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Cork Oak Seedling Growth under Different Soil Conditions from Fertilisation, Mycorrhizal Fungi and Amino Acid Application

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Abstract

Regeneration process deals with some constrains related with the livestock management, pasture rotations, and dependence of a sequence of favourable climatic years, among others, which can lead to a continuous delay in the initiation of the regeneration process. The purpose of this study is to promote an increase of cork oak seedling growth in order to decrease the time required for regeneration and also to contribute to avoid the effect of post-transplant stress on cork oak. With this objective, a study was carried out on a greenhouse, where the effect of fertilisation, mycorrhizal fungi inoculation, and amino acid supply were tested. Results showed that cork oak seedling capability to growth, expressed as total seedling dry weight, was positively affected by treatments, except when only fertilisation was applied. We verified that cork oak seedlings inoculated with mycorrhizal fungi presented better results in terms of aerial structures growth. Any of the treatments was suitable to contribute positively for tap root and total belowground dry weight accumulation. Only fine roots structures were sensitive to treatments effects; it was verified that both inoculated and non-inoculated seedlings subjected to fertilisation were capable to invest largely on the production of these structures (33 and 30%, respectively). To reinforce the cork oak seedling growth, the equilibrium between fertilisers and mycorrhizal fungi inoculation would probably be the better option to enhance the cork oak regeneration process. Thus, the balance between shoot and root systems growth would be guaranteed, fertilisation mainly for root system and mycorrhizal fungi inoculation for shoot growth.

Keywords: cork oak seedlings, regeneration, fertilisation, mycorrhizal fungi, amino acids

1. Introduction

Cork oak tree (*Quercus suber* L.) is one of the main species of Mediterranean ecosystem woodlands - named *Montado* in Portugal - with a life time around 150 years. This species has a high socioeconomic and environmental value; its socioeconomic significance mainly relies on cork production, which is renewable every 9 years. The most typical characteristic of the Portuguese *Montado* is its savanna- like physiognomy, spread throughout a large scale mosaic, with different densities, of cork oak and holm oak (*Quercus rotundifolia* Lam.), according to Pinto-Correia et al. (2011). These typical stands are usually open areas with trees growing on an isolated way or associated with shrubs and/or livestock. Being the woodland system based on trees, it sustainability (continuous crown cover in time) is strongly associated with the natural or artificial trees regeneration (Ribeiro et al., 2010). Thus, Ribeiro et al. (2006; 2010; 2012) referred that the system resilience is based on specific stand structure and densities that are applied, with new trees to compensate natural rates of mortality, allowing the maintenance of a stable crown cover. A crown cover between 30-70% (slope dependent) is fundamental to the woodland ecological sustainability, enhancing the multifunctionality of the system, promoting a protective effect on soil, preventing the erosion risk, and improving the water and nutrient cycles (Ribeiro et al., 2004).

In the last decades it has been observed an increase on cork oak mortality and, at the same time, a lack of natural regeneration (Ribeiro & Surový, 2008), which is one of the major demands to revitalize the *Montado* areas. In

this way, regeneration process deals with some constrains related with the livestock management and pasture rotations, but is also strongly dependent of a sequence of favourable climatic years (at least 10 years). The need of a grazing area to support the existing livestock leads to a continuous delay in the initiation of the regeneration process, which results in the late replacement of the young trees cohort.

Moreover, in the Mediterranean environment, which is characterized by seasonal droughts, the water availability is also a limiting factor that is of key importance in the regeneration of oaks and other woody species (Aranda et al., 2007; Gakis et al., 2004). Hence, it is necessary to understand the strategy of root growth according to the soil environmental matrix for this purpose. In result of the difficulty to access root system of mature trees, studies have been usually relying in experiments with seedlings and young trees (Jonsson et al., 2001; Gogorcena et al., 2001; Rached-Kanouni et al., 2012). The seedling stage is an important and usually critical phase in the regeneration of woody species under natural conditions, since the effect of environmental stress is very high at this stage.

Seedling fertilization is refereed as being relevant in Mediterranean areas influencing seedling resistance to drought (Singh & Sale, 2000; Trubat et al., 2006) by the increase of N and P availability (Sabaté & Gracia, 1994; Sardans et al., 2004). Trubat et al. (2010) results showed that above and belowground cork oak biomass accumulations are reduced by low nutrient availability. Moreover, amino acids are fundamental ingredients, influencing directly or indirectly the physiological activities of the plant (protein synthesis, plant growth, photosynthesis, and nutrients absorption, among others). Under drought conditions, amino acids help the plant to sustain cellular functions and adjust the osmotic process (Khattab et al., 2012); when incorporated them into the soil, its microflora improves and, thereby, also its assimilation of nutrients.

The need to evaluate the success of seedling establishment and also tree transplanting has led the research to complementary studies about the role of mycorrhizal symbiosis. Ectomycorrhizal (ECM) fungal species and fungi networks are mediators between soil processes and plant community, by enhancing nutrient uptake, drought tolerance, and pathogen resistance of their hosts (Azul et al., 2010; Futai et al., 2008). In this way, Smith and Read (1997) pointed out that mycorrhizal symbiosis is essential for oak trees because it promotes water and nutrients uptake under natural conditions in result of a higher absorption surface area. The Mediterranean oaks, specifically cork oak, have been shown to be associated with a wide range of ECM fungi (Ortega & Lorite, 2007). Garbaye and Guehl (1997) also referred that mycorrhizal fungi are more efficient than roots in extracting water at very low soil water potential. Under the Mediterranean climatic conditions, these associations will probably contribute to a major regularity of water absorption during the summer drought season.

These facts lead to the present study, which focuses on the increase of seedling growth in order to decrease the time required for cork oak regeneration. With this objective, a study was carried out where the effect of fertilization, induction of mycorrhizal fungi, and amino acid application were tested on performance of above and belowground systems of the cork oak seedlings. The results of this study will contribute choosing the better treatment that should be applied to reduce the time for seedlings regeneration process and also contribute to avoid the effect of post- transplant stress of cork oak.

2. Material and Methods

2.1 Plant Material and Growing Conditions

The present study was carried out in a pot experiment in a greenhouse at Mitra's campus from University of Évora, South of Portugal. Cork oaks seedlings with one year old and with a mean height of 15cm (seedlings origin from several certified *Montado* areas), were transplanted into individual 30 cm height × 27 cm Ø plastic containers, filled with a Cambissol/Podzol soil collected in the 10-30 cm layer that previously was passed through a 5-mm mesh sieve to separate it from bigger aggregates, dust and residues. *Montado* woodlands in Portugal are commonly observed in this type of soil. First, the soil was sieved through a 5 mm mesh sieve and next a bulk density of 1.66 g cm^{-3} was achieved. The main soil characteristics are described in Table 1.

Table 1. Soil characteristics

Organic matter (%)	pH [H_2O]	NO_3 (mg Kg^{-1})	K_2O (mg Kg^{-1})	P_2O_5 (mg Kg^{-1})
0.58	4.86	38	34	16

The temperature inside the greenhouse ranged between 9-31 ºC and air humidity was over 50%. The plants were subjected to global radiation inside the greenhouse over 275.5 and 138.5 W m^{-2} in spring/summer and autumn/winter seasons, respectively).

More information about climatic conditions in the study area are available at the Centro de Geofísica de Évora (http://www.cge.uevora.pt).

2.2 Experimental Design

Five treatments, including control, were arranged in a randomized complete block design with four replications. Treatments applied were: control (C); fertilization (F); fertilization + mycorrhizal fungi (FM); fertilization + amino acids (FA); and fertilization + amino acids + mycorrhizal fungi (FAM). Considering that there were 9 seedlings per treatment and replication, a total of 180 seedlings were used in this study.

Prior to transplanting, each container was fertilized with 30 mg N, 10 mg NO_3^-, 20 mg NH_4^+, 60 mg P_2O_5, 125 mg K_2O, 12.5 mg MgO, 0.15 mg B, 0.05 mg Cu, 0.1 mg Mn, and 0.15 mg Zn.

For treatments with fertilization (F treatment) were also applied 8.3 mg N, 3.7 mg P_2O_5, 16.0 mg K_2O, 8.1 mg CaO, 4 mg MgO, 7.8 mg SO_3^-, 0.5×10^{-2} mg B, 0.4×10^{-2} mg Cu, 0.1×10^{-2} mg Fe, 2×10^{-2} mg Mn, and 0.1×10^{-2} mg Zn through irrigation water, at each application.

For FM and FAM treatments, a commercial mixture of mycorrhizal fungi (ECTOVIT by Symbiom Ltd; more information at www.symbiom.com) was applied, according instructions, during the transplanting process. The mixture was compound by 4 strains of mycorrhizal fungi on a liquid medium and 2 strains of mycorrhizal fungi on a peat-based carrier with ingredients supporting the development of mycorrhiza (humates, ground materials, extracts from sea organisms), naturally degradable granules of a water-retaining gel. The ECM species were *Cenococcum geophilum*, *Hebeloma sinapizans*, *H. crustiliforme*, *Pisolithus tinctorius*, *Amanita rubescens*, and *Tricholoma acerbum*.

Seedlings under FA and FAM treatments were also subjected to a supply of 0.19 g of amino acids and 0.47 g of vegetable organic matter dissolved in the irrigation water.

Finally, the control treatment (C treatment) was only subject to irrigation.

To indicate that irrigation consisted of 0.5 L of water applied per container at each 10 days along the 18 months of the experiment.

2.3 Data Collection

After 18 months, all seedlings were handled under laboratory conditions for data collection. For each seedling, height (H) was registered. Each of the 180 seedlings was subjected to aerial components separation (leaves, branches, and stem) and each component was labelled and preserved in a cold environment (5 ºC). The root systems were carefully washed out of soil and fine roots (Ø < 2 mm) were removed from tap root. In order to get the remaining fine roots, the entire soil volume of each container was sieved through a sieve of 1mm mesh and fine roots were manually collected and stored in a water and alcohol solution at 5 ºC.

For each sample (individual seedling), fresh leaves, branches and stems, and tap roots were scanned using a HP Scanjet 4850 scanner. For the analysis of scanned images, the ImageJ software was used to calculate the superficial areas occupied by these structures, meaning leaf area (LA), wood area (WA), and tap root area (TRA). After the scanning process, branches and stems and tap roots were dried at 103 ºC and leaves at 75 ºC during 48 hours, and dry weight was obtained as leaf dry weight (LDw), wood dry weight (WDw), and tap root dry weight (TRDw). The seedling growth was also analysed as aerial system dry weight (ADw), aerial system area (AA), root system dry weight (BDw) and root system area (BA), and total seedling dry weight (SDw).

Fresh fine roots were spread on a water-filled transparent plastic tray and scanned with a transmitting light scanner (EPSON Expression 10000XL 3.4). The images were analysed with WinRhizo Reg 2009. Then, total fine root length (FRL) and fine root areas (FRA) were obtained. After image analysis fine roots were dried at 103 ºC during 48 hours, for dry weight (FRDw).

Additionally, the biomass allocation and seedlings growth were evaluated according to the following calculation parameters: root:shoot ratio (root system dry weight/aerial system dry weight), weight of fine roots (fine root dry weight/root system dry weight × 100 (%)), Specific root length (SRL) (fine root length/fine root dry weight (cm g^{-1})), Root length density (RLD) (fine root length/volume of soil (cm^2 g^{-1})), Specific leaf area (SLA) (leaf area/leaf dry weight (cm^2 g^{-1})), fine root area:leaf area ratio (fine root area/leaf area), root length:seedling dry weight ratio (RLR) (fine root length/total seedling dry weight (cm g^{-1})), leaf area:seedling dry weight ratio (LAR) (leaf area/total seedling dry weight (cm^2 g^{-1}).

2.4 Statistical Analysis

Data were analysed using SPSS software (version 20.0, SPSS Inc., Chicago, IL). Distribution was tested for normality by Kolmogorov-Smirnov criterion and homogeneity of variances tested by Levene's test.

According to the main objective of the present study, significant mean differences between plant parameters measured under each typology of treatment were tested using analysis of variance (one-way ANOVA) to compare the means between the 5 treatments and to determine whether any of those means are significantly different from each other. Means were separated at 5% level using Fisher's least significant difference (LSD) test to create confidence intervals for all pairwise differences between factor level means. Fisher's LSD method uses the individual error rate and number of comparisons to calculate the simultaneous confidence level for all confidence intervals.

3. Results

The effect of treatments on seedling growth (seedlings height and dry weight) is shown on Table 2. Seedlings height and dry weight were only significantly affected when fertilisation was associated with mycorrhizal fungi (FM treatment) and amino acid application (FA treatment), with a significant increase in seedling height of 54.53 and 56.54 cm/seedling, respectively. For total seedling dry weight, the major increase (46%) was also obtained for treatment with fertilization+ amino acids+ mycorrhizal fungi (FAM treatment).

Mycorrhizal fungi treatments (FM and FAM treatments) also increased leaf and wood dry weight. The highest values were observed for FMA seedlings, with increases of 108%, 81%, and 146% for aboveground, leaves, and wood, respectively. Although the highest growth expressed through dry weight for aboveground structures is verified on treatments subjected to mycorrhizal inoculation, it is not directly related to a higher root dry weight. For belowground dry weight and tap root dry weight no significant effect from treatments compared to control was verified (Table 2). However it was observed a significant positive effect of FM and FA treatments in fine root dry weight (9.44 g/seedling). As seen in Figure 1, when the percentage of fine roots on the entire root system was analysed, it was verified that F and FM treatments presented the highest percentages (30% and 33%, respectively).

Table 2. Effect of treatment on seedling height and dry weight (leaf, wood, aerial system, tap root, fine root, root system, and total seedling)

Treatments	Height (cm)	Dry weight (g/seedling)						
		Leaf (LD_w)	Wood (WD_w)	Aerial system (AD_w)	Tap root (TRD_w)	Fine root (FRD_w)	Root system (BD_w)	Total Seedling (SD_w)
C	44.58c	12.85cb	9.06d	21.91cb	26.28ab	7.02b	33.30ab	55.21c
F	47.90bc	14.79cb	10.94cd	25.72cb	27.28ab	9.08ab	35.35ab	61.07bc
FM	54.53ab	21.34a	18.22b	39.57a	22.94b	9.44a	31.75b	71.31ab
FA	56.54a	16.04c	12.66c	28.70c	30.16a	9.44a	39.60a	68.29b
FAM	51.72abc	23.27a	22.32a	45.59a	27.73ab	7.50ab	35.23ab	80.82a
F	*3.165**	*29.255***	*24.038***	*37.106***	*3.501***	*4.446***	*31.530***	*10.188***
P-value	*0.015*	*0.000*	*0.000*	*0.000*	*0.009*	*0.002*	*0.000*	*0.003*

Note. Mean. *F-values* for ANOVA test. $^{ns.}$ No significant, * Significant at 0.05 level, ** at 0.01 level. Means with different letters are significantly different (n = 180; $P < 0.05$). C, control; F, fertilisation treatment; FM, fertilization + mycorrhizal fungi treatment; FA, fertilisation+ amino acids treatment; FAM, fertilization + amino acids + mycorrhizal fungi treatment.

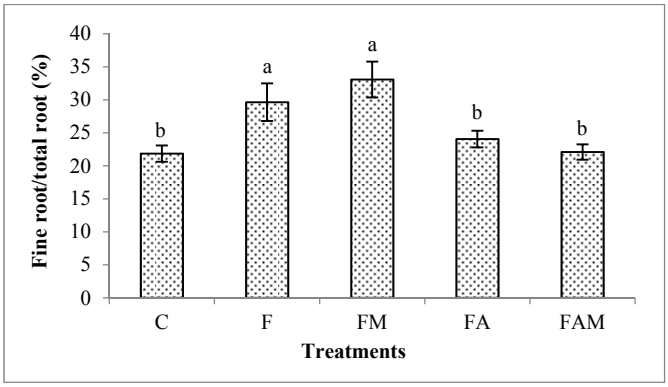

Figure 1. Effect of treatment on fine root dry weight to total root dry weight percentage. Means with different letters are significantly different (n = 180; $P = 0.000$). C, control; F, fertilisation; FM, fertilisation + mycorrhizal fungi; FA, fertilisation + amino acids; FAM, fertilization + amino acids + mycorrhizal fungi

For growth evaluation, expressed through area the effect of treatments, results are shown on Table 3. Treatments where mycorrhizal fungi were present increased significantly the area of aboveground (AA) and leaves (LA) in relation to control and fertilisation treatments. The treatment where mycorrhizal fungi were associated with amino acids (FAM treatment) presented the best results for these parameters; thus, increases of 75% in aboveground area, 71% in leaf area, and 149% in wood area were verified (Table 3). Root system area (BA) and fine roots surface area (FRA) were positively affected by all treatments. However, tap root area was only affected by fertilisation and fertilisation with amino acids. The highest increases for tap root, fine root, and belowground areas were verified in FA (34%, 44.3% and 44.1%, respectively).

Fine root length (FRL) and specific root length (SRL) were significantly affected by F, FM and FA treatments (Tables 3 and 4, respectively). Fertilisation treatment (F treatment) presented the highest values for the abovementioned parameters; increases of 58% for FRL and 37% for SRL were obtained. Concerning to fine root volume, which is also directly related to fine root functions, mainly to water and nutrient transport capacity, it was verified that all treatments affected positively this parameter with mycorrhizal fungi treatment presenting the highest increase (42%), as Table 3 shows. For specific leaf area parameter none of the treatments promoted significant effects.

Table 3. Effect of treatment on seedling area (tap root, fine roots, belowground, leaf, wood, and aboveground)

Treatments	Area (cm^2)						Fine root Length (FRL) (cm)	Fine Root Volume (FRV) (cm^3)
	Leaf (LA)	Wood (WA)	Aerial system (AA)	Tap root (TRA)	Fine root surface area (FRA)	Root system Area (BA)		
C	1102.40b	58.37d	1160.77b	48.54c	1704.57b	1753.12b	9537.53c	25.18b
F	1252.73b	66.42dc	1319.15b	61.07ab	2413.17a	2474.24a	15062.90a	32.02a
FM	1867.77a	117.42b	1985.19a	53.54bc	2364.53a	2418.07a	13057.25ab	35.82a
FA	1397.35b	80.20c	1477.55b	66.46a	2461.14a	2527.60a	13385.92a	35.42a
FAM	1886.32a	145.34a	2031.66a	55.20bc	2178.68a	2233.88a	10849.42bc	33.58a
F	*10.265***	*26.443***	*11.412***	*3.842***	*4.255***	*4.347***	*6.083***	*3.745***
P-value	*0.000*	*0.000*	*0.000*	*0.005*	*0.003*	*0.002*	*0.000*	*0.006*

Note. Mean. *F-values* for ANOVA test. $^{ns.}$ No significant, ** Significant at 0.01 level. Means with different letters are significantly different (n = 180; $P < 0.05$). C, control; F, fertilisation treatment; FM, fertilization + mycorrhizal fungi treatment; FA, fertilization + amino acids treatment; FAM, fertilization + amino acids + mycorrhizal fungi treatment.

Table 4. Effect of treatment on parameters used to evaluate the seedling growth (specific root length and specific leaf area)

Treatments	Specific Root Length (SRL) (cm g^{-1})	Specific Leaf Area (SLA) (cm^2 g^{-1})
C	1395.96c	84.89
F	1917.48a	84.14
FM	1474.16ab	86.00
FA	1514.97a	85.96
FAM	1486.24bc	78.82
F	*3.323**	*$4.98^{n.s.}$*
P-value	*0.012*	

Note. Mean. *F-values* for ANOVA test. $^{ns.}$ No significant, * Significant at 0.05 level, ** Significant at 0.01 level. Means with different letters are significantly different (n = 180; $P < 0.05$). C, control; F, fertilisation treatment; FM, fertilization + mycorrhizal fungi treatment; FA, fertilization + amino acids treatment; FAM, fertilization + amino acids + mycorrhizal fungi treatment.

The root:shoot dry weight ratio was also affected by all treatments (Figure 2). Yet, it is verified that seedlings grown within associations of mycorrhizal fungi (FM treatment) and mycorrhizal fungi + amino acids (FAM treatment) allocated less biomass to roots ($p < 0.001$). These results allowed assuming that production of aerial structures (leaves, branches, and stems) was highly reinforced by the root symbiosis with mycorrhizae.

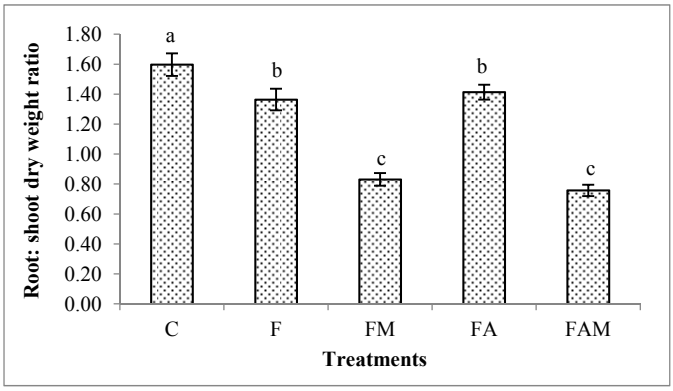

Figure 2. Effect of treatment on root:shoot dry weight ratio. Means with different letters are significantly different (n = 180; $P = 0.000$). C, control; F, fertilisation; FM, fertilization + mycorrhizal fungi; FA, fertilization + amino acids; FAM, fertilization + amino acids + mycorrhizal fungi

The data for the two parameters calculated to evaluate the capability of seedlings to acquire belowground and aboveground resources, RLR and LAR parameters, are presented in Figures 3 and 4, respectively. Compared to control, fertilization treatment was the only treatment affecting positively root length per seedling dry weight (RLR), as Figure 3 shows; a mean value over 284 cm g^{-1}, reporting an increase of 59%, was verified. Although not significant, the treatment subjected to fertilisers, mycorrhizal fungi, and amino acids (FAM treatment) presented a decrease of about 23% when compared to control. For leaf area per seedling dry weight (LAR), only mycorrhizal fungi treatment (FM treatment) affected positively this parameter (Figure 4).

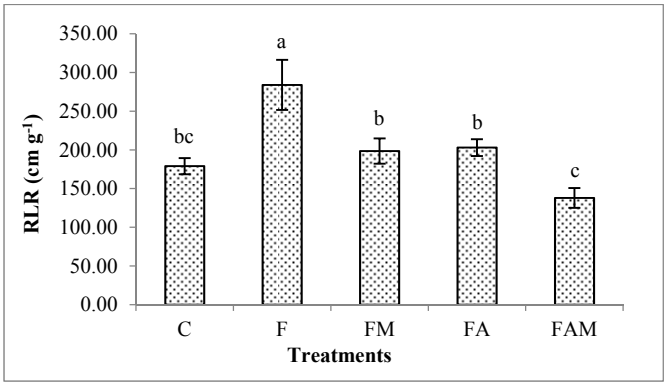

Figure 3. Fine root length per seedling biomass ratio (cm g^{-1}). Means with different letters are significantly different (n = 180; *P* = 0.000). C, control; F, fertilisation; FM, fertilization + mycorrhizal fungi; FA, fertilization + amino acids; FAM, fertilization + amino acids + mycorrhizal fungi

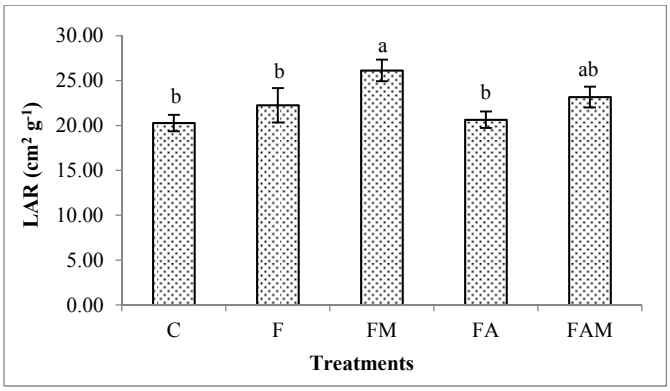

Figure 4. Leaf area per seedling biomass ratio (cm^2 g^{-1}). Means with different letters are significantly different (n = 180; *P* = 0.011). C, control; F, fertilisation; FM, fertilization + mycorrhizal fungi; FA, fertilization + amino acids; FAM, fertilization + amino acids + mycorrhizal fungi

Carbon investments, expressed through exchange surfaces between leaf/atmosphere and soil/root interactions (FRA:LA ratio), were only significantly affected by fertilization (Figure 5). Is important to point out that FMA treatment presented a decrease of 19% compared with control one, probably due to the higher leaf production verified on this treatment.

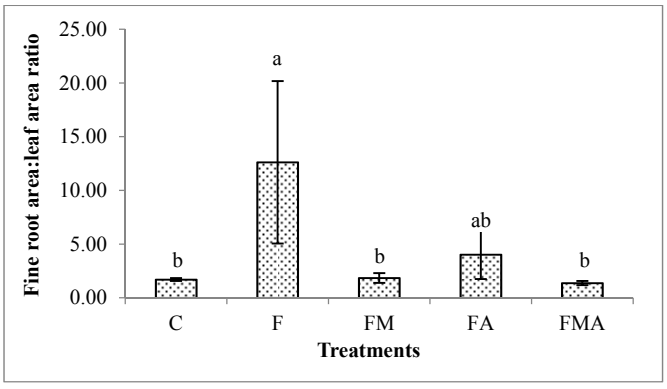

Figure 5. Fine root surface area leaf area ratio. Means with different letters are significantly different (n = 180; P = 0.000). C, control; F, fertilisation; FM, fertilization + mycorrhizal fungi; FA, fertilization + amino acids; FAM, fertilization + amino acids + mycorrhizal fungi

4. Discussion

Due to the fact that there is no extensive literature dealing with cork oak species, it was decided to make a more functional approach for discussion. This study showed that seedling capability to growth, expressed as total seedling dry weight (SD_w), was positively affected by treatments, except when only fertilisation was applied (F treatment). The most effective treatment concerning to seedling growth was composed by fertilisers, mycorrhizal fungi inoculation, and amino acids (FAM treatment), justified by the 46% increase on SD_w. The other results of morphological parameters, as leaves, wood, and aerial system dry weight and area (Tables 2 and 3, respectively), reinforced this positive effect of FMA treatment on $2^{½}$ years-old cork oak seedling growth.

The second higher production of aerial organs expressed, in terms of dry weight and area, was observed for the inoculated seedlings subjected to fertilisation (FM treatment). As hypothesized, mycorrhizal inoculation promoted a higher growth of aerial structures of cork oak seedlings, which is in agreement with Moussain et al. (2009) and Sebastiana et al. (2013) works. Moussain et al. (2009) observed significant aboveground biomass increments of 18 months-old cork oak seedlings inoculated with an ECM fungi (*Pisolithus arrhizus*). In the case of Sebastiana et al. (2013), they evaluated the effect of *Pisolithus tinctorius* inoculation on shoot systems of nursery cork oak seedlings; again, the positive effect of mycorrhizal fungi inoculation in seedling growth was verified mainly through a significant increase in leaf area and dry weight. Our outcomes were also in accordance with results observed for other tree species (e.g., Diagnea et al. (2013) for *Acacia mangnium* seedlings and Wu et al. (2011) for peach seedlings). However in our study, data for aboveground evaluation showed no significant differences between inoculated and fertilised seedlings (FM treatment) and inoculated and fertilised seedlings subjected to a supply of amino acids (FAM treatment). This would indicate that the effect of amino acid supply, to facilitate the nutrients assimilation, was not relevant for the growth rate increase of cork oak seedlings, at least until the 30 months-old.

Taking into account that all treatments applied in this study where subjected to a supply of nutrients (N, P, K) through fertilisation, the results obtained were in with the ones obtained Trubat et al. (2010). In their greenhouse experiment, these authors subjected cork oak seedlings of 18-months to different supplies of N, P, and K nutrients and evaluated the relationship between seedling morpho-functional traits and field performance of cork oak seedlings. They observed that aboveground accumulations were reduced by low nutrient availability in cork oak seedlings. However, we did not verified any significant effect on biomass parameter (expressed through dry weight) of seedlings subjected only to fertilizers through irrigation water (F treatment) during the 18 months growing period.

The evaluation of cork oak seedling root system area (BA), a morphological parameter which gives information about the quantity of root surface in contact with the soil, showed that all treatments increased significantly the root surface contact (values higher than 2,200 cm^2) compared to control (1,753 cm^2). Yet, the application of fertilisers plus amino acids proved to be the more efficient treatment to increase significantly the root surface contact. Results of 34% for tap root and 44% for fine roots were verified on seedlings subjected to this treatment. Comparing results from above and belowground system areas, we verified that the amount of root system area was higher than shoot area after 30 months of growth in all treatments, including control. However, any of the treatments was suitable to contribute positively for tap root and total belowground dry weight accumulation.

Only fine roots structures were sensitive to treatments effects. Significant differences in dry weights were observed when fertilisers and amino acids (FA treatment) and fertilisers with mycorrhizal fungi (FM treatment) were present. Equal result was obtained for both treatments (9.44 g/seedling). But, through the analysis of mean fine root distribution per seedling (Figure 1), we verified that both inoculated and non-inoculated seedlings subjected to fertilisation (FM and F treatments, respectively) were capable to invest largely on the production of these structures (33 and 30%, respectively). This is an important issue because fine roots are major contributors to carbon inputs because of their rapid turnover, despite fine root biomass contributes relatively little to total tree biomass (Kucbel et al., 2011). In addition, for these important structures of root system, we also verified that the abovementioned treatments and yet fertilisation plus amino acids application (FA treatment) promoted significant positive effects in fine root length (FRL), as seen in Table 3, and, specifically, in root length (SRL). This parameter is usually applied in prognoses of the capacity of root systems to changed nutrient availability in soils; the bigger the SRL parameter, the better is the adaptability of root systems to the changing environment (Zeleznik et al., 2007). By so, we could assume that the interactions between roots/soil interface and, consequently, caption and absorption of nutrients, were positively reinforced when F, FM and FA treatments were applied in cork oak seedlings. Particularly for fertilisation treatment, we expected these results, hence this treatment only have the aid of one product and by so, needs to promote the growth in length of fine roots to allow a major capability to explore all the soil available, looking for available water and nutrients. It is

noteworthy that the length of the fine roots in all treatments exceeded 9.5 m, which showed that soil exploration capacity of cork oak seedlings during the first two growing seasons, was high. In contrast and although all treatments affected positively fine root volume non-inoculated seedlings subjected to fertilisation presented the lower value when compared to control (32.02 cm^3). This allowed us to assume that, besides application of fertilisers on cork oak seedlings promoted longer roots (higher FRL), they were thinner than the ones from other treatments.

According to Eissenstat and Yanai (1997), the root length is assumed to be proportional to resource acquisition (benefit) and the root dry weight to be proportional to construction and maintenance (cost). Struve et al. (2009) referred that generally higher fine root production present a slower growth. We disagree with the authors hence inoculated seedlings subjected to fertilisation treatment also presented a significant growth of the upper system in our study (9.44 and 39.57 g/seedling for FRD_w and AD_w, respectively, on FM treatment, compared to 7.02 and 21.91 g/seedling for FRD_w and ADw, respectively, on control treatment). Instead, our results were partly in accordance with Mohammadi et al. (2011); they defended that mycorrhizal fungi frequently stimulated plants to reduce root biomass while simultaneously expanding nutrient uptake capacity by extending far beyond fine root surfaces. Although not significantly for mean total root system dry weight of seedlings subjected to FM treatment, we observed a decrease when compared to control (Table 3), but when the evaluation of fine roots was made a significant increase was observed. However for the inoculated seedlings subjected to fertilisation and amino acids (FAM treatment), a slightly increase was observed on root biomass, expressed through dry weight. In contrast, Diagnea et al. (2013) observed that mycorrhizal fungi enhance root biomass for 4 months-old *Acacia mangnium* seedlings. In our study, we verified that root biomass after 30 months-old cork oak seedlings were not affected by none of the treatments applied. More studies should focus on this subject to verify the influence of ECM inoculation on cork oak root seedlings by itself.

The relative allocation of resources to roots or shoots has been considered a key factor in plant strategies regarding water and it is very important for seedling performance and survival in the field (South, 2000; Kostopoulou et al., 2011). In our study, root:shoot dry weight ratio (R:S) was enhanced by all the treatments tested (Figure 2). Expectedly, lower values of R:S ratio were observed for inoculated seedlings (FM and FAM treatments). This allowed us to assume that cork oak seedlings during the first two growing seasons under FM and FAM treatments were able to transpire more water than the ones subjected to other treatments. Unlike us, Scagel and Linderman (1998), for Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and Lodgepole pine (*Pinus contorta* Douglas) seedlings at the end of the first growing season, and Jonsson et al. (2001) for Scots pine (*Pinus sylvestris* L.) found that inoculation with mycorrhizal fungi had little effect on this ratio. Also, an increase in soil fertility is commonly associated with a reduction in the root:shoot ratio (Harris, 1992), which we confirmed with our results comparing seedlings from control treatment (1.60 ± 0.08) with other treatments applied (1.36 ± 0.07 for F treatment, 1.41 ± 0.05 for FA treatment, 0.83 ± 0.04 for FM treatment, and 0.76 ± 0.04 for FAM treatment), as Figure 2 shows. However, no differences between fertilisation (F treatment) and fertilisation plus amino acids addiction (FA treatment) were found in this work.

For a future field establishment of cork oak plants, we believe that fertilized inoculated seedlings would be better adapted to a faster growth and survival. Villar-Salvador et al. (2004) observed that the field performance of holm oak (*Quercus ilex* L.) seedlings, previously fertilized during 10 months on nursery, was higher on seedlings with larger shoots and lower R:S ratio during the first two growing seasons. They verified that plants with these attributes presented lower mortality and grew faster in the field than those with smaller shoots and high R:S ratio. As in our case, the lower R:S value was due to an increase in shoot growth, but not to a reduction in the biomass allocated to roots. This response has also been observed in other species of the genus Q*uercus*, suggesting that these species had a conservative pattern of root mass in response to variations in mineral nutrients (Villar-Salvador et al., 2004).

Specific leaf area (SLA), as a measure of leaf thickness, is used to evaluate the drought resistance of the plants; an elevated SLA would indicate a better adaptability to dryness environments. In a previous study, Makita et al. (2012) investigated how colonization by different ectomycorrhizal fungal species affected the physiology and morphology of seedlings of *Quercus serrata*. They observed a positive effect of ECM on specific leaf area (SLA) in their 9-month-old oak seedlings inoculated with *Pisolithus tinctorius*, *Scleroderma citrinum*, *Laccaria amethystea*, and *Astraeus hygrometricus*. We could not confirm the same results for cork oak seedlings; after 30 months of growth, any effect on SLA was observed in our inoculated seedlings (Table 4). However when leaf area per total seedling dry weight (LAR) was analysed (Figure 4), we verified that fertilised inoculated treatment enhanced significantly the capacity of 30 months-old cork oak seedlings to acquire resources from the atmosphere. This would be in conformity with results obtained by Merouani et al. (2005). These authors also

subjected cork oak seedlings to the effect of mycorrhizal fungi inoculation (with *Pisolithus tinctorius*) and fertilised them once a week, during 6 weeks, with 50 ± 7.4 mL/seedling of NPK solution; after 18 months growth, they moreover observed that SLA was not affected by this treatment. But, such as us, they observed a positive effect of this treatment on LAR parameter. Similar LAR results were obtained between our 30 and 18 months-old seedlings (26.13 and 29.2 $cm^2 g^{-1}$, respectively).

On the other side, the enhancement capacity of cork oak seedlings to acquire resources from soil (RLR) was only sensitive to fertilisation through irrigation (RLR), as Figure 3 shows. Non-inoculated and fertilised seedlings (from F treatment) presented the better result with a mean value of 283.97 $cm^2 g^{-1}$. It is important to refer that although not significantly mean differences were obtained, a decrease on RLR was observed for FAM treatment when compared to control one. Yet, we verified that during the 30 months-old growing period only non-inoculated and fertilised cork oak seedlings (from F treatment) presented a growth strategy more focused on root functioning (water and nutrients absorption) than on photosynthesis function (leaf atmosphere exchanges). This assumption was made based on the results of the carbon investments balance of cork oak seedlings, expressed through the evaluation of exchanges surfaces between leaf/atmosphere and soil/root interactions (Figure 5). Significant increase compared to control was observed when root leaf area ratio was calculated; cork oak seedlings of control presented a ratio of 1.71 ± 0.13 and for non-inoculated and fertilised seedlings a mean value of 12.62 ± 7.56 was observed. However, when focusing on our main objective, from our understanding it is of the outmost importance to maintain equilibrium between shoot and root system growth, preserving the success of future growth stages (juvenile and mature) in the field. Then, a similar carbon investment in both compartments of the seedling must be guaranteed. Through the results (Figure 5), we assume that mycorrhizal fungi inoculation with fertilisation would be the treatment that better could promote this balance (mean value of 1.85 ± 0.45 for this ratio).

5. Conclusions

Conclusively, this work showed that mycorrhizal fungi inoculation with fertilisation and mycorrhizal fungi inoculation with fertilisation plus amino acids were the treatments capable to enhance greatly shoot production of cork oak seedlings. Mycorrhizal fungi were probably responsible for this effect (major aboveground biomass allocation in comparison to belowground) improving the roots efficiency in extracting water and nutrients for aboveground maintenance. Although it was not verified any significant effect on biomass parameter of seedlings subjected only to fertilisation through irrigation water, this treatment promoted the higher root production expressed through fine root length. The application of fertilisers plus amino acids proved to be the more efficient treatment to increase significantly the root surface contact and fine root dry weight. It is important to refer that with this study it was possible to attest that the amount of root system area is higher than shoot area after 30 months of growth in all treatments, including control and that root:shoot dry weight ratio (R:S) evaluated for cork oak seedlings was enhanced by all the treatments tested. Accordingly to the results obtained we believe that the best management option for regeneration process in terms of reinforcement of cork oak seedlings growth is to maintain the balance between shoot and root system growth through fertilisation and mycorrhizal fungi inoculation (FM treatment). Fertilisers mainly for root system and mycorrhizal fungi inoculation for shoot growth promoting through this balance a major efficiency on survival strategy of trees in post- stress plantation. Although the results presented in this study bring new highlights about the advantage of induction treatments to promote an increase in the rate of cork oak seedling growth, this research lead to some open questions that have to be subject of future works. With this study, it would be not clear whether inoculation with mycorrhizal fungi by itself allows a rate of success in seedling growth. Future work should test the effect of products separately.

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