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Efficient Callus Induction through Anther Culture in Cultivars of *Brassica campestris* var. Brown Sarson

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A B S T R A C T

In the present study, anther of three varieties and their cross combinations of *Brassica campestris*, namely HPBS-1, KBS-3, HPKM-04-1, HPBS-1 x HPKM-04-1 and KBS-3 x HPKM-04-1 were cultured in vitro to observe their callus induction frequency. The effect of two basal media i.e. MS and N6, two different sucrose concentrations i.e. 3% and 4%, three combinations of growth hormones viz. HM1, HM2, HM3 and their callus induction frequency were analyzed by CPCS software. Out of all factors and their interactions, the genotype HPBS-1 performed better in N6 medium with HM2 [0.5 mg/l 2, 4-D + 1.0 mg/l NAA] and 3 per cent sucrose for callus induction frequency. This media and hormonal combination can be successfully utilized for induction of haploids and double haploids in future.

Introduction

Oilseed crops occupy an important place in world's economy. The cultivation of oilseeds (*Brassica* sp.) has increased tremendously from last few years and occupies a prominent position in daily diet, being a rich source of fats and vitamins. *Brassica napus, Brassica juncea* and *Brassica campestris* constitute the important source of edible oils (Gupta and Partap, 2007). Among oilseeds, rapeseed-mustard contributes 28.6% in the total oilseeds production and ranks second after groundnut sharing 27.8% in the India's oilseed economy (Shekhawat *et al.*, 2014).

Conventional methods for breeding crop plants require more than six to seven years of continuous efforts to get true breeding lines after following hybridization approach. However, the anther culture technique holds a great promise in accelerating the pace of breeding programme (Guha and Maheshwari, 1964). This system provides an unparallel opportunity to shorten the breeding cycle and fix agronomic traits in homozygous state instantly. The information pertaining to these parameters in brown sarson is limited.

Therefore, to harvest the multifarious merits of anther culture, the present research work was planned and carried out. Main objectives were establishment of a suitable and reproducible protocol for *in vitro* regeneration of callus through anther culture, optimization of the suitable combination and concentration

of hormones on selected media for regeneration of *Brassica* genotypes.

Materials and Methods

The experiment was carried out in the Molecular Cytogenetics and Tissue culture Laboratory of Department of Crop Improvement, CSK HPKV, Palampur during *rabi* 2016-17. The material used for anther studies comprised of three elite genotypes and their cross combination (Table 1).

Methods

Plant material for anther culture

Sufficient numbers of plants of aforementioned three genotypes and their cross combinations were raised in the pots. In order to have availability of anthers over a long period of time, plants were raised in five lots at an interval of 15 days each.

Media used for anther culture

Two basal media *viz.*, MS (Murashige and Skoog, 1962) and N_6 (Chu, 1978) were used for callus induction. Each of these medium was supplemented with two different sucrose concentrations *i.e.* 3 per cent and 4 per cent sucrose and each of these sucrose concentrated media was also supplemented with three combinations of hormones *viz.*, HM_1, HM_2 and HM_3 (Table 2). All the media were supplemented with 0.8 per cent agar based upon the earlier studies (Kumari, 2010).

Anther culture technique

For anther culture, florets from plants were clipped off when the size of bud was about 2-4 mm. The bud size was earlier established on the basis of presence of majority of the microspores at late uninoculate to early binucleate stage as studied by squashing of anthers in a drop of 1 per cent acetocarmine. The florets of appropriate size were collected in 50 ml test tubes containing distilled water.

The florets collected at aforementioned stages were treated with 70 per cent ethanol for 10-15 seconds under aseptic conditions in a laminar air flow chamber. The florets were then surface sterilized with 0.1 per cent $HgCl_2$ for 3-5 minutes with intermittent shaking followed by three washings with sterile distilled water. Florets were blot dried and opened under aseptic conditions with the help of sterile forceps and the six anthers were clipped off from each floret without damaging the anther wall. About 60 anthers were cultured in each pre-sterilized petri plate containing about 25 ml of culture medium. The experiments on different callus induction media were replicated thrice involving different media, sucrose concentrations and plant growth hormones. Anthers of all three genotypes and their crosses were plated in a replicated fashion. All the cultured plates were sealed with parafilm wax and kept under dark at 25 ± 1°C until calli were developed.

Data analysis

The experimental data was analysed in Factorial Completely Randomized Design (CRD) using statistical CPCS software to determine the effect of various genotypes, hormones, media, sucrose and their interactions on callus induction frequency.

Statistical analysis

Callus induction frequency (%) was calculated as follows:

$$\text{Callus induction frequency (\%)} = \frac{\text{Number of calli forming anthers}}{\text{Number of anthers plated}} \times 100$$

Results and Discussion

Analysis of variance for callus induction frequency in anthers cultured *in vitro* on two media supplemented with two different sucrose concentrations and each of these sucrose concentrated media supplemented with three combinations of hormones is presented in Table 3. All four factors *viz.*, genotypes, hormones, media and sucrose had significant effect on callus induction frequency. Eight out of eleven interactions *viz.*, hormones x genotypes, media x genotypes, hormones x media, hormones x genotypes x media, sucrose x hormones x genotypes, media x sucrose, media x sucrose x genotypes and hormones x genotypes x media x sucrose showed significant effect on callus induction frequency. The results are in conformity with the findings of Singh (2006), Kumari (2010) and Philem and Chadha (2010) in respect of media, hormones and hormones x media in *Brassica* species.

Response of different genotypes on different media

The experiment conducted to study the response of different genotypes on different media indicated that N_6 gave highest callus induction frequency (68.42 %) and was found significantly superior than MS medium. Out of the five genotypes used for anther culture, HPBS-1 gave highest mean callusing (70.85 %).

In interaction between media x genotypes, the highest callus induction frequency was observed in KBS-3 x HPKM-04-1 (78.95 %) on N_6 medium followed by HPBS-1 (71.87 %) in MS medium and HPKM-04-1 (70.90 %) on N_6 medium. Overall it was observed that N_6 medium was best for callus induction frequency as indicated in Table 4 & Figure 1. The differential response of different genotypes for days to callus induction were also reported by Alam *et al.*, (2009), Khan *et al.*, (2009) and Sayem *et al.*, (2010) in *Brassica* species.

Response of genotypes on different hormones and their combination

It was revealed that callus induction differs from genotype to genotype as indicated in Table 5. Out of the five genotypes, HPBS-1 gave significantly highest callus induction frequency (70.85 %) followed by KBS-3 x HPKM-04-1 (70.44 %) and HPKM-04-1 (69.17 %) while HPBS-1 x HPKM-04-1 exhibited lowest callus induction frequency (60.71 %). Out of the three hormonal combinations tested, HM_2 gave the highest mean callusing (70.71 %). Overall, the genotype HPBS-1 and hormone HM_2 [2,4-D(0.5 mg/l) + NAA (1.0 mg/l)] appeared to be best for callus induction frequency as indicated in Figure 2. Higher percentage of callus induction was observed on a medium with 2 mg/l 2, 4-D and NAA each by Roy and Saha (1997). The result is in consonance with the results of Kumari (2010) and Lone *et al.*, (2017).

Response of genotypes on different sucrose concentrations

The experiment conducted to study the effect of sucrose and genotypes on callus induction frequency is presented in Table 6. Out of two different sucrose concentrations, 3 per cent sucrose gave significantly highest callus induction frequency (68.33 %) as indicated in Figure 3. Among five genotypes, HPBS-1 performed best with callus induction frequency (70.85 %) followed by KBS-3 x HPKM-04-1 (70.44 %), both were found to be statistically at par with each other. Shitole (2012) reported that the concentration of 3% sucrose would be adequate for callus induction in Ethopian mustard.

Effects of sucrose and hormones on callus induction frequency

The perusal of data presented in Table 7 indicated that out of two different sucrose concentrations, 3 per cent sucrose showed highest callus induction frequency (68.33 %) and was found significantly superior than the 4 per cent sucrose concentration. Out of the three hormonal combinations, HM_2 [2, 4-D (0.5 mg/l) + NAA (1.0 mg/l)] gave significantly highest callus induction frequency (70.71 %) than HM_1 and HM_3. Similar findings were also observed by Trivedi and Dubey (2014) and Ullah *et al.*, (2004) in *Brassica* species.

Performance of media with different concentrations of hormones for callus induction frequency

The effect of hormones and media to culture of *brassica* anther undergoing *in-vitro* callusing was investigated. Among three hormonal combinations, HM_2 (0.5 mg/l 2,4-D +1.0 mg/l NAA) showed highest callus induction frequency to (70.71 %) and was found to be significantly superior than HM_3 and HM_1. It was observed that N_6 medium showed highest callus induction (68.42 %) and was found to be significantly superior to the MS medium. The interaction between two factors *i.e.* hormones x media had significant effect on the callus induction frequency. Overall it was revealed that the highest callus induction frequency was observed in N_6 medium supplemented with HM_2 (72.86 %) (Table 8). Roy and Saha (1997) reported higher per centage of callus induction frequency on B_5 medium supplemented with 2 mg/l 2, 4-D and NAA (Table 8). Philem and Chadha (2010) also reported highest callus induction frequency in B_5 medium (24.94 %) when supplemented with HM_2 (0.5 mg/l 2, 4-D + 1.0 mg/l NAA).

Performance of media with different sucrose concentrations for callus induction frequency

It was observed that the callus induction frequency was greatly influenced by media used for callus induction with the best results (68.42%) achieved using N_6 medium supplemented with HM_2 [2,4-D (0.5mg/l) +NAA (1mg/l)] Table 9. Shitole (2012) also observed 3 per cent sucrose concentration was for high callus induction frequency which is in confirmation with the results of present study.

Table.1 List of genotypes and their cross combinations used for anther culture study

Sr. No	Genotype	Salient features
1	HPBS-1	High yielding, dwarf variety with short and sturdy stem which makes it lodging resistant and moderately resistant to *Alternaria* blight
2	KBS-3	High yielding variety with tolerant to frost
3	HPKM-04-1	Local collection from H.P.
4	HPBS-1 x HPKM-04-1	-
5	KBS-3 x HPKM-04-1	-

Table.2 Different media, hormones and sucrose concentration used for callus induction

Medium	Sucrose concentration	Hormone	
		Designation	Name and Concentration
MS	3%	HM_1	2,4-D (0.5 mg/l)
MS	3%	HM_2	2,4-D(0.5 mg/l) + NAA (1.0 mg/l)
MS	3%	HM_3	NAA (1.0 mg/l)
MS	4%	HM_1	2,4-D (0.5 mg/l)
MS	4%	HM_2	2,4-D(0.5 mg/l) + NAA (1.0 mg/l)
MS	4%	HM_3	NAA (1.0 mg/l)
N_6	3%	HM_1	2,4-D (0.5 mg/l)
N_6	3%	HM_2	2,4-D(0.5 mg/l) + NAA (1.0 mg/l)
N_6	3%	HM_3	NAA (1.0 mg/l)
N_6	4%	HM_1	2,4-D (0.5 mg/l)
N_6	4%	HM_2	2,4-D(0.5 mg/l) + NAA (1.0 mg/l)
N_6	4%	HM_3	NAA (1.0 mg/l)

Table.3 ANOVA for Callus induction frequency (%) in different genotypes of *Brassica campestris* and their hybrids involving different media, hormones and sucrose concentration

Source of variation	Df	Mean Squares	CD (5 %)	CV (%)
Genotypes	4	881.73*	4.09	13.3
Hormones	2	1007.90*	3.17	
Hormones x genotypes	8	1198.79*	7.09	
Media	1	610.68*	2.59	
Media x genotypes	4	1080.93*	5.79	
Hormones x media	2	264.60*	4.48	
Hormones x genotypes x media	8	1017.44*	10.02	
Sucrose	1	551.83*	2.59	
Sucrose x genotypes	4	126.82	NS	
Sucrose x hormones	2	13.68	NS	
Sucrose x hormones x genotypes	8	318.61*	10.02	
Media x sucrose	1	360.58*	3.66	
Media x sucrose x genotypes	4	1099.01*	8.19	
Hormones x media x sucrose	2	29.07	NS	
Hormones x genotypes x media x sucrose	8	65.35*	14.18	
Error	120	78.32		

* Significant at $P \leq 0.05$ NS = Non-significant

Table.4 Response of different genotypes on different media

Media	Genotypes						
	HPBS-1	**HPKM-04-1**	**KBS-3**	**HPBS-1 x HPKM-04-1**	**KBS-3 x HPKM-04-1**	**Mean**	**CD (5 %)**
MS	71.87 (57.97)	67.44 (55.21)	67.24 (55.08)	55.20 (47.99)	61.94 (51.91)	64.74 (53.57)	2.59 (Media)
N_6	69.84 (56.69)	70.90 (57.35)	56.21 (48.57)	66.21 (54.46)	78.95 (62.69)	68.42 (55.81)	
Mean	70.85 (57.32)	69.17 (56.27)	61.72 (51.78)	60.71 (51.18)	70.44 (57.07)		

CD (5%) = 4.09 (Genotypes)
CD interaction= 6.56 (Media x genotypes)
Values in parentheses are arc sine transformed values

Table.5 Response of genotypes on different hormones and their combination

Hormonal Combination	Genotypes						
	HPBS-1	**HPKM-04-1**	**KBS-3**	**HPBS-1 x HPKM-04-1**	**KBS-3 x HPKM-04-1**	**Mean**	**CD (5 %)**
HM_1	68.62 (55.93)	62.41 (52.18)	62.16 (52.03)	40.25 (39.38)	78.00 (62.03)	62.29 (52.11)	3.17 (Hormones)
HM_2	78.02 (62.04)	78.43 (62.33)	59.69 (50.58)	71.64 (57.82)	65.75 (54.18)	70.71 (57.23)	
HM_3	65.92 (54.28)	66.67 (54.74)	63.33 (52.73)	70.23 (56.93)	66.46 (54.61)	66.52 (54.65)	
Mean	70.85 (57.32)	69.17 (56.27)	61.72 (51.78)	60.71 (51.18)	70.44 (57.07)		

CD (5 %) = 4.09 (Genotypes)
CD interaction= 7.09 (Genotypes x hormone)
Values in parentheses are arc sine transformed values

Table.6 Response of genotypes on different sucrose concentrations

Sucrose	Genotypes						
	HPBS-1	**HPKM-04-1**	**KBS-3**	**HPBS-1 x HPKM-04-1**	**KBS-3 x HPKM-04-1**	**Mean**	**CD (5 %)**
3%	74.57 (59.72)	72.09 (58.11)	60.53 (51.08)	62.65 (52.33)	71.82 (57.93)	68.33 (55.75)	2.59 (Sucrose)
4%	67.14 (55.02)	66.25 (54.48)	62.92 (52.49)	58.76 (50.05)	69.07 (56.21)	64.83 (53.63)	
Mean	70.85 (57.32)	69.17 (56.27)	61.72 (51.78)	60.71 (51.18)	70.44 (57.07)		

CD (5 %) = 4.09 (Genotypes)
CD interaction = NS (Sucrose x genotypes)
Values in parentheses are arc sine transformed values

Table.7 Effects of sucrose and hormones on callus induction frequency

Sucrose	Hormonal combination				
	HM_1	HM_2	HM_3	Mean	CD (5 %)
3 %	64.48 (53.42)	71.91 (57.99)	68.60 (55.92)	68.33 (55.75)	2.59 (Sucrose)
4 %	60.54 (51.08)	69.50 (56.48)	64.44 (53.40)	64.83 (53.63)	
Mean	62.51 (52.24)	70.71 (57.23)	66.52 (54.65)		

CD (5 %) = 3.17 (Hormone)
CD interaction= NS (Hormone x sucrose)
Values in parentheses are arc sine transformed values

Table.8 Performance of media with different concentrations of hormones for callus induction frequency

Hormonal Combination	Callusing Media			
	MS	N_6	Mean	CD (5 %)
HM_1	58.74 (50.03)	66.28 (54.50)	62.51 (52.24)	3.17 (Hormones)
HM_2	68.55 (55.89)	72.86 (58.60)	70.71 (57.23)	
HM_3	66.92 (54.89)	66.12 (54.41)	66.52 (54.65)	
Mean	64.74 (53.57)	68.42 (55.81)		

CD (5 %) = 2.59 (Media)
CD interaction = 4.48 (Hormones x media)
Values in parentheses are arc sine transformed values

Table.9 Performance of media with different sucrose concentrations for callus induction frequency

Media	Sucrose concentration			
	3%	4%	Mean	CD (5 %)
MS	67.90 (55.49)	61.57 (51.69)	64.74 (53.57)	2.59 (Media)
N_6	68.76 (56.02)	68.08 (55.60)	68.42 (55.81)	
Mean	68.33 (55.75)	64.83 (53.63)		

CD (5 %) = 2.59 (Sucrose)
CD interaction = 3.66 (Media x sucrose)
Values in parentheses are arc sine transformed values

Fig.1 Callus formation of genotype HPBS-1 at N_6 medium

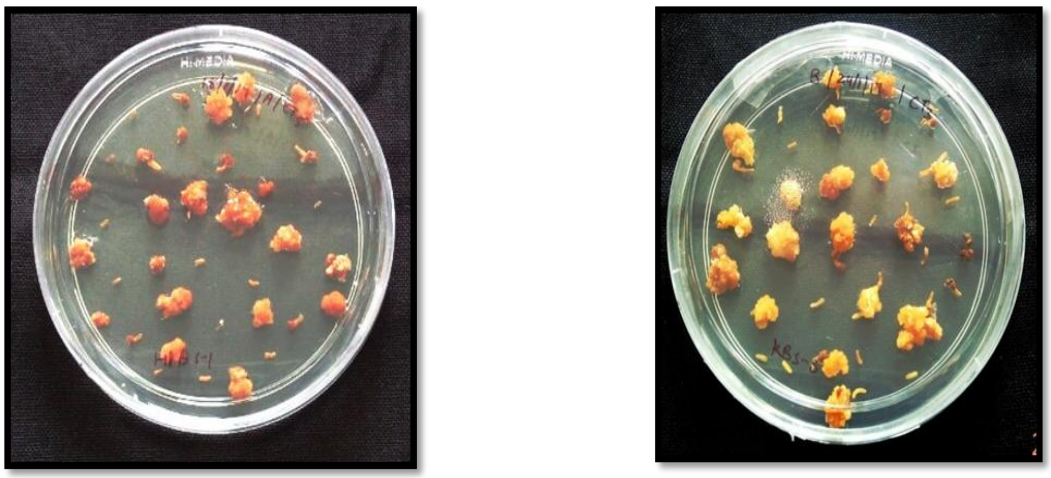


Fig.2 Response of genotype HPBS-1 with HM_2 [2,4-D(0.5 mg/l) + NAA (1.0 mg/l)]

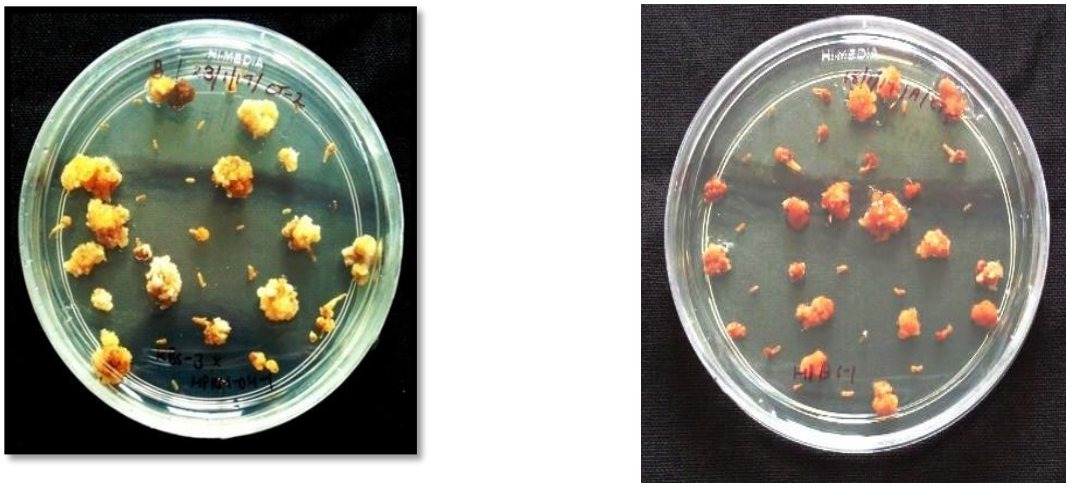
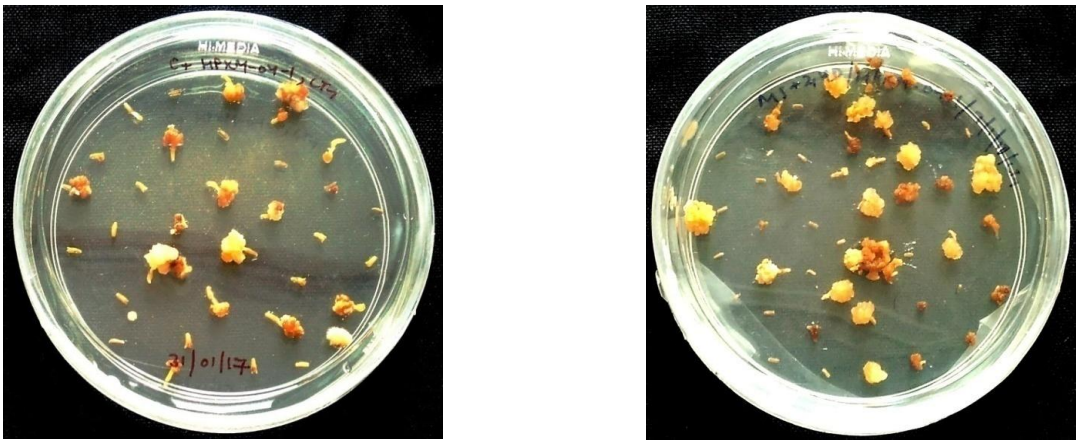


Fig.3 Effect of 3 % sucrose on genotype HPBS-1



Hence concluded, in androgenesis-mediated response, the highest callusing was observed in N_6 medium with HM_2 [0.5 mg/l 2, 4-D + 1.0 mg/l NAA] and 3 per cent sucrose. Overall, genotype HPBS-1 was the most promising for callus induction through anther culture. This media and hormonal combination can be successfully utilized for induction of haploids and double haploids in future.

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