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Efficient microspore embryogenesis for the development of doubled haploid plants in broccoli

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ABSTRACT

The most acceptable strategic tool for creating homozygous inbred lines is the development of doubled haploids (DH's) by microspore culture technique. The current research focuses on developing DH's by microspore culture through optimising important factors limiting its efficiency. The effect of carbon sources, temperature shock for embryogenic response and effect of different plant growth regulators (PGRs) on effective regeneration was studied in four commercial varieties/hybrids of broccoli viz., Palam Vichitra, Lucky, Palam Samridhi and Palam Kanchan. Interaction between variety and carbon source (V × C) revealed the maximum number of embryos per plate (330.00) in genotype V3 (Palam Samridhi) when supplemented with 13% sucrose (C2). At the same time, the interaction between varieties and temperature (V × T) revealed V3 × T3 with significantly highest embryo yield (93.11). Interaction between temperature and carbon sources (T × C) revealed the maximum number of embryos per plate with treatment combination T3 × C2 (382.67). Overall, the highest embryogenic response (715.00) was obtained in Palam Samridhi (V3) genotype when supplemented with 13% sucrose (C2) at temperature treatment T3 (4°C-48 hr + 32°C-24 hr + 25°C-Cont). Further, maximum regeneration of embryos (75.00%) was recorded in genotype V3 when supplemented with 1.0 mg/l BAP (H5). Ploidy analysis of 952 microspore-derived plants from four broccoli genotypes revealed that 52.31% of plants were haploids, 43.17% of plants were doubled haploids, 1.05% of plants were triploids, 2.84% were tetraploids, and 1.20% were mixoploid. The present findings will accelerate doubled haploid based hybrid breeding in broccoli.

Keywords: *Brassica oleracea* var. *italica* L., Haploids, Ploidy analysis, Doubled haploids, Microspore culture

INTRODUCTION

Among the cole crops, broccoli deserves great breeding attention by the seed companies due its anti-carcinogenic properties, and other bioactive compounds. This has led to an increase in the area of production in recent years. At present, most developed countries widely use the technologies for the production of doubled haploids to accelerate breeding. *In vitro* microspore culture for the development of doubled haploid (DH) plants is the leading method among such technologies. The DH technology adds benefit of having homozygous lines after one generation only, as compared to 7-8 generations via conventional breeding. *In vitro* culturing of anthers of Datura by Guha and Maheshwari (9) led the pathway for the development of haploids. Successful microspore culture, in different broccoli genotypes, was described by Takahata and Keller (12).A variety of factors, such as donor plant growth conditions, genotypes, microspore developmental stages, culture medium composition and cultivation conditions affect the efficiency of microspore embryogenesis. An optimal value for the listed hurdle causing factors is the core requirement for effective embryogenesis. However the effective embryogenesis and number of embryos are still the bottleneck in *Brassica oleracea* (Zhang *et al*., 13). Therefore, the main objective of present study was to standardize a protocol for efficient microspore embryogenesis for the development of doubled haploid lines and their use in hybrid breeding of broccoli. The resultant improved protocol took into account the growth conditions, proper stage, pre-treatment, induction treatment, alterations in carbon sources, and alterations in regeneration media.

MATERIALS AND METHODS

Four varieties/hybrids namely: Palam Vichitra, Lucky, Palam Samridhi, and Palam Kanchan were used in the present study and designated as V_1, V_2, V_3, and V_4, respectively. Seeds were sown in greenhouse under controlled conditions (25/20°C, day/night temperature, 16 h photoperiod) at ACSEN HYVEG Pvt. Ltd. situated at Kullu (H.P. India), during August, 2019. Vernalization was done in greenhouse (4°C, 12 h photoperiod) during December to January. For bolting plants were maintained at 15/10°C day/night temperatures with 14/10 hours photoperiod. Buds for experimentation were taken from the first flush of flowering in the month of March 2020.

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Flower buds of size from 3.5 mm, 3.75 mm, 4.0 mm, and 4.75 mm were used. Microspore developmental stage and vitality was checked by squeezing out anthers carefully in 15 µl of DAPI solution and 20 µl of FDA-PI working solution in slides respectively. Staining was observed under fluorescent microscope to select buds having maximal number of microspores at the late uninucleate stage (80-90%), with about 10-20% binucleate and with maximum viability (70:30). In order to correlate the cold shock effect, half of the selected buds of each entry were kept in refrigerator at 4°C for 48 hours. Buds were surface-sterilized with 2-3 drops of Tween 20 for 15 min, 70% ethanol for one minute, 4% sodium hypochlorite solution for 10 minutes, and then rinsed three times in sterile water before experimentation.

To establish the relationship between different carbon sources with effective concentration, liquid NLN media was supplemented with different carbon sources namely sucrose, glucose, and maltose, each source with three different concentrations 6%, 13%, and 15%, respectively. Nine varied media concentrations from C_1 to C_9 were composed (Cristea *et al*., 5). For isolation, buds were squashed in B5 washing media (Gamborg *et al*., 6) using glass rod to obtain microspore suspension which was filtered through 40 µm nylon mesh. The resultant suspension was distributed at equal volumes in three centrifuge tubes and centrifuged three times at 1250 rpm at 4°C for 5 minutes by resuspending in B5 washing media. After third washing, resuspension was done in NLN induction media of respective carbon sources. Microspores were thus suspended in the required amount in different NLN induction medium (C1-C9). Microspore density was determined with Neubauer hemocytometer by adjusting density to 4×10^4 microspores/ml and 10 ml of microspore suspension was plated in 90 mm petri dishes (Bhatia *et al*., 2). 0.1 ml of warm activated charcoal containing low melting point agarose was added to each petri dish (Gland *et al*., 7). Two different temperature regimes were designed i.e., 32°C- 24 hr. + 25°C (T_1) and 32°C- 48 hr. + 25°C (T_2). Three replicas of each temperature were prepared with individual carbon sources from C_1 to C_9 and incubated in regimes as designed. Cold pre-treated buds (4°C) for 48 hours kept in refrigerator were isolated and incubated in similar manner with same number of replicas in two different temperature regimes *i.e.,* 32°C for 24 hours and 25°C (T_3), and 32°C for 48 hours and 25°C (T_4).

Different stages of microspore development for embryogenesis were observed under inverted microscope (Figure 3). After two weeks, plates were transferred to rotary shaker at 70 rpm (gentle) 25°C in dark for one week. Pin shaped embryos after three weeks of start culture were transferred to B5 regeneration media having combination of 3 hormones Zeatin, BAP (Benzyl amino purine), GA_3 (Gibberellic acid) at 6 different concentrations Zeatin: 0.5 mg/l (H_2), Zeatin: 1.0 mg/l (H_3), BAP: 0.5 mg/l (H_4), BAP: 1.0 mg/l (H_5), GA3: 0.1 mg/l (H_6), GA3: 0.2 mg/l (H_7), followed by Control: No hormone addition (H_1), and transferred to MS rooting medium according to Bhattacharya (3). The ploidy levels of *in vitro* microspore-derived plants were analyzed using flow cytometry (FCM) analysis. For sampling, leaves of 2-3 mm were chopped in 400 µl of nuclei extracting buffer (Solution A of CyStain UV Precise P Kit, Sysmex) followed by adding 1,600 µl of the DAPI staining buffer (Solution B of Kit). The suspension was filtered through a 30 µm nylon mesh filter (Cell Trics TM, Sysmex). The rooted plants as per their ploidy level were transferred to the greenhouse for further acclimatization. The experimental data were subjected to analysis of variance (ANOVA) using OPSTAT software and to Duncan's multiple range test with SPSS-16 program for Windows.

RESULTS AND DISCUSSION

It was observed that buds with the highest percentage of microspores at late uninucleate to early binucleate stage along with maximum viable microspores were most responsive towards embryogenesis (Ferrie and Caswell, 10). Figure 1 shows the DAPI staining of microspore stage whereas Figure 2 shows the viable microspores. Genotype V_1 and V_4, contained prerequisite buds at late uninucleate to early bi-nucleate stage with maximum viability when the bud size was in the range of 4.0–4.5 mm while in genotype V_2 and V_3 similar observation were made when the bud size was 4.5–4.75 mm. Different stages of microspore and healthy embryos were observed under an inverted microscope (Figure 3).

Our study confirmed high embryogenic capacity in all four broccoli accessions. Out of four genotypes, V_3 (Palam Samridhi) was a highly responsive genotype in every parameter of optimization undertaken. The significant effect of varieties, temperature treatments and carbon sources was observed on microspore embryogenesis in broccoli (Table 1). The highest embryogenic response (715.00) was obtained in the Palam Samridhi (V_3) variety of broccoli with 13 percent sucrose (C_2) at temperature T_3 (4°C-48 hr + 32°C-24 hr + 25°C-Cont) and this treatment was found significantly superior to rest of the treatment combinations under study.

Significant variations in microspore embryogenesis with three set of interactions undertaken for optimization

Table 1. Effect of varieties, temperature treatments, and carbon sources on microspore embryogenesis in broccoli.

Variety (V)	Temperature (T) / Carbon source (C)	Number of embryos per petri dish				
		T_1 (32°C-24 hr + 25°C-Cont)	T_2 (32°C-48 hr + 25°C-Cont)	T_3 (4°C-48 hr + 32°C-24 hr + 25°C-Cont)	T_4 (4°C-48 hr + 32°C-48 hr + 25°C-Cont)	Mean
Palam Vichitra (V_1)	C_1 (Sucrose-6%)	6.33±0.88 ^{ijklm}	4.33±1.86 ^{g}	15.67±1.76 ^{ij}	5.67±1.67 ^{hijklm}	8.00±0.90 ^{ijk}
	C_2 (Sucrose-13%)	32.67±2.19 ^{g}	36.00±3.61 ^{de}	44.67±1.67 ^{g}	32.33±0.88 ^{de}	36.42±1.37 ^{f}
	C_3 (Sucrose-15%)	36.00±2.89 ^{g}	17.67±1.86 ^{f}	36.67±3.38 ^{h}	28.33±0.88 ^{e}	29.67±1.74 ^{g}
	C_4 (Glucose-6%)	0.33±0.33 ^{m}	0.00±0.00 ^{g}	1.33±1.33 ^{no}	0.00±0.00 ^{m}	0.42±0.30 ^{n}
	C_5 (Glucose-13%)	3.33±0.88 ^{jklm}	0.00±0.00 ^{g}	3.00±0.58 ^{mno}	2.33±0.88 ^{jklm}	2.17±0.36 ^{mn}
	C_6 (Glucose-15%)	0.33±0.33 ^{m}	0.00±0.00 ^{g}	1.33±0.88 ^{no}	0.00±0.00 ^{m}	0.42±0.17 ^{n}
	C_7 (Maltose-6%)	1.33±0.88 ^{lm}	1.00±0.00 ^{g}	6.67±0.88 ^{klmno}	1.33±0.33 ^{klm}	2.58±0.51 ^{mn}
	C_8 (Maltose-13%)	8.33±1.20 ^{hijk}	4.67±0.88 ^{g}	14.67±1.76 ^{j}	6.67±1.20 ^{hijkl}	8.58±0.60 ^{ij}
	C_9 (Maltose-15%)	2.33±0.67 ^{klm}	1.33±0.86 ^{g}	8.00±0.58 ^{klmn}	3.33±0.33 ^{ijklm}	3.75±0.14 ^{klmn}
Lucky (V_2)	C_1 (Sucrose-6%)	31.33±1.86 ^{g}	9.00±1.53 ^{g}	22.33±1.76 ^{i}	11.00±1.73 ^{gh}	18.42±1.62 ^{h}
	C_2 (Sucrose-13%)	196±3.79 ^{b}	48.33±3.76 ^{c}	317.33±5.81 ^{c}	50.67±2.33 ^{c}	153.08±0.36 ^{c}
	C_3 (Sucrose-15%)	86.00±3.06 ^{e}	20.67±2.33 ^{f}	99.67±3.71 ^{e}	15.33±2.03 ^{g}	55.42±1.21 ^{e}
	C_4 (Glucose-6%)	1.33±0.88 ^{lm}	0.00±0.000 ^{g}	2.33±1.20 ^{no}	1.33±0.33 ^{klm}	1.25±0.58 ^{mn}
	C_5 (Glucose-13%)	6.00±0.58 ^{ijklm}	3.00±1.53 ^{g}	5.33±0.33 ^{lmno}	2.00±1.16 ^{jklm}	4.08±0.74 ^{klmn}
	C_6 (Glucose-15%)	1.00±0.58 ^{lm}	1.33±0.33 ^{g}	0.67±0.33 ^{o}	1.00±0.58 ^{lm}	1.00±0.14 ^{mn}
	C_7 (Maltose-6%)	1.00±0.58 ^{lm}	0.33±0.33 ^{g}	2.33±1.20 ^{no}	1.00±0.58 ^{lm}	1.17±0.30 ^{mn}
	C_8 (Maltose-13%)	10.00±1.00 ^{hi}	5.33±0.88 ^{g}	8.00±1.53 ^{klmn}	7.00±1.16 ^{hijk}	7.58±0.82 ^{ijk}
	C_9 (Maltose-15%)	2.33±0.67 ^{klm}	1.00±0.58 ^{g}	3.67±0.88 ^{mno}	1.33±0.67 ^{klm}	2.08±0.44 ^{mn}
Palam Samridhi (V_3)	C_1 (Sucrose-6%)	12.67±1.45 ^{h}	3.67±2.33 ^{g}	9.67±4.81 ^{jklm}	7.33±0.88 ^{hij}	8.33±2.02 ^{ij}
	C_2 (Sucrose-13%)	489.00±6.66 ^{a}	56.67±12.01 ^{b}	715.00±2.65 ^{a}	59.33±4.06 ^{b}	330.00±4.77 ^{a}
	C_3 (Sucrose-15%)	94.00±2.65 ^{d}	40.00±2.89 ^{d}	79.33±1.86 ^{f}	20.33±1.45 ^{f}	58.42±1.09 ^{de}

Contd...

Table 1 contd...

Variety (V)	Temperature (T) / Carbon source (C)	Number of embryos per petri dish				
		T_1 (32°C-24 hr + 25°C-Cont)	T_2 (32°C-48 hr + 25°C-Cont)	T_3 (4°C-48 hr + 32°C-24 hr + 25°C-Cont)	T_4 (4°C-48 hr + 32°C-48 hr + 25°C-Cont)	Mean
	C_4 (Glucose-6%)	1.33±0.88 ^{lm}	0.00±0.00 ^{g}	2.00±1.00 ^{no}	0.00±0.00 ^{m}	0.83±0.44 ^{mn}
	C_5 (Glucose-13%)	7.33±1.20 ^{hijkl}	3.33±1.76 ^{g}	6.67±1.20 ^{klmno}	3.33±0.33 ^{ijklm}	5.17±0.80 ^{jklm}
	C_6 (Glucose-15%)	2.00±0.58 ^{klm}	1.00±1.00 ^{g}	2.67±0.67 ^{no}	0.33±0.33 ^{m}	1.50±0.25 ^{mn}
	C_7 (Maltose-6%)	3.00±1.00 ^{jklm}	2.33±1.20 ^{g}	4.33±1.33 ^{mno}	0.33±0.33 ^{m}	2.50±0.52 ^{mn}
	C_8 (Maltose-13%)	9.33±0.88 ^{hij}	8.67±1.45 ^{g}	12.67±1.86 ^{jk}	9.00±2.31 ^{hi}	9.92±1.31 ^{i}
	C_9 (Maltose-15%)	5.00±1.00 ^{ijklm}	3.00±1.73 ^{g}	5.67±1.45 ^{lmno}	2.00±2.00 ^{jklm}	3.92±1.31 ^{klmn}
Palam Kanchan (V_4)	C_1 (Sucrose-6%)	9.00±1.16 ^{hij}	0.00±0.00 ^{g}	6.67±0.88 ^{klmno}	0.33±0.33 ^{m}	4.00±0.38 ^{klmn}
	C_2 (Sucrose-13%)	135.67±2.96 ^{c}	70.00±4.58 ^{a}	453.67±2.73 ^{b}	80.67±4.18 ^{a}	185.00±2.41 ^{b}
	C_3 (Sucrose-15%)	57.67±1.45 ^{f}	29.00±3.06 ^{e}	114.00±2.52 ^{d}	36.67±4.41 ^{d}	59.33±2.76 ^{d}
	C_4 (Glucose-6%)	2.00±0.58 ^{klm}	0.00±0.00 ^{g}	1.00±0.58 ^{no}	0.00±0.00 ^{m}	0.75±0.25 ^{mn}
	C_5 (Glucose-13%)	6.00±2.08 ^{ijklm}	0.00±0.00 ^{g}	5.67±1.76 ^{lmno}	0.00±0.00 ^{m}	2.92±0.82 ^{lmn}
	C_6 (Glucose-15%)	1.00±1.00 ^{lm}	0.00±0.00 ^{g}	2.33±0.88 ^{no}	0.00±0.00 ^{m}	0.83±0.30 ^{mn}
	C_7 (Maltose-6%)	4.00±1.16 ^{ijklm}	0.00±0.00 ^{g}	2.00±1.00 ^{no}	1.00±1.00 ^{lm}	1.75±0.14 ^{mn}
	C_8 (Maltose-13%)	9.33±1.45 ^{hij}	0.00±0.00 ^{g}	11.67±1.76 ^{jkl}	6.67±3.71 ^{hijkl}	6.92±1.23 ^{ijkl}
	C_9 (Maltose-15%)	6.33±0.88 ^{ijklm}	0.00±0.00 ^{g}	6.00±0.58 ^{klmno}	4.00±1.53 ^{ijklm}	4.08±0.42 ^{klmn}
	Mean	35.57±0.31 ^{b}	10.32±0.81 ^{c}	56.52±0.72 ^{a}	11.17±0.39 ^{c}	

Factors	$C.D._{(0.01)}$	±SE(d)	±SE(m)
Variety (V)	0.97	0.49	0.35
Carbon Source (C)	1.45	0.74	0.52
Temperature (T)	0.97	0.49	0.35
Interaction (V × C)	2.91	1.48	1.04
Interaction (V × T)	1.94	0.98	0.70
Interaction (C × T)	2.91	1.48	1.04
Interaction (V × C × T)	5.82	2.96	2.09

Mean values in each column (for each temperature treatment) and overall mean of each temperature treatment (in row) followed by the same lower case letters were not significantly different at P ≤ 0.05 according to Duncan's multiple range test

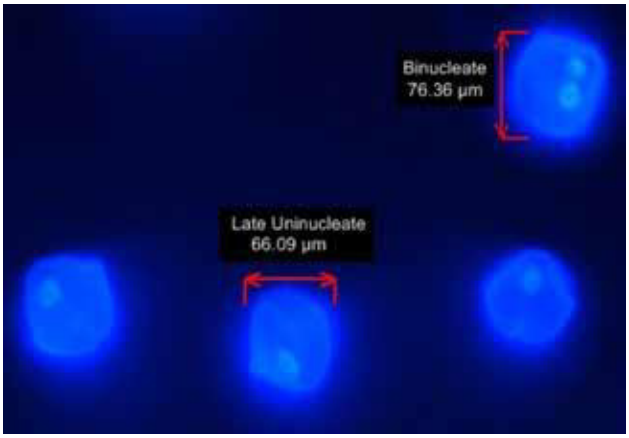

Fig. 1. DAPI staining showing binucleate and late uninucleate developmental stage.

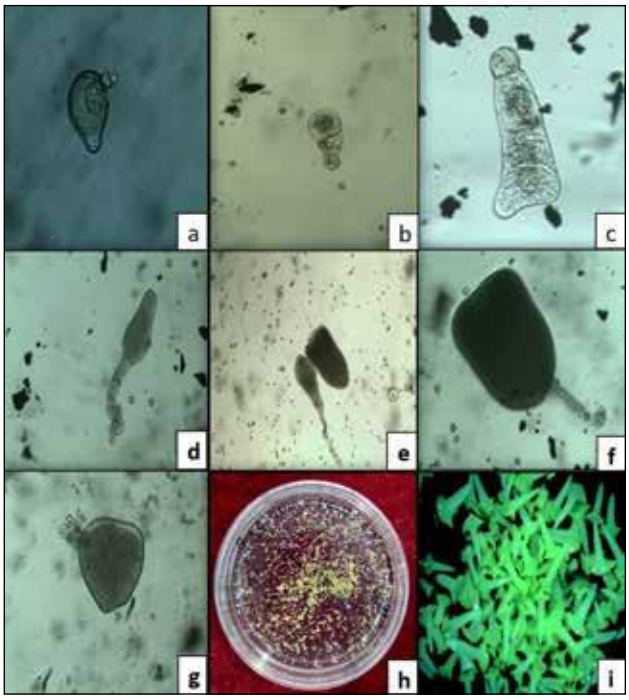

Fig. 3. Different stages of *Brassica oleracea* var. *italica* microspore cultures.

(a) Microspore with broken exine 3-7 days. (b, c, d) multicellular embryo with developing suspensor 7-10 days stage. (e, f, g) Globular, torpedo and heart stage of embryo development 15-20 days. (h) cotyledonary embryo in petri dish 20-25 days. (i) magnified cotyledonary stage.

were observed (Table 1). The significant effects of three carbon sources on their optimal concentrations, as well as their interaction across genotypes, were investigated (V × C). Palam Samridhi (V_3) with 13 percent sucrose (C_2) had the largest number of embryos per plate 330.00 (Figure 4). Different temperature treatment regimes and their interactions

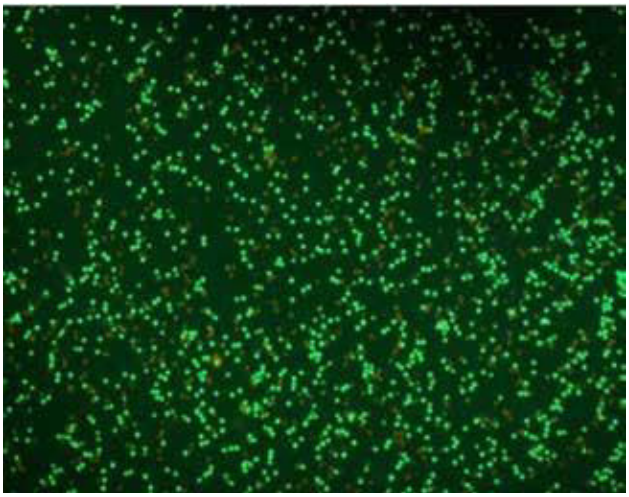

Fig. 2. Viability of cells by FDA/PI staining. (Viable cells - green & dead cells- Red).

between genotypes (V × T) and carbon source (T × C) were observed (Figures 5 and 6). The treatment combination Palam Samridhi (V_3) with 4°C-48 hr + 32°C-24 hr + 25°C-Cont (T_3) yielded the most embryos per plate 93.11, whereas the treatment combination 4°C-48 hr + 32°C-24 hr + 25°C-Cont (T_3) with 13 percent sucrose (C_2) yielded the most embryos per plate 382.67.

Liquid NLN media is reported for its effective efficacy for embryogenesis (Baillie *et al*., 1). The most frequent carbon source used is sucrose (Cristae *et al*., 5). In the present study, embryo yield was enhanced using three different carbon sources namely sucrose, glucose and maltose with three concentrations of each source (6%, 13% and 15%). Sucrose 13% (C_2) resulted in maximum embryogenesis followed by sucrose 15% (C_3). The effective carbon source after sucrose was found to be maltose with an effective concentration of 13% (C_8). Stress patterns also play a critical role for effective embryogenesis (Pechan and Keller, 11; Zhao *et al*., 14). Incubation at 32°C for 24 hours and further incubation at 25°C is reported to be most effective after microspore isolation (Takahata and Keller, 12) and it was found in coherence with our present results as T_1 regime, resulted in effective embryogenesis. Cold pretreatment of flower buds has been used to produce the microspore cultures in *B. napus,* depicting embryonic vigor (Lichter, 10). It was found that cold pre-treated buds at 4°C for 48 hours prior to their microspore isolation resulted in maximum embryogenesis. Hence, T_3 regime was found to be most effective.

Exogenic hormones play important role in embryo regeneration (Charne and Beversdorf, 4). A significant effect of different genotypes and hormonal

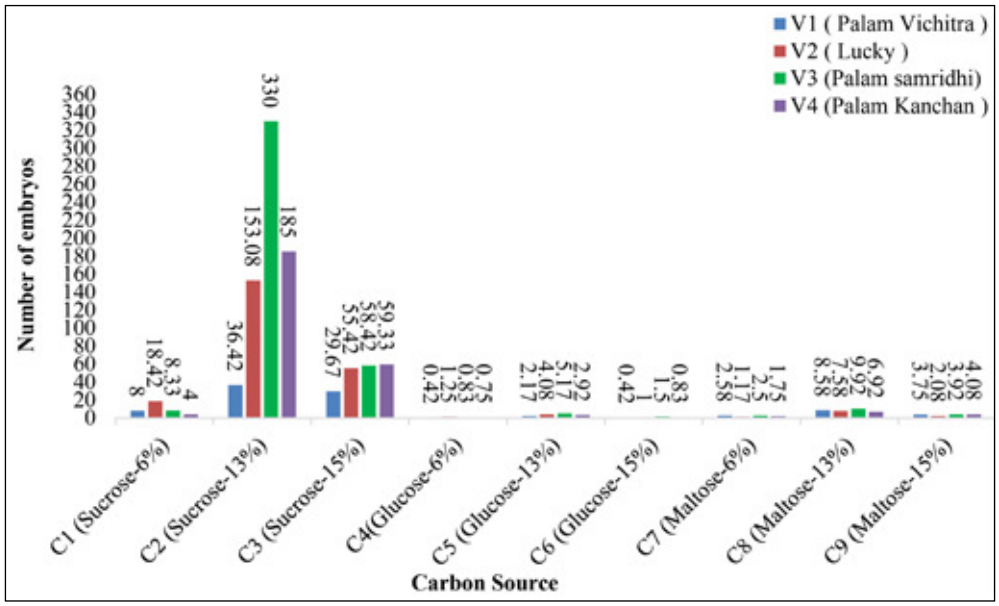

Fig. 4. Interaction between genotype and carbon source for microspore embryogenesis in broccoli.

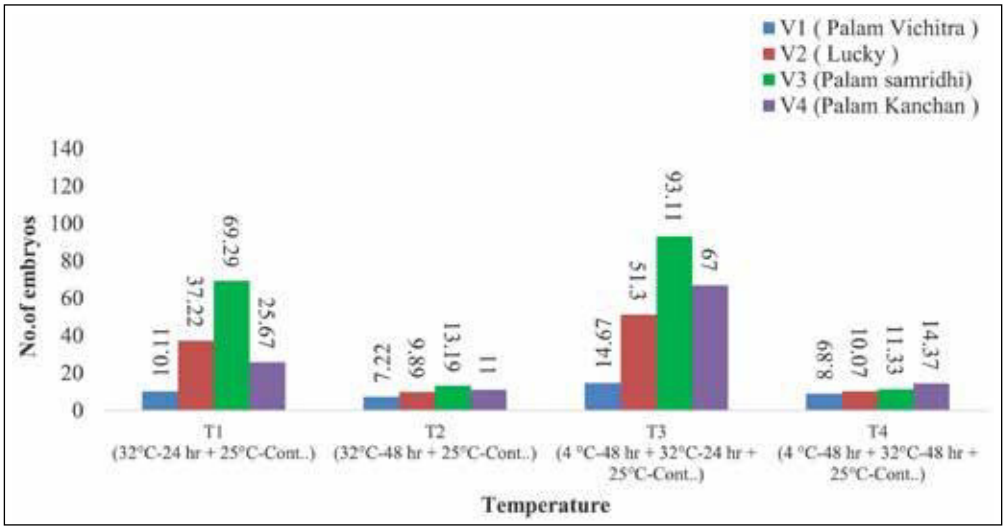

Fig. 5. Interaction between genotype and temperature for microspore embryogenesis in broccoli.

treatments was observed on embryo regeneration in broccoli (Table 2). Among the varieties, the highest regeneration of embryos was recorded in the genotype V_3 (43.29%) followed by V_4 (38.00%). The least response was observed in variety V_2 (24.29%). Highest regeneration of embryos was recorded with BAP (1mg/L) (62.25%). Different stages of embryo regeneration followed by shoot and root formation are shown in Figure 7. Interaction between genotypes and hormonal treatments (V × H) revealed that the highest regeneration of embryos (75.00%) was recorded in the genotype V_3 when supplemented with 1.0 mg/l BAP (H_5). The minimum regeneration response (3.00%) was obtained with the treatment combination (V_2 × H_1).

Cell division activities of the regenerated plants were compared with the control diploid genotype (Figure 8) for ploidy determination. A total of 952 microspore-derived plants of broccoli were used to determine ploidy (Table 3). The FCM analysis revealed that 52.31% plants were haploids, 43.17% were doubled haploids, 1.05% were triploids, 2.84% were tetraploids and 1.20% were mixoploid. Most of the studies cited have shown a high percentage of haploid plants in *Brassica* species (Gu *et al*., 8). In present study more than 50% of the population

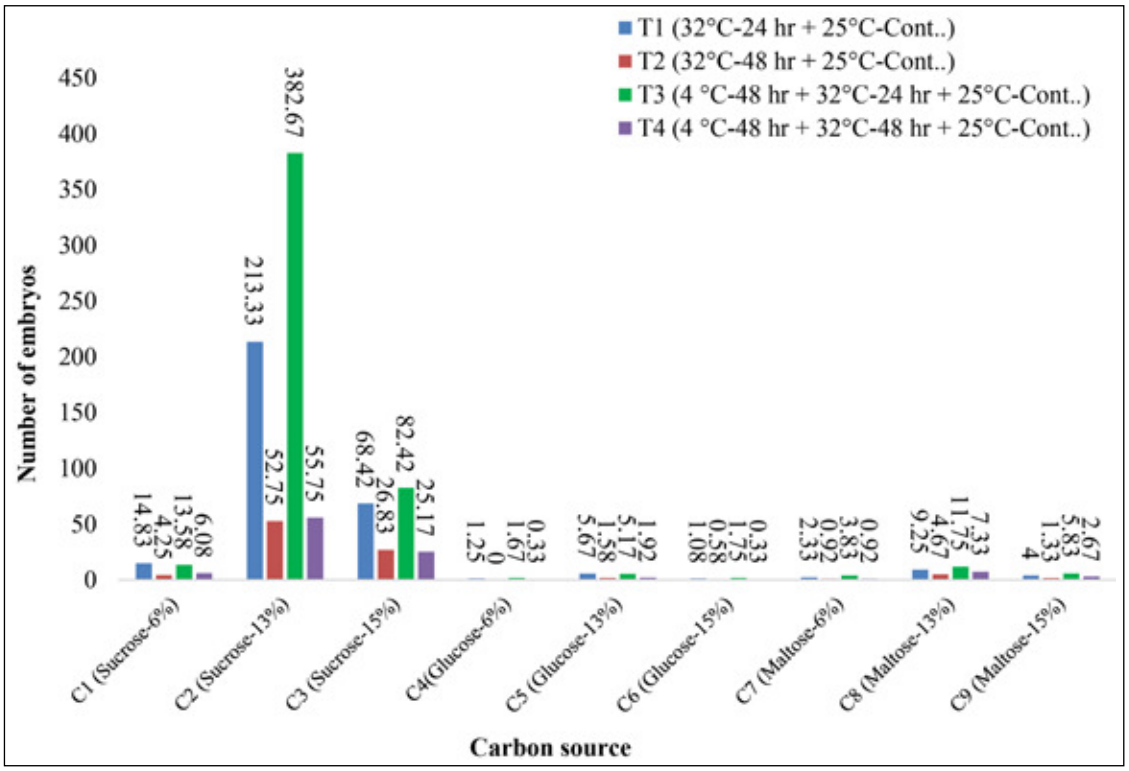

Fig. 6. Interaction between carbon source and temperature for microspore embryogenesis in broccoli.

Table 2. Effect of different hormonal treatments on embryo regeneration in broccoli.

Hormone (H) \ Variety (V)	Regeneration (%)				
	V_1	V_2	V_3	V_4	Mean
H_1(Control: No hormone)	5.00±1.58^{e}	3.00±1.23^{f}	11.00±1.87^{d}	5.00±2.24^{e}	6.00±0.47^{g}
H_2 (Zeatin: 0.5 mg/l)	27.00±2.55^{c}	20.00±1.58^{d}	47.00±4.90^{b}	35.00±3.54^{cd}	32.25±1.07^{d}
H_3 (Zeatin: 1.0 mg/l)	42.00±2.55^{b}	30.00±1.58^{c}	54.00±4.58^{b}	43.00±3.74^{bc}	42.25±0.73^{c}
H_4 (BAP: 0.5 mg/l)	47.00±3.74^{b}	37.00±2.55^{b}	58.00±3.39^{b}	52.00±2.00^{b}	48.50±1.70^{b}
H_5 (BAP: 1.0 mg/l)	58.00±4.36 ^{a}	49.00±1.87^{a}	75.00±3.54^{a}	67.00±4.36^{a}	62.25±1.07^{a}
H_6 (GA3: 0.1 mg/l)	20.00±3.54^{cd}	17.00±3.00^{de}	33.00±3.74^{c}	35.00±5.48^{cd}	26.25±2.34^{e}
H_7 (GA3: 0.2 mg/l)	15.00±1.58^{d}	14.00±1.00^{e}	25.00±4.47^{c}	29.00±1.87^{d}	20.75±1.35^{f}
Mean	30.57±1.79^{b}	24.29±0.68^{c}	43.29±2.25^{a}	38.00±1.97^{a}	

Mean values in each column (for each variety) and overall mean of each variety (in row) followed by the same lower case letters were not significantly different at P ≤ 0.05 according to Duncan's multiple range test.

Table 3. Ploidy level of the microspore derived plants of 4 broccoli genotypes as revealed through flow cytometry.

Variety	No. of tested plants	Haploids (%)	Doubled haploids (%)	Triploids (%)	Tetraploids (%)	Mixoploids (%)
V_1	209	57.42	33.49	2.39	6.22	0.48
V_2	170	64.71	30.00	0.59	1.76	2.94
V_3	307	43.00	51.79	0.98	3.58	0.65
V_4	266	48.87	46.99	0.38	3.01	0.75

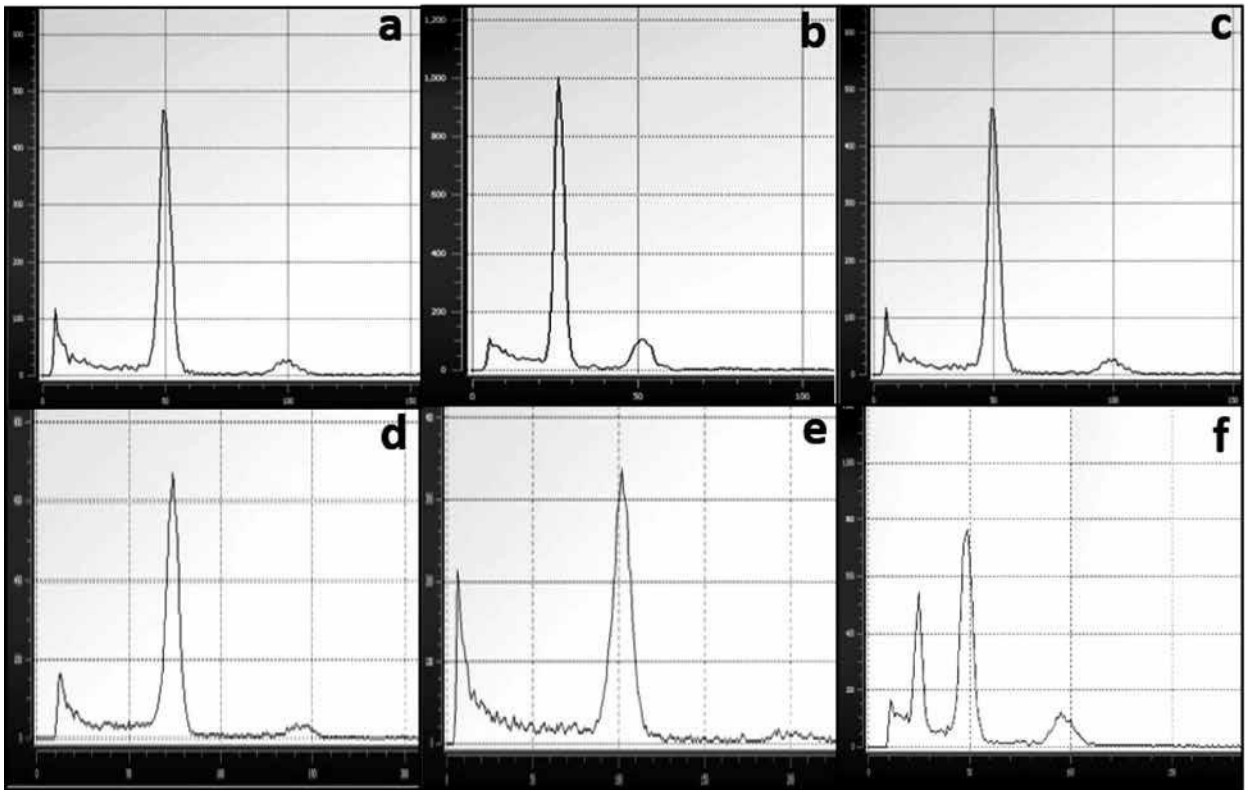

Fig. 8. Flow cytometric analysis of the microspore-derived plants determining ploidy level. (a) Control, (b) Haploid, (c) Doubled haploid, (d) Triploid, (e) Tetraploid, (f) Mixoploid.

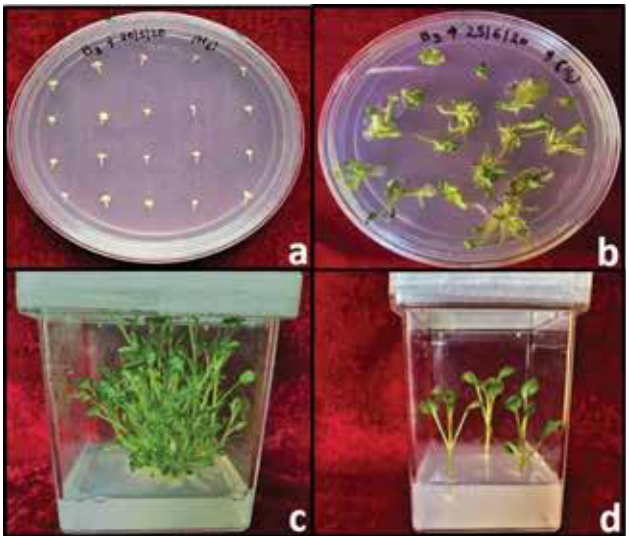

Fig. 7. Embryo regeneration and rooting in Broccoli. (a) Harvested pin shaped embryos. (b) Embryo regeneration. (c) Multiple shoot formation of one embryo. (d) Rooting of plants.

was haploid followed by doubled haploids 43%. The percentage of plants with different ploidy levels varied among genotypes and the most responsive genotype V_3 (Palam Samridhi) exhibited maximum spontaneous duplication (51.79%).

The obtained knowledge may accelerate and guide the application of microspore-derived DH plants as a breeding tool in broccoli. However, the core study is required to further improve the embryo yield and its regeneration frequency in broccoli.

AUTHORS' CONTRIBUTION

Conceptualization (RKS, PKC, AA); Designing of the experiments (RKS, PKC, AA); Contribution of experimental materials (RKS, AA); Execution of lab experiments and data collection (RKS); Analysis of data and interpretation (RKS, PKC, AA); Preparation of the manuscript (RKS).

DECLARATION

The authors declare that they have no conflict of interest.

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