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Characteristics of Four Post *In Vitro*-Conserved Chrysanthemum [*Dendranthema grandiflora* (Ramat.) Kitam.] Varieties

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The vegetative and floral characteristics of chrysanthemum [*Dendranthema grandiflora* (Ramat.) Kitam.] plantlets that had survived after *in vitro* conservation at different periods and in different media were assessed after growing them *in vivo*. The surviving plantlets of varieties 'Pasopati', 'Padma Buana', 'Puspita Nusantara' and 'Tirta Ayuni' previously conserved under low-temperature conditions in Murashige and Skoog (MS) + 2.5% dimethyl sulfoxide (DMSO), full, ½ and ¼ strengths Tsuchiya media for 2, 4, and 6 mo were acclimatized under protected house conditions with standard cultural maintenance until flowering stage.

The initial deviations in plant height, stem diameter, leaf length-width ratio and number of florets within certain culture media and within storage periods were not observed as the storage periods were increased. The size of the flowers, florets and discs, and the color of the adaxial and abaxial parts of all varieties were not affected by the conservation media and length of storage. Cytological alterations in terms of chromosome number, chromosome classification, and grouping were not found within variety and within media. The study showed that *in vitro* conservation of chrysanthemums is an effective alternative method to replace the more expensive and risky maintenance of live and field-grown germplasm without sacrificing genetic stability.

Key Words: Chrysanthemum (*Dendranthema grandiflora*), *in vitro* conservation, flower disc, karyogram, Tsuchiya medium

Abbreviations: DMSO – dimethyl sulfoxide, IAA – indoleacetic acid, IOCRI – Indonesian Ornamental Crops Research Institute, MS – Murashige and Skoog

INTRODUCTION

Chrysanthemum [Family Asteraceae (formerly Compositae)] is the most important cut flower in Indonesia, having replaced roses since 2006. Chrysanthemum production has increased significantly from 108 million stems in 2009 to 387 million stems in 2013, contributing 23.3% of the total floriculture production in 2012. The total export forecast for 2016 was more than USD 2 million based on the increase in productivity and harvest area of production (Indonesian Statistics Bureau 2013). Most of the commercially grown chrysanthemum varieties were developed by the Indonesian Ornamental Crops Research Institute (IOCRI).

Although it is generally considered as a temperate crop, chrysanthemum is also grown in the tropics, where it has to be maintained in the highlands under protected environment to produce high-quality planting materials for the next production season (Budiarto and Marwoto 2007). This practice requires high inputs that entail higher cost of production. Chrysanthemums are asexually propagated and the maintenance/conservation of base collections as source of planting materials under field conditions is very laborious, expensive and risky. The risks that are usually associated with field-grown plants or *in vivo* planting include those caused by pathogens, pests, climatic perturbation and human errors (Ozudogru et al. 2010).

The *in vitro* technique has been used in germplasm conservation, especially for vegetatively propagated crops such as chrysanthemums. It requires less space where a large number of accessions can be stored, and the cultures in sterile conditions can be shipped (Englemann 1991) anywhere. It has been successfully used in carnation

(Budiarto and Marwoto 2011) and in chrysanthemums (Budiarto et al. 2008).

Four chrysanthemum varieties conserved *in vitro* for 6 mo in Tsuchiya medium (Tsuchiya 1954) with nutrient modifications were studied by Budiarto and Rosario (2017) to assess the performance of the varieties while in storage. A number of plantlets survived in each medium after the storage period.

A desirable *in vitro* conservation method should allow not only storage of the plant propagules for longer periods with minimal maintenance cost, but also preservation of the plant's genetic constitution after conservation. The selected medium should be capable of maintaining the potential growth rate of the plantlets during *in vitro* and *in vivo* management without somaclonal variation (Mandal 1997). Although somaclonal variation *in vitro* has been extensively studied in many crops, the mechanism by which it occurs remains largely either unknown or at the level of theoretical speculation. Several factors have been identified to be related to the occurrence of somaclonal variations, such as the regeneration system, type of tissue, explant source, media component and the duration of *in vitro* cultures (Bairu et al. 2011).

Somaclonal variations have been reported in plantlets of grapevine (Baránek et al. 2010), potato (Bordallo et al. 2004), banana and plantain (Cote et al. 1993) and citrus (Hao and Deng 2002) when they were grown *in vivo* after *in vitro* conservation. Their presence becomes an obstacle in the standardization of *in vitro* conservation of the crops. In addition, they are a nuisance in the commercial growing of uniform chrysanthemum cut flowers, interfering in the application of standard production practices (Budiarto et al. 2008).

The protocol for *in vitro* conservation would affect the performance of the plantlets after storage, that is, the growth in induction medium, the characteristics of the plants and quality of the flowers when grown in the greenhouse. This study aimed to assess the horticultural and cytological characteristics of four outstanding and commercially grown chrysanthemum varieties when they were grown *in vivo* after they were conserved *in vitro* for 2, 4, and 6 mo. It also aimed to determine the influence of the conservation media and length of storage on fidelity of the plantlets.

MATERIALS AND METHODS

Experiment Site

The study was conducted at the Indonesian Ornamental Crops Research Institute (IOCRI), Cipanas, Cianjur, West Java, Indonesia. The field is a highland area, well suited for chrysanthemum production, with an elevation of 1100 m above sea level, and located at 107° 02′ eastern and 06° 46 southern latitudes. Normal precipitation is 3145 mm annually and an average of 263 rainy days. The maximum recorded temperature in the area is 24.9 °C and the minimum is 16.2 °C; relative humidity is 64–90% and the average sun radiation is 246 cal/cm-day.

Source of Planting Materials

In a previous experiment conducted at IOCRI, four chrysanthemum varieties were conserved *in vitro* for 2, 4, and 6 mo in four different culture media at low temperature (Budiarto and Rosario 2017). Plantlets of 'Pasopati', 'Padma Buana', 'Puspita Nusantara' and 'Tirta Ayuni' were conserved *in vitro* in ½ Murashige and Skoog (MS) + 2.5% dimethyl sulfoxide (DMSO) (which served as the control for conserved plantlets), full strength Tsuchiya, ½ strength Tsuchiya and ¼ strength Tsuchiya media. The surviving plantlets of the four varieties conserved in each medium in that study served as the planting materials to be grown *in vivo*. Another set of cultures was used as control for gathering data on horticultural and cytological studies.

All plant cultures were transferred into a growth chamber where the temperature was gradually increased every 2–3 d until it reached 16–18 °C. They were then subcultured onto ½ MS + 0.1 mg L^{-1} indoleacetic acid (IAA) to induce further root development in preparation for acclimatization to the field environment.

Plantlet Acclimatization and Field Planting

After 15 d, the plantlets were taken out from the culture flasks, cleaned of the agar medium under flowing tap water, and planted in porous plastic trays with sterile carbonized hull as the growing medium. The plantlets were then acclimatized for 3 wk under protected and shaded conditions. Air humidity inside the plastic house was maintained by spraying the plants and media with sterile water twice a day up to 10 d. Water spraying was reduced to once a day or every 2 d depending on the conditions of the plant after a new leaf has developed from the plantlet.

Three weeks thereafter, 2–3 young plant samples per replication were planted in 15-cm pots containing a mixture of carbonized rice hull and bamboo humus (1:1 v/v). The plants that were used for microscopic observation were maintained in a glass house and were provided with standard cultural practices and long-day conditions for 2 wk. The samples were then taken for cytological studies. The remaining acclimatized plants were transplanted in beds under a plastic house for morphological assessment. There were 7–8 young plants per entry per replication planted in 1.0 m-wide row beds, 20 cm high, with three

replications. The distance between hills within a plot was 20 cm, between plots within a row, 20 cm and between beds, 60 cm. A randomized complete block design with three replications was set up for the experiment.

Light Control and Flower Induction

LED lamps (11 watts) were provided to simulate long-day conditions. The lamps were arranged 1.5–2.0 m above the beds with a distance of 2 x 2 m^2 between them. Lights were turned on for 4 h per day (10:00 p.m. to 2:00 a.m.) for 30 d. Standard cultural practices were performed to maintain the plants throughout the experiment.

After 30 d under long-day conditions or when the average plant height reached 50 cm, short-day conditions were imposed by removing the supplemental lamp lighting (neutral day conditions) to stimulate flowering.

The growth trend of the plantlets across media and storage period and the characteristics of the fully grown plants at 6 mo conservation period were described. The data on vegetative and reproductive characteristics were taken when at least 80% of the plant samples had flowered and had at least two fully opened flowers. Plant height was measured from the basal end of the stem above the ground to the tip of the terminal flower. The stem diameter is the average diameter of the center area of the second 1/3 portion at flowering stage. Only the fully opened leaves were counted. The flower type and characterization were based on classification of chrysanthemums by UPOV (2016). Quantitative data were taken from healthy and fully bloomed flowers. The Royal Horticultural Color Chart was used to describe the abaxial and adaxial color of the flowers.

All the quantitative data gathered were analyzed using ANOVA, and the mean comparisons were tested using LSD ($p < 5\%$) and student-T test ($\alpha = 5\%$). Pertinent photographs were also taken as additional visual descriptive data.

Cytological Analysis

For cytological analysis, root tips were collected and placed in small vials containing Carnoy's fixative (a mixture of 95% ethyl alcohol, chloroform and glacial acetic acid; 6: 3, v/v). The vials were then warmed up at 60 °C for 15 min to soften the root tissues. They were then cooled for a couple of minutes.

Carnoy's fixative was rinsed off with 70% ethyl alcohol and this procedure was repeated twice. Samples were observed cytologically at 1500x magnification. Photomicrographs were taken on the targeted cells and karyotype analyses were conducted based on the procedure of Levan et al. (1964). A karyogram of each variety in each culture medium and storage period was made.

RESULTS AND DISCUSSION

Height and Stem Diameter

In the early development stage of 'Pasopati', there was temporary growth stagnation in the acclimatized young plants that were conserved for 2 mo in ½ MS + 2.5% DMSO (Fig. 1). The plantlets were the shortest (65.85 cm) and differed significantly from those conserved for 4 mo (86.33 cm) and 6 mo (90.3 cm). The inhibitory effect of DMSO in the culture was initially a carry-over from the 2-mo storage *in vitro* of the chrysanthemum plantlets previously studied (Budiarto and Rosario 2017). The effect of DMSO, however, dissipated when the plantlets were conserved for longer periods and then grown i*n vivo*. The conserved plantlets were given a boost when they were transferred to the induction medium before acclimatization. An early, short and temporary effect of DMSO has been reported in the inhibition of pollen tube growth (Dickinson and Cochran 1968).

The sensitivity to the inhibitory effect of DMSO was observed to be genetically controlled. A similar effect was also reported in papaya where the degree of inhibition was determined not only by the concentration but also by genotype responses as well (Wang et al. 2005). In the present study, the heights of 'Padma Buana' plants conserved in media with DMSO were across storage periods. The tallest plants were those conserved for 4 mo in ½ strength Tsuchiya and the value (83.07 cm) was significantly higher than that for 6 mo (69.25 cm) but not from those of plants previously stored for 2 mo (76.24 cm). The heights of 'Puspita Nusantara' and 'Tirta Ayuni' did not differ across media and storage period.

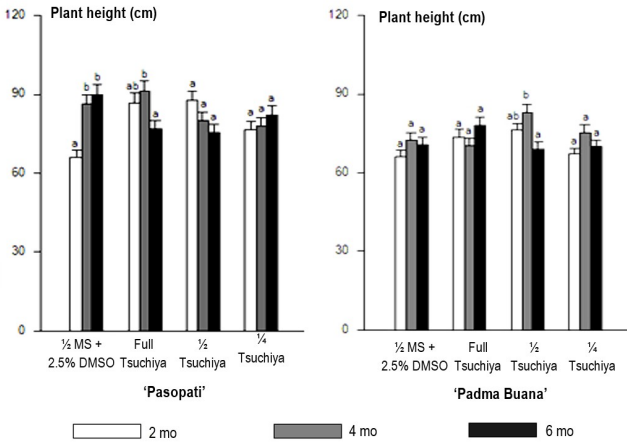

Fig. 1. Plant height of chrysanthemum 'Pasopati' and 'Padma Buana' grown *in vivo* after conservation *in vitro* for 2, 4 and 6 mo in four different media. Means with common letters are not significantly different within media in each variety.

Stem Diameter

Only 'Puspita Nusantara' plants conserved in full strength Tsuchiya medium showed variation in terms of stem diameter, which was 8.87 mm at 6 mo (Fig. 2). The value is significantly higher than that recorded for conserved plants for 4 mo (7.8 mm) but comparable to that for the 2-mo storage (8.27 mm). Within varieties 'Pasopati', 'Padma Buana' and 'Tirta Ayuni', the stem diameters were comparable across media and storage.

Number of Leaves

Initially, there were fewer leaves in 'Pasopati' plants previously conserved for 2 mo in medium with DMSO, but the number increased with increase in conservation or storage time. The number of leaves produced by 'Pasopati' for 2 mo was the least (15.03) and it was significantly lower than that produced by plants conserved for 4 and 6 mo (18.34 and 17.83, respectively, Fig. 3). In 'Tirta Ayuni', the number of leaves from plants conserved in ¼ strength Tsuchiya for 6 mo (19.23) was significantly higher than that from plants conserved for 2 mo (17.20). The variation in the number of leaves in 'Padma Buana' and 'Puspita Nusantara' in any conservation medium and the length of storage was negligible.

The role of DMSO in the reduction of the number of leaves during *in vitro* conservation and consequently during *in vivo* growth has been observed in carnation where the effect was still manifested up to 4 mo of storage (Budarto and Marwoto 2011).

Size of Leaves

The leaf length-width ratio of 'Pasopati' conserved for 4 mo in media with DMSO was significantly higher (2.05) than that of plants conserved for 2 and 6 mo, 1.84 and 1.88, respectively (Fig. 4). No trending and changes in leaf size were found across variety and conservation period in the other varieties. The inhibitory effects of DMSO during *in vitro* conservation on the *in vivo* plant growth, specifically on the stem diameter and leaf length-width ratio, had been reported in heavenly bamboo, *Nandica domestica* (Ozudogru et al. 2013), where conservation at 4 °C hyperhydric-smaller plantlets were observed in post-conservation of shoots. Smaller leaves were also found in Vanda (Haiyan and Kondo 1996) and navel orange (Sakai et al. 1990). The effects of DMSO on the leaf size were also temporary. The normal growths were reportedly expressed after the removal of DMSO from the media (Ozudogru et al. 2010). In this study, there was no consistency on its effect on leaf length-width ratio especially in 'Pasopati'.

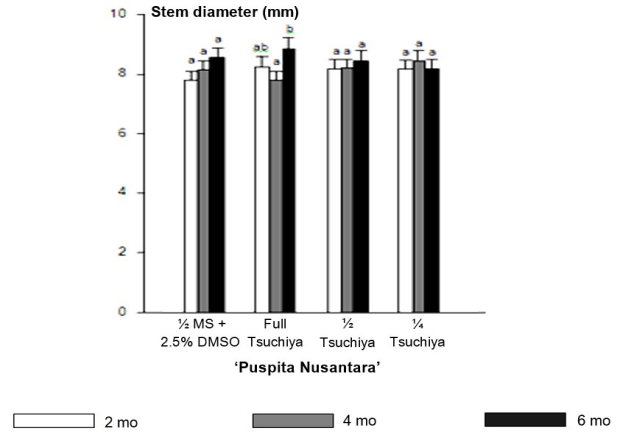

Fig. 2. Stem diameter of chrysanthemum 'Puspita Nusantara' grown *in vivo* after their *in vitro* conservation for 2, 4 and 6 mo in four different culture media. Means with common letters are not significantly different within media.

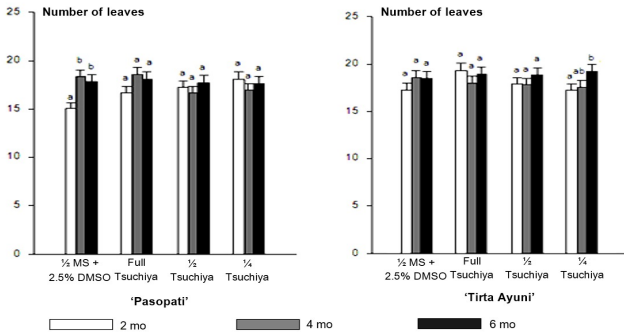

Fig. 3. Number of leaves of chrysanthemum 'Pasopati' and 'Tirta Ayuni' grown in *vivo* after their conservation *in vitro* in four different media for 2, 4 and 6 mo. Means with common letters are not significantly different within media in each variety.

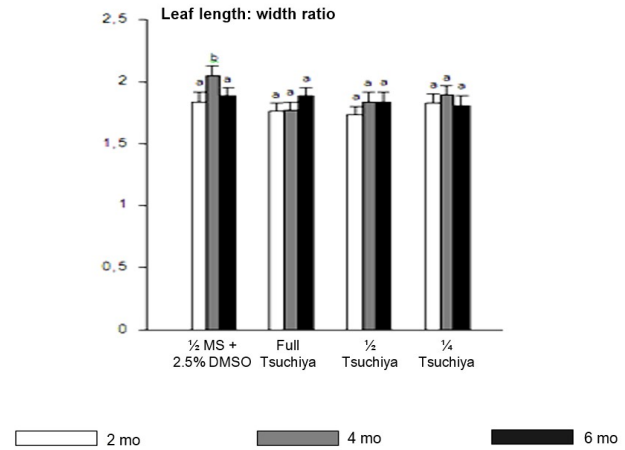

Fig. 4. Size (leaf length: width ratio) of leaves of chrysanthemum 'Pasopati' grown in *vivo* after their conservation *in vitro* in four different media for 2, 4 and 6 mo. Means with common letters are not significantly different within media.

In carnation, the conservation media without DMSO resulted in less hyperhydric plantlets in early storage duration; however, higher plantlet mortality was found after 6 mo of storage. The hyperhydric plantlets showed paler leaves with smaller stem and higher death rate during acclimatization (Budiarto 2009). In another study, there was hyperhydricity on plantlets conserved in medium with DMSO, which in most cases, contributed to lower chlorophyll content in the plantlet leaf, decrease in viability, and smaller stem size when planted under *in vivo* condition (Budiarto and Marwoto 2011).

Number of Florets

The number of florets of the conserved chrysanthemum varieties grown under *in vivo* conditions was not affected by the type of media and the conservation period in 'Puspita Nusantara' and 'Tirta Ayuni'. In 'Pasopati', however, there were fewer florets (28.47) in those conserved for 2 mo in ½ MS + 2.5% DMSO than in those conserved for 4 and 6 mo (31.55 and 31.80, respectively) as shown in Fig. 5. In 'Padma Buana', increasing the storage period led to increase in number of florets in plants previously conserved in full strength Tsuchiya medium for 4 and 6 mo; plants also had significantly more florets (39.3) than those conserved for 2 mo (36.33).

Flower and Disc Diameter

Within a variety, conservation media and storage periods did not show significant differences in the flower and disc diameter. All tested varieties in any of the media treatments under *in vivo* conditions were not affected by the different *in vitro* conservation periods.

Each chrysanthemum variety has its own flower characteristics. In terms of size, 'Tirta Ayuni' was smallest, but the flower disc was bigger than those of the other varieties. 'Padma Buana' had the smallest flower disc size and the flower size was comparable to that of 'Puspita Nusantara' which had the biggest flower and flower disc size. These findings were in accordance with the studies on flower characteristics (Budiarto et al. 2008) although different chrysanthemum varieties and non-DMSO conservation media were used.

Flower Color

The varieties conserved *in vitro* maintained their original dominant flower color in both adaxial and abaxial parts. No large visual changes were observed compared with the control regardless of media and storage period. Based on the Royal Horticultural Society (RHS) color chart, 'Pasopati' was Red 53A; 'Padma Buana' was Red Purple 64B; 'Puspita Nusantara', Yellow 12A and 'Tirta Ayuni', White 155B (Fig. 6). The color of the abaxial and adaxial parts of the flowers likewise, was the same as that of the control.

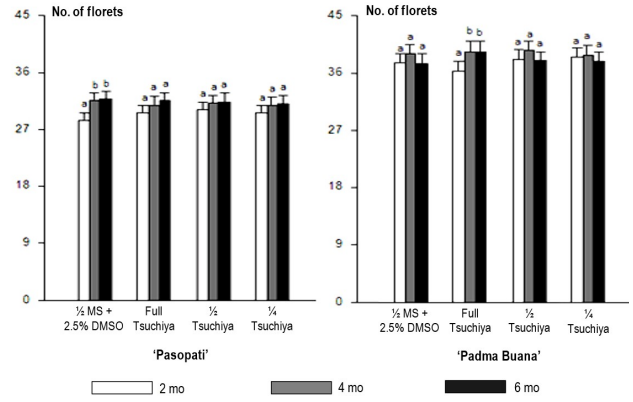

Fig. 5. Number of florets produced by chrysanthemum 'Pasopati' and 'Padma Buana' grown *in vivo* after conservation *in vitro* in four different media for 2, 4 and 6 mo. Means with common letters are not significantly different within media in each variety.

The stability of the flower type and floret color in the spray type of chrysanthemum after *in vitro* conservation was also reported by Martin and Gonzales-Benito (2009). In the current study, the four chrysanthemum varieties used are of the spray type and the flower and floret types were not affected. On the other hand, deviations have been found on the standard, decorative and spider types of chrysanthemum after prolonged duration of *in vitro* conservation and cycles of subcultures (Wang et al. 2007; Jevremovic et al. 2006).

Varietal Characteristics *In Vivo* after 6 mo of Storage *In Vitro*

The performance of plantlets conserved for the longest period is a test of the feasibility of storing them *in vitro*. In this study, the varietal characteristics of plants stored at 6 mo were compared with the control (Table 1). The height of 'Pasopati' conserved in ½ MS + DMSO was 90.3 cm, which is significantly different from that of the other treatments including the control or unconserved plants. No deviations from the control were observed in number of leaves, stem diameter, and leaf length-width ratio. In 'Padma Buana', there was reduction in stem diameter in the control but the value (6.32 mm) was comparable with that for ¼ strength Tsuchiya (6.37 mm). The stem diameter was largest (8.87 mm) in 'Puspita Nusantara' treated with full strength Tsuchiya while the largest stem diameter was observed in 'Tirta Ayuni' treated with ½ strength Tsuchiya (8.43 mm). The flower characteristics within variety showed no variations across conservation media.

Among varieties at 6 mo, 'Puspita Nusantara' had the largest stem (8.87 mm) and flower (7.36 cm) diameters. It was also the tallest (102.89 cm), and had the most number of leaves (20.67) and more rounded leaves and florets

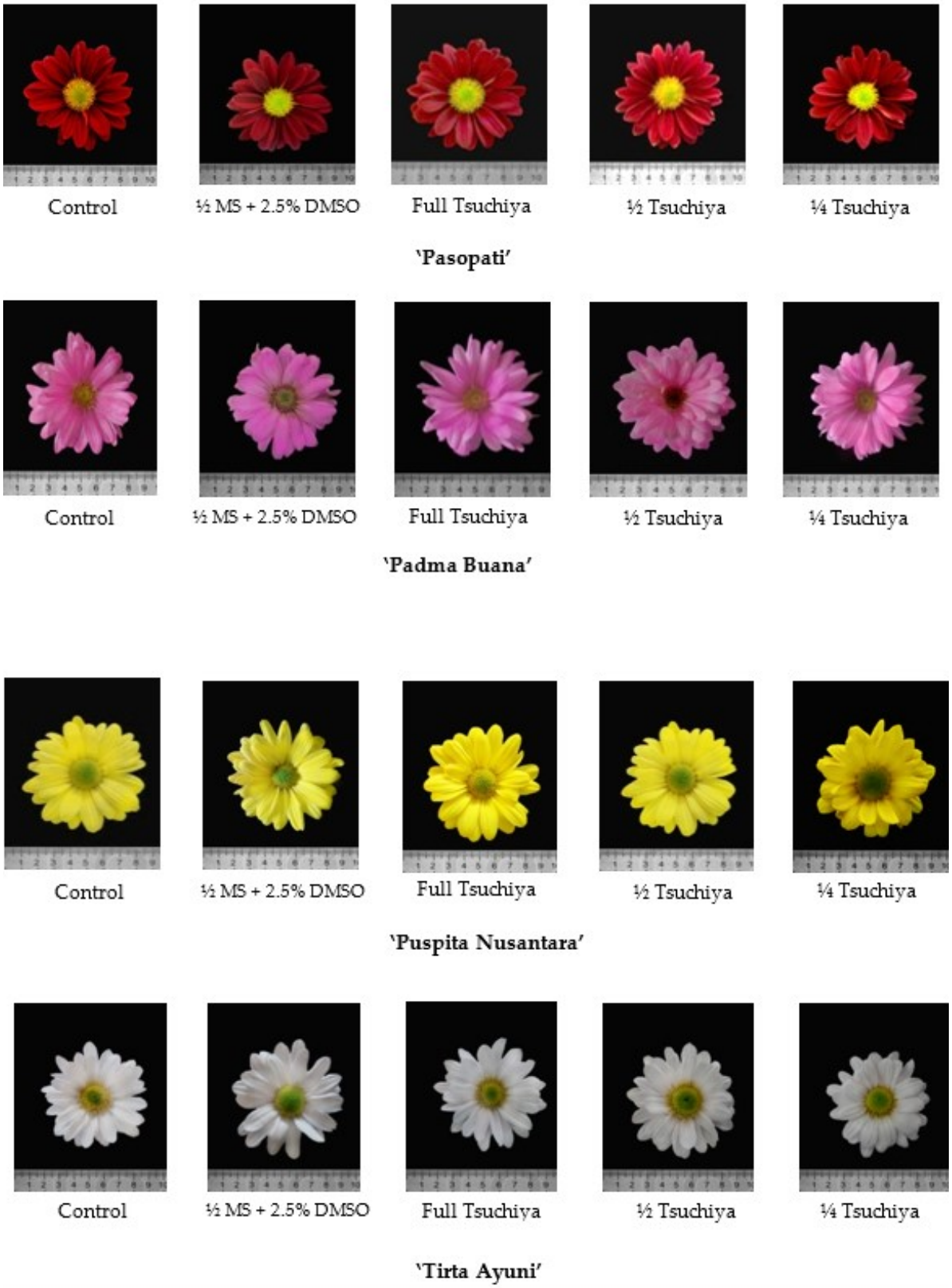

Fig. 6. Adaxial color of flowers of chrysanthemum varieties grown *in vivo* after *in vitro* conservation in different media for 6 mo.

Table 1. Vegetative and flower characteristics of four chrysanthemum varieties grown *in vivo* after a 6-mo conservation period *in vitro* in different media[1].

Variety	Conservation Medium[2]	Plant Height (cm)	No. of Leaves	Stem Diameter (mm)	Leaf Length: Width Ratio	Flower Diameter (cm)	Flower Disc Diameter (cm)	No. of Florets	Floret Length: Width Ratio
'Pasopati'	½ MS + 2.5 % DMSO	90.03 e	17.83 abcd	8.20 fg	1.88 cde	6.99 ab	1.44 a	31.80 ab	2.85 ab
	Full strength Tsuchiya	76.79 abcd	18.08 abcd	8.27 hi	1.88 cde	7.10 abcde	1.47 a	31.45 a	2.86 abc
	½ strength Tsuchiya	75.48 abcd	17.72 abc	8.43 j	1.84 bcde	7.08 abcde	1.42 a	31.42 a	2.90 abc
	¼ strength Tsuchiya	82.37 cde	17.63 abc	8.07 e	1.81 bcd	7.06 abcd	1.44 a	32.07 ab	2.96 bc
	Untreated (control)	78.26 abcd	18.17 abcd	8.30 hi	1.83 bcde	7.03 abcd	1.45 a	31.93 ab	2.87 abc
'Padma Buana'	½ MS + 2.5 % DMSO	70.87 ab	17.25 ab	6.50 d	2.12 de	7.12 abc	1.37 a	37.58 def	3.49 g
	Full strength Tsuchiya	72.13 abc	17.07 ab	5.67 a	2.14 e	7.04 abcd	1.42 a	39.42 f	3.18 de
	½ strength Tsuchiya	69.25 a	16.69 a	6.38 c	2.15 cde	7.08 abcde	1.42 a	37.93 def	3.46 g
	¼ strength Tsuchiya	69.84 a	17.05 ab	6.37 bc	2.13 de	7.11 abcde	1.37 a	37.86 def	3.39 fg
	Untreated (control)	71.37 ab	16.89 ab	6.32 b	2.12 de	7.07 abcd	1.39 a	38.60 ef	3.22 ef
'Puspita Nusantara'	½ MS + 2.5 % DMSO	102.89 f	20.57 e	8.57 k	1.42 a	7.41 f	1.82 b	36.13 cdef	3.03 cd
	Full strength Tsuchiya	102.51 f	19.98 cde	8.87 l	1.45 a	7.29 def	1.79 b	35.53 bcdef	3.00 bc
	½ strength Tsuchiya	102.29 f	19.83 cde	8.44 j	1.36 a	7.18 cdef	1.80 b	34.27 abcd	2.99 bc
	¼ strength Tsuchiya	102.52 f	20.67 e	8.18 f	1.48 a	7.26 bcdef	1.85 b	36.50 cdef	3.03 cd
	Untreated (control)	102.18 f	20.16 de	8.45 j	1.37 a	7.36 ef	1.82 b	35.07 abcde	3.02 bcd
'Tirta Ayuni'	½ MS + 2.5 % DMSO	89.25 e	18.50 abcde	8.31 l	1.62 abc	6.94 a	1.87 b	33.52 abc	2.75 a
	Full strength Tsuchiya	82.15 cde	18.93 abcde	8.25 gh	1.53 ab	6.89 a	1.87 b	34.71 abcde	2.78 a
	½ strength Tsuchiya	88.83 e	18.83 abcde	8.43 j	1.55 ab	6.96 a	1.89 b	35.30 abcde	2.78 a
	¼ strength Tsuchiya	81.20 bcde	19.23 bcde	8.28 hi	1.54 ab	7.05 abcd	1.82 b	35.54 bcdef	2.74 a
	Untreated (control)	84.41 de	19.19 bcde	8.29 hi	1.57 abc	6.95 a	1.87 b	34.62 abcde	2.76 a

[1]Values within a column followed by different letters differ significantly at LSD 5%.
[2]All media were supplemented with 15 g L^{-1} sucrose + 15% mannitol.
DMSO – dimethyl sulfoxide

compared with the other varieties. 'Padma Buana' was the shortest (69.25 cm), and had the least number of leaves (16.89), the least stem and flower disc diameters (5.57 and 1.37 cm, respectively), but had the most number of florets (38.60). 'Pasopati' had the least number of florets (31.42); 'Tirta Ayuni' had the largest disc diameter (1.89 cm) and had more ovate florets compared with those of 'Puspita Nusantara'. The genotype of the four varieties was responsible for the descriptive variations shown by the chrysanthemums.

Storage of plantlets in any of the conservation media did not affect the inherent horticultural characteristics of each variety. Deviations from the control and among the plants previously stored *in vivo* at different periods were not found in the vegetative and flower characteristics within each variety. The nonsignificant differences indicated that *in vitro* conservation did not influence further plant growth and development under *in vivo* conditions. Budiarto and Rosario (2017) reported that DMSO, nutrient modifications of the media in combination with osmotic substances (mannitol), and low temperature retarded the plantlet growth *in vitro*; however, this was not permanent as shown in the current study. Sub-culturing the conserved plantlets into growth medium (½ MS + 0.1 mg L^{-1} IAA) for 3 wk for root induction before acclimatization had eliminated the suppressive effect of the conservation treatments on the plantlets. The succeeding normal growth of plantlet was an indication of the plantlets' recovery from the suppressive conditions (Maryam et al. 2014). Genetic stability in all varieties was maintained after conserving the plantlets for 6 mo.

Cytology of the Conserved Varieties

The chromosome number (2n) of 'Pasopati' is 54; 'Padma Buana', 61; 'Puspita Nusantara', 59 and 'Tirta Ayuni', 63. 'Pasopati' has 38 median, 14 sub-median and 2 sub-terminal chromosomes, while 'Padma Buana' has 44 median, 14 sub-median and 3 sub-terminals. 'Puspita Nusantara' has 40 median, 18 sub-median and 1 sub-terminal chromosome types, and 'Tirta Ayuni' showed 46 median, 14 sub-median and 3 sub-terminal chromosomes (Table 2). The respective untreated varieties have the same chromosome number.

The karyograms of the conserved varieties are presented in Fig. 7. Conserving the chrysanthemum varieties *in vitro* did not produce any alteration in chromosome number, classification of chromosome, and number of chromosomes in each group. These findings supported the non-significance of the results of the observations on vegetative and reproductive parameters of the four chrysanthemum varieties.

In somaclonal variations in some crops, chemicals including DMSO were suspected to have influenced their occurrence. They also have effects on the low recovery capability and inconsistent genetic fidelity of the conserved protocol (Anwar et al. 2010; Rout et al. 2006). Somaclonal variation has been found in chrysanthemum during low-temperature storage when the DMSO concentration is above 5% (Martin and Gonzáles-Benito

2009). The use of DMSO in various concentrations (5–10%) resulted in vitrification, morphological changes in apical structures, decrement in shoot multiplication ability up to 60%, and change in flower color from the original up to 70% (Fukai et al. 1991; Hitmi et al. 2000; Sakai et al. 2000). The concentration of DMSO used in this experiment was only 2.5%, and was considered insufficient to induce chromosomal alteration and variation on the phenotypic performances of the varieties.

The lower number of subculture cycles in this experiment is another possible reason for the absence of variation. The plantlets were subcultured only once for plantlet establishment from the explants, once for plantlet inoculation in different media treatments, and the last was at the induction medium before acclimatization. In every conservation period, the establishment of plantlets was developed through induction of new shoot tip explants from *in vivo* mother stock from the respective variety. By this scheme, the conserved plantlets were not derived from the long high-hormone induction protocols. Jevremović et al. (2006) indicated that long-term subcultures in conservation media might be the cause of the genetic instability in chrysanthemum.

Murashige and Skoog (MS) is the most commonly used medium for *in vitro* mass propagation and conservation of plants including chrysanthemums. The use of Tsuchiya medium for conservation of chrysanthemums has never been reported. The microelement, Fe (iron), in MS medium was also reported to induce somaclonal variation in flower color of Torenia (Nhut et al 2012). In our study, this element is not included in the Tsuchiya medium and could have lessened the possibility of somaclonal variation in the varieties studied.

The decrease in height and change in flower type were observed in chrysanthemum cv. Spider after long exposure of the plantlets to high hormone content medium along with the subculture cycles.

CONCLUSION

Four chrysanthemum varieties stored *in vitro* for 2, 4 and 6 mo in four different conservation media were grown *in vivo* to assess their vegetative and floral characteristics. Initially, there were significant interaction effects of storage period and conservation media on plant height, stem diameter, number of florets, number of leaves, and leaf length-width ratio within varieties. These were dissipated and eliminated as the storage period was increased.

The size of the flowers, florets and discs, and the color of the adaxial and abaxial parts of all varieties were not affected by the conservation media and length of storage. Cytological alterations in terms of chromosome number, chromosome classification, and grouping were not found within variety and within media.

This study has demonstrated that *in vitro* conservation of chrysanthemums for 6 mo is an effective alternative method to replace the more expensive and risky maintenance of live and field-grown germplasm without sacrificing genetic stability. The conservation media did not affect the vegetative and floral characteristics of chrysanthemums.

Table 2. Karyotype data of chrysanthemum varieties stored in various *in vitro* conservation media.

Variety	Conservation Medium	Chromosome No. (2n)	Karyotype Formula[1, 2]	Chromosome Group and No. of Chromosome[2]		
				1	2	3
'Pasopati'	MS + 2.5 % DMSO	54	38m + 14sm + 2st	38	16	-
	Full strength Tsuchiya medium	54	38m + 14sm + 2st	38	16	-
	½ strength Tsuchiya medium	54	38m + 14sm + 2st	38	16	-
	¼ strength Tsuchiya medium	54	38m + 14sm + 2st	38	16	-
	Unconserved (control)	54	38m + 14sm + 2st	38	16	-
'Padma Buana'	MS + 2.5 % DMSO	61	44m + 14sm + 3st	34	24	3
	Full strength Tsuchiya medium	61	44m + 14sm + 3st	34	24	3
	½ strength Tsuchiya medium	61	44m + 14sm + 3st	34	24	3
	¼ strength Tsuchiya medium	61	44m + 14sm + 3st	34	24	3
	Unconserved (control)	61	44m + 14sm + 3st	34	24	3
'Puspita Nusantara'	MS + 2.5 % DMSO	59	40m + 18sm + 1st	34	24	1
	Full strength Tsuchiya medium	59	40m + 18sm + 1st	34	24	1
	½ strength Tsuchiya medium	59	40m + 18sm + 1st	34	24	1
	¼ strength Tsuchiya medium	59	40m + 18sm + 1st	34	24	1
	Unconserved (control)	59	40m + 18sm + 1st	34	24	1
'Tirta Ayuni'	MS + 2.5 % DMSO	63	46m + 14sm + 3st	50	13	-
	Full strength Tsuchiya medium	63	46m + 14sm + 3st	50	13	-
	½ strength Tsuchiya medium	63	46m + 14sm + 3st	50	13	-
	¼ strength Tsuchiya medium	63	46m + 14sm + 3st	50	13	-
	Unconserved (control)	63	46m + 14sm + 3st	50	13	-

[1]Chromosome classification based on centromere location; M = median point, m = median region, sm = sub median region, st = subterminal region, T = terminal point

[2]Chromosome classification and grouping based on Levan's chromosome nomenclature (Levan et al. 1964)

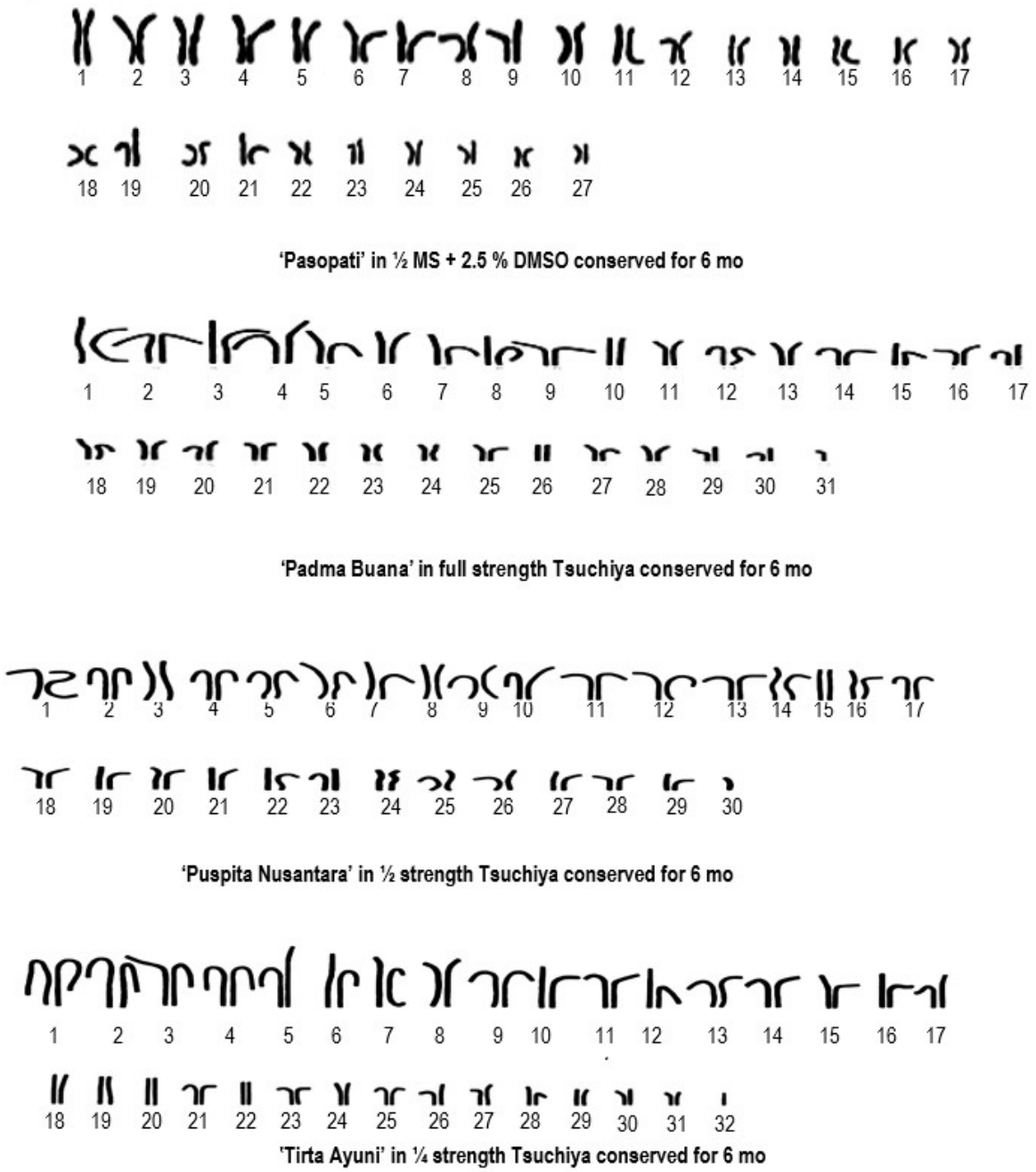

Fig. 7. Karyogram of four chrysanthemum varieties grown *in vivo* after conservation *in vitro*.

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