Establecimiento *in vitro* de *Hypericum goyanesii* Cuatrec. E *Hypericum juniperinum* Kunth, a partir del cultivo de semillas

Establishing *in vitro Hypericum goyanesii* Cuatrec. E *Hypericum juniperinum* Kunth, from seeds culture

Belkys Adriana Pérez-Martínez*

DOI: 10.15446/rev.colomb.biote.v17n2.54294

Resumen

El propósito de este estudio fue evaluar medios de cultivo de germinación para el establecimiento *in vitro* de *Hypericum goyanesii* e *Hypericum juniperinum* como estrategia de conservación *ex situ,* por medios biotecnológicos de especies vegetales pertenecientes al ecosistema alto-andino. Las semillas se desinfectaron con hipoclorito de sodio y se sembraron en seis tratamientos basados en los medios de cultivo Murashige & Skoog (MS) y MS con reducción del 50% de sus macro y microsales y vitaminas, con y sin la adición de carbón activado y pulpa de banano. La germinación se favoreció por el empleo de pulpa de banano en el medio MS con reducción de sales y suplementado con carbón activado. Fue posible evidenciar que los explantes sexuales de *H. goyanesii* e *H. juniperinum* estuvieron influenciados positivamente en la variable porcentaje de germinación, por el empleo de sustancias orgánicas en reemplazo de reguladores de crecimiento.

Palabras clave: Hypericaceae, cultivo *in vitro*, germinación, desinfección, medios de cultivo.

Abstract

The purpose of this study was to evaluate germination culture media for the *in vitro* establishment *Hypericum goyanesii* e *Hypericum juniperinum* as *ex situ* conservation strategy for biotechnology of plant species belonging to the high-Andean ecosystem. The seeds were disinfected with sodium hypochlorite and seeded into six treatments MS and MS with 50% reduction of its macro and microsales and vitaminas, with and without the addition of activated charcoal and pulp banana. Germination is favored by the use of banana pulp in MS medium with reducing salts and supplemented with activated charcoal. It was possible to show that sexual explants and *H. goyanesii* e *H. goyanesii juniperinum* were influenced positively variable germination percentage, by the use of organic substances to replace growth regulators.

Key words: *Hypericaceae, in vitro* culture, germination, disinfection, culture medio.

Received: March 6, 2015 **Approved:** October 16, 2015

Introduction

Mountain ecosystems around the world are being seriously threatened, the most extreme and significant damages being in the Andes (Okada, 2001) which is the most extensive mountain range in the world and forms part of the páramo, considered to be one of the most anthropically affected ecosystems in the continent (Morales and Estévez, 2006). Colombia has not been an exception to this problem , since the páramos have been suffering serious processes of transformation, alteration and degradation, due to anthropic actions, such as burning; the use of firewood for fuel; the use of hedges; agricultural activities such as grazing and trampling; agricultural waste; shifting of agricultural borders; and urban planning and civil works (Office of the Comptroller General of Colombia, 2012). Therefore, it is necessary to generate scientific research and knowledge due to being essential elements to ensure the conservation of biodiversity (Josse C. *et al*. 2009).

* Ing. de Producción Biotecnológica. Profesional de investigación líder en propagación *in vitro*. Subdirección Científica. Jardín Botánico de Bogotá José Celestino Mutis. baperez@jbb.gov.co

In response to this, the José Celestino Mutis Bogotá botanical garden (JBB, for the Spanish original) uses plant tissue culture as an *ex situ* conservation strategy for promising, native, high-Andean and páramo species. Two of the prioritized species in this line of research are *H. goyanesii* and *H. juniperinum,* because of their potential for conservation, reintroduction or ecological restoration.

Taking into account that for these two species it is necessary to develop studies that determine the aspects that influence their seeds' germination, and that in turn, provide guidelines for the adequate management and use of them, this research was proposed with the aim to assess the *in vitro* germination responses in six culture media. With the methodology described herein, the aim is to generate complete seedlings to be used as sources of explants for micropropagation studies.

Materials and Methods

The field trips to collect plant material were carried out in the department of Cundinamarca. The fruits of *H. goyanesii* were collected in El Tablazo Reserve, on the road to the municipality of Subachoque (5°00´56.5"N - 74°12´34.0"W at an altitude of 3,437 m.a.s.l.). The plant material of *H. juniperinum* was collected in the páramo of Sumapáz at 3,690 m.a.s.l., with the following geographical coordinates: 4°17´24.2"N - 74°12´28.9"W. The collected fruits were transported in plastic bags to the plant tissue culture laboratory of the Scientific Subdivision of the JBB, where the seeds were recovered, cleaned and stored for two months at 4 °C**.**

The mature seeds were subjected to a surface disinfection process, submerging them in constant agitation for ten minutes in a 5% sodium hypochlorite solution with two drops of Tween 20. Subsequently, they were rinsed three times with microfiltered sterile water and placed in Eppendorf tubes in order to carry out another disinfection using the centrifuge. In the centrifugation process, they were rinsed once with 5% sodium hypochlorite and then rinsed three times with sterile microfiltered water. Each one of the rinses was for five minutes and at a rotation of 5,000 rpm (Pérez-M, 2014).

Six germination treatments based on mineral salts and vitamins of the M&S (Murashige and Skoog, 1962) medium were prepared at 100% (M&S) and at 50% (½ M&S) (Table 1). Banana pulp and activated carbon were used in the T2, T3, T5 and T6 treatments in order to assess their influence on the germination of the species under study, taking into account the results that these substances have provided in the *in vitro* germination of other species, especially those belonging to the Orchidaceae family. The supplements used in all the treatments were sucrose (15,000 mg/l) and agar (5,000 mg/l).

The nutrients of the culture medium were weighed in an analytical balance of accuracy and dissolved in microfiltered water. Glass recipients with a capacity of 100 ml were used for the distribution of 20 ml of medium in each one of them. The medium's pH was adjusted to 5.8, and the sterilization was carried out in an autoclave at 15 pounds of pressure per square inch (15 lb/in^2) for 15 minutes at an approximate vapor temperature of 121.5 °C.

Table 1. Treatments to induce germination in seeds of *H. goyanesii* and *H. juniperinum.*

Treatments	Description
T1	½ M&S
T2	½ M&S + 2,000 mg/l of activated carbon
T3	½ M&S + 2,000 mg/l of activated carbon + 30,000 mg/l of banana pulp
T4	M&S
T5	M&S + 2,000 mg/l of activated carbon
T6	M&S + 2,000 mg/l of activated carbon + 30,000 mg/l of banana pulp

Once disinfected, the seeds were planted in the six previously described germination treatments. Four seeds were planted per glass flask with culture medium. Five repetitions per germination treatment were carried out for *H. goyanesii*. The total amount of seeds evaluated for this species was 120. Three repetitions were planted for *H. juniperinum* with a total of 72 seeds evaluated. The flasks were maintained in the incubation room for 18 and 12 weeks, respectively; the time in which the germina-

tion variable was recorded. A natural photoperiod (12/12) was managed with a temperature range between 19 °C and 27 °C, and a humidity of 60% to 80%. The light intensity was between 1,500 lux and 5,000 lux.

Statistical analysis: A completely randomized experimental design was used for each of the two prioritized species. The recorded variable was the germination percentage. The results of this variable were subjected to an analysis of variance (ANOVA) through the SAS statistical program, and Duncan's new multiple range test was applied with a confidence level of 95%.

Results and Discussion

The disinfection system did not cause contamination of the seeds. This system was compared by Pérez-M (2014) with two other disinfection methodologies in which the average contamination recorded in the seeds of *H. goyanesii* and *H. juniperinum* was 47.5%.

It can be established that the fruits collected in their natural environment had good sanitary conditions, which facilitated complete disinfection using sodium hypochlorite. This is one of the commonly recommended substances for the surface disinfection of materials when being introduced to the *in vitro* culture (Abdelnour and Muñoz, 2005). It is useful as a germicide and oxidizing agent, and has the advantages of being very efficient for this purpose, being easily rinsed and very economical (Suárez, 1997).

The germination percentages obtained are displayed below for each evaluated species:

Hypericum goyanesii: The germination variable was monitored for 18 weeks. The percentages were zero (0%) for all the treatments except **T3** (½ M&S supplemented with 2,000 mg/l of activated carbon and 30,000 mg/l of banana pulp), where 20% germination was recorded at the end of the total evaluation time. Therefore, it is observed that the germination curve (Figure 1) was represented by the only treatment that provided optimum conditions for germination. When conducting the ANOVA, significant differences were found between the evaluated treatments ($p = 0.0471$). Duncan's new multiple range test indicated that the treatment that was statistically different to the others was **T3**, producing the only effect on the germination variable.

Hypericum juniperinum: During the 12 weeks of monitoring of the reproductive material of *H. juniperinum*, the germination variable was 0% for the culture media: **T1:** ½ M&S; **T2:** ½ M&S + 2,000 mg/l of activated carbon; **T4**: M&S; and **T5**: M&S + 2,000 mg/l of activated carbon.

For the **T6** medium**:** M&S supplemented with 2,000 mg/l of activated carbon and 30,000 mg/l of banana pulp, the germination processes started in the third week of evaluation with a percentage of 5%, which increased to 41.6% by the tenth week of monitoring. With the **T3** medium: ½ M&S supplemented with 2,000 mg/l of activated carbon and 30,000 mg/l of banana pulp, 25% of the seeds started germination in the third week of evaluation, and 100% by the sixth week of monitoring. Figure 2 shows the germination curve for *H. juniperinum*, where it is only possible to appreciate the germination response in the T3 and T6 treatments, because it was zero (0%) in the other treatments. When conducting the ANOVA, significant differences were found between the evaluated treatments

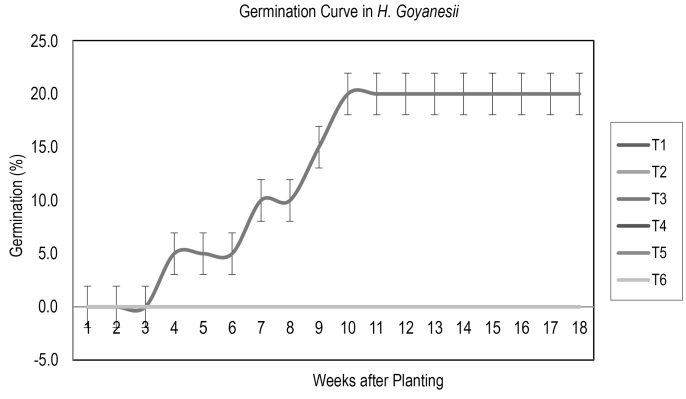

Figure 1. Monitoring of the germination percentage variable in *H. goyanesii* for 18 weeks after planting.

($p < 0.001$). Duncan's new multiple range test determined that in T3, a greater value of the mean (100) was obtained for the evaluated variable.

The *H. goyanesii* and *H. juniperinum* seeds showed a specific requirement of reduction of the concentrations of mineral salts and vitamins present in the M&S medium by 50%. Similar results were obtained by Pérez-M (2012), who reported that the highest percentage of *in vitro* germination (62.5%) for *Hypericum mexicanum* L. in an evaluation time of 15 weeks was recorded with the ½ M&S medium supplemented with 2,000 mg/l of activated carbon.

When this nutrient composition of the M&S medium together with activated carbon was supplemented with banana pulp, the germination percentages could be facilitated in *H. goyanesii* and *H. juniperinum*. Activated carbon is used to absorb toxic substances of the gaseous microatmosphere generated during *in vitro* incubation (Arditti and Ernst, 1993; Pedroza *et al*. 2010). Banana has a high content of sugars, vitamins, amino acids, antioxidants and growth-promoting agents (Arditti, 1993; Kitsaki *et al*. 2004; Moreno and Menchaca, 2007; Yam and Arditti, 2009; Yong *et al.* 2009).

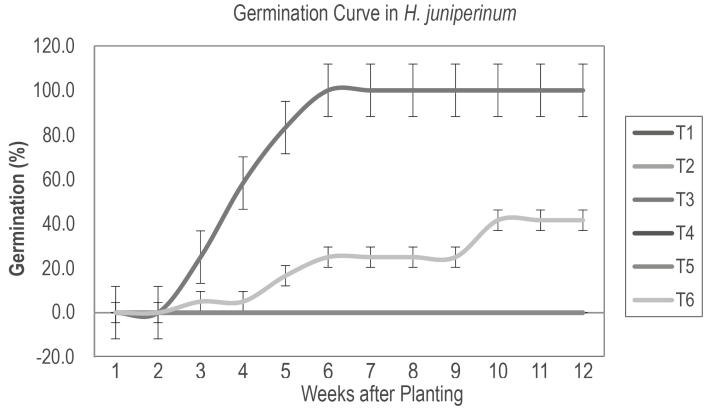

Figure 2. Monitoring of the germination percentage variable in *H. juniperinum* for 12 weeks after planting.

Conclusions

Considering that a key factor in the *in vitro* propagation processes is to achieve the disinfection of explants, on some occasions, achieving this objective becomes a problem when there is no plant material available, or when it is available but has a high degree of contamination for being collected in natural conditions. The use of seeds as a starting material is proposed as a very good alternative for obtaining explants in aseptic conditions when inducing their *in vitro* germination.

The nutritional requirements for the germination of the species were determined. The banana pulp improved the nutrient conditions of the **½** M&S medium, and together with the activated carbon, permitted complete germination in six weeks for *H. juniperinum*. The same nutrient conditions were the only ones that provided total germination of 20%, by the end of 18 weeks for the *H. goyanesii* species.

More studies are needed for both species to explain aspects such as germination by traditional or conventional methods, the nature and concentration of inhibitory substances of germination, light requirements and pretreatments of germination. However, it is considered that the results presented herein may be useful for the development of an *in vitro* production system that allows complete plants to be obtained from the seeds to be reintroduced into their natural habitat.

Acknowledgments

I would like to thank the José Celestino Mutis Bogotá botanical garden (JBB) under the management of the Director Luis Olmedo Martínez Zamora; the Assistant Scientific Director Mauricio Díazgranados, and the Coordinator of Program 2, Sandra Liliana Castañeda Garzón, for giving me the opportunity to form the team of the Scientific Subdivision.

References

Abdelnour, A., Muñoz, A. (2005). Micropropagación de teca (*Tectona grandis* L.f). Kurú: *Revista Forestal*, 2(5), 11 p.

Arditti, J., Ernst, R. (1993). *Micropropagation of orchids*. John Wiley & Sons, Inc., Nueva York. 1–25.

Contraloría General de la República. (2012). Informe del estado de los Recurso Naturales y del ambiente 2012 y 2013. Recuperado de http://www.contraloriagen.gov.co/documents/10136/76600464/INFORME_MEDIO_AMBIENTE_2012_2013_def_web.pdf.

Josse C., Cuesta F., Navarro G., Barrena V., Cabrera E.,Chacón-Moreno E., Ferreira W., Peralvo M., Saito J., Tovar A. (2009). Ecosistemas de los Andes del Norte y Centro. Bolivia, Colombia, Ecuador, Perú y Venezuela. Secretaría General de la Comunidad Andina, Programa Regional ECOBONA-Intercooperation, CONDESAN-Proyecto Páramo Andino, Programa BioAndes, EcoCiencia, NatureServe, IAvH, LTA-UNALM, ICAE-ULA, CDC-UNALM, RUMBOL SRL. Lima.

Kitsaki, C., Zygouraki, S., Ziobora, M., Chintziest, S. (2004). *In vitro* germination, protocorm formation and plantlet development of mature versus immature seeds from several *Ophrys* species (Orchidaceae). *Plant Cell Reports*, *23*, 284–290.

Morales-Betancourt, J. A., Estévez-Varón, J. V. (2006). El Páramo:¿Ecosistema en vía de extinción? *Revista Luna Azul*, 22, 39-51.

Moreno-Martínez, D; Menchaca-García, R. (2007). Efecto de los compuestos orgánicos en la propagación *in vitro* de *Stanhopea trigina* Bateman (Orchiadeace). *Foresta Veracruzana*, 9(2), 27-32.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco cultures. *Physiologia Plantarum,* 15, 473-497.

Okada-Katsuo, A. (2001). La biodiversidad y los peligros que la amenazan. En: Perea Dallos Margarita (ed). *Biotecnología agrícola: Un enfoque hacia el mejoramiento de plantas*. Bogotá: Editora Guadalupe, pp. 29 - 41.

Pedroza-Manrique, J.A., Serrato-Muñoz, L.C., Castaño-Robayo, M. (2010). Efecto del carbón activado y ácido indol acético en el desarrollo de protocormos de *Masdevallia coccinea* Linden ex Lindl. y *Maxillaria nutans* Lindl. *in vitro*. *Revista Colombiana de Biotecnología*, 12 (2), 86-102.

Pérez-Martínez, B.A. (2014). Protocolos de propagación por técnicas biotecnológicas de dos (2) especies priorizadas (*Hypericum goyanesii* Cuatrec. e *Hypericum juniperinum* Kunth) para la conservación, reintroducción, restauración y ecología urbana y su validación masiva. Informe técnico inédito contrato 600-2013. Jardín Botánico José Celestino Mutis–Subdirección Científica. Bogotá D.C.

Pérez-Martínez, B.A. (2012). Protocolos de propagación por técnicas biotecnológicas de dos especies priorizadas en el proyecto 318 *(Hypericum mexicanum y Diplostephium rosmarinifolium)* y apoyo en actividades de transferencia de información a la comunidad. Informe técnico inédito contrato 209-2012. Jardín Botánico José Celestino Mutis–Subdirección Científica. Bogotá D.C.

Suárez, A. E. (1997). Métodos de asepsia y esterilización. En: M. Perea y J. Cedeño (Eds.). Cultivo de Tejidos Vegetales y sus Aplicaciones en la Agricultura. Curso: UDO-OIEA, Maturín, Venezuela. Pp. 33-40.

Yam, Tim., Arditti, J. (2009). History of orchid propagation: a mirror of the history of biotechnology. *Plant Biotechnology Reports*, *3* (1), 1-56.

Yong, J., Ge, L., Yan, F., Ngin, S. (2009). The chemical composition and biological properties of coconut (*Cocos nucifera* L.) water. *Molecules*, 14, 5144-5164.