Research Article

Efficient in Vitro Plant Regeneration Through Somatic Embryogenesis from Callus Induction Method for *Brassica carinata*

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Abstract | A study was conducted for optimizing basal medium through somatic embryogenesis (cotyledon and hypocotyl) for callus induction followed by shoot and root regeneration of *Brassica carinata*. Seeds were sterilized and germinated, of which one week cotyledon and hypocotyl were used for callus formation and shoot regeneration at five different level of 1-naphthaleneacetic acid NAA (0.05, 0.1, 0.5, 1.0 and 1.5 mg/l) and 6-benzyl amino purine BAP (0.1, 0.5, 0.7, 1.0 and 1.5 mg/l), and root regeneration at four levels of indole butyric acid IBA (0.05, 0.1, 0.2 and 0.5 mg/l). Results showed that at NAA 0.05 mg/l + BAP 0.07 mg/l supplements in MS medium significantly effective in callus induction and days to callus in cotyledon and for both cotyledon and hypocotyl were observed. For shoot regeneration efficiency and days to shoots, the optimum phytohormone were observed at NAA 0.1 mg/l + BAP 1.0 mg/l along with $AgNO_3$ supplements. The optimum regeneration efficiency of root and days to root initiation was observed with 0.2 mg/l supplement. As cotyldeon and hypocotyl slightly varied in response to these phytohormones, but overall callus induction along with shoot and root regeneration both were equally susceptible to the present experiment applied phytohormones.

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Keywords | Somatic embryogenesis, *Brassica carinata*, NAA, BAP, IBA, Phytohormone, Hypocotyl, Cotyledon

Introduction

Ethiopian mustard (*Brassica carinata*) is an amphi-diploid species with its origin rising of interspecific hybridization between *Brassica oleracea* and *Brassica nigra*. *B. carinata* is famous for possessing many desirable traits like high rusticity and adaptability, strong resistance to disease and water scarcity, low pod dehiscence or delayed pod shattering, most studied plant physiology, extended crop residue and simple insertion in cereal rotation, drought and salt tolerance, and large seed size (Lazzeri and Avino, 2009; Rakow, 2004). Other advantages of the crops include cultivation on boundaries of agriculture fields as cover crops by suppressing diseases, nematodes and insects (Al-khatib and Boydston, 1999).

Studies have proven several factors such as genotype and various growth conditions (biotic and abiotic) that influence in vitro culture (Vincente and Dias, 1996). *Brassica carinata* seeds were known for containing high content of toxic compounds, erucic acid, but through various tissue culture techniques this toxic content has deceased within the present cultivars of *B. carinata*. Through tissue culture, there have been developed inbred lines with complete homozygosity, which have facilitated other biotechnological approaches like genetic engineering for production of new varieties for selection of stable resistant lines against biotic and abiotic stresses (Galli et al., 1998; Velasco et al.,

2004). Recent report declared *B. carinata* seed oil can be easily diverted for production of biodiesel and the toxic compounds derivatives can be used as chemical additives in tannery, cosmetic and plastic industries (Bozzini et al., 2007).

To overcome these hurdles in cultivating the crop by modern biotechnologies techniques such as somatic embryogenesis, a suitable protocol is necessary. Thus, for these reason, the present study was conducted for assessment of plant regeneration of *Brassica carinata* by optimizing basal medium for callus formation and regeneration of shoot and root.

Materials and Methods

Plant material and germination procedure

Brassica carinata seeds were obtained from the Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar. Experimental work was accomplished in laminar flow for prevention of contamination. First step included sterilization of seeds (Bano et al., 2010). In laminar flow, the media was poured into plates and left for solidification. Sterilized seeds when properly dried were transferred to the media and incubated under standard conditions (Figure 1).

Callus induction medium

Germinated plants hypocotyls and cotyledons of about one week were exercised under sterile condition (Liu et al., 2015). These explants of 0.5 to 1 cm were transferred to MS medium supplemented with various concentrations of 1-naphthaleneacetic acid (NAA) and 6-benzyl amino purine (BAP).

Shoot induction

The callus of various phytohormones formation was subcultured further on shoot induction media supplemented with similar concentrations of 1-naphthaleneacetic acid (NAA) and 6-benzyl amino purine (BAP) with addition of silver nitrate ($AgNO_3$) (Khan et al., 2010).

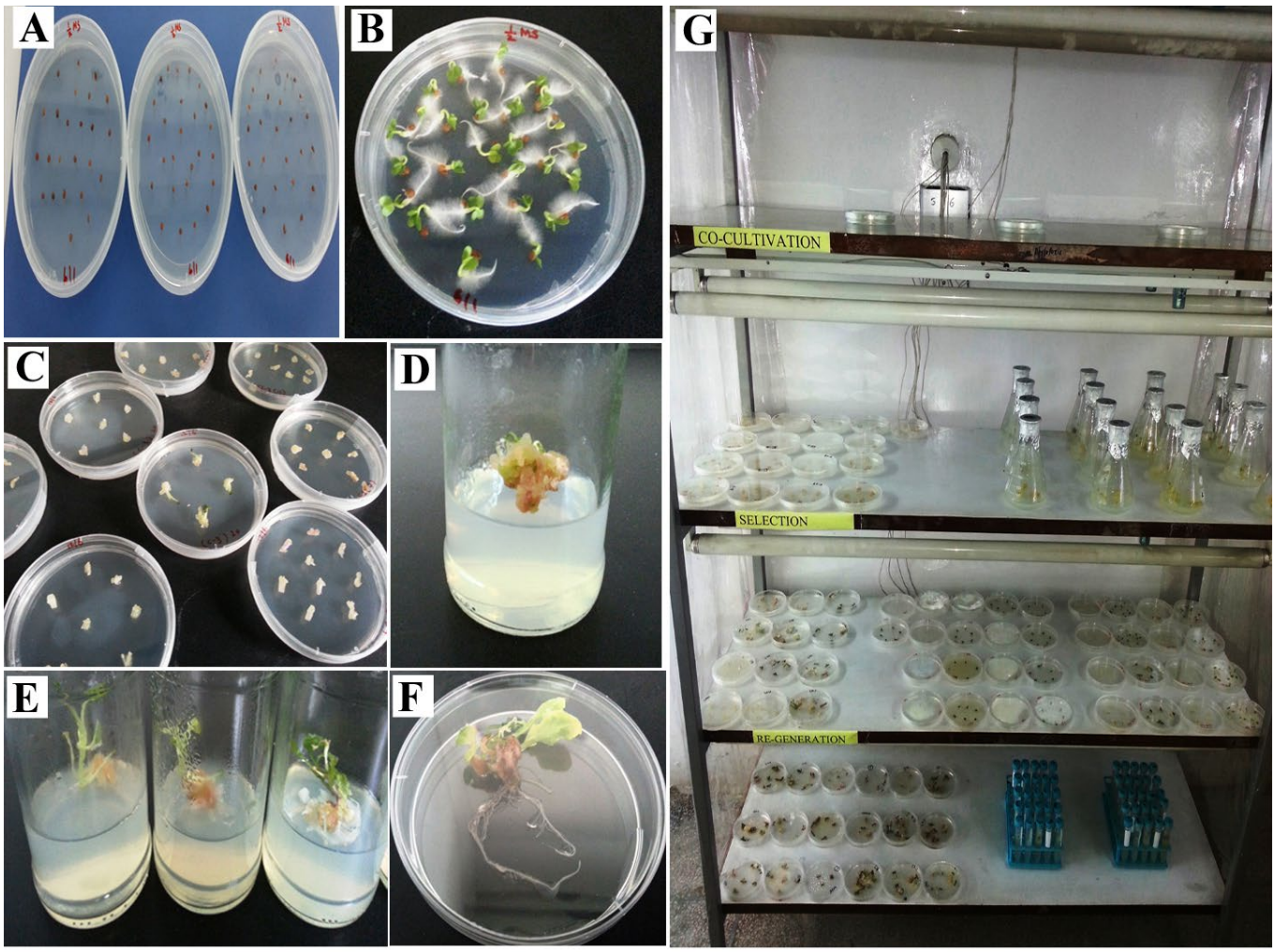

Figure 1: *Callus, Shoots and adventitius roots production by Brassica carinata cotyledon and hypocotyl;* **A:** *Half medium seed germination,* **B:** *Half medium seedlings (cotyledon and hypocotyl),* **C:** *Callus formation at various level of NAA and BAP,* **D** *and* **E:** *Regenaration efficiency of shoot,* **F:** *Regeneration efficiency of root.*

Root induction

Elongated shoots were transferred to root induction medium containing different level of indole butyric acid (IBA) (Ravanfar et al., 2009).

Results and Discussion

Percent of callus formation

Results of percent callus formation from cotyledon and hypocotyl of *B. carinata* by different level of NAA and BAP showed that maximum percent was observed with NAA 0.05 level and BAP 0.7 mg/l in both cotyledon (61.11%) and hypocotyl (88.89%). These results were followed by significant different percent of callus formation within NAA0.05 along with BAP 1.5mg/l in cotyledon (50.00%) whereas BAP 1mg/l in hypocotyl (55.56%). As the NAA level increase the percent of callus formation of cotyledon and hypocotyl dropped highly with first and last level of BAP, whereas the middle level of BAP showed fair percent of callus formation. The percent of callus formation of both cotyledon and hypocotyl showed significant variation with least significant difference (LSD) value of 6.84 at p<0.05 of cotyledon and 6.08 at p < 0.05 of hypocotyl (Table 1).

Days to callus formation

Mean days to callus formation was observed with NAA0.05 mg/l and BAP 0.7 mg/l both in cotyledon (21.67 days) and hypocotyl (23.00 days). These results were followed by higher level of NAA (0.1 mg/l) however similar level of BAP (0.7 mg/l) with 23.67 days that of cotyledon and 24.00 days of hypocotyl. The analyzed data showed significant variation with application of hormones level (NAA and BAP) where the LSD value of 0.78 at p < 0.05 was calculated for cotyledon and 0.67 at p < 0.05 for hypocotyl.

Regeneration efficiency of shoot

Regeneration efficiency of Shoot was observed maximum with NAA 0.1 mg/l and BAP 1mg/l in both cotyledon (72.22) and hypocotyl (83.33). A drastic decrease in regeneration efficiency of shoot was observed in all the other treatments level in the present study. In cotyledon NAA 0.5mg/l with BAP 0.7 mg/l and BAP 1mg/l showed fair regeneration efficiency of shoot (38.89). On the other hand, NAA 0.5mg/l with combination of BAP 1mg/l in hypocotyl showed fair regeneration efficiency (44.44). The LSD recorded for shoot regeneration efficiency in cotyledon and hypocotyl were 6.48 and 8.31 at p <0.05 (Table 2).

Table 1: *Effect of different level of phytohormones (NAA and BAP) on callus induction by cotyledon and hypocotyls of B. carinata.*

Different level of NAA/BAP		Cotyledon		Hypocotyl	
		Callus percent	Days to callus	Callus percent	Days to callus
NAA0.05	BAP0.1	0i	0.00n	5.56ef	36.00abc
	BAP0.5	27.77def	24.67l	33.33c	25.33kl
	BAP0.7	61.11a	21.67m	88.89a	23.00m
	BAP1	44.44bc	27.00jk	55.56b	27.33ij
	BAP1.5	50ab	27.67ijk	50.00b	29.33h
NAA0.1	BAP0.1	5.55hi	27.67ijk	5.56ef	28.67hi
	BAP0.5	22.22efg	26.33k	22.22cd	26.33jk
	BAP0.7	44.44bc	23.67l	61.11b	24.00lm
	BAP1	33.33cde	28.00ij	50.00b	27.67ij
	BAP1.5	16.66fgh	31.67gh	33.33c	33.00efg
NAA0.5	BAP0.1	5.55hi	34.33cde	5.56ef	33.33def
	BAP0.5	16.66fgh	28.67i	11.11def	28.33hi
	BAP0.7	38.88bcd	27.00jk	33.33c	29.33h
	BAP1	22.22efg	34.00def	11.11def	32.00fg
	BAP1.5	11.11ghi	32.67fg	5.56ef	33.33def
NAA1	BAP0.1	5.55hi	31.67gh	0.00f	0.00n
	BAP0.5	5.55hi	28.33ij	11.11def	27.33ij
	BAP0.7	22.22efg	26.33k	22.22cd	25.67k
	BAP1	16.66fgh	31.00h	16.67de	31.67g
	BAP1.5	5.55hi	35.67abc	0.00f	0.00n
NAA1.5	BAP0.1	5.55hi	36.33ab	0.00f	0.00n
	BAP0.5	5.55hi	33.00efg	11.11def	34.00de
	BAP0.7	11.11ghi	27.67ijk	22.22cd	27.33cd
	BAP1	11.11ghi	35.33bcd	0.00f	0.00n
	BAP1.5	5.55hi	37.00a	0.00f	0.00n
LSD		6.84	0.78	6.08	0.67

Means followed by different letter (s) are significantly different from each other (P < 0.05).

Days to shoot initiation

The days to shoot initiation was observed the least with NAA 0.1mg/l and BAP 1mg/l in cotyledon (25.67 days), which was followed by NAA 0.1/ BAP0.7 mg/l and NAA 0.5/BAP1 mg/l (27.67 days). As for hypocotyl, the least days to shoot initiation (25 days) was observed with similar levels of hormones as cotyledon least days to shoot initiation. The days to shoot initiation of cotyledon and hypocotyl to all the various levels of hormones had significant differences with LSD value of 0.81 and 0.75 at p < 0.05, respectively.

Regeneration efficiency of root

The regeneration efficiency of root response to

different levels of IBA from cotyledon callus formation showed the highest regeneration efficiency at IBA 0.2 mg/l (61.11) followed by IBA 0.5 mg/l (38.89). As for hypocotyl, the highest significant regeneration efficiency of root was observed at IBA 0.2 mg/l (66.67) which was followed by IBA 0.5 mg/l (44.44). Of the others combination that were IBA 0.5 and IBA 0.1 mg/l showed similar results in both cotyledon and hypocotyl (5.56 and 22.22). The results significantly varied with LSD value of 7.85 in cotyledon callus and 6.80 in hypocotyl callus at p <0.05 (Table 3).

Table 2: *Effect of different level of phytohormones (NAA and BAP) on regeneration of shoot by cotyledon and hypocotyls of B. carinata.*

Different level of NAA/BAP		Cotyledon		Hypocotyl	
		RES	DSI	RES	DSI
NAA0.05	BAP0.1	0.00g	0l	0.00f	0.00k
	BAP0.5	16.67def	35.33bc	5.56ef	34.33cd
	BAP0.7	22.22cde	31.33ghi	16.67cdef	30.00gh
	BAP1	33.33bc	28.33j	33.33bc	27.00i
	BAP1.5	16.67def	33.33de	16.67cdef	33.33de
NAA0.1	BAP0.1	5.56fg	32.67efg	5.56ef	32.67ef
	BAP0.5	22.22cde	30.00i	16.67cdef	30.00gh
	BAP0.7	27.78bcd	27.67j	33.33bc	26.67i
	BAP1	72.22a	25.67k	83.33a	25.00j
	BAP1.5	27.78bcd	30.33hi	27.78bcd	32.67ef
NAA0.5	BAP0.1	5.56fg	36.00ab	5.56ef	36.67ab
	BAP0.5	22.22cde	31.33ghi	11.11def	32.67ef
	BAP0.7	38.89b	32.67efg	33.33bc	31.33fg
	BAP1	38.89b	27.67j	44.44b	28.67h
	BAP1.5	11.11efg	31.67fgh	16.67cdef	33.33de
NAA1	BAP0.1	0.00g	0.00l	5.56ef	35.33bc
	BAP0.5	5.56fg	33.00ef	11.11def	32.33ef
	BAP0.7	16.67def	32.67efg	22.22cde	32.00ef
	BAP1	27.78bcd	30.67hi	27.78bcd	30.00gh
	BAP1.5	5.56fg	35.00c	0.00f	0.00k
NAA1.5	BAP0.1	0.00g	0.00l	5.56ef	37.33a
	BAP0.5	5.56fg	34.67cd	11.11def	33.33de
	BAP0.7	16.67def	30.67hi	22.22cde	30.00gh
	BAP1	22.22cde	35.00c	27.78cdef	34.67cd
	BAP1.5	5.56fg	37.00a	0.00f	0.00k
LSD		6.48	0.81	8.31	0.75

Means followed by different letter (s) are significantly different from each other (P < 0.05).

Days to root initiation

Results showed that the least days to root initiation which was observed at IBA 0.2 mg/l was recorded both in cotyledon (20.33 days) and hypocotyl (19.33 days). These results were followed by IBA 0.1 mg/l (25.00 days and 25.33 days) and IBA 0.5 mg/l (27.00 days and 26.67 days) in cotyledon and hypocotyl, respectively. The LSD recorded were 0.94 at p < 0.05 of both cotyledon and hypocotyl callus, respectively.

Table 3: *Effect of different level of IBA on regeneration of root by cotyledon and hypocotyls of B. carinata.*

Levels of IBA	Cotyledon		Hypocotyl	
	RER	DRI	RER	DRI
IBA 0.05	5.56c	29.00a	5.56d	28.67a
IBA 0.1	22.22bc	25.00b	22.22c	25.33b
IBA 0.2	61.11a	20.33c	66.67a	19.33c
IBA 0.5	38.89b	27.00ab	44.44b	26.67ab
LSD	7.85	0.94	6.80	0.94

Means followed by different letter (s) are significantly different from each other (P < 0.05).

In the present study, explant (cotyledon and hypocotyl) showed highest response of callus formation with NAA 0.1mg/l and BAP 0.7mg/l, whereas hypocotyl response higher than cotyledon regard to callus formation from explant as well as days to callus formation. Similar findings were determining by Ali et al. (2007), who studied effect of 2,4 D effect on callus induction of hypocotyl and cotyledonary leaves of B. napus. According to Cheng et al. (2001), high regeneration capacity is highly dependent up on tissue type which give variant output at various levels of treatments. The present study findings were also supported by Zeynali et al. (2010) and Khan et al. (2010). Zeynali et al. (2010) determine hypcotyl explants being more suitable than cotyledon for somatic embryogenesis. Khan et al. (2010) studied various *Brassica* genotypes callus induction at different level of NAA and BAP, along with $AgNO_3$. The results with NAA 0.5mg/l and BAP 1mg/l in the study were in line with the findings of Munir et al. (2008), who studied effect of different level of callus media and regeneration media supplement with *B. napus*.

The highest regeneration efficiency and days to shoot initiation from cotyledon and hypocotyl was observed with 0.1 mg/l NAA, 1mg/l BAP and 0.1 mg/l AgNO3, however overall results showed that cotyledonous callus had higher efficiency compare to hypocotyl callus in developing shoot. These results were in line with the finding of Bano et al. (2010), who evaluate

in vitro response of three genotypes of *Brassica juncea* callogenesis and organogenesis at different level of phytohormones. Moreover, the study also indicated that hypocotyls needed longer callus phase for shoot production and were produced with higher concentration of plant growth regulators (PGRs) of 3 mg/l BAP and 0.5 mg/l NAA. Neha and Ashutosh (2014) observed BAP 0.5-1.0 mg/l and NAA 0.5-1.0 mg/l showed highest number of shoots produce from callus. Similar results were also observed by Kamboj et al. (2015), who determine highly efficient and reproducible plant regeneration and transformation system in *Brassica juncea* genotypes. According to Tang et al. (2003) addition of $AgNO_3$ very benefits shoot regeneration but $Ag_2S_2O_3$ have shown superior effect over $AgNO_3$ when used in developing efficient regeneration protocol of *Brassica* species.

In the study, results showed that IBA 0.2 mg/l was efficient level in regenerating roots from callus of cotyledon and hypocotyl. These findings were in support of Ali et al. (2007), Singh et al. (2009), Ravanfar et al. (2009), Das et al. (2010) and Liu et al. (2015). Ali et al. (2007) and Ravanfar et al. (2009) showed that the highest efficiency regeneration of root was observed with IBA 0.3 mg/l, where others reported that at 0.5 mg/l IBA root regeneration from callus was efficient.

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Author's Contribution

Rizwan Ullah Shah: Principal Author who conducted study and research. Analyzed the data.
Iqbal Munir: Major Supervisor who perceived the study. Analyzed the data and wrote final draft of the manuscript.

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