Davies, J. H.; Davis, R. H.; Kirby, P. *J. Chem. Soc. C* **1968**, 431.
Donninger, C.; Schofield, J. A.; Regan, P. D. British Patent 1 101 785, 1968.
Ellman, G. L.; Courtney, K. D.; Andus, V.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88.
Felton, J. C. *J. Sci. Food Agric. Suppl.* **1968**, 33.
Magee, T. A.; Limpel, L. E. *J. Agric. Food Chem.* **1977**, *25*, 1376.
Metcalf, R. L.; Fukuto, T. R. *J. Agric. Food Chem.* **1967**, *15*, 1023.
Payne, L. K., Jr.; Stansbury, H. H., Jr.; Weiden, M. H. J. *J. Agric. Food Chem.* **1966**, *14*, 356.
Payne, L. K., Jr.; Stollings, H. W., and Strow, C. B., Jr. *J. Agric. Food Chem.* **1967**, *15*, 883.
Pearson, D. E.; Keaton, O. D. *J. Org. Chem.* **1963**, *28*, 1557.
Seebach, D.; Geiss, K. H.; Beck, A. K.; Graf, B.; Daum, H. *Chem. Ber.* **1972**, *105*, 3280.
Speziale, A. J. *J. Am. Chem. Soc.* **1956**, *78*, 2556.
Waile, M. G.; Sim, G. A. *J. Chem. Soc. B* **1971**, 752.

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# Insecticide Inhibition of Growth and Patulin Production in *Penicillium expansum*, *Penicillium urticae*, *Aspergillus clavatus*, *Aspergillus terreus*, and *Byssochlamys nivea*

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Since patulin is produced by a wide variety of fungi under natural conditions, experiments were undertaken to determine (1) if the inhibition of naled, Sevin, pyrethrum, and methoxychlor was species dependent and (2) if the breakdown products of naled were active inhibitors of patulin production. At 100 ppm, naled completely inhibited patulin production and growth by the fungi *Penicillium expansum*, *Penicillium urticae* NRRL 1952, *Penicillium urticae* NRRL 994, *Byssochlamys nivea*, *Aspergillus clavatus*, and *Aspergillus terreus*. Sevin (100 ppm) inhibited patulin production by 16.6, 80.3, 100, 81.7, 31.5, and 89.5%, respectively, by these fungi. Pyrethrum (100 ppm) inhibited production of patulin in these fungi by 33.3, 1.2, 59.4, 34.9, 59.2, and 90.9%, respectively. Methoxychlor did not significantly inhibit patulin production. Six breakdown products of naled were important contributors to the effectiveness of naled as an inhibitor of patulin production.

The mycotoxin patulin [4-hydroxy-4*H*-furo[3,2-*c*]-pyran-2(6*H*)-one] is a carcinogenic heterocyclic lactone produced by species of *Aspergillus*, *Penicillium*, and *Byssochlamys*. Originally isolated as an antibiotic (Karow and Foster, 1944), patulin is toxic to experimental animals and carcinogenic to rats (Dickens and Jones, 1961). Patulin is stable during processing in products such as cooked cornmeal mush (Lieu and Bullerman, 1977) and grape and apple juices (Scott and Somers, 1968). Patulin is not stable in high protein products (Ciegler et al., 1972) although Ciegler et al. (1976) reported that teratogenicity of patulin adducts may be retained. Patulin represents a potential health hazard to humans because of its widespread occurrence as a contaminant of agricultural products, especially apples and apple products (Stoloff, 1975).

The presence of patulin and other toxic and carcinogenic mycotoxins in foods and feedstuffs has led to extensive research concerning chemicals which inhibit fungal growth and/or mycotoxin production. Draughon and Ayres (1978) reported that several commonly used insecticides inhibit production of the mycotoxin citrinin. The insecticide dichlorvos completely inhibits aflatoxin production at a concentration of 10 ppm (Rao and Harein, 1973; Hsieh, 1973), and zearalenone production and patulin production are completely inhibited by 100 ppm of the insecticide naled (Berisford and Ayres, 1976; Draughon and Ayres, 1979). In addition, the effectiveness of various acids and salts such as the benzoates, malonates, propionates, and acetates as antifungal agents is well documented (Uriah and Chipley, 1976; Stewart et al., 1977).

Table I. Fungal Strains Tested for Inhibition of Patulin Production

| organism | strain | media[b] | source |
|---|---|---|---|
| *P. (urticae)*[a] *patulum* | NRRL 994 | PDB | University of Georgia, Athens, GA |
| *P. (urticae) patulum* | NRRL 1952 | PDB | A. Ciegler, NRRL, Peoria, IL |
| *P. expansum* | NRRL 2304 | PDB | A. Ciegler |
| *A. clavatus* | NRRL 1980 | YES | A. Ciegler |
| *A. terreus* | NRRL 255 | YES | A. Ciegler |
| *B. nivea* | NRRL 2615 | Czapek-Dox | University of Georgia, Experiment, GA |

[a] The culture *P. patulum* will be referred to as *P. urticae* since the designation *P. patulum* is no longer considered a valid species by taxonomists. [b] See the text for formula modifications.

The organophosphate family of insecticides has been studied as potential inhibitors of mycotoxin production due to the effectiveness of certain of its members and because they have a very short half-life in the environment. Subsequently, they do not accumulate or persist in foodstuffs for any lengthy period of time and the problems associated with toxicity are avoided. However, the organophosphate insecticide naled demonstrated complete inhibition of patulin production in cultures treated for 30 days at an initial concentration of 100 ppm in stationary culture media (Draughon et al., 1980). After 7 days, less than 1 ppm of naled was present in the culture media. Since the prolonged inhibition of patulin production following the breakdown of naled in the culture was unexplained, studies were undertaken to determine if the breakdown products of naled are the active inhibitors of

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Table II. Effect of the Insecticides Sevin, Naled, and Pyrethrum at a Concentration of 100 mg/L on Patulin Production by Selected Fungal Species

| | | sevin | | naled | | pyrethrum | |
|---|---|---|---|---|---|---|---|
| fungal strain | control patulin, mg | patulin, mg | inhibn, % | patulin, mg | inhibn, % | patulin, mg | inhibn, % |
| *A. clavatus* | 135.44 a[a] | 92.74 b | 31.5 | 0 c | 100 | 55.23 d | 59.2 |
| *A. terreus* | 46.48 a | 4.86 b | 89.5 | 0 c | 100 | 4.23 b | 90.9 |
| *B. nivea* | 16.21 a | 2.96 b | 81.7 | 0 c | 100 | 10.54 c | 34.9 |
| *P. expansum* | 0.06 a | 0.05 b | 16.6 | 0 c | 100 | 0.04 b | 33.3 |
| *P. urticae* 1952 | 146.92 a | 28.90 b | 80.3 | 0 c | 100 | 145.11 a | 1.2 |
| *P. urticae* 994 | 2.39 a | 0.00 b | 100.0 | 0 c | 100 | 1.42 d | 59.4 |

[a] Means followed by the same letter (reading across) are not significantly different at the 0.01 level of significance by using Duncan's Multiple Range Test.

patulin production after naled is decomposed in the culture medium. In addition, since patulin is produced by a wide variety of fungi under natural conditions, experiments were performed to determine if the inhibition caused by naled was species dependent. Representative insecticides from the carbamate (Sevin), chlorinated hydrocarbon (methoxychlor), and plant product (pyrethrum) groups were also tested for species-dependent inhibition.

## MATERIALS AND METHODS

The six patulin-producing fungi used throughout this study are listed in Table I together with strain numbers, incubation media, and sources. The microbiological test media consisted of either potato-dextrose broth (Difco Laboratories, Detroit, MI), Czapek-Dox broth (Difco), or YES (2% yeast extract and 20% glucose). Czapek-Dox broth was supplemented with a 0.2% yeast extract and 0.8% glucose. Fifty milliliters of the test medium was dispensed into 250-mL Erlenmeyer flasks and autoclaved at 121 °C for 15 min. Spore suspensions of *Byssochlamys nivea* were prepared for use as an inoculum according to a procedure by Rice et al. (1977). Spore suspensions for species of *Aspergillus* and *Penicillium* were prepared by flooding a 14-day-old slant culture with 5 mL of sterile 0.005% Triton X-100 (Sigma Chemical Co., St. Louis, MO). All test media were inoculated with 1 mL of a spore suspension containing $10^7$ spores/mL to measure growth and toxin production.

Insecticides were dissolved in 95% ethanol or in dimethyl sulfoxide ($Me_2SO$) and added to make a final insecticide concentration of 100 mg/L when added to the broth culture medium immediately after inoculation with the given fungus. Less than 1 μg/mL carrier was added. All insecticides and chemicals tested were of technical grade and were supplied by the manufacturer. The following insecticides were used: naled (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate; Chevron Chemical Co., Richmond, CA), pyrethrum (from *Chrysanthemum cinariaefolium*; FMC, Middleport, NY), methoxychlor [1,1,1,-trichloro-2,2-*bis*(*p*-methoxyphenyl)ethane; E. I. du Pont de Nemours and Co., Wilmington, DE], and Sevin (1-naphthalenol methylcarbamate; Shell Development Co., Modesto, CA). Controls consisted of cultures to which only ethanol or $Me_2SO$ carrier was added at a concentration of 1 μg/mL. Breakdown products of naled were supplied by Chevron Chemical Co.: dimethyl phosphate, mercaptoacetic acid, *S*-methyl-L-cysteine, urea, hippuric acid, and dichlorovinyl methyl phosphate. The breakdown products of naled were each tested at a concentration of 100 mg/L with the test organism *Penicillium urticae* NRRL 994.

Each of the insecticides shown above was tested at a concentration of 100 mg/L for its capacity to inhibit growth and patulin production by *Penicillium urticae* NRRL 1952, *P. urticae* NRRL 994, *Aspergillus clavatus*, *Aspergillus terreus*, *Penicillium expansum*, and *B. nivea*. All data for each treatment are the mean of 10 replicates with 5 observations per replicate (50 high-pressure liquid chromatography samples total per treatment). An analysis of variance was performed and significant differences between means were determined by using Duncan's Multiple Range Test.

After stationary incubation for 14 days at 25 °C, cultures were gravity filtered by using weighed Whatman No. 1 filter paper. Mycelial mats were collected and dried at 100 °C for 24 h to measure growth (biomass) as dried mycelial weight. The extent of inhibition of biomass production in treated samples was calculated as the percentage of control. The broth was placed in a 500-mL separatory funnel and shaken for 1.5 min with an equal volume of ethyl acetate. The ethyl acetate (top) layer was collected and the extraction was repeated twice with equal volumes of ethyl acetate. The combined extracts were dried by stirring for 30 min with 25 g of anhydrous sodium sulfate. The extract was filtered through Whatman No. 1 filter paper and evaporated to $^1/_{10}$ its original volume by flast evaporation at 45 °C.

Patulin was quantitated by the use of a high-pressure liquid chromatograph (LC) (Waters Associates, Inc., Milford, MA) equipped with a Model 440 UV detector (278-nm filter), M6000 pump, U6K septumless injector, and a reversed-phase μBondapak $C_{18}$ column. The patulin was eluted from the column by using an acetonitrile–water–glacial acetic acid (55:34:2) solvent system (pH 3.85) developed by Engstrom et al. (1977) at a flow rate of 1.0 mL/min. By use of this system, the retention time for patulin was 3.6–3.7 min. The amount of patulin in each sample was calculated by using the formula

$$\text{mg of patulin/50 mL of culture filtrate} = C_{std}H_{sam}A_{sam}V_{std}V_{sam}/(H_{std}A_{std}V_i)$$

where $C_{std}$ = concentration of standard (mg/mL), $H_{sam}$ = peak height of sample (mm), $A_{sam}$ = attenuation of sample reading (from high-pressure LC), $V_{std}$ = volume of standard injected (μL), $V_{sam}$ = total volume of sample (mL), $H_{std}$ = peak height of standard (mm), $A_{std}$ = attenuation of standard reading (from high-pressure LC), and $V_i$ = volume of sample injected (μL).

## RESULTS AND DISCUSSION

Since patulin is produced by a wide variety of fungi in foods and feedstuffs, the effect of naled on one particular species of fungi under natural conditions was of limited value. When patulin-producing fungi of three different genera (five different species) were subjected to treatment with a concentration of 100 mg/L naled, growth and patulin production was completely inhibited in all species tested (Table II). Sevin was highly inhibitory to patulin production by *P. urticae* NRRL 994 and NRRL 1952, *B. nivea*, and *A. terreus* but demonstrated only slight inhibition in patulin production by *P. expansum* and *A. clavatus*. The insecticide methoxychlor caused less than 10% inhibition in patulin production in all species tested.

Table III. Effect of the Breakdown Products of Naled on Growth and Patulin Production by *P. urticae* NRRL 994 at a Concentration of 100 mg/L

| treatment,[a] 100 mg/L | patulin production, mg/50 mL | % inhibn of patulin | mycelial growth, mg/50 mL | % inhibn of growth |
|---|---|---|---|---|
| dichlorovinyl phosphate | 0.811 ± 0.105 a[b] | 59.2 | 2576 ± 120 a | 39.0 |
| DCVP-control | 1.987 ± 0.231 b | 0 | 4223 ± 248 b | |
| dimethyl phosphate | 0.531 ± 0.083 a | 0 | 2773 ± 263 a | 13.1 |
| DMP-control | 0.532 ± 0.120 a | 0 | 3192 ± 282 b | |
| mercaptoacetic acid | 1.507 ± 0.49 a | 22.3 | 2669 ± 95 a | 3.8 |
| MA-control | 1.939 ± 0.292 b | 0 | 2776 ± 88 a | |
| *S*-methyl-L-cysteine | 2.349 ± 0.685 a | 16.3 | 4010 ± 388 a | 30.8 |
| SMLC-control | 2.807 ± 0.399 a | 0 | 4589 ± 521 b | |
| urea | 1.146 ± 0.106 a | 16.8 | 3229 ± 114 a | 28.4 |
| U-control | 1.378 ± 0.123 b | 0 | 4509 ± 198 b | |
| hippuric acid | 1.488 ± 0.354 a | 31.6 | 4300 ± 612 a | 0 |
| HA-control | 2.175 ± 0.208 b | 0 | 3751 ± 582 b | |

[a] All data for each treatment are the mean of 10 replicates with 5 observations per replicate (50 high-pressure LC samples total per treatment). [b] Means followed by the same letter (reading down for each treatment) are not significant at the $P < F = 0.01$ level of significance by using Duncan's Multiple Range Test.

Since it was not a good inhibitor of patulin production, studies with methoxychlor were not continued.

The insecticide pyrethrum did not inhibit patulin production by *P. urticae* NRRL 1952; however, it did inhibit patulin production by the other fungi by 33.3–90.9%. The effect of the insecticides Sevin (chemically a carbamate) and pyrethrum (from the plant *C. cinariaefolium*) was species dependent. Pyrethrum was less effective than Sevin which, in turn, was much less effective an inhibitor of patulin production than naled.

The effect of the breakdown products of the insecticide naled on growth and patulin production by *P. urticae* NRRL 994 is shown in Table III. Dimethyl phosphate did not inhibit patulin production, although it did inhibit growth by 13.1%. The two chemicals inhibiting patulin production the greatest were hippuric acid (31.6%) and dichlorovinyl phosphate (59.2%). These two compounds are interesting since dichlorovinyl phosphate significantly inhibited growth as well as patulin production whereas hippuric acid did not inhibit growth and actually stimulated growth significantly. Hippuric acid may have inhibited patulin production by interfering with secondary metabolism. The fungus could then enhance its survival by conserving the energy customarily extended during secondary metabolism. This energy would then be available for reproduction and growth which would account for the increased mycelial weight. Urea and *S*-methyl-L-cysteine significantly inhibited growth of *P. urticae* NRRL 994. Inhibition in patulin production was approximately half that of inhibition in growth and probably resulted as a direct effect of the inhibition in growth. Like hippuric acid, mercaptoacetic acid inhibited patulin production by 22.3% but caused little or no inhibition in growth. These breakdown products of naled may be responsible for the long-term inhibition in patulin production while permitting growth of the fungus. Earlier studies have shown that patulin production was inhibited twice as long as growth (Draughon et al., 1980).

Naled not only inhibits growth and patulin production of all species and strains examined in this study but also has been shown to strongly inhibit production of the mycotoxins zearalenone, aflatoxin, and citrinin. Evidently, some characteristics of naled makes it highly toxic to a diverse group of fungi. Organophosphates exhibit toxicity to man and insects by phosphorylating an important neurotransmitter enzyme acetycholinesterase. Various workers have reported that the organophosphate groups of insecticides vary in their ability to inhibit production of aflatoxin, citrinin, and zearalenone. Additional study is needed since this type of chemical would be extremely useful in preventing mold growth and mycotoxin production when wet weather conditions exist during harvest or improper and inadequate storage facilities must be utilized.

## LITERATURE CITED

Berisford, Y. C.; Ayres, J. C. *J. Agric. Food Chem.* **1976**, *24*, 973.
Ciegler, A.; Beckwith, A. C.; Jackson, L. K. *Appl. Environ. Microbiol.* **1976**, *31*, 644.
Ciegler, A.; Mintzlaff, H. J.; Weisleder, D.; Leistner, L. *Appl. Microbiol.* **1972**, *24*, 114.
Dickens, F.; Jones, H. E. H. *Br. J. Cancer* **1961**, *15*, 85.
Draughon, F. A.; Ayres, J. C. *J. Food Sci.* **1978**, *43*, 576.
Draughon, F. A.; Ayres, J. C. *J. Food Sci.* **1979**, *44*, 1232.
Draughon, F. A.; Link, M. A.; Ayres, J. C. *J. Food Prot.* **1980**, *43*, 575.
Engstrom, G. W.; Richard, J. L.; Cysewski, S. J. *J. Agric. Food Chem.* **1977**, *25*, 833.
Hsieh, D. P. H. *J. Agric. Food Chem.* **1973**, *12*, 468.
Karow, E. O.; Foster, J. W. *Science* **1944**, *99*, 265.
Lieu, F. Y.; Bullerman, L. B. *J. Food Sci.* **1977**, *42*, 1222.
Rao, H. R. G.; Harein, P. K. *Bull. Environ. Contam. Toxicol.* **1973**, *10*, 112.
Rice, S. L.; Beuchat, L. R.; Worthington, R. E. *Appl. Environ. Microbiol.* **1977**, *34*, 791.
Scott, P. M.; Somers, E. *J. Agric. Food Chem.* **1968**, *16*, 483.
Stewart, R. G.; Wyatt, R. D.; Kiker, J. *Poult. Sci.* **1977**, *56*, 1664.
Stoloff, L. S. *Spec. Rep.—N.Y. State Agric. Exp. Stn., Geneva* **1975**, *No. 19*, 51.
Uriah, N.; Chipley, J. R. *Microbios* **1976**, *17*, 51.

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