# Use of the Insecticide Naled to Control Zearalenone Production

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Naled, applied as a fumigant or in a liquid preparation at levels of 30 and 100 μl/l., completely inhibited zearalenone (F-2) production by *Fusarium graminearum* in liquid media and on corn. When applied prior to inoculation, naled at 100 μl/l. effectively inhibited F-2 production in liquid media for 15 and for 6 and 3 days at concentrations of 30 and 10 μl/l., respectively. Although naled did not significantly reduce F-2 production when applied to 12-day or older cultures, it did inhibit F-2 production by 45–92% when applied at concentrations of 10–100 μl/l. to 3–9-day old cultures. F-2 production was completely inhibited only when naled was applied prior to inoculation of the culture media. Although naled does partially inhibit additional fungal growth and F-2 production by actively growing cultures, it is a potential protectant only when applied prior to initial growth of the fungus.

Zearalenone, a compound with estrogenic activity in swine which ingest moldy grain, is produced by the fungus *Fusarium graminearum* Schwabe and several other members of the *Gibberella zea* complex (Caldwell and Tuite, 1968; Eugenio et al., 1970). The chemical and biological activities of zearalenone have been reviewed by Mirocha et al. (1971) and so will not be discussed here. Inhibition of zearalenone (hereafter referred to as F-2) biosynthesis by the insecticide dichlorvos (*O,O*-dimethyl 2,2-dichlorovinyl phosphate) was first reported by Wolf et al. (1972) and Wolf and Mirocha (1973). Using a different strain of *F. graminearum*, we found that although dichlorvos did strongly inhibit F-2 production by 85%, it did not completely prevent its biosynthesis, even when used at a concentration of 100 μl/l.

Dichlorvos is one of the metabolic breakdown products of another widely used insecticide, naled (1,2-dibromo-2,2-dichloroethyl dimethyl phosphate). Naled is used against a wide variety of insects on vegetable crops, citrus, cotton, grapes, hops, ornamentals, peaches, walnuts, and for adult fly and mosquito control. In these uses, naled is applied in concentrations of 20, 6600, 30 000, and 40 000 ppm (w/w) (Ortho Division, Chevron Chemical Co., personal communication). Strong and Sbur (1961) reported 100% mortality of 3 species of common stored product insect pests when naled at levels of 5 and 10 μg/g was applied to infested grain. Although naled is not registered for use on grain, its potential use as a fungicide or antimycotoxigenic agent warrants further research.

The following investigation was conducted to determine (1) the effective life of naled as an inhibitor of F-2 production, (2) the effect of naled on F-2 production in media already visually contaminated with *F. graminearum*, (3) the critical time during the growth of the fungus when naled is most effective, and (4) the effectiveness of naled used as a fumigant or in a liquid preparation, when applied to a natural (corn) substrate.

## MATERIALS AND METHODS

*Fusarium graminearum* NRRL 3376, a known producer of zearalenone, was the organism used in this study. The fungus was maintained on potato dextrose (PD) agar slants at 4 °C and transfers were made every 5 weeks. Since the fungus did not sporulate satisfactorily in our laboratory, an 8-mm (diameter) PD agar (Stevens, 1974) mycelial core was added to each culture flask containing liquid media and six 3-mm (diameter) mycelial cores were added to flasks containing corn. For use with corn, the flasks were rotated so as to better distribute the cores throughout the depth of the corn. By adding 6 small cores to the corn, the total mycelial basal surface areas of inoculum for corn and PD broth cultures were approximated. Cores were cut from 6-day old cultures grown at 27 °C in 100 × 15 mm petri plates containing 20 ml of PD agar.

The strain of *F. graminearum* used throughout this study did not require cold treatment for toxin production at levels of at least 100 ppm (weight of toxin/weight of substrate). In a preliminary study, we found that PD broth cultures incubated for 2, 4, 6, 8, and 10 weeks at 27 °C produced ca. 5, 7, 11, 18, and 22 × $10^3$ μg of F-2, respectively, while cultures which were incubated at 27 °C for 2 weeks, followed by additional incubation at 12 °C for 2, 4, 6, and 8 weeks, produced only ca. 1.7, 1.9, 2.8, and 2.8 × $10^3$ μg of F-2, respectively. In cultures incubated at constant 12 °C for 2, 4, 6, 8, and 10 weeks, only 0, 0.2, 1, 1.6, and 1 × $10^3$ μg of F-2 were produced, respectively. From these experiments, we concluded that neither cold treatment nor a solid substrate was necessary for the fungus to produce sufficient F-2 (100 ppm) to provide a significant test condition for the insecticide treatments. Previously, Mirocha et al. (1971), using different strains of *F. graminearum*, reported that solid substrate and cold treatment were necessary for significant F-2 production. In preliminary testing, we found that our strain of *F. graminearum* produced 2–3 times more F-2 on corn than in liquid media, but the level of F-2 production in liquid media was still sufficiently high to use PD broth cultures for routine screening of pesticides. Pesticides which proved effective in liquid media were further tested on fungus cultures grown on corn.

For toxin production, *F. graminearum* was grown at 27 °C for 2 weeks in 250-ml cotton-stoppered Erlenmeyer flasks containing 1 inoculum core and 50 ml of autoclaved PD broth per flask. Appropriate amounts of naled (technical grade, Chevron Chemical Co.) measured to make final concentrations of 1, 10, 30, or 100 μl/l. pesticide–medium were dissolved in 0.5 ml of 95% ethanol and added to the cultures (1) at the time of inoculation, (2) 3, 6, 9, 12, 15, 18, 21, 25, 28, and 30 days prior to inoculation (to determine the effective life of naled as an inhibitor of F-2 production), or (3) at 3-day intervals for 2 weeks following inoculation (to determine if naled can inhibit F-2 production on material already visually contaminated). For culturing on corn, six 3-mm (diameter) mycelial cores were added to 50 g of autoclaved corn (white hybrid corn having a moisture content of 8–10% and grown without the aid of pesticides at the University of Georgia Horticulture Farm) to which 20 ml of sterile distilled water and the appropriate amount of naled had been added. To test

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the fumigant activity of naled, filter papers containing appropriate amounts of the pesticide (v/v jar) were suspended 4 cm above the media in tightly sealed pint Mason jars. All corn cultures were grown at 27 °C for 3 weeks. Eight replications of each treatment (in PD broth and on corn) were set up simultaneously and the test procedure was repeated, thereby making 16 replications.

After the incubation period, each culture was transferred into a 1-pt Mason jar, homogenized with 100 ml of chloroform, and transferred into a 250-ml separatory funnel and the lower chloroform layer drawn off through anhydrous sodium sulfate into a 500-ml round-bottomed boiling flask. This procedure was repeated with another 100 ml of chloroform. The extracts were concentrated in a rotary flash evaporator and diluted to 5 ml with chloroform. Extracts and standards were spotted on standard thin-layer (250 μm) chromatography plates coated with silica gel GHR and separated with benzene–acetic acid (90:10, v/v) as the developing solvent. A Photovolt fluorodensitometer (Photovolt Corp., New York, N.Y.) was used to compare the intensity of fluorescence of the sample spots with those of the standard. For dry weight determination, the mycelium from each culture flask was rinsed twice with distilled water, drained overnight on preweighed Whatman No. 41 filter paper, oven dried at 90 °C for 24 h, and weighed. Trace (<100 μg) amounts or no F-2 were present in the washed mycelia.

## RESULTS

Except in 6-day old cultures, naled's effectiveness as an inhibitor of growth and F-2 production decreased with increasing culture age and complete inhibition occurred only when the insecticide was added to media just prior to inoculation (Figure 1a,b). In 3- and 6-day old cultures, F-2 production was limited to 9% when treated with 100 μl/l., to 36% at 30 μl/l., and to 38% at 10 μl/l. (Figure 1a). There was no significant change in toxin production in either control or treated cultures from day 3 to day 6 (Figure 1a). Only trace amounts of toxin are formed during this time. Under our culturing conditions, *F. graminearum* exhibits a logarithmic growth pattern between day $3^+$ and 9 (Figure 1b). During this time, cells are rapidly dividing and are very sensitive to changes in the external environment. The addition of naled to 3-day old cultures had little effect on growth, only partially inhibiting it by 8–15% (10–100 μl/l.). The addition of naled to actively growing 6-day old cultures, however, inhibited growth by 25–40% and F-2 production by 62–91% (Figure 1a,b). Although naled does strongly inhibit F-2 production in $3^+$–9-day old cultures, it has no value as a detoxicant. Naled does not destroy F-2 but does strongly inhibit additional production of F-2 in cultures that are ≤9 days old. Hsieh (1973), in examining the mechanism of dichlorvos induced inhibition of aflatoxin production, similarly found that the insecticide effectively stopped toxin synthesis only when added to media prior to the initiation of toxin production.

Added prior to inoculation, naled at 100 μl/l. was effective as a complete inhibitor of mycelial growth for 15 days and only trace amounts of F-2 were produced after 25 days (Figure 2). After 30 days, mycelial growth was restricted by only 5% and F-2 production was decreased by 51%. At 30 μl/l., naled completely inhibited F-2 production for 5 days and at 21 days, limited F-2 production to 50% while decreasing mycelial growth by only 10%. Naled at 10 μl/l. was effective for 3 days and limited toxin production to 48% at 17 days.

Naled has a half-life of 1.4–4 h in nonsterile soil (Chevron Chemical Co., private communication). At 21 °C in aqueous buffered solutions, its half-life is ca. 25 h

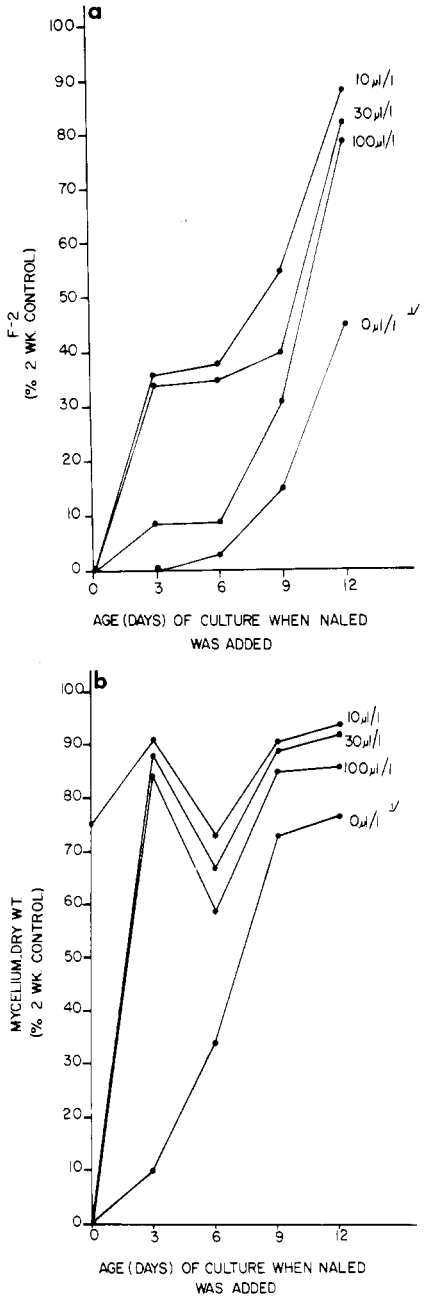


**Figure 1.** Inhibition of (a) F-2 production and (b) mycelial growth by naled added to cultures of *Fusarium graminearum* at different times following inoculation. (Mean quantity of F-2 in 2-week control cultures = 4771 ± 622 μg/50 ml of PDB). Figures marked with a superscript 1 represent the amount (percent of 2-week old cultures) of (a) F-2 or (b) growth already present at the time naled was added to the cultures.

at pH 5, 16 h at pH 7, and 13 min at pH 9. At 37 °C, the half-life is reduced to ca. 6 h at pH 5, 4.5 h at pH 7, and 3 min at pH 9 (Chevron Chemical Co., private communication). The pH of our media ranged from 5.2 to 5.7 at 27 °C. Theoretically then, only trace amounts (1 μl/l.) of naled (100 μl/l.) should be present in our culture media after 5 days, yet naled was still effective in completely inhibiting fungal growth after it had been in the culture media for 15 days. This suggests that naled either breaks down very slowly in our culturing systems or that its decomposition products are also inhibitory. By hydrolysis, naled is converted to dimethyl phosphoric acid, HBr, and dichlorobromoacetaldehyde. Dichlorobromoacetaldehyde further decomposes to oxalic acid (Melnikov, 1971). By

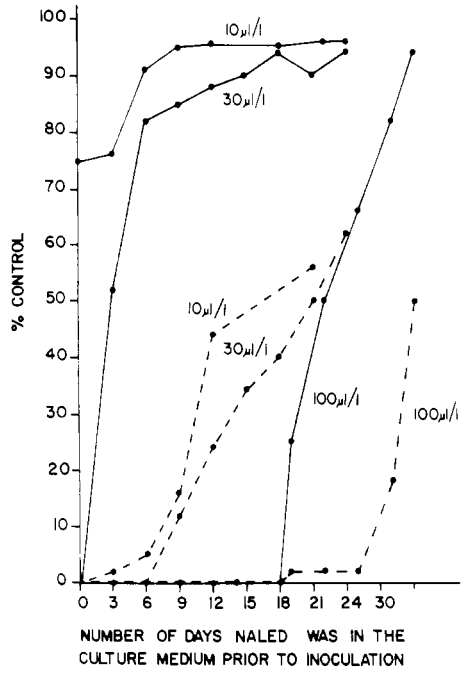


**Figure 2.** Effect of naled on mycelial growth and F-2 production when naled was added to the culture medium at different times prior to inoculation: (- - -) F-2; (—) mycelium dry weight. (Mean mycelial weight in 2-week control cultures = 0.2455 ± 0.008 g/50 ml of PDB.)

hydrolysis, dichlorvos also breaks down to form dimethyl phosphoric acid in addition to dichloroacetaldehyde which rapidly decomposes in water (Melnikov, 1971). By metabolism, naled is converted to dichlorvos and HBr (Casida et al., 1962). Dichlorvos has already been shown to inhibit growth and F-2 production in our culturing system and other investigators have shown that it inhibits ochratoxin production (Wu and Ayres, 1974) and aflatoxin biosynthesis (Rao and Harein, 1972; Rao, 1974; Hsieh, 1973). Dichlorvos, then, may account for some of the naled related inhibition in our culturing systems. The rate of decomposition of naled and the effects of its breakdown products on growth and F-2 production are unknown and warrant further investigation.

As shown in Table I, naled at levels of 30 and 100 μl/l. completely inhibited fungal growth in both liquid media and on corn. The fungus grew in all cultures treated at levels of 10 and 1 μl/l.; however, there was a significant difference in the inhibition of F-2 production in liquid media and on corn. F-2 was not produced in liquid cultures treated with 10 μl/l.; however, F-2 production was reduced by 29% in the corn cultures similarly treated. At 1 μl/l., naled reduced F-2 production by 54% in liquid media and by 12% on corn. Naled, then, has a greater inhibitory effect on F-2 production in liquid media than on corn. In liquid media, naled inhibited mycelial growth and subsequently F-2 production was also decreased. Based on visual comparisons of mycelial growth in treated and nontreated corn cultures, naled at 1 and 10 μl/l. also depressed mycelial growth by 5–10%.

When applied as a fumigant, naled at 10, 30, and 100 μl/l. completely inhibited growth in liquid cultures for 3 weeks. Although no F-2 was produced after 3 weeks incubation in corn cultures, mycelial growth was visually

**Table I. Effects of Naled on Growth and F-2 Production in a Liquid Medium and on Corn after 3 Weeks Incubation at 27 °C**

| Medium | Naled, μl/l. | Mycelial growth,[b,c] % control | F-2,[d] % control |
|---|---|---|---|
| PDB[a] | 1 | 84 | 46 |
| | 10 | 75 | 0 |
| | 30 | 0 | 0 |
| | 100 | 0 | 0 |
| Corn | 1 | | 85 |
| | 10 | | 71 |
| | 30 | 0 | 0 |
| | 100 | 0 | 0 |

[a] Potato dextrose broth (Stevens, 1974). [b] Average of eight replications. [c] Mean mycelial weight of control cultures = 0.2671 ± 0.034 g in PD broth. [d] Mean F-2 in control cultures = 5144 ± 1218 μg in PD broth, and 10 780 ± 2146 μg in corn.

reduced by 75–90% in cultures treated with 100 and 30 μl/l., respectively, and by 25–40% in those treated at the 10 μl/l. level. Even when applied as a fumigant, naled also inhibits growth with a subsequent decrease in F-2 production.

Naled applied as a liquid or a fumigant inhibited mycelial growth and F-2 production in liquid media and on corn. Although naled did partially inhibit F-2 production in cultures that were 3 ± 9 days old, it is a potential protectant only when applied prior to germination or growth of the fungus. Used in this manner, naled in liquid media was active as a growth inhibitor for 15 days at the 100 μl/l. level, for 6 days at 30 μl/l., and 3 days at 10 μl/l. The long term effectiveness of naled residues on F-2 production may be important in commodities when conditions favorable to germination or growth of the fungus occur. At this time, naled residues may prevent or partially inhibit growth so that F-2 production is effectively prevented or significantly reduced.

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