The African Journal of Plant Science and Biotechnology ©2008 Global Science Books

In Vitro Regeneration and Molecular Characterization of Sugar Beet

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ABSTRACT

A successful protocol for the *in vitro* regeneration of sugar beet was established. Leaf and shoot base explants excised from *in vitro* germinated seedlings of sugar beet (*Beta vulgaris*) were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of auxin and cytokinin. Shoot base explants showed higher percentage of direct organogenesis (93%) when cultured on MS medium supplemented with 0.5 mg/l of 6-benzyladenine (BA) and 0.5 mg/l of 1-naphtaleneacetic acid (NAA) than leaf explants cultured on MS medium containing 0.5 mg/l of 6-furfurylaminopurine (kin) and 0.5 mg/l of 2,4-dicholorophenoxyacetic acid (2,4-D) , which showed more efficient indirect regeneration (80%). Although maximum shoot proliferation was observed with MS medium containing 1 mg/l BA and 50 mg/l adenine sulfate (AS), shoot length reached 4 cm with MS medium containing 1 mg/l kin and 50 mg/l AS. Regenerated shoots rooted on indole-3-butyric acid (IBA) at 2 mg/l that seemed more effective than indole-3-acetic acid (IAA) and NAA in root formation. Protein pattern (SDS-PAGE) evidenced no variations between sugar beet cultures proliferated directly and indirectly. RAPD analysis was carried out to identify the differences amongst *in vitro* regenerated plantlets. PCR analysis showed slight differences between *in vitro* and *in vivo* sugar beet plantlets. Acclimatization was achieved by transferring the obtained plantlets to a mixture of peat-moss and vermiculite (1:1) under high humidity conditions.

Keywords: acclimatization, *Beta vulgaris* L., DNA (RAPD), propagation
Abbreviations: AS, adenine sulfate; **BA**, 6-benzyladenine; **2,4-D**, 2,4dicholorophenoxyacetic acid; **IAA**, indole-3-acetic acid; **IBA**, indole-3-butyric acid; **2iP**, N^6-(2-isopentyl) adenine; **Kin**, 6-furfurylaminopurine; **MS**, Murashige and Skoog medium; **NAA**, 1-naphthaleneacetic acid; **PCR**, polymerase chain reaction; **PGR**, plant growth regulator; **RAPD**, Randomly Amplified Polymorphic DNA; **SDS-PAGE**, sodium dedocyl sulphate polyacrylamide gel electrophoresis

INTRODUCTION

Sugar beet (*Beta vulgaris* L.), is the most important cultivated plant used for sugar production in Europe and in temperate zones of other regions of the world (Mishutkina and Gaponenko 2006). Beet sugar and cane sugar constitute 25 and 75%, respectively, of the world sucrose production of about 145 × 106 t $year^{-1}$ (Joersbo 2007).

Due to its large biomass production capacity, it has been estimated that 5.7 m^3 ha^{-1} bio-ethanol can be produced from sugar beet, compared with 2.6 m^3 ha^{-1} from wheat (Anon 2005).

Moreover, it is feasible to obtain a considerable amount of leaves, root slices, which are of value as animal feed (Owens and Eberts 1992). *B. vulgaris* has also attracted further attention, by virtue of the potential to accumulate novel or valuable specific metabolites in the storage tissues (green bioreactors). In Egypt, sugar beet is considered as the second sucrose producing crop. About 26% of Egypt sugar production comes from sugar beet which is grown mainly in the northern part of delta (Rady and Ali 1999). Cultivation of sugar beet in Egypt has been met with several field problems, this has resulted from biotic (weeds, pests, and pathogenic microorganisms) and a biotic (droughts, and soil salinity) stresses.

In view of the fact that sugar beet is biennial plant and that modern cultivars are highly heterozygous, being naturally cross-pollinated, the generation of new varieties by conventional breeding is difficult (Atanassov 1986). A biotechnology strategy for sugar beet therefore would be expected to aid the breeder introducing specific traits into commercially valuable genotypes. The development of an *in vitro* regeneration protocol and a micropropagation system of sugar beet are considered critical steps for its genetic manipulation using modern biotechnology application. In this respect, several attempts have been achieved to make progress in sugar beet regeneration. Organogenesis in *B. vulgaris* has been reported from callus (Tetu *et al*. 1987; Ritchie *et al*. 1989), leaf tissue (Ferytag *et al*. 1988), suspension culture (Van Geyt and Jacobs 1985) and protoplast (Bhat *et al*. 1986). Also, *in vitro* differentiation from various cultured tissues of sugar beet such as axillary buds (Mezei *et al*. 1990; Mezei and Kovacev 1991), apical meristems (Goska and Szota 1992) and inflorescence (Zhong *et al*. 1993) had been reported. Nowadays, molecular techniques were progressively employed by plant breeders to develop improved plant cultivars. In this regard SDS-PAGE as a molecular marker technique was used to distinguish the differences among resulting tissue culture plantlets (Roberts *et al*. 1989; Feirer and Simon 1991). In recent years a new DNA-based marker methods such as restriction fragment length polymorphism (RFLP) analysis, and those utilizing the polymerase chain reaction (PCR) such as RAPD techniques has been established. RAPD technology has been used successfully for measuring diversity in plants, and the patterns of variation observed have been shown to closely resemble those obtained using more classical characters (Howell *et al.* 1994; Virk *et al.* 1995). Moreover, RAPD analysis is sensitive enough to detect genetic variability in somaclones. Herein we report an improved method for *in vitro* direct and indirect regeneration of sugar beet and execute molecular characterization of the regenerates using SDS-PAGE and RAPD techniques.

Received: 30 July, 2008. Accepted: 30 September, 2008.

Original Research Paper

MATERIALS AND METHODS

Establishment of sterilized cultures

Beta vulgaris (sugar beet), seeds cv. 'Pleno' were purchased from Delta Sugar Company, Cairo, Egypt. Seeds were washed with distilled water and then immersed in 70% (v/v) ethanol for 1 min followed by 30% commercial Clorox (5.25% sodium hypochlorite) for 20 min and finally washed three times with sterilized distilled water. The disinfected seeds were sited in jars containing 50 ml of full strength MS basal medium (Murashige and Skoog 1962) with 30 g/l sucrose. Forty-day old seedlings were taken and re-cultured on fresh medium of the same component to improve their vegetative growth (**Fig. 1A**). Regeneration experiments were conducted using leaf segments and shoot bases cultured on MS medium and supplemented with different concentrations and combinations of cytokinins and auxins as follows:

1. 0.5 mg/l BA + 0.1 mg/l NAA
2. 0.5 mg/l BA + 0.5 mg/l NAA
3. 0.5 mg/l BA + 0.1 mg/l 2,4-D
4. 0.5 mg/l BA + 0.5 mg/l 2,4-D
5. 0.5 mg/l kin + 0.1 mg/l NAA
6. 0.5 mg/l kin + 0.5 mg/l NAA
7. 0.5 mg/l kin + 0.1 mg/l 2,4-D
8. 0.5 mg/l kin + 0.5 mg/l 2,4-D

The percentage direct and indirect organogenesis (after callus initiation) was recorded from 15 replicates after six weeks of culture.

In vitro multiplication

For multiplication of sugar beet propagules, the leaves of proliferated shoots were excised and cultured on media containing different types of cytokinins either alone or in addition to adenine sulfate (AS) as follows:

1. MS + 1 mg/l BA
2. MS + 1 mg/l kin
3. MS + 1 mg/l 2ip
4. MS + 1 mg/l BA + 50 mg/l AS
5. MS + 1 mg/l kin + 50 mg/l AS
6. MS + 1 mg/l 2ip + 50 mg/l AS

The number of proliferated shoot buds, shoot length (cm) and the number of leaves/shoot were registered after four weeks of culturing.

In vitro rooting and acclimatization

For rooting, single dark green shoots (3 cm length) were cultured on MS medium amended with 0.03% of activated charcoal and different types of auxins as follows:

1. MS (hormone free)
2. MS + 1 mg/l IBA
3. MS + 2 mg/l IBA
4. MS + 1 mg/l NAA
5. MS + 2 mg/l NAA
6. MS + 1 mg/l IAA
7. MS + 2 mg/l IAA.

Root formation, the number of roots/shoot and root length (cm) were recorded from 10 replicates after five weeks' culture on rooting media.

For acclimatization, *in vitro* rooted plantlets were gently washed with tap water and disinfected by soaking in benlate solution (1 g/l) for 20 min. Then plantlets were transferred to plastic pots containing sterile peat-moss and vermiculite (1:1). The pots were covered with clear polyethylene bags containing a few pores to allow gas exchange and sprayed with water to maintain a high relative humidity. Covers were completely removed after four weeks.

All tissue culture media were solidified with 0.7% agar (Fluka, Switzerland), supplemented with 30 g/l sucrose and adjusted to pH 5.8 before autoclaving at 121°C and 1.5 Ib/M² for 25 min. Cultures were normally incubated at 25 ± 2°C and 16 hr photoperiod (3000 lux) provided by tubular white fluorescent lamps (Philips 40 W LVF 6500 K). Plant growth regulators (PGRs) were purchased from Sigma-Aldrich, USA.

Statistical analysis

The results presented are the mean values ± standard errors obtained from at least five replicates. Statistical significance between mean values was assessed using the F-test according to Steel and Torrie (1960). Analysis of variance was determined and the value of least significant difference was calculated at 5% level to compare different treatment.

Electrophoresis (SDS-PAGE)

Proteins were extracted from leaf tissues (0.5 g) by homogenizing in sodium phosphate buffer (pH 6.8). Protein was separated in 10% SDS-PAGE under reduction conditions (Laemmli 1970). The separation was carried out using EC mini gel unit at 60 V for 4 h. Gels were stained with Coomassie brilliant blue (R-250), distained with 40% methanol in 10% acetic acid, photographed and the molecular weights of polypeptide bands were estimated from a low molecular weight marker standards of Pharmacia.

DNA extraction and RAPD analysis

DNA isolation was performed using the Cetyl Trimethyl Ammonium Bromide (CTAB) method of Doyle and Doyle (1987). Half grams of fresh samples was ground to powder in liquid nitrogen with a pre-chilled pestle and mortar, suspended in 5 ml preheated CTAB buffer, and incubated at 65°C for 1 h with occasional shaking. The suspension was then mixed with 1/3 vol of chloroform, mixed gently, centrifuged and the upper phase was transferred to a new sterilized tube. Extraction was repeated with an equal volume of chloroform. The aqueous layer was transferred to a new tube, 2/3 vol of isopropanol was added and nucleic acids were either spooled using a Pasteur pipette or precipitated by centrifugation. The pellet was washed carefully twice with 70% ethanol, dried at room temperature and re-suspended in 0.5 ml TE buffer. The enzyme, RNAse (20 µg), was added to the re-suspended mixture to digest any contaminating RNA and the tube was incubated at 37°C for 30 min. To remove the enzyme and other contaminating protein, phenol/chloroform extraction was performed. The polymerase chain reaction (PCR) mixture (25 µl) consisted of 0.8 U of Taq DNA polymerase, 25 pmol dNTPs, and 25 pmol of random primer, and 50 ng of genomic DNA. Thermocycler programmed for 45 cycles as follow: 94°C for 5 min (1 cycle), 94°C for 30 sec, 36°C for 30 sec, 72°C for 2 min (45 cycles) and 72°C for 7 min (1 cycle), then held at 4°C. The amplified DNA fragments were separated on 2% agarose gel and stained with ethidium bromide. Five oligonucleotide primers 10-base (Operon Technologies Inc., Alameda, California) were randomly selected and used in RAPD analysis: K1 (TGGCGACCTG), K2 (GAGGCGTCGC), K3 (CCCTACCGAC), K4 (TCGTTCCGCC) and K5 (CACCTTT CCC). A 100 bp DNA ladder (Promega) was used as a marker. The amplified pattern was visualized on a UV transilluminator and photographed.

RESULTS AND DISCUSSION

Regeneration and differentiation

Successful application of gene transfer techniques for improvement of crop plants is dependent on efficient and reliable tissue culture regeneration systems. Due to many biological complexities such as allogamy and incompatibility, as well as a relatively limited pool of useful genes, sugar beet improvement by conventional breeding has been a difficult task. Crop improvement using biotechnology has been limited due to its recalcitrance to both regeneration *in vitro* and genetic transformation (Snyder *et al*. 1999; Zhang *et al*. 2001).

In this part of work, two types of explants and different combinations of PGRs added to culture medium were examined for direct and indirect regeneration of sugar beet. Results obtained reveal that, shoot base explants gave a significantly higher percentage of direct organogenesis (**Fig. 1B**). However in leaf explants, shoots were formed after callus initiation which deemed as indication of indirect

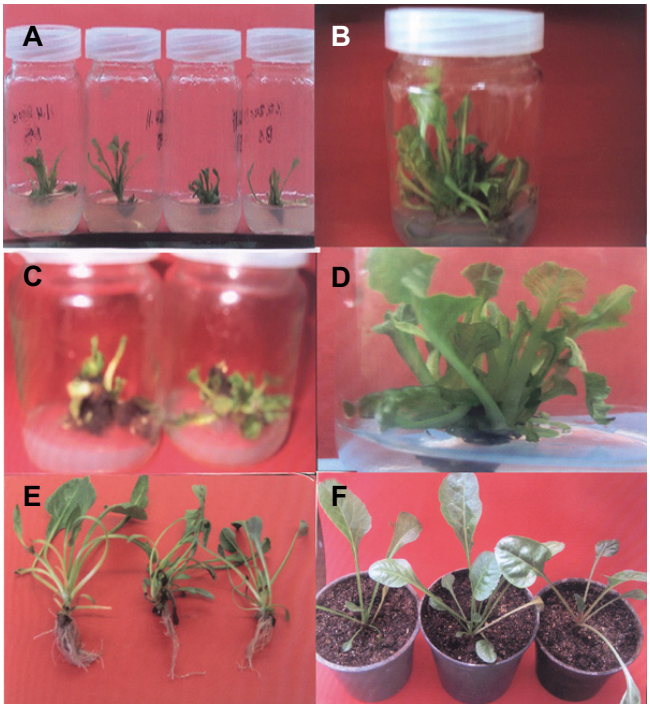

Fig. 1 Micropropagation and acclimatization stages of sugar beet. (**A**) *In vitro* grown seedlings; (**B**) Direct shoot formation from shoot base explants cultured on MS-medium containing 0.5 mg/l BA and 0.5 mg/l NAA. (**C**) Indirect shoot formation on MS-medium containing 0.5 mg/l kin and 0.5 mg/l 2,4-D. (**D**) Abundant shoots obtained from MS-medium containing 1 mg/l kin and 50 mg/l adenine sulfate. (**E**) Root formation on MS-medium containing 2 mg/l of IBA, NAA or IAA from left to right. (**F**) Acclimatized plant observed 5 weeks after their transfer from *in vitro* rooting medium to soil.

differentiation (**Fig. 1C**). Concerning PGRs added to culture media, cytokinins (BA and kin) in combinations with NAA were more effective for direct regeneration. However, 2,4-D was more suitable for indirect organogenesis. The highest percentage of direct shoot initiation from shoot base explants (93%) were registered with MS medium containing 0.5 mg/l BA + 0.5 mg/l NAA. On the other hand, the highest frequency of indirect regeneration (80%) was obtained when leaf explants were cultured on MS-medium containing 0.5 mg/l kin + 0.5 mg/l 2,4-D (**Table 1**). Similar results were reported by several researchers. Direct shoot regeneration in sugar beet was obtained from various explants including petiole (Freytag *et al*. 1988; Krens and Jamar 1989); leaf (Miedema 1982) and shoot base (Rady 1998). Kolodiazhnaia and Deĭneko (2002) examined the effect of various concentrations of synthetic hormones, such as cytokinin (6-benzaminopurine) and auxin (NAA) in the presence of inhibitor of auxin transport in plants (2,3,5-triiodobenzoic acid). Direct organogenesis from the leaf tissues of *B. vulgaris* was obtained.

Genetic variability of callus initiation and plant regeneration has been investigated among three sugar beet genotypes (Golovko 2001). It was found that TDZ has a genotype-independent effect on callus initiation and is responsible for more than a two-fold increase in the friable callus induction rate and more than a three-fold increase in the shoot regeneration rate from this callus. Along with the genotype-independent organogenesis, regeneration from callus occasionally went through the process of somatic embryogenesis in a highly genotype-specific manner. Comparing with other cytokinins, low concentrations of TDZ provide higher level of adventitious shoot formation without tissue hyperhydricity (Golovko 2001). Indirect differentiation was also achieved from callus derived from leaf explants (Doley and Saunders 1989; Owens and Eberts 1992).

Multiplication

Proliferated shoots of *B. vulgaris* were achieved on MS-medium supplemented with 1 mg/l each of BA, Kin or 2iP either alone or with addition of 50 mg/l AS. Results presented in **Table 2** indicate that a single addition of BA was effective for shoot multiplication compared to kin or 2iP. Moreover, addition of AS to culture media significantly enhanced both the number of proliferated shoots and shoot length. MS-medium containing 1 mg/l BA + 50 mg/l AS gave the highest number of shoots (5.00) as well as the highest number of leaves (3.00). However, the maximum shoot length (4.00 cm) was observed when 1 mg/l kin was combined with 50 mg/l AS (**Table 2, Fig. 1D**). The problem of vitrification was not observed. From the above mentioned results, it could be concluded that BA is the most suitable cytokinin for shoot bud proliferation of sugar beet *in vitro* and kin can be used for shoot elongation. Moreover, AS in 50 mg/l should be added to culture medium for enhancing both of the number of proliferated shoots and elongation. Our results are in line with those reported by Rady (1998) which indicated that the highest number of shoots occurred when shoot tip of sugar beet was grown on MS medium supplemented with 0.25 mg/l NAA and 1 mg/l BA. Mezei *et al*. (1990) added 0.5 mg/l BA to MS medium for plantlet formation from flower buds of sugar beet. However, Goska and Szota (1992) stated that shoot proliferation was induced from apical meristems of different types of sugar beet when cultured on MS medium supplemented with 0.09 mg/l NAA and 0.22 mg/l BA. Moreover, Wiśniewska and Majewska-Sawka (2007) reported that arabinogalactan protein-rich extracts isolated from media of embryogenic and non-embryogenic suspension cultures of *B. vulgaris* were able to enhance the organogenesis of guard protoplast-derived callus and to increase the number of shoots formed, in comparison to control cultures the positive role of AS as an important stimulation purine in tissue culture medium was reported by several researchers (Okasha *et al*. 1996; Bekheet 1999).

Table 1 Organogenesis percentage of leaf (L) and shoot base (Sb) explants of sugar beet as affected by different combinations of cytokinin and auxin.

Growth regulators (mg/l)	Direct organogenesis (%)		Indirect organogenesis (%)	
	L	Sb	L	Sb
0.5 BA + 0.1 NAA	26 c	86 a	13 c	6 d
0.5 BA + 0.5 NAA	33 c	93 a	20 c	6 d
0.5 BA + 0.1 2,4-D	0 d	40 c	60 b	33 c
0.5 BA + 0.5 2,4-D	0 d	46 c	66 b	40 c
0.5 kin + 0.1 NAA	20 c	33 c	33 c	20 c
0.5 kin + 0.5 NAA	26 c	40 c	40 c	26 c
0.5 kin + 0.1 2,4-D	6 d	26 c	73 b	46 c
0.5 kin + 0.5 2,4-D	13 c	20 c	80 a	53 b

Leaf explants (L), Shoot base explants (Sb).
Numbers in the same column followed by the same letter are not significantly different ($P \leq 0.05$)

Table 2 multiplication of *in vitro* regenerated sugar beet plantlets as affected by growth regulators and adenine sulfate (AS).

Culture medium	Proliferated shoots (mean ± S.E.)	Shoot length (cm) ± SE	Leaves/shoot (mean ± S.E.)
MS + 1 mg/l BA	4.30 ± 0.25 a	1.90 ± 0.09 b	2.50 ± 0.21 b
MS + 1 mg/l kin	3.90 ± 0.20 b	3.10 ± 0.20 a	2.00 ± 0.10 b
MS + 1 mg/l 2iP	2.50 ± 0. 15 b	2.40 ± 0.11 b	2.00 ± 0.27 b
MS + 1 mg/l BA + 50 mg/l AS	5.00 ± 0. 33 a	2.10 ± 0.17 b	3.00 ± 0.12 b
MS + 1 mg/l Kin + 50 mg/l AS	4.30 ± 0.22 a	4.00 ± 0.30 a	2.75 ± 0.19 b
MS + 1 mg/l 2iP + 50 mg/l AS	3.00 ± 0.30 b	3.00 ± 0.20 a	2.50 ± 0.15 b

Table 3 Rooting of *in vitro* regenerated sugar beet shoots as affected by different types of auxin.

Rooting media	Root formation (%)	№ of roots (mean ± S.E.)	Root length (cm) ± S.E.
MS + 1 mg/l NAA	10 b	2.20 ± 0.12 b	1.90 ± 0.19 b
MS + 2 mg/l NAA	20 b	2.50 ± 0.15 b	3.10 ± 0.13 a
MS + 1 mg/l IBA	60 b	4.00 ± 0.22 a	1.70 ± 0.11 b
MS + 2 mg/l IBA	85 a	4.60 ± 0.19 a	2.10 ± 0.15 b
MS + 1 mg/l IAA	30 b	3.40 ± 0.25 b	1.00 ± 0.12 b
MS + 2 mg/l IAA	40 b	3.00 ± 0.10 b	1.50 ± 0.20 b

Numbers in the same column followed by the same letter are not significantly different ($P \leq 0.05$). Standard Error: S.E.

In vitro root formation

Root formation is an obligatory phase for micropropagation of plants reproduced *in vitro*. Some of them initiate roots without special treatments while others require a medium supplemented with different PGRs essentially of an auxin nature. Different plant species might vary in their requirement of auxin type for adventitious root formation. In this part of work, the effect of three types of auxins (IBA, IAA and NAA) added separately in two concentrations (1 and 2 mg/l) on *in vitro* rooting of sugar beet shoots was investigated. There were significant differences in response to root formation, root number and length. Data of **Table 3** indicates that increasing of auxin concentration showed promoting effect on rooting parameters. IBA was the most suitable type of auxin for *in vitro* rooting of sugar beet compared with IAA and NAA. The highest percentages of root formation (85%) as well as number of roots (4.60) were noticed when 2 mg/l IBA and 0.03% of activated charcoal were added to culture medium However, the highest value of root length (3.10 cm) was obtained when 2 mg/l of NAA was incorporated into the medium (**Table 3, Fig. 1E**). The present results are in accordance with those reported by Goska and Rogozinska (1990). Their results indicated that MS medium with 2 mg/l IBA was the best for root development of sugar beet *in vitro*. On the other hand, Mezei *et al*. (1990) mentioned that the best rooting of sugar beet plantlets produced *in vitro* was achieved on auxin-free medium.

Adaptation and acclimatization

The success of *in vitro* methods in plant propagation depends not only on the number of plantlets produced but also on their survival rate upon transfer to nursery and field conditions. In our study, complete plantlets of sugar beet with good root system were easily adapted to the free environmental conditions. High percentage of survival was obtained after five weeks of transplanting. Three weeks after transfer, new leaves were produced. At the end of the fifth week, the plantlets grew into plants of normal appearance (**Fig. 1F**). As we observed, the most essential requirement for successful transplantation is to maintain the plants under a very high humidity especially in the first 15 days by covering them with transparent plastic bags. Small holes were pored in the bags for air circulation. Moreover, partial defoliation of plantlets at the time of transplantation is beneficial. The regenerated plants showed no morphological differences from those grown *in vivo*.

SDS-PAGE protein analysis

Total soluble proteins of three types of regenerates of sugar beet in addition to their parents (growing *in vivo*) were extracted and subjected to protein electrophoresis to detect their variation. The protein profiles were analyzed by SDS-PAGE under reducing conditions. The electrophoretic protein banding patterns of different regenerated cultures in addition to *in vivo* grown plants (control) of sugar beet illustrated in **Fig. 2** showed three bands (35, 60 and 80 KD) which were clearly expressed in different regenerated tissue cultures. Similar electrophoretic migration rate was observed

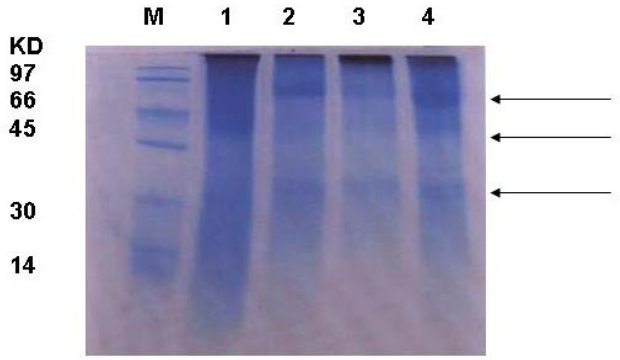

Fig. 2 SDS-PAGE pattern of protein extracted from different sources. Lane 1: *in vivo* grown plants. Lane 2: plantlets derived from proliferation of shoot tips. Lane 3: indirect organogenesis. Lane 4: direct organogenesis. Lane M: control treatment. Molecular weight marker is indicated in kD at the right side of the figure, while protein variations are indicated on the left side by arrows.

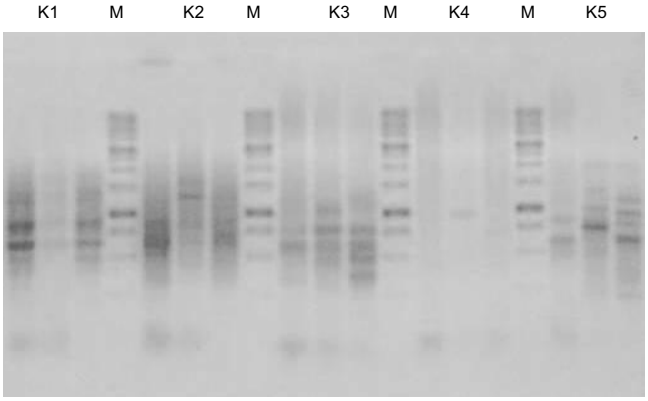

Fig. 3 RAPD profile of proliferated shoots, direct organogenesis and indirect organogenesis. From left to right respectively using primers (k1-k5) and DNA ladder Marker of molecular weight, i.e. 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp (M).

with the three types of tissue cultures. In conclusion, plantlets originated from proliferated shoots, via direct and indirect organogenesis are identical in their polypeptides and similar in protein pattern with those *in vivo* grown. The present results are in accordance with those reported by El-Kazzaz and Taha (2002) and Bekheet (2004) on broccoli and garlic, respectively. On the other hand, Metry *et al*. (2003), in their study on genetic stability of transgenic potato expressing *cry1Aa7* gene reported that, protein banding profiles were not sufficient to detect variations among transgenic and non-transgenic lines.

RAPD analysis

DNA isolated from proliferated shoots, via direct and indirect organogenesis was subjected to RAPD analysis. Five random primers (K1-K5) were screened in RAPD analysis and the result generated by PCR amplification was presented in **Fig. 3**. The number of fragments generated per primer varied between 4 to 12 (**Table 4**). The total number of bands was 46 and the average percentage of polymorphism was 19.2. The primers K3 and K5 gave the largest number of amplified bands (12) and the highest percentage of polymorphism (28.0) was observed with primer K2. However, primer k1 gave the most similar patterns of bands. In general, the results of banding reveal that there is a slight genetic variation between the three types of *in vitro* regenerates of sugar beet. This finding may suggest that the protein banding profile were not sufficient to detect variation among the *in vitro* regenerated plants of sugar beet. The present results are in accordance with those reported by Toldi *et al*. (1996). They mentioned that shoot regeneration through di-

Table 4 The sequence of selected random primers, total number of amplification products per primer, number of polymorphic bands and percentage of polymorphism

Primer	Sequence (5′→3′)	№ of bands	№ of polymorphic bands	Polymorphism (%)
K1	TGGCGACCTG	11	1	9.0
K 2	GAGGCGTCGC	7	2	28.6
K 3	CCCTACCGAC	12	2	16.7
K4	TCGTTCCGCC	4	1	25.0
K5	CACCTTTCCC	12	2	16.7

rect organogenesis is the most efficient way to produce true-type regenerates in sugar beet. Moreover, Jacq *et al*. (1992) found that organogenesis in sugar beet is less genotype dependent and regenerates are genetically stable. In this respect, genetic marker analysis has been used to study the degree of genetic change in plants regenerated *in vitro* such as pea (Cecchini *et al*. 1992), sugar beet (Sabir *et al*. 1992) and wheat (Brown *et al*. 1993).

ACKNOWLEDGEMENTS

This work was sponsored by ICGEB collaborative research program, Project No. CRP/EGY05-04.

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