THE PRESERVATION OF *GOODYERA MACROPHYLLA* LOWE BY *IN VITRO* GERMINATION

By F. M. Fernandes [1], Salomé Soares Pais [2] & M. Costa Neves [3]

With 6 figures

ABSTRACT. Ripe seeds of the endangered madeiran endemic orchid *Goodyera macrophylla* were grown on MS/2 solid media in darkness. Protocorm development was occurred under reduced photon flux conditions. The average of plantlets obtained per capsule was 279.7 ± 44.45. Plantlets were subcultured on MS/2, KC and K 43 medium (modified KC). Plantlets growth was highest on K43 medium. The plantlets obtained after nine months *in vitro* culture, were transferred to field conditions in the Jardim Botânico da Madeira, with 100% success. After one year acclimatisation at the Jardim Botânico da Madeira, 30 plants were transferred to this species natural habitat. After six years in the wild conditions, 6 of the reintroduced specimens remain growing in good conditions, while the others were lost by unknown factors.

Key words: Endemic orchid, *in vitro* germination, orchid conservation.

RESUMO. Sementes maduras da orquídea endémica madeirense ameaçada, *Goodyera macrophylla*, foram semeadas ao escuro em meio sólido MS/2. O desenvolvimento dos protocormos ocorreram em condições de baixa luminosidade. Obteve-se uma média de 279,7 ± 44.45 plântulas por cápsula. As plântulas foram inoculadas em meio MS/2, KC e K43 (KC modificado), verificando-se maior crescimento no meio K43. Após nove meses de cultura *in vitro*, as plântulas aclimatizadas no Jardim Botânico da Madeira tiveram 100% de sucesso. Após um ano de aclimatização, 30 plântulas foram reintroduzidas no seu habitat natural. Após seis anos sobreviveram 6 especimens, sendo os restantes perdidos devido a factores desconhecidos.

Palavras-chave: Orquídea endémica, germinação *in vitro*, conservação de orquídeas.

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[1] Jardim Botânico da Madeira, Quinta do Bom Sucesso, 9050 Funchal, Madeira, Portugal; CCBG (Centro de Ciências Biológicas e Geológicas da Universidade da Madeira).

[2] Centro de Biotecnologia Vegetal, Dept. Biologia Vegetal, F.C.U.L. Bloco C2, Campo Grande, 1700 Lisboa, Portugal.

[3] Centro de Floricultra - Lugar de Baixo, 9360 Ponta do Sol, Madeira, Portugal; CCBG.

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INTRODUCTION

It cannot be stressed enough that *in situ* conservation is the only long-term strategy to conserve flora and fauna. Pressure on Europe's flora is great and, in many cases, it will not be possible to protect the most species *in situ*. *Ex situ* conservation, endangered especially of threatened taxa, is essential to back up populations of native, semi-natural or traditionally managed plant communities. However it cannot substitute genetic and evolutionary processes that operate in the wild conditions (JACKSON & AKEROYD, 1994). Living material preserved in a local botanic garden may supplement a failure to preserve the plant in the wild (LESOUEF, 1985). Plant biodiversity is Europe's most precious resource, and botanical gardens should have a central role in the protection of native plants for the use and enjoyment of future generations. An *ex situ* reserve collection can be maintained by means of seed banks, and *in vitro* material, pollen banks, botanical gardens and other living collections, some of which will be on public display. Micropropagation provides a basis for the recovery of orchids, which more than any other group of European plants has suffered from habitat loss and the interest of rapacious collectors (STEWART, 1989). Micropropagation of orchids has become a standard technique in some tropical gardens, and European orchids micropropagated at the Royal Botanic Gardens, Kew, have already been reintroduced to nature reserves (FAY & MUIR, 1991).

Goodyera macrophylla (Fig. 1, A & B) is a very rare terrestrial endemic orchid of Madeira and was last reported in 1889 by CARLOS MENEZES (Jardim Botânico, Universidade de Lisboa). Since then it was considered an extinct species. However MANUEL DE NÓBREGA (1957) and HENRIQUE COSTA NEVES (1975) (personal communications), found this orchid at Chão da Ribeira in Madeira island. *G. macrophylla* is listed on Red Data Book edited by (IUCN, 1983,1985) and EC Directive (92/43/CEE). The aim of this work was to germinate this orchid *in vitro* as a strategy for the recovery of this threatened species.

MATERIAL AND METHODS

Plant material

To guarantee the production of viable seeds, *ex situ* flowering plants in Ribeiro Frio were hand pollinated in October and the seed capsules were collected in December. Different degrees of maturation were recovered from the base to the top of the inflorescences. When the capsules at the base are mature, those from above must be collected and used for culture initiation.

Culture establishment

Solid half strength MS (MURASHIGE & SKOOG, 1962) medium (500 ml) supplemented with sucrose at 10g/l was transferred to Erlenmeyer flasks (1000 ml). The pH was adjusted to 5.7 before adding 7.0 g/l agar. The medium was autoclaved at 121° C (1 atm.) for 20 min.

A

B

Fig. 1, A & B - *G. macrophylla* in natural habitat.

Capsules were surface sterilised for 15 min in domestic bleach (20%=1% active chlorine), washed 3 times in sterile destilled water, and immediately dissected and aseptically transferred (one capsule/flask).

Seed cultures were kept in culture chambers at 22° ± 1°C, in darkness during the first two months. After this period cultures were transferred to a 16 h/day photoperiod with a photon flux 4μE. m^{-2}. s^{-1}. Three to four months later, liquid medium with the same composition as the original one was added to the cultures, to restore the metabolised nutrients.

Culture growth

To test the best media for growth, plantlets (Fig. 2) were subcultered to 200ml of the following media: 1) half strenght MS medium; 2) K43 (modified Knudson (KC) medium containing homogenised banana (80 g/l) prepared with unripe bananas without tegument, bacteriological meat extract - Biokar Diagnostics (1 g/l), fish emulsion fertilizer - Fogg It Nozzle Company (2 g/l), charcoal (2 g/l), pepton (2 g/l) and coconut milk (10 ml/l)) and 3) Knudson (KC) (KNUDSON, 1964). All media was supplemented with sucrose at 10g/liter. The pH was adjusted to 5.7 before adding 7.0 g/l agar and autoclaved at 121° C (1 atm.) for 20 min. The subcultured plantlets were kept under reduced photon flux (4μE. m^{-2}. s^{-1}) in 500 ml flasks.

To test the influence of light on plant growth, plantlets were subcultured in glass flasks (9×8 cm) containing 40 ml of half strength MS medium under 50μE. m^{-2}. s^{-1} and 4μE. m^{-2}. s^{-1}.

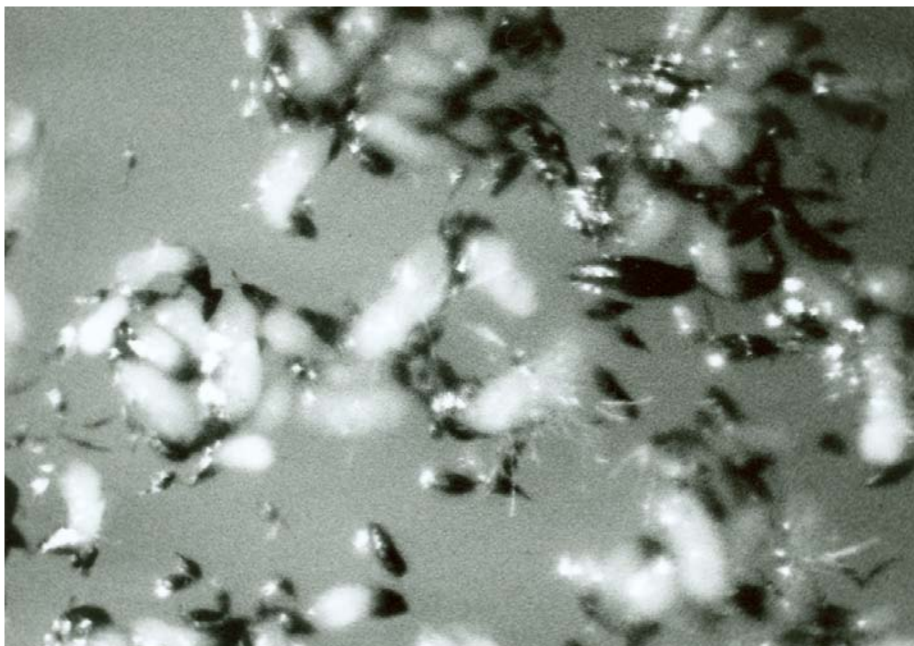

Fig. 2 - Protocorms of *G. macrophylla*, 2 months *in vitro* (10 x).

Acclimatisation

In vitro obtained plants (Fig. 3) were washed in running tap water to remove the agar and transferred to pots containing peat and humus from *Erica arborea* (50/50) and acclimatised in a Phytotron unit with a high relative humidity (100%), which was gradually decreased (10% a week), during a month. The acclimatised plants were transferred to a greenhouse in the Jardim Botânico da Madeira. Another group of plants was transferred without acclimatisation, directly to the greenhouse.

Fig. 3 - Plantlets of *G. macrophylla*, 9 months *in vitro*.

Statistical analysis

Statistical analyses were done using 30 samples. The percentage of germination was expressed as the nº of plants acclimatised per nº seeds/capsule x 100. Selection of the best culture medium and best photon flux for growth was based on the mean of plant growth ($\pm$SE), with an approximate 95 percent confidence interval for μ. To determine the number of seeds/capsule, a capsule content was suspended in 2ml water. Ten samples of each capsule were taken and repeated for ten capsules. The total number of seeds/sample was determined using an hemacytometer. The number of seeds/capsule was calculated using the formula ($X \times 8 \times 10^4 N \div Z$), with X=estimated mean, N = volume (ml) of water suspended seeds, Z=no of square counted in the hemacytometer. Estimated mean of all samples is presented with standard error, 95 percent confidence intervals for μ.

RESULTS AND DISCUSSION

Europe has a rich resource of botanical gardens that play a variety of roles in scientific research, amenity, education and conservation of threatened plants. Globally, much of the conservation activity of botanical gardens is co-ordinated by BGCI (Botanical Gardens Conservation International). This institution (BGCI) has developed a worldwide database on threatened plants already maintained in cultivation, particularly those conserved by botanic gardens.

The BGCI database lists those institutions cultivating 75% of the rare and endangered Macaronesia species (JACKSON & AKEROYD, 1994). According to the BGCI report of the known occurrence of rare and threatened plants (pteridophytes included) in botanical gardens (personal communication), there are listened, for the Madeira archipelago, 97 species (43 rare, 31 vulnerable, 20 endangered, 1 neither rare or threatened, 1 insufficiently known, 1 indeterminate). *Goodyera macrophylla* is in the Royal Botanic Garden, Kew and the Conservatoire Botanique National de Brest.

During the last years *in vitro* culture has emerged as a powerful tool for the effective propagation of many plant species. In some cases it enables the production of many individuals from just a small piece of tissue. The potential of such a technique in the recovery of endangered species is obvious (WOCHOK, 1981). Recovery programmes to perpetuate endangered species should, where possible, bear in mind genetic diversity, minimising selection, avoiding hybridisation and continuity (LESOUEF, 1985).

Orchid seeds are produced by mother plants in high amounts (10^2 to 10^5 seeds per capsule). In *G. macrophylla* the n_o seeds/capsule was about 12700±2300. These seeds can not be sown in the usual way because they are exceptionally small and lack reserves. Therefore, they need to establish a symbiotic association with a fungus (mycorrhiza). This happens, especially in terrestrial orchids and, to a lesser extent in tropical orchids as well (PENNINGSFELD, 1985). The germination rate obtained for seeds from ripe capsules of *G. macrophylla* was 2.2±0.35 %, which gives rise to a total of 279.7±44.45 plantlets per capsule, higher than that reported for other orchid species (ARDITTI, *et al.*, 1981). The K43 medium, the best for *G. macrophylla* growth (Fig. 4), has already been reported as the best for *in vitro* germination and growth of some cultivated orchids (MARGARIDA COSTA NEVES, personal communication). *Goodyera* seedlings develop faster (less than one year) than those from other orchids species (ARDITTI, *et al.*, 1981), and require photon flux reduction for good growth (FERNANDES, 1993). Plantlets maintained on half strength MS medium (Fig. 5), when kept under 4μE. $m^{-2}.s^{-1}$, showed a better growth rate (75±7.3 mm) when compared to those kept under 50μE. $m^{-2}.s^{-1}$ (55±4.62 mm). *In vitro* light conditions reproduce natural environment were seeds start germination. Nine months after sowing, plantlets could be successfully acclimatised because plants produced well developed roots *in vitro*. The success on the acclimatisation of plantlets was 100%, either when the Phytotron unit was used or when plantlets were transferred directly to the greenhouse.

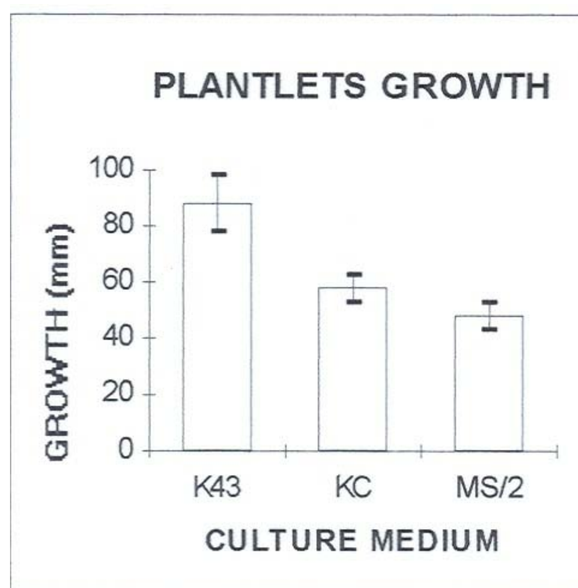

Fig. 4 - Effect of different culture medium on plant growth.
K43- Modified KC
KC - Knudson medium
MS/2 - MS medium (half strenght)

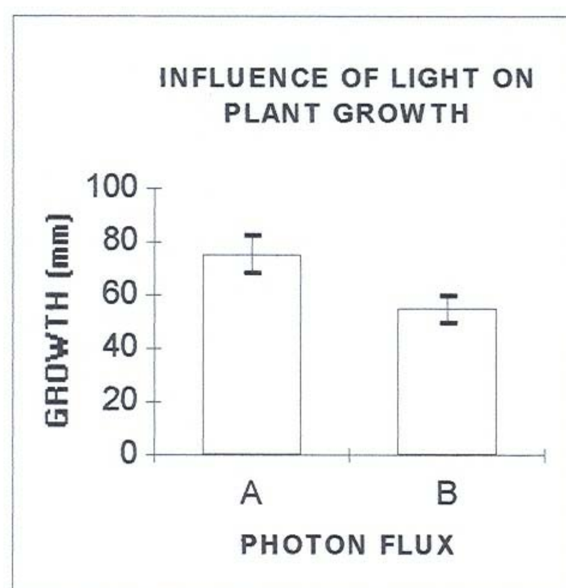

Fig. 5 - Photon flux (μ E. m^{-2}. s^{-1}) and plant growth (A - 4 μ E. m^{-2}. s^{-1}; B - 50 μ E . m^{-2}. s^{-1}).

Seeds of some terrestrial orchids are difficult to germinate asymbiotically (ARDITTI, 1967), and others are not (HADLEY, 1970). Our findings suggest that *in vitro* germination of *G. macrophylla* can be successfully and rapidly achieved (Fig. 6), enabling the recovery of this endangered species, and re-establishment in wild conditions. First results on reintroduction behaviour revealed that 20% plantlets survived after 6 years in the wild. It will be, however, important to follow the complete life cycle and performance of these plants *in situ*. Re-established populations should be able to reproduce in their natural environment in order to guarantee the completion of the life cycle and to ensure their permanence in their own wild conditions (RUBLUO, *et al*., 1993).

Future research should address the light level requirements for germination (MCKINLEY, *et al*., 1997). Studies should also be done to determine the actual mycorrhizal association in the natural habitat of *G. macrophylla* and ascertain if it is the absence of certain fungi which is restricting multiplication of the species.

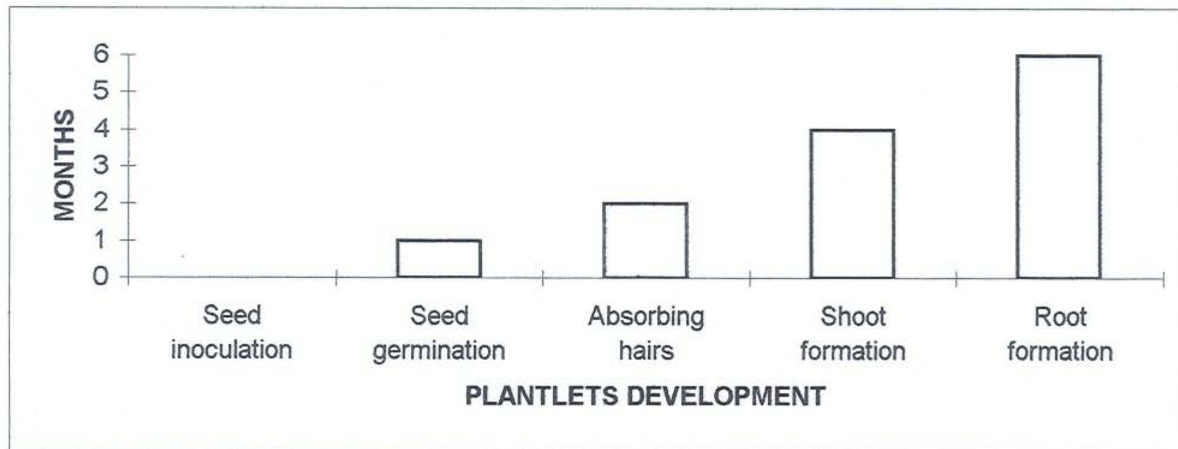

Fig. 6 - Chronology in months of seed germination and plantlets development of *G. macrophylla* in MS half strengh medium.

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