

## Enzymes of Glyoxylate Cycle in Conifers<sup>1, 2</sup>

A. M. Firenzuoli, P. Vanni, E. Mastronuzzi, A. Zanobini,  
and V. Baccari

Department of Biochemistry, University of Florence, Florence, Italy

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**Abstract.** The high level of lipids in seeds of some species of conifers suggested that the glyoxylate cycle might have a role in conifer seed metabolism.

Six species (*Pinus pinea*, *Pinus pinaster*, *Pinus canariensis*, *Pinus strobus*, *Abies alba*, and *Cupressus sempervirens*) were investigated for their lipid content and malate synthase and isocitrate lyase level. The fatty acid composition of the triglyceride fraction was also investigated. The correlation between lipid content of germinating seed with the presence of the cycle was confirmed. The enzymes of the glyoxylate cycle were not detected in *Cupressus sempervirens* where the lipid content is very low.

The presence of the glyoxylate cycle, first demonstrated by Kornberg and Krebs (14), has been shown in bacteria (13, 15), algae (10, 11), fungi (17), and angiosperms (3, 4, 19). Particularly in higher plants it has been detected only in tissues in which fats are being utilized.

The glyoxylate cycle provides a mechanism for synthesis of C<sub>4</sub> intermediates of the tricarboxylic acid cycle from acetyl CoA. Its role in the conversion of fats to carbohydrate has been extensively investigated particularly during the germination of fat-containing seeds (1). Since the seed endosperm of some species of conifers appears particularly rich in lipid content (5, 6), it was supposed that the glyoxylate cycle, which has not previously demonstrated in the systematic group of conifers, might also have a role in the germinating seed of these species. The lipid content of seeds and the levels of isocitrate lyase and malate synthase have been studied. In enzyme extraction Tween 80 was used as a protective agent since the use of this polymer is essential in working with adult tissues (8, 20, 21). The characterization of fatty acids of triglyceride fraction was also investigated, because a study of this composition would probably provide a relation between the enzymes assayed and the material by them utilized as substrate (5, 6, 13).

## Materials and Methods

**Seed Germination.** Seeds of *Pinus pinea*, *Pinus pinaster*, *Pinus canariensis*, *Pinus strobus*, *Abies alba*, and *Cupressus sempervirens* were cultivated on moistened sand at 20° during the 9 hour photoperiod. *Pinus strobus* and *Abies alba* were stratified at 3 to 5° for 3 weeks prior to germination. After a 14 day germination period, the material consisting of endosperm and enclosed cotyledons was collected and used for determination of enzymes and proteins.

**Lipid Extract and Determination.** A portion of dry seeds (time zero of germination) was extracted in an MSE homogenizer with 19 parts of a mixture of chloroform-methanol 2:1 (v/v) by the procedure described by Folch *et al.* (9). A small aliquot of lipid extract was withdrawn and evaporated in an oven at 60° for 24 hours to obtain the weight of total lipids. The separation of the lipid classes in the extract was performed by thin layer chromatography as described by Malins and Mangold (16), identification being achieved by comparison with a pure known standard mixture. Plates 20 × 20 cm were spread with a slurry of 30 g of Silica gel HF 254 (Merck) + 65 ml H<sub>2</sub>O to obtain 250 μ thickness. The developing mixture was petroleum ether-ethyl ether-glacial acetic acid (85:15:1). When the solvent front reached 1 inch from the top, the plates were air-dried and the lipid fractions were visualized by iodine vapor (1% iodine in methanol). When necessary, the fractions were permanently stained by spraying the plate with 50% sulphuric acid containing 1.2% potassium bichromate. The triglyceride fraction was scraped from the plate, eluted with ethyl ether and analyzed for

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fatty acid composition by gas-liquid chromatography of the methyl esters. These were prepared from the lipids dissolved in 0.2 ml benzene by transesterification with 5%  $H_2SO_4$  in methanol under reflux at 70° for 5 hours. Analysis was carried out at 180° on one-eighth inch foot length column packed with 15% DGS on 100 to 120 mesh Gas-Chrom Z, using an Aerograph model 1520 gas chromatograph equipped with a flame ionization detector. Areas under peaks were calculated by triangulation and percentage distributions are given in terms of peak areas. Identification was achieved by comparison with the retention times of known standard and with published values.

**Preparation of Enzyme Extract.** The samples (endosperm with enclosed cotyledons) were homogenized with 1.5 times their weight of a medium containing 100 mM tris pH 7.4, 2 mM EDTA, 10 mM mercaptoethanol and Tween 80 1% (v/v) in an Ultra-Turrax apparatus for 30 seconds in a glycerol bath at -15°. The homogenate was subsequently extracted at 0° for 30 minutes with magnetic stirring. The whole homogenate was centrifuged at 12,000 rpm in a Lourdes centrifuge at 0° and the insoluble fraction was discarded. The fluid supernatant was centrifuged again in a Spinco centrifuge at 45,000 rpm at 0° for 60 minutes. The supernatant was withdrawn and used for protein and enzyme determination.

**Protein Determination.** The total proteins of the enzyme extract were determined by the biuret method according to Beisenherz *et al.* (2). To avoid possible interference of pigments or lipid substances on the final color, the protein precipitate was washed with *n*-butanol before being dissolved in the cupric reagent.

**Enzyme Assays.** Isocitrate lyase and malate synthase were assayed at 25° according to the continuous optical method of Dixon and Kornberg (7) using a Beckman model DB spectrophotometer equipped with a thermoregulated cell-holder and provided with a Sargent model SR recorder. In the determination of isocitrate lyase, any possible interference due to formation of other ketoacid phenylhydrazones in the mixture was checked. In fact

before starting the reaction by the addition of isocitrate, we were sure that no extinction change could be recorded in our experimental condition. Besides, in the isocitrate lyase assay a slight modification of the procedure was represented by use of GSH as activating agent instead of cysteine-HCl (12, 18).

**Materials Used.** Tris and EDTA were purchased from Boehringer and Soehne G.m.b.H., Mannheim; Tween 80 and mercaptoethanol from Fluka AG., Buchs; DL-isocitric acid lactone (allo-free), glyoxylic acid, and acetyl CoA from Sigma Chemical Company, St. Louis, Missouri; DGS (diethylenglycol succinate) and Gas Chrom-Z from Applied Science Laboratories; all other products were obtained from Merck.

DL-Isocitric acid lactone was transformed into isocitrate by saponification with KOH before use.

## Results

Values for the total lipid content of the dry seeds of 6 species are reported in table I. The absence of lipids in *Cupressus sempervirens* is evident.

A chromatogram of the lipid fractions obtained by TLC is reported in figure 1. The more abundant

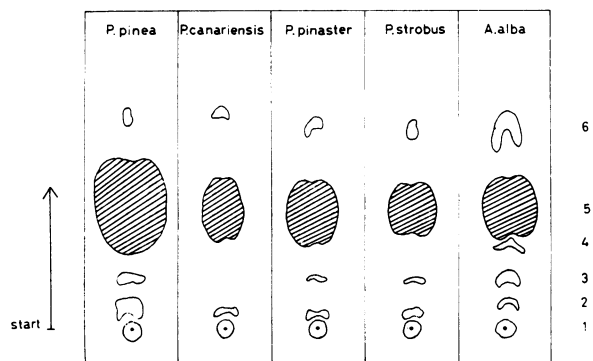


FIG. 1. Chromatogram of total lipids in 5 species of conifers. The spots are identified as follows: 1, phospholipids; 2, sterols; 3, free fatty acids; 4, unknown fraction; 5, triglycerides; 6, sterols esters.

Table I. *Lipid Content, Glyoxylate Cycle Enzymes and Proteins in Seeds of Conifers*

Lipid content was determined at zero time, the enzyme and protein assays were carried out after the 14th day of germination.

Species	Total lipid g/fat-free dry wt	Enzyme activity		Proteins mg/g fr wt
		Isocitrate lyase $\mu$ moles glyoxylate formed per min $\times$ g fr wt	Malate synthase $\mu$ moles acetylCoA utilized per min $\times$ g fr wt	
<i>Pinus pinea</i>	1.05	4.005	3.78	27.40
<i>Pinus pinaster</i>	0.61	0.667	4.25	33.55
<i>Pinus canariensis</i>	0.53	0.89	4.48	41.95
<i>Pinus strobus</i>	1.01	1.065	7.053	58.24
<i>Abies alba</i>	1.00	0.265	5.04	80.08
<i>Cupressus sempervirens</i>	0	0	0	9.10

Table II. Fatty Acid Composition of the Triglyceride Fractions of Five Species of Conifers

Fatty acid		<i>Pinus</i>	<i>Pinus</i>	<i>Pinus</i>	<i>Pinus</i>	<i>Abies</i>
		<i>pinca</i>	<i>pinaster</i>	<i>canariensis</i>	<i>strobus</i>	<i>alba</i>
		%	%	%	%	%
Myristic	(14:0)	0.19	0.11	0.19	0.50	0.37
Pentadecanoic	(15:0)	0.15	0	0	0	0
Palmitic	(16:0)	6.65	4.80	7.11	5.30	3.78
Palmitoleic	(16:1)	0.38	0	0	0	0.71
Stearic	(18:0)	3.44	3.50	5.50	2.60	2.18
Oleic	(18:1)	37.58	20.30	28.50	17.80	30.31
Nonadecanoic	(19:0)	0	0.40	0	1.20	4.92
Linoleic	(18:2)	46.80	52.10	50.20	44.70	42.14
Arachidic	(20:0)	0.68	7.20	1.20	25.00	13.13
Linolenic	(18:3)	1.37	1.70	3.20	0.50	0.63
Eicosadienoic	(20:2)	0	0.37	0	0	0
Eicosatrienoic	(20:3)	0.72	0.50	0.28	0.60	0
Arachidonic	(20:4)	1.99	8.64	3.60	1.30	1.78

fraction is represented by triglycerides, the typical depot fats. Phospholipids, sterols, alcohol esters, diglycerides, and free fatty acids are also present. An unidentified band immediately behind the triglycerides is observed in *Abies alba* chromatogram. The fatty acid composition of the triglyceride fractions is shown in table II. The relative values are reported as percentages. Overall we can observe that the saturated fatty acids are quantitatively less represented. The higher values are those of palmitic and stearic acids. Among unsaturated fatty acids, the predominant ones are almost always linoleic and oleic acids. Only small amounts of other unsaturated fatty acids are present. An exception is *Pinus strobus* where the most abundant saturated fatty acid is arachidic (20:0). This acid also has consistently high values in *Pinus pinaster* and *Abies alba*. The presence of 2 odd-chain fatty acids, C 15:0 and C 19:0, must also be noted. While the first is present only in *Pinus pinca*, the second is found in *Pinus strobus*, *Pinus pinaster* and particularly in *Abies alba*.

The activities of isocitrate lyase and malate synthase and protein content are recorded in table I for the germinating seeds of the 6 species examined at the fourteenth day of germination. While appreciable isocitrate lyase and malate synthase are detectable in *Pinus pinca*, *Pinus strobus*, *Pinus canariensis*, *Pinus pinaster*, and *Abies alba*, they are virtually absent in *Cupressus sempervirens*; this parallels the absence of lipid. Furthermore, the values of malate synthase are nearly constant in all species. The isocitrate lyase values are more variable; the highest values are those of *Pinus pinca*, the lowest of *Abies alba*.

From these results, it is evident that the technique for enzyme extraction employed by us has shown the presence of the glyoxylate cycle enzymes in 5 of the 6 species studied. The data obtained on the lipid content of the seeds, confirm and reinforce the close correlation between the fat content of seed and the presence of the cycle. *Cupressus semper-*

*virens* which is lacking in fats is also lacking in the cycle. At the same time the high content of lipids in *Pinus pinca* agrees with the higher enzyme levels found by us. There was not correlation between enzyme activity and level of a particular one fatty acid. We can only observe that linoleic acid has a higher concentration in all species studied. The presence of the glyoxylate cycle in the systematic group of conifers extends and confirms the large distribution of this metabolic pathway in the plant kingdom.

From a comparative point of view the presence of the cycle in conifers, could confirm that it has a primitive character. Apart from these observations, which necessitate further support, the presence of the glyoxylate cycle in conifers establishes a new systematic level of the same, which could be of considerable importance as a marker in biochemical taxonomy.

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