Plant Cell Reports (1996) 15:695–698

Plant Cell Reports

The effects of proline, thioproline and methylglyoxal-bis-(guanylhydrazone) on shoot regeneration frequencies from stem explants of *B. napus*

C. M. O'Neill, A. E. Arthur, and R. J. Mathias

Brassica and Oilseeds Research Department, John Innes Centre, Norwich Research Park, Norwich NR4 7UJ, UK

Received 22 November 1993/Revised version received 20 November 1995 – Communicated by M. R. Davey

Abstract

Internode segments from aseptic shoot cultures are the most prolific explants for the regeneration of Brassica shoots *in vitro*. These explants also have the advantage of not being subject to the genotypic variations in regeneration response observed in hypocotyl and cotyledon explants. Despite reports of 80-100% shoot regeneration from stem explants, observed frequencies are typically 50-60%. Three media additives, proline, thioproline and methylglyoxal-bis-(guanylhydrazone) (MGBG), were tested for their efficacy in promoting shoot regeneration from stem explants of two *B. napus* varieties, Westar and Cobra. The effects of proline and thioproline on both varieties were neutral or deleterious. In Cobra the MGBG treatments caused a uniform reduction in explant regeneration. However, at low concentrations (0.35μM) MGBG resulted in a 50% increase, to 92%, in regeneration from Westar. The potential of MGBG in promoting explant regeneration in *B. napus* is discussed in the light of its interaction with the explant genotype.

Abbreviations: ABA, abscisic acid; BAP, benzylaminopurine; MGBG, methylglyoxal-bis-(guanylhydrazone); NAA, naphthalene acetic acid; thioproline, thiazolidine-4-carboxylic acid.

Introduction

Improved shoot regeneration from complex explants of *B.napus* is of interest principally because these systems are used in *Agrobacterium*-based transformation protocols. High shoot regeneration frequencies are necessary, both to increase the probability of selecting transformed shoots and because *Agrobacterium* treatment and antibiotic selection for transformed tissue reduces the regeneration frequency of treated explants. Shoots of *B. napus* have been regenerated from several explant types, cotyledons (Narasimhulu and Chopra 1988, Moloney et al. 1989), hypocotyls (Dietert et al. 1982, Radke et al. 1988), flowering internodes (Klimaszewska and Keller 1985) and stem sections (Kartha et al. 1974, Stringham 1977, Pua et al. 1987, Khehra and Mathias 1992). The responsiveness of the various explants is markedly different and covers a range from 23% in hypocotyls (Radke et al. 1988) and 26% in cotyledons (Narasimhulu and Chopra 1988) to 80-100% in stem segments (Weber et al. 1990, Pua et al. 1987). Despite reported regeneration of 80-100% from stem segments we routinely observe frequencies of 50-60%. Khehra and Mathias (1992) examined the relative contributions of explant source, genotype and growth regulator regime to shoot regeneration response in *B. napus*. Their study established that explant type was the dominant effect on regeneration, accounting for 44%-95% of the observed variation in response and that stem segments are much superior to hypocotyls or cotyledons as explants. Genotype also had a significant effect but the variation in response between varieties that was observed in hypocotyl and cotyledon explants, was much reduced when using stem internode explants. The same study found the effect of the growth regulator regime on regeneration was small. However, there are reports of increased regeneration from *B. oleracea* and *B. rapa* hypocotyl callus cultures when metabolic inhibitors (including ethylene antagonists and inhibitors of ethylene synthesis) are included in the media (Sethi et al. 1990b, Williams et al. 1990, Chi and Pua 1989, Palmer 1992). Methylglyoxal-bis-(guanylhydrazone) (MGBG), an inhibitor of spermidine biosynthesis, was reported to increase regeneration frequencies from 7% to 63% (Sethi et al. 1990a).

In tissue cultures of maize (Armstrong and Green 1985), alfalfa (Shetty and McKersie 1993) and orchard grass (Shetty and Asano 1991) proline enhanced the development of embryogenic cells and callus and in explants of *Cucumis melo* increased regeneration frequencies, especially multiple shoot formation (Shetty et al. 1992). To try to further improve the shoot regeneration frequency of *B. napus* stem internode explants we investigated the effect of MGBG, proline and its analog thioproline on regeneration response.

Materials and Methods

Plant material

The two *B. napus* varieties, one winter (Cobra) and one spring (Westar) were provided by Dr AE Arthur, JI Centre, Norwich. Stem explants were prepared from 3-4 week old shoot tip cultures as described by Khehra and Mathias (1992). Five explants were plated per Petri dish and 10 replicate dishes were prepared for each treatment.

Correspondence to: C. M. O'Neill

Culture conditions

The explant culture medium was MS (Murashige and Skoog 1962) medium with 30g/l^{-1} sucrose and 0.8g/l^{-1} agarose containing 1.8 μM NAA + 17μM BAP. The media were autoclaved at 120°C for 20 mins. Growth regulators and other additives were filter sterilised and added to autoclaved medium immediately before pouring 25ml aliquots into 9cm Petri dishes. The germinating seedlings, shoot and explant cultures were incubated under a photoperiod of 16 hours at 25°C ± 1°C.

After 4 weeks in culture shoot regeneration was recorded, both as the total number of calli with shoots >1cm in length and as those explants producing one shoot and those producing multiple shoots. The explant regeneration data was analysed using a standard analysis of variance. A standard χ^2 test was used to test the significance of changes in the ratio of single to multiple shoot regeneration events.

Results and Discussion

The effects of proline, thioproline and MGBG on shoot regeneration were tested on cultured stem segments from shoot tip cultures of two *B. napus* varieties, Cobra and Westar. The explants typically showed some swelling and callusing of the cut surfaces in the first week of culture and within 3 weeks regenerating shoots were seen. The effect of proline and thioproline on the regeneration of explants is presented in figure 1 and table 1. The overall analysis of variance for this data demonstrated that treatment was the only significant effect (significant at the 0.1% level). The inhibitory effects of both proline and thioproline on shoot regeneration were significant and resulted in a reduction of between 2 and 5-fold in response. The only exception was the effect of 0.5mM thioproline on explants of Cobra where the treatment reduced regeneration but the effect was not significantly different from the control treatment at the 5% level. There were no variety or variety x treatment interaction effects. Regeneration efficiency reflects both the total number of regenerating explants and the ratio between explants producing single and multiple shoots. In *B. napus* up to 80% of regenerating stem explants produce multiple shoots (tables 1 and 2). The percentage of regenerating explants that produced one shoot, or more than one shoot, is given in table 1.

In Westar only the 1.0mM thioproline treatment significantly reduced the number of explants with multiple shoots while the number of Cobra explants producing multiple shoots was significantly reduced by all the proline and thioproline treatments. This may partially reflect the control values in this experiment. Reference to the controls (for both genotypes) in tables 1 and 2 show that the ratio of single to multiple shoots in the Cobra control, in this experiment, was slightly abnormal. If this control resembled the others then the 10mM proline and 0.5mM thioproline treatments would probably not be significantly different from the control. Nonetheless, the reduced multiple shoot formation on the higher concentrations of these chemicals would remain significantly different from the controls. In both varieties the total number of regenerating explants and the ratio of single to multiple shoots were either unaffected or adversely affected by proline and thioproline treatments. Thioproline, a proline analog that interferes with proline metabolism, was included in the cultures to test the effect of reduced proline metabolism on shoot regeneration. Its observed inhibitory effect was not unexpected on the basis of previous reports. However, the negative effect of proline was in contrast to the positive effects on regeneration and embryogenesis reported in cultures of several other species (Armstrong and Green 1985, Shetty and McKersie 1993, Shetty, and Asano 1991). In tissue culture proline metabolism is affected by osmotic and salt stress (Pandey and Ganapathy 1985) and interacts with ABA in affecting culture responses (Duncan and Widholm 1987). The interaction with ABA and osmotic stress is interesting as both have been described as increasing embryogenesis and enhancing regeneration in culture (Sethi et al. 1990a, Armstrong and Green 1985, Brown et al. 1989, Close and Ludeman 1987, Kavi Kishor and Reddy 1986a, Kavi Kishor and Reddy 1986b, Rengel 1986). Proline does appear to have a specific role in culture as other amino acids do not substitute for it (Armstrong and Green 1985, Shetty and Asano 1991). This role may be as a regulated source of precursors for both protein phosphorylation and ATP (Shetty and McKersie 1993, Shetty et al. 1992). The mechanism of proline action in culture is unknown but the inhibitory effects of both thioproline and proline demonstrate that proline metabolism does affect the differentiation/regeneration response in *B. napus*.

Table 1. The effect of proline and thioproline treatment on regeneration of single and multiple shoots from stem explants of *B. napus* cvs. Cobra and Westar. (χ^2 values greater than 3.84 are significantly different from the control at the 5% level)

	Cobra			Westar		
Treatment	% of explants with 1 or >1 shoots per callus		χ^2	% of explants with 1 or >1 shoots per callus		χ^2
	1	>1		1	>1	
Control	4	96		17	83	
10 mM Proline	15	85	4.39	20	80	0.12
20 mM Proline	75	25	52.51	0	100	1.00
0.5mM Thioproline	29	71	28.59	25	75	0.40
1.0mM Thioproline	60	40	40.83	60	40	6.76

Table 2. The effect of MGBG on the regeneration of single and multiple shoots from stem explants of *B. napus* cvs. Cobra and Westar. (χ^2 values greater than 3.84 are significantly different from the control at the 5% level)

	Cobra			Westar		
	% of explants with 1 or >1 shoots per callus		χ^2	% of explants with 1 or >1 shoots per callus		χ^2
μM MGBG	1	>1		1	>1	
0 (Control)	21	79		19	81	
0.35	20	80	0.01	17	83	0.03
0.7	10	90	0.78	15	85	0.18
1.4	10	90	0.78	50	50	11.54

MGBG effects on explant regeneration are presented in figure 2 and table 2. The overall analysis of variance for the data demonstrated that variety and variety x treatment interactions were significant (at the 0.1% and 0.5% levels respectively). The overall regeneration of Cobra explants was inhibited, by about one-third as compared to the control, by all the MGBG treatments. In contrast regeneration from Westar explants was increased by 50%, to 92%, in 0.35μM MBGB and to 80% in the 0.73μM treatment. The regeneration response in the 1.4μM MGBG treatment was not significantly different from the control. The interaction of MGBG with variety is also apparent when considering its effect on multiple shoot formation (table 2). In Cobra the ratio of single to multiple shoots was not affected by MGBG. In Westar 0.35μM and 0.7μM MGBG also had no effect but the 1.4μM treatment significantly reduced the number of explants producing multiple shoots. MGBG blocks S-adenosylmethionine decarboxylase and specifically inhibits biosynthesis of spermidine (Slocum and Galston 1985).

As polyamines have been implicated in plant cell division and differentiation processes (Sethi et al. 1988, Fienberg et al. 1984) any interference in polyamine metabolism might be expected to affect the regeneration response in culture. In our experiments the effect of MGBG on the allotetraploid *B. napus* was not so dramatic as that reported for hypocotyl callus of the parental diploid species *B. oleracea* (Sethi et al. 1988). This may, in part, reflect the much higher efficiency of our basal *B. napus* culture system which almost equals that of the MGBG enhanced *B. oleracea* hypocotyl callus. Also, as we found intervarietal differences in response it is to be expected that there will be genetically determined interspecific differences, as well as subtleties in the effect of the inhibitor on different explants. However, 0.35μM MGBG promoted the regeneration from Westar to almost 100%, with no negative effect on multiple shoot formation, by increasing the number of regenerating explants by 50%.

Figure 1: Percent of stem explants of Cobra □ and Westar ■ regenerating shoots on proline and thioproline.

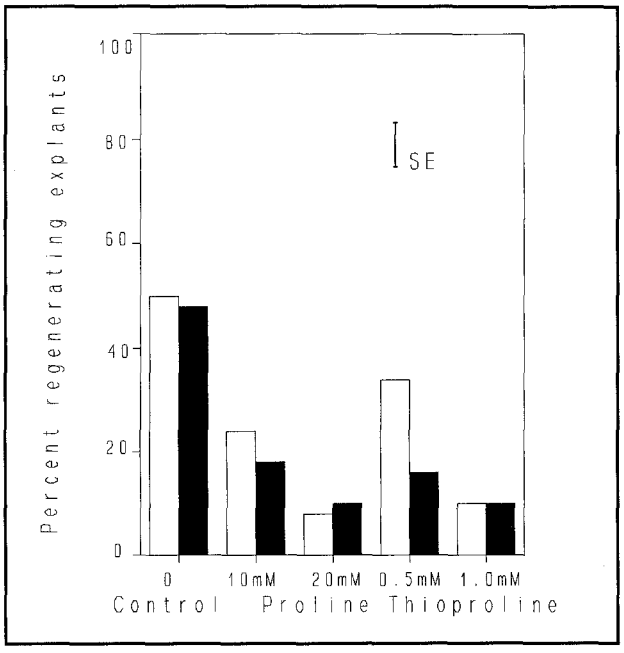
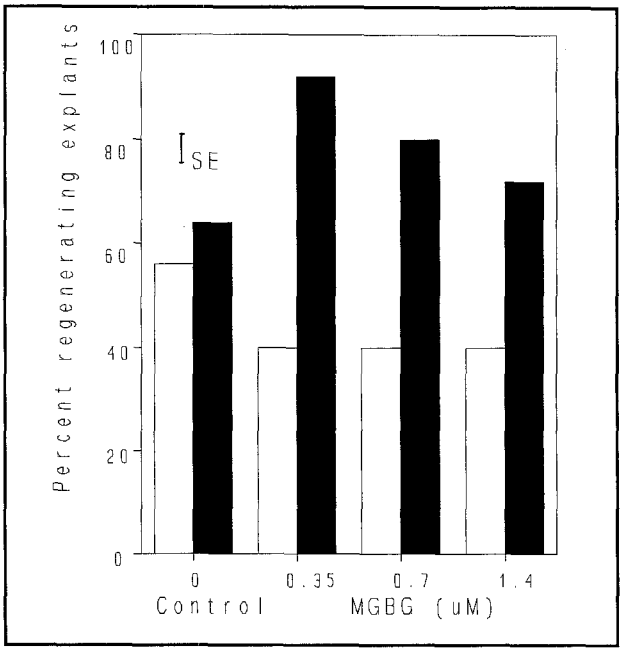


Figure 2: Percent of stem explants of Cobra □ and Westar ■ regenerating on MGBG (0.35, 0.7 and 1.4μM).



Stem internodes from shoot tip cultures are very responsive explants for regenerating shoots of *B. napus* and their use has the advantage that they are less affected by genotypic effects on culture response than other explants (Khehra and Mathias 1992). The experiments reported here, on attempts to increase regeneration frequencies from stem explants, have demonstrated that proline and thioproline do not improve, and indeed may have deleterious effects on, regeneration from stem internode explants. The effect of MGBG on shoot regeneration was genotype dependent. In the winter variety Cobra the overall explant regeneration was reduced at all concentrations of MGBG and at high concentrations multiple shoot formation was inhibited. However, in the spring variety Westar low concentrations of MGBG increased the number of regenerating explants, by 50%, to 92% of the total without an effect on multiple shoot production. As the beneficial effect of MGBG is genotype dependent it will not be suitable for all varieties of *B. napus*. However, where varieties respond positively to MGBG, major improvements in regeneration frequencies can be expected from its use.

Acknowledgements: The authors acknowledge the support of the BBSRC.

References

Armstrong CL, Green CE (1985) Planta 164: 207-214
Brown C, Brooks F, Pearson D, Mathias RJ (1989) J Plant Physiol 133: 727-733
Chi G-L, Pua E-C (1989) Plant Sci 64: 243-250
Close KR, Ludeman LA (1987) Plant Sci 52: 81-98
Dietert MF, Barron SA, Yoder OC (1982) Plant Sci Lett 26: 233-240
Duncan DR, Widholm JM (1987) Plant Physiol 83: 703-708
Fienberg AA, Choi JH, Lubich WP, Sung ZR (1984) Planta 162: 532-539
Kartha KK, Gamborg OL, Constabel F (1974) Physiol Plant 31: 271-220
Kavi Kishor PB, Reddy GM (1986a) Plant Cell Rep 5: 391-393
Kavi Kishor PB, Reddy GM (1986b) Plant Physiol 126: 49-54
Khehra GS, Mathias RJ (1992) J Exp Bot 43: 1413-1418
Klimaszewska K, Keller WA (1985) Plt Cell Tiss Org Cult 4: 183-197
Moloney MM, Walker JM, Sharma KK (1989) Plant Cell Rep 8: 238-242
Murashige T, Skoog F (1962) Physiol Plant 15: 473-497
Narasimhulu SB, Chopra VL (1988) Plant Cell Rep 7: 104-106
Palmer CE (1992) Plant Cell Rep 11: 541-545
Pandey R, Ganapathy PS (1985) Plant Sci 40: 13-17
Pua EC, Mehra-Pelta A, Nagy F, Chua NH (1987) Bio/Technology 5: 815-817
Radke SE, Andrews BM, Moloney MM, Crouch ML, Kridl JC, Knauf VC (1988) Theor Appl Genet 75: 685-694
Rengel Z (1986) Biochem Biophys Pflanzen 181: 605-610
Sethi U, Basu A, Guha-Mukherjee S (1988) Plant Sci 56: 167-175
Sethi U, Basu A, Mukherjee GS (1990a) Plant Cell Rep 8: 598-600
Sethi U, Basu A, Mukherjee SG (1990b) Plant Sci 69: 225-229
Shetty K, Shetty GA, Nakazaki Y, Yoshioka K, Asano Y, Oosawa K (1992) Plant Sci 84: 193-199
Shetty K, Asano Y (1991) J Plant Physiol 139: 82-85
Shetty K, McKersie BD (1993) Plant Sci 88: 185-193
Slocum RD, Galston AW (1985) Plant Cell Physiol 26: 1519-1526
Stringham GR (1977) Plant Sci Lett 9: 115-119
Weber G, Monajembashi S, Wolfrum J, Greulich KO (1990) Physiol Plant 79: 190-193
Williams J, Pink DAC, Biddington NL (1990) Plant Cell Tiss Org Cult 21: 61-66