IN VITRO PROPAGATION OF MIYAMASUKASHI-YURI (*LILIUM MACULATUM* Thunb. var. *bukosanense*), AN ENDANGERED PLANT SPECIES

PROPAGACIÓN *IN VITRO* DE MIYAMASUKASHI-YURI (*LILIUM MACULATUM* Thunb. var. *bukosanense*), UNA ESPECIE VEGETAL AMENAZADA

Amaury-M. Arzate-Fernández [1,2*], Makoto Miwa[1], Tomohide Shimada[1], Tetsushi Yonekura[1] and Kazuo Ogawa[1]

[1]Center for Environmental Science in Saitama, Environmental Biology Division. Kamitanadare 914, Kisai-Town, Kitasaitama-gun, Saitama, 347-0115, Japan. Tel: (81) 48073-8370, Fax: (81) 48070-2031. [2]Dirección actual: Centro de Investigación y Estudios Avanzados en Fitomejoramiento, Facultad de Ciencias Agrícolas, Universidad Autónoma del Estado de México-Campus Universitario "El Cerrillo". Carr. Toluca-Ixtlahuaca, entronque al Cerrillo, Toluca, Estado de México. Tel. y Fax: 01 (722) 296-5518 y 296-5531.
[*]*Autor para correspondencia* (amaury@uaemex.mx)

RESUMEN

***Lilium maculatum* var. *bukosanense* (Honda) Hara, called Miyamasukashi-yuri in Japanese, is a wild lily, considered as critically endangered in Japan. Before *L. maculatum* var. *bukosanense* is lost, attempts for *in vitro* propagation can be performed to provide information for its rescue, efficient *ex situ* conservation as well as for possible use in breeding programs to be used as ornamental plant. For bulblet induction, five scales from aseptic bulbs were used for each treatment and placed in the MS (half-strength) medium, supplemented with sucrose 3 %, agar 0.8 %, at pH 5.8, and the effect of two plant growth regulators (PGR): α-naphthaleneacetic acid (NAA) at 0.0, 1.0, and 2.0 mg L^{-1}, in combination with thidiazuron (TDZ) at 0.0 and 0.5 mg L^{-1}, were studied. Two illuminating conditions (light or darkness), and the presence or absence of activated charcoal (AC) in the medium were also tested. A total of 16 treatments were tested. Explants cultured under light and AC did have a favorable effect on bulblet induction. The average number of induced bulblets under light condition was the double (4 per explant) than those induced under darkness, both in presence of AC, irrespective of PGR level. The best treatments were those with NAA 2 mg L^{-1} + TDZ 0.5 mg L^{-1}, and without PGR, all of them with AC and under light. After 12 weeks of culture, an average of 2.4 well-formed bulblets was obtained per responsive explant. Six months after culture initiation, more than 640 regenerated plantlets from 320 explants, with roots and leaves were acclimatized and transferred to greenhouse conditions with 100 % success of survival. All the regenerated plantlets were morphologically similar among them, and had those characteristic feature of the flowers of this species. Moreover, no chromosome number ($2n=24$) variation was observed among scale-regenerated plantlets tested. The micropropagation procedure described here did not require a callus phase, thus we can state that the simple culture of scales could be applied successfully as a strategy for *in vitro* propagation in 12 months and thus contribute to the rescue and conservation of this plant species.**

Index words: *Lilium maculatum*, α-naphthaleneacetic acid, thidiazuron, *in vitro* culture of scales, *in vitro* propagation.

RESUMEN

***Lilium maculatum* var. *bukosanense* (Honda) Hara, llamada en japonés Miyamasukashi-yuri, es una especie silvestre que está amenazada en Japón. Antes de que desaparezca, el establecimiento de estrategias de propagación *in vitro* podrían coadyuvar a proveer información para su rescate, su conservación *ex-situ* y futura explotación comercial, así como su posible uso en el mejoramiento genético. Para la inducción de bulbillos se usaron cinco escamas de bulbos (catáfilos) asépticos por cada tratamiento, las cuales fueron sembradas en el medio de cultivo de MS a la mitad de su concentración, suplementado con sacarosa 3 %, agar 0.8 %, a un pH de 5.8, y se evaluaron dos reguladores del crecimiento vegetal (RCV): ácido α-naftalenacético (ANA) a 0.0, 1.0 y 2.0 mg L^{-1} en combinación con tidiazurón (TDZ) a 0.0 y 0.5 mg L^{-1}. También se evaluaron dos condiciones de iluminación (luz y oscuridad) y la presencia/ausencia en el medio de cultivo de carbón activado (CA), para un total de 16 tratamientos. El cultivo de escamas en condiciones de luz y en presencia de CA mostraron un efecto favorable en la inducción de bulbillos. El número promedio de bulbillos inducidos en condiciones de luz fue el doble (4 por explante) de los inducidos en oscuridad, ambos en presencia de CA, e independientemente del tratamiento hormonal. Los tratamientos que dieron mejor respuesta fueron ANA 2 mg L^{-1} + TDZ 0.5 mg L^{-1} , y sin RCV, todos ellos con CA y luz. En general, después de 12 semanas de cultivo se obtuvo un promedio de 2.4 bulbillos por explante, y seis meses más tarde se regeneraron más de 640 plantas de un total de 320 explantes, con raíces y hojas; todas éstas fueron aclimatadas y transferidas a condiciones de invernadero. Todas las plantas regeneradas fueron morfológicamente similares entre ellas y mostraron los rasgos distintivos que caracterizan la flor de esta especie. Además, los análisis citológicos revelaron el mismo número de cromosomas ($2n=24$) en todas las plantas examinadas. El procedimiento descrito aquí no requirió de una fase de callo, por lo que el simple cultivo de escamas se pudo aplicar exitosamente como una estrategia para la propagación *in vitro en* 12 meses, y así lograr el rescate y conservación de esta especie.**

Palabras clave: *Lilium maculatum,* ácido α-naftalenacético, tidiazurón, cultivo *in vitro* de escamas, propagación *in vitro*.

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INTRODUCTION

The world's biodiversity is declining at an unprecedented rate. During the period 1996-2004, a total of 8321 plant species were added to the The International Union for the Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species (Sarasan *et al*., 2006).

Although species conservation is achieved most effectively through the management of wild populations and natural habitats (*in situ* conservation), *ex situ* techniques can be used to complement *in situ* methods and, in some species it may be the only option (Sarasan *et al*., 2006). *In vitro* techniques have been found to be useful in the propagation of a large number of threatened plants (Dhar *et al*., 2000).

Lilies (*Lilium* spp.) are usually propagated in two ways: sexually by seeds and vegetatively by scaling. Propagation by seeds is not a practical method because of the extraordinarily slow growth of the bulbs until flowering, and at large scale propagation it is difficult to obtain a large number of bulbs in a short time (Arzate-Fernández, 2002).

Successful *in vitro* culture depends of several factors such as: culture media, light conditions, concentrations and combinations of plant growth regulators, other media constituents and explant type. Several *Lilium* species have been successfully propagated *in vitro* through direct regeneration or by intermediate callus formation (Simmonds and Cumming, 1976; Novak and Petru, 1981; Takayama and Misawa, 1983; Priyadarshi and Sen, 1992; Wickremesinhe *et al*., 1994; Arzate-Fernández *et al*., 1997; Niimi *et al*., 1999; Pelkonen and Kauppi, 1999; Varshney *et al*., 2000; Nhut, 1998, Nhut *et al*., 2001). For example, as many as 9.68×10^5 bulblets of lily Asiatic hybrids can be produced in one year, starting from a single scale segment (Varshney *et al*., 2000). Therefore, the *in vitro* tissue and cell culture will continue being a good technique for the massive propagation of new lily genotypes (Arzate-Fernández, 2002).

L. maculatum var. *bukosanense* (Honda) Hara, called Miyamasukashi-yuri in Japanese, is a wild lily that grows up in Mountain Buko, Saitama, Japan, at altitudes between 600 and 1050 m (Arzate-Fernández *et al*., 2005). Populations of this species usually consist of relatively distinct groups of individuals that are geographically isolated from one another, since each group occupies a well-defined habitat in Mountain Buko. This mountain is characterized by its rocky, limestone soils and extreme weather conditions. Natural population size of this species have been reduced drastically mainly because of changes in land use, and by damages caused by vermins and other animals.

L. maculatum var. *bukosanense* is an herbaceous perennial monocot that usually has a single stem arising from a bulb. One to three (8–10 cm in diameter) cup-shaped flowers are borne at the tip of the stem. Flowers are colorful, bright orange with red–black spots on the opened petals, which are arranged separately from one another. The hermaphrodite flowers are mainly pollinated by insects and wind. It is a self-compatible diploid with chromosome number $2n = 24$ (Noda, 1987). At the end of Summer, the plant produces many light seeds, which are mainly dispersed by gravity and wind; however, the germination rate of these seeds is less than 60 % (personal observation).

In small populations, inbreeding is generally assumed to result in low levels of genetic variation because of increased homozygosity (Persson *et al*., 1998). This is most obvious for species with a selfing mating system. Since *L. maculatum* var. *bukosanense* is a self-compatible plant and is spread in small populations, it has a low diversity value (Arzate-Fernández *et al*., 2005) and its breeding efficiency may be low because of the scarcity of parent plants.

L. maculatum var. *bukosanense* is listed in the Saitama Prefecture Red Data Book as a critically endangered plant, and rescuing information regarding its micropropagation is rather limited. On the other hand, lily breeders are interested in producing a variety of flower colours and shapes of Asiatic and Oriental lily types. *L. maculatum* var. *bukosanense* could be used for ornamental purposes because its pendulous stem with erect and beautiful flower color, features that are rare in lily plants. On this regard, the application of *in vitro*-micropropagation procedures might help to obtain large numbers of uniform plants and to get an efficient *ex situ* conservation, as well as to contribute for its future use in breeding programs and commercial propagation.

The purpose of this study was to evaluate the effect of two plant growth regulators, α-naphthaleneacetic acid (NAA) in combination with thidiazuron (TDZ), the absence or presence of activated charcoal (AC) and illuminating conditions, for the *in vitro* propagation of *L. maculatum* var. *bukosanense*. The morphological and genetic fidelity of regenerated plantlets were also analyzed.

MATERIALS AND METHODS

Plant material and explant source

Due to restricted permission for collecting plants of *L. maculatum* var. *bukosanense* in its natural habitat, 60 *in vitro* cultured bulbs were obtained from the Chichibu Taiheiyo Cement Research Center and from the Chichibu's Agriculture and Engineering High School, Japan. For disinfection, the bulbs (2-4 cm in diameter) were washed and cleaned under running tap water for 1 h, and peripheral damaged scales were removed. Clean inner scales were excised and surface sterilized sequentially, first in ethanol 70 % (v/v) (1 min), sodium hypochlorite 1 % (15 min), and three washes with sterilized distilled water. Then the scales were cut into middle sections of 1 x 1 cm^2 . Five sections of the bulb scale, with the dorsal side in contact with the medium, were placed in 200 mL culture glass flask containing 50 mL MS (Murashige and Skoog, 1962) medium, supplemented with sucrose 2 % (SigmaTM, Japan), gelrite 0.2 % (MerckTM, Rahway, N. J.) and agar 0.4 % (Wako Pure Chemical IndustriesTM, Ltd., Japan). The explants were subcultured every two months in the same conditions. After 7-8 months of scale-culture, many bulbs (1-2 cm in diameter) with leaves and roots could be regenerated and used as experimental material for this present study.

Medium and culture conditions at initiation stage

To standardize the starting experimental materials, the leaves and roots of bulbs were removed; then the bulbs were placed in 200 mL culture glass flask containing 50 mL half-strength MS (Murashige and Skoog, 1962) medium, supplemented with sucrose 3 % (SigmaTM, Japan), agar 0.8 % (Wako Pure Chemical IndustriesTM, Ltd., Japan) and activated charcoal 0.1 % (AC) (SigmaTM), without any plant growth regulator (PGR). The culture medium was adjusted to pH 5.8 before autoclaving at 120 °C and 1.1 kg cm^{-2} for 20 min. The cultures were maintained under growth-chamber conditions (16/8 h light/dark, about 40 μmol m^{-2} s^{-1} provided by cool white fluorescent light) at 25±1 °C, during four months. Scales (1-1.5 cm in length) from these bulbs were used as explants for *in vitro* bulblet induction and plant regeneration.

In vitro bulblet induction and plant regeneration

Six to seven healthy scales (explants) were separated from each *in vitro* grown bulb and five of them were placed with the dorsal side in contact with the MS (half-strength) medium, supplemented with sucrose 3 %, agar 0.8 %, at pH 5.8. For bulblet induction, the effect of α-naphthaleneacetic acid (NAA) at three concentrations (0.0, 1.0 and 2.0 mg L^{-1}) in combination with two concentrations of thidiazuron (TDZ) (0.0 and 0.5 mg L^{-1}) were studied, except NAA 1.0/TDZ 0.0 and NAA 2.0/TDZ 0.0. The absence or presence of AC 0.1 % were also assayed. All explants were tested either under dark or light/dark conditions (16/8 h light/dark, about 40 μmol m^{-2} s^{-1}) and cultured for 12 weeks at 25±1 oC. Thus, a total of 16 treatments were tested (Table 1). For each treatment, 20 explants (five explants per flask) were tested.

Rooting

For rooting test (length and number of roots), regenerated bulblets on explants were excised and transferred to MS (full- and half-strength) medium without plant growth regulators, containing sucrose 3 %, AC 0.1 % or without AC, at pH 5.8. The cultures were incubated for two months at 25±1 °C under a 16-h photoperiod (same conditions as described above).

Acclimatization and transfer to greenhouse conditions

Each regenerated plantlet with roots, green leaves and bulbs was potted in a previously autoclaved field soil and acclimatized in a growth chamber under a mist system for three weeks, then transferred to controlled greenhouse conditions at 22.0 °C and 70 % RH for 4-5 months. The characteristic features of the flowers (form and colour) among regenerated plantlets were observed to check for phenotypical variations.

Cytological analysis

For ploidy determination, root tips of 50 scale-regenerated plantlets were excised and pretreated with chilled water for 20 h at 0 °C, fixed in 3:1 ethanol:acetic acid mixture and stored at –20 °C until used. The used plantlets were randomly selected. The number of chromosomes at metaphase was counted after staining with acetocarmine 2 % by using a squash technique (Arzate-Fernández, 2002). Observations were made with the aid of a phase contrast microscope (Olympus BH-2) with 100 X oil immersion objectives and 10 X compensating oculars.

Statistical analysis

An analysis of variance (ANOVA) and Tukey test were performed to analyze the number of bulblet regeneration and plantlet rooting, according to a randomized-block design. Each flask contained five explants and was regarded as a block. These tests were conducted using the Statistical

Package for the Social Sciences (SPSS 12.0.1J, SPSS, Japan).

RESULTS AND DISCUSSION

In vitro bulblet induction and plant regeneration

The response of explants to illuminating conditions (light and dark), PGR levels and absence-presence of activated charcoal (AC), on the bulblet induction of *L. maculatum* var. *bukosanense* is shown in Table 1. After cultured for two weeks, small adventitious bulblets (1-5 mm in length) appeared *de novo* in the basal portion of the explants in some treatments (Figure 1A). In these treatments, the explants maintained its organogenic capacity for more than three months of culture. Adventitious bulblet formation was more pronounced on cultures supplemented with AC than in those without AC, irrespective of the rate of PGR and of the illuminating conditions (Table 1). However, explants cultured with AC and under light proved to be more inductive than in any other treatment. None or scarce bulblet formation was observed when explants were cultured in absence of AC in light with 0.0 and 2.0 mg L^{-1} NAA in combination with 0.5 mg L^{-1} TDZ, or in dark with 2.0 mg L^{-1} NAA and 0.5 mg L^{-1} TDZ.

Figure 1. Plantlet regeneration of *Lilium maculatum* Thunb. var. *bukosanense* through scaling and bulblets development. (A) Small bulblets (arrows) of 1-5 mm in length developing in the basal portion of the scale, two weeks after culture in darkness on hormone-free MS medium without activated charcoal (AC). Lily roots developing from bulb-scale regenerated plantlets, five months after culture initiation in light on MS (half-strength) medium: (B) in the absence of AC, and (C) in the presence of AC. (D) A completely regenerated plantlet six months after transfer to hormone-free MS (half-strength) medium with AC, prior to transfer to soil. (E) A flower of a regenerated plantlet showing the cup-shaped, showy and bright orange color with red-black spots on the petals, a characteristic of this species. (F) Flowering plantlets 4-5 months after transplantation. (G) Chromosomes at metaphase of a scale-regenerated plantlet ($2n=24$).

In explants cultured in a hormone-free level, under light and presence of AC, four bulblets per responsive explant were induced (Table 1). Pelkonen and Kauppi (1999) also reported higher regeneration rates of *L. ragale* in auxin-free and light treatments than those regenerated under darkness. They suggested that the endogenous auxin/cytokinin rates in lily scales are enough to promote bulblet induction, in light or darkness but better responses might be obtained under light conditions. Nhut *et al*. (2001) reported that the addition of AC to the culture medium improved the efficiency of bulblet regeneration in *L. longiflorum*, probably because AC absorbs vitamins, cytokinins, auxins and inhibitory substances, thus altering the ratios of medium components and subsequently influencing plant regeneration. The response of explants to different light conditions might be probably due to differences in explant size and thus to differences in endogenous levels of hormones in plant tissues (Niimi *et al*., 1999).

Table 1. Number of bulblet regeneration in *L. maculatum* var. bukosanense, in response to plant growth regulators, light conditions and activated charcoal. Values were obtained 12 weeks after culture initiation and are means ± standard deviations (n ≥ 4).

PGR; NAA : TDZ (mg L^{-1})	Light		Dark	
	Activated charcoal			
	Yes	No	Yes	No
0.0 : 0.0	4±0.2 b	1.6±0.1 e	1.8±0.1 e	1.7±0.2 ef
0.0 : 0.5	2.8±0.1 c	0 h	1.9±0.2 de	1.4±0.1 ef
1.0 : 0.5	3±0.3 c	1.8±0 e	1.9±0.2 de	1.2±0.1 efg
2.0 : 0.5	6±0.8 a	0 h	2.6±0.3 cd	0.5±0.2 gh

PGR = Plant growth regulators; NAA = Naphthalene acetic acid; TDZ = Thidiazuron. Values followed by different letters are significantly different (Tukey, 0.01).

It has been reported that lilies propagated by scaling can produce three to five bulbs per scale, depending on the species or cultivar, but especially depends on the scale size (Stimart and Ascher, 1978). In contrast, the *in vitro* regeneration may produce two to seven bulblets per scale on an MS medium supplemented with auxins and cytokinins (Wawrosch *et al*., 2001). We obtained similar results since a range of 0.5 to 6 well-formed bulblets per responsive explant were registered in this study. This slightly lower bulblet induction rate is probably due to differences in scale size (0.5-0.8 cm in length), and consequently in the endogenous PGR and tissue differentiation levels among the explants (Niimi *et al*., 1999; Pelkonen and Kauppi, 1999; Varshney *et al*., 2000; Nhut *et al*., 2001). The explant size may also affect the nutrient uptake by the scale from the medium, leading to variation in composition and quality of the scale cells. We conclude that the procedure described here might constitute an effective *in vitro* mass propagation method, since we estimated that 168 bulblets can be produced from one scale considering an average of 14 scales per bulb of 1-1.5 cm in diameter in one year, although further experiments on the explant size will be required to improve bulblet induction.

Regarding the effect of AC on the *in vitro* culture of lily, Nhut *et al*. (2001) pointed out that AC adsorbs inhibitory substances accumulated in the culture medium, and thus it is often used to reduce oxidation of phenolic compounds and to improve cell growth and development. In spite of the interaction among variation factors observed in the current study, it may be inferred that the variance among treatments was caused by the poor performance of bulblet regeneration in treatments without AC, irrespective of illuminating conditions. The best treatments were those having AC and light, either with NAA 2 mg L^{-1} and TDZ 0.5 mg L^{-1} or without PGR.

Rooting test, acclimatization and transfer to greenhouse conditions

Active charcoal (AC) have been used to improve shoot formation, plant regeneration and rooting *in vitro* (Nhut *et al*., 2001). Nhut (1998) pointed out that rooting in *L. longiflorum* was a slightly enhanced by cultivation in half-strength MS medium containing AC and free of plant growth regulators. In contrast, no root development was observed in *L. nepalense* when a full-strength MS medium was used (Wawrosch *et al*., 2001), but on half-strength MS medium very short roots were formed on 30 % of the bulblets, and it was necessary to use auxins to obtain 100 % rooting. Our results are in partial agreement with Nhut (1998) and Wawrosch *et al*. (2001), since in the present study rooting occurred in the absence of AC (Figure 1B) and on full- or half-strength MS medium; however, a higher rooting quality was obtained in the presence of AC and with a half-strength MS medium, as indicated by the increase in the number and length of roots per plantlet (Figure 1C, Table 2). As it was reported by Nhut *et al*. (2001), this result might be partly due to the adsorption of inhibitory substances, thus altering the ratio of medium components and subsequently influencing on rooting.

Six months after culture initiation, regenerated plantlets (10-13 cm in length) with roots (3-8 cm in length), 7-9 green leaves, and bulbs (1-1.5 cm in diameter) were obtained (Figure 1D). With a 100 % success rate, more than 640 plants of a total of 320 explants, were acclimatizated by the procedure describe here.

Table 2. Effect of the presence or absence of activated charcoal (AC) and medium strength on plantlet rooting. Values were obtained at the moment of transplant and are means ± standard deviations (n ≥ 10).

MS medium strength	Root length (±SD)	Root number (±SD)
Full + AC 0.1 %	6.3±0.1	4.9±0.1
Full - AC	3.7±0.1	3.2±0.1
Half + AC 0.1 %	8.1±0.1	9.2±0.1
Half - AC	5.6±0.1	5.1±0.1

MS = Murashige and Skoog medium; SD = Standard deviation.

L. maculatum var. *bukosanense* is characterized by cup-shaped, colorful and bright orange flowers with red-black spoted petals. All regenerated plantlets were morphologically similar among them, and had those characteristic feature of the flowers, representing the typical phenotype of this species (Figure 1E).

In vitro-raised bulblets have proved to be an efficient explant for inducing early flowering, and are comparable with conventional bulbs as they flower within the first season (Varshney *et at*., 2000). In this study, after 4-5 months of transplanting regenerated plantlets grew normally and more than 60 plants reached flowering (Figure 1F). Thus, *in vitro* formed bulblets can be considered a good planting stock of disease-free and uniform planting material, besides early flowering. This technology can be helpful for the commercial exploitation of new species and hybrids.

Cytological analysis

Lassner and Orton (1983) reported that genetic variation arising in somatic tissue is not easily observed. Arzate-Fernández *et al*. (1997), Godo *et al*. (1998) and Wawrosh *et al*. (2001) did not find abnormal morphological variation among plantlets of *Lilium* ssp. obtained through tissue culture methods. In the present study no morphological variation was observed either among a scale-regenerated plantlets (Figure 1F). These results were confirmed by the cytological analysis performed to regenerated plantlets after six months of *in vitro* culture, irrespective of treatment. Chromosome observations of root tip cells indicated that all the tested plantlets derived from scale-bulblet were diploid ($2n=24$) (Figure 1G). These findings are similar to those reported by Sheridan (1968), Simmonds and Cumming (1976), Bennici (1979), Priyadarshi and Sen (1992) and Arzate-Fernández *et al*. (1997), who considered that *Lilium* is a very stable monocotyledon plant species because the diploid chromosome number can be maintained even after long periods of *in vitro* culture. This fact constitutes an advantage because the *in vitro* propagation procedure used here minimized the risk of chimeric plant development.

CONCLUSIONS

Optimal conditions for the *in vitro* bulblet production of *L. maculatum* var. *bukosanense* was the treatment with NAA 2 mg L^{-1} and TDZ 0.5 mg L^{-1} in presence of AC and under light condition. As many as 168 bulblets can be produced from one scale considering an average of 14 scales per bulb of 1-1.5 cm in diameter in one year, by following the procedure described here. Small bulblets were induced as early as 2-3 weeks after culture initiation, but root development required five additional months. Thus, a completely regenerated plantlet was produced six months after culture initiation, and a regenerated plantlet reached flowering 4-5 months after transplant. All the regenerated plantlets were morphologically similar among them, and had those characteristic feature of the flowers of this species. Moreover, no chromosome number ($2n=24$) variation was observed among scale regenerated plantlets. Since this procedure does not require a callus phase, the scale culture could be successfully used as a strategy for this *in vitro* mass propagation as well as for rescuing and germplasm multiplication of this endangered plant species.

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