Journal of Chemical Ecology, Vol. 19, No. 7, 1993

PHENOLIC ACIDS AFFECT PHOTOSYNTHESIS AND PROTEIN SYNTHESIS BY ISOLATED LEAF CELLS OF VELVET-LEAF[1]

WONDIMAGEGNEHU MERSIE and MEGH SINGH*

University of Florida
Institute of Food and Agricultural Sciences
Citrus Research and Education Center
700 Experiment Station Road
Lake Alfred, Florida 33850

(Received August 29, 1988; accepted February 8, 1993)

Abstract—The effects of *p*-coumaric, ferulic, chlorogenic, and vanillic acids on photosynthesis and protein synthesis by isolated leaf cells of velvetleaf (*Abutilon theophrasti* Medik) were investigated. Photosynthesis and protein synthesis were measured in cell suspensions by the incorporation of $^{14}CO_2$ and [^{14}C]leucine, respectively. None of the tested phenolic acids except vanillic reduced photosynthesis by more than 50% at the highest concentration and 30 min of incubation. At 100 μM concentrations and 60-min incubation periods, *p*-coumaric, ferulic, chlorogenic, and vanillic acids inhibited photosynthesis by 33, 37, 57, and 65%, respectively. Ferulic acid was the most inhibitory to protein synthesis and reduced the incorporation of [^{14}C]leucine by 50% at about 1.0 μM after 60 min of incubation. At the highest concentrations tested in this study, vanillic and ferulic acids were inhibitory to photosynthesis and protein synthesis, respectively, whereas chlorogenic and *p*-coumaric acids did not inhibit either physiological process. The maximum inhibition of protein synthesis by chlorogenic acid was 19% and by vanillic acid was 28% at 100 μM concentrations. Chlorogenic, vanillic, and *p*-coumaric acids at 0.1 μM caused increased protein synthesis over the untreated control. Overall, photosynthesis was more sensitive than protein synthesis to the four phenolic acids tested.

*To whom correspondence should be addressed.
[1] Florida Agricultural Experiment Station Journal Series No. 9228.

0098-0331/93/0700-1293$07.00/0 © 1993 Plenum Publishing Corporation

Key Words—Chlorogenic acid, *p*-coumaric acid, ferulic acid, vanillic acid, growth inhibition, velvetleaf, *Abutilon theophrasti*.

INTRODUCTION

Of all the growth-inhibiting compounds released by living or decaying plant parts, phenolics and the associated compounds are the most common (Horsley, 1977; Rice, 1979; Whittaker, 1970). Phenolic compounds are widely found in different plant tissues (Abdul-Wahab and Rice, 1967; Guenzi and McCalla, 1966; Jackson and Willemsen, 1976; Moje, 1966; Wang et al., 1967; Whitehead, 1964) and occur either in a free state or conjugated with sugars as glycosides. Numerous studies have shown that phenolics are allelopathic, as they inhibit seed germination and plant growth (Borner, 1960; Guenzi and McCalla, 1966; Lodhi, 1979a,b). Einhellig et al. (1970) reported that scopoletin, a coumarin derivative, inhibited dry matter production, leaf area expansion, and photosynthesis in tobacco (*Nicotiana tobacum* L.), sunflower (*Helianthus annuus* L.), and redroot pigweed (*Amaranthus retroflexus* L.). Chlorogenic acid also reduced stomatal aperture in tobacco and sunflower (Einhellig and Kuan, 1971), while ferulic acid and *p*-coumaric acid reduced leaf water potential and stomatal diffusive conductance in grain sorghum [*Sorghum bicolor* (L.) Moench.] and soybean [*Glycine max* (L.) Merr.] (Einhellig and Stille, 1979; Einhellig et al., 1985). Patterson (1981) reported that 1000 μM *p*-coumaric, ferulic, and vanillic acids severely reduced the photosynthesis of soybean, whereas 100 μM had no effect. Coumarins and cinnamic acids also suppressed photosynthesis of *Lemna minor* L. at concentrations corresponding to their individual threshold for growth inhibition (Einhellig, 1986).

These studies investigated the impact of phenolics on photosynthesis and other macromolecular processes using whole plants. However, by treating whole plants with phenolic acids and measuring growth responses, a researcher is faced with many inherent limitations and problems. For example, the type of application (root or foliar), the site of uptake, and the pattern of translocation of a given chemical are all variables that could complicate such studies. These limitations encountered in allelopathic research with intact plants can be circumvented by the use of isolated plant cell preparations.

Several researchers (Ashton et al., 1977; Hatzios, 1982; Hatzios and Howe, 1982; Malakonduiah and Fang, 1978) have used plant cells isolated from the leaves of higher plants for determining the mechanism of action of herbicides. In this study, we employed similar techniques to examine the effects of four phenolic acids on stromal-associated CO_2 fixation (Calvin cycle) reactions and protein synthesis. The objectives of this study were to determine the effects of

p-coumaric, ferulic, chlorogenic, and vanillic acid on photosynthesis and protein synthesis of enzymatically isolated leaf cells of velvetleaf.

METHODS AND MATERIALS

Plant Material. Velvetleaf was grown from seed in moist vermiculite in a greenhouse with day and night temperatures of 26 and 21 ± 1°C, respectively. Plants were grown under natural light supplemented with a fluorescent light with 150 $\mu E/m^2/sec$ for two weeks.

Chemicals. The herbicide diuron [*N'*-(3,4-dichlorophenyl)-*N,N*-dimethylurea] with 99% purity and the fungicide metalaxyl [*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-DL-alanine, methyl ester] with 98% purity were used as standard inhibitors for photosynthesis and protein synthesis, respectively. Analytical grade phenolic compounds *p*-coumaric, ferulic acid, chlorogenic, and vanillic acids were then tested. Each compound was prepared by dissolving the appropriate amount in acetone and diluting with distilled water to a final concentration of 0.1, 1.0, 10, and 100 μM in all suspensions. The concentration of acetone in solution was less than 0.5%.

Cell Isolation. Detached mature leaves from velvetleaf plants were rinsed with distilled water, blotted, deveined, and cut into 1 mm × 1 cm strips. Four to five grams of leaf tissue were then vacuum infiltrated with 30 ml of infiltration medium containing 20 mM MES, pH 5.8, 0.1% macerase, 2% pectinase, and 0.3% potassium dextran sulfate (Hatzios, 1982). After vacuum infiltration, the leaf tissue was filtered through a 242-μm mesh nylon net, the filtrate was discarded, and the leaf tissue remaining was transferred to a beaker with 30 ml of a maceration medium containing 20 mM MES, pH 5.8, 0.2% pectinase, 2% cellulase, 0.3% potassium dextran sulfate, and 0.6 M mannitol. The tissue was stirred slowly on a magnetic stirrer for 10 min. The suspension was filtered through the same nylon net and the filtrate discarded. The leaf tissue was transferred to another 30 ml of fresh maceration medium and was stirred for 2 hr. The mesophyll cells released during this period were filtered again through the nylon net and washed three times with 10 ml of incubation medium by centrifugation at 60*g* for 3 min. The incubation medium contained 0.5 M mannitol 5 mM KNO_3, 2 mM $Mg(NO_3)_2$, 1 mM $CaCl_2$, 50 mM HEPES-KOH buffer, at pH 7.8. The cells were resuspended with incubation medium. For chlorophyll determination, three 1-ml samples of the final cell suspension were placed in three 15-ml centrifuge tubes. To each tube, 4 ml of 80% acetone were added and the tubes were stirred using a vortex mixer; then the tubes were kept in the dark for 2 hr. At the end of this time, the tubes were centrifuged at 150*g* for 5 min, and the supernatant fluid was then assayed spectrophotometrically for its chlorophyll content according to the method of Arnon (1949). The chlorophyll

content of cell preparations used in this study was 18–30 μg/chlorophyll/ml photosynthesis assay medium.

Photosynthesis. Two milliliters of the cell preparation suspended in incubation medium were placed into a 25-ml Erlenmeyer flask. To each flask, 0.1 ml containing 0.1 μCi [^{14}C]$NaHCO_3$ and 0.05 ml of diuron or the phenolic acids were added. The flasks were placed on a shaker illuminated with a fluorescent light (145 $\mu E/m^2/sec$) and incubated for 30 and 60 min at 24 $\pm$ 1°C. At the end of the incubation periods, 200 μl of the samples were removed from each flask and placed on a 2.3-cm-diameter filter paper disk (Whatman 3 mm). The disks were acidified with 0.1 ml of 90% formic acid to release any unfixed $^{14}CO_2$ and dried in a hood. Radioactivity was determined by placing the disks in scintillation vials containing 10 ml of scintillation fluid and quantified by liquid scintillation counter (LSC). The counts per minute (cpm) were converted to disintegration per minute (dpm) based on counting efficiency of the counter ($>$97%). The results were then converted to dpm/mg chlorophyll (chl) and expressed as percent of the untreated control. Data were subjected to analysis of variance and means were separated using LSD (0.05).

Protein Synthesis. Protein synthesis was determined by adding 0.1 μCi of L-[U-^{14}C]leucine (specific activity 290 mCi/mmol), metalaxyl, and the phenolic acids to 2 ml of cell suspension in 25-ml flasks. The flasks were placed on a shaker at 24 $\pm$ 1°C under a fluorescent light (145 $\mu E/m^2/sec$) and incubated for 30 and 60 min. At the end of the incubation periods, a 500-μl sample was collected from each flask and placed in 20-ml vials. To each vial, 2 ml of ice-cold trichloroacetic (TCA) at 10% w/v was added and the vials were left overnight at 4°C in a refrigerator. The TCA-insoluble protein precipitates were then collected by filtering each sample through a 2.1-cm glass fiber filter disk. The disks were then washed successively with 6 ml (3 $\times$ 2 ml) of ice-cold TCA (10% w/v), 6 ml (3 $\times$ 2 ml) of 80% ethanol, 2 ml of acetone, and 4 ml (2 $\times$ 2) of diethyl ether. The disks were then dried, placed into vials with 20 ml of scintillation fluid, and radioassayed.

The results were converted to dpm/mg chl and data were subjected to analysis of variance. There were three replications in each experiment, and all experiments were conducted twice. Although all data were calculated in dpm/pmg of chlorophyll, these values were converted to percentage of control (no chemical present) for uniform graphic presentations of the data.

RESULTS AND DISCUSSION

Photosynthesis. Diuron has been a standard research tool in photosynthesis and was included in this study as a reference. Inhibition of photosynthesis by diuron reached 50% of the untreated control between 0.1 and 1.0 μM after both

30 and 60 min of incubation (Figure 1), which is similar to values reported elsewhere (Fedtke, 1982; Moreland, 1980). The observed responses of the cells to diuron indicate that the cells were physiologically active throughout this study.

None of the tested allelochemicals were as inhibitory as diuron to photosynthesis (Figures 1–5), but the inhibition increased with an increase in the concentration of the phenolic acids at both incubation times. At 100 μM concentrations, *p*-coumaric, ferulic, chlorogenic, and vanillic acids inhibited pho-

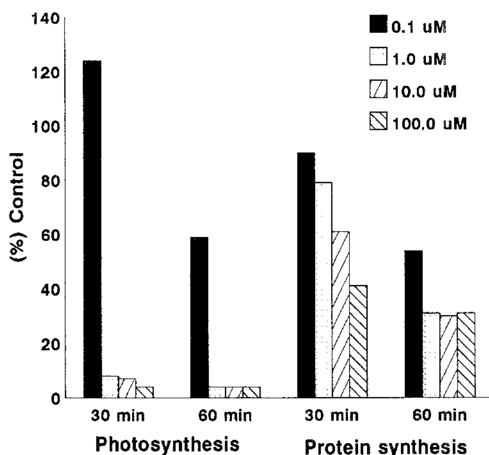

FIG. 1. The effects of diuron on photosynthesis and metalaxyl on protein synthesis.

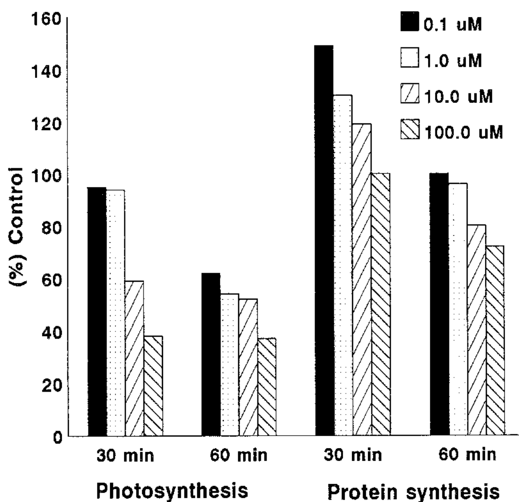

FIG. 2. The effects of coumaric acid on photosynthesis and protein synthesis.

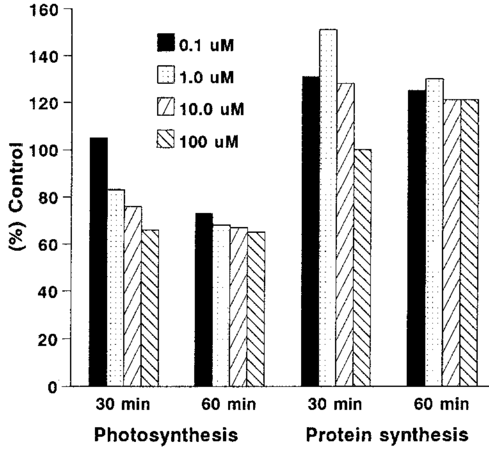

FIG. 3. The effects of ferulic acid on photosynthesis and protein synthesis.

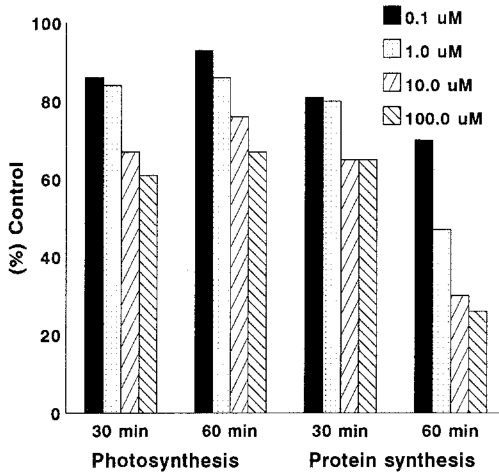

FIG. 4. The effect of chlorogenic acid on photosynthesis and protein synthesis.

tosynthesis by 33, 37, 57, and 65% at 60 min, respectively (Figures 2–5). These four phenolic acids can be compared as to their relative effect on the inhibition of photosynthesis: vanillic > chlorogenic > ferulic > *p*-coumaric acids.

It is not known whether these compounds reach concentrations as high as 100 μM inside mesophyll cells, but bulk soil extractions have indicated soil solution concentrations of 4.9×10^{-5}, 4.2×10^{-5}, and 3.2×10^{-5} M for vanillic, *p*-coumaric, and ferulic acids, respectively (Whitehead, 1964). More-

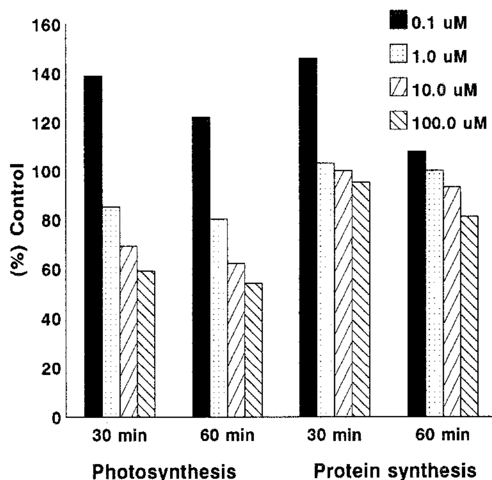

FIG. 5. The effect of vanillic acid on photosynthesis and protein synthesis.

land and Novitzky (1987) reported that several phenolic acids, including vanillic and ferulic acids, inhibited CO_2-dependent oxygen evolution in isolated chloroplasts. However, the I_{50} concentrations ranged between 1 and 10 mM and are much higher than used in this research. Patterson (1981) reported that 1000 μM *p*-coumaric and ferulic acids severely reduced photosynthesis of soybean, whereas 100 μM had no effect. Concentrations above 1000 μM are considered too high to establish a direct effect of a xenobiotic on any physiological process using isolated cells or organelles (Ashton et al., 1977).

Protein Synthesis. The fungicide metalaxyl belongs to the group of chemicals known as acylalanines, and several members of this group are reported to inhibit protein synthesis (Ashton et al., 1977). Metalaxyl was included in this research as a standard inhibition treatment for protein synthesis. Metalaxyl inhibited protein synthesis more at 60 min than at 30 min (Figure 1). At 30 min, there was a progressive reduction in the incorporation of $[^{14}C]$leucine with an increase in concentration. At 60 min, maximal inhibition occurred at 1.0 μM of metalaxyl, and there was no further reduction at higher concentrations. Protein synthesis was reduced by 50% between 10 and 100 μM at 30 min and between 0.1 and 10 μM after 60 min incubation.

Similar to metalaxyl, the degree of protein inhibition by ferulic and vanillic acids was greater at 60 than 30 min of incubation. This suggests either a delay in induction in absorption or subcellular transport, a delay in the induction of the inhibition, or that the chemicals were inhibiting a metabolic site that supplied an essential factor for protein synthesis rather than affecting it directly. It is not

possible to determine which of these factors is responsible for the delayed inhibition of the chemicals from the data presented.

None of the allelochemicals except ferulic acid inhibited protein synthesis by as much as 50%. Chlorogenic, vanillic, and *p*-coumaric acids at 0.1 μM caused increased incorporation of [^{14}C]leucine above the rate of the untreated cells. The maximum inhibition by chlorogenic acid was 19% and for vanillic acid it was 28%, both at 100 μM concentrations. Ferulic reduced [^{14}C]leucine incorporation by 50% at about the 1.0 μM concentration after 60 min incubation, and this was comparable to the effect of the standard treatment (metalaxyl).

On the whole, photosynthesis was more sensitive than protein synthesis to the allelochemicals tested. Protein synthesis is not considered to be the most sensitive site of action for herbicides (Ashton et al., 1977). Among the phenolics tested, vanillic acid was inhibitory to photosynthesis while ferulic acid reduced the incorporation of [^{14}C]leucine by cells. Chlorogenic and *p*-coumaric acids do not have a direct effect on either photosynthesis or protein synthesis. However, it is important to note that results reported here were for individually applied phenolic acids. Under natural conditions, additive or synergistic inhibitory effects of such allelochemical compounds may become a more determinable event at lower concentrations than the effects of individual compounds. It would be worthwhile if future studies address this by investigating the effects of several allelochemicals alone or in combination on selected plant processes.

Acknowledgments—The authors would like to thank Kevin Hostler for his valuable technical assistance throughout the investigation. We also thank Ciba-Geigy Corp. and E.I. duPont de Nemours & Co., Inc. for providing technical metalaxyl and diuron, respectively.

REFERENCES

ABDUL-WAHAB, A.S., and RICE, E.L. 1967. Plant inhibition in old field succession. *Bull. Torrey Bot. Club* 94:486–497.

ARNON, D.I. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris* L. *Plant Physiol.* 24:1–7.

ASHTON, F.M., DEVILLIERS, O.T., GLENN, R.K., and DUKE, W.B. 1977. Localization of metabolic sites of action of herbicides. *Pestic. Biochem. Physiol.* 7:122–141.

BORNER, H. 1960. Liberation of organic substances from higher plants and their role in the soil sickness problem. *Bot. Rev.* 26:393–424.

EINHELLIG, F.A. 1986. Mechanisms and modes of action of allelochemicals, pp. 170–188, *in* A.P. Putnam and C.S. Teng (eds.). The Science of Allelopathy. John Wiley & Sons, New York.

EINHELLIG, F.A., and KUAN, L. 1971. Effects of scopoletin and chlorogenic acid on stomatal aperture in tobacco and sunflower. *Bull. Torrey Bot. Club* 98:155–162.

EINHELLIG, F.A., and STILLE, M.L. 1979. Effects of ferulic and *p*-coumaric acids on plant water status. *Abstr. Bot. Soc. Am., Misc. Ser. Publ. No.* 157:40–41.

EINHELLIG, F.A., RICE, E.L., RISSER, P.G., and WENDER, S.H. 1970. Effects of scopoletin on growth, CO_2 exchange rates and concentration of scopoletin, scopolin and chlorogenic acids in tobacco, sunflower and pigweed. *Bull. Torrey Bot. Club* 97:22–23.

EINHELLIG, F.A., MUTH, M.S., and SCHON, M.K. 1985. Effects of allelochemicals on plant-water relationship, pp. 170–195, *in* A.C. Thompson (ed.). The Chemistry of Allelopathy. *American Chemical Society*, Washington, D.C.

FEDTKE, C. 1982. Biochemistry and Physiology of Herbicide Action. Springer-Verlag, New York.

GUENZI, W.D., and MCCALLA, T.M. 1966. Phytotoxic substances extracted from soil. *Proc. Soil Sci. Soc. Am.* 30:214–216.

HATZIOS, K.K. 1982. Use of isolated leaf cells of *Abutilon theophrasti* to localize the action of two amino-triazinone herbicidal derivatives. *Z. Naturforsch* 370:276–281.

HATZIOS, K.K., and HOWE, C.M. 1982. Influence of the herbicide hexazinone and chlorsulfuron on the metabolism of isolated soybean leaf cells. *Pestic. Biochem. Physiol.* 17:207–214.

HORSLEY, S.B. 1977. Allelopathic interference among plants. II. Physiological modes of action, pp. 93–136, *in* H.E. Wilcos and A.F. Hamer (eds.). Proceedings, Fourth North American Forest Biology Workshop, School of Continuing Education, College of Environmental Science, Suny, Syracuse, New York.

JACKSON, J.R., and WILLEMSEN, R.W. 1976. Allelopathy in the first stages of secondary succession on the piedmont of New Jersey. *Am. J. Bot.* 63:1015–1023.

LODHI, M.A.K. 1979a. Allelopathic potential of *Salsola kali* L. and its possible role in rapid disappearance of weedy stage during revegetation. *J. Chem. Ecol.* 5:429–437.

LODHI, M.A.K. 1979b. Germination and decreased growth of *Kochia scoparia* in relation to its autoalleopathy. *Can. J. Bot.* 57:1083–1088.

MALAKONDUIAH, N., and FANG, S.C. 1978. Influence of monuron on photosystem II and light dependent $^{14}CO_2$ fixation in isolated cells of C_3 and C_4 plants. *Pestic. Biochem. Physiol.* 10:268–274.

MOJE, W. 1966. Organic soil toxins, pp. 533–569, *in* H.D. Chapman (ed.). Diagnostic Criteria for Plants and Soils. University of California Press, Berkeley.

MORELAND, D.E. 1980. Mechanism of action of herbicides. *Annu. Rev. Plant Physiol.* 31:597–638.

MORELAND, D.E., and NOVITZKY, W.P. 1987. Effects of phenolic acids, coumarins and flavonoids on isolated chloroplast and mitochondria, pp. 247–261, *in* G.R. Waller (ed.). Symposium on Allelochemicals: Role in Agriculture, Forestry and Ecology. American Chemical Society, Washington, D.C.

PATTERSON, D.T. 1981. Effects of allelopathic chemicals on growth and physiological responses of soybean (*Glycine max*). *Weed Sci.* 29:53–59.

RICE, E.L. 1979. Allelopathy—an update. *Bot. Rev.* 45:15–109.

WANG, T.S.C., YANG, T.K., and CHUANG, T.T. 1967. Soil phenolic acids as plant growth inhibitors. *Soil Sci.* 103:239–246.

WHITEHEAD, D.C. 1964. Identification of *p*-hydroxybenzoic, vanillic, *p*-coumaric and ferulic acids in soils. *Nature (London)* 202:417–418.

WHITTAKER, R.H. 1970. The biochemical ecology of higher plants, pp. 43–70, *in* E. Sondheimer and J.B. Simeone (eds.). Chemical Ecology, Academic Press, New York.