Annals of Botany **80**: 23–28, 1997

Gametophytic and Sporophytic Regeneration from Bud Scales of the Fern *Platycerium bifurcatum* (Cav.) C.Chr. *In Vitro*

J. AMBROŽIČ DOLINŠEK* and M. CAMLOH†

* *Department of Biology, University of Maribor, Koroška 160, 2000 Maribor, Slovenia*
† *National Institute of Biology, Karlovška 19, 1000 Ljubljana, Slovenia*

Received: 16 September 1996 Accepted: 16 December 1996

Regeneration from *Platycerium bifurcatum* scales *in vitro* was observed for the first time. Regeneration was obtained from scales detached from *in vitro* grown juvenile shoots. Regeneration began as rhizoids or as a proliferation of one or more scale cells into undifferentiated outgrowths distinguishable from adjacent cells by their shape and colour. Outgrowths developed into adventitious buds, aposporous gametophytes, or remained undifferentiated. Numerous outgrowths formed rhizoids on their surfaces. Aposporous gametophytes produced gametangia. Sucrose enhanced scale viability and regeneration, and promoted rhizoid development on outgrowths, but sucrose concentrations greater than 0·1% tended to inhibit apospory.

Key words: *Platycerium bifurcatum* (Cav.) C.Chr., staghorn fern, scale, regeneration, organogenesis, sucrose, apospory.

INTRODUCTION

Scales cover the entire surfaces of the rhizome and extend up the phyllopodia of the staghorn fern, *Platycerium bifurcatum*. On buds, they are tightly overlapped and appressed to the surfaces. Scales develop from superficial apical cell derivatives which surround the bud apex (Richards, Beck and Hirsch, 1983). Their development begins as uniseriate hairs which divide longitudinally, transversely, and clinally at the thickened centre to form two-dimensional scales. They protect the bud from desiccation, animals and excessive water (Hoshizaki, 1970). Their features have been used in describing and identifying some groups of ferns (Leena and Madhusoodanan, 1993), and have also helped in the determination of evolutionary trends within the genus *Platycerium* (Hoshizaki, 1972).

Regeneration is well established *in vivo* and *in vitro*, from explants of some *Platycerium* species (Hoshizaki, 1977; Hennen and Sheehan, 1978; Richards *et al*., 1983; Thentz and Moncousin, 1984; Wee, Kwa and Loh, 1992; Camloh, Gogala and Rode, 1994). However, scales have never been included in regeneration studies of *Platycerium* or any other fern species.

Scales which cover the juvenile buds of *in vitro* grown *P. bifurcatum* shoots vary in colour, bear rhizoids and some develop outgrowths while still attached to buds (J. Ambrožič Dolinšek, unpubl. res.). These observations led us to examine whether or not unproliferated scales detached from shoots and cultured on the medium also have this capacity. Since some studies indicate the importance of carbohydrates in the developmental pathway of some ferns *in vitro* (Hirsch, 1975; Sheffield and Bell, 1981) the effect of sucrose on the type of scale regeneration was also examined.

MATERIALS AND METHODS

Spores of the staghorn fern *P. bifurcatum* (Cav.) C.Chr. (Polypodiaceae) were isolated, sterilized and cultured as described previously (Camloh, 1993). Juvenile plants were raised in aseptic cultures from spores according to the method of Camloh and Gogala (1992), except that sporophyte development occurred on media solidified with 0·8% Difco-Bacto agar and not on sterilized soil. Approximately 5 months after spore sowing, the size of developed sporophytes was convenient for initiating leaf culture. Entire 7–10 mm leaves were detached from these *in vitro* grown sporophytes and placed on the surface of modified Murashige and Skoog (1962) medium (MS) with 2% sucrose to induce adventitious bud development. After 2 months, adventitious buds were excised and elongated for an additional 4 weeks (Camloh *et al*., 1994). From these shoots, scales composed of a single layer of cells were detached and used as the initial explant. They had a triangular shape with an entire margin, peltate base and acute apex with papillae at the top (Fig. 1A). Fifty to 60 scales were placed flat on the surface of 15 ml of MS medium, modified by Hennen and Sheehan (1978) without adenine sulphate, supplemented with 0·8% Difco-Bacto agar and sucrose at concentrations of 0·01, 0·1, 1, 2 or 3%. Medium without sucrose was used as the control.

Media were adjusted to pH 5·7–5·8 and, after autoclaving, placed in plastic 7 cm Petri dishes. Cultures were kept at 23 ± 2 °C, with a photoperiod of 16 h at 8–11 Wm^{-2} (Osram L 65W/20S—cool white lamps).

The viability of scales was assessed after 30 and 60 d of culture. Scales were defined as viable when at least a few cells were light green. Development of new structures—

0305-7364/97/070023+06 $25.00/0 bo960383

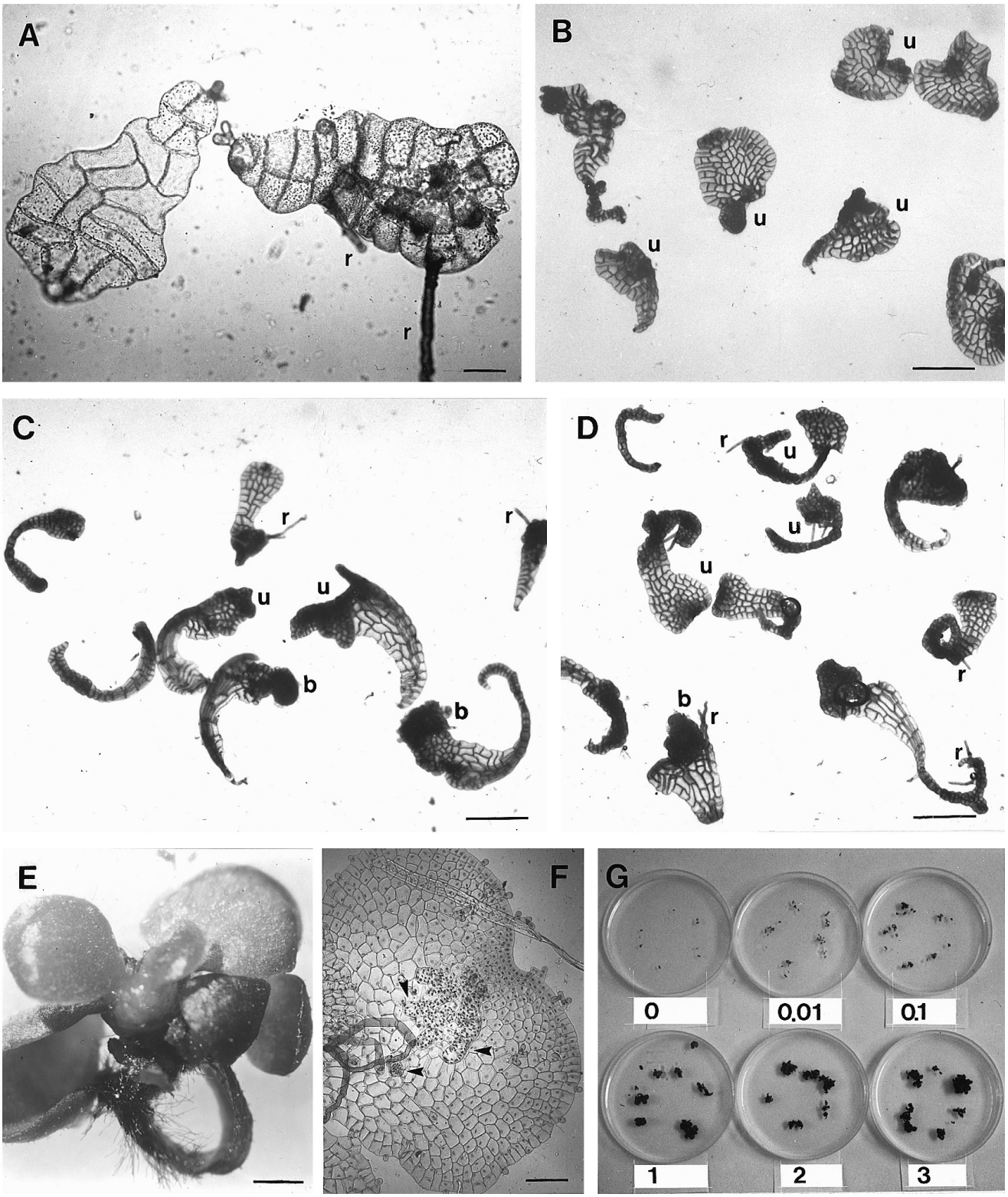

FIG. 1. A, Scales after detachment showing rhizoids (r) and variations in staining intensity. Bar = 0·1 mm. B–D, Scales after 30 d of culture on media with different concentrations of sucrose, showing different types of outgrowths. Note also differences in staining intensity and shape between scale cells and newly proliferated cells. B, 0·01 % sucrose; C; 1 % sucrose; D, 2 % sucrose. b, buds, r, rhizoids, u, undifferentiated outgrowths. Bar = 0·5 mm. E, Regeneration of a whole plant from scales after 90 d of culture. Bar = 2 mm. F, The development of gametangia (arrows) on an aposporous outgrowth developed on scales 4 months after culture initiation. Bar = 0·2 mm. G, Outgrowths regenerated on scales after 90 d of culture on media with 0–3 % sucrose.

outgrowths—on scales was observed after 30, 60 and 90 d of culture. Scales were examined using a stereomicroscope. The numbers of undifferentiated outgrowths, buds and aposporous gametophytes were determined. Outgrowths bearing rhizoids were counted and the number of rhizoids on outgrowths was determined. For each treatment, 100–120 scales were cultured on each medium. After 90 d of culture some scales with aposporous outgrowths were transferred to fresh media supplied with 0·01 % sucrose to observe their further development.

Aposporous gametophytes were fixed and stained with acetocarmine-chloral hydrate according to Edwards and Miller (1972). Photomicrographs were taken by a zoom stereomicroscope OLYMPUS SZH 10 and a ZEISS JENAMED 2 microscope.

The two × two Chi-squared test (χ^2) was used for evaluating levels of statistical significance (P) between the data obtained in control media and those with different sucrose concentrations. All experiments were repeated twice and the examples given represent typical results.

RESULTS

Morphology of scale outgrowths

Scales which covered the buds of shoots grown *in vitro* varied from nearly colourless to light green, bore rhizoids (Fig. 1A) and some of them began to proliferate before detachment from the bud.

The regeneration of outgrowths on the majority of scales began during the first 30 d of culture, as the proliferation of several cells, rarely one, at different sites on the scale. New cells were distinguishable from adjacent scale cells by their shape and colour (Fig. 1B–D). During culture, some scale cells and sometimes also complete scales became brown, necrotic and died. Other scales or scale cells maintained their light green colour or changed to a slightly darker green. Newly proliferated zones, which always occurred only on viable parts of the scale became darker green, and were composed of more isodiametric cells. They formed three-, rarely two-, dimensional groups of cells, undifferentiated outgrowths, which developed into buds, aposporous gametophytes, or remained undifferentiated. Numerous outgrowths formed rhizoids on their surfaces. During the experiment their development was strictly limited to newly proliferated zones. Rhizoids were already observed on some undifferentiated outgrowths during the first 30 d of culture (Fig. 1C and D), but were more frequent after their development into buds. Rhizoids formed on outgrowths had a morphology identical with that of rhizoids developed on gametophytes. Adventitious buds originated from two- and three-dimensional groups of cells, while aposporous gametophytes formed only from the two-dimensional groups

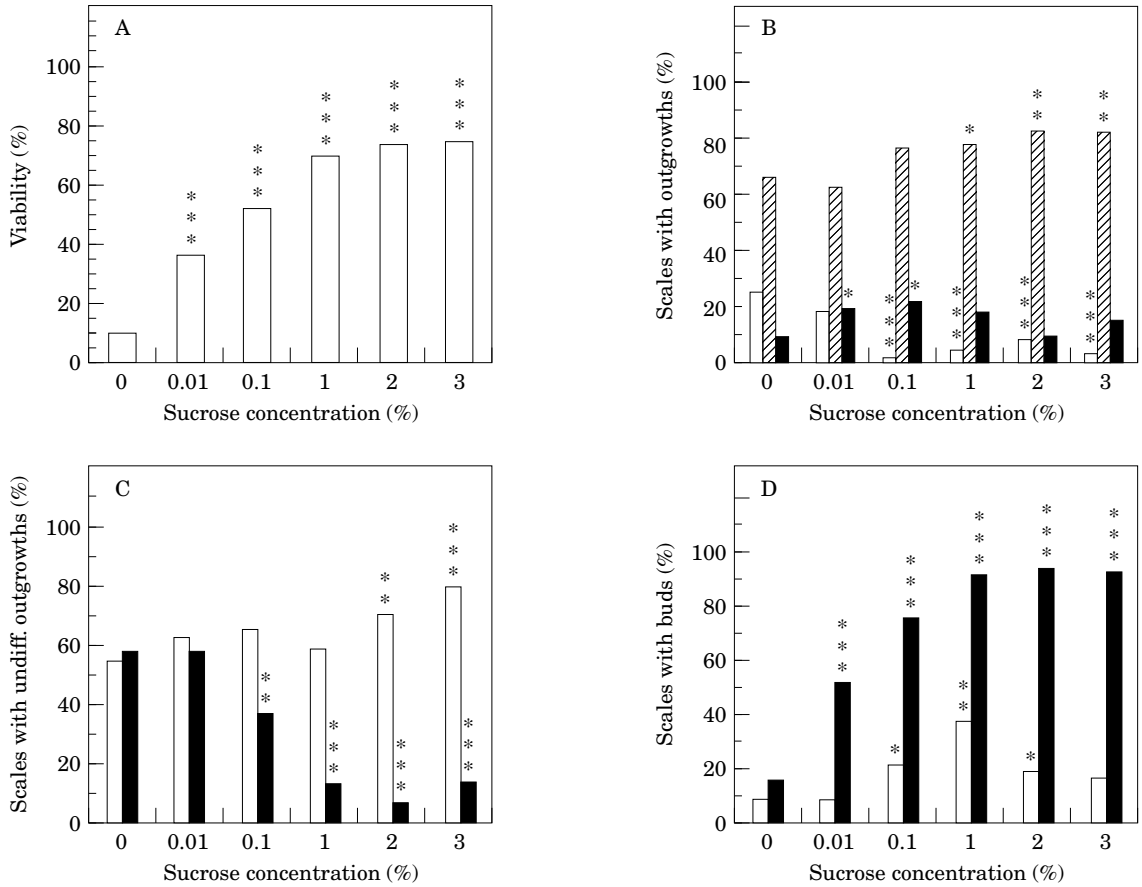

FIG. 2. The effect of sucrose on (A) the viability of scales after 30 d of culture, (B) the regeneration on scales after 30 d of culture; (□) scales with no outgrowth, (▨) scales with one outgrowth, (■) scales with more outgrowths (C), the development of undifferentiated outgrowths after 30 (□) and 60 (■) d of culture, and (D) the development of buds on scales after 30 (□) and 60 (■) d of culture. ***$P < 0{\cdot}001$, **$P < 0{\cdot}01$, *$P < 0{\cdot}05$.

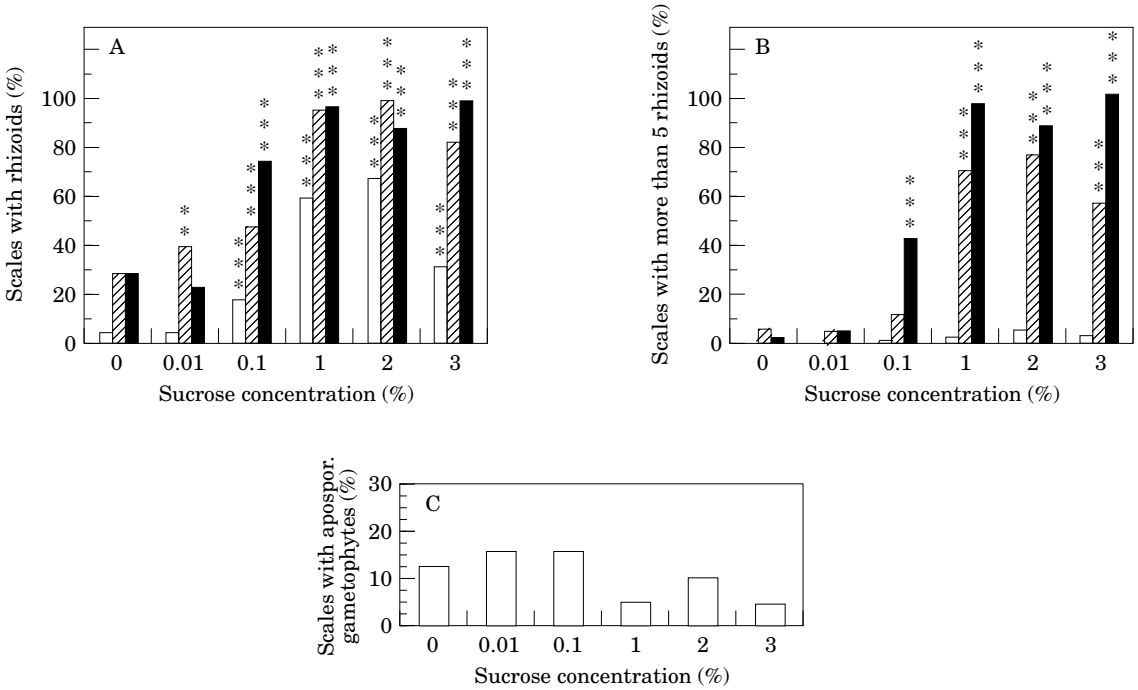

FIG. 3. The effect of sucrose on (A) rhizoid development on scale outgrowths, after 30 (□), 60 (▨) and 90 (■) d of culture (B) the number of rhizoids per outgrowth after 30 (□), 60 (▨) and 90 (■) d of culture and (C) the development of aposporous gametophytes after 30 d of culture. $***P < 0{\cdot}001$, $**P < 0{\cdot}01$.

of cells. After 90 d of culture, whole plants developed from buds (Fig. 1E). Aposporous gametophytes produced gametangia (Fig. 1F). The first antheridia were noticed 4 months after culture initiation, while archegonia usually developed 2 months later. The simultaneous development of antheridia and archegonia was observed only rarely.

Effect of sucrose on scale regeneration

The effect of sucrose on scale viability and regeneration was assessed after 30 and 60 d of culture; regeneration was also studied after 90 d of culture. After 30 d, significantly higher viability was observed in all sucrose-enriched media when compared to the control (Fig. 2A). The same result was also observed after 60 d of culture (data not shown).

After 30 d of culture, 75–97% of explants in all treatments had regenerated outgrowths (Fig. 2B). Their average number per scale varied from 0·9–2·2 in different media. Fully viable scales usually produced only one structure each, while scales with some necrotic cells usually regenerated more than one.

Sucrose strongly stimulated the regeneration of scales. This was evident after 30 d of culture (Fig. 2B; see also Fig. 1B–D), but was more pronounced after 60 and 90 d of culture (Fig. 1G). Figure 2B shows that after 30 d the percentage of scales with one outgrowth was significantly higher on media with 1–3% sucrose, than on control medium. It was also evident that media with lower sucrose concentrations (0·01 and 0·1%) stimulated the development of significantly more outgrowths on scales. Similar results were also obtained after 60 d of culture. Sucrose also affected the type of regeneration: after 30 d of culture the majority of scale outgrowths were undifferentiated (Fig. 2C, see also Fig. 1B–D). The percentage of scales with such outgrowths was significantly higher on media with 2–3% sucrose. However, after 60 d this percentage was significantly lower at 0·1–3% of sucrose in the medium, when compared to other treatments. Sucrose stimulated bud organogenesis (Fig. 2D). This was evident after 30 d of culture (see also Fig. 1B–D), but after 60 d there was a far greater number of buds. These results indicate that the differentiation of outgrowths to buds mainly occurred between 30 and 60 d of culture. When scales produced many outgrowths, usually only one of them formed a bud.

Sucrose promoted rhizoid development on outgrowths (Fig. 3A). Increasing the sucrose concentration dramatically increased the percentage of scales with rhizoids (see also Fig. 1B–D). A similar sucrose effect was also observed when the number of rhizoids on scales was determined. This effect was particularly evident after 60 and 90 d of culture (Fig. 3B).

More apospory occurred on media with no, or low, sucrose concentrations (0–0·1%) than on media containing sucrose concentrations exceeding 0·1% (Fig. 3C).

DISCUSSION

Regeneration of scales

It has been shown for the first time that scales detached from *in vitro* grown shoots of the fern *P. bifurcatum* have the potential to develop different structures. Scales regenerated buds on media lacking growth regulators. Similar results were obtained by Camloh *et al.* (1994) with juvenile leaves of *P. bifurcatum*. These authors concluded that the formation of adventitious buds, without exogenous growth

regulators, might reflect high endogenous levels. This may also be the case for scales of this species. Growth regulators have been used to promote bud induction on the shoot apex of the genus *Platycerium* (Hennen and Sheehan, 1978; Thentz and Moncousin, 1984). Since it is known that variation in hormone-regulated growth patterns occurs at the cell, tissue, organ, individual plant and species level (Bradford and Trewavas, 1994), the differences in regeneration capability obtained by different authors working with the same species might depend on the age, type, and initial size of explants. Regeneration and organogenesis from scales occurred without involvement of an intermediary callus stage, as reported by Camloh *et al*. (1994) for regeneration from leaf tissue.

An interesting observation was the formation of apparently gametophytic rhizoids on outgrowths which was contemporaneous with sporophyte shoot organogenesis. Their number per explant also increased during differentiation of outgrowths to buds. Rhizoids were also observed on cultured leaves and, as we found earlier (Camloh *et al*., 1994), their development coincided with bud induction. Raghavan (1989) described growth of rhizoids from single sporophytic cells, plated on a medium lacking sucrose. Asymmetric division of this cell gave rise to a rhizoid and protonema initials. Such a model of rhizoid inception might describe the origin of rhizoids in our outgrowths, but further investigations are required because our undifferentiated outgrowths did not develop into aposporous gametophytes as Raghavan (1989) reported for sporophytic cells.

Besides bud regeneration, we observed the development of aposporous gametophytes on a few explants. Antheridia and archegonia also appeared on these gametophytes. Usually antheridia developed before archegonia; only rarely was there simultaneous development of both. This is probably due to artificial laboratory conditions which are known to induce the formation of 'bisexual' gametophytes in some fern species (Sheffield, 1994). *In vitro* apospory has also been induced in *P. bifurcatum* on detached leaves, but the formation of gametangia was not observed (Camloh *et al*., 1994).

Effect of sucrose on scale regeneration

Sucrose affected the viability and regeneration of scales. Such regeneration can be induced without sucrose, but sucrose significantly promoted further development. Organ initiation is associated with utilization of accumulated starch in the tissue and also with free sugars in the medium (Welander and Pawlicki, 1994). Perhaps mobilization of accumulated starch in the tissue is enough to initiate regeneration, but more energy is required from the culture medium for further differentiation.

White (1979), in his review of the role of organic nutrition in cultured fern leaf primordia, established that with an increase in sucrose concentration there is a parallel increase in the final height of primordia. He explained that high sucrose concentrations increased respiratory rates and thereby enhanced growth. Histological examination revealed that these increases were due to increases in the amount and duration of cell division activity in the cultured primordium. Although this experimental system was different to ours, the sucrose-induced organogenesis could be explained in the same way.

Our investigations indicated that media containing high concentrations ($> 0{\cdot}1\,\%$) of sucrose inhibit apospory. Similar results have been obtained for some other fern species (White, 1971; Hirsch, 1975; Sheffield and Bell, 1981; Raghavan, 1989; Materi and Cumming, 1991). On the basis of many investigations of apospory made by different researchers, Raghavan (1989) proposed an explanation of apospory induction. According to this explanation, the genetic blue-print of the sporophyte cells is activated under unfavourable conditions to produce structures that are nutritionally less demanding than the sporophyte itself, in order to prolong the life of the plant to the genetically permissible extent. This could also explain the effect of sucrose on apospory in our work.

ACKNOWLEDGEMENTS

We thank Dr E. Sheffield and Dr J. Žel for constructive criticism of an earlier draft of this manuscript and Dr B. J. Hoshizaki for providing spores of *P. bifurcatum*. This work was supported by the Slovene Ministry of Science and Technology.

LITERATURE CITED

Bradford KJ, Trewavas AJ. 1994. Sensitivity thresholds and variable time scales in plant hormone action. *Plant Physiology* **105**: 1029–1036.

Camloh M. 1993. Spore germination and early gametophyte development of *Platycerium bifurcatum*. *American Fern Journal* **83**: 79–85.

Camloh M, Gogala N. 1992. *In vitro* culture of *Platycerium bifurcatum* gametophytes. *Scientia Horticulturae* **51**: 343–346.

Camloh M, Gogala N, Rode J. 1994. Plant regeneration from leaf explants of the fern *Platycerium bifurcatum in vitro*. *Scientia Horticulturae* **56**: 257–266.

Edwards ME, Miller JH. 1972. Growth regulation by ethylene in fern gametophytes. III. Inhibition of spore germination. *American Journal of Botany* **59**: 458–465.

Hennen GR, Sheehan TJ. 1978. *In vitro* propagation of *Platycerium stemaria* (Beauvois) Desv. *Hort Science* **13**: 245.

Hirsch AM. 1975. The effect of sucrose on the differentiation of excised fern leaf tissue into either gametophytes or sporophytes. *Plant Physiology* **56**: 390–393.

Hoshizaki JH. 1970. Rhizome scales of *Platycerium*. *American Fern Journal* **60**: 144–160.

Hoshizaki JH. 1972. Morphology and phylogeny of *Platycerium* species. *Biotropica* **4**: 93–117.

Hoshizaki JH. 1977. Staghorn ferns today and tomorrow. *Gardens' Bulletin* **30**: 13–15.

Leena KR, Madhusoodanan PV. 1993. Dermal appendages of South Indian thelypteroid ferns. *Journal of Economic and Taxonomic Botany* **17**: 187–194.

Materi DM, Cumming BG. 1991. Effect of carbohydrate deprivation on rejuvenation, apospory and regeneration in ostrich fern (*Matteuccia struthiopteris*) sporophytes. *Canadian Journal of Botany* **69**: 1241–1245.

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

Raghavan V. 1989. *Developmental biology of fern gametophytes*. Cambridge: Cambridge University Press.

Richards JH, Beck Z, Hirsch AM. 1983. Structural investigations of asexual reproduction in *Nephrolepsis exaltata* and *Platycerium bifurcatum*. *American Journal of Botany* **70**: 993–1001.

Sheffield E. 1994. Alternation of generations in ferns: mechanisms and significance. *Biological Review* **69**: 331–343.

Sheffield E, Bell PR. 1981. Experimental studies of apospory in ferns. *Annals of Botany* **47**: 187–195.

Thentz M, Moncousin C. 1984. Micropropagation *in vitro* de *Platycerium bifurcatum* (Cav.) C.Chr. *Revue Horticole Suisse* **57**: 293–297.

Wee YC, Kwa SH, Loh CS. 1992. Production of sporophytes from *Platycerium coronarium* and *P. ridleyi* frond strips and rhizome pieces cultured *in vitro*. *American Fern Journal* **82**: 75–79.

Welander M, Pawlicki N. 1994. Carbon compounds and their influence on *in vitro* growth and organogenesis. In: Lumsden PJ, Nicholas JR, Davies WJ, eds. *Physiology, growth and development of plants in culture*. Dordrecht: Kluwer Academic Publishers, 83–93.

White RA. 1979. Experimental investigation of fern sporophyte development. In: Dyer AF, ed. *The experimental biology of ferns*. London: Academic Press, 505–549.