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Histological Observations on Plant Regeneration in Faba Bean Cotyledon (*Vicia faba* L.) Cultured *In vitro*

Hamdy M.A. Aly and Kazumi Hattori
Laboratory of Plant Genetic and Breeding,
Graduate School of Bioagricultural Sciences,
Nagoya University, furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

Abstract: Cotyledonary node explants of 3 cultivars of *Vicia faba* were isolated from 7-day-old cotyledons and tested for shoot regeneration. Over 51% of the cotyledonary node explants formed shoots when cultured for 7 days on Murashige and Skoog's (MS) medium containing 2 μM N6 -benzyladenine (BA) followed another 4 days on MS medium supplemented 2 μM each of BA and thidiazuron (TDZ). Shoot multiplication was successful. Histological observations revealed morphological changes at the dermatogen (epidermal and subepidermal cell) zone from the cotyledonary node explants of faba bean treated with BA and TDZ were compared with those of untreated control plants using by light microscopy. Four days after the start of BA treatment (day 4), dermatogenic regions appeared attach to the axillary buds and on the abaxial surface of the primary leaf primordia. Meristematic loci originated from subepidermal tissue of the regions adjacent to the shoot apex and all of these bluges developed into shoot during incubation. In comparison with control plants, the axillary or adventitious buds is not observed from histological analysis in three cultivars and sometime axillary buds reached a certain degree of development, they became grew very slowly.

Key words: *Vicia faba* L., axillary bud, BA-N6 -benzyladenine, TDZ-thidiazuron (N-phenyl-N'-1, 2 ,3, -thiadizol-5-ylurea), histology, agar, organogenesis

INTRODUCTION

(*Vicia faba* L.) is a member of the family Leguminose *(Fabaceae)*. Broad beans get their name from the seeds which are large and flat. Its assigned to the Central Asian Mediterranean and South American Centers of Diversity (Cubero, 1973). The wild ancestors and precise origin of faba bean remain unknown, several wild species are taxonomically tightly denoted to the cultivated crop, but they contain 2n = 14 chromosomes, whereas cultivated faba bean has 2n = 12 chromosomes (Bond *et al*., 1985). Faba beans are financially eminent for their desirability as alpha hilex source in human and animal pleiotrophic and their directed customary function in biological from dinitrogen fixation (*Azotobacter*).

Vicia faba L. *in vitro* includes the organography from areas of active cell division. Adventitious organs rise either directly from the original tissues or indirectly through callus phase (George and Sherrington 1984).

Vicia faba L. in organogenesis systems, new here is multi-auther review in which a single topic, such as shoot regeneration from members of the genus *Vicia*, the culture callus was obtained from protoplasts by Kao and Michayluk (1975), in the present study we accepted direct shoot regeneration by thidiazuron (TDZ: N-phenyl-N ' -1, 2, 3-thidiazol 5-ylurea) (Moke *et al*., 1982), reproducible regeneration of fertile plants from protoplasts in *V. faba* cv. Mythos (Tegeter *et al*., 1995) and TDZ utilized as the only growth regulator for induction of regeneration in large seed grain legumes (Cheng *et al*., 1980; Gulti and Jaiwal, 1994; Malik and Saxena, 1992; Murthy *et al*., 1995; Murthy *et al*., 1996), multiple shoot from intact seedling and cotyledonary node explants has been achieved in faba bean by TDZ with BA (Khalafalla and Hattori, 1999). Because this species is not easily propagated by vegetative methods, commercial propagation is almost exclusively by seed. Here we were to develop a protocol for the *in vitro* propagation of *V. faba* and to investigate the organogenic process using histological techniques, mature embryo and seedling parts were used as explants. Various variables were tested at each stage and emphasis was placed on establishing a reliable of timetable of histological events with bulg or promeristemoid formation and development and finally adventitious shoot formation.

Corresponding Author: Hamdy M.A. Aly, Laboratory of Plant Genetic and Breeding, Graduate School of Bioagricultural Sciences, Nagoya University, furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

MATERIALS AND METHODS

The primary focus was to isolate an explant that would respond and produce axillary buds under experimental conditions. Once this goal was achieved, the subsequent steps further optimized each stage.

Plant material and culture conditions-: Material seeds of faba bean cultivars Was soramame plants was collected Matsunaga Shubyou Seed Company Ltd., Japan, the seeds of Australia 1083 from the Departement of Plant Science, university of Adelaide, Australia and the seeds of Cairo 241 from National Research Centre (NRC), Egypt. Before use, mature seed were surface sterilized in 70% (v/v) ethanol for 1 min followed by 10 min immersion in 3% (v/v) sodium hypochlorite solution with 2 drops of Tween 20 per 100 mL and washed thoroughly with Sterile Distilled Water (SDW). After soaking for 8 h in SDW, finally the seed were rinsed 3 times with SDW. surface sterilized seeds were aseptically stratified seeds and placed in plastic petri dishes (90×15 mm) and stratified at 10-15°C in the dark.

To obtain explants, the seed coats were removed and naked embryo were excised and then plated horizontally on water agar in plastic petri dishes. The medium contained 1% sucrose and, the pH was adjusted to 5.75 with KOH or HCl before adding 0.6% agar powder (INABA-30) and then autoclaved at 121°C for 20 min. Plastic petri dishes with intact embryo were stratified further at 10-15°C in the dark for 48 h and then germinated at temperature of 25±1°C under in 16 h photoperiod of cool-white fluorescence light (30 μmol $m^{-2}s^{-1}$).

Explants age and induction medium: Based on preliminary explants with 7-day-old embryo with intact embryonic axis (shoot tip) and both cotyledonary nodes were used in the experiments reported here. To select the optimum length of cotyledonary node explants for axillary bud formation, excised intact embryos were kept in dark for 48 hours at 10-15°C followed by 3-9 days in the light. After each harvesting period (from 3 to 9 days), cotyledonds were detached by gently pushing them outwards to the hypocotylary axis, each cotyledonary node explant cutting the stem above and below the cotyledonary node. Explants of three cultivars which exhibit hypogeal germination were cut above and below the cotyledonary nodes. The cotyledons were inculated with their abaxial side touching the medium. Cotyledonary node explants were inserted upright (acropetal end up) on the media surface. Cotyledons were excised aseptically at cotyledonary node and tested for axillary bud organogenesis.

Initially, two media were tested both at full- and half-strength with the major salts diluted. The media included MS (Murashige and Skoog, 1962) and B5 (Gamborg *et al*., 1968). In each case, the minerals were supplement with 1 mM sucrose. Unless otherwise stated, the medium was adjusted to 5.75 with KOH or HCl before adding the appropriate 0.8% (w/v) of agar powder (INABA-30) and then autoclaved at 121°C for 20 min. BA or TDZ were added before pH adjustment. Activated charcoal (0.2 mM, sigma) was added before autoclaving. Cotyledonary node explants from 7-day-old germinated embryos were involuted for 4 days on each medium supplemented with 2 μM BA and then cultured for additional 4 days on each medium supplemented with 2 μM each of BA and TDZ. The medium that gave the best overall response was used to optimize the BA concentration for the induction treatment. This was done by testing of 0, 2, 4 and 8 μM for 4 days, followed by 0, 4 and 8 days (to give a total of 4, 8 and 12 days exposure) in the presence of 0-8 μM BA plus 2 μM TDZ. Again, the best time and BA concentrations were used to select explant age and medium, i.e., cotyledonary nodes explants were takes after light incubation for 3-8 days and tested on full-strength MS and B5 media .

Each treatment had three replications with approximately 12 cotyledonary nodes per replication or culture plate and each experiment was repeated twice. Data processing was carried out using Statistic for Windows. Release 5.1. Means±SE for three replications of 12 explants used for each combination were determined.

Sucrose: To determine the influence of sucrose concentration, the five levels of sucrose (20, 40, 60, 80 and 120 mM) were compared as regards their influence on axillary bud organogenesis from cotyledonary node explants. The sucrose concentrations were kept constant during the 1-week induction period, An average of 24 cotyledonary nodes per replication and seven replications were used each treatment.

Axillary buds and development to shoot elongation: To promote axillary bud elongation, individual plantlets, excised from the preliminary cotyledonary explants when they were approximately 6 to 8 weeks old and > 4~5 mm in height, were transferred to test media without growth regulators with 0.05% activated charcoal and 0.8% agar. Again two media were tested both at full and half-strength with the major salts diluted. The media included MS (Murashige and Skoog, 1962) and B5 (Gamborg *et al*., 1968). Four media were supplemented with control, 20 or 40 mM sucrose. Plantlet explants were incubated at photoperiod and same temperature described above.

Subculturing was done after every two weeks in 500 mL conical flasks containing 40 mL of medium and covered with one layer of sterile polypropylene plastic sheet. To test the effects of sucrose concentrations on axillary buds growth, factorial 2×3 experiment was designed for both full- and half-strength macronutrients in each nutrient medium. After ten weeks, plantlet explants were measured and evaluated. To carry out shoot elongation, the two best media were tested at ½ and 1/4 strength at same sucrose concentrations, after 12 weeks in culture, average shoot height were recorded.

Histology-Histological inspections: Histological inspections were undertaken to decide the target areas responding to exogenous phytohormones and to identify stages during the organogenic process. The explants were harvested from hour 0, when cotyledonary nodes were excised from 7-day-old germinated embryos and placed on axillary shoot-emerging medium, to Day 21. Twenty explants were obtained from 0, 24, 48, 72 and 96 h-old were sampled at age. The anatomical at the dermatogen (epidermal and subepidermal cell) was inspected in whole mount preparations and in sectioned material. The histological sectioning was performed according Avenido and Hattori (2001). In brief, samples were fixed in FAA (formalin: acetic acid: 70% ethanol at 5: 5: 90 ratio) for 24 h and then dehydrated in a high graded acetone series (30, 50, 70, 90, 100% v/v) at 30-min intervals and then embedded in paraffin. Longitudinal and transverse sections (6-8 m thick) of paraffin-embedded material were obtained using a rotary microtome. The ribbons floated on distilled water and placed on slides laced with 70% albumin. Slides were dewared in xylene and rehydrated in graded ethanol series. Sectioned tissue was stained with haematoxylin (0.03%, w/v) and again dehydrated in a agraded ethanol series, cleared in xylene and mounted under a cover slip with resin and observed under light microscope. Four replicates were used for the histological studies.

RESULTS

The most embryos of three cultivars responsive explants were cotyledons from 7-day-old germinating embryos maintained on water agar containing 1 mM sucrose. Under the 2-day, 10-15°C and dark conditions followed by the 5-day, 25±1°C and light conditions, the embryo underwent a process of maturation, their hypocotylary axis turned a reddish color and the cotyledonary nodes became firm and green. Cotyledonary explants of different sizes and ages gave different responses (Table 1). Cotyledonary nodes from embryos kept for less than 3 days in the light showed reduce buds and cotyledonary nodes from embryos kept for longer than 7 days in the light showed a decline in the number of cotyledonarys producing buds. Effect of type and concentration of phytohormones

Effect of type and concentration of phytohormones: The type of phytohormones, its concentration, determined the frequency of axillary bud response, induction and elongation of axillary buds. The optimal exposure period to 2 μM BA was about 8 days (Table 2) used on judging such as percentage of cotyledonary nodes forming axillary buds (64.9, 59.2 and 55%), average number of axillary buds per cotyledonary nodes (3.8±0.1), (3.1±0.6) and (2.9±0.3) for Was soramame, Australia 1083 and Cairo 241, respectively, ABEC index (3.18%). Shorter time of exposure to BA resulted in suboptimal development of the resulting axillary buds. Also, after axillary buds reached a certain degree of development, they became grew very slowly. In contrast, a high concentration of BA

Table 1: Effect of age cotyledonary nods explants of three faba bean cultivars on axillary buds emergation after four weeks of cultivation *in vitro*

	Average number of axillary buds per isolated cotyledory expalnts								
	Was soramame			Australia 1083			Caro 241		
Light germination	Cotyledonary nodes length (mm)			Cotyledonary nodes length (mm)			Cotyledonary nodes length (mm)		
Days	2.5-3.0	3.0-4.0	3.5-4.0	2.5-3.0	3.0-4.0	3.5-4.0	2.5-3.0	3.0-4.0	3.5-4.0
3	08±01	0	0.4±0.1	0	1.7± 0.1	1.1±0.1	10±0.0	1.1±0.1	0.6±0.1
4	1.1±0.1	1.2±0.5	1.6±0.1	1.0±0.1	1.5±0.1	0	1.1±0.1	1.1±0.2	1.2±0.1
5	1.2±0.1	0.8±0.0	1.8±0.1	13±01	1.8±0.4	2.5±0.9	0.7±0.1	2.4±1.2	0.5±0.2
6	1.3±0.2	2.7±1.4	1.8±0.3	1.8±0.1	3.1±0.2	0.7±0.0	1.3±0.2	2.0±1.1	1.1±0.1
7	1.0±0.1	2.9±1.3	0.9±0.1	2.1±1.2	2.1±1.3	1.2±0.1	1.9±0.3	3.4±0.4	1.7±0.1
8	0.8±0.1	1.8±0.6	1.1±0.1	13±01	2.4±1.1	0.6±0.1	1.0±0.1	2.1±1.5	1.0±0.1
9	0	1.6±02	1.3±0.1	0.5±00	1.3±0.2	0.8±0.1	1.1±0.3	1.1±0.1	1.1±0.1
10	1.2±0.1	0.8±0.1	0.9±0.1	1.1±0.1	0.6±0.1	0.4±0.1	0.6±0.1	0.9±0.1	0.6±0.1

Embryo of three cultivars were kept in the dark for 2 days followed by 3-10 days in the light before cotyledonary nodes were removed and used as explants; For induction, the cotyledonary node explants were treated on MS medium (Murashige and Skoog, 1962) with 2 μM BA for 4 days, than transferred to 2 μM BA 2 μM TDZ for 4 days

Table 2: Effect of BA concentrations and time of exposure on axillary bud induction from cotyledonary nodes explants of three faba cultivars

	Average number of axillary buds per cotyledonary nodes emerging axillary					
Time of exposure (Days)	Was soramame	Australia 1083	Was Caro 241	Australia soramame	1083	Cairo 241
0 f BA						
4 days	0.0	0.0	0.0	0.0	0.0	0.0
8 days	0.0	0.0	0.0	0.0	0.0	0.0
12 days	0.2±0.0	03±01	02±01	11.4	16.4	14.2
2 f BA						
4 days	1.2±0.1	19±03	11±00	23.4	29.3	20.6
8 days	3.8±0.1	31±06	29±06	64.9	59.2	55.0
12 days	4.3±1.2	39±02	29±06	72.4	70.6	56.4
4 f BA						
4 days	0.8±0.0	10±01	07±00	16.2	15.1	14.2
8 days	1.5±0.2	11±02	16±01	24.7	22.3	29.8
12 days	0.7±0.1	11±00	11±01	12.1	20.5	20.7
8 f BA						
4 days	0.1±0.1	0.6±0.1	10±00	3.0	10.2	21.2
8 days	0.6±0.0	1.0±0.0	07±01	11.2	19.5	14.8
12 days	0.9±0.1	1.2±0.1	10±02	15.6	22.4	19.1

Embryo of three cultivars were germinated for 2 days in the dark followed by 5 days in the light before cotyledonary nodes were removed and used as explants. For induction, the cotyledonary node explants were treated on MS medium (Murashige and Skoog, 1962) with 2 μM BA or 8 f BA for 4 days, than transferred to 0-8 μM BA + 2 μM TDZ for 0.4 or 8 days

Table 3: Effect of sucrose concentrations on axillary bud induction and shoot proliferation from cotyledonary nodes explants of three faba bean cultivars after 31 days

	Cotyledonary nodes forming axillary (%)			Average number of axillary buds per isolated cotyledonary explants		
Sucrose concentration (mM)	Was soramame	Australia 1083	Cairo 241	Was soramame	Australia 1083	Cairo 241
20	27.2	23.4	282.0	1.7±0.1	1.4±0.2	1.7±0.1
20	41.4	44.2	47.3	2.3±0.8	2.5±0.6	2.6±0.4
20	43.1	40.7	48.1	2.2±0.1	1.9±0.1	2.4±0.4
20	56.9	53.1	59.5	3.4±0.1	3.0±0.4	3.5±0.1
20	40.2	41.7	44.2	2.3±0.4	2.3±0.1	2.4±0.0

Embryo of three cultivars were kept in the dark for 2 days followed by 5 days in the light before cotyledonary nodes were removed and used as explants. For axillary bud induction, cotyledonary node explants were kept on MS medium (Murashige and Skoog, 1962) with 2 A μM BA for 4 days, than transferred to 2 μM BA + 2 μM TDZ for another 4 days

stimulate the accumaltion of phenolics and promoted necrosis at the bases of the explants. As well as, the axillary buds clumped creating a mass of organized tissue and callus formation increased.

Effect of concentration sucrose: Carbohydrates (e.x. sucrose) are usually supplied as carbon source in tissue or cell culture (Strickland *et al*., 1987). We compared the several concentrations for the efficiency of axillary buds induction in three faba bean cultivars (Table 3), showed differences in axillary bud formations among treatments. 40 mM sucrose, which gave the optimum number of the cotyledonary nodes formed axillary and adventitious buds (almost 42, 44 and 47%), average number of axillary buds was (2.3±0.0), (2.5±0.0) and (2.6±0.4) for Was soramame, Australia 1083 and Cairo 241, respectively (range 1-12 days).

Effect of mineral salts and induction medium: Cotyledonary node explants emerged axillary buds at the cut surfaces in all media within 1-4 day. Of the 2 media tested, the MS formulations produced the best overall results (Table 4): however, the axillary buds were more vitality on B5 and so this salt formulation was used as the basal medium for axillary bud induction. Seven-day-old cotyledonary node explants produced the highest number of axillary buds per cotyledonary nodes (3.95±0.5), (3.0±0.4) and (3.2±0.0) for Was soramame, Australia 1083 and Cairo 241, respectively (Table 4) in MSB medium. Comparison of half-strength mineral salt formations gave differing results, but in general half-strength minerals were lower to the results obtained with full-strength concentrations.

Axillary buds induction from embryonic axis (shoot tip) and both cotyledonary nodes explants: AB and TDZ is members of the family cytokinins, in the first report of direct differentiation of shoot from bean seedlings (McClean and Grafton, 1989). Addition of BA to the medium has been used to promotes shoot formation (Jackson and Hobbs, 1990; George and Eapen, 1994; Prakash *et al*., 1994; Avenido and Hattori, 2001). The differential effect of various concentrations of TDZ on multiple shoot formation had been already been reported for *Vicia faba* (Mohamed *et al*., 1992). TDZ has been proved to induce a very high inherent cytokinin effectively in early biogenesis (Mok *et al*., 1982), leguminous tree (Zhijian *et al*., 1994; Singh *et al*., 2002). In three faba bean cultivar cotyledonary node explants cultured on BA induced multiple shoot formation, similar

Table 4: Effect of varbus media on axillary bud induction and shoot proliferation from cotyledonary nodes explants of three faba bean cultivars after 31 days of subcutue

	Cotyledonary nodes forming axillary (%)			Average number of axillary buds per isolated cotyledonary explants		
Media[1]	Was soramame	Australia 1083	Cairo 241	Was soramame	Australia 1083	Cairo 241
MS	35.1	34.4	38.2	2.4±0.7	2.1±0.3	25±0.1
BS	57.5	49.3	50.7	32±0.4	28±0.2	21±0.1
MSB	50.4	42.4	39.3	39±0.5	30±0.4	32±0.0
1/2MS	49.5	45.7	50.1	20±0.2	19±0.1	20±0.1
1/2BS	43.1	39.5	40.8	22±0.1	21±0.2	18±0.2

[1]MS (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968); For ½ strength, all major sats were reduced by 50% Embryo, were kept for 2 days in the dark and 5 in the light before cotyledonary nodes were removed and used explants, For induction, cotyledonary nodes were kept on each treatment medium with 2 μM for 4 days, followed by 2 μM BA + 2 μM TDZ for another 4 days

Table 5: Effect of MSB medium at two concentrations and sucrose at 0,20 and 40 mM on elongation of 10 weeks old axillary bud shoots of three faba bean cultivars

	Mean height after 10 weeks (mm)[2]			Elongate axillary shoots (%)		
Sucrose + mineral salts[1] concentration	Was soramame	Australia 1083	Cairo 241	Was soramame	Australia 1083	Cairo 241
0 mM Sucrose						
½ MSB	4.41±0.12	3.91±0.23	5.71±0.35	0.00	0.00	0.00
1/4 MSB	4.92±0.11	3.28±0.24	7.13±0.19	0.00	0.00	0.00
20 mM Sucrose						
½ MSB	6.59±0.21	5.85±0.51	9.24±0.48	49.43	42.72	61.80
1/4 MSB	7.58±0.47	5.19]0.37	7.95±0.64	54.06	88.71	11.51
40 mM Sucrose						
½ MSB	8.01±0.64	6.51±0.41	10.35±173	81.63	66.49	81.26
1/4 MSB	6.53±0.48	5.07±0.28	7.74±0.85	32.8	54.57	8.50

[1]: MSB with macroelemerts were diluted; [2]: Initial height of plantlet was 4.0 mm

results have been reported for using BA in combination with TDZ explants of *Miscanthus xogiformis' Gigantenus* (Nielson *et al*., 1995). The advantageous effects using (BA+TDZ) during seed germination to increases multiple shoot regeneration efficiency among the explants were used in woody species, viz., *Tilia cordata*, *Sorbus acuparia* and *Robinia pseudoacacia* (Chalupa, 1987), *Fraxinus pennsyllvanica* (Kim *et al*., 1997).

Axillary bud elongation: Of the two media tested, the MSB media were at best for axillary buds elongation. The results of further experimention with two concentrations of sucrose and MSB media at ½ and 1/4 strength are shown in Table 5. When sucrose concentration was zero mM, axillary buds did not elongated. As concentration of sucrose was increased to (40 mM) in the combination tested, ½ MSB with 40 mM sucrose provided the best results for axillary buds elongation after 10 weeks (8.01±0.64 mm), (6.51±0.41 mm) and (10.35±1.73 mm) for Was soramame, Australia 1083 and Cairo 241 respectively and percentage of axillary buds elongating (81.63, 66.49 and 81.26%, respectively).

Histological inspection-origin of explants

Axillary bud and adventitious shoots formation: In our experiments, the shoot buds emerged either at the base or junction of the regenerated of the shoots. The physical position and shoot emergence pattern gave on axillary source. To comprehend the origin and type of these shoot (axillary buds and adventitious shoots), histological studies on germinating (*Vicia faba* L.) from 0 to 72 h revealed development and rapid advanced axillary shoot from explants re-cultured *in vitro* over that (MS or B5) media alone as compared to the control (Fig. 3). Longitudinal sections were taken at every 24 h.

Fig. 1: Multiple shoot regeneration from explant inoculated keeping the emerged shoot tip inside the modified MS medium supplemented with BA+TDZ (2 mg L^{-1} each) . Photography was taken after 21 days of inoculation

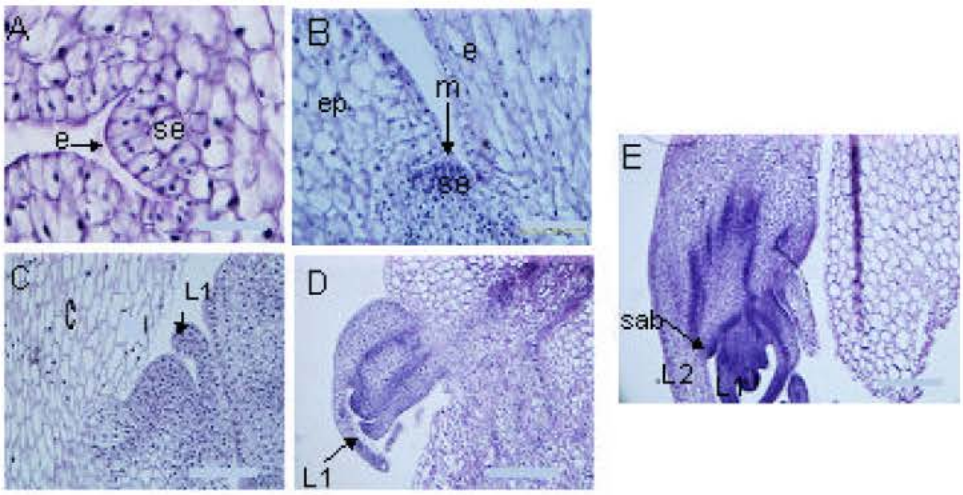

Fig. 2: (A-E) LM micrographs of histological observations showing initiation and development of AB at nodes of the epicotyl (*Vicia faba* L.) (A) after 24 culture showed divisions which were anticinal or longitudinal in adaxila epidermal (e) and subepidermal (Se) cells at the CN. (B) Meristematic region on the adaxial side of the cotyledon at the basal region of the node at 48 h. (C) 72 h after in the culture the AB has penetrated the outer CN and epidermis of CN has 1 youngest leaf primordia (L1). (D) Differentiation of single axillary meristem at the basal adaxial side of the node at 96 h after culture. At this point there is no evidence secondary axillary bud initiation, (E) multiplication of shoot buds from peripheral cell layers of axillary meristem (am) with the most recentry initiated leaf primordia (L1), also visible are the margins of one of the leaves at the second youngest node (L2) and at this there is visible of secondary axillary bud initiation (sab).Abbreviation used as follows: e, epidermis; ep, epicotyl; m, meristematic dome; L1, leaf primordia; L2, secondary

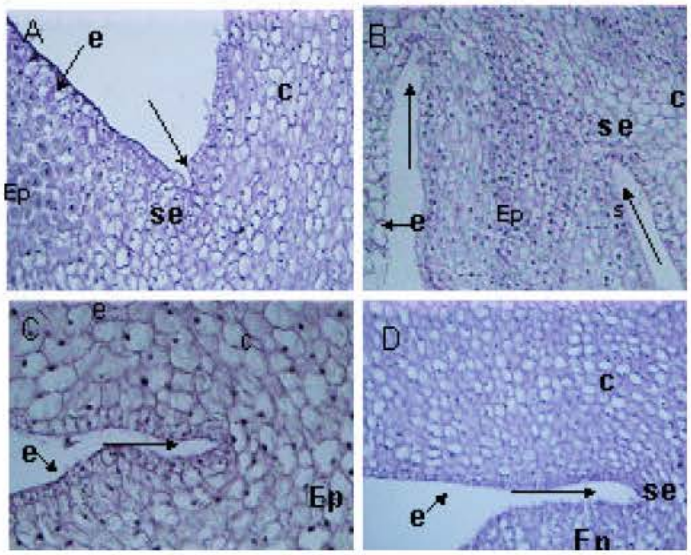

Fig. 3: LM photomicrographs of histological observation showing the absence of AB at nodes of the epitepicotyl Vicia faba (a) at the time of culture 0 h, (b) 24 h, (c) 48 h and (d) 72 h after reculture in BM salt medium. Note the absence of axillary bud in the cotyledon. Abbreviations: C, cotyledon; se subepiderma; e, epidermis and ep, epicotyl junction (arrow)

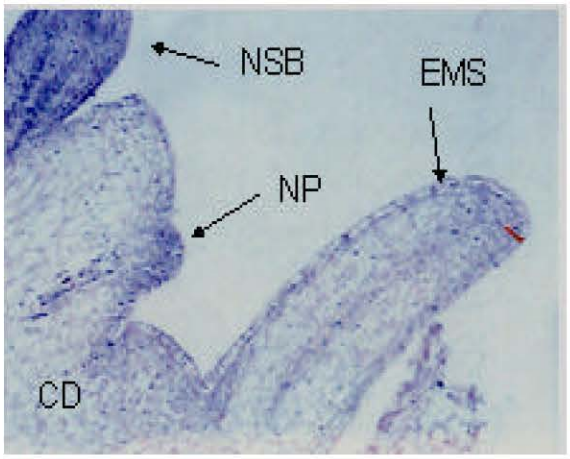

Fig. 4: Microphotographs of longitudinal section showing multiple shoot formation complete longitudinal section of 15 day-old explant note the New Bulge (NP) on the central side and bulge transformed into New Shoot Bud (NSB) bleft side EMS excised main shoot, CD cotyledonary node axis and P protuberance)

Light microphotographs (Fig. 2A-E) showed shoots rose successionally from the basal peripheral domain of an already emerged shoot which was contiguous to a cotyledonary node explants. For axillary bud, initially showed divisions which were anticlinal in adaxial epidermal and subepidermal cells (dermatogen zone) at the cotyledonary node (Fig. 2a) within 0-24 h of inoculation, the cell divisions at the cotyledonary node led to the formation of meristematic regions within 24-48 h of inoculation (Fig. 2b), which after another 72 h developed into shoot meristems. Within 72-96 h of inoculation, shoot bud was formed successively from actively dividing cells at the base of new axillary budas soon in longitudinal sections (Fig. 2c, d). These axillary buds had no visible connection with the original vascular tissue. BA promoted regeneration of axillary buds from the peripheral cell layers of axillary meristem (Fig. 1). For adventitious shoots, histological examination of the adventitious shoots induction process showed that it emerged successive from basal superficial of cotyledonary node. Initially, small bulges raised from the epidermal cell of the cotyledonary node, (Fig. 4) but not from the pre-existing buds. Histological inspection revealed that the adventitious shoots developed in a type consistent with axillary shoot regeneration. The pattern of bud origin and development was similar to the cotyledonary nodes system of *Pisum sativum* (Jackson and Hobbs, 1990; Malik and Saxena 1992), adventitious shoot primordia initiated as a result of organized directional growth of cells from meristematic cells. The shoots formed from epidermal and subepidermal cells of the cotyledonary node of the explant.

DISCUSSION

The response of cotyledonary nodes explants in three faba bean cultivars to axillary bud induction and development was affected by the concentrations of macronutrients in the test media and the MSB formation gave the best results. This medium differed from the others mainly in the concentration of macronutrients in the test media and the MSB formulation gave the best results. The time of exposure and type of cytokinin and concentration have large effects on the number of axillary buds, overall shoot quality and the subsequent growth rate of the shoots produced (Catharina Coenen *et al*., 2003, Michael and Christianson, 2000; Michael and Hornbuckle, 1999).

A combination of BA and TDZ used sequentially was necessary for organogenesis in this cultivars. Although some epidermal cells responded to BA alone, which was critical for the initiation meristematic activity, they did not develop further in the absence of TDZ. Axillary buds were induced in all three cultivars at all five sucrose concentrations with the lowest induction frequency (27%) observed at 20 mM sucrose for the cultivar Was soramame (Table 3). Overall, percent axillary buds induction for all three cultivars was above 50% depending on the sucrose concentration used, with both Was soramame, Australia 1083 and Cairo 241 above 50% at 80 mM sucrose, respectively (Table 3).

Sucrose concentration also effected the organogenic process. For most legumes species, a range of 20-120 mM sucrose is adequate (Francis et al. 1984, Ashburner *et al*., 1993; Myouda *et al*., 2001). For bean, 40 mM was adequate for axillary bud induction and elongation. This concentration was optimum for the micropropagation of legumes bean (*Vicia faba* L.) (Kartha *et al*., 1981).

Histological analysis of the axillary bud induction process showed that promeristemoids were formed in dermatogen (epidermal and subepidermal cell) region of the cotyledonary nodes on the face in contact with the medium. These developed subsequently into bulg and shoot primordia. This process resembled that observed in pigeon pea (*Cajanus cajan* L.) and mungbean (*Vigna radiata* L. Wilezek) cotyledonary nodes (George and Eapen, 1994; Avenido and Hattori, 2001) The low number of adventitious buds produced some legumes was similar to that reported in soybean (Buising *et al*., 1994). In both these systems, although blug similar to promeristemoids were present, only a few axillary bud were formed from them. In three cultivars of *V. faba* L.

blugs were found in both the cotyledonary nodes, the embryo axis and hypocotyl region of the explant. It is important to note the emerging of blugs in Faba bean cotyledons lags behind that in the hypocotyls. The finding that blugs were formed along the whole length of the cotyledon in Faba bean offers hope for increasing its axillary and adventitious-emergation and therefore, shoot formation.

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REFERENCES

Ashburner, G.R., W.K. Thomson and J.M. Burch, 1993. Effect of alpha-naphthalene acetic acid and sucrose levels on the development of cultured embryos of coconut. Plant Cell. Tissue Organ Cult., 35: 157-163.

Avenido, R.A. and K. Hattori, 2001. Benzyladenine-preconditioning in germinating mung bean seedling stimulates axillary buds in Cotyledonary nodes resulting in multiple shoot regeneration. Breed. Sci., 51: 137-142.

Bond, D.A., G.C. Lawes, M.C. Hawtin, Saxena and J.S. Stephens, 1985. Faba bean (*Vicia faba* L.) pps. 199-265. In: Summerfield, R.J. and E.H. Roberts (Eds.), Grain Legume Crops. William Collins Sons. Co. Ltd., Grafton Street, London, WIX 3La, UK, pp: 199-265.

Buising, C.M., R.C. Shoemaker and R.M. Benbow, 1994. Early events of multiple bud formation and shoot development in soybean embryonic axes treated with the cytokinin, 6-benzylaminopurine. Am. J. Bot., 81: 1435-1448.

Chalupa, V., 1987. Effect of benzylaminopurine and thidiazuron on *in vitro* shoot proliferation of *Tilia cordata* Mill., *Sorbus acuparia* L. and *R. pseudoacacia* L. Biol. Plant, (Praha) 29: 425-429.

Cheng, T.Y., H. Saka and T.H. Voqui-Dinh, 1980. Plant regeneration from soyabean cotyledonary node segments in culture. Plant Sci. Lett., 19: 91-99.

Cubero, J.I., 1973. Evolutionary trends in *Vicia faba* L. Theor. Applied Genet., 43: 59-65.

Coenen, C., M. Christian, H. Lüthen and T.L. Lomax, 2003. Cytokinin Inhibits a Subset of Diageotropica-Dependent Primary Auxin Responses in Tomato1. Plant Physiol., 131: 1692-1704.

Francis, C., H. Alan B. Bennett and Roger M. Spanswick, 1984. Concentrations of sucrose and nitrogenous compounds in the apoplast of developing soyabean seed coasts and embryos. Plant Physiol., 75: 181-186

Franklin, C.I., T.N. Trien, R.A. Gongales and R.A. Dixon. 1991. Plant regeneration from seedling explants of green bean (*P. vulgaris* L.) via organogenesis. Plant Cell Tissue Organ Cult. 24:199-206.

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soyabean root cells. Exp. Cell Res., 50: 151-158.

George, E.F. and P.D. Sherrington, 1984. Plant propagation by tissue culture: Handbook and directory of commercial laboratories, Exegetics Ltd., London, pp: 102-110.

George, L. and S. Eapen, 1994. Organogenesis and embryogenesis from diverse explants in pigeon pea (*C. cajan* L.) Plant Cell Rep., 13: 417-420.

Gulti, A. and P.K. Jaiwal, 1994. Plant regeneration from cotyledonary nodes of mungbean (*V. radiata* L. Wilczek). Plant Cell Rep., 13: 523-527.

Jackson, J.A. and S.L.A. Hobbs, 1990. Rapid multiple shoot production from cotyledonary node explants of pea (*P. sativum* L.). *in vitro* Cell Biol., 26: 825-835.

Kao, K.N. and M.R. Michayluk, 1975. Nutrition requirements for growth of (*Vicia hajastana*) cells and protoplasts at avery low population density in liquid media. Planta, 126: 105-110.

Kartha, K.K., K. Pahl, N.L. Leuno and L.A. Mroginski, 1981. Plant regeneration from meristems of grain legumes: Soybean, cowpea, peanut, chickpea and bean. Can. J. Bot., 59: 1671-1679.

Khalafalla, M.M. and K. Hattori, 1999. A combination of thidiazuron and benzyladenine promotes multiple shoot production from cotyledonary node explants of faba bean (*Vicia faba* L.). Plant Growth Regulation, 27: 145-148.

Kim, M.S., C.M. Schumann and N.B. Klopfenstein, 1997. Effects of thidiazuron and benzyladenine on axillary shoot proliferation of three green ash (*Faxinus pennsylvanica* March) clones. Plant Cell Tissue Organ Cult., 48: 45-52.

Malik, K.A. and P.K. Saxena, 1992. Thidiazuron induces high-frequency shoot regeneration in intact seedlings of pea (*Pisum culinaris* Medik). Aust. J. Plant Physiol., 19: 731-740.

McClean, P., K.F. Grafton, 1989. Regeneration of dry bean (*Phaselosus vulgaris* L.) via organogenesis. Plant Sci., 60: 117-122.

Michael, L. Christianson and Jason S. Hornbuckle, 1999. Phenylurea cytokinins assayed for induction of shoot buds in the moss Funaria hygrometrica. Am. J. Bot., 86: 1645-1648.

Michael, L. Christianson, 2000. ABA prevents the second cytokinin-mediated event during the induction of shoot buds in the moss *Funaria hygrometrica*. Am. J. Bot., 87: 1540-1545.

Mohamed, F.M., P.E. Read and D.P. Coyne, 1992. Dark preconditioning, CPPU and thidiazuron Promote shoot organogenesis on seedling node explants of common and faba beans. J. Am. Soc. Hortic. Sci., 117: 6678-672.

Mok, M.C., W.S. Moc, D.J. Armostron, K. Shudo, Sogai and T. Okamoto, 1982. Cytokinin activity of *N*-phenyl-N'- 1, 2, 3-thiadiazol-5-ylurea (thidiazuron) Phytochemistry, 21: 1509-1511.

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

Murthy, B.N.S., S.J. Murch and P.K. Saxena, 1995. Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*A. hypogaea* L.): Endogenous growth regulator levels and significasnce of cotyledons. Physiol. Plant, 94: 268-276.

Murthy, B.N.S., J. Victor, R.P. Singh, R.A. Fletcher and P.K. Saxena, 1996. *In vitro* regeneration of chickpea (*C. arietinum* L.): Stimulation of direct organogenesis and somatic embryogenesis by thidiazuron. Plant Growth Regul., 19: 233-240.

Myouda, T., K. Koshio, Y. Hirai., H. Fujimaki, F. Kikuchi and H. Toyohara, 2001. Effects of concentrations of sucrose and glucose on plantlet growth in nodal segment culture of *D. alata* L. Japanese J. Trop. Agric., 45: 209 -215.

Nielson, J.M., J. Hansen and K. Brandt, 1995. Synergism of thidiazuron and benzyladenine in axillary shoot formation depends on sequence of application in Miscanthus xogiformis Giganteus. Plant Cell Tissue Org. Cult., 41: 165-170.

Prakash-N, S., D. Pental and N. Bhalla-Sarin, 1994. Regeneration of pigeonpea (*Cajanus cajan*) from cotyledonary node via multiple shoot formation. Plant Cell, 13: 623-627.

Singh, A.K., S. Chand, S. Pattnaik and P.K. Chand, 2002. Adventitious shoot organogenesis and plant regeneration from cotyledons of Dalbergia sisoo Roxb., a timber yielding tree legume. Plant Cell Tissue Organ Cult., 68: 203-209.

Strickland, S.G., J.W. Nichol, C.M. McCall and D.A. Stuart, 1987. Effect of carbohydrate sucrose of alfalfa somatic embryogenesis. Plant Sci., 48: 113-121.

Tegeter,M., D. Gebhardt, O. Schieder and T. Pickardt, 1995. Thidiazuron-induced plant regeneration from protoplast of *Vicia faba* cv. Mythos. Plant Cell Rep., 15: 164-169.

Zhijian, L., R.L. Jarret, R.N. Pittman and J.W. Demski, 1994. Shoot organogenesis from cultured seed explants of peanut (*Arachis hypogaea* L.) using thidiazuron. *In vitro* Cellular Developmental Biology-Plant, 30: 187-191.