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Instructions for use

CLONAL PROPAGATION OF SUGAR BEET PLANTS BY APICAL MERISTEM CULTURE[1]

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Introduction

With a biennial and allogamous species such as sugar beet, there is often a need to multiply and conserve the superior genotypes for breeding purposes or genetic studies. The cutting and/or division using vegetative organs has been done exclusively for clonal propagation of sugar beet[7]. These conventional techniques, however, are laborious and unsuitable for rapid multiplication on a large scale.

Recently, HUSSEY and HEPHER[4] and other investigators[1,2,6] demonstrated that tissue culture provided an alternative and efficient method of asexual propagation in sugar beet. Three possible approaches are available for *in vitro* propagule multiplication : 1) enhancement of axillary shoot formation, 2) production of adventitious shoots through organogenesis, and 3) somatic embryogenesis[3]. The advantage of using apical meristem proliferation as a means of regeneration is that the incipient shoots have already been differentiated *in vivo*. Consequently, only shoot elongation and root formation are required to establish a whole plant. Moreover, plants derived from apical meristem culture display generally homogeneous phenotypes, thereby indicating genetic stability. In this paper, we examined the regenerative capacity of *in vitro* cultured flower buds or axillary buds of sugar beet. The subsequent multiplication of shoots and their rooting were also described.

Materials and Methods

Plant materials. Recently, KINOSHITA et al.[5] have succeeded in inducing cytoplasmic reversion to male fertility after streptomycin treatment (1,500 ppm) in two cytoplasmic male-sterile (CMS) lines of sugar beet, TK 76–MS and TK 81–MS. The materials first used in this study were inflorescence explants from 39 male fertile mutants and 4 complete sterile plants (field-grown) which appeared in the M_1 generation of TK 76–MS (Table 1). Our investigations also included two sets of CMS and its maintainer lines, a triploid cultivar and a tetraploid tester line (Table 2). The latter plant materials were grown in a greenhouse with supplementary lighting for about 3 months.

Sterilization and culture conditions. Axillary buds or flower stalks (1–1.5 cm long) with young closed buds were surface-sterilized by immersion in 70% ethanol (30 sec) and in 2% sodium hypochlorite solutions (20 min), followed by washing sufficiently in distilled sterile water. Culture experiments with inflorescence explants were performed essentially as described by MIEDEMA[6].

1) Medium-SF (for shoot formation); the basic nutrient medium consisted of a salt mixture of MURASHIGE and SKOOG[9] (MS) used at half strength together with 30 g/l sucrose, 8 g/l agar and 10 μmol BA (benzyladenine).

2) Medium-SM (for shoot multiplication); differing from medium-SF in that half-strength MS organic compounds (50 mg/l meso-inositol, 0.25 mg/l nicotinic acid, 0.05 mg/l thiamine-HCl, 0.25 mg/l pyridoxine-HCl and 1 mg/l glycine) were included and the BA concentration was reduced to 1 μmol.

3) Medium-RF (for root formation); composition as medium-SM but with 10 μmol IBA (indolebutyric acid) or 1 μmol NAA (α-naphthaleneacetic acid) instead of BA.

The pH of the medium was adjusted with 1.0 N NaOH or 1.0 N HCl to 5.7–5.8. Cultures were kept at a constant temperature of 24°C in the lighting condition.

Results

Shoot formation and multiplication

Flower bud cluster or axillary buds were taken from plants at various stages of development and were placed on medium-SF to stimulate shoot formation. As shown in Table 1, in the experiment with explants from field-grown plants, one half of the cultures failed to establish vegetative shoots due to bacterial or fungal contamination. On the contrary, up to *ca.*

85% propagules proved to be free from such contamination when green-house-grown plants were used as materials (Table 2).

The surviving explants began to elongate within 5 days, and rarely formed white or pale green callus masses at the cut surface. Two to 6 weeks after culture initiation, the first shoots arose at the apical portion of the bud clusters, suggesting that they developed from pre-existing axillary meristem (Plate Ia). In addition, some of the shoots were observed to grow out directly on the basal site of flower buds. They probably originated from

TABLE 1.   Apical meristem culture in male fertile mutants and
            male sterile plants which appeared in M_1 generation
            of TK76-MS after streptomycin treatment

Phenotype	No. of plants	Shoot formation			Rooting	
		No. of explants	Surviving explants	Plantlets with shoot	No. of explants	Rooting
Male fertile	39	560	272 (48.6)	192 (70.6)	442	207 (46.8)
Male sterile	4	40	20 (50.0)	12 (60.0)	23	10 (43.5)
Total	43	600	292 (48.7)	204 (69.9)	465	217 (46.7)

Plants were cultivated in the field. Shoot formation and rooting were estimated 7 and 5 weeks after incubation of inflorescence explants and shoots, respectively. The percentage is given in brackets.

TABLE 2.   Apical meristem culture in 6 sugar beet lines

Line	Ploidy	No. of plants	Shoot formation			Rooting	
			No. of explants	Surviving explants	Plantlets with shoot	No. of explants	Rooting
T K81-O	2x	3	357	341 (95.5)	228 (66.9)	230	46 (20.0)
T K81-M S	2x	3	404	364 (90.1)	265 (72.8)	300	44 (14.7)
N K169-O	2x	3	274	246 (89.8)	177 (72.0)	195	57 (29.2)
N K169-M S	2x	3	349	326 (93.4)	175 (53.7)	185	23 (12.4)
Monohope	3x	2	213	191 (89.7)	50 (26.2)	56	4 (7.1)
H-4002	4x	3	497	367 (73.8)	196 (53.4)	212	52 (24.5)
Total		17	2094	1835 (87.6)	1091 (59.5)	1178	226 (19.2)

Plants were grown in the greenhouse. Shoot formation and rooting were esti-mated 7 and 5 weeks after incubation of inflorescence explants and shoots, re-spectively. The percentage is given in brackets.

TABLE 3.   The effect of the developmental stage of
the flowering plants on shoot formation
in 6 sugar beet lines

Stage of development	% clusters with shoot					
	TK81-O	TK81-MS	NK169-O	NK169-MS	Monohope	H-4002
Onset of flowering	73.9 (88)	70.2 (114)	88.7 (62)	49.0 (83)	28.2 (39)	64.1 (145)
One week later	68.2 (85)	92.0 (87)	89.1 (46)	55.1 (89)	35.6 (45)	58.0 (69)
Two weeks later	63.5 (85)	77.0 (87)	58.5 (82)	78.9 (95)	20.3 (59)	52.7 (74)
Three weeks later	61.4 (83)	50.0 (76)	58.9 (56)	22.0 (59)	22.9 (48)	30.4 (79)

Shoot formation was estimated 7 weeks after incubation. The number of ex-
plants used for shoot formation is given in brackets.

the receptacle or the peduncle, though definite anatomical proof remains to
be seen.   In none of the cases was adventitious shoot induction from callus
noted.

After 7 weeks of culture, 53 to 73% of explants yielded shoots in most
genotypes examined, whereas a triploid cultivar, Monohope exhibited a lower
rate of shoot production (Tables 1 and 2).   These variations are likely to be
related to the vigour of the mother plants rather than genotypic differences.
The shoot forming ability seems to be also affected by the developmental
stage of the mother plants.   As indicated in Table 3, shoot production rate
was highest in the cultures of explants taken from plants at the early stage
of flowering.

The primary shoots were further multiplied by axillary shoot prolifera-
tion on a fresh medium-SF or -SM (Plate I b).   Repeated subculture allowed
the development of 1 to 6 axillary shoots per explant, but gave rise to leaf
malformation which may be attributed to BA in our media (Plate I a).

Rooting

Axillary shoots from multiple shoot cultures were dissected and trans-
ferred to the medium-RF supplemented with 10 μmol IBA or 1 μmol NAA.
The regeneration of fascicular root system started 2 to 3 weeks after in-
cubation (Plate I c).   Tables 1 and 2 present rooting data in a total of 1,643
axillary shoots.   The percentage of rooted plantlets varied from 7% to 47%,
depending on the strains or genotypes, when adventitious root formation was
assessed 5 weeks after incubation of shoots.   In addition, no significant dif-

ference on root formation was found between the two culture media contain-
ing different auxins.

　　During this time, the shoots continued to grow and newly formed leaves
had no BA malformations.　This suggests that BA probably promotes shoot
initiation but affects normal leaf development.　Thus, such malformation may
be readily avoided by transferring propagules to a BA-free medium after
shoot initiation[6].

　　The rooted plantlets were washed to remove adhering agar, and success-
fully planted out in vermiculite.　Several clones were obtained in this way
(Plate I d).　A detailed examination is in progress, in order to ascertain if
any mutations might occur during *in vitro* culture.

Discussion

　　Since the pioneering work of rapid multiplication of orchids by shoot
meristem culture[8], there has been an increasing interest in the application
of tissue culture technique as an efficient method of asexual propagation of
valuable breeding forms[3].　The success in this cloning procedure appears
to be associated mainly with the choice of suitable material and conditions
of nutrient medium.　Our results indicate that flower buds and axillary buds
are good starting materials for the *in vitro* production of shoots in sugar
beet.

　　Micropropagation technique consists of the three main steps, namely 1)
shoot initiation from the explants, 2) axillary bud proliferation *via* dissection
of the primary shoots and subculture and 3) rooting and hardening for
planting into soil.　From the present data, culture conditions for shoot
formation and subsequent multiplication cycle seem to be applicable in various
genotypes of sugar beet such as CMS strains, and tri- and tetra-ploid lines.
In this way very large numbers of shoots could be built up by serial sub-
culture.　It is also relevant to emphasize that contamination with bacteria
or fungi can be avoided considerably by using greenhouse-grown plants
instead of field-cultivated plants.

　　Numerous examples[3] reveal that *in vitro* formation of adventitious roots
depends on a low cytokinin to a high auxin ratio.　In our case, IBA and/or
NAA promoted root development, although elaboration is required to define
more efficient culture conditions for rooting.　It has been frequently discussed
that mutations may occur in callus culture, but can be avoided by apical
meristem culture[3,6].　Nevertheless, this problem will be entirely assessed only
after the genetical characterization of the clones obtained is completed.

　　Abundant clonal materials of single genotype could be very useful for

breeding purposes as well as for physiological and pathological studies. The highly reproducible technique also would probably given some advantages in establishing low temperature preservation of sugar beet germplasm[4,6].

Summary

In vitro clonal propagation of sugar beet has been undertaken by culturing of flower buds or axillary buds. Explants were shown to be capable of developing vegetative shoots on a half-strength MS medium supplemented with 10 µmol BA. The primary shoots were successfully multiplied by axillary bud proliferation on a medium with 1 µmol BA and forced to initiate adventitious roots on a medium with 10 µmol IBA or 1 µmol NAA. BA caused leaf malformations. However, such malformation can be readily avoided by transferring propagules to a BA-free medium after shoot formation. In addition, the rate of bacterial or fungal contamination was significantly reduced when greenhouse-grown plants were used as material source instead of field-cultivated plants. This micropropagation technique proved to be applicable in various genotypes of sugar beet, although it is necessary to improve the culture conditions for rooting.

Acknowledgements

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Legend for Plate I

a. Shoot formation on half-strength MS medium containing 10 μmol BA.
   Left: normal shoot development. Right: leaf malformation.

b. Shoot multiplication on half-strength MS medium containing 1 μmol BA.

c. Rooting on half-strength MS medium containing 10 μmol IBA.

d. Rooted plantlet transferred to soil.