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Effects of Various Growth Regulators on Callus Formation and Regeneration in *Brassica napus* Cv. Oscar

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Abstract: Hypocotyl explants from *in vitro* seedlings were cultured aseptically on Gamborg (B_5) medium supplemented with various concentrations and combinations of dichlorophenoxyacetic acid, indoleacetic acid, naphthalene acetic acid and benzylaminopurine for callus formation and shoot regeneration. 2,4-D alone @ 2 mg/l gave the highest callus formation (96%). The results revealed that BAP @ 2 mg/l with IAA 0.5 mg/l was the most appropriate combination to produce the multiple shoots (14.5 shoots per culture). Efficient rooting was achieved on half-strength MS medium supplemented with 0.250mg/l IBA and 0.125mg/l IAA. *In vitro* raised plantlets were transferred to potted soil and finally to experimental fields to assess their acclimatizing potential.

Key words: *Brassica napus* L., *in vitro*, explant, Gamborg medium, callus, regeneration

Introduction

The genus *Brassica* includes several very important crop species. *Brassica napus* is one the world's most important source of vegetable oil and protein-rich meal. Therefore, *Brassica napus* has become a model of extensive tissue culture studies and breeding. Genetic engineering techniques have been efficiently applied to *Brassica napus* for introducing new genes (Knutzon *et al*., 1992). Regeneration in *Brassica napus* is highly variable and genotype specific. A number of papers have reported the regeneration of shoots from seedlings or mature plant derived explants of *Brassica napus* (Dunwell, 1981). To date, organogenesis has been achieved in a variety of explants such as stem sections (Pua *et al*., 1991; Stringam, 1977), stem thin-cell layer (Klimaszewska and Keller, 1985), leaf discs (Dunwell, 1981), roots (Sharma and Thorpe, 1989), cotyledons (Moloney *et al*., 1989; Narasimhulu and Chopera, 1988) and hypocotyls (Dietert *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 (Riemenschiender *et al*., 1988). High frequency shoot regeneration has been reported from *Brassica napus* var. Westar and consequently this material has been used extensively for genetic transformation studies (DeBlock *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 consequence, 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 our work is to establish an efficient system for callus induction and shoot regeneration from hypocotyl explants in *B. napus* var. Oscar by exploiting specific combinations of growth regulators. Here we report the callus induction and shoot regeneration frequency of canola cv. Oscar.

Materials and Methods

Research work was conducted at National Agricultural Research Center during September 2001 to January 2002.

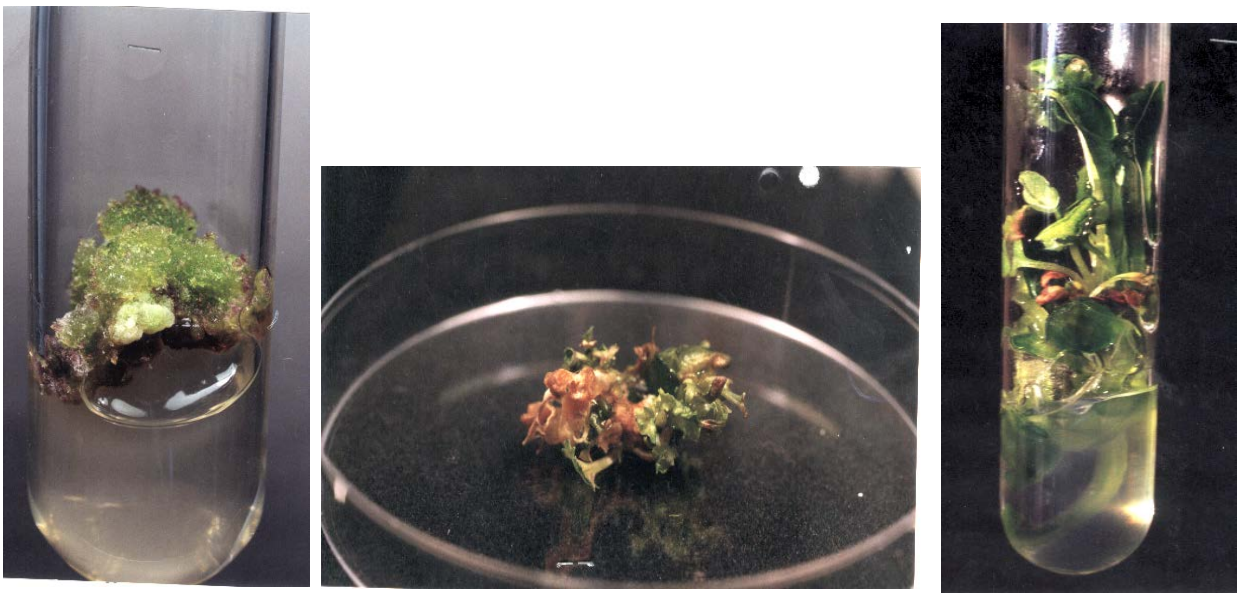
Seeds of *Brassica napus* var. Oscar were surface sterilized in a sequential manner with 70% ethanol for 1min, 0.1% mercuric chloride for 5min followed by treatment with sodium hypochlorite (2.5% active chlorine) for 5min and subsequently rinsed 4-5 times with sterilized water. Treated seeds were germinated aseptically on half-strength MS medium (Murashige and Skoog, 1962) without vitamins and 1.5% sucrose (physical conditions: 500 lux for 3 days followed by 2000 lux for 3-10 days, 16 hr light/8 hr dark cycle 23± 1 ℃). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121℃ for 20 min. For solidification gelrite of Sigma Chemical Co. was used @ 2 g/l.

Hypocotyls from 10-14 days old plants were cut in 5-10 mm segments and inoculated for callus induction in B_5 basal medium (Gamborg *et al*., 1968). The callus induction medium contained different levels of 2,4-D ranging from 0 to 8mg/l. Regenerative calli were transferred to shoot regeneration medium (B_5 salts and vitamins) supplemented with different combinations of BAP, IAA and NAA. For efficient rooting, regenerated plantlets were transferred to half-strength MS medium supplemented with 0.250 mg/l IBA and 0.125mg/l IAA. *In vitro* raised plantlets were transferred to potted soil and finally to experimental fields to assess their acclimatizing potential.

Results and Discussion

The explants exhibited an initial swelling followed by callus formation within two weeks of incubation. It was noted that callus proliferation started from cut ends of the hypocotyls on B_5 medium supplemented with 1.0 mg/l 2,4-D. Higher concentrations of 2,4-D inhibited callus proliferation and low concentrations allowed morphogenesis to occur, which affected both callus quantity and quality. Hence medium containing 2mg/l 2,4-D was found to be optimum for high callus induction frequency which was recorded 96%. Whereas callus induction frequency was lower (75-89%) at 2 and 4mg/l 2,4-D. Profuse callusing was observed when the concentration of 2,4-D was 2mg/l. Increasing 2,4-D level beyond 2 mg/l suppressed the callus induction as reported earlier by Murata and Orton (1987). Profuse callusing was also recorded at 4 mg/l of 2,4-D but callus was brown and non-embryogenic. No callus was observed at 0 and 8 mg/l of 2,4-D (Table 1). A growing unorganized collection of embryoids or embryoid-like structure, which proliferated from the hypocotyl sections, was recognized as embryogenic callus (E callus). This callus was compact, soft, nodular and greenish in color and was capable of proliferating embryoid or embryoid-like structures in subsequent sub-cultures (Fig. 1a). Embryogenic calli were separated from non-embryogenic calli and maintained in subsequent sub-cultures. Good embryoid production was observed during fourth and fifth week of sub-cultures in our study. 2,4-D @ 2 mg/l was chosen as the optimum level for callus proliferation and growth in sub-culture media. It was interesting to note that 2,4-D supplemented media sometimes induced shoot regeneration.

Hypocotyl explants-derived calli were placed on regenerating media containing various concentrations of growth regulators. The nodular structures developed into shoot buds when the embryogenic calli were sub-cultured in the medium supplemented with BAP alone or in combination with 0.5 mg/l NAA or IAA. It lashes that the presence of an auxin accelerated the regeneration process and of the two auxins, IAA was more effective. In higher



a b c

Fig. 1: Callus formation and plant regeneration from hypocotyl explants in canola. a) Embryogenic calli obtained from hypocotyl explant after 5 weeks of culture in B_5 medium supplemented with 2 mg/l of 2,4-D. b) Development of adventitious shoots from hypocotyl-derived calli in B_5 medium supplemented with 2 mg/l BAP and 0.5 mg/l IAA. c) Multiple shoot regeneration from hypocotyl-derived calli on the medium mentioned above in the b.

Table 1: Effects of 2,4-D concentrations on callus formation from hypocotyl explants of *Brassica napus* cv. Oscar on B_5 medium

2,4-D concentration (mg/l)	Days	Callus (%)	Remarks
0.0	20	0.00	No callus
	40	-Do-	-Do-
1.0	20	0.00	Callusing absent and explant dried up
	40	75.0± 3.2	Formation of off white callus in little amount
2.0	20	96.0± 5	Formation of green callus at the base
	40	-Do-	Profuse callusing followed by embryoid formation
4.0	20	89.4± 2.3	Formation of brown callus at the base
	40	-Do-	Profuse callusing
8.0	20	0.00	No callus
	40	-Do-	-Do-

± Represents standard error of the mean

Table 2: Effects of various cytokinin and auxin combinations on regeneration from hypocotyl explants of *Brassica napus* cv. Oscar after 30-40 days of culture B_5 medium

Plant growth regulators (mg/l)			Shoot	Number of shoots per	Root
BAP	NAA	IAA	%	culture	%
1	-	-	43	2.8± 0.6	-
2	-	-	59	5.7± 0.1	-
4	-	-	47	3.8± 0.2	-
1	0.5	-	57	4.5± 0.8	-
2	0.5	-	78	10.2± 1.4	10
4	0.5	-	49	8.9± 0.1	-
1	-	0.5	75	9.8± 0.5	12
2	-	0.5	84	14.5± 1.2	20
4	-	0.5	64	11.2± 1.3	20

± Represents standard error of mean

concentration 4 mg/l of BAP and low level 0.5 mg/l of NAA or IAA, only a few shoots proliferated inhibiting the elongation of other shoots. Shoot production efficiency was higher when the medium contained higher concentration of BAP (2-4 mg/l). Lower levels of hormones, had an adverse effect on shoot formation and process was extremely slow as earlier reported by Narasimhulu and Chopra (1988). The highest frequency of shoot regeneration (84%) and number of shoots per culture (14.5) was achieved on the medium with 2.0 mg/l BAP and 0.5 mg/l IAA (Fig. 1b, Table 2) using hypocotyl-derived calli. BAP alone at 2.0 mg/l induced shoot development in more than 50% cultures. At this concentration the hypocotyl-derived calli developed tiny patches of pale green callus bearing several hump-like structures within period of 20-30 days. Subsequently, these differentiated into shoot buds. These buds were somewhat irregular in appearance but became gradually developed into normal slender shoots measuring 4.5-5.5 cm. The developing shoots had normal and simple leaves (Fig. 1c).

Shoot production and plantlets formation could be increased when auxin was completely removed from the medium in later sub-cultures. Best shoot elongation was achieved when regenerating segments of the callus were cut into smaller pieces containing three or four shoots and sub-cultured in the basal medium supplemented with 0.1 mg/l BAP.

Shoots developing from callus were rooted easily in ½ strength MS medium with exogenous auxins at 0.250 mg/l IBA + 0.125 IAA mg/l. In some cases, BAP supplemented medium induced rooting even in the absence of NAA and IAA (Table 2).

It may be noted that among three auxins tested (2,4-D, IAA and NAA) 2,4-D was clearly the choice for callus induction and proliferation in *Brassica napus* var Oscar. This is probably due to 2,4-D is chemically much more stable, less rapidly inactivated than other instance, NAA and IAA (Jha and Roy, 1982). The growth rate of callus was found to be largely dependent upon the concentration of growth regulators and the type of explant used. In the present investigation, it was interesting to note that in *Brassica napus* profuse callusing and somewhat regeneration of

plantlets resulted in the presence of 2,4-D alone. According to Dodds and Roberts (1982), one unusual characteristic feature of 2,4-D is its ability to perform some extent the functions of both auxin and cytokinin. It is not known in what manner 2,4-D duplicates the function of cytokinin.

Plant regeneration through hypocotyl (Shi and Zhou, 1998; Phogat *et al.*, 2000) explant has been reported in canola. However the shoot multiplication rate in present research is much higher than those reported earlier. Under optimum culture condition 10-20 shoots were obtained from the primary established culture after fifth week of incubation, and the subsequent multiplication rate of adventitious shoots from shoot clumps was 4-5-fold per month sub-culture. The procedure presented here is simple, efficient and reproducible.

In conclusion, hypocotyl explants showed high frequencies of shoot regeneration under the influence of different auxins and cytokinin. We are currently attempting 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 the country in future.

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