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MICROPROPAGATION OF ORNAMENTAL GESNERIACEAE SPECIES AND GENETIC UNIFORMITY ASSESSMENT OF *IN VITRO* PLANTS USING SCOT MARKERS

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Abstract

A micropropagation protocol via direct shoot organogenesis from leaf explants of four commercial cultivars of ornamental Gesneriaceae was established in this study. The shoot induction was successfully achieved on Murashige and Skoog (MS) media supplemented with 0.2 mg L^{-1} indole-3-acetic acid (IAA) and 0.5 mg L^{-1} benzylaminopurine (BA). In proliferation stage, the effects of two combinations of PGRs (V1-0.2 mg L^{-1} IAA + 0.2 mg L^{-1} BA and V2-0.2 mg L^{-1} NAA +1 mg L^{-1} BA) on shoot number and length were examined for each species. The results suggest that PGR's combinations significantly influenced shoot proliferation in all analyzed species and among the treatments 0.2 mg L^{-1} NAA in combination with 1 mg L^{-1} BA was the most effective for in vitro shoot multiplication. The in vitro rooting percentage was 76.86-96.66% and was species-dependent. In vitro-raised plants showed a very high rate of survival (92.59-95.24%). The genetic fidelity between the selected vitro-plants and mother plants were confirmed by SCoT markers and, therefore, the propagation method proposed in this study could be applied for commercial purposes as well.

Key words*: Sinningia speciosa, Kohleria hirsute, Streptocarpus hybridus, Saintpaulia ionantha, genetic fidelity.*

INTRODUCTION

Over the years, the commercial propagation of new species and varieties of ornamental plants was determined by the improvement of decorative traits of the plants, mainly the color and shape of flowers, using classical breeding methods together with modern biotechnological techniques (Davies & Schwinn, 2006; Burchi et al., 2010; Zhao and Tao, 2015).

In addition, obtaining the varieties with a long shelf life of flowers was also a very important objective of plant breeders, as this characteristic of ornamental plants was usually considered a key feature for assessing their value on flower market (Serek et al., 2006; Noman et al., 2017).

Gesneriaceae is known as a family of flowering plants that includes approximately 3540 species (Christenhusz & Byng, 2016) originated from tropical and subtropical areas of Afro-Eurasia and America (Weber, 2004). Many of these species are grown worldwide as potted plants and among them *Sinningia speciosa*, *Kohleria hirsuta*, *Streptocarpus x hybridus* and *Saintpaulia ionantha* are highly valued by plant growers for their colorful and showy flowers.

Sinningia speciosa, known as gloxinia, is a tuberous plant native to Brazil with a spectacular, single or double flower corolla, which presents a variety of colors and patterns (Macas-Palacios et al., 2015).

Kohleria hirsuta, the wooly kohleria, is a species of flowering plants in the family Gesneriaceae originated from northern South America with very attractive dark olive green and hairy leaves. The tubular-shaped hairy flowers have a spectacular spotted throat with bright colors (especially orange and red) and bloom all year round (Kvist & Skog, 1992).

Streptocarpus x hybridus, commonly known as Cape primrose, is an indoor plant native to Afro-mountainous areas of Central, East and South Africa, including Madagascar and the Comoro Islands (Nishii et al., 2015). The long leaves (25-40 cm) of Streptocarpus form a rosette and the trumpet-shaped flowers appear on long peduncles. Flowering is from spring to early fall and the spectacular flowers last a long time on the plant (Cantor et al., 2004).

African violets, with their new botanical name Streptocarpus sect. *Saintpaulia ionanthus* (Nishii et al., 2015), are attractive houseplants native to tropical East Africa, Tanzania, and Kenya (Harrison, 1999). Due to the high demand for this species, there are many varieties of African violets available on the market of ornamental plants that are characterized by different colors (from white, pink, red to purple) and flower shapes (Ghorbanzade & Ahmadabadi, 2014; Buta et al., 2015).

According to previous reports, the Gesneriads have been propagated mainly by seeds and leaf cuttings from mature plants, but in this case, only a limited number of plants have been regenerated over a long period of time (Preece, 2003). Therefore, the development of valuable techniques that allow the rapid multiplication of these plant species in a short amount of time and limited space can be an important benefit for the plant material production of these ornamental plants (Maghami, 2003; Kozak et al., 2007; Ioja-Boldura & Ciulca, 2013; Hârţa et al., 2018).

As a modern method of large-scale plant propagation, tissue culture has been used for rapid multiplication of ornamental species (Rout et al., 2006). However, the effectiveness of the protocols used was strongly influenced by the species (genotype) and also by *in vitro* culture conditions, such as culture media, plant growth regulators (PGRs) and explant type (Debergh & Maene, 1981; do Valle Rego & de Faria, 2001; da Silva, 2013; Datta et al., 2017; Faleiro et al., 2019).

Moreover, another important objective of tissue culture is to obtain true-to-type plants, due to the fact that during tissue culture somaclonal variations might occur (Mujib et al., 2013; Eeckhaut et al., 2020). Thus, the evaluation of the genetic fidelity of micropropagated plants with mother plants using the Start codon targeted (SCoT) markers can be a valuable method to confirm the uniformity of *in vitro* raised plants at molecular level.

Due to the high ornamental value of Gesneriads, the trend of growers in recent years have been to produce high quality planting material for the flower market. In this context, the objectives of this research were (i) to determine the influence of plant growth regulators (PGRs) on the in vitro proliferation of four varieties of Gesneriaceae and (ii) to evaluate the genetic fidelity of micropropagated plants with the mother plants after the 6th successive subculture.

MATERIALS AND METHODS

The plant material used in this study was represented by four hybrid cultivars of *Gesneriaceae*: Avanti Blue (*Sinningia speciosa*), Carnival (*Kohleria hirsuta*), Shannon (*Streptocarpus x hybridus*) and Ivona Specta Adele (*Saintpaulia ionantha*). The mother plants were selected based on flower characteristics and were purchased from two certified nurseries from North Wales, UK. These mother plants were used as explant sources for the initiation and establishment of the *in vitro* culture and were grown as potted plants in greenhouse conditions at 23 °C (Figure1).

To initiate *in vitro* culture, young leaves from mother plants were collected and then were washed with running tap water using a magnetic stirrer to eliminate all the dust and impurities. Thereafter, the leaves were cut in fragments of approximately 10 x 10 mm in size under a laminar flow hood in aseptic conditions and were sterilized with 20 % commercial bleach ACE solution (Procter & Gamble, Romania; <5 % active ingredient) for 20 min followed by triple-rinse with sterile deionized water. The explants were then aseptically inoculated into glass test tubes (11.5 cm x 2 cm ø) containing 5 mL of sterile Murashige & Skoog (1962) medium (MS) supplemented with 2% (w/v) sucrose, 0.2 mg L^{-1} IAA and 0.5 mg L^{-1} BA and gelled with 0.6% agar (Sigma-Aldrich Inc). The pH of culture medium was adjusted to 5.9 before autoclaving at 121°C for 20 min. The cultures were grown in culture room at 24 ± 1°C under 16/8 h light/dark cycle. Light was provided by white fluorescent lamps. After 56 days of culture on the initiation medium, the regenerated shoots from leaf explants were further multiplied at 21 days intervals by two passages on MS medium supplemented with 0.2 mg L^{-1} IAA and 0.5 mg L^{-1} BA for ensuring the stock of plants for subsequent in vitro multiplication experiments.

After *in vitro* culture establishment, in the proliferation stage, adventitious shoots were

divided and individually transferred to 720 ml (v/v) culture jars containing MS medium supplemented with two variants of PGRs: V1- 0.2 mg L^{-1} IAA+0.2 mg L^{-1} BA and V2- 0.2 mg L^{-1} NAA+1mg L^{-1} BA and solidifed with 0.6% Plant agar (w/v). Adventitious shoots were subsequently transferred in aseptic condition to culture jars and cultured (30 days/subculture) at 24 ± 1°C under fluorescent white light (33,6 µmol m^{-2} s^{-1}) conditions with a photoperiod of 16/8 h light and dark cycles. The average multiplication rate and shoot length were recorded after the 6th successive subculture.

After the 6th successive subculture, the proliferated shoots were individually separated and then were rooted on aseptic conditions using ½ MS medium supplemented with 1 mg L^{-1} IAA. The mean values of rooting percentages were calculated after 35 days.

The rooted plants were thereafter subjected to acclimatization process in laboratory conditions for 6 weeks. For this, the plantlets were taken out from the culture jars, rinsed carefully with sterile deionized water and then planted into transparent plastic containers with lids filled up with humidified perlite. After 42 days, the plantlets were individually transplanted to plastic pots with a diameter of 6 cm and filled up with a potting mixture made of peat, vermiculite and perlite (2:1:1) and hardened in greenhouse conditions. The mean values of the survival rate were recorded after 21 days of growth in greenhouse.

To assess the genetic fidelity of micropropagated plants with mother plants, DNA was isolated from both the mother plant and seven in vitro-raised plants of each species that were randomly selected after 21 days of growth in greenhouse. The harvested leaves were dried, grounded into fine powder (TissueLyser II, Qiagen, Germany) and then kept at 4°C until the genetic analyses were performed.

The extraction of total genomic DNA (gDNA) was performed using a CTAB (cetyl trimethylammonium bromide)-based protocol described by Lodhi et al. (1994) and improved by Pop et al. (2003) and Bodea et al. (2016).

The quality and concentration of DNA were determined with a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific, USA). Prior to SCoT analysis, DNA samples were diluted to 50 ng μL^{-1} using sterile double distilled water.

Two SCoT primers were used for each analyzed cultivars to confirm the genetic uniformity of vitro-plants with the mother plant. These primers generated scoreable fragments in all analyzed samples, and to ensure the reproducibility of the results all PCR reactions were repeated twice.

For SCoT analysis, PCR reactions were carried out with a total volume of 15 µL consisting of 3 µL gDNA, 5.6 µL distilled H2O for the PCR reactions, 2.5 µL GoTaq Flexi Green buffer, 2.5 µL MgCl2, 0.25 µL dNTP mix (Promega, USA), 1 µL SCoT primer (GeneriBiotech, Czechia), and 0.15 µL of GoTaq polymerase (Promega, USA). The PCR temperature cycling conditions were: (a) initial denaturation at 94°C for 5 min, (b) 35 cycles of denaturation at 94°C for 1 min, annealing at 50 °C for 1 min and elongation at 72°C for 2 min, and (c) the final elongation step of 5 min at 72°C. Separation of the amplified products was performed by electrophoresis on 1.6% agarose gels (Promega, USA) stained with RedSafeTM Nucleic Acid staining solution (iNtRON Biotech, South Korea) in 1X TAE (Tris-acetate-EDTA buffer), at 100V and 176mA for 2,5-3 hours. The electrophoretic profiles were visualized in UVP Biospectrum AC Imaging System (UVP BioImaging Systems, Germany).

Data analysis

The *in vitro* experiments were carried out in a completely randomized design (4 replication x 5 inoculums/ each treatment). One-way ANOVA was performed to check the differences between the experimental variants. When the null hypothesis was rejected, Duncan test ($p<0.05$) was used to determine the differences between the means. The values shown are means ± S.E.

SCoT gel images were analyzed using TotalLab TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to determine the number and the range size of the amplified bands. Intensity of the bands were not considered while scoring.

(a) (b) (c) (d)

Figure 1. The commercial cultivars used in this study for tissue culture initiation: Avanti Blue-*Sinningia speciosa* (a), Carnival-*Kohleria hirsuta* (b), Shannon-*Streptocarpus x hybridus* (c) and Ivona Spectra Adele-*Saintpaulia ionantha* (d)

RESULTS AND DISCUSSIONS

Shoots induction and proliferation

The induction of adventitious shoots from leaf explants of four cultivars of *Gesneriaceae* was done using MS medium supplemented with 0.2 mg L^{-1} IAA and 0.5 mg L^{-1} BA. Our findings are consistent with previous reports that highlighted the high capacity of *in vitro* shoots proliferation of Gesneriad plants from leaf explants (Naz et al., 2001; Afkhami-Sarvestani et al., 2006; Shukla et al., 2012). Another type of explant, such as shoot tips was used to initiate the in vitro culture of *Kohleria amabilis* (Kozak et al., 2007), but in this study, *Kohleria hirsuta* shoots were successfully initiated from leaf fragments. On the other hand, we observed that the proliferated epidermal cells produced shoots directly, without callusogenesis.

In this study, MS medium with two different combinations of plant growth regulators (PGRs) were tested to choose the best variant of culture medium. The PGRs combinations significantly affected shoot proliferation in all Gesneriad cultivars (Table1).

The results show that the addition of citokinins (BA) and auxins (IAA and NAA) in MS medium had a stimulating effect on the rate of multiplication of the adventitious shoots (Table 1). As shown in Table 1, among the treatments, MS medium supplemented with 0.2 mg L^{-1} NAA and 1mg L^{-1} BA proved to be the best variant for shoot proliferation and significantly improved the number of shoots/explant for Avanti Blue (14.97 ± 0.35) and Carnival (18.15 ± 0.31). On the same variant of the culture medium (V2), non-significant differences were recorded between Shannon (16.71 ± 0.19) and Ivona Spectra Adele (16.91) cultivars.

Our results are in agreement with those reported by other authors (Park et al., 2012; Ghorbanzade and Ahmadabadi, 2014; Macas-Palacios et al., 2015) who stated that MS medium containing BA in concentrations between 0.2 to 2.0 mg L^{-1} and NAA (0.1-0.2 mg L^{-1}) led to the highest efficiency of shoot regeneration per explant. High shoot regeneration rates were observed for all varieties, indicating the genotype dependence of the protocol used (Figure 2).

The data presented in Table 2 show also that the longest shoots (4.15 cm) were recorded at *Streptocarpus x hybridus* cv. Shannon on MS medium containing V2-PGRs combination, while the shortest (1.33 cm) were recorded at *Sinningia speciosa* cv. Avanti Blue proliferated on MS medium containing V1-PGRs combination. Regardless of the PGRs variants used, significant differences were observed between the three varieties cultured on the same PGRs combination as shown in Table 2.

Our results show that for all analyzed cultivars, V2-PGRs (MS + 0.2 mg L^{-1} NAA+1mg L^{-1} BA) proved to be the most effective for shoots proliferation. The recorded value of the average length of the shoots was 1.67 cm for *Sinningia speciosa* cv. Avanti Blue. Compared to our results, Park et al. (2012) reported that the highest value of shoot length (1.7-1.8 cm) was obtained when the MS medium was supplemented with 2.0 mg L^{-1} BA+0.1 mg L^{-1} NAA, and either 7.0 mg L^{-1} $AgNO_3$ or 50 mg L^{-1} putrescine. In addition, Chae et al. (2013) reported that the highest value of shoot length (1.27 cm) was recorded when the regeneration medium (MS +2.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA) was supplemented with 5.0 mg L^{-1} STS (silver thiosulphate).

Table 1. The influence of PGRs on the rate of multiplication of proliferated shoots (number of shoots/inoculum) of the analyzed cultivars of *Gesneriaceae*

Cultivars	Multiplication rate	
	V1	V2
	MS+0.2 mg L^{-1} IAA+0.2 mg L^{-1} BA	MS + 0.2 mg L^{-1} NAA+1 mg L^{-1} BA
Avanti Blue	9.46 ± 0.23 B*	14.97 ± 0.35 D
Carnival	9.96 ± 0.32 C	18.15 ± 0.31 F
Shannon	7.21 ± 0.21 A	16.71 ± 0.19 E
Ivona Spectra Adele	9.31 ± 0.23 B	16.91± 0.28 E

*The values shown are means ±SE. Different letters indicate significant differences between the treatments according to Duncan's test.

(a) (b) (c) (d)

Figure 2. *In vitro* shoot proliferation of *Gesneriaceae* cultivars: Avanti Blue (a); Carnival (b); Shannon (c) and Ivona Spectra Adele (d) on MS medium supplemented with 0.2 mg/L^{-1} NAA and 1mg/L^{-1} BA after 6th successive subculture

Table 2. The influence of PGRs on the length of proliferated shoots of the analyzed cultivars of *Gesneriaceae*

Cultivars	Shoot length (cm)	
	V1	V2
	MS+0.2mg L^{-1} IAA+0.2 mg L^{-1} BA	MS + 0.2 mg L^{-1} NAA+1mg L^{-1} BA
Avanti Blue	1.33 ± 0.01 A	1.67 ± 0.02 C*
Carnival	1.54 ± 0.02 B	1.94 ± 0.01 D
Shannon	3.74 ± 0.02 E	4.15 ± 0.01 F
Ivona Spectra Adele	1.53 ± 0.02 B	1.86 ± 0.02 D

*The values shown are means ± SE. Different letters indicate significant differences between the treatments according to Duncan's test.

The results of this study show that also for *Kohleria hirsuta* (cv. Carnival) the MS medium supplemented with the V2-PGRs combination was the best option for the proliferation of shoots. The average length of the shoots (1.94 cm) was significant higher compared with that obtained by Kozak et al. (2007) at *Kohleria amabilis* (0.45 cm) grown on hormone-free MS medium.

Moreover, the results of this research support our previous findings (Hârţa et al., 2018) regarding the influence of cultivars and different combinations of PGRs on shoots regeneration of *Streptocarpus x hybridus*. Thus, the variant of MS medium supplemented with PGRs combination that included BA and IAA was found to be more effective in producing the higher number of rooted shoots (5.03) than the one supplemented with NAA and TDZ (4.5).

On the other hand, a few research groups reported that the addition of PGRs, such as zeatin (Daud et al., 2008), BA (Sunpui & Kanchanapoom, 2002) and TDZ (Mithila et al. 2003, Shukla et al., 2012) on the culture media of commercial varieties of *Saintpaulia ionantha* played a key role in the regeneration of shoots. For example, Daud & Taha (2008) reported that regeneration of African violet shoots from floral explants was stimulated when MS medium was supplemented with 1 mgL^{-1} BA and 2 mgL^{-1} NAA. Ghasemi et al. (2012) observed that the longest Saintpaulia shoots induced from the leaf disc (with an average of 7.6 mm) belonged to media containing 0.2 or 0.5 mgL^{-1}BA along with 0.5 mgL^{-1} IBA, while the shortest belonged to the media containing only 0.5 mgL-1 BA, (with an average of 2.8 mm). In this study, MS + 0.2 mg L^{-1} NAA+1mg L^{-1} BA was the best option for the proliferation of Ivona Spectra Adele cv. shoots. These results indicated the important role of auxin (NAA) in promoting both number and length of the regenerated shoots.

Rooting and acclimatization of vitro-plants

In vitro rooting of multiple shoots was recorded on ½ MS+1mg L^{-1} IAA for all of the four cultivars of *Gesneriaceae*. The mean values of the percentage of rooting of the shoots and also the survival rate (recorded after 21 days of growth in greenhouse) are presented in Table 3. The results summarized in Table 3 show that ½ MS supplemented with IAA stimulated the rooting of regenerated shoots for all four cultivars of Gesneriaceae. Moreover, there were significant differences between cultivars in term of *in vitro* rooting percentage (Avanti Blue 76.86 % vs. Carnival 85.66 %).

It is worth mentioning that roots induction and elongation is one of the most important steps for the successful production of in vitro regenerated plants (Ghorbanzade & Ahmadabadi, 2014). In the case of the analyzed cultivars of Gesneriaceae, the rooting process was successful.

Regarding the acclimatization process, it is noteworthy that more than 92% of *in vitro* raised plants of the four analyzed cultivars survived (Table 3). Rooted plants were successfully transferred in greenhouse, followed by normal plant growth and flower development.

Table 3. The rooting percentage and survival rate (%) calculated for each of the four Gesneriaceae cultivars. The values shown are means ±SE

Species	**Cultivars**	***In vitro* rooting** (%)	**Survival rate** (%)
Sinningia speciosa	**Avanti Blue**	76.86 ± 0.08 A*	95.24 ± 0.03 A
Kohleria hirsuta	**Carnival**	85.66 ± 0.06 B	94.93 ± 0.02 A
Streptocarpus x hybridus	**Shannon**	94.33 ± 0.06 C	95.05 ± 0.03 A
Saintpaulia ionantha	**Ivona Spectra Adele**	96.66 ± 0.03 C	92.59 ± 0.03 A

*Different letters indicate significant differences among the treatments according to Duncan's test.

Evaluation of genetic uniformity of acclimatized plants

In order to confirm whether or not somaclonal variation was detectable in *in vitro* raised plants, SCoT markers were employed to analyze the genetic fidelity of seven randomly selected acclimatized plants from each species, as well as their corresponding mother plants. Both SCoT primers used generated clear and scorable bands for all samples analyzed (Table 4).

Genetic analysis with SCoT markers led to a total of 10, 20, 11 and 19 monomorphic bands for Avanti Blue, Carnival, Shannon and Ivona Spectra Adele cv. respectively (Table 4).

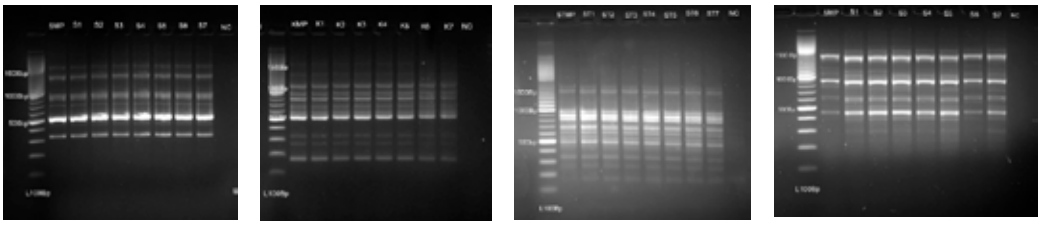
Figure 3 shows that *in vitro* raised plants have the same banding patterns as those of the mother plants, indicating their uniformity at DNA molecular level. Each SCoT markers generated monomorphic bands and their length ranged between 528 and 2036 bp. Moreover, no genetic variability was observed in any of the analyzed cultivars.

Despite the many advantages of clonal propagation through tissue culture techniques, the occurrence of somaclonal variation is a serious limitation in the practical applications of plant micropropagation (Soliman et al., 2014). In fact, the variability detected in regenerated plants from tissue cultures is a combined effect of genetic and epigenetic variations that may occur during different stages of *in vitro* culture protocols and genetic heterogeneity of explant cells (Bhojwani & Dantu, 2013). The genotype, explant type, the combination and concentrations of PGRs, the number of the successive subcultures and methods used for regeneration may also influence the genetic stability of *in vitro* raised plants (Martin et al. 2004). Even so, in this study, no genetic variability was observed between mother plants (MP) and the seven randomly selected acclimatized clonal plants of each *Gesneriaceae* cultivars and provided from 6^{th} successive subculture (Figure 3).

Our results support previous findings reported by other authors refers to the fact that SCoT molecular markers were effective and gave reproducible results in evaluation of genetic fidelity of *in vitro* raised plants at *Dendrobium nobile* (Bhattacharyya et al., 2014) and *Chrysanthemum morifolium* (Nasri et al., 2018). The findings of our study further suggest that SCoT molecular markers are useful in assessing the genetic stability of *in vitro* propagated *Gesneriaceae* cultivars.

Table 4. Sequences of SCoT primers and the number of monomorphic bands generated in the analyzed Gesneriaceae cultivars

Primer name	Primer sequence 5'-3'	Number of monomorphic bands				Range size of bands (bp)
		Avanti Blue	Carnival	Shannon	Ivona Spectra Adele	
SCOT16	ACCATGGCTACCACCGAC	5	9	5	9	528-1712
SCOT25	ACCATGGCTACCACCGGG	5	11	6	10	201-2036
Total bands		10	20	11	19	-



(a) (b) (c) (d)

Figure 3. PCR banding patterns obtained with SCoT16 primer at *Gesneriaceae* cultivars: Avanti Blue (a); Carnival (b); Shannon (c) and Ivona Spectra Adele (d). Lane L-100 bp DNA step Ladder; SMP, KMP, STMP and SPMP - mother plant for each species; L1-L7 SCoT profile of acclimatized vitro-plants; NC-negativ control

CONCLUSIONS

As conclusions, the combinations of PGRs used in this research stimulated the shoot induction and direct shoots regeneration in devoid of the callus phase, considering that direct regenerated plants may exhibit greater genetic stability than those produced *via* callus. Based on ScoT molecular markers analysis, the genetic uniformity of the regenerated plants with the mother plants were confirmed and, therefore, the propagation method presented in this study could be applied also for commercial purposes.

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