Title

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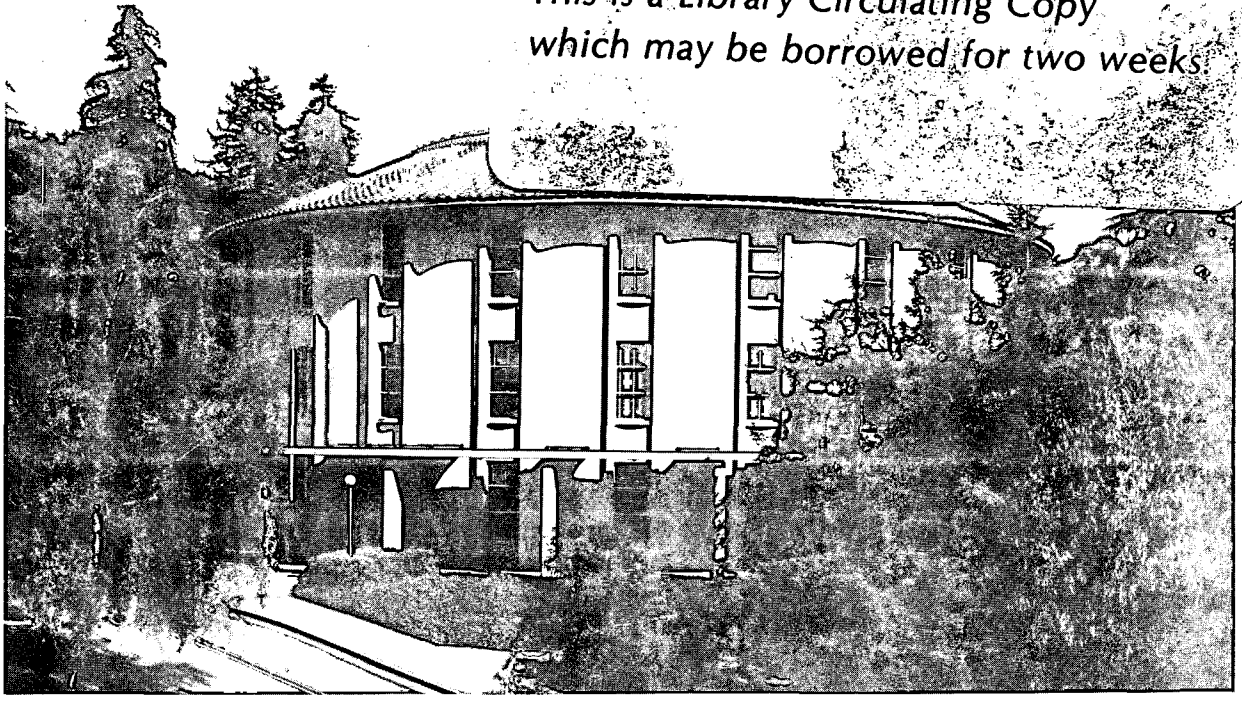

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DISCLAIMER

THE EFFECT OF SALINITY ON THE ALLOCATION OF CARBON TO ENERGY-RICH COMPOUNDS IN EUPHORBIA LATHYRIS

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ABSTRACT

Hydroponically-grown Euphorbia lathyris plants were exposed to increasing levels of NaCl to study the effect of salinity on carbon allocation within the plant. Salinization caused a decrease in overall growth and an increase in the percentage of both hydrocarbons and sugars. The hydrocarbon fraction, containing mostly triterpenoids, increased by 50% and the sugar fraction, containing mostly sucrose, was increased by 88%. This resulted in a shift of available biomass from lignocellulose to the more usable sugars and hydrocarbons.

A two-fold increase in the activity (per leaf area) of the enzyme β-Hydroxymethylglutaryl-Coenzyme A Reductase was also observed with increased salinity. This enzyme is involved in the biosynthesis of the triterpenoids, and its response to increased salinity indicates a role for this enzyme in the regulation of plant hydrocarbon productivity.

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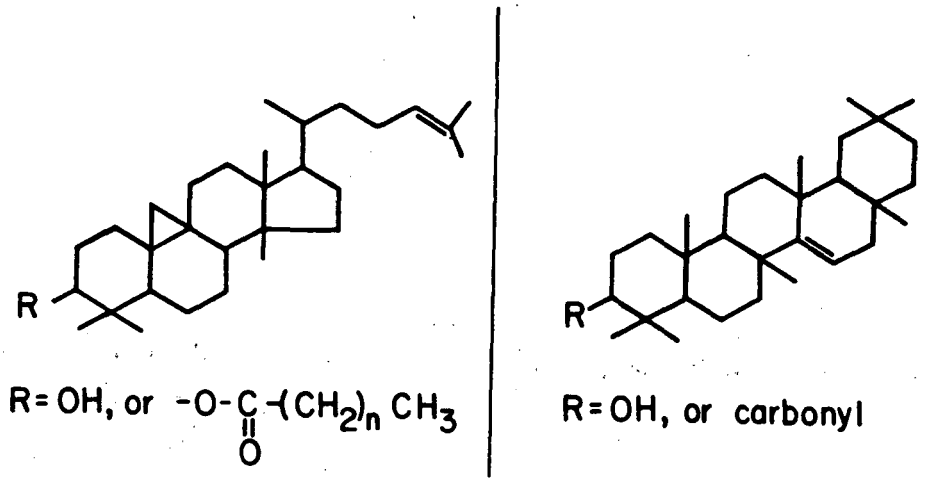
INTRODUCTION

We are studying the possibility of using plants as a source of renewable liquid fuels to replace petroleum. Although alcohol fuels can be generated by the fermentation of plant sugars or lignocellulose, our major concern has been with the exploitation of plants naturally high in hydrocarbons (1).

The plant hydrocarbon fraction, made up of isoprenoids, triglycerides, and free alkanes is lower in oxygen content than the other plant constituents and thus has a higher energy value (on a per weight basis). The isoprenoids and free alkanes have about 2.5x and the triglycerides have about 2x the energy content of cellulose (10). These components are also easily converted into gasoline- or diesel-like fuels. For example, the major hydrocarbon components of the Euphorbia lathyris plant, the pentacyclic and tetracyclic triterpenoids, can be converted to gasoline-type fuels by cracking on a zeolite catalyst column (Figure 1).

To increase the yields of plant hydrocarbons to the point that they offer an economical alternative to petroleum fuels, we must first understand the basic mechanisms that control plant hydrocarbon production. Our laboratory has been studying the biosynthesis of isoprenoids from photosynthetically reduced carbon. Specifically, we are interested in a) the conversion of acetate through the isoprenoid biosynthetic pathway to the triterpenoids (Figure 2), and b) the mechanism that regulates carbon flow into this pathway. It is possible that these two processes are related and that the rate of carbon flow through the pathway will control the rate of carbon allocated to the pathway. The experiments described in this paper were performed to examine this possibility.

We utilize Euphorbia lathyris plants for most of our studies. These latex-bearing plants contain high levels of low molecular weight, reduced isoprenoids (approx. 5%), in addition to high levels of free sugars (approx. 20%). Latex can be tapped from these plants, and it maintains its biologial activity, providing a convenient material for the study of isoprenoid biosynthesis. This plant has been proposed as a candidate for cultivation on energy farms (1).



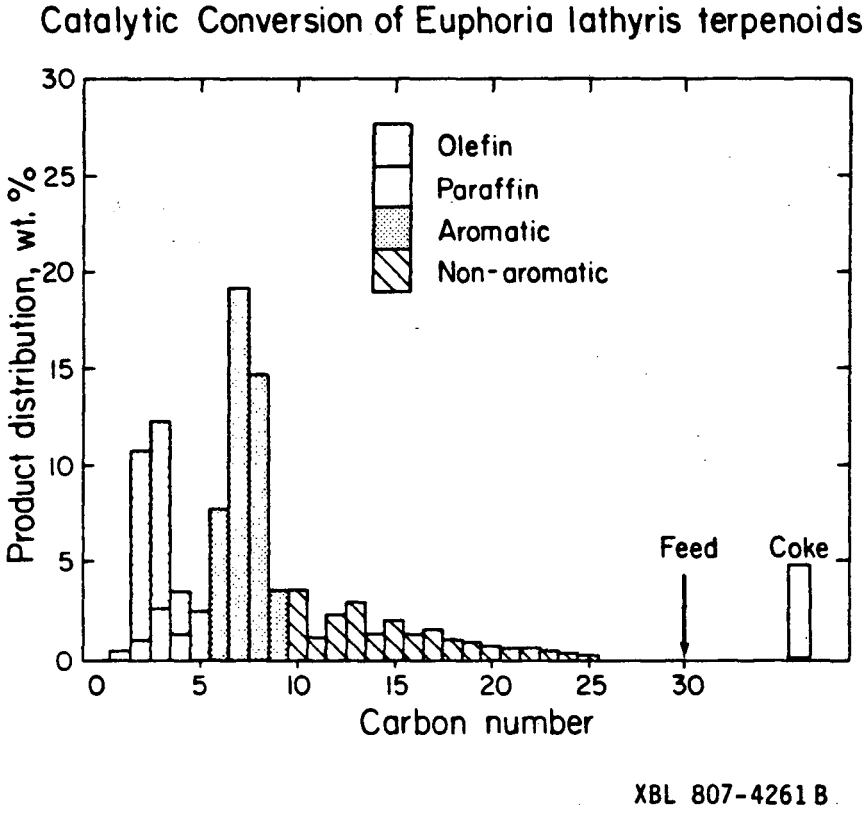


Figure 1. (top) Representative structures of the tetracyclic and pentacyclic triterpenoids found in Euphorbia lathyris. The structures shown are cycloartenol (left) and taraxerol (right). (bottom) Catalytic conversion of triterpenoids on a zeolite column. (figures from reference 6).

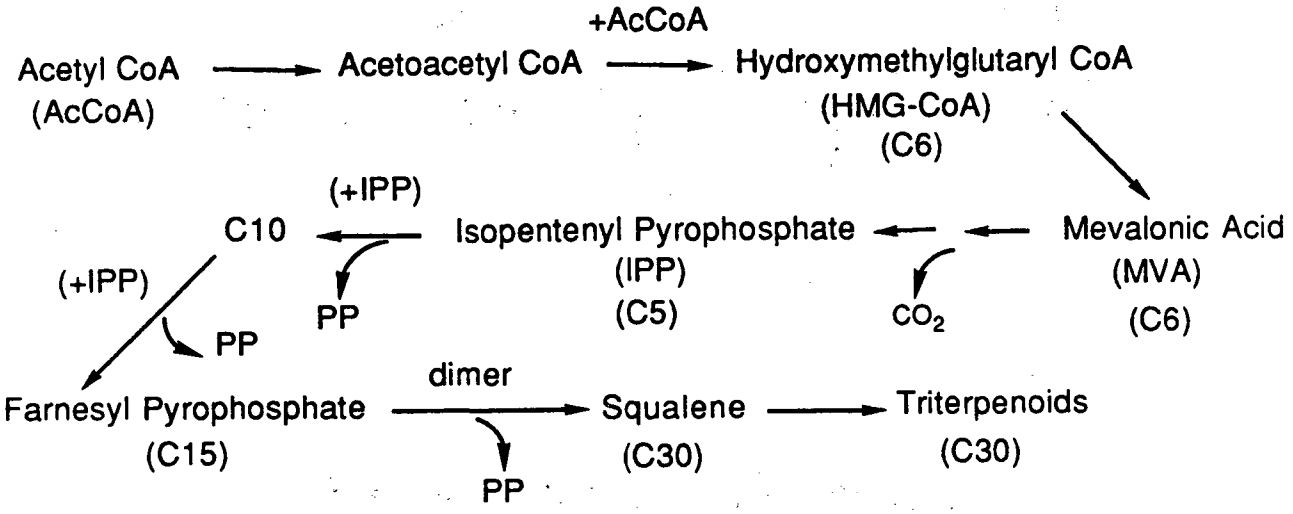


Figure 2. The pathway of triterpenoid biosynthesis from acetyl-CoA.

We have used salinity stress to study carbon allocation because previous reports have shown that carbon allocation to plant secondary metabolites can be increased by the application of environmental stress (2). In many stress situations, the growth processes are affected before photosynthesis is reduced. Such a condition would result in a decrease in carbon demand by the growing regions while photosynthesis remained high, making more carbon available for allocation to secondary plant products. We utilized such conditions to increase carbon allocation to the plant isoprenoids and studied the changes in metabolism that accompanied increased hydrocarbon production. By using this approach we hoped to identify the underlying processes that control hydrocarbon biosynthesis. Such information is necessary before we can manipulate the plant genome to engineer high hydrocarbon-producing varieties.

MATERIALS AND METHODS

Growth Conditions

Euphorbia lathyris seeds were germinated in sand and were watered daily with half-strength Johnson's nutrient solution. The seedlings were then transferred to pots in growth chambers and were grown hydroponically in a modified Johnson's nutrient solution (the phosphate level was reduced to 0.5 mM). The plants were exposed to 16 hours of light at 25°C followed by 8 hours of darkness at 19°C. Plants were grown for 10 weeks before salinity treatments were started.

Salinity stress was applied by the addition of 25 mM increments of NaCl to the nutrient solution every four days. The final concentrations of the treatments were 0 mM (control), 25 mM, 50 mM, 100 mM and 200 mM added NaCl. Three plants were used for each treatment. The plants were harvested two weeks after the final NaCl addition.

Analysis of Plant Material

The effects of salinity on growth were determined from changes in shoot length, total fresh weight, and total and shoot dry weights. Dry weights were obtained by oven-drying plants at 70°C for four days. Changes in the levels of energy-rich compounds were determined as described previously (5,6). Dried plant material was ground in a mill and extracted in a Soxhlet apparatus with boiling heptane for 24 hours to remove the hydrocarbon fraction. Remaining sugars and amino acids were then extracted with boiling methanol for 8 hours. Solvents were removed from the extracts by flash evaporation. Only the shoots were used for these

extractions, as they would be the only part of the plant available for harvest.

β-Hydroxymethylglutaryl Coenzyme A Reductase Assay

Fresh shoots were harvested from undried plants, cut into small sections, mixed 1:5 (w/w) with cold buffer (100 mM potassium phosphate, 10 mM DTE, 30 mM EDTA, pH 6.8), and ground in a hand homogenizer. The extract was incubated for two hours with ^{14}C-HMG-CoA (0.6 mM, 2.2 mCi/mmole) and 2.5 mM NADPH, then quenched with HCl. The resulting product, mevalonolactone, was separated by silica gel TLC chloroform,acetone 2:1)and further purified by HPLC using an organic acid column in 0.0025 N H_2SO_4. Incorporation was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Salinity and Growth

The results indicated that growth of E. lathyris plants was very sensitive to salinity, as levels as low as 50 mM NaCl resulted in reduced growth (Figure 3). Shoot length decreased about 20% and dry weights decreased 30-50% over the range of salinity treatments, while the most abrupt change occurred between 25 and 50 mM NaCl. An examination of the photosynthetic capacity of the plants showed that salinity had a much smaller effect on photosynthesis than it did on growth (unpublished data, manuscript in preparation). These data suggest that the demand for carbon for growth would decrease, while photosynthetic carbon production would remain high, resulting in increased carbon available for allocation to the hydrocarbon and free sugar fractions.

Such a change in carbon allocation patterns did occur. Salinity treatments caused an increase in both the methanol (sugar and amino acid) fraction and the heptane (hydrocarbon) fraction, with a concurrent decrease in the residue, or bagasse fraction (Figure 4). These results differ from those of Ventas et al. (9), who reported that exposure to water stress increased the sugar content but not the hydrocarbon content of E. lathyris plants.

Hydrocarbon Production

The heptane fraction increased by 1.5x, from 4.57% to 6.86% of the total plant dry weight. Though the actual energy increase per plant is small, these results indicate that the plant can regulate its hydrocarbon production. Nemethy et al. (5,6) reported that the major components of

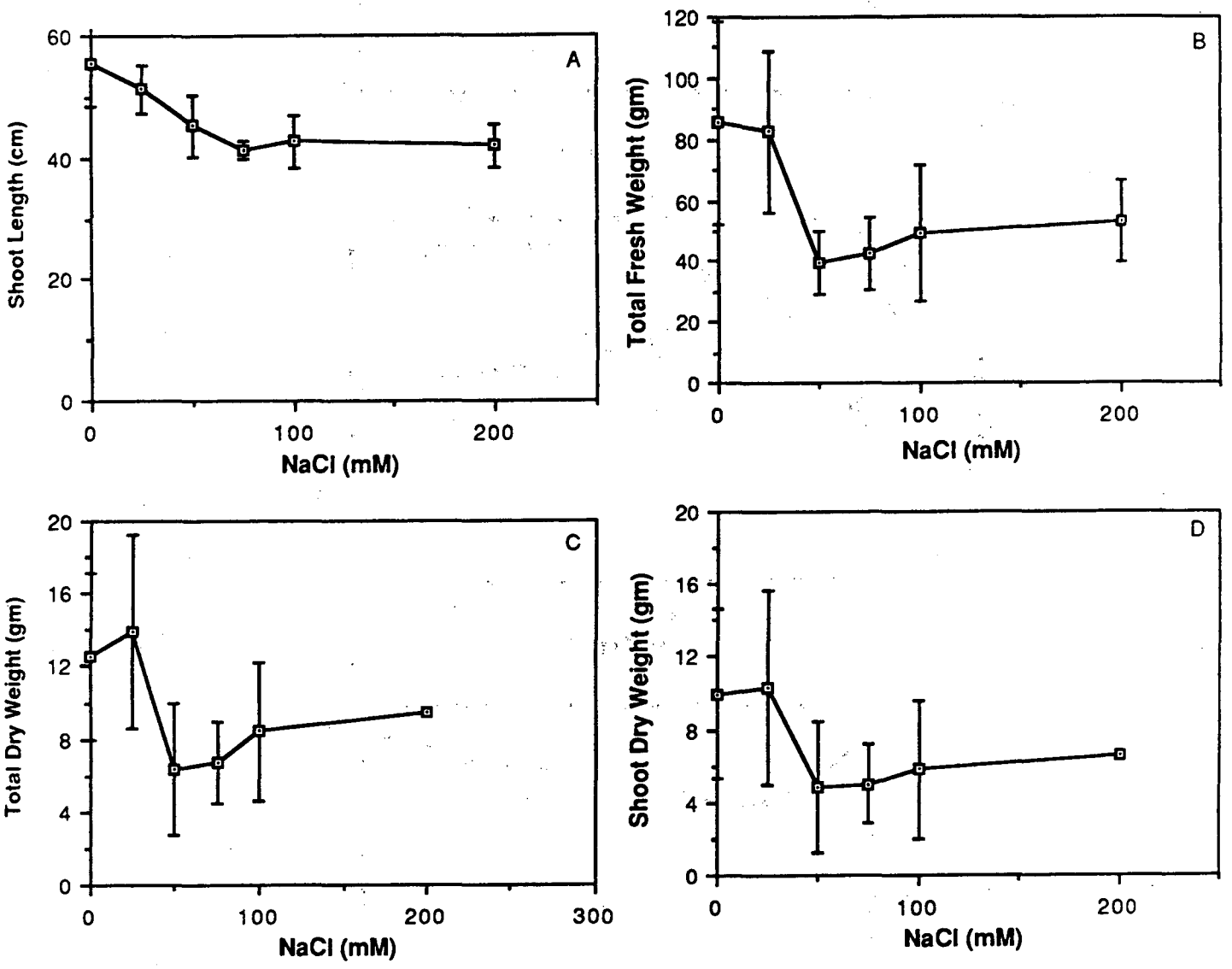
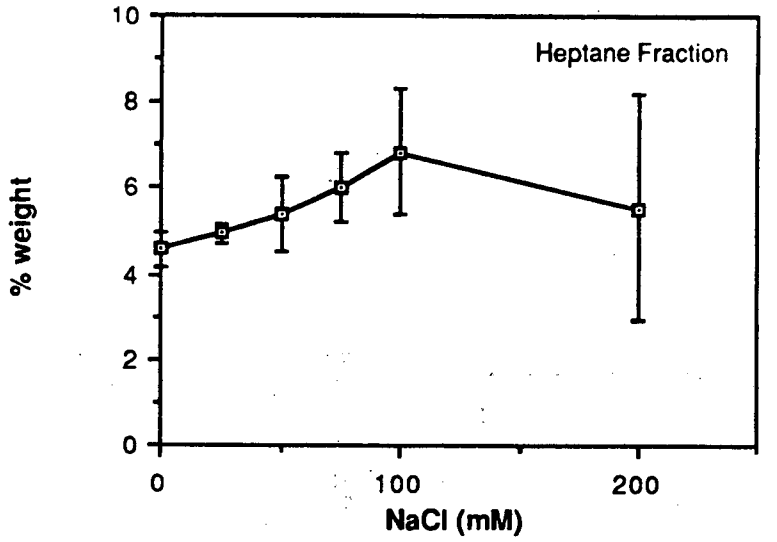
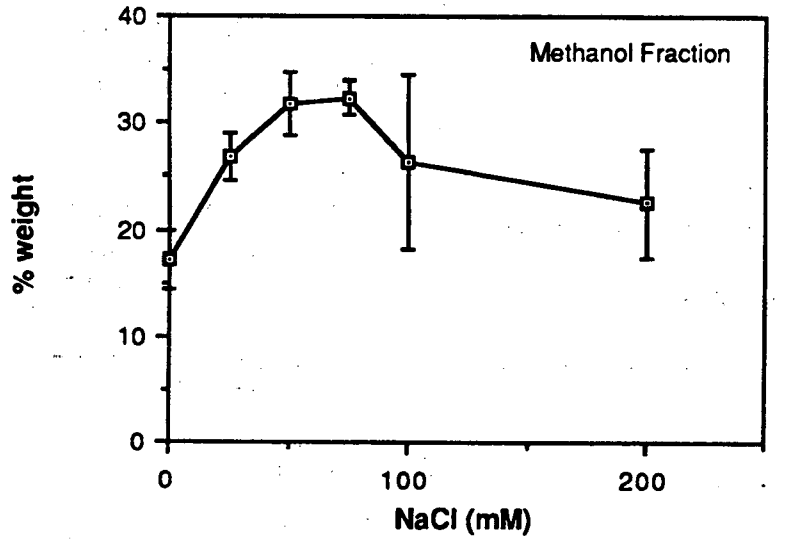


Figure 3. The effect of salinity treatments on the growth of Euphorbia lathyris plants. A) Shoot length, measured from top of nutrient solution to growing point on top of stem; B) Total fresh weight of plants, including both roots and shoots; C) Total dry weight of plants, measured after drying at 70 C for 4 days; D) Shoot dry weight, comparable to the portion of the plant that would be harvested in a field situation. (Error bars represent standard deviations, n=3)





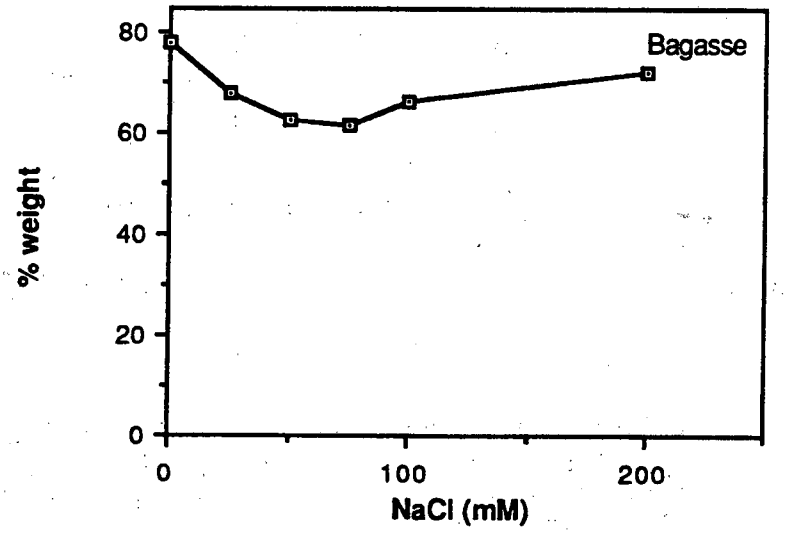


Figure 4. The effect of salinity on carbon allocation. The major constituents of the heptane extraction are hydrocarbons. The major constituents of the methanol extraction are sugars and amino acids. The bagasse fraction was calculated from the total weight minus the combined weights of the heptane and methanol fractions.

the heptane fraction were triterpenoids and their fatty acid esters. We have examined the processes involved in biosynthesis of the triterpenoids to determine how production is controlled and how salinity affects these controls.

Previous work by our laboratory (8) (Figure 5) has shown that a key rate-limiting step in triterpenoid biosynthesis is the conversion of β-Hydroxymethylglutaryl-Coenzyme A (HMG-CoA) to mevalonic acid (MVA) (see Figure 2). This step is catalyzed by the enzyme HMG-CoA Reductase (HMGR). We determined the activity of this enzyme in extracts made from shoots of plants exposed to 0, 25 and 50 mM NaCl. We observed that increased salinity caused an increase in the activity of HMGR, especially between 25 and 50 mM NaCl (Table I). We interpret these results as indicating a strong correlation between the activity of HMGR and the rate of hydrocarbon production in E. lathyris plants.

We are currently involved in a project to isolate and purify HMGR, with an ultimate goal of isolating the gene(s) coding for it. The work presented here and the work of Maurey et al. (4) with the alga Ochromonas mathamensis indicate that it may be possible to increase hydrocarbon production by increasing the expression and activity of this enzyme.

Carbohydrate Production

The methanol fraction has been found to contain 67% sugars (sucrose, galactose, glucose and fructose) and 10% amino acids. (5,6) Elemental analysis of the methanol fractions isolated from the salinized plants showed that there were no significant changes in the C/N ratio, indicating that the relative concentrations of sugars and amino acids remained unaffected by salinity. Since we have previously shown that this fraction is easily converted into ethanol with no further purification (6), the shift of carbon allocation from the cellulose to the free sugars is an improvement in the usability of the biomass.

Total Energy Content

The total energy content of the plant (on a weight basis) remained unchanged. Energy content can be calculated from an elemental analysis of the plant material. A determination of the amount of oxygen required to burn a gram of sample is made (the "R" value), and this can be converted to joules per unit weight by comparison with known standards(7). This was done with 0, 25 and 50 mM NaCl samples, and little change was seen in their energy content (Table II).

Steps along Pathway	Substrate Incorporation (nmol/100µl/hr)	Triterpenoid Equivalents (nmol/100µl/hr)
Acetate----------Sterols	0.02	0.001
MVA------Sterols	0.55	0.09
HMG---MVA	0.02	0.003

Figure 5. The rates of various steps along the triterpenoid (sterol) biosynthetic pathway. To help compare rates, substrate incorporation was converted into the equivalent rate of triterpenoid biosynthesis (Triterpenoid Equivalents). (figure from values reported in reference 8)

Table I Effect of Salinity on the Activity of β–Hydroxymethylglutaryl Coenzyme A Reductase (HMGR)

Treatment (added NaCl)	HMGR Activity (m moles m^{-2} hr^{-1})
0 mM	1.57 ± 0.10
25 mM	1.31 ± 0.04
50 mM	2.91 ± 0.52

(extracts were made from whole leaves; n=2;
± values are standard deviation)

Table II Effect of Salinity Treatment on the Energy Content of Dried Plant Shoots

Treatment (added NaCl)	"R" Value (g oxygen g fuel^{-1})	Agronomic value (GJ ton^{-1})
0 mM	1.33 ± .03	18.5
25 mM	1.34 ± .03	18.6
50 mM	1.36 ± .04	18.9

(shoots were ground in a mill and C, H, N, and ash content was determined. R values were determined from this elemental analysis as described in (7); n=3; ± values are standard deviations)

This occurred because most of the difference in carbon allocation resulted from a reduction in the bagasse fraction (high in lignocellulose) and an increase in the methanol fraction (high in sugars). Lignocellulose and sugars have the same energy value, though the limitations of present conversion technologies make the sugars a more valuable product than the lignocellulose.

It is difficult to apply the results obtained with plants grown in hydroponic solutions in growth chambers to field grown plants, but this system can be used as a model to demonstrate how changes in carbon allocation can result in large increases in available liquid fuel. For example, a projection of liquid fuel yields can be made for a field of control plants (0 mM NaCl) and a field of slightly stressed plants (25 mM NaCl). Since the data indicate that little change occurs in growth between these two treatments, we can use Kingsolver's values (3) for optimum E. lathyris biomass yields of 15 tons of dry biomass per hectare for both treatments. We then assume that the liquid fuel will be generated from only the extractable hydrocarbons and sugars. The total hydrocarbon content of the control and 25 mM treatments was determined to be 4.57% and 4.95%, or 0.686 and 0.743 metric tons per hectare, respectively. From elemental analysis of the heptane extract (7), we can determine that this fraction has an average energy content of 3.18 g oxygen per g fuel, or 44.4 GJ per metric ton. Thus the energy value of the hydrocarbons in the control plants would be 0.686 tons per hectare x 44.4 GJ per ton, or 30.4 GJ per hectare, and the yield from the 25 mM plants would be 33.0 GJ per hectare.

The same type of calculations can be made for the extractable sugars. The methanol extractable content of the control and 25 mM treated plants was 17.2% and 26.9%, or 2.58 and 4.03 tons per hectare, respectively. We have previously determined the efficiency of conversion of the methanol fraction to ethanol (6), and can thus estimate the yield of ethanol production for the 0 mM plants to be 0.69 tons per hectare and for the 25 mM plants to be 1.08 tons per hectare. Ethanol has an energy content of 1.96 g oxygen per g fuel, or 27.6 GJ per ton, so the ethanol energy yields would be 18.8 GJ per hectare for the 0 mM plants and 29.4 GJ per hectare for the 25 mM plants. The total liquid fuel yield (hydrocarbons + ethanol) for the 0 mM plants would be 49.2 GJ per hectare, and for the 25 mM plants the yield would be 62.4 GJ per hectare. So if one can extend the growth chamber results to a field situation, it should be

possible to increase liquid fuel yields by the appropriate application of environmental stress.

In terms of yield per plant the increases in sugars and hydrocarbons was negated by the decrease in above-ground biomass. Only the 25 mM treated plants showed an increase in the total yield of hydrocarbons and carbohydrates (Table III). If salinity stress were to be used as an agronomic technique to increase sugar and hydrocarbon yields in E. lathyris, it should be applied at low levels and probably no more than a few weeks before harvesting.

CONCLUSIONS

We have been able to alter carbon partitioning within a E. lathyris plant by exposure to increased NaCl levels. Growth was reduced and carbon allocation to both hydrocarbon, sugar and amino acid production was increased. The activity of the enzyme -Hydroxymethylglutaryl Coenzyme A Reductase was correlated with the increased hydrocarbon production, indicating a role for it in the control of isoprenoid biosynthesis.

Although there was little change in the total energy content of the plant, the shift of carbon from the bagasse fraction to the hydrocarbon and sugar fraction resulted in an improvement of the yield of liquid fuels from the plant.

ACKNOWLEDGMENTS

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Table III Effect of Salinity on Yields per Plant of Heptane and Methanol Extracables and Residual Bagasse

Treatment (added NaCl)	Heptane Fraction gm/plant	Methanol Fraction gm/plant	Residual Bagasse gm/plant
0 mM	0.46	1.71	7.82
25 mM	0.51	2.77	7.02
50 mM	0.27	1.56	3.08
75 mM	0.30	1.61	3.09
100 mM	0.40	1.53	3.87
200 mM	0.37	1.48	4.75

(values represent the product of the shoot dry weights from Figure 3 and the percentage weights from the fractions in Figure 4)

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