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Research article

Generation of doubled haploids in cauliflower

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ABSTRACT

Hybrids of cauliflower are in high demand world over due to their high yield potential, earliness, better quality, better resistance to biotic and abiotic stresses. Conventionally, hybrids are developed from the intercrossing of two diverse inbred parental lines which are developed through continuous inbreeding for 8–10 generations and still don't attain complete homozygosity. Doubled haploid technology on the other hand generate completely homozygous inbred lines in a single step. Therefore, a study was undertaken at Punjab Agricultural University, Ludhiana, to develop a protocol for the development of doubled haploid lines in cauliflower. The anthers were excised from the flower buds of different genotypes viz. Jyoti, Pusa Sharad, Kartiki, CAUMH-2, CAUMH-10, LS-2, LS-3, and LS-5 followed by their culture on five different callus induction media compositions. Genotypes differed significantly in the ability to induce callus which was maximum in Jyoti followed by LS-2. Different media compositions also varied significantly in callus induction efficiency which was maximum on MS media+1.5 mg/L 2,4-D +1.0 mg/L NAA. Maximum shoot regeneration was recorded in genotype Kartiki followed by LS-2 when cultured on MS media+3.0 mg/L BAP+2.0 mg/L Kin. The regenerated shoots thus obtained were rooted on ½ MS media +1.0 mg/L IBA. Ploidy analysis of root tips revealed that 22.2% of the regenerated plantlets were haploids, 27.8% were spontaneous doubled haploids, 16.7% were tetraploids and remaining 33.3% were mixoploids.

1. Introduction

The genus *Brassica* comprises of 39 different plant species therefore occupies a prominent place in the *Brassicaceae* family [1]. Most popular crops of the family are cauliflower, cabbage, broccoli, Knolkhol, Brussels sprout and kale (also known as 'cole' crops) which are different morphotypes of *B. oleracea* [2]. The regular consumption of these vegetables is reported to reduce the risk of chronic diseases like cancer, cardiovascular diseases [3, 3, 4, 4, 5, 5]. Among the cole crops, cauliflower (*B oleracea var. botrytis* L.) is one of the most economical and nutritionally important vegetable crops worldwide grown from temperate to tropical climatic conditions in different cropping seasons therefore available round the year in the market [6]. Cauliflower is a self-incompatible and cross-pollinated crop [7]. It is grown for its white tender flower buds, called 'curd'. China leads in the production of cauliflower followed by India, collectively both the countries represent about 74% of the total world production.

In cauliflower, hybrids are in high demand due to high yield, earliness, better resistance to biotic and abiotic stresses [8]. An ideal hybrid is produced by crossing two diverse inbred lines which are produced either through self-pollination or doubled haploid (DH) technology. But in cauliflower, self-pollination is restricted due to self-incompatibility and needs a large number of generations to attain homozygosity. Thus, the viable option to produce inbred lines is through haploid production. The development of inbred line through DH technique will require less time and have complete homozygosity as compared to an inbred line produced through self-pollination. Haploid plants can either be produced from male floral part i.e. androgenesis or female floral part i.e. gynogenesis. In *Brassica*, androgenesis is preferred for haploid production [9]. Basic theory behind androgenesis is to induce a sporophytic pathway of development in immature pollen grains through different chemical and physical shock treatments [10]. Anther culture is a relatively easy and quick method to produce haploids and doubled haploids [11]. Anthers having microspores at the late uninucleate to early binucleate stage are preferred for haploid production [12]. DH lines may be straightly used as cultivars if found suitable or used as parental lines for hybrids. In addition to inbred development in the shortest possible time span, the DH technology has many other practical applications in agricultural biotechnology like gene

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mapping, genomics, mutation induction, cytological studies etc. In *B oleracea*, plants obtained through anther culture contain numerous ploidy stages i.e. haploid, diploid, triploid, tetraploid, octoploid and aneuploid [13, 14]. The reason behind different ploidy levels is spontaneous doubling and abnormal polyploidization at the time of culture and subsequent growth stages [15]. The successful production of androgenic plantlets largely depends on the type of species, genotypes, physiology of the donor plants, stage of development of microspores/pollen, composition of culture medium, carbon source, sucrose levels, plant growth hormones and pretreatment temperatures [16]. Favorable growth conditions of donor plants and modifications in *in vitro* conditions may reduce the effect of genotype up to some extent [17, 18, 19]. Cultural media composition influences the androgenic process in a great way. A complete nutrient media composition containing all the mineral salts, sucrose and vitamins are required for androgenesis. Most commonly used basal media compositions in androgenesis are MS media [20], B5 medium [21], Nitsch and Nitsch [22] and N6 [23]. Sucrose is being generally utilized as a carbon and energy source in most of the culture media which also acts as a regulator of the osmotic pressure [24]. To enhance embryogenesis anthers are subjected to various kinds of pretreatment i.e. cold or heat shock, water stress, high humidity, anaerobic treatment, sucrose and nitrogen starvation, gamma radiation, ethanol microtubule disruptive agents, electro-stimulation, heavy metal pre-treatments etc. [15, 25, 26]. The pretreatments are believed to switch the gametophytic pathway of development to sporophytic pathway in microspores. Among all the pretreatments, temperature is most effective to elicit embryogenesis.

In present investigation, the evaluation of callus induction potential of eight cauliflower genotypes with respect to different cultural conditions has been done.

2. Material and methods

2.1. Donor plant material

In present study, total 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. The source of these genotypes is given in Table 1. These genotypes were grown in the field at the Vegetable Research Farm, Punjab Agricultural University, Ludhiana following all the recommended cultural practices [27].

2.2. Media used for anther culture

Murashige and Skoog media [20] was used as basal media for the anther culture. The growth regulators were added as per the treatments listed in Table 2**.** pH of the media was adjusted to 5.8 by adding 1N NaOH/1N HCL dropwise. Agar (0.8%) was added while boiling for solidification of media. Callus induction and shoot regeneration potential of cauliflower genotypes was evaluated over five different media compositions while root regeneration capability was tested over four media compositions (Table 2).

2.3. Anther culture technique

The unopened floral buds ranging from 4.0-5.0 mm in size, were collected from the field in the icebox. The buds were surface sterilized in the laminar air flow cabinet with 0.1% (w/v) $HgCl_2$ solution containing 0.1 % (v/v) Tween 20 for 10 min followed by washing with the sterile distilled water. Individual flower buds were then cut at the base with sharp surgical sterilized scissors to free the anthers from the filaments. These excised anthers were cultured on the callus induction media and incubated in dark at 25 °C. After six to seven weeks of inoculation, callus induction was observed in the cultured anthers and it had attained a convenient size. These calli were further sub cultured to new containers containing shoot regeneration media. The cultured jars were incubated under cool white fluorescent light in dark/light conditions for 16/8 h, respectively at 25±2 °C. The subcultured calli differentiated into shoots and attained a size of 3–4 cm in six to seven weeks. To regenerate roots, the regenerated shoots were rescued aseptically and again cultured in a jar which contained freshly prepared root induction media and incubated under light/dark period of 16/8 h, respectively, at 25±2 °C.

Table 1. The genotypes used in the study along with their source.

S. No.	Genotype	Source
1	Jyoti	Private Seed Company
2	Kartiki	Private Seed Company
3	Pusa Shard	IARI
4	CAUMH-2,CAUMH-10	AICRP-ICAR
5	LS-2, LS-3, LS-5	PAU, Ludhiana

Table 2. Different culture media used for anther culture.

Type of medium	Media Code	Composition of media
Callus Induction	CIM1	MS + 1.5 mg/L 2,4-D + 1.0 mg/L NAA
	CIM2	MS + 1.0 mg/L 2,4-D + 0.5 mg/L NAA
	CIM3	MS + 0.5 mg/L 2,4-D + 1.0 mg/L NAA
	CIM4	MS + 0.5 mg/L NAA
	CIM5	MS + 1.5 mg/L BAP
Shoot Generation	SRM1	MS + 3.0 mg/L BAP + 2.0 mg/L Kin
	SRM2	MS + 2.0 mg/L BAP + 0.5 mg/L NAA
	SRM3	MS + 3.0 mg/L BAP + 0.5 mg/L NAA
	SRM4	MS + 2.0 mg/L Kin
	SRM5	MS + 2.0 mg/L BAP

2.4. Ploidy analysis

The ploidy analysis of the root tips of regenerated plantlets was carried out as per the method suggested by Schwarzacher and Heslop-Harrison [28]. For slides preparation root tips were initially washed with a citrate buffer (40 mM citric acid, 60 mM trisodium citrate; pH 4.8) for 5 min followed by washing with water for 1 min. These roots were transferred for digestion in an enzymatic solution containing 0.1 % (w/v) cytohelicase, 0.1 % (w/v) cellulase Onozuka RS and 0.1 % pectolyase Y23 in 10 mM citrate buffer, pH 4.8 for 90 min at 37 °C. To stop digestion, the enzyme solution was replaced with a citrate buffer. These root tips were then cleaned followed by maceration in in 60 % (v/v) acetic acid and placed on clean glass slide. A slide containing macerated root tip(s) was then covered with a coverslip and heated by passing it back and forth through the gentle flame of an alcohol lamp. Following the

Table 3. Callus induction frequency and days taken to callus initiation in different genotypes of cauliflower.

Genotype	Callus Induction Frequency (CIF) (%)	Days taken to Callus Induction (CI)
LS-2	50.3 b	18.2 b
LS-3	40.0 c	16.3 a
LS-5	26.7 e	19.8 c
Kartiki	32.7 d	20.2 cd
Jyoti	55.6 a	18.3 b
CAUMH-2	25.3 e	22.7 e
CAUMH-10	21.9 f	22.7 e
Pusa Sharad	25.1 e	20.9 d
CD (5%)	2.4	1.01

Mean values in each column having the same lower-case letter were not significantly different ($p \leq 0.05$) according to Duncan's multiple range test.

Table 4. Response of different media compositions for callus induction.

Media Code	Media Composition	CIF (%)	Days taken to CI
CIM1	MS + 1.5 mg/L 2,4-D + 1.0 mg/L NAA	44.3 a	16.5 a
CIM2	MS + 1.0 mg/L 2,4-D + 0.5 mg/L NAA	40.0 b	18.4 b
CIM3	MS + 0.5 mg/L 2,4-D + 1.0 mg/L NAA	36.0 c	18.0 b
CIM4	MS + 0.5 mg/L NAA	29.3 d	22.2 c
CIM5	MS + 1.5 mg/L BAP	23.9 e	24.5 d
CD (5%)		1.8	0.8

Mean values in each column having the same lower-case letters were not significantly different ($p \leq 0.05$) according to Duncan's multiple range test.

heating process, thumb pressure was applied to flatten the chromosomes. The slide was observed in a fluorescent microscope.

2.5. Observations recorded

The observations were recorded on callus induction frequency and shoot regeneration frequency by counting total number of cultured anthers which is given below.

$$\text{Callus induction frequency (CIF)} : \frac{\text{Number of anthers showing callus induction}}{\text{Total numbers of anthers cultured}} \text{X } 100$$

$$\text{Shoot Regeneration Frequency (\%)} : \frac{\text{Number of calli showing regeneration}}{\text{Number of calli cultured}} \text{X } 100$$

2.6. Statistical analysis

Analysis of variance (ANOVA) was done in a completely randomized design (CRD) using SPSS software version 13. Duncan's multiple range tests was carried out at $P \leq 0.05$ level of significance for the comparison of mean values [29].

3. Results and discussions

Genotypes, media composition and their interaction had a significant effect on the callus induction frequency and days taken to initiate callusing.

3.1. Callus induction

Callus induction frequency as shown in Table 3 was recorded in eight genotypes i.e. LS-2, LS-3, LS-5, Kartiki, Jyoti, CAUMH-2, CAUMH-10 and Pusa Sharad. Maximum percent callus induction was observed in genotype 'Jyoti' i.e. 55.6% (Plate 1 A) followed by LS-2 (50.3%) and LS-3 (40.0%) while minimum callus induction was observed in Pusa Sharad (25.1%). Genotypes also differed statistically from each other in the days taken to initiate callusing. Minimum days for callus initiation were taken by genotype LS-3 (16.3 days) followed by LS-2 (18.2 days) while maximum number of days were taken by the genotype CAU MH-2 and CAU MH-10 i.e. 22.7 days each. Maximum callus induction was reported in Jyoti genotype. Many studies have revealed that percent androgenesis varied with genotypes [30, 11, 31] and in *Brassica* too similar reports are available [32, 33, 34, 35].

Among 5 media combinations, maximum callus induction was observed in CIM1 (44.3%) followed by CIM2 (40.0%) (Table 4). Minimum callus induction (%) was observed in CIM5 (23.9) followed by CIM4 (29.3). Minimum days for callus initiation were reported in CIM1 (16.5 days) followed by CIM2 (18.4 days). Maximum days for callus initiation were reported in CIM5 (24.5 days) followed by CIM4 (22.2 days). Our results are in concurrence with the findings of Nagoo [36] who also observed a positive correlation among 2, 4-D and NAA growth hormones for callus induction in *Brassica*. Shyam *et al* [34] also reported maximum callus induction in *Brassica juncea* on MS media supplemented with 2,4-D (3 mg/L) while Lone *et al* [37] reported maximum callus induction upon MS media having 2,4-D (2.0–2.5 mg/L).

Among the 40 combinations of media and genotypes, 5 media × genotype combinations performed superior than the rest of combinations (Figure 1). These combinations included Jyoti × CIM1 (63.3%), LS-2 × CIM1 (62.7%), Jyoti × CIM2 (60.0%), LS-2 × CIM2 (59.3%), Jyoti × CIM3 (60.7%). There was also a significant difference in the days taken for callus initiation on different media × genotype combi-

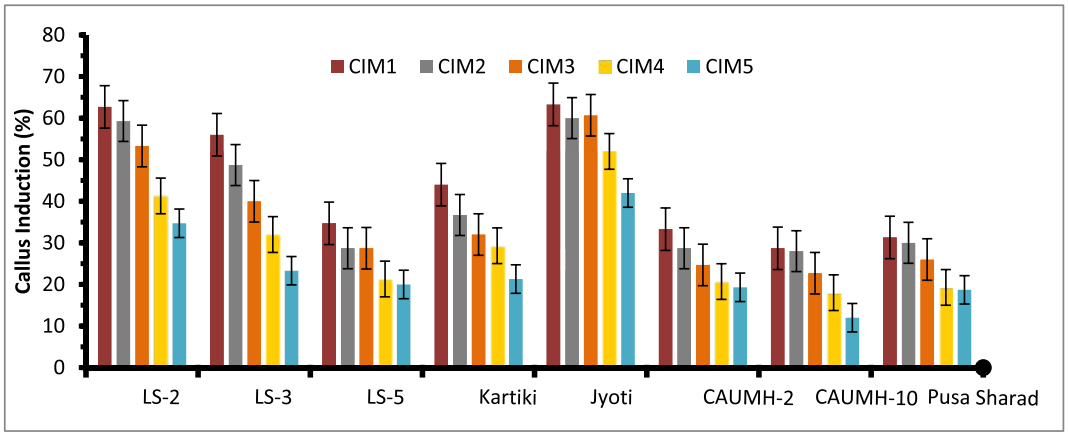

Figure 1. Interactive effect of genotype and media combinations on callus induction frequency.

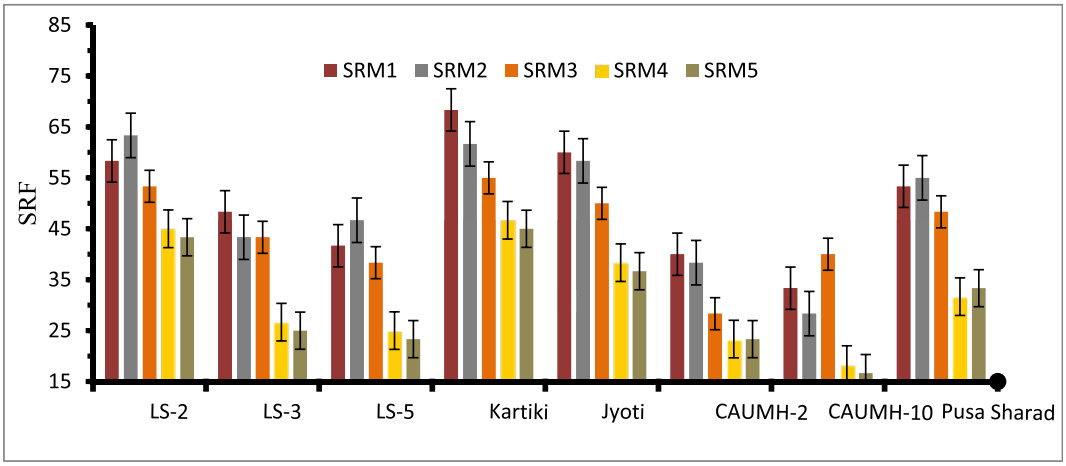

Figure 2. Interactive effect of genotype and media combinations on shoot regeneration frequency.

nations. Among all the media × genotype combinations, 5 combinations i.e. LS-2 × CIM1 (15.7 days), Jyoti × CIM1 (14.6 days), LS-5 × CIM1 (15.4 days), LS-3 × CIM1 (13.0 days), LS-3 × CIM2 (15.2 days) took less time to initiate callusing. These five media × genotype combinations were statistically at par for days taken to initiate callusing. Similar results were reported by Reetisana [38] in which a significant effect of genotype and media interaction was observed for days taken to initiate callusing.

3.2. Shoot regeneration

Shoot regeneration frequency of different genotypes differed significantly from each other (Figure 2). which was highest in genotype 'Kartiki' followed by 'LS-2' (Plate 1 b & c). Shoot regeneration frequency was at par for Kartiki and LS-2 genotypes (Table 5). Minimum shoot regeneration frequency was observed in CAUMH-2 and CAUMH-10. Among all the genotypes investigated in our study, minimum days for shoot emergence were taken by Jyoti and Kartiki (Plate 1d). Maximum days for shoot regeneration were observed in CAUMH-2 and CAUMH-10. The results of the present investigation are in concurrence with the findings of Romeijn and Lammeren [39], Zamani *et al* [40] and Klima *et al* [41]. All these workers witnessed the genotypic dependence in *Brassica* and other crops (barley, soyabean etc.) for shoot regeneration, thus the shoot regeneration frequency was controlled by genetic factors. The genetic control of shoot regeneration frequency was also claimed by Lelu and Bollon [42] in head cabbage and brussels sprouts.

Highest shoot regeneration frequency was observed in SRM1 and SRM 2 (Table 6). The performances of SRM1 and SRM2 were at par for shoot regeneration frequency. Shoot regeneration media also differed significantly among each other for days taken to shoot emergence. Among the all media combinations, least time for shoot induction was taken by SRM1 and maximum time for the same was taken by SRM3, SRM4 and SRM5. It is evident from our study that media and growth hormone combinations play a significant role in the shoot regeneration. Our results are in concurrence with the findings of Rudolf *et al* [43], all these workers witnessed the importance of regenerative media combinations and amount and concentrations of growth hormones for shoot regeneration. Chaudhary *et al* [16] also reported the maximum shoot regeneration in broccoli with BAP (1 mg/L) + Kin (2.0 mg/L) + IAA (0.25 ml/L) growth hormone combination. Similarly, Mousa *et al* [44] also reported highest shoot regeneration in *Brassica oleracea* var *italica* with BAP (1 mg/L) and 2,4-D (0.5 mg/L). Ravanfar *et al* [45] concluded that BAP was the most effective plant growth regulator used for shoot regeneration and multiplication for broccoli.

After sufficient shoot development, healthy green looking shoots were cultured on rooting media i.e. ½ MS medium supplemented with 1.0 mg/L IBA. When there was ample development of roots, plantlets were hardened on wet cotton for a week before shifting to greenhouse (Plate 1e).

3.3. Ploidy analysis

The meristematic root tips of the regenerated plantlets of different genotypes were subjected to ploidy analysis to confirm the presence of

Table 5. Response of different genotypes of cauliflower for shoot regeneration and days taken to initiate shoot regeneration.

Genotype	Shoot Regeneration Frequency (%)	Days taken to initiate shoot regeneration
LS-2	52.7 ab	24.3 e
LS-3	37.3 d	42.2 a
LS-5	35.0 d	29.1 c
Kartiki	55.3 a	22.2 f
Jyoti	48.7 b	23.0 f
CAUMH-2	30.7 e	30.8 b
CAUMH-10	27.3 e	31.6 b
Pusa Sharad	44.3 c	25.8 d
CD (5%)	5.1	0.9

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

Table 6. Response of different media compositions for shoot regeneration and days taken to initiate shoot regeneration.

Media Code	Media Composition	Shoot Regeneration Frequency (%)	Days taken
SRM1	MS + 3.0 mg/L BAP + 2.0 mg/L Kin	50.4 a	23.4 a
SRM2	MS + 2.0 mg/L BAP + 0.5 mg/L NAA	49.4 a	25.9 b
SRM3	MS + 3.0 mg/L BAP + 0.5 mg/L NAA	44.6 b	28.5 c
SRM4	MS + 2.0 mg/L Kin	31.9 c	28.4 c
SRM5	MS + 2.0 mg/L BAP	30.8 c	28.1 c
CD (5%)		4.1	0.7

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

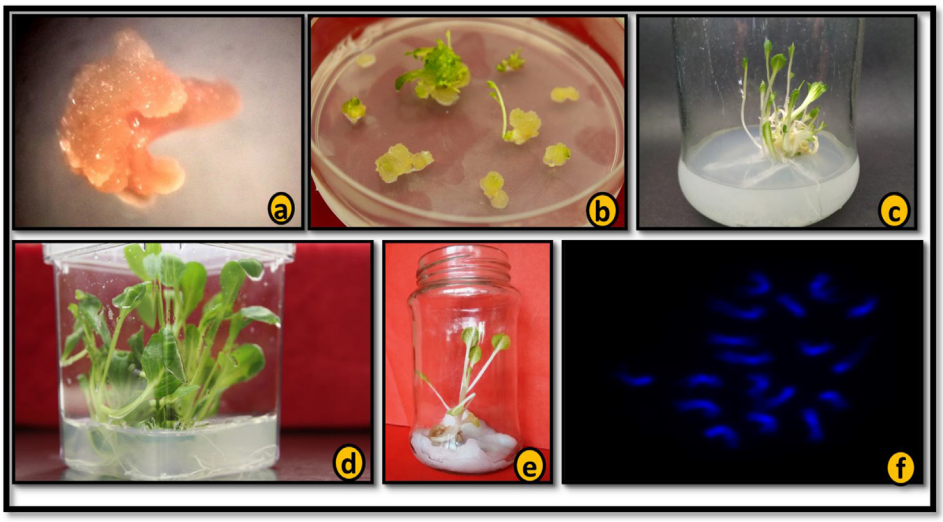

Plate 1. Different stages of doubled haploid development in cauliflower genotype "Jyoti"; (a-Embryogenic callus formation, b-c: Shoot regeneration from embryogenic calli, d: Plantlet development, e: Hardening of plantlets, f: Doubled chromosome number in the root meristematic cells (2n=18).

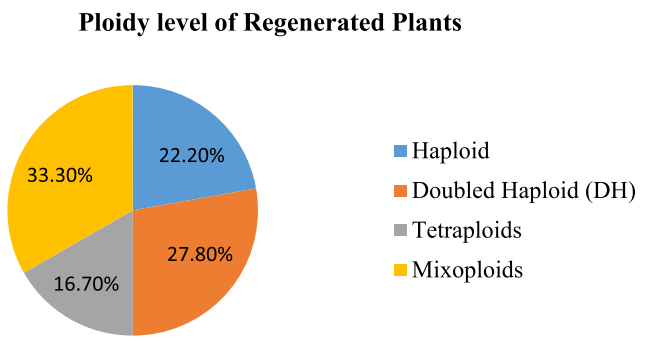

Figure 3. Ploidy level of regenerated plants.

spontaneous doubled haploid plants. The slides were prepared from the root tips of regenerated plantlets to count the mitotic chromosome number. Since diploid chromosome number of cauliflower is 2n = 18, the haploid plantlet root tips contained 9 chromosomes while doubled haploids had exactly double of haploids i.e. 18 chromosomes (Plate 1). A mixed population of haploids, doubled haploids, tetraploids, and mixoploids was obtained in genotype 'Jyoti' which were 22.2%, 27.8%, 16.7% and 33.3%, respectively (Figure 3). Keller and Armstrong [46] and Prabhudesai and Bhaskaran [47] also generated haploid plants through the anther culture technique in cauliflower. Genotypic differences were recorded in obtaining the percent ploidy among different genotypes. Wang *et al.* [14] also said that ploidy levels were genotype and species specific. However, it is still unknown why some genotypes/species produce more haploids than others. Among other factors pollen development stage i.e. uninucleate or binucleate, at the time of culturing also affects ploidy level regenerants [48, 49].

4. Conclusion

Conventionally, development of homozygous inbred lines takes years while doubled haploid technology is a single step route. But it is dependent on many factors like genotype and media compositions. Therefore, it has to be standardized for each genotype to be used in the breeding programme. We found that 'Jyoti' gave maximum androgenic response when cultured upon MS+ 1.5 mg/L 2,4-D+1.0 mg/L NAA. Maximum plant regeneration frequency was observed in 'Kartiki' genotype upon MS+3.0 mg/L BAP + 2.0 mg/L Kin which indicated that cytokinins played an important role in for shoot regeneration. The ploidy analysis confirmed the presence of 22.2% haploids, 27.8% doubled haploids, 16.7% tetraploids and 33.3% mixoploids. The protocol thus developed will help the research community to produce inbred lines in relatively less time.

Declarations

Author contribution statement

Ramandeep Singh: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Ruma Devi: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Navraj Kaur Sarao: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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Data availability statement

Data associated with this study has been deposited at Punjab Agricultural University, Ludhiana.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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