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Protoplast production and isolation from *Etlingera elatior*

Jessé Marques da Silva Júnior[1], Renato Paiva[1], Ana Carolina Atala Lombelo Campos[1], Marcelo Rodrigues[1*], Milene Alves de Figueiredo Carvalho[1] and Wagner Campos Otoni[2]

*[1]Departamento de Biologia, Setor de Fisiologia Vegetal, Universidade Federal de Lavras, Cx. Postal 3037, 37200-000, Lavras, Minas Gerais, Brazil. [2]Departamento de Biologia Vegetal, Laboratório de Cultura de Tecidos, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil. *Author for correspondence. E-mail: marcel.or.7@hotmail.com*

ABSTRACT. The technique of hybridization using plant protoplasts is widely used in plant breeding programs. The purpose of our study is to further characterize the process of protoplast isolation from the ornamental species *Etlingera elatior* (Jack) R. M. Smith. Protoplasts were isolated from different tissues: *in vitro* leaves, *in vitro* pseudostem, and leaves from plants cultivated hydroponically. We tested six enzymatic combinations, four incubation time periods, the rotary system (40 rpm) or steady in the dark, and three concentrations of mannitol (0.5, 0.6 and 0.7 M). The diameter and viability of obtained protoplasts were evaluated. The best source of explants used for protoplast isolation was the *in vitro* leaves, which yielded $22x10^5$ protoplasts g^{-1} of fresh matter. The optimal incubation period was 15 hours. The *in vitro* leaves presented a greater viability (96%) and larger protoplasts (36.7 μm diameter). Greater yields were obtained using a rotatory system with protoplasts incubated in the dark. The best enzymatic combination was 3% Cellulase "Onozuca" R-10 + 2% Meicelase + 1% Driselase + 1% Dextran + 5 mM MES, followed by the addition of 0.6 M mannitol.

Keywords: FDA, ornamental plant, enzymatic combinations, incubation period.

Produção e isolamento de protoplasto de *Etlingera elatior*

RESUMO. Com o objetivo de realizar hibridações que auxiliam em programas de melhoramento genético de flores ornamentais, protoplastos foram isolados a partir de diferentes tecidos (folhas *in vitro*, pseudocaules *in vitro* e folhas em sistema hidropônico) de *Etlnigera elatior* (Jack) R. M. Smith. Foram testados seis diferentes combinações enzimáticas, quatro períodos de incubação, sistema rotatório (40 rpm) ou estacionário no escuro, concentrações de manitol (0,5; 0,6 e 0,7 M), o diâmetro e a viabilidade dos protoplastos isolados. A melhor fonte de explante utilizado no isolamento de protoplastos foi folha *in vitro*, com rendimento de 22 $x10^5$ protoplastos g^{-1} MF. O melhor tempo de incubação foi 15 horas, pois períodos superiores a este causavam diminuição no rendimento e viabilidade dos protoplastos. Protoplastos de folhas *in vitro* apresentaram viabilidade de 96% e diâmetro de 36,7 μm. Maiores rendimentos foram alcançados em sistema rotatório e no escuro. A melhor combinação enzimática utilizada no atual trabalho foi a 3% Cellulase "Onozuka" R-10 + 2% Meicelase + 1% Driselase + 1% Dextran + 5 mM MES. A melhor concentração de manitol foi de 0,6 M.

Palavras-chave: FDA, planta ornamental, combinações enzimáticas, período de incubação.

Introduction

Etlingera elatior (Jack) R. M. Smith is an ornamental plant extensively commercialized in the flower market. In Brazil, it is used as a cut flower and in landscapes of parks and stands (LAMAS, 2002). Its propagation is mainly made by *in vitro* cultivation, due to the occurrence of several diseases that affect this species. Plant breeding programs are, therefore, attempting to increase *E. elatior* pathogen resistance.

Somatic hybridization by protoplast fusion is a promising technique for breeding ornamental species and requires reliable *in vitro* protocols. Somatic hybridization can fuse two complete genomes, which is an alternative to sexual reproduction (WU et al., 2009). This technique was successfully used to breed citrus, sunflower, brassica and wheat (DAVEY et al., 2005).

Somatic hybridization offers the following possible genomic manipulations: (1) overcoming sexual incompatibility; (2) producing amphyploids; (3) transferring part of one species genome to another (cybrids); (4) transferring cytoplasmatic DNA to produce male-sterile plants; and (5) producing plants resistant to environmental stresses, pests and diseases (WU et al., 2009). In addition, the use of protoplasts have been mentioned in the study of protein subcellular localization, protein interactions, gene

expression in *Arabidopsis* (WU et al., 2009) and the production of secondary metabolites used commercially (FONTES et al., 2010).

According to Wu et al. (2009), a protoplast is a transitory state of a cell lacking its cell wall and can be obtained using pectocelulolitics enzymes. Without cell walls, protoplasts can incorporate materials such as DNA and fuse. Somatic hybrids can be obtained when protoplasts of different species are fused.

For protoplast isolation, tissues should be pre-plasmolysed with enzymatic solutions followed by washing with CPW-Cell Washing Protoplasts and 13% mannitol. The most-used enzymes for protoplast isolation are Cellulase "Onozuka" R-10 (Yakult Honsha), Macerozyme R-10 (Yakult Honsha), Cellulase Cellulysin, hemicellulase Rhozyme and pectinase Pectoyase Y-23 (DORNELAS et al., 1995). In well-established cultures, protoplasts can maintain cell totipotency; rebuilding their walls, dividing, forming callus, and regenerating plants through embryogenesis or organogenesis (WU et al., 2009). Therefore, this study further characterizes the conditions used for isolating protoplasts from *E. elatior* because different tissues (*in vitro* leaves, *in vitro* pseudostems and leaves from a hydroponic system), different combinations of enzymes, different incubation periods, using a rotating or stationary system in light or dark, and mannitol concentrations can affect the diameter and viability of isolated protoplasts.

Material and methods

Plant material

For protoplast isolation, three plant tissues were used based on their different cell wall structures: *in vitro* leaves, *in vitro* pseudostems, and leaves from plants cultivated hydroponically.

To isolate *in vitro* leaves and pseudostems, we grew seedlings in Ms medium (MURASHIGE; SKOOG, 1962) combined with the B5-Gamborg vitamin mixture. The culture medium was supplemented with 3.0 mg L^{-1} of BAP (6-benzilaminopurine, 3% (w v^{-1}) sucrose and 0.7% (w v^{-1}) of agar; Sigma Chemical Co., USA). The pH was adjusted to 5.7 ± 0.1, before autoclaving. Plants were maintained in a growth chamber for 30 to 45 days under 36 μmol m^{-2} s^{-1} of photon irradiance at 26 ± 2°C and 16 hours of light per day. Plants were grown hydroponically using a solution with 35% of its ionic strength under to isolate protoplasts from leaves.

The protoplasts' diameters from the plant material used was determined based on digital images (Canon PowerShot A710 7MP). The images were analyzed using Sigma Scan Pro 5® software. For each plant material, 200 protoplasts were evaluated, and the percentage of the protoplasts belonging to different diameter categories was determined. For this experiment we used protoplasts incubated in 9 M CPW solution.

Protoplast isolation

Under aseptic conditions, the *in vitro* leaves were sectioned parallel to the middle vein, resulting in 1 - 1.5 mm wide pieces. The pseudostems were transversally sectioned into pieces of 50 mm. For hydroponically cultivated plants, leaves were sterilized with 70% ethanol for 2 minutes, followed by 40% sodium hypochlorite for 20 minutes, and rinsed with autoclaved demi-water (5 times). The epidermis was removed from the leaves using tweezers (*peeling*) to maximize infiltration of the enzymatic solution in the tissue.

The plant material obtained was transferred to 60 x 15 mm Petri dishes containing 10 mL of CPW solution. Three mannitol concentrations were tested: 0.5 M (9 g 100 mL^{-1} CPW), 0.6 M (11 g 100 mL^{-1} CPW), and 0.7 M (13 g 100 mL^{-1} CPW). Approximately 0.5 g of plant material was pre-plasmolysed with this solution for one hour in the dark. Next, the 0.5, 0.6 and 0.7 M CPW solutions were discarded using Pasteur pipettes, followed by the addition of 10 mL of the enzymatic mixture.

Six enzyme combinations were used: A - 3% (p v^{-1}) Cellulase "Onozuka" R-10 (Yakult Honsha) + 1% (w v^{-1}) Pectolyase (Seishim Pharmaceutical., USA) + 0.5% (w v^{-1}) Driselase; B - 3% (w v^{-1}) Cellulase "Onozuka" R-10 + 1% (w v^{-1}) Pectolyase + 1% (w v^{-1}) Driselase (Sigma London Chemical Co Ltd); C – 3.75% (w v^{-1}) Cellulase "RS" (Yakult Honsha) + 1% (w v^{-1}) Driselase; D - 2% (w v^{-1}) Rhozyme HP150 (Rohm & Haas Co., USA) + 1% (w v^{-1}) Macerozyme R-10 + 0.5% (w v^{-1}) Driselase; E - 3% (w v^{-1}) Cellulase Onozuka R-10 + 2% (w v^{-1}) Meicelase (Meiji Seika Haisha Ltd., Japan) + 1% (w v^{-1}) Driselase; and F - 3% (w v^{-1}) Cellulase *Aspergillus niger* (Fluka Chemicals Ltd) + 1% (w v^{-1}) Pectinase *Aspergillus niger* (Fluka Chemicals Ltd.). The enzymatic solutions were buffered with 5 mM MES and 1% dextran followed by dilution in three mannitol concentrations (0.5, 0.6 and 0.7 M). The pH of the mannitol solutions was adjusted to 5.6.

Each plant material was incubated with the enzyme combinations for 20 hours in shaking and stationary systems at 25ºC in the dark. The effectiveness of the enzymatic solutions for protoplast isolation was monitored every 5 hours, and the number of protoplasts released was

evaluated. The experimental design was randomized, with a factorial of 6 x 3 (6 enzymatic solutions, 3 concentrations of mannitol); each replicate was constituted by a single Petri dish.

After the incubation phase, the suspension obtained (isolated protoplasts and tissues that were not digested) was filtered using a 64 μm nylon mesh (Wilson Sieves, Nottingham, UK) and centrifuged at 700 rpm for 5 minutes (3x). The precipitate was re-suspended and transferred to a new centrifuge tube, being the volume completed with the following CPW solutions with different sucrose gradients: 30, 25, 20, 21, 18, and 15S (5 mL for each solution sucrose). Finally, the suspension was centrifuged at 700 rpm for 5 minutes. The purified protoplasts, localized in the interface between the two media, were collected with a Pasteur pipette and transferred to new tubes.

The number of isolated protoplasts was determined using a Fuchs-Rosenthal-B.S. 74B hemacytometer (Weber Scientific Int. LTD., Sussex, U.K.) and an optic microscope (Hausser Scientific, USA). The viability of the protoplasts was determined based on staining with diacetate of fluorescein (FDA). For this test, a mixture of equal volumes of protoplast suspension and the FDA solution (0.01%) was incubated at room temperature for 3 to 5 minutes. The solution was observed using an inverted optic microscope 40 x (Olympus IMT 2) under UV light (with a blue filter). The viable protoplasts were indicated by a green fluorescence, and viability was defined by the percentage of observed fluorescent protoplasts (ADITYA; BAKER, 2003). The experimental design consisted of 2 replicates, each corresponding to the observation of 200 protoplasts. Data were analyzed by ANOVA and the separation of means test SNK (5%).

Results and discussion

The enzyme combinations B and E after 15 hours produced the best results for protoplast isolation and were significantly different according to the SNK test (5%) compared to other enzyme combinations. *In vitro* leaves of *E. elatior* incubated for 15 hours with shaking yielded 22.0 x 10^5 and 12.30 x 10^5 protoplasts g^{-1} of fresh tissue for the enzyme combinations E and B, respectively (Table 1 and Figure 1).

Incubation periods longer than 15 hours resulted in a decrease in yield of isolated protoplasts caused by increased membrane instability and nonselectivity of the enzymatic solution. Similar results were obtained by Costa et al. (2002) using an enzymatic solution of 1% Cellulase "Onozuca" R-10 + 0.2% Macerozyme R-10 and 0.1% Driselase, with a yield of 23.68 x 10^6 protoplasts 500 g^{-1} of callus from a variety of citrus. Previous studies with protoplast regeneration from leaf explants of *Robinia pseudoacacia* L. was obtained using an enzyme combination of 2% Celulose + 0.3% Macerozyme and incubated for 20 hours (KANWAR et al., 2009). Kanchanapooma et al. (2001) isolated protoplasts from *Dendrobium pompadour* with an enzyme mixture of 1% Cellulase "Onozuka" + 1% Macerozyme + 0.5% Driselase in a 0.4 M mannitol, which yielded 22.0 x 10^5 (light) and 21.7 x 10^5 (dark) of protoplasts g^{-1} of leaf tissue with a diameter of 50 to 80 μm.

Table 1. Efficiency of different enzyme combinations and incubation periods for *E. elatior* protoplast isolation from *in vitro* leaves (average ± standard deviation).

Incubation period (h)	Protoplasts income[a] (x10^5 g^{-1} MF)	
	B*	E **
5	0.38 ± 0.18 b	1.87 ± 0.73 a
10	3.23 ± 0.61 b	13.76 ± 1.22 a
15	12.30 ± 1.26 b	22.00 ± 2.37a
20	9.25 ± 2.45 b	19.5 ± 1.51 a

*3% (w v^{-1}) Cellulase "Onozuka" R-10 + 1% (w v^{-1}) Pectolyase + 1% (w v^{-1}) Driselase (Sigma London Chemical Co Ltd) + 0.6 M (mannitol) + 1% Dextran + 5 mM MES. **3% (w v^{-1}) Cellulase Onozuka R-10 + 2% (w v^{-1}) Meicelase (Meiji Seika Haisha Ltd., Japan) + 1% (w v^{-1}) Driselase + 0.6 M (mannitol) + 1% Dextran + 5 mM MES. [a]Number of protoplasts obtained by enzymatic digestion of 0.5 g of tissue. Average of 200 protoplasts (2 replicates). Same letters in a row indicate values that do not differ for the SNK test (5%).

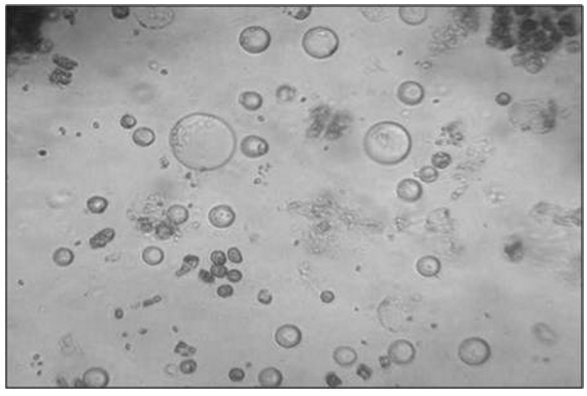

Figure 1. *E. elatior* protoplasts isolated after 15 hours of incubation in enzyme solution E (40x).

Castelblanque et al. (2010) isolated protoplasts from leaf explants of the ornamental species *Kalanchoe blossfeldiana* with the enzyme mixture of 0.4% of Cellulase "Onozuka" R-10 + 0.2% of Driselase, which yielded 6.0 x 10^5 protoplasts per gram of fresh tissue.

The isolation of protoplasts was described in several studies with the gender *Passiflora* (DORNELAS et al., 1995). Protoplast yields varied according to the species genotype and explant used.

After incubation in enzyme solution E with 0.6 M mannitol, different sources of plant materials resulted in protoplasts with different diameters. *In vitro* leaves yielded protoplasts with the greatest

average diameters (36.7 μm) followed by leaves from plants grown hydroponically (32.28 μm) and *in vitro* pseudostems (27.6 μm) (Table 2).

Table 2. Average diameter of protoplasts isolated from 3 different explants of *E. elatior*. Enzyme solution E: 3% (w v^{-1}) Cellulase "Onozuka" R-10 + 2% (w v^{-1}) Meicelase (Meiji Seika Haisha Ltd., Japan) + 1% (w v^{-1}) Driselase + 0.6 M (mannitol) + 1% Dextran + 5 mM MES. (average ± standard deviation).

Type of tissue	Medium diameter [a]
in vitro leaf	36.7 ± 6.4
Pseudostem	27.6 ± 3.4
Hydroponic system	32.28 ± 5.2

[a]Average of 200 protoplasts (2 replicates).

According to Rodríguez and Dallos (2004), using protoplasts isolated from leaf mesophyll from *Passiflora edulis* var. flavicarpa, different diameters were observed depending on the tissue. Fully expanded leaves had protoplasts of a greater average diameter (19.45 ± 0.50 µm) when compared to cotyledons (28.90 ± 0.62 µm). Oliveira et al. (1995), using two citrus species, observed a variation of 4.8 to 16.8 μm in the diameter of isolated protoplasts. Protoplast diameter is information that can be used in hybridization studies using electriofusion so that an inverse relationship exists between protoplast diameter and the voltage necessary to promote protoplast fusion.

According to Dornelas et al. (1995), the average size of protoplasts depends on the species analyzed and the explant used and varies from 19 to 47 mm, when isolated from leaf tissues, or from 30 to 60 mm, when derived from cotyledon tissues. Protoplasts obtained from the mesophyll of dicotyledons tend to be smaller than those isolated from callus or cell suspensions (OCHATT, 1993). In monocotyledons, the protoplasts size is, in general, less than 30 μm, independent of tissue source.

Two systems were used for the protoplast incubation: stationary and shaking (40 rpm) in the dark. For this experiment, *in vitro* leaves of *E. elatior* were incubated in the enzymatic solution E. A higher yield of protoplasts was obtained for leaves incubated in the shaking system for 15 hours (22.0 x 10^5 protoplasts g^{-1} of fresh tissue) (Table 3).

Expressive results were obtained by Monteiro et al. (2003) when they isolated protoplasts from the alfalfa *Medicago sativa* using the system of continuous shaking (35 rpm) in the dark.

From the different mannitol concentrations used in this study, a greater yield (19.95 x 10^5 protoplasts g^{-1} of fresh matter of protoplasts) was obtained with 0.6 M (11 g 100 mL^{-1}) mannitol in combination with enzyme solution E incubated for 15 hours; however, no differences resulted as determined by the SNK test (5%) for protoplast isolation with 20 hours of incubation (19, 21 x 10^5 protoplasts g^{-1} of fresh matter of protoplasts). In addition, these protoplasts had the highest viability percentage (96.7%; Table 5).

Table 3. The protoplast isolation efficiency from *E. elatior* for tissues incubated in the shaking (40 rpm) and stationary systems for different periods of time (average ± standard deviation). Enzymatic solution E: 3% (w v^{-1}) Cellulase Onozuka R-10 + 2% (w v^{-1}) Meicelase (Meiji Seika Haisha Ltd., Japan) + 1% (w v^{-1}) Driselase + 0,6 M (mannitol) + 1% Dextran + 5 mM MES.

Incubation period (h)	Protoplasts yield [a] (x10^5 g^{-1} MF) ST★	SH★★
5	4.53 ± 0.32 a	4.87 ±0.73 a
10	15.20 ± 0.48 a	15.75 ± 1.22 a
15	20.30 ± 0.26 b	22.00 ± 1.37 a
20	19.25 ± 0.45 a	19.74 ± 0.51 a

★Stationary (dark) ★★Shaking (40 rpm in the dark). [a]Number of protoplasts obtained from the enzymatic digestion of 1 g of tissue. Average of 200 protoplasts (2 replicates). Same letters in a row indicate values that do not differ for the SNK test (5%).

Table 4. Protoplast isolation efficiency from *E. elatior* for tissues incubated in enzyme solution E with different concentrations of mannitol for different incubation periods (average ± standard deviation).

Incubation period (h)	Protoplasts yield [a] (x10^5 g^{-1} MF) 0.5★	0.6★★	0.7★★★
5	1.38 ± 0.18 b	4.29 ± 1.73 a	4.24 ± 2.75 a
10	5.21 ± 0.61b	13.76 ± 3.22 a	12.2 ± 3.12 a
15	12.30 ± 1.26c	19.95 ± 3.37a	16.45 ± 3.75b
20	15.65 ± 2.45c	19.21 ± 2.51a	17.33 ± 1.75 b

★Mannitol 0.5 M (9 g 100 mL^{-1} CPW). ★★Mannitol 0.6 M (11 g 100 mL^{-1} CPW). ★★★Mannitol 0.7 M (13 g 100 mL^{-1} CPW). [a]Number of protoplasts obtained from the enzymatic digestion of 1 g of tissue. Average of 2 replicates. E: 3% (w v^{-1}) Cellulase "Onozuka" R-10 + 2% (w v^{-1}) Meicelase (Meiji Seika Haisha Ltd., Japan) + 1% (w v^{-1}) Driselase + 0.6 M (mannitol) + 1% Dextran + 5 mM MES. Average of 200 protoplasts (2 replicates). Same letters in a row indicate values that do not differ for the SNK test (5%).

The CPW 13 solution is commonly used for pre-plasmolysis, dissolution of enzymes and protoplast washing of tissues from *Passiflora*. This solution is composed of CPW medium salts and 13% mannitol (DORNELAS et al., 1995).

The highest viability percentages were obtained for protoplasts incubated with the enzyme solution E and purified with 0.6 or 0.5 M mannitol, with 96.7 and 81.8% of viable protoplasts, respectively (Table 5 and Figure 2).

Table 5. Viability of *E elatior* protoplasts isolated from *in vitro* leaves using three concentrations of mannitol after 15 hours of isolation in enzyme solution E: 3% (w v^{-1}) Cellulase "Onozuka" R-10 + 2% (w v^{-1}) Meicelase (Meiji Seika Haisha Ltd., Japan) + 1% (w v^{-1}) Driselase + 0.6 M (mannitol) + 1% Dextran + 5 mM MES. (average ± standard deviation).

Mannitol	Viability[b]
0.5 M	81.8 ± 2.7 b
0.6 M	96.7 ± 3.9 a
0.7 M	30.2 ± 1.2 c

[b]Percentage of protoplasts with green fluorescence compared to total protoplasts. Average of 200 protoplasts (2 replicates). Same letters in a row indicate values that do not differ for the SNK test (5%).

The SNK test (5%) showed that these values differed from the average value for the highest concentration of mannitol (0.7 M) with (30.2%) viability of protoplasts.

Previous studies with protoplasts isolated from *Dendrobiun pompadour* using three concentrations of mannitol (0.4, 0.5 and 0.6 M) revealed that 0.4 M yielded 19.89 x 10^5 protoplasts, which was greater than from the other concentrations tested, 13.59 x 10^5 and 6.95 x 10^5, respectively (3-hour incubation).

Greater values for protoplasts viability (89%) were observed when 0.4 M mannitol solution was used (KANCHANAPOOMA et al., 2001).

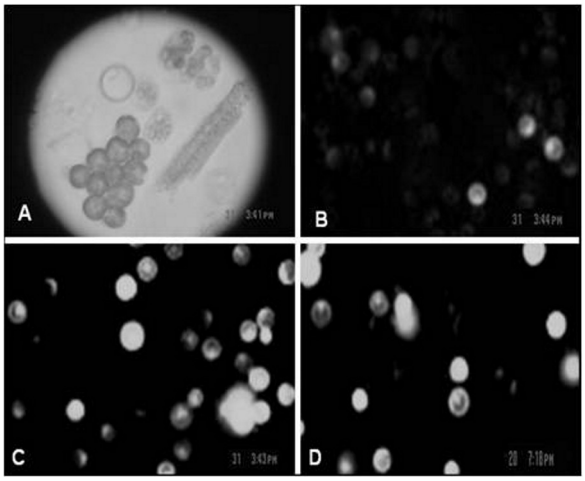

Figure 2. (A) Protoplasts recently isolated. (B) Viability test using 0.7 M mannitol. (C) Viability test using 0.6 M mannitol. (D) Viability test using 0.5 M mannitol. Enzyme solution E: 3% (w v^{-1}) Cellulase "Onozuka" R-10 + 2% (w v^{-1}) Meicelase (Meiji Seika Haisha Ltd., Japan) + 1% (w v^{-1}) Driselase + 0.6 M (mannitol) + 1% Dextran + 5 mM MES.

Evaluating viability after isolation is important for determining the plating density to use for protoplast cultivation, which influences cell division and differentiation.

Conclusion

The optimal condition to isolate *E. elatior* protoplasts is enzyme solution E composed of 3% Cellulase "Onozuca" R-10, 2% Meicelase, 1% Driselase, 1% Dextran and 5 mM MES combined with 0.6 M mannitol and incubated for 15 hours with a shaking system (40 rpm) in the dark.

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