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ORIGINAL PAPER

The effect of cytokinins on growth and sexual organ development in the gametophyte of *Blechnum spicant* L.

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Abstract In this study, we report the role of exogenous and endogenous cytokinins on growth and sexual organ development in the fern *Blechnum spicant* L. Spore-derived gametophytes (SG) were cultured in full-strength Murashige and Skoog (1962) liquid medium supplemented with (a) 4.44 μMN^6-benzyladenine (BAP), (b) a crude extract from mature female gametophytes, and (c) 4.44 μM BAP in combination with the crude extract from mature gametophytes, respectively. Both BAP and the crude extract delayed the gametophyte development, and this effect was increased when they were added together. With respect to sexual organ development, BAP inhibited the sexual organ formation, while the crude extract favored antheridia formation; however, when added together, the percentage of antheridia decreased. The endogenous level of the cytokinins *cis*-zeatin (cZ), *cis*-zeatin-riboside (cZR), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR), isopentenyl adenine (iP), isopentenyl adenosine (iPR), isopentenyl-9-glucoside (iP9G), *trans*-zeatin (tZ), and *trans*-zeatin riboside (tZR) were analyzed in female and male gametophytes of *B. spicant* L. The endogenous levels of cytokinins tZ, cZ, DHZ, cZR, iP, and iPR were higher in female gametophytes than in male gametophytes, with the endogenous iP and iPR content being increased more than 300 and 400 times, respectively.

Keywords Antheridiogen · *Blechnum spicant* · Cytokinin · Fern · Gametophyte · Sex

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Abbreviations

BAP	N^6-benzyladenine
cZ	*cis*-zeatin
cZR	*cis*-zeatin riboside
DHZ	Dihydrozeatin
DHZR	Dihydrozeatin riboside
HG	Gametophytes regenerated from homogenized mature gametophytes
HPLC-MS/MS	High-performance liquid chromatography-tandem mass spectrometry
iP	Isopentenyladenine
iP9G	Isopentenyl adenine-9-glucoside
iPR	Isopentenyladenosine
MS	Murashige and Skoog nutrient medium (1962)
SG	Spore-derived gametophytes
tZ	*trans*-zeatin
tZR	*trans*-zeatin riboside

Introduction

The control of sex expression in plants has been mainly studied on species with unisexual flowers, either monoecious or dioecious, although dioecious plants are particularly appropriate because all the processes involved in an induced program of genetic expression cannot interfere with the development of the other sex. In line with it, the gametophyte of the ferns has been proposed as an interesting model for studying several processes related to the plant growth and development, particularly the mechanisms underlying sex determination (Banks 1994).

The differentiation of plant sexual organs can be determined by chromosomes as it has been reported in *Marchantia polymorpha* and *Silene latifolia*, by the endogenous plant growth regulators (PGR) balance in *Zea mays* and *Cucumis sativa*, or by the epigenetic action of pheromones in some homosporous ferns (Tanurdzic and Banks 2004). In the last case, it was Döpp (1950) who observed that the culture medium of maturing prothallia of the bracken fern *Pteridium aquilinum* hastened the onset of antheridium formation in juvenile prothallia of *Dryopteris filix-mas* as well as *P. aquilinum.* Döpp concluded that the maturing prothallia produced a hormonal substance regulating antheridial formation, which was active in many species extending to over at least five families, and termed it as antheridiogens (Näf 1966). All the antheridiogens characterized so far are gibberellins-related compounds (Yamane 1998).

Blechnum spicant L. belongs to one of the most ancient groups of ferns. Sexual development in the gametophyte of this species is carried out through epigenetic action of the unknown antheridium-inducing substances. When cultured in vitro, initially the gametophyte develops female sexual organs or archegonia, and produces and excretes antheridiogens into the medium, which induce the formation of male sexual organs or antheridia in the youngest gametophytes, which are filamentous or spatulate-shaped (Klekowski 1969; Cousens 1979; Fernández et al. 1997).

The possible effects of plant growth regulators (auxins, cytokinins, and gibberellins) on the sexual organ development mediated by antheridiogen in *B. spicant* L. have been investigated in two types of gametophytes: spore-derived gametophytes (SG) and gametophytes regenerated from homogenized mature gametophytes (HG) (Menéndez et al. 2006a). It had been observed that in the absence of exogenous growth regulators, SG became predominantly females, while HG were mainly asexuals or males. The maleness observed in HG might be associated with the release of antheridiogen from the mature gametophytes into the culture medium during homogenization (Menéndez et al. 2006b). Of all the phytohormones tested (Menéndez et al. 2006a), only the cytokinin BAP strengthened the antheridiogen extract activity in the HG. However, SG cultured with BAP and without antheridiogen extract remained as females.

To examine whether the stimulatory effect of an antheridiogen-rich medium on male gametophyte production is amplified by BAP application, the SG were cultured in the presence of BAP, antheridiogens, and their combination, respectively. Apart from this preliminary study related to the effect of exogenous cytokinins on the sexual organ development, the endogenous levels of cytokinins in gametophytic cultures that were mostly male or female, were analyzed.

Materials and methods

Spores (5 mg) of *B. spicant* L. obtained from sporophytes growing in the forest of Turón (Asturias, Spain) were soaked in water for 2 h, and then sterilized for 10 min with a solution of NaClO (0.5%) containing Tween-20 (0.1%). Subsequently, they were rinsed thrice with sterile distilled water and centrifuged at 2,000 rpm for 3 min between the rinses, and cultured in 500 ml Erlenmeyer flasks containing 100 ml full-strength Murashige and Skoog (MS) (1962) liquid medium supplemented with (a) 4.44 μM BAP, (b) a crude extract from 0.5 fresh weight mature female gametophytes, and (c) 4.44 μM BAP plus the crude extract, respectively. The pH was adjusted to 5.7 and, unless otherwise noted, the cultures were maintained at 25°C under cool-white fluorescent light (40 μmol/m^2/s) with a 16 h photoperiod. The cultures were grown on a gyratory shaker (75 rpm).

The crude extract added to the culture medium was prepared as follows: 0.5 g of fresh gametophytes was homogenized with an Ultraturrax in 5 ml sterile distilled water for 10 s. Then, 1 h after the extraction, the liquid without the gametophytic tissue was recovered with a sterile Pasteur pipette and incorporated into a fresh MS medium.

The endogenous cytokinin content was analyzed in two types of cultures of *B. spicant* L. gametophytes, labeled A and B. Culture A was compounded by 2-year-old gametophytes, approximately 1 cm in length and mostly females, which were maintained on solid MS medium; culture B was compounded by 40-day-old gametophytes obtained from homogenized cultures of mature female gametophytes, and were mostly males (Table 1). Homogenized cultures from the mature gametophytic tissue were prepared according to the protocol by Fernández et al. (1993). For this purpose, mature female gametophytes (0.3 g) that had been maintained in culture for 2 years were mechanically fragmented using an Ultraturrax for 5 s under aseptic conditions. The homogenized tissue was washed thrice with sterile distilled water. Then, the gametophytic tissue was separately cultured in 250 ml Erlenmeyer flasks containing 50 ml of liquid MS medium supplemented with 2% sucrose (w/v).

Morphological studies were carried out under an optical microscope (Nikon Eclipse E600). Fresh gametophyte samples were taken and classified according to morphology of the gametophyte: filamentous (one-dimensional growth), spatulate (initial bidimensional growth), spatulate-heart (apical notch in progress), and heart (well-defined apical notch and lobes), and sexual development (male, female, hermaphrodite, and asexual).

Samples of the gametophytes (200 mg dry weight) were extracted overnight in 1.8 ml of Bieleski solvent (chloroform/

Table 1 Percentage of sexual phenotypes in the two types of cultures of gametophytes of *Blechnum spicant* L. A. Gametophytes cultured on solid MS medium. B. Gametophytes regenerated from mature homogenized gametophytes cultured in liquid MS medium

Sexual condition (%)	Types of cultures	
	A	B
Male	28	65
Female	60	10
Hermaphrodite	10	2
Asexual	2	23

methanol/formic acid/water—25:60:5:10, Bieleski 1964) at 4°C. Then, 10 pmol of deuterated cytokinins ([$^2H_5$]tZ, [$^2H_5$]tZR, [$^2H_5$]DHZ, [$^2H_5$]9RDHZ, [$^2H_6$]iP, [$^2H_6$]iPR; OldChemIm®, Olomouc, Czech Republic) were added. The samples were centrifuged (20,000*g*, 20 min, 4°C), re-extracted for 1 h in 1 ml 80% MeOH, and re-centrifuged. Both the supernatant fractions were pooled and passed through a C18 SPE (500 mg) to remove the pigments. Organic solvents were evaporated under reduced pressure at 40°C, the pH of the water phase was adjusted to 7, and distilled water was added to a final volume of 20 ml. The sample was applied onto a preequilibrated DEAE-Sephadex A-25 (Amersham Pharmacia Uppsala, Sweden) (2 ml NH_4HCO_3 form) with a preequilibrated C18 SPE cartridge (BondElut®. Varian, Hamburg, Germany) coupled underneath. The cartridges were rinsed with an additional 20 ml of distilled water. Then, the C18 cartridge was removed and eluted with 10 ml of 80% methanol to recover the free bases, ribosides, and 9-glucosides. These cytokinins were further purified using an immunoaffinity column according to the manufacturer's instructions (OldChemIm®, Olomouc, Czech Republic). The cytokinins purified by this way were dissolved in 20% methanol and analyzed by micro liquid chromatography coupled to electrospray tandem mass spectrometry (LC-(ES+)-MS/MS). The LC system used was a Kontron 325 pump, and the samples (30 µl) were injected using a Kontron 465 injector. The LC system was coupled to a Quattro II triple quadruple mass spectrometer (VG Micromass, Manchester, UK) equipped with an electrospray interface [(+)ES LC-MS/MS) and a Z-spray source (source temperature: 80°C, capillary voltage; +3.5 kV, cone voltage: 20 V]. The column was a Phenomenex Synergy Max-RP 80 Å (10 cm × 1 mm, 4 µm). Cytokinins were eluted using a gradient from 20% MeOH in 0.01 M ammonium acetate to 90% MeOH for 2 min, held in those conditions for 3.5 min, and returned to the initial conditions in isocratic mode for 5 min (flow rate: 60 µl/min). Quantification was done using Multiple Reaction Monitoring of $[M + H]^+$ ion (dwell time: 0.1 s) and the appropriate product ion. All the data were processed by Masslynx software (VG Micromass, Manchester, UK).

One hundred gametophytes were collected for each treatment and the experiments were repeated twice. Statistical analyses of the data were carried out using SigmaStat® v3.1 software. Nonparametric data (morphotype and sexual condition) were analyzed using the χ^2 test of frequency of gametophytes showing a particular morphological or sexual phenotype. The level of significance was set at $\alpha = 0.05$ for all the tests.

Results

Spores of *B. spicant* germinated in all the media assayed in 1 week, and the gametophyte development occurred (Fig. 1a). Significant differences with respect to the gametophyte development were observed among the treatments ($\chi^2_3 = 116.527$, $P < 0.01$). In the control medium, initially the gametophytes were filamentous, then spatulate, and finally, grew up until they achieved a typical heart shape. On the other hand, those cultured with BAP or the crude extract did not reach the typical heart shape and were mostly spatulated ($\chi^2_1 = 54.192$, $P < 0.001$; $\chi^2_1 = 64.826$, $P < 0.01$), and no significant differences were observed between these two treatments ($\chi^2_1 = 0.294$, $P = 0.588$). The addition of both BAP and crude extract together into the culture medium increased the presence of the spatulate morphology in the gametophytes with respect

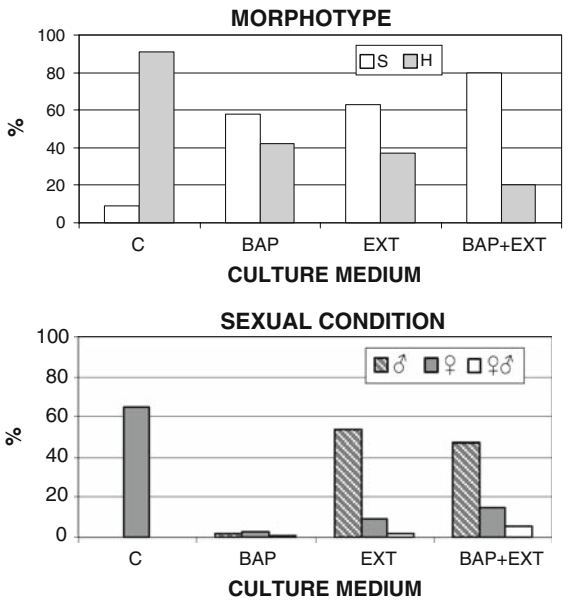

Fig. 1 Data of growth and gender in spore-derived gametophytes of *Blechnum spicant* L. cultured in liquid MS medium for 75 days. S = spatula, H = heart-shaped gametophytes, C = control medium, BAP = medium supplemented with 4.4 µM N^6-benzylaminopurine, EXT = medium with an extract from mature female gametophytes, antheridiogen-rich; BAP + EXT = medium with 4.4 µM BAP plus extract from mature female gametophytes

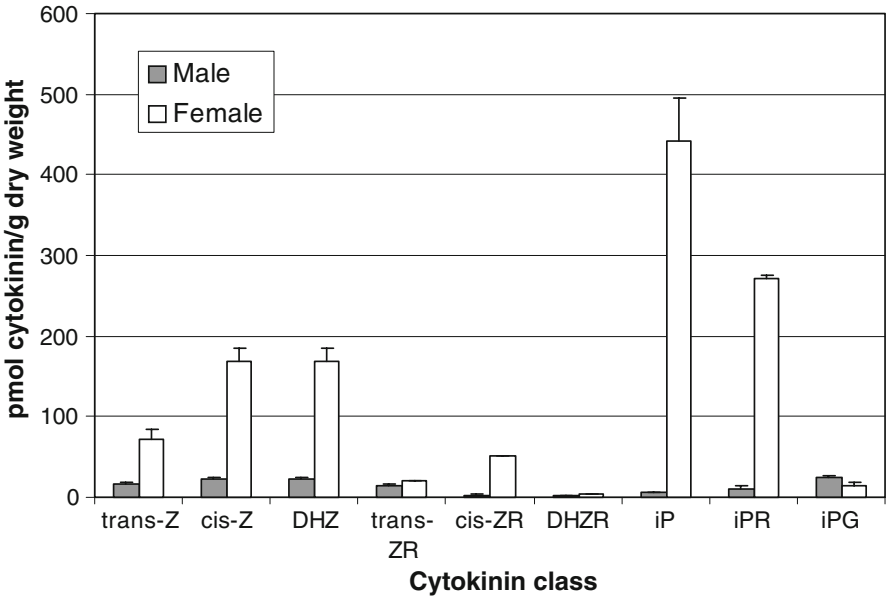

Fig. 2 Endogenous cytokinin content in female and male gametophytes of *B. spicant* L. Female gametophytes were cultured in solid MS medium and male gametophytes were obtained from mature homogenized gametophytes cultured in liquid MS medium

to the control ($\chi^2_1 = 113.916$, $P < 0.001$). Significant differences were also observed when comparing the percentage of spatulate gametophytes present in the MS medium with both the crude extract and BAP together, with respect to the addition of BAP ($\chi^2_1 = 9.841$, $P = 0.002$) or crude extract ($\chi^2_1 = 6.362$, $P = 0.012$) separately.

In terms of sexual organ development, significant differences were observed among the treatments (Fig. 1b) ($\chi^2_3 = 243.152$, $P < 0.001$). The SG cultured in liquid MS medium were mostly female; the addition of BAP diminished the archegonia formation significantly with respect to the control ($\chi^2_2 = 83.946$, $P < 0.001$), while the addition of the extract promoted maleness, either alone ($\chi^2_1 = 64.826$, $P < 0.001$) or with BAP ($\chi^2_2 = 14.275$, $P < 0.001$), even though the percentage of antheridia significantly decreased when added together ($\chi^2_3 = 81.106$, $P < 0.001$).

The endogenous levels of several cytokinins were analyzed by HPLC-MS/MS in cultures of gametophytes that were predominantly males or females. The co-existence of more than one sexual condition—male, female, hermaphrodite, and asexual—in the gametophytes cultured in either in vivo or in vitro conditions, makes it difficult to carry out this type of analyses in ferns. Nevertheless, we can obtain gametophytic cultures in which one type of sexual phenotype is predominant, by adequately handling the culture. The results of endogenous cytokinin concentration (pml/g dry weight) are shown in Fig. 2. Significant differences were found in the endogenous levels of *trans*-zeatin (tZ), *cis*-zeatin (cZ), dihydrozeatin (DHZ), *cis*-zeatin-riboside (cZR), isopentenyl adenine (iP), and isopentenyl adenosine (iPR) in female and male gametophytes. The endogenous levels of these cytokinins were higher in female gametophytes than in males. The endogenous content of iP and iPR detected in the female gametophytes strongly increased—the level of iP and iPR was around 300 and 400 times higher, respectively, in female than in male gametophytes of *B. spicant*.

Discussion

In this study, a possible role of cytokinins on both the growth and sexual organ development mediated by an antheridium-inducing hormonal substance, known as antheridiogen, in the gametophyte of the fern *B. spicant* is reported.

In an earlier work done in our laboratory, the effect of the exogenous plant growth regulators: indol-3-acetic acid (IAA), BAP, and the gibberellins, GA_3 and GA_{4+7}, on the growth and sexual organ development in the SG and HG of *B. spicant* was reported (Menéndez et al. 2006a). The results obtained revealed that HG cultured in a medium without exogenous plant growth regulators showed an inhibitory effect on their development, as well as a stimulatory effect on the antheridia formation (presumably owing to the fact of being cultured in an antheridiogen-rich medium), which was enhanced by the addition of the cytokinin, BAP, to the culture medium. When comparing these results with those obtained with SG presented in this work, particularly, those referring to the BAP+ crude extract treatment, the differences between them were obvious. In HG, the addition of BAP to the antheridiogen-rich medium in which they were cultured (Menéndez et al.

2006b) enhanced the antheridia formation. On the contrary, in SG, the addition of BAP to the medium with or without crude extract (which also includes antheridiogens), did not enhance the antheridia formation, and moreover, the sexual organ development diminished or strongly diminished, respectively. Furthermore, converse effects were detected for the cytokinin, BAP, on the antheridiogen activity in each type of the gametophytes.

It has been extensively reported that the same plant growth regulator may have a diametrically opposite effect on sex, depending on the species studied (Duran and Duran 1990). Our study went further and demonstrated how the same plant growth regulator could have different effects on the sexual development in the gametophytes of different origins within the same species. The cytokinin, BAP, enhanced the positive effect of the antheridiogens on antheridia formation in HG and inhibited the sexual organ formation (mainly archegonia) in SG, or diminished the effect of antheridiogens on antheridia formation when these compounds (BAP and antheridiogens) present in the crude extract were added together to the culture medium. These findings also contribute to study on the lability of sex expression reported in the more ancient plant forms, such as mosses and ferns, when compared with many angiosperms (Korpelainen 1998).

The fact that exogenous cytokinins, such as BAP, affect the sexual organ development mediated by antheriogens in the gametophyte of *B. spicant*, inspired us to analyze the endogenous levels of several cytokinins in the gametophytes of different sexes. The endogenous level of the cytokinins cZ, cZR, DHZ, dihydrozeatin riboside (DHZR), iP, iPR, isopentenyl-9-glucoside (iP9G), tZ, and trans-zeatin riboside (tZR) were analyzed, and the results revealed that the endogenous levels of these cytokinins were higher in female gametophytes than in the male gametophytes, with the endogenous iP and iPR content being increased more than 300 and 400 times, respectively.

The organogenesis of sexual organs can be considered as resulting from the expression of differentiation programs in which the plant growth regulators are considered to be involved. To determine the importance of plant growth regulators in sex expression, two different approaches have been employed: the exogenous administration of growth regulators to the developing plants and the quantitative analyses of endogenous phytohormones (Duran and Duran 1984; Chailkyan Kh and Khryanin 1987). In the light of these data, gibberellins and cytokinins have been reported as the major plant growth regulators responsible for expressing the male or female sex in the flowering plant species. In particular, the predominance of cytokinins favored female-sex expression and that of gibberellins contributed to male-sex expression (Khryanin 2002).

No differences in the endogenous content of the gibberellins GA_1, GA_3, GA_4, GA_7, GA_9, and GA_{20} were found between the sexes in the gametophytes of *B. spicant* (Menéndez et al. 2006b); however, differences in the endogenous content of the cytokinins mentioned earlier were observed. Not much is known about the influence of particular cytokinin metabolites on sex expression. In the dioecious species, *Leuceadendron rubrum*, a correlation between the increased content of the cytokinin, iP, and the development of female plants was reported (Dekock et al. 1994). In the most extensively studied system, *Mercurialis*, where a number of genes control the sex expression, specific cytokinins have been linked with sex differentiation in comparisons done between the wild-type male and female plants. The results showed that the nucleotide iP, although shared by both the sexes, was accumulated in females. Besides, the zeatin-free base was in higher quantity in females, whereas the males lacked it (Duran and Duran 1990). Furthermore, a direct link between the feminizing alleles and the female-specific cytokinin metabolite, tZ, was also suggested. In males, the zeatin pathway stops at its immediate precursor, tZR, whereas the tZ mononucleotide gets accumulated (Yang et al. 1998). In addition, Yang et al. (1998) reported on a female-specific DNA and the existence of a female-specific enzyme capable of converting tZR into the specific feminizing cytokinin, tZ. More recently, studies on over-expressed zeatin O-glucosylation genes demonstrated the formation of tassel seed in the homozygous transformants in maize, revealing a link between cytokinins and sex-specific floral development in monocots (Pineda Rodó et al. 2008).

In summary, our data revealed that the effect of an exogenous plant growth regulator, such as BAP, may change depending on the origin of the plant material, in our case, the spores and homogenized mature gametophytes. It is possible to find either a synergistic or an antagonistic action among different growth substances as presented in this study for antheridiogen and cytokinins. Further research is required to understand the antagonistic effect of BAP on the antheridiogen activity in each type of gametophytes. However, analysis of the endogenous hormonal level would be a desirable starting point. On the other hand, the analyses of endogenous content of these compounds in different sexes revealed, for the first time, that the male and female individuals have significant differences in their endogenous cytokinin levels in this species, with female gametophytes being several time richer in tZ- and cZ-free bases, especially in iP and its riboside. The analyses of molecular markers expressed by the action of antheriodiogens and cytokinins are in progress in our experimental systems of *B. spicant* gametophytes.

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