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Morphological and molecular characterization of some Chrysanthemum (*Dendranthema grandiflora*) cultivars

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Key words: Flower yield traits, gas exchange, pigments, SSR markers, vegetative growth traits.

Abstract: The diversity and genetic relationships among seven commercial chrysanthemum cultivars were analyzed using morphological and molecular markers. Vegetative growth, flowering, flower yield, and flower quality parameters were evaluated to assess genetic variability across the cultivars. Cultivars Crystal Red, Kodiack, and Crystal White exhibited superior vegetative growth, while Abrun, Crystal Red, and Kodiack displayed better flowering characteristics, particularly in terms of the number of inflorescences per plant and mass of colored flowering. Crystal White, Coca Bleach, and Crystal Red cultivars demonstrated the highest inflorescence stalk length, while cvs. Crystal Red, Crystal Yellow, Crystal Pink, and Kodiack Yellow recorded the maximum number of ray floret inflorescences. Other quality parameters such as inflorescence diameter and ray floret length were found to be optimal in Kodiack, Crystal White, Crystal Pink, Coca Bleach, and Crystal Yellow cultivars. Simple Sequence Repeat (SSR) markers were employed to distinguish and identify standard-type chrysanthemum cultivars, utilizing twelve SSR markers from the chrysanthemum SSR database. The results suggest that these SSR markers hold promise for identifying additional chrysanthemum cultivar types and assessing genetic relationships among them. Association studies combining morphological and molecular data offer a valuable approach to identifying informative markers for plant breeding purposes.

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1. Introduction

Chrysanthemum (mums) (*Dendranthema grandiflora* Tzvelev, formally, *Chrysanthemum morifolium* Ramat.) is one of the most important ornamental crops grown worldwide. It belongs to the family Compositae (Asteraceae) and has been commonly cultivated in gardens for more than 2500 years (Bose *et al*., 2003). It is produced on a large scale as a cut flower or as a potted plant due to its commercial significance (Van Der

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Ploeg and Heuvelink, 2006).

Analyzing genetic variability in chrysanthemum is essential for breeding programs as it can provide data on genetic relationships among different genotypes of this genus. Among the available strategies for assessing genetic variability, molecular markers are the most widely applicable, as they are best suited for understanding the genome and can be used for genetic variability characterization, paternity testing, elucidating genetic relationships between genotypes, developing methods for maintaining genetic variability in germplasm banks, and identifying genes or combinations of features related to key biological and agronomic traits (Hayden *et al*., 2010).

Another approach to studying genetic variability is the analysis of morphological and phenotypic characters, as these methods are relatively simple to perform. However, analysis based solely on morphological features may not be conclusive due to the limited number of characters and the strong influence of plant development stage and environmental factors. Morphophenological characterization does not replace molecular analyses but may complement both characterization and genetic variability studies and cultivar development (Fufa *et al*., 2005). In contrast, molecular markers based on DNA sequence polymorphisms are unaffected by environmental factors and exhibit high rates of polymorphism.

While morphological markers reflect variation in the coding regions of the genome, DNA-based molecular markers represent variations occurring in various regions of the genome, including coding and non-coding regions. Thus, molecular markers provide a rapid and reliable method to estimate genetic relationships between genotypes (Tatikonda *et al*., 2009). Molecular characterization has been widely used to quantify genetic variability among different accessions comprising germplasm banks (Glaszmann *et al*., 2010), enabling researchers to elucidate the genetic structure and diversity in a wide range of plant species (Kilian *et al*., 2007; Leišová *et al*., 2007).

Various methods have been employed to evaluate genetic diversity, with morphological character measurement being a commonly used index due to its simplicity in quantifying genetic variation while simultaneously assessing genotype performance under normal growing conditions (Fu *et al*., 2008). However, investigating morphological traits is labor-intensive, and the phenotypic plasticity of plants poses challenges due to environmental variation (Van Beuningen and Busch, 1997). In contrast, molecular markers offer several advantages over morphological measurement for assessing genetic diversity.

Assessing genetic variability is crucial in breeding programs, with molecular markers providing a direct means to access genome sequences and enabling the isolation of genetic differences from environmental influences (Ferrão *et al*., 2007). Simple Sequence Repeats (SSR) markers have shown potential in assessing genetic diversity among chrysanthemum species, cultivars, and germplasm bank collections, as well as determining geographical origin, level of domestication, dispersal history, species and cultivar identification, and genealogy (Lopez-Gartner *et al*., 2009; Hong *et al*., 2013; Hong *et al*., 2016).

SSR markers offer several advantages over other markers such as RAPD, AFLP, SRAP, and ISSR, including co-dominance, multi-allelic nature, abundance, and wide distribution across the genome, making them easy to score (Powell *et al*., 1996; Feng *et al*., 2016). Various SSR databases have been constructed and utilized for purposes such as cultivar identification, seed purity tests, and determining parent-offspring relationships in crops like citrus and pear (Kim and Nou, 2016; Nguyen *et al*., 2019). Recent studies have also employed SSR markers for variety identification in chrysanthemum and other crops (Caramante *et al*., 2011; Zhang *et al*., 2014).
Chrysanthemum, particularly standard-type cultivars with long, sturdy stems and large flowers, is a commercially significant crop, valued highly as cut flowers and for flower arrangements. Therefore, accurate genetic identification and fingerprinting of these cultivars are crucial for safeguarding breeders' intellectual property rights (Manjulatha *et al*., 2020). The promising potential of SSR markers in assessing chrysanthemum diversity has prompted this research endeavor.

The objectives of this study were to: (a) compare morphological analysis and molecular markers (SSR) of seven commercial chrysanthemum cultivars and provide molecular data to assess genetic relationships among accessions, and (b) declare the genetic diversity among cultivars.

2. Materials and Methods

Plant material and experimental site

Seven commercial chrysanthemum (*Dendran-*

thema grandiflora Tzvelev) cultivars were selected for morphological and molecular characterization: C1 - Crystal Pink (Violet), C2 - Crystal White (White), C3 - Crystal Yellow (Yellow), C4 - Crystal Red (Dark Purple Red), C5 - Kodiack (Yellow), C6 - Coca Bleach (Brown Red), and C7 - Abrun (Violet) (Table 1 and Fig. 1).

18°C, with the ventilators opening at 22°C. Short days (10 hrs light) and daytime relative humidity were set at 70%, and the area was maintained shaded with black polythene sheets.

Once the cuttings were established, they were decapitated (pinched) above the 3rd - 4th leaf from the base to encourage the production of lateral shoots.

Table 1 - List of chrysanthemum cultivars used in this study and their inflorescence colors

No.	Code	Cultivars	Royal Horticultural Society color chart No.	Inflorescence color
1	C1	Crystal pink	77D	Violet, light center
2	C2	Crystal white	N155D	White, yellow center
3	C3	Crystal yellow	5C	Yellow
4	C4	Crystal red	53A	Dark purple red
5	C5	Kodiack	4B	Yellow
6	C6	Coca bleach	179A	Brown red, yellow green center
7	C7	Abrun	N78A	Dark violet, yellow green center

Fig. 1 - Standard-type chrysanthemum cultivars used in this study.

The investigation was conducted in a greenhouse at the nursery of the Plant Production Department, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia, during the period 2019-2020.

Planting method and experimental design

Uniform rooted cuttings of the seven chrysanthemum cultivars, each measuring 7 cm in height with 5-6 true leaves, were selected. Nine rooted cuttings per cultivar were then planted on February 2nd, 2019 (first growing season), and February 4th, 2020 (second growing season), by placing them in 6-inch diameter plastic pots (one cutting per pot) filled with a mixture of peat and perlite growing media (2:1 by volume). The minimum day and night temperatures in the greenhouse were

The pinching procedure ensured that the apical meristems of all plants started active growth at the same time under the same conditions, increasing the uniformity of flowering (Cockshull, 1976).

A chemical growth retardant, B-Nine (Crompton Uniroyal Chemical Co., Washington, DC, USA), was applied as an aqueous solution with concentrations of 2000 ppm via foliar spray until runoff. This treatment was repeated three times at one-week intervals, starting three weeks after planting on February 23rd, March 2nd, and March 9th, respectively. A slow-release fertilizer, Osmocote (The Scotts Co., Marysville, OH, USA), was applied at the rate of 140 mg Kg^{-1} soil of media, which contained Nitrogen (N), Phosphorus (P_2O_5), and Potassium (K_2O), in the ratios of 17:11:10, respectively (El-Nashar, 2013).

The experiment was conducted using seven chrysanthemum cultivars in a Completely Randomized Design (CRD) with three replications. Each cultivar represented one treatment, and each replication included three plants of a cultivar.

Morphological characteristics

Plant characterization took place in late April, and the morphological evaluation was carried out when the plants were in full bloom. Observations regarding the color of the flowers were recorded with the assistance of the Royal Horticultural Society (RHS) color chart (RHS, 1966; Dorling, 2008). All cultivars were observed and divided into two parts: the vegetative parts and the inflorescence parts. The number of characters that differed from each other was scored to determine the distinctiveness and uniformity of the plant under investigation. The data from all plants were compared to identify any variations between cultivars.

Plant development and growth were recorded per pot/plant unit during both the 2019 and 2020 growing seasons by measuring the following parameters: plant height (cm) (PH), number of branches (NL), number of leaves (NL), leaf area (cm^2) (LA), using a leaf area meter (Li-Cor, Lincoln, 404, NE), shoot fresh and dry weights (g) (SFW and SDW), root fresh and dry weights (g) (RFW and RDW), root length (cm) (RL), leaf area of one leaf 10 cm from plant height (cm^2) (LA10), leaf width (cm) (LW), and leaf length (cm) (LL). Additionally, flower production per pot/plant unit was monitored, taking into account the following traits: number of inflorescences (flower yield) (NI), inflorescence diameter (cm) (ID), total inflorescences fresh and dry weights (g) (IFW and IDW), inflorescence stalk length (cm) (ISL), number of inflorescences per branch (NIB), number of ray florets per inflorescence (NRFI), length of ray florets per inflorescence (cm) (LRFI), and fresh and dry weights of a single inflorescence (g) (SFWI and SDWI) using a precision balance (KERN, 440-47N, Balingen, Southern Germany). Fresh weight was carefully recorded after removing the plants (88 days from planting). Dry weight trait was determined after drying the plant material in a dry oven for 48h at 70°C until the weight became constant.

Photosynthetic pigments

Extraction of photosynthetic pigments (chlorophyll (Chl.) A and B) from leaves were implemented using N, N- dimethylformamide (DMF) method. Chls A, B, Chls A+ B concentrations in µmol L^{-1} and Chls ratio were then estimated utilizing the equations of Porra *et al*. (1989) as follows:

$$\text{Chl A} = 13.43\ w^{663.8} - 3.47\ w^{646.8}$$

$$\text{Chl B} = 22.90\ w^{646.8} - 5.38\ w^{663.8}$$

$$\text{Total chlorophyll (Chl A+B)} = 19.43\ w^{663.8} - 8.05\ w^{646.8}$$

Chls a and b concentrations were estimated spectrophotometrically using UV Spectrophotometer (Pharmacia Biotech Ultrospec 2000).

Moreover carotenoid = (1000 w^{480} – 0.89 Chl A – 52.02 Chl B)/245 (Wellburn, 1994; Vicaş *et al*. 2010) and anthocyanin = w^{530} - 0.25 w^{657} were taken into account (Mancinelli, 1994).

Gas exchange

The assessment of the leaves' gas exchange was conducted using a portable photosynthesis system, known as the Li-COR 6400, manufactured by LI-COR Inc., based in Lincoln, U.S.A. The evaluation of net photosynthetic rate (P_n), stomatal conductance of water (g_s), transpiration rate (E), and the intercellular CO_2 concentration (C_i) was carried out on fully expanded fourth leaves between 10:20 and 11:30 am on a sunny day with a humidity level of approximately 60±5%. The measurements were taken at an ambient temperature of 27°C and under a photosynthetic photon flux density (PPFD) of about 720 µmol m^{-2} s^{-1}. The CO_2 concentrations were compared to the reference levels present in the growth chamber.

DNA extraction

Fresh young leaf tissues were collected from all chrysanthemum cultivars, then frozen in liquid nitrogen in a mortar, and stored at -80°C. The genomic DNA was extracted utilizing the DNeasy Plant Mini Kit, manufactured by QIAGEN in Germany. Subsequently, the quality of the DNA was assessed through electrophoresis on a 1% agarose gel, while the DNA concentration was determined using Quick Drop, a product of Molecular Devices in the United States. The DNA was then appropriately diluted to a concentration of 25 ng μL^{-1} and employed for SSR analysis.

SSR analysis

Seven standard-type chrysanthemum cultivars were classified and identified using a total of twelve SSR markers. Detailed information about these

markers is provided in Table 2. Each SSR marker was amplified separately in a reaction volume of 25 µL. This reaction volume included 8 µmol of each forward (5' FAM labelled) and reverse primer, 50 ng of total genomic DNA, 2 mM $MgCl_2$, 10 mM tris-HCl (pH = 7.5), 50 mM KCl, and 0.3 U/µL of Taq DNA polymerase in 1 X PCR supplied buffer. The PCR reaction took place in a thermocycler (Applied Biosystems, Veriti, C.A., U.S.A.) following these cyclic parameters: one cycle of 3 min at 94°C, 45 cycles of about 1 min at 94°C, 1 min at 50 to 60°C, 2 min at 72°C, and a final extension for 10 min at 72°C. The PCR product was then analyzed by checking 2 µL of it on a 2% agarose gel electrophoresis. To detect the product, DNA Loading STAR (Dyne Bio, S. Korea) was used. Images were captured and photographed following the application of ethidium bromide stain on a gel deposition apparatus.

Data analysis

Concerning morphological and physiological analyses, the average and standard deviation values were calculated by One-way Analysis of Variance (ANOVA) using the statistical analysis software computer program (SAS Institute Inc., Cary, NC). The Least Significant Difference (LSD) procedure was used to determine significant differences among the means of cultivars at the 0.05 significance level (Steel *et al*., 1997).

Regarding genetic data analyses, Power Marker was used to calculate the total number of alleles, genetic diversity, heterozygosity, allele frequency, and polymorphism information content (PIC) for each SSR locus (Liu and Muse, 2005). The SSR amplification bands were assigned a score of 0 for absence and 1 for presence. A Simple Matching similarity index was utilized to calculate the similarity of the qualitative data. The genetic similarity data were subjected to cluster analysis using the unweighted pair group method of arithmetic averages (UPGMA), and a dendrogram was generated using DendroUPGMA software. Principal Component Analysis (PCA) was conducted using the software PAST (version 3.14).

3. Results

Plant vegetative growth

Significant differences were observed among the various cultivars in terms of plant height, number of leaves per plant, leaf area, and leaf length in both seasons (Table 3). During the first season, the mean plant height ranged from 18.03 to 23.76 cm, while in the second season, it ranged from 17.98 to 23.03 cm. The cultivar Crystal Red recorded the highest plant height, followed by Coca Bleach, while Crystal Pink exhibited the shortest plant height, followed by 'brun' (Table 3).

The mean number of branches per plant and leaf width was not significantly affected by the compared cultivars in both seasons. The number of leaves per plant ranged from 27.33 to 33.07 for cv. Coca Bleach and 52.02 to 60.00 for cv. Crystal Red respectively. 'Abrun' exhibited the lowest leaf area (231.39 and 230.02 cm^2) in both seasons, while the largest leaf area was detected for cv. Crystal Red (390.53 and

Table 2 - List of twelve SSR primers that were screened to distinguish the seven standard-type chrysanthemum cultivars

S. No.	SSR Primers	Primer sequence	
		Forward	Reverse
1	Xcfd1	5' ACCAAAGAACTTGCCTGGTG 3'	5' AAGCCTGACCTAGCCCAAAT 3'
2	Xgwm205	' CGACCCGGTTCACTTCAG 3'5	5' AGTCGCCGTTGTATAGTGCC 3'
3	Xgwm133	5' ATCTAAACAAGACGGCGGTG 3'	5' ATCTGTGACAACCGGTGAGA 3'
4	Xcfd9	5' TTGCACGCACCTAAACTCTG 3'	5' CAAGTGTGAGCGTCGG 3'
5	Xcfd46	5' TGGTGGTATAGTCGTTGGAGC 3'	5' CCACACACACACACCATCAA 3'
6	Xgwm181	'TCATTGGTAATGAGGAGAGA 3'5	5' GAACCATTCATGTGCATGTC 3'
7	Xcfd49	5' TGAGTTCTTCTGGTGAGGCA 3'	5' GAATCGGTTCACAAGGGAAA 3'
8	Xgwm174	5' GGGTTCCTATCTGGTAAATCCC 3'	5' GACACACATGTTCCTGCCAC 3'
9	Xcfd18	5' CATCCAACAGCACCAAGAGA 3'	5' GCTACTACTATTTCATTGCGACCA 3'
10	Xcfd183	5'ACTTGCACTTGCTATACTTACGAA3'	5' GTGTGTCGGTGTGTGGAAAG 3'
11	Xgwm210	5' TGCATCAAGAATAGTGTGGAAG 3'	5' TGAGAGGAAGGCTCACACCT 3'
12	Xcfd66	5' AGGTCTTGGTGGTTTTGGTG 3'	5' TTTTCACATGCCCACAGTTG 3'

Table 3 - Plant height, number of branches, number of leaves, leaf area, leaf width and leaf length of the seven studied chrysanthemum cultivars

Cultivars	Vegetative growth character											
	Plant heigh (cm)		Branches (No.)		Leaves (No.)		Leaf area (cm^2)		Leaf width (cm)		Leaf length (cm)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Crystal pink	18.03 c	17.98 b	3.73 a	3.37 a	37.67 bc	42.07	308.80	256.10 c	4.71 a	4.80 a	6.90 bc	6.80 d
Crystal white	20.77 bc	20.83 ab	4.37 a	4.73 a	42.01 ab	42.03	380.88 a	377.17	4.43 a	5.11 a	8.20 ab	8.83 ab
Crystal yellow	18.23 c	20.23 ab	3.74 a	3.73 a	38.67 bc	44.34 bc	244.65 b	289.96	3.91 a	4.60 a	6.71 c	7.43 bcd
Crystal red	24.43 a	23.03 a	4.36 a	4.77 a	52.02 a	60.00 a	390.53 a	455.31 a	5.23 a	4.97 a	9.30 a	9.23 a
Kodiack	22.43 ab	20.27 ab	3.43 a	4.03 a	49.01 ab	36.67 cd	380.24 a	313.16	4.67 a	4.96 a	6.86 bc	7.10 cd
Coca bleach	23.76 a	22.93 a	3.07 a	2.76 a	27.33 c	33.07 d	252.00 b	270.12	4.43 a	4.73 a	7.1 bc	8.66 abc
Abrun	19.01 c	18.04 b	3.70 a	3.37 a	47.32 ab	49.77 b	231.39 b	230.02 c	4.56 a	4.23 a	7.5 bc	8.01 a-d

Values in each column followed by the different letter(s) are significantly different at $P \leq 0.05$. Least Significant Difference.

455.31 cm^2) in both seasons. 'Crystal Red' recorded the maximum leaf area followed by cv. Crystal White, while the least leaf area was recorded in 'Abrun' followed by 'Coca Bleach'. Leaf length ranged from 6.90 to 6.80 cm for 'Crystal Pink' and 9.30 to 9.23 cm for 'Crystal Red' respectively (Table 3).

The mean values of the leaf area of one leaf 10 cm from the plant height did show significant differences among plant cultivars in both seasons (Table 4 and Fig. 2). 'Crystal Pink' exhibited the lowest leaf area (9.34 and 13.10 cm^2) in both seasons, while the largest leaf area was detected in 'Crystal Red' (19.99 and 20.46 cm^2) in both seasons. The mean values of the compared cultivars did not show any significant

Table 4 - Leaf area, shoots fresh weight, shoots dry weight, root fresh, root dry weight and root length of the seven studied chrysanthemum cultivars

Cultivars	Vegetative growth characters											
	LA10 (cm^2)		SFW (g)		SDW (g)		RFW (g)		RDW (g)		Root length (cm)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Crystal pink	9.34 e	14.78 bc	17.17 a	16.27 a	1.72 a	1.67 a	4.21 b	4.95 b	0.74 a	1.02 a	22.43 a	22.63 a
Crystal white	17.98 ab	19.91 a	19.80 a	18.92 a	2.15 a	2.02 a	5.17 b	5.50 b	1.39 a	1.06 a	22.14 a	21.60 a
Crystal yellow	11.13 de	13.75 bc	16.67 a	19.77 a	1.74 a	2.61 a	9.94 a	8.94 a	1.71 a	2.13 a	24.63 a	26.20 a
Crystal red	19.99 a	20.46 a	18.53 a	22.65 a	2.15 a	2.46 a	5.42 b	5.80 b	1.21 a	1.45 a	22.40 a	24.67 a
Kodiack	15.52 bc	18.49 ab	17.87 a	14.76 a	2.04 a	1.88 a	4.11 b	4.48 b	0.84 a	0.99 a	24.11 a	25.80 a
Coca bleach	14.71	17.67	15.17 a	20.31 a	1.75 a	2.40 a	3.65 b	4.46 b	0.82 a	1.14 a	24.13 a	19.41 a
Abrun	12.01	13.10 c	17.41 a	18.16 a	2.24 a	2.71 a	6.33 b	6.25 b	1.31 a	1.45 a	18.53 a	20.04 a

LA10= Leaf area measured 10 cm from plant height; SFW= Shoot fresh weight per pot unit; SDW= Shoot dry weight per pot unit; RFW= Root fresh weight per pot unit; RDW= Root dry weight per pot unit.
Values in each column followed by the different letter(s) are significantly different at P≤0.05 (Least Significant Difference).

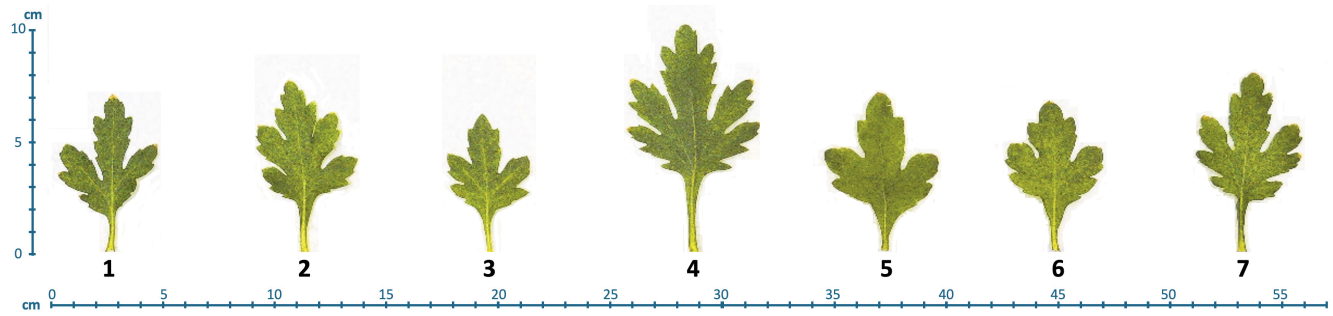

Fig. 2 - Leaf morphology of the studied chrysanthemum cultivars. 1) 'Crystal pink', 2) 'Crystal white', 3) 'Crystal yellow', 4) 'Crystal red', 5) 'Kodiack yellow', 6) 'Coca bleach', 7) 'Abrun'. Refer to Table 1 cultivars

differences in plant shoot fresh and dry weights per plant in both seasons (Table 3). Regarding the cultivar's effect on root characteristics in both seasons, the highest root fresh weight was recorded for cv. Coca Bleach (3.65 and 4.46 g), whereas the lowest root fresh weight (9.94 and 8.94 g) was detected for cv. Crystal Yellow. The mean values of the compared cultivars did not show any significant differences in root dry weight and root length per plant in both seasons.

Flower characteristics

The plants comparison cultivars had highly significant effects on number of inflorescence per plant, inflorescence diameter and inflorescence stalk length in both seasons. In the first season, the mean number of inflorescence per plant varied from 10.07 to 29.40, while the mean number height varied from 11.06 to 26.71 in the second season. The cv. Abrun recorded maximum number of inflorescence per plant followed by Crystal red and Crystal pink cultivars recorded the least height followed by cv. Crystal white (Table 5 and Fig. 3). The inflorescence diameter ranged from cv. Abrun (6.07 and 6.13 cm) to cv. Kodiack (9.93 and 10.47 cm), respectively (Fig. 3). A highest inflorescence stalk length was recorded at cv. Crystal white (6.37 and 6.77 cm), whereas the shortest inflorescence stalk length (2.77 and 2.43 cm) was detected at the cv. Crystal Yellow.

Insignificant differences were detected between the first and second seasons in fresh inflorescence weight. Significant differences in chrysanthemum inflorescence dry mass per plant were detected in the second season. The lower value resulted in an increase in inflorescence dry weight with cv. Crystal Pink (1.36 g). On the other hand, the highest value of inflorescence dry weight was observed with cv. Abrun (2.86 g). No significant differences were detected among the first season in chrysanthemum inflorescence dry weight per plant (Table 5).

The compared cultivars had highly significant effects on the number of inflorescences per branch, number of ray florets per inflorescence, ray floret length, and one inflorescence fresh weight in both seasons. In the first season, the mean number of

Table 5 - Number of inflorescences, inflorescence diameter, Inflorescence stalk length, inflorescences fresh weight and inflorescences dry weight of the seven studied chrysanthemum cultivars

Cultivars	Flower characteristics									
	Inflorescences (No.)		Inflorescences diameter (cm)		Inflorescence stalk length (cm)		Inflorescences fresh weight (g)		Inflorescences dry weight (g)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Crystal pink	10.07 c	11.06 b	9.33 a	9.37 ab	5.20 abc	3.90 cd	25.36 a	23.04 a	1.58 a	1.36 c
Crystal white	11.03 c	13.46 b	9.01 a	9.97 ab	6.37 a	6.77 a	20.00 a	23.27 a	1.85 a	1.89 bc
Crystal yellow	12.13 bc	12.40 b	7.23 bc	7.70 c	2.77 d	2.43 d	23.04 a	28.22 a	1.75 a	2.65 ab
Crystal red	24.07 a	22.03 a	6.17 c	6.13 d	4.46 bcd	4.23 bc	17.18 a	26.47 a	1.53 a	3.01 a
Kodiack	14.77 bc	12.73 b	9.93 a	10.47 a	3.63 cd	2.96 cd	20.70 a	21.10 a	2.01 a	2.03 abc
Coca bleach	17.03 b	14.77 b	8.60 ab	8.87 bc	6.23 ab	5.57 ab	14.03 a	22.24 a	1.11 a	2.05 abc
Abrun	29.40 a	26.71 a	6.07 c	6.13 d	4.1 cd	4.40 bc	19.10 a	23.76 a	2.20 a	2.86 ab

Values in each column followed by the different letter(s) are significantly different at P≤0.05 (Least Significant Difference).

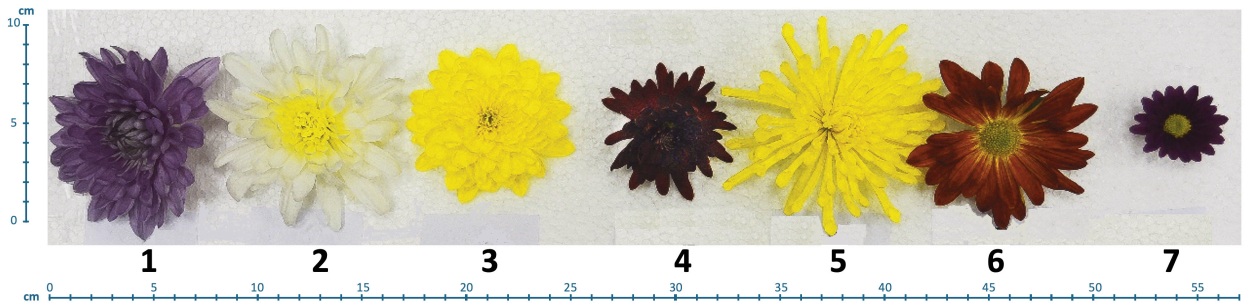

Fig. 3 - Standard-type inflorescences of chrysanthemum cultivars used in this study. 1) Crystal pink, 2) Crystal white, 3) Crystal yellow, 4) Crystal red, 5) Kodiack yellow, 6) Coca bleach, 7) Abrun. Refer to Table 1 cultivars.

inflorescences per branch varied from 4.37 to 9.73, while the mean height varied from 3.80 to 8.73 in the second season. The cultivar Crystal Red recorded the maximum number of inflorescences per branch followed by cv. Abrun; however, Crystal Yellow cv. recorded the least height followed by cv. Kodiack (Table 5). The number of ray florets per inflorescence ranged from cv. Coca Bleach (32.33 and 28.30) to cv. Crystal Red (121.00 and 126.66) in both seasons, respectively. The ray floret length ranged from cv. Abrun (2.03 and 2.30 cm) to cv. Kodiack (4.13 and 4.83 cm). The one inflorescence fresh weight ranged from (0.92 and 0.95 g) in cv. Abrun to (5.94 and 4.26 g) in cv. Crystal Pink On the other hand, one inflorescence dry weight, as affected by the compared cultivars, showed negative effects in both seasons (Table 6).

Photosynthetic pigments

The mean values of leaf chlorophyll contents are presented in figure 4A, while figure 4B shows the mean values of carotenoid and anthocyanin contents in leaves. The levels of chl a and chl b showed negligible effects, but significant changes were observed in total chlorophyll and the chl a/b ratio. Moreover, there were significant differences in carotenoid and anthocyanin contents. 'Crystal Pink' showed an increase in total chlorophyll content, while cv. Kodiack exhibited a significant reduction in total chlorophyll content compared to all other cultivars. In the present study, the levels of carotenoid and anthocyanin in the compared cultivars increased in cv. Crystal Pink, whereas cv. Kodiack had lower levels of carotenoid and anthocyanin.

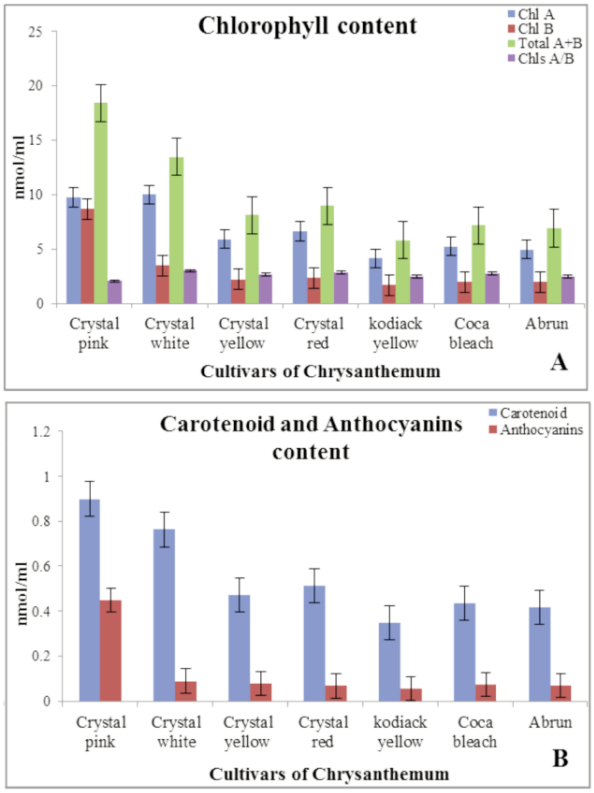

Fig. 4 - Leaf pigments of the chrysanthemum cultivars under study A) Chlorophyll (Chl) A, B, total and A/B Chls content; B) Carotenoid and anthocyanins content. Means are given with standard error.

Gas exchange

Gas exchange parameters [net photosynthetic rate (P_n), stomatal conductance (g_s), transpiration (E), and intercellular CO_2 concentration (C_i)] of the seven chrysanthemum cultivars under study showed significant variation and are depicted in figure 5.

Table 6 - Number of inflorescences per branch, number of ray floret per inflorescences, ray floret length, one inflorescence fresh weight and one inflorescence dry weight of the seven studied chrysanthemum cultivars

Cultivars	Flower characteristic									
	NIB		NRFI		LRFI (cm)		SFWI (g)		SDW (g)	
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Crystal pink	4.47 c	4.70 bc	108.67 ab	115.00 ab	3.87 ab	4.30 ab	5.94 a	4.26 a	0.40 a	0.38 a
Crystal white	6.36 bc	6.73 ab	84.65 c	91.33 c	4.06 a	3.93 bc	3.32 b	3.41 b	0.32 a	0.35 a
Crystal yellow	4.37 c	3.80 c	114.68 a	106.32 bc	3.20 b	3.47 c	2.79 c	3.36 b	0.47 a	0.35 a
Crystal red	9.73 a	8.73 a	121.00 a	126.66 a	2.43 c	2.83 d	2.01 d	1.89 c	0.23 a	0.24 a
Kodiack	4.40 c	4.03 c	98.64 b	100.67 bc	4.13 a	4.83 a	2.08 d	3.83 ab	0.21 a	0.34 a
Coca bleach	6.03 bc	5.66 bc	32.33 d	28.30 d	3.53 ab	3.93 bc	2.16 d	1.69 cd	0.26 a	0.17 a
Abrun	8.13 ab	8.41 a	33.32 d	30.34 d	2.30 c	2.03 e	0.92 e	0.95 d	0.23 a	0.25 a

NIB= Number of inflorescences per branch; NRFI= Number of ray florets per inflorescence; LRFI= Length of ray florets per inflorescence; SFWI= Fresh weight of a single inflorescence; SDWI= Dry weights of a single inflorescence.
Values in each column followed by the different letter(s) are significantly different at P≤0.05 (Least Significant Difference).

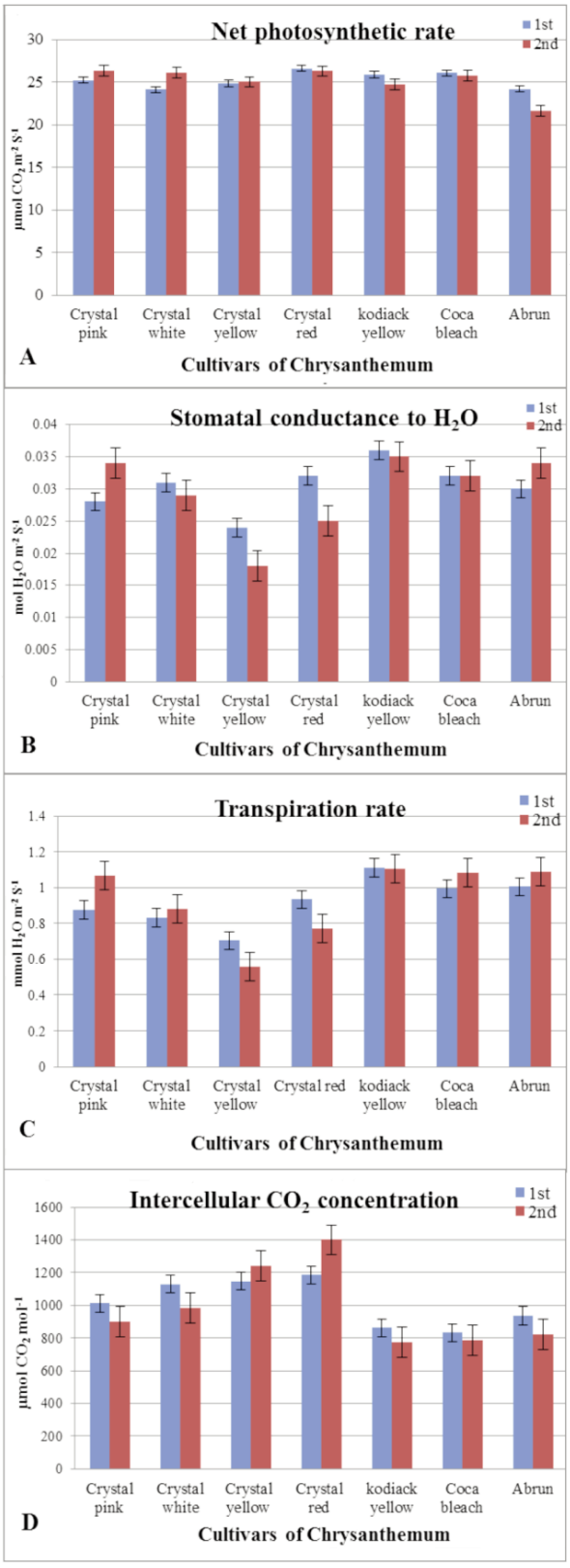

Fig. 5 - Gas exchange parameters of the chrysanthemum cultivars under study (a) Net photosynthesis rate, (b) Stomatal conductance to H_2O, (c) Transpiration rate, and (d) Intercellular CO_2 concentration). Means are given with standard error.

Cultivar 'Crystal Red' had the highest P_n, while 'Abrun' had the lowest P_n compared to other cultivars (Fig. 5A). 'Crystal Yellow' exhibited the lowest g_s and E, whereas 'Kodiack Yellow' had the highest values under the given conditions (Figs. 5B and C). C_i increased in response to 'Crystal Yellow', while it decreased in 'Kodiack Yellow', compared to other cultivars (Fig. 5D). Cultivars showed significant differences in net photosynthetic activities; however, these variations were only evident under controlled conditions.

SSR analysis

PCR amplification of DNA using 12 primers for SSR analysis resulted in a total of 40 amplified bands, all of which were polymorphic bands with a 100% polymorphism rate (Table 7; Figs. 6A and B). These results also demonstrated the presence of uniquely amplified bands in the genomic DNA of the seven chrysanthemum cultivars, which were used as molecular markers to identify each of these seven different chrysanthemum cultivars. The number of amplified bands varied from two in primers Xgwm205, Xgwm133, Xgwm181, and Xgwm210, three in primers Xcfd49, Xcfd18, and Xcfd66, four in primers Xcfd9, Xcfd46, and Xgwm174, and five in primer Xcfd1, with a total of 40 bands and DNA lengths ranging from 100 to 600 bp. Additionally, six bands were found in primer Xcfd183, with DNA lengths ranging from 900 to 1000 bp.

The results obtained from the phylogenetic tree, based on twelve SSR primers as displayed in figure 7, indicated that the seven different chrysanthemum cultivars were separated into two main clusters. Cluster A consisted of the C1 (Crystal Pink) and C5 (Kodiack Yellow) cultivars, whereas cluster B was further divided into two sub-clusters. Sub-cluster B1 contained only the C2 (Crystal White) cv., while sub-cluster B2 was also divided into two sub-clusters. The first sub-cluster included the C6 (Coca Bleach) and C7 (Abrun) cultivars, forming a closely related group, whereas the second sub-cluster consisted of the C3 (Crystal Yellow) and C4 (Crystal Red) cultivars. The similarity matrix indicated a range of values from 0.18 to 0.39, with Crystal White standing out as a distinct cultivar among the seven cultivars analyzed.

4. Discussion and Conclusions

Vegetative growth parameters such as plant

Table 7 - Total numbers of amplified fragment and polymorphic fragments generated by PCR using SSR primers

S. No.	Primer name	Total number of bands	Monomorphic bands	Polymorphic bands	Unique bands	Percent of polymorphism %
1	Xcfd1	5	0	5	0	100
2	Xgwm205	2	0	2	0	100
3	Xgwm133	2	0	2	0	100
4	Xcfd9	4	0	4	0	100
5	Xcfd46	4	0	4	0	100
6	Xgwm181	2	0	2	0	100
7	Xcfd49	3	0	3	0	100
8	Xgwm174	4	0	4	0	100
9	Xcfd18	3	0	3	0	100
10	Xcfd183	6	0	6	(1) 900-1000 bp C6	100
11	Xgwm210	2	0	2	0	100
12	Xcfd66	3	0	3	0	100
Total		40	0	40	1	100

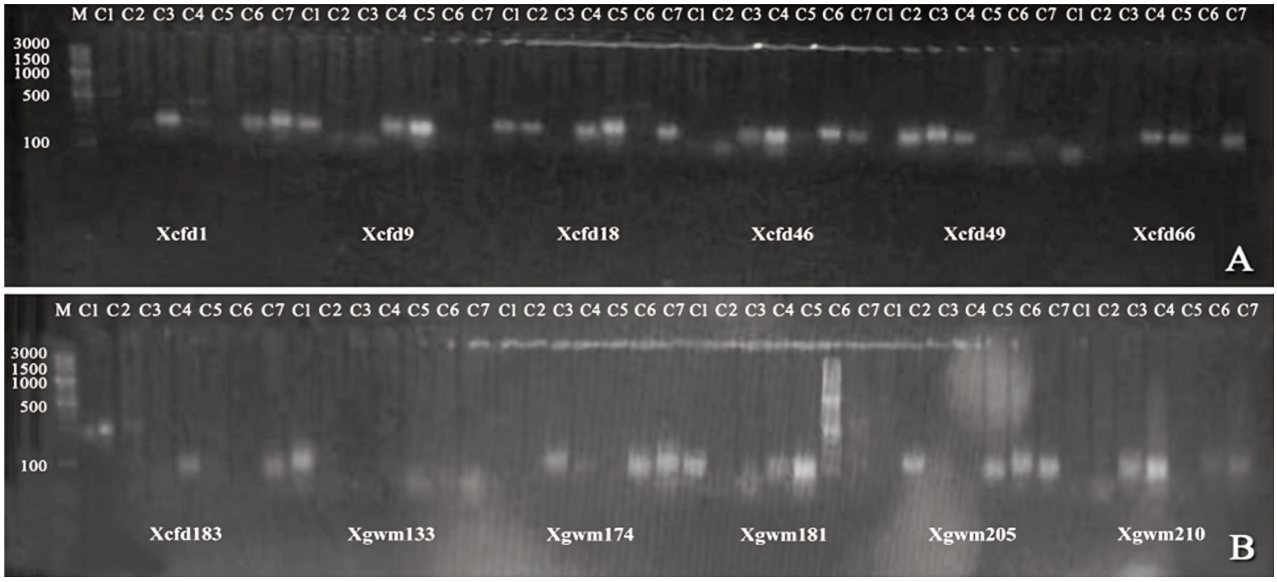

Fig. 6 - Banding of SSR patterns of seven chrysanthemum cultivars using twelve selected random primers, C1 - Crystal pink, C2 - Crystal white, C3 - Crystal yellow, C4 - Crystal red, C5 - Kodiack yellow, C6 - Coca bleach, C7 - Abrun. A) first six primers, and B) second six primers of the list primers used in this study.

height, number of branches, number of leaves per plant, leaf area, and dry weight accumulation play a crucial role in determining the overall crop yield. In this study, cultivars Crystal Red, Coca Bleach, Kodiack, and Crystal White exhibited vigorous growth, while Abrun and Crystal Yellow cultivars displayed medium growth, and Crystal Pink was characterized by dwarfism, recording the shortest plant height. The observed variations in plant height among the cultivars may be attributed to a combination of genetic factors, environmental conditions during growth, and plant management practices (Sirohi and Behera, 2000; Gharge *et al*., 2009). The increased number of leaves per plant in these cultivars was associated with higher plant height and number of branches per plant. Similar results were reported by Tarannum and Naik (2014) and Prasanth *et al*. (2020). These variations in growth characteristics may contribute to higher leaf area and ultimately increased dry weight production per plant

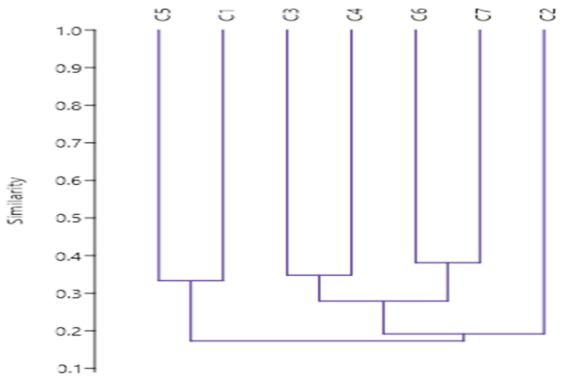

Fig. 7 - Dendrogram of relationship between seven chrysanthemum cultivars using Jaccard's (1908) index for SSR primers. C1 - 'Crystal pink', C2 - 'Crystal white', C3 - 'Crystal yellow', C4 - 'Crystal red', C5 - 'Kodiack yellow', C6 - 'Coca bleach', C7 - 'Abrun'.

in superior cultivars. These findings align with the conclusions of Barigidad *et al*. (1992) and Yoon-Jung *et al*. (2013) in chrysanthemum. The differences in growth characteristics between genotypes may be attributed to their inherent genetic traits, as all plants were subjected to similar practices under the same environmental conditions; Baskaran *et al*. (2016) also reported comparable findings.

Flower yield is a crucial factor in determining the suitability of specific genotypes for commercial cultivation, which directly affects the cost of cultivation. The maximum number of inflorescences per plant was recorded in the cultivars Abrun and Crystal Red, while Crystal Pink and Crystal White cultivars exhibited the lowest numbers. The study revealed that larger leaf area, more number of leaves and branches per plant, along with increased dry weight accumulation, resulted in higher photosynthetic activity, contributing to the production of more and larger flowers. These results are consistent with the findings of Tarannum and Naik (2014), Reddy *et al*. (2016), Palai *et al*. (2018), Singh *et al*. (2019), and Prasanth *et al*. (2020).

Flower stalk length is a critical quality characteristic that influences the quality of chrysanthemum cut flowers and extends their post-harvest life. The variation in stalk length among genotypes may be attributed to inherent genetic factors and growing environmental conditions, as reported by Dalal *et al*. (2009) and Tarannum and Naik (2014).

Flower diameter, being a genetically controlled trait, was found to be superior in cultivars Kodiack Yellow, Crystal Pink, Crystal White, and Coca Bleach, possibly due to the presence of more petals per inflorescence. However, Abrun cultivar produced smaller-sized flowers, which may be attributed to the fewer number of ray florets in its flower buds. The variation in inflorescence size could be attributed to the genetic makeup of the genotypes (Reddy *et al*., 2016; Neelam *et al*., 2018; Prabhu *et al*., 2018). Cultivar Crystal Red exhibited superiority in terms of inflorescence dry weight, followed by Abrun, while it was lower in cultivar Crystal Pink. Variations in inflorescence weight could be expected among different cultivars due to differences in genetic structure (Gharge *et al*., 2009). Carbohydrates serve as an energy source for growing buds, inflorescence opening, and longevity, ultimately resulting in strong and long inflorescence stalks and large-sized buds or inflorescences. These variations may be attributed to varietal characteristics, as reported by Halvey and Mayak (1979). Similar variations have been observed in chrysanthemum and carnation by several researchers, such as Sirohi and Behera (2000), Singh and Sangama (2003), and Uddin *et al*. (2015).

The yield and growth of any flower crop are influenced by various factors, including environment, season, and varieties. Among these factors, varieties play a significant role in the evolution of any flower crop, particularly in selecting varieties with high inflorescence production. Therefore, the selection of appropriate varieties is crucial for successful floriculture cultivation (Palai and Rout, 2011).

Photosynthetic pigments

The values of chlorophyll, carotenoid, and anthocyanin contents in the leaves of chrysanthemum cultivars are presented. Results clearly distinguish cultivars with high pigment content (Crystal Pink, Crystal White, Crystal Red, and Crystal Yellow) from those with low pigment content (Kodiack Yellow, Coca Bleach, and Abrun).

Photosynthesis in plants relies on capturing light energy using the pigment chlorophyll (Blankenship, 2014). Differences in chlorophyll a, b, carotenoid, and anthocyanin contents are indicators of damage to the photosynthetic apparatus, stress, or senescence and affect the normal course of plant biological processes (Filimon *et al*., 2016). Having a higher amount of chlorophyll could lead to increased light absorption, which is advantageous for photosynthesis in Rosa hybrida (Terfa *et al*., 2013).

The genotype of a plant affects pigment

accumulation by influencing the morphology and anatomy of the leaves (Hopkins and Hüner, 2009). Leaf area has been identified as a factor that can limit the photosynthetic capacity of plants, as reported by Petrie *et al*. (2000). However, it is important to note that the intensity of net photosynthesis (P_n) is not necessarily correlated with chlorophyll content, with differences potentially arising from variations in intracellular spaces and gaseous conductivity (Patakas *et al*., 2003). Chlorophyll loss is often linked to environmental stress, and changes in the Chlorophyll/Carotenoid ratio can serve as an indicator of stress in plants (Netto *et al*., 2005). The specific cultivar of a plant can also impact the accumulation of photosynthetic pigments by influencing the morphology and anatomy of the leaves, including factors like mesophyll thickness, area, and perimeter (Salem-Fnayou *et al*., 2011).

Gas exchange

Light affects not only the photosynthetic rates but also the stomatal function. Previous research has focused on studying the impact of long-term acclimation to specific wavelength light on stomatal morphology, density, and opening rates, as demonstrated by studies conducted by Wang *et al*. (2016) and Zheng and Van Labeke (2018). Numerous studies have also shown that light has the ability to induce stomatal opening, as observed in research conducted by Shimazaki *et al*. (2007). Stomatal conductance (g_s) is influenced by the density of stomata on the leaf surface as well as how wide the stomata are open. When plants have abundant water, high g_s levels can lead to more transpiration, resulting in reduced leaf water content. Closing stomata can help maintain leaf water content by reducing transpiration. In the case of Crystal Yellow cv. leaves, the low stomatal conductance and leaf transpiration can mainly be attributed to a decrease in stomatal conductance compared to other cultivars.

The impact of different types of light on the process of photosynthesis and transpiration in chrysanthemums at various stages of growth is uncertain. Scientists conducted experiments to measure the exchange of CO_2 and H_2O in the leaves and entire plants of chrysanthemums under long-day and short-day conditions. It was observed that all light sources effectively stimulated leaf photosynthesis, regardless of whether it was a long or short day (Leonardos *et al*., 2019).

Molecular analysis

Chrysanthemum cultivars pose challenges in terms of genetic backgrounds and similar morphological features, making it difficult to distinguish among them. Various molecular markers like sequence-related amplified polymorphism (SRAP) (Fei *et al*., 2011), inter simple sequence repeats (ISSR) (Shao *et al*., 2010), and simple sequence repeats (SSR) (Chang *et al*., 2018) have been employed to identify and classify chrysanthemum cultivars. Between these markers, SSRs offer advantages such as co-dominance, high variability, and reproducibility. SSR markers have also been utilized in the construction of molecular maps, analysis of genetic diversity, and assessment of intellectual property rights in different plants (Feng *et al*., 2016; Mekapogu *et al*., 2020). Previous studies have employed SSRs for genetic analysis of chrysanthemum and related genera (Chang *et al*., 2018). This investigation introduces a method for identifying standard-type seven cultivars in chrysanthemum.

Previous research has already established a database consisting of SSR markers that can be used to identify different cultivars of chrysanthemum. In two separate studies conducted by Shim *et al*. (2015) and Olejnik *et al*. (2021), a total of 28 SSR markers from the chrysanthemum DNA profile database were utilized to analyze the genetic relationship among a vast number of chrysanthemum cultivars, specifically 147 and 97, respectively. However, it is worth noting that very few studies have delved into the potential use of SSR markers for distinguishing standard-type chrysanthemum cultivars, as highlighted by Han *et al*. (2018) and Thakur *et al*. (2023).

In the current study, a set of twelve SSR markers was employed to distinguish and classify seven different standard-type chrysanthemum cultivars. It was determined that out of the twelve SSR markers utilized, there was noticeable genetic variation observed in the seven standard-type cultivars.

The evaluation of genetic relationships between populations serves as the foundation for both selective breeding and cultivar identification. By analyzing the SSR data and constructing a UPGMA-based dendrogram, it was observed that the tested chrysanthemum cultivars could be divided into two main groups. The similarity matrix indicated a range of values from 0.18 to 0.39, with Crystal White standing out as a distinct cultivar. Among the seven

cultivars analyzed (Crystal Pink, Crystal White, Crystal Yellow, Crystal Red, Kodiack Yellow, Coca Bleach, and Abrun) which formed cluster I, Coca Bleach and Abrun cultivars showed a moderate level of distinction and displayed complete genetic similarity. This suggests that there is a relatively low genetic diversity between these cultivars and that they may have been developed from a limited genetic background. In this study, it was found that Kodiack Yellow and Crystal White cultivars exhibited genetic divergence, which aligns with the findings of Shim *et al*. (2015), Olejnik *et al*. (2021), and Thakur *et al*. (2023), who also observed a distant relationship between Kodiack Yellow and Crystal White cultivars.

SSR genetic diversity does not necessarily match morphological differences. However, in the present investigation, to a certain extent, the clustering of genotypes in the sub-clusters seemed to correspond with a few phenotypic traits. Crystal pink and Kodiack, with the same shape of inflorescences (spoon-type) and without disk florets; the leaves are three-lobed, and both have an average inflorescence diameter, root fresh weight per pot unit, length of ray florets per inflorescence, and number of inflorescences per branch, formed sister relationships within cluster A. Within the second sub-cluster B2, two genotypes with the same shape of inflorescences (reflex-type); the leaves are five-lobed, and both have a light fresh weight of a single inflorescence, length of ray florets per inflorescence, number of ray florets per inflorescence, and inflorescence diameter (Crystal yellow and Crystal red), formed the sister relationships. Coca bleach and Abrun have the same shape of inflorescences (single/sami-double-type) and disk florets color (yellow-green center), and both have a low number of ray florets per inflorescence, leaf area, and leaf length, forming sister relationships within the first sub-cluster B1. The genotype Crystal white remained as a single cultivar in a separate sub-cluster within the major (B), which is the only white color cultivar in the group (B). Inflorescence was the type of irregular-type chrysanthemums. Buldewo *et al*. (2012) performed clustering based on spathe color in *Anthurium andraeanum* to group phenotypic traits/colors. Dai *et al*. (2012) used SSR markers to classify chrysanthemum germplasm based on inflorescence. The economic uses of various cultivars were observed to be linked with different clusters within the primary groups. In a study by Minano *et al.* (2009), chrysanthemum cultivars were grouped according to their inflorescence type and cultural characteristics.

The use of twelve SSR primers resulted in 100% polymorphism in seven chrysanthemum cultivars, demonstrating a remarkably high level of diversity. This confirms the effectiveness of these SSR markers in analyzing the genetic characteristics of chrysanthemum cultivars (Mekapogu *et al*., 2020; Olejnik *et al*., 2021).

Chrysanthemums possess various traits, including diverse flower shapes and colors, plant sizes, forms, and flowering periods, which are extensively utilized in landscaping. The study identified notable distinctions among the different cultivars. This research reveals that our method of using SSR markers is effective in assessing the genetic connections between closely related chrysanthemum cultivars and distinguishing between them. These microsatellites can be employed for certifying protected varieties and conducting pedigree analysis.

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