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# Do fern gametophytes have the capacity for irradiance acclimation?

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## Abstract

Ferns present two alternant generations: sporophyte and gametophyte. In the present work we address the question of whether fern gametophytes have the potential to acclimate to different irradiances as vascular plants do. We studied the gametophytes of three different fern species belonging to the *Aspleniaceae* family with different ecological requirements (*Asplenium trichomanes*, *Asplenium scoloprendrium* and *Ceterach officinarum*). Fern spores were germinated and the gametophytes cultivated under photon flux density (PFD) of 10, 50 or 100 μmol $m^{-2}$ $s^{-1}$. From the early stages of spore germination (the formation of the 5-celled germinal filament), photosynthetic apparatus acclimates showing the typical patterns of photochemical responses to high or low PFD. In agreement with the photochemical pattern of acclimation, higher contents of xanthophyll cycle pigments and α-tocopherol was observed in plants grown under high PFD. The α/β-carotene ratio, used as indicator of the acclimation of the photosynthetic apparatus, also sustained the initial hypothesis except for *A. trichomanes*. We conclude that fern gametophytes display a complete array of photosynthetic and photoprotective traits that allow an effective acclimation to PFD.

*Additional key words*: *Asplenium scoloprendrium, Asplenium trichomanes*, *Ceterach officinarum*, chlorophyll fluorescence imaging, HPLC, tocopherols, xanthophylls.

## Introduction

In plants and algae there is an alternation of generations: a diploid sporophyte and a haploid gametophyte. It is well established that there is an evolutionary tendency for a reduction in the size of the gametophyte, which in flowering plants is reduced to the ovule or the pollen. In this context, ferns represent an intermediate evolutionary stage, with the presence of free-living sporophytes and gametophytes. Fern sporophytes are typical vascular plants with laminate photosynthetic structures, but fern gametophytes are very small and lack tissue organization. After germination of the spore, a protonemal structure is formed. In the genera *Asplenium* and *Ceterach* belonging to the *Aspleniaceae* family (Smith *et al*. 2006), the filament of few cells becomes a bidimensional prothallus and ultimately reaches a heart-shaped stage where archegonia and antheridia are formed (Muccifora and Gori 1995). From the initial steps of development, most gametophytes are photosynthetically active and their environmental requirements, which may differ from those of the sporophyte, frequently restrict fern distribution (Johnson *et al*. 2000). Photosynthetic responses have been characterised in a wide representation of fern sporophytes (*i.e*., Brach *et al*. 1993, Hietz and Briones 2001, Lösch *et al*. 2007, Saldaña *et al*. 2005). Gametophyte contribution to sporophyte growth has also been studied (Sakamaki and Ino 1999, 2007). However, very few works have focused on photosynthesis in fern gametophytes (Hagar and Freeberg 1980, Johnson *et al*. 2000, Watkins *et al*. 2007a).

All photosynthetic organisms must have a positive carbon balance for long-term survival, which implies that they must acclimate to prevailing environmental conditions. In general, acclimation to PFD involves two main sets of physiological modifications: optimization of radiation absorption and use (Lüttge 1997) and protection from the damaging effects of excess radiation (Müller

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*Abbreviations*: A - antheraxanthin; Car - carotenoid; FM - fresh mass; L - lutein; N - neoxanthin; NPQ - non photochemical quenching; PFD - photon flux density; Toc - tocopherol; V - violaxanthin; Z - zeaxanthin; $\Phi_{PS2}$ - quantum yield of PS 2 photochemistry.

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*et al*. 2001). Both sets of physiological traits are strongly interconnected, since all the energy that is absorbed but not used must be dissipated to avoid damage. Acclimation to high PFD involves upregulation of the carbon fixation capacity, the potential for thermal dissipation of the excess energy absorbed and antioxidant capacity. On the other hand, low PFD acclimation favours the maximum of energy capture and the downregulation of photoprotective capacity.

Since species success in different environments is directly or indirectly associated with their ability of photosynthetic acclimation (Cai *et al*. 2010), the present work aims to verify whether gametophytes are able to acclimate their photosynthetic apparatus to different PFD. To address this question we studied the composition of photoprotective compounds and the photochemical responses to increasing PFD from the early stages of gametophytes development. Gametophytes from three fern species with contrasting ecological requirements, grown under different PFD, were studied. We also tested whether ecological differences in the sporophytes are based on the potential of gametophytes for PFD acclimation.

## Materials and methods

Three fern species belonging to the family *Aspleniaceae* with different ecological requirements were used for this study: *Asplenium scolopendrium* L., which grows in moist and shady habitats; *Asplenium trichomanes* L., which requires a less shade environment; and *Ceterach officinarum* Willd, which can grow under a wide range of PFD (Ellenberg *et al*. 1992). Mature fronds from the three species were collected from the field in November 2008. *A. scolopendrium* plants were harvested from the understorey of a beech (*Fagus sylvatica*) forest in Cadagua (Burgos, Spain, N 43º4', W 3º21', alt. 400 m). *A. trichomanes* and *C. officinarum* were collected from a stone wall in Las Machorras (Burgos, Spain, 43º7', 3º36', alt. 800 m). Fronds from the sporophytes were used for pigment analysis. Spores of the three species were gently removed from mature fronds with a brush. The removed spores were covered with water for 24 h and then surface-sterilized with sodium hypochlorite (1 %) for 3 min. Finally, the spores were washed twice with deionised water. Sterile spores from each fern were sown onto mineral Thompson agar (Klekowski 1969) in 5 cm diameter Petri dishes and cultured in a growth chamber at 25 ºC and 12-h photoperiod with high, medium or low PFD of 10, 50 and 100 μmol $m^{-2}$ $s^{-1}$, respectively. These irradiances were obtained by using neutral filters. Spores were watered every two weeks with sterile deionised water. Two different developmental stages were studied: the initial filament-shaped stage reached at about 15 d after sowing and the more developed heart-shaped stage reached at about two months after the sowing (Fig. 1). When grown under high PFD, enough biomass for pigment analysis of heart-shaped gametophytes from *A. trichomanes* was not obtained.

Dark-acclimated gametophytes, collected before lamps in the chamber were switched on, were put over a microscope slide with a wet filter paper, covered with a cover slip, and adapted at 50 μmol $m^{-2}$ $s^{-1}$ for 15 min to activate photosynthetic responses before fluorescence measurements. Chlorophyll *a* fluorescence was measured with an *Imaging PAM Microscopy Version* fluorometer (*Walz*, Effetrich, Germany). Each individual measurement was performed on a single cell from each sample (see Fig. 1.) A middle-sized single cell of the focused field, avoiding the meristematic region, was always selected for chlorophyll fluorescence measurements. The increasing actinic PFD contained 8 progressive steps of 20 s each ranging from 4 to 211 μmol $m^{-2}$ $s^{-1}$ separated by a 0.2 s saturating pulse (1 000 μmol $m^{-2}$ $s^{-1}$). The actual quantum yield of PS 2 ($\Phi_{PS2}$) was calculated as $(F_m'-F)/F_m'$, with $F_m'$ being the maximal fluorescence under saturating pulse and F the steady-state fluorescence under the actinic PFD.

For pigment analyses, heart-shaped gametophytes were taken from Petri dishes, frozen in liquid $N_2$ and stored at -80º C until use. For sporophytes, frond discs were frozen in the same way. Frozen samples were homogenized with a tissue homogenizer (*Tearor model 395*, Dremel, Mexico) in a pure acetone solution buffered with $CaCO_3$. The extracts were centrifuged at 16 100 *g* for 20 min. Supernatants were filtered with 0.2 mm *PTFE* filters (*Teknokroma*, Barcelona, Spain). Pigment separation was performed using HPLC with a reverse phase C18 column (*Waters Spherisorb ODS1*, 4.6 x 250 mm, Massachusetts, USA) following the method described by García-Plazaola and Becerril (1999) but including the modifications described in García-Plazaola and Becerril (2001). During processing in the HPLC, samples were maintained at 4º C in a refrigerated compartment. Identification and quantification was carried out with a photodiode array (PDA) detector. Retention times and conversion factors for pigments were the same as described by García-Plazaola and Becerril (1999, 2001). The relative de-epoxidation state of the xanthophyll cycle pigments (DEPS) and the total xanthophyll cycle pool expressed per chlorophyll were estimated.

We used one-way *ANOVA* to test for differences in pigments contents in response to PFD during the growth of gametophytes. Duncan post-hoc test was performed to discriminate among different treatments, after Cochran test to check for homogeneity of variances. The differences in the photosynthetic quantum yield were checked by the LSD test. All analyses were performed using *SPSS 17.0* statistical package.

## Results

The content of photosynthetic pigments was analyzed in heart-shaped fern gametophytes grown under medium PFD and in field-grown sporophytes of the same species (Table 1). Gametophytes of the three species showed the pigment composition typical of green plants, with six major carotenoids (neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and β-carotene) plus α-carotene in some conditions. Qualitatively, carotenoid composition was the same in gametophytes and sporophytes of all species. The content of xanthophylls (neoxanthin, violaxanthin, antheraxanthin, lutein and zeaxanthin), however, was higher in gametophytes as compared to sporophytes while β-carotene showed the opposite pattern. These quantitative differences have to be considered with care because they could be due to the different environments during the growth of both the gametophytes and the sporophytes. Gametophytes of *A. scolopendrium* showed the highest de-epoxidation of xanthophyll cycle pigments (DEPS; 0.325) whereas *C. officinarum* had the lowest DEPS (0.101). Compared with the rest of the gametophytes, *A. trichomanes* showed the lowest pigment content (note the small amount of chlorophylls), although the proportion of carotenoids per chlorophyll was similar to the other species (Table 1).

Table 1. Pigment composition of fern gametophytes (G) in the heart-shaped stage grown under PFD 50 μmol $m^{-2}$ $s^{-1}$ and of sporophytes (S) grown in the field: chlorophyll (Chl) *a* and Chl *b* contents [nmol $g^{-1}$(f.m.)], contents of main carotenoids neoxanthin (N), violaxanthin (V), antheraxanthin (A), lutein (L), zeaxanthin (Z), α-carotene (α-Car), β-carotene (β-Car) [mmol $mol^{-1}$(Chl)] and DEPS [(0.5 A + Z)/(V + A + Z)] of xanthophyll cycle pigments. Means ± SE, *n* = 3 - 5. Plant material was kept in darkness at 100 % RH during 12 h before sampling.

| | Species | Chl *a* | Chl *b* | N | V | A | L | Z | α-Car | β-Car | DEPS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| G | *A. scoloprendrium* | 285.8±16.8 | 146.8±9.3 | 55.0±1.8 | 75.6±3.6 | 19.9±0.7 | 200.9±5.2 | 16.6±0.9 | 2.1±0.5 | 86.6±0.9 | 0.325 |
| | *A. trichomanes* | 120.7±32.5 | 69.7±19.8 | 55.8±1.1 | 84.3±9.0 | 14.7±1.9 | 195.7±4.4 | 14.5±0.9 | 2.5±1.2 | 71.3±5.2 | 0.226 |
| | *C. officinarum* | 208.3±6.0 | 118.0±3.1 | 56.2±2.6 | 77.7±3.5 | 3.8±0.8 | 218.7±10.4 | 5.3±1.9 | 0.8±0.1 | 70.6±6.7 | 0.101 |
| S | *A. scoloprendrium* | 2002.5±114.7 | 740.2±35.4 | 46.2±1.2 | 67.0±1.7 | 3.2±0.4 | 162.2±1.8 | 2.5±0.6 | 1.1±0.0 | 91.2±1.0 | 0.078 |
| | *A. trichomanes* | 1900.4±53.3 | 744.9±20.2 | 48.3±0.0 | 62.2±0.0 | 4.4±0.0 | 138.8±0.0 | 3.4±0.0 | 1.3±0.0 | 78.1±0.0 | 0.114 |
| | *C. officinarum* | 268.0±25.9 | 268.0±25.9 | 44.8±0.6 | 69.4±5.0 | 5.1±1.1 | 161.5±4.0 | 4.4±1.2 | 1.5±0.2 | 96.8±13.1 | 0.120 |

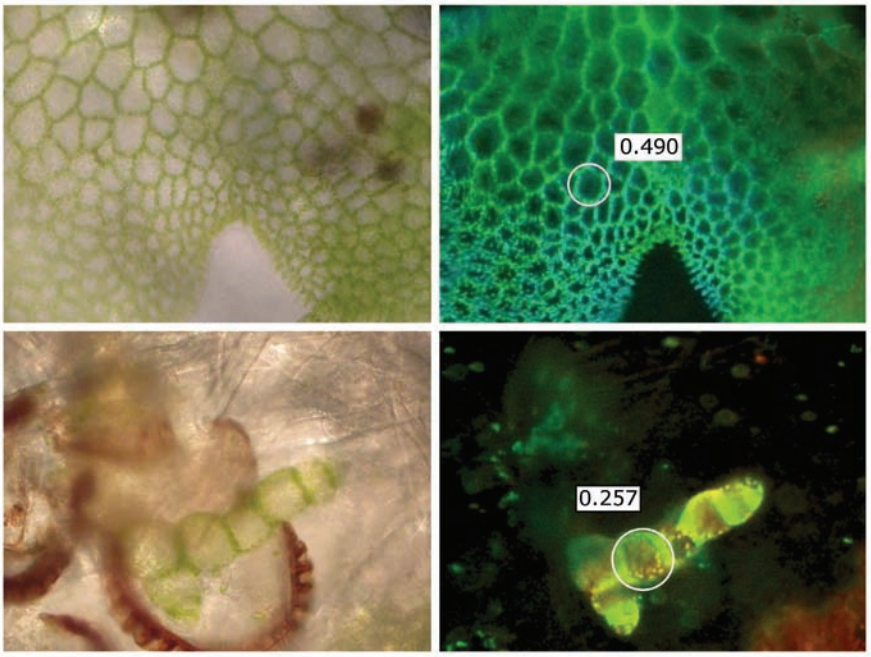


Fig. 1. An example of the fluorescence measurements with the Imaging PAM microscopy fluorometer. *On the left*, photographs of heart-shaped gametophyte (*upper panel*) and filament-shaped fern gametophytes (*lower panel*) taken with the optical microscopy. *On the right*, the fluorescence images of the same two samples. The numbers show the average value of the maximum photochemical efficiency of PS 2 for a particular selected cell (*circle areas*).

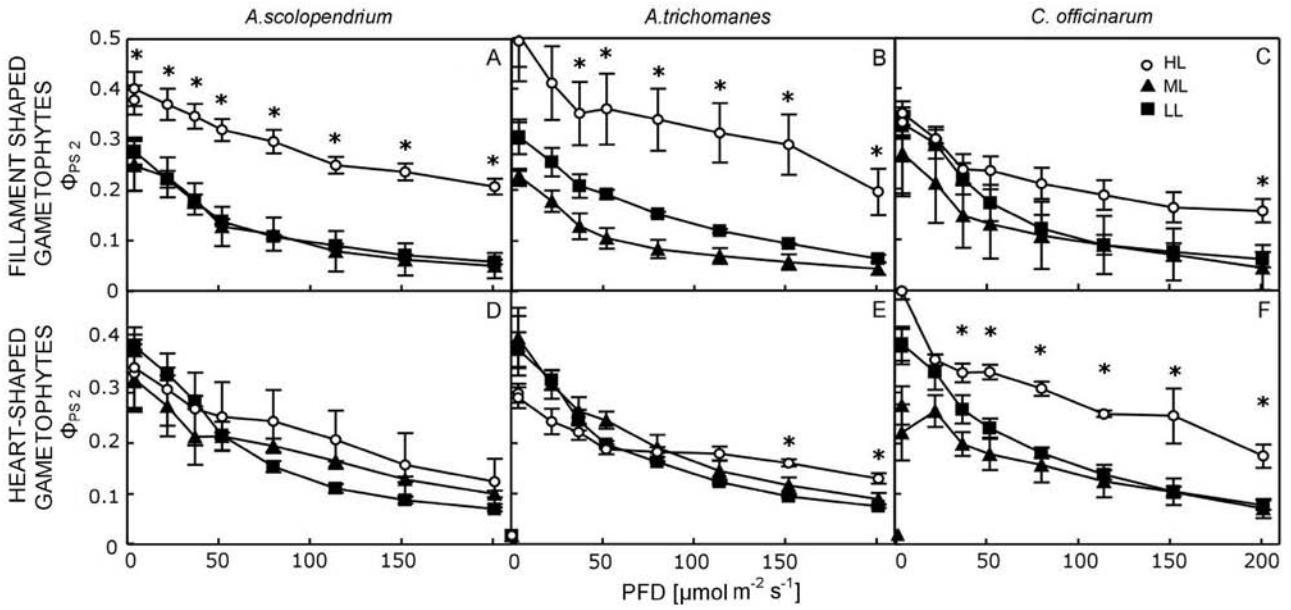


Fig. 2. Quantum yield of PS 2 of the filament stage of fern gametophytes (*A*,*B*,*C*) and of heart-shaped stage gametophytes (*D*,*E*,*F*) grown at different PFD of 10 (LL), 50 (ML) and 100 (HL) µmol m$^{-2}$ s$^{-1}$. Means ± SE, $n$ = 3 - 6. *Asterisks* indicate significant differences between high PFD and, at least, one of the other PFDs ($P < 0.05$).

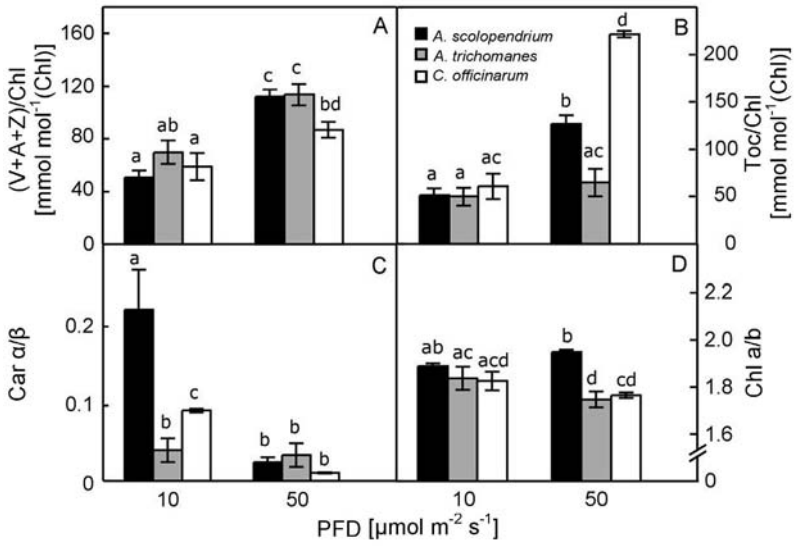


Fig. 3. Pigment composition of heart-shaped gametophytes grown at 10 and 50 µmol m$^{-2}$ s$^{-1}$. Means ± SE, $n$ = 3. Different letters indicate significant differences in the pigment content between growth PFDs ($P < 0.05$). Data for high PFD (100 µmol m$^{-2}$ s$^{-1}$) are not shown because it was not obtained enough material for pigment analysis.

The capacity for PFD acclimation was analyzed in the initial developmental stages of all three fern species germinated and grown at 10, 50 or 100 µmol m$^{-2}$ s$^{-1}$ (Fig. 2). Irrespective of the species, no differences were found in the photosynthetic response of the gametophytes when comparing the low and medium PFD treatments (Fig. 2). However, the quantum yield ($\Phi_{PS2}$) was significantly higher in gametophytes grown at high PFD than in those grown at lower PFD. This symptom of acclimation was remarkable in *A. scolopendrium* and *A. trichomanes*, in which the differences among treatments were statistically significant. These results were unexpected because of the assumption that recently germinated gametophytes of a particular species would show similar responses to increasing irradiances, irrespective of PFD during spore germination. Nevertheless, we observed differences in PDF acclimation from the initial developmental stage of fern gametophytes.

Acclimation to PFD was also studied on heart-shaped fern gametophytes (Fig. 2). In this more developed stage, differences in the $\Phi_{PS2}$ were attenuated among the

growing PFD treatments and no general pattern was found except for *C. officinarum* which showed clear symptoms of PFD acclimation. In *A. trichomanes*, high PFD treatment induced higher $\Phi_{PS2}$ values only when exposed to PFDs higher than 100 μmol $m^{-2}$ $s^{-1}$ (Fig. 2*E*), while *C. officinarum* grown at high PFD, showed the highest $\Phi_{PS2}$ through all the measured PFDs (Fig. 2*F*).

The $\Phi_{PS2}$ decreased gradually for the three ferns in both the filament and heart-shaped stages when they were exposed to increased PFD (Fig. 2). Higher differences among PFD acclimation were found in early stages of filament-shaped gametophytes for *A. scolopendrium* and *A. trichomanes*, whereas major acclimation patter for *C. officinarum* was showed in the more developed heart-shaped gametophytes (Fig. 2*F*).

The composition of pigments and photoprotective compounds was studied in heart-shaped gametophytes of the three species cultured at 10 or 50 μmol $m^{-2}$ $s^{-1}$. The xanthophyll pigment cycle pool (V + A + Z)/Chl responded positively to growth irradiance in all the three species (Fig. 3*A*). Higher α-tocopherol content was found in gametophytes grown at 50 μmol $m^{-2}$ $s^{-1}$, with the exception of *A. trichomanes* (Fig. 3*B*) while the carotene α/β ratio increased with increasing PFD (Fig. 3*C*), also with the exception of *A. trichomanes*. The response of the chl *a/b* ratio to PFD was erratic, with no effect in *A. scolopendrium* and in *C. officinarum,* and a negative trend in *A. trichomanes* (Fig. 3*D*). In summary, the composition of photoprotective pigments was less responsive to irradiance in *A. trichomanes* than in the other species. *A. scolopendrium*, with the highest chl *a/b* and Car α/β at low PFDs, was apparently the most shade-adapted gametophyte, while *C. officinarum* showed the best acclimation capacity for high PDF as evidenced by the strong accumulation of α-tocopherol.

## Discussion

Gametophytic generation is essential in the fern life cycle, however, very little is known about its ecology and physiology (Greer *et al*. 1999, Johnson *et al*. 2000, Watkins *et al*. 2007a,b). In fact, to the best of our knowledge this study is the first report to show the carotenoid and tocopherol composition of fern gametophytes. Present results show that gametophyte pigment composition does not qualitatively differ from that of fern sporophytes (Tausz *et al*. 2001) or from other vascular plants. Thus, the six major carotenoids (Esteban *et al*. 2009) were also present in the three fern species analyzed. Sporophytes of the three species were also compared with gametophytes, showing that there were some slight but consistent quantitative differences in pigment composition between both generations. Basically, chlorophyll content was much higher in sporophytes, which is in agreement with their higher photosynthetic rates (Hagar and Freeberg 1980).

The gametophyte pigment composition was modulated by growth irradiance, as it was reflected by the lower values of (V+A+Z)/Chl and the higher car α/β ratio in plants grown at low PFD (Fig. 3*B*,*C*). This trend is consistent with the typical pattern of PFD acclimation observed in vascular plants (Demmig-Adams 1998, Thayer and Björkman 1990, Zhang *et al.* 2009), which consists of an up-regulation of light-harvesting systems under low PFD and a maximization of energy dissipation under high PFD. Not only pigments but also other photoprotective compounds such as α-tocopherol changed with growth PFD, with the lowest contents at low PFD (Fig. 3*D*). This observation agrees with the reported significance of α-tocopherol in photoprotection in epiphytic ferns (Tausz *et al*. 2001). Interestingly, they found the highest potential for α-tocopherol synthesis in other species of the same family (*Asplenium cuspidatum*).

It has been demonstrated that PFD affects the germination of spores (Hiendlmeyer and Randi 2007) and density of terrestrial gametophytes (Watkins *et al*. 2007b). However, photosynthetic responses to PFD remained to be established. In fact, the Chl *a* fluorescence of fern gametophytes has been reported only in a couple of studies (Johnson *et al*. 2000, Watkins *et al*. 2007a). Aiming to fill this gap, we have tested whether differences in pigment composition at different PFD were also reflected in photosynthetic performance. In this sense, acclimation in PS 2 activity was observed from the very early stages of gametophyte development. Besides, no evidence of saturation in filament-shaped gametophytes was observed until an irradiance of 200 μmol $m^{-2}$ $s^{-1}$. This contrasts with previous reports in which saturation occurred at PFDs below 100 μmol $m^{-2}$ $s^{-1}$ as measured by the use of gas exchange (Hagar and Freeberg 1980).

Filament-shaped gametophytes showed the maximum $\Phi_{PS2}$ when grown under high PFD (Fig. 2*A*,*B*,*C*), just as it usually occurs in other vascular plants (Chowdhury *et al*. 2009), thus representing clear symptoms of PFD acclimation in the three species. Once the gametophytes reached the heart-shaped stage, the typical pattern of PFD acclimation (Lüttge 1997) was attenuated but still observed in the case of *C. officinarum*. In our study, the $\Phi_{PS2}$ was in the range reported by other works for gametophytes (Johnson *et al*. 2000), but it is noticeable that $\Phi_{PS2}$ was highest in gametophytes of *C. officinarum*, which is in concordance with the high PFD in the environments where it usually grows. Considering that gametophytes grew in unlimited water and mineral supply, these results should be taken with care. Nevertheless, the strong dependence of photosynthetic performance on growth irradiance shown in this paper confirms the pivotal role of PFD in gametophyte survival during their long life span.

In conclusion, according to our knowledge, this is the first study showing the capacity of the photosynthetic apparatus of fern gametophytes to acclimate to a range of irradiances beginning in the early stages of development immediately after spore germination. The gametophytes of the all three ferns studied showed the typical acclimation pattern. Thus, photosynthetic plasticity in gametophytes allows them to optimize their carbon gain and successfully compete with co-occurring phototrophs, and explains the wide ecological amplitude of the sporophytes of most fern species.

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