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Incorporation of ^{14}C-Photosynthate into Major Chemical Fractions of Leaves and Bark of *Ceratonia siliqua* L. at Different Seasons

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Summary

Parts of branches of *Ceratonia siliqua* (L.) were exposed to $^{14}CO_2$ for 3 h during the growth period in April, after termination of growth (end of May), in the summer drought period (August) and in the cool winter (February). Twigs were harvested immediately after the end of the feeding period as well as after 48 and 144 h. Simultaneously with the last harvest of exposed plant parts, controls from outside the exposure-chamber were also gathered. The total ^{14}C-incorporation into leaves and bark as well as the labelling and contents of soluble sugars, starch, total lipids and other precipitable soluble compounds were determined.

Total ^{14}C-incorporation immediately after termination of $^{14}CO_2$ application is a measure of the assimilation rate; it was high and nearly identical in April and May, but low (only about 5% of the April value) in summer and winter. The incorporation of ^{14}C into soluble structural components was highest in April. Uptake of total ^{14}C and labelling of soluble compounds during the 6-day period of the experiments show that metabolic rates were very low in summer but high during spring. The turnover of soluble compounds in February was remarkably high regarding the low CO_2-fixation rate. The labelling of soluble sugars in leaves decreased in all experiments; the specific activity of starch remained rather stable. The incorporation of ^{14}C into the lipid fraction was weak in all seasons and significant alterations took place only during the growing period.

Variation in assimilation rates of *Ceratonia* at different seasons was comparable to that found for other mediterranean species using IRGA methods. There was no indication that lipids in *Ceratonia* are true storage compounds and participate in cyclic metabolic processes preventing stress effects. The weak turnover of lipids leads to the conclusion that maintenance costs for these compounds, as calculated by Merino et al. (1984), perhaps are too high, at least for *Ceratonia*. Our results do not suggest a specific adaptation of the metabolism of storage compounds to summer drought in *Ceratonia*.

Key words: Ceratonia siliqua L., assimilation rate, $^{14}CO_2$-application, drought stress, lipids, maintenance cost, sugars, starch, storage.

Introduction

The carob-tree *Ceratonia siliqua* is a very drought-tolerant, but rather mesomorphic-leaved species growing in most thermophilic plant-communities of the Mediterranean region (Lo Gullo et al., 1986). The stomata behaviour and assimilation rates are those of a true sun plant (Rhizopoulou and Nunes, 1981). The evergreen leaves have a life-time of about 2–2½ years (Diamantoglou and Mitrakos, 1981). Diamantoglou and Meletiou-Christou (1978) found no accumulation of soluble carbohydrates and no change in the osmotic potential in the leaves during the summer drought period, which was confirmed by Rhizopoulou et al. (1989) in investigations using extracted sap. The sugar content of the leaves

is highest at the end of the growth period in May. In the bark of twigs there is an accumulation of sugars and lipids during the summer and autumn. The lipid content of the leaves is highest during the winter months and in the late growth period (Diamantoglou and Meletiou-Christou, 1977). To obtain further information on the accumulation of sugars, starch and lipids and their function as storage substances, we performed experiments to trace the incorporation and distribution of the photosynthetically fixed $^{14}CO_2$. The role of lipids as storage substances is equivocal (Nelson and Dickson, 1981; Hetherington et al., 1984; Beeson and Proebsting, 1988; Chapin et al., 1990). Our earlier speculation on a possible role of the allocation of lipids in Mediterranean evergreen species (Diamantoglou and Kull, 1982) therefore needs confirmation or refutation. For this reason the labelling experiments were undertaken during different seasons: the growth period, the cool winter and the hot summer drought period.

Material and Methods

Material

A middle-sized tree of *Ceratonia siliqua* L., about 40–50 years old, growing on the edge of the wilderness area of the Botanical Garden Diomidis, Athens, Greece was chosen for the labelling experiments. The branches used for the investigations were partly shaded when the sun was high in the sky.

Methods

Labelling procedures

The experiments took place from 10^{00}–14^{00}. Labelling with $^{14}CO_2$ was performed according to Kull and Baitinger-Haardt (1977), with some modifications for branches of trees as described by Distelbarth (1982). The terminal part (about 60 cm) of a branch (length > 1 m) with several twigs was placed in an air-tight plastic-foil chamber and equilibrated for 1 h before labelling took place. Transpiration of the enclosed leaves in the chamber caused the air humidity to rise to about 80–90 % during summer and 100 % during the other seasons. The temperature reached maximum values about 4–5 °C higher than the air temperature outside the chamber. As observed microscopically, stomata of the leaves were open in all experiments. The twigs enclosed in the chamber were allowed to only bear fully developed leaves; therefore, in the April experiment about 25 % of the leaves present had to be removed and in the May experiment about 10 %. This is important because growing leaves form sinks and this would lead to problems in interpretation of data (compare Kriedemann, 1969; Roberts and Menary, 1990).

After the equilibration period, 7.4 MBq (200 μCi) $^{14}CO_2$ were generated in the chamber (11 a.m.). After a 3-h supply of $^{14}CO_2$ to the branch, the chamber was opened, ventilated and then removed.

Harvesting

Immediately after removing the chamber some twigs of the branch were harvested and placed in plastic bags. In the following chase-period further harvesting took place 48 h and again 144 h after the labelling experiment. Simultaneously with the last harvest, some twigs of the branch that had remained outside the exposure-chamber were also gathered to find out how much photosynthate was exported from the labelling area.

The branch used in the experiment was next cut at its base. In the May experiment, very little radioactivity was found at the base of the branch. No further investigation of the labelling of the branch-bases took place in the other experiments.

Quantitative estimation of substances

The plant material was separated into leaf-blades, rhachis and petioles (not investigated further), and bark. It was placed in plastic bags, dipped into boiling water for 10 min and brought to the laboratory, about 60–90 min after harvesting the material was dried in a ventilation oven at 60 °C. Prior to analysis, the plant material was freeze-dried and ground to a fine powder.

Soluble sugars were analyzed by TLC and total sugar content by the anthrone method (compare Distelbarth et al., 1984). Starch content was measured using the anthrone method of McCready et al. (1950) and total lipids were quantified according to the gravimetric method of Bligh and Dyer (1959). The lipid fraction contained membrane lipids and triacylglycerols, as well as chlorophylls, carotenoids and perhaps other isoprenoids. The isoprenoid lipids constitute a rather small portion of the total lipid fraction (compare Diamantoglou and Kull, 1988), but the amount of waxes perhaps also included is not known. Mucilage and proteins were precipitated with acidic ethanol (Naglschmid et al., 1982) and determined by gravimetry.

As a gross value of (drought-)stress (compare Diamantoglou and Kull, 1988; Rhizopoulou et al., 1990), the proline content of the leaf-blades was measured according to the method of Bates et al. (1973) in the version modified for freeze-dried material by Huq and Larher (1984). In the leaves harvested in August the content was about 25 % higher than in May, in those harvested in February about 15 % higher.

Estimation of labelling

Total activity

Samples of 100 mg fine powdered plant material were dissolved in a scintillation vial in 1 mL tissue solubilizer (Solubilizer TS-1; 0.6 M solution in toluene, Zinsser Analytic, Frankfurt). The vials were incubated with slow shaking at 40 °C for 12 h, then decolorized by adding 1 mL isopropanol and 1 mL H_2O_2 (30 %) according to the method described by Benzioni and Mills (1991). After 10 h, 0.5 mL concentrated acetic acid and 10 mL scintillation fluid (Quicksafe A, Zinsser Analytic) were added. The activity was determined in a liquid scintillation counter (Packard Liquid Scintillation Analyzer Tri-Carb, Model 1600 CA). Quench correction was accomplished using an internal standard.

Labelling of the fractions

After removing the spots from the TL plates they were extracted with 80 % ethanol (30 min, 50 °C, shaking) and separated from the silica gel by centrifugation in Eppendorf microsample tubes. After separation and evaporation to dryness scintillation fluid (Quicksafe A, as above) was added.

Labelling of starch (and of the mucilage-protein-fraction) was measured by adding Quicksafe A (a scintillation fluid suitable for aqueous probes) to an aliquot of the solubilized material.

For quantification of the activity of the total lipid fraction, the lipid was weighed, dissolved in chloroform-methanol and scintillation fluid (also Quicksafe A, for reasons of comparison) added.

Standard deviation was calculated from several (2–4) separate investigations of the same sample. This could not be accomplished with all samples because of lack material, in which case the standard deviations obtained were generalized.

Table 1: Total ^{14}C-activity (Bq/mg dry material) in leaves and bark after feeding $^{14}CO_2$ to twigs of *Ceratonia* at different seasons.

Date of experiment		0 h	48 h	144 h	control from outside the exposure chamber (144 h after termination of the feeding)
		after termination of the feeding			
April	leaves	15.1	12	7.3	5.6*
(24. 4.)	bark	4.9	7.9	8.5	0.02
May	leaves	14.5	11.8	3.6	0.3
(28. 5.)	bark	12.8	15.0	4.5	0.2
August	leaves	0.4	0.3	0.4	0.04
(20. 8.)	bark	0.02	0.3	0.3	<0.01
February	leaves	0.7	0.5	0.7	0.03
(10. 2.)	bark	0.05	0.5	0.6	<0.01

* Controls contained growing leaves.
Standard deviation measured from 8 of the samples by 3, resp. 4, separate investigations is in the range ± (0.08–0.15 Bq/mg).

Results

Total ^{14}C-incorporation at different seasons

Total ^{14}C-incorporation (Table 1) immediately after termination of the $^{14}CO_2$-feeding, which can be taken as a measure of the assimilation rate, was highest in the April experiment during the main growth period of the tree. When the fixation rate of this experiment is considered as 100 %, 4 weeks later (end of May) we found 96 %, during the summer drought period in August 3 % and in winter (February) 5 %. The CO_2-uptake was very low in the summer experiment in spite of the cuvette-climate existing in the exposure chamber during the 3 h feeding period. Also, in the cool season the assimilation rate was remarkably low and less than what we expected. In the bark, the initial activities were higher in May than in April; for summer and winter the results were comparable to those found for leaves.

To obtain some information on the incorporation of ^{14}C into structural components of the leaf-tissue, the activity of water soluble compounds, total lipids and starch was summed up. The total initial activity of these compounds was highest in the May experiment. If for reasons of comparison we consider the activity of this fraction in April as 100 %, the May value reached about 150 %. In April, the twigs of our sample contained growing tissues (the young leaves were removed, but growing tips were still present). Therefore, a greater part of the produced assimilates was metabolized to structural components, e.g. new cell walls. In May, growth had terminated in the twigs used for the experiment. In the summer experiment the activity of soluble compounds and starch was 19 %, and in February it reached 58 % of the April value. From a comparison of relative values (percentages) of the total activities, one can conclude that in summer and winter the labelling of the structural components is weaker than during the growth period in April.

The dynamics of labelling during the 6-day-period of the experiments showed that metabolic rates were very low during the summer drought and low in the cool, though rather humid winter, but high during spring. In April, in the main growth phase, the activity in the leaves during the course of 6 days declined to about 50 %, whereas in the bark, due to export from the leaves, the activity doubled in this period. A high translocation rate was also recognized from the labelling of the control leaves outside the exposure chamber. About 30 % of these control leaves were growing and therefore acting as sinks for assimilates. In May, the decrease of ^{14}C in the leaves was still more distinct (to about 25 % in 6 days). The leaves were then predominantly producing for export. In the bark, we initially found a rise and then a decline of activity, which may be explained by the transport function. In the summer experiment, the weak initial activity in the leaves was not altered significantly. The same is true for the experiment in February, but labelling was somewhat higher than in August in all samples; hence, we may infer that the reduction of metabolism is more distinct in the summer than in winter.

Specific activities of labelled compounds

The contents of sugars, starch and lipids in the samples were in general similar to those reported by Diamantoglou and Meletiou-Christou (1977, 1978), but some minor differences should be mentioned. In leaves, the starch content in our experiment was approximately the same in February and April (2.7–2.9 %). The total lipid content of leaves in February (about 5 %) and in April (about 5 %) was less than reported earlier. In May, the content of sugars in the bark was higher and that of starch was lower than described.

The specific activities of the compounds investigated are shown in Fig. 1. To calculate rough values of the relative specific activities of the fraction of «other soluble compounds», mucilages and proteins were precipitated; the remaining activity in the supernatant was then very low. Because mucilages (carob gum) share a relatively great part of this fraction but were labelled weakly (apart from the growth period), the specific activity of this fraction should be taken only as a rough value.

A comparison of the labelling pattern in the different seasons shows that in May and August sucrose had the highest activity of all compounds, but in February and April glucose and fructose both reached higher values. The incorporation of ^{14}C in the lipid fraction was weak in all cases. The «other compounds» showed a distinct labelling in April and also in February, but a very low value in August.

Looking at the dynamics of the specific activities during the period of 6 days, we found the expected decrease in sucrose in all cases, which was least distinct in April. In summer the monosaccharides showed only a weak decline. The specific activity of starch remained remarkably stable, not only during one experiment (that may be expected for a long-term storage compound), but also throughout the different seasons. Starch in the control leaves outside the chamber after 144 h had approximately the same activities as the starch of the leaves inside. In August, there seemed to be no metabolic turnover of the starch after it had been synthesized (perhaps the rather high rate of synthesis in August is a consequence of the chamber climate). In the total lipids, significant variations of the specific activities are observed only during the growing season. The low activities in August and

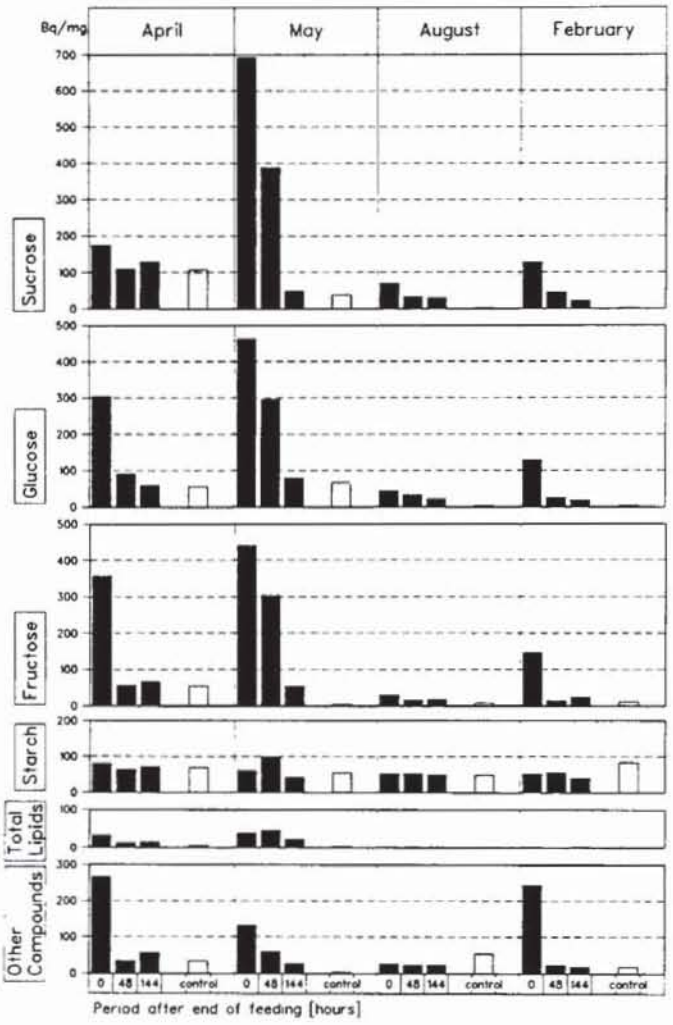

Fig. 1: Specific ^{14}C-activity (Bq/mg) of several compounds in leaves of *Ceratonia* at different periods after the end of feeding $^{14}CO_2$ to twigs. Feeding period: 3 h. Control: leaves from outside the exposure chamber, 144 h after termination of feeding. The fraction of «other compounds» contains mucilages and proteins. Standard deviations are too small to be presented in the figure (s.d. as Bq/mg: sucrose ± 8.2; glucose ± 14; fructose ± 13.5; starch ± 5.5 lipid fraction ± 6.3). In August and February the activity of the lipid fraction was in the range of standard deviation.

February (less than 1 % of the initial total activity) point to a very weak metabolic rate. There was no correlation to the total lipid content, which was high in February and low in August. The heterogeneous group of other soluble compounds in all seasons except summer showed a rapid loss of activity due to compounds with rather intense metabolism. In August, the turnover of this fraction was very low.

A similar conclusion to a weak turnover in August can be drawn from calculation of the total activities of soluble compounds expressed as percentages of the initial values after the 6 days of experiment. The decrease in April led to about one third of the initial value, in May to about 15 %, in February to 20 % and in August to 65 %. In April, the retention of growing tissues seems to be important. The turnover in February was remarkably high regarding the low CO_2-fixation rate.

In bark (Table 2), only soluble sugars attained a high labelling rate. In April, the activity seemed to be retained, whereas

Table 2: Specific ^{14}C-activity (Bq/mg) of several compounds in bark of *Ceratonia* at different periods after the end of feeding $^{14}CO_2$ to twigs.

experiment	period	total sugars	starch	total lipids
April	0 h	132	39	n.s
	48 h	131	69	19
	144 h	123	73	23
	control	n.s.	7	n.s.
May	0 h	225	21	n.s.
	48 h	237	29	12
	144 h	42	10	9
	control	n.s.	n.s.	n.s.
August	0 h	n.s.	n.s.	n.s.
	48 h	5	n.s.	n.s.
	144 h	6	9	n.s.
	control	n.s.	8	n.s.
February	0 h	n.s.	n.s.	n.s.
	48 h	10	n.s.	n.s.
	144 h	10	6	n.s.
	control	n.s.	6	n.s.

n.s.: Difference between measured activity and background less than standard deviation.

in May a peculiar decline from the 2nd to the 6th day was observed. The total lipids – as found for the leaves – showed a significant turnover only in April and May. The labelling patterns in the bark and their variations can be explained by the transport processes. The transport rates were low during the periods of reduced metabolic activity. From the data obtained for leaves and bark in February one may conclude that there are rather intense metabolic processes occurring within the leaves. However, transport out of the leaves was very weak, which is in accordance with the observed low CO_2 fixation rate.

Labelling patterns of compounds (as percentage of total activity)

Calculation of percentages of specific activities in the allocated compounds gave further information on storage processes at different seasons. The initial labelling of starch was higher in August (40 % of total ^{14}C of soluble compounds) than in the other seasons (8 – 13 %). For the lipid fraction, in May a rise in the percentage of ^{14}C during the 144 h experimental period from 5 % to 15 % was found; however, in the other experiments the percentage of activity was not altered significantly. Soluble sugars showed a decline of activity (as percentage) during the 6 days, but it was distinct only in May, probably caused by a more effective transport of the leaves. As expected, the percentage of labelling of starch rose in all experiments, predominantly during the first 2 days.

Discussion

Ceratonia, in spite of growing in the driest areas of the mediterranean region, is not a very sclerophyllous species.

The young leaves are vulnerable to water stress, so that leaf growth has to be completed before the dry season commences (Lo Gullo et al., 1986). We found high rates of photosynthesis during the growth period and low values in winter and summer, thus confirming the results from *Arbutus* obtained by Beyschlag et al. (1986) and very recent results from *Ceratonia* (Nunes et al., 1992) using an IRGA-method. From their data these authors conclude that in winter the photosynthetic rate may be low due to low temperature and in summer due to the severe drought. We also observed the highest rate of photosynthesis in adult leaves of *Ceratonia* during the period of intense growth. During the feeding-experiment in August, the stomata of the leaves inside the exposure chamber were open (as proved by microscopy) and transpiration took place (seen from a rise of air humidity in the chamber); therefore, the low assimilation rate cannot be attributed to a tight stomatal closure. Non-stomatal mechanisms of the reduction of photosynthetic rates already were discussed by Larcher et al. (1981) for *Olea* and then, in a more general way comprising the data from literature, by Farquhar and Sharkey (1982) and Farquhar et al.. (1989). A shift in the physiological response of photosynthesis to drought is not influenced by short time changes of leaf water potential (Gollan et al., 1985; Tenhunen et al., 1985), which in the August experiment could have been effective in the exposure chamber. However, there seem to be major influences of soil water content (Sharp and Davies, 1989). Nunes et al. (1992) showed that in *Ceratonia* a marked reduction in photosynthesis rate occurred only after depletion of all the available water in the upper soil layer. According to Tenhunen et al. (1990), such physiological adjustments lead to large savings of water and to a more efficient water use under severe stress; if so, they may be substantial in terms of carbon gain.

Variations in the labelling of sugars in *Ceratonia* leaves are comparable to those described for leaves of growing and non-growing *Populus* plants (Dickson and Nelson, 1982). During the growth period, most of the ^{14}C initially found in sugars is metabolized or exported. Therefore, in *Ceratonia* after 144 h the activity of sugars in non-exposed leaves of the branch was as high as in the $^{14}CO_2$ fed leaves.

A function of lipids as storage compounds is not clear and an accumulation, as described for *Ceratonia* by Diamantoglou and Meletiou-Christou (1977), is not in all cases a true storage (Hetherington et al., 1984; Chapin et al., 1990). Fully expanded leaves of *Glycine* show a slow lipid turnover (Kagawa and Wong, 1985) and we obtained comparable results for *Ceratonia* in spring. The metabolic rates of lipids in *Ceratonia* in the summer and winter experiments almost may be neglected. Perhaps only a fairly small portion of the lipid fraction (e.g. the membrane lipids) has a high turnover rate and other components are rather inert. Therefore, an important portion of the fraction may not be used as readily available resources. Larcher and Thomaser-Thin (1988) showed that the lipid accumulation in sclerophylls is not influenced by environmental factors. From our experiments, a function of components of the lipid fraction as long term storage substances with only a weak metabolism cannot be fully ruled out. However, in any case they are less important as an energy storage than carbohydrates. Because of their weak turnover, the values for maintenance costs for the total lipids as calculated by Merino et al. (1984) seem to be too high for *Ceratonia*, especially in the cool and dry seasons. Perhaps this may also be true for other sclerophyllous species.

Severe stress conditions may cause a reinforcement of cyclic metabolism. Under conditions of drought and high light intensity in sclerophylls, a recycling of CO_2 by photorespiration could maintain carbon metabolism behind closed stomata. This mechanism provides a sink for the dissipation of photochemical energy, preventing damage of the photosynthetic system (Osmond et al., 1980). Anoxic stress may cause cyclic metabolic processes with participation of lipids (Kennedy et al., 1992). The decrease in lipid content in leaves of *Ceratonia* during the drought period led us to suppose that degradation of lipids could increase the CO_2-production for recycling (Diamantoglou and Kull, 1982). Our labelling experiments described here do not give any indication of such a process. Our results in *Ceratonia* do not suggest a specific adaptation of the metabolism of storage compounds to summer drought. Biophysical mechanisms may be important for drought stress tolerance of leaves (Wyn Jones and Pritchard, 1989). According to recent results from *Ceratonia* leaves (Nunes et al., 1989) such mechanisms, as e.g. a change in the elastic modulus of cell walls, may be efficacious for avoiding the results of severe water stress.

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