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An efficient shoot regeneration system for medicinally important *Elephantopus scaber* Linn.

Jyothi Abraham^{1,2} and T. Dennis Thomas²

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Abstract – An efficient protocol for the rapid micropropagation of medicinally important *Elephantopus scaber* has been standardized using cotyledonary node explants. Direct multiple shoot induction was observed when the cotyledonary node explants at various age groups were cultured on MS medium supplemented with various plant growth regulators. The highest shoot induction was obtained when the cotyledonary node explants from 20-day-old seedlings were cultured on MS medium supplemented with 1.5 mg L⁻¹ TDZ and 0.5 mg L⁻¹ NAA. On this medium, 98% of the cultures responded, with an average number of 33.7 shoots per explant. The highest frequency of rooting (100%) and mean number of roots (3.3 per shoot) were observed when the shoots were transferred to MS medium supplemented with 1.0 mg L⁻¹ IBA. The plantlets raised in vitro were acclimatized and transferred to soil with a 92% success rate. The protocol described here may be utilized for multiplication and conservation of elite clones of *E. scaber*.

Key words: Acclimatization, cotyledonary node, *Elephantopus scaber*, multiple shoots, rooting.

INTRODUCTION

Elephantopus scaber L., popularly known as “Elephant’s foot”, is a valuable medicinal herb belonging to the family Asteraceae. It is distributed in the tropical regions of Southeast Asia and Latin America (Cabrera and Klein 1980). The plant is characterized by a forked, stiff stem, with a purple inflorescence (flower head). *E. scaber* has been used in traditional medicine to treat various diseases in several countries (for a review see Hiradev and Rangari 2014). It is used against cardiovascular diseases, bronchitis, and smallpox (Kiritikar and Basu 1991). The healing properties of the roots of this plant are useful for heart and liver problems, and a hot water extract of the roots is used in combating filariasis and diarrhea (Hammer and Johns 1993). The fresh roots are used in the treatment of leucorrhoea, metrorrhagia, menorrhagia, and dysmenorrhoea (Behera and Misra 2005). The root part is used to cure headaches, a leaf paste to treat menorrhagia and paste from the entire plant applied externally to cure tetanus (Rajakumar and Shivanna 2010). Furthermore, the roots are used externally as an anti-venom, as an antiseptic for cuts and wounds, and on lesions for chicken pox. It is also used against dysentery, diarrhea, stomach troubles, and tuberculosis (Hammer and Johns 1993).

The medicinal properties of *E. scaber* are due to its phytochemical components, and several important constituents have been isolated from various parts of the plant. These important phytochemicals include deoxyelephantopin, isodeoxyelephantopin, scabertopin, elescaberin, scabertopinol, hexadecanoic acid, octadecadienoic acid, n-tetradecane, tetramethyl hexadecanol, isopropyl dimethyl tetrahydro-naphthalenol, phytol, stigmasterol, tricin, lupeol, betulinic acid, and luteolin (Chang et al. 2011, Geetha et al. 2012). The compounds deoxyelephantopin and isodeoxyelephantopin isolated from *E. scaber* exhibited strong anticancer properties (Geetha et al. 2012, Lee and Shyur 2012). Destructive harvesting of this plant for medicinal purposes, lack of knowledge of proper cultivation methods, habitat destruction, and deforestation have resulted in rapid depletion of the natural stock of this plant. Its natural propagation is through seeds, but low rate of seed set, poor seed viability, and early seedling death are the major hurdles for its natural propagation (Parashurama et al. 2013).

With the advent of plant tissue culturing and associated biotechniques, supplementary conservation strategies have now been employed for protecting valuable germplasm. In vitro propagation, an ex situ conservation strategy, contrib-

¹ Research and development center, Bharathiar University, Maruthamalai Road, Coimbatore, Tamil Nadu PIN-641046, India

² Postgraduate and Research Department of Botany, St. Thomas College, Palai, Arunapuram (P.O), Kottayam, Kerala 686 574, India. *E-Mail: den_thuruthiyil@yahoo.com

utes to new means for conservation and mass propagation of economically important plants. In addition, in vitro plant systems, combined with other complementary strategies, serve as an alternative approach for effective and sustainable conservation. In vitro micropropagation for commercial production and ex situ conservation has been successfully applied to various plant species (Rodrigues et al. 2015, Cheruvathur et al. 2013, Cheruvathur and Thomas 2014). In vitro propagation and conservation of several Asteraceae species, including endangered and threatened medicinal plants, have been reported. These species include *Eclipta alba* (Baskaran and Jayabalan 2005) and *Crepis novoana* (Corral et al. 2011). There are some preliminary studies on micropropagation of *E. scaber* using seed- and leaf-derived callus organogenesis (Jagadeesh Singh et al. 2004, Rout and Sahoo 2013). However, direct shoot regeneration in *E. scaber* has not been reported. The aim of the present study was to establish a rapid in vitro regeneration system through multiple shoot induction using cotyledonary node explants. This study is useful because it provides a reliable source of *E. scaber* plants to replenish declining populations in the wild and to be used for biocompound extraction, reducing pressure on wild stocks.

MATERIAL AND METHODS

Plant material and culture medium

The mature seeds of *E. scaber* were collected from field grown plants and surface sterilized with 3% (v/v) sodium hypochlorite solution for 10 min, followed by three rinses in autoclaved double-distilled water. The seeds were then transferred to a sterile Petri plate with sterile filter paper (Whatman no. 1) soaked with sterile MS basal medium (Murashige and Skoog 1962). The seeds (about 50-70 seeds/Petri plate) were placed on the filter paper under aseptic conditions in a laminar air flow chamber and incubated for germination in the culture room. The cotyledonary node explants (which included 0.5-1.5 mm epicotyl and hypocotyl regions with 2 attached cotyledons), after removing the radicle, were excised from 15-, 20-, and 25-day-old seedlings maintained in vitro and cultured vertically in 25×150 mm test tubes containing MS medium supplemented with various concentrations of 6-benzylaminopurine (BA; 0.5-3.0 mg L⁻¹) and thidiazuron (TDZ; 0.5-3.0 mg L⁻¹) alone or in combination with naphthalene acetic acid (NAA; 0.3-1.0 mg L⁻¹).

Multiple shoot induction, rooting, and transfer to soil

The response was expressed in terms of per cent response,

mean number of shoots, and mean shoot length, and data was recorded 45 d after culturing. Induced multiple shoots were taken out of the culture and each shoot was isolated and cultured on MS medium supplemented with 0.5 mg L⁻¹ BA for shoot elongation. The shoots with a size greater than 2.5 cm from the primary culture as well as from the elongation medium were used for rooting experiments. The shoots were cultured on half-strength MS medium with the addition of different concentrations of NAA (0.5-2.0 mg L⁻¹), IBA (0.5-2.0 mg L⁻¹), or IAA (0.5-2.0 mg L⁻¹) for rooting. Percentage of shoots rooted, mean number of roots, and mean root length were recorded 45 d after culturing.

For hardening, the regenerated shoots with vigorous root systems were transferred to small plastic cups (6 cm diameter) containing a sterile sand:soil (1:1) mixture and placed in a greenhouse under high humidity (90%). The plantlets were irrigated initially with sterile MS basal medium for 14 d, followed by tap water. Initially the plants were kept under reduced light (20 μmol m⁻² s⁻¹) intensity and slowly shifted to comparatively high light intensities (40 μmol m⁻² s⁻¹). After 2 months, the plants were moved out of the greenhouse and placed in shade (approximately 60% shade) under natural conditions. The acclimatized plants were transferred to an outdoor environment after three months.

Culture conditions

All media were supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, and the pH was adjusted to 5.8 with 1.0 N HCl or 1.0 N NaOH. All culture media were autoclaved at 121 °C for 20 min. All the cultures were incubated in a culture room at 25 ± 2 °C under a 16-h photoperiod with a photosynthetic photon flux density of 60 μmol m⁻² s⁻¹ supplied by Philips TL 40W fluorescent tubes. Each treatment consisted of 20 tubes with one explant each, and all experiments were repeated 3 times. The data were presented as mean values and their standard deviations (mean ± SD) and comparisons of mean values were carried out through Duncan's multiple range test (DMRT; Duncan 1995) at 0.05% significance.

RESULTS AND DISCUSSION

Seed germination and cotyledonary node culture

The seeds of *E. scaber* are comparatively small and dark brown in colour. The seeds were stored at 4 °C in a refrigerator. The seeds cultured in Petri plates lined with filter paper and soaked with MS medium showed initial signs of germination 3-4 days after culturing. The seeds increased in size and started germination one week after culturing. Fifty per cent of cultured seeds germinated after

30 d when fresh seeds were used (Figure 1A). However, the germination rate was low when stored seeds were used, suggesting a decrease in seed viability after storage (data not shown). The germinating seedlings showed well developed hypocotyls, radicals, and cotyledons after 15 d of culture. In the present study the cotyledonary node explants were isolated from the germinating seedlings at 15, 20, and 25 d of culturing.

The multiple shoot induction from cotyledonary node explants varied with the type and concentration of plant growth regulators, as well as seedling age (Table 1). The cotyledonary node explants failed to produce shoots on the MS basal medium. Incorporation of cytokinin into the medium was essential to induce multiple shoot proliferation from cotyledonary node explants. Explants obtained from 20-day-old seedlings (Figure 1B) produced a higher number of shoots, compared to those obtained from 15- or 25-day-old seedlings. Explant age plays a crucial role in multiple shoot induction from cotyledonary node explants, as evidenced by several previous reports for species, such as *Morus alba* (Thomas 2003) and *Cassia sophera* (Parveen and Shahzad 2010). Similar observations were also made for Asteraceae species like *Eclipta alba* (Baskaran and Jayabalan 2005) and *Vernonia anthelmintica* (Subhan and Agrawal 2011). The percentage of cultures responding and the mean number of shoots increased when the highest concentration of 2.0 mg L⁻¹ BA was used. The optimum response (66%) and number of shoots (12.3 per explant) was observed on the MS medium

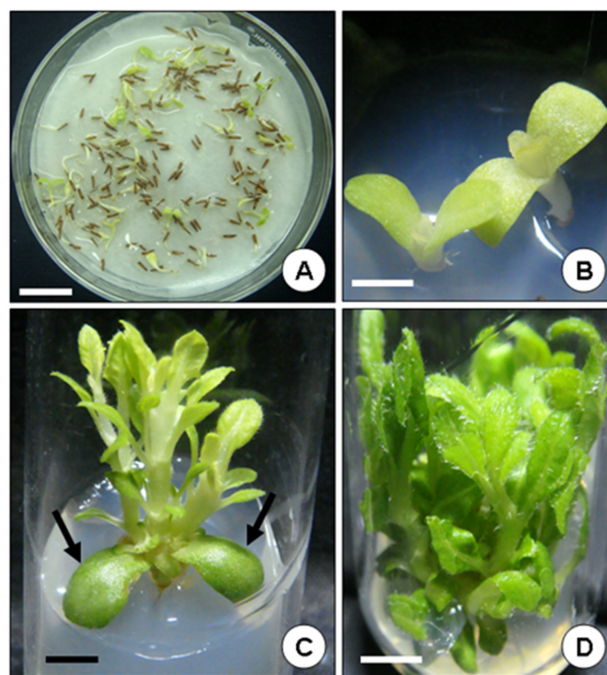


Figure 1. Various stages of shoot induction from cotyledonary node explants of *E. scaber*. A. *In vitro* seed germination on filter paper soaked in sterilized distilled water at 20 d of culturing. About 50% of the seeds germinated. Bar = 3.0 mm; B. The cotyledonary node just after culturing on MS medium supplemented with TDZ (1.5 mg L⁻¹) and NAA (0.5 mg L⁻¹). Bar = 0.3 cm; C. Multiple shoot induction from cotyledonary node explants 3 weeks after culturing on MS medium supplemented with TDZ (1.5 mg L⁻¹) and NAA (0.5 mg L⁻¹). Two cotyledons (arrows) are also visible. Bar = 0.6 cm; D. Same as in Figure 2C, 30 d after culturing. More multiple shoots have emerged from the explants. Bar = 0.7 cm

Table 1. Effect of various plant growth regulators on multiple shoot induction from 15, 20, and 25-day-old cotyledonary node explants of *E. scaber*. Medium: MS; Culture period: 45 days

Plant growth regulators (mg L ⁻¹)			% response			Mean no. of shoots*			Mean shoot length (cm)*		
BA	TDZ	NAA	15d	20d	25d	15d	20d	25d	15d	20d	25d
0.0	0.0	0.0	0.0f	0.0g	0.0g	0.0g	0.0g	0.0h	0.0b	0.0b	0.0b
0.5	-	-	45e	52f	49f	2.0±0.6f	2.3±0.6f	2.1±0.8g	0.6±0.2a	0.7±0.3a	0.5±0.3a
1.0	-	-	48d	58f	52e	5.6±1.7e	7.8±2.1e	4.8±1.4f	0.7±0.3a	0.7±0.4a	0.8±0.4a
1.5	-	-	51d	61e	57e	6.2±1.2d	8.3±2.3d	5.3±1.9e	0.8±0.2a	0.8±0.3a	0.9±0.2a
2.0	-	-	54d	66d	61d	8.1±2.1d	12.3±1.8d	6.6±1.8e	0.6±0.3a	0.7±0.4a	0.8±0.3a
3.0	-	-	51d	63e	58d	5.3±1.0e	8.4±2.3d	5.5±1.4e	0.8±0.3a	0.8±0.3a	0.7±0.4a
-	0.5	-	52d	64d	57e	6.9±1.3d	8.3±1.9d	5.9±2.3e	0.6±0.2a	0.9±0.2a	0.7±0.3a
-	1.0	-	56d	68d	59d	8.3±2.4d	9.3±2.8d	6.8±1.9d	0.9±0.3a	0.8±0.4a	0.8±0.4a
-	1.5	-	59d	71c	68d	10.3±2.6c	16.3±2.7c	8.8±2.3c	0.8±0.2a	0.8±0.3a	0.8±0.2a
-	2.0	-	55d	65d	64d	8.9±1.9d	9.6±2.5d	8.1±2.4c	0.8±0.3a	0.6±0.3a	0.9±0.3a
-	3.0	-	54d	66d	62d	7.7±1.2d	9.1±2.1d	7.8±2.7d	0.7±0.3a	0.6±0.4a	0.9±0.4a
2.0	-	0.3	59d	61e	64d	10.3±1.8c	16.7±2.8c	9.8±2.8c	0.6±0.4a	0.7±0.2a	0.8±0.3a
2.0	-	0.5	61c	67d	68d	12.7±2.1c	19.2±2.7c	14.8±3.4b	0.8±0.3a	0.7±0.3a	0.8±0.4a
2.0	-	1.0	66c	79c	76c	16.4±3.3b	22.7±3.2b	16.2±3.6b	0.8±0.4a	0.8±0.4a	0.9±0.3a
-	1.5	0.3	71b	83b	80b	15.3±3.1b	27.6±4.5b	11.5±3.2b	0.9±0.3a	0.9±0.4a	0.8±0.3a
-	1.5	0.5	78a	98a	91a	18.4±4.7a	33.7±6.2a	22.6±5.3a	0.9±0.3a	0.9±0.3a	0.9±0.4a
-	1.5	1.0	73b	91b	85b	14.6±3.2b	25.2±4.2b	10.8±4.8b	0.7±0.4a	0.8±0.2a	0.8±0.2a

*The values represent the means (±SE) of three independent experiments. At least 20 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test (P ≥ 0.05)

supplemented with 2.0 mg L⁻¹ BA. The BA concentration beyond 2.0 mg L⁻¹ did not enhance shoot production; rather, there was a decline in the number of shoots. Among the various concentrations of TDZ used, 1.5 mg L⁻¹ TDZ gave the optimum response (71%) and number of shoots (16.3 shoots per explant) when 20-day-old seedlings were cultured (Table 1). Simultaneous addition of NAA (0.3-1.0 mg L⁻¹) and a cytokinin significantly improved the shoot induction efficiency of cotyledonary node explants. The cotyledonary node explants started to enlarge and produce direct multiple shoots within 2-3 wk after culturing (Figure 1C). Within one month, several multiple shoots with many leaves had emerged from the cotyledonary node (Figure 1D). There was no callus formation from the explants. Ninety eight per cent of the cultures produced multiple shoots with an average of 33.7 shoots per explant when 20-day-old cotyledonary node explants were cultured on MS medium supplemented with 1.5 mg/l TDZ and 0.5 mg L⁻¹ NAA after 45 d of culture (Figure 2A; Table 1). The superiority of TDZ, a substituted phenyl urea, over other adenine type cytokinins for shoot induction in cultures has been well documented in several systems (Cheruvathur et al. 2013, Cordeiro et al. 2014). In the present study, a combination of TDZ and NAA produced the highest response. Synergistic effect of TDZ and NAA on shoot induction has been reported in several plant systems, such as *Gentiana kurroo* (Sharma et al. 2014) and *Artemisia vulgaris* (Sujatha and Kumari 2007).

Shoot elongation rooting and field transfer

Although a large number of shoots was formed from the cotyledonary node explants on various media, the mean shoot length was low. The average shoot length ranged from 0.5-0.9 cm on various shoot induction media (Table 1). Therefore, each shoot was excised from the multiple shoot clusters and cultured on MS medium supplemented with 0.5 mg L⁻¹ BA for shoot elongation. On this medium, the shoots showed rapid growth and reached an average length of 3.5 cm after 30 d of culturing (Figure 2B).

Shoots of 3.5-4.0 cm were transferred to half-strength MS medium supplemented with various concentrations of NAA (0.5-2.0 mg L⁻¹), IBA (0.5-2.0 mg L⁻¹), or IAA (0.5-2.0 mg L⁻¹) for root induction. No root formation was observed when shoots were cultured on MS basal medium. Although root induction was observed on all auxins tested, IBA was comparatively better than other auxins (Table 2). The highest root induction ($P \geq 0.05$) was observed on half-strength MS medium supplemented with 1.0 mg L⁻¹ IBA. At this concentration, 100% of the shoots rooted, with an average number of 3.2 roots per shoot (Table 2; Figure 2C). The use of half-strength MS medium for root induction has

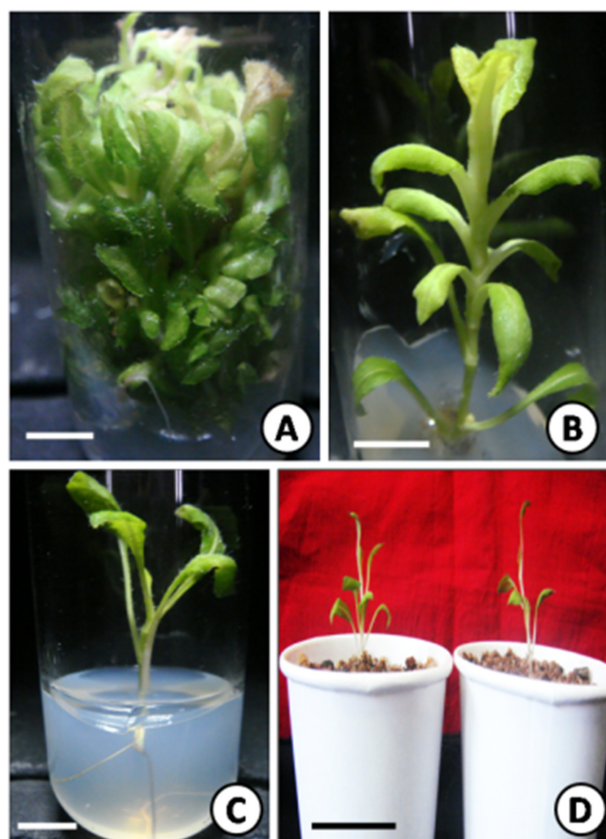


Figure 2. Multiple shoot induction, shoot elongation, rooting, and transplantation of plants in *E. scaber*. A. Multiple shoots induced 45 d after culturing on MS medium, augmented with TDZ (1.5 mg L⁻¹) and NAA (0.5 mg L⁻¹). A large number of shoots were formed. Bar = 0.7 cm; B. Elongation of shoots on MS medium supplemented with 0.5 mg/l BA. The shoots reach an average size of about 3.5 cm in 30 d. Bar = 1.2 cm; C. Rooting of shoots on half-strength MS medium supplemented with 1.0 mg L⁻¹ IBA after 45 d. Whitish roots have emerged from the basal cut end of the shoot. Bar = 1.0 cm; D. Two transplanted plants in expanded polystyrene cups 2 months after transfer. Bar = 3.0 cm

been reported in several systems, including *Rhinacanthus nasutus* (Cheruvathur and Thomas 2014) and *Caesalpinia bonduc* (Cheruvathur et al. 2012). In the present study, the addition of IBA to the medium produced a higher response than the other auxins. This observation is in agreement with other systems, where IBA induced optimum rooting (Beena et al. 2003)

The rooted shoots were transferred to expanded polystyrene cups containing autoclaved garden soil and sand (1:1 v/v) and placed in a greenhouse under high humidity. The plants were watered every day and were transferred to shade after two months (Figure 2D). Of the 66 plants transferred to soil, 61 survived.

In conclusion, the protocol described here provides an efficient method for the rapid multiplication of *E. scaber*.

Table 2. Effect of various auxins on rooting of shoots on half-strength MS medium after 45 d of culture

Auxins (mg L ⁻¹)			% shoots rooted	Mean no. of roots*	Mean root length (cm)*
NAA	IBA	IAA			
0.0	0.0	0.0	0.0g	0.0d	0.0b
0.5	-	-	45f	1.4±0.3c	3.1±0.3a
1.0	-	-	68d	1.7±0.2b	3.3±0.4a
1.5	-	-	71c	2.4±0.4b	3.6±0.6a
2.0	-	-	66d	1.9±0.4b	3.0±0.7a
-	0.5	-	78c	1.9±0.5b	3.2±0.6a
-	1.0	-	100a	3.2±0.3a	3.4±0.5a
-	1.5	-	90b	2.1±0.5b	3.3±0.8a
-	2.0	-	65d	1.8±0.6b	3.5±0.5a
-	-	0.5	55e	1.7±0.5b	3.1±0.4a
-	-	1.0	67d	1.9±0.4b	2.9±0.8a
-	-	1.5	78c	2.1±0.5b	3.8±0.6a
-	-	2.0	53e	1.2±0.6c	3.2±0.7a

*The values represent the means (±SE) of three independent experiments. At least 20 cultures were raised for each experiment. Mean values within a column followed by the same letter are not significantly different by Duncan's multiple range test ($P \geq 0.05$)

Cotyledonary node explants from 20-day-old seedlings may be used to obtain high frequency direct shoot regeneration. A combination of 1.5 mg L⁻¹ TDZ and 0.5 mg L⁻¹ NAA produced the best shoot induction from cotyledonary node explants. This protocol also has the advantage of establishing long-term cultures with a high multiplication coefficient. In addition to being used for extraction of bioactive compounds to reduce pressure on wild stocks, the regenerated plantlets could be used to replenish declining populations in the wild,

making a valuable contribution to preservation of the species. Thus, the method of multiplication reported here shows great potential for recovering the species, for propagation and conservation, production of useful phytochemicals, and genetic manipulation of this valuable medicinal plant.

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Sistema eficiente de regeneração de brotos para a espécie medicinal *Elephantopus scaber* Linn.

Resumo – Um protocolo eficiente para a rápida micropropagação da planta medicinalmente importante *Elephantopus scaber* foi padronizado a partir de explantes de nós cotiledonares. Indução de brotações múltiplas diretas foi observada quando os explantes de várias idades foram cultivadas em meio MS suplementado com vários reguladores de crescimento. A maior indução de brotos foi obtida quando os explantes de mudas de 20 dias foram cultivadas em meio MS suplementado com 1,5 mg L⁻¹ TDZ e 0,5 mg L⁻¹ NAA. Neste meio, 98% dos cultivos responderam, com um número médio de 33,7 brotos por explante. A maior frequência de enraizamento (100%) e número médio de raízes (3,3 por broto) foram observados quando os brotos foram transferidos para meio MS suplementado com 1,0 mg L⁻¹ de IBA. As mudas cultivadas in vitro foram aclimatizadas e transferidas para solo com sucesso de 92%. Este protocolo pode ser utilizado para a multiplicação e conservação de clones elite de *E. scaber*.

Palavras-chave: Aclimação, nó cotiledonar, *Elephantopus scaber*, brotos múltiplos, enraizamento.

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