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Studies of *In Vitro* Propagation Systems for Sugar Beet

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Abstract

Commercial cultivars of *Beta vulgaris* (sugar beet) and an annual diploid line, as well as some breeder's materials developed in Ireland, were used in studies on several *in vitro* culture procedures. An efficient micropropagation system, based on cytokinin-mediated release of apical dominance in axenic shoot cultures, was established for all genotypes. Callus was initiated by several combinations of naphthalene acetic acid and benzyl aminopurine, and was maintained indefinitely on a defined medium. Shoot regeneration from callus developed from petiole explants was achieved using two different protocols but somatic embryogenesis was not observed. The best frequency of shoot regeneration achieved was 16% of calli responding with 1 to 30 adventitious buds developing on responsive calli. A number of apparently normal, rooted plants were obtained from regenerants. Out of 200 ovaries dissected from flowering spikes of cv. Hilma, two ovules developed into plantlets after a two-step culture procedure.

Keywords: Micropropagation; sugar beet; tissue culture

Introduction

Sugar beet is an important crop in Ireland, despite the sub-optimal climate which results in yields which are generally lower than those in most continental European countries. This has led to the recognition that research needs to focus on more efficient utilisation of solar radiation for the production of root storage sugar, either by breeding cultivars suited to Irish conditions or by altering husbandry practices (Burke, O'Connor and Herlihy, 1986). Currently, late sowing, needed to minimise frost damage and vernalisation, results in plants which are too small to utilise the peak solar radiation of May and June. Earlier sowing depends on either the development of cold-tolerant and bolting-resistant cultivars, or seedling protection (e.g. germination in a protected environment followed by transplanting). Considerable progress has been made in both these areas. Bolting-resistant cultivars have been produced in Ireland (O'Connor and Fitzgerald, 1987) and efficient transplanting technology is now available for sugar beet (Burke *et al.*, 1986).

In vitro culture technology has the potential to contribute to these developments in two ways. Firstly, there is considerable support (Nabors, 1990) for the selection *in vitro* of germplasm with enhanced tolerance to environmental stresses as an adjunct to conventional breeding practices. Secondly, clonal propagation of sugar beet, either through axenic shoot cultures, or through the encapsulation of somatic embryos to produce "artificial seeds" (Redenbaugh *et al.*, 1986) could provide uniform propagules for integration with transplanting

technology in a new production system. An attraction of this approach lies in the clonal uniformity obtainable using elite genotypes in this crop in which commercial cultivars are triploid hybrids and seed production is costly.

The type of tissue culture system required for genome modification *in vitro* clearly depends on the kind of genetic manipulation undertaken (Collin and Dix, 1990). For example, both mutant selection and genetic transformation can be carried out using a variety of systems, ranging from protoplasts (e.g Bourgin, 1983; Negrutiu *et al.*, 1987) to organogenic explants (Horsch *et al.*, 1985; McCabe, Timmons and Dix, 1989), while genetic recombination via cell fusion is dependent on an efficient and reproducible protoplast culture system (Gleba and Shlumukov, 1990). Clonal propagation can also proceed through techniques which exploit existing meristems or adventitious shoots or embryos, which in turn can develop from organised explants or undifferentiated cultures (Hussey, 1986).

Clearly, a range of *in vitro* culture procedures could have valuable applications in sugar beet production and breeding. Additional to these applied objectives, tissue cultures constitute valuable research tools which can contribute to understanding of the physiological basis of environmental stress injury (Dix, Plunkett and Toth, 1983; Van Swaaij *et al.*, 1986). It is unfortunate, therefore, that as for many other agronomically important crops, only a limited repertoire of facile *in vitro* culture techniques are available for sugar beet. Shoot cultures can be maintained *in vitro* (Hussey and Hepher, 1978), but regeneration from callus (Saunders and Daub, 1984; Tétu, Sangwan and Sangwan-Norreel, 1987; Freytag *et al.*, 1988; Ritchie, Short and Davey, 1989) tends to be erratic, strongly cultivar dependent, and occurs at too low a frequency to commend it either for clonal propagation, or for *in vitro* selection. Exceptionally, however, it has been possible, for certain cultivars, to establish continuously regenerating lines from hormone-treated autonomous cell cultures (Van Geyt and Jacobs, 1985). Where regeneration from undifferentiated cultures has been accomplished this generally proceeds through adventitious shoot initiation, somatic embryos being observed only occasionally (Freytag *et al.*, 1988). Successful protoplast culture, including subsequent plant regeneration, is also highly problematic and genotype dependent. It is encouraging that the first successful report has appeared (Krens *et al.* 1990), but so far this is restricted to diploid beet and two responsive accessions.

Clearly, considerably more research is needed on *in vitro* culture of sugar beet. The present report describes the results of investigations into clonal propagation of sugar beet, aimed at providing uniform material for physiological studies on cultivars differing in cold tolerance, and regeneration from undifferentiated cultures. This is a first step towards longer-term goals of breeding for enhanced stress tolerance and development of artificial seed technology for this species.

Materials and Methods

Plant material

Seeds and whole plants of sugar beet cultivars, as well as a monogerm diploid (annual) beet, were obtained from Oak Park Research Centre, Carlow.

Culture media

The basal culture media used in these investigations are described in Table 1. The media based on Murashige and Skoog (1962) were prepared using MS salt mix (Flow Laboratories) as a supply of all the mineral components.

Plant growth regulators (indole acetic acid (IAA), naphthalene acetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), indole butyric acid (IBA), kinetin, 6-benzyl amino purine (BAP) and tri-iodo benzoic acid (TIBA)) were all obtained from Sigma Chemical Company. Concentrations used are described in the relevant Results section.

Sterilisation of plant material

Seeds, with seed coat removed, unopened flowers or explants, were dipped in 70% ethanol, transferred to 20% domestic bleach (Domestos) for 20 min and washed three times in sterile distilled water. Seeds were placed on RM medium (Table 1) in 9 cm petri dishes, sealed with parafilm and incubated at 25°C. These, and all other cultures, were maintained under a 16-hour photoperiod (2,000 lux, cool-white plus warm-white tubes). The initiation of shoot cultures from the seedlings, as well as details of callus initiation and shoot regeneration experiments, are described in the appropriate Results sections.

Ovule culture

Flowering spikes were obtained from plants which had bolted in the field. Spikes were surface-sterilised and ovaries dissected out of unopened flowers, using a dissecting microscope. Ovaries were placed, cut surface downwards, on MS

TABLE 1: Composition of basal culture media (mg l^{-1})

Medium	RV[a]	RM[d]	MS[b]	P_{GO}
Inorganic salts	MS[b]	MS[b]	MS[b]	§
Meso-inositol			100	
P-amino benzoic acid	0.2			
Ascorbic acid	0.4			
D-biotin	0.00025			
Choline chloride	0.2			
Folic acid	0.015			
Nicotinic acid	0.5		0.5	1.0
D-pantothenic acid	0.4			
Pyridoxine HCl	0.5		0.5	1.0
Riboflavin	0.015			
Thiamine HCl	0.5		0.1	10
L-arginine	40			
L-asparagine	40			
L-glycine	20		2.0	
L-glutamine	60			
L-phenylalanine	20			
L-trytophan	40			
Sucrose	25,000	30,000	30,000	30,000
Agar (Difco)[e]	6,500	7,000	8,000	7,500

§Inorganic salts of P_{GO} (mg l^{-1}):

NaH_2PO_4	250	H_3BO_3	0.62	Na_2EDTA	37.3
KCL	600	$MnSO_4.H_2O$	1.68	$FeSO_4.7H_2O$	27.8
$(NH_4)_2SO_4$	400	$Zn\ SO_4.7H_2O$	1.06		
$MgSO_4.7H_2O$	500	KI	1.58		
KNO_3	2,000	$Na_2MoO_4.2H_2O$	0.0025		
$CaCl_2.2H_2O$	300	$CuSO_4.5H_2O$	0.0025		
		$CoCl_2.6H_2O$	0.0025		

[a]Freytag *et al.* (1988); [b]Murashige and Skoog (1962); [c]Modified DeGreef and Jacobs (1979); [d]Maliga (1984); [e]pH values adjusted to 5.6 before addition of agar

TABLE 2: Effect of different combinations and concentrations of plant growth hormones[1] on the growth of callus and on adventitious root production on petiole pieces of sugar beet cv. Hilma on MS medium. Observations made 4 weeks after initiation

	2,4-D (mg l^{-1})			NAA (mg l^{-1})			IAA (mg l^{-1})		
	0.1	0.3	1.0	0.1	0.3	1.0	0.1	0.3	1.0
Kinetin (mg l^{-1})									
0.1	+	+	+ +	−	+ (R)	+ (R)	+	+ (R)	+ (R)
0.3	+ +	+ +	+	+	+	+ + (R)	+ (R)	+ (R)	+ (R)
1.0	+ + +	+ + +	+ +	+	+ +	+ + + (R)	+	+ + (R)	+ + (R)
BAP (mg l^{-1})									
0.1	+ +	+ +	+ +	+	+	+	+	+ (R)	+ (R)
0.3	+ +	+ + +	+ +	+	+ + (R)	+ + (R)	+	+	+
1.0	+ + +	+ + +	+ + +	+	+ +	+ + + (R)	+	+	+

[1]2,4-D = 2,4-dichlorophenoxyacetic acid; NAA = naphthalene acetic acid; IAA = indole acetic acid; BAP = 6-benzyl amino purine

− = no callus; + = callus < 2 times size of explant; + + = callus 2 to 3 times size of explant; + + + = callus > 3 times size of explant; R = root production

medium (Table 1) containing 0.5 (w/v) activated charcoal and supplemented with BAP (3.0 mg l^{-1}), NAA (0.1 mg l^{-1}) and 2,4-D (0.5 mg l^{-1}). After 2 to 3 weeks ovaries turned brown and ovules were dissected out and placed on MS medium containing BAP (1.0 mg l^{-1}) and NAA (1.0 mg l^{-1}).

Results

Shoot cultures

Shoots from 2-week-old seedlings (see Materials and Methods) were excised above the cotyledons and placed on MS medium containing 0.3 mg l^{-1} BAP. Axillary buds rapidly developed into shoots and after 3 to 4 weeks could either be transferred to 50 ml of the same medium in 250 ml plastic containers (Irish Merchants, Dublin) for further multiplication or to MS medium without hormones to give single rooted plantlets. The same process could be carried out with nodal cuttings from shoot cultures. Rooting of shoots or nodal cuttings was rare on medium containing BAP.

Callus initiation and organogenesis on explants

In all these experiments, seedlings or axenic shoot cultures grown on RM or MS medium, respectively, without hormones, were used. In the case of petioles, the basal 1 cm was excluded to ensure the absence of axillary buds. Petiole explants (0.5 to 1.0 cm long), leaves (0.3 cm wide), hypocotyls and cotyledons, from axenic seedlings of cvs. Hilma, Monofeb and Winter Hybrid, were placed on MS medium containing different combinations of growth hormones (Table 2). A similar response was obtained with all three cultivars and the most responsive tissues, for callus and adventitious root initiation, were hypocotyl and petiole explants. Very little callus developed on leaves or cotyledons. Detailed results are given for petiole segments of Hilma in Table 2. Generally, poor callusing was achieved with IAA, although it was effective in inducing adventitious roots, particularly in association with kinetin. Highest callus yields (without roots) were obtained with 2,4-D, although these could be approached with the highest level (1 mg l^{-1}) of NAA in combination with 1 mg l^{-1} of either kinetin or BAP (Table 2). Under the latter conditions, roots were also produced.

In view of the responsiveness of petioles and the abundant production of this material in shoot cultures, this tissue was chosen for further experiments with the diploid beet, in which the effectiveness of the P_{GO} and RV basal media (Table 1) were compared. Petiole explants (1 to 2 cm) were placed on media containing a range of NAA and BAP concentrations. Visual observations were recorded after 2 weeks (Table 3) and fresh weight determinations made after 4 weeks (Table 4). A supply of both NAA and BAP was essential for callus initiation and RV supported much more rapid growth than P_{GO}. The actual concentrations of NAA and BAP had only a small influence on callus proliferation on RV but the lowest level of NAA (0.1 mg l^{-1}) appeared the best. Root initiation depended on the presence of NAA and tended to be reduced by inclusion of BAP, except at the highest NAA level on P_{GO} and some intermediate treatments on RV medium.

Callus was subcultured onto fresh medium after 4 weeks. The callus on P_{GO} medium grew poorly and could not be maintained indefinitely. On RV medium, however, vigorous growth was maintained after subculture and continuous cultures have been established. The best hormone levels were 0.1 mg l^{-1} NAA and any of the three BAP concentrations. In none of these experiments was shoot regeneration observed.

TABLE 3: Effect of different plant growth hormone[1] concentrations and basal media on callus initiation from petiole explants of diploid sugar beet on P_{GO} and RV media. Observations made 2 weeks after initiation

	NAA (mg l^{-1})			
BAP (mg l^{-1})	0	0.1	0.3	1.0
		P_{GO} medium		
0	–	–	–	–
0.1	–	+	+	+ +
0.3	–	+	+	+ +
1.0	–	+	+	+ +
		RV medium		
0	–	–	–	–
0.1	–	+ + + +	+ + +	+ + +
0.3	–	+ + + +	+ + +	+ + +
1.0	–	+ + +	+ + +	+ + +

[1]See footnotes Table 2
– = no callus; + = callus less than size of explant
+ + = callus 1 to 2 times size of explant
+ + + = callus 2 to 3 times size of explant
+ + + + = callus >3 times size of explant

TABLE 4: Effect of different plant growth hormone concentrations and basal media on fresh weight accumulation and adventitious root initiation from petiole explants of annual sugar beet, 4 weeks after initiation[1]

	NAA (mg l^{-1})			
BAP (mg l^{-1})	0	0.1	0.3	1.0
		P_{GO} medium		
0	0.044 ± .005	0.045 ± .002(R)	0.044 ± .009(R)	0.066 ± .02
0.1	0.057 ± .005	0.087 ± .006	0.054 ± .006	0.159 ± .02(R)
0.3	0.074 ± .009	0.098 ± .01	0.142 ± .012	0.172 ± .017(R)
1.0	0.109 ± .027	0.115 ± .016	0.152 ± .013	0.278 ± .024(R)
		RV medium		
0	0.044 ± .007	0.041 ± .004(R)	0.092 ± .015(R)	0.160 ± .02(R)
0.1	0.154 ± .014	0.472 ± .038	0.387 ± .032(R)	0.368 ± .043
0.3	0.121 ± .021	0.443 ± .042	0.467 ± .042(R)	0.441 ± .03
1.0	0.145 ± .012	0.462 ± .034	0.290 ± .045	0.432 ± .063

[1]Values are mean fresh weight of explants plus callus, of 30 replicates, and initial fresh weight was 0.05 g. R indicates the presence of adventitious roots on some explants.

Shoot regeneration from callus

These studies centred on two experimental protocols based on Tétu *et al.* (1987) and Freytag *et al.* (1988). Initially, these were successfully used to recover shoots from callus of cv. Hilma and the diploid beet, respectively. The protocols were as follows:—

(a) Hilma: Callus was initiated by placing petioles on MS medium containing BAP (5.0 mg l^{-1}) and NAA (1.0 mg l^{-1}) and was then excised and transferred (after 4 to 6 weeks) to MS medium containing TIBA (1.0 mg l^{-1}) and zeatin (3.0 mg l^{-1}). Much of the callus blackened but 11 out of 70 pieces (16%) retained green knobs which developed into buds. To multiply the buds, these pieces were transferred to P_{GO} medium containing BAP (1.0 mg l^{-1}) and NAA (1.0 mg l^{-1}). Between 1 and 30 buds could be obtained from each callus in this way. For rooting, the resulting shoots were placed on MS medium with NAA (1.0 mg l^{-1}).

(b) Diploid beet: A critical step was the transfer of the donor shoot cultures to RV medium supplemented with IBA (0.1 mg l^{-1}) and BAP (0.4 mg l^{-1}) for 6 weeks prior to initiation of cultures. Without this step, no regeneration was obtained from the resulting cultures. Petiole explants (1 cm) were then placed on the same RV medium. Callus rapidly developed at one end of the explant and was subcultured to fresh medium every 3 to 4 weeks. Thirteen weeks after initiation some calli were observed to develop shoots. Eleven shoots were rooted after transfer to RV medium with 1.0 mg l^{-1} IAA.

This latter procedure (Freytag *et al.*, 1988) was then successfully extended to six additional cultivars, Rex, Regina, Hilma, Primo, Monofeb and Bingo. The effect of prior growth of shoot cultures on half-strength MS medium, or on supplemented RV medium (as described in (b) above) was investigated, as was the formation of different types (friable v. compact) of callus, and the origin of adventitious shoots (Table 5). Monofeb responded like diploid beet in requiring prior cultivation on RV medium and in this case gave one of the highest frequencies of regeneration from callus observed so far. However, this requirement was not universal. Hilma and Primo gave low regeneration frequencies but only from shoots grown on half-strength MS medium. The growing conditions of the donor shoots clearly influenced the type of callus developing with a much higher incidence of friable callus from RV-grown plants. This did not greatly affect the frequency of shoot

TABLE 5: Callus initiation and shoot regeneration (%)[1], using the method of Freytag *et al.* (1988), from shoot culture — derived petiole explants of six cultivars of sugar beet

	Donor shoots on half-strength MS medium				Donor shoots on RV medium			
	Callus		Regeneration from		Callus		Regeneration from	
Cultivar	Friable	Compact	Explant	Callus	Friable	Compact	Explant	Callus
Rex	9.3	87.7	0	7.0	36.4	29.5	0	2.3
Regina	0	100	6.7	0	15.6	62.5	6.2	0
Hilma	3.4	89.7	0	3.4	0	44.0	0	0
Primo	0	97.8	4.4	0	37.5	22.4	0	0
Monofeb	3.8	92.3	0	0	40.0	13.3	0	13.3
Bingo	0	100	3.1	0	19.0	50.0	0	5.2

[1]Values are percentages of total explants in each case and between 25 and 60 explants were scored for each treatment 6 weeks after initiation

regeneration although most callus-derived shoots (with exceptions for Monofeb and Bingo) did arise from friable callus. A cultivar dependence of the origins of adventitious shoots was detected with shoots in most cases, being associated either with the explant or the callus, but not both.

A number of rooted, regenerated shoots were obtained from all the above experiments and appeared to have normal sugar beet morphology, both *in vitro* and after transfer to potting compost in the glasshouse.

Ovule culture

Following the protocol described in Materials and Methods only two ovules developed into plantlets out of 200 ovaries dissected from unopened flowers. The ploidy status of these plants was not determined.

Discussion

The results obtained show that sugar beet is not very amenable to the application of tissue culture technology. There are, however, some promising indications and it is worth noting the uses to which the current procedures, while limited, may be put.

Rapid *in vitro* clonal propagation of shoot cultures through nodal cuttings is clearly a simple and convenient technique. Its cost effectiveness as a procedure for bulk production of propagules for the grower, however, is extremely doubtful. This must await development of an efficient and reproducible system for induction of somatic embryogenesis, which preferably should be synchronous, to facilitate automation of encapsulation and artificial seed production. The propagation system described here would, however, be useful for bulking up of single genetically-modified (by mutagenesis and selection, or transformation) shoots to allow rapid production of sufficient seed for subsequent field assessment. Additionally, shoot cultures can provide a good source of morphologically and genetically uniform material for physiological or biochemical investigations.

It is encouraging that shoot regeneration from callus can be achieved using two different protocols. The frequencies of success, however, are much too low for certain applications, particularly the use of undifferentiated cultures for mutagenesis and selection. It is unlikely that any line resistant after repeated cycles of selection in the presence of an antimetabolite or stress would retain its limited potential for shoot regeneration (Dix, 1986). Better regeneration frequencies have been reported directly from explants, with minimal callusing, and there are already indications that this is a suitable system for production of transgenic plants by *Agrobacterium*-mediated transformation (Lindsey and Gallois, 1990).

Tétu *et al.* (1987) obtained shoots from 32% of calli with a mean value of four per callus by comparison with the results reported here of 16% with 1 to 30 buds per callus. The difference in frequency is likely to reflect cultivar effects, Tétu *et al.* (1987) having chosen to work with a cultivar (Monosvaloff) they had previously found to respond well to tissue culture.

Generation of somaclones, for assessment of somaclonal variation, is not dependent on highly prolific organogenesis, since no lethal selection pressure is applied and only a limited number of somaclones can be subjected to field assessment. Clearly, the regeneration frequencies achieved in the present investigation are sufficient to provide ample material for the initiation of a search for somaclonal variation for a number of useful agronomic characters. All the plants regenerated so far had

normal sugar beet morphology, both *in vitro* and after transfer to the soil, and no obvious evidence for somaclonal variation has been seen.

One important use for haploid material is the provision of cultures in which mutagenesis and selection can be realistically expected to pick up recessive mutations. This is another area where high frequencies are not paramount. For example, a number of mutagenesis studies in *Nicotiana plumbaginifolia* have employed a haploid plant line originally derived from a single pollen grain by androgenesis (Sidorov, Menczel and Maliga, 1981; Marton *et al.*, 1982). Even the low success rate of ovule culture reported here should be able to provide a haploid source for comparable work with sugar beet.

Finally, a range of tissue-culture techniques can contribute to fundamental physiological studies. Axenic shoot cultures have already been used in comparative studies on three sugar beet cultivars differing in tolerance to low temperatures (Dix, Finch and Burke, 1993) and the callus cultures now available should facilitate investigation of the cellular and whole plant components of this complex phenomenon.

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