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THIOPHENE ACCUMULATION IN HAIRY ROOTS OF *TAGETES PATULA* IN RESPONSE TO FUNGAL ELICITORS.

Usha Mukundan[1] and Martin Hjortso*
Chemical Engineering, Louisiana State University, Baton Rouge, LA 70803

SUMMARY

Hairy roots of *Tagetes patula* were treated with fungal extracts from 3 different fungi. Treated roots were found to accumulate thiophenes at a higher level than untreated control cultures. The kinetics of thiophene formation varied with the fungus from which the elicitor was prepared, elicitor concentration and duration of exposure.

INTRODUCTION

Asteraceae is the largest family of flowering plants, representing 10% of the species of this group. Plants of this family accumulate polyacetylenes and their sulphur derivatives. Thiophenes belong to the later group and accumulate mainly in the roots of some of the plants belonging to this family. Recent findings suggests that they may play a role in protection of the plant (Towers, 1987). This is further suggested by their toxicity at low concentrations to phytopathogenic fungi (DiCosmo et al., 1982; Towers, 1980), insects (Champagne, 1984; Downum et al., 1984) and nematodes (Gommers, 1972). Thus thiophenes may provide an environmentally safe alternative to conventional pesticides.

Hairy roots are obtained from dicotylenodous plant species by infection with the bacterium *Agrobacterium rhizogenes*. Hairy roots are fast growing, genetically stable and produce the secondary metabolites characteristic of normal roots at levels similar to or even exceeding those of the normal root (Kamada et al., 1986). The fact that thiophenes accumulate mainly in the roots in intact plants makes hairy roots particularly well suited as a system for production of these compounds.

Fungal elicitation is a well established tool for increasing the productivity of plant cell cultures (Eilert, 1987) and has also been found to be effective with hairy root cultures (Mukundan and Hjortso, 1990). In

1 On leave from Department of Biological Sciences, R.J. College, Bombay 86, India

this paper we examine the effect of several fungal derived elicitors on thiophene accumulation in hairy roots of *Tagetes patula* (French marigold). The effect of elicitor concentration as well as the exposure time is investigated.

EXPERIMENTAL METHODS

Hairy root cultures were initiated from the clone *Tagetes patula* T5, established by Dr. Hector Flores (Flores et al., 1986). Four 1cm tips were grown in 50ml of hormone free MS medium (Murashige and Skoog, 1962) in 125ml erlenmeyer flasks on a rotary shaker at 150rpm at 25°C under dim light conditions.

Cultures, maintained on potato-dextrose agar, of the fungi *Botrytis cinerea*, *Cercospora lingustr* and *Pythium ultimum* were obtained from the Department of Plant Pathology and Crop Physiology, LSU. *B. cinerea* and *P. ultimum* were transferred to 1 liter of potato-dextrose medium. *C. lingustr* did not grow well in this medium and was transferred to a defined medium containing NH_4NO_3, 2 $g \cdot l^{-1}$; KH_2PO_4, 1 $g \cdot l^{-1}$; $MgSO_4 \cdot 7H_2O$, 0.5$g \cdot l^{-1}$; KCl, 0.5$g \cdot l^{-1}$; $FeSO_4 \cdot 7H_2O$, 0.01$g \cdot l^{-1}$; $ZnSO_4 \cdot 7H_2O$, 0.01 $g \cdot l^{-1}$; $CuSO_4 \cdot 5H_2O$, 0.005$g \cdot l^{-1}$ and 2% sucrose. The cultures were swirled initially to aerate the medium and then incubated in dark at 30°C without shaking for 15 days.

Cell wall filtrates used for elicitation studies were prepared as described previously (Mukundan and Hjortso, 1990). Total carbohydrate in the extract was measured by the dinitrosalicylic acid reagent method (Miller, 1959). Filter sterilized elicitor was added at different concentrations to 20 day old hairy root cultures in 125ml erlenmeyer flasks. Control cultures received sterile, distilled water. Thiophenes were extracted from roots 48 hours after addition of elicitor. In time course experiments, thiophenes were extracted from roots 6, 24, 30, 48 and 72 hours after the addition of a specific amount of elicitor. Thiophenes were assayed by extraction of air dried roots with dichloromethane and quantified by HPLC as described previously (Mukundan and Hjortso, 1990).

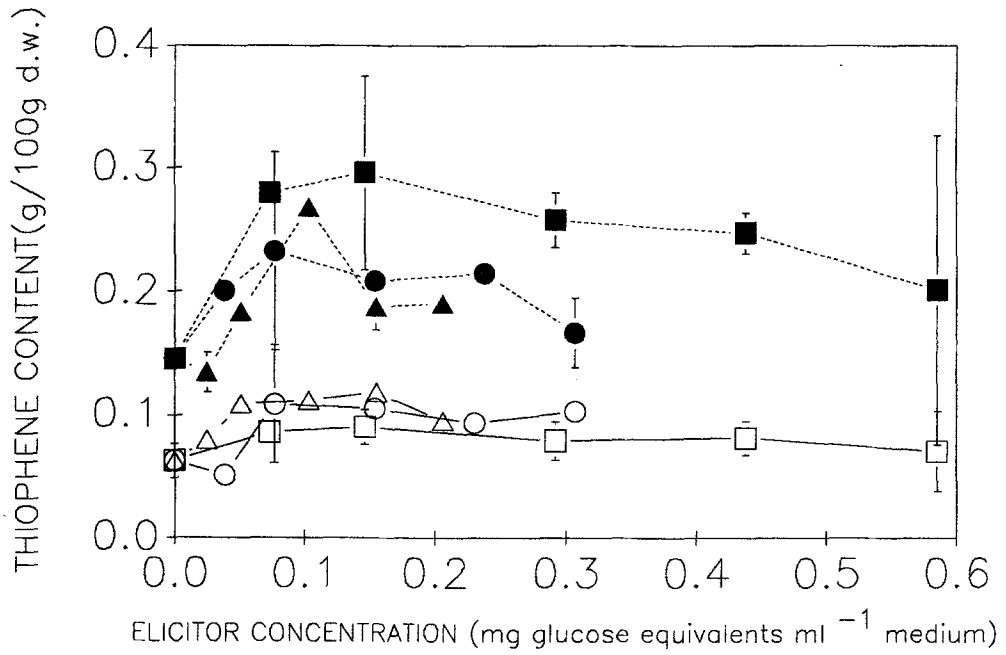

Figure 1. Effect of a 48 hour exposure to different elicitor concentrations on thiophene accumulation in 20 day old *T. patula* hairy root cultures. Filled symbols, dotted line: BBT. Open symbols, solid line: BBTOAc. Circles: *C. lingustr*. Triangles: *P. ultimum*. Squares: *B. cinerea*.

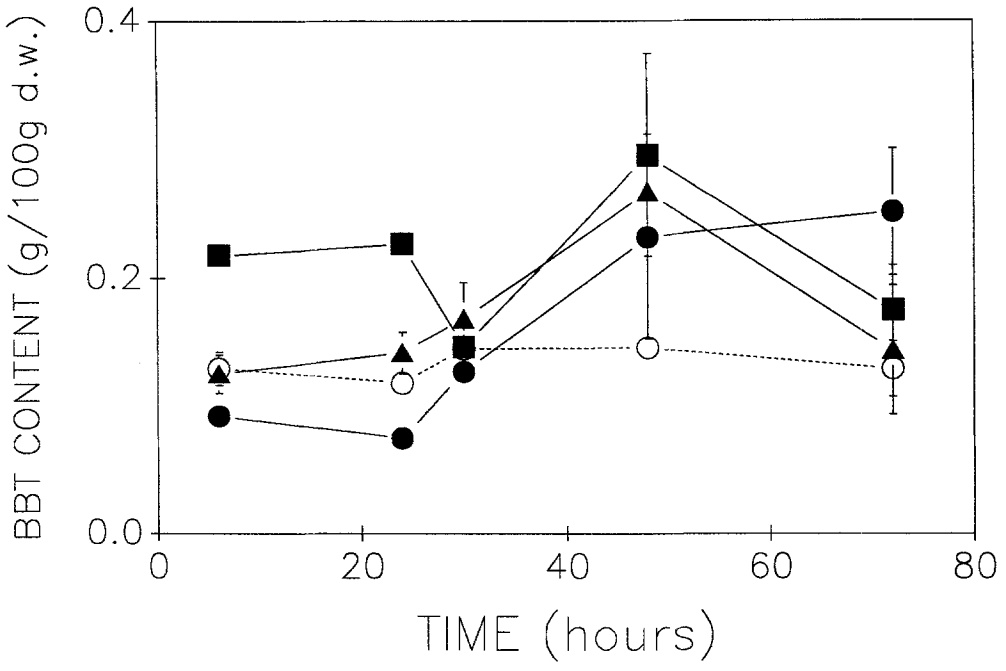

Figure 2. Effect of incubation time on BBT accumulation in *T. patula* hairy roots. Controls are open symbols, dotted line. Treated cultures are filled symbols, solid line. Circles: *C. lingustr*, 0.1032 mg glucose equivalents·ml^{-1}. Triangles: *P. ultimum*, 0.0768 mg glucose equivalents·ml^{-1}. Squares: *B. cinerea*, 0.1462 mg glucose equivalents·ml^{-1}.

RESULTS

Both the medium and the roots were assayed, but thiophenes were only detected in the roots. Two thiophenes were detected, acetoxybutinylbithiophene (BBTOAc) and bithienylbutinen (BBT). All experiments were done in triplicates and bars on figures represent standard deviation.

The amount of these two thiophenes in the roots is shown versus the elicitor concentration in figure 1. Concentration of elicitor is indicated by mg glucose equivalents per ml of medium. This is not done to imply that the active compound(s) in the fungal extracts must be carbohydrates, but is a convenient way of representing the amount of elicitor used. All the curves pass through a maximum at an intermediate value of the elicitor concentration. For a given elicitor, the maximum accumulation of the two thiophenes take place at the same elicitor concentration, or, for *P. ultimum*, at neighboring values of the concentration. The maximum accumulation of BBT is observed when the elicitor is derived from *B. cinerea*, 0.296% of dry weight as compared to 0.145% for the control. An increase of more than 200%. However, this elicitor has the least effect on the BBTOAc content, 0.09% of dry weight at the maximum versus 0.063% for the control. A 143% increase. For the two other elicitors, the effect on BBT and

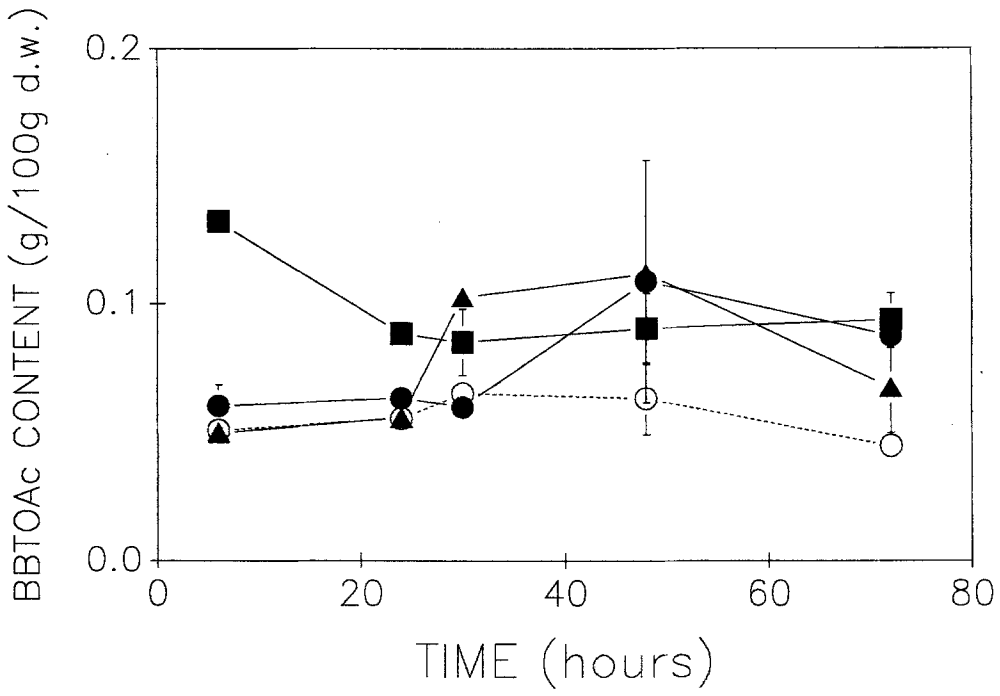

Figure 3. Effect of incubation time on BBTOAc accumulation in *T. patula* hairy roots. Control are open symbols, dotted line. Treated cultures are filled symbols, solid line. Circles: *C. lingustr*, 0.1032 mg glucose equivalents·ml^{-1}. Triangles: *P. ultimum*, 0.0768 mg glucose equivalents·ml^{-1}. Squares: *B. cinerea*, 0.1462 mg glucose equivalents·ml^{-1}.

BBTOAc is very similar. For *P. ultimum* derived elicitor the maximum BBT accumulation is 185% of the control, while the maximum BBTOAc accumulation is 189% of the control. For *C. lingustr* the values are 160% (BBT) and 173% (BBTOAc) respectively.

The results from the time course experiments are shown in figures 2 and 3. All experiments were done in triplicates and bars on figures represent standard deviation. The specific amount of elicitor used was 0.1032, 0.0768 and 0.1462 mg glucose equivalents·ml^{-1} medium derived from *C. lingustr.*, *P. ultimum* and *B. cinerea* respectively.

Elicitors derived from *P. ultimum* and *C. lingustr*, show no significant response until hour 30 with the maximum thiophene accumulation occurring at 48 hours or later. *B. cinerea* elicitation, on the other hand, resulted in a rapid response with an approximate doubling of both thiophenes after 6 hours. The response at later times was erratic with no clear common trend between the two thiophenes.

DISCUSSION

Hairy root cultures of *T. patula* accumulated two naturally occurring thiophenes in response to elicitor treatment. The amount of accumulation varied with elicitor concentration, the fungi from which the elicitor was derived and the period of incubation. The maximum elicitation was obtained using elicitor derived from *B. cinerea*, 204% in BBT and 259% in BBTOAc, while the two other fungi tested gave maximum elicitations of 160-185%. Cultures elicited with *B. cinerea* also exhibited the fastest response to the elicitor. From a technological point of view this is desirable as a fast response will help to minimize the time required in a batch process.

It would be very desirable to know a priori what fungi would be good candidates to test for elicitation. In other words, which fungi would cause the greatest accumulation of metabolites in a given plant tissue culture. There are several theories that attempt to explain ways in which elicitors influence accumulation of metabolites in plants and these could serve as guides for choosing the best fungi. According to one view, fungi contain specific elicitors which causes accumulation of phytoalexins in resistant plants but not in susceptible cultivars (Keen, 1974; Keen and Legrand, 1980). Alternatively it has been suggested that elicitors are not specific, affecting all cultivars and also possibly non-host species (Albersheim, et al., 1981; Albersheim and Valent, 1978). The biological activity of thiophenes and their induction in natural plant pathogen systems has previously been shown (Eilert, 1987; Kourany and Thorarnason, 1988) and they can thus be considered as phytoalexins. However, our results do not corroborate either of the two above mentioned views regarding elicitation. *P. ultimum* is pathogenic causing root rot of *T. patula*, *C. lingustr* is non-pathogenic and *B. cinerea* has a broad host range (USDA, 1960). In spite of these differences, all the elicitors were successful in increasing accumulation of thiophenes in the hairy root cultures. Thus the results indicate, that one should not restrict oneself when screening fungi for elicitation of hairy roots.

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