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Carob productivity as affected by inorganic nitrogen fertilization

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Executive Summary

Review and summary

This research project was developed to provide the much needed scientific background on the nutritional and growth characteristics of carob, which is essential for further improvement of carob utilization both as an important ecosystem component and as a productive regional crop. The primary aim of this project was to provide necessary basic information on the effects of combined fertilization and irrigation management techniques applied to existing carob trees. The results obtained, published in international scientific journals, constitute a **unique body of knowledge on the assimilation of inorganic nitrogen by slow growing trees** in general and carob in particular. Our extensive greenhouse and laboratory research with carob, which included research of its primary production, water use efficiency and nitrogen use efficiency constituted the basis of our achievements in the present project. The effects of different environmental parameters such as rhizosphere temperature, atmospheric CO_2 enrichment and water supply on the uptake and assimilation of nitrate and ammonium have been investigated. Studies were carried out on the critical role of mineral nutrient supply under stress conditions limiting growth and development (drought, salinity, root temperatures). The application of combined irrigation and nitrogen fertilization management was proved to be successful for the improvement of millions of existing carob trees in Portugal. This had a significant ecological and economical impact on the vast zones of Portugal in which the trees have been planted over the centuries.

Project accomplishments in the overall context of international development

Understanding the productivity limits of plants as controlled by the type of inorganic nitrogen supplied are presently a major research aim of numerous agrobiological research groups in the world. The cost of fertilizers, the increasing demands of the farmers to avoid the undesirable environmental implications of excessive fertilization, the need of developing Mediterranean agriculture to produce high quality produce - all of these reasons increased the importance of this research project.

The results obtained may increase carob productivity in the entire Mediterranean basin and contribute to the preservation of the trees, rational planting of new orchards and to provide a steady source of income for regional farmers. This research focused on a better control of inputs and a reduction in production costs for carob production, giving special attention to the needs of less-favored and marginal rural Mediterranean areas. The stabilization of carob production levels through optimal use of fertigation, to which we have significantly contributed, will help to satisfy the increasing demand of the market stabilizing carob price fluctuations.

This project also represented a major effort to understand the response of primary assimilation processes in plants to reduced (ammonium) or oxidized (nitrate) nitrogen. Up to now the major

considerations to supply either one of these nitrogen forms were cost considerations. The use of different forms of inorganic nitrogen to enhance plant productivity at different developmental stages constitutes a new and important aspect of nitrogen assimilation by woody species at the physiological level and on the design of modern fertilization management at the agricultural level.

What skills or awareness will remain in the developing country as a result of the project ?.

The present research project taught us how to provide cost-efficient and balanced nutrition to the trees, allowing better orchard management, enhanced and stabilized productivity. It will contribute to minimize nitrogen pollution by over fertilization and define the efficient water use in Mediterranean areas in which desertification is a very real threat. Carob, as a well adapted and hardy Mediterranean tree crop, can reduce land degradation and allow both intensification and diversification of crop and stock production.

Research Objectives

Carob has a considerable potential as a tree crop for restoring vegetation and improving the productivity of marginal dry lands. It is a traditional crop in poorer regions of the Mediterranean basin. Mediterranean farmers are familiar with it because it is a multi-purpose tree crop whose fruits and seeds may be used for food, fodder, sugar and syrup, industrial production of gums and alcohol. In contrast to other traditional crops in the area, its cultivation can be expanded at low input costs. Since modern agriculture has become more competitive and sophisticated, critical changes were demand on the agricultural practices of countries such as Portugal, Spain, Greece, Turkey, Egypt, Algeria, Tunis, Morocco, etc. for which agriculture production constitutes a major income of vast sectors of the population. Learning to use more efficiently water and fertilizers, especially in relation to carob trees may be of great importance for the modernization of traditional agriculture in Portugal.

Problems addressed and their importance to development ?

The main problem that considered in this work was to learn the effects of inorganic nitrogen assimilation on the growth rate of carob trees and how to enhance assimilate translocation from source to sink increasing productivity and fruit quality. This problem was and still is very important to scientists concerned with the advancement of agriculture in developing countries and in marginal areas. Moreover it contributed to link basic laboratory research to applied field trials while having a considerable ecological impact by determining ecological-safe management of carob orchards.

Relation to other projects

The present research blends with a number of other projects supported by AID/CDR (C5-001, C5-224, C11-131, C11-134, C12-157) in which the impact of inorganic nitrogen assimilation on the growth rate and productivity of crops under normal and stress (salinity, drought, high rhizosphere temperature) have been studied. The work with carob has been of significant value to develop present

models of the regulation of biomass production and the determination of growth rate of crops and wild species. These model (Fig. 1) is already widely used by crop- and eco-physiologists and may be one of the key elements for the industrialization of agricultural production during the next century.

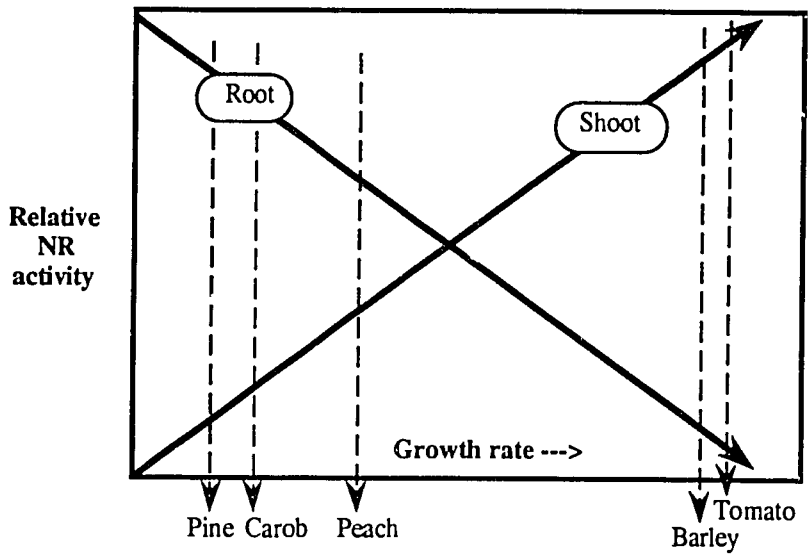

Figure 1: The relationship between growth rate, yield or biomass production and the relative distribution of nitrate assimilation sites in several plant species

Innovative aspects

The innovative aspects of this project were the physiological/biochemical studies of nitrogen uptake and assimilation systems in carob, photosynthesis and translocation of assimilates in a woody perennial and also the findings about the importance of a combined fertilization and irrigation management with very low inputs to stimulate high carob productivity. The cooperative research was, consequently, important for the introduction of modern aspects of biological and agricultural research to enhance productivity of carob and other tree crops as well as to establish the basis for the selection of sites for successful reforestation.

Other organizations supporting the project

The project was supported by the following organizations:

(a) The Dept. of Ecology of Faculty of Sciences of the Univ. of Lisbon.

(b) AIDA - Associação Interprofissional para o Desenvolvimento e Valorização da Alfarroba - which put at our disposal the carob orchards in which our field work was carried out.

(c) The Blaustein International Center (Ben-Gurion University of the Negev) which supported the work of Dr. Cristina Cruz and Dr. M. A. Martins-Louçao in Sede Boqer.

(d) ALGARVERDE put at our disposal additional field facilities.

(e) JNICT offered financial support for the work of C. Cruz

Methods and Results

The following are the last four publications of the project

Seasonal variations of leaf water potential and growth in fertigated carob trees (*Ceratonia siliqua* L.)

P. J. Correia and M. A. Martins-Loução*

Abstract

Variations of predawn and midday leaf water potential and relative growth rates were studied in mature carob trees (*Ceratonia siliqua* L. cv "Mulata") submitted to a fertigation experiment. Three levels of irrigation were tested: 0%, 50% and 100%, based on daily standard evaporation values. For each irrigation level two nitrogen amounts were applied - 21 and 63 kg N ha^{-1} $year^{-1}$ - as ammonium nitrate. The experiment was run between July 91 and August 93. Measurements of leaf water potential and absolute branch length increments were made at monthly intervals, respectively during all the experimental period or during seasonal growth. Leaf water potential was related to soil volumetric water content, maximum and minimum air temperature and daily evaporation. Predawn leaf water potentials were always higher than -1.1 MPa. Midday leaf water potential values presented very large seasonal variations and very low values independent of treatments. The low leaf water potentials observed for the fertigated trees during summer, suggests that this parameter may be related not only to the evaporative demand but also to growth investment. The amount of fertigation was positively correlated with vegetative growth increment and fruit production. Practical implications for irrigation schedules of leaf water potential patterns together with drought adaptation mechanisms of carob tree are discussed.

Introduction

Carob tree (*Ceratonia siliqua* L.) is known to possess several types of drought resistance mechanisms (Nunes et al., 1989; Lo Gullo and Salleo, 1988; Salleo and Lo Gullo, 1989; Nunes et al., 1992). Like in other Mediterranean species, with long-lived leaves (Diamantoglou and Mitrakos, 1981) and an extended flowering period (Martins-Loução and Brito de Carvalho, 1991) mechanisms have evolved that optimize water, carbon and nitrogen use for reproduction. It is also a high value cash crop and a valuable resource for reforestation to prevent erosion processes in marginal lands. However, some agronomic practices need to be improved to guarantee optimization as well as reliable carob productivity and maintain the economical viability of this species.

Information relating yield and rainfall is scarce. Marti and Caravaca (1990) presented some references which indicate that a rainfall range of 400-500 mm per year is needed to obtain a good fruit production.

An important attempt for introducing irrigation practices in carob plantations in marginal lands was made in the Negev Desert (Israel) by Herwitz et al. (1988). They showed that the use of runoff water on a micro-catchment system could sustain carob-trees having leaf surface areas over 13 m^2 and Merwin (1980) reported the use of runoff water and drip-irrigation of carob-trees in Mexico.

Since Correia and Martins-Loução (1990) showed the importance of drip-irrigation during plantation of young carob in Portugal, in 1991 a study was initiated concerning the possibilities of improving growth and yield in existing mature orchards through a fertigation system. It is, thus, necessary to obtain data concerning the relationship between carob production and tree water status in order to optimize the establishment of irrigation schedules. Several relationships have been found between atmospheric parameters and plant water status (Nel and Berliner, 1990; Peñuelas et al., 1992) mainly to optimize irrigation regimes and prevent water stress. Leaf water potential is one of the most widely studied plant parameters concerning those relationships.

In this work we investigate if leaf water potential is a good parameter for the diagnosis of the factors affecting soil-plant-atmosphere continuum in trees submitted to different fertigation regimes. The relationship between nitrogen gain and water status was also studied in order to verify if there were any variations in tree response due to nitrogen effects on the balance between carbon gain and water loss.

Materials and Methods

Experimental site and soil parameters

The orchard used for the experiment presented 20-30 years old mature trees (cv. "Mulata") with 2-3 m height and 14.9 ± 0.4 cm trunk diameter, established in a 12x12 m spacing (69 trees ha^{-1}). It is located in the southern region of Portugal (37° 13' N; 7° 28'W). The soil is a sandy loam, with a large amount of gravel throughout the profile, with low levels of organic matter (1.5%), C/N (7-11) and poor in N (0.15 %). Annual rainfall, temperature and evaporation of the site are shown in Fig. 1, showing characteristics of a typical Mediterranean climate (Mitrakos, 1981).

The field trial consisted of three irrigation (I) levels (0%, 50% and 100%) based on water loss by evaporation (E_{pan} measured with a class A pan; Doorenbos, 1976) according to the formula $I=E_{pan} \times CF$. The conversion factor, CF, (8.4 m^2) was calculated as the average of the projected canopy area of all the trees in the experiment. In that formula the cultural coefficient (K_c) was not used, since no K_c is known for carob. Two N-fertilizer levels were used: 21 kg ha^{-1} and 63 kg ha^{-1}. The N concentration was 0.3 kg $tree^{-1}$ and 0.9 kg $tree^{-1}$, corresponding to 1.5 kg $tree^{-1}$ and 4.5 kg $tree^{-1}$ of total fertilizer, respectively. The fertilizer contained 15% calcium and 20.5% nitrogen, with equal

parts of nitrate and ammonium. The fertilizer was applied once per year and spread all over the area under the canopy. The water was applied daily, during the morning through the dry season, between June and August of 1991, 1992 and 1993. The irrigation consisted of a micro-sprinkler system, one per tree and closed to the tree trunk, delivering 40 l h^{-1}, with a range of 360 °. Border effects and runoff water are not expected due to the wide tree spacing and a small hill slope (<10%). As the experimental trees are relatively small, considering their age, we also do not expect water uptake from adjacent irrigated trees.

A total of six treatments were tested. Each treatment consisted of 3 replicates of 4 trees in a total of 12 trees per treatment distributed in randomized design: 1.5/0, 1.5/50, 1.5/100, 4.5/0, 4.5/50 and 4.5/100 (N/water). On the 0% water level the fertilizer was applied two months before the other treatments in order to ensure dilution through the last rainfall. For the other water levels the fertilizer was applied at the beginning of the irrigation period (June).

Before irrigation three soil samples per treatment were taken with an auger under tree canopy at a distance of 60-80 cm from the trunk and always in north direction. The samples were taken at 30-40 cm depth, because "in situ" observations, in a well irrigated tree, indicated the presence of active roots at this depth. All the samples were taken during the early hours of the morning. They were weighed and dried in an oven for 48 h at 105 °C for gravimetric water content.

Soil volumetric water content (VWC), in $g.cm^{-3}$, was calculated using the gravimetric method, through the formula:

$$VWC = \left[\left(\frac{W_{ms} - W_{ds}}{W_{ds}}\right)\right] \times bd$$

where W_{ms}, W_{ds} and bd, are the weight of moist and dry soil and the bulk density, respectively. Bulk density used in the formula (1.37 g cm^{-3}) was obtained by taking an undisturbed block of soil with a metal cylinder.

Leaf water potential - LWP

Predawn and midday LWP were determined in the field with a Scholander-type pressure chamber in mature leaves taken from the south side of the canopy. In each treatment, the leaves used to measure LWP were taken off from 3 different trees. Three leaves per treatment were used for the measurements, which were done monthly during the irrigation season. Some measurements were also done during winter. Between dates the same trees were used. A wet filter paper was placed inside the chamber in order to minimize leaf water evaporation.

Meteorological data

Maximum and minimum amidity and precipitation were recorded daily using a thermo-hydrograph and a rain gauge placed within the orchard. Mean month temperature and evaporation were used in the regressions with LWP.

Branch length increment

At the beginning of the experiment (May 1991) 9 trees per treatment, homogeneous both in size and shape, were selected, and 8 branches on each tree were marked on the external side of the canopy. The terminal 100 cm of each branch was labeled and the subsequent shoot length increment of those branches were followed monthly all over the years. During winter time no growth was registered.

The absolute growth length increment was determined in order to evaluate the effects of fertigation treatments. Therefore the values shown were only reported to the growing season.

Fruit production

Fruit was harvested in September of each year from 91 to 93 and expressed as kg per hectare.

Statistical analysis

For each measurement, differences among treatments were compared by analysis of variance, using F-test and Duncan's multiple range test (DMRT). "Water", "Nitrogen" and "Water x Nitrogen" interactions were tested by the same procedure. Relations between predawn and midday leaf water potential and climatic variables, such as air temperature, evaporation and VWC were examined through linear regressions. All the data analysis were made with a computer program Statgraphics version 5.0 STSC.

Results

Meteorological data at the experimental station are shown in Figure 1. Figure 2 shows predawn and midday leaf water potential (LWP) between June 91 and August 93.

In spite of the small seasonal variation between and throughout years for the predawn leaf water potential values, significant differences during irrigation seasons were observed. The highest values were obtained for the well-irrigated treatments, in July 91: -0.73 MPa and -0.83 MPa for 1.5/100 and 4.5/100, respectively (Fig. 2). No nitrogen (N) nor N x water interactions effects were registered. Only in July and August 1992 significant differences were observed between treatments, the water effect being more marked in August than in July (Table 1). During this month a positive interaction between nitrogen and water was registered as well as a greater nitrogen effect predominance. In 1993, predawn values obtained in summer were not statistically different, all the treatments showing slightly higher values than those of the previous year. Apparently, the same trend was obtained all over the year. This fact is probably associated with rainfall events between February 93 and the end of May (Fig.1). No significant differences were observed in predawn LWP during winter and early spring, except on the first year when a N effect was detected (Fig. 2).

Midday LWP were low despite the irrigation levels (Fig. 2). Significant differences between treatments were not evident, although seasonal variations were observed. However statistical differences (Table 1) were detected between treatments for midday leaf water potentials, in June and August 1992 and 1993, clearly pointing out to irrigation effects on LWP patterns. It is possible that the small number of measurements was associated with the absence of more notorious differences. A positive effect was also observed for N, since the highest values of LWP were observed for the more fertilized trees (Fig. 2).

In order to evaluate the driving forces in the soil-plant-atmosphere continuum, a set of regressions were assessed between LWP (predawn and midday), atmospheric parameters and soil volumetric water content (VWC). The results of coefficient of determination (r^2) are presented in Table 2. Although r^2 values are statistically significant they are lower than midday LWP, suggesting that predawn LWP followed a pattern which may eventually be independent of the environmental conditions. Although it is known that predawn LWP is related to soil water availability this fact was not entirely sustained by the regression values obtained for predawn LWP and VWC. Nevertheless, when we consider "non-irrigated" treatments, which were submitted to soil drought during summer, we obtained a slightly significant correlation (r^2 = 0.71 and 0.50), which was similar to the treatments with 50% irrigation levels. For "well-irrigated" treatments (100%) no correlation was obtained, which may be related to the heterogeneity of soil water distribution, due to irrigation effects.

Midday LWP values (Fig. 2) were well correlated (Table 2) with atmospheric conditions, especially with maximum temperature and Epan evaporation. The lowest values were recorded during summer (Fig.2) for all treatments and we may assume that evaporative demand played an important role in LWP patterns. No correlation was obtained between VWC and LWP, except for 1.5/0 treatment. Considering the reasons stated above we may not exclude some effect of VWC on LWP patterns.

Plants growing under well-irrigated conditions (1.5/100 and 4.5/100) exhibited the highest growth increments all over the 3 years, as shown in figure 3. As it can be seen in table 3, branch length increment was statistically significant after full establishment of irrigation (July and August) in 91 and 92. A significant and positive N x water interaction was also observed. In the first and second years the effect of nitrogen and summer irrigation is clear (Fig. 3), since N-high and well-irrigated treatments showed higher growth increments. In 1993 the occurrence of late rainfall in May (Fig. 1) triggered very high initial growth increments (Fig. 3), especially for the non-irrigated treatments.

The increase in fruit yield (Fig. 4) as a response of fertigation is more pronounced in the well-fertigated treatment (4.5/100). It should be noted that the increment from 91 to 92 was different between treatments. There was a larger increment from 92 to 93, but similar for all treatments.

Discussion

Contrary to what occurs for several Mediterranean species in natural habitats or in potted plants under greenhouse conditions (Acherar and Rambal, 1992; Rambal, 1992; Correia and Catarino, 1994) predawn values in the dry season for the non-irrigated treatments do not drop below -1.1 MPa, which may indicate a deep-rooting strategy in our conditions. Similar conclusions were suggested by Nunes et al. (1992) also for mature carob-trees. Rhizopoulou and Davies (1991) also found that, in young carob tree plants submitted to soil water deficit for 3 weeks, deep penetration of some roots was enough to supply substantial amounts of water to shoot growth. On the other hand, the direct response of predawn LWP values to irrigation in 1991 (Fig. 2), associated with the increment of branch length increments exhibited by the well-irrigated trees (Fig. 3), may also indicate an important root development or good infiltration in upper soil layers. The same kind of root adaptation was observed in olive trees submitted to drip-irrigation, and grown under dry-farming conditions for several years (Fernandez et al. 1992). The high values of LWP presented during early summer 1993 (Fig.2), responsible for the high growth increments observed in this period (Fig.3) can be explained by accumulated rainfall during the previous spring (Fig. 1), recharging soil water and reducing the severe summer drought stress.

The low minimum LWP values registered in well-irrigated trees, showing no clear differences compared with the non-irrigated trees (Fig. 2), was also observed in previous works using drip-irrigated carob-trees (Correia and Martins-Loução, 1990). Water effects were only observed at the end of irrigation season (Table 1). Similarly, Gower et al. (1992) reported no differences in midmorning xylem water potential comparing irrigated and non-irrigated *Pseudotsuga* trees and Torrecillas et al. (1989) also found no differences in minimum LWP values for almond trees, submitted to different irrigation regimes. The low values of LWP observed in this study (Fig. 2) may indicate that not enough water had reached the active roots, due to high air evaporative conditions (Fig. 1). However, significant differences were observed for vegetative growth (Fig. 3) showing a significant effect of N, water and Nxwater interactions (Table 3). Similarly, reproductive output (Correia and Martins-Loução, 1992; 1993a) and fruit production (Fig. 4) show a positive effect of fertigation treatments. Lloveras and Tous (1992) also observed a significant increase in carob production after nitrogen application in an old carob tree stand and other experiments registered a positive irrigation effect on fruit production (Esbenshade and Wilson, 1986).

Concerning the "N-effect", the hypothesis was that the cumulative effect of nitrogen fertilization would affect foliar area and consequently whole tree water balance, as it was simulated by Rambal (1993). Although our results do not indicate a clear "N-effect", we might expect that the increase in branch growth (Fig. 3) triggered modifications in tree water balance. Under drought conditions, such as the case of the non-irrigated treatments, and in the presence of high levels of N, the increase in N availability, influences carob productivity (Fig. 4) leading to higher output of leaves (Correia and Martins-Loução 1992) and reproductive organs (Correia and Martins-Loução 1993a). Considering fruit production per unit of available water it is expected that non-irrigated trees would present higher

rates of water use efficiency (Correia and Martins-Loução, 1993b). Although soil drying will eventually reduce root growth (Rhizopoulou and Davies, 1991), it is also established that N accumulation is able to function not only as a N source but also as an osmoticum (Leidi and Lips, 1990). This is in agreement with recent work which shows that osmotic potential can control carob drought stress tolerance (Nunes et al., 1989) and could explain the low values of midday LWP in non-irrigated trees during the summer drought period (Fig. 2). With an increasing water supply a direct N-mobilization contributed to a higher growth increment (Fig. 3). The increment of leaf and, consequently, of canopy area, forces the plant to change water distribution, explaining the lower LWP values observed (Fig. 2) and also non-significant differences in N x water effects (Table 1).

Another driving force may also be present which modifies minimum LWP patterns. The regressions obtained between atmospheric conditions and midday LWP (Table 2) showed that the effect of air vapor pressure deficit is more important than the effect of soil water status for the maintenance of the soil-plant-atmosphere continuum, which is in agreement with other studies (Oliveira et al., 1992). On the contrary predawn LWP seems to have some relations with VWC. The absence of correlation for the highest irrigated treatments may be explained by the association between high soil water status and high predawn LWP. However we must admit that probably the sampling for the VWC might not have been sufficiently representative of the soil water status. The large amount of gravel and the rapidly drying soil, especially during summer, made homogeneous soil sampling very difficult. So, the correlation between LWP (predawn and midday) with VWC did not seem to be an appropriate approach to the analysis of soil-plant-atmosphere continuum in this orchard.

Air evaporative demand (Fig. 1) and branch growth (Fig. 3) increase soil-to-leaf water flux which, in turns, condition LWP patterns maintaining the trees under a moderate drought stress, independently of the treatments (Fig. 2). Therefore, this moderate water stress should be regarded as a "functional" stress which is a natural consequence of tree development.

The results presented here are in agreement with the "water-spending" strategy hypothesis suggested by LoGullo and Salleo (1988), as a drought adaptation mechanism of carob-tree. Carob-tree can maintain stomatal opening and high leaf water content even with low soil water availability (Nunes et al. 1992), which is allowed by sharply dropping LWP in response to small water losses (Lo Gullo and Salleo, 1988).

It is important to note that the major differences in LWP patterns were observed between non-irrigated (0% Epan) and irrigated treatments (50 and 100% Epan). It means that under these conditions 50% Epan was enough to ensure a reasonable vegetative growth (Fig. 3), reproductive output (Correia and Martins-Loução, 1993) and fruit production (Fig. 4). Similarly, these data also suggest that under the normal conditions of this type of Mediterranean vegetation, nitrogen application is effective even without irrigation. These results have important implications for practical and economical purposes and thus must be taken into consideration during the establishment of irrigation schedules, especially when semi-arid Mediterranean climates are involved.

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Legends

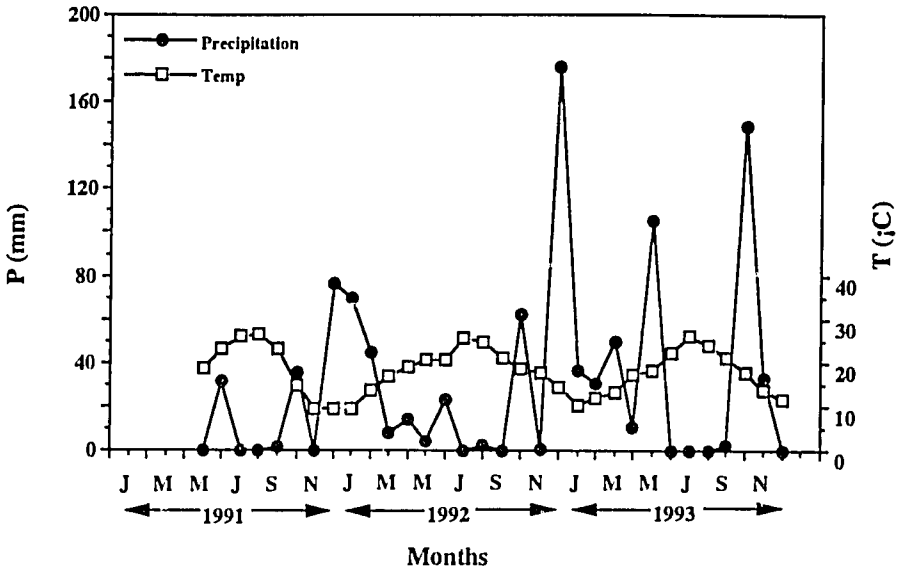
Fig. 1. Seasonal variation of atmospheric conditions at the experimental field. Fertigation periods are indicated by the shade bars in the upper part of the graph. A. Minimum and maximum air temperature and precipitation. B. Air evaporation.

Fig. 2. Seasonal variation of predawn and midday leaf water potential for all treatments. The symbols referred as 1.5/0 - 4.5/100 means N/water application. Fertigation periods are indicated by the shade bars in the upper part of the graph. Each point is an average of 3 measurements per treatment.

Fig. 3. Absolute branch increment for the fertigation treatments during the 3 years of the experiment. Symbols as Fig. 2. Each point is an average of 72 measurements per treatment.

Fig 4 · Number of inflorescences per branch for all treatments. ☐ May 91; ☐ May 92; ▨ 1993. The results are the mean of 72 branches. Dashed line is a reference line, representing the mean inflorescence number for 1991. For each year, values with the same letter are not significantly different at $p \leq 0.05$. The values of the two years were not significantly different at low N levels at $p \leq 0.05$.

Figure 1a


Precipitation
Temp
P (mm)
T (;C)
200
160
120
80
40
0
40
30
20
10
0
J M M J S N J M M J S N J M M J S N
1991
1992
1993
Months

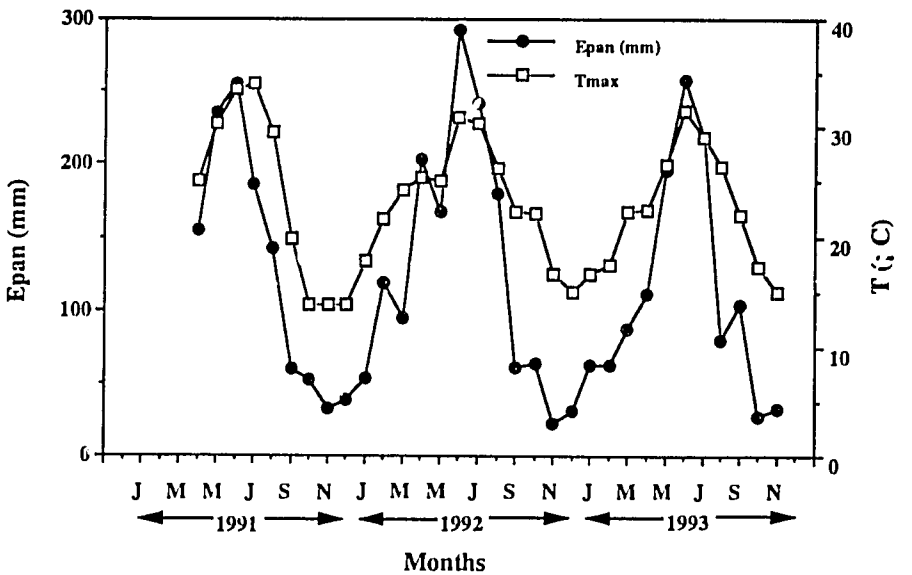

Epan (mm)
Tmax
Epan (mm)
T (; C)
300
200
100
0
40
30
20
10
0
J M M J S N J M M J S N J M M J S N
1991
1992
1993
Months

Figure 1b

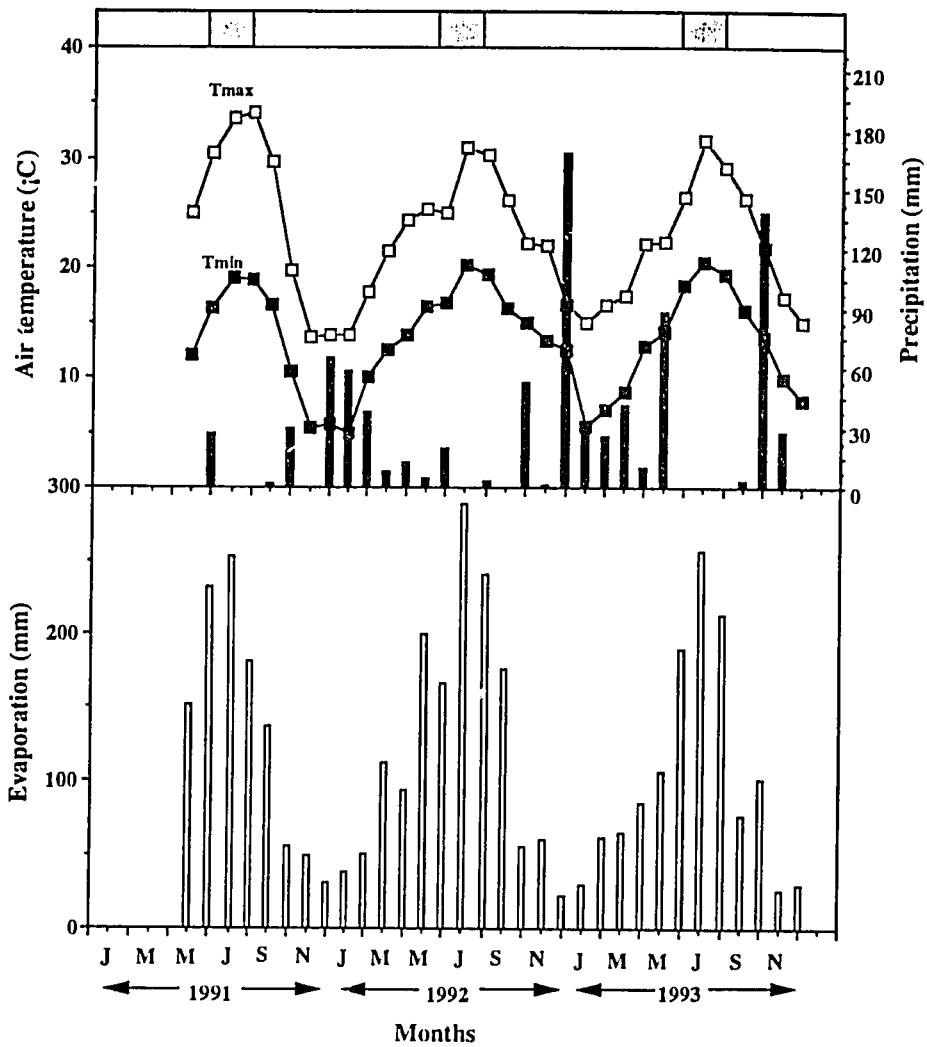

Tmax
Tmin
Air temperature (°C)
Precipitation (mm)
Evaporation (mm)
40
30
20
10
300
200
100
0
210
180
150
120
90
60
30
0
J M M J S N J M M J S N J M M J S N
1991
1992
1993
Months

Figure 2

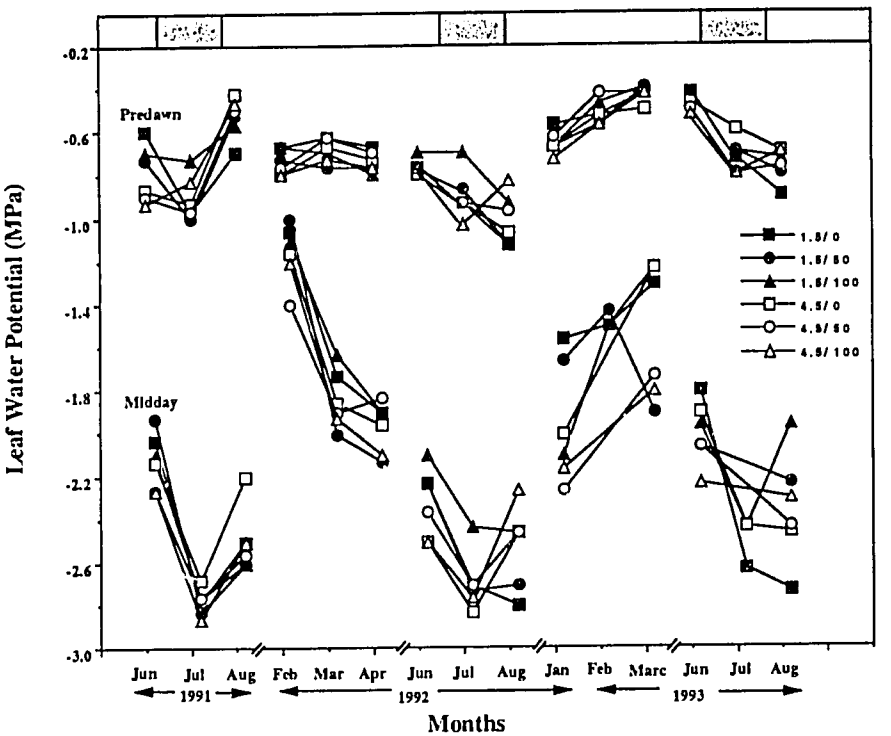

Leaf Water Potential (MPa)
-0.2
-0.6
-1.0
-1.4
-1.8
-2.2
-2.6
-3.0
Predawn
Midday
1.5/0
1.5/50
1.5/100
4.5/0
4.5/50
4.5/100
Jun
Jul
Aug
Feb
Mar
Apr
Jun
Jul
Aug
Jan
Feb
Marc
Jun
Jul
Aug
1991
1992
1993
Months

Figure 3

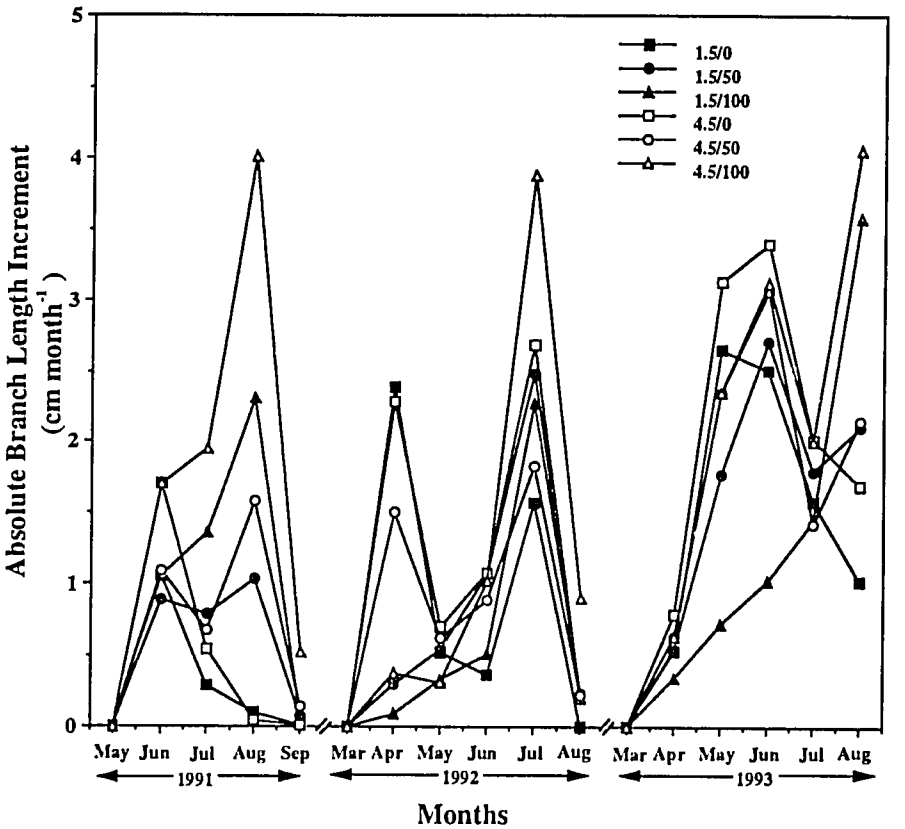
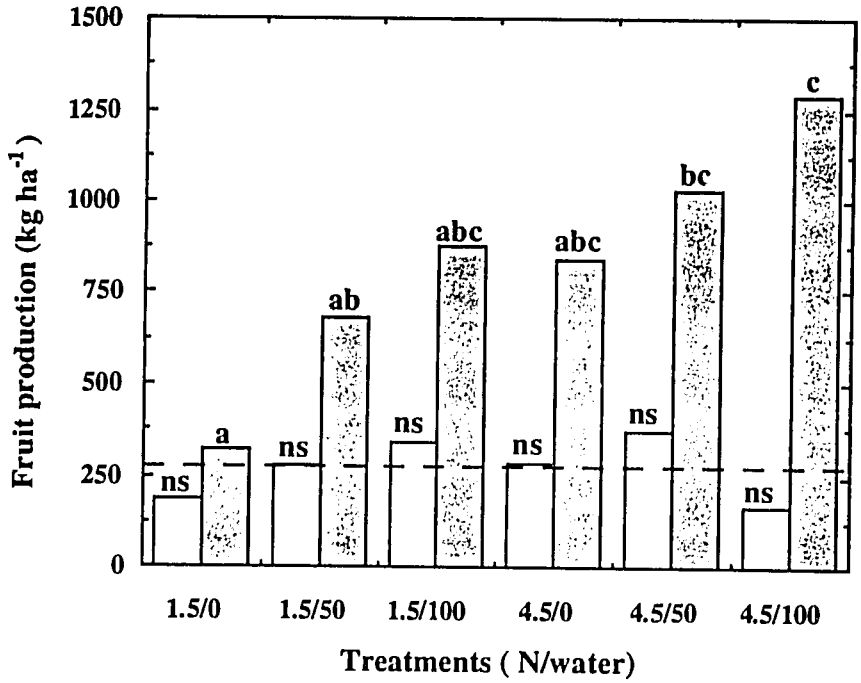

Absolute Branch Length Increment
(cm month^{-1})
0
1
2
3
4
5
1.5/0
1.5/50
1.5/100
4.5/0
4.5/50
4.5/100
May Jun Jul Aug Sep
1991
Mar Apr May Jun Jul Aug
1992
Mar Apr May Jun Jul Aug
1993
Months

Figure 4


1500
1250
1000
750
500
250
0
Fruit production (kg ha-1)
ns
a
ns
ab
ns
abc
ns
abc
ns
bc
ns
c
1.5/0
1.5/50
1.5/100
4.5/0
4.5/50
4.5/100
Treatments (N/water)

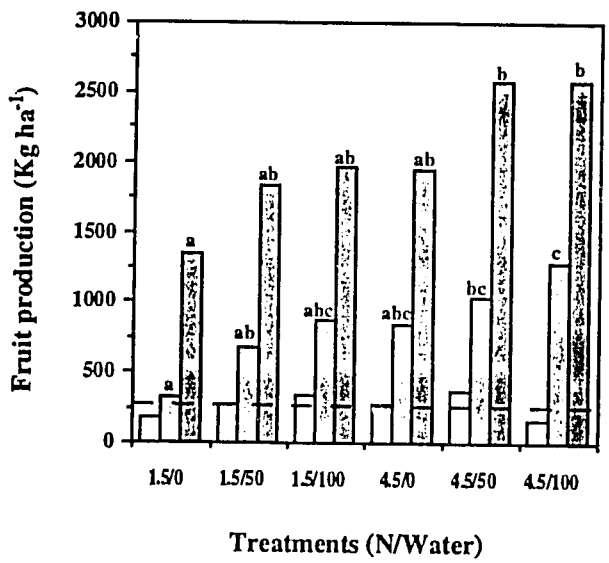

3000
2500
2000
1500
1000
500
0
Fruit production (Kg ha-1)
a
a
ab
ab
abc
ab
abc
ab
bc
b
c
b
1.5/0
1.5/50
1.5/100
4.5/0
4.5/50
4.5/100
Treatments (N/Water)

Table 1. Significance levels of differences among treatments for predawn and midday LWP obtained from multifactorial ANOVA table using F-test. These data are related to Fig. 2, however only the irrigation season was considered for each year. Significant at 95% (*) and 99% (**). Non- (ns).

LWP (Predawn)								
Factors	**Jun 91**	**Jul 91**	**Jun 92**	**Jul 92**	**Au 92**	**Jun 93**	**Jul 93**	**Au 93**
Water (W)	ns	*	ns	ns	*	ns	ns	ns
N	ns	ns	ns	**	ns	ns	ns	ns
N x W	ns	ns	ns	*	ns	ns	ns	ns
LWP (midday)								
Water (W)	ns	ns	*	ns	**	*	ns	**
N	ns	ns	ns	ns	ns	ns	ns	ns
N x W	ns	ns	ns	ns	ns	ns	ns	ns

Table 2 - Coefficients of determination (r^2) between leaf water potentials and atmospheric parameters: Maximum air temperature (Tmax), evaporation pan (Epan), minimum air temperature (Tmin) and volumetric water content (VWC). N = 15, except for VWC were N = 10. All the coefficients were statistically significant at $p<0.05$, except those with superscript ns (non significant).

LWP	Atmospheric parameters	1.5/0	1.5/50	1.5/100	4.5/0	4.5/50	4.5/100
	Tmax	0.45	0.48	0.42	0.38	0.64	0.43
	Epan	0.50	0.55	0.41	0.49	0.75	0.53
Predawn	Tmin	0.45	0.39	0.37	0.26	0.50	0.30^{ns}
	VWC	0.71	0.54	0.24^{ns}	0.50	0.49	0.06^{ns}
	Tmax	0.81	0.73	0.83	0.66	0.82	0.71
	Epan	0.79	0.74	0.78	0.61	0.75	0.67
Midday	Tmin	0.75	0.68	0.68	0.73	0.78	0.63
	VWC	0.71	0.31^{ns}	0.22^{ns}	0.22^{ns}	0.22^{ns}	0.10^{ns}

Table 3. Significance levels of differences among treatments for branch length increment obtained from multifactorial ANOVA table using F-test. These data are related to Fig. 3. Significant at 95% (*) and 99% (**). Non-significant (ns).

Branch length increment			
Dates	**N**	**Water (W)**	**N x W**
June 91	*	ns	ns
July 91	ns	**	ns
August 91	**	**	**
Sept 91	**	**	**
April 92	**	**	**
May 92	ns	**	ns
June 92	**	ns	*
July 92	**	**	**
Aug92	**	**	**
April 93	ns	ns	ns
May 93	**	**	ns
June 93	**	**	**
July 93	ns	ns	ns
Aug93	ns	**	ns

Preliminary studies on mycorrhiza of *Ceratonia siliqua* L.

P.M. Correia and M. A. Martins-Loução

Summary - This is the first work concerning the evaluation of mycorrhizal status of *Ceratonia siliqua L.* in Portugal. This study is able to prove the existence of arbuscular mycorrhiza (AM) colonizing carob roots. No ectomycorrhizal association was found. Apparently, neither climate, soil type nor human intervention were determinant for AM infection and all the soil types tested seem to have enough inoculum to promote carob root infection.

Introduction

Carob (*Ceratonia siliqua* L.), a drought-resistant tree legume, has been a traditional crop along the shores of Mediterranean Sea, although the cultivation of this crop was long neglected. While producing a commercial crop, carob trees contribute to soil conservation and reclamation. It is a multi-purpose tree crop whose fruit may be used for food, fodder, sugar and syrup, industrial gums and alcohol. It is, thus, a high value cash crop which can also serve the goals of environmental conservation, farm diversification, and extensification of agriculture. Since it requires no manage, tolerates poor soils and is long-lived, carob trees are a valuable resource for reforestation of degraded zones where erosion and desertification are a threat. Like most Caesalpinoideae, carob tree does not nodulate and therefore it is not a nitrogen-fixing plant (4). The nitrogenous nutrient pathway need further testing and development in order to understand better the capacity of this plant to survive and produce under, apparently, deficient soil conditions.

Improved growth and crop production must be achieved to make profitable use of existing carob plantations as well as the establishment of new, modern carob orchards. So, it is important to develop modern agrotechniques for the efficient use of these orchards. Because of the fundamental importance of mycorrhiza in key ecosystem processes an understanding of the possible symbiosis is an essential prerequisite of any sustainable agricultural system. Now, the question is if this plant, being able to tolerate different stress conditions, can establish any other mutualistic association.

The objective of this work was to evaluate the mycotrophic status of carob trees in Portugal. The observations were conducted to examine if different physicochemical properties of soils, different types of carob explorations and plant age may influence the mycorrhization of carob roots.

Material and Methods

Roots of mature carob trees were collected in an orchard in Algarve (southern region of Portugal, 37° 13' N, 7° 28' W), in Arrábida, a native ecosystem (center southern region, 38° 27' N, 9° 2' W) and in the Botanical Garden of Coimbra (center northern region, 40° 12'N, 8° 25' W). Root samples were collected in Autumn and Spring at 5-20 cm depth, depending on the site. Roots of plants of different ages (6 months, 3 and 15 years) cultivated in the Botanical Garden of Lisbon were also collected and examined.

To evaluate the potential inoculum of different soils and the ability of carob to mycorrhizal infection, 4 different soils from the above mentioned regions of Portugal and 1 from Alentejo (38° 5' N, 8° 39' W) were used as a natural inoculum. The general characteristics of the site soils and climate are presented in table I. Annual rainfall is presented as a mean of 30 years. Soil pH was determined by use of a water-to-fresh-soil ratio of 5:1.

Carob seeds cv. Mulata from 1992 were used in this study. Seed dormancy was broken by putting the seeds in concentrated sulfuric acid (97%) for 15 min. After this treatment the seeds were washed and imbibed in tap water for 48 h. Seed germination took place in pots containing wet sterilized expanded clay, placed in a growth chamber with 40% of relative humidity, 200 $\mu mol\ m^{-2}\ s^{-1}$ photosynthetic photon flux density and a photoperiod of 12h at 18/24 °C (night/day) air temperature. The seeds were watered every day in order to maintain the humidity of the substrate. After developing the first pair of leaves, 20 days after the beginning of the experiment, seedlings were transferred to plastic pots with the different soils above mentioned. Five replicate pots with one seedling each, were done for each soil. These plants were maintained under greenhouse conditions. Root observations were done every month.

Table I. General characteristics of the sample site soil and climate

Site	Type of human intervention	Type of climate	Mean annual rainfall (mm)	Soil type	Organic matter (%)	pH
Algarve	Orchard	semi-arid	513	silty-clay	1.5	8
Alentejo	"Montado"	sub-humid	574	sandy	3.8	5.5
Arrábida	Semi-natural	humid	750	clay-loam	4.9	7.5
Coimbra	Garden	humid	1038	sandy	-	6.3

Roots were washed in running water, fine roots were separated and immediately observed. Stereomicroscopical observations (4-40x) were used to look for ectomycorrhiza associations and special types of root ramifications. All samples were later cleared with KOH and H_2O_2 treatments and stained in trypan blue in acidified glycerol 50%, adapted to this specific plant, according to the procedure followed by Koske and Gema (3). Arbuscular mycorrhiza were deemed present when typical highly branched arbuscules could be observed.

Incidence of mycorrhizae was rated on a scale of none (0); moderate (1 = 1-50%); and high (2 = 50-100%). The same type of scale was used for arbuscules and vesicles.

Results and Discussion

These preliminary results show no ectomycorrhiza association in this species, either in juvenile or mature plants. Independent of climate or type of soil, carob always present AM infection in a high percentage (Fig. 1, Table II). The attempt made to estimate if the climate, the type of human intervention and the type soil characteristics influence the level of mycorrhization was not found. Apparently, those conditions were not determinant for AM infection and all the soil types tested seem to have enough inoculum to promote carob root infection. The percentage of tree roots colonization

did not differ within each climatic zone and type of human intervention. The observations made with different soil inocula during seedling development suggest that chemical site properties do not influence invasion of the root cortex by indigenous AM fungi (table II). The work done by Diop et al. (2) in different climatic regions and with another legume tree also present no relation between root colonization and soil type, however they found different amount of inoculum according to the soil profile.

Table II. Evaluation of mycorrhizal infection in carob roots of seedlings and mature trees.

Tree age	AM infection	Arbuscules	Vesicles
Mature trees (15-40 years)	2	0	2
Young plants (> 3 years old)	2	1	1
Seedlings (6 month old)	1	2	0

Since the observations made on mature trees were essentially done on old roots the amount of vesicles observed were very high. The low density of more thin lateral roots on carob trees could explain the lack of observation of arbuscules on these plants. Whenever it was possible to observe very young root apices arbuscules were present at a high level.

The pot experiments with seedlings growing in the different soil types showed that after 2 months some infection was already present. However the rate of infection was lower on seedlings cultivated on clay-loam soils than on sandy soils (Table I). It was also observed that this rate of infection was dependent on air temperature, being the plants much more infected in spring than in winter. Since carob roots present a rapid lignification during development only a small portion above the root apices, where the nutrient uptake is highly intense (1), was able to be colonized by AM fungi. Far as long as these observations have been done we have no indications about any root architecture modification due to mycorrhiza infection. We only know that according to nitrogen source addition the type of ramification can change (4, 1)

Further studies are in course concerning the mycorrhizal dependency of carob. Since Mediterranean soils and especially carob orchards in the southern region of Portugal are often deficient in nutrients, mainly nitrogen and phosphorous, AM fungi may improve these conditions. This knowledge can help the development of new inoculum procedures in carob nurseries, promoting a more sustainable agriculture practices in new carob orchards.

Fig. 1. Stereomicroscopical observations of carob roots. a) higher magnification of AM infected root (x 400). b) Detail of arbuscules (x 1000).

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III Uptake regions of inorganic nitrogen in carob seedling roots.

C., Cruz, S.H. Lips and M. A. Martins Loução

Abstract

Three-week-old carob seedlings were grown for 9 weeks under different root temperatures (20, 30 and 40°C), pH (5, 7 and 9), and nitrogen concentrations (1, 2 and 3 mM) of either nitrate or ammonium. Nitrogen uptake rates were determined by its depletion from the medium.

Nitrate and ammonium uptake rates by carob roots decreased with distance from the apex. The decline of nitrogen uptake rates along the roots depended on the form of inorganic nitrogen in the medium as affected by pH and temperature. Nitrate and ammonium ions uptake took place mainly in the younger sections of the root (0-2 cm from root apex). The uptake pattern of nitrate corresponded to nitrate accumulation and in nitrate reductase activity in the root tissue pointing out the very low nitrate uptake in mature (2 -10 cm) regions of the carob seedlings root. Nitrate uptake increased with medium acidity while ammonium/ammonia uptake decreased under similar conditions. Medium pH between 5 and 9 had no effect on the pattern of nitrate uptake along the root. At pH 5, which favors the formation of ammonium ions, uptake of nitrogen was predominantly carried out through the tip and very little, if any, through mature root regions. At pH 9, which favors formation of NH_3, enhanced influx through the mature root areas was observed.

In summary, the ionized NO_3^- and NH_4^+ forms of nitrogen were taken up by carob root seedling essentially by root tips (0-2 cm) through processes requiring energy. The uncharged NH_3 species entered the root passively, through the mature parts of the root (2-10 cm). Root zone temperature and pH affect the NH_4^+ <---> NH_3 equilibrium in the nutrient solution and, consequently, the uptake areas of the root through which used by these ions. Furthermore, while root tip uptake of nitrogen is energy dependent, uptake through mature root areas is essentially passive and seems to depend on a well developed apparent free space.

Key words: Ammonium, Carob, nitrate, nitrogen, uptake.

Introduction

The precise identification and estimation of root areas active on ion uptake have been a topic of interest and controversy. Calculations of uptake rates are erroneous without a clear assessment of the root areas active in uptake. Root uptake rates are generally calculated on the basis of root fresh and dry weight although in some cases they have been expressed on the basis of root length or root surface area. All these methods assume that the entire root system is equally active in ion uptake. But the root is a well organized conglomerate of cells differing in both developmental stage and function with prominent regional differences in nutrient absorption, translocation and assimilation processes (Clarkson 1990, Lazof et al. 1992). Although variations in plant transport rates along the length of

the roots have been observed frequently (Clarkson and Hanson 1980, Luttge 1983, Romheld 1987, Henriksen et al. 1992), the topic is still a matter of polemics.

The absorption of potassium, phosphate and ammonium in barley was reported took place throughout the entire root surface (Clarkson and Hanson 1980). In contrast, the uptake of calcium has been reported to be restricted to the apical region (Mengel and Kirkby 1986). Iron uptake may take place either at the apical region or over the entire root surface, depending on plant species (Kashirad et al. 1973, Romheld 1987).

Contradictory results can be due to plant species diversity, ionic species, energy requirements of the uptake systems and a large spectrum of environmental (pH, temperature, electroconductivity, soil moisture) and developmental (plant age, vegetative/reproductive stages, relative growth rate, etc.) conditions. Structural and physiological variations along the length of the root could cause further diversity (Luttge 1983). Different methodological approaches in assessing the absorption and translocation capacities of defined root regions can also contribute to the different results obtained (Lazof et al. 1992).

Most of the studies on ion uptake have been carried out on roots of cereals and maize seedlings and the results extrapolated to the rest of the plant species. Very little has been done, however, to study uptake characteristics of tree roots such as that of the carob. Gradients of NO_3^- accumulation and nitrate reductase activity along the carob roots have been observed (Cruz et al. 1993a). The NH_4^+ uptake system in carob was reported to consist of active and passive components which are differently affected by root temperature and inhibitors. Several environmental factors such as temperature and pH affect the equilibrium between NH_3 and NH_4^+, each of which is taken up by different components of the ion uptake system of carob (Cruz et al. 1993 b) and barley (Siddiqi et al. 1990) roots. In view of this facts, it was considered that a study of the extent to which root temperature and pH affect uptake of NO_3^-, NH_3 and NH_4^+ through defined regions of the root would be useful to understand carob adaptability to environmental conditions.

The aim of this work was to determine the uptake areas of the carob root through which different forms of inorganic nitrogen (NO_3^-, NH_3 and NH_4^+) are acquired by the plant as affected by several environmental factors, such as root temperature, nitrogen concentration and pH.

Materials and Methods

Plant material

Seeds of carob (*Ceratonia siliqua* L. cv Mulata) harvested in 1992 were obtained from AIDA (Associação Interprofissional para o Desenvolvimento da Produção e Valoração da Alfarroba, Algarve, Portugal) and used in this study. Seed dormancy was broken by placing the seeds in concentrated sulfuric acid (97%) for 15 min. The seeds were then washed and imbibed in tap water for 48 h. Seed germination and plant growth took place in pots containing wet sterilized expanded clay inside a growth chamber with 50-70% relative humidity, a photosynthetic photon flux density of

550 µmol m^{-2} s^{-1} (from a mixture of 7 lamps Philips TLD 18W/84, England, and 1 Sylvania GRO-Lux IF 18 W/GRO, Germany) and a photoperiod of 12 h at 20/24 °C (night/day). The seeds were watered every three days in order to maintain the humidity of the growth substrate. After developing the first pair of leaves, 15 days after the beginning of the experiment, seedlings were transferred to plastic pots (3 seedlings per pot) containing 1.5 l water. Root temperature was kept at 20, 30 or 40°C by thermostatically controlled circulation of hot or cold water around the pots.

One week after their transfer to pots, the seedling cotyledons were removed and the plants transferred to a modified Crone solution (Hewitt 1966) containing: 3 mM $CaSO_4 \cdot 2H_2O$, 2 mM $MgSO_4 \cdot 7H_2O$, 1 mM $Ca_3(PO_4)_2$ and 1 µM EDTA-Fe, at 3 different pH values (5,7 or 9), with (+N) or without (-N) nitrogen for 9 weeks, under the conditions described above.

Nitrogen added to the nutrient solution was either NO_3^- or NH_4^+ at a final concentration of 1, 2 or 3 mM. Plants growing in presence of KNO_3 or $(NH_4)_2SO_4$ received KCl in a way that K concentration in the root medium was maintained constant, independently of the nitrogen concentration in the nutrient medium. The nutrient solutions were aerated vigorously and replaced twice a week.

Experimental procedures

Nitrogen uptake through defined root regions was studied with roots of carob seedlings grown as described above. All the experiments, with the exception of determination of uptake rates of nitrogen by (-N) seedlings, were performed between 10 a.m. and 14 p.m.. The roots were rinsed in water and cut into 1 cm segments at the beginning of the experiments. Five different groups of root segments were prepared, according to distance (cm) from the root apex: 0-1; 1-2; 2-4; 4-10 and >10 cm. Similar root segments obtained from 20 different plants were mixed, a sample of 1 g of segments was weighed and transferred to petri dishes containing 10 ml of 25% Crone solution. Nutrient solutions were gently aerated to maintain optimal oxygen supply and to prevent formation of boundary layers around the root segments. Forty petri dishes were prepared for each group of root sections. Every 30 min., during 2 h, 10 petri dishes were collected and solution volumes and nitrogen concentrations of the medium were determined. Each treatment was repeated, at least, 4 times. The reported values are the mean of the results obtained.

Intact plants grown without nitrogen (-N) at 20 or 40°C were exposed to nutrient Crone solution with 3 mM NO_3^- or NH_4^+. All the other growth conditions were kept constant. Uptake experiments were carried out with root segments, as described above at 0 time (addition of nitrogen to the medium) and after 5 and 15 h.

Measurements of nitrogen uptake

Determination of net uptake rates of NO_3^- and NH_4^+ were based on nitrogen disappearance from the solution per unit time. The rates were expressed as µmol g^{-1} root fresh weight h^{-1}. Rates were

calculated from the changes in nitrogen concentration and solution volumes at consecutive sampling times.

Nitrate reductase activity

Nitrate reductase was extracted by maceration in a chilled mortar with 0.2 g acid washed sand and 1:8 (w/v) ratio of the following medium: 0.1 M phosphate buffer pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 2.2. % of water soluble casein (Lewis et al. 1982).

The extract was filtered through 2 layers of gauze and used immediately. Nitrate reductase was assayed for 20 minutes at 27 ° in the medium described by Bar-Akiva and Sagiv (1967). The final volume of the reaction medium was 2 ml. The reaction was stopped by the addition of 30 mmol phenazine methasulphate (PMS) in order to oxidize any residual NADH in the reaction medium (Scholl et al. 1974). PMS was allowed to react for 20 minutes before determination of nitrite. A calibration curve based of nitrite in the assay medium without NADH was used.

Glutamine synthetase (GS) activity

The GS assay was based on the method described [illegible] (1985). Crude extracts were obtained by homogenizing the tissues in 0.05 M imidazole-HC[illegible] 7.2, 1 mM EDTA, 1 mM mercaptoethanol and 1 mM dithiotreitol. Extracts were filtered through 8 layers of gauze and then centrifuged at 10,000 g for 15 min. GS activity in the supernatant was assayed immediately. The reaction mixture contained: 36 µmol ATP, 90 µmol $MgSO_4$, 12 µmol hydroxylamine, 184 µmol L-glutamate and 100 µmol imidazole-HCl, pH 7.2. The final volume of the reaction mixture was 2 ml. The reaction was initiated by the addition of extract and incubated at 30° for various time periods. The reaction was stopped by the addition of 1.0 ml of ferric chloride reagent (0.37 M ferric chloride, 0.67 M HCl and 0.2 M trichloro acetic acid, TCA) which forms a brown compound with any glutamyl-hydroxamate formed and precipitates out the enzyme protein. The reaction tubes were then centrifuged at 20,000 g for 10 min. to precipitate protein and the absorbency was read in a spectrophotometer (PU 8620 UV/VIS/NIR, Philips Scientific and Industrial equipment, Cambridge, England) at 540 nm. A standard curve of glutamyl hydroxamate was prepared with concentrations up to 3 µmol per 1.0 ml of the assay mixture.

Chemical analyses

Nitrate was determined following its reduction to nitrite (McNamara et al. 1971) and nitrite determined according to Snell and Snell (1949). Ammonium was determined by the blue indophenol reaction (Solozano 1969).

Results

Nitrate ions were preferentially taken up through the youngest 10 cm of the roots of carob seedlings. Nitrate uptake rates in the youngest 2 cm of the root were several fold larger than the rates observed in the next 8 cm (Fig. 1). The pattern of nitrate uptake along the roots did not significantly change when nitrate concentration in the medium was increased from 1 to 3 mM indicating the near saturation of the uptake system involved. Increasing root temperature from 20 to 40°C lead to a general increase of nitrate uptake rates without a significant change on the relative uptake activity of the root areas studied.

Contrary to the limited effect of temperature on net nitrate uptake by the root areas studied, net ammonium/ammonia uptake was extensively affected. At 20°C the rate of ammonium/ammonia uptake in the first cm was 3-4 times higher than that observed in the oldest parts of the root (Fig. 1). However, uptake of reduced nitrogen along the root became very similar at 40°C. Nitrate and ammonium/ammonia were preferentially taken up by the youngest 10 cm of the root, contrary to the very limited uptake by root sections from the area above 10 cm from the root apex (Table 1).

Nitrate uptake rates on the youngest two cm of the roots were slightly higher than the uptake rates of ammonium/ammonia (Fig. 1), while in the mature parts (> 10 cm from the root tip) ammonium/ammonia uptake rates were significantly higher than those of nitrate. These differences became more pronounced as the temperature of the medium increased.

Nitrate and ammonium concentrations in root tissue increase with root temperature (Fig. 2). Seedlings accumulated nitrate preferentially in the youngest part of the roots (0-2 cm) while ammonium accumulated along the entire root, independently of temperature. The accumulation of nitrate and ammonium at 20°C root temperature was similar while at 40°C ammonium accumulation was higher than that of nitrate, especially in the more mature areas of the root (Fig. 2). In other words, temperature did not affect the pattern of uptake of nitrate along the roots, while it had a profound effect on ammonia/ammonium uptake (Fig. 1).

The pH of the medium affected both nitrate and ammonium/ammonia uptake (Fig. 3). Nitrate uptake rates in different root sections decrease with increasing pH. Uptake by the first cm of the root at pH 9 was 70 % of the uptake rate observed at pH 5. A decrease of similar magnitude was observed for the other root sections. Contrary to the response of nitrate uptake to increasing pH, ammonium/ammonia uptake increase with pH. The pH-induced increase of ammonium/ammonia uptake was 30 % in the youngest parts of the roots and 300 % in the oldest root sections (Fig. 3). This changes resulted on a fairly equal uptake of ammonium/ammonia by all root sections at pH 9, while at pH 5 uptake was maximal in the youngest 1 cm section, declining rapidly with root maturity.

Following the initial exposure to nitrate (0 time), nitrate uptake in the different root sections of (-N) seedlings increased with the distance from the root tip (Fig. 4). Five hours later, the uptake rates along the entire root were very low. After 15 h the nitrate uptake rates by the youngest root

sections (0-2 cm) reached 8-9 μmol g^{-1} fresh weight h^{-1}. The rest of the root was increasingly inactive in nitrate uptake. Increasing root temperature from 20° to 40° caused a small increase on nitrate uptake rates although the relative uptake by root sections remained essentially unchanged.

Ammonium/ammonia uptake patterns along the root at 20 or 40°C were very different (Fig. 5) from that of nitrate. Uptake followed the general pattern described for nitrate at 20°C, with a clear preferential uptake through the youngest 2 cm of root. There were no significant differences between ammonium/ammonia uptake rates at 5 or 15 h after the initial exposure to ammonium at 40°C. A considerable increment of ammonium/ammonia uptake rates took place in the oldest root parts at 40°, dissipating the marked uptake of the young 2 cm root sections observed at 20°. Ammonium/ammonia uptake rates decreased only 18% from the root tip to the 10 cm segment at 40°C, while at 20 ° they decreased 62%.

In both nitrate and ammonium fed carob seedlings GS activity increased with root temperature and was higher in ammonium than in nitrate fed seedlings. There was a slight loss of the enzyme activity with distance from the root apex (Fig. 6), a pattern more evident in nitrate than in ammonium fed seedlings.

NR activity was clearly highest in the youngest 2 cm of root (Fig. 7), corresponding to nitrate uptake by different root sections. The increment in nitrate reductase activity due to an increase in root temperature was more noticeable in the youngest part of roots. As it would be expected nitrate reductase activity of ammonium fed seedlings was very low.

Discussion

Uptake rates of nitrogen in carob root decreased with age or with the distance from the apex. The magnitude of the decrease depended on the form of inorganic nitrogen used. Nitrate uptake was clearly carried out preferentially by the 2 cm tip section than the gradient for ammonium/ammonia uptake along the root (Fig. 1). This was due to the fact that NH_4^+ is in equilibrium with NH_3 and while NH_4^+ is taken up actively principally by the youngest root zones (0 -2 cm) of the growing root, NH_3 enters the root by passive diffusion mainly through the more mature sections of the root. Consequently, at a low pH which favors formation of the NH_4^+ ion species, uptake takes place preferentially at the 2 cm tips, while a higher pH which enhances the presence of NH_3

Measurements of net fluxes of nitrate and ammonium/ammonia along the roots can be carried out using several techniques (Henriksen et al. 1992).

The use of root sections to determine changing uptake rates along the roots has several disadvantages: (1) there may be an efflux of nitrogen to the root medium yielding underestimations of the uptake rates; (2) cutting the roots segments increases the area of the root that is exposed to the root medium.

Carob showed a steep gradient of nitrate concentrations along the roots (Fig. 2) with the highest concentrations in the first 2 cm decreasing very drastically along the roots. If this is so, the efflux of nitrate during measurements will be higher in the 2 youngest cm of the root than in the other root parts. Therefore, the uptake rates determined along the roots (Fig. 1) implied a larger underestimation in the youngest part of the roots than on the other root parts. Changes on the area of exposure to the root medium were similar for all root sections since the length of all segments was identical (1 cm) and root diameter changed very slightly. Taking in consideration the maturation of the cells along the root axis it could be expected that the increase in the size of vacuoles and the maturation of the xylem vassels would increase the efflux of nitrate in more mature root segments. Cruz et al (199?) have shown that carob roots offer a large resistance to nitrate transport through the xylem, a fact which determines that most of the nitrate reduction and assimilation in carob takes place in the roots and not in the shoot.

NH_4^+ uptake was reported to take place along the entire root length (Bowling 1973). We conclude that the NH_4^+ ions are taken up preferentially by the tip 2 cm section of the root, while NH_3 is taken up passively along the entire mature root. This conclusion is based by the fact that uptake patterns of NH_4^+/NH_3 along the root are largely affected by factors shifting the equilibrium between NH_3 and NH_4^+ such as temperature and pH (Figs 1 and 5). Ammonium uptake rates along the root at 20°C which favors equilibrium towards NH_4^+ shows preferential uptake by root tips while at 40°C, due to a shift of equilibrium in favor of NH_3, uptake at fairly similar rates takes place along the entire root. Results obtained for net influx rates by the entire root system (Cruz et al. 1993 b) showed a large increase of ammonium/ammonia influx by plants grown at 40°C root temperature for 3 months. The increase in ammonium/ammonia uptake rates at 40°C was due mainly to an increase of the ammonia ion species in the solution leading to its enhanced passive influx through the more mature parts of the root (Fig. 1), richer in apparent free space.

There seems to be a correlation between the development of the AFS with root maturation and the capacity of NH_3 to diffuse into the root. The AFS of the carob root becomes evident at 0 time (Figs. 4 and 5). After 5 h the AFS is saturated and no further uptake of ionized NH_4^+ or NO_3^- was observed at 20°. As we indicated in a previous paper, active uptake systems for ammonium and nitrate seem to be fully induced after about 15 h in the carob root. The developing AFS in mature areas of the root may be a key factor on the enhanced NH_3 diffusion into the maturing root sections of the carob root. AFS development with root maturation may be the result of anatomical changes in the root which allow enhanced exposure of electrostatically charged intracellular surfaces which do not hinder diffusion of neutral compounds such as NH_3. The distribution of inorganic uptake areas of root of the carob seedlings are schematically described in figure 8.

One of the advantages of nitrate as a nitrogen source is that this anion can be accumulated in the vacuole while ammonium can not. Ammonium has to be assimilated as soon as it is taken up, otherwise it can have toxic effects (Mehrer and Mohr 1989, Lips et al. 1990). Ammonium/ammonia uptake at 40 °C was not accompanied by a proportional increase in glutamine synthetase activity (Fig.

6), which may have facilitated ammonia diffusion into the root tissue (Fig. 2). This fact supports our hypothesis that the enhanced influx of reduced nitrogen at 40°C is a result of the increased concentration of the diffusible species of ammonia. The high levels of unassimilated ammonium/ammonia observed at 40°C may have been responsible for the frequent death of the plants observed at this temperature (Cruz at al. 1993 c).

Nitrate uptake rates by the youngest 2 cm carob root segments were higher then those of ammonium uptake rates. This seems to contradict apparently the believe that ammonium uptake rates are larger than those of nitrate (MacDuff and Wild 1989, Cruz et al. 1993b). However the contribution of the root tips to the total nitrogen uptake is relatively small (Table 1), when compared to the ammonium/ammonia uptake by root zones between 2 and 10 cm from the root tip. In this region ammonium/ammonia uptake is higher than nitrate uptake and so it contributes more to the overall uptake rates of the roots, especially when uptake rates are calculated on a total root system basis. Our present interpretation of the results shown is that both ammonium and nitrate ions are actively taken up through the youngest root regions (0 to 2 cm), while passive uptake of NH_3 takes place through the more mature areas of the root (2 to 10 cm) as described in Fig. 8.

Nitrate and ammonium are two of the most abundant nitrogen sources available to plants. However, plants differ in their preference for nitrate or ammonium. Carob plants grow better in hydroponic solutions containing ammonium than nitrate (Martins-Loução and Duarte 1987). Differences in the root morphology of nitrate and ammonium fed plants (Silberbush and Lips 1988) may be the result of adaptation to growth conditions, namely to the nitrogen source available. Carob seedlings grown with ammonium/ammonia produce a root system with more branches and more root tips than nitrate fed plants, contrary to wheat plants which prefer nitrate (Silberbush and Lips, 1991). The wheat root system developed in the presence of ammonium shorter roots and more root tips, allowing a better control of ammonium/ammonia uptake because the relation between the areas of passive uptake of NH_3 and active NH_4^+ uptake decrease. Nitrate induces the formation of short and very branched roots in plants that grow better in nitrate than in ammonium (Ganmore-Neuman and Kafkafi 1980, Lips et al. 1990) where .On carob plants, which are forced to reduce nitrate in the root due to their very limited ability to transport nitrate to the shoot, nitrate induces the formation of longer and less branched root systems than ammonium.

Nitrate uptake rates by the youngest part of carob roots are higher than these of ammonium. This could be in contradiction with the idea that ammonium uptake rates are larger than nitrate uptake rates (MacDuff and Wild 1989, Cruz et al. 1993b). However, the contribution of the root tips to the total nitrogen uptake is quite small, when compared to the contribution of the areas between 2 and 10 cm from the root tip (Table 1). In this region ammonium/ammonia uptake is higher than nitrate uptake in the mature sections of the root (Table 1) which may be related to passive influx of NH_3. Differences in root morphology of nitrate and ammonium fed plants (Lips et al. 1990) can also be another contributing factor for these results. Ammonium fed carob plants also have more lateral branches and more root tips than nitrate fed carob seedlings, which produce longer roots. One may

conclude that shorter and more branched root systems favor active uptake of nitrate and ammonium due to a larger proportion of root tips, while long unbranched roots favor passive uptake of NH_3.

The observed gradients in nitrate content and in nitrate reductase activity in the root tissue (Figs 2 and 7) indicate the low capacity of nitrate uptake in mature (2 -10 cm) regions of the carob seedlings root. Nitrate uptake increased with the acidity of the medium (pH 5) while ammonium/ammonia uptake decreases (Fig. 3). Increasing medium pH to 9 had no effect on the relative nitrate uptake rates by different root areas. At pH 5, which favors the formation of ammonium, uptake of reduced nitrogen was preferentially through the tip, rapidly decreasing in more mature areas of the root. At pH 9, which shifts equilibrium towards NH_3, the uptake gradient observed at pH 5 disappeared, showing enhanced influx through the mature root areas. This can be related to the effect of pH on the NH_4^+<----> NH_3 equilibrium in the nutrient solution (Raven et al. 1992).

The changing patterns of nitrate and ammonium/ammonia uptake with time after the initial exposure to nitrate and ammonium (Figs. 4 and 5), suggests the presence of an inductive or slowly activated uptake component. The patterns of nitrate uptake at 20 or 40°C root zone temperature were similar:

(a) Uptake during the first h (0 time) took place mainly in the older parts of the root. This initial uptake represents the filling of apparent free space (Cruz et al. 1993b) that, due to root morphology, is higher in the most mature parts of the roots.

(b) Five hours after exposure of the (-N) plants, net nitrate uptake rate is very low along the youngest 10 cm of the root. This may correspond to the moment at which the apparent free space (AFS) is already saturated and the process of induction/activation of a nitrate uptake system has not been fully expressed.

(c) Ten hours later (15 -16 h after initial exposure to nitrate) high nitrate uptake rates were observed in the young (0 -2 cm) but were low in the older (2 - 10 cm) root segments. The active uptake system of the carob root seems to be located preferentially in the youngest 2 cm tips. The time schedule for the development of nitrate uptake components is in agreement with previous results obtained for carob seedlings grown under similar conditions (Cruz et al. 1993b).

Ammonium uptake by (-N) plants at 20° was similar to that of nitrate (Fig. 5), presumably due to the fact that both nitrogen ions were actively taken up through the active uptake system of young 2 cm tips. Ammonium/ammonia uptake was drastically changed at 40°(Figs 4 and 5). The ammonium/ammonia uptake rates at 5 or 10 h after the initial exposure to ammonium were high at young root segments but also along the root length studied. This influx is due to passive diffusion of NH_3, whose formation was favored at the high temperature. This NH_4^+ active uptake component becomes marginal with increased temperature and pH, when the equilibrium NH_4^+<---> NH_3 shifts in favor of NH_3. At 40°C root temperature or pH 9, the entire root obtains NH_4^+ and NH_3 which

enter to the root through different regions and are mediated by different mechanisms: active NH_4^+ uptake through the young root tip and passive NH_3 influx through the mature root regions.

We propose that the young 2 cm tip of the root, in which the highest uptake rates of nitrogen were observed (Fig. 1), NO_3^- and NH_4^+ uptake are mediated by active transport systems which requires respiratory energy (Cruz et al. 1993a). These components of nitrate and ammonium uptake appears only about 15 h after the initial exposure of the roots to nitrogen (Figs 4 and 5). These uptake systems are located mainly in the 2 cm tips and decrease along the root becoming negligible after the first 10 cm.

The second uptake component providing nitrogen to the root is related to root areas with a developed AFS. Influx of nitrogen through this mature area of the root is mainly in the form of NH_3, insensitive to respiratory inhibitors (Cruz et al. 1993 a). As it would be expected the adsorption capacity of the AFS is higher for ammonium than for nitrate due to the presence of more negative than positive charges in this space (Marschner 1986).

In summary, we can say that this set of experiments shows that there a clear specific localization of active and passive uptake mechanisms of nitrogen ions. Environmental conditions such as pH and root temperature may permit a relative larger expression of either one of these uptake components.

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Table 1

Contribution of the different root parts for nitrogen uptake 20 h after their first exposure to either nitrate or ammonium. Nitrogen uptake rates were determined by nitrogen depletion in the medium during 4 consecutive periods of 30 min. Each experiment consisted of 10 replicates. The values in the table are the mean of 10 results obtained ± s.d.

Distance from root tip (cm)	% fresh weight	N uptake (mol g^{-1} fresh weight h^{-1})	% uptake
		NH_4^+	
0-1	2.66– *0.21*	13.14– *1.02*	9.95– *0.45*
1-2	3.32– *0.26*	13.34– *1.02*	12.51– *1.02*
2-4	10.37– *1.02*	5.05– *0.45*	14.93– *1.23*
4-10	34.97– *2.30*	5.20– *0.07*	51.95– *3.21*
>10	48.67– *3.21*	0.767– *0.02*	10.66– *1.23*
		NO_3^-	
0-1	3.18– *0.10*	14.74– *1.02*	11.24– *1.23*
1-2	4.11– *0.02*	14.03– *1.02*	14.44– *1.34*
2-4	10.87– *1.02*	5.34– *0.32*	14.55– *1.23*
4-10	36.47– *4.23*	5.55– *0.32*	50.52– *2.34*
>10	45.35– *2.34*	0.82– *0.07*	9.26– *1.23*

LEGENDS

Fig. 1. Nitrogen uptake rates along carob roots in the presence of different nitrate or ammonium concentrations (■ - 1 mM, ⊡ - 2 mM and □ - 3 mM), and at different root temperatures (20, 30 or 40 °). Vertical bars represent the standard deviation.

Fig. 2. Nitrate or ammonium concentration along carob roots in presence of nitrate or ammonium (3 mM), respectively (■ - nitrate, ⊡ - ammonium). Root temperatures were 20 and 40°C. Vertical bars represent the standard deviation.

Fig. 3. Nitrogen uptake rates along carob roots in the presence of nitrate or ammonium (3 mM) at different root medium pH (■ - pH 5, □ - pH 7 and □ - pH 9). Root temperatures were 20 and 40°C. Vertical bars represent the standard deviation.

Fig. 4. Nitrate uptake rates along carob roots of 12-week-old starved carob seedlings grown at different root temperatures (20 or 40°C). Nitrate uptake rates were estimated at 0- □ , 5- ■ and 15 - O h after initial exposure of the roots to nitrate. Vertical bars represent the standard deviation.

Fig. 5. Ammonium uptake rates along carob roots of 12-week-old starved carob seedlings grown at different root temperatures (20 or 40°C). Ammonium uptake rates were estimated at 0- □ , 5- ■ and 15 - O h after initial exposure of the roots to ammonium. Vertical bars represent the standard deviation.

Fig. 6. Glutamine synthetase (GS) activity along carob roots grown with nitrate or ammonium (3 mM) at different root temperatures (20 - ■ and 40°C - ⊡). Vertical bars represent the standard deviation.

Fig. 7. Nitrate reductase (NR) activity along carob roots of seedlings grown with nitrate or ammonium (3 mM) for 3 months at different root temperatures (20 - ■ and 40°C - ⊡). Vertical bars represent the standard deviation.

Fig. 8. Schematic representation of inorganic nitrogen uptake components of nitrate and ammonium uptake in carob seedlings roots.

Figure 1

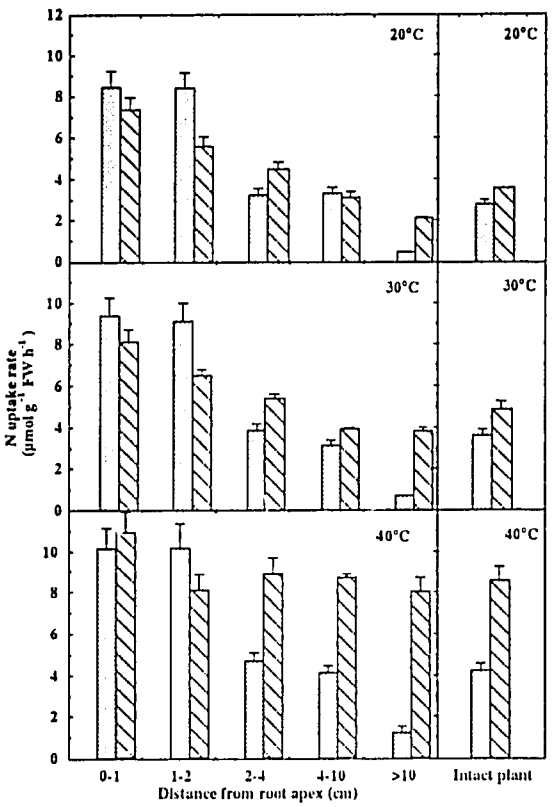


Fig 1

Figure 2

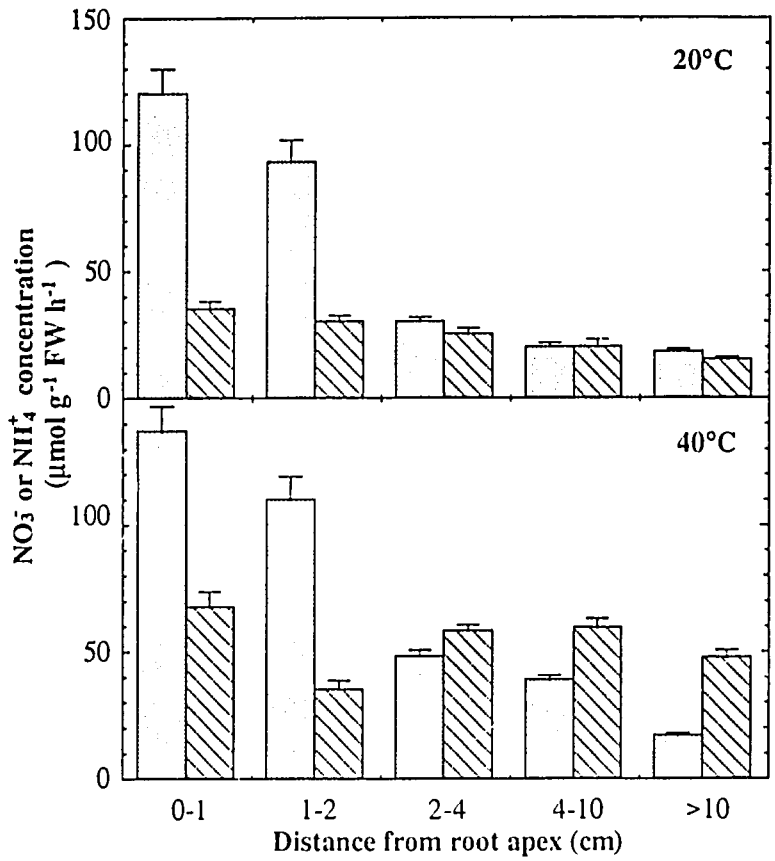

150
100
50
0
20°C
40°C
NO_3^- or NH_4^+ concentration
(µmol g^{-1} FW h^{-1})
0-1
1-2
2-4
4-10
>10
Distance from root apex (cm)

Figure 3

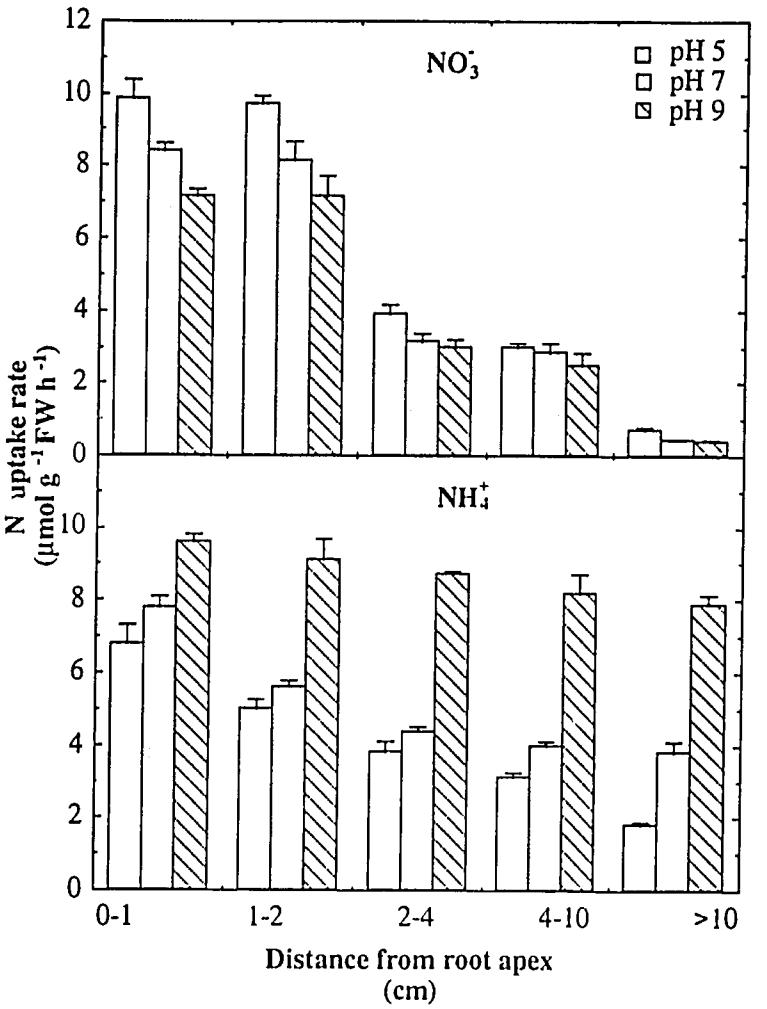

NO3-
pH 5
pH 7
pH 9
NH4+
N uptake rate
(µmol g-1 FW h-1)
0
2
4
6
8
10
12
0-1
1-2
2-4
4-10
>10
Distance from root apex
(cm)

Figure 5

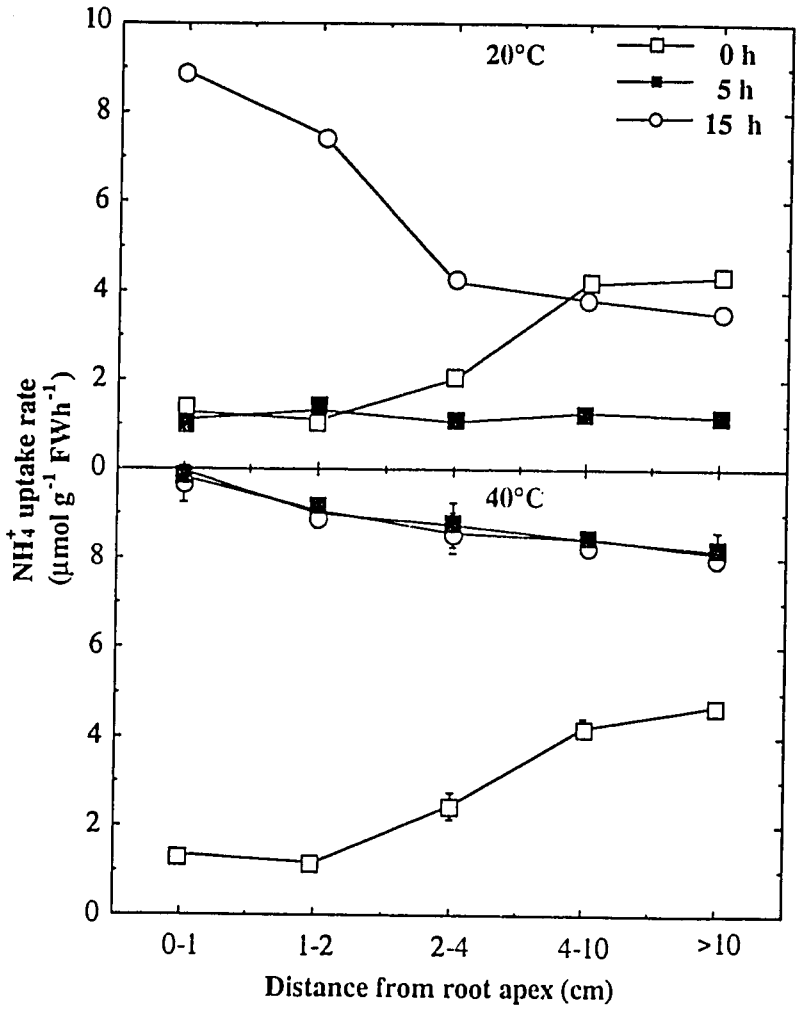

20°C
0 h
5 h
15 h
40°C
NH_4^+ uptake rate
(µmol g^{-1} FWh^{-1})
10
8
6
4
2
0
0-1
1-2
2-4
4-10
>10
Distance from root apex (cm)

Figure 6

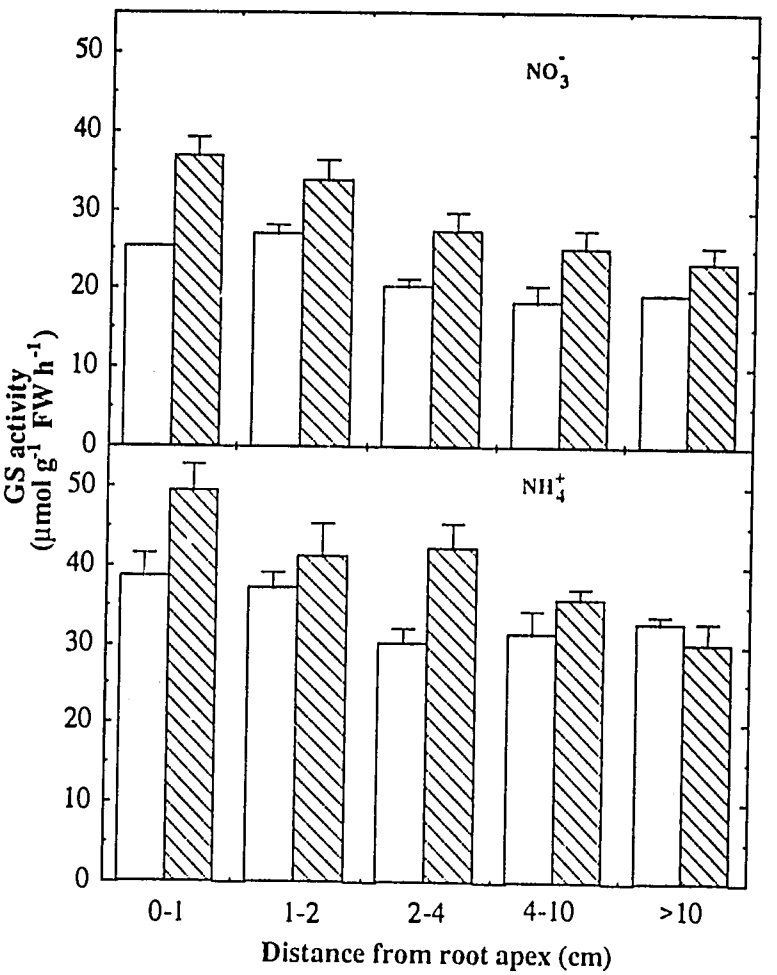

GS activity
(µmol g-1 FW h-1)
NO3-
NH4+
0
10
20
30
40
50
0-1
1-2
2-4
4-10
>10
Distance from root apex (cm)

Figure 7

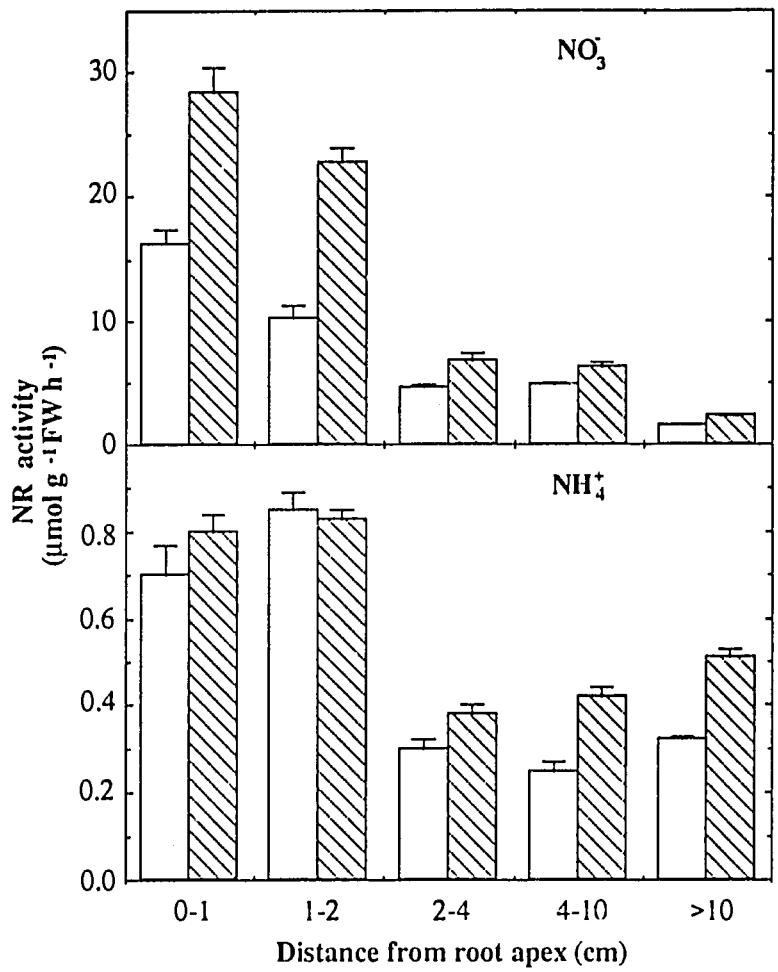
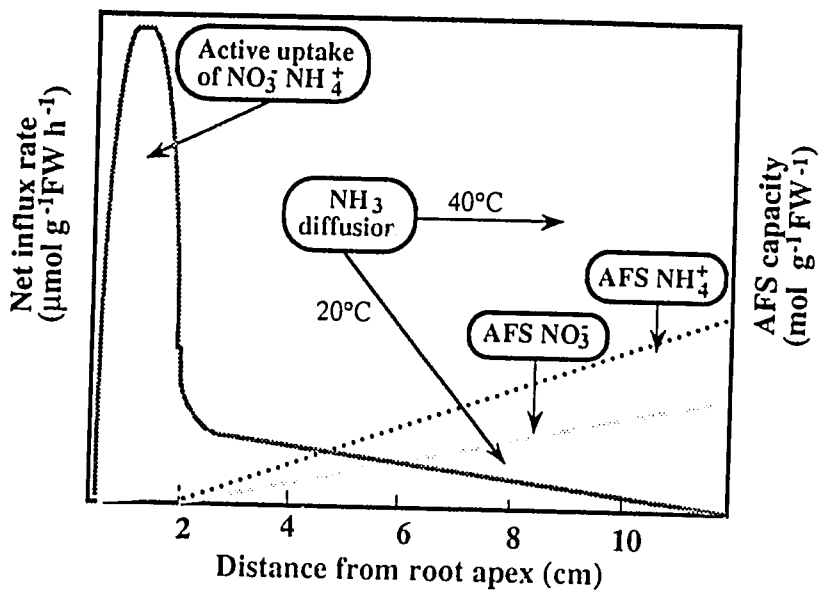

NO3-
NH4+
NR activity
(µmol g-1FW h-1)
30
20
10
0
0.8
0.6
0.4
0.2
0.0
0-1
1-2
2-4
4-10
>10
Distance from root apex (cm)

Figure 8


Active uptake of NO_3^- NH_4^+
NH_3 diffusion
40°C
20°C
AFS NH_4^+
AFS NO_3^-
Net influx rate (μmol g^{-1}FW h^{-1})
AFS capacity (mol g^{-1}FW^{-1})
2
4
6
8
10
Distance from root apex (cm)

IV Nitrogen use efficiency in carob as affected by atmospheric CO_2 concentration and nitrogen source

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Abstract.

Plants grown in air with elevated CO_2 levels show faster growth rates and lower tissue nitrogen concentrations than plants grown in ambient CO_2. Nitrogen tissue concentration was highest in seedlings and decrease during subsequent growth making comparisons between plants growing at different rates, such as plants growing in ambient and elevated CO_2 atmospheres, more difficult to compare. Differences in nitrogen tissue concentration may be the result of either growth rates or different nitrogen assimilation process, each or both of them affected by CO_2 atmospheric concentration. Rather, this comparison may highlight differences in tissue nitrogen concentration between bigger, more developed plants and smaller, less developed plants. In this study we examined whether elevated CO_2 environments reduce plant nitrogen concentrations independently of changes in growth rates.

Introduction

There is substantial evidence that anthropogenic activities such as the combustion of fossil fuels and the burning of forests are increasing the atmospheric CO_2 concentration of the Earth (Clark, 1982; Rotty & Marland, 1886; Houghton, 1988). The present CO_2 concentration of 350 cm^3 m^{-3} is rising at a rate of 1.2 cm^3 m^{-3} per year and is expected to have doubled by the mid- to late 21st century (Conway et al., 1988; Watson et al., 1990). Even if emissions of CO_2 could be kept at current rates, atmospheric CO_2 would increase to about 450 cm^3 m^{-3} by the year 2050 and to 520 cm^3 m^{-3} by the year 2100 (Watson et al., 1990; Retuerto & Woodward 1993). These rising levels of CO_2 will likely have substantial direct and indirect effects on terrestrial ecosystems. Plant responses to elevated CO_2 atmospheric concentrations have been extensively investigated (Strain & Bazzaz, 1983; Bazzaz, 1990; Woodward et al, 1991). Nevertheless the positive and the negative biological feedback in response to CO_2 and the ways in which other environmental factors interact with CO_2 are partially known.. Ackerly et al. (1992) concluded that plant responses to CO_2 are not easily predictable since they depend on interactions with other environmental factors and on the plant species, emphasizing the importance of studying multiple factors simultaneously . Some researchers suggest that since CO_2 is the primary substrate for photosynthesis, increased CO_2 levels may act as a fertilizer and augment plant growth (e.g. Idso, 1984, 1986; Kimball, 1986, Arp & Drake, 1991). Others stress that in natural environments, limited supplies of other plant resources, such as light, nutrients and water, may likely reduce the potential growth enhancement due to increased atmospheric CO_2 (Kramer, 1981; Strain & Bazzaz, 1983; Cure & Acock, 1986; Jarvis, 1989; Bazzaz, 1990; Hunt et al., 1991; Woodward et al., 1991; Bazzaz & Fajer, 1992; McConnaughay, Berntson & Bazzaz, 1993). The knowledge of the responses of the biota to these interactions will be useful to attempt predicting the ecological consequences of the expected change in atmospheric CO_2 levels since, in

spite of timing uncertainties, plants will be living in an environment richer in CO_2, regardless of climate changes (Retuerto & Woodward, 1993). The responses of trees, which cover 1 third of the surface of the earth (Van Oosten, Afil & Dizengremel, 1992), facing elevated CO_2 levels is thus of significant interest (Kramer, 1981).

One of the documented effects of elevated CO_2 is the change of plant tissues composition, particularly of their carbon and nitrogen content (Wong, 1979; Norby et al, 1986; Larigauderie et al, 1988; Brown, 1991; Kuehny et al., 1991). Reductions of tissue nitrogen concentrations in plants grown at high atmospheric CO_2 might indicate physiological changes in the efficiency with which plants use nitrogen to gain biomass or their nitrogen use efficiency (Norby et al., 1986; Hilbert et al, 1991). It is unclear whether the observed increases in plant growth due to high CO_2 atmospheres are sustainable without concomitant increases in nitrogen availability (Eamus & Jarvis, 1989; Bazzaz, 1990). If declines in tissue nitrogen concentrations are a result of increased nitrogen use efficiency, the likelihood that nitrogen availability would severely limit plant growth in high CO_2 environments would be greatly reduced.

Changes in C/N ratio of the natural vegetation could have a profound impact on ecosystem performance, affecting nitrogen use efficiency (Field & Mooney, 1986), herbivory (Scriber, 1984) and nutrient cycling (Melillo et al 1990). Limited data available suggest that C/N ratios of C_3 species could increase substantially with increasing atmospheric CO_2 concentrations (Curtis, Drake & Whigham, 1989).

Carob seedlings (Ceratonia siliqua L.) increased their biomass production under high CO_2 concentrations although the magnitude of the response depended on root temperature and on the nitrogen form available in the root medium (Cruz, Lips & Martins-Loução, 1993 a). In view of this it seemed of interest to study the effect of temperature and nitrogen source on the nitrogen use efficiency of carob seedlings under high CO_2.

Materials and Methods.

Growth conditions

Seeds of carob (Ceratonia siliqua L. cv Mulata) from collected in 1991, were obtained from AIDA (Associação Interprofissional para o Desenvolvimento da Produção e Valoração da Alfarroba, Algarve, Portugal) for use in this study. Seed dormancy was broken with applications of concentrated sulfuric acid (97%) for 15 min. After this treatment the seeds were washed and imbibed in tap water for 48 h. Seed germination took place in pots containing wet sterilized expanded clay in a growth chamber with 50-70% relative humidity, a photosynthetic photon flux density of 550 µmol m^{-2} s^{-1} (obtained with a mixture of 7 lamps Phillips TLD 18W/84, England, and 1 Sylvania GRO-Lux IF 18 W/GRO, Germany) and a photoperiod of 12 h at 20/24 °C (night/day) air temperature. The seeds were watered every three days in order to maintain the humidity of the substrate. After developing the first pair of leaves, 15 days after the beginning of the experiment, the cotyledons were

removed and every 3 seedlings were transferred to plastic pots containing 1.5 dm^3 of water. The experiment consisted of 20 pots per treatment with three replicates per treatment, placed in growth chambers (Fitotron, Aralab, Lisb , Portugal) equipped with automatic control of CO_2 concentration in the air.

Carbon dioxide levels in the growth chambers, ambient (360 cm^3 m^{-3}) and enriched (800 cm^3 m^{-3}), were automatically sampled every 2 min and monitored by an infrared gas analyzer (IRGA, Guardian II, Edinburgh Sensors Ltd, Edinburgh, Scotland). Drops from the desired level in CO_2 concentration were corrected by IRGA-controlled injections of CO_2 into the chamber. The added CO_2 was introduced into an air circulation duct at the back of the chamber, mixed with the air stream, and led into the plant chamber. The rate of air recirculating was such that uniform CO_2 concentrations were assured in the chamber and maintained within ± 20 cm^3 m^{-3} of the desired levels.

One week the water in the pots was replaced with Crone solution (Hewitt, 1966) containing 3 mol m^{-3} $CaSO_4 \cdot 2H_2O$, 2 mol m^{-3} $MgSO_4 \cdot 7H_2O$, 1 mol m^{-3} $Ca_3(PO_4)_2$ and 1 mol m^{-3} EDTA-Fe. Plants growing in presence of 3 mol m^{-3} KNO_3 received 7 mol m^{-3} KCl and plants growing with 1.5 mol m^{-3} $(NH_4)_2SO_4$ received 10 mol m^{-3} KCl. The nutrient solutions were aerated vigorously and replaced twice a week. The final volume of the old nutrient solution was measured during solution changes .Nitrate or ammonium in the nutrient solutions was determined before and after solution changes.

Seedlings were grown for 90 days under these conditions. Sample plants(3 pots with 3 plants each) were collected every 2 weeks, roots and shoots were harvested separately and the plant material analyzed for nitrogen fractions (organic, inorganic and total) and biomass production.

Photosynthesis

Photosynthesis was measured with a portable infrared gas analyzer (LCA-2, manufactured by ADC, Hoddesdone, England) based on an open air circuit system. Measurements were taken on the youngest fully expanded leaf, between 10.00 and 12.00 h. Leaf area was determined using a Li-3000 (Lambda Instruments Inc., Lincoln, NE) portable leaf area meter. Measurements were made on 5 plants per treatment during the last 4 days prior to harvest. The results presented are the mean of the values obtained for each treatment.

Nitrate reductase activity

Nitrate reduction in vivo was assayed as described by Soares et al. (1985 a). Fully expanded leaflets or roots of several 15 week-old seedlings, grown as described above, were cut with a new razor blade into 0.5 cm segments and thoroughly mixed to minimize variability in nitrate content of plant segments. Tissue samples weighing 0.3 to 0.5 g were placed in nylon net bags, submerged in the infiltration solution and subjected to a vacuum of 2.5 Pa for 1.5 min; the vacuum was released and the procedure repeated twice. The infiltration medium consisted of 0.1 mol dm^{-3} potassium phosphate buffer, pH 7.5 and 0.1 mol dm^{-3} KNO_3. Controls were infiltrated with buffer only. After

infiltration, the bags and their plant sections were thoroughly rinsed in distilled water, blotted on filter paper and incubated for one h at 30 °C, in the dark. Incubation took place in 100 ml Erlenmeyer flasks (3-4 bags per flask) wrapped with aluminum foil and fitted with an inlet (ending near the bottom of the flask) through which humidified N_2 was supplied at a flow rate of 200 cm^3 min^{-1}, and an outlet. Assays were ended by transferring each bag to a glass tube containing 15 cm^3 of boiling distilled water. The tissue was kept in boiling water for 20 min to achieve complete extraction of nitrate (Soares et al., 1985 b). After this time the extraction water was cooled and its volume readjusted to 15 cm^3. Aliquots of the extraction solutions were removed for nitrate determination. Initial levels of nitrate present in tissue were determined by the same method. Nitrate reductase activity could therefore be estimated on the basis of nitrate disappearance from the tissue.

Glutamine synthetase activity

Crude extracts for determination of glutamine synthetase activity were obtained by homogenizing the roots of 15-week-old seedlings, grown as described above, in 0.05 mol dm^{-3} imidazole-HCl, pH 7.2, 1 mol m^{-3} EDTA, 1 mol m^{-3} mercaptoethanol and 1 mol m^{-3} dithiotreitol. After filtration through 8 layers of gauze the extract was centrifuged at 10 000 g for 15 min and glutamine synthetase activity was measured immediately in the supernatant. The reaction mixture contained: 36 µmol ATP, 90 µmol MgSO4, 12 µmol hydroxylamine, 184 µmol L-glutamate and 100 µmol imidazole-HCl, pH 7.2. The final volume of the reaction mixture was 2 cm^3. The reaction was initiated by the addition of the crude extract and carried out at 30°C for 30 min. The consumption of ATP was followed by the decrease in absorbency at 260 nm using a spectrophotometer (PU 8620 UV/VIS/NIR, Philips Scientific and Industrial Equipment, Cambridge, England).

Nitrogen uptake rates

Twelve-week old seedlings were transferred to smaller screens for to study nitrogen uptake rates. They were pretreated in 1 mol m^{-3} $CaSO_4$ for 3 h in order to allow adjustment to the transfer stress and to exchange all nitrate or ammonium in the apparent free space (AFS). Seedlings damaged during transfer were discarded.

Roots were thoroughly rinsed in 25% Crone solution without nitrogen prior to the transfer, then removed from the nutrient solutions and held for 3 min to drain surface solution from the roots, which were neither blotted nor touched. Each seedling of 10 per treatment grown as described above, was placed in a Pyrex culture tube (40 mm x 100 mm) containing 50 cm^3 of the nutrient solution corresponding to the treatment. Tubes without seedlings were kept under the same conditions to assess ammonium losses. Solutions were continuously aerated to maintain optimal oxygen supply and to prevent formation of boundary layers at the root surfaces.

Roots were immersed in Crone solutions with different nitrogen concentrations in the range of 0.1 to 5 mol m^{-3} N. Nitrogen concentrations in the solution were maintained within narrow limits by changing solutions every h. The volume of the removed solution was determined and samples were taken and analyzed for nitrate and ammonium. Net uptake rates were based on nitrogen

disappearance from the solution per unit time. The rates were expressed as μmol N g^{-1} root fresh weight h^{-1} and were calculated from the changes in nitrogen levels and solution volumes. Each treatment had three replicates, and all the experiments were run three times. The results reported are the mean of the values obtained.

Chemical analysis

Nitrate was determined by reduction to nitrite (McNamara et al., 1971) and nitrite analyzed according to Snell & Snell (1949). Ammonium was determined by the indophenol blue reaction (Solozano, 1969). Organic nitrogen was determined according to the Kjeldhal method using a mixture of Se, Cu and K as catalyst. All the analysis were repeated three times.

Calculations

Photosynthetic nitrogen use efficiency was calculated as the ratio between photosynthesis per plant per unit time and nitrogen content per plant.

Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to compare the differences between treatments: nitrogen nutrition and CO_2 enrichment and root temperature. All significant values in the text are at $P<0.05$.

Results

Fifteen-week-old carob seedlings grown for 12 weeks in EC (enhanced CO_2 concentration in air, 800 cm^3 m^{-3}) produced more biomass of seedlings growing in AC (enhanced CO_2 concentration in air, 360 cm^3 m^{-3}) (Fig. 1). With nitrate as the nitrogen source in the nutrient solution differences in plant biomass production, on a fresh weight basis, became noticeable 45 days after the beginning of the CO_2 treatment. By the end of the experiment plants grown at 800 cm^3 m^{-3} CO_2 showed an increase of 100 % than plants grown under the same conditions 360 cm^3 m^{-3} (Fig. 1). All seedlings showed a slow increase in their fresh weight during the first 30 days of treatment..

Ammonium fed plants showed a detectable effect of the treatments after t20 days. At the end of the experiment the increase in fresh weight was of 13 % higher in EC than in AC grown plants (Fig. 1). There were no significant differences on the pattern of biomass production along the time for ammonium grown plants with the exception of the plants grown at a root temperature of 15°C. All the ammonium-grown plants showed low biomass productions till DAT 15 increasing to a faster growth rate thereafter -(Fig. 1).

Comparing the effect of nitrogen source on the growth rate it was noticeable that, although ammonium grown plants produced more biomass at any given condition, EC enhanced biomass production more significantly in plants growing in nitrate than in ammonium.(Fig. 1). This effect of nitrogen source was observed also on the relative proportion of nitrogen to carbon (Fig. 2).

Total nitrogen concentration in nitrate-fed plants decreased with time (Fig. 3). In both EC and AC nitrate-grown plants nitrogen content decreased at a rate of 30±4 μg N g^{-1} FW day^{-1}. When comparisons were made between AC and EC nitrate-grown plants on a plant basis either there were no differences on nitrogen concentration in the tissues, or high CO_2 grown-plants had smaller nitrogen concentrations (Fig. 4).

Ambient CO_2 levels brought about a decrease in the total nitrogen concentration in the tissues of ammonium fed plants. The nitrogen concentration of plant biomass of ammonium fed plants decreased at a rate of 8 ± 2 μg N g^{-1} FW day^{-1} (Fig 3). Plants showed a tendency to maintain their nitrogen concentration on a whole plant basis when growing at 360 or 800 cm^3 m^{-3} CO_2 (Fig. 4).

The higher nitrogen concentration on a plant size basis (mg N g^{-1} fresh weight) of ammonium-fed plants, as compared to nitrate-grown plants, increased with plant size (Fig. 4).

The allometric relation between $\ln_e$ (Total plant nitrogen) versus $\ln_e$ (Total plant biomass-Total plant nitrogen) for nitrate-grown plants showed that total biomass production increased at a faster rate relative to total nitrogen accumulation (Fig. 5). For ammonium grown plants the same allometric relation was linear, $y = 1.6\ x - 5.64$, with no differences among treatments (Fig. 5).

Nitrate concentration in the shoot of nitrate fed plants was stable with time, with root temperatures or ambient CO_2 levels (Fig. 6). While organic nitrogen concentration of the shoots of these plants tend to decrease with time (Fig. 7).

Nitrate in the roots tend to increase from a very low initial value to a maximum value of 35 μmol NO_3^- g^{-1} FW. Plants growing at 360 cm^3 m^{-3} have higher root NO_3^- concentrations than at 800 cm^3 m^{-3} (Fig. 6). Changes in CO_2 levels did not affect the pattern of distribution of organic nitrogen concentration between roots and shoots (Fig. 7).

Nitrate reductase activity (NRA) in vivo, determined at the end of the growth period, increased 50% in the roots due to the rise in atmospheric CO_2 level from 360 to 800 cm^3 m^{-3} (Table 1). Roots in vivo NRA was one order of magnitude higher than that of the shoots, so the differences in nitrate reductase activity at the different CO_2 levels in the shoot did not affect the relative distribution of NRA between roots and shoots in a significant way (Table 1).

Glutamine synthetase (GS) activity, determined at the end of the growth period, increased 55% with the increase in atmospheric CO_2 levels in nitrate grown-plants. The increment in the activity of the enzyme was not significant in ammonium grown-plants. GS activity was always higher in ammonium- than in nitrate-fed plants (Table 1).

Plants grown at 800 CO_2 cm^3 m^{-3} had higher nitrate uptake rates than plants grown at 360 CO_2 cm^3 m^{-3} (Fig. 8). Ammonium-grown plants also increased their ammonium uptake rates under higher conditions of higher CO2 (Fig. 9).

Discussion.

There is much information and little agreement concerning the effects of CO_2 enrichment on plant growth. This may be the result of ~~following~~ secondary rather than primary responses to CO_2. Increased growth rates at high CO_2 concentrations may be caused by a number of factors, such as an increased availability of photosynthetically fixed carbon (Strain & Cure, 1985; Kimball, 1986), increased water potential and turgor due to stomatal closure (Eamus & Jarvis, 1989; Bazzaz, 1990), increased osmoregulation under water stress due to improved carbon supply to roots, decreased dark respiration rates , and/or direct effects of CO_2 on plant growth (Coleman et al., 1991).

Despite these complexities plants respond, almost without exception, to increases in CO_2 concentrations with enhanced rates of biomass production. Cure & Acock (1986) calculated that the average increase in biomass for C_3 grasses grown under twice normal CO_2 concentration was 28 %, but increases as high as 100 % or more in biomass have been reported for some other C_3 plants (DeLucia et al., 1985; Mauney et al., 1979; Wong, 1979).

The increase in biomass production by carob seedlings grown under high CO_2 levels varied between 13 and 100% in relation to plants grown under the same conditions but at ambient concentrations of CO_2, depending on the nitrogen source available in the root medium (Fig. 1 and Cruz et al., 1993 a). Nitrate-fed plants showed a larger relative increase in biomass production in comparison with ammonium-fed seedlings. This may well be related with their increase in photosynthesis (Cruz et al., 1993 a) due to higher CO_2 levels. These explanation is supported also the changes in photosynthetic nitrogen use efficiency observed (Fig. 2) which was very much increased in nitrate-grown plants. The higher PNUE was the result of a much larger of increase of photosynthesis rate than of nitrogen content (Fig. 3)

Although nitrate ions are generally regarded as the major form of inorganic nitrogen available to higher plants, other forms of nitrogen, particularly ammonium ions may predominate in some soil types and at certain succession stages (Rice & Pancholy, 1972; Stewart, 1991). Carob is a tree species typical from climactic Mediterranean ecosystems, known for its preference of ammonium or ammonium nitrate as a nitrogen source than of nitrate (Martins Loução and Duarte, 1987; Cruz et al , 1993 b). The increase in atmospheric CO_2 concentration from 360 to 800 $cm^3\ m^{-3}$ did not affect this preference, the reason of which is the difficulty of the carob roots to load nitrate into the xylem and transport it to the shoots (Cruz et al.). The difference of biomass production between nitrate and ammonium fed plants were smaller at 800 than at 360 $cm^3\ m^{-3}$ CO_2 (Fig. 1).

In this as in many other studies (Neales & Nicholls, 1978; Mauney et al., 1979; Thomas & Harvey, 1983) the period of CO_2 enrichment was at the early stages of growth. This fact makes it difficult to know whether the resulting increased growth was due to some properties of seedling development or to CO_2 enrichment. Despite decreases in photosynthesis and relative growth rate of older plants during CO_2 enrichment, plants grown at high CO_2 concentrations usually continue to grow at a faster rate maintaining biomass production rates higher than control plants throughout the enrichment

period. This effect has been attributed to the fact that increases in growth rate during the early period of growth increase the leaf area over that of the controls which allowed higher rates of photosynthesis per plant and a higher growth rate, despite maintenance of similar rates of photosynthesis per leaf area unit (Spencer & Bowes, 1986; Curtis et al., 1989; Mauney et al., 1979; Mott, 1990).

There was a tendency for tissue nitrogen concentrations to decrease with time, especially in nitrate-grown plants under high CO_2 concentrations (Figs 3 and 4). Several authors observed a decrease in plant nitrogen concentration under CO_2 enrichment during vegetative growth (Williams et al., 1986), but the reasons for this decline remained unclear. Several mechanisms such as the increase in starch content at high CO_2 or reduced N uptake as a result of stomatal closure at high CO_2 (Madsen, 1975) have been proposed. This seems not to be the case in carob since ammonium-fed plants accumulate more starch than nitrate fed plants (Cruz et al., 1993 a) and display the smallest decrease in tissue nitrate concentration with time (Fig. 3). If the decrease in nitrogen concentration was a result of increased photosynthetic nitrogen use efficiency, total biomass production should increase at a faster rate than total nitrogen accumulation in an enriched CO_2 atmosphere. And the slope of the nitrogen to biomass relationship should become shallower under high CO_2, as it was indeed the case for nitrate-fed plants (Fig 5). Nitrogen concentration in nitrate fed plants, on a fresh weight basis, decreases with plant size, suggesting that the observed decrease in nitrogen concentration of these plants in response to elevated CO_2 levels were the result of accelerated growth.

For ammonium fed plants N concentration of the tissue is almost independent of the plant size (Fig. 4). The observed allometric relationship between ln (Total plant biomass) versus ln (Total plant biomass - total plant nitrogen) in ammonium-grown plants for high and low CO_2-grown plants are identical (Fig. 5). Consequently CO_2 levels did not affect the pattern of biomass and nitrogen accumulation over the course of their development. There may be a genetic limitation for the growth rate of a species. If this is the case, then it is possible that ammonium fed carob plants, which have higher growth rates than nitrate-fed carob were much closer to this limit than nitrate fed carob. Consequently their response to high CO_2 concentrations was relatively small and changes in the pattern of nitrogen accumulation could not be detected.

It seems that the nitrogen source affected the allometric relationship described and the response to elevated CO_2 concentrations: nitrate-fed plants gained more biomass per unit in nitrogen, displaying or increased PNUE when grown in high CO_2 levels than ammonium fed plants (Figs 4 and 5). Availability of photosynthates may have determined differences in the plant composition, such as a higher the C/N ratio in nitrate fed plants grown under high CO_2 conditions than in ammonium fed plants. The higher production of soluble carbohydrates (sucrose) by nitrate fed plants grown under high CO_2 resulted (Cruz et al., 1993 a) in enhanced growth . Ammonium-grown carob shift photosynthates to starch rather than sucrose, in such a way that a larger proportion of the fresh assimilates are not expressed in additional growth.

Neither root temperature nor atmospheric CO_2 levels increased nitrate concentrations ~~of~~ in carob shoots grown with nitrate (Fig. 6). The restriction of nitrate transport from the root to shoot in carob may be a limiting factor for the growth of nitrate fed carob plants (Cruz et al., 1993 c). Although nitrate-fed plants showed an higher growth increase due to rising levels of CO_2 than ammonium-fed plants their fresh weight at the end of the experimental period was smaller (Fig. 1). The interactions between CO_2 enrichment and nutrient utilization varied according to the treatments. The distribution of nitrate reductase activity between the shoot and the root of carob (Table 1) was essentially unaffected by atmospheric CO_2 concentration although higher CO_2 levels enhanced the activity of the enzyme in both plant parts but especially in the root. Glutamine synthetase (Table 1) was enhanced both by higher CO2 and by increasing temperature of the rhizosphere, presumably due to the increase in nitrate uptake.

Nitrate accumulation in the roots of carob seedlings reached a plateau 75 days after the beginning of the CO_2 treatments. Nitrate concentration in roots of plant growing at the higher CO_2 was smaller than that of the roots of plants exposed to ambient CO_2 (Fig. 6). This is presumably a consequence of the observed increase in root nitrate reductase activity observed in high CO_2 grown plants (Table 1), which could be a consequence of the higher photosynthetic rates of high CO_2 grown plants resulting in increased availability of transportable carbohydrates such as sucrose (Cruz et al., 1993 a). Concentrations of organic N in all organs of NO_3^--fed carob plants grown under high CO_2 were consistently lower than in ambient CO_2 grown plants (Fig. 7). Roots and shoots of nitrate fed plants showed a similar proportional decrease in organic N concentration due to CO_2 enrichment.

There was indication of a biphasic pattern of NO_3^- accumulation in roots of nitrate grown carob plants. In the early development stages of the seedlings there was an increasing accumulation of nitrate, followed by a decline on the concentration of nitrate (Fig. 6) which coincides with the increase in fresh weight production (Fig. 1). The increase in nitrate reduction (Table 1) was smaller than the increase of photosynthesis induced by high CO concentrations and, consequently, nitrate reserves stored in the tissues declined (Figs 4 and 5). The mechanism of photosynthetic regulation of nitrate reductase activity via a rapid reversible modulation (Kaiser & Brendle-Behnisch, 1991) described for Spinach leaves is presumably inconsequential in carob seedlings in which most of the nitrate is reduced in the roots (Cruz et al., 1991). The physical separation of shoot photosynthesis and root nitrate reduction in carob seedlings could contribute to the incomplete synchronization of CO_2 and nitrate assimilation under changing environmental conditions.

Glutamine synthetase activity increased significantly (27%) in nitrate-fed carob grown under high CO_2 in relation to the plants grown at ambient CO_2 concentrations (Table 1). The activity of glutamine synthetase of ammonium fed plants was not significantly affected (8%) by changes in atmospheric CO_2 concentrations. This constitutes an additional indication that atmospheric CO_2 levels affect more plants grown in nitrate than in ammonium.

The increase of nitrate and ammonium uptake rates (Fig. 1) observed in plants growing in high CO_2 (12-15 %) was much smaller than the increase in biomass production (up to 100%).

Growth in high CO_2 must lead either to changes in the morphology of the root system with the production of more young roots, able to assimilate more nutrients, or to a decline in nitrogen concentration in the plant. This must inevitably lead to severe deficiency as the nutrients in plant are progressively diluted by increasing amounts of carbon and this can not continue indefinitely (Eamus & Jarvis, 1989). To survive, the plant must either extract precious unavailable quantities of the nutrient or to exploit new volumes. Ammonium plants increased their nitrogen uptake rates (12%) (Fig. 9) to an extent similar to that in biomass production (12.9%). Though this is the smallest increase in biomass production it is the one that is more susceptible to persist for longer.

Increases in nitrate uptake by the root system are associated with an increase of cytokinins, a growth regulators while the increase in ammonium uptake is associated with rising levels of abscisic acid, responsible for the restrain of plant growth. The increase in CO_2 levels may be linked to an increase in cytokinin concentrations in nitrate fed plants when grown under high CO_2 which increased nitrate uptake and resulting in the observed growth promotion of in these plants. On the other hand, the limited response of ammonium-fed plants to high CO_2 can be related with to the increase in abscisic acid concentration that is associated with ammonium nutrition. In this case the increase in growth that could be observed under high CO_2 due to an increase in ammonium uptake and carbohydrate production could suffer a negative modulation because of the increase in abscisic acid concentration.

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Figure legends

Figure 1. Plant biomass as a function of time, atmospheric CO_2 level and nitrogen source. Each point represents data collected from 9 plants grown in 3 different pots. Black symbols represent plants grown in 360 cm^3 m^{-3} CO_2 -and open symbols indicate plants grown in 800 cm^3 m^{-3} CO_2. Vertical bars represent the standard deviation. They are not shown when smaller than the symbols.

Figure 2. Photosynthesis rates per unit nitrogen as a function of time, atmospheric CO_2 level and nitrogen source. Each point represents data obtained from 9 plants grown in 3 different pots. Black symbols represent plants grown in 360 cm^3 m^{-3} CO_2 and open symbols indicate plants grown in 800 cm^3 m^{-3} CO_2. Vertical bars represent the standard deviation. They are not shown when smaller than the symbols.

Figure 3. Total plant nitrogen concentration in the plants as a function of time, atmospheric CO_2 level and nitrogen source. Each point represents data obtained from 9 plants grown in 3 different pots. Black symbols represent 360 cm^3 m^{-3} grown-plants and open symbols 800 cm^3 m^{-3} grown-plants. Vertical bars represent the standard deviation. They are not shown when smaller than the symbols.

Figure 4. Percent total plant nitrogen concentration as a function of plant size, CO_2 level and nitrogen source. Each point represents data collected from the destructive harvest of 9 plants (3 pots). Black symbols represent 360 cm^3 m^{-3} grown-plants and open symbols 800 cm^3 m^{-3} grown-plants. Vertical bars represent the standard deviation. They are not shown when smaller than the symbols.

Figure 5. The allometric relationship of the natural logarithm of total nitrogen vs. the natural logarithm of (plant biomass - total nitrogen) as a function of time , atmospheric CO_2 level, nitrogen source. Black symbols represent 360 cm^3 m^{-3} and open symbols 800 cm^3 m^{-3} grown-plants. Each point represents data collected from the destructive harvest of 9 plants (3 pots). Vertical bars represent the standard deviation and are not shown when smaller than the symbols.

Figure 6. Nitrate concentrations in roots and shoots of nitrate fed-plants as a function of time and atmospheric CO_2 level. Black symbols represent 360 cm^3 m^{-3} grown-plants and open symbols 800 cm^3 m^{-3} grown-plants. Each point represents data collected from the destructive harvest of 9 plants (3 pots). Vertical bars represent the standard deviation. They are not shown when smaller than the symbols.

Figure 7. Organic nitrogen concentrations of roots and shoots of nitrate-fed plants as a function of plant age and CO_2 level. Black symbols represent 360 cm^3 m^{-3} grown-plants and open symbols 800 cm^3 m^{-3} grown-plants. Each point represents data collected from the destructive harvest of 9 plants (3 pots). Vertical bars represent the standard deviation. They are not shown when smaller than the symbols.

Figure 8. Concentration dependence of net nitrate uptake rates of 15-week-old carob seedlings on 90 AT (Days after beginning of treatment). Black symbols represent 360 cm^3 m^{-3} grown-plants and open symbols 800 cm^3 m^{-3} grown-plants. The values in the graph are the means of three replicates with three plants each ± SD.

Figure 9. Concentration dependence of net ammonium uptake per unit root fresh weight of 15-week-old carob seedlings, 90 days after starting of each treatment. Black symbols represent 360 cm^3 m^{-3} grown-plants and open symbols 800 cm^3 m^{-3} grown-plants. The values in the graph are the means of three replicates with three plants each ± SD.

Table 1. Rates of nitrate reductase activity (NRA) in roots and shoots of nitrate-fed plants and glutamine synthetase activity (GSA) in roots of nitrate- and ammonium-fed plants. NRA was determined by the method *in vivo*. Values presented in table are the mean of 2 experiments with 3 replicate each. Values of the same column followed by the same letter are not significantly different at P>0.05 by Duncan's test.

CO_2 (cm^3 m^{-3})	N source	NRA (mol NO_3 g^{-1} FW h^{-1})		GSA (mol g^{-1} FW h^{-1})
		Root	Shoot	
360	NO_3^-	12.1^b	2.0^a	26.4^c
	NH_4^+	*	*	38.0^a
800	NO_3^-	19.2^a	2.3^a	33.6^b
	NH_4^+	*	*	41.2^a

* traces

Figure 1

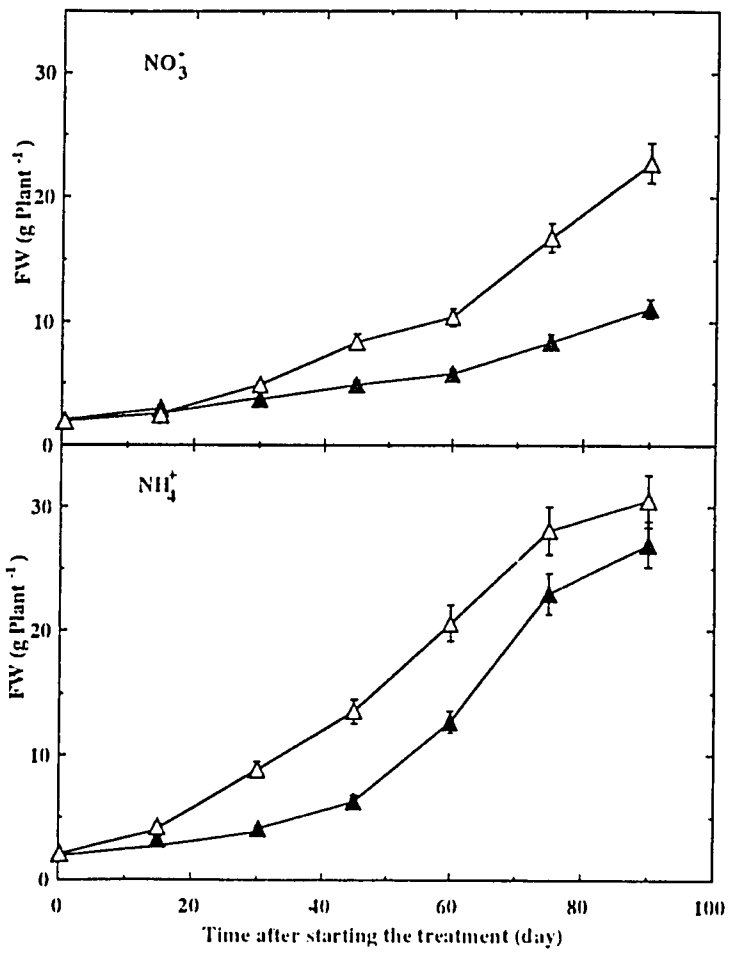


Figure 1. Plant biomass as a function *time*, CO_2 level and nitrogen source. Each point represents data *obtained from* 9 plants, *grown in* 3 pots. Black symbols *AC* grown plants and open symbols EC grown plants. Vertical bars *indicate* standard deviation. They are not shown when smaller than the symbols.

Figure 2

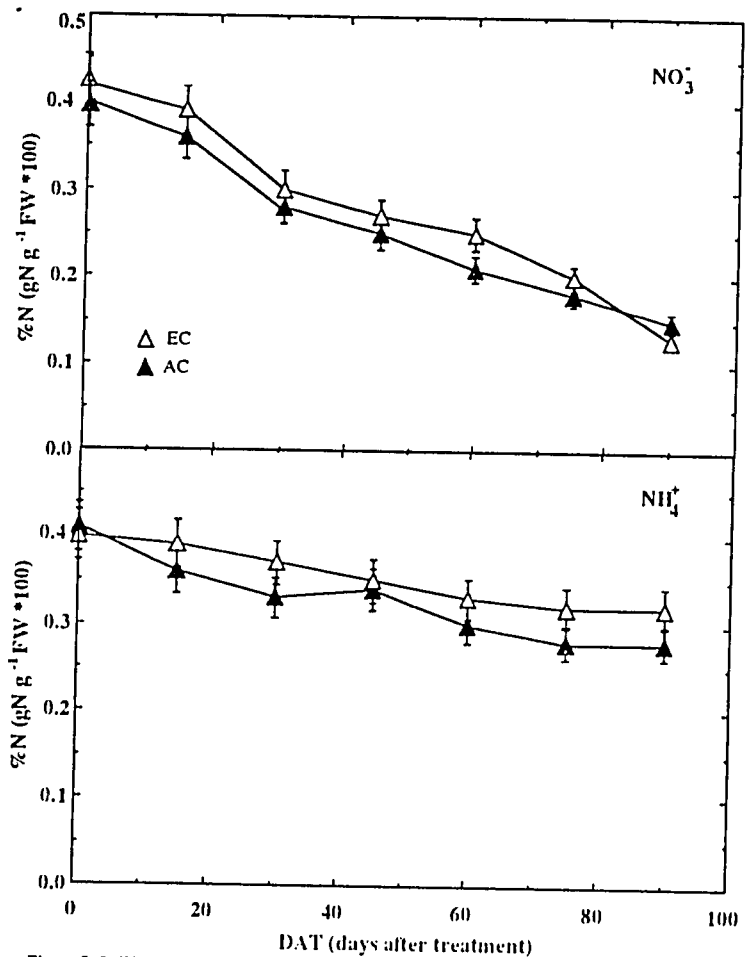


Figure 2. PNUE as a function of time, CO2 level and nitrogen source. Each point represents data *obtained* from 9 plants grown in 3 pots. Black symbols represent 360 cm3 m-3 grown-plants and open symbols 800 cm3 m-3 grown-plants. Vertical barrs represent the standard deviation. They are not shown when smaller than the symbols.

Figure 3

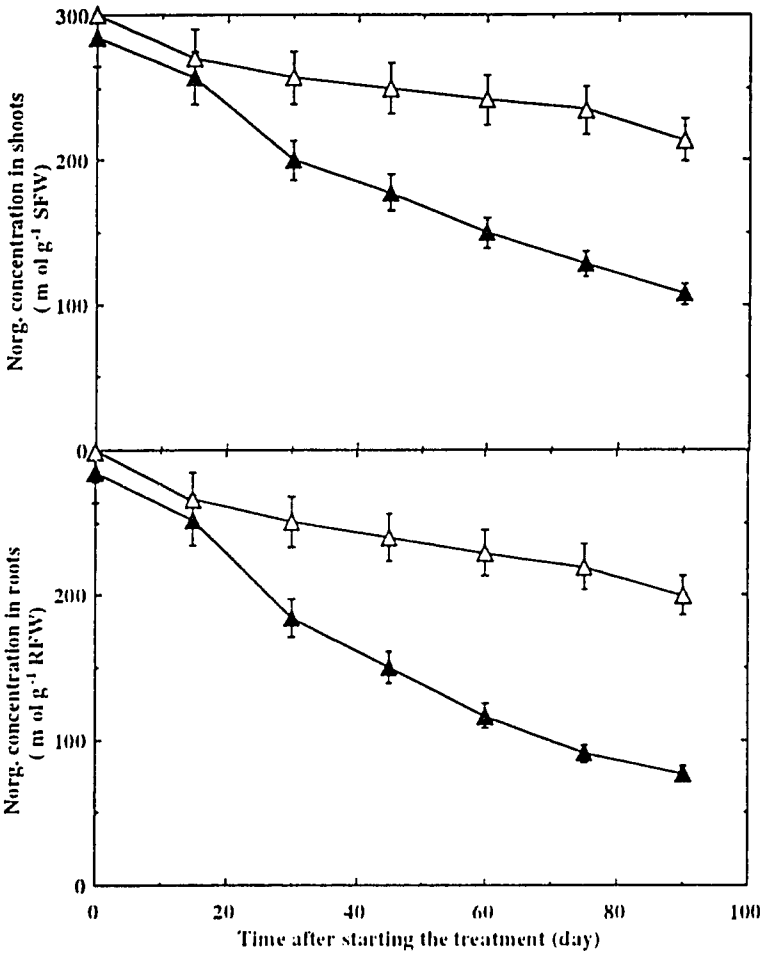

Norg. concentration in shoots
(m ol g-1 SFW)
300
200
100
0
Norg. concentration in roots
(m ol g-1 RFW)
200
100
0
0
20
40
60
80
100
Time after starting the treatment (day)

Figure 4

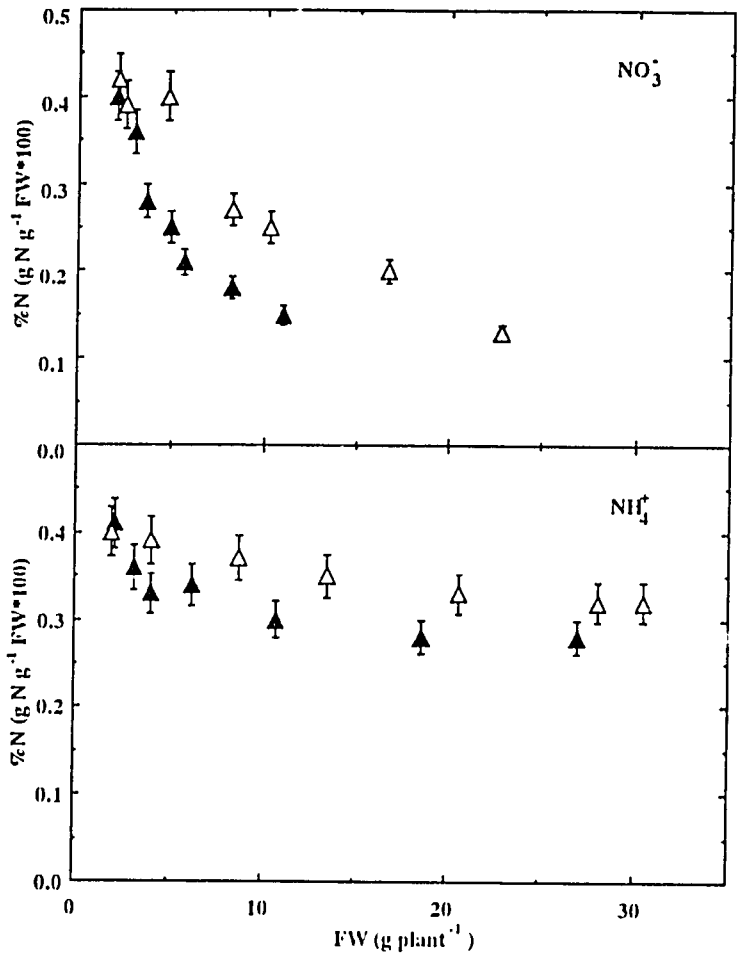


Figure 5

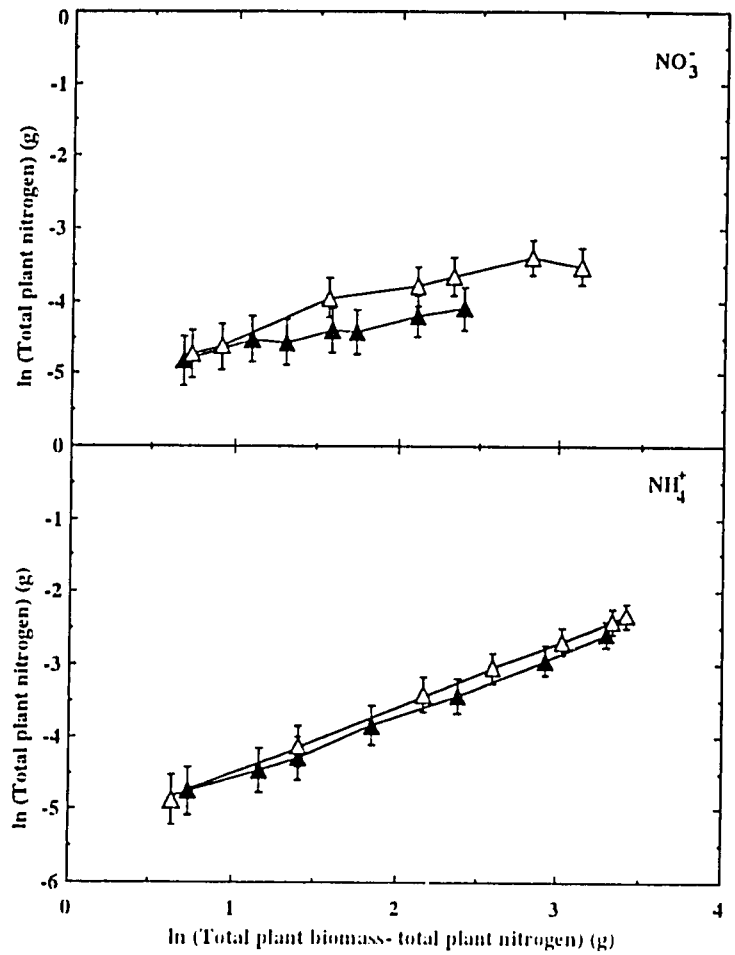


Figure 4. Percent total plant nitrogen as a function of plant size, CO2 level and nitrogen source. Each point represents data collected from the destructive harvest of 5 plants (3 pots). Black symbols represent 360 cm3 m-3 grown-plants and open symbols 800 cm3 m-3 grown-plants. Vertical barrs represent the standard deviation. They are not shown when smaller than the symbols.

Figure 6

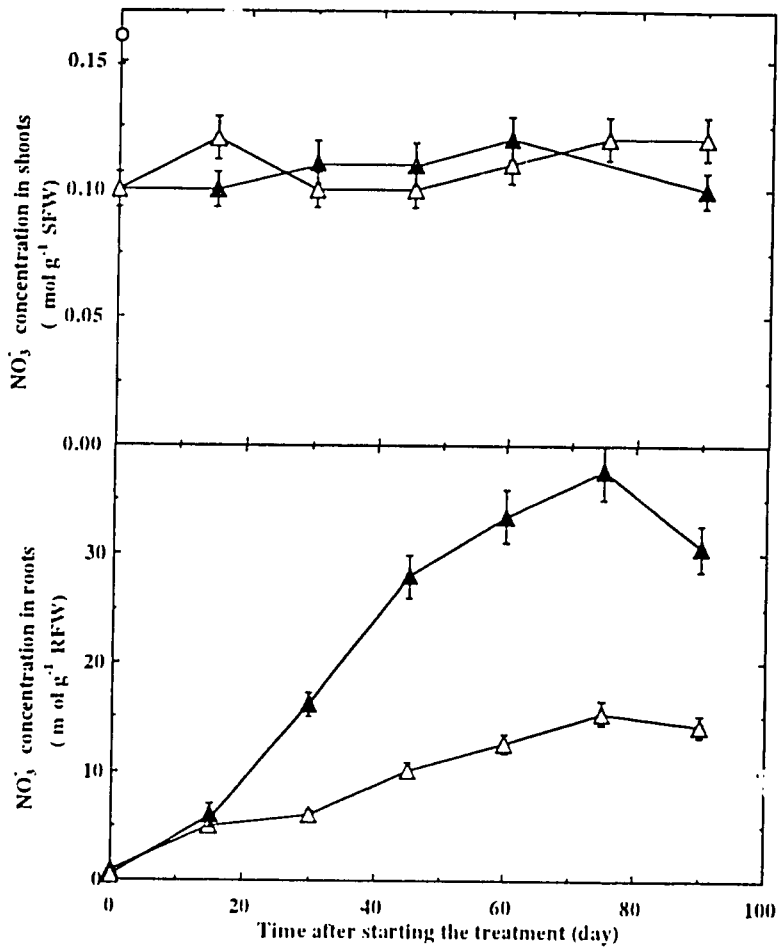
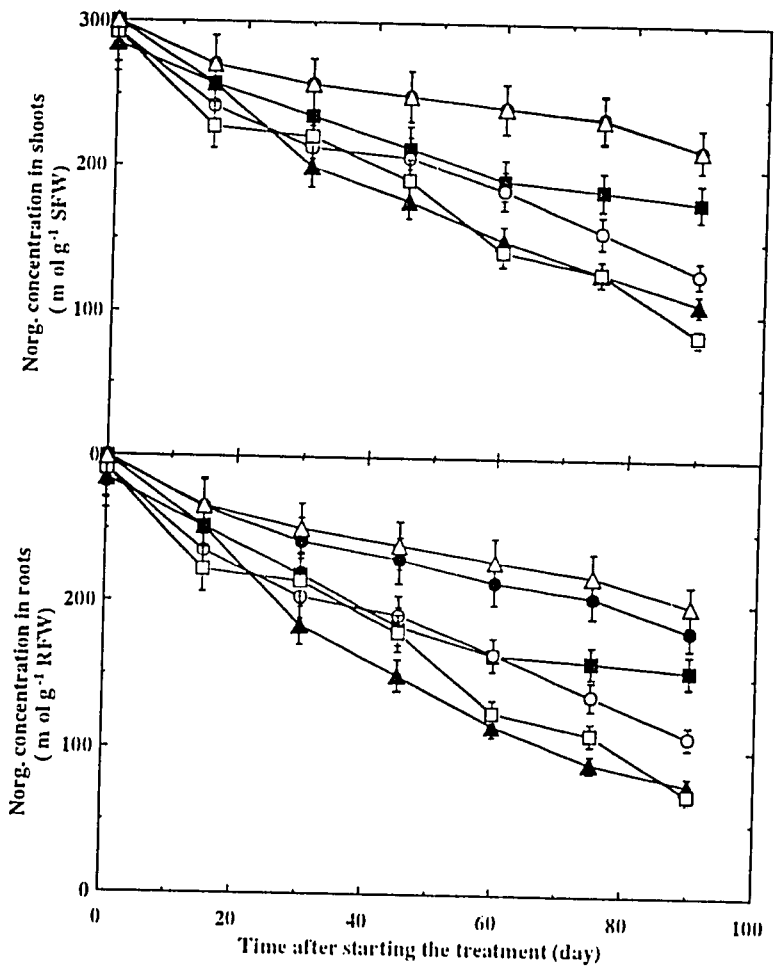
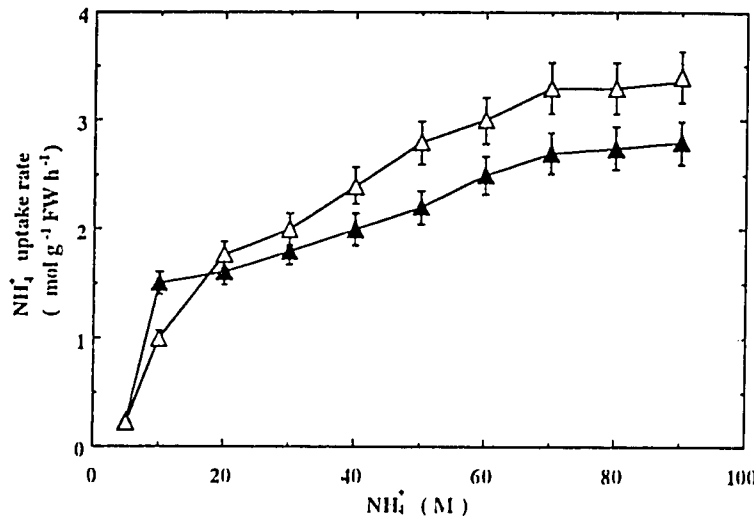
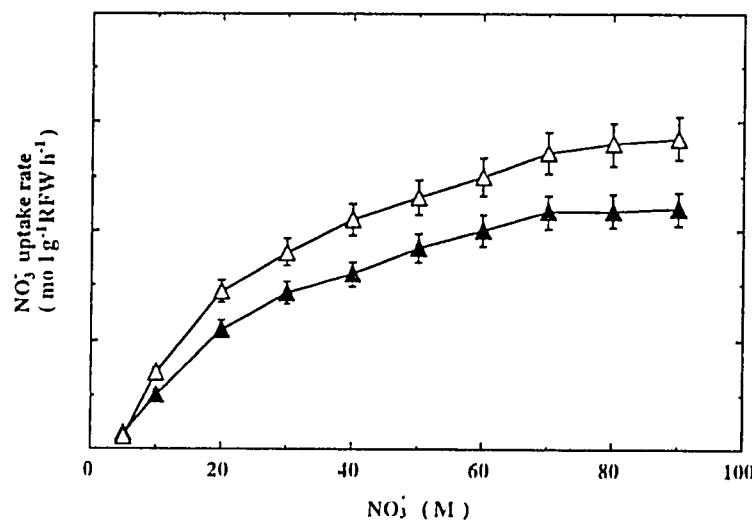

0.15
0.10
0.05
0.00
NO3- concentration in shoots
(mol g-1 SFW)
30
20
10
0
NO3- concentration in roots
(m ol g-1 RFW)
0
20
40
60
80
100
Time after starting the treatment (day)

Figure 7


Norg. concentration in shoots
(m ol g-1 SFW)
300
200
100
0
Norg. concentration in roots
(m ol g-1 RFW)
200
100
0
0
20
40
60
80
100
Time after starting the treatment (day)

Figures 8 and 9





Impact, Relevance and Technology Transfer

The usefulness of the findings in the developing country

All the findings of this research will be useful and will present positive impacts on: 1) reclamation of various areas of the Iberian Peninsula where the standard of living needs to be raised; 2) addressing both economic and ecological aspects of important deforested areas, taking as a high priority the preservation of native Mediterranean plant genetic material; 3) understand (and ultimately manage) the ecological consequences of fertigation, both edaphic and hydrologic.

Our research will lead to an increase in the quantity and quality of carob production. This will provide raw material needed for food, fodder, sugar and syrup industries, as well as for industrial gums and alcohol. The reliability of the production, that we enable to improve, will help to satisfy the increasing demand of the market and will prevent price fluctuations. We also expect that interest in germ will increase because of its high protein and vitamin contents.

Project impact on individuals, laboratories, departments and institutions.

Dr. Cristina Cruz worked in this project which was very important for the successful completion of her doctoral dissertation, recognized as a very good and complete work at the Faculty of Sciences of the University of Lisbon. The research completed by Dr. Cruz has been presented in numerous international Conferences and published in some of the best scientific journals in the field of plant physiology.

Due to her knowledge and scientific work Dr. Cruz could enter the Plant Biology Department staff at Faculdade de Ciências de Lisboa.

Mr. Pedro Correia is almost finishing his Ph.D. work. He has been responsible for the establishment of field experimental work with carob orchards in Algarve with the enthusiastic assistance of AIDA (Associação Interprofissional para o Desenvolvimento e Valorização da Alfarroba). The results are rapidly reaching the local farmers. The advisory work normally offered by AIDA was enhanced and it has been fruitful for the improvement of carob orchards.

The knowledge acquired during this research work improved the research lines of Plant Biology Department through the stimulation of new Ph.D. works and new research projects.

Usefulness of results. Larger scale trials.

This interdisciplinary work is and will be completely addressed to the issues of a better control of inputs and a reduction in production costs in primary production in semiarid areas, contributing to the protection of the environment and the sustainable exploitation of land use under Mediterranean conditions. These studies give basic and technical formation to new researchers reinforcing the

scientific capacity of Portugal in the management of marginal rural areas. Moreover this research will contribute to intensify and diversify the socio-economic capacities of South Portugal.

These studies could also serve as a useful model for the establishment of carob and other leguminous trees in other parts of the world such as the Philippines, Chile, Morocco, and Peru from were we have been approached already to establish cooperative research projects.

New capacity, equipment and expertise left in developing country

This project provided us the knowledge to intensify and diversify marginal dry lands use where the most carob orchards spontaneously grow, all over the Mediterranean area. Moreover, it gave a scientific background to the establishment of new intensive orchards with both soil and crop rehabilitation with rural economical rather than ecological impacts.

Another innovative aspect of our presented work was the use of a woody species for nitrogen studies, uptake, assimilation and transport bringing a new understanding on the overall nitrate versus ammonium interactions. These aspects have had a very important impact at international community level where the basic physiological knowledge of nitrogen metabolism was based in herbaceous crop species.

The equipment and the expertise acquired during this research work improved the scientific knowledge and capabilities of the Portuguese group leading to the development of new Ph.D. works and research projects.

Project Activities/Outputs

Publications:

Cruz, C., Martins-Loução, M.A., Soares, M.I. & Lips, H. (1991). Nitrate reduction in seedlings of carob (*Ceratonia siliqua* L.) . New Phytol. **119**: 413-419.

Cruz, C., Martins-Loução, M.A. & Lips, H. (1991). Efeito da concentração de cálcio no crescimento das plântulas de alfarrobeira. Actas de Horticultura **6**: 399-405

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Correia, P., Martins-Loução M. A. (1992). Effect of N-nutrition and irrigation water on carob-tree (*Ceratonia siliqua* L.) growth responses. Suelo y Planta **2**: 773-785.

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Correia, P., Martins-Loução M. A. (1993). Effect of N-nutrition and irrigation on fruit production of carob (*Ceratonia siliqua* L.). Physiol. Plant. **89**: 669-672.

Martins-Loução, M. A., Cruz, C. & Lips, S. H. (1994). O papel da fonte de azoto na resposta das plantas ao aumento do CO_2 atmosférico. 4ª Conferência Nacional sobre a Qualidade do Ambiente. Vol. I: 1-9

Correia, P.M. & Martins-Loução M. A. (1994). Preliminary studies on mycorrhizar of *Ceratonia siliqua* L. Proceedings of the 4th European Symposium on Mycorrhizas (in press).

Correia, P., Martins-Loução M. A. (1993). Seasonal variation of leaf water potential and growth in fertigated carob trees (*Ceratonia siliqua* L.). Plant and Soil (in press).

Cruz, C., M. A. Martins-Louçao and S.H. Lips. 1994. Nitrogen uptake areas of the carob root. Physiologia Plantarum (in press)

Cruz, C., M. A. Martins-Louçao and S.H. Lips. 1994. Nitrogen use efficiency as affected by high CO_2, root temperature and nitrogen source. Plant Cell and Environment (in press).

Presentation in international scientific conferences

Cruz, C., Martins-Loução, M.A., Soares, M.I. & Lips, H. (1989). Studies of nitrate and carbon assimilation of carob (*Ceratonia siliqua* L.) plants. II Advanced Course on Inorganic Nitrogen Metabolism.

Cruz, C., Lips, H. & Martins-Loução, M.A. (1990). Kinetics of NO_3^- and NH_4^+ uptake by nitrogen-depleted and non-depleted carob seedlings. Nitrogen Metabolism of Plants.

Cruz, C., Lips, H. & Martins-Loução, M.A. (1990). Nitrate reductase activity in carob seedlings. II International Symposium on Nitrate Assimilation. Molecular and Genetic Aspects.

Catarino, F.M. & Martins-Loução, M.A. (1991). Components of biological productivity in carob (*Ceratonia siliqua* L.). NOPROT 91.

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Correia, P. Barros, M. & Martins-Loução, M.A. (1991). Adaptações a condições de escassos recursos hídricos. O exemplo da alfarrobeira. Água e Agricultura - Encontro Técnico APRH - UNESUL.

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Cruz, C., Lips, H. & Martins-Loução, M.A. (1992). Nutrição azotada na alfarrobeira: a interacção NO_3^- /NH_4^+ .IV Simposio Nacional Nutrición Mineral de las Plantas.

Cruz, C., Lips, S. H. & Martins-Loução, M. A. (1992). NO_3^- and NH_4^+ uptake systems. The effect of root temperature. III International Symposium on Inorganic Nitrogen Assimilation.

Cruz, C., Lips, S. H. & Martins-Loução, M. A. (1992). N nutrition under high CO_2. III International Symposium on Inorganic Nitrogen Assimilation.

Cruz, C., Lips, S. H. & Martins-Loução, M. A. (1992). Long-distance nitrogen transport. III International Symposium on Inorganic Nitrogen Assimilation.

Cruz, C., Lips, S. H. & Martins-Loução, M. A. (1992). Effect of previous nitrogen nutrition on the interference of nitrate *versus* ammonium uptake. III International Symposium on Inorganic Nitrogen Assimilation.

Correia, P., Martins-Loução M. A. (1992). Effect of N-nutrition and soil available water on carob (*Ceratonia siliqua* L.) mature tree development. Vegetative growth and fruitification aspects. III International Symposium on Inorganic Nitrogen Assimilation.

Lips, S. H., Cruz, C., Martins-Loução, M. A. & Brunner, R. (1992). Root temperature, nitrogen nutrition and photosynthesis. III International Symposium on Inorganic Nitrogen Assimilation.

Cruz, C., Sebastiana, M., Lips, S. H. & Martins-Loução, M. A. (1993). Efeito da concentração de amónia no desenvolvimento de plântulas de *Ceratonia siliqua* L. X Reunión Nacional de la S.E.F.V.. Pamplona. Espanha. 251 pp.

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Correia, P & Martins-Loução, M.A.(1994). Seasonal variation of leaf water potential in irrigates mature carob-trees (Ceratonia siliqua L.). Biologia Plantarum 36 (suppl.), pp S352.

Correia, P.M. & Martins-Loução, M.A.(1994). Arbuscular mycorrhizas in *Ceratonia siliqua* L.. Structural aspects of morphology. 4th European Symposium on Myxorrhizas. Granada pp. 42

Correia, O., Martins-Loução, M.A., Nunes, M.A. & Catarino, F. (1994). Drought resistance strategies in species from different succession stages in Portuguese mediterranean maquis.

International Conference on Landscape degradation in mediterranean-type ecosystems. Reñaca. (Chile). pp: 17.

Cruz, C., Lips, S. H. & Martins-Loução, M. A. (1994). Plant nitrogen use efficiency under high CO_2. The effect of root temperature and nitrogen source. ECOCRAFT.

Cruz, C., Lips, S. H. & Martins-Loução, M. A. (1994). Water use efficiency of carob seedlings grown under high CO_2. The effect of nitrogen form. ECOCRAFT.

Dr. Cristina Cruz work for nearly two years with Prof. S. H. Lips in Sede Boqer. This period of research was an important element on the successful completion of Dr. Cruz's doctoral dissertation, which was recognized as a very good and complete work at the Faculty of Sciences of the University of Lisbon. The research completed by Dr. Cruz has been presented in numerous international Conferences and published in some of the best scientific journals in the field of plant physiology.

Mr. Pedro Correia learned advanced field research techniques in the research farms of Ben Gurion University of the Negev. This training allowed Correia later to establish through field experimental work with carob orchards in Algarve with the enthusiastic assistance of AIDA (Associação Interprofissional para o Desenvolvimento e Valorização da Alfarroba). The results are rapidly reaching the local farmers and the actual export of Portuguese carob products is steadily increasing for the benefit of local farmers and the food industry.

Numerous invited seminars and lectures have been offered by Prof. S. H. Lips and Dr. M. A. Martins Louçao on the nutritional and developmental mechanisms of the carob tree as affected by environmental conditions at the J. Blaustein Institute for Desert Research (Ben-Gurion University) at Sede Boqer, the Faculty of Sciences (Univ. of Lisbon), the University of Algarve, the Institute for Environmental Science and Management (Univ. of the Philippines at Los Baños), the Faculty of Agriculture of Khon Kaen University (Thailand), etc.

Project Productivity

The project did accomplished all the proposed goals and more that expected, as evident on the large number of publications and meeting presentations of the work carried out.

Future Work

We propose the use of different new physiological and ecological approaches such as the use of ^{15}N in the laboratory experiments and natural abundance levels in the field to get basic information to a better interpretation of carob eco-physiology.

The development of modern agrotechniques for the efficient use of combined irrigation and fertilization to optimize productivity of carob and other fast growing leguminous trees need to be implemented, since according to the new Portuguese Regulation (Port. nº 199/94) carob must be planted within a considerable area in the south of Portugal. A steady carob seed production would allow the supply of carob pods required to stabilize the demands by the food industry limiting present unsettling price fluctuations.