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Full Length Research Paper

The micropropagation of chrysanthemums via axillary shoot proliferation and highly efficient plant regeneration by somatic embryogenesis

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Protocols for axillary shoot proliferation and somatic embryogenesis were developed for *Dendranthema* × *grandiflora* (Ramat.) Kitamura cv. Palisade White. Shoot tips were cultured on a modified Murashige and Skoog (MS) media supplemented with benzyl aminopurine (BA) and gibberellic acid (GA_3) or BA, kinetin (Kin) and indole-3-acetic acid (IAA). The auxins indole -3-butyric acid (IBA) and IAA were used to induce rooting. Direct somatic embryogenesis was induced from leaf, internode's stem and for the first time for chrysanthemums from petiole explants. Modified MS medium supplemented with 1 mg/L naphthalene acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/L BA, 200 mg/L casein hydrosylate (CH) and 290 mg/L proline was used for induction. Proliferation rate of 3.2 new microshoots per one inoculated was achieved when BA (0.1 mg/L) was used in combination with GA_3 (0.5 mg/L). The number of roots per shoot was higher using IBA (0.5 mg/L), but IAA (2 mg/L) promoted longer roots. A high percentage of embryogenesis was induced by both combinations of plant growth regulators (PGRs). Leaf explants were most responsive, demonstrating the highest percentage of embryogenesis (97.9%), followed by petiole and internode's stem explants (56.3 and 35.1%, respectively). The number of somatic embryos per embryogenic explant was also the highest on leaf explants; however, the best conversion rate (53.8%) of somatic embryos to plantlets was observed from petiole explants. For this reason, petiole explants are the most suitable type of explants for plant regeneration of chrysanthemum cv. Palisade White through somatic embryogenesis.

Key words: Chrysanthemum, *Dendranthema* × *grandiflora* (Ramat.) Kitamura cv. Palisade White, micropropagation, direct somatic embryogenesis, explant type.

INTRODUCTION

The chrysanthemum, *Dendranthema* × *grandiflora* (Ramat.) Kitamura, has been cultivated for more than 2000 years and today, it is the world's second most economically important floricultural crop following the rose (Teixeira da Silva, 2003a). In different countries, chrysanthemums are used for different purposes and occasions. Although in Japan the majority of chrysanthemums are used for gifts and commercial purposes (hotels, events), in some European countries such as Poland, France and Croatia chrysanthemums, especially with a white-coloured inflorescence are a symbol of death and are mostly used for the decoration of graves on All Saints Day. The same applies to the ″Palisade″, an old fashioned cultivar belonging to a group of exhibition chrysanthemums of the intermediate incurve type that

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Abbreviations: MS, Murashige and Skoog; **BA,** 6-benzylaminopurine; **GA_3,** gibberellic acid; **Kin,** kinetin; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **NAA,** α-naphthalene acetic acid; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **CH,** casein hydrolysate; **PGR,** plant growth regulator; **SE,** somatic embryo.

Table 1. Media composition for shoot proliferation, rooting and somatic embryogenesis.

Medium	Composition
Shoot proliferation media	
MSSP1	MS*, 0.1 mg/L BA, 0.5 mg/L GA_3, 25 g/L sucrose
MSSP2	MS, 0.5 mg/L BA, 0.5 mg/L Kin, 0.1 mg/L IAA, 25 g/L sucrose
Rooting media	
MSR1	MS, 0.5 mg/L IBA, 25 g/l sucrose
MSR2	MS, 2 mg/L IAA, 25 g/l sucrose
Media for somatic embryogenesis	
MSSE1	MS, 1 mg/L NAA, 0.1 mg/L BA, 200 mg/L CH, 290 mg/L L-proline, 30 g/L sucrose
MSSE2	MS, 1 mg/L 2,4-D, 0.1 mg/L BA, 200 mg/L CH, 290 mg/L L-proline, 30 g/L sucrose
MSHF	MS, 200 mg/L CH, 290 mg/L L-proline, 30 g/L sucrose

MS*- Murashige and Skoog basal salts and vitamins (Murashige and Skoog, 1962), with some modified components (660 mg/L of $CaCl_2.2H_2O$, 41.7 mg/L of $FeSO_4.7H_2O$ and 55.95 mg/L of Na_2EDTA). Media were solidified with 5 g/L of Phytagel (Sigma) and the pH was adjusted to 5.8 before autoclaving.

produces a sizable white or yellow inflorescence. The ″Palisade″ cultivar is grown as a pot plant as well as a cut flower worldwide. It is, like chrysanthemums generally, propagated vegetatively with cuttings and suckers. However, since cuttings are obtained repeatedly from mother plants, they can be subjected to virus infection and degeneration, thereby increasing production costs (Hahn et al., 1998).

Chrysanthemums are susceptible to infection by many viruses, but few, for example chrysanthemum virus B, tomato aspermy virus, tomato spotted wilt virus and chrysanthemum stunt viroid, are considered economically important. The problem of virus infection can be solved by micropropagation methods, which are applied to the clonal propagation of a variety of horticultural plants including the chrysanthemum (Ben-Jaacov and Langhans, 1972; Karim et al., 2002; Waseem et al., 2009). The large-scale propagation of disease-free plants by *in vitro* disease-free shoot proliferation would be costly. In addiiton, the production of mother stock plants by micropropagation (Previati et al., 2008), starting from disease-free shoots could be cost effective and would guarantee a production of healthy cuttings. An alternative way to defend against pathogens, but also insect pests, could be the breeding resistant genotypes. To achieve this purpose, a method such as genetic transformation can be used (Takatsu et al., 1999; Shinoyama et al., 2003; Teixeira da Silva and Fukai, 2003b), but an effective regeneration protocol, which is usually genotype-dependent, should also be available.

Mutation breeding, an established method for breeding ornamentals (Mandal et al., 2000; Barakat et al., 2010b), also depends on plant regeneration from mutated tissue. Chrysanthemums have been regenerated from explants of different sources including the leaf, stem, petal, flower pedicel and shoot tip either by organogenesis or somatic embryogenesis (Teixeira da Silva, 2003a, b, 2004). The single cell origin of somatic embryos eliminates the possibility of regeneration of chimeric plants consisting of both transgenic and non-transgenic tissues. In spite of many attempts, it has been difficult to induce either direct or indirect somatic embryogenesis in the chrysanthemum (Tanaka et al., 2000). May and Trigiano (1991) developed somatic embryos from leaf midrib explants of selected chrysanthemums, Pavingerova et al. (1994) and Oka et al. (1999) from leaves, Tanaka et al. (2000), Mandal and Datta (2005) and Barakat et al. (2010a) from ray florets and Teixeira da Silva and Fukai (2003a) from stem transverse thin cell layers (tTCLs). However, genotypic dependence remains the major limitation for the use of somatic embryogenesis in the chrysanthemum (Rout et al., 2006).

The objectives of this study were to establish a protocol for (1) the *in vitro* propagation of chrysanthemum *Dendranthema* × *grandiflora* (Ramat.) Kitamura cv. Palisade White by axillary shoot proliferation and (2) the direct somatic embryogenesis of this cultivar as a method of *in vitro* plant regeneration from different explant types.

MATERIALS AND METHODS

Shoot proliferation and rooting

Cuttings of *Dendranthema* × *grandiflora* (Ramat.) Kitamura cv. Palisade White were collected from field-grown plants in September 2008. After removing the leaves, cuttings with three to four axillary buds were washed in tap water and surface-sterilised by immersion in 70% ethanol for 1 min, followed by immersion in 5% sodium hypochlorite with the addition of 0.1% Tween 20 and 150 mg L^{-1} ascorbic acid for 10 min. Cuttings were rinsed four times in sterile distilled water. Shoot tips approximately 0.5 to 0.8 mm long were excised from axillary buds under a binocular microscope and transferred to the surface of 12 ml shoot proliferation medium MSSP1 (Table 1) in 9 cm Petri plates. After six weeks, 0.5 to 1 cm

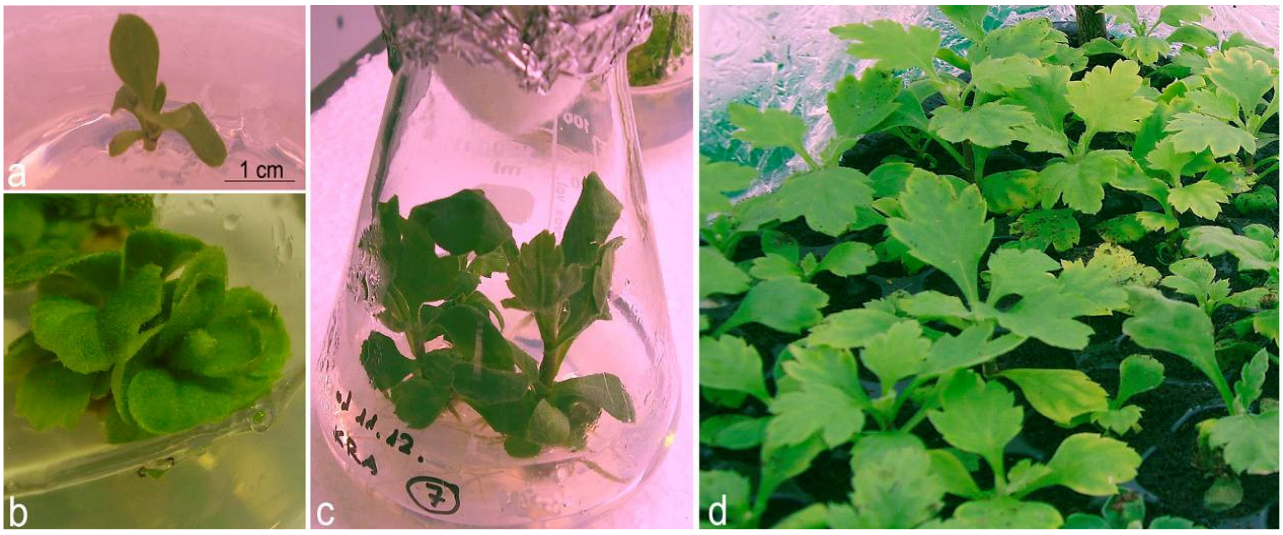

Figure 1. The micropropagation of chrysanthemum *Dendranthema* × *grandiflora* (Ramat.) Kitamura cv. Palisade White. (a) Single microshoot separated from shoot clump and subcultured for further shoot proliferation. (b) Axillary shoot proliferation. (c) Rooted *in vitro* plants after 35 days on rooting medium. (d) Acclimatisation of micropropagated plants in the growth chamber.

long micro shoots were separated if they had developed into clumps, and three of them were transferred into each of 12 Erlenmeyer flasks containing 30 ml MSSP1 or MSSP2 medium (Table 1) (36 micro shoots in each medium). The effect of the medium on axillary shoot proliferation was analysed during two successive sub-cultivations on these media, each time after 30 days in subculture. The efficiency of axillary shoot proliferation was expressed as the mean number of new shoots per one inoculated.

Developed shoots 1 cm long were transferred into MSR1 or MSR2 medium (Table 1) for rooting, taking care that half of them originated from MSSP1 and half from MSSP2 medium. Root number per shoot and root length were determined after 30 days depending on the auxin type in the rooting medium and the previous shoot proliferation medium. All cultures were maintained at 22°C for a 16 h photoperiod of cool white light (40 µE $m^{-2}s^{-1}$). Rooted plants were transplanted to a plastic tray with 96 pots containing peat substrate, covered with plastic dome and raised in a growth chamber at 20°C for a 16 h photoperiod of cool white light (40 µE $m^{-2}s^{-1}$) and 75% relative humidity. After one week, plastic dome was removed and plants were raised next two weeks at the same conditions. Acclimatised plants were planted in a greenhouse and grown under natural light conditions.

Somatic embryogenesis and conversion of somatic embryos to plantlets

In vitro-grown 40-day-old plantlets were used as the explant source for the induction of somatic embryogenesis. Leaves were cut into 3 to 5 mm^2 explants, taking care that each explant retained a rib portion, and placed on their abaxial sides on the medium surface. The internodal stem and petioles were cut into 2 mm long segments and placed horizontally on the medium surface. Fifty explants of each type were placed in five Petri plates onto each of the two media (MSSE1 and MSSE2) to allow somatic embryogenesis (Table 1). Explants were cultured in the dark at 23°C for 35 days, after which the percentage of embryogenic explants and number of somatic embryos (SE) per responding explant were evaluated. Embryogenic explants were then transferred to a hormone-free medium (MSHF; Table 1) and maintained under low light intensity (25 µE $m^{-2}s^{-1}$) for a 16 h photoperiod. Conversion of somatic embryos to plantlets was evaluated one month later.

Experimental design and statistical analysis

All experiments were set up in a completely randomized design. The effect of media composition on shoot proliferation was monitored for two subcultures, each time with 12 replicates (in Erlenmeyer flasks) containing three inoculated microshoots per medium. Data from rooting experiments were collected from 14 replicates, each containing three plantlets per medium, and data on somatic embryogenesis were recorded from five replicates (Petri plates) containing 10 explants for each combination of explant type and medium. ANOVA and Duncan's multiple range test at $P<0.05$ were used for statistical analyses. Analyses were performed using SAS version 9.1.

RESULTS

Shoot proliferation and rooting

Sterilization procedure was 100% efficient and contamination of explants was not observed. During six weeks of culture establishment, shoot tips developed into microshoots (Figure 1a) and frequently proliferated. The effect of the combination of different plant growth regulators (PGRs) in MSSP1 and MSSP2 media on shoot proliferation (Figure 1b) was monitored in two successive experiments. Each microshoot inoculated in MSSP1 media produced an average of 3.2, while those inoculated in MSSP2 produced 2.6 new shoots per one inoculated which does not make statistically significant difference ($P<0.05$) between two media. Axillary proliferated microshoots were normal in appearance and few days after were transferred to rooting media. Besides the rooting, rapid elongation of shoots was observed.

The percentage of rooting was about 95% for both

Table 2. Number of roots per shoot and root length as affected by rooting medium and shoot proliferation medium.

Rooting medium	Number of root	Root length (mm)
MSR1	5.3^{A}	19.3^{B}
MSR2	4.1^{B}	22.4^{A}
Shoot proliferation medium		
MSSP1	5.3^{a}	21.9^{a}
MSSP2	4.4^{b}	19.4^{a}

Values followed by the same letter within columns are not significantly different at $P<0.05$.

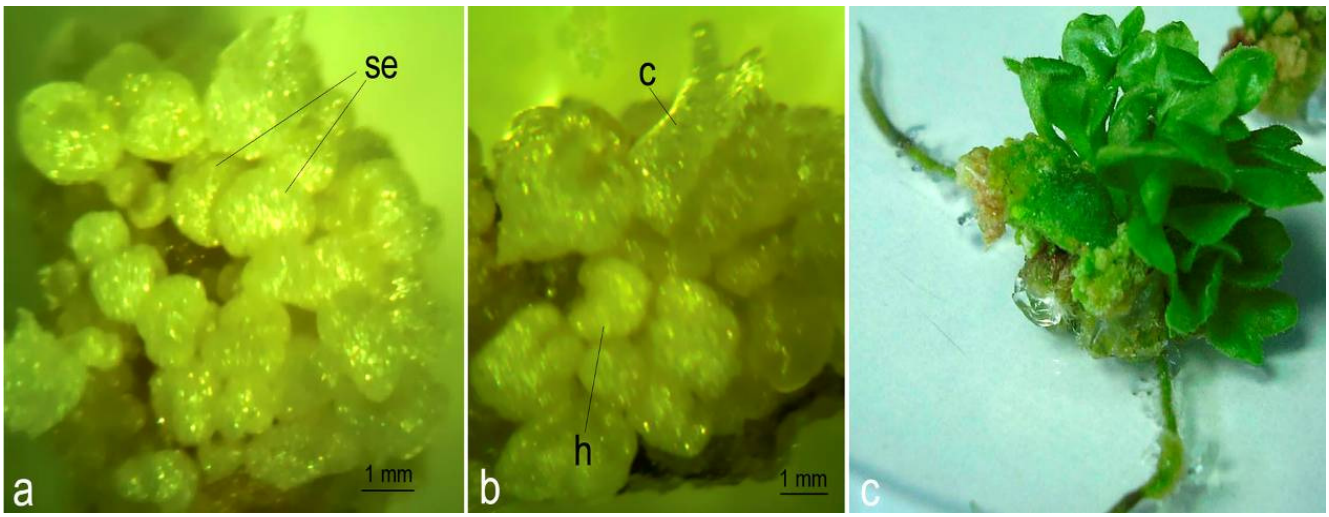

Figure 2. The plant regeneration of chrysanthemum *Dendranthema* × *grandiflora* (Ramat.) Kitamura cv. Palisade White by somatic embryogenesis. (a) Embryogenic leaf explant after 35 days of culture with somatic embryos in the globular stage. (b) Different stages of somatic embryos after transfer to a hormone-free medium. (c) Plantlets regenerated from somatic embryos. se, Somatic embryo; h, heart stage of se; c, cotyledonary stage of se.

rooting media. Auxin type in rooting media and previous shoot proliferation medium significantly influenced the rooting of chrysanthemums. The rooting medium with 0.5 mg/L IBA (MSR1) produced a significantly higher ($P<0.01$) number of roots per shoot than the rooting medium with 2 mg/L IAA (MSR2). By contrast, the length of the longest root was significantly longer in MSR2 (Table 2) (Figure 1c). Shoots originating from the MSSP1 medium developed a higher average number of roots than those from the MSSP2 medium. Moreover, root length was not significantly influenced by previously used shoot proliferation medium (Table 2). Potted plants were acclimatised in the growth chamber with efficiency higher than 90% (Figure 1d) before being transplanted to the greenhouse.

Somatic embryogenesis and conversion of somatic embryos to plantlets

Explants of leaves, petioles and internode's stems were cultured on modified MS medium with two different combinations of PGRs. All explants initially increased in size and after three to four weeks somatic embryos (SE) appeared directly on the explants' surfaces without callus induction. Somatic embryos were white-yellow in appearance (Figure 2a). Percentage of embryogenesis across explant types and media varied between 32 to 100% (Table 3). Significant differences ($P<0.01$) in the percentage of embryogenesis were found between the three explant types. Leaf explants were the most responsive, demonstrating the highest percentage of embryogenesis, followed by petiole and internode's stem explants (Table 3). Differences in auxin type in MSSE1 or MSSE2 media did not significantly influenced mean percentage of embryogenesis. Leaf explants demonstrated also a significantly ($P<0.01$) higher number of SE per responding explant in comparison with the other explant types (Table 4). Mean number of SE across all explant types were similar and amounted to 11.7 and 11.5 for MSSE1 and MSSE2 medium, respectively. The highest number of SE per explant (21.6) was observed on leaf

Table 3. Percentage of embryogenesis as affected by explants type and medium.

Explant type	Embryogenic explant (%)		
	Medium		Explant type mean
	MSSE1	MSSE2	
Leaf	95.8[a]	100.0[a]	97.9[A]
Petiole	64.7[b]	48.0[bc]	56.3[B]
Internode's stem	32.0[c]	38.3[c]	35.1[C]
Medium mean	64.1[A]	62.1[A]	

*Values followed by the same letter are not significantly different at $P<0.05$.

Table 4. Number of somatic embryos per embryogenic explant as affected by explants type and medium.

Explant type	Number of SE		
	Medium		Explant type mean
	MSSE1	MSSE2	
Leaf	16.2[b]	21.6[a]	18.9[A]
Petiole	10.4[c]	4.0[d]	7.2[B]
Internode's stem	8.6[c]	9.0[c]	8.8[B]
Medium mean	11.7[A]	11.5[A]	

*Values followed by the same letter are not significantly different at $P<0.05$.

explants in MSSE2 medium containing 2,4-D.

After the transfer of explants to a hormone-free medium (MSHF), globular embryos asynchronously started to develop to the heart and cotyledonary stages (Figure 2b). The conversion of SE to plantlets was observed in the same medium for all explant types used (Figure 2c). Conversion rate of SE to plantlets across explant types and media varied between 5.7 to 53.8%. Petiole explants showed higher conversion rate of SE to plantlets in comparison with other explant types (Table 5). Mean percentage of SE conversion to plantlets across explant types were significantly better in MSSE1 medium containing NAA than MSSE2 containing 2,4-D. The highest conversion rate (53.8%) of SE to plantlets was observed on the petiole explants previously induced in the MSSE1 medium, followed by petiole explants induced in the MSSE2 medium (50.0%).

DISCUSSION

Shoot proliferation and rooting

Preliminary experiments showed that microshoots developed from shoot tips on standard MS medium solidified with 2.5 g/L of Phytagel were frequently hyperhydrated and yellowish in appearance (results not shown). The modification of $CaCl_2.2H_2O$, $FeSO_4.7H_2O$ and Na_2EDTA to full and half strength (Table 1) as well as increasing Phytagel to 5 g/L precluded these problems. Zalewska et al. (2007) also used modified MS medium with additional $CaCl_2.2H_2O$, $FeSO_4.7H_2O$ and $Na_2EDTA.2H_2O$ for chrysanthemum shoot tip cultivation. Furthermore, we managed to induce a proliferation rate of 3.2 new shoots per one inoculated, with only 0.1 mg/L BA + 0.5 mg/L GA_3 (MSSP1). Similarly, Hui and ShanLin (2004) reported that the optimum medium for chrysanthemum shoot tip propagation was MS with 0.1 mg/L BA + 0.02 mg/L IAA. It is desirable to achieve efficient micropropagation using as low growth regulator concentration as possible. This strategy diminishes the side effects of a high concentration of growth regulators such as bushy, dwarf habit in the case of cytokinins and the genetic instability of propagated plants. Bairu et al. (2006) found that somaclonal variation among *in vitro*-propagated plants increased with an increase in the multiplication cycle and BA concentration. A higher BA concentration can induce adventitious shoots, which have a greater chance of mutating than axillary proliferated shoots. MSSP1 medium, which was slightly, but not significantly better for shoot proliferation, positively influenced later rooting performance (Table 2), which is in agreement with McComb (1995), who reported that hormone concentration in shoot culture medium can have a carryover effect on rooting.

A higher number of roots per shoot induced by IBA (MSR1 medium), in comparison with IAA (MSR2 medium), as revealed in our study is in agreement with the findings of Karim et al. (2002). However, these authors also reported that roots developed in medium with IBA (0.2 mg/L) are longer than those developed in medium with IAA, which was in contrast to our findings.

Table 5. Conversion of somatic embryos to plantlets (%) as affected by explants type and medium.

Explant type	Conversion rate of SE (%)		
	Medium		Explant type mean
	MSSE1	MSSE2	
Leaf	11.7[c]	6.8[c]	9.3[C]
Petiole	53.8[a]	50.0[a]	52.0[A]
Internode's stem	23.4[b]	5.7[c]	29.1[B]
Medium mean	29.6[A]	20.8[B]	

*Values followed by the same letter are not significantly different at $P<0.05$.

The differences observed in root number and length caused by different auxin types was expected. It is known that auxin inhibits the outgrowth of root primordia when continuously applied (Klerk et al., 1990). However, Nissen and Sutter (1990) showed that IAA is photo-oxidised rapidly (50% in 24 h) and IBA slowly (10%) in tissue culture. Since the rooting experiment was carried out for a 16 h photoperiod, we might assume that IAA in the rooting medium degraded soon after initial root induction. This enabled the better outgrowth of the root primordia and consequently longer roots in this media. IBA, although at a lower concentration than IAA, remained active for a longer time, which positively affected the number of new root primordia but at the same time slowed their outgrowth. If grown in optimal soil moisture conditions, a higher number of roots, as obtained on IBA could support the better performance of acclimatized plants.

Somatic embryogenesis and conversion of somatic embryos to plantlets

Somatic embryogenesis as a favoured way of plant regeneration is difficult to achieve for chrysanthemums and it is genotype depended. Our study revealed that efficiency of somatic embryogenesis and especially conversion of somatic embryos to plantlets highly depends also on explant type. Chrysanthemum cv. Palisade White were already used for induction of somatic embryogenesis from ray florets (Barakat et al., 2010a), but the authors reported only about successful callus induction. Embryogenic callus and somatic embryos did not develop for this cultivar in any of four media protocols used in mentioned experiment. In this study, we successfully induced somatic embryogenesis for chrysanthemum cv. Palisade White from three different explant types - leaf, internode's stem and petiole. To the best of our knowledge, this is the first report on somatic embryo development from chrysanthemum petiole explants.

Both PGR combinations used in this study, namely 0.1 mg/L BA + 1 mg/L NAA or 2,4-D, induced equally high percentages of embryogenesis (64.1 and 62.1%, respectively), and equally high mean numbers of somatic embryos per responding explant (11.7 and 11.5, respectively). Oka et al. (1999) obtained 85% embryogenesis from primary leaf explants on media with 0.1 mg/L BA + 1 mg/L NAA, but data on the number of somatic embryos per explants were not shown. May and Trigiano (1991) obtained a lower percentage of embryogenesis and 4.9 somatic embryos per responding midrib explant using 0.1 mg/L BA + 1 mg/L 2,4-D under similar incubation conditions (continuous darkness for 28 days). Somatic embryos in our study, developed directly on explants' surfaces. The same was found by May and Trigiano (1991) and Oka et al. (1999), who both used media constitutions similar to ours. Direct embryogenesis is more suitable for plant regeneration because it diminishes the possibility of somaclonal variation, an undesirable phenomena in the regeneration of specific genotypes.

The efficiency of leaf explant embryogenesis (97.9%), regardless of the media used, is comparable with the results of Teixeira da Silva and Fukai (2003a), who used stem tTCLs and media with 1 mg/L of IAA and Kin. The mean number of somatic embryos per responding leaf explant found in our study (18.9) is similar to that produced from ray florets (15.8) found by Mandal and Datta (2005). Although leaf explants produced significantly higher numbers of somatic embryos than petiole and internode's stem explants (7.2 and 8.8, respectively), the conversion of SE to plantlets was the highest from petiole explants (53.8 and 50% for SE developed in MSSE1 and MSSE2 medium, respectively). This successful conversion of somatic embryos to plantlets has not yet been reported for any other explant type (and/or chrysanthemum cultivar). The reason for such high numbers of somatic embryos per explant, and especially the conversion rate to plantlets could be from using the organic supplements proline and casein hydrolysate (CH) in the media. Proline has been shown to have a positive influence to embryogenic callus formation in Iris species (Jéhan et al., 1994; Kereša et al., 2009), whereas CH has induced somatic embryos from root explants in carrots (Smith et al., 1997). Purohit and Kothari (2007) reported that subculturing bishop's weed somatic embryos in MS medium supplemented with 100

mg/L of CH significantly promoted the maturation of heart and torpedo stage to cotyledonary stage somatic embryos.

Conclusion

The results presented here describe the efficient micropropagation system of chrysanthemum *Dendranthema* × *grandiflora* (Ramat.) Kitamura cv. Palisade White via axillary shoot proliferation as well as rooting of propagated microshoots. This method could be used in nurseries for the production of healthy mother stock plants, from which healthy cuttings could be taken for further propagation. Furthermore, plant regeneration by direct somatic embryogenesis from leaves, petioles and internode's stem explants has been developed for this cultivar. The highest percentage of embryogenesis was obtained by leaf explants, while the best conversion rate (53.8%) of somatic embryos to plantlets was achieved by petiole explants, which makes this kind of explants the most suitable for *in vitro* plant regeneration of cv. Palisade White. To the best of our knowledge, this is the first report on somatic embryo development and successful plantlets regeneration from chrysanthemum petiole explants at all.

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