

Influence of Four Herbicides on Carpogenic Germination and Apothecium Development of *Sclerotinia sclerotiorum*

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Michigan Agricultural Experiment Station Journal Article 11834.

Accepted for publication 27 March 1986 (submitted for electronic processing).

ABSTRACT

Casale, W. L., and Hart, L. P. 1986. Influence of four herbicides on carpogenic germination and apothecium development of *Sclerotinia sclerotiorum*. *Phytopathology* 76:980-984.

Mycelial growth of *Sclerotinia sclerotiorum* on agar was inhibited by metribuzin and diuron at 50 $\mu\text{g}/\text{ml}$. The number of sclerotia that produced stipes was reduced by these compounds at 100 $\mu\text{g}/\text{g}$ in soil; apothecia that did develop appeared normal and produced ascospores. Atrazine and simazine were less inhibitory to mycelial growth and had no effect on the number of sclerotia producing stipes, even at 10 mg/g soil. In the presence of atrazine or simazine, however, apothecia either did not develop from the

stipes or were abnormally shaped and produced no ascospores. Abnormal apothecia formed in soil amended with atrazine (4–500 $\mu\text{g}/\text{g}$) or in solutions of 2–50 μM atrazine. When normal immature apothecia grown in the absence of atrazine were soaked in 50 μM atrazine for 30 min, the hymenia aborted and each gave rise to new stipes. This study indicates a potential use of triazines for investigating apothecial differentiation in *S. sclerotiorum*.

Apothecium formation is a critical stage in the life cycle of *Sclerotinia sclerotiorum* (Lib.) de Bary because of the importance of ascospore inoculum in the initial infection of host plants. This stage is also the most sensitive to external influences. Divalent metal ions, simple sugars, common salts, buffers, and fungicides inhibit carpogenic germination (events leading to the production of apothecia), generally at lower concentrations than are inhibitory to mycelial germination or growth (8,10,15,25). Carpogenic germination and the number of stipes produced per sclerotium were increased by incubating sclerotia in soil amended with atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] or simazine [2-chloro-4,6-bis(ethylamino)-s-triazine], but apothecial disk development was inhibited (19). The specificity of inhibition by atrazine and simazine was in contrast to the effects of metribuzin [4-amino-6-tert-butyl-3-(methylthio)-as-triazine-5(4H)one] and trifluralin [α,α,α -trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine], which increased production of both stipes and apothecia. These triazine compounds are effective herbicides that inhibit photosynthetic electron transport in plants (1).

This is a study of specific effects of atrazine and other compounds with similar effects in plants on *S. sclerotiorum* carpogenic germination and apothecium development.

MATERIALS AND METHODS

Production of sclerotia. Individual sclerotia of *S. sclerotiorum* (isolates A, B, and H) were originally derived from single sclerotia collected in the field. The sclerotia were germinated myceliogenically on potato-dextrose agar, and 5-mm disks were cut from the advancing colony margin after 3–4 days. Autoclaved canned green beans were arranged in a single layer in plastic petri dishes, inoculated with inverted mycelial disks, sealed with Parafilm, and stored in the light at room temperature. After 1 mo, the new sclerotia were harvested. This method gave maximum yield, because new sclerotial initials formed while others were maturing. Sclerotia were separated from macerated bean tissue in a metal strainer under a strong stream of tap water, air-dried for 24 hr, and stored at 5 C in plastic bags until needed.

Mycelial growth on herbicide-amended agar. The herbicides used were atrazine (AAtrex), simazine (Princep), metribuzin (Lexone), and diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea; Karmex). The compounds were diluted to 0, 5, 50, and 500 μg a.i./ml in 1.5% Bacto agar (Difco), autoclaved at 121 C for 20 min, then poured into petri dishes and allowed to cool. A mycelial disk (5 mm in diameter) from the colony margin of *S. sclerotiorum* (isolate B) growing on potato-dextrose agar was placed in the center of each plate with the mycelium in contact with the surface of the medium. Four replicates at each herbicide concentration were placed under fluorescent lights at 23 C. Colony diameters were determined after 3 days by taking the mean of two perpendicular measurements.

Incubation of sclerotia in herbicide-amended soil. Ten milliliters each of atrazine, simazine, and metribuzin (commercial

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formulations) were diluted in distilled water and added to 50 g of oven-dried Capac sandy clay loam soil in each 100 × 15 mm petri dish. Final concentrations were 0, 0.5, 1.0, 5.0, and 10.0 mg a.i./g soil. Ten sclerotia (isolate B) were placed on the soil surface in each dish (two replicates) and incubated in plastic bags in the dark at 15 C. Although carpogenic germination and stipe elongation may occur in the dark, apothecial disk differentiation requires light. Therefore, after 35 days of incubation, the dishes were placed under fluorescent light (2,800 lux, 14-hr photoperiod) at 15 C. Eighteen days after exposure to light (53 days after beginning incubation), the sclerotia in one replicate were washed under running distilled water for 1 min, then placed in a petri dish with 20 ml of distilled water. The water was replaced with fresh distilled water after 24, 48, and 84 hr. All sclerotia were incubated under fluorescent lights at 15 C.

In a second experiment, lower concentrations of herbicides were used; simazine was excluded because of similarity to atrazine and replaced with diuron, a substituted urea that inhibits photosynthetic electron transport. Sclerotia (isolate H) were placed on soil amended with the herbicides at 0, 0.8, 4, 20, 100, or 500 μg a.i./g soil, prepared as for the first experiment. Petri dishes containing the sclerotia (four replicates) were placed in plastic bags in the dark at 15 C. After 28 days, the dishes were sealed with Parafilm and two replicates were placed under fluorescent light at 15 C for apothecial disk development. The other two replicates were left in the dark at 15 C for determination of percentage of germination and stipe production.

Incubation of sclerotia in analytical-grade herbicide solutions.

Because we used commercial herbicide formulations in the first experiments, we tested analytical-grade herbicides to confirm that the observed effects were due to the active herbicide and not to other compounds in the formulations. Five sclerotia (isolate A) were incubated on 25 g of sand (washed and ignited) plus 10 ml of a 1% methanol solution of analytical-grade herbicide in each 100 × 15 mm petri dish (three replicates). These were incubated in the dark at 15 C for 65 days, then placed under fluorescent lights at 15 C for 18 days.

The effect of atrazine exposure subsequent to carpogenic germination was also examined. Sclerotia (isolate A) germinated in the absence of atrazine and bearing stipes without apothecia or with apothecial disks beginning to expand were placed in 0 or 50 μM atrazine in 1% methanol for 30 min. Treated sclerotia were rinsed briefly in distilled water, placed on 10 g of sand plus 4 ml of distilled water in each of several 60 × 15 mm petri dishes (four sclerotia per dish), and incubated under fluorescent light at 15 C.

RESULTS

Mycelial growth on herbicide-amended agar media. Diuron was more inhibitory to mycelial growth of *S. sclerotiorum* than were

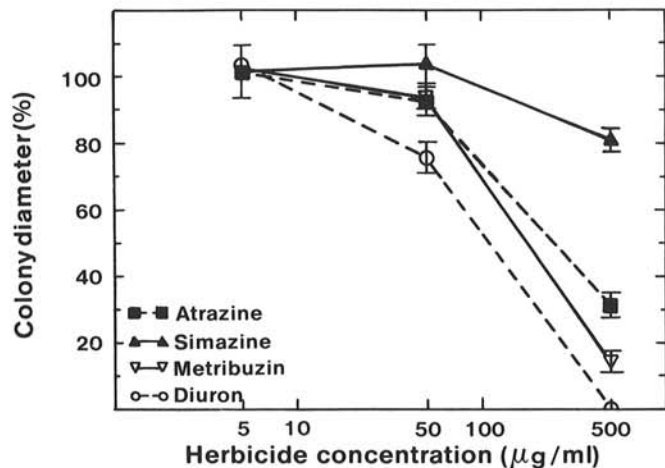


Fig. 1. Colony diameters of *Sclerotinia sclerotiorum* grown on herbicide-amended water agar for 3 days, expressed as percentage of unamended control (5 cm).

the triazines (atrazine, simazine, and metribuzin) (Fig. 1). Colony diameters were not decreased by any of the compounds at 5 μg/ml. At 50 μg/ml, however, diuron reduced colony diameters by 24%, whereas the triazine treatments were not significantly different from the controls. Mycelial growth on agar was completely prevented by diuron at 500 μg/ml and reduced 86, 69, and 19% by metribuzin, atrazine, and simazine, respectively, at 500 μg/ml.

Effect of herbicide-amended soil on carpogenic germination.

Sclerotia in soil containing atrazine or simazine at 0.5–10.0 mg/g soil germinated as well as those in unamended soil. Carpogenic germination was 90–100% after 53 days. Many stipes were produced by these sclerotia, but apothecia were present only on sclerotia in unamended soil (Fig. 2A). In contrast, no carpogenic germination occurred of sclerotia incubated in metribuzin-amended soils, although myceliogenic germination occurred at 0.5 and 1.0 mg/g soil. The mycelium grew across the soil surface, giving rise to smaller “secondary” sclerotia. One of these secondary sclerotia germinated carpogenically after removal to fresh, unamended soil. The “primary” sclerotia in these treatments collapsed with apparently only the dark rind remaining, as though

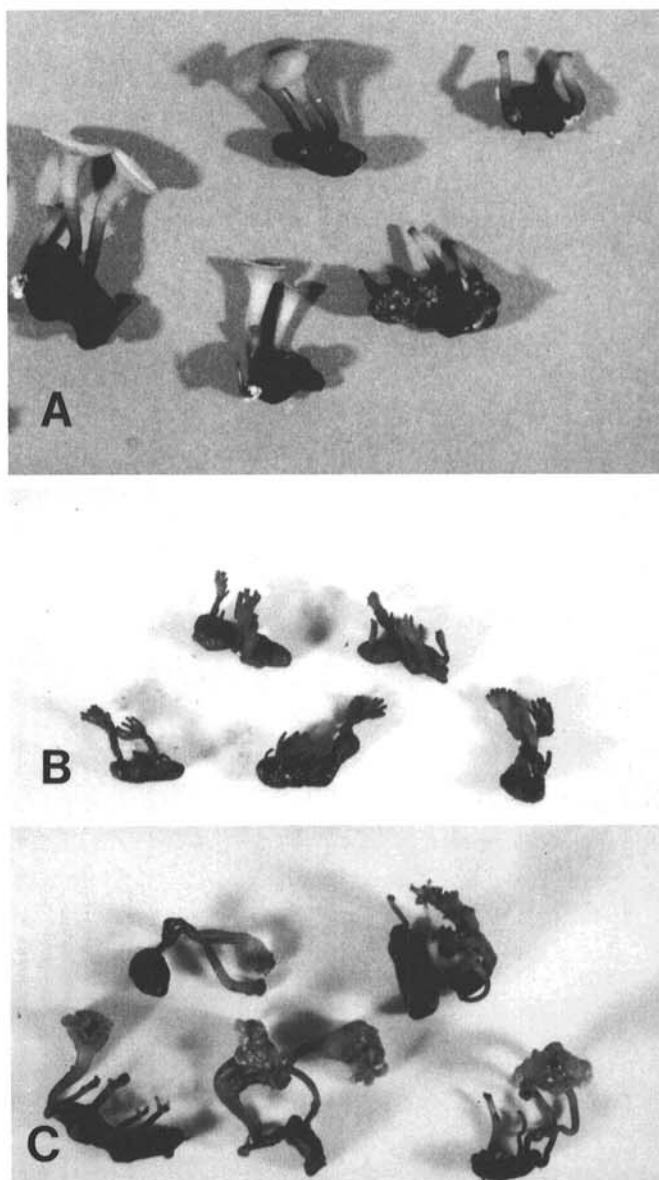


Fig. 2. A, Normal apothecia of *Sclerotinia sclerotiorum* incubated in soil. B, Abnormal stipes produced in 0.5 mg of atrazine per gram of soil. C, Abnormal apothecia formed by sclerotia incubated in 0.5 mg of atrazine per gram of soil for 53 days, then washed and placed in distilled water for 29 days.

their contents were mobilized into production of the secondary sclerotia.

After these observations were made, half of the sclerotia from the atrazine and simazine treatments were removed from the soil and washed. The sclerotia were placed in distilled water, which was changed several times in an attempt to remove the herbicides from fungal tissues. No apothecia developed from the sclerotia that remained in herbicide-amended soil (Fig. 2B). In contrast, by 29

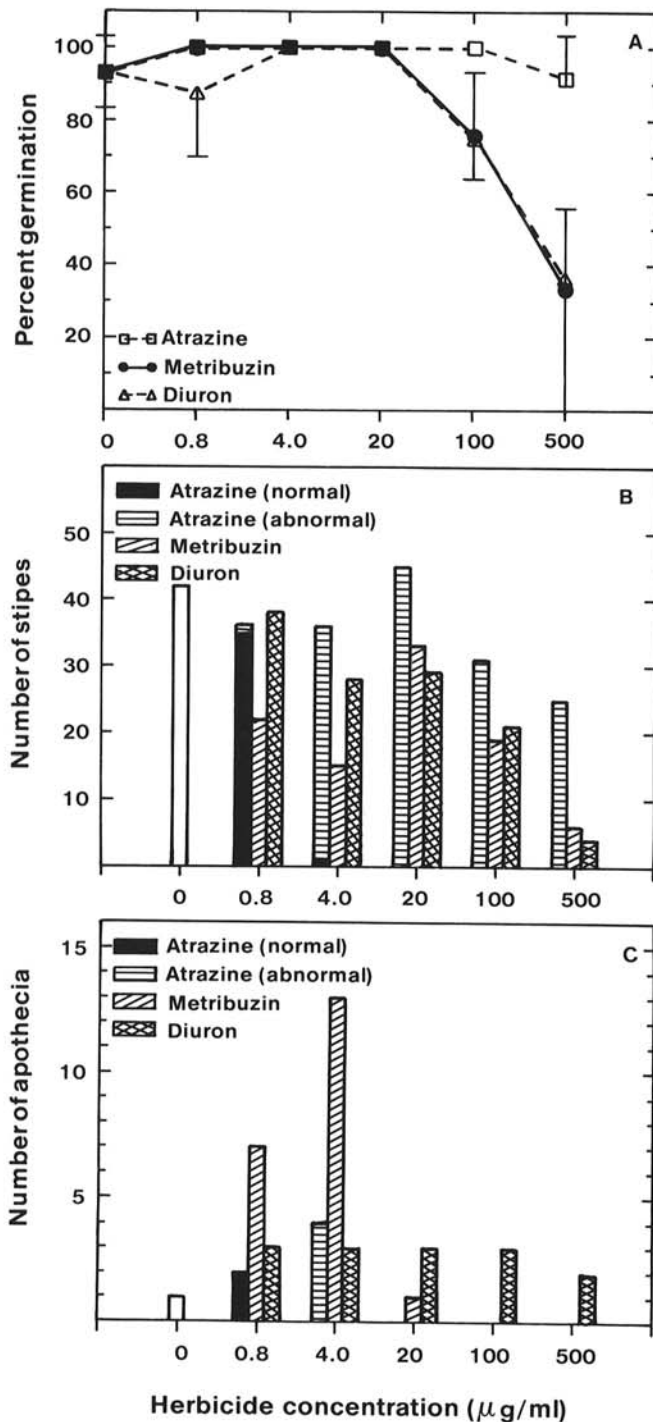


Fig. 3. A, Carpogenic germination of sclerotia (number of sclerotia with at least one stipe per 20 sclerotia) of *Sclerotinia sclerotiorum* incubated in herbicide-amended soil for 77 days in the dark. B, Stipes produced by 20 sclerotia incubated in herbicide-amended soil for 77 days in the dark. Atrazine bars represent total number of stipes in that treatment; countershading shows proportion of total stipes that were normal and abnormal. C, Apothecia produced by 20 sclerotia incubated in herbicide-amended soil for 28 days in the dark, then for 18 days under fluorescent light.

days the washed sclerotia had formed numerous abnormally shaped apothecia that produced no ascospores (Fig. 2C).

Soil amended with lower concentrations of herbicide was used in the second experiment. Half of the sclerotia were exposed to light after 28 days and incubated in the light for 49 days; the other half were left in the dark for the entire 77 days. Metribuzin and diuron at $> 100 \mu\text{g/g}$ soil reduced the percentage of sclerotia that formed stipes, but atrazine had no effect on percentage of germination (Fig. 3A). However, stipes produced by sclerotia incubated in soil containing atrazine at $4\text{--}500 \mu\text{g/g}$ were abnormal, whereas stipes were normal in soil containing metribuzin and diuron (Fig. 3B). After exposure to light, sclerotia incubated in soil containing atrazine at $4 \mu\text{g/g}$ bore abnormal apothecia; at higher concentrations, no apothecia developed (Fig. 3C). Apothecia in metribuzin- and diuron-amended soils appeared normal. Although there was an apparent stimulation of apothecia formation by $0.8\text{--}4 \mu\text{g}$ of metribuzin per gram of soil, no apothecia formed with metribuzin at $> 20 \mu\text{g/g}$ soil.

Effects of analytical-grade herbicides on carpogenic germination. Experiments using analytical-grade herbicides confirmed that the malformations of stipes and apothecia were due to atrazine rather than to another component of the commercial formulation. All stipes were normal in $2\text{--}8 \mu\text{M}$ analytical-grade atrazine ($1 \mu\text{M} = 216 \text{ ng/ml}$), but $50\text{--}92\%$ of apothecia were abnormal over this concentration range. In $10\text{--}50 \mu\text{M}$ atrazine, stipes and apothecia were abnormal (Fig. 4). The degree of apothecial malformation increased with increasing atrazine concentration. Microscopic examination showed no asci in the hymenia of these abnormal apothecia. All stipes and apothecia in metribuzin or diuron up to $100 \mu\text{M}$ were normal. No significant differences were observed among any of the herbicide treatments or controls in the percentage of carpogenic germination.



Fig. 4. A, Normal (right) and abnormal apothecia produced by sclerotia of *Sclerotinia sclerotiorum* incubated in 0 and $10 \mu\text{M}$ atrazine, respectively. B, Abnormal apothecia from sclerotia incubated in $10 \mu\text{M}$ atrazine.

The hymenia of immature apothecia that were placed in 50 μ M atrazine for 30 min darkened after 2 days, although their stipes remained a normal appearance. Numerous stipes grew from the hymenial surface of these apothecia after 10 days, and each new stipe produced a malformed apothecium (Fig. 5A and B). Two days after exposure to atrazine, stipes lacking apothecia appeared normal, but new growth was highly deformed by 10 days (Fig. 5C). Control apothecia treated with 1% methanol developed normally (Fig. 5A).

DISCUSSION

Studies on the effect of herbicides on soil microorganisms and on the plant diseases they cause have been reviewed by Kaiser et al (11) and by Katan and Eshel (12). Atrazine stimulates growth (17,18,20,24), respiration (6,23), and glucose catabolism (26) of some fungi at low concentrations (about 10 μ g/ml) but is often inhibitory at higher concentrations (20–23). Diuron inhibits production of sclerotia by *Sclerotium rolfsii* and *Sclerotinia trifoliorum* (5). Some fungi utilize simazine as a nutrient (13).

None of the herbicides in our study stimulated mycelial growth on agar. In soils containing high concentrations of metribuzin, however, sclerotia germinated myceliogenically, with the mycelium growing across the soil to produce new sclerotia. We did not investigate this phenomenon further but did not observe it when sclerotia were incubated in metribuzin solutions without soil. Metribuzin may act indirectly by suppressing antagonistic soil microorganisms, thereby allowing *S. sclerotiorum* to utilize nutrients in the soil. Metribuzin did not enhance mycelial growth on agar cultures, but a direct stimulation of myceliogenic germination cannot be dismissed.

Metribuzin and diuron inhibited mycelial growth on agar more than did atrazine and simazine, and this pattern was reflected in the

effect of metribuzin and diuron on carpogenic germination. However, stipes and apothecia that did develop in the presence of metribuzin and diuron appeared normal and produced ascospores (Fig. 3B and C). In contrast, atrazine was less inhibitory to mycelial growth and had little or no effect on the number of sclerotia that germinated carpogenically (Fig. 3A). Stipes produced in soils containing atrazine > 4 μ g/g were malformed and produced no apothecia; at 4 μ g of atrazine per gram of soil, distorted, sterile apothecia developed (Fig. 3B and C). Although soil was amended with atrazine as the commercial formulation AAtrex, similar apothecial malformations were elicited by solutions of analytical-grade atrazine. This indicates that atrazine was the compound in AAtrex that produced the observed effects on *S. sclerotiorum*.

The effects caused by high levels of atrazine and simazine were partially alleviated when germinated sclerotia were removed from soil and placed in water, which presumably leached the herbicides from the tissue, because apothecia subsequently differentiated from these stipes. The apothecia were always abnormal (Fig. 2C), however, indicating either incomplete removal of the toxic compound or an irreversible effect on the fungus.

When normal immature apothecia, initially formed in the absence of atrazine, were exposed to atrazine, maturation of the disk ceased and the hymenium darkened and sprouted many new stipes. Only the hymenial surface of the disk appeared to be affected; the stipe and lower portion of the disk retained the usual color and form.

Atrazine appears to have a specific influence on apothecial differentiation rather than a general toxicity to the organism. Mycelial growth and percentage of germination were affected only at concentrations of atrazine much higher than those that generated abnormal apothecia. Moreover, metribuzin and diuron, which were more inhibitory to vegetative growth and the onset of carpogenic germination, caused no malformation of the stipes or

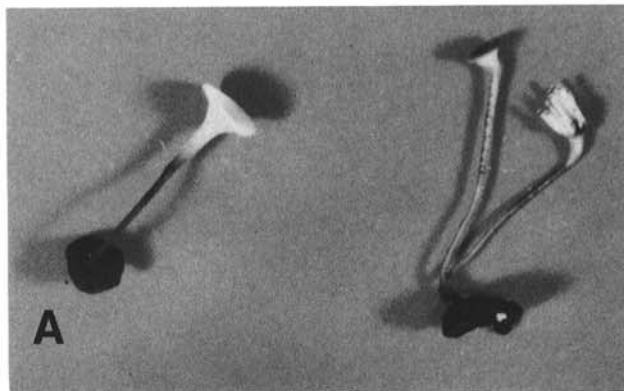


Fig. 5. A, Normal (left) and abnormal development of apothecia of *Sclerotinia sclerotiorum* exposed to 0 and 50 μ M atrazine, respectively, for 30 min. Immature apothecia were produced by sclerotia in the absence of atrazine, then treated and incubated in distilled water for 10 days. B, Close-up of the abnormally developed apothecia in A shows numerous stipes growing from their darkened hymenia. C, Extensive branching and distorted growth of a stipe, lacking apothecial disk, that was exposed to 50 μ M atrazine for 30 min.

apothecia at any concentration. Only three isolates of *S. sclerotiorum* were tested, but the effects of atrazine were similar among these isolates.

Branching of stipes after decapitation (9,14) as well as abnormal development of apothecia exposed to dichloropropene and dichloropropane (7,16), uranium nitrate (2), and cadmium salts (3,4) have been reported previously. But the specific effects on apothecium development by the symmetric triazines reported here, at concentrations apparently not toxic to mycelial growth or initiation of carpogenic germination, make these compounds potentially useful in elucidating the mechanism of apothecial disk differentiation. The abortion of the apothecial disk with subsequent production of stipes from the hymenial surface may be particularly important.

The use of these triazines to control *S. sclerotiorum* in the field is obviously limited by their toxicity to crops. However, rotation of *S. sclerotiorum*-susceptible crops with atrazine-resistant crops, such as corn, might influence the inoculum potential of *S. sclerotiorum* in some fields.

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