Phytochemistry, 1976, Vol 15, pp 129-131. Pergamon Press. Printed in England

PHENOLIC SYNTHESIS IN *PERILLA* CELL SUSPENSION CULTURES

RAGAI K. IBRAHIM and DUART EDGAR

Department of Biological Sciences, Concordia University, Sir George Williams Campus, Montreal H3G 1M8, Que., Canada

(*Revised received* 7 *July* 1975)

Key Word Index—*Perilla ocymoides*; Labiatae; cell suspension culture; caffeyl compounds; phenolic synthesis.

Abstract—Suspension cultures of *Perilla ocymoides* accumulate caffeic acid, both in free and ester forms, as the only phenylpropanoid end metabolite. Increased levels of growth substances influenced the levels of PAL activity and phenolic accumulation so that cytokinin stimulated, while auxin repressed both parameters. The regulatory role of caffeyl compounds is discussed in relation to their accumulation during the early exponential phase of culture growth.

INTRODUCTION

Perilla ocymoides loses the biosynthetic potential for its characteristic anthocyanin, perillanin [1] [cyanidin-3-(6-*p*-coumarylglucoside)-5-glucoside] when grown as cell suspension culture. While several attempts to induce pigment formation *in vitro* were unsuccessful, the suspension culture, however, produced one major phenolic metabolite, caffeic (3,4-dihydroxycinnamic) acid both in the free and ester forms. This remarkable property was considered by itself a valid reason to study its biogenesis in relation to phenylalanine ammonia-lyase (PAL) activity during culture growth. We also report the effect of high levels of growth regulators on the accumulation of caffeyl compounds and their possible role in culture growth.

RESULTS

PAL activity and phenolic accumulation during culture growth

Changes in fresh and dry weights of *Perilla* cells cultured in the control (C) medium were found to follow a reproducible pattern over successive growth cycles (Fig. 1). They were essentially similar to those of the high-auxin (HA) and high-kinetin (HK) cultures, except that the former exhibited a 10–15% increase above the control. The relative increase in protein content was found to reach its maximum during the exponential phase of cell growth. On the other hand, the increase in PAL activity and the accumulation of caffeyl compounds began during the lag phase and attained their maxima during early exponential growth, while both tissue yield and protein content continued to increase for a further period of one week (Fig. 1).

Incorporation of cinnamic acid-2-^{14}C into soluble and insoluble fractions

The pattern of label incorporation into the alcohol-soluble fraction (Fig. 2) is almost consistent with the accumulation of caffeyl compounds during culture growth. The alcohol-insoluble (cell wall) residue, on the other hand, contained relatively low activity especially during early growth. Alkaline hydrolysis of this fraction yielded small but detectable amounts of labelled caffeic acid, indicating some incorporation of the phenolic precursor into cell wall material. Regardless of changes in label incorporation, the specific activity of both caffeyl compounds continued to increase during culture growth, indicating active phenolic synthesis and reflecting the pool size of both compounds.

Effect of growth substances on PAL activity and accumulation of caffeyl compounds

Figure 3 shows that treatment with HA caused a marked reduction of both PAL activity and phenolic accumulation especially during early exponential growth.

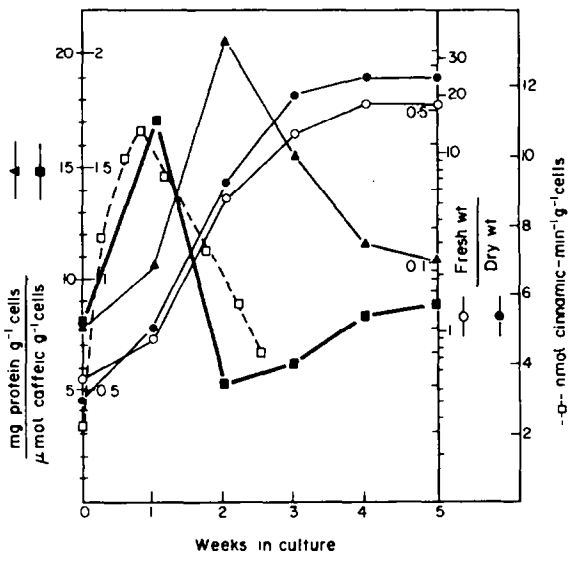

Fig. 1. Growth measurements (Fr. wt., –O–; Dry wt., –●–; protein content, –△–). PAL activity (–□–) and caffeyl content (–■–) of *Perilla* cells cultured in control medium.

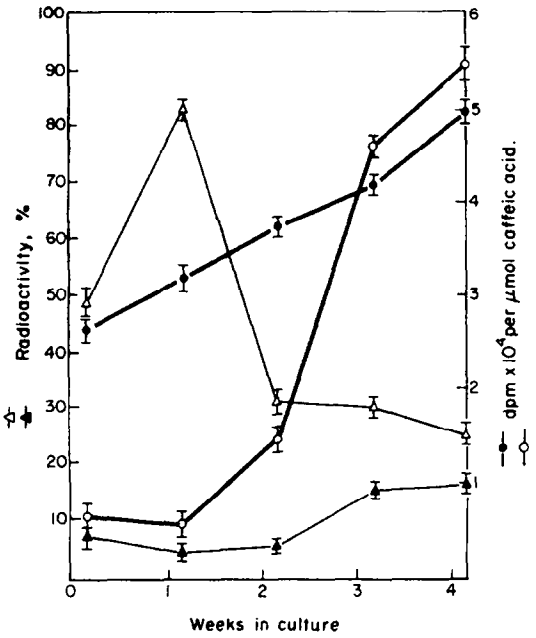

Fig 2. Incorporation of label from cinnamic acid-2-^{14}C into alcohol-soluble (-▲-) and insoluble (-▲-) fractions and specific activity of caffeic acid (-○-) and caffeyl ester (-●-) in *Perilla* cells cultured in control medium.

The HK treatment, on the other hand, produced a significant increase of both parameters as compared with the control. Furthermore, HK resulted in the extension of the period over which phenolic synthesis took place. Regardless of the quantitative changes in phenolic accumulation during culture growth, the amount of caffeyl ester was consistently higher than that of the free acid in all treatments.

DISCUSSION

The results obtained in this investigation clearly demonstrate that the peaks for both PAL activity and phenolic accumulation are almost restricted to lag-phase and early exponential phase cells. This seems to indicate that caffeyl compounds may be essential for the mediation of culture growth [2,3]. These results are in contrast with those reported for Paul's scarlet rose cells where the accumulation of polyphenols was restricted to late growth [4,5]; and for parsley cells where PAL activity attained its peak shortly before the suspension culture reached maximum growth [6].

It is remarkable that HA and HK both affect PAL activity and phenolic accumulation. A comparison between PAL activity and phenol accumulation, however, shows that the former was several times higher than that expected from the amount of caffeyl compounds formed. It should be recognized that the activity of PAL was determined *in vitro* under optimal conditions which are not likely to exist *in vivo*, especially with respect to substrate availability. Furthermore, it was not possible to obtain total recovery of caffeyl compounds since (a) they are unstable under prolonged extraction periods, and (b) some caffeic acid chemically bound to cell wall material was not accounted for. However, the increased rate of PAL activity and phenolic accumulation in the HK-treatment is consistent with the reports on flavonoid [7-9] and lignin [10,11] formation in a number of callus and suspension cultures. In most cases, the appearance of phenolic substance was correlated with induction or activation of PAL. The inhibition of caffeyl synthesis in *Perilla* by HA is comparable with the reports on anthocyanin production in *Haplopappus gracilis* [8,12]. In rose suspension cultures, high auxin levels caused retardation in PAL activity and concomitant delay in polyphenol accumulation [13].

The fact that caffeyl compounds constitute the only detectable intermediates and end metabolites in the pathway of phenylpropanoid biosynthesis seems to imply a functional role in culture growth. It is conceivable that cell growth may actually be regulated by its endogenous auxin (presumably IAA) rather than the synthetic auxin added to the culture medium. The caffeyl moiety by virtue of its *o*-dihydroxy grouping would, therefore, regulate the activity of IAA-oxidase during culture growth [3, 14, 15]. The high accumulation of caffeyl compounds observed during early-exponential phase and preceding active growth supports this view.

EXPERIMENTAL

Plant material. Callus tissue was initiated from 7-day-old cotyledons of *Perilla ocimoides* cv. Crispa (Labiatae) and was brought into suspension in a salt nutrient medium [16] containing 3% sucrose supplemented with α-naphthaleneacetic acid and kinetin, in mg/l respectively, 1 and 0·1 for control; 5 and 0·1 for HA; 1 and 2·0 for HK treatments. Tissue samples were taken for analysis after suspension cultures have been transferred at least 2× to either HA or HK media.

Growth measurements. Growth parameters used in this investigation were (a) fr. wt of cells per culture flask after cells were filtered through a sintered glass funnel using suction and air was allowed to pass through the filter for 1 min., (b) dry wt of cells after standing in a vacuum oven for 5 days at 45° and (c) protein content of the cells as determined by the method of Lowry [27].

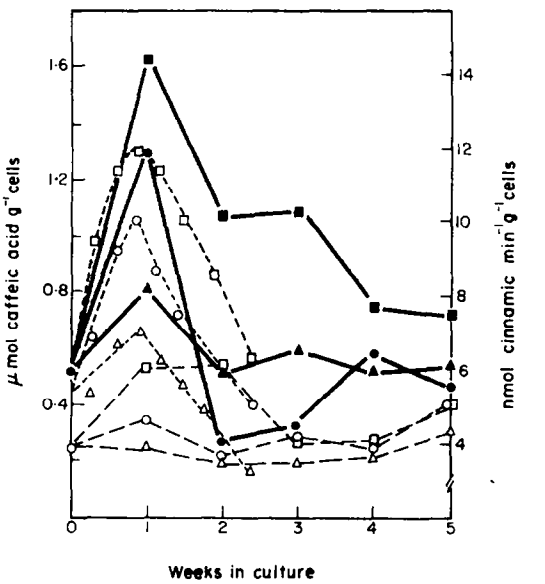

Fig. 3. Caffeyl ester (——), caffeic acid (— —) and PAL activity (- - - -) of *Perilla* cells cultured in control (○ ●), high-auxin (△ ▲) and high-kinetin (□ ■) media.

Isolation and identification of phenolics. Filtered cells were homogenized using a Potter-Elvehjem homogenizer, then transferred to a glass thimble of a Soxhlet extractor and were extracted for 6 hr with alcohol. Extracts were flash evaporated under red pres at 40° and the residue was directly used for chromatographic separation of caffeic acid and the caffeyl ester. Best separation was achieved by using the organic phase of C_6H_6–HOAc–H_2O (15:10:1) on cellulose thin layer plates. R_f values were 0·46 and 0·20 for the acid and ester, respectively. Both caffeic acid and the alkaline hydrolysis product of the ester were identified by (a) R_f values and co-chromatography with authentic sample in various solvents, (b) characteristic fluorescence in UV-light (366 nm), (c) UV maxima in MeOH and MeONa; the latter distinguishes the ester from the glucoside by a bathochromic shift [18] and (d) IR spectra with maxima at 1590, 1440 and 1275 cm^{-1}. The caffeyl ester which was susceptible to mild hydrolysis with alkali (1N NaOH for 2 hr under N_2) yielded no identifiable, non-phenolic moiety such as sugar or cyclic organic acid. It did not co-chromatograph with chlorogenic acid and its identity was not investigated further.

Quantitative determination. Both caffeic acid and its ester were quantitated directly on TLC plates by a fluorometric method [19] using a Turner filter fluorometer model 111 (Palo Alto, Calif.).

Assay for PAL activity. The methods for preparation and assay of enzyme extracts were essentially those of Koukol and Conn [20] after some modification [21].

Isotope experiments. Duplicate suspension cultures were administered, under aseptic conditions, 2 μCi of cinnamic acid-2-^{14}C (sp. act. 1·4 mCi/mmol, ICN Tracerlab, Irvine, Calif.). Label adminstration was carried out with other cultures at different growth stages for 24 hr. The sp. act. of caffeic acid and its ester was determined from triplicate sample per culture flask. Aliquots of extractive-free, cell wall residue were combusted in duplicates by the Schöniger flask method and $^{14}CO_2$ released was absorbed in a mixture of 2-phenethylamine–MeOH–toluene (1:2:1). All radioactivity measurements were carried out by liquid scintillation using 2,5-diphenyloxazole in spectral grade toluene.

Acknowledgements—We wish to thank the National Research Council of Canada for financial support of this work.

REFERENCES

1. Watanabe, S., Sakamura, S. and Obata, Y. (1966) *Agr. Biol. Chem. (Tokyo)* **30**, 420.
2. Zucker, M., Hanson, K. R. and Sonheimer, E. (1967) in *Phenolic Compounds and Metabolic Regulation* (Finkle, B. J. and Runeckles, V. C., eds.) pp. 68–93, Appleton-Century-Crofts, New York.
3. Sondheimer, E. (1964) *Bot. Rev.* **30**, 667.
4. Nash, D. T. and Davies, M. E. (1972) *J. Exp. Botany* **23**, 75.
5. Davies, M. E. (1972) *Planta (Berlin)* **104**, 50.
6. Hahlbrock, K., Ebel, J., Ortmann, R., Sutter, A., Wellman, E. and Grisebach, H. (1971) *Biochim. Biophys. Acta* **244**, 7.
7. Miller, C. O. (1969) *Planta (Berlin)* **87**, 26.
8. Constabel, F., Shyluk, J. P. and Gamborg, O. L. (1971) *Planta (Berlin)* **96**, 306.
9. Ibrahim, R. K., Thakur, M. L. and Permanand, B. (1971) *Lloydia (J. Nat. Products)* **34**, 175.
10. Rubery, P. H. and Fosket, D. E. (1969) *Planta (Berlin)* **87**, 54.
11. Carceller, M., Davey, M. R., Fowler, M. W. and Street, H. E. (1972) *Protoplasma* **73**, 367.
12. Blakely, L. M. and Steward, F. C. (1961) *Am. J. Botany* **48**, 351.
13. Davies, M. E. (1972) *Planta (Berlin)* **104**, 66.
14. Galston, A. W. and Davies, P. J. (1970) *Control Mechanisms in Plant Development.* Prentice-Hall, Englewood Cliffs, New Jersey.
15. Thimann, K. V. (1972) in *Plant Physiology, a Treatise* (Steward, F. C., ed.) Vol. VIB, pp. 3–145, Academic Press, New York.
16. Murashige, T. and Skoog, F. (1962) *Physiol. Plantarum.* **15**, 473.
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
18. Runeckles, V. C. and Woolrich, F. (1963) *Phytochemistry* **2**, 1.
19. Ibrahim, R. K. (1969) *J. Chromatog.* **42**, 544.
20. Koukol, J. and Conn, E. E. (1961) *J. Biol. Chem.* **236**, 2692.
21. Havir, E. A. and Hanson, K. R. (1968) *Biochemistry* **7**, 1896.