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High Frequency Shoot Regeneration from Hypocotyl of Canola (*Brassica napus* L.) cv. Dunkled

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Abstract

Hypocotyl explants of *Brassica napus* L. cv. Dunkled were used to investigate the effects of various plant growth regulators and silver nitrate on morphogenesis *in vitro*. Two types of explants, such as upper and lower portion of hypocotyl were examined. A high frequency of shoot regeneration (96 %) was achieved on MS supplemented with growth regulators. Shoots regenerated earlier (within three weeks) from segments taken from the upper portion of the hypocotyl than those from lower portion (after three weeks), although shoot regeneration frequency was similar in both the cases. Exclusion of silver nitrate from the medium drastically reduced the regeneration potential (50 %). Multiple shoot regeneration was obtained from the periodic subculture of the explants in the same medium. *In vitro* elongated shoots were rooted with a 100 % success by treating them with half-strength MS supplemented with 0.250 mg/l IBA and 0.125 mg/l IAA. Rooted plantlets were successfully established into the soil.

Introduction

The genus *Brassica* includes several important crop species. *Brassica napus* L. is one of the world's most important sources of vegetable oil and protein-rich meal. Therefore, *Brassica napus* has become an object of extensive tissue culture studies and breeding. Genetic engineering techniques have been applied to *Brassica napus* to introduce new genes (Knutzon et al. 1992). Regeneration in *Brassica napus* is highly variable and genotype specific. A number of papers have reported regeneration of shoots from seedlings or mature plant derived explatns of *Brassica napus* (Dunwell 1981). To date organogenesis has been achieved in a variety of explants such as stem sections (Pua et al. 1991;

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Stringam 1977), stem thin-cell layer (Klimaszewska and Keller 1985), leaf discs (Dunwell 1981), roots (Sharma and Thorpe 1989), cotyledons (Moloney et al. 1989) and hypocotyls (Dieter et al. 1982; Phogat et al. 2000).

Efficient *Agrobacterium*-mediated transformation methods require reliable and efficient callus induction and plantlet regeneration procedures. This area needs to be researched for a high frequency shoot regeneration system, as an essential pre-requisite for genetic transformation (Leding and Sederoff 1985; Riemenchucider et al. 1988). De Block et al. (1989) reported that silver nitrate was pre-requisite for efficient shoot regeneration in *Brassica napus.* High frequency shoot regeneration using hypocotyl explants has been reported from *Brassica napus* var. Westar and consequently this material has been used extensively for genetic transformation studies (De Block et al. 1989). However, Westar is a long duration variety, which is ill adapted to agronomic conditions prevailing in the mustard-rapeseed growing regions of Pakistan. As a consequent, both for agronomic improvement and genetic studies callus induction and regeneration protocols are required for *B. napus* varieties that are adapted to this region.

The overall interest in the present work is to establish an efficient system for shoot regeneration in *B. napus* var. Dunkled by exploiting specific combinations of growth regulators. In addition the second most important aim of the present study was to evaluate the silver nitrate for its effect on shoot regeneration frequency. Here the report on the high shoot regeneration frequency of canola var. Dunkled adapted to agronomic conditions prevailing in Pakistan is made.

Materials and Methods

Research work was conducted at National Agricultural Research Centre (NARC) during August, 2001 to January, 2002. Agricultural Biotechnology Institute (ABI), NARC provided the experimental facilities. The seeds of the variety were kindly provided by National Oilseed Development Programme (NODP), NARC, Islamabad, Pakistan.

Seed of *Brassica napus* cv. Dunkled were surface sterilized in a sequential manner with 70 % ethanol for one min, 0.1% mercuric chloride for five min followed by treatment with sodium hypochlorite (2.5 % active achlorine) for five min and subsequently rinsed four - five times with sterilized water. Treated seeds were germinated aseptically on half-strength MS without vitamins and with only 1.5% sucrose (physical conditions : 500 lux for three days followed by 2000 lux for three - ten days, 16 hr light/8 hr dark cycle, 23 ± 1_C).

Hypocotyl segments (0.5 - 1.0 cm) from upper and lower portion were obtained from five - 14-day-old seedlings and used for regeneration experi-ments.

All the media tested for regeneration (RM media) contained MS salts and vitamins with other adjuvants (Table 1). Unless otherwise mentioned, the media were solifidied with 0.6 % agar (Sigma) or 0.2 % gelrite. Adjuvant like silver nitrate was filter sterilized and added to the autoclaved media. All other adjuvants and media were autoclaved at 20 psi for 15 min.

Table 1. Regeneration potential of *B. napus* cv. Dunkled on different media with or without 5 mg/l silver nitrate.

Media[1]		Days	% of induced shoots				No. of shoots/culture with 5 mg/l Ag	
			UPH[2]		LPH[3]		UPH	LPH
			+ Ag	– Ag	+ Ag	– Ag		
RM1	NAA (0.1) + BAP (1.0)	20	96.2	50.8	–	–	16.48 ± 1.7	
	+ $AdSO_4$ (40)+ PVP	30	96.2	50.8	97.2	46.9		18.3 ± 2.4
	+ MES + GA_3							
RM2	2,4-D (0.05)	20	42.5	12.4	–	–	7.23 ± 2.44	
	+ BAP (1.0)	30	42.5	12.4	45.2	24.6		8.21 ± 2.52
RM3	BAP (1.0)	20	31.2	16.8	–	–	4.78 ± 0.91	
	+ NAA (1.0)	30	21.2	16.8	40.8	21.4		6.12 ± 1.46
RM4	NAA (1.0) + BAP (1.0)	20	86.4	35.9	–	–	12.87 ± 1.45	
	+ PVP + MES	30	86.4	35.9	83.9	54.2		12.15 ± 1.86
RM5	NAA (1.5) + BAP (1.0)	20	69.4	35.0	–	–	10.25 ± 1.80	
	+ PVP+ MES	30	69.4	35.0	75.7	29.9		13.81 ± 2.00
RM6	NAA (1.0) + BAP (1.0)	20	78.5	40.8	–	–	14.08 ± 3.42	
	+ PVP + MES	30	78.5	40.8	80.4	39.8		15.27 ± 4.25
RM7	2,4-D (0.05) + BAP (1.0)	20	56.2	19.8	–	–	7.48 ± 2.58	
	+ PVP + MES	30	56.2	19.8	34.8	9.1		6.97 ± 3.01
RM8	NAA (0.5) + BAP (1.0)	20	61.5	40.5	–	–	12.35 ± 2.78	
	+ PVP + MES	30	61.5	40.5	50.8	25.6		10.79 ± 2.20
RM9	NAA (0.5) + BAP (1.0)	20	93.8	46.7	–	–	15.78 ± 3.18	
	+ $AdSO_4$ (40) + PVP	30	93.8	46.7	95.8	43.3		16.45 ± 2.48
	+ MES + GA_3 (0.001)							
RM10	NAA (1.0) + BAP (1.0)	20	82.9	38.2	–	–	13.71 ± 2.46	
	+ $AdSO_4$ (40) + PVP	30	82.9	38.2	86.1	43.2		14.19 ± 2.73
	+ MES + GA_3 (0.001)							

Numbers represent percentage regeneration response calculated from approximately 25 explants/test condition. [1]All the media contained MS salts, vitamins and 3% sucrose. Concentration is given in mg/l for all the adjuvants. [2]Explants from upper portion of the hypocotyl. [3]Explants from lower portion of the hypocotyl. ± represents standard error of the mean.

Hypocotyl segments (0.5 - 1 cm) between cotyledon and the first leaf were excised from aseptically germinated plants. These segments were placed on different media for regeneration and kept in a growth room (light intensity 2000 lux, 16 hr light/8 hr dark cycle, 25 ± 2_C) to determine the regeneration potential of the cultivar used.

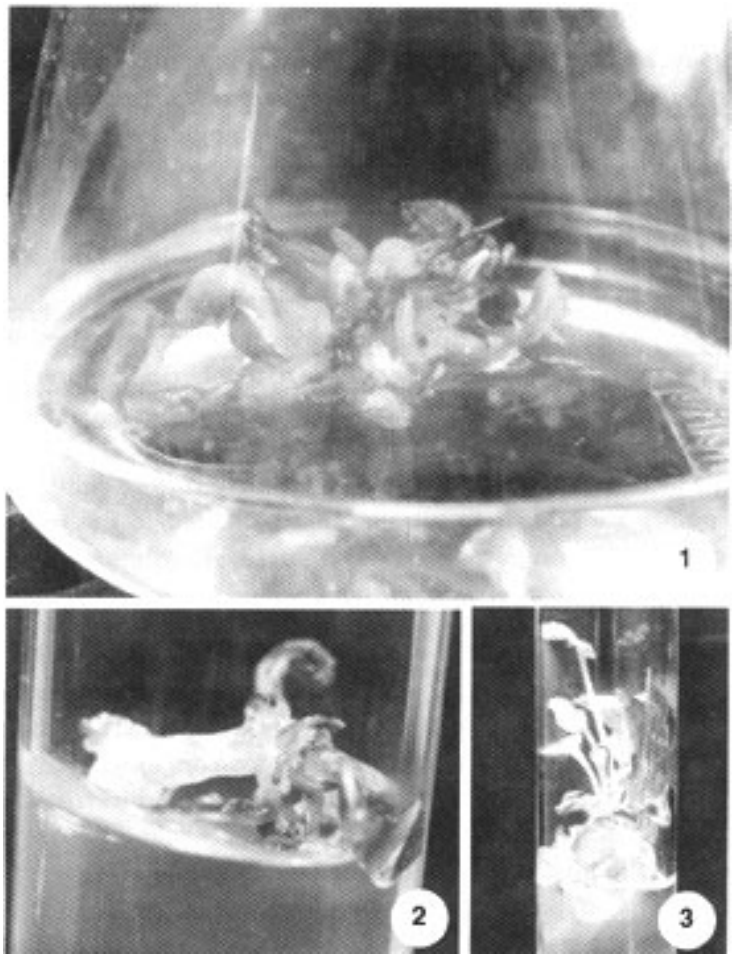
Twenty to 40 cultures were raised for each treatment, and each experiment was conducted twice. Subculture period was maintanied at two - three weeks intervals. Visual observations were taken every week and the effects of different treatments were quantified on the basis of the percentage of explants showing response for plant development and number of shoots/culture for high frequency shoot regeneration. Shoot regeneration was observed on the basis of the cultures showing response for callus induction, plant development and number of shoots/culture.

Results and Discussion

The regeneration in *Brassica napus* is highly variable and genotype specific. There are differences in shoot regeneration ability of various explants in one variety as well as in differnt genotypes. It is also reported that the shoot tips possessed the best shoot regeneration ability (Szule and Drozdowska 1997). In the present study different experiments were conducted with a view to finding the optimum culture conditions for shoot regeneration from cultured explants. Two different modes of regeneration were obtained from hypocotyl explants. The first one was direct whereby shoots originated directly from subepidermal tissues of hypocotyl explants (Fig. 1). Narasimhulu and Chopra (1988) observed similar type of regeneration in *Brassica napus* from immature cotyledonary explants. In the second system, shoot proliferation occurred through a callus phase, which was initiated at the cut end of explants within two weeks (Fig. 2). These findings are in consistent with Turget et al. (1998).

Multiple shoots were found to develop from both types of explants i.e., hypocotyl explants taken from upper and lower protions of *in vitro* plants, when cultured on different media with or without 5 mg/l silver nitrate (Fig. 3). In both the cases, the best shoot regeneration induction was observed on RM1 (96 %) medium followed by RM9 (93 %) and RM4 (86 %), respectively. RM3 was least effective in regeneration response (31 %). It was observed that higher concentrations of an auxin in the medium suppressed the regeneration frequency (86 %) regeneration was noted on RM4 but it reduced to 69 % on RM5 mainly due

to high concentration of NAA. Increased concentration of auxin also enhanced callusing in both types of explants (GA_3 in low concentration stimulated the shoot regeneration (RM1, RM2 and RM3). It may be due to role of GA_3 in breaking the dormancy of explants.



Figs. 1 - 3. Plant regeneration from hypocotyl explants in *Brassica napus*. 1. Direct multiple shoot induction in RM1 supplemented with 5 mg/l silver ntirate after two weeks of subculture. 2. Development of multiple shoots from hypocotyl-derived calli in RM1. 3. Fully developed multiple shoots in RM1.

Initiation of multiple shoots in most of the treatments was observed within three weeks of cultue, in case of explants from upper portion of hypocotyl (Fig. 1). 96.2 % of the explants developed shoot and the number of shoots per explant was 16.48 on RM1. In the absence of silver nitrate the

regeneration frequency was reduced to nearly 50 % on all the media. In the case of hypocotyl from lower portion the highest shoot regeneration was 97.2 % and the maximum number of shoots per explant was 18.3, when the explants were cultured on RM1 with 5 mg/l silver nitrate. But in this case, the initiation of multiple shoots in most of the treatments was observed after three weeks of culture. Similarly, the shoot regeneration frequency was reduced by 50 % in the absence of silver nitrate in this case too. These results indicated that explants taken from upper hypocotyl regenerated earlier, although regeneration frequency and number of shoots per culture was similar in both the cases. The earlier responsiveness of explants from upper then the lower can be attributed to the presence of actively growing cells at the tips. These results are in agreement with Shi and Zhou (1998).

The use of silver nitrate in the medium was a pre-requisite. Exclusion of silver nitrate from the medium drastically reduced the regeneration frequency. Without silver nitrate none or only a very few shoots could be obtained. The stimulating effect of silver nitrate on shoot regeneration has been described (De Block et al. 1989; Shi et al. 1998; Phogat et al. 2000). The addition of silver nitrate in the medium decreases the ethylene content during culture. The most important points are (a) silver nitrate must be added to the medium after autoclaving and (b) silver nitrate should be used as early as possible, when regeneration starts. Once a certain type of non-regenerating callus is formed, it is difficult to revert this process to shoot regeneration.

Effects of gelling agents (agar and gelrite) to shoot regeneration frequency was investigated (data not shown). RM1 medium with varying concentrations ranging from 1 - 4 g/l gelrite and 6 - 9 g/l agar was tested. There was no significant difference for shoot development between gelrite and agar. However, 2 g/l of gelrite was found to be superior over agar for average number of shoots/culture. Shoot growth was declined on medium having either 3 - 4 g/l gelrite or 8 - 9 g/l agar. Small shoots were formed on the medium having 3 g/l gelrite. Among all the treatments 2 g/l gelrite was found to be optimal gelling agent for shoot multiplication, and all the further experiments were conducted on RM1 medium with 2 g/l gelrite. There was no significant difference for shoot development between gelrite and agar. It was also reported that gelrite promoted long term maintenance, differentiation and de-differentiation stages of *Vitis vinfera*.

Shoot production and plantlets formation could be increased when auxin was completely removed from the medium in later subcultures. Best shoot elongation was achieved when regenerating segments of the callus were cut into

smaller pieces containing three or four shoots and subcultured in the RM1 medium supplemented with 0.1 mg/l BAP.

Callus tissues with regenerating shoots were removed from the hypocotyl explants and transferred to root induction medium (RM1) (half strength of MS salts, no vitamins, 1% sucrose, 0.1 mg/l IBA, 0.025 mg/l BAP and 2 g/l gelrite) to recover complete plants.

The rooted micropropagated and regenerated transgenic plants were thoroughly washed to remove the adhering gel and were transferred to pots containing vermiculite. These were kept in a room with low intensity and high relative humidity for one week. Thereafter, they were grown in a green house to assess their acclimatizing potential.

In conclusion, hypocotyl explants from both the upper and lower portions showed high frequencies of shoot regeneration under the influence of differnt media and silver nitrate. Currently attempt to use hypocotyl explants for *Agrobacterium*-mediated transformation impact of such techniques is tremendous and hence demands its integration with conventional plant breeding to meet the needs for crop improvements and to further increase the canola production in future.

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