In Vitro Multiplication of Primary Trisomic Sugarbeets

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ABSTRACT

Seed multiplication is difficult for several of the recently developed sugarbeet primary trisomics. To assure the preservation of these genetic stocks, in vitro shoot multiplication and root induction of eight of the nine trisomic sugarbeet types was investigated and accomplished. Only type 5 was not successfully cloned. Significant differences were found among trisomic types for multiplication rate and weight increase of in vitro clonal cultures. Morphology of in vitro plantlets resembled that of trisomics grown from seed. The methods described in this research provide an effective means of maintaining and multiplying most of the sugarbeet primary trisomics.

Additional index words: *Beta vulgaris*, cloning, asexual reproduction, vegetative propagation, *in vitro* culture.

A primary trisomic series has been developed in homozygous sugarbeet by Romagosa et al. (1986). Seed multiplication of several trisomic types, especially 5, 6, 7, and 8, is difficult. The purpose of this research was to develop methods of cloning the trisomics as an efficient system of trisomic maintenance and multiplication.

Since Margara (1970) reported in vitro propagation of sugarbeet by means of flower buds, a number of successful techniques and explant types have been described (Hussey and Hepher, 1978; Atanassov, 1980; Coumans-Gilles et al., 1981; Saunders, 1982; M. S. Gibson, M. C. G. Middelburg, G. A. Smith, and A. C. Van Spijk, personal communications, 1985).

MATERIALS AND METHODS

The nine primary trisomic sugarbeets isolated by Romagosa et al. (1986) were used. A minimum of three plants of each type and five apical bud explants per plant were used as starting material.

Two experiments were conducted, starting in the springs of 1985 and 1986, respectively. In 1985 only six trisomic types were available. The 1986 experiment included the complete trisomic set. In both experiments, diploid plant materials were used as checks. All source plants were photothermally induced to promote bolting, then grown in the greenhouse.

Apical buds, 0.3 to 0.5 cm long, were collected, then surface sterilized by soaking them in 1% mercuric chloride (1 min) followed by three rinses in sterile distilled water (2, 30, and 30 min), and, finally, soaked in 1% calcium hypochlorite (10 min) with no rinse.

Throughout this text, we use the Murashige (1974) three stage terminology, i.e., shoot establishment (I), shoot multiplication (II), and rooting (III).

Murashige and Skoog (1962) medium with a modified vitamin content (Saunders and Daub, 1984) was the basal medium (BM).

The media were solidified with agar (6 g L^{-1}) adjusted to pH 5.6, and dispensed into test tubes (25 X 150 mm) covered with Kap-uts (Bellco). Twenty ml per tube, was used for stages I and II. The rooting medium (stage III), was dispensed into 500 ml beakers (100 ml per beaker). Ten to 15 shoots were transferred into each beaker, then the beakers were covered with aluminum foil and sealed with Parafilm. Growth regulators were added before autoclaving (15 min, 121° C, 1.2 kg cm^{-2}). All cultures were placed in a growth chamber at 25° C with a 16 h photoperiod of fluorescent light (15 $\mu E\ m^{-2}s^{-1}$).

The initial apical bud explants, one per tube, were placed on BM. Hormone contents differed between experiments for stage I. The first experiment received 0.44 μM benzyladenine (BA), whereas, the second experiment received 4.44 μM BA, 0.53 μM naphthaleneacetic acid (NAA), and 0.29 μM gibberellic acid (GA_3).

In stage II, the buds developed on stage I cultures were transferred to PGoB medium (De Greef and Jacobs, 1979) supplemented with 0.44 μM BA. This medium was used in an attempt to reduce shoot vitrification, based on our experience with diploid sugarbeets (unpublished). New shoots developed on this medium were multiplied on BM with 0.44 μM BA. After two to three subcultures, the shoots were transferred to rooting medium (stage III), consisting of BM (half strength) supplemented with 5.37 μM NAA.

The rooted plantlets were rinsed to remove adhered agar,

planted into soil (sterilized mixture 2.5:1 compost:sand, no fungicides), and placed in clear plastic boxes at 20-25° C. Relative humidity was maintained near 100% for 10 days, in a controlled environment, and during later acclimatization in a greenhouse.

Multiplication rate, number of viable shoots (larger than 1 cm) formed per bud, and fresh weight increase (g), were determined after 28 days in each subculture. The percentage of rooted shoots was recorded after 5 weeks.

Statistical analyses were performed using SPSS programs (Norusis, 1986).

RESULTS AND DISCUSSION

Due to the incidence of vitrification (described in a subsequent section), all the following data analyses were made only on the nonvitrified materials.

Stage I

The analysis of variance for the seven trisomic types present in both experiments showed significant differences between experiments and among types (Table 1). The interaction of type x experiment was significant for both multiplication rate and fresh weight increase. Therefore, independent analyses were performed for each experiment.

Diploid explant responses (Table 2) were similar in multiplication rate for both experiments, but weight increase was greater in the second experiment, which had the higher hormone level. This observation, in conjunction with the presence of all the trisomic types in experiment 2, caused us to use only the data of this second experiment for the remainder of the analyses.

The development and growth of the trisomic types during stage I are presented in Table 3. The poor establishment of types 5 and 7 is apparent.

Table 1. Combined analyses of variance (experiments 1 and 2) of multiplication rate and weight increase (g) of in vitro culture of sugarbeet trisomics (Stage I).

Source of variation	DF	Mean squares	
		Mult. rate	Weight increase
Trisomics	6	2.545 **	.339 **
Exp.	1	1.632 **	4.359 **
Trisomics x Exp.	6	1.359 **	.595 **
Residual	235	.232	.066
Total	248	.319	.105

** Significant at $\alpha = 0.01$.

Table 2. Mean multiplication rate and weight increase (g) of in vitro cultures (stage I) of diploid sugarbeets.

	Number of explants	Mult. rate	Weight increase
Exp. 1	43	0.95	0.29
Exp. 2	15	0.93	0.73 **

** Significantly different from Exp. 1 (α = 0.01)

Table 3. Mean multiplication rate and weight increase (g) of in vitro cultures (stages I and II) of nine sugarbeet trisomics and a diploid, experiment 2.

	STAGE I			STAGE II - PGoB + 0.4 μM BA		
Type	N	Mult. rate	Weight increase	N	Mult. rate	Weight increase
1	10	1.00	0.57	9	1.22	1.26
2	13	0.85	0.39 *	6	1.00	1.38
3	15	0.87	0.29 *	14	1.43	0.71
4	15	1.00	0.67	9	1.78	0.90
5	18	0.28 *	0.20 *	5	0.00 *	0.08 *
6	5	0.80	0.48	4	2.00	1.22
7	16	0.69 *	0.62	5	0.00 *	0.07 *
8	12	1.00	0.83	8	1.62	1.15
9	11	0.73	0.29 *	8	2.37 *	1.73 *
2x	15	0.93	0.73	10	1.50	0.88

N = Number of individual cultures.
* Significantly different from the diploid (α = 0.05)

Stage II

Axillary shoot multiplication which started in stage I was executed in Stage II. Within 4 weeks one to six new shoots usually had arisen from axillary buds. They were separated and transferred to fresh medium every 28 days.

Shoots which had been formed in stage I were planted on PGoB medium supplemented with 0.44 μM BA. All trisomics had relatively similar multiplication rates and weight increases, except trisomics 5 and 7 which failed to multiply (Table 3). All these cultures of trisomic 5 were lost. One culture of trisomic 7 was maintained via a vitrified shoot that multiplied.

Shoots then were transferred to BM with 0.44 μM BA, where they were subcultured every 4 weeks. The analyses of the three subcultures in this stage showed a significant type x subculture interaction for both variables measured. Hence, we presented results for each subculture (Table 4).

For multiplication rate there were no significant differences among types in subcultures 1 and 2. However, subculture 3 showed significant differences among types. It appeared that the trisomics could be grouped into three clusters, one group in-

Table 4. In vitro multiplication rates and weight increase (g) of eight sugarbeet trisomics and a diploid through three stage II (BM + 0.44 μM BA) subcultures, experiment 2.

	1st Subcul.			2nd Subcul.			3rd Subcul.		
Type	N	Mult. rate	Weight inc.	N	Mult. rate	Weight inc.	N	Mult. rate	Weight inc.
1	11	1.64	0.86 *	18	1.67	0.41 *	30	1.70 *	0.26 *
2	8	1.62	1.14	13	1.15	0.43 *	15	1.27 *	0.18 *
3	20	1.75	0.44 *	35	1.77	0.16	62	1.32 *	0.21 *
4	16	1.19	0.37 *	18	1.83	0.49 *	32	1.72 *	0.39 *
6	8	2.25	0.48 *	18	1.89	0.33	32	4.56 *	0.68 *
7	1	1.00	0.63	1	1.00	0.14	1	4.00	0.63
8	17	1.70	0.59 *	28	1.75	0.43 *	45	3.20	1.32
9	22	1.00	0.45 *	22	1.59	0.20	34	3.41	1.12
2x	17	1.65	1.21	27	1.56	0.27	39	3.28	1.57

N = Number of individual cultures.
* Significantly different from the diploid ($\alpha = 0.05$).

cluded types 1, 2, 3, and 4 (lower multiplication rates), a second group included the diploid and types 8 and 9, and a third group included type 6, with its uniquely high multiplication rate.

The analysis for fresh weight increase showed significant differences among types in all the three subcultures. Nevertheless, the results were somewhat erratic, with large differences between subcultures. Clusters similar to the ones developed for multiplication rate could be deduced from the third subculture, with the exception of type 6, which had a large number of shoots but little weight increase. This may be related to the peculiar aspect of type 6 plants grown from seed, since at older stages they produce a large number of small shoots.

The analysis of variance of the mean number of shoots produced per explant at the end of stage II showed significant differences among types, but there was considerable variability among explants within types (analysis not shown). Explants of the same size are not necessarily of the same physiological state. These results could be related to different physiological responses which could have produced unanticipated differences prior to the start of the culture.

Stage III

Rooting occurred with variable efficiency in all the surviving trisomic types (Table 5). In stage III cultures, the first roots appeared after 12-16 days for types 1 and 9, and after 21-28 days for the others.

When the rooted plants were transferred to soil, 89 trisomic plants survived from the 210 transplanted.

Table 5. Percent rooting of in vitro cultures (stage III) of eight sugarbeet trisomics and a diploid, experiment 2.

Type	Number of plantlets	Percent rooting
1	43	41.1
2	40	15.0
3	69	46.5
4	48	33.3
6	140	36.8
7	24	33.3
8	128	27.3
9	110	37.1
2x	122	38.9

Table 6. Percent vitrification of in vitro cultures (stages I and II) of nine sugarbeet trisomics and a diploid, experiment 2.

	Stage I		Stage II			
			PGoB + 0.4 μM BA		BM + 0.4 μM BA	
Type	N	Percent vitrif.	N	Percent vitrif.	N	Percent vitrif.
1	10	0.0 *	10	10.0 *	59	0.0 *
2	13	0.0 *	11	45.4	39	7.7
3	15	0.0 *	14	0.0 *	117	0.0 *
4	15	33.3	12	25.0 *	68	2.9
5	21	14.3 *	9	44.4		
6	5	0.0 *	4	0.0 *	58	0.0 *
7	25	36.0	18	72.2 *	5	40.0 *
8	15	20.0 *	15	46.7	98	8.2 *
9	14	21.4 *	11	27.3 *	79	1.3
2x	25	40.0	21	52.4	88	4.5
Total	158	20.9	125	37.6	611	3.3

N = Number of individual cultures.
* Significantly different from the diploid ($\alpha = 0.05$)

In a preservation technique test, explants from each of the eight trisomic types were maintained at 4° C, under a 16-h photoperiod, and low fluorescent light intensity (5 μE $m^{-2}s^{-1}$). They were subcultured every 4 months, and after a year, the average survival was near 85%.

The morphology of in vitro plants resembled that of trisomics grown from seed (Romagosa et al., 1986), e.g., type 1 plants had small dark green, glossy leaves, type 4 had broad, triangular, dark green leaves folded at the top and base of the lamina, and type 8 had small, thick, dark green leaves with white and parallel venation. Type 8 plants also developed in a rosette form.

Vitrification

Vitrification of shoots occurred in varying amounts during in vitro culture, showing thickened leaves, translucence, and frequently etiolation, as described by Miedema (1984) and Keimer (1985).

Numerous hypotheses have been put forward to explain the causes of vitrification, e.g., high concentrations of cytokinins (Pasqualetto et al., 1986) and high ammonium level of the MS medium (Daguin and Letouze, 1986).

The incidence of vitrification in stages I and II of experiment 2, with all the trisomic types, is presented in Table 6. There was a high proportion of vitrification during establishment and first multiplication, and a decrease of vitrification following culture on the PGoB medium with 0.44 μM BA. In stage II cultures grown on BM with 0.44 μM BA, only 3% of the plantlets became vitrified. It is not apparent whether this was due to medium, hormones, or stage of development.

The nine trisomics showed different frequencies of vitrification (Table 6). No vitrification occurred in types 3 and 6. On the other hand, type 7 and the diploid material had the highest vitrification frequency.

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