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Hormone-free medium will support callus production and subsequent shoot regeneration from whole plant leaf explants in some sugarbeet (*Beta vulgaris* L.) populations *

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ABSTRACT

Sugarbeet plants representing 14 of 16 germplasm sources (4 to 5 plants per source) produced callus from leaf disks on a hormone-free Murashige and Skoog based medium. Overall, 49.2% of explants from partially expanded leaves of whole plants initiated callus (53 of 74 plants tested), in an average time of 96.7 days. The time to callus was considerably longer than the 4-6 weeks observed when 1 mg/L N^6-benzyladenine has been used in the medium. Shoots were regenerated on the hormone-free medium without subculture from callus of eight individual genotypes, representing 3 of the 14 populations that produced callus. Shoots produced by 'Gartons White Knight' and 'L53' appeared to be of somatic embryo origin. Rhizogenic calli were also produced by the same three populations that regenerated shoots. Significant differences among populations were found for frequency of root formation from leaf disks and time to callus. Variation among plants within a population was significant for four of the five traits examined. The results indicate the ease of hormone autonomization in sugarbeet, and should be of value in designing regeneration media for a wider range of beet germplasm.

ABBREVIATIONS

BA, N^6-benzyladenine
MS, Murashige and Skoog basal medium

INTRODUCTION

Following early preliminary reports of shoot regeneration from sugarbeet callus (De Greef and Jacobs, 1979; Hooker and Nabors, 1977; Saunders and Daub, 1984), high frequency regeneration has been demonstrated across a range of sugarbeet germplasm (Freytag et al., 1988; Saunders and Doley, 1986; Saunders and Shin, 1986; Tetu et al., 1987; Van Geyt and Jacobs, 1985). The simplest method for obtaining callus and subsequent shoot regeneration of several unrelated genotypes of sugarbeet consisted of placing leaf explants on a slightly modified MS medium with 1.0 mg/L BA (Saunders and Doley, 1986; Saunders and Shin, 1986).

In previous experiments (Saunders and Doley, 1986), leaf explants of the somaclonal variant 'N' produced callus and shoots on hormone-free medium, but the source plant, 6926-0-3, and three other regenerants did not produce any callus on hormone-free medium. Therefore, the habituation response of this somaclonal variant might be unique. Jarl and Bornman (1986) reported genotypic variation for callus proliferation with several combinations of auxin and cytokinin, and Saunders and Shin (1986) found germplasm effects on callus and shoot formation using only a cytokinin. There has been no report of the response of a range of beet germplasm on hormone-free medium. We report here an assessment of the in vitro response of leaf explants from 16 sugarbeet populations cultured on a hormone-free MS-based medium.

MATERIALS AND METHODS

Sugarbeet (Beta vulgaris L.) plants used as donors of leaf explants were grown in pots in a glasshouse without supplemental lighting. Plants were fertilized every 2 weeks with Peters 20-20-20 water soluble commercial nutrient mix and once a month with Snyder's (1974) nutrient formulation. All plants were grown from seed and represent a diverse sample of USA monogerm and multigerm sugarbeet germplasm, including several parental lines used in hybrid seed production. Four to five plants were sampled from each of eight monogerm and eight multigerm populations. Characteristics and seed sources for these populations are listed in Table 1. With the exceptions of Owen's Annual (OA) and 84M5-20, each of these lines is a genetically heterogeneous population.

Explants were taken in December 1986 from approximately one-yr-old donor plants, varying in root diameter at ground level from 5-15 cm. Two small partially expanded leaves, varying in size from 5-15 cm, per plant were used as the source of explants. Detached leaves were surface sterilized with two 20 min soakings in 15% commercial hypochlorite bleach solution with 0.01% sodium laurylsulfate, followed by six rinses with sterile distilled water. Circular explants were cut with a No. 3 cork borer (7 mm inner diameter).

The culture medium consisted of MS (Murashige and Skoog, 1962), mineral salts, 3% sucrose, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 1.0 mg/L thiamine HCl and 0.9% (w/w) Difco Bacto agar, without growth regulators. The pH

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Table 1. Characteristics and seed sources of populations studied.

Population	Genotypes Sampled	Germness[a]	Comments
EL 36[b]	4	mm	Breeding line
EL 40[b]	5	MM	Parent of cultivar
EL 44[b]	4	mm	Parent of cultivar
EL 45/2[b]	5	mm	Breeding line
EL 48[b]	5	mm	Breeding line
F1003[c]	5	MM	Breeding line
FC 506[d]	5	mm	Breeding line
FC 607[e]	5	mm	Breeding line
FC 701/5[e]	5	MM	Breeding line
FC 708[e]	5	mm	Breeding line
GWK[f]	4	MM	Mangel beet
L53[h]	4	MM	Breeding line
OA[bg]	4	MM	Annual cms tester
SP 6822[b]	5	MM	Parent of cultivar
SP 6926[i]	4	mm	Parent of cultivar
84M5-20[h]	5	MM	Genetic stock

[a] MM denotes multigerm, mm denotes monogerm.
[b] from JW Saunders, East Lansing, MI, USA
[c] from DF Cole, Fargo, ND, USA
[d] from R Zielke, Carrollton, MI, USA
[e] from GA Smith & RJ Hecker, Fort Collins, CO, USA
[f] Garton's White Knight from I Linde-Larson, Roskilde, Denmark
[g] Owen's Annual
[h] from JC Theurer, East Lansing, MI, USA
[i] from G Coe, Beltsville, MD, USA

was adjusted to 5.95 prior to autoclaving. Thirty-five mL of medium was dispensed into each 20 x 100 mL plastic Petri dish after autoclaving. Six explants were taken per leaf and a single explant was placed in each dish. Cultures were maintained at 31 C in an incubator with dim light (5-10 $\mu Em^{-2}s^{-1}$) from cool-white fluorescent bulbs.

Data on callus and shoot production was recorded weekly for 6 months. Statistical Analysis System (SAS Institute, 1985) was used for most analyses. Data was unbalanced and was handled by the General Linear Models (GLM) procedure. The experimental design was completely randomized. Sampling was hierarchical and completely nested, i.e. plants within populations and leaves within plants. Populations were treated as fixed effects, while plants and leaves were treated as random effects. To avoid deflation of error terms, populations which gave no response for a variable were not included in the analysis for that variable. With the exception of time to callus, the experimental unit was the leaf and response frequencies were calculated as the mean of the samples within the leaf. All frequency variables displayed variance heterogeneity and were transformed using the arcsine function (Steel and Torrie, 1980). For time to callus, the experimental unit was the Petri dish and samples within leaves were an additional source of variation. Approximate F tests involving synthesized mean squares were necessary in some cases (Satterthwaite, 1946).

RESULTS AND DISCUSSION

The overall frequency of callus formation on hormone-free medium was 49.2% (Table 2). Callus was produced by 53 of the 74 plants sampled, representing 14 of the 16 populations. No significant differences between populations were found for callus frequency (Table 3), but there was highly significant variation among plants within a population. Populations with

Table 2. Mean frequencies of callus and root production from leaf disks cultured on hormone-free medium, and number of genotypes which produced callus or root from leaf disks.

Population	N[a]	Frequency (%) Callus	Frequency (%) Root	Number of genotypes Callus	Number of genotypes Root
GWK	41	100.0	0.0[b]	4/4	0/4
EL 45/2	57	77.2	0.0	5/5	0/5
EL 48	60	76.7	15.0	5/5	3/5
L53	43	69.8	39.5	4/4	4/4
FC 701/5	60	68.3	5.0	5/5	2/5
84M5-20	60	68.3	0.0	5/5	0/5
SP 6822	59	64.4	10.2	4/5	2/5
FC 708	59	47.5	0.0	4/5	0/5
FC 607	58	46.6	12.1	4/5	2/5
F1003	60	40.0	8.3	2/5	3/5
SP 6926	48	33.3	0.0	3/4	0/4
EL 40	54	31.5	3.7	2/5	2/5
FC 506	60	30.0	1.7	3/5	1/5
EL 36	48	25.0	2.1	3/4	1/4
OA	46	0.0[b]	0.0	0/4	0/4
EL 44	47	0.0	2.1	0/4	1/4
Total	860	---	---	53/74	21/74
Mean	---	49.2	6.0	---	---

[a] number of explants sampled
[b] populations with no response were not included in the analysis of variance

callus frequencies ranging from 0 to 100% suggest that true differences exist between populations but were difficult to detect due to the large within population variance. In some cases the within population variance seemed qualitative. For example, of the five plants sampled from EL 40, three produced no callus while the other two produced callus at frequencies of 83.3% and 100%.

The moist, white, friable callus was morphologically similar to that produced when 1 mg/L BA has been used in the medium (Saunders and Doley, 1986; Saunders and Shin, 1986). In each of several cases tested, callus was capable of continued growth after transfer to fresh hormone-free medium. The gradual reduction in exogenous hormone levels used by Van Geyt and Jacobs (1985) to achieve the habituated state is apparently not a requirement.

Three populations, Garton's White Knight (GWK), L53 and EL 45/2, also produced some shoots from the habituated callus (Table 5). These populations were among the top four for callus frequency (Table 2). In the cases of GWK and L53, the shoots appeared to be of a somatic embryo origin (Figure 1). Tetu et al. (1987) achieved somatic embryogenesis from sugarbeet callus but a sequence of three media and several subcultures was used. Although the frequency here was low, somatic embryogenesis may be achieved in some

Table 3. Analysis of variance of frequency of callus production from leaf disks cultured on hormone-free medium.

Source	df	Mean squares(arcsine)
Populations	13	0.99246
Plants/Population	52	0.65583**
Leaves/Plant	66	0.07597

**Significant at the 0.01 probability level.

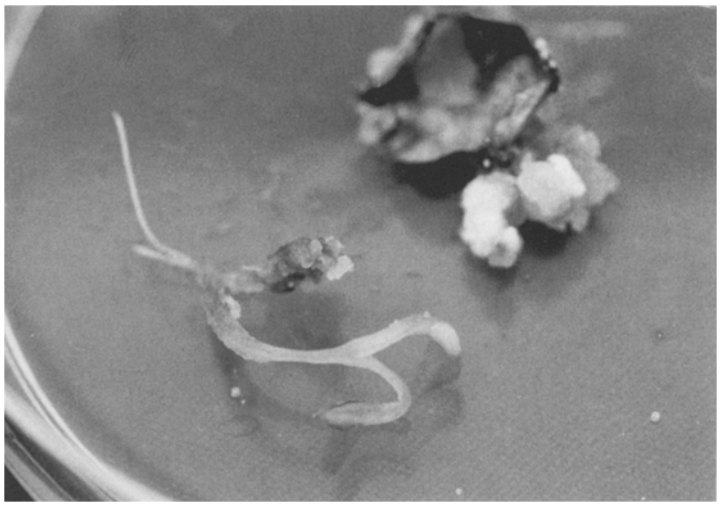

Figure 1. Somatic embryo-derived plantlet of GWK on original plate.

genotypes with a simple system.

Shoot regeneration by L53 was interesting because, in several previous experiments, no shoots had been obtained from explants of these same plants on an MS-based medium containing 1 mg/L BA (Doley and Saunders, unpublished). Genotype x medium interaction for in vitro response apparently is operative in sugarbeet and, as suggested by Jarl and Bornman (1986), may be partially responsible for the recalcitrance observed in previous reports.

Rhizogenic callus was only produced by the three populations that regenerated shoots (Table 5). In the cases of GWK and L53, three of the four plants sampled produced some rhizogenic calli, while there was only an isolated case within EL 45/2. The rhizogenic calli produced many short stubby roots even after subculture to fresh medium. Roots arising directly from the explants, particularly from cut vascular tissue, occurred at about three times the overall frequency of the rhizogenic calli, and in 10 of the 16 populations (Table 2). Unlike those produced by the rhizogenic calli, these roots tended to be long and slender. Populations differed significantly for frequency of leaf disk rooting (Table 4), but not for frequency of root regeneration from callus (Table 6).

EL 45/2 was the only population which gave relatively high shoot regeneration frequency on the callus arising on hormone-free medium. Four of the five plants sampled produced shoots from callus for a regeneration frequency of 29.5% (Table 5). When explants of EL 45/2 have been placed on medium containing 1 mg/L BA, shoot formation was prolific while little callus was obtained (Doley and Saunders,

Table 4. Analysis of variance of frequency of root production from leaf disks cultured on hormone-free medium.

Source	df	Mean squares(arcsine)
Populations	9	0.14571*
Plants/Population	37	0.05280*
Leaves/Plant	47	0.03209

* Significant at the 0.05 probability level.

Table 5. Mean frequencies of shoot and root regeneration from callus on hormone-free medium, and number of genotypes which regenerated shoot or root from callus.

Population	N[a]	Frequency (%) Shoot	Frequency (%) Root	Number of genotypes Shoot	Number of genotypes Root
EL 45/2	44	29.5	2.3	4/5	1/5
L53	30	10.0	16.7	3/4	3/4
GWK	41	2.4	22.0	1/4	3/4
EL 36	12	0.0[b]	0.0[b]	0/3	0/3
EL 40	17	0.0	0.0	0/2	0/2
EL 48	46	0.0	0.0	0/5	0/5
F1003	24	0.0	0.0	0/2	0/2
FC 506	18	0.0	0.0	0/3	0/3
FC 607	27	0.0	0.0	0/4	0/4
FC 701/5	41	0.0	0.0	0/5	0/5
FC 708	28	0.0	0.0	0/4	0/4
SP 6822	38	0.0	0.0	0/4	0/4
SP 6926	16	0.0	0.0	0/3	0/3
84M5-20	41	0.0	0.0	0/5	0/5
EL 44	0	---	---	---	---
OA	0	---	---	---	---
Total	423	---	---	8/53	7/53
Mean	---	4.0	3.5	---	---

[a] number of explants which produced callus
[b] populations with no response were not included in the analysis of variance

unpublished). When explants of EL 45/2 were placed on hormone-free medium, it was common to find several shoots arising on the callus from a single explant. We speculate that this is due to a high level of endogenous cytokinin.

The lag period between inoculation and callus production was longer than the 4-6 weeks observed with MS + 1 mg/L BA (Saunders and Doley, 1986). The overall mean for time to callus was 96.7 days (Table 7), with a range of 42 to 185 days (the experiment was terminated at 185 days). Significant differences between populations were found for this trait (Table 8), and there was also significant variation among plants within a population and among leaves within a plant. Only one population, GWK, had a mean time to callus less than 10 weeks, which is frequently the time at which related experiments employing BA are terminated in our lab. It was common for an explant to appear 'dead' at the time of callus initiation. When using population means, the correlation between time to callus and callus frequency was not significant ($r = -0.16$), but the correlation was highly significant ($r = -0.43^{**}$) when examined at the level of individual leaf means. Thus, leaves which produced callus at high frequency were faster to callus, but this relationship was not observed at the population level.

Table 6. Analysis of variance of frequency of shoot and root regeneration from callus on hormone-free medium.

Source	df	Mean squares(arcsine) Shoot	Mean squares(arcsine) Root
Populations	2	0.14216	0.43226
Plants/Population	10	0.05970	0.36108**
Leaves/Plant	13	0.03670	0.00838

** Significant at the 0.01 probability level.

Table 7. Mean values of time to callus from leaf disks on hormone-free medium.

Population	N[a]	Time(days)
GWK	41	57.6
EL 45/2	44	78.5
SP 6926	16	83.1
EL 36	12	83.9
EL 40	17	84.4
F1003	24	85.2
SP 6822	38	92.2
FC 506	18	98.7
FC 701/5	41	99.5
FC 607	27	108.9
L53	30	108.9
EL 48	46	110.5
FC 708	28	119.3
84M5-20	41	129.7
OA	0	---
EL 44	0	---
Total	423	---
Mean	---	96.7

[a] number of explants which produced callus

The behavior of explants on hormone-free medium was different than that observed in previous studies using medium containing 1 mg/L BA (Doley and Saunders, unpublished). The presence of BA in the medium resulted in explant expansion, sometimes up to 20-fold, and increased the duration of chlorophyll retention. The explants on hormone-free medium remained small and became brown sooner. There also appeared to be fewer sites of callus initiation per explant on the hormone-free medium.

Variation among plants within a population was significant for 4 of the 5 traits examined (Tables 3,4,6 and 8), and leaves within a plant varied significantly for the one trait where this information was available (Table 8). This variation highlights the need for sampling more than one leaf per plant and several plants per population, particularly when demonstration of population differences for in vitro behavior is an objective. Although differences among populations were difficult to detect due to the large variation of plants within a population, sampling more plants per population should allow detection of the differences which seem to exist.

Population differences for frequency of root formation from leaf disks and for time to callus on hormone-free medium provide further demonstration of the extensive variation for in vitro behavior within the sugarbeet germplasm pool. This study also demonstrates that the actual requirements for callus production are quite minimal. The shoot regeneration by L53 on hormone-free medium but not on medium containing 1 mg/L BA suggests that examination of genotype x medium interaction is warranted and may allow regeneration media to be designed for a wider range of beet germplasm.

Table 8. Analysis of variance of time to callus from leaf disks on hormone-free medium.

Source	df	Mean squares
Populations	13	12913.3**
Plants/Population	39	2670.4**
Leaves/Plant	46	1235.1**
Samples/Leaf	324	601.2

** Significant at the 0.01 probability level.

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