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Micropropagation of *Homalomena pineodora* Sulaiman & Boyce (Araceae): a new species from Malaysia

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ABSTRACT

Homalomena pineodora (family Araceae) is a species found to have impressive foliage characteristics which remain evergreen throughout the year. Therefore, *H. pineodora* can be grown as an ornamental plant. Generally *H. pineodora* needs 3-5 years to propagate and multiply. However, the demand for new ornamental plants is increasing worldwide and the quality of planting material is a basic need for boosting productivity. Therefore an efficient micropropagation protocol for large-scale production of *H. pineodora* was developed. *In vitro* shoot cultures were initiated from the rhizomatous buds on MS basal medium. The best conditions for propagating *H. pineodora* was found to be MS medium supplemented with 3% sucrose and 0.5 mg L^{-1} BA (6-benzyladenine) under 24 h of cool fluorescent light which produced an average of 3.8 shoot per explant. Presence of an auxin was not necessary for plantlet production. Liquid MS medium supplemented with 0.5 mg L^{-1} BA, enhanced the shoot production of *H. pineodora* as compared to agar-gelled medium with same composition. All the *in vitro* plantlets of *H. pineodora* were successfully acclimatized with 100% survival rate. Scanning electron microscopy confirmed the similarity of leaf microstructures between the *in vitro* and mother plants of *H. pineodora*.

Keywords: *Homalomena pineodora*, acclimatization, scanning electron microscopy, cytokinin, *in vitro* culture, shoot proliferation.

RESUMO

Micropropagação de *Homalomena pineodora* Sulaiman & Boyce (Araceae): uma nova espécie da Malásia

Homalomena pineodora (família Araceae) é uma espécie que apresenta como característica a folhagem perene durante todo o ano. Portanto, H. pineodora pode ser cultivada como planta ornamental. Geralmente H. pineodora necessita 3-5 anos para sua propagação e multiplicação. No entanto, a demanda por novas plantas ornamentais vem aumentando no mundo e a qualidade do material de plantio é uma necessidade básica para aumentar a produtividade. Com essa finalidade foi desenvolvido um eficiente método de micropropagação para produção em larga escala de *H. pineodora*. Foram desenvolvidos cultivos *in vitro* de gemas dos rizomas em meio MS. As melhores condições para propagação de *H. pineodora* foram obtidas com o emprego do meio MS, acrescido de 3% de sacarose e 0,5 mg L^{-1} de BA (6-benziladenina) sob 24 h de luz fluorescente, sendo produzidas uma média de 3,8 brotos por explante. O uso de auxina se mostrou dispensável na produção de plântulas. O uso do meio MS na forma líquida, suplementado com 0,5 mg BA L^{-1}, foi mais eficiente na produção de explantes de *H. pineodora*, em comparação com o mesmo meio na forma de agar-gelificado. Todas as plântulas de *H. pineodora* foram aclimatizadas com successo, com 100% de taxa de sobrevivência. A microscopia eletrônica de varredura confirmou a semelhança da microestrutura de folhas das plântulas obtidas *in vitro* e das plantas mãe de *H. pineodora*.

Palavras-chave: *Homalomena pineodora*, aclimatização, microscopia eletrônica de varredura, citocinina, cultivo *in vitro*, reprodução de explantes.

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The family Araceae is most abundantly represented by the genus *Homalomena* which comprises approximately 120 species. Most of the *Homalomena* species are distributed in South and Central America, the Mexican lowlands, the Caribbean islands, southern Florida and the Asian tropical areas (Hay & Herscovitch, 2002; Bown, 2000). Most of the *Homolomena* species are found in tropical climates. However, many aroids, including *Homalomena* species, are native to temperate climates (Mayo *et al.,* 1997). Species of genus *Homalomena* have been used for traditional medicinal purposes. *H. coerulescens* was used to treat various kinds of skin diseases. *H. griffltthii* was found to accelerate childbirth and relieve backache. *H. purpurascens* was used to relieve hoarseness while *H. sagillifolia* was reported to treat fever and abdominal flatulence. *H. occulta* was commonly used to relieve pain and edema due to traumatic injury (Sulaiman & Mansor, 2002; Bensky & Gamble, 1993). The oil extracted from the rhizome of *H. aromatica* showed significant antifungal activity against *Fusarium graminearum* (Singh *et al.*, 2000). The natives of Papua New Guinea eat the leaves of *Homalomena* species combined with the leaves and bark of *Galbulimima belgraviana* as a narcotic (Schultes, 1970). A few species, such as *H. philippinensis* and *H. rubescens* are used as ornamental plants.

Homalomena pineodora, a dwarf and creeping aroid plant of about 25 cm in height, was found in Peninsular Malaysia and described as a new species by Sulaiman & Boyce (2005). Leaves of *H. pineodora* are dark green in color, heart shaped with smooth and glossy appearance and clustered towards the shoot tip. *H. pineodora* was recommended as an ornamental plant because of its impressive large

leaves. This plant was found to remain evergreen throughout the year. They also observed that *H. pineodora* did not propagate and multiply for up to 3-5 years. The demand for new ornamental plants is increasing worldwide and the quality of planting material is a basic need for boosting productivity. If *H. pineodora* is to be recommended as an ornamental plant, the growers will face the problem of getting sufficient plants due to its slow propagation rate. If this plant is to be harvested from its natural habitat without control, it will lead to eventual extinction of this species. Therefore, development of an efficient micropropagation protocol was proposed in this paper for the conservation and rapid propagation of this species.

MATERIAL AND METHODS

Plant materials and culture conditions - Plants of *H. pineodora* were grown under greenhouse conditions at the School of Biological Sciences, Universiti Sains Malaysia, Penang. Rhizomatous stem explants (2 cm) were excised from the mother plants and washed thoroughly with detergent and kept under running tap water for one hour. The explants were immersed in 90% ethanol for 30 seconds and rinsed with sterile distilled water. They were then surface-sterilized with 100 mg L^{-1} (w/v) $HgCl_2$ solution for 15 minutes, followed by rinsing three times with sterile distilled water. They were again surface-sterilized with 15% Clorox® (containing 5.3 % sodium hypochlorite, NaClO) solution added with 2-3 drops of Tween-20 for another 10 minutes and again rinsed three times with sterile distilled water. The surface-sterilized rhizomatous stems were trimmed to remove the damaged and dead tissue and cut into small pieces, each piece containing one bud. The explants were inoculated into 350 mL glass jars containing Murashige & Skoog (MS) medium (1962), supplemented with 30 g L^{-1} sucrose and 0.8% agar (Algas, Chile) for four weeks. The pH of the medium was adjusted to 5.7-5.8 prior to autoclaving at 121°C at 1.06 kg cm^{-2} for 11 minutes. All the cultures were maintained at 25±2°C in a culture room with continuous lighting provided with cool, white fluorescent tubes at 35 µmol m^{-2} s^{-1}.

Effect of 6-benzylaminopurine (BA) on shoot proliferation of *H. pineodora* - In order to determine the effect of BA on shoot proliferation of *H. pineodora,* the aseptic shoot explants were inoculated into 350 mL glass jars containing MS medium supplemented with different concentrations of BA (0.0-10 mg L^{-1}) in a complete randomized design. Six replicates were used for each concentration of BA and the experiment was repeated twice. Number of shoots produced from each explant was recorded after 4, 8 and 12 weeks of culture.

Since the result obtained indicated that the addition of 2 mg L^{-1} BA was sufficient to induce multiple shoot formation from the bud explants, another experiment was carried out to effect the low concentration of BA on shoot proliferation. The shoot explants were inoculated in 350 mL glass jars containing MS medium supplemented with different concentrations of BA (0.0-2.0 mg L^{-1}) in a complete randomized design for 4 weeks.

Effect of combination of BA and 3-indole butyric acid (IBA) on shoot growth and rooting - The multiple shoots were separated into single shoots and were used as explants. The shoot explants were inoculated in 350 mL glass jars containing MS medium supplemented with 0.5 mg L^{-1} BA and different concentrations of IBA (0.0-2 mg L^{-1}) in a complete randomized design. Six replicates were used for each combination treatment and the experiment was repeated twice. Parameters, such as average shoot height, number of shoots, leaves and roots produced from each explant, were recorded after 4 weeks of culture.

Comparation of the efficiency of liquid and gelled medium for shoot proliferation - The shoot explants were inoculated in a 100 mL Erlenmeyer flask containing MS medium supplemented with 0.5 mg L^{-1} BA with or without the addition of a gelling agent. Each flask contained 3 shoot explants and six vessels were used for each medium type. The experiment was repeated twice. Average shoot height, number of shoots, leaves and roots produced from each explant were recorded after 4 weeks of culture. The obtained data were analyzed using student t-test at $p \leq 0.05$ with SPSS version 13.

Acclimatization - Four-week old *in vitro* plantlets of *H. pineodora* with shoot height of 3-4 cm, 3-4 roots and at least 2-3 leaves were selected for the acclimatization process. The *in vitro* plantlets (n=50) were removed from the culture vessels, washed thoroughly with tap water and transplanted into plastic pots (25 x 25 cm) containing a mixture of organic soil and sand (1:1). They were placed under greenhouse conditions with relative humidity of 80-90%, temperature of 28±2ºC during day and 24±2ºC during night time. The plantlets were watered twice a day (morning and evening) with tap water. Shoot height was measured from the base of the plant to the tip of the leaves and the percentage of surviving plants was recorded after four weeks of growing in the plant house.

Comparison of leaf morphology of mother and *in vitro* clone - For scanning electron microscopy (SEM), healthy and mature leaves of *H. pineodora* were collected from *in vitro* as well as from mother plants of *H. pineodora*. Leaf segments were secured onto brass stubs with carbon conductive tape and the observations on leaf surface morphology were carried out using a Leo Supra 50 VP Field Emmision Scanning Electron Microscope (Carl-Zeiss SMT, Oberkochen, Germany) operated at 12 kV, with a working distance of 15 mm.

RESULTS AND DISCUSSION

Only 50% aseptic rhizomatous stems of *H. pineodora* was established using $HgCl_2$ and Clorox® surface-sterilization technique. This method was found to be more effective for the establishment of aseptic explants of other plant species such as Zingiberaceae species (Chan & Thong, 2004), banana (Kulkarni *et al.,* 2006), *Cymbopogon nardus* (Chan *et al.,* 2005) and *Maranta leuconeura* cv kerchoviana (Ebrahim & Ibrahim,

Table 1. Effect of Murashige & Skoog medium (MS) supplemented with BA (0.0-10.0 mg L^{-1}) on the shoot proliferation of *H. pineodora* explants over a duration of 12 weeks of culture (efeito do meio Murashige e Skoog (MS) suplementado com BA (0,0-10,0 mg L^{-1}) na produção de explantes de *H. pineodora* durante 12 semanas de cultivo). Malaysia, USM, 2010.

Concentration of BA (mg L^{-1})	Shoots/explant (nº)[1]		
	4th week	8th week	12th week
0.0	1.8 ± 0.4 a	1.8 ± 0.4 a	2.5 ± 0.6 a
2.0	2.8 ± 0.8 b	2.8 ± 0.8 b	3.5 ± 0.5 b
4.0	2.5 ± 0.8 b	2.5 ± 0.8 b	3.2 ± 1.0 b
6.0	2.5 ± 0.8 b	2.5 ± 0.8 b	3.5 ± 0.8 b
8.0	2.8 ± 0.4 b	2.8 ± 0.4 b	2.8 ± 0.4 b
10.0	3.2 ± 0.8 b	3.2 ± 0.8 b	3.2 ± 0.8 b

Mean values ± SD (standard deviation) within the same column followed by same letter are not significantly different (Tukey test, p≤0.05) (valores médios ±SD (desvio padrão) dentro da mesma coluna seguidos de mesma letra não são significativamente diferentes (Tukey, p≤0,05)).

2000). After four weeks of culture on the MS basal medium, the axillary buds remained green in color while rhizomatous stem tissues turned brown and necrotic. The axillary bud explants were subcultured into fresh MS medium every four weeks for culture stabilization and uniform shoot production and subsequently used for further studies.

Preliminary results revealed that MS medium, supplemented with 2.0 -10.0 mg L^{-1} BA, did not show any significant difference in the shoot multiplication rate of *H. pineodora*. MS medium supplemented with as low as 2.0 mg L^{-1} BA was sufficient to induce multiple shoot formation and the number of shoots produced was not found to be significantly different from MS medium supplemented with 4–10 mg L^{-1} BA. An average of 2.8-3.2 shoots was formed from each shoot explant on MS medium, supplemented with 2.0 -10.0 mg L^{-1} BA after four weeks of culture. Maintaining the shoots on the same culture media for 12 weeks did not show much increase in multiple shoots formation (Table 1). However the *in vitro* shoots of *H. pineodora,* cultured on MS medium supplemented with 10.0 mg L^{-1} BA, turned pale green in color after 12 weeks of culture. This might indicate that high concentration of BA was detrimental to the shoot cultures of *H. pineodora.*

Subsequently, shoot explants of *H. pineodora* were cultured in MS medium, supplemented with lower concentration of BA (0.5-2.0 mg L^{-1}). Shoot explants that were cultured on MS medium, supplemented with 0.5 mg L^{-1} BA, produced significantly higher number of shoots (3.8 shoots per explant) as compared to other concentrations of BA (1.0-2.0 mg L^{-1}) added into the culture medium. MS medium, supplemented with low concentration of BA (0.5 mg L^{-1}), also induced significantly higher root length as compared to those shoots cultured on other concentrations of BA. However, shoot explants cultured on MS medium devoid of plant growth regulators, produced less shoots with the longest roots. There was no significant difference in the shoot height, number of roots and number of leaves produced by the shoots explants cultured on MS medium supplemented with different concentration of BA (Table 2).

When the shoot explants were cultured on MS medium supplemented with 0.5 mg L^{-1} BA in combination with different concentrations of IBA (0.0-2.0 mg L^{-1}), there was no significant difference in shoot height, and the number of shoots, roots, and leaves produced from each shoot. The presence of higher concentration of IBA (1.0–2.0 mg L^{-1}) had significantly reduced the root length (Table 3). This indicated that IBA was not necessary and the addition of only low concentration of BA (0.5 mg L^{-1}) was sufficient to promote shoot and roots multiplication of *H. pineodora*. Thus MS medium, supplemented with 0.5 mg L^{-1} BA, was chosen as the optimum proliferation medium for production of *H. pineodora* plantlets.

Result indicated that liquid MS medium, supplemented with 0.5 mg L^{-1} BA, induced a significantly higher number of shoots as compared to the agar-gelled MS medium with same composition. The shoots also produced longer roots in liquid medium, as compared to the solid medium. However, there was no significant difference in

Table 2. Effect of Murashige & Skoog medium (MS) supplemented with BA (0.0-2.0 mg L^{-1}) on shoot proliferation, shoot growth and root production of *H. pineodora* after four weeks of culture (efeito do meio Murashige & Skoog (MS) suplementado com BA (0,0-2,0 mg L^{-1}) sobre a proliferação e crescimento de ramos e raizes de *H. pineodora* após quatro semanas de cultura). Malaysia, USM, 2010.

Concentration of BA (mg L^{-1})	Shoots/explant (nº)	Shoot height (cm)	Leaves/explant (nº)	Roots/explant (nº)	Length of roots (cm)
0.0	1.8 ± 0.4 a	4.6 ± 0.5 a	2.3 ± 0.5 a	5.6 ± 1.0 a	1.5 ± 0.6 b
0.5	3.8 ± 0.5 c	4.8 ± 0.3 a	2.5 ± 0.5 a	5.0 ± 1.3 a	1.2 ± 0.3 b
1.0	2.5 ± 0.3 b	4.8 ± 0.5 a	2.3 ± 0.5 a	4.8 ± 1.7 a	0.8 ± 0.2 a
1.5	2.8 ± 0.6 b	4.9 ± 0.5 a	2.3 ± 0.5 a	5.2 ± 1.2 a	0.8 ± 0.1 a
2.0	2.8 ± 0.5 b	4.7 ± 0.2 a	2.3 ± 0.5 a	4.7 ± 1.2 a	0.8 ± 0.2 a

Mean values ± SD (standard deviation) within the same column followed by same letter are not significantly different (Tukey test, p≤0.05) (valores médios ±SD (desvio padrão) dentro da mesma coluna seguidos de mesma letra não são significativamente diferentes (Tukey, p≤0,05)).

Table 3. Effect of Murashige & Skoog medium (MS) supplemented with BA (0.5 mg L^{-1}) and IBA (0.0-2.0 mg L^{-1}) on the shoot proliferation, shoot growth and root production of *H. pineodora* after four weeks of culture (efeito do meio Murashige & Skoog (MS) suplementado com BA (0,5 mg L^{-1}) e IBA (0,0-2,0 mg L^{-1}) sobre a proliferação e crescimento de ramos e raizes de *H. pineodora* após quatro semanas de cultura). Malaysia, USM, 2010.

Growth regulators (mg L^{-1})		Shoots/explant (nº)	Shoot height (cm)	Leaves/explant (nº)	Roots/explant (nº)	Mean length of roots (cm)
BA	IBA					
0.0	0.0	1.8 ± 0.4 a	4.6 ± 0.5 a	2.3 ± 0.5 a	5.6 ± 1.0 b	1.5 ± 0.2 b
0.5	0.0	3.8 ± 0.5 b	4.8 ± 0.3 a	2.5 ± 0.5 a	5.0 ± 1.2 b	1.2 ± 0.3 b
0.5	0.5	3.3 ± 0.8 b	4.7 ± 0.4 a	2.5 ± 0.5 a	5.6 ± 1.0 b	1.2 ± 0.5 b
0.5	1.0	2.9 ± 0.7 b	5.2 ± 0.5 a	2.5 ± 0.5 a	3.3 ± 0.5 a	0.7 ± 0.2 a
0.5	1.5	3.4 ± 0.8 b	5.2 ± 0.7 a	2.5 ± 0.5 a	4.3 ± 1.2 ab	0.5 ± 0.2 a
0.5	2.0	2.9 ± 1.0 b	5.3 ± 0.6 a	2.7 ± 0.5 a	4.7 ± 1.4 ab	0.7 ± 0.2 a

Mean values ± SD (standard deviation) within the same column followed by same letter are not significantly different (Tukey test, $p \leq 0.05$) (valores médios ±SD (desvio padrão) dentro da mesma coluna seguidos de mesma letra não são significativamente diferentes (Tukey, $p \leq 0,05$)).

Table 4. Effect of culture medium type on the shoot proliferation, shoot growth and root production of *H. pineodora* after 4 weeks of culture (efeito do tipo de meio de cultura na proliferação de ramos, crescimento de brotos e produção de raizes de *H. pineodora* após 4 semanas de cultura). Malaysia, USM, 2010.

Type of medium	Shoots/explant (nº)	Shoot height (cm)	Leaves/explant (nº)	Roots/explant (nº)	Length of roots (cm)
Solid	3.3 ± 1.0 a	4.8 ± 0.3a	2.5 ± 0.5 a	5.0 ± 1.3 a	1.2 ± 0.2 a
Liquid	5.9 ± 0.2 b	4.7 ± 0.3a	2.7 ± 0.5 a	5.2 ± 0.7 a	1.7 ± 0.1 b

Mean values ± SD (standard deviation) within the same column followed by same letter are not significantly different (Tukey test, $p \leq 0.05$) (valores médios ±SD (desvio padrão) dentro da mesma coluna seguidos de mesma letra não são significativamente diferentes (Tukey, $p \leq 0,05$)).

Figure 1. Micropropagation of *H. pineodora* (A) *in vitro* plant of *H. pineodora* in MS medium supplemented with 0.5 mg/L BA after four weeks of culture. (B) multiple shoots of *H. pineodora in vitro* plantlet and (C) mother plant (micropropagação de *H. pineodora* (A) *in vitro* das plantas de *H. pineodora* em meio MS acrescido de 0,5 mg/L de BA após quatro semanas de cultura. (B) de brotações múltiplas de *H. pineodora in vitro* das plântulas e (C) planta-mãe). Malaysia, USM, 2010.

the shoot length, number of roots and the number of leaves produced by *H. pineodora* produced in solid as well as liquid proliferation medium (Table 4). The *in vitro* plantlets, produced either in the solid and liquid media were normal and healthy. More shoots produced in liquid medium than agar-gelled medium were also observed in other plant species such as zingiberaceae species (Chan & Thong, 2004), *Centaurium erythraea* (Piatczak *et al.*, 2005) and *Eucalyptus* hybrid (Whitehouse *et al.*, 2002). They explained that the high rate of proliferation of shoots in liquid medium was due to the fact that the shoots were completely submerged in the liquid medium, presenting a large surface area for the uptake of nutrients and plant growth regulators.

After 4 weeks of acclimatization in the greenhouse, all the *in vitro* plantlets (100%) survived. The tissue cultured plantlets of *H. pineodora* were morphologically similar to their mother plants. The four week old acclimatized plantlets were found to be fairly uniform with an average height of 6.7±0.5 cm.

There was no variation in leaf morphology of the *in vitro* plantlets

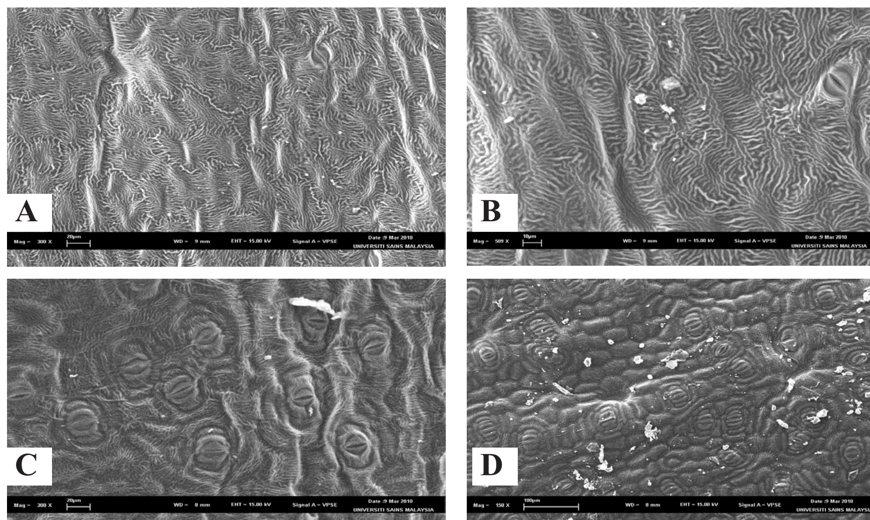

Figure 2. Scanning Electron Microscopy (SEM) micrographs showing leaf surface morphology of *H. pineodora*. The adaxial leaf surface showing distinctive patterned or ornamented cuticle in the form of striae (A); The striae in the form of deep ridges forming a rosette pattern (B). Distribution of stomata with well developed guard cells on adaxial leaf surface (C) abaxial leaf surface (D) (microscopia eletrônica de varredura (SEM) micrografias mostrando a morfologia da superfície foliar de *H. pineodora*. A face inferior das folhas mostra cutícula ou modelado ornamentado distinto, sob a forma de estrias (A); As estrias sob a forma de riscas profundas que formam um padrão de roseta (B). Distribuição de estômatos com células guarda bem desenvolvidas na face inferior das folhas (C) face abaxial (D)). Malaysia, USM, 2010.

as well as the mother plants of *H. pineodora* when observed under the scanning electron microscope. The adaxial leaf surface has a distinctive patterned or ornamented cuticle in the form of striae (Figure 1A). Also, the striae are in the form of deep ridges sometimes forming a rosette pattern in certain areas of the leaf (Figure 1B). These ridges could probably allow the run off of water droplets which increased water repellency and reduced the adhesion of the contaminating particles on the leaf surface. This phenomenon was called the 'lotus' effect (Barthlott & Neinhuis, 1997). Stomata were in level with the epidermis and occurred on both leaf surfaces. It appeared that the density of stomata was lower on adaxial leaf surface as compared to the abaxial leaf surface. Both leaf surfaces showed the presence of stomata with well developed guard cells (Figures 1C, 1D). The stomata regulate the gases exchange and water vapor between the outside air and the interior of the leaf. Typically, the stomata are more numerous over the abaxial (lower) epidermis of the leaf than the (adaxial) upper epidermis. The similar trends of stomata distribution was reported in *Persea americana* (Blanke & Lovatt, 1993). The lack of abundant stomata on the adaxial surface could be correlated with low gas-exchange rates on abaxial surfaces. The dissimilarity in the adaxial and abaxial leaf surface morphology could be an interesting feature and can also be used as a marker for the identification of *H. pineodora*.

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