1   # Shrub species affect distinctively the functioning of scattered

2   ## *Quercus ilex* trees in Mediterranean open woodlands

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11   Running head: Effects of shrub understory on oaks functioning

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22   **Abstract**

23   A low tree stand density has been showed as necessary to thrive with summer drought in semiarid

24   Mediterranean open woodlands. Shrub encroachment of these open woodlands is currently

25   recommended to guarantee the persistence of the system, due to the nursery effect of shrubs on tree

26   seedling. However, the increase in abundance and cover of a shrub understory in these water limited

27   woodlands could bring consequences to tree overstory functioning. The present study analyzes the

28   physiological status of scattered *Quercus ilex* L. trees in paired adjacent plots with and without the

29   presence of a shrubby understory in CW Spain. Two contrasting shrub strategies were addressed in

30   order to take into account possible species-specific effects: a dense-shallow rooting shrub (*Cistus*

31   *landanifer* L.) and a sparse-deep rooting shrub (*Retama sphaerocarpa* (L.) Boiss). Leaf water

32   potential (at predawn and midday), leaf gas exchange parameters (net photosynthetic rate and

33   stomatal conductance), leaf nitrogen content and chlorophyll fluorescence transients (maximum

34   photochemical efficiency and performance index, *sensu* Strasser et al., 2004) were measured during

35   three consecutive summers. Trees growing with *Cistus* as understory showed significant lower leaf

36   water potential, leaf gas exchange parameters, leaf nitrogen content and chlorophyll photochemical

37   efficiency than trees growing without shrub competence. However, the presence of the legume

38   *Retama* did not affect significantly the physiological state of *Quercus ilex*. Thus, we conclude that the

39   presence of a shrubby understory has the potential to modify the functioning of scattered trees, but

40   these effects are species-specific.

41   Keywords: Plat-to-plant interaction, OJIP, *Cistus ladanifer*, *Retama sphaerocarpa*, Iberian dehesa,

42   Encroachment.

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1. Introduction

Ecosystems with scattered trees occur throughout the world. The prevailing ecological usage of these systems denotes a continuous grass layer as understory and scattered trees layer as overstory. They comprehend both natural ecosystems, as savannas and sparse woody steppes, and man-made open woodlands (Scholes and Archer, 1997; Manning et al., 2006; Moreno and Pulido, 2009). Open woodlands are the result of the simplification, in terms of structure and woody species number, of former close forests. These practices have been the origin of well-established cultural landscapes as European wood-pasture (Eichhorn et al., 2006; Bergmeier et al., 2010), Mediterranean open woodlands (e.g. Iberian dehesas; Vicente and Ales, 2006) or some scattered oak systems of North America (McEwan and McCarthy, 2008) which have been sustained over a long period of time. Furthermore, tree clearance in water-limited ecosystems of semiarid regions have important consequences for remnant trees which, due to a higher soil volume available, enhance their production and functioning (Pulido and Díaz, 2005; Moreno and Cubera, 2008). Indeed, scattered trees could play an important role in facilitating climate change adaptation. They are keystone structures because of the disproportionally large ecological values and ecosystem services that they provide relative to the area they occupy in these landscapes (Manning et al., 2009).

However, there is a great concern worldwide about regeneration failure in open woodlands (Plieninger, 2006; Tyler et al., 2006; Alijanpour and Mahmoudzadeh, 2007; Campos et al., 2007; Gibbons et al., 2008; Manning et al., 2009). At this point, some authors have argued that seedling survival is related to ''safe sites'' offered by the presence of shrubs in Mediterranean climates (Castro et al., 2004; Gómez-Aparicio et al., 2004; Gómez-Aparicio et al., 2005; Pulido and Díaz, 2005; Smit et al., 2008; Gómez-Aparicio, 2009). Consequently the shifting from two-layered to three-layered systems (tree, shrub and pasture), commonly referred as 'shrub encroachment', has been recommended to ensure tree regeneration and so that long-term persistence of the Mediterranean open woodlands (Ramírez and Díaz, 2008; Plieninger et al., 2010; Pulido et al., 2010).

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70 But shrub encroachment is also seen as a potential threat both natural and human-dominated scattered

71 trees ecosystems worldwide (Fensham et al., 2005; Manning et al., 2006; Acacio et al., 2008; Wigley

72 et al., 2010). A great variety of causes, ranging from climate change to grazing and fire suppression

73 are implicated in this global phenomenon which can have the potential to alter thoroughly the

74 structure and functioning of scattered-trees systems due to the introduction of a new woody vegetation

75 layer (van Auken, 2000). Trees in semiarid open woodland depend on soil resources which take up far

76 away from interstitial areas between trees (Joffre and Rambal, 1993; McPherson, 1997) thanks to their

77 long lateral roots (Schenk and Jackson, 2002; Moreno et al., 2005). Thus, competition for soil

78 resources between shrubs and trees is likely to affect the functioning and persistence of the tree layer

79 in water-limited open woodlands.

80 Barnes & Archer (1999) described strongly asymmetrical tree-shrub interaction. Understory shrubs,

81 whose establishment is facilitated by the overstory trees (*Prosopis*), impressed a pronounced negative

82 effect on *Prosopis* after their ingress in the system. Soil resource depletion by shallow-rooted

83 understory shrubs appears to be a primary factor contributing to the demise of the deeply rooted

84 overstory *Prosopis* plants. Kume et al. (2003) found that the understory woody vegetation had

85 negative physiological effects on the overstory pine needles through the competition of roots for water

86 and nutrients. Cubera and Moreno (2007) and Pulido et al. (2010) reported a clear competition for

87 soil nutrients and water between trees and shrubs in Mediterranean open woodlands; mature *Quercus*

88 *ilex* trees growing with a pasture understory showed a significantly improved nutritional and

89 physiological status, faster growth, and higher fruit productivity, than trees growing with a shrub

90 understory.

91 According to Canadell et al. (1996), who reviewed the maximum rooting depth of the major terrestrial

92 biomes, mean maximum rooting depth for trees from the various water-limited regions of the world is

93 12.6 ± 3.4 m while for shrubs is 3.5 ± 0.3 m. Thus, although most studies indicate the existence of a

94 strong competence for belowground resources between trees and shrubs, according to their rooting

95 profile, a certain complementarity in the use of soil resources could also exist between these two

96   functional plant groups. The coexistence of functional groups in scattered-tree systems have often

97   been attributed to a rooting-niche separation, and several published studies have reported a

98   complementary use of soil resources among plant functional types around the world (Weltzin et al.,

99   1997; Midwood et al., 1998; Dulormne et al., 2004; Moreno et al., 2005; Bucci et al., 2009;

100   Kulmatiski et al., 2010). However most of them analyzed the coexistence between trees and grasses

101   and little is known about the coexistence and the nature of interaction between woody vegetation

102   layers .

103   In order to clarify the effects of shrubs on trees under water-limited conditions, we analyzed the

104   ecophysiologycal state of scattered *Quercus ilex* trees in shrub encroached open woodlands in western

105   Iberian Peninsula. Each open woodland was composed by two habitats, one shrub encroached and one

106   adjacent control without shrub layer. Additionally, due to the great variety of structural,

107   morphological and functional types of shrubs present in Mediterranean woodlands, we selected two

108   widely spread shrub species as a representative of two common plant functional types (*sensu* Gitay

109   and Noble, 1997), one deep rooted (*Retama sphaerocarpa* (L.) Boiss) and one shallow rooted (*Cistus*

110   *ladanifer* L.). These two basic types of rooting profile correspond to two main rooting profile

111   strategies to cope with highly seasonal water shortage (Schwinning and Ehleringer, 2001; Schenk and

112   Jackson, 2002; Silva et al., 2002; Bucci et al., 2009).

113   We hypothesized that the presence of a shrub layer in the Mediterranean open woodland may affect

114   the ecophysiological state of the tree layer due to the interaction for soil resources and this effect

115   could be dependent of the functional shrub type present. Therefore, we try to answer the following

116   specific questions (i) can a shrubby understory compete with the tree overstory for belowground

117   resources (N and water), and so that affect their ecophysiological state?, and (ii) can different rooting

118   strategies of shrub understory perform different responses in the ecophysiological tree state?. Given

119   the sparse and deep rooting system of *Quercus ilex* (Moreno et al. 2005), we expect that *Cistus, a*

120   shallow rooted shrub (< 50 cm, Silva et al., 2002), could use the soil water complementarily and

121   compete strongly for N. By contrast, *Retama, a* deep rooted leguminous shrub (> 10 m, Haase et al.,

122   1996), may facilitate N acquisition but compete for deep soil water, so that their effects on the

123   ecophysiological state of trees would be more conspicuous at the end of the summer when this

124   resource gets limiting and becomes essential for *Quercus ilex* (Cubera and Moreno, 2007).

125   **2. Material and Methods**

126   *2.1 Study system and species*

127   The study was carried out in Iberian dehesas, savanna-like open woodlands dominated by scattered

128   oak (10-40 trees ha^{-1}) with a native pasture understory. This man-made landscape occupies 3.1 million

129   ha in SW Iberian Peninsula (Moreno and Pulido, 2009), and is considered to be the most extended

130   silvopastoral system in Europe (Eichhorn et al., 2006). Similar open woodlands extend both in

131   southern Europe and northern Africa (Eichhorn et al., 2006). Dehesas are a simplification of former

132   Mediterranean forests and shrublands in terms of their structure and woody species number in order to

133   favoring the pasture layer. Traditionally, pasture layer was maintained through management practices

134   as: (a) regular thinning and pruning of trees; (b) livestock grazing for prevention of shrub

135   encroachment; and (c) periodical mechanical clearance of shrubs (Moreno and Pulido, 2009).

136   Currently, two main divergent trends seem to threat the system, extensification and intensification

137   being the former process more widespread and dominant than the latter which is more localized.

138   Extensification is associated to certain abandonment allowing shrub encroachment of grasslands and

139   open woodlands (Pinto-Correia and Mascarenhas, 1999).

140   The study is focused on *Quercus ilex* (*Q.ilex* hereafter), a small- to medium-sized trees (3-20 m in

141   height, usually 5-10 m in Iberian dehesas), that grow in dry subhumid climate in the Mediterranean

142   basin. This sclerophytic specie has morphological and physiological features with moderate capacity

143   and plasticity to face severe drought conditions (Martínez-Vilalta et al., 2002; Mereu et al., 2009).

144   *Cistus ladanifer* (*Cl*, hereafter) is a shallow rooted shrub (< 50 cm, Silva et al., 2002) that grows in

145   dense population and with allelopathic compounds (Chaves and Escudero, 1997). *Retama*

146   *sphaerocarpa* (*Rs*, hereafter) is a leafless N-fixing, deep rooted shrub (> 10 m, Haase et al., 1996) that

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147 grows in more sparse population and with positive effects on soil nitrogen content (Rodríguez-

148 Echeverría and Pérez-Fernández, 2003).

2.2 Study area

150 The study was performed in the north of Extremadura region, Central-Western Spain (39.54 - 40.02

151 N, 05.58 - 06.15 W). Extremadura represents the primary area for dehesa landscape, with 1.03 million

152 ha dominated by these open oak woodlands (MMARM 2007). The climate of the area is

153 Mediterranean, with hot, dry summers, and mild, rainy winters. Mean annual precipitation and

154 temperature are 552 mm and 16 ºC. All woodland stands were flat or gently sloping areas. Soils were

155 fine-textured for oak woodlands with *Cl* understory, and coarse-textured for oak woodlands with *Rs*

156 understory, being oligotrophic and acidic in all cases.

2.3 Experimental layout

158 We selected twelve open woodland stands for our study, which were grouped in function of the type

159 of shrubby understory present. In six stands the understory was composed only by *Cl* and in the rest

160 six stands the understory was composed only by *Rs*. In each stand two adjacent plots (habitats) were

161 selected, being similar in soil, slope and tree cover but contrasting in understory cover, one habitat

162 with shrubby understory (shrub encroached (*SE*), either with *Cl* (SE_{Cl}) or with *Rs* (SE_{Rs})) and the

163 adjacent one without shrubby understory (control, *CT*). Shrub cover was function of the type of shrub

164 present. In this way, *Rs* cover ranged from 20% to 30%, 1.75 m height on average, while *Cl* cover

165 ranged from 60 to 95%, 2.2 m height on average. Tree layer was only composed by *Q.ilex* in all plots.

166 Canopy size of mature trees ranged from 5 to 20 m width and stem diameter at breast height ranged

167 from 30 to 90 cm (Table 1). Tree cover ranged from 10 % to 31 %; although tree cover was generally

168 lower in SE plots than in their respective *CT* plots (Table 1), this difference was assumed to have not

169 any effect on results because in all cases tree cover was well below the threshold of tree-tree

170 competition for soil resources (Moreno and Cubera 2008).

171 In each of 24 plots (6+6 stands x 2 habitats), six matures similarly-sized trees (around 40 cm of DBH)

172 were selected to conduct measures (144 trees in total). Measures were taken in three consecutive

173 summers (2007-2009). The first summer was considered as average respect to water availability

174 according to the rainfall accumulated in that hydrological year (21th sept 2006 to 20th sept 2007), 527

175 mm compared with the decade average of 552 mm. The following two years were qualified as dry and

176 very dry, with 445 and 364 mm, respectively, for the whole hydrological year.

177 *2.4 Leaf water potential*

178 Tree leaf water potential (ψ) was measured in the 144 selected trees during the dry season (from July

179 to September) of the three consecutive years. Measurements were made by means of a Scholander

180 chamber (Skye Instr., UK, model SKPM 1400) once per month (around mid-month) . In 2007 and

181 2009 two measurements were taken each day: predawn potential before sunrise (ψ_{pd}), and midday

182 potential (ψ_{md}). In 2008 just ψ_{pd} was measured due to technical constraints. Measurements were

183 conducted on two current-year twigs per tree (around 1–2 mm in diameter and 2–4 leaves)

184 immediately after excision. A third twig was sampled if the observed difference in the water potential

185 between the two measurements was higher than 0.2 MPa. Twigs were excised from the outer portion

186 of peripheral branches in the mid height of the canopy.

187 *2.5 Leaf Gas exchange.*

188 Net leaf photosynthesis (A, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}) and

189 intracellular CO_2 concentration (Ci, μmol mol^{-1}) of *Q.ilex* leaves were measured by means of a

190 portable differential infrared gas analyzer (IRGA model LCi, ADC BioScientific Ltd., UK) and a

191 broadleaf chamber (area: 6.25 cm^2). Measurements were made on the same trees and days as ψ in

192 2007 and 2009. Two sun-exposed current-year leaves per tree were measured between 9:00 and 11:00

193 am. A red/blue light emitting diode (LC pro, ADC) was used to measure gas exchange at saturating

194 light (1500 mmol m^{-2} s^{-1}). Intrinsic water use effiency (iWUE) was calculated as neat leaf

195 photosynthesis rate divided by stomata conductance (Medrano et al., 2009). Apparent soil-to-leaf

196   hydraulic conductance (K_L) was calculated as leaf transpiration rate (E, mmol H_2O m^{-2} s^{-1}) divided by

197   leaf water potential gradient ($\Delta\psi = \psi_{pd} - \psi_{md}$) (Sperry, 2000). Additionally, in July and August of 2009

198   in two stands (one with *Cl* and one with *Rs*) a daily track of gas exchange was measured in the same

199   trees (six trees in SE_{Cl} plot, six in SE_{Rs} plot, and six in each of their respective *CT* plots).

200   Measurements were made at intervals of one hour from sunrise till midday and at intervals of two

201   hour from midday till sunset. In all cases leaves were harvested after each measurement to determine

202   their leaf area, and then to recalculate the gas flux parameters according to the amount of the leaf area

203   that was included in the chamber.

204   *2.6 Nitrogen content*

205   Leaf nitrogen content (N, mg g^{-1}) was sampled in the 144 trees two different years, in 2007 and 2009.

206   Full-extended leaves of 40 current- year twigs (10 per aspect) were harvested per tree by the end of

207   July in both years. Leaves were removed from twigs and grounded in order to make a composite

208   sample per tree. Then, N content was analyzed by Kjeldhal procedure.

209   *2.7 Chlorophyll Fluorescence measurement*

210   Maximum PSII photochemical efficiency (Fv/Fm ratio), the most widely used PSII efficiency

211   indicator, was determined to check the functional state of photosynthetic apparatus. Many studies

212   have used this ratio as an indicator of photosynthesis performance under different kind of stress as

213   low water availability or low temperatures (García-Plazaola et al., 2000; Ogaya and Peñuelas, 2003;

214   Baquedano and Castillo, 2006). However, some studies have shown this parameter to be quite

215   insensitive to change at low or moderate stressful conditions (e.g., Thach et al., 2007). Force et al.

216   (2003) demonstrated the advantage of using a number of JIP test-derived fluorescence parameters to

217   evalutate the photosynthetic apparatus functioning due to their effectiveness and sensivity, rather than

218   using only the Fv/Fm ratio. The JIP-test analyzes the fluorescence transient to derive a number of

219   biophysical parameters that quantify the energy flow through PSII. The performance index (PI) on an

220   absorption basis (Strasser et al., 2004) has been introduced as a multi-parametric expression of the

221   three independent steps which contribute to the initial stage of photosynthetic activity of the reaction

222   center (RC) complex, namely absorption of light energy (ABS), trapping of excitation energy (TR),

223   and conversion of excitation energy to electron transport (ET). In addition, the PI has been found to be

224   sensitive to different kind of stress, i.e. excess light or drought (Bussotti, 2004; Thach et al., 2007).

225   Thus, the PI was also used as an indicator of photosynthetic apparatus functioning

226   Chl fluorescence transient of *Q. ilex* trees were measured by means of a Multy-Mode Chlorophyll

227   Fluorometer OS5p (Multi-mode Chlorophyll Fluorometer OS5p, Opti-science Inc., USA).

228   Measurements were made on the same trees and days as ψ between 7:00 am and 9:00 am in July and

229   August of 2009. All measurements were performed on the upper surface of three current-year fully

230   expanded leaf per tree following a dark adaptation period of 30 min using the leaf clip provided by the

231   manufacture. The data used from the original measurement were: maximal fluorescence intensity

232   (Fm), minimal fluorescence intensity (Fo), fluorescence intensity at 300 µs (F300 µs), and

233   fluorescence intensity at 2 ms (F2 ms). From the original data Fv/Fm ratio was calculated as: (Fm −

234   Fo)/Fm; and PI was calculated as described in Thach et al., (2007) who redefined the original

235   formulae of Strasser et al., (2004):

236                  $PI = (\gamma_{RC}/(1- \gamma_{RC}))((\phi_{Po}/(1-\phi_{Po}))((\psi o/(1- \psi o))$

237   Where γ_{RC} represents the fraction of reaction center Chlorophylls per total Chlorophyll content; ϕ_{Po}

238   represents the maximum quantum yield of primary photochemistry and ψ_o represents the probability

239   that a photon trapped by the PSII reaction center enter in the electron transport chain. From here,

240   Thach et al., (2007) defined $(\gamma_{RC}/(1- \gamma_{RC}))$ = RC/ABS, what represents the active RC density on a Chl

241   basis and can be estimated from the original fluorescence measurements as RC/ABS = [(F2ms − Fo) /

242   4(F300µs −Fo)]·(Fv/Fm); $((\phi_{Po}/(1-\phi_{Po}))$ = P_{TR}, what represents the performance due to the trapping

243   probability and is estimated as P_{TR} = Fv/Fo; and $((\psi o/(1- \psi o))$ = P_{ET}, what represents the performance

244   due to the conversion of excitation energy to electron transport and is estimated as P_{ET} = (Fm - F2ms)

245   / (F2ms − Fo). Accordingly, PI = (RC/ABS) * P_{TR} * P_{ET}.

2.8 Statistical Analysis

247 Comparison of mean values of Ψ_{pd}, Ψ_{md}, A, gs, Ci, iWUE, K_L, N, Fv/Fm and PI (as variables

248 responses) were analyzed by means of generalized linear mixed models with random effects. All

249 factors, namely Type of stand (*Cl* or *Rs*), Habitat (*SE* or *CT*), Year (*Y*) and Month (*M*) and their

250 interactions, where treated as independent fixed effects, whereas the stand was included as a random

251 effect nested within. Data were checked for normality and homogeneity of variance, and were

252 transformed, when necessary, to correct deviations from these assumptions. Additionally variables

253 relationships were analyzed using linear models, and then the homogeneity of slopes between

254 Habitats for each Type of stand was tested through Factor x Covariate interactions in respective

255 ANCOVAs. Differences in daily curves of gas exchange parameters between Habitats were compared

256 per hour and Type of stand and analyzed with *t-test*. All analyses were performed with Statistica 7.0.

257 **3. Results**

258 *3.1 Leaf water potential*

259 The seasonal evolution of air temperature and rainfall during the study period are shown in Fig. 1.

260 Measurements of predawn leaf water potential (Ψ_{pd}) reflected rainfall differences among years with

261 higher values in 2007 and lowest in 2009 ($P < 0.001$; $F = 418.1$) (Table2; Fig. 2). Indeed, lowest

262 values of single measurement of tree leaf water potential were also found in 2009 (-4.6 and -3.7 MPa

263 both in SE_{Cl} and SE_{Rs}, respectively). Ψ_{pd} was significantly lower in trees with understory than without

264 ($P < 0.001$; $F = 57.9$), being this differences function of the type of shrub present (significant Type x

265 Habitat interaction; $P < 0.001$; $F = 16.0$) and year (significant Type x Habitat x Year interaction; $P <$

266 0.001; $F = 8.3$) (Table 2). Trees of SE_{Cl} plots showed a significant lower value of Ψ_{pd} during 2008 and

267 2009 compared with their *CT* ($P < 0.001$ in both years), while trees of SE_{Rs} just showed significant

268 differences in 2008 ($P = 0.02$). The highest reductions in Ψ_{pd} between Habitats within years were in

269 August of 2008 and July 2009 with a mean diminution of 37 and 40 %, respectively, in trees of SE_{Cl}

270   plots respect to their *CT*. In trees of SE_{Rs} plots the higher diminution respect to their *CT* was, on

271   average, of 19 % in August of 2008 (Fig. 2; Table 3).

272   With regard to midday leaf water potential (Ψ_{md}), results showed the same trend as Ψ_{pd} with lower

273   values in trees with understory than without, being these differences function of the type of understory

274   present and year (marginally significant Type x Habitat x Year interaction; $P < 0.061$; $F = 3.52$)

275   (Table 2). In this way, in 2009 Ψ_{md} was significantly lower in trees of *SE* plots with both types of

276   understory respect to their *CT*, while in 2007 just trees of SE_{Cl} plots showed a significant lower value

277   respect to their *CT* (Fig. 2; Table 3).

278   *3.2 Gas exchange parameters*

279   According to Ψ values, mean values of net CO_2 assimilation rate (A) and stomatal conductance (gs)

280   were significant lower in 2009 than in 2007 ($P < 0.01$ each one; $F = 97.9$ and 91.2; respectively)

281   (Table 2). For both parameters, differences among *SE* and *CT* plots were shrub species-dependent,

282   with a significant Type x Habitat interaction ($F = 7.6$; and 7.1 for A and gs, respectively; $P < 0.01$ in

283   both cases) (Table 2). Trees of SE_{Cl} plots showed significantly lower values of gs in both years, with a

284   significant reduction of A just in 2009, respect to their *CT*. By contrast, trees of SE_{Rs} plots did not

285   show significant differences respect to their *CT* (Fig. 3, Table 3). The highest differences between

286   Habitats within years were in August of 2009 with a diminution in trees of SE_{Cl} plots respect to their

287   CT of 37 and 40 % for A and gs, respectively (Fig. 3). With regard to intracellular CO_2 concentration

288   (Ci), trees of SE_{Cl} plots showed significantly lower values of in both years, whereas trees of SE_{Rs} plots

289   exhibited just a significant reduction in 2009 (Table 3).

290   Furthermore, daily curves of A and gs showed the same trend as above. Thus, there were significant

291   lower values in trees of SE_{Cl} plots respect to *CT* ones during whole day in each parameter, while in

292   trees of SE_{RS} plots there was just a significant reduction of gs in the first part of the morning, respect

293   to their *CT*, and differences were not significant for A at any hour (Fig. 4).

294   *3.3 Intrinsic water use efficiency (iWUE) and apparent soil to leaf hydraulic conductance (K_L)*

295   Trees of SE_{Rs} plots showed a significant higher value of iWUE than their CT in 2009, whereas in 2007

296   there were no differences. By contrast, trees of SE_{Cl} plots showed a significant higher value of iWUE

297   both years (Table 3). This result was the consequence of a higher reduction of gs than A values in

298   trees with understory than without. Additionally, when plotting iWUE data in function of Ψ_{pd}, as an

299   indicator of water stress, a significant linear relationship was detected (r ~ -0.33, $P < 0.05$), increasing

300   iWUE as Ψ_{pd} decrease, except for trees of SE_{Cl} (r = -0.02; $P =0.84$) (Fig. 5A,B). Indeed, test of

301   homogeneity of slopes was not significant for SE_{Rs} compared to their CT trees ($P = 0.37$), but for

302   SE_{Cl} the test resulted significant ($P < 0.01$). In all cases, Ci increased significantly with the decrease

303   of iWUE (r < -0.50, $P < 0.01$; Fig. 5c). With regard to K_L, on average was reduced by a 60% the less

304   favorable year (2009) compared to the average year (2007). Trees of SE_{Cl} plots showed a significant

305   lower value of K_L compared to their CT in 2007, whereas in 2009 there were no significant

306   differences, despite the consistent reduction (Table 3). Trees of SE_{Rs} did not exhibit any significant

307   effect on K_L neither 2007 nor 2009.

308   *3.4 Leaf nitrogen content*

309   Differences in N between CT and SE were function of the year and the type of shrub present

310   (significant Type x Habitat x Year interaction; $P < 0.001$; $F = 7.54$) (Table 2). In this way, N was

311   lower in 2009 than 2007 (12.5 and 14.2 mg g^{-1} respectively; $P < 0.01$; $F = 142.9$). Trees of SE_{Cl} plots

312   showed a lower N than their CT, being this reduction significant only in 2007 (Table 3). In contrast, it

313   is noteworthy a slight but consistent N increase in trees of SE_{Rs} plots respect to their CT, although

314   differences were not significant in both years of study (Table 3).

315   *3.5 Fluorescence*

316   Differences for Fv/Fm ratios and PI among Habitats were dependent of the shrub species, with a

317   significant Type x Habitat interaction ($F = 10.1$ and 31.4 for Fv/Fm ratio and PI, respectively, P <

318   0.001 in both cases) (Table 2). Trees of SE_{Cl} plots showed a significant lower value of Fv/Fm than CT

319   (2.5 % decrease; $P < 0.001$), while trees of SE_{Rs} plots did not show differences respect to their CT

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(Table 3). PI showed the same trend than Fv/Fm values (Table 3). Thus, PI was reduced by 30% in trees of SE_{Cl} respect to CT ones ($P < 0.001$; F = 31.35), with no differences in tress of SE_{Rs} (Table 3). The strongly decline in PI in trees of SE_{Cl} plots was mostly accounted for by the decrease in electron transport function and trapping function with a reduction of 20 and 10 % respectively compared with CT. Additionally, the active RC density on a Chl basis (RC/ABS) was also slightly reduced (Fig. 6). Although no significant differences were found in the PI of trees of SE_{Rs} plots compared with their CT, is noteworthy a slight reduction mostly due to a decrease in the trapping function (Fig. 6).

4. Discussion

The presence of the shrub layer in the open woodland studied had marked consequences in the physiological state of the tree layer. The response of the tree layer was function of the shrub specie present, a consistent competitive relation for soil resources was found with the presence of Cl as understory, while Rs exerts a less intense competition for soil resources, even become neutral. Cl with a shallower rooting profile competed more strongly for soil resources with $Q. ilex$ than $Rs,$ despite the higher overlapping of rooting profiles. Therefore, the resource partitioning hypothesis based just on rooting profiles is unlikely to be a general mechanism explaining plant-to-plant interaction in mixed systems (van Noordwijk et al., 1996; Weltzin et al., 1997: Ong et al., 1999; Moreno et al., 2005; Mulia and Dupraz, 2006). Moreover, the spatial partitioning of belowground resources in two-layered systems could not be enough for full understanding of plant interaction and coexistence which may be also driven by demographic processes (Cipriotti and Aguiar, 2010).

Trees in presence of Cl reached significant lower Ψ_{pd} than control trees, and differences were higher in drier years (2008 and 2009), when the rainfall was under the decade average. By the end of the summer of 2009, the less favorable year, Ψ_{pd} of trees encroached with Cl (-2.1 MPa) was 40 % lower than control trees (-1.5 MPa), suggesting that Cl compete for soil water with the tree layer, and that this effect increase as the abiotic stress increase. This result seems to contradict the "so-called" stress gradient hypothesis, which postulates that the importance of facilitation in plant communities increases with increasing abiotic stress. Also Maestre et al. (2005, 2006), through a meta-analysis of

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346 results of many studies, have recently showed that the stress gradient hypothesis cannot be

347 generalized neither in arid nor in semiarid environments. Moreover, this hypothesis applies for

348 combination of plants in equilibrium, but our results indicate that the strength of competitive effects

349 of *Cl* on *Q. ilex* could compromise the long survival of trees in stands densely encroached with *Cl*,

350 due to their detrimental effects to water and nutritional (N) tree status, with negative consequences for

351 stomatal conductance and the photosynthetic apparatus. The negative consequences of *Cl* presence on

352 *Q.ilex* surely will be reinforced with the increasingly recurrence of severe drought episodes in

353 Mediterranean region (IPCC, 2007).

354 Contrary to trees of SE_{Cl} plots, trees encroached with *Rs* did not show significant differences with

355 control trees for Ψ_{pd}, even in drier years (Table 2). The differential consequences of two shrub species

356 studied on tree water relations leaded, consequently, to differential responses in the gas exchange

357 parameters. It is known that *Q. ilex* have a high stomatal control when face water stress, closing the

358 stomata at relatively high water potentials in order to avoid dangerous losses of conductivity in the

359 xylem caused by low water potentials (Martínez-Vilalta et al., 2003). In this way, in agreement with

360 the stomatal control of water deficit, trees growing with *Cl* as understory showed a sharp reduction in

361 gs. Daily curves measured in 2009, also evidence this trend, showing a significant reduction of gs

362 during whole day. Trees growing with *Rs* as understory also showed a slight reduction of gs during

363 the morning despite the absence of significant differences in Ψ_{pd} (Table 2). Accordingly, the

364 significant reduction of K_L in trees of SE_{Cl} plots suggests that certain level of xylem embolism might

365 have occurred (Martínez-Vilalta et al., 2003).

366 The reduction in gs of trees encroached with *Cl* could have leaded to a significant, but lower,

367 reduction in A, and hence an increase in iWUE, both in 2007 and 2009. In trees growing with *Rs* there

368 was also a significant increase in iWUE in 2009. In principle, our average values support the idea that

369 with increasing water stress there is a higher reduction in gs relative to the reduction in A leading,

370 consequently, to an increase of iWUE (Aranda et al., 2005). The significant lower Ci values of

371 encroached trees suggest that the photosynthetic machinery is not damage and non-stomatal

372 limitations are not likely (Flexas and Medrano, 2002). However, looking deeper, our results

373 contradicted partly this assumption when plotting the response of iWUE in function of Ψ_{pd}, as

374 indicator of water stress. Whereas trees encroached with *Rs* presented an increase in iWUE with

375 increasing Ψ_{pd}, trees encroached with *Cl* seemed to exhibit an increase in iWUE at low Ψ_{pd} but a

376 steep decrease at high Ψ_{pd} (Fig. 5A). This decrease in iWUE was parallel to an increment in Ci (Fig.

377 5C), suggesting the predominance of non-stomatal limitation of photosynthesis beyond certain water

378 stress threshold (Flexas and Medrano, 2002).

379 Trees with *Cl* as understory showed a clear diminution of N in the two years analyzed, what suggests

380 that *Cl* competes for this soil resource, as expected given the dense and shallow rooting profile of *Cl*.

381 It is well known that there is a strong correlation between photosynthetic capacity and leaf N

382 concentration (Gulías et al., 2003), indeed half of leaf N is invested in the photosynthetic apparatus.

383 Thus, the lower N content in trees growing with *Cl* could have compromised their photosynthetic

384 machinery, especially under a severe water stress. On the other hand, tress growing with *Rs* had a

385 consistent, but not significant, increase in N respect to control trees both years. Pasture growing under

386 *Rs* has been shown to have higher N content (Puignaire et al., 1996), benefited by the N-fixing

387 behavior of the *Rs*, although there is no evidence of direct transfer (Rodríguez-Echeverría and Pérez-

388 Fernández, 2003). The clear benefit of N-fixed by *Rs* for pasture but not for trees is in agreement with

389 the high and low root density of pasture and trees, respectively, in the uppermost soil layer of

390 Mediterranean open woodland (Moreno et al., 2005). Overall, results would indicate a low

391 competitive capacity of *Q. ilex* for soil N compare to plants with dense shallow rooting profile

392 (pasture and *Cl*). This limitation to compete for soil N in nutrient-poor ambient could be mitigated for

393 the high internal N-use efficiency of *Q. ilex* (Silla and Escudero, 2004)

394 The presence of an understory composed by *Rs* did not seem to have any significant effects on the

395 Fv/Fm ratio and PI in agreement with the low differences found for N leaf and leaf Ψ between trees of

396 *SE$_{Rs}$* and *CT* ones. By contrast, and as consequence of the detrimental hydric and nutritional status of

397 *Q. ilex* in presence of *Cl*, trees of *SE$_{Cl}$* plots showed a significant lower value of Fv/Fm and a drastic

398 diminution of PI compared to their *CT* (Table 2). Leaf photoprotection in plants subjected to

399 excessive light, as in the Mediterranean during summer time, and water stress depends primary to

400 their capacity to thermally dissipate absorbed light by non-radiative process (Chaves et al., 2003).

401 Under this condition, the result of sustained energy dissipation activity could reduce the

402 photochemical efficiency (Fv/Fm) due to a down-regulation of photochemistry (Flexas and Medrano,

403 2002). Regarding to PI, trees of SE_{Cl} plots exhibited a significant reduction of its three defining

404 components (Fig. 6), showing electron transport (P_{ET}) the highest reduction with a diminution around

405 20% compared with control trees. A decrease in P_{ET} could be a sign that energy produced in the light

406 reactions cannot be used for CO_2 fixation (Strasser et al, 2000). To sum up, the results suggest that the

407 presence of *Cl* as understory can compromise the functioning of *Q.ilex*, however trees seem to face

408 plant-to-plant competition through certain photosynthetic and hydric adjustments.

409 **5. Conclusions**

410 The effects of the presence of a third vegetation layer (shrub) on the ecophysiological status of trees in

411 the Mediterranean open woodland studied depend on the shrub species. Moreover, the differential tree

412 performance under contrasting shrub strategies varies among years in function of the water

413 availability (annual precipitation), what exemplifies the complexity of plant-to-plant interactions in

414 the system studied. Whereas there was a slight interaction for soil resources between *Rs* and tress, and

415 consequently, on their functional status, *Cl* compete hugely affecting trees widely. In addition,

416 Mediterranean open woodlands are likely to experience more frequent and intense droughts with the

417 on-going climate change. So that, long term water shortage could also lead to a carbon starvation, that

418 could affect tree survivorship (McDowell et al., 2008), when trees are growing with a dense layer of

419 *Cl*.

420 Moreno & Pulido (2009) proposed periodical shrub encroachment of managed Mediterranean open

421 woodland to favor natural tree regeneration and thus the persistence of the system. This study

422 highlights the differential behavior of different shrub layer where each functional shrub type would

423 need a different planning to minimize the potential negative consequences of shrub understory-tree

424 overstory interaction. Certainly, integrated overviews are needed to clarify interactions among

425 different tree-shrub types along successive life stages in order to optimize management practices of

426 currently threatened Mediterranean open woodlands.

427 **Acknowledgements**

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611

612   Table 1.

613   Main characteristics of the tree and shrub layer of paired plots without (Control) and with an

614   understory layer (Shrub Encroached) composed either of *Cistus ladanifer* (Cl) or *Retama*

615   *sphaerocarpa* (Rs).

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		Habitats					
		Control		Shrub Encroached			
		Tree Layer		Tree Layer		Shrub layer	
Stand	Type	%[1]	DBH[3]	%[1]	DBH[3]	%[2]	Height
1	CL	16	40	12	44	95	2
2	CL	19	50	15	47	60	2
3	CL	26	40	22	37	70	2,3
4	CL	26	46	19	32	80	2,7
5	CL	26	47	16	38	95	1,8
6	CL	27	45	19	42	90	2,5
7	RS	29	60	16	73	30	2,5
8	RS	13	50	27	56	30	0,8
9	RS	18	40	10	44	30	1,7
10	RS	26	50	20	54	20	1,8
11	RS	15	43	31	41	25	2
12	RS	22	45	12	40	25	1,7

617   [1,2] %: Cover estimated as the percentage of land cover by tree canopy[1] or shrubs[2].

618   [3] DBH: Diameter at breast height

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634   Table 2.

635   Results from a Generalized Linear Mixed Model on a nested ANOVA design testing for the effects of

636   Type of stand (either Cistus or Retama), Habitat (either with or without shrub understory), Year and

637   Month as Factors, and tree leaf water potential (MPa) measure at predawn (Ψ_{pd}) and midday (Ψ_{md}),

638   net photosynthesis rate (A, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (gs, mol H_2O m^{-2} s^{-1}),

639   intercellular CO_2 concentration (Ci, µmol mol^{-1}), intrinsic water use efficiency (iWUE, µmol CO_2

640   mol $^{-1}$ H_2O), apparent soil-to-leaf hydraulic conductance (K_L, mmol H_2O MPa^{-1} m^{-2} s^{-1}), leaf nitrogen

641   content (N, mg g^{-1}), photoquemical efficiency (F_v/F_m) and Performance Index (PI), as response

642   variables. Significant differences: ***$P < 0.01$, ** $P < 0.05$, * $0.05 < P < 0.1$

	Ψ_{pd}	Ψ_{md}	A	gs	Ci	iWUE	K_L	N	F_v/F_m	PI
	F	F	F	F	F	F	F	F	F	F
Type (T)	0.12	0.89	0.00	0.34	0.11	0.20	5.59***	0.91	0.92	0.07
Habitat (H)	57.85***	11.06***	3.66*	15.11***	8.28***	16.06***	4.23**	10.14***	13.59***	35.61***
Year (Y)	418.1***	6.12**	97.85***	91.18***	114.1***	2.01	105.3***	142.9***	-	-
Month (M)	345.4***	52.71***	18.89***	44.64***	12.24***	18.86***	32.49***	-	18.45***	11.15***
T * H	15.95***	2.50	7.55***	7.09***	0.08	0.67	4.13**	24.42***	10.12***	31.35***
T * Y	2.41*	60.29***	4.25**	0.07	0.01	0.39	26.66***	0.53	-	-
T * M	1.59	6.59***	7.53***	17.84***	11.12***	7.72***	8.36***	-	9.75***	0.01
H * Y	5.69***	0.81	1.80	0.35	1.44	1.19	0.04	5.63**	-	-
H * M	4.70***	0.74	1.12	0.12	1.94	0.75	0.01	-	0.01	8.11***
Y * M	31.03***	27.10***	3.19**	6.46***	6.47***	5.60***	11.70***	-	-	-
T *H* Y	8.30***	3.52*	1.76	0.49	0.83	0.18	3.58*	7.54***	-	-
T *H* M	1.89	1.98	0.11	1.01	1.25	1.06	1.28	-	6.41**	14.90***
T *Y* M	2.92**	4.21**	7.18***	6.85***	8.59***	7.44***	24.74***	-	-	-
Z *Y* M	1.21	0.64	2.31	0.06	0.60	0.81	0.92	-	-	-
T*H*Y*M	0.57	1.10	1.29	0.40	0.86	0.50	0.12	-	-	-

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656 Table 3.

657 Comparison of the mean values (standard error in parenthesis) of tree leaf water potential (MPa)

658 measure at predawn (Ψ_{pd}) and midday (Ψ_{md}), net photosynthesis rate (A, μmol CO_2 m^{-2} s^{-1}), stomatal

659 conductance (gs, mol H_2O m^{-2} s^{-1}), intercellular CO_2 concentration (Ci, μmol mol^{-1}), intrinsic water

660 use efficiency (iWUE, μmol CO_2 mol^{-1} H_2O), apparent soil-to-leaf hydraulic conductance (K$_L$, mmol

661 H_2O MPa^{-1} m^{-2} s^{-1}), photochemical efficiency (F$_v$/F$_m$), performance Index (PI) and leaf nitrogen

662 content (N, mg g^{-1}) of trees with (SE) and without (CT) understory in fields encroached with *Cistus*

663 *ladanifer* or *Retama sphaerocarpa*. Between stands differences: ***$P < 0.01$, ** $P < 0.05$.

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|		2007		2009	
		CT	SE	CT	SE
Cistus ladanifer	Ψ_{pd}	-0.9 (0.04)	-0.9 (0.04)	-1.4 (0.06)	-1.9 (0.07)[**]
	Ψ_{md}	-2.6 (0.07)	-2.8 (0.09)[***]	-2.8 (0.05)	-2.9 (0.04)[**]
	A	10.1 (0.6)	9.1 (0.6)	8.4 (0.3)	6.1 (0.3)[***]
	gs	0.14 (0.01)	0.11 (0.01)[***]	0.10 (0.01)	0.06 (0.01)[***]
	Ci	207.6 (8.3)	182.7 (10.4)[***]	283.9 (7.3)	264.3 (8.4)[**]
	iWUE	90.0 (4.8)	106.2 (4.0)[***]	90.1 (2.4)	106 (3.4)[***]
	K$_L$	2.73 (0.3)	2.11 (0.19)[***]	1.4 (0.08)	1.19 (0.07)
	F$_v$/F$_m$	-	-	0.80 (0.002)	0.78 (0.003)[***]
	PI	-	-	28.01 (0.9)	20.4 (0.6)[***]
	N	15.0 (0.6)	13.2 (0.2)[***]	12.5 (0.2)	12.1 (0.2)
Retama sphaerocarpa	Ψ_{pd}	-0.9 (0.04)	-0.9 (0.03)	-1.7 (0.06)	-1.8 (0.06)
	Ψ_{md}	-3.1 (0.06)	-3.1 (0.05)	-2.6 (0.05)	-2.8 (0.06)[**]
	A	10.3 (0.6)	9.8 (0.6)	6.6 (0.4)	6.7 (0.4)
	gs	0.12 (0.01)	0.12 (0.01)	0.08 (0.01)	0.07 (0.01)
	Ci	203.5 (7.2)	212.7 (9.0)	292.9 (8.4)	257.0 (9.4)[***]
	iWUE	94.3 (4.0)	94.6 (4.6)	96.5 (3.1)	110.3 (3.4)[***]
	K$_L$	1.6 (0.09)	1.73 (0.10)	1.39 (0.11)	1.18 (0.08)
	F$_v$/F$_m$	-	-	0.78 (0.003)	0.78 (0.002)
	PI	-	-	22.8 (0.8)	22.5 (0.8)
	N	14.1 (0.2)	14.4 (0.2)	12.6 (0.2)	12.8 (0.2)

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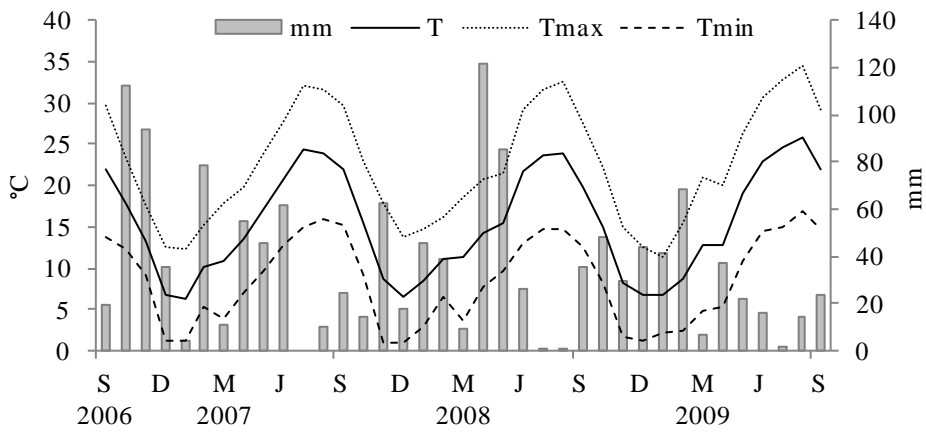
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676 Figure 1. Monthly average values of maximum (T_{max}), minimum (T_{min}) and mean temperature (T) and

677 monthly accumulated rainfall at the study site from September 2006 to September 2009. Data from

678 the nearest weather station (Aldehuela del Jerte, 40.00 N, 6.13 W)
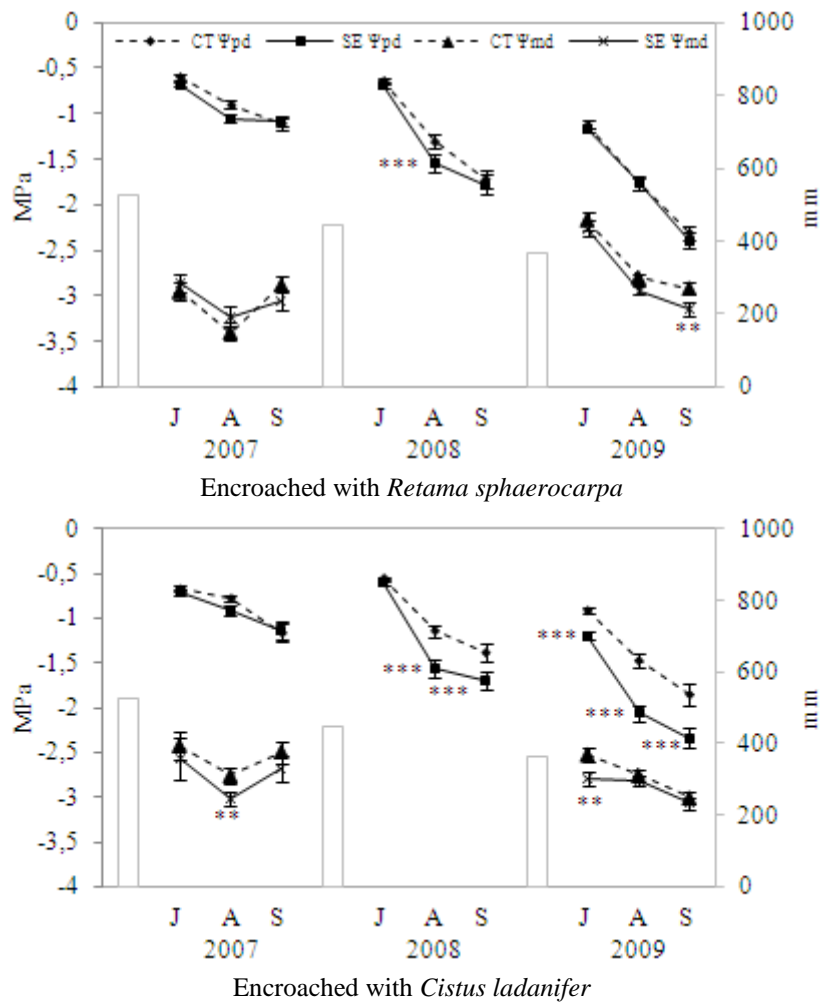
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Encroached with *Retama sphaerocarpa*

Encroached with *Cistus ladanifer*

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697   Figure 2. Time evolution of tree leaf water potential (MPa) at predawn (Ψ_{pd}) and midday (Ψ_{md})

698   measured during summer of 2007, 2008 and 2009 in trees with (SE) and without (CT) shrub

699   understory in plots encroached with *Cistus ladanifer* or *Retama sphaerocarpa* (mean values and

700   standard error). Vertical bars represent annual accumulated rainfall from September 21th till following

701   September 21th. Between Habitats (SE vs CT) differences per Year and Month: ***$P < 0.01$, ** $P <$

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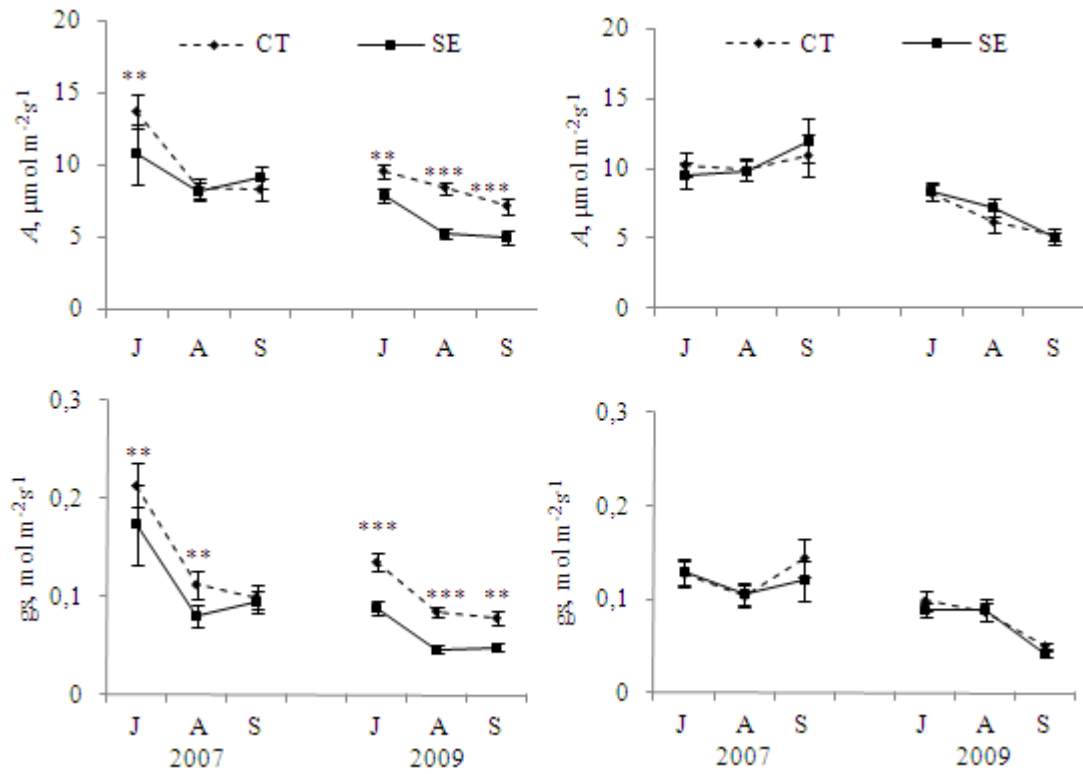
Encroached with *Cistus ladanifer*   Encroached with *Retama sphaerocarpa*

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714   Figure 3. Time evolution of *A* and gs measured in trees with (SE) and without (CT) shrub understory

715   in plots encroached with *Cistus ladanifer* or *Retama sphaerocarpa* in July, August and September of

716   2007 and 2009 (mean values and standard error). Between Habitats (SE vs CT) differences per Year
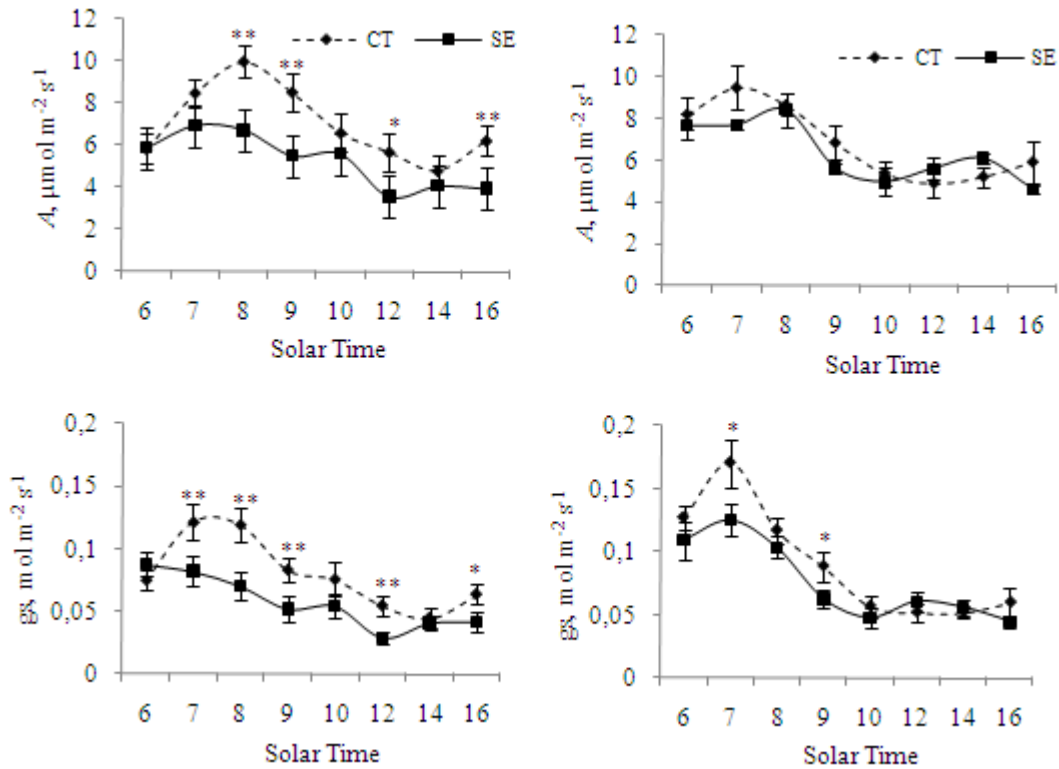
717   and Month: ***$P < 0.01$, ** $P < 0.05$.

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Encroached with *Cistus ladanifer*   Encroached with *Retama sphaerocarpa*

Figure 4. Daily evolution of A and gs measured in trees with (SE) and without (CT) shrub understory in plots encroached with *Cistus ladanifer* or *Retama sphaerocarpa* (mean values and standard error). Values were recorded hourly till 10 h and then each two hour till 16 h (solar hour). Comparison between Habitats (SE vs CT) per Type of shrub and Hour, ** $P < 0.05$, * $0.05 < P < 0.10$.
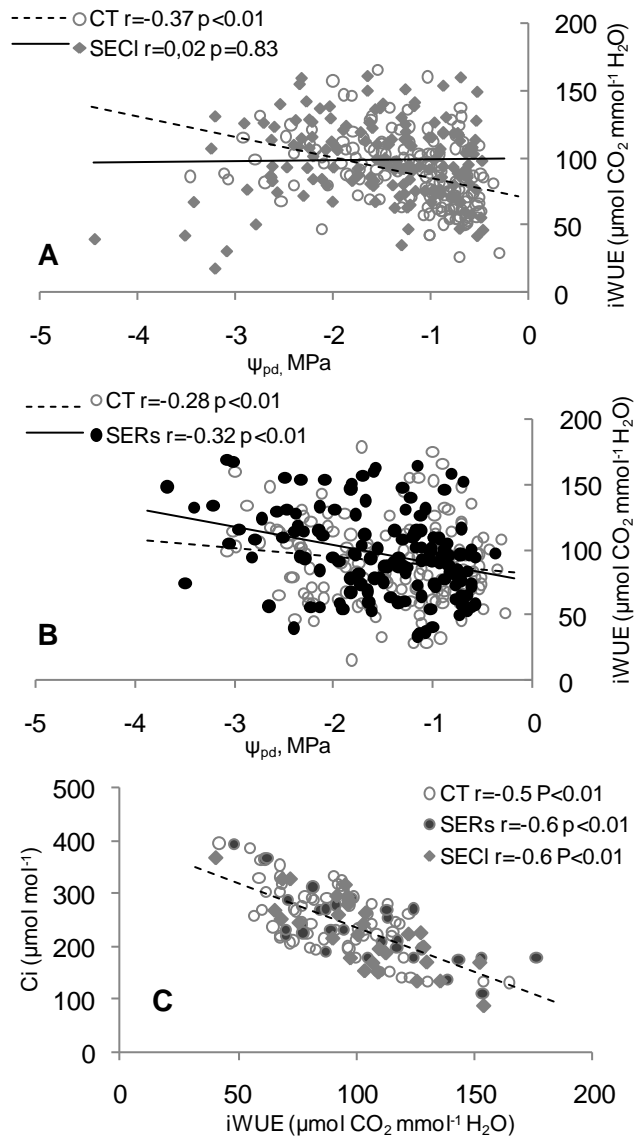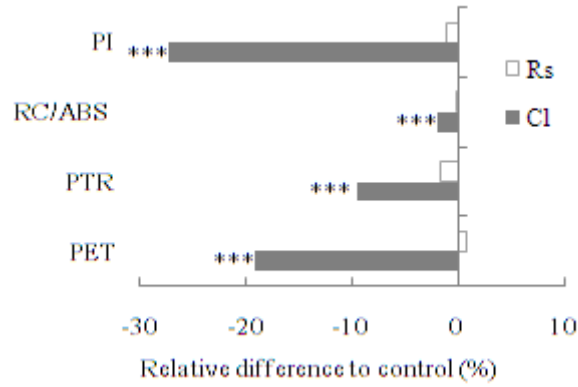
Figure 5. Relationship between leaf water potential (Ψ_{pd}, MPa) and intrinsic water use efficiency (iWUE, μmol CO_2 mol $^{-1}$ H_2O) of trees with (SE) and without (CT) shrub understory in plots encroached with *Cistus ladanifer* (A) or *Retama sphaerocarpa* (B). Figure C depicts the relationship between intrinsic water use efficiency and intracellular CO_2 concentration. A linear model was fitted for data points.

Figure 6. Relative difference among trees with shrub understory and their respective control in plots encroached with *Cistus ladanifer* (Cl) or *Retama sphaerocarpa* (Rs) in the PI and its components: active RC density on the Chl basis (RC/ABS), performance due to trapping probability (P_{TR}), and performance due to electron transport probability (P_{ET}). *** $P < 0.01$.

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