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IAA-induced apogamy in *Platycerium coronarium* (Koenig) Desv. gametophytes cultured *in vitro*

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Abstract

Apogamous sporophytes were produced on *Platycerium coronarium* gametophytes cultured in the presence of indole-3-acetic acid (IAA). The percentage of apogamy as well as the total number of apogamous sporophytes produced per gametophyte clump were highest in the presence of 40 µM IAA. When ethylene was allowed to accumulate in the culture vessel in the presence of an optimum level of IAA, the percentage and total number of apogamous sporophyte production decreased significantly. Using light microscope and confocal laser scanning microscope we have shown that nuclear size can be used as a quick parameter to estimate the ploidy level of *P. coronarium*.

Abbreviations: CLSM: confocal laser scanning microscope, IAA: Indole-3-acetic acid, MS: Murashige and Skoog.

Introduction

The production of sporophytes from vegetative cells of a gametophyte without the intervention of sex organs is termed apogamy. In the early days, the induction of apogamy in ferns was carried out using soil-grown gametophytes (Duncan, 1941) but in later years, studies have been conducted mainly on gametophytes grown on sterile culture media (Bristow, 1962). Whittier (1964) reported that apogamy in *Pteridium* was induced by light whereas the actual development of apogamous sporophytes was controlled by light, succinic acid or sugar.

Developing gametophytes have been reported to produce ethylene (Miller *et al.*, 1970). This gaseous phytohormone promoted the induction of apogamy in *Pteridium aquilinum* gametophytes (Elmore and Whittier, 1973) but inhibited cell division in *Onoclea sensibilis* (Edwards and Miller, 1972). Ethylene production from the gametophytes of *P. aquilinum* was also stimulated by IAA (Tittle, 1987).

In this paper, we describe the role of IAA in the production of apogamous sporophytes from gametophytes of *Platycerium coronarium* cultured *in vitro* as well as the inhibitory effect of ethylene during this process.

Materials and methods

Induction of apogamous sporophytes: Gametophytes obtained from spores cultured under aseptic conditions (Kwa *et al.*, 1991) were used. These gametophytes did not produce sporophytes either through syngamy or apogamy even after 6 months of culturing. Development of sex organs did not occur in such gametophytes grown in a crowded condition. The gametophytes were macerated into fine pieces using a scalpel and clumps of about 70-75 mg were cultured individually in petri dishes (9 cm diameter). Murashige and Skoog (1962) culture medium supplemented with 2 % (w/v) sucrose was used. The pH was adjusted to 5.6 and 0.2 % (w/v) Gelrite (Merck and Co., Inc., USA) was added prior to autoclaving the medium for 20 min at 121°C. Indole-3-acetic acid (IAA; at 0, 20, 40, 60, 80 and 100 µM) was filter-sterilized before adding to the autoclaved medium. Six petri dishes, each containing 10 gametophytic clumps, were used for each concentration of IAA tested. All dishes were sealed with a layer of Nescofilm (Bando Chemical Ind. Ltd., Kobe, Japan) and the surface of the medium was kept dry throughout the culture period. Cultures were incubated at 25±1°C under a 12 hour photoperiod using cool-white fluorescent lamps generating photosynthetically active radiation of 20 µmol m^{-2} s^{-1} at the level of the gametophytes. The number of explants producing sporophytes as well as the total number of apogamous sporophytes produced per explant were counted at the end of 50 days. The experiment was repeated twice.

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Effect of different seal types: To study the effect of ethylene on apogamous sporophyte production, gametophytic clumps were cultured in 100 ml Erlenmeyer flasks containing 25 ml medium. Each flask contained 12 gametophytic clumps and there were 5 flasks per seal type for each experiment. The medium and culture conditions were similar to that used for the induction of apogamous sporophytes, except that IAA was used only at 40 µM. Three types of seals, namely, aluminum foil, foam bung and serum caps were used. To absorb ethylene produced by the explants during the culture period, 1 ml of 0.25 M mercuric perchlorate was put into a presterilized glass vial placed partly embedded in the center of each flask (Kumar *et al.*, 1987). These flasks were sealed with serum caps. The flasks with aluminum foil were sealed with a layer of Nescofilm while those with serum caps were wrapped around the rim with Glad-wrap (First Brands Corporation, USA). The number of explants producing sporophytes as well as the total number of apogamous sporophytes produced per explant were also counted at the end of 50 days. All experiments were repeated twice.

Ethylene measurement: Two types of seals were used to compare their effects on ethylene production. Five flasks were sealed with serum caps throughout the experiment while another five were sealed with aluminum foil. On every tenth day, a 1 ml gas sample was withdrawn using a hypodermic syringe from the headspace of these flasks for ethylene measurement. Flasks sealed with aluminum foil were then aired in the laminar flow for 15 min and resealed with new aluminum foil. This would give an estimate of ethylene accumulation during the entire 50 day culture period for flasks sealed with serum caps. For flasks sealed with aluminum foil the data correspond to accumulation of ethylene during successive 10 day periods.

The amount of ethylene produced was measured using a gas chromatograph (Hewlett Packard model 5890) equipped with a 180 cm long Porapak N (80/100 mesh) column, a flame ionization detector and integrator (Hewlett Packard 3390A). The operating temperatures were set at 100, 150 and 200°C for the oven, injector and detector, respectively. Nitrogen flowing at a rate of 35 ml min^{-1} was used as the carrier gas.

Measurement of nuclear size: The apogamous sporophytes produced were transferred to MS medium supplemented with 2 % (w/v) sucrose to allow them to grow. After about 2 months, these sporophytes developed fronds that were about 1.5 cm long (Fig. 1C). Such fronds were used to obtain epidermal peels. Pieces of fresh epidermal peels were placed on a glass slide and a drop of the fluorescent dye, acridine orange (0.025 % w/v), was added to the specimen before viewing them under an MRC500 confocal laser scanning microscope (CLSM; BioRad Microscience Ltd., England). The CLSM consists of a computer driven laser scanning assembly attached to a Nikon Optiphot microscope. The argon ion laser was of class 3b (25 mW), which ran in multi-line mode. Filter assemblies were used to select the 488 nm (blue) wavelength for scanning. The specimens were scanned at 1 % laser transmittance and the lens used was a 60X oil immersion lens (N.A.=1.4, Nikon, Japan). The fluorescent signals detected by the photomultiplier tube of the CLSM were then assembled in an integral image processor and the confocal image displayed on a high resolution video monitor. The images were photographed with a Polaroid Freeze-frame Recorder using Kodak TMX100 film. Epidermal peels of sporophytes obtained as a result of syngamy as well as cells from the gametophytes were also subjected to scanning under the CLSM. This was to enable comparison of the sizes of their respective nuclei. A total of 20 nuclei from 4 peels and 5 fields of view were measured per specimen type. In addition, the nuclear size in the epidermal peels were also measured using a light microscope. 2 % acetoorcein was used and 150 nuclei from 3 peels and 6 fields of view were measured per specimen type.

Statistical analysis: Data obtained from experiments on induction of sporophytes as well as effects of different seal types were subjected to one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test. Prior to analysis, data obtained were subjected to arcsine transformation.

Results and Discussion

The macerated gametophytic tissue regenerated into clumps of prothalli after 2 weeks (Fig 1A). Subsequently, sporophytes arose mainly from the center of the newly regenerated gametophyte clumps after 3 weeks of culture and appeared as bud-like structures initially (Fig. 1B). Treatment with IAA at 40 µM was found to induce the highest percentage of gametophyte clumps to form sporophytes as compared to the other concentrations (Table 1). In addition, this treatment resulted in 2- to 3-fold higher number of sporophytes per gametophyte clump when compared to the other treatments. With IAA at 80 µM and above, extensive rhizoid development was observed on the under surface of the gametophyte clumps (Table 1), the center of which appeared brownish.

The optical sectioning power of the confocal laser scanning microscope (CLSM) enabled a quick check on the nuclear size of the sporophytic cells produced and the picture produced is clear and free from interference from other cell structures (Fig. 2). The sporophytes produced as a result of IAA treatment and the gametophytes from which they arose both had nuclei of comparable sizes,

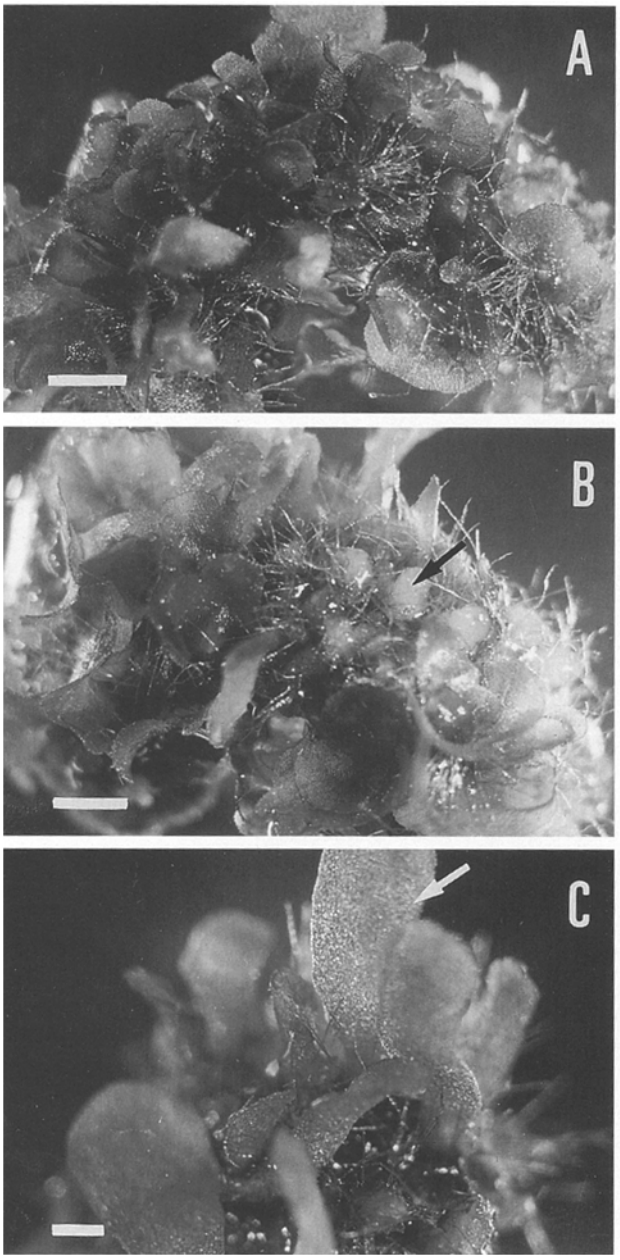

Fig. 1: Production of apogamous sporophytes from gametophytes cultured *in vitro*. (A) Explants showing reorganization into gametophytes. (B) Production of apogamous buds (arrow) from the newly regenerated gametophytes. (C) Sporophytes (arrow) used for epidermal peels. Scale bar = 2.5 mm.

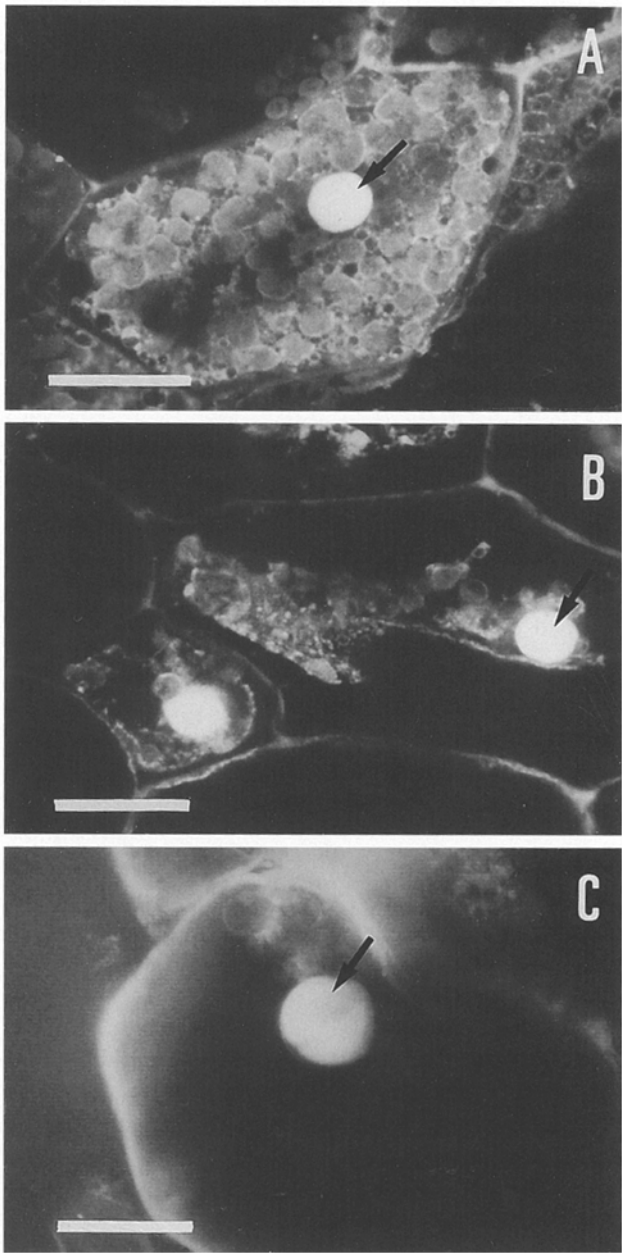

Fig. 2: Representative nuclei from cells of gametophyte (A), apogamous sporophyte (B) and sporophyte produced as a result of syngamy (C). Under the confocal laser scanning microscope, the acridine orange-stained nuclei appeared as bright round spheres (arrows). The cell wall and chloroplasts can be identified by their autofluorescence. Scale bar = 25 µm.

suggesting that the sporophytes were apogamous (Fig. 2). The mean diameter of 20 nuclei measured by CLSM was found to be 10.46±0.23 µm for the gametophytes, 11.79±0.20 µm for the apogamous sporophytes and 16.43±0.27 µm for the sporophytes produced by syngamy. All of the nuclei observed under the light microscope were spherical. The mean diameter of 150 nuclei was 8.87±0.08 µm for the gametophytes, 11.08±1.17 µm for the apogamous sporophytes and 16.07±0.92 µm for the sporophytes produced by fusion of gametes. Light microscopic measurement of nuclear size enabled us to sample a larger number of cells in a relatively short time. Moreover, the values obtained were comparable to that from CLSM, suggesting that light microscopy can be used routinely for this purpose. The hypothesis that cell size is correlated with ploidy in the ferns was first proposed by Lawton (1932) and further supported by Barrington and Paris

Table 1: Effect of indole-3-acetic-acid (IAA) and different seal types on the percentage of apogamous sporophyte production (calculated based on the number of explants forming sporophytes as a percentage of the total number of explants), number of sporophytes and extent of rhizoids produced from gametophyte explants after 50 days in culture. Data from three independent experiments were pooled.

Treatment	Mean percentage of explants producing sporophytes[x]	Mean number of sporophytes per explant	Extent of rhizoid production[y]
IAA (μM)			
0	4.2[a]	1.1±0.1	+
20	17.6[bc]	1.1±0.1	+
40	34.3[d]	3.5±0.1	++
60	20.4[b]	1.7±0.2	++
80	18.5[bc]	1.3±0.1	+++
100	14.4[bc]	1.4±0.1	+++
Seal Type			
Foam bung	74.4[a]	1.3±0.0	++
Foil	58.3[b]	1.3±0.1	++
Serum cap+mercuric perchlorate	54.5[b]	1.2±0.0	++
Serum cap	32.8[c]	1.1±0.0	++

[x]Means with the same letter in the same column per treatment type do not differ significantly (P=0.05) as indicated by one way ANOVA followed by Duncan's Multiple Range Test.
[y]Extent of rhizoid production was ranked from the lowest (+) to the highest (+++).

(1986) in their studies on three genera of ferns. Based on the assumption that doubling of the chromosome number should result in a corresponding increase in the nuclear size, we measured the latter from cells of gametophytes and sporophytes. Our results suggest that the ploidy level of the apogamous sporophytes of *P. coronarium* produced as a result of IAA treatment is similar to that of the gametophytes from which they developed, confirming the apogamous origin of the sporophytes. Cell size may vary depending on the growth environment and nutrition. However, the nuclear size may not be dependent on such factors. Hence, we propose that measurement of nuclear size rather than other parameters should be used for a quick estimation of the ploidy levels. This will facilitate such studies on plants with very high chromosome numbers like *Platycerium* (n=37) (Hennipman and Roos, 1982) where counting of the chromosome number can be very tedious.

The components of media, sugars and light have previously been demonstrated to affect apogamy (Bristow, 1962; Whittier, 1964) but to date there has been no report on the effect of auxin on apogamy in ferns. In our study, the addition of IAA to the medium containing sucrose could have enhanced the effect of the latter in inducing apogamy. Whittier (1964) proposed that supply of sugar to the gametophytes caused an increase in the availability of the respiratory substrate and hence an increase in availability of energy. This might have led to a change in the developmental pathway from gametophytes to sporophytes without the involvement of syngamy.

The presence of ethylene together with IAA in the culture vessels significantly decreased the percentage of apogamous sporophyte production. When serum caps were used to seal the flasks, there was ethylene accumulation (Fig. 3), which suppresses the promotory effect of IAA on apogamy. The percentage of apogamous sporophyte production in flasks sealed with serum caps increased significantly when ethylene was absorbed by setting up mercuric perchlorate traps (Table 1). Accumulation of ethylene in the culture vessels could have inhibited the polar and lateral transport of IAA in the gametophytes as reported for higher plants (Suttle, 1988). The inhibitory effect of ethylene was partially overcome by the use of mercuric perchlorate as has been reported in angiosperms (Kumar *et al.*, 1987; Abeles *et al.*, 1992). The use of aluminum foil or foam bungs as seals allowed a relatively low level of ethylene to remain in the flasks as these seals are not airtight, thus allowing a certain level of gaseous diffusion. This can also affect apogamy in our culture system (Table 1).

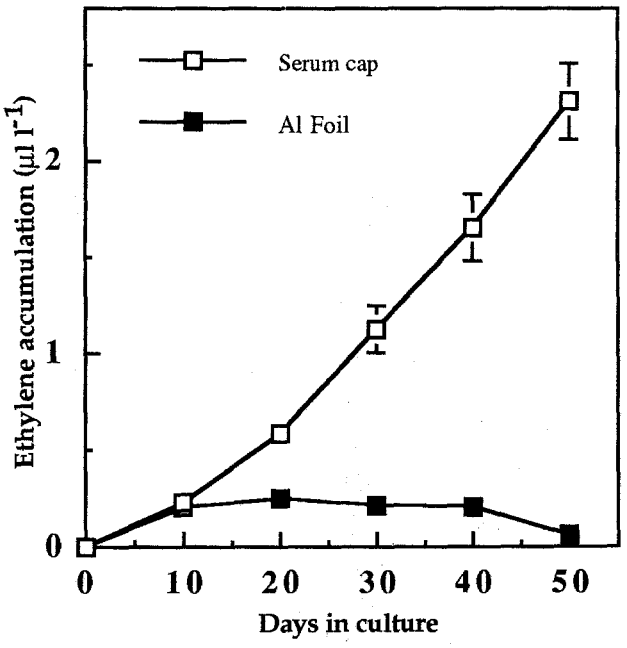

Fig. 3: Effect of two different seal types on the amount of ethylene accumulation in cultures of gametophyte clumps. Data from 3 independent experiments were pooled. Vertical bars represent SE of means.

Our present study shows that while IAA induces sporophyte production from the gametophytes of *P. coronarium* cultured *in vitro*, ethylene inhibits the process. The inhibitory effect of ethylene is contrary to past reports (Whittier and Pratt, 1971; Elmore and Whittier, 1973). The formation of apogamous sporophytes from the parenchymatous cells of the gametophytes involves cell division and organized growth (Whittier, 1962). Ethylene has been reported to have an inhibitory effect on cell division and organization of meristematic cells into organ primordia in some species (Miller *et al.*, 1970; Edwards and Miller, 1972; Reid 1987; Pearce *et al.*, 1991). Thus, inhibition of apogamous sporophyte production in the fern *P. coronarium* by ethylene is in line with the inhibitory role of ethylene on both cell division and organized growth in most systems.

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