MORPHOGENIC RESPONSE FROM LEAF EXPLANT OF *Dendranthema grandiflora* VAR MICROMARGARA †

[RESPUESTA MORFOGÉNICA A PARTIR DE EXPLANTE DE HOJA DE *Dendranthema grandiflora* VAR MICROMARGARA]

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SUMMARY

Background: Chrysanthemum is one of the most popular landscape plants worldwide. Introducing desirable traits into this plant by conventional breeding has limitations due to cross-incompatibility. An approach to this problem is the introduction of resistance or tolerance traits via plant genetic transformation. *In vitro* regeneration of plants is necessary for implementing genetic transformation systems; therefore, regeneration via morphogenesis is required before any genetic transformation attempt. **Objective**: To induce morphogenesis from the leaf explant of *Dendranthema grandiflora* var Micromargara. **Methodology**: Micropropagation from node cuttings was induced from node cuttings of 3 cm in length from 6 months-old plants that were used as explants to produce seedlings as source explants. Morphogenesis experiments were done using leaf segments of 1 cm^2 from 8-weeks-old *in vitro* seedlings. The explants were transferred to basal medium Murashige and Skoog at 4.4 gL^{-1} supplemented with different plant growth regulators. During the morphogenic process, leaves samples were collected to detect which morphogenic process was induced. **Results:** Node cuttings culture on Murashige and Skoog medium at 3.3 gL^{-1} supplemented with benzylaminopurine at 2.2 µM yielded a vegetative growth increase from 1 to 7 shoots, 2 to 12 leaves per node cutting, and the stem length of 3 to 9.5 cm. The leaf explant induced three ways of *in vitro* morphogenesis: direct and indirect roots organogenesis (rhizogenesis) and, shoot organogenesis. Direct rhizogenesis was induced in plant growth regulator-free Murashige and Skoog medium (18 roots/explant). Indirect rhizogenesis from leaf explant was less efficient than direct rhizogenesis, get obtaining six roots per leaf explant cultured on Murashige and Skoog medium at 4.4 g L^{-1} supplemented with 0.4 µM thidiazuron and 4.5 µM 2,4-dichlorophenoxyacetic acid, and three roots per leaf explant cultured on Murashige and Skoog medium supplemented with 0.4 µM thidiazuron and 9.05 µM 2,4-dichlorophenoxyacetic acid. Indirect shoot organogenesis was induced from leaf explants cultured on Murashige and Skoog at 4.4 g L^{-1} supplemented with 13.32 µM benzylaminopurine and 4.83 µM naphthalene acetic acid; 50% of explants with callus formed shoots (2 shoots/leaf explant). Through histological analysis it was possible to verify that the morphogenic response obtained was organogenesis. **Implications:** Regeneration of *Dendranthema grandiflora* var. Micromargara established herein will allow improvement of the variety through techniques of genetic transformation mediated by Agrobacterium and any other strategy to incorporate or silence genes of interest in the plant. **Conclusion**: The high capacity to induce roots of *Dendranthema grandiflora* var Micromargara, may provide an efficient model for further investigating the mechanism of rhizogenesis from leaf explants cultured without plant growth regulators.

Keywords: Rhizogenesis; thidiazuron; chrysanthemum; adventitious shoots; adventitious roots.

RESUMEN

Antecedentes: El crisantemo es una de las plantas de paisaje más populares en el mundo. La introducción de rasgos deseables en esta planta mediante mejoramiento convencional tiene limitaciones debido a la incompatibilidad cruzada. Una solución a este problema es la introducción de rasgos de resistencia o tolerancia a través de la transformación genética de plantas. La regeneración *in vitro* de plantas es un paso necesario en la implementación de sistemas de transformación genética; por lo tanto, se requiere la regeneración a través de la morfogénesis antes de cualquier intento de transformación genética. **Objetivo**: Inducir morfogénesis a partir de explante de hoja *Dendranthema grandiflora* var Micromargara. **Metodología:** Con la finalidad de obtener plántulas *in vitro* para fuente de explantes de hoja para los experimentos de morfogénesis, se llevó a cabo la micropropagación de esquejes de nudos de 3 cm de longitud

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obtenidos de plantas de seis meses de edad. Los experimentos de morfogénesis se realizaron utilizando segmentos de hoja de 1 cm^2 de plántulas *in vitro* de 8 semanas de edad. Los explantes de hoja se transfirieron a medio de cultivo con 4.4 g L^{-1} de medio basal Murashige y Skoog suplementado con diferentes reguladores de crecimiento vegetal. Durante el proceso morfogénico, se recolectaron muestras de explantes de hoja para determinar mediante análisis histológicos cuál fue el proceso morfogénico inducido. **Resultados:** Los explantes de hoja se obtuvieron a partir de plántulas obtenidas de esquejes nodales cultivados en 3.3 gL^{-1} de medio Murashige y Skoog suplementado con 2.2 µM de bencilaminopurina. Los esquejes nodales cultivados tuvieron crecimiento vegetativo de 1 a 7 brotes, 2 a 12 hojas por esqueje nodal, y el largo del tallo de 3 a 9.5 cm. El explante de hoja indujo tres vías de morfogénesis *in vitro*, la organogénesis directa e indirecta de raíces (rizogénesis) y la organogénesis indirecta de brotes. La rizogénesis directa fue inducida en medio Murashige y Skoog libre de reguladores de crecimiento vegetal (18 raíces/explante de hoja). La rizogénesis indirecta fue menos eficiente que la directa, se obtuvieron seis raíces por explante cultivado en 4.4 g L^{-1} de medio Murashige y Skoog con 0.4 µM tidiazuron y 4.5 µM de ácido 2,4-diclorofenoxiacético; y tres raíces por explante de hoja cultivado en 4.4 g L^{-1} de medio Murashige y Skoog suplementado con 0.4 µM tidiazuron y 9.05 µM de ácido 2,4-diclorofenoxiacético. La organogénesis indirecta de brotes se indujo a partir de explante de hoja cultivado en 4.4 g L^{-1} de Murashige y Skoog suplementado con 13.32 µM bencilaminopurina y 4.83 µM ácido naftaleno acético, 50% de los explantes con callo formaron 2 brotes/explante. Mediante análisis histológico se pudo comprobar que la respuesta morfogénica obtenida fue organogénesis. **Implicaciones**: La regeneración de *Dendranthema grandiflora* var. micromargara aquí establecida, permitirá la mejora de la variedad mediante técnicas de transformación genética mediada por Agrobacterium, y/o cualquier otra estrategia para incorporar o silenciar genes de interés en la planta. **Conclusión:** La alta capacidad para inducir raíces de *Dendranthema grandiflora* var Micromargara, puede proporcionar un modelo eficiente para futuras investigaciones sobre el mecanismo de rizogénesis a partir de explantes de hojas cultivadas sin reguladores de crecimiento.
Palabras clave: Rizogénesis; tidiazuron; crisantemo; brotes adventicios; raíces adventicias.

INTRODUCTION

Chrysanthemum (D*endranthema grandiflora* Tzvelev) is the number one flower crop in many countries, including India, the United States, and Japan (Ranadev, Kumari and Praveen Ranadev, 2019). *Dendranthema grandiflora* is a synonym of *Chrysanthemum morifolium* Ramat (Lowe *et al*., 1993; Sjahril *et al*., 2016). Abiotic and biotic stresses affect the chrysanthemum crop, so breeders have always aimed to develop new cultivars with increased tolerance. Biotic stress is caused mainly by the fungus causing degradation of flower quality and resulting in up to 100% yield loss (Bety and Pangestuti, 2021), Drought, salinity, and other abiotic stresses have severe effects on plant photosynthesis, respiration, and the natural growth and development process (Liu *et al*., 2019), Chrysanthemum is highly sensitive to several abiotic stresses, including drought, heat, salinity, heavy metal pollution, and nutrient deficiency. Heat stress is known to be particularly damaging to the growth and end-use quality of the chrysanthemum (Qi *et al*., 2018). On the other hand, manipulating plant architecture increases the ornamental value and hence, the marketability of a commercial species (Su *et al*., 2019). Thus, desirable traits have been introduced into Chrysanthemum by conventional breeding.

However, there are limitations to such programs due to the limited gene pool and cross incompatibility. Therefore, Chrysanthemum breeders have begun to exploit Agrobacterium-mediated transformation methods to develop novel cultivars to satisfy current market trends (Xu *et al*., 2022). Thus, a genetic transformation mediated by Agrobacterium can be an alternative. However, this method requires *in vitro* regeneration protocol, which depends on the variety. *In vitro* regeneration can be done through morphogenesis. Differentiated somatic cells may dedifferentiate in plants' morphogenesis process and subsequently redifferentiate into (adventitious) shoots, roots, or embryos. Root formation from non-root tissues is known as adventitious roots (Verstraeten *et al*., 2014; Steffens and Rasmussens, 2016). Rhizogenesis is essential for obtaining diverse secondary metabolites (Simao *et al*., 2016; Babich *et al*., 2020).

Various secondary metabolites were detected in a study on Chrysanthemum morifolium (Zhang *et al*., 2020). The formation of adventitious shoots and adventitious roots (rhizogenesis) is related to the term adventitious organogenesis, whereas somatic embryogenesis refers to the formation of adventitious somatic embryos (Chowdhury, Hoque and Sarker, 2021). Organogenesis can be indirectly generated from the callus or direct over the explant without previous callus formation. Direct shoot organogenesis was reported in Chrysanthemum from leaf, petiole, stem, and ray floret explants (Lim *et al*., 2012; Naing *et al*., 2014; Kumar *et al*., 2017; Kazeroonian *et al*., 2018; Chowdhury *et al*., 2021; Prathyusha *et al*., 2021). Also, there are recent reports of indirect shoot organogenesis from leaf and petal explants (Ghosh *et al*., 2018; Din *et al*., 2022). A histological analysis of the chronological sequence of morphological events during ontogeny is a way to know what morphogenic process was induced (Delporte *et al*., 2014). Somatic embryogenesis and/or organogenesis has been described and confirmed using histological analysis in

different species (Delgado-Aceves *et al*., 2021; Mamgain *et al*., 2022; Xu *et al*., 2022; García-Hernández *et al*., 2022).

Morphogenesis is a complex process that involves the use of plant growth regulators (PGRs). Therefore, most of the reports evaluate their effect; for example, rhizogenesis from petals was reported using phloroglucinol (PG), indole butyric acid (IBA), indol acetic acid (IAA), and naphthalene acetic acid (NAA) (Teixeira Da Silva, 2014); 2,4-dichloro phenoxy acetic acid (2, 4-D) and benzyl amino purine (BAP) were effective for callus induction from stem explant of *Chrysanthemum indicum* (Jin *et al*., 2017), and leaf explant of *Dendranthema grandiflora* (Babiker *et al*., 2021). While that callus from petals of *Dendranthema grandiflora* was formed in the presence of individual action of 6-(γ,γ-Dimethylallyl-aminopurine (2ip), picloram, 2,4-D, coconut water, and thidiazuron (TDZ) (Teixeira Da Silva, 2014). Even though auxin and cytokinin have been the most extensively used and studied hormones in callus formation and subsequent organ regeneration (Ikeuchi *et al*., 2013), the potential of TDZ has been reported in *Hedychium coronarium*, *Caryopteris terniflora*, and *Gaylussacia brasiliensis* (Verma *et al*., 2014; Wu *et al*., 2021; Fritsche *et al*., 2022). TDZ has shown effects both auxin and cytokinin-like. Reports indicate that TDZ may modify endogenous plant growth regulators, directly or indirectly, and produce reactions in cells/tissue necessary for its division/regeneration (Ali *et al*., 2022). BAP induced direct shoot organogenesis from Chrysanthemum, either individually or through combinations of BAP and NAA (Hodson De Jaramillo *et al*., 2008; Sjahril *et al*., 2016; Kazeroonian *et al*., 2018; Ghosh *et al*., 2018). Kinetin (Kin) plus IAA is ideal for regenerating petal explants in calli-producing shoots and shoot number per explant (Din *et al*., 2022). This work aimed to test the effect of the PGRs on the morphogenic capacity of leaf explant of *Dendranthema grandiflora* var Micromargara.

MATERIALS AND METHODS

Micropropagation from node cuttings

The node cuttings of 3 cm in length from 6 months-old plants were used as explants to produce seedlings as source explants. The nodes cuttings were washed with running tap water for 30 min and sterilized with a 70% (v/v) ethanol solution for one min and 0.1% mercury chloride for three minutes, followed by three rinses with sterile distilled water. The node cuttings were transferred to basal medium Murashige and Skoog (MS) at 2.2, 3.1, and 4.4 gL^{-1} without plant growth regulators (PGRs) as controls or supplemented with BAP at 1.11, 2.2, and 4.4 µM (Table 1). Ten explants per treatment were cultured in Magenta boxes; each treatment was replicated twice (n = 20). Response to treatments was evaluated by counting the percentage of survival explants, the stem length, and the number of leaves and roots for a seedling. All media were supplemented with sucrose at 3% (w/v) and solidified with Gelrite at 0.2% (w/v). The pH was adjusted to 5.7 before sterilization by autoclave at 121°C for 15 minutes. The cultures were incubated under photoperiod conditions for two months, with 16/8 h photoperiod (light/dark; photosynthetic photon flux density = 60 µmol m^{-2} s^{-1}), 23 ± 2°C temperature, and 60% relative humidity.

Morphogenesis

Morphogenesis experiments were done using leaf segments of 1 cm^2 from 8-weeks-old *in vitro* seedlings. The explants were transferred to basal medium MS at 4.4 gL^{-1} without PGRs (control) or supplemented with PGRs (Table 2). Leaf explants were placed on the medium, with the abaxial surface facing downwards in Magenta boxes containing 25 mL culture medium in each treatment. Ten explants per treatment were cultured in Magenta boxes; each treatment was replicated twice (n = 20). All media were supplemented with sucrose at 3% (w/v) and solidified with Gelrite at 0.2% (w/v). The pH was adjusted to 5.7 before sterilization by autoclave at 121°C for 15 minutes. The cultures were incubated under total darkness for a month for callus induction. Posteriorly, the cultures were transferred to photoperiod conditions for two months to induce morphogenesis. Photoperiod consists of 16 hours of light/8 hours of darkness photosynthetic photon flux density = 60 µmol m^{-2} s^{-1}), 23 ± 2°C temperature, and 60% relative humidity.

Statistical analysis

The results were analyzed with one-way ANOVA. The Tukey test assessed significant differences among PGR treatments at a $p \leq 0.05$ using Statgraphics® Centurion XVI statistical software.

Histological studies

During the morphogenic process, leaves samples were collected and kept in Formalin-Aceto-Alcohol (FAA) solution containing 10% formaldehyde, 5% acetic acid, 50% ethanol, and 35% distilled water. The explants were kept in the FAA solution for 48 hours. Dehydration was carried out inside the desiccator, using ethanol at different concentrations, starting with 30% ethanol, to gradually increase the ethanol concentration, passing through 50%, 70%, 85%, 96%, and absolute ethanol. After dehydration, ethanol residues were eliminated using liquid paraffin and xylene for 24 hours. After, a series of changes with new paraffin allowed for the elimination of the xylene residues. After, six changes with liquid paraffin were permitted to eliminate the xylene residues. Then, a

deparaffinization was carried out, making three changes with xylene. Posteriorly, tissue rehydration was performed, starting with absolute ethanol, passing through 96%, 85%, 70%, and 50%. The samples were acidified with periodic acid for 20 min. Slides containing samples were spiked with Schiff's reagent for 8 min. Subsequently, the cell walls were stained using naphthol blue for 7 minutes. The prepared samples were cut 5 µm thick with a rotary microtome. A Nikon Eclipse 2000 optical microscope was used to observe plant tissues, and the INFINITY Analyze software was used to take computerized photographs.

RESULTS AND DISCUSSION

Micropropagation from nodes cuttings

The nodes cuttings culture is a method used for the micropropagation of selected genotypes (Scalzo *et al*., 2016; Prathibhani *et al*., 2020; Trigiano *et al*., 2021). In this study, node cuttings formed bud shoots, leaves, and roots, a significant difference among the treatments was observed in the survival percentage, number of bud shoots, length of the stem, number of leaves, and roots generated per treatment. Node cuttings had the best response in 3.3 g L^{-1} MS + 2.2 µM BAP treatment (Table 1); in this treatment, the survival percentage was 100%, The advantage in this report is that the vegetative growth occurred in the same media treatment, and the vegetative growth increase from 1 shoot to 7 shoots, and from 2 to 12 leaves per node cutting, and an average stem growth was from 3 to 9.5 cm, seven roots was developed for node cutting (Figure 1A-C). In contrast, the vegetative growth of node cutting cultured in MS media supplemented with 18.6 µM Kin and 2.94 µM IBA of *Chrysanthemum indicum* plant increased from 3.3 to 7.2 shoots, 5.3 to 17 leaves, stem growth was 3.81 to 5. 27 cm; and 12.5 roots per explant rooting occurred on 2.2 g L^{-1} MS medium supplemented with 4.9 µM IBA for rooting (Alsoufi *et al*., 2021). On the other hand (Imtiaz *et al*., 2019) reported that MS media supplemented with 44.39 µM BAP induced maximum shoot buds (12) and shoot length (6.06 cm)/ node explant of *Chrysanthemum morifolium*. The advantage of the present report is that only one medium is necessary to develop bud shoots, leaves, and roots, using only BAP at a low concentration (2.2 µM).

Morphogenesis

Different ways observed the acquisition of morphogenic competence in *Dendranthema grandiflora* var Micromargara. Indirect and direct rhizogenesis from leaf explant, and indirect organogenesis of shoots from leaf explant. Friable callus formation was observed in leaf explant in 53% of treatments tested (Table 2). It is essential to highlight that the PGR-free medium induced direct rhizogenesis from leaf explant without calli formation (Figure 2A-B), indicating that *Dendranthema grandiflora* var Micromargara has a high capacity for generating rhizogenesis; a similar behavior was observed in *Salvia miltiorrhiza* (Tsai *et al*., 2016).

Thidiazuron in combination with 2,4-D, at all concentrations tested, induced callus, two of them developing roots from leaf explant (0.4 µM TDZ + 4.5 µM 2,4-D, and 0.4 µM TDZ + 9.05 µM 2,4-D) (Figure 2C-D), a similar effect had 2,4-D and TDZ for callus and organogenesis induction from leaf explants of *Salvia miltiorrhiza* (Tsai *et al*., 2016). Babiker *et al*. (2021) reported callus induction from leaf explant of *Dendranthema grandiflora* var. Zembla yellow in the presence of 2.26 µM 2,4-D combined with 0.88 µM BAP but not registered organogenesis. This result is because 2,4- D is a PGR type auxin that induces callus, and TDZ causes rhizogenesis from callus. On the other hand, rhizogenesis from petals explants of *Dendranthema grandiflora* was induced in individual action of PG, IAA, IBA, and NAA (Teixeira Da Silva, 2014).

Table 1. Micropropagation of seedlings from node cuttings of *Dendranthema grandiflora* var Micromargara.

Treatment	% Survival	Stem length (cm)	Number of leaves/Node cutting	Number of roots/Node cutting	Number of shoots/Node cutting
2.2 gL^{-1} MS, 1.1 µM BAP	66.66±3.33^{a}	1.40±0.10^{a}	6.33±0.30^{a}	1.33±0.20^{ab}	4.00±0.50^{b}
4.4 gL^{-1} MS, 1.1 µM BAP	83.33±5.20^{a}	2.00±0.30^{a}	6.66±0.20^{a}	2.66±0.20^{bc}	2.00±0.20^{a}
2.2 gL^{-1} MS, 4.4 µM BAP	100.00±0.00^{a}	2.25±0.20^{a}	8.50±0.30^{ab}	4.00±0.20^{c}	2.00±0.20^{a}
4.4 gL^{-1} MS, 4.4 µM BAP	100.00±0.00^{a}	1.11±0.10^{a}	6.16±0.10^{a}	0.66±0.30^{a}	2.00±0.20^{a}
3.3 gL^{-1} MS, 2.2 µM BAP	100.00±0.00^{a}	9.50±2.10^{b}	12.00±2.00^{b}	6.50±0.50^{d}	7.00±0.30^{c}

* 2.2, 3.33, and 4.4 g L^{-1} MS (controls) did not show growth. Means with different letters denote significant differences at P <0.05 between treatments.

Table 2. *In vitro* morphogenesis from the leaf of *Dendranthema grandiflora* Var. Micromargara.

PGRs concentration (µM)	% Explants with callus	No. Adventitious shoot/explant leaf. Direct shoot organogenesis	No. Adventitious roots/explant leaf. Direct rhizogenesis	No. Adventitious roots/explant leaf. Indirect rizhogenesis
2.22 BAP, 0.57 IAA	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
4.44 BAP, 1.71 IAA	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
13.32 BAP, 4.83 IAA	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
2.22 BAP, 0.53 NAA	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
4.44 BAP, 1.61 NAA	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
13.32 BAP, 4.83 NAA	50.00±2.00^{b}	2.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}
2.22 BAP, 2.26 2,4-D	100.00±0.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
4.44 BAP, 4.50 2,4-D	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
13.32 BAP, 9.05 2,4-D	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
0.40 TDZ, 2.26 2,4-D	90.00±3.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
0.40 TDZ, 4.5 2,4-D	100.00±0.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}	6.00±1.00^{b}
0.40 TDZ, 9.05 2,4-D	100.00±0.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}	3.00±0.05^{a}
0.44 TDZ, 0.22 ZEA	80.00±5.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
0.44 TDZ, 0.44 ZEA	80.00±2.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
0.44 TDZ, 0.66 ZEA	80.00±2.00^{c}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Without PGRs	00.00±0.00^{a}	0.00±0.00^{a}	18.00±2.00^{b}	0.00±0.00^{a}

All treatments contain MS at 4.4 g L^{-1}. Means with different letters denote significant differences at P <0.05 between treatments.

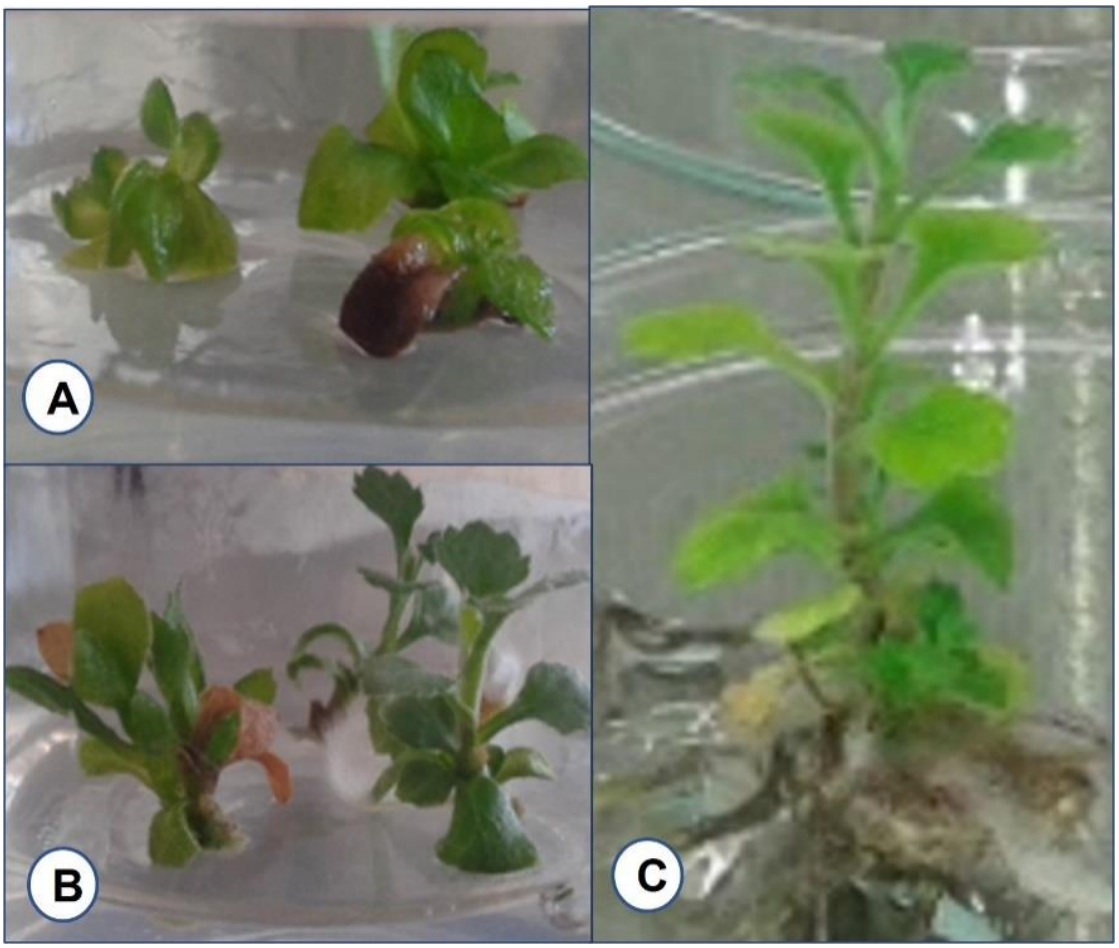

Figure 1. Plant micropropagation from nodes cuttings of *Dendranthema grandiflora* var Micromargara. A) Node cutting 15 days after culture; B) Node cutting 30 days after culture; C) Plant of two months age. *All processes occurred in 3.3 g L^{-1} MS + 2.2 µM BAP.

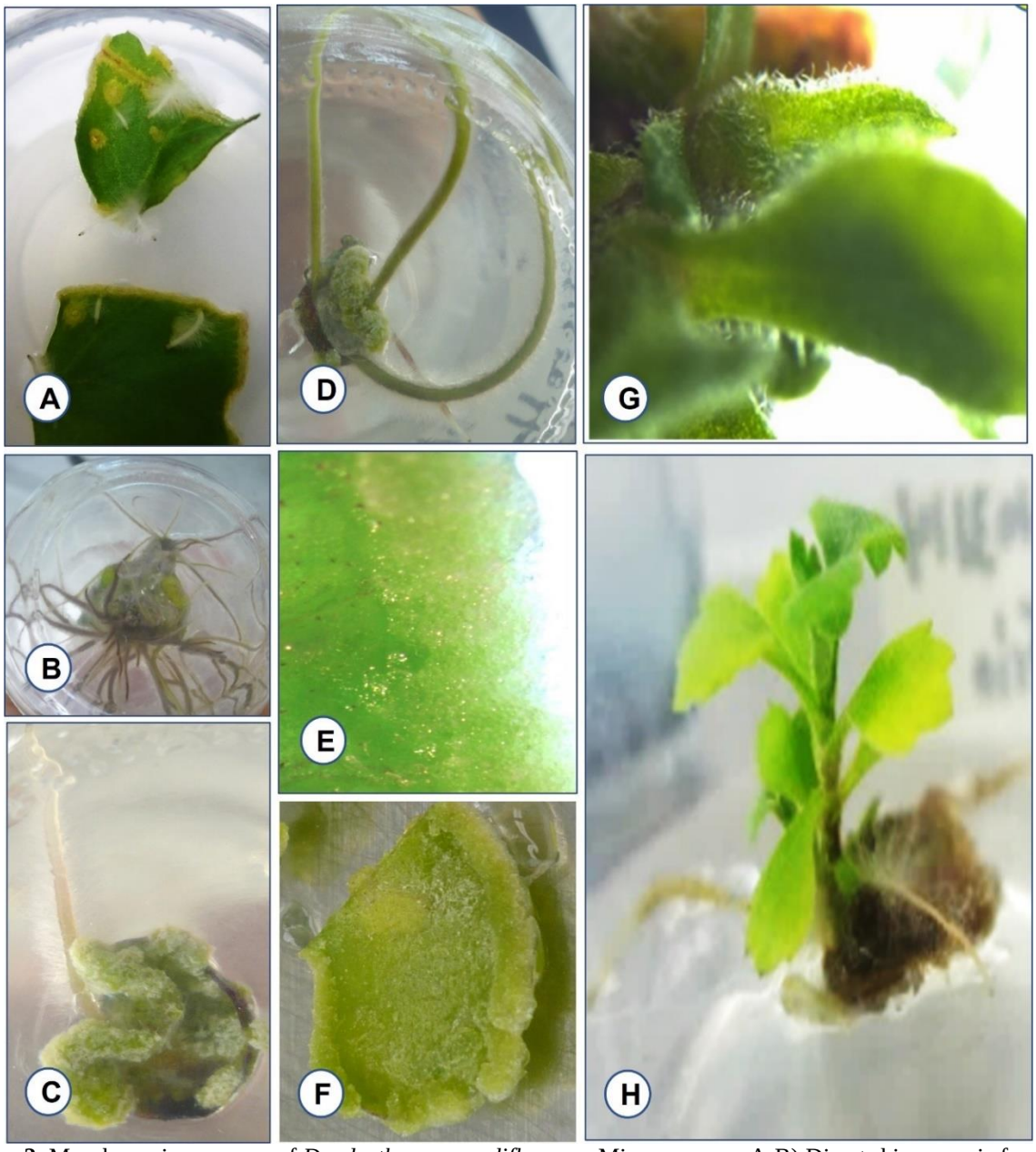

Figure 2. Morphogenic response of *Dendrathema grandiflora* var Micromargara. A-B) Direct rhizogenesis from leaf explant on MS free PGR (30-60 dpi respectively); C) Indirect adventitious roots induction from leaf explant on MS + 0.4 TDZ µM + 4.5 2,4-D µM (45-60 dpi respectively); E-F) Calli from leaf explant (15 and 30 dpi respectively); G) Adventitious shoot from leaf explant at 60 dpi; H) Regenerated plant from leaf explant 90 dpi. *Calli, shoot induction, elongation, and regeneration occur in media with 4.4 g L^{-1} of MS + 13.32 µM BAP + 4.83 µM NAA. dpi: days post-induction.

De novo shoot organogenesis in *Dendranthema grandiflora* var Micromargara was an indirect process involving the previous callus formation. A friable translucent callus was observed, then converted to a green callus on leaf explant cultured in media with 4.4 g L^{-1} MS supplemented with 13.32 µM BAP and 4.83 µM NAA. The participation of NAA and BAP in this process could relate to the response of the explant to exogenous PGRS. The callus progressively acquired competence to respond to differentiation into shoots, shoot elongation, and rooting using the same treatment. Callus response in this treatment was reproducible; 50% of induced calluses formed shoots (2 shoots/explant, Table 2). This result is according to those reported by Hodson De Jaramillo *et al*. (2008), which yielded adventitious shoots from leaves of two varieties of *Dendrathema grandiflora*, Escapade (1.2 leaves), White albatros (3.7 leaves), and Yellow albatros (1.2 leaves) on MS supplemented with 4.83 µM NAA and 13.32 µM BAP.

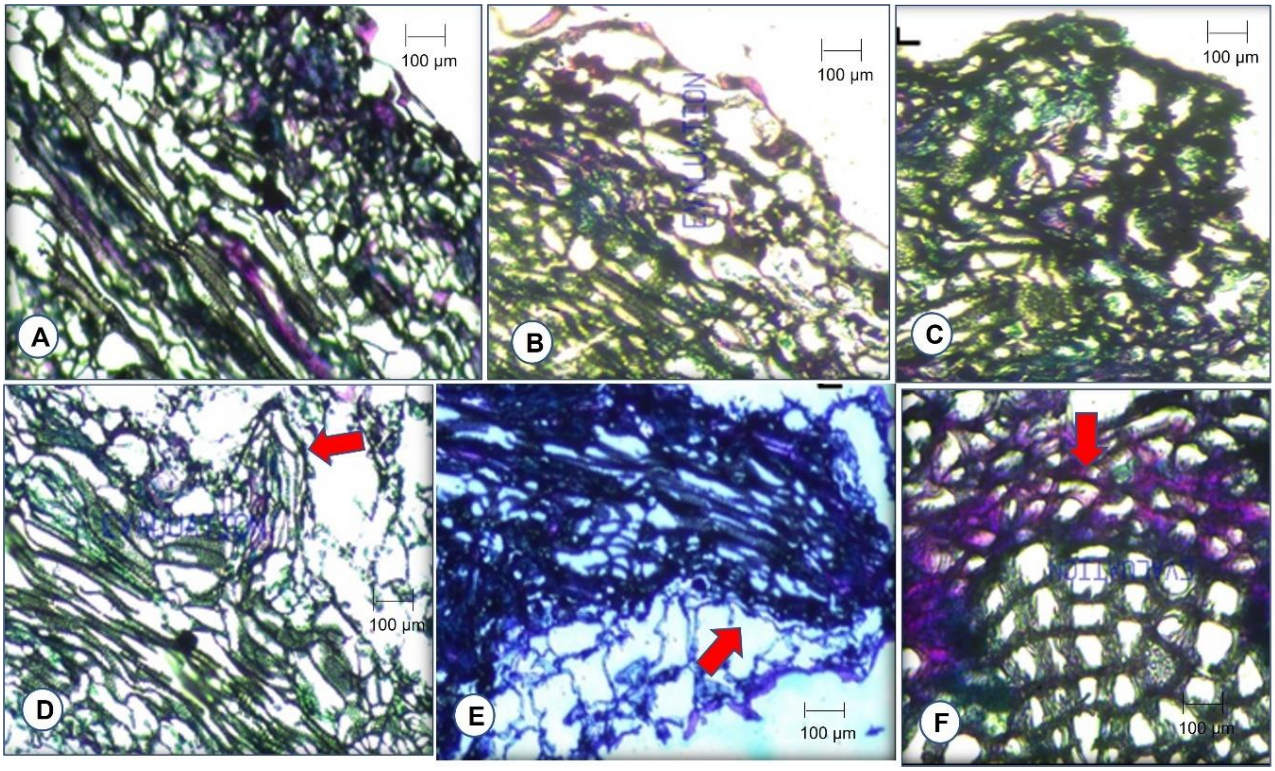

Figure 3. Histological characterization of *in vitro* morphogenesis process from leaf explant of *Dendranthema grandiflor*a. A) Disaggregation of leaf epidermis, 20X, 5 dpi; B) Adventitious shoot 20X, 15 dpi. C) Adventitious shoot with advanced development 20X, 25 dpi; D) Adventitious root 20X, 15 dpi; E) Adventitious root 20X, 30 dpi; F) Meristematic center of the adventitious root, 20X, 45 dpi. Dpi: days post-induction.

Furthermore, the protocol in this report has the advantage that it did not need to change the treatment for shoot elongation and rooting. For example, Din *et al*. (2022) reported indirect shoot organogenesis from petal explant of two varieties of *Dendrathema grandiflora*, 3.25 shoots for candid and 2.75 for Flirt varieties. Callus induction occurred on MS media supplemented with 4.44 µM BAP and 8.56 µM NAA to induce callus, but shoot regeneration was necessary for another medium (MS+ 4.65 µM Kin + 2.85 µM IAA). Another example is a report on two varieties of *Chrysanthemum morifolium* Ramat, in which shoot organogenesis from leaf explant was induced using BAP at a low concentration (2.22 µM) with another auxin (IAA at 11.42 µM). Treatment-induced 7.6 shoots for the BARI Chry-2 variety and 8.6 for the Y variety. Posteriorly, it was necessary to transfer to MS-free PGR for shoot elongation and to root (Chowdhury *et al*., 2021).

Proliferating cells possess a higher ratio of the nucleus: cytoplasm wherein cell separation with a prominent nucleus was observed (Figure 3 A); this is a character during the morphogenesis and was reported by (Haradzi *et al*., 2021). Histological analysis during the morphogenic process of leaves shows that cell protuberances were formed on the epidermis, cell activity was observed, and the growth of structures indicates the process of organogenesis of shoots in the explants (Figure 3 A and 3 C) and rhizogenesis process (Figure 3 D-F). Similarly, the organogenesis and anatomical study of the chrysanthemum were described (Verma and Prasad, 2019). This work confirms that we obtain organogenesis due to a vascular tissue connection between the regenerating structure and explant (Figure 3 B-C), which distinguishes organogenesis (Zhao *et al*., 2022).

CONCLUSIONS

The leaf explant induced three ways of *in vitro* morphogenesis: indirect shoot organogenesis and direct and indirect organogenesis of roots (rhizogenesis). Direct rhizogenesis was induced in plant growth regulator-free MS medium. Shoot organogenesis from leaf explant, shoot elongation, and shoot rooting occurred in the same treatment resulting in an easy and practical protocol for plant micropropagation. Shoot organogenesis from leaves is helpful for plant regeneration from genetically transformed tissues. Alternatively, this variety of chrysanthemum could propagate through node cuttings cultivation. Finally, the ability to form roots directly

without adding PGR is attractive as a study model for understanding the process of rhizogenesis.

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Conflict of Interest
All authors declare that they have no conflict of interest.

Compliance whit ethical standards. The authors have not to declare, due to the nature of this publication.

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