# Title: Facilitating the afforestation of Mediterranean polluted soils by nurse shrubs

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## Abstract

The revegetation of polluted sites and abandoned agricultural soils is critical to reduce soil losses and to control the spread of soil pollution in the Mediterranean region, which is currently exposed to the greatest soil erosion risk in Europe. However, events of massive plant mortality usually occur during the first years after planting, mainly due to the adverse conditions of high irradiance and drought stress. Here, we evaluated the usefulness of considering the positive plant-plant interactions (facilitation effect) in the afforestation of polluted agricultural sites, using pre-existing shrubs as nurse plants. We used nurse shrubs as planting microsites for acorns of *Quercus ilex* (Holm oak) along a gradient of soil pollution in southwestern Spain, and monitored seedling growth, survival, and chemical composition during three consecutive years. Seedling survival greatly increased (from 20% to more than 50%) when acorns were sown under shrub, in comparison to the open, unprotected matrix. Facilitation of seedling growth by shrubs increased along the gradient of soil pollution, in agreement with the stress gradient hypothesis that predicts higher intensity of the facilitation effects with increasing abiotic stress. Although the accumulation of trace elements in seedling leaves was higher underneath shrub, the shading conditions provided by the shrub canopy allowed seedlings to cope with the toxicity provoked by the concurrence of low pH and high trace element concentrations in the most polluted sites. Our results show that the use of shrubs as nurse plants is a promising tool for the cost-effective afforestation of polluted lands under Mediterranean conditions.

**Keywords**: nurse plant; soil remediation; soil pH; *Quercus ilex*; *Retama sphaerocarpa.*

**Highlights:**

- We assessed the potential use of nurse shrubs for the afforestation of Mediterranean polluted soils.
- Holm oak acorns were sown in different planting microsites along a gradient of soil pollution
- Shrub cover greatly increased oak seedling survival during the first three years after sowing.
- The facilitation of oak seedling growth by shrubs increased along the pollution gradient.
- Using nurse shrubs is a promising tool for the afforestation of polluted agricultural lands.

## 1. Introduction

The revegetation of degraded sites and abandoned agricultural soils is critical to reduce soil losses in the Mediterranean region, which is currently exposed to the greatest soil erosion risk in Europe (Grimm et al., 2002; Panagos et al., 2015). Among degraded sites, the revegetation of polluted sites should be prioritised, as the increase in soil erosion could lead to the spread of pollutants from polluted spots. However, establishing a woody plant cover in these degraded sites is a challenging task, given that the environmental conditions in these sites are, in general, far from similar to those in which natural regeneration occurs.

In barren polluted sites, such as those affected by mining activities, vegetation is often poorly developed and the soil surface is exposed to high irradiance; soils are usually poor in organic matter, and their structure is frequently altered (Tordoff et al., 2000; Walker, 2002), leading to a decreased water holding capacity (Stocking and Murnaghan, 2001). All these factors strongly reduce seedling survival during the dry season, which constitutes one of the most limiting demographic processes for regeneration in Mediterranean woody plant species (Pérez-Ramos et al., 2012; Pulido and Díaz, 2005; Rey and Alcántara, 2000). Consequently, mortality rates during the first years after planting are usually very high in Mediterranean degraded sites (Gómez-Aparicio et al., 2004; Navarro-Cerrillo et al., 2005; Pausas et al., 2004), and the afforestation of large degraded areas poses a huge cost for local and regional authorities.

The need for alternative afforestation techniques prompted a number of studies during the last decade to explore the potential application of positive plant-plant interactions for the restoration of degraded sites (Castro et al., 2004; Gómez-Aparicio et al., 2004; Maestre et al., 2001). Based on results obtained in these studies, many authors have called for a change in the paradigms of traditional afforestation techniques towards a new conceptual framework that considers the spatial heterogeneity of vegetation structure and promotes these positive plant-plant interactions (Gómez-Aparicio, 2009; Padilla and Pugnaire, 2006; Rey-Benayas et al., 2008). Many studies in Mediterranean ecosystems have reported that the presence of pioneer shrub species (often called nurse plants) facilitates the establishment of other late-successional species under their canopies, mainly due to the amelioration of extreme temperature conditions and the improvement of plant water status (Callaway, 1992; Castro et al., 2004; Gómez-Aparicio et al., 2005; Padilla and Pugnaire, 2009), but also by the concurrence of better soil conditions under the shrubs (Pugnaire et al., 1996). In addition, facilitation by nurse plants may also be mediated by indirect underlying mechanisms when the nurse species promotes other mutualistic or beneficial interactions with soil microorganisms, such mycorrhizal species (Goberna et al., 2007; Gonzalez-Polo et al., 2009; Duponnois et al., 2011; Martinez-Garcia et al., 2011). The target species may also benefit from the release of herb competition for water and nutrients under the nurse shrubs (Cuesta et al., 2010).

Positive plant-plant interactions are expected to be especially beneficial under high abiotic stress (Callaway et al., 2002; Lortie and Callaway, 2006), and therefore the application of the facilitation effect could be particularly useful in highly disturbed environments (Brooker et al., 2008; Pueyo et al., 2008; Zsereva and Kozlov, 2007). Surprisingly, there are very few examples of the application of these techniques in the restoration of polluted sites. In these sites, the presence of nurse shrubs might have additional benefits to the target species, given that the higher levels of soil organic matter detected under the shrub canopy (Ginocchio et al., 2004) as well as the stabilisation of pollutants in the nurse root system (Domínguez et al., 2009; Frèrot et al., 2006) could reduce the levels of bioavailability of some pollutants. Conversely, airborne pollutants can be captured and accumulated more intensively under the shrub canopy than in open gaps, resulting in a better performance of the target species at a certain distance from the shrub canopy (Eränen and Kozlov, 2007). To date, very few studies have assessed the role of the chemical stress (i.e. high concentrations of toxic elements in the soils) in the intensity of the facilitation by shrubs. According to the abiotic stress gradient hypothesis (Bertness and Callaway, 1994; Callaway et al., 2002; Pugnaire and Luque, 2001), the intensity of the facilitation effect enhances with increasing stress. Therefore, given similar levels of water and light conditions, it would be expected that the facilitation effect provided by shrubs will be higher as the chemical stress (i.e. soil pollution) increases. Here, we aimed to test this hypothesis.

In this study, we evaluated the effectiveness of using nurse shrubs as planting microsites for acorns of *Quercus ilex* (Holm oak) along a gradient of soil pollution in the Guadiamar River Valley (southwestern Spain). Soils in this area were affected by a mining accident that polluted them with trace elements, mainly As, Cd, Cu, Pb, and Zn (Domínguez et al., 2008). We followed a two-phase restoration strategy, first selecting shrubs that were planted during the initial afforestation of the area (after the accident), and secondly, planting acorns under the shrubs as potential facilitators of oak recruitment. This multi-phase technique, which attempts to mimic the natural sequence of the successional process (i.e. herb-shrub-tree), could result in a valuable restoration tool of degraded areas, as previously proposed for other Mediterranean non-polluted forests (Gómez-Aparicio et al., 2004; Siles et al., 2008). Specifically, we were interested in testing that: 1) the presence of shrubs have a significant effect on soil properties, particularly organic matter, pH, and nutrient content, which results in a better nutritional status of the target plant and in a lower accumulation of trace elements in the aboveground biomass; 2) microsites under shrubs are more favourable for emergence, survival, and growth of oak seedlings than the open, unprotected microsites; 3) the intensity of facilitation increases along a gradient of soil pollution (chemical stress). As a secondary objective, we aimed to test whether the intensity of the facilitation is higher when a pioneer shrub species (*Retama sphaerocarpa*), rather than a late-successional shrub (*Phillyrea angustifolia*), is used as a nurse in these harsh environments. Understanding the effectiveness of different nurse plants constitutes an issue of major interest for the conservation and restoration of degraded ecosystems (Gómez-Aparicio, 2009; Rolo et al., 2013).

## 2. Material and Methods

### *2.1. Study site and species*

The Guadiamar River Valley is located in southwestern Spain. The climate is Mediterranean-type with an annual average of about 2900 h of sunshine and maximum values of solar radiation exceeding 1000 W $m^{-2}$. The average annual temperature is 19 ºC, the average annual rainfall is 610 mm, and potential evapotranspiration is 774 mm.

The area was affected by a large mining accident in 1998, contaminated the soils, mostly under agricultural production, with As, Cd, Cu, Pb, Tl, and Zn (Cabrera et al., 1999; Garralón et al., 1999). Although sludge and contaminated topsoils were removed after the accident, the underlying soils still contained high concentrations of trace elements (Moreno et al., 2001). In 1999–2001 the affected area was afforested using native Mediterranean shrub and tree species as part of a large soil remediation and phytomanagement programme (Domínguez et al., 2008). Plantations followed the traditional technique using regular planting grids, with densities ranging from 480 to 980 plants per hectare. The success of these plantations was very irregular; plant species in the higher terraces showed higher mortality rates, which was positively related to the drought stress and the high levels of soil pollution, while riparian species showed the highest survival and growth rates (Domínguez et al., 2010a). A monitoring survey carried out eight years after the plantations showed that shrub species contributed the highest percentage of cover (Rodríguez et al., 2009).

We selected Holm oak (*Quercus ilex* subsp. *ballota* Desf. Samp), which is the most common late-successional tree in the native forests in the area, as the target species to afforest. We tested the potential of two of the most common shrub species planted in the area as nurse plants for the establishment of Holm oak seedlings: *Retama sphaerocarpa* and *Phillyrea latifolia*. In one of the experimental sites where *P. latifolia* was absent (Site 2, see below), wild olive saplings (*Olea europea*) of similar age and height were used instead as late-successional nurse plants.

### *2.2. Experimental design*

Four sites were selected along a gradient of soil pollution in the Guadiamar Valley, determined by the distance to the pollution source, based on previous surveys of the spatial distribution of soil and plant trace elements along the Valley (Domínguez et al., 2008). All these sites were under agricultural or pasture production before the accident (1998), and were afforested between 1999 and 2001 during the implementation of the remediation programme.

Holm oak acorns were collected in October–December 2005 from native, non-contaminated forests in the Valley. Unhealthy acorns were discarded using the flotation method (Gribko and Jones, 1995), and the rest of the acorns were stored at 2–4 ºC until used. In December 2005 the selected acorns were sown in 15 experimental units per site. Each experimental unit consisted of a pair of planting microsites, separated by a maximum distance of 10 m: a) SHRUB, under the canopy of the selected shrub species, and b) OPEN, in the open sites, without the protection of any plant cover. From the 15 experimental units per site, 8 had *R. sphaerocarpa* individuals as nurse shrub, and 7 had *P. angutifolia*/*O. europaea* individuals. In each of the planting microsites, 8 acorns were sown horizontally, protected by wire cages (25 × 25 × 25 cm) to exclude seed predators. Previously, acorns were weighed individually to ensure homogeneity in the size of the used acorns. A total of 960 seeds were sown. Under shrubs, acorns were systematically sown at the southeast face of the shrubs.

### *2.3. Microsite environment characterisation*

To analyse the effect of shrubs on microenvironmental conditions, we characterised some key above and belowground properties in each experimental unit. Light availability was quantified by hemispherical photography using a digital camera (Coolpix 4500, Nikon) aimed at the zenith, with a fish-eye lens with 180 degree field of view (FCE8, Nikon). Photographs were taken at dawn, sunset, or under cloudy conditions. Images were analysed using Hemiview canopy analysis software ver. 2.1 (1999, delta-T Devices, Cambridge, UK). Global Site Factor (GSF) ranging from 1 (lack of plant cover) to 0 (complete cover) was used as an integrative index of light availability at the ground level.

Soil moisture (soil volumetric water content, SVWC) was measured at every routine visit to the experimental microsites (see details below) using a time-domain reflectometer (TDR, Campbell Scientific Inc., Logan, UT, USA) with 12 cm depth rods. At least two measurements were taken and averaged in each experimental unit at each time. Soil samples (0–10 cm) were collected from each of the experimental microsites in spring 2008 using an auger of 3 cm diameter. Several samples were collected and mixed at each planting microsite to produce a composite soil sample per microsite and experimental unit (a total of 120 soil samples). Samples were air dried and sieved to < 2 mm to analyse standard chemical and physical properties: pH, organic matter content (Walkley and Black, 1934), total organic-N content (Kjeldahl digestion; Kammerer et al. 1967), available P (Olsen et al. 1954), and available K (1M ammonium acetate extraction and determination by atomic emission spectroscopy, Bower et al. 1952). A fraction of each soil sample was also ground in an agate mortar to <1 mm for trace elements analysis. Soil samples were digested using 'aqua regia' (1:3 concentration $HNO_3$:HCl) in a microwave oven (Microwave Laboratory Station Mileston ETHOS 900, Milestone s.r.l., Sorisole, Italy) and analysed for trace elements concentrations (As, Cd, Cu, Mn, Pb, Tl, and Zn) by ICP-MS (inductively coupled plasma-mass spectroscopy, Perkin Elemer, Sciex-Elan 5000). In a subset of 10 of the 15 experimental units per microsite (a total of 80 soil samples), soil microbial biomass was also analysed in fresh samples within 48 h after collection using the fumigation-extraction method (Gregorich, 1990).

Because the presence of shrubs might also improve oak seedling performance by releasing competition with herbs for water and nutrients (Cuesta et al., 2010), we estimated the abundance of herbaceous plant species in each experimental unit and microsite in the late spring of 2007 by harvesting the aboveground biomass contained in two 25 × 25 cm quadrats per microsite. Samples were oven dried at 70 ºC for 48 h and further weighed.

### 2.4. *Seedling monitoring and growth analysis*

We monitored seedling emergence, growth, and survival over three consecutive years (from January 2006 to December 2008). During the first three months after sowing the experimental units were visited fortnightly to record seedling emergence and survival. Afterwards, the experimental units were monitored once per month during the first year, and once per season during the second and the third year. The height of the emerged plants was also measured at each visit using a ruler.

In order to conduct a detailed analysis of seedling growth and morphology, we performed non-destructive measurements of stems and leaves on a subsample of the emerged seedlings. For each experimental unit and microsite, a seedling was randomly selected and marked (N = 15 seedlings per microsite and site) at the beginning of the growing season of the second year (March 2007). We preferred not to quantify seedling growth for the first year due to their recognised primary dependence on seed mass (Pérez-Ramos et al., 2010). Length and diameter at the base and the top of each stem and branch were recorded to calculate stem/branch volume. The number of leaves was counted, and the length and width of each leaf was measured using a digital caliper. These measurements were taken at the beginning (March–April 2007) and end (June–July 2007) of the growing season in order to estimate relative growth rates (see below). The same measurements were conducted one year later (2008) on the same marked seedlings to estimate growth rates during the third growing season.

To estimate plant biomass from these non-destructive measurements, a subset of seedlings (four per site and microsite) was harvested and transported to the laboratory in a chilled container. There, a calibration between the non-destructive and destructive biomass measurements was conducted. Allometric relationships between dry biomass measured in the laboratory and field measurements were established for each type of microsite ($r^2 > 0.93$). Total aboveground biomass (M) of each marked seedling in the field was then calculated for each sampling date, and aboveground relative growth rates (RGR) were calculated as $RGR = (\ln M_1 - \ln M_0)/t$, where $M_0$ and $M_1$ are the total biomass values at the beginning and end of each growing season, respectively, and t is the time interval (number of days) between the two dates (Hunt, 1978).

### *2.5. Plant chemical analysis*

At the end of the experimental period (autumn 2008), a subset of the surviving seedlings (five per site and microsite) was harvested to measure their final biomass and analyse the chemical composition of their roots and leaves. For the collection of the root samples, the surrounding soil was carefully excavated, as deep as possible, and the maximum depth of the rooting system was recorded. For chemical analyses, only the top 10 cm of the root length were considered. Leaves, stems, and roots were washed thoroughly with distilled water, dried at 70 ºC for at least 48 h, weighed, and ground using a stainless-steel mill. Plant material was digested in a microwave oven using concentrated $HNO_3$. Macronutrients (except N) were analysed by ICP-OES (Inductively Coupled Plasma Optical Emission Spectrophotometry; Thermo Jarrel Ash Corporation). Trace elements were analysed by ICP-MS. The quality of the analysis was assessed using reference materials NCS DC 73350 (white poplar leaves, China National Analysis Center for Iron and Steel) and INCT-TL-1 (tea leaves, Polish Institute of Nuclear Chemistry and Technology). Our experimental values showed recoveries from the certified values of 81 to 105%. Isotopic analyses of C and N in the leaf samples were performed using a continuous flow elemental analyser-isotopic ratio mass spectrometer (EA Thermo 1112-IRMS Thermo Delta V Advantage). The precision for both the $\delta^{13}C$ and $\delta^{15}N$ analyses was approximately 0.2 per mil.

### *2.6. Data analysis*

A principal component analysis (PCA) was applied to explore the patterns of covariation among all the studied environmental variables. Previously, variables were log-transformed. To test for the effect of nurse shrubs on environmental properties (soil characteristics, herb biomass and light availability, previously log-transformed), a mixed-linear model was applied, with microsite type (shrub vs. open) as a fixed factor and site as a random factor. Likewise, similar models were applied to test for the effect of nurse shrubs on seedling growth rates and chemical composition.

We applied generalised linear models (GLZ) to analyse the effect of shrubs on seedling emergence. Emergence was considered as a binomial variable, whereas emergence time (period of time between the sowing date and the date of emergence) followed a gamma distribution. GLZ models were also applied to study the influence of the different microsite environmental conditions (seasonal soil moisture values, light availability, soil physico-chemical properties, and herb biomass) on emergence time, as well as on different parameters related with seedling growth (aboveground biomass and RGR). In all these analyses, soil Cd concentration was used as an index of soil pollution, given that the concentrations of all the metals deposited on the soil by the mine spill are largely interrelated (Domínguez et al., 2008), and that Cd showed the highest levels of bioavailability after the remediation of the area (Domínguez et al., 2009). Soil moisture values were averaged by season to have some indicators of the seasonal moisture levels at each experimental unit.

The distribution of the seedling survival times was analysed by Kaplan-Meier function estimations using the Gehan’s Wilcoxon test for the comparison of the survival curves between microsites and across sites. We used the Cox's Proportional Hazard Model as a regression model to estimate the influence of the environmental variables on survival times. All the mentioned analyses were performed using STATISTICA v.7 (StatSoft Inc., Tulsa, OK, USA).

To summarise the effect of shrub presence on the demographic processes and traits considered in this study (emergence, survival, growth, and accumulation of trace elements), we used a modified Relative Neighbour Effect index (RNE, Markham & Chanway, 1996). The original RNE ranges from -1 to 1, with negative values indicating facilitation and positive values indicating competition. In our case, we calculated the inverse of this index so that facilitation effect could be indicated by a positive index for an easier interpretation (Gómez-Aparicio et al., 2004). For seedling emergence ($E$), RNE was calculated for each site as $\mathrm{RNE}_E = (E_{sb} - E_{op})/E_{max}$, where $E_{sb}$ is emergence rate under nurse shrubs (*sb*), $E_{op}$ is the emergence rate in the open microsites (*op*), and $E_{max}$ is the emergence rate in the site with the greatest emergence in the pair. Similarly, for seedling survival ($S$), $\mathrm{RNE}_S$ was calculated as $\mathrm{RNE}_S = (S_{sb} - S_{op})/S_{max}$. For seedling growth ($G$), $\mathrm{RNE}_G$ was calculated as $\mathrm{RNE}_G = (RGR_{sb} - RGR_{op})/RGR_{max}$, where $RGR_{sb}$ is the mean relative growth rate (RGR) in the shrub microsite, $RGR_{op}$ is the mean relative growth rate in the open microsite, and $RGR_{max}$ is the maximum growth value in each site. We also used Zn and Cd leaf concentrations (C) to calculate a $\mathrm{RNE}_A$ for trace element accumulation ($A$) in each site: $\mathrm{RNE}_A = (C_{sb} - C_{op})/C_{max}$.

## 3. Results

### *3.1. Soil properties and effect of shrub cover on microsite*

Soils from the different sites had contrasted pH and trace element concentrations. In the most contaminated site (Site 3), extremely acid soils (pH < 4) were frequently found, as the result of the oxidation of the remnants of sludge deposited on the soil during the mining accident (Table S1). Main pollutants were As, Cd, Cu, Pb and Zn, for which concentrations in the most polluted site were up to 133, 0.86, 180, 250 and 247 mg $kg^{-1}$, respectively (Table S1).

Some of the aboveground and belowground microsite properties showed a strong pattern of covariation. A PCA applied to all the studied environmental variables, with all sites and microsites pooled, revealed that those soils located in polluted sites (i.e. with higher values of soil Zn and Cd) tended to have lower soil pH and lower soil moisture during spring and summer. Herb biomass was more closely associated to winter soil moisture and soil organic matter than to light availability, and soil microbial biomass was basically driven by soil pH (Figure 1, Appendix Table S2).

The presence of shrub had a limited influence on soil properties. Most of the variability in the studied soil variables was determined by the differences across sites, and not between microsites (shrub vs. open, Appendix Table S3). Only organic matter content was significantly higher in the soils under shrub than in the open microsites (mixed-linear model, microsite effect: $F$ =143.15, $p$ = 0.023). For organic N content, there was a significant site × microsite interaction ($F$ = 6.52, $p$ < 0.001). Light availability, as expected, was clearly lower under shrubs (mixed-linear model, microsite effect: $F$ =117.39, $p$ =0.002).

When distinguishing between nurse types, we found that under the pioneer shrub (*R. sphaerocarpa*) the amount of light reaching the ground level tended to be higher ($F$ = 7.33, $p$ =0.07) than under the late-successional shrub (*P. angustifolia*). In general, soils underneath *P. angustifolia* individuals tended to be richer in phosphorus ($F$ = 7.1, $p$= 0.07) than soils underneath *R. sphaerocarpa.*

### *3.2. Seedling emergence and survival*

Seedling emergence was similar between microsite types (GLZ; microsite effect: Wald statistic = 2.06, $p = 0.151$, Supplementary Information, Table S4). The final percentage of emerged seedlings was not significantly influenced by any environmental factor, including soil moisture, light availability, or soil pollution (data not shown). However, the timing of emergence (period of time between the sowing date and the average date of emergence) was significantly influenced by soil moisture (Supplementary Information Table S5).

Nurse shrubs had a clear positive effect on seedling survival (Gehan's Wilcoxon test = -11.7, $p < 0.0001$; Figure 2a). In the open microsites, massive seedling mortality occurred during the first summer, when nearly 50% of the emerged seedlings died. Under the shrub cover, the number of seedlings surviving the first summer was around 90%, and afterwards the mortality rates were still much lower than in the open microsites. At the end of the experimental period (three years after seed sowing), the probability to survive under shrub was higher than 50%, while in the open sites it was lower than 20% (Figure 2a). When comparing across sites, survival curves were also significantly different ($Chi^2 = 27.2759$, $p = 0.0024$), with the lowest survival rates (when both microsites were pooled) found in the most polluted site (Site 3, Figure 2b). When distinguishing between shrub species (*R. sphaerocarpa* vs. *P. angustifolia*), a significant site × shrub species interaction was found (GLZ, Wald statistic = 10.1; $p = 0.018$). In general, seedling survival was similar or slightly higher under *R. sphaerocarpa* individuals than *P. angustifolia*, except in Site 3, where the mortality under the *R. sphaerocarpa* shrubs was comparatively higher (Figure 2c).

Survival regression analysis confirmed that, when all microsites were pooled together, light availability at the ground level was the environmental factor with the greatest influence on seedling survival (Table 1). In addition, soil pH also influenced survival dynamics significantly. Thus, those seedlings that emerged in extremely acidic soils, which could be found in the most polluted site due to the oxidation of the metal residues, had a lower probability to survive the dry periods. When the regression analysis was performed for each microsite type individually, soil pH was the only influential factor for seedling survival under shrub. In the open microsites, soil pH and light influenced survival curves significantly. Interestingly, light was positively associated to the survival of the seedlings in the open microsites, as opposed to the general pattern observed when both microsite types (spanning a broader range of light availability values) were pooled.

### *3.3. Seedling growth*

The intensity of the facilitation of growth by shrubs was enhanced with plant age, but only in those sites where soil conditions were more stressful for plants. After the second growing season (two years after sowing), seedling aboveground biomass was similar or slightly higher under shrub than in the open microsites, except in the most polluted site (Site 3, Figure 3a). Relative growth rates were heterogeneous across sites, without any clear pattern of facilitation of growth under shrub (Figure 3b). However, during the third growing season, RGR was clearly higher under the shrubs in those spill-affected sites, while in the non-affected sites RGR remained similar between microsites (mixed-linear model; site × microsite interaction: $F = 2.56$, $p = 0.06$; Figure 3d). Moreover, there was a relationship between the level of soil pollution and the facilitation intensity (the relative increase in seedling growth under shrub, in comparison to the open microsites) during the third growing season (Figure 4).

### *3.4. Plant chemical composition*

The presence of shrubs did not have a strong influence on the nursed plant nutritional status (Table 2). Only in the two polluted sites were there some significant differences between microsites. In these sites, leaf Mg concentration tended to be higher in those shrub-nursed plants (Table 2; Supplementary Information Table S6). When distinguishing between shrub species, plants under *R. sphaerocarpa* benefited from the N-fixing capacity of this shrub species, as indicated by a higher leaf N content and a lower $^{15}N$ signature of the seedling, in comparison with plants growing under *P. angustifolia* (data not shown).

Trace element composition in the oak seedlings was driven basically by the site location, as soil pollution levels were very different across sites (Table 2). The type of microsite significantly influenced leaf Mn and Tl only; plants under shrub tended to accumulate more Mn and less Tl in their leaves (Supplementary Information Table S7). Leaf Zn concentrations also tended to be greater in those seedlings under shrub (mixed-linear model, microsite effect: $F = 8.40$; $p = 0.059$). In roots, Pb concentrations were significantly higher in those plants growing in open microsites (Table 2; Supplementary Information Table S7). In all cases, leaf trace element concentrations were within the normal ranges for higher plants, and below the phytotoxic threshold values (Supplementary Information Table S7).

### *3.5. Relative neighbour effects*

The analysis of relative neighbour effects (RNE) allowed the comparison of the facilitation intensity among different plant processes. Among the studied processes, the intensity of the facilitation by shrubs was, by far, higher for plant survival than for seedling emergence or growth (highest positive RNE, Figure 5). The balance of the shrub-seedling interaction on seedling growth depended on the site and seedling age, and, as revealed above, the facilitation of growth by shrubs increased with the plant age. In contrast to one of our initial hypotheses, the accumulation of the two most labile soil pollutants (Cd and Zn) in oak seedlings was also enhanced under the cover of shrubs.

## 4. **Discussion**

### *4.1. Shrub effects on soil properties and trace element accumulation*

In natural Mediterranean shrublands, shrubs exert a significant influence on soil physico-chemical properties, promoting the accumulation of soil organic matter and enhancing soil microbial activity (Duponnois, 2011; Goberna et al., 2007, Gonzalez-Polo et al., 2009), thereby resulting in greater nutrient availability and moisture retention in the soils underneath (Boeken and Oresten, 2001; Gómez-Aparicio et al., 2005; Pugnaire et al., 1996). This shrub influence on soil is a key driver for the dynamics of plant communities inhabiting these water-limited environments (Armas and Pugnaire, 2005; Boeken and Oresten, 2001; Shachak et al., 2008), as well as for the colonisation of new species in highly disturbed ecosystems (Párraga-Aguado et al., 2013; 2014). Therefore, we expected that in the studied afforested sites, where shrubs had been planted nine years before soil sampling, the presence of the shrubs would result in significant changes in some key soil properties in comparison to the open matrix. However, most of the variability in soil parameters was determined by the differences across sites, and only organic matter levels were significantly greater in the soils underneath shrub. Similarly, Cuesta et al. (2010) did not find a significant increase in nutrient content in soils located under young *R. sphaerocarpa* individuals in comparison to open microsites six years after their plantation. It is possible that at this successional stage after agriculture abandonment and afforestation, when ruderal species still contribute significantly to total aboveground biomass (Madejón et al., 2009), the influence of shrubs on soil nutrient availability is overruled by the presence of ruderal herbs, in comparison to natural Mediterranean shrublands. In the future, it is quite likely that the influence of shrubs on soil organic matter and nutrient availability will become more evident as ruderal plant species are replaced by other herbs with other suites of functional traits (Kazakou et al., 2006; Kazakou et al., 2009; Pywell et al., 2003) and as the shrub cover increases. In addition, soil contamination may be hampering the decomposition of the shrub litter to a certain extent, slowing the processes of organic matter formation and differentiation of physico-chemical properties between microsites. A recent work in the same study area showed that microbial biomass and activity were strongly affected by the soil acidification and the high availability of trace elements in the polluted soils (Madejón et al., 2012), affecting tree litter decomposition (Ciadamidaro et al., 2014).

The presence of shrubs did not have a strong influence on the patterns of trace element accumulation in the target species. In contrast to one of our hypotheses, the accumulation of Mn and Zn was enhanced, and not suppressed, by the presence of shrubs, and the RNE index showed a positive effect of the shrubs on Zn and Cd accumulation in the oak leaves. On the one hand, the solubilisation of these cationic trace elements could be enhanced under shrubs due to the wetter soil conditions. In addition, as shrubs (particulary *R. sphaerocarpa*) are able to explore deeper soil layers than herbs and promote the hydraulic lift to shallow layers (Prieto et al., 2010), it is possible that they enhanced the mobilisation and transport of these two labile elements from deeper layers. On the other hand, the litter quality of shrub species might have also influenced the solubilisation of trace elements in the soils underneath. The studied species are evergreen shrubs with a low SLA and high LDMC. In Mediterranean woody species, these traits are usually associated to low Ca concentration in the leaf (Domínguez et al., 2012), which usually promotes the acidification of the soils underneath (Reich et al., 2005). Despite the greater accumulation of Zn and Cd in the oak seedlings growing underneath shrubs, trace element accumulation in leaves was always within the normal ranges reported for higher plants, and below the phytotoxic thresholds. Therefore, this unexpected enhancement of trace element accumulation under shrub is not likely to pose a disadvantage for the oak seedlings growing in this type of microsite in comparison to those without the protection of shrubs.

### *4.2. Seedling performance in polluted areas: the role of nurse shrubs*

#### *4.2.1 Seedling emergence and survival*

Nurse shrubs had a clear facilitation effect on oak seedling establishment, strongly decreasing oak seedling mortality over the dry season. Our results indicate that the underlying mechanisms of facilitation were mainly related to the improvement of microclimatic conditions derived from shading, as indicated by the selection of light as the best predictor of seedling survival in our regression models. Shading prevented plants from the damaging effects of high irradiance, extreme temperatures, and water loss during the summertime, with *Q. ilex* seedlings likely being less water stressed and photoinhibited under shrubs than in the open sites (Baquedano and Castillo, 2006; Cuesta et al., 2010). Soil nutrients apparently had a minor role in the facilitation process, given that soil nutrient concentrations were not increased under shrub, as discussed in detail above. When distinguishing between nurse types, seedling survival rates were slightly higher under the pioneer shrub (*R. sphaerocarpa*) than under the late successional shrubs (*P. angustifolia* and *O. europaea*), although this pattern was not observed in the most polluted site. Seedlings growing under *R. sphaerocarpa* might benefit from the higher light availability in comparison to the conditions under the canopy of the late-successional shrubs. The moderate shading conditions provided by the pioneer, leafless shrub would allow seedlings to maintain a relatively high C gain during the growing season, potentially helping them to tolerate drought stress during the dry season (Valladares et al., 2004).

Our results also highlight the importance of soil acidity (low pH) for oak mortality in polluted sites. For both types of microsites, those seedlings that emerged in more acidic soils had a lower probability to survive the dry season. In these soils, the availability of cationic trace elements, and consequently, the risk of toxicity to plants, is basically driven by soil pH (Domínguez et al., 2009). Previous studies with woody plant species have reported root damage by trace elements, but low contaminant transport from roots to shoots (Arduini et al., 1996; Fuentes et al., 2007; Wisniewski and Dickinson, 2003). Therefore, despite the fact that trace element accumulation in seedling aboveground biomass was always below the phytotoxic thresholds, toxicity at the root level cannot be excluded. Metal toxicity might cause multiple direct and indirect effects that concern plant-water relations (reviewed by Barceló and Poschenrieder, 1990). Damages in the root system might be critical for seedling survival, given that the development of an extensive root system is crucial to cope with water stress and survive the dry season (Castro, 2006; Urbieta et al., 2008). In addition, oak plants growing in acidic soils from this area are more prone to show nutritional deficiencies, particularly of phosphorus (Domínguez et al., 2010b).

In contrast to the clear effect on seedling survival, the presence of shrubs did not exert any influence on seedling emergence, likely because this process was more dependent on initial seed reserves than on external resources (Pérez-Ramos et al., 2010; Quero et al., 2007). In fact, the final percentage of emerged seedlings was not significantly influenced by any environmental factor, including soil moisture, light availability, or soil pollution. Soil water heterogeneity however influenced the timing of emergence, seedlings planted in moister microsites emerging earlier than those sown in more water-limited environments. A shortened time to emergence is commonly interpreted as an advantageous regeneration strategy for Mediterranean tree species, likely because it enables seedlings to develop more extensive root systems for a longer period of time, and thus enhances their probability of survival during the dry season (e.g. Castro, 2006; Urbieta et al., 2008). The contrasting effects we found on seedling survival and emergence highlight the necessity of considering several performance estimators to obtain complete and robust conclusions of the role played by these nurse shrubs in the recovery of plant community structure in degraded ecosystems (Gómez-Aparicio, 2009 and references therein).

### *4.2.2 Seedling growth*

The use of shrubs as nurses not only increased oak seedling survival but also enhanced seedling growth in the long term. Thus, the positive effects of shrubs on seedling growth were more marked after three growing seasons, likely due to the strong dependence on seed reserves during early growth in large-seeded species such as oaks. Interestingly, the facilitation intensity (in terms of growth) was only evident in polluted soils, where seedlings growing in open microsites were likely more stressed by the combined negative effects imposed by a high irradiance, a high level of acidity, and, potentially, a high availability of trace elements. In these open and spill-affected habitats, the three-year-old seedlings even reached negative values of RGR as a consequence of a drought-escape strategy involving leaf shedding during summer and further regrowth (i.e. resprouting) with the arrival of the rainy season. The enhanced benefits of shrub facilitation in the polluted sampling sites supports our initial hypothesis stating higher facilitation effects with increasing chemical stress, which was in turn based on the common assumption that positive plant interactions are expected to be more important in more stressful environments (Bertness and Callaway, 1994; Callaway and Walker, 1997). In some polluted environments, the additional benefits provided by shrubs are derived from the role of their litter as a good cation-chelating agent (thus reducing soil phytotoxicity), as well as a good source of macronutrients (e.g. Frèrot et al., 2006; Ginocchio et al., 2004). In our study, however, we did not find a strong influence of the shrubs in the soil factors related to trace element bioavailability (i.e. soil pH). Thus, the increase in the neighbour effect in the polluted sites could be mainly derived from the release of high-irradiance stress under shrub, which allowed the plants to better cope with the toxicity induced by low pH and high availability of trace elements.

### 4.3. *Conclusions: use of nurse shrubs for the afforestation of polluted soils*

Our results clearly show that the use of shrubs as nurse plants is a promising tool for the afforestation of polluted lands with Holm oak. By sowing acorns underneath shrubs, emerged seedlings benefited from shading conditions that, in turn, helped them to cope better with additional abiotic stresses (such as those derived from a low pH and a high presence of potentially toxic trace elements). Therefore, considering the presence of pre-existing shrubs in the design of planting schemes would considerably reduce the costs of achieving a significant number of survivors during the first years after planting, when massive mortalities occur. If pre-existing shrubs are not available in the area to afforest, we recommend applying a multi-phase approach, considering the plantation of shrub species first, and then the introduction of late-successional trees in the microsites created by shrubs. This approach attempts to accelerate the natural sequence of the successional process, and it is particularly recommended for semi-arid systems, where neighbour effects are in general more positive than in mesic temperate systems (Gómez-Aparicio et al., 2009). Because shrubs are able to develop certain plant cover faster than slow-growing, late-successional trees, the early establishment of shrub species positively contributes to the enhancement of the aesthetic value of the site and to the improvement of the social perception of the afforestation programme during the initial years of the restoration process.

Our results also highlight the importance of controlling soil pH conditions during the restoration programme. Soil pH was the most important soil factor influencing survival of oak seedlings, and a strong soil acidification was observed in the most polluted soils at the time of sampling, almost ten years after the application of soil amendments. Thus, restoration programmes in similar polluted sites should consider the long-term monitoring and correction of soil pH in order to ensure the maintenance of soil conditions that maximise oak survival over time, and to reduce the risks associated to the leaching of soluble trace elements from acidic soils.

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## Tables

**Table 1**. Results of the regression models (Cox's Proportional Hazard Models), applied to seedling survival times, with both microsites types pooled (all microsites), and analysed separately (open vs. shrub). Those environmental factors with a significant influence on survival times ($p < 0.05$) are indicated in bold letters. In these models, those factors explaining the *hazard rates* have positive beta values. Thus, positive beta values indicate a positive relationship between an environmental factor and seedling mortality. GSF: global site factor (light availability at the ground level); OM: organic matter.

| Environmental Factor | All microsites | | Open | | Shrub | |
|---|---|---|---|---|---|---|
| | Beta | p | Beta | p | Beta | p |
| Winter soil moisture | 0.015 | 0.239 | 0.010 | 0.525 | 0.051 | 0.141 |
| Summer soil moisture | 0.010 | 0.688 | 0.031 | 0.404 | -0.042 | 0.428 |
| GSF | **2.481** | **<0.001** | **-3.466** | **0.003** | -0.741 | 0.412 |
| Herb biomass | 0.009 | 0.025 | 0.001 | 0.930 | 0.014 | 0.111 |
| pH | **-0.208** | **0.001** | **-0.202** | **0.005** | **-0.498** | **0.001** |
| Soil OM | -0.106 | 0.271 | -0.077 | 0.496 | 0.040 | 0.840 |
| Soil P | 0.001 | 0.929 | **0.027** | **0.023** | -0.015 | 0.529 |
| Soil K | 0.000 | 0.758 | 0.000 | 0.676 | 0.000 | 0.914 |
| Soil Cd | 0.272 | 0.165 | 0.343 | 0.167 | -0.091 | 0.809 |

**Table 2.** Results of the mixed-model analysis, analyzing the effect of the microsite type (open vs. shrub, fixed factor) and the site location (random factor) on leaf and root chemical composition (*F* statistic indicated). Significance level is indicated as follows: $p<0.001$***, $p<0.01$**, $p<0.05$*.

|  | Microsite effect | Site | Microsite × Site |  | Microsite | Site | Microsite × Site |
|---|---|---|---|---|---|---|---|
| *Leaf* |  |  |  | *Root* |  |  |  |
| Ca | 0.01 | 0.47 | 3.01* | Mn | 1.32 | 32.21** | 0.51 |
| Fe | 2.13 | 1.75 | 1.50 | Cu | 1.12 | 22.39* | 0.58 |
| K | 0.34 | 0.44 | 1.37 | Zn | 3.88 | 2.18 | 1.02 |
| Mg | 2.56 | 2.68 | 3.05* | As | 2.09 | 17.93* | 1.60 |
| P | 3.24 | 1.03 | 0.82 | Cd | 0.71 | 5.84 | 4.17* |
| S | 3.24 | 1.03 | 0.82 | Tl | 0.05 | 5.30 | 1.98 |
| N | 0.58 | 0.30 | 2.80 | Pb | 12.30* | 7.53 | 0.55 |
| $\delta^{15}$N | 0.03 | 0.35 | 1.07 |  |  |  |  |
| $\delta^{13}$C | 0.38 | 0.53 | 1.01 |  |  |  |  |
| C:N | 0.62 | 0.30 | 2.68 |  |  |  |  |
| N:P | 0.02 | 0.54 | 1.08 |  |  |  |  |
|  |  |  |  |  |  |  |  |
| Mn | 26.01** | 49.31*** | 0.13 |  |  |  |  |
| Cu | 0.01 | 1.47 | 2.14 |  |  |  |  |
| Zn | 8.40 | 16.16* | 0.57 |  |  |  |  |
| As | 1.51 | 38.16** | 0.73 |  |  |  |  |
| Cd | 0.45 | 5.45 | 2.38 |  |  |  |  |
| Tl | 30.30** | 46.97** | 0.13 |  |  |  |  |
| Pb | 4.12 | 26.94 | 0.76 |  |  |  |  |

## Figure legends

**Fig. 1**. Result of a PCA analysis applied to the microsite environmental variables. GSF: global site factor (light availability at the ground level); MB: soil microbial biomass; OM: soil organic matter; SpringM: average spring soil moisture; SummerM: average summer soil moisture; WinM: average winter soil moisture; Herb: herb biomass.

**Fig. 2**. Survival curves of the emerged seedlings, distinguishing between microsites types (shrub vs. open) and across sites (b). Survival rates distinguishing between shrub species (*R. sphaerocarpa* vs. *P. angustifolia*) are also indicated (c). In figure b, open and filled symbols correspond to non-polluted and polluted sites, respectively.

**Fig 3**. Aboveground biomass and Relative Growth Rates (RGR) of the oak seedlings growing under shrub and in open microsites during the second (a ,b) and the third (c, d) growing season after sowing, across the four studied sites.

**Fig. 4**. Relative increase in seedling survival and growth under shrub, in comparison to plants growing in the open microsites across the explored gradient of soil pollution (indicated by total soil Cd concentration).

**Fig. 5**. Relative neighbour effect (RNE) on seedling emergence, survival and growth, and on Zn and Cd accumulation in leaves.