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In vitro spore culture and reproductive aspects of the annual fern *Anogramma chaerophylla* (Pteridaceae)

MARÍA L. LUNA[1,2], AGUSTINA YAÑEZ[1,3,4], JUAN PABLO RAMOS GIACOSA[1,4], DANIEL GORRER[1,4], PEDRO C. BERRUETA[1,4] and GABRIELA E. GIUDICE[1]

Summary: *Anogramma chaerophylla* belongs to a fern genus with annual sporophytes and potentially perennating gametophytes. In the studied area, Natural Reserve Punta Lara, the plants grow stressed mainly by pollution and the invasion of exotic species. As a part of a project on conservation of ferns inhabiting Buenos Aires province, the objectives of this work were to evaluate in *Anogramma chaerophylla* the conditions for *in-vitro* spore germination and to analyse the different stages of its reproductive cycle. Spores were sterilized in an aqueous solution 10 % of NaClO during different times and then sown in Petri dishes with Murashige & Skoog medium, without the addition of sucrose. The dishes were kept under laboratory conditions, at 12 h light/ darkness photoperiod and a temperature of 22 (± 2) °C. After two weeks, the percentage of germination was 80%. The spore germination pattern corresponds to the *Vittaria* type and the prothallus development was *Ceratopteris* type. Gametangia developed first in the bended thalloid region of the prothallus and then bisexual tubercles originated near this zone. The sporophytes developed only in association with the tubercles. During culture in plastic pots the sporophytes gave origin to a second generation of prothalli. Our preliminary findings demonstrate that the *in vitro* culture technique is suitable for *A. chaerophylla* propagation as a strategy for *ex-situ* conservation.

Key words: *Anogramma chaerophylla*, micropropagation, gametophyte, tubercle, *ex-situ* conservation

Resumen: El cultivo *in vitro* de esporas y los aspectos reproductivos del helecho anual *Anogramma chaerophylla* (Pteridaceae). *Anogramma chaerophylla* pertenece a un género de helechos con esporofitos anuales y gametofitos potencialmente perennes. En el área de estudio, la Reserva Natural Punta Lara, la vegetación crece bajo estrés debido principalmente a la contaminación y la invasión de especies exóticas. Como parte de un proyecto de conservación de helechos que habitan en la provincia de Buenos Aires, los objetivos del trabajo fueron evaluar en *Anogramma chaerophylla* las condiciones para la germinación de esporas *in-vitro* y analizar las distintas etapas de su ciclo reproductivo. Las esporas fueron desinfectadas en solución 10 % de NaClO durante distintos tiempos y sembradas luego en medio de cultivo Murashige – Skoog, sin agregado de sacarosa. Las cápsulas se incubaron con un fotoperíodo de 12 h luz/oscuridad y una temperatura de 22 (± 2) °C. A las dos semanas germinaron el 80% de las esporas. El patrón de germinación fue tipo *Vittaria* y el desarrollo del protalo tipo *Ceratopteris*. Los gametangios aparecieron primero en la región engrosada del protalo donde posteriormente se originaron los tubérculos bisexuales. Los esporofitos se desarrollaron solo en asociación con los tubérculos. Durante su cultivo en macetas, éstos dieron origen a una segunda generación de protalos. Nuestros resultados demuestran que la técnica de cultivo *in vitro* es apropiada para la propagación de *Anogramma chaerophylla* y como estrategia para su conservación *ex-situ.*

Palabras clave: *Anogramma chaerophylla*, micropropagación, gametofito, tubérculo, conservación *ex-situ*.

[1] Cátedra Morfología Vegetal, Facultad de Ciencias Naturales y Museo (UNLP). Boulevard 120 y 61, 1900, La Plata, Argentina, lujanluna@fcnym.unlp.edu.ar.
[2] Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (CIC-BA).
[3] Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Av. Ángel Gallardo 470, C1405DJR, Ciudad Autónoma de Buenos Aires, Argentina.
[4] Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET).

Introduction

The genus *Anogramma* Link (Pteridaceae) comprises seven species of homosporous ferns with typically annual small sporophytes and potentially perennating gametophytes (Nakazato & Gastony, 2003). These species grow often in environments with alternating wet and dry seasons (Tryon & Tryon, 1982).

Anogramma is found mainly in the tropics of Mexico and Central and South America, although *Anogramma leptophylla* (L.) Link inhabits also in Africa, southern Europe to northern India, Australia and New Zealand (Tryon & Tryon, 1982; Nakazato & Gastony, 2003).

A striking feature of *Anogramma* is that prothalli are considered perennial, because they carry tubercles that can become dormant during stressful seasons and support the expansion of a latent embryo when favorable conditions arrive (Goebel, 1889; Baroutsis, 1976; Mehra & Sandhu, 1976; Page, 2002). Additionally, tubercles contain starch grains and other food reserves that may be consumed by the sporophyte as it grows (Goebel, 1969; Pangua et al. 2011).

In Argentina, *Anogramma chaerophylla* (Desv.) Link grows from Misiones and Jujuy provinces in the north through the centre-east, reaching its southernmost distribution in Buenos Aires province (Zuloaga *et al*., 2008). The Natural Reserve Punta Lara is located on the Coast of La Plata River and constitutes the core area of the Biosphere Reserve Parque Pereyra Iraola. In this zone *A. chaerophylla* grows associated with gallery forests that border internal streams, where vegetation and in particular ferns are stressed by urbanization, furtive pulling out and invasion of exotic species (Giudice *et al*., 2011).

In vitro spore germination is a reliable method for the propagation of ferns and is often employed for their conservation (Fay, 1994; Barnicoat *et al*., 2011; Soare, 2008; Baker *et al*., 2014).

In the genus *Anogramma* most of the studies on spore germination and prothallus development were conducted in the broadly distributed species *A. leptophylla* (Mehra & Sandu, 1976; Pangua & Vega 1996; Hagemann, 1997).

As a part of a project on conservation of ferns inhabiting Buenos Aires province, the objectives of this work were to evaluate in *Anogramma chaerophylla* the conditions for *in-vitro* spore germination and to analyse the different stages of its reproductive cycle.

Material and Method

Sampling zone: The Natural Reserve Punta Lara is located on the coast of Río de la Plata, Buenos Aires, Argentina (34º47' S 58º01' W), where diverse environments like marginal forest, espinal, flooded scrublands and grasslands occur. The weather data for the region (expressed in mean annual values) are: precipitation 994 mm, temperature 16.5 °C and humidity 80%. The warmest month of the year is January with an average maximum temperature of 30.5 °C whereas July is the coldest, with an average minimum of 7.3 °C (www.estadistica.laplata.gov.ar/paginas/climasueloLP.htm).

During hikes in the Reserve only ten individuals of *A. chaerophylla* were registered in an area of 40 ha, growing in isolation on fallen tree trunks or in the soil (Fig.1A-B). They were found in fertile stage from November to May. Material was collected from the months of January to March, over three seasons. Vouchers were deposited at Herbario Museo de La Plata (LP), Universidad Nacional de La Plata.

***In vitro* propagation:** Assays were carried out with fresh spores. For this, portions of pinnae with mature closed sporangia were placed in paper envelopes and set to dry at room temperature (ca. 25 °C) until spore release. Then, material was sieved with a metallic mesh with pores 44-88 µm in diameter to eliminate the remains of sporangia.

The spores were transferred to paper envelopes and sterilized through immersion in an aqueous solution 10 % of NaClO (5 gl/1) during 1- 3 and 5 minutes. Afterwards the envelopes containing the spores were rinsed three times with distilled water.

Spores were sown in Petri dishes 5 cm in diameter containing Murashige & Skoog medium (Phyotechnology Laboratories™) and Difco Bacto-agar (7 g/l) without the addition of sucrose, previously autoclaved at 120 ºC for 20 minutes. One percent nystatin (Denver Farma, 100000Ul /g) was added to the culture medium as a fungicide (Ranal, 1999).

The dishes were kept under laboratory conditions, at 12 h light/darkness photoperiod (white fluorescent illumination 28 µmol m^{-2} s^{-1}) and a temperature of 22 (±2) °C, commonly used in the cultivation of *Anogramma* (Pangua & Vega, 1996; Pangua *et al*.

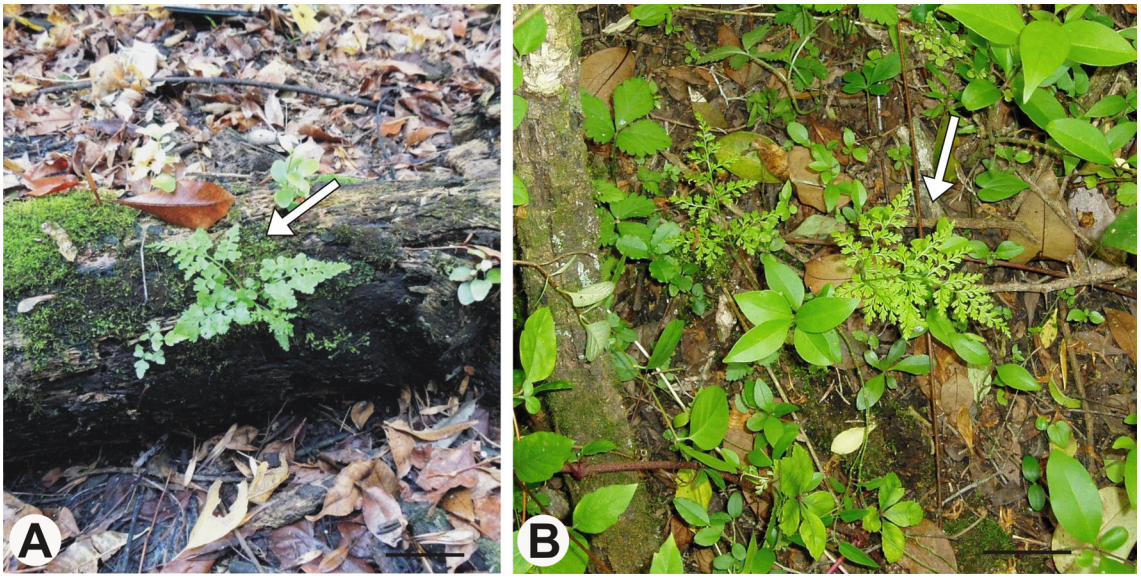

Fig. 1. A-B: *Anogramma chaerophylla* growing in Natural Reserve Punta Lara. A: an individual growing on a fallen tree trunk (arrow). B: specimen growing in the soil among leaf litter (arrow). Scales: A: 10 cm; B: 5 cm.

2011; Baker *et al*. 2014). The number of capsules used was twenty.

The germination percentage was calculated from a random sample of 50 spores per capsule. Petri dishes were observed every two days under stereoscopic microscope Nikon SMZ 1000. Different stages of prothallus, tubercle and sporophyte development were documented under stereoscopic microscope and Nikon Eclipse E 200 light microscope.

When the development of sporophytes was noticed, material was transferred to plastic pots with fertile soil and brought to natural conditions of temperature, light and humidity.

Results

Spore germination and gametophyte development: After spores are sterilized during different times, only those immersed for 1 minute in the aqueous solution of sodium hypochlorite remain viable. Germination begins six days after inoculation onto the culture medium and it is evidenced by the emergence of a rhizoid (Fig. 2 A). Two weeks after sowing 80% of the spores germinate.

Successive mitotic divisions give rise to a uniseriate filament, 3-4 cells in length (Fig. 2 B). In a next step the apex is divided into two directions (Fig. 2 C) and as development progresses (near two months after spore culture) the prothallus acquires an asymmetrical spatula shape (Fig 2 D-F). One month later, the prothallus bends around the growth zone and is curved into a helix, acquiring a funnel shape (Fig. 2 G).

Five months after spore culture archegonia as well as antheridia are developed in the meristematic region of the prothallus (Fig. 3A-B). Then, tubercles appear next to the multi-layered portion where gametangia originated previously (Fig. 4 A). The tubercles can be distinguished in early stages as tiny white bulges, that acquire a rounded shape as they increase in size (ca. 2 mm in diameter). Archegonia as well as antheridia are originated on their surfaces (the former in greater amount), among numerous rhizoids (Fig. 4 B). Sometimes propagation is registered in smaller tubercles.

Sporophyte development: About six months after spore germination, sporophytes emerge from the archegonia located in the tubercles, in a number of one by each (Fig. 4 C). The first leaves develop

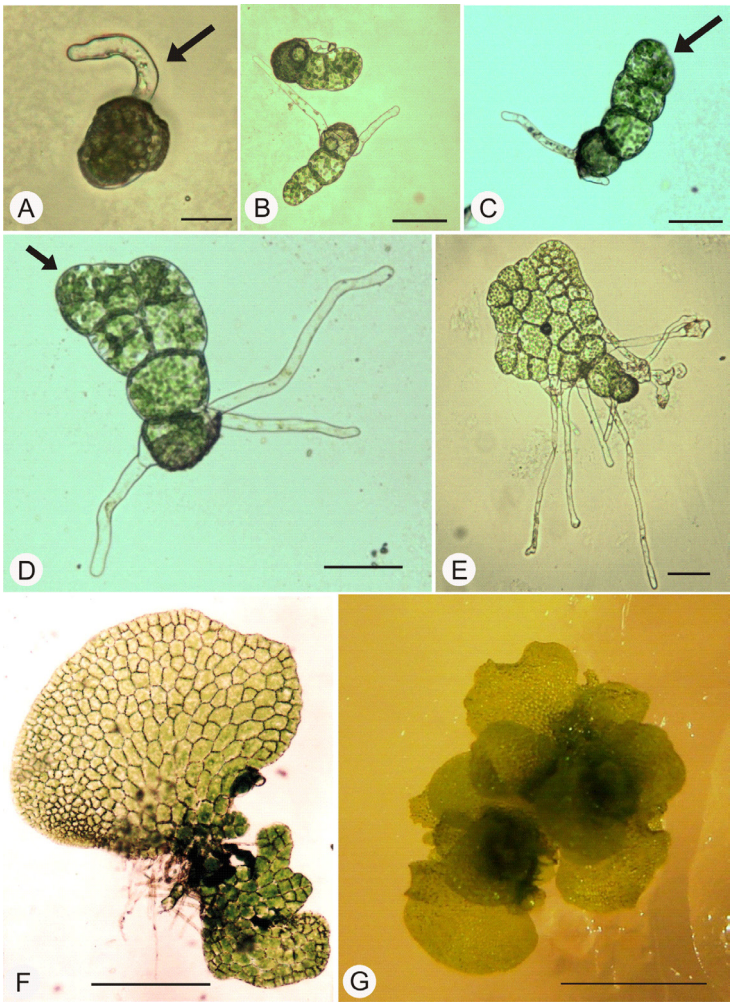

Fig. 2. A-G: *In vitro* prothallus development. A: germinated spore showing the emerged rhizoid (arrow). B: filamentous uniseriated stage of prothallus development. C: later step showing divisions at different directions in the apex of a filament (arrow). D: early stage of asymmetrical spatula-shaped prothallus (the arrow points to the meristematic zone). E: a more advanced stage of asymmetrical development. F: last stage of spatula- shaped prothallus before starting to bend. G: funnel-shaped prothalli. Scales: A, C, D-E-F: 50 µm; B-G: 100 µm.

soon as photosynthetic organs (Fig. 5 A-B). Near two months later the sporophyte is made up by four-five leaves originated from the short shoot that remains attached to the tubercle (Fig. 5 C). At this stage sporangia are already developed. Continued growth of the sporophytes gives rise to healthy plants 15-20 cm height (ca. ten months after spore culture), whose viable spores fall into the pots and germinate originating a new generation of prothalli (Fig 5 D-E).

Discussion and Conclusions

Our preliminary findings demonstrate that the *in vitro* culture technique is suitable for *A. chaerophylla* propagation, since many prothalli and sporophytes were obtained in laboratory conditions. Sporophytes developed only from the tubercles, the perennating reservant organ exclusive of the genus *Anogramma*, which shelter the embryo until favorable conditions

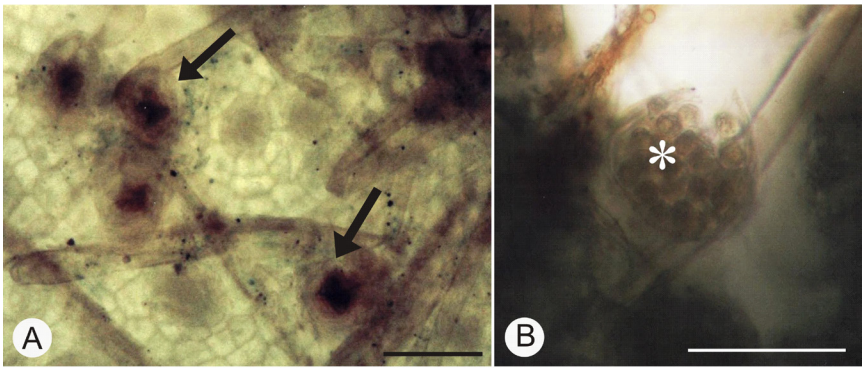

Fig. 3. A-B: Gametangia developed in the green prothallus. A: archegonia (arrows) surrounded by rhizoids. B: antheridium with antherozoids (asterisk). Scales: A: 50 µm; E: 40 µm.

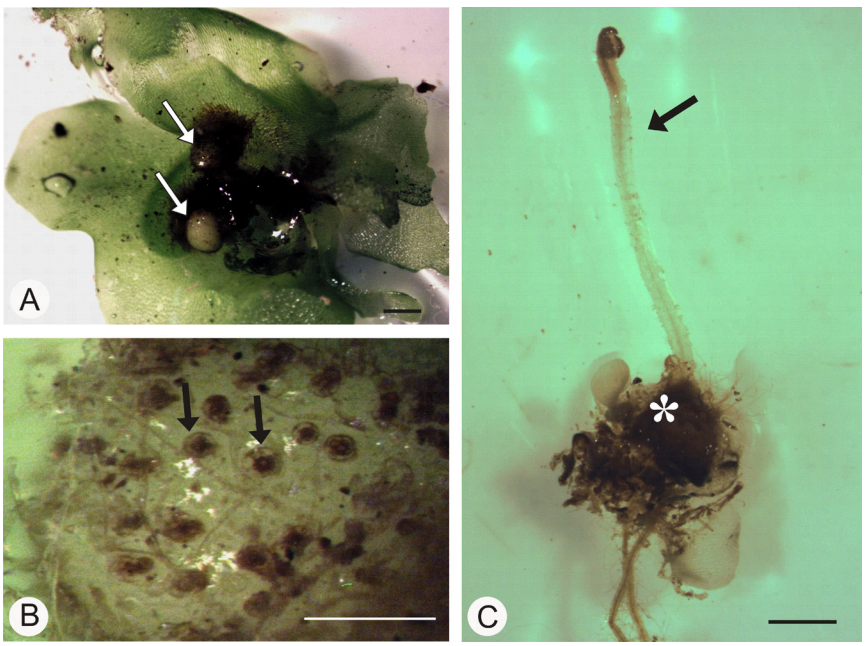

Fig. 4. A-C: Tubercles of *Anogramma chaerophylla*. A: tubercles developed on prothalli's bottom surface covered by profuse rhizoids (arrows). B: detail of tubercle with numerous archegonia (arrows). The scarce antheridia are not shown. C: a young sporophyte (arrow) emerging from the tubercle (asterisk). Scales: A, C: 2mm; B: 200 µm.

for sporophyte emergence arrive (Goebel, 1889; Baroutsis, 1976; Hagemann, 1997). The use of controlled conditions in our assays (temperature, humidity and photoperiod) appears to reduce the adverse factors, such as pollution or drought periods, that prevent sporophyte development and setting of larger populations in the Reserve. However, this hypothesis remains to be proved.

The spore germination pattern of *A. chaerophylla* followed the *Vittaria* type of Nayar & Kaur (1971) whereas the prothallus development was *Ceratopteris* type. Huckaby *et al.* (1981) found

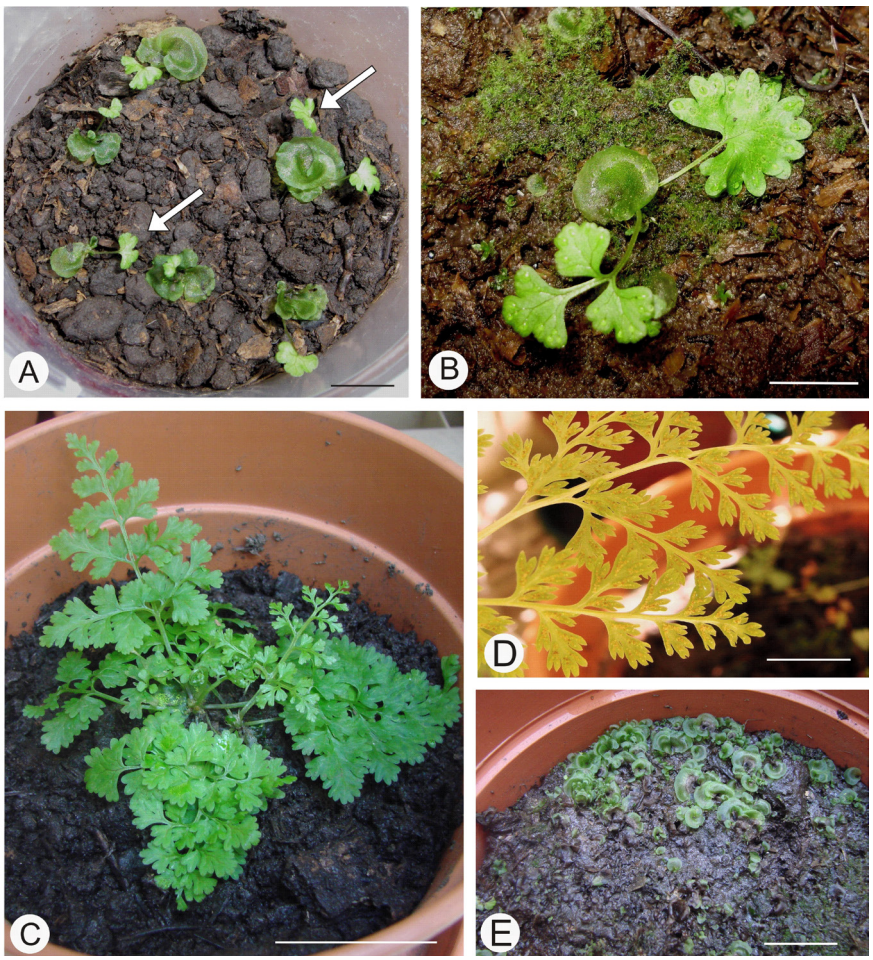

Fig. 5. A-E: *Anogramma chaerophylla* sporophytes and a second generation of prothalli grown under culture conditions. A: young sporophytes emerging from tubercles located on prothalli's bottom side (arrows). B: detail of sporophyte with two early photosynthetic leaves. C: near six-month old sporophytes grown under culture conditions. D: portion of a leaf showing sporangia. E: new generation of prothalli originated from spores that fell in the pots. Scales: A-B: 0.5 cm; C: 5 cm; D: 1 cm; E: 3 cm.

for *A. chaerophylla* kept under cultivation in USA the same germination pattern, but in this case employing Knop's medium and red light. Although it is known that culture conditions, like substrate or light, can bring modifications in the germination pattern (Hagemann, 1997), it seems that different situations conduce to the *Vittaria* type. Concerning prothallial development, it coincides with findings in *A. leptophylla* (Pangua & Vega, 1996), being the *Ceratopteris* type in both species.

The sporophytes of *A. chaerophylla* were obtained in our assays in the absence of sucrose. Kuriyama *et al*. (2004) and Baker *et al*. (2014) found that the aggregate of sucrose in MS medium inhibited sporophyte production in *Adiantum capillus-veneris* L. and in *Anogramma ascensionis* (Hook.) Diels, respectively. Thus our findings are in agreement with these authors.

The sporophytes of *A. chaerophylla* became fertile in the pots and the viable spores gave rise to a second generation of prothalli. In this manner, periodically watering seems to favor natural fertilization and the production of numerous embryos. Hence, under greenhouse conditions the

sporophytes of *A. chaerophylla* arose at any time in the year, in coincidence with Molnár *et al.* (2008) observations in *A. leptophylla*.

Our preliminary findings encourage us to continue applying this methodology to promote *ex-situ* conservation of *A. chaerophylla*. Recently, Baker *et al.* (2014) proved that *in vitro* culture of spores together with *ex situ* cultivation of the sporophytes resulted successful for the survival of the critically endangered species *A. ascensionis*.

Currently, the sporophytes *A. chaerophylla* are being rusticated to be brought to suitable areas of the Reserve Punta Lara, as strategy of reinforcement of the natural populations as well as the spore soil bank.

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