


RESEARCH PAPER

Water deficit disrupts male gametophyte development in *Quercus ilex*

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ABSTRACT

- Tree species distribution, and hence forest biodiversity, relies on the reproductive capacity of trees, which is currently affected by climate change. Drought-induced pollen sterility could increase as a consequence of more intense and more frequent droughts projected for temperate and Mediterranean regions, and threaten the sexual regeneration of trees in these regions. To evaluate this possibility, we examined the effect of long-term partial rainfall exclusion (−27% precipitation) on male reproductive development in holm oak, *Quercus ilex*, one of the most important and widespread tree species of the Mediterranean region.
- We examined anther area, pollen production, pollen abortion as well as viable pollen production in control and dry treatments. Microscopic examinations revealed significant differences in pollen development between trees in the dry and the control treatments, even though anthesis occurred before the onset of annual drought.
- Our results demonstrate that anthers collected from *Q. ilex* trees in the dry treatment, which experienced long-term increased drought stress especially during the summer, were the same size as anthers in the control treatment, but displayed 25% pollen abortion and almost 20% reduction in pollen production. Subsequently, the number of viable pollen grains in anthers from dry treatment was 35% less than in control.
- These results suggest a carry-over effect of drought stress on pollen production that could reduce the reproductive success of *Q. ilex*. The results have broad implications for better understanding of the determinants of tree reproduction by masting and anticipate the outcomes of expected drought increase in the Mediterranean on forest dynamics.

INTRODUCTION

Drought stress is known to reduce reproductive output in a number of economically important species, such as cereals, through a reduction in pollen grain number and/or pollen abortion (e.g. Saini *et al.* 1984; Manjarrez-Sandoval *et al.* 1989; Sheoran & Saini 1996). Abscisic acid (ABA) accumulation and reduced carbohydrate reserves disrupt meiotic and mitotic divisions, eventually leading to reductions in pollen emission. In trees, however, the deleterious effect of water deficit on male gametophyte development has not yet been empirically demonstrated. If a reduction in water availability negatively affects male gametophyte development in woody species, as it does in cereals, chances are that predicted climate changes will alter their reproductive success, causing changes in forest composition. Drought sensitivity of reproduction could affect fitness and geographic distributions of woody species, especially in areas where drought is severe enough to approach sterility thresholds. With predicted reductions in rainfall, populations operating close to their threshold for reproduction could experience selection for drought tolerance of reproduction, but also most probably changes in range distribution, leading to regional biodiversity and ecosystem changes.

In this study we looked at the effect of rainfall exclusion on male gametophyte development in holm oak (*Quercus ilex*). The distribution of *Q. ilex*, one of the most important and widespread tree species of the Mediterranean region, might severely contract following projected temperature rises and reductions in precipitation. Based on multi-model assembled results, its habitat loss in Spain by 2050–2080 is projected to be 40.4% (Keenan *et al.* 2011). Despite its high drought tolerance as an adult tree, *Q. ilex* seems to be sensitive to water availability during its early life-cycle stages. Although the amount of summer precipitation is considered to be the best predictor of reproductive success in *Q. ilex* (Pérez-Ramos *et al.* 2010), spring rainfall can also play an important role in its seed production through effects on male and female gametophyte development (Alejano *et al.* 2008; Pérez-Ramos *et al.* 2010). The number of days with torrential rain in spring and spring rainfall were found to be positively correlated with seed production in *Q. ilex*, revealing a causal link between water availability and initial stages of its reproductive cycle (Alejano *et al.* 2008; Pérez-Ramos *et al.* 2010). Sanchez-Humanes & Espelta (2011) demonstrated that spring and summer drought mediated by an experimental 15% rainfall exclusion not only reduced the number of female flowers in *Q. ilex*, but also decreased the

number of fertilised flowers, which could be a result of disruptions at the gametophyte or/and zygote stages.

The limited knowledge of drought sterility in wild plants, particularly long-lived woody species, represents a gap in our understanding of how natural vegetation will be impacted by climate changes. The objective of this study was to examine the effect of long-term partial rainfall exclusion on male reproductive development in Mediterranean *Q. ilex*. Unlike many cultivated trees, this species is wind-pollinated, which slightly simplifies the comparison of our study and previous findings on cereals. Two questions were addressed: (i) does drought trigger changes in *Q. ilex* anther size; and (ii) does it affect total pollen production and viability in *Q. ilex*? The implication of these results for the future distribution of this species is subsequently discussed.

MATERIAL AND METHODS

Site description

Our study was performed at the experimental site of Puéchabon located 35 km northwest of Montpellier in southern France, on a flat plateau in the Puéchabon State Forest. The site represents an evergreen forest, largely dominated by a dense overstorey of *Q. ilex* ssp. *ilex* (80% canopy cover) and an understorey of *Buxus sempervirens*, *Phyllirea latifolia*, *Pistacia terebinthus*, *Pistacia lentiscus* and *Juniperus oxycedrus* that compose a sparse shrubby layer. All the *Q. ilex* trees are of the same age as they resprouted after a clear cut performed in 1942. Their top canopy height is approximately 5.5 m. Development of male and female inflorescences occurs from April to May and is asynchronous (Misson *et al.* 2011). Mature acorns drop between October and December. *Q. ilex* trees growing at this site are characterised by high inter-annual reproductive variability (Pérez-Ramos *et al.* 2010, 2013).

The Puéchabon experimental site is located at 270 m a.s.l. The climate is Mediterranean, with a mean annual temperature of 13.2 °C and a mean annual precipitation of 916 mm. Rainfall accumulated between September and April accounts for more than 80% of the annual precipitation in this region. Mean spring precipitation between 2005 and 2015 was 226 mm with a range of 94–510 mm. The soil is formed on Jurassic limestone and has high volumetric rock content comprising 75% within the top 0–50 cm layer and 90% below. The stone-free topsoil fraction consists of homogeneous silt clay loam soil. Due to the high soil permeability, surface runoff rarely occurs at the site (Limousin *et al.* 2009), making it an ideal location for a rainfall exclusion experiment.

Rainfall exclusion experiment and water stress measurement

A partial rainfall exclusion experiment was established at the Puéchabon site in March 2003. Two 140-m² plots located next to each other were subjected to two distinct precipitation regimes. In 2015, the density of resprouted stem at both plots was 4700 stems ha⁻¹. Trees grown on both plots did not differ in age or in mean stem diameter. The control treatment experiences natural drought conditions, while the dry treatment had been exposed to 27% rainfall exclusion since 2003, achieved through the installation of PVC gutters hanging under the canopy (Fig. 1a). Identical PVC gutters were installed in the

control plot, but upside down (Limousin *et al.* 2008, 2009). The effect of the rainfall exclusion experiment on tree water stress has been verified regularly since 2003 by measuring predawn leaf water potential (Ψ_{pd}). Measurements were carried out four to 12 times a year from 2003 to 2009, in 2011, and again from 2015 to 2017. Two leaves were sampled before dawn on four trees per treatment and Ψ_{pd} was immediately measured with a pressure chamber (PMS1000; PMS Instruments, Corvallis, OR, USA). A third leaf was sampled when the observed difference between two leaves from the same tree was > 0.2 MPa. Ψ_{pd} was assumed to be a surrogate for soil water potential, given a constant 0.45 MPa disequilibrium taken as the average Ψ_{pd} value when soil water content was at field capacity. The soil water balance model described in Cabon *et al.* (2018) was used to simulate Ψ_{pd} in years when field measurements were not available. The relationship between measured and simulated Ψ_{pd} had an $R^2 = 0.88$ for the control treatment and $R^2 = 0.89$ for the dry treatment.

Sample collection and microscopy analysis

To analyse the effect of increased drought on male gametophyte development we randomly selected 16 *Q. ilex* trees from the two treatments (eight from control treatment and eight from dry treatment) and sampled male catkins for microscopy analysis just prior to anther dehiscence between 4 and 20 May 2015, depending on the precocity of flowering of each tree. The number of pollen grains per anther, pollen viability and anther area were measured on 27 anthers from each tree (three anthers per catkin, three catkins per branch and three branches per tree). We were not interested in the total number of male flowers produced per tree, since this parameter does not affect male gametophyte development. Anthers were stored in Carnoy fixative solution (alcohol:chloroform:acetic acid, 6:3:1) immediately after sampling and analysed using a simplified method for differential staining of aborted and non-aborted pollen grains introduced by Peterson *et al.* (2010). Pollen viability was evaluated with modified Alexander's triple stain (Peterson *et al.* 2010), which colours the pollen wall blue-green and living cytoplasm purple-red (Fig. 1b, c). Aborted pollen grains lack cytoplasm and therefore show little staining. Anther area was assessed using Image J software (National Institutes of Health, Bethesda, MD, USA). In order to calculate the number of pollen grains in the anther we counted pollen grains within a known view area and extrapolated this using the area of locules in the anther.

Statistical analyses

Data were analysed using SIGMASTAT (version 3.0.1; SPSS, Chicago, IL, USA). Treatment differences in predawn water potential for a given measurement date were assessed with Student *t*-tests. A logit function between the Ψ_{pd} treatment difference simulated with the soil water balance model and the statistical significance at $P < 0.05$ of the measured treatment difference in Ψ_{pd} was used to evaluate if the model adequately detected the periods with significant treatment differences in Ψ_{pd} . Treatment and tree effects on anther area, pollen production, percentage of pollen abortion and number of viable pollen grains were assessed with two-way ANOVA followed by Holm-Sidak *post-hoc* tests. A regression analysis was performed

to determine the relationship between anther area and pollen production.

RESULTS

The rainfall exclusion has resulted in significantly more intense summer water stress than in the control treatment in 9 years of the 11 years when it has been measured between 2003 and 2017 (Fig. 2), thus demonstrating the efficiency of the experimental set-up in increasing tree water stress in summer. From 2003 to 2015, the year when we conducted our anther sampling, the minimum predawn water potential recorded in summer varied between -2.12 MPa and -4.07 MPa in the control treatment, and between -2.82 MPa and -4.82 MPa in the dry treatment. Winter 2015 was relatively dry in Puéchabon with only 110 mm of precipitation falling from January to March, which is approximately half of the average winter precipitation at the site. This was, however, compensated by above average precipitation in April that received 98 mm of precipitation. Consequently, when catkins were collected in May 2015, trees had not yet suffered from water stress in any of the treatments, as the predawn water potential did not start to decrease before mid-June. Predawn leaf water potential was not measured in 2014, the year prior the anther measurements, but the model predicted significant treatment differences in Ψ_{pd} on 25 consecutive days from 12 June 2014 to 6 July 2014 (treatment

difference greater than the logit threshold 0.34 MPa, $P < 0.001$ for the logit function). The minimum Ψ_{pd} in 2014 was reached in late June with a simulated Ψ_{pd} of -2.54 MPa in the control treatment and -2.92 MPa in the dry treatment (see Supporting information).

Between 2003 and 2016, the dry treatment also significantly reduced acorn production. Mean annual acorn production in the control and dry treatments was 24.8 and 11.2 g m^{-2} of dry matter (DM) m^{-2} , respectively, ranging from 1.9 to 57.8 g m^{-2} of DM for control and 1.3 to 29.79 g m^{-2} of DM for dry treatment (data not shown).

Anther size was not affected by the rainfall exclusion ($P = 0.918$); yet there were significant tree effect ($P < 0.001$; Fig. 3a). Mean area of anthers developed under control and dry treatments were 1.26 mm² and 1.31 mm², respectively. Under control conditions *Q. ilex* anthers produced on average 1943 ± 753 pollen grains anther⁻¹ (Mean \pm SD; $n = 216$). Previously reported number for southwestern subspecies, *Q. ilex* ssp. *ballota*, is 3402 pollen grains anther⁻¹. In the dry treatment, anthers contained 1595 ± 604 pollen grains on average ($n = 216$), which is almost 20% less than in control treatment ($P < 0.001$; Fig. 3b).

Anthers collected from trees in the control treatment contained predominantly viable pollen grains and displayed only 6.08% abortion rate (Fig. 3c). Mean percentage of pollen abortion in anthers developed in dry treatment was, in contrast,

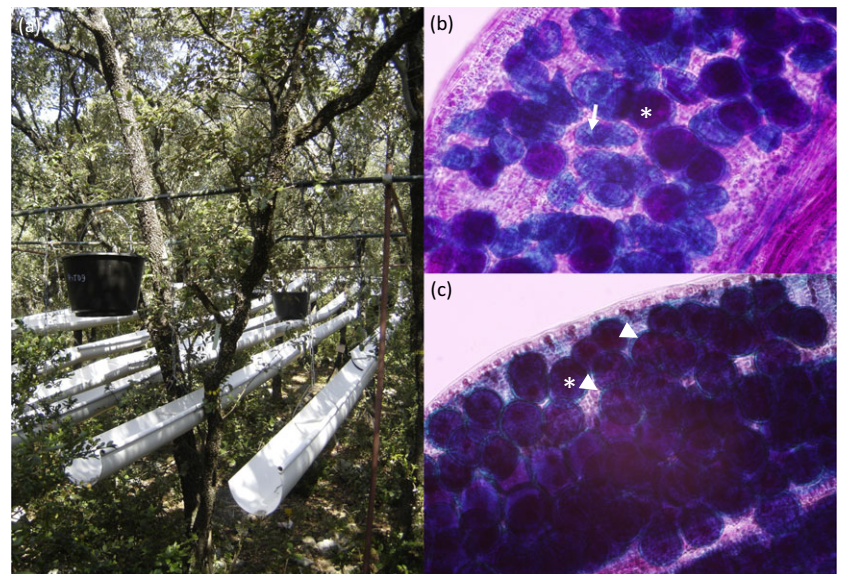


Fig. 1. *Q. ilex* grown in dry treatment at the Puéchabon experimental site (a). A 27% rainfall exclusion in dry treatment was achieved through installation of PVC gutters hanging under the canopy. Anthers of *Q. ilex*, developed in the dry (b) and control (c) treatments, stained with modified Alexander's triple stain (Peterson *et al.* 2010). Pollen wall stains blue-green (arrowhead). Cytoplasm of non-aborted pollen grain stains purple-red (asterisk). Cytoplasm of aborted pollen grain is absent, therefore no purple-red stain is present (arrow).

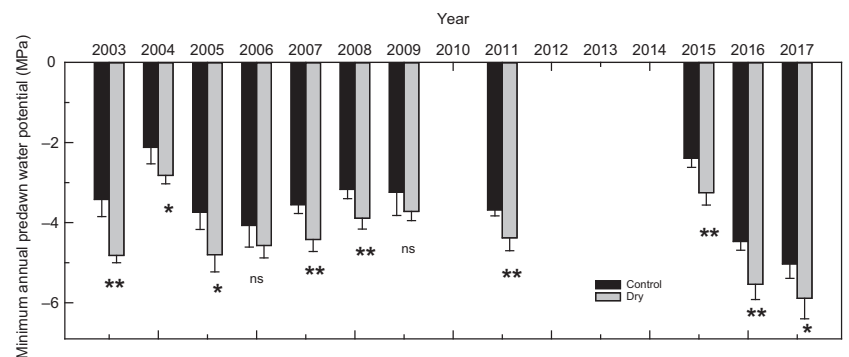


Fig. 2. Minimum predawn water potential (Ψ_{pd}) for *Q. ilex* grown in control (black bars) and dry (grey bars) treatment, measured between 2003 and 2017. Measurements were carried out four to 12 times a year. No measurements were obtained in 2010, 2012–2014. Asterisks indicate significant difference between dry and control treatments at $***P < 0.001$ and $*P < 0.05$. Means \pm SD; $n = 4$

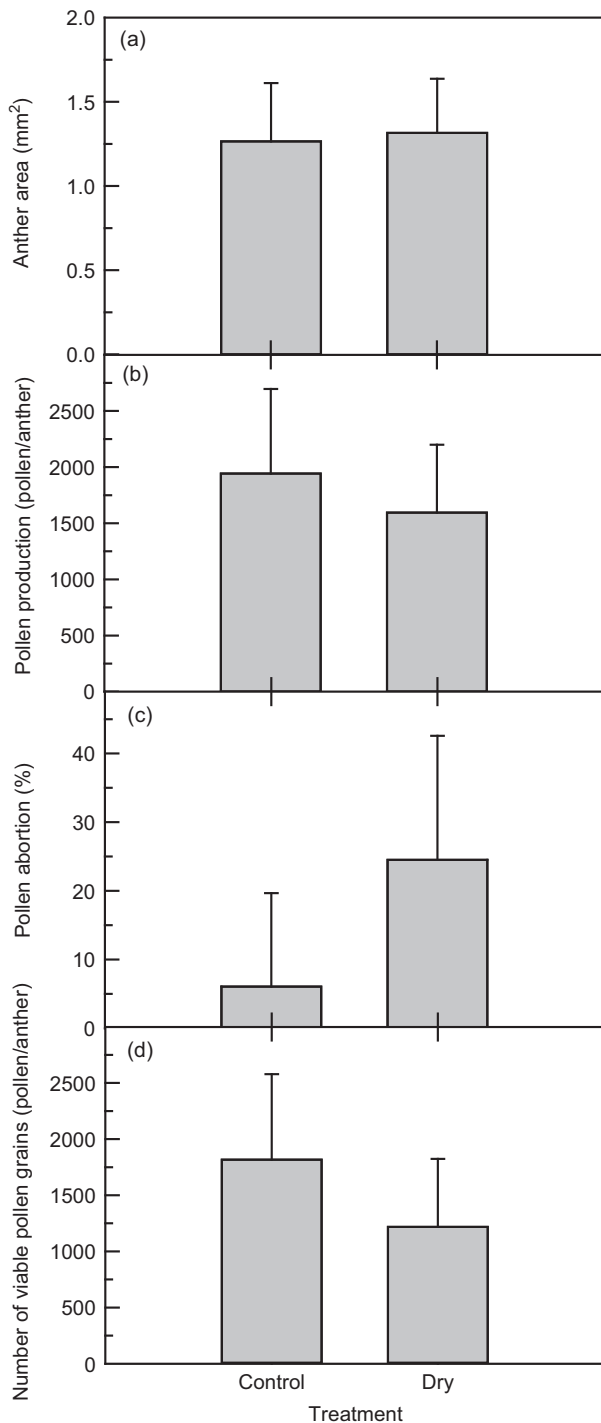


Fig. 3. Effect of drought on anther area (a), pollen production (b), pollen abortion (c) and viable pollen grains (d) in *Q. ilex*. Treatment had no effect on anther area ($P = 0.918$, two-way ANOVA), but a significant effect on pollen production ($P < 0.001$, two-way ANOVA), pollen abortion ($P < 0.001$, two-way ANOVA) and viable pollen production ($P < 0.001$, two-way ANOVA). Means \pm SD; $n = 216$.

four times higher (24.5%; Fig. 3c). In addition, a small number of these anthers (<5%) had locules that lacked evidence of meiosis. Although there was some variation within the treatments due to the individual tree effect ($P < 0.001$), the effect of treatment on the percentage of pollen abortion was significant

($P < 0.001$). Reduction in the total number of pollen grains as well as pollen abortion significantly decreased the number of viable pollen grains in dry treatment ($P < 0.001$). While in the control treatment viable pollen production was on average 1817 grains anther⁻¹, in the dry treatment it was 1218 grains anther⁻¹, an almost 35% decline (Fig. 3d). No relationship was found between anther area and pollen production in dry and control treatments ($R^2 = 0.0004$ and $R^2 = 0.129$, respectively; Fig. 4).

DISCUSSION

Trees monitored in this study experienced significantly increased drought stress for several years (Fig. 2). Although they were not experiencing drought at the time of anthesis, our microscopy observations demonstrate that increased long-term drought negatively affected their male reproductive development. This suggests that impact of water stress in long-lived species like *Q. ilex* may last for several growing seasons and represent a substantial limitation to pollen production. The 35% decline in the number of viable pollen grains triggered by reduced pollen differentiation and increased rate of pollen abortion was most likely a result of drought stress experienced by these trees during the previous growing season.

Pollen production and sterility

The size of the anthers did not vary between treatments, but pollen production in dry treatment was 20% lower than in control. Moreover, 25% of pollen developed in drought plot was non-viable and contained no cytoplasm. Increased pollen abortion rates observed in the dry treatment indicate that male gametophyte development in trees can be as vulnerable to drought as it is in cereals. Primary factors responsible for the observed defects are considered to be a reduction in carbohydrate reserves and ABA accumulation in reproductive organs (Saini 1997; Yang *et al.* 2001). Changes in anther sugar reserves have long been associated with stress-induced sterility; yet it is not known whether these alterations are linked to reductions in photosynthetic activity or to the suppression of sugar use and metabolism (Sheoran & Saini 1996; Sudhir & Murthy 2004; Dai *et al.* 2007). While a number of studies have shown reductions of carbohydrate influxes to anthers due to reduced photosynthetic activity; others revealed drought-induced down-regulation of enzymes involved in carbohydrate synthesis (acid invertase and soluble starch synthase; Sheoran & Saini 1996; Tezara *et al.* 1999; Chaves *et al.* 2002). Since we did not detect lower leaf water potential in the dry treatment than in the control at the time of anthesis, changes observed during this study seem to be a carry-over effect of the previous year's water limitation and not a direct effect of drought on anther sugar reserves or photosynthetic activity. Floral primordia in oak species are initiated before winter, typically at the end of summer after growth cessation, and remain dormant until the following spring (Boavida *et al.* 1999; Peter *et al.* 2009), which could explain the existence of a carry-over effect. *Q. ilex* trees experience drought mainly during the summer. Drought-induced transcriptional changes, initiated during the formation of flower primordia in late summer, may affect the cell division process during anthesis and result in meiotic and mitotic disruptions. Moreover, insufficient resource storage in dry

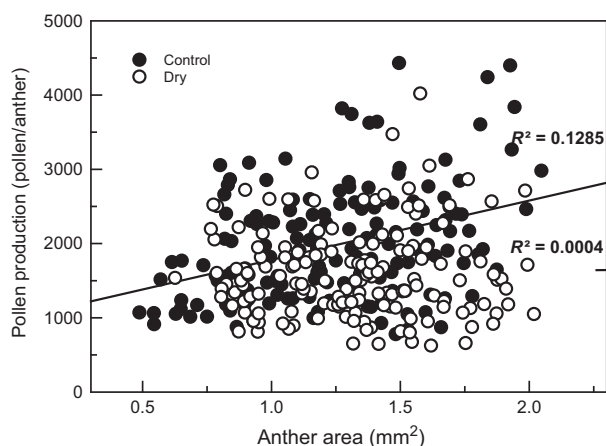


Fig. 4. Relationship between pollen production and anther area in *Q. ilex* grown under control (filled circle, solid line) and dry (open circle, dashed line) treatments. $n = 216$.

treatment might accelerate this effect. Overall, the carry-over effect of drought on pollen production in *Q. ilex* is similar to that observed on leaf and lateral shoot production. A study conducted between 2003 and 2009 at the same experimental site showed that rainfall exclusion induced a significant reduction in the number of leaves and new ramifications produced (Limousin *et al.* 2012). Since the newly emerging organs in oaks are pre-formed in buds during the previous summer, the organ development of these species can be influenced by drought with a 1-year lag (Boavida *et al.* 1999; Kuster *et al.* 2014).

The absence of a direct drought stress during the time of anthesis explains differences between our study and previous findings. In contrast with studies showing drought-induced deformation of anthers and significant reductions in their size (Sheoran & Saini 1996), we did not detect any changes in anther formation. Regardless of the decline in pollen differentiation (20%), we found no evidence for cell proliferation suppression. In addition, no correlation between anther size and pollen production was identified, which also contrasts with previous reports on herbaceous and woody species (Beri & Anand 1971; Trivedi & Verma 1975; Mondal *et al.* 1992; Torno Molina *et al.* 1996; Bhowmik & Datta 2013). The number of pollen grains produced per anther usually increases with increasing anther size; however, *Q. ilex* did not follow this pattern independently of the type of treatment.

Quercus ilex reproductive success and pollen limitation

The timing and duration of drought stress largely determine its effect on male reproductive processes. While short-term spring and autumn rainfall exclusions have no effect on male floral development processes in *Q. ilex* (Misson *et al.* 2011), a year-round drought stress negatively affects its male flower production (Ogaya & Peñuelas 2007). A long-term experiment conducted in southern Catalonia demonstrated that a 15% reduction in soil moisture occurring throughout the year (20% in spring/autumn and 10% during summer/winter) reduced the number of male flowers in *Q. ilex* by 30% (Ogaya & Peñuelas 2007). These findings match our detailed microscopy analysis, which shows that long-term reductions in water availability and a subsequent drought stress during

floral primordia development have a negative effect on viable pollen production.

Pollen limitation can influence plant reproductive success and population dynamics (Culley *et al.* 2002). Although this phenomenon is usually discussed in the context of animal-pollinated plants, its role in the reproductive success of wind-pollinated species, like *Q. ilex*, could be quite substantial. Contrary to previous assumptions, the dispersal distance of their pollen sometimes is even shorter than that for large seeds (Koenig & Ashley 2003). Pollen can limit seed production in these species due to the negative effect of phenological asynchrony as well as abiotic factors, like wind and precipitation that directly influence pollen release and transfer (Pearse *et al.* 2015). Moreover, reduction in viable pollen production can also lead to an inadequate pollen receipt by ovules and result in production of seed output below its maximum potential value. Several studies demonstrate that pollen emissions play a crucial role in *Quercus* seed production (García-Mozo *et al.* 2007; Koenig *et al.* 2012). A strong correlation between the airborne pollen counts and final seed yield was found for *Q. ilex* in southern Spain, and pollen production for those trees was positively affected by January and March rainfall occurring 1 to 3 months prior to male flower anthesis (García-Mozo *et al.* 2007).

Our findings suggest that the dependence of *Q. ilex* reproductive output on male gametophyte development might increase with expected climate changes (IPCC 2013). Reductions in spring precipitation projected for the Mediterranean region (Giorgi & Lionello 2007; Beniston *et al.* 2007; Bravo *et al.* 2008) might significantly reduce *Q. ilex* pollen and seed production, and thus threaten the sexual regeneration of this species. Despite relatively large morphological, physiological and phenotypic plasticity, as well as genetic differentiation (Lumaret *et al.* 2002; Vernesi *et al.* 2012) reflected in its wide distribution range (Gratani 2014), *Q. ilex* phenotypic plasticity in flowering time is limited (Michaud *et al.* 1992), making it particularly vulnerable to abiotic stress during the gametophyte stage. This effect might become even more pronounced in fragmented and low-density stands, since pollen limitation in self-incompatible species, like *Q. ilex*, is known to increase with decreasing population density (Burd 1994).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. *T*-test *P* value of measured difference in predawn water potential between the dry and control treatments and the simulated treatment difference obtained by the model.

Figure S2. Logit function between the significance of the measured treatment effect on water potential and the simulated treatment difference obtained by the model.

Figure S3. Simulated predawn leaf water potential of *Q. ilex* between May 2014 and August 2014. The minimum Ψ_{pd} in

2014 was reached in late June with a simulated Ψ_{pd} of -2.54 MPa in the control treatment and -2.92 MPa in the dry treatment.

Table S1. Two-way ANOVA results on anther size, pollen production and pollen viability in *Q. ilex*.

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