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GENETICS AND PLANT BREEDING

In Vitro Regeneration Studies in *Brassica Napus* with Response to Callus Induction Frequency and Regeneration Frequency

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Abstract

The present study entitled "*In vitro* Regeneration Studies in *Brassica napus*, variety GSL- 1 and DGS-1 was carried out in the Biotechnology Laboratory, Division of Genetics and Plant Breeding SKUAST-J. *Brassica napus*, variety GSL 1 and DGS-1 were cultured on MS Media supplemented with different concentrations of growth regulators. The sterilization treatment with $HgCl_2$ (0.1%) for 3 minutes resulted in highest per cent of aseptic seeds and survival of seedlings. Highest callus induction was observed in GSL-1. MS medium augmented with 2,4-D at 2-2.5 mg/l. Different concentrations of growth regulators in media showed a significant difference in the callus induction frequency (CIF) and regeneration frequency (RF) for both the genotypes tested. MS medium supplemented with BAP (5.0 mg/l) and 2, 4-D (0.5 mg/l) showed highest shoot regeneration frequency in GSL-1.

Highlights

- Seed Sterilisation with $HgCl_2$ (0.1%) for three minutes resulted (100%) aseptic callus.
- Highest callus induction frequency was observed in GSL-1.
- MS medium supplemented with BAP (5.0 mg/l) and 2, 4-D (0.5 mg/l) showed highest shoot regeneration frequency in GSL-1.

Keywords: *In vitro*, *Brassica*, callus induction frequency, regeneration frequency

Brassica is economically most important genus in the Brassicaceae family (syn. Cruciferae). Brassica species are widely used in the cuisine of many cultures and recognized as a valuable source of dietary fibre. Brassica vegetables contain little fat, and are sources of vitamins, minerals, and fibre. They also contain a large number of novel phytochemicals, some of which are anti-carcinogenic (Steinmetz and Potter, 1996). Among the three cultivated species, *Brassica napus* (AACC 2n=38) is the most important oleiferous crop and it is gaining importance because of its high yield potential of 20 q/ha (Anonymous, 1991), wide adaptability and high oil content (44.6 %) of good quality Rai *et al.* (2007).

Brassicas are the third most important oilseed crop in the world after soybean and palm. China, India, Canada, Japan and Germany are the major rapeseed-mustard growing countries (Prajapat *et al.*, 2015). India is the third largest rapeseed-mustard producer in the world after China and Canada with 12 per cent of world's total production. India holds a premier position in rapeseed-mustard economy of the world with 2nd and 3rd rank in area and production respectively. This crop accounts for nearly one-third of the oil produced in India making it country's key edible oilseed crop. Due to the gap between domestic availability and actual consumption of edible oils,

In order to diversify the varietal profile of this

crop, development of diverse cultivars with high yielding ability, high oil content and early maturity is thus necessitated. The use of such varieties would enable the farmers to achieve high production and consequently change the edible oil seed scenario of the state. Genetic variation in cruciferae 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 plant through conventional breeding methods is slow, time consuming and labour intensive. Non-conventional genetic improvement programmes based on tissue culture and molecular genetics are essential complement to standard breeding Lichtenstein and Draper (1985).

Conventional breeding approaches can be done to improve the new trait within the species. But Conventional breeding programmes alone were not successful enough in Brassica due to high degree of segregation (the separation of allelic genes that occurs typically during meiosis) upon cross-pollination and unavailability of suitable wild germplasm Enrichment of genetic variability through mutation, somaclonal variation, and protoplast fusion contributed only a little in the production of disease and pest resistant plants to overcome incompatibility barriers as well as plants with better agronomic characters in Brassica spp. In this regard, in vitro regeneration and transformation have prospects to fulfill breeding needs (Khan *et al.*, 2010).

Plant tissue culture technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites. Small pieces of tissue (explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis. Endangered, threatened and rare species have successfully been grown and conserved by micro propagation because of high coefficient of multiplication and small demands on number of initial plants and space (Hussain *et al.*, 2012).

Substantial research has been conducted in tissue culture, transformation and molecular breeding of the Brassicas. Transformation in Brassica has been reviewed by (Poulson 1996), Earle *et al.* (1996), Earle and Knauf (1999). *In vitro* regeneration and transgenic plant selection efficiency are two important elements in molecular breeding Cardoza and Stewart (2003). Therefore the current study was conducted to standardise optimal concentrations and combinations of plant growth regulators in the medium for obtaining *in vitro* callus induction and shoot regeneration in *Brassica napus*.

Materials and Methods

The present study was carried out at Biotechnology laboratory, Division of Genetics and Plant Breeding, Sher-e-Kashmir University of Agricultural Sciences and Technology, Faculty of Agriculture, Chatha, Jammu. The certified seeds of cultivated species namely *Brassica napus*, were procured from the Division of Genetics and Plant Breeding , FOA, Chatha for the present investigation. Before preparation of media, glassware was washed with laboratory detergent, (Teepol), rinsed several times with tap water and, finally with purified water. The washed glassware was kept stored in drying oven at temperature of 150 ^{o}C for twenty minutes to use for tissue culture purposes. The MS basal media in readymade form was procured from Hi media labs. Mumbai for the present study. The media was used at the rate 4.41 mg/l and supplemented with sucrose (3%). The pH of the media was adjusted at 5.8 using 1 N NaOH and 1 N HCL.

It was solidified with agar (0.8%) after dissolving the agar, the media was poured in the culture vessels (test tubes, conical flasks) of different sizes and autoclaved at 15 psi for 20 minutes. This media was used for germination of sterilized seeds of different *Brassica* species. The stock solutions for growth regulators were prepared in distilled water and stored in refrigerator at 4 ^{o}C . The growth hormones were added to the media before autoclaving and finally media was used for callus induction. The growing calli from the established cultures were separated from the initial explants and sub cultured on to fresh medium. Visual observations with respect to growth, type and colour of the callus were recorded. On the basis of these observations, the callus was again sub cultured onto the medium

giving the best results for further multiplication. The stock solution of growth regulators were prepared in the distilled water and stored in refrigerator at 4 °C the growth hormones were added to the media before autoclaving and this media was used for organogenesis. To optimize the culture medium for shoot regeneration, the calli were divided into small pieces and cultured on modified MS medium supplemented with various combinations and concentration of growth regulators *viz*. BAP and NAA. The regenerated shoots were then multiplied on the medium showing best shoot multiplication. All the subsequent culture operations were conducted under strict aseptic conditions in a laminar air flow cabinet, fitted with UV light. The inoculation chamber and surgical instruments etc. were thoroughly wiped with ethanol (70 %) prior to use and UV light was kept on for 20 minutes. Routine precautions were followed to maintain sterile conditions inside the cabinet. All metal instruments and other accessories were wrapped in aluminum foil and sterilized by autoclaving at 15 psi for 20 minutes. Tips of scalpel and forceps were kept sterilized by periodically dipping them in ethanol and air drying at the time if inoculation. Subsequently the cultures were incubated in the culture room at 25 ± 2 °C under 16 hours photoperiod regime and about 2500 lux light intensity.

The seeds of *Brassica napus* were raised under *in vitro* conditions. For this purpose, seeds were thoroughly washed under tap water, the seeds were then treated with Bavastin at the rate (2 mg/l) and 2 to 3 drops of tween 20 .The treatment was given by constant shaking of the glass jar for 10 – 20 minutes. The seeds were washed in running tap water to remove traces of Bavastin and tween 20. Inside the Laminar air flow chamber seeds were surface sterilized by treatment with 0.1 per cent mercuric chloride ($HgCl_2$) for 3-5 minutes. The seeds were finally washed 3-4 times with sterilized distilled water in a laminar air flow cabinet, and inoculated on Murashige and Skoog (1962) full strength basal medium containing 3 per cent sucrose with pH 5.8. Twenty days old seedlings were used as a source of explants. The cotyledon sections measuring 0.5 to 1.0 cm of *in vitro* grown seedlings were used as explants. They were excised in small sections with sterilized blades and used to inoculation. The cultures were kept in dark chamber with the temperature at 25 ± 2 °C for establishment of callus cultures. The establishment of cultures was done by inoculating the cotyledon sections of *In vitro* grown seedlings on modified MS medium supplemented with various combinations of growth regulators (2,4-D). The cultures were then incubated for callus initiation and the days taken for proliferation were recorded. For organogenesis the callus were kept in light with photoperiod of 16 hours light and 8 hours dark with the light intensity of 2400 lux. to optimize the culture medium for shoot regeneration, the calli were divided into small pieces and cultured on modified MS medium supplemented with various combinations and concentration of growth regulators *viz*. BAP, NAA and 2,4-D. For every combination, 8 flasks with 5 pieces of callus were inoculated. The regenerated shoots were then multiplied on the medium showing best shoot multiplication

The relative response of *Brassica napus* for callusing and regeneration was observed for the following parameters as per the formulae followed by Moghaieb *et al.* (2006).

$$CIF = \frac{\text{No. of calli produced explant}}{\text{Total no. of explants in the culture}} \times 100$$

$$RF = \frac{\text{No. of shoot initiated in the culture}}{\text{Total no. of calli used}} \times 100$$

Results and Discussion

The present study was conducted to standardise optimal concentrations and combinations of plant growth regulators in the medium for obtaining *in vitro* callus induction and shoot regeneration in *Brassica napus*. The results obtained from the present study are presented below:

Surface sterilization

The seeds when surface sterilized with $HgCl_2$ (0.1%) for three minutes resulted into 100 per cent aseptic callus. Maximum survival of seedlings was recorded in variety GSL-1 after four weeks in this treatment while lowest per cent of aseptic seed cultures and seedling survival was observed when seeds were surface sterilized in $HgCl_2$(0.1%) for one minute duration after one and four weeks after treatment. These findings are in conformity with the findings of

Bajaj and Nietesch (1975), Singh *et al.* (1981), Dieter (1982), Kamal *et al.* (2007) and Moghaieb *et al.* (2006) who reported that seed explants of Brassicas can be surface sterilised by using different sterilents such as 0.5% and 0.1% Hg cl_2, 0.5% sodium hypochlorite or 70% ethanol. Dubey and Gupta (2014) also reported that Surface Sterilization - Combination of 70% Alcohol + 0.1% $HgCl_2$ for 5 minutes was recorded as best surface sterilization with lowest rate of contamination.

For initiation of callus cultures, cotyledons of *Brassica napus* (DGS I and GSL I) were cultured on MS media supplemented with 2,4-D and NAA at different concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 mg/l). It was observed that callus proliferation started from the cut ends of cotyledon sections on MS media supplemented with 2,4-D at 2.0 and 2.5 mg/l. Very high response of callus induction was observed in *B. napus* inoculated on MS medium supplemented with 2,4-D, 2.0 and 2.5 mg/l. Moderate response was observed at 2,4-D 1.5 mg/l while as no response was found at 0.5 and 1.0 mg/l of 2,4 –D (Table 1). Ullah *et al.* (2004) reported that the concentration of 1.5 mg/ L 2,4-D would be adequate for callus induction from hypocotyle segments of *Brassica napus* on MS medium. Vyvadilova and Zelenkova (1987) also observed that best callus induction from auxiliary and terminal meristems, leaves, hypocotyles, cotyledons and roots was on MS medium supplemented with 0.5 to 2.0 mg/l 2,4-D. Khan *et al.* (2010) reported that among the different explants used, cotyledonary leaf with petiole and hypocotyl were found to be the most responsive in terms of percentage of shoot regeneration as well as the number of shoots per explant in all the varieties tested. Mollika *et al.* (2011) also investigated that a considerable variation in shoot regeneration from cotyledon explants was observed in all the varieties used.

Among the three varieties used, *Brassica juncea* var. BARI Sarisha-11 was most responsive. It was also noticed that *Brassica juncea* showed higher response towards *in vitro* regeneration than that of *Brassica campestris* (Tori-7). It showed maximum percentage of response in terms of shoot regeneration as well as highest number of shoots per explants. Sharma *et al.* (2014) showed positive results on callus formation and initiation of shoot regeneration from broccoli hypocotyl explants.

Effect of growth regulators on Callus Induction Frequency (CIF) and Regeneration Frequency (RF) of Brassica napus

Cotyledonary explant sections exhibited on initial swelling followed by callus formation within two weeks of incubation in MS media supplemented with different concentrations (1.5, 2.0 and 2.5 mg/l of 2,4-D). Data given in Table 2 showed that in GSL 1maximum callus induction frequency of 85.71 per cent was obtained in media containing 2.5 mg/l of 2,4-D, followed by 71.43 and 42.86 per cent, at 2.0 and 1.5 mg/l of 2,4-D, respectively. However, in case of DGS 1 maximum callus induction frequency of 85.71 per cent was observed at 2.0 and 2.5 mg/l of 2,4- D, followed by 57.14 per cent at 1.5 mg/l. No significant differences were observed among Gobhi sarson cvs. DGS 1 and GSL 1 with respect to callus induction frequency at 5 per cent level of significance. This matches with the findings of Khan *et al.* (2002) who reported 2 mg/l as best calli producing concentration using only 2,4-D for the callus induction in *Brassica napus* L. Cultivar Oscar also found no significant difference between the different explants of a cultivar and among the cultivars for callus induction ($P<0.05$). the cotyledonary explants generally gave calli with more mean weight and mean length the same results were obtained by Zhang and Bhalla (1999).

Calli induced were transferred to regeneration media containing various concentrations of growth regulators i.e MS+ 2,4-D (0.5mg/l) + BAP (1, 2, 3, 4 and 5 mg/l). Results depicted in Table 3 showed that the cultivars differed in the number of regenerated plants. Maximum of 100 per cent regeneration frequency was obtained in both GSL 1 and DGS 1 on the media containing 0.5 mg/l 2,4-D and 5.0 mg/l BAP. However, minimum regeneration frequency of 50 per cent was recorded in GSL 1 and 66.67 per cent in DGS 01 at 3.0 mg/l BAP. Both the cultivars showed no shoot regeneration in the media containing 1.0 and 2.0 mg/l BAP. No significant difference was observed among the varieties with respect to regeneration frequency (Plate 1 and Plate 2).This was also matching with the findings of Sharma and Srivastava (2014) they reported that the highest regeneration efficiency was obtained on MS basal medium containing combinations of BA-IAA and Kn-IAA. The best response in terms of the percentage of shoot regeneration (77%) was

obtained from petiole explant cultured on MS basal medium supplemented with 1.0 mg/l Kn + 1.0 mg/l IAA.

Table 1: Effect of different concentrations of growth regulatorson callus induction for two varieties of *Brassica napus*

Medium	Concentration (mg/l)	*Brassica napus*	
		GSL 1	DGS 1
MS+2,4-D	0.5	-	-
	1	-	-
	1.5	+	+
	2	++	+
	2.5	++	++
MS+NAA	0.5	-	-
	1	-	-
	1.5	-	-
	2	-	-
	2.5	-	-

_ = No response, += Moderate response, ++= High response, +++ = very high response

Table 2: Effect of different concentration of growth regulators on Callus induction frequency of *Brassica napus*

Genotype	Concentration of MS+ 2,4-D(mg/l)	No. of cultured explants	Callus inducing explants	CIF (%)
GSL I	0.5	14	0.00	0.00 (1.00)
	1.0	14	0.00	0.00 (1.00)
	1.5	14	6.00	42.86 (6.56)
	2.0	14	10.00	71.43 (8.42)
	2.5	14	12.00	85.71 (9.24)
DGS I	0.5	14	0.00	0.00 (1.00)
	1.0	14	0.00	0.00 (1.00)
	1.5	14	8.00	57.14 (7.59)
	2.0	14	12.00	85.71 (9.29)
	2.5	14	12.00	85.71 (9.29)
C.D. (p=0.05)				1.05
S.E. m (±)				0.36
C.V.				16.03

Figures in parenthesis represent square root transformed values.

Table 3: Regeneration frequency of *Brassica napus* at different concentration of Growth Regulators

Geno-type	Conc. of MS+ 2,4-D+BAP (mg/l)	No. of calli used	No. of shoots regene-rated	No. of regene-rated plants	RF (%)
GSL I	0.5+1.0	12	0	0	0.00 (1.00)
	0.5+2.0	12	4	2	33.33 (5.74)
	0.5+3.0	12	6	4	50.00 (7.08)
	0.5+4.0	12	10	8	83.33 (9.08)
	0.5+5.0	12	12	12	100.00 (9.97)
DGS-I	0.5+1.0	12	0	0	0.00 (1.00)
	0.5+2.0	12	0	0	0.00 (1.00)
	0.5+3.0	12	8	6	66.67 (8.14)
	0.5+4.0	12	10	10	83.33 (9.15)
	0.5+5.0	12	12	12	100.00 (10.05)
C.D. (p=0.05)					1.24
S.E. m (±)					0.42
C.V.					16.54

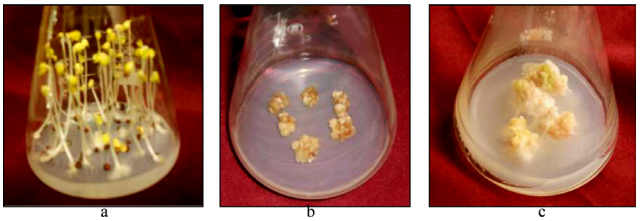
Figures in parenthesis represent square root transformed values.

a b c

Plate - 1

Brassica napus, variety GSL 1

a) = seed germination, b) = callus induction, c) = organogenesis (shoot proliferation)



a b c

Plate - 2

Brassica napus, variety DGS 1

a) = seed germination, b) = callus induction, c) = organogenesis (shoot proliferation)

Conclusion

The seeds when surface sterilized with $HgCl_2$ (0.1%) for three minutes resulted in 100 per cent aseptic callus. Maximum survival of seedlings was recorded in variety GSL-1. Callus proliferation was initiated from the cut ends of cotyledon sections on MS media supplemented with 2,4-D at 2.0 and 2.5 mg/l. High response of callus induction was observed in *B. napus* cultured on MS medium supplemented with 2,4-D, 2.0 and 2.5 mg/l. Moderate response was observed at 2,4-D 1.5 mg/l while as no response was found at 0.5 and 1.0 mg/l of 2,4 –D. In GSL 1maximum callus induction frequency of 85.71 per cent was obtained in media containing 2.5 mg/l of 2,4-D, followed by 71.43 and 42.86 per cent, at 2.0 and 1.5 mg/l of 2,4-D, respectively. However, in case of DGS 1 maximum callus induction frequency of 85.71 per cent was observed at 2.0 and 2.5 mg/l of 2,4- D, followed by 57.14 per cent at 1.5 mg/l.

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