Effect of irradiance, sugars and nitrogen on leaf size of *in vitro* grown *Ceratonia siliqua* L.

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Abstract

Carob (*Ceratonia siliqua* L.) has compound pinnate leaves consisting of 4 - 6 pairs of leaflets. However, in conditions of *in vitro* culture only one pair of leaflets develops. With increasing irradiance from 9.3 to 74.1 μmol m^{-2} s^{-1}, leaf area increased 5-fold. Sucrose also significantly increased leaf area and the maxima were at concentration 147 mM at high irradiance and 233.6 mM at low irradiance. Sucrose was superior to fructose, glucose and combination of both in increasing leaf area. Decreasing concentration of KNO_3 and NH_4NO_3 caused a 3-fold decline of leaf area.

Additional key words: carob, leaf area, sucrose, fructose, glucose.

Introduction

Carob (*Ceratonia siliqua* L.) is an indigenous, helioxerophytic Mediterranean species adapted to high irradiance and long periods of drought. It is considered a neglected subtropical fruit species which bears pods rich in valuable dietary compounds. Leaves are 10 - 20 cm long compound and pinnate, usually consisting of 4 - 10 pairs of leaflets 3 - 4 cm in length (Batlle and Tous 1997)

In vitro propagation of carob has been elaborated (Thomas and Mehta 1983, Sebastian and McComb 1986, Alorda and Medrano 1986). In our own studies on the *in vitro* propagation of carob, we showed this species to be prone to various physiological disorders (Vinterhalter and Vinterhalter 1992, 1999). Apart from lenticel hypertrophy, apical necrosis and vitrification (hyperhydricity), we reported changes in leaf size triggered by high irradiance in the growth room leading to formation of unusually large leaves (Vinterhalter and Vinterhalter 1992).

It is well known that leaves on plants cultured *in vitro* are much smaller than leaves on plants grown outdoors. In white birch the leaf area of *in vitro* plants was 83 times smaller than in micropropagated plants grown in the glasshouse (Smith *et al.* 1986). Leaf area decrease was mostly due to reduced cell division, since the decrease of individual cell size was only 40 %. This was the starting point in our research in which we investigated the size of carob leaves in relation to non-hormonal factors, including irradiance and changes in sugars and nitrogenous salts in the medium.

Materials and methods

Carob shoot cultures were maintained on Murashige and Skoog (1962, MS) medium with 58.4 mM sucrose, 0.64 % agar, 0.5 mg dm^{-3} 6-benzyladenine (BA) and 0.1 mg dm^{-3} indole-3-butyric acid (IBA). The procedure for establishment, and conditions for maintenance of carob shoot cultures have been published previously (Vinterhalter and Vinterhalter 1992). The temperature in the growth room was 25 ± 2 °C, 16-h photoperiod and the standard irradiance used for maintenance of cultures 32.75 - 46.5 μmol m^{-2} s^{-1} was provided by 20 and 65 W white fluorescent lamps (*Tesla*, Pančevo, Yugoslavia). In all experiments 5 single isolated shoots, 15 - 20 mm long, were cultured on 40 cm^3 of agar solidified medium in 100 cm^3 wide neck Erlenmeyer flasks closed with cotton

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Abbreviations: I - irradiance; HC - high sugar concentration, MS - Murashige and Skoog; SC - standard sugar concentration.
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wool plugs. Treatments, consisting of 30 - 35 shoots, were repeated three to six times. Data were pooled, means and standard errors were calculated for leaf area and shoot multiplication and their variance was analyzed by Duncan's multiple range test to establish statistical significance.

Leaf area was determined for the larger of the two uppermost well developed leaves for every shoot in the treatment. Leaflets of compound leaves were carefully spread on a glass plate, moistened with glycerol and covered with transparent foil. Plates were then photocopied using a *Canon NP 3825* copier (*Canon*, Tokyo, Japan). The photocopies were scanned with a *HP 4C* scanner (*Hewlett-Packard*, Palo-Alto, USA) and the images analyzed using an *UTHSCSA Image Tool* program (University of Texas Health Science Center, San Antonio, USA) available from the Internet by anonymous FTP from maxrad6.uthscsa.edu.

For experiments in which effect of irradiance was investigated a special shelf was constructed on which positions with desired irradiance for every single culture vessel was measured in advance using a *Li-Cor 1000 Data Logger* with an *LI 190SA* quantum sensor (Lincoln, USA). Flasks comprising the same irradiance treatment were well-spaced on the shelf.

The multiplication index calculated by dividing the number of axillary shoot buds 2 mm or more in length at the end with the number of buds at the beginning of subculture illustrates the growth vigour of shoot cultures.
Experimental treatments:
1. Effect of irradiance (9.3, 16.5, 20.4, 32.7, 46.5 and 74.1 µmol $m^{-2} s^{-1}$) on media with 58.5 mM (2 %) sucrose.
2. Effect of sucrose concentration (29.2, 58.4, 87.6, 148, 233.6 and 292 mM) at low (9.3 µmol $m^{-2}s^{-1}$) and high (74.1 µmol $m^{-2}s^{-1}$) irradiance.
3. Effect of different sugars in concentration 58.5 or 146 mM (sucrose, fructose, glucose and a fructose + glucose in combination) at irradiance 74.1 µmol $m^{-2} s^{-1}$.
4. Effect of different concentrations of nitrogenous macronutrient salts (KNO_3 and NH_4NO_3) corresponding to 1/1, 1/2, 1/4, 1/10 and 1/20th of the full MS formulation at irradiance 74.1 µmol $m^{-2} s^{-1}$.

Results

Carob shoot cultures grown on a basal medium are characterized by fast shoot and leaf development. As a consequence leaves used for area determination were always those which expanded in the current subculture.

Most of the leaves consisted of a single pair of leaflets. Leaves with an additional terminal leaflet (3 leaflets) or two pairs of leaflets formed occasionally, but such phenotypes with an increased number of leaflets could not be maintained by shoot subculturing. Conditions leading to the formation of additional leaflets could not be connected to internal or external culture factors. After acclimation, plantlets propagated by *in vitro* methods developed large compound leaves consisting of several pairs of leaflets.

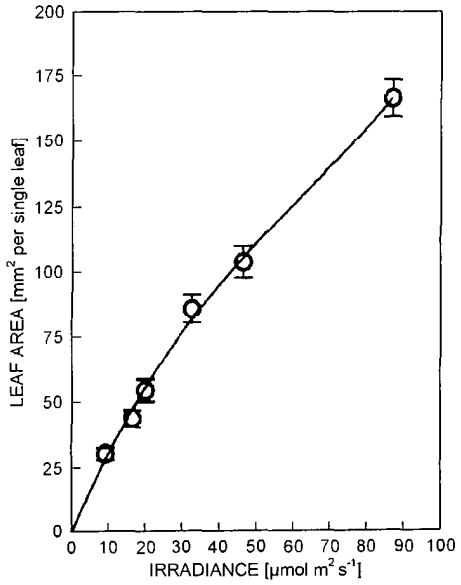

Fig. 1. Effect of irradiance on leaf area. Media supplemented with 58.4 mM sucrose, inorganic salts at full MS concentration. Means of 180 - 220 leaves.

Effect of irradiance (I): The size of the leaflets on carob shoot cultures was markedly increased with the irradiance (Fig. 1). At the highest I leaves became very large and it appeared as if the cultures mobilized all available resources in the formation of these gigantic leaves. In darkness the carob shoot cultures formed etiolated shoots on which leaves were present only as primordia.

It is evident that the low I generally used for maintenance of *in vitro* cultures (32.5 µmol $m^{-2} s^{-1}$) in case of helioxerophytic carob shoot cultures is suboptimal.

Effect of sucrose, glucose and fructose: At high I increased concentration of sucrose stimulated development of leaf lamina (Table 1) with a maximum at 146 mM (5 %) sucrose. In treatments in which sucrose concentrations were above 87.6 mM leaves were tough, leathery and reddish in colour. On low sucrose concentration (29.2 mM) leaflets were not only small but often failed to unfold creating a problem for leaf area determination.

At low I leaf area was always smaller than at corresponding sucrose concentrations at high I. At low I, the leaf area maximum occurred at a higher sucrose

Table 1. Effect of sucrose concentration on leaf area and shoot multiplication at high (74.1 μmol m^{-2} s^{-1}) and low (9.3 μmol m^{-2} s^{-1}) irradiance (I). Means ± SE, n = 70 - 183 shoots. Within each column means followed by the same letter were not significantly different according to Duncan's multiple range test at $P \leq 0.05$. nm - area not measured due to the small size and incomplete opening of leaflets.

Sucrose [mM]	Leaf area [mm^2] high I	low I	Multiplication index high I	low I
29.2	87.8 ± 5.3 a	nm	3.9 ± 0.1 b	0.5 ± 0.1 a
58.4	172.6 ± 6.9 b	35.9 ± 2.6 a	4.8 ± 0.2 c	1.9 ± 0.2 b
87.6	225.2 ± 7.4 c	71.4 ± 5.8 b	5.2 ± 0.2 d	2.9 ± 0.2 c
146.0	247.8 ± 8.4 c	120.7 ± 6.6 c	4.4 ± 0.2 c	2.7 ± 0.2 c
233.6	224.4 ± 9.8 c	167.0 ± 8.0 d	3.6 ± 0.2 b	1.0 ± 0.1 a
292.0	189.2 ± 10.2b	151.1 ± 6.8 d	2.8 ± 0.2 a	0.6 ± 0.1 a

Table 2. Effect of sucrose replacement with glucose and fructose on leaf area and shoot multiplication at high irradiance. Standard sugar concentration (SC, 58.4 mM), or high sugar concentrations (HC, 146 mM) were used. Means ± SE, n = 90 - 108 shoots. Within each column means followed by the same letter were not significantly different at $P \leq 0.05$.

Sugar type	Leaf area [mm^2] SC	HC	Multiplication index SC	HC
sucrose	161.2 ± 7.9 c	221.1 ± 10.5 c	4.2 ± 0.1 b	4.1 ± 0.1 b
fructose	45.0 ± 2.2 a	59.7 ± 3.1 a	3.6 ± 0.1 a	3.8 ± 0.1 ab
glucose	67.1 ± 5.2 b	107.4 ± 6.9 b	3.6 ± 0.1 a	3.7 ± 0.1 ab
fructose + glucose	61.1 ± 3.9 b	93.5 ± 6.6 b	3.7 ± 0.1 a	3.5 ± 0.1 a

concentration (233.6 mM) than at higher I. It seems that in leaf area development light can partly compensate for insufficient sucrose supply.

Glucose and fructose applied alone or in combination were far less efficient in promoting leaf development than sucrose at the same concentration. Glucose was superior to fructose at both concentrations tested and the glucose + fructose combination was intermediate (Table 2).

Effect of nitrogenous salts: Another factor which affect leaf development was the concentration of nitrogenous salts in the medium. Decreasing the concentration of both KNO_3 and NH_4NO_3 in the medium resulted in a rapid decline of leaf area. Thus, at a concentration equal to 25 % of the full MS formulation leaf area decreased to less then one third of the area measured when full-strength MS medium was used (Table 3).

Table 3. Effect of reduced concentration of nitrogenous salts (KNO_3 and NH_4NO_3) on leaf area and shoot multiplication. The medium was supplemented with 58.4 mM sucrose and irradiance was high (* - full strength MS). Means ± SE, n = 87 - 93 shoots. Within each column means followed by the same letter were not significantly different at $P \leq 0.05$.

N salts [mM]	Leaf area [mm^2]	Multiplication index
60*	167.4 ± 9.1 c	5.0 ± 0.2 c
30	107.5 ± 6.8 b	4.7 ± 0.2 c
15	50.7 ± 3.2 a	4.7 ± 0.2 c
6	42.4 ± 1.9 a	3.6 ± 0.2 b
3	41.3 ± 2.0 a	1.7 ± 0.2 a

Discussion

Light seems to be the major factor for leaf development in carob shoot cultures since, in its absence, development of leaf primordia was arrested. In carob we showed a five fold increase of leaf area in the I range 9.8 - 74.1 μmol m^{-2} s^{-1}. Leaf area has a tendency to increase even further at I higher then those which were tested. According to Marks and Simpson (1999) I decreased the growth of shade tolerant *Rhododendron* and *Disanthus* but not of *Crataegus* which is more light tolerant. In potato a stimulatory effect of I on leaf area was demonstrated in

the 30 - 120 μmol m^{-2} s^{-1} range (Kitaya *et al.* 1995). It should, however, be mentioned that the highest I tested is a small fraction of that of full sunshine, 1900 μmol m^{-2} s^{-1} (Dale 1988) for which carob is well adapted. Thus, the irradiances obtained using fluorescent lamps under *in vitro* conditions in this and other similar investigations are more comparable to shade conditions than to full sunshine.

Influence of light quality on leaf area development *in vitro* has been also investigated and species-dependent reactions were encountered. Blue light increased leaf area in potato (Aksenova *et al.* 1994) and birch (Saebo *et al.* 1995) but not in *Azorina* (Moreira da Silva and Debergh 1997).

Sucrose also strongly stimulated leaf area development in carob with a maximum at 146 mM sucrose for high I and 233.6 mM sucrose for low I respectively. Both, sucrose concentrations were higher than standard 58.4 - 87.6 mM concentrations used for maintenance of *in vitro* cultures of most plant species. It is interesting that even in conditions of high I sucrose stimulates leaf area indicating a synergistic effect of light and sucrose on leaf development under *in vitro* conditions. Sucrose affected the multiplication index which was highest at 87.6 mM sucrose both at high and low I.

Sucrose was a far superior carbon source than glucose and fructose or a combination of both these sugars. Previously, we showed sucrose to be indispensable source of sugar for the formation and growth of etiolated carob shoots (Vinterhalter and Vinterhalter 1999) which had much higher multiplication rates than in light-grown cultures (Vinterhalter 1998).

The decrease of the nitrogenous salt content of the MS medium resulted in a rapid decline of leaf area. It should be noted that the concentration of nitrogenous ions (NO_3^- and NH_4^+) in the MS medium is more then twice than that in Gamborg B5 (Gamborg *et al.* 1968) and more then four times than in WPM (woody plant) medium (Lloyd and McCown 1981). The multiplication index in these treatments decreased together with leaf area but it was not statistically significant until a 1/10 dilution of nitrogenous salts was reached.

We, therefore, showed that a combination of nutritional factors and I can be used efficiently to regulate leaf size in carob shoot cultures.

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