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Exploiting double haploidy in cauliflower (*Brassica oleracea* var. *botrytis* L.) for crop improvement

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Abstract

A study was conducted to augment a viable protocol for the development of double haploid system in cauliflower, using in-house genotypes, by anther culture. A total of 13,680 anthers were cultured on different media combinations across nine genotypes. Genotype-to-(segregating) genotype variations in anther culture to plant regeneration were observed, chiefly, segregating genotype-9, had highest frequency of regeneration (26.43%) whereas, segregating genotype-2, had the lowest frequency (0.2%). Two segregating genotypes remained unresponsive. Anther cultured on modified media showed highest embryogenic callus induction potential as compared to other media compositions. Regeneration media, RM1 and RM2, showed good differentiation and regeneration potential. In total, 711 haploid / double haploid plants were developed across seven genotypes, with an average regeneration efficiency of 7.6%. This simple protocol reported here, can be used for DH line development in cauliflower, through anther culture, resulting in embryogenic callus induction and plant regeneration. Further, protocol developed and discussed in the paper, will help to exploit DH technology in cauliflower for rapid inbred line development, and to speed-up cauliflower breeding for faster cultivar and better hybrid development.

Key words: Cauliflower, anther culture, double haploid, embryogenic callus, MS (Murashige and Skoog) media, plantlet regeneration

Introduction

Cauliflower (*Brassica oleracea* var. *botrytis* L.) is an important vegetable crop in Asia. India is the second major producer of *Brassica* species. In *Brassica ssp*., area under cultivation of cabbage and its production is higher than cauliflower in the world, whereas in India, it is *vice versa*. Cauliflower is grown in India from 11^0 N to 35^0 N, both on hills and plains, and between the temperature range of 5-8^0 C to 25-28^0 C, at latitudes 11^0 N to 60^0 N. In India, states such as Punjab, Bihar, Mysore, Uttar Pradesh and West Bengal are the major producer of cauliflower (Swarup and Chatterjee, 1972).

Cauliflower was originated from *Brassica oleracea* group, and is a distinct botanical variety, *var.* Botrytis. It is diploid (2n = 18), self incompatible and cross pollinated crop (Ferdinando, 2008). Member of Brassicaceae family comes under *Brassica* genus, which consists of 150 species, of annual and biannual herbs. Researchers reported six species with different chromosome number of which, three are *B. nigra* (n = 8), *B. oleracea* (n = 9), *B. compestris* (n = 10) and intercrossing among these species gave rise to *B. carinata* (n = 17), *B. juncea* (n = 18) and *B. napus* (n = 19) (Kimber and McGregor, 1995; Prakash and Hinata, 1980). This speciation event resulted in triangle of U (evolution and relationship among members of *brassica*) (Singh *et al.*, 2013).

During the period, 2004-2013, cauliflower and broccoli production in India was increased from 4.9 MT to 7.9 MT, and was the second major producer after China. Asia contributes up to 80.8% of total production of cauliflower and broccoli in the world, as compared to Europe (11.9%), America (4.9%), Africa (1.7%) and Oceania (0.7%), (FAO STAT 2004-2013).

Double haploid line development in cauliflower through gynogenesis, androgenesis or microspore culture is an important tool to speed up breeding technology and faster inbred line development. A sporophyte or plant with gametophytic chromosome number (n) is known as haploid (Kimber and Riley, 1963). Monoploids are the haploids obtained from diploid species (2n = 2X) and sporophyte with single set of chromosome (2n = X) are sterile due to chromosome un-pairing and having less plant vigor.

Haploids are produced through the process of gynogenesis (ovule, ovary culture- maternal haploid) and androgenesis (immature anther or microspore culture – paternal haploids), which are extensively studied in various crops like maize, wheat, rice, barley and brassica species and well documented world wide. Still there is a need and scope for improvement of double haploid line development through improved cultural practices, such as growth conditions of donor materials, media and phyto-hormone combinations. In tissue culture, spontaneous doubling occurs, in sufficient proportion which, ultimately reduces the need for chemical induced colchicine doubling and is observed in various crops. DH technology has various applications in commercial cultivar development (Klima *et al.*, 2004 and Babbar *et al.,* 2004) which includes inbred line production in shortest period of time, genetic studies, gene mapping, genomics, mutation, cytological studies etc. Ploidy detection can be done by various direct and indirect approaches. Indirect method includes grow-out-studies, which are also referred as segregation analysis (used in the present study, as pure DH line shows uniform phenotype), morphological studies between regenerants and donor plants, chloroplast count, guard cell size measurement, whereas direct methods, include chromosome counting and DNA content by flow cytometry. Other

approaches include progeny testing and isozyme profiling (Jana and Bohanec, 2012).

Completely homozygous plant can be developed in a single generation through DH technology. Induced haploids can spontaneously double their chromosome number and resultant regenerants are called homozygous double haploids. Anther culture / androgenesis is one of the haploid inducing techniques, whereby, *in vitro* culture of immature anthers, under aseptic conditions following surface sterilization and pre-treatment of flower buds is performed (Maluszynski *et al.* 2003). Guha and Maheshwari reported pollen plant through androgenesis in *Datura annoxia* in the year 1964 and 1966, which received undivided attention from plant breeders, as an important tool, for developing inbred line in the shortest period of time. *Brassica oleracea* var. *botrytis* L. need improved protocol for which many researchers made efforts (Yang *et al.*, 1992; Gu *et al.*, 2014; Takahata and Keller, 1991). Many morph-types of *B. oleracea* have been studied for their androgenesis potential (Keller & Armstrong, 1981; Ockendon 1984; Ockendon 1988).

To improve crop productivity, it is important to speed up breeding technology with recent development in biotechnology tools such as *in vitro* tissue culture techniques like androgenesis (an anther culture technique) for inbred line /homozygous line/double haploid line development in shortest period of time *i.e.* in a single generation, which can speed up *Brassica ssp.* breeding. Breeder needs best inbred line (homozygous) for better cultivar or hybrid development. As cauliflower is long duration crop, it takes long time for inbred line development by conventional breeding method which can be reduced to a single generation through anther or microspore culture.

Materials and methods

Experiments were conducted in the tissue culture laboratory of Mahyco Seeds Pvt. Ltd., Jalna. Nine segregating genotypes of cauliflower were used in the present study for anther culture protocol optimization and double haploid line development (Table 1). Plant materials were grown in controlled conditions. One of the critical factors includes growing condition of the donor materials and thus, the plants were grown at 10-15 ^{o}C temperature in the culture rooms. Unopened flower buds were collected from donor plants in which majority of microspores were at early or mid uni-nucleate stage (samples were observed under microscope with 1% acetocarmine stain) before anther culture was initiated. To overcome the problem of recurrent anther staining, flower buds were staged according to their length (measured in mm) and proportion of microspores at proper stage was recorded for DH work (Fig. 1 A and B). Stringent conditions were maintained for donor parent materials. Use of agro-chemicals, such as, insecticides and fungicides, were strictly avoided.

Buds were surface sterilized with 4 % Na-hypochlorite for 15 min followed by three rinses in pure sterile distilled water. Anthers were excised from the buds without filament under stereomicroscope and plated on different media combinations (Table 1 and Table 2). Anther culture plates were then incubated in dark at 25 ^{o}C till heart shaped embryo / embryogenic callus induction was observed (Dias, 1999). Anthers with embryogenic callus sub-cultured were differentiated and regenerated on RM1 and RM2 media. Well regenerated shoots were sub-cultured on plain MS media for rooting, and well developed DH plants were hardened into soil cups (Fig. 1). Thereafter, seeds were individually collected from individual DH lines developed. A grow-out-test was conducted on the developed DH materials. Uniform and stable lines were selected based on phenotype. Subsequently, stable DH lines were used for further downstream studies.

Results and discussion

A total of 13680 anthers were cultured across nine segregating genotypes on different media compositions (Table 1 and Table 2) ranging from 180-6720 anthers per media, and 720-2190 anthers per segregating genotypes, depending upon availability of flower buds during the period of study. Initially, thirteen published media combinations with or without modifications were used for three

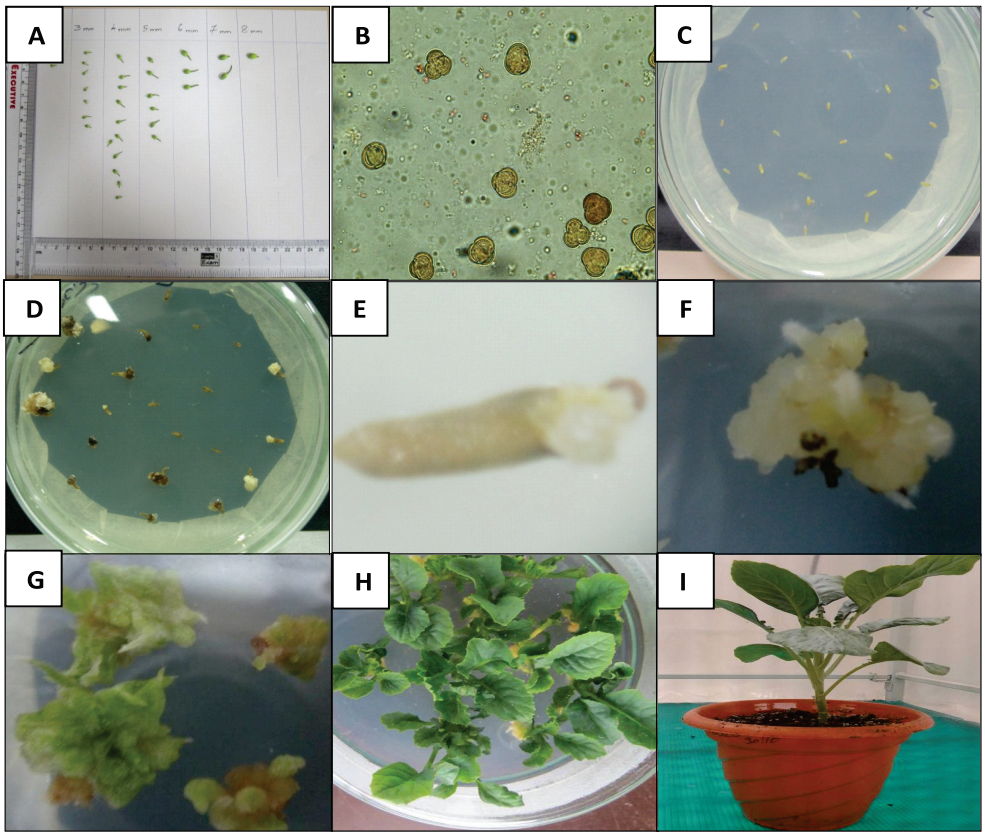

Fig. 1. Cauliflower Double Haploid Developmental Stages (A- Cauliflower bud length, B- Pollen staining (Acetocarmine), C- Anther culture, D- Callus induction, E- Anther bursting, F and G- Callus proliferation & differentiation, H- H/DH shoot induction, I- Cauliflower H/DH plant)

Table 1. Media, segregating genotype and DH regeneration frequencies in cauliflower

Media	Genotype 1	Genotype 2	Genotype 3	Media-wise total number of anthers cultured	Media-wise number of H/DH plants developed	Media-wise percent anther to plant regeneration
CACM-1	120	180	180	480	0	0.00
CACM-2	120	180	180	480	0	0.00
CACM-3	120	180	180	480	0	0.00
CACM-4	120	180	180	480	0	0.00
CACM-5	60	90	90	240	0	0.00
CACM-6	60	90	90	240	0	0.00
CACM-7	60	90	90	240	0	0.00
CACM-8	60	90	90	240	0	0.00
CACM-9	60	90	90	240	0	0.00
CACM-10	60	90	90	240	0	0.00
CACM-11	360	420	570	1350	27	2.00
CACM-12	150	150	150	450	0	0.00
CACM-13	150	150	150	450	0	0.00
Genotype-wise Total number of anthers cultured	1500	1980	2130	5610	27	0.48
Genotype-wise number of H/DH plants developed	23	4	0	27	-	-
Genotype-wise percent anther to plant regeneration	1.53	0.20	0.00	0.48	-	-

CACM = Cauliflower anther culture medium
CACM 1 to CACM 5- MS salts + vitamin + 3% sucrose + 0.7% agar and 0.1mg/L to 0.5mg/L 2,4-D, respectively (pH-5.8) [Sayem *et al.,* 2010]
CACM 6 to CACM 10- B5 salts + vitamin + 3% sucrose + 0.7% agar and 0.1mg/L to 0.5mg/L 2,4-D,respectively (pH-5.8) [Sayem *et al.,* 2010]
CACM 11- MS salts + vitamin + 0.3mg/L 2,4-D + 2.0mg/L BAP + 3% sucrose + 0.7% agar (pH- 5.8) [Sayem *et al.,* 2010]
CACM 12- NLN Salts + vitamin + 0.5 mg/L 2,4-D + 3% sucrose + 0.8% Agar (pH- 5.8) [Lichter, 1982 with modification]
CACM 13- NLN Salts + vitamin + 0.5 mg/L 2,4-D + 0.5 mg/L NAA + 3% sucrose + 0.8% Agar (pH- 5.8) [Lichter, 1982 with modification]

segregating genotypes for developing a robust DH system (Table 1). Among the tested media combinations, CACM 11 media proved to be the best with anther to plant regeneration frequency of 2% (Table 1).

In order to increase the scope of the present study, new eight published media combinations with or without modifications and six more segregating genotypes were further studied (Table 2). The same protocol was also used in our routine in-house DH cauliflower project involving other segregating genotypes (data not shown). In the protocol reported here, anther staging is important. Therefore, unopened flower buds were divided into several categories in accordance with their size (measured in mm). Best results were obtained with flower buds with average size of 6 mm, which corroborated with the highest frequency of plant regeneration, and therefore was used as a standard. The flower bud at this stage also contained highest frequency of early or mid-nucleate microspores (which was confirmed by acetocarmine staining). Therefore, in the later part of protocol development, focus was emphasized on media composition. We found that segregating genotype 9 had good regeneration potential and androgenic response with 222 developed lines, whereas segregating genotype 2 showed low androgenic response. However, two genotypes; segregating genotype 3 and segregating genotype 6 were found non-responsive (Table 1 and Table 2).

In cauliflower, haploid induction by anther culture, is affected by many factors such as culture conditions, media, genotype, donor plant growth conditions, incubation temperature, anther or bud pre-treatment (Yang *et al.*, 1992; Phippen and Ockendon, 1990). Phippen and Ockendon (1990) reported variation in embryo response between plants of same genotype, bud size acting as a critical factor for successful anther culture.

Several studies reported haploid plant generation through anther culture in cauliflower *i.e.* androgenic plants (Ockendon, 1988; Fuller and Turton, 1990).

In our study, we found that modified medium showed best embryogenic callus induction (Dias and Martins, 1999); further, better regeneration (callus to plants) was observed in RM1 and RM2 medium. We found variation in anther to plant regeneration with respect to segregating genotypes as well as media used in the present study. Segregating genotype 9 showed highest anther to plant regeneration frequency of 26.43%, as compared to other segregating genotypes studied. Segregating genotype 2 showed low DH inducibility of around 0.2% whereas, segregating genotype 3 and segregating genotype 6 were found non-responsive (Table 1 and Table 2).

We developed 711 DH lines across nine segregating genotypes used on different media combinations with an average frequency of 7.6%. These developed lines were grown till maturity for

Table 2. Media, segregating genotypes and DH regeneration frequencies in cauliflower

Media	Genotype 4	Genotype 5	Genotype 6	Genotype 7	Genotype 8	Genotype 9	Media-wise total number of anthers cultured	Media-wise number of h/dh plants developed	Media-wise percent anther to plant regeneration
CACM-11	1500	1680	2010	60	60	60	5370	341	6.35
CACM-14	0	0	0	30	120	120	270	0	0.00
CACM-15	0	0	0	120	180	180	480	0	0.00
CACM-16	120	180	180	0	0	0	480	0	0.00
CACM-17	0	0	0	120	120	120	360	0	0.00
CACM-18	0	0	0	240	120	120	480	19	3.96
CACM-19	0	0	0	60	90	90	240	0	0.00
CACM-20	0	0	0	30	90	90	210	0	0.00
CACM-21	0	0	0	60	60	60	180	324	180.00
Genotype-wise, total number of anthers cultured	1620	1860	2190	720	840	840	8070	684	8.48
Genotype-wise number of h/dh plants developed	65	87	0	96	214	222	684	-	-
Genotype-wise percent anther to plant regeneration	4.01	4.68	0.00	13.33	25.48	26.43	8.48	-	-

CACM 14- MS salts + Vitamin + 0.5mg/L 2,4-D + 0.3mg/L BAP + 3% sucrose + 0.7% agar (pH- 5.8) [Sayem *et al.,* 2010 with modification]
CACM 15- Gamborg's Basal Salts + 0.1 mg/L NAA + 0.1 mg/L 2,4-D + 14% sucrose + 125mg/L $AgNO_3$ + 0.7% agar (pH- 5.8) [Yang *et al.,* 1992]
CACM 16- Gamborg's Basal Salts + 0.1 mg/L NAA + 0.1 mg/L 2,4-D + 14% sucrose + 0.7% agar (pH- 5.8) [Gamborg's B5 medium (1968) modified by Keller (1975) and Yang *et al.* (1989)]
CACM 17- Gamborg's Basal Salts + 0.1 mg/L NAA + 0.1 mg/L 2,4-D + 14% sucrose + 72.5mg/L $AgNO_3$ + 0.7% agar + (pH- 5.8) [Yang *et al.,* 1992 with modification]
CACM 18- MS Salts + vitamin + 2mg/L BAP + 0.5mg/L NAA + 3% sucrose + 0.7% agar (pH- 5.8) [Sayem *et al.,* 2010]
CACM 19- MS Salts + vitamin + 0.2mg/L BAP + 0.2mg/L IAA + 3% sucrose + 0.7% agar (pH- 5.8) [Alam *et al.,* 2009 with modification]
CACM 20- MS Salts + vitamin + 1mg/L BAP + 0.6mg/L 2,4-D + 3% sucrose + 0.7% agar (pH- 5.8) [Sayem *et al.,* 2010 with modification]
CACM 21- MS salts + vitamin + 0.5mg/L 2,4-D + 1.5 mg/L BAP + 3% sucrose + 0.7% agar (pH- 5.8) [Sayem *et al.,* 2010 with modification]
Regeneration Media
RM1- MS Salts + vitamin + 0.5mg/L NAA + 3% Sucrose + 0.8% Agar (pH-5.8) [Kumari *et al.,* 2015]
RM2- MS Salts + vitamin + 2mg/L IBA + 3% Sucrose + 0.8% Agar (pH-5.8) [Kumari *et al.,* 2015 with modification]

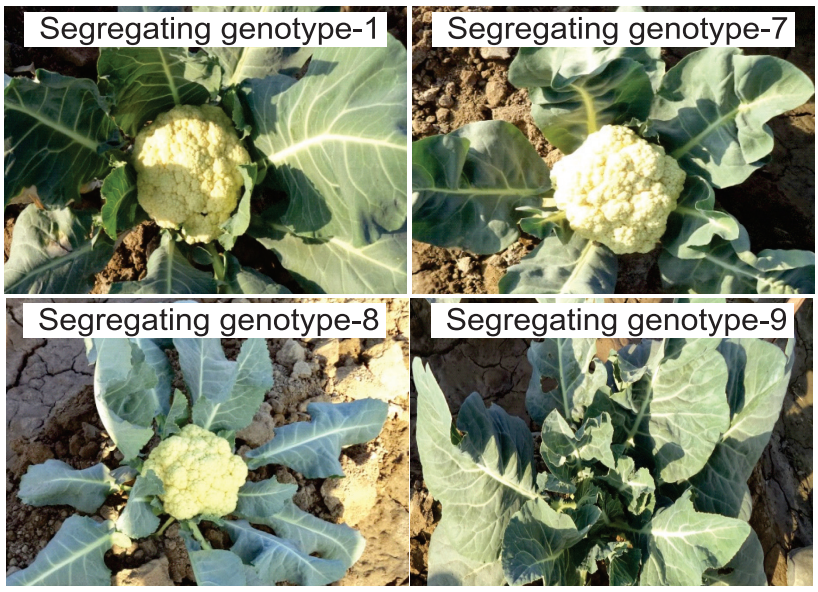

Fig. 2. Representative segregating genotypes of cauliflower DH plants in field for phenotypic evaluation.

seed collection and, further segregation analysis were conducted (Fig. 2). High frequency of spontaneous doubling was reported in Cauliflower by Gu *et al.* (2014). Further, they reported natural development of 50% DH, 25% tetraploid and less than 7% haploid in the studied regenerants. Segregating genotype to segregating genotype variation in embryo response was reported in this present investigation, which is again in conformity with the results published by Ockendon (1988).

Since, several techniques are available for detecting ploidy in cauliflower including direct or indirect methods, we choose indirect method namely, grow out test (GOT) / segregation analysis as it is more practical way of analysis for commercial DH work. One of the advantages of the method is that screening for robust phenotype and uniformity in developed DH population can be done simultaneously, thus, saving time and resource. Another advantage is that in cauliflower, naturally occurring DH phenomenon happens at a high frequency (up-to 80%, in cauliflower), therefore,

colchicines applications are largely unwarranted and may result in polyploids with a relatively large frequency (*pers comm*. Dr. Klima) and developed lines will tend to remain unstable. Our experience with cauliflower DH also confirms that colchicine treatment is not necessary and lines developed (without colchicines) are largely stable double haploids.

To summarize, our optimized protocol for cauliflower DH development using modified medium, for double haploid line development along with optimized regeneration media composition (RM1 & RM2), can be used to exploit DH technology commercially, for faster cultivar and better hybrid development.

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