



# The effect of simulated damage by weevils on *Quercus ilex* subsp. *Ballota* acorns germination, seedling growth and tolerance to experimentally induced drought



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## ABSTRACT

The main aim of this study is to assess the effect of moderate to low level of cotyledon damage (simulated weevils infection) on holm-oak seedling growth and physiological performance under conditions of soil water stress, a recurrent constraint in Mediterranean and other seasonally dry environment. Three levels of artificial damage were applied to the acorns (no damage NoD, low damage LD, and medium damage MD), and the germination, emergence and early seedling growth under controlled conditions were studied during ca. 1.5 months. On the other hand drought effect on seedling growth, leaf gas-exchange, PSII efficiency, photosynthetic pigments and electron transport energy fluxes was analyzed in a set of older seedlings (i.e., 6.5 months old) that also derived from treated acorns and were exposed to two irrigation treatments (well water WW and water stress WS) for 1.5 months. The results showed that LD and MD acorns germinated earlier than NoD ones but final seedling emergence was lowest in the MD treatment. Cotyledons exhibited increased level of necrotic tissue as physical damage (drilling) increased while seedling biomass and size-related traits tended to decrease. Under WS conditions seedlings derived from LD and MD exhibited higher decrease in above and belowground biomass, as well as on net photosynthetic rate ( $A_n$ ), stomatal conductance ( $g_s$ ), intercellular  $CO_2$  concentration ( $C_i$ ) and PSII efficiency than those derived from NoD. These differences were much less conspicuous under WW treatment where all traits reached higher values. In view of the interactive effect of drought and acorn damage it is concluded that under natural conditions acorn infection by weevils and other insects may represent a highest limitation to holm-oak seedling recruitment than previously considered. In addition this limitation may become more important in the future whether warming and drought increase.

## 1. Introduction

Seed predation is a generalized phenomenon in both temperate and tropical forests (Vallejo-Marín et al., 2006). In the Mediterranean oak forests the guild of acorn predators is very wide, consuming the seeds before (pre-dispersal predation) or after (post-dispersal predation) acorn dropping (Crawley and Long, 1995; Siscart et al., 1999; Espelta et al., 2009a). Pre-dispersal acorn predation is mostly carried out by weevils (*Curculio* spp.) and moths (*Cidya* spp.) (Crow, 1988; Siscart et al., 1999) and may affect a significant proportion of the acorn crop (Bonal et al., 2007; Espelta et al., 2009b). Different studies have estimated that the fruits attacked by these insects may represent a 16–18% of the acorn crop in Mediterranean Holm-oak (*Quercus ilex*) forests

(Pulido and Díaz, 2003; Leiva and Fernández-Alés, 2005) while it varied from 17 to 68% in *Q. suber* forests in southern Portugal (Branco et al., 2002) and, from 5 to 40% in *Q. robur* forests in southern Sweden (Andersson, 1992). Weevils and moths generally predate the acorn cotyledons partially. The females lay their eggs inside developing fruits during summer and the larvae feed inside the acorn for several weeks until the seed drop in early autumn. Then, larvae emerge and bury themselves into the soil (Leiva and Fernández-Alés, 2005; Johnson et al., 2009).

Different studies have demonstrated that weevils infected acorns may lose their ability to germinate (Andersson, 1992; Fukumoto and Kajimura, 2000; Leiva and Fernández-Alés, 2005; Sone et al., 2016). However, weevils attack is not always lethal especially whether vital

Abbreviations:  $A_n$ , net photosynthetic rate; ETR, electron transport rate;  $g_s$ , stomatal conductance;  $C_i$ , intercellular  $CO_2$  concentration;  $C_a$ , atmospheric  $CO_2$  concentration surrounding the leaf; NPQ, non-photochemical quenching; PPF, photosynthetic photon flux density;  $iWUE$ , intrinsic water use efficiency;  $F_0$ , minimal fluorescence;  $F_m$ , maximum fluorescence;  $F_v/F_m$ , maximum quantum efficiency of PSII photochemistry;  $\Phi_{PSII}$ , quantum efficiency of PSII; DES, De-Epoxidation State

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embryo parts are not affected what is frequent in big-sized species and when the larvae develop in the distal end of the acorn (Weckerly et al., 1989; Hou et al., 2010). In spite of that partial cotyledon loss may indirectly affect the embryo by a decrease in delivery of reserve and nutrients from cotyledons (Hou et al., 2010; Sone et al., 2016). Moreover, exit holes left by larvae in the acorn coats make the cotyledons accessible to pathogens, fungi and bacteria, once the acorn falls into the soil what is another source of risk for acorn viability under natural conditions (Andersson, 1992; Johnson et al., 2009). In opposition to the aforementioned negative effects of natural or artificial partial cotyledon loss, positive effects have also been detected: the speed up of acorn germination (Branco et al., 2002; Yi and Zhang, 2008) and, the synchronization of germination process, both of interest when seedlings are grown in containers for forestry activities or other purposes (Giertych and Suszka, 2011; Liu et al., 2015).

By the other hand partial cotyledon loss by natural predation or artificial scission usually affect seedling net growth thus decreasing biomass, shoot height, root length, stem diameter, etc. (Branco et al., 2002; Yi and Zhang, 2008). In fact, an extraction of non-vital parts of cotyledons mimics somehow a decrease in seed size (Mancilla-Leyton et al., 2013), a trait that is broadly recognized to have big effect on absolute and relative seedling growth (e.g., Jurado and Westoby, 1992; Bonfill, 1998; Milberg and Lamont, 2008).

Under the Mediterranean and other seasonally dry climate, plant species have to withstand severe water stress, high temperatures and high irradiance (Flexas et al., 2014). Despite this fact, most of the aforementioned studies on the effect of acorn damage on acorn germination and seedling development have been carried out under controlled, not-limiting soil conditions. Thus we hypothesized that the availability of water in soil could alter the impact of acorn infection by insects on oak seedling recruitment and development. Thus this study was designed and carried out in order to fill this gap of knowledge and our aims were: (I) to assess the effect of simulated acorn infection by weevils on germination, emergence and early seedling growth in Holm-oak, a big-sized species that occupy a broad area (ca 3 million ha) in the Mediterranean Basin. (II) To analyze whether seedlings derived from different levels of acorn damage show differential growth and physiological responses to a decrease in soil water content as surrogate of drought conditions.

## 2. Material and methods

### 2.1. Seed source and experimental damage treatments

In December 2015 acorns from the same holm-oak (*Quercus ilex* subsp. *ballota*) mother tree were collected in a representative Mediterranean savannah-like forest ecosystem in SW Spain (37°14'33"N; 6°19'37"W, Villamanrique de la Condesa Municipality. See Leiva and Díaz-Maqueda, 2016, for detailed site description). The acorns were selected for apparent soundness (float method, Gribko and Jones, 1995) and similar size (10–24 mm in diameter, 25–32 mm in length) and were stored in a cold chamber (5 °C). After one month the seeds were moved to the laboratory and were immersed in a 10% sodium hypochlorite solution for 10 min to ensure surface sterility and were rinsed in distilled water. Then the seeds were distributed in three lots to apply different damage treatments.

Experimental damage treatments consisted in simulating weevil hurt by drilling the acorns with an electric 2-mm in diameter drill. Drills were done in the distal part of the acorn to avoid embryo damage. Three levels of damage were applied: No damage (intact seeds, NoD hereafter), low damage (seeds drilled once exhibiting an entry and an exit hole, LD hereafter), and medium damage (seeds drilled twice exhibiting two drills with the same characteristic as in low damage, MD hereafter). To avoid potential interference of fresh weight loss in our results due to applied damage treatments, the seeds included in the LD and MD were slightly larger than the Control seeds (NoD) based on

visual selection. And to assess potential changes in seed fresh weight the acorns were weighted in a microbalance immediately before and after drilling and compared with NoD acorns. Fresh weight after drilling did not significantly vary among different damage levels, showing values of  $3.5 \pm 0.1$ ,  $3.6 \pm 0.1$  and  $3.5 \pm 0.1$  g in NoD, LD and MD, respectively.

### 2.2. Acorn germination and early seedlings development experiment

In January 2016 seeds derived from the three damage levels were placed into 20 × 15 cm plastic trays filled with pieces of filter paper saturated with distilled-water in the bottom (14 acorns/tray × 3 damage treatments × 4 replicates per pre-treatment; n = 168) in order to analyze damage treatment effect in germination capacity. The trays were placed in a germination chamber (12–25 °C, 12 h photoperiod, ASL Aparatos Científicos M-92004, Madrid, Spain) and were daily inspected and seed germination considered after radicle appearance. Two germination characteristics were determined: kinetic of germination after 5 and 9 days and final germination percentage. Furthermore damage treatment effect on seed hydration capacity was obtained by analyze weight gain due to hydration after 24 h from the beginning of the experiment, thus a random subsample of acorns from each specific tray treatment (12 seeds per treatment) was selected and each seed was individually reweighted.

The acorns included in the germination experiment were later individually sown (buried 5 cm) in black polypropylene bags (30 cm in depth, 3000 cm<sup>3</sup> total volume) filled with commercial peat and perlite and were moved to the greenhouse (University of Seville, Agrarian Research Service) in order to know damage effect on seedlings emergency capacity. Bags were firstly irrigated at field capacity and bi-weekly irrigated during the experimental period. In addition all bags were redistributed at random within the greenhouse-table to avoid border effect. During this time greenhouse temperature evolved from 14 to 22 °C (mean daily temperature) and photoperiod 10.5 to 11.5 h. On February 25th (i.e., 35 days after sowing) a subset of 39 seedlings (13 seedlings per damage level) were harvested in order to analyze the effect of acorn damage on early seedling development. Thus each specific bag was longitudinally cut and the whole root system extracted. Roots, shoots and cotyledons attached to the stem base were separated. Coatless cotyledons were cut longitudinally into quarters or smaller pieces and were carefully prospected into the binoculars for potential necrotic tissue. Cotyledons were rated into 6 categories according the fraction that exhibited signs of necrosis (0 = no necrosis, 6 ≥ 97.5% necrosis; Method from Swiecki et al., 1991). The middle point of each class was used to calculate averaged necrosis proportion within each treatment. In addition, the length and diameter (at the base) of the taproot and the shoot height and diameter (at collar) of the seedlings were measured on fresh material using a rule and a digital caliper. Finally all biomass fractions were oven dried (80 °C during 48 h) and weighted. Another set of seedling for each damage treatment was kept under previous described growth conditions for ca 5 months until the onset of the irrigation stress experiment, in order to gain a more thorough understanding of damage treatment effect in plant performance in relation with soil water availability.

### 2.3. Plant drought tolerance response experiment

On May 23th, after the last irrigation at field capacity, seedlings of each specific damage treatment were divided in two irrigation regimens (well water, WW and water stress, WS hereafter) of eight individuals per group and arranged in a randomized plot (3 damage treatment × two irrigation level × 8 replicates, n = 48). The WW conditions consisted in adding weekly 500 ml water per pot. The WS conditions consisted in withholding completely the irrigation until the end of the experiment (i.e., 1.5 months).

Soil water content (SWC) was monitored through the experimental

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