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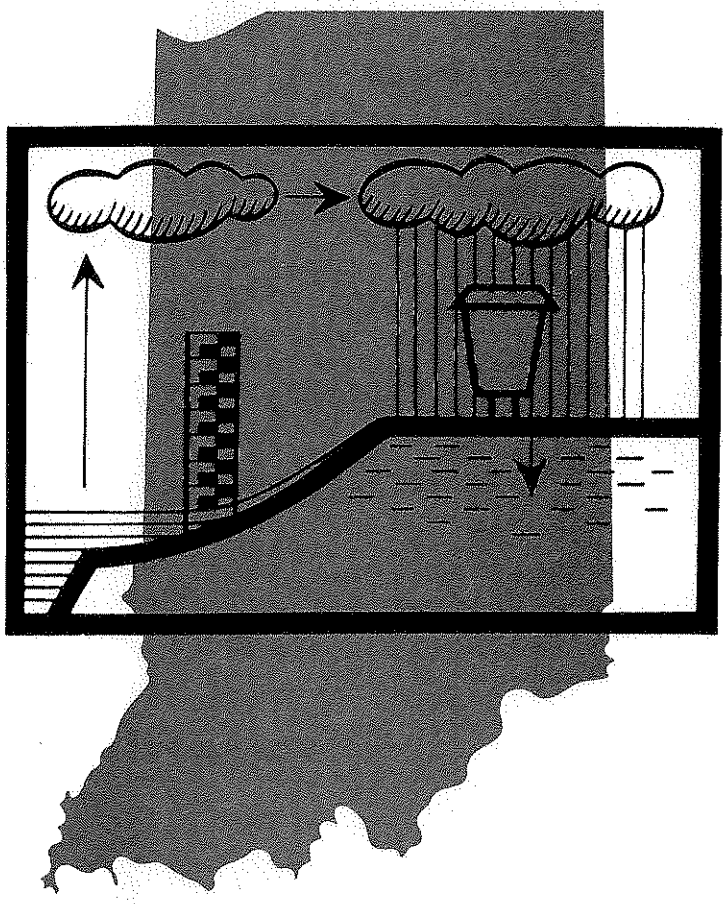
MYCOHERBICIDE CONTROL OF EURASIAN WATERMILFOIL



by

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MYCOHERBICIDE CONTROL OF EURASIAN WATERMILFOIL

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ABSTRACT

Aquatic vegetation is a major environmental concern. Throughout the northern states the most important aquatic weed is Eurasian watermilfoil (*Myriophyllum spicatum* L.). Aquatic weeds are controlled primarily with herbicides. However, techniques that are less threatening to the environment are preferred. One technique is the use of mycoherbicides. Mycoherbicides are fungi that have the capacity to kill unwanted plants. The use of mycoherbicides against aquatic weeds has received little attention and there has been no emphasis on understanding how the plant protects itself from infection. Plants use secondary metabolic products, especially phenolic compounds, to protect themselves from fungal invasion.

The focus of this research was to determine the class of secondary products synthesized by milfoil and used possibly for protection against fungal pathogens. Analyses of leaves and stems showed that phenylpropanoid phenols, known to be toxic to fungi, accumulate in tissue as the initial response to infection. These compounds become esterified to the plant cell wall and present a barrier to fungal growth. The accumulation of pigmented materials in response to infection suggested that early in the infection process the phenols are oxidized, a factor that increases their toxicity. Analyses of peroxidase levels in inoculated tissue showed that the activity of this enzyme increased dramatically as a result of inoculation indicating that significant phenolic oxidation occurs. Thus, milfoil synthesizes phenylpropanoid phenols to protect itself from fungal infection.

INTRODUCTION

Excessive growth of aquatic vegetation in the lakes and ponds of Region IV is a major management concern. Aquatic weeds prevent the use of a body of water for recreation, fisheries, irrigation, and as a source of potable water. Throughout the northern states the major aquatic weed is Eurasian watermilfoil (*Myriophyllum spicatum* L.). Although it has been the major weed in Indiana and surrounding states, its recent invasion of Minnesota lakes and the Okanagan and Columbia River basins of British Columbia and Washington has prompted concern for its control.

Eurasian watermilfoil, a dicot, is a rooted perennial plant with a long flexible stem reaching up to four meters or more in length. It is rooted in bottom sediments and grows up through the water column concentrating the majority of its biomass in dense canopies near the water surface [1]. It is an extremely competitive plant, having crowded out native aquatic species in many lakes, reservoirs, and rivers in the United States, Canada, Europe, and Asia Minor [2, 3, 4, 5]. It spreads rapidly by means of underground rhizomes, seed production and fragmentation. A single fragment has the theoretical potential to produce 250 million new plants in a single year under appropriate environmental conditions [6]. The plant is found throughout Indiana lakes and reservoirs and is a major target of control efforts.

Luxuriant growth of the plant results in waters that are unusable for recreation, have a lower aesthetic value, have reduced flow for irrigation, drainage, and hydroelectric power generation, and support reduced quantities of fish in spawning areas [1, 7]. Control methods have included herbicides, habitat manipulation such as dredging, mechanical harvesting, and biological control agents such as herbivorous fish [1, 8, 9, 10]. Although a number of control methods are available, none has proven both effective and environmentally safe.

Theoretically, there are advantages for using mycoherbicides to control weeds, and the process has had considerable, although limited success, in terrestrial weed control [11, 12]. The potential importance of such control strategies has increased as evidenced by the recent interest of the American Chemical Society in microbes and microbial products to deal with environmentally significant problems [13].

Aquatic weeds are controlled primarily with herbicides. However, management techniques that present less of a threat to the environment are preferred. One technique, the use of mycoherbicides, has considerable potential for weed control and presents a minimal of environmental damage. Mycoherbicides are plant pathogenic fungi that can cause disease and kill

an unwanted weed species. However, success to date has been minimal in aquatic situations. Few pathogens are known to attack milfoil, and when introduced into the environment their virulence appears to decrease substantially. Testing of mycoherbicides with aquatic weeds has been limited to aquaria or small scale field tests in which plants have been artificially stressed to enhance disease development. In addition, testing programs involve the screening of large numbers of fungi for the potential to cause disease. No emphasis has been place on the susceptibility of the target weed under varying conditions of age, season, or environmental parameters.

The ability of a target plant to defend itself from a mycoherbicide is based on the plant's biochemical and morphological characteristics. Therefore, the mechanisms by which a weed defends itself must be understood if mycoherbicides are to be developed as effective control agents. Plants primarily use secondary products to protect themselves from fungal pathogens. However, little information is available on the specific types of compounds produced in milfoil and which of these compounds might be toxic to fungi. How and when milfoil is most capable of synthesizing such compounds, or even if the compounds are synthesized, is unknown. This basic information, which was the objective of the present study, must be obtained to determine the conditions, such as plant age, season of growth, or environmental conditions, under which milfoil would be most susceptible to a mycoherbicide. Therefore, the physiological basis of resistance of milfoil to fungal pathogens was investigated. This is needed since the use of mycoherbicides has had limited success and only with terrestrial weed species [13, 11, 12, 14].

For weed control, and especially the control of aquatic weeds, a concern for the mechanisms through which the plant protects itself has not been addressed [13]. Rather, research has been directed to a search for pathogens with the potential to cause disease [13]. This approach lacks several important aspects. First, it is important to understand the natural defense mechanisms that the plant uses to protect itself once the pathogen has gained entry. Such mechanisms include the presence of preformed fungitoxic compounds and the synthesis of fungitoxic secondary products that occurs specifically in response to fungal infection [15, 16, 17, 18, 19]. Second, it is necessary to understand the constraints placed on successful infection by the environment. These include the effects of the environment on plant growth, growth and survival of the pathogen, and on the ability of the plant to employ its natural defense mechanisms [15, 16, 17, 20, 21, 22, 23].

The potential for success of mycoherbicides is based on biological principles of plant growth and physiology, disease development, and the ability or not, of plants to protect themselves from successful attack by pathogens. For example, there are many instances in which susceptibility to fungi varies with the plant's developmental stage. The following scenario

highlights a few simple examples of these interrelationships. Plants often produce compounds that are toxic to plant pathogens. Importantly, the synthesis of such compounds typically occurs only during specific stages of the growth of the plant. For example, tulip bulbs resist a rot caused by the fungus *Fusarium oxysporum* f. *tulipae*, a significant problem in tulip production, as a result of the presence of compounds known as tuliposides that are toxic to the fungus [24]. For a brief period of two weeks before the commercial harvest of bulbs the concentration of tuliposides falls to such low levels that it is not sufficient to inhibit the development of the rot disease. Just after harvest the concentration again rises and the bulbs are once again protected [25]. Thus, there is a period in the plant's normal growth during which the fungus can successfully attack bulb tissue. A similar situation exists in grain sorghum. These plants contain a cyanogenic glycoside, called dhurrin, but it is only present in juvenile leaf tissue [26]. As the tissue matures the concentration of the compound falls to undetectable levels [26] and the plant becomes extremely susceptible to a fungus that causes an important leaf blight and reduces grain yield. It is presumed that the precipitous drop in concentration of this highly toxic compound allows the fungus to gain entry and cause the death of the plant tissue [27]. Perhaps of greater significance, is the fact that the compound also acts as an important deterrent of insect feeding during the juvenile stages of plant growth. Again, this is an example of a preformed compound that is present only during a specific stage of plant growth and accounts for the protection of the plant from certain fungal and insect depredations.

Few studies have been reported that deal with phenolic compounds present in aquatic weeds that could act as natural deterrents of disease development. One exception is the study of phenolics in the salt marsh grass *Spartina alterniflora*. Certain phenols (especially ferulic acid) present at various stages of the plant's growth cycle have been suggested to act as deterrents to feeding by snails and wild geese [28, 29, 30]. Although such herbivores are not disease-causing organisms, the similarity of the relationship suggests that phenols are also natural components of the mechanisms used by weedy species for protection against fungal pathogens.

There are few literature citations that deal with phenolic constituents of aquatic weeds [31, 32]. These reports dealt with phenolic compounds in another aquatic weed, *Hydrilla verticillata*, that is a significant problem from Florida to the Potomac River [33, 34, 35]. In our own studies of Hydrilla we demonstrated that the previous reports on the phenolic composition of leaves and internodes of this plant were incorrect. We showed that all tissues of the plant contain significant levels of a single phenolic compound, an unusual caffeic acid ester [36]. This is important in that no known fungal pathogens of hydrilla exist that are capable of causing significant disease development in the plant. Importantly, the compound we have identified is fungitoxic. As an

example of what can be done to render a plant such as hydrilla susceptible to fungal attack we have performed experiments to inhibit the synthesis of phenols in the tissue, reducing the level of the toxic caffeic acid ester, and increasing the potential for the plant to become diseased.

MATERIALS AND METHODS

Growth of Milfoil: Milfoil was grown in an unstressed state in growth chambers at optimal light intensities and temperatures (photosynthetic photon fluence rate of 400 $\mu E\ m^{-2}s^{-1} \pm 10\%$, 16:8 h light:dark photoperiod, 25 °C). Plants were cultured in 3 L Florence flasks containing 2 L of sterile 10% Hoaglands solution [37].

Fungal Pathogen and Inoculations: Cultures of *Colletotrichum gloeosporioides*, previously demonstrated to have significant potential as a pathogen of milfoil were grown on potato dextrose agar in the light at 23 °C. This fungus had been initially isolated from stem lesions of milfoil at the University of Wisconsin Aboretum [38]. Conidial suspensions were obtained from 2-week-old cultures. Conidia were suspended in sterile, distilled water and used as inoculum (4×10^6 conidia per ml). The high inoculum level was used to ensure that sufficient infection sites could be obtained and to optimize the isolation of representative phenolic profiles.

Sterile, plastic petri dishes were lined with a sterile filter paper and 6 ml of sterile, distilled water were added. Individual stem segments of milfoil plants were transfered to the petri dishes so that the entire plant section was submerged. Plant segments were typically 8 to 10 cm in length and included the apical meristem. Conidia of *C. gloeosporioides* were atomized onto the surface of the water in which plants were submerged and the dishes were swirled to distribute conidia. The dishes were sealed with Parafilm to maintain high humidity and incubated under constant fluorescent light (60 μE m-2s-1, 23 °C) until harvested for extraction of phenols .

Isolation of Phenolic Compounds: Phenolic compounds were selectively isolated from leaves and stem internodes of vigorously growing plants. Extractions were performed first on uninoculated tissues to determine whether significant levels of phenolics were present prior to the time of inoculation. Leaves and internode segments were separated under a dissecting microscope. The tissues (approximately 25 mg for each replicate) were blotted dry, weighed, and submerged in 4 ml of absolute HPLC grade methanol at 4 °C. Free, esterified, and glycosylated phenolics were allowed to leach into the methanol overnight. The methanol extract was placed in a round bottom flask for evaporation. An additional 4 ml of methanol were added and the tissue was homogenized. The homogenate was centrifuged (12,000 g, 7 min) and the methanol added to the

original methanol extract. This combined methanol extract represented the portion of phenolics that would be considered "free" forms, that is either glycosylated, esterified, or the free unglycosylated, unesterified forms of the compounds. The combined methanol extract was reduced to dryness under vacuum and the residue was dissolved in 1 ml of HPLC grade methanol for subsequent HPLC analysis.

Separate analyses indicated that acid hydrolysis of the methanol extract showed no significant differences after HPLC analysis from the unhydrolyzed material indicating that few glycosylated forms of phenols were present in the extract. Thus, subsequent analyses of tissue extracts included only base hydrolysis for the determination of phenolic esterification.

The methanol extract was also base hydrolyzed to determine if significant proportions of phenols in the extract were present in the form of esters. Five hundred µl of the methanol extract were placed in a glass centrifuge tube and the volume was brought to 1 ml with double distilled water. The preparation was purged with N_2 to remove the methanol. An additional 500 µl of water were added and the sample was vortexed and purged again with N_2. One ml of 3N NaOH was added and the sample was placed in a desiccator for 3 h to ensure the absence of oxygen. After incubation 1 ml of 6 N HCl was added, and the sample was vortexed. The preparation was then partitioned three times with 3 ml of ethyl acetate. The ethyl acetate phases were pooled, dried under vacuum, and the residue redissolved in 500 µl of HPLC grade methanol for HPLC analysis. This final extract represented the portion of the phenolic compliment present as esters and the data provided information on the parent acids, typically phenylpropanoids, of the compounds.

Homogenized tissue residues of both leaves and stems were also analyzed for the presence of phenols esterified to cell wall polysaccharide polymers. Tissue homogenates, originally prepared in methanol, were centrifuged and the pellets were resuspended and centrifuged repetitively in methanol to remove free phenolic materials. The final washed pellet was suspended in 500 µl of distilled water and the sample was mixed, purged with N2, and 500 µl of 3 N NaOH were added. The sample was mixed again, purged with N2 and placed in a desiccator for 3 h. After incubation 500 µl of 6 N HCl were added and the sample was vortexed and centrifuged. The supernatant was decanted and partitioned 3 times with equal volumes of ethyl acetate. The ethyl acetate fractions were pooled, dried under vacuum, and the residue was resuspended in 500 µl methanol. The final methanol soluble preparation, which represented those phenols which had been esterified to the cell wall materials, was analyzed by HPLC chromatography.

HPLC Analyses: For HPLC analyses samples of extracts were injected onto a Beckman, Ultrasphere, C-18 Reverse Phase column (4.5x250 mm). For the separation of components of the methanol extract of tissues (the "free" phenolic components) the mobile phase consisted solvent A (100 % HPLC grade methanol) and solvent B (1.0% HPLC grade acetic acid in water). The initial conditions for separation were 80 % solvent B and the phenolic compounds were separated with a linear gradient to 50 % B over 30 min. The program was returned to 80% B in 5 min after which an additional sample could be run. The program for determination of phenolics in the base hydrolyzed preparations (both the base hydrolyzate of the methanol extract and the base hydrolyzate of the tissue residue) was a linear gradient of 85 % B to 70 % B over 10 min. It was run isocratically at 70 % B for an additional 10 min and was returned to the initial starting conditions in 5 min. Because of the small size of samples assayed, the HPLC sensitivity was set at 0.005 OD full scale for methanol extracts and at 0.001 OD for analysis of base hydrolyzates.

Peroxidase Assays: Stem internodes that exhibited a host response were selectively isolated under the microscope and compared with uninoculated controls for alterations in the level of peroxidase enzyme activity. Replicates of approximately 50 mg each of internode tissue were homogenized in 5 ml of 0.05 M sodium phosphate buffer, pH 6.0, with a polytron homogenizer. The homogenate was centrifuged at 20,000 g for 30 min at 4 °C. The supernatant, the fraction that contained soluble peroxidase activity, was assayed for enzyme activity. The reaction was performed as as a modification of that described previously [39]. Reaction mixtures consisted of 1350 µl of a freshly prepared buffer-substrate solution, 50 µl of 0.05 M sodium phosphate buffer pH 6.0, and 100 µl of enzyme preparation. The buffer-substrate solution was a mixture of 0.05 M sodium phosphate buffer pH 6.0 (100 ml), 1 % guaicol in 50% ethanol (10 ml) and 0.03% hydrogen peroxide (10 ml). The buffer-substrate solution was incubated at 25 °C and the reaction was initiated with the addition of 100 µl of the enzyme preparation. One unit of peroxidase activity was defined as a change of 1 OD unit at 470 nm min^{-1}.

Microscopy: Preliminary studies were performed to determine if evidence of the success of infection could be detected microscopically. Tissues were inoculated as described and at 3 h intervals samples were observed to determine conidium germination, appressorium formation, evidence of host penetration, and evidence of a host response.

RESULTS

Microscopy: Microscopic investigations revealed that the fungus began to germinate by the direct formation of appressoria at approximately 12 h following inoculation. No evidence of penetration

of the fungus into the tissue was observed until more than 72 h post inoculation when intercellular mycelia were detected.

Leaves did not provide a suitable substratum for the fungus. Few conidia appeared to attach and adhere to leaf surfaces. This could be a function of the morphology of the leaf which is tubular in form, possibly resulting in the conidia failing to become attached on the surface. It is also possible that the surface of the leaf does not present a suitable infection court due to a lack of receptor sites for conidial adhesion, a factor well established as important to the initiation of the infection process of *Colletotrichum* species [40]. In contrast, conidia formed appressoria readily on stem internodes.

Within an additional 8 to 12 h following appressorium formation on stem internodes the host tissue was found to exhibit a visible response to attempted penetration. This was evident as the appearance of extensive areas of orange-brown pigmentation within cells directly associated with appressoria. Attempts to isolate the pigmented material as a distinct chromophore failed, suggesting that the material represented oxidized products of the interaction.

Peroxidase assays: A comparison of peroxidase levels in inoculated stem internodes with levels in uninoculated controls demonstrated that within 24 h of inoculation there was a substantial increase in enzyme activity. Controls exhibited no detectable enzyme activity whereas inoculated samples had levels of activity that exceeded 42 units. That the level of activity increased so dramatically indicated that oxidation of phenolic materials was a component of the host response.

Phenolic compound profiles: The methanol soluble fractions of phenolics isolated from leaves, either inoculated or uninoculated, did not exhibit distinct peaks representative of known compounds (data not shown). Rather, the samples consistently exhibited small peaks that were not integrated for peak area by the instrument. In contrast, when the methanol extracts from inoculated leaves were base hydrolyzed the presence of substantial levels of phenylpropanoids was revealed. Figure 1 shows a comparison of the extracts taken from control and inoculated tissues at 72 and 96 h post inoculation. The phenylpropanoids caffeic, p-coumaric and ferulic acid were readily apparent in extracts from inoculated tissue but only present in trace amounts in extracts from uninoculated controls (Fig. 1).

Figure 2 is a representative profile of the pattern of phenolics obtained after base hydrolysis of tissue residues (cell wall materials) from leaves. The examples given are for samples taken at 48 and 96 h after inoculation. The profiles demonstrate that throughout the time interval studied no

phenylpropanoids or other phenolic residues could be detected in uninoculated tissue. However, caffeic acid was a primary component of the cell wall residue from inoculated tissue (Fig. 2). The caffeic acid content increased as the time after inoculation increased indicating that the esterification of phenylpropanoids to the cell wall was an ongoing component of the infection process.

In contrast to methanol extracts from leaf tissues, extracts from stem internodes exhibited numerous unidentified peaks of phenolic materials in both uninoculated and inoculated tissues. Figure 3 shows a representative profile for samples taken at 96 and 120 h after inoculation comparing extracts from inoculated and uninoculated tissues. As the duration after inoculation increased the number of phenolic components in extracts increased dramatically and in all cases the number of peaks present in inoculated tissues were substantially greater that in controls.

Base hydrolysis of the methanol extracts from inoculated stem internodes demonstrated the presence of substantial levels of the phenylpropanoids caffeic, p-coumaric and ferulic acid in comparison to uninoculated controls. Figure 4 shows representative profiles of base hydrolyzed methanol extract samples taken at 72 and 96 h after inoculation. The substantial level of caffeic acid in this preparation indicates that this phenylpropanoid represents a primary component for the diversion of carbon in the phenylpropanoid biosynthetic pathway as a response to fungal infection.

As was the case when leaf tissue extracts were base hydrolyzed (Fig. 2), the base hydrolysis of stem tissue residues exhibited the presence of phenylpropanoids only in residues from inoculated tissues (Fig. 5). This indicates that esterification of phenolics to the cell wall is a primary response of the plant to infection.

Representative examples of time course studies to evaluate the amounts of the three common phenylpropanoids in base hydrolyzed methanol extracts of stem tissues are shown in Figures 6, 7, and 8. The high level of variance in the data, as shown by the error bars, is a reflection of the difficulty in obtaining uniformly diseased samples of the tissue. Notably Figure 6 shows that substantial levels of caffeic acid esters accumulated in inoculated tissues (350 to 400 μM per gram fresh weight) whereas the level is barely detectable in uninoculated control tissue. Importantly, the level of caffeic acid remained high throughout the period studied. As the assays were initiated 48 h after inoculation the initial response had to have begun substantially earlier. In contrast, the level of p-coumaric acid appeared to rise in response to inoculation and then to fall as the infection process continued (Fig. 7). In the case of ferulic acid, this compound appeared to be present in considerable amounts in uninoculated tissues but as the infection process progressed

these levels fell significantly (Fig. 8) and by 96 h the compound could never be detected. In contrast, the levels of ferulic acid in the inoculated tissue extracts appeared to remain constant.

DISCUSSION

The results of the present investigation demonstrate that Eurasian watermilfoil produces a significant level of phenolic intermediates in response to infection with the fungus *Colletotrichum gloeosporioides*. In contrast, the levels of phenols in uninoculated tissues are barely detectable suggesting that the synthesis of phenolic intermediates is the primary response of the plant to protect itself from the stress of infection.

Two significant observations have been made. First is that leaf tissue is a poor substratum for fungal infection in that the pathogen essentially fails to adhere to the leaf and to penetrate. In contrast the stem internode is quite suitable for the fungus. This is important as it must be taken into consideration as we continue to search for pathogens that will serve as aggressive agents of disease. Second is the fact that the plant readily accumulates phenylpropanoid phenolics in all tissues, both leaves and stem internodes, as a response to infection. Thus, leaf tissue does respond to infection when the fungus makes successful contact. Because leaves do not represent a means through which the plant can multiply it is probably unwise to attempt to destroy this tissue through the use of a mycoherbicide. Because it is stem tissue, and particularly the nodes, that can give rise to new plants through vegetative propagation, it is this tissue that must be the target of disease initiation. Second is the fact that substantial levels of phenylpropanoids, in particular caffeic acid, were found to have become esterified to cell walls of both leaf and stem internodes. In contrast, no phenylpropanoids were found esterified to cell walls of uninoculated tissues. This suggests that the potential for containment of the pathogen is through the esterification of phenolics to wall materials, a factor which would present a barrier to successful intercellular mycelial growth.

The observation that pigmented materials appear in tissues within 24 h of inoculation is an indication that oxidation of phenolic materials occurs as an early response to attempted infection. The numerous unidentified peaks observed in HPLC analyses of phenolic extracts is a further indication that oxidation of phenols has occurred. In addition the substantial increase in peroxidase enzyme activity following inoculation confirms the importance of the phenolic oxidation to the resistance mechanism used by the plant to protect itself. The present results indicate that successful control of milfoil might be realized through strategies that alter the ability of the plant to synthesize phenols. Because phenolic synthesis typically occurs under high light intensity and is often a characteristic of plant age and stage of ontogeny, the results also indicate that future studies should

evaluate the capacity of the plant to synthesize phenolics at different stages of development. Such observations could lead to logical conclusions concerning the appropriate time for the application of mycoherbicide treatments for milfoil control.

REFERENCES

1. Grace, J., and Wetzel, R. 1978. The production biology of Eurasian watermilfoil (*Myriophyllum spicatum* L.): a review. J. Aquat. Plant Manage. 16:1-11.

2. Reed, C.F. 1977. History and distribution of Eurasian watermilfoil in the United States and Canada. Phytologia 36:417-436.

3. Aiken, S.G. 1981. A conspectus of *Myriophyllum* (Halagoraceae) in North America. Brittonia 33:57-69.

4. Cook, C.D.K. 1985. Worldwide distribution and taxonomy of *Mryiophyllum* species. In: Anderson, L. (Ed.), Proceedings of the First International Symposium on Watermilfoil (*Myriophyllum spicatum*) and related Haloragaceae species. Vancouver, British Columbia, pp. 1-7.

5. Rawson, R.M. 1985. History of the spread of Eurasian watermilfoil through the Okanagan and Columbia River Systems (1978-1984). In: Anderson, L. (Ed.), Proceedings of the First International Symposium on Watermilfoil (*Myriophyllum spicatum*) and related Haloragaceae species. Vancouver, British Columbia, pp. 35-38.

6. Stanley, R.A., and Gangstad, E.O. 1987. Biological parameters of the TVA Eurasian watermilfoil management program. In: Gangstad, E.O. and Stanley, R.A. (Eds.), Environmental Management of Water Projects. CRC Press, Boca Raton, FLA. pp. 35-37.

7. Newroth, P.R. 1985. A review of Eurasian water milfoil impacts and management in British Columbia. In: Anderson, L. (Ed.), Proceedings of the First International Symposium on Watermilfoil (*Myriophyllum spicatum*) and related Haloragaceae species. Vancouver, British Columbia, pp. 139-153.

8. Thompson, T.W., and Hartwig, H. 1973. Control of watermilfoil in large Wisconsin lakes. Hyac. Cont. J. 11:20-23.

9. Rawls, C.K. 1975. Mechanical control of Eurasian watermilfoil in Maryland with and without 2,4-D application. Chesapeake Sci. 16:266-281.

10. Nicholson, S.A. 1981. Effects of uprooting on Eurasian watermilfoil. J. Aquat. Plant Manage. 19:57-59.

11. Templeton, G.E., TeBeest, D.O., and Smith, R.J., Jr. 1979. Biological weed control with mycoherbicides. Annu. Rev. Phytopathol. 17:301-310.

12. Templeton, G.E., TeBeest, D.O., and Smith, R.J., Jr. 1984. Biological control in rice with a strain of *Colletotrichum gloeosporioides* (Penz.) Sacc. used as a mycoherbicide. Crop Protect. 3:409-422.

13. Hoagland, R.E. 1990. Biological Weed Control Using Microbes and Microbial Products as Herbicides. American Chemical Society Symposium Series 439. American Chemical Society, Washington, DC.

14. US Army Corps of Engineers. 1989. Proceedings, 23rd Annual Meeting, Aquatic Plant Control Research Program. Aquatic Plant Control Research Program Miscellaneous Paper A-89-1. 316 p.

15. Bailey, J.A., and Mansfield, J.W. (Eds.). 1982. Phytoalexins. Blackie, Glasgow.

16. Ebel, J. 1986. Phytoalexin synthesis: the biochemical analysis of the induction process. Annu. Rev. Phytopathol. 24:235-264.

17. Friend, J. 1979. Phenolic substances and plant disease. Recent Adv. Phytochem. 12:557-588.

18. Nicholson, R.L., Kollipara, S.S., Vincent, J.R., Lyons, P.C., and Cadena-Gomez, G. 1987. Phytoalexin synthesis by the sorghum mesocotyl in response to infection by pathogenic and nonpathogenic fungi. Proc. Natl. Acad. Sci. USA., 84:5520-5524.

19. Overeem, J.C. 1976. Pre-existing antimicrobial substances in plants and their role in disease resistance. In: J. Friend and D.R. Threlfall, (eds.). Biochemical aspects of plant-parasite relationships. Academic Press, London. pp. 195-206.

20. Leonard, K.J., and Thompson, D.L. 1976. Effects of temperature and host maturity on lesion development of *Colletotrichum graminicola* on corn. Phytopathology 66:635-639.

21. Rupe, J.C., Siegel, M.R., and Hartman, J.R. 1982. Influence of environment and plant maturity on grey leaf spot of corn caused by *Cercospora zeae-maydis*. Phytopathology 72:1587-1591.

22. Nicholson, R.L., Butler, L.G., and Asquith, T.N. 1986. Glycoproteins from *Colletotrichum graminicola* that bind phenols: Implications for survival and virulence of phytopathogenic fungi. Phytopathology 76:1315-1318.

23. Nicholson, R.L., and Moraes, W.B.C. 1980. Survival of *Colletotrichum graminicola*: Importance of the spore matrix. Phytopathology 70:255-261.

24. Bergman, B.H.H. 1966. Presence of a substance in the white skin of young tulip bulbs which inhibits growth of *Fusarium oxysporum*. Neth. J. Plant Pathol. 72:222-230.

25. Schonbeck, F., and Schlosser, E. 1976. Preformed substances as potential protectants. In: R. Heitefuss and P.H. Williams (eds.). Physiological Plant Pathology. Springer-Verlag, Berlin. pp. 653-678.

26. Loyd, R.C., and Gray, E. 1979. Amount and distribution of hydrocyanic acid potential during the life cycle of plants of three sorghum cultivars. Agron. J. 62:394-397.

27. Ferreira, A.S., and Warren, H.L. 1982. Resistance of sorghum to *Colletotrichum graminicola*. Plant Dis. 66:773-775.

28. Valiela, I., and Rietsma, C.S. 1984. Nitrogen, phenolic acids, and other feeding cues for salt marsh detritivores. Oecologia 63:350-356.

29. Valiela, I., Koumjian, L., Swain, T., Teal, J.M., and Hobbie, J.E. 1979. Cinnamic acid inhibition of detritus feeding. Nature 280:55-57.

30. Buchsbaum, R., Valiela, I., and Swain, T. 1984. The role of phenolic compounds and other plant constituents in feeding by Canada geese in a coastal marsh. Oecologia 63:343-349.

31. Woodward, R.E., Silver, W.S., and Mansell, R.L. 1974. Herbicide-related changes in phenolic acid content of field-grown hydrilla. Hyac. Contr. J. 12:35-37.

32. Rao, G.G., Rao, K.N., and Rao, G.R. 1980. Biochemical studies of Hydrilla and its leachates. Indian J. Exp. Biol. 18:771-772.

33. Harlan, S.M., Davis, G.T., and Pesacreta, G.J. 1985. Hydrilla in three North Carolina lakes. J. Aquat. Plant Manage. 23:68-71.

34. Steward, K.K., Van, T.K., Carter, V., and Pieterse, A.H. 1984. Hydrilla invades Washington, D.C. and the Potomac. Am. J. Bot. 71:162-163.

35. Yeo, R.R., Falk, R.H., and Thurston, J.R. 1984. The morphology of hydrilla (*Hydrilla verticillata* (L.F.) Royle). J. Aquat. Plant Manage. 22:1-17.

36. Hipskind, J., Wood, K.V., Chand, T., Lembi, C.A., and Nicholson, R.L. 1990. Phenolic compounds in *Hydrilla verticillata.* (in review, Biological Control).

37. Hoagland, D.R., and Arnon, D. 1950. The water-culture method for growing plants without soil. Calif. Agric. Exp. Sta. Circ. 347.

38. Smith, C.S., Chand, T., Harris, R.F., and Andrews, J.H. 1989. Colonization of a submersed aquatic plant, Eurasian water milfoil (Myriophyllum spicatum), by fungi under controlled conditions. Appl. Environ. Microbiol. 55:2326-2332.

39. Kahn, V., Goldschmidt, S., Amir, J., and Granit, R. 1981. Some biochemical properties of soluble and bound potato tuber peroxidase. J. Food Sci. 46:756-764.

40. Nicholson, R.L. and Epstein, L. 1991. Adhesion of fungi to the plant surface. In: G.T. Cole and H.C. Hoch, (eds.), The Fungal Spore and Disease Initiation in Plants and Animals. Plenum, New York. pp. 3-23.

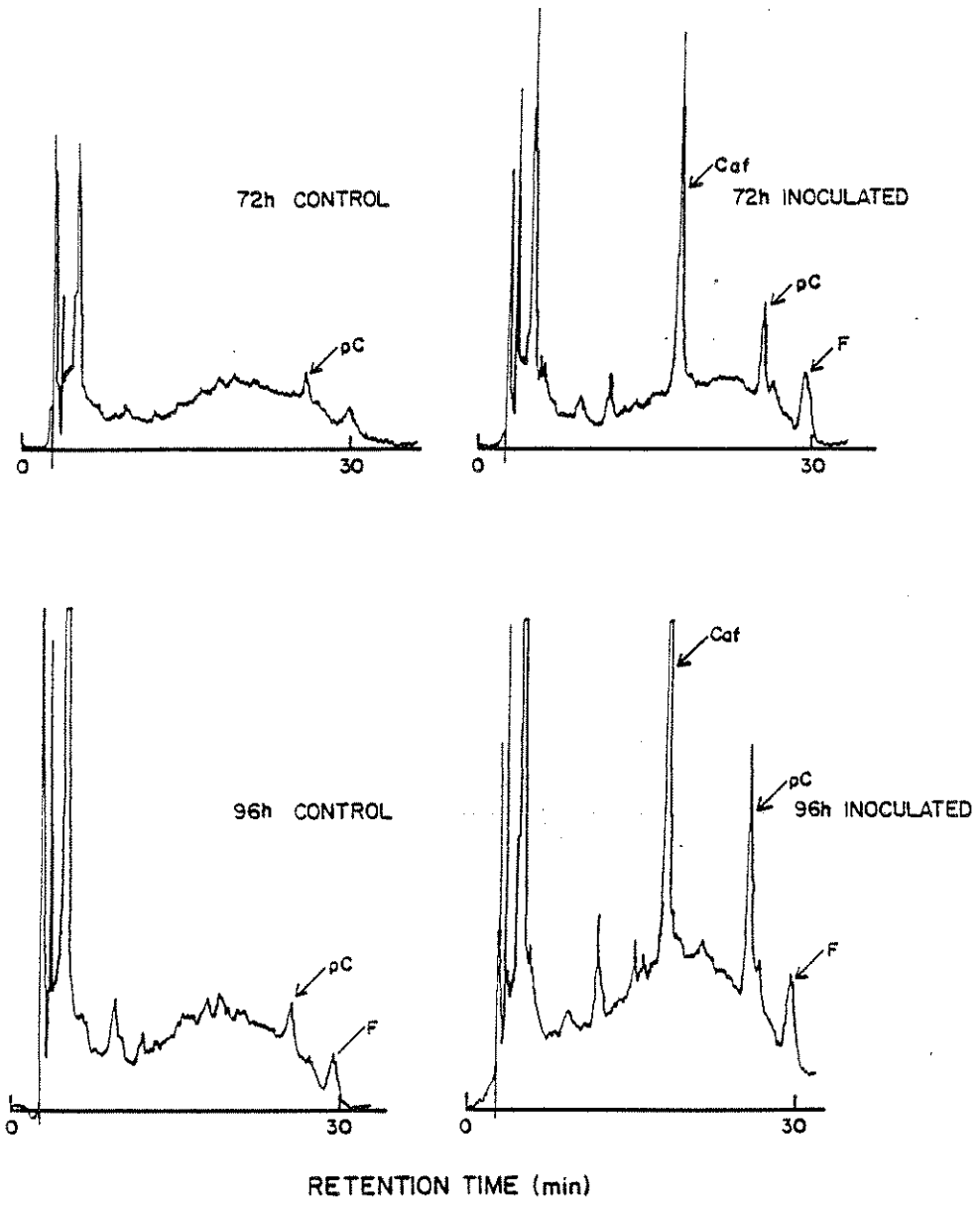

Fig. 1. HPLC profiles of phenolic materials present in a base hydrolyzate of a methanol extract from leaf tissue. A comparison of phenolics is shown for samples taken from control and inoculated tissues at 72 and 96 h after inoculation. Caf = caffeic acid; p-C = p-coumaric acid; F = ferulic acid.

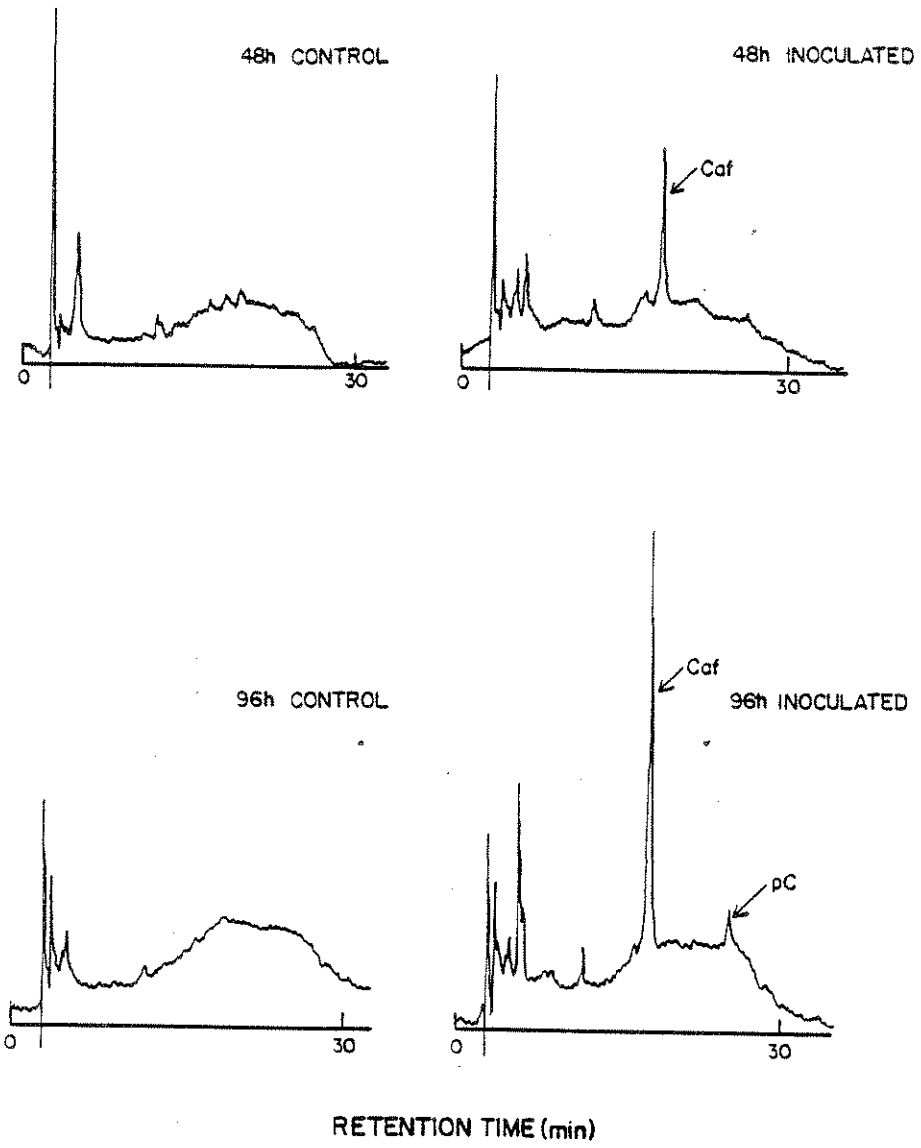

Fig. 2. HPLC profiles of phenolic materials present in a base hydrolyzate of leaf cell wall tissue. A comparison of phenolics is shown for samples taken from control and inoculated tissues at 48 and 96 h after inoculation. Caf = caffeic acid; p-C = p-coumaric acid.

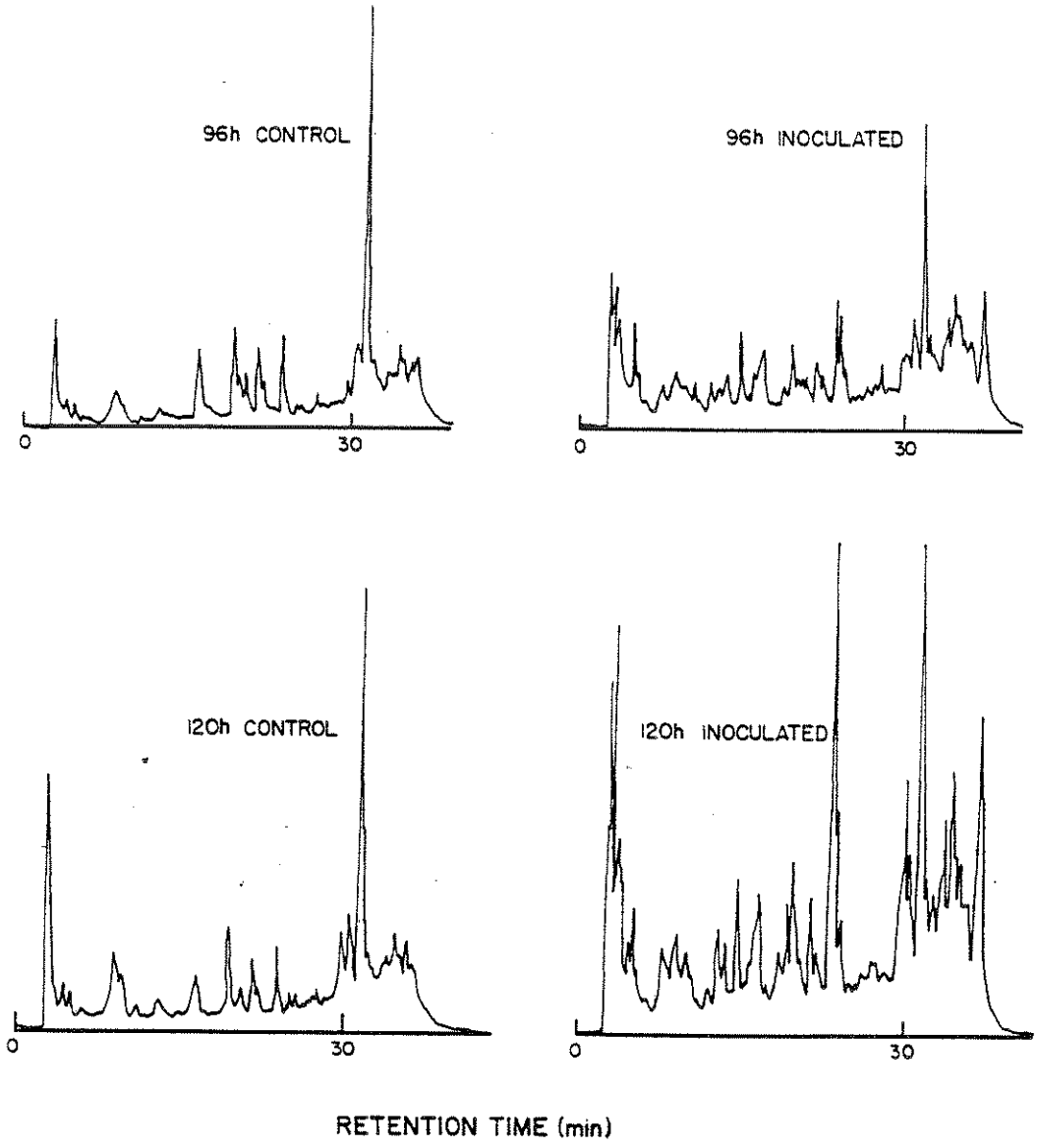

Fig. 3. HPLC profiles of phenolic materials present in a methanol extract from stem internodes of milfoil. A comparison of phenolics is shown for samples taken from control and inoculated tissues at 96 and 120 h after inoculation.

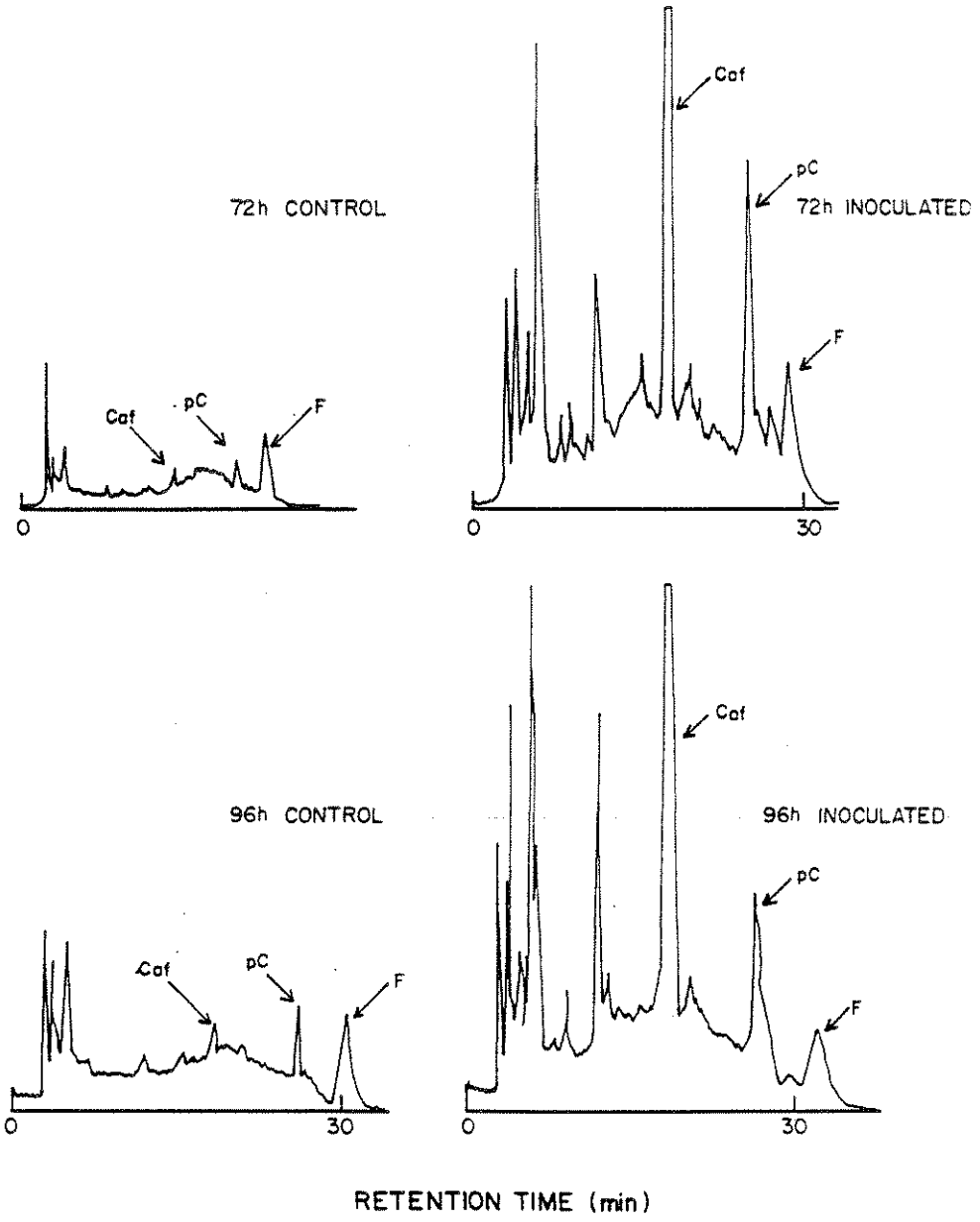

Fig. 4. HPLC profiles of phenolic materials present in a base hydrolyzate of a methanol extract from milfoil stem internodes. A comparison of phenolics is shown for samples taken from control and inoculated tissues at 72 and 96 h after inoculation. Caf = caffeic acid; p-C = p-coumaric acid; F = ferulic acid.

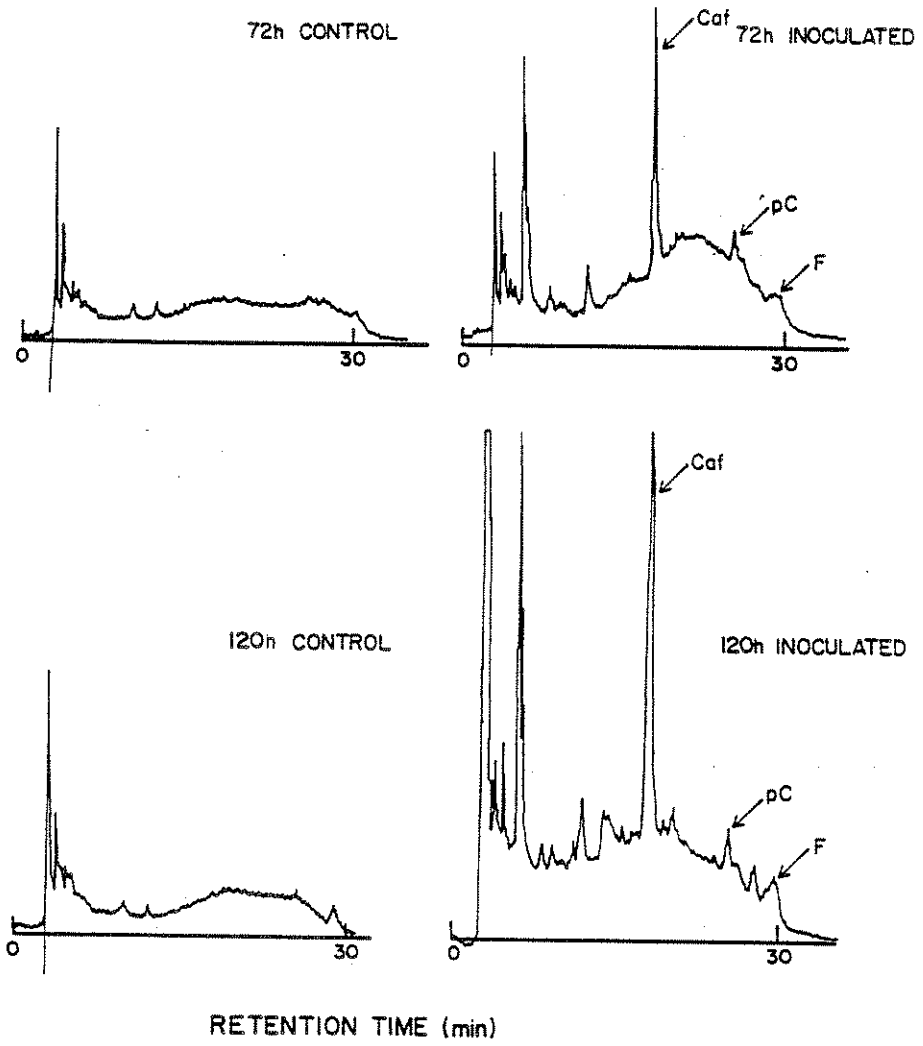

Fig. 5. HPLC profiles of phenolic materials present in a base hydrolyzate of milfoil stem internode cell wall material. A comparison of phenolics is shown for samples taken from control and inoculated tissues at 72 and 120 h after inoculation. Caf = caffeic acid; p-C = p-coumaric acid; F = ferulic acid.

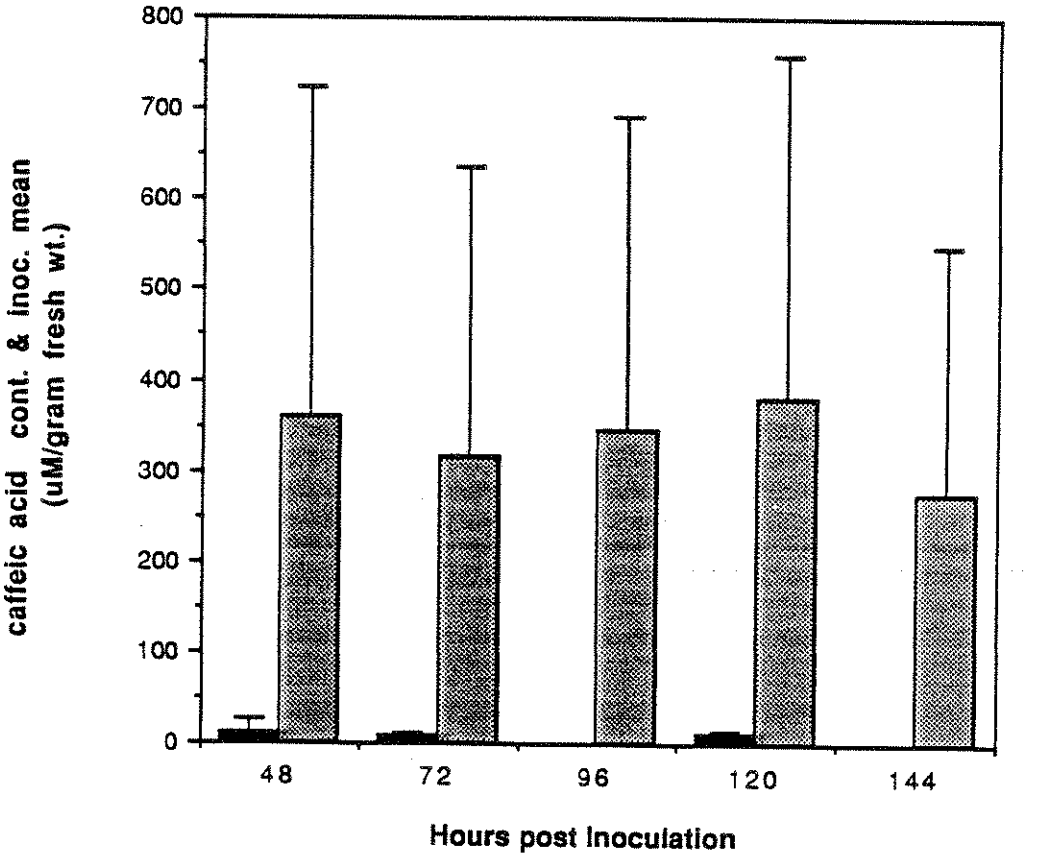

Fig. 6. Time study of the presence of caffeic acid in base hydrolyzed methanol extracts of milfoil internodes inoculated with C. gloeosporioides.

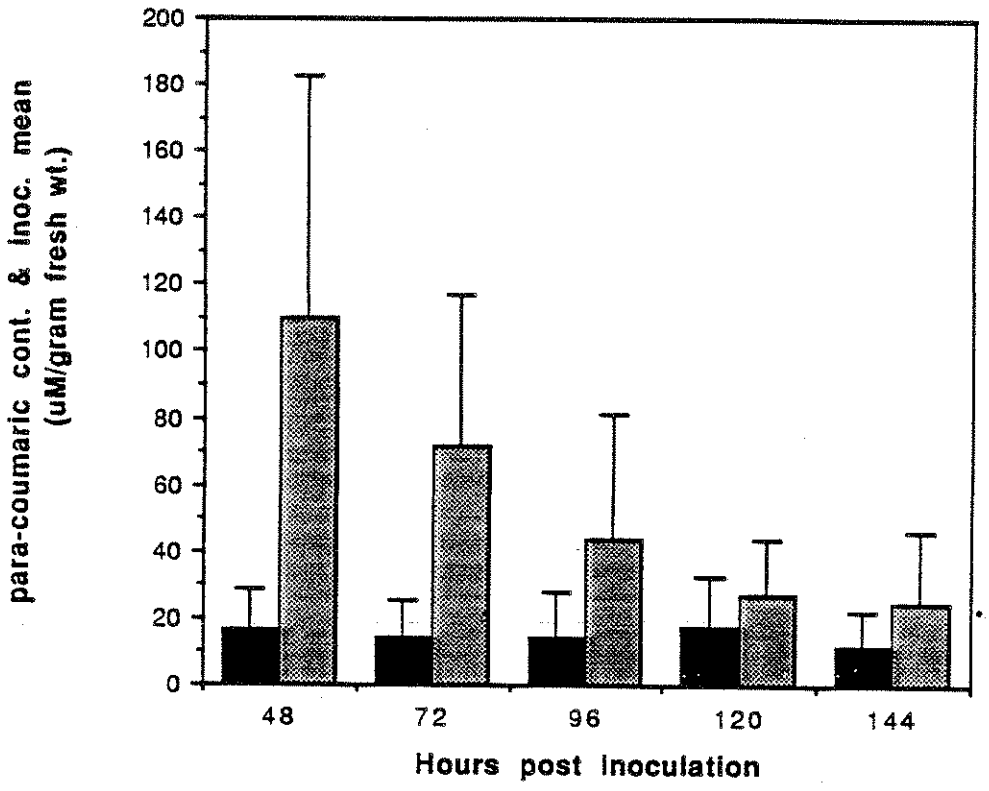

Fig. 7. Time study of the presence of p-coumaric acid in base hydrolyzed methanol extracts of milfoil internodes inoculated with C. gloeosporioides.

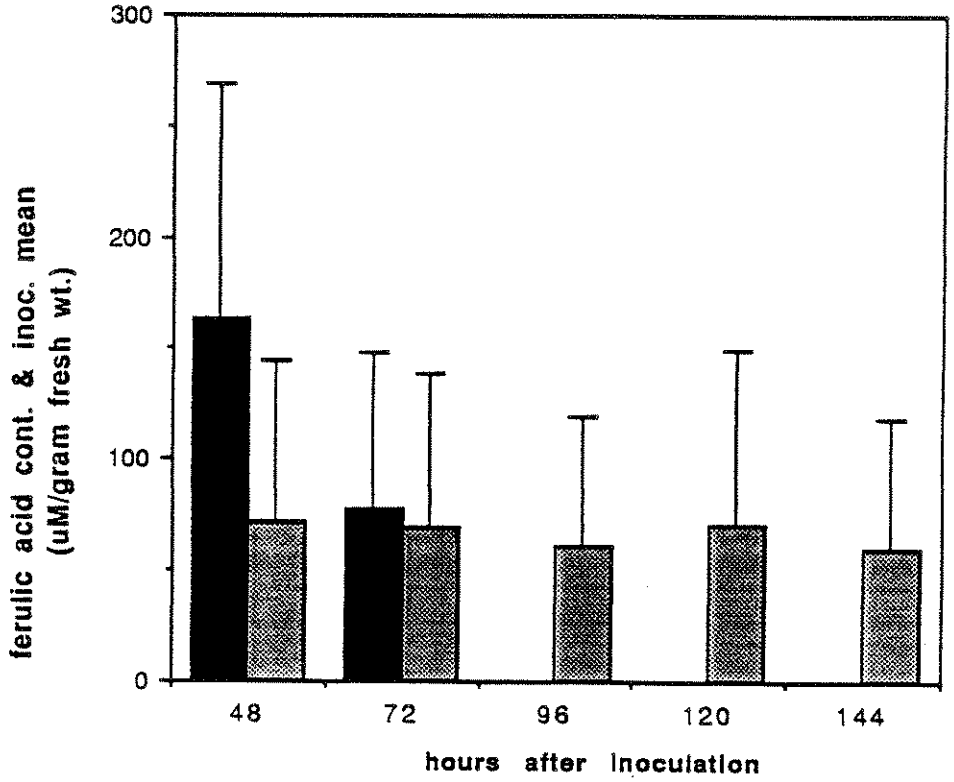

Fig. 8. Time study of the presence of ferulic acid in base hydrolyzed methanol extracts of milfoil internodes inoculated with C. gloeosporioides