


ORIGINAL RESEARCH

Leaf structure and water relations of an allotetraploid Mediterranean fern and its diploid parents

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Abstract

Allopolyploidy is a common speciation mechanism in plants; however, its physiological and ecological consequences in niche partitioning have been scarcely studied. In this sense, leaf traits are good proxies to study the adaptive capacity of allopolyploids and diploid parents to their respective environmental conditions. In the present work, leaf water relations (assessed through pressure–volume curves) and structural and anatomical traits of the allotetraploid fern *Oeosporangium tinaii* and its diploid parents, *Oeosporangium hispanicum* and *Oeosporangium pteridioides*, were studied under controlled conditions in response to a water stress (WS) cycle. *O. hispanicum* showed the lowest osmotic potential at turgor loss point ($\pi_{t_{lp}}$) and leaf capacitance, together with higher leaf mass per area (LMA), leaf thickness (LT), leaf density (LD), and leaf dry matter content (LDMC), whereas *O. pteridioides* presented the opposite set of traits (high $\pi_{t_{lp}}$ and capacitance, and low LMA, LT, LD, and LDMC). *O. tinaii* showed an intermediate position for most of the studied traits. The responsiveness (osmotic and elastic adjustments) to WS was low, although most of the traits explained the segregation of the three species across a range of drought tolerance according to the rank: *O. hispanicum* > *O. tinaii* > *O. pteridioides*. These trait differences may underlie the niche segregation among coexisting populations of the three species in the Mediterranean basin.

1 | INTRODUCTION

Polyploidy, or whole-genome duplication, is a very common speciation mechanism in plants, especially in ferns (Wood et al., 2009). In the case of allopolyploidy, genome duplication occurs after interspecific hybridization, and this combination of two divergent parental genomes gives rise to novel traits that may allow colonization of previously unoccupied ecological niches (Soltis et al., 2014). Some allopolyploids have

“transgressive” traits (Coate et al., 2013), which are outside the variation range of parent diploids and can favor niche novelty (Wang et al., 2022), that is, a niche largely different from those of the diploids. Others show intermediate traits (Sessa & Givnish, 2014) and can occupy intermediate abiotic niches between those of the parents (Marchant et al., 2016). Both niche intermediacy and niche novelty reduce competition between cytotypes and thus enhance the establishment and persistence of polyploids among their diploid parents (Fowler

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& Levin, 2016; Rodríguez, 1996). In this context, the most studied transgressive trait is cell size, which is larger in polyploids than in related diploids for diverse cell types. This increase in cell size has long been interpreted as a “nucleotypic” effect of polyploidy, that is, a consequence of a larger bulk DNA amount irrespective of its genic content (Doyle & Coate, 2019). In seed plants, this cell enlargement occurs, for example, in guard and mesophyll cells (Ohri, 2021; Snodgrass et al., 2017; Warner & Edwards, 1993) and modifies leaf function (Coate et al., 2012; Wilson et al., 2021). In ferns, the enlargement of guard cells and spores is well-documented in taxonomic studies as it allows distinguishing polyploids from their diploid ancestors (e.g., van den Heede et al., 2002). However, the effects of polyploidy on the cell size of leaf tissues have been largely ignored in fern research.

Oeosporangium Vis. (Pteridaceae: Cheilanthesoideae), one of the most diverse and widespread fern genera in dry, rocky habitats throughout the Mediterranean basin, including several allopolyploid species. *Oeosporangium tinaei* (Tod.) Fraser-Jenk. is the allotetraploid derived from *Oeosporangium hispanicum* (Mett.) Fraser-Jenk. & Pariyar and *Oeosporangium pteridioides* (Reichard) Fraser-Jenk. & Pariyar, both diploid species. The fronds of the three species show “resurrection” ability, that is, they can withstand almost complete water loss for long periods of time (longer than 3 months during dry summers) and then rehydrate and recover their function after the first autumn rains (Gaff & Oliver, 2013; Quintanilla et al., 2023). “Resurrection” or desiccant-tolerant ferns are able to sustain and recover from much lower water potentials ($\Psi < -100$ MPa) than the most drought-tolerant ferns (which can sustain minimum Ψ of -8 MPa; Holmlund et al., 2016), although there is notable diversity in the degree of desiccation tolerance even within resurrection ferns (Gaff & Oliver, 2013). In the *Oeosporangium* species, the resurrection capacity is higher in *O. hispanicum* than in *O. pteridioides* and intermediate in *O. tinaei* (own unpublished data). In the Iberian Peninsula, the three species can grow sympatrically on the same rocky slope, but they show niche divergence that is explained by differences in their leaf (or frond) functional traits (Quintanilla et al., 2023). Specifically, *O. hispanicum* has fronds that present a more “conservative” set of traits in terms of carbon acquisition per investment (Díaz et al., 2016; Reich, 2014) when compared to *O. pteridioides*, including much higher leaf mass per area (LMA), lower carbon assimilation rate per unit mass, higher non-enzymatic antioxidant capacity, higher carbon content and lower contents of nitrogen, phosphorus, and other macronutrients. This combination of leaf traits allows it to grow in microhabitats with less availability of water and nutrients (rock crevices) and to have a greater capacity for sustained cycles of desiccation and recovery, and thus, a longer leaf lifespan. On the other hand, *O. pteridioides* presents fronds with a more “acquisitive” set of traits (Díaz et al., 2016; Reich, 2014), which allow it to occupy more humid and nutrient-rich microhabitats (shallow soils). Leaf traits of *O. tinaei* are mostly intermediate between those of the diploid parents and explain the intermediate abiotic niche of this allotetraploid in terms of water and nutrient availability (Quintanilla et al., 2023).

An additional aspect that can help in the characterization of the *Oeosporangium* species regarding their leaf traits and niche partitioning is the study of leaf water relations, that is, the relationship

between leaf water potential and water content (Tyree & Jarvis, 1982). Drought tolerance has been observed in ferns in response to water stress (WS) under Mediterranean conditions (Campany et al., 2021; Holmlund et al., 2016; Lo Gullo et al., 2010); nonetheless, there are differences across species displaying different degrees of tolerance in terms of water relations parameters such as osmotic and pressure potentials. Distinct water relations explain part of the variability in the capacity to endure dry periods (Holmlund et al., 2016) and even segregation throughout microenvironmental conditions differing in water availability (Hietz & Briones, 1998; Holmlund et al., 2016). Pressure–volume isotherms, or P – V curves as commonly referred to, are one of the best experimental approaches for studying leaf water relations and their relationship with drought tolerance in leaves (Bartlett et al., 2012; Lenz et al., 2006; Turner, 1986). Among the parameters derived from P – V curves, leaf turgor loss point (π_{tlp}) and leaf capacitance (C) are important physiological traits related to plants’ withering and drought tolerance (Bartlett et al., 2012; Lenz et al., 2006; Xiong & Nadal, 2020; Zhu et al., 2018). π_{tlp} is especially relevant for its link with other processes such as regulation of stomata closure thresholds (Bartlett et al., 2016; Brodribb et al., 2003; Meinzer et al., 2016), maintenance of growth (Mitchell et al., 2008), or hydraulic safety margins (Fu & Meinzer, 2019; Zhu et al., 2018). High leaf capacitance contributes to maintain optimal water status under increasing transpiration (Xiong & Nadal, 2020) and is associated with leaf survival after stomatal closure in ferns (McAdam & Brodribb, 2013). Recently, P – V parameters such as π_{tlp} and capacitance have been related to the anatomical features of leaves, particularly saturated water content and cell size (Nadal et al., 2023); hence, it could be expected a different set of water relations in the allotetraploid *O. tinaei* compared to its parental species. Few studies have characterized water relations in ferns (e.g., Campany et al., 2021; Holmlund et al., 2016; Lo Gullo et al., 2010) and, to the best of our knowledge, no study has focused on water relations and their potential role in determining the ecophysiology of an allopolyploid system such as *Oeosporangium* spp.

In this study, we compared the anatomical and water relations traits in the fronds of these three *Oeosporangium* species in an experiment of followed by rehydration. We hypothesize that: (1) *O. hispanicum* has morphological and water relations traits more related to drought tolerance than those of *O. pteridioides*, (2) traits of *O. tinaei* are mostly intermediate between those of the diploid parents and contribute to explaining the intermediate habitat requirements and resurrection capacity of the allopolyploid, and (3) cell sizes of leaf tissues are larger in *O. tinaei* than in diploid parents due to genome doubling in the allopolyploid.

2 | MATERIALS AND METHODS

2.1 | Plant material and experiment conditions

The studied *Oeosporangium* species will hereafter be referred to by the genome constitution of the sporophyte: $HH = O. hispanicum$,

PP = *O. pteridioides*, and *HHPP* = *O. tinaei*, where *H* = *hispanicum* genome and *P* = *pteridioides* genome. Mature individuals (i.e., with sporangia) of *HH*, *HHPP*, and *PP* were obtained in the Picadas reservoir (central Spain, 40°20' N, 4°15' W, altitude 530 m) in early April 2021. For each species, 30 individuals were collected and placed in Ziploc bags, with the root ball wrapped in a moistened paper towel. Individuals were planted in 0.5-L pots with a 1:3 mixture of perlite:natural substrate. This substrate was also collected at the Picadas reservoir, on a schist slope where the studied species were growing. Plants were then placed in a growth chamber for acclimation for 2 months before the experiment (PAR intensity 470 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 12 h/12 h photoperiod, 25°C/20°C, and 40%–60% RH). After acclimation, different plants were subjected to a WS cycle: well-watered (WW) with daily irrigation, WS subjected to watering withholding for approximately 10 days, and recovery (REC) after rewatering for 2 days plants previously subjected to WS. A total of 8–10 individuals were used per species and treatment. Volumetric soil water content (SWC) at full water holding capacity in WW plants was 15%–20% (Probe Type GS1; Decagon Devices, Inc.). The soil moisture at the peak of the WS was, on average, 5% across species.

2.2 | Frond bulk structure

LMA was measured in 8–10 fronds from different individuals under WW conditions. Leaf area was measured from photographs of the frond lamina just before dehydration using ImageJ software (Wayne Rasband/NIH; Schneider et al., 2012). In addition, for comparison with LDMC obtained from *P*–*V* curves (see below), “rehydrated” leaf dry matter content (LDMC_{rt}) was measured on an independent set of fronds from WW plants as LDMC = DW/SW, where the saturated weight (SW) was measured after overnight rehydration in distilled water (Vile et al., 2005) instead of being inferred from the *P*–*V* relationship.

2.3 | Frond anatomy

Light microscopy was used for anatomy analysis in 5–6 fronds from different individuals under WW conditions. Small pieces (2 × 2 mm) were cut from fresh fronds, avoiding major veins and sporangia. Cuttings were immediately fixed under vacuum with 4% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (0.1 M, pH 7.2) and post-fixed in 2% buffered osmium tetroxide. Samples were embedded in London White resin (Electron Microscopy Science) after sequential dehydration with ethanol. Transversal semi-thin (1 μm) sections were cut with an ultramicrotome (Leica UC7) and stained with epoxy tissue stain (Electron Microscopy Science). Samples were observed at ×200 magnifications under light microscopy (Olympus) and photographed with a Moticam 3 (Motic Electric Group Co.). Images were analyzed with the ImageJ software. The following parameters were obtained, as means of 10 measurements per sample: leaf thickness (LT), upper and lower epidermis thickness,

palisade and spongy mesophyll thickness, cross-section area of upper and lower epidermis cells (UEA_{cell} and LEA_{cell}) and palisade and spongy mesophyll cells (PMA_{cell} and SMA_{cell}), and intercellular air space fraction in palisade and spongy mesophyll (PM_{ias} and SM_{ias}). In addition, the following parameters were calculated: ratio of mesophyll to epidermis thickness (M:E), weighted average (accounting for different tissues) cross-section cell area (A_{cell}), and the fraction of intercellular air spaces (f_{ias}).

2.4 | P–V curves

One frond from 8–10 plants subjected to each treatment was harvested just before the beginning of the light period. The cut section of the distal part of the fronds was maintained in rehydration in distilled water for 1 h before *P*–*V* curves were constructed. The drop in water potential (Ψ) and fresh weight (FW) were recorded from close to full hydration in a progressive bench dehydration process of the fronds until completing 10–12 pairs of weight and pressure records. Fronds were weighed before and after completion of each Ψ recording at each round of measurement, and average weight was considered for posterior *P*–*V* curve construction (Aranda & Pardos, 1996; Robichaux, 1984). Water potential was measured using a pressure chamber (Model 600D; PMS Instrument Company). The relationship between FW and Ψ from the initial 4–6 sampling points was used to infer SW. The dry weight (DW) was estimated after drying samples for 2 days at 65°C. Relative water content (RWC) was calculated from FW, SW, and DW as $\text{RWC} = (\text{FW} - \text{DW}) / (\text{SW} - \text{DW})$. The following functional traits were calculated from *P*–*V* curves: osmotic potential at full (π_o) and zero turgor or turgor loss point (π_{tip}), relative water content at zero turgor (RWC_{tip}), bulk modulus of elasticity (ϵ), the apoplastic water fraction (or relative water content in the apoplast, RWC_a), and leaf dry matter content (calculated using the inferred SW, LDMC_{pv}). The ϵ was estimated considering the full range of RWC over the points before turgor was lost. In addition, leaf capacitance before ($C^*_{o,\text{mass}}$) and after ($C^*_{\text{tip},\text{mass}}$) zero turgor were calculated as the slope of the quasilinear relationship between water potential and water content normalized by the frond DW.

2.5 | Statistical analysis

Differences between species under WW conditions were assessed using one-way ANOVA and Tukey's HSD test for post hoc comparisons; the “treatment” effect (WW, WS, and REC) for *P*–*V* data was assessed using two-way ANOVA with “species” and “treatment” as main factors. Values are presented as mean ± standard error unless indicated otherwise. Correlations among variables were tested using Pearson comparison. All analyses were conducted with R (R Core Team, 2022); the functions used were: *lm* for ANOVAs (“stats” package), function *HSD.test* for post hoc (“agricolae”; de Mendiburu, 2021), and *rcorr* for correlations (“Hmisc”; Harrell, 2022).

3 | RESULTS

3.1 | Structural and anatomical characteristics of fronds

The three species present similar sizes but distinct frond characteristics, especially in terms of frond thickness and air spaces (Figure 1). Frond bulk structure differed among the species in WW plants (Table 1): LMA was highest in *HH* (mean of $143 \pm 12 \text{ g m}^{-2}$) and lowest in *PP* ($58 \pm 7 \text{ g m}^{-2}$), whereas *HHPP* presented intermediate values ($102 \pm 9 \text{ g m}^{-2}$). These differences in LMA can be explained by variations in both thickness and density. *HH* possessed the thickest fronds (mean LT of $231 \pm 17 \mu\text{m}$) and *PP* the thinnest ($159 \pm 12 \mu\text{m}$), while *HHPP* showed intermediate LT ($176 \pm 21 \mu\text{m}$). Leaf density (LD) was calculated from the aforementioned LMA and LT, and again, LD indicates an intermediate position for *HHPP* (0.58 g cm^{-3}) compared to *HH* (0.62 g cm^{-3}) and *PP* (0.36 g cm^{-3}). LDMC_{rh} (assessed independently of *P-V* curves) was higher in *HH* ($0.39 \pm 0.01 \text{ g g}^{-1}$) than in the other two species, although LDMC_{rh} in *HHPP* was closer to that of *PP* (0.32 ± 0.01 and $0.29 \pm 0.01 \text{ g g}^{-1}$, respectively) when compared to the LD values.

The possible anatomical basis of the differences in the two components of LMA (i.e., thickness and density) were explored through differences in tissue thickness, cell size, and the air fraction among the species in WW plants (Table 1). The higher LT in *HH* can be mainly

attributed to greater palisade mesophyll thickness ($113 \pm 11 \mu\text{m}$) compared to the other two species (66 ± 8 and $53 \pm 3 \mu\text{m}$ for *HHPP* and *PP*, respectively). The upper epidermis is thinner in *HH* ($19 \pm 1 \mu\text{m}$), especially compared to *PP* ($30 \pm 3 \mu\text{m}$), while *HHPP* presents intermediate values ($26 \pm 3 \mu\text{m}$). These features result in a differentiated ratio of mesophyll to epidermis (M:E) across species, where *HH* presents the highest M:E (5.6 ± 0.7) compared to *HHPP* (2.9 ± 0.2) and *PP* (2.3 ± 0.2). In turn, *HH* presented a lower fraction of intercellular air spaces in the mesophyll (f_{ias} of $15 \pm 1\%$) compared to the other two species, although in this case, *PP* showed the intermediate values ($22 \pm 3\%$) and *HHPP* the highest f_{ias} ($29 \pm 3\%$). These differences in f_{ias} emerge from the air fraction of the spongy mesophyll (highest in *HHPP*), not the palisade mesophyll (Table 1). On the other hand, the cross-section cell area (a proxy of cell size) of all frond tissues did not differ significantly among species (Table 1). The only exception was the upper epidermis ($p = 0.090$), with marginally significant differences mainly due to smaller cells in *HH*.

3.2 | Water relations from *P-V* curves

The imposition of drought for 2–3 weeks resulted in an extensive decrease of SWC, from SWC of $0.200 \pm 0.024 \text{ m}^3 \text{ m}^{-3}$ in WW plants to $0.053 \pm 0.007 \text{ m}^3 \text{ m}^{-3}$ in WS plants (pooled data across species, no significant “species” effect in any case), with a significant recovery of

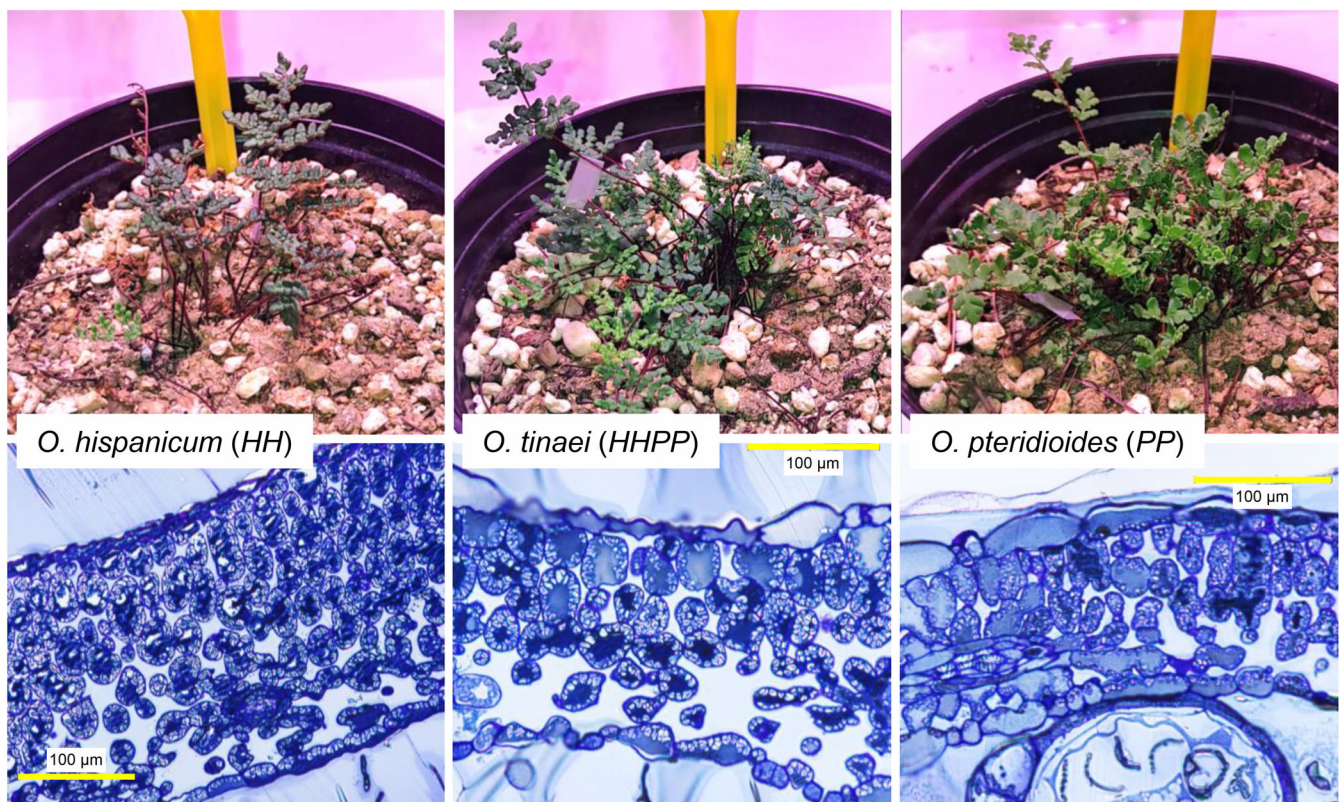


FIGURE 1 Representative plants and frond anatomy images of the three *Oesporangium* species studied. Scale bars for frond cross-sections represent $100 \mu\text{m}$.

TABLE 1 Comparison of frond structure and anatomy of the three *Oesporangium* species in well-watered (WW) plants.

Parameters	Species			One-way ANOVA	
	HH	HHPP	PP	F	p Value
Bulk structure					
LMA (g m ⁻²)	143.0 ± 12.2a	102.1 ± 8.5b	57.8 ± 6.9c	20.24	<0.001
LDMC _{rh} (g g ⁻¹)	0.392 ± 0.012a	0.318 ± 0.009b	0.287 ± 0.012b	24.44	<0.001
LT (μm)	231 ± 17a	176 ± 21ab	159 ± 12b	4.48	0.031
LD (g cm ⁻³)	0.620	0.581	0.364		
Tissue thickness					
UET (μm)	19 ± 1b	26 ± 3ab	30 ± 3a	4.55	0.030
PMT (μm)	113 ± 11a	66 ± 8b	53 ± 3b	17.07	<0.001
SMT (μm)	86 ± 6a	71 ± 12a	63 ± 9a	1.49	0.260
LET (μm)	17 ± 1a	20 ± 2a	24 ± 2a	2.23	0.144
M:E	5.56 ± 0.67a	2.93 ± 0.19b	2.25 ± 0.17b	20.59	<0.001
Cell size					
UEA _{cell} (μm ²)	735 ± 69a	1187 ± 218a	1245 ± 129a	2.87	0.090
PMA _{cell} (μm ²)	978 ± 107a	1290 ± 207a	1004 ± 138a	1.16	0.342
SMA _{cell} (μm ²)	899 ± 126a	815 ± 156a	865 ± 107a	0.10	0.906
LEA _{cell} (μm ²)	534 ± 62a	633 ± 127a	732 ± 71a	1.06	0.374
A _{cell} (μm ²)	919 ± 97a	1064 ± 159a	1019 ± 129a	0.59	0.756
Air fraction					
PM _{ias} (%)	9.2 ± 1.3	11.2 ± 2.8	9.3 ± 1.5	0.31	0.741
SM _{ias} (%)	22.5 ± 1.4b	46.0 ± 4.4a	33.1 ± 3.3b	11.29	0.001
f _{ias} (%)	15.0 ± 1.3b	28.7 ± 3.1a	22.1 ± 2.8ab	6.40	0.011

Note: Values indicate mean ± SE of leaf mass per area (LMA), leaf thickness (LT), “rehydrated” leaf dry matter content (LDMC_{rh}), leaf density (LD; calculated using species means for LMA and LT), upper and lower epidermis thickness (UET and LET, respectively), palisade and spongy mesophyll thickness (PMT and SMT, respectively), ratio of mesophyll:epidermis thickness (M:E), cross-section area of upper and lower epidermis cells (UEA_{cell} and LEA_{cell}, respectively) and palisade and spongy mesophyll cells (PMA_{cell} and SMA_{cell}, respectively), average cross-section area weighted across tissues (A_{cell}), intercellular air space fraction in palisade and spongy mesophyll (PM_{ias} and SM_{ias}), and average air fraction weighted across both tissues (f_{ias}). Species effect tested using one-way ANOVA; letters denote significant differences according to Tukey’s HSD test; n = 8–10 for LMA and LDMC_{rh}, n = 5–6 for the rest.

SWC in REC plants after 48 h of rewatering (0.155 ± 0.010 m³ m⁻³). SWC of WS plants was close to the exponential decrease in predawn water potential in the relationship between predawn Ψ versus SWC (Figure S1).

Most differences in all P - V parameters were explained by species, with only relatively minor effects of watering treatments (Figures 2 and 3). There were clear differences in the osmotic potential at full turgor in WW plants (Figure 2A): π_{tip} was lowest in HH (−1.74 ± 0.07 MPa), intermediate in HHPP (−1.65 ± 0.07 MPa) and highest in PP (−1.44 ± 0.10 MPa). π_{tip} was affected by treatments ($P = 0.013$ for “treatment” effect), showing a general decrease in WS plants compared to WW (average decrease by 0.15 MPa across all species) and an increase in REC plants (Figure 2A), although no clear recovery occurred after drought in PP. No such clear differences in RWC_{tip} were found in WW plants, although PP tended to present lower values (Figure 2B); on the other hand, the “treatment” effect was less significant ($p = 0.074$), with a tendency for lower RWC_{tip} in WS plants and an increase in REC plants (except for PP). No clear drought effect

was observed for π_o ($p = 0.401$), although there were significant differences among species ($p < 0.001$); in particular, PP presented higher values across different treatments (Figure 2C). There was a correlation between both π_{tip} and π_o across species and treatments (Table 2). The modulus of elasticity was significantly different among species ($p < 0.001$), although no clear differences were present in WW plants; on the other hand, the significant interaction between species and treatment ($p = 0.041$) indicated a different response of ϵ to drought and recovery depending on species. Indeed, WS and REC plants of PP showed a decrease in ϵ , while the other two species did not show such response, and REC plants presented similar values to those of WW (Figure 2C).

LDMC was the P - V parameter that more clearly differentiated the species ($p < 0.001$), with no significant effect of drought ($p = 0.227$) or the interaction of both factors ($p = 0.971$) (Figure 3A). In WW plants, HH showed the highest values (0.412 ± 0.009 g g⁻¹) and PP the lowest (0.271 ± 0.013 g g⁻¹), with HHPP showing an intermediate position (0.361 ± 0.010 g g⁻¹). RWC_a showed a similar

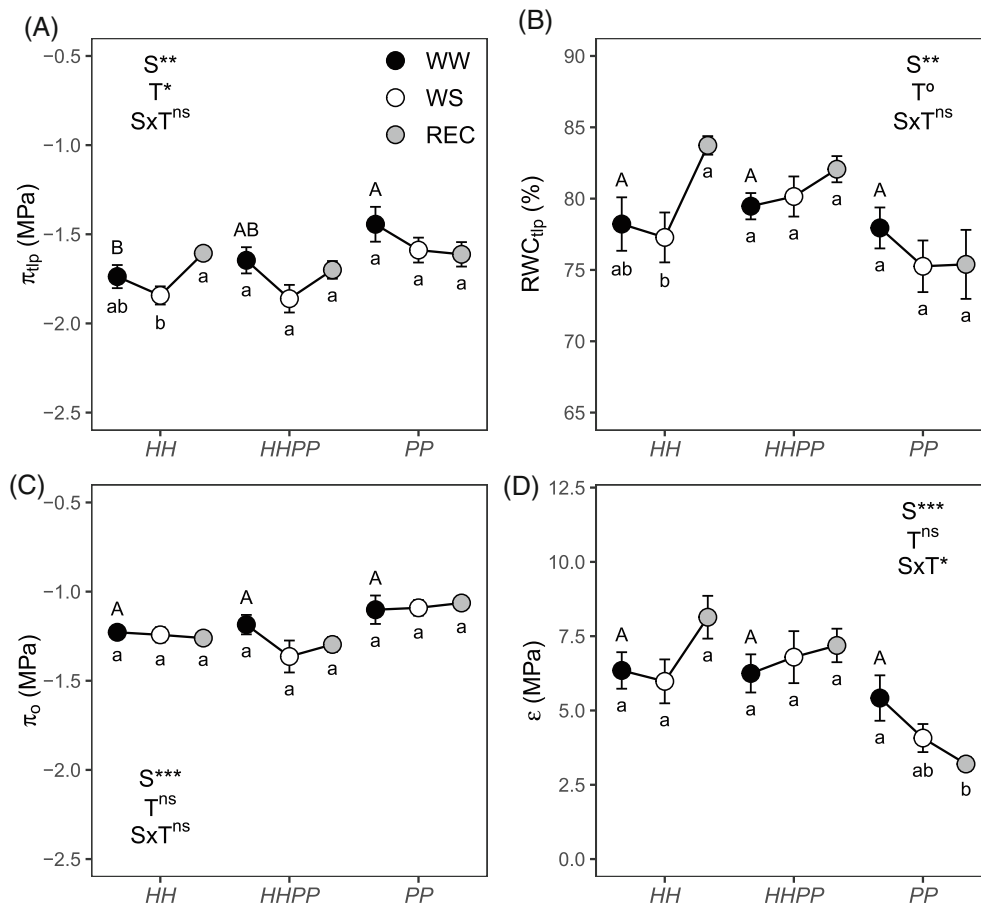


FIGURE 2 Pressure–volume parameters in the three *Oesporangium* species subjected to soil drought and recovery. WW, well-watered plants; WS, water-stressed plants; REC, plants recovered after rewatering. Values indicate mean \pm SE of the following frond parameters: (A) osmotic potential at the turgor loss point (π_{tip}), (B) relative water content at the turgor loss point (RWC_{tip}), (C) osmotic potential at full turgor (π_o), and (D) bulk modulus of elasticity (ϵ). Species (S) and water treatment (T) effects—and their interaction—were evaluated by two-way ANOVA; significance indicated as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ^o $p < 0.1$; ^{ns} $p > 0.1$. Uppercase letters denote differences across species among WW plants and lowercase letters indicate differences in treatments within each species (Tukey's HSD test); $n = 6$ –8.

trend in WW plants (Figure 2B), with HH and HHPP showing the highest values ($26 \pm 3\%$ and $27 \pm 2\%$, respectively) and PP the lowest ($10 \pm 2\%$). Only a minor “treatment” effect was detected ($p = 0.081$), where PP tended to show higher values in WS and REC plants compared to WW (Figure 2B). Overall, leaf capacitance (both before and after the turgor loss) was inversely related to LDMC_{pv} in WW plants (Table 2), where HH and HHPP followed a similar pattern, with only PP showing a distinct response after turgor loss (Figure 3C,D). PP presented the highest capacitance values and HH the lowest, with HHPP occupying an intermediate position. $C^*_{o,\text{mass}}$ showed a differential response to drought depending on species ($p = 0.016$ for the interaction term), whereas PP showed an increase in WS, especially in REC plants (Figure 3C). This pattern of $C^*_{o,\text{mass}}$ was also associated (inversely) to ϵ (Table 2). On the other hand, there were no clear differences in the response of $C^*_{\text{tip},\text{mass}}$ to stress across species, with only a minor decrease in WS and REC plants ($p = 0.095$ for the “treatment” effect).

4 | DISCUSSION

In the present study, we show how closely related species of the fern genus *Oesporangium* differ in their frond structure, water relations, and capacity to respond to WS and later recovery. Most notably, the results support both our first hypothesis that the two parent diploids

show distinct frond traits linked to water availability, as discussed below, and our second hypothesis that the allopolyploid has mainly an intermediate position in most of the studied traits. These findings are consistent with previous studies on the effects of allopolyploidy on functional traits (e.g., photosynthetic capacity) in other fern species (Sessa & Givnish, 2014) and in the same *Oesporangium* species under field conditions (Quintanilla et al., 2023). Our study species are also of interest given their desiccation tolerance (Quintanilla et al., 2023), which is associated with a particular set of frond traits in previously studied resurrection ferns, namely high LMA and low ϵ (Nadal, Brodrribb, et al., 2021; Nadal, Perera-Castro, et al., 2021).

LMA in the three species was in the range of previous reports for ferns (Dani et al., 2021; Poorter et al., 2009). The LMA of HH (143 g m^{-2}) and HHPP (102 g m^{-2}) were in the range of those reported for resurrection ferns (86 – 142 g m^{-2}) in Nadal, Perera-Castro, et al. (2021), whereas PP showed lower LMA (58 g m^{-2}), similar to that associated with desiccation-sensitive ferns. However, it must be noted that LMAs obtained from plants of the same populations (Picadas reservoir) under natural conditions were much higher (HH: 186 g m^{-2} , HHPP: 140 g m^{-2} , PP: 97 g m^{-2} ; Quintanilla et al., 2023). These differences indicate that, during acclimation, our cultivated plants produced fronds with reduced LMA, possibly associated with lower light intensity under growth chamber conditions, since the light regime is a strong driver of LMA variation (Aranda et al., 2004; Poorter et al., 2009). On the other hand, the three

FIGURE 3 Additional pressure–volume parameters in the three *Oesporangium* species subjected to soil drought and recovery. WW, well-watered plants; WS, water-stressed plants; REC, plants recovered after rewatering. Values indicate mean \pm SE of the following frond parameters: (A) leaf dry matter content from P–V ($LDMC_{pv}$), (B) relative water content at the apoplast fraction (RWC_a), (C) leaf specific capacitance at full turgor on a mass basis ($C^*_{o,mass}$), and (D) leaf specific capacitance at turgor loss on a mass basis ($C^*_{tip,mass}$). Species (S) and water treatment (T) effects—and their interaction—were evaluated by two-way ANOVA; significance indicated as *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; $^{\circ}p < 0.1$; $^{ns}p > 0.1$. Uppercase letters denote differences across species among WW plants and lowercase letters indicate differences in treatments within each species (Tukey's HSD test); $n = 6-8$.

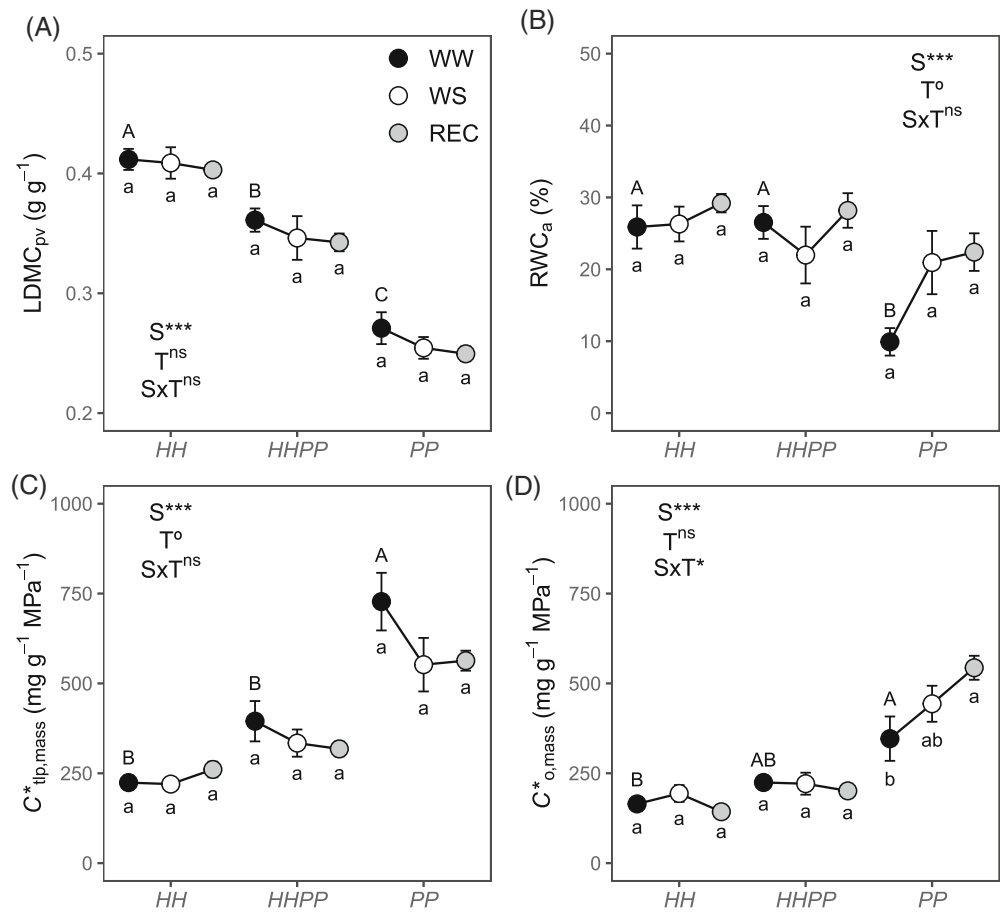


TABLE 2 Pearson correlation matrix of pressure–volume parameters using pooled data (individual replicates) of the three *Oesporangium* species and all treatments.

	π_{tip}	RWC_{tip}	π_o	ϵ	$LDMC_{pv}$	RWC_a	$C^*_{o,mass}$	$C^*_{tip,mass}$
π_{tip}	-	0.406	<0.001	0.300	<0.001	0.357	0.011	< 0.001
RWC_{tip}	0.106	-	0.001	<0.001	0.004	0.026	<0.001	0.115
π_o	0.710	-0.407	-	<0.001	<0.001	0.897	<0.001	0.007
ϵ	-0.132	0.754	-0.632	-	<0.001	0.139	<0.001	0.002
$LDMC_{pv}$	-0.419	0.356	-0.540	0.541	-	0.004	<0.001	<0.001
RWC_a	-0.117	0.278	0.016	0.187	0.353	-	0.007	<0.001
$C^*_{o,mass}$	0.317	-0.544	0.617	-0.743	-0.818	-0.333	-	<0.001
$C^*_{tip,mass}$	0.407	-0.199	0.334	-0.381	-0.726	-0.524	0.619	-

Note: Upper side shows p values and lower side shows r coefficients (bold when $p < 0.001$). The studied P–V parameters are: osmotic potential at the turgor loss point (π_{tip}), relative water content at the turgor loss point (RWC_{tip}), osmotic potential at full turgor (π_o), bulk modulus of elasticity (ϵ), leaf dry matter content from P–V ($LDMC_{pv}$), relative water content at the apoplast fraction (RWC_a), leaf specific capacitance at full turgor on a mass basis ($C^*_{o,mass}$), and leaf specific capacitance at turgor loss on a mass basis ($C^*_{tip,mass}$).

species present relatively low ϵ compared to desiccation-sensitive ferns (Holmlund et al., 2016; Nadal et al., 2018) and to other fern species from Mediterranean habitats (Lo Gullo et al., 2010). Their ϵ are in line with the value reported for the desiccation-tolerant fronds of *Anemia cafferorum* (Nadal, Brodribb, et al., 2021), possibly due to the high elasticity required for coping with the extensive mechanical stress associated with desiccation (Moore et al., 2008; Oliver et al., 2020).

The differences in LMA can be attributed to LT and density, which constitute the two components that define LMA (Poorter et al., 2009) and can vary independently in ferns (Vasheka et al., 2016). In this sense, HH fronds were both the thickest, mainly due to very thick palisade mesophyll, and the densest (Table 1). This high density was reflected by both LD and the independent measurement of LDMC (both from P–V and from “rehydrated” samples), as dry mass accumulation per SW constitutes a good proxy for LD

(Vile et al., 2005). Denser leaves can be the product of either smaller, denser cells or a reduction of the air fraction (John et al., 2017); here, the increased density of *HH* compared to *HHPP*, and especially *PP*, appears to be mostly driven by a reduced air fraction (Table 1), since no clear differences in cell size were detected (although *HHPP* and *PP* presented slightly bigger epidermis cells, which could contribute to their lower LD and LDMC given the lower mesophyll to epidermis ratio in these species). The differences in LMA in the three species could explain the presence of *HH* under the most stressful conditions in crevices of rocky walls, whereas *PP* is more often present in shallow soils (Quintanilla et al., 2023). Air fraction (SM_{ias}) was the only transgressive trait we found in *HHPP*, which had higher values than both diploid parents (Table 1). By contrast, cell sizes of all the frond tissues studied (upper and lower epidermis, palisade and spongy mesophylls) were similar in the three species. Thus, our hypothesis that *HHPP* shows cell enlargement due to genome doubling is not supported by these results. Consistent with this finding, the cell size of *HHPP* gametophytes is within the range of variation of *HH* and *PP*, while spores are larger in the allopolyploid (Pangua et al., 2019).

The analysis of *P-V* traits under different drought conditions indicates a limited degree of adjustment of water relations in response to WS and points mainly to constitutive differences among species as the main source of their capacity to withstand limited water conditions (Figures 2 and 3). The species presented clear differences in both π_{tip} and leaf capacitance, two aspects of water relations that reflect the degree of drought tolerance in plants. The turgor loss point is a key indicator of drought tolerance, with more negative values associated with more arid environments (Bartlett et al., 2012; Lenz et al., 2006; Zhu et al., 2018). On the other hand, higher capacitance is associated with the capacity of drought avoidance in ferns (McAdam & Brodribb, 2013) and provides a buffer against sudden decreases in water potential at the leaf level (Blackman & Brodribb, 2011; Xiong & Nadal, 2020). The high capacitance in *PP* could indicate a higher degree of isohydry compared to the other two species, although this is somewhat reduced under WS given its elastic adjustment, which leads to a lower capacitance in WS plants (Figure 3). Despite being associated with drought avoidance, high capacitance has also been reported in the desiccation-tolerant fronds of *A. cafferorum*, possibly due to its association with increased elasticity (Nadal, Brodribb, et al., 2021). Constitutively, π_{tip} and capacitance are related to water content, here reflected in $LDMC_{pv}$ and $LDMC_{rh}$ (Nadal et al., 2023). Indeed, $LDMC_{pv}$ is negatively correlated with both $C^*_{o,mass}$ and $C^*_{tip,mass}$ and, to a lesser extent, with π_{tip} (Table 2). The π_{tip} is mainly driven by the osmotic potential at full turgor (Bartlett et al., 2012; Lenz et al., 2006); in the studied species, we find a strong association between π_{tip} and π_o (Table 2). However, the observed changes in π_{tip} during drought are most likely due to elastic rather than osmotic adjustments, contrary to the common pattern observed in most plant species (Bartlett et al., 2012). Nonetheless, it is worth noting that the degree of turgor loss point change is rather small (here, the mean difference of 0.15 MPa across all species between WW and WS plants) when compared to larger datasets (Bartlett et al., 2012). Hence, this further points towards constitutive water relations traits,

rather than the capacity of osmotic or elastic adjustments in response to WS, as being the main determinants of the overall drought tolerance of the studied species.

In conclusion, we found a combination of constitutive traits regarding frond structure and water relations that could allow for niche partitioning of the three sympatric *Oeosporangium* species. On the one hand, *HH*, which lives in rock crevices, presents a set of traits more associated with low water and nutrient availability and lower carbon returns (Aerts & Chapin, 1999; Díaz et al., 2016; Nadal et al., 2023; Reich, 2014), such as thicker and denser fronds, with higher carbon content, lower nitrogen content and lower photosynthetic rate per mass (Quintanilla et al., 2023). On the other hand, *PP* shows a set of traits at the opposite extreme of nutrient and water availability (Díaz et al., 2016; Nadal et al., 2023; Reich, 2014), including lower LMA and higher π_{tip} and leaf capacitance, and a certain capacity for elastic adjustment under drought. In turn, these traits could restrain *PP* to more humid microhabitats on deeper soils with greater water retention and nutrient availability, as it is found in its natural environment. Compared to both diploid parents, *HHPP* shows mostly intermediate traits, which may explain its intermediate abiotic niche. These intermediate phenotypes can be the consequence of both allopolyploidy per se (i.e., hybridization and chromosome doubling) and other evolutionary processes (e.g., natural selection, genetic drift) subsequent to polyploid formation (Bretagnolle & Lumaret, 1995). In any case, our results support the idea that *Oeosporangium* allopolyploids are intermediate “fill-in” taxa that contribute to a more intensive occupation of the ecological space available (Ehrendorfer, 1980; Marchant et al., 2016).

AUTHOR CONTRIBUTIONS

Conceptualization and research design by Miquel Nadal, Luis G. Quintanilla, Ismael Aranda, and Jorge Gago. Measurements were performed by all authors. Data analysis by Miquel Nadal and Ismael Aranda. First manuscript draft by Ismael Aranda and Miquel Nadal. Manuscript writing, review, and editing by Miquel Nadal, Luis G. Quintanilla, Ismael Aranda, and Jorge Gago. Supervision and project administration by Jorge Gago. Funding acquisition by Luis G. Quintanilla, Ismael Aranda, and Jorge Gago.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as all new created data is already contained within this article.

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