Journal of Oilseed Brassica, 9 (2) : 155-163, July 2018

Response of different genotypes and their cross combinations to anther culture in Ethiopian mustard (*Brassica carinata* A. Braun)

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(Received: 07 March 2018; Revised: 14 May 2018; Accepted: 11 June 2018)

Abstract

The effects of seven genotypes and their cross combinations, two basal media *i.e.* B_5 and MS, two different sucrose concentrations *i.e.* 3% and 4% and three combinations of hormones *viz.,* HM_1, HM_2 and HM_3 and their interactions on callus inductions frequency in *Brassica carinata* were analyzed by using CPCS software. Mean sum of squares due to all factors were significantly revealed the effects of genotypes, media, hormones, sucrose and their interactions on callus induction frequency. Out of all factors and their interactions, the genotype P-51 performed better in B_5 medium supplemented with HM_2 (0.2mg/l BAP + 2.0 mg/l NAA) and 3% sucrose concentration for high callus induction frequency.

Key words: *Callus, hormones, sucrose*

Introduction

Oilseed crops are the backbone of Indian agricultural economy and occupy an important position in daily diet, being a rich source of fats and vitamins. India is the second largest rapeseed-mustard growing country and accounts for 21.7% area in the world after China. Among oilseeds, rapeseed-mustard is the second most important oilseed crop of the country after groundnut and plays a significant role in Indian oil economy by contributing about 28.6% to the total oilseed production (Shekhawat *et al.*, 2014).

Over the last decades, researchers have made great efforts in developing biotechnology methods to facilitate the breeding of *Brassicas*. Research studies indicated that the modern biotechnology will have a major impact in two areas. Firstly, it provides a new range of techniques enabling the efficient selection of favourable variants in plant breeding programmes. Secondly, it provides the opportunity to improve germplasm by increasing its diversity beyond conventional genetic limitations. Due to the relative ease of genetic transformation, *Brassica* oilseed crops have been amongst the first to be subject to the full range of modern biotechnology methods (Abraha *et al.*, 2008).

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, a time consuming process (Morrison and Evans, 1988). Hence, biotechnological tools including anther culture hold a great promise in accelerating the pace of breeding programme (Guha and Maheshwari, 1964). *In vitro* technique of anther culture helps to achieve homozygosity very quickly (Snape, 1989). Anther culture of potential F_1 generation genotypes can be used to facilitate regeneration of stable recombinant inbreds in one to two years thereby saving time and resources for their further use directly as commercial cultivars and/or in structural and functional genomics. Keeping this in view, the present study was conducted to investigate the androgenesis-mediated response of different genotypes and their cross combinations in Ethiopian mustard (*Brassica carinata* A. Braun).

Materials and Methods

The anther culture work was carried out in the Molecular Cytogenetics and Tissue culture Laboratory of Department of Crop Improvement, CSK HPKV, Palampur during *Rabi* 2010-11. The material used and methodology adopted to achieve the objectives of the investigation are given below.

Experimental material

The material used for anther culture studies comprised of four elite genotypes and their three cross combinations (Table 1).

Plant material for anther culture

Sufficient numbers of plants of aforementioned four 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.

Table 1: List of genotypes and their cross combinations under anther culture study

Sr.	Genotype	Parentage	Salient features
1	Jayanti	Developed through irradiation from the parent variety HC-1	Moderately susceptible to *A. brassicae*, Tall
2	P-18	Advanced generation mutant obtained through treatment of Jayanti seeds with 0.3% EMS (Pre-Soaked)	Moderately resistant to *A. brassicae*, Dwarf
3	P-51	Advanced generation mutant obtained through treatment of Jayanti seeds with 0.3% EMS (Pre-Soaked)	Moderately resistant to *A. brassicae*, Dwarf
4	$P_{(2)2}$	Advanced generation mutant obtained through treatment of Jayanti seeds with 90 kR dose of gamma radiations	Moderately resistant to *A. brassicae*
5	Jayanti x P-18	-	-
6	Jayanti x P-51	-	-
7	Jayanti x $P_{(2)2}$	-	-

Stage of explants

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 uninuclate to early binucleate stage as studied by squashing of anthers in a drop of 1% acetocarmine. The florets of appropriate size were collected in 50 ml test tubes containing distilled water.

Plating of anthers in callus induction media

The florets collected at aforementioned stages were treated with 70% ethanol for 10-15 seconds under aseptic conditions in a laminar air flow chamber. The florets were then surface sterilized with 0.1% $HgCl_2$ 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.

Two basal media *viz.*, B_5 (Gamborg *et al*., 1968) and MS (Murashige and Skoog, 1962) were used for callus induction. Each of these medium was supplemented with two different sucrose concentrations *i.e.* 3% and 4% 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% agar.

The experiments on different callus induction media were replicated thrice involving different media and plant growth hormones. Anthers of all four genotypes and their crosses were plated in a replicated fashion. If there was any contamination, replating of the particular treatment was done to complete the experiment under uniform conditions. All the cultured plates were sealed with parafilm wax and kept under dark at 25 ± 1°C until calli were developed.

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

Medium	Sucrose concentration	Hormone	
		Designation	Name and Concentration
B_5	3%	HM_1	NAA (1.0 mg/l)
B_5	3%	HM_2	BAP (2.0 mg/l) + NAA (2.0 mg/l)
B_5	3%	HM_3	2, 4-D (0.5 mg/l) + NAA (1.0 mg/l)
B_5	4%	HM_1	NAA (1.0 mg/l)
B_5	4%	HM_2	BAP (2.0 mg/l) + NAA (2.0 mg/l)
B_5	4%	HM_3	2, 4-D (0.5 mg/l) + NAA (1.0 mg/l)
MS	3%	HM_1	NAA (1.0 mg/l)
MS	3%	HM_2	BAP (2.0 mg/l) + NAA (2.0 mg/l)
MS	3%	HM_3	2, 4-D (0.5 mg/l) + NAA (1.0 mg/l)
MS	4%	HM_1	NAA (1.0 mg/l)
MS	4%	HM_2	BAP (2.0 mg/l) + NAA (2.0 mg/l)
MS	4%	HM_3	2, 4-D (0.5 mg/l) + NAA (1.0 mg/l)

Statistical analysis

The Callus induction frequency (%) was calculated as follows:

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

Data analysis

The data pertaining to different parameters were subjected to appropriate transformation using arc sine transformation wherever necessary. Data on callus induction frequency were analyzed in Factorial Completely Randomized Design (CRD) to obtain the effect of various treatments and their interactions using statistical CPCS software.

Results and Discussion

Effects of different parameters on callus induction frequency

Analysis of variance for callus induction frequency in anthers of seven genotypes 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 and Plate I. Mean sum of squares due to all factors were significant revealing thereby significant effects of genotypes, media, hormones, sucrose and their interactions on callus induction frequency.

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

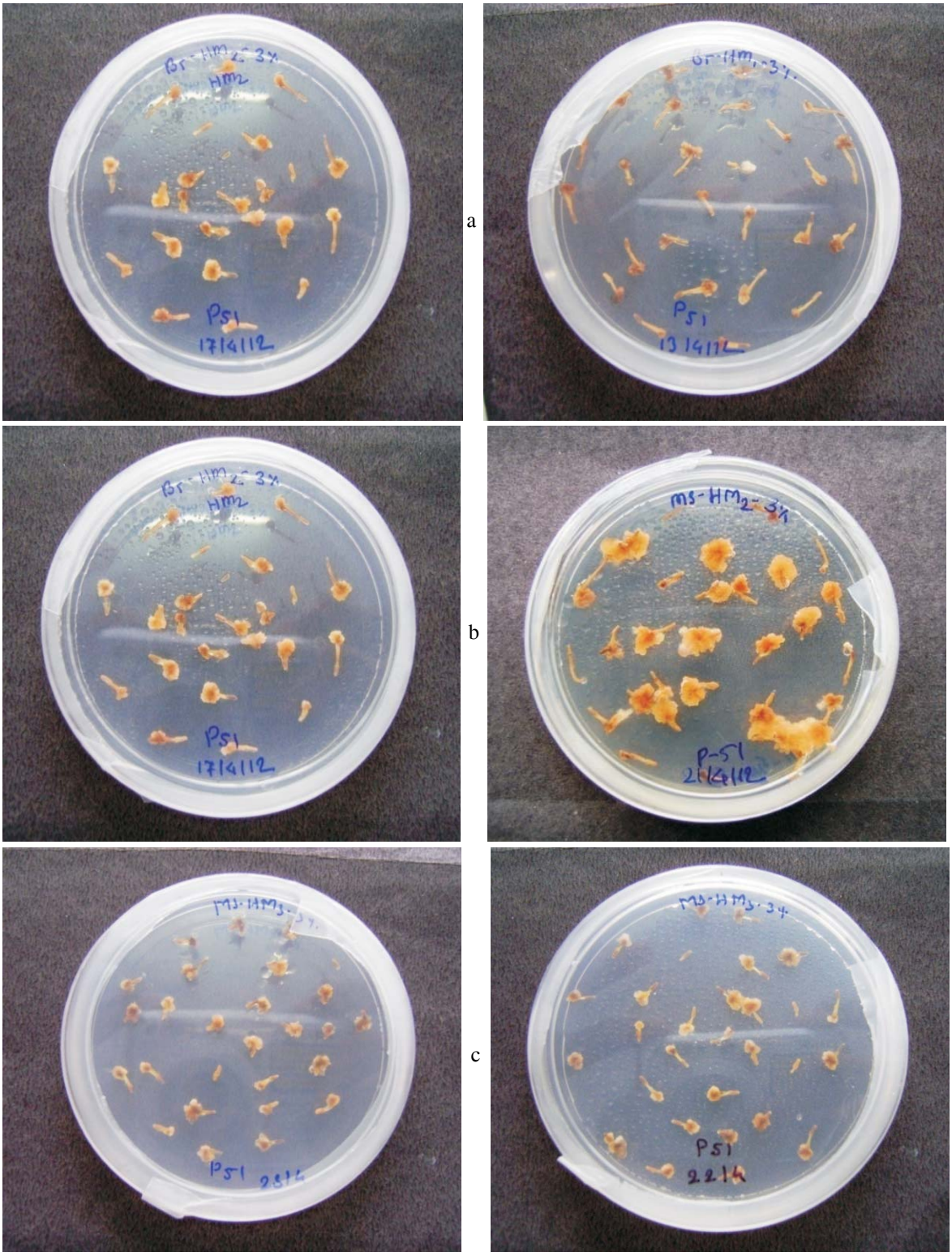
Source of variation	df	MeanSquares	CD(5%)
Genotypes	6	146.91**	2.96
Hormones	2	3375.03**	1.94
Genotypes x Hormones	12	826.23**	5.13
Media	1	10768.06**	1.58
Genotypes x Media	6	824.45**	4.18
Hormones x Media	2	576.26**	2.74
Genotypes x Hormones x Media	12	198.70**	7.25
Sucrose	1	2483.36**	1.58
Genotypes x Sucrose	6	156.31**	4.18
Hormones x Sucrose	2	3901.22**	2.74
Genotypes x Hormones x Sucrose	12	283.26**	7.25
Media x Sucrose	1	2410.23**	2.24
Genotypes x Media x Sucrose	6	913.80**	5.92
Hormones x Media x Sucrose	2	17017.98**	3.87
Genotypes x Hormones x Media x Sucrose	12	339.16**	10.25
Error	168	40.01	

** Significant at P = 0.01

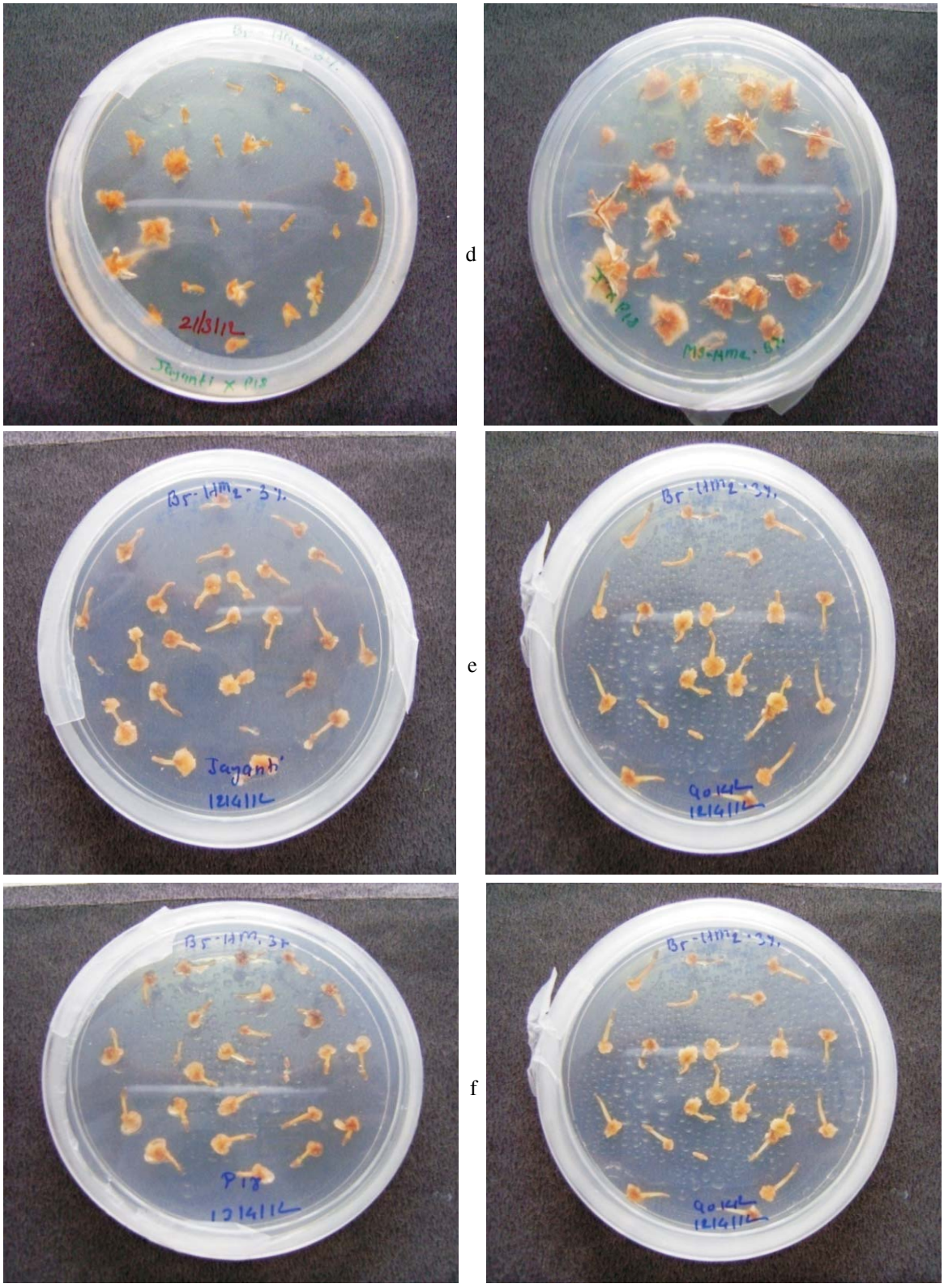
Effects of media and genotypes on callus induction frequency

The data pertaining to effects of media and genotypes on callus induction frequency is presented in Table 4a. Out of two media tested, B_5 gave highest callus induction frequency (77.5 %) and was found significantly superior than MS medium. Out of the seven genotypes used for anther culture, P-51 gave highest mean callusing (75.8 %) and was statistically at par with Jayanti x P-18. On the other hand, Jayanti and Jayanti x $P_{(2)2}$ showed least callus induction frequency (71.2 % each). In genotypes x media interaction, the highest callus induction frequency was recorded in P-51 (87.3 %) on B_5 medium followed by P-18 (81.4 %) and Jayanti x $P_{(2)2}$ on B_5 medium (77.40 %) whereas P-51 exhibited lowest callus induction frequency on MS medium (64.3 %). Overall B_5 medium was best for callus induction frequency. Superiority of B_5 over MS has also been reported by Lelu and Bollon (1990) for *Brassica oleracea* var. *capitata* and *Brassica oleracea* var. *gemmifera.* The same madia have also been successfully used to induce *in vitro* callusing in cultured anthers of *Brassica juncea* by various workers (Goel et al., 1990 and Agarwal and Bhojwani, 1993). Apart from B_5 medium, KA and N_6 media have also been successfully used to induce *in vitro* callusing in anther culture of *Brassica carinata* by various workers (Sharma and Bhojwani, 1985 and Arora and Bhojwani, 1988).

Plate I. Effects of different media, hormones and sucrose concentration on callus induction frequency



a) B_5 + P-51
b) P-51 + HM_2 (0.2 mg/l BAP+2.0 mg/l NAA)
c) 3% + P-51



d) 3% + HM_2 (0.2 mg/l BAP+2.0 mg/l NAA)

e) HM_2 (0.2 mg/l BAP+2.0 mg/l NAA) + B_5

f) B_5 + 3%

Effects of hormones and genotypes on callus induction frequency

The perusal of data presented in Table 4b indicated that out of the three hormonal combinations tested, HM_2 gave the highest mean callusing (81.80 %) and was found to be significantly superior to HM_1 and HM_3. Hormonal combination HM_1 showed the least callus induction frequency (59.70 %). Out of the seven genotypes, P-51 gave highest callus induction (75.80 %) followed by Jayanti x P-18 (75.60 %) and both were found to be statistically at par with each other. Jayanti and Jayanti x $P_{(2)2}$ exhibited lowest callus induction frequency (71.20 % each). The interaction genotypes x hormones had significant effect on the callus induction frequency. Considering interaction between these two factors, the highest callus induction frequency was observed for genotype P-51 with HM_2 (86.20 %) and was statistically at par with Jayanti on HM_2 (84.30 %) and Jayanti x P-51 on HM_2 (84.00 %). Overall, the genotype P-51 and hormone HM_2 (0.2 mg/l BAP+2.0mg/l NAA) appeared to be best for callus induction frequency. Sayem et al. (2010) found that the highest number of shoots/callus was found in media combined with 2.0 mg/l BAP + 0.5 mg/l NAA. Roy and Saha (1997) also reported higher percentage of callus induction on a medium with 2 mg/l 2, 4-D and NAA each.

Effects of sucrose and genotypes on callus induction frequency

The data pertaining to effects of sucrose and genotypes on callus induction frequency is presented in Table 4c. Out of two different sucrose concentrations *i.e.* 3% and 4% sucrose tested, 3% sucrose gave highest callus induction frequency (74.27 %) and was found significantly superior than 4% sucrose. Out of the seven genotypes, P-51 gave highest callus induction frequency (75.77 %) followed by Jayanti x P-18 (75.64 %), both were found to be statistically at par with each other while Jayanti showed least callus induction frequency (71.17 %). In Genotypes x Sucrose interaction, the highest callus induction frequency was recorded for $P_{(2)2}$ (78.46 %) followed by Jayanti x P-18 (77.89 %) on 3% sucrose whereas Jayanti x P-51 exhibited lowest callus induction frequency on 4% sucrose (69.00 %). Overall, 3% sucrose and the genotype P-51 was best for callus induction frequency. The concentration of sucrose also plays an important role in induction of pollen plants. Dunwell and Thurling (1985) found that a higher concentration of sucrose was beneficial for initial growth and development. Narasimhulu and Chopra (1987) reported the induction of shoots when sucrose was supplemented at two% in *Brassica carinata*.

Table 4a: Effects of media and genotypes on callus induction frequency (%)

Media	Genotypes							
	Jayanti	$P_{(2)2}$	P-51	P-18	Jayanti x $P_{(2)2}$	Jayanti x P-51	Jayanti x P-18	Mean	CD (P=0.05)
MS	67.50 (55.24)	78.30 (62.24)	64.30 (53.31)	64.50 (53.43)	64.90 (53.67)	65.70 (57.15)	75.90 (60.60)	68.70 (55.98)	1.58 (Media)
B_5	74.90 (59.93)	69.30 (65.35)	87.30 (69.12)	81.40 (64.45)	77.40 (61.61)	77.00 (61.34)	75.30 (60.20)	77.50 (61.68)	
Mean	71.20 (57.54)	73.80 (59.21)	75.80 (60.53)	72.90 (58.63)	71.20 (57.54)	71.40 (57.67)	75.60 (60.40)		

CD (P=0.05) = 2.96 (Genotypes); CD interaction= 4.18 (Genotypes x Media); Values in parentheses are arc sine transformed values

Table 4b: Effects of hormones and genotypes on callus induction frequency (%)

Hormonal	Genotypes							
Combination	Jayanti	$P_{(2)2}$	P-51	P-18	Jayanti x $P_{(2)2}$	Jayanti x P-51	Jayanti x P-18	Mean	CD (P=0.05)
HM_1	57.70 (49.43)	69.70 (56.60)	63.40 (52.77)	64.30 (53.31)	56.80 (48.91)	45.10 (42.19)	60.50 (51.06)	59.70 (50.59)	1.94 (Hormones)
HM_2	84.30 (66.66)	80.90 (64.09)	86.20 (68.19)	78.00 (62.03)	78.10 (62.10)	84.00 (66.42)	81.00 (64.16)	81.80 (64.75)	
HM_3	71.50 (57.73)	70.80 (57.29)	77.70 (61.82)	76.40 (60.94)	78.60 (62.44)	85.00 (67.21)	85.50 (67.62)	77.90 (61.96)	
Mean	71.20 (57.54)	73.80 (59.21)	75.80 (60.53)	72.90 (58.63)	71.20 (57.54)	71.40 (57.67)	75.60 (60.40)		

CD (P=0.05) = 2.96 (Genotypes); CD interaction= 5.13 (Genotypes x Hormone); Values in parentheses are arc sine transformed values

Table 4c: Effects of sucrose and genotypes on callus induction frequency (%)

Sucrose	Genotypes							
	Jayanti	$P_{(2)2}$	P-51	P-18	Jayanti x $P_{(2)2}$	Jayanti x P-51	Jayanti x P-18	Mean	CD (P=0.05)
3%	72.29 (58.24)	78.46 (62.35)	74.09 (59.40)	73.68 (59.13)	69.69 (56.60)	73.76 (59.19)	77.89 (61.95)	74.27 (59.52)	1.58 (Sucrose)
4%	70.05 (56.82)	69.15 (56.26)	77.46 (61.66)	72.17 (58.16)	72.67 (58.48)	69.00 (56.17)	73.39 (58.95)	71.98 (58.04)	
Mean	71.17 (57.52)	73.81 (59.22)	75.77 (60.51)	72.93 (58.65)	71.18 (57.53)	71.38 (57.66)	75.64 (60.43)		

CD (P=0.05) = 2.96 (Genotypes); CD interaction = 4.18 (Genotypes x Sucrose); Values in parentheses are arc sine transformed values

Effects of sucrose and hormones on callus induction frequency

The perusal of data presented in Table 5 indicated that out of two different sucrose concentrations *i.e.* 3% and 4% sucrose tested, the former gave highest callus induction frequency (74.27 %) and was found significantly superior than the latter. Out of the three hormonal combinations, HM_2 (0.2 mg/l BAP+2.0 mg/l NAA) gave significantly highest callus induction frequency (81.79 %) than HM_1 and HM_3. The interaction between two factors *i.e.* sucrose x hormones had significant effect on the callus induction frequency. Considering interaction, the highest callus induction frequency was observed in 3% sucrose supplemented with HM_2 (88.22 %) followed by 4% sucrose supplemented with HM_3 (75.55 %).

Effects of hormones and media on callus induction frequency

The data pertaining to effect of hormones and media on callus induction frequency is presented in Table 6. Out of the three hormonal combinations tested, HM2 (0.2 mg/l BAP+2.0 mg/l NAA) gave highest callus induction frequency to (81.80 %) and was found to be significantly superior than HM3 and HM1. Out of two media, B5 medium gave highest callus induction (77.50 %) 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. Considering interaction, the highest callus induction frequency was observed in MS medium supplemented with HM2 (82.90 %) followed by B5 medium supplemented with HM2 (80.70 %).

Table 5: Effects of sucrose and hormones on callus induction frequency (%)

Sucrose	Hormonal combination				
	HM_1	HM_2	HM_3	Mean	CD (P=0.05)
3 %	54.3	88.2	80.3	74.3	1.58
	(47.4)	(69.9)	(63.7)	(59.5)	(Sucrose)
4 %	65.0	75.4	75.6	72.0	
	(53.8)	(60.2)	(60.4)	(58.0)	
Mean	59.7	81.8	77.9		
	(50.6)	(64.7)	(62.0)		

CD (P=0.05) = 1.94 (Hormone)

CD interaction= 2.74 (Hormone x Sucrose)

Values in parentheses are arc sine transformed values

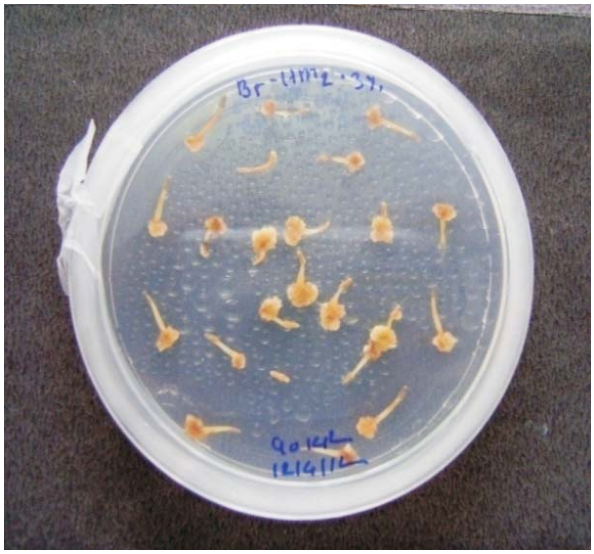


Table 6: Effects of hormones and media on callus induction frequency (%)

Hormonal	Callusing Media			
Combination	MS	B_5	Mean	CD (P=0.05)
HM_1	43.70	72.20	58.00	1.94
	(41.38)	(58.18)	(49.60)	(Hormones)
HM_2	82.90	80.70	81.80	
	(65.57)	(63.94)	(64.75)	
HM_3	76.20	79.60	77.90	
	(60.80)	(63.15)	(61.96)	
Mean	67.60	77.50		
	(55.30)	(61.68)		

CD (P=0.05) = 1.58 (Media)
CD interaction = 2.74 (Media x Hormone)
Values in parentheses are arc sine transformed values

Effects of media and sucrose on callus induction frequency

The perusal of data presented in Table 7 revealed that out of two media tested, B_5 gave highest callus induction frequency (77.60 %) and was found significantly superior than MS medium. Out of two different sucrose concentrations tested, 3% sucrose gave highest callus induction frequency (74.30 %) and was found to be significantly superior than 4% sucrose. The interaction between two factors *i.e.* media x sucrose had significant effect on the callus induction frequency. Considering interaction, the highest callus induction frequency was observed in B_5 medium supplemented with 3% sucrose (82.30 %) followed by B_5 medium supplemented with 4% sucrose (72.80 %). Keller et al. (1975) used B_5 and MS media with 2% sucrose for *Brassica campestris* and *Brassica napus.*

Table 7: Effects of media and sucrose on callus induction frequency (%)

Media	Sucrose			
	3%	4%	Mean	CD(P=0.05)
MS	66.30	71.20	68.80	1.58
	(54.51)	(57.54)	(56.04)	(Media)
B_5	82.30	72.80	77.60	
	(65.12)	(58.56)	(61.75)	
Mean	74.30	72.00		
	(59.54)	(58.05)		

CD (P=0.05) = 1.58 (Sucrose)
CD interaction = 2.24 (Sucrose x Media)
Values in parentheses are arc sine transformed values

Conclusion

The quality and callus induction frequency of cultured anthers ultimately depend on genotypes, culture medium, hormonal combination and sucrose concentration. Therefore, selection of efficient culture medium and culture conditions like B_5 medium supplemented with HM_2 (0.2mg/l BAP + 2.0 mg/l NAA) and 3% sucrose concentration and cultivation at 25 ± 1°C under dark condition for genotype P-51 would offer great promise for the higher callus induction frequency.

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