Plant Regeneration Through Somatic Embryogenesis in Two Cultivars of Pineapple (Ananas Comosus L.)

Gilles Habib Todjro CACAÏ (caghat@yahoo.fr)
Universite d'Abomey-Calavi,
Brunith A.M. Ahokpossi
University of Abomey-Calavi
Serge S. Houédjissin
University of Abomey-Calavi
Jerome Anani Houngue
University of Abomey-Calavi
Bienvenu T. Badou
University of Abmey-Calavi
Corneille Ahanhanzo
University of Abmey-Calavi

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Abstract

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. 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 plant growth regulators combinations. BAP and / or 2,4-D have been added to base medium for calli maturation. BAP and GA3 have been added for plant elongation. The results indicated a significant influence of type of explant and copper on callus induction in pineapple cultivars. Likewise, the medium MS with NAA (0.5 mg/l) + BAP (1mg/l) has a highly significant influence with 8.8 mature somatic embryos per explant. Also, the growth regulator combinations and the cultivars have significantly influenced somatic embryos regeneration with a high rate of 55.25% plantlets using the hormonal combination BAP (3 mg/l) + GA_3 (2 mg/l) for the smooth Cayenne. Leaves from 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.

Introduction

Pineapple (*Ananas comosus*) is one of fruit crops widely produced in the tropics and with global production around 24.8 million tons in 2017 (Sukruansuwan and Napathorn, 2018). It is the third world tropical fruit crop after banana and citrus (Hemalatha and Anbuselvi, 2013). It is a self-sterile plant, and the propagation methods widely used is from suckers (Yapo et al., 2011). 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 (Luan et al., 2000; Badou et al., 2017). Therefore, tissue culture have become an alternative methods for mass production of healthy pineapple planting material with homogeneity of morphotypes (Danso et al., 2008; Zuraida et al., 2011). However, the micropropagation by organogenesis, mostly used for mass production, (Acheampong et al., 2015; Badou et al., 2018) but didn't offer a number of applications as alternative pathways for mass multiplication within a short time, germoplasm conservation and genetic (Wu et al., 2007; Gonzalez-Arnao et al., 2008).

In this regard, somatic embryogenesis constitutes a palliative for *in vitro* multiplication (Piatti, 1988), and constitute the most rapid *in vitro* multiplication method for many types of plants and trees that also request the industrialization (Gaj, 2001; Luan et al., 2020). It is reported that developmental pathways of somatic embryogenesis and plant regeneration greatly depends on different factors, such as: genotype, explant type, and age of explant (Gogate and Nadgauda, 2003; Hegde et al., 1994). Moreover, the external environment which includes composition of media and physical culture conditions (light, temperature)

are not fully clear (Jimenez, 2005; Jimenez and Thomas, 2006). Interaction between all these factors leads to the induction and expression of a specific mode of cell differentiation and development. In pineapple, explants origin, its age and plant genotypes highly determine the quality of callus induced (Stefenon et al., 2020). The determining of adequate explants and its age for embryogenic callus production in the local varieties constitute an advantage for pineapple transformation. Furthermore, plant growth regulators such as auxins (2,4.D and Pi) / cytokinins (BAP and Kinetin) action in the different step on somatic embryogenesis in pineapple need a deepen knowledge for their utilization and their effects on callus induction, callus colors, and more on somatic embryos induction, their maturation and embryos regeneration. Copper and picloram constitute also an excessive additive used in somatic embryogenesis of pineapple (Méndez-Hernández et al., 2019), but their action is highly depended by the plant growth regulators used alone or in combination. The microscopic structure of leave stomata in the regenerated plant through somatic embryogenesis 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 somatic embryos induction, maturation, and regeneration. Here, we have studied somatic embryogenic response in two cultivars of pineapple (Sugar loaf and Smooth cayenne) and the information provided are efficient for reproductive protocol.

Materials And Methods

Plant material

The plant material was composed of 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., (2013). 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) (Kouadio et al., 2017). 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, (Kouadio et al., 2017) 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 2 mg/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.

Cells observation in somatic embryogenesis and organogenesis regenerated plantlets

Leaf diaphanization 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 covered with a glass by a coverslip on a slide maintained with a drop water. Optical microscope (MOTIC) was used for cells observations of sections in order to make epidermis cells difference of different type of leaves (Tomlinson and Zimmermann, 1969; Lindorf, 2005).

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 impact of somatic embryogenesis technique on the regenerated plantlets (Mize et al., 2005).

Results

Effects of explants origin and genotypes on embryogenic 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 Sugar loaf (27.78%) **(Figure 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)**.

Rate of callus induced

The response of explants on the media different in their growth regulators combination and copper concentrations have been grouped in six lots according to callus induced rate (Table 2). The medium MS without growth regulators and copper was used as control in the experiment and classified in the lot A according to the callus induced rate (Table 2).

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 (1mg/l) + Copper (2mg/l) + Picloram (6 mg/l or 12 mg/l) within the cultivars **(Lot F)**.

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 plant growth regulators combination **(Table 3)**. However, the average of fresh calli weight varied according to the plant growth regulators combinations and the cultivars **(S1 File)**. The highest weight of callus (604.7 mg) whatever the hormonal combination was obtained in the absence of copper in Sugar loaf cultivar and the lowest weight of callus (345.1 mg) in the presence of copper with Smooth Cayenne.

Effects of NAA and BAP / Kinetin on somatic embryos development

Different combination of plant growth regulators combinations had a highly significant influence (P <0.0001) on the rate of formed and matured embryos in the different cultivars **(S2 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 3)**. The developmental stages of somatic embryos included the embryos induction, embryos formation, and embryos maturation are described in the figure 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 embryos while no influence of the interaction culture media X cultivars was noted) **(S3 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 4)**. 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 **(S4 File)**. The plantlets regeneration process from mature embryos is described in figure 5.

Histological impact of somatic embryogenesis of the regenerated plantlets

The microscopic analysis of leaf section from plantlets obtained through organogenesis and somatic embryogenesis 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 organogenesis plantlets and somatic embryogenesis plantlets **(Figure 6)**. Abaxial epidermis had more stomata. In Sugar loaf, the stomata obtained from organogenesis plantlets (149) is higher than those of somatic embryogenesis 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 significant influence (P < 0.0001) **(S5 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 per leaf area analyzed 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 5)**.

Discussion

Callus induction, embryos maturation, and plantlets regeneration constitute essential steps in indirect somatic embryogenesis regeneration technique developed in *Ananas comosusvar. 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 dedifferentiation and callus induction. Cells were more directed to morphogenesis in tissue culture (Firoozabady and Moy, 2004). 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 (2003) 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 (Sahrawat and Chand, 1999). 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 (Roostika et al., 2012a). This is explained by the easy transport of picloram compared to 2,4-D participating for rapid and large proliferation cells (Roostika et al., 2012b). 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 (Yapo et al., 2011). 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 (Cacaï et al., 2012, 2013). 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 GA3 (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 (Alexandro and Conger, 2002; Ansaldi, 2002).

The somatic embryogenesis 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., (1990). 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.

Conclusions

The study highlighted some information necessary for the somatic embryogenesis of pineapple. 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.

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 declarations

Conflict 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 PCTOC.

Availability of data and materials

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

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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.

References

1. Acheampong S, Galyuon IK, Asare AT. (2015). Effects of sterilization protocols, benzylaminopurine and type of explants on growth initiation of pineapple [Ananas comosus (L.) Merr.] Cultures. *J Basic Appl Sci*, 1(3), 50-65
2. Alexandro KS and Conger BV. (2002). Isolation of two somatic embryogenesis related genes from Dactylis glomerata. *Plant Sci.*, 162: 301-307.
3. Ansaldi C. (2002). Recherche de descripteurs des phases précoces de l'embryogenèse somatique chez le café, Univ. Toulouse Paul Sabatier, Mém. DESS, Toulouse, France, 26 p.
4. Badou BT, Agbidinoukoun A, Cacaï GT, Dossoukpèvi RC, Ahanhanzo C. (2018) 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). 2 (9), 1-15.
5. Badou BT, Agbidinoukoun A, Nondichao L, Dossoukpèvi R, Cacaï GTH, Houédjissin SS, Ahanhanzo C (**2017)**. 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*. 3(10), 94-105.
6. Bukhori MM and Khalid N. (**2013**). Potential Embryogenic Callus Induction Protocol through Cell Suspension Culture for High Frequency Plant Regeneration of Maspine Pineapple (*Ananas comosus*

L.).BJRST, 3(2), 40-45.

7. Cacaï GHT, Ahanhanzo C, Adjanohoun A, Houédjissin S, Azokpota P, Agbangla C. (2013). Effets de l'acclimatation sur le comportement en milieu réel de quelques variétés améliorées de manioc (*Manihot esculenta*, Crantz) cultivées. *Afr. J. Biotechnol.* 12(13), 1475-1481.
8. Cacaï GHT, Ahanhanzo C, Dangou J, Houedjissin S, Agbangla C. (2012) 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 and Chemical Sciences 6(4): 1593-1607.
9. Danso KE, Ayeh KO, Oduro V, Amiteye S, Amoatey HM. (2008) Effect of 6-Benzylaminopurine and α-Naphthalene Acetic Acid on In vitro Production of MD2 Pineapple Planting Materials. World Applied Sciences Journal, 3(4): 614-619.
10. Daquinta M, Cisneros A, Rodriguez Y, Escalona M, Perez M, Luna I, Borroto C. **1997).** Somatic embryogenesis in pineapple (Ananas comosus L.) Merr.). Acta Hort., 425:251–257.
11. Firoozabady E and Moy Y. (**2004**). Regeneration of pineapple plants via somatic embryogenesis and organogenesis. In Vitro Cell Dev Biol Plant, 40:67–74
12. Firoozabady E, Heckert M, and Gutterson N. (2006). Transformation and regeneration of pineapple. Plant Cell Tiss Organ Cult. 84:1–16. doi:10.1007/s11240-005-1371-y
13. Gaj MD, (**2001).** Direct somatic embryogenesis as a rapid and efficient system for in vitro regeneration of Arabidopsis thaliana. *Plant Cell Tiss. Org. Cult.* 64: 39–46.
14. Gangopadhyay G, Roy SK, Basu GS, Mukherjee KK. (2009). Agrobacterium-mediated genetic transformation of pineapple var. Queen using a novel encapsulation-based antibiotic selection technique. Plant Cell Tiss Organ Cult 97:295–302
15. Gogate S, Nadgauda R. (**2003).** Direct induction of somatic embryogenesis from immature zygotic embryo of cashewnut (Anacardium occidentale L.) Scientia horticulturae. 97(1): 75-82.
16. Gonzalez-Arnao MT, Panta A, Roca WM, Escobar RH, Engelmann F (2008) Development and large-scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. Plant Cell Tiss Organ Cult 92:1–13
17. Hegde M, Kulasekaran M, Jayasankar S, and Shamnungavelu KG. (1994). In vitro embryogenesis in cashew (*Anacardium occidentale L.)*. Ind. Cashew J, 1994, 21(4), 17-25.
18. Hemalatha R and S Anbuselvi (2013). Physicochemical constituents of pineapple pulp and waste. J. Chem. Pharmaceutical Res. 5(2):240-242.
19. Jimenez VM, Thomas C. (2006). Participation of plant hormones in determination and progression of somatic embryogenesis. In: Mujib, A., Samaj, J. (Eds) Plant Cell Monograph (Somatic embryogenesis), Vol. 2, Springer-Verlag, Berlin Heidelberg, pp. 103–118.
20. Jimenez VM. (2005). Involvement of plant hormone and plant growth regulators on *in vitro* somatic embryogenesis. Plant Growth Regulation, 47: 91-110.

21. Kouadio O, Yapo E, Kouassi K, Silue O, Koffi, E, Kouakou T. (**2017**). Improved callogenesis and somatic embryogenesis using amino acids and plant growth regulators combination in pineapple [Ananas comosus (L.) merr. (Bromeliaceae)]. *Eur. J. Biotechnol. Biosci.* 5 (5), 06-16.
22. Lindorf H. Historia de la anatomía vegetal en la facultad de ciencias de la universidad central de venezuela. Acta Botánica Venezuelica, 28(2), 409-450.
23. Luan A, Chen C, Xie T, He J, He Y. (**2020).** Methylation Analysis of CpG Islands in Pineapple SERK1 Promoter. Genes, 11, 425; doi:10.3390/genes11040425
24. Méndez-Hernández HA, Ledezma-Rodríguez M, Avilez-Montalvo RN, Juárez-Gómez YL, Skeete A, Avilez-Montalvo J, De-la-Peña C and Loyola-Vargas VM (2019) Signaling Overview of Plant Somatic Embryogenesis. Front. Plant Sci. 10:77. doi:10.3389/fpls.2019.00077
25. Mize WC, Kenneth JK, Compton CE (1999). Considérations statistiques pour la recherche in vitro: II - données pour la présentation. Cellule in vitro. Dev. Biol. Plante 35: 122–126
26. Nirwan RS and Kothari SL (2003). High copper levels improve callus induction and plant regeneration in Sorghum bicolor (L.) Moench. In Vitro Cell. Dev. Biol. Plant 39: 161–164.
27. Piatti,MF (**1988)**. Embryogenèse somatique et synchronisation du développement embryonnaire. Thèse de doctorat d'état en pharmacie. Paris VI, 130.
28. Rahman KW, Ahmed MB, Rahman MM, Amin MN, Hossain MS, Ahmed R (2005). Large scale plant regeneration in vitro from leaf derived callus cultures of pineapple [Ananas comosus (L.) Merr. cv. Giant Kew]. Int J Bot. 1(2):128–132
29. Roostika I, Mariska I, Khumaida N, Wattimena GA. (2012a). Indirect organogenesis and somatic embryogenesis of pineapple induced by dichlorophenoxy acetic acid. J Agrobiogen 8(1): 8-18.
30. Roostika I, Mariska I, Khumaida N, Wattimena GA. (2012b). The effect of picloram and light on somatic embryogenesis regeneration of pineapple. IJAS 13(2): 43-53.
31. Sahrawat AK and Chand S. (1999). Stimulatory effect of copper on plant regeneration in indica rice (Oryza sativa L.). *J. plant physiol*, 154(4), 517-522.
32. Shackel KA, Novello V, and Sutter EG. (**1990)**. Stomatal Function and Cuticular Conductance in Whole Tissue-cultured Apple Shoots. J. Amer. Soc.Hort. SCI. 115(3):468-472.
33. Stefenon VM, Pinheiro MVM, de Freitas FR, da Silva VJB, Vieira PB, dos Santos DD, Guerra MP (*2020*). *In vitro* callogenesis for the induction of somatic embryogenesis and antioxidant production in *Eugenia uniflora. Biotecnología Vegetal (20) 2: 135 – 146.*
34. Stefenon VM, Pinheiro MVM, de Freitas FR, da Silva VJB, Vieira PB, dos Santos DD, Guerra MP (*2020*). *In vitro* callogenesis for the induction of somatic embryogenesis and antioxidant production in *Eugenia uniflora. Biotecnología Vegetal (20) 2: 135 – 146.*
35. Sukruansuwan V and Napathorn SC (2018). Use of agro-industrial residue from the canned pineapple industry for polyhydroxybutyrate production by Cupriavidus necator strain A-04. *Biotechnol biofuels*, 11(1), 202

36. Tomlinson PB and Zimmermann MH. (**1969).**Vascular anatomy of monocotyledons with secondary growth-An introduction. J. Arnold Arbor. 50: 159-179.
37. Wu YJ, Huang XL, Chen QZ, Li XJ, Engelmann F (2007). Induction and cryopreservation of embryogenic cultures from nucelli and immature cotyledon cuts of mango (Mangifera indica L. var Zihua). Plant Cell Rep 26:161–168
38. Yapo E, Kouakou T, Kone M, Kouadio J, Kouame P, Merillon JM. (2011). Regeneration of pineapple (*Ananas comosus* L.) plant through somatic embryogenesis. J P B B. 20(2): 196-204.
39. Zuraida A R, Nurul Shahnadz A H, Harteeni A, Roowi S, Che Radziah C M Z, Sreeramanan S. (2011). A novel approach for rapid micropropagation of maspine pineapple (Ananas comosus L.) shoots using liquid shake culture system. African Journal of Biotechnology, 10(19): 3859-3866.

Tables

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	12.6726	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.*

WAS = Week after sowing.

Table 2: Effect of plant growth regulators combination and copper on calli induction rate

Lots	PGRs	Calli induction rate	Groupes
	K0.5D12	0	A
Lot 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
	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.L-1], Dx = 2.4-D [mg.L-1], Cop = copper, Px = Picloram [mg.L-1], Bx = Benzylaminopurine [mg.L-1], M0 = Medium without plant growth regulators.

Table 3: Effect of plant growth regulators combination and copper 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

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

Table 4: Effects of different concentrations of BAP and GA3 on plant regeneration.

Cultivars	GA3+BAP (mg/l)	Nber.Emb/ Treatment	Nber.Plantlets /Treatment	Nber.Plts / Emb. /Treatment
Smooth Cayenne	2 mg/l+5 mg/l	7ab	15a	3.92a
	2 mg/l+1 mg/l	2.25a	9.5a	5.21a
	2 mg/l+0.5 mg/l	1.25a	16.25a	9.58ab
	1 mg/l+ 1 mg/l	4ab	16a	10.36b
	1 mg/l+3 mg/l	2.25a	24.5ab	7.92ab
	1 mg/l+5 mg/l	0.50a	12.75a	5.25a
	1 mg/l+0.5 mg/l	9ab	22.25ab	4.88a
	2 mg/l+3 mg/l	12.75b	55.25b	5.29a
Sugar Loaf	2 mg/l+5 mg/l	0.25a	1.75a	1.75a
	2 mg/l+1 mg/l	4.5ab	7.75a	2.21a
	2 mg/l+0.5 mg/l	0.25a	2.25a	2.25a
	1 mg/l+1 mg/l	2a	2.75a	0.58a
	1mg/l+ 3 mg/l	0.75a	5.25a	3.37a
	1 mg/l+5 mg/l	6ab	23ab	3.36a
	1 mg/l+0.5 mg/l	8.25ab	2.75ab	4.80a
	2 mg/l+3 mg/l	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

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

Table 5: 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^2	0.816	0.975	0.967	0.911	0.787
Pr > F	0.0197*	< 0.0001***	< 0.0001***	< 0.0001***	< 0.0001***

Figures

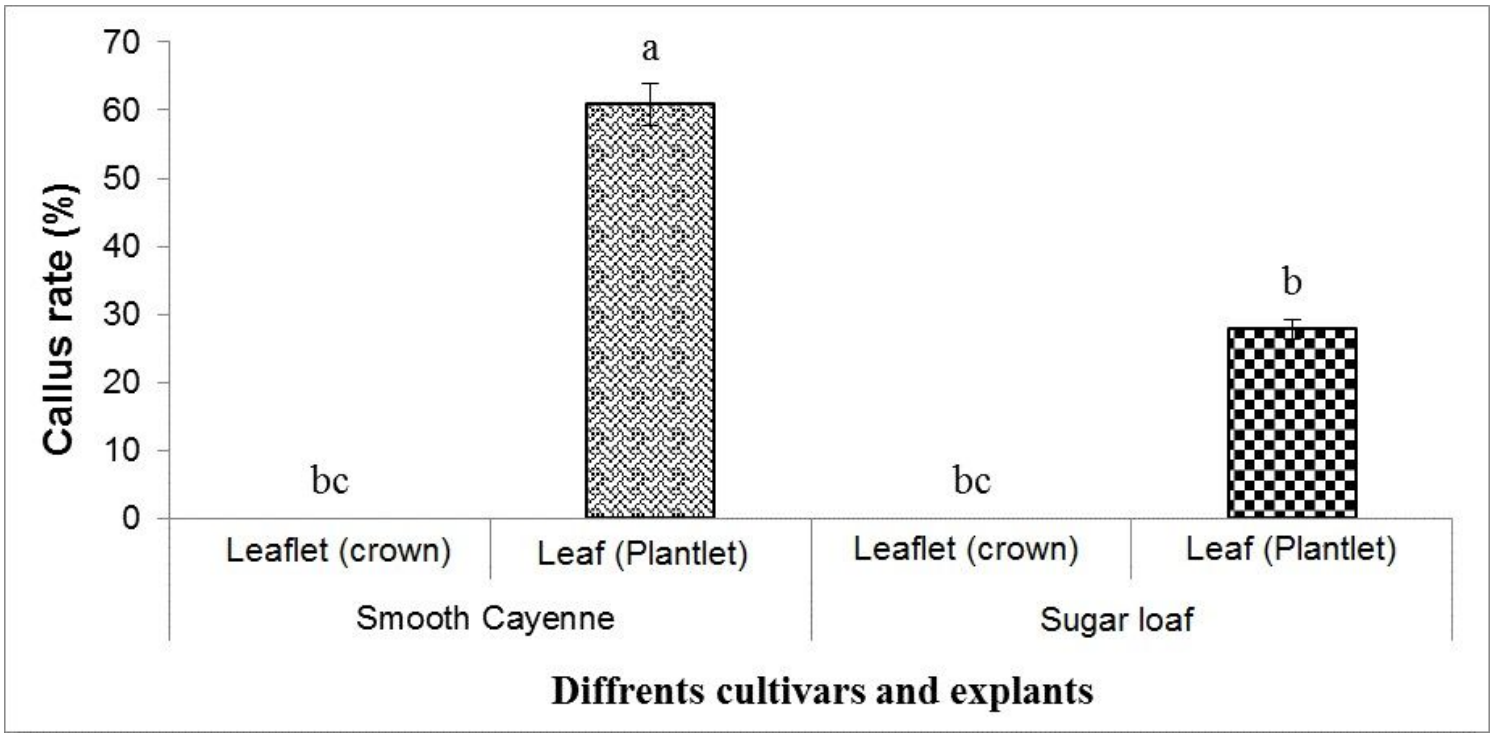

Figure 1

Effect of explants origin on calli formation in pineapple.

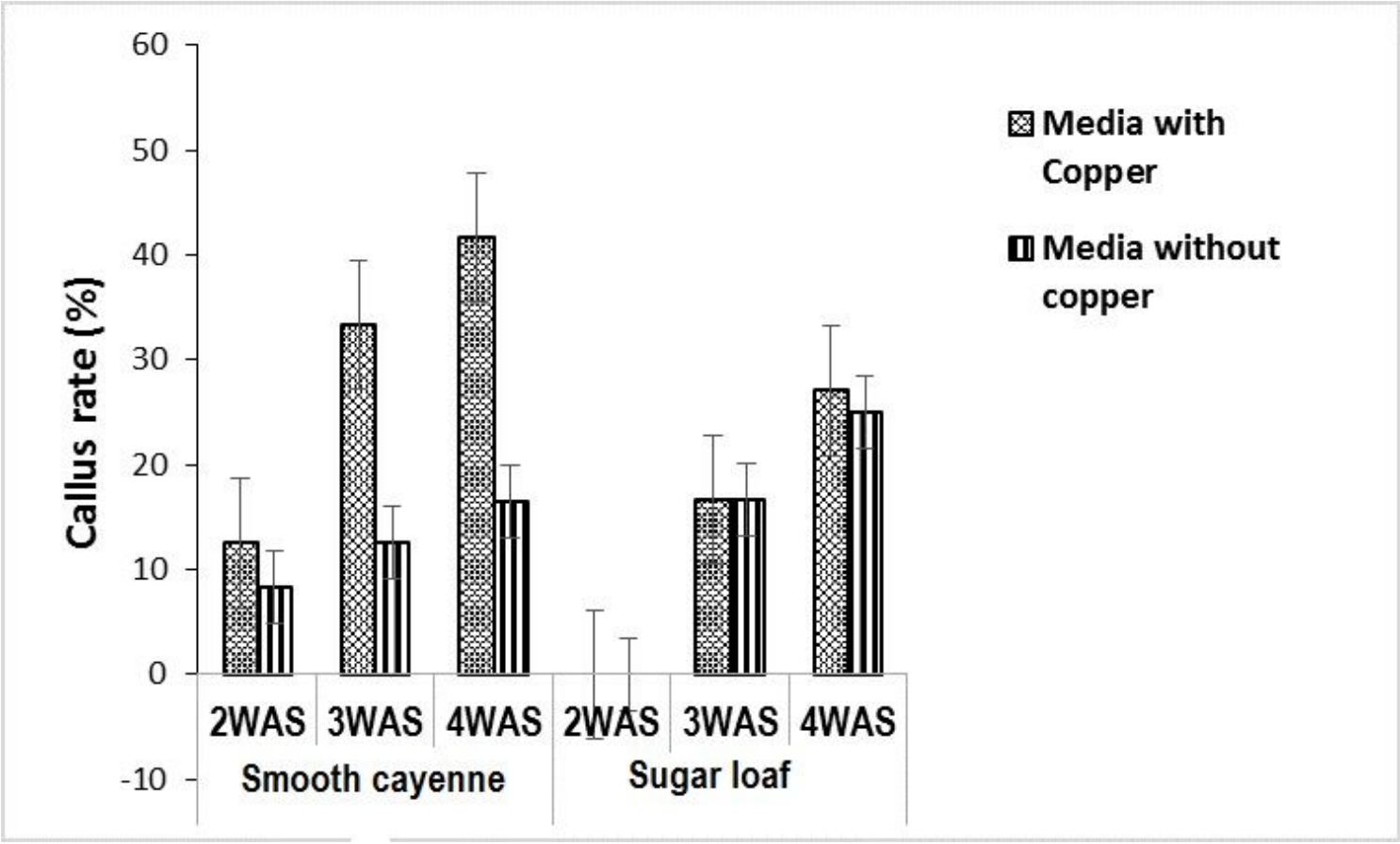

Figure 2

Effect of copper on callus formation; (WAS) Week after seeding.

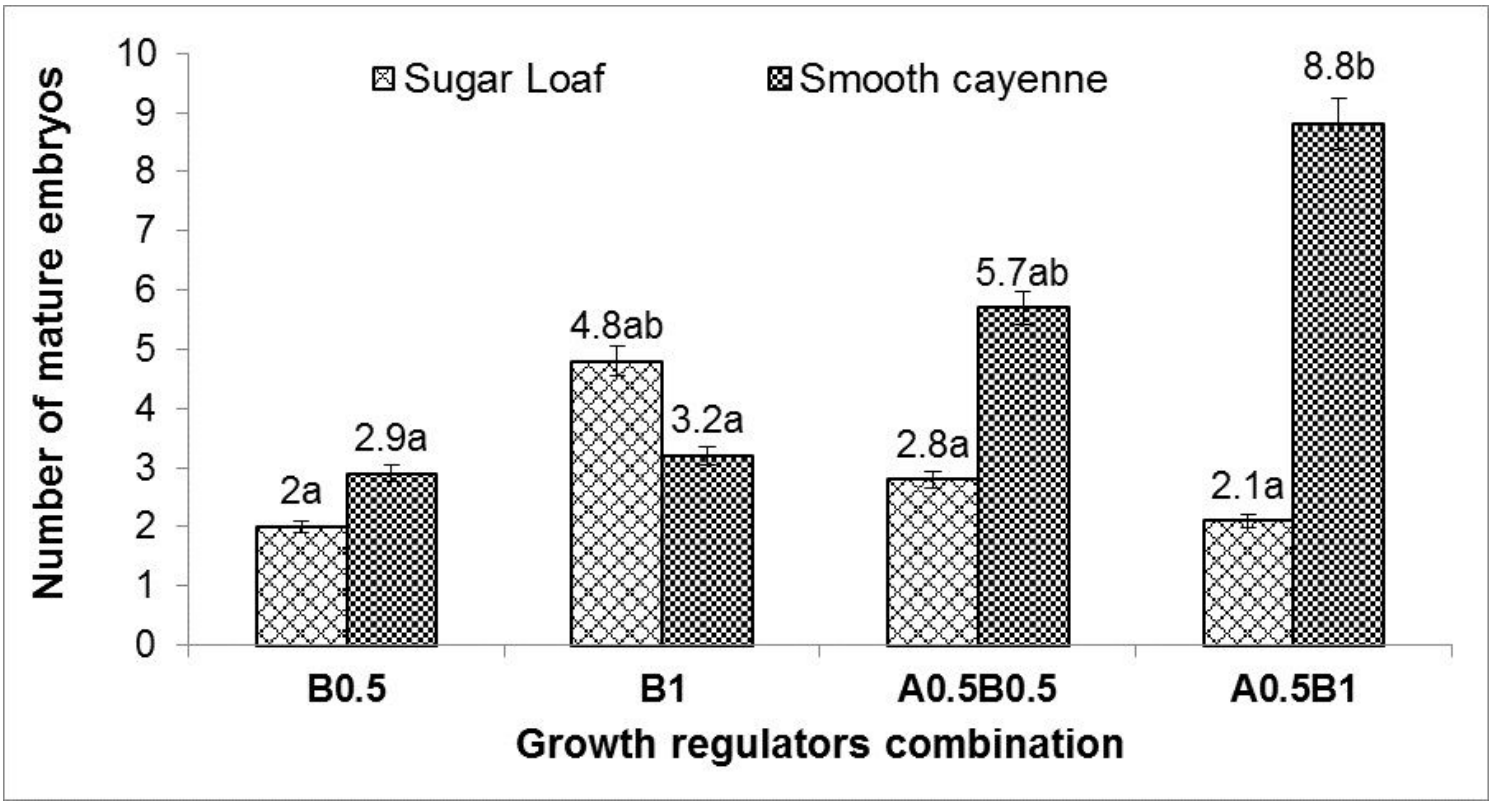

Figure 3

Effect of growth regulators combination on somatic embryos maturation

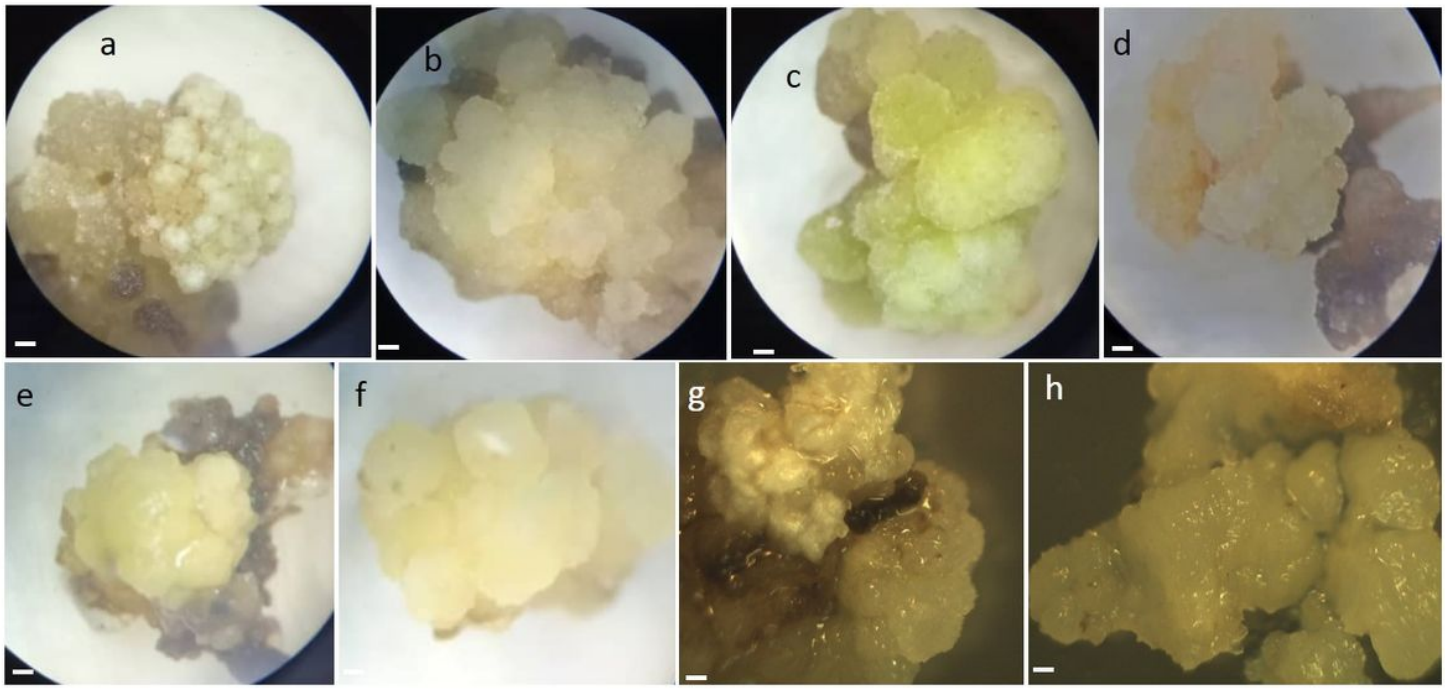

Figure 4

Somatic embryos development stages in pineapple

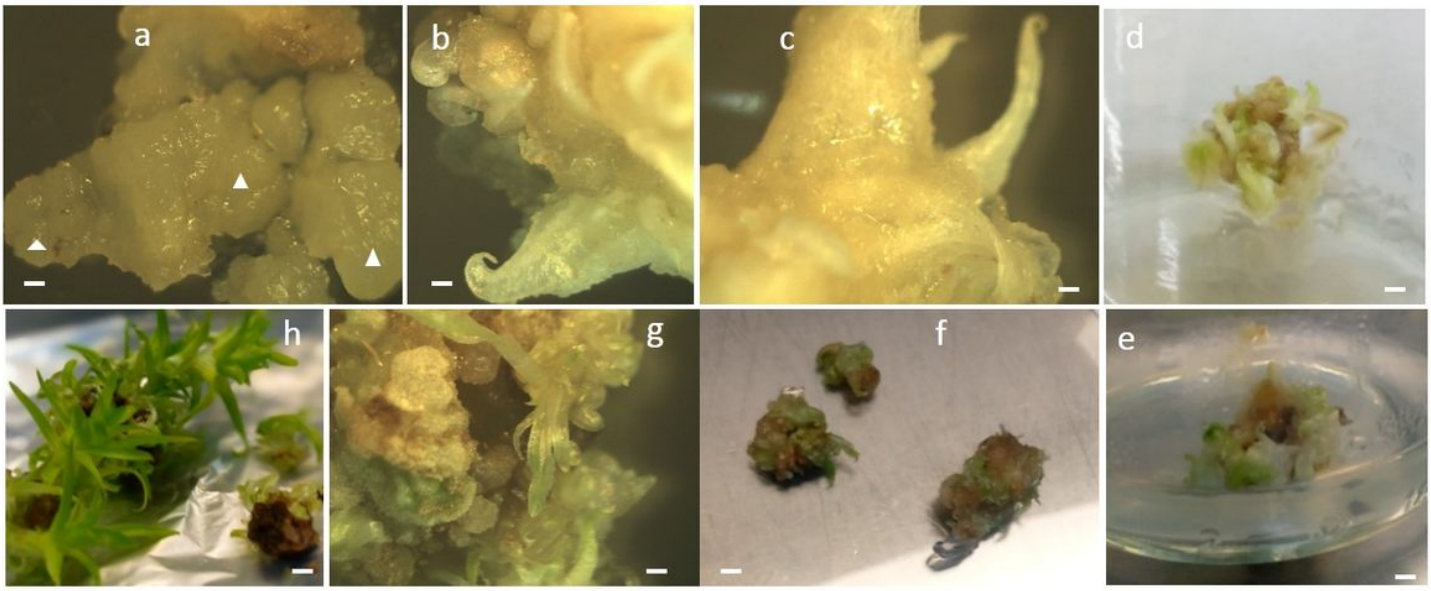

Figure 5

Plant regeneration steps in somatic embryogenesis of pineapple.

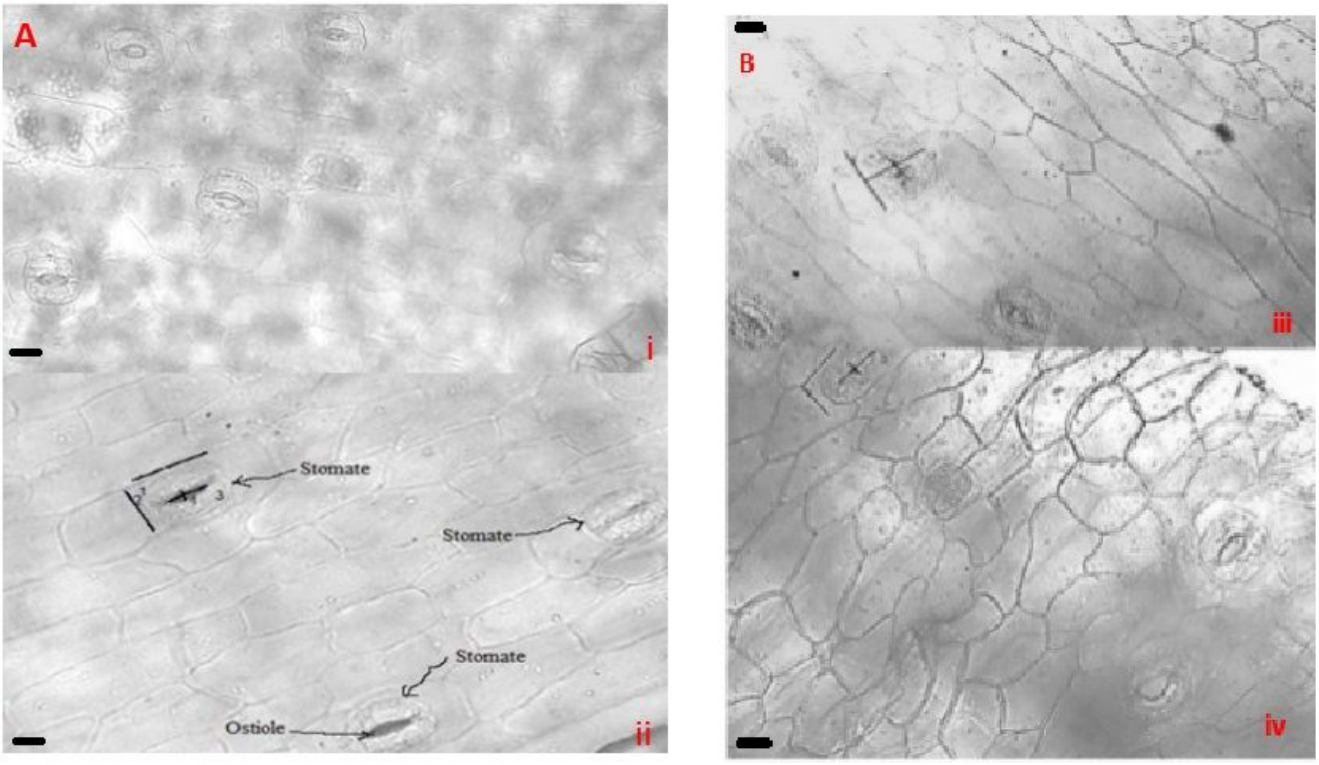

Figure 6

Microscopic structure of pineapple leaves.

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