

Effect of Density on Mediterranean Pine Seedlings Using the Nelder Wheel Design: Analysis of Survival and Early Growth

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23 **ABSTRACT**

- 24 • Background: There is growing interest in mixed-species forests but a lack of studies that
25 analyse them for regeneration phases or any stage other than mature stands. Information
26 is scarce about relatively unproductive species such as *Pinus pinaster* and *Pinus*
27 *halepensis* in Mediterranean ecosystems. The objective of this study was to investigate
28 inter- and intra-specific interactions of both species in different tree densities during the
29 first years of establishment. Five Nelder wheel plots were planted to test densities
30 ranging from 1000 to 80000 seedlings/ha and simulate establishment sub-processes at
31 high densities. *Pinus pinaster* and *Pinus halepensis* were mixed along the spokes, to
32 obtain three mixture levels in which 100%, 80% or 60% of the seedlings were of the
33 same species. Cox proportional-hazards models and binomial logistic regressions were
34 fitted to analyse seedling survival. Early growth (basal diameter and height at one and
35 four years after plantation) was analysed by fitting linear mixed-effects models.
- 36 • Results: *Pinus halepensis* showed higher survival rates and basal diameter increments
37 but more time is needed to know how *Pinus pinaster* responds to density and mixture.
- 38 • Conclusions: Both competitive and facilitating seedling interactions were observed at
39 higher densities, which facilitate seedling survival but decrease early growth. *Pinus*
40 *halepensis* showed higher survival rates and basal diameter increments but more time is
41 necessary to determine *Pinus pinaster* response to density and mixture.

42

43 **Keywords**

44 Establishment; mixed stands; Mediterranean ecosystem; experimental design; *Pinus pinaster*;
45 *Pinus halepensis*

46

47 **BACKGROUND**

48 Germination and establishment are key sub-processes of natural regeneration because seedlings
49 are especially liable to suffer high mortality due to water stress (Grime 1979). This is critical in
50 Mediterranean ecosystems, where high interannual climate variability and strong drought
51 during summer are increasing as a consequence of climate change (IPCC 2007). Environmental
52 conditions for seedlings are especially harsh, so seedling establishment has been studied in
53 terms of scarce natural regeneration (Hanssen 2003; González-Alday et al. 2009; Ruano et al.
54 2009; Lucas-Borja et al. 2012; Manso et al. 2013...). However, there are also cases of excessive
55 regeneration, for instance in post-fire regeneration of the *Pinus* genus (Vega et al. 2009;
56 Rodríguez-García et al. 2008; Ruano et al. 2013); so it is interesting to analyse seedling
57 responses to different densities, including high densities.

58 In mature stands, high densities should cause competition for resources and reduce tree growth,
59 but competitive and facilitating effects are sometimes found simultaneously in other phases of
60 stand development, such as seedling establishment. For example, seedlings might compete for
61 water or light with their own or other species, even as wind velocity or transpiration are reduced
62 by mutual shading or as other species provide protection against herbivores (Jactel and
63 Brockerhoff 2007; Zamora et al. 2008; Uhl et al. 2015). Moreover, positive interactions can
64 become negative as seedlings grow (Callaway et al. 1996; Callaway and Walker 1997; Zamora
65 et al. 2008; Uhl et al. 2015). Some models predict that facilitation and competition interactions
66 vary across abiotic stress gradients and that facilitation interactions will be dominant under
67 stressful conditions, though this is debated (Maestre et al. 2005, 2006; Lortie and Callaway
68 2006). Therefore, the net effect of intra- and inter-specific interactions among seedlings under
69 high densities is a key issue to analyse.

70 In the present work, two of the most common pine species in Mediterranean ecosystems were
71 studied: *Pinus pinaster* Aiton and *Pinus halepensis* Mill. Both species cover extensive areas in
72 the western Mediterranean basin, while *Pinus halepensis* populations can be also found in

73 eastern Mediterranean zones. From timber, food and energy to soil protection, cultural services,
74 hydrological control, habitat for biodiversity, or climate regulation, forests provide vital
75 ecosystem services (Science for Environment Policy 2015). Pine plantations have been
76 questioned in semi-arid areas in terms of soil fertility, erosion or ecological succession, but they
77 provide more ecosystem services than grasslands, abandoned agricultural fields or other
78 unrestored landscapes (Derak and Cortina 2014). *Pinus pinaster* and *Pinus halepensis* have
79 high ecological importance because of their ability to grow in extremely poor soils and survive
80 multiple droughts (Alía and Martín 2003; Fady et al. 2003). The two species share niches, but
81 there are no studies about inter-specific interactions between them. Climate projections for
82 Mediterranean forests predict negative effects at different levels in the future, including
83 reduction of forest and site productivity, increment of disturbances (pests, pathogens, fires...) and
84 shifting species distributions or even extinctions (Resco De Dios et al. 2007, Lindner and
85 Calama 2013). The changes in species distribution and structure that are expected in
86 Mediterranean areas (Thuiller 2003) make it especially timely to study how the selected species
87 will behave together. During seedling development, interactions between coexisting species are
88 key to understanding and predicting specific responses to different densities and mixture ratios.

89 Inter-specific interactions have been analysed between seedlings and other weed or shrub
90 species (Helliwell and Harrison 1979; Scholes and Archer 1997; De las Heras et al. 2002;
91 Gómez-Aparicio et al. 2005; Rodríguez-García et al. 2011). Despite the increasing interest in
92 mixed-species forests, little research has been done to analyse mixed stands or to compare pure
93 and mixed stands on similar sites, so the discussion is usually theoretical (Pretzsch 2009). Most
94 studies analyse intra- and inter-specific interactions among productive species in mature stands
95 from a productive point of view (Piotto et al. 2004; Kuehne et al. 2013; Vanclay et al. 2013),
96 but there is scarce information about relatively unproductive species and ecosystems or other

97 forest stages. The regeneration stage in particular is crucial to stand regeneration and long-term
98 stability.

99 One of the main issues in this kind of research is the experiment design. The most frequent
100 designs for analysing mixed stands consist of growing two species in varying proportions while
101 keeping stand density constant (Vanclay 2006). Mixed mature stands have been studied in
102 recent years thanks to long-term experimental plots (Pretzsch et al. 2015, 2019a, b; Riofrío et
103 al. 2019; Aguirre et al. 2019...) but little is known about seedling stands or young forests. The
104 Nelder wheel design makes it possible to test multiple densities in a single plot (Nelder 1962;
105 Kerr 2003; Uhl et al. 2015). It consists of a circular plot containing concentric rings radiating
106 outward with spokes that connect the centre with the furthest ring (Figure 1 – experimental
107 design). At the intersections of spokes and arcs, a tree is planted. This creates variable tree
108 densities along the length of the spokes within a single plot.

109 The main objective of this experimental site was to analyse inter- and intra-specific interactions
110 of *Pinus pinaster* and *Pinus halepensis* seedlings in different stand densities. For this, five
111 Nelder wheel plots were planted to test mixed-species densities ranging from 1000 to 80000
112 seedlings/ha. Results from the analysis of seedling survival and early growth, defined in terms
113 of basal diameter and total height, are presented here. We expected that: (1) seedling survival
114 rates and early growth would be different for *Pinus halepensis* and *Pinus pinaster*; (2) seedling
115 survival rates and early growth would be affected by density and mixture levels; (3) the effect
116 of density and mixture levels would be different for each species. The data obtained from this
117 study will contribute to our understanding of inter- and intra-specific interactions during the
118 first years at different stand density levels.

119 **MATERIAL AND METHODS**

120 **Experimental design**

121 The experimental design consists of four permanent and one temporary Nelder (1962) wheel
122 plots for testing 10 densities. The temporary Nelder wheel plot was installed to harvest the
123 seedlings on two occasions (half each time), for dry biomass analysis. (Ruano et al 2021). All
124 the Nelder wheel plots have 26 spokes, constant angles of 13.85° and 12 rings, housing a total
125 of 312 seedlings per wheel (Figure 1, experimental design). Trees in the outermost and
126 innermost rings served as buffers for edge effects and were not included in the study sample,
127 leaving 260 seedlings per wheel to analyse. The plots were established during the autumn of
128 2013 in Calabazanos (Palencia – central Spain), on approximately 1 ha of land located on an
129 old forest nursery site belonging to the Castile-and-Leon Government. The site currently houses
130 the Forest Health Centre (Figure 1). The experimental design was implemented according to
131 the explanations of Parrott et al. (2012).

132 Ten densities were tested, ranging from 1000 to 80000 seedlings/ha (Table 1). Minimum and
133 maximum densities were also defined, to measure the effects of low and high regeneration
134 densities (Matney and Hodges 1991; Calvo et al 2007; Orozco et al 2007; Ruano et al 2013).
135 Matney and Hodges (1991) identified a recruit density of 2000 seedlings/ha as the minimum
136 requirement for successful natural regeneration. However, a density of 1000-1500 seedlings/ha
137 can be considered satisfactory in stands with abiotic stress (Rodríguez-García et al. 2010), so
138 the minimum density tested was 1000 seedlings/ha. At the other end, high densities have been
139 reported in studies on post-fire natural regeneration in Mediterranean ecosystems (Calvo et al.
140 2007; Orozco et al. 2007; Ruano et al. 2013). Ruano et al. (2013) observed densities of 80000
141 seedlings/ha after a fire in *Pinus halepensis* stands, which served to establish the maximum
142 density at 80000 seedlings/ha in the present study. The same densities were tested for all
143 seedlings of each concentric ring. This will be referred to as the “growing space”, which is
144 related to stand density (Table 1).

145 Nelder wheel design can also be used to analyse inter-specific interactions. In the present study,
146 *Pinus pinaster* and *Pinus halepensis* seedlings were mixed along the spokes (Figure 2). Two
147 regions of provenance – the ‘Meseta Castellana’ (Castilian High Plains) for *Pinus pinaster* and
148 ‘Repoblaciones de la Meseta Norte’ (North Plains Reforestation) for *Pinus halepensis* – were
149 selected to avoid the site effect. The temporary Nelder wheel plot was installed to harvest the
150 seedlings on two occasions for dry biomass analysis. The design made it possible to look at
151 three mixture levels taking the surrounding seedlings into account: 100 indicates that the central
152 seedling and the four surrounding seedlings are of the same species (100% of the seedlings are
153 of the same species); 80: the central seedling and three surrounding seedlings are of the same
154 species (80% of the seedlings are of the same species); 60: the central seedling and two
155 surrounding seedlings are of the same species (60% of the seedlings are of the same species).

156 One of the aims of the study was to simulate natural regeneration establishment, but the
157 seedlings had to be protected from rabbits given their high population density on the site. We
158 used plastic mesh protectors measuring 60 cm high, which were as light as possible to avoid
159 changes in micro-climatic conditions. One weed control intervention was also applied during
160 the first summer, to eliminate weed species.

161 The seedlings were one growing season old when the plantation was established. Basal diameter
162 and total height (length of the main stem) were measured for each plant at that time. The same
163 measurements (basal diameter and total height) were repeated after one year (2014) and after
164 four years (2017) and seedling survival was determined at both times.

165 **Effect of mortality in the experimental design**

166 When a seedling dies, the growing space of surrounding seedlings changes and thereby alters
167 the initial experimental design. Some authors have done double plantings, but this was not
168 considered in the present study because one of our aims was to analyse seedling survival.

169 Another possibility is to remove the seedlings surrounding dead seedlings from the analysis to
170 preserve the initial design (Parrott et al. 2012; Kuehne et al. 2013). In the present work, 984
171 seedlings survived after a year. If the four seedlings surrounding every dead seedlings had been
172 removed, 52.3% of the original seedlings would have been eliminated, leaving only 620
173 seedlings for analysis. We evaluated two options to solve the problem of mortality: i) Hegyi's
174 competition index, which has been used to estimate local competition in Nelder rings
175 (Weiskittel et al. 2011; Uhl et al. 2015); and ii) recalculating the growing space using Voronoi
176 polygons. Hegyi's index is harder to relate to stand density and the optimal radius must be
177 defined to estimate it correctly (Linares et al. 2009), so Voronoi polygons were calculated in
178 the present study using R Core Team 2020 software with the 'deldir' package (Turner 2020)
179 for 2014 and 2017 (Figure 3). Since the mixture levels defined in the initial design had also
180 changed, mixture levels were recalculated considering surrounding seedlings based on Voronoi
181 polygons. Though four surrounding seedlings were considered in the initial design, the final
182 number of surrounding seedlings depended on the Voronoi polygon. As in the initial design,
183 however, they were estimated as a percentage of surrounding seedlings.

184 **Analysis of seedling survival**

185 Seedling survival was recorded one year and four years after planting (2014 and 2017) and dead
186 seedlings were found during this period. In 2013, 1300 seedlings (650 of each species) had been
187 planted in the initial design. Approximately 80% of *Pinus pinaster* seedlings were alive after a
188 year (2014) and about 40% were alive after four years (2017). For *Pinus halepensis*,
189 approximately 89% of the seedlings were alive after one year and 84% after four years (2017).
190 Two methods were fitted to analyse which factors affected seedling survival. First, Cox
191 proportional-hazards models (Cox 1972) were fitted to investigate the association between
192 seedling survival time and one or more predictors. The Cox model is expressed by the hazard

193 function denoted by $h(t)$ and can be interpreted as the risk of dying at time t . It can be estimated
194 according to the formula:

$$195 \quad h(t) = h_0(t)\exp(\sum_{i=1}^n \beta_i X_i) \quad (1)$$

196 Where: $h(t)$ is the hazard function at time t ; $h_0(t)$ is the baseline hazard at time t ; and X_i are
197 explanatory variables. This expression gives the hazard rate at time t for object i with covariate
198 vector (explanatory variables) X_i .

199 In the present study, different Cox proportional-hazards models were fitted to define better
200 predictors. Model significance was confirmed by the Likelihood-Ratio test, Wald test and Score
201 test. Finally, proportional hazards assumptions were tested for the selected Cox regression
202 model.

203 Basal diameter at time of planting (2013), total height at time of planting (2013), height to
204 diameter ratio (h/d ratio) at time of planting (2013), species (*Pinus pinaster*, *Pinus halepensis*),
205 seedling growing space and mixture levels were considered as candidate covariates. Growing
206 space for the seedling and mixture levels were defined in the initial design but had to be
207 recalculated using Voronoi polygons due to mortality. Accordingly, growing space and mixture
208 level were defined as the initial design if the seedling was dead in the 2017 inventory and
209 recalculated using 2014 data and Voronoi polygons if the seedling was alive in 2017.

210 Second, binomial logistic regressions were fitted to model survival probability. Logistic models
211 are very useful for fitting binary events such as survival or mortality probability, so they have
212 been used in many forestry studies (Yao et al. 2001; Bravo et al. 2001; Trasobares et al. 2004;
213 Bravo-Oviedo et al. 2006; González et al. 2007...). The response variable was a binary variable
214 denoted as "success" (live seedling coded as 1) and "failure" (dead seedling coded as 0). The
215 survival model is as follows:

$$P = \frac{1}{1 + e^{-(\beta_0 + \sum_{i=1}^n \beta_i X_i)}} \quad (2)$$

Where: P is the probability of survival; β_0 is the intercept term; and X_i are the explanatory variables. In this case, the same candidate explanatory variables as those of the Cox proportional-hazards models were considered: basal diameter at time of planting (2013), total height at time of planting (2013), height to diameter ratio (h/d ratio) at time of planting (2013), species (*Pinus pinaster* and *Pinus halepensis*), seedling growing space and mixture levels.

Cox proportional-hazards models were fitted using the ‘survival’ package (Therneau 2020). Alternative fits in both analyses were evaluated based on Akaike Information Criterion (AIC) (Akaike 1973) and they were performed using R Core Team 2020 software. The AIC differences (Δ_i) were also estimated for all candidate models to determine the empirical support of each model. Thus, Δ_i is estimated as: $\Delta_i = AIC_i - AIC_{\min}$ and models with a $\Delta_i > 10$ have essentially no support and might be omitted (Burnham and Anderson 2002). The two selected models were validated graphically.

229 **Analysis of early growth**

Basal diameter and total height were measured at the time of planting, at one year and at four years after plantation (2013, 2014 and 2017). Early growth was analysed considering increments of measured variables over one year (2013-2014) and four years (2013-2017). The data were processed by analysis of variance in a split-plot design model, with the density factor associated to whole plots (rings into the wheels) in a randomized block design with five blocks (the wheel plots), and two factors associated to the split-plots (trees in the rings) in a 2x3 factorial design (two species and three mixture proportions) with replicates (four trees for 40/60 and 20/80 mixtures, and five trees for no mixture). In this case, when growing space for the

238 seedling and mixture levels changed from initial design, they were recalculated again to be
239 considered as the initial design.

240 The mathematical formulation of the model is given by:

$$241 \quad y_{ijm(kl)} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \delta_k + \eta_l + \delta\eta_{kl} + \beta\delta_{jk} + \beta\eta_{jl} + \beta\delta\eta_{jkl} + \varepsilon_{ijm(kl)} \quad (3)$$

242 with $i=1,\dots,5$ for the wheels; $j=1,\dots,10$ for the rings in the wheels; $m=1,\dots,26$ for the trees in the
243 rings, $k=1,2$ for the species and $l=1,2,3$ for the mixture levels

244 where: $y_{ijm(kl)}$ = observed increment of basal diameter or height of the tree m (with species k
245 and mixture l) in ring j of wheel i ; μ = general mean effect; α_i = main effect of the block (wheel
246 I ; β_j = main effect of density j ; γ_{ij} = random effect of ring j in wheel i (whole-plot error); δ_k =
247 main effect of species k ; η_l = main effect of the mixture proportion l ; $\varepsilon_{ijm(kl)}$ = random error
248 in the observed growth for the tree m (with species k and mixture l) in ring j of wheel i .

249 The other terms are the usual double and triple interactions between factors. The assumptions
250 for the model were:

- 251 • $\gamma_{ij} \sim N(0, \theta^2)$, with θ^2 =random variance for whole plots (rings).
- 252 • $\varepsilon_{ijm(kl)} \sim N(0, \sigma_k^2)$, with σ_k^2 = random variance for errors in the trees of species k .
- 253 • $\text{Cov}(\gamma_{ij}, \gamma_{i'j'}) = \text{Cov}(\varepsilon_{ijm(kl)}, \varepsilon_{i'j'm'(k'l')}) = \text{Cov}(\gamma_{ij}, \varepsilon_{i'j'm'(k'l')}) = 0$ for all values of the
254 subscripts.

255 Therefore, the linear mixed model included three variance parameters, which were estimated
256 using the Restricted Maximum Likelihood Method (REML).

257 Comparisons between the estimated LS-means were performed using a pairwise t-test with a
258 0.05 significance level. The normality of the studentized residuals in the model was analysed
259 using the Kolmogorov-Smirnov test.

260 Although the statistical design included 1300 trees, only 1101 trees were used at the first date
261 and 799 at the second one, due to mortality. Furthermore, the density and mixture proportion
262 classes of each tree were reassigned based on mortality.

263 All statistical analyses of early growth were developed using SAS/STAT[®] software (SAS
264 Institute Inc. 2020). Figures 4 and 5 were developed using R Core Team 2020 software with
265 the ‘ggplot2’ package (Wickham 2016) from the data obtained in SAS/STAT[®].

266 **RESULTS**

267 **Analysis of seedling survival**

268 Seedling survival was analysed using two statistical methods: Cox proportional-hazards
269 regressions and binomial logistic regressions. Same models were fitted for both methods to
270 obtain better conclusions. Based on the AIC differences (Δ_i), models 12, 13 and 35 for the Cox
271 proportional-hazards regression and models 13, 21, 28 and 35 for the binomial logistic
272 regression could be considered useful (Supplementary Table 1). In both cases, the Akaike
273 information criterion was lower for models in which the initial size, species, seedling growing
274 space and mixture level were significant (Supplementary Table 1). Model 13, where the basal
275 diameter at time of planting was significant, gave the lowest AIC for the Cox proportional-
276 hazards regression. Model 21, where the basal diameter and the total height at time of planting
277 were significant, gave the lowest AIC for the binomial logistic regression model. However, a
278 similar AIC (1405.14 vs 1405.90; $\Delta_i=0.75$) was obtained with Model 13 and significance levels
279 for tested variables were higher (Supplementary Table 2). Thus, Model 13 was considered the
280 better model for both methods.

281 A summary of the selected Cox proportional-hazards regression is shown in Table 2. The
282 coefficients in a Cox regression are related to hazard: the negative coefficient of basal diameter
283 indicates a positive effect for seedling survival and the positive coefficients of the other

284 significant variables indicate a worse prognosis. The hazard ratios represent instantaneous risk
285 over the study time period for the increment of each explanatory variable by one unit, keeping
286 the other covariates constant. In this case, the expected hazard is 4.86 times higher for *Pinus*
287 *pinaster* than *Pinus halepensis*, when the rest of the variables are fixed. Incrementing the basal
288 diameter by 1 mm at the time of plantation (one year old) and keeping the other covariates fixed
289 reduces the hazard by a factor of 0.79. For seedling growing space, hazard increases by a factor
290 of 1.029 when the growing space increases 1 m² and the other covariates are kept constant.
291 Growing space is related to stand density, so hazard increases with lower densities. Finally, the
292 mixture effect works in a similar way. The hazard increases by a factor of 2.83 when the mixture
293 level increases by 1 unit and other covariates remain constant. In other words, the hazard
294 increases as the species proportion becomes more mono-specific.

295 For the binomial logistic model, a summary of the selected model is shown in Table 3.
296 According to Equation (2), the fitted survival model is:

$$297 \quad P = \frac{1}{1 + e^{-(1.700 + 0.383 * \text{basaldiameter} - 2.096 * \text{species} - 0.055 * \text{growingspace} - 1.365 * \text{mixture})}}$$

298 The estimated parameters indicate that seedlings with smaller basal diameter at the time of
299 planting (one year old) have less probability of survival and that the survival probability of
300 *Pinus pinaster* is lower than that of *Pinus halepensis*. Also, survival rate decreases as the
301 growing space for a seedling increases. Finally, when species proportion is near mono-specific,
302 the survival probability is lower.

303 **Analysis of early growth**

304 The mixed linear model of equation (3) was fitted four times to analyse the following response
305 variables: Model 1: increment in basal diameter over one year (2013-2014); Model 2: increment
306 in basal diameter over four years (2013-2017); Model 3: increment in total height over one year

307 (2013-2014); Model 4: increment in total height over four years (2013-2017) (Table 4). It was
308 not possible to consider time as a repeated measurement because the model did not converge.

309 The blocks (wheels) and the species were significant with a significance level of 0.001 for all
310 models. In Model 1 (one-year basal area increment), the mixture level was significant, with a
311 significance level of 0.05 (Table 5). The one-year increment in basal diameter was higher for
312 *Pinus halepensis* than for *Pinus pinaster*. The mixture level was significant and the one-year
313 increment in basal diameter was higher when half of the surrounding seedlings were of the same
314 species as the analysed seedling, and lower when all the surrounding seedlings were of the same
315 species as the analysed seedling (Supplementary Table 2).

316 In Model 2 (four-year basal area increment), growing space and the interaction between species
317 and growing space were significant, with a significance level of 0.001. The interaction between
318 the species and the mixture level was also significant, with a level of significance of 0.05 (Table
319 5). The four-year increment in basal diameter was higher for *Pinus halepensis* than for *Pinus*
320 *pinaster* when growing space remained constant. Higher basal diameter increment values were
321 obtained with 2.32 m² growing space (approximately 4300 seedlings/ha), though non-
322 significant differences appeared with growing spaces above 0.88 m², indicating that higher
323 basal diameter increments were found in densities of approximately 1000-11400 seedlings/ha.
324 Lower basal diameter increment values were also found with a growing space of 0.13 m² but
325 were not significantly different compared to a growing space of 0.2 m² (approximately 49000-
326 80000 seedlings/ha) (Supplementary Table 3).

327 Results for the interaction between species and growing space showed higher basal diameter
328 increments over four years for *Pinus halepensis* than for *Pinus pinaster*, but each species had
329 higher and lower values with different growing spaces. *Pinus halepensis* had a higher basal
330 diameter increment with a growing space of 1.43 m², but without significant differences

331 compared to larger growing spaces (approximately 1000-7000 seedlings/ha) and lower basal
332 diameter increment with smaller growing spaces (0.13 m², or 80000 seedlings/ha). Like *Pinus*
333 *halepensis*, basal diameter increments for *Pinus pinaster* were also higher with a growing space
334 of 1.43 m² (approximately 7000 seedlings/ha), but significant differences compared to other
335 growing space levels were less clear. Curiously, lower values were found with a growing space
336 of 6.15 m² (approximately 1600 seedlings/ha) (Supplementary Table 3 and Figure 4).

337 Interactions between species and mixture levels for the four-year basal diameter increment
338 again showed higher values for *Pinus halepensis* than *Pinus pinaster*. Differences between
339 mixture levels were non-significant for *Pinus pinaster*. In contrast, *Pinus halepensis* had higher
340 basal diameter increment when half of the surrounding seedlings were also *Pinus halepensis*
341 (Supplementary Table 4 and Figure 5).

342 Finally, species was significant in Models 3 and 4, where the total height increment was
343 analysed over one and four years, respectively. In both cases, total height increment was higher
344 for *Pinus halepensis* than for *Pinus pinaster*, but none of the factors were significant enough to
345 subsequently analyse differences in growing spaces or mixture levels (Supplementary Table 5).

346 **DISCUSSION**

347 Seedling establishment has been studied for different species and places, but mostly in natural
348 forests (Gray and Spies 1997; González-Martínez and Bravo 2001; Hunziker and Brang
349 2005...). In the present study, seedling establishment with high densities was analysed for two
350 species using the Nelder design. This design is more common in agricultural experiments
351 (Hanson et al. 2005; Gaines et al. 2008; Bagavathiannan and Norsworthy 2014...) because
352 crops are annual and mortality problems do not arise. It has not been used much in forestry
353 experiments.

354 As with any other method, the Nelder design has different strengths and weaknesses. Most of
355 the traditional designs require much larger areas to analyse different stand densities compared
356 to the Nelder design (Vanclay et al. 2013). Moreover, while monospecific stands can be
357 described at stand level, mixed stands need more complex approaches at tree level. The Nelder
358 design provides an advantage over factorial designs, because the experimental unit is the tree
359 rather than the plot. From a statistical point of view, however, there is high spatial and temporal
360 correlation along with a lack of independence and randomization. This weakness has been a
361 problem for years, but today new statistical methods such as mixed models or generalized
362 additive models can be used (Parrott et al. 2012; Kuehne et al. 2013; Uhl et al. 2015). The main
363 weakness for this study is mortality, but double planting was not feasible because one of our
364 aims was to analyse the effect of density on seedling survival. In this case, the growing space
365 was re-calculated using Voronoi polygons to avoid removing the seedlings surrounding dead
366 seedlings. Therefore, the Nelder design allows for optimal analysis of tree dynamics but
367 requires some conceptual and statistical adaptations (Uhl et al. 2015).

368 Seedling survival was analysed using two different statistical methods. In both cases, the same
369 model was chosen, and the effect of explanatory variables was similar. Higher survival
370 probabilities were obtained for higher basal diameters in both species. This means that seedling
371 size is important from the beginning. When studying natural regeneration of *Pinus sylvestris* in
372 Sweden, Ackzell (1993) found that height was significant for survival, based on a mean height
373 difference of 40 cm between surviving and dead trees.

374 Both species in this study are defined as well-adapted to summer drought (Alía and Martín
375 2003; Fady et al. 2003) and high densities have been recorded for both species after fires (Calvo
376 et al. 2007; Ruano et al. 2013). In the present work, however, the survival probability of *Pinus*
377 *halepensis* was higher than that of *Pinus pinaster*. Rodrigo et al. (2004) simulated stochastic
378 models to predict forest dynamics after fire for different Mediterranean species (including *Pinus*

379 *pinaster* and *Pinus halepensis*), observing similar behaviour for the two species. Density values
380 of both species were high just after the fire, then decreased considerably during the early post-
381 fire years, but had high resilience. This was not the case for mixed stands, however, where the
382 probability of transition to other forest types is high. In the present work, however, seedling
383 survival probability was lower in monospecific stands and points to a facilitating effect between
384 species.

385 Competition for resources between seedlings may be expected, but our results also indicated a
386 facilitating effect because survival probability was higher with less growing space and higher
387 stand density. Facilitation may occur if wind velocity or transpiration are reduced through
388 mutual shading or if other species provide protection against herbivores (Rodríguez-García et
389 al. 2008; Zamora et al. 2008; Uhl et al. 2015). Bertness and Callaway (1994) considered
390 competition less important than facilitation in low-productivity stands such as the one studied
391 here.

392 Early growth was analysed based on the increment in basal diameter over one and four years
393 and the increment in total height over one and four years. Differences between species were
394 found in all analyses, with higher values for *Pinus halepensis*. *Pinus pinaster* and *Pinus*
395 *halepensis* are considered as pioneer and invasive species in terms of ecological succession;
396 they also have similar behaviour in terms of relative growth (Davis et al. 2000; Grotkopp et al.
397 2002). Grotkopp et al. (2002) analysed relative growth for 29 pine species, including *Pinus*
398 *pinaster* and *Pinus halepensis*. They observed similar relative growth rates between both
399 species, while *Pinus halepensis* had higher basal diameter increments and total height than
400 *Pinus pinaster* in the present study. This may be due to a negative relationship between seed
401 weight and relative growth rate. The corresponding theory suggests that species with lighter
402 seeds would have higher relative growth (Marañón and Grubb 1993; Grotkopp et al. 2002). Our
403 analysis supports this theory: *Pinus halepensis* seeds weigh less than *Pinus pinaster* seeds

404 (Grotkopp et al. 2002) and we observed higher diameter and height increments for *Pinus*
405 *halepensis*.

406 Only species was significant in the results for total height increment, which confirms Eichhorn's
407 rule (Eichhorn 1902). It states that the total production of a species at a given height should be
408 identical for all site classes. Thus, production is independent of age and site (Skovsgaard and
409 Vanclay 2008). In this early stage of development, we observed concordance with Eichhorn's
410 empirical rule, which is applied regularly to mature stands.

411 Another explanation of the difference between the two species in terms of early growth might
412 be found in the specific characteristics of *Pinus halepensis*. Puértolas et al. (2005) analysed
413 early growth and physiological parameters of *Pinus pinea* and *Pinus halepensis* over one year.
414 Apparently, *Pinus halepensis* could grow higher because it is well-adapted to summer drought.
415 The key moment for this species occurs in May, when water availability is higher. Olivar et al.
416 (2012) also found rainfall during spring to be a key variable for *Pinus halepensis* growth.

417 Non-significant influences were found for the other factors and interactions, except in the case
418 of basal diameter. Only mixture level was significant over one year while growing space and
419 interactions between species, mixture levels and growing space were significant over four years.
420 Competition can have an immediate effect on diameter but a delayed effect on height growth
421 (Richardson et al. 1999). Similarly, other factors such as growing space and mixture were non-
422 significant over one year but substantial over four years.

423 The effect of thinning on post-fire natural regeneration has been widely analysed in the
424 Mediterranean basin (Madrigal et al. 2004 for *Pinus pinaster*; Tsitsoni et al. 2004 for *Pinus*
425 *brutia*; Navarro et al. 2010 and Ruano et al. 2013 for *Pinus halepensis*...). Most studies showed
426 higher diameter increments after a thinning when post-fire natural regeneration was high,
427 indicating that reducing density increases diameter growth. However, the seedling growth

428 conditions in those studies were different than those of the present work because apart from
429 mortality, density is constant over the time in Nelder wheel design. In general terms, basal
430 diameter increments over four years were higher where density was lower (between 1000 and
431 7000 seedlings/ha for *Pinus halepensis*). In contrast, survival results were higher when seedling
432 density was higher. These results point to simultaneous facilitation and competition effects:
433 survival was higher at higher densities but early growth was lower. So we see that competition
434 and facilitation interactions are complex in the early years.

435 Regarding stand mixture, *Pinus pinaster* had similar basal diameter increments over four years
436 regardless of the mixture level. However, basal diameter increments for *Pinus halepensis* were
437 higher in 60% mixtures.

438 We can confirm our first hypothesis that the species studied would have different seedling
439 survival and early growth behaviour. In this case, survival rates and basal diameter increments
440 after four years were higher for *Pinus halepensis*. We can also confirm the second hypothesis
441 about the effect of density and mixture on survival and early growth: higher densities facilitated
442 seedling survival but basal diameter increments after four years were higher at lower densities.
443 This indicates a complex effect of density: higher densities can be considered a facilitating
444 effect during establishment, when survival is key, but competition interactions emerge during
445 early growth. Species mixture improved both seedling survival rates and basal diameter
446 increments after four years but mixture had no effect on early growth for *Pinus pinaster*. This
447 confirms our third hypothesis, that the mixture effect would be different for each species.

448 **CONCLUSIONS**

449 There are competitive and facilitating interactions for seedlings at higher densities. Higher
450 densities facilitate seedling survival but decrease early growth. *Pinus halepensis* showed higher
451 survival rates and basal diameter increments but more time is necessary to determine *Pinus*

452 *pinaster* response to density and mixture. Further research is needed on mixed stands, especially
453 during seedling establishment, because little is known about inter- and intra-specific
454 interactions at this key moment for ensuring a successful stand.

455 **DECLARATIONS**

456 Consent to participate: yes

457 Ethics approval: not applicable

458 Consent for publication: not applicable

459 Availability of data and material: The datasets used and/or analysed during the current study
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467 **Authors' contributions:**

468 Irene RUANO: designed the experiment, conducted field measurements, ran the first version
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470 from the other authors.

471 Valentín PANDO: ran the data analysis and collaborated in the first version of the manuscript.

472 Felipe BRAVO: defined the hypothesis, designed the experiment, supervised the work,
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477

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657

658 **TABLES**

659 Table 1. Radial length, potential growing space and associated tree density along each spoke of
 660 the Nelder wheel plots.

Ring	Radial length (m)	Growing space for seedling (m ²)	Densities tested (seedlings/ha)
0	1.14	-	-
1	1.45	0.13	80000
2	1.85	0.20	49162.68
3	2.36	0.33	30212.11
4	3.01	0.54	18566.36
5	3.84	0.88	11409.65
6	4.90	1.43	7011.61
7	6.25	2.32	4308.87
8	7.97	3.78	2647.94
9	10.17	6.15	1627.25
10	12.97	10.00	1000
11	16.55	-	-

661

662

663 Table 2. Cox proportional-hazards regression fitted to analyse seedling survival: significant
 664 covariates, estimated parameters, standard errors, p-values and hazard ratios with their
 665 confidence intervals in brackets, 0.95 probability.

Covariates	Parameter	Standard error	p-value	Hazard ratio
Basal diameter	-0.233536	0.081144	0.00400	0.791729 [0.6753, 0.9282]
Species	1.581141	0.112366	<0.001	4.860497 [3.8997, 6.0580]
Growing space	0.028870	0.009088	0.00149	1.029291 [1.0111, 1.0478]
Mixture	1.039063	0.194849	<0.001	2.826567 [1.9293, 4.1411]

666

667 Table 3. Binomial logistic regression fitted to analyse seedling survival: explanatory variables,
 668 estimated parameters, standard error and p-value.

Explanatory variable	Parameter	Standard error	p-value
Intercept	1.70059	0.45242	>0.001
Basal diameter	0.38284	0.12210	0.0017
Species	-2.09603	0.13909	<0.001
Growing space	-0.05497	0.01622	<0.001
Mixture	-1.36514	0.28757	<0.001

669

670 Table 4. Characteristics of the early growth variables analysed for each species: increment
671 estimates in years (1: one year (2013-2014); 4: four years (2013-2017)) (time); sampling size
672 (n), mean, maximum, minimum and standard deviation.

Species	Variable	Time	n	Mean	Max	Min	Standard deviation
<i>P. pinaster</i>	Increment of basal diameter (mm)	1	522	0.47	3.07	0.00	0.68
	Increment of basal diameter (mm)	4	254	5.76	35.95	0.21	4.75
	Increment of total height (cm)	1	522	5.91	5.91	0.00	5.59
	Increment of total height (cm)	4	254	31.55	31.55	0.00	22.93
<i>P. halepensis</i>	Increment of basal diameter (mm)	1	579	2.22	7.39	0.00	1.51
	Increment of basal diameter (mm)	4	545	25.82	68.23	2.66	12.73
	Increment of total height (cm)	1	579	12.40	43.50	0.00	10.03
	Increment of total height (cm)	4	545	125.54	241.60	0.00	42.81

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Table 5. P-values and significant values for early growth factors. Variables: Diameter: basal diameter increment; Height: increment of total height; Time: increment estimates in years (1: one year (2013-2014); 4: four years (2013-2017)). Significance values: ***: p-value <0.001; **: p-value <0.01; *: p-value < 0.05; .: p-value < 0.1; ns: non-significant effect.

Model	Variable	Time	Wheel	Growing space	Species	Mixture	Sp*Mixture	Sp*Space	Space*Mixture	Sp*Space*Mixture
1	Diameter	1	<0.001 ***	0.1091 ns	<0.001 ***	0.0314 *	0.7707 ns	0.1123 ns	0.6635 ns	0.4752 ns
2	Diameter	4	<0.001 ***	<0.001 ***	<0.001 ***	0.0817 .	0.0022 **	<0.001 ***	0.0776 .	0.8178 ns
3	Height	1	<0.001 ***	0.1753 ns	<0.001 ***	0.0545 .	0.3339 ns	0.4854 ns	0.8748 ns	0.4607 ns
4	Height	4	<0.001 ***	0.0988 .	<0.001 ***	0.118 ns	0.377 ns	0.4512 ns	0.0764 .	0.5415 ns

FIGURES

Figure 1. Location of the experimental site with a climate diagram (data from Sistema de Clasificación Bioclimática Mundial, 1996–2009). Nelder experimental design (1962): below: dots represent seedlings; distances along spokes and angles between spokes, measured from the centre of the plot.

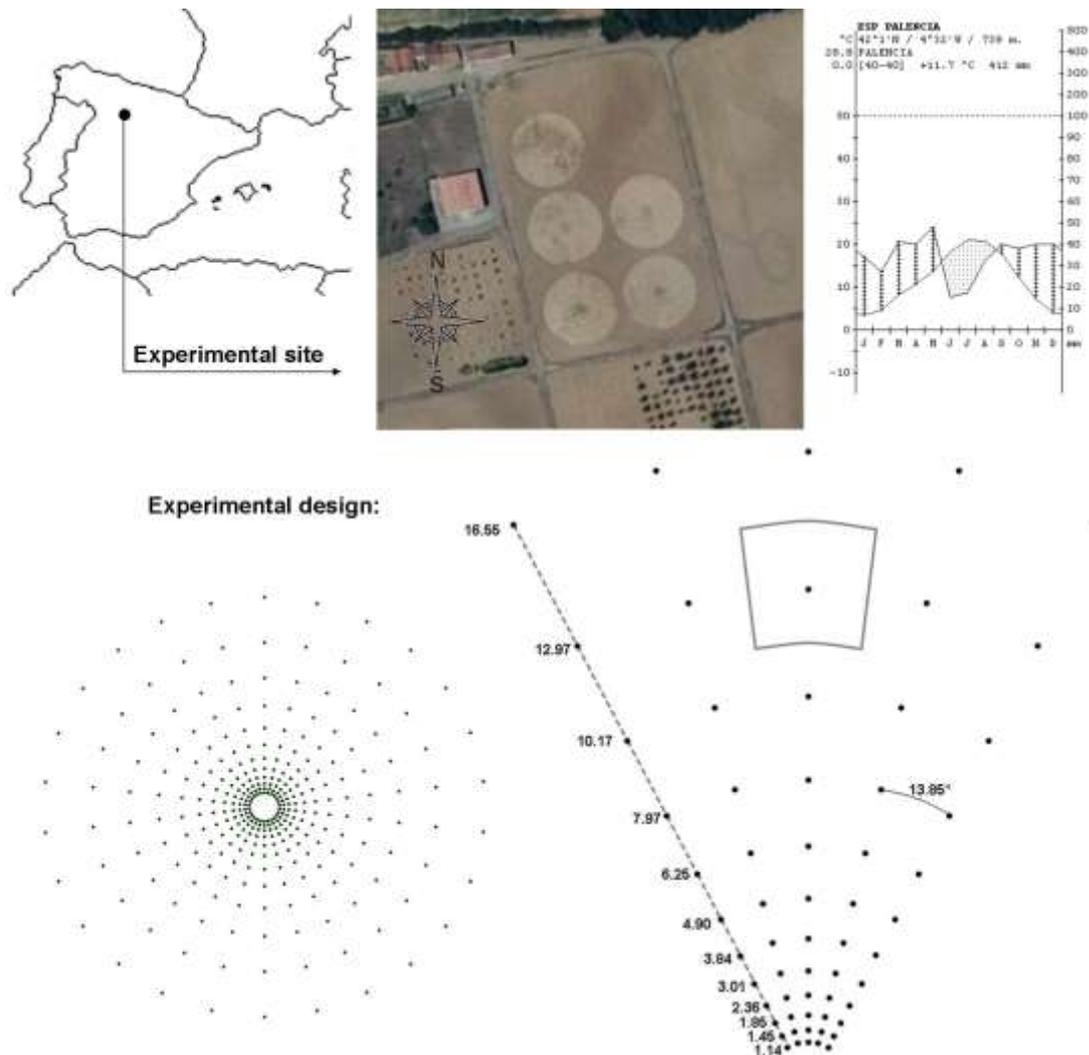


Figure 2. Species distribution along the spokes of the permanent (left plot) and temporal (right plot) Nelder wheels. Mixture levels are shown by percentage examples. Black dots are *Pinus pinaster* seedlings and white dots are *Pinus halepensis* seedlings. Grey dots in temporal wheel plot serve as buffers for harvests.

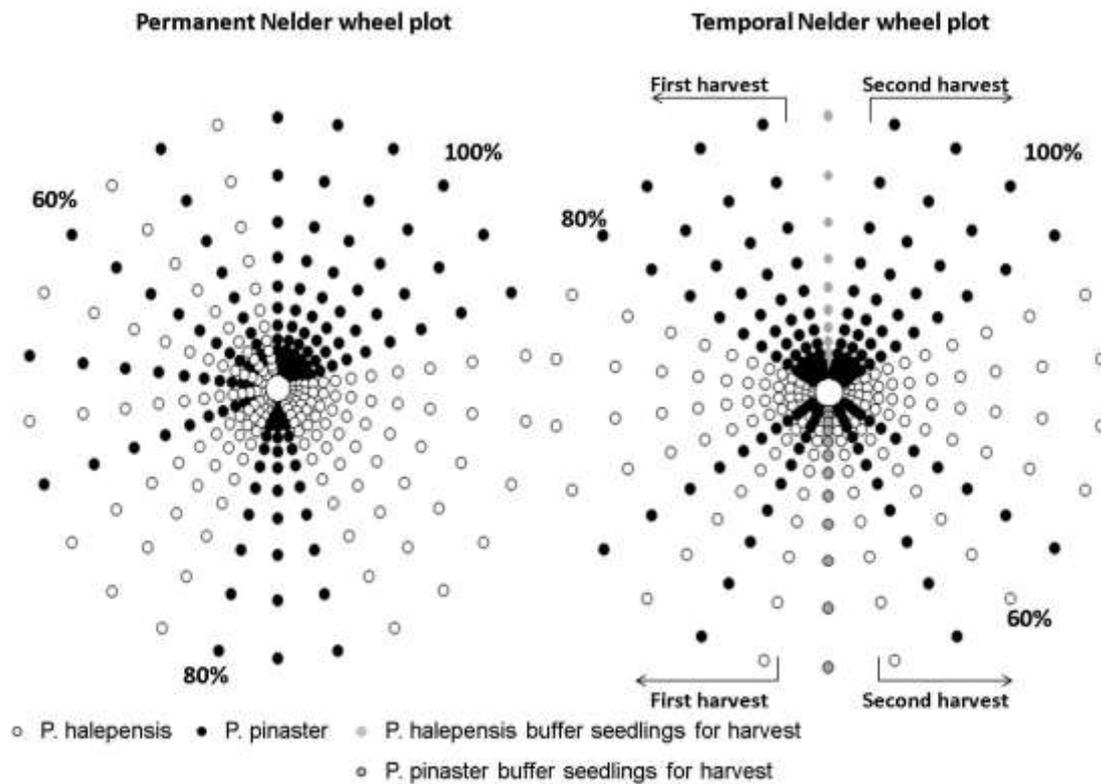


Figure 3. Effect of mortality in the experimental design: initial design, evolution and re-calculation of growing spaces using Voronoi polygons.

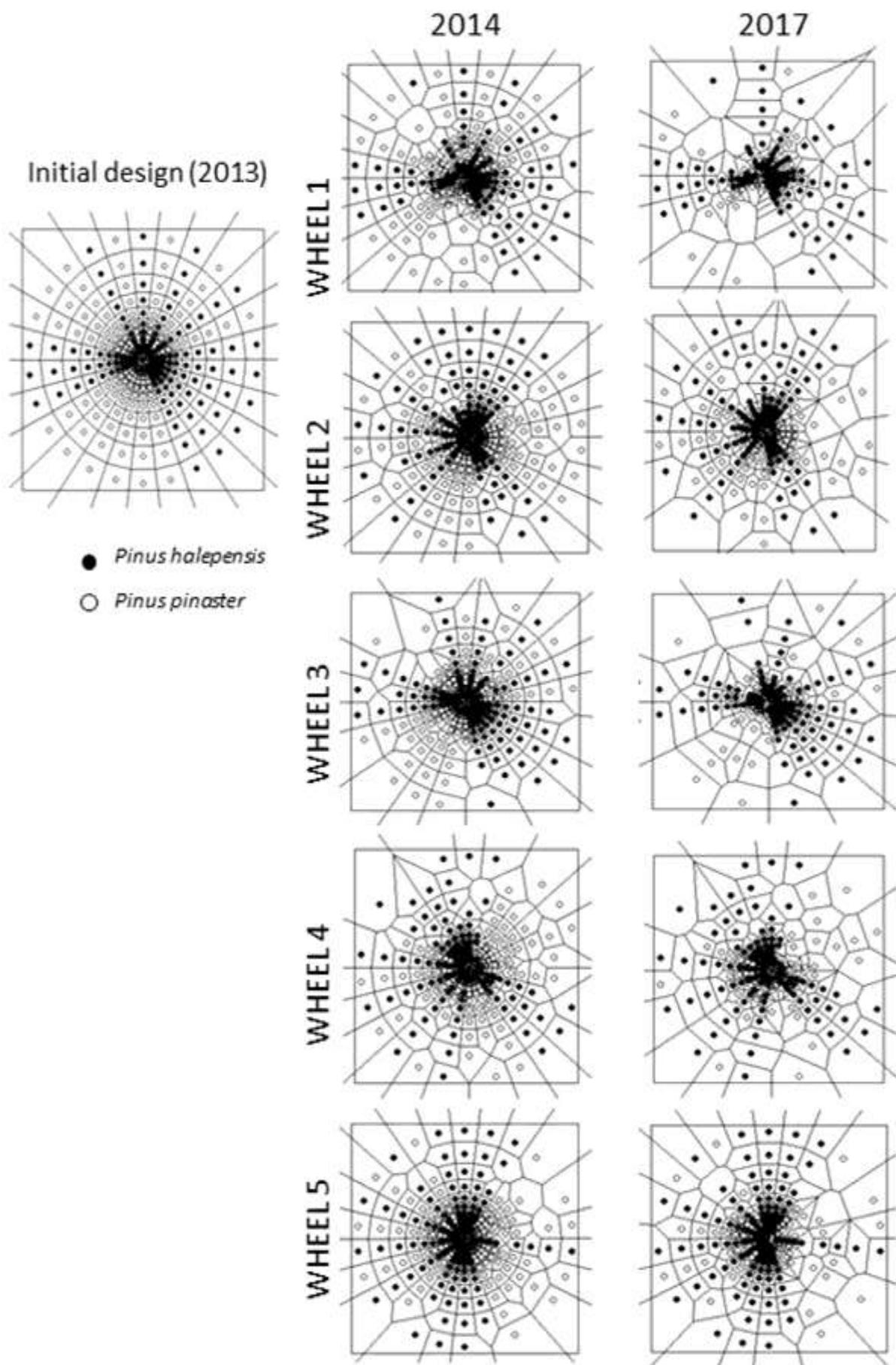


Figure 4. Mean values and confidence intervals for basal diameter over four years, based on the growing space/density of each species and factor level comparison: levels with same letters indicate no significant differences ($P>0.05$). Numeric details in Supplementary Table 3.

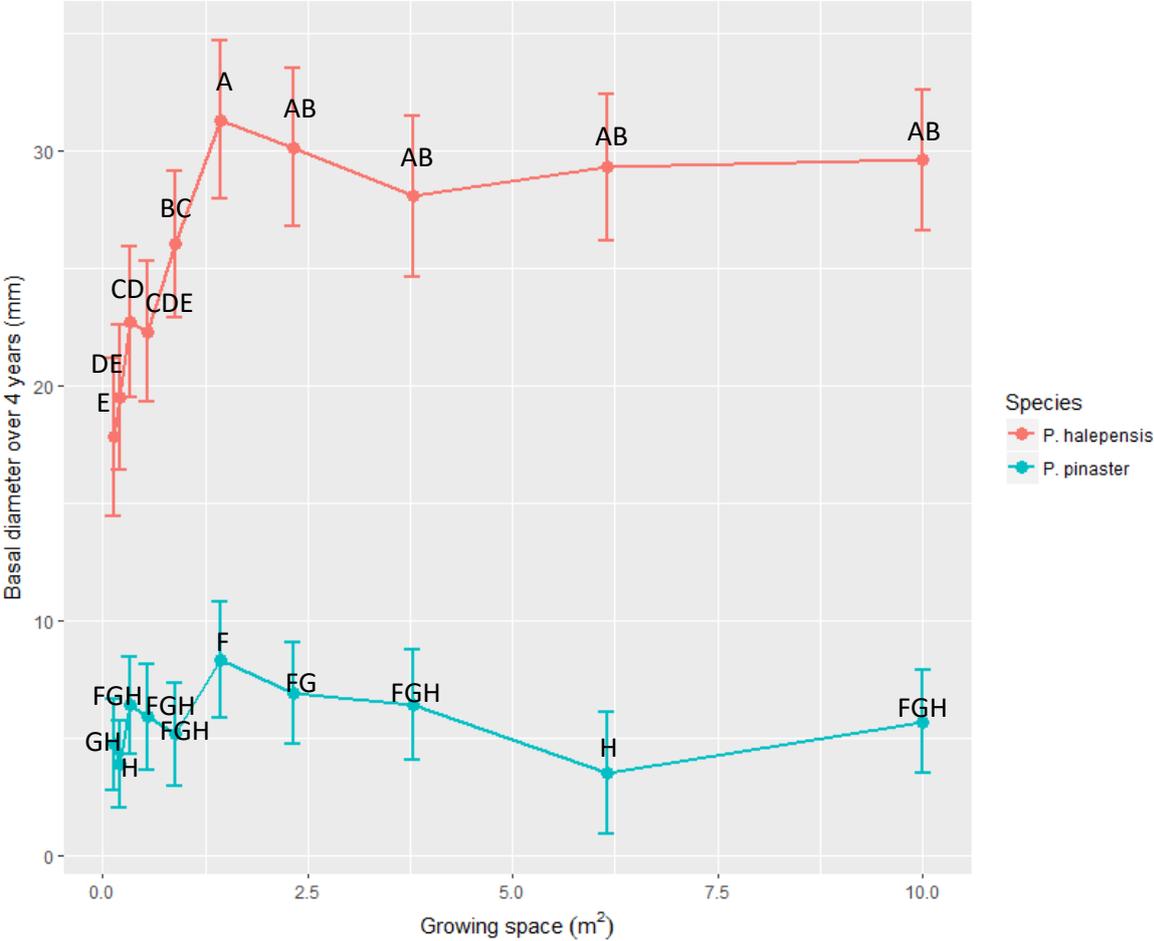
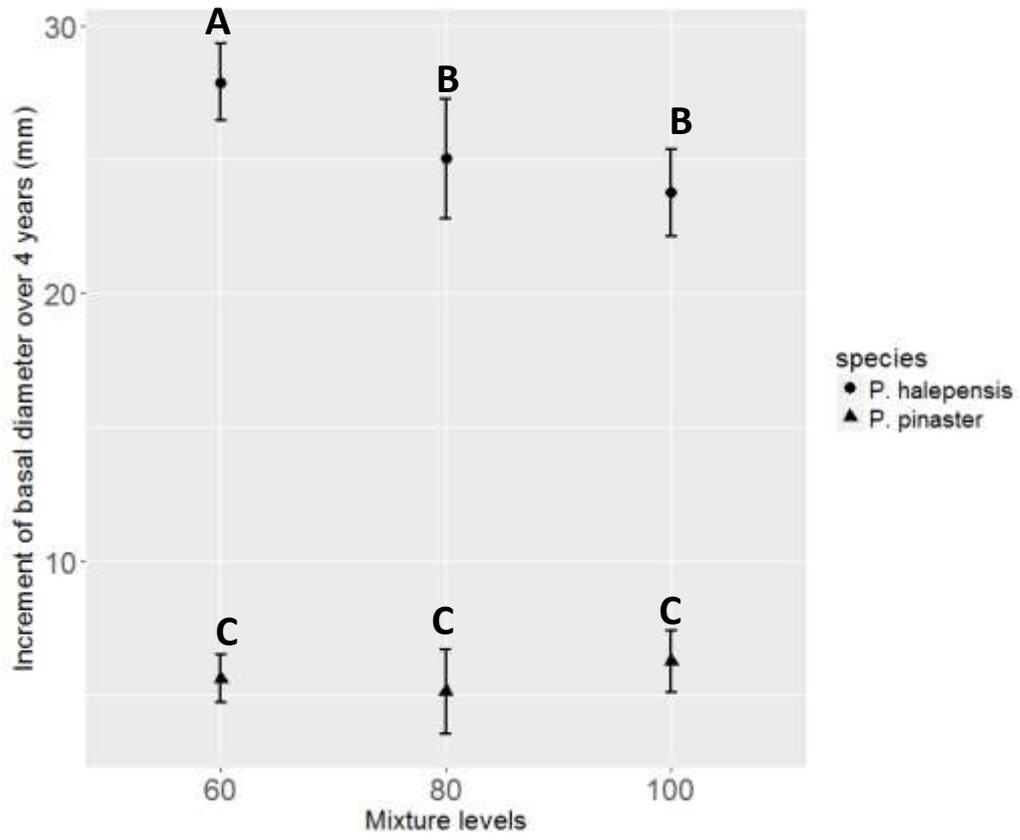


Figure 5. Mean values and confidence intervals for basal diameter over four years based on mixture levels for each species and factor level comparison: levels with same letters indicate no significant differences ($P>0.05$).



SUPPLEMENTARY MATERIAL

Supplementary Table 1. Significance of parameters of fitted models for analysing seedling survival: Cox proportional-hazards regressions and binomial logistic regressions. Explanatory variables: d13: basal diameter at time of planting (2013); H13: total height at time of planting (2013); H/d: height to diameter ratio at time of planting (2013); sp: species (*Pinus pinaster* and *Pinus halepensis*); gs: growing space for the seedling; mx: mixture level. AIC: Akaike’s information criterion. Significance values: ***: p-value <0.001; **: p-value <0.01; *: p-value < 0.05; ∙: p-value < 0.1; ns: non-significant effect.

model	Cox proportional-hazards regressions							Binomial logistic regressions									
	d13	H13	H/d	sp	gs	mx	AIC	Δ _i	b0	d13	H13	H/d	sp	gs	mx	AIC	Δ _i
1	***						6911.22	269.32	***	***						1694.33	289.18
2		***					6849.16	207.26	***		***					1615.07	209.93
3			***				6872.19	230.29	***			***				1656.74	251.59
4				***			6699.16	57.27	***				***			1448.69	43.55
5					**		6953.35	311.46	***					***		1725.92	320.78
6						***	6942.46	300.57	***						***	1721.48	316.33
7	**			***			6675.82	33.93	ns	**			***			1436.70	-1405.14
8	***				**		6904.14	262.24	***	***				***		1685.10	279.96
9	***					***	6892.30	250.41	*	***					***	1679.89	274.75
10	**			***	**		6669.08	27.19	.	**			***	***		1427.02	21.88
11	***				**	***	6885.45	243.55	.	***				***	***	1670.58	265.43
12	**			***		***	6648.96	7.06	***	***			***		***	1415.79	10.65
13	**			***	**	***	6641.89	0.00	**	***			***	***	***	1405.90	0.75
14	ns	***					-		***	ns	***					-	
15	ns	.		***			-		ns	*	ns		***			-	
16	ns	***			***		-		***	ns	***			***		-	
17	ns	***				***	-		**	ns	***				***	-	
18	ns	***				***	-		ns	.	ns		***	***		-	

19	ns	***		**	***	-	*	ns	***		***	***	-	
20	ns	.	***		***	-	***	.	ns		***	***	-	
21	ns	.	***	**	***	-	***	.	.		***	***	***	1405.14 0.00
22		**	***			6691.89	50.00	**	**	***			1441.55	36.41
23		***		**		6841.51	199.61	***	***		***		1604.42	199.28
24		***			***	6825.98	184.09	**	***			***	1598.74	193.60
25		**	***	**		6684.87	42.98	***	**	***	***		1431.22	26.08
26		***		**	***	6818.26	176.37	*	***		***	***	1587.98	182.84
27		**	***		***	6665.17	23.28	***	**	***		***	1420.79	15.64
28		**	***	**	***	6657.73	15.84	***	**	***	***	***	1410.21	5.06
29			ns	***		-		***		ns	***		-	
30			***		**	6864.68	222.79	***		***		***	1645.80	240.66
31			***		***	6849.35	207.46	*		***		***	1640.37	235.22
32		.	***	**		6675.19	33.30	***		ns	***	***	-	
33		***		***	***	6841.52	199.62	ns		***		***	-	
34		.	***		***	6654.78	12.89	***		ns	***		-	
35		.	***	**	***	6647.29	5.40	***		.	***	***	***	1413.13 7.98
36			***	**		6692.15	50.26	***			***	***	1438.66	33.51
37				**	***	6934.92	293.02	***			***		1428.04	22.90
38			***		***	6672.28	30.38	***			***	***	1417.74	12.59
39			***	**	***	6664.83	22.94	***				***	1711.96	306.82

Supplementary Table 2. Model 1: significant factors affecting basal diameter increment over one year and comparison of means. Values: LS means, standard error and confidence intervals. Comparison: factor levels with same letters indicate no significant differences ($P>0.05$).

Significant factor	Factor levels	Values	Comparison
Species	<i>P. halepensis</i>	2.2047 (0.06049) [2.0860, 2.3234]	a
	<i>P. pinaster</i>	0.4518 (0.03305) [0.3869, 0.5167]	b
Mixture	0.6	1.4380 (0.05074) [1.3384, 1.5376]	A
	0.8	1.2695 (0.07473) [1.1229, 1.4162]	AB
	1	1.2315 (0.05847) [1.1167, 1.3462]	B

Supplementary Table 3. Model 2: Significant factors affecting basal diameter increment over four years and comparison of means (I): species, growing space (m^2) and interaction between them. Values: LS means, standard error and confidence intervals. Comparison: factor levels with same letters indicate no significant differences ($P>0.05$).

Growing space	Species			With constant species		
	<i>Pinus halepensis</i>	<i>Pinus pinaster</i>				
0.13	17.8261 (1.7147) [14.4595, 21.1927]	<i>E</i>	4.7491 (1.0009) [2.7839, 6.7143]	<i>GH</i>	11.3465 (1.0767) [9.1628, 13.53029]	<i>E</i>
0.2	19.4885 (1.5698) [16.4063, 22.5706]	<i>DE</i>	3.8915 (0.9455) [2.0351, 5.7478]	<i>H</i>	11.7504 (1.0051) [9.7121, 13.7888]	<i>DE</i>
0.33	22.7209 (1.6238) [19.5328, 25.9089]	<i>CD</i>	6.423 (1.0543) [4.3530, 8.4930]	<i>FGH</i>	14.7033 (1.0560) [12.5616, 16.8451]	<i>BCD</i>
0.54	22.3139 (1.5240) [19.3217, 25.3061]	<i>CDE</i>	5.9166 (1.1373) [3.6837, 8.1496]	<i>FGH</i>	14.4189 (1.0453) [12.2989, 16.5390]	<i>CD</i>

0.88	26.0136 (1.5798) [22.9119, 29.1153]	BC	5.1811 (1.1162) [2.9896, 7.3727]	FGH	16.0208 (1.0610) [13.8691, 18.1725]	ABC
1.43	31.3030 (1.7118) [27.9421, 34.6640]	A	8.3631 (1.2491) [5.9106, 10.8156]	F	18.2732 (1.1002) [16.0418, 20.5045]	A
2.32	30.1340 (1.7020) [26.7924, 33.4756]	AB	6.9137 (1.1010) [4.7521, 9.0753]	FG	18.7981 (1.1027) [16.5617, 21.0344]	A
3.78	28.0618 (1.7368) [24.6518, 31.4718]	AB	6.4328 (1.1862) [4.1040, 8.7617]	FGH	17.5344 (1.1360) [15.2306, 19.8383]	AB
6.15	29.2815 (1.5928) [26.1542, 32.4088]	AB	3.5257 (1.3229) [0.9284, 6.1230]	H	16.4936 (1.1148) [14.2328, 18.7545]	ABC
10	29.6052 (1.5263) [26.6086, 32.6018]	AB	5.7301 (1.1054) [3.5598, 7.9004]	FGH	17.3867 (1.0209) [15.3163, 19.4572]	AB
With						
constant	25.7930 (0.5147)		5.7191 (0.3712)			
growing space	[24.7824, 26.8035]	a	[4.9903, 6.4479]	b		

Supplementary Table 4. Model 2: Significant factors affecting basal diameter increment over four years and comparison of means (II): interaction between species and mixture levels. Values: LS means, standard error and confidence intervals. Comparison: factor levels with same letters indicate no significant differences ($P>0.05$).

Mixture	Species	
	<i>P. halepensis</i>	<i>P. pinaster</i>
60	27.8840 (0.7299) [26.4510, 29.3171]	A 5.6138 (0.4450) [4.7401, 6.4874]
80	25.0300 (1.1401) [22.7915, 27.2685]	B 5.1147 (0.7999) [3.5442, 6.6851]
100	23.7532 (0.8230) [22.1374, 25.3690]	B 6.2593 (0.5899) [5.1010, 7.4176]

Supplementary Table 5. Models 3 and 4: Significant factors affecting total height increment over one and four years and comparison of means (II): species. Values: LS means, standard error and confidence intervals. Comparison: factor levels with same letters indicate no significant differences ($P>0.05$).

		Species			
model	<i>Pinus halepensis</i>		<i>Pinus pinaster</i>		
3	12.3377 (0.4434) [11.4676, 13.2078]	A	5.7906 (0.2875) [5.2263, 6.3548]	B	
4	124.30 (1.8077) [120.75, 127.85]	a	30.5963 (1.7874) [27.0870, 34.1057]	b	