus tissue (Giza 5 and Giza 461) derived from immature embryos showed that the major amino acids components were glutamate, aspartate, alanine, leucine and lycine (Tables 4-7). Proline percentage was increased as the salinity levels increased in both cultivars Giza 5 and Giza 461callus tissue. However, the percentage of proline was higher in the callus tissue of the cultivar Giza 461 as compared to Giza 5 cultivar. In the cultivar Giza 461, an increase of 80% was recorded in proline concentration, when the callus tissue was kept on 4000 ppm NaCl as compared to its control treatment. Whereas, this increase was almost 60% in the callus tissue of the cultivar Giza 5. When the salt mixture of both NaCl and $CaCl_2$ in the ratio of 3:1 was used, proline concentration was similar to that of using NaCl alone, while it was increased in Giza 5 cultivar. Similar results were obtained by Sumaryati *et al.* (1992) on tobacco and Vajrabhaya *et al.* (1989) on rice. Stewart (1972) emphasized that proline accumulation may be due to the influence of NaCl stress on the activity of certain enzymes. He also reported that proline accumulation could be eliminated by hydration or converted to protein through carbohydrates metabolism. Proline may play a role as a compatible cytoplasmic solute of low molecular weight existing in the cytoplasm apparently counteracts the osmotic potential of the vacuole state (Dove, 1987).

Primary experiments were conducted to establish a transformation system for *V. faba* L. by using the *Agrobacterium* mediated transformation. The binary vector pBI121, which is carrying the NPT II-GUS cassette, was used in all the transformation experiments. Kanamycin used at different concentrations showed a significant negative correlation inhibition of *V. faba* L. callus tissue derived from immature embryos; i.e. as the kanamycin concentration increased, survival percentage of the callus tissue significantly decreased (Table 8). These results indicated that 75 mg/l kanamycin is enough to kill almost 98% of the tissue and it can be used as a selectable marker. It was observed that when the succinopine-chracterized strain EHA105 was used, GUS transient expression was significantly higher, almost two folds in the case of cultivar Giza 5 (6.1% transformation) as compared to the nopaline strain LBA4404 (Table 9). Generally, the mean average of the transformation percentage was higher (about 4 folds) in the callus tissue of the cultivar Giza 5 as compared to that of the cultivar. Giza 461.

Table 8: Effect of different levels of kanamycin (mg/l) on the survival percentage of *Vicia faba* L. callus tissue derived from immature tissues.

*Kanamycin level	Survival percentage		Mean
	Giza 5	Giza 461	
Control (zero)	90.0	93.3	91.7
25	43.3	53.3	48.3
50	10.0	13.3	11.7
75	2.0	3.3	2.7
100	0.0	0.0	0.0
Mean	29.1	32.6	

*callus tissues were sub-cultured on the media containing kanamycin for 3 weeks.
L.S.D. (0.05): Cultivar=N.S. treatments=12.4

Table 9: Effect of interaction between *V. faba* L. cultivars Giza 5 and Giza 461 and *A. tumefaciens* strains on the percentage of transformation of the callus tissue derived from immature embryos .

Cultivar	*Agrobacterium* strain	% Transformation
Giza 5	EHA105	6.10
	LBA4404	3.20
	EHA105 + C58	1.40
	LBA105 + C58	2.20
Mean		3.23
Giza 461	EHA105	1.00
	LBA4404	0.60
	EHA105 + C58	1.00
	LBA105 + C58	0.60
Mean		0.80

Also, *Agrobacterium* mediated gene transfer was achieved in Egyptian clover (legume) using *A. rhizogenes* (Aly and Tanaka, 1995). Plants of birdsfoot trefoil (*Lotus corniculatus*) and alfalfa (*Medicago sativa*) and tobacco (*Nicotiana tabacum*) were transformed by Agrobacterium with binary vectors harbouring genes that code either for wild-type and γ-zein:KDEL, or for β-zein:KDEL (Bellucci1 *et al*., 2001). The maize seed storage proteins γ-zein and β-zein are rich in sulphur amino acids, to improve alfalfa and birdsfoot trefoil forage quality. Significant levels of zeins were detected in leaves of birdsfoot trefoil transformants, ranging up to 0.055% and 0.30% of total extractable protein for γ-zein and β-zein:KDEL, respectively. In leaves of alfalfa, the γ-zein:KDEL expression level was up to 0.026% of the total extractable protein. From a F1 population of transgenic tobacco, a plant was selected in which the amount of zeins (γ-zein:KDEL plus β-zein:KDEL) accounted for 1.1% of the total extractable protein.

Thus, the established callus formation as well as transformation systems for *V. faba* may offer a solution for gene transfer by adding new desirable genes such as zein gene which plays a role in improving the

nutritional values of seed storage protein. Also, osmo-protectant genes can be added to increase resistance of plants to draught and salinity. Resistance to herbicides or diseases can be added through gene transfer and consequently improving the qualitative and quantitative plant productivity.

REFERENCES

Abd Alla, M. H.; A.E. El-Enany; A. M. Hamada and A. M. Abdel Wahab (2001). Element distribution in faba bean root nodules under salinity and its effects on growth, nodulation and nitrogen fixation. Rostlinna-Vyroba., 47(9): 399-404.

Aly, M. A. M. and N. Tanaka (1995). Transformation of berseem (*Trifolium alexandrinum* L.) by *Agrobacterium rhizogenes*. Egypt. J. Genet. Cytol, 24: 233-242.

Aly, M. A. M. and M. M. Saker (1998). Somaclonal variations detected in somatic embryos-derived faba bean plants by RAPD-based fingerprinting. Proc. 3rd Arab Conf. on Modern Biotechnology and its Applications. Cairo, Egypt. Dec. 14-17. pp 697-707.

A.O.A.C. (1975). Official Methods of Analysis of the Association of Official Agricultural Chemists. Washington, D.C. 12th Ed.

Anderson, R.; D. Annette and N. Jackson (1977). Ion-exchange chromatographic study of amino acid degradation during hydrolysis of avoin protein. J. Chromatog., 135:447-454.

Bellucci1, M.; A. Alpini1 and S. Arcioni1 (2001). Zein accumulation in forage species (*Lotus corniculatus* and *Medicago sativa*) and co-expression of the γ-zein:KDEL and β-zein:KDEL polypeptides in tobacco leaf. Plant Cell Rep., 20:33-38.

Bernaerts, M. J. and J. De Ley (1960). Microbial formation and preparation of 3-ketoglycosides from disaccharides. Gen. Microbiol., 42:572-606.

Bernaerts, M. J. and J. De Ley (1963). A biochemical test for crown gall bacteria. Nature ,197:406-407.

Bressan, R. A.; A. K. Handa; S. Handa and P. M. Hasegawa (1982). Growth and water relation of cultured tomato cells after adjustment to low external water potentials. Plant Physiol., 70:1303-1309.

Caplan, A. B.; B. Claes; R. Dekeyser and M. Van Montagu (1990). Salinity and drought stress in rice. In: The Impact Of Biotechnology In Agriculture, vol 8. Sangwan, R. S.and Sangwan-Norreel B. S. (eds.). Kluwer Academic Publ., The Netherlands. Pp391-402.

Ditta, G.; S. Stanfield; D. Corbin and D. R. Helinksi (1980). Broad host range DNA cloning system for gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti.* Proc. Natl. Acad. Sci., U.S.A. 77:7347-7351.

Dov, P. (1987). Salt tolerance and crop production. Ann. Rev. Phytopathol. 25:271-292.

El-Shafey, Y. H.; O. M. El shihy and S. M. Salem (1994). Production of salt tolerant line through wheat calli treatment with fast neutron. Bull. Fac. Agric., Cairo Univ., 45(1):1-31.

El shihy, O. M.; S. M. Salem and M. K. Khalil (1994a). Production of salt tolerant line of *Vicia faba* induced by protoplast irradiation with fast neutrons and gamma rays. Egypt. J. Appl. Sci., 9(4):393-418.

El shihy, O. M.; S. M. Salem and M. K. Khalil (1994b). Production of salt tolerant line of alfalfa and Egyptian clover through protoplasts fast neutrons irradiation treatment. Egypt. J. Appl. Sci., 9(6):129-155.

Emam, S. G; H. A. Nafee; M. A. M. Aly and M. H. Rashad (2000). Evaluation of salt tolerance in tissue culture of mungbean (*Vigna radiata* L.) using randomly amplified polymorphic DNA (RAPD). Egypt. J. Genet. Cytol., 29 (2): 149-169.

Forrest, G. L. and D. S. Bendall (1969). The distribution of polyphenols in the tea plant *Camilia seninsis* L. Biochem. J., 113:741-755.

Gulati, A. and P.K. Jaiwal (1992). Comparative salt responses of callus cultures of *Vigna radiata* to various osmotic and ionic stresses. J. Plant Physiol., 141:120-124.

Gulati, A. and P. K. Jaiwal (1994). Selection and characterization of *Vigna radiata* callus line resistant to NaCl, KCl and Na2SO4. Biol. Plant., 36:21-28.

Jefferson, R. A.; T. A. Kavanagh and M. Bevean (1987). GUS Fusion:β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., 6:3901-3907.

Khalafalla, M.M. and K. Hattori (2000). Ethylene inhibitors enhance *in vitro* root formation on faba bean shoots regenerated on medium containing thidiazuron. Plant Growth Regulation, 32(1):59-63.

Murashige, T. and P. Skoog (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15:473-497.

Pollard, A. and R. G. Jones (1979). Enzyme activities in concentrated solutions of glycine-betaine and other solutes. Planta, 144:291-298.

Saker, M. M.; A. A. Reda; A.E. Mohamed; M. K. El-Bahr and A. A. El-Kazzaz (1999). Induction of embryogenic callus and plant regeneration in faba bean. Egypt. J. Agron., 21:87-97.

Sambrook, J.; E. F. Fritsch and T. Maniatis (1989). Molecular Cloning: A Laboratory Manual, Cold Spring Habor Laboratory, Cold Spring Harbor, T.N., U.S.A., pp 306-309.

Santarius, K. A. (1973). The protective effect of sugars on chloroplast membranes during temperature and water stress and its relationship to frost, desiccation and heat resistance. Planta, 113:105-114.

Snedecor, G.W. and W.G. Cochran (1980). Statistical Methods 7th ed. Iowa State Univ. Press, Ames., Iowa, U.S.A.

Strognov, B. (1970). Structure and function of plant cells under salinity conditions. Nauka Publishing House, Moscow pp 233-270.

Steward, C. R. and J. A. Lee (1974). The role of proline accumulation in halophytes. Planta, 120:279-289.

Stewart, C. R. (1972). Effects of proline and carbohydrates on the metabolism of exogenous proline by excised bean leaves in the dark. Plant Physiol., 50:551-555.

Sumaryati, S.; I. Negruti and M. Jacobs (1992). Characterization and regeneration of salt and water stress mutants from protoplast culture of *Nicotiana plumbaginnfolia* (Viviai). Theor. Appl. Genet., 83:613-619.

Tal, M. (1984). Physiological genetics of salt resistance in higher plants. Studies on the level of the whole plant and isolated organs, tissue and cells. In: Salinity Tolerance In Plants, Strategies For Crop Improvement, Staples, R. and Toenniessen, C. (Eds.), John Wiley, New York. Pp 269-276.

Vajrabhaya, M.; T. Thanapaisal and T. Vajrabhaya (1989). Development of salt tolerant lines of KDML and LPT rice cultivars through tissue culture. Plant Cell Rep., 8:411-414.

تأثير الملوحة والتحوير الوراثى عتى نسيج الكالس لنبات الفول البلدى

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تم استخدام الأجنة غير الناضجة لنبات الفول البلدى صنفى جيزة ٥ و جيزة ٤٦١ للحصول على نسيج الكالس لأستخدامة لدراسة تأثير الملوحة وتحويرة وراثيا باستخدام بكتريا الأجروباكتريم المحتوية عتى البلازميد pBI121. لوحظ نقص كتل من الوزن الطازج والوزن الجاف لنسيج الكالس لنبات الفول البلدى بزيادة مستوى الملوحة. أما عند استخدام مخلوط الأملاح المكون من كلوريد الصوديوم + كلوريد الكالسيوم فقد كانت حدة النقص فى كل من الوزن الطازج والوزن الجاف أقل من حدتة فى حالة استخدام ملح كلوريد الصوديوم منفردا. وعموما فان صنف الفول البلدى جيزة ٥ أدى الى الحصول على وزن طازج وجاف اكبر بالمقارنة بصنف جيزة ٤٦١. أدت زيادة مستوى الملوحة الى زيادة تركيز كل من السكريات الكلية ومواد الفينولية الذائبة والبرولين فى نسيج الكالس لنبات الفول البلدى. زاد تركيز الحامض الأمينى برولين فى نسيج الكالس لنبات الفول البلدى وكانت تلك الزيادة أكبر قيمة فى صنف جيزة ٤٦١ مقارنة بصنف جيزة ٥. كان نسيج الكالس لنبات الفول البلدى حساسا لاضافة المضاد الحيوى kanamycin فى البيئة المغذية حيث أدى تركيز ٧٥ مجالتر الى موت حوالى ٩٨% من نسيج الكالس وكانت نسبة النجاة ٢%. كانت نسبة التحوير الوراثى لنسيج الكالس لنبات الفول البلدى صنف جيزة ٥ أعلى من نظيرة جيزة ٤٦١. وبناءا على ذلك فان النجاح فى الحصول نسيج الكالس لنبات الفول البلدى والنجاح فى تحويرة وراثيا فانة يمكن من خلالة اضافة جينات وراثية مرغوبة لتحسين القيمة الغذائية لبذور الفول البلدى. كذلك يمكن زيادة مقاومة تلك النباتات للامراض وبعض مبيدات الحشائش وبالتبعية تحسين الانتاجية الاقتصادية لنبات الفول البلدى.

Table 4: Effect of different salinity levels as NaCl on total amino acids concentration (mg/g D.W.) and percentage in callus tissue derived from immature embryos of Giza 5 cultivar of *Vicia faba* L.

Amino acids	Salinity level (ppm)									
	Control		500		1000		2000		4000	
	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%
Aspartate	16.84	28.9	10.02	27.2	8.92	18.5	7.15	16.1	5.79	13.2
Threonine	1.93	3.3	1.54	4.20	4.33	9.0	2.63	5.9	1.27	2.9
Serine	1.89	3.2	1.81	4.9	4.86	10.1	3.22	7.2	1.37	3.1
Glutamate	7.88	13.5	4.40	11.4	4.75	9.8	4.26	9.5	4.87	11.1
Proline	0.89	1.5	0.61	1.6	0.89	1.8	0.89	2.0	1.10	2.5
Glycine	2.92	5.0	1.84	5.0	2.60	5.5	1.80	4.0	1.91	4.3
Alanine	4.52	7.7	2.42	6.6	2.85	5.9	2.86	6.4	3.46	7.9
Valine	3.52	6.0	3.39	6.5	3.17	6.6	3.59	8.0	4.23	9.6
Isoleucine	2.25	3.9	1.75	4.7	2.67	5.6	1.97	4.4	2.53	5.8
Leucine	3.31	5.7	2.48	6.7	3.18	6.6	3.54	7.9	3.60	8.2
Tyrosine	1.79	3.1	1.36	3.7	1.25	2.6	2.15	4.8	2.49	5.7
Phenylalanine	1.98	3.4	1.46	4.0	2.37	4.9	2.96	6.6	3.05	6.9
Histidine	1.53	2.6	0.91	2.5	1.09	2.3	1.25	2.8	1.44	3.3
Lysine	4.84	8.3	1.91	5.2	2.10	4.3	2.78	6.2	3.09	7.0
Arginine	2.12	3.6	2.15	5.8	3.11	6.5	3.68	8.2	3.75	8.5
Total	58.19		36.87		48.14		44.82		43.95	

Table 5: Effect of different salinity levels as NaCl and $CaCl_2$ in the ratio of 3:1 on total amino acids concentration (mg/g D.W.) and percentage in callus tissue derived from immature embryos of Giza 5 cultivar of *Vicia faba* L.

Amino acids	Salinity level (ppm)									
	Control		500		1000		2000		4000	
	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%
Aspartate	16.84	28.9	7.04	34.1	9.31	25.7	8.33	19.5	10.19	30.4
Threonine	1.93	3.3	0.85	4.1	1.64	4.5	2.24	5.2	1.24	3.7
Serine	1.89	3.2	0.99	4.8	2.11	5.8	3.05	7.1	1.61	4.8
Glutamate	7.88	13.5	1.92	9.3	4.70	12.9	5.54	13.0	3.00	8.9
Proline	0.89	1.5	0.35	1.6	0.64	1.8	0.96	2.3	0.98	2.9
Glycine	2.92	5.0	0.62	3.0	2.04	5.6	2.02	4.8	3.79	11.3
Alanine	4.52	7.7	1.17	5.7	1.82	5.1	2.61	6.1	1.35	4.0
Valine	3.52	6.0	1.33	6.4	2.37	6.5	3.13	7.3	1.60	4.7
Isoleucine	2.25	3.9	0.99	4.8	1.59	4.4	1.85	4.3	1.00	3.0
Leucine	3.31	5.7	1.27	6.1	2.47	6.8	2.87	6.7	2.02	6.0
Tyrosine	1.79	3.1	0.79	3.8	1.47	4.0	1.93	4.5	1.89	5.5
Phenylalanine	1.98	3.4	0.72	3.5	1.54	4.3	1.77	4.1	1.00	3.0
Histidine	1.53	2.6	0.53	2.6	1.19	3.3	1.01	2.5	0.84	2.5
Lysine	4.84	8.3	1.01	4.9	2.18	6.0	2.06	4.9	1.48	4.4
Arginine	2.12	3.6	1.09	5.3	1.76	4.8	3.28	7.7	1.64	4.9
Total	58.19		20.67		36.30		42.65		33.54	

Table 6: Effect of different salinity levels as NaCl on total amino acids concentration (mg/g D.W.) and percentage in callus tissue derived from immature embryos of Giza 461 cultivar of *Vicia faba* L.

Amino acids	Salinity level (ppm)									
	Control		500		1000		2000		4000	
	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%
Aspartate	11.2	20.8	4.83	10.5	4.92	12.3	7.46	24.0	8.07	28.9
Threonine	1.2	2.6	2.45	5.3	1.73	4.3	1.43	4.5	1.16	4.1
Serine	1.7	2.7	2.40	5.2	2.17	5.4	1.50	4.8	1.39	5.0
Glutamate	14.62	25.6	9.46	20.5	8.57	21.5	2.57	8.2	2.95	10.5
Proline	1.01	1.8	0.89	1.9	0.99	2.6	0.99	3.1	0.90	3.2
Glycine	1.99	3.6	2.32	5.0	1.86	4.6	2.06	6.5	1.93	6.9
Alanine	4.19	7.4	3.58	7.5	3.17	7.9	2.06	6.5	1.93	6.9
Valine	2.70	4.7	3.33	7.2	2.75	6.9	2.41	7.6	1.60	5.7
Isoleucine	1.72	3.6	1.90	4.1	1.70	4.3	1.83	5.8	1.32	4.7
Leucine	4.27	7.5	3.10	6.7	2.77	6.9	2.42	7.6	1.65	6.0
Tyrosine	1.63	2.8	1.65	3.6	1.92	4.8	1.16	3.7	0.95	3.7
Phenylalanine	2.95	5.2	1.82	3.9	1.75	4.4	1.34	4.3	1.04	3.7
Histidine	1.09	1.9	1.80	3.9	1.22	3.1	0.75	2.4	0.69	2.5
Lysine	3.75	6.5	2.53	5.4	2.26	5.7	1.56	5.0	1.18	4.2
Arginine	2.35	4.1	3.69	8.0	2.11	5.3	1.87	6.0	1.63	5.8
Total	52.28		46.15		39.89		31.42		27.96	

Table 7: Effect of different salinity levels as NaCl and $CaCl_2$ in the ratio of 3:1 on total amino acids concentration (mg/g D.W.) and percentage in callus tissue derived from immature embryos of Giza 461 cultivar of *Vicia faba* L.

Amino acids	Salinity level (ppm)									
	Control		500		1000		2000		4000	
	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%	mg/g F.W.	%
Aspartate	11.2	20.8	3.27	9.7	3.94	11.2	5.65	14.8	10.04	29.7
Threonine	1.2	2.6	1.40	4.1	1.61	4.6	1.67	4.4	1.24	3.7
Serine	1.7	2.7	2.70	8.0	1.97	5.6	2.20	5.7	1.51	4.5
Glutamate	14.62	25.6	7.90	23.5	8.05	22.9	8.70	22.8	3.10	9.2
Proline	1.01	1.8	0.90	2.6	1.00	2.8	1.10	2.9	1.05	3.1
Glycine	1.99	3.6	1.51	4.5	1.79	5.1	1.84	4.8	3.79	11.2
Alanine	4.19	7.4	1.97	5.8	2.44	6.9	2.36	5.9	1.41	4.2
Valine	2.70	4.7	2.06	6.1	2.29	6.5	2.23	4.9	1.84	5.4
Isoleucine	1.72	3.6	1.42	4.2	1.53	4.3	1.48	3.9	1.38	4.1
Leucine	4.27	7.5	3.30	9.8	2.60	7.4	2.94	7.7	1.94	5.8
Tyrosine	1.63	2.8	1.33	3.9	1.06	3.0	1.50	3.9	1.09	3.2
Phenylalanine	2.95	5.2	1.40	4.1	1.58	4.5	1.30	3.4	1.23	3.6
Histidine	1.09	1.9	0.92	2.7	1.15	3.3	0.99	2.6	0.80	2.4
Lysine	3.75	6.5	1.83	5.4	2.0	5.7	2.13	5.6	1.61	4.8
Arginine	2.35	4.1	1.80	5.3	2.18	6.2	2.11	5.5	1.72	5.1
Total	52.28		33.71		35.19		38.20		33.75	