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Studies of protoplast culture and plant regeneration from commercial and rapid-cycling *Brassica* species

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Abstract. Protoplasts were isolated from aseptic shoot cultures of commercial cultivars of *Brassica napus*, *B. oleracea* and *B. campestris*, and from the six 'rapid-cycling brassica species'. Of the rapid-cycling species, only *B. napus* responded well to the culture conditions used; 2% of protoplasts formed calli and up to 5% of calli regenerated shoots. Regeneration was also achieved from commercial cultivars of *B. napus* and *B. oleracea*. For these two species the plating density, time of dilution with fresh medium and the composition of the shoot-inducing medium were all found to have an important influence on the efficiency of plant regeneration. Both responded better to maltose than to sucrose-based media. Under the optimum conditions *B. napus* showed a plating efficiency of 7.8% and shooting efficiency of 17%; for *B. oleracea* the figures were 2% and 56%, respectively.

Abbreviations: BAP – 6-benzylaminopurine; NAA – α-naphthaleneacetic acid; 2,4-D – 2,4-dichlorophenoxyacetic acid

Introduction

The genus *Brassica* contains a number of crops of considerable economic importance. These include the oilseed cultivars of *B. napus* and *B. campestris* (rape or canola) and the various vegetable varieties of *B. oleracea* [1].

This is one of the few groups of crop species for which the techniques of tissue culture and genome transformation are well advanced [2, 3]. Several different protocols have been devised for the regeneration of whole plants from brassica protoplasts [4, 5, 6, 7, 8, 9], and there are several examples of intra- and inter-specific hybridizations involving brassica species [6, 10, 11, 12, 13, 14].

The aim of the present project was to study aspects of protoplast culture and plant regeneration in a number of brassica lines. The 'rapid-cycling brassica populations' [1] were included in this investigation. These were developed for research purposes by P.H. Williams at the University of Wisconsin. They flower within 4 weeks of sowing and stand only 25 cm tall at maturity, and lines are now being developed carrying various genetic markers. Their small size and, above all, their short generation time make them ideal subjects for genetic studies. Preliminary investigations have shown them to respond well to conditions in vitro and protocols have already been developed for anther and microspore culture of some of the species concerned [15]. The development of protoplast culture and regeneration techniques for these lines could prove very useful for investigations into various aspects of somatic hybridization and genome transformation.

Leaf mesophyll was chosen as the source material for all protoplast isolations since it can be easily grown in aseptic culture and can be generated in large amounts. However, a major drawback of using this material is that under conditions of stress mesophyll protoplasts produce a brown phenolic precipitate that is inhibitory to further growth [5]. Experiments reported here resulted in the establishment of an efficient protocol for plant regeneration in which such colony browning was avoided.

Materials and methods

1 Plant material, protoplast isolation and culture, plant regeneration

1.1 Plant material

The following plant material was used:

- *B. napus* ssp. *oleifera* cv. Brutor (spring oilseed rape), obtained from The Nickerson Seed Company Ltd., Cambridge, UK, and cv. Crack (forage rape), obtained from the National Seed Development Organization, Newton, Cambridge, UK.
- *B. oleracea* var. *capitata* cv. January King (cabbage) and cv. Red Drumhead (red cabbage), obtained from Sanders Seed Ltd., Cambridge, UK, and cv. Kinkei (cabbage), obtained from Sakata Seed America Inc., Salinas, California, USA;
  B. oleracea var. *acephala* cv. Maris Kestrel (kale), obtained from the National Seed Development Organization, Newton, Cambridge, UK.
- *B. campestris* ssp. *oleifera* cv. Tyko (oilseed rape), obtained from The Nickerson Seed Company Ltd., Cambridge, UK;

B. campestris ssp. *rapifera* cv. Appin (turnip), obtained from the National Seed Development Organization, Newton, Cambridge, UK. *B. campestris* ssp. *pekinensis* cv. Seikai (chinese cabbage), obtained from Sakata Seed America Inc., Salinas, California, USA, and cv. Pe Tsai (chinese cabbage), obtained from Sanders Seed Ltd., Cambridge, UK.

— Rapid-cycling *B. napus*, *B. oleracea*, *B. campestris*, *B. nigra*, *B. carinata* and *B. juncea*, obtained from the Crucifer Genetics Co-operative, University of Wisconsin, USA.

1.2 Shoot cultures

Seeds were surface-sterilized by a 30 min immersion in 50% sodium hypochlorite, rinsed in 100 ml of sterile distilled water then plated on MS basal medium (consisting of the vitamins and inorganic salts of Murashige & Skoog [16], supplemented with 9.5 gl^{-1} Difco Bacto agar, pH 5.6). After 7 days the top 2 cm of each seedling was excised and transferred to fresh medium designated MS20 (with the constituents of MS basal medium, supplemented with 20 gl^{-1} sucrose) in 250 ml Beatson jars (Fisons Ltd., Loughborough, UK), covered with the lids from 9 cm plastic Petri dishes and sealed with Nescofilm (Bando Chemical Ind. Ltd., Kobe, Japan). These, and all subsequent cultures, were incubated at 25 °C under lights in a controlled temperature room (16 h photoperiod, 59.5 $\mu E m^{-2} s^{-1}$). Plant material was subcultured onto fresh MS20 every 4 weeks.

1.3 Protoplast isolation

After 4 to 5 weeks of growth approximately 1 g of leaf material was excised and placed in 10 ml of W5 solution [17](9 gl^{-1} NaCl, 18.375 gl^{-1} $CaCl_2$, 0.373 gl^{-1} KCl, 0.901 gl^{-1} glucose, pH 5.6) in a 12 cm glass Petri dish, and scored on one surface with a six-bladed scalpel. After incubation for 30 min at room temperature the W5 was replaced with 20 ml of an enzyme solution consisting of MS basal medium supplemented with 4 gl^{-1} cellulase R10, 2 gl^{-1} pectolyase Y23 (both from Unwin and Co. Ltd., Welwyn, UK) and 140 gl^{-1} mannitol at pH 5.6.

Digestion took place over 3 to 4 h in the dark at 25 °C on an orbital shaker at 40 rpm. The crude digest was then diluted with an equal volume of W5 and filtered through 100 μm and 50 μm steel sieves (Endecott Ltd., Caldecote, UK). Protoplasts were pelleted by centrifugation at 200 *g* for 7 min in four 10 ml glass culture tubes (Fisons Ltd., Loughborough, UK). The supernatant was discarded from each tube and pellets resuspended in 10 ml W5 before being centrifuged at 100 *g* for a further 5 min. Each pellet was then resuspended in 1.5 ml W5 and gently layered over 2.5 ml Percoll solution (30% Percoll, 70% 720 mosmole mannitol). After centrifugation at

100 *g* for 5 min, the protoplast band was re-isolated from the Percoll/W5 interface. Protoplasts were then resuspended in W5 and washed twice more (100 *g* for 5 min).

1.4 Protoplast culture and plant regeneration

Freshly isolated protoplasts were plated in 3 ml of a culture medium, based on that of Glimelius [5], designated KG1 (consisting of the minerals and vitamins of Nitsche & Nitsche [18] supplemented with 72 gl^{-1} glucose, 8.7 gl^{-1} mannitol, 1 mgl^{-1} 2,4-D, 0.5 mgl^{-1} BAP, 0.1 mgl^{-1} NAA and 1 gl^{-1} sea plaque agarose (Gibco-BRL Ltd., Paisley, UK), pH 5.6) in a six-well multidish (Nunc Ltd., Kamstrup, Denmark) and placed in the dark in an incubator at 25 °C. After 7 days the cultures were diluted with 1 ml of KG2 dilution medium (with the constituents of KG1 but without NAA and with 34.2 gl^{-1} sucrose replacing glucose and mannitol) and placed under lights. Developing cultures were diluted every other day with 1 ml of KG2 and transferred to 9 cm Petri dishes when their volume exceeded 10 ml. When calli reached a diameter of approximately 300 μm they were plated over the solid proliferation medium of Bidney et al. [4] (designated DBCl) in a small amount of the original culture medium. This was done by adding 2.5 ml of protoplast-derived cell suspension onto a 9 cm Petri dish of DBCl, then swirling the plate until the liquid evenly covered the solid. Calli stayed on proliferation medium until they reached a diameter of about 3 mm, when they were individually plated onto fresh DBCl to stimulate shoot morphogenesis. They were transferred to fresh medium every 4 weeks.

When shoots arising on DBCl had reached a length of approximately 5 mm they were excised from the callus and plated on MSF medium (consisting of MS basal medium with the addition of 5 gl^{-1} sucrose, 0.25 mgl^{-1} BAP, 0.05 mgl^{-1} NAA and 6 gl^{-1} sea plaque agarose at pH 5.6) for elongation. After attaining a length of approximately 10 mm they were transferred to MS20 medium for root production. Rooted plantlets were placed in Jiffy 7 peat blocks (Jiffy Products Ltd., Stange, Norway) inside 350 ml sterile jars (Medfor Products Ltd., Fleet, UK). After approximately 2 weeks of growth in a growth cabinet (Fisons Ltd., Loughborough, UK) at 25 °C and a 16 h photoperiod of 200 $\mu E m^{-2} s^{-1}$, plants were placed in Levington Potting Compost (Fisons Ltd., Ipswich, UK) in a glasshouse under daylight supplemented with artificial light from 'daylight' fluorescent tubes to maintain a 16 h photoperiod, and grown to maturity.

2 Optimization of culture conditions

Of the genotypes assessed, the two most responsive were *B. napus* cv. Brutor

and *B. oleracea* cv. January King. Thus these cultivars were chosen for use in experiments to optimize the regeneration protocol.

2.1 The effect of protoplast plating density on division frequency
Freshly isolated protoplasts were plated at 5, 8, 10 and 25×10^4 protoplasts ml^{-1} in 3 ml of KG1 medium in a six-well multidish. Four to six replicates were set up for each plating density. Cultures were placed in the dark in an incubator at 25 °C for 8 days, then examined under an inverted light microscope (Olympus Ltd., West-Germany) at 400 × magnification. Three hundred cells were selected at random from each replicate and the numbers undergoing divisions were counted.

2.2 The effectiveness of maltose as a carbon source for protoplast culture
The protocol described above is based on that of Glimelius [5], in which protoplasts are initially plated in a glucose-based medium, then subsequently maintained on sucrose. In these studies the effect of replacing glucose and sucrose with maltose was investigated.

2.2.1 The comparative effectiveness of glucose and maltose in supporting the initial development of protoplast cultures. Freshly isolated protoplasts of cv. Brutor were plated at 8×10^4 protoplasts ml^{-1} in 3 ml of culture medium in six-well multidish. Two different media were used:
— KG1 medium (see above), containing 72 gl^{-1} (0.4 M) glucose;
— KG1 (M) medium with the constituents of KG1 medium, but with 144 gl^{-1} (0.4 M) maltose replacing the glucose.

Two replicates were set up in each medium. Cultures were placed in the dark in an incubator at 25 °C for 8 days, then examined and the division frequency counted as described above.

2.2.2 The comparative effectiveness of sucrose and maltose in supporting the sustained division of protoplast-derived cells. Freshly isolated protoplasts were plated at optimum density (see above) in 3 ml of KG1 medium in a six-well multidish. They were then cultured under one of two different regimes:
— diluted with KG2 medium (see above) containing 34.2 gl^{-1} (0.1 M) sucrose, and subsequently plated over DBCl proliferation medium (see above) containing 2.5 gl^{-1} (0.007 M) sucrose;
— diluted with KG2 (M) medium, with the constituents of KG2 but with 36 gl^{-1} (0.1 M) maltose replacing the sucrose; they were subsequently plated over DBCl (M) proliferation medium, with the constituents of DBCl, but with 2.63 gl^{-1} (0.007 M) maltose replacing the sucrose.

Cultures of cv. Brutor were diluted with 1 ml aliquots of dilution medium on day 7 of culture and every other day thereafter. Cultures of cv. January King were diluted with 3 ml aliquots of KG1 on day 3 of culture, then were maintained as described for cv. Brutor. All cultures were transferred to 9 cm Petri dishes and then onto proliferation medium as described above.

Three replicates were set up for each culture regime. After approximately 3 weeks on proliferation medium the number of calli produced was counted. From these results the plating efficiencies (number of calli formed/number of protoplasts plated × 100) were calculated.

2.3 The effectiveness of different shoot-inducing media

Protoplasts were plated in KG1, diluted with KG2, then plated on DBCl proliferation medium as described in Section 2.2.2. When calli reached a diameter of 3 mm they were individually plated on shoot-inducing medium. For *B. napus*, three different shoot-inducing media were compared, designated DBCl [4], KG4 [5] and TB [7]. For *B. oleracea*, two different media were used, DBCl [4] and RT [11]. Calli were transferred to fresh medium every 4 weeks. The total number of calli producing shoots on each medium was counted, and from these results the shooting efficiencies (number of calli producing shoots/number of calli plated) were calculated.

Results

1 A comparison of the behaviour of different genotypes

Protoplasts of sixteen different genotypes were cultured (Table 1). Whole plant regeneration was achieved for commercial lines of *B. napus* and *B. oleracea*, and for rapid-cycling *B. napus*. Cultivars of commercial *B. oleracea* showed a wide range of responses. Protoplasts of cv. January King regenerated whole plants with relatively high frequency (see below), while cv. Kinkei and cv. Red Drumhead showed high plating efficiencies of 1% and 4%, respectively, when maintained on the maltose-based dilution and proliferation media, but did not produce shoots. Protoplasts of cv. Maris Kestrel laid down cell walls but did not develop further. A low proportion (less than 0.1%) of protoplasts of *B. campestris* cvs. Seikai and Pe Tsai developed as far as callus, but these did not produce shoots on DBCl or RT medium.

Of the rapid-cycling species, only *B. napus* responded well to the protoplast culture conditions described. Approximately 2% of protoplasts developed as far as callus when maintained on maltose-based dilution and

Table 1. The responses of various genotypes of *Brassica* species to the protoplast culture conditions used in the present study (see 'Materials and methods' for details).

Brassica species		Response[1]		
		Cell division	Callus formation	Shoot formation
B. napus	cv. Brutor	+	+	+
	cv. Crack	+	+	+
B. oleracea	cv. January King	+	+	+
	cv. Kinkei	+	+	−
	cv. Red Drumhead	+	+	−
	cv. Maris Kestrel	−	−	−
B. campestris	cv. Tyko	−	−	−
	cv. Appin	−	−	−
	cv. Pe Tsai	+	+	−
	cv. Seikai	+	+	−
Rapid-cycling	*B. napus*[2]	+	+	+
	B. carinata	+	+	−
	B. oleracea	−	−	−
	B. campestris	−	−	−
	B. nigra	−	−	−
	B. juncea	−	−	−

[1] + = specified response obtained − = specified response not obtained
[2] Reference [1]

proliferation media. Shoot induction on DBCl medium was low with only 1% of calli producing shoots. The highest shooting efficiency obtained for this material was on KG4 on which 5% of calli underwent shoot morphogenesis. These shoots were subsequently established as whole plants growing in soil.

2 Optimization of protoplast culture methods

2.1 The effect of plating density on division frequency

B. napus cv. Brutor and *B. oleracea* cv. January King showed markedly different patterns of response to different plating densities (Table 2). Protoplasts of cv. Brutor were able to undergo divisions over a wide range of plating densities, with the best results being obtained at 8×10^4 protoplasts ml^{-1}. Protoplasts of cv. January King plated at 5 to 10×10^4 protoplasts ml^{-1} did not initiate sustained divisions. However, at 2.5×10^5 protoplasts ml^{-1} 53% of cells underwent division and a high proportion of these continued development to form calli.

Table 2. The effect of plating density on division frequency of protoplast cultures of *Brassica napus* cv. Brutor and *B. oleracea* cv. January King. Figures show the mean division frequencies (DF) and 5% confidence limits (CL) of cells plated in KG1 medium at different densities.

Plating density (protoplasts ml^{-1})	*B. napus* cv. Brutor		*B. oleracea* cv. January King	
	DF (%)	CL (%)	DF (%)	CL (%)
5×10^4	66.6	3.4	0	-
8×10^4	70.8	4.3	0	-
1×10^5	51.2	12.0	0	-
2.5×10^5	-	-	53.4	8.0

2.2 Time of dilution

The time of dilution and the amount of fresh medium added were of critical importance. If cultures were diluted too soon or with too much medium, development was inhibited. Cultures which were not diluted soon or often enough produced a brown precipitate that was inhibitory to further growth.

Cell division of cv. Brutor was inhibited if cultures were diluted before day 5. Cultures which were left undiluted exhibited browning by day 12. Sustained development was obtained if cultures were diluted with 1 ml of KG2 medium on day 7 and every other day thereafter.

Protoplasts of cv. January King had to be plated at relatively high density (2.5×10^5 protoplasts ml^{-1}) to initiate cell division. At this density, cells began to produce brown precipitate from about day 5 of culture. Once cell division had been initiated, cultures could be maintained at lower density. Best results were obtained when cultures were diluted with an equal volume of KG1 on day 3 of culture, and then with 1 ml of KG2 3 days later. Thereafter they were given the same treatment as cultures of cv. Brutor.

2.3 The effectiveness of different carbon sources

Glucose proved to be superior to maltose in supporting the initial development of protoplast cultures of cv. Brutor. Protoplasts plated in the glucose-based KG1 medium showed an average division frequency of 72.8% with 5% confidence limits of 11.9%, while those plated in maltose-based KG1 (M) medium showed an average of 39.7% with 5% confidence limits of 9.8% (three replicates for each treatment).

Cvs. Brutor and January King showed similar responses to the different dilution and proliferation media. Cultures maintained on the maltose-based media showed higher plating efficiencies than those on the sucrose-based ones. *B. napus* cv. Brutor showed a plating efficiency of 4.87% (5% confidence limits of 2.14%) when maintained on sucrose, compared to 7.83% (5% confidence limits of 4.02%) on maltose. For *B. oleracea* cv. January King the plating efficiencies were 1.30% (5% confidence limits of 0.37%) on

Table 3. The number of shoots of *Brassica napus* cv. Brutor and *B. oleracea* cv. January King produced by protoplast-derived calli on four different media: TB [7], RT [11], DBCl [4] and KG4 [5].

Brassica genotype	Medium	Number of calli plated	Number of calli producing shoots	Shooting efficiency
Brutor	TB	1207	193	16.0%
	DBCl	2939	510	17.4%
	KG4	986	79	8.2%
January King	DBCl	285	86	30.0%
	RT	305	170	55.7%

sucrose, and 1.97% (5% confidence limits of 0.22%) on maltose. These differences are significant at the 1% level when the results from both cultivars are transformed under the arc sin square root transformation and pooled for an analysis of variance.

2.4 The comparative effectiveness of different shoot-inducing media

The results are shown in Table 3. For cv. Brutor the most effective media were DBCl and TB with shooting efficiencies of 17.4% and 16%, respectively. For cv. January King the optimum was RT medium with a shooting efficiency of 55.7%. Nearly all calli of cv. Brutor formed only one shoot; calli of cv. January King formed an average of 4 shoots per callus.

Discussion

This paper presents the first report of plant regeneration from protoplast cultures of the rapid-cycling brassica material. A relatively high proportion of protoplasts of rapid-cycling *B. napus* developed to form calli. Plant regeneration occurred at relatively low frequency, but was routine. Hence protoplasts of this material could prove to be a useful tool in studies of various aspects of genetic manipulation in vitro.

One of the most noticeable features of the studies involving protoplast culture of different brassica cultivars was the wide range of responses observed (Table 1). It has been widely reported that regeneration from protoplast culture is dependent on genotype [5]. Clearly the culture conditions used in this study were only suitable for a narrow range of brassica genotypes, and different protocols need to be devised for unresponsive lines.

The optimum plating density of 8×10^4 protoplasts ml^{-1} for cv. Brutor is typical for this species [8]. However, cv. January King showed an unusual requirement for high plating density for the initiation of cell division. Once

this had been achieved the cultures needed to be diluted to continue development. Presumably the initial high plating densities are necessary to precondition or detoxify the culture medium, allowing it to support lower cell densities after dilution. Similar effects have been observed in cultures of haploid tobacco protoplasts [20].

The optimum division frequencies, plating efficiencies and shooting efficiencies obtained with *B. napus* cv. Brutor and *B. oleracea* cv. January King are shown in Table 4. These results compare well with those obtained by other workers. The plating efficiencies obtained are the highest yet reported for brassica material. We consider this success to be mainly due to the establishment of the correct time to dilute developing cultures. Once this had been established, colony browning, widely reported to be a feature of brassica mesophyll protoplast cultures [5, 9], was prevented and healthy callus proliferation was maintained.

Plating efficiencies were further increased by the substitution of sucrose for maltose. Maltose has already been shown to be greatly superior to sucrose for somatic embryogenesis in alfalfa [21], for the protoplast culture of a range of winter and spring cultivars of *B. napus* (R. Loose et al., in preparation) and for barley anther culture [19]. The biochemical reasons for these effects are not clear. In pea chloroplasts incubated in the dark there is evidence that starch is broken down to maltose [22]. Hence, at least in this species, maltose is probably the substrate for respiration in non-photosynthesizing photosynthetic tissues. In tissues which are engaged in active photosynthesis the main carbon source for metabolism is probably triose phosphate exported from the chloroplasts [23]. Thus tissues may have a low inherent ability to break down sucrose, since it is not a major substrate for respiration, but a relatively high rate of maltose metabolism (T. ap Rees, pers. communication). If this is true it will be specific to photosynthetic tissues and to species, since glucose, not maltose, has been found to be the major breakdown product of starch in chloroplasts of spinach [23]. More basic biochemical research needs to be done to establish the pathways of carbon metabolism in brassicas before any firm explanations can be offered for these results.

Table 4. The division frequency, plating efficiency and shooting efficiency of *Brassica napus* cv. Brutor and *B. oleracea* cv. January King under the optimum culture regime (see 'Materials and methods' for details).

Brassica genotype	Division frequency (%)	Plating efficiency (%)	Shooting efficiency (%)
Brutor	71	7.8	17
January King	54	2.0	56

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References

1. Williams PH, Hill CB (1986) Rapid-cycling populations of *Brassica*. Science 232: 1385–1389
2. MacDonald MV, Ingram DS (1984) The use of tissue culture in oilseed rape breeding. Aspects Appl Biol 6: 37–48
3. Charest PJ, Holbrook LA, Gabard J, Iyer VN, Miki BL (1988) *Agrobacterium*-mediated transformation of *Brassica napus* L. Theor Appl Genet 75: 438–445
4. Bidney DL, Shepard JF, Kaleikau E (1983) Regeneration of plants from mesophyll protoplasts of *Brassica oleracea*. Protoplasma 117: 89–92
5. Glimelius K (1984) High growth rate and regeneration capacity of hypocotyl protoplasts in some Brassicaceae. Physiol Plant 61: 38–44
6. Pelletier G, Primard C, Vedel F, Chetrit P, Remy R, Rouselle P, Renard M (1983) Intergeneric cytoplasmic hybridisation in Cruciferae by protoplast fusion. Mol Gen Genet 191: 244–250
7. Barsby TL, Yarrow SA, Shepard JF (1986) A rapid and efficient alternative procedure for the regeneration of plants from hypocotyl protoplasts of *Brassica napus*. Plant Cell Rep 5: 101–103
8. Klimaszewska K, Keller WA (1987) Plant regeneration from stem cortex protoplasts of *Brassica napus*. Plant Cell Tissue Organ Culture 8: 225–233
9. Xu ZH, Davey MR, Cocking EC (1982) Plant regeneration from root protoplasts of *Brassica*. Plant Sci Lett 24: 117–121
10. Schenck HR, Robbelen G (1982) Somatic hybrids by fusion of protoplasts from *Brassica oleracea* and *B. campestris*. Z Pflanzenzuchtg 89: 278–288
11. Terada R, Yamashita Y, Nishibayash S, Shimamoto K (1987) Somatic hybrids between *Brassica oleracea* and *B. campestris*: selection by the use of iodoacetate inactivation and regeneration ability. Theor Appl Genet 73: 379–384
12. Toriyama K, Hinata K, Kameya T (1987) Production of somatic hybrid plants 'Brassicomoricandia' through protoplast fusion between *Moricandia arvensis* and *Brassica oleracea*. Plant Sci 48: 123–128
13. Toriyama K, Kameya T, Hinata K (1987) Selection of a universal hybridiser in *Sinapis turgida* Del. and regeneration of plantlets from somatic hybrids with *Brassica* species. Planta 170: 308–312
14. Gleba YY, Hoffmann F (1980) '*Arabidobrassica*': A novel plant obtained by protoplast fusion. Planta 149: 112–117
15. Aslam F (1988) Haploid Production in Rapid-cycling *Brassica napus* and *B. campestris*. PhD thesis, University of Cambridge, Cambridge

16. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
17. Menczel L, Wolfe K (1984) High frequency of fusion induced in freely suspended protoplast mixtures by polyethylene glycol and dimethylsulfoxide at high pH. Plant Cell Rep 3: 196–198
18. Nitsche JP, Nitsche C (1969) Haploid plants from pollen grains. Science 163: 85–87
19. Hunter C (1988) Plant Regeneration from Microspores of Barley, *Hordeum vulgare*. PhD thesis, University of London, Wye College, Ashford, Kent
20. Caboche M (1980) Nutritional requirements of protoplast-derived haploid cells grown at low densities in liquid media. Planta 149: 7–18
21. Strickland SG, Nichol JW, McCall CM, Stuart DA (1987) Effect of carbohydrate source on alfalfa somatic embryogenesis. Plant Sci 48: 113–121
22. Kruger NJ, ap Rees T (1983) Maltose metabolism by pea chloroplasts. Plant 158: 179–184
23. Stitt M, Steup M (1985) Starch and sucrose degradation. In: Douce R, Day DA (Eds) Encyclopedia of Plant Physiology, Vol. 18. Springer-Verlag, Berlin