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Satendra Chaudhary
Department of Vegetable Science and Floriculture, CSK Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh, India

Viveka Katoch
Department of Vegetable Science and Floriculture, CSK Himachal Pradesh Agricultural University, Palampur, (H.P.), India

Rakesh Kumar Kapila
Department of Vegetable Science and Floriculture, CSK Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh, India

Akhilesh Sharma
Department of Vegetable Science and Floriculture, CSK Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh, India

Shweta Sharma
Department of Vegetable Science and Floriculture, CSK Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh, India

Correspondence
Viveka Katoch
Department of Vegetable Science and Floriculture, CSK Himachal Pradesh Agricultural University, Palampur, Himachal Pradesh, India

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Response of Sucrose, PGRs and pretreatments on another culture efficiency in broccoli (*Brassica oleracea* L. var. *italica* Plenck.)

Satendra Chaudhary, Viveka Katoch, Rakesh Kumar Kapila, Akhilesh Sharma and Shweta Sharma

Abstract
The present investigation was undertaken to assess the effect of different plant growth regulators (2,4-D and NAA), sucrose concentrations (10, 12, 14 and 16 %) on callus induction frequency and different pretreatment temperatures (cold and heat shock) *viz.*, 0-4°C, 25°C, 27.5°C, 30°C, 32.5°C, 35°C and 37.5°C along with three different incubation periods *viz.*, 24, 48 and 72 hours on Anther culture efficiency in four hybrids of broccoli namely, BR-60, Kendy, Lucky and Fiesta. The highest callus induction frequency was recorded in BR-60 in the treatment T_5 (B_5 + 0.10 mg/l NAA and 0.50 mg/l 2,4-D + 10 % sucrose). The best response of callus induction frequency was obtained in hybrid BR-60 at 12 per cent concentration of sucrose. The highest anther culture efficiency was observed in BR-60 when anthers were subjected to 35°C for 48 hours using best hormonal combination (T_5) and sucrose (12 per cent). The highest calli regeneration frequency was obtained in hybrid Kendy when calli were transferred to HM_5 combination (B5 + 0.25 mg/l IAA + 1.0 mg/l BAP + 2.0 mg/l Kin + 3 % sucrose). The rhizogenesis was induced in all the hybrids of broccoli when their calli derived meristemoids were sub-cultured on rooting medium (B_5 + 0.5 mg/l NAA + 3 % sucrose). The anther culture response was recorded on the basis of meristemoids. The highest meristemoids were recorded in hybrid Kendy followed by BR-60.

Keywords: Broccoli, Anther culture, plant growth regulators, sucrose, pre-treatment

Introduction
Broccoli (*Brassica oleracea* L. var. *italica* Plenck) a member of family Brassicacea is one of the nutritious cole crop. It is known for its taste, flavor, nutritive and medicinal properties. Broccoli contains the compound namely, glucoraphanin, which can be processed into an anti-cancerouus compound sulphoraphane (Kumari and Katoch 2016) [10]. Being a native of Mediterranean region, the diversity of broccoli in India is very less. One of the major bottlenecks for broccoli breeders is the availability of germplasm. Broccoli is cross-pollinated crop and to develop a new inbred through conventional breeding usually takes at least five generations of inbreeding and backcrossing to reach the same goal of recovering genetically stable lines through conventional breeding approaches. For the development of hybrid(s), inbred lines are required and the development of inbred lines is a time consuming task. Androgenesis *via* anther/microspore culture offers an alternative for the development of new inbred lines in comparatively short-time thereby, reducing the cost incurred in the conventional breeding. The successful proportion of androgenic plantlets production depend on some critical factors including genotype, physiology of the donor plant, microspores/pollen grains stage, culture medium, sucrose concentrations, plant growth regulators and pretreatment temperatures. The present study intends to investigate the effects of different plant growth regulators, different concentrations of sucrose content and various pretreatment temperatures and incubation periods on Anther culture efficiency of broccoli.

Material and Methods

The F_1 hybrids of broccoli *viz.*, Kendy, BR-60, Lucky and Fiesta were raised at the Research Farm of the Department of Vegetable Science and Floriculture, CSK HPKV, Palampur.

Bud collection and surface sterilization

The immature flower buds of 3-5 mm were collected from donor plants for anther culture. The flower buds were kept under running tap water for 30 minutes followed by washing with Tween-20 and washing, surface sterilization of buds by dipping the buds in 70 per cent ethanol for 25 seconds in the laminar air flow hood. After that, the flower buds were surface sterilized in 0.1 per cent $HgCl_2$ for 2-3 minutes with intermittent shaking and finally followed by three washings of florets with sterilized double distilled water.

Culture medium, anther excision and plating

The culture medium B_5 (Gamborg *et al*. 1968) [7] was used for callus induction. Media was supplemented with different concentrations of plant growth regulators (NAA and 2,4-D), 10 per cent sucrose and 0.8 per cent agar for an initial callus induction. The pH of media was adjusted to 5.7 to 5.8 with concentrated HCl or NaOH and then autoclaved for 20 minutes at 121°C to 130°C and 15 psi. About 20 ml media was poured into sterilized petri plates under controlled conditions in a laminar flow cabinet. From the sterilized flower buds, anthers along with their filaments were excised under aseptic conditions with the help of sterilized forceps. About 20 to 25 anthers were cultured in each pre-sterilized petri plate and all the cultured plates were sealed with parafilm and kept under dark at 25 ± 1°C until callus induction.

Determination of the effect of growth regulators on callus induction

The experiment on different concentrations and combinations of auxins (2,4-D and NAA) added to the B_5 medium supplemented with 10 per cent sucrose was designed in a Completely.

Table 1: Different concentrations and combinations of growth regulators used for an initial callus induction

Basal Medium	Sucrose Concentration (%)	Designation	Concentrations and combinations of growth regulators (mg/l)
B_5	10	T_1	0.10 NAA + 0.02 2,4-D
B_5	10	T_2	0.10 NAA + 0.05 2,4-D
B_5	10	T_3	0.10 NAA + 0.10 2,4-D
B_5	10	T_4	0.10 NAA + 0.25 2,4-D
B_5	10	T_5	0.10 NAA + 0.50 2,4-D
B_5	10	T_6	0.25 NAA + 0.02 2,4-D
B_5	10	T_7	0.25 NAA + 0.05 2,4-D
B_5	10	T_8	0.25 NAA + 0.10 2,4-D
B_5	10	T_9	0.25 NAA + 0.25 2,4-D
B_5	10	T_{10}	0.25 NAA + 0.50 2,4-D
B_5	10	T_{11}	0.50 NAA + 0.02 2,4-D
B_5	10	T_{12}	0.50 NAA + 0.05 2,4-D
B_5	10	T_{13}	0.50 NAA + 0.10 2,4-D
B_5	10	T_{14}	0.50 NAA + 0.25 2,4-D
B_5	10	T_{15}	0.50 NAA + 0.50 2,4-D
B_5	10	T_{16}	1.00 NAA + 0.02 2,4-D
B_5	10	T_{17}	1.00 NAA + 0.05 2,4-D
B_5	10	T_{18}	1.00 NAA + 0.10 2,4-D
B_5	10	T_{19}	1.00 NAA + 0.25 2,4-D
B_5	10	T_{20}	1.00 NAA + 0.50 2,4-D

Randomized Design (CRD) with three replications (Table 1). Each treatment was represented by nine petri dishes along with the control treatment to evaluate the effect of different growth regulator combinations on callus induction in broccoli.

Determination of the effect of different sucrose concentrations on callus induction

The experiment was conducted to investigate the effects of different sucrose concentrations *viz.*, 10, 12, 14 and 16 per cent on callus induction frequency in broccoli. The B_5 medium supplemented with best hormone combination T_5 (0.10 mg/l NAA + 0.50 mg/l 2,4-D) was supplemented with different sucrose concentrations (10, 12, 14 and 16 %). The experiment was set up in a Completely Randomized Design with three replications. Each replication consisted of three petri dishes of the respective treatment and control consisted of B_5 medium supplemented with one per cent sucrose to evaluate the effect of different sucrose concentrations on callus induction.

Table 2: Different concentrations and combinations of growth regulators for shoot regeneration from callus

Medium	Sucrose concentration (%)	Designation	Concentrations and combinations of growth regulators (mg/l)
B_5	3	HM_1	0.5 2, 4-D + 1.0 BAP
B_5	3	HM_2	0.5 2, 4-D + 2.0 BAP
B_5	3	HM_3	0.5 NAA + 1.0 BAP
B_5	3	HM_4	0.5 NAA + 2.0 BAP
B_5	3	HM_5	0.25 IAA + 1.0 BAP + 2.0 Kin
B_5	3	HM_6	0.25 NAA + 1.0 BAP + 2.0 Kin

Effect of incubation temperature regimes for enhancing anther culture efficiency

Anthers of donor plants were subjected to six different heat shock temperatures *viz.*, 25°C, 27.5°C, 30°C, 32.5°C, 35°C and 37.5°C and a cold temperature pretreatment at 0-4°C for 24, 48 and 72 hours. These pretreatments consisted of B_5 medium supplemented with best sucrose concentration *i.e.* 12 per cent + 0.10 mg/l NAA + 0.50 mg/l 2,4-D and subsequently, maintained at 25±1°C in the dark. Experiment was laid out in Completely Randomized Design (CRD) with three replications for each treatment including control (at 25±1°C).

Regeneration of plantlets from calli

Anthers producing calli (about 3 mm in diameter) in 15-20 days of culturing were transferred into 250 ml culture flask containing 100 to 125 ml regeneration medium. The regeneration B_5 medium was supplemented with sucrose three per cent, agar 0.8 per cent, different concentration of auxins (2,4-D, NAA and IAA) and cytokinins (BAP and Kinetin) (Table 2). Flasks containing calli were incubated at 25±1°C with a 16 hours photoperiod.

Rooting of plantlets

The green shoots regenerated from callus were subsequently separated and sub-cultured in 250 ml culture flasks containing rooting medium (B_5 + 0.5 mg/l NAA + 3 % sucrose). The sub-cultured green shoots were incubated at 25±1°C under 18/6 light/dark cycle till the plantlets developed roots.

Observation recorded

Data was recorded on the callus induction frequency (%), regeneration frequency (%) and anther culture response (%).

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

$$\text{Regeneration frequency (\%)} = \frac{\text{Number of shoots regenerated}}{\text{Number of calli kept for regeneration}} \times 100$$

$$\text{Anther culture response (\%)} = \frac{\text{Number of plants with shoot and roots}}{\text{Number of anthers plated}} \times 100$$

Results and Discussion

Effect of plant growth regulators on callus induction frequency

Analysis of variance revealed that the mean sum of squares due to F_1 hybrids, plant growth regulators and F_1 hybrids × plant growth regulators interaction on callus induction frequency were found to be significant. Out of 20 growth regulator combinations, T_5 gave significantly high mean callusing (84.51%) followed by T_{20} (80.39 %). The growth regulator treatment T_{20} (80.39 %) was significantly higher than T_{10} (76.83 %), T_{15} (76.05 %) and T_{19} (76.03 %). The growth regulator treatment T_{10} (76.83 %), T_{15} (76.05 %) and T_{19} (76.03 %) were found to be *at par* with each other. Lowest callusing was obtained in the treatment T_1 (50.81 %) (Table 3). Out of the four hybrids, BR-60 gave significantly high mean callusing (75.26 %) while lowest callusing mean was observed in Fiesta (65.45 %). The hybrid Fiesta was *at par* with Kendy (66.66 %). In F_1 hybrids × plant growth regulators interaction, the highest callus induction frequency was recorded in BR-60 (90.70 %) followed by Lucky (86.48 %), Fiesta (80.48 %) and Kendy (80.38 %) in the treatment T_5. The broccoli hybrid 'BR-60' was found to be significantly superior over Lucky, Kendy and Fiesta in the treatment T_5.

The broccoli hybrids had appreciable callus induction frequency in four treatments, T_5 (0.10 mg/l NAA and 0.50 mg/l 2,4-D), T_{20} (1.0 mg/l NAA and 0.50 mg/l 2,4-D), T_{10} (0.25 mg/l NAA and 0.50 mg/l 2,4-D) and T_{15} (0.50 mg/l NAA and 0.50 mg/l 2,4-D). It has been concluded that, NAA and 2,4-D are required for increasing callus induction frequency in broccoli. Androgenic response is known to vary considerably from plant to plant even in individual genotype (Keller and Armstrong, 1983 and Arnison and Keller, 1990) [9, 1] and furthermore, the influence of plant growth regulators such as auxins (2,4-D and NAA) and cytokinins (BAP) (Arnison *et al*. 1990b; Paksoy, 1995 and Ming *et al*. 1996) [3, 14, 11].

Effect of sucrose concentrations on callus induction frequency

In order to find the optimal sucrose concentration for callus induction, four different concentrations of sucrose *i.e.* 10, 12, 14 and 16 per cent were tested by using B_5 medium supplemented with best growth regulator combination T_5 (0.10 mg/l NAA and 0.50 mg/l 2,4-D). From the analysis of variance, the observed mean of square of F_1 hybrids, sucrose concentrations and their interaction *i.e.* F_1 hybrids × sucrose concentrations were found to be significant with.

Table 3: Effect of plant growth regulators (PGR's) on androgenic callus induction frequency (%) in four hybrids of broccoli

Hybrids (B)\GR (A)	Kendy	Lucky	BR-60	Fiesta	Mean (A)	CD (P ≤ 0.05) (A)
T1	45.45 (42.37)	51.64 (45.93)	48.40 (44.06)	57.75 (49.44)	50.81 (45.45)	1.87
T2	54.32 (54.32)	58.62 (49.95)	64.90 (53.65)	59.43 (50.42)	59.32 (50.37)	
T3	55.80 (55.81)	62.38 (52.16)	65.75 (54.18)	61.72 (51.78)	61.41 (51.61)	
T4	65.25 (65.25)	64.86 (53.63)	67.31 (55.12)	67.39 (55.27)	66.20 (54.49)	
T5	80.38 (80.38)	86.48 (68.42)	90.70 (72.22)	80.48 (63.79)	84.51 (67.04)	
T6	59.87 (50.68)	59.24 (50.33)	66.32 (54.51)	48.46 (44.10)	58.51 (49.90)	
T7	58.39 (49.81)	63.34 (52.74)	77.68 (61.84)	65.55 (54.09)	66.24 (54.62)	
T8	62.88 (52.48)	68.32 (55.74)	77.45 (61.81)	65.28 (53.89)	68.48 (55.98)	
T9	72.90 (58.70)	69.22 (56.39)	79.26 (62.91)	68.57 (55.89)	72.49 (58.47)	
T10	77.07 (61.51)	74.60 (59.91)	82.67 (65.90)	72.99 (58.67)	76.83 (61.50)	
T11	67.82 (55.47)	60.88 (51.34)	73.69 (59.12)	57.48 (49.32)	64.97 (53.81)	
T12	68.77 (56.00)	61.08 (51.38)	78.48 (62.76)	63.05 (52.57)	67.85 (55.68)	
T13	71.79 (57.90)	62.03 (51.94)	73.03 (59.40)	65.37 (54.42)	68.49 (55.92)	
T14	73.25 (58.87)	69.45 (56.44)	80.00 (63.52)	68.73 (56.00)	72.86 (58.71)	
T15	75.60 (60.50)	78.42 (62.41)	79.77 (63.25)	69.53 (57.09)	76.05 (60.81)	

T16	71.18 (57.51)	71.64 (57.80)	73.53 (58.72)	62.01 (52.84)	69.86 (56.72)	
T17	73.03 (58.70)	68.43 (55.84)	77.59 (61.67)	64.03 (54.03)	71.12 (57.56)	
T18	73.85 (59.26)	74.31 (59.56)	79.56 (63.12)	66.62 (54.72)	73.58 (59.16)	
T19	75.50 (60.31)	77.05 (61.36)	82.94 (65.64)	68.64 (55.93)	76.03 (60.81)	
T20	77.55 (61.70)	81.96 (65.02)	86.07 (68.06)	76.03 (60.70)	80.39 (63.87)	
Mean (B)	66.66 (55.57)	67.46 (55.67)	75.26 (60.18)	65.45 (54.14)	69.30 (56.35)	
Control	38.28 (38.22)	40.74 (39.66)	41.47 (40.09)	39.20 (38.76)	39.92 (39.18)	

* Values in parentheses are arc sine transformed values

regard to frequency of callus induction. The callus induction frequency of broccoli hybrids due to sucrose concentrations was found to be non-significant over the mean based on paired *t*-test. The highest mean callusing was obtained in 12 per cent concentration of sucrose (89.19 %) and was found to be significantly superior to 14 per cent (84.91 %) and 10 per cent (84.33 %) sucrose concentrations (Table 4). Out of the four hybrids of broccoli, the highest mean callusing (86.94 %) was observed in BR-60 followed by Lucky (86.19 %) and both of them were statistically *at par* with each other (Table 4.4). However, lowest callusing mean was obtained in Kendy (82.18 %). In F_1 hybrids × sucrose concentrations interaction, the highest callus induction frequency (90.83 %) was recorded in hybrid BR-60 and Lucky (89.36 %) at 12 per cent concentration of sucrose and these were found to be *at par* with each other.

In the present study, the sucrose concentrations influenced the callus induction frequency wherein, 12 per cent sucrose resulted in better callusing (in terms of brown callusing). However, high concentration of sucrose (16 %) led to abnormalities in callus development. High concentration of sucrose 15 per cent (Na *et al*. 2011) [13] and low concentration of sucrose six per cent (Mousa *et al*. 2014b) [12] have also been reported to increase the androgenic capacity of broccoli.

Table 4: Effect of sucrose concentrations on androgenic callus induction frequency (%) in four hybrids of broccoli

Hybrids (B)\ Sucrose concentrations (A)	Lucky	Kandy	BR-60	Fiesta	Mean (A)	CD (P ≤ 0.05) (A)
10 %	88.51 (68.45)	80.27 (63.63)	88.23 (69.94)	82.31 (65.13)	84.33 (66.68)	2.05
12 %	89.36 (70.96)	88.47 (70.15)	90.83 (72.37)	88.11 (69.83)	89.19 (70.81)	
14 %	85.58 (67.68)	82.28 (65.11)	86.57 (68.51)	85.21 (67.38)	84.91 (67.14)	
16 %	83.31 (65.89)	77.71 (61.82)	82.12 (64.99)	80.03 (63.46)	80.79 (64.01)	
Mean (B)	86.19 (68.18)	82.18 (65.03)	86.94 (68.81)	83.92 (66.36)	84.81 (67.06)	
Control	77.24 (61.51)	74.55 (59.70)	82.12 (64.99)	76.26 (60.84)	77.54 (61.71)	

Effect of pretreatment temperatures and durations on Anther culture efficiency

The analysis of variance on Anther culture efficiency revealed that the mean of squares due to F_1 hybrids, temperature pretreatments and durations were found to be significant. However, interaction effects between temperatures, durations and F_1 hybrids were also found to be significant except for duration × F_1 hybrids, temperatures × durations × F_1 hybrids interaction. Out of six heat and one cold pretreatment temperatures, the maximum anther culture efficiency was obtained at 35°C (76.14 %) followed by 32.5°C (56.53 %) and 30°C (21.70 %) (Table 4). The pretreatment temperature at 35°C was found to be significantly superior over 32.5°C and 30°C. In temperatures × durations interaction, the highest anther culture efficiency was observed at 35°C for 48 hours (80.46 %) followed by 35°C for 72 hours (74.24 %), 35°C for 24 hours (74.04 %), 32.5°C for 72 hours (62.92 %), 32.5°C for 48 hours (56.18 %) and 32.5°C for 24 hours (50.30 %). The pretreatment temperature with duration, 35°C for 48 hours was found to be significantly superior to the pretreatments, 35°C for 72 hours, 35°C for 24 hours, 32.5°C for 72 hours, 32.5°C for 48 hours and 32.5°C for 24 hours. However, no response was observed at 0-4°C (0 %) and 25°C (0 %) for 24, 48 and 72 hours. The results clearly indicate that the anther culture efficiency of all the hybrids of broccoli got enhanced when anthers were subjected to optimal pretreatment temperatures along with increased pretreatment durations.

Out of the four hybrids, the highest anther culture efficiency was observed in BR-60 (27.99 %) followed by Fiesta (26.34 %) and Kendy (24.40 %) (Table 5). In temperatures × F_1 hybrids interaction, the highest anther culture efficiency was observed at 35°C in BR-60 (79.49 %) followed by at 35°C in Fiesta (79.38 %), at 35°C in Kendy (73.09 %), at 35°C in Lucky (72.67 %), at 32.5°C in BR-60 (69.17 %), at 32.5°C in Fiesta (64.09 %), at 32.5°C in Kendy (55.97 %) and at 32.5°C in Lucky (36.87 %). BR-60 and Fiesta were found to be *at par* with each other at 35°C pretreatment temperature with regard to Anther culture efficiency. However, lowest anther culture efficiency was observed at 0-4°C (0 %) followed by 25°C (0 %) in four hybrids of broccoli. These results indicate that the optimum pretreatment temperature in Anther culture of broccoli has also been influenced by the genotype *i.e.* hybrids.

Table 5: Effect of pretreatment temperatures (cold and heat shock) and different incubation periods on Anther culture efficiency (%)

Durations (hours) (B)\Pretreatments (°C) (A)	24 hours	48 hours	72 hours	Mean (A)	CD (P ≤ 0.05) (A)
0°C	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	1.0
25°C	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	
27.5°C	16.24 (23.76)	19.86 (26.47)	22.35 (28.21)	19.44 (26.16)	
30°C	18.13 (25.20)	21.61 (27.70)	25.34 (30.23)	21.70 (27.76)	
32.5°C	50.30 (45.17)	56.18 (48.55)	62.92 (52.49)	56.53 (48.75)	
35°C	74.04 (59.37)	80.46 (63.77)	74.24 (59.50)	76.14 (60.76)	
37.5°C	02.85 (09.71)	01.37 (06.72)	00.32 (03.26)	01.63 (07.34)	

Mean (B)	23.08 (28.71)	25.64 (30.42)	26.45 (30.95)	25.06 (30.40)	
CD (P ≤ 0.05) (A×B)	0.48				

Values in parentheses are arc sine transformed values

Out of three durations of pretreatment, the maximum anther culture efficiency was observed for 72 hours (26.45 %) followed by 48 hours (25.64 %) and 24 hours (23.08 %). The pretreatment duration of 72 hours and 48 hours were found to be statistically *at par* with each other (Table 5). In durations × F_1 hybrids interaction, the highest anther culture efficiency was observed for 72 hours in BR-60 (28.61 %) followed by 48 hours in BR-60 (28.37 %), for 72 hours in Fiesta (27.44 %), for 48 hours in Fiesta (27.12 %), for 24 hours in BR-60 (26.90 %) and for 72 hours in Kendy (26.53 %).

In temperatures × durations × F_1 hybrids interaction, the highest anther culture efficiency (84.12 %) was observed in BR-60 at 35°C for 48 hours followed by Fiesta (82.17 %) and Lucky (79.25 %) (Table 6). However, exposure of anthers to 0-4°C and 25°C for different durations *viz.*, 24, 48 and 72 hours showed no effect on anther culture efficiency of all the F_1 hybrids of broccoli under study. Anther culture efficiency was comparatively reduced on increasing.

Table 6: Effect of pretreatment temperatures (cold and heat shock) and four hybrids of broccoli on anther culture efficiency (%)

Hybrids (C)\Pretreatments (°C) (A)	BR60	Fiesta	Kendy	Lucky	Mean (A)
0°C	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)
25°C	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)	00.00 (00.00)
27.5°C	20.60 (26.99)	18.31 (25.33)	19.10 (25.91)	19.76 (26.39)	19.44 (26.16)
30°C	25.03 (30.02)	20.56 (26.96)	21.20 (27.42)	19.99 (26.56)	21.70 (27.76)
32.5°C	69.17 (56.27)	64.09 (53.18)	55.97 (48.43)	36.87 (37.39)	56.53 (48.75)
35°C	79.49 (63.28)	79.38 (62.99)	73.09 (58.75)	72.67 (58.48)	76.14 (60.76)
37.5°C	01.64 (07.36)	02.03 (08.19)	01.47 (06.96)	01.39 (06.77)	01.63 (07.33)
Mean (C)	27.99 (31.94)	26.34 (30.88)	24.40 (29.60)	21.53 (27.65)	25.06 (30.40)
CD (P ≤ 0.05) (C)	0.93				
CD (P ≤ 0.05) (A×C)	0.55				

Values in parentheses are arc sine transformed values

Table 7: Effect of different incubation periods and four hybrids of broccoli on anther culture efficiency (%)

Hybrids (C)\Durations (hours) (B)	BR60	Fiesta	Kendy	Lucky	Mean (B)	CD (P ≤ 0.05) (B)
24 hours	26.90 (31.24)	24.56 (29.71)	21.93 (27.92)	18.92 (25.78)	23.08 (28.71)	0.93
48 hours	28.37 (32.18)	27.12 (31.38)	24.68 (29.79)	22.42 (28.26)	25.64 (30.42)	
72 hours	28.61 (32.34)	27.44 (31.59)	26.53 (31.00)	23.24 (28.82)	26.45 (30.95)	
Mean (C)	27.99 (31.94)	26.34 (30.88)	24.40 (29.60)	21.53 (27.65)	25.06 (30.40)	
CD (P ≤ 0.05) (B×C)	NS					

Values in parentheses are arc sine transformed values

Duration and temperature of pretreatment. These results are in conformity with results obtained by Keller and Armstrong (1983) [9] who found that the anther culture efficiency of broccoli hybrids increased at optimal temperature (at 35°C for 48 hours) and decreased at higher temperature (at 40°C for 24 hours). Arnison *et al*. (1990a) [2] also reported that the optimal pretreatment temperature at 35°C for 48 hours was required to induce androgenesis in broccoli.

Effect of plant growth regulators on calli regeneration and root formation

The regeneration of callus *via* organogenesis was attempted on B_5 medium supplemented with three per cent sucrose, 0.8 per cent agar and different plant growth regulator combinations *viz.*, auxins (2,4-D, NAA and IAA) and cytokinins (BAP and Kinetin) (Table 3). The calli regeneration frequency of broccoli hybrids due to plant growth regulator combinations and the interaction effects between F_1 hybrids and plant growth regulator combinations were found to be significant. Calli regeneration frequency of all the four hybrids of broccoli in combination HM_5 was found to be significant over the mean based on paired *t*-test.

Of the six combinations of plant growth regulators used, the highest regeneration frequency of calli (36.88 %) was observed in combination HM_5 (B5 + 0.25 mg/l IAA + 1.0 mg/l BAP + 2.0 mg/l Kin) followed by HM_6 (B5 + 0.25 mg/l NAA + 1.0 mg/l BAP + 2.0 mg/l Kin) (28.76 %) and HM_3 (B5 + 0.50 mg/l NAA + 1.0 mg/l BAP) (28.39 %) (Table 7). It indicated that shoot differentiation may be obtained by the addition of cytokinins (Kinetin and BAP) in combination with a small quantity of auxins (NAA and IAA). Out of four broccoli hybrids, Kendy gave maximum calli regeneration frequency (25.21 %) (Table 7).

Plant growth regulators × F_1 hybrids interactions revealed the highest calli regeneration frequency in hybrid Kendy (42.86 %) when calli were transferred to HM_5 combination (B5 + 3 % + 0.25 mg/l IAA + 1.0 mg/l BAP + 2.0 mg/l Kin), followed by Lucky (37.50 %) in HM_5, BR-60 (35.71 %) in HM_5, Kendy (32.50 %) in HM_3 and Fiesta (31.43 %) in HM_5 (Table 4.7). Daniela *et al*. (2005) [5]; Gorecka *et al*. (2007) [8] and Qin *et al*. (2007) [15] found that BAP and Kinetin were most effective in enhancing shoot multiplication and elongation in *Brassica oleracea*. However, Mousa *et al*. (2014b) [12] observed that when only BAP at different concentrations was used it was found to stimulate shoot regeneration from the callus. It may be concluded that the shoot initiation frequency may vary across genotypes to species.

The mean of broccoli hybrids was found to be statistically non-significant over the mean when compared by paired *t*-test (Table 8). It is concluded that all the four hybrids of broccoli exhibited similar response to rhizogenesis. Thin, long and well spread roots (rhizogenesis) were observed when meristemoids were transferred on B5 medium supplemented with 0.5 mg/l NAA and three per cent sucrose (Plate IV). Highest non-rhizogenesis frequency was observed in hybrid Lucky (16.67 %) followed by Fiesta (15.38 %). However, broccoli hybrids exhibited 14.57 % non-rhizogenesis from these green calli meristemoids.

Table 8: Effect of pretreatment temperatures (cold and heat shock) with different incubation periods on Anther culture efficiency (%) in four hybrids of broccoli

Pre-treatments (°C)	Durations (hours)	F_1 hybrids								Mean (A×B×C)	Arc	Control (25°C)	CD (P ≤ 0.05) (A×B×C)
		BR60	Arc	Fiesta	Arc	Kendy	Arc	Lucky	Arc				
0°C	24 h	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	NS
	48 h	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	
	72 h	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	
25°C	24 h	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	
	48 h	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	
	72 h	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	(00.00)	00.00	
27.5°C	24 h	18.35	(25.36)	16.55	(24.01)	13.60	(21.64)	16.45	(23.93)	16.24	(23.76)	00.00	
	48 h	20.17	(26.69)	18.41	(25.41)	20.48	(26.91)	20.39	(26.84)	19.86	(26.47)	00.00	
	72 h	23.78	(29.19)	19.97	(26.54)	23.23	(28.81)	22.42	(28.26)	22.35	(28.21)	00.00	
30°C	24 h	22.74	(28.48)	19.30	(26.06)	16.26	(23.78)	14.22	(22.15)	18.13	(25.20)	00.00	
	48 h	24.66	(29.77)	20.40	(26.85)	21.05	(27.31)	20.32	(26.79)	21.61	(27.70)	00.00	
	72 h	27.69	(31.75)	21.98	(27.96)	26.27	(30.83)	25.43	(30.28)	25.34	(30.23)	00.00	
32.5°C	24 h	66.37	(54.56)	54.35	(47.50)	48.81	(44.32)	31.65	(34.23)	50.30	(45.17)	00.00	
	48 h	68.6	(55.92)	67.35	(55.15)	53.18	(46.82)	35.60	(36.63)	56.18	(48.55)	00.00	
	72 h	72.54	(58.40)	70.57	(57.15)	65.21	(53.86)	43.37	(41.19)	62.92	(52.49)	00.00	
35°C	24 h	78.09	(62.09)	78.17	(62.15)	71.95	(58.02)	67.93	(55.51)	74.04	(59.37)	00.00	
	48 h	84.12	(66.52)	82.17	(65.02)	76.31	(60.87)	79.25	(62.90)	80.46	(63.77)	00.00	
	72 h	76.26	(60.84)	78.87	(62.63)	70.99	(57.41)	70.83	(57.31)	74.24	(59.50)	00.00	
37.5°C	24 h	02.59	(09.26)	03.93	(11.43)	02.67	(09.40)	02.19	(08.51)	02.85	(09.71)	00.00	
	48 h	00.90	(05.44)	01.48	(06.99)	01.74	(07.58)	01.36	(06.70)	01.37	(06.72)	00.00	
	72 h	00.00	(00.00)	00.68	(04.73)	00.00	(00.00)	00.61	(04.48)	00.32	(03.26)	00.00	
Mean		27.95	(31.91)	26.39	(30.91)	24.37	(29.58)	21.52	(27.64)	25.06	(30.40)	00.00	

Values in parentheses are arc sine transformed value

Table 9: Effect of different plant growth regulator combinations on calli regeneration frequency (%) in four hybrids of broccoli

Hybrids (A)\Regeneration medium (B)	Callus regeneration frequency (%)				Mean (B)
	BR-60	Kendy	Lucky	Fiesta	
HM_1 (0.5 mg/l 2, 4-D + 1.0 mg/l BAP)	15.00 (22.79)	12.00 (20.27)	10.00 (18.43)	15.00 (22.79)	13.00 (21.13)
HM_2 (0.5 mg/l 2, 4-D + 2.0 mg/l BAP)	15.00 (22.79)	16.00 (23.58)	13.33 (21.41)	15.00 (22.79)	14.83 (22.65)
HM_3 (0.5 mg/l NAA + 1.0 mg/l BAP)	30.00 (33.21)	32.50 (34.76)	28.57 (32.31)	22.50 (28.32)	28.39 (32.20)
HM_4 (0.5 mg/l NAA + 2.0 mg/l BAP)	16.67 (24.10)	17.14 (24.46)	14.29 (22.21)	30.00 (33.21)	19.53 (26.22)
HM_5 (0.25 mg/l IAA + 1.0 mg/l BAP + 2.0 mg/l Kin)	35.71 (36.70)	42.86 (40.90)	37.50 (37.76)	31.43 (34.10)	36.88 (37.39)
HM_6 (0.25 mg/l NAA + 1.0 mg/l BAP + 2.0 mg/l Kin)	28.57 (32.31)	30.77 (33.69)	28.57 (32.31)	27.14 (31.40)	28.76 (32.43)
Mean (A)	23.49 (28.99)	25.21 (30.14)	23.51 (28.00)	22.04 (29.01)	27.18 (31.42)

1. CD (P ≤ 0.05) = 2.14 (PGR's)
2. CD (P ≤ 0.05) = 2.03 (F_1 hybrids)
3. CD (P ≤ 0.05) = 1.49 (Interaction)
4. Values in parentheses are arc sine transformed values

Table 10: Rhizogenesis from meristemoids in four hybrids of broccoli by using B_5 medium supplemented with three per cent sucrose and 0.50 mg/l NAA

F_1 hybrids	No. of meristemoids regenerated	No. of meristemoids without rhizogenesis	Non-regenerants frequency (%)
BR-60	68	8	11.76
Kendy	76	11	14.47
Lucky	72	12	16.67
Fiesta	65	10	15.38
Mean	70.25	10.25	14.57
CD (P ≤ 0.05)			NS
S.E. (m) ±			01.05

Significant at *t*-value (P ≤ 0.05) = NS

Anther culture response

The best hormonal combination T_5 (0.10 mg/l NAA and 0.50 mg/l 2,4-D) and best concentration of sucrose 12 per cent were subjected to pretreatment temperature at 35°C for 24, 48 and 72 hours for the stimulation of anthers. These stimulated calli were transferred to the regeneration medium for shooting and rooting.

Data revealed that the highest frequency of meristemoids was recorded in Kendy (19.29 %) followed by BR-60 (18.23 %) and Fiesta (18.16 %) and Lucky (15.86 %) (Table 9). It may be concluded that the mode of development of meristemoids from calli is highly genotype specific. This variation might be due to physiological status of the donor plants at the time of bud collection, stage of pollen development, growth conditions and genetic background of the donor plant (Arnison and Keller, 1990; Duijs *et al*. 1992; Takahata *et al*.

1993; Paksoy *et al*. 1995; Vyvadilova *et al*. 1998 and Chanana *et al*. 2005) [1, 6, 16, 14, 17, 4]. The analysis of variance revealed that the contribution of all the four hybrids of broccoli was statistically significant. However, the mean of broccoli hybrids was found to be statistically non-significant over the mean when compared by paired *t*-test.

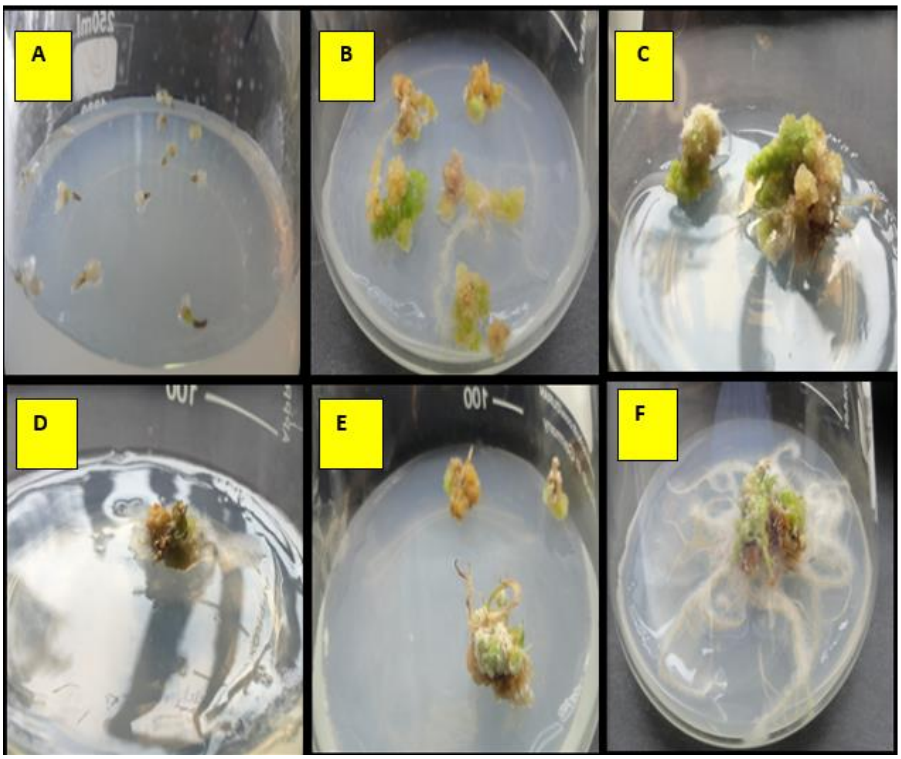

Fig 1: a. Brown callus (B5 + 0.5 mg/l 2,4-D + 1.0 or 2.0 mg/l BAP)
b. Green callus (B5 + 0.5 mg/l NAA + 1.0 or 2.0 mg/l BAP)
c. Green calli regenerates with few roots (B5 + 0.25 mg/l NAA + 1.0 mg/l BAP + 2.0 Kin)
d. Initiation of shooting (0.25 mg/l IAA + 1.0 mg/l BAP + 2.0 Kin)
e and **f**. Initiation of rooting (B5 + 0.5 mg/l NAA)

Table 11: Overall response of anther culture in four hybrids of broccoli

F_1 hybrids	No. of anthers plated	No. of stimulated anthers (%)	Callus induction frequency (%)	No. of calli cultured (%)	No. of meristemoids regenerated (%)	Anther culture response (%)
BR 60	373	297 (79.62)	290 (77.75)	250 (67.02)	68 (27.20)	18.23
Kendy	394	287 (72.84)	280 (71.07)	260 (65.99)	76 (27.14)	19.29
Lucky	454	330 (72.69)	320 (70.48)	280 (61.67)	72 (25.71)	15.86
Fiesta	358	284 (79.33)	275 (76.82)	250 (69.83)	65 (26.00)	18.16
Overall response	1579	1198 (75.87)	1165 (73.78)	1040 (65.86)	281 (27.02)	17.80

Significant at *t*-value ($P \leq 0.05$) = NS (Anther culture response)
Values in parentheses are frequencies of parameters

Statement of Conflict: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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