African Journal of Biotechnology Vol. 6 (7), pp. 861-867, 2 April 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Effects of genotype, explant type and nutrient medium components on canola (*Brassica napus* L.) shoot *in vitro* organogenesis

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Accepted 5 March, 2007

The objective of the study was to develop an efficient method for shoot regeneration of canola (*Brassica napus* L.) and to compare the regeneration capacity of different explants on MS medium with several combinations of plant growth regulators. The experiments showed that the morphogenetical potential of canola depends on genotype, primary explant, hormonal structure and concentration of nutrient medium. Cotyledons possessed higher regeneration ability in comparison to hypocotyls and roots. The best regeneration capability was exhibited by the cultivar 'Quantum'. Its frequency with cotyledonary explants reached 68.8% on all used media. Addition of 3 mg/l ABA in nutrient medium considerably increased the regeneration frequency. The highest shoot regeneration (100%), however, took place when cotyledonary explants were cultivated on medium, containing 1.0 mg/l NAA, 8.0 mg/l BAP and 3.0 mg/l ABA. Precultivation of explants on callus induction medium did not affect the shoot regeneration frequency. Vitrification of regenerants was promoted by increasing the auxin NAA or cytokinin BAP, and ABA in the nutrient medium.

Key words: *Brassica napus*, shoot regeneration, cotyledonary explants, nutrient medium, seedlings, vitrification.

INTRODUCTION

Canola (*Brassica napus* L.) is an important oil crop grown in Canada, India, China, Europe and other regions of the world, and is ranked third in global production of oil crops (Kazan et al., 1999; Cardoza et al., 2003). In Iran, the area under cultivation of canola increases annually.

The increase in the transformation efficiencies is desirable in order to decrease the amount of resources needed to produce transgenic plants, and to potentially provide a higher baseline for subsequent transformation of other canola varieties. Two important factors governing the efficiency of transgenic plant recovery are obtaining healthy shoots that are not hyperhydrated and having a good rooting efficiency (Cardoza et al., 2003).

In the development of new forms of transgenic plants by genetic transformation methods, shoot regeneration frequency has a great value. Considerable progress has been accomplished in the cellular and molecular biology of *Brassica* species in the recent years. Plant regeneration has been increasingly optimized via organogenesis and somatic embryogenesis using various explants and by tissue culture improvements focusing on factors such as age of explant, genotype and media additives. In this study, we report an increase in the regeneration efficiency

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Table 1. Components of different nutrient media for organogenesis of canola.

Components (mg/l)	Media									
	No. 1	**No. 2**	**No. 3**	**No. 4**	**No. 5**	**No. 6**	**No. 7**	**No. 8**	**No. 9**	**No. 10**
Macrosalts Microsalts $MgSO_4.7H_2O$ $CaCl_2.2H_2O$ Iron chelate	½ MS	Based on MS								½ MS
Vitamins	-	Based on B_5								
BAP	-	-	4	4	4	8	4	0.5	6	-
NAA	-	2	-	1	2	1	2	-	0.6	-
Kinetin	-	4	-	-	-	-	-	-	-	-
2,4-D	-	0.1	-	-	-	-	-	-	-	-
$AgNO_3$	-	-	-	-	-	-	10	5	-	
IBA	-	-	-	-	-	-	-	-	-	0.5
Sucrose (g/l)	5	30	10	10	10	10	10	10	10	20
pH 5.7 – 5.8										
Agar (g/l)	7	6	6	6	6	6	7	7	6	7

of canola. Improved efficiency was achieved through altering the shoot regeneration ability of 5-day-old cotyledonary leaves, hypocotyles and roots of Iranian canola cultivars of 'Sarigol', 'Quantum' and 'Option 500' by manipulating the nutrient media components required for organogenesis.

MATERIALS AND METHODS

All experiments with the plant tissues were carried out *in vitro* on media based on MS (Murashige and Skoog, 1962) and vitamins based on B_5 medium (Gamborg et al., 1968), plus sucrose and plant growth regulators in different concentrations (Table 1). The media were solidified with 6 - 7 g/l agar (Bacto-agar "DIFCO". USA). For regeneration of canola, four variants of phytohormones BAP and NAA were used, with and without addition of abscisic acid (ABA). Canola seeds of cultivars 'Sarigol', 'Quantum' and 'Option 500' were rinsed 1 min with 96% ethanol, then surface sterilization was followed for 15 min with 0.5% sodium hypochlorite, and 0.2% Tween 20 was added as a surfactant. The seeds were thoroughly washed with sterile distilled water (3 - 5 times for 15 min). The disinfected seeds were germinated on MS medium No. 1 (Table 1). The cultures were grown for 5 days in growth chamber at 20 - 22°C, under a 16/8-h (light/dark) photoperiod with light supplied by cool-white daylight fluorescent lights.

Cotyledonary leaves with a small petiole, whose size did not exceed 2 - 5 mm, hypocotyl (5 - 10 mm) and root (2 - 5 mm) segments that were used as explant, were excised from 5-day-old seedlings. The explants were precultivated at 24°C in darkness on the MS medium No. 2. After 2 days, they were transferred into media No. 3 - 6 and were cultivated in growth chamber within 3 weeks at 20 - 22°C under a 16/8-h (light/dark) photoperiod.

The regenerated explants transferred to medium containing silver nitrate ($AgNO_3$) for shoot development and shoot elongation. The elongated shoots were cut out and transferred into rooting medium No. 10. The rooted plantlets were washed and transferred to the autoclaved soil in pots. The pots were covered with clear bags to provide 100% relative humidity. They were placed in an acclimatization room under a 16/8 h photoperiod at 20 - 23°C. After 2 weeks, acclimatizied plants were transferred to greenhouse and allowed to grow to maturity. They normally passed generative phase and produced seeds.

Regeneration frequency was calculated 21 days after precultivation as percentage of explants capable to shoot regeneration on the media No. 3 - 6. The data were analyzed by ANOVA (analysis of variance). In the tables, the means with a standard error were shown. The means were compared using the Duncan multiple comparison test at $P < 0.05$.

RESULTS

Estimation of *in vitro* morphogenesis

The morphogenesis potential of cotyledon, hypocotyl and root explants of canola cultivars was estimated. Occurrence of the first regenerated shoots was observed 13 - 18 days after precultivation, irrespective of the studied genotypes. The result established that for shoot regeneration of canola, cotyledons isolated from 5-day-old seedlings were the best explants (Table 2). Depending on genotype, the average morphogenesis frequency ranges from 27.1 to 35.9%. As 'Quantum' and 'Option 500' cultivars had the higher levels of morphogenesis, they were selected for transformation studies.

Table 2. Dependence of shoot regeneration frequency on genotype and primary explant (in %).

Genotype	Explant type			Mean of genotype ($S.E._{05} = 1.6$)
	Cotyledon	Hypocotyl	Root	
Sarigol	51.6	23.4	6.3	27.1
Quantum	68.8	31.3	7.8	35.9
Option 500	62.5	26.6	3.1	30.7
Mean of explant type ($S.E._{05} = 1.6$)	60.9	27.1	5.7	-

$S.\ E._{05}$ for individual differences = 2.9. Precultivation of explants for 2 days on medium No. 2 with subsequently followed by shoot regeneration. Values are average on all nutrient media.

Influence of nutrient medium components on *in vitro* shoot formation

The change of cytokinin to auxin ratio in a nutrient medium allows the induction of morphogenesis and obtaining the regeneration of shoots or roots, depended on the object of the experiment. The influence of various concentrations of phytohormones on the organogenesis was studied with the purpose of increasing its efficiency. Cotyledon, hypocotyl and root explants were used on media No. 3 - 6 (Table 3). The investigated parameters affected the phytohormonal component of nutrient medium, genotype, type of primary explant and the interaction of nutrient media, plant genotype and explant type.

For all explants and genotypes, the greatest frequency of shoot regeneration (39.6%) was observed on the medium No. 5, while the least (17.3%) was obtained on the medium No. 3. Media No. 5 and 6 had similar frequencies of shoot regeneration (37.5 and 39.6%, respectively). It is necessary to note that cotyledonary explants possessed high morphogenesis potential in comparison with hypocotyl and root segments on all used nutrient media. Regeneration efficiency of cotyledons was 60.9%, whereas that of hypocotyls was 27.1%, and that of roots was 5.7% (Table 3). Therefore, it is suggested that in the further experiments, cotyledons must be selected as primary explant.

Also from Table 3, cotyledons on the medium No. 6 containing the highest concentration of cytokinins (8 mg/l BAP) in combination with 1 mg/l NAA possessed the greatest regeneration frequency of shoots (85.4%). However, medium No 5 was the best nutrient medium for hypocotyls and roots (41.7 and 12.5%, respectively).

Influence of ABA on shoot formation

Addition of ABA to morphogenesis induction medium caused significant effects (at 5% level) on the shoot formation, and considerably increased the regeneration frequency in all investigated types of explants on all nutrient media. The highest shoot formation frequency (100%) was observed in the cultivation of cotyledons on the medium containing 8 mg/l BAP, 1 mg/l NAA and 3 mg/l ABA (Table 4).

Effect of precultivation

The influence of precultivation of canola explants was investigated for 2 days on media No. 2 and No. 5. From the given results in Table 5, it follows that although shoot regeneration frequency on the callus induction, medium No. 2 (50.4%) was better than the control on morphogenesis medium No. 5 (47.1%), in this parameter were not observed significantly differences at the 5 % level.

Influence of growth regulatores on vitrification of canola explants

In our research, we also observed vitrificant shoots (Table 6). Depending on hormonal concentration of medium, the average vitrification frequency ranges from 5.6 to 16.7%. The greatest frequency of vitrificant shoots was observed on the medium No. 5 containing the highest concentration of NAA (2 mg/l) in combination with 3 mg/l ABA.

Hardening of the plantlets and transfer to soil

The shoots obtained from cotyledonary explants on root formation medium (No. 10) formed powerful root system. The plantlets were potted up and hardened off by gradually decreasing the humidity. Plants were grown in the vegetative vessels in greenhouse. They were fertilized, passed generative phase normally and produced seeds (Figure 1).

DISCUSSION

Efficiency of plant regeneration is one of the main limiting conditions influencing frequency of genetic transformation.

Table 3. Dependence of shoot regeneration frequency of canola cultivars on phytohormonal components of nutrient medium, primary explant and genotype (in %).

Medium	Cultivar	Explant			Mean of medium and genotype ($S.E._{.05}$=3.3)	Mean of medium ($S.E._{.05}$=1.9)
		Cotyledon	Hypocotyl	Root		
3	Sarigol	31.2	6.3	-	12.5	17.3
	Quantum	43.7	18.8	-	20.8	
	Option 500	43.7	12.5	-	18.7	
	Mean of explant type and medium ($S.E._{.05}$=3.3)	39.5	12.5	0.00	-	
4	Sarigol	43.7	25.0	-	22.9	30.5
	Quantum	56.3	43.7	12.5	37.5	
	Option 500	62.5	31.2	-	31.2	
	Mean of explant type and medium ($S.E._{.05}$=3.3)	54.2	33.3	4.2	-	
5	Sarigol	56.2	43.7	12.5	37.5	39.6
	Quantum	75.0	43.8	18.7	45.8	
	Option 500	62.5	37.5	6.3	35.4	
	Mean of explant type and medium ($S.E._{.05}$=3.3)	64.6	41.7	12.5	-	
6	Sarigol	75.0	18.7	12.5	35.4	37.5
	Quantum	100	18.7	0.1	39.6	
	Option 500	81.3	25.0	6.2	37.5	
	Mean of explant type and medium ($S.E._{.05}$=3.3)	85.4	20.8	6.3	-	
Mean of explant type ($S.E._{.05}$=1.6)		60.9	27.1	5.7	-	-

S. $E._{.05}$ for individual differences = 5.7. Precultivation of explants for 2 days on medium No. 2 followed by shoot regeneration.

Table 4. Influence of ABA on shoot regeneration frequency of canola cultivars (in %).

Medium	ABA (mg/l)	Explant			Mean of medium and ABA ($S.E._{.05}$ = 2.7)	Mean of medium ($S.E._{.05}$ = 1.9)
		Cotyledon	Hypocotyl	Root		
3	-	25.0	8.3	-	11.1	17.3
	3	54.2	16.7	-	23.6	
4	-	41.7	29.2	-	23.6	30.5
	3	66.7	37.5	8.3	37.5	
5	-	58.3	29.2	12.5	33.3	39.6
	3	70.8	54.2	12.5	45.8	
6	-	70.8	16.7	-	29.2	37.5
	3	100	25.0	12.5	45.8	
Mean of explant type ($S.E._{.05}$ = 1.6)		60.9	27.1	5.7	-	-

S. $E._{.05}$ for individual differences = 4.7. Precultivation of explants for 2 days on medium No. 2 followed by shoot regeneration. Average values of the 3 cultivars are shown in the table.

The numerous factors influencing canola *in vitro* organogenesis includes the plant genotype, age of plant (donor of explant), explant type and nutrient medium components (Raldugina and Sobolkova, 1995; Cardoza et al., 2003;

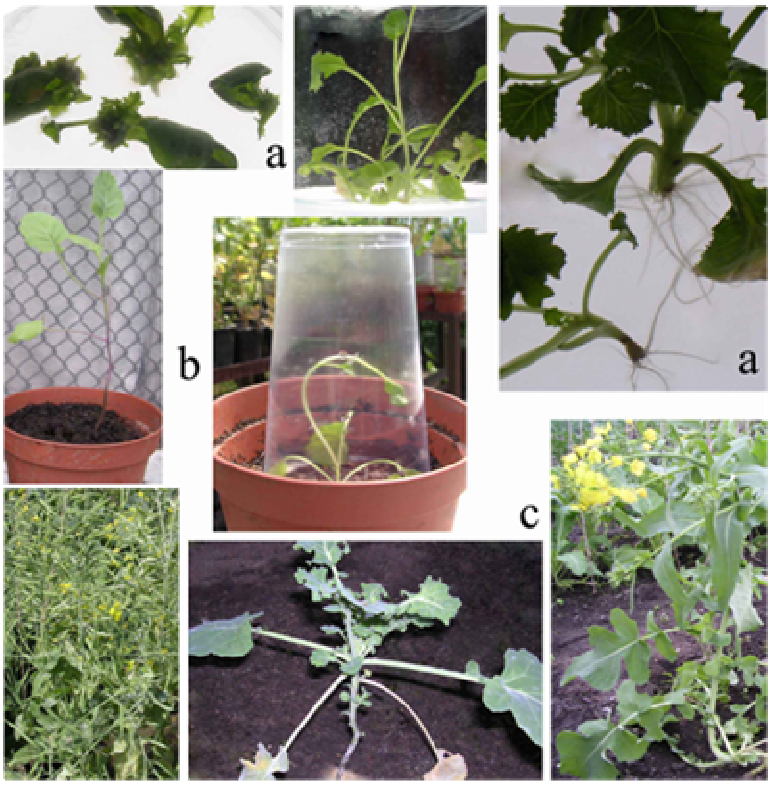

Figure 1. Development of regeneration and obtaining normal plants of canola (cultivar 'Quantum') from cotyledon isolated from 5-day-old seedlings: **a**) rooting of shoots on nutrient medium No. 10; **b**) potting up and hardening off the plantlets; and **c**) cultivation of canola plants in greenhouse and seed formation.

Table 5. Precultivation and comparison of shoot regeneration frequency in canola cultivar of 'Quantum' (in %).

Precultivation medium	Morphogenesis medium		Mean of media for precultivation ($S.E._{.05}$=1.77)
	No. 5	No. 9	
No. 2	54.2	46.6	50.4
No. 5	52.8	41.4	47.1
Mean of media for morphogenesis ($S.E._{.05}$=1.77)	53.5	44.0	-

$S.E._{.05}$ for individual differences = 2.5. Precultivation of explants for 2 days followed by morphogenesis.

Malishenko et al., 2003; Jonoubi et al., 2004, 2005; Halina et al., 2005; Reda et al., 2006; Wang et al., 2006). Therefore, the first step to develop an effective technique for plant regen-eration is to find an optimum combination of four factors above.

To choose the primary explant, it is necessary to study the organogenesis process. In the experiments, explants capable of regenerating only roots were not taken into account, because the roots possess low frequency of stem regeneration.

From comparison of the results obtained on media No 3 - 6, it is possible to conclude that the presence of 1 mg/l NAA in nutrient medium essentially increases the organogenesis efficiency. Besides, the results obtained on media No. 5 and 6 testify that high con-centration of both BAP and NAA simultaneously leads to increase in the quantity of regenerated explants.

These results indicate that canola organogenesis depends on the ratio and concentration of cytokinins and auxins in the nutrient medium as have been obse-

Table 6. Frequency of vitrificant shoots in cotyledonary explants of 'Quantum' cultivar (in %).

Medium	ABA (mg/l)	Vitrification (%)	Mean of medium ($S.E_{05}$ = 0.47)
3	-	5.6	6.0
	3	6.4	
4	-	6.3	6.9
	3	7.6	
5	-	13.9	14.8
	3	16.7	
6	-	10.8	12.1
	3	13.5	

S. $E_{.05}$ for individual distinctions = 0.66. Precultivation of explants for 2 days on medium No. 2 followed by morphogenesis.

rved in other studies (Raldugina and Sobolkova, 1995; Maisurian et al., 2005; Jonoubi et al., 2005; Reda et al., 2006). For example, medium No. 3 containing 4 mg/l BAP in the absence of auxins results in the least relative shoot regeneration frequency (17.3%), while presence of NAA together with 4 or 8 mg/l BAP in media No. 4 - 6 results in greater relative frequency of shoot regeneration.

As observed in Table 3, cotyledons on the medium No. 6 containing 1 mg/l NAA possessed the greatest regeneration frequency of shoots, while medium No. 5 containing 2 mg/l NAA was the best nutrient medium for hypocotyls and roots regeneration. It is known that cotyledons are capable of independently synthesizing auxins, but the content of this phytohormone decreases in plants from the top towards the stem base (Sparrow et al., 2004).

As mentioned, preliminary cultivation of explants on the callus induction medium considerably increases the ability of explants for stem formation (Halina et al., 2005). In addition, the study of precultivation was necessary for further joint cultivation of explants with *Agrobacterium* in transformation studies, which is usually carried out on the induction medium for callusogenesis. Based on the studies of Raldugina and Sobolkova (1995) on canola, the increase of precultivation time of cotyledonary explants increased shoot regeneration frequency. However, our results on precultivation of canola explants for 2 days on media No. 2 and No. 5 before proceeding to the morphoge-nesis media show-ed that although shoot regeneration frequency on the callus formation medium was more than the control on morphogenesis medium, this parameter was not significantly differences at the 5% level. The precultivation was limited to 2 days, since longer contact time of explants with *Agrobacterium* complicates their release from bacteria at the subsequent regeneration stages.

In the cultivation of plants *in vitro*, the vitrification phenomenon is often observed, which is found in the strong hyperhydrated leaves and stems. Thus, in plants, are formed leaves with abnormal morphology, with expanded basis of stalk. As a rule, such plants gradually perish. There is no common opinion about the reasons of this phenomenon. The factors responsible for vitrificant shoots formation includes high humidity in cultivation vessels due to enveloping them by parafilm or foil that entails sharp deterioration of gas exchange. Other factors are accumulation of ethylene and carbonic gas-rich nutrient media containing significant amounts of ammonium salts, sucrose and vitamins, reduced gel concentration and increase temperature, high doses of exogenous cytokinin and $AgNO_3$ and high CO_2 concentration (Curtis and Shetty, 1996; Lim et al., 1998; Popadin, 2002; Kadota and Niimi, 2003; Tisserat, 2005).

From comparison of the data with nutrient medium components, it is possible to conclude that the high content of auxin NAA or cytokinin BAP in the nutrient medium increases vitrification. By analyzing the influence of nutrient medium components on *Brassica oleracea*, Popadin (2002) reported that high content of NAA in the nutrient medium promoted vitrification. Kadota and Niimi (2003) also observed the stimulation of stem vitrification of pear due to high concentration of cytokinins in the nutrient medium. The same tendency was observed with addition of ABA in the medium. In the full absence of ABA, the frequency of vitrificant shoots was lower than on the medium with 3 mg/l ABA.

Finally, genotype, type of explant, components of nutrient medium (ratio of various concentrations of cytokinins and auxins in nutrient media, influence of ABA

on regeneration), and the interaction of these factors affected morphogenesis of canola.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support of the Agricultural Research and Education Organisation of the Islamic Republic of Iran and Department of Agricultural Biotechnology and the Molecular Biotechnology Center of the Russian State Agrarian University - MTAA after K. A.Timiriazev, Russia. We also thank Professor V. S. Sheveloukha, Professor E. A. Kalashnikova and Dr. G. N. Raldugina for assisting in this project as well as our laboratory colleagues for their support.

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