MICROPROPAGATION OF *KHAYA SENEGALENSIS*, AN AFRICAN MAHOGANY FROM DRY TROPICAL ZONES

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DANTHU, P., DIAITÉ-SANOGO, D., SAGNA, M., SAGNA, P. & DIA-GASSAMA, Y. K. 2003. Micropropagation of *Khaya senegalensis*, an African mahogany from dry tropical zones. An *in vitro* cloning protocol was set up on seedlings of *Khaya senegalensis* then adapted to more mature plants. *In vitro* shoot multiplication was conducted by using a culture medium rich in mineral salts (MS medium) in the presence of IBA 0.26 µM-added BAP 2.2 µM. The rooting of microcuttings was favoured by a less concentrated medium (MS/2) and a weak auxin concentration (IBA 5.2 µM) or by a 1- to 7-day-long induction on a medium with IBA 260 µM, followed by transfer to a regulator-free medium. A method of micrografting of *K. senegalensis* was developed. It consisted of grafting apices or buds taken from young shoots onto the epicotyl of young seedlings grown *in vitro*. The survival and/or growth of the grafts were improved if the rootstock was grown in light and if the graft was an apex. The height at which grafting was performed (low down or high up on the epicotyl) had little influence. On the basis of these findings, a protocol for the rejuvenation and cloning of *K. senegalensis* has been proposed.

Key words : Microcutting - micrografting - rejuvenation - Sahel - Senegal

DANTHU, P., DIAITÉ-SANOGO, D., SAGNA, M., SAGNA, P. & DIA-GASSAMA, Y. K. 2003. Pembiakan mikro *Khaya senegalensis*, sejenis mahogani Afrika dari zon tropika kering. Protokol pengklonan *in vitro* dibangunkan untuk anak benih *Khaya senegalensis* yang kemudian digunakan pada pokok yang lebih matang. Penggandaan pucuk secara *in vitro* dijalankan menggunakan kultur media yang kaya dengan garam galian

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(media MS) bersama-sama media IBA 0.26 µM ditambah BAP 2.2 µM. Pengakaran keratan mikro lebih baik dalam media dan kepekatan auksin yang kurang pekat (masing-masing MS/2 dan IBA 5.2 µM) atau dengan aruhan 1 hingga 7 hari pada media yang mempunyai IBA 260 µM, lalu dipindahkan ke media yang tiada pengawal atur. Kaedah cantuman mikro *K. senegalensis* dibangunkan. Apeks atau tunas yang diambil daripada pucuk muda dicantum ke atas epikotil anak benih muda yang tumbuh secara *in vitro*. Kemandirian dan pertumbuhan cantuman bertambah baik jika stok akar bertumbuh dalam cahaya dan juga jika apeks digunakan. Aras cantuman (jauh di bawah atau tinggi di atas epikotil) tidak banyak mempengarui keputusan. Berdasarkan penemuan ini, protokol bagi rejuvenasi dan pengklonan *K. senegalensis* telah dicadangkan.

Introduction

Khaya senegalensis, belonging to the family Meliaceae, is one of the main timber wood species of dry Africa. Its distribution area corresponds to the Sudanian zone, which falls between 650 and 1300 isohyets and stretches from Senegal to Sudan (Anonymous 1988).

This mahogany species can reach up to 30 m in height. Its hard, dense, red wood has good technological properties (Normand & Sallenave 1958) and is fungi- and termite-resistant (Von Maydell 1990). It is widely used in woodwork and in cabinet making. *Khaya senegalensis* is also used as a shade and as an ornamental tree. Its generous foliage provides poor-quality but frequently-used fodder (Anonymous 1988) and it also has many medicinal uses (Kerharo & Adams 1974). The oil extracted from its seeds is much sought after by local populations (Seignobos 1982, Okieimen & Eromosele 1999).

However, when young, *K. senegalensis*, like most African or American mahoganies, is commonly parasitised by a lepidopterous shoot borer, *Hypsipyla robusta*; this cause severe impoverishment of the tree value (Grijpma & Gara 1970, Brunck & Fabre 1974, Yamazaki *et al.* 1992, Brunck & Mallet 1993). Several methods have been used to fight this parasite, but neither chemical nor biological treatments have given convincing and viable results on a large scale (Singh & Misra 1988, Brunck & Mallet 1993, Newton *et al.* 1993). The only good compromise seems to be planting *K. senegalensis* in combination with species resistant to the shoot borer (Brunck & Mallet 1993, Dupuy & M'Bla Koua 1993).

Nevertheless, within natural populations, there are individuals that show apparent tolerance to the shoot borer and these could form a reference population for a programme aimed at the improvement of this species which should allow, *in fine*, the planting of selected or improved plants (Brunck & Mallet 1993, Newton *et al.* 1993, 1994).

Mastering the methods of rejuvenation and cloning of selected trees, therefore, appears to be a prerequisite. Different paths can be explored, through the association of horticultural and *in vitro* techniques (Bonga 1987, Franclet *et al.* 1987, Aitken-Christie & Connett 1992, Arnaud *et al.* 1993).

Several studies of plant propagation have already been conducted on different species of mahogany. Propagation by cuttings of *Swietenia mahogani* and *Khaya ivorensis* has been developed (Howard *et al.* 1990, Tchoundjeu & Leakey 1996)

and some research has been carried out on the grafting of *Swietenia macrophylla* (Zabala 1977). Micropropagation from young shoots of various species belonging to the genera *Swietenia, Cedrela* and *Khaya* has been the object of a few papers (Venketeswaran *et al.* 1988, Maruyama *et al.* 1989, Newton *et al.* 1994). Nevertheless, there seems to be no published works on *K. senegalensis.*

Drawing inspiration from the work of Pliego-Alfaro and Murashige (1987) as well as Huang *et al.* (1992a, b), our paper seeks to develop a method of rejuvenation and cloning of selected *K. senegalensis.* Our research was directed, first, towards developing methods of microcutting and micrografting of juvenile shoots. The final part of our research was aimed at applying and adapting the protocols developed on juvenile material to the rejuvenation and cloning of older *K. senegalensis.*

Material and methods

The development of these methods was done with plantlets grown *in vitro.* The seeds came from Bambey station (Senegal). Following harvesting, they were sun-dried, then hermetically stored at 5 °C until use (Danthu *et al.* 1999). They were then disinfected by soaking for 10 minutes in mercuric chloride solution ($HgCl_2$, 0.1%), thoroughly rinsed with sterile water and sown in culture tubes (diameter 22 mm) containing agar (7 g l^{-1}). After three weeks, the shoots that emerged from the seedlings were collected and used in the different tests.

In microcutting experiments (experiments 1 to 4), the main culture medium was that of Murashige and Skoog (1962), referred to as MS. Two other media were used, one whose concentration in macroelements was halved (referred to as MS/2) and the other one whose macroelements were replaced by those of Woody Plant Medium, referred to as WPM (Lloyd & McCown 1980). When growth regulator or sugar was added, this is mentioned in the course of the paper.

In micrografting experiments (experiments 5 and 6), the rootstocks used were three-week-old seedlings grown *in vitro* on 3-cm Milcap® lumps (polypropylene woven fibres) imbibed with 12 ml of MS/2 medium. The micrografting technique was adapted from Huang *et al.* (1992a, b) and Palma *et al.* (1997). The technique is a miniaturisation of cleft grafting. Grafting was done onto the epicotyl of the rootstock, and the scion was a 10-mm apex or axillary bud. The graft was ligatured with Parafilm® sterile tape. Experiment 5 was a preliminary test. It studied the effect of some parameters (micrografting level, rootstock light conditioning, scion origin) on survival and growth of micrografts, with young scions (aerial part of the rootstocks, without self-graft). Experiment 6 was a validation of the protocol; it was conducted on six-year-old ortets originating from Nioro du Rip (Senegal) mobilised in a greenhouse by horticultural grafting.

All media were sterilised by autoclaving for 20 min at 110 °C. Cultures were allowed to grow in long days (day length of 16 hours) under a light source that provided a photon flux of 40 µE m^{-2} s^{-1}. Temperature was 30 °C in daytime and 27 °C at night.

Each experiment was replicated three times on 12 to 24 plants. When null hypothesis (equality of means) was rejected by variance analysis, the means were compared by the Newman-Keuls test, with $p < 0.05$. Means are given together with CI (confidence interval) ($p < 0.05$). The meaningfulness of the χ^2 independence test is also reported.

Results

Microcutting, shoot multiplication

The main factors studied were the mineral components of the culture medium and the growth regulator balance.

Table 1 shows that the average number of shoots sprouted by microcuttings of *K. senegalensis* in the presence of 2.2 µM BAP was significantly higher on the MS medium than on MS/2 or WPM media (experiment 1). The same applies to the growth of these shoots; after one month, their length was significantly greater when cultivated on MS medium.

In experiment 2 the average number of shoots produced by each explant was close to 1 when no cytokinin was used (Table 2). The number of shoots per explant was always higher in the presence of BAP than with kinetin or 2iP at the same concentration (2.2 µM). The addition of a small amount of auxin helped shoot multiplication. When IBA 0.26 µM was used together with BAP 2.2 or 8.8 µM, each explant sprouted two or three shoots (Figure 1).

Table 1 Effect of mineral composition of media on the shoot multiplication of *Khaya senegalensis* mononodal microcuttings from seedlings, after one month (experiment 1)

Medium composition*	Number of shoots per microcutting	Mean shoot length (cm)
MS	2.5 a	2.3 a
MS/2	1.9 b	1.7 b
WPM	1.7 b	1.8 b

*medium with BAP 2.2 µM
Newman-Keuls test, $p < 0.05$

Table 2 Effect of hormonal supplies on the shoot multiplication of *Khaya senegalensis* mononodal microcuttings from seedlings, after one month (experiment 2)

Cytokinin*	Concentration (µM)	IBA concentration (µM) 0	0.26	2.6
Control	0	1.0 ± 0.2	1.1 ± 0.3	1.2 ± 0.3
Kinetin	2.2	1.3 ± 0.1	1.2 ± 0.2	1.0 ± 0.1
2iP	2.2	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.2
BAP	2.2	1.7 ± 0.3	2.3 ± 0.4	1.5 ± 0.3
	8.8	-	2.4 ± 0.5	-
	22.0	-	1.3 ± 0.4	-

* MS medium
± CI, $p < 0.05$

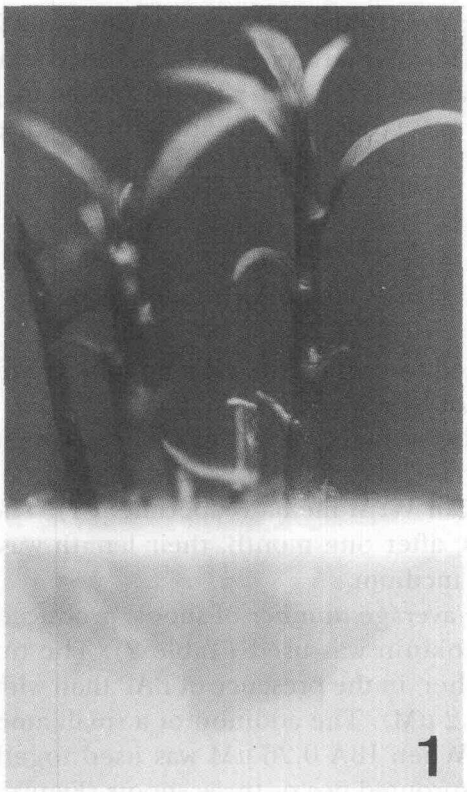

Figure 1 Shoot multiplication of *Khaya senegalensis* on medium with BAP 2.2 µM + IBA 0.26 µM

Microcutting rooting

Results showing the influence of mineral composition, sucrose concentration and hormonal supplies of the medium on the rooting of young *K. senegalensis* microcuttings are given in Tables 3 and 4 (experiments 3 and 4). The percentage of well-rooted microcuttings was significantly higher on MS/2 medium than on MS or WPM media. A sucrose supply in concentrations ranging from 10 to 50 g l^{-1} resulted in rooting of about three-quarters of the microcuttings. Below and beyond these doses, the rooting rate fell significantly (Table 3). Table 4 shows that microcutting rooting is improved by IBA supply. A brief stimulation (1 to 7 days) in a medium with high doses of IBA (260 µM) followed by transfer to a growth regulator-free medium resulted in rooting of about 90% of the microcuttings. These microcuttings developed four or more thin, long roots (Figure 2). Continuous cultivation in the presence of IBA 2.6 or 5.2 µM resulted in rooting of only two thirds of the microcuttings.

After greenhouse acclimatisation, the rooted microcuttings were transplanted to the fields. There, they showed the same positive development as that of seedlings (Figure 3).

Table 3 Effect of mineral composition of media and sucrose concentration on rooting percentage of *Khaya senegalensis* microcuttings from seedlings, after one month (experiment 3)

	Percentage of rooted microcuttings (%)
Culture medium *	
MS	58 b
MS/2	75 a
WPM	56 b
Sucrose concentration (g l^{-1})**	
0	11 c
10	75 a
20	70 a
50	77 a
100	55 b

* medium with 10 g l^{-1} of sucrose and IBA 2.6 µM
** MS/2 medium and IBA 2.6 µM
Newman-Keuls test, $p < 0.05$

Table 4 Effect of different rooting stimulation on rooting of *Khaya senegalensis* microcuttings from seedlings, after one month (experiment 4)

Rooting stimulation (µM)*	Percentage of rooting (%)	Number of roots per microcutting	Root length (cm)
0	11 c	1.0 b	2.5 ab
IBA 2.6	71 b	1.3 b	3.2 a
IBA 2.6 + NAA 0.26	72 b	2.1 b	1.7 b
IBA 5.2	62 b	1.8 b	2.7 ab
IBA 260 (1 day)	87 a	4.5 a	3.5 a
IBA 260 (7 days)	96 a	4.2 a	3.0 a

*MS/2 medium and sucrose 10 g l^{-1}
Newman-Keuls test, $p < 0.05$

Micrografting technique

The germination of *K. senegalensis* was both hypogeous (its hypocotyl is very short) and cryptocotyledonous (the cotyledons remain shut within the integument of the seed) (Figure 4). The epicotyl was 4 to 5 cm long. Table 5 shows that the micrografting of *K. senegalensis* can be achieved with close to 100% success rate whatever the level of grafting was, i.e. lower down on the epicotyl (above the cotyledonous node) or higher up on the epicotyl (underneath the first pair of leaves) (experiment 5). In either case, the growth of the graft was the same. On the other hand, the survival of graft and the growth of scion were influenced by growth conditions of the stock. If the rootstock was constantly grown in long days, 100% of the grafts survived, whereas only 83% of the grafts performed on stocks grown in darkness were still alive one month after grafting ($\chi^2 = 8.7$, 1 df, $p < 0.01$). The size of the scion and number of buds were higher when the stock was grown under light. Finally, Table 5 shows that the growth of graft was significantly improved if the scion was an apex rather than an axillary bud.

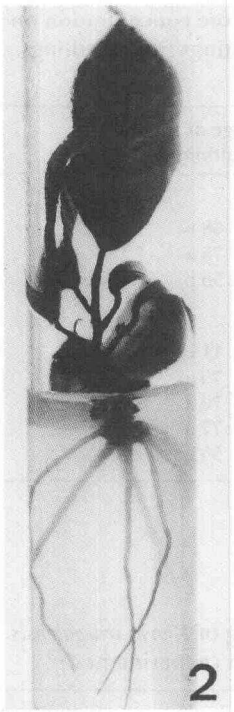

Figure 2 Microcutting of *Khaya senegalensis* on rooting medium (IBA 5.2 μM)

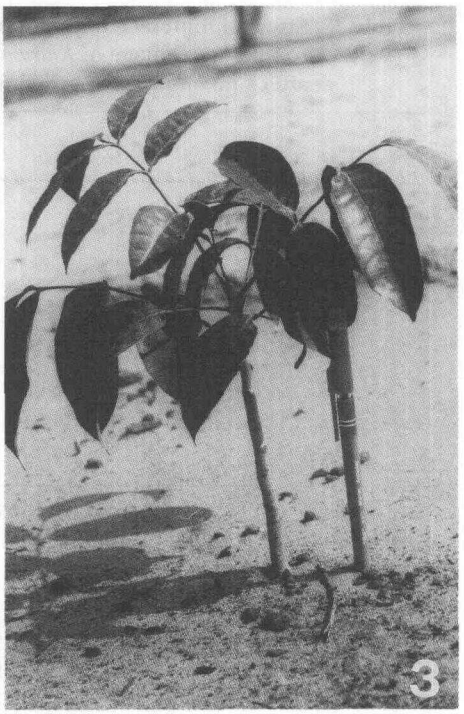

Figure 3 Field acclimatised *Khaya senegalensis* microcutting

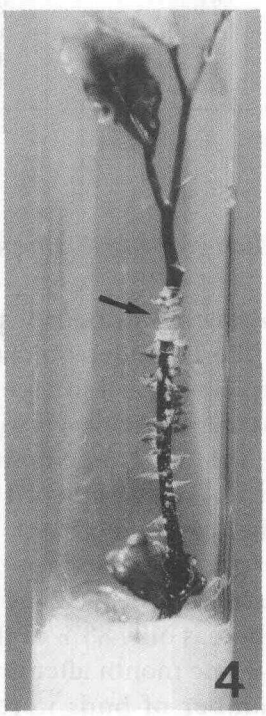

Figure 4 *Khaya senegalensis* micrograft (→), one month after grafting

Table 5 Effect of some parameters on survival and growth of *Khaya senegalensis* micrografts (measured one month after grafting) (experiment 5)

	Surviving grafts (N = 48) (%)	Scion growth (cm)	Number of nodes per scion
Micrografting level			
Lower part of epicotyl	98	3.1 ± 0.3	6.3 ± 0.5
Upper part of epicotyl	100	3.2 ± 0.2	6.4 ± 0.6
Rootstock light conditioning			
Darkness (1 week)	83	2.5 ± 0.3	4.4 ± 0.7
Long days	100	3.4 ± 0.2	5.5 ± 0.6
Scion nature			
Apex	100	3.2 ± 0.4	5.5 ± 0.6
Axillary bud	100	1.5 ± 0.5	4.5 ± 0.8

± CI, $p < 0.05$

Rejuvenation and cloning of mature trees

Table 6 gives the reactivity of microcuttings originating from six-year-old *K. senegalensis* mobilised by horticultural grafting in the greenhouse (Figure 5) (experiment 6). The microcuttings were either taken directly from these ortets, or were micrografted onto young seedlings. The rooting ability and the shoot elongation of microcuttings resulting from the micrografting cycle were significantly higher than that of microcuttings directly removed from ortets. In the former, 68% of the microcuttings rooted and one-third of the shoots elongated (Figure 6).

Table 6 Comparison of rooting behaviour of *Khaya senegalensis* microcuttings kept on six-years-old trees (mobilised in nursery by grafting) according to their origin* (experiment 6)

Microcutting origin	Number of microcuttings rooted (%)	Number of microcuttings with shoot elongation (%)
Ortet (horticultural bud)	15 (6/40)	8 (3/40)
Micrograft	68 (30/44)	36 (16/44)
	χ^2 24.2, $p < 0.001$	9.9, $p < 0.01$

*sampled directly in nursery or micrografted beforehand on young seedling

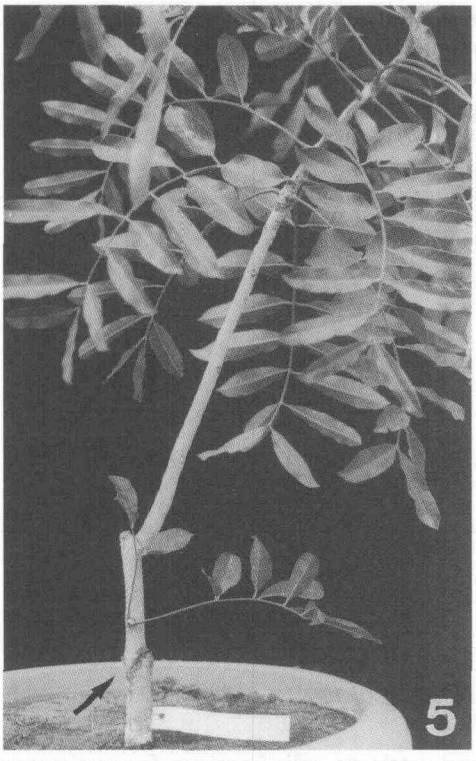

Figure 5 Six-years-old *Khaya senegalensis* mobilised in nursery by horticultural grafting (cleft budding, →)

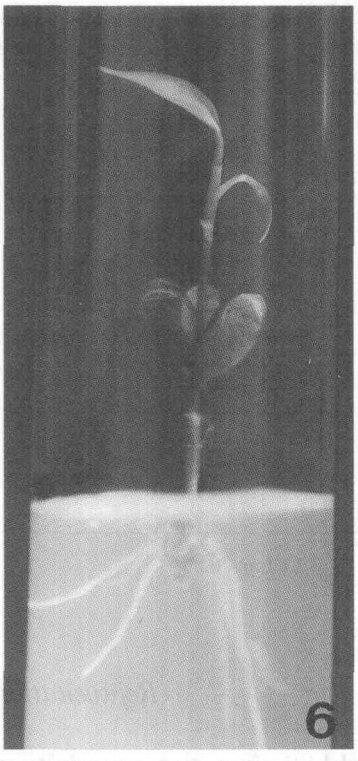

Figure 6 Rooted vitroplant of *Khaya senegalensis* rejuvenated by micrografting on juvenile rootstock

Discussion and conclusions

Our study has made it possible to develop a micropropagation method of *K. senegalensis* through the use of micrografting and microcutting.

The micrografting method is a miniaturisation of horticultural techniques. It optimises and adapts to *K. senegalensis* the protocols used on *Sequoia sempervirens* by Huang *et al.* (1992b) or on various *Acacia* species by Detrez (1994) or Palma *et al.* (1997). The scion is not a meristem as in virus eradication attempts (Murashige *et al.* 1972, Navarro *et al.* 1975) but a bigger (a few millimetres) apex or axillary node, which makes micromanipulations easier as they can be conducted with the naked eye. A high rate of success was reached regardless of the level at which the grafting was made and regardless of the nature of the scion. However, unlike in *Acacia senegal* (Palma *et al.* 1997), the use of rootstocks etiolated by growth in darkness was detrimental to the survival and growth of *K. senegalensis* grafts.

This study also showed that microcutting of *K. senegalensis* was possible as long as a two-step protocol was observed: a phase of shoot multiplication in the presence of cytokinins followed by a phase of rooting induced by the presence of auxin. The shoot multiplication of *K. senegalensis* was optimal in the presence of a low

concentration of BAP (2.2 or 4.4 μM). It is worth noting that the supply of a small dose of auxin (IBA 0.26 μM) into the multiplication medium improved the production of shoots, as has already been shown in the case of *K. ivorensis* (Newton *et al.* 1994). The rooting was promoted by a brief stimulation with a high level of IBA (260 μM). We observed that the association of IBA and NAA used by Maruyama *et al.* (1989) for another Meliaceae (*C. odorata*) was not effective for *K. senegalensis.*

Our results showed that shoot multiplication was favoured by the use of MS medium, while rooting was better on a medium whose macroelements were diluted (MS/2). The need to lower the mineral salt concentration of the culture medium to improve rooting is well documented (Chalupa 1983) and has been widely applied to ligneous tropical species (Maruyama *et al.* 1989, Badji *et al.* 1993). In all cases, our study showed that WPM produced inferior results. The ionic concentration of WPM was half that of MS and equalled that of MS/2. One can, therefore, assume that the behavioural difference of *K. senegalensis* microcuttings did not depend on the total ionic concentration of the media but rather on the relative concentrations of some elements. For instance, MS/2 medium contains two times more nitrate than WPM. On the other hand, WPM contains 2.1 times more sulfate than MS/2 (George *et al.* 1987, Sellmer *et al.* 1989). Definite conclusions, however, cannot be drawn from our experiments.

The use of these two techniques of micropropagation, i.e. micrografting followed by microcutting, was successfully applied to clone six-year-old *K. senegalensis* trees. However, it must be noted that our work was based on the cloning of relatively young material. Before this protocol is used in any selection and improvement programme, it should be checked whether it is adaptable to mature subjects which can be more than 40 years old. In particular, it must be ascertained whether rejuvenation achieved through horticultural grafting and micrografting is enough to guarantee a good reactivity of the shoots. It is possible to contemplate resorting to several cycles of grafting as has already been done to rejuvenate some ligneous species (Huang *et al.* 1992a, b).

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