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Explanatory ecological factors for the persistence of desiccation-sensitive seeds in transient soil seed banks: *Quercus ilex* as a case study

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• **Background and Aims** Dominant tree species in northern temperate forests, for example oak and beech, produce desiccation-sensitive seeds. Despite the potentially major influence of this functional trait on the regeneration and distribution of species under climate change, little is currently known about the ecological determinants of the persistence of desiccation-sensitive seeds in transient soil seed banks. Knowing which key climatic and microsite factors favour seed survival will help define the regeneration niche for species whose seeds display extreme sensitivity to environmental stress

• **Methods** Using the Mediterranean Holm oak (*Quercus ilex*) forest as a model system, an *in situ* time-course monitoring of seed water status and viability was performed during the unfavourable winter season in two years with contrasting rainfall, at an instrumented site with detailed climate records. In parallel, the characteristics of the microhabitat and their influence on the post-winter water status and viability of seeds were investigated in a regional survey of 33 woodlands representative of the French distribution of the species.

• **Key Results** Time-course monitoring of seed water status in natural conditions confirmed that *in situ* desiccation is the main abiotic cause of mortality in winter. Critical water contents could be reached in a few days during drought spells. Seed dehydration rates were satisfactorily estimated using integrative climate proxies including vapour pressure deficit and potential evapotranspiration. Seed water status was therefore determined by the balance between water uptake after a rainfall event and water loss during dry periods. Structural equation modelling of microhabitat factors highlighted the major influence of canopy openness and resulting incident radiation on the ground.

• **Conclusions** This study provides part of the knowledge required to implement species distribution models which incorporate their regeneration niche. It is an important step forward in evaluating the ecological consequences of increasing winter drought and environmental filtering due to climate change on the regeneration of the most dominant Mediterranean tree species.

Key words: Recalcitrant seeds, oak acorn, *Quercus ilex*, seed survival, desiccation, drought, environmental filtering, germination, regeneration niche, Mediterranean climate, microhabitat, structural equation modelling.

INTRODUCTION

Seed persistence, i.e. the survival of seeds from the time they reach maturity on the parent plant until they germinate or die (through ageing, predation or decay), enables the dispersion of plant populations in time as well as in space, thereby increasing the likelihood that some seeds of a plant's cohort encounter favourable environments for germination and establishment (reviewed in Long *et al.*, 2015). The ability of so-called 'orthodox' seeds to tolerate desiccation and persist for long periods in a dry quiescent state enables them to survive adverse environmental conditions after dispersal, such as periods of cold or drought (Buitink and Leprince, 2008). Such long-lived seeds may form a reserve of seeds, named persistent soil seed banks, which remain viable in the soil for several years (Thompson and Grime, 1979; Walck *et al.*, 2005). In natural conditions, seed persistence in the soil is important for the sexual recruitment and regeneration of local plant populations, and plays a key role in population dynamics and vegetation composition

(Saatkamp *et al.*, 2013; Robledo-Arnuncio *et al.*, 2014). Several authors have pointed out that this role will become even more crucial in the near future because seed to seedling transition is critically sensitive to climate change (Lloret *et al.*, 2004; Svensson *et al.*, 2005; Millar *et al.*, 2007).

However, >8% of the world's flowering plants produce short-lived seeds that are non-orthodox (Tweddle *et al.*, 2003), i.e. they either only withstand partial drying (intermediate seeds) or are extremely sensitive to dehydration (recalcitrant seeds) (Roberts, 1973; Ellis *et al.*, 1990). Most of these non-orthodox seeds remain fully hydrated at maturity and are shed in a metabolically active state (Berjak and Pammenter, 2008). Non-orthodox seed species prevail in certain habitats, for example in tropical rain forests where they represent >45% of plant species (Tweddle *et al.*, 2003; Hamilton *et al.*, 2013).

Desiccation-sensitive seeds are also present in temperate forests. Indeed, dominant tree species such as oak (*Quercus*) and beech (*Fagus*) belong to the non-orthodox category (Finch-Savage, 1992; Leon-Lobos and Ellis, 2002). The seeds of these

autumn dispersers and spring germinators display short-term persistence (Walck *et al.*, 2005), in which the role of persistence can be summarized as shifting germination from one side of the unfavourable season to the other. Seed desiccation sensitivity is potentially a high-risk regeneration strategy for these plants, because a prolonged dry spell between shedding in the autumn and the return of favourable conditions for germination in the spring could result in the death of an entire annual cohort of seeds. However, to date, very few empirical studies have analysed the environmental factors encountered during the unfavourable season and their effects on seed desiccation and survival in natural vegetation systems. Using the evergreen Holm oak (*Quercus ilex*, one of the most dominant Mediterranean tree species, estimated to cover >80 000 km²; Le Houerou 1981) as a model system, we recently analysed the relationships between seed water contents and seed survival rates after the winter period in different woodlands across the French distribution area of the species (Joët *et al.*, 2013). The tight link between survival and water status of seeds at the end of winter clearly revealed that, after predation, desiccation is the major cause of *in situ* mortality.

Interest in seed persistence has recently increased due to concerns about the maintenance of plant populations and species under global climate change with more intense predicted periods of seasonal drought (Ooi *et al.*, 2009; Walck *et al.*, 2011; Ooi, 2012). Improved understanding of seed persistence could help define regeneration niches (Grubb, 1977) for different species and, ultimately, help optimize species distribution process-based models (Mok *et al.*, 2012). As described in a general resistance–exposure model (Long *et al.*, 2015), the persistence of a given seed population in the field (ecological longevity) is theoretically determined by the combination of environmental conditions and seed characteristics such as their intrinsic longevity and their level of defences against biotic/abiotic stresses. Since non-orthodox seeds display very short potential longevity and limited intrinsic tolerance to dehydration or cold stress (the hydrated state conferring frost sensitivity), persistence in transient soil seed banks is thought to be mostly influenced by the environmental conditions encountered during the unfavourable season, i.e. the intensity and frequency of stressful events, as well as by seed ultrastructure in relation to desiccation. Indeed, the large seeds generally produced by non-orthodox species could reduce their dehydration rate (Dickie and Pritchard, 2002; Daws *et al.*, 2006; Hill *et al.*, 2012), while anatomical barriers such as the seed coat/fruit pericarp could reduce water loss until the coat becomes more permeable as it deteriorates due to weathering (Hill *et al.*, 2010; Xia *et al.*, 2012). Adaptive phenological synchronization of seed shedding with the wet season is a stress avoidance strategy which is also observed in seasonal tropical forests (Dussert *et al.*, 2000; Pritchard *et al.*, 2004; Daws *et al.*, 2005). However, empirical studies of seed persistence in transient soil seed banks in relation to environmental factors are still rare. Such studies require the identification of the environmental drivers of seed persistence and an understanding of how they inter-relate, which would make it possible to predict seed persistence under particular conditions.

We recently showed that in Mediterranean forest ecosystems, the water content of Holm oak seeds on the soil, and hence their viability at the end of winter, is dramatically influenced by

winter climate factors such as cumulative rainfall and average maximum temperatures, as well as by microhabitat characteristics (Joët *et al.*, 2013). However, in this previous report, topographical parameters were simply recorded as qualitative descriptors with a limited number of categories. Considering the significance of these results, further characterization of microsites using quantitative variables (e.g. canopy openness, incident radiation, litter amount and soil texture) was needed to improve our understanding of the effects of microhabitat characteristics on seed water status. Similarly, our previous report focused on the relationship between winter climate, topography, seed water status and viability after the winter period without assessing water loss dynamics during the winter period. However, how quickly desiccation-sensitive seeds lose water is an important determinant of their ability to tolerate periods of seasonal drought.

To improve our understanding of the effects of climate and local topographic factors on the water status of seeds that remain on the soil throughout the winter period, the aims of the present study were to (1) explore and inventory the climate and microsite factors which affect the persistence of desiccation-sensitive seed; (2) quantify the probabilities of the different factors through structural equation modelling, and identify their inter-annual variability, and (3) provide ecological time-course evaluations of the survival and water content of these seeds, in relation to microclimate factors and the environmental drivers of seed desiccation.

MATERIALS AND METHODS

Post-winter seed sampling campaign: study area, climate data and microhabitat characterization

The water status and viability of seeds after winter were investigated in two consecutive years in 33 woodlands, representative of the French distribution of *Q. ilex* (from latitude 42°27'08''N to 44°51'08''N and from longitude 02°07'24''E to 06°48'26''E), and analysed with respect to local winter climate factors and microhabitat characteristics (the whole data set is presented in [Supplementary Data Table S1](#)). The Holm oak woodlands were sampled from 28 March to 2 April 2012 and from 2 to 6 April 2013, using the same plots and sampling protocols. These plots were identical to those analysed previously (Joët *et al.* 2013).

Acorns of *Q. ilex* are abiotically dispersed by gravity in autumn. They may also be biotically dispersed, mainly by rodents, and cached in the close vicinity of their original drop point, either in the litter or buried in the superficial soil layer (Gómez *et al.*, 2008). Our study focused on acorns which remained in the litter and the superficial soil layer, i.e. those that had not been removed by long-distance dispersers (European jays), damaged by insects or consumed by rodents and ungulates such as the wild boar *Sus scrofa*. At each sampling site, at least 200 sound acorns were collected from the relatively dense seed bed underneath at least five randomly selected *Q. ilex* trees. Acorns were collected from at least five randomly selected quadrats (50 × 50 cm). Acorns were immediately placed in a hermetically sealed plastic bag, and stored in the dark at ambient temperature for a maximum of 2 d until viability tests and water content measurements.

The climate conditions (daily rainfall, minimum and maximum temperatures) experienced by acorns from shedding to the collection date were estimated using climate data from 1 November 2011 to 31 March 2012 and from 1 November 2012 to 31 March 2013, recorded by the nearest meteorological station (data were purchased from Météo France public library, <https://public.meteofrance.com/>). The distance between a sampling site and the meteorological station was always less than 5 km. Temperature data were corrected using the international standard atmosphere adiabatic lapse rate of 0.65 °C per 100 m and the difference in altitude between each sampling site and its corresponding meteorological station.

To characterize the environmental conditions at the micro-site, the amount of soil litter cover, expressed on a dry weight per area basis, was assessed in each of the 33 experimental plots using three randomly selected quadrats (50 × 50 cm) within the sampling plot. The superficial (0–10 cm) soil texture was characterized by the volumetric fractional content of stones and by the gravimetric composition (sand, clay and silt) of the stone-free fraction (Saxton and Rawls, 2006). Light availability was estimated by hemispherical canopy photography. Photographs were taken at ground level in the centre of each acorn harvesting plot (10 × 10 m), using a horizontally levelled digital camera (DMC-LX5, Panasonic, Osaka, Japan) with a fish-eye lens with a 180° field of view (FCE8, Nikon). WinScanopy software (Regent Ltd, Canada) was used to analyse the images (taking into account geographical position, elevation, slope and exposure, and corrected for magnetic north) to calculate incident radiation on the ground ($\text{J m}^{-2} \text{d}^{-1}$) and the site factor, which represents the fraction of total radiation above the canopy that penetrates below the canopy.

Instrumented site for time-course monitoring of the seeds over the winter

A dense time-course monitoring of seed water status was performed in an instrumented site with fine micro- and mesoclimate records to refine the respective influence of these factors (the whole data set is presented in [Supplementary Data Table S1](#)). The experimental site (<http://puechabon.cefe.cnrs.fr/>), a *Q. ilex*-dominated evergreen forest, is located 35 km north-west of Montpellier (southern France) in the Puéchabon State Forest on a flat plateau (3°35'45''E, 43°44'29''N, 270 m a.s.l.). All the trees grew after a clear-cut made in 1942. The maximum canopy height is 5.5 m and the density of re-sprouts is around 6400 stems ha^{-1} . Understorey evergreen species (*Buxus sempervirens*, *Phyllirea latifolia*, *Pistacia terebinthus*, *Pistacia lentiscus* and *Juniperus oxycedrus*) make up a sparse shrubby layer with <25% cover and <2 m in height. The soil is extremely rocky and of hard Jurassic limestone origin; on average, the volumetric fractional content of stones and rocks in the top 0–50 cm layer is 0.75 on average, the stone-free fraction being a homogeneous silty clay loam.

Around 4000 freshly shed mature acorns of *Q. ilex* were collected in November 2012 and November 2013, in a single plot located near the instrumented plot and close to Montpellier (43°6'29''N, 03°89'00''E), which was protected from predation by wild boars, and transferred to the Puéchabon site. The acorns were placed on the ground, one half in a shady micro-habitat under shrubs and trees (understorey, down to 5% full

sun) and the other half in an open space with up to 90% full sun. The acorns were protected by wire cages (100 × 100 × 50 cm, 1.3 cm mesh size) to protect them from seed predators. One hundred seeds were collected from both halves of the plot weekly to check viability and measure seed water content, as well as to monitor *in situ* germination. The experimental plot is a flat 100 m² area (10 × 10 m) with no lateral water flow. Superficial soil volumetric water content (0–5 cm) was measured using three EC-5 frequency domain reflectometry probes (Decagon Devices Inc., Pullman, WA, USA) surrounding the wire cage (from 1 to 3 m). The maximum water content was 25.1% of the soil volume. Soil temperature was measured at a depth of 3 cm using three thermocouples (Type T with a stainless steel sheath; TC SA, Dardilly, France) also surrounding the wire cage. Both parameters were measured continuously during the winter season, and recorded at a 30 min time step on a CR10X datalogger (Campbell Scientific Ltd, Shepshed, Leicestershire, UK).

Weather variables [rainfall, wind, air temperature and air relative humidity (RH)] were recorded using a standard weather station located in a tree-free area close (100 m) to the experimental plot. Global and net radiations were recorded on an 11 m tall scaffolding tower about 5 m taller than the dominant trees. Meteorological data were continuously recorded by a datalogger (model 21X, Campbell Scientific Ltd) and averaged at a 30 min time step. Air temperature and air humidity were measured with an MP100 sensor (Rotronic, Bassersdorf, Switzerland). A three-dimensional sonic anemometer (Solent Model R3A; Gill Instruments, Lymington, UK) was used to measure wind velocity and friction velocity. Global radiation was measured with a pyranometer (SKS 1110; Skye Instrument Ltd, Powys, UK) and net radiation was measured with a Kipp Zonen CNR1 net radiometer (Kipp & Zonen BV, Delft, The Netherlands). Soil heat flux was measured with five soil heat plates (HFP01SC; Hukseflux, Delft, The Netherlands) buried at a depth of 5 cm near the tower. Potential evaporation (PET) was estimated using the Penman–Monteith equation.

Seed water content and viability

All experiments were carried out using sound acorns, which were sorted from unsound ones by visual screening after removal of the pericarp. The moisture content, expressed on a fresh weight basis, and the dry mass of the seed and the pericarp were determined gravimetrically after oven drying for 17 h at 103 °C, using 15 seeds per sample.

Seed viability was assessed by both percentage germination and the percentage of normal seedling development. For each condition and location sampled, the pericarp was removed and six batches of six seeds were placed on 18 g of vermiculite fully imbibed with 45 mL of sterile water in closed plastic [®]Magenta boxes and kept at 25 °C in the dark. Germination was recorded when the radicle had grown at least 10 mm after 2 weeks of culture, therefore ensuring that protrusion was not due to mechanical elongation of the hypocotyl but to true growth of the radicle. Normal seedling development was recorded using the criteria of radicle geotropic growth and the opening of primary leaves after 6 weeks of culture.

Seed and acorn dehydration time under controlled conditions

Prior to monitoring seed water status under natural winter conditions, the basic desiccation properties of the acorns (i.e. maximal dehydration rates) were assessed under controlled conditions using equilibrium drying at two rates of RH (45 and 62%) frequently experienced *in situ*. Batches of ten holm oak naked seeds or acorns were desiccated by equilibration over K_2CO_3 (45% RH) and NH_4NO_3 (62% RH) saturated solutions for 20 d in the dark as previously described (Dussert *et al.*, 2000). Dehydration rates were measured at 25 °C, a temperature close to maximal temperatures frequently observed *in situ* (at the Puéchabon site) in March and April. Water loss was assessed gravimetrically on a daily basis.

Statistical analyses and structural equation modelling

Correlations between environmental variables and seed water content were analysed by linear regression using Pearson's correlation coefficient. A significance threshold of $P = 0.01$ was retained. The effects of environmental variables on seed water content were also tested using linear models. For each model tested, a year effect was added, to take into account the fact that measures were repeated in two consecutive years.

Principal component analysis (PCA) was used to analyse the respective contributions of mesoclimatic and microtopographic variables to the variation in seed water content and viability. A structural equation model (SEM; Mitchell, 1992) was used to test for possible causal links within the transient soil seed bank system, and especially to investigate whether seed viability was related to explanatory variables such as climate. For each separate year (2012 and 2013), we explored whether seed survival was explained by the relationships between seed water content and climate variables (rainfall and solar incident radiation). Since climatic conditions were highly contrasted between the 2011–2012 and 2012–2013 winter seasons, we aimed at testing whether the contributions of environmental variables display interannual variations. Furthermore, we also tested the importance of seed mass in the model, or whether the pericarp:seed mass ratio explained differences in seed desiccation better. Note that pericarp:seed mass ratio data were not available in 2012; therefore, we were only able to test a simple model. We used two SEM 'goodness of fit' measures, the standard χ^2 test and also the Bentler's comparative fit index (CFI; Bentler, 1990), for which a model is regarded as acceptable if the CFI exceeds 0.93 (Byrne, 1994). All analyses were carried out with R statistical software (R Core Team, 2014). SEM analysis was conducted with the R package lavaan (Rosseel, 2012).

RESULTS

Influence of climate and microsite factors on post-winter seed water content and survival

Interannual variability of cumulative winter rainfall was very high: the 2011–2012 winter was the driest winter in Languedoc since rainfall has been recorded (approx. 100 years), whereas the 2012–2013 winter was mild and wet, with rainfall exceeding seasonal norms. Relationships between seed water content

and environmental factors were analysed by linear regression (Fig. 1). A highly significant ($P < 10^{-3}$) positive correlation was observed between seed water content and winter cumulative rainfall from December to March, without any significant interannual effect on the slope (Supplementary Data Table S2). This climate variable explained a significant proportion (50%) of variance for seed water content (Fig. 1A). Independently of cumulative rainfall, seed water status was also affected by the duration of the drought (Fig. 1B), but with a significant interannual variation (Table S2). Finally, microhabitat features including canopy openness (site factor) and amount of direct solar radiation reaching the transient soil seed bank appeared to have an influence on seed water content (Fig. 1C, with significant variation between years as shown in Table S2). Such an effect appeared to be additive to that of other dominant effects since fitted lines for the two consecutive surveys displayed similar slopes with a y-intercept offset.

To examine the respective contribution of all climate and microhabitat variables to seed status after winter, all the variables were analysed by PCA. The four main principal components (PC1–PC4) explained 78.5% and 69.7% of the variance of the whole data set for winter 2011–2012 and winter 2012–2013, respectively (Supplementary Data Table S3). Variances of seed viability and water content were mainly explained by PC1 and PC3; these two components accounted for 48.4 and 37.8% of the overall variance, respectively, in 2012 and 2013 (Fig. 2). Independently of the winter season analysed, seed viability and water content vectors were collinear. The correlation circles demonstrate that the mesoclimate variables shown to influence seed water content individually (i.e. cumulative rainfall and drought duration, as assessed by linear regression, see above) mainly contributed to PC1, while a microhabitat parameter such as incident solar radiation was associated with PC3. These key findings suggest that both climate and microhabitat features are required to explain between-site variation in seed survival.

Although PCA is useful to identify covariations between ecological factors and seed status, it cannot be used to detect whether there is a particular structural organization among variables, such as cause–effect relationships. An SEM was thus used to test the fit of data to prior causal hypotheses concerning the functioning of the transient soil seed bank system. The first, and simplest, hypothesized model included only one key factor for climate (rainfall) and one for microhabitat (incident solar radiation). This model fitted the data well [$P(\chi^2) > 0.647$; d.f. = 13; CFI = 0.99], with all paths being significant (Fig. 3A). A more complex theoretical structure, including additional factors, also fitted the data well [$P(\chi^2) > 0.325$; d.f. = 11; CFI = 0.987], but was not retained since other paths from maximum temperature to seed water content, and from drought stress index (maximum number of consecutive days without rainfall) to seed water content, were not significantly different from zero (Supplementary Data Fig. S1). Similarly, no significant effect was detected for soil texture or litter biomass (data not shown). Structural equation modelling therefore confirmed that seed survival and seed water content were under relatively simple controls when approached at the seasonal time scale. Indeed, using the 2012–2013 data set, 46% of the variance of seed water content (i.e. $r^2 = 0.46$) was explained by rainfall, incident radiation and seed coat ratio, while 50% of the variation in seed survival was explained by seed water

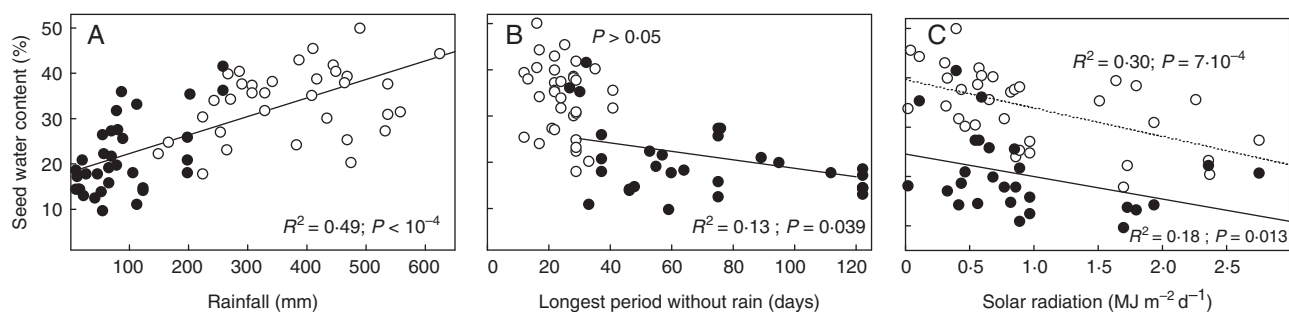


Fig. 1. Correlations between the seed water content of *Quercus ilex* seeds collected in 33 woodlands in the South of France in spring 2012 (filled circles) and in spring 2013 (open circles), and winter climatic parameters used to describe the intensity and length of drought events: rainfall (A) and number of days without rain (B). Descriptors of microhabitat including direct solar radiation ($\text{MJ m}^{-2} \text{d}^{-1}$) are also presented (C). R and P are Pearson's linear correlation coefficient and probability of significance, respectively.

content and seed mass (whereby seed survival increased with seed mass; Fig. 3A). The final structural model explained 50% of the variation in seed survival (estimated by germination rate) during the wet 2012–2013 winter, and 70% of the variation during the dry 2011–2012 winter (Fig. 3B). The influence of incident solar radiation on seed survival was consequently quite low in the 2011–2012 dry winter, whereas rainfall was the main limiting factor and the influence of rainfall was therefore greater in the 2011–2012 dry winter than during the wet 2012–2013 winter. Finally, the SEM supported a slight indirect causal relationship between seed mass and seed survival through subtle changes in the seed:seed coat ratio (Fig. 3A). The negative relationship between seed water content and pericarp:seed ratio suggests a trade-off between seed mass and pericarp mass, small seeds being copiously coated. However, the direct effect of seed mass was limited, and did not reach significance in the dry 2011–2012 winter season (Fig. 3B).

Kinetics of dehydration under controlled conditions

Visualized by the decrease in fresh mass, the dehydration kinetics of both acorns and naked seeds fitted a simple exponential decay function (Supplementary Data Fig. S2A). Fresh Holm oak acorns equilibrated relatively quickly with environmental moisture under these adverse conditions (i.e. low relative humidity and warm temperatures) which are relatively frequently observed in March–April in Mediterranean regions. The time required to reach the critical water content (WC_{50} values of approx. 30%, i.e. the seed water content at which half of the initial viability is lost; Joët et al., 2013) was 2.5 and 3.5 d for naked seeds at 43 and 62% RH, respectively, while pericarp-protected seeds reached this critical value after 11 and 14 d, at 43 and 62% RH, respectively (Fig. S2B). These results highlight the fact that the pericarp is relatively water permeable and does not provide efficient long-term protection against desiccation.

Seed water content and survival over the course of the season

The experimental site was characterized by a mild and rainy winter during the first monitoring period, from 1 December 2012 to 15 April 2013 (Fig. 4C, I). The rainfall pattern was relatively regular, with a maximum of only six consecutive days

without rain, and rain fell on half the 136 monitored days. Moreover, the rainfall record was exceptionally high (with 365 mm in March), including five rainfall events with >40 mm rainfall per day. As a result, the relative water content (RWC) of the superficial soil layer remained high (Fig. 4K), oscillating between 50 and 100% of the soil retention capacity. It is worth noting that even small rainfall events (5–10 mm) had noticeable impacts on the superficial soil water content, maintaining favourable humidity conditions in the vicinity of the soil seed bank. In parallel, the vapour pressure deficit (VPD) of the atmosphere remained low but stable throughout the season (Fig. 4E), as did the PET, a synthetic variable which reflects the energy available to evaporate water by including temperature, solar radiation, wind and VPD (Fig. 4G). These winter climate conditions were highly favourable for the transient soil seed bank located in the forest understorey. Indeed, seed water content remained relatively stable, oscillating between 36.5 and 43.2%. The seed survival remained unchanged for most of the monitoring period (survival rate >95%), followed by only a 10% drop during the last 2 weeks, which were the warmest (Fig. 4M). Substantial *in situ* germination was observed. The onset of *in situ* germination coincided with a major rainfall event at the end of January, while the maximal germination rate was observed after the major rainfall events in March (asterisks in Fig. 4O). The final germination reached 50%. Much lower seed survival and germination were observed in the soil seed bank located in the open microhabitat (exposed to the sun) than in the forest understorey. There, despite the favourable climatic conditions, germination was only half that observed in shady conditions (25% final germination) while survival was critically affected and decreased to around 30% (data not shown, Supplementary Data Table S1).

The second monitoring period, from 1 December 2013 to 15 April 2014, was characterized by far less rainfall than the first monitoring period (Fig. 4J). March was particularly dry (5.4 mm), with up to 17 consecutive days without rain. This resulted in low soil RWC that ranged from 24.8 to 56.7% of the soil retention capacity (Fig. 4L). The driest warmest periods were associated with high values of VPD and PET, which peaked in April (Fig. 4F, H). Consequently, seed water content gradually decreased and fell below WC_{50} values (approx. 30%; Joët et al., 2013; Fig. 4N). These climatic conditions were extremely detrimental for seed survival, which was zero in the sunny open microhabitat (Supplementary Data Table S1) and

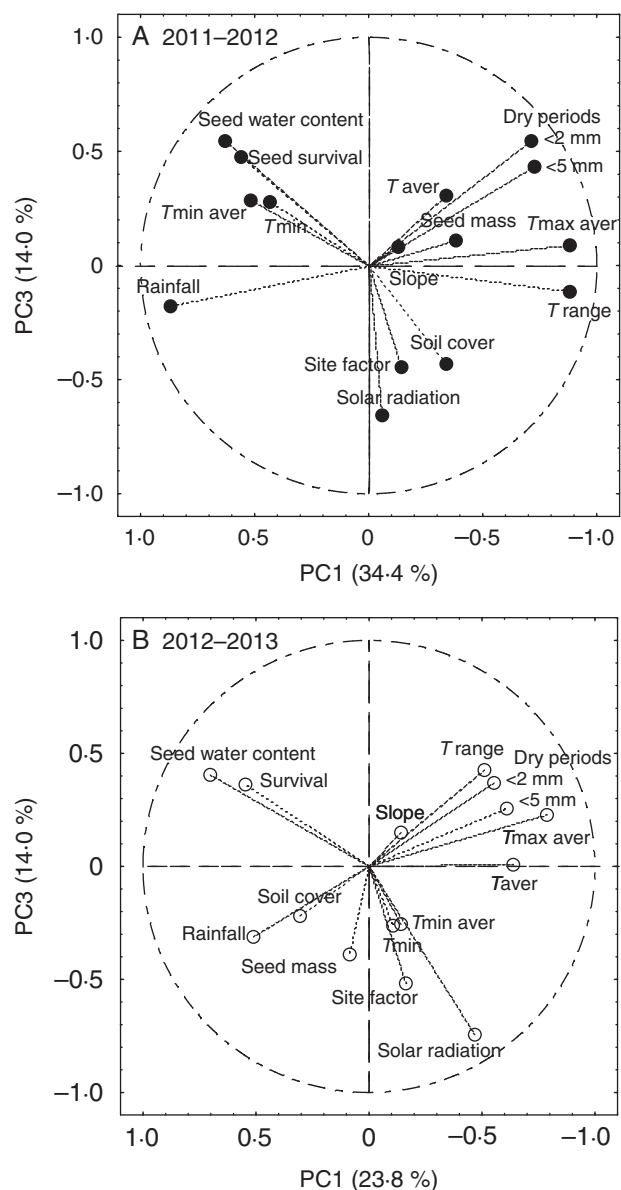


Fig. 2. Correlation circle of all seed, climatic and microhabitat variables using the first and third component (PC1 \times PC3) resulting from the principal component analysis (PCA) of data collected in 33 locations distributed throughout the French distribution area of *Quercus ilex* in 2012 (A) and 2013 (B). Dry periods, number of consecutive days without significant rainfall (daily rainfall not more than 2 and 5 mm); site factor, the fraction of total radiation above the canopy which penetrates below the canopy; soil cover, litter biomass (g m^{-2}); solar radiation, estimated incident radiation on the ground ($\text{MJ m}^{-2} \text{d}^{-1}$); Taver, average daily temperatures; Tmax aver, average maximum temperatures; Tmin aver, average minimum temperatures; Tmin, absolute minimum temperature; T range, average daily temperature range.

was as low as 15% in the forest understorey in the final stages (Fig. 4P). These dry conditions also prevented the seeds from germinating in either microhabitat.

Seed water dynamics and effects of climate variables

The regular rainfall events during the first monitoring period (winter 2012–2013), and the subsequent refilling to levels close

to maximum soil water retention capacity, made it possible for us to compare changes (in amplitudes and in dynamics) in water content in the different compartments of the experimental system: the superficial soil layer in the close vicinity of the soil seed bank (Fig. 4K), in the pericarp and in the seed itself (Fig. 4M). Over this exceptionally rainy winter period, the coefficient of variation in soil water content (13.8%) was higher than that in the pericarp (8.5%) and much higher than that in the seed itself (4.1%). These variations in the amplitude of water content suggest that water exchanged between the seed and the atmosphere is significantly buffered by the pericarp barrier compared with water exchange rates of the superficial soil. However, the three rainfall events with >40 mm rainfall per day in mid-December, mid-January and at the beginning of March had a major impact not only on soil RWC and on pericarp water content, but also on the seed water content (arrows in Fig. 4I, K, M). This result suggests relatively rapid water exchanges between the seed and its environment, with significant water uptake in the first few days after a rainfall event. The second monitoring period (winter 2013–2014) was exceptionally dry and enabled us to identify the dynamics of soil and seed water losses (Fig. 4L, N). The driest, warmest episodes (high PET values, arrows in Fig. 4H) were associated with rapid marked decreases in soil RWC as well as seed water content (arrows in Fig. 4L, N), with a decrease of $>1\% \text{d}^{-1}$ in seed water content (approx. 3.5% RWC), linked with decreases in seed viability levels (arrows in Fig. 4P). However, as mentioned above, the coefficient of variation in soil water content (23.1%) was higher than that observed for pericarp (9%) and seed (10.4%), confirming that water losses from the seed, which was partially protected by the pericarp, were slower than from the superficial soil layer.

The marked climatic variations between the two consecutive winter seasons provided suitable conditions to identify the main environmental drivers of seed desiccation using bivariate analysis and linear regression between seed water content and climate factors (Table 1; see Supplementary Data Table S4 for correlations computed for each annual data set). First, it is worth noting that the pericarp and seed water content were clearly correlated with soil water content. Among climate variables, maximum temperature, total solar radiation, VPD and PET had the most highly significant ($P < 0.001$) negative correlations with both pericarp and seed water content (Table 1; Table S4), suggesting that they are directly (environmental drivers) or indirectly (covariables associated with the environmental drivers) involved in seed water loss. These correlations were observed in acorns placed on the ground in both microhabitats, but Pearson coefficients were significantly higher in sunny open microhabitats than in the understorey. To better understand water exchanges, seed water balance (i.e. the net water gain or loss between two consecutive sampling campaigns) was also calculated and correlations with climatic data analysed (Table 1). Among climatic factors, only VPD and PET were also correlated at a highly significant level ($P < 0.001$) with the seed water balance, suggesting a simple nearly straight-line relationship between these environmental drivers and the rate of seed water evaporation (Fig. 5). Seed water content appears to be a balance between water uptake after a rainfall event and water loss driven by evaporative demand during dry periods. Indeed, an integrative view was obtained using the water

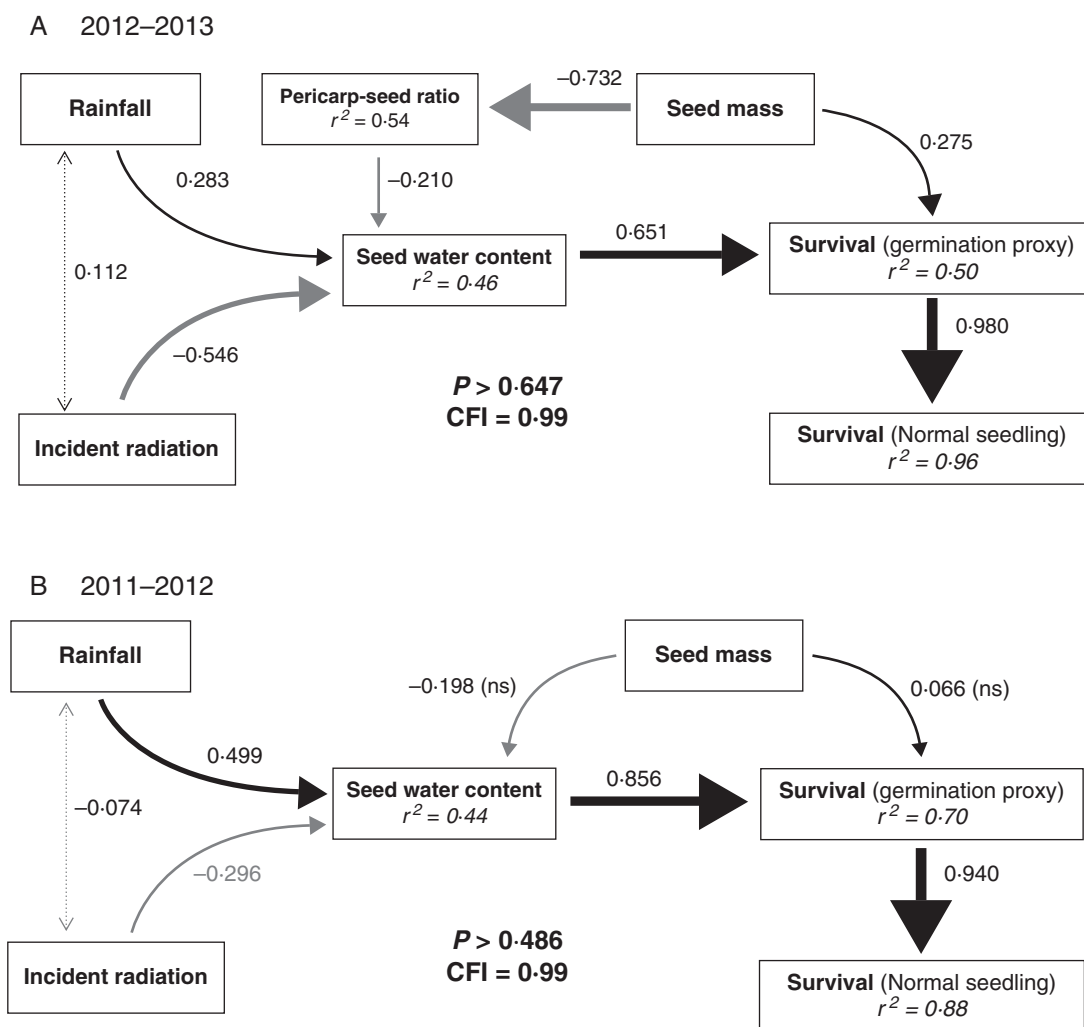


FIG. 3. Structural equation models of the influence of the selected explanatory variables on seed survival in transient soil banks. The best and simplest model derived from data acquired in 2012–2013 (A). $P(\chi^2) > 0.647$; d.f. = 13; comparative fit index (CFI) = 0.99. The part of the variance explained by the model (r^2) is given under the variable name. Path coefficients between variables are standardized partial regression coefficients. Line widths are proportional to the standardized path coefficients. All paths shown are significant. Positive and negative effects are represented by black and grey arrows, respectively. Direct paths are shown with straight single-headed arrows, whereas correlations are shown with dotted double-headed lines. Structural equation models for seed survival in transient soil banks derived from data acquired in 2011–2012 (B). $P(\chi^2) > 0.486$; d.f. = 15; CFI = 0.99.

balance (difference between precipitation and PET) (Fig. 5). Seed water content decreased progressively when the evaporative demand was greater than rainfall (negative water balance). Conversely, when the water balance was positive, seed water content stabilized at around 40%, a fully hydrated state (Fig. 5). Similar trends were observed using seed net water gain or loss (Fig. 5).

DISCUSSION

In consecutive years with contrasted precipitation in winter, the time-course monitoring of seed water status in natural conditions enabled the identification of key environmental drivers of seed dehydration. Seed water losses were satisfactorily described using the integrative variable PET, which represents the environmental demand for evaporation. Seed water status is

therefore under relatively simple controls, and can be represented by the balance between water uptake after rainfall and water loss during dry periods. Monitoring the seed water status during the winter season also enabled us to identify the critical periods for seed survival. PET, the proximal cue for seed water status, was most influenced by solar radiation and high temperatures, which both reach the maximum in spring. Indeed, during drought spells of similar length, the intensity of stress experienced by Holm oak acorns was higher in March–April than earlier during the winter period. Our data therefore highlighted the March–April period as a critical time window for seed survival, since regular significant rainfall was required to prevent seed dehydration and to trigger *in situ* germination. It is worth mentioning that, during this period, only a few warm sunny days sufficed to cause water loss at rates that are critical for seed survival. The highest rate of water loss observed in natural conditions (a decrease of 1% d⁻¹ in seed water content) is close to

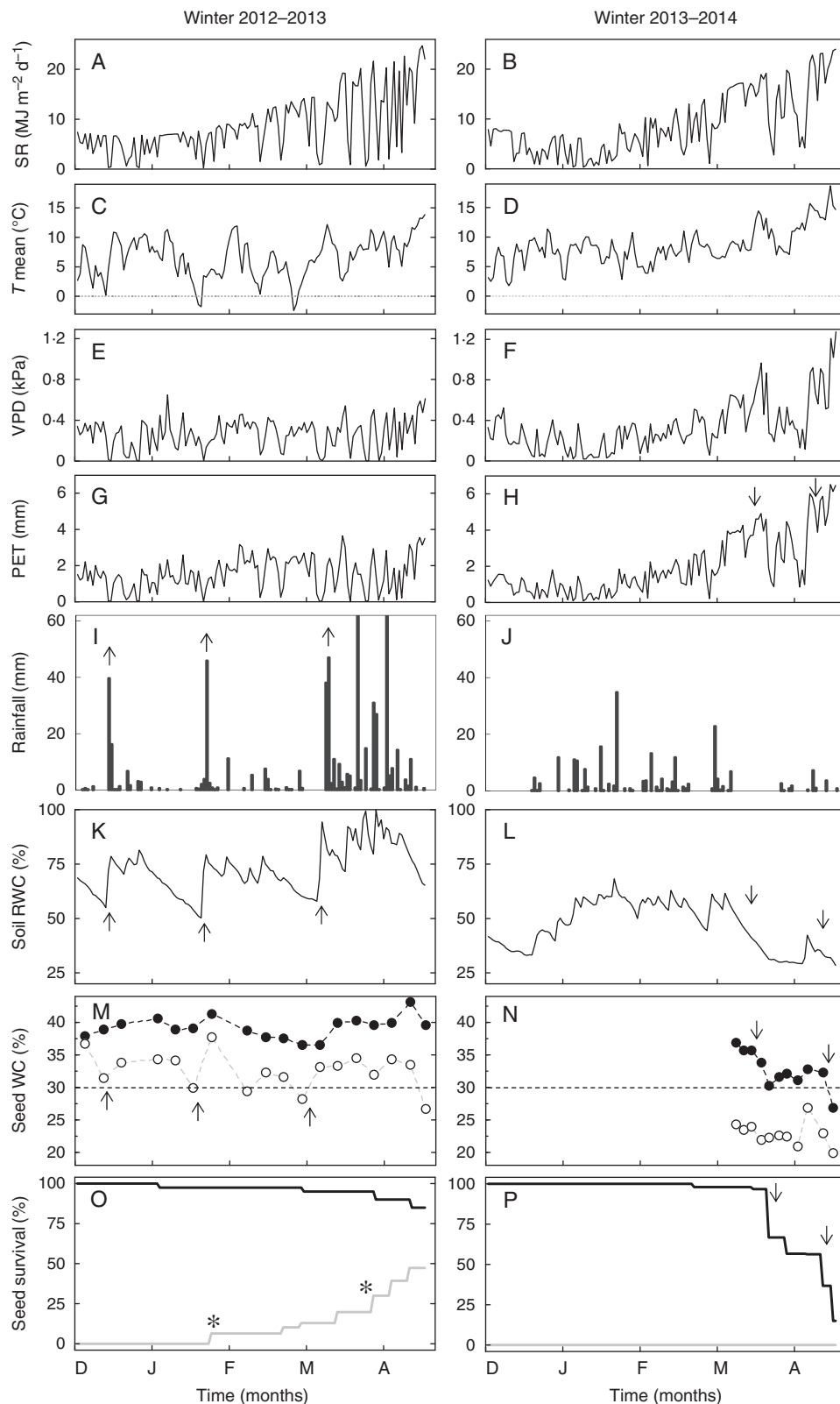


FIG. 4. Seasonal time course of seed water content, seed survival and *in situ* germination in relation to the climatic variables at the instrumented site of Puéchabon during winter 2012–2013 and 2013–2014. The *x*-axis represents months, from December to mid-April. The illustrative climatic variables are the average daily solar radiations (A, B), temperatures (C, D), VPD (vapour pressure deficit; E, F), PET (potential evapotranspiration; G, H), rainfall (I, J) and superficial soil water content (K, L). (M, N) Pericarp and seed water content (open and filled circles, respectively). (O, P) Seed survival and *in situ* seed germination (black and grey lines, respectively). Major rainfall events are illustrated by arrows pointing up and intense drought periods by arrows pointing down. Rainfall events associated with the onset of *in situ* germination are illustrated by asterisks.

TABLE 1. Correlation matrix between pericarp water content, seed water content and seed water balance, and climatic factors for acorns monitored on a weekly basis at the Puéchabon site during the two consecutive winter seasons 2012–2013 and 2013–2014 (n = 30)

	Forest understorey			Sunny conditions		
	Pericarp WC	Seed WC	Seed water balance	Pericarp WC	Seed WC	Seed water balance
T (°C)	<u>-0.67</u>	<u>-0.59</u>	-0.43	<u>-0.66</u>	<u>-0.68</u>	-0.38
Tmin (°C)	-0.32	-0.30	-0.10	-0.25	-0.33	-0.00
Tmax (°C)	<u>-0.79</u>	<u>-0.67</u>	<u>-0.52</u>	<u>-0.81</u>	<u>-0.77</u>	<u>-0.52</u>
T soil (°C)	<u>-0.74</u>	<u>-0.71</u>	-0.44	<u>-0.70</u>	<u>-0.78</u>	-0.38
RH (%)	<u>0.49</u>	0.42	<u>0.52</u>	<u>0.54</u>	0.44	<u>0.58</u>
RH min (%)	<u>0.65</u>	<u>0.52</u>	<u>0.58</u>	<u>0.73</u>	<u>0.59</u>	<u>0.67</u>
RH max (%)	0.21	0.23	0.33	0.14	0.15	0.37
SR (MJ m ⁻²)	<u>-0.77</u>	<u>-0.62</u>	-0.55	<u>-0.90</u>	<u>-0.80</u>	<u>-0.63</u>
Wind (m s ⁻¹)	<u>0.59</u>	<u>0.48</u>	0.20	<u>0.59</u>	<u>0.53</u>	0.21
VPD	<u>-0.77</u>	<u>-0.68</u>	<u>-0.62</u>	<u>-0.82</u>	<u>-0.78</u>	<u>-0.68</u>
Rain (mm week ⁻¹)	<u>0.51</u>	0.46	<u>0.50</u>	0.38	0.32	0.36
Soil RWC (%)	<u>0.88</u>	<u>0.88</u>	0.37	<u>0.72</u>	<u>0.76</u>	0.30
PET (mm d ⁻¹)	<u>-0.86</u>	<u>-0.80</u>	<u>-0.59</u>	<u>-0.90</u>	<u>-0.88</u>	<u>-0.66</u>
Water balance (rainfall – PET)	<u>0.57</u>	<u>0.51</u>	<u>0.56</u>	0.45	0.40	0.44
Water balance (negative values; n = 22)	0.51	0.41	<u>0.69</u>	<u>0.66</u>	<u>0.64</u>	<u>0.84</u>

Seed water balance is the net water gain or loss between two consecutive sampling campaigns.

R = Pearson's linear correlation coefficients. R-values in bold are P-values < 0.01, and underlined R-values are P-values < 0.001.

PET, potential evapotranspiration; RH, relative humidity; RWC, relative water content; SR, solar radiation; T, temperatures; VPD, vapour pressure deficit; WC, water content.

and even higher than maximal rates observed in fresh mature fruits under experimental equilibration drying conditions at 60% RH, 25°C (Supplementary Data Fig. S2). Such differences suggest that the pericarp barrier becomes more permeable to water exchanges over the course of the winter season due to deterioration, as already suggested in other oak acorns (Xia *et al.*, 2012). Finally, time-course monitoring of seed water status in two different locations, i.e. the forest understorey and open sun-exposed conditions, further demonstrated the influence of microhabitat conditions on seed water status, the rate of water loss being significantly higher in open sunny conditions. Conversely, one may hypothesize a lower rate of water loss for locally dispersed seeds that have been cached in the litter or buried in the soil by rodents. However, our study clearly established a highly significant correlation ($r^2 = 0.77$; $P < 10^{-4}$) between the water content of the superficial soil layer (0–5 cm) and that of seeds on the ground. Therefore, the water loss dynamics of cached seeds should be driven by the same climate proxies (i.e. PET and water balance) as unburied ones.

The seed sampling campaign conducted at the end of winter throughout the French distribution range of Holm oak enabled the fine characterization of explanatory factors for seed water status and survival at the microsite level. Several microsite descriptors including soil texture and litter biomass were found to have little influence on seed water content, suggesting a rather limited effect of seed caching in the litter on the water status and fate of Holm oak seeds. In contrast, the openness of the canopy (site factor) and, consequently, the direct incident radiation on the ground were found to be of utmost importance. Direct incident radiation explained most of the seed water content variance associated with microsite characteristics. Seed water status and survival in natural conditions was explained through very few ecological correlates, the most important ones being rainfall and incident solar radiation. These are major

contributors to the water balance, the proximal cue that best explained variations in seed water content during time-course monitoring of the seeds. The relative contributions of climatic and microsite factors were subject to strong interannual variations depending on the winter climate scenario concerned. The harsh climatic conditions in the 2011–2012 winter, with exceptional drought spells, resulted in high seed mortality throughout the French area of distribution of the species. Under these conditions, climate explained most of the between-site variation in seed survival, and microhabitat features explained most of the residual part. Indeed, seed survival was mostly associated with a few sites with highly favourable microhabitat characteristics including low incident radiation. On the other hand, under the more favourable climate conditions in the 2012–2013 winter, seed mortality was associated with harsh microhabitat conditions. Our findings on Holm oak transient soil seed banks corroborate the results of an increasing number of studies which established the crucial importance of the microhabitat for Holm oak seed germination, seedling survival and growth (Gómez *et al.*, 2004; Monnier *et al.*, 2012; Perez-Ramos *et al.*, 2012, 2013), as well as of biotic interactions through facilitation (Cuesta *et al.*, 2010; Muhamed *et al.*, 2013).

This study also highlighted the role of seed mass in promoting seed survival up to the end of the winter season. Seed mass has long been recognized as a key plant functional trait which defines a species' ecological strategy (Westoby, 1998), affects seedling survival in particular (Moles and Westoby, 2004) and even influences species distribution (Morin and Chuine, 2006). Our results confirmed the role of seed mass in seedling survival in Holm oak. However, our results also showed that seed mass is less important than water content. This suggests that common functional traits alone cannot always explain fine physiological processes at the intraspecific level, but need to be completed by other eco-physiologically appropriate measurements. In that

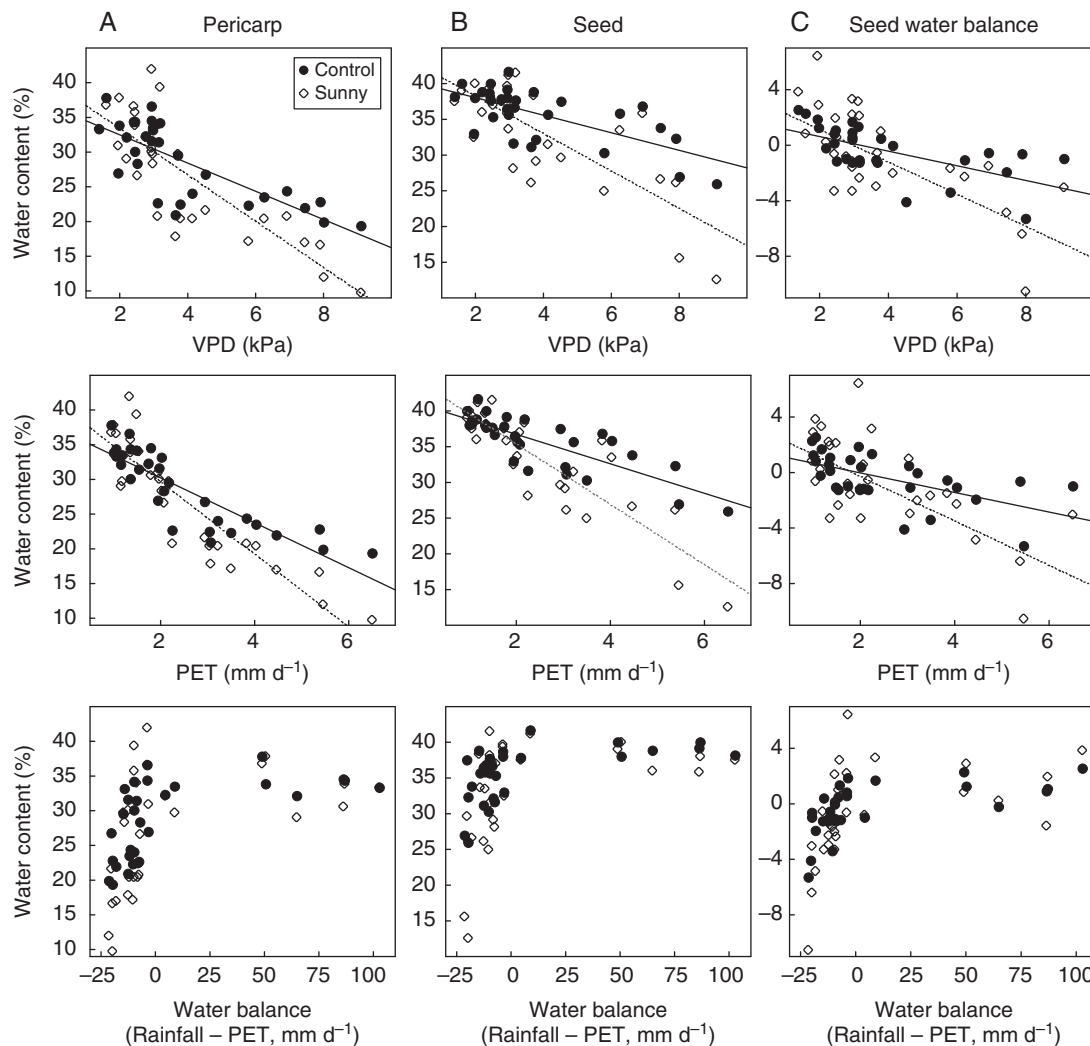


FIG. 5. Effects of the integrative synthetic climatic variables such as air vapour pressure deficit (VPD), potential evapotranspiration (PET) and water balance (rain – PET) on pericarp water content (A), seed water content (B) and seed net water gain or loss (C). The control treatment (forest understorey conditions) is represented with filled circles and the solid line, and results for the sunny open microhabitat are represented by open diamonds and the dotted line. Regression lines are shown ($P < 0.001$).

sense, the seed:seed coat ratio and the degree of sensitivity to desiccation are interesting proxies (and are complementary to seed mass) for non-orthodox seed survival (e.g. Daws *et al.*, 2006; Hill *et al.*, 2012).

For long-lived species such as Holm oak, a low regeneration rate by seeding could be balanced by long-term maintenance of established plants and vegetative reproduction (García and Zamora, 2003). However, a long-standing increase in the frequency of prolonged drought spells in winter and spring and a rise in temperatures are likely to compromise the regeneration of temperate non-orthodox seed species in certain areas. The sensitivity of plants to environmental changes when they are in their early developmental stages makes regeneration the most critical stage for survival in a Mediterranean-type community (Lloret *et al.*, 2004; Perez-Ramos *et al.*, 2013). However, the seed and seedling stages are currently rarely taken into account in species distribution models [both correlative (e.g. Thomas *et al.*, 2004) and process-based models (e.g. Morin *et al.*, 2007)] used to predict shifts in species ranges in response to

climate change. There is thus an urgent need to estimate the role of seed desiccation sensitivity in the processes which determine the distribution of individual tree species, i.e. to define the combination of climate regimes and microhabitat conditions which exceed the environmental tolerance of Holm oak seeds. The present study is the first step towards this goal, as it advances our understanding of the climatic drivers of seed survival. Moisture availability and drought intensity have already been shown to have a major influence on the distribution of plant species and plant community structure in various biomes (Silvertown *et al.*, 1999; Engelbrecht *et al.*, 2007). Whether the sensitivity of seeds to desiccation is a key functional trait, that also plays a role in shaping species distribution, remains to be determined. This role should be better studied and integrated since, in a similar vein, the minimum temperature required for seed germination has recently been proposed as a direct proxy for predicting plant species distribution ranges along a temperature gradient (Rosbakh and Poschlod, 2015).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. **Table S1**: seed mass and water content, and percentages of germination and of normal seedling development in different batches of *Quercus ilex* acorns. **Table S2**: effect of rainfall, number of days without rain and direct solar radiation, as well as their interannual variation, on the water content of *Quercus ilex* seeds. **Table S3**: correlation of the first four principal components resulting from principal component analysis of the whole data set with individual seed traits, climatic and microtopographic variables. **Table S4**: correlation matrix between pericarp, seed water content, seed water balance and climatic factors for acorns monitored on a weekly basis in the Puéchabon site during the two consecutive winter seasons 2011–2012 and 2012–2013. **Figure S1**: structural equation models of the influence of the selected explanatory variables on seed survival in transient soil banks. **Figure S2**: time to seed desiccation in controlled conditions.

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