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

Belkys Adriana Pérez-Martínez*

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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.

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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).
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/in²) 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-
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.](image-url)
In Vitro Germination of Hypericum

(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).

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.

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