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Original article

Positive effect of *in vitro* embryo rescue on breaking the dormancy of wild banana seeds compared to direct sowing in the greenhouse

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Summary

Introduction – **The genetic diversity of wild bananas can conveniently be preserved in seed form. However, depending on the species and experimental sowing conditions, such seeds give uncertain, highly variable germination rates in the greenhouse. One hypothesis would be that the integuments of banana seeds contain dormancy factors preventing good germination when sown naturally. The aim of this work was to ascertain whether the germination rate for banana could be increased if embryos were removed from the influence of the integuments by culturing isolated embryos *in vitro*.** *Materials and methods* – **We present here our results for over 12,000 sowings of a vast panel of *Musa acuminata* and *M. balbisiana* genotypes grown in a collection at CARBAP in Cameroon. After harvesting at maturity from self-pollinated banana plants, fresh seeds were immediately sown on two contrasting sterile substrates in a greenhouse at CARBAP and were also cultured *in vitro* using embryos excised from the same seed batches.** *Results and discussion* – **We found substantial variations in germination percentages in the greenhouse depending on the genotypes studied. *M. balbisiana* seeds germinated well, with an average rate of 80%, or even peaks of 90% for some batches. On the other hand, the different *M. acuminata* accessions germinated poorly, suggesting dormancy factors in the seeds. We found that *in vitro* embryo culture eliminated all these differences between the genotypes, with a strong increase in germination rates for the genotypes studied.** *Conclusion* – **These results support the hypothesis that dormancy factors in *Musa* seeds are located in the seed integuments, rather than in the actual embryos. The implications of these results are numerous for collecting and managing batches of *Musa* seeds, and for conserving *Musa* genetic resources.**

Keywords
dormancy breakage, embryo rescue, genetic resources, *Musa*, seed germination

Significance of this study

What is already known on this subject?

- Banana seeds are known to germinate poorly in greenhouses, particularly the *Musa acuminata* subspecies. Consequently, *in vitro* culture of banana zygotic embryos has been used to improve germination rates significantly. All these past results have been reported in various publications, usually involving small samples. A real comparison of the two methods on plant material from the same source is lacking.

What are the new findings?

- By comparing the germination rates of over 12,000 seeds from a wide range of *Musa* genetic diversity, we found that *in vitro* embryo culture led to consistently high germination rates regardless of the genotype, while greenhouse seed germinations remained subject to erratic results.
- These comparisons of germination rates between *in vivo* and *in vitro* conditions also confirmed the hypothesis of dormancy factors in the integuments of *Musa acuminata* seeds, but not in *M. balbisiana*.

What is the expected impact on horticulture?

- This work is part of a project that aims to facilitate the circulation and conservation of banana genetic resources in seed form. It showed *in vitro* embryo culture to be very effective in ensuring seed germination in such a way as to preserve wide allelic diversity in the genus *Musa,* or to support banana genetic improvement.

Introduction

Banana originates from Southeast Asia and the Pacific region, and it is thought to have first been domesticated more than 7,000 BP ago (Perrier *et al.*, 2011). East Africa and central Africa are the secondary centres of diversity. A distinct group of cultivars, Fe'i bananas, developed independently in the Pacific region.

It is estimated that around 1,000 cultivated banana varieties exist today. Most domesticated bananas are triploids, with three sets of chromosomes, and their parthenocarpic

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fruits do not contain seeds. Apart from a few exceptions, triploid varieties are more vigorous and productive than diploid varieties, which are now rarer.

On the other hand, wild bananas are diploids and produce non-edible fruits full of seeds. Over 50 species of wild banana are known to exist, but two species, *Musa acuminata* and *Musa balbisiana*, are known for their role in the domestication of most edible bananas (Simmonds and Shepherd, 1955; Perrier *et al.*, 2009). The natural range of *M. acuminata* extends from West to East, from eastern India to New Guinea, passing through the Philippines and Indonesia. Along that axis, *M. acuminata* displays substantial morphological variability, structured in subspecies (Cheesman, 1947b). The wild forms vary in height and are often slender, but they display great variations in bunch and fruit shapes and lengths. Up to nine subspecies have been defined depending on the authors (Simmonds, 1966; De Langhe and Devreux, 1960).

Musa balbisiana is also a wild, uncultivated species. Although it originates from Southeast Asia, from regions much further north than the *M. acuminata* subspecies, it now exists throughout the tropical zones of the world. It is probably the most common and most widespread of all the species of the genus *Musa* (Cheesman, 1947a). They are very vigorous and tall plants, displaying strong vegetative vigour, strong suckering and a good root system. The species *M. balbisiana* displays less variability than *M. acuminata*.

Edible varieties are usually sterile and long-term conservation options are therefore limited by the vegetative nature of their reproductive system. These clones have to be conserved in plant form in the field or in greenhouses, *in vitro* as *in vitro* plantlets under slow growth conditions, or cryopreserved.

For their part, wild bananas have kept both reproductive systems, firstly sexual as they are perfectly fertile and readily produce seeds, and secondly vegetative through suckers, like edible varieties. Wild species can, of course, be conserved as suckers under the same conditions as cultivated forms. However, this form is not really adapted to the conservation challenges of plant population diversity. Indeed, whilst it is feasible to use vegetative propagation to maintain a particular cultivated genotype arising from a long history of domestication, or even particular wild clones for description/botanical characterization purposes, it is not conceivable to preserve populations for the future in the form of particular individuals. Indeed, such preservation must be designed in the form of gene banks (*i.e.*, large-sized seed batches), where the major concern will be inexpensively collecting and conserving allele representativeness of genes that is as close as possible to that of the wild populations from which they were collected (Hardy-Weinberg, 1908).

Little is known about seed germination in the genus *Musa* (Stotzky and Cox, 1962), apart from the fact that germination rates are extremely variable, and often quite poor under controlled conditions. In *Musa*, the hardness of the seed integument embodies a conflict between seed protection and germination (Graven *et al.*, 1996). For instance, these authors showed that the walls of banana seed integuments contain compounds, notably polyphenols, that protect seeds, but which are also likely to inhibit embryo germination.

The purpose of our work was to ascertain whether embryo dormancy could be "broken" and germination promoted by removing zygotic embryos from the influence of their seed integuments. To that end, we cultured *in vitro* the excised embryos of seeds from a vast panel of genetic diversity representative of the diversity of *M. acuminata* and *M. balbisiana* wild populations, and we compared the results to sowing on horticultural substrates.

Materials and methods

Plant materials

The germination study on seed-bearing bananas was carried out on clones representative of the subspecies *Musa acuminata* and *Musa balbisiana* (Figure 1). In this work we studied five representatives of the species *M. acuminata* from different origins (Table 1).

We also tested three representatives of the species *Musa balbisiana*:
- the Pisang Klutuk Wulung clone (PKW);
- the Cameroon clone (CAM);
- the Honduras clone (HON).

Unfortunately, however, the species *M. balbisiana* was only represented by a single accession (Cameroon accession – CAM), due to the poor quality of seeds obtained from self-pollination of the "Pisang Klutuk Wulung" and "Honduras" accessions (see Results).

The seeds used were genetically uniform batches for each accession, as they all came from self-pollinations under controlled conditions at CARBAP (*Centre Africain de Recherche sur Bananiers et Plantains*) and not from open pollinations of uncertain origins, as is often the case in this type of study. CARBAP research station is located in Njombé, Cameroon, at lat. 7°22'10.74"N, long. 12°20'40.51"W, 80 m a.s.l. The climate is humid tropical with an 8-month rainy season from mid-March to mid-November and a 4-month dry season.

All the germination tests were carried out on fresh seeds following the recommendations of Simmonds (1952, 1959), *i.e.*, seeds were extracted from ripe fruits, carefully washed and sown immediately. All the seeds underwent the flotation test and only good quality seeds, sinking to the bottom of the water, were sown.

Methods

1. Pollination. Self-pollinations were carried out by pollinating female flowers with the pollen of male flowers taken from another banana plant of the same accession, but having flowered earlier.

Table 1. Description of the five subspecies representative of the *Musa acuminata* species complex.

Musa acuminata subspecies	Accession name	Code	Place of origin	Characteristic feature
M. acuminata ssp. *zebrina*	Zebrina clone	ZEB	Java, Indonesia	Zebra-like striping of red pigmentation on the leaves
M. acuminata ssp. *truncata*	Truncata clone	TRU	Kelantan Highlands, Malaysia	Close to ssp. *malaccensis*, adapted to cooler temperatures
M. acuminata ssp. *microcarpa*	Microcarpa clone	MIC	Borneo, Indonesia	Bunches bearing a large number of small fruits
M. acuminata ssp. *malaccensis*	THA018	MAL	Thailand	Slender bunches with top-shaped carmin male bud
M. acuminata ssp. *burmannica*	Cal4	CAL	Myanmar	Compact pendulous bunch with blue-violet male bud

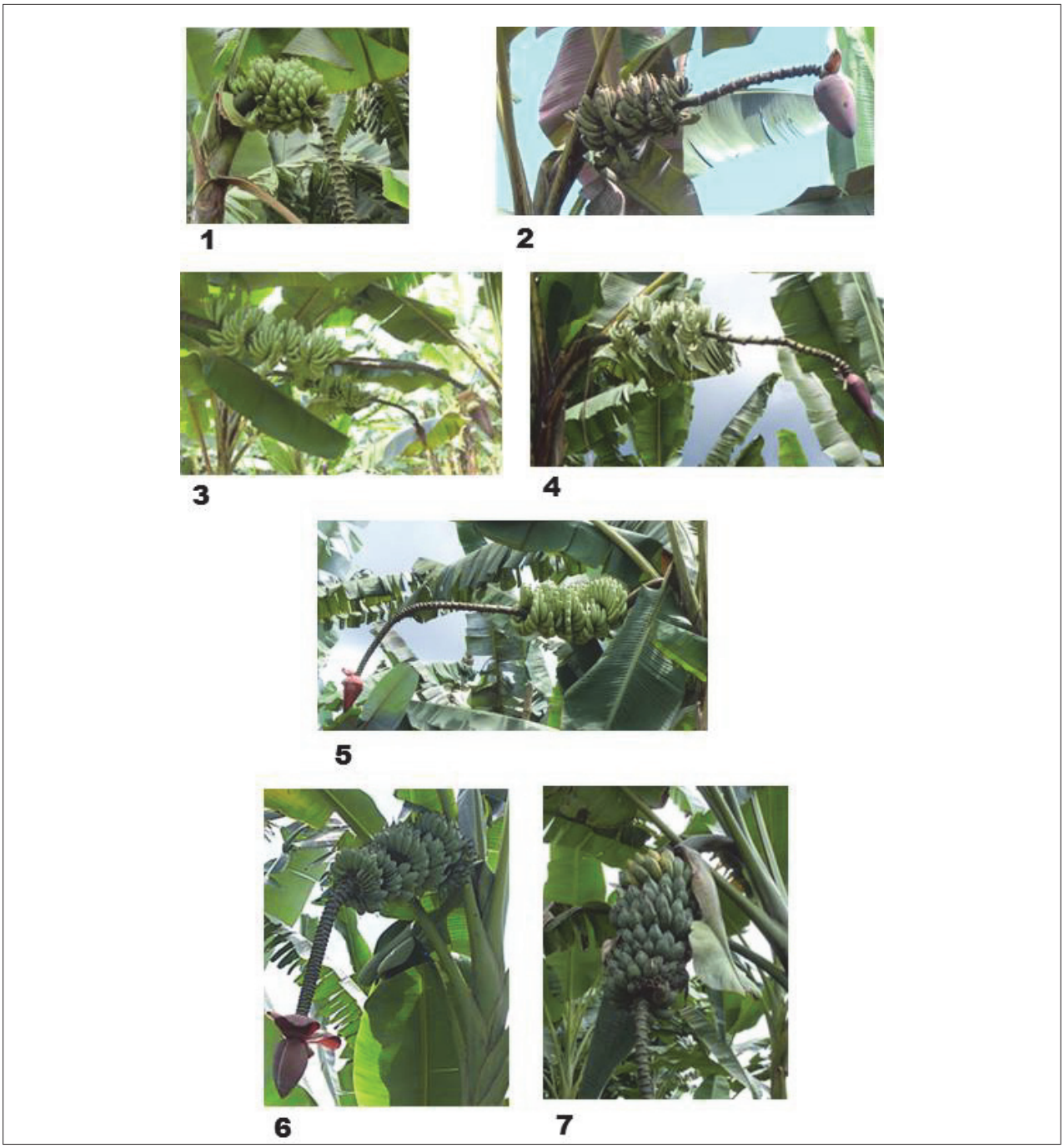

Figure 1. Morphological diversity of self-pollinated bunches of the different genotypes. 1: *M. a.* ssp. *burmannica*, Calcutta 4 clone; 2: *M. a.* ssp. *zebrina*, Zebrina clone; 3: *M. a.* ssp. *microcarpa,* Microcarpa clone; 4: *M. a.* ssp. *truncata*, Truncata clone; 5: *M. a.* ssp. *malaccensis*, THA018 clone; 6: *M. balbisiana*, Cameroon clone; 7: *M. balbisiana*, Honduras clone.

2. Number of ovules per fruit. The mean number of ovules per ovary was calculated from 10 female flowers taken randomly on hands No. 2 and No. 3 at flowering, from a bunch representative of each genotype.

3. Number of seeds per fruit. This was estimated from the total number of seeds per bunch divided by the number of fruits per bunch.

Several sources of variation likely to affect germination were studied:

1. Seed sampling. Inherent physiological factors linked to the position of the seeds in the fruits, or even the position of the fruits in the bunch were studied. The aim was to ascertain whether all the seeds in a bunch developed uniformly or, on the contrary, might be subject to variations due to varying access to nutrient resources (elaborated sap). To that end, we compared the germination rate for seeds located in the proximal, median and distal sections of fruits taken from the median hand of the bunch. We also compared the germination rate for seeds taken from the median fruits of the first hand, median hand and last hand of the bunch.

2. Horticultural germination protocols and *in vitro* germination. Factors extrinsic to the plant material, notably the germination substrates, were investigated. In this study, we sought to determine whether a germination substrate comprising 40% black volcanic soil (Table 2) and 60% coffee parchment (substrate S2) would help to significantly increase the germination rate compared to unmixed black volcanic soil alone (100%) (substrate S1). These results were

Table 2. Composition of volcanic soil and coffee parchment (Percentage on dry matter).

Components	Volcanic soil (CIRAD analysis)	Coffee parchment (Pinon and Godefroy, 1973)
N	0.74	2.74
P	0.003	0.105
K	0.052	2.75
Mg	0.058	0.30
Ca	0.24	1.14
Organic matter	9.92	78

then compared to those obtained with *in vitro* culturing of embryos (substrate S3). The embryos were cultured according to the protocol published by Bakry (2008).

3. Genetic variations. Lastly, variations in genetic origin were studied, working on a vast panel of genotypes representative of the diversity of wild bananas (five genotypes of *M. acuminata* and one genotype of *M. balbisiana*).

Statistics

This work involved large numbers, with systematic replications to effectively pinpoint any variations between the different treatments. For each of the modalities, each unit batch of seeds contained 25 seeds and each batch was replicated four times in a split-plot experimental design.

All the data gathered were processed simultaneously in an analysis of variance to determine the variables having an impact on seed germination. Within the variables, significant differences between modalities were tested at the 5% threshold by the Newman and Keuls test.

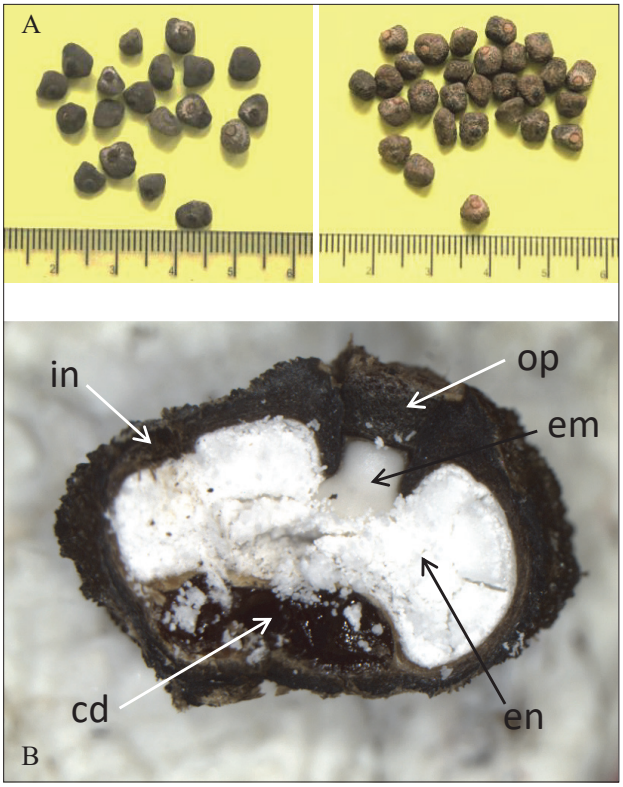

Figure 2. Mature *Musa* seeds. A: Morphology of seeds – Left: flattened *M. acuminata microcarpa* seeds; right: rounded *Musa balbisiana* seeds (Cameroon clone). Scale ruler in cm. B: Anatomy of *M. balbisiana* seed (Cameroon clone). Cd, chalazal disc; em, embryo; en, endosperm; in, integuments; op, operculum.

Results and discussion

Seed production of self-pollinated bananas

The pollinated bunches were harvested when the fruits of the first hand started turning yellow. The time lapse between pollination and bunch harvesting proved to be quite constant, from 120 to 136 days depending on the clones (Table 3). Under the conditions at the Njombé station, we did not find any differences in the flowering-harvest time interval between the *acuminata* and *balbisiana* species. The bunches were then left to ripen in a shed for about 4–5 days, at ambient temperature, prior to harvesting seeds from uniformly yellow fruits.

When mature, seeds are black with a hard coat. A mealy endosperm fills the whole of the seed cavity, blocking the embryo below the micropylar plug (Figure 2). Mature seeds were obtained from all the self-pollinated *M. acuminata* accessions. However, for *M. balbisiana*, mature seeds were only obtained from the Cameroon clone, because the fruits of the Honduras and Pisang Klutuk Wulung genotypes only produced aborted seeds. The abortion seemed to be the result of self-pollination, as the Honduras clone pollinated by the Cameroon clone produced some perfectly normal, non-aborted seeds. The reason for this abortion, which only occurred in these two *M. balbisiana* clones, remains a mystery at this stage (recessive lethal gene?).

With self-pollination, the gross number of seeds obtained per bunch proved to vary considerably from one accession to another (Table 3). We classed these accessions in three categories:

- Genotypes producing few seeds, around 4,000 to 8,000 seeds bunch^{-1}. These were the Zebrina, Calcutta 4 and Truncata clones. The bunches comprised from 14 to 17 hands, with an average number of seeds/fruit varying from 30 to 60.

Table 3. Seed production from self-pollinated wild genotypes. DFH: Duration from flowering to harvest at maturity (in days).

Species	Accession name	DFH (days)	No. hands bunch^{-1}	No. fruits hand^{-1}	No. fruits bunch^{-1}	No. seeds bunch^{-1}	No. seeds fruit^{-1}	No. ovules fruit^{-1}	Seed-set rate (%)
M. acuminata	ZEB	120	9	14	129	3,886	30	126 (e)	24
	CAL	124	8	17	142	6,798	48	134 (de)	36
	TRU	130	9	14	133	8,308	62	174 (c)	36
	MIC	129	10	23	238	12,750	54	148 (d)	36
	MAL	124	9	17	160	15,925	100	189 (b)	53
M. balbisiana	CAM	136	13	17	231	42,700	184	353 (a)	52
	HON	128	17	18	285	77,242	271	–	–

- Genotypes producing an average number of seeds, *i.e.*, two or three times more than the above genotypes, at around 12,000 to 16,000 seeds bunch^{-1}. These were the *microcarpa* and *malaccensis* genotypes.
- The *balbisiana* genotypes producing a very large number of seeds, *i.e.*, over 40,000 seeds bunch^{-1}.

For *M. acuminata*, Lassoudière (2010) and Vineesh *et al.* (2015) reported that fruits can contain from 60 to 100 seeds and that, in particular, the average number for *M. acuminata* subsp. *burmannica* is 80. Overall, our own data (from 30 to 100 seeds per fruit) corroborated those results. However, with self-pollination, we were led to class the different accessions in three categories, and these variations could be attributed to different reasons, as explained below.

For an average number of around 50 seeds per fruit, the high seed production per bunch for the *microcarpa* accession resulted from a larger number of fruits hand^{-1} (around 23) and, consequently, a clearly larger number of fruits bunch^{-1}, which is one of the botanical particularities of that clone and of the other accessions of the subspecies associated with it (Champion, 1967). Such was not the case for the *malaccensis* accession, which only bore an average number of fruits bunch^{-1}, but whose particularity was to contain almost 100 seeds fruit^{-1}, *i.e.*, almost double the other *M. acuminata* accessions (larger fruits than the other accessions).

Lastly, the high seed production of *M. balbisiana* resulted from a much larger number of hands bunch^{-1} than the *M. acuminata* accessions, but also a much larger number of seeds fruit^{-1}.

An analysis of the number of ovules per ovary first revealed differences between the subspecies within the *acuminata* species, and always a smaller number than that of *M. balbisiana* (Table 3): about 130–140 ovules fruit^{-1} for the CAL, MIC and ZEB accessions, close to 180 for MAL and TRU, and up to 350 for the CAM accession. A comparison of the number of ovules and fully seed-filled fruits showed that seed-set varied from 24% up to 50% within the fruits.

This variable number of seeds per fruit may reflect a variation between the different subspecies for the number of ovules per fruit that are potentially fertilizable on flowering, or quite simply the result of competition between fertilized ovules developing in small fruits. Our results revealed that the average number of seeds in *M. balbisiana* fruits was at least twofold, but quite often fivefold to sevenfold that found in *M. acuminata*. This last result is hardly surprising when considering that the ovules are inserted in a single row in axillary placentation in the three carpels of the ovary in *M. acuminata* (Simmonds and Shepherd, 1955), while the ovules are inserted in two rows in the species *M. balbisiana*. For the same fruit length, it can therefore easily be concluded that the fruits of *M. balbisiana*, which are plumper, statistically contain twice as many seeds as *M. acuminata* fruits, provided of course that all the ovules have been fertilized.

Description of banana seed germination

1. Description of seed germination in the greenhouse. An illustration of seed sowing and germination under our conditions at the CARBAP station in Njombe is given in Figure 3.

The first visible sign of germination, usually 10 to 30 days after sowing, was expulsion of the micropylar plug beneath which the primary root emerged. The primary root arose from an irregularly swollen hypocotyl on which adventitious roots and the shoot were differentiated (Figure 3A). The first two leaves took the form of sheathes without laminas; the third, which was produced around two or three weeks after the start of germination, bore a clearly deployed lamina. After four weeks, all visible traces of endosperm disappeared, but the coat of the seeds remains for at least five weeks, often longer, before falling off or rotting.

The root system of a young seedling comprised fine adventitious roots, usually pointing downwards, somewhat branching. At around one month old, a new type of root appeared suddenly and quickly replaced the juvenile system; the new type of root was clearly thicker, longer, and less branched than the juvenile type. The new root type occurred without any transition and was the first indication of the shape that the mature root system would later take (Figure 3A d).

2. Description of *in vitro* embryo germination. Under our culture conditions, embryos generally began to germinate in the dark at 27 °C around 10–15 days after their extraction and culturing on "embryo medium" (Figure 4A). The first visible sign of germination was a change in embryo colour, turning from opaque white to yellowish. Then, germination in the dark continued with elongation of the coleoptile taking the form of a white spindle, from the middle of which the first

Table 4. Effects of intrinsic and extrinsic factors on seed germination in *Musa* wild species.

Variation factors	D.F.	F value	Pr value (>F)	Significance
Genotype	5	45.11	2.00E-16	***
Effect of the position of the seed in the fruit on germination - PSF	2	0.065	0.937	
Effect of the position of the seeds in the bunch on germination - PSB	2	0.925	0.398	
Interaction effect: Germination substrate S1 *vs.* S2 - S1/S2	1	0.346	0.557	
Interaction effect: Germination substrate (S1-S2) *vs. in vitro* culture (S3) - (S1-S2)/S3	1	221.5	2.00E-16	***
Interaction effect: Genotype / PSF	12	0.863	0.585	
Interaction effect: Genotype / PSB	12	0.664	0.785	
Interaction effect: Genotype *vs.* Germination substrate (S1-S2)	6	0.764	0.599	
Interaction effect: Genotype / Germination substrate (S1-S2) *vs. in vitro* culture (S3)	6	145	2.00E-16	***
Interaction effect: PSF *vs.* PSB	1	0.007	0.931	
Interaction effect: PSF *vs.* Germination substrate (S1-S2)	3	0.065	0.978	
Interaction effect: PSF / Germination substrate (S1-S2) *vs. in vitro* culture (S3)	3	23.981	2.19E-13	***
Interaction effect: PSB *vs.* Germination substrate (S1-S2)	3	0.074	0.974	
Interaction effect: PSB / Germination substrate (S1-S2) *vs. in vitro* culture (S3)	3	58.27	2.00E-16	***

Statistical ANOVA analysis: D.F.: degrees of freedom; Pr: probability (Pr) of absence of effect; Significance code: 0 '***' – 0,001 '**' – 0,01 '*' – 0,05 '.' - 1* 0'***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1.

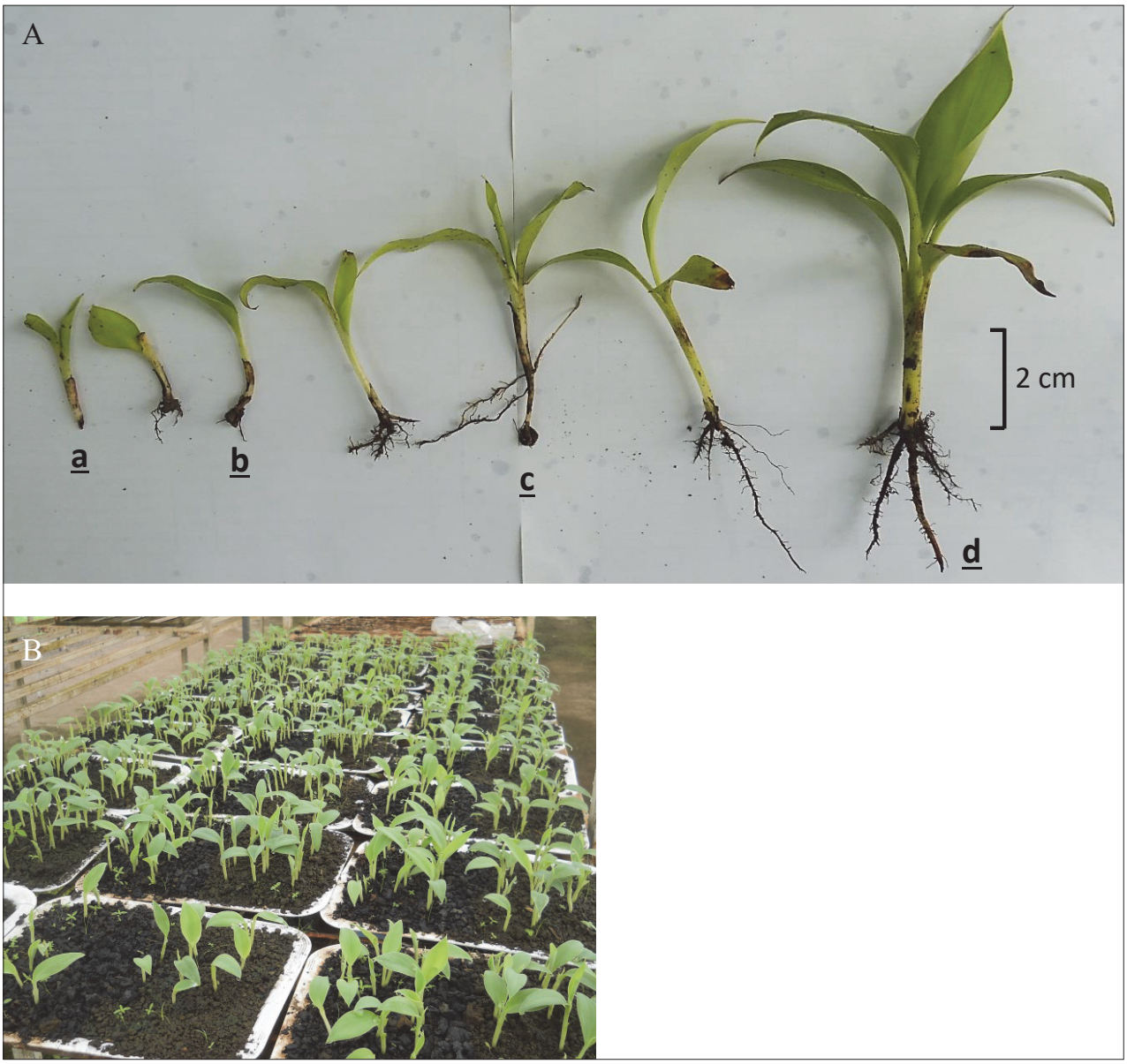

Figure 3. *In vivo* germination of banana seeds. A: Different stages in the germination of banana seeds (*M. acuminata microcarpa*) – a: about 6–10 days after sowing; b: about 15 days after sowing; c: four weeks old, two leafless sheaths, two expanded leaves and one rolled leaf visible. Adventitious roots and remnant of the seed coat present; d: about 8 weeks after sowing, four expanded leaves and one rolled leaf, thicker and less ramified expanded root system. B: Overall view of sowing replicates under greenhouse conditions at CARBAP (*Musa balbisiana*, Cameroon clone).

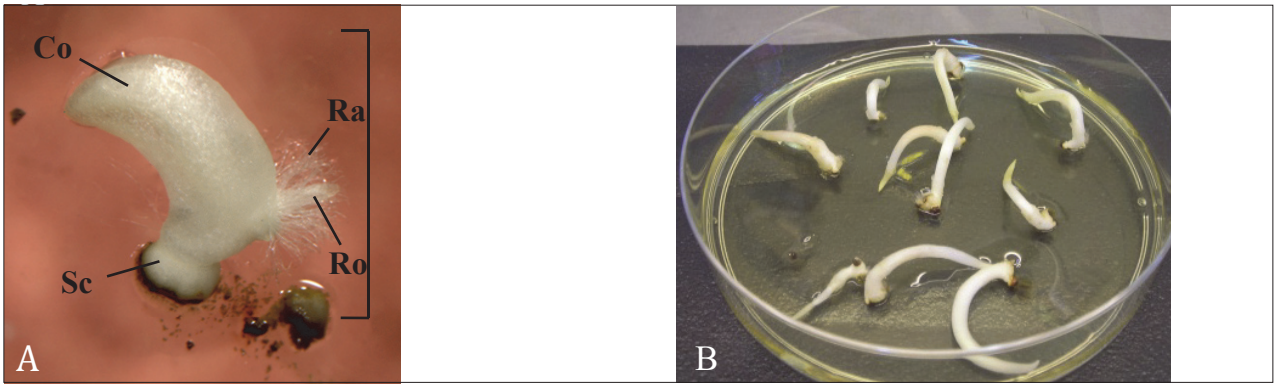

Figure 4. *In vitro* germination of banana embryos. A: Ten-day-old *in vitro* cultured embryo (*M. balbisiana*, Cameroon clone). Detailed observations show coleoptile development, and sometimes the emergence of radicles and one primary rootlet. Co: coleoptile; Sc: scutellum; Ro: primary root; Ra: radicles. Bar size: 5 mm. B: Twenty-day-old *in vitro* cultured embryos (*M. balbisiana*, Cameroon clone).

Table 5. Average germination rate of seeds on natural substrates (S1/S2) according to their position in fruits and bunches.

Species	Genotype	Positioning of seeds in fruit			Positioning of seeds in bunch		
		Proximal section	Median section	Distal section	Proximal hand	Median hand	Distal hand
AA	CAL	17.5	13.5	21.0	18.0	25.0	20.5
AA	MIC	63.0	45.0	68.0	58.0	62.0	63.5
AA	THA	61.0	65.5	50.0	44.0	31.5	28.0
AA	TRU	12.5	15.5	14.5	16.0	19.5	18.0
AA	ZEB	20.0	22.5	27.5	25.5	26.0	23.0
BB	CAM	86.0	82.0	86.5	75.5	84.5	77.0

ZEB: *M. acuminata* ssp. *zebrina*; CAL: M. *acuminata* ssp. *burmannica*, Calcutta 4 clone; TRU: *M. acuminata* ssp. *truncata*; MIC: *M. acuminata* ssp. *microcarpa*; THA: *M. acuminata* ssp. *siamea* KhaePhrae clone; CAM: *M. balbisiana*, Cameroon clone; HON: *M. balbisiana*, Honduras clone. No significant differences in germination rates were detected between the different seed batches on S1/S2 substrates for the same genotype.

Table 6. Germination rates on natural substrates and *in vitro* culture.

Species	Genotype	Germination conditions				Germination rate increase
		S1/S2 substrates		*In vitro* embryo culture		
		Number of seeds	Germination rate (%)	Number of seeds	Germination rate (%)	
AA	CAL	1,200	19.0	600	82.5	+ 334%
AA	MIC	1,200	60.0	600	75.0	+ 25%
AA	ZEB	1,200	24.0	600	73.0	+ 204%
AA	THA	1,200	46.8	600	86.5	+ 85%
AA	TRU	1,200	16.0	300	80.0	+ 400%
BB	CAM	1,200	81.8	200	93.8	+ 15%

leaf protruded around three weeks after the start of culturing (Figure 4B).

Unlike the descriptions made by some other authors (Afele and De Langhe, 1991; Asif *et al.*, 2001), the embryos developed very few or no radicles and roots under our culture conditions. This type of reaction must have been due to the presence of cytokinin in the culture medium (1 mg L^{-1} of BAP), which is known to inhibit root growth in plants. After 30–40 days' culturing, the germinated plantlets were transferred to a growth medium without growth regulator and placed in the light to encourage rooting.

3. Quantitative data on seed germination. All the germination data gathered were processed simultaneously in a nested ANOVA to ascertain which variables had an impact on seed germination. The results are summarized in Table 4. *Banana seed germination in the greenhouse.* Out of more than 12,000 sowings carried out with fresh seeds of highly varied genetic origin, we never found any differences in germination in the greenhouse or *in vitro* that could be attributed to the positioning of the seeds in the fruit or even to the "hand" positions in the bunches (Tables 4 and 5). These results indicated that there was no competition for access to carbon resources (from elaborated sap) within the fruits and bunches of seed-bearing bananas and that all the seeds harvested from the same bunch had identical viability.

In addition, the ANOVA analysis also highlighted that our substrates had no influence on the germination rate under greenhouse conditions (Table 4). In a study carried out in 1952, Simmonds concluded that greenhouse soil is better than sand for banana seed germination, that deep sowing gives better results than shallow sowing and, lastly, that drained containers give better results than undrained containers. It was these preliminary results of Simmonds that led us in this study to rule out sand as a germination substrate and only study variations in germination between a pure volcanic soil (substrate S1) and a mixture of the same volcanic soil (40%) and coffee parchment (60%) with better drainage (substrate S2). The choice of substrate S1 arose from the fact that we had seen natural germination at the foot of certain clones grown on this black volcanic soil of the CARBAP collection at Njombé. It was thus that we thought of using that soil as it was, after sterilization, or mixed with coffee parchment for aeration reasons (substrate S2). It turned out that growth substrates S1 and S2 both produced good results (Table 5) compared to work undertaken by other authors, who reported that germination was extremely variable and relatively difficult under natural conditions (Chin, 1996; Uma *et al.*, 2012). Usually, *Musa* spp. seeds germinate at an extremely low rate in soil (Dayarani *et al.*, 2014), especially as little is known about the factors that affect seed germination rates in this genus (Asif *et al.*, 2001).

Unlike the previous two variables, the genotype of the different accessions proved to be a source of variation in the germination rate in the greenhouse (Table 4). Under natural conditions (substrates S1 and S2), *M. balbisiana* CAM achieved a high mean germination rate of 79%, with peaks of 88% for some batches, though without ever reaching 100% germination (Tables 5 and 6). On the other hand, seed germination on natural substrates for all the subspecies of *M. acuminata* studied was always poorer than for the CAM accession. These rates also proved to be highly variable from one genotype to another: average rates for the MIC accession (mean: 58.5%; max.: 80%) and the THA accession (mean: 46.75%; max.: 76%), low for ZEB (mean: 25%; max.: 40%) and even surprisingly low for CAL4 (mean: 19%; max.: 40%) and TRU (mean: 15.75%; max.: 28%).

Our results for *M. acuminata* tallied with the findings of other authors, who described some highly variable results for this species. Asif *et al.* (2001) and Rashid *et al.* (2013) reported germination rates amounting to only 2–3% for fresh

seeds of *M. a. malaccensis*, and Vineesh *et al.* (2015) under 5% for seeds of the subspecies *burmannica*. We considered our results rather satisfactory given the other work published, as under our own experimental conditions we never found germination rates under 15% in the *acuminata* species. However, as highlighted by Simmonds (1952), germination rates for *M. acuminata* are highly variable and erratic from one batch to another, suggesting seed dormancy, defined by most authors as the physiological status of seeds or embryos which, despite being placed under environmental conditions propitious to their germination, are incapable of germinating or germinate poorly.

Of course, germination success depends first and foremost on seed quality (physical, physiological and health qualities). In *Musa*, seed malformation usually involves an absence of embryos or their poor conformation, or an absence of endosperm (Shepherd *et al.*, 1987; Bakry and Horry, 1992), or the mass of chalaza (Pancholi, 1995), or even underdeveloped integuments. The low and sporadic germination rate in substrates S1 and S2 for some of the *acuminata* genotypes studied may have been due to this quality problem, which may have resulted from the self-pollinated origin of the seeds. However, that did not appear to be the case here for the *M. acuminata* clones, as all the seeds passed flotation tests prior to sowing, indicating, in theory, that they were normally formed. In addition, these seeds were systematically opened for *in vitro* embryo cultures, without any particular abnormalities being found.

One possible explanation of seed dormancy might be that the low germination rate of the seeds was due to specific inhibition of the seed integuments. The integuments, which are first of all a physical barrier (mechanical resistance, impermeability to water) are also a chemical barrier, because they contain phenolic compounds that trap oxygen, or germination inhibitors (Pancholi *et al.*, 1995; Baskin and Baskin, 2004; Puteh *et al.*, 2011). Moreover, the last hypothesis is backed up by the results of Graven *et al.* (1996), who showed that the macromolecular fingerprint of banana seed integuments is rich in aromatic phenolic compounds likely to promote seed dormancy.

Faced with these seed germination difficulties under controlled conditions, Pushkaran (1989) reported achieving an increase in the germination rate in *M. acuminata* (subspecies not specified) from 0% to almost 90% with mechanical scarification of the seeds prior to sowing. In 1962, Stotzky *et al.* had already scarified the lateral surfaces of *Musa balbisiana* seeds, which helped to increase the germination percentage to 80% and shortened the germination time from 3 ± 6 weeks to 6 ± 10 days. All these authors suggested that water penetration and gas exchanges were enhanced by breaking the silica layer, the cuticle and the mesotesta. Yet, on other batches, while water had penetrated as far as the embryos, there was no improvement in germination. Moreover, our own results on *M. balbisiana* Cameroon were equivalent to those of these authors, without needing to scarify the seeds.

The strong immediate germination of fresh *M. balbisiana* seeds under our natural greenhouse conditions showed that, unlike *acuminata* accessions, there was no dormancy phase in this species. This observation had already been reported by several authors (Simmonds, 1952, 1959; Stotzky and Cox, 1962; Afele and De Langhe, 1991; Bhat *et al.*, 1994). This strong germination in *M. balbisiana* is no doubt the reason why most studies on banana seed storage and germination have focused on the *balbisiana* species for the Eumusa section. It may also be the reason for the strong expansion of this very hardy species, which, by combining high seed production and easy germination, has become an invasive plant in some regions of the world.

Another hypothesis might be linked to light, which is important in plants for photosynthesis. Some rare studies (not available) would seem to indicate that light has a negative effect on *Musa* germination. This result tallies with the literature, whereby the presence of light acts as a negative photoblastism on the germination of zygotic embryos in other species more distant from *Musa* (Hisajima *et al.*, 1991). This factor is involved in secondary embryo dormancy (Le Page-Degivry and Garello, 1973), which corresponds to a loss of viability when the embryo, in an imbibed state, is placed under conditions incompatible with its germination (excessively high temperatures, lack of oxygen, presence of light).

However, all these possible causes do not explain the substantial reaction disparity observed between all the genotypes within *Musa* and, at this stage, the germination of banana seeds remains an exercise that is doomed to erratic results and difficult to rely on as part of a vast programme to conserve banana genetic resources in seed form. This explains why we wished to study the potential offered by *in vitro* germination on the same seed batches.

In vitro *germination of banana embryos.* The general analysis of variance (Table 4) revealed a highly significant difference in germination rates between "traditional sowing" and "*in vitro* embryo culture".

Under *in vitro* conditions, the overall germination rate for all the accessions reached an average value approaching 82% and, in all cases, was never under 73% (Table 6). This rate was much higher than for seeds germinated in the greenhouse, which was only 41%. For *M. acuminata*, the average germination rate rose from 33% in the greenhouse to 79% *in vitro*, *i.e.*, an increase of 140%. More specifically, we found an increase in germination rate of around 200% for the Zebrina clone, and even more than 300% for the Calcutta 4 and Truncata clones.

In addition, the *in vitro* germination rates for *M. acuminata* were also more uniform: the coefficient of variation for the means of the five subspecies was only 7% *in vitro*, while it was 58% in the greenhouse (on an identical sample). *In vitro* culture helped to increase embryo germination significantly, but also in a more regular and uniform way.

Our *in vitro* germination results were equivalent to (or even better than) those reported in the literature and they covered, in a single study, wider genetic diversity than all the other authors combined (Stotzky *et al.*, 1962; Afele and De Langhe, 1991; Asif *et al.*, 2001; Ahmed *et al.*, 2006; Uma *et al.*, 2011; Rashid *et al.*, 2013; Arun *et al.*, 2013; Barrudin and Kayat, 2015). These results on a vast panel of diversity lent credence to the technique we present here, based on a medium enabling the germination of all sorts of genotypes. The culture medium used had a balanced formulation, well adapted to juvenile banana tissues. It enabled the germination of wild species, but also the good germination of embryos taken from the seeds of polyploid varieties, which are often abnormal or immature, and sometimes devoid of endosperm (Bakry and Horry, 1992). It was the same medium that led to the production of androgenetic plants from banana anther cultures (Bakry *et al.*, 2008).

The beneficial effect of *in vitro* culture very clearly appeared to be linked to a breakage of dormancy. It is true that adding cytokinin to the "embryo medium" may have helped

to increase the germination rate after breaking dormancy. However, it is possible to obtain germination just as quickly without adding growth regulators to the germination medium (Cox *et al*., 1960; Bakry, *pers. commun.*). In *Musa*, the hardness of the seed integument embodies a conflict between seed protection and seed germination (Graven *et al.*, 1996). The hard walls offer effective protection during maturation, dispersal and dormancy. However, it hinders germination and the embryo requires great strength to break through the seed walls, with the micropylar plug opening after elongation of the root-hypocotyl axis (McGahan, 1961). Our results clearly seemed to indicate that, even in an imbibed state, dormancy phenomena reside more in the tissues surrounding the embryo (integuments, chalaza, albumen, *etc*.) than in the embryo itself.

Thus, more than an increase in germination rates, the main advantage of *in vitro* embryo culture lies in securing germination for recalcitrant or "dormant" genotypes. Indeed, we found that *in vitro* culture tended to render the germination rates of different genotypes more uniform, within a range of 70 to 95%, with minimal subsequent losses (less than 5%) after hardening in greenhouses. This positive effect of *in vitro* germination was all the more marked in that germination rates for traditional sowing were low (Calcutta 4, Zebrina, THA18).

Conclusion

Beyond genetic aspects, conservation practices within the wild compartment of *Musa* need to take on board a set of technical issues linked to the collection, germination and storage of seeds from these seed-bearing bananas. This is a point already highlighted by Simmonds as early as 1952, taken up again much later at a colloquium devoted to banana genetic resources (Musa Net/Trust Joint Meeting, 2012) and today by the FAO Crop Trust project. They all agree that seed conservation for crop wild relatives (CWR) is an attractive option, but still requires prior studies to solve some technical problems, notably good knowledge of factors affecting seed viability and the development of reliable germination protocols that are repeatable over time. For banana, the aim is not so much to achieve a maximum germination rate, as to obtain sufficient germination to reach the thresholds that have been fixed: five to ten plants for conservation *in situ* and *ex situ* collection, and 200 to 300 individuals for studies on population genetics (allele frequency search), or formal genetics.

Of all the factors investigated in this study, our results showed that only the genotype of the accessions, along with the contribution of *in vitro* culture compared to natural sowing, had a real impact on seed germination. *In vitro* germination overcomes dormancy constraints, whose origin remains fairly unknown in banana, and secures the germination of individuals owing to a minimum rate of 50% with fresh seeds. It is a sound technique, perfectly adapted to conserving representative allele distributions taken from *in situ Musa* populations because it has been shown, from a genetic viewpoint, not to induce allele disruption in wild holotypes (Hippolyte *et al.*, 2010; D'Hont *et al*., 2012; Dupouy *et al.*, 2019).

Lastly, for the conservation of wild *Musa* genetic resources, we would today recommend, from a summarized practical viewpoint:

- Firstly, harvesting fruits at maturity and mixing all the seeds from the same bunch, but in view of erratic germination results, not mixing seeds from different bunches of the same genotype, even if they come from self-pollination under controlled conditions.
- Secondly, using embryo *in vitro* culture, which is admittedly a sophisticated and expensive method, but it is also very effective in promoting banana seed germination. This method can only be implemented if an *in vitro* culture laboratory is available and if these techniques have been well mastered, so as not to lose plant material through culture medium infections.

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