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Photosynthesis limitations in three fern species

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Maximum photosynthesis rates in ferns are generally lower than those of seed plants, but little is known about the limiting factors, which are crucial to understand the evolution of photosynthesis in land plants. To address this issue, a gas exchange/chlorophyll fluorescence analysis was performed in three fern species spanning high phylogenetic range within Polypodiopsida (*Osmunda regalis*, *Blechnum gibbum* and *Nephrolepis exaltata*) to determine their maximum net photosynthesis (A_N), stomatal (g_s) and mesophyll (g_m) conductances to CO_2, and the maximum velocity of carboxylation ($V_{c,max}$). The in vitro Rubisco specificity factor ($S_{C/O}$) was also determined. All three species had values for $S_{C/O}$ similar to those typical of seed plants, but values of A_N, g_s, g_m and $V_{c,max}$ were within the lowest range of those observed in seed plants. In addition, g_s was unresponsive to light and CO_2, as already described in other fern species. On the contrary, g_m varied with changes CO_2. A quantitative photosynthesis limitation analysis suggested that early land plants (ferns) presented not only stomatal limitations – which were less adjustable to the environment – but also restricted g_m and $V_{c,max}$, resulting in limited maximum photosynthesis rates.

Introduction

Photosynthetic characteristics are key traits in plants for the adaptation to the environment. In land plants (embryophytes), evolutionary patterns have been described for traits related to photosynthesis, including the photosynthetic type (Keeley and Rundel 2003, Sage 2004, Keeley et al. 2012), the leaf shape (Beerling 2005, Brodribb et al. 2007, Flexas and Keeley 2012), the stomatal morphology and function (Carpenter 2005, Franks and Farquhar 2007, Brodribb and McAdam 2011) and photoprotection mechanisms (Esteban et al. 2009). However, studies concerning how the photosynthetic capacity has evolved in land plants are scarce (Feild et al. 2009, Soni et al. 2012). In particular, there is a surprising scarcity of studies concerning the photosynthetic

Abbreviations – α, leaf absorptance; β, partitioning of absorbed quanta between photosystems II and I; ϕ_{PSII}, actual photochemical efficiency of photosystem II; Γ^*, CO_2 compensation point in the absence of respiration; A_N, maximum net photosynthesis; C_a, ambient CO_2 concentration; C_c, CO_2 concentration at the site of the chloroplast; C_i, CO_2 concentration at sub-stomatal cavities; g_c, cuticular conductance; g_m, mesophyll conductance; g_s, stomatal conductance; J_{flu}, electron transport rate; J_{max}, electron transport rate; K_c, Michaelis constant for CO_2 of Rubisco; $k_{cat}{}^c$, catalytic constant for the Rubisco carboxylase reaction; l_b, l_m, l_s, limitations and S_L, M_{CL}, B_L relative limitations imposed by biochemistry, mesophyll and stomatal conductances, respectively; LMA, leaf mass area; PPFD, photosynthetically photon flux density; P_r, photorespiration rate; PSI, photosystem I; PSII, photosystem II; RH, relative humidity; R_d, non-photorespiratory CO_2 release in the light; R_n, mitochondrial respiration; $S_{C/O}$, in vitro Rubisco specificity factor; $V_{c,max}$, maximum velocity of carboxylation; VPD, vapor pressure deficit.

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capacity of plants other than Spermatophyta, i.e. seed plants (Hagar and Freeberg 1980, Gildner and Larson 1992, Volkova et al. 2011, Soni et al. 2012). Among these, ferns (i.e. monilophytes *sensu* Pryer et al. 2004) are a key group within the radiation event of land plants, and the closest group to seed plants (Kenrick and Crane 1997, Soltis et al. 1999, Smith et al. 2006). Hence, understanding their photosynthetic characteristics would be crucial to unravel the evolution of photosynthetic traits in land plants. In general, ferns have been reported to have a much lower range of photosynthetic capacities than seed plants (Hariri and Prioul 1978, Durand and Goldstein 2001, Wright et al. 2005, Brodribb et al. 2007). In a global comparison by the Glopnet group (Wright et al. 2004, 2005), a limited number of fern species were considered, and they presented significantly lower photosynthetic capacities than any functional group within seed plants for similar ranges of leaf mass per area and nitrogen contents, i.e. low nitrogen use efficiency. Lower photosynthesis for a given stomatal conductance – i.e. lower intrinsic water use efficiency – has also been reported for ferns, as compared to seed plants (Gratani et al. 1998, Volkova et al. 2010, McAdam and Brodribb 2012a). While, in part, these differences could be caused by the fact that many ferns present shade-specific traits (Hariri and Prioul 1978, Johnson et al. 2000), this cannot be generalized and, even focusing on sun ferns only, their photosynthetic capacity and resource use efficiency are lower than in seed plant (Volkova et al. 2009a, 2011, Haworth et al. 2011). The underlying causes of these photosynthetic differences between ferns and seed plants are still unknown due to lack of specific studies focused in this topic. Lower CO_2 assimilation rates could potentially be due either to biochemical or diffusional limitations, the latter involving both stomatal and mesophyll conductance to CO_2 (Grassi and Magnani 2005).

Regarding diffusional limitations, most ferns present stomata in their fronds (Brodribb et al. 2009). However, in contrast to seed plants, fern stomata do not present well-defined companion epidermal cells and therefore do not exhibit the associated mechanical advantage of epidermal cells (Franks and Farquhar 2007). In consequence, ferns display high stomatal sensitivity to changes in guard cell turgor, for which aperture–closure is much less tuned in than it is in seed plant stomata (Brodribb and Holbrook 2004, McAdam and Brodribb 2012b). Moreover, it has been recently pointed out that ferns mostly lack stomatal responses to ABA, CO_2 and blue light (Doi et al. 2006, Doi and Shimazaki 2008, Brodribb et al. 2009, Brodribb and McAdam 2011, McAdam and Brodribb 2012a), and a delayed stomatal closure under transitions from high light to low light (McAdam and Brodribb 2012b). Altogether, the absence of finely tuned stomatal regulation and active stomatal responses to environmental changes imply that fern stomata remain open even under conditions not favoring photosynthesis, such as under low light; instead, they can close suddenly in response to mild desiccation conditions. The consequence is that ferns lack a fine adjustment of photosynthesis and stomatal conductance. In other words, stomata in ferns do not close under many conditions in which seed plants stomata close, for which it is unlikely that stomatal limitations exert the most important role in setting fern low photosynthesis under those conditions, in contrast to seed plants, in which stomatal limitations to photosynthesis are quantitatively the most important (Flexas et al. 2012).

Besides stomata, CO_2 diffusion across the frond mesophyll, from sub-stomatal cavities to chloroplast stroma, – i.e. the so called mesophyll conductance (g_m) – can also impose a significant limitation to photosynthesis. The few estimates of mesophyll conductance available for the most primitive land plants (liverworts and hornworts) are several orders of magnitude below the typical values for seed plants (Meyer et al. 2008). Unfortunately, data on g_m are not available for ferns, lycophytes and mosses (Flexas et al. 2012), with the exception of a single estimate for the fern *Dicksonia antarctica* (Volkova et al. 2009b). According to the authors of this article, their estimate was subject to large variability, probably owing to the low accuracy of the curve fitting method used. Still, the average value fell within the range of values described for seed plants. The low nitrogen and water use efficiencies in ferns are indeed an indicative of diffusional limitations. In addition, in *Selaginella* (a Lycophyte, a close group to ferns; Smith et al. 2006) data on gas exchange and chlorophyll fluorescence were provided by Soni et al. (2012) that allow a rough g_m estimation of approximately 0.04 mol m^{-2} s^{-1}, i.e. a very low value. Overall, several lines of evidence lead us to hypothesize that g_m might be generally low in ferns, exerting a significant limitation of photosynthesis. Finally, biochemical limitations could also play a role in setting the low photosynthesis values displayed by ferns. Early studies showed that ferns present a basic photosynthetic metabolism similar to that of seed plants, both at the level of stromal biochemistry (Norris et al. 1955) and thylakoid photochemistry (Ludlow and Wolf 1975, Leong et al. 1985). Nevertheless, little is known about Rubisco kinetics in ferns. The Michaelis constant for CO_2 of Rubisco (K_c) has been described for only five species belonging to the genera *Pteris*, *Pteridium*, *Polypodium*, *Pellaea* and *Ceratopteris* (Yeoh et al. 1981, Bird et al. 1982, Jordan and Ogren 1983); whereas the catalytic constant for the

carboxylase reaction (k_{cat}^{c}) has been reported for only two species, *Pteris aquilina* (Bird et al. 1982) and *Platycerium coronarium* (Kwa et al. 1997). So far, a single value of Rubisco specificity factor (82 mol mol^{-1}) has been reported for two fern species, *Polypodium aureum* and *Equisetum* sp. (Jordan and Ogren 1983). Overall, the few reported Rubisco kinetic constants for ferns are within the range of values reported for seed plants of the C_3 type (Galmés et al. 2005). Values for the apparent in vivo carboxylation activity, $V_{c,max}$, have been reported for two fern species (Volkova et al. 2009a, 2009b, 2010), and these were within the lower range of values reported for seed plants (Wullschleger 1993, Manter and Kerrigan 2004), suggesting low Rubisco concentration and/or activation state. This is confirmed by the few reported in vitro data for ferns (Yeoh et al. 1981, Bird et al. 1982, Jordan and Ogren 1983). Therefore, it is likely that biochemical limitations exerted by low $V_{c,max}$ on photosynthesis are important, at least in the few ferns studied.

Following Smith et al. (2006), there are about 9000 extant fern species (i.e. monilophytes), classified in three minor classes (Psilotopsida, Equisetopsida and Marattiopsida; together with only ca. 260 species) and the Polypodiopsida (i.e. leptosporangiates), the latter including most fern species. In turn, the leptosporangiales include seven orders, from which Osmundales and Polypodiales are the basal and most modern groups, respectively. Within Polypodiales (ca. 6000 spp.), a recent molecular phylogeny (Rothfels et al. 2012) recognizes two clear lineages, namely eupolypods I and eupolypods II, with ca. 3300 and ca. 2600 species, respectively. The main aim of this work is to provide the first complete and systematic characterization of quantitative photosynthesis limitations in some fern species. To achieve this goal, we selected representative sun-adapted fern species from the most phylogenetically distant groups: *Osmunda regalis* (basal ferns), *Blechnum gibbum* (eupolypods II) and *Nephrolepis exaltata* (eupolypods I). We determined g_s and g_m, as well as $V_{c,max}$, and compared them with values measured in the seed model plant *Nicotiana tabacum*, presenting somewhat similar life form (non-woody) and leaf structure (similar leaf mass per area) to the selected fern species. Additional aims were to assess the short-term response to CO_2 of g_s and g_m, as well as to determine $S_{C/O}$ for the selected fern species.

Materials and methods

Plant material and growing conditions

Three leptosporangiate fern species (Polypodiopsida) were used in this study: *O. regalis* L. (Osmundales: Osmundaceae), *B. gibbum* (Lab.) Mett. (Polypodiales: Blechnaceae) and *N. exaltata* (L.) Schott (Polypodiales: Lomariopsidaceae). *B. gibbum* (40 cm tall) and *N. exaltata* (80 cm tall) were purchased from established commercial cultures in nurseries in Mallorca, while *O. regalis* (50–70 cm tall) was collected in Galicia (northwest of Spain).

Nephrolepis exaltata was grown inside a greenhouse in 5-l pots and the two other species were grown outdoors in 2-l pots in the experimental field of the University of the Balearic Islands, under typical Mediterranean conditions during 3 months from April to June 2012, with the following meteorological conditions: a mean temperature over the experimental period of 26.7 and 27.0°C outdoors and inside the greenhouse, respectively; an average daily photosynthetically active photon flux density (PPFD) of 450 and 370 µmol m^{-2} s^{-1} (semi-shade, with maximum values at midday of 1500 and 850 µmol m^{-2} s^{-1}) and mean relative humidity (RH) between 27.2 and 48.8% for this period outdoors and inside the greenhouse, respectively. The pots were filled with horticultural substrate Prohumin® (Projar SA, Valencia, Spain) with additional fertilization [5 g of slow-release fertilizer Multigreen® (Haifa Chemicals, Madrid, Spain) per liter of substrate]. The plants were watered daily to field capacity to ensure an optimal plant water status and plant vigor.

Purification of Rubisco and measurements of the specificity factor

Leaves (ca. 100 g) of each species were collected at mid-morning and immediately frozen in liquid nitrogen. The leaf material was ground to a powder in a mortar, 400 ml of extraction buffer (100 m*M* Bicine pH 8.0, 11 m*M* Na-DIECA, 6% PEG_{4000}, 50 m*M* 2-mercaptoethanol, 2 m*M* benzamidine, 2 m*M* ε-amino-*n*-caproic acid, and 2 m*M* PMSF) was added and grinding continued as the mixture thawed. Thereafter, all purification steps were carried out at 4°C. Fully thawed but still cold homogenates were filtered through butter muslin and centrifuged at 11 000 rpm for 20 min. The supernatant liquid was decanted through 50-ml nylon mesh and PEG_{4000} was added to a final concentration of 20% (w/v). Also, $MgCl_2$ was added to a final concentration of 2 m*M*, followed by gentle mixing. After standing for 15 min, the mixture was centrifuged at 1352.8 *g* for 20 min. The pellet was then re-suspended in 60 ml of column buffer (20 m*M* TRIS pH 8.0 with 10 m*M* $MgCl_2$, 10 m*M* $NaHCO_3$, 1 m*M* EDTA and 1 m*M* KH_2PO_4) containing 1 m*M* each of Dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), benzamidine and ε-amino-*n*-caproic acid. The suspension was centrifuged at 82 508.4 *g* for 30 min in a 70.1 Ti rotor (Beckman, High Wycombe, UK).

The supernatant liquid was applied to two 5-ml HiTrap Q HP columns (Amersham Biosciences, Uppsala, UK) connected in series previously equilibrated with column buffer and operated at 1 ml min^{-1}. The proteins were eluted using a step gradient from 0.1 *M* to 0.8 *M* NaCl in column buffer and fractions were collected in 2-ml intervals. Total soluble protein content in fractions was confirmed using the Bradford assay (Bradford 1976). Those fractions with high protein concentration were transferred to two 4-ml Amicon Ultra-4 centrifugal filters (100 kDa membrane) (Merk Milipore, Darmstadt, Germany) and centrifuged at 4000 *g* to concentrate Rubisco (>mg ml^{-1}). Finally, glycerol was added to the retained fraction to a final concentration of 20% (w/v), and stored at −70°C.

On the day of $S_{C/O}$ measurement, high-concentrated Rubisco solutions were desalted by centrifugation through G25 Sephadex columns previously equilibrated with CO_2-free 0.1 *M* Bicine (pH 8.2) containing 20 m*M* $MgCl_2$. The desalted solutions were made 10 m*M* with $NaH^{14}CO_3$ (0.5 Curies/mol) and 4 m*M* NaH_2PO_4, to activate Rubisco by incubation at 37.5°C for 40 min. Reaction mixtures were prepared in oxygen electrodes (Oxygraph, Hansatech instruments Ltd., Norfolk, UK) by first adding 0.95 ml of CO_2-free assay buffer (100 m*M* Bicine pH 8.2, 20 m*M* $MgCl_2$, containing 1.5 mg of carbonic anhydrase per 100 ml buffer). After the addition of 0.02 ml of 0.1 *M* $NaH^{14}CO_3$, the plug was fitted to the oxygen electrode vessel, and enough activated Rubisco (20 µl) was added for the reaction to be completed in 5 min. When the signal from the electrode was steady, the reaction was started by the addition of 10 µl of 25 m*M* RuBP. RuBP oxygenation was calculated from the oxygen consumption, and carboxylation from the amount of ^{14}C incorporated into PGA when all the RuBP had been consumed (Parry et al. 1989). A total of eight to nine replicates were measured at 25°C for each species. A sequence of reaction mixtures containing pure wheat Rubisco were interspersed with those containing fern Rubiscos, and the results were normalized to the average value obtained for wheat Rubisco (125.7). Provided that measurement of $S_{C/O}$ by the radioactive plus oxygen-electron method is highly dependent on the pH, such standardization procedure is a common practice to allow the obtained values to be comparable themselves and to others in literature (Parry et al. 1989; Galmés et al. 2005).

Gas exchange and chlorophyll *a* fluorescence measurements

Measurements for the three species were performed in June in young but fully expanded fronds were clamped into the cuvette of an open gas-exchange system with an integrated fluorescence chamber head (Li-6400-40; Li-Cor Inc., NE) for simultaneous measurements of gas-exchange and chlorophyll fluorescence. Block temperature was kept at 25°C during all measurements (registered leaf temperatures ranging 24–28°C) and vapor pressure deficit (VPD) at around 1.5 kPa. Given the low photosynthetic rates of these species and the small area (2 cm^2) of the leaf cuvette, the flow rate was adjusted to 150 mmol air min^{-1} to ensure that CO_2 differentials between the reference and the sample IRGAs were >4 µmol mol^{-1} air. These deltas were achieved in all cases at 400 µmol CO_2 mol^{-1} air, and in most cases at other CO_2 concentrations except around the CO_2 compensation point.

Photosynthetic light response curves were performed at a CO_2 concentration (C_a) of 400 µmol mol^{-1}, and varying PPFD from 0 to 2000 µmol m^{-2} s^{-1}. Desired levels of PPFD were achieved using the Li-6400 led source with a 90% red and a 10% blue light. The response of net CO_2 assimilation (A_N) to varying C_a were performed at saturating PPFD of 1000 µmol m^{-2} s^{-1}, and consisted in 16 different CO_2 concentrations between 50 and 1800 µmol mol^{-1}, following the procedures described in Galmés et al. (2011). CO_2 leakage into and out of the leaf cuvette was assessed as in Flexas et al. (2007a), and the data corrected accordingly.

From steady-state measurements at C_a of 400 µmol mol^{-1} and PPFD of 1000 µmol m^{-2} s^{-1} A_N, stomatal conductance (g_s) and sub-stomatal CO_2 concentration (C_i) were recorded. Owing to the low g_s values, cuticular conductance (g_c) could result in wrong estimates of C_i (Boyer et al. 1997). The three ferns studied are hypostomatous and therefore, g_c was determined measuring g_s in equal conditions of $A_N - C_i$ curves at 400 µmol CO_2 mol^{-1} air, but with the frond abaxial surface covered with silicone grease and a polyethylene filter to prevent stomatal gas exchange. These data were used to re-calculate g_s and C_i as described previously (Boyer et al. 1997, Flexas et al. 2002).

Mitochondrial respiration (R_n) rates were measured after darkening the plants for 30 min. Non-photorespiratory CO_2 release in the light (R_d) was considered as half of R_n (Niinemets et al. 2005, Gallé et al. 2012). Photorespiration rate (P_r) was calculated according to Valentini et al. (1995) combining gas and chlorophyll fluorescence measurements.

From chlorophyll fluorescence recordings, the actual photochemical efficiency of photosystem II (ϕ_{PSII}) was obtained as $\phi_{PSII} = (Fm' - Fs)\ Fm'$, being Fs the steady state fluorescence, and Fm′ the maximum fluorescence during a light-saturating pulse of ca. 8000 µmol m^{-2} s^{-1} (Genty et al. 1989). From this, the electron transport rate (J_{flu}) was calculated as $J_{flu} = (\phi_{PSII}) \cdot PPFD \cdot \alpha \cdot \beta$, where

α is leaf absorptance and β reflects the partitioning of absorbed quanta between photosystems II and I. The product $\alpha \cdot \beta$ was determined from the relationship between ϕ_{PSII} and ϕCO_2 under non-photorespiratory conditions (Valentini et al. 1995, Flexas et al. 2007b). α was double checked by additional measurements of frond absorptance to the Li-6400 LED light using a spectroradiometer (HR2000CG-UV-NIR; Ocean Optics Inc., Dunedin, FL), as described (Flexas et al. 2007b).

Because pinnate compound leaves of ferns (fronds) did not cover the entire leaf cuvette surface (2 cm^2) in a few cases, a digital photograph of the frond was taken immediately after the measurement using an equal foam gasket located in the same measured area previously marked; GOOGLE SKETCHUP® software was used to estimate the actual frond area. Gas exchange values given by Li-6400 were corrected using the ratio cuvette area/actual frond area as a correction factor. After the measurements of gas-exchange, fronds were placed in a drying oven at 60°C until reaching constant weight, which was taken for the estimation of the leaf mass area (LMA) as dry weight/area.

Estimations of mesophyll conductance to CO_2 (g_m), the maximum velocities of carboxylation ($V_{c,max}$) and electron transport (J_{max}), and quantitative limitation analysis

Two different methods were used to estimate g_m and retrieve $V_{c,max}$ and J_{max} after the photosynthesis model by Farquhar et al. (1980). In the first, the three parameters were obtained simultaneously by the iterative least-squares curve-fitting method described in Sharkey et al. (2007). In the second, g_m was estimated after Harley et al. (1992) as: $g_m = A_N\ (C_i - (\Gamma^*\ (J_{flu} + 8(A_N + R_d)))(J_{flu} - 4(A_N + R_d)))$. All parameters, except Γ^*, were estimated as described above, and Γ^* was calculated from the above described in vitro determinations of $S_{C/O}$, as $\Gamma^* = 0.5^* O/S_{C/O}$ (von Caemmerer, 2000). The obtained g_m values were used to convert the A_N–C_i curves into A_N–C_c curves using the following equation: $C_c = C_i - (A_N/g_m)$. Finally, $V_{c,max}$ and J_{max} were calculated by fitting the Farquhar et al. model to A_N–C_c curves, for which the temperature dependence of kinetic parameters of Rubisco described on a C_c basis by Bernacchi et al. (2002) was used.

Using values of A_N, g_s, g_m and $V_{c,max}$, the quantitative photosynthesis limitation analysis by Grassi and Magnani (2005) was performed to evaluate the limitations imposed by stomatal conductance (l_s), mesophyll conductance (l_m) and leaf biochemistry (l_b) for each of the three studied fern species. In addition, as our aim was to know what are the most important factors that limit photosynthesis in ferns *as compared to seed plants*, we employed previously published photosynthesis values for tobacco (*N. tabacum* L.) as a standard reference to assess the *relative* limitations imposed by stomatal conductance (S_L), mesophyll conductance (M_{CL}) and leaf biochemistry (B_L) in the ferns studied. In this last approach, a total limitation (TL) for net assimilation is calculated as $((A_{N\ fern} - A_{N\ tobacco})/A_{N\ tobacco} \times 100)$, and then the three individual limitations are expressed as percentages of this total. Tobacco data were published previously in the literature, grown either inside growth chamber with a 12-h photoperiod (25°C day/20°C night), 40–60% RH and a photon flux density at plant height of about 800–1000 µmol m^{-2} s^{-1} (Flexas et al. 2006) or outdoors during spring with peak PPFDs > 1500 µmol m^{-2} s^{-1} and maximum temperature reaching 28°C (Gallé et al. 2009). Tobacco was chosen because it presents a life form (non-woody, short height annual plant) comparable to that of the three ferns studied and has a similar LMA to these three ferns, and the two extreme growing conditions were used to confirm that the differences encountered between ferns and tobacco were constitutive of the species, i.e. independent of the precise growing conditions used in each case.

Statistical analysis

Univariate analysis of variance (ANOVA) and Duncan multiple comparisons test ($P < 0.05$) were employed to determine significant differences between the species in the parameters measured. These analyses were performed using the SPSS 21.0 software package (SPSS Inc., Chicago, IL). Regression analyses were performed with the 11.0 Sigma Plot software package (Sigma, St Louis, MO).

Results

Values for the product of leaf absorptance by PSII/PSI partitioning ($\alpha \cdot \beta$) measured by gas exchange and chlorophyll fluorescence, α measured optically and calculated β, i.e. the fraction of absorbed irradiance that reaches PSII, are shown in Table 1. No significant differences were found between species in these parameters, ranging under typical described values: 0.91–0.94 for α, 0.4–0.51 for $\alpha \cdot \beta$ product and 0.49–0.54 for calculated β.

With the aim of modeling the photosynthetic activity of these three fern species several related traits were evaluated. The three species showed no significantly different values of LMA and Rubisco specificity factor ($S_{C/O}$), ranging 41.8–36.9 g m^{-2} and

Table 1. Frond absorptance measured (α), product $\alpha \cdot \beta$ from the relationship between ϕ_{PSII} and ϕ_{CO2} under non-photorespiratory conditions and β calculated, fraction of absorbed irradiance that reaches PSII, for the three ferns studied (Valentini et al. 1995, Flexas et al. 2007b). No significant differences between species were found in these parameters using Duncan's multiple comparison test (0.05).

Species	α	$\alpha \cdot \beta$	β calc
Osmunda regalis	0.94 ± 0.1	0.51	0.54
Blechnum gibbum	0.91 ± 0.1	0.53	0.49
Nephrolepis exaltata	0.93 ± 0.1	0.48	0.52

99.2–105.1 mol mol^{-1}, respectively (Table 2). The cuticular conductance (g_c) of *O. regalis* was statistically higher than *B. gibbum* and *N. exaltata*, with values of 12, 6 and 4 mmol H_2O m^{-2} s^{-1}, respectively.

As for gas exchange, in some cases the variability between replicates was larger than desirable, as indicated by large standard errors, which was probably due to the use of the measurement equipment close to its technical limits. Despite of this, significant observations could be made. The CO_2 assimilation rate (A_N) was light-saturated at PPFD below 500 µmol m^{-2} s^{-1} in all three ferns. However, in *O. regalis* light-saturated A_N was about twofold higher than in *B. gibbum* and *N. exaltata* (Table 3). The ratio between A_N and photorespiration rate (P_r) ranged between 2.5 for *O. regalis* and 4.9 for *N. exaltata* (Table 2). In all three species, stomatal conductance (g_s) was < 0.1 mol CO_2 m^{-2} s^{-1}, and remained constant at any light intensity (Fig. 1) and fairly constant in response to varying CO_2 (Fig. 2). Only a slight increase in g_s was observed in *B. gibbum* and *O. regalis* when C_a dropped from 400 to 50 µmol mol^{-1}, while in *N. exaltata* no changes were observed. On the contrary, when C_a was increased from 400 to 1800 µmol mol^{-1}, none of the three ferns modified their g_s. Comparing the responses of g_s and g_m to changes in C_i, it was observed that g_m behaved differently to g_s in all species. Hence, while g_s slightly increased as C_i was reduced, g_m showed an opposite trend decreasing to minimum values (Fig. 3). Conversely, while increases in C_i induced almost no change in g_s, g_m was decreased up to 30% of its value at ambient C_a.

Table 2. Leaf mass area (LMA), cuticular conductance (g_c) and Rubisco specificity factor at 25°C ($S_{C/O}$). Values are average ± SE of three replicates per species. *Statistically significant differences between species using Duncan's multiple comparison test ($P < 0.05$).

Species	LMA (g m^{-2})	g_c (mmol H_2O m^{-2} s^{-1})	$S_{C/O}$ (mol mol^{-1})
Osmunda regalis	41.8 ± 4.5	$12.5 \pm 1^*$	101.5 ± 4.5
Blechnum gibbum	36.9 ± 5.0	5.8 ± 0.4	105.1 ± 4.6
Nephrolepis exaltata	41.3 ± 2.5	4.0 ± 0.4	99.2 ± 9.5

The three species displayed different responses of A_N to varying C_i (Fig. 4A) and C_c (Fig. 4B). The solid lines represent the Farquhar et al. modelization for the average of each species. On a C_c basis, and depending on the method used for its estimation, statistically significant differences were observed between species for $V_{c,max}$, ranging from 20–25 µmol m^{-2} s^{-1} in *N. exaltata* to 60–70 µmol m^{-2} s^{-1} in *O. regalis* and for J_{max} values the same behavior was observed to those obtained for $V_{c,max}$ and J_{flu} (Table 3).

A photosynthetic quantitative limitation analysis was performed to evaluate the components related to stomatal conductance (l_s), mesophyll conductance (l_m) and leaf biochemical characteristics (l_b). The three imposed limitations shared similar ranges for the three ferns (i.e. a proportion similar to 0.33:0.33:0.33), although l_b was the largest limitation for *B. gibbum* and *N. exaltata*, with values of ca. 0.4 and 0.5, respectively, while l_m was the most important in *O. regalis*, with a value of 0.4. In contrast to ferns, maximum A_N in tobacco – a standard for seed plants – ranges 18–22 µmol CO_2 m^{-2} s^{-1} regardless of being grown indoors or outdoors. Hence, when the three ferns studied here were compared with tobacco, the TLs revealed strong photosynthesis limitations in ferns, of 53–61%, 75–80% and 78–82% for *O. regalis*, *B. gibbum* and *N. exaltata*, respectively. It is important to note that the sum of non-stomatal limitations (MCL + BL) accounts for up to 70% of photosynthesis limitation in these three species.

Discussion

This study aims to reveal limitations in the main photosynthesis determinants in some ferns, in order to compare modern ferns (polypods) with data available in seed plants, and also to reveal possible differences between a basal fern (*O. regalis*) to two modern ferns (the eupolypod I *N. exaltata* and the eupolypod II *B. gibbum*).

Frond traits and characteristics

The values of LMA measured on the three selected species are within the range reported for ferns, from 29 g m^{-2} in *Dennstaedtia punctilobula* (Brach et al. 1993) to 135 g m^{-2} in *Cibotium glaucum* (Durand and Goldstein 2001), and similar to those reported for some herbaceous plants, like tobacco (Flexas et al. 2006) or tomato (Galmés et al. 2011). They also showed frond absorptances >90% for the red and blue LED light provided by Li-6400, just as described for many seed plants (Niinemets et al. 2005, Flexas et al. 2007b).

Table 3. Photosynthetic characteristics of the studied ferns at PPFD of 1000 μmol m^{-2} s^{-1}, 25°C and 400 μmol CO_2 mol^{-1} air. g_m, $V_{c,max}$ and J_{max} were calculated following the methodologies proposed by Farquhar et al. 1980, Harley et al. 1992 and the iterative curve-fitting method proposed by Sharkey et al. 2007. Values are means ± SE of four to six replicates per species measured on different individuals. Different letters denote statistically significant differences through Duncan test ($P < 0.05$). J_{flu}, electron transport rate; P_r, photorespiration.

Species	*Osmunda regalis*	*Blechnum gibbum*	*Nephrolepis exaltata*
A_N (μmol CO_2 m^{-2} s^{-1})	8.5 ± 0.4a	4.5 ± 0.2b	3.9 ± 0.1b
R_d (μmol CO_2 m^{-2} s^{-1})	0.24 ± 0.04a	0.08 ± 0.01b	0.18 ± 0.03c
g_s (mol CO_2 m^{-2} s^{-1})	0.077 ± 0.01a	0.041 ± 0.01b	0.025 ± 0.01b
g_m Harley (mol CO_2 m^{-2} s^{-1})	0.073 ± 0.30a	0.05 ± 0.01b	0.03 ± 0.01c
g_m curve-fitting (mol CO_2 m^{-2} s^{-1})	0.112 ± 0.01a	0.06 ± 0.01b	0.03 ± 0.01b
$V_{c,max}$ (μmol CO_2 m^{-2} s^{-1})	69.8 ± 3.7a	40.2 ± 3.3b	17.4 ± 0.9c
$V_{c,max}$ curve-fitting (μmol CO_2 m^{-2} s^{-1})	57.9 ± 8.5a	47.1 ± 4.9a	24.9 ± 5.9b
J_{max} (μmol electrons m^{-2} s^{-1})	69.7 ± 4.4a	50.0 ± 3.6b	22.2 ± 1.4c
J_{max} curve-fitting (μmol electrons m^{-2} s^{-1})	63.1 ± 4.9a	46.6 ± 4.6b	28.7 ± 3.1c
J_{flu} (μmol electrons m^{-2} s^{-1})	73.3 ± 3.0a	40.0 ± 1.7b	21.6 ± 0.2c
P_r (μmol CO_2 m^{-2} s^{-1})	3.1 ± 0.1a	1.8 ± 0.1b	0.8 ± 0.1c

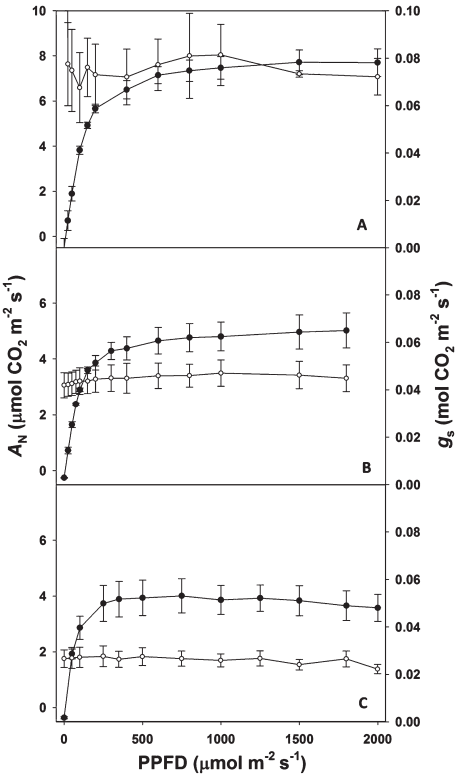

Fig. 1. Response of net photosynthesis (A_N) and stomatal conductance (g_s) to varying photosynthetically active photon flux density (PPFD) in (A) *Osmunda regalis*, (B) *Blechnum gibbum* and (B) *Nephrolepis exaltata*. Values are averages ± SE of three to six replicates performed in different plants depending on the species.

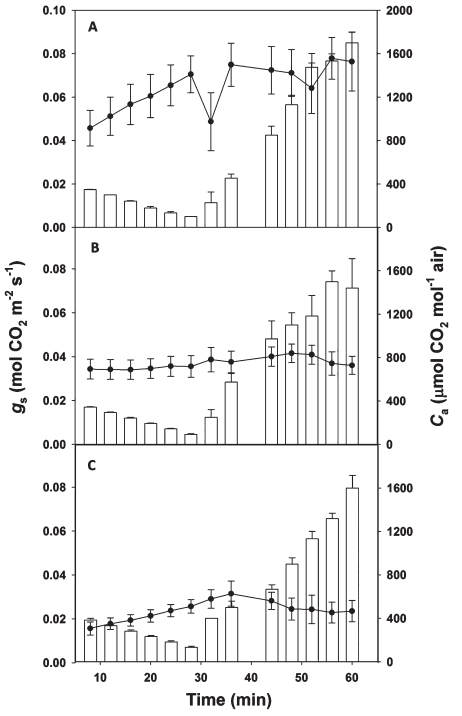

Fig. 2. Time courses of stomatal conductance (g_s) during changes in ambient CO_2 concentration (C_a) from 100 to 1700 μmol CO_2 mol^{-1} air for (A) *Osmunda regalis*, (B) *Blechnum gibbum* and (C) *Nephrolepis exaltata*. Values are averages ± SE of three to six replicates performed in different plants depending on the species.

Likely as a consequence of similar absorptance, the slope of the relationship between ϕ_{PSII} and ϕ_{CO2} under non-photorespiratory conditions was also within the range of values usually reported for seed plants, i.e. 8–12, the $\alpha \cdot \beta$ product and so that the calculated β (Valentini et al. 1995, Flexas et al. 2007b, Miyazawa et al. 2008). The measured variability in g_c matched with values typically reported for both ferns (McAdam and Brodribb, 2012b) and seed plants (Galmés et al. 2006, and references therein). The dark respiration rates (R_n) measured in these three species were low (<0.5 μmol m^{-2} s^{-1}), but still within the range reported for ferns (Hollinger 1987,

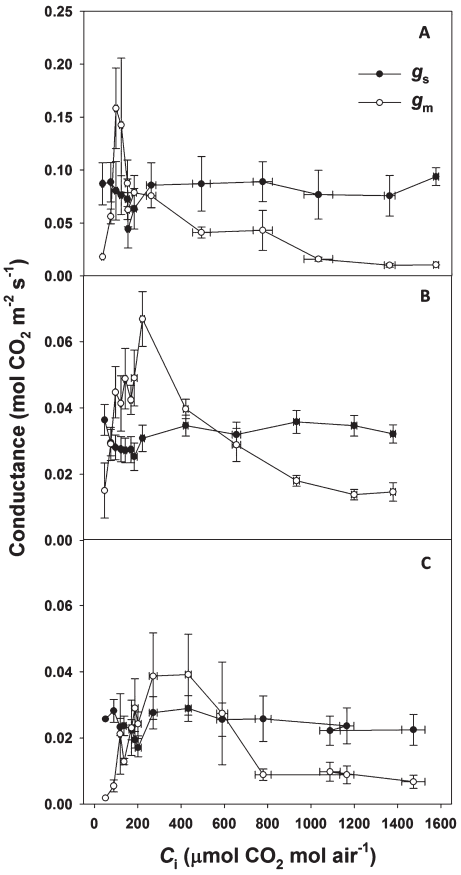

Fig. 3. Response of stomatal conductance (g_s) and mesophyll conductance (g_m) to sub-stomatal CO_2 concentration (C_i) for (A) *Osmunda regalis*, (B) *Blechnum gibbum* and (C) *Nephrolepis exaltata*. Values are averages ± SE of three to six replicates performed in different plants depending on the species. Notice the different scale in plot (A).

Durand and Goldstein 2001, Saldaña et al. 2005) and slow-growing seed plants (Wright et al. 2004). Finally, measured $S_{C/O}$ was slightly higher than values previously reported for ferns (Jordan and Ogren 1983, Kent and Tomany 1995), but similar to values reported for many seed plants (Galmés et al. 2005). In summary, most of the studied physiological characteristics of fern fronds are in agreement with values previously published for other fern species, and resemble those typical of leaves of seed plants.

Stomatal and mesophyll conductance

Stomatal conductance measured on the three fern species was low as compared to typical values for seed species (Schulze et al. 1994, Wright et al. 2004), and fitted within the range of values typically reported in ferns (Doi and Shimazaki 2008, Zhang et al. 2009, McAdam and Brodribb, 2012b). However, measured g_s values were somewhat lower in relation to values previously published for the same or closely related species (Wright et al. 2004, Franks and Farquhar 2007, Brodribb et al. 2009, Volkova et al. 2009a, 2009b, 2011, McAdam and Brodribb, 2012b), probably due to differences in the growing conditions. Extreme sensitivity of fern stomata to desiccation has been described (Brodribb and Holbrook 2004, Franks and Farquhar 2007, McAdam and Brodribb 2012b), and hence the low VPD endured during the growth period may have caused some influence on the stomatal morphology, leading to somewhat lower g_s than expected. For instance, Franks and Farquhar (1999) reported that increasing VPD from 1 to 2 kPa resulted in up to 30% decrease of g_s in *O. regalis* and other fern species.

Stomatal conductance of the three fern species behaved as expected in response to light and CO_2. It remained at almost constant values during the entire light response curves of photosynthesis (Fig. 2). The insensitivity of stomata of other fern species to light intensity has already been reported (Hollinger 1987, Hunt et al. 2002, Volkova et al. 2011), and constitutes a major inefficiency in terms of optimizing the photosynthetic water use efficiency (McAdam and Brodribb 2012a, 2012b). Stomatal conductance also showed limited response to decreasing CO_2 and no response to increasing CO_2, as previously reported (Doi and Shimazaki 2008, Brodribb et al. 2009). In contrast, g_m showed a marked response to changes in the CO_2 concentration, as has been shown in some seed plant species (Flexas et al. 2007b, Hassiotou et al. 2009, Vrábl et al. 2009, Bunce, 2010, Douthe et al. 2011), but not in others (Tazoe et al. 2011). A recent study has demonstrated that the observed response to low CO_2 is actually an artifact, related to the partially recycled (photo)respired CO_2 by photosynthesis (Tholen et al. 2012). Hence, the apparent decline of g_m when C_i drops from about 200 to 50 μmol mol^{-1} (Fig. 4) is, at the least, debatable. In contrast, under high C_i photorespiration is low, so that the consequences of CO_2 recycling (Tholen et al. 2012). Therefore, the observed decrease of g_m at high CO_2 is in principle trustable, just as it does in seed plants (Flexas et al. 2007b, Hassiotou et al. 2009, Vrábl et al. 2009, Bunce et al. 2010). In seed plants, g_s and g_m often co-vary in response to environmental changes like light intensity, water stress, temperature or CO_2 (reviewed in Flexas et al. 2008), and it has been related to adjustments in the intrinsic water use efficiency (Flexas et al. 2010, Galmés et al. 2011, Peguero-Pina et al. 2012). Therefore, it is likely that the inability of ferns to close stomata under high CO_2 while still decreasing g_m is the main cause for the observed inability to increase water use efficiency under high CO_2 (Brodribb et al. 2009).

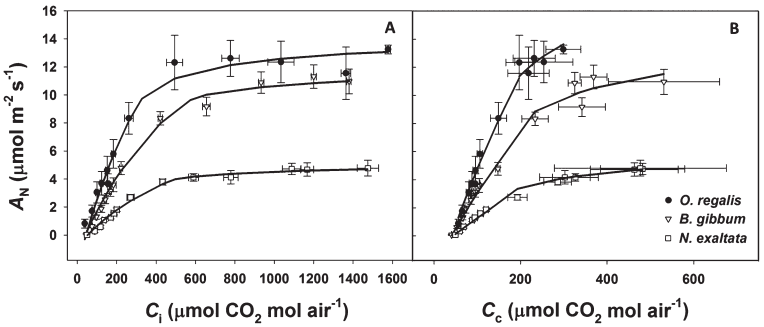

Fig. 4. Response of net photosynthesis (A_N) to sub-stomatal (C_i) and chloroplast (C_c) CO_2 concentrations in (A) *Osmunda regalis*, (B) *Blechnum gibbum* and (C) *Nephrolepis exaltata*. Values are averages ± SE of three to six replicates performed in different plants depending on the species. Lines show the Farquhar model fitting for these response curves.

Photosynthesis limitations

The causes for the lower photosynthetic capacity of ferns as compared to seed plants could be due to stomatal, mesophyll, photochemical and/or biochemical causes. It has been already discussed that the absolute values of g_s measured in this study are somewhat lower than those previously reported for the same species, and much lower as compared to the range for non-woody seed plants. Irrespective of the method used, g_m values were lower than the unique value reported so far for a fern species, 0.15 mol CO_2 m^{-2} s^{-1} for *D. antarctica* (Volkova et al. 2009a, 2009b), and clearly below average values reported for non-woody seed plants, of about 0.2–0.4 mol CO_2 m^{-2} s^{-1} (Flexas et al. 2008, 2012). Averaging the three values reported here with that for *D. antarctica*, gives a mean g_m in ferns of 0.076 mol CO_2 m^{-2} s^{-1}, which is below the lowest values reported for any phylogenetic group within seed plants, i.e. conifers, which average 0.105 mol CO_2 m^{-2} s^{-1} (reviewed by Flexas et al. 2012). The underlying causes of this restricted g_m in ferns remain to be determined, but according to literature the presence or activity of aquaporins (Flexas et al. 2006) and frond ultrastructural traits (Peguero-Pina et al. 2012, Tosens et al. 2012) are candidates deserving accurate exploration. Similarly, the measured values for $V_{c,max}$ and J_{flu} are among the lowest reported for higher plants (Manter and Kerrigan 2004, Flexas et al. 2007b). Overall, low g_m, $V_{c,max}$ and J_{flu}, in addition to low g_s, set the reduced photosynthetic capacities of ferns as compared to seed plants.

Using the limitation analysis described by Grassi and Magnani (2005), the relative limitations to photosynthesis exerted by the stomata, the frond mesophyll and Rubisco capacity (using $V_{c,max}$ as a proxy, because at ambient CO_2 all three species were Rubisco-limited and not electron transport-limited, confirm Fig. 4) were similar in the three species. Non-stomatal limitations accounted for up to 70% of photosynthesis limitation in these species. Using J_{flu} or J_{max} as a proxy for biochemical limitations instead of $V_{c,max}$ led to similar results (data not shown). The absolute limitation analysis described by Grassi and Magnani (2005) accounts for short-term variations in photosynthesis *within* a given species, e.g. for short-term responses to an environmental change. Still, it can be roughly used to compare photosynthetic limitations *between* species, if one species is taken as the reference (i.e. a species presenting 0% total photosynthetic limitation). In order to compare photosynthesis limitations in ferns with those of seed plants, we have chosen tobacco as the reference, as this species is also non-woody and shows almost identical LMA to those determined in all three ferns studied. The limitation analysis has been run twice, using values for tobacco growing inside a growth chamber (Flexas et al. 2006), for which air RH was similar to that experienced by ferns in this study, or growing outdoors (Gallé et al. 2009), for which irradiance and temperature were similar to those endured in the present study. Both comparisons yielded similar results, total photosynthesis limitation ranging from about 50–60% in *O. regalis* to >80% in *N. exaltata*. In both cases, more than 2/3 of TLs were accounting for non-stomatal factors. In other words, the causes for these three ferns displaying on average less than half the maximum photosynthesis of a seed plant like tobacco are mostly found in their low g_m and $V_{c,max}$, with only a minor contribution of g_s, especially considering that the measured g_s values in this study were below the potential maxima.

Concluding remarks

We show for the first time that mesophyll conductance to CO_2 in the three studied ferns is within the lowest range of values reported for seed plants, and provide the first

systematic quantitative limitation analysis of photosynthesis in ferns. Even under climatic conditions likely forcing stomatal closure (i.e. leading to overestimated stomatal limitations), it is shown that non-stomatal factors (i.e. mesophyll conductance and carboxylation activity) set most of the limitations to photosynthesis in these species, resulting in maximum rates less than half those exhibited by seed plants with similar leaf structure. The causes for such low mesophyll conductance and carboxylation capacity in these basal vascular plants remain to be elucidated. In addition, the present study also extends the knowledge on photosynthesis and gas exchange in ferns, it reports values of the Rubisco specificity factor similar to those of seed plants, and confirms that their stomatal conductance does not respond to changes in light intensity and CO_2 concentration, in contrast to seed plants.

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