Herbicide-Related Changes In Phenolic Acid Content Of Field-Grown Hydrilla[1]

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ABSTRACT

Two lakes infested with hydrilla (*Hydrilla verticillata* Royle) were treated with 6,7-dihydrodipyrido[1,2-*a*:2',1'-*c*]-pyrazinediium ion (diquat) using the bivert system. Five phenolic acids were identified in the apical meristem portion of these plants and were assayed quantitatively prior to and for 5 months after herbicide treatment. Vanillic, protocatechuic, *p*-coumaric, and ferulic acids were present at μg/g dry weight whereas caffeic acid was present in mg/g dry weight concentrations. The caffeic acid concentration showed large fluctuations after herbicide treatment, but the other phenolic acids remained relatively unchanged. The possible relationships of these observations to infestation and potential methods of controlling hydrilla are discussed.

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INTRODUCTION

A variety of naturally occurring phenolic compounds have been implicated in the resistance of plants to an assortment of parasites including viruses (2), bacteria and fungi (5), and insects (10, 15). Classes of compounds involved include flavonoids, phenolic acids, coumarins, and phytoalexins (11).

These compounds, which are all classified as phenolics, are synthesized from carbohydrates by the shikimic acid pathway (6). Cinnamic and benzoic acids are important intermediates and derivatives of this pathway and have been implicated directly in disease resistance in other vascular plants (11, 15). A literature survey failed to produce data on the content of these acids in hydrilla.

Diquat is known to affect the production of carbohydrates and also photosynthesis by interferring with electron transport. In these reactions the herbicide inhibits NADP reduction competitively. The action of diquat is catalytic in that the reduced form of the herbicide, which is a free radicle, can be oxidized by molecular

oxygen, thereby reforming the herbicide. During the reoxidation process, these free radicles can yield hydrogen peroxide or other reactive hydroxyl radicles which are capable of destroying plant cells (3).

Thus, herbicides which can affect phenolic acid metabolism or the concentrations of these compounds thereby causing a reduction in disease resistance, may be valuable when used in conjunction with biological controls. We have undertaken this study as an investigation of the effects of diquat applied as a bivert on the concentrations of phenolic acids in hydrilla.

METHODS AND MATERIALS

Plant material was collected from Lake Lipsey (Hillsborough Co.) and Lake Bell (Pasco Co.), both of which were sprayed with diquat and copper triethanolamine complex (Cutrine) by the bivert method (4). These lakes were sprayed by the Maintenance Division of the Southwest Florida Water Management District, and the diquat and Cutrine were applied at the rates of 4 and 12 liters per 0.4 ha, respectively. An unsprayed lake on the University of South Florida campus was sampled over the same period and was used as the control.

Samples were taken at weekly intervals during the first 5 weeks and then monthly until the 19th week after spraying.

Tissue was transported to the laboratory, washed thoroughly with tap water, and the apical meristem sections isolated. Each sample was frozen, lyophilized, powdered, and stored at minus 20 C until extracted.

Extraction of Tissue and Identification of Phenolic Acids

The powdered tissue was washed three times with petroleum ether (b.p. 60 to 110 C) to remove interfering lipids, then filtered through fiberglass filters in Gooch crucibles. The residue was extracted by refluxing twice with 250 ml of 80% ethanol for 1.5 hr, filtered and the ethanol-soluble fraction was concentrated at 40 C in a rotary evaporator to approximately one-tenth the original volume.

Initially, the ethanol-soluble fraction was divided into three equal portions for acid, base, or neutral hydrolyses (7). However, acid and neutral hydrolyses yielded only trace amounts of free phenolic acids and were discontinued. For alkaline hydrolysis, extracts were adjusted to 2N with NaOH, boiled for 3 minutes, cooled in ice and acidified with HCl to pH 1. This fraction was extracted with anhydrous diethyl ether in a liquid-liquid extractor for at least 9 hr. The ether-soluble fractions were taken to dryness under vacuum in a rotary evaporator, redissolved in 5 ml 95% ethanol and stored at 4 C.

The plant substances were compared to reference compounds (vanillic, ferulic, *p*-coumaric, protocatechuic, and caffeic acid) according to the following criteria: Rf values and co-chromatography in two solvents, benzene:acetic acid: water (10:7:3 v/v, upper phase) and 2% formic acid; UV absorption spectrophotometry; color development with diazotized *p*-nitroaniline oversprayed with 2N NaOH (13); color development with diazotized sulfanilic acid (1), and observation of color and fluorescence under 366 and 254 nm UV light before and after exposure to ammonia vapor.

For quantitative analysis, each acid was assayed in triplicate tissue samples and four determinations of each were made. Redissolved ether solubles 0.045 ml in volume were spotted on Whatman No. 1 chromatographic paper and developed two dimensionally in the solvents described above. The compounds were located under UV light and eluted with 95% ethanol. The eluate was brought to a volume of 0.9 ml and 0.1 ml of 0.06 M NaOH was added. The absorbance at the following wavelengths was measured: protocatechuic-302 nm, ferulic-345 nm, *p*-coumeric-333 nm, vanillic-295 nm, and caffeic-350 nm. The caffeic acid ion was stabilized by the addition of sodium borohydride (9). Standard concentration curves were determined from commercially available compounds using identical chromatographic and spectral procedures.

RESULTS

An analysis of the phenolic acids present in alcohol extracts of hydrilla apical sections revealed that five different compounds could be identified: ferulic, *p*-coumaric, vanillic, protocatechuic, and caffeic acids. The results obtained from base hydrolysis of these extracts show clearly that derivatives of these compounds were the main forms present in the tissues.

Quantitative analyses of these compounds in unsprayed tissue showed an average concentration of 70 μg/g dry weight for ferulic and vanillic acids and 128 μg/g dry weight for *p*-coumaric and protocatechuic. Caffeic acid, however, was present at an average concentration of 6.6 mg/g dry weight, which is approximately 100 times greater than ferulic or vanillic and 50 times greater than *p*-coumaric and protocatechuic.

During the early period of the experiments, the plants were separated into the roots, stems, leaves, and turions. However, definite separation of these parts became increasingly difficult as plant degradation proceeded due to herbicide treatment. Since apical meristem tissue was easiest to identify and isolate, only results for this tissue are reported here.

During the duration of the experiment, the concentrations of ferulic, vanillic, *p*-coumaric and protocatechuic acids showed variations of usually less than 10%. Caffeic acid on the other hand showed a dramatic decrease in concentration during the first 2 weeks after spraying, followed by a slower but continued decline over the next 5 weeks (Fig. 1). After this period there was observed a gradual increase, which by the end of the 19th week was nearing the levels found in the control. The data obtained from the two sprayed lakes are quite similar with the exception of the sample taken 7 days after treatment. At this point Lake Bell tissue showed a marked decrease followed by a sudden rise at the 14 day sample. Thereafter, the changes in concentration of caffeic acid in both lakes were nearly parallel.

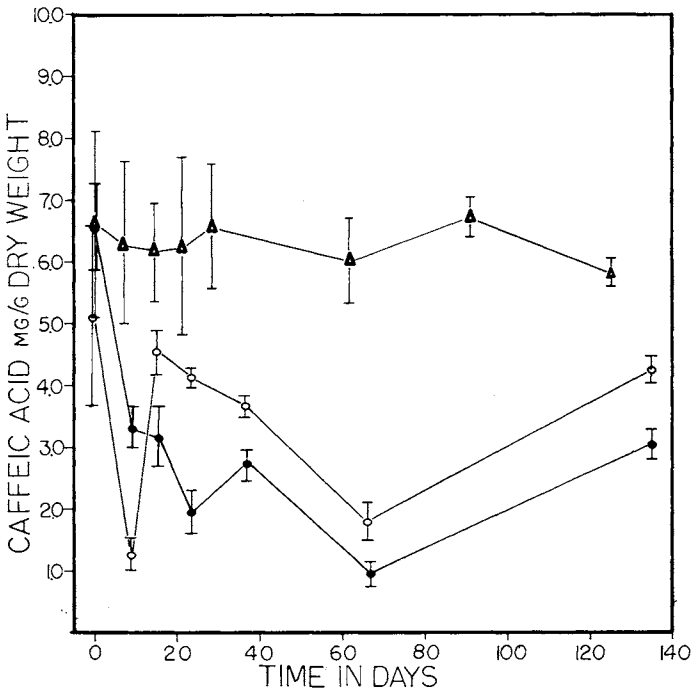

Figure 1. Effect of biverted diquat on caffeic acid in hydrilla under field conditions. △, Control lake; O, Lake Bell; •, Lake Lipsey. Bars indicate ±2 standard deviations (95% confidence interval). Treatment made on day 2.

DISCUSSION AND CONCLUSIONS

This study reveals a large decrease in the concentration of at least one phenolic acid in apical sections of hydrilla after the application of diquat. In unsprayed tissue this compound was most likely present as an ester derivative and its concentration decreased more than 50% within 1 week after spraying.

Fluctuations of this magnitude have at least two possible interpretations. First, it is possible that diquat affects the synthesis of caffeic acid by limiting the production of the acid itself or of the ester derivatives which increase the stability of caffeic acid in plant tissue (14). Since the synthesis of caffeic acid and its esters requires carbohydrate precursors, any response in hydrilla resulting in a decrease in the concentration of the carbon moieties would lead to a reduced level of synthesis. In *Chlorella*, diquat causes an increase in the rate of carbohydrate utilization for respiratory demands, thereby lowering the availability of carbohydrate for other metabolic requirements (12).

A second possibility is that the herbicide affects cell integrity sufficiently to cause the release of esterases, oxidases, or other hydrolytic enzymes which would result in an effective lowering of the endogenous levels of the compounds which serve as substrates of these enzymes. In this manner diquat could act as electron acceptor during the oxidation of caffeic acid or its esters. It has been reported (9) that these oxidized esters (orthoquinones) are unstable and rapidly decompose. The diquat would then become reoxidized by molecular oxygen and could again participate in this reaction.

At present it is not known which of these mechanisms operate in hydrilla. It is also possible that both may participate to some degree.

Another important aspect arising from this study is the rather high concentration of caffeic acid derivatives which have been found in this plant. Caffeic acid and its derivatives have been implicated in the disease resistance of a number of plants (11, 8) and the high concentrations of these compounds in hydrilla may be responsible, at least in part, for the rarity of disease found in this plant. Further studies are planned to determine how many derivatives are present and to elucidate their structures. These compounds could then be tested for their antimicrobial activities and assessed as to whether they indeed contribute to disease resistance in hydrilla. It will also be important to study the kinetics of change in phenolic content and hydrolytic enzyme activities after diquat application.

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