

Full Length Research Paper

Efficient micropropagation of *Epidendrum secundum* Jacq. from leaves and protocorms

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An efficient method for the large-scale propagation of *Epidendrum secundum* using protocorms and leaves from *in vitro* germination was established. Explants were inoculated in Woody Plant Medium (WPM) supplemented with 6-benzyladenine (BA) (1.0–40.0 μM) for 60 days and cultured in a growth room in darkness (30 days) or with a 16 h photoperiod. Protocorms were most effective for regenerating protocorm-like bodies (PLBs; 100.0%), independent of the presence or absence of light. The leaves were less responsive, and the best results were obtained with the youngest leaves (58.0% PLB induction). BA influenced the responses of protocorms and young leaves, and medium supplemented with 1.0 μM BA was most effective for inducing and regenerating PLBs. The shoots were rooted (100.0%) in WPM without any growth regulator and were transplanted to Isopor trays containing Plantmax florestal[®] and vermiculite (2:1), with 100.0% survival after 60 days. A histological analysis demonstrated that for both the explants, leaves and protocorms, PLBs were formed from successive divisions of epidermal cells. This simple protocol will be useful for the large-scale propagation of *E. secundum*.

Key words: Orchidaceae, epiphytic orchid, *in vitro* propagation, micropropagation.

INTRODUCTION

Epidendrum secundum Jacq. is a species belonging to the Orchidaceae. The species is epiphytic, terrestrial, or rupicolous, and it flowers throughout the year (Stancik et al., 2009). This species is distributed in areas of the Atlantic Forest that due to continuous destruction of natural habitats, unauthorized trade and ruthless collection by orchid lovers; many orchid species in nature are disappearing at an alarming rate (Hossain, 2008).

The seeds of these plants have great difficulty in germinating because they generally have no food reserves, require a symbiotic association with mycorrhizal fungi, and grow slowly (Dressler, 1981). Furthermore, conventional propagation of this plant is hindered by its slow growth and lack of production of sufficient numbers of clones in a short period of time (Martin and Madassery, 2006). Tissue culture techniques have been used for the

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Abbreviations: BA, 6-Benzyladenine; PLBs, protocorm-like bodies; WPM, woody plant medium.

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propagation of different species of terrestrial and epiphytic orchids (Díaz and Alvarez, 2009).

The formation of protocorms from *in vitro*-germinated seeds and the subsequent induction of protocorm-like bodies (PLBs) from protocorms, nodal segments, leaf apices, leaves, root apices, and other explants has become a reliable method for the propagation of orchids (Ng and Saleh, 2011). Foliar explants represent an effective alternative for micropropagating orchids because they are easy to obtain, they do not require the sacrifice of the mother plant, and they offer opportunities to raise large numbers of true-to-type plants (Murthy and Pyati, 2001). The use of leaf explants to induce PLBs has been reported for *Dendrobium* hybrids (Martin and Madassery, 2006), *Aranda* × *Vanda coerulea* hybrids (Gantait and Sinniah, 2012), and *Renanthera imschootiana* (Wu et al., 2012). Propagation using protocorms has been studied for *Aerides crispum* (Sheelavanthmath et al., 2005), *Phalaenopsis gigantea* (Murdad et al., 2006), and *Esmeralda clarkei* (Paudel and Bijaya, 2012).

Cytokinins and auxins are required for shoot induction and development. 6-Benzyladenine (cytokinin) has been reported to be effective in promoting the regeneration of a number of orchid species (Nayak et al., 2002; Martin and Madassery, 2006; Naing et al., 2011). Plant growth regulator-free media have been used for the conversion of PLBs into complete plantlets (Murthy and Pyati, 2001). In addition to growth regulators, the photoperiod influences the formation of PLBs of some orchid species, such as *Oncidium flexuosum* (Mayer et al., 2010). Thus, the goal of this study was to test different explants at different ages and assess the influence of BA and the photoperiod on the formation of PLBs of *E. secundum* to establish an *in vitro* propagation protocol.

MATERIALS AND METHODS

Plant materials, surface sterilization, and culture conditions

Hand pollination of the *E. secundum* flowers was performed at the Botanical Institute of São Paulo, Brazil (São Paulo, SP, plant numbers 17,480 and 17,672). Fruit harvesting began six months after pollination, and seeds were removed from mature capsules, immersed in 0.75% (v/v) sodium hypochlorite plus 0.1% Tween 20[®] for 10 min and rinsed four times with sterile, distilled water. Seeds were dried on filter paper and then inoculated in Petri dishes (150 mm in diameter and 20 mm in height) containing 40 ml of Woody Plant Medium (WPM) (Lloyd and McCown, 1980) supplemented with 3% sucrose (w/v) and 5.6 g L⁻¹ agar Himedia[®]. pH was adjusted to 5.8 with 0.1 N NaOH or HCl before the addition of agar. Medium was autoclaved at 120°C for 20 min. The cultures were maintained in a growth room with a temperature of 26 ± 2°C/18 ± 2°C (day/night), under a 16 h photoperiod provided using white fluorescent tubes at an intensity of 40 μmol m⁻² s⁻¹.

The experiments were performed with the protocorms and leaves obtained from *in vitro* germination of seeds. Leaves (0.3 and 1.0 cm in length) from two and six-month old, respectively, and protocorms from two-month old were used as plant material. Leaves were placed in Petri dishes with the abaxial surface in contact with the

culture medium and protocorms had their leaf apices completely removed, and they were placed (cut side up) in Petri dishes.

Induction of protocorm-like bodies (PLBs) from leaves and protocorms

The explants were cultured in WPM supplemented with 3% (w/v) sucrose, 100 mg L⁻¹ (w/v) myoinositol and gelled using agar (5.6 g L⁻¹) Vetec[®]. The medium was supplemented with 1.0, 2.5, 5.0, 10.0, 20.0, or 40.0 μM 6-benzyladenine (BA), in addition to a control without growth regulators. Cultures were maintained for 60 days with a photoperiod of 16 h or for 30 days in the dark followed by 30 days with a 16 h photoperiod in a growth chamber at a temperature of 26 ± 2°C/18 ± 2°C (day/night) and a light intensity of 40 μmol m⁻² s⁻¹. After 60 days, individual shoots were transferred to WPM without growth regulators for rooting and plant growth.

Acclimatization of regenerated plants

After 120 days of culture, plantlets (1.5 cm long) with two or three expanded leaves were transplanted into Isopor trays containing Plantmax florestal[®] and vermiculite (2:1). The plants were maintained in a greenhouse at room temperature (25 ± 3°C), and they were manually irrigated every three days. After 60 days, the percent survival was recorded.

Experimental design and statistical analysis

The experimental design was completely randomized, with a 2 × 2 factorial scheme (age of the explants and photoperiod), and each type of explant was evaluated separately for treatment with BA and the photoperiod (7 × 2). The treatments consisted of five Petri dishes containing 10 explants each. After 60 days, the percentage of explants forming PLBs and the average number of PLBs regenerated per explant were evaluated. Means were transformed by the square root of x + 0.5 when necessary, and analysis of variance and comparison of means by the Tukey's test were performed using the SISVAR[®] program.

Histological analysis

The leaves and protocorms at various stages of PLB formation were fixed in Karnovsky's fixative solution (Karnovsky, 1965) at room temperature for 24 h. The samples were dehydrated in an alcohol series and were subsequently infiltrated and polymerised using hydroxyethylmethacrylate (Histo-resin, Leica[®]) according to the manufacturer's instructions. The cross sections of 8 μm in thickness were cut using a steel blade in a rotary microtome and stained with toluidine blue (O'Brien et al., 1964). The stained slides were mounted with Permout[®] and analyzed on a digital equipment connected to a Zeiss microscope[®].

RESULTS

Induction of protocorm-like bodies (PLBs) from leaves and protocorms

The PLBs regeneration was observed for all explants tested, and the best results occurred when protocorms were used, followed by younger and older leaves (Table 1). The protocorms exhibited high regenerative potential

Table 1. Effect of the explant type and photoperiod of *Epidendrum secundum* on the regeneration of protocorm-like body (PLB) after 60 days of culture in Woody Plant Medium (WPM), containing different concentrations of 6-benzyladenine (BA)

Explant	PLB regeneration (%)			Average number of PLBs per responsive explants		
	Light ^a	Dark/light ^a	Mean ^b	Light	Dark/light	Mean
2-month-old leaf	56.8	59.2	58.0b	1.7	1.6	1.7b
6-month-old leaf	26.6	20.4	23.5c	1.5	1.3	1.4b
2-month-old protocorm	97.7	96.0	96.9a	3.8	3.0	3.4a
Mean ^b	60.4 A	58.5A		2.3A	2.0B	

^aLight: 16-h photoperiod for 60 days. Dark/light: dark for 30 days, followed by a 16-h photoperiod for 30 days. ^bMeans within a column followed by the same lowercase letter and means on the same line followed by the same upper case letter do not differ significantly according to Tukey's test ($P \leq 0.05$).

by inducing direct PLB formation in the two light regimes to which they were submitted. The results indicated that younger leaves (2-month-old) were more responsive than the older (6-month-old) leaves. For the average number of PLBs per explant, protocorms were more effective than leaves independent of age ($P \leq 0.05$) (Table 1).

The frequency of PLB regeneration and the average number of PLBs regenerated from the leaves (2-month-old) of *E. secundum* were not influenced by the light regime. However, the concentration of 6-benzyladenine (BA) influenced the responses of explants; leaves cultured in medium supplemented with 1.0-5.0 μM BA regenerated more PLBs than the control medium ($P \leq 0.05$; Figure 1a). However, the average numbers of PLBs per explant were similar between the control explants and those exposed to the highest concentration of BA (40.0 μM) (Table 2).

The photoperiod did not influence PLB regeneration from leaves (6-month-old; Table 3). Regarding the percentage of explants that regenerated PLBs, explants cultured in medium containing 2.5 μM BA were more responsive than those cultured in the presence of other BA concentrations as well as the control ($P \leq 0.05$). Therefore, there was no difference in the average number of PLBs between the control and different concentrations of BA. Under a 16 h photoperiod, the optimal concentration of BA for PLB induction and regeneration was 2.5 μM , as 46% of explants were responsive with an average of 1.7 PLBs produced per explant (Table 3).

Protocorms lacking the leaf apex were more responsive than leaves, independent of the light regime. The percentage of explants regenerating PLBs was high (Table 4), with the lowest percentage obtained in the presence of 40.0 μM BA, which was statistically lower than that observed for the other concentrations under a 16 h photoperiod. The average number of PLBs per explant was highest in the presence of 2.5 and 5.0 μM BA, as well as for the control. Due to the response in the presence of light, the optimal and most economical treatment was growth in the presence of 1.0 μM BA

under a 16 h photoperiod, with all explants regenerating PLBs and an average of 4.4 PLBs produced per explant (Figures 1b and c and Table 4).

According to the results, we can estimate the production of new plants from the established protocol of *E. secundum*. Initially, 1000 seeds germinated in WPM, and 1000 seedlings were produced after 60 days. Then, protocorms lacking leaf apices were cultured in WPM containing 1.0 μM BA using a 16 h photoperiod for 60 days, and all protocorms regenerated PLBs at an average of 4.4 per explant. The 1000 leaves (2-month-old) withdrawn from protocorms were placed in WPM containing the same concentration of BA, first in the dark for 30 days followed by 30 days of incubation with a 16 h photoperiod, resulting in PLB regeneration by approximately 70.4% of the explants at an average of 2.0 PLBs per explant. Then, individual PLBs were transferred to WPM lacking a growth regulator for elongation and rooting. After 60 days, the plants were acclimatized in forest Plantmax[®] substrate and vermiculite (1:2) with 100% survival in the greenhouse, yielding 5808 plants after 4 months.

Histological analysis

Direct PLB regeneration was obtained from protocorms and leaf explants. Histological observations revealed that the PLBs originated from the epidermis and subjacent layers of the epidermis (Figure 1d). In the regeneration of PLBs, globular structures formed first followed by the normal development of protocorms of this species, including the differentiation of the leaf apex and the formation of the first leaf. Meanwhile, protocorms lacking leaf apices were induced to form PLBs, displaying meristematic centers and numerous cells throughout the vascular structure (Figure 1e).

DISCUSSION

In this study, protocorm and leaf explants were able to

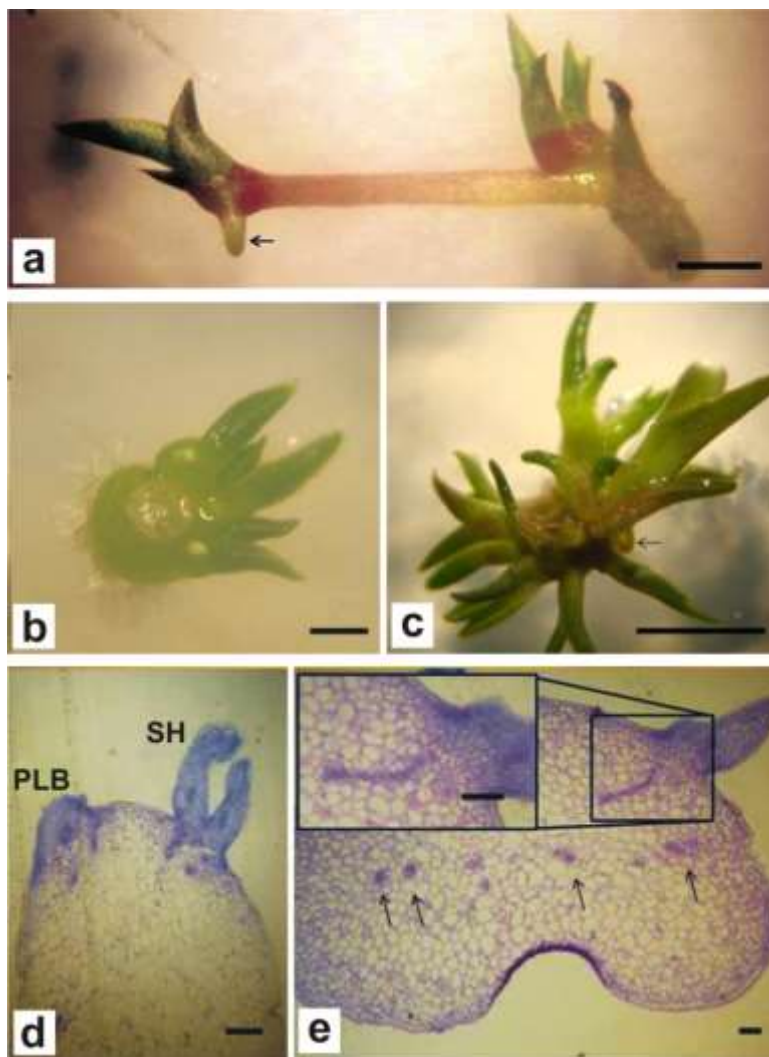


Figure 1. Protocorm-like body (PLB) formation from the leaves and protocorms of *Epidendrum secundum*: **a**, PLBs regenerated from leaves cultured in WPM supplemented with 6-benzyladenine (BA), including the start of root growth (arrow), after 60 days (bar = 1.0 mm). **b**, PLBs from protocorms after 30 days of culture in WPM containing BA (bar = 1.0 mm). **c**, PLBs developing from protocorms cultured in WPM medium containing BA. The start of root growth (arrow), was noted after 60 days (bar = 10.0 mm). **d**, Direct induction of PLBs and shoots from protocorms (bar = 0.2 mm). **e**, Protocorms exhibiting formation of PLBs and procambium (arrows) (bar = 0.1 mm): PLB, protocorm-like body; SH, shoot.

Table 2. Effect of 6-benzyladenine (BA) and the photoperiod on the regeneration of protocorm-like body (PLB) from the 2-month-old leaves of *Epidendrum secundum* after 60 days of culture in Woody Plant Medium (WPM)

BA (μM)	PLB regeneration (%)			Average number of PLBs per responsive explants		
	Light ^a	Dark/Light ^a	Mean ^b	Light	Dark/light	Mean
0.0	48.8	33.0	40.9b	1.3	1.0	1.2c
1.0	69.8	70.4	70.1a	1.8	2.0	1.9ab
2.5	62.8	74.0	68.4a	2.0	1.9	2.0ab
5.0	61.4	79.0	70.2a	2.1	1.9	2.0a

Table 2. Contd.

10.0	59.4	44.0	51.7ab	2.0	2.0	2.0a
20.0	54.0	68.0	61.0ab	1.6	1.6	1.6abc
40.0	41.4	46.0	43.7ab	1.3	1.0	1.2bc
Mean ^b	56.8A	59.2A	-	1.7 A	1.6A	-

^aLight: 16-h photoperiod for 60 days. Dark/light: dark for 30 days, followed by a 16-h photoperiod for 30 days. ^bMeans within a column followed by the same lowercase letter and means on the same line followed by the same upper case letter do not differ significantly according to Tukey's test ($P \leq 0.05$).

Table 3. Effect of 6-benzyladenine (BA) and the photoperiod on the regeneration of protocorm-like body (PLB) from the 6-month-old leaves of *Epidendrum secundum* cultured in Woody Plant Medium (WPM) for 60 days.

BA (μM)	PLB regeneration (%)			Average number of PLBs per responsive explants		
	Light ^a	Dark/light ^a	Mean ^b	Light	Dark /light	Mean
0.0	20.0	17.0	18.5b	1.8	1.6	1.7a
1.0	33.0	21.0	27.0b	1.8	1.5	1.7a
2.5	46.0	38.0	42.0a	1.8	1.6	1.7a
5.0	25.0	19.0	22.0b	2.3	1.6	2.0a
10.0	22.0	14.5	18.3b	1.2	1.2	1.2a
20.0	20.0	14.0	17.0b	1.4	1.1	1.3a
40.0	19.5	12.5	16.0b	1.3	1.0	1.2a
Mean ^b	26.5A	19.4A		1.7A	1.4A	

^aLight: 16-h photoperiod for 60 days. Dark/light: dark for 30 days, followed by a 16-h photoperiod for 30 days. ^bMeans within a column followed by the same lowercase letter and means on the same line followed by the same upper case letter do not differ significantly according to Tukey's test ($P \leq 0.05$).

Table 4. Effect of 6-benzyladenine (BA) and the photoperiod on the regeneration of protocorm-like body (PLB) from 2-month-old protocorms of *Epidendrum secundum* cultured in Woody Plant Medium (WPM) for 60 days

BA (μM)	PLBs regeneration (%)			Average number of PLBs per responsive explants		
	Light ^a	Dark/light ^a	Mean ^b	Light	Dark/light	Mean
0.0	92.0	98.0	95.0a	2.3	2.1	2.2c
1.0	100.0	100.0	100.0a	4.4	2.9	3.7b
2.5	100.0	100.0	100.0a	4.4	3.5	4.0ab
5.0	100.0	100.0	100.0a	5.2	4.3	4.8a
10.0	100.0	96.0	98.0a	3.9	3.1	3.5b
20.0	100.0	96.0	98.0a	3.4	2.9	3.2bc
40.0	92.0	82.0	87.0b	3.3	2.5	2.9bc
Mean ^b	97.7A	96.0A		3.8A	3.0B	

^aLight: 16-h photoperiod for 60 days. Dark/light: dark for 30 days, followed by a 16-h photoperiod for 30 days. ^bMeans within a column followed by the same lowercase letter and means on the same line followed by the same upper case letter do not differ significantly according to Tukey's test ($P \leq 0.05$).

regenerate protocorm-like bodies (PLBs) directly in woody plant medium (WPM) supplemented with BA. The type of explant and BA concentration influenced the

responses concerning the induction and regeneration of PLBs. The explants with the best responses were protocorms lacking leaf apices. They responded readily in

WPM irrespective of the presence growth regulators, as also reported for *Esmeralda clarkei* (Paudel and Bijaya, 2012). The protocorms are the result of the germination of orchids, and they are composed of parenchymal cells surrounded by epidermis. As noted in the anatomy of the seedling growth of *E. secundum*, the structure of the protocorm is meristematic, especially the upper pole of the protocorm, which originates the leaf apex. Meristematic parts are rich in auxins and consist of cells with high metabolic activity; the removal of the leaf apex overcome the apical dominance, which combined with the addition of a cytokinin in the culture medium promoted the regeneration of PLBs. Similar results were also obtained for a hybrid *Cymbidium*, for which the best explants for PLB regeneration were also protocorms lacking the apical region as compared with protocorms cut in half and thin-layer cells (Silva and Tanaka, 2006).

The results indicate that younger leaves (2-month-old) of *E. secundum* were more responsive, generating nearly two-fold more PLBs than the older leaves (6-month-old). The initiation of meristematic activity in foliar explants is directly related to the juvenility of the donor tissues besides the chemical stimulus to which they are subjected (Kaur and Buthani, 2009). According to Chugh et al. (2009), several studies illustrated this same variation in the competence of regeneration, which is the frequency of response and the number of PLBs regenerated by leaf explants. Similar responses were also obtained by Wu et al. (2012), in which PLB formation occurred only in juvenile leaves of *Renanthera imschootiana*. Murthi and Pyati (2001) also reported that the explants from mature leaves of *Aerides maculosum* did not respond to any culture medium, while proliferation occurred in those from juvenile leaves, which developed PLBs. The differential responses of the explants from mature and juvenile leaves under identical nutritional conditions seem to indicate the importance of their source and physiological age of the explant.

The absence of a growth regulator had little effect on the induction of PLB regeneration, and the highest concentration of cytokinin exerted an inhibitory effect on the leaf explants of *E. secundum*, as was also reported by Gantait and Sinniah (2012). The addition of a cytokinin to the medium was essential to induce PLB regeneration from *E. secundum* leaf explants. This was also observed for the leaf explants of *Dendrobium* hybrids (Martin and Madassery, 2006), and *A. maculosum* (Murthy and Pyati, 2001). The addition of a low concentration of BA (1.0 μM) to WPM was recommended to induce regeneration of PLBs by 70.0% of explants, with an average of two PLBs produced per leaf explant of *E. secundum*. In the study by Gow et al. (2009) using the younger leaves of *Phalaenopsis amabilis* cultivated *in vitro*, 25% of explants formed PLBs, and the average number of PLBs formed per explant was 3.4 in MS/2 medium containing 13.3 μM BA after 60 days of induction. This study corroborates the results obtained for *E. secundum*, in which PLBs formed

from the leaves used as explants. However, the regeneration of the PLBs of *E. secundum* was superior to the results obtained for the aforementioned species, as 70.4% of explants formed PLBs in the presence of 1.0 μM BA.

The presence of light appeared to influence the induction of PLBs in the first weeks of growth for the explants of *E. secundum*. Similar findings occurred with the foliar explants of *Phalaenopsis bellina* (Khoddamzadeh et al., 2011). The optimal light condition for the leaves and protocorms of *E. secundum* was a 16 h photoperiod. Mayer et al. (2010) observed different result for the leaves of *Oncidium flexuosum*, in which the effect of light on the direct regeneration of PLBs was decisive. In the presence of constant light, the regeneration rate was extremely low, and the average number of PLBs was lower than that produced by explants grown in the dark. Different results were also found by Gow et al. (2009), finding that the light regime influenced the responses of the explants of *P. amabilis* and *P. nebula*. In the absence of light, there was a higher percentage of responsive explants.

Histological observations using thin sections revealed direct PLB regeneration from both protocorm and leaf explants without mediation of the callus, and PLBs developed from the epidermal cell layers of *E. secundum* explants and converted into plantlets through sequential organogenesis. Similar responses were observed for the protocorm sections of *Aerides crispum* (Sheelavanthmath et al., 2005) and *Doritaenopsis* hybrids, for which PLBs developed directly from the subepidermal layers near the wounded region of leaf sections (Park et al., 2002). The PLBs consisted of multiple meristematic centers that gradually differentiated into shoots, leaves, and then plantlets, as also described for the hybrid orchid *Aranda x Vanda coerulea* (Gantait and Sinniah, 2012).

Conclusions

On the basis of this study reported, it can be concluded that an efficient protocol for the direct regeneration of *E. secundum* using protocorms and young leaves (*in vitro* germination at 2 months of age) was established. For inducing and regenerating PLBs, it is recommended to add 1.0 μM BA to WPM. Rooting and plant growth also occurred in medium lacking a growth regulator. The plants were acclimatized (100% survival) with the substrate forest Plantmax[®] and vermiculite (2:1). This protocol is simple and easy to utilize, and it can provide large numbers of plants for mass propagation in a short period. This efficient and reliable protocol could be useful for the mass multiplication and germoplasm conservation of this orchid.

Conflict of interests

The author(s) did not declare any conflict of interest.

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