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# Seasonal Changes in $CO_2$ Assimilation in Leaves of Seedlings and Micropropagated Plants of Carob Tree Established in Field

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**Abstract**

**In this communication we evaluate the field performance of two micropropagated Portuguese carob cultivars (Galhosa and Mulata) throughout the season, particularly at extreme conditions of light and temperature. Two irrigated plots were established in the field: 1) micropropagated plants, *vs* 2) seedlings. During the first year following transplantation to the field, we followed net photosynthetic rate, stomatal conductance, transpiration rate, chlorophyll *a* fluorescence and leaf contents in chlorophyll, carotenoids and protein. No significant differences were detected between seedlings and micropropagated plants along the year. However, at the end of summer, despite irrigation, the photosynthetic rate (*NP*), the quantum yield of PSII ($\phi_{PSII}$) and the intrinsic efficiency of open PSII reaction centers ($F'_v/F'_m$) declined, concomitantly with the increase of the thermal energy dissipation at the PSII (NPQ). As the maximal photochemical efficiency of PSII ($F_v/F_m$) was maintained high (0.82), these results indicate that regulated thermal dissipation in light harvesting complexes was promoted in order to avoid photoinhibition. After the first growth period in the field, data from micropropagated plants did not differ from seedlings, and those plants showed the characteristic behaviour of plants well adapted to Mediterranean climates. So, *in vitro* propagation could be use as a promising alternative to traditional propagation and establishment of carob orchards.**

## INTRODUCTION

Carob tree (*Ceratonia siliqua* L.), a medium (10-20 m) evergreen polygamo-dioecious tree of slow growth and great longevity, is one of the most economically important tree species of the Mediterranean basin. The use of micropropagated plants would avoid time lasting procedures of grafting, with high rate of unsuccessful, to obtain the desired varieties. Although carob tree (*Ceratonia siliqua*) is well adapted to Mediterranean climates, micropropagated plants tend to display shade characteristics and suffer from light stress when exposed to natural environmental conditions. In a previous paper we reported on the establishment of a field trial of seedlings and micropropagated plants of two Portuguese carob cultivars (Galhosa and Mulata). After three months, clonal trial has been established with 100% of success and plants showed good photosynthetic performance and growth (Osório *et al.* 2007). However, the performance of micropropagated plants at the field can be affected throughout the season, since they will be coping with substantial higher light and extreme temperatures which are potential stressful conditions to them. It has been demonstrated that the combination of these factors, that predispose plants to photoinhibition or down-regulation process, contributes to the reduction in carbon assimilation that will further affect the ability of young plants for growth and survival (Chaves *et al,* 2002). In particular, a $CO_2$ deprivation at the chloroplast level by stomatal closure could enhance the sensitivity of the photosynthetic apparatus to high irradiance (Flexas et al. 1998). Protection mechanisms against excess light are thus an important strategy under Mediterranean conditions and may be achieved by several mechanisms such as the regulated thermal dissipation at the light harvesting complexes (Demmig-Adams and Adams 1996). This photoprotective mechanism competes with photochemistry for the absorbed energy, leading to a decrease in quantum yield of PSII (Genty et al. 1989). Understanding the plant's potential to acclimate to new environments is of particular importance to predict and improve performance and survival of micropropagated plants during the process of acclimation. The aim of this study was to find out how photosynthetic capacity of micropropagated plants of *C. siliqua* acclimates to environmental changes along the year. Thus, we provide data to evaluate the field performance of these plants of *C. siliqua* throughout season, particularly at extreme conditions of light and temperature, highlighting possible differences between micropropagated plants and seedlings.

## MATERIAL AND METHODS

### Experimental site and plant material

The experiment was carried out in a field trial near Tavira in Algarve at south of Portugal. Two irrigated plots of *Ceratonia siliqua* (L.) were established: 1) micropropagated plants; 2) seedlings. The study was conducted by means of concurrent gas exchange and chlorophyll fluorescence measurements in sun-exposed leaves. The measurements were carried out, at the middle of the light period, in the youngest fully expanded leaf, sampled from five plants of each set. Physiological performance has been followed throughout the first year after transplantation into field and plants were irrigated twice a week during summer. Plant survival and growth were also evaluated.

### Gas exchange and chlorophyll *a* fluorescence measurements

Gas exchange measurements were performed under natural conditions of photosynthetic photon flux density (PPFD), air temperature, vapour pression deficit (VPD) and $CO_2$ concentration, using a portable Minicuvette System HCM 1000 (Walz, Effeltrich, Germany) in current-year, non-senescent, leaves. Leaf net photosynthetic rate ($P_N$) and stomatal conductance ($g_s$) were calculated according to the equations of von Caemmerer and Farquhar (1981). Chlorophyll *a* fluorescence measurements were performed in the same leaves using a portable pulse amplitude modulation fluorometer (PAM-2000 system, Walz, Germany). The maximal photochemical efficiency of PSII ($F_v/F_m$) was estimated from $F_o$ (basal fluorescence) and $F_m$ (maximal fluorescence) which values were taken before dawn. The intrinsic efficiency of open PSII reaction centers ($F'_v/F'_m$) was calculated from basal and maximal fluorescence measured under natural irradiance ($F'_o$ and $F'_m$) and the quantum yield of PSII in light-adapted leaves ($\phi_{PSII}$) was evaluated by the ($F'_m$-$F_s$)/$F'_m$ ratio (Genty et al. 1989). The photochemical quenching ($q_p$), which was used as an estimate of the fraction of open centres, was calculated as: $q_p$= 1-($F_s$-$F'_o$)/ ($F'_m$-$F'_o$) (Bilger and Schreiber 1986) and the thermal energy dissipation at the PSII as: NPQ = ($F_m$/$F'_m$)-1 (Cornic 1994).

**Photosynthetic pigments and total soluble protein**

Leaf discs were collected and quickly frozen in liquid nitrogen Chl *a* and *b*, as well as total carotenoids (xantophylls and carotenes) extraction was performed in dim light by grinding the frozen samples in the mortar with 100% acetone. The levels of pigments were determined using the extinction coefficients and equations predetermined by Lichtenthaler (1987). Soluble proteins were extracted by homogenizing leaf samples with 50 m*M* HEPES containing 0.1% Triton X-100. Determination of leaf total soluble protein concentration was performed according to Bradford (1976), using the Bio-Rad Protein Assay Dye (Bio-Rad, Hercules, CA) and bovine serum albumin as a standard.

**Statistical analysis**

Statistical analysis and graphic display were performed using SPSS® for Windows (release 15.0, SPSS Inc. Chicago, IL) and SigmaPlot (Version 9, SPSS Inc., Chicago, IL) software packages, respectively. Values shown are mean ± standard error of five replicates. All pairwise comparisons of individual means were done by the Duncan test. Differences were considered significant at $P \leq 0.05$.

## RESULTS AND DISCUSSION

**Survival and Growth**

Micropropagated plants survived transplanting with 100% of success compared with the 97% of the seedlings and in general, plants showed good growth and uniformity one year after transfer to the field. Micropropagated plants showed higher shoot length and number of branches than seedlings, although these differences were not statistically significant, (Table 1). Bigger increases in shoot length and new branches production were observed in seedlings that were smaller at transplantation (Fig. 1).

**Gas Exchange and Chlorophyll Fluorescence**

After transplantation (Jun 04), all gas exchange parameters were improved in all plants, particularly net photosynthesis rate (Fig. 2). No significant differences were detected between seedlings and micropropagated plants along the year either in gas exchange or in chlorophyll fluorescence (Fig. 3). However, at the end of summer, despite irrigation, the photosynthetic rate (*NP*), the quantum yield of PSII ($\phi_{PSII}$) and the intrinsic efficiency of open PSII reaction centers ($F'_v/F'_m$) declined, concomitantly with the increase of the thermal energy dissipation at the PSII (NPQ) (Fig. 3). As the maximal photochemical efficiency of PSII ($F_v/F_m$) was maintained high (0.82), these results indicate that regulated thermal dissipation in light harvesting complexes was promoted in order to avoid photoinhibition. This downregulation of photosynthetic activity expected to be quickly reversible following the relief of seasonal summer stress.

**Photosynthetic pigments and soluble protein**

The increase on photosynthetic capacity of micropropagared plants after Nov 04 can be associated with the increase in the concentration of chlorophyll (Fig. 2). Moreover, total soluble protein content decrease in micropropagated plants to values analogous to seedlings and no significant differences were detected after the acclimation period (4 Nov) and during the year, which may suggest that Rubisco, that usually comprises 40-60% of leaf soluble protein, is at least present in comparable quantities in micropropagated and seedlings of carob tree, under the natural growing conditions.

## Conclusions

This study shows that micropropagated plants of carob tree have excellent field survival. After the first growth period in the field, data from micropropagated plants did not differ from seedlings, and micropropagated plants showed, in the absence of water deficit, the characteristic behaviour of well adapted plants to Mediterranean climates. So, the similar field performance revealed by micropropagated young plants and seedlings, pointing out the use of *in vitro* propagated plants as promising alternative to traditional propagation and establishment of carob orchards. In fact, this methodology as compared to traditional propagation offer certain advantages for large scale production of carob trees, particularly, avoiding time lasting procedures of grafting and supplying plants genetically identical to the mother plants

## ACKNOWLEDGEMENTS

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## Tables

Table 1. Main shoot length, trunk diameter and number of branches of micropropagated 'Mulata and 'Galhosa' and seedlings of carob tree one year after transplantation at field.

| Plant Type | Survival (%) | Shoot length (cm) | Diameter (cm) | Nº of branches |
|---|---|---|---|---|
| 'Mulata' | 100 a | 130.4 ± 5.61 a | 10.0 ± 0.9 a | 22.0 ± 3.4 a |
| 'Galhosa' | 100 a | 143.8 ± 5.68 a | 15.5 ± 1.8 a | 18.7 ± 3.6 a |
| Seedling | 97 a | 116.0 ± 14.3 a | 12.4 ± 1.5 a | 14.8 ± 1.3 a |

The values shown are means ± SE from five samples. Values followed by the same letter are not significantly different at P≤0.05 (one-way ANOVA).

## Figures

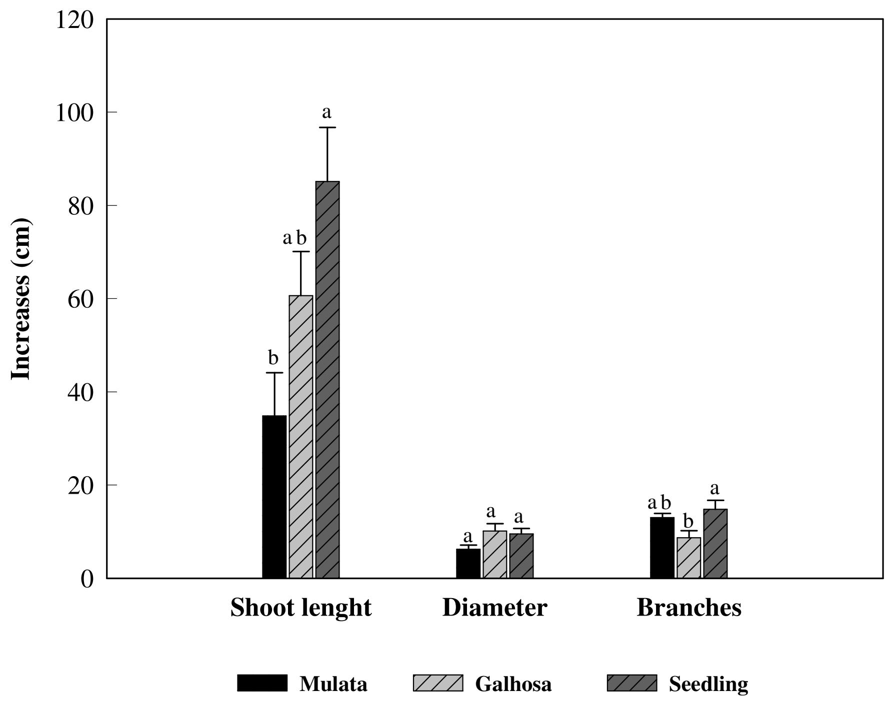


Fig. 1. Increases in shoot length, trunk diameter and number of branches of micropropagated 'Mulata and 'Galhosa' and seedlings of carob tree from transplantation until one year after. The values shown are means ± SE from five samples. Values followed by the same letter are not significantly different at P≤0.05 (one-way ANOVA, Duncan test).

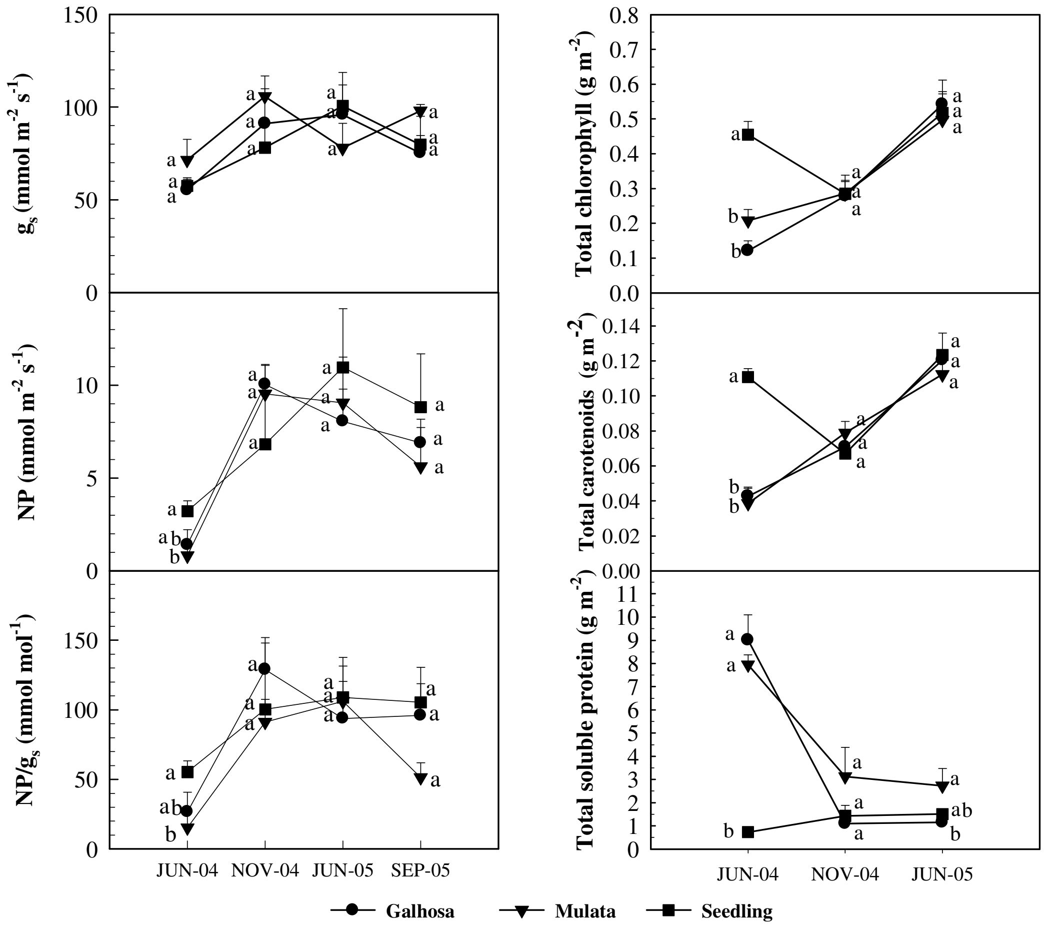


Fig. 2. Net photosynthesis rate (NP), stomatal conductance ($g_s$), instantaneous water use efficiency NP/$g_s$, chlorophyll, carotenoids and protein of micropropagated plants and seedlings of carob tree. The values shown are means ± SE from five samples. Values followed by the same letter are not significantly different at P≤0.05 (one-way ANOVA, Duncan test).

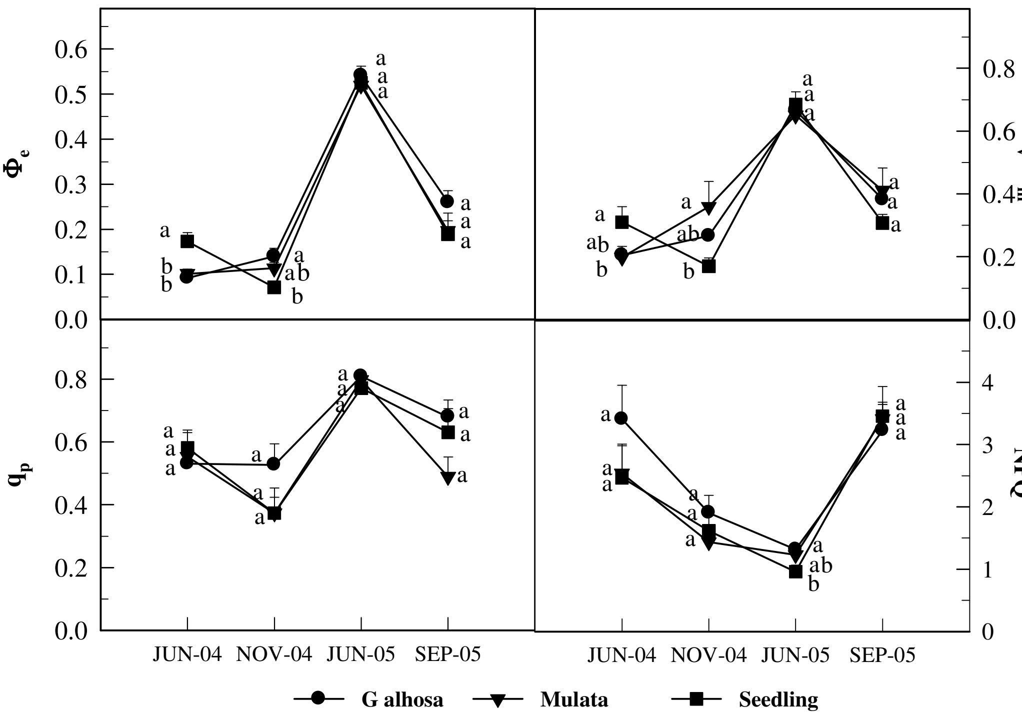


Fig. 3. Quantum yield of PSII ($\phi_{PSII}$), intrinsic efficiency of open PSII reaction centers ($F'_v/F'_m$), photochemical quenching ($q_p$) and non-photochemical quenching ($NPQ$) of chlorophyll fluorescence of micropropagated plants and seedlings of carob tree The values shown are means ± SE from five samples. Values followed by the same letter are not significantly different at P≤0.05 (one-way ANOVA, Duncan test).