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Callus Induction and Shoot Organogenesis in Two Sugar Beet (*Beta vulgaris* L.) Breeding Lines *in vitro* Cultured

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Abstract: An applicable *in vitro* callus initiation and shoot regeneration of commercial sugar beet breeding lines was investigated. Procedures are described for producing different types of callus and competent callus formation able to produce shoots. Leaf, cotyledon and hypocotyl explants taken from *in vitro* grown seedlings were subjected to Murashige and Skoog (MS) medium containing different combination of plant growth regulators. The leaf explants of genotype 436 showed the highest competent callus production able to form shoot when 0.1 mg L^{-1} α-Naphthaleneacetic (NAA) acid was used in combination with 1 mg L^{-1} thidiazuron (TDZ). Hypocotyl explants of IC genotype showed the highest callus formation when 0.1 mg L^{-1} NAA was combined with 0.3 mg L^{-1} 6-Benzylaminopurine (BAP). Cotyledon explants of IC genotype showed the high callus production when 0.3 mg L^{-1} TDZ was combined with 0.1 mg L^{-1} NAA. The result evaluation demonstrated that cotyledon explants of genotype 436 produced more callus than IC genotype which able to produce shoot and the best callus induction medium was MS medium in combination with 0.1 mg L^{-1} NAA and 1 mg L^{-1} TDZ. In conclusion, TDZ was more effective cytokinin than BAP and NAA was more efficient auxin than both Indol-3-Acetic Acid (IAA) and 2, 4-Dichlorophenoxyacetic acid (2, 4-D).

Key words: Callus, shoot regeneration, *in vitro*, sugar beet, *Beta vulgaris*

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is the only species of agricultural importance crop belongs to Chenopodiaceae family. A cultivated plant of *Beta vulgaris* grown in temperate zone is a plant by the main root contains a high quantity of sucrose. Among the recalcitrant plants sugar beet is well known for *in vitro* propagation and genetic transformation (Krens *et al*., 1996). The development of an efficient protocol for plant regeneration *in vitro* from cultured cells, tissues and organs is a prerequisite for the genetic transformation and plant improvement by modern biotechnology methods (Garcia-Gonzales *et el*., 2010). Sugar beet breeding is mainly carried out conventionally but the developing of new varieties by conventional breeding is difficult (Atanassov, 1986). In the past couple of decades, the use of molecular techniques, more specifically genetic transformation technologies, has drastically increased (Mannerlof *et al*., 1997; Ivic *et al*., 2001; Sohail *et al*., 2012; Karimi *et al*., 2013). Several efforts have been achieved for development of *in vitro* tissue culture and plant regeneration from callus (Ritchie *et al*., 1989), leaf tissue (Freytag *et al*., 1988), suspension culture cells (Van Geyt and Jacobs, 1985) and protoplast (Bhat *et al*., 1986). Mezei and Kovacev (1991) reported the upstanding segregation of vegetative cultures was proliferated *in vitro* from axillary buds, apical meristems (Goska and Szota, 1992) and inflorescence pieces (Zhong *et al*., 1993). Shoot regeneration from different cultured explants of sugar beet tissues have been carried out for the production of suoerior sugar beet genotypes (Zhong *et al*., 1993; Grieve *et al*., 1997). In sugar beet the regeneration of plant is in low frequency and highly depends on genotype (Krens and Jamar, 1989). Adventitious shoot organogenesis has been obtained from callus cultures of cotyledon, hypocotyl, leaf blade and petiole (Freytag *et al*., 1988; Ritchie *et al*., 1989; Catlin, 1990; Jaco *et al*., 1993). Callus can be obtained from various parts of sugar beet comprising hypocotyls, cotyledons, leaves, petioles, roots, flower stalks, anthers, embryos and seeds (Brown *et el*., 1995). *In vitro* callus induction and shoot regeneration from cotyledon explants using MS medium (Murashig and Skoog, 1962) was reported by Catlin (1990). Tetu *et al*. (1987) obtained shoot morphogenesis from petiole, root and shoot tip callus applying 2,3,5- triiodobenzoic acid (TIBA) in the regeneration regime. Freytag *et al*. (1988) obtained callus induction and shoot regeneration from petioles in MS

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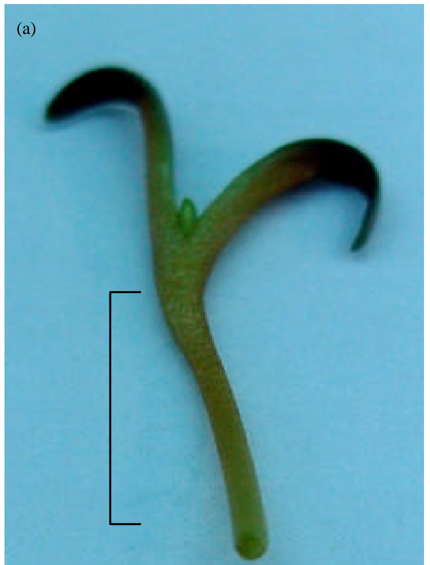
medium with several amino acids and vitamins combinations (termed RVIM medium) using 0.4 mg L^{-1} 6-benzyladenin (BA) and 0.1 mg L^{-1} Indol-3-Butric Acid (IBA) as the growth regulators. Genotype diversity is important in relation to callus production and subsequent organogenesis, some genotypes being more amenable to organogenesis than others and young tissues being more responsive than olders (Mikami *et al*., 1989). The ability of explants in efficient callus formation is an advantage in using *Agrobacterium*-mediated for genetic transformation and regeneration of whole transformed plant easily.

The present study aims to develop *in vitro* callus induction of two different sugar beet cultivars and subsequent shoot organogenesis from different seedling explants cultured in medium and a variety of plant growth regulators affecting callus induction and shoot regeneration.

MATERIALS AND METHODS

Plant material and seed germination: Diploid sugar beet 436 and triploid IC genotypes provided by the Sugar Beet Seed Institute (SBSI), Karaj, Iran. To improve germination efficiency, seeds were etching in concentrated H_2SO_4 (95-97%) about 40 min for both genotypes with gentle stirring. Then, the seeds were rinsed with distilled water to remove sulphuric acid traces. Sterilization process was done with rinsing of seeds in 70% (v/v) EtOH for 2 min followed by sterilizing in 0.1% $HgCl_2$ for 1 min. Final sterilization process was done with immersing of seeds in 20% commercial bleach containing 5.25% active sodium hypochlorite and 0.25 mL Tween 20, 100 mL^{-1} of solution about 25 min. Finally, the sterilized seeds washed 3 times using distilled sterilized water and dried on filter paper under laminar flow for about 12 h. Then, the sterilized seeds were cultured in glass cultural tubes containing 40 mL of Germination Medium (GM) a solidified compound of MS basal salts, B5 Vitamins (Gamborg *et al*., 1968), 3% (w/v) sucrose, 0.8% (w/v) agar-agar, 0.5 mg L^{-1} TIBA and 1 mg L^{-1} TDZ. The pH was adjusted to 5.75±0.05 with NaOH and HCl before adding agar. Germination medium was autoclaved for 20 min in 121°C at 15 lb sq^{-1}. For primary seed germination the cultural tubes were kept in 21±2°C in dark condition 3 days. After 3 days, the tubes were transferred into incubator under 16:8 h light and darkness period and kept at the same temperature.

Explant preparation for callus induction: For callus induction, three types of explants including hypocotyl, cotyledon and leaf explants were chosen. Hypocotyl and cotyledon explants were excised from 12-14 day-old seedlings. The exact site of hypocotyl and cotyledon explants was shown in Fig. 1. The explants were cut into 3-4 mm in length for both cotyledon and hypocotyls. The leaf explants were taken from 30-35 day-old seedlings cultured *in vitro* while they had 4-5 true leaves. For excision of leaf explants into 5-6 mm in length, the main vein surrounded tissues shown in Fig. 2 were used as explant because this section has more meristematicsimilar cells than others sites.



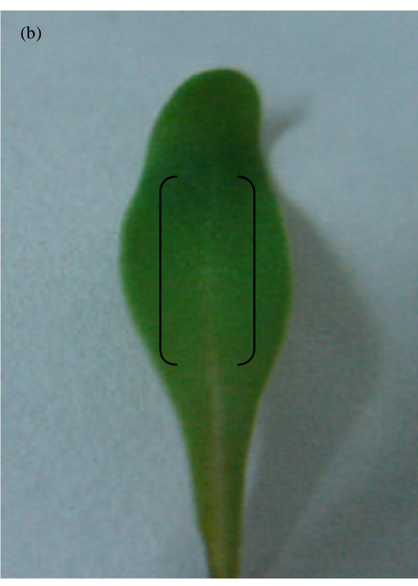


Fig. 1(a-b): Hypocotyl and cotyledon explants excised sites (a) Upper half of hypocotyl and (b) Central section of cotyledon

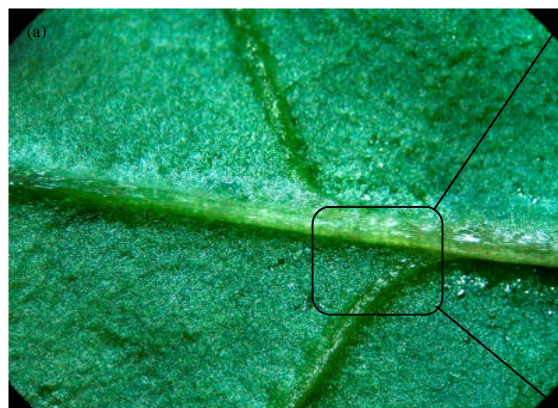


Fig. 2(a-b): Leaf explant preparation (a) Leaf tissue and (b) Exact site of excised leaf explants main vein surrounded tissue

Preparation of callus induction medium: The media including MS and B1 (Doley and Saunders, 1989) were applied. NAA, 2,4-D, IAA, TDZ and BAP from auxin and cytokinin types were used as the plant growth regulators. Three types of explants were cultured in 18 MS medium with different concentration of plant growth regulators and Gamborg's vitamins was combined at all mediums (Table 1). Hormone-free MS (CIM_0) was considered as control and B1 medium was prepared according to its formulation. Five explants cultured in each Petri dish containing 30 mL of medium were regarded as one treatment and each treatment was repeated three times. The primary results were obtained after three weeks of culture and the average of data were calculated.

The environmental condition for all samples was stable in 23±2°C in darkness for three weeks. Petri plates were checked per week and the contaminated plates were removed and were replaced with fresh cultured explants and exact date recording. All samples were assessed.

Table 1: Callus induction medium and plant growth regulator types and concentrations used for callus induction in sugar beet genotypes

Callus induction medium (treatment)	Plant growth regulators (mg L^{-1})				
	NAA	2, 4-D	IAA	TDZ	BAP
CIM_0	-	-	-	-	-
CIM_1	-	-	-	0.5	-
CIM_2	-	-	-	1	-
CIM_3 (Roussy *et al*., 1996)	-	-	-	2.2	-
CIM_4	-	-	-	-	0.5
CIM_5	-	-	-	-	1.0
CIM_6	-	-	-	-	2.2
CIM_7	0.1	-	-	0.3	-
CIM_8	-	0.1	-	1	-
CIM_9	0.1	-	-	1	-
CIM_{10}	0.3	-	-	-	0.1
CIM_{11} (Jaco *et al*., 1993)	0.1	-	-	-	0.3
CIM_{12}	0.5	-	-	-	0.5
CIM_{13}	0.1	-	-	-	1.0
CIM_{14}	-	0.3	-	-	0.1
CIM_{15}	-	0.1	-	-	0.3
CIM_{16}	-	0.5	-	-	0.5
CIM_{17} (Roussy *et al*., 1996)	-	0.1	-	-	1.0
CIM_{18}	-	-	0.1	-	0.3
B_1 (Doley and Saunders, 1989)	-	-	-	-	1.0

CIM: Callus induction medium, CIM_0: Hormone-free (control), CIM_1-CIM_{18}: Medium contain MS basal salts+B5 (Gamborg's) vitamins with different phytohormone concentrations mentioned in table, B1: MS basal salts (difference in some inorganic salt concentration in MS: 16.8 mg L^{-1} $MnSO_4.7H_2O$, 10.6 mg L^{-1} $ZnSO_4.7H_2O$, 0.88 mg L^{-1} KI, 1 mg L^{-1} Thiamine+1 mg L^{-1} BAP, pH = 5.95)

White and friable calluses were collected for extension of growth because this kind of calluses containing of large and succulent cells were able to produce shoots and roots (Shimamoto *et al*., 1993; Konwar and Coutts, 1990; Saunders and Daub, 1984).

Observational consequent and statistical analysis: The size, quality and degree (amount) of calluses were measured exactly by direct observation with binocular. The number of shoots developed from the callus was counted. All statistical analyses were performed using Microsoft Excel 2010. Standard errors were calculated and the mean comparison was determined by Duncan's Multiple Range Test.

RESULTS

Callus formation from leaf, cotyledon and hypocotyl explants: Callus induction trend from aseptic *in vitro* grown tissues of sugar beet is considered one of the important aspects in genetic manipulation by gene transfer as well as production of hybrid plants. In this experiment, the early results were observed according to the effect of different growth regulator concentration on callus formation in three types of explants in two genotypes of sugar beet (Table 1). The first obvious object in leaf explants cultured was the initiation of roots

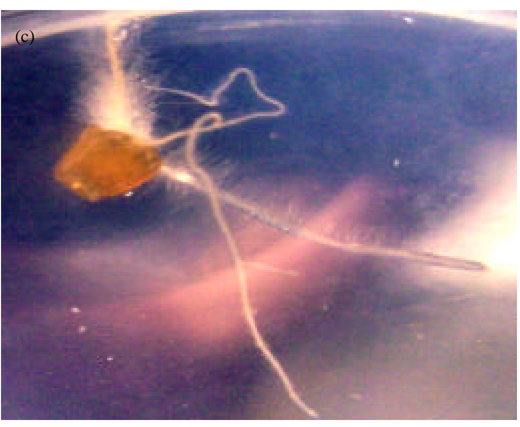


Fig. 3(a-c): Root initiation in callus induction medium (MS) hormone free (a) Root formation in leaf explants of 436 genotype and (b-c) Root formation in hypocotyl explants of 436 and IC genotypes, respectively

from brown and compact mass of tissue treated by hormone-free (CIM_0) in genotype 436. There was not any root initiation in IC genotype leaf explants in similar medium and almost all tissues were necrotized. Figure 3 shows the root initiation was also observed in Hypocotyl explants cultured in both IC and 436 varieties cultured in CIM_0.

Table 2-4 show the early results of callus formation from leaf, hypocotyl and cotyledon explants, respectively. All primary parameters of callus formation were observed and calculated were recorded in mentioned tables. Figure 4 shows the callus induction in genotype 436 was significantly more than IC genotype in leaf explants in CIM_9 when 0.1 mg L^{-1} NAA was combined with 1 mg L^{-1} TDZ. According to the Figure 4, effective culture medium hormone concentrations upon IC leaf explants was CIM_8 with 0.1 mg L^{-1} 2, 4-D+1 mg L^{-1} TDZ but the induced callus masses were compact and unable to form shoot based on results on Table 2. Figure 4 shows the significant callus initiation from cotyledon explants of IC genotype in CIM_1 with 0.5 mg L^{-1} TDZ only as well as callus formation in hypocotyl explants of IC genotype was significant in $CIM_{11.}$ The produced callus inducted by CIM_1 in cotyledon explants of IC cultivar was soft and compact callus which was unable for shoot organogenesis. Figure 4 showed callus formation was significantly high in cotyledon and leaf explants of genotype 436 than IC genotype. Callus induction from hypocotyl explants of two sugar beet genotype was almost equal overall. Table 5 shows the mean callus formation in genotype 436 was more than IC overall. According to Table 5, cotyledon and leaf explants of genotype 436 produced more callus than IC genotype but hypocotyl explants of IC produced more callus than genotype 436 overall. Based on observation and early results of Table 2-4, the genotype 436 was more eligible in callus production than IC genotype particularly in cotyledon and leaf explants cultured *in vitro*. CIM_9 was able to induced high amount of competent callus able to produce shoot in leaf explants of genotype 436; therefore this medium was selected as the best growth regulator combination in MS medium for competent callus production in this experiment. Figure 5 shows different types of initiated callus including friable, compact and greenish callus masses.

The effect of hormone concentrations in medium were affected on the number of explants produced callus masses. Figure 6 shows, CIM_9, CIM_7 and CIM_1 were more effective in number of leaf explants produced callus in genotype 436 respectively. In IC genotype CIM_8, CIM_3 and CIM_1 concentration were shown more effective on leaf explants callus formation (Fig. 6). Hypocotyl cultured-explants in IC genotype showed the higher number of callus production in CIM_{11}, CIM_{12}, CIM_{13} and CIM_0. In genotype 436, CIM_0 and CIM_9 showed the higher

Table 2: Effect of different concentration of plant growth regulators in MS and B1 medium on callus induction from cultured leaf explants of two sugar beet genotypes. Data were recorded after three weeks of culture

Culture medium	Av. No. of induced callus (a)		Explants induced callus (b) (%)		Callus color		Callus degree		Callus size (mm)		Callus quality	
	IC	436	IC	436	IC	436	IC	436	IC	436	IC	436
CIM$_{0}$	0.0±0.00	1.0±0.58	0.0±0.00	20±0.12	-	LB	-	+	-	2	-	SC
CIM$_{1}$	3.0±1.15	3.6±0.88	60.0±0.23	72±0.18	B/LY	LB/B	+	++	6	10	CC	CC
CIM$_{2}$	1.3±0.33	1.6±0.67	26.0±0.07	32±0.13	LB	C/W	+	+++	6	16	CC	FC/SC
CIM$_{3}$	3.6±0.67	2.0±0.58	72.0±0.13	40±0.12	B/LB	W/LB	++	+++	8	13	CC	FC/SC
CIM$_{4}$	0.0±0.00	1.6±0.33	0.0±0.00	32±0.07	-	B	-	++	-	8	-	CC
CIM$_{5}$	0.3±0.33	1.0±0.58	6.6±0.07	20±0.12	LB	C/W	+	+	1	3	CC	FC
CIM$_{6}$	0.0±0.00	1.0±1.00	0.0±0.00	20±0.20	-	LB	-	+	-	3	-	CC
CIM$_{7}$	1.0±0.58	4.3±0.67	20.0±0.12	86±0.13	G/LB	LB/LY	+	+++	4	13	CC	CC/SC
CIM$_{8}$	4.0±0.58	2.0±0.33	80.0±0.12	40±0.07	B/LB	B	++	+	8	5	CC	CC
CIM$_{9}$	0.7±0.33	4.6±0.33	13.0±0.07	92±0.07	B	LB/W	+	+++	6	19	CC	FC
CIM$_{10}$	0.7±0.33	2.0±0.58	13.0±0.07	40±0.12	LB	W/C	+	+++	5	16	CC	FC/SC
CIM$_{11}$	2.0±1.53	0.7±0.33	40.0±0.31	13±0.07	B/C	B	+	+	2	3	CC	FC
CIM$_{12}$	2.0±1.53	0.7±0.67	40.0±0.31	13±0.13	LB	B	+	+	6	2	CC	CC
CIM$_{13}$	0.3±0.33	2.0±0.00	6.6±0.07	40±0.00	B	B	+	+	2	6	CC	CC
CIM$_{14}$	2.3±1.20	0.7±0.33	47.0±0.24	13±0.07	LB/B	B	+	+	5	4	CC	CC
CIM$_{15}$	0.3±0.33	2.7±1.45	6.6±0.07	53±0.29	LB	B	+	+	2	5	CC	CC
CIM$_{16}$	1.6±0.33	0.3±0.33	32.0±0.07	7±0.07	B	B	++	+	7	2	CC	CC
CIM$_{17}$	1.0±0.58	1.3±1.33	20.0±0.12	26±0.27	LB	LB	+	+	3	4	CC	CC
CIM$_{18}$	0.0±0.00	0.3±0.33	0.0±0.00	7±0.07	-	B	-	+	-	1	-	CC
B1	0.3±0.33	2.0±1.53	6.6±0.07	40±0.31	B	LB	+	+	1	1	CC	CC

CIM: Callus induction medium (Table 2), Callus color: B: Brown, LB: Light brown, C: Creamy, G: Greenish, LG: Light green, DG: Dark green, W: White, Y: Yellow, LY: Light yellow, -: No callus (necrosis tissue), Callus degree: +: Slight callus, ++: Moderate callus, +++: Massive callus 0 and -: Tissue necrosis, Callus quality: FC: Friable callus, SC: Soft callus, CC: Compact callus. Each (a) and (b) value represents the Mean±SE of three replicated experiments with 5 explants per treatment in each experiment

Table 3: Effect of different concentration of growth regulators in MS and B1 medium on callus induction from cultured hypocotyl explants of two sugar beet genotypes. Data were recorded after three weeks of culture

Culture medium	Av.No. of explants induced callus (a)		Explants induced callus (b) (%)		Callus color		Callus degree		Callus size (mm)		Callus quality	
	IC	436	IC	436	IC	436	IC	436	IC	436	IC	436
CIM$_{0}$	3.3±1.50	3.3±1.20	66.0±0.18	66.0±0.24	LB	B	+	+	5	6	CC	CC
CIM$_{1}$	1.0±0.58	2.3±0.33	20.0±0.12	46.0±0.07	B	LB/B	+	++	4	9	CC	CC
CIM$_{2}$	1.3±0.88	1.6±0.33	26.0±0.18	32.0±0.07	C	LB/W	+	++	6	9	SC	FC
CIM$_{3}$	1.3±0.67	1.3±0.33	26.0±0.13	26.0±0.07	B	LB	+	+	5	4	CC	CC
CIM$_{4}$	1.3±0.33	0.3±0.33	26.0±0.07	6.6±0.07	B	W	+	++	3	9	CC	FC
CIM$_{5}$	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	-	-	-	-	-	-	-	-
CIM$_{6}$	1.3±0.33	0.0±0.00	26.0±0.07	0.0±0.00	B	-	+	-	5	-	CC	-
CIM$_{7}$	2.6±0.33	1.3±0.88	52.0±0.07	26.0±0.18	W/LB	B	++	+	8	4	SC	CC
CIM$_{8}$	0.3±0.33	1.0±0.58	6.6±0.07	20.0±0.12	B	B	+	+	2	3	CC	CC
CIM$_{9}$	1.0±0.33	2.6±1.20	20.0±0.07	52.0±0.24	B	LB	+	+	2	6	CC	SC
CIM$_{10}$	0.0±0.00	0.3±0.33	0.0±0.00	6.6±0.07	-	B	-	+	-	1	-	CC
CIM$_{11}$	4.0±0.58	2.3±0.33	80.0±0.12	46.0±0.07	LB/C	LB	+	+	6	7	CC	CC
CIM$_{12}$	3.6±1.33	1.3±0.88	72.0±0.27	26.0±0.18	LB/C	B	++	+	9	4	CC	CC
CIM$_{13}$	3.3±0.88	1.6±0.33	66.0±0.18	32.0±0.07	C	LB	++	+	10	6	CC	CC
CIM$_{14}$	1.0±0.58	2.3±0.33	20.0±0.12	46.0±0.07	LB/W	LB/W	+	++	6	9	FC/SC	CC
CIM$_{15}$	1.0±0.58	2.3±0.88	20.0±0.12	46.0±0.18	W/C	W/LB	+	++	8	7	FC	FC/SC
CIM$_{16}$	0.3±0.33	1.6±0.33	6.6±0.07	32.0±0.07	C	LB/B	+	++	1	7	SC	CC
CIM$_{17}$	1.3±0.33	1.3±0.88	26.0±0.07	26.0±0.18	C	LB/C	+	+	3	5	CC	SC/CC
CIM$_{18}$	1.6±0.33	2.3±0.33	32.0±0.07	46.0±0.07	LB/W	W/C/B	++	++	9	10	CC	FC/SC
B$_{1}$	1.3±0.33	1.6±0.67	26.0±0.07	32.0±0.13	W	LY/W	+++	+++	13	13	FC	FC

CIM: Callus induction medium (Table 2), Callus color: B: Brown, LB: Light brown, C: Creamy, G: Greenish, LG: Light green, DG: Dark green, W: White, Y: Yellow, LY: Light yellow, -: No callus (Necrosis tissue), Callus degree: +: Slight callus, ++: Moderate callus, +++: Massive callus 0 and -: Tissue necrosis, Callus quality: FC: Friable callus, SC: Soft callus, CC: Compact callus. Each (a) and (b) value represents the Mean±SE of three replicated experiments with 5 explants per treatment in each experiment

number of hypocotyl explants produced callus. Figure 6 shows; the number of cotyledon explants produced callus in IC genotype was higher in CIM$_{1}$ and CIM$_{7}$ respectively. Higher number of cotyledon explants produced callus in genotype 436 was in CIM$_{4}$, CIM$_{3}$ and CIM$_{7}$ respectively (Fig. 6).

It was clearly intelligible that the composition of the culture medium, plant growth regulators concentration and explants type are three important factors beside genotype and Ploidy effect in callus formation and subsequently, developing the adventitious shoot regeneration in *Beta vulgaris* plants.

Table 4: Effect of different concentration of growth regulators in MS and B1 medium on callus induction from cultured cotyledon explants of two sugar beet genotypes. Data were recorded after 3 weeks of culture

Culture medium	Av. No. of explants induced callus (a) IC	436	Explants induced callus (b) (%) IC	436	Callus color IC	436	Callus degree IC	436	Callus size (mm) IC	436	Callus quality IC	436
CIM_0	1.6±0.33	2.0±0.58	32.0±0.07	40.0±0.12	G	Y	+	+	5	6	SC	SC
CIM_1	4.3±0.67	2.3±0.67	86.0±0.13	46.0±0.13	LG	LB	+	++	6	7	SC	CC
CIM_2	0.7±0.33	2.3±0.33	13.0±0.07	46.0±0.07	LG	B	+	++	5	8	CC	CC
CIM_3	2.0±0.58	3.3±0.67	40.0±0.12	66.0±0.13	LB	W/B	++	++	8	10	CC	FC/SC
CIM_4	0.7±0.33	3.6±0.33	13.0±0.07	72.0±0.07	B	LB	+	++	4	12	CC	CC
CIM_5	2.0±1.15	1.6±0.33	40.0±0.23	32.0±0.07	LG	B/LY	+	++	6	10	CC	FC/CC
CIM_6	2.6±0.33	0.7±0.33	52.0±0.07	13.2±0.07	Y	LB	++	+	10	3	SC	CC
CIM_7	4.0±1.00	3.3±1.67	80.0±0.27	66.0±0.33	LY/W	C	++	++	12	8	FC/CC	CC/SC
CIM_8	1.6±0.67	1.3±0.88	32.0±0.13	26.0±0.18	B	B	++	+	8	4	CC	CC
CIM_9	2.3±0.33	2.0±0.58	46.0±0.07	40.0±0.12	LB/W	B	++	++	11	8	CC	CC
CIM_{10}	1.6±0.33	1.0±0.58	32.0±0.07	20.0±0.12	LY	B	++	+	7	4	CC	SC
CIM_{11}	0.3±0.33	1.0±0.58	6.6±0.07	20.0±0.12	LY	W/LB	+	++	2	9	CC	FC
CIM_{12}	2.3±0.33	0.7±0.33	46.0±0.07	13.2±0.07	W/B	LB	+++	+	13	4	FC/CC	CC
CIM_{13}	2.6±0.88	3.0±1.00	52.0±0.18	60.0±0.20	LB/W	LB/W	++	++	11	11	FC/SC	FC/SC
CIM_{14}	0.3±0.33	1.6±0.33	6.6±0.07	32.0±0.07	LB	B	+	+	2	6	CC	CC
CIM_{15}	0.3±0.33	1.0±0.58	6.6±0.07	20.0±0.12	B	LB	+	+	2	5	FC	FC/CC
CIM_{16}	0.0±0.00	0.7±0.33	0.0±0.00	13.2±0.07	-	B	-	+	-	4	-	CC
CIM_{17}	1.0±0.58	1.6±0.67	20.0±0.12	32.0±0.13	LB	B/LY	+	++	5	8	CC	CC
CIM_{18}	1.3±0.88	2.0±0.58	26.0±0.18	40.0±0.12	LB/W	W/LB	+	+++	5	13	SC/FC	FC/SC
B1	1.6±1.00	2.0±0.58	32.0±0.20	40.0±0.12	W/LB	W/LB	++	++	9	11	SC	SC/FC

CIM: Callus induction medium (Table 2), Callus color: B: Brown, LB: Light brown, C: Creamy, G: Greenish, LG: Light green, DG: Dark green, W: White, Y: Yellow, LY: Light yellow. -: No callus (Necrosis tissue), Callus degree: +: Slight callus, ++: Moderate callus, +++: Massive callus 0 and -: Tissue necrosis, Callus quality: FC: Friable callus, SC: Soft callus, CC: Compact callus. Each (a) and (b) value represents the Mean±SE of three replicated experiments with 5 explants per treatment in each experiment

Table 5: An overall comparison of the genotypes in terms of the mean callus formation from leaf, hypocotyl and cotyledon explants. Means are not significantly different at p≤0.05 according to Duncan's multiple range test

Explant type	Mean callus initiation SE Genotype	No.	(%)	No.	(%)
Leaf	IC	1.22^b	24.47	0.30^b	0.30
	436	1.77^a	35.30	5.00^a	5.53
Hypocotyl	IC	1.54^a	3.86	0.27^a	5.31
	436	1.53^b	30.66	0.20^b	4.00
Cotyledon	IC	1.65^b	33.09	0.20^b	4.10
	436	1.85^a	36.88	0.26^a	5.20

Effect of plant growth regulator concentration on the No. of explants produced callus

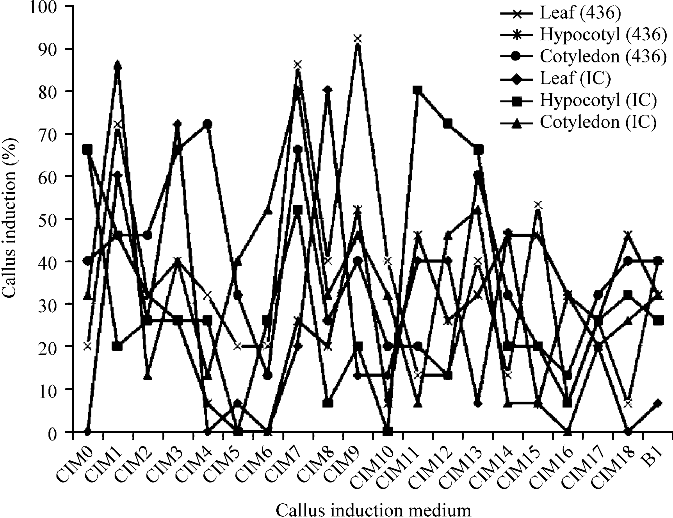


Fig. 4: Percentage of callus induction in leaf, hypocotyl and cotyledon explants of 436 and IC genotypes. Genotype 436 shows the highest callus induction in leaf explants on callus induction medium number 9 (CIM_9). IC genotype shows the highest callus induction in cotyledon explants on callus induction medium number 1 (CIM_1)

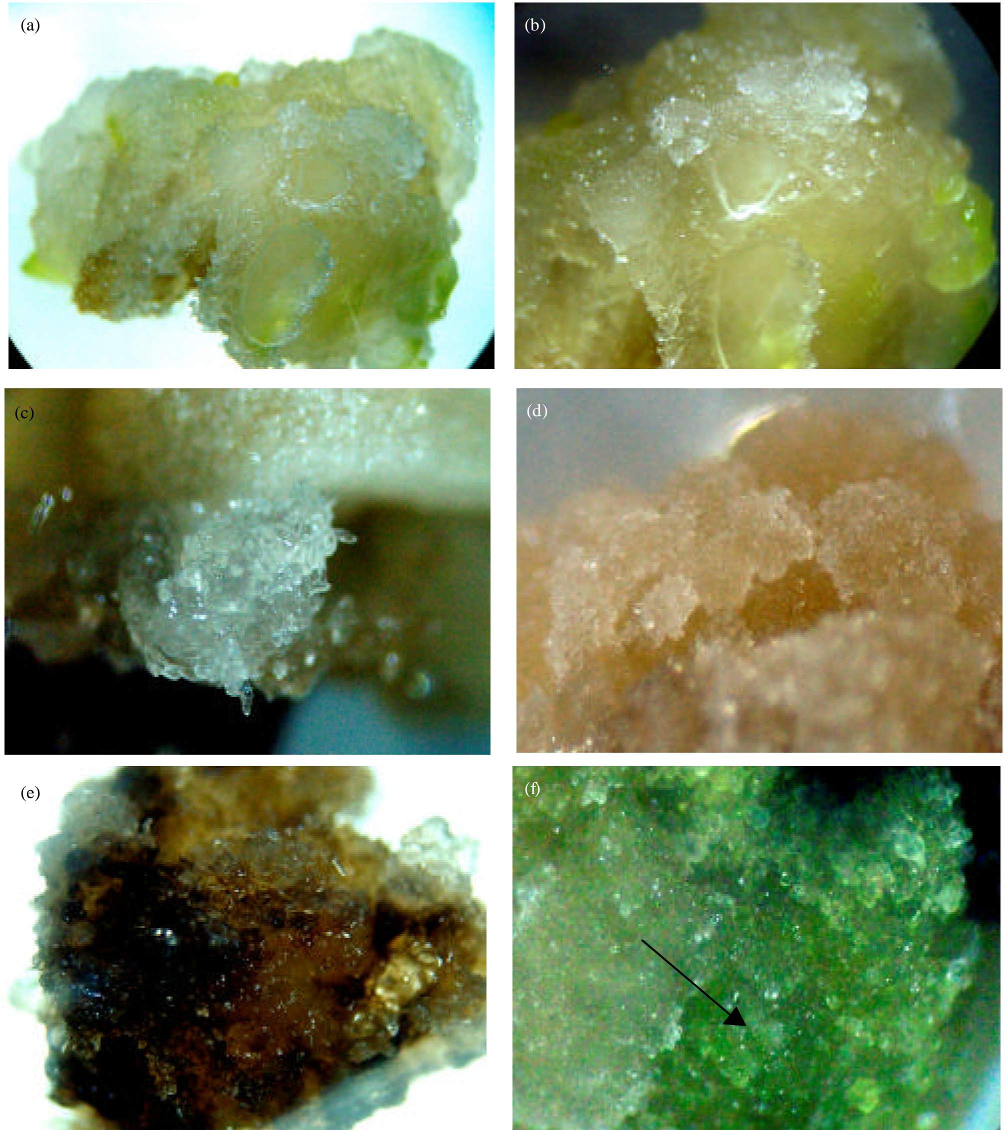


Fig. 5(a-f): Initiated callus types from *in vitro* cultured explants. (a-b) Callus derived from genotype 436, (c) Callus derived from IC genotype, (d) Light brown callus, (e) Dark brown callus and (f) Greenish compact callus with soft cells above shown by arrow

Shoot regeneration from callus: After three weeks of culture, in order to propagation of competent callus masses, the white and friable calluses (regardless the explant type) were collected and sub-cultured in MS medium CIM_9 for extra 2 weeks in dark place, then samples were transferred to photoperiod condition with 16:8 h lightness-darkness. Figure 7 shows the first presage of shoot initiation. Regenerated shoots can be transferred for rooting and soil acclimatization.

For root formation the amount of auxins have significant effect on rooting of shoots obtained from callus and 2 mg L^{-1} NAA in MS medium have the best result. This experiment was just focused on callus formation from different explant type and the effect of various hormone concentrations on callus initiation for shoot regeneration from competent callus as well.

DISCUSSION

Effect of sucrose concentration on tissue samples: During callus induction experiments there were several necrosis symptoms in cultured explants and it was

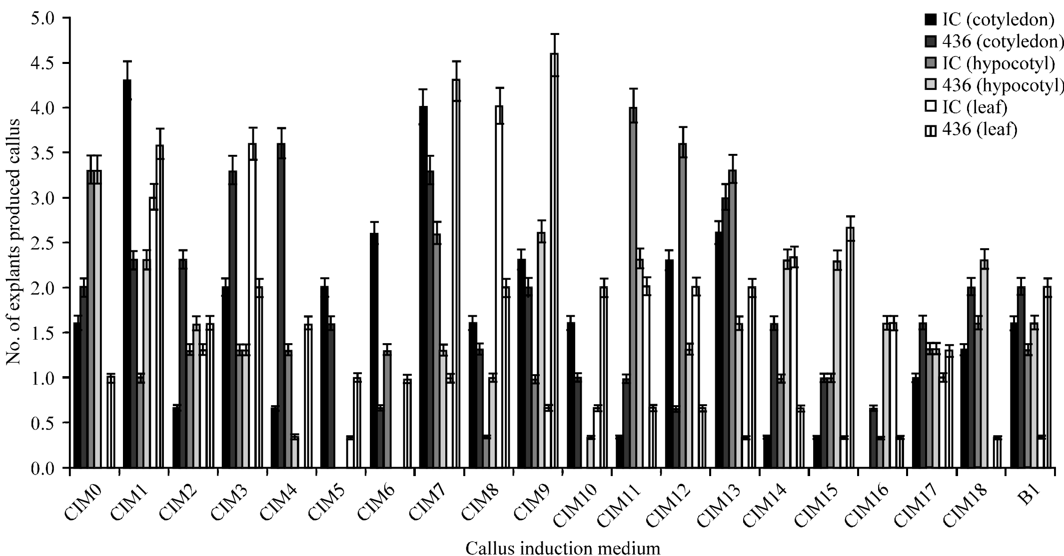


Fig. 6: Effect of different plant hormones concentration in MS medium on the number of cotyledon, hypocotyl and leaf cultured-explants of IC and 436 genotypes in callus production. Leaf explants in Medium 9 (CIM9) and cotyledon explants in medium 1 (CIM1) show the highest number of explants in callus induction

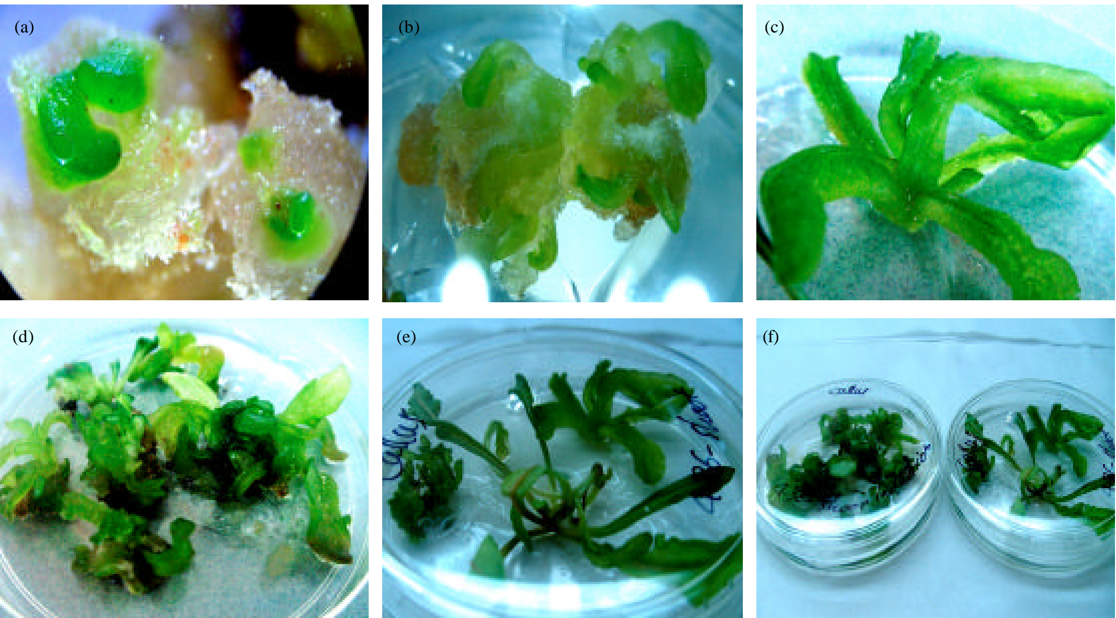
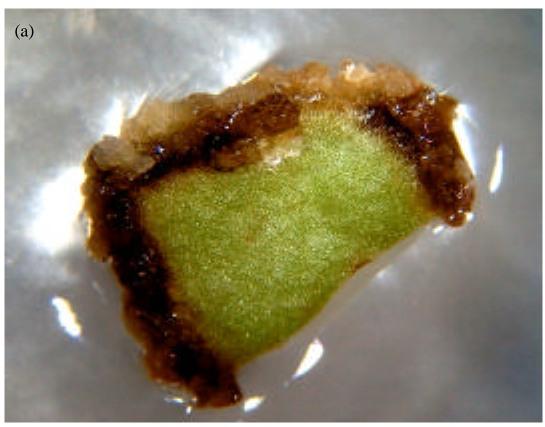


Fig. 7(a-f): Shoot regeneration from callus (a-b) Shoot initiation from white and friable callus, (c-d) Developing of shoots from callus and (e-f) Developed shoots for transfer into rooting medium

affected on the callus quality and quantity, therefore many necrotic tissue samples have been observed. Yildizi *et al.* (2007) has been reported that sucrose can cause the tissue blackening and browning by the oxidation of phenolic compounds which are exuded from cut surfaces of explants has caused serious problems in establishing *in vitro* culture and the exudates inhibit growth and tissue necrosis. In this experiment, sucrose concentration was decreased from 3 to 1-2% and necrosis was significantly decreased in tissue samples. Figure 8 shows the necrosis tissues caused by sucrose phenolic compound effect.



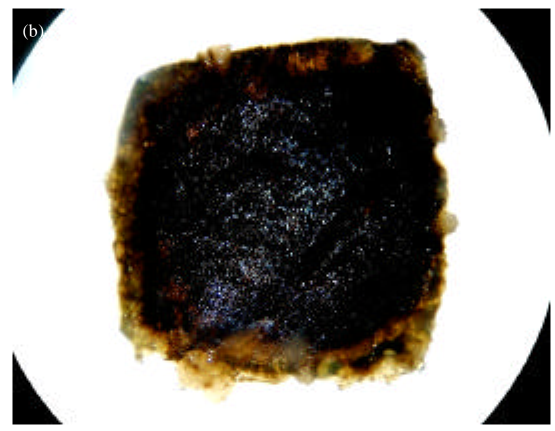


Fig. 8(a-b): Explants necroses caused by phenolic compounds produced by sucrose (a) Explant cut surface cell necrosis and (b) Whole explant necrosis

This problem can affect cultured explants in *vulgaris* family or other species of plants which are sensitive to phenolic compounds leakage.

Effect of different plant growth regulators on callus induction and shoot regeneration: In present experiment, two types of cytokinins (BAP and TDZ) and three kinds of auxin (NAA, 2,4-D and IAA) were used; however the IAA was used in only one cultural reaction in CIM_{18}. From the all culture medium, CIM_1 to CIM_3 were treated with TDZ only and CIM_4 to CIM_6 were treated with single BAP (Table 1). The effects of single-used cytokinins in callus induction from cotyledon explants, TDZ played a prominent role in compare with single-used BAP in both 436 and IC, however more effect was observed in genotype 436. Unlikely, the single-used TDZ and BAP were not very effective in callus induction of hypocotyl explants; however TDZ was effective than BAP in 436 when its concentration was 0.5 mg L^{-1}. In callus induction of hypocotyl explants the cytokinins were more effective in combine with auxins. The great amounts of callus were obtained in leaf explants when 1 mg L^{-1} TDZ was combined with 0.1 mg L^{-1} NAA and 2, 4-D in 436 and IC respectively (Fig. 4). The effect of TDZ with auxins on callus induction from hypocotyl explants was normal and it was obvious that the effect of BAP with auxins was greater than TDZ+auxins. TDZ played a significant role in callus induction of leaf and cotyledon explants of sugar beet than BAP when was used single or combined with auxins particularly NAA; however in callus formation of hypocotyl the effect of BAP was prominent in both sugar beet genotypes. IAA almost was 2-fold effective on callus induction trend in 436 than IC genotype. B1 and CIM_5 were equal in single BAP concentration but B1 was very pivotal and it may be because of high concentration of thiamin or some mineral salts. The productions of callus masses in 436 were higher than IC overall; however the number of callus-produced explants is not directed to the amount and quality of calluses which produced shoots.

Heretofore, callus induction has been reported from many types of tissues of sugar beet on various media formulation, different types of plant growth regulators and concentration (Gurel, 1997; Saunders and Shin, 1986; Saunders and Tsai, 1999). Subsequently, *in vitro* shoot regeneration has been reported from callus and various source of explants (Zhang *et al*., 2004; Mishutkina and Gaponenko, 2006) in different sugar beet lines. Plant growth regulators played a very important role in callus induction from different explants of all species of plants. Roussy *et al*. (1996) obtained favorable media for callus induction of sugar beet explants contained a combination of 0.1 mg L^{-1} 2, 4-D and 1 mg L^{-1} BAP or 2.2 mg L^{-1} TDZ alone. Regardless of the explants type the most effective media were recorded for CIM_9 (1 mg L^{-1} TDZ+0.1 mg L^{-1} NAA) and CIM_8 (1 mg L^{-1} TDZ+0.1 mg L^{-1} 2,4-D) in compare with CIM_{13} (1 mg L^{-1} BAP+0.1 mg L^{-1} NAA) and CIM_{17} (1 mg L^{-1} BAP+0.1 mg L^{-1} 2,4-D) respectively. It shows the importance of high yield of TDZ cytokinins hormone in callus formation of all types of explants tested. Organogenic callus from hypocotyl explants of sugar beet was initiated at concentrations of 0.3 mg L^{-1} BAP and 0.1 mg L^{-1} NAA (Jaco *et al*., 1993). Similarly, in this experiment the same reported combinations (0.3 mg L^{-1} cytokonin+0.1 mg L^{-1} auxin) were used for all types of explants in two sugar beet lines with different types of auxins and cytokinins. Subsequently, BAP+NAA were better than TDZ+2, 4-D in callus induction only in hypocotyl explants. IAA was combined only in one media (CIM_{18}) and was not efficient in leaf-cultured explants

callus induction; however it was effective mostly on hypocotyl and cotyledon explants particularly in friable and soft callus initiation. White and friable callus mass with large cells was able to produce shoots while compact callus with small cells showed no organogenesis capacity (Tetu *et al.*, 1987; Ritchie *et al.*, 1989). Genotype 436 was the most productive than IC genotype and it was affected by genotype influences on callus initiation. De Greef (1978) reported a genotypic variation in the callusing ability of leaf explants of sugar beet, with some genotypes being more responsive than others. This may suggest that there is a considerable variation in the distribution of the genes responsible for this response among different population of beet (Bhat *et al.*, 1985). Shoot formation from sugar beet is often induced when callus is cultured on medium containing BAP (Shimamoto *et al.*, 1993), TDZ (Roussy *et al.*, 1996), IBA and BAP (Freytag *et al.*, 1988). Development of an efficient callus initiation protocol and *in vitro* shoots regeneration of sugar beet is difficult because it is highly recalcitrance and heterogeneous plant. In conclusion, recognition of highly regenerative explant types, suitable hormone type and concentration in medium for shoot regeneration of sugar beet can help to be successful in production of transgenic sugar beet plants; however the genotype effect is very important in both *in vitro* regeneration and genetic transformation.

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