Scientia Horticulturae 272 (2020) 109524

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Induction of tetraploids in 'Red Flash' caladium using colchicine and oryzalin: Morphological, cytological, photosynthetic and chilling tolerance analysis

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ARTICLE INFO

Keywords:
Chromosome counting
Chromosome doubling
Flow cytometry
Photosynthesis
Variants

ABSTRACT

In vitro pre-cultured leaf segments of 'Red Flash' Caladium (*Caladium* × *hortulanum* Birdsey) were treated with 0.1%, 0.2% and 0.3% (w/v) colchicine, or 0.001%, 0.002% and 0.003% (w/v) oryzalin for 2, 4 and 6 days, respectively, with the aim to develop an efficient polyploid induction protocol for caladium, and to identify promising caladium variants for cultivar development and chilling tolerance breeding. A total of 206 out of 723 plants were found to exhibit stable and remarkable morphological changes, and were grouped into 10 variant types based on differences in leaf shape, color, and/or coloration. As many as 93 plants were identified preliminary as tetraploids by flow cytometry, and the most efficient way for chromosome doubling seemed to be exposed to 0.002% oryzalin for 6 days. Chromosome counting were performed to further determine the ploidy level of the variants as extensive variation of mean fluorescence intensity (MFI) were recorded among them, and results showed that chromosome gains or losses occurred frequently in the established variants. As compared to the wild type, tetraploidization resulted in plants with rounder and thicker leaves, larger petiole diameter, higher plant height, lower leaf number per plant, and lower stomatal density, and significantly increased the net photosynthesis rate, transpiration rate, and stomatal conductance. Furthermore, enhanced chilling tolerance were observed in the tetraploids (T1), as evidenced by their higher superoxide dismutase (SOD) activity, peroxidase (POD) activity and proline (Pro) content, and a lower relative electrical conductivity (REC) and malondialdehyde (MDA) content in the leaves compared with those of the diploid counterparts and the diploid aneuploids (SVT1) during chilling stress. The variants associated with valuable phenotypic traits including the tetraploids, diploid variants, diploid aneuploids, and tetraploid aneuploids hold considerable potential for cultivar development, genetic study and chromosome engineering in caladium.

1. Introduction

Caladium Vent is an important perennial foliage plant of the Araceae family. They are often forced in containers or utilized in the landscape for their colorful and variably shaped leaves (Wilfret, 1993; Deng, 2012). Commercial caladium cultivars are very sensitive to low temperature and chilling stress, and minimum temperatures below 15°C can delay tuber sprouting, damage leaves and roots, and stop plant growth (Wilfret, 1993; Deng, 2018). Efforts have been dedicated to develop chilling-tolerant caladium cultivars *via* conventional cross breeding (Deng and Harbaugh, 2006; Deng et al., 2008), while it has encountered challenges such as lack of chilling-tolerance parents. Hence, it is imperative to develop new methods to create novel caladium germplasm with desirable ornamental traits and chilling tolerance.

Polyploidization has profound effects on dosage-regulated gene expression, resulting in diverse phenotypic and genetic variation (Osborn et al., 2003), and thus plays an important role in plant genetic improvement and breeding. Compared with their diploid progenitors, polyploids often exhibit broader environmental adaptability, such as enhanced abiotic stress tolerance (Liu et al., 2011; Denaeghel et al., 2018; Jiang et al., 2019), disease resistance (Wang et al., 2018; Šedivá et al., 2019), insect tolerance (Hannweg et al., 2016), and photosynthetic performance (Zhou et al., 2017; Cao et al., 2018), which in turn may result in polyploids with increased growth vigor (Zahumenická et al., 2018), nutritional value (Hannweg et al., 2016), and secondary metabolite production (Javadian et al., 2017; Shmeit et al., 2020). Furthermore, polyploids are reported to often have valuable phenotypic traits, such as larger leaves (Javadian et al., 2017), thicker and broader leaves (Podwyszyńska et al., 2017; Wang et al., 2018), thicker stems (Kermani et al., 2003; Jiang et al., 2019), deeper

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https://doi.org/10.1016/j.scienta.2020.109524
Received 28 January 2020; Received in revised form 18 April 2020; Accepted 27 May 2020

green leaves (Kermani et al., 2003; Podwyszyńska et al., 2017), bigger or more showy flowers (Wang et al., 2018; Šedivá et al., 2019), earlier or delayed flowering (Zahumenická et al., 2018; Podwyszyńska et al., 2018), extended vase life (Manzoor et al., 2018), and a more compact plant shape (Denaeghel et al., 2018) when compared to their diploid counterparts. For these benefits, polyploidization has been considered an efficient and prominent strategy to create a wide germplasm base for genetic improvement in a wide range of horticultural crops including ornamental plants (Kermani et al., 2003; Cai et al., 2015; Manzoor et al., 2018; Fu et al., 2019).

Recent progress revealed that antimitotic agents such as colchicine and oryzalin can induce autopolyploids in an efficient and reliable way. Colchicine is an alkaloid that can prevent spindle microtubule formation at the early anaphase stage during cell division, and has been well established for polyploid induction reviewed by Eng and Ho (2019). Besides, oryzalin is referred as a preferable alternative for chromosome doubling, due to its lower toxicity and much higher affinity to plant microtubules at much lower concentrations as compared to colchicine (Thao et al., 2003; Chung et al., 2014). Presently, chromosome doubling by application of oryzalin has increasingly become a promising ploidy manipulation tool in a variety of plant species (Thao et al., 2003; Chung et al., 2014; Zahumenická et al., 2018; Shmeit et al., 2020). *In vitro* system has been successfully established in an increasing number of plant species for polyploid induction, due to its higher efficiency, shorter term and controlled conditions (Eng and Ho, 2019). Various explant types are usually used for *in vitro* polyploidization, such as shoots (Podwyszyńska et al., 2017), shoot tips (Kermani et al., 2003; Thao et al., 2003) and leaf segments (Cai et al., 2015). Certainly, concentrations and exposure time of antimitotic substances are needed to be optimized to increase the survival rate and polyploidization efficiency of different explant types.

Although several studies have been conducted on *in vitro* chromosome doubling in ornamental aroids (Thao et al., 2003; Chen et al., 2011) including caladium (Cai et al., 2015) in recent years, few reports are available regarding the effects of polyploidization on the ornamental values and stress tolerance of these plants. Herein, one-month-cultured leaf cultures of 'Red Flash' caladium were treated by different concentrations of colchicine or oryzalin for different time periods, respectively, and relative DNA content analysis, chromosome counting, morphological comparison, stomatal analysis, photosynthesis comparison as well as chilling tolerance evaluation were performed to characterize the regenerated morphological variants, with the aim to develop an efficient *in vitro* polyploidization technique and identify promising variants holding enhanced ornamental values and/or chilling tolerance for future caladium breeding.

2. Materials and methods

2.1. Plant material preparation

In vitro plantlets of 'Red Flash' caladium (*Caladium* × *hortulanum* Birdsey) were kindly provided by Dr. Zhanao Deng (the University of Florida's Gulf Coast Research and Education Center, Wimauma, FL, USA.) and subcultured three times a year on Murashige and Skoog (MS) (1962) basal medium. Plant materials were prepared by *in vitro* culture of young leaves of the micropropagated plants of 'Red Flash' caladium according to Cai and Deng (2016). Briefly, the leaves were cut into small segments (about 0.5 × 0.5 cm) using a surgical blade, and then cultured on callus induction medium (CIM), *i.e.*, MS (Murashige and Skoog, 1962) medium supplemented with 2 mg/L thidiazuron (TDZ), 1 mg/L 6-benzyladenine (6-BA), 4% (w/v) sucrose and 0.7 % (w/v) agar, pH 5.8. One month later, the induced leaf cultures were collected for subsequent treatments.

2.2. Colchicine and oryzalin treatments

The induced leaf cultures were treated with 0.1%, 0.2% and 0.3% (w/v) colchicine (Sinapharm Chemical Reagent Co., Ltd, China), or 0.001%, 0.002% and 0.003% (w/v) oryzalin (Shanghai Yuanye Bio-Technology Co., Ltd., China) for 2, 4 and 6 days, respectively, and those cultured in liquid CIM for 6 days were used as a control. Different concentrations of colchicine and oryzalin were prepared by dissolving them in 2% (V/V) dimethylsulfoxide (DMSO) and then incorporated into a certain volume of fresh CIM, respectively, and were sterilized by filtration through 0.22 μm membrane filters. For each treatment, a total of 20 leaf cultures were picked out and submerged in 40 mL of treatment solution in an Erlenmeyer flask, and all these flasks were placed in a shaker at 100 rpm and 25 ± 1°C in the dark. When the treatment was completed, the leaf cultures were rinsed with sterile water five times and placed horizontally on solidified MS medium supplemented with 1 mg/L 6-BA, 1 mg/L 1-naphthylacetic acid (NAA), 3% (w/v) sucrose and 0.7 % (w/v) agar (pH 5.8) for plantlet regeneration. Five treated leaf cultures were cultured in a glass jar containing about 100 mL of the medium, and incubated in a culture room at 25 ± 2°C under cool white fluorescent lamps (about 55 μmol $m^{-2}s^{-1}$, 12 h: 12 h light: dark cycle) for 4 months before planting *ex vitro*. All treatments were repeated three times.

2.3. Plant establishment and visual screening

Regenerated plantlets were transplanted individually to cell trays (6 × 6 × 11 cm/cell) filled with a commercial potting mix (Nursery Soil Mixture, Jiangsu Peilei Biotechnology CO., LTD., China) and maintained in an artificial climate chamber with a temperature of 26–28°C, a 12 h: 12 h light (about 65 μmol $m^{-2}s^{-1}$): dark cycle, and a relative humidity of 70–75%. After one month of acclimatization, the survived plantlets were then transplanted into plastic containers (Top inside diameter: 8.4 cm, bottom diameter: 6.0 cm, height: 14.5 cm) filled with the similar potting mix, and were grown in a growth room with 12 h light of approximately 87 μmol $m^{-2}s^{-1}$ at 20–28°C for further plant growth. After another 3 months of culture, the plants were observed weekly and any plants exhibiting obviously different morphological characteristics from the wild were flagged and monitored closely. When the plants were 5-months-old after transplantation from the glass jars, the variants were selected and transplanted into plastic pots (15 cm in diameter and 17.5 cm in height) contained the above mentioned culture mix, and were grown under the similar conditions as described above. Four months later, leaf shape and leaf color patterns including color of main veins, leaf margins, and leaf spots were visually examined and compared, and stable morphological variants were used for the following study.

2.4. Flow cytometry analysis

Ploidy level of the wild type and the screened variants was estimated preliminarily by using a CytoFLEX flow cytometer (Beckman Coulter, USA). For releasing nuclei, approximately 50 mg of fresh leaves were chopped with a sharp single-sided blade in a plastic Petri dish containing 350 μL of cold WPB lysis buffer (Loureiro et al., 2007) on ice, and then filtered through a 40 μm nylon mesh filter into a 5 mL sample tube. Thereafter, 200 μL RNase A solution (100 μg/mL) and 200 μL propidium iodide (PI, 100 μg/mL, Shanghai Yuanye Bio-Technology Co., Ltd., China) were added to the sample tube and maintained in dark for 30 min at room temperature for staining the released nuclei, and then shaken gently for 5 s before sample analysis. Three runs were performed for each plant according instructors of the manufactory, and at least 3000 nuclei were analyzed in each run.

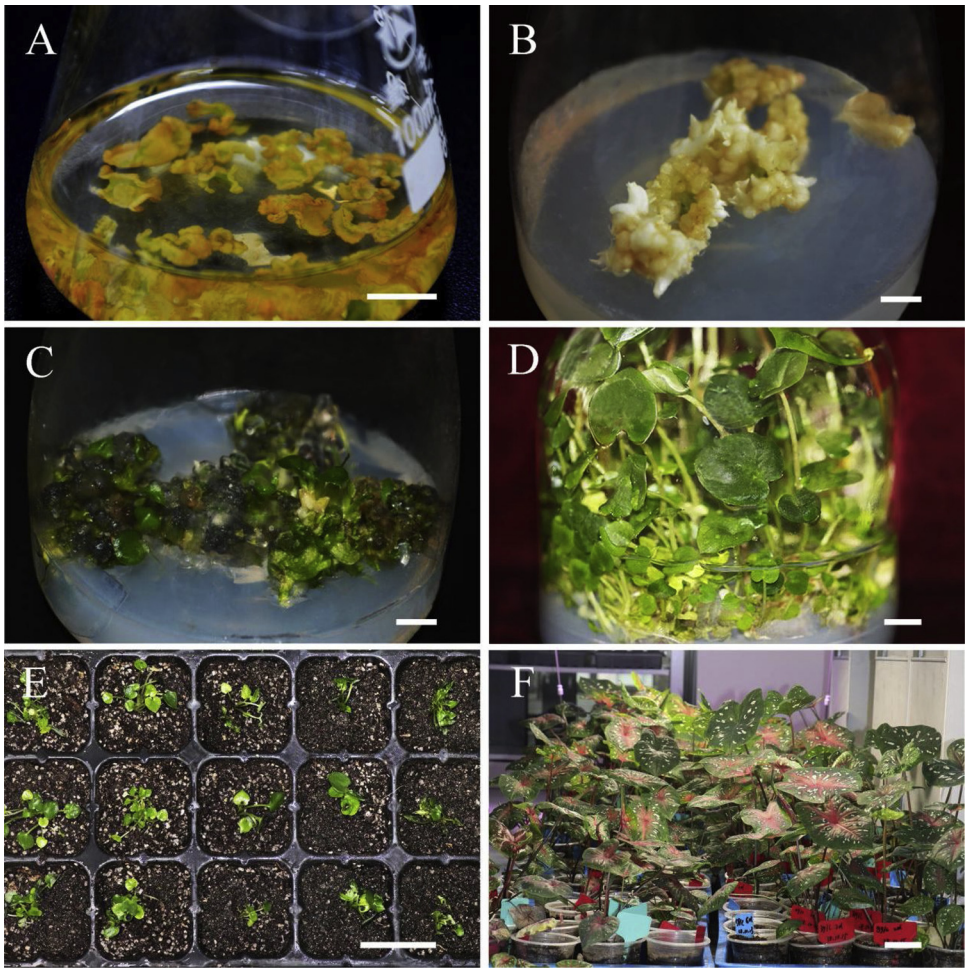

Fig. 1. *In vitro* regeneration and transplantation of plants from colchicine or oryzalin treated leaf cultures of 'Red Flash' caladium (*Caladium* × *hortulanum* Birdsey). (A) Caladium leaf cultures soaked in the liquid callus induction medium containing colchicine or oryzalin in the dark, (B) Initiated shoots or roots from the leaf cultures treated by 0.002% oryzalin for 6 days after one month of culture on MS basal medium supplemented with 1 mg/L 6-BA and 1 mg/L NAA, (C) Dark green shoots regenerated from the treatment of 0.1% colchicine for 4 days after 2 months' culture, (D) Plantlets regenerated from the leaf cultures treated with 0.002% oryzalin after 4 months' culture, (E) Regenerated plantlets grown in cell trays during acclimation, (F) Growth of the caladium plants 4 months after transplantation (*bar* = 1 cm for A-D, and 5 cm for E and F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.5. Chromosome counting

Chromosome counting was carried out *via* root tip squash technique according to Cao et al. (2016) with minor modification. Actively growing root tips (about 1 cm long) were excised at 9:00–11:00 AM and instantly immersed in 0.002 M 8-hydroxyquinoline solution for at least 3 h in dark at 4°C. After washing with distilled water for 10 min, the pretreated root tips were fixed in fresh Carnoy solution (absolute ethanol: glacial acetic acid = 3:1, v/v) at room temperature for 24 h in dark. The fixed root tips were rinsed under running water for 10 min, and then hydrolyzed in 1 *N* HCl at a water bath of about 60°C for 5 min. After hydrolysis, the root tips were rinsed with deionized water for 10 min again. After removal of the root caps with a sharp blade on a glass slide, the remaining tissues were stained in a drop of carbol-fuchsin stain (Solarbio, Beijing, China) for approximately 10 min at room temperature. Thereafter, the samples were covered with a cover slide and squashed gently to spread the stained cells, and were examined and photographed under a bright field microscope (Panthera L, Motic, China) at 1000 times magnification.

2.6. Morphological characterization, stomatal analysis and gas exchange measurements

With an electronic digital caliper, thickness of leaf blades was taken between major leaf veins, and petiole diameter was measured at the middle part. At least five plants of each selected variation type and two mature leaves per plant were used for analysis of leaf characteristics including plant height, leaf number per plant, leaf length and width, leaf thickness, and petiole diameter. Stomata length, width and density were measured according to Cai et al. (2015), and three independent counts were carried out on each mature leaf. Three photosynthesis parameters including net photosynthetic rate, stomatal conductance, and transpiration rate were measured on three replications per plant from 9:30 and 11:00 in a sunny morning using the LI-6400 (Li-Cor, Lincoln, NE, USA) portable photosynthesis system following the instructions of the manufacturer.

2.7. Evaluation of chilling tolerance

Prior to chilling treatments, the wild and the variant plants were watered thoroughly, and then maintained in an artificial climate box for 3 days with similar conditions to those adopted during acclimatization to alleviate the differences of the physiological status of these plants. Three plants of each type were placed in each climate box. Afterwards, the temperature of the climate boxes was stepwise reduced to 15°C, 10°C, 5°C and 0°C to avoid sudden chilling damage, respectively, with 28°C as a temperature control. After these plants were maintained at constant temperatures of 28°C, 15°C, 10°C, 5°C ant 0°C for 3 days, respectively, leaves were detached and placed in a 10 mL centrifuge tube, and then immersed in liquid nitrogen. These samples were stored at –80°C for subsequent evaluation of physiological and biochemical parameters related to chilling tolerance, and all measurements were repeated three times. For assessing the damage level of the leaf cell membrane, leaf relative electrical conductivity (REC) was measured following the protocol of Zhou and Leul (1998). Malondialdehyde (MDA) is one of the most important products of lipid peroxidation, which has been found at elevated levels when plants are exposed to low temperatures (Liu et al., 2011). Enhanced activity of superoxide

dismutase (SOD) and peroxidase (POD) in plants can alleviate oxidative damages induced by chilling stress (Ashraf and Ali, 2008), and accumulation of proline (Pro) is a widespread protective response in plants when suffered from environment stress (Delauney and Verma, 1993; Liu et al., 2011). For determination of the SOD activity, POD activity, MDA content and Pro content, the corresponding assay kits (Nanjing Jiancheng Bioengineering Ins., Nanjing, China) were used according to the manufacturer's protocols.

2.8. Statistical analysis

Data of different parameters were analyzed statistically by using one-way ANOVA in SPSS statistical software (version 23 for Windows). The data were expressed as the mean value ± standard deviation, and significant differences between the means were evaluated using Duncan's multiple range test at $P < 0.05$.

3. Results

3.1. Plant establishment

Small amount of callus was observed around the cut surfaces of the leaf segments after culture on the CIM for one month. After treatment with colchicine and oryzalin, some of the leaf explants turned dark yellow (Fig. 1A). Shoots and roots began to initiate from the leaf cultures after about one month of culture on MS basal medium supplemented with 1 mg/L 6-BA and 1 mg/L NAA (Fig. 1B), and the shoots turned dark green after another month of culture (Fig. 1C). Four months later, a number of well-rooted plantlets were regenerated (Fig. 1D). These plantlets were transplanted to cell trays individually (Fig. 1E) and maintained in an artificial climate chamber, and over 80% of the plantlets were survived after one month's acclimatization. Then they were transplanted to plastic containers for further growth, and each plant was labeled with a treatment code followed by a sequential number. Five months after transplantation from culture vessels, large quantities of plants with different leaf color patterns were established (Fig. 1E). At last, a total of 723 plants were produced from all treatments, and 206 plants showed stable morphological changes in leaf shape, color, and/or coloration from the wild type were established in this study (Table 1).

3.2. Types of variants

Remarkable morphological differences were observed among the 206 variants (Table 1 and Fig. 2). Based on the differences in leaf shape, and color of main veins, leaf margins and leaf spots, the variants were grouped into 10 variant types, *i.e.*, 5 somaclonal variation types (SVT) from SVT1 to SVT5, and 5 tetraploid types (T) including T1 to T5 (Table 1; Fig. 2), and the naming was based on subsequent ploidy and chromosome counting analysis. As shown in Fig. 2, wild plants developed large heart-shaped leaves, and each leaf was characterized with red main veins, multiple white spots between veins, and medium-green margins. Leaves of the SVT1 were drastically different from the wild caladium, with green main veins and leaf margins, and the total number of this variant type accounted for 50% (103/206) of the variants. Green main veins were also observed in the SVT4 and the T2, while the leaves of the SVT4 were mostly green and much smaller, and had no leaf spots as compared to the T2. All the rest variant types exhibited red main veins, but their leaf color patterns were obviously different from each other. The leaves of the SVT2 were significantly smaller with dark-green and curly leaf margins when compared to the wild type, and rugose leaves were also observed in the SVT4 and T4. The leaves of the SVT3 was triangular heart-shaped and exhibited a brighter red color with small pinkish red spots, while the leaves of the SVT5 had purple red veins and large white spots. For the tetraploid types, the leaf color pattern of the T1 plants seemed to be similar to the wild caladium, while the leaves was rounder and thicker than the wild type. Similar results were also observed between the SVT1 and the T2. The leaves of the T3 were drastically different from the other tetraploid variants, with rounder heart-shaped leaves and several small white leaf spots. The T4 and the T5 had much smaller leaves, showing small pinkish red and white spots between main veins, respectively. Especially for the T5, the leaves were smallest among all the variant types with a dull color, as could be attributed to their slow growth.

Table 1
Main foliar characteristics of the 'Red Flash' caladium (wild type) and the 10 variation types grown in plastic pots for 3 months.

Plant types	No. of the plants in the group	Leaf color characteristics		
		Main veins	Leaf margins	Leaf spots
Wild type	497	Red	Green	White
SVT1[1]	103	Green	Green	Light green
SVT2	2	Red	Dark green	No spots
SVT3	6	Red	Green	Pinkish red
SVT4	1	Green	Green	No spots
SVT5	1	Purple red	Green	White
T1[2]	57	Red	Green	Big and white
T2	27	Green	Green	Big and white
T3	1	Purple red	Green	White
T4	1	Red	Green	Pinkish red
T5	7	Purple red	Dark green	Pinkish red

[1] SVT: Somaclonal variation type.
[2] T: Tetraploid type.

3.3. Effects of colchicine or oryzalin on polyploid and variant induction

As shown in Fig. 3, all the variants and the wild type showed one main peak of relative fluorescence intensity during ploidy analysis, suggesting that they were not chimeras or mixoploids. The 2C peak of the diploid wild caladium was situated at a value of about 120 in the histogram, and the morphological variants including the SVT1–SVT4 all had a main peak at the diploid area, indicating they were potential diploids. The 2C peak of the T1–T5 was at about 240, which was double that of the wild type, indicating that these variant types appeared to be tetraploids.

The effects of different treatments on tetraploid induction were summarized in Table 2. According to the preliminary ploidy analysis, all colchicine and oryzalin treatments yielded tetraploids, and the largest number of tetraploids (21) and the highest tetraploid induction rate (46.67%) were achieved when the leaf cultures were exposed to 0.002% oryzalin for six days, followed by the treatment of 0.003% oryzalin for 6 days. Among the colchicine treatments, the highest doubling rate (15.00%) was obtained when treated by 0.2% colchicine for 4 days, while only 3 plants were identified as tetraploids in this treatment. The results indicated that oryzalin at the concentrations used had a much higher efficiency in polyploid induction than colchicine in caladium. Furthermore, morphological variation rate varied in a wide range among these treatments, and the highest (77.78%) was also observed in the treatment of 0.002% oryzalin for 6 days (Table 2), as might be due to its highest induction efficiency of tetraploids with considerable morphological changes (Table 1 and Fig. 2). Unexpectedly, ten out of 59 plants from the control were also found to be morphological different from the wild caladium.

3.4. Variation of relative DNA content and chromosome number

As shown in Table 3, extensive variation of mean fluorescence intensity (MFI) from 1296264.78/2C (the SVT4) to 2514211.12/2C (the T2) were recorded among the 10 variation types, which was equivalent to 3.43% and 100.60% increase compared with the wild, respectively.

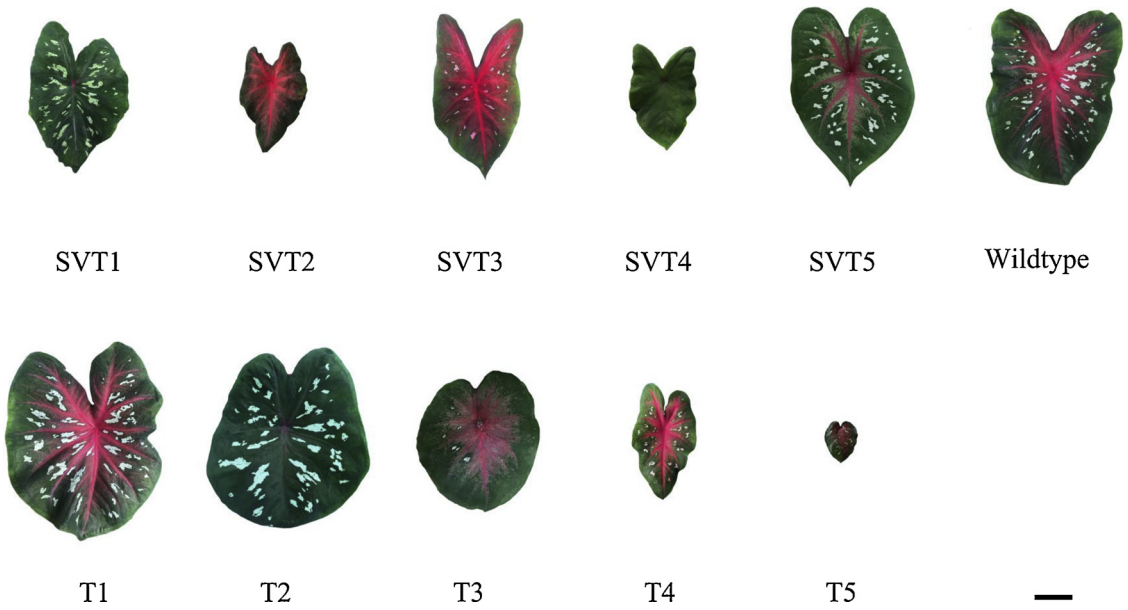

Fig. 2. Typical leaves of the wild type and the 10 variation types including five somaclonal variation types (SVT) and five tetraploid types (T) of 'Red Flash' caladium regenerated from leaf cultures treated with colchicine or oryzalin solution at different concentrations and durations. The regenerated plants were established in plastic pots filled with a commercial potting mix and grown in a greenhouse for 4 months (*bar* = 5 cm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The MFI of the four variation types (SVT1–SVT4) was similar to that of the wild type, while the SVT5 had 36.96% increased MFI compared with that of the wild type. The MFI of T1, T2, T3, T4 and T5 showed significantly increased MFI by 94.29%, 100.06%, 94.20%, 88.60% and 93.15% compared with that of the wild type, respectively.

Results of chromosomes of the wild type and 10 variation types were shown in Fig. 4 and Table 3. By chromosome counting, the wild caladium had 30 chromosomes (Fig. 4A) as previously reported by Cao et al. (2016). The chromosome number was $2n = 2x + 2 = 32$ in the SVT1, SVT2 and SVT3 (Fig. 4B–D), as might be explained by their increased MFI compared with the wild type. The chromosome numbers of the SVT4 were similar to the wild type ($2n = 2x = 30$) (Fig. 4E), which was consistent with their MFI. The SVT5 had four more chromosomes than the wild type ($2n = 2x + 4 = 34$) (Fig. 4F), as might be attributed to its significantly increased MFI as compared to the wild type. The chromosome number of T1, T2 and T4 was $2n = 4x = 60$, 64 and 58 in the cells, respectively (Fig. 4G–I), which was largely consistent with their measured MFI. No data were obtained for the T3 and T5, as they grew poorly and could not provide enough vigorously growing roots for chromosome counting.

3.5. Variations of morphological, stomatal and gas exchange parameters

Morphological, stomatal and gas exchange parameters were compared among the wild type, the T1 (tetraploid, $2n = 4x$) and the SVT1 (diploid aneuploid, $2n = 2x + 2$) (Table 4). The SVT1 was analyzed in this study as this type accounted for a higher proportion of the established variants (Table 1). As shown in Table 4, the T1 had a significantly higher plant height, leaf width, leaf thickness and petiole diameter, and a significantly lower leaf number per plant as well as leaf length/width ratio as compared to the wild type. No significant differences between the wild type and the SVT1 in terms of all the analyzed morphological traits besides the plant height. For the stomatal parameters, the stomatal density of the T1 was significantly lower than that of the wild type and the SVT1, while the stomatal guard cell length of the T1 was significantly higher than the other two types. These results indicated that polyploidization leaded to rounder and thicker leaves, thicker petiole diameter, lower leaf number per plant, and lower stomatal density in caladium.

It could be seen from Table 4 that the net photosynthesis rate, transpiration rate, and stomatal conductance of the T1 and the SVT1 were all significantly higher than that of the wild type, respectively, and there were also significant differences between them for these gas exchange parameters except the net photosynthesis rate. These results indicated that polyploidization significantly increased the photosynthetic capability in caladium. Unexpectedly, the net photosynthesis rate of the SVT1 reached 8.73 μmol CO_2 m^{-2} s^{-1} and was almost twice of that of the wild type, significantly higher than that of the other analyzed plants.

3.6. Evaluation of chilling tolerance of tetraploid and diploid plants

Two variant types including the SVT1 (diploid aneuploid) and the T1 (tetraploid) were used for chilling tolerance testing (the wild caladium as a control) as shown in Fig. 5. When the temperature was decreased from 28°C to 15°C, all the caladium plants could still grow normally. At 10°C, the upper petioles of the wild type and the SVT1 were slightly wilted and the leaf margins of them began to curl towards midrib, while obvious changes were not observed in the T1 and their petioles still stood upright. At 5°C, chilling damages to the three caladium types was obviously observed, such as greatly wilted and curled leaves, especially for the diploid plants. At 0°C, the whole plants of the wild type and the SVT1 were withered completely with very severe necrosis of the leaves and the petioles, while the leaves of the T1 did not wilted entirely after chilling stress for 3 days. These results indicated that polyploidization increased chilling tolerance of caladium at least to a certain degree.

The damage level of the membrane based on the REC showed nearly the same pattern in chilling treatments in the three types of caladium, *i.e.*, the REC of them was all increased with the decrease of temperature (Fig. 6A). A significant increase of the REC was detected both in the SVT1 when the temperature decreased from 28°C to 15°C and in the wild type when it decreased from 10°C to 5°C, while there were no significant differences of the REC for the T1 within the two chilling ranges (Fig. 6A). Although no significant differences of the REC were measured among the three plant types at 5°C and 0°C, the REC of the T1 was always lower than that of the wild type and the SVT1 (Fig. 6A). The MDA content was measured in this study as an indicator of the damage

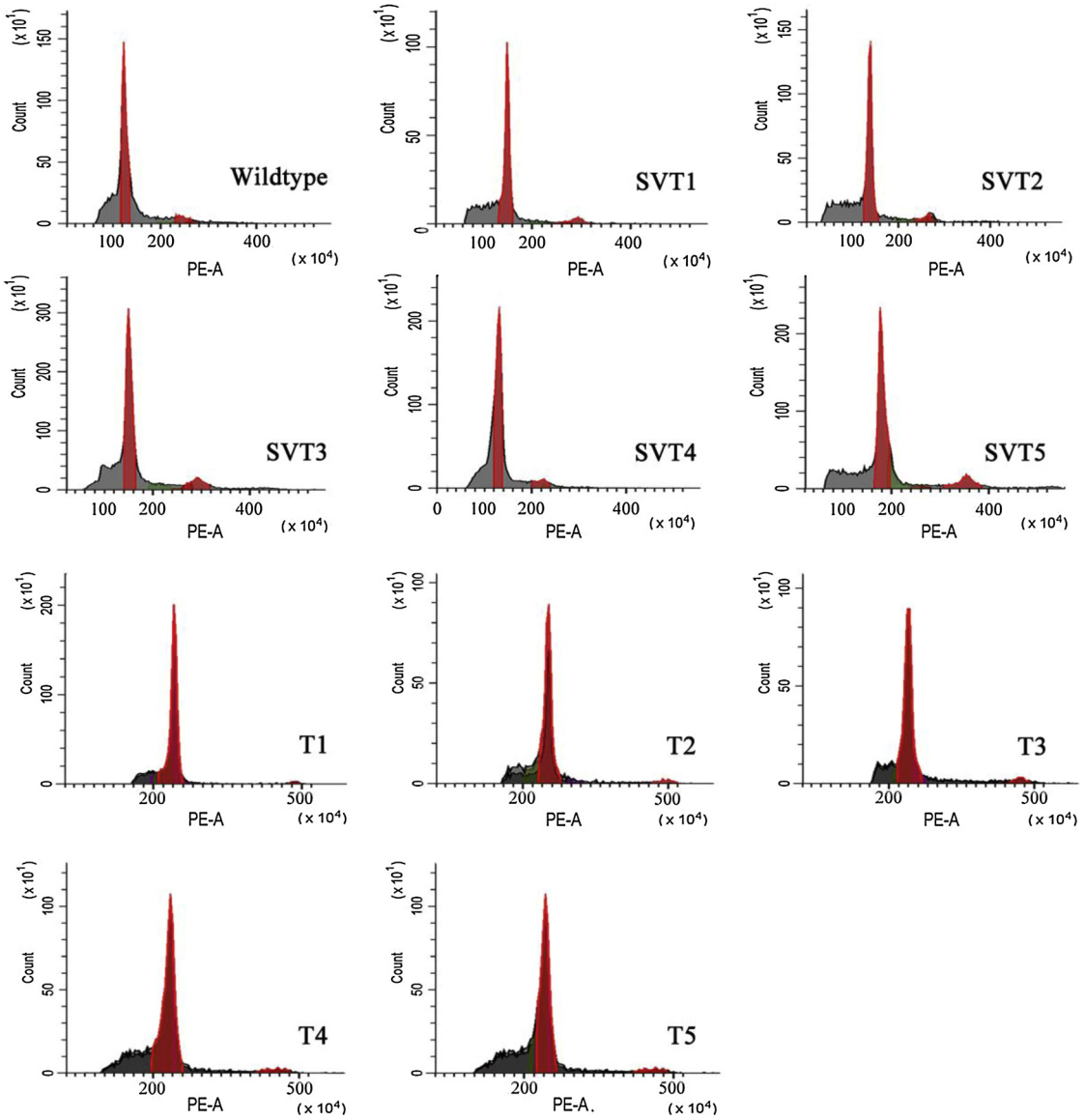

Fig. 3. Flow cytometry analysis of the wild type and the 10 variation types of 'Red Flash' caladium regenerated from leaf cultures treated with colchicine or oryzalin at different concentrations and durations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

to the plasma membrane. As shown in Fig. 6B, the MDA content in the T1 leaves was always lower than the other two types when suffered from chilling stress, and was significantly lowest especially at 5°C among the three types. For the antioxidant enzymes, the activity of SOD and POD in the three types all tended to increase initially (from 28°C to 5°C) and then decrease (from 5°C to 0°C) (Fig. 6C and D). When the temperature was reduced from 15°C to 10°C, the SOD activity of the SVT1 increased significantly, while no significant change was detected either in the wild caladium and the T1. From 10°C to 0°C, no significant difference was observed among the three caladium types for the SOD activity. However, the T1 always had higher SOD activity than the wild type and the SVT1 during the chilling stress (Fig. 6D). For the POD activity, no significant increase was recorded in each type when the temperature was reduced from 28°C to 15°C, while there were a significant increase both in the wildtype and the T1 when the temperature decreased from 15°C to 5°C (Fig. 6D). When the temperature was decreased from 5°C to 0°C, a significant decline occurred in all the three types with respect to the POD activity, and no significant difference was found between the wild and the SVT1, while their POD activity was both lower than the T1. For the Pro content, a slight decline (from 28°C to 15°C), an obvious increase (from 15°C to 5°C), and a significant decrease (from 5°C to 0°C) were found in the T1 and the wildtype, while there were almost no significant changes in the STV1 during the chilling stress besides the temperature range from 28°C to 15°C (Fig. 6E). The Pro content in the T1 was also found to always be higher than that of the wild type and the SVT1 during the chilling treatments. In conclusion, the tetraploids (T1) always had a higher SOD activity, POD activity and Pro content, and a lower REC and MDA content as compared to those of the wild type and the SVT1 during chilling stress, indicating that the tetraploids (T1) had enhanced chilling tolerance as compared to the wild type and the diploid aneuploid plants (SVT1).

4. Discussion

4.1. *In vitro* polyploid induction

Chromosome doubling continues to contribute greatly to genetic improvement and utilization in numerous crops. Recently, *in vitro* polyploid induction using antimitotic agents such as colchicine and oryzalin has gained enormous advances in a number of plant species (Thao et al., 2003; Chung et al., 2014; Cai et al., 2015; Podwyszyńska et al., 2018; Eng and Ho, 2019). In caladium, *in vitro* regeneration

Table 2
Summary of morphological variants and tetraploids regenerated from 'Red Flash' caladium leaf cultures treated by three concentrations and three durations of colchicine and oryzalin, respectively.

Treatment	Concentration (%) (w/v)	Treatment time (days)	No. of plants established in containers	No. of morphological variants	Morphological variation rate (%)[1]	No. of estimated tetraploids	Polyploidization rate (%)[2]
Control	–	–	59	10	16.95	0	0.00
Colchicine	0.1	2	27	8	29.63	3	11.11
		4	35	5	14.29	4	11.43
		6	24	8	33.33	2	8.33
	0.2	2	24	2	8.33	2	8.33
		4	20	4	20.00	3	15.00
		6	25	2	8.00	2	8.00
	0.3	2	20	8	40.00	2	10.00
		4	20	3	15.00	2	10.00
		6	21	5	23.81	1	4.76
Oryzalin	0.001	2	71	13	18.31	3	4.23
		4	40	19	47.50	3	7.50
		6	49	4	8.16	5	10.20
	0.002	2	69	18	26.09	2	2.90
		4	48	12	25.00	4	8.33
		6	45	35	77.78	21	46.67
	0.003	2	43	15	34.88	10	23.26
		4	36	7	19.44	6	16.67
		6	47	28	59.57	18	38.30

[1] Morphological variation rate = number of the morphological variants/number of the established samples × 100.
[2] Doubling rate = number of the tetraploids/number of the established samples × 100.

systems have been well established from several explant types, such as fully expanded or young leaves, petioles, and apical meristem (Cai and Deng, 2016; Cao et al., 2016; Zhang et al., 2019). However, there are few reports concerning ploidy manipulation in caladium up to now (Cai et al., 2015), and production and characterization of polyploids induced by oryzalin treatment is not available in caladium. In this study, the leaf cultures of 'Red Flash' caladium were exposed to different concentrations of colchicine or oryzalin for different durations, respectively, and a total of 93 plants were identified preliminary as tetraploids by ploidy analysis. Most of these tetraploid plants showed prominent changes in plant height, leaf shape, leaf thickness, petiole diameter, and leaf color characteristics, resulting in novel ornamental values which may be valuable for caladium breeding. Moreover, these tetraploids grew upright, sturdier and vigorously, and were expected to hold improved container performance and chilling tolerance. Therefore, the described procedure of *in vitro* polyploid induction here and the availability of these tetraploids provide new opportunities for cultivar development in caladium.

For *in vitro* chromosome doubling, concentrations and types of antimitotic agents, and exposure durations are critical factors affecting induction and regeneration of polyploids. For polyploid induction, although colchicine is most frequently used, many reports have proved that oryzalin was more efficient and less toxic than colchicine (Thao et al., 2003; Chung et al., 2014; Zahumenická et al., 2018; Shmeit et al., 2020). In this study, exposure of the leaf cultures to oryzalin at a relatively higher concentration (0.002% or 0.003%) for 6 days yielded much higher doubling rate as compared to all the colchicine treatments, indicating oryzalin was also more efficient for chromosome doubling than colchicine at the concentration studied in caladium. However, contrary results were reported by Feng et al. (2017) and Podwyszyńska et al. (2017), who found that oryzalin treatments induced fewer tetraploids and lower polyploidization rate than colchicine. This may be due to the application of an inappropriate concentration range of oryzalin or the different genotype adopted (Dhooghe et al., 2011). Besides, antimitotic agents accumulated in the treated explants have a side effect on their differentiation and dedifferentiation, and it will even be lethal if the explants were exposed to excessively high doses of antimitotic agents for a prolonged duration (Dhooghe et al., 2011). Generally, relatively high dosage of colchicine is needed to hinder microtubule formation as colchicine shows low affinity to plant microtubules, which will greatly reduce the survival rate of the treated materials due to its high toxicity (Thao et al., 2003; Podwyszyńska et al., 2018). In this

Table 3
Relative DNA content and chromosome counting of the wild type and the 10 variant types of 'Red Flash' caladium grown in plastic pots for 3 months.

Plant types	Mean fluorescence intensity (MFI)	MFI change compared to the wild type (%) [1]	Somatic chromosome numbers ($2n$)	Chromosome number changes
Wild type	1253329.76 ± 11690.65c [2]	–	30	$2x$
SVT1	1335618.01 ± 84472.79c	+6.57	32	$2x + 2$
SVT2	1376668.06 ± 31678.69c	+9.84	32	$2x + 2$
SVT3	1459035.64 ± 34179.5c	+16.41	32	$2x + 2$
SVT4	1296264.78 ± 79410.76c	+3.43	30	$2x$
SVT5	1716613.6 ± 86140.59b	+36.96	34	$2x + 4$
T1	2435047.49 ± 185442.19a	+94.29	60	$4x$
T2	2514211.12 ± 95160.95a	+100.60	64	$4x + 4$
T3	2434020.05 ± 42943.94a	+94.20	N [3]	N
T4	2363832.85 ± 55695.05a	+88.60	58	$4x - 2$
T5	2420859.84 ± 179689.12a	+93.15	N	N

[1] MFI change compared to the wild type = [(MFI of the variant type – MFI of the wild type) ÷ MFI of the wild type] × 100%, and "+" indicated MFI of the variant was increased compared to the wild caladium.
[2] Means ± standard deviation within a column followed by the same letters are not significantly different according to Duncan's range test at $P < 0.05$.
[3] N: no data were obtained for the plants grew poorly thus could not provide enough actively growing roots.

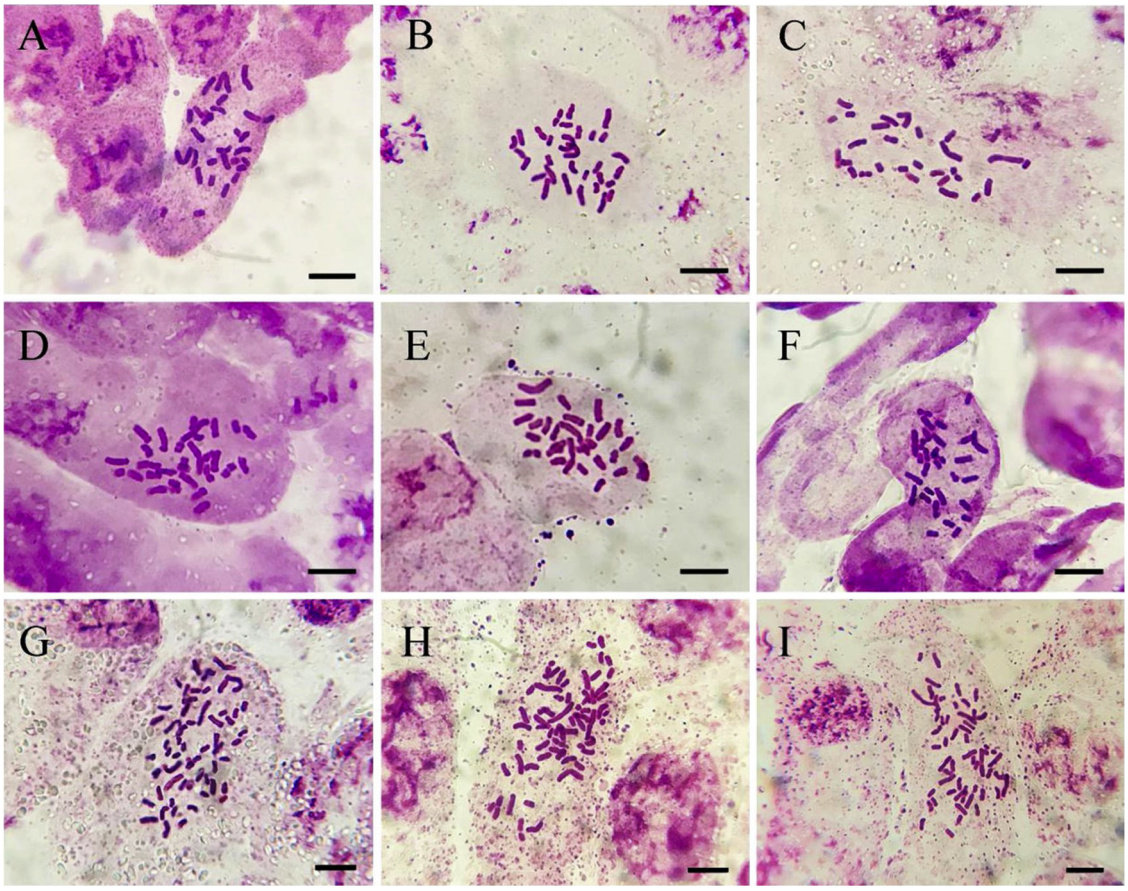

Fig. 4. Micrographs of somatic chromosomes in the root tips of the wild type and the variation types. (A) Wild type ($2n = 2x = 30$), (B) SVT1 ($2n = 2x + 2 = 32$). (C) SVT2 ($2n = 2x + 2 = 32$), (D) SVT3 ($2n = 2x + 2 = 32$), (E) SVT4 ($2n = 2x = 30$), (F) SVT5 ($2n = 2x + 4 = 34$), (G) T1 ($2n = 4x = 60$), (H) T2 ($2n = 4x + 4 = 64$), (I) T4 ($2n = 4x - 2 = 58$) (*bar* = 10 μm).

study, oryzalin was also found to be less toxic than colchicine during the *in vitro* chromosome doubling of caladium, as the number of the established plants treated by oryzalin was always more than that of the colchicine treatments.

4.2. Morphological characteristics and screening of variants

When large quantities of plants were obtained from antimitotic agents treated explants, an accurate and rapid method for screening potential tetraploids is required to avoid unnecessary labor and expense in subsequent identification. Caladiums are foliage plants and their ornamental values depend largely on their intriguing leaf traits including leaf shape, leaf color and leaf color pattern, while those except main vein color are very susceptible to growth environment and development stages in caladium (Deng, 2012). Therefore, all the regenerated plants, the screened morphological variants and the wild plants were cultured under the similar conditions from the beginning, and only stable morphological variants were selected and used for the following analysis. Subsequent study proved that our screening of the variants was very necessary and efficient, as a large number of plants (723) were regenerated in this study. Additionally, previous reports suggested that chromosome doubling is often accompanied by increased leaf thickness and reduced leaf length/width ratio in the regenerated polyploids (Cai et al., 2015; Podwyszyńska et al., 2017; Wang

Table 4
Morphological, stomatal and photosynthetic characteristics of the wild type, the tetraploids (T1), and diploid aneuploids (SVT1) established in this study.

Characteristics	Wild type ($2n = 2x$)	Diploid aneuploids (SVT1, $2n = 2x + 2$)	Tetraploids (T1, $2n = 4x$)
Plant height (cm)	21.40 ± 18.54b	32.23 ± 1.46a	44.33 ± 5.87a
Leaf number per plant	3.33 ± 0.58a	3.67 ± 0.58a	2.17 ± 0.41b
Leaf length (cm)	21.03 ± 3.56a	19.82 ± 4.62a	23.34 ± 4.21a
Leaf width (cm)	13.65 ± 2.38b	13.28 ± 3.40b	17.82 ± 3.08a
Leaf length/width ratio	1.54 ± 0.04a	1.51 ± 0.19a	1.31 ± 0.09b
Leaf thickness (cm)	0.20 ± 0.04b	0.21 ± 0.05b	0.27 ± 0.04a
Petiole diameter (mm)	4.35 ± 1.40b	4.27 ± 0.70b	6.66 ± 0.75a
Stomatal guard cell length (μm)	19.37 ± 3.03b	13.11 ± 1.74c	25.75 ± 2.66a
Stomatal guard cell width (μm)	12.95 ± 1.77a	4.30 ± 1.43b	15.42 ± 4.71a
Stomatal density (no./mm^2)	246.32 ± 38.50a	357.89 ± 38.44a	138.95 ± 30.08b
Net photosynthesis rate (μmol CO_2 m^{-2} s^{-1})	4.43 ± 0.62c	8.73 ± 1.83a	7.11 ± 0.95b
Transpiration rate (mmol H_2O m^{-2} s^{-1})	1.03 ± 0.25c	2.71 ± 0.01b	4.00 ± 0.22a
Stomatal conductance (mol H_2O m^{-2} s^{-1})	0.08 ± 0.02c	0.27 ± 0.00b	0.38 ± 0.03a

Means (± standard deviation) within rows followed by different letters are significant different at 5% level by Duncan's range test.

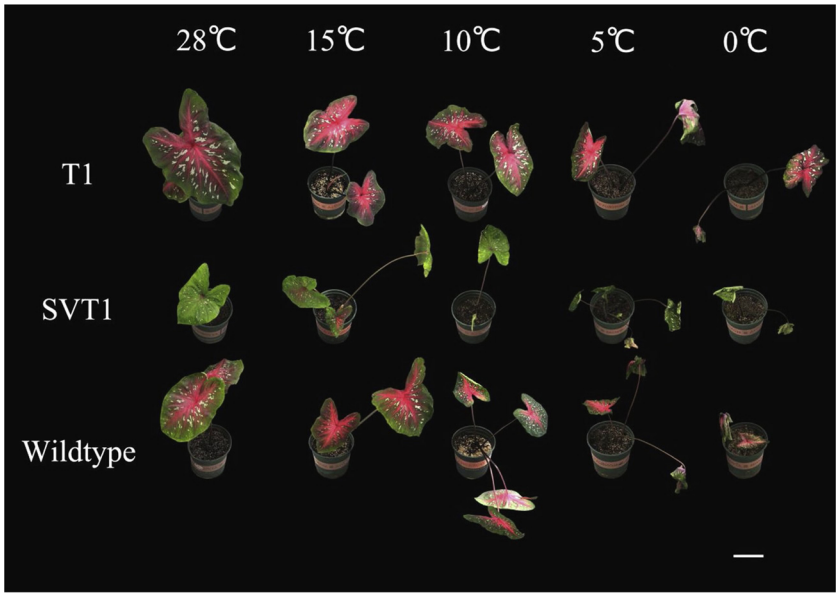

Fig. 5. Effects of different chilling treatments on the plant performance of the T1 (tetraploids), the SVT1 (diploid aneuploids) and the wild type (*bar* = 10 cm).

et al., 2018). In this study, most of the tetraploids including the T1 and T2 had rounder and thicker leaves, and larger petiole diameter as compared to the wild type, and ploidy analysis showed they were tetraploids. These results also indicated that visual screening of polyploids based on leaf length/width ratio and leaf thickness was easy, fast and quite accurate in caladium.

4.3. Somaclonal variation and aneuploidy

Besides tetraploid plants, another concern was that the diploid variants (SVT4), the diploid aneuploids (SVT1–SVT3, SVT5), and the tetraploid aneuploids (T2 and T4) occurred frequently in this study (Table 3 and Fig. 4). Besides chromosome doubling, colchicine has been reported to may generate chromosome losses or rearrangements, and gene mutations due to its mutagenic effects (Luckett, 1989). A relatively high frequency of aneuploids was also observed in the plants derived from colchicine-treated leaf cultures of 'Tapestry' caladium (Cai et al., 2015), and colchicine-treated somatic embryos and scales of *Lilium distichum* and L.*cernuum* (Fu et al., 2019). Besides, It is well known that somaclonal variation is common in tissue-cultured caladium plants (Deng, 2012; Cao et al., 2016), and previous reports suggested that several cytological variations including chromosome losses, chromosome gains, and chromosome doubling were involved in somaclonal variation in caladium (Cai et al., 2015; Cao et al., 2016). Therefore, some of the diploid and aneuploid variants arise from the colchicine or oryzalin treatments in this study might be caused by somaclonal variation during tissue culture. Aneuploids can lead to change in global gene expression, gene structure and phenotype in organisms (Huettel et al., 2008), and thereby these aneuploid plants hold great potential for plant genetic study and chromosome engineering in caladium.

4.4. Gas exchange parameters

Previous reports found that polyploids showed higher photosynthetic capacity than their original diploids (Zhou et al., 2017; Cao et al., 2018). The present study also found that the net photosynthetic rate, stomatal conductance and transpiration rate of the tetraploids (T1) were significantly higher than those of the wild type, respectively. It was worth to notice that the photosynthetic rate, transpiration rate and stomatal conductance of the SVT1 were significantly higher than that of

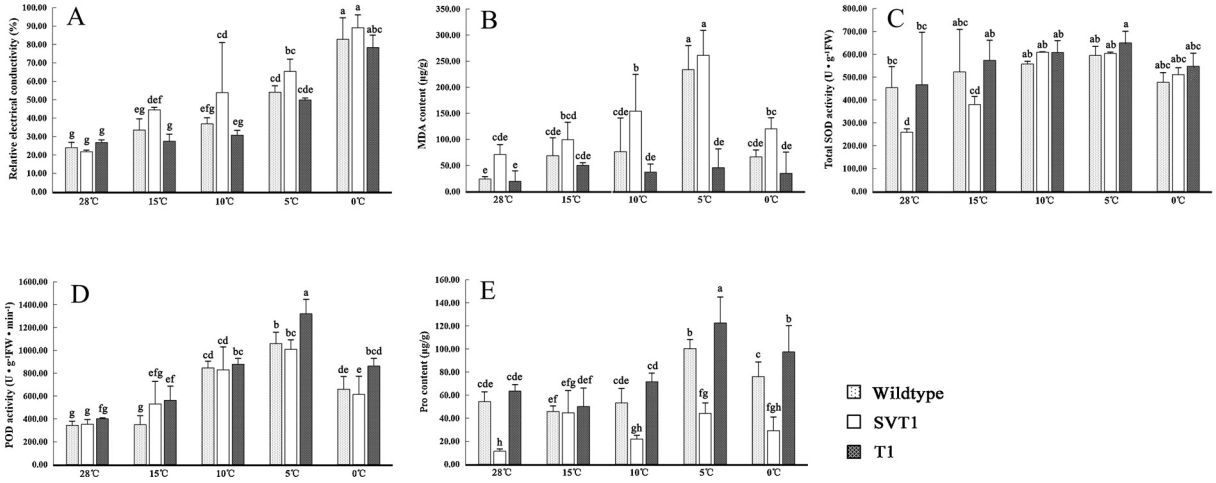

Fig. 6. Effects of different chilling treatments on the relative electrical conductivity (REC) (A), malondialdehyde (MDA) content (B), superoxide dismutase (SOD) activity (C), peroxidase (POD) activity (D), and proline (Pro) content (E) in the leaves of the T1 (tetraploids), the SVT1 (diploid aneuploids) and the wild type. The *error bars* represent the standard deviation (± SD) for three replicates, and different letters indicate significant differences at 5% level by Duncan's range test.

the T1 and the wild type, respectively, as might be attributed to their higher content of photosynthetic pigment. Cao et al. (2018) found that polyploidization caused significantly increased photosynthetic pigment content, thicker epidermal and spongy tissues, more and thicker thylakoid lamellae, which in turn improve the light absorption and conversion capacity of the tetraploids and further enhance their photosynthetic performance. Further pigment content analysis and anatomical study should be carried out to determine the differences in photosynthesis among the three caladium types.

4.5. Chilling tolerance evaluation

Plant growth and ornamental value of caladiums are usually limited when cultivated in open fields in most of China due to their chilling sensitive nature, and thus development and creation of chilling-tolerant caladiums continues to be one of the most important breeding objects. Most polyploids can adapt better under stress conditions than their corresponding diploids, such as low temperature stress (Liu et al., 2011; Denaeghel et al., 2018; Jiang et al., 2019). In this study, chilling hardiness of the tetraploids and the diploid variants were evaluated and analyzed, and results showed that the tetraploids (T1) had enhanced chilling tolerance compared with the diploid wild type and the diploid aneuploids (SVT1). Similar results were also observed when comparison of cold tolerance between tetraploid and diploid *Escallonia rubra* (Denaeghel et al., 2018), and between tetraploid and diploid *Escallonia rubra* (Jiang et al., 2019).

In general, plants will readjust their biochemical makeup to adapt and survive when confronted to low temperature damage (Ruelland and Zachowski, 2010). Increasing of cell membrane permeability is considered as the primary damage of low temperature injury, which will lead to increased electrolyte leakage and metabolic dysfunction (Zhou and Leul, 1998; Ashraf and Ali, 2008). Results of this study showed that less damage of cell membrane of the T1, as was evidenced by its lower REC during the chilling stress as compared to the wild caladium and the SVT1. MDA is the final product of lipid peroxidation when plants suffered from abiotic stress, and its content is positively correlated with the degree of damage to plants exposed to low temperatures (Liu et al., 2011; Zhou and Leul, 1998). In this study, an enhanced accumulation of MDA in the leaves of the wild and SVT1 suggests that lipid peroxidation was more active in the two types than that of the T1 when suffered from the chilling stress. In addition, antioxidant enzymes such as SOD and POD can alleviate oxidative damages of plants imposed by chilling stress, and these enzymes have an important role in cellular defense against increased active oxygen under stressed conditions (Ashraf and Ali, 2008). The results showed that the T1 always had higher activity of SOD and POD than the wild type and the SVT1, indicating that the T1 could achieve greater defensive ability compared with the wild type and the SVT1 during the chilling treatments. Furthermore, enhanced Pro accumulation content is thought to be a widespread protective response against environment stress (Delauney and Verma, 1993; Liu et al., 2011). In this study, almost no significant changes of the Pro content in the STV1 suffered from the chilling stress, indicating that the STV1 had poor protective response against the chilling treatments. On the contrary, an obvious increase of the Pro content was observed in the wild caladium and the T1 when the temperature was reduced from 15°C to 5°C, and the T1 always exhibited a higher Pro content than the wild type and the SVT1 during the chilling treatments, which means that the T1 could better adapt to low temperature, followed by the wildtype. Therefore, enhanced chilling tolerance of the tetraploids (T1) in the study might be due to the increased SOD activity, POD activity and Pro content, and accordingly reduced REC and MDA content in their leaves as compared to the diploid wild caladium and the diploid aneuploid (SVT1).

5. Conclusion

In summary, the present study demonstrates that tetraploids could be efficiently induced in caladium when the leaf cultures were treated with 0.002% oryzalin for 6 days. Chromosome doubling resulted in significant changes in morphological, stomatal and photosynthetic characteristics, and enhanced chilling tolerance as compared to the wild caladium. Moreover, results of the DNA content analysis and chromosome counting revealed that chromosome gains or losses occurred frequently in the established morphological variants. These variants including the tetraploids, diploid variants, diploid aneuploids, and tetraploid aneuploids hold great potential for cultivar development, genetic study and chromosome engineering in caladium.

CRediT authorship contribution statement

Yuan-Shan Zhang: Conceptualization, Methodology, Software, Investigation, Writing - original draft. **Jin-Jin Chen:** Validation, Formal analysis, Visualization, Software. **Yun-Mei Cao:** Validation, Visualization. **Jia-Xin Duan:** Data curation. **Xiao-Dong Cai:** Resources, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Scientific Research Project of Hubei Education Department (No. B2018024). We are grateful to Dr. Zhanao Deng (the University of Florida's Gulf Coast Research and Education Center, Wimauma, FL, USA.) for providing the 'Red Flash' caladium.

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