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Improved *in vitro* shoot proliferation and rooting of two banana varieties (FHIA-21 and PITA-3)

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Abstract
In the present study, shoot tip explants were used for shoot proliferation and rooting of two banana varieties (FHIA-21 and PITA-3). Shoot-tip cultures were maintained on Murashige and Skoog (MS) medium containing different levels of cytokinins combination [benzylaminopurine (BAP), kinetin (Kin) and 2-isopentenyl (2-iP)], auxins [indole acetic acid (IAA) and naphthaleneacetic acid (NAA)] and antioxidants [Silver nitrate (AgNO3), Citric acid (CA), Ascorbic acid (AA) and Cystein (C)] for shoot proliferation. The best response in terms of shoot number per explant was obtained from MS medium supplemented with BAP (3 mg/L) + Kin (0.25 or 0.5 mg/L) and with IAA (0.5 or 1 mg/L). The elongating shoots were cut to prepare further shoot proliferation. The addition of antioxydant such as Citric acid at 25 mg/L to the proliferation medium significantly improved the number and shoot length. Regenrated shoots were excised and rooted; the highest number and longest root were obtained with MS medium with 2-iP (1 mg/L) + NAA (0.5 mg/L + AgNO3 (10 or 15 mg/L) after three weeks. The rooted shoots were acclimatized and transfered to field conditions.

Keywords: antioxidants, auxins, banana, cytokinins, *in vitro* shoot proliferation, rooting, shoot tip

Introduction
Bananas and plantains (Musa spp.) are among the most important fruit crops in the world and are staple food for millions across the globe (FAO, 2010) [1]. Presently banana is grown in around 150 countries across the world on an area of 50.34 million hectare producing 106.84 million tonnes (FAO, 2015) [2]. World total banana and plantain production ranks at the 5th place after cereals, and there is still much scope for yield improvement (Jain *et al.*, 2004) [3]. They are at the second place after cassava (Manihot esculenta Crantz) in sub-Saharan Africa where they provide diet to million people (FAOSTAT, 2005) [4]. Plantains account for about 32 % of total Musa production, from mostly Central and West Africa (Lescot, 2008) [5]. It represents an essential source of nutrients for millions of people, particularly in tropical and subtropical regions, as well as a cash crop in many developing countries. In general, banana cultivars are considered as good sources of carbohydrates, proteins, vitamins and minerals (Anbazhagan *et al.*, 2014) [6].

Despite the importance of bananas and plantains, the crop is threatened by many pests and diseases (Viljoen, 2010) [7]. Based on the high importance of bananas and plantains in food security and human's health, their production need to be improved.

Banana is generally propagated vegetatively through suckers, which grow from lateral buds originating from corms and suckers. Thus, banana propagation through conventionnal method using young shoots or part of the tuber is not an ideal method. This process is very slow as the rate of multiplication of suckers through conventional vegetative means has been found to express several negative impacts which include transmission of diseases, low production and poor preservation of original plant genetic material (Hussein, 2012) [8]. Moreover, 5 to 10 suckers can be obtained per plant over a year, The traditional clonal propagation method appears to be unable to supply the increasing demand for disease free and healthy planting materials of banana. In order to increase conventional propagation and to avoid pathogens related constraints, *in vitro* approach has been considered (Tripathi, 2003) [9]. Methods developed using this approach offers more effective and better controlled condition for banana cultivation.

However, several problems still need to be solved and currently under study, high content of phenolic compounds and its accumulation in culture media which affects vitality of explants and survival percentage and after a while, explants color exchanges in brown and subsequent death of the cultured explants appears. To resolve toxic effect of phenolic compounds in plant tissues cultures, different attempts had been done in order to overcome *in vitro* explant browning and

increase shoot multiplcation and rooting such as including pretreatment of explants with antioxidants, incorporation of antioxidants into the culture medium, incubation of cultures in the dark and frequent subculture to fresh medium (Ahmad *et al.*, 2013) [10]. In addition, several papers have shown the influence of antioxidants on shoot multiplication (Bais *et al.*, 2000 ; Giridhar *et al.*, 2001 ; Akhtar *et al.*, 2016) [11, 12, 13].
Thus, present study aimed to develop a rapid and high frequency shoot regeneration protocol for elite plants of Two banana varieties suitable for mass propagation by manipulating growth regulators and antioxidants.

Materials and methods

Explant Source

Suckers as explants source were collected from the banana collection of Azaguie research station of National Agronomic Research Center (CNRA) of Côte d'Ivoire. All the experiments were conducted at the Central Laboratory of Biotechnology of CNRA at Adiopodoume.

Selection of explant and surface sterilization

Healthy meristematic shoot tip from sword suckers were initially washed thoroughly with soap water and then were surface presterilized with 15 % (v/v) calcium hypochloride containing few drops of 0.01 % tween-20 for 10 to 15 minutes. In the laminar air flow cabinet, shoot tip were sterilized with ethanol (98 %v/v) for 5 min followed by three time rinsing in sterile distilled water. Then explants were again sterilized with commercial bleach (3.8 % of active chloride) containing few drops of 0.01 % tween-20 for 20 minutes. At the end, shoot tip were rinsed thoroughly with sterile distilled water to remove any traces of commercial bleach.

Effect of differents auxins on shoots proliferation

In the first stage of our study, microshoots initially induced in 200 mL glass bottles (Figure 1a, 1b) containing 30 mL of induction medium consisted of MS (Murashige and Skoog, 1962) medium supplemented with BAP (5 mg /L) and sucrose (30 g /L). The pH of media was adjusted to 5.7 prior to adding agar (7 g /L). After 4 weeks, in order to test the effect of auxins on shoot proliferation, microshoots were cut and cultured on basal MS medium supplemented separately with different concentrations of IAA and NAA (0, 0.5, 1.0, 1.5 and 2 mg/L).

Effect of different cytokinins combination on shoot proliferation

In the second stage, according to the results of our previous experiments, microshoots were isolated and inoculated in media supplemented with BAP (3 mg /L) in combination with Kin and 2-iP at various concentrations (0.25, 0.5 and 1 mg/L) for shoot proliferation. After 4 weeks, mean number of shoots per explant and shoot length of shoot per explant were recorded.

Effect of different antioxidants on shoot proliferation

In the third stage, To obtain high proliferation of shoots, the microshoots were cultured on the best medium in following experiments supplemented with different antioxidants (Ascorbic acid, Citric acid, Cystein and Silver nitrate) at various concentrations (10, 25, 50 and 100 mg/L). After one month, the number of shoot /explants and shoot length (cm) were recorded.

Rooting of *in vitro* grown microshoots culture using different antioxidants and hardening

In vitro grown microshoots were inoculated into MS media supplemented with 2-iP (1 mg/L), NAA (0.5 mg/L) and different antioxidants (Ascorbic acid, Citric acid and Silver Nitrate) at various concentrations (0, 5,10 and 15 mg/L). After three weeks, the cultures were evaluated considering of the mean number and lenght of roots
Rooted plantlets were carefully taken out from the rooting medium and washed gently with tap water to remove any traces of medium. These plantlets were transferred to perforated plastic bag containing forest soil and sawdust from dead tree (1:1) and kept in the greenhouse for hardening. One month old hardened plants were finally transferred to the open field.

Culture conditions

All explants were cultured on different types of media and transferred to growth room in dark for shoots proliferation or under 16 : 8 h (light : dark) photoperiod at 25 ± 2 °C and with light intensity of 20 µmol m-2 s-1 for rooting.

Statistical analysis

All the experiments in this study were prepared in completely randomized design (CRD) with three replications and a maximum care was taken to minimize any variation in the laboratory conditions among treatments for each of the experimental materials. Statistical data analysis was done by using STATISTICA 7.1 version. To detect the significance of differences among treatments at $p \leq 0.05$, analysis of variance (ANOVA) was used. Means of different treatments were compared by using Least Significance Difference (LSD) test at a 5% confidence interval.

Fig 1: *In vitro* shoot proliferation and rooting of two banana varieties. **(a)** Healthy meristematic shoot tip. **(b)** Initiation of healthy meristematic shoot tip on MS + 5 mg/L BAP medium. **(c)** FHIA-21 shoot proliferation on MS + 3 mg/L BAP + 0.5 mg/L IAA. **(d)** PITA-3 shoot proliferation on MS + 3 mg/L BAP + 1 mg/L IAA. **(e)** FHIA-21 shoot proliferation on MS + 3 mg/L BAP + 0.25 mg/L Kin. **(d)** PITA-3 shoot proliferation on MS + 3 mg/L BAP + 0.5 mg/L Kin. **(g)** FHIA-21 shoot proliferation on MS + 25 mg/L CA. **(h)** PITA-3 shoot proliferation on MS + 25 mg/L CA. **(i)** *in vitro* rooting in presence of different antioxidants.

Results

Effect of differents auxins on shoots proliferation

Two auxins were examined for the proliferation of shoot number and shoot length from cultured microshoots of 2 banana varieties Table 1.

Microshoots cultured onto proliferation medium gave the higest mean number of shoots per explant when BAP (5 mg/L) + IAA (0.5 mg/L) and BAP (5 mg/L) + IAA (1 mg/L) were added to MS (Murashige and Skoog, 1962) basal medium respectivelly for FHIA-21 (5.30 shoots) (Figure 1c) and PITA-3 (5.10 shoots) (Figure 1d).

Among various concentrations best response in terms of shoot length was observed on MS supplemented with NAA (1 mg/L) for FHIA-21 (2.60 cm) and NAA (0.5 mg/L) for PITA-3 (2.13 cm) (Table 1).

Table 1: Effect of differents auxins on shoot proliferation of two banana varieties

Auxins (mg/L)		FHIA-21		PITA-3	
IAA	NAA	Mean number of shoots	Mean length of shoots (cm)	Mean number of shoots	Mean length of shoots (cm)
0	0	2.20 ± 0.32^{d}	1.24 ± 0.07^{c}	2.00 ± 0.25^{d}	1.15 ± 0.04^{d}
0.5	-	5.30 ± 0.51^{a}	1.78 ± 0.16^{b}	3.30 ± 0.61^{bcd}	1.67 ± 0.15^{bc}
1	-	3.60 ± 0.47^{bc}	1.65 ± 0.09^{bc}	5.10 ± 0.70^{a}	1.77 ± 0.19^{ab}
1.5	-	3.10 ± 0.67^{bcd}	1.92 ± 0.14^{b}	3.50 ± 0.61^{bc}	1.30 ± 0.08^{cd}
2	-	2.80 ± 0.20^{bcd}	1.60 ± 0.10^{bc}	3.20 ± 0.51^{bcd}	1.71 ± 0.16^{b}
-	0.5	3.90 ± 0.65^{b}	1.27 ± 0.09^{c}	2.60 ± 0.37^{cd}	2.13 ± 0.15^{a}
-	1	3.00 ± 0.29^{bcd}	2.60 ± 0.25^{a}	4.00 ± 0.53^{ab}	1.84 ± 0.11^{ab}
-	1.5	2.80 ± 0.35^{bcd}	1.92 ± 0.22^{b}	3.00 ± 0.44^{bcd}	1.62 ± 0.18^{bc}
-	2	2.60 ± 0.26^{cd}	1.84 ± 0.17^{b}	2.70 ± 0.33^{bcd}	1.87 ± 0.09^{ab}

Mean values within a column followed by the same letters are not significantly different at $p < 0.05$ according to Least Significance Difference (LSD).

Effect of different cytokinins combination on shoot proliferation

After one month of culture, the number of shoots ranged from 3.20 to 7.50 shoots in FHIA-21 and 3.30 to 6.80 shoots in PITA-3 (Table 2). The maximun number of shoots per explant FHIA-21 (7.50 shoots) was obtained on medium containing Kin (0.25 mg/L) (Figure 1e) and with Kin (0.5 mg/L) for PITA-3 (6.80 shoots) (Figure 1 f). Meanwhile, the length of shoots FHIA-21 (3.51 cm) was enhanced in the presence of Kin (0.25 mg/L) and PITA-3 (2.45 cm) with Kin (0.5 mg/L) (Table 2)

Table 2 : Effect of cytokinins combination on shoot proliferation of two banana varieties.

Cytokinins (mg/L)			FHIA-21		PITA-3	
BAP	Kin	2-iP	Mean number of shoots	Mean length of shoots (cm)	Mean number of shoots	Mean length of shoots (cm)
3	-	-	5.40 ± 0.54^{b}	2.73 ± 0.21^{b}	4.80 ± 0.44^{bcd}	2.20 ± 0.20^{ab}
3	0.25	-	7.50 ± 0.74^{a}	3.51 ± 0.13^{a}	5.90 ± 0.88^{ab}	1.85 ± 0.20^{b}
3	0.5	-	5.80 ± 0.69^{b}	2.31 ± 0.33^{bc}	6.80 ± 0.67^{a}	2.45 ± 0.26^{a}
3	1	-	3.40 ± 0.40^{d}	1.98 ± 0.17^{c}	3.30 ± 0.30^{e}	1.96 ± 0.14^{ab}
3	-	0.25	5.20 ± 0.74^{bc}	2.10 ± 0.21^{c}	5.10 ± 0.37^{bc}	1.89 ± 0.17^{ab}
3	-	0.5	3.70 ± 0.30^{cd}	1.94 ± 0.16^{c}	3.90 ± 0.27^{cde}	1.83 ± 0.17^{b}
3	-	1	3.20 ± 0.38^{d}	1.82 ± 0.19^{c}	3.50 ± 0.34^{de}	1.87 ± 0.20^{b}

Mean values within a column followed by the same letters are not significantly different at $p < 0.05$ according to Least Significance Difference (LSD).

Effect of different antioxidants on shoot multiplication

Various concentrations of different antioxidants were evaluated (Table 3). The maximum capacity for shoot multiplication was observed on the medium containing CA (25 mg/L) both for FHIA-21 (11.00 shoots) (Figure 1 g) and PITA-3 (8.00 shoots) (Figure h). The longest induced shoots were obtained in presence of CA (25 mg/L). The mean shoot length was 2.68 cm (FHIA-21) and 2.90 cm (PITA-3) (Table 3).

Table 3: Different antioxidants Effect on shoot proliferation of two banana varieties

Antioxidants (mg/l)		FHIA-21		PITA-3	
		Mean number of shoots	Mean length of shoots (cm)	Mean number of shoots	Mean length of shoots (cm)
Silver nitrate	10	6.00 ± 0.89^{bc}	1.30 ± 0.10^{c}	6.50 ± 0.56^{ab}	1.26 ± 0.10^{f}
	25	5.50 ± 0.92^{bc}	1.46 ± 0.14^{bc}	6.00 ± 0.89^{abc}	1.30 ± 0.10^{f}
	50	4.50 ± 0.50^{bcd}	1.53 ± 0.15^{bc}	4.00 ± 1.12^{cde}	1.63 ± 0.09^{ef}
	100	4.00 ± 1.09^{cd}	1.73 ± 0.09^{bc}	2.50 ± 0.76^{de}	2.13 ± 0.32^{cde}
Citric acid	10	10.50 ± 1.05^{a}	2.20 ± 0.22^{ab}	6.50 ± 0.99^{ab}	2.68 ± 0.39^{abc}
	25	11.00 ± 0.51^{a}	2.68 ± 0.20^{a}	8.00 ± 0.57^{a}	2.90 ± 0.18^{a}
	50	6.00 ± 0.77^{bc}	2.15 ± 0.11^{ab}	4.50 ± 0.42^{bcd}	2.81 ± 0.31^{ab}
	100	4.50 ± 0.61^{bcd}	2.15 ± 1.01^{ab}	3.50 ± 0.34^{de}	2.28 ± 0.24^{bcd}
Ascorbic acid	10	6.66 ± 0.55^{b}	1.41 ± 0.17^{bc}	4.00 ± 0.68^{cde}	1.31 ± 0.11^{f}
	25	6.50 ± 0.42^{b}	1.23 ± 0.12^{c}	4.50 ± 0.42^{bcd}	2.20 ± 0.27^{cde}
	50	4.50 ± 0.99^{bcd}	1.60 ± 0.15^{bc}	4.00 ± 0.73^{cde}	2.15 ± 0.23^{cde}
	100	4.00 ± 0.44^{cd}	1.45 ± 0.17^{bc}	3.00 ± 0.63^{de}	1.11 ± 0.07^{f}
Cystein	10	4.00 ± 0.67^{cd}	1.16 ± 0.10^{c}	2.50 ± 0.61^{de}	1.26 ± 0.12^{f}
	25	4.50 ± 0.67^{bcd}	1.20 ± 0.11^{c}	4.50 ± 1.11^{bcd}	1.33 ± 0.10^{f}
	50	4.00 ± 0.89^{cd}	2.13 ± 0.05^{ab}	4.00 ± 0.85^{cde}	2.11 ± 0.25^{cde}
	100	3.00 ± 0.51^{d}	1.70 ± 0.25^{bc}	2.00 ± 0.51^{e}	1.73 ± 0.28^{def}

Mean values within a column followed by the same letters are not significantly different at $p < 0.05$ according to Least Significance Difference (LSD).

Rooting of *in vitro* grown microshoots culture using different antioxidants and hardening

The supply of AgNO3 and AA increased the number of roots in the microshoots. Conversely, the CA treatment had no effect or decreased rooting. Maximum mean number of root was 16.65 roots/shoot with AgNO3 (10 mg/L) for FHIA-21 and 14.65 roots/ shoot with AgNO3 (15 mg/L) for PITA-3 (Table 4). The AgNO3 (10 mg/L) resulted in significantly the longest root both FHIA-21 (6.24 cm) and PITA-3 (6.32 cm) (Table 4) (Figure 1i). The rooted shoots were transferred to perforated plastic bag containing forest soil and sawdust from dead tree in the ratio of 1:1. These plants were successfully kept in the green house. All the shootlets regenerated survived (100%).

Table 4: Effect of antioxidants incorporated in MS medium on root number and length in two banana varieties after three weeks in culture.

Antioxidants (mg/l)			FHIA-21		PITA-3	
Silver nitrate	Ascorbic acid	Citric acid	Mean number of roots	Mean length of roots (cm)	Mean number of roots	Mean length of roots (cm)
-	-	-	11.35 ± 0.29^{de}	5.10 ± 0.18^{cd}	10.75 ± 0.28^{cde}	4.59 ± 0.22^{def}
5	-	-	14.40 ± 0.53^{b}	6.04 ± 0.24^{a}	11.85 ± 0.41^{b}	5.30 ± 0.19^{c}
10	-	-	16.65 ± 0.27^{a}	6.24 ± 0.12^{a}	12.20 ± 0.50^{b}	6.32 ± 0.33^{a}
15	-	-	13.70 ± 0.64^{b}	6.01 ± 0.15^{a}	14.65 ± 0.27^{a}	6.01 ± 0.27^{b}
-	5	-	12.45 ± 0.33^{cd}	5.31 ± 0.17^{bc}	11.80 ± 0.25^{bc}	5.13 ± 0.24^{cd}
-	10	-	13.60 ± 0.58^{bc}	5.36 ± 0.16^{bc}	11.70 ± 0.33^{bc}	4.96 ± 0.20^{cde}
-	15	-	11.70 ± 0.35^{de}	5.87 ± 0.21^{ab}	11.40 ± 0.43^{bcd}	4.76 ± 0.14^{cdef}
-	-	5	10.75 ± 0.22^{e}	4.97 ± 0.25^{cd}	10.50 ± 0.45^{def}	4.47 ± 0.18^{ef}
-	-	10	10.60 ± 0.51^{e}	4.98 ± 0.19^{cd}	10.05 ± 0.31^{ef}	4.43 ± 0.17^{ef}
-	-	15	8.60 ± 0.41^{f}	4.72 ± 0.22^{d}	9.50 ± 0.48^{f}	4.24 ± 0.16^{f}

Mean values within a column followed by the same letters are not significantly different at $p < 0.05$ according to Least Significance Difference (LSD).

Discussion

The experimental results of the role of auxins (IAA and NAA) on shoot number and length produced on multiplication medium are shown on Table 1. All of auxins concentrations examined on two banana varieties showed to produce an average of higher number of shoots compared to the control. This declares the necessity of auxins presence in proliferation medium. The results of our study revelated that IAA (0.5 or 1 mg/L) were found to be suitable auxin to shoot proliferation and NAA (0.5 or 1 mg/L) for shoot elogation. The results are in line with those of prior researchers (Muhammad *et al.*, 2007; Asmare *et al.*, 2012; Choudhary *et al.*, 2015) [14, 15, 16], who used IAA combined with BAP in shoot multiplication medium. In addition, Ahmed *et al.* (2014) [17] mentioned that IAA (2.00 mg/L) was necessary for shoot multiplication. In terms of shoot elongation, our results are in disagreement with those for banana (Rahaman *et al.*, 2013; Ahmed *et al.*, 2014) [17]. But in concordance with those obtained in *Prunella Vulgaris* (Rafia *et al.*, 2009) and *Vanda tessellata* (Bakul *et al.*, 2014).

It was found in the present study that when the low Kin concentration was supplemented with BAP, both the number of shoots per explant and shoot length increased. Optimum response was obtained at (0.25 or 0.5 mg/L) Kin plus 3 mg/L BAP. In similar experiments, proliferation of higher number of shoots per explant due to the synergistic effect of the two cytokinins (BAP and Kinetin) was demonstrated for many *in vitro* propagated plants, for example banana (Bikram *et al.*, 2016) [21], who obtained obtained the same result with BAP (1.5 mg/L) + Kin (1.5 mg/L). Similar results have been reported in sugarcane (Ali and Afghan, 2001 ; Khan *et al.*, 2009; Adilakshmi *et al.*, 2014) who reported that medium supplemented with BAP and Kin resulted in rapid multiplication of shoots. Concerning shoot length, The best result obtained in our study is in agreement with the result reported by Ismail *et al.* (2012) who were obtained maximum shoot length *Acacia auriculiformis* with BAP (0.5 mg/L) plus Kin (0.1 mg/L) and by Shimelis *et al.* (2014) who were obtained maximum shoot length on MS medium supplemented with BAP (1.5 mg/L and Kin (0.5 mg/L) in *Saccharum officinarum L.*

Antioxidants control browning of culture medium and also plays a significant role in the multiplication of shoots in many plants. Hence, their efficiency on shoot multiplication was examined in two banana varieties. Citric acid (25 mg/L) enhance number of shoot in FHIA-21 (11.00 shoots per explant) and PITA-3 (8.00 shoots per explant). In addition, our study shown all media supplemented with CA gave elongated shoots. These results agree with results obtained by Sanjaya *et al.* (2005) on *Pseudoxytenthera stocksii* observed effectiveness of Citric acid on enhancing number of shoots per explant. Similary, Abdelwahd *et al.* (2008) [28] with *Vicia faba*; they suggest that ascorbic acid enhanced shoot number (9.50 shoots per explant). Consistent with our results, Gehan *et al.* (2015) [29] also reported that Ascorbic acid (150 mg/L) or Citric acid (150 mg/L) or combination both are very effective for banana shoots multiplication.

Rooting is crucial step. Success of the protocol also depends on frequency of root induction (e.g., number of roots and root length). In the current study antioxidants influenced rooting in both banana varieties (FHIA-21 and PITA-3) in terms of roots number per shoot and root length. Several studies shown that silver ions in the form of nitrate, such as AgNO3, play a major role in efficient root formation. In our study the addition of AgNO3 to the culture medium enhanced rooting of both banana varieties than other antioxidants used. In other hand, Citric acid reduced rooting compared to the control. These results corroborate with those of *Gentiana lutea* (Mariya *et al.*, 2011) [30] and *Dillenia indica* (Essam *et al.*, 2012) [31], who found that silver nitrate improved *in vitro* root formation.

Conclusion

The appropriate conditions for *in vitro* shoot proliferation and rooting of two banana varieties (FHIA-21 and PITA-3) were established. The optimum concentration of auxins for shoot proliferation of both banana was AIA (0.5 mg/L) in FHIA-21 and AIA (1 mg/L). The use of BAP plus Kin in combination with IAA was found to enhance shoot proliferation. Thus, BAP (3 mg/L) plus Kin (0,25 mg/L) in FHIA-21 and BAP (3 mg/L) plus Kin (0.5 mg/L) in PITA-3 were best for high rates of shoot proliferation. Likewise, the use of antioxidants revealed that citric acid 25 mg/L was fovorable for shoot multiplication of both varieties. MS medium supplemented with 10 or 15 mg/L AgNO3 increased the number and length

of roots formed in FHIA-21 and PITA-3. From this study we can conclude that the developed protocol provides rapid shoot multiplication technique that enables to minimize the time required for large-scale propagation of newly evolved and high yielding banana varieties.

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