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Influence of Cytokinins on the Shoot Proliferation and Subsequent Rooting in Rose

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ABSTRACT

The single-node explant of rose cvs. 'Bianca' and 'El Torro' were cultured in *in vitro* conditions to investigate the influences of different cytokinins in the multiplication and subsequent rooting phases. The *in vitro* growth of explant was affected by the type of cytokinins used in the culture media. The best results regarding shoot proliferation was found in the medium containing 8.87 μM BA. Although 2-iP (8.87 μM) showed the better response in shoot elongation but due to their lower multiplication ability, it cannot ensure rapid micropropagation in rose cvs. 'Bianca' and 'El Torro'. In addition, kinetin (8.87 μM) had virtually no effect on multiplication phase. Although, higher concentration of BA proliferated maximum shoots per culture but there were a risk of hyperhydricity symptom in plantlets. One milligram per liter BA was found the best medium in shoot multiplication phase by securing the good proliferation rate, greater shoot elongation, maximum number of leaves and highest fresh and moderate percentage of dry weight per culture. But 1.0 mg L^{-1} BA could not maintain the best performance in the rooting phase. On the other hand, 2.0 mg L^{-1} BA was found to be optimum concentration for better formation of longer roots and can be recommended for rooting of rose cvs. 'Bianca' and 'El Torro'. Regarding the rose cultivars tested in the experiments, 'Bianca' showed the higher response in both multiplication and rooting phases than 'El Torro'.

Key words: *In vitro*, rose, cytokinin, nodal explant, proliferation, rooting

INTRODUCTION

Rose is one of the most economically important flowering ornamental in the world. It has been used for its beauty and fragrance since the dawn of civilization. In Germany, the genus *Rosa* took the first and the third places of the top 10-list in the commerce of cut flowers and flowering pot plants, respectively in 2013. In Germany, the rose import in 2013 amounted 281.38 million euro which was 10 fold higher than the export value of 28.34 million euro. Generally, the most ornamental roses are heterozygous and do not breed true to type, those are, therefore, propagated vegetatively. Commercially, roses are propagated by cutting, budding and grafting which are difficult, undesirable and tedious processes (Horn, 1992). This conventional process is not satisfactory in multiplication of rose species (Roy *et al*., 2004). Whereas, *in vitro* has higher multiplicative capacity within a relatively short time, production of healthy and disease free plants and its ability to generate propagules round the year (Dhawan and Bhojwani, 1986).

The success of micropropagation involves several factors viz., the composition of the culture medium, culture environment, genotype etc. In plant micropropagation, type and concentration of plant growth regulator play major role in cell division, differentiation and morphogenesis. Under *in vitro* conditions, the axillary buds can be activated by supplementing cytokinins to the culture medium. In addition to promoting bud break by reducing the dominance of the apical bud, cytokinins delay senescence, stimulate chloroplast development and nutrient metabolism and enhance the resistance to various stresses (McGaw and Burch, 1995). The successful regeneration of shoots and their subsequent rooting is essential for the commercial exploitation of micropropagation technology. Since the first report of Hasegawa (1979) on micropropagation of *Rosa hybrida* L. by proliferation of axillary buds, several studies regarding plant shoot multiplication (Ibrahim and Debergh, 2001; Hameed *et al*., 2006; Senapati and Rout, 2008; Kanchanapoom *et al*., 2010), rooting (Bressan *et al*., 1982; Asadi *et al*., 2009), genotypes (Carelli and Echeverrigaray, 2002; Kim *et al*., 2003; Misra and Chakrabarty, 2009) have been published. However, there is limited commercial uses of the technique because of contradictory results and the low multiplication rate achieved with the most important rose cultivars. So, the aim of the present study was to investigate the influence of cytokinin on the shoot multiplication and their subsequent rooting of roses.

MATERIALS AND METHODS

Plant material: Two cultivars of rose namely, 'Bianca' and 'El Torro' were used in this study. About 20 cm long young healthy flowering shoots of roses were collected from the Greenhouse Laboratory Center, Technische Universität München, Freising, Germany.

Culture establishment: About 5-7 cm apical and basal portion of each shoots were discarded and only the axillary buds from middle portion of stem were taken. After removing leaves and thorns, the shoots were neatly cut into nodal segments (3-4 cm long) each bearing a quiescent axillary bud with a fragment of the petiole. Explants were surface-sterilized by immersing in 70% ethanol for 1 min, after then in 1% sodium hypochloride solution with some drops of Tween 20 for 15 min. The nodal segments were rinsed in 3 changes of sterile deionized and distilled water, each time 5 min to remove all the traces of sodium hypocholoride. Following sterilization, about 0.5 cm was trimmed from both the ends of each nodal segment to remove damaged tissues and about 1.5-2.0 cm long single-bud stem segments were used as the explant source.

In proliferation phase, modified MS medium (Davies, 1980) containing 40 g L^{-1} of sucrose and 7 g L^{-1} agar were used in the study. The pH of the medium was adjusted to 5.8 prior to the addition of agar. The glass test tubes (size 150×25 mm) containing 15 mL of modified MS media were autoclaved at 1.2 kg cm^{-2} pressure and 121°C temperature for 15 min. The tools like scalpels, forceps, needles etc were also pre-sterilized by autoclaving and subsequent sterilization was done by flaming and cooling method inside the laminar air-flow cabinet before use. The cabinet was usually started half an hour before using and wiped with 70% ethyl alcohol to reduce the chances of contamination. Hands were also sterilized by wiping with the mixture of 0.26% glycerine and 70% ethyl alcohol solution. The necks of the test tubes were flamed before opening and closing those with the glass caps. The explants were planted vertically into the culture tubes and were capped with glass cap. The culture tubes containing the explants were kept under 16 h photoperiod and at 24±1°C and 70% relative humidity. A photosynthetic photon flux density of 60 μmol min^{-2} sec^{-1} was maintained at the plant level by using white fluorescent tubes installed above the culture.

Adventitious shoot proliferation: In the preliminary experiment, equimolar (8.87 μM) of different type of cytokinins viz., 6-benzylaminopurine (BA), 2-isopentenyladenine (2-iP) and kinetin were used in the MS medium for proliferation of shoots. In the second experiment, the better performing cytokinin BA was supplemented in different concentrations: 1.0, 2.0, 5.0 and 10.0 mg L^{-1} (equivalent to 4.43, 8.87, 22.17 and 44.35 μM, respectively). Data on shoot development and proliferation were evaluated after 5 weeks of culture induction.

Root induction: For induction of roots, regenerated microshoots were excised and transferred to half of the concentration of macronutrients supplemented with 1 μM (equivalent to 0.176 mg L^{-1}) IAA and 30 g L^{-1} sucrose to the MS medium. Cultures were incubated in the same growth conditions as provided during the multiplication phase. Root formation was evaluated after 4 weeks of culture.

Statistical analyses: Both the proliferation and rooting experiments were laid out in the Completely Randomized Design (CRD). Each of the treatments was replicated thrice and 10 culture tubes were used per replication. The data were analyzed using Statgraphics Plus Version 2.1 statistical program (STSC Inc., 1987) and the means were compared using Fisher's Least Significant Difference (LSD) test. All analyses were regarded as significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Effect of cytokinins on shoot proliferation: Among the cytokinins tested, BA showed a good response on shoot multiplication compared to 2-iP and kinetin (Fig. 1a). In both the cultivars, the maximum number of shoots per culture (3.4 in 'Bianca' and 3.16 in 'El Torro') were achieved from the medium containing BA whereas only 1 or rarely 2 shoots were formed per culture from the media with 2-iP and kinetin (Fig. 1a). Similar findings in different cultivars of roses has also been reported by Carelli and Echeverrigaray (2002), Kim *et al*. (2003) and Misra and Chakrabarty (2009) where they found BA enhanced more shoots per explant compared to 2-iP and kinetin. But in other studies, the maximum number of shoots per culture were obtained from the MS medium supplemented with kinetin in the range of 1-2 mg L^{-1} than BA (Ibrahim and Debergh, 2001; Kanchanapoom *et al*., 2010). Genotypical differences may be responsible for this contradictory results. It was also observed that 2-iP enhanced the elongation of shoots than BA. On the other hand, kinetin was unable to elongate the shoot and even the length of the shoots were shorter than the shoot developed from the medium lacking of cytokinin (Fig. 1b). The minimum length of the shoot in BA might be due to their higher proliferation of shoot which inhibited their elongation. Similar results in rose had been reported by Carelli and Echeverrigaray (2002) where the presence of BA significantly reduced the elongation of shoot compared to 2-iP and kinetin. The result is in accordance with those obtained in other rose cultivars by Horn (1992). Due to the ineffectiveness of kinetin, those did not elongate shoot in both the cultivars. This result is in contrary with that of Ibrahim and Debergh (2001) as they observed the maximum elongation of shoot in 1-2 mg L^{-1} of kinetin than BA. In the present study, kinetin had virtually no effect on shoot proliferation, even it inhibited elongation of shoots. Although 2-iP showed better shoot elongation but failed in the proliferation of shoots. This characteristic does not ensure rapid propagation of rose. Oppositely, the medium containing BA was found more effective in shoot proliferation and also showed moderate vigorous growth. Based on these results, BA was adopted and was tested in different concentrations in the following experiments.

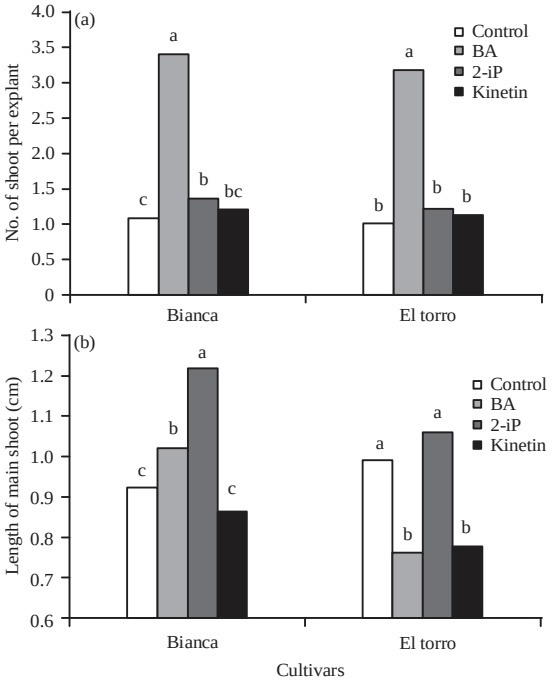

Fig. 1(a-b): (a) No. of shoots per explant and (b) Length of shoot of 'Bianca' and 'El Torro' in the multiplication phase grown in different types of cytokinins. Means followed by the same letter are not significantly different according to Fisher's least significant difference test ($p \leq 0.05$)

Effect of different concentrations of BA on shoot proliferation: Ability of multiplication of shoots in both the cultivars was significantly affected by different concentrations of BA (Table 1 and Fig. 2). Inclusion of BA in the medium enhanced the rate of multiplication and at 1.0 mg L^{-1} BA, on an average 2.81 and 1.52 shoots per culture were formed by 'Bianca' and 'El Torro', respectively. After increase in the concentration of BA, a gradual increase of shoots per culture was observed (Table 1 and Fig. 2). In 'Bianca', the maximum number of shoots per explant (4.22) was formed in the medium with 10.0 mg L^{-1} BA which was about 4 fold higher than the control treatment. Barna and Wakhlu (1995) also reported the maximum number of microshoots from the medium at the same concentration of BA (10.0 mg L^{-1}). But in 'El Torro' the highest proliferation of shoot (3.12) was obtained from 5.0 mg L^{-1} BA and with the further increase in the concentration of BA, the rate of multiplication was decreased (Table 1). This decrease in the shoot multiplication with the increased concentration of BA followed the same pattern of Kim *et al*. (2003) and Baig *et al*. (2011). Some roses are relatively more resistant to high concentration of BA but the desirable concentration mostly ranges between 1.0-2.0 mg L^{-1} (Roberts and Schum, 2003; Hameed *et al*., 2006; Senapati and Rout, 2008).

Effect of different concentrations of BA on the length of shoot: Considering the length of shoot, both the cultivars produced the tallest shoots from the medium containing 1.0 mg L^{-1} BA and 'Bianca' formed nearly 2 fold taller shoot than 'El Torro' in the same treatment (Table 1 and Fig. 2). It may be possible as genotypic differences could have perceptible influence on the growth performance of rose cultures (Al-Khalifah *et al*., 2005) with further increase in the concentration

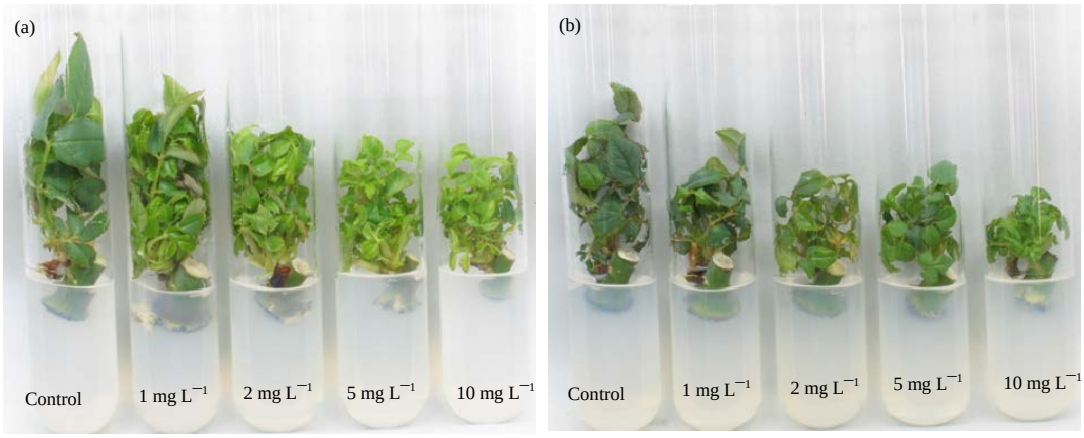

Fig. 2(a-b): Development of plantlets of (a) Bianca and (b) El Torro in the multiplication phase as affected by the different concentration of BA in the media

Table 1: Effect of different concentration of BA in MS medium

Concentration of BA (mg L^{-1})	No. of shoots per explant	Length of shoot (cm)	No. of leaves in the main shoot
Bianca			
Control	1.07^{e}	1.08^{d}	5.22^{c}
1.0	2.81^{d}	2.07^{a}	7.44^{a}
2.0	3.33^{c}	1.66^{b}	6.67^{b}
5.0	3.81^{b}	1.19^{c}	5.33^{c}
10.0	4.22^{a}	0.84^{e}	4.11^{d}
Lsd (0.05)	0.25	0.10	0.31
El Torro			
Control	1.00^{e}	1.01^{b}	5.52^{c}
1.0	1.52^{d}	1.11^{a}	7.72^{a}
2.0	2.08^{c}	0.83^{c}	6.60^{b}
5.0	3.12^{a}	0.73^{d}	6.24^{b}
10.0	2.80^{b}	0.67^{d}	5.08^{c}
Lsd (0.05)	0.20	0.07	0.45

In each column, means followed by the same letters are not significantly different according to Fisher's least significant difference ($p \leq 0.05$)

of BA, the length of the shoot decreased gradually which is in accordance with the findings of Carelli and Echeverrigaray (2002), Waseem *et al*. (2009) and Baig *et al*. (2011) where they mentioned that higher the concentration of BA reduced the length of shoot in different cultivar of roses. Generally, cytokinin stimulate shoot proliferation and inhibits their elongation of shoot and BA being a strong cytokinin depresses shoot length by an increase in number of axillary buds (Hameed *et al*., 2006) as all the nutrients are utilized for the formation of lateral shoots (Yakimova *et al*., 2000). In another report, Ahmad *et al*. (2003) explained that the higher concentration of BA increase the ethylene level in plants which block the basipetal transport of endogenous auxin in the shoots resulting in the minimum shoot length.

Effect of different concentrations of BA on the number of leaves in main shoot: Supplementation of BA was found to be synergistic for producing new leaves in both the cultivars and 1.0 mg L^{-1} of BA was the optimum concentration for obtaining the higher number of leaves in the main shoot (Table 1). The ability of producing new leaves of the culture decreased dramatically when the concentration of BA exceed 1.0 mg L^{-1} and the minimum number of leaves were obtained at higher concentration of BA (10.0 mg L^{-1}) in both the cultivars (Table 1). Hence, the higher

concentration of BA in the MS medium had adverse effects on the number of leaves per culture in both the cultivars. This result confirms the findings of Marcelis-van Acker and Scholton (1995) and Khosravi *et al*. (2007) as they also noticed a descending order in leaf number after exceeding the optimum level of BA in the MS medium.

Effect of different concentrations of BA on the fresh and percentage of dry weights per explant: Fresh and percentage of dry weight of explants were significantly influenced by the different concentration of BA used in the MS medium (Table 2). In both the cultivars, the maximum fresh weight was gained from the medium with 1.0 mg L^{-1} BA (Table 2). It is logical, since the higher number of leaves and the maximum elongation of shoot occurred in the this treatment (1.0 mg L^{-1}). A similar response to fresh weight of roses has been reported by Baig *et al*. (2011). As the concentration of was BA raised, the fresh weight was decreased and the lowest fresh weight was found at the higher concentration of BA. (10.0 mg L^{-1}, Table 2) which confirms the report of Marcelis-van Acker and Scholton (1995). Percentage of dry weight accumulation also showed synergism to fresh weight and best response in dry matter accumulation as was observed in the medium lacking BA followed by the culture supplemented with 1.0 mg L^{-1} BA (Table 2). Considerable declining in the percentage of dry matter was observed when explants were grown on the higher concentration of BA (Table 2). This is not surprising as higher concentration of cytokinin is known to induce hyperhydricity in *in vitro* raised culture which reduce the percentage of dry matter in the plant. It was observed that the incidence of hyperhydricity only occurred in the plantlet grown on higher concentration of BA in both the cultivars while no evidence of hyperhydricity was found in the control and lower concentrations of BA (1.0 mg L^{-1}, Table 2). Although increasing level of BA enhanced the shoot proliferation but had an inhibitory effects on shoot elongation and number of leaves in the plantlet. Moreover, the high level of BA also favoured the risk of hyperhydricity in rose plants and might be less effective in subsequent rooting of the multiplied shoots. Therefore, an experiment was subsequently conducted to determine the optimal concentration of BA for rooting in multiplied shoots.

Residual effect of BA on the percentage of rooting: In 'Bianca' all the treatments except the MS medium with higher concentration of BA (10.0 mg L^{-1}) used during multiplication phase produced roots but 'El Torro' did not response at both 5.0 and 10.0 mg L^{-1} BA (Table 3). In fact, the optimum level of each type of growth regulator in the culture medium may differ greatly according to the kind of plant or culture being used, the culture conditions and the compounds used. However,

Table 2: Effect of different concentration of BA on shoot growing parameters of 'Bianca' and 'El Torro'

Concentration of BA (mg L^{-1})	Fresh weight per plantlet (mg)	Dry weight per plantlet (%)	Hyperhydricity (%)
Bianc			
Control	473.31^{d}	14.34^{a}	0.00^{d}
1.0	1006.42^{a}	9.34^{b}	0.00^{d}
2.0	903.57^{b}	9.31^{b}	10.48^{c}
5.0	788.76^{c}	8.58^{c}	22.40^{b}
10.0	472.93^{d}	8.23^{c}	35.65^{a}
Lsd (0.05)	24.89	0.36	0.49
El Torro			
Control	274.67^{c}	21.98^{a}	0.00^{d}
1.0	361.73^{a}	17.32^{b}	0.00^{d}
2.0	288.71^{b}	16.76^{b}	15.64^{c}
5.0	237.29^{d}	15.91^{b}	25.33^{b}
10.0	236.70^{d}	13.60^{c}	48.22^{a}
Lsd (0.05)	11.90	1.38	0.27

In each column, means followed by the same letters are not significantly different according to Fisher's least significant difference ($p \leq 0.05$)

Table 3: Residual effect of BA on root growing parameters of 'Bianca' and 'El Torro'

Concentration of BA (mg L^{-1})	Rooting (%)	No. of roots	Total length of roots (cm)
Bianca			
Control	31.25^{d}	1.67^{c}	4.02^{bc}
1.0	71.25^{b}	2.00^{b}	4.59^{b}
2.0	91.25^{a}	2.56^{a}	5.84^{a}
5.0	36.25^{c}	1.44^{d}	3.24^{c}
10.0	0.00^{e}	0.00^{e}	0.00^{d}
Lsd (0.05)	3.37	0.04	0.79
El Torro			
Control	21.25^{c}	1.00^{c}	0.75^{b}
1.0	26.25^{b}	1.04^{b}	1.30^{a}
2.0	51.25^{a}	1.35^{a}	1.35^{a}
5.0	0.00^{d}	0.00^{d}	0.00^{c}
10.0	0.00^{d}	0.00^{d}	0.00^{c}
Lsd (0.05)	2.92	0.05	0.34

In each column, means followed by the same letters are not significantly different according to Fisher's least significant difference ($p \leq 0.05$)

the medium without BA during the multiplication phase gained lowest percentage of rooting (31.25%) in 'Bianca' and after that the rate of rooting in plantlets was increased up to 91.25% in the medium with 2.0 mg L^{-1} BA. Similarly, 'El Torro' also produced higher (51.25%) and lower percentages of roots (21.25%) from the medium containing 2.0 mg L^{-1} and without BA used during multiplication phase, respectively. The results indicated that different concentrations of BA applied during the multiplication phase markedly influenced the rooting of plantlet and the percentages of rooting decreased in both the cultivars with an increased level of BA added during the multiplication phase (Table 3). This result supported the findings of Bressan *et al.* (1982) where the number of roots per explants decreased with the increasing level of BA as the multiplication phase. They attributed this effect to the accumulated in the tissue, since root initiation may be inhibited if the endogenous cytokinin level is too high. Podwyszynska and Hempel (1988) also reported similar result where shoot of roses formed on the media with smaller amount of BA, rooted and better than shoots from the media with higher amount of this compound. Earlier Hasagawa (1980) reported that BA concentration which are commonly used to attain optimum shoot multiplication in roses inhibited root formation even at the lower concentration. But De Klerk *et al.* (2001) suggested that the low level of cytokinin could be useful for rooting in apple shoots.

Residual effect of BA on the number and the total length of roots per plantlet: The development of roots, as evaluated by the number and total length of roots, was significantly affected by the different concentrations of BA used during the multiplication phase (Table 3). It was observed that the regeneration and growth of roots were higher in 'Bianca' compared to 'El Torro' (Table 3). The result also showed that during the multiplication phase, the minimum amount of BA was necessary for the formation of roots in plantlets but the higher concentration of BA inhibited the formation of roots in both the cultivars (Table 3). Plantlets of both the cultivars grown in the medium with 2.0 mg L^{-1} BA during the multiplication phase showed the maximum number of roots and higher length of roots than plantlets grown in other concentrations of BA (Table 3). These results do not support the findings of Asadi *et al.* (2009) where they did not observed any root of rose cv. 'Morrasia' in the medium with 2.0 mg L^{-1} BA but found higher number of longer roots in the medium without BA used during the multiplication phase. However, the number of roots per plantlet linearly decreased with the increase of BA concentration used in the multiplication phase. The effect may be due to the persistence of cytokinins in the tissues creating an unsuitable hormonal balance in this phase (Kanakis and Demetriou, 1993).

Based on the results concerning the multiplication as well as the rooting phase, it could be concluded that the medium supplemented with 1.0 mg L^{-1} BA was appropriate for the cultivation of 'Bianca' and 'El Torro' in the multiplication phase. However, in the rooting phase, plants grown in this concentration of BA (1.0 mg L^{-1}) produced lower number of roots as well as total root length per plantlet compared to 2.0 mg L^{-1} BA used in medium during the multiplication phase. Therefore, 2.0 mg L^{-1} level of BA was found to be better for root formation among all concentrations of BA used during the shoot multiplication phase in both the cultivars. Concerning the two cultivars observed in the experiments, 'Bianca' developed roots easily in *in vitro* conditions and also it showed a better growth in the *in vitro* condition compared to the cv. 'El Torro', being a cultivar easy-to-micropropagate. In contrast, the cv. 'El Torro' could be considered a cultivar difficult-to-root in *in vitro* conditions as well as a cultivar difficult-to-micropropagate.

ABBREVIATIONS

2-iP	:	2-isopentenyladenine
ANOVA	:	Analysis of Variance
BA	:	6-Benzylaminopurine
cvs.	:	Cultivars
IAA	:	Indole Acetic Acid
MS	:	Murashige Skoog

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