

Interaction between sucrose and pH during *in vitro* culture of *Nephrolepis biserrata* (Sw.) Schott (Pteridophyta)

Sandra Tereza Ambrósio¹ and Natoniel Franklin de Melo^{1,2}

Received: April 15, 2003. Accepted: April 24, 2004

RESUMO – (Interação da sacarose e níveis de pH durante o cultivo *in vitro* de *Nephrolepis biserrata* (Sw.) Schott (Pteridophyta)). O presente trabalho relata o efeito de diferentes valores de pH e concentrações de sacarose na propagação *in vitro* de *Nephrolepis biserrata*. Frondes obtidas assepticamente a partir do cultivo de estolões foram inoculadas em meio semi-sólido MS, suplementado com 0, 15, 30, 45 e 60g.L⁻¹ de sacarose e pH ajustado para 3, 5, 7 e 9, em arranjo fatorial. Foram avaliados: número e comprimento das frondes, número de pinas, comprimento e diâmetro da raque e biomassa fresca e seca da parte aérea. Em geral, houve inibição da regeneração de brotos e folhas em todos os tratamentos de pH na ausência de sacarose. Por outro lado, a presença de sacarose no meio induziu 51,25 e 38,25 brotos por explante no pH 5 e 7, respectivamente. Concentrações entre 15 e 45g.L⁻¹ de sacarose aumentaram o comprimento e o diâmetro das folhas e o pH 9 não afetou o peso foliar seco, sendo inadequado para o desenvolvimento de novas frondes. Os esporófitos jovens foram submetidos, com sucesso, ao processo de aclimação.

Palavras-chave: pteridófitas, plantas ornamentais, cultura de tecidos

ABSTRACT – (Interaction between sucrose and pH during *in vitro* culture of *Nephrolepis biserrata* (Sw.) Schott (Pteridophyta)). The present work reports the effect of different pH and sucrose concentrations on *in vitro* propagation of *Nephrolepis biserrata*. Fronds aseptically obtained from stolon segment culture were cultivated in MS semi-solid medium supplemented with 0, 15, 30, 45 and 60g.L⁻¹ sucrose and pH adjusted to 3, 5, 7 and 9, in a factorial design. Frond number and length, pinnae number, rachis length and diameter, fresh and dry matter weight were measured. Inhibition of shoot and leaf regeneration was observed in all the pH treatments in the absence of sucrose. On the other hand, when sucrose was added to the medium, the shoot number increased, reaching the maximum average values of 51.25 and 38.25 shoot per explant at pH 5 and 7, respectively. Sucrose concentrations from 15 to 45g.L⁻¹ increased leaf length and diameter and the pH 9 did not affect the dry matter weight, and was still not adequate for development of new fronds. Young sporophytes were successfully acclimated.

Key words: ferns, ornamental plants, tissue culture

Introduction

In the sporophytic phase, pteridophytes commonly present large and complex fronds, with elaborate leaves, which, besides facilitating spore dispersion and supporting asexual reproduction for the spore presence, also provide excellent ornamental qualities (Jones 1987). Therefore, the pteridophytes fronds are known worldwide due to their ornamental value, and several studies have been conducted for optimizing *in vitro* conditions, not only to contribute for the propagation of ornamental species in commercial scale, but also to understand the species biology (Fernández *et al.* 1999). Among the pteridophytes species used as ornamental foliage in commercial and residential environments, we can find those of the genus *Nephrolepis* (Morton 1958; Zimmerman & Jones 1991). It is a highly

distinctive genus characterized by abundant stolons, disarticulate, inequilateral and usually entire pinnae, hydathodes in the upper surface of the pinnae and spores with a relative thin and tuberculate-rugose perispore (Tryon & Tryon 1982).

Several difficulties imposed by specificity of fern life cycle interfere on *in vitro* cultivation protocol establishment (Morini 2000). According to Stokes (1984), *in vitro* propagation of pteridophytes species can require prolonged periods of time, which sometimes might extend to several years, so that the frond and plant size can reach the uniformity demanded by the market of ornamental plants. During *in vitro* regeneration of ferns species, some factors affect the multiplication efficiency and many aspects have been examined, such as explant donor source (Atmane *et al.* 2000; Whittier & Braggins 2000), nutrient type (Padhya

¹ Embrapa Semi-Árido, C. Postal 23, CEP 56302-970, Petrolina, PE, Brasil

² Corresponding Author: natonielf@cpatsa.embrapa.br

& Mehta 1982; Fernández *et al.* 1999) and physical culture conditions (Lin & Yang 1999). Sporophyte fragments have been used as explants source, which are usually the most practical and viable form of micropropagation of ornamental species in commercial scale (Bertrand *et al.* 1999).

Since the 1970's, *in vitro* multiplication protocols of *Nephrolepis* species have been reported. Loescher & Albrecht (1979) observed the effect of environmental, nutritional and hormonal conditions on *in vitro* organogenesis of *N. exaltata*, var. *bostoniensis*, using juvenile segments of stolon. Lê (1983) reported the influence of sucrose concentrations on sporophyte propagation of *N. exaltata* (L.) Schott using gametophytic tissue as explant source, while Pasqual *et al.* (1994) propagated the same fern by shoots, in order to study *in vitro* multiplication under the effect of sucrose and mineral salt association. Higuchi *et al.* (1987) described the effect of growth regulators and sucrose on histological differentiation and fresh matter weight of *N. cordifolia* Prsel. The association of these variables with pH is important because it could influence the multiplication efficiency, establishment and quality of the propagated sporophytes (Handreck 1992; Koedam *et al.* 1992; Pevalek-Kozlina 1996).

In the present work four pH treatments and five levels of sucrose concentrations were studied, in order to evaluate *in vitro* induction, regeneration and establishment of *N. biserrata* sporophytes.

Material and methods

The material studied was obtained from cultivated plants and identified as *Nephrolepis biserrata* (Sw.) Schott (Davalliaceae/Filicopsida) (Tryon & Tryon 1982). The plants were maintained in the Experimental Glasshouse of Embrapa Semi-Árido, Petrolina-PE, for sanitary treatment.

Initially, young sporophytes cultivated in a glasshouse, in vases with substrate (organic matter and vermiculite 1:1), were treated with a solution containing Benomyl (0.5%), Nuvacron (1mg.L⁻¹) and Extravon (1mg.L⁻¹) solution once a week. Stolon segments were collected, washed in water containing 1-3 drops of commercial detergent, and re-washed in running distilled water. The disinfection process consisted of immersion in 70% alcohol (v/v) for 1-2 minutes, followed by 20 minutes of immersion in a 2% sodium hypochloride solution and rinsed in sterile distilled water.

A hundred stolon segments were cut into pieces of 2cm length, placed in MS semi-solid medium (Murashige & Skoog 1962) supplemented with 30g.L⁻¹ sucrose, with pH adjusted to 5.9. Juvenile sporophytes were obtained after 90 days. Single shoots initiated from juvenile sporophytes were used as explants for the experimental assays, as previously described (Byrne & Caponetti 1992).

Under sterile condition, explants were transferred to MS semi-solid medium supplemented with factorial combinations of sucrose at 0, 15, 30, 45 and 60g.L⁻¹ and pH adjusted to 3, 5, 7 and 9. Each treatment consisted of four replicates and was maintained in a growth chamber at 25 ± 1°C and 16 h light photoperiod. All media were solidified with 7g.L⁻¹ agar and autoclaved at 120°C, 1kg.cm⁻² pressure, for 20 minutes. After autoclaving, the final pH shifted downwards around 0.2.

After 45 days of cultivation, frond and pinnae numbers, frond length, rachis diameter, and fresh and dry matter weight of aerial parts were evaluated. The Duncan test ($p \leq 0.05$) was used for evaluating the data obtained with different sucrose concentrations and pH levels after ANOVA assay (Statsoft Inc 1997). Plants from *in vitro* culture were transferred to plastic boxes (veg-box), using substrate composed by vermiculite, sand and organic matter in the ratio 1:1:1, previously sterilized. The acclimatization was carried out in a glasshouse at an average temperature of 27±2°C, 85% relative humidity and 250-560µmol.m⁻².s⁻¹ light intensity.

Results and discussion

The sporophyte sanitary state was considered a critical factor for *in vitro* cultivation. Explants with endophytic microorganisms displayed growth and differentiation into primary leaves, but the development was interrupted around the 20th day after inoculation. This kind of explant was not viable for establishment or regeneration experiments. However, new shoots from health explants were obtained 10 to 15 days after inoculation. There was 80% of shoot regeneration and 20% showed both necrosis and contamination. According to Morini (2000), the main problem for establishment of *in vitro* propagation protocols in pteridophytes is the presence of microorganism in the plants, hindering the disinfection process and affecting their survival.

Frond and pinnae numbers and fresh matter weight were significantly affected by the interaction

Table 1. Effects of sucrose and pH on frond number, pinnae number and fresh matter weight of 45 day-old *Nephrolepis biserrata* cultivated *in vitro*.

pH	Sucrose (g.L ⁻¹)	Frond number	Pinnae number	Fresh weight (g)
3	0	6.25 c	10.25 c	0.053 bc
3	15	15.00 b	24.25 b	0.088 bc
3	30	11.75 b	27.75 ab	0.064 bc
3	45	12.75 b	37.00 ab	0.078 bc
3	60	21.00 b	36.25 ab	0.432 ab
5	0	6.75 c	19.00 bc	0.355 ab
5	15	18.50 b	47.50 a	0.142 bc
5	30	21.00 b	45.50 a	0.192 bc
5	45	19.50 b	26.50 ab	0.615 a
5	60	51.25 a	30.25 ab	0.290 bc
7	0	15.50 b	11.00 bc	0.027 c
7	15	20.00 b	33.00 ab	0.430 ab
7	30	31.75 b	47.50 a	0.257 bc
7	45	26.20 b	36.00 ab	0.190 bc
7	60	38.25 a	31.75 ab	0.080 bc
9	0	-	-	-
9	15	31.00 b	23.50 bc	0.035 c
9	30	26.25 b	38.00 ab	0.098 bc
9	45	32.50 b	26.25 ab	0.057 bc
9	60	-	-	-

Means followed by the same letter in the same column do not differ at 5% level by the Duncan's test. - Values not observed.

between sucrose concentration and pH (Table 1). Regarding frond and pinnae numbers, satisfactory results were obtained with the increase of sucrose concentration in all pH treatments. The increase of sucrose concentration significantly improved frond production. Maximum values of 51.25 and 38.25 fronds per explant were obtained, with 60g.L⁻¹ sucrose at pH 5 and 7, respectively. Sucrose at 45g.L⁻¹ and pH 5 also increased the fresh matter weight. On the contrary, an increase in sucrose concentration reduced the frond induction in *N. exaltata* (Pasqual *et al.* 1994). It was also reported that sucrose concentrations higher than 50g.L⁻¹ caused a decrease in the formation of new prothallias colonies in this species (Lê 1983).

Length, diameter and dry matter weight of aerial parts were not significantly affected by the interaction between sucrose concentration and pH. In this case, the Figure 1 shows data analyzed separately, because pH and sucrose levels in ANOVA were significant. For simplicity, however, we have presented the graphs with levels pooled (Figure 1). Responses were obtained at sucrose concentration of 30 and 45g.L⁻¹ regarding frond length, rachis diameter and dry matter weight. The absence or higher sucrose concentration (60g.L⁻¹)

significantly reduced these parameters. Data suggested inhibition of plant development at pH 9, in which reduction of frond length and rachis diameter was also observed. Similar response was observed for dry matter weight, although statistical differences were not observed among pH levels 3, 5 and 9. Thus, pronounced growth responses were obtained at pH 5 and 7. This intense growth is possibly due to increase in cell division with subsequent frond production, as reported by Pevalek-Kozlina (1996) in *Platyserium bifurcatum*. In this species, neutral or acid pH stimulates *in vitro* frond production. Under natural conditions, pH also appears to have a selective effect in the establishment of many ornamental species (Symonds *et al.* 2001). Some pteridophytes have been reported to prefer alkaline soils (Handreck 1992), whereas others are acidiphilous (Koedam *et al.* 1992) or yet generalists (Ranal 1995). Under *in vitro* conditions, the medium pH may have significant effects on growth and differentiation of cells, although there are many reports showing that cells in culture can significantly alter the pH of the external medium through differential uptake of nutrients and/or through H⁺ fluxes (Minocha 1987).

Some factors, such as limited photosynthetic activity due to *in vitro* conditions, may explain the significant decrease of regeneration rate under sucrose absence. However, high sucrose level in the culture medium might result in chlorophyll degradation and reflect on the frond development (Fernández *et al.* 1999). Despite of *in vitro* plant regeneration can be successful without sucrose, its presence usually promotes a significant increase of new regenerated structures, since in the induction phase, the carbon source accumulated in the plant tissue is enough to start the development (Lalonde *et al.* 1999). Meanwhile, during the development phase, large and available carbon amounts are necessary for structural growth, differentiation and development. In general, sucrose in a medium can serve as an osmotic agent and as a carbon and energy source. Many cultures, especially embryo and some shoot cultures, require a medium with high osmotic potential (Tremblay & Tremblay 1995).

In the present study, all *in vitro* regenerated plants did not show any observable morphological abnormalities. During acclimatization process, the regenerated sporophytes produced new fronds quite well distributed in the apex, and show a more intense green color when compared to the original sporophyte source. In conclusion, the results described here provided efficient approaches for *in vitro* multiplication of *N. biserrata*.

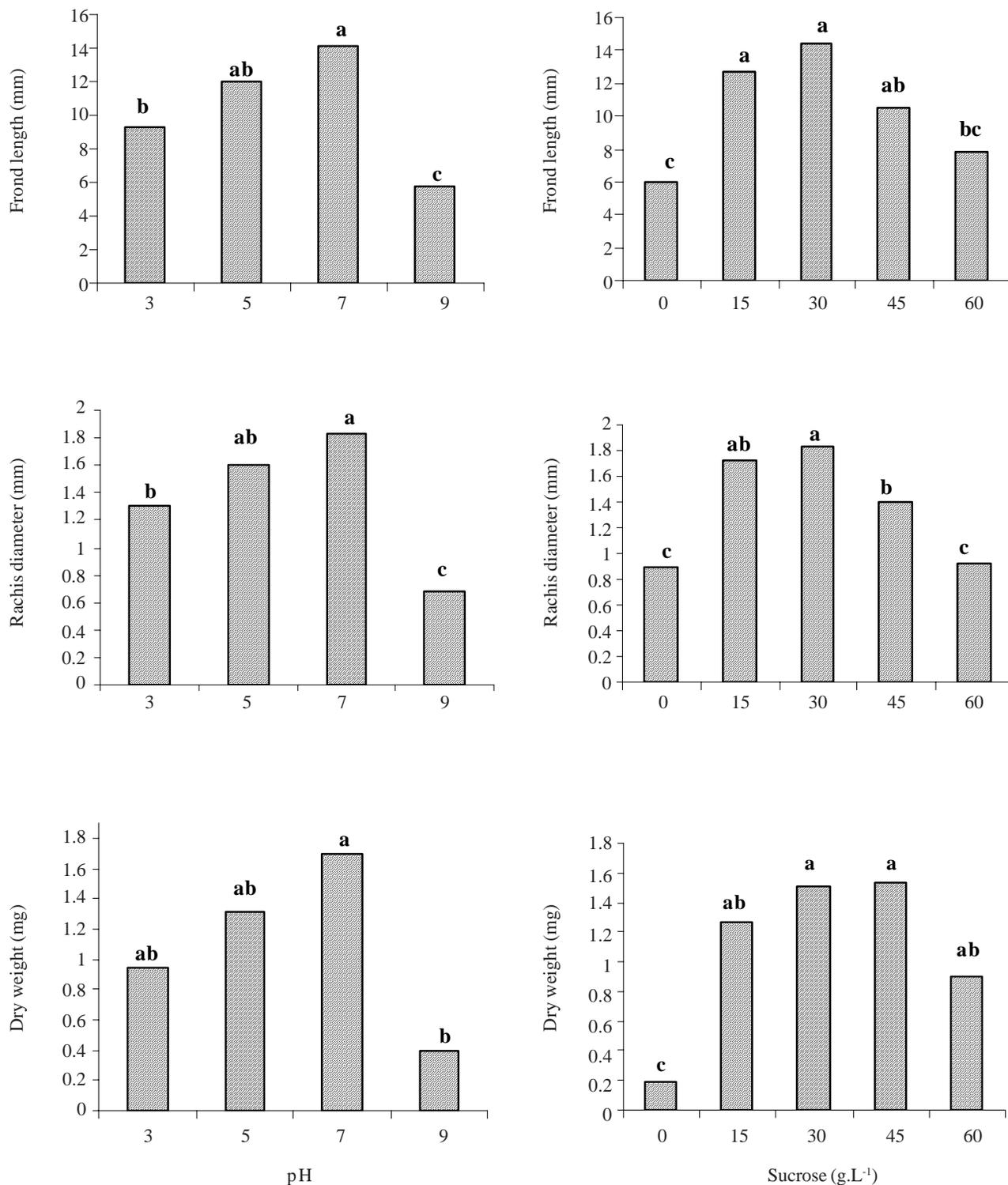


Figure 1. Influence of sucrose and pH on frond length, rachis diameter and dry matter weight of *Nephrolepis biserrata* plants cultivated *in vitro* for 45 days. The data were analyzed separately because only pH and sucrose levels in ANOVA were significant. Same letter indicate no difference at 5% level by the Duncan's test.

Acknowledgments

The authors are very thankful to Maria Tereza Ataíde, for her technical assistance; to Adriana Mayumi Yano-Melo, for the statistical analyses and help in the manuscript; to CNPq (Proc. 300392/96-6) and Embrapa Semi-Árido, for their financial support.

References

- Atmane, N.; Blervacq, N.; Michaux-Ferriere, N. & Vasseur, J. 2000. Histological analysis of indirect somatic embryogenesis in the Marh Clubmoss *Lycopodiella inundata* (L.) Holub (Pteridophytes). **Plant Science** **156**: 159-167.
- Bertrand, A.M.; Albuérne, M.A.; Fernández, A.; González, A. & Sánchez-Tamés, R. 1999. *In vitro* organogenesis of *Polypodium cambricum*. **Plant Cell, Tissue and Organ Culture** **57**: 65-69.
- Byrne, T. & Caponetti, J.D. 1992. Morphogenesis in three Cultivars of Boston Fern II. **American Fern Journal** **82**(1): 1-11.
- Fernández, H.; Bertrand, A.M. & Sánchez-Tamés, R. 1999. Biological and nutritional aspects involved in fern multiplication. **Plant Cell, Tissue and Organ Culture** **56**: 211-214.
- Handreck, K.A. 1992. Growth of ferns in soil-less media, as affected by pH, iron and calcium/magnesium ratio. **Scientia Horticulturae** **50**(1-2): 115-126.
- Higuchi, H.; Amaki, W. & Suzuki, S. 1987. *In vitro* propagation of *Nephrolepis cordifolia* Prsel. **Scientia Horticulturae** **32**: 105-113.
- Jones, O.L. 1987. **Encyclopedia of ferns: An introduction to fern, their structure, biology, economic importance, cultivation and propagation**. British Museum, **2**: 12-22.
- Koedam, N. Buscher, P. & Speybroeck, D.V. 1992. Acidiphily in pteridophytes: assessment of the role of root cation-exchange properties. **Journal of Plant Nutrition** **15**(12): 2605-2619.
- Lalonde, S.; Boles, E.; Hellmann, H.; Barker, L.; Patrick, J.W.; Frommer, W.B. & Ward, M.F. 1999. The dual function of sugar carriers: transport and sugar sensing. **The Plant Cell** **11**(4): 707-726.
- Lê, L. 1983. Essai de multiplication de *Nephrolepis exaltata* par culture "in vitro" de tissu gamétophytique. **Revue Suisse de Viticulture, Arboriculture et Horticulture** **15**(3): 189-192.
- Lin, B. & Yang, W. 1999. Blue light and abscisic acid independently induce heterophyllous switch in *Marsilea quadrifolia*. **Plant Physiology** **119**: 429-434.
- Loescher, N.H. & Albrecht, C.N. 1979. Development "in vitro" of *Nephrolepis exaltata* var. *bostoniensis* runner tissues. **Physiologia Plantarum** **47**(4): 250-254.
- Minocha, S.C. 1987. pH of the medium and the growth and metabolism of cells in culture. Pp. 125-141. In: J.M. Bonga & D.J. Durzan (eds.). **Cell and tissue culture in forestry**. Martinus Nijhoff Publishers.
- Morini, S. 2000. *In vitro* culture of *Osmunda regalis* fern. **Journal of Horticultural Science & Biotechnology** **75**(1): 31-34.
- Morton, C.V. 1958. Observations on cultivated ferns. V. The species and forms of *Nephrolepis*. **American Fern Journal** **48**: 18-27.
- Murashige, T. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiologia Plantarum** **15**: 473-497.
- Pasqual, M.; Ishida J.S. & Hoshida, E. 1994. Influência de diferentes concentrações de sacarose e sais minerais sobre a multiplicação "in vitro" de *Nephrolepis exaltata* - uma samambaia ornamental. **Pesquisa Agropecuária Brasileira** **29**(11): 1681-1684.
- Padhya, M.A. & Mehta, A.R. 1982. Propagation of fern (*Nephrolepis*) through tissue culture. **Plant Cell Reports** **1**: 261-263.
- Pevalek-Kozlina, B. 1996. Effects of sucrose and agar concentration and medium pH on staghorn fern [*P. bifurcatum* (Chr.) C. Cav.] shoot multiplication. **Horticultural Science** **28**: 18-20.
- Ranal, M.A. 1995. Estabelecimento de pteridófitas em mata mesófila semidecídua do estado de São Paulo. 2. Natureza dos substratos. **Revista Brasileira de Biologia** **55**(4): 583-594.
- Stokes, P. 1984. Hardy ferns. **International Plant Propagators' Combined Proceedings** **34**: 332-333.
- Symonds, W.L.; Campbell, L.C. & Clemens, J. 2001. Response of ornamental *Eucalyptus* from acidic and alkaline habitats to potting medium pH. **Scientia Horticulturae** **88**: 121-131.
- Statsoft Inc. 1997. **Statistica for Windows. Computer Program Manual**. Tulsa.
- Tremblay, L. & Tremblay F.M. 1995. Maturation of black spruce somatic embryos: Sucrose hydrolysis and resulting osmotic pressure of the medium. **Plant Cell, Tissue and Organ Culture** **42**: 39-46.
- Tryon, R.M. & Tryon, A.F. 1982. **Ferns and allied plants, with special reference to tropical America**. Springer-Verlag.
- Whittier, D.P. & Braggins, J.E. 2000. Observations on the mature gametophyte of *Phylloglossum* (Lycopodiaceae). **American Journal of Botany** **87**: 920-924.
- Zimmerman, R.H. & Jones J.B. 1991. Commercial micropropagation in North America. In: P.C. Debergh & R.H. Zimmerman (eds.). **Micropropagation: Technology and Application**. Kluwer Acad.