

Is *Leptoglossus occidentalis* entirely responsible for the high damage observed on cones and seeds of *Pinus pinea*? Results from a fertirrigation trial in Portugal

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ABSTRACT

The uncertainty surrounding the part played by an invasive North American seed bug, *Leptoglossus occidentalis*, in the observed decrease in seed production of the Mediterranean pine, *Pinus pinea*, is a limiting factor for its management. Furthermore, the possibility of increasing cone production through irrigation and fertilization regimes is gaining interest among landowners, but its effects on insect pests are still unknown.

Using bagged branches in the field, we aimed at evaluating the impact of *L. occidentalis* on young and mature cones of stone pine trees submitted to fertirrigation (FR) compared to trees with no treatment (C). For two consecutive years (2015 and 2016), we carried out both an insect-exclusion trial and an insect-bagged trial. In the first one, polyester-covered branches, excluding insects, were compared to branches exposed to natural insect infestation. In the second trial, bags included either two adult bugs or 3–4 third-instar nymphs, placed there for one month during mid-summer, or were kept without insects. Branch protection resulted in a significant decrease in the mortality of second-year conelets which dropped to 6% compared to 30% in exposed branches. Seed damage also decreased from 60% on exposed branches to 10% on protected ones. The presence of nymphs in the bags resulted in a mortality of second-year conelets 63% higher than in exclusion bags, whereas adults had no effect. In contrast, bags with adults presented the highest seed damage. The partial damage of the kernel can be a signature of *L. occidentalis* feeding since such damage was not observed in exclusion bags. Additionally, another type of seed damage, showing a wholly shrunk and dry embryo without remaining endosperm, and a reduction in the number of extractable seeds may also be attributed to this seed bug. Overall, seed damage per mature cone reached up to 12% in bags with two adult bugs enclosed for a month, i.e., twice the seed damage in protected cones. Overall, FR trees were more susceptible to both conelet mortality and seed damage. In the particular case of *L. occidentalis*, FR regime influenced the consumption positively by the nymphs but not by adults.

1. Introduction

Since its introduction in Europe in 1999, the invasive insect pest Western Conifer Seed Bug, *Leptoglossus occidentalis* Heidemann, (Hemiptera: Coreidae), has been gaining increasing attention by the Mediterranean Basin countries. This interest is mainly due to the putative impact of *L. occidentalis* on the edible, and of high commercial value seed of the Stone Pine, *Pinus pinea* L. with current retail prices

that can exceed 100 euros/Kg (Mutke et al., 2014). The tree species, *P. pinea*, commonly known as Stone pine or Umbrella pine, is native to the Mediterranean region (Costa et al., 2008). Cones of this species have a 3-yr reproductive development cycle, which begins with the burst of the female strobili (conelet) in the spring of the first year, with wind pollination occurring a few weeks after that. Strobili fertilization, however, takes place only in the spring/summer of the third and last year of cone maturation when the nutritious edible seeds are thus formed

Abbreviations: FR, fertirrigation; C, control

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(Valdivieso et al., 2017). The total area of *P. pinea* represents now nearly 1 million hectares in the Mediterranean Basin resulting from new plantations during the last decades (Mutke & Calama, 2016). Great investigation efforts have been made to disentangle the drivers of cone production. Irrigation and fertilization, for example, are becoming a common practice in young plantations aiming to increase the number and weight of the harvested cones (Calama et al., 2007; Montero et al., 2004).

Several countries recently report a significant decrease in cone production per hectare and substantial cone yield losses (percentage of commercial kernels per kg of the fresh cone) (Mutke et al., 2005, 2014; Mutke & Calama, 2016). Biotic factors like the invasive *L. occidentalis* have been pointed out as most plausible cause (Bracalini et al., 2013; Mutke & Calama, 2016; Parlak 2017; Roversi, 2009) although climate change is also often referred (Calama et al., 2011; Mutke et al., 2005). This bug can damage the seeds of *P. pinea* last-year cones (Bracalini et al., 2015; Calama et al., 2017; Elvira-Recueno et al., 2016; Farinha et al., 2018). First-year and second-year conelets although without developed seeds are also susceptible to be fed upon by *L. occidentalis* (Mutke et al., 2014; Parlak, 2017).

Assigning damage to this bug is not as easy as no visible external signs of feeding can be detected in cones where the bug pierced. Antibody (Lait et al., 2001) and DNA techniques (Bracalini et al., 2015) were able to track seeds fed by *L. occidentalis* although limited by the time that has elapsed since the feeding and also by the high costs associated which makes these techniques not expeditious.

The slow maturation process of the cones means that the same cone may be exposed to the insects for two to three years, resulting in an accumulation of damage which is difficult to track. This fact, along with the asymptomatic damage, makes the task of assessing the impact of the bug extremely difficult. One alternative to characterize and quantify the damage by *L. occidentalis* is force-feeding mature seeds under controlled laboratory conditions (Bates et al., 2000; Farinha et al., 2018; Koerber, 1963; Lesieur et al., 2014). However, extrapolating these results to the natural environment where live conelets and cones are the primary food source becomes a questionable exercise. Another relevant setback is the absence of specific traps able to capture the insect and estimate its density in the field. This methodological limitation makes it difficult to correlate the damage found on cones with the natural insect density and distribution. In fact, up to the present, it has never been demonstrated that the current increase in damage to *P. pinea* cones and seeds reported by several Mediterranean countries is directly attributed to an increase in the population of *L. occidentalis*.

In an attempt to overcome these limitations, and to obtain a field assessment of bug damage, several studies used branches bearing cones covered by bags, into which insects were then either added (Bates et al., 2001, 2002a, 2002b; Connelly & Schowalter, 1991; Lesieur et al., 2014; Schowalter & Sexton, 1990; Strong et al., 2001; Strong, 2006;) or excluded (Blatt & Borden, 1996; Elvira-Recueno et al., 2016; Strong, 2016). Variables such as the host species, the period and duration of the experiments as well as the density of the insect inside the bags vary significantly between studies. *Pinus contorta* Douglas and *Pseudotsuga menziesii* (Mirb.) Franco are the most referenced hosts which comes from most publications being from the North American continent where these species are of relevant importance, especially in seed orchards (Bates et al., 2001, 2002a, 2002b; Blatt & Borden, 1996; Lesieur et al., 2014; Schowalter & Sexton, 1990; Strong et al., 2001; Strong, 2006, 2016). Regrettably, the reported damages caused by *L. occidentalis* on these two hosts vary greatly depending on the experimental and local conditions (Bates et al., 2001, 2002a, 2002b; Blatt & Borden, 1996; Lesieur et al., 2014; Schowalter & Sexton, 1990; Strong et al., 2001; Strong, 2006, 2016;) making it difficult to compare impact estimates or even extrapolate damages between different hosts.

In Europe, publications on *L. occidentalis* are mostly from the Mediterranean Basin countries and are essentially concerning *P. pinea* (Bracalini et al., 2015; Calama et al., 2017; Farinha et al., 2018; Mutke

& Calama, 2016). Lesieur et al. (2014) however, provided a typology of seed bug damage on many European conifers, but the study did not include *P. pinea*. Moreover, accurate quantification of seed bug damage on *P. pinea* is not yet available (Kenis et al., 2017).

There is also a lack of knowledge on how tree growing conditions will influence tree susceptibility to the bug. In fact, site productivity is one of the critical factors influencing the colonization and establishment of insect pests in a specific area (Liebhold & Tobin, 2008). It is known, for example, that changes in water and nutrient availability affect, not only the plant productivity but also its susceptibility to herbivores (Ayres, 1993; Moon & Stiling, 2000; Netherer & Schopf, 2010; Rusch et al., 2010). For many invertebrate herbivores it has been demonstrated they could discriminate, for the same host species, plants of high nutritional quality from those of low quality (Awmack & Leather, 2002; Firempong & Zalucki, 1989; Kareiva, 1982; Santiago Lastra et al., 2006).

The pine, *P. pinea*, is known to be quite resistant to drought. Nonetheless, trees growing in arid sites tend to show a lower leaf area index and lower cone production than trees growing with irrigation (Bono & Aletà, 2013; Calama et al., 2007, 2011; Correia et al., 2017; Loewe-Muñoz et al., 2016; Mutke et al., 2005).

Concerning fertilization, nitrogen (N) is the most widely used fertilizer (Chen et al., 2010). The increase in N availability can enhance host attractiveness to an herbivore-insect by altering herbivore-induced plant volatiles (Holopainen & Gershenzon, 2010; Saha et al., 2012) or by modifying the plant structure (Chen et al., 2010).

The expansion of *P. pinea* orchards for cone production using irrigation and fertilization (Calama et al., 2007; Loewe et al., 2017; Mutke, 2017) are excellent opportunities to study plant-insect interactions related to the tree vulnerability status.

Thus, in this work we had two main objectives: (i) characterization and assessment of *L. occidentalis* impact on cone and seeds of *P. pinea* under natural conditions, using branches covered with mesh insect-exclusion bags and (ii) analyze the effect of tree fertirrigation in *L. occidentalis* feeding activity.

This study allows us to isolate the type of damage caused by *L. occidentalis* feeding on mature seeds of *P. pinea* during part of its active period, which is essential to the future development of impact quantification methodologies related with this bug.

2. Methods

2.1. Study site and management protocols

The study site is located in Coruche, in center Portugal, in a ten years old pure *P. pinea* stand grafted at the age of four. The climate is Mediterranean with a cold and moist winter and dry and hot summers, with annual precipitation of 641 mm and a mean annual temperature of 15.1 °C. Within site, an area of 3 ha was selected and eight randomized plots, corresponding to 2 treatments with four repetitions each, were installed during the summer of 2014. The two plot treatments were Control (C) and Fertirrigation (FR). Each plot comprehends 15–21 trees spaced by 3 × 8 m. FR trees were irrigated with diluted fertilizer (40 kg N * ha⁻¹) every day from March until June. The trees were also irrigated for 3.5 h during October of both years. Besides the natural precipitation occurring each year, this irrigation regime corresponded to an additional water input to the system of approximately 400 mm ha⁻¹ year⁻¹.

Climatic data were obtained from a local meteorological station on site. The year of 2015 was a dry year, totaling 315 mm of precipitation from January to December and with an unusually dry spring. In 2016, total precipitation was above the annual average (529 mm). Regarding air temperature, August 2016 was particularly warm with 17 days with maximum temperatures above 35 °C contrasting with only four days observed in 2015. No rain events were recorded during August 2016 and in 2015 only rained 3 mm. August corresponds to the insect trial establishment in the field.

2.2. Tree physiological traits

Trees from the C and FR treatments were characterized by their ecophysiological traits regarding leaf and crown structure during the two years of the experiment. For that, we selected harvested trees from both treatments for component biomass separation and weighing. All the aboveground components were then separated and weight according to the methodology described in [Correia et al. \(2010\)](#). The total tree needle area, used as an indicator of the primary photosynthetic production, was calculated using the tree needle biomass and an average of the specific needle area by age cohort. For the needles' morphological characterization, we randomly selected one branch from each node. A sample of current year needles (2015 and 2016) was collected in each branch, scanned in a flatbed scanner and analyzed with Winseedle software (Regent Instruments Inc.) for the needles' length and projected area calculations. The needles were then oven dried and, together with the needles' projected area, used for specific needle area calculations.

2.3. Cone and seed production

All mature cones (third-year cones) in the sampled branches of the field trials were harvested in the winter of the sampled year (November/December), dried in the laboratory (at 45 °C until opening) and weighted. We then evaluated the average weight of cones and number of seeds per cone. The average number of cones per tree in each year and plot treatment was estimated using all trees from the site (n = 246).

2.4. Field trials

The experiment occurred during two consecutive years: 2015 and 2016. Ten trees from each treatment, C, and FR, bearing at least six branches with second and third-year cones simultaneously, were randomly selected each year. Two trials were conducted on the same trees: (i) an exclusion trial and (ii) a bagged insect trial.

Branches bearing second year conelets and third-year cones were selected for both trials. Bagged branches were enclosed from the base of the fork to the tip within a mesh of 0.5 mm, about 50–90 cm long depending on the branch length, leaving room for it to grow. All bags had



Fig. 1. Damage categories of *P. pinea* kernels. Bar corresponds to 1 cm.

an inner wire structure to keep the cones away from the bag walls thus preventing insects from outside from feeding on the cones, and also to provide more space for the insects in the case of the bagged insect trial. In both trials, bags were set up in April, before the emergence of the overwintering adult bugs, and removed in December of the same year, when the third-year cones were collected.

All second-year conelets and third-year cones present on the selected branches were counted in spring and their mortality assessed by December of the same year. Conelets of the second-year were considered dead when they appeared dried or if they broke easily when touched. Last year cones were classified as dead when more than 1/3 of the scales were damaged thereby making them non-marketable. Conelets of the first-year were not followed because by the time the bags were set up they were not formed yet.

Mature cones (third-year cones) were opened after drying and the number of seeds counted. Seed health status was then evaluated using X-ray (Philips Practix 300) and by a manual opening. Seed kernels were counted and separated into the four following categories: (i) apparently sound (kernel intact); (ii) kernel partially damaged/with a different colour/serous appearance (named as “damage type I”); (iii) completely shrunken and dry embryo with no endosperm (named as “damage type II”); and, (iv) empty shell without embryo (named as “damage type III”). This classification follows [Calama et al. \(2017\)](#), but we changed the description of type I damage by type II, and vice versa, because it seemed to be more evident to consider an increasing level of damage ([Fig. 1](#)).

2.4.1. Exclusion trial

Branches enclosed by bags were set up in April, in two consecutive years, 2015 and 2016 ([Fig. 2](#)). Simultaneously 2–3 branches on the

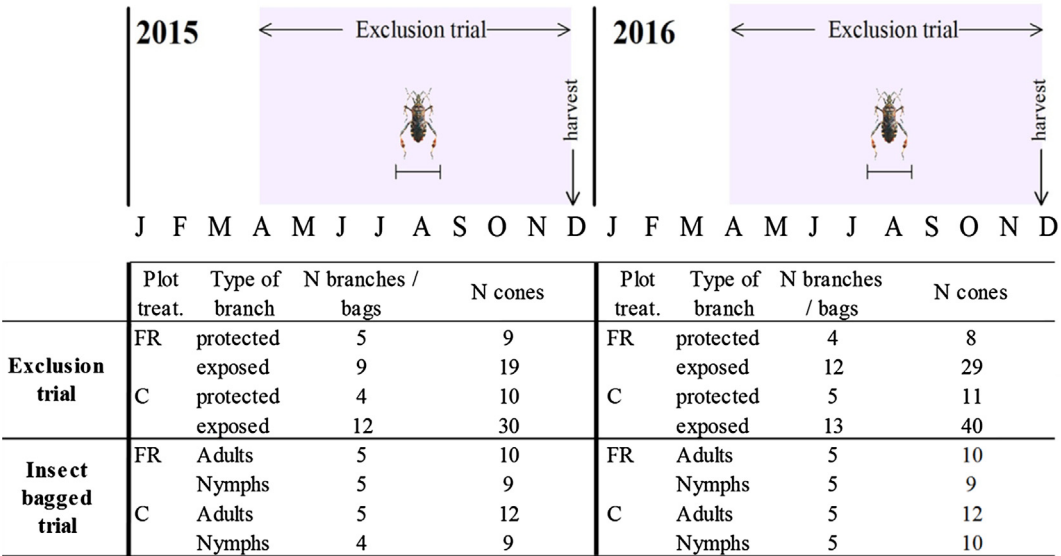


Fig. 2. Field trials. The top diagram concerns field trials methodology: shade corresponds to the time during which the sampled branches were covered with bags (Exclusion trial), and the insect represents the time when the adults and nymphs of *L. occidentalis* were placed inside sampled bags (Bagged insect trial). The bottom table shows the number of branches and cones sampled by treatment and year.

same trees were marked with a rubber ring and left exposed throughout the year. Chosen branches were preferably from the same and most recent fork of the tree to minimize variations of abiotic factors such as solar exposition, wind or humidity. The exclusion trial tested the effect of the protection of the cones of *P. pinea* from April until December in comparison with exposed ones.

Two bags were removed from the analysis, one from each treatment, FR, and C, respectively, because they were found unsealed at the time of removal. Also, three exposed cones from FR were removed from the analysis for presenting symptoms of *Diploidia pinea*.

2.4.2. Bagged insect trial

To determine the number of insects to use inside bags we conducted a pilot study in 2014 aiming to estimate the density of *L. occidentalis* in the experimental site. Monthly observations were carried out spending, at least, 3 min in each tree ($n = 20$), observing all cones and tree crown. This pilot study showed a maximum average density of 1.6 bugs/tree during July–August. Taking into account that visual monitoring techniques may underestimate real insect density (Richardson, 2013) we rounded the ratio obtained by excess, and we use it by branch rather than by tree. Therefore, in each bagged branch with insects, either two adults, one male and one female, or 3–4 nymphs, were added to simulate a high natural impact.

Three types of bags were randomly assigned to three cone-bearing branches: Adults (bag with one couple), Nymphs (bag with 3–4 nymphs of third-instar) and Exclusion bag (a branch with a bag but without insects).

Insects were placed inside the bags at the beginning of August and removed after four weeks (Fig. 2). All bugs used in one year were from the F1 generation of wild adults captured in May /June of that same year and kept in the laboratory under natural temperature and photoperiod. Bags were monitored twice a month. Any dead or missing individuals were replaced whenever possible with other F1 individuals from the lab colony.

The bagged insect trial tested the effect of the bugs on *P. pinea* for one month (August). From March till December, these branches remained covered by the exclusion bags. Data were compared with exclusion bags with no bugs.

One bag with nymphs from a C tree was removed from the analysis because it was found with a hole at the time of removal.

2.5. Statistical analysis

To examine the differences among the ecophysiological traits, we used a two-way ANOVA: factors were tree treatment (2 levels, FR and C) and year (2 levels, 2015 and 2016). Tested variables were the needle's maximum length and the total leaf area following Normal Distribution. Post-hoc pairwise comparisons were performed using the Student-Newman-Keuls Method.

For the differences on the cone and seed production between the two plot treatments, C and FR, and years, the t-student test or the Mann-Whitney test was used whenever normality of the data was not accomplished. Tested variables were the number of cones per tree, the cone weight and the number of seeds per cone.

For both the exclusion trial and the bagged insect trial, statistical analysis was performed using generalized linear models (GLM) with a Binomial distribution or Negative Binomial distribution, according to the variable distribution and a link model binary probit. The following variables were analyzed: (i) mortality of second-year conelets; (ii) proportion of seeds from mature cones with total damage and proportion of seeds with damage type I and II, and (iii) number of extractable seeds per cone. Type III damage were rare and analyzed with Mann-Whitney test. Tested factors were the plot treatment and type of bag and its interaction. A separate analysis was done to test the factor year. The Wald Chi-Square ($W \text{ Chi}^2$) was used to test the effect of the factors in the model. Post-hoc pairwise comparisons were performed with the

Least Significant Difference (LSD) test between all pairs of groups.

All statistical analyses were performed using SPSS, version 24.0 (IBM Corp., Armonk, New York) with a statistical significance level of 0.05.

3. Results

3.1. Tree physiological traits

Trees from FR plots presented longer needles and a higher total leaf area in both years. FR trees showed better vegetative growth conditions when compared with C trees (Table 1). Trees from control group also exhibited better vegetative physiological parameters in 2016 than in 2015 (Table 1).

3.2. Cone and seed production

A total of 237 last year cones were harvested in the two years with all seeds inside (20,849) being checked and categorized. In 2015, the overall average cone fresh weight was lower than in 2016, and no significant differences were found between C and FR trees in this first sampled year. By contrast, in 2016 cones from C trees were significantly heavier than cones from FR trees (Table 2). The number of seeds per cone did not differ between the two plot treatments in each year, but differences were found between the years. The heavier cones in 2016 lead to a significantly higher number of seeds per cone in that year (Table 2).

In respect to the number of cones per tree, it did not differ significantly between treatments or years although in 2016 a higher number of cones was observed in FR trees (Table 2).

3.3. Field trials

3.3.1. Exclusion trial

3.3.1.1. Cone mortality. In both years of experiment, the branches exposed to natural infestation presented mortality of second-year conelets almost six times higher than the protected ones ($W \text{ Chi}^2 = 51.246$; $df = 1$, $p < 0.001$) (Fig. 3A). Furthermore, mortality was higher in 2015 compared to 2016, regardless of plot treatment ($W \text{ Chi}^2 = 13.730$; $df = 3$, $p = 0.003$). Overall mortality of young conelets

Table 1

Leaf and canopy characteristics in the Control (C) and Fertirrigated plots (FR) by treatment and year (average \pm standard error). Differences between average values for each tree characteristic are indicated by different letters.

Year	Tree treatment	Needles maximum length (cm)	Total leaf area (m ²)
2015	C	10.4 \pm 0.6 a	31.6 \pm 2.9 a
	FR	15.6 \pm 0.6 b	55.2 \pm 5.5b
2016	C	14.2 \pm 0.6 b	40.3 \pm 3.7 ab
	FR	15.5 \pm 0.4 b	57.3 \pm 5.7b

Table 2

Characterization of the site trees and cones regarding production (average \pm standard error) in control (C) and in fertirrigated (FR) plots. Differences between averages in each column are indicated by different letters.

Year	Tree treatment	Fresh weight per cone (g)	Number of cones per tree	Number of seeds per cone
2015	C	249.4 \pm 11.1 a	3.6 \pm 2.7	68.1 \pm 4.3 a
	FR	254.48 \pm 13.2 a	2.6 \pm 1.2	76.5 \pm 3.7 a
2016	C	361.2 \pm 11.7 b	6.2 \pm 3.2	99.9 \pm 4.1 b
	FR	310.0 \pm 8.7 c	12.4 \pm 6.1	95.9 \pm 6.1 b

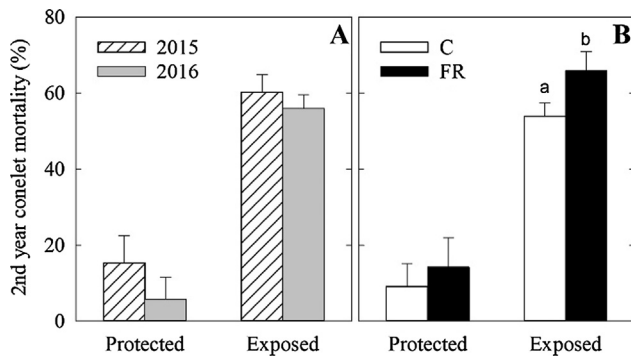


Fig. 3. Mortality (\pm SE) of second-year conelets per year (A) and per plot treatment (B) in the protected and exposed branches of the exclusion trial. C - control trees; FR - fertirrigated trees. Whenever there are significant differences ($P < 0.05$) between years (A) or between treatments (B) they are indicated by different letters.

was higher in FR trees than in C ($W \text{ Chi}^2 = 4.531$; $df = 1$, $p = 0.033$). However, in pairwise comparisons, this difference was only significant in the exposed branches ($p = 0.047$) (Fig. 3B).

Mortality of mature cones (third-year cones) happened only in 2015 on exposed branches, still corresponding uniquely to 4.3% ($n = 6$) of all exposed cones collected ($n = 137$). Accordingly, the year and the type of bag influenced the mortality of last year cones ($W \text{ Chi}^2 = 36.736$, $df = 1$, $p < 0.001$ and $W \text{ Chi}^2 = 5.089$, $df = 1$, $p = 0.024$, respectively).

3.3.1.2. Seed damage. Exposed cones presented on average ca. 30% damaged seeds while on protected ones it was only 6% (Fig. 4). Neither the protected nor the exposed cones showed differences in the proportion of damage per cone between years (Fig. 1 – supplementary material). In respect to the plot treatment, FR trees always had a higher proportion of damaged seeds than C trees whether they were exposed or protected ($p < 0.001$) (Fig. 1 – supplementary material).

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foreco.2018.07.014>.

Most of the damage found on cones was of type II with 20.6% and 4.6% seeds damaged in exposed and protected cones, respectively (Fig. 4). A statistically significantly higher proportion of damage in FR trees was found when compared to C ($p < 0.001$) (Fig. 4).

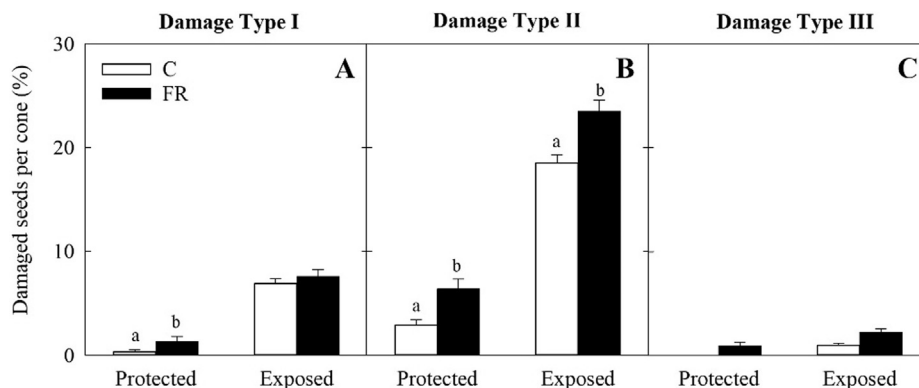


Fig. 4. Mean percentage (\pm SE) seeds per cone per damage category and type of bag in each of the plot treatment in the exclusion trial. C - control trees; FR - fertirrigated. Whenever there are significant differences ($P < 0.05$) between treatments, they are indicated by different letters.

Nearly all the remaining damaged seeds per cone were of type I (7.1% and 0.8% of total seeds, respectively for exposed and protected cones). In respect to the plot treatment, the proportion of type I damaged seeds was similar on FR and C cones from exposed cones ($p = 0.396$) but differed in the protected ones ($p = 0.039$).

Type III damaged seeds, also known as empty seeds, were residual, corresponding to less than 2% of the total number of seeds per cone and did not differ between treatments, FR and C trees, or between protected and exposed cones (Fig. 4).

In respect to the average number of extractable seeds per cone, significant differences were noted between exposed (80.4 ± 1.3) and protected cones (88.8 ± 2.3) ($p = 0.005$). When comparing the FR and C cones, although the former presented slightly lower mean values of extractable seeds, these were not significant, regardless of the type of branch.

3.3.2. Bagged insect trial

3.3.2.1. Cone mortality. Overall mortality of second-year conelets was in average 18.4% in the bags with adults, 31.6% in the bags with nymphs and 11.7% in the exclusion bags, with only the last two being significantly different between them ($p < 0.001$). However, we observed high variability in the conelet mortality (Fig. 5).

None of the three types of bags presented different second-year conelet mortality between the two sampled years. Likewise, no significant differences were noted between FR and C cones in each type of bag (Fig. 5).

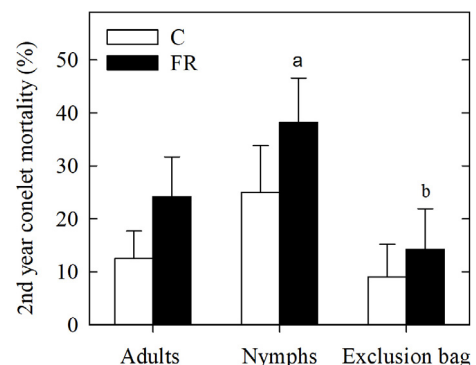


Fig. 5. Average mortality (\pm SE) of second-year conelets in the two years, per plot treatment in the bagged insect trial. C - control trees; FR - fertirrigated trees.

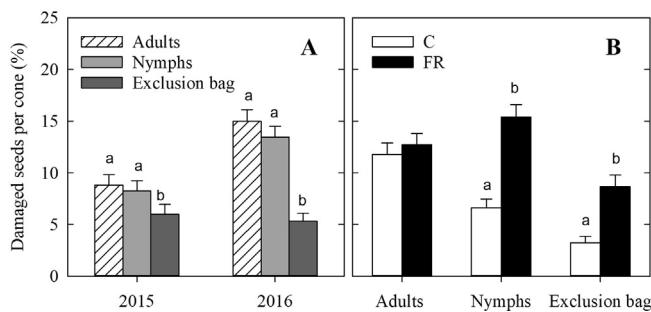


Fig. 6. Mean percentage (\pm SE) of seeds damaged per cone in each year (A) and plot treatment (B) in the bagged insect trial. C - control trees; FR - fertirrigated trees. Whenever there are significant differences ($P < 0.05$) between types of branches in each sampled year or between treatments, they are indicated by different letters.

We did not observe mortality of third-year cones, except two cones which died with *Diplodia pinea* and were removed from the analysis.

The mean percentage of seeds damaged per cone was highest in the bags with adults (11.9%), followed by those containing nymphs (10.8%) and finally by exclusion bags with no bugs (5.7%). These proportions were significantly different between the exclusion bags and both types of bags with insects ($p < 0.001$) but not between these last two ones ($p = 0.374$).

Total damage per cone in the bags with adults and with nymphs was significantly higher in 2016 than in 2015 ($p < 0.001$). However, total damage in the exclusion bags was similar in both years (Fig. 6).

Regarding the two plot treatments, the damage was higher on FR compared to C on bags with nymphs ($p < 0.001$) and exclusion bags ($p < 0.001$), but not on bags with adults ($p = 0.551$).

No differences were found on the number of extractable seeds per cone from the three types of bags (Adults: 89.0 ± 2.1 ; Nymphs: 88.5 ± 2.1 ; Exclusion bag: 88.8 ± 2.3). Regarding the plot treatment, none of the types of bags had different values between FR and C.

Analyzing damage seed by category, the overall average proportion of type I damaged seeds per cone was significantly different between the three types of bags ($p < 0.001$). Bags with adults had the highest proportion (6.9%), followed by bags with nymphs (4.1%) and lastly by the exclusion bags with only 0.7%. On the contrary, the average proportion of type II damaged seeds was similar between cones from all types of bags with exclusion bags presenting damage as well (Adults – 5.2%; nymphs – 5.7%; exclusion bags – 4.3%) ($p > 0.05$). Also, the proportion of type III damaged seeds in cones was not different between the three types of bags and was always below 1.5%.

Differences between damaged seed in cones from FR and C trees were observed in bags with nymphs and in exclusion bags for both type I (nymphs' bag: $p < 0.001$; exclusion bag: $p = 0.039$) and type II (nymphs' bag: $p < 0.001$; exclusion bag: $p = 0.002$) damaged seeds by cone (Fig. 7). FR cones always presented greater damage (Fig. 7). The average percentage of seeds with damage type III was not different between the two plot treatments in the bags with insects. In the exclusion bags, the cones from FR were the only ones showing type III damages (Fig. 7C).

4. Discussion

During the two years of the experiment, we found differences in the ecophysiological performance of the trees from the FR and C plots, with fertirrigated trees showing denser canopies and a higher cone production average. However, with only two years of data and having in mind that cone production shows a very high interannual fluctuation (Calama et al., 2008, 2011; Mutke et al., 2005), the conclusions regarding cone production should be carefully analyzed. What has already been demonstrated in other studies on *P. pinea* is that water can

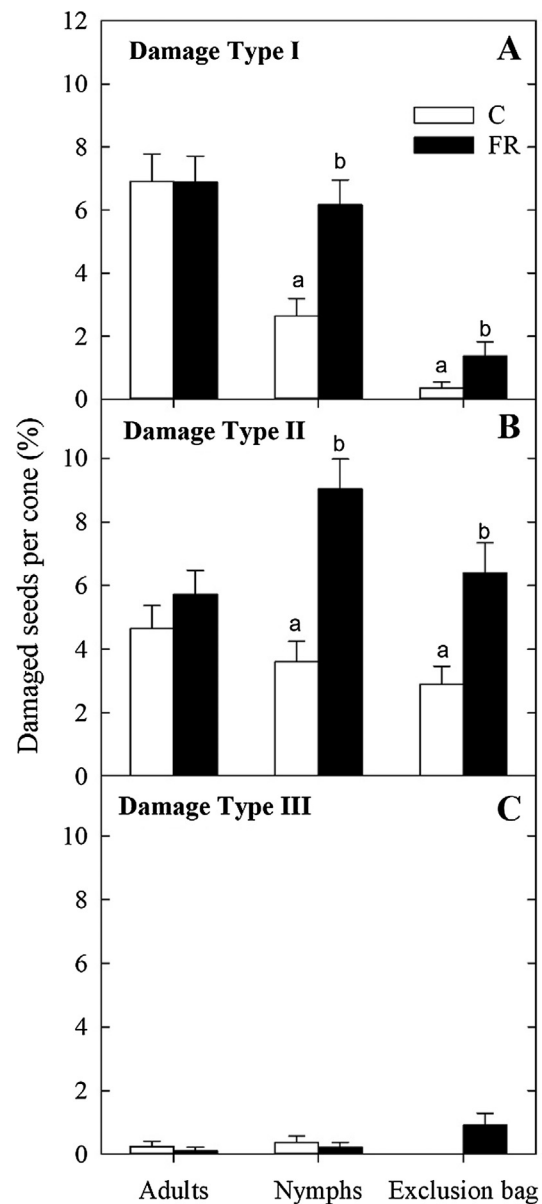


Fig. 7. Percentage (\pm SE) of type I (A), type II (B), and type III (C) seeds damaged per cone in each type of bag and plot treatment in the bagged insect trial. C - control trees; FR - fertirrigated trees. Whenever there are significant differences ($P < 0.05$) between years or between treatments are indicated by different letters.

be a limiting factor for *P. pinea* growth in some arid Iberian Peninsula regions (Natalini et al., 2015) and that artificial watering can increase the induction of first-year conelets, the survival of second-year conelets, the yield in ripening cones and the area and number of leaves (Bono & Aletà, 2013; Correia et al., 2017; Loewe et al., 2017; Mutke et al., 2005, 2014). Our study confirms a high vigor in FR trees, but a lower survival of second-year conelets, apparently contradicting the results from other studies.

4.1. Second-year conelet mortality

Overall, the mortality rate of second-year conelets on site was very high, with each tree losing on average more than half of the cones (ca. 60%) between spring and the harvest of matured cones in December. Protecting branches with a bag reduced second-year conelets mortality rate by 83%, leading to the conclusion that biotic agents such as the

insects *L. occidentalis* (Hemiptera), *Dioryctria mendacella* (Lepidoptera), the fungus *Diplodia pinea* and other unknown agents are, most certainly, involved in the considerable mortality observed in young cones of *P. pinea*.

The highest mortality of young cones was recorded in 2015. The spring of 2015, with only 84 mm of rain from February to May (tree times lower than the recorded in 2016), may have compromised the trees' physiological performance. The observed decrease in total needle length expansion and overall leaf area affected the photosynthetic activity (data not shown) and therefore the available carbon resources for growth. Concomitantly, it is likely that the spring drought influenced the insect's activity, along with other biotic agents, probably making them more active in a context of exacerbated tree vulnerability.

It must be stressed that the high number of aborted conelets of *P. pinea* reported here does not come as a surprise. Several Mediterranean countries have revealed alarming data on an unusually high abortion rate of unripen conelets in the last decade in a phenomenon known as the Dry Cone Syndrome (DCS) for which the cause is not yet completely understood (Mutke et al., 2017). Although *L. occidentalis* is pointed out as one of the most plausible causes, we were not able to support this hypothesis straightforwardly in this study. More research is needed to fully understand this phenomenon. Bagged branches with bugs inside showed that both adults and nymphs of *L. occidentalis* could feed on second-year conelets, but only nymphs caused significant damage in one of the sampled years. The impact of the bagged bugs on these young cones was always much lower than on the exposed ones. We must note that the second-year conelets were exposed to the insects for only one month, whereas exposed cones were vulnerable to the seed bug for more than six months. We thus hypothesize that nymphs of *L. occidentalis* might be, in part, responsible for the mortality of conelets. These findings are following field observations where nymphs were very often spotted feeding on conelets, unlike the adults which were almost always found feeding on last year cones. The impact of adults on second-year conelets was negligible in our trials with these insects showing a preference for mature cones, at least during August, when the trial was set up. It is important to note, however, that the variability in the mortality of second-year conelets found inside the bags, including in the insect exclusion bag, was very high (around 10% in the exclusion bags) which might be due to physiological or reproductive causes which we were not able to track properly. Effectively, our data suggest that *L. occidentalis* is most certainly not the only responsible for the high mortality rate of young conelets in *P. pinea*. A multiple cause scenario combining climatic effect, tree physiological responses as well as biotic agents other than *L. occidentalis* are more plausible hypothesis according to our findings.

Other studies showed that differences in preference between young and mature cones of adults and nymphs of *L. occidentalis* are not consistent between hosts. In *Pinus contorta*, nymphs only feed on conelets if cones are not available and even then, fail to develop to the next instar (Bates et al., 2002a). In *Pinus monticola*, both nymphs and adults cause considerable damage on conelets (Bates et al., 2002a) and in *P. sylvestris* no difference in young cones mortality was found between bags with adults and with nymphs and control ones (Lesieur et al., 2014). In addition, *L. occidentalis* also has different feeding behavior according to the season (Bates et al., 2001, 2002a, 2002b; Calama et al., 2017; Connelly & Schowalter, 1991; Schowalter & Sexton, 1990; Schowalter 1994; Strong, 2006). All reveal great adaptability of *L. occidentalis* to different hosts and climates.

In opposition, mortality of all last year cones (mature cones) analyzed summing the two sampled years was negligible (less than 5%) with these cones being much less vulnerable than second-year cones.

4.2. Seed damage

This study shows nearly one-third of damaged seeds in mature cones that were exposed.

The protection of the cones inside a bag from early spring until harvest resulted in an 80% reduction (from 30% on exposed cones to 6% on protected ones) of damage per cone. Thus, suggesting that biotic agents and not physiological or environmental factors are involved in this ratio. Still, damaged seeds were also found in protected cones (5–7% of all seeds) which we attribute to both physiological causes and biotic factors affecting the cones in the previous years of the cone development when a bag did not protect it. Studies in *P. pinea* seed orchards in Central Spain where cones protected by mesh bags were compared to unprotected cones shows very similar ratios of damage per cone in exposed (36%) and protected cones (6%) (Elvira-Recuenco et al., 2016).

In addition to the higher damage in matured seeds of the exposed cones, the number of extractable seeds per cone was 10% lower in these cones when compared to the protected ones. The reduction in the total number of extractable seeds per cone has been related to *L. occidentalis* feeding of seeds not yet fully formed, of several different conifers: *Pinus monticola* (Connelly & Schowalter, 1991), *P. contorta* (Bates et al., 2002b; Strong et al., 2001, 2006), *Pseudotsuga menziesii* (Bates et al., 2000; Schowalter & Sexton, 1990) and *P. sylvestris* and *P. nigra* (Lesieur et al., 2014). Our results reveal that, most probably, the same fused seeds phenomenon happens for pine cones of *P. pinea*. Cones from the bags that had insects inside had an average of extractable seeds similar to cones that were always protected, but we only bagged insects during one month in August. Feeding by *L. occidentalis* in August, when the seed is already fully formed, does not cause fused seeds. The lower number of extractable seeds on the exposed cones presumably resulted from the feeding of the bug before the end of July, when the seed is not yet fully formed (Calama et al., 2016).

When seeds were separated by damage categories, we note that type II damage (totally shrunken embryo) was the most represented, corresponding to around 80% of all the damaged seeds per cone inside the protected bags and 70% of all the damaged seeds in exposed cones. The type II damaged seeds on protected cones suggest it can be either climatic, physiological or caused by biotic agents before the settlement of the bag, that is, in the previous years of cone development. Results from the bagged insect trial support, in part, this hypothesis since cones from this trial presented a similar overall proportion of type II damaged seeds per cone whether they came from a bag with *L. occidentalis* adults or nymphs during one month or from an exclusion bag that had no insect at all. However, in 2016 when the total damage per cone was higher than in 2015, there were some differences. Cones from bags with insects, especially with nymphs, had two times more type II damaged seeds per cone than cones from the exclusion bags indicating that this bug may also be responsible for part of the seeds with this type of damage in the tested period. We admit that if the insects were allowed to feed during a more extended period, the type II damage could have been higher. In fact, this utterly shrunken seed, classified as damage type II, was observed in laboratory forced feeding studies when one or more adults of *L. occidentalis* feed over an extended period on the same mature seed (Farinha et al., 2018). However, we still do not know precisely how the seeds of *P. pinea* that have not yet been fertilized, that is, in the years before harvest, develop after *L. occidentalis* feed on it. Studies on the impact of *L. occidentalis* on other hosts, particularly on *P. menziesii*, have shown differentiated damage accordingly to the period when the feeding occurs (Bates et al., 2001; Schowalter & Sexton, 1990).

Concerning damaged type I seeds (partially damaged kernel), the almost nil percentage observed in the protected cones, which contrasts with the consistent percentage of 7% in the exposed cones, indicates that this type of damage is due to biotic agents. The results from the bagged insect trial strongly support the hypothesis that *L. occidentalis* adults are responsible for type I damage. A study using exclusion bags hypothesized that also damage type I could be a signature of *L. occidentalis* feeding since no seeds with this damage were recorded on the cones protected from the bug (Elvira-Recuenco et al., 2016). In

practical terms, this means that we may use type I damage to assess damages caused by *L. occidentalis* among sites and years, being an accurate indicator of the seed bug activity.

Lastly, the proportion of seeds with damage type III (empty seeds) per cone was constantly negligible regardless if the cone was protected, fully exposed or exposed uniquely to *L. occidentalis* for one month. These results showed unambiguously that *L. occidentalis* or other biotic agents do not cause this type of damage. In fact, studies on other pines have shown that it is the lack of fertilization that leads to empty seeds (Owens, 2006).

4.3. Host management

From a plant protection point of view, several studies showed that insects perform nonrandom foraging, being able to distinguish plant quality in a heterogeneous patch of the host (Awmack & Leather, 2002; Firempong & Zalucki, 1989; Kareiva, 1982; Santiago Lastra et al., 2006). A higher total leaf area together with a higher average production of pine cones in the FR plots leaves little doubt for this study site to be considered heterogeneous about the quality of the host in the period that the field experiments occurred. It is expected that seed feeders like *L. occidentalis* choose the tree to colonize by the number and quality of the available cones. Nevertheless, the canopy should also be a critical factor as it serves as a refuge to weather conditions, to natural enemies (Chen et al., 2010) and is also a source of water. In fact, whereas second-year conelets protected by a bag revealed no differences in mortality between C and FR trees, exposed ones had higher mortality rates in the FR trees. This result may indicate that a preference for FR conelets or trees by the biotic agents may be occurring. However, other factors should be considered. Bagging *L. occidentalis* for one month in mid-summer revealed differences only for the nymphs in FR trees with a mortality of second-year conelets 63% higher in nymphs' bags compared to the insect exclusion bags. Mortality in the bags with adults was not significantly different from the exclusion bags whether it was FR or C trees suggesting that adults had a similar feeding rate in both types of trees.

In the matured cones, the number of extractable seeds per cone did not vary between the two plot treatments in any of the trials. However, looking at the proportion of damaged seeds per cone, we could see that the influence of the plot treatment was noteworthy. Both exposed and protected cones from FR trees had a higher damage percentage (more 22% and 67%, respectively) than cones from C trees. This result suggests that the cause for such increase should be related with the management regime although we cannot identify which particular factors or processes are involved in it.

This same pattern of higher damage on FR cones was observed in the bags with nymphs of *L. occidentalis* but not on adults. We hypothesize that for the nymphs, the cone and seed wall tissue on FR trees are more suitable for piercing and feeding, with nymphs being more demanding for nutritional and water food requirements for growth, whereas an adult would not be so sensitive to food quality at this level. However, for detailed and proven knowledge of such an effect, more experimental work is needed.

Furthermore, cones from the nymphs' bags had a much higher proportion of type II and type I seeds than the exclusion cones which indicates that nymphs were responsible for part of these damage types.

Finally, type III damaged seeds (empty seeds) appear in similar proportions in cones from C and FR, which reinforces that causes are probably physiologic and ecologic and not biotic.

5. Conclusion

The production of the edible seed of *P. pinea* is now facing a challenging reality with the rate of production falling. Researchers are struggling to understand the impacts of *L. occidentalis* and its relation to the Dry Cone Syndrome. Our study shows that type I damage is a

signature of *L. occidentalis* and therefore can be used in future studies to assess its impact on seed damage. However, seed type II damage must also be partly attributed to *L. occidentalis* feeding in mid-summer. Moreover, this bug may also have a significant impact on the number of extractable seeds per cone when feeding of seeds not yet fully formed (before late July) causing them to fuse to the seed coat. Hence, inferring the impact of this insect using just type I damage is underestimating its real impact.

The increasing market demand for this seed has been leading producers to invest in management programs using artificial watering and fertilization in an attempt to increase production. The present study is a first attempt to evaluate the impact of *L. occidentalis* on young and ripen cones in trees that are fertirrigated compared with trees growing in natural conditions. In general, fertirrigated trees were more susceptible to seed damage and cone mortality, which appear to be driven by biotic agents. It is possible that these trees are more attractive to the seed bug *L. occidentalis*, as well as to other seed and cone pests, like *D. mendacella*. In the particular case of the seed bug, nymphs demonstrate a higher feeding activity on fertirrigated cones compared to control ones, when forced to feed inside a bag.

Overall, we found that the activity of two adult insects in one month in mid-summer could contribute to as much as 6% of seed damage. Still, efficient field monitoring techniques are needed to determine an economic threshold density to *L. occidentalis* in this host. The high price of *P. pinea* seed implies that this threshold will be low, thus justifying prophylactic management.

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