CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Development and characterization of elite doubled haploid lines of ornamental kale

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Abstract: *The ornamental kale, scientifically known as Brassica oleracea var. acephala, stands out as an attractive plant admired for its vibrant leaves that grace the winter season. Notably, the hybrid KTOK-28-1 × KTOK-40-1 demonstrated the highest embryogenesis rate, yielding 56.12 embryos per Petri dish. This efficiency largely hinges on the characteristics of the donor plants used. Through flow cytometry analysis, approximately 61% of spontaneous doubled haploids (DH) were identified. These individuals exhibited normal flower development and successfully produced seeds when subjected to bud pollination. The compatibility index values exhibited variation among different DH lines, ranging from 1.0 to 13.12. This diversity is promising for the development of superior hybrids. Noteworthy among the DHs were KTDH-52, KTDH-56, and KTDH-57, exhibiting suitability for various horticultural traits. The emergence of these novel DH lines suggests their potential contribution to future breeding programs aimed at producing superior-quality hybrids.*

Keywords: *Ornamental kale, embryogenesis, doubled haploids, self-compatibility, hybrids*

INTRODUCTION

The Brassicaceae family encompasses over 372 genera and 4060 species, with considerable significance in terms of agronomy, science, aesthetics, and economics (Bayer et al. 2018). Among these species, *Brassica oleracea* var. *acephala* DC., commonly known as ornamental kale, stands out as a remarkable ornamental foliage plant, showcasing a diverse array of vibrant colours (including brilliant white, red, pink, and lavender blue) and leaf shapes. The vivid leaves and remarkable cold resistance of this plant contribute to its high value as an ornamental specimen (Liu et al. 2020). It serves various commercial purposes, finding use as a potted plant, a bedding plant, and a source of cut flowers during the winter season (Wang et al. 2011).

In its natural state, this species is primarily cross-pollinated, resulting in considerable genotypic heterozygosity due to self-incompatibility (Han et al. 2018, Chen et al. 2019, Yao et al. 2019, Esin et al. 2021). Consequently, traditional breeding methods encounter challenges in producing homozygous inbred lines (Dickson and Wallace 1986, Cristea 2013). Many commercial ornamental kale varieties are F_1 hybrids, developed by crossing inbred lines. Traditional breeding methods require several generations of self-pollination and stepwise selection, a process that can take six to eight generations to establish homozygous parental

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lines for F_1 hybrid production (Niazian and Shariatpanahi 2020). Microspore culture has proven effective in accelerating breeding procedures (Henderson and Pauls 1992, Wang et al. 2011), with chromosome doubling serving as a highly efficient method to generate doubled haploids (DH) with exceptional uniformity (Sharma et al. 2004, Ferrie and Möllers 2011). Ferrie and Mollers (2011) extensively explored the application of haploids and DH in brassica breeding programs. These DHare useful in genetic engineering, precise selection of desirable traits through mutation, and studies concerning metabolic changes in plants (Liu et al. 2005, Brew-Appiah et al. 2013, Zur et al. 2014).

Over the past two decades, microspore embryogenesis in *B. oleracea* has been investigated by various researchers, with varying degrees of success (Lemonnier-Le Penhuizic et al. 2001, Zhang et al. 2008, Winarto and Silva 2011, Gu et al. 2014). However, application of the technique in kale remains limited, due to challenges such as high embryo mortality at early stages and low induction rates (Varnier et al. 2009). Numerous factors influence the efficiency of microspore embryogenesis induction, including donor plant growth conditions, genotypes, microspore development stages, culture media composition, and culture environments (Yu et al. 1995, Liu et al. 1997, Niu et al. 1999). Stress factors, such as high-temperature pre-treatments of anthers and microspores, appear to enhance embryogenesis in *Brassica* species (Zhang et al. 2006). Nevertheless, effective embryogenesis and embryo quantity continue to be challenges in *Brassica oleracea* (Takahata and Keller 1991, Zhang et al. 2008, Winarto and Silva 2011).

In the realm of ornamental kale, no attempts in India have been reported regarding haploid or doubled haploid plant development through microspore culture. After conducting experimental research for 2-3 years, we successfully generated DH plants using the protocol established by Bhatia et al. (2016) in cauliflower. The objective of the research was to produce elite DHs from two F_1 hybrids resulting from a cross between "KtOK 29-1 × KtOK 28-1" and "KtOK 29-1 × KtOK 40-1", followed by evaluation for diverse ornamental traits.

MATERIAL AND METHODS

Donor plant growth conditions for ornamental kale

The donor plant genotypes chosen for microspore isolation were meticulously nurtured under controlled environmental conditions at the ICAR-Indian Agricultural Research Institute, Regional Station, Katrain, Kullu, Himachal Pradesh, India. Situated in the mid-Himalayan region, this station boasts a reputation for breeding and seed production of temperate vegetables and flower crops. For generation of the F_1 hybrids, three ornamental kale inbred lines, namely KTOK-29-1, KTOK-28-1, and KTOK-40-1were used. KTOK-29-1 and KTOK-28-1 are both long-stem types used as sources of cut flowers with pink-coloured inner leaves, and KTOK-40-1 is a bedding type with purple leaves. The cross combinations "KTOK-29-1 × KTOK-28-1" and "KTOK-28-1 × KTOK-40-1 were attempted to develop F_1 hybrids.

After being raised in the nursery, the seedlings were transplanted into 12-inch pots filled with a mixture of soil, sand, and coconut peat (1:1:1). The plants were then transferred to controlled climatic conditions (glasshouse), maintaining minimum temperature above 5 °C and maximum temperatures below 20 °C. Insecticide and pesticide were not applied on the plants, as this would affect microspore viability. Infected leaves were manually collected as and when required. Plants were watered regularly and liquid fertilizer was applied (N:P:K; 19:19:19) fortnightly. The genotypes were then allowed to bolt under similar conditions. Flower buds (4-5 mm in size) from both main and lateral inflorescences were selected for microspore culture according to the size of buds that was previously standardized in cabbage to obtain maximum embryogenesis (Bhatia et al. 2017). Upon harvesting, whole inflorescences were promptly placed under refrigeration to prevent exposure to elevated temperatures.

Microspore isolation and culturing

A microspore suspension culture was set up following the method outlined by Cousin and Nelson (2009), with some adaptations. Buds of the desired size (4-5 mm) were carefully extracted from the inflorescences using forceps. A 10-minute surface sterilization was carried out with a solution containing mercuric chloride (0.1% w/v) (Merck, Mumbai, India) and 0.1% (v/v) Tween-20 as a detergent (Merck, Mumbai, India). Subsequently, the buds were rinsed three times with cold sterile distilled water to eliminate excess mercuric chloride solution. A further sterilization step involving cold 4% NaOCl solution for 10 minutes was conducted, followed by rinsing with cold sterile autoclaved distilled water for 5 to 10 minutes to remove excess NaOCl.

Around 20-30 flower buds were gently crushed individually in a 100 ml beaker containing B5 washing media using a sterilized glass rod (Gamborg et al. 1968). The resulting microspore suspension was filtered through a 40 µm nylon filter (Merck Millipore, USA), then centrifuged at 250 ×g (40 °C) for 5 minutes. The final volume was adjusted to 30 ml with B5 medium. The washed pellet was suspended in aNLN-13 solution [NLN medium (Duchefa, Netherlands) containing 13% sucrose (w/v), pH 5.8] (Bhatia et al. 2016). Microspore density was determined using a Neubauer haemocytometer counting chamber (Bioanalytic, GmbH, Germany). Microspore suspension was appropriately diluted to achieve a density of 4×10^4 microspores mL^{-1} (Cristea 2013). After adjusting the density, 10 mL of suspension was dispensed per 90 mm Petri dish (Tarson, India). Petri dishes were sealed with Parafilm (Tarsons, India), and cultures were incubated at 32 °C for 48 hours followed by 25 °C under continuous darkness until embryo formation.

Examination of cell division and embryo formation

The Petri dishes were thoroughly examined under an inverted microscope (Olympus CX41RF) to observe cell division, with the initial division becoming visible within 3-5 days after culture initiation. After a two-week period, the cultures were transferred onto a rotary shaker operating at a gentle 70 rpm in darkness for an additional week, maintaining a temperature of 25 °C. This period allowed for proper development of pin-shaped embryos, which were easily visible (Figure 1).

Embryo regeneration and plant development

The cotyledonary embryos, measuring about 2 mm in size, were transitioned to solid B5 medium containing diverse hormone concentrations (such as Zeatin, BAP, and GA_3), as outlined by Bhatia et al. (2016) and Bhatia et al. (2017). These encapsulated dishes were placed under fluorescent white light (47 µmol/m^2/s) with a photoperiod of 16 hours light to 8 hours darkness at a temperature of 25 ± 1 °C, fostering germination and multiplication of the embryos. To initiate root formation in the regenerated embryos, the procedure outlined by Bhatia et al. (2016) in cauliflower was adopted.

Ploidy determination through flow cytometry analysis

Ploidy levels of the shoots derived from microspores were determined using Flow Cytometry (FCM) under the methodology detailed by Mishiba et al. (2000). At the three-leaf stage, prior to acclimatization in a growth chamber, the ploidy level was assessed. Leaves of 2-3 mm were finely chopped in a 60 mm Petri dish containing 400 µL of extraction buffer (Solution A in the CyStain UV Precise P Kit, Sysmex, Japan). After the leaves were chopped, 1,600 mL of the 4, 6-diamidino-2-phenylindole (DAPI) staining buffer (Solution B of the kit) was introduced. The resulting suspension was filtered through a 30 µm nylon mesh filter (CellTricsTM, Sysmex, Japan). Subsequently, the sample underwent analysis using a Sysmex flow cytometer with UV excitation (Sysmex Partec GmBH, Germany). The acquired sample data were

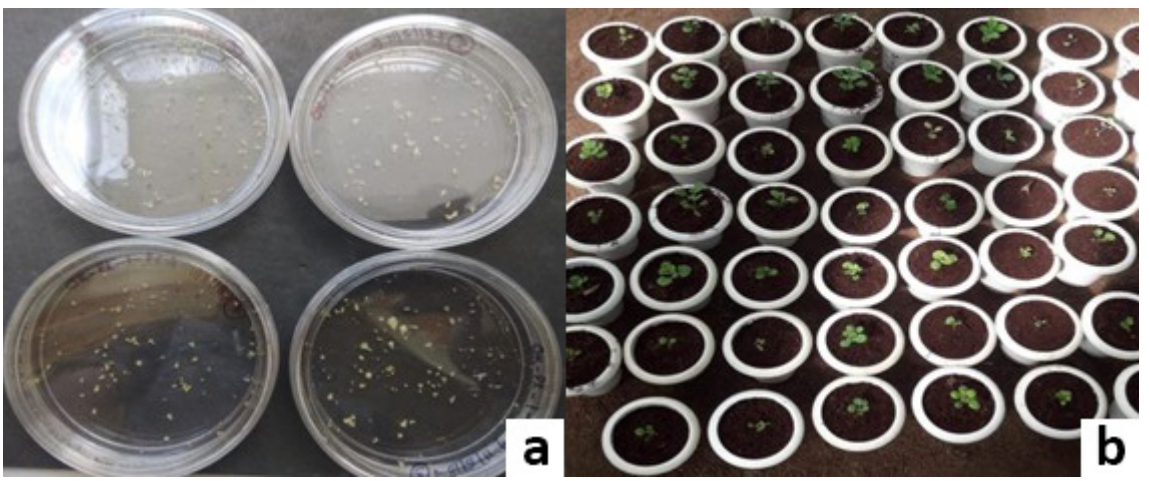

Figure 1. a) Plant development from microspore derived embryos of ornamental kale. b) fully developed plants from microspore derived embryos transplanted for hardening in soilless media.

processed using BD FACS Diva V6.1.1 (BD Biosciences), and FlowJo V7.2.5 (Tree Star Inc., Ashland, OR, USA) analysis software was used for data analysis and coefficient of variation (CV) calculations. Noteworthy plants from varying ploidy levels were transplanted in a naturally ventilated glasshouse. The morphological (shape, leaf and pod size) and floral (flower shape and size) traits were documented from the plants grown in the field.

Self-compatibility test of DH lines

The assessment of the self-compatible phenotype relied on the Compatibility Index (CI), a measure determined by the procedure outlined by Fang et al. (1983). Self-pollination was conducted manually on individuals 1 or 2 days after full flower opening. The CI was calculated at the podding stage, using the following formula:

Compatibility Index = (Number of self-pollinated seeds) / (Number of pollinated flowers or buds).

Self-incompatibility was indicated by CI < 1, moderate self-incompatibility by 1 ≤ CI ≤ 4, and self-compatibility by CI > 4.

Morphological characterization of DH lines

Individual plants from various ploidy levels were transplanted into a naturally ventilated glasshouse to assess the ploidy level of the DH_0 population through diverse morphological parameters (plant height, spread, colour, head size, etc.). The DH_0 lines progressed to the DH_1 population through either bud pollination or selfing. Based on their performance within the DH_1 generation, twelve promising lines were further advanced to the DH_2 generation for thorough evaluation, alongside two private commercial hybrids (Nagoya F_1 mix and Crane Red) serving as controls. Data pertaining to distinct ornamental traits were recorded, with five plants chosen at random from the plots.

Statistical analysis

The number of embryos within each Petri dish was tallied using a trinocular stereo zoom microscope (RSM-9). Mean and standard error (SE) values were calculated. To determine the significance of differences between means, analysis of variance (ANOVA) was performed using SPSS (2007) version 16.0 software, followed by the LSD test (P< 0.05).

RESULTS AND DISCUSSION

Impact of genotype on microspore embryogenesis

The genotype of the donor plant emerged as a pivotal factor in determining the success of microspore culture in ornamental kale. Consequently, notable distinctions surfaced in the microspore embryogenesis levels across different genotypes. Notably, the hybrid "KTOK-28-1 × KTOK-40-1" had the highest embryogenesis rate, yielding 56.12±6.26 embryos per Petri dish, compared to 42.46±2.34 embryos forKTOK-29-1 × KTOK-28-1 (Figure 1). The efficacy of an isolated microspore culture clearly hinged upon the specific donor plant genotypes (Hiramatsu et al. 1995, Zhang et al. 2008, Bhatia et al. 2017), subsequently affecting embryogenesis frequency, embryo quality, and plant regeneration capacity.

Flow cytometry (FCM) analysis for ploidy identification of microspore regenerated plants

Prior to field transplantation, the ploidy levels of microspore-regenerated plants were assessed using a flow cytometry ploidy analyser. Haploid, diploid, triploid, tetraploid, and chimeric individuals were identified using flow cytometric analysis (Supplementary file).

Further examination of plants regenerated with varying ploidy levels included diverse ornamental traits. FCM analysis unveiled that among the microspore-derived plants, 8% were haploids, 61% were spontaneous doubled haploids, 6% were triploids, 9% were tetraploids, and 8% exhibited mixoploidy or aneuploidy (Figure 2). This reveals that a significant proportion of ornamental kale plants underwent spontaneous chromosome doubling during microspore culture, corroborating findings by other researchers (Rao and Suprasanna 1996, Dai et al. 2009). In *Brassica* species, this phenomenon of spontaneous doubling may be influenced by factors such as genotype, microspore developmental stage, culture conditions, and temperature treatments (Charne et al. 1988, Chen and Beversdorf 1992).

Ploidy levels were also determined through observation of morphological traits, including plant form, flower structure,

pollen fertility, and seed-setting behaviour. This assessment highlighted the diversification of plant morphology, flower structure, pollen fertility, and seed-setting behaviour across different ploidy levels (Supplementary file). Haploid plants had small flowers with withered anthers and diminutive pods, while they failed to set seeds upon selfing. Conversely, DHs showed normal flower development and effectively produced seeds through bud pollination (Supplementary file). Buds of these regenerated plants were subsequently pollinated to yield corresponding seeds (DH_1 generation).

Influence of ploidy on plant morphology

Zhang et al. (2010) documented that tetraploid plants tend to exhibit thicker stems, larger leaves, increased stomatal size, and a higher number of chloroplasts per guard cell. The distinct morphological differences between polyploids and diploids are widely recognized (Otto and Whitton 2000), with variations in pollen size being considered a straightforward marker for tetraploid identification in the field (Cohen and Yao 1996, Kadota and Niimi 2002, Hodgson et al. 2010, Yang et al. 2013).

Self-Compatibility testing of developed doubled haploid lines

The compatibility index (CI) values among various DH lines exhibited a range from 1.0 to 13.12. Notably, the results indicated that out of the newly developed doubled haploid lines, 10 demonstrated moderate self-incompatibility, characterized by CI values ranging between 1 and 4. Additionally, 21 DH lines showcased a high level of self-compatibility (SC), specifically, KTDH-3, KTDH-13, KTDH-26, KTDH-27, KTDH-30, KTDH-46, KTDH-47, KTDH-

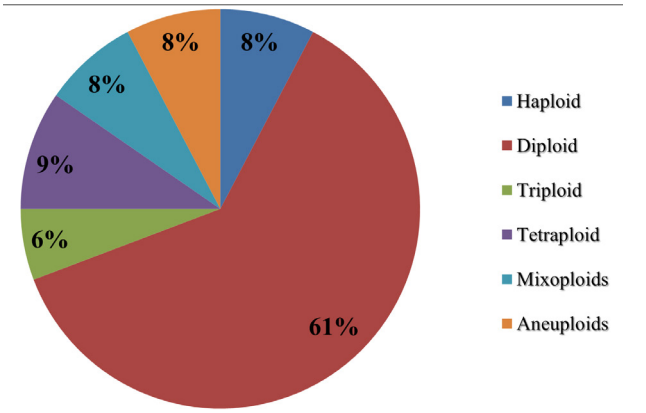

Figure 2. Average number of plants with different ploidy levels determined through flow cytometer analysis of microspore derived plants.

Table 1. Evaluation of elite doubled haploid (DH) lines of ornamental kale for horticultural traits

DH Lines	Plant height (cm)	Plant spread (cm)	Diameter of central coloured portion (cm)	Stem thickness (mm)	Average head size (cm)	Colour of leaf margins	Inner leaf colour	Leaf pattern	Potential use
KtDH-10	28.00±0.87	24.48±1.00	8.63±0.21	12.96±1.10	16.87±0.48	Green	Purple	Wavy	Cut
KtDH-13	18.73±0.27	27.20±0.67	9.17±0.24	11.74±1.08	9.90±0.35	Green	Purple	Wavy	Bedding
KtDH-26	33.55±1.48	17.03±0.15	8.67±0.24	15.26±0.83	11.42±0.62	Green	Purple green	Wavy	Cut
KtDH-27	28.13±0.86	22.74±1.04	7.73±0.30	14.01±0.41	13.40±1.22	Green	Pink	Wavy	Cut
KtDH-29	29.40±0.65	15.85±0.81	8.55±0.36	20.47±0.83	11.67±0.43	Green	Pink	Cabbage	Cut
KtDH-30	29.23±0.58	15.83±0.99	6.70±0.08	19.55±0.66	11.05±0.28	Green	Purple green	Wavy	Cut
KtDH-37	22.74±1.15	30.97±0.35	4.82±0.25	12.57±0.46	26.43±0.07	Purple green	Violet pink	Wavy	Bedding
KtDH-45	27.03±0.87	28.23±0.52	7.60±0.35	19.13±0.32	26.83±1.67	Green	Yellow green	Wavy	Cut
KtDH-52	16.10±0.95	37.19±0.69	10.47±0.52	13.12±0.12	30.27±1.97	Purple	Light yellow	Wavy	Bedding
KtDH-55	14.99±1.04	34.21±3.26	9.63±0.07	13.19±0.63	22.80±0.95	Green	Pink	Fringed	Bedding
KtDH-56	16.77±0.27	41.00±0.75	9.20±0.17	10.81±0.55	11.28±0.39	Green	Pink	Wavy	Bedding
KtDH-57	49.87±0.95	25.17±0.44	10.07±0.56	24.69±0.65	24.17±0.67	Green	White pink	Cabbage	Cut
Crane red	27.38±0.55	30.10±1.43	8.10±0.48	15.16±1.00	15.67±0.13	Purple	Dark purple	Wavy	Cut
Nagoya F1 Mix	13.90±1.68	40.25±1.99	13.03±0.18	16.33±0.90	26.52±1.78	Green	White purple	Fringed	Bedding
C.D.	2.83	3.54	0.94	2.18	2.84	-	-	-	-
SE (m)	0.97	1.21	0.32	0.75	0.97	-	-	-	-
SE (d)	1.37	1.71	0.45	1.06	1.37	-	-	-	-
C.V.	6.61	7.53	6.35	8.27	9.12	-	-	-	-

55, KTDH-56, KTDH-57, and KTDH-59. These self-compatible lines exhibited CI values exceeding 7 (Supplementary file). Remarkably, these self-compatible lines exhibited strong seed-setting capabilities, which positions them favourably for future use in developing new hybrids within the ornamental kale realm.

		
KtDH-10	**KtDH-13**	**KtDH-26**
		
KtDH-27	**KtDH-30**	**KtDH-37**
		
KtDH-45	**KtDH-52**	**KtDH-55**
		
KtDH-56	**KtDH-57**	**Nagoya mix**
		
Crane Red		

Figure 3. Horticultural characteristics of elite ornamental kale doubled haploid lines

Horticultural characteristics of elite ornamental kale DH lines

The regenerated DH lines, encompassing both cut and bedding types, exhibited a diverse range of horticultural changes. A comprehensive assessment of these DH_1 lines was conducted, evaluating various horticultural traits, such as plant height, plant spread, size of coloured portions, stem thickness, head size, and diverse foliage attributes like leaf margin colour, inner leaf colour, and leaf type. Notably, significant variations were observed among these lines for the horticultural characteristics under study. Over the course of the study, specific lines had distinct attributes, with KTDH-57 showcasing maximum plant height (49.87±0.95 cm), a central-coloured portion (10.07±0.56 cm), and stem thickness (24.69±0.65 mm). KTDH-56 exhibited the widest plant spread (41.00±0.75 cm), while KTDH-52 displayed the largest head size (30.27±1.97 cm) (Table 1). These findings are consistent with those reported by Liu et al. (2022).

The outer leaf colours ranged from green to purple, while the inner leaves of the DH lines (Xiaoping et al. 2020) exhibited a spectrum of hues, including pink, purple, violet-pink, yellow-green, light yellow, and whitish purple (Figure 3). Additionally, these lines displayed diverse leaf patterns, including wavy, cabbage-type, and fringed leaves, making them versatile candidates for a variety of ornamental purposes, including cut flowers, potted plants, and bed plantings.

CONCLUSION

Our study showed that Flow Cytometry (FCM) analysis effectively determined the ploidy levels. A substantial proportion of doubled haploids (>61%) were generated, making them readily applicable in breeding efforts. We successfully identified several outstanding DH lines with varying levels of self-incompatibility; notably, around 10 lines exhibited moderate self-incompatibility, making them valuable for inclusion in breeding programs. Additionally, a significant number of self-compatible progenies were observed, a crucial attribute for hybrid-breeding initiatives. Furthermore, our research unveiled considerable diversity among the DH lines across all the horticultural traits assessed. These promising lines can serve as essential parental candidates for generating a diverse range of hybrids. This extensive pool of potential hybrid combinations holds the promise of expediting and enhancing efficient breeding strategies within the realm of ornamental kale.

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