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Plant Regeneration through Indirect Organogenesis in Two Cultivars of Pineapple (*Ananas comosus* L.)

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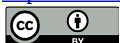

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Abstract

Unavailability of performant planting material of pineapple constitutes a major problem of its cultivation in Africa. For this purpose, indirect organogenesis technique is used to evaluate the *in vitro* responses of two cultivars of pineapple during the explant's regeneration. Calli were induced from crown leaf and plantlets leaf of "Smooth Cayenne" and "Sugarloaf cultivars". Murashige and Skoog medium with vitamins B5 supplemented with different growth regulators combinations were used. BAP and/or 2,4-D have been added to base medium for calli cells' differentiation while BAP and GA3 have been added for plant elongation. The results indicated that explants from regenerated plantlets leaves cultivated on MS supplemented with copper (II) sulphate 5-hydrate concentrations incorporated had significant ($p < 0.0001$) influence on callus induction in pineapple cultivars. Likewise, MS medium with NAA (0.5 mg/l) + BAP (1 mg/l) had a highly significant influence with 8.8 differentiated Calli. Also, MS medium supplemented with BAP (3 mg/l) + GA3 (2 mg/l) for the "smooth Cayenne" had significantly influenced ($p < 0.0001$) Calli regeneration with a high rate of 55.25% plantlets. MS medium containing 0.5 mg/l of NAA + 0 mg/l IBA produced a high number of roots in Sugarloaf whereas the medium containing 1.5 mg/l NAA + 0.5 mg/l (IBA) produced high number of roots in smooth Cayenne. We have established an efficient and reproducible protocol for mass propagation and genetic transformation of pineapple though indirect organogenesis. This protocol may be used in genetics engineering studies for pineapple breeding.

Keywords

Callus Induction, Plant Growth Regulators, Stomata Structure, Rhizogenesis,

Pineapple

1. Introduction

Pineapple (*Ananas comosus* var. *comosus*) is one of fruit crops widely produced in the tropics and with global production around 24.8 million tons in 2017 [1]. It is the third world tropical fruit crop after banana and citrus [2]. It is a self-sterile plant, and the propagation methods widely used are from suckers [3]. The traditional propagation of suckers facilitates the production of pineapple but has enormous disadvantages, such as low rate of multiplication, long period of production, high variation in size, weight and age, and non-uniform vegetative cycle [4] [5] [6]. Therefore, tissue culture is a modern method for mass production of healthy pineapple planting material with homogeneity of morphotypes [7] [8]. In vitro regeneration by organogenesis and embryogenesis is developed in pineapple [9]. The most important question is the genetic variability that may be occurred in the seedlings by using somatic embryogenesis. It has been reported that the regeneration through somatic embryogenesis favored somaclonal variants in the regenerated plants [10]. Thus, the regeneration through organogenesis has become the convenient method to reproduce genetic identical plants' material. Direct organogenesis through buds' regeneration, mostly used for seed production, [11] [12] did not supply a sufficient number of plant material for mass multiplication within a short time, with maximual number of multiple axillary shoots proliferated on MS basal medium containing 1.0 mg/l BA and 0.1 mg/l NAA [13]. In this regard, indirect organogenesis has become one of palliative for in vitro multiplication [14], and indicated as rapid in vitro multiplication method for many species of plants that also request the industrialization [15]. It is reported that developmental pathways of indirect organogenesis greatly depend on different factors, such as: genotype, explant type, and age of explant [16]. Moreover, the external change to environment which includes composition of media and physical culture conditions (light, temperature) influenced the plant regeneration [17] [18]. Interaction between all these factors leads to the induction and expression of a specific mode of cell differentiation and development. The determining of adequate explants and its age for callus production in the local varieties constitute an advantage for pineapple transformation. Furthermore, plant growth regulators such as auxins (2,4-dichlorophenoxyacetic Acetic and Picloram)/cytokinins (6-benzylaminopurine and Kinetin) act in the different steps of indirect organogenesis in pineapple. The roles of 2,4-D and plicloram in callogenesis are well known. Picloram, compared to 2,4-D, is known for its easier assimilation and transportation character into all parts of the plant in large numbers of cells [6]. BAP plays a decisive role during the somatic embryogenesis in pineapple, particularly, in the proliferation of embryogenic cells of the calli [3]. Kinetin, a natural cytokinin, has a particular effect on stem development. Thus, the specific auxin-cytokinin combinations for en-

hanced callus formation are yet determined. Regarding the concentrations, they are defined on the basis of Auxin/Cytokinin ratios in organogenesis. MS supplemented with Picloram (3.0 mg/l) + BAP (0.05 mg/l) gave the highest rate of callus formation in pineapple whereas MS supplemented with Picloram + KIN at the same concentration gave the lowest rate of callus formation [3]. Also, Copper (II) sulphate constitutes also an excessive additive that is also used [19], but their action is highly depended on cytokinin and auxin used alone or in combination. The addition of copper has greatly optimized the frequency of appearance of calli and stimulate tissue sensitivity particularly in competent cells [10]. For this technique, some physiological characteristics depending on stomata structure must be controlled. However, the use of MS medium supplemented with Auxin/cytokinin and additives need a deepen knowledge for their concentration utilization and their effects on callus induction, callus colors, and more on callus conversion to plants. Much research input and further refinement considering different key factors for devising efficient protocol with particular reference to indirect organogenesis pathway in pineapple is required. The microscopic structure of leave stomata in the regenerated plant through indirect organogenesis as compared to the original are things to investigate. However, the full understanding of these internal and external factors influence in the process remained the key point to be addressed for the effective callus induction, cell multiplication through the subculturing, and plant regeneration. This work presents an effective and reproductible procedure of regeneration of pineapple through indirect organogenesis from leaf explants. We studied the effect of 2,4-D, picloram, cytokinin, and copper on two cultivars of pineapple ("Sugarloaf" and "Smooth cayenne"), and the finding may facilitate the use of indirect organogenesis technique for transformation and mass propagation reproductive protocol of pineapple.

2. Materials and Methods

2.1. Plant Material

The plant material was composed of plantlets and fruits of two cultivars, "Sugarloaf" and "Smooth cayenne". Both cultivars of pineapple were obtained respectively from University of Abomey-Calavi in Central Laboratory of Biotechnology and Plant Breeding Germplasm, and the laboratory field station located at Wawata in Benin Republic.

2.2. Methods

2.2.1. Explants Sterilization

The leaflets from crowns of the fruits and those from plantlets of each cultivar were harvested in a beaker and washed with tap water for 1 min. The leaf explants were sterilized with copper hydroxide (45 mg/l) for 45 min following by three successive rinsing with sterile distilled water, and thereafter soaked in 70% v/v commercial bleach Jik (15% NaOCl) and rinsed with sterile distilled water three times successively as described by Bukhori and Khalid [20]. The explants

were then transferred under laminar flow hood and soaked in ethanol 96˚ solution for 10 min before being rinsed three times with sterile distilled water.

2.2.2. Callus Induction, and Plant Regeneration/Morphogenesis

The sterilized leaf explants from crown and those from plantlets were excised aseptically and cut into pieces of 1 to 2 cm before being cultured Firoozabady basal medium [21] (MS medium (1962) basal salts) supplemented with 30 g/l of sucrose, vitamins B5, glycine (2.0 mg/l), glutamine (1000 mg/l), casein hydrolysate (100 mg/l) and $MgCl_2$ (0.75 g/l) (Sigma, France) [22]. The media were different by supplying BAP (0.5 mg/l or 1 mg/l), Kinetin (0.5 mg/l or 1 mg/l) and copper (II) sulphate 5-hydrate (2 mg/l). Each cytokinin was combined with 2,4-D or Picloram at the concentrations of 3 mg/l, 6 mg/l, 9 mg/l or 12 mg/l. Cultures were maintained in the dark at 27˚C ± 1˚C with unmonitored light interruptions during daily observation with a relative humidity of 80% for four to six weeks.

Calli obtained were removed from plates six weeks after and fragmented into cell aggregate subunits. Each subunit was plated on subculturing media having the same basic elements with MS but containing double concentration of KNO_3 and half of NH_4NO_3, [22] supplemented with 6-benzylaminopurine (0.5 mg/l or 1 mg/l) and/ Naphthaleneacetic Acid (0.5 mg/l) for two eight weeks.

Calli obtained were transferred on MS media supplemented with combined Naphthaleneacetic Acid (0.5 mg /l), BAP (0.5 mg/l, 1 mg /l, 3 mg/l, and 5 mg/l), and Gibberellic Acid (1 mg/l and 2 mg/l) and incubated for one week in the dark, before placed in a 10/14 h light/dark cycle at 27˚C ± 1˚C for plantlets regeneration/morphogenesis. The pH of the media was adjusted to 5.7 ± 0.1 using 0.1M HCl or 0.1M NaOH, and the media was gelled with 0.7% phytagel. The media were dispensed in 10 ml aliquots into culture vessels and then autoclaved at 1.1 kg·cm^2 and 121˚C for 20 min. The calli were cultivated in this medium for four weeks after incubation for plantlets regeneration.

2.2.3. Rooting of Plantlets of Two Cultigroups of Pineapple

Vigorous seedlings without roots over 2 cm [23] [24] and weighing more than 100 mg of fresh material obtained after four (04) weeks each were transferred to the different rooting environments. Six rooting media varying on the basis of the hormonal combination of NAA (N0640-25G; Sigma) and AIB (I5386-25G; Sigma) were tested in liquid and solid (8 g agar) consistencies during four weeks (**Table 1**).

2.2.4. Cells Observation in Direct and Indirect Organogenesis Regenerated Plantlets

Leaf diaphanization was made freehand on the leaves of indirect organogenesis regenerated plantlets and direct organogenesis regenerated plantlets of two cultivars. The sections were transferred to NaOCl (30%) solution for 15 min and rinsed with tap water followed by soaking in green carmine solution for 5 min and rinsed again with tap water. The sections were covered with a glass by a

Table 1. Growth regulator composition of different rooting media.

Media	Growth regulators (Auxins)	
	NAA (mg/l)	IBA (mg/l)
ANA*AIB	00	0.5
ANA*AIB	0.5	00
ANA*AIB	0.5	1
ANA*AIB	0.5	1.5
ANA*AIB	1	0.5
ANA*AIB	1.5	0.5

coverslip on a slide maintained with a drop water. Optical microscope (MOTIC) was used for cell observations of sections in order to make epidermis cells difference of different types of leaves [25] [26].

2.2.5. Acclimatization Des Vitroplants

The rooted plantlets were removed from tubes and successfully rinsed in clean water and planted in a horticultural socket containing a sterilized substrate of sawdust and compost as described by Agbinoukoun *et al.* [27]. The plantlets were maintained under greenhouse respecting plant growing conditions (T = 29˚C; Humidity of 70.2%) for eight weeks. Plantlets were periodically watered with Shive solution as described by Agbinoukoun *et al.* [27].

2.2.6. Data Collection and Statistical Analysis

The rooting rate was calculated after observing the presence or absence of root at the end of the fourth week according to the formula:

$$\text{Rooting rate} = \frac{\text{Nomber of rooted plants}}{\text{Total number of plants}} \times 100$$

The average number of roots was determined by counting the roots emitted by each tissue culture plant at the end of the fourth week. A logistic regression was performed according to the concentration of auxin to evaluate its effect on the rooting rate. An ANOVA was used to compare the different means (number and length of roots) which were discriminated by the Student Newman and Keuls t test at the 5% threshold. The Khi^2 independence test was performed to evaluate the type of explant responding best to indirect organogenesis and Binary logistic regression was performed on the callus induction rate of the explants. The analysis of variance was carried out with General Linear Model (GLM) procedure to evaluate the variability of induced callus rate, the fresh mass of callus, the kinetic of callus induction, during the different treatments. The test of Student Newman and Keuls (SNK) at 5% threshold was performed to compare the length and width of stomata and ostioles in the cultivars. The normality of distribution and the equality of variances of the dataset were verified by Ryan-Joiner test and Levene test, respectively, before the comparisons. Poisson regression was performed to determine first, the effect of genotype and

hormonal combinations on the variability of regenerated plantlets number and second, the impact of indirect organogenesis technique on the regenerated plantlets.

3. Results

3.1. Effects of Explants Origin and Genotypes on Callus Induction

The type of explant and cultivar significantly influence ($P < 0.0001$) callus induction. Thus, only leaf explants of regenerated plantlets were able to induce calli with higher rate in "Smooth cayenne" (60.87%) than "Sugarloaf" (27.78%) (**Figure 1**).

3.2. Effects of Combined Auxins (2,4-D and Pi)/Cytokinins (BAP and Kinetin) and Copper on Callus Induction

3.2.1. Kinetics of Callus Induction

There was a significant difference ($p < 0.0001$) in the time of appearance and callus rate within cultivars of pineapple (**Table 2**). Media supplemented with copper produced higher callus rate in "Smooth Cayenne" than media without copper. Explants of "Smooth cayenne "induced calli from second week after incubation while those of "Sugarloaf induced" calli from third week after incubation in presence or not of copper (II) sulphate (**Figure 2**).

3.2.2. Rate of Callus Induced

The response of different media has been grouped in six lots according to callus induced rate (**Table 3**). The medium MS without growth regulators and copper

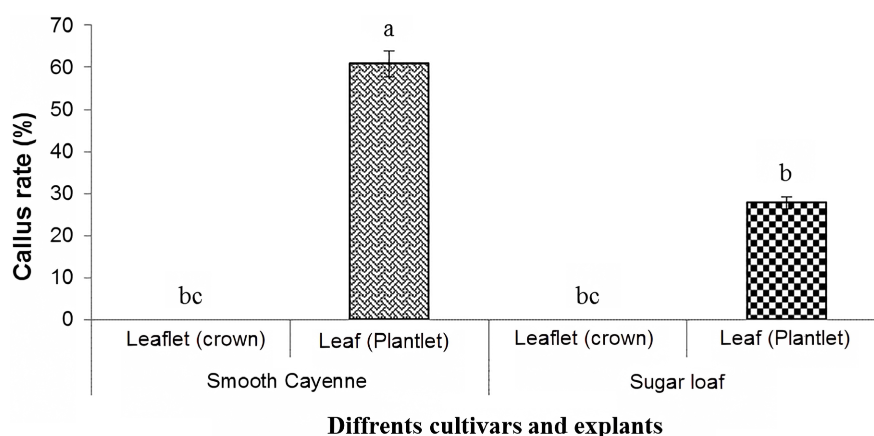

Figure 1. Effect of explants origin on calli formation in pineapple.

Table 2. Khi² test on time of appearance of callus and callus rate within pineapple cultivars.

Source	DDL	Khi² (Wald)	Pr > Wald	Khi² (LR)	Pr > LR
Cultivars	1	12.6726	0.0004	12.6291	0.0001***
Nber.WAS	2	95.2701	<0.0001	90.6280	<0.0001***

*** = highly significant difference ($p < 0.0001$); Nber = Number; WAS = Week after sowing.

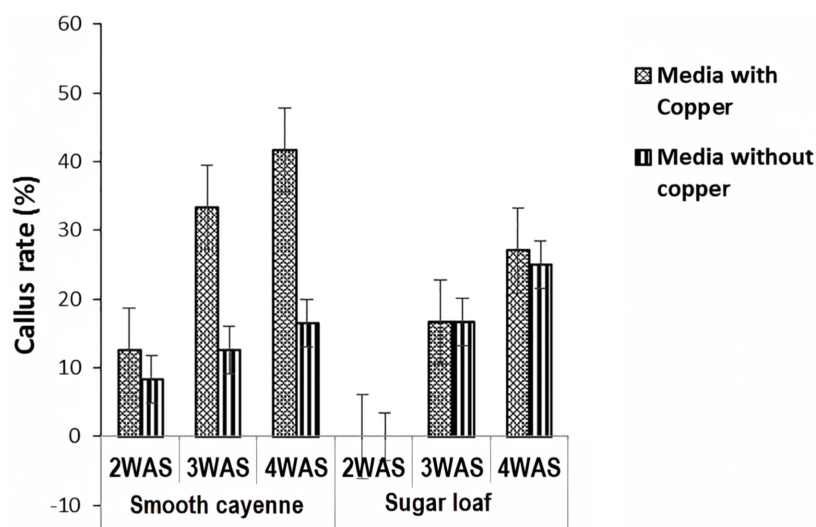

Figure 2. Effect of copper on callus formation; (WAS) Week after seeding. WAS = Week after sowing.

Table 3. Effect of plant growth regulators combination and copper on calli induction rate.

Lots	PGRs	Calli induction rate	Groupes
Lot A	K0.5D12	0	A
	K0.5D3 + Cop	0	A
	B0.5P6	0	A
	K1D6	0	A
	B1D6	0	A
	M0	0	A
	B0.5P12	0	A
	B0.5D9	0	A
Lot B	B1D3 + Cop	16.66	AB
	K1P12 + Cop	16.66	AB
	K1P3	16.66	AB
	B0.5P6 + Cop	16.66	AB
	B0.5P9	16.66	AB
	K0.5D12 + Cop	16.66	AB
	K1P9	16.66	AB
	B0.5D9 + Cop	16.66	AB
	K1P12	16.66	AB
	K1P6 + Cop	16.66	AB
	B1P12	16.66	AB
	K0.5P3	16.66	AB
Lot C	B1P6	33.33	AB
	K0.5P12	33.33	AB

Continued

	K0.5P12 + Cop	33.33	AB
	K1P6	33.33	AB
	B0.5P12 + Cop	33.33	AB
	B0.5P3	33.33	AB
	B0.5P3 + Cop	33.33	AB
	K0.5P9	33.33	AB
	B0.5D6	33.33	AB
Lot D	B0.5D3	50	AB
	B0.5D3 + Cop	50	AB
	B0.5D6 + Cop	50	AB
	B1D6 + Cop	50	AB
	B1P3 + Cop	50	AB
	B1P9 + Cop	50	AB
	K0.5D3	50	AB
	K0.5P3 + Cop	50	AB
	K0.5P6	50	AB
	K0.5P9 + Cop	50	AB
	K1D6 + Cop	50	AB
	K1P3 + Cop	50	AB
	K1P9 + Cop	50	AB
Lot E	B0.5P9 + Cop	66.66	AB
	B1D3	66.66	AB
	B1P9	66.66	AB
	K0.5P6 + Cop	66.66	AB
Lot F	B1P6 + Cop	100	B
	B1P12 + Cop	100	B

PGRs = plant growth regulators, Kx = Kinetin [$mg \cdot L^{-1}$], Dx = 2.4-D [$mg \cdot L^{-1}$], Cop = copper, Px = Picloram [$mg \cdot L^{-1}$], Bx = Ben-zylaminopurine [$mg \cdot L^{-1}$], M0 = Medium without plant growth regulators.

was used as control in the experiment and classified in the lot A according to the callus induced rate (Table 3).

Formation of callus was significantly influenced by plant growth regulators combination and copper concentrations incorporated in the medium ($p < 0.0001$). The average rate of callus formation varied from 0% to 100%. Maximum callus formation (100%) was obtained for both cultivars on a medium containing the combination of BAP (1 mg/l) + Copper (2 mg/l) + Picloram (6 mg/l or 12 mg/l) within the cultivars **(Lot F)**.

3.2.3. Weight of Callus Induced

There was not a significant difference ($P > 0.05$) of copper on the callus weight

within the cultivars whatever the plant growth regulators combination (**Table 4**). However, the average of fresh calli weight varied according to the plant growth regulators combinations and the cultivars. The highest weight of callus (604.7 mg) whatever the hormonal combination was obtained in the absence of copper in “Sugarloaf” cultivar and the lowest weight of callus (345.1 mg) in the presence of copper with “Smooth Cayenne”.

3.3. Effects of NAA and BAP/Kinetin on Cells Differentiation

Different combination of plant growth regulators combinations had a highly significant influence ($P < 0.0001$) on calli multiplication and differentiation in the different cultivars. Excepted the medium supplemented with BAP (1 mg/l), “Smooth cayenne” produced calli with important differentiate cells than “Sugarloaf” on any other. The highest number (8.8) of differentiate cells was obtained with the medium supplemented with NAA (0.5 mg/l) + BAP (1 mg/l) in “Smooth Cayenne” and the lowest (2) with BAP (0.5 mg/l) in “Sugarloaf”. In “sugarloaf”, the highest number (4.8) of calli with differentiate cells was obtained on the medium supplemented with BAP (1 mg/l) (**Figure 3**). Different step of callus induction and cells differentiation were stated in **Figure 4**.

3.4. Effects of BAP and GA_3 on Plant Regeneration

The cultivars and plant growth regulator combination had a highly significant difference ($P < 0.0001$) on the number of plantlets formed per explant while no influence of the interaction culture media X cultivars was noted). Thus, the highest average number (10.36) of plantlets per explant was obtained with “Smooth Cayenne” on the medium supplemented with GA3 (1 mg/l) + BAP (1 mg/l) and the lowest (0.58) was obtained on the same medium with the “Sugarloaf” (**Table 5**). Thus, except the media supplemented with GA3 (1 mg/l) + BAP (0.5 mg/l; 1 mg/l), “Smooth Cayenne” had more plantlets than “Sugarloaf” with all other combinations. Medium supplemented with growth regulator combination GA3 (2 mg/l) + BAP (3 mg/l) was favorable to obtain the highest number

Table 4. Effect of plant growth regulators combination and copper on induced calli weight.

Cultivars	Culture media	Calli weigth (mg)
“Sugarloaf”	MWC	432.7 ± 0.7
	MSC	604.6 ± 0.7
“Smooth Cayenne”	MWC	345.1 ± 0.7
	MSC	378.3 ± 0.7
P-Value	Cultivars	0.037*
	Culture media	0.345NS
	Cultivars x Culture Media	0.166NS

MWC: Media without copper; MSC: Media supplemented with copper; NS: No significant difference (5%); * significant difference (5%).

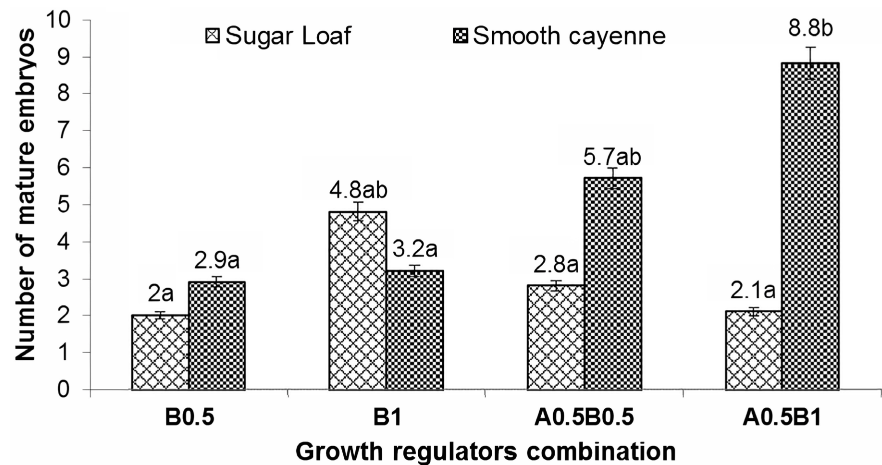

Figure 3. Effect of growth regulators combination on calli differentiation. B0.5: 0.5 mg/l (BAP); B1: 1 mg /l (BAP); A0.5B0.5: 0.5 mg/l (ANA) 0.5 mg/l (BAP); A0.5B1: 0.5 mg/l (ANA) 1 mg/l (BAP).

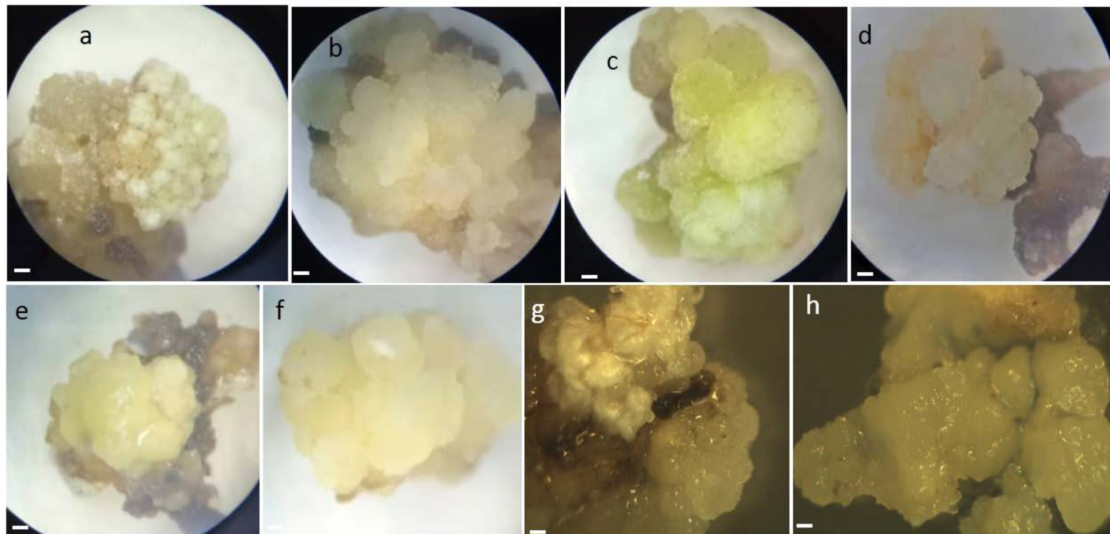

Figure 4. Callus induction and cells differentiation in pineapple. (a and b) callus induced by explants; (c and d) Sub-culture of callus for cells differentiation; (e) Early differentiate callus; (f and g) callus with differentiate cells; (h) Differentiate cells ready to be transferred to regeneration medium. Scale bars = 2 mm.

(55.25) of plantlets with "Smooth Cayenne", while this same medium gave a low number (5) of plantlets with "Sugarloaf". The plantlets regeneration process from mature embryos is described in Figure 5.

3.5. Effect of Hormonal Combinations on Root Number and Root Length

There was a significant difference ($P < 0.0001$) among the number and length of roots formed in the two cultigroups according to the hormonal combinations (Table 6). The number of roots was higher (18.73 ± 1.11) on medium containing 0.5 mg/L of NAA + 0 mg/L IBA in the Sugarloaf cultivar, whereas the lowest (04.67 ± 1.11) was obtained with 0.5 mg/L NAA + 1 mg/L IBA. In the smooth

Table 5. Effects of different concentrations of BAP and GA3 on plant regeneration.

Cultivars	GA3 + BAP (mg/l)	Nber. Plantlets/Treatment
"Smooth Cayenne"	2 mg/l + 5 mg/l	15a
	2 mg/l + 1 mg/l	9.5a
	2 mg/l + 0.5 mg/l	16.25a
	1 mg/l + 1 mg/l	16a
	1 mg/l + 3 mg/l	24.5ab
	1 mg/l + 5 mg/l	12.75a
	1 mg/l + 0.5 mg/l	22.25ab
	2 mg/l + 3 mg/l	55.25b
"SugarLoaf"	2 mg/l + 5 mg/l	1.75a
	2 mg/l + 1 mg/l	7.75a
	2 mg/l + 0.5 mg/l	2.25a
	1 mg/l + 1 mg/l	2.75a
	1mg/l + 3 mg/l	5.25a
	1 mg/l + 5 mg/l	23ab
	1 mg/l + 0.5 mg/l	2.75ab
	2 mg/l + 3 mg/l	14a
P. Value	Culture Media	<0.0001***
	Cultivars	<0.0001***
	Culture Media X Cultivars	0.089NS

Nber. Plantlets/Treatment: Number of plantlets per treatment; *** Highly Significant difference (0.01%); NS: not significant at 5%.

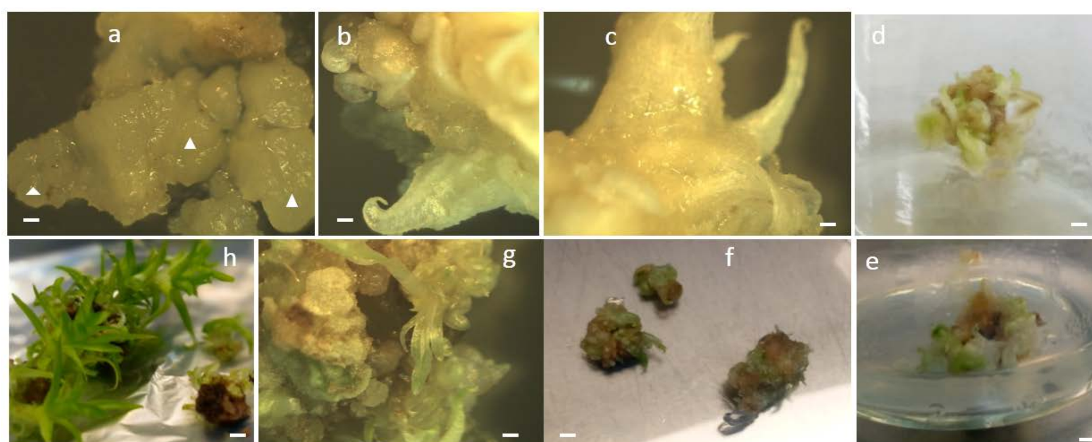

Figure 5. Morphogenesis steps of indirect organogenesis in pineapple. (a) Differentiate callus; (b and c) Early stage of cells conversion to plant; (d, e, and f) Plants regenerated and early developmental stages; (g) Young plants to be transfered to growing medium; (h) Plantlets of "Smooth cayenne" cultivar obtained on the growing medium. Scale bars = 2 mm.

Cayenne cultivar, the highest number of roots (18.40 ± 1.11) is obtained with the hormonal combination 1.5 mg/l NAA + 0.5 mg/L IBA and the lowest (6.93 ±

1.11) with the hormonal combination 0 mg/l NAA + 0.5 mg/L IBA (Table 7) (Figure 6). The strong average length (3.03 ± 0.12) of roots was obtained on the medium containing 0.5 mg/L of NAA and 0 mg/L of IBA while the weakest (0.65 ± 0.12) was obtained with the hormonal combination 1.5 mg/l NAA + 0.5 mg/L IBA in Sugarloaf. In Cayenne Smooth, the highest root length (2.12 ± 0.12) was obtained by using 0.5 mg/l of NAA + 1 mg/L IBA while the lowest was obtained using 1.5 mg/l NAA + 0.5 mg/L IBA (Table 7) (Figure 6).

3.6. Histological Impact of Indirect Organogenesis of the Regenerated Plantlets

The microscopic analysis of leaf section from plantlets obtained through direct organogenesis and indirect organogenesis presented a similar structure of epidermis cells tissues. The tissues presented guard cells and unequal distribution of opened ostioles on the both faces of the sections in direct and indirect organogenesis plantlets (Figure 7(a) and Figure 7(b)). Abaxial epidermis had more stomata. In "Sugarloaf", the stomata obtained from direct organogenesis plantlets (149) is higher than those of indirect organogenesis techniques (141). By comparing the stomata and ostioles in plantlets from different techniques, there was a significant difference ($P < 0.0001$) in stomata and ostioles development of the plantlets obtained through the both techniques. Moreover, cultivars had a

Table 6. Analysis of variance on the root number and root length.

Source of variation	Ddl	Sum of Scare	Scare Mean	Fisher valor	P > F
Comb. Horm.	15	14283.2	952.21	51.28	0.000***
Cultivars	2	707.4	353.71	19.05	0.000***
Comb. Horm.*Cultivars	30	4671.9	155.73	8.39	0.000***

Table 7. Variation of root number and length through different hormonal combinations in two cultigroups of pineapple.

Base medium	Comb. Hormo.	Cultivars	TE(%)	Number of roots	Length of roots (cm)
MS	NAA 0 mg/L + IBA 0.5 mg/L	Smooth Cayenne'	100 a	06.93 ± 1.11 mq	1.80 ± 0.12 e
		Sugarloaf	100 a	08.27 ± 1.11 kq	2.33 ± 0.12 cd
	NAA 0.5 mg/L + IBA 0 mg/L	Smooth Cayenne'	100 a	09.73 ± 1.11 jo	2.06 ± 0.12 de
		Sugarloaf	100 a	18.73 ± 1.11 ae	3.03 ± 0.12 ab
	NAA 0.5 mg/L + IBA 1 mg/L	Smooth Cayenne'	100 a	12.13 ± 1.11 hl	2.12 ± 0.12 d
		Sugarloaf	100 a	04.67 ± 1.11 oq	1.32 ± 0.12 fg
	NAA 0.5 mg/L + IBA 1.5 mg/L	Smooth Cayenne'	100 a	17.40 ± 1.11 ah	2.09 ± 0.12 d
		Sugarloaf	100 a	10.27 ± 1.11jn	1.74 ± 0.12 e
	NAA 1 mg/L + IBA 0.5 mg/L	Smooth Cayenne'	100 a	13.60 ± 1.11 ek	1.27 ± 0.12 g
		Sugarloaf	100 a	11.00 ± 1.11 im	1.14 ± 0.12 gh
	NAA 1.5 mg/L + IBA 0.5 mg/L	Smooth Cayenne'	100 a	18.40 ± 1.11 af	0.95 ± 0.12 h
		Sugarloaf	100 a	18.53 ± 1.11 ae	0.65 ± 0.12 hi

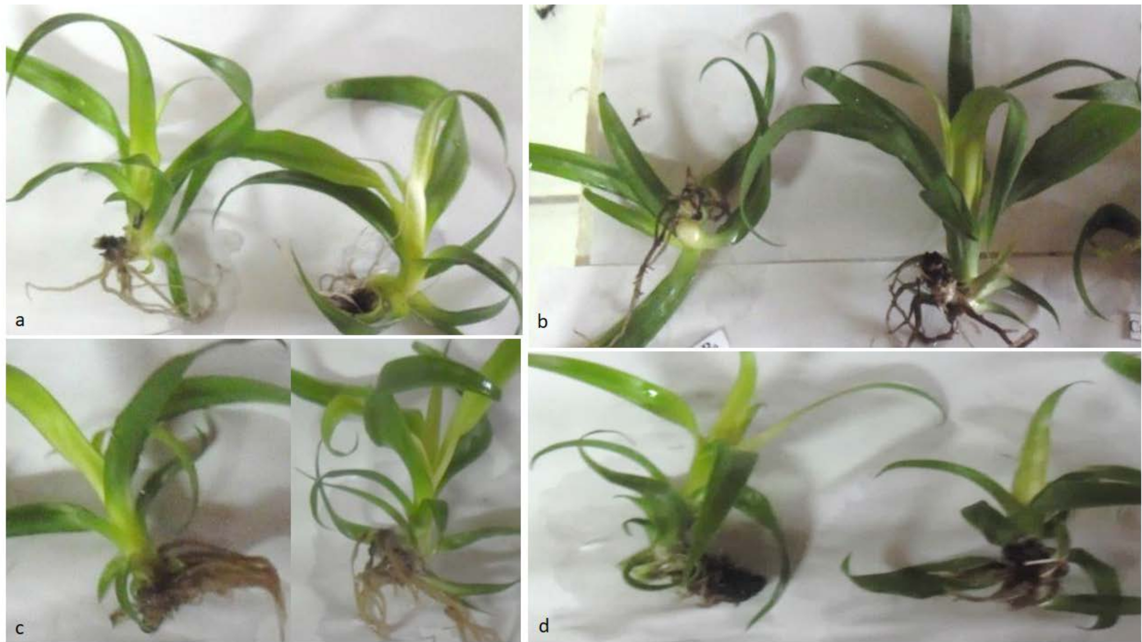

Figure 6. Roots development of the regenerated plantlets of pineapple. (a) Rooting on MS + 0.5 mg/l of IBA; (b) Rooting on MS + 1.5 mg/l of NAA + 0.5 mg/l of IBA; (c) Rooting on MS + 0.5 mg/l of NAA; (d) Rooting on MS + 1 mg/l of NAA + 0.5 mg/l of IBA.

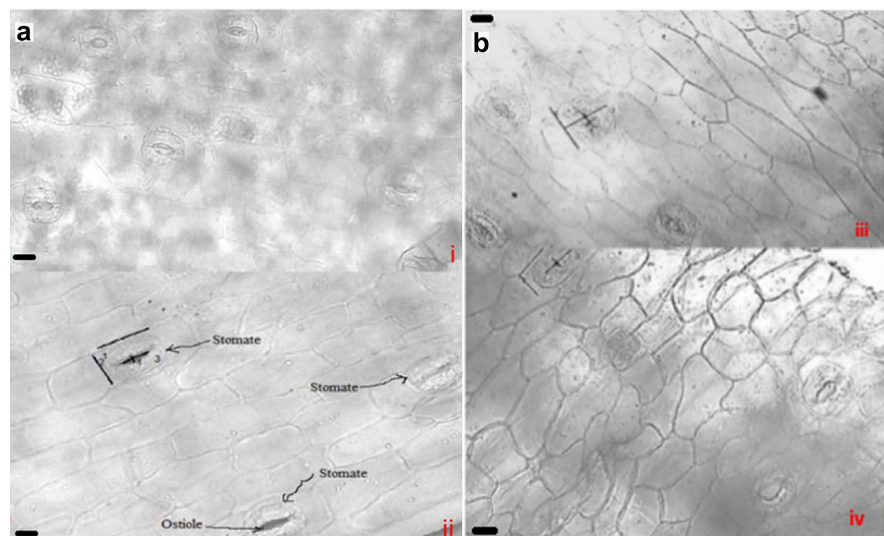

Figure 7. Microscopic structure of regenerated pineapple leaves. (a) Microscopic structure of "Smooth cayenne" leaves: (i) "Smooth cayenne" leaf from plantlet obtained by organogenesis; (ii) "Smooth cayenne" leaf from plantlet obtained by indirect organogenesis). (b) Microscopic structure "Sugarloaf "leaves (iii) Sugar "loafleaf" from plantlet obtained by direct organogenesis; (iv) "Sugarloaf leaf" from plantlet obtained by indirect organoogenesis). Scale bars: 100 µm.

significant influence ($P < 0.0001$) on the number, width and length of stomata; the length and width of ostioles by both direct and indirect organogenesis. Thus, in "Smooth cayenne", 134 stomata were counted per leaf area analyzed against 141 in "Sugarloaf" through indirect technique. The length, and width of the

stomata and ostioles are respectively 26.6 µm; 19.82 µm for the stomata, 16.23 µm and 5.45 µm for the ostioles in "Smooth cayenne" versus 33.5 µm; 22 µm for the stomata and 19.8 µm and 8.3 µm for the ostioles in "Sugarloaf" (**Table 8**).

3.7. Plantlets Growth in Acclimatization

The results obtained initially showed high rate (100%) of survival and growth of the plantlets watered with Shive solution (**Figure 8(a)**). In addition, the plantlets with spread pores exhibiting a good characteristic of which the number, weight, size leaves were favored for its development. The good junction between the aerial

Table 8. Stoma frequency and dimensions of stomata and ostioles in "Smooth cayenne" and "Sugarloaf".

Variables	Nber Sto/µm²	LoSto (µm)	LaSto (µm)	LoOS (µm)	LaOS (µm)
CayFVPT	134 ± 0.31	46.6 ± 0.5	35.6 ± 0.8	32 ± 1	5.87 ± 0.1
Cay FVPEmS	134 ± 0.63	26.6 ± 0.5	19.82 ± 0.4	16.23 ± 0.5	5.45 ± 0.47
PSFVPT	149 ± 1.26	36.43 ± 0.4	30.82 ± 0.2	19 ± 1.26	10.48 ± 0.48
PSFVPEmS	141 ± 2.21	33.5 ± 0.8	22 ± 0.7	19.8 ± 0.86	8.3 ± 0.8
R^2	0.816	0.975	0.967	0.911	0.787
Pr > F	0.0197*	<0.0001***	<0.0001***	<0.0001***	<0.0001***

CayFVPT: Control sheets of plantlets in the "Smooth Cayenne"; **CayFVPEmS**: Control sheets of plantlet from Indirect organogenesisogenesis in "Smooth Cayenne"; **PSFVPT**: Control sheets of plantlet in the "SugarLoaf" cultivar; **PSFVPEmS**: Control sheets of plantlet from indirect organogenesis in "Sugarloaf"; **R2**: coefficient of determination; **Nbr of Sto**/µm²: Number of stomata per square micrometer leaf area; **LoSto** (µm): Length of stomata in micrometer; **LaSto** (µm): Width of the stomata in micrometer; **LaOS** (µm): Width of the ostiole in micrometer; **LoOS** (µm): Length of the ostiole in micrometer; * = significant difference ($P < 0.05$), *** = highly significant difference ($p < 0.0001$).

Figure 8. (a) Plantlets transferred to socket and watered with Shive solution under greenhouse; (b) Plantlets transplantation into shaded pots for development.

part and the root system were those which were successfully grown in acclimatization phase under greenhouse, unlike plantlets with erect pores having lower success rate (**Figure 8(b)**).

4. Discussion

Callus induction, cells differentiation, and plant morphogenesis constitute essential steps in indirect organogenesis regeneration technique developed in *Ananas comosus* var. *comosus*. Unlike the collected leaves from crown of the fruit, those from the plantlets lead the development more to callogenesis, whatever the plant growth regulators combinations tested. Indeed, the leaves of conventional plants being in advanced differentiation compared to the younger plantlets; should require special conditions favoring their callus induction and differentiation. Cells were more directed to morphogenesis in tissue culture [9]. The effect of copper was found to be significant on callus induction in both cultivars. This result suggested that the copper is an essential additive witch activate cells of the explants for multiplication. Nirwan and Kothari [28] have also shown the positive effect of copper on plant calli production that stimulate the tissue sensitivity. As copper plays a fundamental role in the activation of several enzymes involved in electron transport, the biosynthesis of proteins and carbohydrates, the metabolism of polyphenols is thought to be involved in the rapid proliferation of cells and explants regeneration [29]. Also, a significant influence of cultivars was noted especially with regard to the kinetics of callogenesis with an earliness callus induction in "Smoothcayenne" (two weeks) compared to "Sugarloaf" (three weeks). Despite the delay in "Sugarloaf", the addition of copper (2 mg/l) and growth regulator in the combinations BAP (1 mg/l) + Picloram (6 mg/l or 12 mg/l) to the media induced 100% of calli in both cultivars. These findings revealed the importance of picloram in inducing callogenesis and maintaining strong callus growth in pineapple cultivars. This may be explained by the easy transport of picloram compared to 2,4-D participating for rapid and large proliferation cells [30]. The media tested as well as the cultivars have a significant influence on the cell's differentiation. By increasing BAP (0.5 mg/l - 1 mg/l), number of differentiate cells have been increased. This growth regulator plays a determining role by acting on the cell's proliferation on the calli [3]. Also, it was found that by increasing the number of cells differentiation by adding of NAA (0.5 mg/l) to medium with an optimal rate of initiation (88%) on MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) [31]. The results of the present study also revealed that obtaining plantlets from differentiate cells in "Smooth cayenne" and "Sugarloaf" cultivars of pineapple required the use of auxins and cytokinins, as this has been indicated in our previous studies on cassava [31] [32]. However, the most favorable combination for Smooth cayenne was very unfavorable in "Sugarloaf" cultivar with a rate of 21% showing the non-significant effect between the media factors and cultivar factors in cells differentiation.

The types of medium and cultivar had significant influence ($P < 0.05$) on the plantlet's regeneration. Thus, there are more plantlets obtained per calli (10.36) by adding to the medium, growth regulator combination BAP (1 mg/l) + GA3 (1 mg/l). Indeed, if a lower concentration of GA3 (0.05 mg/l) with the same dose of BAP favored the conversion of differentiate cells in plantlets with "Smooth cayenne", we deduce from the lower plantlets obtained with 2 mg/l of GA3, that a high concentration of GA_3 slow down the conversion of differentiate cells into plantlets. The influence of cultivar with the ability of differentiate cells from "Smooth cayenne" to produce plantlets clearly indicates the involvement of specific genetic factors not only to cultivars but also to the conversion into plantlets [33] [34].

The indirect organogenesis technique did not affect the structure of leaf cells, the ostioles opening, and their uneven distribution between the faces in the cultivars. This constant openness, a particularity of plantlets, has already been found by Shackel *et al.* [35]. Compared to indirect organogenesis, the stomata in leaves of plantlets produced by direct organogenesis were large in both cultivars. This difference could be explained by the juvenility of the material obtained from indirect organogenesis technique. In addition, the stay in the darkness of the material during the process of indirect organogenesis could also impact their dimensions, unlike plantlets exposed to light radiation during direct organogenesis.

By using NAA and IBA in MS medium, all media induced roots and the number of roots increased with high auxin concentrations, especially with NAA, which strongly induced root emission compared to IBA. Hamad *et al.* [36] showed that MS medium without growth regulator could induce root but the number was limited. At this level, it is noted that Sugarloaf respectively emitted more roots than Smooth cayenne. Therefore, the concentration of endogenous auxin varied among two cultigroups of pineapple. Badou *et al.* [5] have noted a similar result in Sugar Loaf and suggesting that Sugar Loaf contains more endogenous auxins than Smooth cayenne. It is also found that the watering solution and its frequency make possible to establish an optimal dose of irrigation that can be apply for plantlets for successful acclimatization.

5. Conclusion

The study highlighted the key step from the explants choosing to plant regeneration of pineapple using indirect organogenesis technique. Plantlets leaves have been shown to be the material of choice in callus induction. Also, the addition of BAP (1 mg/l) + copper (2 mg/l) + Picloram (6 mg/l or 12 mg/l) to Murashige and Skoog (MS) medium with vitamins B5 was favorable for differential cells regeneration in pineapple. MS medium supplemented with the growth regulators combination NAA (0.5 mg/l) + BAP (1 mg/l) was efficient for cell differentiation in the two cultivars. The combination for GA_3 (2 mg/l) + BAP (3 mg/l) in the same medium was favored for shoot induction with stable histological

structure of leaves cells. MS containing 0.5 mg/l of NAA gave high number of roots in Sugarloaf whereas MS containing 1.5 mg/l NAA + 0.5 mg/l gave high number of roots in smooth Cayenne. With this study, we have established an efficient and reproducible protocol of plant regeneration through indirect organogenesis. The protocol may serve as mass propagation of pineapple and also use in the genetics transformation of pineapple. We believe that this regeneration protocol could be useful in the micropropagation of pineapple suckers. The capacity of regeneration from leaf section into plantlets via callus may constitute a reliable tool for genetic engineering studies.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

Consent for Publication

This manuscript is not under consideration for publication elsewhere. All authors have read, corrected and approved the manuscript submission to Open Journal of Applied Science

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

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