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Effects of system benzylaminopurine-adenine sulphate in combination with naphthalene acetic on *in vitro* regeneration and proliferation of pineapple (*Ananas comosus* (L.) Mill var. *comosus*)

Bienvenu T. Badou[1*], Arnaud Agbidinoukoun[1], Gilles T.H. Cacaï[1], René C. Dossoukpèvi[1,2] and Corneille Ahanhanzo[1,2]

[1.] Central Laboratory of Plant Biotechnology and Plant Breeding, Department of Genetic and Biotechnology, Faculty of Science and Technology, University of Abomey-Calavi (Benin); [2.] Beninese Center of Scientific Research and Innovation

ABSTRACT

In vitro micropropagation is now used in the production of healthy and homogenous pineapple planting materials. In order to increase the in vitro proliferation of pineapple, the synergetic effect of adenine sulphate (AdS) and benzylaminopurine (BAP) in presence or not of naphthalene acetic acid (NAA) was evaluated. Median axillary buds of crowns bursted 6 to 8 weeks old were cultured on Murashige and Skoog medium supplemented with BAP (0, 1 and 2 mg/l), NAA (0 and 0.5 mg/l) with or without AdS (40 mg/l). Five buds per treatment with four replicates were used. The addition of BAP to the medium stimulated regeneration (65 to 82%) and multiplication (3.04 to 6.13 plantlets/explant) which were further enhanced by the addition of NAA (98%; 6.42 plantlets/explant). AdS highly stimulated proliferation in presence of 2 mg/l BAP and NAA (14.96 plantlets/explant), plantlet mass (1.143g) but limited plantlet growth in height which was more induced in the absence of regulators (4.88 cm) and with AdS alone (5.63 cm). Thus, BAP is necessary for the proliferation of pineapple and the addition of 0.5 mg/l NAA to 2 mg/l BAP and 40 mg/l AdS optimizes the multiplication and growth parameters of pineapple plantlets. These results allow the mass propagation of pineapple's homogeneous healthy planting materials.

Key words: Pineapple, micropropagation, adenine sulphate, benzylaminopurine, naphthalene acetic acid, shoots.

***Correspondence to Author:**
Bienvenu T. Badou
Central Laboratory of Plant Biotechnology and Plant Breeding, Department of Genetic and Biotechnology, Faculty of Science and Technology, University of Abomey-Calavi (Benin)

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1. Introduction

Pineapple, one of the main tropical fruit crops, ranks third in the world after bananas/plantains and citrus fruits. It ranks first in international trade [1]. In Benin, in 2006, pineapple contributed 13 billion FCFA ie 1.2% of national GDP and 4.3% of agricultural GDP [2], reducing poverty in peasants areas and bringing foreign currency to the state. The increase of the demand for pineapple fruit because of its organoleptic qualities and its rich composition of vitamins and minerals is faced by the extension of plantation. Besides, the main problem of extending plantations for massive production of pineapple is lack of the uniform planting materials in large quantities [3]. Indeed, pineapple naturally produces very few propagules and the different conventional techniques of multiplication of propagules (crown, slips, hapas, suckers and ratoons) do not permit to cover the demand of planting materials which often are poor quality [3,4]. The crown permit to obtain homogeneous fruits in taste quality, weight and height [5] but pineapple has only one. It has a greater potential to develop a root system [6]. Unfortunately, the fresh pineapple's trade is losing the crown. It is therefore essential to find an alternative to palliate these difficulties inherent in pineapple grown. The discovery of totipotency in plants and the establishment of tissue culture in recent decades is therefore a perfect alternative. Micropropagation, one of the applications of tissue culture, has advantage to produce rapidly large number and uniform pineapple propagules [7]. It is used in the mass production of healthy cassava planting material [8,9], yam [10], banana [11-13] and pineapple [14,15]. For pineapple *in vitro* multiplication, liquid cultures showed best results compared to solid media [14-18]. It favors better salts absorption due to large contact surface offered to explants and the uniform distribution of salts [19]. In addition, liquid media disperses phenolic exudates from explants, consequently, resulting in faster growth rate [20]. Also, the absence of agar in medium permit to reduce cost of shoots producing. The explants showing the best results of conformity and homogeneity of shoots are axillary crown buds [15,21-25]. Beyond the type of explant and consistency of medium, the effect of growth regulators on the regeneration and *in vitro* propagation of pineapple is a very important factor. Thus, cytokinin (BAP) and auxin (NAA) are widely used and given best results [18,24,25]. Various studies have shown that best multiplication rate in pineapple were obtained with BAP between 1 and 4 mg/l [15,17,18,21,26,27]. However, some authors noted that the addition of NAA to BAP enhances multiplication [18,28]. Adenine sulphate (AdS) has been used in several species like *Cichorium intybus* L. [29], *Jatropha curcas* L. [30], *Trifolium repens* L. [31], *Stevia rebaudiana* Bertoni [32] with a beneficial effect on multiplication. In addition, when it combined with cytokinins such as BAP or kinetin, it further enhances multiplication [33]. Nevertheless, few studies investigated the effect of AdS alone, combined with cytokinins or auxins on *in vitro* multiplication of pineapple for optimizing the multiplication rate. Dutta *et al.* [34] and Ibrahim *et al*. [23] using AdS at 50 mg/l and 40 mg/l respectively but have not evaluated its effect on pineapple micropropagation. In fact, the present study aims to evaluate the synergic effect of AdS and BAP in combination with NAA on *in vitro* proliferation of beninese pineapple cultivars.

2. Material and Methods

2.1 Plant Material

Pineapple crowns of Smooth Cayenne (*Hilo* and *Saint Michael*) and Sugarloaf (*Green Sugarloaf*) cultivars were used. These are the mains pineapple cultivars produced in Benin and exported [35]. It was collected from the experimental garden of the Central Laboratory for Plant Biotechnology and Plant Breeding located in the township of Abomey-calavi, one of the major pineapple growing areas in Benin.

2.2 Sterilization of the explant

Disinfection of the explants was done according to Badou *et al.* [36]. Explants used are median crown tips in order to obtain homogeneity of explants structure and age.

2.3 Culture media and culture conditions

After excision, buds were inoculated into Murashige and Skoog (MS) salts [37] modified with glycine 10 g, nicotinic acid 2.5 g, pyridoxine 2.5 g, thiamine hydrochloride 0.5 g, myo-inositol 0.2 g and supplemented with sucrose 30 g, BAP 5 mg and agar 8 g (initiation medium). The buds burst after six to eight weeks were transferred to different regeneration/multiplication media (Table 1). Media were designated with letters (‘‘B’’ for BAP, ‘‘A’’ for NAA and ‘‘S’’ for AdS) followed by a number which indicates its concentration in mg/l.

Table 1: Composition of regeneration and proliferation media

Media / Composition	$B_0A_0S_0$	$B_1A_0S_0$	$B_1A_{0,5}S_0$	$B_2A_0S_0$	$B_2A_{0,5}S_0$	$B_0A_0S_{40}$	$B_1A_0S_{40}$	$B_1A_{0,5}S_4$	$B_2A_0S_{40}$	$B_2A_{0,5}S_4$
BAP (mg)	0	1	1	2	2	0	1	1	2	2
NAA (mg	0	0	0,5	0	0,5	0	0	0,5	0	0,5
Adenine Sulphate (mg)	0	0	0	0	0	40	40	40	40	40

To avoid the total immersion of the buds that could become asphyxiated, 5 ml of the nutritive solution (first and second subcultures) and 15 ml (third subculture) were distributed per 200 ml jar (Sigma 5.5 x 9.5). The media were autoclaved at 121°C for 15 minutes after adjusting the pH to 5.7±0.1 using solutions of 1N NaOH or HCl. The jars were incubated on rotary shaker at 80 rpm to maintain homogeneity of media during the three subcultures (each subculture tested 4 weeks) (figure 1). All cultures were maintained in the growth room at 27±1°C in light (photoperiod 12h) under a light intensity of 5000 lux and a relative humidity of 80%. Shoots were then rooted and transferred to soil. Regeneration and proliferation parts of this study took place from June to November 2015.

Figure 1: Shoots at proliferation stage on rotary shaker

2.4 Parameters evaluated and data analysis

In order to evaluate the effect of different treatments on pineapple micropropagation, the following parameters were measured at each subculture. The rate of regeneration (at the end of 1st subculture), the multiplication rate (determined by shoots counting), the height of shoots (obtained by measuring the shoots with a graduated ruler) and the fresh mass (obtained by weighing of shoots). The percentage of explants which gave microshoots was calculated and their abundance has been estimated.

$$\text{Regeneration rate} = \frac{\text{Number of buds regenerated}}{\text{Number of buds bursted}}$$

$$\text{Multiplication rate} = \frac{\text{Number of plantlets obtained}}{\text{Number of plantlets initiated}}$$

The experiments followed a completely randomized design and five buds were used for each treatment. All experiments were repeated four times. After regeneration, five flasks were raised per treatment with three replicates and each flask contained five shoots. Each treatment consists to the hormonal combination and the cultivar.

A descriptive analysis assessed the rate of regeneration and the average number of shoots. A binary logistic regression was carried out to discriminate the regeneration rates according to the treatments. Analysis of the variance (ANOVA) at two factors (hormonal combination and cultivar) at the 5% threshold was carried out to evaluate the effect of the treatments at each subculture on the number of shoots on the one hand, the height and mass fresh of shoot on the other hand respectively after a logarithmic transformation (log (x+1)) and square root ($\sqrt{x}$). Significant differences between treatment means were compared using Low Significant Difference (LSD) test at 5%. Graphs were generated by the Excel spreadsheet version 2010. The XLSTAT version 2014 software was used for the various analyzes performed.

3. Results

3.1 Regeneration rate

The binary logistic regression on the behavior of buds according to treatments showed that hormonal combination strongly influenced the regeneration ($p<0.0001$) but the cultivar had a hardly significant influence on the regeneration ($p=0.05$). Thus, Hilo cultivar showed better regenerability (86%) while Sugarloaf cultivar showed the lowest regeneration rate (77%). As for growth regulators, the addition of 1 mg/l BAP to the control medium (hormone free MS) improved regeneration from 65% to 81.67% ($p=0.039$). The combination of 0.5 mg/l NAA to 1 mg/l BAP ($B_1A_{0,5}S_0$) promoted bud regeneration (98.33%) while high dose of BAP (2 mg/l) limited bud recovery (60%). The combination of NAA with BAP and/or AdS improved bud burst (86.67%) compared to their alone presence (79%). Although slightly improving regeneration (65% to 80%), AdS did not show a significant difference at 5% ($p=0.067$) to control medium. Similarly, its presence in general slightly improved the regeneration (77% to 84.33%) without however the difference being significant ($p=0.151$). Finally, the interaction between cultivar and hormonal combination did not show any significant difference ($p=0.692$). In sum, St Michael and Hilo cultivars on $B_1A_{0,5}S_0$ and Sugarloaf on $B_1A_0S_{40}$ had the highest rate of regeneration (100%) (Figure 2).

3.2 Propagation rate and plantlet height

At the first subculture a significant difference at 1% was noted between cultivars and different hormonal combinations based on the multiplication rate. However, their interaction was not significant ($p=0.388$) showing that the two factors can be studied separately. Thus, for hormonal combinations, the addition of NAA (0.5 mg/l) and AdS (40 mg/l) to BAP at 1 and 2 mg/l ($B_1A_{0,5}S_{40}$ and $B_2A_{0,5}S_{40}$) gave the highest multiplication rates, respectively 13.98 and 14.96 shoots/explant while the control medium ($B_0A_0S_0$) gave the lowest multiplication rate

(3.04 shoots/explant). BAP at 1 mg/l ($B_1A_0S_0$) showed a significant effect at 1% threshold on the multiplication of shoots (6.13 shoots/explant) compared with the control medium. The addition of NAA (0.5 mg/l) to BAP (1 mg/l and 2 mg/l) increased the multiplication rate (respectively 6.42 and 10.60 shoots/explant) compared to BAP alone (6.13 and 4.07 shoots/explant). Similarly, its addition to BAP-AdS combination (9.89 and 11.98 shoots/explant) gave the highest multiplication rates. At the third subculture, the highest multiplication rate (6.6 shoots/plantlet) was obtained with $B_2A_{0,5}S_{40}$, whereas control medium ($B_0A_0S_0$) further limited the multiplication of shoots (1.44 shoots/plantlet) (Table 2). AdS with 1 mg/l BAP ($B_1A_0S_{40}$) strongly induced the formation of microshoots (35.56%) (Figure 3) whereas control medium and the combination of the three growth regulators did not show any microshoots. The highest rate of microshoot induction (60%) was obtained with Hilo cultivar on AdS and 1 mg/l BAP combination. Thus, Hilo cultivar showed a strong predisposition to induce microshoots (20.67%) compared to Sugarloaf (1.33%) (Table 3).

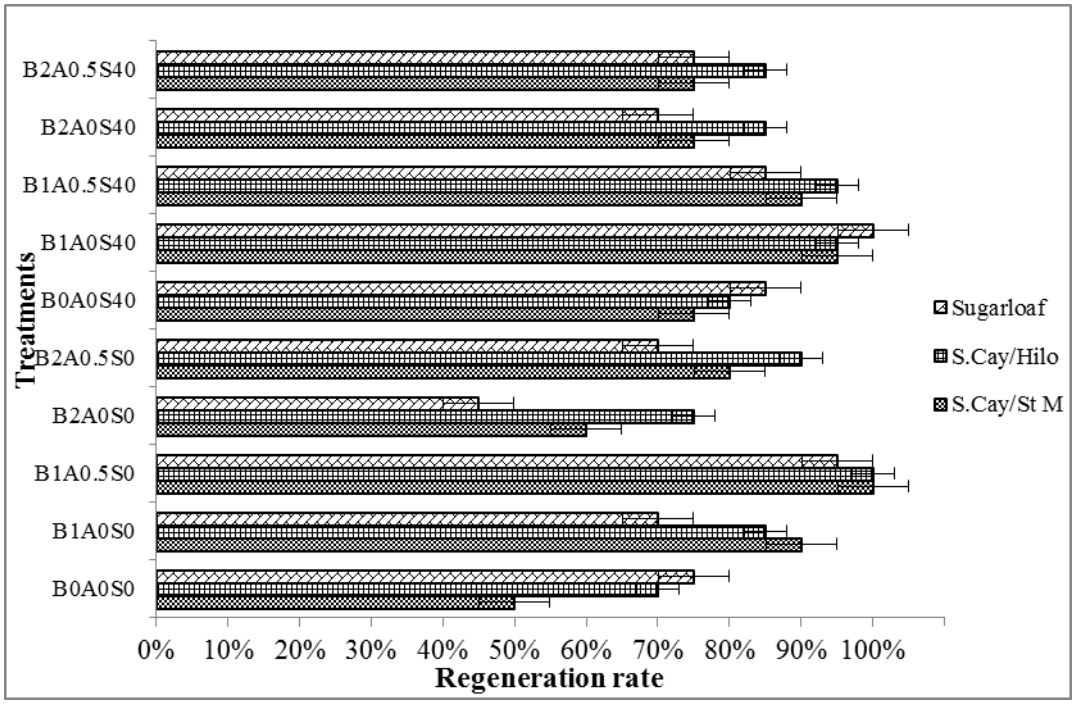

Figure 2: Regeneration rate according to treatments
S.Cay/Hilo: Smooth Cayenne (Hilo); S.Cay/StM: Smooth Cayenne (St Michael)

For the height of plantlets, at the first subculture, a significant difference at 1% was recorded for cultivar, hormonal combination and their interaction. The medium supplemented with AdS and the control medium favored plantlet growth. Thus, St Michael cultivar in presence of AdS ($B_0A_0S_{40}$) had the largest height (5.04 cm) while the presence of all growth regulators ($B_2A_{0,5}S_{40}$) limited plantlet elongation especially in Sugarloaf (1.13 cm). At the third subculture, the same trend was noted and the largest height (7.28 cm) was recorded for St Michael with AdS ($B_0A_0S_{40}$) so an average growth of 2.24 cm while the smallest average height (2.01 cm) was obtained for Sugarloaf with 2 mg/l BAP ($B_2A_0S_0$) so an increase of 0.86 cm (Figure 4).

The steps of pineapple micropropagation are showed in figure 5.

3.3 Evolution of fresh biomass

The results obtained showed a significant variation at 1% threshold of fresh biomass production of plantlets according to the hormonal combination and the cultivar. It is

Table 2: Multiplication rate of plantlets at different subcultures

Growth Regulators	St. Michael		Hilo		Sugarloaf	
BAP+NAA+AdS (mg/l)	Number of Shoots	Multiplication Rate	Number of Shoots	Multiplication Rate	Number of Shoots	Multiplication Rate
Subculture 1						
0.0+0.0+0.0	55	3.67±0.25	45	3.00±0.19	37	2.47±0.26
1.0+0.0+0.0	120	8.00±0.81	91	6.07±0.46	65	4.33±0.51
1.0+0.5+0.0	135	9.00±1.02	79	5.27±0.37	75	5.00±0.24
2.0+0.0+0.0	83	5.53±0.51	55	3.67±0.36	45	3.00±0.29
2.0+0.5+0.0	203	13.53±0.80	135	9.00±1.06	139	9.27±1.03
0.0+0.0+40.0	95	6.33±0.63	75	5.00±0.58	60	4.00±0.57
1.0+0.0+40.0	158	10.53±1.21	145	9.67±1.26	142	9.47±0.65
1.0+0.5+40.0	283	18.87±1.99	170	11.33±0.80	176	11.73±0.61
2.0+0.0+40.0	215	14.33±1.35	189	12.60±1.75	135	9.00±0.74
2.0+0.5+40.0	250	16.67±1.30	232	15.47±1.83	191	12.73±1.25
Subculture 2						
0.0+0.0+0.0	77	1.4±0.45	81	1.8±0.45	57	1.54±0.45
1.0+0.0+0.0	376	3.16±0.45	316	3.47±0.45	235	3.62±0.45
1.0+0.5+0.0	645	4.78±0.45	458	5.8±0.45	429	5.72±0.45
2.0+0.0+0.0	249	3.00±0.45	275	5.00±0.45	300	6.67±0.45
2.0+0.5+0.0	920	4.53±0.45	751	5.56±0.45	741	5.33±0.45
0.0+0.0+40.0	147	1.55±0.45	190	2.53±0.45	165	2.75±0.45
1.0+0.0+40.0	748	4.73±0.45	1005	6.93±0.45	480	3.38±0.45
1.0+0.5+40.0	1254	4.43±0.45	595	3.5±0.45	551	3.13±0.45
2.0+0.0+40.0	802	3.73±0.45	554	2.93±0.45	431	3.19±0.45
2.0+0.5+40.0	1225	4.9±0.45	984	4.24±0.45	764	4.00±0.45
Subculture 3						
0.0+0.0+0.0	84	1.09±0.45	101	1.25±0.45	114	2.00±0.45
1.0+0.0+0.0	1341	3.57±0.45	1580	5.00±0.45	872	3.71±0.45
1.0+0.5+0.0	1333	2.07±0.45	2203	4.81±0.45	2106	4.91±0.45
2.0+0.0+0.0	620	2.49±0.45	968	3.52±0.45	710	2.37±0.45
2.0+0.5+0.0	3951	4.29±0.45	3114	4.15±0.45	3120	4.21±0.45
0.0+0.0+40.0	341	2.32±0.45	357	1.88±0.45	361	2.19±0.45
1.0+0.0+40.0	2747	3.67±0.45	4651	4.63±0.45	1712	3.57±0.45
1.0+0.5+40.0	5058	4.03±0.45	1554	2.61±0.45	1991	3.61±0.45
2.0+0.0+40.0	1954	2.44±0.45	1935	3.49±0.45	1230	2.85±0.45
2.0+0.5+40.0	7579	6.19±0.45	3980	4.04±0.45	7312	9.57±0.45

Means following by same letter at a subculture are not significantly different at least of 5% by LSD test.

Table 3: Microshoots induction rate and abundance

Sub.	G.Regulators / Cultivars	$B_0A_0S_0$	$B_1A_0S_0$	$B_1A_{0.5}S_0$	$B_2A_0S_0$	$B_2A_{0.5}S_0$	$B_0A_0S_{40}$	$B_1A_0S_{40}$	$B_1A_{0.5}S_{40}$	$B_2A_0S_{40}$	$B_2A_{0.5}S_{40}$
Sub.1	St Michael	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	40 (+)	0 (-)	0 (-)	0 (-)
	Hilo	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	26.67(++)	0 (-)	0 (-)
	Sugarloaf	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
	Mean	**0 (-)**	**0 (-)**	**0 (-)**	**0 (-)**	**0 (-)**	**0 (-)**	**13.33 (+)**	**08.89(+)**	**0 (-)**	**0 (-)**
Sub.2	St Michael	0 (-)	0 (-)	0 (-)	33.33(+)	0 (-)	0 (-)	33.33(++)	0 (-)	0 (-)	0 (-)
	Hilo	0 (-)	0 (-)	46.67(++)	0 (-)	0 (-)	0 (-)	40 (+)	40 (+)	06.67(+)	0 (-)
	Sugarloaf	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
	Mean	**0 (-)**	**0 (-)**	**15.56(+)**	**11.11(+)**	**0 (-)**	**0 (-)**	**24.44(++)**	**13.33(+)**	**02.22(+)**	**0 (-)**
Sub.3	St Michael	0 (-)	06.67(+)	26.67(+)	26.67(+)	26.67(+)	0 (-)	46.67(+++)	0 (-)	0 (-)	0 (-)
	Hilo	0 (-)	0 (-)	13.33(++)	13.33(+)	13.33(+)	53.33(+)	60 (+++)	46.67(+)	06.67(+)	0 (-)
	Sugarloaf	0 (-)	0 (-)	0 (-)	06.67(+)	06.67(+)	0 (-)	0 (-)	0 (-)	0 (-)	0 (-)
	Mean	**0 (-)**	**02.22(+)**	**13.33(+)**	**15.56(+)**	**15.56(+)**	**17.78(+)**	**35.56(++)**	**15.56(+)**	**02.22(+)**	**0 (-)**

Abundance: (-) = absent ; (+) = present ; (++) = abundant ; (+++) = very abundant.

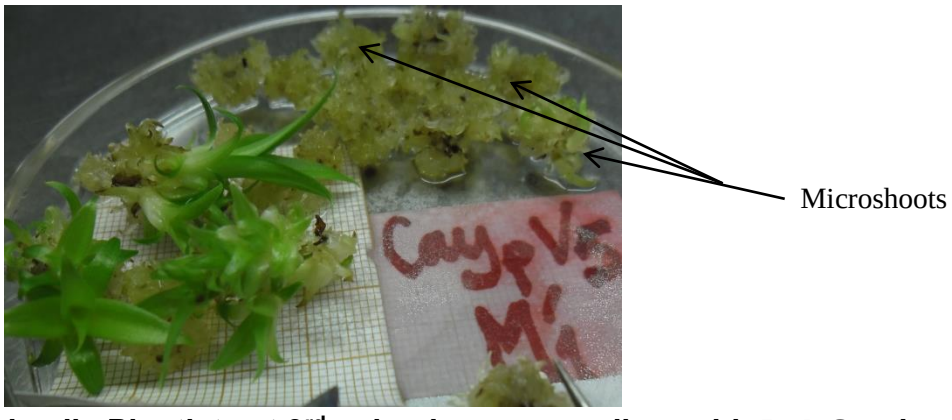

Figure 3: St Michael's Plantlets at 3rd subculture on medium with $B_1A_0S_{40}$ showing microshoots

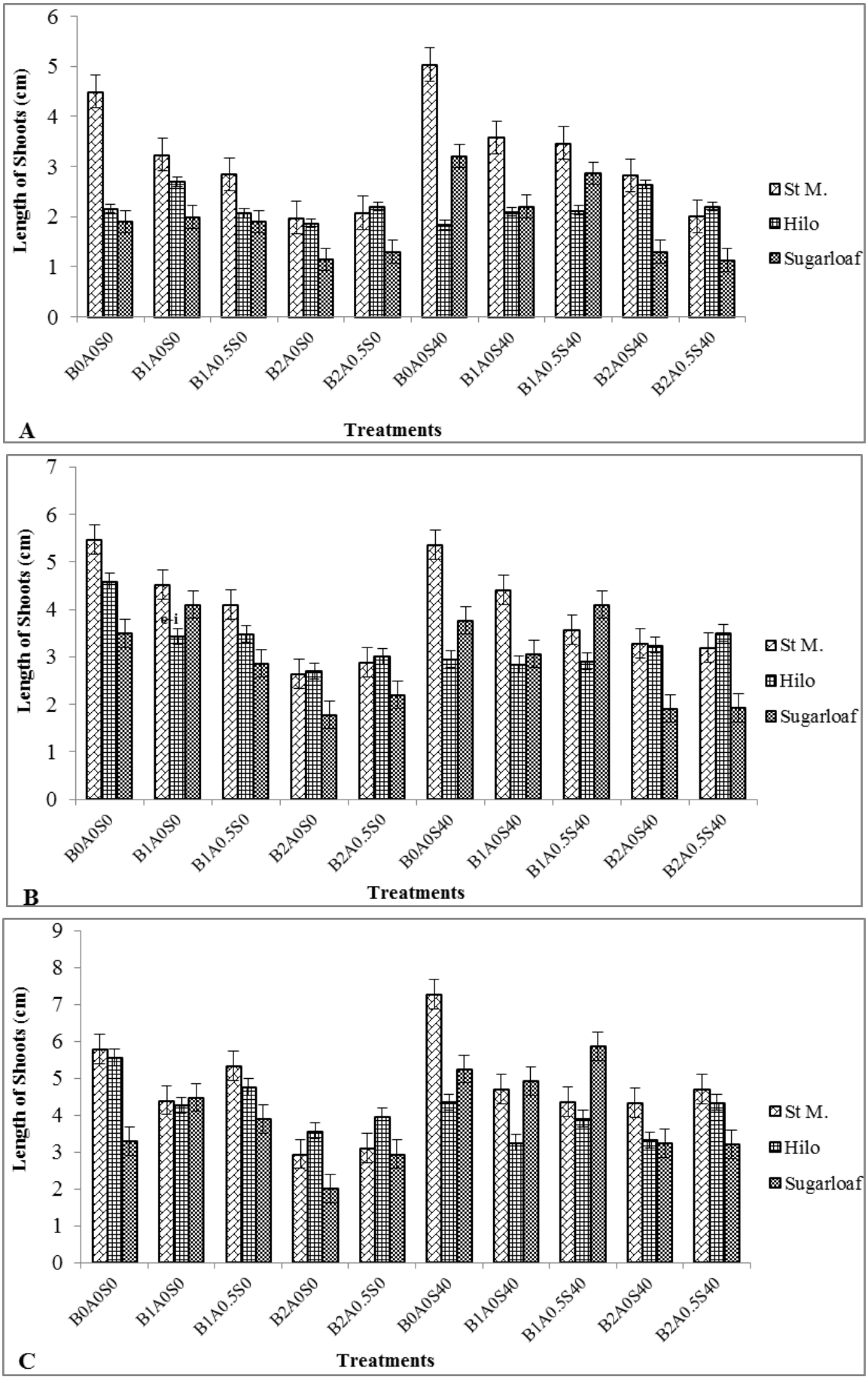

Figure 4: Effect of hormonal combinations on shoots height: (A) at 1st subculture; (B) at 2nd subculture; (C) at 3rd subculture. Means followed by the same letter, for each subculture, are not significantly different, according to LSD test, at 5%

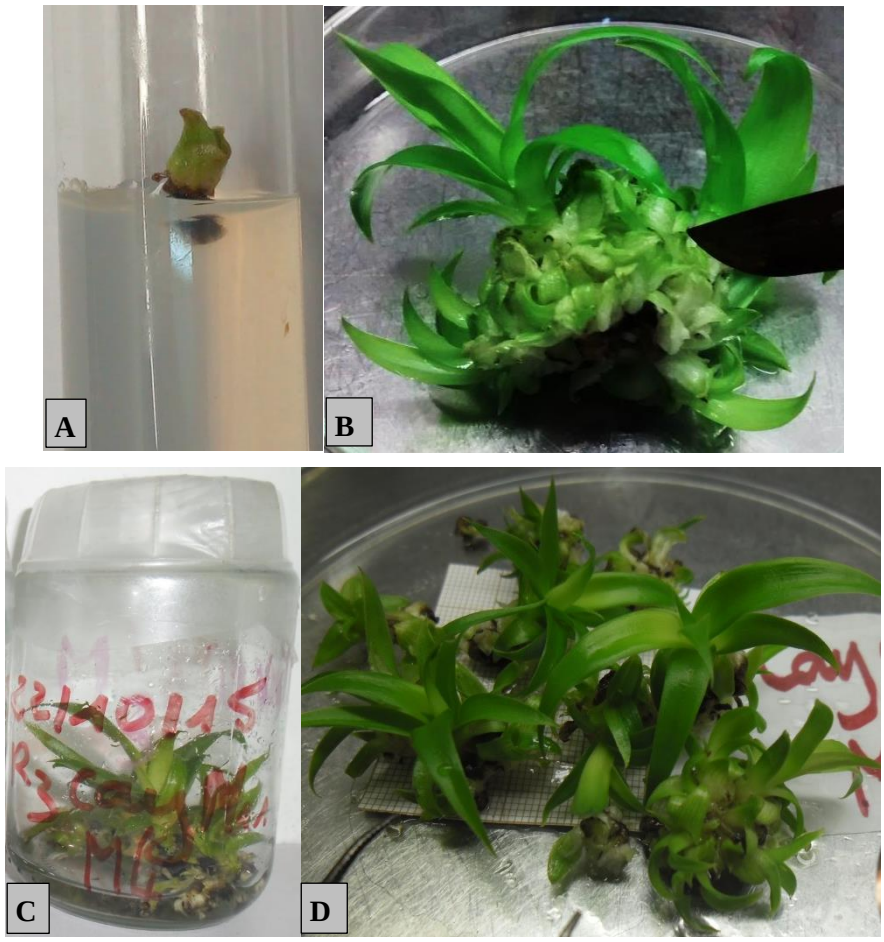

Figure 5: Shoots multiplication stages (A: Bud burst after 6 weeks on initiation medium; B: Regenerated shoots at 1st subculture on medium with $B_2A_{0.5}S_{40}$; C and D:Micropropagation of shoots at 3rd subculture on medium with $B_2A_{0.5}S_{40}$ into and out glass)

noted at the first subculture that 1 mg/l BAP stimulated the fresh biomass production with an average of 0.466 g against 0.157 g obtained with 2 mg/l BAP which, with Sugarloaf presented the lowest fresh biomass (0.114 g).In contrast, the best treatment favoring biomass production is 1 mg/l BAP with St Michael (0.632 g). The addition of NAA to 1 mg/l BAP with AdS (0.401 g) or without AdS (0.424 g) generally favored biomass production. For the genotype, St Michael plantlets showed better biomass growth (0.447 g) compared to Hilo plantlets (0.285 g). At the third subculture, a significant difference at 1% was also noted between the different treatments. Sugarloaf on 1 mg/l BAP and AdS produced a high biomass (1.617 g) so an increase of 1.166 g from the first to the third subculture. In contrast, the combination of BAP (2 mg/l) and NAA limited biomass production (0.302 g) with Sugarloaf so an increase of 0.188 g from the first to the third subculture. Also, at the first subculture (p=0.042) and at the third (p = 0.045), the addition of AdS to different media showed a significant difference at the 5% level. It induced a an biomass increase of 0.040 g (0.334 g to 0.374 g) at the first subculture and 0.120 g (0.809 g at 0.929 g) at the third subculture (Figure 6).

At the end of this study, the plantlets obtained were rooted and acclimated (Figure 7).

4. Discussion

This study was initiated in order to test the synergistic effect of BAP and AdS in combination with NAA on the *in vitro* regeneration and multiplication of pineapple cultivars produced in Benin. The results showed that addition of BAP to medium favored regeneration. Indeed, BAP belong to cytokinins which are known to stimulate cell division and

axillary bud proliferation [20], thereby resulting to significant regeneration of plantlet from bud. The presence of BAP has been shown to be necessary for the regeneration of pineapple during the work of Firoozabady and Gutterson [18]. In addition, low dose of NAA (0.5 mg/l) with BAP showed a synergistic effect on bud burst. Danso *et al*. [14] and Usman *et al.* [24] showed that addition a low dose of NAA to BAP has improved pineapple regeneration and multiplication.

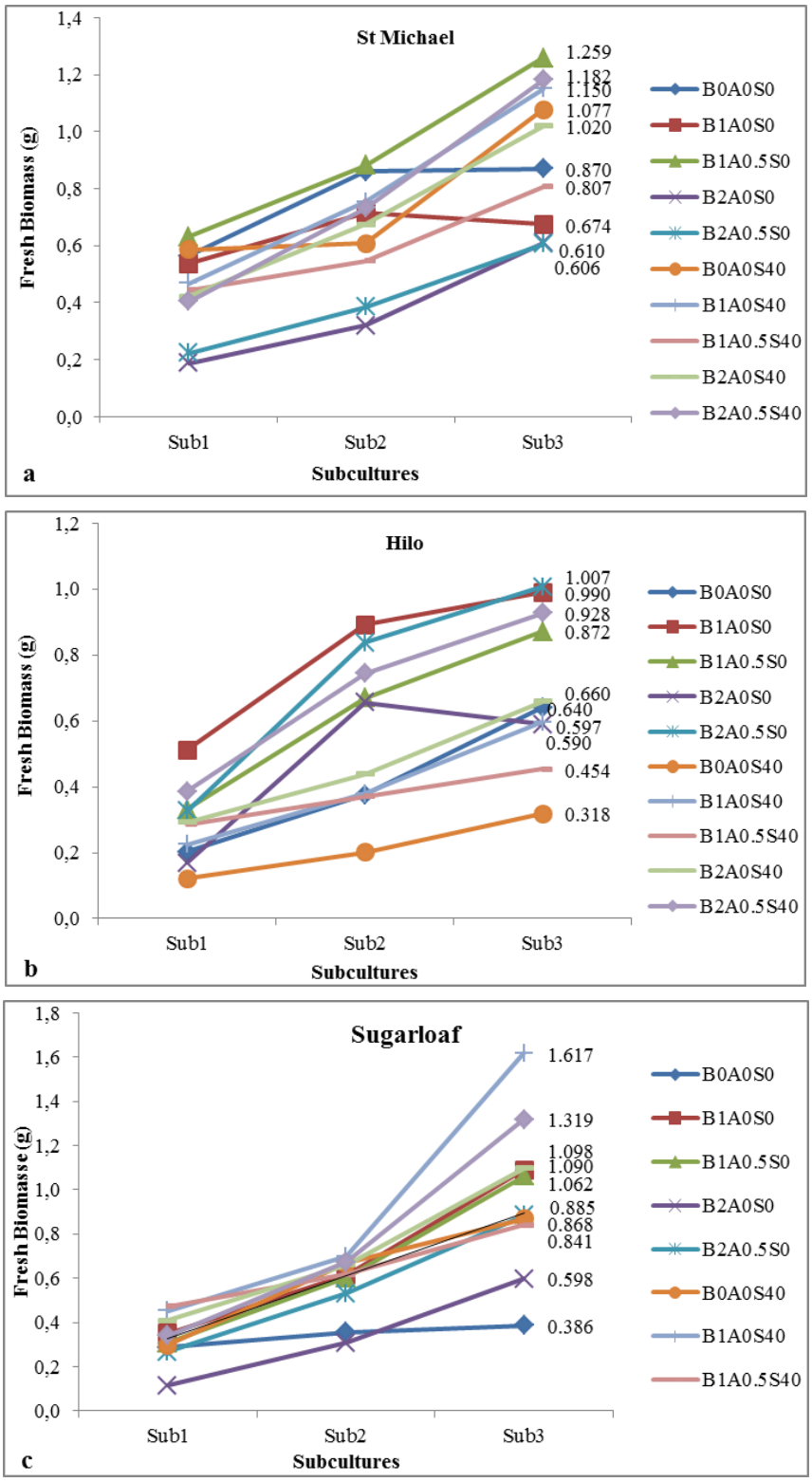

Figure 6: Evolution of fresh biomass according to treatments: St Michael (a) ; Hilo (b) ; Sugarloaf (c)

Figure 7: Acclimatized pineapple plantlets ready for transplanting to field

On the other hand, Be and Debergh [26], Zuraida *et al*. [15] and Acheampong *et al.* [38] argued that regeneration of pineapple can be done effectively with BAP alone. The addition of AdS at 40 mg/l did not show for all hormonal combinations a significant additive effect on regeneration.

For multiplication, control medium highly limited the proliferation of plantlets showing the need of exogenous hormones, especially BAP, for *in vitro* proliferation of pineapple. In the absence of AdS, NAA enhanced the mitotic action of BAP (1 and 2 mg/l). The simultaneous presence of BAP, NAA and AdS strongly favored the multiplication of shoots. In general, the addition of AdS to different hormonal combinations (BAP, BAP+NAA) has improved the multiplication of shoots. Khan *et al*. [32] showed that AdS is a precursor of natural cytokinins synthesis and it increase cytokinin biosynthesis. AdS is from adenine like BAP and majority of molecules which have cytokinic effect. The addition of a low concentration of NAA to these cytokinins then stimulates the induction of axillary buds in the axils of plantlet leaves and thus the production of shoots. Similar observations were done by Vicaş [31] where although AdS at 40 mg/l did not positively influence regeneration, it strongly induced the multiplication of *Trifolium repens* by its addition to 2 mg/l of Zeatin and 1 mg/l of indole butyric acid. Raha and Roy [39] put forward that AdS plays an important role in organogenesis; in synergy with BAP, it stimulates cell growth and promotes stem formation. Indeed, the NAA belonging to auxin family by its action of cell elongation by weakening of the pecto-cellulose wall optimizes the cell multiplication action of BAP-AdS complex. Boxus *et al*. [40] showed that auxins promote the induction and maintenance of cell proliferation. They are essential for the synthesis of DNA and play a decisive role in tissue culture because of their stimulating power for cell proliferation [41,42]. Cytokinins allow division of cells and act with auxins to stimulate their multiplication [41]. Thus, the combination of both is very beneficial for multiplication. Cytokinins compounds produced by AdS addition may give a more effective physiological response than the addition of BAP [32]. This was observed on plantlet height growth where AdS alone stimulated elongation over the action of BAP alone or control medium. Like pineapple, it increases *in vitro* proliferation of *Jatropha curcas* [30], *Cichorium intybus* [29] but reduces multiplication in red and black raspberry [43]. Its effect therefore varies from one species to another. Indeed, the BAP-AdS complex stimulated the massive induction of microshoots with structures similar to those of calli. These last ones to the following subcultures regenerate thus giving a large number of plantlets. This pineapple

micropropagation pathway by the induction of microshoots reduce the production costs of pineapple plantlets [44,45] which use gibberellin (GA_3) at 10 µM in a temporary immersion bioreactor. This equipment is expensive for laboratories in developing countries and the use of liquid media with shaker it possible to obtain similar results. Similarly, the positive effect of BAP on stimulating the multiplication of pineapple shoots has been reported by Zuraida *et al*. [15] where 1 mg/l BAP gave a multiplication rate of 6.4 shoots/plantlet at the third subculture. Bhatia and Ashwath [46] and Al-Saif *et al*. [27] obtained with 2 mg/l BAP the best multiplication rates in pineapple, respectively 7 and 10 shoots/explant at the first subculture, but they did not evaluate its synergistic effect with NAA. The average of 18.87 plantlets/explant obtained with St Michael cultivar (Smooth Cayenne) on combination of 1 mg/l BAP, 0.5 mg/l NAA and 40 mg/l AdS at the 1st subculture is largely superior to 10 shoots/explant [18] with 3 mg/l BAP; 11.5 shoots/explant [24] with 1.125 mg/l BAP; 12 shoots/explant [47] with BAP (2.25 and 3.5 mg/l); 12 shoots/explant [27] with BAP (1.75; 2.25 and 3.5 mg/l) and 14 shoots/explant [21] with 4 mg/l BAP. The cultivar has influenced the proliferation of shoots at all subcultures and St Michael's plantlets highly increased compared to those of Sugarloaf. This result is contrary to the trend observed in the field where Sugarloaf outside the suckers presents a large number of slips. This is therefore an advantage for the massive production of smooth Cayenne propagules which naturally produce very little propagules. This influence of genotype has also been noted in pineapple by Wald *et al*. [48] where Smooth Cayenne had a low multiplication rate compared to the Red Spanish and Perolera cultivars. AdS alone and lack of growth regulators stimulated plantlet length growth at all subcultures, while 2 mg/l BAP limited height growth. Hamad and Taha [47] noted that pineapple plantlets on control medium grow better in length. They obtained a length of 2 cm at the first subculture against 0.8 cm with BAP (1.75 to 3.75 mg/l). This finding is justified by the role of BAP which cancels apical dominance due to auxin synthesis in the apical zone. Thus, the endogenous auxin production by the plantlets in the absence of exogenous hormones favored the apical dominance allowing more height growth of plantlets, due to auxesis, than a multiplication. Referring to the work of Quoirin [49] and Druart [50] who have shown that, in many cases, it suffices to allow the plantlets to grow sufficiently on the multiplication medium before they are rooted, the plantlets obtained at the third subculture have reasonable heights allowing to rooting for acclimation. Also, control medium could serve as a growth buffer medium for microshoots before rooting-acclimation. Regarding the mass of plantlets, although the purpose of this study is the production of shoots and that this parameter is negligible in such study [27], the plantlets with complex 2 mg/l BAP+0.5 mg/l NAA+ 40mg/l AdS showed the highest fresh mass (1.143 g) whereas control medium negatively influenced mass growth (0.632 g). In fact, cell proliferation induced by the synergistic action of NAA with cytokinins (BAP and AdS) allowed the increase of fresh biomass. Low dose NAA in the presence of cytokinin is therefore beneficial for *in vitro* micropropagation of pineapple across all growth parameters. The cultivar also influenced fresh biomass production with St Michael, which at all subcultures favored biomass construction. Indeed, St Michael belonging to Smooth Cayenne cultigroup is characterized by large fruits and large mass (2.25-2.75 kg) compared to Hilo (1-2 kg) [51].

5. Conclusion

It appears from the present study that the addition of exogenous cytokinin such as BAP is necessary for *in vitro* regeneration and multiplication of the pineapple. The low dose of NAA (0.5 mg/l) enhances the *in vitro* response of pineapple initiated by BAP. In addition, it

induces height growth of plantlets. AdS, stimulates pineapple proliferation and also induce a highly production of fresh biomass based on it mitotic action. Thus, the addition of 0.5 mg/l NAA to 2 mg/l BAP and 40 mg/l AdS gives a significant synergistic action on the multiplication rate of plantlets having a height average to be rooted and acclimatized.

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