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Micropropagation and phytopathology of calla lily (*Zantedeschia* spp.)

Micropropagación y fitopatología de la cala (*Zantedeschia* spp.)

White calla lily (*Zantedeschia aethiopica* [L.] Spreng.) seedling obtained from embryo isolation and *in vitro* culture.

Photo: A. Martínez-Hernández

AMPARO MARTÍNEZ-HERNÁNDEZ[1]
JOSÉ LUIS RODRÍGUEZ DE LA O[1]
JUAN GUILLERMO CRUZ-CASTILLO[2, 3]
JOSÉ OSCAR MASCORRO-GALLARDO[1]
MA. DE JESÚS JUÁREZ-HERNÁNDEZ[1]
LUIS ÁNGEL BARRERA-GUZMÁN[2]
HÉCTOR TECUMSHÉ MÓJICA-ZÁRATE[2]

ABSTRACT

Calla lily (*Zantedeschia* spp.) is an herbaceous flowering plant that belongs to the Araceae family, with worldwide distribution. Native to the swampy or mountainous regions of South Africa, it is valued as an ornamental plant due to its extraordinary spathe and decorative leaves. However, its production has decreased due to the presence of diseases. *In vitro* culture of plant cells and tissues has been successfully applied to precisely diagnose and control diseases to produce disease-free plants. This study analyzes information from *in vitro* techniques applied to this crop, highlighting phytopathological aspects. It considers the different stages of *in vitro* culture and the process of obtaining and propagating healthy or pathogen-free plants. *In vitro* culture has proven to be an effective tool for rapid clonal propagation and multiplication of *Zantedeschia* spp. Sanitary management before the *in vitro* culture is recommended.

Additional key words: tissue culture; embryogenesis; organogenesis; totipotency; *Pectobacterium.*

[1] Universidad Autónoma Chapingo, Departamento de Fitotecnia, Texcoco (Mexico). ORCID Martínez-Hernández, A.: https://orcid.org/0000-0003-0733-9720; Rodríguez de la O., J.L.: https://orcid.org/0000-0002-2331-9984; Mascorro-Gallardo, J.O.: https://orcid.org/0000-0001-9713-4758; Juárez-Hernández, M.J.: https://orcid.org/0000-0002-5043-4240

[2] Universidad Autónoma Chapingo, Centro Regional Universitario Oriente, Huatusco (Mexico). ORCID Cruz-Castillo, J.G.: https://orcid.org/0000-0002-8687-6235; Barrera-Guzmán, L.A.: https://orcid.org/0000-0001-8057-2583; Mójica-Zárate, H.T.: https://orcid.org/0000-0002-9067-3983

[3] Corresponding author. jcruzc@chapingo.mx

RESUMEN

La cala lily (*Zantedeschia* spp.) es una planta herbácea con flores que pertenece a la familia Araceae, con distribución mundial. Originaria de las regiones pantanosas o montañosas de Sudáfrica, es valorada como planta ornamental por su extraordinaria espata y hojas decorativas. Sin embargo, su producción ha disminuido por la presencia de enfermedades. El cultivo *in vitro* de células y tejidos vegetales se ha aplicado con éxito para diagnósticar y controlar enfermedades con precisión y producir plantas libres de enfermedades. Este estudio analiza información de las técnicas *in vitro* aplicadas a este cultivo, destacando aspectos fitopatológicos. Considera las diferentes etapas del cultivo *in vitro* y el proceso de obtención y propagación de plantas sanas o libre de patógenos. El cultivo *in vitro* ha demostrado ser una herramienta eficaz para la rápida propagación clonal y multiplicación de *Zantedeschia* spp. Se recomienda manejo sanitario antes del cultivo *in vitro*.

Palabras clave adicionales: cultivo de tejidos; embriogénesis; organogénesis; totipotencia; *Pectobacterium*.

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INTRODUCTION

In Mexico, calla lily production is concentrated in the provinces of Veracruz, Puebla, Jalisco, Chiapas, Oaxaca, Colima, and the State of Mexico (Cruz-Castillo and Hernández-Montes, 2022). Its production is limited by *Pectobacterium carotovorum,* also known as *Erwinia carotovora* subsp. *carotovora* (Trejo-Téllez *et al*., 2013; Cruz-Castillo and Torres-Lima, 2017) and other unknown pathogens (Delgado-Paredes *et al*., 2021). Calla lilies are popular in Mexico and are used to decorate weddings and other celebrations (Cuellar-Mandujano *et al*., 2022). Most calla lilies produced in Mexico are perennial with white spathes. In Latin

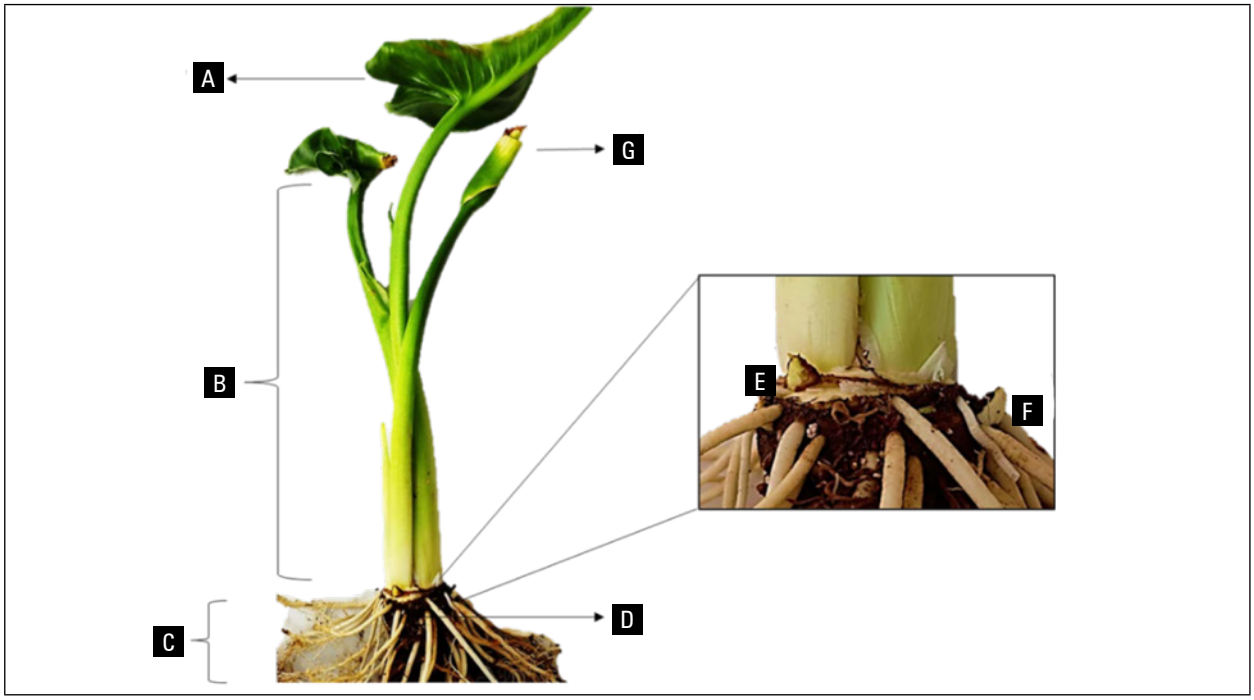

Figure 1. Calla lily (*Zantedeschia aethiopica* L. Spreng.) var. Utopia. A, leaf; B, petiole; C, roots; D, rhizome; E, apical bud; F, adventitious bud; G, immature spathe.

America, this calla lily grows in Argentina, Bolivia, Brazil, Colombia (Casierra-Posada *et al*., 2014), Ecuador, and Guatemala (Cruz-Castillo, 2022).

The calla lily (*Zantedeschia aethiopica* [L.] Spreng.) is the most well-known species (Fig. 1), but there are other species with flowers with various colors from pink to brown. The genus *Zantedeschia* is divided into two sections or botanical groups: the *Zantedeschia* section and the *Aestivae* section (He *et al*., 2020). The *Zantedeschia* section comprises the species *Z. aethiopica* ([L.] Spreng.) and *Z. odorata* Perry, while the *Aestivae* section includes *Z. albomaculata* ([Hook.] Baill.), *Z. rehmannii* (Engl.), *Z. elliottiana* ([Watson] Engl.), *Z. jucunda* (Letty), *Z. valida* (Singh.), and *Z. pentlandii* ([Watson] Wittm.) (Wei *et al*., 2017). It has been demonstrated that the *Aestivae* section is more susceptible to soft rot caused by bacteria of the genus *Pectobacterium* due in part to the ecological adaptation of each section (Guttman *et al*., 2021). This susceptibility has reduced the production of calla lilies, leading to the introduction of *in vitro* cultivation techniques to obtain pathogen-free plants (Nery *et al*., 2018).

A review regarding tissue culture of *Zantedeschia* was published (Sun *et al*., 2023), but rot causal agents of explants were not considered. The micropropagation of *Z. aethiopica* has often been difficult due to contamination of cultured rhizome tissue (Martínez-Hernández, 2022). Therefore, this review focuses on *in vitro* techniques in *Zantedeschia*, highlighting phytopathological aspects to reduce the contamination of the explants.

Phytopathology of the *Zantedeschia* genus

The main factor limiting the production of calla lilies is disease. Soft rot, caused by *Pectobacterium carotovorum* subsp. *carotovorum* poses a significant worldwide threat to *Zantedeschia* crop (Githeng'u *et al*., 2016). Other associated pathogens include *Pseudomonas marginalis* (Brown) Stevens (Krejzar *et al*., 2008; Yan *et al*., 2014), *Pseudomonas veronii* Elomari *et al*., *Chryseobacterium indologenes* (Yabuuchi *et al*.) Vandamme *et al*. (Mikiciński *et al*., 2010), *Cellvibrio zantedeschiae* sp. Nov. (Sheu *et al*., 2017), *Pectobacterium zantedeschiae* Waleron *et al*. (Waleron *et al*., 2019), and *Pectobacterium aroidearum* Nabhan *et al*. (Chen *et al*., 2020). The main symptoms of this disease include dwarfing, chlorosis along the leaf margin progressing in a "V" shape that may turn brown, stem bending, and eventual desiccation (Ciampi *et al*., 2009).

Viruses that often attack *Zantedeschia* include dasheen mosaic virus (DsMV), classified as the most prevalent among Aroids (Cafrune *et al*., 2011). Its distribution was recorded in Bosnia and Herzegovina, where crops presented symptoms such as yellowing and distortion of leaves as well as mosaic and chlorotic ring symptoms (Grausgruber-Gröger *et al*., 2016).

For the konjac mosaic virus (KoMV), *Myzus persicae (Sulzer)* and *Aphis gossypii* (Glover) were identified as main vectors, along with vegetative propagation and seed transmission. Symptoms appear as mosaics, green islands, interveinal chlorosis, leaf deformity, short peduncles, and discolored pigmented spots on the spathe (Liao *et al*., 2020).

The calla lily latent virus (or zantedeschia mild mosaic virus [ZaMMV]) is a potyvirus isolated from the Black Magic cultivar. It manifests as yellow spots and stripes, green islands, and an unusually mild mosaic (Rizzo *et al*., 2015). For the detection of viruses, *Zantedeschia* has been cultured *in vitro* (Purmale *et al*., 2023). However, with this type of propagation the viruses were not eliminated. Therefore, studies on thermotherapy, chemotherapy, cryotherapy or electrotherapy (Szabó *et al*., 2024) to eliminate virus in *Zantedeschia* could be evaluated.

Pathological control and eradication methods

Endogenous contamination is a recurring issue in *in vitro* establishment, particularly in rhizome tissue. Sanitary management before *in vitro* cultivation is recommended. Species of the *Pectobacterium* family propagate easily through decaying plant material, and there are no efficient control agents for the disease they cause (van der Wolf *et al*., 2021; Niyokuri and Nyalala, 2023). Soil fumigation with metam sodium and the application of antagonistic bacteria CAE 01 of the Enterobacteriaceae family reduced the percentage of infection in rhizomes (García-López, 2010). As a biological control for *P. carotovorum* subsp. *carotovorum*, the high specificity of the bacteriophage virus PP1 of the Podoviridae family was studied (Lim *et al*., 2013). The use of *Bacillus velezensis* (Ruiz-García *et al*.) (other scientific name *Bacillus amyloliquefaciens* subsp. *plantarum*) reduced the growth of the phytopathogen in both *in vitro* and field conditions (He *et al*., 2021). Protein profiles from *in vitro* and *in vivo* conditions for *P. carotovorum* following interaction with *Z. elliottiana* 'Black Magic' were also generated. Using two-dimensional electrophoresis, 53 essential

proteins for their virulence were identified, showing differential expression *in vitro*, *in vivo*, or both compared to the control (Wang *et al*., 2018).

Methods of incorporation to *in vitro* conditions

The eradication of pathogens prior to calla lily *in vitro* establishment has been a challenge. The use of mercury chloride ($HgCl_2$) at 0.1-0.2% (w/v) for 15 min (Xiao-Chun, 2010) or for 30 min for leaf and rhizome at doses of 0.1 and 0.2% (w/v) respectively with prior treatment with benomyl at 1% (w/v) in shaking for 30 min (Hlophe *et al*., 2015) yielded good results. Even doses of 0.5% of $HgCl_2$ (w/v) for 15 min were effective at this stage in *Z. rehmannii* with pre-treatment of immersion in 70% ethanol (v/v) for 30 s (Kulpa, 2016). Despite the effectiveness of these treatments, a concentration of 0.25 mg L^{-1} has negative environmental consequences on amphibian development (Muñoz-Escobar and Palacio-Baena, 2010).

Hernández-Villaseñor *et al*. (2018) disinfected with a 15% (w/w) chlorine solution followed by 500 mg L^{-1} of streptomycin, 500 mg L^{-1} of oxytetracycline, and 500 mg L^{-1} of carboxamide for 60 min. Finally, the explants were placed into a 70% (v/v) ethanol solution for 30 s. Similarly, the application of chlorine dioxide (ClO_2) at a concentration of 60 to 180 mg L^{-1} (v/v) for 5 min followed by hydrogen peroxide (H_2O_2) at 5% (v/v) for 15 min provided a survival rate >75% for *Z. aethiopica* (Chen *et al*., 2017).

Due to the need to implement disinfestation protocols that have less environmental impact, studies based on antibacterial activity of *Coptis chinensis* (Franch.) extract has been developed against soft rot. 100% *C. chinensis* made an inhibition effect comparable to streptomycin sulphate (Githeng'u *et al.,* 2016), an antibiotic widely used in the 90's. This protocol has not been tested on *Zantedeschia* explants. Hashemidehkordi *et al*. (2021) evaluated the effect of hot water in the disinfection of rhizome explants and achieved a survival rate of 90% at 45°C for 35 min followed by immersion in 70% ethanol for 30 s and 1% (v/v) sodium hypochlorite (NaOCl) for 10 min. This procedure represents a good alternative to reduce the use of previously mentioned products and their impact in terms of environmental, economic impact, and antibiotic resistance. Table 1 shows the control methods for the *in vitro* culture of calla lilies.

In vitro propagation of *Z aethiopica* cultivars in Mexico has not been easy due to contamination of the explants. The antibiotic Agrimycin® (2 g L^{-1}) added to the fungicide Benomyl® (2 g L^{-1}) reduced endophytic contamination by fungi and bacteria in explants from rhizomes of the cultivars Deja Vú and Utopia. For seed germination, *in vitro* contamination was not observed when using 70% ethanol for 1 min followed by 10% sodium hypochlorite for 15 min, and 10 min of 30% hydrogen peroxide (Martínez-Hernández, 2022).

Table 1. Methods for controlling contaminants in *Zantedeschia* spp. cultured *in vitro*.

Plant material	Origin of explant	Control method	Survival rate (%)	Reference
Z. hybrida	Rhizome	$HgCl_2$ at 0.1-0.2% for 15 min for rhizome segments	Not reported	Xiao-Chun, 2010
Z. aethiopica	Leaf and rhizome	$HgCl_2$ at 0.2% for rhizome segments and 0.1% for leaves for 30 min	28% in rhizome and 78.3% in leaf	Hlophe *et al*., 2015
Z. rehmannii	Rhizome	Immersion in 0.5% $HgCl_2$ for 15 min	73%	Kulpa, 2016
Z. aethiopica	Rhizome	15 % chlorine + 500 mg L^{-1} streptomycin, 500 mg L^{-1} oxytetracycline, and 500 mg L^{-1} captan for 60 min + 70 % ethanol (v/v) for 30 s	Not reported	Hernández-Villaseñor *et al*., 2018
Z. aethiopica	Rhizome-buds	ClO_2 at 60 to 180 mg L^{-1} (v/v) for 5 min + 5% H_2O_2 (v/v) for 15 min	>75 %	Chen *et al*., 2017
Zantedeschia spp. 'Orania' and 'Sunclub'	Rhizome-buds	Hot water bath at 45°C for 35 min + immersion in 70 % ethanol for 30 s and 1 % sodium hypochlorite (v/v) for 10 min	>90 %	Hashemidehkordi *et al*., 2021
Z. aethiopica 'Déjà vu' and 'Utopia'	Rhizome	Agrimicyn® (2 g L^{-1}) added to the fungicide Benomyl® (2 g L^{-1})	'Utopía': 60%; 'Déjà vu'; 80 %	Martínez-Hernández, 2022
Z. aethiopica	Seed	70 % ethanol for 1 min + 10% NaOCl for 15 min, and 10 min of 30% H_2O_2	100 %	Martínez-Hernández, 2022

Direct organogenesis

The process of organogenesis provides the basis for asexual plant propagation largely from nonmeristematic somatic tissues (Schwarz and Beaty, 2000). Through this method, pathogen-free plants have been obtained via meristem culture (Tab. 2). In *Z. aethiopica*, the addition of benzylaminopurine (BAP) at 22.19 μM produced 2.6 shoots per explant (Ribeiro *et al*., 2014). Rhizomes and tubers have been the main organs targeted for obtaining explants despite the likelihood of presenting endogenous contamination. Singh *et al*. (2009) obtained 5 to 6 shoots of *Z. aethiopica* 10.0 mg L^{-1} of BAP supplemented with 50 mg L^{-1} of ascorbic acid. In *Z. rehmannii*, culture was obtained using a concentration of 3 mg L^{-1} BAP, and a rate of 4.3 shoots per explant was obtained with 2.5 mg L^{-1} BAP (Kulpa, 2016).

Indirect organogenesis

Shin *et al*. (2020) reconstructed the process of regeneration of new shoots from callus and differentiated four stages: acquisition of pluripotency (in a medium rich in auxins), formation of pro-meristems, establishment of shoot progenitor, and shoot growth. Some species from the genus *Zantedeschia* have been propagated through this pathway (Tab. 3).

In vitro dedifferentiation in Z. *aethiopica* and *Z. elliottiana* was observed in petiole, leaf, and spathe segments, achieving over 80% callus formation on Murashige and Skoog (MS) medium supplemented with indoleacetic acid (IAA) at 0.5 mg L^{-1} + BAP 2.0 mg L^{-1} for leaves; IAA (2.0 mg L^{-1}) + BAP (2.0 mg L^{-1}) for spathes; and IAA (1.0 mg L^{-1}) + BAP (2.0 mg L^{-1}) for petioles. The petioles showed the greatest dedifferentiation capacity among the organs mentioned. Furthermore, *Z. elliottiana* demonstrated a higher differentiation capacity than *Z. aethiopica*, as well as the ability to generate callus at high doses of BAP (4.0 mg L^{-1}) combined with NAA (0.1 mg L^{-1}) (Jonytienè *et al*., 2017). In *Zantedeschia* spp., callus formation was induced by culturing leaf segments on MS medium supplemented with 2,4-D (2.0 mg L^{-1}) and BA (2.0 mg L^{-1}) or with naphthaleneacetic acid (NAA) (2.0 mg L^{-1}) and BA (2.0 mg L^{-1}) (Gong *et al*., 2008).

The hormonal balance between auxins and cytokinin is crucial in the indirect organogenesis of *Zantedeschia in vitro* (Cheng *et al*., 2013). Lee and Ko (2005) achieved up to 56.7% callus formation on a medium containing BA (2.0 mg L^{-1}) and a shoot regeneration rate of 70% at 3.0 mg L^{-1} of BA. Cytokinin induced the formation of multiple shoots; 2ip (1.0 mg L^{-1}), BA (5.0 mg L^{-1}), and BA (1.0 mg L^{-1}) brought on 16, 14, and 12 multiple shoots in the cultivars Sunlight, Chiante,

Table 2. Research relating to the direct organogenesis of *Zantedeschia* spp.

Plant material	Origin of the explant	Medium	Growth regulators	Multiplication rate (shoots per explant)	Reference
Z. aethiopica	Rhizome	MS	10.0 mg L^{-1} BAP, 50 mg L^{-1} ascorbic acid, 25 mg L^{-1} adenine sulfate, L-arginine and citric acid	5-6	Singh *et al*., 2009
Z. rehmannii	Rhizome-buds	MS	3 mg L^{-1} and 2.5 mg L^{-1} BAP + 100 mg L^{-1} ascorbic acid	4.3	Kulpa, 2016
Z. aethiopica	Shoot tip	MS	22.19 μM BAP	2.6	Ribeiro *et al*., 2014

Table 3. Research related to indirect organogenesis of *Zantedeschia* spp.

Plant material	Origin of the explant	Medium	Growth regulators	Reference
Z. hybrida 'Feng Yang'	Rhizome	MS	BA (2.0 mg L^{-1}) + NAA (0.1 ~ 0.2 mg L^{-1})	Xiao-Chun, 2010
Zantedeschia spp. 'Sunlight', 'Chiante' and 'Pink 'Persuasion'	Apices derived from callus shoots and adventitious shoots	MS + 70-90 g L^{-1} sucrose	BA (2-3 mg L^{-1})	Lee and Ko, 2005
Zantedeschia sp. 'Pink Giant'	Leaf	MS	2,4-D (2.0 mg L^{-1}) and BA (2.0 mg L^{-1}) or NAA (2.0 mg L^{-1}) and BA (2.0 mg L^{-1})	Gong *et al*., 2008

MS, Murashige and Skoog.

and Pink Persuasion, respectively. Xiao-Chun (2010) promoted callus formation on an MS medium + 2.0 mg L^{-1} BA + 0.1-0.2 mg L^{-1} NAA and achieved 100% rooting with an MS medium + indole butyric acid (IBA) at a concentration of 0.3 mg L^{-1}.

Somatic embryogenesis

Few studies on induced somatic embryogenesis in *Zantedeschia* exist (Tab. 4). The first reported research on somatic embryogenesis in calla lilies was conducted on three hybrids with explants derived from rhizomes and anthers. However, the formation of somatic embryos was only achieved from rhizome segments. The best results were obtained in media supplemented with plant growth regulators (Nic-Can *et al*., 2016), as NAA (2.0 mg L^{-1}) and BA (0.6 to 2.0 mg L^{-1}). Conversion to normal seedlings was achieved in MS medium supplemented with vitamins, micro and macronutrients, 1.0 mg L^{-1} 2iP, 3% sucrose, and 0.7% agar (Duquenne *et al*., 2006).

Stem segments containing apical meristems derived from the cultivar Gag-si were cultured on MS medium. A 25% induction rate of yellow embryogenic calli was obtained with MS + 0.5 mg L^{-1} NAA and 1.5 mg L^{-1} BA. In the regeneration experiments from embryogenic calli, the MS medium supplemented with 0.5 mg L^{-1} IAA and 2.0 mg L^{-1} BA showed the highest rates, approximately 85 ~ 90%. Table 4 summarizes research related to somatic embryogenesis of *Zantedeschia* species (Han and Kim, 2019).

Plant mass propagation through bioreactor

Research with *Zantedeschia* grown in liquid media has focused on determining the optimal frequency of immersion, comparing temporary immersion systems (TIS) with other culture systems such as semi-solid and agitated liquid, and the effect of growth regulators. A multiplication coefficient of 9.7 shoots per explant was obtained using active buds from *Zantedeschia* spp rhizomes in a modified MS medium with 100 mg L^{-1} myo-inositol, 1 g thiamine, 4 mg L^{-1} BAP, and paclobutrazol at 0.3 mg L^{-1} with an immersion frequency of 4 h every 4 min (Sánchez *et al*., 2009). Sánchez *et al.* (2010) achieved multiplication rates of 7.93, 10.66, and 11.30 shoots per explant in an MS medium + 1, 2, and 4 mg L^{-1} BA and an immersion frequency of 4 h every 3 min.

In vitro zygotic embryos germination

Mature embryos from *in vitro* seeds have also been used to generate transgenic plants of *Zantedeschia* (Sun *et al*., 2022). Studies on the *in vitro* germination of embryos (seeds) of species in the genus *Zantedeschia* determined that temperatures of 14-20°C, high concentrations of sucrose and agar (Ngamau, 2001), fruit maturity, and GA_3 can influence the germination rate (Nery *et al*., 2015). *In vitro* germination of *Zantedeschia* studies focused on early flowering, and salt and/or temperature tolerance. Seedlings from seeds established at low temperatures had greater growth under lower night temperatures than those germinated at high temperatures, and clones from seeds exposed to high levels of sodium chloride (NaCl) achieved greater growth. Also, early seeds showed better growth, early flowering, and a higher number of spathes (Ngamau, 2008). The germination of *Z. aethiopica* seeds *in vitro* was enhanced in a medium supplemented with 3.0 mg L^{-1} of GA_3 and 0.3 mg L^{-1} of BAP, with germination occurring 35 d after establishment (Martínez-Hernández, 2022) (Fig. 2).

Martínez-Hernández *et al.* (2022) isolated zygotic embryos from mature and immature white calla lily fruits. The seeds were pretreated with sterile water and 30% (v/v) H_2O_2. The isolated embryos were cultured in a medium with MS salts (100%) supplemented with BAP (0, 0.3 and 3 mg L^{-1}) and GA_3 (0, 0.3 and 3.0 mg L^{-1}). The embryos' development was not affected by the composition of culture media, the seed pretreatments or the fruit ripening stage (Fig. 3).

Table 4. Research related to somatic embryogenesis of *Zantedeschia* spp.

Species	Origin of explant	Medium	Growth regulators	Reference
Zantedeschia hybrids	Rhizomes and anthers	MS	BA (0.22 mM)	Duquenne *et al*., 2006
Zantedeschia hybrida	Petiole	MS	0.5 mg L^{-1} NAA, 1.5 mg L^{-1} BA, 0.5 mg L^{-1} IAA, 2.0 mg L^{-1} BA	Han and Kim, 2019

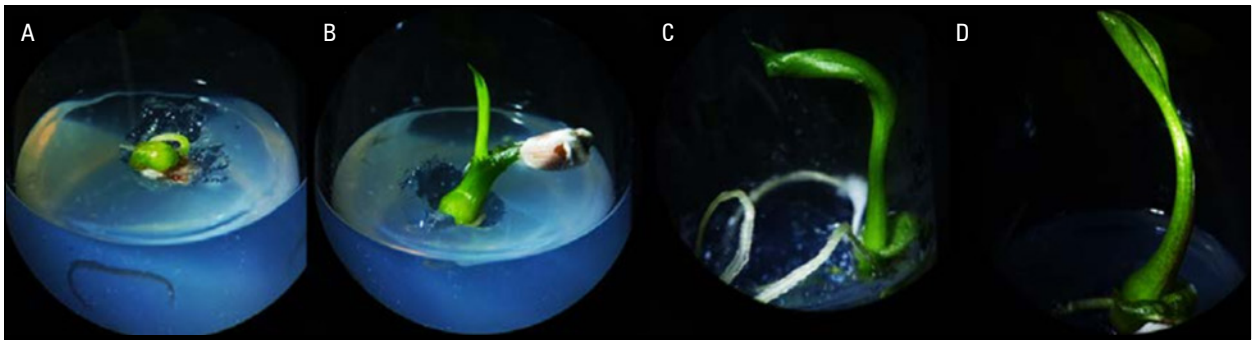

Figure 2. Growth *in vitro* of white calla lily (*Zantedeschia aethiopica* L. Spreng) seed cultured. A, development of the radicle and emergence of the plumule; B and C, elongation of the root and plumule; D., first leaf.

Figure 3. White calla lily (*Zantedeschia aethiopica* L. Spreng.) seedling obtained from embryo isolation and *in vitro* culture.

In vitro flowering

Naor *et al*. (2004a) investigated the hormonal control of floral induction and the development of inflorescence *in vitro* in day-neutral plants of *Zantedeschia* spp., as well as the effects of GA_3 and BA on the development of inflorescences in regenerated tissue culture seedlings. The seedlings were immersed in GA_3 and BA solutions before replanting in new media. GA_3 was found to be essential for the transition from the vegetative to the reproductive stage. Inflorescence development in the apical bud was observed after 30-50 d in seedlings treated with GA_3 grown *in vitro*. However, the resulting inflorescences had incomplete male and female flowers. BA did not affect flower induction but in the presence of GA_3, BA at 4.4 M enhanced inflorescence differentiation. They concluded that the floral initiation was not influenced by environmental stimuli and that the inflorescence development in the apical bud resembled natural conditions (Naor *et al*., 2004a). GA_3 had a dual action in the flowering process, inducing inflorescence differentiation and promoting floral stem elongation. They inferred that the flowering pattern might be a gradient in the distribution of GA, likely controlled by the apical dominance of the primary bud (Naor *et al*., 2004b).

Future studies

Genotype influences the growth and development of explants *in vitro*. Thus, the propagation of new varieties will need specific protocols. The evaluation of artificial light sources for illumination to improve the quality of the explants may be explored. The use of nanomaterials in modulating *in vitro Zantedeschia* tissue growth and development could also be evaluated. Additionally, organic additives in micropropagation to enhance the growth and health protection of the explants could be tested.

CONCLUSION

This review provided a historical overview of the applications of *in vitro* cultivation of *Zantedeschia* and the phytopathological issues that limit the propagation of this ornamental crop. The establishment of *Zantedeschia* in the field free from pathogens is ensured using healthy *in vitro* propagated explants. *In vitro* culture has proven to be an effective tool for rapid clonal propagation and multiplication of *Zantedeschia* spp. However, the propagation *in vitro* of *Z. aethiopica* has faced challenges in some cultivars due to the contamination of the explants.

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