Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(7):540-546

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Germplasm conservation of four species of native bromeliads at risk of extinction in the Farm El Prado-IASA I-ESPE region, Pichincha province-Ecuador

Evelyn Martínez Rueda[1], Darwin Rueda Ortiz[1*], Pedro Romero Saker[1], Juan Ortiz Tirado[1], Bangeppagari Manjunatha[1], Maddela Naga Raju[2], Mariadoss Selvanayagam[2,3] and Selvaraj Thangaswamy[4]

[1]*Department of Life Sciences, Universidad de las Fuerzas Armadas-ESPE, Sangolquí, Quito, Ecuador, South America*
[2]*Department of Environmental Engineering, Universidad Estatal Amazonica, Puyo, Ecuador, South America*
[3]*Loyola -ICAM College of Engineering and Technology (LICET), Loyola campus, Chennai, India*
[4]*Centro de Biologia Cellular Y Molecular (CBCM), Universidad Technica Particular de Loja (UTPL), San Cayetano Alto s/n. Loja, Ecuador, South America*

ABSTRACT

Bromeliads are epiphytes which are distributed in subtropical and tropical latitudes of South America. In Ecuador there are about 455 species with inflorescence's with a unique beauty of intense colors and long lasting, reason has been of great interest in many countries. But human activities such as the introduction of new species, over exploitation and habitat loss have resulted in the negative impact on bromeliads and leads to the extinction of species. Hence, the objective of this research is the implementation of a pilot germplasm bank by an ex situ process of adaptation and development of biotechnological tools such as in vitro culture of cryopreservation and minimal growth for the conservation of the four bromeliads species. Thus the species that have shown positive results with minimal growth of cryopreservation obtained viable and hydrated plants.

Keywords: Bromeliads, Germination *in vitro* cryopreservation, Minimal growth, Over exploitation.

INTRODUCTION

Bromeliaceae is one of the most distinctive families of epiphyts with approximately 2885species found in subtropical and tropical region of South America [1]. They are monocots, epiphyts or terrestrial plants, herbaceous or perennial, having leaves in rosettes, trimere flowers, colorful bracts and seeds with pappus. Most bromeliads live as epiphytes associated with certain trees, which are used for support. They can grow one or more in the same tree and manufacture their food with the help of sunlight and damp air. Others feed on organic matter and water found in the leaves, in rain or absorb water from the environment [2].

Currently bromeliads are used for various applications, namely edible use as *Ananas comosus* (the pineapple or pineapple), manufacture of fabrics like Chevalliera magdalenae possessing high quality fiber, manufacture of births as Till and so usneoides known as old man's beard and for making flower arrangements and decoration of gardens thanks to the beauty they possess in florescence's having exotic colors and long lasting nature. Hence, the bromeliads are in great demand in the international market.

At present in many countries bromeliads are affected due to various anthropogenic activities such as; the introduction of new species, Environmental pollution, demographic, overfishing or habitat loss are real negative

consequences, leads to the extinction of species [1,3,4]. Because of these problems, there is an urgent need to develop biotechnological methods for mass reproduction of bromeliads, with sustainable farming to achieve the effective conservation.

Germination Bromeliads *in vitro*

In vitro germination is a technique carried out in fully aseptic conditions, from a small segment of tissue, seeds, embryos, organs or protoplasts may be obtained from plants genetically identical to the mother plant. This methodology is a tool that allows propagation in large numbers of plants in less time. The main element to implement in this methodology is the nutrient agar containing all the minerals and sugars needed for seed germination, growth and development. The average nutrient agar best known is the MS medium [5].

Similarly, *in vitro* germination allows easy handling in small spaces and obtaining pathogen free plants. It is important to know that the conditions in which germination is performed *in vitro* are aseptic, free of any contaminant, and the plant nutrient should be disinfected before planting. The *in vitro* germination is a resource for the preservation and conservation of species that are part of the floristic diversity of ecosystems [6].

Cryopreservation

One of the fundamental and essential tools to carry out the *ex situ* cryopreservation is the *in vitro* culture that preserves large portions of biodiversity areas and small volumes [7]. It is important to note that the success of this process will depend on the treatments used with plant material before and after being preserved. That is why it is essential to make a selection of the material and if it comes from a culture *in vitro*, the medium being held should have a good composition of macronutrients and micronutrients [8].

Mínimum growth

One of the methods used to establish a germplasm bank is the minimum growth, in which the maintenance of plant material through *in vitro* culture methods is obtained by performing optimal changes in environmental conditions for cultivation. That is why it is necessary to lower the temperature, reduce the lighting conditions, change the culture medium by adding osmotic inhibitors or growth retardants, dehydrating tissue or modify the gas phase of the culture vessel [9]. As mentioned above, the aim of this method is to reduce cell division and plant metabolism, thus achieving the increased longevity *in vitro* plant material without causing any genetic change. This means there is no cellular processes, on the contrary, the speed with which they occur and the period of transfer to a fresh culture medium decreases [10]. In this process antigiberélicos agents like CCC Cycocel which reduces metabolism of the plant and the plants provides resistance against stress [11].

This study will be a great contribution to the knowledge of strategies and potential adaptation in *ex situ* conservation of plant material of native bromeliads which are endangered. To avoid losing this important genetic material it is necessary to establish germplasm banks using techniques of *in vitro* culture, cryopreservation and minimal growth. This will enable us to conserve important plant material and subsequently it could help us in the regeneration of whole plants [8].

Furthermore, this research will benefit researchers, students and entrepreneurs who are interested in the conservation of biodiversity and pave way for the possibility of national and international commercial exploitation of bromeliads.

EXPERIMENTAL SECTION

The research was carried out in two phases:

Phase 1-Field study

The four species of bromeliads were collected from three nearby localities to the El Prado's farm; Pinllocoto river, Pita river and San Nicolás river located in the San Fernando–Sangolquí parish–Rumiñahui canton-Pichincha Province, Quito, Ecuador. For the collection of bromeliads machete, ropes, plastic bags were used.

An adaptation of bromeliads collected

The adaptation is performed in the secondary forest IASA. For this purpose a wire mesh was used and moss was collected from the trees, and a kind of maseta developed around the plant. Thus, they were placed on the branches of trees. Adaptation was also carried out in the laboratory by using plastic bottles with coal, blond moss and limestone.

Collection of capsules
The collected capsules were identified based on the taxonomic characters and confirmed the nature of the capsules young, mature or are in the process of putrefaction. The transports of the capsules were made with newsprint, since the plastic bags caused them sweat, in turn affect the germination *in vitro.*

Phase 2- Laboratory study
Disinfection
The capsules were disinfected by putting them in a small vial containing distilled water and proceeded to rinse capsule for disinfection. Then the capsules were placed them on a paper towel to remove the remaining moisture. Finally the bottles with capsules were taken to the laminar flow chamber for the next procedure namely forced maturation of capsules.

Forced maturation of capsules
It is important to sterilize the work area, for which we were cleaned using the laminar flow chamber, subsequently proceeded to force maturation of capsules with alcohol and fire and then they were placed in sterile containers and sealed them eventually. Finally the containers were moved to maturation chamber.

Preparation of culture medium
For culturing the capsules, medium of Murashige and Skoog was prepared with the following concentrations (Table1). The medium was dispensed into transparent plastic recipients, planting and easy handling.

Solution of macroelements	½ ml
Fe EDTA	2.5 ml
Organic substances	4 ml
Sucrose	30 g
Myo-inositol	0.1 g
Agar	4g

Seeding seed in vitro
The vials containing capsule were opened by keeping them in the laminar airflow chamber and the seeds were sowed in the culture medium with the help of sterile forceps, scissors. Finally the seeds containing medium were kept in the germination chamber under controlled conditions of light and temperature.

Cryopreservation
Preconditioning plant material
After seven months the germinated bromeliads, were transferred into a solid culture medium supplemented with 0.3M sucrose for 24 hours [12].

Loading treatment
According to Hirai and Sakai [13], the main objective of the loading solution is dehydrated, thus prepared the tissue for the next stages of freezing. The plant material was placed in the loading solution (0.4 M sucrose + 2M glycerol) and kept in the room temperature for 20 to 30 minutes. This was carried out inside the laminar flow chamber [12].

Treatment with cryoprotectants
Once the plant material complied with the time set in the loading solution, with the help of the clamps, the plant material placed one by one in each cryovial containing from 1-2 ml of Vitrificadora PSV2 solution (15% DMSO, 30% glycerol, 15% ethylene glycol and 0.04 M sucrose). This procedure was performed at room temperature in the laminar flow chamber [12].

Cryofreezing
Cryovials with plant material were taken to cryofreezer at -80°C. Cryovials remained there for a period of 15 days and 30 days.

Heating step
Once cryovials with plant samples complied with the time set in the freeze dryer were extracted and taken to a water bath at 40°C for 2-3 minutes.

Washing of cryoprotectants
The Cryoprotectants washing was carried out in the laminar flow chamber. Once cryovials were removed from the water bath, they were moved to the laminar flow chamber where the washing of cryoprotectants was performed. For

this, a Pasteur pipette was used to remove the cryoprotectant solution from each of cryovials and plant samples was placed in the wash solution (liquid culture medium supplemented with 1.2 M sucrose) for 20 minutes [12].

Re-cultivation
Once the plant samples were in the wash solution, then dried plant samples, the surplus of this solution with filter paper in a Petri dish. Finally the plant samples in glass tubes (10 x 2 cm) containing 2 inches of MS solid culture medium of multiplication. It is important to know that the samples during the first week remained dark phase and then transferred them to photoperiod conditions established [12].

Viability test
The viability test involved the observation of plant material by stereomicroscopy which evaluated the viability, texture and color consistency of the plant. Furthermore, the longitudinal growth of the samples was also evaluated by a digital gauge (NONIO). This procedure was performed after 15 days being sown in the solid culture medium and then 30 days.

Minimum growth
For this phase, plant samples that were already germinated in *in vitro* was chosen, then the solid MS culture medium was prepared with concentrations of 0 ppm and 10 ppm chloride (2-chloroethyl) trimethyl ammonium chloride (CCC). Following this, in laminar flow chamber grown plant material test tubes containing culture medium. Once the plant samples were cultured, they were evaluated at 15, 30 and 60 days of planting and feasibility and longitudinal growth was measured by a digital caliper (NONIO).

Statistical analysis
An exploratory data analysis was performed which were represented as descriptive graphs developed in Microsoft Office Excel 2007 and then a statistical analysis was performed using the statistical software Info Statand the Kruskal–Wallis test was performed with species of bromeliads and treatment of PVS2 and CCC.

RESULTS AND DISCUSSION

Cryopreservation
The species *Racinaea pectinata Andre* (Rp) with a cryoprotectant solution (PVS2) presented the highest longitudinal variation followed by the species *Tillandsia lajensis Andre* (Tl) (Figure 1). In these species, plant age, environmental conditions and the effect of PVS2 were favorable factors that helped to maintain adequate hydration of tissues [14]. According to Westendorp and Encina [14] one factors influencing the resistance to low temperatures is age. Although all species are aged seven months, *Racinaea pectinata Andre* (Rp) and *Tillandsia lajensis Andre* (Tl)) are those that showed higher resistance to cryofreezing. This occurs because a cryoprotectant applies regardless of dependent "capacity extracellular spaces to control the volume of the crystal and the ability to resist protoplast dehydration" [14]. *Tillandsia pastensis* (Tp) and *Tillandsia incarnate Kunth* (Ti) with PVS2, showed growth equal to 30 days of cryofreezing, while *Andre Racinaea pectinata* (Rp) and *Tillandsia lajensis Andre* (Tl) had a high longitudinal variability. The fastest growing *Racinaea pectinata Andre* (Rp) and *Tillandsia lajensis Andre* (Tl) was due to previously described by Westendorp and Encina [14].

It should be shown to the plants *in vitro* cultive supplemented with sucrose in order to prevent formation of intracellular ice upon freezing should be shown to the plants in a medium rich *in vitro* sucrose, in order to prevent formation of intracellular ice upon freezing culture, thereby allowing the plant physiology remains feasible. Thus, as stated above with the species *Racinaea pectinata Andre* (Rp) and *Tillandsia lajensis Andre* (Tl) rather pastens is *Tillandsias pecies* (Tp) [14].

Bromeliad species not being exposed to PVS2 showed a lower longitudinal variation than the species that was protected. This is because the control plants, not being subjected to treatments such as dehydration and not be contacting plants with cryoprotectants, which are substances that covers the plant material protecting and facilitating the passage of water through the cell makes ice crystals are formed intracellular causing tissue death or cell lysis and decreased oxygen requirements (Figure 1) [14,15].

The four species of bromeliads not treated with PVS2, namely *Racinaea pectinata Andre* (Rp) and *Tillandsia incarnate Kunth* (Ti), achieved minimal longitudinal growth when compared with cryoprotected species, because their leaves have a thick cuticle that provides resistance to cold, but not plants with thin leaves, like *Tillandsia pastensis* (Tp) and *Tillandsia lajensis* Andre (Tl) that are more susceptible to cell damage caused by cold (Figure 1) as mentioned by Westendorp and Encina [14]. The species *Racinaea pectinata Andre* (Rp) after 15 days of evaluation had longitudinal growth almost equal when used 0 ppm CCC, however, after 60 days of evaluation found

that using 10 ppm CCC, its longitudinal variation was 0.53mm while with 0 ppm CCC was 0.96mm. Thus achieving the effect of CCC in this species have positive results (Figure 2). According to Weaver [16], Cycocels one of the most active growth retardants group of quaternary ammonium compounds which are used in plants as an antigiberélicoagent not only can reduce plant metabolism but also provides the plant resistance to stress.

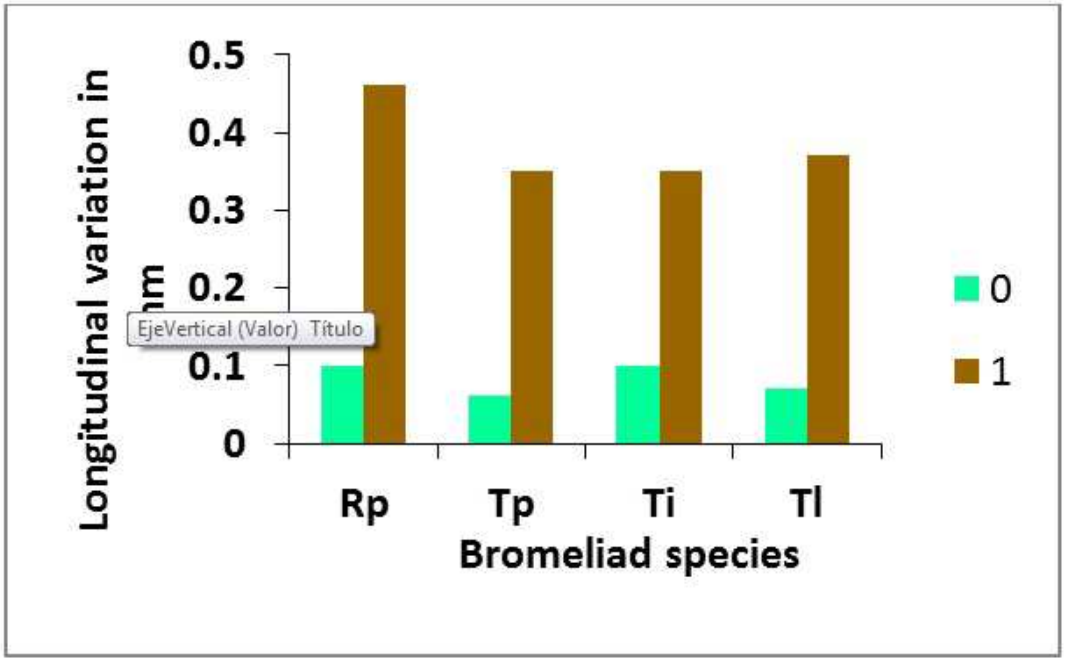

Figure1. Longitudinal variation of the four species of bromeliads with and without PVS2 30 days cryo freezing.

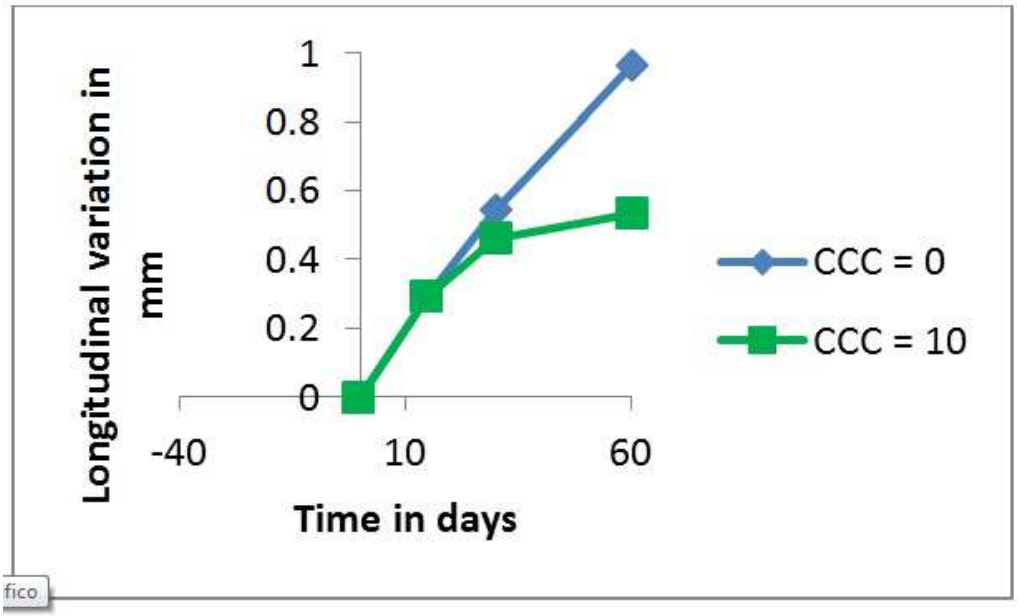

Figure 2. Longitudinal variation versus time.

The species *Racinaea pectinata Andre* (Rp) despite having thick and elongated leaves, showed signs of stress and yellowing of leaves, because the Cycocel have contact with young leaves produce a slight yellowing at the edges and the tip of the leaves. This coloration is related to the amount of CCC used in the culture. Therefore, although this reagenthasa wetting agent and is used in various crops, it is important to calculate the optimum amount of CCC

to be used and the frequency with which it is applied and such observations were made in herbaceous perennials or as bromeliads [17]. Furthermore, the high concentration of CCC applied to plants can cause brown spots or necrotic effect on the leaves preventing the plant to recover the green color as observed in *Racinaea pectinata Andre* (Rp).

The species *Tillandsia incarnata Kunth* (Ti) showed positive results from the beginning. At the time of evaluation (0, 15, 30 and 60 days) it was evident the effect of 10ppm CCC, which enabled to decrease the speed with which the plant grows. Thus, at 60 days with the concentration of 10 ppm has a longitudinal variation of 0.95mm while with a concentration of 0 ppm CCC longitudinal variation was1.97mm (Figure 3).

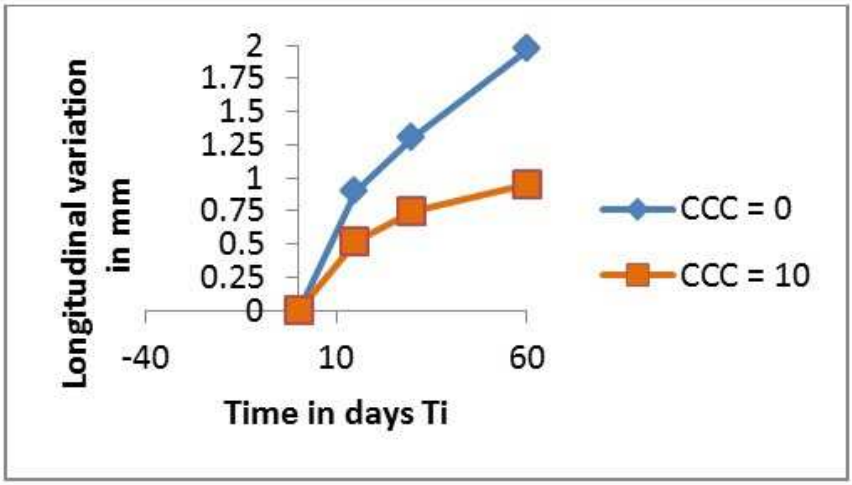

Figure 3. Ti longitudinal variation versus time.

According to [17], plants with mature leaves are not affected by the CCC reagent, as in Tillandsia incarnate Kunth (Ti) after seven months old and has long and thick leaves. Therefore the optimal concentration for this species CCC was 10 ppm. *Tillandsia lajensis Andre* (Tl) at 30 days showed the effect of the concentration of 10 ppm CCC presenting a variation of longitudinal growth of 0.28mm while the concentration of 0ppm longitudinal variable was 0.4mm indicating positive results. However, after 60days of evaluation longitudinal variation was not significant (Figure 4). By using high concentrations of CCC it inhibits the photosynthesis in the leaf of plants, leading to cell lysis [17]. At 60 days *Tillandsia lajensis Andre* (Tl) showed de pigmentation in their leaves which reduced the viability of the plant and thus its longitudinal variation was not significant.

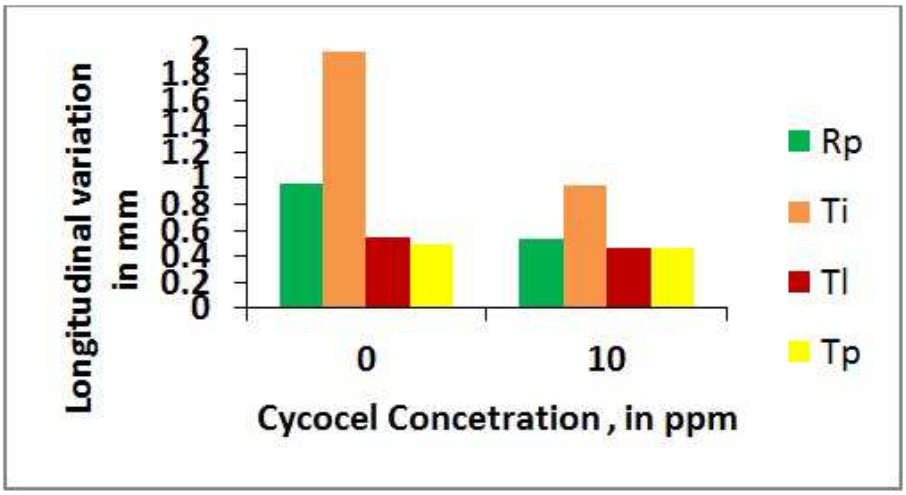

Figure 4. Longitudinal variation of the four species of bromeliads at 60 days.

Tillandsia pastensis (Tp) at 60 days of evaluation showed at 0 ppm concentration of CCC, the longitudinal variation was 0.49 mm while the at 10 ppm concentration of CCC the longitudinal variation was 0.46 mm which has a significant decrease (Figure 4). Figure 4 shows that the species *Tillandsia pastensis* (Tp) has a homogeneous growth of the two treatments with 0 and 10 ppm. According to [17], CCC phytotoxicity reagent affects the plant by not using the optimum concentration of CCC, which caused cell death preventing normal physiological growth of the species. This phenomenon was observed in most of them, thus causing, decrease in the viability and one only survived (Figure 4).

CONCLUSION

The process of *ex situ* adaptation and development of *in vitro* cryopreservation techniques and minimal growth allowed the implementation of a pilot germplasm bank for four species of bromeliads (*Racinaea pectinata Andre, Tillandsia incarnate Kunth, Tillandsia lajensis Andre* and *Tillandsia pastensis*). With forced ripening capsule technique into sterile containers obtaining contamination free seeds and 100% viable and ensured that they sowed in MS nutrient medium enabled multiplication in vitro all the four species of bromeliads germinated with sexual characters with seeds. The cryopreservation protocol developed in the laboratory could establish bromeliads cryo-storage pilot bank and avoided the toxicity of PVS2 plants (15% DMSO, 30% glycerol, 15% ethylene glycol and 0.04M sucrose). The minimum growth technique used in the laboratory (10 ppm Cycocel) allowed successfully to obtain collections of bromeliads in test tubes because efficiently decreases the speed of plant growth. In the technique of cryopreservation, the species that obtained the best results, when exposed to cryoprotectant solution PVS2 30 days was *Racinaea pectinata Andre* (Rp) and *Tillandsia lajensis Andre* (Tl) (p-value=0.0001), as its variation longitudinal and viability was greater than the other species.

In the technique of minimal growth, *Tillandsia incarnate Kunth* (Ti) obtained the best results when exposed to a concentration of 10 ppm Cycocel (CCC) for 60 days (p value=0.0001), as it managed to decrease the speed of longitudinal growth and remain a viable and hydrated plant. The four species of bromeliads are not adapted to the conditions of cryopreservation were *Tillandsia pastensi*s (Tp) and *Tillandsia incarnate Kunth* (Ti) (p-value=0.8613), whereas in the technique of minimal growth were *Racinaea pectinata Andre* (Rp) (p-value = 0.0008), *Tillandsia pastensis* (Tp)) (p value = 0.9999) and *Tillandsia lajensis Andre* (Tl)) (p value = 0.8887). The best method for implementing the pilot germplasm bank was the technique of minimal growth.

REFERENCES

[1] JM Manzanares. Biodiversity notebooks, **2001**, 38: 9-12.
[2] L Bohs, JME Miranda, MJJ Arellano, ABZ Salazar, MF Hernández, CR Quero, SL Pérez, Bases for community management of ornamental bromeliads, **2007**, pp . 98.
[3] J Pilco, EN Sandoval-Bucio, A Flores-Cruz y, Martínez-Bernal, Useful bromeliads, **2004**, 49 (4), 100-115.
[4] A Lowe; D Boshier; M Ward; C Bacles; C Navarro, *Heredity.*, **2005**, 95, 255-273.
[5] T Murashige; F Skoog, *Physiologic Plantarum.*, **1962**, 15, 473-497.
[6] N Acosta Molina, MA García, Dolores, Thesis. Metropolitan Autonomous University, Iztapalapa, **1999**, Mexico.
[7] OH Frankel, ME Soulé, Cambridge University Press, Cambridge, UK. **1992**, pp. 327.
[8] A Mroginski; W Roca; K Kartha, *Tissue culture in agriculture*., **2011**, 32: 1-16.
[9] A Medina, F Serrano, Biodiversity notebooks, **2012**, 38, 9-12
[10] WM Roca, R Escobar, G Mafla, International Center for Tropical Agriculture. CIAT, Cali. Colombia, **1994**, pp. 44.
[11] A Blanco, *Horticultural Physiology.*, **2013**, 36.
[12] MT González-Arnao; F Engelmann, *Horticultural Physiology*., **2013**, pp. 36.
[13] D Hirai; A Sakai, *Plant Cell Reports*, **1999**, 19, 150-155.
[14] N Westendorp, C Encina, Meeting in biology, **2008**.
[15] G Fernández; M Johnston, *Plant Physiology*., **2006**, 20: 21-24.
[16] R Weaver, University of California, Davis. Mexico DF. **1989**, pp. 622.
[17] OHP, *Product Information Bulletin*, **2008**, 301, 1-2.