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Effects of Two Disinfectants and Two Growth Regulators on *in vitro* Propagation of Smooth Cayenne and Sugarloaf Cultivars of Pineapple (*Ananas comosus* (L) Mill var. *comosus*)

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Abstract: Pineapple is the first fruit crop that greatly contributes to poverty reduction for population in Benin. However, the lack of plant material is the main factor that limits field extension. The aim of this work was to optimize the production of plant material for the two pineapple cultivars by tissue culture technique. Disinfection of the buds was evaluated by using antibiotics (gentamicin at 300 mg/l for 12 hours), and disinfectants (sodium hypochlorite and mercuric chloride) at different doses and immersion duration. Axillary buds of crown were seeded on Murashige and Skoog medium (MS) supplemented with agar (0.8%). Benzylaminopurine (BAP) was tested at different concentrations (5 mg/l and 10 mg/l) or combined with naphthalene acetic acid (NAA) (4.5 mg/l BAP + 0.7 mg/l NAA). Six to eight weeks after initiation, buds were transferred to MS medium supplemented with 1 mg/l BAP + 40 mg/l adenine sulfate. The lowest infection rate (26.07%) was recorded by 15% of Sodium hypochlorite when buds were immersed for 5 min. The best bud burst rate was obtained on BAP combined with NAA (65.09%) with Sugarloaf (64.95%). After the first subculture, an average of 12.70 and 15.30 shoots per plantlet were obtained respectively for Smooth Cayenne and Sugarloaf. The number of rooted plantlets and the length of roots varied depending on the cultivar.

Keywords: Pineapple suckers; Disinfection; *in vitro* micropropagation; Adenine sulphate; Benzylaminopurine; Naphthalene acetic acid.

1. Introduction

The cultivated pineapple (*Ananas comosus* var. *comosus*) are the forth tropical fruits crop in the world respectively after bananas and plantains, mango and citrus but take the first place in international trade. In Benin, pineapple was classed as the first fruit crop with 315,795t according to FAOSTAT [1]. Part of the pineapple produced in Benin is exported to European Union (EU) and the subregion. Indeed, the quality of beninese pineapple is highly appreciated on the international market, which has an important export potential but not exploited yet [2].

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Main cultivars are produced, Smooth Cayenne and Sugarloaf, but cultivars such as Queen, Spanish and Perolera are also found [3, 4].

Despite of its economic role, the culture of pineapple, especially Smooth Cayenne, is faced a real problem of the lack of suckers due to their low rate of multiplication by conventional methods. Pineapple is vegetative propagated plants. This gap requires many farmers to purchase suckers from different provenances, varying in size and weight, with a tendency to increasingly accept smaller sizes and thus longer vegetative cycles with a risk on the quality of product [5]. It was showed that, pineapple culture is facing several diseases among which the most dangerous is wilt induced by Pineapple Mealybug Wilt associated Virus (PMWaV) and transmitted by mealybugs (*Dysmicoccus brevipes* and *D. neobrevipes*). It causes considerable losses of production [6, 7]. There is no curative method and therefore prevention of infestations [8]. The conventional multiplication favors diseases multiplication that is transmitted every season with accumulations of resistances. An infected sucker generates less productivity and the impossibility to have exploitable suckers. Thus, improved conventional methods of multiplication of healthy suckers such as castration, stem fragmentation have been developed but were unable to significantly increase the rate of multiplication of suckers [9, 10]. The production of healthy shoots by biotechnology methods is therefore a real alternative.

In vitro culture through micropropagation is recently used for mass production of healthy and homogenous shoots, thus optimizing the result of Floral Induction Treatment. Tissue culture techniques have been used to produce healthy planting materials for several species such as cassava, pineapple and banana [11-13]. However, *in vitro* regeneration of pineapple like other species is influenced by several factors including medium composition [11, 14, 15]. Nature and concentration of growth regulators used are the factors that determine duration of culture and the number of potentially regenerated plantlets [16]. *In vitro* regeneration is also influenced by genotype [17, 18]. Escalona, *et al.* [19] showed that the best multiplication rates are obtained on liquid media compared to solid media but with the temporary immersion technique, these multiplication rates are even higher. Considerable variability exists among genera, species, and even cultivars in the type and amount of growth regulators required for induction of morphogenesis. The type of morphogenesis that occurs in a plant tissue culture largely depends upon the concentrations of growth regulators present in the medium. Zuraida, *et al.* [20] reported that the dose of Benzylaminopurine was the mainly factor which increased the multiplication rate of pineapple depending of its dose on Murashige and Skoog base medium [21]. For the establishment of *in vitro* mass propagation of pineapple cultivars produced in Benin, it is important to determinate the type and dose of disinfectants and the concentration of Benzylaminopurine for the proliferation of crown axillary buds.

2. Material and Methods

2.1. Plant Material

Two cultivars (Smooth Cayenne and Sugarloaf) of pineapple were produced at stations of Central Laboratory of Biotechnology and Plant Breeding (LCBVAP) at University of Abomey-calavi (Benin). These stations are located in Zinvié and Wawata (Department of Atlantique), two areas of pineapple production in Benin. The present work took place from August 2012 to October 2013.

3. Methods

3.1. Disinfection of Crowns

Fruit freshly harvested were rinsed and crows were delicately removed and washed under running tap water for 30 minutes. After reduction of the crown leaves, half of axes obtained (Fig. 1) was soaked in a gentamicin solution (bactericide) at 300 mg/l for 12 hours. After rinsing in distilled water, axes are placed in a fungicide solution (Agriete 80 WP) at 30 g/l for 1 hour followed by a second rinse with sterile distilled water.

3.2. Axillary Buds Removal

The crowns leaves were delicately removed under laminar flow for axillary buds exposure. Then, cubic explants (size 90 mm^3) were excised from the crown's axes using scalpels (Fig. 2).

Fig-1. Axes and crowns of cultivars:
(a) Sugarloaf; (b) Smooth Cayenne

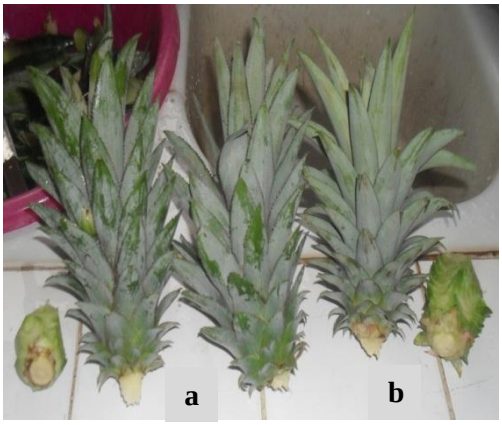
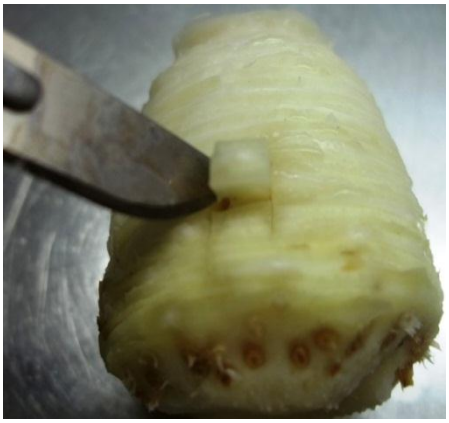


Fig-2. Crown axillary bud removal



3.2.1. Disinfection of Axillary Buds and Media

Modified protocol of Almeida, *et al.* [22] was used. Explants were rinsed with sterile distilled water for 2 minutes to get rid of debris. Surface sterilization was done by sequential immersion in 70% (v/v) ethanol for 3 min with slight agitation followed by a rinse of 3 minutes with sterile distilled water. Subsequently, the explants were treated in various dose of sodium hypochlorite solutions (NaOCl containing 8° of active chlorine) or mercuric chloride solutions containing 3 drops of Tween 20 at different durations (Table 1) followed by three (03) rinses for 5 minutes each.

Table-1. Different disinfection treatments

Treatments	Types and Concentrations of Disinfectant	Immersion Time
T_1	Overnight of Gentamicin 300 mg/l + NaOCl 10%	15 mn
T_1'	NaOCl 10%	15 mn
T_2	Overnight of Gentamicin 300 mg/l + NaOCl 12%	10 mn
T_2'	NaOCl 12%	10 mn
T_3	Overnight of Gentamicin 300 mg/l + NaOCl 12%	12 mn
T_3'	NaOCl 12%	12 mn
T_4	Overnight of Gentamicin 300 mg/l + NaOCl 15%	5 mn
T_4'	NaOCl 15%	5 mn
T_5	Overnight of Gentamicin 300 mg/l + $HgCl_2$ 0,1%	5 mn
T_5'	$HgCl_2$ 0,1%	5 mn
T_6	Overnight of Gentamicin 300 mg/l + $HgCl_2$ 0,01%	5 mn
T_6'	$HgCl_2$ 0,01%	5 mn
T_7	Overnight of Gentamicin 300 mg/l + $HgCl_2$ 0,001%	10 mn
T_7'	$HgCl_2$ 0,001%	10 mn

After excision of necrotic external tissues, a second disinfection was carried out in 5% sodium hypochlorite for 3 minutes. Buds were then rinsed with sterile distilled water for 3 minutes and were seeded into three different media. Murashige and Skoog medium [21] (MS) supplemented to 30 g/l sucrose, 7 g/l agar, and various doses of growth regulators (BAP and NAA). M1 = MS + 4.5 mg/l BAP and 0.7 mg/l NAA, M2 = MS + 5 mg/l BAP and M3 = MS/2 + 10 mg/l BAP. The media M1 and M2 were respectively used by Danso, *et al.* [23] for MD2 cultivar and Zuraida, *et al.* [20] using Smooth Cayenne. The media were adjusted to pH= 5.7 before adding agar. 20 ml of each media were dispensed in test tube (5 x 15 cm) which were covered with an autoclaveable plastic lid and then autoclaved at 121°C and 1.5 bar for 15 minutes. After sterilizing, explants were seeded into media in sterile conditions under laminar flow.

3.2.2. Environmental conditions

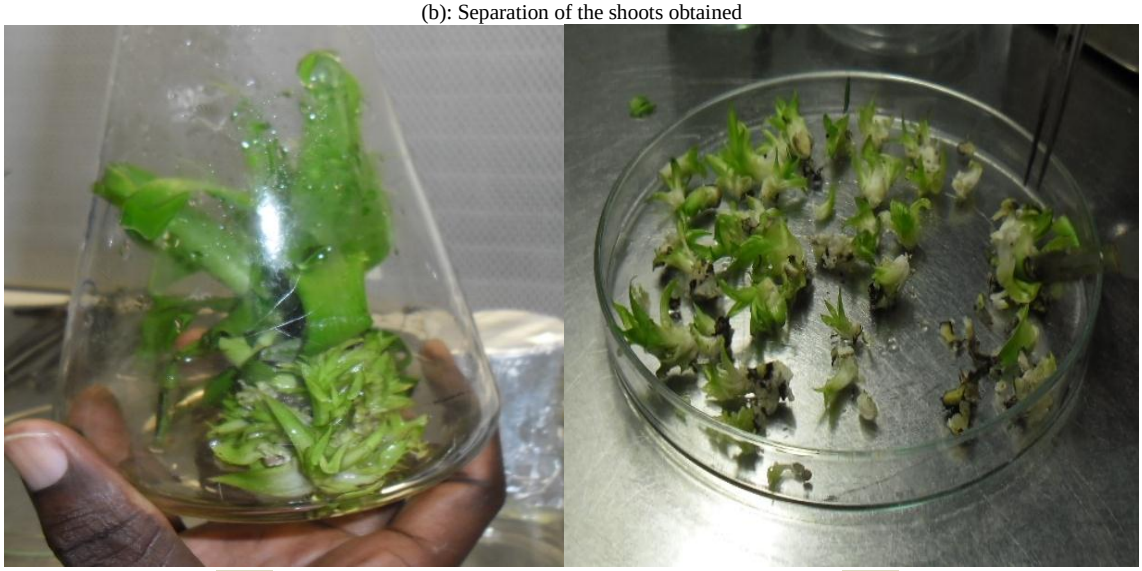
Test tube were kept in the growth room at 26°C ± 1, 80% relative humidity with 12 hours photoperiod using cool white florescent bulbs under 4000-5000 lux light intensity.

3.2.3. Multiplication of Shoots

After 6-8 weeks, the shoots were transferred to the regeneration medium composed by MS supplemented to 30 g/l sucrose, 1 mg/l BAP, 40mg/l adenine sulfate. 10 ml is distributed in 100 ml erlenmeyer flasks and subcultures were done each 5 weeks. The shoots regenerated were then separated and cultured on same medium for subcultures.

In order to against the buds asphyxia during their stay in liquid medium and to maintain homogeneity of nutrients in the medium, the erlenmeyers were placed on shakers at 80 rpm.

Fig-3. (a): Shoots at the 3rd subculture on MS + 1mg/l BAP + 40mg/l Adenine sulphate;
(b): Separation of the shoots obtained



a **b**

The determination of infection and necrosis rates were made after eight (08) weeks and the regeneration of buds of the two cultivars was done on the various media after two (02) weeks. The strength of the plantlets of the two pineapple genotypes was assessed through the phyllogenesis, rooting, fresh mass and color of these plantlets obtained during successive subcultures.

3.3. Data Editing and Analysis

A completely randomized device was adopted and 10 explants were used per medium and genotype with three repeats. Analysis of variance (ANOVA) was done to test the effect of treatments on disinfection and survival of buds, medium and genotype on regeneration, genotype and subcultures on growth parameters. Then, the Student Newman and Keuls test were used to structure the averages. The coefficient of Pearson at the 5% allowed the evaluation of the correlation between the length, width of leaves and fresh weight of plantlets. The various tests were carried out with MINITAB 16 software.

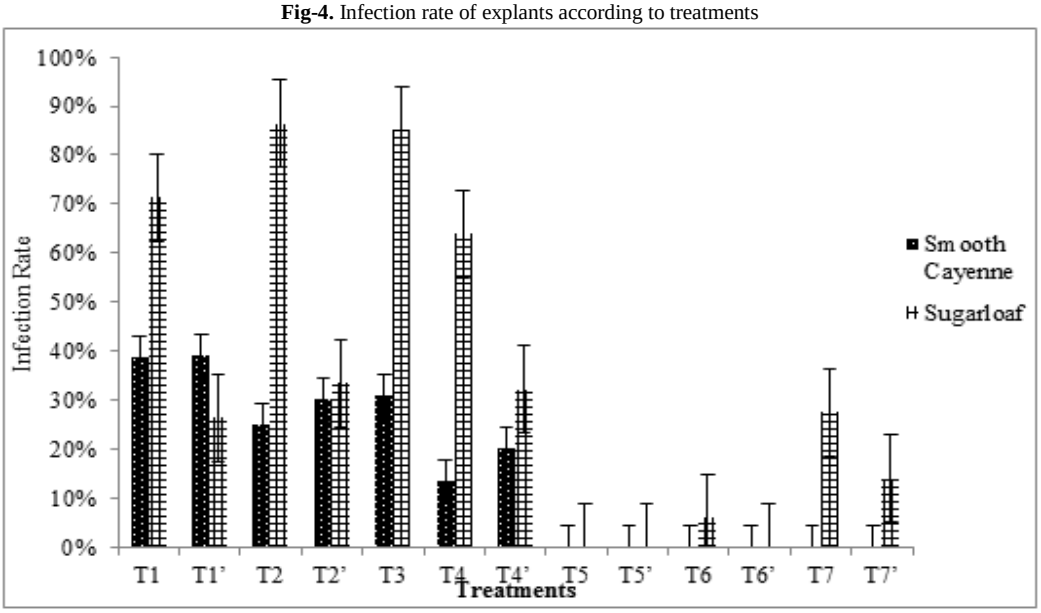
4. Results

4.1. Effect of Disinfectant Doses and Immersion Duration on Buds Surface Sterilization

The infection rate varied with treatment for the two cultivars tested. With sodium hypochlorite treatments, the lowest and highest rates of infection for Smooth Cayenne were 13.33% and 38.88% respectively for 15% NaOCl with gentamicin (T4) and 10% NaOCl (T1') compared with 26.31% and 86.36% respectively for 10% NaOCl (T1') and 12% NaOCl with Gentamicin (T2) for Sugarloaf.

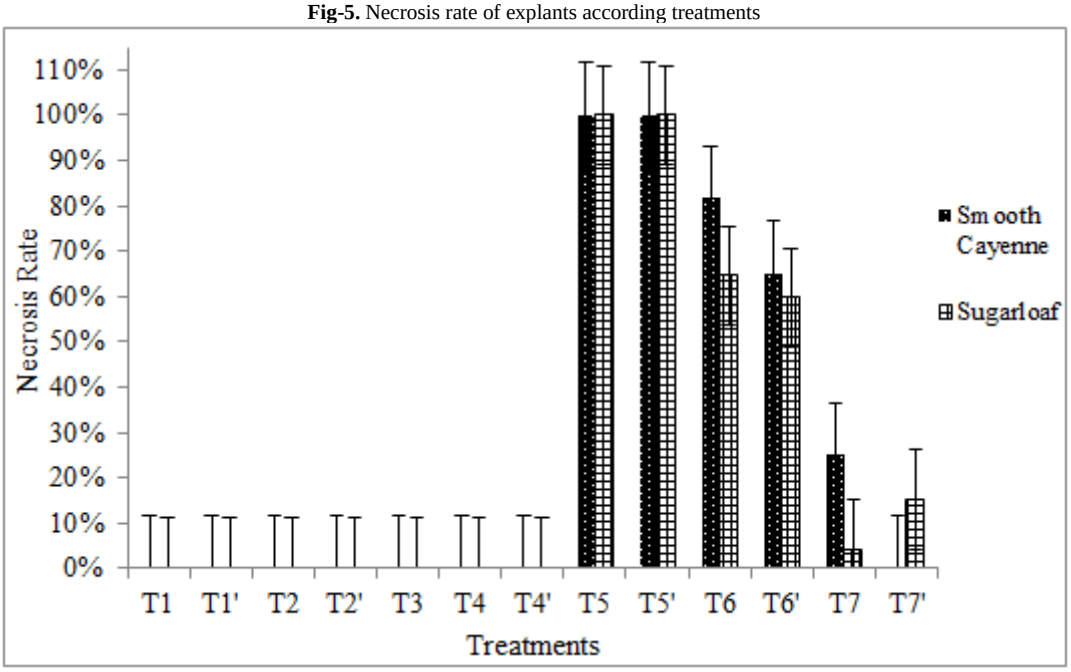
No infection was noted for treatments of mercuric chloride in Smooth Cayenne. But, low rates of infection were noticed in Sugarloaf (Fig. 4).

Fig-4. Infection rate of explants according to treatments



T_1 to T_4': Treatments with sodium hypochlorite T_5 to T_7': Treatments with mercuric chloride

Fig-5. Necrosis rate of explants according treatments



Analysis of comparison of averages revealed a highly significant difference between the different treatments for each cultivar ($p = 0.005$ and $p = 0.002$) respectively for Smooth Cayenne and Sugarloaf. However, analysis of variance showed that there was no significant difference ($p = 0.082$) between cultivars. Disinfection rate therefore depends more on the nature and the dose of disinfectants than the genotype.

4.2. Comparative Study of Treatments on the Explants Necrosis

The necrosis of explants according to the treatments (Fig.5) showed that treatments based on sodium hypochlorite did not cause any necrosis in the both cultivars. All treatments based on mercuric chloride have induced necrosis with Sugarloaf and the highest rate of necrosis (100%) was recorded by 0.1% $HgCl_2$ (T5 and T5') and the lowest rate of necrosis (04.16%) was recorded by overnight of 300 mg/l Gentamicin + 0.001% $HgCl_2$ (T7). The same observation was achieved with Smooth Cayenne. T5 and T5' treatments induced the highest rate of necrosis (100%) and T7 treatment not induced necrosis (0%). Sodium hypochlorite has therefore, contrary to mercuric chloride, favored the survival of explants.

4.3. Effect of Growth Regulators and Genotype on Bud Regeneration

Regeneration was observed as early as 12^{th} day in both cultivars independently of media. The rate of bud regeneration varied according to the media. Thus, medium contain 4.5 mg/l BAP with 0.7 mg/l NAA (M1) gave an

average of 65.09 ± 0.50 regeneration whereas 5 mg/l BAP (M2) gave 48.89 ± 0.50 and 10 mg/l BAP gave 57.78 ± 0.50. However, the analysis showed that there was no significant difference (p = 0.275) between media (Fig. 6a).
The ability of regeneration of buds varied according also to genotype. It reveals that Sugarloaf has a rate of 64.95 ± 0.40 whereas Smooth Cayenne gave 49.56 ± 0.40 (Fig. 6b) but the difference is not significant (p = 0.082).
The high concentration of Benzylaminopurine improved the regeneration especially for Sugarloaf and the interaction between the media and the genotype showed a significant difference (p=0.029).

Fig-6. Comparative effect of medium (a) and genotype (b) on bud burst

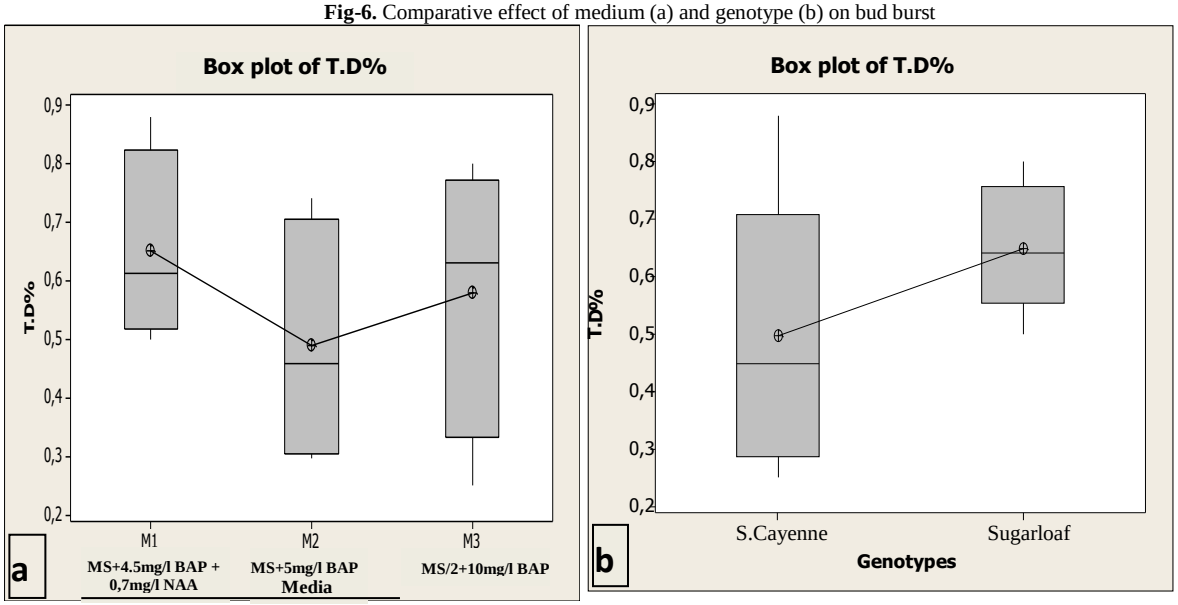
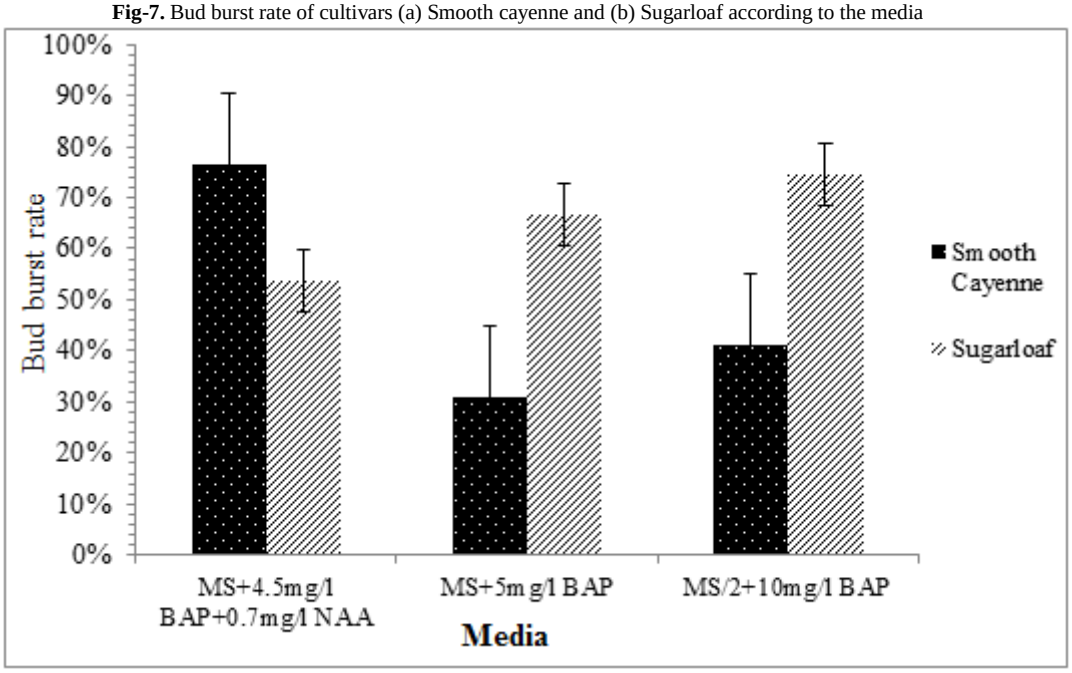


Fig-7. Bud burst rate of cultivars (a) Smooth cayenne and (b) Sugarloaf according to the media



4.3.1. Evaluation of Growth Parameters of Plantlets in Proliferation Stage

a) Evolution of Shoot Number per Subculture

The mean number of shoots of cultivar Smooth Cayenne varied from 12.70 (1st subculture) to 6.58 (2nd subculture) while the high mean for the cultivar Sugarloaf (23.80) was recorded at the 3rd subculture and the low one (9.10) at the 2nd subculture. The cultivar Sugarloaf produced more shoots than the cultivar Smooth Cayenne trough the subcultures.

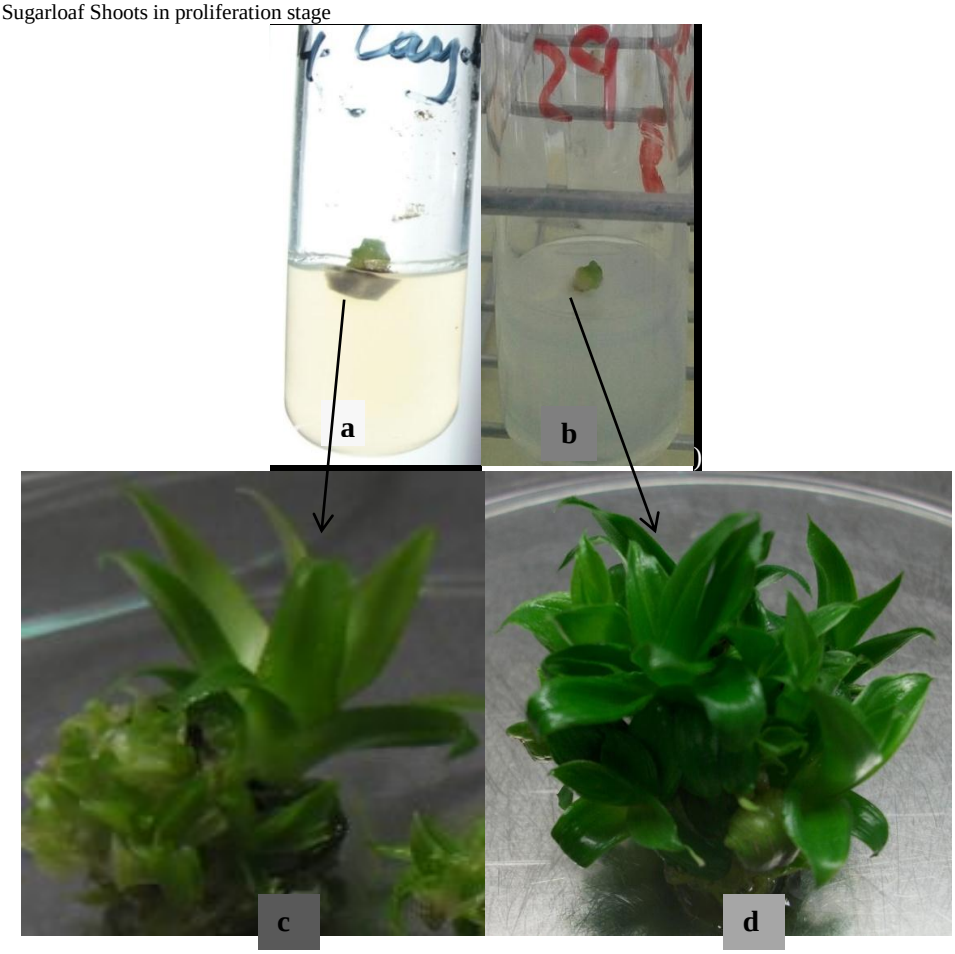
There was no significant difference at first subculture (p = 0.064) between the cultivars but at the 2nd, 3rd and 4th subcultures, the difference was significant (p = 0.000). This showing that the genotype influences the induction of shoots (Table 2).

Table-2. Shoots number per cultivar at each subculture

Sub.	1st		2nd		3rd		4th	
Cult.	S.Cay.	Sugarloaf	S.Cay.	Sugarloaf	S.Cay.	Sugarloaf	S.Cay.	Sugarloaf
Var.	08-18	10-20	5-11	6-15	05-21	15-35	5-13	12-27
Means	12.70±0.97**a**	15.30±0.97**a**	6.6±0.39**b**	9.10±0.54**a**	08.95±0.85**b**	23.80±1.40**a**	8.45±0.43**b**	17.65±0.95**a**
p	0.064		0.001		0.000		0.000	
Cov.	18.64		5.77		37.43		18.13	

Sub.= Subcultures; Cult.= Cultivars ; Var.= Variation; S. Cay.=Smooth cayenne; Cov.=Covariance
At each subculture, means of the same row followed by the same letter were not significantly different at p = 0.005 using Newman and Keuls test (SNK).

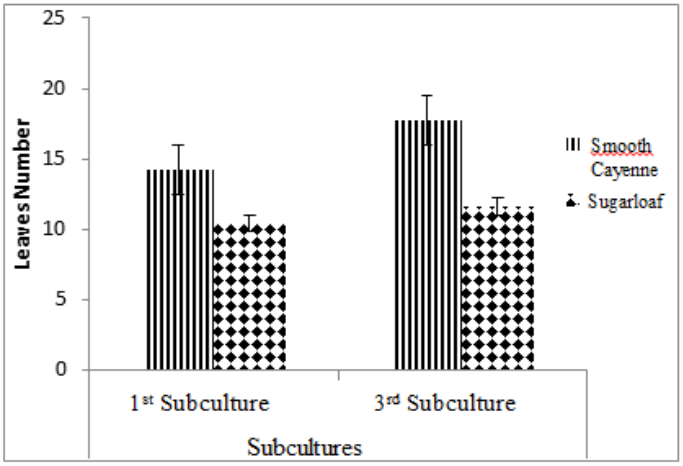
Fig-8. Pineapple tissue culture: (a) Smooth Cayenne and (b) Sugarloaf buds after 6 weeks on MS+4,5mg/l BAP+0,7mg/l NAA; (c) Smooth cayenne and (d) Sugarloaf Shoots in proliferation stage



b) Leaves Differentiation

The evolution of leaves number of the both cultivars is illustrated in Fig. 9.

Fig-9. Evolution of leaves number according to cultivar and subculture



Smooth Cayenne's plantlets gave at the first subculture an average of 14.25 ± 0.96 leaves/plantlet with a variation of 08 to 27 leaves while Sugarloaf recorded an average of 10.40 ± 0.49 leaves/plantlet with a variation of 7 to 13 leaves for Sugarloaf. At the 3rd subculture, a mean of leaves was 17.75 ± 0.80 leaves/plantlet with a variation of 12 to 25 leaves/plantlet was observed with Smooth Cayenne whereas it obtained an average of 11.60 ± 0.93 leaves/plantlet with a variation of 8 to 20 leaves/plantlet for Sugarloaf.

Leaves formation was varied according to genotype with a significant difference at the 1st subculture (p = 0.004) and at the 3rd subculture (p = 0.000) showing that Smooth Cayenne induced more leaves than Sugarloaf whatever of stage.

For leaf development (Table 3), Smooth Cayenne showed an average length of 5.80 cm at the 3rd subculture compared to 6.76 cm for Sugarloaf with a significant difference. The average width is 1.08 cm versus 0.99 cm respectively for Smooth Cayenne and Sugarloaf but there is no significant difference.

Pearson's correlation between length and width of leaf of Smooth Cayenne showed a positive relationship (0.263) between these two parameters. However this relationship was not significant (p = 0.065) ie different to 0. The same trend is noted with Sugarloaf (r = 0.311, p = 0.131). Whatever the cultivar, the correlation between length and width of the leaves of plantlets is small. The length doesn't therefore influence the width although the two are moderately correlated.

Table-3. Length and Width of Leaves at 3rd Subculture

Cultivars	Leaves length (cm)		Leaves width (cm)	
	Var.	Means	Var.	Means
Smooth Cayenne	3.10-8.70	5.80±0.30b	0.50-1.50	1.08±0.05a
Sugarloaf	4.10-10.20	6.76±0.33a	0.40-1.40	0.99±0.05a
p	***	0.034	***	0.255
Cov	***	2.664	***	0.067

Root Differentiation

Root number at the 1st subculture was nil in both cultivars but at the 3rd subculture, 85% of Smooth Cayenne plantlets were rooted with a variation between 0 and 9 roots. But 100% of Sugarloaf plantlets were rooted with a variation of 1 to 11 roots. An average of 3.45 roots/plantlet was observed in Smooth Cayenne versus 6.05 roots/plantlet for Sugarloaf with a significant difference (p = 0.015). This mean that Sugarloaf induced more roots than Smooth Cayenne and that genotype influenced the root induction in *Ananas comosus* var. *comosus*. For roots length, it was noted that Smooth Cayenne have longer roots (2,72±0.36 cm) (with variation between 0.5 and 7.5 cm) than Sugarloaf (2.03 ± 0.29 cm) whose lengths varied between 0.4 and 5.1 cm (Table 4).

c) Fresh Mass

Fresh biomass increased from 0.81 g at first subculture to 2.89 g at 3rd subculture and from 0.82 g at first subculture to 2.73 g at 3rd subculture respectively for Smooth Cayenne and Sugarloaf. There was therefore a gain of 2.08 g and 1.91 g respectively for Smooth Cayenne and Sugarloaf after a period of 10 weeks. The fresh mass gain from 1st to 3rd subculture was therefore significant (p = 0.000) for both cultivars. But, there was no significant difference between cultivars at any stage (Table 4).

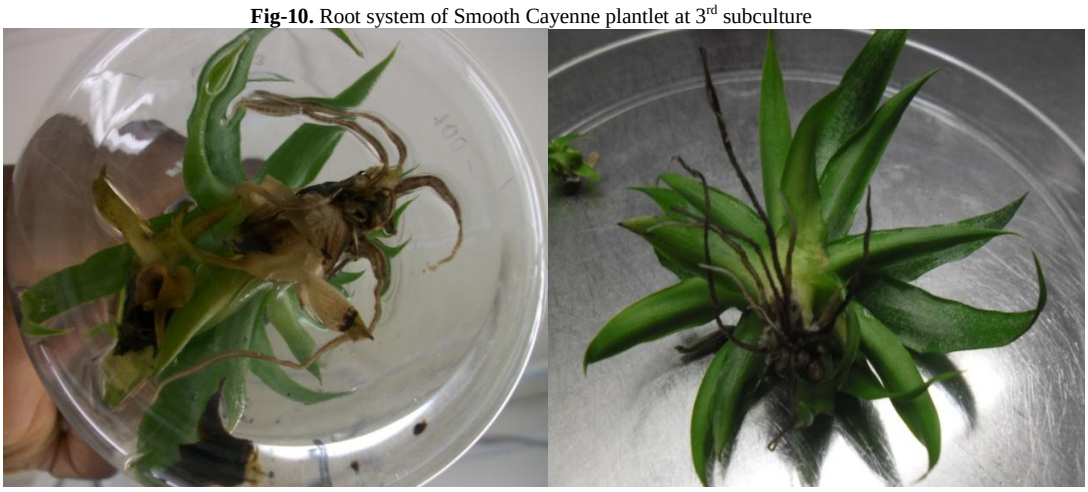
The Pearson Correlation of leaf number and the fresh mass in Smooth Cayenne at 3rd subculture showed that these two parameters were positively correlated (0.442) but p = 0.051. This showed that there was no dependence between them. The same observation was noted with Sugarloaf (0.215) with p = 0.362.

Table-4. Roots Differentiation and Fresh Mass at 1st and 3rd Subcultures.

Subcultures	Cultivars	Roots Number		Roots length (cm)		Fresh weight (g)	
		Var.	Means	Var.	Means	Var.	Means
1st	*Smooth Cayenne*	0	0	-	-	0.60-1.11	0.81±0.04a
	Sugarloaf	0	0	-	-	0.66-1.16	0.82±0.04a
p		***	***	***	***	***	0.876
Cov		***	***	***	***	***	0.04
3rd	*Smooth Cayenne*	00-09	3.45±0.45b	0.50-7.50	2.03±0.29a	01.38-5.08	02.89±0.25a
	Sugarloaf	01-11	6.05±0.82a	0.40-5.10	2.03±0.29a	0.89-4.98	02.73±0.38a
p		***	0.015	***	0.146	***	0.721
Cov		***	13.73	***	1.707	***	2.89

At each subculture, means of the same column followed by the same letter were not significantly different at p=0.05 using Newman and Keuls test (SNK). Var. = variation

Fig-10. Root system of Smooth Cayenne plantlet at 3rd subculture



5. Discussion

The use of new biotechnology techniques through tissue culture is an alternative for a large production of pineapple propagules. In the present study, several parameters were studied to evaluate the pineapple tissue culture conditions.

Before their seeding, the explants must be disinfected. Results showed that the infection rate of the explants varied according to the treatment and revealed that the best treatments were those based on sodium hypochlorite (0% necrosis). Treatments based on mercury chloride induce high levels of necrosis due to their high ability to penetrate tissues. The best treatment for Smooth Cayenne is T4 (Overnight of Gentamicin 300mg + NaOCl 15% for 5mn) and T1' (NaOCl 10% for 15mn) for Sugarloaf. This showed that more time is needed for Sugarloaf so that the disinfectant enters the tissues and proceeds to a better disinfection. This would be due to the steady structure of the axillary buds of Sugarloaf, also noted on the fruit which is less juicy than Smooth Cayenne.

The present work reveals that Sugarloaf is more susceptible to infections than Smooth Cayenne. Even mercuric chloride based on the treatments that are very strong and prevented any infection in Smooth Cayenne have not been able to eliminate the infections in Sugarloaf. This would be due to its high sugar content which would thus promote the proliferation of endogenous germs. This observation is also done in field on fruits where there is noticed more mealybugs, ants and nematodes, all vectors of fungal and viral diseases on Sugarloaf. It's therefore its capacity to contain more germs without showing disease signs which then reveals in tissue culture. The effect of gentamicin in Sugarloaf testifies to a high accumulation of bacteria in this cultivar. Similar observations were made by Ummey, *et al.* [24] on banana, where explants were immersed in screened antibiotics (ampicillin, gentamicin and tetracycline) to remove endogenous bacteria.

Treatments based on mercuric chloride have generally shown that the immersion time has little influence on the disinfection of pineapple explants. Mercuric chloride is more effective for destruction of microorganisms when its concentration is high and the percentage of necrosis increases as its concentration increases. Similar observations were reported by Ahanhanzo, *et al.* [25] showing that mercuric chloride significantly induced necrosis on *Tectona grandis* L. f. by the increase of the doses (0.05% to 0.20%) than the immersion duration.

According to the bud regeneration, both of cultivars buds regenerated the 12th day indicating that the bud regeneration is neither influenced by the genotype nor by the medium. Omokolo, *et al.* [26] noticed the regeneration for the 14th day using whole crowns of Smooth Cayenne cultivar on MS medium supplemented with 4 mg/l BAP. *In vitro* regeneration of pineapple is influenced by factors such as the composition of medium [14, 15] and also in other species such as *Manihot esculenta* Crantz [11]. Specifically, the nature and concentration of growth regulators used [16] are factors that determine the duration of culture and the number of potentially regenerated shoots.

Indeed, the direct contact of the buds on media improves the mineral and carbonaceous absorption. The slight variation of bud regeneration rate according to BAP indicates that it is little influence on regeneration. Moreover, Be and Debergh [27] report showed that the rate of bud regeneration increased with the concentration of BAP without, however, revealing the genotype influence. The trend is noticed for Sugarloaf where the regeneration rate was increased slightly as a function of BAP concentration. Indeed, although the regeneration rates according to the media ($p = 0.275$) and the genotype ($p = 0.082$) did not show a significant difference, this works shows that the interaction between medium and genotype gave a significant difference ($p = 0.029$). Similar results were obtained by Cacaï, *et al.* [28] who assessed cassava (*Manihot esculenta* crantz) regeneration and showed that it depends on both the genotype and medium.

A multiplication rate of 8.95 was obtained at the 3rd subculture more than 6.4 obtained by Zuraida, *et al.* [20] who also used 1mg/l BAP except that the medium used did not contain adenine sulphate but also subcultures were spaced 4 weeks apart. This improvement in the multiplication rate should be due to the addition of adenine sulphate, a cell multiplication cofactor and the difference of one week between subcultures. The time difference then allowed the plantlets to further improve their axillary bud development potential. Also, the increase of the shoots induction

from the 1st subculture to the 3rd is due to the increase in the surface of the plantlets at the 3rd subculture which presenting more axillary buds in order to induce the formation of many shoots.

The significant difference of shoots production for the two cultivars tested showed that genotype is the main factor for shoots proliferation. These results support the trend observed on field where Smooth Cayenne gives only suckers and at most five suckers per direct maintenance of stump. Moreover, Sugarloaf gives more suckers, many slips and does not present enough problems of shoots.

A significant increase in the number of leaves is also noted from the first to the third subculture for the both cultivars but it is more pronounced for Smooth Cayenne showing a high influence of genotype on the foliar emission in *Ananas comosus* var. *comosus*. Several studies have shown, apart the influence of medium on leaves differentiation, that of the genotype as in cassava [18]. Omokolo, *et al.* [26] obtained an average of 12.1 leaves per plantlet for Smooth Cayenne on 4 mg/l BAP with whole crowns. This increase in the number of leaves from the first to the third subculture confirms the increase in the number of shoots because the buds are born in the axils of the leaves and consequently more leaves would induce more potential buds that will give shoots.

The average size of the longest leaf of plantlets obtained at the first subculture is 5.58 cm compared to 5.0 cm and 6.0 cm obtained by Zuraida, *et al.* [20] respectively on the MS medium supplemented with 1 mg/l BAP and MS alone. The mean number of leaves obtained per plantlet for Smooth Cayenne at the first subculture was 14.29 compared to 12.1 obtained by Omokolo, *et al.* [26]. For foliar development, the genotype was a determining factor in the length of the leaves and Sugarloaf showed longer leaves but for the width of leaves, there was no significant difference. From the Pearson correlation between length and width of plantlets leaves at the 3rd subculture, it is apparent that the length does not influence the width although both are moderately correlated.

The presence of roots for Smooth Cayenne plantlets (85%) and Sugarloaf (100%) at the 3rd subculture, without the addition of auxins was also reported by Zuraida, *et al.* [20] for Smooth Cayenne on 1 mg/l BAP without specifying the length of these roots and their proportion on the plantlets. This root emission is due to the action of endogenous auxins which at the first subculture would not have reached a threshold triggering the root emission in view of the very juvenile condition of the plantlets that have just emitted their first leaves as a result of budding. Likewise, the medium being composed only of cytokinin (1 mg/l BAP and 40 g/l adenine sulphate), the conditions were therefore favorable only to multiplication. As auxins were formed from the apex, it was therefore necessary to first obtain plantlets of reasonable size before the induction of its synthesis and the observation of its activity. Smooth Cayenne with fewer roots than Sugarloaf but with longer lengths show that Sugarloaf would have more endogenous auxins than Smooth Cayenne. Indeed, Hamad, *et al.* [29] showed that the presence of auxins such as NAA, IAA or IBA in the medium induces rooting but increasing doses reduces root elongation in Morris and Smooth Cayenne cultivars.

The number of roots obtained and their length are acceptable for acclimatization because respectively greater than 3 and 2 cm as recommended by Saifullah, *et al.* [30].

It noticed a fresh biomass increase at the 3rd subculture for both cultivars. This increase shows that the growth does not depend to the genotype. Differences between the 1st and 3rd subcultures for the leaves and roots differentiation confirm this significant gain in fresh biomass. This would therefore be parameters that highly determine the mass of plantlets.

6. Conclusion

Through this work focusing on the initiation and micropropagation of the two main cultivars of *Ananas comosus* var. *comosus* produced in Benin it is clearly identified that sodium hypochlorite using at 15% for 5 min allows successful disinfection and Sugarloaf shows more infection than Smooth Cayenne.

The bud burst of two cultivars is noted as early as 12th day after seedling whatever the medium. M1 (MS + 4 mg/l BAP + 0.7 mg/l NAA) favored better budding which was also slightly influenced by the genotype.

Sugarloaf showed better growth and production of shoots to all subcultures. None of the cultivars gave root at the first subculture but almost all plantlets gave roots to the third subculture. 12.70 plantlets per bud were regenerated after 15 weeks for Smooth Cayenne and 15.30 for Sugarloaf. If Smooth Cayenne yields only 5 propagules after 14 months by conventional methods, this study makes it possible to obtain from a single bud about 381000 shoots and about 3000000 shoots per plant, which is considerably higher than the multiplication rates generally obtained by traditional techniques of multiplication of propagules. All this work shows the advantage of plant biotechnology for producing of suckers for pineapple grown.

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