UNIVERSIDAD POLITÉCNICA DE MADRID

Escuela Técnica Superior de Ingeniería de Montes, Forestal y del Medio Natural

SOIL IMPROVEMENT THROUGH ECOLOGICAL TREATMENTS (LIQUID AND SOLID) IN ORDER TO PROTECT OAKS AGAINST "INK DISEASE" (SECA) IN A SPANISH SOUTHERN DEHESA

AUTHORS:

Aida López Sánchez

Ramón Perea García-Calvo

March 2020

Index

Acknowledgements:

We thank Mariana Yuan Ribeiro, Lavinia de Lucchi, Juan de Dios, Johanna Thiede and all Erasmus students from Erasmus+KA1 movilities for their help with fieldwork and data collection. We thank David Medel, Jorge Pallarés and Adrián for their help in lab work and data collection.

1. Introduction

Mediterranean scattered oak woodlands have undergone important anthropogenic impacts, especially climate disruption, socio-economic shifts, land conversion and land-use changes since mid of 20th century (Blondel and Aronson, 1999; Underwood *et al.*, 2009). They are currently facing unprecedented threats from oak decline due to some novel and emergent diseases (Sánchez *et al.*, 2002; Branco and Ramos, 2012). Previous research has demonstrated that dominant plant species affected drastically by pests and pathogens can alter a wide range of ecosystem functions such as net primary productivity, decomposition and nutrient cycling (Waring *et al.*, 1987; Kranz, 1990; Eviner and Likens, 2008; de Sampaio e Paiva Camilo-Alves *et al.*, 2013).

The 'Ink Disease' or 'Seca' (caused by the oomycete *Phytophthora cinnamomi* a soil-borne root pathogen) damages native oaks, including the two most prominent oak species in Mediterranean scattered oak woodlands: the cork oak -*Quercus suber*- and the holm oak -*Quercus ilex*- (Moreira *et al.*, 2006; Aronson *et al.*, 2012). Complex interactions exist among drivers of Seca, such as habitat destruction, mismanagement, extreme climate events, and infestation by pests such as bark and wood borers (Branco and Ramos, 2012). The 'Seca' virulence attack is influenced by site characteristics (e.g. edaphic and physiographic factors, land-use) and tree condition (e.g. size, age and vitality); which contribute to increase the probability of i.e. *P. cinnamomi* occurrence (Moreira and Martins, 2005). Previous research has shown that prolonged droughts and water-logging periods may interact with the association of *P. cinnamomi* with oak decline in Spain and southern Portugal (Brasier, 1993; Brasier *et al.*, 1993).

Among Mediterranean scattered oak woodlands, Spanish dehesas are recognized for their ecological, socioeconomic and cultural importance and support a high rich biological diversity (Plieninger and Wilbrand, 2001; López-Sánchez, 2015). The objective of this work is to improve soil conditions through ecological treatments (liquid and solid forms) in order to protect oaks from 'Seca' disease. In doing so, we have selected a dehesa (a scattered oak woodland) which is severely affected by 'Seca'. We, then, have developed four experiments (3 in the field and 1 in the laboratory), which tested the efficacy of some ecological treatments. The final aim of these experiments was to search applied measures that invigorate trees, allowing them to resist 'Seca' virulence. Most trees affected by 'Seca' usually die after their infection

along with other drivers mentioned above (de Sampaio e Paiva Camilo-Alves *et al.*, 2013). We have categorized different vegetation layers to evaluate the efficacy of treatments. In the first experiment, we have focused on adult tree layer. In the second experiment, we have focused on the established oak regeneration. In the third experiment, we examined oak seedlings planted in the field. These seedlings come from acorns that were collected in fall 2018 from healthy trees within the dehesa. Finally, in the fourth experiment, we have focused on some lab tests where all variables are perfectly controlled under greenhouse conditions at the School of Forestry (Universidad Politécnica de Madrid).

2. Study area

The study was conducted over a year (November 2018- October 2019) in a traditionally managed dehesa ecosystem. The "Dehesa de San Francisco" (515 ha) is located in Huelva province (Spain) in the Southwest of the Iberian Peninsula (37°52' N, 6°14' W; 445 m asl; Fig. 1).

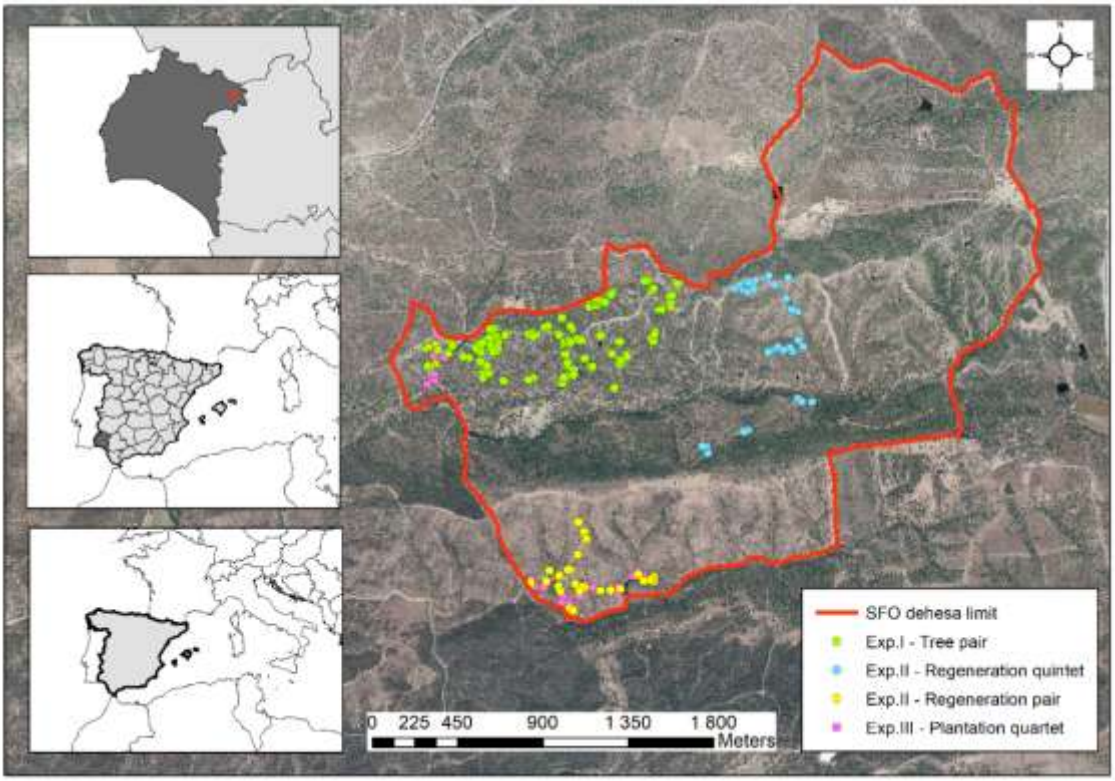


Fig. 1. Map of study area location and the four different experiment locations.

The climate is Mediterranean pluviseasonal oceanic, with hot and dry summers and precipitation concentrated in the spring and fall. Mean annual temperature is 17.2 ºC and mean annual rainfall (September-August period) is 677.1 mm (n = 21 years; weather station IQ102 "Santa Olalla de Cala" 37º54'N, 6º13'W). Annual rainfall of study year was 326.5 mm and spring rainfall (from March to May) was 106 mm.

Parent material of soil formation at the research area is schist. Due to the strongly dissected topography, some soil material has moved down the slopes and is forming colluvic material (IUSS 2014) at the footslopes. Predominantly, continuous rock (schist) starts ≤ 25 cm from the soil surface. Hence, these soils belong to the Reference Soil Group (RSG) of Leptosols (IUSS 2014).

The tree layer is co-dominated by holm oak (*Quercus ilex subsp. ballota* (Desf.) Samp.) and cork oak (*Quercus suber* L.), locally interspersed with gall oaks (*Q. faginea* subsp. *broteroi* Cout. A.Camus). Mean tree density is around 82 trees ha^{-1}.The shrub layer is low and dominated by evergreen xerophytes (e.g. *Cistus salviifolius* L., *Cistus ladanifer* L., *Lavandula stoechas* Lam., and *Genista hirsuta* Vahl). As in other dehesas systems, the herbaceous layer mainly comprises therophytic oligotrophic communities (*Tuberarietalia guttatae)* and sub-nitrophilous Mediterranean annual communities *(Thero-Brometalia)*; see Rivas-Martínez *et al.* (2001). Grasslands belonging to the *Poetea bulbosae* community are also common; they are promoted by intense and continuous livestock grazing and dominated by small perennial grasses and nutritious forbs (Rivas-Martínez *et al.*, 2001).

A traditional rotational grazing system with typical dehesa livestock species (cattle, sheep, goats, and Iberian pigs) has been re-established from 2004 onwards, and the farm has been subject to organic farming since then. The cattle breeds "Retinta", "Berrenda en rojo", "Berrenda en negro", and "Limusín" are kept year-round on the farm with stocking rates of 0.042 LU ha^{-1}. Sheep (mainly the breed "Merina") are kept with stocking rates of 0.104 LU ha^{-1}, while goats have stocking rates of 0.004 LU ha^{-1}. The pig breed is "Ibérica"; stocking rates are 0.090 LU ha^{-1}. In addition, wildlife such as red deer (*Cervus elaphus* L.), fallow deer (*Dama dama* L.) and wild boar (*Sus scrofa* L.) are present on the farm with overall densities of ca. 0.081 LU ha^{-1}.

3. Description of the products

3.1. OptiFer

OptiFer is a biological trace element fertilizer that can be liquid or solid form. For the experiments here, we have used the liquid form. It contains 6% Fe, 1.5% Mn, 1.5% Mg and the remainder is water. The trace elements are extracted from the bark of trees with *Tsuga canadensis* as their main source. Their application is recommended for 3 years once every year) and in areas with stunted plants every 2 years. For plants with deep roots, OptiFer can also be sprayed in the morning (when the leaf openings are open) from below onto the leaves. Their effect is in the revival of the soil organisms in order to restore or produce the needed symbiosis with plants through roots. The luminescent bacteria which hardly survive in arid soils, are supplied with Fe. These bacteria make possible (by using a plant-compatible form) to transfer iron, manganese and magnesium into the hair roots of the trees. These trace elements are in the tree, specifically in the leaves, which represent the basis for the formation of chlorophyll (leafy green) as well as sunscreen thanks to thicker cell walls of the leaves and ultimately for the promotion of sufficient fruit (here acorns). Another effect of "thicker" leaves is the resistance to pests. A better soil flora with balanced bacteria and fungi in symbiosis with the main plant also prevents the aggressiveness of destructive fungi of all kinds. We may assume that this fertilizer can limit the spread of the dreaded *Phytophthora cinnamomi*.

3.2. OptiPlus

OptiPlus is a biological humic fertilizer that can be liquid or solid. We have used here the liquid form. It contains 0.8% N organic, 0.3% P, 0.2% P organic, 0.5% S, 1% Ca, 0.18% Mg, 0.3% trace elements (Fe, Mn, B, Zn), 4.5% organic substance, 20% humic fulvic acids, and the remainder is water. Humic substance is an effective nutritional fertilizer without NPK, which is immediately receptive to the plants. Humic matter often occurs in nature, even in rivers.

3.3. Biohumim

Biohumim is a natural soil regenerator establishing the general balance of the soil trace element circulation. It can be liquid or solid form. For the purpose of this study we

have used the solid form. It contains 0.5% N organic, 0.3% P, 0.5% S, 1% Ca, 0.2% Mg, 0.3% trace elements (Fe, Mn, B, Zn), 55% organic substance, 20% humic fulvic acids, and the remainder is water.

4. Experiment I – Tree Layer

4.1. Hypothesis

For this experiment, we hypothesize that tree health condition will be improved after liquid product (OptiFer and OptiPlus) application.

4.2. Experimental design and data collection

4.2.1. Tree selection in pairs

We selected 120 holm oaks (*Q. ilex*) in a site ("San Francisco" including "Pan de Pobres" zone) in 107 ha severely affected by ink disease, within the dehesa of San Francisco during November 2018 (Fig. 2).

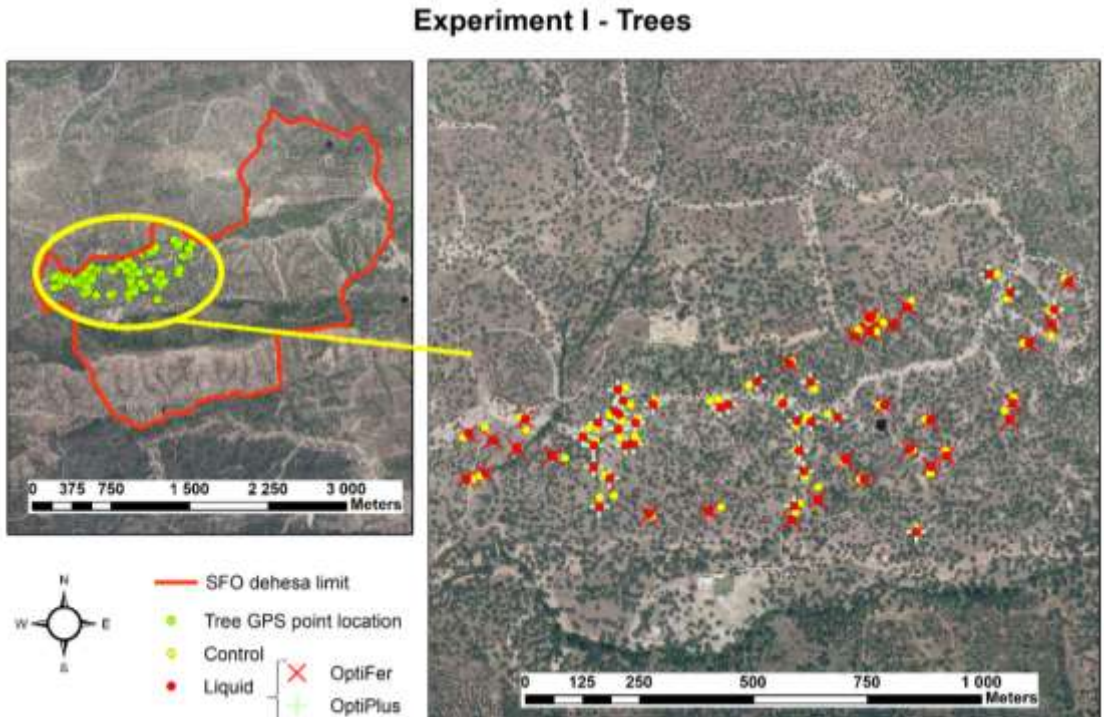


Fig. 2. Map of the 120 selected holm oaks in San Francisco site. Yellow odd numbers are the control trees and red even numbers are the treated trees with liquid product.

Within the site, we selected trees in pairs (Fig. 3). Each pair is comprised of two holm oak trees with very similar size (similar diameter at breast height), ecological conditions, and crown damage. Trees of each pair were distributed in the same hillside following a slope gradient. We assigned yellow odd numbers for control oaks (no

treatment) within each pair and red even numbers for treated oaks with liquid product (Fig. 2).

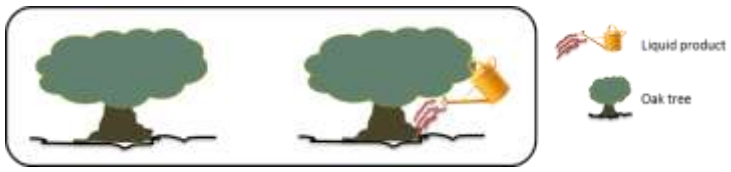


Fig. 3. Tree pair scheme with the control and the treated tree of similar size and crown damage.

We established four categories of crown damage (Table 1). Only trees clearly affected (>10% crown defoliation) were selected for the experiment to ensure that damage is not only due to possible insect defoliation, which typically affect <10% of the crown biomass.

Table 1 Categories of crown damage

Categories of damage	Label	Crown defoliation
I	Healthy	0 to 10 %
II	Light damage	11 to 30 %
III	Medium damage - diseased	31 to 60 %
IV	High damage - severe diseased	61 to 90 %

We avoided pairs with more than 90% crown defoliation since they are almost dead. Figure 4 is an example of one selected tree categorized as high damage or severely diseased:

Fig. 4. Example of one selected tree categorized as high damage or severely diseased.

4.2.2. Liquid product application

One of the trees within each pair received a liquid treatment and the other was the control (Fig. 2 and 3). We applied the product in a season without drought (November 2018). Thus, we assure that improvement of tree condition is due to product and not due to the "needed" water contribution. A total of 60 trees received treatment.

We used two different liquid products: OptiPlus (1000 l available) and OptiFer (1000 l available). Each tree received 100 l of mix (30% product + 70% water) in order to assure a significant amount of product per tree since some liquid losses might happen during mix preparation, mix transportation to each tree and tree watering. The mix is composed of 30 l liquid product and 70 l of water. In total, for each product (OptiPlus and OptiFer) we had 3000 l of mix distributed in three containers of 1000 l capacity (Fig 5.i). Table 2 summarizes the mix preparation.

Table 2 Summary of the preparation.

Measurements	**OPTIPLUS**	**OPTIFER**
Total pure liquid:	1000 l	1000 l
Container capacity:	1000 l	1000 l
Mix:	300 l O-plus + 700 l water	300 l O-Fer + 700 l water
Three containers :	3 × 1.000 l mix	3 × 1.000 l mix
Total of mix:	3000 l mix	3000 l mix
Total of mix per tree:	3000 / 30 = **100 l**	3000 / 30 = **100 l**

Then, three containers with the mix were carried to the study sites within the San Francisco site. From these points, we filled four containers of 25 l capacity (Fig 5.ii) and transported them by hand to each selected tree for the treatment (red even numbers). We gently watered the 100 l of mix on the base of the trunk from the uphill side of the tree to avoid liquid losses as less as possible (Fig. 5.iii). Thirty trees received OptiPlus liquid, 30 trees received OptiFer liquid and 60 acted as control.

i)

ii)

iii)

Fig. 5. Pictures about the watering process: i) tanks of 1000 l each with OptiFer and OptiPlus products; ii) process of transferring the liquid from the tank to the 25 l containers; iii) four containers of 25 l each were used to treat each tree.

4.2.3. Data collection

The sample size is **120 holm oaks** (60 treated vs. 60 control). In each tree, we measured some fieldwork variables in the two study years: GPS coordinates, altitude, dbh (diameter at breast height), total crown diameters (d_1, d_2), life crown diameters (d_1, d_2) and crown defoliation. Total crown diameters are those that include all branches, both foliated and completely defoliated. For each tree, we measured the longest total crown diameter (d_1) and its perpendicular (d_2). Life crown diameters are those that include only foliated branches. Crown defoliation is standardized per species by estimating the percentage defoliation degree in comparison with a fully foliated (healthy) reference tree of the same species (Müller and Stierlin, 1990). Later, in laboratory, we were able to calculate new variables obtained from fieldwork data (2 perpendicular diameters) such as percentage of crown area loss which is a percentage of life crown area regarding to the total crown area. We, then, calculated the difference of defoliation between two annual visits (November 2018 and October 2019) obtaining thus the **crown defoliation rate** and the **crown area loss rate.** Physiographical variables were also collected for each tree (e.g. slope, aspect) using satellite images.

4.3. Statistical analysis

4.3.1. Software used

Data processing and statistics were performed using R 3.6.0 (R Development Core Team, 2019) with the modules " lme4" (Bates *et al.*, 2015),"car" (Fox and Weisberg, 2011), "MuMIn" (Barton, 2015).

4.3.2. Variables

Response variables were **% crown defoliation rate** and **% of crown area loss rate**. Fixed effects were treatment (control vs. treated with liquid product). Covariates such as slope and aspect were also included. Pair was considered as a random effect. In addition, we repeated the analysis using liquid product (Control vs. OptiFer vs. OptiPlus) instead of treatment variable in order to obtain which product worked best. The rest of predictors were the same as above.

4.3.1. Statistics

We developed four maximal Generalized Linear Mixed Models -GLMMs- (Venables and Ripley, 2002) to analyze the data of this experiment. Maximal models are summarized in Table 4.3.a. Box-Cox transformations (Box & Cox 1964) were applied when needed in order to calculate the transformation lambda that maximizes the likelihood. Thus, some of the response variables were fitted to Gamma error distribution with their corresponding power lambda link function (Table 3). When monotonic transformations were not necessary, the response variables were fitted to Gaussian error distribution with identity function. For all models, the analyses included each site (pair of trees) as the random effects structure (Table 3).

Table 3 Summary of maximal models performed for data analysis in this experiment

Model	Response variable	Fixed effect[1]	Random effect	Error distribution (power lambda link function) [2]
I	Crown defoliation rate (ΔCD)	T×S + T×A	1|Pair	Gamma (0)
II	Crown defoliation rate (ΔCD)	P×S + P×A	1|Pair	Gamma (0)
III	Crown area loss rate (ΔCAL)	T×S + T×A	1|Pair	Gaussian (1)
IV	Crown area loss rate (ΔCAL)	P×S + P×A	1|Pair	Gaussian (1)

[1]T: Treatment (control vs. treated); P: Product used in the treatment; S: Slope; A: Aspect

[2]Power lambda link function [$g(\mu) = \mu^{\lambda}$] is the lambda (λ, numeric value inside the brackets) used for the monotonic transformations.

When two or more fixed effects were in the model, we used the model averaging approach (Burnham and Anderson, 2002). We first fitted the maximal model, containing all predictors. Then, we ranked through AIC weights all possible models derived from the maximal model by using the "dredge" function within the "MuMIn" package of R and selected those with the best AIC weight (hereafter top models) which had ΔAIC < 2 (Burnham and Anderson, 2002). Finally, we obtained the model-averaged coefficients of top models as well as the relative importance of each predictor (from 0 to 1) by using the "model.avg" function of "MuMIn" (Burnham and Anderson, 2002). Residuals were visually checked for heterogeneity in selected top models and the explained deviance (Crawley, 2012).

4.4. Results

4.4.1. Crown defoliation rate

Treatment (control vs. treated) and type of product were the only significant variables in the top linear mixed models (Table 4). Thus, control trees (without treatment) had higher crown defoliation rate than those treated trees with liquid product (Fig. 6.i). In addition, trees treated with OptiPlus liquid showed a significant reduction of crown defoliation rate compared to those trees that received OptiFer liquid product (Fig. 6.ii).

Table 4 Summary of the top linear mixed models (ΔAIC < 2) to analyze the crown defoliation rate depending on I) treatment and II) product (covariates: slope and aspect are also included)

Model	Fixed effects	*Importance*	Levels	*Coeff.*	*SE*	*z-value*	*P*
	Intercept			0.694	0.270	2.543	**0.011**
	Treatment (T)	1.00	Liquid	-1.049	0.191	5.443	**<0.001**
I	Slope (S)	0.41	Slope	0.116	0.145	0.791	0.429
	Aspect (A)	0.55	Aspect	0.226	0.175	1.280	0.201
	T × S	0.18	T_{Liquid} × S	-0.159	0.204	0.772	0.440
	Intercept			0.729	0.268	2.687	**0.007**
	Product (P)	1.00	OptiFer	-0.656	0.232	2.800	**0.005**
II			OptiPlus	-1.647	0.289	5.640	**<0.001**
	Slope (S)	0.43		0.173	0.159	1.077	0.282
	Aspect (A)	0.45		0.106	0.140	0.752	0.452

Importance: Importance of predictor variable in the model averaging.
Bold type indicates statistical significance (P<0.05).
Results from Treatment and Product type are against control trees.

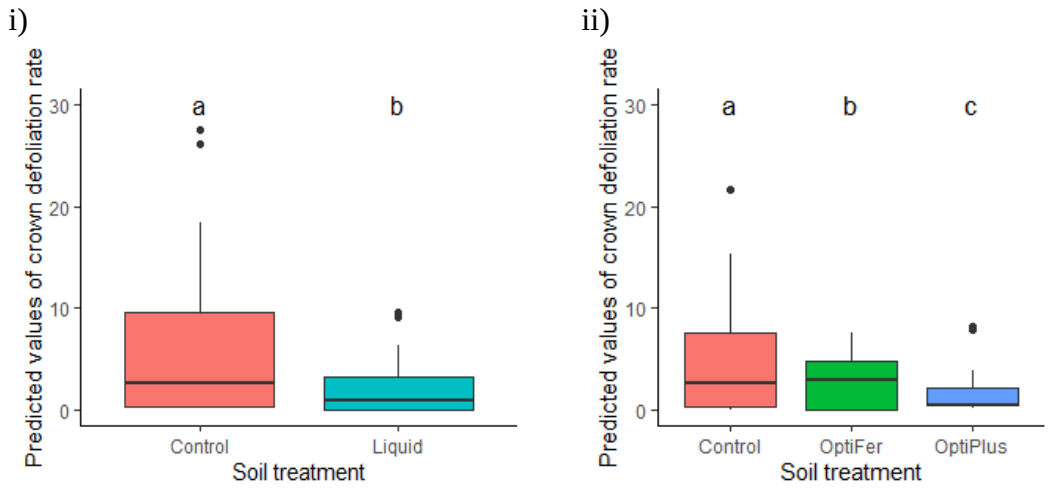


Fig. 6. Predicted values of crown defoliation rate depending on i) treatment and ii) liquid product (N=120 trees). Control= trees within each pair that received no liquid treatment, Liquid= trees within each pair that received liquid treatment. OptiFer= trees that received OptiFer liquid treatment, OptiPlus= trees that received OptiPlus liquid treatment. Different letter above the boxes indicate significant differences (P<0.05).

4.4.2. Crown area loss rate

We only detected significant differences in % of crown area loss rate depending on treatment when interacting with slope (Table 5). In the case of slope absence (0 to 10º) we did not find significant differences in % of crown area loss rate among treated and control trees (Fig. 7). However, as the slope increased, the % of crown area loss rate also increase for controls whereas, for treated trees, the soil liquid treatment applied reduced the stress generated by the slope increase (Fig. 7). We did not find any significant differences for trees treated with OptiFer liquid product interacting with slope due to most of trees that received OptiFer liquid treatment had similar slope (0 to 10; Fig. 8).

Table 5 Summary of the top linear mixed models (ΔAIC < 2) to analyze the % of crown area loss rate depending on III) treatment and IV) product (covariates: slope and aspect are also included)

Model	Fixed effects	*Importance*	Levels	*Coeff.*	*SE*	*z-value*	*P*
III	Intercept			35.276	4.707	7.397	<0.001
	Treatment (T)	0.87	Liquid	7.015	6.983	0.994	0.320
	Slope (S)	0.66	Slope	0.631	0.333	1.855	0.064
	T × S	0.51	T_{Liquid} × S	-1.107	0.509	2.129	**0.033**
IV	Intercept			34.132	4.010	8.512	**<0.001**
	Product (P)	1.00	OptiFer	8.388	6.564	1.278	0.207
			OptiPlus	13.742	7.912	1.737	0.088
	Slope (S)	0.82	Slope	0.568	0.322	1.763	0.084
	T × S	0.75	$P_{OptiFer}$ × S	-0.449	0.612	-0.734	0.466
			$P_{OptiPlus}$ × S	-1.818	0.713	-2.549	**0.014**

Importance: Importance of predictor variable in the model averaging. Bold type indicates statistical significance (P<0.05). Results from Treatment and Product type are against control trees.

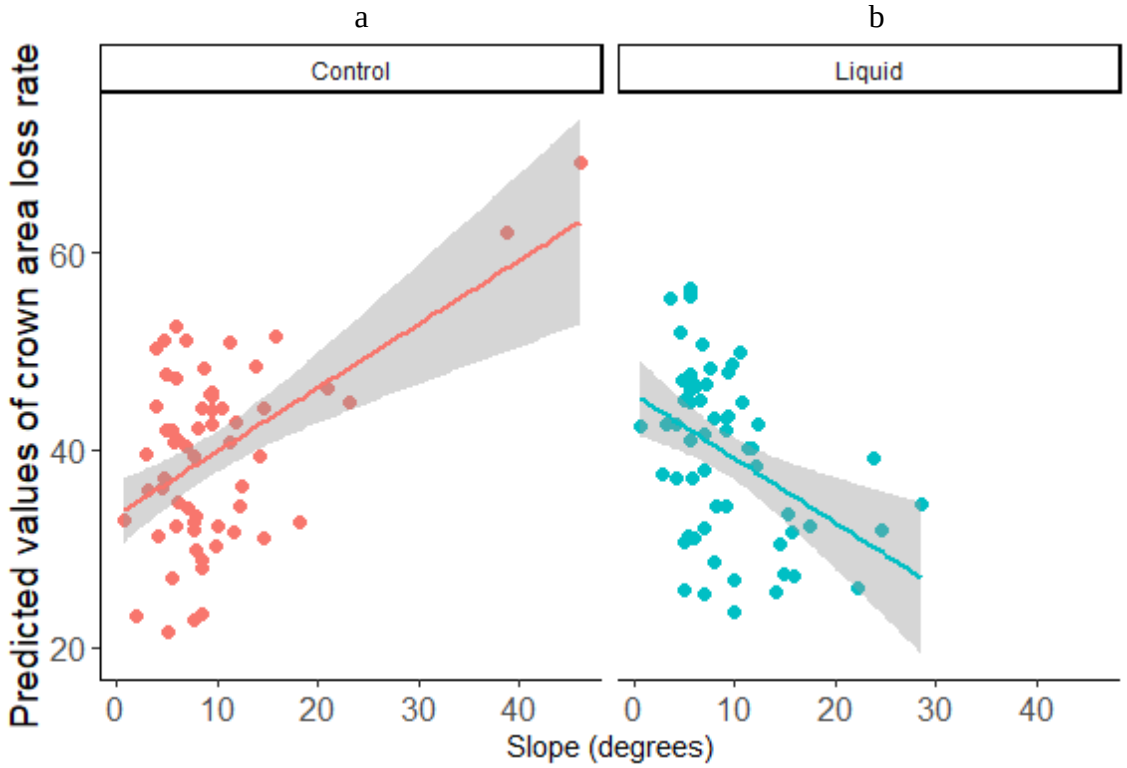


Fig. 7 Predicted values of crown area loss rate depending on treatment-slope interaction (N=120 trees). In legend, Control= trees within each pair that received no liquid treatment, Liquid= trees within each pair that received a liquid treatment. Different letter above the graphs indicate significant differences (P<0.05).

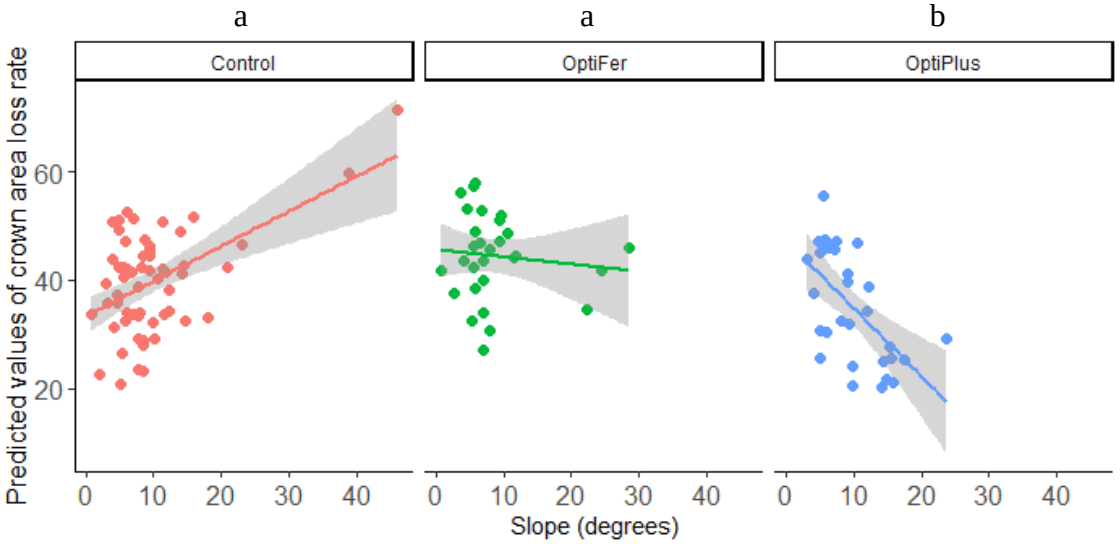


Fig. 8 Predicted values of crown area loss rate depending on product-slope interaction (N=120 trees). In legend, Control= trees within each pair received no liquid treatment, OptiFer= trees within each pair that received OptiFer liquid treatment, OptiPlus= trees within each pair that received OptiPlus. Different letter above the graphs indicate significant differences (P<0.05).

5. Experiment II – Oak regeneration

5.1. Hypothesis

For this experiment, we hypothesize that oak recruit condition will be improved after liquid/solid product application.

5.2. Quintets: Experimental design and data collection

5.2.1. Selection of oak recruit quintets

We selected 150 oak recruits (dbh > 5 cm) grouped in quintets (total: 30 quintets) of two different oak species - holm and cork oaks (*Q. ilex* and *Q. suber*, respectively) - in two sites of Dehesa San Francisco ("La Vieja" and "Umbría del Cuervo") where ink disease is widespread (Fig. 9). Oak recruit selection and experiments were carried out in November 2018.

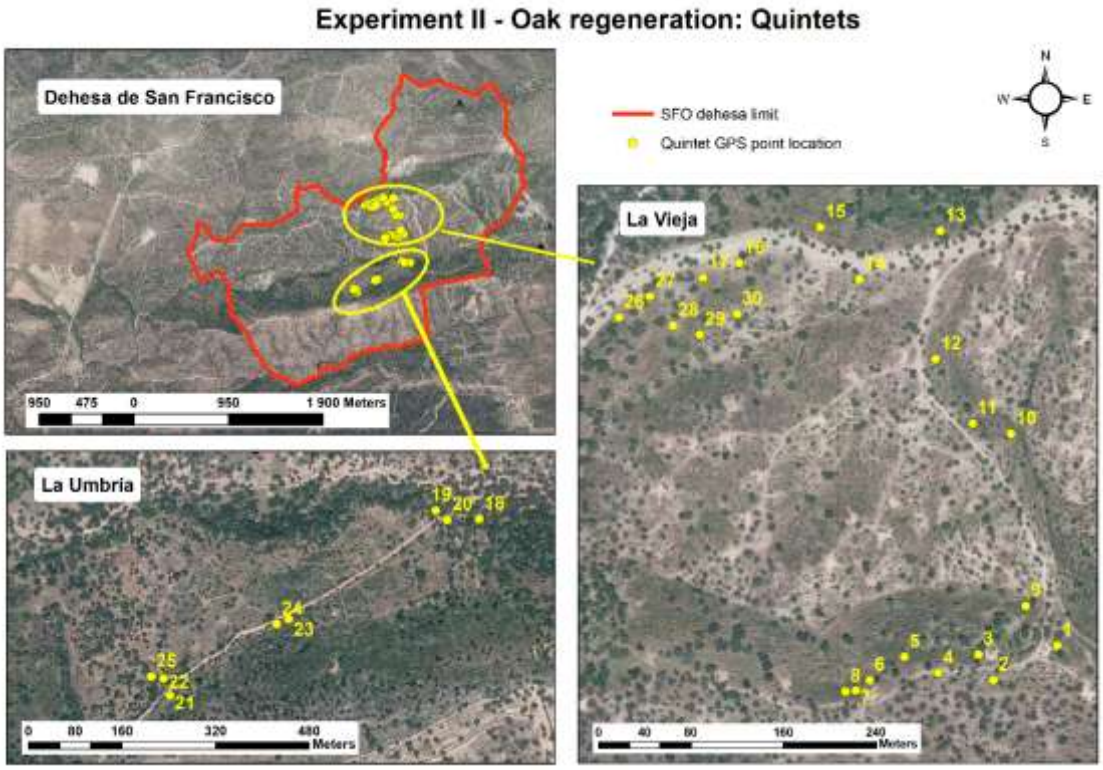


Fig. 9 Map of the 30 selected quintets in "La Vieja" and "Umbría del Cuervo" sites.

Each quintet (approximately 25-100 m2 each; Fig. 10) contained five oak recruits of the same species and with very similar size (basal diameter and crown), ecological conditions, and crown damage (if possible). However, most of the oak recruits did not have any apparent damage; thus, we selected all of them with similar defoliation (mostly light damage; 10-30% defoliation due to the lack of oak recruits with heavier damage). When light-damaged recruits were rare, we used healthy recruits to analyze the effect of the product in preventing future possible damage since ink disease is present in the study areas. Recruits of each quintet were distributed on the same hillside and fairly close to each other (4-10 m for most recruits). Control recruits were always located uphill of the treated recruits to avoid product invasion. We also separated those treated with solid product from those treated with liquid products (Fig. 10). We assigned clamps of different colors to each recruit in order to distinguish treatments: white for control recruits with protector, black for Biohumim recruits with protector, green for OptiPlus recruits with protector, red for OptiPlus recruits without protector and yellow for control recruits without protectors.

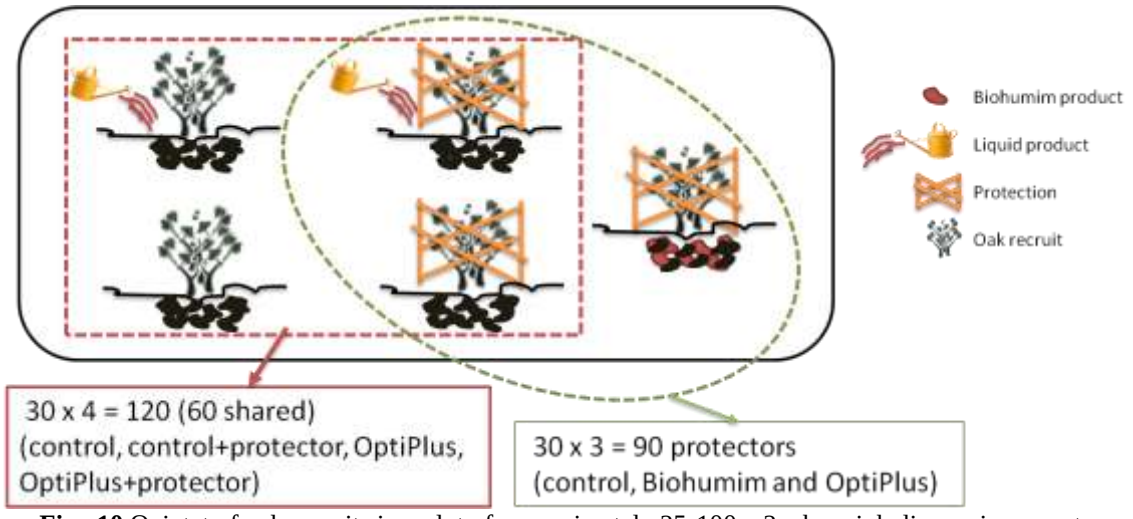


Fig. 10 Quintet of oak recruits in a plot of approximately 25-100 m2 where ink disease is present.

5.2.2. Liquid and solid product application

Three of the oak recruits within the quintets received a treatment (one recruit Biohumin and two recruits OptiPlus, protected and non-protected; Fig. 10) and the other two were the controls.

For liquid product (1000 l available), we used 50 l of mix (product + water) per oak recruit in order to assure a significant amount of product per recruit since some liquid

losses might happen during mix preparation, mix transportation to each recruit and watering. The mix contained 15 l of OptiPlus and 45 l of water. In total, we had 3000 l of mix distributed in three containers of 1000 l capacity. Table 6 summarizes the mix preparation.

Table 6 Summary of the preparation

Measurements	**OPTIPLUS**
Total pure liquid:	1000 l
Container capacity:	1000 l
Mix:	300 l O-plus + 700 l water
Three containers (1000 l capacity):	3 × 1.000 l mix
Total of mix:	3000 l mix
Total of mix per recruit:	3000 / 60 = **50 l**

Then, three containers with the mix were carried to "La Vieja" and "Umbría del Cuervo". From this point location, we filled four containers of 25 l capacity and transported them by hand to each two selected oak recruits within the quintet for the treatment (green and red clamp oak recruits). A total of 60 oak recruits received OptiPlus liquid (Fig. 11.i).

For solid product, we used 2.5 kg of Biohumin per oak recruit. We distributed the Biohumim around the base of stems on the surface and watered each oak recruit after Biohumim application (Fig. 11.ii and 11.iii). We did not dig up in order to avoid damages on established roots of oak recruits. A total of 30 oak recruits received Biohumim product (Fig. 11.iv). In addition, three of the oak recruits of quintets were protected against herbivores and omnivores with protectors (Fig. 10) since Biohumim is very attractive to ungulates, especially to pigs.

i)

ii)

iii) iv)

Fig. 11 Process of product application: i) container of 25 l (OptiPlus product) used to treat each oak recruit; ii) Biohumim application to each oak recruit; iii) watering each oak recruit after Biohumim application; iv) oak recruit with Biohumim.

5.2.3. Data collection

The total of sample size is **150 oak recruits**. In each oak recruit, we measured the following variables in the two annual visits: GPS coordinates of the quintet, basal diameter, plant height, plant crown diameters (d_1, d_2). For crown diameters, we measured the longest total crown diameter of each recruit (d_1) and its perpendicular (d_2). We also recorded ungulate herbivory damage. For that, we selected ten top twigs of the recruit and marked those browsed by herbivores obtaining a damage percentage which might be categorized (Table 7), following Perea *et al.* (2015). We, then, calculated the difference of plant size (height and crown area) and herbivory between two visits (November 2018 and October 2019 -study year-) obtaining thus the **plant height rate (hereafter growth rate)**, **recruit crown area rate** and **herbivory rate.**

Table 7 Categories of herbivory damage

Categories of herbivory	Label	Leaves damages
1	Light damage	<10% of twigs damaged
2	Low damage	11-30% of twigs damaged
3	Intense damage	31-60% of twigs damaged
4	Heavy damage	61-90% of twigs damaged
5	Maximum damage	>90% of twigs damaged

5.3. Pairs: Experimental design and data collection

5.3.1. Selection of oak recruit pairs

We selected 60 oak recruits grouped in pairs (total: 30 pairs) of two different oak species - holm and cork oaks (*Q. ilex* and *Q. suber*, respectively) – in one site of Dehesa

San Francisco ("La Solana del Cuervo") where ink disease is widespread (Fig. 12). Oak recruit selection and experiments were carried out in January 2019.

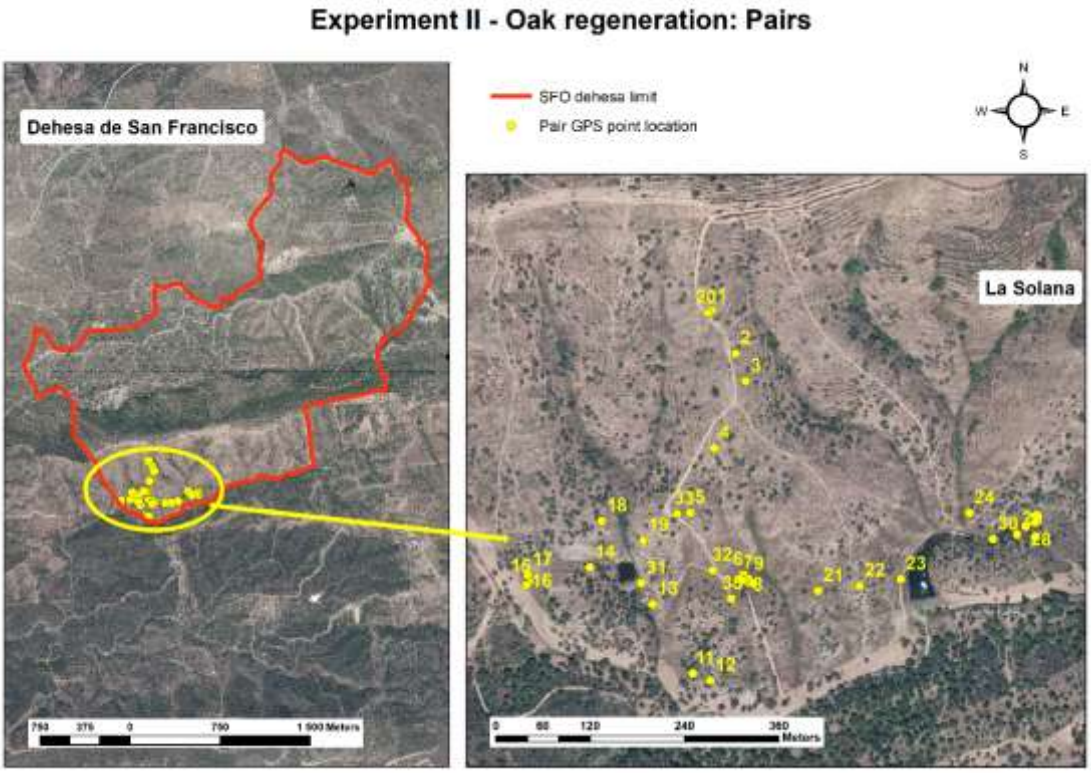


Fig. 12 Map of the 30 selected pairs in "La Solana del Cuervo" site.

Each plot (approximately 25-100 m2 each; Fig. 13) contained two oak recruits of the same species and with similar size (basal diameter and crown diameter), ecological conditions, and crown damage (if possible). However, most of the oak recruits did not have any apparent damage; thus, we selected all of them with similar defoliation (mostly light damage; 10-30% defoliation due to the lack of oak recruits with heavier damage). When light-damaged recruits were rare, we used healthy recruits to analyze the effect of the product in preventing future possible damage since ink disease is present in the study areas. Recruits of each pair were distributed on the same hillside and close to each other (< 5 m). Control recruits were always selected uphill of the treated recruits to avoid product invasion. We assigned clamps of different colors to each recruit in order to distinguish treatments: white for control recruits with protector and black + yellow for Biohumim recruits with protector.

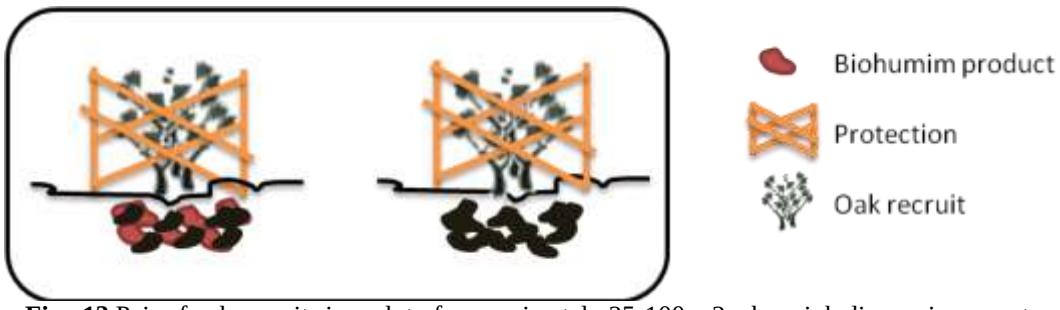


Fig. 13 Pair of oak recruits in a plot of approximately 25-100 m2 where ink disease is present.

5.3.2. Solid product application

One of the recruits within each pair received a solid treatment (Biohumim) and the other was the control (Fig. 13). We used 2.5 kg of Biohumin per oak recruit. We distributed the Biohumim around the base of stems on the surface and watered each oak recruit after Biohumim application (Fig. 14.i and 14.ii). We did not dig in order to avoid damages on established roots of oak recruits. In addition, all recruits were protected against herbivores and omnivores with protectors (Fig. 11.iii) because Biohumim is very attractive to ungulates, especially to pigs. A total of 30 oak recruits received Biohumim product (Fig. 14.iv).

i) ii) iii)

iv)

Fig. 14 Process of product application: i) Biohumim distribution to each oak recruits; ii) watering each oak recruit after Biohumim application; iii) protector establishment after watering; iv) Established oak pair.

5.3.3. Data collection

The sample size is **120 oak recruits**, 60 oak recruits come from quintets (Fig. 10; see 5.2.1 section) and 60 oak recruits come from pair experiment (Fig. 13).

In each oak recruit, we measured the following variables: GPS coordinates of the pair, basal diameter, plant height, and plant crown diameters (d_1, d_2). We measured the longest total crown diameter of each recruit (d_1) and its perpendicular (d_2). We also recorded ungulate herbivory damage (Table 7). We, then, calculated the difference of plant size (height and crown area) and herbivory between two visits (November 2018 and October 2019 -study year-) obtaining thus the **plant height rate (hereafter growth rate)**, **recruit crown area rate** and **herbivory rate.**

5.4. Statistical analysis

5.4.1. Software used

Data processing and statistics were performed using R 3.6.0 (R Development Core Team, 2019) with the modules " lme4" (Bates *et al.*, 2015),"car" (Fox and Weisberg, 2011), "MuMIn" (Barton, 2015).

5.4.2. Variables

The quintet design allows us to have three separate analyzes on which we have the following variables:

5.4.2.1. Effectiveness of OptiPlus vs. Biohumim vs. control (n=90). All protected with the wire cage

The response variables are: **oak plant survival, growth rate**, and **recruit crown area rate**. The predictor is product type (control vs. OptiPlus vs. Biohumim). Each plot containing the "Triplet" is considered as random effect.

5.4.2.2. Effectiveness of Biohumim vs. control (n=120) in contrasting sites. Both protected

The response variables are: **oak plant survival, growth rate** and **recruit crown area rate**. The predictors are product type (control vs. Biohumin) and contrasting sites ("La Vieja" vs "La Solana"). "Pair" is considered as random effect.

5.4.2.3. Effectiveness of OptiPlus vs. control and with protection vs. without protection (n=120)

The response variables are: **oak survival** and **herbivory rate.** The predictors are product type (control vs. OptiPlus) and fence protection (protection vs. non-protection). Each plot containing the "Quartet" is considered as random effect.

5.4.3. Statistics

We developed four maximal Generalized Linear Mixed Models -GLMMs- (Venables and Ripley, 2002) to analyze the data of this experiment. Maximal models are summarized in Table 8.c Box-Cox transformations (Box & Cox 1964) were applied when needed in order to calculate the transformation lambda that maximizes the likelihood. Thus, some of the response variables were fitted to Binomial and Gamma error distribution with their corresponding power lambda link function (Table 8). When monotonic transformations were not necessary, the response variables were fitted to Gaussian error distribution with identity function (linear mixed models). For all models, the analyses included different random effects structures according to the analysis (Table 8).

Table 8 Summary of maximal models performed for data analysis in the oak recruit experiments (quintets)

Model	Response variable	Fixed effect[1]	Random effect	Error distribution (power lambda link function) [2]	Sample size (n)
I	Oak survival	$P_t \times S$	1|Triplet	Binomial ()	90
II	Oak survival	$P_t \times S + P_t \times CS$	1|Pair	Binomial ()	120
III	Oak survival	$P_t \times S + P_t \times P$	1|Quartet	Binomial ()	120
IV	Plant growth rate	$P_t \times S$	1|Triplet	Gamma (0.222)	90
V	Plant growth rate	$P_t \times S + P_t \times CS$	1|Pair	Gamma (0.200)	120
VI	Recruit crown area rate	$P_t \times S$	1|Triplet	Gamma (-0.101)	90
VII	Recruit crown area rate	$P_t \times S + P_t \times CS$	1|Pair	Gamma (-0.061)	120
VIII	Herbivory rate	$P_t \times S + P_t \times P$	1|Quartet	Gamma (-0.263)	120

[1] P_t: Product type; CS: Contrasting sites; S: Species (cork oak vs. holm oak); P: Protection (fenced vs. non-fenced)
[2]Power lambda link function [$g(\mu) = \mu^{\lambda}$] is the lambda (λ, numeric value inside the brackets) used for the monotonic transformations.

When two or more fixed effects were in the model, we used the model averaging approach (Burnham and Anderson, 2002). We first fitted the maximal model, containing all predictors. Then, we ranked through AIC weights all possible models derived from the maximal model by using the "dredge" function within the "MuMIn" package of R and selected those with the best AIC weight (hereafter top models) which had ΔAIC < 2 (Burnham and Anderson, 2002). Finally, we obtained the model-averaged coefficients of top models as well as the relative importance of each predictor (from 0 to 1) by using the "model.avg" function of "MuMIn" (Burnham and Anderson, 2002). Residuals were visually checked for heterogeneity in selected top models and the explained deviance as well as the dispersion parameter of each model was calculated to evaluate its fit and ensure no overdispersion (Crawley, 2012).

5.5. Results

5.5.1. Oak survival

The survival was very high for the three different analyses (97% for Triplets, 100% for Pairs and 97% for Quartets). Hence, we did not find significant differences in survival depending on product type (Triplets: z =0.002, P=0.999; Pair: z=0.000, P=1.000; Quartets: z =0.004, P=0.997), species (Triplets: z=0.000, P=1.000; Pair: z=0.000, P=1.000; Quartets: z=0.004, P=0.997), sites (Pair: z=0.000, P=1.000) or protection (Quartets: z=1.060, P=0.284).

5.5.2. Plant size rate

5.5.2.1. Plant growth rate

We did not find any significant differences in plant growth rate depending on product type (Table 9). However, we found significant differences depending on species for triplets (located in the same site) and for pairs (located in contrasting sites), with lower plant growth rate values for holm oak than for cork oak in both analysis (Table 9). In addition, we found significant differences between sites, with lower plant growth rate values in the south-facing site as compared to the north-facing site (Table 9).

Table 9 Summary of the top linear mixed models (ΔAIC < 2) to analyze the plant growth rate depending on product, oak species, site and protection

Model	Fixed effects	*Importance*	Levels	*Coeff.*	*SE*	*z-value*	*P*
IV	Intercept			1.875	0.079	23.593	**< 0.001**
	Product type (P_t)	0.35	Biohumin	-0.130	0.109	1.173	0.241
			OptiPlus	0.144	0.117	1.215	0.225
	Species (S)	0.68	Holm Oak	-0.287	0.100	2.829	**0.005**
V	Intercept			1.671	0.067	24.633	**<0.001**
	Product type (P_t)	0.40	Biohumin	0.026	0.075	0.340	0.734
	Species (S)	0.63	Holm Oak	-0.223	0.100	2.208	**0.027**
	Contrasting site(CS)	0.74	South site	-0.254	0.095	2.644	**0.008**

Importance: Importance of predictor variable in the model averaging.
Bold type indicates statistical significance (P<0.05).
Results from Product type are against control plants, results for species refer to holm oak against cork oak and results for contrasting sites refer to south-facing site against north-facing site.

5.5.2.2. Recruit crown area rate

We did not find any significant differences in recruit crown area rate depending on product type, oak species and contrasting sites (Table 10).

Table 10. Summary of the top linear mixed models (ΔAIC < 2) to analyze the recruit crown area rate depending on depending on product, oak species, site and protection

Model	Fixed effects	*Importance*	Levels	*Coeff.*	*SE*	*z-value*	*P*
VI	Intercept			6.415	0.320	19.767	**< 0.001**
	Species (S)	0.30	Holm Oak	-0.344	0.618	0.548	0.584
VII	Intercept			5.898	0.358	16.302	**< 0.001**
	Product type (P_t)	0.40	Biohumin	0.109	0.490	0.221	0.825
	Species (S)	0.63	Holm Oak	0.011	0.561	0.019	0.985
	Contrasting site(CS)	0.74	South site	-0.715	0.502	1.410	0.159

Importance: Importance of predictor variable in the model averaging.
Bold type indicates statistical significance (P<0.05).
Results from Product type are against control plants, results for species refer to holm oak against cork oak and results for contrasting sites refer to south-facing site against north-facing site.

5.5.3. Herbivory

We did not find significant differences in plant herbivory rate depending on product type itself (Table 11). Holm oaks showed in general less herbivory than cork oaks; however, herbivory was higher on holm oaks when OptiPlus was applied on recruits (Fig. 15). In addition, for protected oaks, the herbivory was increased in OptiPlus treated-oaks compared to controls, but they still maintain lower herbivory than non-protected oaks (Fig. 15).

Table 11 Summary of the top linear mixed models (ΔAIC < 2) to analyze plant herbivory rate depending on product, oak species, site and protection.

Model	Fixed effects	*Importance*	Levels	*Coeff.*	*SE*	*z-value*	*P*
VIII	Intercept			0.028	0.533	0.053	0.958
	Product type (P_t)	1.00	OptiPlus	-0.614	0.578	-1.063	0.288
	Species (S)	0.97	Holm Oak	-1.561	0.714	-2.185	**0.029**
	Protection (P)	1.00	Yes	-2.559	0.488	-5.241	**<0.001**
	P_t × S	0.96	OptiPlus×HolmOak	2.421	0.694	3.488	**<0.001**
	P_t × P	0.99	OptiPlus×Yes	1.562	0.654	2.389	**0.017**

Importance: Importance of predictor variable in the model averaging.
Bold type indicates statistical significance (P<0.05).
Results from Product type are against control plants, results for species refer to holm oak against cork oak and results for protection refer to protected plants against non-protected plants.

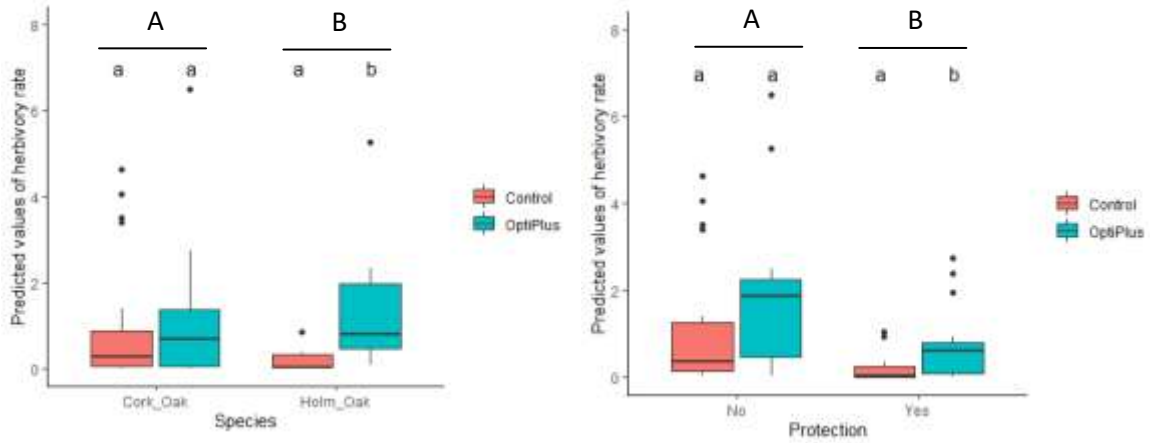


Fig. 15 Predicted values of herbivory rate depending on a) product-species interaction, and b) product-protection interaction (N=120 recruits). In legend, Control= recruits that received no liquid treatment, OptiPlus= recruits that received OptiPlus treatment. Different letter above the boxes indicate significant differences (P<0.05).

6. Experiment III – Field plantation

6.1. Hypothesis

The hypothesis of this experiment is based on the prediction that oak treated oak seedlings (planted in the field with application of product) will better resist ink disease infection.

6.2. Experimental design and data collection

6.2.1. Acorn selection

We selected 8 healthy trees of two different oak species - 4 holm and 4 cork oaks (*Q. ilex* and *Q. suber*, respectively) - in two sites of Dehesa San Francisco ("San Francisco" including "Pan de Pobres" zone and "La Solana del Cuervo") where ink disease is widespread. These trees had very similar size (dbh), ecological conditions, and crown damage. In each tree we collected a sufficient number of acorns (n>50) to be planted. Acorns of each species did not have any insect damage (no oviposition perforation and larva exit hole), and were tested for viability using a flotation method (Perea *et al.*, 2012).

6.2.2. Selection of plantation location in quartets

Acorns were germinated inside a plastic bag during 45 days in a fridge at 8ºC. Only successfully-germinated acorns, with a clear radicle, were used for plantation (Fig. 16).

Fig. 16 Successfully-germinated acorns

We then selected 30 adult trees of the two oak species (*Q. ilex* and *Q. suber*) severely affected by ink disease (class III) and located in the highest infected areas ("Pan de Pobres" and "La Solana del Cuervo"; Fig. 17). We used a portable fridge to move acorns to each selected tree in order to reduce the possible damages caused by transportation.

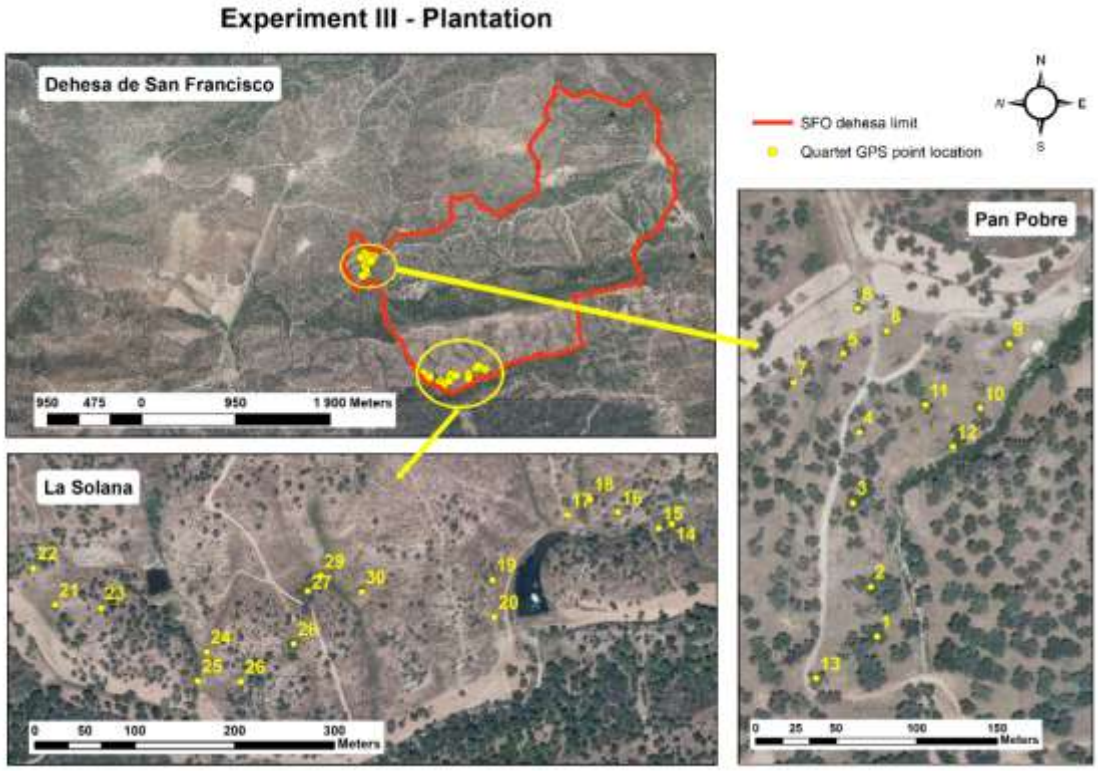


Fig. 17 Map of the 30 selected quartets in "La Solana del Cuervo" and "Pan Pobres" sites for acorn plantation experiments.

6.2.3. Plantation methodology and Biohumin product application

In each selected tree, we planted four germinating acorns at the same distance (>5m) to the infected tree and in open areas, and were protected with grazing exclosure cages (Fig. 18). We used more than 5 m from the infected oak tree to avoid tree shading on the seedlings (same open microsite for all seedlings since microsite strongly affects survival and performance of seedlings). We planted two oak seedlings (1 holm oak and 1 cork oak) without any treatment (controls) separated 2 m from each other. Then, at enough distance (>4m), we planted two oak seedlings (1 holm oak and 1 cork oak) with Biohumim treatment separate 2 m from each other (Fig. 18).

For controls, the plantation followed this methodology: we first dug up a hole with enough depth (15 cm depth). Then, we mixed soil and filled the hole. All acorns were planted at 5 cm depth following the natural pattern of acorn dispersers (Perea *et al.*, 2011). Finally, we watered the plant.

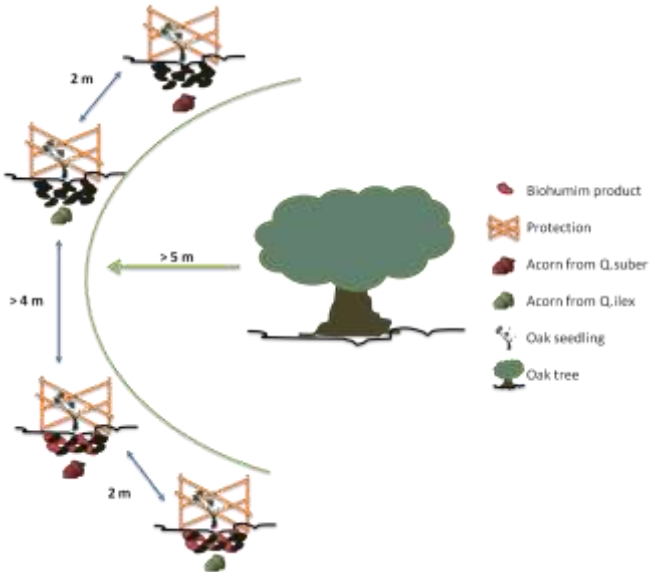


Fig. 18 Experimental design of each quartet of planted acorns in an area where ink disease is present

For Biohumim plants, the plantation followed this methodology: we first dug up a hole with enough depth to establish Biohumin on the bottom (25 cm depth; Fig. 19.i). We used 2.5 kg of Biohumin per oak seedling. Most of the Biohumin (2 kg) was established at the bottom of the hole (Fig. 19.ii). The rest of Biohumin (0.5 kg) were mixed with the extracted soil and filled the hole, locating the acorn 5 cm depth. Finally, we watered the plant (Fig. 19.iii). A total of 60 oak seedlings received Biohumim product.

All plants were protected in order to avoid animal consumption (Fig. 19.iv). In each protector, we marked the plants following this way: for controls (plants without treatment) holm oak plants received a yellow clamp and cork oak plants received a red clamp in the protectors. For plants with Biohumim we added to each species a black clamp.

i)

ii)

iii) iv)

Fig. 19 Plantation example: i) hole of 25 cm depth; ii) Biohumim distribution on the bottom; iii) preparation of the watering pit; iv) protector establishment after watering.

6.2.4. Data collection

The sample size is **120 oak seedlings**. In each oak seedling, we measured the following variables: GPS coordinates of the quartet, oak survival, and plant height. Physiographical variables (e.g. altitude, slope, aspect) were also collected for each plot using remote sensing techniques.

6.3. Statistical analysis

This experiment failed. Hence, statistics could not be done, only descriptive values such as percentage of emergence and survival rates, were obtained.

6.4. Results

6.4.1. Oak seedling survival

This experiment failed. Only 10 oak seedlings emerged from 120 planted oak seedlings (8% emergence), but in addition only one oak seedling survived after summer. Figure 20 shows the development of one cork oak seedling that emerged and died during summer. Hence, no statistical analysis could be done due to the lack of data.

i) ii) iii)

Fig. 20 Development of one emerged-died oak seedling (cork oak species): i) picture in May 2019; ii) picture in June 2019; iii) picture in August 2019.

6.4.2. Oak seedling height

The average height (± SD) achieved for the 10 emerged seedlings was 6.45 ± 4.57 cm.

7. Experiment IV– Laboratory oak seedlings

7.1. Hypothesis

The hypothesis of this experiment is based on the prediction that oak seedlings infected by *P. cinnamomi* will better resist and tolerate the infection with applied product under controlled conditions (greenhouse). In addition, we tested whether water stress had an effect on the disease prevalence and the efficacy of the Biohumin product.

7.2. Experimental design and data collection

7.2.1. Acorn selection

We selected 5 adult trees of holm oaks (*Q. ilex*) in areas where ink disease is present within the dehesa of San Francisco in November 2018. Trees had very similar size (dbh), ecological conditions, and their crown damage was categorized as healthy. In each tree we picked up 100 sound acorns of similar size. A total of 500 acorns were collected.

Acorns were germinated inside a plastic bag during 60 days at 5ºC in the School of Forestry (Universidad Politécnica de Madrid).

7.2.2. Plantation methodology and Biohumim product application

For the whole experiment we used 360 acorns. First of all we prepared the pots (3 dm^3) filled with sterilized substrate and same weight (1.6 kg). We prepared the substrate mix as follows: a) control plants had substrate of 25% sand and 75% peat; b) 25% Biohumim plants had substrate of 25% sand, 50% peat and 25% Biohumim; and c) 12.5% Biohumim plants had a substrate of 25% sand, 62.5% peat and 12.5% Biohumim. We established two pipes within each pot, which will be use for infecting the plant with *P. cinnamomi* in order to avoid possible root damages when we inoculate with *P. cinnamomi.*

All selected acorns were weighed. We selected acorns with similar size (8-10 g) and with no damage (no oviposition perforation and larva exit hole), and tested for viability using a flotation method (Perea *et al.*, 2012). They were planted in each prepared individual pot (3 dm^3) and were labelled with their corresponding code.

We marked the seedling emergence date and waited for the adequate oak seedling development (approximately 2 months). After oak seedling establishment, we applied the treatments: drought vs. watering and infected vs. no infected.

i) ii) iii)



iv) v) vi)

Fig. 21 Greenhouse pictures: i) burying selected acorns in each pot (3 dm3); ii) pot control; iii) pot with pipes where *P. cinnamomi* will be inoculated; iv) pots located within the greenhouse; v) emerged seedling in a pot control; vi) emerged seedling in a pot with pipes where *P. cinnamomi* will be inoculated.

7.2.3. Drought treatment

Additionally, half of the total number of seedlings was treated with drought treatment (low watering). On May 17th, 2019 we cut the watering for this selected group. To induce a moderate drought stress, we controlled soil relative water content (SRWC) to be around 40% of soil water content at field capacity. For that, every 3 days, we weighed the same 18 pots subjected to drought treatment to check the SRWC. We also weighed the same 6 pots with no drought treatment to check that they maintain the SRWC at 90-100%. SRWC was estimated as:

$$SRWC = \frac{Mi - DM}{SM - DM} \times 100$$

Mi = soil mass in each pot

DM = mean soil dry mass measure in 5 pots

SM = mean saturated mass measured in 18 pots after excess water had drained for 48 h such as field capacity (Wang *et al.*, 2014).

In total, we applied three drought cycles before *P. cinnamomi* inoculation.

7.2.4. Seedling infection

Half of each substrate group was artificially inoculated with *P. cinnamomi* on June 24th, 2019. We filled two swimming pools (measurements) and wait for 2 days to evaporate the chlorine in water. Then, we start the inoculation following this protocol:

- Addition of 200 and 800 ml of distilled water for 1 and 4 l flask capacity, respectively, which contained *P. cinnamomi* inoculums and mixed (Fig. 22.i).
- Extraction and drainage of mix until homogenized all in trays. Watered with distilled water to remove sugars and avoid bacterial contamination (Fig. 22.ii).
- We add the inoculums in all pots with two pipes which were extracted to deliver the inoculums in the empty volume. The amount of inoculums was approximately a soup spoon.
- Finally, we immersed the pots in one of the swimming pools. The other one was for controls, to avoid differences due to flooding.

i)

ii)

iii)

Fig. 22 Greenhouse pictures: i) mix of inoculums and distilled water; ii) homogenized mix and cleaning with distilled water; iii) flooding pots within swimming pools.

7.2.5. Data collection

The sample size is **360 oak seedlings**. In each oak seedling, we measured some variables: emergence date, seedling survival and plant height. Plants were checked every 2-3 days from May to August.

7.3. Statistical analysis

7.3.1. Software used

Data processing and statistics were performed using R 3.6.0 (R Development Core Team, 2019) with the modules " lme4" (Bates *et al.*, 2015),"car" (Fox and Weisberg, 2011), "MuMIn" (Barton, 2015).

7.3.2. Variables

The response variables are **seedling emergence**, **seedling survival** and **plant height**. The predictors for seedling emergence are soil treatment (control, Biohumim 12.5% vs. Biohumim 25%). The predictors for the other 2 variables are: soil treatment (control, Biohumim 12.5% vs. Biohumim 25%), drought treatment (drought vs. watering) and *Phytophtora* treatment (infected vs. no infected).

7.3.3. Statistics

We developed three maximal models: two Kaplan-Meier non-parametric models for oak emergence and survival (I and II, respectively) and one Generalized Linear Mixed Model for plant height (III) following the structure shown in Table 12. All the models included mother tree as the random effects structure.

Table 12 Summary of maximal models performed for data analysis in this experiment

Model	Response variable	Fixed effect[1]	Error distribution (power lambda link function) [2]	Sample size (n)
I	Oak emergence	$S \times A_W$	--	360
II	Oak survival	$S \times A \times B$	--	320
III	Plant height	$S \times A \times B$	Gaussian (1)	60

[1] S: soil treatment; A_W: Acorn weight; A: Abiotic stress drought treatment; B: Biotic stress *Phythoptora* infection treatment

[2]Power lambda link function [$g(\mu) = \mu^{\lambda}$] is the lambda (λ, numeric value inside the brackets) used for the monotonic transformations.

When two or more fixed effects were in the model, we used the model averaging approach as we did in the above mentioned experiments (Burnham and Anderson, 2002).

7.4. Results

7.4.1. Oak seedling emergence

The emergence was very high (89%), only 11% of acorns did not emerge (Fig. 23). In addition, we did not find any statistically significant differences in seedling emergence depending on soil treatment (χ^2 =2.700, *P*=0.300; Fig. 23).

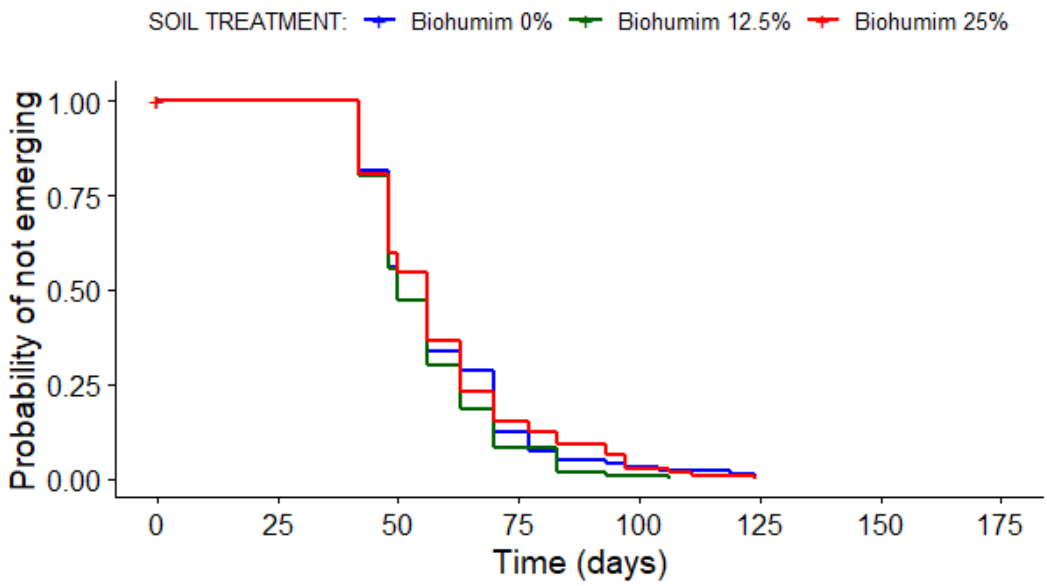
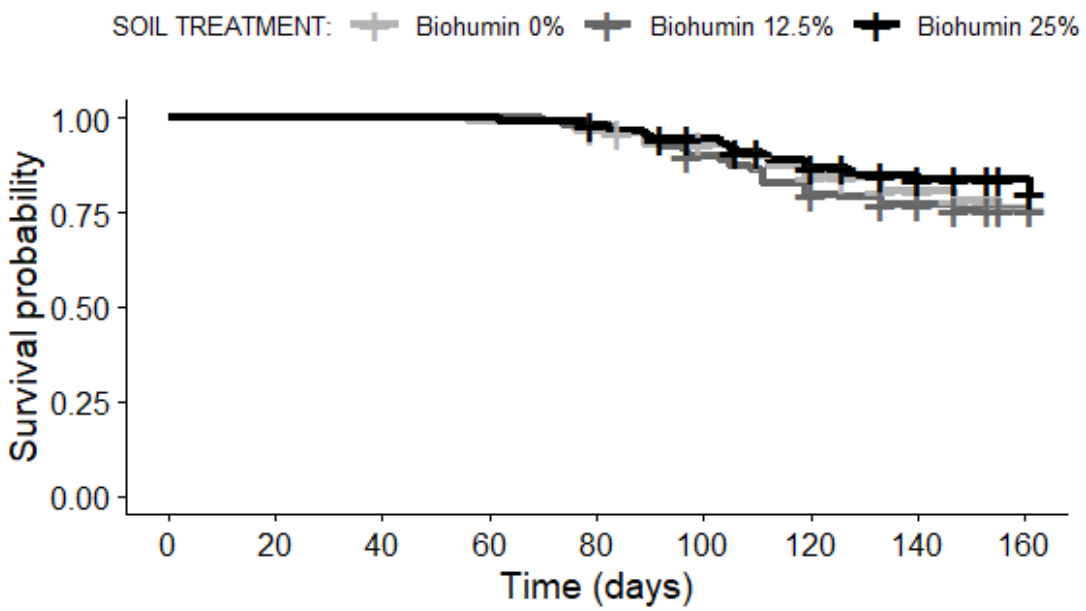


Fig. 23 : Emergence of oak seedling depending on soil treatment (N= 360 acorns). In legend, Biohumim 0%= seedlings that did not received Biohumim treatment; Biohumim 12.5% = seedlings that received 12.5% Biohumim treatment; Biohumim 25% = seedlings that received 25% Biohumim treatment.

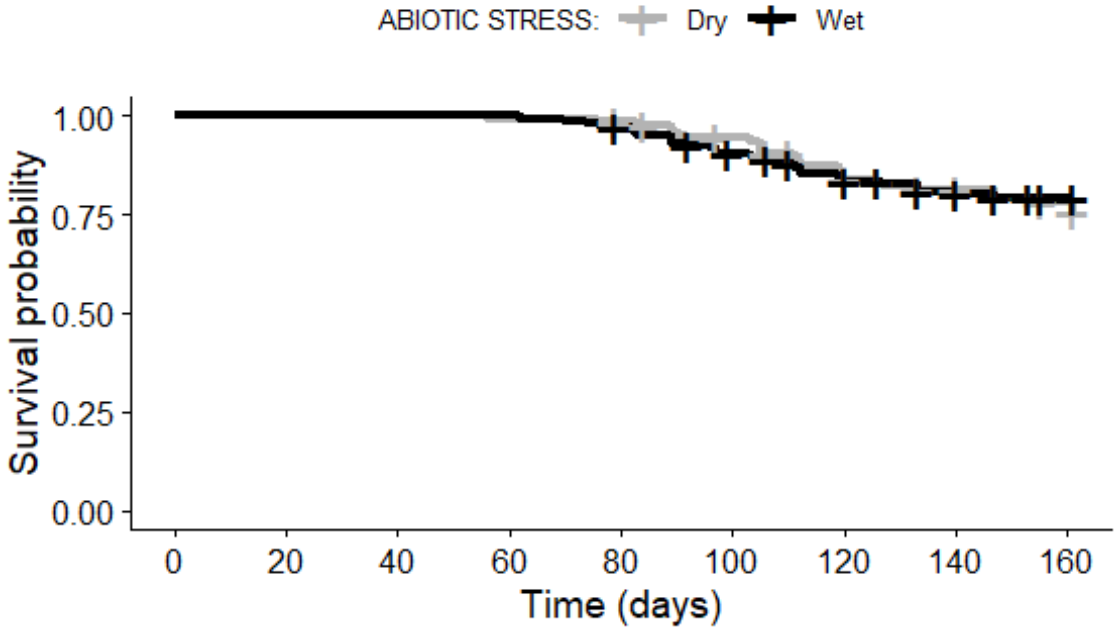
7.4.1. Oak seedling survival

The survival was 79.69% of plants at the end of August (Fig. 24). We did not find significant differences in seedling survival depending on soil treatment (χ^2 =1.700, P=0.400; Fig. 24.i) and abiotic stress (χ^2 =0,001 P=1.000; Fig. 24.ii)). However, we found significant differences in seedling survival depending on biotic stress (χ^2 =72.200, P<0.001; Fig. 24.iii) with higher survival for controls than for those seedlings infected by *P. cinnamomi*.

i)


SOIL TREATMENT:
Biohumin 0%
Biohumin 12.5%
Biohumin 25%
Survival probability
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
120
140
160
Time (days)

ii)


ABIOTIC STRESS:
Dry
Wet
Survival probability
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
120
140
160
Time (days)

iii)

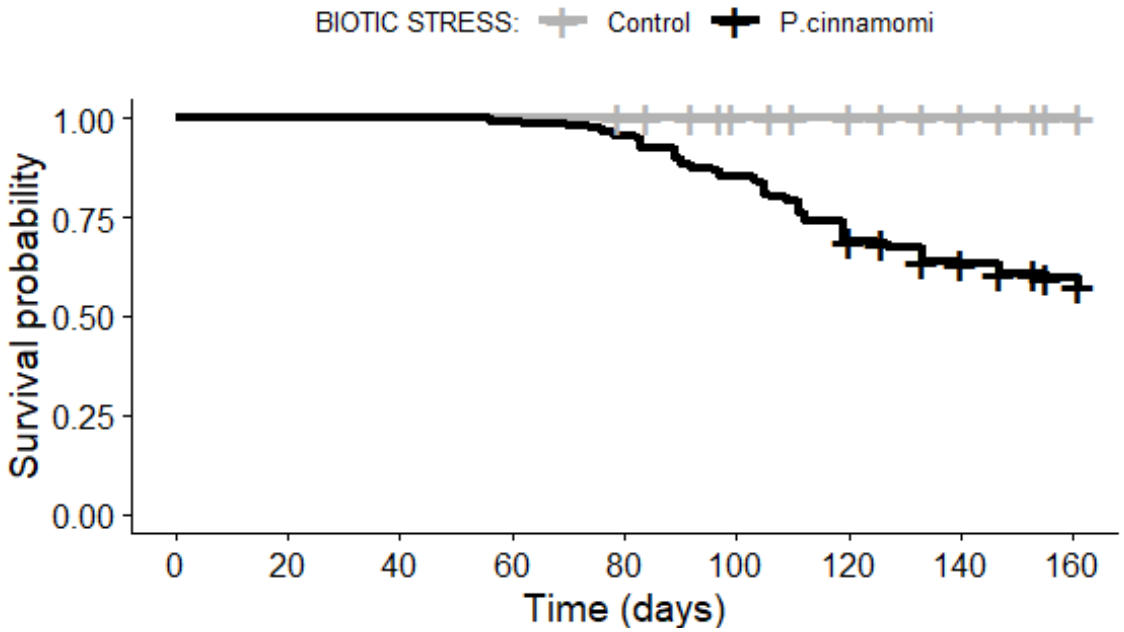


Fig. 24 : Survival of oak seedling depending on: i) soil treatment; ii) abiotic stress; iii) biotic stress (N= 320 seedlings). In legend, i) Biohumim 0%= seedlings that did not received Biohumim treatment, Biohumim 12.5% = seedlings that received 12.5% Biohumim treatment, Biohumim 25% = seedlings that received 25% Biohumim treatment; ii) Dry = seedlings subjected to a drought cycle, Wet = seedlings watered throughout the experiment; iii) Control = seedlings not infected by *P. cinnamomi*, *P. cinnamomi* = seedlings infected by *P. cinnamomi*

7.4.2. Plant height

We find significant differences in plant height depending on soil treatment, abiotic stress and their interaction (Table 13). Seedlings that received drought cycles showed, in general lower heights than those without drought cycles; however, we did not find differences when seedlings received low proportion of Biohumim (12.5%; Fig. 25). Control seedlings had larger heights than seedlings treated with Biohumim at 25%.

Table 13 Summary of the top linear mixed models (ΔAIC < 2) to analyze the plant height depending on soil treatment, abiotic stress and biotic stress

Variables	*Importance*	*d.f.*	*LR χ2*	*P*
Soil treatment (S)	1.00	2	12.943	**0.002**
Abiotic stress (A)	1.00	1	5.318	**0.021**
Biotic stress (B)	1.00	1	0.522	0.470
S × A	1.00	2	9.365	**0.009**
S × B	1.00	2	0.836	0.658
A × B	1.00	1	2.268	0.132
S × A × P	0.99	2	4.073	0.131

Importance: Importance of predictor variable in the model averaging.
Bold type indicates statistical significance ($P<0.05$).

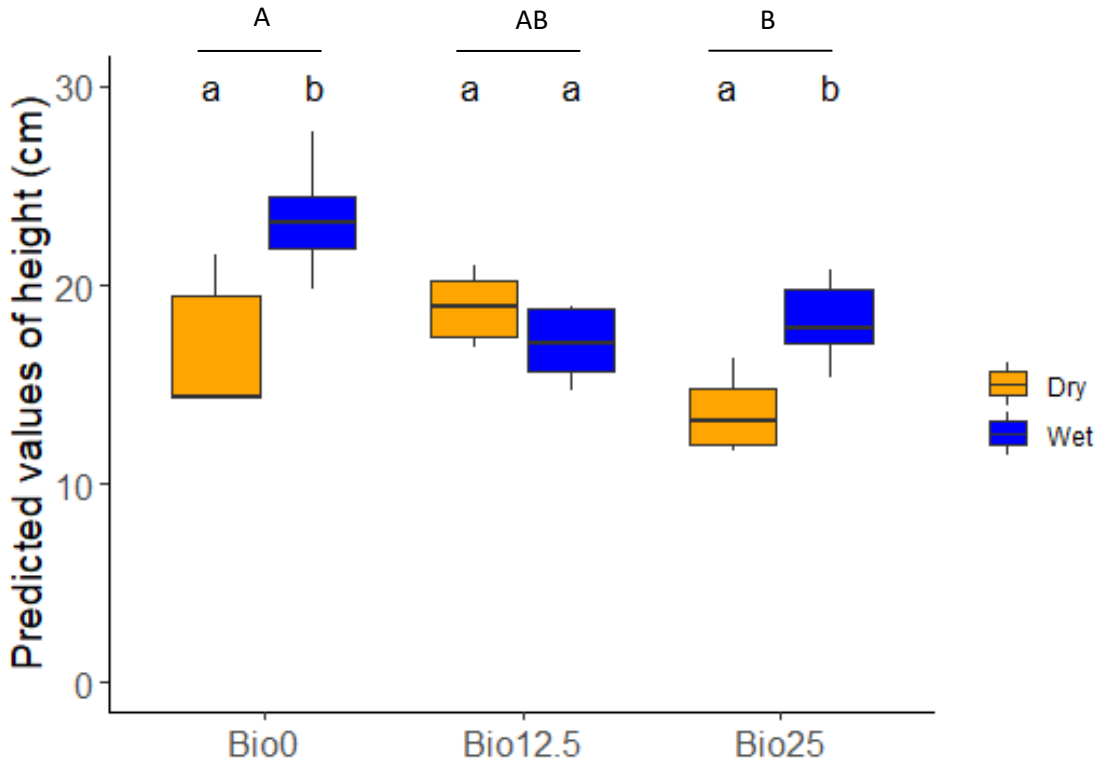


Fig. 25 : Predicted values of height depending on soil treatment-abiotic stress interaction (N= 60 seedlings). In X axis, Biohumim 0%= seedlings that did not received Biohumim treatment, Biohumim 12.5% = seedlings that received 12.5% Biohumim treatment, Biohumim 25% = seedlings that received 25% Biohumim treatment. In legend, Dry = seedlings subjected to a drought cycle, Wet = seedlings watered throughout the experiment.

8. Discussion

8.1. Tree layer

Our results have shown some effects of ecological substrates (e.g. OptiPlus) in soil improvement reducing crown defoliation of adult trees. We have used two variables to identified the 'Seca' desease in canopies: crown defoliation rate and the crown area loss rate. 'Seca' acelerates progressive defoliation (Duque-Lazo *et al.*, 2018). However, the massive crown defoliation is not only linked to an oomycete presence (e.g. *P.cinnamomi*); but also to other environmental factors (de Sampaio e Paiva Camilo-Alves *et al.*, 2013). For example, soil characteristics and water deficit in soil are important drivers of canopy decline (Sapp *et al.*, 2019). Hence, it is important to find

adequate tools to improve soil condition, minimizing both the abiotic and the biotic sources of stress. A high oak mortality at landscape level prompt an individual selective removal or, in many cases, over-harvesting, which causes high economic cost of oak treatment, removal and replacement in response to Seca infestation (Kovacs *et al.*, 2011).

We found a positive effect of OptiPlus product as a soil improvement treatment which reduced canopy defoliation; however, for crown area loss rate variable, this amelioriation was only effective in high slopes. Thus, OptiPlus product treatment buffered the stress generated by an increasing slope, which, in turn, causes shallower and less fertile soils. Regarding OptiFer product, we did not find better results when compared with OptiPlus. Nevertheless, further research is needed, since many factors (e.g. runoff, drought, management) are involved in the study area that can modify the effects of these products between pairs of trees. It might be interesting to analyze the application of these products with different types of management well identified and develop soil analysis to examine the possible changes in soil composition after the application of these ecological products. An adequate grazing management may be a tool to improve soil properties along with the application of soil ecological treatments; and therefore to avoid oak decline as a consequence of long-term inadequate land use (Asner et al. 2004), increasing ecosystem resilience to 'Seca'.

In addition, we suggest extending the experiment for several years, with multiple times of product application per year (e.g. one in spring and one in autumn) to avoid liquid losses over time and to reinforce the positive effects observed in this preliminary short-term experiment. We also expect that the time of application (e.g. summer vs. winter) will have a significant effect on the response of trees to defoliation.

8.2. Oak regeneration

In general, at oak regeneration level, our results showed no 'Seca' symptoms despite they are located in an area severely affected by this disease. At the end of experiment, recruit survival was higher than 97%. In addition, we have analysed two variables related to plant size over the study year: plant height rate and crown area rate. Ecological treatments (Biohumim) did not change or improve the plant size of recruits. We only found differences in plant growth (plant height) between oak species (higher

for holm oak species) and between contrasting sites (higher in north-facing sites). We suggest, once more, extending the experiment for several years. A year is probably not enough time to see changes in the application of solid ecological treatments. The distribution of Biohumim at the plant base (it was not buried to avoid root damage) needs some time to infiltrate and mix with the soil. We suspect that this was the main reason why we did not find any statistically significant differences.

In addition, we have also evaluated the herbivory rate on oak recruits. For this variable, as expected, we found that protectors strongly reduced herbivory on recruits. López-Sánchez *et al*. (2019) found a herbivory reduction on seedlings protected in Californian oak woodlands. However, protected recruit plants that received OptiPlus treatment in soil were heavily browsed thant those protected recruits without liquid soil treatment. Physiological compounds generated on treated plants might atract or repel herbivores. In this particular case, Optiplus is a nutrient supplement for plants, which contains important deficietary minerals [organic N, P, organic organic, S, Ca, Mg, and trace elements (Fe, Mn, B, Zn)], for both plants and animals (Gambín *et al.*, 2017), which probably increased the palatability of the plant for the main herbivores (wild and domestic ungulates). However, OptiFer, a poorer fertilizer with only three main elements (Fe, Mn, Mg) showed no significant effect on herbivory intensity.

In addition, we found less herbivory damage on holm oaks (*Q. ilex*) than on cork oaks (*Q. suber*) in line with previous research (Bugalho and Milne, 2003). Over the last decades, populations of wild ungulates have increased dramatically in the Northern Hemisphere (Gordon *et al.*, 2004; San Miguel *et al.*, 2010) and intensification of domestic ungulates has been exerted in some rangelands (Pulido *et al.*, 2001; Plieninger *et al.*, 2003; Plieninger *et al.*, 2011), causing a significant and widespread increase of herbivore pressure. The stressing effect of herbivory could produce instability on the performance and development of plants during their growth (Møller and Shykoff, 1999). Since herbivory pressure (biotic stress) is expected to increase in the coming decades as long as ecosystems are still managed inadequately, further studies should be conducted to analyse the interaction between herbivory and the ecological treatments applied in the soil.

Regarding oak acorn plantation, we could not properly develop the experiment due to a severe spring-summer drought (106 mm), which strongly affected the germination

and survival of plants. The annual rainfall of study year was 50% lower than the mean of the last 20 years (see section 2). Anthropogenic climate change is strengthening many sources of **abiotic stress**, such as extreme temperature, water deficit and water-logging periods (IPCC, 2007), generating a strong stress in many plant species (Valladares, 2004). The increase of frequency and intensity of severe climate events are particular risk factors in the 'Seca' disease context (Brasier, 1996; Duque-Lazo *et al.*, 2018). Drought is an important factor of failed oak recruitment (Rey-Benayas, 1998; Pulido and Díaz, 2005) and here we confirmed that only 8% of acorns emerged and only one single oak seedling survived after summer. Therefore, we could not really check the efficacy of Biohumim due to the extremely low sample size.

The perpetuation of any natural ecosystem mainly depends on its regeneration. Those species unable to regenerate jeopardize their continuity and presence in the ecosystem (Schemske *et al.*, 1994). Therefore, oak recruitment is necessary for population regeneration (Gibbons *et al.*, 2008) to avoid the desertification process. In areas affected by 'Seca' the probability of oak regeneration will be lower because little by little adult trees (source of acorns) are gradually dying. A low numbers of young oaks compared to adults suggests that populations are not demographically balanced (Callaway and Davis, 1998).

The lab (greenhouse) experiments represent a very promising line as all ecological conditions are under control (same soil, temperature, water, light, etc.). In addition, we were able to succesfully inoculate the pathogen (*Phytophtora*), which ensured the presence and development of the disease, with clear associated signs. However, we did not find clear significant results in relation to a possible increase resistance or tolerance of treated plants (with Biohumim) to the disease. We obtained some intriguing results that deserve further long-term and deeper research. For instance, Biohumim 12.5% had a significant effect on reducing the water stress caused during the drought treatment. In contrast, and surprisingly, we did not find a significant effect when Biohumim 25% (a higher dosis) was applied. Here, we strongly recommend further studies in the lab (greenhouse) with the three products to compare their possible effects in the short and the long term. The disease inoculations procedures are known and, therefore, experiments can be now conducted in laboratory conditions more efficiently.

9. Conclusions

The 'Seca' disease causes strong damages on oaks (e.g. loss of vitality or strong defoliation) as has been recorded through multiple research studies (Moreira and Martins, 2005; Moreira *et al.*, 2006; Aronson *et al.*, 2012; Sapp *et al.*, 2019). Damaged oaks might change the local environment on which a variety of other species depend (e.g. reductions in insect and plant populations, modifications in insectivorous birds forage and nest, reductions of acorn production influencing vertebrate populations; (Rizzo, Garbelotto 2003). In addition, a declined oak layer reduce ecosystem services strongly linked to oak canopy layer such as climate regulation (including C sequestration), water regulation, soil stability and fertility; and lightly linked to oak canopy layer such as fiber provision, natural hazard prevention, fodder provision, etc (de Bello et al. 2010).

We found some positive effects of OptiPlus liquid on the decaying (defoliated) adult trees, which may contribute to increase tree resistance and tolerance against the Seca disease. However, the study period was very short and further studies are needed to properly analyze the long-term effects and the mechanisms behind this positive response of trees to Optiplus addition. However, the fertilizer Optifer (also liquid), which contained fewer nutrients and minerals did not apparently cause any significant effect on reducing adult tree defoliation. Both products were applied only once a year and further long-term and more intense studies (e.g. with 2-3 application per year) are desirable to scientifically validate the preliminary results obtained here.

Interestingly, in general, oak recruits did not show any significant "Seca" symptoms, neither before nor after the application of the different products. However, some of them (Optiplus and Biohumin pers. Comm.) increased the herbivory damage on the recruits probably as a result of an increase in plant palatability, which reinforces the idea of protecting the recruits with wire cages, even more when these products are applied. We did not obtain a significant positive effect of Biohumim product in the field (plant growth and survival) but it might be a good ecological treatment according to the preliminary results in the lab. However, further studies are needed as some results were quite intriguing (e.g. with the 25% Biohumin) and, therefore, it was not possible to obtain a clear response pattern of plants to the addition of the product and to decipher the mechanisms behind this possible response. Again, it is necessary to implement new

greenhouse studies, with more information on the effects of Biohumim on soil conditions, and to run experiments for, at least, 1-2 years to ensure theta the product is causing a significant effect on the plant. These greenhouse experiments represent a very promising research line due to the controlled conditions of the lab (same soil, temperature, water, light, etc.) and the assurance that plants have been infected (inoculated with the fungi).

In sum, the results of this project have lightly visualized the importance of soil improvement through ecological treatments and the possibility and need to explore more in depth this research line. Further research efforts should be done not only for conservation and restoration of Mediterranean oak woodlands but also to make them more resilient against global change. Hence, the solutions should consider society needs, life style and preferences of the majority; otherwise these ecosystems will progressively disappear as we know them today, losing a highly valuable heritage.

10. References

Aronson, J., Pereira, J.S., Pausas, J.G., 2012. Cork oak woodlands on the edge: ecology, adaptive management, and restoration. Island Press, Washington.

Barton, K., 2015. MuMIn: Multi-model inference. R package version 1.15.1. In.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 67, 1-48.

Blondel, J., Aronson, J., 1999. Biology and wildlife of the Mediterranean region. Oxford University Press.

Branco, M., Ramos, A.P., 2012. Coping with pests and diseases. In: Aronson, J., Pereira, J.S., Pausas, J.G. (Eds.), Cork oak woodlands on the edge: ecology, adaptive management, and restoration. Island Press, Washington, USA, pp. 103-113.

Brasier, C.M., 1993. Phytophthora cinnamomi as a contributory factor in European oak declines. In: Luisi, N., Lerario, P., Vannini, A.B. (Eds.), Recent Advances in Studies on Oak Decline. Proc. Int. Cong. Selva di Fasano. Tipolitografia-Putignano, Putignano, Italy, pp. 49-57.

Brasier, C.M., 1996. Phytophthora cinnamomi and oak decline in southern Europe. Environmental constraints including climate change. In, Annales des Sciences Forestieres. EDP Sciences, pp. 347-358.

Brasier, C.M., Moreira, A.C., Ferraz, J.F.P., Kirk, S., 1993. High mortality of cork oak in Portugal associated with Phytophthora cinnamomi. In: Luisi, N., Lerario, P., Vannini, A.B. (Eds.), Recent Advances in Studies on Oak Decline. Proc. Int. Cong., Selva di Fasano. Tipolitografia-Putignano, Putignano, Italy, pp. 461-462.

Bugalho, M.N., Milne, J.A., 2003. The composition of the diet of red deer (Cervus elaphus) in a Mediterranean environment: a case of summer nutritional constraint? For. Ecol. Manag. 181, 23-29.

Burnham, K.P., Anderson, D.R., 2002. Model selection and multi-model inference: a practical information-theoretic approach. Springer Verlag, New York, USA.

Callaway, R.M., Davis, F.W., 1998. Recruitment of *Quercus agrifolia* in central California: the importance of shrub-dominated patches. J. Veg. Sci. 9, 647-656.

Crawley, M.J., 2012. The R book. John Wiley & Sons.

de Sampaio e Paiva Camilo-Alves, C., da Clara, M.I.E., de Almeida Ribeiro, N.M.C., 2013. Decline of Mediterranean oak trees and its association with *Phytophthora cinnamomi:* a review. European Journal of Forest Research 132, 411-432.

Duque-Lazo, J., Navarro-Cerrillo, R.M., van Gils, H., Groen, T.A., 2018. Forecasting oak decline caused by *Phytophthora cinnamomi* in Andalusia: Identification of priority areas for intervention. For. Ecol. Manag. 417, 122-136.

Eviner, V.T., Likens, G.E., 2008. Effects of pathogens on terrestial ecosystem function. In: Ostfel, R.S., Keesing, F., Eviner, V.T. (Eds.), Infectious Disease Ecology: Effects of Ecosystems on Disease and of Disease on Ecosystems. Princeton University Press, Princeton, New York, USA, pp. 260-283.

Fox, J., Weisberg, S., 2011. An {R} Companion to Applied Regression. Sage, Thousand Oaks CA.

Gambín, P., Ceacero, F., Garcia, A.J., Landete-Castillejos, T., Gallego, L., 2017. Patterns of antler consumption reveal osteophagia as a natural mineral resource in key periods for red deer (Cervus elaphus). Eur. J. Wildl. Res. 63, 39.

Gibbons, P., Lindenmayer, D.B., Fischer, J., Manning, A.D., Weinberg, A., Seddon, J., Ryan, P., Barrett, G., 2008. The future of scattered trees in agricultural landscapes. Conserv. Biol. 22, 1309-1319.

Gordon, I.J., Hester, A.J., FESTA-BIANCHET, M., 2004. The management of wild large herbivores to meet economic, conservation and environmental objectives. J. Appl. Ecol. 41, 1021-1031.

IPCC, 2007. Climate Change 2007: Synthesis Report. An Assessment of the Intergovernmental Panel on Climate Change. IPCC, Cambridge.

Kovacs, K., Václavík, T., Haight, R.G., Pang, A., Cunniffe, N.J., Gilligan, C.A., Meentemeyer, R.K., 2011. Predicting the economic costs and property value losses attributed to sudden oak death damage in California (2010–2020). J. Environ. Manag. 92, 1292-1302.

Kranz, J., 1990. Fungal diseases in multispecies plant communities. New Phytol. 116, 383-405.

López-Sánchez, A., 2015. Balancing management and preservation of Mediterranean scattered oak woodlands (dehesas) in human-dominated landscapes. In. Universidad Politécnica de Madrid (Spain).

López-Sánchez, A., Peláez, M., Dirzo, R., Fernandes, G.W., Seminatore, M., Perea, R., 2019. Spatio-temporal variation of biotic and abiotic stress agents determines seedling survival in assisted oak regeneration. J. Appl. Ecol.

Møller, A.P., Shykoff, J.A., 1999. Morphological developmental stability in plants: patterns and causes. Int. J. Plant Sci. 160, S135-S146.

Moreira, A.C., Martins, J.M.S., 2005. Influence of site factors on the impact of *Phytophthora cinnamomi* in cork oak stands in Portugal. For. Pathol. 35, 145-162.

Moreira, A.C., Medeira, C., Maia, I., Quartin, V., Matos, M., Cravador, A., 2006. Studies on the association of the Quercus suber decline disease with Phytophthora cinnamomi in Portugal. In.

Müller, E., Stierlin, H.R., 1990. Sanasilva: Kronenbilder: mit Nadel-und Blattverlustprozenten. Eidgenössische Forschungsanstalt für Wald, Schnee und Landschaft, Birmensdorf, Switzerland.

Perea, R., Perea-García-Calvo, R., Díaz-Ambrona, C.G., San Miguel, A., 2015. The reintroduction of a flagship ungulate Capra pyrenaica: Assessing sustainability by surveying woody vegetation. Biol. Conserv. 181, 9-17.

Perea, R., San Miguel, A., Gil, L., 2011. Leftovers in seed dispersal: ecological implications of partial seed consumption for oak regeneration. J. Ecol. 99, 194-201.

Perea, R., San Miguel, A., Martínez-Jauregui, M., Valbuena-Carabaña, M., Gil, L., 2012. Effects of seed quality and seed location on the removal of acorns and beechnuts. European Journal of Forest Research 131, 623-631.

Plieninger, T., Pulido, F.J., Konold, W., 2003. Effects of land-use history on size structure of holm oak stands in Spanish dehesas: implications for conservation and restoration. Environ. Conserv. 30, 61-70.

Plieninger, T., Schaich, H., Kizos, T., 2011. Land-use legacies in the forest structure of silvopastoral oak woodlands in the Eastern Mediterranean. Regional Environmental Change 11, 603-615.

Plieninger, T., Wilbrand, C., 2001. Land use, biodiversity conservation, and rural development in the dehesas of Cuatro Lugares, Spain. Agrofor. Syst. 51, 23-34.

Pulido, F.J., Díaz, M., 2005. Regeneration of a Mediterranean oak: a whole-cycle approach. Ecoscience 12, 92-102.

Pulido, F.J., Díaz, M., de Trucios, S.J.H., 2001. Size structure and regeneration of Spanish holm oak Quercus ilex forests and dehesas: effects of agroforestry use on their long-term sustainability. For. Ecol. Manag. 146, 1-13.

R Development Core Team, 2019. R: A Language and Enviroment for Statistical Computing. In. Foundation for Statistical Computing, Vienna, Austria.

Rey-Benayas, J.M., 1998. Growth and survival in Quercus ilex L. seedlings after irrigation and artificial shading on Mediterranean set-aside agricultural land. In, Annales des Sciences Forestieres. EDP Sciences, pp. 801-807.

Rivas-Martínez, S., Fernández-González, F., Loidi, J., Lousã, M., Penas, A., 2001. Syntaxonomical checklist of vascular plant communities of Spain and Portugal to association level. Itinera geobotanica.

San Miguel, A., Perea, R., Fernández-Olalla, M., 2010. Wild ungulates vs extensive livestock. Looking back to face the future. Options Méditerr. A (92), 27-34.

Sánchez, M., Caetano, P., Ferraz, J., Trapero, A., 2002. Phytophthora disease of Quercus ilex in south-western Spain. For. Pathol. 32, 5-18.

Sapp, M., Tyborski, N., Linstädter, A., López Sánchez, A., Mansfeldt, T., Waldhoff, G., Bareth, G., Bonkowski, M., Rose, L.E., 2019. Site-specific distribution of oak rhizosphere-associated oomycetes revealed by cytochrome c oxidase subunit II metabarcoding. Ecology and Evolution 9, 10567-10581.

Schemske, D.W., Husband, B.C., Ruckelshaus, M.H., Goodwillie, C., Parker, I.M., Bishop, J.G., 1994. Evaluating approaches to the conservation of rare and endangered plants. Ecology 75, 584-606.

Underwood, E.C., Viers, J.H., Klausmeyer, K.R., Cox, R.L., Shaw, M.R., 2009. Threats and biodiversity in the mediterranean biome. Divers. Distrib. 15, 188-197.

Valladares, F., 2004. Ecología del bosque mediterráneo en un mundo cambiante. Ministerio de Medio Ambiente Madrid.

Venables, W.N., Ripley, B.D., 2002. Modern applied statistics with S. Springer.

Wang, X., Vignjevic, M., Jiang, D., Jacobsen, S., Wollenweber, B., 2014. Improved tolerance to drought stress after anthesis due to priming before anthesis in wheat (Triticum aestivum L.) var. Vinjett. J. Exp. Bot. 65, 6441-6456.

Waring, R.H., Cromack, K., Matson, P.A., Boone, R.D., Stafford, S.G., 1987. Responses to pathogen-induced disturbance: decomposition, nutrient availability, and tree vigour. Forestry 60, 219-227.