PLANT REGENERATION FROM COTYLEDONARY EXPLANTS IN A RANGE OF BRASSICA SPECIES AND GENOTYPES

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INTRODUCTION

Genetic transformation of Brassica using Agrobacterium tumefaciens has been reported using various explants, including: stem sections, thin cell layers, microspore derived embryos and hypocotyls (Fry et al. 1987; Pua et al, 1987, Charest et al.1988; Pechan 1989; De Block 1989). For large scale transformation programmes it is necessary to have an efficient method of transformation. One of the main prerequisites for this is a high level of regeneration from the chosen explant. A particularly efficient method of transformation uses cotyledonary explants (Moloney 1989) which have the advantage of being able to produce a large number of aseptic explants in a few days without having to wait for flowering. In order to determine how widely applicable cotyledonary explants are for use in our research programme, we tested 23 lines including 11 genotypes of B. napus, along with genotypes of B. oleracea, B. campestris and Sinapis alba. The effect of explant age and the influence of an ethylene antagonist, silver nitrate, are also analyzed to determine their importance for plant regeneration.

MATERIALS AND METHODS

The various species and cultivars used are listed in Table 1. Seeds were surface sterilized in 1% sodium hypochlorite (to give 0.14% w/v available chlorine) for 20 min followed by three washes in sterile distilled water. Seeds were sown into sterile disposable plastic tubs 100mm diameter and 60mm high (Neoplants Ltd, Preston, UK) at a density of approximately 15 seeds per container. The culture medium and conditions used for germination were MS medium (Murashige and Skoog 1962), 30g/l sucrose, adjusted to pH 5.8, solidified with 7g/l agar (Sigma, Cat. no. A1296) and autoclaved for 15 min at 121°C. Seeds were germinated at 25°C in darkness for 24 hours followed by 25°C in continuous cool white fluorescent light at an intensity of 125uE $m^{-2}s^{-1}$.

Cotyledons were excised from seedlings at the cotyledonary node taking care to eliminate the apical meristem. The excised cotyledons, cleanly across their petiole, were placed in MS medium, 30g/l sucrose, 4.5mg/l 6-benzylaminopurine (BAP) and prepared as for the germination medium. The cotyledonary petioles were excised at 5 days old (unless stated otherwise) and embedded to a depth of c.2mm (Moloney et al. 1989) into the medium at a density of 10 explants in the same type of plastic container as used for germination. It is important not to damage the cotyledonary lamina as this reduces regeneration frequency. The regeneration rate was determined for each genotype after 4 weeks and expressed as the percentage of the explants regenerating shoots.

Ethylene, the gaseous phytohormone, has been observed to influence the growth of Brassica plants and cultures. Silver nitrate, an ethylene

antagonist, can be used to reduce ethylene effects and in some cases give a significant improvement in the regeneration response in culture (Biddington et al. 1988; Chi et al 1990; Sethi et al. 1990). Silver nitrate was included at concentrations of 0, 2, 5, and 10 gm/l into the growth and regeneration medium in the 16 combinations. Regeneration was determined after 4-6 weeks.

RESULTS

Plant Regeneration from various Species and Subspecies

During the first 3 days on the regeneration medium the cotyledonary lamina enlarged to approximately twice its original size at culturing. The first regenerating shoot buds appeared at the cut end of the petiole at approximately 10 days. There was a wide range in regeneration rate between the lines tested (Table 2) and the overall differences were highly significant ($P = < 0.001$). The rate of regeneration ranged from 5.8-94.4% of explants with shoots. Because the cut surface of the petioles has a small surface area it is difficult to determine the precise number of shoots from each explant, but 2-3 shoots were frequently observed. The number of genotypes from each species varied but B. campestris had the lowest overall regeneration rate with a mean of 20.7% and a range of 5.8-41.7%. B. oleracea had a mean of 53.7% with a wide range of 20.3-94.3%. These compare with the single genotype values of 50.0% for Sinapis alba and 73.1% for B. napus. These data suggest that there is a considerable genetic variation for regeneration response among the genotypes tested.

Plant Regeneration within B. napus

Similar cotyledonary explants were cultured for eleven Brassica napus genotypes. Nine were cultivars of oilseed rape (two spring and seven winter cultivars) and their was one swede variety and a rapid cycling experimental line. There were again highly significant differences in regeneration frequency between genotypes ($P < 0.001$) with a range of 0-98.6% (Table 3). Cotyledons from the cultivar Olga regenerated shoots significantly better than any other cultivar. The swede cultivar Marian did not regenerate any shoots. The amount of callusing varied with genotype and there tended to be an inverse relationship between callus formation and shoot regeneration. Cultivar Marian (swede) produced the most callus at the cut surface and Westar the least. Callus formation was also associated with retarded shoot development. The cultivars with the highest rates of regeneration tended to regenerate shoots most quickly with clearly visible shoots after 10 days in Westar and Olga.

The Effect of Explant Age on Regeneration Frequency

An effect of seedling age on the ability of cotyledonary explants to regenerate shoots has been reported in B. juncea (Sharma et al. 1990). As the optimum seedling age is likely to depend on the environmental conditions used for germination, seeds were germinated from the lines: rapid cycling (No. 21), Westar, Olga, (B. napus) and All Year Round (B. oleracea) under the conditions described, and cultured cotyledonary explants were cultured onto plant regeneration medium. Regeneration rates in the range of 47-90% were obtained when cotyledons were cultured 3-5 days after plating out the seeds, and regeneration fell sharply after 5

days. The best regeneration was observed when cotyledons were cultured as soon as possible after germination, but very young explants are difficult to handle and dissect and a compromise has to be achieved between ease of handling and rate of regeneration.

The Effect of Silver Nitrate on Plant Regeneration

Silver nitrate was included in the germination medium and the regeneration medium at 0, 2, 5 and 10 mg/l in the 16 combinations. From the results of earlier experiments two cultivars of oilseed rape were selected, Westar with relatively high regeneration (54% and 73%) and Libravo (10%) with low. Sixty explants were cultured on each of the media combinations. No statistically significant differences were observed for Westar and hence there was no advantage from adding silver to this variety which has a relatively high regeneration rate. Libravo showed no significant improvement from including silver nitrate into the regeneration medium but did ($P = <2\%$) when incorporated into the germination medium, with 5 mg/l giving significantly better (64%) regeneration than 0, 2 or 10mg/l (29, 35 and 29% respectively). It appears from these data that silver nitrate added to the germination medium but not the regeneration medium, can be beneficial for increasing the regeneration rate in poorly responding cultivars.

DISCUSSION

Substantial differences in regeneration rate from cotyledonary explants were observed between the genotypes tested. Murata and Orton (1987) also found differences when they used seedling explants but their regeneration rates were considerably less. This may have been due in part to their use of 7day old seedlings. In our studies, regeneration declined sharply after 5 days and as a general rule better results were observed when explants were cultured early; as soon as they can be handled with forceps without undue difficulty.

Murata and Orton (1987) proposed that B. oleracea, which carries the C genome, may have genes which predispose it to regenerate in culture. On average B. oleracea gave a higher regeneration rate (54% range 20-94%) than B. campestris with the A genome (21% range 6-42%) or B. napus with the AC genome (30% range 0-99%; Table 3). But there were substantial differences between genotypes within species, and hence there appears to be more genetic variation within these species than between them.

There is anecdotal evidence that winter oilseed rape cultivars regenerate less readily than spring. This is supported by the present study. The mean regeneration rate for the 7 winter cultivars tested was 23% (range 2-43%) and for the 2 spring cultivars 76% (54-99%). This is, however, not a good test because the two spring cultivars are not a random pair; they were chosen after previous studies showed them to be responsive. Westar is the most widely used cultivar for regeneration and transformation studies.

The use of the ethylene antagonist, silver nitrate, has been found to give improved regeneration in various types of in vitro systems and species (see above). There was an improvement in regeneration in these studies when used on a poorer responding oilseed rape cultivar. This was only

observed when silver nitrate was incorporated into the germination medium (5mg/l) rather than the regeneration medium. The effect of this component in the medium probably needs to be determined empirically for each genotype and set of culture conditions as it is likely to be influenced by type of culture vessel, amount of ventilation, air volume to agar volume ratio and other variables.

Variability in rate of germination can make it difficult to obtain sufficient explants at a uniform developmental stage. It was found important to use recently harvested seeds (< 1 year) and the germination rate and uniformity can be improved by selecting the heaviest 25% of the seeds using a seed blower designed for cleaning seed samples.

CONCLUSIONS

1. There are substantial differences in the regeneration rate from cotyledonary explants between Brassica species and subspecies and between cultivars of B. napus.

2. On average B. oleracea had a higher frequency of regeneration than B. campestris or B. napus but there was more variation in regeneration rate within these species than between them. Regeneration was also observed from Sinapis alba.

3. Cotyledonary explants should be cultured as soon as possible after germination, to give maximum regeneration. Regeneration falls sharply after 5 days.

4. Silver nitrate added to the germination medium and not the regeneration medium, improved regeneration rate from a lower regenerating oilseed rape cultivar. The benefit of this ethylene antagonist probably needs to be determined empirically for a particular culture system.

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Table 1. Genotypes used to determine the regeneration rate from excised cotyledonary explants

No.[a]	Species	Common name/cultivar
1	B. oleracea spp botrytis	cauliflower/All Year Round
2	B. oleracea spp selensia	dwarf curly kale/Borecole
3	B. oleracea spp italica	green broccoli/Autumn calabrise
4	B. oleracea spp gemmifera	brussels sprout/Peer Gynt
5	B. oleracea spp acephala	curly kale/Pentland Brig
6	B. oleracea spp sabauda	savoy cabbage/Ormskirk
7	B. oleracea	rapid cycling line/CRGC-3
8	B. campestris spp rapifera	turnip/Vetch's Red Globe
9	B. campestris spp pekinensis	chinese cabbage/Green Rocket
10	B. campestris	rapid cycling line/CRGC-1
11	B. napus spp oleifera	oilseed rape/Westar
12	B. napus spp oleifera	oilseed rape/Olga
13	B. napus spp oleifera	oilseed rape/Bienvenu
14	B. napus spp oleifera	oilseed rape/Cobra
15	B. napus spp oleifera	oilseed rape/Mikado
16	B. napus spp oleifera	oilseed rape/Ariana
17	B. napus spp oleifera	oilseed rape/Pasha
18	B. napus spp oleifera	oilseed rape/Rafal
19	B. napus spp oleifera	oilseed rape/Libravo
20	B. napus spp napobrassica	swede/Marian
21	B. napus	rapid cycling line/CRGC-5
22	Sinapis alba	mustard/Fine White

[a] Genotype number also used in Tables 2 & 3.

Table 2. The frequency of explants giving shoot regeneration from various species and subspecies

No.[a]	Species	No. of explants cultured[b]	Regeeneration rate[c]	Homogeneous groups[d]
9	B.c pekinensis	70	5.8	a
8	B.c.rapifera	50	14.6	ab
6	B.o. sabauda	30	20.3	abc
7	B.o.	130	26.5	bc
10	B.c.	60	41.7	bcd
5	B.o. acephala	40	42.0	bcd
1	B.o.botrytis	40	46.3	cd
22	S.a.	30	50.0	cde
2	B.o.selensia	60	61.5	def
11	B.n.oleifera	90	73.1	efg
4	B.o.gemmifera	50	84.8	fg
3	B.o.italica	30	94.3	g

[a]Genotype number from table 1. [b]Approximate. [c]The percentage of explants regenerating shoots. [d]Values followed by the same letter do not differ significantly from each other (LSD=0.05 P).

Table 3. The frequency of hypocotyl explants giving shoot regeneration from various spring and winter Brassica napus cultivars

No.	Cultivar[a]	Winter/ spring	No. explants cultured	Regenera- tion rate	Homogeneity group
20	Marian	W	60	0	a
13	Bienvenu	W	110	2.2	a
17	Pasha	W	150	5.4	ab
19	Libravo	W	40	9.8	abc
21	Rapid cycling	S	90	17.4	bc
15	Mikado	W	90	22.9	cd
18	Rafal	W	100	33.4	de
14	Cobra	W	120	42.4	ef
16	Ariana	W	90	43.0	ef
11	Westar	S	70	53.7	f
12	Olga	S	80	98.6	g

Footnotes as for Table 2. [a]All are commercially available oilseed rape cultivars except Marian (swede) and the rapid cycling experimental line.