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Clonal Propagation of Different Cultivars of *Pelargonium graveolens* (L' Herit.) viz., Reunion, Bourbon and Egyptian

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Abstract: *Pelargonium graveolens* is a highly valued aromatic plant mainly grown for its rose like essential oil called Geranium oil. In India different cultivars of *Pelargonium graveolens* are cultivated in varied agroclimatic zones. Algerian cultivar is grown in North Indian plains while Bourbon cultivar is grown in hilly regions of Southern India. These varieties are very difficult to differentiate morphologically. But they could be differentiated by Gas chromatography on the basis of relative proportion of Geraniol and Citronellol. Always Geraniol rich varieties are preferred which impart rosy smell to the essential oil. The germplasm collection of Kelkar Education Trust's Scientific Research Centre has identified a Geraniol rich variety called Reunion which is suitable for commercial cultivation in the plateau region of Maharashtra. A protocol for clonal propagation for Reunion cultivar was established using nodal explant. Requirement of growth regulators for shoot initiation, shoot proliferation and root induction for all the three cultivars viz., Reunion, Bourbon and Egyptian was compared. Further the *in vitro* raised plantlets were hardened successfully.

Key words: *Pelargonium graveolens*, clonal propagation, nodal explants, shoot cultures, root induction, hardening

INTRODUCTION

Pelargonium graveolens, also known as rose scented Geranium (Family-Geraniaceae) is highly valued aromatic plant native to Cape Province of South Africa. It is mainly grown for its essential oil called Geranium oil which has pronounced rose like odor (Gunther, 1950). The rosy odor of the oil is primarily due to chief constituents Geraniol and Citronellol often known as rose alcohols that occur in varying proportions depending upon the origin of oil (Mahindru, 1992). Secondary constituents, which contribute to the olfactory value of commercial Geranium oil are 6, 9-guaidiene and 10-epi-γ-eudesmol which are ranked highest (Lawrence, 1984).

Geranium oil is one of the top 20 essential oils in the world which has wide application in perfumery, cosmetics and flavour industry. It forms a part of many high grade perfumes (Douglas, 1969). It has antifungal (Wollman *et al*., 1973), insect repellant (Osmani *et al*., 1974; Osmani and Sighamony, 1980) and antibacterial (Aggarwal *et al*., 2000) activity because of which it finds extensive use in medicinal and agrochemical field. The oil has a wide application in aromatherapy (Ranade, 1988) and food product industry (Leung, 1980).

Against its own requirement of approximately 200 tonnes, India produces less than 20 tonnes of Geranium oil annually and meets its requirement by imports (Navale and Mungse, 2002). Mild climate with low humidity is ideal for its growth. On the other hand high humidity, heavy rainfall with mist, fog and water logging are found to be detrimental (Ram and Kumar, 1996).

In India three cultivars of *P. graveolens* namely Algerian (Hemanti), Bourbon (Bipuli) and Egyptian (Kunti) are cultivated commercially (Rajeswara-Rao and Bhattacharya, 1992). Among these cultivars Algerian variety is grown in Northern Plains of Uttar Pradesh while Bourbon variety is recommended for plantation in Southern India especially Nilgiris (Rajeswara-Rao, 2000). Gas chromatographic analysis of the three cultivars show variation in the composition of the essential oil. The Geraniol:Citronellol (G/C) ratio in Algerian cultivar is 1:3 which imparts strong citrusy note to the oil due to which it resembles Chinese type of oil. This ratio decreases to 1:2 in Bourbon oil giving it the desired rosy note that makes the oil similar to African type of oil. In Egyptian cultivar this ratio still decreases to 1:1. The other components that are affecting the rosy note of oil were 6-9-guiadiene and 10-epi-γ-eudesmol (Gupta *et al*., 2002).

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In Maharashtra, the plateau region with low humidity is suitable for Geranium cultivation. Germplasm collection of Kelkar Education Trust's Scientific Research Center, Mulund (West), Mumbai, has identified a Geraniol rich variety of *P. graveolens* called Reunion which could be propagated by tissue culture and could be made available to farmers for its commercial cultivation in Maharashtra. Chemically Reunion was found to be the best clone with 1:1 G/C ratio and showed absence of 10-epi-γ-eudesmol. This cultivar was also successfully differentiated at molecular level with RAPD analysis (Tembe, 2009). Hence it was decided to undertake large scale cultivation of Reunion cultivar.

Though there are several reports on *in vitro* regeneration of *P. graveolens* (Charlwood and Charlwood, 1991; Lakshmana-Rao, 1994; Rajan *et al*., 1997) many of them consists of indirect regeneration through intervention of callus. The resulting calliclones lead to somaclonal variation (Saxena *et al*., 2000, 2008). Present communication discusses a protocol for clonal propagation of three cultivars of *P. graveolens* viz., Reunion, Bourbon and Egyptian.

MATERIALS AND METHODS

Plant material: The three cultivars viz., Reunion, Bourbon and Egyptian (Fig. 1a) of *P. graveolens* were collected from Germplasm collection of Medicinal and Aromatic Plant Garden, Kelkar Education Trust's Scientific Research Center, Mulund (West), Mumbai (March 2006-April 2009). Nodal explants collected from these cultivars were used for tissue culture experiments.

Surface sterilization: The nodal segments were first thoroughly washed under tap water. The explants were then immersed in Teepol® for 5 min followed by Dettol® for 5 min. The explants were then thoroughly washed and kept under running tap water for half an hour to remove traces of detergents. The subsequent treatment was carried out in the laminar airflow cabinet. The explants were first treated with 0.1% (w/v) systemic fungicide Bavistine® (BASF India Ltd, India) for 20 min followed by 3 successive washes of sterile distilled water. The next treatment was of 0.1% (w/v) mercuric chloride for 5 min followed by 3 successive washes of sterile distilled water. The exposed parts of the explants were trimmed. They were then treated with Ciplox ® for 1 min and were further used for inoculation.

Media: The presterilised nodal segments were cultured on Nitsch and Nitsch medium (NN) (Nitsch and Nitsch, 1965) supplemented with 3% (w/v) sucrose and growth regulator viz., 6-benzylaminopurine (BAP) (0-15.4 μM). The pH of the medium was adjusted to 5.7±0.02 with 0.1N NaOH or 0.1N HCl as per the requirement. The medium was solidified using 0.8% (w/v) agar (Bacteriological grade, Qualigens, Mumbai). All the chemicals used were from Loba Chemie and of AR grade. About 25 mL media was dispensed in Pyrex glass Borosil® culture tubes (25×150 mm). The tubes were covered with tight fitting plastic caps. The culture tubes were autoclaved at 1.05 kg cm^{-2} for 15 min.

Culture conditions: The cultures were maintained at 25±2°C under 16 h photoperiod provided by the cool-white fluorescent tubes.

Establishment of *in vitro* cultures

Establishment of multiple shoot cultures: For shoot initiation, nodal segments of all the three cultivars viz., Reunion, Bourbon and Egyptian were inoculated on NN medium supplemented with different concentrations of BAP (0-15.4 μM). They were subcultured after every 30 days for 5 months. At the end of 12 weeks, the number of shoots produced per explant in each concentrations was scored and the data was analyzed by ANOVA.

Establishment of rooted plantlets: After about 5 subcultures in shoot multiplication medium, the regenerated shoots about 0.5-1.0 cm in length were transferred to ½ NN medium supplemented with Indole-3-butyric acid (IBA) (0-2.45 μM). The rooting behaviour was noted. The rooted plantlets were then subjected to acclimatization.

Establishment of hardened plants: The rooted plantlets of all the three cultivars were removed from tubes and were washed under running tap water to remove traces of agar. They were treated with 1% (w/v) Bavistine® for 1 min. Then they were potted in potting mixture containing cocopeat and sand (1:1), thoroughly drenched with water. The potted plants were covered with polycaps to prevent dessication and were kept in the green house where the humidity was 80-90% and the temperature was 26±2°C. The plants were watered after 4 days and at the same time water from the polycaps was drained to avoid water logging. After about 12 days the polycaps were removed and the plants were sprayed with 1% (w/v) Bavistine®. Then the plants were shifted in the green house where the humidity was 70-80% and the temperature was 30±2°C. After 20 days the plants were repotted in bigger pots containing mixture of sand, soil and compost (2:2:1). The plants were watered and sprayed with fungicide Bavistine® and fertilizer Biozyme® alternately after every 4 days for 2 months. After 2 months the plants were

transferred to shade house. They were maintained in the shade house for 1 more month under similar conditions. After 1 month they were transferred to bigger earthen pots. Here they were maintained for 2 months under similar conditions.

RESULTS AND DISCUSSION

Initially Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) was tried for establishment of shoots. However the regenerated shoots showed browning in MS medium (Fig. 1b). Whereas in NN medium the regenerated shoots appeared healthy (Fig. 1c). Hence, for further experiments, instead of MS medium, we have used NN as basal medium.

Chemically NN medium shows variation in concentration of different components as compared to MS medium. The concentration of macro nutrients in NN medium is low as compared to MS medium. Among micro nutrients Iodine and Cobalt are absent in NN medium. Similarly the amount of vitamins like Nicotinic acid and Thiamine is high as compared to MS medium. Also NN medium shows presence of Biotin and Folic acid which are totally absent in MS medium.

For studying the effect of growth regulators on shoot initiation a range of BAP (0-15.4 μM) was selected. After

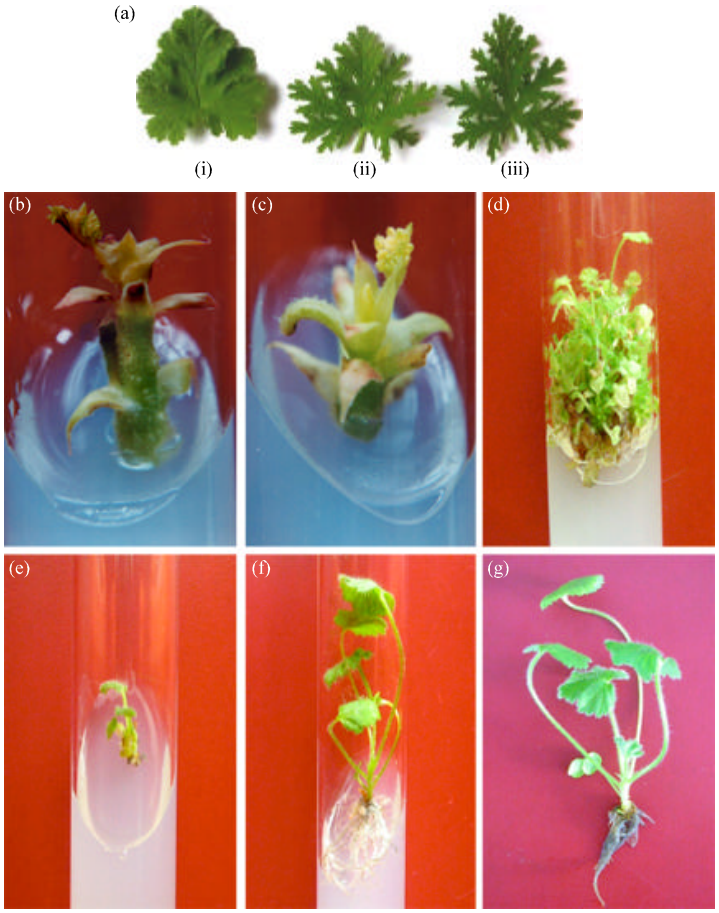

Fig. 1: (a) Difference in leaf morphology of various cultivars of *Pelargonium graveolens* (L' Herit.). (i) Cultivar: Reunion. (ii) Cultivar: Bourbon. (iii) Cultivar: Egyptian. (b) Effect of basal medium viz., MS + 8.80 μM BAP on *in vitro* shoot regeneration from nodal explants of *Pelargonium graveolens* (L' Herit.). (c) Effect of basal medium viz., NN + 8.80 μM BAP on *in vitro* shoot regeneration from nodal explants of *Pelargonium graveolens* (L' Herit.). (d) Multiple shoot proliferation from nodal explants of *Pelargonium graveolens* (L' Herit.); Cultivar: Reunion on NN + 8.80 μM BAP at the end of 3 months. (e) *In vitro* shoot of *Pelargonium graveolens* (L' Herit.); Cultivar: Reunion just inoculated in rooting medium viz., ½ NN + 0.49 μM IBA. (f) *In vitro* root induction in *Pelargonium graveolens* (L' Herit.); Cultivar: Reunion on ½ NN + 0.49 μM IBA. (g) Fully grown plantlet of *Pelargonium graveolens* (L' Herit.); Cultivar: Reunion at the end of 2 months

Table 1: Effect of various concentrations of BAP on shoot regeneration in different cultivars of *Pelargonium graveolens* (L' Herit) viz., Reunion, Bourbon and Egyptian

	Mean number of shoots per explant±SE		
BAP (μM)	Reunion	Bourbon	Egyptian
0	0±0	1.8±0.491	1.2±0.223
2.20	20.1±0.215	19.2±0.374	18.8±0.491
4.40	20.4±0.240	23.2±0.491	20.4±0.245
6.60	29.1±0.564	28.2±0.665	30.2±0.374
8.80	35.4±0.449*	31.4±0.424*	34.2±0.584*
11.0	25.0±0.579	21.4±0.245	26.8±0.736
13.2	18.7±0.201	18.0±0.317	25.4±0.412
15.4	20.3±0.402	20.8±0.374	22.4±0.814

Data recorded after 12 weeks (n = 30). *Significantly different ($p<0.05$) compared to response in other culture media

about 3 subcultures in the same medium the mean number of shoots per explants was scored and tabulated (Table 1). It was observed that in the medium without BAP, a single axillary shoot developed from nodal explant. With the incorporation of BAP in the basal medium, this axillary shoot shriveled up and many shoot buds developed from axillary meristem. For Reunion cultivar in medium containing 2.20 μM BAP the mean number of shoots was 20.1. As concentration of BAP in the medium was increased from 2.20 μM to 8.80 μM, there was significant increase in mean number of shoots per explant from 20.1 to 35.4 (Table 1). However further increase in BAP concentration from 8.80 μM upto 15.4 μM resulted in significant decrease in mean number of shoots from 35.4 to 20.3. For all cultivars, 8.80 μM BAP was found to be the most favourable concentration for shoot initiation and proliferation. At this concentration after 3 months of incubation the mean number of shoots observed in Reunion was 35.4, in Bourbon it was 31.4 and in Egyptian it was found to be 34.2. Figure 1d exhibits the *in vitro* multiple shoot cultures of *P. graveolens*; cultivar Reunion.

Satyakala *et al.* (1995) required Indole-3-acetic acid (IAA) (5.71 μM) and Gibbrellic acid (GA3) (1.44 μM) supplementation along with Kinetin (Kin) (4.60 μM) for maximum shoot proliferation. Similarly Gupta *et al.* (2002) added 1-naphthalene acetic acid (NAA) (0.54 μM) and Adenine disulphate (ADS) (5.43 μM) along with BAP (11 μM) for optimum shoot proliferation i.e., 36.6 shoots per explant. However in our experiments we found that BAP (8.80 μM) alone is favourable for optimum shoot initiation and multiplication producing 35.4, 31.4 and 34.2 shoots per explant in Reunion, Bourbon and Egyptian cultivar respectively. Supplementation of auxins like NAA resulted in callus formation at nodal region and shoot initiation occurred through the callus intervention. Also the callus formation increased with increase in NAA concentration from 0 to 5.37 μM (Tembe, 2004).

Table 2: Effect of various concentrations of IBA on root induction in different cultivars of *Pelargonium graveolens* (L' Herit) viz., Reunion, Bourbon and Egyptian

Cultivar	IBA (μM)	Rooting response (%)	Mean No. of roots±SE	Mean length of roots (cm)±SE
Reunion	0	0	0	0
	0.25	50	2±0.231	1.5±0.467
	0.49	90	5±0.563*	2.5±0.325*
	0.98	50	2±0.435	1.5±0.224
	2.45	28	1±0.332	1.0±0.235
Bourbon	0	0	0	0
	0.98	91	12±0.564*	1.8±0.325*
	2.45	86	10±0.676	0.5±0.536
Egyptian	0	0	0	0
	0.98	83	10±0.347	0.7±0.673
	2.45	90	14±0.651*	2.2±0.453*

Data recorded after 8 weeks (n = 30). *Significantly different ($p<0.05$) compared to response in other culture media

In our initial experiments (Tembe, 2004) for Bourbon, root induction occurred in ½ NN medium with 0.98 μM IBA after about 8 days, whereas for Egyptian comparatively more concentration of IBA (2.45 μM) was required for rooting (Table 2). Period required for root initiation was also more i.e. 12 days. About 91 and 90% shoots of Bourbon and Egyptian cultivar showed rooting, respectively. Hence similar concentrations were tried for rooting in Reunion cultivar.

After about 5 subcultures in shoot multiplication medium, *in vitro* shoots of Reunion cultivar about 0.5-1.0 cm in length were transferred to rooting medium (Fig. 1e). Initially ½ NN with 0.98 and 2.45 μM IBA was tried. At 2.45 μM IBA only 28% of the shoots rooted, the mean number of roots was also very less i.e. 1. However, at 0.98 μM IBA rooting response was 50% and mean number of roots was 2.

Hence, for further studies lower concentrations of IBA viz., 0, 0.25 and 0.49 μM were tried. Root initiation was best seen in ½ NN with 0.49 μM IBA. About 90% shoots showed rooting after 15 days. At the end of 2 months the mean number of roots was 5 per shoot and mean length of roots was 2.5 cm (Table 2) (Fig. 1f). When concentration of IBA was still reduced to 0.25 μM only 50% of shoots rooted. Also the mean number of roots per shoot was 2 and mean length of roots was 1.5 cm. Further at 0 μM of IBA there was no rooting. Figure 1g depicts the fully grown rooted plantlets of Reunion cultivar of *P. graveolens*.

For root induction ½ NN medium supplemented with 2.45 μM IBA was the most favourable medium for Egyptian variety. At this concentration 90% rooting was observed. For Bourbon rooting occurred well at comparatively lower concentration i.e., 0.98 μM IBA. At this concentration rooting response was 91% whereas at 2.45 μM IBA for Bourbon rooting response was 86%. For Reunion, amount of IBA required was still lower. The

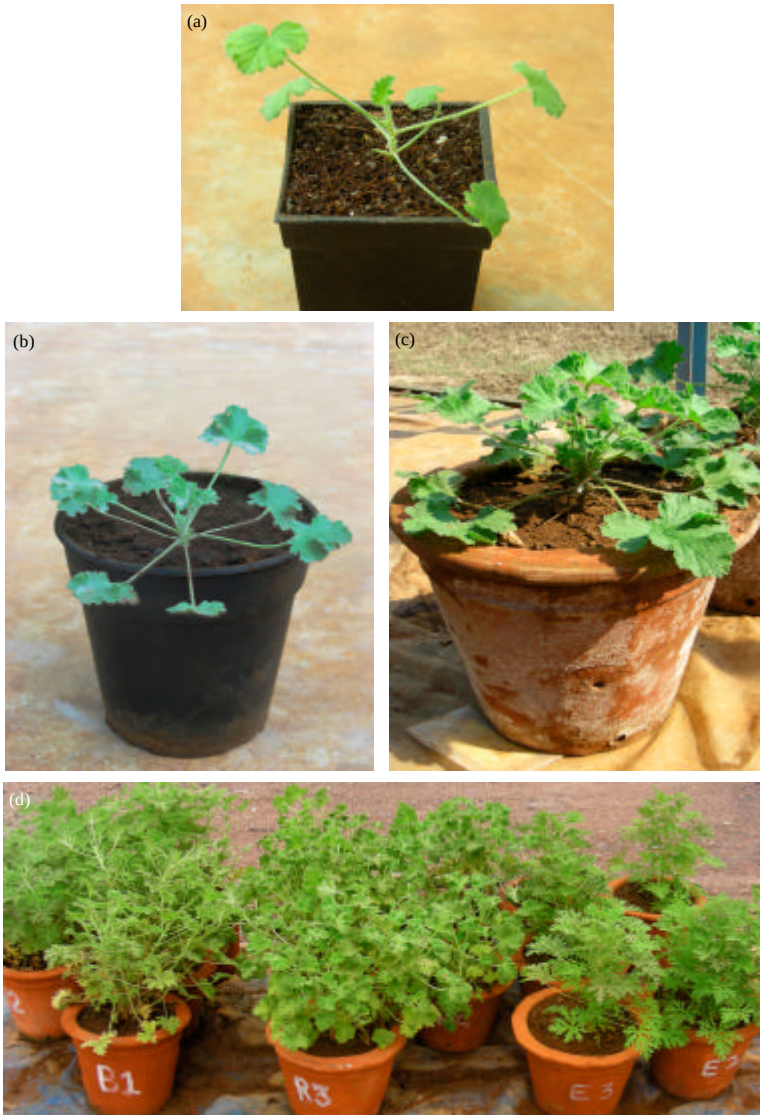

Fig. 2: (a) Twenty days old hardened plant of *Pelargonium graveolens* (L' Herit.); Cultivar: Reunion growing in cocopeat and sand (1:1). (b) 3 months old hardened plant of *Pelargonium graveolens* (L' Herit.); Cultivar: Reunion transferred to sand, soil and compost (2:2:1). (c) 4 months old hardened plant of *Pelargonium graveolens* (L' Herit.); Cultivar: Reunion transferred to shade house in pots containing sand, soil and compost (2:2:1). (d) 6 months old hardened plants of *Pelargonium graveolens* (L' Herit.) ready for field transfer

highest rooting response i.e., 90% was observed at 0.49 μM. As the concentration of IBA increased, comparatively less rooting was observed. At 0.98 μM rooting response was 50% and at 2.45 μM it was 28%. Similar observations were made by Gupta *et al.* (2002). For Bourbon variety, as the concentration of IBA was increased from 2.45 to 4.90 μM the number of shoots that rooted decreased from 80 to 40%. Contrary to this in case of Egyptian, as concentration of IBA increased from 2.45 to 4.90 μM the number of rooted shoots increased from 73% to 88%.

Gupta *et al.* (2002) required comparatively higher concentration of IBA for rooting. It was as high as 2.45 and 4.90 μM. Percentage rooting was also not more than 80%. Similarly, Satyakala *et al.* (1995) in their experiments observed that 2.45 μM IBA was most favourable for rooting of *in vitro* shoots in *P. graveolens*. In our experiments a lower concentration of auxin was

required for rooting. This might be because of no auxin supplementation and presence of comparatively less concentration of cytokinin in shoot establishment medium.

For all the three cultivars it was observed that before transferring to rooting medium the shoot initials were very small (0.5-1.0 cm). However the internodal length and leaf lamina size increased substantially after the emergence of first root (Fig. 1e, f). This was prominently observed in case of Reunion.

Compared to Bourbon and Egyptian, Reunion cultivar required more number of days for root initiation and internodal elongation *in vitro*. The entire plantlet of Reunion cultivar required 2 months of maintenance on rooting medium (Fig. 1g) unlike that of Bourbon and Egyptian where the entire plantlet regeneration was achieved by 1 month maintenance on rooting medium.

A mixture of cocopeat and sand (1:1) was used for initial acclimatization of plants. The plants survived and acclimatized well in the above potting mixture (Fig. 2a). The survival percentage was 98, 95 and 90% for Reunion, Bourbon and Egyptian respectively. Later the plants were maintained in sand, soil and compost (2:2:1) for further growth with 100% survival rate (Fig. 2b). On transferring to shade house the plants showed vigorous growth (Fig. 2c). At the end of 6 months they were ready for field cultivation (Fig. 2d).

It was observed that though Reunion showed slow growth as compared to Bourbon and Egyptian in *in vitro* conditions, its hardening phase was more vigorous (Fig. 2a). The first new leaf emergence was observed after 4 days in Reunion whereas for Bourbon and Egyptian it was seen after 8 days of hardening.

Watering the plants at an interval of 4 days resulted in fast growth of plants. Water logging due to excess watering and transpiration was found to be detrimental for plant growth. Shifting of plants from higher humid area (80-90%) to a lower humid area (70-80%) within the green house also facilitated the healthy growth of plants (Fig. 2b). The foliage growth increased drastically as soon as the plants were shifted to shade house where the conditions were dry (Fig. 2c). The plants were ready for field transfer at the end of 6 months (Fig. 2d).

Thus in the present study a protocol for clonal propagation of selected elite cultivar of *P. graveolens* viz., Reunion was established so that these could be made available for commercial cultivation to the farmers in Maharashtra.

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