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Field assessment of dihaploid *Solanum tuberosum* and *S. brevidens* somatic hybrids

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Summary. Following both chemical and electrical fusion of protoplasts of a dihaploid line of potato (*Solanum tuberosum*), (PDH40), with those of the wild species, *Solanum brevidens*, 11 and 40 somatic hybrid plants, respectively were obtained. Fifteen of these somatic hybrid genotypes and the two parents were studied further in a small field trial to assess field performance and phenotypic variability. In the UK, somatic hybrid plants are classified as genetically engineered organisms, and the UK Advisory Committee on Genetic Manipulation have imposed various restrictions on field experiments. Examination of the somatic hybrids in the field showed extensive phenotypic variability, and no two genotypes were identical. Some of the variation reflected changes in chromosome numbers, but other factors were also involved. Half the somatic hybrid genotypes produced tubers in the field, although the tubers were smaller and differed morphologically from those of PDH40. The results of the study suggest that the extent of somaclonal variation manifested in somatic hybrids is greater than that found in protoplast-derived plants of potato. The implications of this observation and the current regulations concerning field experiments of somatic hybrid plants in the UK are discussed.

Key words: *Solanum tuberosum* – *Solanum brevidens* – Somatic hybrids – Planned release – Field trial

Introduction

The application of protoplast fusion technology in practical potato breeding programmes is becoming more widespread (Jones 1987; Fish 1988). Integration of protoplast fusion techniques can give the breeders new opportunities to incorporate novel germplasm and to produce improved varieties. For example, protoplast fusion will allow the direct synthesis of novel somatic hybrids between the potato (*Solanum tuberosum*) and its sexually incompatible wild relatives (e.g. *S. brevidens*, Austin et al. 1985a, 1986; Fish et al. 1987, 1988). The use of dihaploid and monohaploid potato genotypes is also being examined as a means of simplifying potato breeding (de Maine 1978, 1982; Ross 1986). Protoplast fusion will enable the best dihaploid and monohaploid lines to be combined systematically into superior tetraploid genotypes (Wenzel et al. 1979; Austin et al. 1985b; Uijtewaal et al. 1987).

Successful application of protoplast fusion methods in plant breeding will depend on the production of fertile, vigorous, somatic hybrid plants that have useful agronomic characters. During protoplast fusion, parental protoplasts are mixed, fused, and the resulting heterokaryons regenerated to plants. Thus, somatic hybrids will be expected to show a range of phenotypic characters, as a result of somaclonal variation (Larkin and Scowcroft 1981; Karp and Bright 1985), segregation of parental organelles and recombination between the parental mitochondrial genomes (Kemble et al. 1986). Recent studies have indicated that the range of phenotypic characters in somatic hybrids may be extensive (Austin et al. 1986; Evans et al. 1983), and it has been suggested that agronomic characters (e.g. virus resistance) may, to some extent, be subject to tissue culture-induced variation (Gibson et al. 1988). However, more detailed assessments of the type and range of variation present in somatic hybrids and its transmission through subsequent sexual generations are still needed.

We have generated 51 somatic hybrid plants between a dihaploid potato (PDH40) and the diploid wild species

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S. brevidens, following both chemical and electrical techniques of protoplast fusion (Fish et al. 1987, 1988). Examination of these hybrids revealed a range of chromosome numbers, including the tetraploid level, and greenhouse-grown plants showed different phenotypic characters. To assess this variation further, a small field experiment was planned at Rothamsted Experimental Farm in which individual somatic hybrid genotypes could be studied in detail. However, at present in the UK, somatic hybrid plants are classified as 'genetically manipulated organisms' (ACGM/HSE/Note 3). Consequently, approval for the field trial was sought from a government committee, the Advisory Committee on Genetic Manipulation (ACGM, part of the Health and Safety Executive), with responsibility to oversee and advise on release of genetically manipulated organisms. In this paper we report the results of the field trial, which was the first such experiment involving somatic hybrid plants to be carried out in the UK.

Materials and methods

Planning

As noted above, since the somatic hybrid plants are classified as genetically manipulated organisms, it was necessary to submit a proposal to the ACGM before the experiment could proceed. After consultation with ACGM and the Rothamsted Biological Safety Committee, and following greenhouse testing for fertility, approval for the field trial was given by the ACGM, but with the following restrictions: (1) the experimental plot was to be a minimum distance of 50 m from other potato plants. (2) A non-solanaceous guard crop was required. (3) All flower buds of somatic hybrids and parental lines were to be removed before they opened. (4) All harvested plant material was to be autoclaved after the experiment. (5) The plot was to be inspected over the following year, ploughed in the autumn following the experiment and sprayed with a total herbicide. Potato plants were not to be grown on the plot for 4 years subsequently. (6) Access to the site was to be restricted. (7) The local chief Environmental Health Inspector, town council and general public (via the local press) were to be informed of the experiment. During the experiment, the above restrictions were followed carefully. The experiment was checked daily during the flowering period, and all developing flower buds were removed.

Experimental design and field measurements

The field trial was conducted at Rothamsted Experimental Farm during the 1987 growing season. The experiment consisted of 10 replicates each of *S. tuberosum* PDH40 and *S. brevidens,* 5 replicates of somatic hybrid plants derived from chemical fusion (70064, 65003, 65009, 65013, 65014, 70067, 70023, 70027, 65006) and from electrical fusion (81011, 81012, 84042, 81045, 84111 and 84140). The plot consisted of four rows of plants spaced 76 cm apart. The plants within the rows were spaced at 46 cm intervals in a totally randomized design. Two 1-metre-wide spray paths were left through the middle of the plot, and the plants were sprayed following a standard regime, at two weekly intervals, against aphids and potato blight. The entire experimental plot was surrounded by a field of spring barley as the non-solanaceous guard crop. In addition, the experiment was positioned 54 metres away from the nearest potato plants on Rothamsted Farm.

All the genotypes studied were maintained as in vitro shoot cultures on MS20 + 0.05 $mg l^{-1}$ NAA (Fish and Karp 1986). Plants were established in pots in EFF compost (EFF products, Guildford, Surrey), and after an initial period in a greenhouse, they were 'hardened off' in an open-sided greenhouse for 3 weeks. The plants were transferred to the field on May 27, 1987 during a spell of warm weather. The morphological characters of the parental and somatic hybrids plants were measured 58 days after planting. The criteria used for assessing plant morphology was based on those of the NIAB guide for identifying potato varieties (NIAB 1975). The following characters were measured: (a) general appearance, (b) habit, (c) canopy denseness, (d) plant height, (e) number of stems, (f) mean internode distance, (g) terminal and lateral leaf shape, (h) lateral leaflet surface and margin, (i) number of lateral leaves and secondary leaflets, (j) foliage colour, (k) glossiness, (1) anthocyanin pigmentation, (m) stem wing size and shape.

The experiment was harvested September 1 (99 days after planting), and the following tuber characters were measured: (a) tuber shape, (b) number of tubers per plant, (c) total tuber weight per plant, (d) average tuber weight, (c) number of eyes per tuber, (f) tuber skin colour, (g) tuber flesh colour, (h) sprout colour.

All the tubers were harvested and stored at 4 °C in the dark. The remaining plant material was killed by autoclaving, and the site carefully inspected for any remaining tubers. The plot will be left fallow for the next 3 years, sprayed with the herbicide glyphosate during 1988, and inspected for any residual *Solanum* plants.

Statistical analysis

The statistical analysis was kindly carried out by Dr. H. Spechter (Statistics Department, Rothamsted Experimental Station, UK). The leaf and shoot characters were compared by an analysis of variance (ANOVAR) to find significant differences in the measurements for the parental and somatic hybrid plants. In the analysis, the somatic hybrids were compared either individually to each parent or to a combined parental mean. The scored tuber characters were compared by ANOVAR between the somatic hybrids and PDH40 alone.

The data were then analysed further to determine if there were any significant differences in the morphological characteristics of somatic hybrids derived from either chemical or electrical protoplast fusion. Finally, to examine the effect of ploidy on somatic hybrid morphology, the somatic hybrids were subdivided further into three classes: (a) tetraploids (48), (b) aneuploids at the tetraploid level (48 ±) and (c) aneuploids at the hexaploid level (48 + +), and the data examined.

Results

The 1987 growing season in the UK was colder and wetter than normal, and consequently, the conditions were ideal for growing potatoes. As a result, all somatic hybrid plants except for three genotypes (70027, 65006, 84111) established well and grew quickly in the field (Fig. 1). Since the weather was relatively cool, the conditions were, in general, unsuitable for aphid multiplication or for development of potato blight: there was no

Table 1. Measurements of somatic hybrid and parental plant morphology under field conditions

Genotype	Chromosome no.[a]	No. of plants	Average plant height (cm)	Mean internode distance (cm)	Average no. of stems	Terminal leaf shape[b]	Lateral leaf shape[b]	No. of laterals (mode)	No. of secondary leaflets (mode)
S. brevidens	24	9	39.9 ±2.54	4.43±0.22	12 ±0.5	1.86	2.27	8	12
PDH 40	24	10	23.8 ±0.96	2.4 ±0.17	12.5±1.25	1.87	2.6	4	4
65003	47	5	20.2 ±1.39	3.57±0.38	19.8±2.13	1.14	1.47	4	0
65006	48	5	10 ±0.70	2.19±0.81	3.5±0.5	1.13	1.87	2	0
65009	71	5	26.2 ±2.22	2.46±0.18	4.8±0.37	1.07	1.42	4	2
65013	48	5	33.6 ±4.39	4.3 ±0.22	18.6±1.44	1.19	1.50	6	4
65014	47	5	39.0 ±1.35	4.06±0.42	13 ±1.58	1.23	1.55	6	4
70023	48	5	27.6 ±3.4	3.33±0.38	10.6±0.87	1.1	1.44	4	4
70027	46	5	19.75±3.14	2.2 ±0.29	3.5±1.32	1.08	1.66	4	2
70064	48	5	33.2 ±3.58	4.55±0.7	11 ±1.6	1.08	1.64	4	4
70067	47	5	34.6 ±1.4	3.38±0.16	10.2±1.0	1.26	1.87	4	4
81011	47	5	28.6 ±1.32	4.39±0.28	13 ±0.63	1.15	1.80	4	4
81012	46	5	28.8 ±2.37	2.68±0.21	8 ±0.7	1.38	1.69	4	0
81045	78	5	25.8 ±1.56	3.06±0.19	7.2±0.37	1.03	1.56	4	2
84042	48	5	34.4 ±1.49	2.97±0.19	18.4±1.4	1.21	1.44	4	4
84111	71	5	13 ±0.58	3.22±0.59	2.4±0.33	1.04	1.23	4	4
84140	48	5	44 ±1.58	4.1 ±0.25	16.4±2.0	1.12	1.38	6	6

[a] Chromosome data from Fish et al. (1987, 1988)

[b] Leaf shape = length ÷ width; 1 = broad shape, 2 = narrow shape

Table 2. Morphological characters of somatic hybrid and parental plants under field conditions

Genotype	General appearance	Habit	Canopy denseness	Lateral leaf margin	Lateral leaf surface	Foliage colour	Foliage gloss
S. brevidens	H	B	Av	Pl/Kink	Rib	GRG	L
PDH 40	H	B	C	W	Rou	LG/MG	M
65003	H	Dom	C	Kink	Rou	MG	L
65006	P	Prst	O	Kink	Rou	YG	L
65009	Wk	E	O	Kink	Rou	MG	L
65013	H	B	Av	Kink	Rou	MG	L
65014	H	Str	O	Kink	Rou	MG	L
70023	H	E	Av	Kink	Rou	MG	L
70027	P	E	O	Pl	Rou	YG	Hea
70064	H	Spr	O	Kink	Rou	MG	L
70067	H	B	O	W	Rou	LG	L
81011	H	Spr	O	Pl	Rou	MG	L
81012	H	E	C	W	Rou	LG	L
81045	H	E	O	Kink	Rou	DG	M
84042	H	B	A	W	Rou	MG	M
84111	P	E	O	Kink	Rou	LG	L
84140	H	B	Av	W	Rou	MG	L

Key

H = Healthy
B = Bushy
O = Open
W = Wavy
Rou = Rough
LG = Light green
L = Light gloss
E = Erect
C = Close

Spr = Spreading
Kink = Kinked
MG = Mid green
Dom = Domed
DG = Dark green
M = Mid gloss
Av = Average
Wk = Weak

P = Poor
Pl = Plain
YG = Yellow green
Hea = Heavy
Rib = Ribbed
GRG = Grey green
Str = Straggly
Prst = Prostrate

apparent incidence of blight or aphid-transmitted diseases during the field trial.

The data from the field trial show that the somatic hybrids differed in a wide range of phenotypic characters (Tables 1–3), and no two appeared to be the same. They possessed phenotypic characters that were either similar to one parent, intermediate between those of the two parents, or that were unique. For example, some expressed an anthocyanin pigmentation pattern which was intermediate between that of *S. brevidens* (pink anthocyanin pigmentation present on stem, axils and rachides), and PDH40 (no anthocyanin pigmentation) (Table 3). However, other characters such as leaf shape, plant habit and canopy denseness were quite distinct and typical only of the somatic hybrids (Tables 1 and 2). Other phenotypic characters resembled those of either *S. brevidens* or PDH40; for example the lateral leaf surfaces of the somatic hybrids were similar to that of PDH40, while the extent of foliage gloss and stem wing shape were the same as *S. brevidens* (Tables 2 and 3).

Fig. 1. Field trial 8 weeks after planting; note cereal guard crop. Somatic hybrid and parental plants were grown in a randomized design

The data also show that many of the characters scored in the somatic hybrids can exhibit patterns of variation that differ from both parents. The height values for somatic hybrid plants, and their mean internode distances, ranged between those of PDH40 and *S. brevidens*. However, the values for the average number of stems per somatic hybrid plant extended both above and below those of the parents (Table 1). Finally, the somatic hybrids typically produced much broader leaves when compared with PDH40 and *S. brevidens* which both have narrow leaves (Table 1, Fig. 2).

Statistical analysis of the data showed that many of the observed differences between the somatic hybrids and the parents were significant. Significant differences ($P < 0.001$) were found for all the characters measured in Table 1 when the somatic hybrids were compared individually with either parent. In addition, significant differences ($P < 0.001$) were found for terminal and lateral leaf shape and for the numbers of laterals and secondary

Table 3. Morphological characters of the parental and somatic hybrid plants under field conditions continued

Genotype	Anthocyanin on stem	Anthocyanin on leaf axil	Anthocyanin on leaflet axil	Anthocyanin on rachides	Stem wing size	Stem wing shape
S. brevidens	Mot	Mot	Mot	Pr	S	Stg
PDH 40	A	A	A	A	S	W
65003	T	T	A	Pr	SS	S
65006	T	T	A	A	–	–
65009	Mot	T	T	T	SS	Stg
65013	T	T	T	T	S	Stg
65014	Mot	Mot	T	T	S	Stg
70064	T	T	T	A	S	Stg
70067	Mot	T	T	T	S	W
70023	T	T	T	T	S	Stg
70027	T	T	T	A	S	Stg
81011	T	T	T	T	S	Stg
81012	T	T	T	T	S	Stg
81045	T	T	T	T	S	Stg
84042	T	T	T	T	S	Stg
84111	T	T	Ob	Ob	S	Stg
84140	Pr	T	T	T	Med	W

Key

Pr = Present
T = Trace
Med = Medium
W = Wavy
Ob = Obvious

S = Small
Stg = Straight
A = Absent
Mot = Mottle
SS = Very small

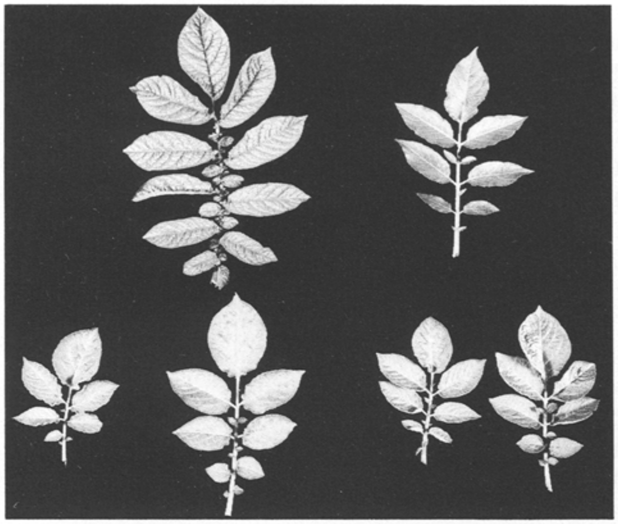

Fig. 2. Leaf morphology. Top left, *S. brevidens*; top right, PDH40; bottom row, leaves from four different somatic hybrids (left to right: 65013, 81011, 84140, 70067)

leaflets when the somatic hybrids were compared to the combined parental mean. No significant differences were found between the somatic hybrids derived from either chemical or electrical fusion. However, significant differences were found between the ploidy and height, mean internode distance, leaf shape and the number of secondary leaflets of individual somatic hybrid plants. These data suggest that the 48 and 48± categories of somatic hybrid plants were similar, while the 48++ group were generally shorter with broader leaves and fewer secondary leaflets. The three genotypes which grew poorly in the field (70027, 65006 and 84111) had 46, 48 and 71 chromosomes, respectively. Thus, chromosome number and/or ploidy appears not to be the only reason for poor growth, and other factors are involved.

Of the 15 somatic hybrids examined, only 7 (65013, 70023, 70064, 70067, 81011, 84042 and 84140) produced tubers in the field (Table 4). Tuberization of the somatic hybrids depended on ploidy level since 5 of the tuberizing genotypes had 48 chromosomes, and the remaining 2 had 47 chromosomes. None of the hexaploid somatic hybrids tuberized. The somatic hybrid tubers were typically more elongated than those of PDH40 (Fig. 3, Table 4). The total and average tuber weights and the number of tubers per plant were significantly less ($P<0.001$) for the somatic hybrids compared to PDH40. However, tuber flesh colour, skin colour and the number of eyes per tuber were the same for the somatic hybrids and PDH40. When in the field, the somatic hybrid tubers tended to sprout while still attached to the parental plant. Thus, the tuberizing hybrids had more stems per plant than those hybrids without tubers.

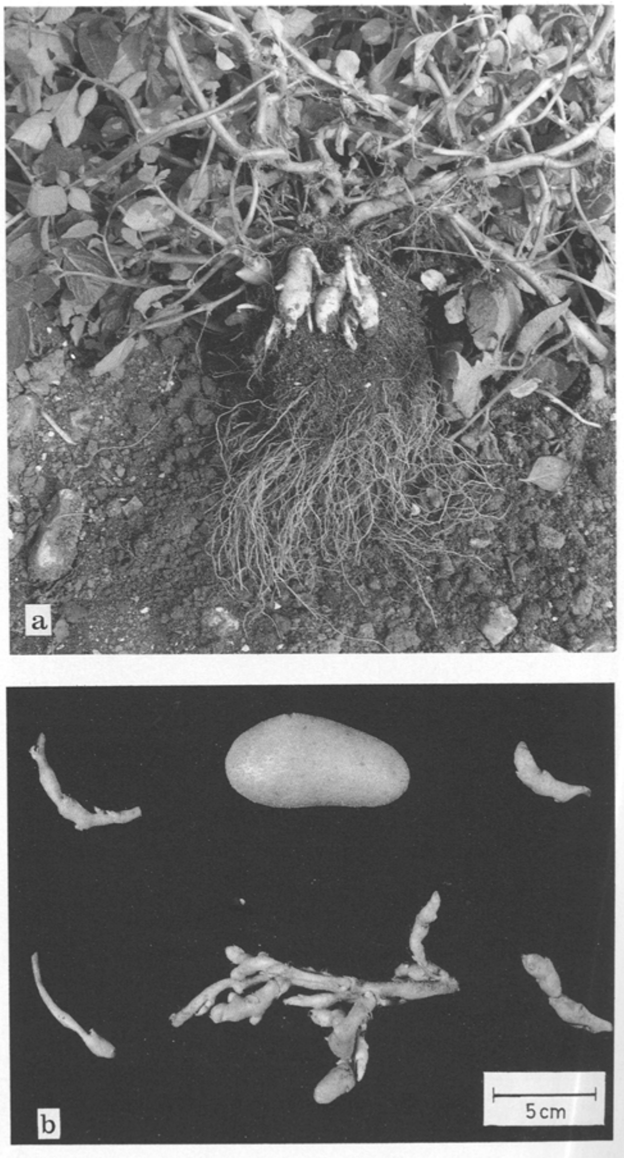

Fig. 3. **a** Harvesting tubers from somatic hybrid 70064; **b** tuber morphology. Top row, left to right: 81011, PDH40, 84042; bottom row, left to right: 65013, 84140, 70064

Discussion

In this paper, we have described extensive phenotypic variability in somatic hybrid potato plants grown in the field. The somatic hybrids exhibited many different phenotypic characteristics, some intermediate between those of either parent, and some unique. Thus, our observations agree with the findings of Austin et al. (1986), and by examining individual somatic hybrid plants in detail, it was also apparent that no two genotypes of somatic hybrid plants appeared to be identical. Phenotypic differences between individual somatic hybrid genotypes have been observed before in somatic hybrids of *Solanum*

Table 4. Tuber characteristics of the parental and somatic hybrid plants under field conditions

Genotype	No. of plants with tubers	Tuber shape[a]	No. of tubers per plant	Total tuber weight per plant (g)	Average tuber weight (g)	No. of eyes per tuber	Tuber skin colour[b]	Tuber flesh colour[b]	Sprout colour[b]
PDH 40	10	1.67 ± 0.08	49.7 ± 4.43	574 ± 72.3	12.3 ± 1.47	3.8 ± 0.2	Wt	Wt	Gr
65013	5	6.4 ± 0.67	6.2 ± 0.66	14.9 ± 2.7	2.33 ± 0.26	4.2 ± 0.37	Wt	Wt	Pr
70023	5	4.24 ± 0.27	17.8 ± 3.76	26 ± 5.1	1.6 ± 0.27	4.92 ± 0.79	Wt	Wt	Pr
70064	5	3.39 ± 0.27	16.8 ± 5.5	126.5 ± 41.6	7.73 ± 2.13	4.6 ± 0.25	Wt	Wt	Pr
70067	3	4.1 ± 0.32	11 ± 4.04	31.46 ± 9.8	3.1 ± 0.22	4.33 ± 0.33	Wt	Wt	Pr
81011	5	3.8 ± 0.37	21.4 ± 5.8	48.74 ± 8.1	2.9 ± 1.1	4.6 ± 0.24	Wt	Wt	Pr
84042	5	6.42 ± 1.6	32.8 ± 3.24	65.28 ± 9.8	1.97 ± 0.16	5.4 ± 0.51	Wt	Wt	Pr
84140	5	4.3 ± 0.6	18 ± 6.4	49.2 ± 20	2.57 ± 0.37	4.86 ± 0.35	Wt	Wt	Pr

[a] Tuber shape = length ÷ width. 1 = round; 2 = narrow, elongated

[b] Wt = white; Pr = purple; Gr = green

(Austin et al. 1986) and *Nicotiana* (Evans et al. 1983). However, it would appear that the range of phenotypic variation was greater in the somatic hybrids than would normally be expected from potato protoclones (Shepard et al. 1980; Secor and Shepard 1981; Thomas et al. 1982; Fish and Karp 1986; Austin et al. 1986). Some of this variability can clearly be attributed to changes in chromosome number (Table 1), as the hexaploid somatic hybrids differed phenotypically in various characters from the tetraploid (48) and tetraploid aneuploid (48 ±) somatic hybrids. However, other changes, such as recombination between the parental organelle genomes (Kemble et al. 1986), changes in gene copy number (Landsmann and Uhrig 1985), changes in DNA methylation (Quemada et al. 1986) and point mutations (Evans and Sharp 1986) may have occurred. In addition to these effects, there may be some incompatibility between the parental genomes, which could result in the loss of the chromosomes of one parent to produce asymmetric hybrids (Hoffman and Adachi 1980). It is interesting to note that none of our hexaploid somatic hybrids produced tubers, whereas those of Austin et al. (1986) tuberized well. The latter hybrids were the result of fusing a tetraploid potato line with *S. brevidens*. This observation may indicate that the constitution of the hexaploid somatic hybrids examined in this study could be two copies of the *S. brevidens* (non-tuberizing) genome and one of *S. tuberosum*, which could well lead to the absence of tuber production. This aspect is being studied further.

It is possible that the effect of somaclonal variation on somatic hybrids may be more extensive than that on normal protoplast-derived regenerants. This may have further implications in the use of protoplast fusion to resynthesize tetraploid potatoes from dihaploid genotypes (Ross 1986; Austin et al. 1985b). Plant regeneration via tissue culture, and in particular through a callus phase, may depress useful agronomic characters in crop species (Secor and Shepard 1981; Powell et al. 1986; Pfeiffer and Bingham 1984) although useful variants have also been found (Matern et al. 1978; Evans et al. 1984, 1986; Gavazzi et al. 1987). The successful application of protoplast fusion to potato breeding will depend on efficient protoplast fusion and regeneration techniques by which large numbers of somatic hybrids can be produced (Fish et al. 1988). In most cases, fertile somatic hybrids are required, and initial tests have shown limited female fertility of our somatic hybrids. By screening these hybrids, it should be possible to identify genotypes whose performance is not reduced by tissue culture induced variation. It is also possible that some hybrids may have characters that are enhanced by somaclonal variation, and this potentially new source of variability would need to be assessed further through subsequent sexual generations.

In the UK, the planting of somatic hybrids in the field is overseen by the government committee, the ACGM. This contrasts with regulations in some other countries. For example, in the USA, at present there are no regulations to control field release of somatic hybrid plants (Austin et al. 1986). The UK Government has taken a cautious approach and decided to regulate all such experiments and to assess any possible risk on a case by case basis until the environmental implications have been fully assessed (Hauptlii et al. 1985). Because of the regulations involved, considerable interest in the press, both national and scientific, was generated by the experiment. During the field trial, the imposed restrictions were carefully followed. The most time-consuming part was the daily removal of flower buds. This was required despite the fact that greenhouse tests had shown the hybrids plants to be essentially male sterile. The harvested tubers lose viability at 4 °C more rapidly than those of standard potato varieties, and so it is unlikely that tubers of somatic hybrid plants could persist in the soil through the winter. Nevertheless, the site is being monitored for any *Solanum* shoots and will be sprayed with a total herbicide. Further field experiments of somatic hybrids are needed to evaluate fully any environmental hazards and that may enable the ACGM regulations to be relaxed or removed completely. Copies of our applications to the ACGM are available to those specifically interested in the field evaluation of somatic hybrid plants in the UK.

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