Isolates Of *Penicillium, Aspergillus,* And *Trichoderma* Toxic To Aquatic Plants[1]

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ABSTRACT

Species of *Penicillium, Aspergillus,* and *Trichoderma* were commonly found associated with rotten or chlorotic specimens of hydrilla (*Hydrilla verticillata* Royle) in Florida and India. In laboratory inoculation tests with 30 isolates of these genera, 12 caused chlorotic damage on hydrilla and subsequent lysis. All 12 produced toxic substances in liquid cultures; in four of them, oxalic acid was identified as the toxin. The nature of toxins in the other eight isolates is being investigated. At the minimal concentration needed to kill hydrilla, pure as well as fungal oxalic acids were also toxic to four species of fish (lethal) and twelve species of aquatic plants tested. The degree of damage varied with the type of plant. The possible usefulness of phytotoxic microbial metabolites in biocontrol of aquatic weeds is discussed.

INTRODUCTION

During our search for plant pathogenic biocontrol agents of hydrilla in Florida and India, several isolates of *Penicillium, Aspergillus,* and *Trichoderma* species were repeatedly obtained. The consistent association of these fungi with diseased hydrilla plants suggested that they were pathogenic or toxigenic in aquatic habitats. Results of laboratory inoculation trials on hydrilla with species of these fungi were reported earlier (2). It was suggested that these fungi might produce toxic metabolites which killed or weakened tissues of hydrilla rendering them suitable for saprophytic colonization (2). This study was intended to investigate metabolites of these fungi and assess their potential as controls of hydrilla.

METHODS AND MATERIALS

Isolation of fungi, their maintenance and pathogenicity tests on hydrilla were as described earlier (2). Pathogenicity and toxicity tests were done in the quarantine greenhouse at the University of Florida, using hydrilla plants in the test tube assembly mentioned previously (2). The fungal inoculum for pathogenicity tests consisted of a block of agar containing mycelium (2). For toxicity tests the fungi were grown as standing cultures for 7 days at 28 C on a liquid medium containing KNO_3, 10 g; KH_2PO_4, 5 g; $MgSO_4$ $7H_2O$, 2.5 g; $FeCl_3$, 0.02 g; and sucrose, 50 g per liter of deionized water. The culture solutions of fungi were collected by passing them through Whatman No. 1 filter papers. Toxicity was tested by adding 3 ml of a culture solution to 40 ml of sterile deionized water in a tube into which was introduced a sprig of hydrilla of 8 cm length. The toxicity of culture solutions was evaluated and

1 This research was funded by Florida Department of Natural Resources Contract No. 3. and supported in part by Army Corps of Engineers Contract No. DACW 73-71-C0002 and by the U.S. Department of the Interior, Office of Water Resources Research as authorized under the Water Resources Act of 1969. Florida Agricultural Experiment Station Journal Series No. 5007.

rated on the basis of the degree of yellowing of hydrilla plants. Generally, results were recorded within 60 hr. Control tubes with hydrilla received 3 ml of uninoculated liquid medium per 40 ml of water.

The culture solutions were dialysed in 2-cm diameter cellophane tubing for 36 hr at 4 C against 100 volumes of deionized water. At the end of dialysis, 3-ml samples per 40 ml of sterile deionized water of the following were tested for toxicity to hydrilla: i) dialysed culture solution, ii) nondialysed culture solutions, iii) nondialysed and non-inoculated medium and iv) deionized water. The toxicity of water containing the diffusate from the dialysis bag was also tested by using 43 ml of this solution per tube.

Toxicities of culture solutions and reagent chemical were tested on other aquatic plants and fish by incorporating such substances in tubes or 38-L aquarium tanks, respectively, at rates proportional to the minimum dose needed to kill hydrilla in tubes. Controls received deionized water or deionized water acidified with dilute H_2SO_4 equalling the volume of test samples. Deionized, nonsterile water and water from Crystal River, Florida were used for tests on fish. Except water lettuce (*Pistia stratiotes* L.) and waterhyacinth (*Eichhornia crassipes* [Mart.] Solms), which were floated in toxin solutions, all aquatic plants, namely, hydrilla, alligatorweed (*Alternanthera philoxeroides* [Mart.] Griseb.) coontail (*Ceratophyllum demersum* L.), cryptocoryne (*Cryptocoryne neville* Trimen), Amazon sword plant (*Echinodorus brevipedicellatus* [O. Kuntze] Buchen.), Canadian elodea (*Elodea canadensis* Michx.), common duckweed (*Lemna minor* L.), Eurasian watermilfoil (*Myriophyllum spicatum* L.), bladderwort (*Utricularia foliosa* L.), unicellular green algae and filamentous alga (*Oedogonium* sp.), were immersed completely in water containing the toxic substances. The piscine species tested included *Gambusia affinis* Baird and Girard, *Mollienesia latipinna* La Screur and *Jordanella floridae* Goode and Bean from the wild and an unidentified cyprinid from a local game fishing supplies company. Toxicity to plants and fish was recorded respectively at 60 and 24 hr after mixing toxic samples.

RESULTS

In pathogenicity tests, 12 of 30 fungal isolates tested on hydrilla induced yellowing reaction within 3 weeks after inoculation. The fungi were found to sporulate and/or proliferate throughout the tube in mycelial stage during this time. After turning yellow, the plants either lysed (Figure 1) or remained yellow, rarely producing healthy sideshoots. The fungi used for inoculations could be reisolated from yellowed hydrilla sprigs. No fungi were isolated from surface sterilized healthy controls or the symptomless inoculated plants. The 12 chlorosis inducers included eight isolates of *Penicillium,* two of *Trichoderma* and one each of *Aspergillus* and an unidentified fungus.

Culture solutions of each of the 12 fungi causing yellowing reactions in pathogenicity tests were also toxic to sprigs of hydrilla. The toxicity symptoms induced by culture solutions were similar to the reaction of the respective isolates

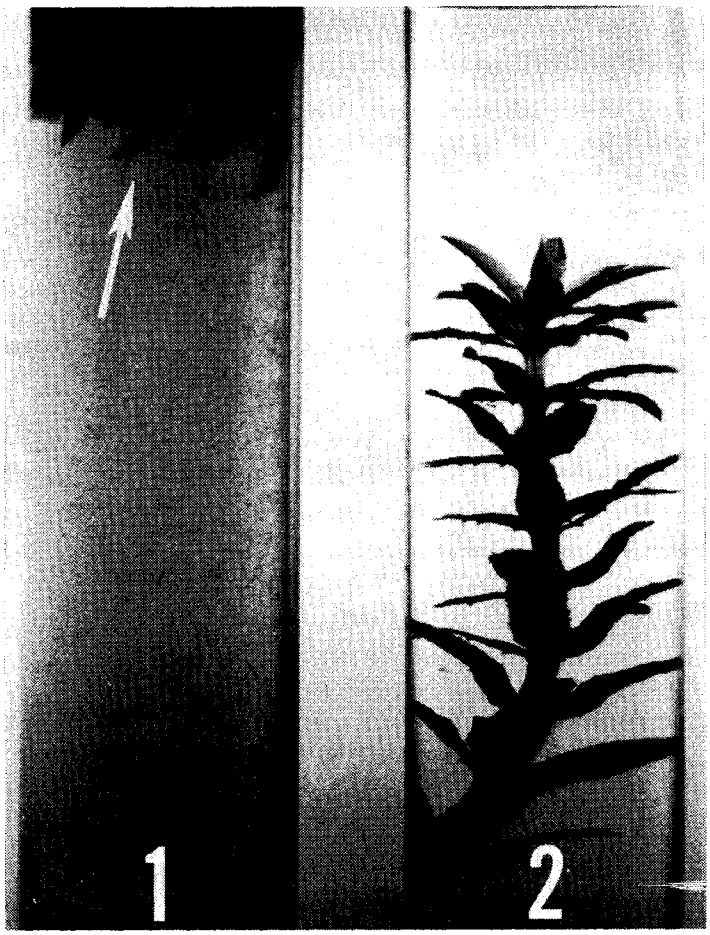

Figure 1. Lysis of hydrilla caused by toxic culture solution of *P. oxalicum*. 1. Tube with toxin and lysed hydrilla (arrow). 2. Control tube without toxin.

in pathogenicity tests except that symptoms developed much earlier. Toxicity of culture solutions could be seen as early as 48 hr after mixing the test samples and invariably within 6 days. Plants killed by toxic culture solutions lysed within 3 weeks.

In another experiment, culture solutions of seven isolates were subject to dialysis and the various fractions tested in order to determine if any macromolecules were involved in toxicity. These seven, including toxic and nontoxic isolates, were selected at random from the 30 isolates tested in pathogenicity trials. Controls in this experiment included the noninoculated culture medium as well as deionized water. Four of the seven isolates produced toxic culture solutions. The toxic damages were produced by the nondialysed culture solutions as well as the diffusates. Neither of the controls caused any damage to hydrilla (Figure 2). That the toxic substance passed through the dialysis membrane into the dialysis medium indicated that it was a relatively low molecular weight substance.

The culture solution from one of the toxic isolates (tentatively identified as *Penicillium oxalicum* Currie and Thom and originally from India) was tested further to identify the toxin. Preliminary chemical, chromatographic, and spectrophotometric tests have indicated that the toxin from this isolate is oxalic acid. Three others among the

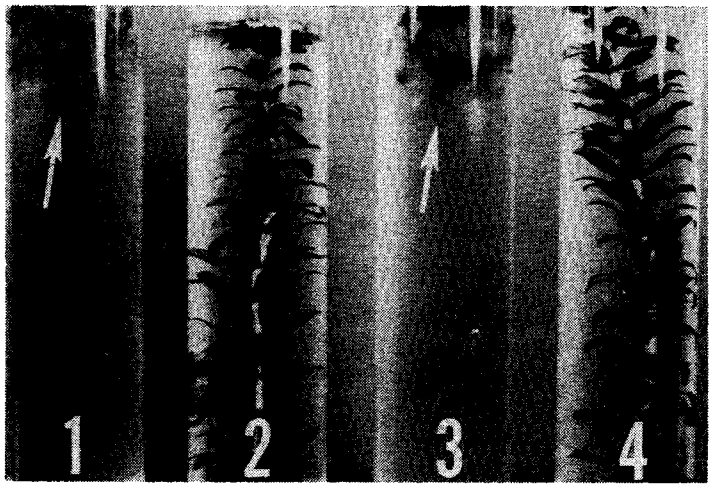

Figure 2. Lysis of hydrilla due to toxic culture solution of *P. oxalicum* subject to dialysis. Nondialysed toxic culture solution (1); dialysed culture solution (2); diffusate (3); and control consisting of non-dialysed, uninoculated culture medium (4). Arrows point to remnants of lysed hydrilla.

twelve toxic isolates also produced oxalic acid in cultures. The remaining eight isolates, though toxic, apparently did not produce oxalic acid in culture solutions, since no oxalate was detected in them by chemical tests (1).

The minimum dose of reagent grade oxalic acid needed to kill hydrilla in tube tests was found to be 1.0 mM or roughly 126 ppm. Toxicity of purified fungal oxalate and reagent grade oxalic acid to other aquatic plants and fish was, therefore, tested at this concentration. The culture solution, the purified fungal oxalate and pure oxalic acid were nonspecifically toxic to a number of aquatic plants and fish tested (Table 1). Alligatorweed and cryptocoryne,

TABLE 1. TOXICITY OF OXALATE FROM ISOLATE 31 (*Penicillium oxalicum* Currie and Thom) AND PURE OXALIC ACID TO AQUATIC PLANTS AND FISH.

Test organism	Culture[a] solution	Purified[b] fungal oxalate	Pure[b] oxalic acid
Hydrilla	+++	+++	+++
Alligatorweed	–	+	+
Coontail	NT	+++	+++
Cryptocoryne	–	+	+
Amazon sword plant	–	+++	+++
Waterhyacinth	–	+	+
Canadian elodea	+++	NT	NT
Common duckweed	–	+++	+++
Eurasian watermilfoil	+++	++	++
Water lettuce	–	+	+
Bladderwort	+++	NT	NT
Unicellular green algae	+++	NT	NT
Filamentous alga	+++	+++	+++
Fish[c]	Alive	Dead	Dead

–represents no visible difference from controls (no toxicity). + to +++ represent the degree of yellowing of test plants from slight chlorosis to total yellowing and death of plant. Plants were kept under observation for 6 days. NT = not tested.

[a]Tested at 3 ml culture solution per 40 ml of sterile deionized water.

[b]Tested at approximately 1.0 mM concentration. Fungal oxalate was dissolved first in small volume of dilute H_2SO_4. Pure oxalic acid was dissolved in water.

[c]Included species mentioned under Methods and Materials.

which have relatively thick cuticle and water lettuce and waterhyacinth that floated in toxic solutions were fairly resistant to oxalic acid at 1.0 mM concentration compared to other plants including hydrilla. Only root damage was noticeable in waterhyacinth and water lettuce at 1.0 mM concentration of oxalic acid. Water lettuce was more susceptible to oxalic acid at this concentration than waterhyacinth.

DISCUSSION

To our knowledge, so far the potential of fungal toxins in aquatic weed control has not been sufficiently evaluated. Plant pathogens produce a variety of substances toxic to plants, several of which have been identified including some that are chemically complex (4). Some of them are highly host-specific and damage susceptible plants in a manner similar to the pathogens from which they were isolated (7). Species of *Penicillium, Aspergillus,* and *Trichoderma* also produce a variety of phytotoxins (3, 6, 8). The production of an apparently narrow host spectrum phytotoxin by *Alternaria eichhorniae* was demonstrated by Nag Raj and Ponnappa (5). This toxin, which induced necrotic symptoms on leaves of waterhyacinth comparable to those caused by the fungus, was considered one of the factors in the usefulness of this pathogen in biocontrol of waterhyacinth (5). Toxins produced by microorganisms might be used to control aquatic weeds if they are found to be efficient and selective. The use of purified phytotoxin substances of microbial origins could have certain advantages over living pathogens in biocontrol of aquatic weeds in their handling such as quantitation, application, and biodegradability in the field.

The isolates that caused lethal damage on hydrilla in pathogenicity tests could be reisolated from the plant tissue. It cannot be stated with certainty if these fungi were true parasites or merely colonized saprophytically tissues that were weakened or killed by the toxin. It is possible, in an aquatic medium the microbial metabolites permeate and affect the plant tissue in advance of the invading mycelium. In pathogenicity tests it was likely that a minimum toxic concentration of the metabolite was reached in the 3-week incubation, before the damage to hydrilla was visible.

Culture solutions of only 12 of the 30 isolates of *Penicillium, Aspergillus,* and *Trichoderma* were toxic to hydrilla. The twelve isolates appear to be significant in their toxicity to hydrilla when compared with the nontoxic isolates of these genera. Only four of these produced oxalic acid in cultures. It appears that substances other than oxalic acid are involved in toxicity of the other eight isolates. Attempts are being made to identify these toxins.

The apparent extreme toxicity and nonspecificity of the purified fungal oxalate and pure oxalic acid to aquatic plants and fish seemingly preclude their use in biocontrol of hydrilla. Also, the minimum dose of oxalate needed to kill hydrilla (approximately 126 ppm) is perhaps excessive for use in field conditions. However, we are exploring the feasibility of using spores of toxic molds in controlling hydrilla under experimental conditions along with our search for phytotoxins from plant pathogens that are host-specific.

The striking effect of the microbial toxins studied presently in causing total lysis of hydrilla suggests a possible efficient method of clearing obstructions in waterways caused by this submersed plant.

ACKNOWLEDGMENT

Thankful appreciation is extended to Mrs. Phyllis Habeck, Laboratory Technician for her assistance in this work.

LITERATURE CITED

1. Baker, C. J. L. 1952. The determination of oxalates in fresh plant material. Analyst 77:340-344.
2. Charudattan, R. 1973. Pathogenicity of fungi and bacteria from India to hydrilla and waterhyacinth. Hyacinth Contr. J. 11:44-48.
3. Gibson, I. A. S. 1953. Crown rot, a seedling disease of groundnuts caused by *Aspergillus niger*. *Trans. Brit. Mycol. Soc.* 36:198-209.
4. Kalyanasundaram, R. and R. Charudattan. 1966. Toxins and plant diseases. J. Sci. Ind. Res. (India) 25:63-73.
5. Nag Raj, T. R. and K. M. Ponnappa. 1970. Blight of water-hyacinth caused by *Alternaria eichhorniae* sp. nov. Trans. Brit. Mycol. Soc. 55:123-130.
6. Raper, K. B. and C. Thom. 1949. A Manual of the Penicillia. The Williams and Wilkins Co., Baltimore. 875 pp.
7. Scheffer, R. P. and O. C. Yoder. 1972. Host-specific toxins and selective toxicity. In "Phytotoxins in Plant Diseases." R. K. S. Wood, A. Ballio and A. Graniti (Eds.) pp. 251-269. Academic Press, London.
8. Weindling, R. and O. H. Emerson. 1936. The isolation of a toxic substance from the culture filtrate of Trichoderma. Phytopathology 26:1068-1070.