

## Evaluation of the in vitro growth of perolera pineapple (*Ananas comosus*) explants using organogenesis technique

### Evaluación del crecimiento in vitro de explantes de piña perolera (*Ananas comosus*) usando la técnica de organogénesis

Christian Andrei Chacin Zambrano <sup>1</sup>, Yessica Paola Quintero Avila <sup>2</sup>,  
Leydy Gabriela Rodríguez González <sup>3</sup>, Johanna Alexandra Gómez Santos <sup>4</sup>.

<sup>1</sup>Universidad de Santander. ✉ [cchacin@udes.edu.co](mailto:cchacin@udes.edu.co)

<sup>2</sup>Universidad de Santander. ✉ [yessicaquintero2019@gmail.com](mailto:yessicaquintero2019@gmail.com)

<sup>3</sup>Centro industrial del diseño y de la manufactura. ✉ [lgrdriguez96@misena.edu.co](mailto:lgrdriguez96@misena.edu.co)

<sup>4</sup>Centro Industrial de Mantenimiento Integral, SENA, Girón. ✉ [jagomez0742@misena.edu.co](mailto:jagomez0742@misena.edu.co)

Recibido: 29-10-2020 Aceptado: 21-11-2020

**ABSTRACT** From an economic standpoint, pineapple (*Ananas comosus*) is one of the most important fruits in Colombia. A decade ago, the Perolera variety was the most cropped cultivar of the Santander department, however, the variety has been displaced considerably due to the lack of technical extension services and the introduction of new varieties. This research project was carried out with the intention to conserve the species through the development of in vitro pineapple explants using the organogenesis technique. Meristems that have been extracted from the crown of the Perolera pineapple variety were used for this purpose. Four disinfectant treatments were evaluated by looking at the different kinds of disinfectant exposure times. The treatment that gave the best results in terms of contaminant-free explants was the T2: Commercial detergent + Tween 80 for 8 minutes, ethyl alcohol at 70% for 1 minute and sodium hypochlorite at 1,5% over 10 minutes, with a contamination rate of 7% and 93% of the explants thriving. For the establishment phase, it was found that the medium MS MEP1 with 100% solid salts supplemented with 2000 µl/L BAP - 1000 µl/L ANA - 1000 µl/L AIA and 500 µl/L thiamine enabled 90 % of the pineapple explants to continue developing four weeks after planting. Similarly, the medium containing 3000 µl/L of BAP for the multiplication phase permitted an average proliferation of 4.62 shoots with 9.12 leaves per shoot and a length of 2.25 mm.

**Key Words:** Apical sprout; Meristem; Micropropagation; Organogenesis

**RESUMEN** Desde el punto de vista económico, la piña (*Ananas comosus*) es una de las frutas más importantes de Colombia. Hace una década, la variedad Perolera era la variedad más cultivada del departamento de Santander, sin embargo, la variedad se ha desplazado considerablemente debido a la falta de servicios de extensión técnica y la introducción de nuevas variedades. Este proyecto de investigación se llevó a cabo con la intención de conservar la especie mediante el desarrollo de explantes de piña in vitro mediante la técnica de organogénesis. Para ello se utilizaron meristemas extraídos de la corona de la variedad de piña Perolera. Se evaluaron cuatro tratamientos desinfectantes observando los diferentes tipos de tiempos de exposición a los desinfectantes. El tratamiento que mejores resultados dio en cuanto a explantes libres de contaminantes fue el T2: Detergente comercial + Tween 80 durante 8 minutos, alcohol etílico al 70% durante 1 minuto e hipoclorito de sodio al 1,5% durante 10 minutos, con una contaminación tasa de crecimiento del 7% y 93% de los explantes. Para la fase de establecimiento, se encontró que el medio MS MEP1 con 100% de sales sólidas suplementado con 2000 µl / L BAP - 1000 µl / L ANA - 1000 µl / L AIA y 500 µl / L tiamina permitió que el 90% de los explantes de piña se desarrollaran cuatro semanas después de la siembra. Asimismo, el medio que contenía 3000 µl / L de BAP para la fase de multiplicación permitió una proliferación promedio de 4.62 brotes con 9.12 hojas por brote y una longitud de 2.25 mm.

**Palabras clave:** Brote apical; Meristemo; Micropropagación; Organogénesis

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## INTRODUCTION

Pineapple belongs to the Bromeliaceae family which grows in tropical and subtropical climates. The plant reaches about 1 m in height, while the width and concave leaf formation allow the plant to collect and store water. After bananas and citrus, pineapple is the third most important tropical fruit presenting very good organoleptic and morphologic properties (López- Herrera et al., 2014). Pineapple is considered to be one of the most important commercial crops in Colombia given that the country is the tenth largest producer of pineapple in the world (FAOSTAT, 2016).

Pineapple crops are primarily located in the departments of Valle del Cauca, Risaralda, Cauca and Santander, the latter being the lead producer with 50% of the national production. The crop is an important component in sustaining the local agricultural economy in the region where the Perolera variety is the one most grown and harvested. Even so, in recent years the hectares dedicated to cultivating Perolera pineapple have diminished notably as a result of limited technical extension services for controlling pests and diseases, as well as other limiting factors like soil depletion, incorrect fertilization practices, aphids, scale insects, *Dysmicoccus brevipes* among others, that have directly and indirectly led to a drop in production in the region. Added to this, the appearance of new varieties like Golden Honey have begun to replace the Perolera endemic to the department.

Given the concern to conserve the variety in the region while keeping the organoleptic properties that make pineapple so sought after by consumers, this project of implementing a micropropagation technique using organogenesis came about. The goals were to radically increase the number of plants, reduce multiplication time, produce material on an ongoing basis, and allow for multiplying large numbers of plants in a small space to simplify costs, facilitate transportation of the material, create better possibilities for rapid

multiplication of a variety with few remaining individuals, and have greater control over the health of the propagated material. In addition, this project is part of establishing an in vitro and ex situ germplasm bank for the propagation of agroforestry systems of native timber species that are threatened, as well as promising crops for the department of Santander.

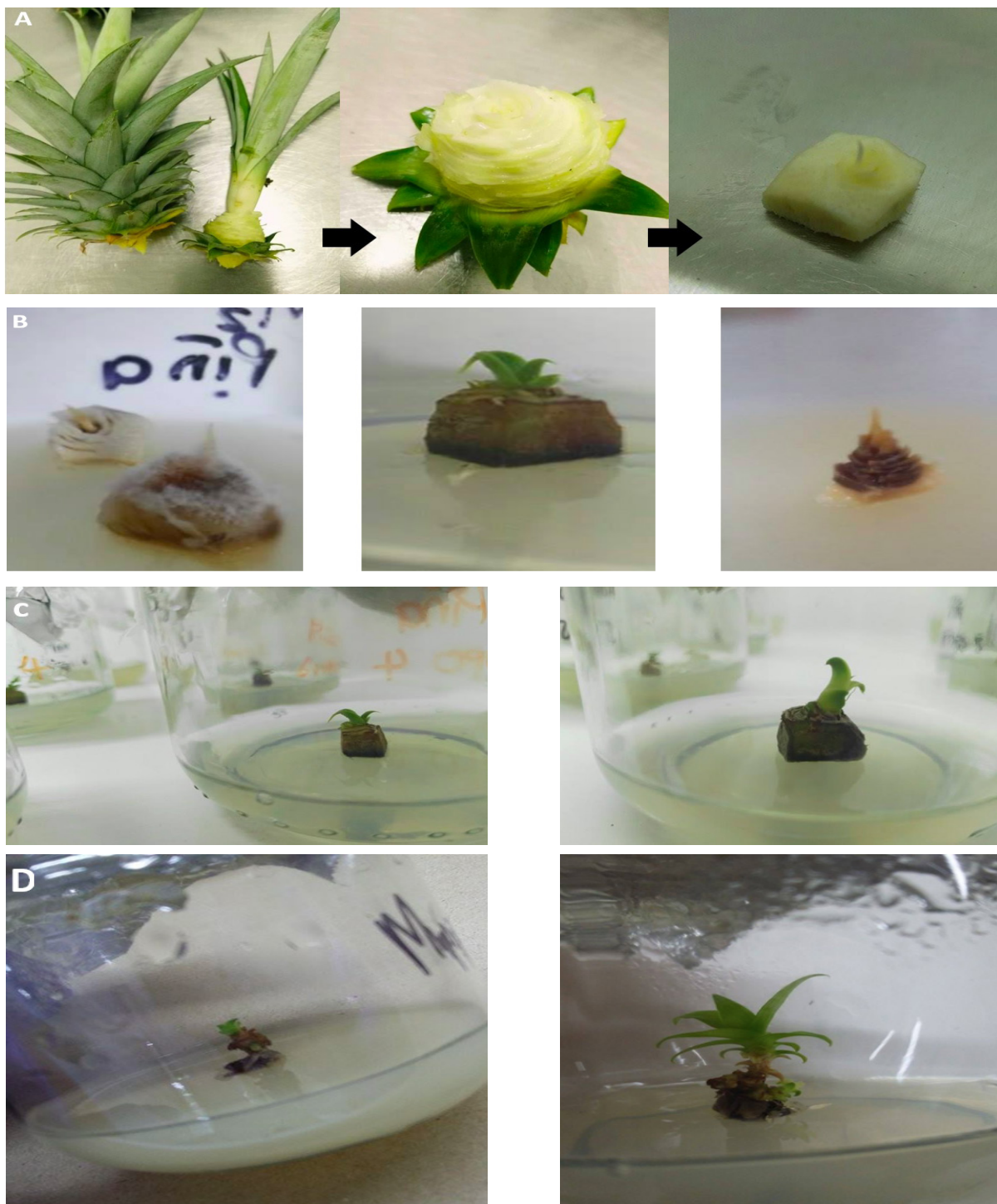
## MATERIAL AND METHODS

The research took place in the University of Santander Plant Tissue Laboratory located on the Lagos del Cacique campus in the city of Bucaramanga. Perolera pineapple plants collected from the field of the Luz Helena farm located in Lebrija, Santander were used. The plants were selected by phytosanitary state and phenotypic traits such as vigour, and pest free. The meristems of the pineapple were extracted from the crown of the fruit. Selected crowns were carefully plucked until the apical sprout covered with only one leaf was exposed. The base surrounding the sprout was cut leaving a 1cm x 1cm x 1cm cube (Figure 1A).

### Evaluation of the disinfection treatments

The meristems were washed using different concentrations of disinfectants and exposure times. Four treatment regimens were established as shown in Table 1. For each one of the changes of exposure to disinfectants, they were washed with sterile distilled water for 1 minute. Following this, the explants were taken to the laminar airflow (LAF) bench that had been disinfected with 70% ethyl alcohol and then sterilized by 15 minutes of ultraviolet light under aseptic conditions. Here they were washed with sterile distilled water for 3 minutes in the planting area under aseptic conditions.

To evaluate the efficacy of the disinfection treatment, observations were made for each treatment 4 weeks after planting and the percentages of oxidized pineapple explants (%EPO; Eq. 1), thriving pineapple explants



**Figure 1.** A) Extraction of pineapple meristems from the crown. B) Results obtained during the disinfection phase: Contaminated, thriving and oxidized explant. C) Developing pineapple explants during the establishment phase. D) Development of a pineapple explant at the multiplication phase. Source: own elaboration

(%EPP; Eq. 2), and contaminated pineapple explants (%EPC; Eq. 3) were analysed, using the following relationships:

$$\%EPO = \frac{\#Oxidized\ explants}{\#Total\ explants} * 100 \quad (Eq.1)$$

$$\%EPC = \frac{\#Contaminates\ explants}{\#Total\ explants} * 100 \quad (Eq.2)$$

$$\%EPS = \frac{\#Healthy\ explants}{\#Total\ explants} * 100 \quad (Eq.3)$$

The presence of microorganisms was visually assessed 7 days after planting in the growing medium. For each treatment, the percentages of bacterial and fungal contamination were calculated, as well as the percentage of healthy plants.

### Development of the establishment phase

The disinfected explants were planted in the solid medium Murashige y Skoog (1962). The supplements of hormones and vitamins were varied according to the nutritional requirements of the plant, leading to the use of three growing mediums (MEP1-MEP3) for the establishment phase as outlined in Table 2. After 40 days from planting, the percentage of response was calculated by expressing the relation between explants that responded well and the total number of explants planted.

### Development of the multiplication phase for pineapple shoots

Pineapple meristems with an outstanding response percentage were used for the multiplication phase, meaning sprouts with an average size of 1-2 centimetres and no evidence of contamination after two months. The growth mediums that were used are shown in Table 2. Two different ratios of growth regulators (3:1 y 1:1) were assessed in order to determine the optimum concentrations for in vitro multiplication of pineapple. All the growing mediums were sterilized in an autoclave for 30 minutes at a temperature of 121°C. The explants that were planted were taken to an incubation room with a temperature of 19°C, relative humidity of 68% and a photoperiod of 16 hours of light and 8 hours of darkness.

## RESULTS

### Disinfection treatment

The results of this first phase are shown in Figure 2, where the percentages of contaminated, oxidized, and thriving explants are shown for

each of the different proposed disinfection treatments. In the same way, Figure 1B shows the appearance of contaminated, thriving, and oxidized pineapple explants observed in the research.

### Establishment phase

The development of the Perolera pineapple variety during the establishment phase can be seen in Figure 1C. The growth and appearance of the first shoots can be observed. The growing medium used had a significant influence on growth takeoff of and development as can be seen in the results. The treatments MEP1, MEP2 and MEP3 achieved a 90%, 70% and 60% response rate, respectively.

### Multiplication Phase of the pineapple shoots

The goal of this phase was to achieve a massive increase in the shoots in mediums with higher

**Table 1** Disinfection treatments

Treatment	Solution composition		
T1	Neutral detergent 2% + tween 80 2%, 8 minutes	Ethyl Alcohol 70%, 1 minute	HClO 1,5%, 5 minutes
T2	Neutral detergent 2% + tween 80 2%, 8 minutes	Ethyl Alcohol 70%, 1 minute	HClO 1,5%, 10 minutes
T3	Neutral detergent 2%, 8 minutes	Ethyl Alcohol 70%, 1 minute	HClO 1,5%, 5 minutes
T4	Neutral detergent 2%, 8 minutes	Ethyl Alcohol 70%, 1 minute	HClO 1,5%, 10 minutes

**Table 2** Growing mediums used in the establishment phase (MEP1-MEP3) and the multiplication phase (MMP1-MMP2) of the pineapple explants

Growing medium	MEP1 (µl/L)	MEP2 (µl/L)	MEP3 (µl/L)	MMP1 (µl/L)	MMP2 (µl/L)
Cytokines	BAP 2000	2000	2500	3000	2000
Auxins	ANA 1000	500	1000	1000	2000
	AIA 1000	500	-	-	-
Supplements / Vitamins	My-inositol 100 mg/L, Thiamine 500 µl/L, Sucrose 30 g/L, pH 5,7 – Agar Agar 10 g/L				



concentrations of growth regulators than those used in the establishment phase. Different ratios of cytokines/auxins were used in two different mediums as proposed in the methodology (Table 2) and then the number of shoots (NB), number of leaves (NH) and plant length (LP) were evaluated four months after planting. The results can be seen in Table 3. During the evaluation, it was possible to observe uniformity in the shoot growth and morphological and physiological aspects (Figure 1D).

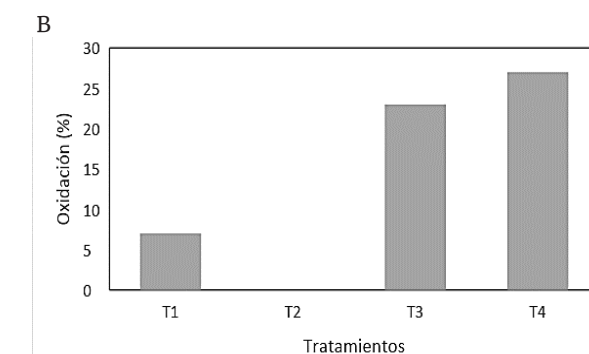
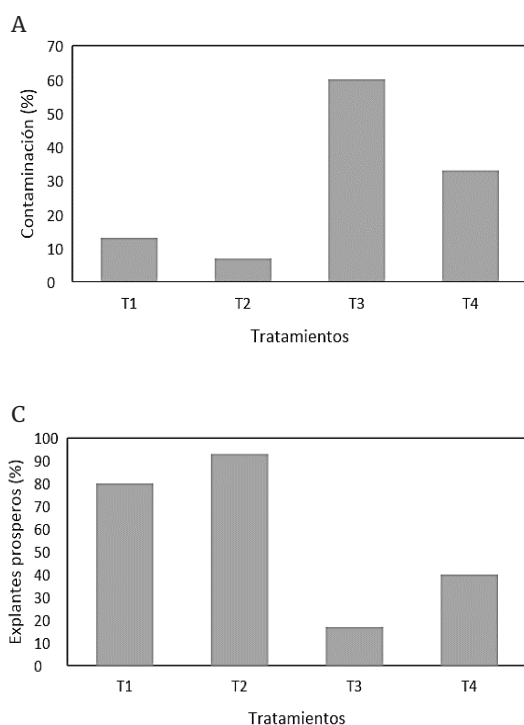
## DISCUSSION

Diverse methods to establish in vitro pineapple crops have been developed (Kiss et al., 1995; Escalona et al., 1999; Garita and Gomez, 2000; Roostika and Mariska, 2003; Firoozabady and Gutterson, 2003; Saucedo et al., 2008; Blanco et al., 2011; Pineda et al., 2012; Medina-Rivas et al., 2014). They include the preparation of explants and growing them in a Murashige-Skoog (MS) medium with different combinations and concentrations of supplements using benzyl adenine (BAP), kinetin (kn), naphthaleneacetic

acid (ANA), indole-3-butyric acid (IBA), among others. However, results obtained in other latitudes and with other mediums are not always replicable (Garita and Gomez, 2000). For this reason, different disinfection methods were tried and various growing mediums were proposed based upon the results of previous work carried out by the same research team.

## Disinfection treatment

The disinfection phase is the most sensitive in the process since it is here that the largest number of explants can be lost. As such, sodium hypochlorite was used as a disinfectant agent. This substance fosters the disinfection of explants through an action mechanism that permits the destruction of the cell membrane in bacteria, causing lysis in the microorganisms (Sánchez and Sáens, 2005). In the same way, in every case, the plant matter was disinfected using a 70% alcohol solution for 1 minute. In this step, the surface contaminants are eliminated and the waxy layer of the explant is eroded thus enabling contact with the disinfectant solution.



**Figure 2.** Results from the disinfection protocols for the Perolera pineapple variety. A) Contaminated pineapple explants. B) Oxidized pineapple explants. C) Thriving pineapple explants.

**Table 3.** Number of shoots, leaves and plant length with the multiplication medium

TREATMENT	Number of shoots (NB)	Number of leaves (NH)	Plant length (LP)
MMP1	4,62	9,12	2,25
MMP2	0	4,3	0,85

The results show that T2 was the treatment with the best disinfection activity in terms of viability, contamination and mortality, using Tween 80 in the first step followed by the application of 1.5% NaClO for 10 minutes. With this treatment, the least contamination of plant material (7%) was observed with the highest number of explants thriving (93%).

Contamination is a common problem of plant in vitro culture and the origin can be external or internal. In many cases, the methods used for surface disinfection are not completely effective and depend on variables such as the concentration of disinfection agents as well as exposure time. For this work, and following Garita and Gómez (2000), some factors that can favour contamination include the shape and the arrangement of the pineapple leaves, as well as the fact that the material used comes from the field thus favouring the presence of large numbers of microorganisms. The humid environment needed for growing the explants is also a factor.

It can be seen in Figure 2A that the lowest percentages of contamination were attained with treatments T1 and T2. These results could be attributed to the use of Tween 80 in the first step, a mild non-ionic, and a highly hydrophilic surfactant that acts as a moisturizing, dispersing and solubilizing agent that then has multiple benefits when used in combination with sodium hypochlorite. This surfactant helps to moisten the plant material and/or significantly reduce the angle of contact and in so doing allows the NaOCl to spread uniformly providing effective treatment.

Moreover, it can be seen that the percentage of contamination diminished when the exposure time of the explants to sodium hypochlorite was increased, both in the absence of (T4) and the presence of Tween 80 (T2). This matches with the findings of various authors who demonstrate that the effectiveness of sterilization using hypochlorite increases with added exposure time (Bedoya-Pérez et al., 2016).

Furthermore, in this research, it was found that the main cause of contamination to the explants was fungi. Examination of the structures under a microscope allowed for the identification of the most representative genera: *Penicillium* sp. and *Mucor* sp. Both are contaminating fungi characteristically present in fruit and the environment as a whole. These findings concur with those published by Rodríguez et al (2008) and Das & Pal (2005), who found that the most frequent contaminants during the in vitro establishment of explants taken from adult plants are fungi that normally occur in the natural crop conditions.

Disinfection treatments imply coming to some compromise between contamination-free explants and explants that maintain their growth and development capacity in vitro (Garita and Gomez, 2000). In general, the oxidation evident in each of the different disinfection treatments was found to be relatively low, and in the case of T2 was practically nil (Figure 1B). Even so, with treatments T3 and T4, the oxidation was 3 to 4 times higher than that observed for T1 and T2. The low percentage of oxidation in the pineapple explants for each of the disinfection treatments coincides with that presented by Litz and Jaiswal (1991) who indicate that pineapple does not produce phenols and other substances that alter the oxidation of the explant as they do in other crops. They also show that this modification in the meristem is possibly due to the age of the parent plant.

The highest percentage of explant oxidation is seen with treatments 3 and 4. In this case, increasing the exposure time to hypochlorite from 5 to 10 minutes led to a decrease in the number of contaminated explants but a higher number of explants that died. Such behaviour has been observed in different plant materials (Moncada et al., 2014), where the tendency towards contamination decreases as exposure time is increased but necrosis in explants also increases. This result was ratified for the in vitro cultivation of Perolera pineapple

Concerning the thriving explants, the T2 treatment was the one that achieved the best results with 93%, while T3 did not exceed 17% due to the high levels of contamination and oxidation observed with it.

## Establishment phase

In all cases, the factors that contribute to the *in vitro* response of the meristems are the appropriate concentrations of salts in the MS and the addition of other important components like thiamine and other vitamins.

Given the results obtained, the medium that provided the most optimum conditions for the explants was the MEP1 where 90% of the explants developed shoots, unlike mediums MEP2 and MEP3 with a respective response of 70% and 60%. The difference in the treatments had to do with the ratio of cytokinin/auxins. In the first case, the ratio was 1:1, while for MEP2 and MEP3 it was 2:1 and 2.5:1.

## Multiplication phase of the pineapple shoots

Just as was seen in the establishment phase, the growing medium affected the rate of multiplication of the pineapple explants mirroring the findings of Garita and Gómez (2000). Of the mediums under consideration, medium 1 (MMP1) is that which shows the best results, higher numbers of shoots, leaves and plant length, due to a greater concentration of cytokines as well as the auxin/cytokinin ratio (3:1).

With the addition of the cytokine BAP, the medium achieved a proliferation of shoots since cytokines are a growth hormone with the capacity to break down the apical dominance and stimulate the sprouting of the meristems. As a general rule propagation medium contains cytokines but the concentration is varied depending on the endogenous balance of auxins and cytokines in the explants (Pérez-Bernal et al., 2007).

Despite the fact that the ratios of auxins / cytokines used in the two mediums were destined to multiply shoots, the MMP2 medium

did not show a proliferation of shoots in the pineapple explants. Only longitudinal growth of the initial shoot was evident. This could be due to the BAP-ANA balance that was created. According to Rojas et al. (2004), the presence of cytokines in the medium is beneficial because their primary function is to promote cell differentiation and division, and because they act in concert with auxins that promote cell growth and attract nutrients. According to Zamora y Juárez (2008), maintaining the correct balance between auxins and cytokines is important because in combination they promote apical regeneration and primordia multiplication from the meristems, tips or buds.

## CONCLUSION

The results show that a combination of commercial detergent, Tween 80, 70% alcohol for 1 minute and sodium 1,5% hypochlorite for 10 minutes is a great alternative for disinfecting pineapple explants of the Perolera variety.

It was determined that the best MS medium for the establishment phase (MEP1) contains salts that are 100% in solid phase and is supplemented with 2000 µl/L BAP – 1000 µl/L ANA – 1000 µl/L AIA y 500 µl/L thiamine, glycine and myo-inositol. This made possible the development of 90% of the pineapple explants within four weeks of planting.

Finally, the best concentration of 6-Benzylaminopurine (BAP) used in the multiplication phase of the shoots was 3000 µl/L combined with 1000 µl/L 1-Naphthaleneacetic acid (ANA), giving an average of 4,62 shoots per explant.

## ACKNOWLEDGEMENTS

The authors acknowledge the Universidad de Santander and Servicio Nacional de Aprendizaje SENA Bucaramanga and SENA Girón: Centro de Servicios Empresariales y Turísticos and Centro Industrial de Mantenimiento Integral;

Departamento Administrativo de Ciencia, Tecnología e Innovación, COLCIENCIAS for a doctoral scholarship (Francisco Jose de Caldas, grant No. 511, 2010).

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