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CALLUS FORMATION AND PLANTLETS REGENERATION FROM HYPOCOTYL OF *BRASSICA NAPUS* BY USING DIFFERENT MEDIA COMBINATIONS

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Abstract

The genus *Brassica* includes several important crop species. Canola (*Brassica napus* L.) is considered as the most important source of vegetable oil and protein rich meal worldwide. Effect of 5 different callusing media (CIM_1 to CIM_5) and regeneration media (RM_1 to RM_{10}) supplemented with different concentrations of hormones were studied for callus induction and regeneration of four cultivars of *Brassica napusc* viz., Dunkeld, Oscar, H-19 and Rainbow. The experimental work was carried out at the Agricultural Biotechnology Program, National Agricultural Research Centre, Islamabad during March 2004 to January 2005. The results indicated that among the 5 media combinations, MS media supplemented with 0.5mg /l IAA, 1mg /l BAP, 0.5mg/l NAA and 1 mg /l Kinetin (CIM_2) was the most responsive media and gave best results for callus induction followed by CIM_3, CIM_1, CIM_4 and CIM_5. As the concentration of IAA was increased further there was a decrease in callus induction. The cultivar Oscar formed relatively more calli at higher rates closely followed by cultivar H-19. The cultivar Rainbow showed minimum calli formation among the 4 cultivars. In case of regeneration, all the 10 media combinations showed significantly different regeneration response except RM_1 and RM_6 media combinations that showed almost no regeneration. The best results were obtained when explants were cultured on a regeneration medium containing 1mg Zeatin/l and 0.1 mg IAA/l. Maximum regeneration percentage was recorded with RM_8 followed by RM_3. The results indicated that cultivar Oscar showed regeneration relatively at higher rates. The cultivar Rainbow showed minimum regeneration capability among the four cultivars.

Introduction

In Pakistan, the critical shortage of edible oil is increasing rapidly and the country is facing an adverse economic affect by the deficiency of edible oil. More than 75% of the total foreign exchange allocated for the import of food items is used for the purchase of edible oil. Presently domestic production of oil meets only 30% of the country's requirements. After cotton, rapeseed-mustard is second most important source of oil in Pakistan. *B. napus* (*Gobhi Sarsoon*) is an important rapeseed-mustard crop in the world as well as in Pakistan.

Tissue culture technique in combination with molecular techniques has been successful to incorporate specific traits through gene transfer called DNA recombinant technology (Brown & Thorpe, 1995). Tissue culture is a process where by small pieces of living tissues called explants are isolated from an organism and grown on semi-refined medium aseptically. The explants may be as large as a seedling or an organ such as ovule, embryo etc., and as small as single cells and protoplasts. Success in plant tissue culture can pivot on two critical factors i.e., choice of an explant and culture medium used.

Cell and tissue culture relating to variability and selection efficiency are essential. Genetic variation in canola is required to breed cultivars that are high yielding and resistant to several biotic and abiotic stress conditions. It is well known that improvement of plants through conventional breeding method is relatively slow, time consuming and labour intensive. On conventional genetic improvement programmes based on tissue culture and molecular genetics is essential as a complement to standard breeding (Lichtenstein & Draper, 1985). Regeneration in *Brassica napus* is highly variable and genotype specific (Dunwell, 1981).

High frequency shoot regeneration using hypocotyls explant 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). As a consequence, both for agronomic improvement and genetic studies, callus induction and regeneration protocol are required for *B. napus* varieties that are adapted to this region.

The present work was conducted to establish a reproducible protocol for callus induction and regeneration of 4 rapeseed (*Brassica napus*) cultivars viz., Dunkeld, Oscar, Rainbow and H-19 using hypocotyls as explant source. The objectives were to select the best media combination for callusing and regeneration and to find out the best responsive cultivars on selected media.

Material and Methods

The seeds of *Brassica napus* cultivars viz., Dunkeld, Oscar, H-19 and Rainbow were provided by National Oilseed Program, National Agricultural Research Centre (NARC) Islamabad. Hypocotyls were used as explant source from *In vitro* seedlings. Research work was carried out at Agricultural Biotechnology Program, NARC, Islamabad during March 2004 to January 2005.

Sterilization of plant materials: selected healthy seeds of all four genotypes were washed with commercial detergent under tap water following surface sterilization with brief rinse with 95 % ethanol. Seeds were then treated with 20% (v/v) "Clorox" bleach (Sodium hypochlorite) for 10 min, with occasional vigorous swirling, and rinsed 3 times (15 min each) in sterile distilled water. They were rinsed thoroughly with water added with Tween-20 (0.05%) for 2-3 minutes. Then they were washed with distilled water till the foam was completely removed. All manipulations were executed aseptically in a laminar flow cabinet. After completing the process the seeds were placed separately in four sterilized Petri dishes.

Seed germination: Seeds were sterilized and placed in Petri dish with filter paper (Whatman No.1), moistened with sterilized water on 4^{o}C for 72 hours to synchronize their germination. Then sterilized seeds were placed on the MS (Murashige & Skoog 1962) plain medium and placed in the dark at 26 ± 3^{o}C, after that the germinated seeds were transferred to light in growth room with 16 hours photoperiod of a light intensity of 1500 lux.

Culture media: MS (Murashige & Skoog, 1962) culture media were used for seed germination while different hormones were added in this medium for callusing. Media based on MS salts were made using commercially available preparations (Flow Laboratories Ltd., Irvine, Scotland, U.K.) to which additions of a carbon source and growth regulators were prepared from refrigerated stock solutions. Sugars were added as solids.

After the addition of growth regulators and other components to the basal solution, the pH was adjusted using 1.0M HCl or 0.5M NaOH. Distilled water was added to the desired final volume. To solidify media, 0.6% (w/v) agar (Type V, Sigma, U.S.A.) was added and dissolved by steaming in the medium after adjustment of the pH 5.7-5.8, prior to sterilization. The media was then autoclaved for 15 min., at 121^{o}C. After autoclaving the media was left to cool in an upright position for 24 hours.

Explant and their source: After germination hypocotyls of the *In vitro* plants were used as the source of explants (Pushpa *et al.*, 2002). The hypocotyls of uniform sizes (1 cm) were kept. Hypocotyls below the first true leaf from 3-4 weeks old *in vitro* seedlings were taken.

Initiation of callus: The media evaluated are shown in Table 1. The experiments were performed in test tubes using 5-7 ml agar medium per test tube. Explants were removed from *In vitro* grown seedlings and were cultured in test tubes. Each treatment was repeated three times with 3 racks per repeat. Results were observed according to the presence of callus after 28 days.

Growth conditions: The temperature ranged from 25 ± 3^{o}C in growth chamber, while a photoperiod of about 16 hours was kept.

Media assessments for plant regeneration

Media combinations: The maintained calli were sub-cultured on regeneration media. Ten plant regeneration media, viz., RM_1 to RM_{10}, were used for determination of regeneration capacity of the *Brassica* cultivars. The composition is shown in Table 2. Explants were cultured in test tubes containing regeneration media. Each cultivar was assessed with 1 rack of test tubes per replication and 3 replications were cultured at 25 ± 3^{o}C under a 16 h day length with illumination of 125 uE/m^2/s (daylight fluorescent tubes). After 28 days, percentage of regenerated plants giving one or more shoots per responsive medium and species were calculated. All regenerated shoots (3-4 cm in length) were excised from the explants and cultured for rooting on rooting media containing 0.2 mg/1 IBA (4 shoots per jar; each jar with 50 ml of medium). Ten regenerates of each *Brassica* cultivars were transferred from tissue cultures to the glasshouse.

Regenerations: Ten regenerated shoots (3-4 cm in length) of each variety were excised from calli and rooted on media containing 0.2 mg/l IBA (4 shoots per jar on 50 ml of medium). Rooted regenerates (10 plants of each variety) were transferred from tissue culture to the glass house. Two factors CRD statistical design was applied on the present studies.

Results and Discussion

***In vitro* seed germination:** It was observed that the seeds started germination in the second week after placing on the medium. Complete seedlings with two leaves appeared in the 3rd week. Similar results were reported by Patil *et al.*, (2002) that seeds of the varieties germinated in 7-10 days after sowing and seedlings were ready to transplant after 25-30 days.

Table 1. Composition of callus induction media used for the *B. napus* cultivars.

Media	Composition
CIM_1	MS salts and 3% sucrose, 0 mg/l IAA, 1 mg/l BAP, 0.5 mg/l NAA, 1 mg/l Kinetin, 0.6% Agar, pH 5.75.
CIM_2	MS salts and 3% sucrose, 0.5 mg/l IAA, 1 mg/l BAP, 0.5 mg/l NAA,1mg/l Kinetin, 0.6%Agar, pH 5.75.
CIM_3	MS salts and 3% sucrose, 1 mg/l IAA, 1 mg/l BAP, 0.5 mg/l NAA, 1 mg/l Kinetin, 0.6% Agar, pH 5.75.
CIM_4	MS salts and 3% sucrose, 1.5 mg/l IAA, 1 mg/l BAP, 0.5 mg/l NAA, 1 mg/l Kinetin, 0.6% Agar, pH 5.75.
CIM_5	MS salts and 3% sucrose, 2 mg/l IAA, 1 mg/l BAP, 0.5 mg/l NAA, 1 mg/l Kinetin, 0.6% Agar, pH 5.75.

Table 2. Compositions of media used for regeneration.

Media	Composition
RM_1	MS salts 3% sucrose, (without hormone), 0.6% Agar
RM_2	MS salts 3% sucrose, 2 mg/l BAP, 0.6% Agar
RM_3	MS salts 3% sucrose, 2 mg/l BAP, 1 mg/l Kinetin, 0.6% Agar
RM_4	MS salts 3% sucrose, 2 mg/l BAP, 2 mg/l Kinetin, 0.6% Agar
RM_5	MS salts 3% sucrose, 2 mg/l BAP, 5 mg/l Kinetin, 0.6% Agar
RM_6	MS salts 3% sucrose, 1 mg/l IAA, 0.6% Agar
RM_7	MS salts 3% sucrose, 0.5 mg/l IAA, 1 mg/l Zeatin, 0.6% Agar
RM_8	MS salts 3% sucrose, 1 mg/l IAA, 1 mg/l Zeatin, 0.6% Agar
RM_9	MS salts 3% sucrose, 1.5 mg/l IAA, 1 mg/l Zeatin, 0.6% Agar
RM_{10}	MS salts and Vitamins, 3% sucrose, 2 mg/l IAA, 1 mg/l Zeatin, 0.6% Agar

Table 3. Callus induction (percentage) of four *Brassica napus* cultivars on different media combinations.

Callus induction media	Cultivars				Mean
	Dunkeld	Oscar	Rainbow	H-19	
CIM_1	28.70k*	39.81f	42.12e	38.42g	37.26C
CIM_2	49.53c	69.90a	55.09b	50.46b	45.85A
CIM_3	35.18h	44.90d	40.27f	32.87i	38.31B
CIM_4	26.28l	30.09j	31.94i	24.07m	28.10D
CIM_5	25.00l	30.55j	21.75n	19.90o	24.30E
Mean	32.89C	41.45A	38.23B	26.48D	

*The values in the Table having similar alphabets are not significantly different from one another at 0.05 probability level.

LSD at 0.05 probability level

Media combinations = 0.65, Cultivars = 0.58, Interaction = 1.31

Callus induction (%): The results varied with respect to treatments and the cultivars (Table 3). Among five callus induction media, CIM_2 (MS media supplemented with 0.5mg /l IAA, 1mg /l BAP, 0.5mg/l NAA and 1 mg /l Kinetin) was the most responsive medium with an average of 45.85% callus induction. It was significantly higher than all other media combinations. It was followed by the CIM3, CIM1, CIM_4 and CIM_5 with average of 38.31, 37.26, 28.10 and 24.30% callus induction respectively. As the concentration of IAA was increased further there was a decrease in callus induction. The minimum percentage of callus induction (24.30%) was recorded with CIM_5. Similar results were reported by Khan *et al.,* (2003). Callus induction in the *B. napus* genotypes is shown in Figs. 1 and 4.

Fig. 1. Callus induction in *Brassica napus* cv. Oscar.

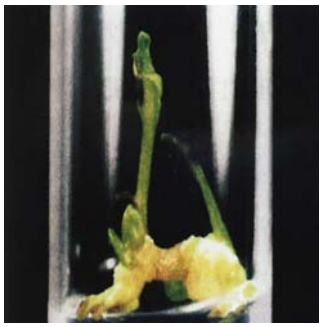

Fig. 2. Callus induction in *Brassica napus* cv. H-19.

Fig. 3. Callus induction in *Brassica napus* cv. Dunkeld.

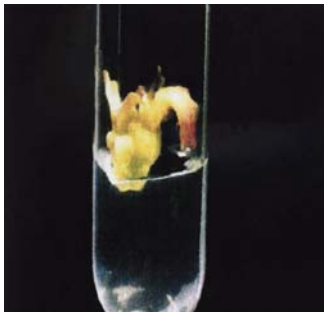

Fig. 4. Callus induction in *Brassica napus* cv. Rainbow

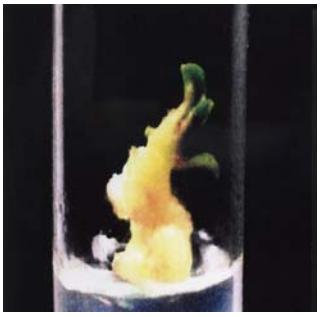

Fig. 5. Callus regeneration of *Brassica napus* cv. Dunkeld. Fig. 6. Callus regeneration of *Brassica napus* cv. Oscar.

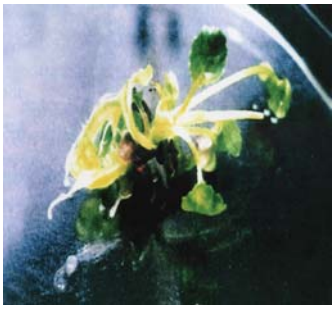

Fig. 7. Callus regeneration of *Brassica napus* cv. H-19.

Table 4. Callus regeneration (percentage) of four *Brassica* cultivars on different media combinations.

Regeneration media	Cultivars				Mean
	Dunkeld	Oscar	Rainbow	H-19	
RM_1	00.00o*	00.00o	00.00o	00.00o	0J
RM_2	20.83j	33.33f	16.66k	25.00h	24.20D
RM_3	41.66d	58.33b	33.33f	29.16g	40.62B
RM_4	25.00h	45.83d	25.00h	33.33f	34.54C
RM_5	16.66k	25.00h	16.66k	8.33m	16.66F
RM_6	0.00o	0.00o	0.00o	4.16n	1.04I
RM_7	16.66k	29.20g	20.83j	23.00i	22.43E
RM_8	37.50e	70.83a	45.83c	37.50e	50.17A
RM_9	8.30m	12.50l	4.20n	8.33m	8.33G
RM_{10}	4.20n	12.50l	0.00o	4.16l	5.21H
Mean	17.18B	29.65A	17.15B	17.29B	

*The values in the table having same alphabets are not significantly different from one another at 0.5 probability level.

LSD at 0.05 probability level

Media combinations = 0.254**,** Varieties = 0.160**,** Interaction = 0.507

The results also indicated that the Oscar produced the maximum average calli i.e., 41.45%, which was significantly higher than all other varieties used during the present experiment (Table 3). It was followed by H-19 and Dunkeld, which gave callus induction 38.23 and 32.89% respectively. The minimum callus induction of (26.48%) was observed in Rainbow. Similar results were reported by Lu *et al.,* (1997) who observed that different genotypes differed significantly in callus induction and regeneration. In case of interaction between varieties and media combinations, it was observed that Oscar gave the maximum callus induction (69.90%) on CIM_2, whereas on CM_1 and CM_4, H-19 gave the maximum callus induction (42.12 and 31.94% respectively). All the four cultivars gave the best results on CIM2. H-19 was the second best cultivar, which produced maximum calli (55.09%) on the same media combination.

Regeneration (%): The results are shown in Table 4. According to the results, all ten regeneration media combinations showed significantly different behavior in regeneration response except RM_1 (without growth hormone) and RM_6 (1 mg/l IAA only without cytokinin), therefore these two media combinations showed almost no regeneration. Maximum regeneration percentage was recorded with RM_8 containing MS plus 1 mg/l IAA and 1 mg/l Zeatin followed by RM_3 which contained (MS plus 2 mg/l BAP, 1 mg/l Kinetin). The best results were obtained when explants were cultured on a regeneration medium containing 1 mg Zeatin/l and 1 mg IAA/l which was the best media combination among all ten combinations used during the present studies. RM_9 (MS plus 2 mg/l IAA and 1 mg/l Zeatin) and RM_{10} (MS plus 1.5 mg/l IAA and 1 mg/l Zeatin) showed the poorest results because of higher concentration of auxins. In case cultivars, all the four cultivars showed their best results on RM_8. On this media combination Oscar showed maximum regeneration percentage i.e., 70.83%. While Rainbow showed minimum regeneration percentage i.e., 33.33% on this medium. The results revealed that there was no regeneration in all of the four varieties on the media devoid of growth hormones, and the media containing only auxin and no cytokinin. All the four varieties showed significant differences for their regeneration capacity; Oscar showed maximum mean value for regeneration capacity 29.65% (Table 4) and Rainbow showed minimum average

regeneration capability 17.15% (Table 4). These results are in close conformity to that of Ajay *et al*., (1997), who studied the effect of *Brassica* napus genotypes and explants source and culture medium growth regulation composition on callus formation and plantlet regeneration rates using two cultivars. Regeneration in the genotypes studied is shown in Figs. 5 and 7. During present study, it was revealed that the varieties varied significantly for their regeneration capacity on the similar medium. This might be due to their genetic differences.

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