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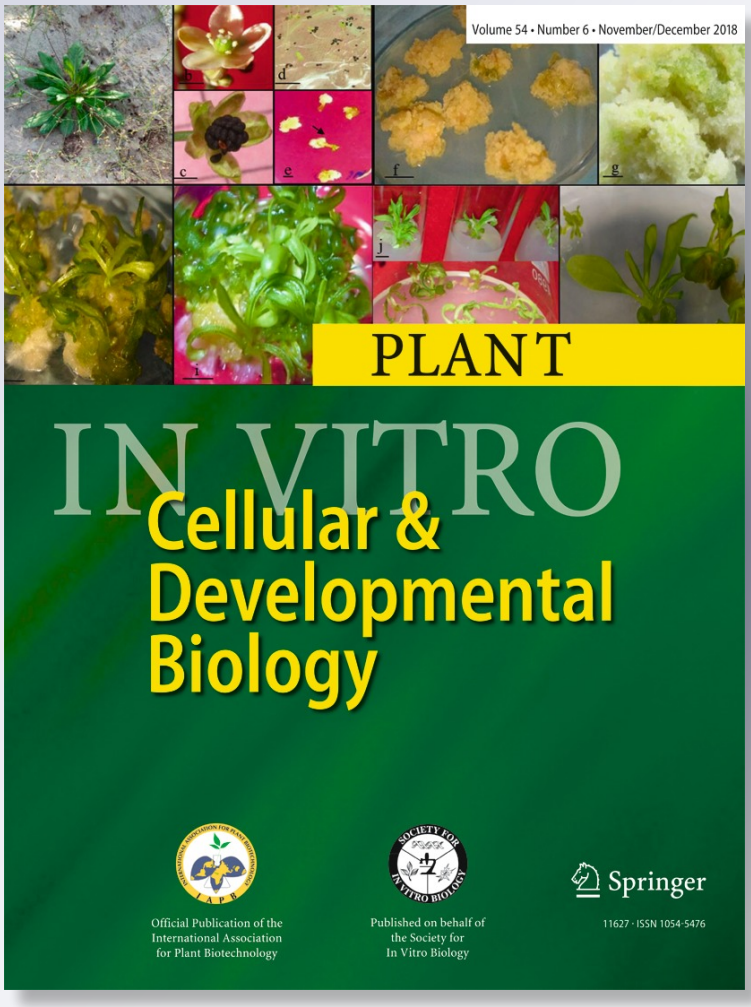

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MICROPROPAGATION

In vitro preservation and micropropagation of *Oreocharis mileense* (W.T. Wang) M. Möller & A. Weber (Gesneriaceae) through shoot organogenesis

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Abstract

Oreocharis mileense (W.T. Wang) M. Möller & A. Weber is endemic to China and was considered to be extinct because it had not been seen in the wild since the first collection in 1906. In 2006, the species was rediscovered in Shilin County, Yunnan Province. *Oreocharis mileense* was considered critically endangered for its narrow geographic range and extremely small population. An efficient method to preserve plant germplasm by *in vitro* culturing of *O. mileense* has not been reported. In this study, an orthogonal array with three factors (6-benzyladenine, BA; α-naphthaleneacetic acid, NAA; and sucrose), at four levels was performed, and shoot induction as well as shoot proliferation were recorded. The results were analyzed to determine the most significant components and the optimum combination for micropropagation of *O. mileense*. The results showed that: (1) organogenesis was easily induced by different combinations of plant-growth regulators and sucrose; (2) NAA and sucrose had the most significant effect on shoot induction and shoot multiplication, and (3) the optimum induction and proliferation media were 0.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA + 30 g L^{-1} sucrose and 1 mg L^{-1} BA + 0.1 mg L^{-1} NAA + 30 g L^{-1} sucrose, respectively.

Keywords *Oreocharis mileense* · Rediscovery · Critically endangered · *In vitro* preservation · Micropropagation

Paraisometrum mileense W. T Wang (Gesneriaceae), described by Wang *et al.* (Wang *et al.* 1997) as the sole member of the genus, was first collected in Mile County, Yunnan Province, China, in 1906. In the following 100 yr it was not collected again. In 2011, *P. mileense* was included in the expanded genus *Oreocharis* Benth. as *Oreocharis mileensis* (W.T. Wang) M. Möller & A. Weber (Möller *et al.* 2011).

In 2006, Dr. Yumin Shui rediscovered *O. mileense* in Shilin County in Southeastern Yunnan (Shui 2006; Shui and Cai 2007). Xu *et al.* (2009b) and Gao and Xu (2011) discovered two additional populations in Longlin County, Guangxi Province and in Xingyi, Guizhou Province. The rediscoveries are of special significance in studies of the biodiversity of the Karst regions of Southwestern China, the origin and differentiation of the Gesneriaceae in China, and the protection of the native flora in the Karst region. It also provides an opportunity to understand its evolution, conservation, and potential utilization (Shui and Cai 2007).

Oreocharis mileense has a diploid chromosome number of 2n = 34 (Tan *et al.* 2011). Its sister groups are *Briggsia rosthornii* and the *Ancylostemon* clade (Tan *et al.* 2011), and its closest relative is *Ancylostemon hekouensis* (Chen *et al.* 2014). The populations of *O. mileense* contain relatively low levels of genetic diversity (Chen *et al.* 2014). According to the International Union for Conservation of Nature (IUCN), *O. mileense* can be considered a critically endangered (CR) species because of its narrow geographic range and extremely small population size (101–1000 individuals in a single population; Ma *et al.* 2013). According to the latest report, 630

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mature plants are in Yunnan (the sum of the ten populations), 150 in Guangxi, and 60 in Guizhou. In Longlin County, the population in Dahongbao Nature Reserve were well protected (Xu *et al*. 2009b), whereas the two other populations were outside naturally preserved areas. In Yunnan Province, due to the short distances from dense settlements, some habitats of *O. mileense* are quite vulnerable to human activities (Chen *et al*. 2014).

A previous study showed that *O. mileense* is a resurrection plant. The seedlings of *O. mileense* can recover from a 3.7% relative water content (Li *et al*. 2014). However, 70%–80% of the seedlings and young plants died and 50–60% of mature plants sustained damage after the extreme drought from 2010 to 2011 in Yunnan (Chen *et al*. 2014), which indicated that it is quite vulnerable to climate fluctuations.

Additional experiments indicated that germinating seedlings and plantlets derived from shoot organogenesis showed extraordinary drought tolerance (data not shown). An efficient procedure was also established for the cryopreservation of *O. mileense* (Lin *et al*. 2014). *Oreocharis mileense* was among the first groups of plants with extremely small population size used to establish small–scale protected areas in China (Ma *et al*. 2013). Collecting wild plants and seeds of *O. mileense* has been forbidden since 2011. Micropropagation of *O mileense* is therefore the prerequisite for additional studies of this species. All *in vitro* grown adventitious shoots of *O. mileense* used for cryopreservation in the study of Lin *et al*. (2014) originated from *in vitro* culture. *In vitro* culture is an important and effective method for preserving plant germplasm. However, studies on micropropagation and preservation of *O. mileense* using *in vitro* culture have not been reported. In the present study, an efficient method was developed for micropropagation and preservation of this CR species.

Dry mature seeds of *O. mileense* were collected from Shilin County, Yunan Province, in January 2008 and used as explants to induce adventitious shoots. Seeds were surface sterilized with 75% (*v/v*) ethanol (Xilong Chemicals, Shantou, China) for 1 min followed by a 5-min immersion in 5% (*v/v*) Enox® (Enox, Changshu, China), a commercial liquid bleach containing 5.2% (*w/v*) sodium hypochlorite, then rinsed three times with sterile distilled water. Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 0.1% (*w/v*) activated charcoal (AC) (Reagent grade, powder; Sangon Biotech, Shanghai, China) was used for seed germination. The pH of the medium was adjusted to 5.8 with 1 M KOH before the addition of 3% (*w/v*) Phytagel™ (P8169; Sigma-Aldrich®, St. Louis, MO). All media were autoclaved at 121 °C for 25 min. Seed-containing culture jars (10.5 cm high × 6.5 cm diameter) with 50-mL MS medium and sealed with polypropylene rigid caps were maintained in an incubation room at 22 °C with a 12-h photoperiod, and a light intensity of 80 μmol m^{-2} s^{-1} supplied by cool-white fluorescent lamps (Philips, Shanghai, China).

Seed germination was observed after 20 d of culture. *Oreocharis mileense* showed a high germination percentage

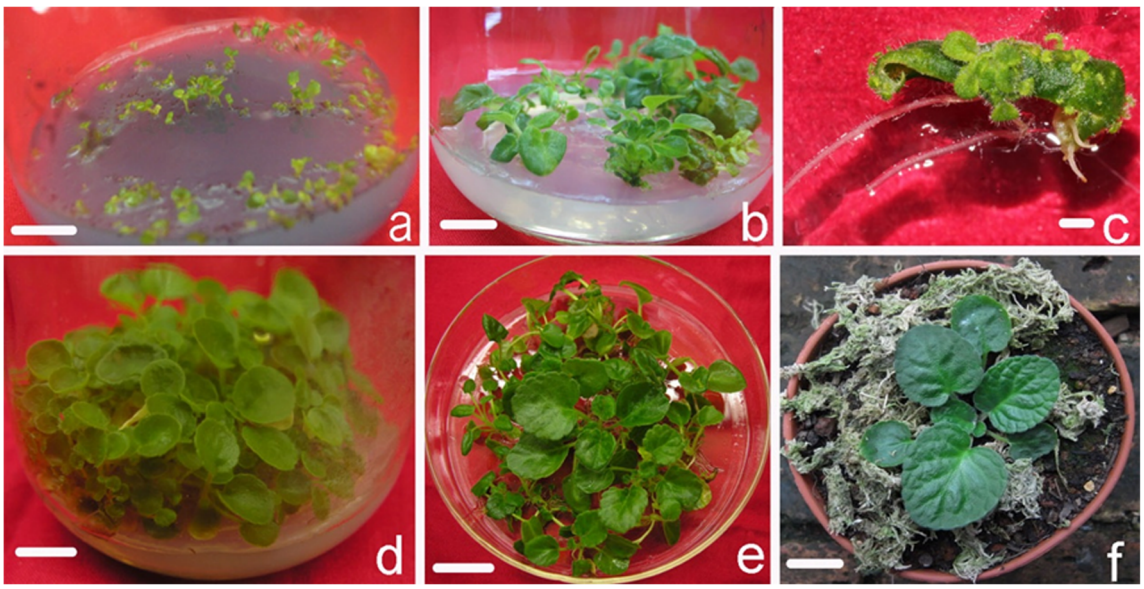

Figure 1. Regeneration of *Oreocharis mileensis* (W.T. Wang) M. Möller & A. Weber on Murashige and Skoog (MS) medium (Murashige and Skoog 1962). (*a*) Seed germination on MS medium containing 0.5% activated charcoal after culture for 10 wk. (*b*) Sterile plantlets derived from aseptic seed germination on MS medium after culture for 9 wk. (*c*) Adventitious shoots derived from leaf explant on MS medium containing 2 mg L^{-1} 6-benzylaminopurine (BA) and 0.1 mg L^{-1} α-naphthaleneacetic acid (NAA) after culture for 1 mo. (*d*) Plantlets developed from adventitious shoots grown on MS medium containing 2.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA after culture for 14 wk. (*e*) Well-developed plantlets were detached and ready for transplanting. (*f*) Plantlets were transplanted to a pot containing a 2:1 (*v/v*) soil mixture of humus:red soil. Bars represent 1 cm.

and vigorous growth of plantlets (Fig. 1*a*). After 10 wk of growth, seedlings were transplanted to new MS medium for another 9 wk (Fig. 1*b*). To determine the optimum medium for shoot induction and adventitious shoot proliferation, an $L_{16}(4^3)$ orthogonal array was constructed at four levels with three factors A: 6-benzyladenine (BA), B: α-naphthaleneacetic acid (NAA), and C: sucrose (Table S1) in MS medium and inoculated with 0.5 × 0.5 cm leaf explants excised from the 19-wk-old seedlings. 6-benzyladenine and NAA were purchased from Sigma-Aldrich® (St. Louis, MO) and were added to the medium before autoclaving. Sucrose was purchased from Sangon Biotech.

Shoot induction and the number of adventitious shoots per explant were recorded after 75 d of continuous culture. Complete adventitious shoots and shoot bud protuberance were considered signs of successful shoot organogenesis. Shoot induction was observed in all treatments (Table 1). Treatments 6 and 14 (Table 1) exhibited 100% shoot induction. In this study, the mean value of the sum of the evaluation indexes of all levels ($\bar{K}_{ji}$, $i = 1, 2, 3, 4$; j = A, B, C) in each factor, was calculated to determine the optimum level and the optimum combination of factors. There was a positive correlation between $\bar{K}_{ji}$ and induction due to the level of a factor. For example, to estimate the induction efficiency of BA (factor A), the value of $\bar{K}_{j2}$ was the largest among the four $\bar{K}_{ji}$s. Thus, it was inferred that A_2 (0.5 mg L^{-1} BA) had the most significant effect on shoot induction. Based on the analysis of the orthogonal array, for shoot induction, A_2, B_3, and C_3 were the optimum levels of BA, NAA, and sucrose, respectively. Thus, $A_2B_3C_3$ was determined to be the optimum combination for shoot induction (Table 1).

Adventitious roots formed at the cut edges and on the surface of leaf explants within 1 mo (Fig. 1*c*). Although the initiation of shoots was later than for roots, the induction frequency and development of shoots was higher and faster than for roots (Fig. 1*d*). No callus was observed during the process. Adventitious shoot induction was observed in all treatments, including treatment 1, which was devoid of any growth regulators (Table 2). Analysis of the orthogonal array showed that $A_3B_2C_3$ was the best combination for shoot proliferation (Table 2). Analysis of variance (ANOVA) was performed by R software (https://www.r-project.org/). The results showed that the interaction of the three factors was the most important for shoot

Table 1. The results and analysis of orthogonal test of shoot induction (%) from *Oreocharis mileensis* (W.T. Wang) M. Möller & A. Weber leaf tissue. Values are mean of six independent experiments with 10 leaf explants per culture jar

Treatments	Factors			Shoot induction (%)
	(A) BA (mg L^{-1})	(B) NAA (mg L^{-1})	(C) Sucrose (g L^{-1})	
1	0	0	10	75.0 ± 28.8
2	0	0.1	20	70.0 ± 21.6
3	0	0.2	30	96.0 ± 8.9
4	0	0.5	40	72.0 ± 8.4
5	0.5	0	20	90.0 ± 12.6
6	0.5	0.1	10	100.0 ± 0
7	0.5	0.2	40	98.0 ± 4.5
8	0.5	0.5	30	86.7 ± 8.2
9	1.0	0	30	90.0 ± 12.2
10	1.0	0.1	40	98.0 ± 4.5
11	1.0	0.2	10	88.3 ± 11.7
12	1.0	0.5	20	64.0 ± 45.1
13	2.0	0	40	72.5 ± 5
14	2.0	0.1	30	100.0 ± 0
15	2.0	0.2	20	88.3 ± 14.7
16	2.0	0.5	10	44.2 ± 27.3
$\bar{K}_{j1}$	78.2	81.9	76.9	
$\bar{K}_{j2}$	93.7	92.0	78.1	
$\bar{K}_{j3}$	85.1	92.6	93.2	
$\bar{K}_{j4}$	76.2	66.7	85.1	
Optimal level	A_2	B_3	C_3	
Optimum conditions	$A_2B_3C_3$ (0.5 mg L^{-1} BA +0.2 mg L^{-1} NAA + 30 g L^{-1} sucrose)			

BA, 6-benzyladenine; NAA, α-naphthaleneacetic acid

Table 2. Results and analysis of orthogonal test of shoot proliferation from *Oreocharis mileensis* (W.T. Wang) M. Möller & A. Weber cultures (mean number of adventitious shoots per explant). Values are mean of six independent experiments with 10 leaf explants per culture jar

Treatments	Factors			Mean number of adventitious shoots per explant
	(A) BA (mg L^{-1})	(B) NAA (mg L^{-1})	(C) Sucrose (g L^{-1})	
1	0	0	10	28.8 ± 18
2	0	0.1	20	32.3 ± 9.1
3	0	0.2	30	37.0 ± 13.1
4	0	0.5	40	27.5 ± 9.1
5	0.5	0	20	38.0 ± 14.8
6	0.5	0.1	10	21.2 ± 8.9
7	0.5	0.2	40	76.7 ± 40.3
8	0.5	0.5	30	37.8 ± 11.3
9	1.0	0	30	74.0 ± 40.2
10	1.0	0.1	40	62.0 ± 18.7
11	1.0	0.2	10	19.0 ± 11
12	1.0	0.5	20	39.4 ± 36.9
13	2.0	0	40	26.5 ± 7.2
14	2.0	0.1	30	91.5 ± 46.9
15	2.0	0.2	20	40.6 ± 17.5
16	2.0	0.5	10	16.3 ± 7.7
$\bar{K}_{j1}$	31.4	41.8	21.3	
$\bar{K}_{j2}$	43.4	51.7	37.6	
$\bar{K}_{j3}$	48.6	43.3	60.1	
$\bar{K}_{j4}$	43.7	30.2	48.2	
Optimal level	A_3	B_2	C_3	
Optimum conditions	$A_3B_2C_3$ (1 mg L^{-1} BA +0.1 mg L^{-1} NAA + 30 g L^{-1} sucrose)			

BA, 6-benzyladenine; NAA, α-naphthaleneacetic acid

induction, followed by factor B, whereas factors A and C, interactions of BA and NAA, BA and sucrose, and NAA and sucrose, did not show any significant effect on shoot induction (Table 3). As for shoot proliferation, only factor C and the interaction of the three factors significantly affected the number of adventitious shoots (Table 3). After

Table 3. Variance analysis by R software (*$p < 0.05$, **$p < 0.01$) of shoot induction and shoot proliferation of *Oreocharis mileensis* (W.T. Wang) M. Möller & A. Weber cultures

Biological parameter	Source of variance	Degree of freedom	Sum of squares of deviations	*F* value	*P* value
Shoot induction (%)	A	1	94.3	0.99	0.350
	B	1	882.5	9.23	0.016*
	C	1	317.6	3.32	0.106
	A:B	1	206.0	2.15	0.180
	A:C	1	246.9	2.58	0.147
	B:C	1	27.8	0.29	0.604
	A:B:C	1	1154.1	12.07	0.008**
Shoot proliferation	A	1	242.1	0.97	0.353
	B	1	569.6	2.28	0.169
	C	1	2123.9	8.51	0.019*
	A:B	1	309.9	1.24	0.297
	A:C	1	64.4	0.26	0.625
	B:C	1	380.6	1.53	0.252
	A:B:C	1	1799.8	7.21	0.028*

A: 6-benzyladenine (BA), B: α-naphthaleneacetic acid (NAA), and C: sucrose

14 wk of growth (Fig. 1*d*), well-developed plantlets were detached (Fig. 1*e*), and transplanted into pots (10 cm in diameter) containing a 2:1 (*v/v*) soil mixture of humus:red soil (Fig. 1*f*). The duration of the procedure took about 7 to 8 mo. The plantlets grew well in the greenhouse, and some tissue culture seedlings flowered in the third year.

Shoot multiplication was achieved by repetitive subculture of adventitious shoots on MS medium supplemented with 30 g L^{-1} sucrose, and subsequent induction of adventitious shoots on MS medium containing 30 g L^{-1} sucrose, 1.0 mg L^{-1} BA, and 0.1 mg L^{-1} NAA. After several rounds of subculture and induction, a relatively large number of plants were obtained for the preservation experiment.

For the preservation experiment, plantlets cultured on complete MS medium grew better than those on half strength MS (1/2 MS). Plantlets grown on 1/2 MS medium showed stunted growth and smaller chlorotic leaves with longer petioles (Fig. S1). Plantlets of *O. mileense* exhibited slower growth, even under the optimum conditions. Plant growth retardants or osmoprotectants therefore, were not necessary for *in vitro* preservation of *O. mileense*. Retarding growth can be accomplished by reducing the supply of nutrients, such as using 1/2 MS.

In the Gesneriaceae, shoot organogenesis has been successfully induced in some species. In the resurrection plant *Haberlea rhodopensis*, leaf explants can be induced to form shoots on woody plant medium (WPM) (Lloyd and McCown 1980), without any growth regulators (Djilianov *et al*. 2005). In *Primulina tabacum*, more adventitious shoots than somatic embryos were induced when BA or kinetin (KIN) concentrations exceeded thidiazuron (TDZ), and the opposite trend was observed when the TDZ concentration exceeded BA or KIN concentrations (Ma *et al*. 2010). Leaf explants of *Primulina tabacum* were induced to form adventitious shoots when cultured on MS medium containing 5.0 μM TDZ for 30 d, followed by culturing on MS medium containing 5.0 μM BA, whereas somatic embryos were obtained when the order of the addition of TDZ and BA was reversed (Yang *et al*. 2012). In *Chirita medica*, adventitious shoots were induced from leaf explants cultured on MS medium supplemented with 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BA (Li *et al*. 2009). For *Sinningia speciosa*, MS medium containing 2.0 mg L^{-1} BA and 0.2 mg L^{-1} NAA was the most effective for shoot induction and produced 5.53 buds per explant (Xu *et al*. 2009a). In *Metabriggsia ovalifolia*, high activity cytokinins (TDZ or BA), combined with low activity auxins (NAA, indole-3-acetic acid, or indole-3-butyric acid) induced shoot organogenesis from leaf or petiole explants (Ma *et al*. 2011). Shoot organogenesis was obtained in African violet (*Saintpaulia ionantha*) by applying ≤1.2 mg L^{-1} TDZ (Mithila *et al*. 2003; Taha *et al*. 2009). The highest shoot induction rate of *Chirita pumila* was obtained when 0.5 mg L^{-1} BA and 0.1 mg L^{-1} NAA were applied (Liu *et al*. 2014). These studies indicate that different species of Gesneriaceae respond differently to different concentrations, combinations, and sequences of plant growth regulators (PGRs) for shoot organogenesis.

In this study, based on the analysis of the orthogonal data, 0.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA + 30 g L^{-1} sucrose, and 1.0 mg L^{-1} BA + 0.1 mg L^{-1} NAA + 30 g L^{-1} sucrose, were the most effective combinations for shoot induction and shoot proliferation, respectively. These two combinations can be used for additional shoot induction and shoot proliferation experiments.

Interestingly, adventitious shoots can be obtained even without the addition of PGRs, which indicated that active endogenous PGR metabolism was sufficient to activate organogenesis. Shoot induction is affected by many factors, including leaf age, wounding, and leaf orientation (Geier 1986; Lo 1997; Ma *et al*. 2010; Corral *et al*. 2011; Cardoso and Habermann 2014; Padmanabhan *et al*. 2015). In the present study, effects of leaf age, leaf orientation, and wounding on shoot induction were not studied due to the limited plant material. Thus, great variation in shoot induction and mean shoot number among individual leaf explants was observed (Tables 1 and 2). Unknown sources of variation added difficulty to the statistical analysis and data interpretation. Additional studies are needed to identify the sources of variation in shoot induction. *O. mileense* showed high rooting ability, thus, a protocol for root induction was not developed. However, the addition of low concentrations of auxin can facilitate root growth of *O. mileense*.

Drought stress has a profound impact on crop productivity. Understanding how resurrection plants respond to and survive desiccation is of great importance to improve crop drought tolerance (Xiao *et al*. 2015). In the present study, an efficient *in vitro* shoot micropropagation protocol was developed for the long-term preservation and proliferation of the critically endangered resurrection plant, *O. mileense*. Adventitious shoots of *O. mileense* maintained their organogenesis and proliferation capacities after 8 yr of subculture. This is the first report of successful *in vitro* preservation, micropropagation, and regeneration of *O. mileense* using direct shoot organogenesis. This proliferation method may facilitate additional cryopreservation and resurrection studies of this plant.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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