Somatic Embryos from Callus of Sugarbeet Biotechnology Clone REL-1

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ABSTRACT

Somatic embryos could be used for proliferative propagation or for gene transfer procedures in sugarbeet (*Beta vulgaris* L.) if adequate methods for initiation and development could be devised. With sugarbeet model clone REL-1, plating of fresh suspension culture cells grown with hormone-free Murashige and Skoog (MS) medium onto further hormone-free MS medium elicited a low frequency of somatic embryogenesis, about one embryo per ml of suspension used. The inclusion of 0.1 or 0.3 mg/l abscisic acid (ABA) in the plating medium increased the number of somatic embryos in this system. A combination of naphthaleneacetic acid (NAA) (1 mg/1) and ABA (0.1 mg/1) gave the highest somatic embryo yield, 15 embryos per ml of suspension. After 22 to 40 days, embryos at various stages, ranging from globular, heart, and torpedo-shaped embryos to mature opaque white embryos with cotyledons and radicles, were present at the callus surface. The external morphology of several somatic embryos was examined by scanning electron microscopy. The somatic embryos developed into normal plantlets, including betalain pigmentation on hypocotyls, after being transferred onto hormone-free MS medium. The conversion rate of somatic embryos of different lengths (1, 2, 3 mm) into complete plantlets was similar (78, 81 and 86%, respectively). Secondary embryogenesis, which would be useful in providing somatic embryos for gene transfer purposes, was not observed in this study.

Additional Key Words: *Beta vulgaris* L., benzyladenine, tissue culture, triiodobenzoic acid, callus, betalain

Somatic embryogenesis is the process of embryo initiation and development from somatic, i.e., non-germ, cells. In plant tissue culture, somatic embryos have most often been produced from callus or suspension culture cells under appropriate culture conditions, with genotype dependency being common. Somatic embryos currently are under investigation in species such as alfalfa and celery for use in the production of artificial seeds (Gray and Purohit, 1991). Elite highly productive individual genotypes from genetically heterogeneous cultivars, or superior-combining male-sterile clones, can be vegetatively reproduced on a large scale as somatic embryos and delivered to the field in a seedlike fashion following conditioning and coating, or, alternatively, by fluid drilling. Somatic embryos also can be used in genetic transformation applications as exemplified in gene transfer with *Agrobacterium* in walnut (McGranahan et al. 1990), grape (Le Gall et al.) and *Datura innoxia* (Ducrocq et al. 1994). Synthetic seed production, as well as gene transfer, would be more efficient if somatic embryogenesis also occurred via immediate subsequent cycles wherein multiple new embryos arose from existing ones. This self-replicating production is termed secondary somatic embryogenesis.

Somatic embryos in sugarbeet were first reported by Atanasov (1976) in suspension cultures. Tetu et al. (1987) concluded that multiple hormonal sequences were necessary for the induction and development from callus of somatic embryos they noticed. A low frequency of somatic embryogenesis in callus was reported by Freytag et al. (1988) in all six germplasm sources tested. Kubalakova (1990) described embryogenic callus that had maintained its embryogenic ability for three years on media without growth regulators, from at least one genotype. Somatic embryos were directly produced on sugarbeet zygotic embryos by Tenning et al. (1992), who observed some direct secondary embryogenesis. Doley and Saunders (1989) reported the simple production and germination of somatic embryos from callus from leaf discs of a fodder beet cultivar cultured on medium without the use of growth regulators. D'Halluin et al. (1992) used embryogenic callus from seedlings in an *Agrobacterium*-mediated genetic transformation system for sugarbeet.

REL-1 is a self-fertile diploid sugarbeet clone bred for ease of tissue culture manipulations, with high frequency of leaf disc callusing, shoot regeneration from callus, dispersed suspension cultures and resistance to shoot vitrification. REL-1 is also heterozygous for monogermness (*Mm*), annualism (*Bb*), and red hypocotyl (*Rr*). REL-1 has been used for the recovery of monogenic dominant sulfonylurea herbicide resistance by somatic cell selection (Saunders

et al. 1992) and of other mutant traits (Saunders et al. 1990).

We report here (1) the initial success in obtaining somatic embryos from REL-1, (2) converting somatic embryos into plantlets, and (3) examination of later stages of somatic embryos under scanning electron microscopy.

MATERIALS AND METHODS

Abbreviations.

MS: Murashige and Skoog inorganic salts (1962)
aba: (±) *cis, trans*-abscisic acid
NAA: 1-naphthaleneacetic acid
BA: 6-benzyladenine
TIBA: 2,3,5-triiodobenzoic acid
SEM: scanning electron microscope

Plant materials. All experiments were performed with the diploid sugarbeet (*Beta vulgaris* L.) clone REL-1, released to the public in 1987. REL-1 has been maintained in shoot culture (Saunders, 1982) and is available upon request, either as in vitro shoots, whole plants, or S_1 seed.

Culture media. The culture media contained MS mineral salts (Murashige and Skoog, 1962), 100 mg/l myo-inositol, 1.0 mg/l thiamine·HCl, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine·HCl, and 30 g/l sucrose. Media used for plating were gelled with 3.5 g/l phytagel. Growth regulators used were BA (1.0 mg/1), TIBA (1.0 mg/1), NAA (0.25-1.0 mg/1), and/or ABA (0.1-1.0 mg/1). The pH was adjusted to 5.95 prior to autoclaving. ABA was filter-sterilized and added to previously autoclaved and partially cooled media. Culture vessels were 125 ml Erlenmeyer flasks or 20 x 100 mm Falcon (Becton Dickinson, Lincoln Park NJ) disposable plastic Petri plates. Medium volume per vessel was 35 ml. Flasks were closed with foam caps and aluminum foil. Petri plates were sealed with one layer of Parafilm (American National Can, Neenah WI) wax film.

Initiation of callus and maintenance of cell suspensions. Callus was initiated from leaf discs (8 mm diameter) from partially expanded leaves of greenhouse-grown REL-1 plants. Discs were cultured on MS medium with 1.0 mg/l BA (Saunders et al. 1992) and 0.9 % Bacto (Difco, Detroit MI) agar in Petri plates at 30 C in the dark. Callus first appeared after one month. After another month, 2 to 3 g of leaf-disc callus was transferred to hormone-free liquid MS medium. Flasks were

incubated at 21 ± 2 C in the dark on rotary shakers at 120 rpm to aerate the cultures and to reduce cell cluster size.

Induction of somatic embryos. After 14 days, suspension cultures used as inoculum were pushed through a stainless steel sieve with 830 μm openings. Sieved suspension cells were washed with hormone-free liquid medium and plated onto MS media with no growth regulators or with combinations of BA & NAA, or of NAA, ABA, & TIBA. Each Petri plate received 1 ml of log phase suspension preparation containing two to three hundred cell clusters (about 0.1 g fresh weight), and was incubated in dim light (less than 5 μmol m^{-2} s^{-1} from fluorescent lamps) at 25 C. Minimum size for an embryo to be counted was 0.5 mm.

Germination and conversion of somatic embryos. Somatic embryos were transferred onto hormone-free MS medium and placed under light (20-50 μmol m^{-2} s^{-1} from fluorescent lamps) at 25 C. The proportion that developed into normal plantlets with roots constituted the conversion rate of somatic embryos into complete plantlets.

Scanning electron microscopy. According to the procedures of Flegler et al. (1993), somatic embryos were fixed in 2 % glutaraldehyde in 0.1 M cacodylate buffer for 1 hour, and washed with two changes of buffer for 10 minutes each. Dehydration was carried out gradually by immersion for 10 minutes each in 25, 50, 75, 95 and 100 % ethanol with two additional final changes of 100 % ethanol. Fixed somatic embryos were dried in a Balzers critical point dryer (Balzers A.G., Fuerstentum, Liechtenstein) and then mounted and sputter coated with gold, and examined with a JSM-6400 scanning electron microscope (Japanese Electron Optics Laboratory, Peabody, MA).

Data analysis. Analysis of variance was based on a random complete block design. The average number of somatic embryos per plate for each media treatment was subjected to ANOVA and the least significant difference (LSD) test ($P = 0.05$) was performed to permit individual treatment comparisons.

RESULTS AND DISCUSSION

Induction of somatic embryos. A low frequency of somatic embryogenesis, almost one embryo per Petri plate, was obtained following plating of suspension culture cells on hormone-free MS medium. NAA and BA tested either individually or in combination did not

permit production of significantly more somatic embryos than with the hormone-free medium. Rather, the presence of BA at 0.5 or 1.0 mg/l markedly reduced the occurrence of somatic embryos relative to the hormone-free medium (Table 1). On the other hand, ABA at 0.1 or 0.3 mg/l in the media increased the number of somatic embryos per plate up to eight fold (Fig. 1). The highest somatic embryo yield of 15 per plate was attained with the combination of 1 mg/l NAA and 0.1 mg/l ABA (Fig. 1). ABA is best known for its positive effects in somatic embryogenesis where it normalizes development and inhibits precocious germination (Ammirato, 1974, 1983). TIBA was not stimulatory to somatic embryogenesis obtained with REL-1 and the described procedure, even though Doley (1990) found that TIBA promoted bud formation with callus of REL-1. Tetu et al. (1987) reported a stimulatory effect of TIBA on bud formation from callus of another genetic background.

Development of somatic embryos. During the first two weeks following plating-out, white or light yellow callus grew. From the third to the sixth week, various late stages of somatic embryos from torpedo to mature opaque white embryos with cotyledons were clearly present at the callus surface (Fig. 2A and B). The simultaneous occurence of embryos of different lengths (0.5 to 4 mm) (Fig. 2C) indicated that somatic embryogenesis was not uniform and synchronous. After 40 days, most of the somatic embryos with cotyledons were around 2-3 mm long. Each somatic embryo could be easily separated from the surrounding callus.

Conversion of somatic embryos into plantlets. After being transferred onto hormone-free MS medium, the cotyledons and radicle of the bipolar embryos gradually developed simultaneously and germination into normal plantlets occurred (Fig. 2D). The germination rate of somatic embryos longer than 2 mm was 100 percent. However, the proportion of conversion into complete plantlets was somewhat less because of subsequent callusing of some embryos or the formation of abnormal plants. The conversion rate of somatic embryos of different sizes into complete plantlets was up to 86 % (Table 2).

Embryo morphology could have affected subsequent germination and conversion of somatic embryos into plantlets. Abnormal somatic embryos are common in other species, for example, caraway (Ammirato, 1974) and soybeans (Buchheim et al. 1989). Achieving a high degree of normal morphology usually involves optimizing the culture medium components and environmental conditions.

Table 1. The effect of BA and NAA concentrations on somatic embryogenesis. One ml of suspension cells was plated onto media with various combinations of BA and NAA and scored 68 days after plating. Means marked with same letter are not significantly different according to LSD with $P < 0.05$.

		Number of somatic embryos per plate			
		BA			
	(mg/l)	0	0.25	0.5	1.0
NAA	0.0	0.75 ab	0.25 bc	0.0 c	0.0 c
NAA	0.25	1.0 a	0.25 bc	0.08 c	0.0 c
NAA	0.5	0.67 ab	0.0 c	0.0 c	0.0 c
NAA	1.0	1.08 a	0.08 c	0.0 c	0.08 c

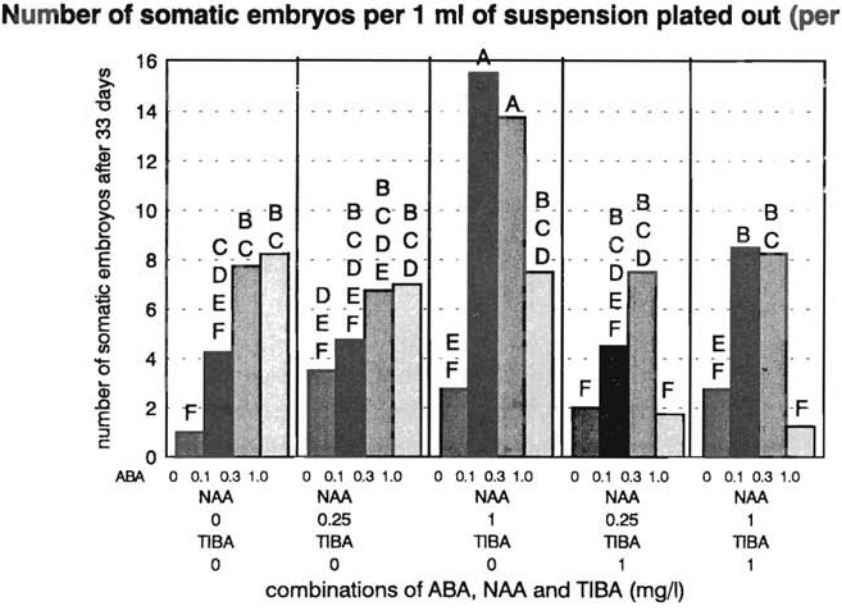

Figure 1. The effect of ABA, NAA, and TIBA concentrations on somatic embryogenesis. One ml of suspension cells was plated onto media with combinations of ABA, NAA and TIBA and scored 33 days after plating. Means marked with same letter are not significantly different according to LSD with $P < 0.05$.

Scanning electron microscopy. The development of somatic embryos from REL-1 callus resulted in torpedo (Fig. 3A and B) and cotyledonary (Fig. 3C) stage embryos. Although somatic embryogeny is reported to mimic zygotic embryogeny in many aspects (Crouch, 1982; Sharp et al. 1980), this has yet to be demonstrated in sugarbeet. Artschwager (1927) and Artschwager and Starrett (1933) have described the anatomy of embryo and seed development in sugarbeet. A more detailed comparison of somatic with zygotic embryogenesis would involve detection of earlier stages in or on callus, or the use of liquid cultures.

With SEM, trichomes could be seen on the cotyledonary surface of some abnormal embryos (Fig. 3D). Gray et al. (1993) reported trichome development on precociously germinating cotyledonary stage embryos from *Cucumis melo.*

Based on the initial research reported here, model clone REL-1 is capable of at least moderate intensities of production of somatic embryos, which then are easily collected and converted into plantlets. This is the first report of sugarbeet somatic embryos that describes the use and promotive effect of ABA on embryo number. In order for REL-1 embryos to be useful in gene transfer research, conditions for more prolific embryo production must be developed, perhaps through secondary embryogenesis. If somatic embryos are to find a direct application in the field, conditions must be found for their massive proliferation in other genotypes. It is encouraging to note that both Tenning et al. (1992) and Kubalakova (1990) reported some secondary embryogenesis in their cultures, using procedures which were not tried in this work with REL-1.

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Table 2. Effect of embryo length on the proportions of somatic embryos germinating and converting into complete plantlets.

Length of somatic embryos (mm)	Germination rate (%)	Conversion rate (%)
0.5 -1.9	88 (61/69)	78 (54/69)
2.0 -2.9	100 (96/96)	81 (78/96)
3.0 -3.9	100 (28/28)	86 (24/28)

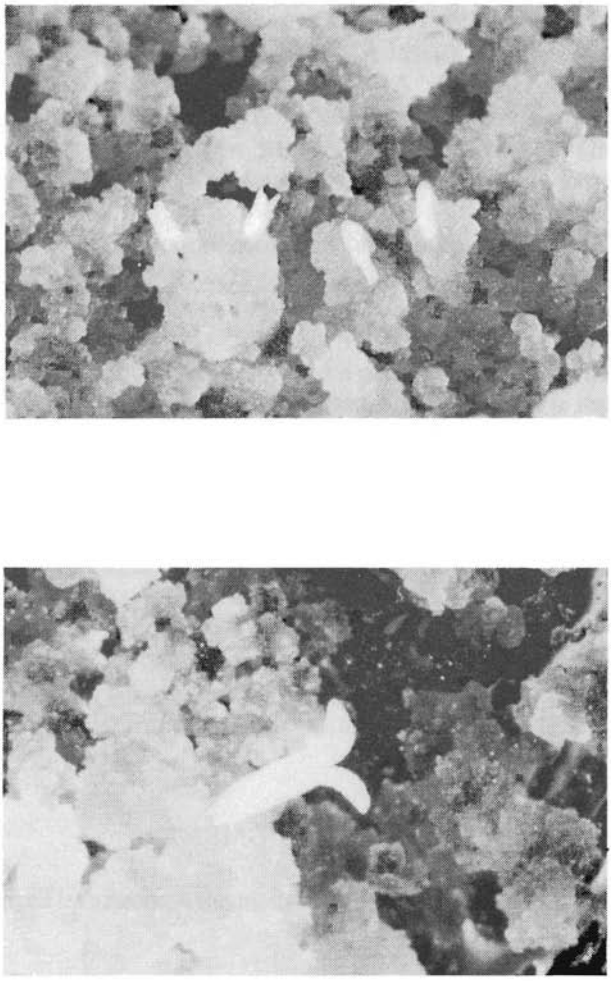


Figure 2. A) Opaque white somatic embryos (1.5 mm long) on the surface of callus tissue 22 days after suspension plating. B) A 3 mm long tricotyledonary somatic embryo 30 days after suspension plating.

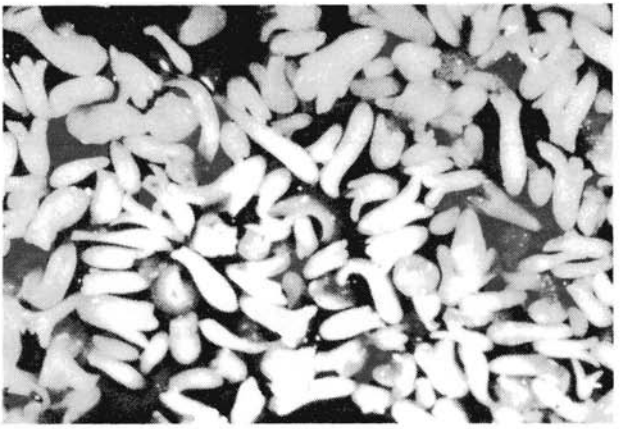


Figure 2, *continued.* C) Isolated somatic embryos (length: 0.5 - 4 mm). D) Somatic embryo-derived complete plantlet, 10 days after being transferred to fresh hormone free MS medium (length: 20 mm).

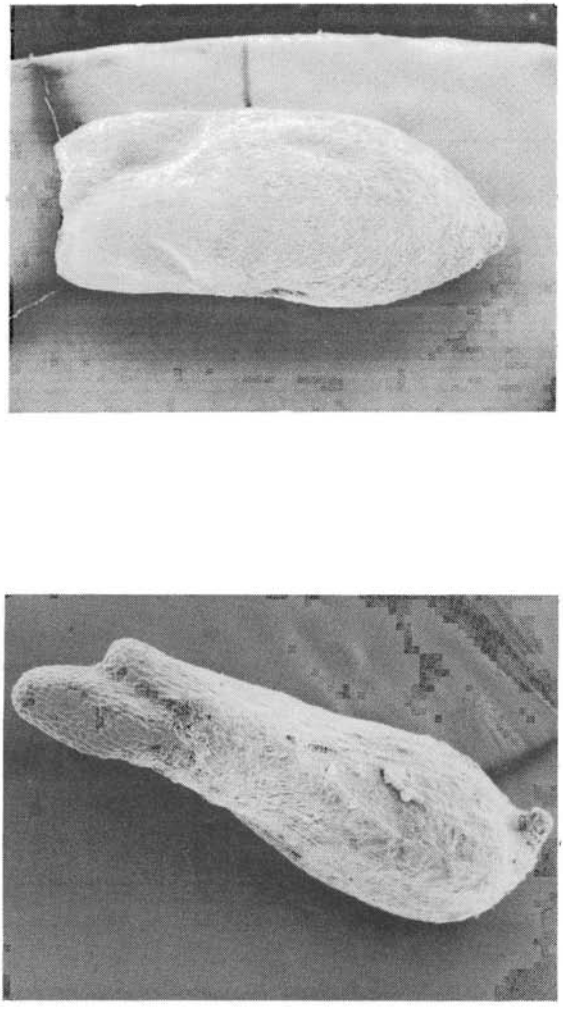
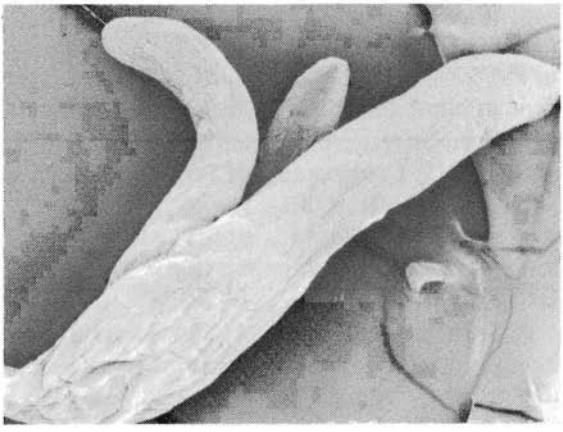


Figure 3. A) SEM photograph of an early torpedo stage somatic embryo (length: 0.5 mm). B) SEM photograph showing a torpedo stage somatic embryo (length: 2 mm).



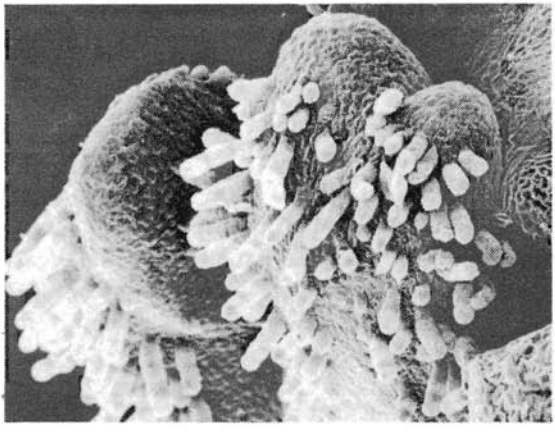


Figure 3, *continued.* C) SEM photograph showing a late cotyledonary stage somatic embryo (length: 3 mm). D) SEM photograph showing a somatic embryo with trichomes on the cotyledonary surface.

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