# Plant regeneration through somatic embryogenesis in two cultigroups of Pineapple (Ananas comosus L.)

**Gilles H.T. Cacaï** ( cagnat@yahoo.fr )
Central Laboratory of Plant Biotechnology and Plant Breeding, Department of Genetics and Biotechnology, Faculty of Science and Technique, University of Abomey-Calavi, Abomey-Calavi, Cotonou

**Brunith A.M. Ahokpossi**
Central Laboratory of Plant Biotechnology and Plant Breeding, Department of Genetics and Biotechnology, Faculty of Science and Technique, University of Abomey-Calavi, Abomey-Calavi, Cotonou

**Serge S. Houédjissin**
Central Laboratory of Plant Biotechnology and Plant Breeding, Department of Genetics and Biotechnology, Faculty of Science and Technique, University of Abomey-Calavi, Abomey-Calavi, Cotonou

**Jerome Anani Houngue**
Central Laboratory of Plant Biotechnology and Plant Breeding, Department of Genetics and Biotechnology, Faculty of Science and Technique, University of Abomey-Calavi, Abomey-Calavi, Cotonou

**Bienvenue T. Badou**
Central Laboratory of Plant Biotechnology and Plant Breeding, Department of Genetics and Biotechnology, Faculty of Science and Technique, University of Abomey-Calavi, Abomey-Calavi, Cotonou

**Corneille Ahanhanzo**
Central Laboratory of Plant Biotechnology and Plant Breeding, Department of Genetics and Biotechnology, Faculty of Science and Technique, University of Abomey-Calavi, Abomey-Calavi, Cotonou

**Research Article**

**Keywords:** Embryogenic callus induction, plant growth regulators, somatic embryos regeneration, stomata structure, Pineapple

**Posted Date:** February 26th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-244247/v1

# Abstract

## Background

Pineapple production is mostly constrained by unavailability of high-performance suckers. However, somatic embryogenesis (SE) have been revealed the most rapid and controllable method for Pineapple propagation than conventional sucker production methods. The aim of this study was to evaluate the responses of two cultivars of pineapple regenerated through somatic embryogenesis.

## Methods

Thus, calli were induced from crown leaf and plantlets leaf of Smooth Cayenne and Sugar Loaf cultivars. *Murashige and Skoog* base medium supplemented with vitamins B5 and different in hormonal combinations: Auxins / Cytokins. BAP and / or 2,4-D have been added to base medium for calli maturation and BAP and GA3 for plant regeneration.

## Results

The results indicated a significant influence of type of explant and copper on callus induction in pineapple cultivars. Likewise, The medium MS with growth regulator combination NAA (0.5 mg/l) + BAP (1mg / l (BAP) has a highly significant influence with 8.8 mature somatic embryos. Also, the growth regulator combinations and the cultivars have significantly influenced somatic embryos regeneration with a high rate of 55.25% shoots by using the hormonal combination BAP (3 mg/l) + $GA_3$ (2 mg/l) for the smooth Cayenne.

## Conclusion

Leaves from organogenesis plantlets constitute the explants to be used for callus induction in pineapple. The combination of BAP (1 mg/l) + copper (2 mg/l) + Picloram (6 mg/l or 12 mg/l) on Murashige and Skoog medium supplemented with vitamins B5 was favorable somatic embryos regeneration of pineapple. The protocol developed is a key study for successful mass propagation and genetics transformation of pineapple.

# Background

Pineapple (*Ananas 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 is from suckers [3]. The traditional propagation of suckers facilitate 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 [3–4]. Therefore, tissue culture have become an alternative methods for mass production of healthy pineapple planting material with homogeneity of morphotypes.

However, the micropropagation by organogenesis, mostly used for mass production, [5–6] have shown limited multiplication rate for a permanent availability of propagules.

In this regard, somatic embryogenesis constitutes a palliative for in vitro multiplication [7]. It is the most rapid in vitro multiplication method for many types of plants and trees that also request the industrialization [3, 8]. Several preview studies have been focused on the regeneration through somatic embryogenesis in pineapple [9–13]. It is reported that developmental pathways of somatic embryogenesis of pineapple and plant regeneration greatly depends on different factors, such as: genotype, explant type, and age of explant [14, 15]. Moreover, the external environment which includes composition of media and physical culture conditions (light, temperature) are not fully clear [16, 17]. Interaction between all these factors leads to the induction and expression of a specific mode of cell differentiation and development. The role of growth regulators (Auxins and Cytokinins) combination with other additive (copper and picloram) in each step of somatic embryogenesis process and the histological structure of the regenerated plantlets are things to be underlined. However, the full understanding of these internal and external factors influence in the process remained the key point to be addressed for the effective somatic embryos induction, maturation, and regeneration. Here, we have extended studies on somatic embryogenic response in two cultivars of pineapple and the information provided are efficient for reproductive protocol.

# Results

### Effects of explants origin and genotypes on embryogenic callus induction

The type of explant and cultivar significantly influence ($P < 0.05$) callus induction. Thus, only explants from organogenesis regenerated plantlets leaves were able to induce calli with higher rate in Smooth cayenne (60.87%) than Sugar loaf (27.78%) (Fig. 1).

### Effects of combined Auxins (2,4.D and Pi) / Cytokinins (BAP and Kinetin) and copper on callus induction.

### 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 1)**. 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 Sugar loaf induced calli from third week until fourth week in presence or not of copper **(Figure 2)**.

**Table 1:** Khi$^2$ 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 | 12.6726234 | 0.0004 | 12.6291589 | 0.0001*** |
| Nber.WAE | 2 | 95.2701409 | < 0.0001 | 90.6280087 | < 0.0001*** |

*** = highly significant difference ($p < 0.0001$)

### Rate of callus induced

Formation of callus was significantly influenced by growth regulators combination and copper concentrations incorporated in the medium ($p < 0.001$). The average rate of callus formation varied from 0 to 100% **(S1 File)**. Maximum callus formation (100%) was obtained for both cultivars on a medium containing the combination of BAP (1mg/l) + Copper (2mg/l) + Picloram (6 mg/l or 12 mg/l) within the cultivars **(Lot F)**. It was observed that the same hormonal combination without copper gave callus rate varying from 0 to 66% in Smooth cayenne and from 0 to 33% in Sugar loaf **(Figure 3)**.

### Weight of callus induced

There was not a significant difference ($P \geq 0.05$) of copper on the callus weight within the cultivars whatever the hormonal combination **(Table 2)**. However, the average of fresh calli weight varied according to the hormonal combinations and the cultivars **(S2 File)**. The highest weight of callus (610 mg) was obtained in the absence of copper in Sugar loaf cultivar and the lowest weight of callus (330 mg) in the presence of copper with Smooth Cayenne.

**Table 2:** Effect of growth regulators combination on induced calli weight.

| Cultivars | Culture media | Calli weigth (mg) |
|---|---|---|
| Sugar loaf | 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 |

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

### 3-2-4. Callus color, texture and structure

Whitish and friable calli have been obtained regardless growth regulator combination (Figure 4 A and B). The Figure 4 C and Figure 4 D showed cell structures of calli in Sugar loaf, and Smooth cayenne after two months, respectively. Bulges were clearly seen, indicating complete transformation of the explant and cell

proliferation. In Smooth cayenne (Figure 4 C), the calli had an external origin because they are nearly parenchyma, while in Sugar loaf, the origin is internal near conductive tissues (Figure 4D).

### Effects of NAA and BAP / Kinetin on somatic embryos induction and maturation

Different combination of growth regulators combinations had a highly significant influence ($P < 0.0001$) on the rate of induced and matured embryos through the cultivars **(S3 File)**. Excepted the medium supplemented with BAP (1 mg/l), Smooth cayenne produced embryos with more degree of maturation than Sugar loaf on any other. The highest number (8.8) of mature embryos 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 Sugar loaf. In sugar loaf, the highest number (4.8) of mature somatic embryos was obtained on the medium supplemented with BAP (1 mg/l) **(Figure 5)**.

### Effects of BAP and $GA_3$ on somatic embryos regeneration

The cultivars and growth regulator combination had a highly significant difference ($P < 0.0001$) on the number of plantlets formed per embryos, somatic embryos formation and number of embryos, while no influence of the interaction culture media X cultivars was noted) **(S4 File)**. Thus, the highest average number (10.36) of plantlets per embryo was obtained with Smooth Cayenne on the medium supplemented with GA3 (1mg/l) + BAP (1mg/l) and the lowest (0.58) was obtained on the same medium with the Sugar loaf **(Table 3)**. Thus, except the media supplemented with GA3 (1 mg/l) + BAP (0.5 mg/l; 1mg/l), Smooth Cayenne had more plantlets than Sugar loaf 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 (55.25) of plantlets with Smooth Cayenne, while this same medium gave a low number (5) of plantlets with Sugar loaf **(Figure 6) (S5 File)**.

**Table 3**: Effects of different concentrations of BAP and GA3 on somatic embryos regeneration.

| Cultivars | Culture Media | Nber.Emb | Nber.Plantlets | Nber.Plts /Emb. |
|---|---|---|---|---|
| Smooth Cayenne | G2.B5 | 7ab | 15a | 3.92a |
| | G2.B1 | 2.25a | 9.5a | 5.21a |
| | G2.B0.5 | 1.25a | 16.25a | 9.58ab |
| | G1.B1 | 4ab | 16a | 10.36b |
| | G1.B3 | 2.25a | 24.5ab | 7.92ab |
| | G1.B5 | 0.50a | 12.75a | 5.25a |
| | G1.B0.5 | 9ab | 22.25ab | 4.88a |
| | G2.B3 | 12.75b | 55.25b | 5.29a |
| Sugar Loaf | G2.B5 | 0.25a | 1.75a | 1.75a |
| | G2.B1 | 4.5ab | 7.75a | 2.21a |
| | G2.B0.5 | 0.25a | 2.25a | 2.25a |
| | G1.B1 | 2a | 2.75a | 0.58a |
| | G1.B3 | 0.75a | 5.25a | 3.37a |
| | G1.B5 | 6ab | 23ab | 3.36a |
| | G1.B0.5 | 8.25ab | 2.75ab | 4.80a |
| | G2.B3 | 2.75a | 14a | 5a |
| P. Value | Culture Media | < 0.0001*** | < 0.0001*** | < 0.0001*** |
| | Cultivars | 0.0003*** | < 0.0001*** | < 0.0001*** |
| | Culture Media X Cultivars | 0.212NS | 0.089NS | 0.821NS |

***Caption:*** *Nber.Emb: Number of embryos; Nber.Plantlets: Number of vitroplants; Nber.Plts /Emb: Number of plantlets per embryo; *** Highly Significant difference (0.01%); NS: not significant at 5%.*

### Histological impact of somatic embryogenesis of the regenerated plantlets

Semi-thin sections from plantlets obtained through organogenesis and somatic embryogenesis had a similar anatomy for the presence of guard cells, the unequal distribution of ostioles faces and their opening **(Figure 7)**. The underside of the epidermis had more stomata, and Sugar loaf cultivar had more stomata (149) by organogenesis technique than embryogenesis technique (141). Concerning the size of stomata and ostioles, there was a significant difference ($P < 0.0001$) between organogenesis and somatic embryogenesis. Thus, the leaves collected from plantlets produced by organogenesis presented more developed stomata with more open ostioles than those taken from plantlets regenerated by somatic embryogenesis. Moreover, cultivars had a significant influence ($P < 0.0001$) **(S6 File)** on the number, width and length of stomata; the length and width of ostioles by both somatic embryogenesis and organogenesis. Thus, in Smooth cayenne cultivar, 134 stomata were counted against 141 in Sugar loaf by somatic embryogenesis technique. The length, and width of the stomata and ostioles are respectively 26.6 µm; 19.82 µm for the stomata, 16.23µm and 5.45µm for the ostioles in Smooth cayenne versus 33.5µm; 22µm for the stomata and 19.8µm and 8.3µm for the ostioles in Sugar loaf. **(Table 4)**.

**Table 4:** Stoma frequency and dimensions of stomata and ostioles in Smooth cayenne and Sugar loaf.

| 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 | 0.816 | 0.975 | 0.967 | 0.911 | 0.787 |
| Pr > F | 0.0197* | < 0.0001*** | < 0.0001*** | < 0.0001*** | < 0.0001*** |

# Discussion

Callus induction, embryos maturation, and plantlets regeneration constitute essential steps in indirect somatic embryogenesis 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 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 dedifferentiation and callus induction. Cells were more directed to morphogenesis in tissue culture [10]. 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 et al. [18] have also shown the positive effect of copper on embryogenic 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 [19]. Also, a significant influence of cultivars was noted especially with regard to the kinetics of callogenesis with an earliness callus induction in Smooth cayenne (two weeks) compared to Sugar loaf (three weeks). Despite the delay in Sugar loaf, 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 [20]. This is explained by the easy transport of picloram compared to 2,4-D participating for rapid and large proliferation cells [21]. The media tested as well as the cultivars have a significant influence on the somatic embryos. By increasing BAP (0.5 mg/l – 1 mg/l), number of mature embryos have been increased. This growth regulator plays a determining role by acting on the pro-embryogenic cells and the proliferation of embryogenic globules on the surface of calli [2]. Also, it was found the increase of the number of mature embryos by addition of NAA (0.5 mg/l) to medium with an optimal rate of initiation and maturation of embryos (88%) on Murashige and Skoog medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l). This indicates that BAP alone is not sufficient to optimize somatic embryos induction. The results of the present study also revealed that obtaining plantlets from somatic embryos in Smooth cayenne and Sugar loaf cultivars of

pineapple required the use of auxins and cytokinins, as this has been indicated in our previous studies on cassava [22, 23]. However, the most favorable combination for Smooth cayenne was very unfavorable in Sugar loaf cultivar with a rate of 21% showing the non-significant effect between the media factors and cultivar factors in embryos maturation.

The types of medium and cultivar had significant influence ($P < 0.05$) on the germination of somatic embryos and the plantlets regeneration. Thus, there are more plantlets obtained per embryo (10.36) by adding to the medium, growth regulator combination BAP (1 mg/l) + GA3 (1 mg/l). Indeed, if a lower concentration of $GA_3$ (0.05 mg/l) with the same dose of BAP favored the conversion of embryos 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 embryos into plantlets. The influence of cultivar with the ability of somatic embryos from Smooth cayenne to produce plantlets clearly indicates the involvement of specific genetic factors not only to cultivars but also to the conversion of somatic embryos into plantlets [24, 25].

The somatic embryogenesis technique did not affect the anatomy 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. [26]. Compared to embryogenesis, the stomata in leaves of plantlets produced by organogenesis were large in both cultivars. This difference could be explained by the juvenility of the material obtained from somatic embryogenesis technique. In addition, the stay in the darkness of the material during the process of somatic embryogenesis could also impact their dimensions, unlike plantlets exposed to light radiation during organogenesis.

## Conclusion

The study highlighted some information necessary for the somatic embryogenesis of pineapple cultivars from Benin. Plantlets leaves have been shown to be the material of choice in callus induction. Also, the combination of BAP (1 mg/l) + copper (2 mg/l) + Picloram (6 mg/l or 12 mg/l) on Murashige and Skoog medium supplemented with vitamins B5 was favorable somatic embryos regeneration of pineapple. As for embryos maturation, MS medium supplemented with the growth regulators combination NAA (0.5 mg/l) + BAP (1 mg/l) was efficient for two cultivars. The combination for $GA_3$ (2 mg/l) + BAP (3 mg/l) in the same medium was favored for shoots induction from somatic embryos of pineapple with stable histological structure of leaves cells. This is a key study for mass propagation of the pineapple and genetics transformation of pineapple.

## Materials And Methods

# Plant material

The plant material used was composed to plantlets and fruits of two cultivars, Smooth Cayenne and Sugarloaf of pineapple obtained respectively from University of Abomey-Calavi in Central Laboratory of

Biotechnology and Plant breeding Gemoplasm, and the laboratory field station located at Wawata in Benin Republic.

## Methods

### 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 mn. 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 et al. [27]. 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, maturation and somatic embryos regeneration

The sterilized leaf explants from crown and those from organogenesis obtaining plantlets were excised aseptically and cut into pieces of 1 to 2 cm before being cultured on MS medium (1962) basal salts (MSBS) supplemented with 30 g/l of sucrose, vitamins B5, glycine (2.0 mg/l), glutamine (1,000 mg/l), casein hydrolysate (100 mg/l) and MgCl2 (0.75 g/l)—(Sigma, France) [28]. 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 (2 mg/l). Each cytokinin were 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±1°C with unmonitored light interruptions during daily observation with a relative humidity of 80% for four to six weeks.

Embryogenic calli obtained were removed from plates and fragmented into cell aggregate subunits. Each subunits were plated on embryos induction media having the same basic elements with MSBS but containing double concentration of KNO3 and half of NH4NO3, [28] supplemented with BAP (0.5 mg/l or 1 mg/l) and/or NAA (0.5 mg/l). The plated calli were incubated in the darkness with monthly subculture.

Somatic embryos obtained were transferred MSBS media supplemented with combined NAA (0.5 mg / l), BAP (0.5 mg/l; 1 mg / l; 3 mg/l and 5 mg/l), and $GA_3$ (1 mg/l and 2mg/l). The embryos were incubated for one week in the dark, then placed in a 10/14 h light/dark cycle at 27±1°C for plantlets regeneration.

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.

### 2-2-3- Histological analysis of somatic embryogenesis regenerated plantlets

Cross sections were made freehand on the leaves of somatic embryogenesis regenerated plantlets and organogenesis regenerated plantlets of two cultivars. The sections were transferred to sodium hypochlorite (30%) solution for 15 min and rinsed with tap water follow by soaking in green carmine solution for 5 min and rinsed again with tap water. The sections were fixed by a coverslip on a slide

maintained with a drop water. Optical microscope (MOTIC) was used for histological observations of sections in order to make a comparative study of anatomy of different type of leaves [29, 30].

### 2-2-4- Data collection and statistical analysis

Data analyzes were performed based on a completely random block design making each explant a repetition. The $Khi^2$ independence test was performed to evaluate the type of explant responding best to somatic embryogenesis and Binary logistic regression (BLR) 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 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 histological impact of somatic embryogenesis technique [31].

## List Of Abbreviations

NAA: Acid α Naphthalele Acetic; 2,4-D: 2,4- Dichlorophenoxylacetic acid; Pi : Picloram or; 4-amino 3,5,6-trichloropicolinic acid; BAP: 6-benzylaminopurine; $GA_3$: Gibberellic Acid.

## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

This manuscript does not contain any individual person’s data and further consent for publication is not needed.

### Availability of data and materials

All data generated or analyzed during this study are included in this published article (and its supplementary information files).

### Competing interests

The authors declare that they have no competing interest.

### Funding

This work was supported by “Programme Cadre d'Appui à la Diversification Agricole (ProCAD)” funded by World Bank. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

### Authors’ contributions

**GHTC** and **BAMA** conceptualized the research work and analyzed the data; **GHTC** and **SSH** designed and validated the research methodology; **GHTC** supervised the work; **BAMA** and **BBT** conducted the research and collected data; **BAMA** and **JAH** wrote the manuscript; **JAH** reviewed and edited the manuscript; **CA** acquired the fund, administrated the project and provided the resources; all authors read, corrected and approved the manuscript.

### Acknowledgments

Their deep gratitude and appreciation go to the staff of Central Laboratory of Biotechnology and Plant Breeding, and Ecological Genetics laboratory of the Department of Genetics and Biotechnology.

## References

1. References.
2. -Sukruansuwan V, Napathorn SC. Use of agro-industrial residue from the canned pineapple industry for polyhydroxybutyrate production by Cupriavidus necator strain A-04. Biotechnol biofuels. 2018;11(1):202.
3. -Yapo E, Kouakou T, Kone M, Kouadio J, Kouame P, Merillon JM. Regeneration of pineapple (*Ananas comosus* L.) plant through somatic embryogenesis. J P B B. 2011;20(2):196–204.
4. -Luan A, Chen C, Xie T, He J, He Y. Methylation Analysis of CpG Islands in Pineapple SERK1 Promoter. Genes. 2020;11:425. doi:10.3390/genes11040425.
5. -Badou BT, Agbidinoukoun A, Nondichao L, Dossoukpèvi R, Cacaï GTH, Houédjissin SS, Ahanhanzo C. 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). *J. Biotechnol. Res*. 2017, 3(10), 94–105.
6. -Acheampong S, Galyuon IK, Asare AT. Effects of sterilization protocols, benzylaminopurine and type of explants on growth initiation of pineapple [Ananas comosus (L.) Merr.] Cultures. J Basic Appl Sci. 2015;1(3):50–65.
7. -Badou BT, Agbidinoukoun A, Cacaï GT, Dossoukpèvi RC, Ahanhanzo C. 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). 2018; 2 (9), 1–15.
8. -Piatti MF. Embryogenèse somatique et synchronisation du développement embryonnaire. Thèse de doctorat d'état en pharmacie. Paris VI, 1988, 130.

9. -Gaj MD. Direct somatic embryogenesis as a rapid and efficient system for in vitro regeneration of Arabidopsis thaliana. Plant Cell Tiss Org Cult. 2001;64:39–46.
10. -Daquinta M, Cisneros A, Rodriguez Y, Escalona M, Perez M, Luna I, Borroto C. Somatic embryogenesis in pineapple (Ananas comosus L.) Merr.). Acta Hort. 1997;425:251–7.
11. -Firoozabady E, Moy Y. Regeneration of pineapple plants via somatic embryogenesis and organogenesis. In Vitro Cell Dev Biol Plant. 2004;40:67–74. doi:10.1079/IVP2003494.
12. -Rahman KW, Ahmed MB, Rahman MM, Amin MN, Hossain MS, Ahmed R. Large scale plant regeneration in vitro from leaf derived callus cultures of pineapple [Ananas comosus (L.) Merr. cv. Giant Kew]. Int J Bot. 2005;1(2):128–32.
13. -Firoozabady E, Heckert M, Gutterson N. Transformation and regeneration of pineapple. Plant Cell Tiss Organ Cult. 2006;84:1–16. doi:10.1007/s11240-005-1371-y.
14. -Gangopadhyay G, Roy SK, Basu GS, Mukherjee KK. Agrobacterium-mediated genetic transformation of pineapple var. Queen using a novel encapsulation-based antibiotic selection technique. Plant Cell Tiss Organ Cult. 2009;97:295–302.
15. -Hegde M, Kulasekaran M, Jayasankar S, Shamnungavelu KG. In vitro embryogenesis in cashew (*Anacardium occidentale L.)*. Ind Cashew J. 1994;21(4):17–25.
16. -Gogate S, Nadgauda R. Direct induction of somatic embryogenesis from immature zygotic embryo of cashewnut (Anacardium occidentale L.) Scientia horticulturae. 2003, 97(1): 75–82.
17. -Jimenez VM. Involvement of plant hormone and plant growth regulators on *in vitro* somatic embryogenesis. Plant Growth Regul. 2005;47:91–110.
18. -Jimenez VM, Thomas C. Participation of plant hormones in determination and progression of somatic embryogenesis. In: Mujib A, Samaj J, editors Plant Cell Monograph (Somatic embryogenesis), 2006, Vol. 2, Springer-Verlag, Berlin Heidelberg, pp. 103–118.
19. -Nirwan RS, Kothari SL. High copper levels improve callus induction and plant regeneration in Sorghum bicolor (L.) Moench. In Vitro Cell Dev Biol Plant. 2003;39:161–4.
20. -Sahrawat AK, Chand S. Stimulatory effect of copper on plant regeneration in indica rice (Oryza sativa L.). J plant physiol. 1999;154(4):517–22.
21. -Roostika I, Mariska I, Khumaida N, Wattimena GA. Indirect organogenesis and somatic embryogenesis of pineapple induced by dichlorophenoxy acetic acid. J Agrobiogen. 2012a;8(1):8–18.
22. -Roostika I, Mariska I, Khumaida N, Wattimena GA. The effect of picloram and light on somatic embryogenesis regeneration of pineapple. IJAS. 2012b;13(2):43–53.
23. -Cacaï GHT, Ahanhanzo C, Adjanohoun A, Houédjissin S, Azokpota P, Agbangla C. Effets de l'acclimatation sur le comportement en milieu réel de quelques variétés améliorées de manioc (*Manihot esculenta*, Crantz) cultivées. Afr J Biotechnol. 2013;12(13):1475–81.
24. -Cacaï GHT, Ahanhanzo C, Dangou J, Houedjissin S, Agbangla C. Effets de différentes combinaisons hormonales sur l'organogenèse in vitro de quelques cultivars locaux et variétés améliorées de

Manihot esculenta Crantz (manioc-Euphorbiaceae) cultivées au Bénin. International Journal of Biological Chemical Sciences. 2012;6(4):1593–607.

25. -Alexandro KS, Conger BV. Isolation of two somatic embryogenesis related genes from Dactylis glomerata. Plant Sci. 2002;162:301–7.
26. -Ansaldi C. Recherche de descripteurs des phases précoces de l'embryogenèse somatique chez le café, Univ. Toulouse Paul Sabatier, Mém. Toulouse: DESS; 2002. 26 p.
27. -Shackel KA, Novello V, Sutter EG. Stomatal Function and Cuticular Conductance in Whole Tissue-cultured Apple Shoots. J Amer SocHort SCI. 1990;115(3):468–72.
28. -Bukhori MM, Khalid N. Potential Embryogenic Callus Induction Protocol through Cell Suspension Culture for High Frequency Plant Regeneration of Maspine Pineapple (Ananas comosus L.).BJRST, 2013, 3(2), 40–45.
29. -Kouadio O, Yapo E, Kouassi K, Silue O, Koffi E, Kouakou T. Improved callogenesis and somatic embryogenesis using amino acids and plant growth regulators combination in pineapple [Ananas comosus (L.) merr. (Bromeliaceae)]. Eur J Biotechnol Biosci. 2017;5(5):06–16.
30. -Tomlinson PB, Zimmermann MH. Vascular anatomy of monocotyledons with secondary growth-An introduction. J Arnold Arbor. 1969;50:159–79.
31. -Lindorf H. Historia de la anatomía vegetal en la facultad de ciencias de la universidad central de venezuela. Acta Botánica Venezuelica. 2005;28(2):409–50.
32. -Mize WC, Kenneth JK, Compton CE. Considérations statistiques pour la recherche in vitro: II - données pour la présentation. Cellule in vitro. Dev Biol Plante. 1999;35:122–6.

# Figures

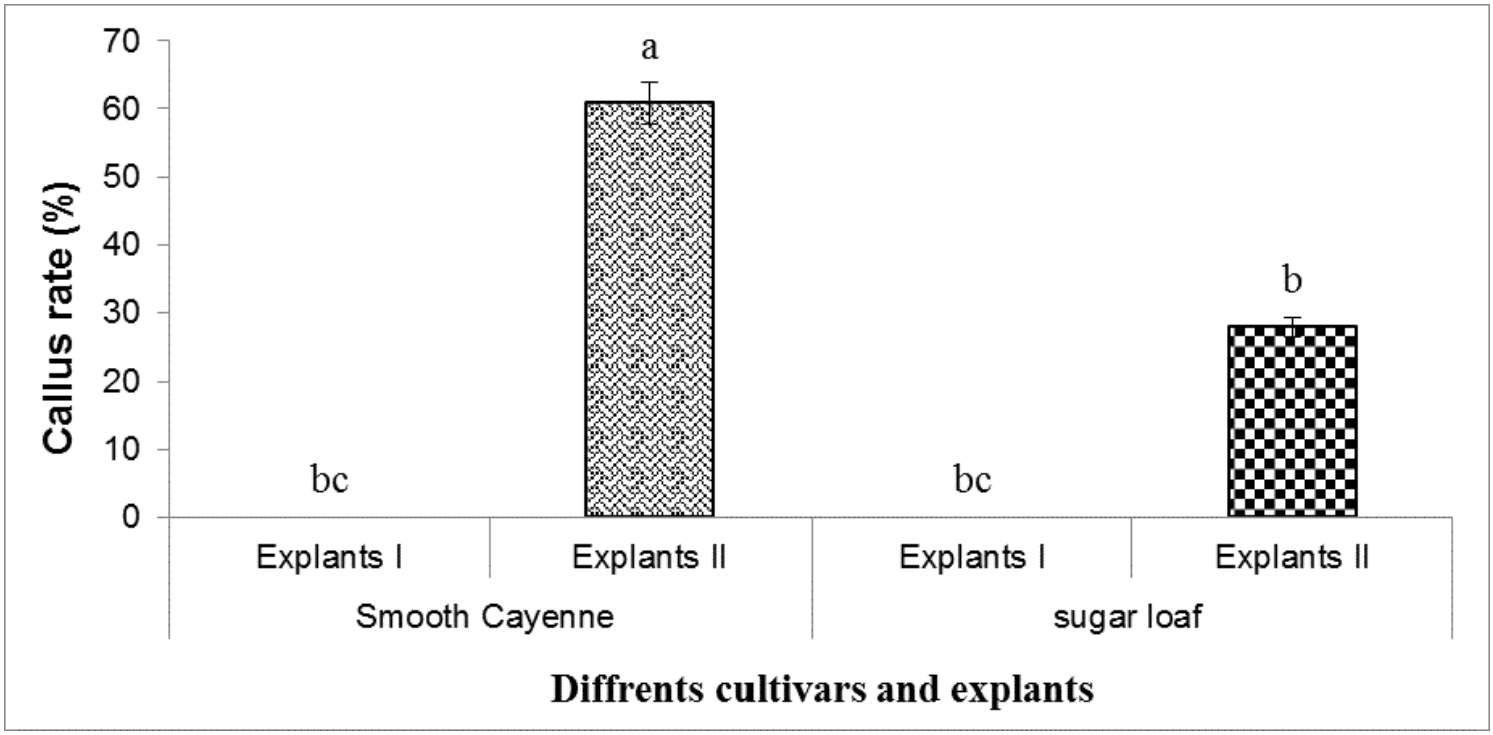


**Figure 1**

Effect of explants origin on calli formation in pineapple.

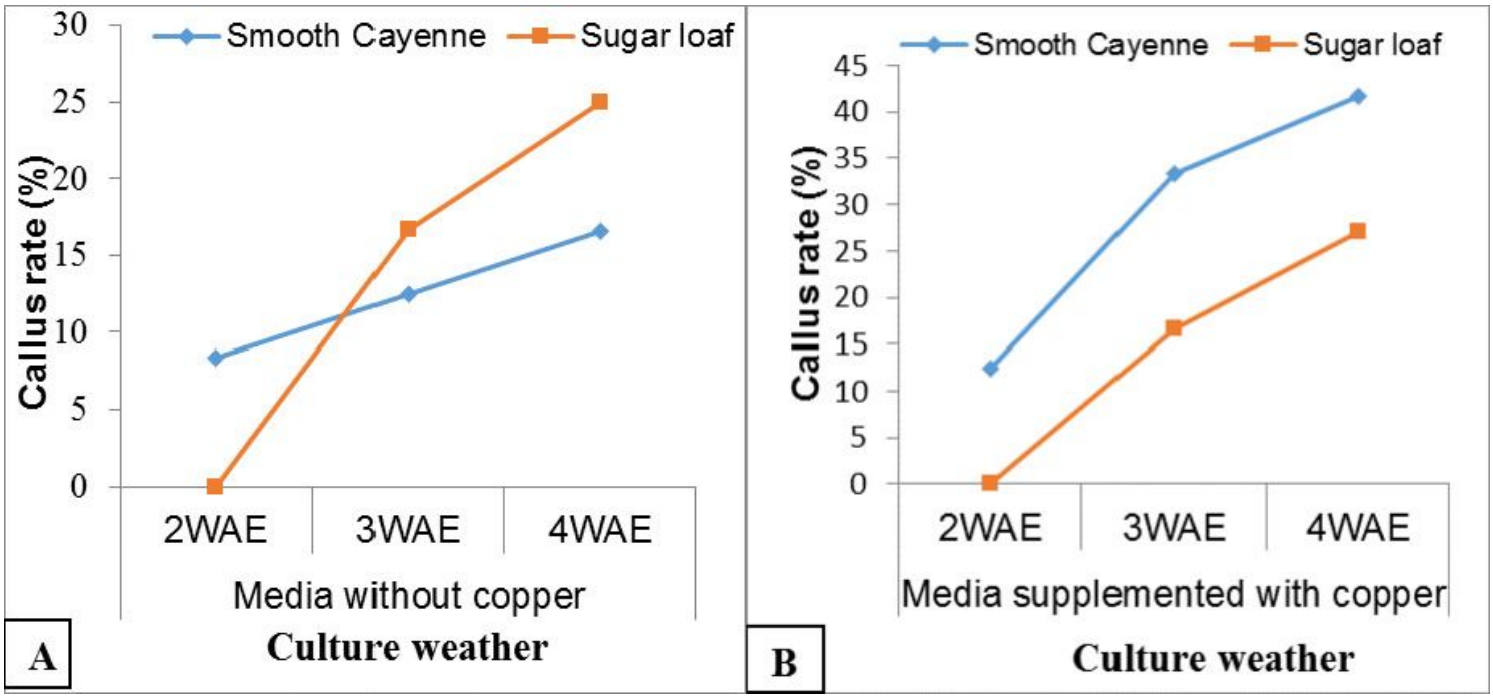



**Figure 2**

Effect of copper on callus formation (A) Media without copper; (B) Media supplemented with copper; (WAE) Week after seeding.

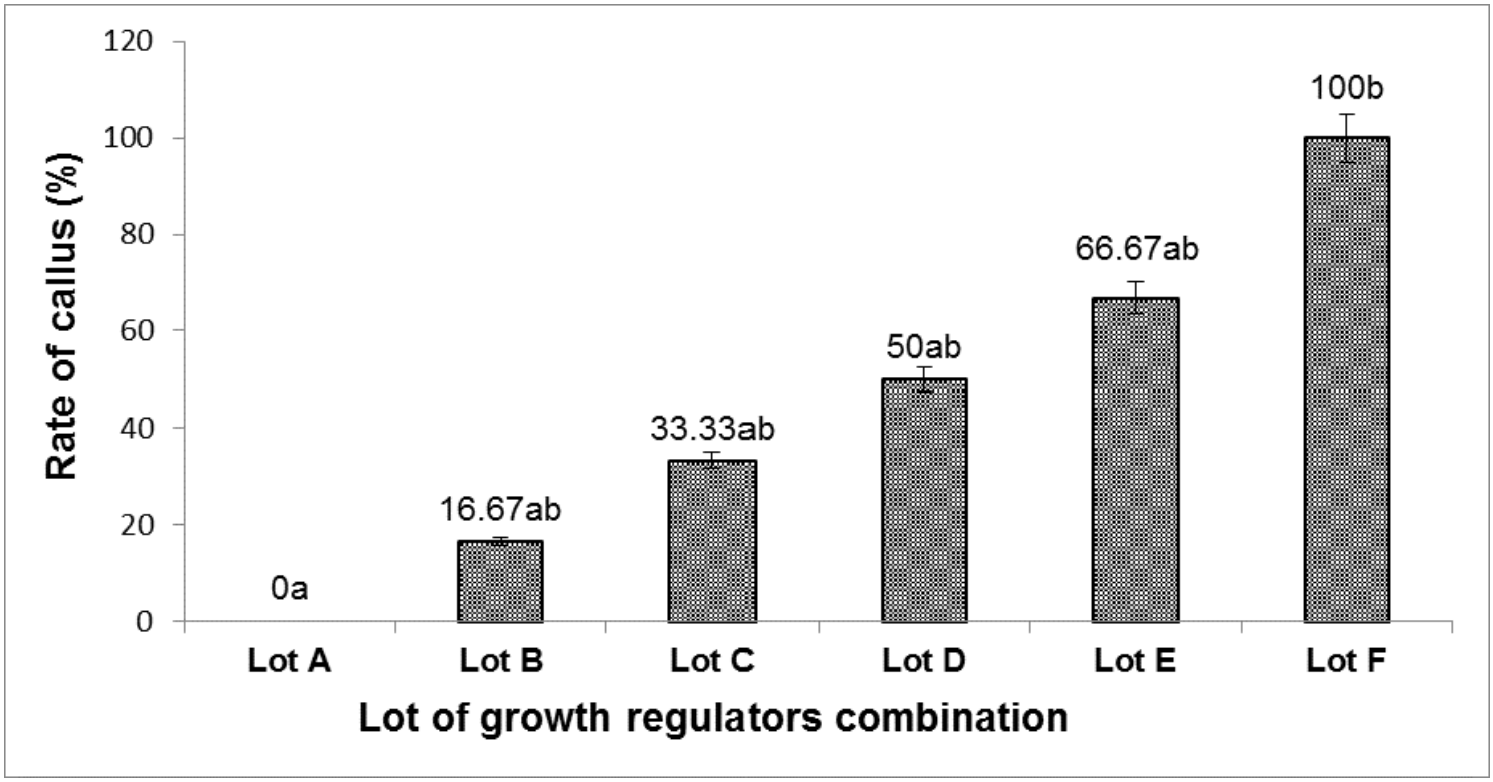
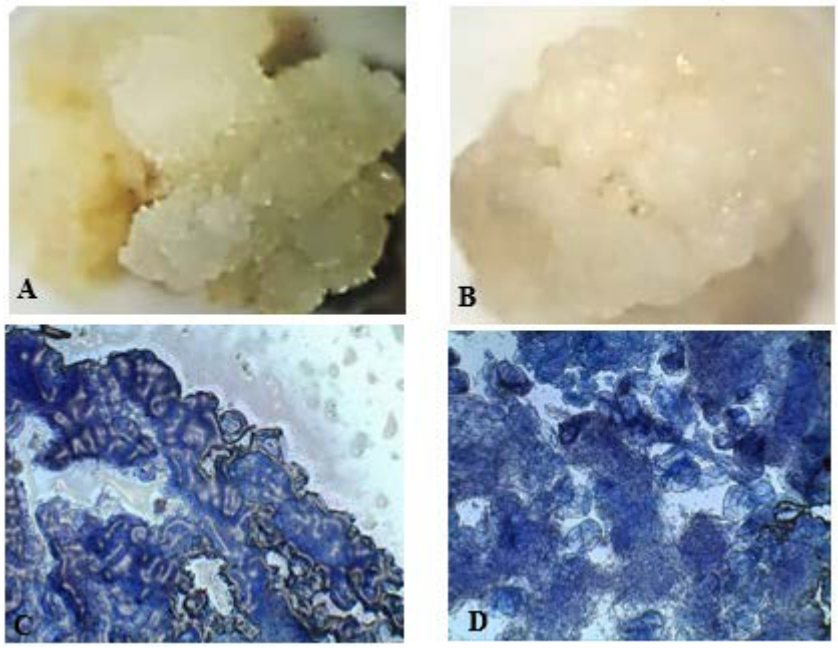



**Figure 3**

Effect of different growth regulators alone or supplemented with copper on callus formation. Caption: Lot A (K0.5D12; K0.5D3 + Cop.; B0.5P6; K1D6; B1D6; B1P3; B0.5P12; B0.5D9); Lot B (B1D3 + Cop; K1P12 + Cop; K1P3; B0.5P6 + Cop; B0.5P9; K0.5D12 + Cop; K1P9; B0.5D9 + Cu; K1P12; K1P6 + Cop; B1P12; K0.5P3); Lot C (B1P6; K0.5P12; K0.5P12 + Cop.; K1P6; B0.5P12 + Cop.; B0.5P3; B0.5P3 + Cop; K0.5P9; B0.5D6); Lot D (B0.5D3; B0.5D3 + Cop.; B0.5D6 + Cop.; B1D6 + Cop.; B1P3 + Cop.; B1P9 + Cop.; K0.5D3; K0.5P3 + Cop.; K0.5P6; K0.5P9 +Cop.; K1D6 + Cop.; K1P3 + Cop.; K1P9 + Cop.); Lot E (B0.5P9 + Cop.; B1D3; B1P9; K0.5P6 + Cop.); Lot F (B1P6 + Cop.; B1P12 + Cop.)




## Figure 4

Characteristics of induced calli on MS supplemented with BAP + Pi + Cu medium after two months. Caption: A: Texture of Smooth Cayenne calli; B: Texture of Sugar loaf calli; C: Cell structure of Smooth Cayenne calli; D: Cell structure of Sugar loaf calli.

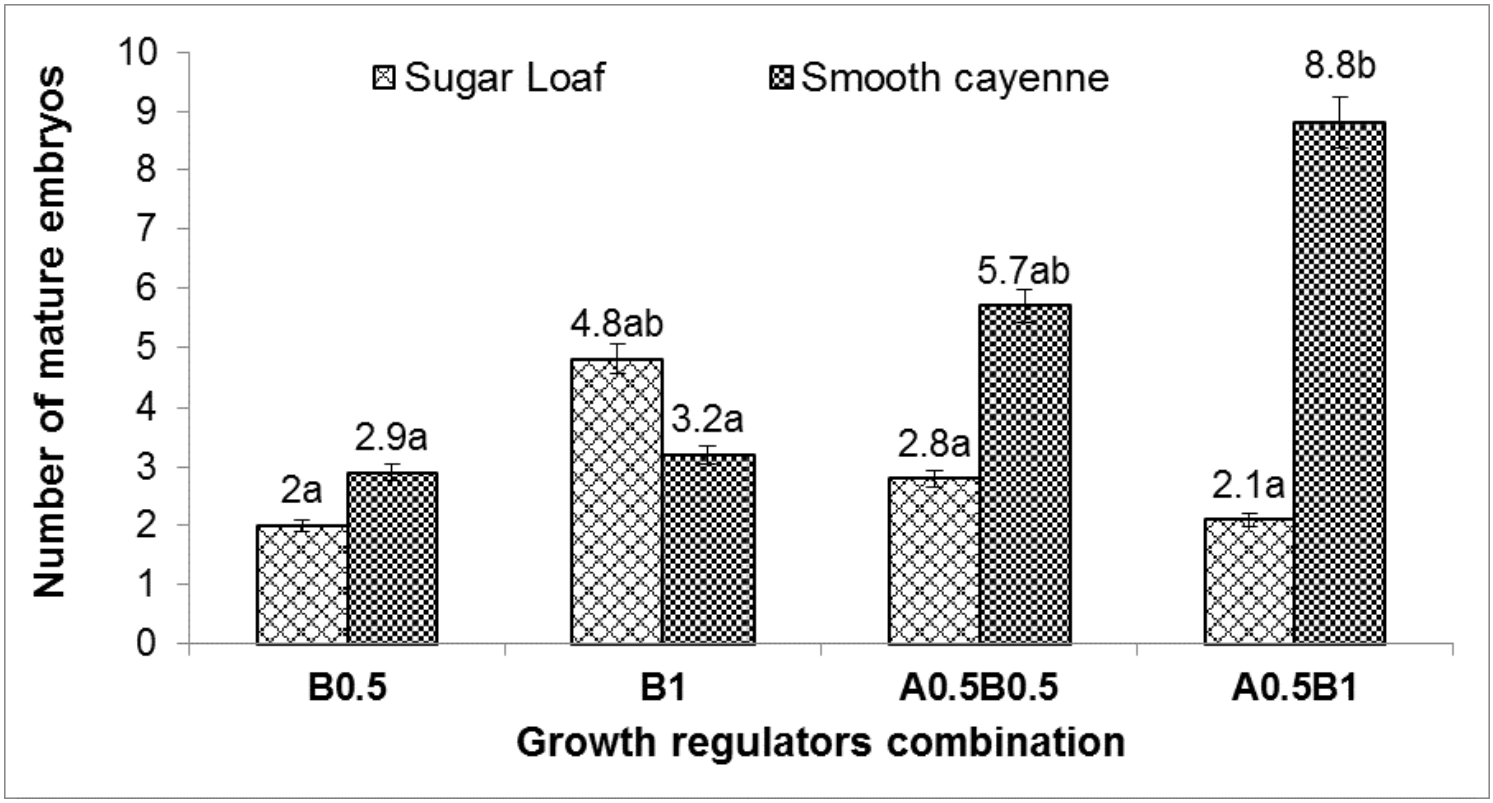
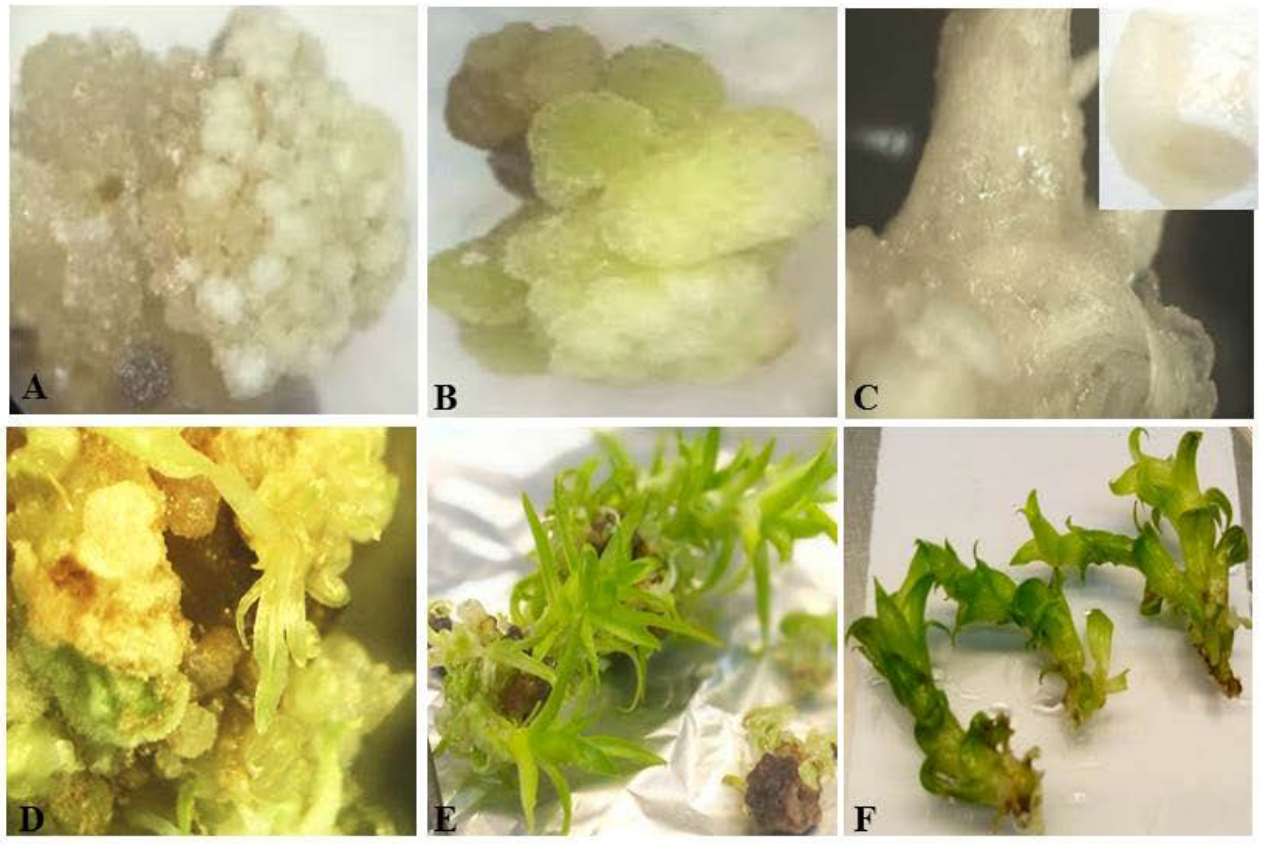



**Figure 5**

Effect of growth regulators combination on somatic embryos maturation. Caption: B0.5: 0.5mg/l (BAP); B1: 1mg /l (BAP); A0.5B0.5: 0.5mg/l (ANA) 0.5mg/l (BAP); A0.5B1: 0.5mg/l (ANA) 1mg/l (BAP).




## Figure 6

Shoots regeneration through somatic embryogenesis of Pineapple. Caption: A and B: Pro-embryos formed from the callus; C: Somatic embryos; D: Beginning of plant regeneration from Somatic embryos; E: Regenerated plantlets of Smooth cayenne cultivar; F: Regenerated plantlets the Sugar loaf cultivar.

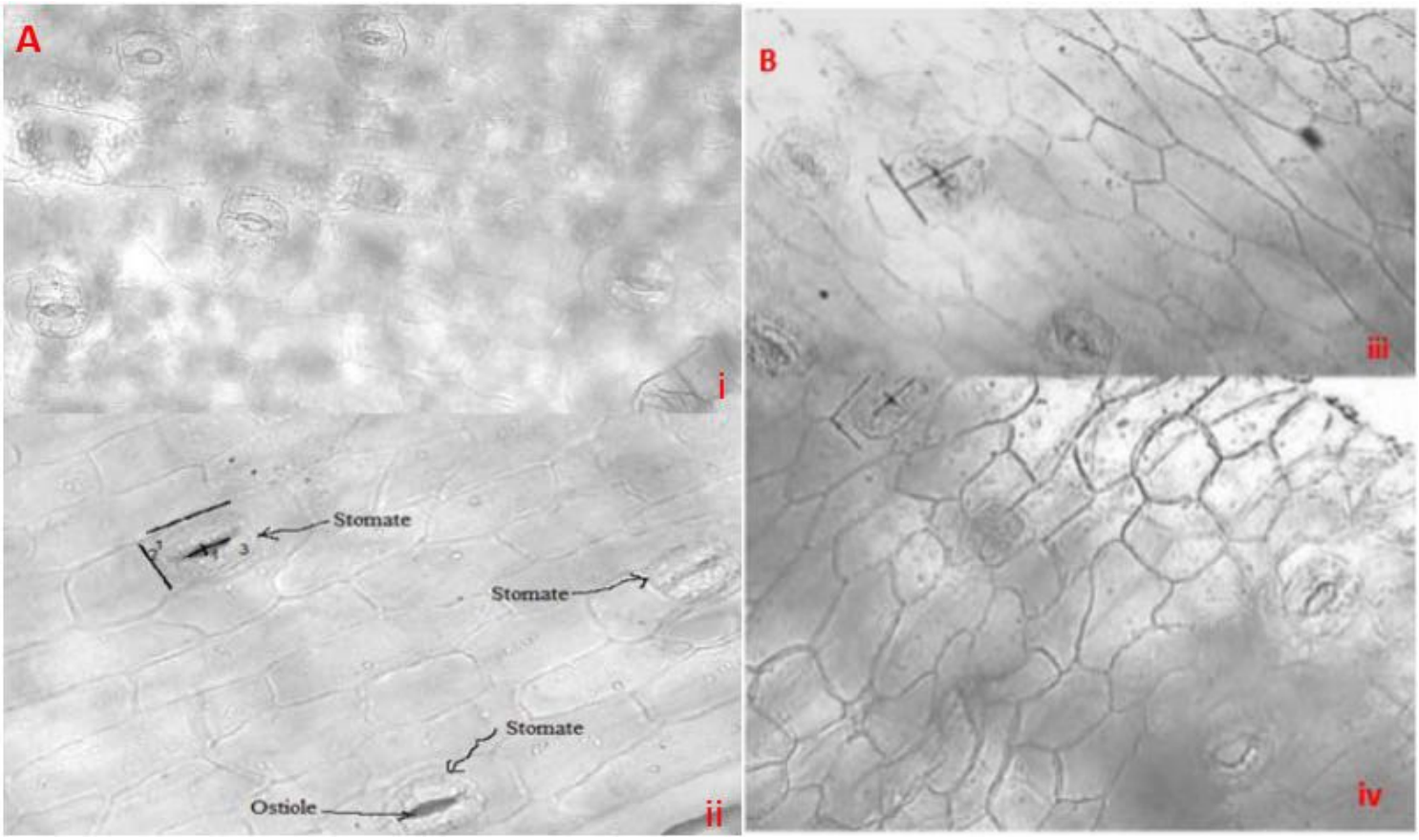



**Figure 7**

Microscopic structure of pineapple leaves. Caption: A: Microscopic structure of Smooth cayenne leaves (i: Smooth cayenne leaf from plantlet obtained by organogenesis; ii: Smooth cayenne leaf from plantlet obtained by somatic embryogenesis). B: Microscopic structure Sugar loaf leaves (iii: Sugar loaf leaf from plantlet obtained by organogenesis; iv: Sugar loaf leaf from plantlet obtained by somatic embryogenesis).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- S1File.xlsx
- S1RawImage.pdf
- S1RawData.xlsx
- S2File.xlsx
- S3File.xlsx
- S4File.xlsx
- S5File.pdf
- S6File.xlsx