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Regeneration of (*Vicia faba* L.) Cultivars from Mature Seed Cotyledons

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Abstract: The objective of this research was to develop recipient system of faba bean cultivars (*Vicia faba* L.) and to obtain 686 Sudan; 1059 Morocco; NEB 367; ILB 752; 724 Norway; 1083 Australia; Wasa soramame and Cairo 241 plants through direct regeneration *in vitro*. The morphogenetic of seeds in selected faba bean cultivars (*Vicia faba* L.) was realized via one way: direct regeneration of plants from mature cotyledons. The cotyledons with ten combinations of growth regulators: (Kin.: 6-Furfurylaminopurine N6-Furfuryladenine $C_{10}H_9N_5O$; BAP: 6-Benzylamino-9-(2-tetrahydropyranyl)-9H-purine $C_{17}H_{19}N_5O$; TDZ: N-phenyl-N'-1,2,3-thiadiazol-5-ylurea; NAA: α-Naphthaleneacetic acid 1-Naphthylacetic acid $C_{12}H_{10}O_2$ and IBA 4-(3-Indolyl) butanoic acid $C_{12}H_{13}NO_2$) were compared. Effect of growth regulator treatments and cotyledon of cultivars were generally small within inter-cultivars, but callus and shoot formation different intra-cultivars. The other cultivars generally did not produce calli. The developed *in vitro* systems of faba bean cultivars (*Vicia faba* L.) plants approve us to use them for different purposes of plant biotechnology: genetic transformation, multiplication and conservation *in vitro* of valuable (*Vicia faba* L.).

Key words: 686 Sudan, 1059 Morocco, NEB 367, ILB 752, 724 Norway, 1083 Australia, Wasa

INTRODUCTION

Adventitious shoot regeneration from cotyledons of faba bean (*Vicia faba* L.) is the chosen route for obtaining plants through genetic transformation (Duc *et al*., 1999; Jelenić *et al*., 2000; Mutasim and Hattori, 1999) and for obtaining plants through genetic transformation of tetraploid parental lines used to breed seedless faba bean (Torres *et al*., 1993; Fuchs and Schubert, 1995; Maria del *et al*., 2000; Miranda *et al*., 2003). Several types of cotyledonary explants have been used for adventitious shoot regeneration of legume plants (Mante *et al*., 1989; Venketeswaran *et al*., 1990; Knittel *et al*., 1991; Ceriani *et al*., 1992; Gray, 2000; Corredoir *et al*., 2002). Regeneration has also been reported from cotyledons of mature seed of *Albizia lebbeck* and (*Psophocarpus tetragonolobus* (L.) DC) by Klu *et al*. (1997), Mamun *et al*. (2004). Virus-resistant transgenic soya bean plants were regenerated from mature seeds using *Agrobacterium*-mediated transformation (Napoli *et al*., 1990; Matzke and Matzke, 1995; Gellatly and Lefebvre, 1990). Mature seeds are convenient explant source although they do not allow a specific genotype to be manipulated. Few faba bean cultivars are proved in *in vitro* culture, so *phyllo*-material is recalcitrant for regeneration and subsesequent transformation of cultivars. However, regeneration and transformation from mature cotyledons represents a convenient tool to introduce novel genes into the flowering faba bean germplasm, and would also be ideal for seed-propagated cultivars that are used as rootstocks. Faba bean culture release considerable amounts of phenol compounds (Prithiviraj *et al*., 2000; Zheng-jun *et al*., 2002). which inhibit the callus growth and make the plants regeneration impossible. Application of explant culture was established on modified Murashige and Skoog (MS) medium containing 250 mg L^{-1} of casein hydrolisate and 100 mg L^{-1} of myo-inositole may one of methods to prevent partly the oxidation of phenols. We report here an attempt to develop a regeneration procedure for dry faba bean which utilizes mature cotyledon and to show that the new shoot development appears to be of an adventitious shoot.

The aim of the study was to develop an efficient shoot regeneration and transformation of *Vicia faba* cultivar seeds by introducing novel genes for disease and pest resistance. This will enable usage of developed plants as parental material in a breeding program. We inspected the regeneration of mature cotyledons from eight faba bean cultivars all grown predominatly for their protein traits and are readily cotyledon propagated. We examined the responses of explants of the mature cotyledons to combinations of growth regulators.

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MATERIALS AND METHODS

Inbreds: Callus and shoot induction capabilities of 8 diverse cultivars, genetic stocks of faba bean were tested using MS medium described above, *V. faba*: 686 Sudan; 1059 Morocco; NEB 367; ILB 752; 724 Norway; 1083 Australia; Wasa soramame and Cairo 241. The cultivar seeds were collected from the department of plant science, university of Adelaide, Australia, (seventh and eighth), collected Matsunaga Shubyou Seed Company Ltd. Japan and Departement of Plant Science, National Research Centre, Egypt, respectively.

Mature seeds were chosen because a consistent, year - round supply of many cultivars is available. They are genetically diverse and distantly related. All of these lines are diploid (2n = 12).

Plant material, culture method and explants: Mature seeds were surface sterilized in 70% (v/v) 2 CH_3CH_2OH (ethanol) for 1 min. followed by 10 min. immersion in 3% (v/v) NaCI (sodium hypochlorite) solution with 2 drops of Tween-20 β per 100 mL and washed thoroughly with Sterile Distilled Water (SDW). After soaking for 8 h in SDW, finally the seed were rinsed incessantly with (SDW) and seed coats were removed and the two cotyledons separated. Embryonic axis and the immediate tissues surrounding the embryonic axis, were excised with a scalpel and discarded.

Effects of cultivars and growth regulators on regeneration : Callus and emerging shoot were compared by two types of explants: Explants types tested included distal or proximal whole-cotyledons and distal or proximal half of cotyledons. The whole cotyledons were excised with a scalpel and used as an explant and the half cotyledons discarded were dissected transversely and the explants (2/vessel) were cultured abaxial side down in deep-sided petri dishes (9×1.5 cm) containing 25 mL of medium. For each treatment 16 distal and proximal whole-cotyledon explants and 32 distal and proximal half-cotyledons were used. This experiment was repeated twice and the explants were placed in the regeneration medium: Murashige and Skoog's (MS) macro-and micro-salts, with sucrose increased to 6%, solidified with 8 g L^{-1} agar powder (INA BA-30) and supplemented the combinations were as follows, with all combinations in mg L^{-1}: (1) 1.6 Kin. + 2.3 IBA; (2) 4.3 Kin. + 2.3 IBA; (3) 4.3 Kin. + 4.1 IBA; (4) 4.3 Kin. + 2.3 IBA transferred to 1.6 Kin. + 3.1 BAP; (5) 4.3 Kin. + 4.1 IBA transferred to 1.6 Kin. + 3.1 BAP; (6) 4.3 Kin. + 4.1 IBA changed to 1.6 Kin. + 5.2 TDZ; (7) 3.1 BAP + 3.5 NAA (8) 3.1 BAP + 3.5 NAA changed to 1.6 Kin. + 3.1 BAP; (9) 5.2 TDZ + 3.5 NAA changed to 1.6 Kin. + 3.1 BAP + 5.2 TDZ; (10) 1 BAP + 7.2 NAA changed to 4.3 Kin. + 1 BAP and then to 3.5 NAA + 4 BAP. Transfers to new medium were made every 3 weeks. One replicate consisted of 80 dishes (ten growth regulator combinations for each of eight cultivars). The cotyledons of NEB 367 and 724 Norway induced no callus or shoots and were not included in the data analysis. Percentage of explants inducing callus per dishes were recorded. After 10 to 15 weeks, percentage of explants emerging shoot were also recorded. The medium was adjusted to 5.75 with KOH or HCl and autoclaved at 121°C for 20 min before pouring into deep-sided sterile petri dishes (9×1.5 cm). Explants were either cultured in the dark at 25°C for 7 days: before being exposed to light, or were transferred directly to light without an initial dark period. Unless otherwise described, cultures were maintained at 25°C for 16 h photoperiod of cool-white fluorescence light of (30 μ mol m^{-2} s^{-1}).

Histological sections: Sixty structures derived from six cultivars were evaluated histologically. All of these formed on MS supplemented ten combinations of growth regulators. Histology was performed according to Mendoza *et al.* (1993) with some modifications. Samples were fixed in FAA (formalin, acetic acid, 70% ethano 15:5:90) dehydrated in a graded acetone series (30, 50, 70, 90, 100%) at 30 min intervals, embeded in resin containing glycol merthacrylate (as main component) and sectioned at 6 μm thickness on a rotary microtome. Sections were stained with toluidine blue (0.05%, 2 min) observed under a light microscope.

Statistical analysis: Data analysis was carried out using statistica for Windows, release 5.1. All the treatments were repeated twice and the standard deviation was calculated. Data on callus induction and shoot formations were statistically analysed using a completely randomized block design and means were evaluated at p = 0.05 level of significance using Duncan's new multiple range test.

RESULTS

The percentage of cotyledons that induced callus did not differ significantly among growth regulator treatments, but there was a significant difference among cultivars, with Wasa soramame and Cairo 241 producing significantly more callus than other cultivars (Table 2 and 3). The type cotyledon explants effect and type cotyledon explants by cultivars interaction for callus formation were also significant. When cultivars from the whole cotyledons were compared ILB 752; 686 Sudan and 1083 Australia all induced significantly more callus than 1059 Morocco. Data for the half cotyledon demonstrated that ILB 752 similar from 1059 Morocco, but 686 Sudan

Table 1: Effect of Kinetin, plant growth regulators and dark on percentage of adventitious shoots development from mature cotyledon of six faba bean cultivars cultured 12-15 weeks MS medium

		Growth regulators (mg L^{-1})					Dark treatment	*Vicia faba* cultivars						
Treatments	Transfers	Kin.	BAP	TDZ	NAA	IBA	(Days)	WS.	Aus.	Su.	Mor.	IL.	Ca.	Means
1	1M	1.6	0.0	0.0	0.0	2.3	-	39.5	13.5	0.0	4.1	0.00	19.5	12.70
		1.6	0.0	0.0	0.0	2.3	7	38.4	9.4	0.0	2.1	1.70	20.7	12.10
2	1M	4.3	0.0	0.0	0.0	2.3	-	17.1	12.1	0.0	0.0	2.90	20.5	8.70
		4.3	0.0	0.0	0.0	2.3	7	16.4	10.8	0.0	0.0	1.80	21.7	8.45
3	1M	4.3	0.0	0.0	0.0	4.1	-	30.5	10.2	1.5	3.6	0.00	8.4	9.00
		4.3	0.0	0.0	0.0	4.1	7	33.4	5.2	2.1	5.2	0.00	9.1	9.10
4	1M	4.3	0.0	0.0	0.0	2.3	-	41.2	21.4	1.9	0.0	0.00	13.7	13.00
	2M	1.6	3.1	0.0	0.0	0.0	-							
	1M	4.3	0.0	0.0	0.0	2.3	7	38.1	18.4	0.0	9.4	7.10	15.7	14.70
	2M	1.6	3.1	0.0	0.0	0.0	7							
5	1M	4.3	0.0	0.0	0.0	4.1	-	21.7	18.1	0.0	4.7	5.30	4.8	9.10
	2M	1.6	3.1	0.0	0.0	0.0	-							
	1M	4.3	0.0	0.0	0.0	4.1	7	27.4	9.4	0.0	9.4	3.70	23.1	12.10
	2M	1.6	3.1	0.0	0.0	0.0	7							
6	1M	4.3	0.0	0.0	0.0	4.1	-	48.7	15.7	3.4	10.1	0.00	40.6	19.80
	2M	1.6	0.0	5.2	0.0	0.0	-							
	1M	4.3	0.0	0.0	0.0	4.1	7	36.9	21.4	1.1	11.2	4.70	38.1	18.90
	2M	1.6	0.0	5.2	0.0	0.0	7							
7	1M	0.0	3.1	0.0	3.5	0.0	-	2.1	5.7	0.0	8.4	0.00	4.8	3.50
	1M	0.0	3.1	0.0	3.5	0.0	7	0.0	2.1	1.7	4.5	4.20	1.3	2.30
8	1M	0.0	3.1	0.0	3.5	0.0	-	37.5		0.0			42.4	13.30
	2M	1.6	3.1	0.0	0.0	0.0	-							
	1M	0.0	3.1	0.0	3.5	0.0	7	33.1	19.3	0.0	10.7	9.40	21.5	15.60
	2M	1.6	3.1	0.0	0.0	0.0	7							
9	1M	0.0	0.0	5.2	3.5	0.0	-	5.3	0.0	0.0	2.4	0.00	7.8	2.50
	2M	1.6	3.1	5.2	0.0	0.0	-							
	1M	0.0	3.1	5.2	3.5	0.0	7	14.7	1.5	1.1	3.1	7.40	11.2	6.50
	2M	1.6	3.1	5.2	0.0	0.0	7							
10	1M	0.0	1.0	0.0	7.2	0.0	-	40.2	3.8	1.5	2.9	12.40	23.7	14.00
	2M	4.3	1.0	0.0	0.0	0.0	-							
	3M	0.0	0.0	4.0	3.5	0.0	-							
	1M	0.0	2.1	0.0	7.2	0.0	7	29.4	7.6	3.5	8.5	9.70	18.5	12.80
	2M	4.3	3.5	0.0	0.0	0.0	7							
	3M	0.0	0.0	4.0	3.5	0.0	7							
							Means	55.1	20.5	1.7	10.0	7.03	36.7	

Cultivar abbreviations: WS. = Wasa soramame, Aus. = 1083 Australia, Su. = 686 Sudan, Mor. = 1059 Morocco, IL. = ILB 752 and Ca. = Cairo 241

and 1083 Australia produced significantly less callus than did ILB 752 and 1059 Morocco.

The percentage of cotyledons that produced shoots differed significantly among cultivars and among growth regulator treatment and interactions between cultivars and treatment and between cultivars and cotyledon types were significant, as was as a three-way interaction among cultivars, treatment, and cotyledon types (Table 2 and 3). Treatments 1 and 5 produced most shoots (12.7, 9.1 and 12.1%, respectively for light/dark) across all cultivars (Table 1). Means for cotyledon types across growth regulators treatments differed significantly for *Vicia faba* cv. Cairo 241 and *Vicia faba cv.* Wasa soramame (Table 1).

The percentage of mature cotyledons with shoots differend significantly among the cultivars over the growth regulator treatments (Table 1 and 2) and Wasa soramame produced the most shoots followed by Cairo 241 although these two cultivars like significantly. Only on a combination of 3.1 mg L^{-1} BAP + 3.5 mg L^{-1} NAA (Treatment 8 in dark) did 1083 Australia significantly than Wasa soramame (Table 1). ILB 752 usually produced litter plantlets than did Wasa soramame and 1083 Australia, but more than 1059 Morocco; 686 Sudan and 1083 Australia all induced significantly more callus than did 1059 Morocco (Table 2). In treatment 10 (1 mg L^{-1} BAP + 7.2 mg L^{-1} NAA changed to 4.3 mg L^{-1} Kin. + 1 mg L^{-1} BAP and then to 3.5 mg L^{-1} NAA + 4 mg L^{-1} BAP) was there no significant difference among cultivars and all cultivars did not well on this combination of growth regulators (Table 1). Wasa soramame cotyledons produced significantly more plantlets on growth regulator combinations with IBA (Treatments 1-6) than on those not with IBA (Treatments 7-10) (Table 1 and 3). This style was also showed in Cairo 241, although differences were not significant in this cultivars (Table 1).

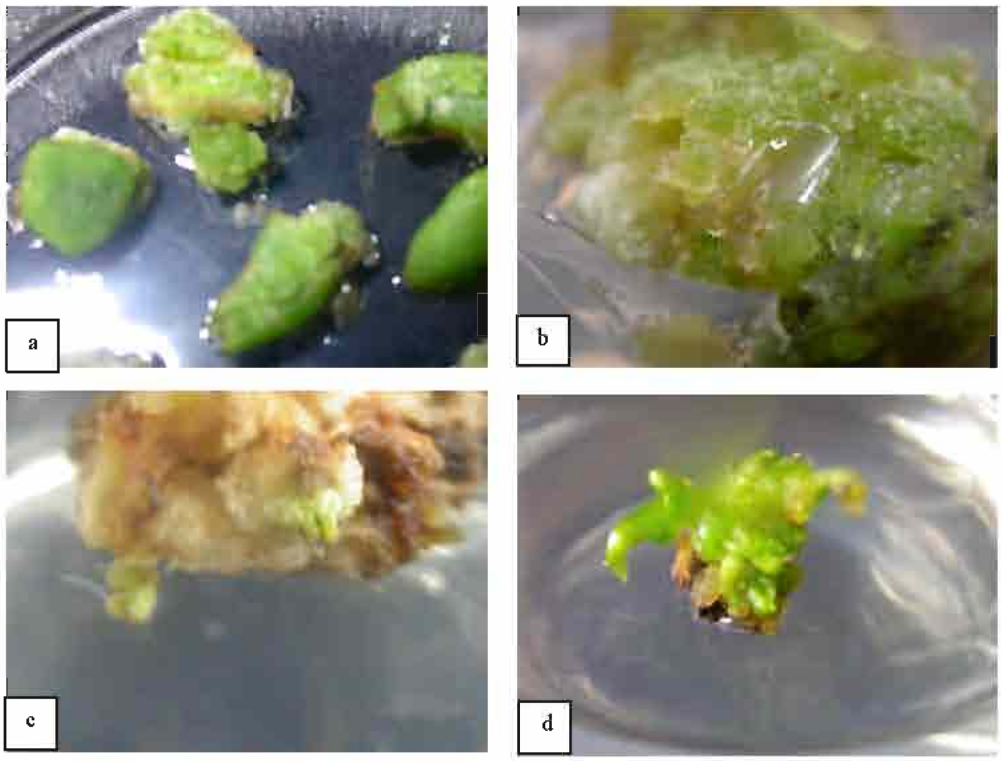

Fig. 1: Plant regeneration via somatic embryogenesis. a) Two type cotyledons derived microculli, b) compact somatic embryogenesis callus on MS/ following regeneration medium (Kin., BAP, TDZ, NAA and IBA) emerging, c) developed of dark green and compact calluses induced in Kin, TDZ and NAA-supplement medium. d) regeneration plantlet vigorous shoot

Table 2 Main effect of cultivars on percentage of (*Vicia faba* L.) cotyledons that induced callus and shoots in cotyledons explants and growth regulators treatment explant experiments

	Growth regulator treatment-	
Cultivars	% Callus	% Shoots
686 Sudan	40 8a	1 7a
1059 Morocco	27 8b	3 07b
ILB 752	13 5b	10 3b
Wasa soramame	64 1c	21 9c
1083 Australia	87 4bc	55 1c
Cairo 241	80 1d	36 71d

Means followed by same letters are significantly different at the 5% level using Duncan's multiple range test

Morph-histological evaluation: Regenerating structures were observed on the cotyledon explants of almost all cultivars, after culture on a medium IBA or NAA and kinetin, all the explants swelled and turned green calli after 1 month (Fig. 1 a-c). The results of histological inspection of prior to the culture , cotyledon explants of all cultivars composed of uniform epidermal and mesophyll cells, interspersed with vascular tissue (Fig. 2a). Multiplication was observed in vascular parenchyma and in mesophyll cells around the vascular system after 7 days culture (Fig. 2b). The multiplication resulted in the uniformness of nodular structures after 21 days of culture (Fig. 2c). The nodular structures were characterized by having meristematic, isodiametric cells in the outer layers and more differentiated, vacuolated cells in the inner layers. No vascular elements were observed in the nodular tissue. A similar tissue specificity response to auxin has been shown in mungbean (Narciso *et al*., 1996; Wei and Ronald., 2005) indicating that totipotency is extrinsic to all plant cells. Bud recovery from friable callus first appeared as compact and smooth bud terminal, whereas larger, vacuolated and parenchyma cells were formed similar to those showed in *Pigeon pea* (Mohan and Krishnamurthy, 1998).

DISCUSSION

Qualitative differences among cultivars were observed when mature cotyledons were used as explants. Wasa soramame cotyledons generally swelled and became identically covered with callus, while 1083 Australia cotyledons typically bulged and

Table 3 Callus and shoots percentage induced from mature cotyledon explants of *Vicia faba* cultivars using ten growth treatment experiments

	Callus (%)			Shoots (%)		
Source of variation	df	F	p-value	df	F	p-value
Types of cotyledon (D)	1	20 03	0 004 *	1	4 31	0 029 *
Cultivars (C)	5	207 02	0 0001 *	5	4 32	0 0541 *
Treatments (Tr)	9	14 39	0 0001 NS	9	2 82	0 0132 NS
D x C	5	28 11	0 0001 *	5	12 98	0 0001 *
D x Tr	9	0 515	0 8311 NS	9	2 83	0 0132 NS
C x Tr	45	1 36	0 2663 NS	45	37 68	0 0001 NS
Tr x C x D	45	0 46	0 6248 NS	45	45 31	0 9322 *
(Kin +IBA vs BAP+NAA)	1	3 29	4 26 NS	1	13 16	4 35 *
(Tr 2,3 vs Tr 4,5)	1	0 1	0 7491 NS	1	6 5	0 016 *

(NS and *) = Nonsignificant or significant at p<0 05, respectively

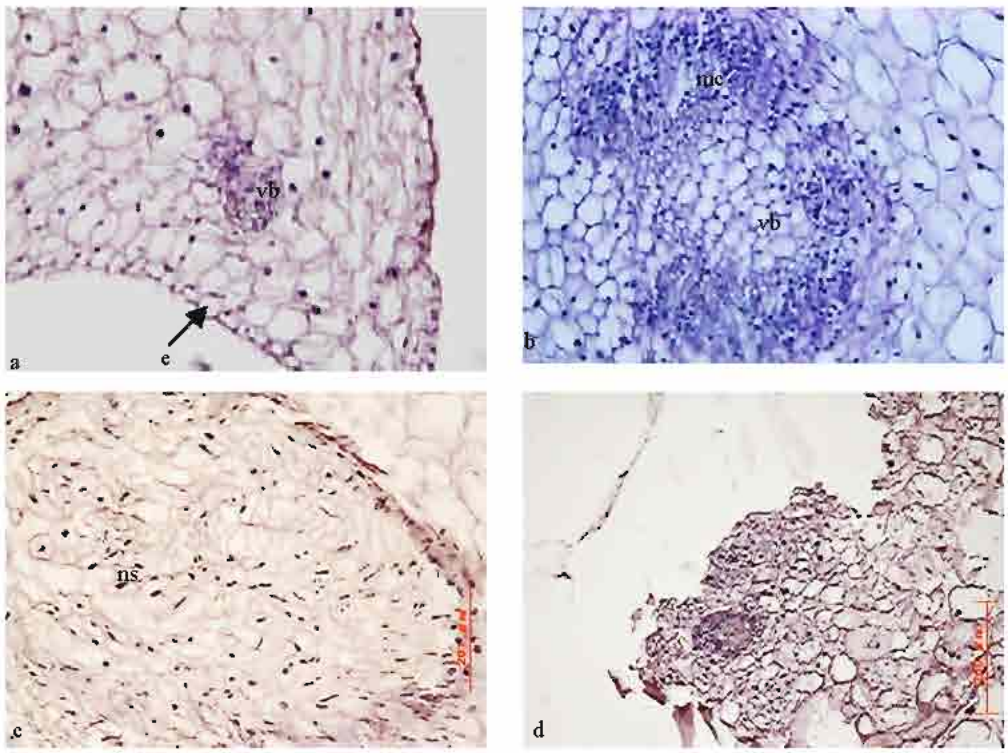

Fig. 2: Transverse sections of cotyledon explants of (*Vicia faba* L.) during 20 days of culture in medium with growth regulators. a) Section of explant at the time excision from the parent plant (bar = 100 μm), b) mitotic activity around vascular bundles after 7 days of culture (bar = 100 μm), c) meristematic nodule developed after 21 days of culture (bar = 200 μm), (bar - 100 μm).

Abbr. e: epidermis, me: mesophyll cells, mc: meristematic cells, ns: nodular structure, vb: vascular bubdies

formed callus at proximal and distal ends. Cairo 241 swelled, elongated and producing callus primarily at the proximal end; 686 Sudan; 1059 Morocco and ILB 752 tended to emerge patches of callus anywhere on the (whole or half) cotyledons. These observations were consistent within cultivars, regardless of growth regulators combination used. Wasa soramame, 1083 Australia and Cairo 241 cotyledons differed in the appearance and number shoots formed. Of three cultivars Wasa soramame shoots were healthiest and most vitality, though there was some differences within this cultivars. Wasa soramame cotyledons typically produced shoots after the fifth week, when they were moved from darkness to a 16 h photoperiod. By the fifteenth week, Wasa soramame cotyledons with shoots had from two to 8 shoots that could be easily regenerated. 1083 Australia cotyledons began to form shoots by third week and formed a large a mount of callus and a number of shoots

about three by the end of the experiment and shoots hyperhydric in appearance and were hard to transfer and maintain. Cairo 241 cotyledons often produced a single main shoot that occupied other smaller, little well-produced shoot and mature cotyledons of this cultivars also appeared to produce roots occasionally on all of the growth regulator combinations.

Nearly all striking result of this experiment is the variability among the faba bean cultivars in regeneration from mature although of how the cotyledons were treated. Four cultivars *V. faba*: 686 Sudan; 1059 Morocco; NEB 367 and 1083 Australia' were not capable to shoot regeneration from mature cotyledons (Torres *et al*., 1993). Unlike in regeneration potential between cotyledons within some cultivars were not surprising, thinking that seeds were harvested from plants in different geographical. Regardless of each cultivar (*V. faba*: 686 Sudan; 1059 Morocco; NEB 367; Waza soramame and Cairo 241) described a single genotype and indirectly some genotypes will regenerate more efficiently under our experiment factors than others, we think trends within a cultivars to be of primary use and importance, since these data will approve us to focus our transformation achievement on cultivars that regeneration well. Established on the percentage of explants forming shoots, three of faba bean cultivars inspected here, Wasa soramame, 1083 Australia and Cairo 241 are possible useful for genetic engineering via *Agrobacterium*. A transformation protocol from mature cotyledon materials is possible to genetically engineer *(Vicia faba* L.*)* germplasm. Multiplication from *phyllo* material should be examined in *Vicia* sp., since such protocol would lead to direct transformation cotyledon cultivars. This is the first published study to report that callus induction and shoot regeneration in faba bean is influenced by mature cotyledon.

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