Phytochemistry, 1978, Vol. 17, pp 581–582. Pergamon Press. Printed in England

OXYGEN INCORPORATION IN THE BIOSYNTHESIS OF *p*-COUMARIC ACID IN SEEDLINGS OF *ZEA MAYS**

GEORGE J. FRITZ† and BRIAN D. ANDRESEN

Departments of Agronomy and Pharmaceutical Chemistry, University of Florida, Gainesville, FL 32611, U.S.A.

(*Received* 5 *July* 1977)

Key Word Index—*Zea mays*; Gramineae; biosynthesis; *p*-coumaric acid; oxygen incorporation.

Abstract—The results of experiments in which seedlings of *Zea mays* were grown in the light in an atmosphere enriched with oxygen-18 indicated that the hydroxyl oxygen atom in *p*-coumaric acid is derived from molecular oxygen.

INTRODUCTION

p-Coumaric acid (4-hydroxycinnamic acid) is widely distributed in plants [1] and appears to have several roles in metabolism. It is an intermediate in the pathway leading to the biosynthesis of coumarins, flavonoids and lignin [2–4], has been shown to serve as a coenzyme for pineapple IAA oxidase [5] and to stimulate the decarboxylation of IAA under conditions where IAA depresses growth [6]. Derivatives of *p*-coumaric acid are present in stigmatic exudates of certain plants [7] and may determine the germination of some pollens [8]. The compound has also been identified as a constituent of cutin [9] and its ester of myo-inositol has been identified in gymnosperm needles, but its role here remains to be elucidated [10].

Although the enzyme which catalyzes the hydroxylation of cinnamic acid to *p*-coumaric acid has been extracted from plant tissues and some of its properties studied [11], no definitive experiments concerning the source of oxygen atoms for the reaction have been reported. This paper is concerned with the demonstration that the hydroxyl oxygen atom in *p*-coumaric acid synthesized by green *Z. mays* is derived from molecular oxygen.

RESULTS AND DISCUSSION

In preliminary experiments, *p*-coumaric acid was isolated from green *Z. mays* which had been grown in an atmosphere containing the natural abundance of oxygen-18. The identity of the isolated compound was confirmed by TLC and MS comparisons with that of authentic material. *p*-Coumaric acid was then isolated from the ethanol-insoluble portion of the shoot–root axes of green *Z. mays* grown in an atmosphere enriched with 10.6 at.% oxygen-18 and analyzed by mass spectrometry. The MS (average of 24 scans) when corrected for natural abundance isotopes indicated that the carboxyl oxygen atoms in *p*-coumaric acid were not labeled and the phenolic oxygen atom was labeled to the extent of 5.3 ± 0.7 at.% The incorporation of an ^{18}O atom into the molecule was apparent by comparison of the labeled and unlabeled MS. The presence of oxygen-18 in the phenolic oxygen atom only was confirmed by comparing the abundance of ^{18}O in the molecular ion region (*m/e* 164, 166) with that in the ($M\text{-}CO_2^+$) ion region (*m/e* 120, 122). Absolute isotopic incorporation determinations were made by averaging and subtracting background noise. Atom per cent enrichment was calculated by standard methods [12].

These results suggest that the phenolic oxygen atom in *p*-coumaric acid is derived directly from molecular oxygen. Because only one atom of each oxygen molecule is incorporated by oxygen fixation into *p*-coumaric acid, the maximum labeling which can be expected in the hydroxyl of *p*-coumaric acid is 5.3 at.% (i.e. one-half of the enrichment of atmospheric oxygen).

To exclude the possibility that the observed enrichment in *p*-coumaric acid was an indirect effect caused by the reduction of $^{18}O_2$ to H_2O during respiration followed by subsequent incorporation of $H_2^{18}O$ into *p*-coumaric acid, it was necessary to analyze the oxygen-18 content of tissue water. The oxygen-18 enrichment

* Florida Agricultural Experiment Station Journal Series No. 647.

†To whom requests for reprints should be addressed.

Table 1. Incorporation of oxygen-18 into tissue water in 7.5-day-old green *Zea mays* grown in an atmosphere enriched with 10.6 at.% $^{18}O_2$

Fraction	Shoot-root axes*		Cotyledons†	
	Quantity of water recovered (ml)	Atom per cent excess‡	Quantity of water recovered (ml)	Atom per cent excess‡
First	0.49	0.1	0.95	0.1
Second	0.35	0.1	0.33	0.0
Third	0.21	0.1	0.22	0.2
Fourth§	0.12	0.0	0.06	0.2

* 3 g fr. wt. † 4 g fr. wt. ‡ The accuracy of measurement was estimated to be ±0.1 at.% excess. § After the fourth fraction was collected, no more water could be recovered from the tissues, even though the vac pump exhausted to a pressure of 0.1 μm Hg (McLeod gauge) and the lyophilization process continued for 12 hr.

in H_2O recovered from both shoot–root axes and cotyledons of *Z. mays* seedlings grown in an atmosphere enriched with 10.6 at.% oxygen-18 were found to be very small, between 0 and 0.2 at.% excess ±0.1 at.%. Therefore it can be concluded that the hydroxyl oxygen atom in *p*-coumaric acid is derived exclusively from molecular oxygen.

EXPERIMENTAL

Growth conditions. Maize grains (*Zea mays* L., Wf9 × 38-11, fertile version) were soaked for 6 hr in H_2O. Then they were sown in vermiculite in a 1 l. conical flask. The vermiculite was wetted with sufficient H_2O at the start of the growth period, so that further additions were not needed. To prevent excessive accumulation of respiratory CO_2, a vial containing 25 ml 40% NaOH was placed upright inside the flask and the contents stirred continuously to prevent the formation of a surface layer of solid Na_2CO_3. The flask was sealed with a rubber stopper fitted with a glass tubing which was sealed with rubber tubing and a clamp. Germination and subsequent growth was under room daylight. O_2 containing 10.6 at.% oxygen-18 was prepared by electrolysis of ^{18}O-enriched H_2O (Miles Laboratories) and mixed with N_2 (vol. 21.79). The gas mixture was introduced into the conical flask, by evacuating the flask to 20 mm Hg and then filling to 1 atm. pressure. Thereafter, O_2 absorbed by the growing seedlings was replaced every 24 hr with pure O_2 containing 10.6 at.% ^{18}O. At the end of the growth period (7.5 days from the time of immersion in H_2O), the coleoptiles (1.5–2 cm) had not been pierced by the primary leaves, which were light green in colour. Cotyledons and endosperm were separated from the shoot–root axes; 23 g (fr. wt) of the latter tissue was harvested, stored frozen and used for the isolation of newly formed *p*-coumaric acid. Preliminary investigation showed that cotyledonary tissue contained relatively large quantities of preformed *p*-coumaric acid.

Isolation and purification of p-*coumaric acid.* Frozen and thawed plant tissue was ground to a slurry in 95% EtOH in a pestle and mortar with sand, the mixture boiled for 20 min and filtered. The ethanol-insoluble residue was then refluxed in N NaOH for 3 hr, cooled, acidified with HCl to pH 2, and was extracted 18 hr with Et_2O. The ether was evapd off and the residue dissolved in 95% EtOH. *p*-Coumaric acid in the extract was isolated by TLC on powdered cellulose (MN 300, Brinkmann Instruments), in 4% HOAc using either diazotised sulphanilic acid or UV for detection. The acid layers were scraped off, eluted with EtOH and re-chromatographed seven times. Only the *trans* isomer (R_f 0.55) was detected on the first plate, but a portion of the trans isomer was converted to the *cis* isomer (R_f 0.7) on each succeeding plate. The *cis* isomer was collected (on the 3rd through 7th plates) and pooled for MS analysis; it was rechromatographed once and then dissolved in $NaHCO_3$, washed with Et_2O, acidified and extracted into Et_2O. The EtOH-soluble fraction also contained *p*-coumaric acid as shown by TLC and MS but this was not examined for ^{18}O incorporation.

Mass spectrometry. Analyses were carried out with a Du Pont 490-F single-focusing magnetic sector spectrometer by distillation of samples directly into the ion source (220°, 70 eV EI).

H_2O analysis. H_2O, present in shoot–root axes and cotyledons at the end of the growth period in $^{18}O_2$, was removed by lyophilization. The lyophilization process was interrupted to give successive fractions of tissue H_2O. Oxygen-18 content of tissue H_2O was determined by direct introduction of H_2O samples into the batch inlet of the spectrometer. Oxygen prepared by electrolysis of ^{18}O-enriched H_2O, was analyzed in the same way. Atom percentages were calculated as in ref. [12].

Acknowledgements—We are grateful to Harold Snellen for his technical assistance and expertise in the isolation procedure.

REFERENCES

1. Robinson, R. (1963) *The Organic Constituents of Higher Plants* p. 49. Burgess, Minneapolis.
2. Neish, A. C. (1965) in *Plant Biochemistry* (Bonner, J. and Varner, J. E. eds) pp. 581–617. Academic Press, New York.
3. Brown, S. A. (1966) *Ann. Rev. Plant Physiol.* **17**, 223.
4. Freudenberg, K. (1968) in *Constitution and Biosynthesis of Lignin* (Freudenberg, K. and Neish, A. C. eds) pp. 45–122. Springer-Verlag, New York.
5. Gortner, W. A. and Kent, M. J. (1958) *J. Biol. Chem.* **233**, 731.
6. Tomaszewski, M. and Thimann, K. V. (1966) *Plant Physiol.* **41**, 1443.
7. Martin, F. W. (1970) *Phyton* **27**, 47.
8. Martin, F. W. (1970) *Bull. Torrey Bot. Club* **97**, 1.
9. Riley, R. G. and Kolattukudy, P. E. (1975) *Plant Physiol.* **56**, 650.
10. Dittrich, P. and Danböck, T. (1977) *Plant Physiol.* **59**, 279.
11. Benveniste, I., Salaun, J.-P. and Durst, F. (1977) *Phytochemistry* **16**, 69.
12. Roboz, J. (1968) *Introduction to Mass Spectrometry Instrumentation and Techniques* pp. 455–456. John Wiley, New York.