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Full Length Research Paper

Efficient one-step tissue culture protocol for propagation of endemic plant, *Lilium martagon* var. *cattaniae* Vis.

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A rapid, one-step method for direct leafy and rooted bulblets regeneration of *Lilium martagon* var. *cattaniae* Vis. was established using seeds as the starting explants for *in vitro* culture initiation. Adventitious bulblets were regenerated from one scale explants on MS (Murashige and Skoog, 1962) medium supplied with various concentrations of plant growth regulators. The most efficient medium for multiplication was MS supplemented with 0.2 mg/l 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA) ranging from 0.25 to 2 mg/l. On average, five bulblets were obtained per explant after six weeks in culture. Upon rooting, about 200 plantlets were successfully hardened in the green house with a 95% survival rate. Preliminary experiments indicated that the plantlets from the greenhouse could be successfully used for field cultivation. *In vitro* propagation of this endemic and protected plant species is of great importance for germplasm conservation.

Key words: Bulblet, endemic, *in vitro*, *Lilium martagon* var. *cattaniae* Vis., protocol.

INTRODUCTION

Lilium martagon L. belonging to the Martagon section of the large Liliaceae family is an endangered and protected species in many European countries, including Serbia (List of plants protected as natural rarities, 1993). *L. martagon* L. shows a significant variability throughout the entire range of areal. In Serbia, there are two varieties of this species: *L. martagon* L. var. *cattaniae* and var. *martagon* (Diklić, 1975). According to Šilić (1990), variety *cattaniae* is a distinct species named *L. cattaniae* Vis. It is widespread in higher mountain areas of littoral Dinarides, from Mt Velebit to Mt Rumija, belonging to the Dinaridi's endemic species (Šilić, 1990). Preliminary results based on molecular data obtained by Sočo et al. (2003) are indicative of possible bifurcation of *L. cattaniae* Vis. from *L. martagon* L.

This perennial plant produces beautiful dark wine-red flowers without spots on petals, and is therefore attractive for the cut-flower and potted-plant industries. Furthermore, secondary metabolites found in some lilies (*Lilium candidum*) Vachálková et al., 2000; Eisenreichová et al., 2004; *L. martagon* - Satou et al., 1996), were shown to have antifungal and antiviral activities (Wang and Ng, 2002; Pieroni, 2000). Bulbs of *L. martagon* L. posses cardiotonic properties and are used in the treatment of dysmenorrhoea (Khare, 2007), liver diseases in both humans and animals in Northern Albania (Pieroni et al., 2005) and for ulcers (Khare, 2007). Therefore, the possible benefits of our research could evolve.

Advances in biotechnology, especially in the field of *in vitro* culture techniques, provide important tools for

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Abbreviations: BAP, 6-Benzylaminopurine; **IAA,** indole-3-acetic acid; **NAA,** α-naphthalene acetic acid; **MS,** Murashige and Skoog.

improved conservation of plant genetic resources. Used for bulb plants, as well as for other plants, *in vitro* techniques as an alternative to the conventional methods have shown a few advantages: the multiplication level is several-fold increased, and obtained material is free of viruses and other pathogens. This is particularly important when endemic and rare plants are used as a source of medically active substances. Tissue culture has been applied for the propagation of lilies since the late 1970's (Takayama and Misawa, 1979, 1983; Rybczyński and Gomolińska, 1989; Mii et al., 1994; Nhut, 1998; Chang et al., 2000) since lilies as well as other bulbous plants exhibit high regeneration potential. Nowadays, lilies are one of the most important bulbous crops produced in tissue culture on a large-scale. This method provides a continuous aand stable source of natural products.

The main goal of this research was to establish the protocol for rapid *in vitro* multiplication of *L. martagon* var. *cattaniae*, thus enabling the germplasm maintenance of this endangered, endemic lily. In addition, sufficient material was obtained for field cultivation, possible introduction and further pharmacological analyses.

MATERIALS AND METHODS

L. martagon var. *cattaniae* seeds were isolated from fruits collected from Orjen Mountain (Montenegro). Seeds were surface sterilized with 20% NaOCl for 15 min and thoroughly washed three times with sterile distilled water. They were then aseptically placed on MS (Murashige and Skoog, 1962) medium and stratified at 4°C for three months to facilitate germination (Nikolaeva et al., 1985). After development of several bulbscales, these were separated and cultured on MS medium supplemented with 0.2 mg/l 6-benzylaminopurine (BAP) and 0.1 mg/l α-naphthalene acetic acid (NAA). Further multiplication was performed using the same medium, until enough material was obtained. After two months of multiplication, bulblets produced from each bulbscale were separated and transferred to hormone-free MS medium. Bulbscales were successively transferred every two months, but only one clone was used as a material for the main screening experiments. As a result, one-scale segments (the leaves differentiated from *in vitro* formed scales were chopped off) were inoculated on MS media supplemented either with various concentrations of BAP ranging from 0.1 to 2 mg/l with constant NAA concentration of 0.1 mg/l or with various concentration of auxins (IAA or NAA) with constant BAP concentration of 0.2 mg/l. All experiments were carried out for 6 weeks. There were 21 explants per treatment and every experiment was repeated three times.

Culture conditions

The pH of all media was adjusted to 5.8 prior to autoclaving at 114°C for 25 min. All cultures were grown in glass vessels of round type (10 cm diameter x 15 cm height) and each vessel contained 100 ml medium. Cultures were maintained in a growth chamber at 25 ± 2°C, under long day conditions (16 h/8 h light/dark cycle). Light was provided by "Tesla" (Pančevo, Serbia) white fluorescent tubes (photon flux density 50 µmol/m^2 s).

Statistical analysis

Statistical analyses were performed using STATGRAPHICS software, version 4.2 (STSC Inc. and Statistical Graphics Corporation, 1985-1989, USA). Data were subjected to analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at the confidence level of $P<0.05$. Data given as percentages were subjected to arcsine ($\sqrt{X}$) transformation prior to statistical analysis.

Acclimatization

Plantlets were washed and transferred to rectangular containers (40 x 30 x 8 cm) containing gardening soil mixture soaked with a 0.1% aqueous solution of fungicide Previcur (Aventis, Germany). Plantlets were kept under high humidity conditions for 15 days; afterwards, the humidity was reduced to the greenhouse level. Plants were maintained in the greenhouse at 24 to 26°C under natural light (47 µmol/m^2 s) and were transferred to the field after two months.

RESULTS

After 3 months of stratification all seeds germinated, buds differentiated directly from the basal plate of the scale. These buds developed into small leafy bulblets 3 weeks after the culture initiation (Figure 1A). Tables 1 and 2, and Figure 2 summarizes the bulblet production from one scale bulb segments on MS medium containing various BAP, NAA or IAA levels. The frequency of the bulblet formation was influenced by growth regulator concentration tested (Tables 1 and 2). Considering both the percentage of explants producing bulblets and the mean number of bulblets per explant, the highest bulblet regeneration was achieved on MS medium supplemented with 0.2 mg/l BAP and IAA in concentrations ranging from 0.25 to 2 mg/l (Table 2 and Figure 2B). The best results, regarding the leaf length, were achieved by addition of 0.2 mg/l BAP and 2 mg/l IAA (Figure 2B). Medium supplemented with BAP (ranging from 0.1 to 0.2 mg/l) and 0.1 mg/l NAA or medium supplemented with NAA (from 0.1 to 0.5 mg/l) in combination with 0.2 mg/l BAP was also favorable for leaf elongation (Figure 2C). The interesting observation was that with increase in NAA concentration, mean number of bulblets with leaves per explant decreased (Figure 2C). In addition, on media supplemented only with 0.1 mg/l NAA without BAP, leaf formation (sprouting) was suppressed (Figure 2A). However, medium supplemented with NAA (from 0.1 to 0.5 mg/l) in combination with 0.2 mg/l BAP showed favorable effects on leaf growth. With regard to mean leaf length, the best regeneration rate was obtained on medium with 0.2 mg/l BAP and 2 mg/l IAA: nearly six bulblets per explant, five of which were sprouted and with mean leaf length of about 25 cm (Figure 2B).

The regenerated shoots subsequently rooted (Figure 1B), to form whole plantlets (Figure 1C). Percentage of rooting, as well as mean root number per explant, differed among treatments (Tables 1 and 2, Figure 3). Adventitious roots were formed on MS medium without plant growth regulators at frequency of 62%. Similar

Figure 1. Micropropagation of *L. martagon* var. *cattaniae*. A) Bulblets formed on the basal plate of the scale on MS medium with 0.2 mg/l BAP and 1 mg/l IAA after 3 weeks of culture initiation (bar = 3 mm); B) The formation of roots on MS medium supplemented with 0.2 mg/l BAP and 0.5 mg/l NAA (bar = 5 mm); C) Well developed plantlets on MS medium with 0.2 mg/l BAP and 0.25 mg/l NAA after 6 weeks period *in vitro* culture (bar = 7 mm); D) General view of successfully acclimatized plantlets in the greenhouse; E) Successful acclimatization of *L. martagon* var. *cattaniae* to the field conditions.

Table 1. Effect of BAP (in the presence of 0.1 mg/l NAA) on bulblet production and rooting efficiency of *L. martagon* var. *cattaniae* Vis. (n = 60).

BAP (mg/l)	Explants with formed bulblets ± SE (%)	Rooting ± SE (%)
0.0	56.51 ± 11.76[a]	52.06 ± 11.21[ab]
0.1	76.82 ± 6.52[ab]	60.95 ± 11.59[b]
0.2	77.78 ± 6.88[ab]	39.05 ± 9.16[ab]
0.5	80.63 ± 7.39[b]	32.38 ± 8.70[ac]
1	71.43 ± 6.49[ab]	11.11 ± 5.20[cd]
2	82.22 ± 5.55[ab]	93.65 ± 2.51[a]

[a]Data were scored after 6 weeks of culture; [b]Data followed by different letters are significantly different (P<0.05); [c]On hormone-free MS medium, the percentage of explants with formed bulblets was 63.98 ± 12.21%, and the rooting percentage was 62.26 ± 7.69%.

results were obtained on medium supplemented with 0.2 mg/l BAP and NAA over the entire concentration range (Table 2). Surprisingly, addition of IAA instead of NAA did not induce root formation (Table 2 and Figure 3B). MS medium supplemented with NAA (from 0.1 to 2 mg/l) and 0.2 mg/l BAP resulted in obtaining about 4 to 5 roots per explant (Figure 3C), as compared to 2.39 on hormone-free MS medium. However, most of the shoots that were rooted on medium with higher NAA (1 to 2 mg/l) concentration had short thick roots and some callus tissue at the base of the scale.

Irrespective of rooting efficiency, the plantlets could be successfully acclimatized. About 200 plantlets were transferred to the green house regardless of the root development. About 95% of these plantlets continued to grow, and no morphological abnormalities were observed in the population (Figure 1D). This demonstrated that all these plants were easily and efficiently acclimatized to the green house conditions. Some of them were transferred to the field after two months, where they continued to grow normally (Figure 1E).

DISCUSSION

Lily tissues have a high regeneration potential. Still, bulbscales have the best capacity to regenerate adventitious bulbs (Takayama and Misawa, 1979), hence they are the most commonly used explants for vegetative propagation and *in vitro* culture initiation of *Lilium* sp. However, seeds are usually recommended for the production of pathogen-free plants (Pelkonen, 1997). Therefore, we used seeds for establishing *in vitro* culture. In earlier studies, seeds were used as starting material for *Lilium formolongi* (Mii et al., 1994), *Lilium regale*, *Lilium bulbiferum* (Pelkonen, 1997), and for *L. martagon* (Pelkonen, 1997; Kędra and Bach, 2005; Glamočlija et al., 2010).

Various methods have been reported for *in vitro* propagation of bulbous plants. *Lilium* species in particular can be efficiently multiplied by bulblet formation or by somatic embryogenesis (Priyadarshi and Sen, 1992; Niimi et al., 1997; Pelkonen, 1997; Chang et al., 2000). Alternatively, leafy bulb scales regeneration from scale

Table 2. Effect of IAA or NAA (in the presence of 0.2 mg/l BAP) on bulblet production and rooting efficiency of *L. martagon* var. *cattaniae* Vis. (n = 60).

Auxins (mg/l)	Explants withformed bulblets ± SE (%)		Rooting ± SE (%)	
	IAA	NAA	IAA	NAA
0	94.70 ± 1.74[a]	94.70 ± 1.74[a]	0[a]	0a
0.1	100.00 ± 0.00[a]	88.62 ± 5.23[ab]	12.70 ± 5.02[b]	60.71 ± 9.84[b]
0.25	92.06 ± 7.94[a]	85.71 ± 7.14[ab]	9.52 ± 5.32[ab]	67.86 ± 8.08[b]
0.5	96.82 ± 2.10[a]	68.25 ± 10.04[bc]	8.93 ± 7.11[ab]	53.87 ± 6.91[b]
1	95.24 ± 3.37[a]	70.90 ± 5.85[bc]	4.76 ± 3.37[ab]	50.39 ± 10.05[b]
2	93.65 ± 2.51[a]	60.84 ± 10.67[c]	20.63 ± 8.94[b]	48.81 ± 10.32[b]

[a] Data were scored after 6 weeks of culture; [b]Data followed by different letters are significantly different (P<0.05); [c] On hormone-free MS medium, the percentage of explants with formed bulblets was 63.98 ± 12.21%, and the rooting percentage was 62.26 ± 7.69%.

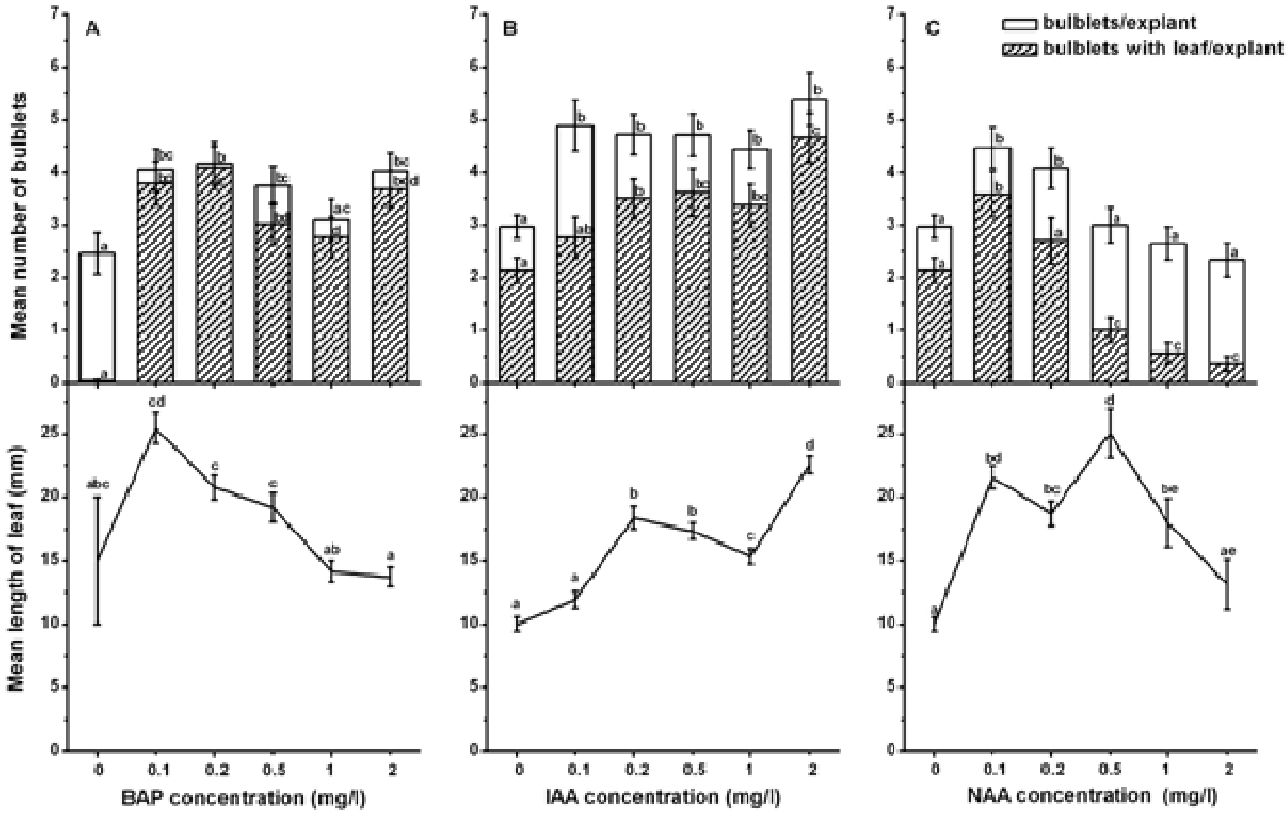

Figure 2. The bulblet production in *in vitro* culture of *L. martagon* var. *cattaniae* on medium with various concentrations of (A) BAP, in the presence of 0.1 mg/l NAA; (B) IAA, in the presence of 0.2 mg/l BAP; (C) NAA, in the presence of 0.2 mg/l BAP (n = 60). [a] Data were scored after 6 weeks of culture; [b]Data followed by different letters are significantly different (P<0.05); [c]On hormone-free MS medium, the mean number of bulblets per explant was 2.61 ± 0.50 (with leaves 0.06 ± 0.04).

explants has been reported for several *Lilium* species, such as *Lilium auratum* (Takayama and Misawa, 1983), *Lilium candidum* (Sevimay et al., 2005), *Calochortus nuttallii* (Hou et al., 1997) and *Urginea maritime* (Allacher et al., 1998). Typically, in most of these protocols, combinations of auxins and cytokinins were used. Among cytokinins, BAP has been extensively used due to its pronounced effect on the adventitious bulblet formation (Takayama and Misawa, 1979; Maesato et al., 1994; Kędra and Bach, 2005). Rybczyński and Gomolińska (1989) described the influence of BAP at low concentration on bulblet formation in *L. martagon*.

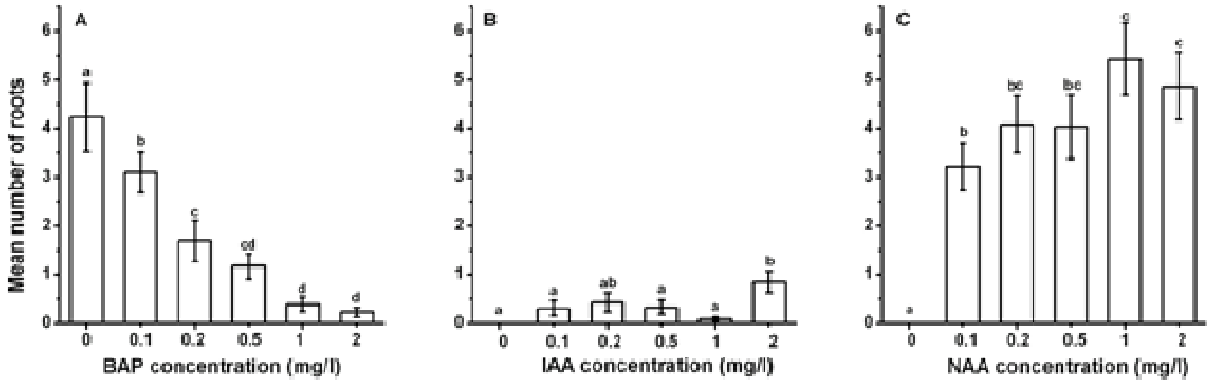

Figure 3. The root production in *in vitro* culture of *L. martagon* var. *cattaniae* on medium with various concentrations of (A) BAP, in the presence of 0.1 mg/l NAA; (B) IAA, in the presence of 0.2 mg/l BAP; (C) NAA, in the presence of 0.2 mg/l BAP (n = 60). [a]Data were scored after 6 weeks of culture; [b]Data followed by different letters are significantly different ($P<0.05$); [c]On hormone-free MS medium, the mean number of roots per explant was 2.39 ± 0.46.

Glamočlija et al. (2010) have used MS medium supplemented with 0.1 mg/l gibberellic acid (GA_3), 0.1 mg/l indole-3-butyric acid (IBA) and 0.5 mg/l BAP only to obtain fresh material for further genotoxic analysis of *L. martagon* var. *cattaniae.* According to Pelkonen (1997), the best way to induce *in vitro* multiplication in *L. martagon* was to use the combination of BAP and NAA. In our experiments, these two plant growth regulators were used for the first multiplication step. Afterwards, the influence of IAA in combination with BAP was also investigated.

According to the obtained results, the addition of BAP enhanced the bulblet production and induced the production of leafy bulblets. Contrary to our initial expectation, the addition of NAA with BAP was less favorable for the induction of bulblet regeneration than the addition of IAA. The addition of 0.2 mg/l BAP in combination with IAA gave rise to 5 bulblets per explant. The use of NAA was less efficient. The increase of NAA concentrations resulted in decrease of bulblets formation to a value of 2 per explant at 0.2 mg/l BAP with 1 to 2 mg/l NAA. Combination of BAP with IAA was also shown to be more efficient for *Lilium nepalense* (Wawrosch, 2001) and *Lilium longiflorum* (Han et al., 2004). In addition, the combination of these two plant hormones has shown the most beneficial effect on proliferation of shoots with small bulbscales from shoot segments of *Lilium oriental* hybrid 'Casablanca' (Han et al., 2005).

Morphological features of differentiated bulblets were markedly affected by BAP, that is, the formation of numerous leafy bulbscales was stimulated by increased concentrations of this cytokinin. Similar effects were reported for kinetin in *Lilium speciosum* (Takayama and Misawa, 1979). Mean leaf length increased significantly when BAP was added to the auxin-containing medium. Similar results were reported for *Charybdis numidica* (Kongbangkerd et al., 2005). An interesting finding in our experiments was that NAA and IAA have exerted opposite effects on leaf formation, that is, formation of scaly leaves was suppressed on medium with increasing concentrations of NAA.

The formation of adventitious roots was observed in all the treatments, although the rooting frequency, as well as the average root number varied. Promoting effects of auxins on rooting have been reported for *Lilium auratum* (Takayama and Mishawa, 1979) and *Lilium nepalense* (Wawroch et al., 2001). However, our results indicated that only NAA was an important factor for the root formation in *L. martagon* var. *cattaniae.* Rooted bulblets have only been induced on medium with combination of BAP and NAA. Although, root formation was stimulated on medium supplemented with NAA, addition of BAP suppressed rooting in terms of rooting percentage and mean number of roots per explant. Similarly, Takayama and Mishawa (1979) reported that kinetin inhibits the effect of NAA on root formation. Actually, lower auxin/cytokinin ratio is favorable for the bulblet formation, whereas the higher ratio of these hormones stimulated rooting (Takayama and Mishawa, 1979). Our results, obtained with different combinations of BAP and NAA, are in accordance with this observation. On medium with higher NAA/BAP ratio, leafy bulblet production was suppressed, and the mean number of roots increased. However, the combination of IAA with BAP had different effects. On medium with higher IAA/BAP ratio, the leafy bulblet formation was induced. In addition, root formation was not stimulated over the entire IAA concentration range. Nhut et al. (2001) reported that IAA was less efficient than NAA for root induction in *L longiforum*. Han et al. (2004) reported that no rooting was recorded on medium supplemented with BAP and various IAA concentrations. On the contrary, Takayama and Misawa (1979) reported root formation over the tested concentration range of IAA and kinetin. In *in vitro* culture of *L. longiflorum*, root formation was inhibited by BAP (Han et al., 2004) as we reported for *L. martagon* var.

cattaniae. Without BAP, or on hormone-free medium, the roots were observed (Han et al., 2004), which is in accordance with our results. Kongbangkerd et al. (2005) have shown that high NAA concentrations caused thickening of the short roots and callus formation at the base of the scale, although they used only NAA without addition of BAP.

Acclimatization was successful regardless of the root formation, as reported for *L. nepalense* (Wawarosch et al., 2001). None of the plants were dormant, and they continued to grow both *in vitro* and in the greenhouse.

In conclusion, this study reports efficient and rapid one-step method for propagation of *L. martagon* var. *cattaniae* without an intermediate callus phase, thus limiting the possibility of somaclonal variation. Within the scope of a domestication strategy, the application of *in vitro* propagation techniques enables the production of a great number of uniform plantlets for the commercial cultivation or introduction. Successful *in vitro* multiplication and subsequent establishment of *ex vitro* culture of this rare plant species provides sufficient amounts of material for further investigations of secondary metabolites.

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