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THE EFFECT OF SUPRAOPTIMAL TEMPERATURES ON THE CONTENTS OF KREBS' CYCLE ACIDS IN *ARABIDOPSIS THALIANA**

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Abstract—The effect of supraoptimal temperatures on the contents of several Krebs' cycle acids in *Arabidopsis thaliana* (L.) Heynh was investigated. Plants grown at the higher temperatures (32° day and 25° night) were lower in dry and fresh weights than those grown at the optimal temperatures (25° day and 18° night). Citric, succinic, fumaric, and malic acid concentrations were measured at 2, 3 and 4 weeks after planting. Although the sum of the amounts of the four acids was generally higher in the shoots of plants grown at supraoptimal temperatures than in those grown at optimal temperatures, at 3 weeks after planting, the reverse was true. This latter result was due largely to changes in the fumaric and malic acid contents. From the second to the third week of growth, the contents of these two acids dropped in higher temperature plants and rose sharply in the optimal temperature plants with an exactly opposite trend occurring from the third to fourth weeks of growth. In the fourth week, the citric acid content decreased to a very low value under optimal conditions and increased under supraoptimal conditions. The results show that malic and succinic acids are strikingly different with respect to compartmentation or to the directness of their association with the Krebs' cycle. The data provide information on changes in organic acid content in shoots through most of the life cycle of a plant, and they further show that temperature can affect organic acid levels at any given time.

INTRODUCTION

ALTHOUGH a large amount of information concerning the effect of supraoptimal temperatures on plant growth and development is available, knowledge of the biochemical effects of such temperatures is less complete. Since much of the relevant data is derived from experiments with algae, fungi, or bacteria, further biochemical studies on higher plants are necessary.

The causes of injuries produced in plants by supraoptimal temperatures are not yet known, although several theories have been proposed.[1] Among those which appear to have some experimental support are: self-toxification by ammonia resulting from intensified proteolysis;[2] uncoupling of respiration and oxidative phosphorylation induced by formation of uncoupling agents;[3] and protein denaturation.[4,5]

In general, very little is known about what specific compounds or, more importantly, about the kinds of metabolism which are particularly sensitive to supraoptimal temperatures in higher plants. Accordingly, in this laboratory we have been examining a number of the major metabolic pathways in higher plants, looking for readily measurable constituents that

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[1] J. LANGRIDGE, *Ann. Rev. Plant Physiol.* **14**, 441 (1963).
[2] N. S. PETINOV and YU. G. MOLOTKOVSKY, *Fiz. Rastenii* **4**, 225 (1957).
[3] YU. G. MOLOTKOVSKY, *Fiz. Rastenii* **8**, 669 (1961).
[4] J. LEVITT, *Ann. Rev. Plant Physiol.* **2**, 245 (1951).
[5] J. LEVITT, *J. Theoret. Biol.* **3**, 355 (1962).

might be consistently and markedly affected by supraoptimal temperatures. In this report attention will be confined to the effect on the amounts of certain Krebs' cycle acids in *Arabidopsis thaliana*.

RESULTS AND DISCUSSION

To determine the proper optimal and supraoptimal temperatures, plants were grown in the different temperature regimes as shown in Fig. 1. It was desired to find a set of day and night temperatures under which the plants would grow best with the other conditions prevailing in our growth chambers (hereafter called optimal) as well as a set of higher temperatures at which the plants would also grow, but demonstrably less well (hereafter called supraoptimal). Fresh weights of plants were determined at 1, 2, 3, and 4 weeks after planting with plants maintained under a variety of night and day temperatures. From the results of

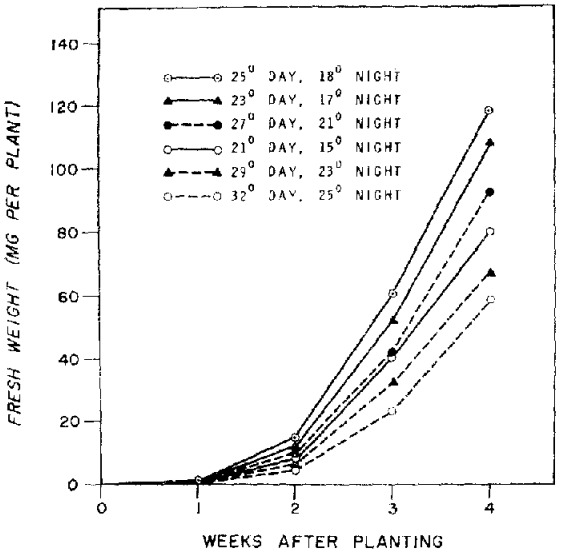

FIG. 1.

these experiments, a regime of 25° day, 18° night temperatures was selected as meeting the above criteria for the optimal, and a regime of 32° day, 25° night temperature was selected for the supraoptimal temperature treatment. Temperatures were maintained within $\pm 1°$; the light intensity was $11{,}900 \pm 2200$ lux.

Supraoptimal temperatures reduced both fresh and dry wt. of the plants at all sampling periods as compared to those of plants grown at optimal temperatures (Table 1).

The effects of supraoptimal temperatures on the amounts of four of the Krebs' cycle acids (citric, succinic, fumaric, malic) in the shoots were measured at 2, 3, and 4 weeks after planting. At each sampling period, the sum of the four acids in the supraoptimal temperature series was different from the amount in plants grown at optimal temperature conditions (Table 2). The differences were significant at the 0·05 level. At the two later sampling periods there were individual acids which were present in significantly different amounts in tissue from the two regimes.

The direction of difference of the total acid concentrations and the identity of the individual

acids differing significantly in amount at the two temperatures was not the same at all sampling periods. The samples taken at 2 weeks and at 4 weeks after planting showed the total concentration (in μmoles/g fr. wt.) of the four acids in the plants at supraoptimal conditions were 21 and 33 per cent higher, respectively, than in the plants at optimal conditions. In contrast, at the 3-week sampling period, the total amount of the four acids was 71 per cent higher in the plants grown at optimal conditions.

TABLE 1. EFFECT OF TEMPERATURE ON FRESH AND DRY WEIGHT OF *Arabidopsis thaliana*

	Average fresh and dry weights per plant			
	Optimal treatment		Supraoptimal treatment	
Weeks after planting	Fresh wt. (mg)	Dry wt. (mg)	Fresh wt. (mg)	Dry wt. (mg)
1	1·0± 0·2	0·08±0·0	0·75±0·1	0·06±0·0
2	14·4± 1·2	1·1 ±0·1	7·2 ±0·7	0·63±0·1
3	62·0± 8·5	4·9 ±0·8	24·9 ±2·5	2·1 ±0·2
4	115·1±12·1	11·9 ±2·1	60·1 ±7·7	7·0 ±1·1

TABLE 2. EFFECT OF TEMPERATURE ON ORGANIC ACID CONTENT OF THE SHOOT OF *Arabidopsis thaliana*

	Weeks after planting					
	2		3		4	
	Treatments		Treatments		Treatments	
Organic acid	Optimal (μmoles/ g fr. wt.)	Supra-optimal (μmoles/ g fr. wt.)	Optimal (μmoles/ g fr. wt.)	Supra-optimal (μmoles/ g fr. wt.)	Optimal (μmoles/ g fr. wt.)	Supra-optimal (μmoles/ g fr. wt.)
Citric	4·8±1·2	5·1±1·8	3·8± 1·0	5·4±1·6	1·1±0·3*	9·2± 2·6*
Succinic	5·1±1·6	5·1±1·7	5·8± 1·6	3·7±1·1	4·3±1·3	5·3± 1·7
Fumaric	8·3±2·6	8·7±2·3	19·1± 4·5*	6·6±1·9*	12·5±3·1	8·9± 2·2
Malic	9·3±1·2	14·4±4·1	12·5± 3·6	8·3±2·1	6·8±2·3*	13·4± 4·3*
Total	27·5±6·7*	33·3±8·5*	41·2±10·9*	24·0±6·8*	27·7±6·3*	36·8±10·3*

* Differences between optimal and supraoptimal significant at the 0·05 level.

The higher total amounts of organic acids seen at two of the three sampling periods in the plants grown at supraoptimal temperatures might have been an indirect consequence of an increase in free amino acids which others have suggested may result from protein breakdown at high temperatures.[2,6,7] Excess amino acids could enter the Krebs' cycle via α-keto acids. These possibilities are now being investigated in this laboratory with another plant species.

One possible explanation for the reversal of the total acid contents under two different temperature regimes between the 2-week and 3-week periods is that flowering and maximum

[6] E. H. SHOKRAII, Ph.D. Dissertation, University of Florida, Gainesville, Florida (1965).
[7] J. B. ANASTASIA, M.S. Thesis, University of Florida, Gainesville, Florida (1966).

increase in the rate of stem elongation in plants grown under optimal temperature occurred at this time. The increase in stem length during the twenty-first day after planting was approximately equal to the total stem elongation obtained during the first 19 days of growth! However, the increase in stem length of the plants grown under supraoptimal temperatures was much lower than those grown under optimal temperatures during this sampling period. It was the plants grown at optimal temperatures, and at the 3-week sampling period, i.e. precisely those just completing the great growth spurt, that were highest in organic acids. This was a little surprising since periods of rapid protein breakdown rather than those of rapid synthesis had been suggested by Webster[8] as being associated with an increase in free amino acids, amides, and Krebs' cycle acids (in etiolated pea seedlings).

At the 3-week sampling time, fumaric acid was higher in the plants grown at optimal temperatures than in those grown at supraoptimal temperatures. In contrast however, at 4 weeks, citric and malic acids were higher in plants grown at supraoptimal temperatures. The fact that some of the acids fluctuate markedly in amounts, whereas succinic remains nearly constant and at a substantially lower level than malic and fumaric acids, agrees with the results of Steer and Beevers.[9] They found succinic acid to be largely in active turnover pools and not in cytoplasmic or other compartments in corn. On the other hand, Lips and Beevers[10,11] interpret their data on malic acid to mean that a large fraction of this acid in certain tissues is in some cytoplasmic or other compartment not in ready equilibrium with the enzymes involved in its metabolism.

One general significance of the data of this report is that they provide information on changes in organic acid content in shoots through most of the life cycle of a plant and they further show that an environmental variable, temperature, can affect organic acid levels at any time. The results cast some doubt on the usefulness of tables of data purporting to give the organic acid composition of the shoots of this or that species of plant. Our results, at least with *Arabidopsis*, suggest that answers to the questions—"At what stage of growth, and at what temperature?"—should also be given.

EXPERIMENTAL

Preparation of Seeds and Agar Medium

Arabidopsis thaliana (L.) Heynh (Variety PI)* (Cruciferae) was grown on agar medium in test-tubes aseptically. The agar medium was prepared by a method modified from that of Langridge.[12] An agar suspension was prepared by addition of 8·0 g of agar to 1·0 l. of Hoagland's solution[13] and liquefied in an autoclave at 125° and 15 lb/in² for 15 min. 10-ml aliquots were added to 25 × 150 mm test tubes and solidified by placing the tubes in a cool room. The seeds were placed between two filter papers moistened with distilled water in a Petri dish and held in a refrigerator for 24 hr at 4° to ensure more uniform germination of the seeds.[12] Following their cold treatment, the seeds were surface sterilized for 3 min in 10% Chlorox solution after which they were washed several times with autoclaved, cooled, distilled water and transferred to the surface of the medium in test tubes under aseptic conditions in a transfer box. The culture tubes were then placed in growth chambers set to provide either a supraoptimal temperature or an optimal temperature regime. The source of light was a combination of fluorescent (cool white) and incandescent bulbs. Fresh and dry weights of the plants were measured at 1, 2, 3, and 4 weeks after planting. A sufficient number of

* We are indebted to Dr. J. Langridge, Division of Plant Industry, C.S.I.R.O., Canberra, Australia for the original supply of seeds of this variety of *Arabidopsis*.

[8] GEORGE C. WEBSTER, *Nitrogen Metabolism in Plants*, p. 124, Row-Peterson Biological Monographs, Row-Peterson and Co., Evanston, Illinois and White Plains, New York (1959).
[9] B. T. STEER and H. BEEVERS, *Plant Physiol.* **42**, 1197 (1967).
[10] S. H. LIPS and H. BEEVERS, *Plant Physiol.* **41**, 709 (1966).
[11] S. H. LIPS and H. BEEVERS, *Plant Physiol.* **41**, 713 (1966).
[12] J. LANGRIDGE, *Australian J. Biol. Sci.* **12**, 243 (1957).
[13] D. R. HOAGLAND and D. I. ARNON, *Calif. Agric. Exp. Sta. Circ.* 347 (1950).

plants was harvested at each of the time periods for determination of organic acid contents under both temperature regimes. Sampling time was 2 hr after termination of the dark period (i.e. after 2 hr of illumination in all samplings). It should be mentioned that the physiological ages as well as the chronological ages of plants grown at optimal and supraoptimal temperatures were approximately the same at all sampling periods as shown by plastochrome indices.[14]

Extraction and Purification of Organic Acids

Alcoholic extracts were prepared from the shoots of plants at 2, 3, and 4 weeks of age for the determination of citric, succinic, fumaric, and malic acids. Whole shoots (1–2 g) were cut in small pieces and extracted for 5 min in 50–100 ml of boiling 80% ethanol. The extract was cooled to room temperature and filtered through Whatman No. 1 filter paper. The plant residue was homogenized in a glass homogenizer with an additional 40 ml of hot 80% ethanol. This extract was again filtered and combined with the first filtrate; the combined filtrate was concentrated in a rotary evaporator at room temperature. The concentrate was centrifuged at 10,300 × *g* for 20 min at 0°. The supernatant was decanted and the pellet resuspended in a small amount of distilled water. The suspension was again centrifuged and the supernatant combined with that from the first centrifugation. The combined supernatants were reduced to 10 ml and passed through a Dowex 50 × 8 (hydrogen form) cation-exchange column. The eluates from the Dowex 50 columns containing the organic acids were then passed through a Dowex 1 × 8 anion-exchange column in the formate form. The organic acids were eluated from the column with 25 ml conc. HCO_2H followed by distilled water. The eluate was then taken to dryness under reduced pressure and the residue was taken up in an appropriate volume of 10% isopropanol to give 1 ml of solution/g original fresh wt. of tissue.

Quantitative Paper Chromatography of Organic Acids

Concentrated tissue extracts were analyzed by a method modified from that of Luke and Freeman.[15] 50-μl samples of the extracts were chromatographed ascendingly on strips of Whatman No. 1 filter paper (50 × 4 cm) in *n*-butanol–formic acid–water (10:2:15 v/v/v, upper phase). The strips were equilibrated for 5 hr with a portion of the solvent before being developed for 36 hr at 24 ± 2°. The developed paper strips were dried at room temperature for 5 hr after which they were subjected to a series of alternate 10-min periods of forced hot (70°) and cool air to remove all traces of solvent. The paper strips were dipped in a solution of 1 g xylose in 1 ml aniline, 3 ml H_2O and 96 ml MeOH,[16] air dried for 30 min, then heated at 60–70° for 20–30 min. The organic acids appeared as reddish-brown spots on a routinely white background. Quantitative measurements were made by eluting the spots in 50% ethanol and determining the absorbance of the solutions at 395 nm in a Spectronic 20 (Bausch and Lomb) spectrophotometer. Per cent recovery of the organic acids was 98, 88, 83 and 83 for citric, succinic, fumaric, and malic, respectively. All values for tissue level of organic acids given in the tables were corrected by using these values.

Effect of Storage on Organic Acids

In preparing standard curves from solutions of known amounts of organic acids which were stored at −15° in 10% isopropanol, a reduction in the slope of the curves with duration of storage was noticed. This suggested some loss (probably via esterification) in the amounts of organic acids. Experiments showed that the loss was higher when acids were stored in 80% ethanol. Accordingly, storage of standards and extracts in 80% ethanol was eliminated and all the analyses were done immediately after harvesting the plants.

[14] R. O. Erickson and F. Michelini, *Am. J. Botany* **44**, 297 (1957).
[15] H. H. Luke and T. E. Freeman, *Phytopathology* **55**, 967 (1965).
[16] I. Smith, *Chromatographic Techniques*, William Reinemann, Medical Books, Ltd., London Interscience Publishers, New York (1958).