Theor Appl Genet (1986) 72:405–412

Improvements in regeneration from protoplasts of potato and studies on chromosome stability

1. The effect of initial culture media

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Accepted January 27, 1986
Communicated by R. Riley

Summary. Regeneration of plants from protoplasts of potato (*Solanum tuberosum*) cv. 'Maris Bard' has been achieved from four different initial culture media (ET2, ET3, CLG, VkCLG). These media differed in their hormone, salt and sugar content. Plating efficiencies were highest in the VkCLG medium, but no correlation was found between plating efficiency and regeneration frequency (i.e. the percentage of calli producing shoots). Regeneration frequencies were high on all four media; up to 95% on ET3. Chromosome counts of up to 50 regenerants selected at random from the four treatments showed no significant differences between any of the treatments, in the proportions of plants which were euploid (48), aneuploid at the tetraploid level (48±), and aneuploid with high chromosome numbers (48++). Highly significant differences were present, however, between shoots which rooted quickly (predominantly euploid) and those which rooted only after transfer to a rooting medium (predominantly 48++). Overall more than 60% of the regenerants were normal (2n=4x=48) and this is a considerable improvement on our earlier work in this cultivar (4% normal). These findings are discussed in relation to factors affecting chromosome stability. Chromosome structural rearrangements are also described.

Key words: Potato – Protoplasts – Culture media – Regeneration – Chromosome variation

Introduction

Somaclonal variation is now recognised as a general phenomenon of regeneration systems involving disorganised (callus) growth (Larkin and Scowcroft 1981). In potato (*Solanum tuberosum*), there has been considerable interest in this variation, particularly in protoplast-derived regenerants, following the initial reports in the American cultivar 'Russet Burbank', that changes could occur in agronomically useful traits such as yield and disease resistance (Matern et al. 1978; Shepard et al. 1980; Secor and Shepard 1981). Cytological studies have since indicated that part of this variation, at least, can be attributed to changes in the chromosomes, and in protoplast-derived potato plants both numerical (Karp et al. 1982; Sree Ramulu et al. 1983) and structural (Creissen and Karp 1985) chromosome variation have been described.

Our early studies indicated that chromosome variation could be quite extensive amongst potato plants regenerated from protoplasts. In cv. 'Maris Bard', regenerated using an early method (Thomas 1981) only 4% of protoplast-derived plants had the normal tetraploid number of 48 chromosomes (2n=4x=48). The remainder were aneuploids with mostly high numbers (2n=67 → 2n=93), suggesting that chromosome doubling followed by loss had occurred during the regeneration procedure (Karp et al. 1982). In contrast, potato plants regenerated from protoplasts of cv. 'Fortyfold' were 30% euploid (2n=4x=48) and the aneuploidy was largely independent of chromosome doubling (mostly 2n=44 → 2n=49).

More recently, in cv. 'Majestic', an improved proportion of normal euploid regenerants was obtained. On average, almost 60% of the regenerated plants had 48 chromosomes and the remaining aneuploids were either limited at about the tetraploid level (i.e. 2n=44 → 2n=49) or else contained plants with high numbers (2n=73 → 2n=96) (Creissen and Karp 1985).

Clearly, the proportions of normal tetraploid regenerants obtained between potato cultivars can vary.

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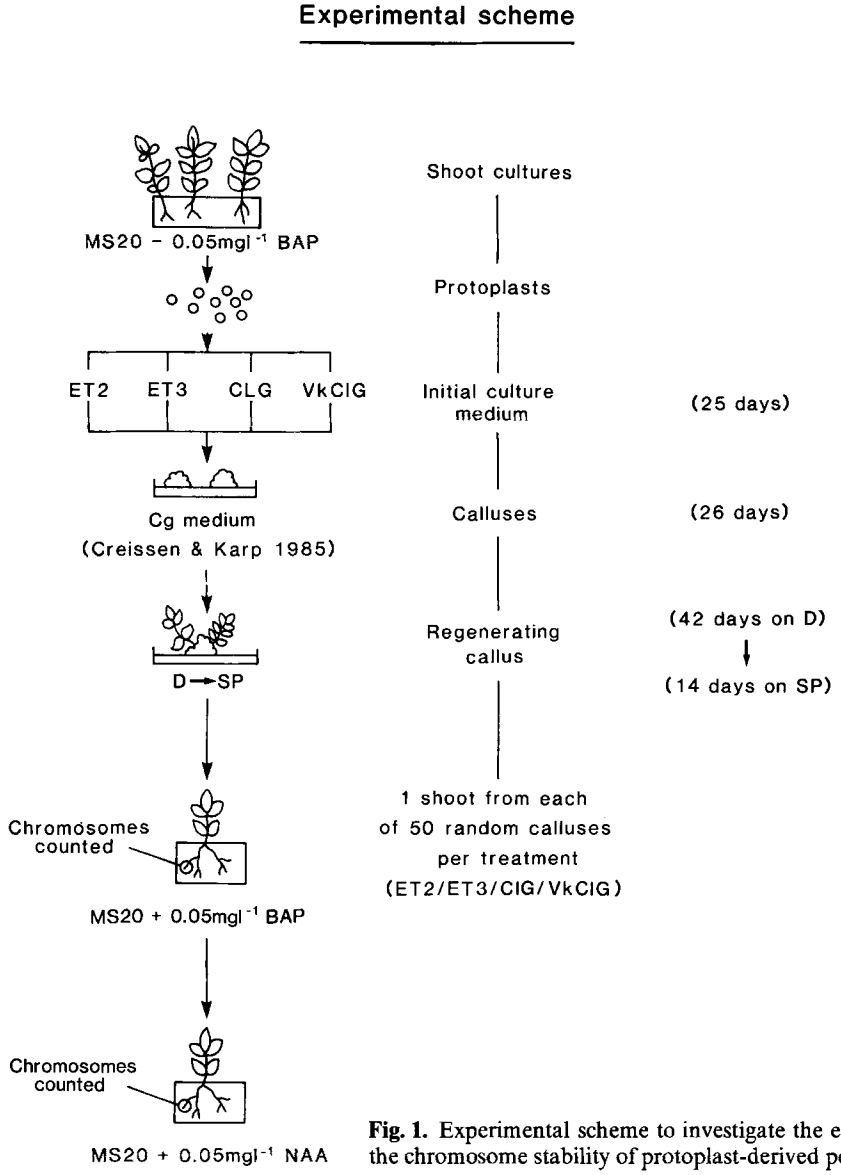

Fig. 1. Experimental scheme to investigate the effect of four different initial culture media on the chromosome stability of protoplast-derived potato plants

This may stem from differences in the media components utilised, from genotypic differences in the stability of the cultivars (Wenzel et al. 1979; Sree Ramulu et al. 1985) or from a combination of both these factors. So far in our studies, the separation of these possibilities has been impeded by the difficulty of regenerating the same cultivar, in sufficient quantities, using a variety of different regeneration protocols. This difficulty has now been resolved.

The present study describes a cytological analysis of 'Maris Bard' plants regenerated from protoplasts using four different initial culture media. 'Maris Bard' was selected in order to establish whether the low frequency (4%) of euploid regenerants obtained in our earlier studies (Karp et al. 1982) was typical of this particular cultivar, or whether it was related to the protocol adopted (Thomas 1981, 1982). In addition, we have changed the media components used in only the initial stages of culture, whilst keeping the later stages identical, because of the numerous reports in the literature indicating that most of the variation occurs during the early stages of protoplast culture (Barbier and Dulieu 1983; Sree Ramulu et al. 1984; Carlberg et al. 1984), and the suggestion that manipulation of hormone levels during initial culture may be one way of avoiding chromosome instability (Sree Ramulu et al. 1984).

Materials and methods

In vitro shoot cultures of *Solanum tuberosum* cv. 'Maris Bard' were established from surface-sterilised axillary buds, as de-

scribed by Thomas (1981). Shoot culture material was maintained on Murashige and Skoog (1962) basal medium containing 20 $g l^{-1}$ sucrose (MS20), 0.05 $mg l^{-1}$ benzyl amino purine (BAP), 6 $g l^{-1}$ Agar (Difco Bacto, Detroit) at pH 5.6 (160–180 $\mu E m^{-2} s^{-1}$ from white daylight fluorescent tubes, 16 h day, 25 °C). Shoot cultures were grown in 8 cm diameter glass jars covered with a 9 cm diameter Petri dish lid. The jars were sealed with Nescofilm and ventilation provided by making seven 0.5 cm slits (Creissen and Karp 1985).

Protoplasts were isolated using the method of Creissen and Karp (1985) with the modifications of Foulger and Jones (1986), except that $CaCl_2 \cdot 2 H_2O$ in the enzyme and wash media was increased to 10 mM. Protoplasts from a single isolation were cultured in four different media (Fig. 1) at a density of 2×10^4 ml^{-1} at 25 °C in the dark. Media ET2 and ET3 have previously been described (Thomas 1981) and contained 2 and 5 $mg l^{-1}$ 2,4-D, respectively, as have CLG and VkCLG which both contained 1.0 $mg l^{-1}$ naphthalene acetic acid (NAA) and 0.4 $mg l^{-1}$ BAP, and differed in their salt content (Foulger and Jones 1986). All culture media were adjusted to 570 mOsm.

After 6 days the protoplast density was reduced by diluting 2.5 ml of culture medium with 4 ml of the same fresh medium. The osmotic pressure of the culture medium was reduced by transferring 6.5 ml aliquots into one compartment of a tri-compartmented 9 cm Petri dish (Sterilin), connected by a horizontal slot to an identical reservoir medium, but at 300 mOsm solidified with 0.4% Agar. Ten days after isolation the cultures were transferred to continuous light (20 $\mu E m^{-2} s^{-1}$ from white daylight fluorescent tubes, 25 °C), and 25 days after isolation protoplast-derived colonies were transferred to Cg medium (0.1 $mg l^{-1}$ NAA, 0.5 $mg l^{-1}$ BAP) for 26 days (100 $\mu E m^{-2} s^{-1}$ from white daylight fluorescent tubes, 24 h day, 20 °C). Plating efficiencies (defined as the percentage of cultured protoplasts giving rise to colonies) were determined 6 days after transfer to Cg medium. Regeneration was achieved by transferring colonies to D medium (0.1 $mg l^{-1}$ IAA, 1.0 $mg l^{-1}$ zeatin) for 42 days and then to SP medium (0.25 $mg l^{-1}$ BAP, 0.1 $mg l^{-1}$ GA_3 for 14 days (Creissen and Karp 1985). Transfer to D medium induces shoot primordia to form on the calli and shoot elongation is then induced by transfer to SP medium.

Fifty regenerating calli were selected at random from all four treatments for cytological analysis. The first regenerating shoot to appear on each callus was excised and transferred to basal Murashige and Skoog medium with 20 $g l^{-1}$ sucrose, 0.05 $mg l^{-1}$ BAP at pH 5.6 in 60 ml plastic tubs (sterilin). Squash preparations were carried out as described previously (Karp et al. 1982). Selected plants (see "Results") were transferred into EFF compost (EFF Products Ltd., Guildford, Surrey) in 25 cm pots and grown inside in a growth chamber (450 $\mu E m^{-2} s^{-1}$ from warm fluorescent and tungsten lights, 12 h day, 20/16 °C, 70/80% RH).

Results

Protoplast isolation and plant regeneration

Regeneration of plants from protoplasts of potato (*Solanum tuberosum*) cv. 'Maris Bard' was successfully achieved from all four initial culture media (ET2, ET3, CLG, VkClG). Protoplast yields of up to 1.4×10^6 g^{-1} fwt were obtained from in vitro shoot cultures.

Protoplasts reformed cell walls and divided 3–4 days after isolation (Fig. 2 a–b) in all four culture media (ET2, ET3, ClG, VkCLG). Small colonies were obtained after 21 days (Fig. 2 b). Large numbers of calli were recovered from all four culture media, VkClG producing nearly 3,000 colonies from 1.5×10^5 protoplasts initially cultured, thereby giving the highest plating efficiency of 2%, whereas ET3 and CLG both had 0.3% and ET2 0.2%. Numerous shoots developed on the calluses (Fig. 2 c) and for all four culture treatments the percentage of calli producing shoots (i.e. the regeneration frequency) was high, up to 95.3% for ET3 (92.6% CLG, 70% ET2 and VKCLG). There was no correlation between plating efficiency in the initial culture medium and the regeneration frequency.

Numerical chromosome variation

The chromosome number of cv. 'Maris Bard' was confirmed as $2n = 4x = 48$, by screening 5 shoot culture controls. Controls rooted on basal medium with 0.05 $mg l^{-1}$ NAA (below) also had 48 chromosomes.

On average, 63.6% of 178 regenerants screened had the normal tetraploid number of 48 chromosomes and were designated '48'. The remaining aneuploids were of two types; 8.4% had lost or gained only a few chromosomes – designated 48 ± (Fig. 3 a), whilst 28.0% had high chromosome numbers designated 48 + + (Fig. 3 b, c).

The proportion of regenerants in the three classes of chromosome numbers (48, 48 ±, 48 + +) for the four different culture treatments are given in Table 1. The differences between the treatments are small. The data were transformed by the binomial error function, using the logit link, and analysed by the GLIM 3 computer package (Baker and Nelder 1978). An analysis of deviance on the total data showed that the differences in the proportion of plants in the three chromosome classes were not significant between any of the four treatments.

During the regeneration of the 'Maris Bard' plants, it was observed that although all the shoots forming on the calli were transferred to MS 20 + 0.05 $mg l^{-1}$ BAP medium on the same day, the roots forming on these shoots took varying times to appear. Some of the shoots formed roots easily within the first month and their

Table 1. The percentage of regenerants in the three chromosome classes (48, 48 ±, 48 + +) for the four different culture treatments

Treatment	48	48 ±	48 + +	Total counted
ET2	62.2	6.7	31.1	45
ET3	59.6	8.5	31.9	47
CLG	62.8	9.3	27.9	43
VkCLG	69.8	9.3	20.9	43

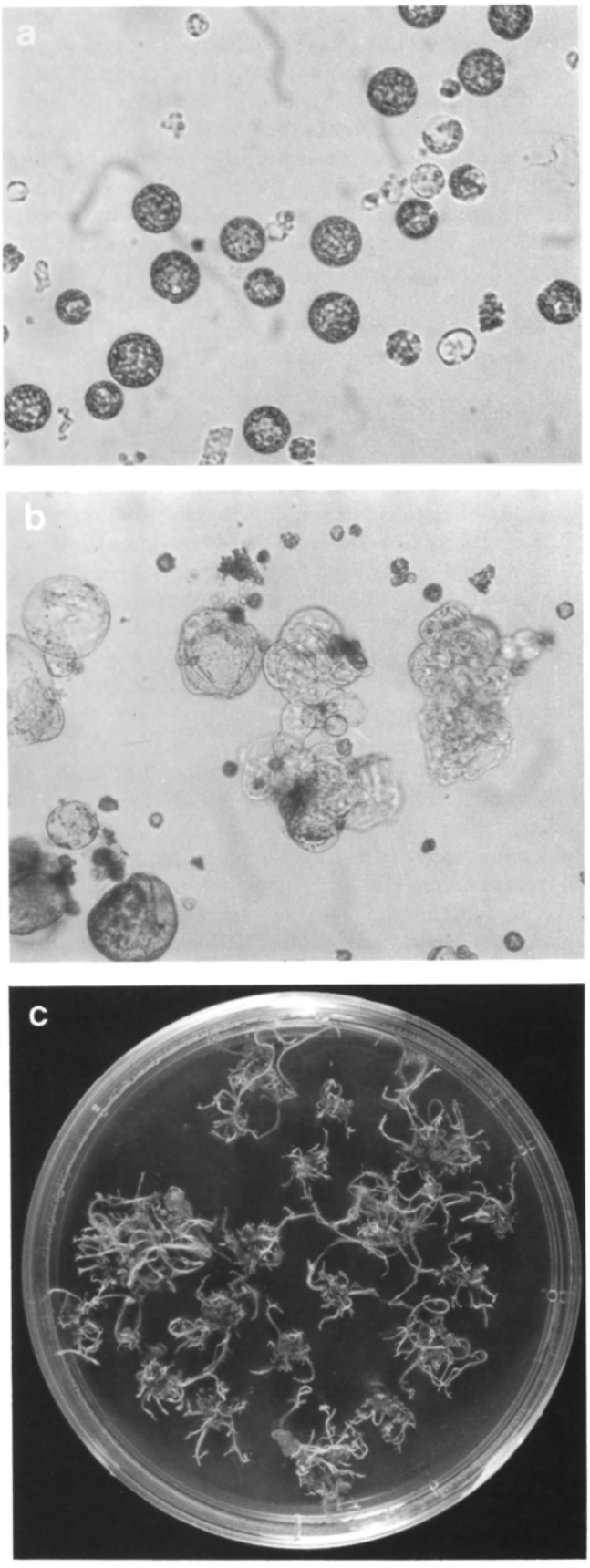

Table 2. The percentage of regenerants in the three chromosome classes (48, 48±, 48++) which rooted before (a) or only after (b) transfer to rooting medium, for the four initial culture treatments

Treatment	48		48±		48++	
	a	b	a	b	a	b
ET2	81.5	33.3	7.4	5.6	11.1	61.1
ET3	79.2	39.1	12.5	4.3	8.3	56.6
CLG	84.6	29.4	15.4	0	0	70.6
VkCLG	80.0	46.2	10.0	7.7	10.0	46.1

chromosome counts were tabulated separately (Table 2a). Other shoots only produced roots if they were transferred to a rooting (auxin-containing) medium (MS 20+0.05 mg l^{-1} NAA). Their chromosome counts are given in Table 2b. A small proportion of plants (12.4%) failed to produce any roots, even 3 months after transfer. They were probably aneuploids, with high chromosome numbers, and have not been included in the data.

The selected data (Table 2a, b) were transformed and analysed as described above. No significant differences in the proportion of plants falling into the three chromosome classes (48, 48±, 48++) were present in either of the two sets of data, between any of the four culture treatments. However, when the average data are compared (Fig. 4a, b), it can be seen that there are large differences in the distribution of regenerants in the three chromosome classes. An analysis of deviance indicated that these differences are highly significant ($P < 0.01$), and are chiefly made up of changes in the proportions of regenerants in the 48 and 48++ classes. Regenerants which rooted quickly and without transfer were predominantly (81.3%) euploid, whereas regenerants which only produced roots after transfer to a rooting medium mostly had high chromosome numbers (only 37% '48').

Structural chromosome variation

Amongst the 178 plants screened, 4 regenerants were identified with structural chromosome changes. Two of these plants had 48 chromosomes (N9212 and N9450), one was aneuploid at the tetraploid level (N9321, 2n=50) and the fourth (N9430) was the only regenerant observed with more than the octoploid complement of chromosomes (2n=97). Regenerants N9212 (Fig. 3d) and N9321 carry a deletion (or possibly a translocation in which the second partner involved cannot be identified) whilst the other two structural

Fig. 2a–c. Plant regeneration: **a** freshly isolated protoplasts; **b** protoplast-derived colonies after 21 days of culture; **c** regenerating calli producing numerous shoots on Sp medium

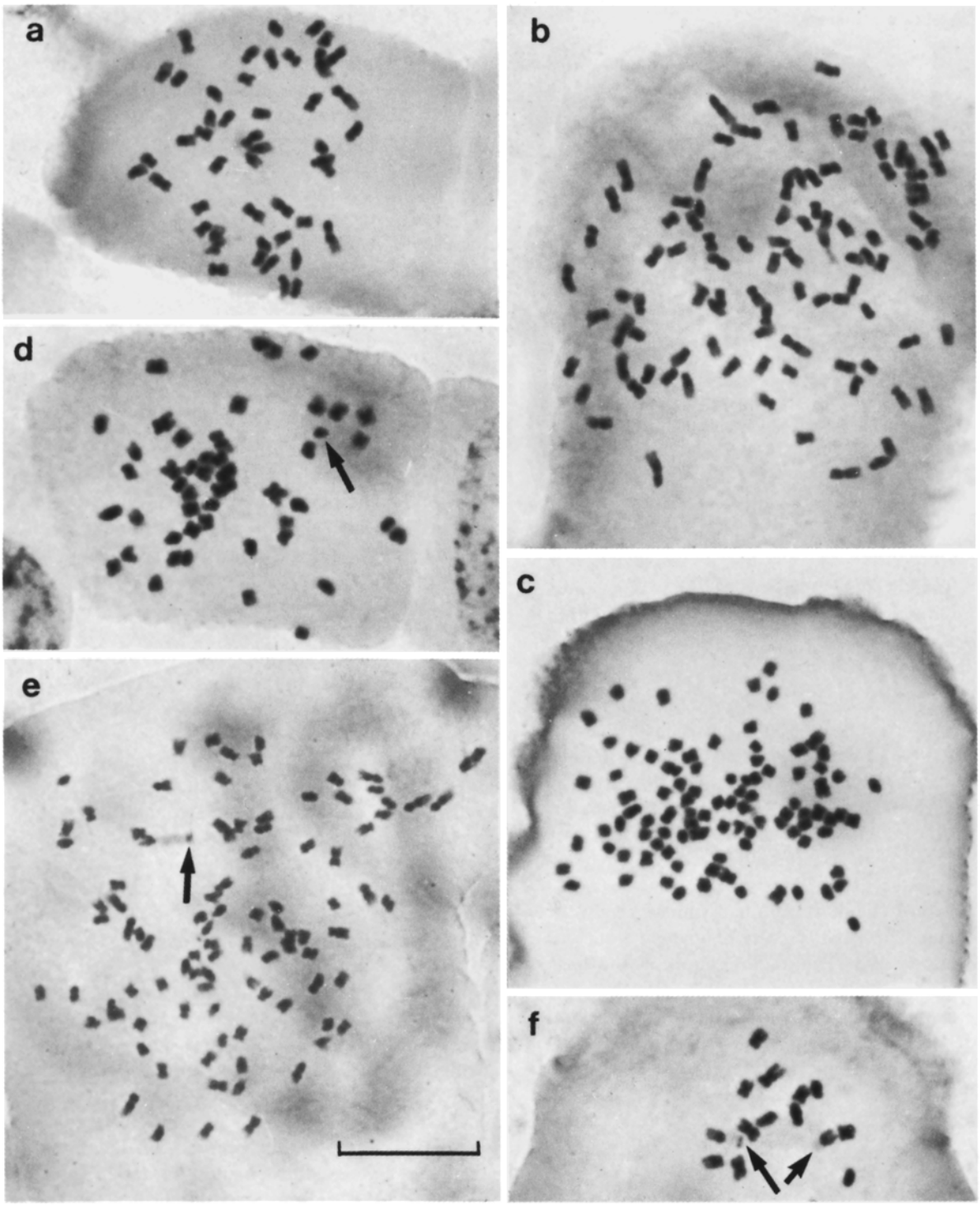

Fig. 3a–f. Chromosome variation in protoplast-derived 'Maris Bard' plants: **a** 48± aneuploid (2n=47); **b** 48++ octaploid (2n=96); **c** 48++ aneuploid (2n=94); **d** euploid (2n=48) with a deletion (*arrowed*); **e** 48++ aneuploid (2n=47) with an amplification of the nucleolus organiser (*arrow*); **f** part of a euploid cell (2n=48) showing another example of an amplification of the nucleolus organiser (*long arrow*). The normal homologue is indicated by a *short arrow.* (Scale bar=10 μm)

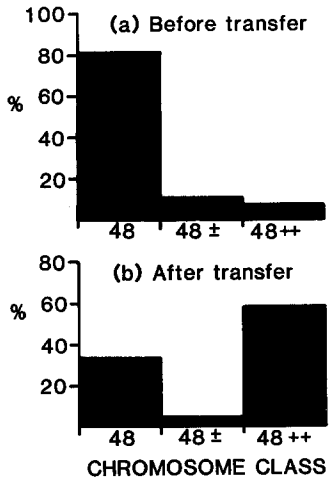

Fig. 4 a, b. The average percentage of regenerants (from all four initial culture treatments) present in the three chromosome classes (48, 48±, 48++) for shoots which rooted before transfer to rooting medium (**a**) and for shoots which rooted only after transfer (**b**)

changes (N9430, N9450) involve the nucleolus organiser, and are both amplifications of the satellite region (Fig. 3 e, f).

General morphology

Seven plants selected at random from each of the 48, 48± and 48++ classes as well as one regenerant carrying a structural change were grown to maturity. An assessment was made on10 morphological characters used in the identification of commercial cultivars (NIAB, 1975). In general terms, the 48 class of regenerants were similar to the 'Maris Bard' control in all characters scored. The 48± class had similar height to the control but with slightly reduced vigour. Some of these regenerants also showed differences in the leaf shape and degree of leaflet overlapping (Fig. 5 a, b). The 48++ class was generally abnormal. Plants in this class showed reduced height and vigour, quite often lateral leaves were absent and there was little leaf overlap (Fig. 5 c, d). The structural change N9450, carrying an amplification of the satellite, resembled the 48 class.

Discussion

Significant improvements have been made from earlier reports (Thomas 1981; Gunn and Shepard 1981) on the isolation and culture of *S. tuberosum* cv. 'Maris Bard' protoplasts.

Over 60% of the potato plants regenerated had the normal euploid chromosome number (2n=4x=48). This frequency is also an improvement on earlier studies in this cultivar, where only 4% of the regenerants were euploid (Karp et al. 1982). There are four possible reasons for the improvement in chromosome stability: 1. the starting material (i.e. in the way that the shoot cultures were grown), 2. the protoplast isolation procedures, 3. the hormones used in the media, 4. the regeneration procedure itself.

Choice of starting material may affect the degree of chromosome variation found amongst regenerated plants, as shown for both the origin of the explant source (Murashige and Nakano 1967; Horak et al. 1975) and the age of the plant tissues (Kasperbauer and Collins 1972; Banks-Izen and Polito 1980). The shoot cultures of 'Maris Bard' used by Thomas (1981) had a high ratio of stem: leaf and it is probable that the protoplasts isolated were not solely from the leaf mesophyll regions. In contrast, our protoplast preparations consisted purely of leaf mesophyll cells (Fig. 2 a) and cytophotometric measurements of such preparations have indicated that the nuclei are predominantly tetraploid with a 4C value of 3.6±0.08 pg of DNA (Jacobsen et al. 1983). It is therefore possible that the earlier instability was present because the source material was heterogenous. This illustrates the necessity of starting with a protoplast population of uniform ploidy, and may become an important consideration where protoplasts are isolated from complex explants, or cell suspension cultures.

A second possibility, is that the conditions of protoplast culture, which were not defined in detail by Thomas (1981), resulted in a greater number of spontaneous fusions which in turn could result in a higher frequency of 48++ plants. Osmolarity is particularly of importance here, and an attempt was made to keep this identical.

Differences in instability could have arisen because of differences in the hormones in the media. Evidence concerning the effects of media components on chromosome stability is both diverse and contradictory (Harvey 1975; Bayliss 1975; Ghosh and Gadgil 1979, Vanzulli et al. 1980; Evans and Gamborg 1982) and has recently been reviewed (Karp and Bright 1985).

In this study the effect of initial culture media was examined. From the results it can be concluded that (i) changing the initial culture medium (as tested here) did not influence the chromosome stability of the regenerated plants (ii) that the high chromosome instabilities described in our earlier report (96% regenerants aneuploid) did not arise from the use of 2,4-D in the initial culture media.

It is possible that hormones are of influence, but in the regeneration medium. Selection is clearly operating in the regeneration stage as more chromosome variation is present in the callus than is found in the

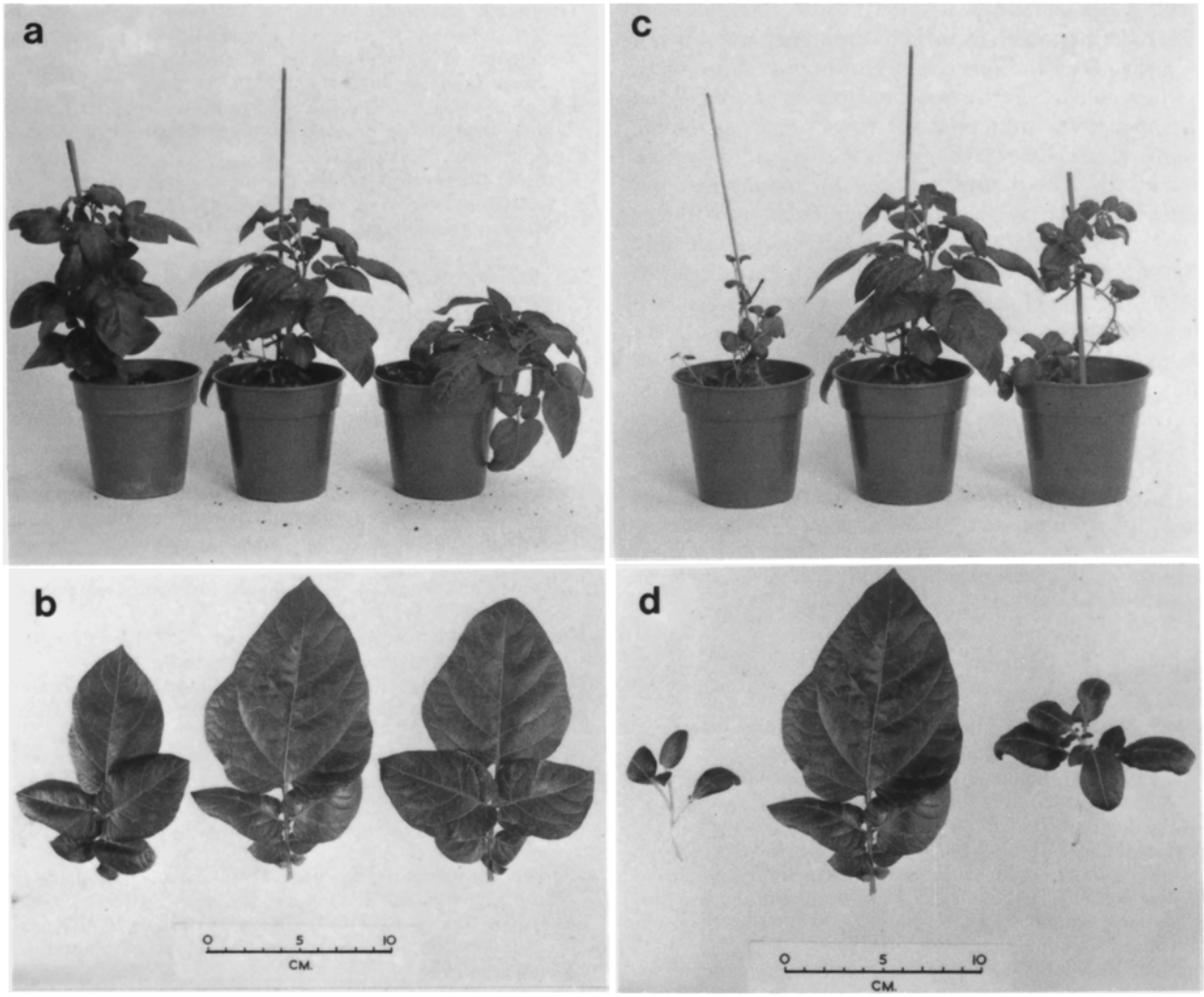

Fig. 5a–d. Morphological variation in regenerated plants: **a** control *centre;* 48± class plants *left* and *right;* **b** corresponding leaves of above; **c** control *centre,* 48++ class plants *left* and *right;* **d** corresponding leaves of above

regenerated plants (Orton 1980; Ogihara 1981; Browers and Orton 1982). Furthermore, indications that the regeneration medium may be important were found in the Dutch cv. 'Bintje', where BAP or BAP+NAA gave a higher percentage of calli that regenerated normal-looking plants than ZR (zeatin riboside) or ZR+NAA (Sree Ramulu et al. 1983). It now remains to test whether chromosome stability is influenced by the selective action of hormones in the regeneration medium. If so, this could be at the stage when shoot primordia form, or at the shoot elongation stage or both. It would also be instructive to establish whether high chromosome stability is related to high regeneration frequency.

The final possible cause of the improvement in chromosome stability lies in the way the regeneration procedure was carried out. In the method of Thomas (1981) the whole regeneration process was achieved essentially on one medium, whereas successive transfer to different media was utilised here. The media used by Thomas (1981) gave no significantly different effect on chromosome stability when included as an initial culture medium, and the total time spent on callusing medium was the same. It is therefore not likely that this is the sole cause of the differences in chromosome stability.

General observations of plants falling in the 48, 48± and 48++ classes indicated that although the 48++ class can be easily recognised, plants in the 48± class could not always be distinguished from 48 regenerants, or control material, grown in the same conditions. This agrees with earlier findings (Karp et al.

1982; Creissen and Karp 1985; Sree Ramulu et al. 1982). Furthermore, distinctions are sometimes difficult to make when the plants are growing as sterile shoot cultures, and are not always a reliable means of picking out aneuploids at this stage. However, chromosome counts for shoots which rooted easily, and for those which only rooted after transfer to a rooting medium were found to be significantly different, the former being predominantly 48 and the latter predominantly 48 + +. This observation indicates that the frequency of normal tetraploid regenerants can be increased to 80% by selecting shoots which root quickly without the aid of rooting hormones. This may be very useful in situations where large numbers of shoots are initially produced and then a selection transferred for growth to whole plants.

Acknowledgements. Neil Fish thanks the PMB for financial support. We are grateful for the helpful advice of Dr. M. G. K Jones and discussions with members of the Biochemistry Department. We also thank Miss S. Steele for technical assistance.

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