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Effect of incubation temperature and sucrose concentration on callus induction and plant regeneration in cauliflower (*Brassica oleracea* var. *botrytis*)

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Abstract

Androgenic and plant regeneration potential from anther culture were investigated in cauliflower genotypes under different incubation temperatures, sucrose concentrations and regeneration media compositions. Anthers were cultured on MS medium supplemented with 1.5 mg/L 2,4-D and 1.0 mg/L NAA growth hormone with four different treatments of sucrose i.e. 3%, 5%, 10% and 13%. Maximum androgenesis has been observed with 10% sucrose concentration. Anthers of all the varieties were incubated under three different temperatures i.e. 25°C, 32°C and 35°C. Among these incubation temperatures, higher androgenesis was observed at 32°C incubation temperature. Callus were cultured on five shoot regeneration media compositions, among which maximum regeneration has been observed on MS media supplemented with 5.0 mg/L BAP and 1.0 mg/L NAA followed by MS media supplemented with 6.0 mg/L BAP and 1.0 mg/L NAA. Maximum root regeneration has been observed in ½ MS medium supplemented with 1.0 mg/L IBA growth hormone followed by ½ MS medium supplemented with 0.4 mg/L IBA. In cytological examination, 20.3% of the regenerated plants were found to be haploid while 29.8% were spontaneous doubled haploids.

Key words: Anther culture, Cauliflower, Incubation temperature, Regeneration, Sucrose

Abbreviations: MS medium: Murashige and Skoog medium; 2,4-D: 2,4-Dichlorophenoxyacetic acid; NAA: 1-Naphthaleneacetic acid; BAP- 6-Benzylaminopurine; IBA: Indole-3-butyric acid

Introduction

Cauliflower (*Brassica oleracea* var. *botrytis;* 2n=2x=18) is one of the most important vegetable crops of the *Brassicaceae* family, which is grown throughout the world. Cauliflower is an herbaceous annual for vegetable production and a biennial for seed production. Globally, cauliflower grows best between the latitudes 11-60 °N with average temperature ranging from 5-8 °C to 25-28 °C (Singh et al. 2018). Cauliflower may tolerate temperature from -10°C to 40°C for a few days during the vegetative growth period. Asian countries contribute the 79.6 % production share in total world's cauliflower and broccoli production. China leads in the production of cauliflower and broccoli followed by India (Anonymous 2018). Generally, an inbred line is produced through continuous sibmatting over 6-8 generations. To speed up the process of inbred line development, doubled haploid technology is employed. In doubled haploid technology, haploid plants are produced either naturally or manually followed by doubling their chromosome number through antimitotic chemicals like colchicine. Haploid plants can either be produced through androgenesis or gynogenesis. In *Brassica,* androgenesis is employed for haploid production. Completely homozygous plant can be developed in a single generation through DH technology.

Androgenesis is influenced by various factors i.e. genotype, cultural media compositions, incubation temperature, donor plant's growth conditions (Chen et al. 2011; Maheshwari et al. 1980). To enhance embryogenesis, anthers are subjected to various kinds of pre-treatments i.e. cold or heat shock, water stress, high humidity, anaerobic treatment, sucrose and nitrogen starvation, centrifugation, gamma radiation, ethanol microtubule disruptive agents, concentrated medium pH, electro-stimulation, heavy metal pre-treatments etc. (Ferrie et al. 1995b; Olmedilla 2010; Shariatpanahi et al. 2006). The pretreatments are believed to switch the gametophytic pathway of development to sporophytic pathway in microspores. In present study, the effects of temperature and sucrose concentration on androgenesis and plant regeneration ability have been evaluated.

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Materials and Methods

In present study, eight genotypes of cauliflower viz. Jyoti, Pusa Sharad, Kartiki, CAUMH-2, CAUMH-10, LS-2, LS-3, and LS-5 were evaluated for androgenic potentials under different temperature and sucrose treatments. Floral buds were collected from Vegetable Research Farm, Punjab Agricultural University, Ludhiana. In tissue culture lab, the buds of all genotypes were subjected to viability test. To evaluate the viability, 1% acetocarmine solution test was performed (Singh 2003). Anthers were crushed over glass slide and a drop of acetocarmine solution was added and seen under microscope. For anther culture, floral buds were surface sterilized by immersing in 0.1% mercuric chloride solution containing 0.1% Tween 20 for 10 mins, followed by three washings with autoclaved distilled water. To remove water from the surface, buds were wrapped in filter paper. Dried buds were dissected with the help of forceps and scalpel blade in autoclaved petri plate. Anthers were placed on callus induction medium i.e. MS + 1.5 mg/L 2,4-D + 1.0 mg/L NAA containing two levels of sucrose i.e. 3% and 4%. The cultured anthers were incubated at two different temperature treatments i.e. 25 °C and 35 °C in complete dark. Calli obtained on this media were sub-cultured on four different compositions of regeneration media (Table 1). After proper shoot development, the shoots were cultured on four different root induction media combinations (Table 1). After sufficient development of root system, the small plantlets were taken out from the culture vessels and washed to remove excess of agar around the roots. The plantlets were transferred to plastic pots containing MS basal medium having half strength and were covered with polyethylene to maintain a condition of high humidity (85% RH). Hardening was done at 25 ± 2 °C under 16/8 h photoperiod and grown for 2 weeks before planting to the green house. After the development of new leaves, the covers were removed, and hardened plants were transferred to earthen pots filled with soil mix (sand, coco peat, vermiculite and well rotten FYM in 1:1:1:1 ratio v/v/v) and grown to maturity. To evaluate the ploidy level of regenerated plants, chromosomes from the root tip were counted (Schwarzacher and Heslop-Harrison 2000).

Table 1: Regeneration Media Compositions

Medium	Composition of media
Shoot Regeneration	MS + 3.0 mg/L BAP + 1.0 mg/L NAA
	MS + 4.0 mg/L BAP + 1.0 mg/L NAA
	MS + 5.0 mg/L BAP + 1.0 mg/L NAA
	MS + 6.0 mg/L BAP + 1.0 mg/L NAA
Root Induction	½ MS + 1.0 mg/L IBA
	½ MS + 0.4 mg/L IBA
	½ MS + 0.2 mg/L IBA
	½ MS

Results and Discussion

Standardization of bud size: In present investigation, the buds of all genotypes were categorized into four groups on the basis of size (Plate 1). In group I buds of size 3.5-4.0 mm, in group II 4.0-4.5 mm, in group III 4.5-5.0 mm and in group IV 5.0-5.5 mm were included. All these four groups were subjected to viability test. The viable pollen grains were stained with red-purplish colour while the non-viable pollen grains remained non-stained and didn't retained any colour (Plate 2). In genotype Cauliflower Jyoti, LS-2, LS-3 and Pusa Sharad maximum viability was observed in buds of group II i.e. buds of 4.0-4.5 mm size. On the other hand, in Kartiki, LS-5, CAUMH-2 and CAUMH-10 maximum viable pollens were observed in group III i.e. buds of 4.5-5.0 mm size (Table 2). In all the genotypes collectively, maximum number of viable pollens were observed in group II and group III, while minimum number of viable pollens were observed in group IV. Thus, in all further experiment's buds of only 4.0-5.0 mm size were used. The results of present study are in concurrence with the findings of Bhatia et al. (2016). Bhatia et al. (2016) claimed maximum number of viable pollens in buds of 4.0-5.0 mm size in four genotypes belonging to different maturity groups of cauliflower. Number of viable pollens in a given group of bud size varies from genotype to genotype. Thus, protocol for favorable group of bud size needs to be standardized for the concerned genotype prior it's uses in any haploid generation program. Similarly, Winarta and Silva (2011) and Gu et al. 2014 also considered bud size as an vital element for any successful haploid generation program.

Callus Induction: Effect of four sucrose concentrations i.e. 3%, 5%, 10% and 4% was evaluated for callus induction capability. Among four sucrose concentrations, maximum callus induction was observed

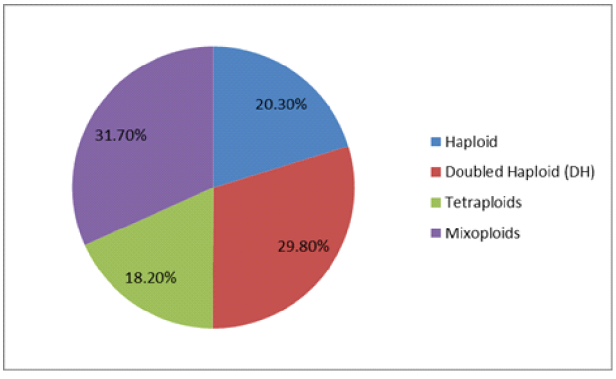

Fig.1: Ploidy level of regenerated plants

Table 2: Viability of different genotypes of cauliflower (%)

Genotype	Grouping of buds on the basis of size			
	Group I (3.5 mm-4.0 mm)	Group II (4.0 mm-4.5 mm)	Group III (4.5mm-5.0mm)	Group IV (5.0mm-5.5mm)
Cauliflower Jyoti	35.3	69.7	49.7	31.3
LS-2	31.3	65.3	45.3	27.3
LS-3	31.3	60.7	44.7	25.3
Kartiki	35.7	51.3	65.7	29.7
LS-5	31.7	49.7	63.3	30.3
CAUMH-2	29.7	45.7	63.7	24.7
CAUMH-10	35.3	51.3	62.3	30.3
Pusa Sharad	35.0	60.7	51.7	26.7
Mean	33.2	56.8	55.8	28.2

in a media which contains 10% sucrose (43.1%) followed by 13% sucrose (29.8%) (Table 3). In a study with *B. campestris*, Kelley et al. (1975) compared media with 2%, 6% and 10% sucrose concentrations and found the highest response with the 10% sucrose concentration. Lichter (1981) compared media with 4%, 6%, 8%, 10 and 12% sucrose for cultured anthers of *B. napus* and reported 8% sucrose concentration to be optimal.

Role of temperature for callus induction was also evaluated in present study. After placing anthers on media, test tubes were incubated at three different incubating temperatures i.e. 35 °C, 32 °C and 25 °C. Number of anthers producing callus was observed for all three incubating temperature treatments. Number of anthers producing callus were significantly different among all the temperature treatments (Plate 3). Anthers produced maximum number of calli at 32°C as compared to 35°C and 25°C (Table 3). It is evident from present study that the androgenic response in *Brassica* increases with increase in incubation temperature. In brussels sprouts, maximum androgenesis has been observed under 35°C incubation temperature, in all the six varieties that has been used in experiment (Krzyzanowska and Gorecka 2008). The temperature stress treatment requirements are genotype specific and need to be optimized before routine application of haploid development program. There is a dire need to explore other novel temperature treatments for more efficient embryogenesis.

Table 3: Effect of Temperature and Sucrose on callus induction (percent)

Sucrose (S)	Temperature (T)			Mean
	35° C	32° C	25° C	
3%	20.0	25.3	18.7	21.3 d
5%	24.7	26.7	19.3	23.6 c
10%	42.0	48.7	38.7	43.1 a
13%	28.0	36.7	24.7	29.8 b
Mean	28.7 b	34.3 a	25.3 c	

CD at 5% Temperature (T): 1.3; Sucrose (S): 1.5;T × S : NS

Mean values in each column/row followed by the same lower-case letters were not significantly different (*p*d" 0.05) according to Duncan's multiple range test.

Shoot Regeneration: A significant effect of media composition has been observed for shoot regeneration frequency and days taken to initiate shoot regeneration. Among the four media compositions, maximum shoot regeneration (52.0%) has been observed in MS media supplemented with 5.0 mg/L BAP and 1.0 mg/L NAA followed by MS media supplemented with 6.0 mg/L BAP and 1.0 mg/L NAA (44.4%) (Plate 4). Minimum days (24.1 days) to shoot regeneration has been observed in MS media supplemented with 5.0 mg/L BAP and 1.0 mg/L NAA (Table 4). Maximum number of shoots emerging from single callus has been witnessed in MS medium supplemented with 5.0 mg/L BAP and 1.0 mg/L NAA and MS medium supplemented with 6.0 mg/L BAP and 1.0 mg/L NAA. The results of present investigation were in line with the findings of Wang et al. (2000) and Alam et al. (2009). The media containing 5 ml/L BAP induced the maximum shoot regeneration, which confirms the statement of George *et al* 2008 i.e. BAP is most effective in promoting differentiation of cell into shoot initials followed by formation of shoot. It should also be noted that the shoot regeneration frequency decreased as the amount of BAP increased from 5 ml/L to 6 ml/L.

Root Regeneration: Rooting was observed on all the genotypes, but rooting frequency varied significantly

Table 4: Response of different media compositions for shoot regeneration and days taken to initiate shoot regeneration

Media Composition	Shoot Regeneration Frequency (%)	No of shoots emerging from callus	Days taken to initiate shoot regeneration
MS + 3.0 mg/L BAP + 1.0 mg/L NAA	37.2 c	3.5 b	29.1 c
MS + 4.0 mg/L BAP + 1.0 mg/L NAA	38.8 c	2.3 c	30.3 d
MS + 5.0 mg/L BAP + 1.0 mg/L NAA	52.0 a	5.5 a	24.1 a
MS + 6.0 mg/L BAP + 1.0 mg/L NAA	44.4 b	4.9 a	26.1 b
CD (5%)	3.53	0.9	0.68

Mean values in each column followed by the same lower-case letters were not significantly different (*p*d" 0.05) according to Duncan's multiple range test.

from genotype to genotype (Plate 5). 'Kartiki' shows the maximum root regeneration frequency followed by 'Jyoti'. Genotypes also differ significantly for days taken to induce rooting (Table 5). Minimum days for root induction were taken by Kartiki followed by Jyoti and LS 2 (Table 5). It was evident from our study that there is a strong genotypic impact on the root regeneration frequency. Similar findings were reported by Reetisana et al. (2018) in which they claimed a significant role of genotype for root initiation in six varieties of Indian mustard. Alam et al. (2009) also reported a genotypic difference for root regeneration in oilseed *Brassica* species.

There was a significant difference observed among the rooting media for root regeneration frequency and days taken to initiate rooting. Maximum rooting frequency was observed in ½ MS medium supplemented with 1.0 mg/L IBA, whereas ½ MS supplemented with 0.2 mg/L IBA and ½ MS shows the minimum rooting frequency (Table 6). Rooting media also differ significantly among themselves for days taken to induce rooting (Table 6). Half MS media supplemented with 1.0 mg/L IBA taken the least days to induce rooting whereas ½ MS supplemented with 0.2 mg/L IBA and ½ MS took the maximum days. Very poor rooting frequency was observed when our media is devoid of any growth hormone (RM4). Our results were in concurrence with the findings of Pavlovic et al. (2010) who also witnessed the positive influence of IBA on root regeneration. Similarly, Zhang et al. (2006) demonstrates the role of physico-chemical conditions to induce rooting in *Brassica.*

Table 5: Response of different genotypes of cauliflower for root regeneration and days taken to initiate root regeneration

Genotype	Root Regeneration Frequency (%)	Days taken to initiate root regeneration
LS-2	43.3 c	22.2 b
LS-3	39.6 c	24.4 c
LS-5	30.8 d	26.9 d
Kartiki	55.8 a	18.8 a
Jyoti	47.9 b	21.0 b
CAUMH-2	16.2 f	29.1 e
CAUMH-10	21.7 e	28.7 e
Pusa Sharad	32.1 d	25.3 c
CD (5%)	4.5	1.38

Mean values in each column followed by the same lower-case letters were not significantly different (*p*d" 0.05) according to Duncan's multiple range test.

Table 6: Response of different media compositions for root regeneration and days taken to initiate root regeneration

Media Code	Media Composition	Root Regeneration Frequency (%)	Days taken
RM1	½ MS + 1.0 mg/L IBA	50.6 a	19.7 a
RM2	½ MS + 0.4 mg/L IBA	39.4 b	22.9 b
RM3	½ MS + 0.2 mg/L IBA	28.3 c	27.4 c
RM4	½ MS	25.4 c	28.1 c
CD (5%)		3.2	0.98

Mean values in each column followed by the same lower-case letters were not significantly different (*p*d" 0.05) according to Duncan's multiple range test.

Ploidy analysis: The ploidy level of regenerated plants was assessed with the root tip analysis prior to planting the plants in field. In root tip analysis the haploid plants were characterized by nine chromosomes while doubled haploid plants were characterized with the presence of eighteen chromosomes (Plate 6). The root tip analysis revealed that the 20.3% of the regenerated plants were haploid in nature, 29.8% spontaneous doubled haploids, 18.2% tertaploids and 31.7% were mixoploids. The occurrence of double haploids spontaneously has been well documented in *Brassica* with up to 10-30% spontaneous diploids being observed with some of genotypes (Chen et al. 1992; Chen and Beversdorf 2001). Among the anther derived plants in Brussels sprouts, half of the regenerants were haploids and the others were diploids (Ockendon et al. 1993). Zhang et al. (2001) reported 12% haploids and 87% diploids in cabbage. Previous anther culture studies in cauliflower reported 41% (Ockendon 1988), 26% (Wang et al. 1999) and 79% spontaneous diploids (Stipic and Campion 1997). In *Brassica napus* 5-50% spontaneous diploids (Moller et al. 1994; Zhou et al. 2002) whereas in *Brassica rapa* 70% (Gu et al. 2003) such diploids have been reported.

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पराग संबर्धन से एंड्रोजेनिक और पौधों के उत्थान की क्षमता को विभिन्न ऊष्मायन तापमान, सुक्रोज सांद्रता और पुनर्जनन माध्यम रचनाओं के तहत फूलगोभी प्रभेदों में जांच की गयी। सुक्रोज के चार अलग–अलग उपचारों यानी 3 प्रतिशत, 5 प्रतिशत, 10 प्रतिशत और 13 प्रतिशत के साथ 1.5 मिलीग्राम/एल 2,4–डी और 1.0 मिलीग्राम/एल एनएए वृद्धि हार्मोन के साथ पूरक एमएस माध्यम पर सुसंस्कृत किया गया था। अधिकतम एण्ड्रोजन को 10 प्रतिशत सुक्रोज सान्द्रता के साथ देखा गया है। सभी किस्मों के पंखों को तीन अलग–अलग तापमानों यदि 25 डिग्री सेल्शियस, 35 डिग्री सेल्शियस के अंतर्गत ऊष्मायन किया गया। इन ऊष्मायन तापमान के बीच, उच्च एण्ड्रोजन को 32 डिग्री सेल्शियस ऊष्मायन तापमान पर देखा गया था। कैलस को पांच शूट पुनर्जनन मीडिया रचनाओं पर सुसंस्कृत किया गया था, जिसके बीच एमएस मीडिया पर 5.0 मिलीग्राम/एल बीएपी और 1.0 मिलीग्राम/एल एनएए के साथ पूरक है। अधिकतम जड़ उत्थान 1/2 एमएस माध्यम +1.0 मिलीग्राम/एल आईबीए विकास हार्मोन, 1/2 एमएस मध्यम +0.4 मिलीग्राम/एलआईबीए में देखा गया है। कोशिकीय परीक्षण में पुनर्जीवित पौधों में से 20.3 प्रतिशत हैप्लॉयड पाए गए जबकि 29.8 प्रतिशत दोगुना हैप्लॉयड थे।

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