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PHYSIOLOGICAL AND ULTRASTRUCTURAL EFFECTS OF STEROL-INHIBITING FUNGICIDES ON APPLE LEAVES AND THE APPLE SCAB FUNGUS

by

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ABSTRACT

The effects of sterol-inhibiting fungicides (SIFs) on the free sterol and free fatty acid composition of apple leaves of Red Delicious and Jonathan cultivars were examined over a 2 year period. Trees were treated in mornings vs evenings throughout each season and samples collected after 24 and 72 hours after each treatment. Generally, SIFs appeared to have an effect on the free sterol content of apple leaves after 24 hours, but the concentrations of free sterol returned to normal after 72 hours in the leaves of both cultivars. Morning versus evening application had little or no influence on leaf free sterol concentrations. There were

increases in unsaturated and total fatty acid concentrations in Red Delicious leaves 24 hours following applications with the SIF, etaconazole, and the non-sterol-inhibiting fungicide (NSIF), metiram, early in the season. There were also increased concentrations of linoleic, linolenic and total free fatty acids in fenarimol and triadimefon-treated Jonathan leaves 72 hours after treatment. Early in the season, the SIF, fenarimol, caused an increase in linolenic acid in both Red Delicious and Jonathan leaves 72 hours after either morning or evening applications. Generally, both the Red Delicious and Jonathan leaves exhibited a decrease in saturated fatty acids following morning application whereas, an increase in saturation following evening application. Although SIFs may have had an effect primarily on the unsaturated fatty acids, particularly linolenic acid, early in the season, particularly linolenic acid, the fatty acid composition of the leaves appeared to return to normal later in the season.

Ultrastructural observations were made of Red Delicious leaves 12, 24 and 72 hours after treatment with the SIF, bitertanol. Twelve hours after treatment thylakoids of chloroplasts appeared swollen and irregular resulting in loss of integrity of the organelles. However, after 24 and 72 hours, thylakoids of chloroplasts of treated leaves were

similar to the controls. Infection of bitertanol-treated Red Delicious leaves by Spilocaea pomi was also examined at the fine structural level. Nuclear envelopes were not well defined and mitochondrial matrices appeared washed-out after 12 and 72 hours post treatment. There was dissolution of normally plate-like cristae of mitochondria, accompanied by the accumulation of minute electron dense bodies around their periphery. Invaginations and proliferations of the plasmalemma were observed as well as increased vacuolization of the cell. Further electron microscopic observations were made of the in vitro conidial state of Venturia inaequalis following the application of fenarimol. Conidia treated 2 hours with the fungicide for had necrotic areas throughout the cytoplasm. The plasmalemma was not well defined, and appeared to be degrading. Increased vacuolization was observed as were numerous lipid bodies and multivesicular complexes (MVCs) which contained vesicles of varying electron densities. Structural integrity of the organelles was such that they were difficult to discern. After 12 hours, the entire fungal cell was necrotic accompanied by the degradation of the cell wall. Detection of a selected number of SIFs in apple leaf tissue using bioassay procedures were also evaluated. It was found that the leaf disk and leaf extract bioassays examined in this study were

ineffective in determining the presence of SIFs in apple leaves.

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CHAPTER 1 - INTRODUCTION AND LITERATURE REVIEW

STEROL-INHIBITING FUNGICIDES (SIFS)

An innovation in the use of chemicals to control plant pathogens is the introduction of compounds that inhibit ergosterol biosynthesis and membrane function in fungi. Almost all of these sterol-inhibiting fungicides (SIFs) exhibit varying degrees of systemicity, and are used to control a wide range of diseases caused by Ascomycetes, Basidiomycetes and Deuteromycetes. Several unique characteristics differentiate them from other systemic agents. They are the largest group of fungicides with the same specific mode of action (Siegel 1981). In addition, SIFs are a diverse group of compounds with respect to chemical structures; and, only recently, has reduced sensitivity to SIFs in field isolates of *Venturia inaequalis* (Cke.) Wint. (= *Spilocaea pomi* Fr.) been reported (Stanis and Jones 1985).

Sterols are required for growth and reproduction of several eucaryotic organisms and serve as structural components of membranes as well as precursors for important steroid components. Sterol biosynthesis is one aspect of general lipid metabolism (Fig. 1.1) in which the basic starting chemical unit, acetate, is converted into mevalonate (Ragsdale 1977). The initial stage of sterol, gibberellin and carotenoid biosynthesis is frequently called the "isoprenoid pathway" (Ragsdale 1977). Isoprenoid refers to the basic five-carbon unit that is formed from mevalonic acid after the loss of one carbon. The condensation of five-carbon isoprenoids eventually leads to the formation of squalene. The cyclization of squalene to form lanosterol, is the first step leading to the formation of ergosterol, a major sterol in higher fungi.

Figure 1.2 illustrates the general pathway of ergosterol biosynthesis (Siegel 1981). This synthesis involves the following reactions:

A. Introduction of a methyl group into lanosterol at C-24 accompanied by a double-bond shift from C-24(25) to C-24(28).

B. Removal of three methyl groups on the steroid nucleus (two at C-4 and one at C-14).

C. Double-bond shift from C-8(9) to C-7.

D. Introduction of a double-bond at C-5(6) and C-22.

E. Reduction of the C-24(28) double-bond.

Many of the reactions have not been completely elucidated and their sequence may vary in different species of fungi.

Sterol biosynthesis occurs in the smooth portion of the endoplasmic reticulum; in part, this involves the microsomal, mixed function, oxidase system (Siegel 1981). Sterol carrier proteins allow the interaction of the sterol and enzymes necessary for various sterol conversions.

Two groups of compounds exist which inhibit ergosterol biosynthesis (Table 1.1). The first group inhibits the biosynthesis of ergosterol, as determined by biochemical analysis of their toxic action in fungal cells (see references in Table 1.1). The second group is presumed to inhibit ergosterol biosynthesis based on the similarity of their structures to those of known sterol-inhibitors (Siegel 1981).

Imidazole derivatives (imazalil, clotrimazole, miconazole and etaconazole), triazole derivatives (triadimefon, fluotrimazole) and the fungicides fenarimol and denmert all reportedly inhibit desmethylation at 24-methylenedihydrolanosterol in ergosterol biosynthesis (Berg et al. 1981). Kerkenaar et al. (1981) determined

that the site of action of tridemorph in ergosterol biosynthesis by Ustilago maydis (DC) Corda was Δ^{14}-reductase which is responsible for 14/15 double-bond reduction in sterol biosynthesis. This was accompanied by the accumulation of ergosta-8,14-dien-3β-ol (ignosterol) as the major sterol intermediate. However, Kato et al. (1980) using Botrytis cinerea. found tridemorph to block the $\Delta^{8} \rightarrow \Delta^{7}$ isomerization resulting in the accumulation of fecosterol (ergosta-8,24(28)-dien-3β-ol). Fecosterol has the same molecular ion (398) and also a fragmentation pattern very similar to that of ignosterol (Kato et al. 1980). However, they identified the structure only by analyzing the mass spectrum after gas liquid chromatography. Therefore, a misinterpretation in the identification of fecosterol by Kato et al. appears quite likely, although inhibition may occur at different sites in U. maydis and B. cinerea.

Several systemic fungicides including triadimefon, triforine, buthiobate, triarimol, fenarimol, nuarimol, imazalil, fluotrimazole, which are active on phytopathogens and clotrimazole, used against animal mycoses, inhibit sterol C-14α-demethylation, thereby causing the accumulation in fungi of ergosterol precursors that retain the 14α-methyl group (Gadher et al. 1983). No sterols are synthesized in the pathway beyond those with a C-14 methyl group;

therefore, the primary site of action of these fungicides appears to be the sterol 14-demethylase complex (Gadher et al. 1983). Clotrimazole and triadimefon, both known to inhibit sterol C-14α-demethylation in pathogenic yeasts and fungi, were also reported by Berg et al. (1981) to inhibit the enzyme 3-hydroxy-3-methyl-glutaryl (HMG)-CoA-reductase. It was concluded that clotrimazole and triadimefon have to be considered as inhibitors of desmethylation reactions involved in the ergosterol synthesis of yeasts and fungi accompanied by a decreased production of mevalonate. Etaconazole (CGA 64251), a triazole fungicide, was observed by Ebert et al. (1983) to inhibit sterol C-14α-demethylation in Ustilago maydis. This resulted in the accumulation of the intermediate 14α-methyl-ergosta-8,24(28)-dien-3β,6α-diol, the structure of which, suggests a hydroxylation-dehydration mechanism for the introduction of a double bond at C-5 during ergosterol biosynthesis. Detailed structure-activity studies by Copping et al. (1984) culminated in the synthesis of N-alkyl-N-phenoxyalkyl substituted compounds and finally, prochloraz which gives excellant broad-spectrum disease control in cereals. Its mode of action is through the inhibition of C-14 demethylation.

Walsh and Sisler (1982) reported that a mutant deficient in sterol C-14 demethylation was similar to the wild-type

sporidia of U. maydis treated with fenarimol, etaconazole and miconazole, supporting the contention that inhibition of sterol C-14 demethylation in U. maydis is the primary mode of toxicity of these sterol inhibitors. Henry and Sisler (1984) suggested that the primary action of miconazole, fenarimol and etaconazole is competitive inhibition of sterol/steroid-type cytochromne P-450 enzymes rather than interference with the function of sterol carrier proteins or enzyme-modulating phospholipids. The mode of action of fungicides of the pyrimidin-5-ylmethanol type (fenarimol) is inhibition of the cytochrome P-450 dependent sterol C-14 demethylation and the C-22(23) desaturation reaction. No evidence for target sites exists in other pathways of comparable sensitivity, although the failure of additional ergosterol to reverse the fungitoxicity suggests the existence of other sites. However, growth inhibition by high concentrations of pyrimidin-5-ylmethanol, not reversible by GA, perhaps involves action at sites outside the GA biosynthesis pathway (Sisler et al. 1984). Sisler et al. (1984) suggested from several studies of the mechanisms of fungitoxicity and growth regulation that any undetected primary targets of the pyrimidin-5-ylmethanols are likely to be haem enzymes, similar to the cytochrome P-450 involved in sterol C-14 demethylation.

The SIFs are characterized by uptake of the active material by the plant followed by translocation within the plant system so that it produces a protective action that is systemic. Systemicity is determined by a number of factors which include the active material itself (structure, polarity, lipophilic and hydrophilic groups), the plant (surface structure, wax layer, cuticle, age of plant, degree of lignification) and environmental conditions (temperature, humidity, rate of transpiration).

Although foliar-applied systemic fungicides are absorbed into the leaf tissue, the quantitative aspects of active ingredient uptake have been investigated in only a few cases. In a report on the biological activity of fenarimol against scab and powdery mildew on apples, fenarimol was absorbed by apple leaves within 30 minutes application (Anonymous 1979). However, quantitative data and methods were not given. Data on triadimefon uptake so far have been published only with respect to its absorption by barley and cucumber leaves (Brandes et al. 1978; Fuhr et al. 1978).

Following treatment of bean leaves (Glycine max L. cv. Evans) with RH 2161, 50% of the compound was absorbed by the leaf after one day (Martin and Edgington 1978). Hisada et al. (1976) applied procymidone to the foliage of cucumber plants and found after one day 45% of the applied dose could

not be removed by washing with acetone. Kraus (1981) using [^{14}C] Bayleton® 5W (triadimefon) found that triadimefon very quickly becomes fixed in the leaf surface and thus is not readily removed from the tissues by rainfall, dew, etc., and then can be translocated in successive phases into the leaf tissue.

Following application of [^{14}C] Bayleton® 5 WP to young stem tissue, Kraus (1981) found that the active ingredient was subsequently translocated into the leaves. Autoradiography revealed that radioactivity was present mainly in the vascular system but was also observed to be evenly distributed in the intercostal spaces of the leaves indicating that the active ingredient had been translocated in a lateral direction. Translocation from the vascular system to the adjacent tissue was more pronounced in young leaves than in old ones. Following application to the leaf surface, triadimefon moves rapidly into the uppermost epidermal layers (wax layer, cuticle) from where it is absorbed successively into the tissue. As a result, washing with water only 45 minutes after foliar application did not recover more than half of the applied dose.

Barak et al. (1983) reported that lignin preparations, derived from stems of pepper (Capsicum annuum L. cv. Pelle California) and cotton (Gossypium hirsutum L. cv. SJ-1)

plants, adsorbed the systemic fungicides carbendazim, triadimefon, nuarimol, triarimol and fenarimol more effectively than bovine serum albumin, cellulose, ethylcellulose or sodium polygalacturonate. They also determined that lignin is the main component which adsorbs pesticides in the apoplast of plants. The more lipophilic fungicides triarimol and fenarimol were adsorbed to the greatest extent while triadimefon and nuarimol were moderately adsorbed (Barak et al. 1983). In barley (Hordeum vulgare L. cv. Fergus), the imidazole fenapanil and several closely related analogs exhibited systemic translocation, which was limited to apoplastic transport (Martin and Edgington 1982).

SIFS: EFFECTS ON MICROORGANISMS

Although the sterol inhibiting fungicides are quite different structurally, their antifungal spectra are considered to have the following general characteristics. They fail to inhibit spore germination or initial cell

growth and dry weights increase (Siegel 1981). They alter cell morphology, subsequently producing abnormal growth patterns, swollen hyphae and/or excessive hyphal branching (Siegel 1981). Sporidia of U. maydis, which normally multiply by budding, become abnormally large, branched and multicellular (Ragsdale and Sisler 1973). None of the fungicides appreciably affect early stages of respiratory metabolism or macromolecule synthesis, and they cause the accumulation of free fatty acids (FFA) and sterol intermediates in cells (Siegel 1981). In addition, fungal mutants selected in the laboratory for resistance usually displayed cross-resistance to other members as well.

These inhibitors rapidly curtail the biosynthesis of ergosterol. However, mycelial growth and various aspects of metabolism (respiration, protein and nuclei acid synthesis) are only mildly affected for a period of time after synthesis of the sterol has stopped. Ergosterol levels in treated mycelium do not decline rapidly which indicates that the rate of use of the sterol in membrane synthesis is slower than its biosynthesis. Interference in membrane synthesis occurs once the level of ergosterol becomes depleted resulting in growth inhibition and changes in morphology and metabolism (Siegel 1981). The use of radiolabeled neutral lipids in treated mycelium shows that

certain sterol intermediates begin to accumulate immediately upon inhibition of ergosterol biosynthesis. However, only after longer incubation periods with inhibitors and after cessation of membrane synthesis will FFA accumulate in treated mycelium. This accumulation results from continued de nova synthesis of FFA, a reduction in triglyceride and polar lipid utilization, and from the degradation of the existing membrane phospholipids (Siegel 1981).

In addition to chemical analysis of the toxic action of an inhibitor on cellular metabolism, other assays can be used to further characterize toxicity. One such method uses fungicide resistant mutants and measures patterns of cross-resistance (Siegel 1981). "Cross-resistance" is used when a change in one genetic factor in the fungal strain results in the development of resistance to different fungicides (Dekker 1977). Usually this occurs only between compounds that have similar modes of action.

Barug and Kerkenaar (1979) studied patterns of cross-resistance with fungicide-resistant mutants of U. maydis to clotrimazole, imazalil, miconazole, fenarimol, nuarimol, triadimefon and tridemorph. They found that cross-resistance was reciprocal in all cases except for triadimefon and imazalil, and for triadimefon and tridemorph. The sterol inhibitors and prochloraz also

exhibited cross-resistance, indicating a similar mode of action for this compound (Barug and Kerkenaar 1979). This was subsequently confirmed by biochemical analysis of the antifungal activity of prochloraz by Pappas and Fisher (1979).

Beginning in 1969, researchers have extensively evaluated new fungicides for efficacy against several major apple diseases. These compounds include triarimol, triforine, triadimefon, fenarimol, fenapanil, prochloraz, etaconazole and bitertanol which are commonly referred to as SIFs. These compounds affect haustorial formation in the powdery mildew fungus, and because they penetrate the leaf cuticle, they often bring curative action. All were significantly more effective than standard fungicides (Hickey and Yoder 1981). This same group of fungicides has shown high activity against apple scab, cedar-apple rust and several fruit decay pathogens (Yoder and Hickey 1981). In addition, the use of these broad-spectrum fungicides should be valuable in combating strains of Venturia inaequalis resistant to benomyl or dodine (Yoder and Hickey 1981). Szkolnik (1981) evalauted all the above SIFs except triarimol on apple in the greenhouse and found that against V. inaequalis, they were more effective in eradication than in protection. They inhibited spore production when applied

before scab symptoms appeared but not when applied to existing scab lesions. In addition, etaconazole, bitertanol and triadimefon sprays gave excellent protection against Podosphaera leucotricha (Ell. and Everh.) Salm. and vapor from etaconazole and triadimefon gave very good protection against mildew on unsprayed trees (Szkolnik 1981). Their efficacy against a broad range of important plant pathogens and their high rate of activity at relatively low concentrations make them highly desirable fungicides.

Ross and Newbery (1981) observed that the 80 mg/L rate of fenarimol caused complete suppression of ascospore discharge by V. inaequalis when applied in five, four or three sprays, but not with two applications. The 40 g rate was less effective as the number of sprays decreased. They also determined that benomyl alone and in combination with fenarimol exhibited complete inhibition. However, fenarimol had no effect on mycelial growth or perithecium formation on inoculated sterile leaf discs, while all benomyl treatments and the combination of benomyl and fenarimol completely inhibited growth (Ross and Newbery 1981).

Kelley and Jones (1981) reported that etaconazole and bitertanol effectively controlled apple scab under greenhouse and orchard conditions. In greenhouse studies, fungicides applied 4.0, 4.5, 5.0 and 5.5 days after

inoculation did not prevent establishment of lesions, but did inhibit their further development as well as sporulation. In orchard studies, two applications, 7 days apart, of etaconazole or bitertanol to lesions either late in the incubation period or beginning 2 days after they appeared, inhibited spore production in chlorotic lesions and suppressed conidial development in sporulating lesions. Fruit infections were also inhibited. In addition, fungicides applied at 7-day intervals suppressed scab development better than those applied at 14-day intervals (Kelley and Jones 1981). The SIFs etaconazole, fenarimol, CGA 71818 and bitertanol plus an adjuvant were highly effective in preventing the formation of apple scab lesions on apple leaves when applied up to 72 hours after the onset of infection periods at 15°C in the greenhouse (Schwabe et al. 1984). Chlorotic lesions developed if applications of bitertanol and triforine were delayed 24 hours or more after inoculation. The SIFs, etaconazole, triforine, CGA 71818, bitertanol in combination with an adjuvant and fenarimol, inhibited the production of conidia, even when applied 120 hours following inoculation. However, the protective activity of these fungicides decreased faster between application and inoculation than that of captan and mancozeb (Schwabe et al. 1984).

O'Leary and Sutton (1986) observed that the SIFs, bitertanol, etaconazole, fenarimol and triforine, applied 120 hours after inoculation of apple (Malus domestica Bork. 'McIntosh') foliage with V. inaequalis conidia, resulted in the formation of chlorotic lesions 2 weeks after application. Sporulation typically occurred around the edges of the chlorotic tissue 5 weeks after application. Some of the chlorotic lesions in the fenarimol, etaconazole, and bitertanol treatments were necrotic after 5 weeks, but sporulation was observed in leaf tissue surrounding the necrotic as well as the chlorotic areas (O'Leary and Sutton 1986). They also determined that neither benomyl nor the SIFs completely eradicated the fungus or prevented sporulation when applied 120 hours after infection.

Previous experiments have shown that postinfection applications of SIFs prevented sporulation, but evaluations were usually made 1-2 weeks after the fungicides were applied (Brandes and Paul 1981; Drandarevski and Schicke 1976; Kelly and Jones 1981; Schwabe et al. 1984; Szkolnik 1981). The studies by O'Leary and Sutton (1986) confirmed the poor postsymptom activity of one application of SIFs against V. inaequalis. These compounds were less effective than benomyl in reducing recovery of the fungus from lesions and preventing sporulation. Szkolnik (1981) also found

benomyl to be more effective than SIFs in a postsymptom application.

Although O'Leary and Sutton (1986) reported that these fungicides do not inhibt germination when they are applied directly to conidia, the germination of mature conidia produced from treated mycelium was reduced. Previous studies have shown that seasonal and postseasonal applications of SIFs and benomyl resulted in the production of fewer pseudothecia (Connor and Heuberger 1968; Ross 1973; Ross and Newbery 1975 and 1981) and the release of fewer ascospores from overwintered leaves (Connor and Heuberger 1968; Drandarevski and Schicke 1976; Gadoury and MacHardy 1984; Ross 1973; Ross and Newbery 1975 and 1981). O'Leary and Sutton (1986) indicated that a single postseason application of the SIFs, bitertanol, etaconazole, fenarimol and triforine, resulted in the production of fewer and smaller pseudothecia that contained fewer ascospores.

Structure-activity relationships of the imidazole, fenapanil and several closely related analogs demonstrated that chlorine or methyl substitutions to the phenyl ring of fenapanil increased both the antifungal and phytotoxic activity (Martin and Edgington 1982). In addition, the presence of a cyano group contributed to antifungal activity. Part of the increased antifungal activity was related to the compound lipophilicity.

Sancholle et al. (1984) determined that propiconazol was an effective growth inhibitor of the 14 fungi tested and may have potential in the control of plant diseases caused by the plant pathogens represented. Rhizopus stolonifer (-)(fr.) Lind, Mucor rouxii and M. mucedo required two to 12 times more fungicide for 50% growth inhibition than the other species supporting the belief that lower fungi are less sensitive to C-14 demethylation inhibitors than higher fungi.

Fungal growth inhibition by C-14 demethylation inhibitors is suggested to be due to a reduction of ergosterol and/or accumulation of C-14 methyl sterols, which lead to the disruption of membrane-associated activities. Both of these responses have been observed by Weete et al. (1983) in plasmalemma fractions of Taphrina deformans (D1), treated with ED50 concentrations of propiconazol. However, it has not been established whether growth inhibition is due primarily to one, a combination or either of these responses for propiconazol or other C-14 demethylation inhibitors. The consequences of these responses may be expressed as changes in cell permeability and cell-wall formation. A decrease in the amount of functional sterol (ergosterol) would be expected to result in reduced growth and increased cell permeabiltiy. Differences in the sterol composition of

plasma membranes from propiconazol-treated cells of T. deformans are accompanied by alterations in cell permeability (Sancholle et al. 1984). The treatment of fungi with C-14 demethylation inhibitors appears to alter the permeability characteristics of fungal cells (Siegel and Ragsdale 1978).

Results of electron microscopic studies of fungicide-treated sporidia of Ustilago avenae (Pers.) Rostr. (Hippe and Grossman 1981 and 1982) and similar results obtained in various organisms treated with triazole, pyrimidine or imidazole derivatives may be explained on the basis of similar modes of action. Different stages of ultrastructural changes to complete cell lysis have been described depending on the toxicity and incubation period of the substances applied (de Nollin and Borgers 1976; Stiers et al. 1980). These changes may be related to functional and structural defects in cellular membranes due to interferences with ergosterol biosynthesis.

Hippe and Grossman (1982) observed ultrastructural changes in sporidia of U. avenae following treatment with the fungicides, nuarimol and imazalil nitrate. Nuarimol caused increased cell wall thickness and invaginated plasmalemma, with accumulations of extracytoplasmic vesicles between the plasmalemma and cell wall. Pores were also

visible in the septa. In addition, nuarimol-treated cells were characterized by vacuolization, accumulation of lipid bodies and enhanced number of mitochondria. The application of imazalil caused only minor ultrastructural changes; most notable, elongated sporidia with enlarged cell diameters, increased thickness of the septa and an increased number of mitochondria with reduced diameters (Hippe and Grossman 1982).

The fungicidal actions of triadimefon, nuarimol and imazalil-nitrate inhibited the budding process of the sporidia of U. avenae, resulting in filamentous promycelium and at the fine structural level, enlarged cell diameters, thickening of the cell walls and septa and increased number of mitochondria (Hippe 1984). Organelles were enlarged and irregular, accompanied by larger amounts of endoplasmic reticula and extensive vacuolization and accumulation of lipid bodies. The destructive effects of the fungicides with extended incubation periods increased the intensity of cell damage in the following sequence: triadimefon ≥ nuarimol > imazalil-nitrate. An increased number of lipid bodies was found to be correlated to an increased lysis of the membranes.

Pring (1984) observed changes in the intercellular mycelia of both Uromyces vicia-fabae Pers. Schroet infecting

leaves of broad bean (Vicia faba cv. Broad Windsor) and Puccinia recondita Rob. ex Dorm infecting wheat (cv. Huntsman) leaves following both root and leaf application of the SIF, triadimefon. Incomplete and multiperforate septa occurred and extensive wall thickening was found. Pring discovered that vesicles of wall-building material may fuse with the plasmalemma to lay down wall material at a greater rate or in larger quantities than normally occurs. A similar wall outgrowth was observed in Candida albicans (strain R. V. 4688) treated with miconazole (de Nollin and Borgers 1974). Pring (1984) suggested that these effects may correspond with the biochemical studies showing the interference of triadimefon and related compounds on ergosterol biosynthesis (Buchenauer and Rohner 1982; Deas and Clifford 1982). Barug et al. (1983) showed alterations in the cell wall of C. albicans following treatment with the sterol C-14 demethylation inhibitor bifonazole. This was thought to be due to an irregular chitin deposition. This may indicate that the location or binding of chitin synthetase, a membrane protein, is affected by the inhibition of sterol biosynthesis.

Richmond (1984) observed that conidia of Botrytis allii Munn, germinated in the presence of the SIF, triadimefon, produced stubby and swollen germ tubes. The most striking

difference was in the process of septum formation which remained incomplete in the presence of triadimefon, but complete in its absence. Septa were not produced in some hyphae, with the wall becoming unevenly thickened. Vesicles occurred close to septal and hyphal walls and within swollen parts of walls. Triadimefon can disrupt septum formation and produce abnormal hyphal wall thickenings by inhibiting sterol biosynthesis, thereby altering the permeability and fusion properties of the microvesicles involved in wall synthesis (Richmond 1984). Richmond suggests that this may account for the large number of vesicles within incomplete septa and wall thickenings. However, ergosterol deficiency may limit the rapid membrane synthesis necessary during septum formation, the resulting imbalance between wall and membrane synthesis leading to wall swellings.

Electron microscopic analyses add further evidence to the dependence of the structure of intracellular components upon the membrane composition. They suggest correlations between membrane instability due to a lack of ergosterol and cell physiology, and indicate morphological and fine structural consequences.

SIFS: EFFECTS ON HIGHER PLANTS

Sterol biosynthesis inhibition by drugs has been studied extensively in animals tissues (Schroepfer 1981) and in microorganisms (Oehlschlager et al. 1980; Leroux 1981). However, very little work has been done with higher plants. Triarimol and ancymidol (a plant growth retardant), both known to interfere with ergosterol biosynthesis in fungi (Ragsdale 1975), retarded the growth of Phaseolus vulgaris L. cv. Contender seedlings but did not noticeably affect the qualitative and quantitative distribution of the main sterols present (Shive and Sisler 1976). Moreover, Compactin®, a potent inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase, a key enzyme of sterol biosynthesis, also inhibited sterol biosynthesis in suspension cultures of sycamore (Ryder and Goad 1980).

The SIF, fenarimol, strongly inhibited the biosynthesis of Δ^5-sterols in bramble cells (Rubus fructicosus L.) resulting in an accumulation of 14α-methyl-Δ^8-sterols: obtusifoliol and 14α-methyl-5α-ergosta-8,24-dien-3β-ol (Schmitt and Benveniste (1979). Schmitt et al. (1982) reported that the main target of this drug was 14α-methyl-sterol-14α-methyl-demethylase. When the concentration of

fenarimol in the culture medium was more that 20 uM, the growth rate increased but remained noticeably lower than that of the control cells. As soon as the cells reached the stationary phase, they became brownish and died while the control cells survived up to two weeks in this state without displaying necrotic symptoms . The sterol pattern obtained was not stable and the relative percentage of the 14α-methyl-Δ^8-sterols decreased gradually. They also found that 25-aza-cycloartanol, tridemorph, 15-azasterol and AY 9944 inhibited the following enzymes, respectively: S-adenosyl methionine-cycloartenol-C-24-methyltransferase, cycloeucalenol-obtusifoliol isomerase, $\Delta^{8,14}$-sterol-Δ^{14} reductase and $\Delta^8 \rightarrow \Delta^7$-sterol isomerase. In most cases, the major sterol sitosterol of control cells disappeared to be replaced by new sterols (Schmitt et al. 1982). Chang et al. (1977) suggested that 14α-methyl-Δ^8-sterols such as lanosterol could be deleterious to membranes. This hypothesis could apply to obtusifoliol which is the major sterol from bramble cells treated with fenarimol (Schmitt et al. 1982). Thus, the toxicity of the drug may be explained by its effect on sterol biosynthesis. Because these SIF's act systemically, antigibberellin activity in plants may be an important consideration in their use as fungicides. Like sterol biosynthesis, the initial stages of gibberellin

synthesis involve a portion of the isoprenoid pathway. Many of the reactions, primarily cyclizations and oxidations that occur in the later stages of sterol synthesis, are similar to those of gibberellin biosynthesis (Ragsdale 1977; Sisler and Ragsdale 1977).

Recent experiments suggested that fenarimol as well as ancymidol and miconazole (van den Bossche et al. 1978), could interact with some species of cytochrome P-450, especially those involved in sterol biosynthesis (Ohba et al. 1978), gibberellin biosynthesis (Coolbaugh et al. 1978) and possibly others. Hence, fenarimol toxicity could result not only from its effect on sterol biosynthesis and consequently on membrane structure and function but from its effect on vital metabolic pathways involving cytochrome P-450 such as gibberellin biosynthesis (Schmitt et al. 1982).

Bladocha and Benveniste (1983) observed that in the roots of maize (Zea mays L. cv. LG 11) seedlings treated with the systemic fungicide, tridemorph, for 3 to 4 weeks from the onset of germination, very few Δ^5-sterols, the major sterols present in the control, were detected in the treated plants, and 9β,19-cyclopropyl sterols accumulated dramatically in both roots and leaves. In addition, Δ^8-sterols were also found when low concentrations of the drug were used

(Bladocha and Benveniste (1983). They also reported that cycloeucalenol-obtusifoliol isomerase, an enzyme opening the cyclopropane ring of cyclopropyl sterols, was strongly inhibited and that the drug diffused readily from the roots to the whole plant to reach its enzymatic targets in most of the leaf cells. Schmitt et al. (1981) also showed that in bramble cells tridemorph effectively inhibits the enzyme cycloeucalenol-obtusifoliol isomerase.

Growth inhibition was observed by Hosokawa (1984) in carrot (Daucus carota cv. Danvers), tobacco (Nicotiana tabacum cv. Sulfur) and soybean (Glycine max cv. Acme) cultures when treated separately with triarimol, tridemorph or triparanol. There was a total sterol increase in triarimol treated cultures of soybean, but a decrease in the cultures of carrot and tobacco. Total sterol increased in tridemorph treated carrot and soybean cultures, but decreased in tobacco cultures. When treated with triparanol, total sterol increased in carrot, tobacco and soybean cultures.

Hosokawa (1984) also determined that the sterol compositions of carrot, tobacco and soybean cultures were changed when treated with each of these three fungicides. There was a tremendous reduction of Δ^5-sterols normally present in the control cultures together with an increase in

the level of cyclopropyl sterols and/or methyl sterols in most treatments.

Triarimol inhibited C-14 demethylation resulting in the accumulation of sterols such as 14α-methyl-5α-ergost-8-en-3β-ol, obtusifoliol and 14α-methyl-5α-(24R)-stigmast-8-en-3β-ol supporting the observation that triarimol is an effective inhibitor of the 14α-demethylase in carrot, tobacco and soybean cultures (Hosokawa 1984). Triarimol also inhibited the second alkylation at C-24 in these cell cultures.

Hosokawa (1984) observed that tridemorph inhibited an enzyme involved in the opening of the 9β,19-cyclopropane ring. The sterols, cycloeucalenol and obtusifoliol, occurred in carrot, tobacco and soybean cultures, but obtusifoliol was not present in tridemorph treated cultures suggesting that tridemorph may inhibit the cycloeucalenol-obtusifoliol isomerase in carrot, tobacco and soybean cultures, resulting in the accumulation of cyclopropyl sterols. Tridemorph also inhibited the second alkylation at C-24 in the side chain (Hosokawa 1984). The sterol 14α-methyl-5α-ergost-8-en-3β-ol accumulated in both tridemorph and triarimol treated soybean cultures. Because triarimol is an effective inhibitor of C-14 demethylation, this indicated that tridemorph may also be an inhibitor of the

14α-demethylase in soybean cultures. A somewhat minor effect of tridemorph was observed by Hosokawa on the reduction of the $\Delta^{24(28)}$-bond of $\Delta^{5,24(28)}$-sterols in carrot and tobacco cultures.

Triparanol inhibited C-14 demethylation as well as the second alkylation at C-24 in carrot and tobacco cultures (Hosokawa 1984). There was an accumulation of the sterol, cycloeucalenol, and an absence of obtusifoliol indicating that triparanol may have inhibited the cycloeucalenol-obtusifoliol isomerase in these cultures. Hosokawa noted that where the concentrations of campesterol and stigmasterol were decreased in soybean cultures treated with triparanol, the concentration of sitosterol increased. It was suggested that triparanol inhibited the introduction of the Δ^{22}-bond required in the synthesis of stigmasterol. The inhibition of stigmasterol synthesis may account for the increase in sitosterol.

Triadimefon and triadimenol reduced growth of coleoptiles, primary leaves and roots of barley (Hordeum vulgare cv. Firlbecks Union) seedlings when grown for 7 days in petri dishes in the dark (Buchenauer and Rohner 1981). Both compounds also retarded the elongation of shoots of tomato and cotton plants while simultaneous application of GA3 nullified the retardation. In addition, extracts of

treated shoot tissue of 10 to 12-day-old barley plants contained substantially lower gibberellin-like activity than control shoots. Both triadimefon and triadimenol interfered with sterol metabolism of shoots of barley seedlings when compared to control plants, resulting in lower levels and altered proportion of C-4,4-desmethyl sterols (Buchenauer and Rohner 1981). Levels of stigmasterol and β-sitosterol were greatly reduced in extracts of treated tissue where triadimefon proved to be more active in reducing C-4-desmethyl sterol concentrations than triadimenol. Incorporation studies by Buchenauer and Rohner (1981) further indicated accumulation of sterol compounds possessing C-4 methyl and C-14-methyl groups. Nuarimol, fenarimol and imazalil interfered with sterol biosynthesis in primary leaves of barley seedlings in a way similar to triadimefon and triadimenol (Buchenauer 1979).

Reduction of C-4-desmethyl sterols may also affect structure and function of membranes culminating in decreased growth rate of plants. Douglas and Paleg (1978) thought that Amo-1618-induced inhibition of sterol biosynthesis in tobacco (Nicotiana tabacum cv. Turkish Samson) seedlings may cause reduced development and abnormal structure of membranes resulting in lower gibberellin levels (Cooke and Saunders 1975).

The altered relative proportions of C-4-desmethyl sterols determined by Buchenauer and Rohner (1981) in shoots of triadimefon and triadimenol-treated barley seedings may also incite physiological effects. For example, it was previously reported that changes in ratios of stigmasterol to sitosterol affected growth and developmental processes (Bush and Grunwald 1973; Geuns and Vendring 1974). In addition, the accumulation of sterol components possessing C-4 and C-14-methyl groups may influence membrane structure and function.

THE DISEASE: APPLE SCAB

Apple scab, caused by the fungus, *Venturia inaequalis* (Cke.) Wint., is most severe in the cool, moist, apple-growing regions of the world. Its primary effect is the reduction of quality of fruit; however, it also reduces fruit size and the length of time infected fruit can be kept in storage. Infection of the young developing fruit is generally severe enough to cause premature drop, or leave

the fruit misshapen, and often cracked. Later infections are usually less severe and superficial, but reduce the market appeal of the fruit because of its scabby appearance. Severe leaf infections result in reduction of the functioning leaf surface, defoliation and poor fruit bud development for the next year's crop.

As described by Agrios (1978), first symptoms appear as light, somewhat olive-colored irregular spots on the under surface of sepals or young leaves of the flower buds. The lesions subsequently become olive green with a velvety grayish-dark surface and more circular in appearance. Later, the velvety surface disappears, and the lesions appear metallic black in color and may be slightly raised. Lesions present on leaves that have already unfolded are generally on the upper surface of the leaves. Severity of infection varies as a result of the number of lesions per leaf which may remain distinct or coalesce.

Fruit infections appear as distinct, almost circular scab lesions which initially are velvety and olive green, subsequently becoming darker, scabby and sometimes cracked. The cuticle of the fruit is ruptured at the margin of the lesions. Late season infections result in small lesions which may not be visible at harvest, but develop into dark scab spots during storage.

The mycelium of V. inaequalis is initially light in color, but later turns brown in the host tissues (Agrios 1978). In young leaf lesions the mycelium develops radially in branched ribbons of hyphae; however, in older leaves and on fruit the mycelial strands are compact, thick, and layered. The mycelium is located only between the cuticle and the epidermal cells of living tissues and produces short, erect, brownish conidiophores which successively give rise to several, one-or-two-celled, reddish brown conidia. In dead leaves the mycelium grows through the leaf tissues. Fertilization occurs by means of ascogonia and antheridia and perithecia form. The perithecia, when mature, are dark brown to black with a slight beak and an ostiole. Within the perithecium 50 to 100 asci are formed, normally containing eight ascospores with each ascospore consisting of two cells of unequal size, which are hyaline at first, changing to brown when mature (Agrios 1978).

Early in the spring the asci, which are elongated sacs located within the perithecia, appear to contain unstructured cytoplasm, but as time passes, the cytoplasm aggregates and is converted into mature ascospores. The first mature ascospores generally are produced at approximately the green tip stage of bud development (Arneson et al. 1978). The peak occurs at about full

bloom, and the last ascospores mature about two weeks after petal fall.

Asci containing mature ascospores accumulate in the perithecia until sufficient moisture is present which triggers an active discharge mechanism. Approximately two hours of wetness in the field is required for discharge (Arneson et al. 1978). When this occurs, the ascospores are forcibly discharged from the perithecia, and dispersed by wind to susceptible sites such as exposed green tissue in newly opened buds, newly expanding leaves, or developing fruit.

Primary infection by ascospores only occurs in water droplets. Therefore, if infection is to take place, susceptible sites must remain wet long enough for spore germination and infection. The time required for infection depends upon temperature with a longer period necessary at lower than at higher temperatures (Arneson et al. 1978).

Once infection takes place, an incubation period is necessary before the lesion becomes visible and conidia (secondary spores) are produced. In addition, the length of the incubation period depends on temperature and varies from nine days at the optimum temperature (19°C) to greater than 18 days at low temperatures (Arneson et al. 1978).

Secondary infection is characterized by hundreds of thousands of conidia produced from each lesion throughout its rather long period of fertility. Dispersal is by splashing rain and, to a lesser extent, by wind. Like ascospores, conidia require free water for germination and infection, but the length of the wetting period is shorter. Generally, rain is necessary for infection to occur; however, when temperatures are high enough so that infection can occur in less than six hours, a heavy dew can provide a sufficient wetting period for infection (Arneson et al. 1978).

Under favorable conditions, the disease level can rapidly increase due to repeated cycles of secondary infection by conidia. If primary infections occur very early, both conidia and ascospores will be present in the early part of the season. If primary infections can be prevented, little or no secondary infection will occur once the supply of ascospores is exhausted (Arneson et al. 1978). Generally, only young, expanding leaves are susceptible, and once the terminal bud is set, no new leaf infections occur. However, in late summer and early fall, there is a rapid proliferation of tiny new lesions in close proximity to older lesions. These numerous new lesions have little effect on the current season's crop, but contribute

significantly to the overwintering inoculum. The apple scab disease cycle is summarized in Figure 1.3.

INFECTION AND COLONIZATION

In an ultrastructural study of host-parasite interaction in the apple scab disease, Maeda (1970) demonstrated that a membrane-bound "infection sac" was present in appressoria of Spilocaea pomi, the imperfect state of V. inaequalis, and in contact with the host cuticle at the site of penetration. Corlett and Chong (1977) observed that the appressorium, after penetrating the apple leaf cuticle, possessed a two layered wall, an electron-dense outer layer and an electron-transparent inner layer. An electron-dense outer layer bordered the pore of the appressorium. The penetration hypha was derived from the infection sac within the appressorium (Corlett and Chong 1977). Furthermore, the sac appeared to be the first component of the fungal protoplast to contact the cuticle. Maeda (1970) suggested that the sac might function as a reservoir of fungal products, including

enzymes, necessary for penetration. Based on the discovery of the infection sac, Nicholson et al. (1972) detected histochemically that esterase activity was in V. inaequalis at the spore apex prior to germination. Activity disappeared from the elongating germ tube, and then reappeared in the appressorium. Morphological development and the pattern of esterase activity were similar for all isolates studied.

It was suggested that the activity in the apex of the ungerminated spore was to facilitate germination by a rupturing of the spore wall components, since the protoplast of the ungerminated spore of V. inaequalis is enclosed by a rigid wall (Maeda 1970) which must be ruptured in the germination process. The conidium is delimited by a two-layer transverse septum, and prior to conidium secession, a new two-layered inner wall is laid down around the entire conidiogenous cell adjacent to the plasma membrane (Corlett et al. 1976). The apical region of the new inner wall proliferates beyond the annellation scar left by the seceded conidium, subsequently producing another conidium. Bracker (1971) demonstrated vesicle accumulation at sites of germination in sporangiospores of Gibberella persicaria and suggested that germination, like hyphal tip growth, occurs by vesicular additions at the site of cell expansion (Grove

and Bracker 1970). Maeda (1970) observed apical vesicles in germinating conidia of V. inaequalis. Localization of the cytoplasm or association with vesicles or other cytoplasmic components, could aid in rupturing the wall layers and thus facilitate emergence of the germ tube. In addition, activity in the appressorium may represent the localization of hydrolytic enzymes necessary for host penetration (Nicholson et al. 1972).

Maeda (1970) demonstrated the degradation of apple cuticle at the site of penetration by V. inaequalis and suggested that enzymic dissolution of the cuticle had occurred. Hence, the appressorial "infection sac" of V. inaequalis could be a site of localization of cuticle-dissolving enzymes, possibly of the cutin esterase type, since it or its content contact the cuticle throughout penetration. Corlett et al. (1976) also reported that the fungus enzymatically degraded the cuticle and epidermal cell wall. The epidermal cells and the palisade mesophyll cells below a sporulating lesion (susceptible reaction) were destroyed or severely altered.

The chemical nature of the interface in which the fungus exists beneath the cuticle and above the epidermal cell walls is not clearly defined (Williams and Kuc 1969). Probable components include proteins, lipids, pectic

substances, hemicelluloses and cellulose. The fungus can obtain nutrients in this stratum by solubilizing components, by absorbing nutrients diffusing into the stratum from underlying cells, or by a combination of both. Franke (1961) demonstrated the existence of cytoplasmic links (called ectodesmata) which extend through outer epidermal walls. These in, Malus, could provide intimate contact between host cytoplasm and fungus and serve as a bridge with underlying host tissue.

Extracellular fungal melanoprotein isolated from culture filtrates of V. inaequalis had biological activity when injected into test plants (Hignett and Kirkham 1967; Kirkham and Hignett 1966). Solute transport in xylem of healthy plants was interrupted after injection of melanoproteins into the petioles, and tracer solutes were contained within vascular tissues in test leaves. Similar distribution patterns of tracer solutes were observed in leaves after inoculation with the fungus. The patterns changed as the lesions matured, with tracer compounds accumulating at the lesion sites. The percentage of leaf coverage by lesions was increased by application of melanoprotein with spore inoculum to susceptible plants. These effects were not duplicated by numerous other proteins. Hignett and Kirkham (1967) suggested that the pathogen redirects host metabolism

in favor of the developing lesion by keeping nutrients within the leaf. Thus, a mechanism for susceptibility is suggested, explaining the availability of nutrients to the fungus in at least the latter stages of its development. Injection of melanoproteins at concentrations higher than that required to localize solutes in the leaf vein caused cupping of the leaves followed by interveinal desiccation and necrosis.

Raa (1968) found that both the nonsporulating and avirulent isolate and the virulent isolate of V. inaequalis formed toxins, but the toxins of the former acted less specifically than those of the latter. This suggested that the collapse of cells in resistant hosts triggers a series of metabolic events which produce metabolites that inhibit extracellular enzymes produced by the fungus and thereby inhibit its growth.

Hignett et al. (1979) found that ribonuclease, deoxyribonuclease, acid phosphatase and phenoloxidase were detected in preparations of extracellular melanoprotein isolated from cultures of V. inaequalis. The high initial levels of activity declined within a few days of inoculation, to a relatively constant level. About six times as much compound was released by conidia germinating in a medium enriched with wood extract than was released in

basal medium. After 5 days incubation, the rate of production of enzymically active melanoprotein reflected the rate of growth of the fungus. Isolated melanoprotein stored at 4°C for 3 months showed up to 190% more hydrolase activity than was measured originally, but longer storage caused a subsequent decrease in activity. Only phenoloxidase decreased continuously during storage.

Thus, loss of virulence during storage is associated with decreased ribonuclease, deoxyribonuclease and acid phosphatase production in subsequent cultures, and an increase in the specific activity of phenoloxidase (Hignett et al. 1979). Phenoloxidase has been implicated in general disease resistance (Stahmann and Demorest 1973). The role of phenoloxidase in disease is usually correlated with the generation of phenolic oxidation products toxic to the pathogen. Production of extracellular phenoloxidase by V. inaequalis would tend to enhance this effect, thereby inhibiting its growth. The fungus would also be deprived of phenolic substrates, which would hinder its capacity to synthesize melanoprotein which is active in the apple scab disease (Hignett 1973).

RESISTANCE

A significant problem in the study of disease resistance is the determination of the stage of pathogen development at which it is first affected by the host's resistance mechanisms. This is important especially in investigations built upon the hypothesis that stress compounds or phytoalexins constitute the primary resistance response of the host. If resistance is to be attributed solely to phytoalexins, their appearance in the host should coincide with an adverse effect on the pathogen.

The resistance of apple to V. inaequalis has been attributed to phytoalexin-like oxidation products of phloridzin (the major phenolic glycoside in apple) or its aglucone, phloretin (Noveroske et al. 1964). In several studies phloridzin, 3-hydroxy-phloridzin, phloretin, and various derivatives of these compounds were shown to be inhibitory to V. inaequalis (Barnes and Williams 1961). Barnes and Williams (1961) demonstrated that phloridzin and phloretic acid stimulated, and that phloretin inhibited, growth of V. inaequalis at concentrations between 5 x 10^{-3} and 10^{-2}. Hunter (1975) later showed that phloretin (2 x 10^{-3}M) as well as phloridzin and phloretic acid stimulated

the growth of V. inaequalis. However, these reports were based on the effect of these compounds on in vitro growth of the pathogen and their location in the cell and the quantities available in vivo for conversion to inhibitory oxidation products (either by the host or pathogen) is unknown. Therefore, the importance of phloridzin and phloretin to apple scab resistance is questionable (Nicholson et al. 1977).

Phenolic oxidation in apple scab results in the characteristic browning of the hypersensitive reaction and has been suggested to be responsible for inhibition of V. inaequalis (Kuc 1972). However, Nicholson et al. (1977) found that for each fungus race studied, cellular browning of the host was not observed until 8, 12 and 36 hours before symptoms were observed macroscopically. Their histological data indicated that inhibition of V. inaequalis occurs close to the time of penetration and prior to 24 hours after inoculation. This would require a change in phloridzin and/or phloretin at an extremely early time in disease development if changes in the levels of these compounds or their oxidation products primarily are responsible for inhibition of the pathogen. However, they found no substantial changes in either phloridzin or phloretin in the hypersensitive response prior to 24 hours after inoculation.

In addition, host cell granulation which precedes cell browning in the hypersensitive response (Nicholson et al. 1973) did not occur until after cessation of fungal development. Therefore, they suggested that inhibition of the fungus occurs just after penetration of the cuticle and that neither phloridzin and phloretin nor their oxidation products represent the primary means of host resistance in the hypersensitive response. These compounds may, however, represent a secondary source of resistance since they and/or their oxidation products have been shown to inhibit in vitro growth of V. inaequalis (Noveroske et al. 1964).

Phenolics found in apple leaves, fruit, bark and roots include phloridzin, leucoanthocyanins, epicatechins, catechins, quercetin, cyanidin, 3-hydroxyphloridzin, p-coumarylglucose, kaempferol, p-coumarylquinic acid, isochlorogenic acid and chlorogenic acid (Williams and Kuc 1969). Phloridzin and chlorogenic acid are the principle phenolics of leaves and fruits, respectively. However, aside from metabolic changes occurring to phloridzin, little is known concerning the qualitative or quantitative changes of other phenolic compounds following inoculation.

Two types of scab resistance in Malus are available for use by the breeder (Williams and Kuc 1969). These are quantitative or multiple factor and qualitative or single

major factor determination. The former type is sensitive to the effect of environment, host conditioning or both, while the latter is expressed under all conditions, usually as single dominant genes. Both types may condition field immunity in that no macroscopic evidence of infection is present, or resistance may be expressed as a reduced number and size of sporulating lesions.

The low to moderate level of scab resistance exhibited by many commercial varieties (Kotecov 1966) is apparently of the multiple factor type. This resistance usually results in fewer and smaller sporulating lesions when mass inoculum is used, and in the formation of nonsporulating necrotic flecks when selected single isolates are used (Williams and Kuc 1969). The major problem encountered in utilizing the resistance present in existing commercial varieties in breeding programs for the development of scab immune varieties is the necessity of growing and screening large numbers of seedlings to secure a few showing the desired level of immunity. However, it was suggested that multiple factor resistance may be more valuable in the long run than the relatively easy-to-use qualitative type.

Although several apple cultivars are available that are resistant to apple scab, and more are becoming available, the fruit quality has not yet matched the popular commercial

cultivars. Therefore, resistant cultivars are not yet an acceptable alternative to fungicide sprays for apple scab control (Arneson et al. 1978). However, the commonly grown apple cultivars do vary in their degree of susceptibility to apple scab. Generally, fungicide recommendations have been developed for the most susceptible cultivars. Therefore, lower rates or fewer fungicide applications are necessary for adequate control when working with the less susceptible varieties.

If resistance to a fungicide being used occurs in the fungus population, that mutant will be able to grow and sporulate while the susceptible "wild-type" will be inhibited (Arneson et al. 1978). Therefore, a "selection pressure" is being imposed on the fungus population, and continued use of that fungicide will cause the proportion of resistant individuals in the population to increase to the point where that fungicide will no longer effectively control the disease. Resistant mutants are far more likely to occur with the systemic fungicides that have highly specific biochemical modes of action than with the conventional protectant fungicides that generally interfere with a broad range of biological functions in the fungus.

OBJECTIVES

Sterol-inhibiting fungicides are extremely important in controlling plant diseases and are often applied throughout a growing season on numerous field crops, ornamentals and fruit crops. However, relatively little research has been done on the effects of these fungicides on sterol metabolism and their uptake and residual activity in higher plants. The purpose of the present study is to determine the effects of several SIFs on the sterol and fatty acid concentrations of apple. This plant was chosen because any significant change in its sterol metabolism can have a profound effect on the aesthetic value and quality of the fruit.

An important question is concerning the activity of these sterol-inhibiting chemicals to their relationship to poor fruit shape and firmness. This is a problem which has been observed and which might be the result of the fungicide's quantitative or qualitative alteration of certain metabolites. Therefore, systemicity tests will be conducted to determine the uptake of SIFs and their residual activity in leaves of different apple varieties. Changes in concentrations will be determined following selective extraction, thin layer and gas liquid chromatography. The

presence of specific compounds will be confirmed by GC analysis. Isolates of Venturia inaequalis will be obtained from trees that have never been sprayed with SIFs. An electron microscopic analysis will be made on apple tissues to determine any ultrastructural changes which may occur in the host and in the apple scab pathogen (V. inaequalis) due to the activity of the SIFs. The apple scab fungus was chosen because it is of major economic importance in apple production and is relatively difficult to control with other fungicides.

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Table 1.1: Inhibitors of ergosterol biosynthesis (Siegel 1981)

Chemical Class	Common Name	Other Names	Reference
Triazole	Triadimefon	Bay MEB 6447; Bayleton	Buchenauer, H. 1977
	Triadimenol	Bay KWG 0519; Baytan	Buchenauer, H. 1977 Gasztonyi, M. et al. 1979
	Bitertanol	Bay KWG 0599; Baycor	Kraus, P. 1979
	Fluotrimazole	Bay 6683; Persulon	Buchenauer, H. 1978
	Diclobutrazol[a]	ICI 296; Vigil	Buchenauer, H. 1978
	Propiconazol[a]	Tilt; CGA 64250	
	Etaconazole[b]	Vangard; CGA 64251	
Imidazole	Imazalil	Fungaflor	Buchenauer, H. 1977 Leroux, P. et al. 1979
	Miconazole[b]		Henry, J. M. et al. 1979 van den Bossche, H. et al. 1978
	Clotrimazole[b]	Bay L 5097; Canestan	Buchenauer, H. 1978
	Prochloraz	BTZ 40502	Leroux, P. et al. 1979 Pappas, A. C. et al. 1979
	Fenapanil[a]	RH 2161; Sisthane	
	XE 326[b]		
	Dodecylimidazole[b]		Henry, J. M. et al. 1979
Pyrimidine	Triarimol[b]	EL-273	Ragsdale, N. N. 1975 Ragsdale, N. N. et al. 1973

Table 1.1. (Continued)

Chemical Class	Common Name	Other Names	Reference
	Fenarimol	EL-222; Rubigan Bloc	Buchenauer, H. 1977 de Waard, M. A. et al. 1977
	Nuarimol	EL-228; Trimidal	Buchenauer, H. 1977
Morpholine	Tridemorph	Calixin	Kato, T. et al. 1980 Kerkenaar, A. et al. 1979
	Fenpropemorph[a]	BAS 42100; Corbel	
Piperazine	Triforine	Cela W-524; Fungi-nex; Saprol	Sherald, J. L. et al. 1973 & 1975
Pyridyl	Buthiobate	S-1358; Denmert	Kato, T. et al. 1976 & 1980
Misc.	Azasterol[b]	A 25822 B	Woloshuk, C. P. et al. 1979

[a]Suspected inhibitors of sterol biosynthesis based on structural similarities with known inhibitors.

[b]Nonagricultural use.

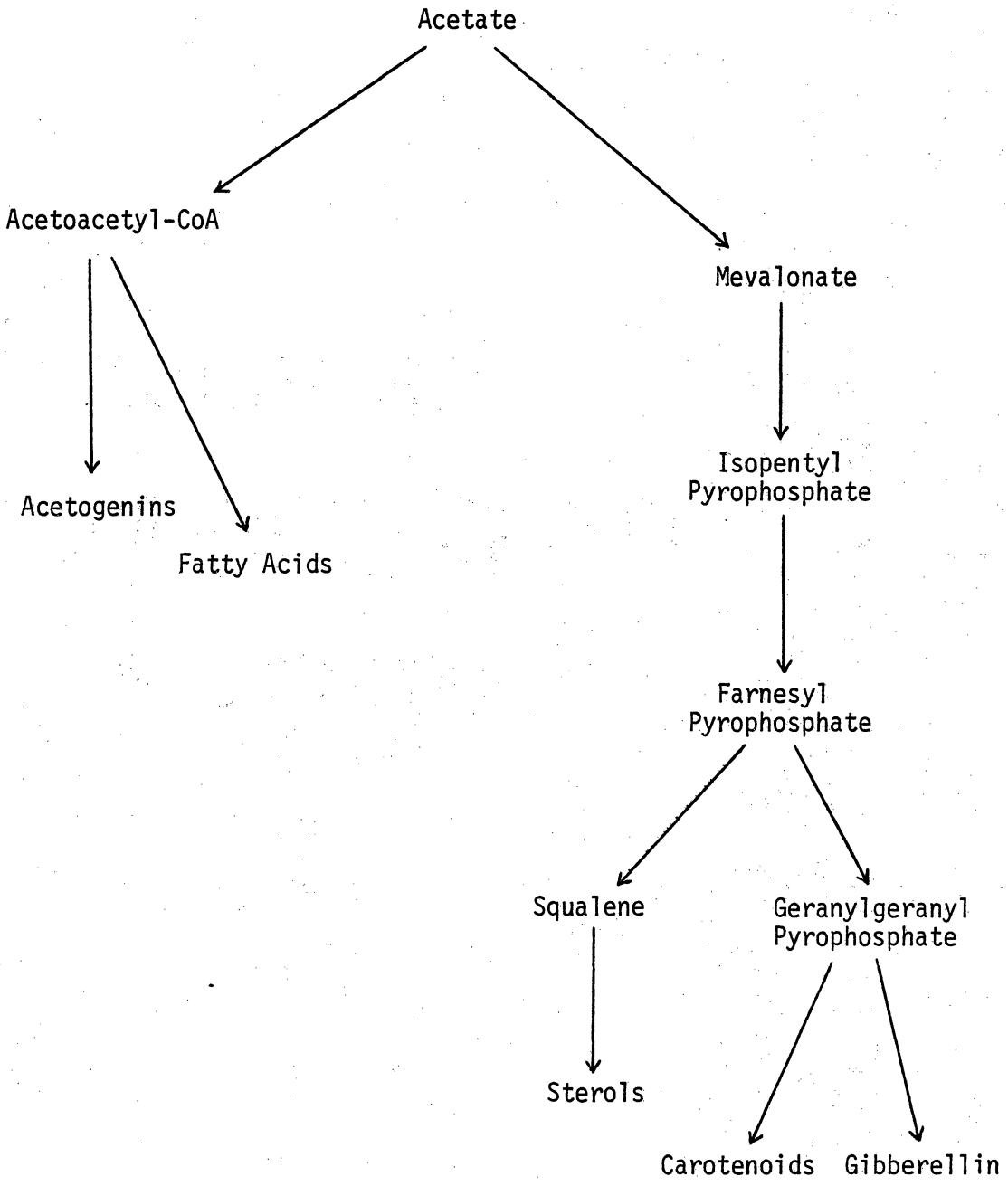

Fig. 1.1. Scheme of lipid biosynthesis (Ragsdale 1977)

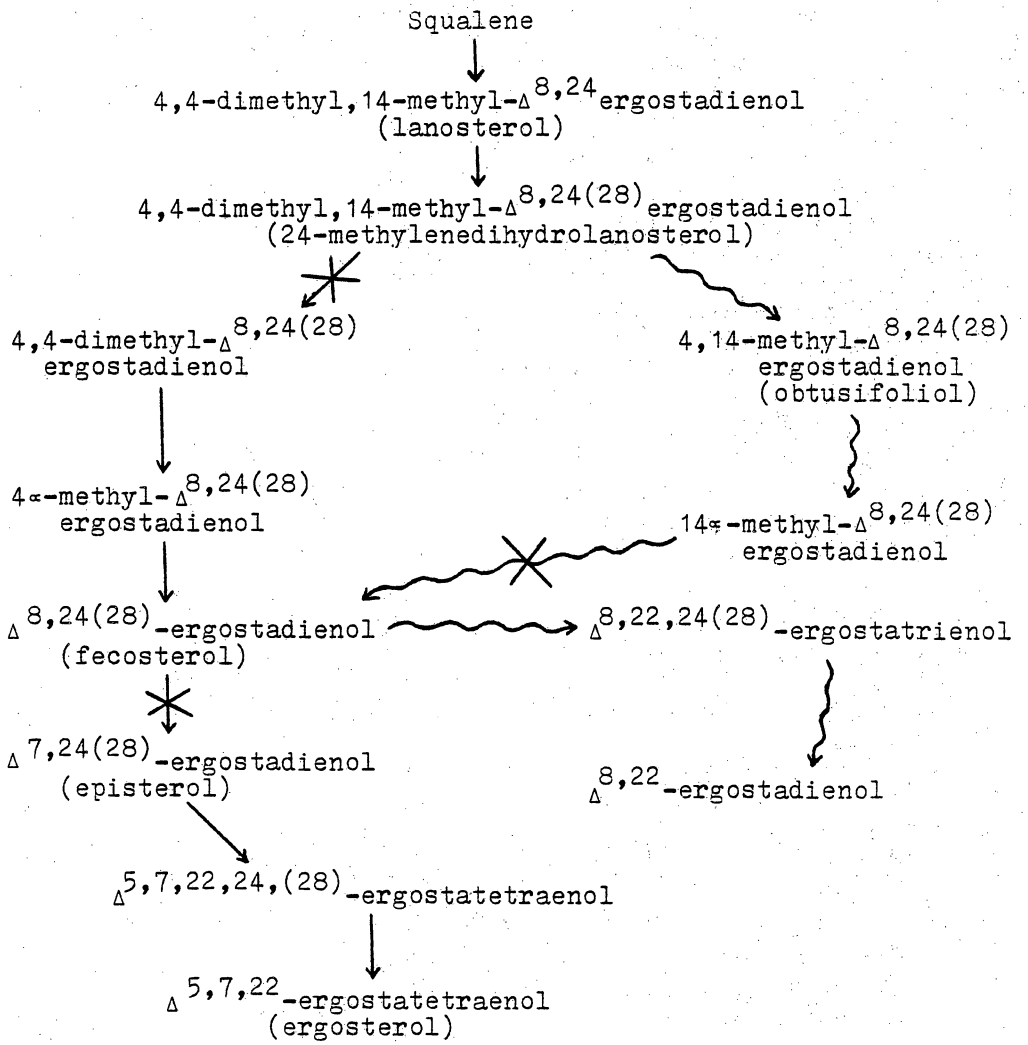

Fig. 1.2. Scheme of sterol biosynthesis. Straight arrows represent normal sterol biosynthetic pathway; wavy arrows represent alternate or abnormal pathways reduced by fungicides. X's indicate major points of inhibition by ergosterol-inhibition fungicides (Siegel 1981)

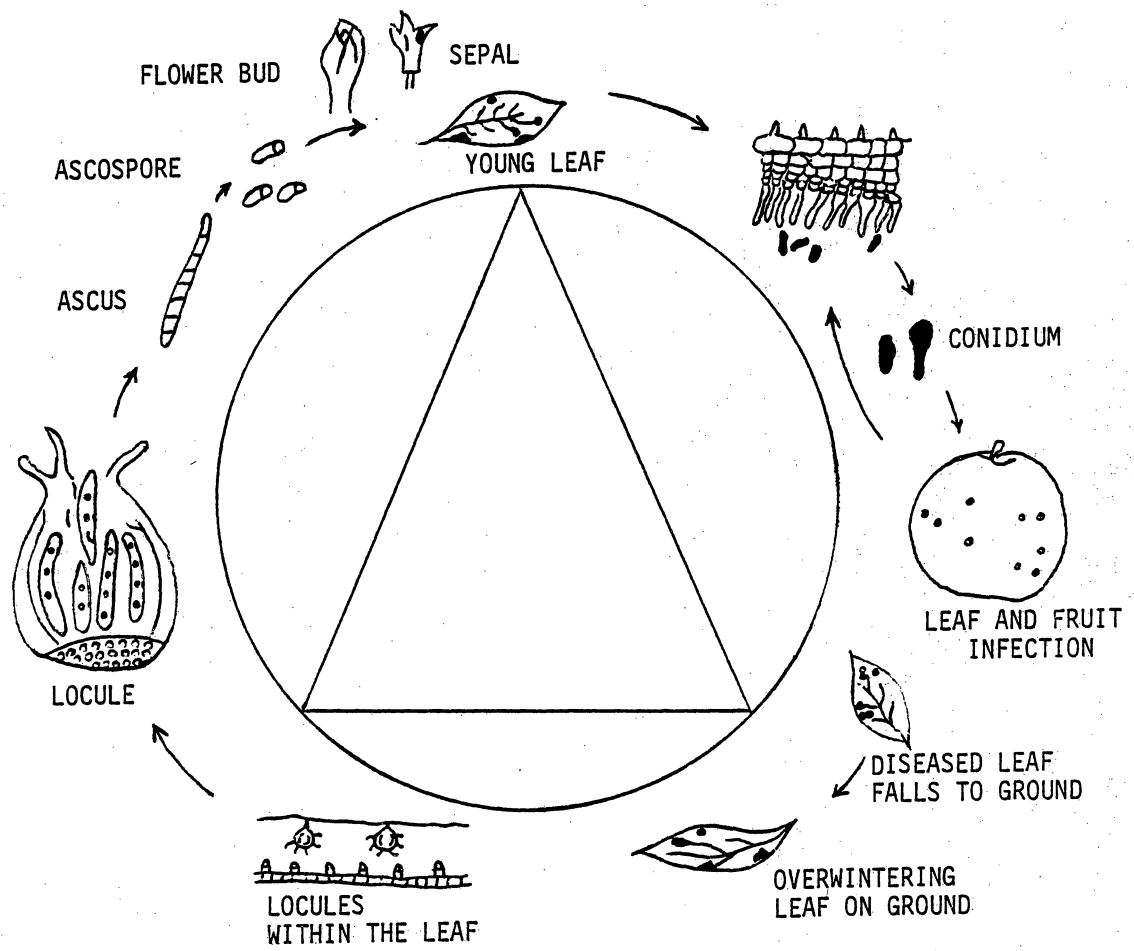

Figure 1.3. Disease cycle of apple scab (Arneson et al. 1978)

CHAPTER 2

THE INFLUENCE OF STEROL-INHIBITING FUNGICIDES ON THE FREE STEROL COMPOSITION IN APPLES

ABSTRACT

The effects of several sterol-inhibiting fungicides (SIFs) on the free sterol content of apple leaves of Red Delicious and Jonathan cultivars were examined over a 2 year period. Trees were treated in the morning and evening and samples collected after 24 and 72 hours. Sitosterol and total sterol concentrations were greatly reduced in etaconazole and metiram-treated Red Delicious leaves 24 hours after treatment in test 1-May, 1982. In addition, in test 1 campesterol of metiram-treated leaves and stigmasterol of etaconazole, fenarimol, bitertanol and metiram-treated leaves decreased significantly. Reduced sterol concentrations were not evident in either test 2 or test 3-1982, but stigmasterol, sitosterol and total free sterol concentrations of fenarimol and metiram-treated leaves decreased significantly in test 4-1983 24 hours after treatment. Campesterol levels of metiram-treated leaves was also reduced. In test 5-May, 1983 campesterol concentrations of Jonathan leaves treated with fenarimol and triadimefon were significantly higher after 24 hours. Although there may be an effect by fenarimol and triadimefon on campesterol in test 5-1983, this difference appeared to

be cultivar related and not time dependent. SIFs may have an effect on the free sterol concentrations of apple leaves 24 hours after treatment, but the levels of free sterol appeared to return to normal by 72 hours after treatment in both Red Delicious and Jonathan leaves, with the exception of sitosterol and total sterol concentrations of triadimefon-treated leaves in test 5. Generally, morning versus evening application had no effect on the free sterol levels of apple leaves. No consistent differences existed among the SIFs and the non-sterol-inhibiting fungicide, metiram.

INTRODUCTION

A recent innovation in the use of chemicals to control plant pathogens is the introduction of compounds that inhibit the biosynthesis of ergosterol, which is a major fungal component and possibly involved in membrane function. Almost all sterol-inhibiting fungicides (SIFs) exhibit varying degrees of systemicity, and are useful in controlling a wide range of fungal diseases. There are several unique characteristics which differentiate SIFs from other systemic agents: they are the largest group of fungicides with the same specific mode of action (Siegel 1981); they are a diverse group of compounds with respect to chemical structures; and, reduced sensitivity to SIFs in field isolates of Venturia inaequalis have only been recently reported (Stanis and Jones 1985).

Schmitt and Benveniste (1979) demonstrated that the SIF, fenarimol, strongly inhibited the biosynthesis of Δ^5-sterols in bramble cells (Rubus fructicosus). Moreover, Schmitt et al. (1982) reported that in most cases, the major sterol, sitosterol, disappeared to be replaced by new sterols following the use of 25-azacycloartanol, tridemorph, 15-azasterol and AY9944.

Buchenauer and Rohner (1981) reported that both triadimefon and triadimenol interfered with sterol metabolism of shoots of barley seedlings. Levels of stigmasterol and β-sitosterol were greatly reduced in extracts of treated tissue where, triadimefon proved to be more active in the reduction of C-4-desmethyl sterol concentrations than triadimenol.

Although SIFs are often applied throughout a growing season on numerous field crops, ornamentals and fruit crops, relatively little work has been done on the effects of SIFs on sterol metabolism in higher plants. The purpose of the present study is to determine the effects of several SIFs on the free sterol (FS) composition of apple leaves in Red Delicious and Jonathan cultivars. Free sterols influence the permeabiltiy characteristics of membranes and a change in permeability may be reflected by a change in the composition or arrangement of membrane lipids, possibly affecting the shape of the fruit. Morning versus evening applications were examined to determine if there was any effect of time of application on the levels of FS. Fungicide application during different times of the year was also studied. Apple trees were chosen because any significant change in its sterol metabolism could have a profound effect on the aesthetic value and the quality of the fruit (Yoder et al. 1982; Yoder et al. 1983a and b).

MATERIALS AND METHODS

Effect of time of application:

During 1982 and 1983 Red Delicious apple trees grown in an orchard near Winchester, VA were treated with the SIFs etaconazole, fenarimol, or bitertanol or with a non-sterol-inhibiting fungicide (NSIF), metiram, in addition to an untreated control. Also in 1983, apples from Jonathan trees grown near Winchester, VA were treated with the SIFs etaconazole, fenarimol and triadimefon. Rates of application and formulations are listed in Table 2.1. Test trees, uniform in age, were spaced differently in each orchard. Treatments were arranged in a randomized complete block design with five single-tree replicates. Fungicides were suspended in water and applied until mist formation occurred by an airblast sprayer driven at 2.0 mi/hr (935 L/ha).

Four different tests were conducted on Red Delicious with leaf samples collected at 24 hours after application in 1982

and at 24 and 72 hours in 1983. In 1982 successive applications were made on the same trees in the morning or evening of May 5 (petal fall, 2nd spray); in the evening of May 13 or morning of May 14 (1st cover, 3rd spray); and in the evening of July 15 or morning of July 16 (5th cover, 7th spray). Applications in 1983 were made in the evening of May 23 or morning of May 24 (2nd cover, 3rd spray). In 1983 two additional tests (Table 2.2) were conducted on Jonathan trees. Collections of samples were made 24 and 72 hours after the applications of treatments. Applications in 1983 were made in the evening of May 24 or morning of May 25 (2nd cover, 5th spray), and in the morning or evening of July 7 (5th cover, 8th spray), respectively. Samples were collected from Red Delicious and Jonathan trees used in season-long disease control and fruit effects evaluations (Yoder et al. 1982; Yoder et al. 1983a and b). All applications were made at 7 AM or 7 PM. Applications were coordinated so that rapid drying would be expected for morning applications and slow-drying for evening applications.

For each treatment/replication fifteen leaves including petioles, exhibiting no signs of disease or phytotoxicity and chosen for uniform size and age, were removed from each tree. Samples were placed in Whirl-Pak bags, quick frozen

in an ethanol-dry ice mixture, kept on ice throughout the collection, and then stored at 0°C until lyophilization.

Collection and analysis of various apple tissues:

Entire leaf samples were lyophilized, ground to pass through a 40 mesh screen, and stored at room temperature until analyzed. At the time of extraction coprostanol (0.01 ug) was added as an internal standard to 200 mg of dried leaf sample.

The tissue was initially extracted with 100 parts by weight of isopropanol and filtered through Whatman #1 paper and the filtrate saved. The extraction was repeated, the two filtrates combined and stored at 0°C.

The remaining tissue residue was further extracted in erlenmyer flasks with 40 ml of chloroform:isopropanol (1:1 by vol.), overnight at room temperature using a rotary shaker (Nichols 1963 and 1964).

The tissue was filtered and all three filtrates combined, taken to dryness using a rotary evaporator and resuspended in 8 ml of chloroform:methanol (2:1 by vol.). The solution was transferred to a preweighed vial and 2.0 ml of 0.88 percent potassium chloride in water was added. The mixture was capped, shaken thoroughly and allowed to settle. The

upper layer was removed and discarded. Three ml of water:methanol (1:1 by vol.) was added and the washing repeated leaving the lipid in the bottom layer (Folch et al. 1957). The lipid was concentrated under N2 at 55°C and total lipid determined gravimetrically. The samples were stored in screw capped vials under nitrogen at 0°C.

Free sterols were separated from the lipid extract using Silica gel G TLC plates and a solvent system of hexane-diethylether-acetic acid (80:20:1 v/v/v). Sterols were identified by cochromatography with sterol standards and visualized under UV light after spraying with dichlorofluoscein.

Free sterols were recovered from the TLC silica gel plates by eluting them with diethyl ether through sintered glass filters. Trimethylsilyl ether (TMS) derivatives of the sterols were formed by adding 30 ul of BSA [N,O-bis(trimethylsilyl)acetamide] (Supelco) and heating at 55°C for 1 hour.

Individual sterols were identified and quantified by gas liquid chromatography (model 2600 Bendix Gas Chromatograph) isothermally at 275°C on a 28.3 cm x 4 mm (ID) glass column packed with 3% SE-30. Detector and injector temperatures were 320° and 275°C, respectively. The carrier gas was N2, set at a flow rate of 65 ml/min. Quantitation was based on

an internal standard of coprostanol and identification of the sterol components compared to authentic sterol standards was expressed in ug/mg dry weight.

Data analysis:

Morning and evening applications were examined alone and in combination at 24 and 72 hours. One-way anovas were calculated to determine if significant F tests existed. Where the F test was significant , a Duncan's multiple range test at $p \leq 0.05$ was used to determine significant differences between group means.

RESULTS

In test 1 which was conducted early in the season, there were significant decreases in sitosterol and in total sterol concentrations of etaconazole and metiram-treated Red Delicious leaves 24 hours after treatment (Table 2.3). Levels of campesterol in metiram-treated leaves and

stigmasterol in etaconazole, fenarimol, bitertanol and metiram-treated leaves were also reduced. The greatest decrease appeared to occur after treatment with the NSIF, metiram. However, this was not observed in tests 2 or 3-1982. In test 4-1983, stigmasterol, sitosterol and total sterol concentrations of fenarimol and metiram-treated leaves were significantly lower than the control, as well as campesterol of metiram-treated leaves; however, cholesterol levels in these two treatments were higher 24 hours after treatment. No significant differences were observed in the free sterol content of Red Delicious leaves 72 hours after treatment in test 4 (Table 2.4).

After 24 hours, campesterol levels were higher in Jonathan leaves treated with fenarimol and triadimefon when compared to the control in test 5 (Table 2.5). However, no other significant differences were observed in test 5 or in test 6, 24 hours after treatment. In test 5, sitosterol and total sterol of leaves treated with triadimefon increased significantly after 72 hours (Table 2.6). No significant changes were found in the FS composition in test 6, 72 hours after treatment (Table 2.6).

Only in test 1 was there a difference related to time of fungicide application. Stigmasterol was significantly lower in Red Delicious leaves for all treatments regardless of

time of applications. This was not observed in any of the other tests in 1982, nor did time of application affect sterol levels in 1983 (tests 5 and 6) in Jonathan leaves.

DISCUSSION

The SIFs etaconazole, fenarimol, bitertanol and triadimefon have been shown to be potent inhibitors of 14α-demethylation in ergosterol biosynthesis (Ebert et al. 1983; Gadher et al. 1983; and Kraus 1981), and are used in this study to examine their effects on sterol composition in apples. In test 1 of May, 1982, sitosterol and total sterol concentrations were reduced in etaconazole and metiram-treated Red Delicious leaves after 24 hours. In addition, campesterol of metiram-treated leaves and stigmasterol of etaconazole, fenarimol, bitertanol and metiram-treated leaves also decreased. Likewise, Schmitt and Benveniste (1979) found that fenarimol strongly inhibited the biosynthesis of Δ^5-sterols in bramble cells (Rubus fructicosus) resulting in an accumulation of 14α-methyl-

Δ^8-sterols: obtusifoliol and 14α-methyl-5α-ergosta-8,24-dien-3β-ol. Bladocha and Benveniste (1983) observed that very few Δ^5-sterols, the major sterols of the control, were detected in the roots of tridemorph-treated maize seedlings, and that 9β,19-cyclopropyl sterols accumulated dramatically in both roots and leaves. Tridemorph, a SIF, inhibits $\Delta^8 \rightarrow \Delta^7$ isomerization and prevents Δ^{14} reduction as another site of action (Kerkenaar et al. 1981). Hosokawa (1984) also determined that the sterol compositions of carrot, tobacco and soybean cultures were changed when treated with the 14α-demethylation inhibitors triarimol, triparanol and tridemorph. There was a tremendous reduction of Δ^5-sterols normally present in the control cultures together with an increase in the cyclopropyl sterols and/or methyl sterols in most treatments. Hosokawa (1984) also found a total sterol reduction in triarimol-treated cultures of carrot and tobacco, but an increase in the total sterol content of soybean cultures. Total sterol decreased in tridemorph-treated tobacco cultures, but increased in carrot and soybean cultures. When treated with triparanol, total sterol increased in carrot, tobacco and soybean cultures (Hosokawa 1984).

The reduced sterol concentrations that occurred in test 1 of May 5, 1982, however, were not evident in either test 2 of May 13 and 14, 1982 or test 3 of July 15 and 16, 1982. Moreover, only fenarimol and metiram-treated leaves in test 4 of May 23 and 24, 1983 showed significant reductions in stigmasterol, sitosterol and total sterol concentrations after 24 hours. The reduction in sterol concentrations in Red Delicious leaves may be due to higher metabolic activity during the time of year that the fungicide was applied. When soybean cultures were treated with triparanol (Hosokawa 1984), concentrations of campesterol and stigmasterol decreased, accompanied by an increase in the sitosterol level. In this study the inhibition of stigmasterol synthesis did not cause an increase in the level of sitosterol. Because sitosterol is a C29-sterol with a Δ^5-bond, stigmasterol may be synthesized by the dehydrogenation of sitosterol at C-22 and C-23. If the pathway leading to stigmasterol biosynthesis is blocked, synthesis of sitosterol may occur. Buchenauer and Rohner (1981) determined that the altered relative proportions of C-4-desmethyl sterols in shoots of triadimefon-treated barley seedlings may also incite physiological effects. For example, it has been reported that changes in ratios of stigmasterol to sitosterol were connected with effects on

growth and developmental processes (Bush and Grunwald 1973; Geuns and Vendring 1974). Although some changes existed in the free sterol content of Red Delicious leaves 24 hours after treatment, no significant differences were observed in the free sterol 72 hours after treatment. Regardless of the effect SIFs had on the FS content 24 hours after treatment, the metabolism of the plant appears to return to normal 72 hours after treatment.

The increases observed in campesterol in test 5 may be explained by the fact that this test was conducted on Jonathan leaves, whereas, test 4 of May, 1983 was done with Red Delicious leaves in which campesterol of fenarimol-treated leaves decreased relative to the control instead of increasing. The differences found appear to be cultivar related.

Sitosterol and total sterol concentrations of triadimefon-treated leaves increased significantly after 72 hours in test 5. However, no significant differences in the levels of FS occurred in test 6. Even though these changes in sitosterol and total sterol in Jonathan leaves were observed 72 hours after treatment in test 5, the sterol composition of the plant appeared to be returning to normal after 72 hours as was observed in the Red Delicious cultivar.

Significantly lower stigmasterol levels were found among the treatments in both the morning and evening applications in test 1. No significant differences were observed in the levels of FS in any other test. Generally, time of day of application appeared to have little effect on the FS composition of apple leaves.

No differences in sterol concentrations were observed between the SIFs and the NSIF, metiram. Metiram, a member of the dithiocarbamates, may act by being metabolized to the isothiocyanate radical (-N=C=S), which inactivates the -SH groups in amino acids, proteins, and enzymes contained within the individual pathogen. A more recent theory suggests that ethylene thiuram, not the isothiocyanate radical, is the toxic moiety.

Early in the season, it is important to note that 24 hours after application SIFs may have an effect on the FS composition of apple leaves. Such changes may affect return bloom, a reflection of an apple tree's vigor the previous year. Latham et al. (1985) reported that the proportion of spurs blooming on Redspur Delicious apples in 1983 was significantly lower after season-long applications in 1982 of the SIFs, bitertanol and etaconazole, and some fenarimol-captan treatments, than with benomyl and carbamate fungicides. Latham et al. (1985) also found that return

bloom in 1984 was significantly less after season-long applications of bitertanol or etaconazole than after benomyl, suggesting detrimental effects to apple buds by the former fungicides. However, Strydom and Honeyborne (1981) reported that the SIF, triadimefon, applied at a higher rate (100 versus 25 ppm) to 'Starking Delicious' apple trees significantly increased fruit set and seed content, and increased yield 30% without any detrimental effect on fruit size. Kolbe (1978 and 1982) also observed increased yield and fruit numbers when apple powdery mildew was controlled with triadimefon. It was reported by Spotts and Cervantes (1986) that SIFs, triadimefon and etaconazole, when applied to mature Newtown apple trees, did not affect percent floral buds or fruit set, size or shape. Yield of trees treated with etaconazole was greater than triadimefon-treated or check trees (Spotts and Cervantes 1986).

Although it was reported in the present study that SIFs may have an effect on the FS content of apple leaves within 24 hours of treatment, the FS composition of the plant appears to return to near normal after 72 hours. Therefore, SIFs do not appear to have a long lasting effect on the plant sterol concentrations after 72 hours. Within the present study, there was no measurable accumulative effect of sterol concentrations from the treatments on the same

trees later in the year. However, one must be aware that SIFs may have an effect on the plant's metabolism after continued use over an extended period of time (i.e. years) or perhaps on other parameters such as hormone synthesis.

Szkolnik (1981) observed a plant growth regulator-like response when multiple applications of the fungicide, etaconazole, were made to apple seedlings. It was also reported by Kelley and Jones (1981) that plant growth regulator-like responses occurred on apple trees sprayed with etaconazole. The apple leaves developed three to five layers of palisade cells from trees sprayed with etaconazole, whereas, those from unsprayed trees had only two to three layers.

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Table 2.1. Fungicide treatment rates applied to apple cultivars

Fungicides	Common Names	Formulated Amt/Ha
---------- Red Delicious ----------		
Untreated	--	--
Vangard® 10W	Etaconazole	114.5 g
Rubigan® 1EC	Fenarimol	143.4 ml
Baycor® 50W	Bitertanol	183.3 g
Polyram® 80W	Metiram	1.2 kg
---------- Jonathan ----------		
Untreated	--	--
Vangard® 10W	Etaconazole	85.9 g
Rubigan® 1EC	Fenarimol	71.7 ml
Bayleton® 50W	Triadimefon	68.7 g

Table 2.2. Date and time of fungicide application to each apple cultivar

--------- Red Delicious ----------	
Test 1 =	5/5/82 AM[a] and PM
Test 2 =	5/13/82 PM - 5/14/82 AM
Test 3 =	7/15/82 PM - 7/16/82 AM
Test 4 =	5/23/83 PM - 5/24/83 AM
---------- Jonathan ----------	
Test 5 =	5/24/83 PM - 5/25/83 AM
Test 6 =	7/7/83 AM and PM

[a]Applications made at 7 AM or 7 PM

Table 2.3. Free sterol concentrations of Red Delicious leaves 24 hours after treatment with sterol-inhibiting fungicides

Fungicide	Free sterol (µg/mg DW) Chol[a]	Camp	Stig	Sit	Total
			Test 1[b]		
Untreated	0.073 A[c]	0.207 A	0.033 A	1.771 A	2.084 A
Etaconazole	0.052 A	0.172 AB	0.001 B	1.396 BC	1.622 BC
Fenarimol	0.112 A	0.175 AB	0.001 B	1.714 AB	2.002 AB
Bitertanol	0.088 A	0.237 A	0.001 B	1.456 ABC	1.782 AB
Metiram	0.039 A	0.136 B	0.001 B	1.181 C	1.357 C
			Test 2		
Untreated	0.089 A	0.098 A	0.001 A	1.651 A	1.839 A
Etaconazole	0.150 A	0.134 A	0.026 A	1.695 A	2.005 A
Fenarimol	0.133 A	0.120 A	0.032 A	1.479 A	1.764 A
Bitertanol	0.163 A	0.106 A	0.001 A	1.689 A	1.959 A
Metiram	0.147 A	0.173 A	0.001 A	1.779 A	2.100 A
			Test 3		
Untreated	0.101 A	0.032 B	0.005 A	1.017 A	1.155 A
Etaconazole	0.093 A	0.031 B	0.001 A	0.919 A	1.044 A
Fenarimol	0.108 A	0.035 B	0.001 A	1.080 A	1.224 A
Bitertanol	0.107 A	0.029 B	0.001 A	1.103 A	1.240 A
Metiram	0.088 A	0.073 A	0.001 A	0.868 A	1.030 A
			Test 4		
Untreated	0.006 B	0.018 AB	0.007 A	0.861 A	0.892 A
Etaconazole	0.004 B	0.021 A	0.009 A	0.902 A	0.936 A
Fenarimol	0.030 A	0.013 BC	0.001 B	0.301 B	0.345 B
Bitertanol	0.006 B	0.018 AB	0.004 AB	0.880 A	0.908 A
Metiram	0.036 A	0.009 C	0.001 B	0.334 B	0.380 B

[a]Chol = cholesterol, Camp = campesterol, Stig = stigmasterol, Sit = sitosterol.

[b]Test 1 = plants treated on 5/5/82 AM and PM; Test 2 = plants treated on 5/13/82 PM-5/14/82 AM; Test 3 = plants treated on 7/15/82 PM-7/16/82 AM; and Test 4 = plants treated on 5/23/83 PM-5/24/83 AM.

[c]Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 2.4. Free sterol concentrations of Red Delicious leaves 72 hours after treatment with sterol-inhibiting fungicides

Fungicide	Free sterol (µg/mg DW) Chol[a]	Camp	Stig	Sit	Total
			Test 4[b]		
Untreated	0.007 A[c]	0.001 A	0.000 A	0.081 A	0.089 A
Etaconazole	0.006 A	0.002 A	0.000 A	0.090 A	0.098 A
Fenarimol	0.013 A	0.004 A	0.001 A	0.073 A	0.091 A
Bitertanol	0.010 A	0.003 A	0.000 A	0.102 A	0.115 A
Metiram	0.009 A	0.001 A	0.000 A	0.089 A	0.099 A

[a]Chol = cholesterol, Camp = campesterol, Stig = stigmasterol, Sit = sitosterol.

[b]Test 4 = plants treated on 5/23/83 PM-5/24/83 AM.

[c]Numbers in a column followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 2.5. Free sterol concentrations of Jonathan leaves 24 hours after treatment with sterol-inhibiting fungicides

Fungicide	Free sterol (µg/mg DW) Chol[a]	Camp	Stig	Sit	Total
			Test 5[b]		
Untreated	0.164 A[c]	0.025 C	0.042 A	0.828 A	1.059 A
Etaconazole	0.104 A	0.027 BC	0.051 A	0.900 A	1.082 A
Fenarimol	0.138 A	0.034 AB	0.058 A	0.841 A	1.071 A
Triadimefon	0.122 A	0.036 A	0.051 A	0.885 A	1.094 A
			Test 6		
Untreated	0.095 A	0.009 A	0.034 A	0.808 A	0.946 A
Etaconazole	0.111 A	0.011 A	0.038 A	0.730 A	0.890 A
Fenarimol	0.103 A	0.013 A	0.036 A	0.854 A	1.006 A
Triadimefon	0.104 A	0.019 A	0.042 A	0.895 A	1.060 A

[a]Chol = cholesterol, Camp = campesterol, Stig = stigmasterol, Sit = sitosterol.

[b]Test 5 = plants treated on 5/24/83 PM-5/25/83 AM; and Test 6 = plants treated on 7/7/83 AM and PM.

[c]Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 2.6. Free sterol concentrations of Jonathan leaves 72 hours after treatment with sterol-inhibiting fungicides

Fungicide	Free sterol (µg/mg DW) Chol[a]	Camp	Stig	Sit	Total
			Test 5[b]		
Untreated	0.111 A[c]	0.029 A	0.045 A	0.947 B	1.132 B
Etaconazole	0.117 A	0.023 A	0.053 A	0.919 B	1.112 B
Fenarimol	0.110 A	0.037 A	0.055 A	0.957 B	1.159 AB
Triadimefon	0.128 A	0.036 A	0.061 A	1.129 A	1.354 A
			Test 6		
Untreated	0.129 A	0.016 A	0.042 A	1.145 A	1.332 A
Etaconazole	0.106 A	0.022 A	0.052 A	1.144 A	1.324 A
Fenarimol	0.120 A	0.029 A	0.054 A	1.208 A	1.411 A
Triadimefon	0.125 A	0.028 A	0.050 A	1.074 A	1.277 A

[a]Chol = cholesterol, Camp = campesterol, Stig = stigmasterol, Sit = sitosterol.

[b]Test 5 = plants treated on 5/24/83 PM-5/25/83 AM; and Test 6 = plants treated on 7/7/83 AM and PM.

[c]Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

CHAPTER 3

THE INFLUENCE OF STEROL-INHIBITING FUNGICIDES ON THE FREE FATTY ACID COMPOSITION IN APPLES

ABSTRACT

Selected sterol-inhibiting fungicides (SIFs) were chosen to determine their effects on the free fatty acid (FFA) composition of leaves in Red Delicious and Jonathan apple cultivars over a 2 year period. Trees were treated in the morning and evening and samples collected after 24 and 72 hours. Results of morning and evening application were evaluated alone and in combination at 24 and 72 hours. There were increases in unsaturated and total fatty acid concentrations in Red Delicious leaves 24 hours following applications with the SIF, etaconazole, and the non-sterol-inhibiting fungicide (NSIF), metiram, early in the season. The SIFs etaconazole, fenarimol and bitertanol and the NSIF, metiram, appeared to have similar effects on the fatty acid composition of Red Delicious leaves even though their modes of action are different. Although these SIFs may affect the FFA concentration of Red Delicious and Jonathan leaves, the levels of FFAs, except for increased concentrations of linoleic, linolenic and total FFAs of fenarimol and triadimefon-treated Jonathan leaves in one test, returned to normal after 72 hours. Linolenic acid of Red Delicious leaves increased after 24 hours following morning

application of the SIFs etaconazole and fenarimol; however, stearic acid increased when fenarimol was applied in evening sprays. Early in the growing season, there does not appear to be any effect of the SIFs, etaconazole and fenarimol, on the free fatty acid composition of Jonathan leaves after either morning or evening applications. However, early in the season, the SIF, fenarimol, caused an increase in linolenic acid in both Red Delicious and Jonathan leaves 72 hours after either morning or evening applications. No significant differences were observed later in the season. The large reduction in the ratio of saturated to unsaturated fatty acids was primarily due to a decrease in saturated fatty acids as a percent of the total, and also an increase in unsaturated fatty acids. This resulted in a high double bond index (DBI). Increases observed in different unsaturated fatty acids, resulting in higher DBIs, appeared to be cultivar related. Generally, both the Red Delicious and Jonathan leaves exhibited a decrease in saturation following morning application, whereas, there was an increase in saturation following evening application. Although it was reported in the present study that SIFs may have an effect on the FFA composition early in the season, particularly unsaturated fatty acids, the fatty acid composition of the plant appeared to return to normal later in the season.

INTRODUCTION

Sterol-inhibiting fungicides (SIFs) prevent the biosynthesis of ergosterol and alter membrane function in fungi. Almost all SIFs demonstrate some degree of systemicity, and are useful in controlling a wide range of fungal diseases. Several unique characteristics differentiate the SIFs from other systemic agents: they are the largest group of fungicides with the same specific mode of action (Siegel 1981); they are diverse with respect to chemical structures; and only recently, has reduced sensitivity to SIFs in wild type isolates of Venturia inaequalis been reported (Stanis and Jones 1985).

Permeability characteristics of lipoprotein membranes are primarily influenced by their lipid component (Collander 1959) and a change in fluidity-permeability is often reflected by a change in either the composition or arrangement of membrane lipids. With this change in membrane fluidity there can be a corresponding change in membrane function (Eletr et al. 1973; Shinitzky and Barenholz 1978).

Lurie and Ben-Arie (1983) examined the changes in leakage and viscosity of microsomal membranes from apple fruit at

different stages of ripening and found that in the transition from climacteric to postclimacteric, an increase in saturated fatty acids contributed to increased membrane viscosity. The DBI reflected changes throughout the ripening process with a large increase in the ratio of saturated to unsaturated acids at later stages of ripening. Madhosingh et al. (1976) demonstrated the inhibition of yeast hydroxymethylglutaryl coenzyme A reductase (HMGCoA reductase) by unsaturated fatty acids. Hydroxymethylglutaryl coenzyme A reductase is a critical rate-limiting enzyme in the biosynthesis of sterols. Therefore, changes in the fatty acid composition may have a profound effect on membranes, thereby affecting many membrane associated physiological processes.

Although SIFs are often applied throughout a growing season, relatively little research has been conducted on the effects of SIFs on the FFA composition of higher plants. An important question is that concerning the activity of these SIFs and their relationship to poor fruit shape and firmness. This is a problem which has been observed and which might be the result of the fungicide's quantitative or qualitative alteration of certain metabolites.

The purpose of the present study is to determine the effects of several SIFs on the FFA composition of apple

leaves of Red Delicious and Jonathan cultivars. Morning versus evening application and times following application were examined. Fungicide application during different times of the year were also studied. Apples were chosen because any significant change in their FFA components could have a profound effect on their aesthetic value and the quality of the fruit.

MATERIALS AND METHODS

Effect of time of application:

Times of applications and collections of samples were described previously in Chapter 2.

Collection and analysis of various apple tissues:

Leaf samples were lyophilized, ground to pass through a 40 mesh screen and stored at room temperature until analyzed. At the time of extraction, N-heptadecanoic acid

(0.01 ug) was added as an internal standard to 200 mg of dried leaf sample. The tissue was extracted and purified as described previously in Chapter 2.

Free fatty acids were separated from the lipid extract using Silica gel G TLC plates and a solvent system of hexane-diethyl-ether-acetic acid (80:20:1 v/v/v). Free fatty acids were identified by cochromatography with standards, and visualized under UV light after spraying with dichlorofluoscein.

The band consisting of FFAs was scraped from the plate, dissolved in diethyl ether and filtered through a sintered glass column. When ready to derivatize, the sample was taken to dryness under N2 in a 35°C water bath. Derivatives were prepared by adding 1.5 ml of boron-trichloride-methanol to dry samples followed by heating at 55°C for 5 minutes. After cooling, an equal amount of hexane was added and vortexed. The top hexane layer was drawn off with a pasture pipette and saved. This was repeated three times. The hexane extracts were combined, evaporated almost to dryness under N2, transferred to reaction vials and taken to complete dryness. Sixty ul of hexane was added as the final step in preparing for gas chomatography.

Samples were run on a Bendix model 2600 Gas Chromatograph (GC) isothermally at 170°C on a 28.3 cm x 4 mm (ID) glass

10% EGS column on 80/100 chromosorb W AW (Supelco). Detector and inlet temperatures were 320° and 185°C, respectively. The carrier gas was N2 with a flow rate of 65 ml/min. Methyl stearate was the external standard with an internal standard of C-17:0. Data were analyzed as described previously in Chapter 2 and expressed in ug/mg dry weight.

RESULTS

Significant increases were observed in palmitic acid (16:0) of etaconazole-treated Red Delicious leaves after 24 hours in test 1-1982 (Table 3.1). Similar increases were also found in linoleic (18:2), linolenic (18:3) and total FFA concentrations of etaconazole-treated leaves. In test 2-1982, oleic (18:1), linoleic, linolenic acid and total FFA concentrations of metiram-treated leaves were significantly higher than the control. There were no significant changes in the saturated FFAs in test 2 or in any of the FFA concentrations of Red Delicious leaves 24 hours after

treatment in test 3-1982. In test 4-1983, there was a significant increase in the 18:3 of etaconazole-treated leaves and a significant reduction in 18:3 with fenarimol and metiram treatments. However, by the end of 72 hours after treatment there were no significant differences in the FFA concentrations of Red Delicious leaves in test 4-1983 (Table 3.2).

No significant differences were observed in the concentrations of FFAs of Jonathan leaves 24 hours after treatment in test 5-1983 (Table 3.3). Although, in test 6-1983, a significant reduction was found in the stearic acid (18:0) concentrations of fenarimol-treated leaves. After 72 hours, there were significant increases in 18:2, 18:3 and total FFA concentrations of fenarimol and triadimefon-treated leaves in test 5 (Table 3.4). However, no significant differences were observed in any of the FFA concentrations in test 6 at 72 hours.

Upon examination of the effect of sterol-inhibiting fungicides on the FFA composition 24 hours after morning versus evening applications, significant increases were observed in the 18:3 concentrations of etaconazole, fenarimol and metiram-treated Red Delicious leaves in the morning in test 4-1983 (Table 3.5). Stearic acid concentration was significantly higher in fenarimol-treated leaves in the evening in test 4.

There were no significant changes in the FFA concentrations of Jonathan leaves 24 hours after morning applications in test 5 (Table 3.6). The only significant difference that occurred in test 6 was an increase in the 18:3 concentrations in triadimefon-treated leaves. A significant increase was observed in the 18:3 concentrations of triadimefon-treated Jonathan leaves in the evening in test 5 (Table 3.7). Stearic and 18:2 concentrations were significantly lower in fenarimol and triadimefon-treated leaves in test 6.

The fatty acid concentrations were statistically analyzed to determine whether there were significant differences at 24 and 72 hours following application either after morning or evening treatments. Results obtained from analysis made 24 hours after treatment for both the morning or evening application were inconclusive. However, in test 4-1983, there were significant increases in 18:3 of fenarimol and metiram-treated Red Delicious leaves 72 hours after morning application (Table 3.8). Yet no significant differences were observed for the evening application (Table 3.10). There was a large reduction in the ratio of saturated to unsaturated fatty acids in fenarimol and metiram treatments. (Table 3.9). This was a reflection of decreases in both saturated fatty acids as a percent of the total and of

increases in all three of the unsaturated 18 carbon chain fatty acids (Table 3.9). The best reflection of the change in the components of the FFAs acids was the double bond index (DBI) which showed a large increase in the degree of unsaturation of fenarimol and metiram-treated leaves.

In test 5, which was conducted relatively early in the year, significant increases were found in 18:3 concentrations of fenarimol and triadimefon-treated Jonathan leaves 72 hours after treatment in the morning (Table 3.8). In addition, there was a large reduction in the ratio of saturated to unsaturated fatty acids in both of these treatments, reflecting decreases in the saturated fatty acids and increases in 18:3 (Table 3.9). However, no significant differences were observed among treatments made later in the season in test 6 (Table 3.8).

There were significant increases in 18:2, 18:3 and total FFA concentrations of fenarimol and triadimefon-treated Jonathan leaves 72 hours after evening application in test 5-1983 (Table 3.10). Moreover, a large reduction was observed in the ratio of saturated to unsaturated fatty acids in both of these treatments (Table 3.11). This was due to a decrease in the percentage of saturated fatty acids and an increase in the percentage of 18:3. A large increase in the degree of unsaturation was observed in their DBIs.

Later in the season, in test 6, there were no significant differences in the FFAs 72 hours after evening treatment (Table 3.10).

DISCUSSION

There appeared to be an effect of these SIFs, particularly etaconazole, on the FFA concentration of Red Delicious leaves early in the season, but not later in the year. The tendency was for an increase in unsaturated fatty acids, particularly 18:3, 24 hours after application. Little or no effect was observed on the saturated fatty acid concentrations. These changes in FFA may be due to the higher metabolic activity of the plant at the time of year of fungicide application. The SIFs etaconazole, fenarimol and bitertanol and the non-sterol-inhibiting fungicide (NSIF), metiram, appeared to have similar effects on the fatty acid composition of Red Delicious leaves although their modes of action are different. Metiram, a member of the dithiocarbamates, may act by being metabolized to the

isothiocyanate radical (-N=C=S), which inactivates the -SH groups in amino acids, proteins and enzymes contained within the individual pathogen. A more recent theory suggests that ethylene thiuram, not the isothiocyanate radical, is the toxic moiety. No significant differences were observed in the FFA concentrations of Red Delicious leaves 72 hours after treatment in test 4-1983.

Relatively little research has been conducted on the effects of SIFs on the FFA composition of higher plants although much work has been done with microorganisms. Henry and Sisler (1981) reported an accumulation of FFAs in the sporidia of Ustilago maydis treated with the SIF, etaconazole. Walsh and Sisler (1982) compared an ergosterol-deficient mutant of U. maydis to a wild type with regard to morphology, growth rate, lipid content and sensitivity to ergosterol biosynthetic inhibitors, and found that the concentration of FFAs was higher in the mutant than in wild-type cells. The sterols of the mutant were identical to those which accumulated in wild-type sporidia treated with the SIFs fenarimol, etaconazole and miconazole. Manners and Gay (1982) discovered that the fatty acids, 18:2 and lauric, were readily accumulated by haustorial complexes isolated from Pisum sativum infected with Erysiphe pisi following treatments with the SIFs nuarimol, fenarimol,

dimethirimol, ethirimol and other pyrimidine-based analogues.

Early in the growing season, significant increases occurred primarily in unsaturated fatty acids of fenarimol and triadimefon-treated Jonathan leaves 72 hours after treatment. The trend was for an increase in unsaturated fatty acids, particularly 18:3, in other treatments. However, there were no significant differences in the FFA composition of Jonathan leaves later in the season. Although these SIFs may have an effect on the FFA concentrations of Red Delicious and Jonathan leaves early in the season, the FFA concentration of the plant appeared to return to normal later in the year. There also appeared to be a quicker response of the Red Delicious cultivar to the SIFs.

There were also differences in morning versus evening applications in fenarimol-treated Red Delicious leaves. The plant may be compensating for the increase in membrane viscosity in fenarimol-treated leaves by synthesizing more polyunsaturated fatty acids overnight to enhance membrane fluidity and maintain the integrity of the membrane, as evidenced by the increased 18:3 of fenarimol-treated leaves in the morning. Ferrari-Iliou et al. (1984) suggested that a decrease in the long chain polyunsaturated fatty acids may

cause a decrease in chloroplast membrane fluidity and alter the permeability of the membrane, thereby affecting the compartmentation of the cells.

Generally, both the Red Delicious and Jonathan cultivars exhibited a decrease in saturation after morning application and an increase after evening treatment. Relatively little fungicide will be lost in the morning because of faster drying, while in the evening some of the fungicide will be lost due to slower drying and accumulation of moisture (dew) on the leaf surface and subsequent runoff. In addition, the fungicide may become diluted in the moisture on the leaf surface and not have the same effect as when applied in the morning. An appreciable increase in unsaturated fatty acids, particularly 18:3, was more apparent after morning application than evening application for both Red Delicious and Jonathan cultivars. This may be seen in the present study as a change in membrane fluidity. Permeability characteristics of lipoprotein membranes are primarily influenced by the lipid component (Collander 1959) and a change in permeability should be reflected by a change in either the composition or arrangement of membrane lipids. Several investigations have been reported indicating a correspondence between changes in membrane function and changes in membrane fluidity (Eletr et al. 1973; Shinitzky and Barenholz 1978).

Early in the year, the differences observed after 72 hours following treatment of Jonathan leaves in the evening may be cultivar related. No significant differences occurred later in the season either after morning or evening applications. It appeared that these fungicides, particularly the SIF, fenarimol, caused an increase in 18:3 in both Red Delicious and Jonathan leaves early in the growing season when the plant's metabolism is at its highest.

The large reduction in the ratio of saturated to unsaturated fatty acids in fenarimol and metiram-treated Red Delicious leaves was due to decreases in both saturated fatty acids as a percent of the total, and also to increases in all three of the unsaturated fatty acids. In fenarimol and triadimefon-treated Jonathan leaves, the reduction was seen as decreases in the saturated fatty acids as a percent of the total, and increases in 18:3. The increase in different unsaturated fatty acids, resulting in reduced saturated to unsaturated ratios and higher DBIs in both cultivars, appears to be cultivar related.

A previous study demonstrated the inhibition of yeast HMGCoA reductase by unsaturated fatty acids (Madhosingh et al. 1976). This enzyme is a critical rate-regulating enzyme which is involved in all sterol biosynthesis. Therefore, an

increase in unsaturated fatty acid biosynthesis may inhibit the production of sterols (free sterols) which have been shown to affect processes vital to the plant's metabolism. As previously noted, the SIFs, fenarimol and triadimefon, caused an increase in unsaturated fatty acids in both Red Delicious and Jonathan leaf tissue 72 hours after treatment early in the year. In test 1-1982, the authors previously observed decreases in stigmasterol of etaconazole, bitertanol, fenarimol and metiram-treated Red Delicious leaves, and in sitosterol and total sterol concentrations of etaconazole-treated leaves 24 hours after treatment. Similar reductions occurred in stigmasterol, sitosterol and total sterol concentrations of fenarimol and metiram-treated Red Delicious leaves 24 hours after treatment in test 4-1983. No such decreases were found in Jonathan leaf tissue. However, sterol concentrations in Red Delicious leaves appeared to return to normal 72 hours after treatment.

In the present study, within the first 72 hours depending on the statistical analysis (separate or in combination), the trend was for an increase in unsaturated fatty acids, particularly 18:3, early in the season. The plant may be synthesizing more polyunsaturated fatty acids to enhance membrane fluidity and maintain the integrity of the

membrane. It has been suggested that the unsaturated fatty acid metabolism provides a natural and dynamic basis for the maintenance of fluidity in membranes at lower temperatures (Barran et al. 1976; Singh et al. 1975) which would be present early in the growing season.

Early in the season, SIFs had an effect on the FFA composition, particularly unsaturated fatty acids, in both Red Delicious and Jonathan cultivars. However, fatty acid concentrations returned to normal later in the season. The authors also observed ultrastructurally thylakoid swelling and disruption of membranes in chloroplasts of bitertanol-treated Red Delicious leaves after 12 hours. However, 24 and 72 hours following application of the fungicide, the chloroplasts regained their integrity and appeared normal. This suggests that the SIF, bitertanol, had an immediate effect upon the fine structure of chloroplasts which may contribute to the accumulation of FFAs, particularly 18:3, of Red Delicious and Jonathan leaves after 24 hours. Although there was some difference between the cultivars, SIFs did not appear to have a long lasting effect on the plant FFA concentrations later in the season. However, one must be aware that SIFs may have an effect on the plant's metabolism after continued use over an extended period of time (i.e. years).

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Table 3.1. Free fatty acid concentrations of Red Delicious leaves 24 hours after treatment with sterol-inhibiting fungicides

Fungicide	Free fatty acid (ug/mg DW) 16:0[a]	18:0	18:1	18:2	18:3	Total
Test 1[b]						
Untreated	1.174 B[c]	0.313 AB	0.210 A	0.887 B	0.902 B	3.486 B
Etaconazole	1.998 A	0.474 A	0.259 A	1.499 A	1.604 A	5.834 A
Fenarimol	0.998 B	0.266 B	0.151 A	0.681 B	0.705 B	2.801 B
Bitertanol	1.340 AB	0.378 AB	0.198 A	0.870 B	1.036 B	3.822 B
Metiram	1.288 AB	0.383 AB	0.201 A	0.944 B	1.028 B	3.844 B
Test 2						
Untreated	0.849 A	0.278 A	0.273 B	1.116 BC	0.961 B	3.477 B
Etaconazole	0.863 A	0.291 A	0.313 B	0.888 C	0.726 B	3.081 B
Fenarimol	1.067 A	0.298 A	0.325 B	1.492 B	1.117 B	4.299 AB
Bitertanol	1.024 A	0.286 A	0.263 B	1.175 BC	0.907 B	3.655 B
Metiram	1.000 A	0.273 A	0.529 A	2.018 A	1.687 A	5.507 A
Test 3						
Untreated	0.308 A	0.113 A	0.125 A	0.071 A	0.178 A	0.795 A
Etaconazole	0.337 A	0.106 A	0.114 A	0.108 A	0.315 A	0.980 A
Fenarimol	0.262 A	0.083 A	0.081 A	0.061 A	0.222 A	0.709 A
Bitertanol	0.290 A	0.092 A	0.102 A	0.062 A	0.165 A	0.711 A
Metiram	0.352 A	0.096 A	0.087 A	0.063 A	0.245 A	0.843 A
Test 4						
Untreated	0.310 A	0.066 A	0.026 AB	0.067 A	0.092 B	0.561 A
Etaconazole	0.375 A	0.081 A	0.004 B	0.071 A	0.129 A	0.660 A
Fenarimol	0.337 A	0.111 A	0.050 A	0.063 A	0.051 CD	0.612 A
Bitertanol	0.369 A	0.073 A	0.024 AB	0.062 A	0.073 BC	0.601 A
Metiram	0.334 A	0.108 A	0.031 AB	0.060 A	0.042 D	0.575 A

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Test 1 = plants treated on 5/5/82 AM and PM, Test 2 = plants treated on 5/13/82 PM -5/14/82 AM, Test 3 = plants treated on 7/15/82 PM - 7/16/82 AM, and Test 4 = plants treated on 5/23/83 PM - 5/24/83 AM.

[c] Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 3.2. Free fatty acid concentrations of Red Delicious leaves 72 hours after treatment with sterol-inhibiting fungicides

	Free fatty acid (ug/mg DW)					
Fungicide	16:0[a]	18:0	18:1	18:2	18:3	Total
			Test 4[b]			
Untreated	0.268 A[c]	0.121 A	0.004 A	0.037 A	0.046 A	0.476 A
Etaconazole	0.333 A	0.108 A	0.031 A	0.065 A	0.075 A	0.612 A
Fenarimol	0.320 A	0.097 A	0.025 A	0.057 A	0.096 A	0.595 A
Bitertanol	0.307 A	0.083 A	0.046 A	0.057 A	0.070 A	0.563 A
Metiram	0.344 A	0.092 A	0.029 A	0.082 A	0.123 A	0.670 A

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Test 4 = plants treated on 5/23/83 PM - 5/24/83 AM.

[c] Numbers in a column followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 3.3. Free fatty acid concentrations of Jonathan leaves 24 hours after treatment with sterol-inhibiting fungicides

Fungicide	Free fatty acid (ug/mg DW) 16:0[a]	18:0	18:1	18:2	18:3	Total
			Test 5[b]			
Untreated	0.458 A[c]	0.272 A	0.086 A	0.198 A	0.265 A	1.279 A
Etaconazole	0.401 A	0.217 A	0.089 A	0.235 A	0.301 A	1.243 A
Fenarimol	0.401 A	0.192 A	0.101 A	0.237 A	0.319 A	1.250 A
Triadimefon	0.424 A	0.192 A	0.099 A	0.241 A	0.388 A	1.344 A
			Test 6			
Untreated	0.288 AB	0.141 A	0.132 A	0.082 A	0.210 A	0.853 A
Etaconazole	0.298 A	0.132 AB	0.133 A	0.075 A	0.323 A	0.961 A
Fenarimol	0.263 B	0.118 B	0.114 A	0.059 A	0.195 A	0.749 A
Triadimefon	0.300 A	0.133 AB	0.136 A	0.073 A	0.302 A	0.944 A

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Test 5 = plants treated on 5/24/83 PM - 5/25/83 AM, and Test 6 = plants treated on 7/7/83 AM and PM.

[c] Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 3.4. Free fatty acid concentrations of Jonathan leaves 72 hours after treatment with sterol-inhibiting fungicides

	Free fatty acid (ug/mg DW)					
Fungicide	16:0[a]	18:0	18:1	18:2	18:3	Total
			Test 5[b]			
Untreated	0.359 A[c]	0.134 A	0.110 A	0.230 B	0.320 B	1.153 B
Etaconazole	0.357 A	0.125 A	0.114 A	0.260 AB	0.445 A	1.301 AB
Fenarimol	0.397 A	0.141 A	0.121 A	0.275 A	0.486 A	1.420 A
Triadimefon	0.383 A	0.134 A	0.123 A	0.303 A	0.510 A	1.453 A
			Test 6			
Untreated	0.220 A	0.150 A	0.161 A	0.059 A	0.113 A	0.703 A
Etaconazole	0.253 A	0.163 A	0.153 A	0.043 A	0.097 A	0.709 A
Fenarimol	0.234 A	0.105 A	0.139 A	0.039 A	0.121 A	0.638 A
Triadimefon	0.218 A	0.109 A	0.126 A	0.039 A	0.129 A	0.621 A

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Test 5 = plants treated on 5/24/83 PM - 5/25/83 AM, and Test 6 = plants treated on 7/7/83 AM and PM.

[c] Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 3.5. Free fatty acid concentrations of Red Delicious leaves 24 hours after morning or evening treatment with sterol-inhibiting fungicides

	Free fatty acid (ug/mg DW)					
Fungicide	16:0[a]	18:0	18:1	18:2	18:3	Total
Morning - Test 4[b]						
Untreated	0.284 AB[c]	0.112 A	0.021 A	0.040 A	0.041 B	0.498 A
Etaconazole	0.371 A	0.101 A	0.010 A	0.057 A	0.085 A	0.623 A
Fenarimol	0.272 B	0.081 A	0.030 A	0.052 A	0.069 A	0.504 A
Bitertanol	0.302 AB	0.081 A	0.027 A	0.061 A	0.062 AB	0.533 A
Metiram	0.291 AB	0.089 A	0.026 A	0.060 A	0.070 A	0.536 A
Evening - Test 4						
Untreated	0.294 A[c]	0.075 B	0.008 A	0.064 A	0.096 A	0.538 A
Etaconazole	0.336 A	0.088 AB	0.024 A	0.079 A	0.119 A	0.647 A
Fenarimol	0.385 A	0.127 A	0.045 A	0.067 A	0.077 A	0.702 A
Bitertanol	0.374 A	0.075 B	0.043 A	0.058 A	0.081 A	0.630 A
Metiram	0.386 A	0.112 AB	0.034 A	0.081 A	0.095 A	0.708 A

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Test 4 = plants treated on 5/23/83 PM - 5/24/83 AM.

[c] Numbers in a column followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 3.6. Free fatty acid concentrations of Jonathan leaves 24 hours after morning treatment with sterol-inhibiting fungicides

	Free fatty acid (ug/mg DW)					
Fungicide	16:0[a]	18:0	18:1	18:2	18:3	Total
			Test 5[b]			
Untreated	0.435 A[c]	0.213 A	0.104 A	0.210 A	0.273 A	1.235 A
Etaconazole	0.395 A	0.227 A	0.100 A	0.240 A	0.331 A	1.293 A
Fenarimol	0.440 A	0.185 A	0.109 A	0.254 A	0.391 A	1.379 A
Triadimefon	0.432 A	0.189 A	0.118 A	0.255 A	0.418 A	1.412 A
			Test 6			
Untreated	0.270 A	0.154 A	0.134 A	0.057 A	0.161 B	0.776 A
Etaconazole	0.273 A	0.148 A	0.143 A	0.054 A	0.245 AB	0.863 A
Fenarimol	0.284 A	0.130 A	0.132 A	0.048 A	0.186 AB	0.780 A
Triadimefon	0.285 A	0.136 A	0.133 A	0.065 A	0.269 A	0.888

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Test 5 = plants treated on 5/24/83 PM - 5/25/83 AM, and Test 6 = plants treated on 7/7/83 AM and PM.

[c] Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 3.7. Free fatty acid concentrations of Jonathan leaves 24 hours after evening treatment with sterol-inhibiting fungicides

	Free fatty acid (ug/mg DW)					
Fungicide	16:0[a]	18:0	18:1	18:2	18:3	Total
			Test 5[b]			
Untreated	0.382 A[c]	0.193 A	0.092 A	0.218 A	0.311 B	1.196 A
Etaconazole	0.363 A	0.143 A	0.102 A	0.255 A	0.415 AB	1.278 A
Fenarimol	0.358 A	0.149 A	0.113 A	0.257 A	0.414 AB	1.291 A
Triadimefon	0.375 A	0.137 A	0.104 A	0.289 A	0.480 A	1.385 A
			Test 6			
Untreated	0.238 A	0.136 A	0.160 A	0.084 A	0.162 A	0.780 AB
Etaconazole	0.278 A	0.146 A	0.142 A	0.064 B	0.175 A	0.805 A
Fenarimol	0.213 A	0.093 B	0.121 A	0.049 B	0.130 A	0.606 B
Triadimefon	0.233 A	0.106 B	0.128 A	0.047 B	0.162 A	0.676 AB

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Test 5 = plants treated on 5/24/83 PM - 5/25/83 AM, and Test 6 = plants treated on 7/7/83 AM and PM.

[c] Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 3.8. Free fatty acid concentrations of Red Delicious and Jonathan leaves 72 hours after morning treatment with sterol-inhibiting fungicides

	Free fatty acid (ug/mg DW)					
Fungicide	16:0[a]	18:0	18:1	18:2	18:3	Total
Test 4[b] (Red Delicious)						
Untreated	0.244 A[c]	0.160 A	0.000 A	0.022 A	0.000 C	0.426 A
Etaconazole	0.318 A	0.104 A	0.016 A	0.044 A	0.038 BC	0.520 A
Fenarimol	0.259 A	0.079 A	0.024 A	0.047 A	0.081 AB	0.490 A
Bitertanol	0.254 A	0.080 A	0.047 A	0.068 A	0.041 BC	0.490 A
Metiram	0.254 A	0.063 A	0.020 A	0.061 A	0.110 A	0.508 A
Test 5 (Jonathan)						
Untreated	0.392 A	0.134 A	0.122 A	0.235 A	0.306 B	1.189 A
Etaconazole	0.367 A	0.128 A	0.110 A	0.249 A	0.422 AB	1.276 A
Fenarimol	0.427 A	0.142 A	0.121 A	0.273 A	0.464 A	1.427 A
Triadimefon	0.409 A	0.146 A	0.123 A	0.290 A	0.490 A	1.458 A
Test 6 (Jonathan)						
Untreated	0.240 A	0.160 A	0.145 A	0.050 A	0.109 A	0.704 A
Etaconazole	0.235 A	0.159 A	0.165 A	0.044 A	0.088 A	0.691 A
Fenarimol	0.287 A	0.126 A	0.149 A	0.041 A	0.168 A	0.771 A
Triadimefon	0.248 A	0.126 A	0.133 A	0.046 A	0.179 A	0.732 A

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Test 4 = plants treated on 5/23/83 PM - 5/24/83 AM,
Test 5 = plants treated on 5/24/83 PM - 5/25/83 AM, and
Test 6 = plants treated on 7/7/83 AM and PM.

[c] Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 3.9. Free fatty acid saturation of Red Delicious and Jonathan leaves 72 hours after morning treatment with sterol-inhibiting fungicides

Fungicide	16:0[a]	18:0	18:1	18:2	18:3	Saturated / Unsaturated	DBI[b]
Test 4[c] (Red Delicious)							
Untreated	57[d]	38	0	5	0	18.36	0.10
Etaconazole	61	20	3	9	7	4.31	0.42
Fenarimol	53	16	5	10	16	2.22	0.73
Bitertanol	52	16	10	14	8	2.14	0.62
Metiram	50	12	4	12	22	1.66	0.94
Test 5 (Jonathan)							
Untreated	33	11	10	20	26	0.79	1.28
Etaconazole	29	10	9	19	33	0.63	1.46
Fenarimol	30	10	8	19	33	0.66	1.45
Triadimefon	28	10	8	20	34	0.61	1.50
Test 6 (Jonathan)							
Untreated	34	23	21	7	15	1.32	0.80
Etaconazole	34	23	24	6	13	1.33	0.75
Fenarimol	37	17	19	5	22	1.15	0.95
Triadimefon	34	17	18	6	25	1.04	1.05

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Double bond index = Σ(peak area % x no. double bonds)÷100.

[c] Test 4 = plants treated on 5/23/83 PM - 5/24/83 AM,
Test 5 = plants treated on 5/24/83 PM - 5/25/83 AM, and
Test 6 = plants treated on 7/7/83 AM and PM.

[d] % of total

3.10. Free fatty acid concentrations of Red Delicious and Jonathan leaves 72 hours after evening treatment with sterol-inhibiting fungicides

	Free fatty acid (ug/mg DW)					
Fungicide	16:0[a]	18:0	18:1	18:2	18:3	Total
			Test 4[b] (Red Delicious)			
Untreated	0.293 A[c]	0.083 A	0.007 A	0.053 A	0.092 A	0.528 A
Etaconazole	0.348 A	0.112 A	0.045 A	0.086 A	0.112 A	0.703 A
Fenarimol	0.381 A	0.115 A	0.026 A	0.067 A	0.110 A	0.699 A
Bitertanol	0.360 A	0.085 A	0.045 A	0.046 A	0.098 A	0.634 A
Metiram	0.433 A	0.121 A	0.039 A	0.102 A	0.136 A	0.831 A
			Test 5 (Jonathan)			
Untreated	0.326 A	0.135 A	0.097 A	0.226 B	0.333 B	1.117 B
Etaconazole	0.348 A	0.122 A	0.117 A	0.271 AB	0.468 A	1.326 AB
Fenarimol	0.367 A	0.140 A	0.120 A	0.276 A	0.508 A	1.411 A
Triadimefon	0.358 A	0.122 A	0.123 A	0.316 A	0.530 A	1.449 A
			Test 6 (Jonathan)			
Untreated	0.200 A	0.140 AB	0.177 A	0.069 A	0.118 A	0.704 A
Etaconazole	0.270 A	0.166 A	0.140 A	0.041 A	0.106 A	0.723 A
Fenarimol	0.181 A	0.084 B	0.129 A	0.036 A	0.074 A	0.504 A
Triadimefon	0.188 A	0.092 B	0.119 A	0.032 A	0.079 A	0.510 A

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Test 4 = plants treated on 5/23/83 PM - 5/24/83 AM,
Test 5 = plants treated on 5/24/83 PM - 5/25/83 AM, and
Test 6 = plants treated on 7/7/83 AM and PM.

[c] Numbers in a column for each test followed by the same letter are not significantly different ($\alpha = 0.05$).

Table 3.11. Free fatty acid saturation of Red Delicious and Jonathan leaves 72 hours after evening treatment with sterol-inhibiting fungicides

Fungicide	16:0[a]	18:0	18:1	18:2	18:3	Saturated / Unsaturated	DBI[b]
			Test 4[c] (Red Delicious)				
Untreated	56[d]	16	1	10	17	2.47	0.72
Etaconazole	50	16	6	12	16	1.89	0.78
Fenarimol	54	16	4	10	16	2.44	0.72
Bitertanol	57	13	7	7	16	2.35	0.69
Metiram	52	15	5	12	16	2.00	0.77
			Test 5 (Jonathan)				
Untreated	29	12	9	20	30	0.70	1.39
Etaconazole	26	9	9	21	35	0.55	1.56
Fenarimol	26	10	8	20	36	0.56	1.56
Triadimefon	25	8	8	22	37	0.50	1.63
			Test 6 (Jonathan)				
Untreated	28	20	25	10	17	0.93	0.96
Etaconazole	37	23	19	6	15	1.52	0.76
Fenarimol	36	17	25	7	15	1.11	0.84
Triadimefon	37	18	23	6	16	1.22	0.83

[a] 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, 18:3 = linolenic acid.

[b] Double bond index = Σ(peak area % x no. double bonds)÷100.

[c] Test 4 = plants treated on 5/23/83 PM - 5/24/83 AM,
Test 5 = plants treated on 5/24/83 PM - 5/25/83 AM, and
Test 6 = plants treated on 7/7/83 AM and PM.

[d] % of total

CHAPTER 4

THE FINE STRUCTURE OF BITERTANOL-TREATED RED DELICIOUS LEAVES

ABSTRACT

Ultrastructural observations were made of Red Delicious leaves 12, 24 and 72 hours following treatment with the sterol-inhibiting fungicide, bitertanol. Thylakoids of chloroplasts appeared swollen and irregular after 12 hours, resulting in loss of integrity of the organelles. Occasionally, mitochondria were washed-out; however, no other changes in membrane or organelle structure were observed. After 24 and 72 hours, thylakoids of chloroplasts were similar to those of the controls. However, the amount and number of starch granules in the chloroplasts increased after 12, 24 and 72 hours.

INTRODUCTION

Sterol biosynthesis inhibition by chemicals has been studied considerably in animal tissues (Schroepfer 1981) and in microorganisms (Oehlschlager et al. 1980; Leroux 1981). However, very little work has been done with higher plants. Most of the compounds used in agriculture reduce the growth of plants (Shive and Sisler 1976; Buchenauer and Grossman 1977; Buchenauer 1977; Buchenauer and Rohner 1981) and the effects were partly or in some cases fully reversible with gibberellins. The sterol-inhibiting fungicide (SIF), triarimol and ancymidol, a plant growth retardant, both known to interfere with ergosterol biosynthesis in fungi (Ragsdale 1975), were reported to retard the growth of Phaseolus vulgaris L. cv. Contender seedlings but did not noticeably affect the qualitative and quantitative distribution of the main sterols present (Shive and Sisler 1976). Sterol-inhibiting fungicides nuarimol, triadimefon, triadimenol and imazalil all inhibited the incorporation of [^{14}C] acetate into lipids of barley plants and the effects on the sterol fractions were analogous to those reported to occur on fungal systems (Buchenauer and Rohner 1981; Buchenauer 1977). However, much higher concentrations were

required to achieve this as compared with fungi. Schmitt and Benveniste (1979) similarly found that sterol C-14 demethylation was sensitive to the SIF, fenarimol, in suspension cultures of bramble cells.

Although SIFs are often applied throughout a growing season on numerous field crops, ornamentals and fruit crops, relatively little work has been done on the effects of these compounds ultrastructurally in higher plants. The purpose of this study is to examine the effects of the SIF, bitertanol, on the fine structure of apple leaves in Red Delicious cultivar.

MATERIALS AND METHODS

The youngest six leaves fully expanded on actively growing shoot apices of 4 Red Delicious trees, located at the Winchester Fruit Research Center, were used in this study. The fungicide, Baycor® 50W (bitertanol), at 1.134 g/gal of water, was applied until runoff occurred to abaxial and adaxial leaf surfaces using a hand-held sprayer.

Several 1-3mm rectangular strips intermediate between the 1° vein and margin of leaves, untreated and treated with the fungicide, were removed with a razor blade 12, 24 and 72 hours after fungicide application. These were then processed for electron microscopy. This involved the sequential fixation of specimens in 4% glutaraldehyde and 2% osmium tetroxide. Rectangular strips of leaf tissue were prefixed in 4% glutaraldehyde in 0.1M cacodylate buffer for five hours at 0°C, washed in three changes of 0.1M sodium cacodylate buffer for one hour and postfixed in sodium cacodylate buffered 2% osmium tetroxide for 16 hours at 0°C. After osmium fixation, specimens were washed in three changes of 0.1M sodium cacodylate buffer for 45 minutes. Specimens were dehydrated in a graded ethanol series (30 min each) followed by an additional change of absolute alcohol for one half hour and then by acetone (2 changes of 10 min each). The specimens were infiltrated overnight and embedded in Spurr's low-viscosity resin (Spurr 1969) the next day. Blocks were cured at 70°C for 24 hours. Sections cut on a Sorvall MT2B ultramicrotome with a Dupont diamond knife were collected on 200-mesh copper grids and double stained with 2% uranyl acetate for 30 min and Reynold's lead citrate (Reynolds 1963) for 8 min.

RESULTS

Untreated Red Delicious leaves possessed a uniform cuticular layer that covered the outer surface of the epidermis (FIG. 4.1). Beneath the epidermis were the palisade and spongy parenchyma which make up the mesophyll. Chloroplasts were found primarily in the palisade and spongy mesophyll cells of the leaf (FIGS. 4.2, 4.3 & 4.4). Palisade parenchyma consisted of long columnar cells (FIGS. 4.3 & 4.4) where most of the photosynthesis takes place. Located around the periphery of these cells were numerous spherical to elongate chloroplasts which measured approximately 2 x 6um (FIGS. 4.3 & 4.4). The internal structure of these choloroplasts was characterized by a system of membranes, the basic subunit of which was a flattened vesicle surrounded by a single membrane which is called a thylakoid (FIGS. 4.3 & 4.4). Tightly packed stacks of thylakoids parallel to each other, were present throughout the stroma of the chloroplasts. In addition, lipid and starch granules were located within the chloroplasts (FIGS. 4.2, 4.3 & 4.4).

Twelve hours following treatment of Red Delicious leaves with the SIF, bitertanol, the thylakoids of chloroplasts appeared swollen and irregular (FIGS 4.5, 4.6 & 4.7). This gave the chloroplast a somewhat distorted appearance. Mitochondria were occasionally washed-out (FIG. 4.7) although nuclei did not appear to be affected (FIG. 4.8). No other changes in membrane or organelle structure were observed. After 24 (FIG. 4.9) and 72 hours (FIG. 4.10) the thylakoids were tightly packed and no longer swollen. Both mitochondria and nuclei (FIGS. 4.9 & 4.10) appeared normal. There was, however, an increase in the amount and number of starch granules 12, 24 and 72 hours following treatment with the fungicide (FIGS. 4.5, 4.9 & 4.10).

DISCUSSION

It appears that the SIF, bitertanol, may cause a reduction in photosynthesis initially after application. This was evident by the swollen and irregular thylakoid membranes and loss in chloroplast integrity observed 12

hours following treatment of Red Delicious leaves with bitertanol. Ashton et al. (1963) observed the disorganization of chloroplasts and cellular membranes induced by the triazine herbicide, atrazine, an electron transport inhibitor. The structure of atrazine is similar to that of bitertanol in that both have N-containing rings. This suggests that bitertanol may possess photosynthetic inhibitor capabilities. Whether or not this is the case needs further investigation. However, the chloroplasts appear to have recovered from the initial effect of the fungicide and returned to normal after 24 and 72 hours.

It was also observed by these authors that in 1982 the stigmasterol level of bitertanol-treated Red Delicious leaves decreased significantly after 24 hours. This may be the result of the breakdown in the integrity of the chloroplasts. Although it was reported that bitertanol may have an effect on the free sterol content of apple leaves after 24 hours, the free sterol composition of the plant appears to return to normal after 72 hours. This correlates well between membrane destabilization due to changes in sterol composition and cell physiology, where the thylakoids are no longer swollen and the chloroplasts have regained their integrity. Bitertanol does not appear to have a long lasting effect on the plant sterol concentration after 72

hours. However, one must be aware that SIFs such as bitertanol may have an effect on the plant's metabolism after continued use over an extended period of time (i.e. years) or perhaps on other parameters such as hormone synthesis.

Starch granules were observed to increase in size and number within the chloroplasts throughout the collection periods following application of the fungicide. This suggests that glucose transport to regions of active growth was being inhibited and subsequently converted to the storage form of starch. Again, this may be another mechanism by which the SIF, bitertanol, exerts its influence on the physiology of the plant.

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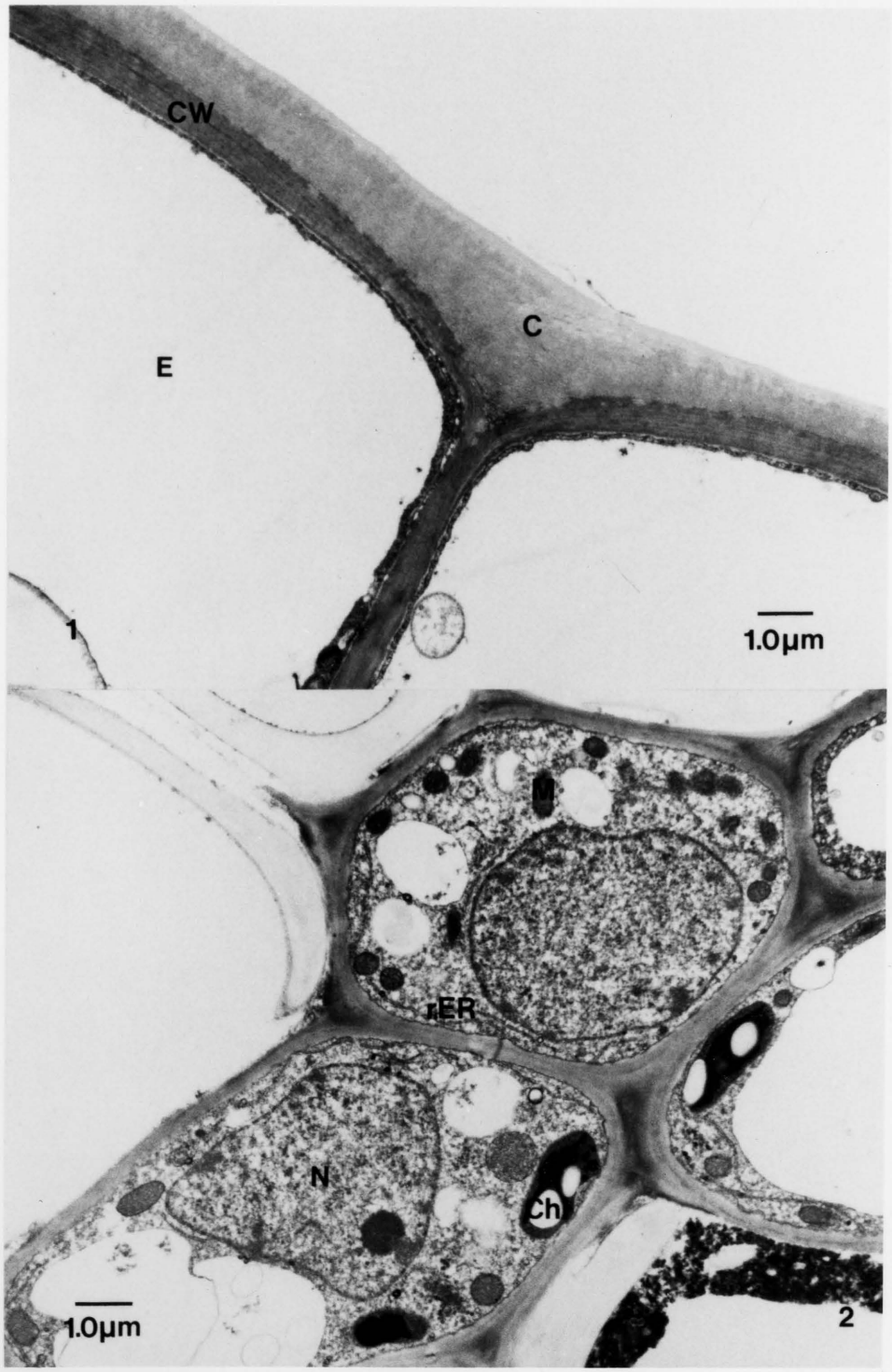
FIGS. 4.1-4.2. Untreated Red Delicious leaves. 1. Transmission electron micrograph of an untreated Red Delicious leaf showing a uniform cuticular layer with underlying epidermal cells; cuticle (C), cell wall (CW), epidermis (E), x8,000. 2. Untreated parenchyma cells showing internal arrangement of organelles; chloroplast (Chl), mitochondrion (M), nucleus (N), rough endoplasmic reticulum (rER), x8,000.


CW
C
E
1
1.0µm
M
rER
N
Ch
1.0µm
2

FIGS. 4.3-4.4. Untreated Red Delicious leaves. 3. Transmission electron micrograph of untreated palisade parenchyma cells showing peripheral elongated chloroplasts; chloroplast (Chl), x8,000. 4. Untreated palisade parenchyma cells showing numerous chloroplasts containing minute electron dense lipid bodies and starch granules. Note spherical to elongated mitochondria; mitochondrion (M), starch (S), x8,000.

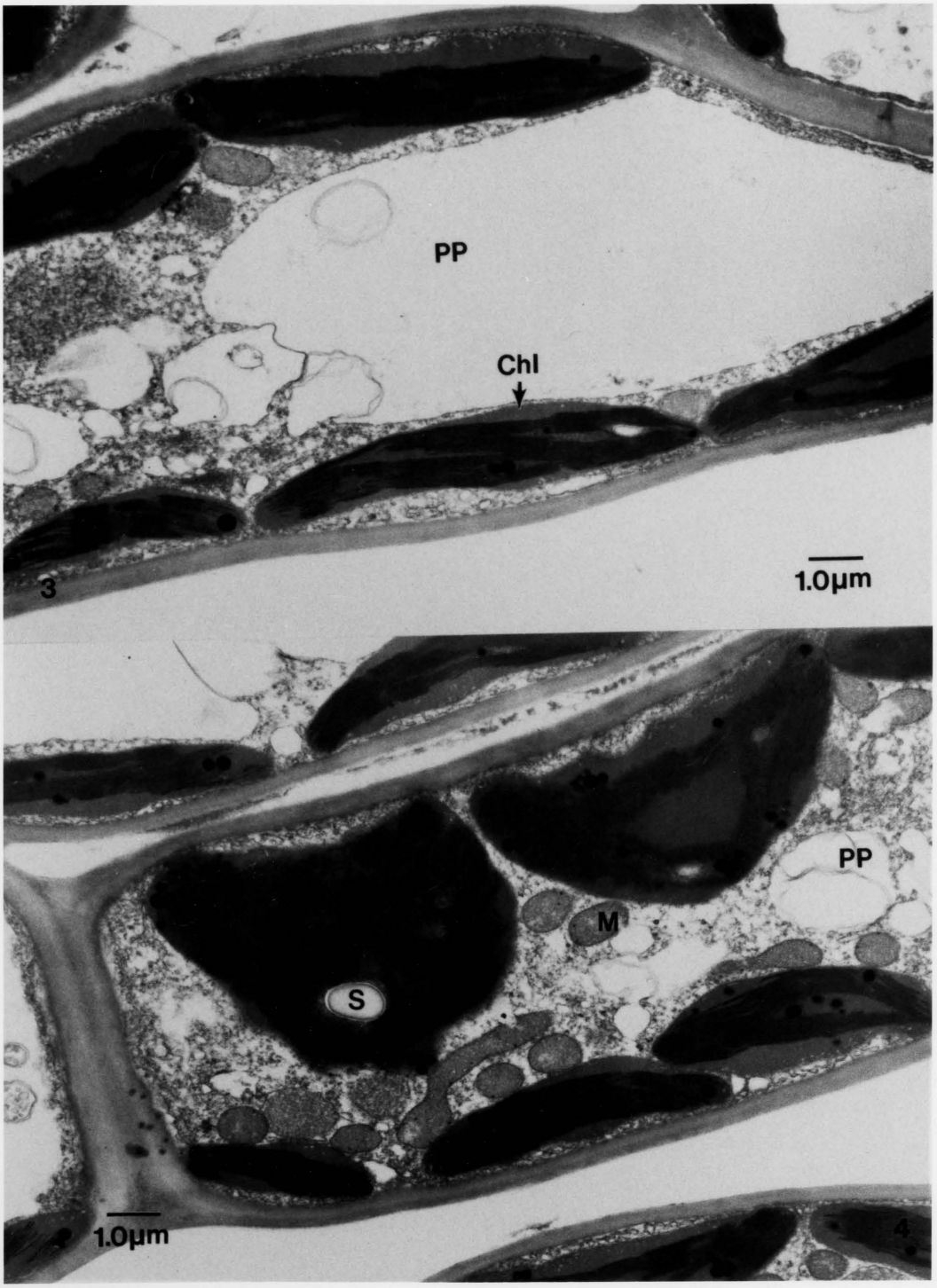
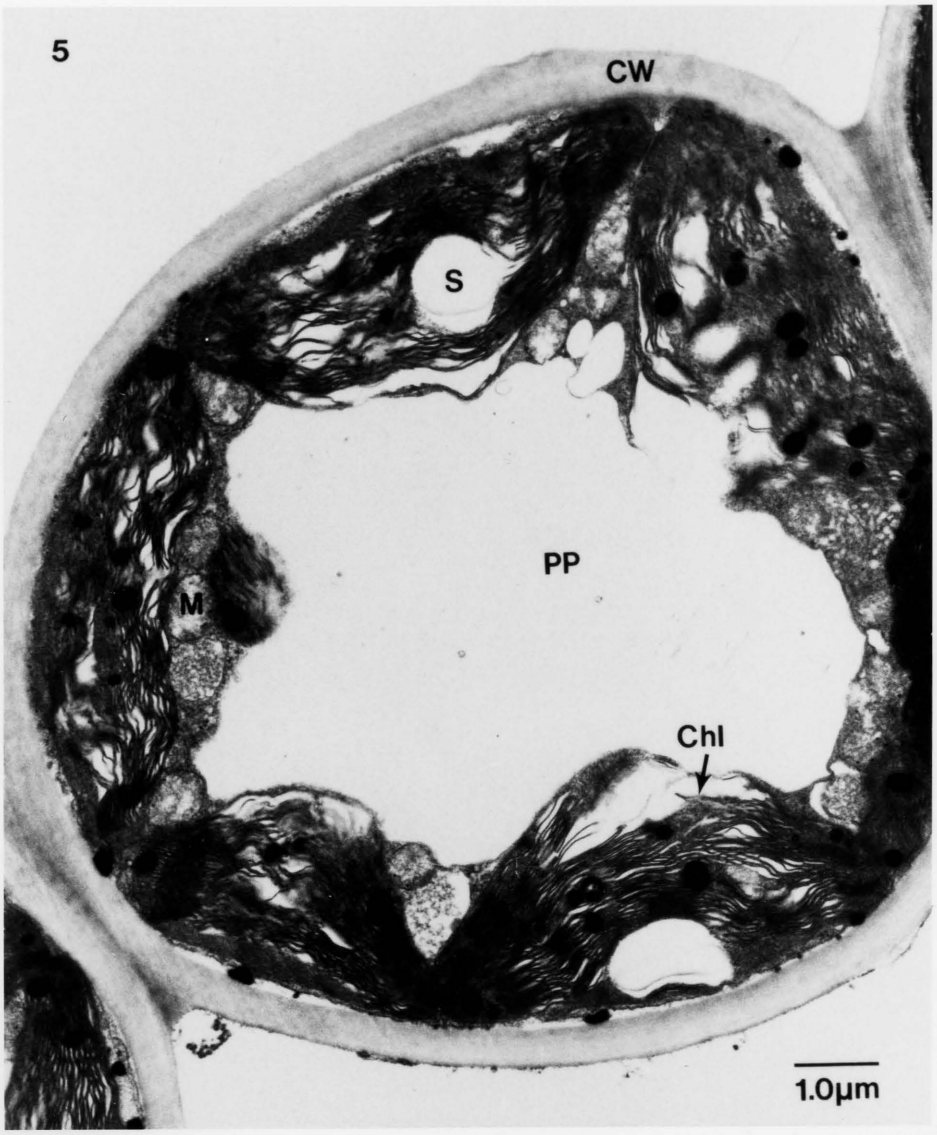
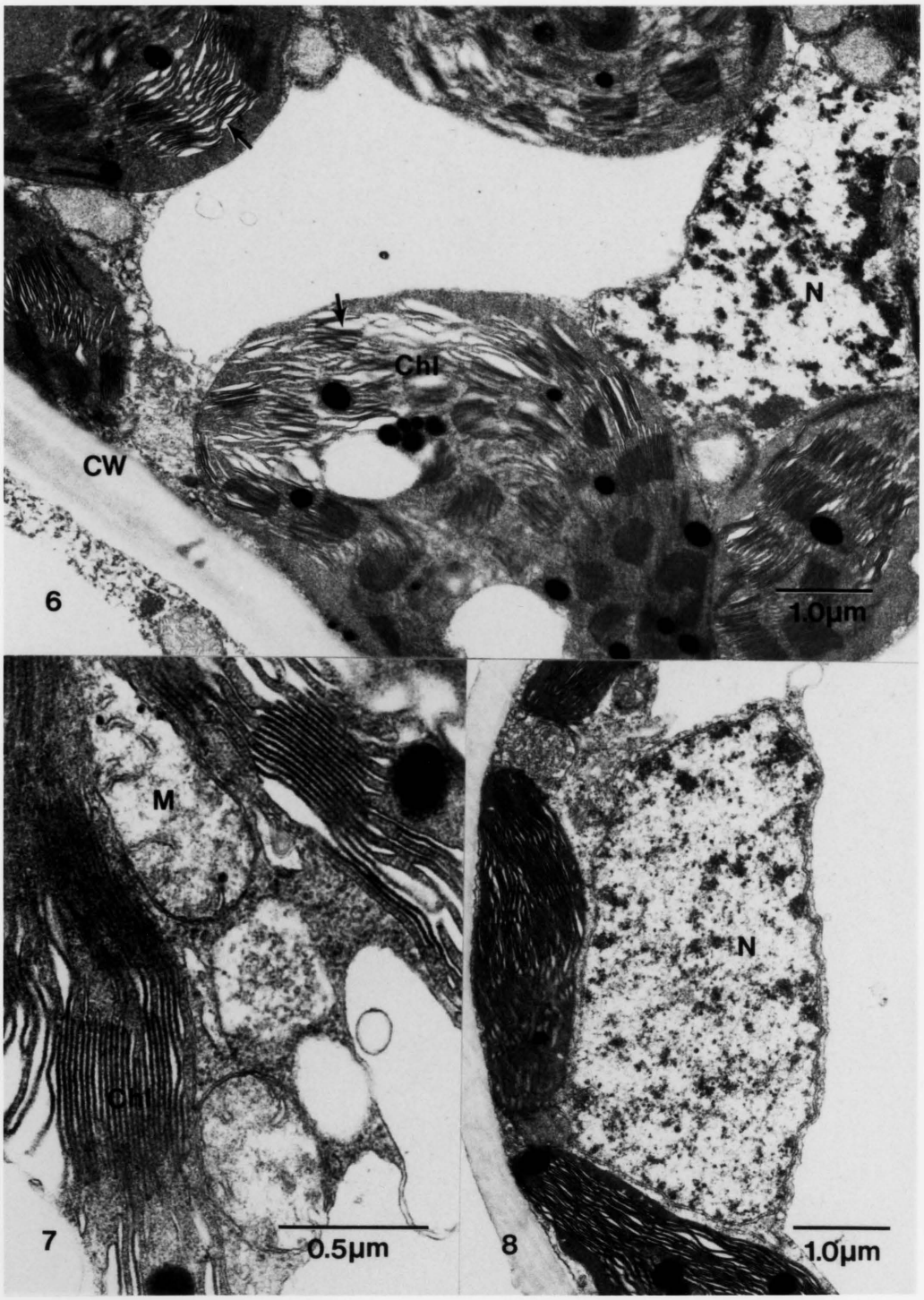

PP
Chl
3
1.0µm
PP
M
S
1.0µm
4

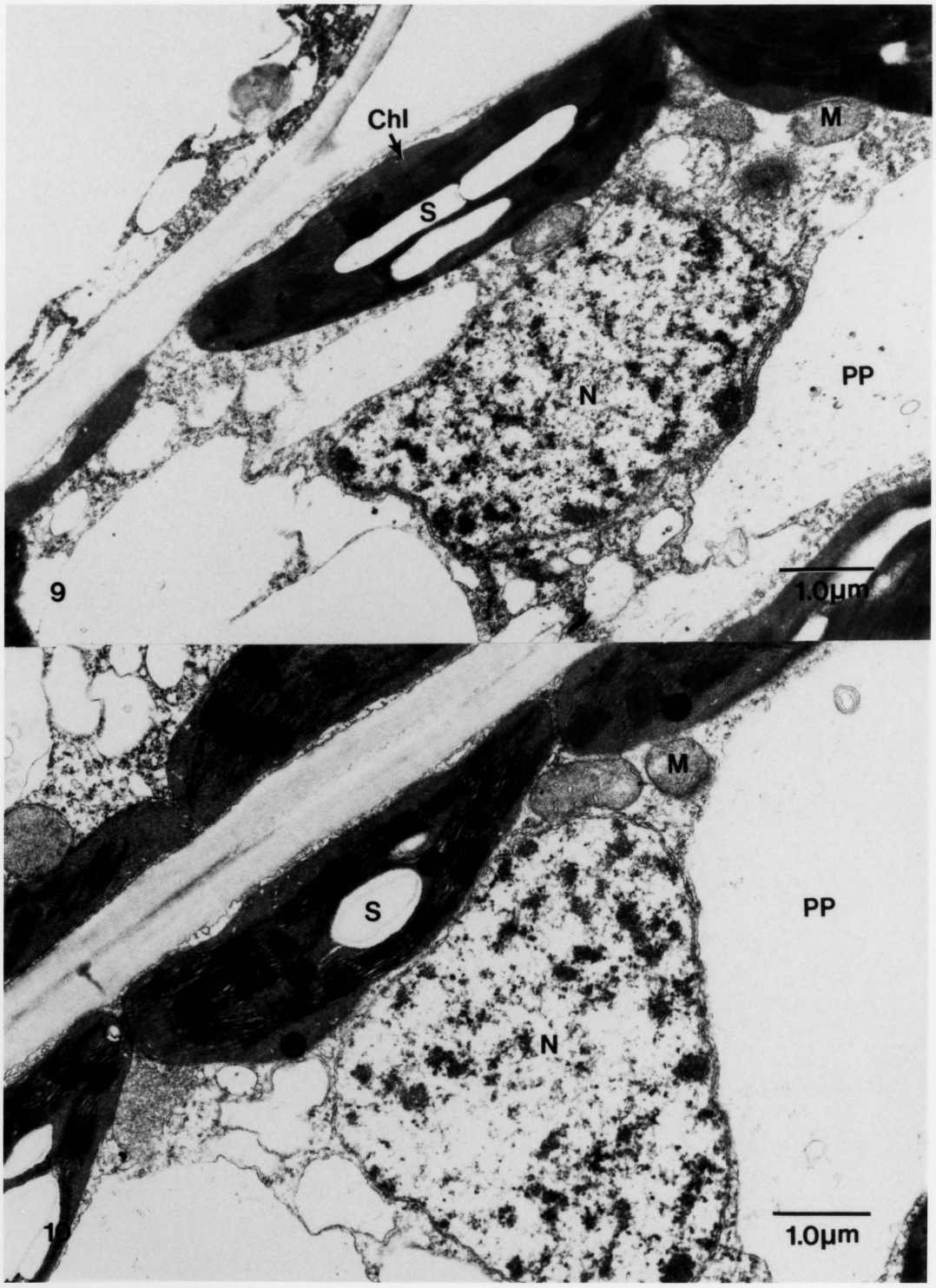
FIG. 4.5. Red Delicious leaves 12 hours after fungicide application. 5. Palisade parenchyma cell 12 hours after treatment with the SIF, bitertanol. Note membrane disruption and swelling of the thylakoids in chloroplasts as well as an increase in the number of starch granules; chloroplast (Chl), cell wall (CW), mitochondrion (M), starch (S), x11,000.


5
CW
S
PP
M
Chl
1.0µm

FIGS. 4.6-4.8. Red Delicious leaves 12 hours after fungicide application. 6. Higher magnification of chloroplasts 12 hours after fungicide application showing membrane disruption and thylakoid swelling (arrows); chloroplast (Chl), cell wall (CW), nucleus (N), x19,000. 7. Mitochondria 12 hours after treatment with the SIF, bitertanol. Note washed-out appearance; chloroplast (Chl), mitochondrion (M), x44,000. 8. Nucleus 12 hours after fungicide application. Nucleus appears normal with a uniform nuclear envelope; nucleus (N), x14,000.


N
Chl
CW
6
1.0µm
M
Chl
7
0.5µm
N
8
1.0µm

FIGS. 4.9-4.10. Red Delicious leaves 24 and 72 hours after fungicide application. 9. Transmission electron micrograph of a palisade parenchyma cell showing internal organelle arrangement 24 hours after treatment with the SIF, bitertanol. The nucleus, chloroplasts and mitochondria appear normal. Note increase in number of starch granules within the chloroplast compared to the control; chloroplast (Chl), mitochondrion (M), nucleus (N), starch (S), x14,000. 10. Bitertanol-treated palisade parenchyma cells after 72 hours showing internal arrangement of organelles. Nucleus, chloroplasts and mitochondria appear normal; mitochondrion (M), nucleus (N), starch (S), x14,500.


Chl
S
M
N
PP
9
1.0µm
M
S
PP
N
1.0µm

CHAPTER 5

THE ULTRASTRUCTURE OF *Spilocaea pomi* IN RED DELICIOUS LEAVES AFTER TREATMENT WITH THE STEROL-INHIBITING FUNGICIDE, BITERTANOL

ABSTRACT

Infection of bitertanol-treated Red Delicious leaves by Spilocaea pomi was examined at the fine structural level. Fungal hyphae of S. pomi encased within an extracellular matrix or slime layer were observed in infected Red Delicious leaves not treated with the fungicide. There appeared to be enzymatic degradation of both the cuticle and epidermal cell walls. Extensive granulation of fungal cytoplasm was evident during degradation of the cuticle and cell walls. This may have resulted from the accumulation of phenolic compounds and their oxidative products. However, extracellular matrices which enclosed the hyphae of S. pomi in untreated Red Delicious leaves were not observed in infected leaves treated with the fungicide. In addition, nuclei and mitochondria were both affected 12 and 72 hours following the application of the compound. Nuclear envelopes were not well defined and mitochondrial matrices were washed-out. There was also dissolution of normally plate-like cristae of mitochondria, accompanied by the accumulation of minute electron dense bodies around their periphery. Invaginations and proliferations of the plasmalemma were also observed as well as increased

vacuolization. Although mesosomes are not found in eukaryotic organisms, mesosome-like structures were found in hyphae 12 hours following application of the fungicide. Numerous effects of the SIF, bitertanol, were observed on the fine structure of S. pomi although a number of these were secondary. Different stages of ultrastructural change, including lysis, was found depending upon the toxicity and treatment period of the SIF.

INTRODUCTION

Bitertanol is a sterol-inhibiting fungicide (SIF) of the triazole type which has been reported to effectively control apple scab under greenhouse and orchard conditions (Kelley and Jones 1981). Bitertanol has shown high activity against cedar-apple rust and several fruit decay pathogens (Yoder and Hickey 1981) and also gave excellent protection against Podosphaera leucotricha (Ell. and Everh.) Salm. (Szkolnik 1981). The mode of action of this compound is inhibition of sterol C-14 demethylation (Gadher et al. 1983), causing the accumulation in fungi of ergosterol precursors that retain the 14α-methyl group, which appears to lead to the disruption of membrane-associated activities. Its efficacy against a broad range of important plant pathogens and high rate of activity at relatively low concentrations makes bitertanol a highly desirable fungicide.

Ultrastructural examinations of fungicide treated-sporidia of U. avenae (Hippe and Grossman 1981 and 1982) and similar results obtained in the research of cell pathology in various organisms treated with triazole, pyrimidine or imidazole derivatives have been explained on the basis of similar modes of action. Different stages of degradation,

including cell lysis have been described (deNollin and Borgers 1974 and 1976; Stiers et al. 1980), depending on the toxicity and incubation period of the compounds. Hoch and Szkolnik (1979) reported progressive necrosis of the protoplasm of Venturia inaequalis along hyphal profiles 24 hours following applications of the pyrimidine fungicide, fenarimol, at 50 and 100 hours after inoculation of leaves of Malus sylvestris (Mill.) plants. The entire subcuticular fungal stroma in the chlorotic regions was necrotic three weeks after fenarimol application.

Results obtained through electron microscopy may add further evidence to dependence of the structure of intracellular components upon membrane composition. Correlations may also exist between membrane destabilization due to a lack of ergosterol and cell physiology, and indicate morphological and fine structural consequences. Relatively little is known about the causal relationship between biochemical and structural changes in phytopathogenic fungi after fungicide treatment. Therefore, the purpose of the present study is to examine the effects of the SIF, bitertanol, on the fine structure of the Spilocaea state of V. inaequalis.

MATERIALS AND METHODS

The youngest six leaves fully expanded on actively growing shoot apices of 4 Red Delicious plants, located at the Winchester Fruit Research Center, were used in this study. A hand-held sprayer was used to apply the fungicide, Baycor® 50W (bitertanol), at 1.134 g/gal of water, until run-off occurred to abaxial and adaxial leaf surfaces infected with the fungus, Venturia inaequalis. Several 1-3mm rectangular strips from leaves, untreated and treated with the fungicide, were removed with a razor blade from the periphery of lesions containing green and chlorotic tissue 12 and 72 hours after fungicide application. Samples then were processed for electron microscopy as described previously in Chapter 4.

RESULTS

Cytoplasmic organization and structural integrity of various cellular components of Spilocaea pomi in untreated Red Delicious leaves are similar to that previously described (Corlett et al. 1976; Hoch and Szkolnik 1979; Maeda 1970). Fungal hyphae encased within an extracellular matrix or slime layer were observed adjacent to the cuticle (FIGS. 5.1 & 5.2). There appeared to be enzymatic degradation of both the cuticle and epidermal cell walls (FIG. 5.1). Mechanical separation may also be involved due to the irregular appearance of the lower surface of the cuticle and the upper surface of the epidermal cells where gaps occur (FIG. 5.1). Extensive granulation of fungal cytoplasm was evident during degradation of the cuticle and cell walls (FIG. 5.3), resulting in the accumulation of fibrillar material (FIG. 5.4). Spherical to elongated mitochondrial profiles with electron dense matrices and plate-like cristae were seen in the cytoplasm (FIGS. 5.2 & 5.5). The nucleus contains finely granular nucleoplasm surrounded by a uniform nuclear envelope (FIG. 5.5). Ribosomes were evenly distributed throughout the cytoplasm while vacuoles occupied only a small portion (FIGS. 5.2 & 5.5).

Infected Red Delicious leaves, sampled 12 hours following the application of the SIF, bitertanol, revealed a less dense cytoplasm and increased vacuolization of fungal hyphae (FIG. 5.6). Invagination of the plasmalemma was also observed (FIG. 5.6). The nuclear envelope was not as well defined compared to the control (FIG. 5.6). Mitochondrial matrices appeared washed-out and were accompanied by a reduction in their plate-like cristae (FIGS. 5.6 & 5.7). There was also the accumulation of minute electron dense bodies around the periphery of the mitochondria (FIGS. 5.6 & 5.7). Mesosome-like structures derived from the plasmalemma by invagination were observed in the form of double membrane-bound concentric rings (FIGS. 5.8 & 5.9).

Seventy-two hours following application of the fungicide, nuclear (FIG. 5.10 & 5.11) and mitochondrial profiles (FIGS. 5.11, 5.12 & 5.13) appeared similar to those described previously for 12 hours. In some instances, degradation of part or all of the mitochondrion was observed (FIGS. 5.12 & 5.13). Varying degrees of vacuolization, ranging from small increases (FIG. 5.10) to almost complete vacuolization of the fungal cell were also seen as well as invagination and proliferation of the plasma membrane (FIG. 5.14). In addition, areas containing glycogen-like rosettes were found in the cytoplasm (FIG. 5.15).

DISCUSSION

Fungal hyphae of Spilocaea pomi encased within an extracellular matrix or slime layer were observed in infected Red Delicious leaves not treated with the SIF, bitertanol. Palmer et al. (1983) observed in brown-rot fungi, a hyphal sheath enclosing released cellular contents which surrounded the hyphae or the cellulose fiber away from the hyphae. This suggested that the sheath or slime layer was a structure that maintains and provides translocation of cellular contents which may contain a depolymerizing agent required for the initial attack on cellulose. Extensive granulation of fungal cytoplasm was evident during degradation of the cuticle and cell walls. This may have resulted from the accumulation of phenolic compounds and other oxidative products. Release of cellular contents from hyphae into these extracellular matrices may provide nutrients for growing hyphae. Both proteinaceous materials and polysaccharides, which could be used immediately or stored, have been reported in sheaths (Bullock et al. 1980;

Olah and Reisinger 1974; Saito 1974; Van der Valk et al. 1977). Cytoplasmic organization and structural integrity of various organelles of S. pomi in untreated tissue were similar to that previously described (Corlett et al. 1976; Hoch and Szkolnik 1979; Maeda 1970).

Extracellular matrices or sheaths which enclosed the hyphae of S. pomi in untreated Red Delicious leaves, were not observed in infected leaves sprayed with the SIF, bitertanol. This suggests that the fungicide was effective against the structure which may house a depolymerizing agent necessary for the initial attack on cellulose as well as providing nutrients for growing hyphae. Nuclear and mitochondrial profiles of fungal hyphae both appear to be affected at 12 and 72 hours following the application of the fungicide to infected Red Delicious leaves. Nuclear envelopes were not well defined and may have broken down. Mitochondrial matrices appeared washed-out together with the dissolution of their plate-like cristae and accumulation of minute electron dense bodies around their periphery. These minute electron dense bodies are similar in size to ribosomes. Because of their close association with the mitochondria, these structures may more readily obtain energy in the form of ATP so that vital cellular processes may continue. Biochemical studies have suggested indirect

influence of ergosterol biosynthesis inhibitors on protein and nucleic acid metabolism as well as respiration processes (Buchenauer 1977a; Buchenauer 1977b; Siegel and Ragsdale 1978; Ragsdale and Sisler 1973).

Sterol-inhibiting fungicides rapidly curtail the biosynthesis of ergosterol. However, mycelial growth and various aspects of metabolism (respiration, protein and nucleic acid synthesis) are only mildly affected for a period of time after synthesis of the sterol has stopped. Ergosterol levels in treated mycelium do not decline rapidly which indicates that the rate of use of the sterol in membrane synthesis is slower than its biosynthesis. Interference in membrane synthesis occurs once the level of ergosterol becomes depleted, resulting in growth inhibition and changes in morphology and metabolism (Siegel 1981). The ultrastructural modifications observed in the nuclei and mitochondria in the present study may be the initial stages that influence protein and nucleic acid metabolism and respiration processes that result subsequently in growth inhibition. Hippe and Grossman (1982) found a large number of mitochondria in sporidia of Ustilago avenae treated with the SIFs, nuarimol and imazalil nitrate, accompanied by fragmentation of the internal structure of mitochondria as well as constriction of the organelles, resulting in mitochondria of small diameters.

The plasmalemma exhibited both invaginations and proliferations accompanied by increased vacuolization 12 and 72 hours following the application of bitertanol. Hippe and Grossman (1982) observed invaginations in the plasmalemma and tonoplast and proliferations at the tonoplast of vacuoles in sporidia of Ustilago avenae following treatment with the fungicides nuarimol and imazalil-nitrate. Altered composition and structure of the plasmalemma appear to be directly correlated to effects on membrane bound transport systems. Changes in permeability are connected to a variety of cytopathological symptoms which may be seen as invaginations and intramembrane modifications of the plasma membrane. Richmond (1984) also observed an increase in vacuolation and the number of lipid vesicles in triadimefon-treated germinating conidia of Botrytis allii. Nuarimol-treated sporidia were characterized by vacuolization and accumulation of lipid bodies (Hippe and Grossman 1982). These effects are consistent with the mode of action of SIFS, resulting in an interference with membrane synthesis and resulting accumulation initially of sterol intermediates and then FFAs (Siegel 1981). However, lipid accumulation was not evident in the present study. This may be due to ergosterol levels that do not decline rapidly in treated hyphae.

Numerous mesosome-like structures, which are not interpreted as mitochondria primarily because of their size and continuous double membrane-bound rings, are present in hyphae 12 hours following application of the fungicide. Since these structures increase the surface area, this may have an effect on the transport of materials into and out of the cells. Hall et al. (1974) previously reported these as membranous structures and suggested that they may be involved in polysaccharide synthesis during cell wall function. This suggests that the increased metabolic activity may be a dysfunction of the membranes.

Numerous effects of the SIF, bitertanol, have been observed on the fine structure of S. pomi in the present study; however, a number of these are secondary. Hoch and Szkolnik (1979) reported that phenylmercuric acetate, 10% applied at 0.5ml/L of water to infected leaves of Malus sylvestris and fenarimol 12.5% applied at 0.37ml/L of water, affected subcuticular hyphae and individual cells of V. inaequalis in much the same way. In 24 hour samples most mitochondria were swollen and their flattened cristae were inflated and tubular in appearance. Cisternae of nuclear envelopes were occasionally swollen. Eventually, entire cells and hyphae became necrotic. The entire subcuticular fungal stroma was necrotic three weeks after application of

the SIF, fenarimol (Hoch and Szkolnik 1979). Depending on the toxicity and incubation period of the applied substance, different stages of ultrastructural changes up to complete lysis may be observed.

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FIG. 5.1. Untreated Spilocaea pomi. 1. Transmission electron micrograph of untreated fungal hyphae of Spilocaea pomi encased within a slime layer adjacent to the cuticle of a Red Delicious leaf. Note degradation of both the cuticle and epidermal cell walls (arrows); cuticle (C), epidermis (E), slime layer (sl), x8,000.

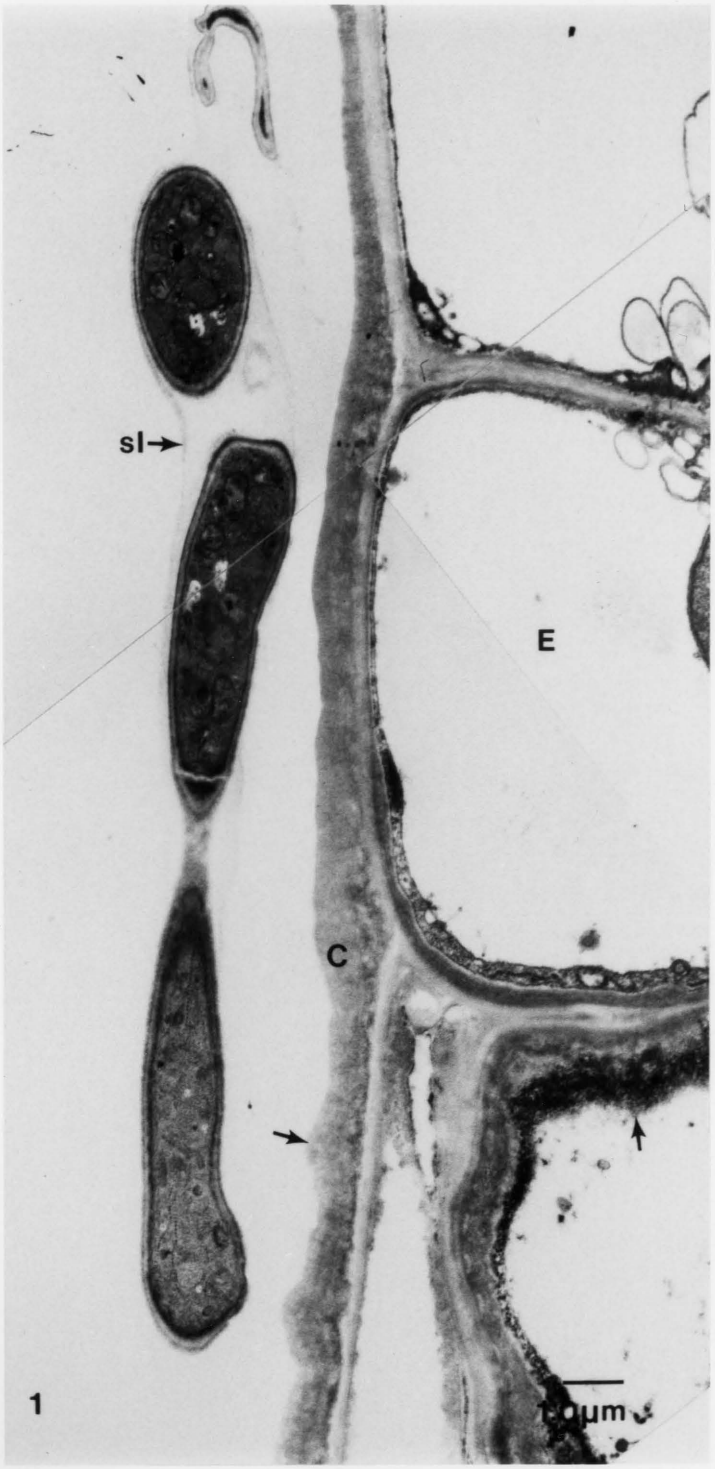
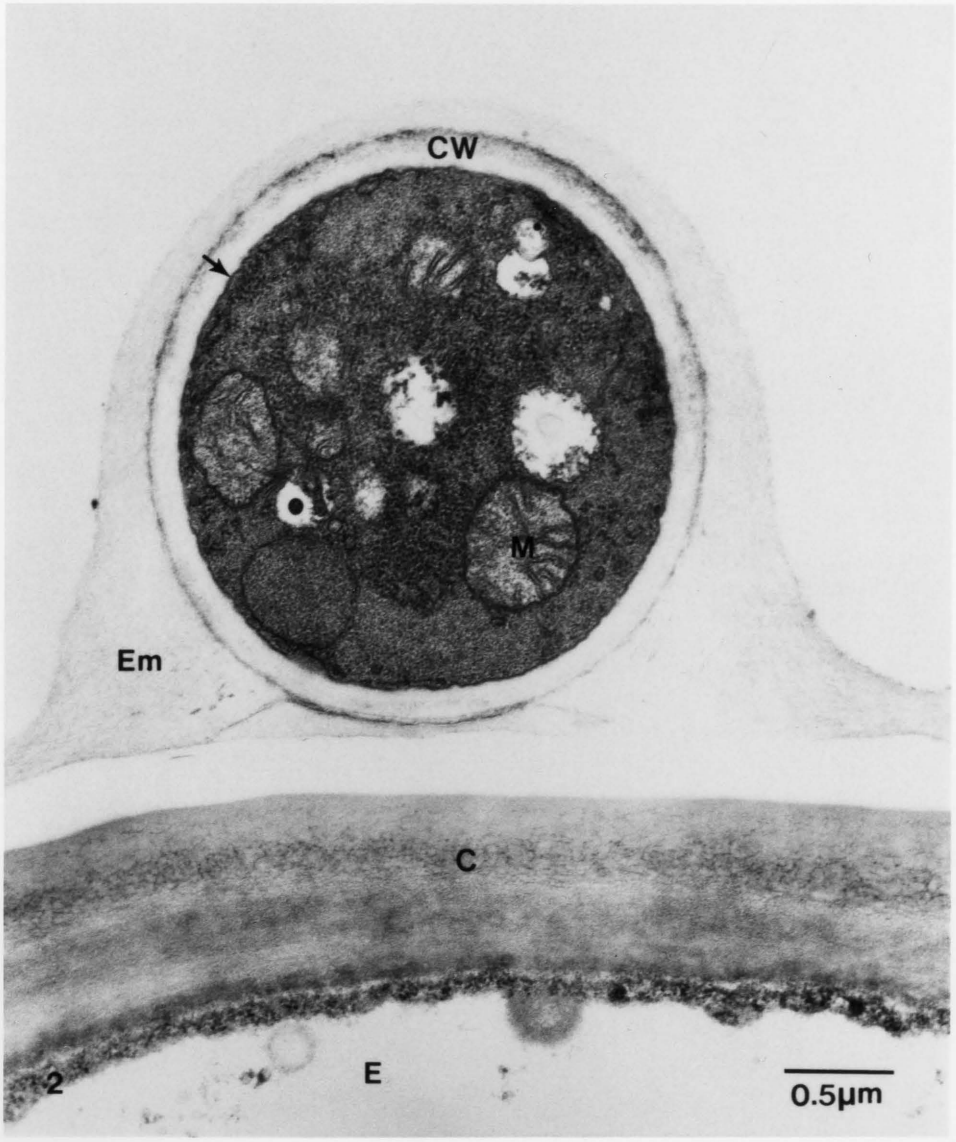
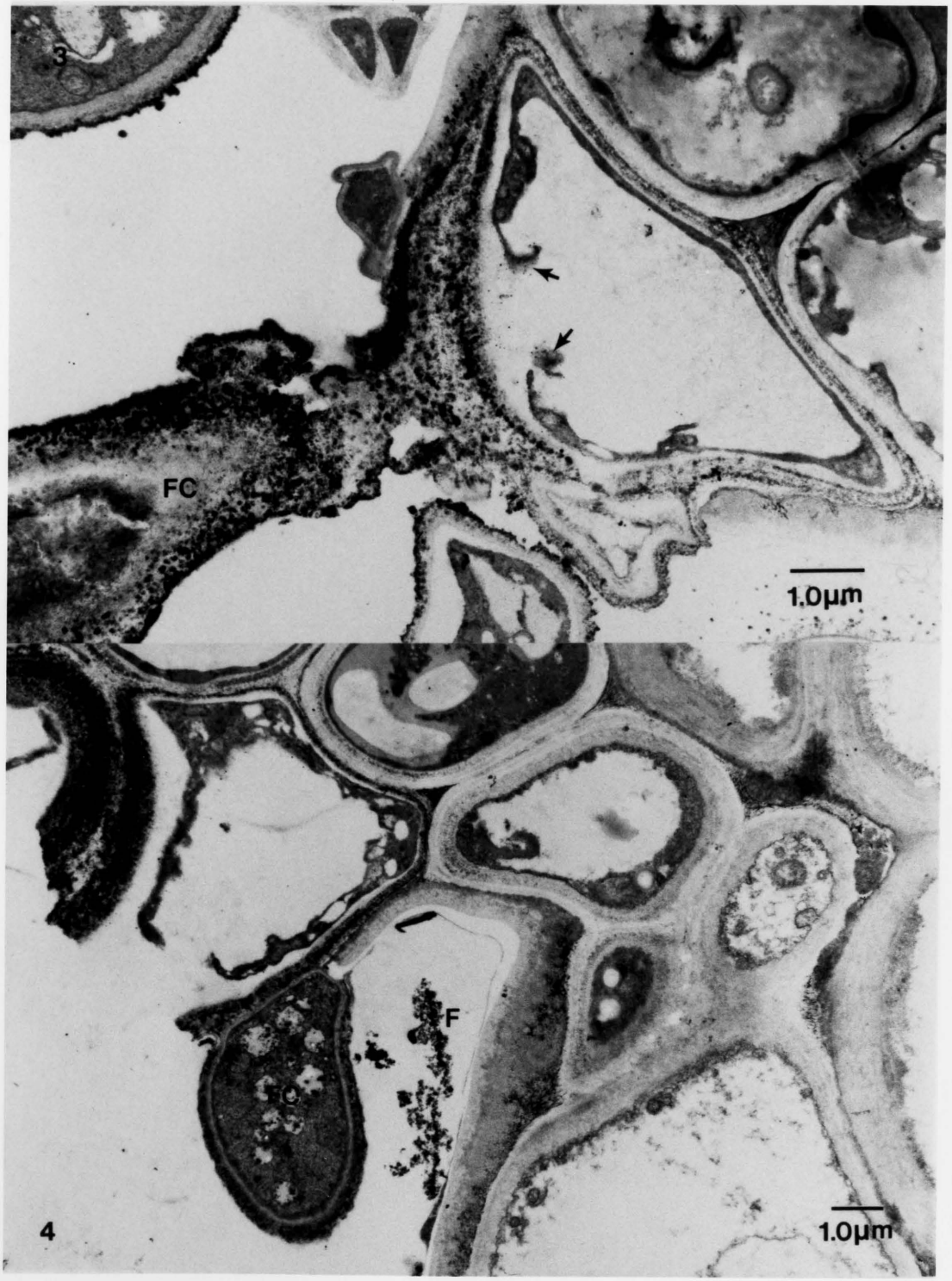

sl
E
C
1
1 µm

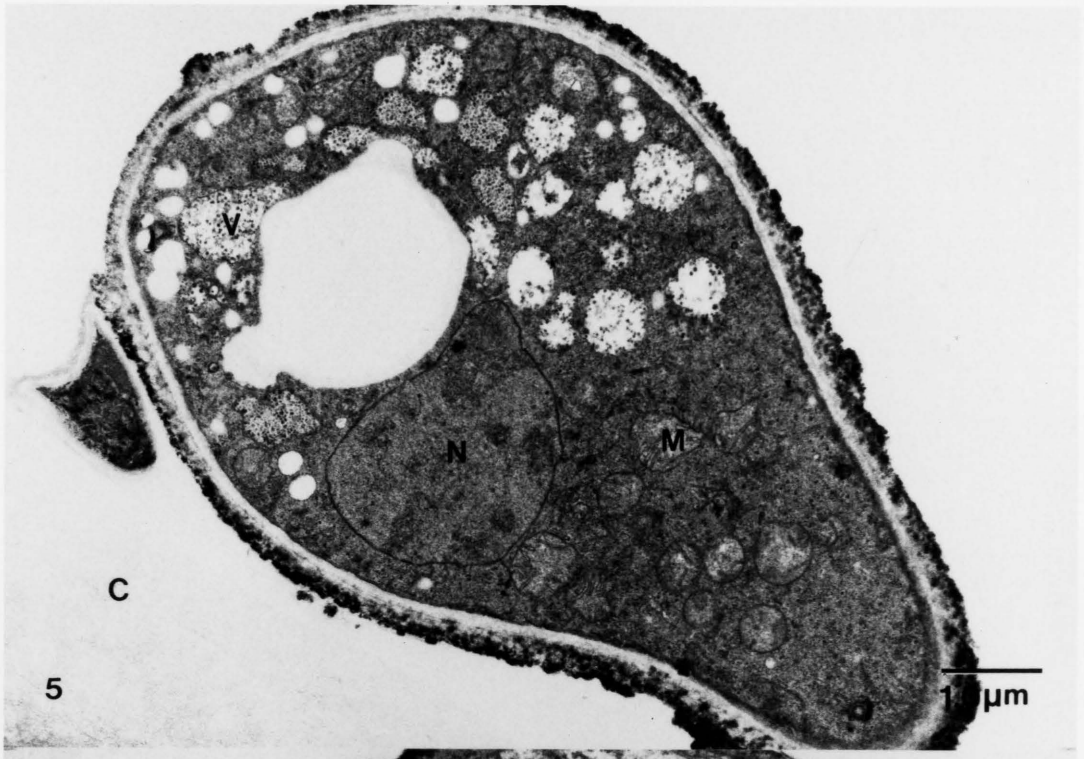
FIG. 5.2. Untreated Spilocaea pomi. 2. Untreated fungal hyphae encased within an extracellular matrix or slime layer showing internal arrangement of organelles. Note undulating plasma membrane (arrow) surrounded by a thick cell wall and plate-like cristae of mitochondria; cuticle (C), cell wall (CW), epidermis (E), extracellular matrix (Em), mitochondrion (M), x28,000.

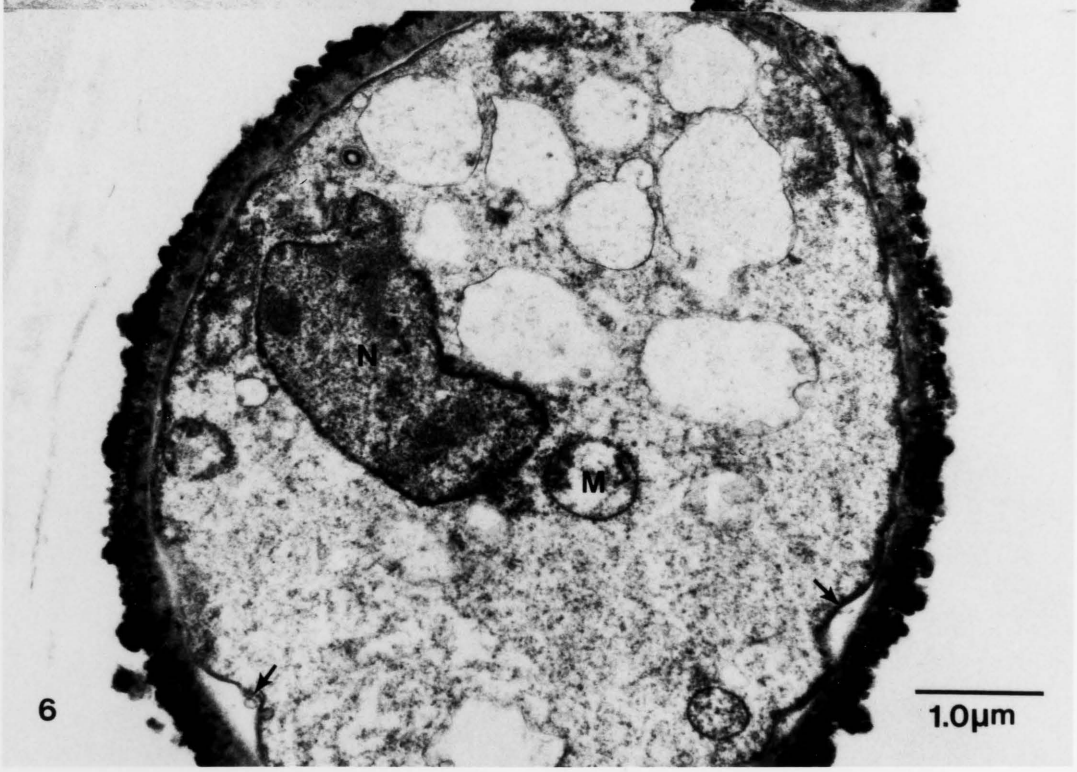

CW
M
Em
C
E
2
0.5µm

FIGS. 5.3-5.4. Untreated Spilocaea pomi. 3. Degradation of the cuticle and epidermal cell walls (arrows) showing extensive granulation of fungal cytoplasm in untreated tissue; fungal cytoplasm (FC), x11,500. 4. Untreated tissue showing the accumulation of fibrillar material during degradation of the cuticle and epidermal cell walls; fibrillar material (F), fungal cytoplasm (FC), x8,000.

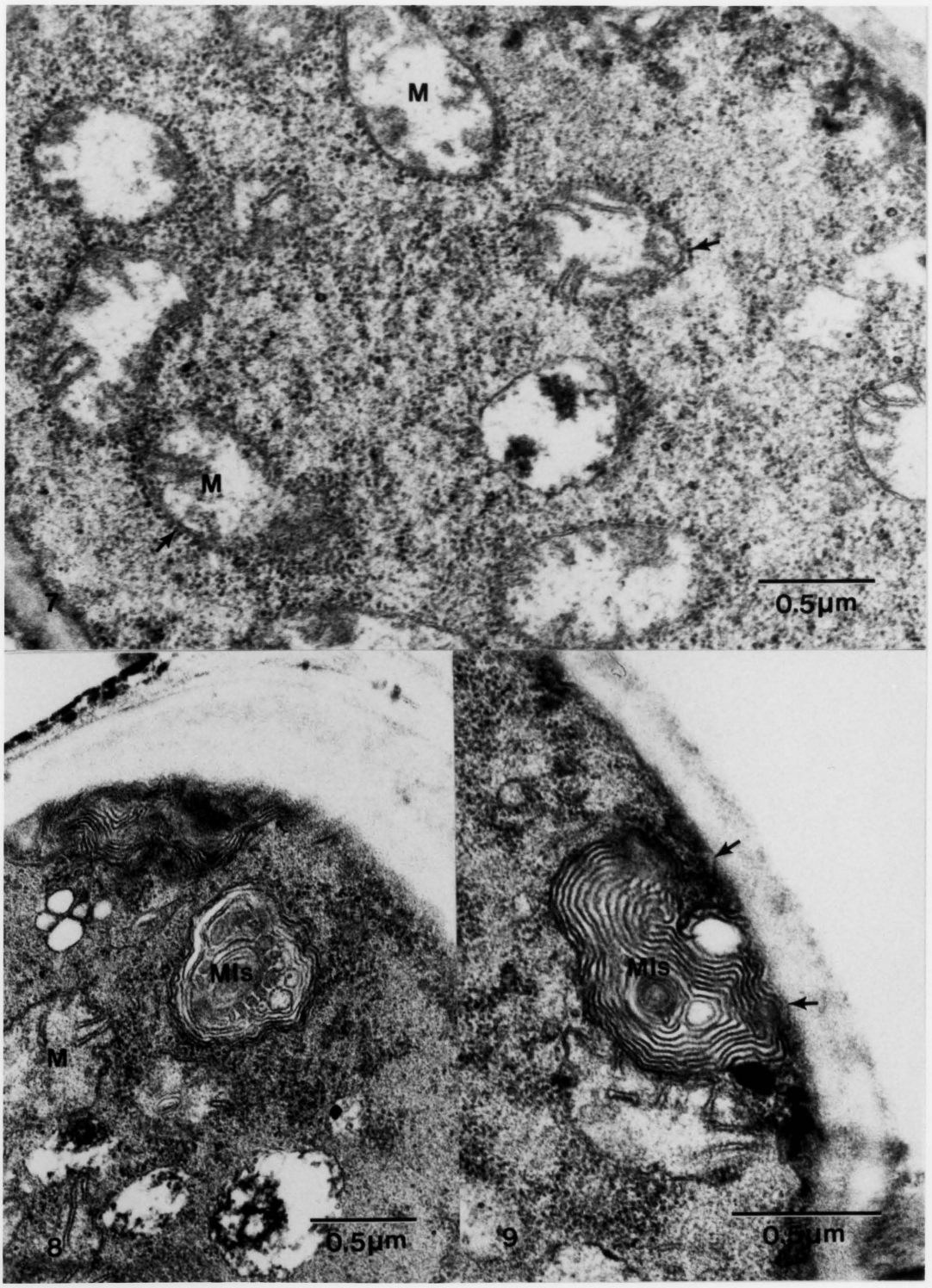

3
FC
1.0µm
F
4
1.0µm

FIGS. 5.5-5.6. Spilocaea pomi. 5. Transmission electron micrograph of fungal hyphae not treated with the fungicide showing internal organelle arrangement. Note nucleus with uniform nuclear envelope; cuticle (C), mitochondrion (M), nucleus (N), vacuole (V), x13,000. 6. Fungal hyphae 12 hours after treatment with the SIF, bitertanol. Note invagination of the plasmalemma (arrows), washed-out appearance of the mitochondria and possible degradation of the nucleus as evidenced by the accumulation of electron dense material around the nuclear envelope; mitochondrion (M), nucleus (N), x16,000.

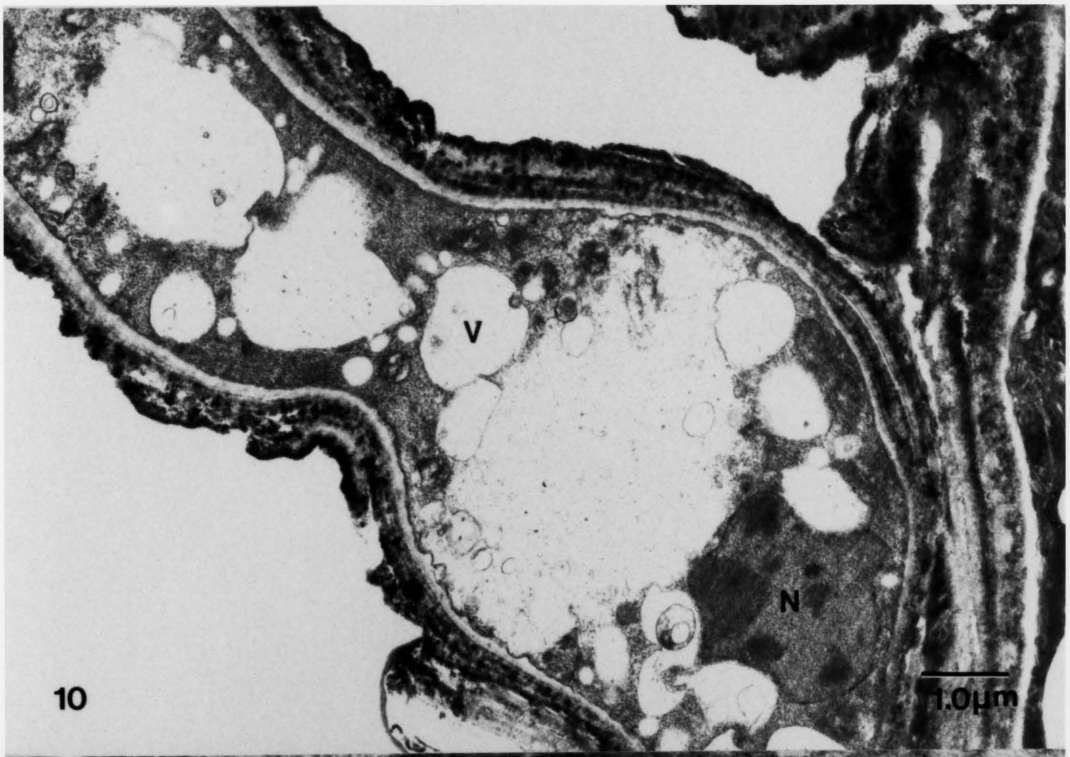

V
N
M
C
5
µm


N
M
6
1.0µm

FIGS. 5.7-5.9. Spilocaea pomi 12 hours after fungicide application. 7. Fungal mitochondria 12 hours after treatment with the fungicide. Note degradation of mitochondrial profiles as well as the accumulation of minute electron dense bodies around their periphery (arrows); mitochondrion (M), x35,000. 8. Bitertanol-treated hyphae after 12 hours. Note formation of mesosome-like structures; mitochondrion (M), mesosome-like structure (Mls), x32,000. 9. Higher magnification of mesosome-like structure 12 hours after fungicide application. Note formation of mesosome-like structure via invagination of plasma membrane (arrows); mesosome-like structure (Mls), x45,000.


M
M
0.5µm
7
MIs
M
0.5µm
8
MIs
9

FIGS. 5.10-5.11. Spilocaea pomi 72 hours after fungicide application. 10. Transmission electron micrograph of fungal hyphae 72 hours after treatment with the SIF, bitertanol. Note internal organelle arrangement as well as increased vacuolization; nucleus (N), vacuole (V), x11,500. 11. Nucleus and mitochondrion 72 hours following fungicide application. Note possible nuclear degradation (arrows) and loss of structural integrity of the mitochondrion; mitochondrion (M), nucleus (N), x22,000.


V
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10
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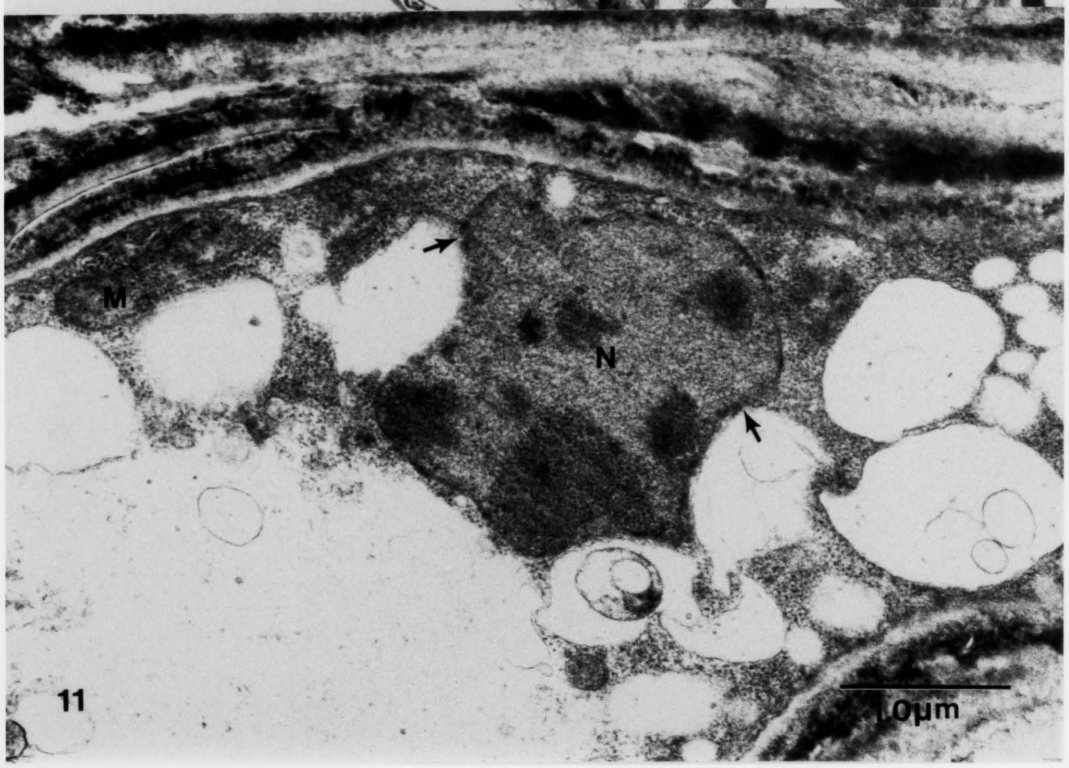
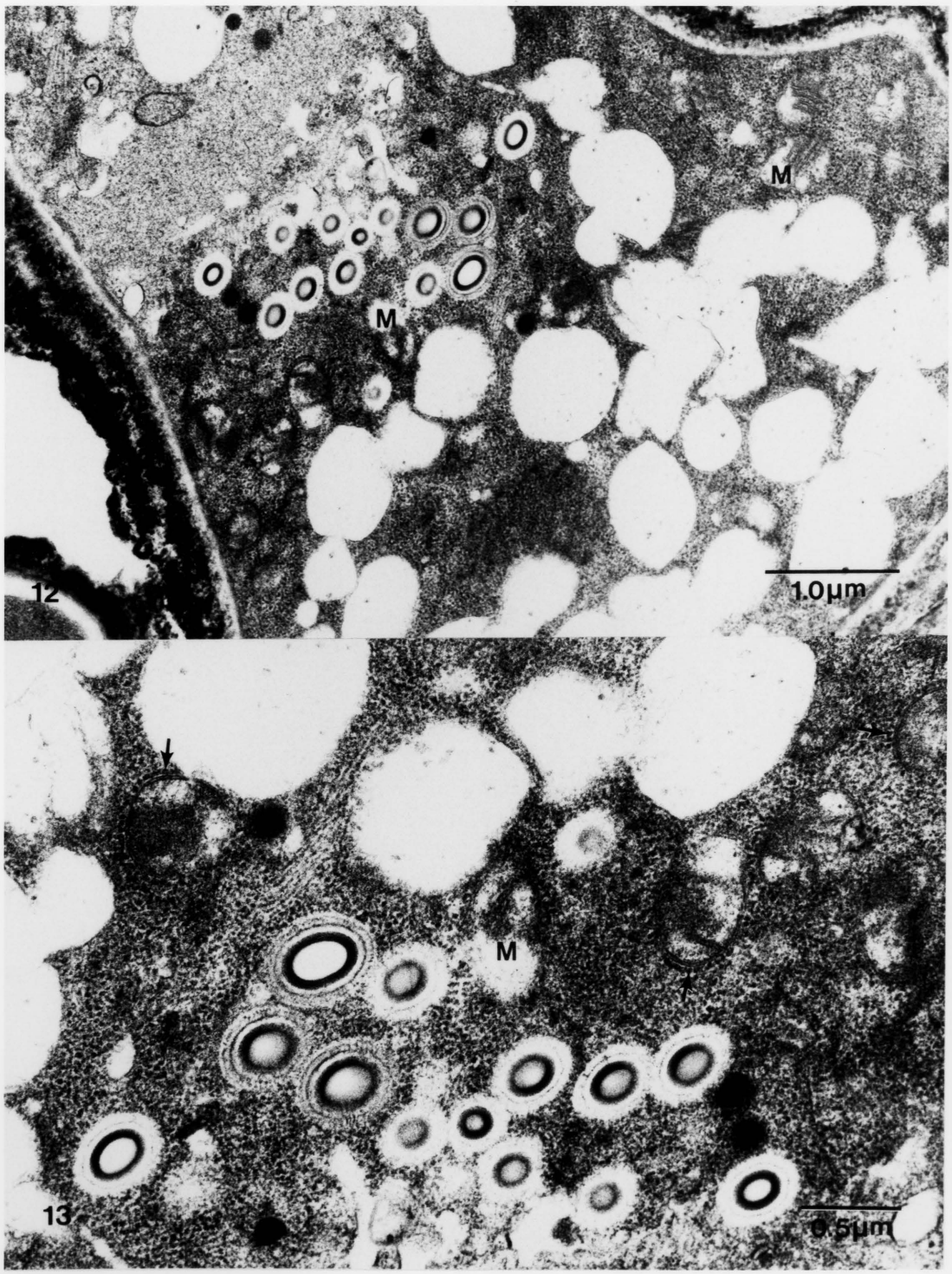
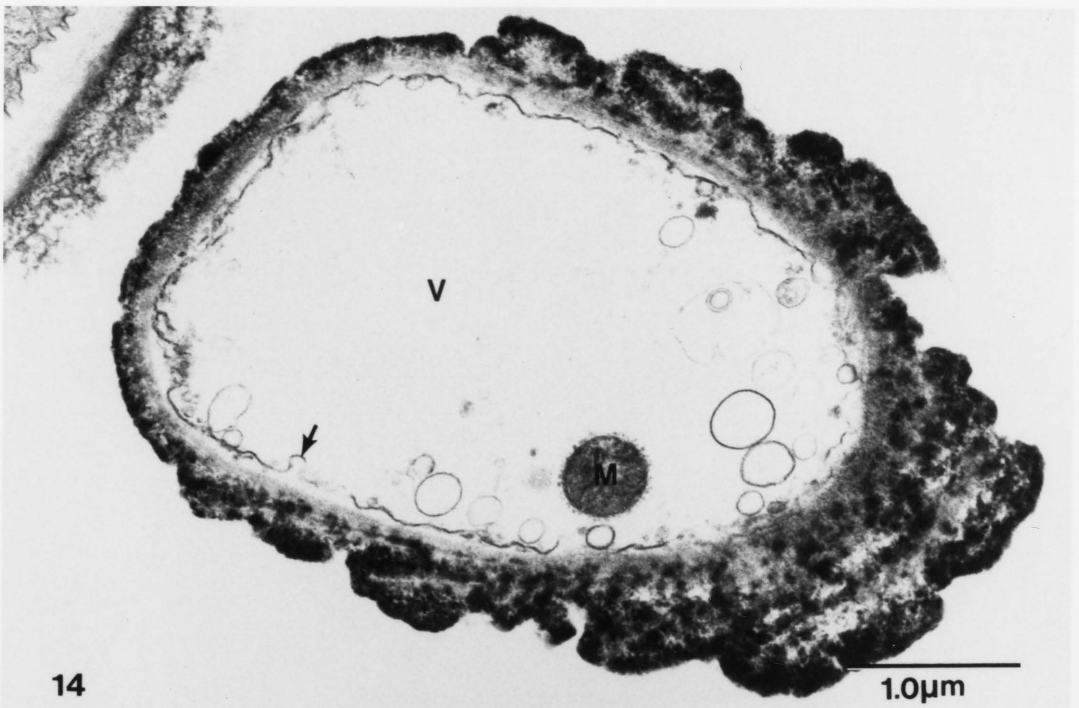

M
N
11
1.0µm

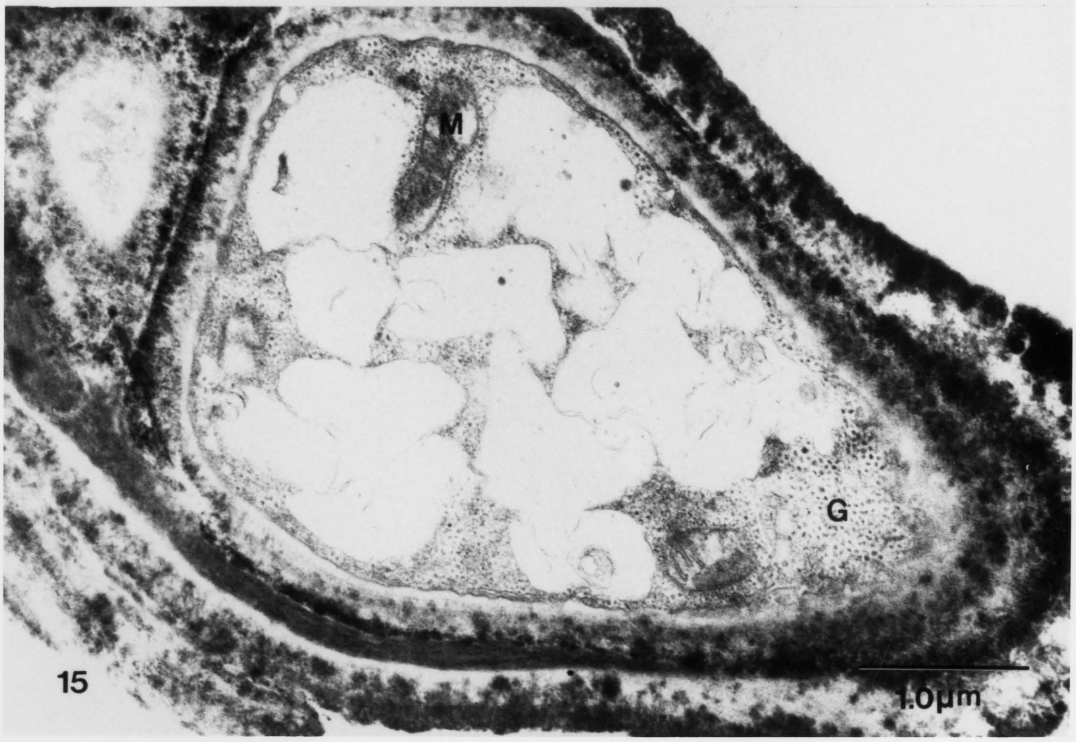
FIGS. 5.12-5.13. Spilocaea pomi 72 hours after fungicide application. 12. Bitertanol-treated hyphae after 72 hours. Note mitochondrial degradation and increased vacuolization; mitochondrion (M), x20,000. 13. Higher magnification of mitochondria 72 hours after treatment with the fungicide. Note degradation of mitochondrial profiles and their plate-like cristae as well as the accumulation of minute electron dense bodies around their periphery (arrows); mitochondrion (M), x30,000.


M
M
12
1.0µm
M
13
0.5µm

FIGS. 5.14-5.15. Spilocaea pomi 72 hours after fungicide application. 14. Almost complete vacuolization of a fungal cell 72 hours after fungicide application. Note invagination and proliferation of the plasmalemma; mitochondrion (M), vacuole (V), x22,000. 15. Formation of glycogen-like rosettes 72 hours after treatment with the SIF, bitertanol; glycogen (G), mitochondrion (M), x22,000.



14



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CHAPTER 6

THE FINE STRUCTURE OF FENARIMOL-TREATED CONIDIA OF *Spilocaea pomi*

ABSTRACT

Electron microscopic observations were made of the conidial state of Venturia inaequalis following the application of the sterol-inhibiting fungicide, fenarimol. Conidia treated with the fungicide for 2 hours revealed necrotic areas throughout the cytoplasm. The plasmalemma was not well defined and in the process of degradation. Increased vacuolization was also observed as well as numerous lipid bodies and multivesicular complexes which contained vesicles of varying electron densities. Structural integrity of the organelles was such that they were difficult to discern. Ultrastructural observations of conidia exposed to the fungicide for 4 hours were similar to those for 2 hours. However, after 12 hours, the entire fungal cell was necrotic accompanied by the degradation of the cell wall.

INTRODUCTION

Fenarimol is a sterol-inhibiting fungicide (SIF) which specifically inhibits sterol C-14α-demethylation, thereby causing the accumulation in fungi of ergosterol precursors that retain the 14α-methyl group which leads to the disruption of membrane-associated activities (Gadher et al. 1983). Henry and Sisler (1984) suggested that the primary action of fenarimol is competitive inhibition of sterol/steroid-type cytochrome P-450 enzymes rather than interference with the function of sterol carrier proteins or enzyme-modulating phospholipids. No evidence for target sites exists in other pathways of comparable sensitivity, although the failure of additional ergosterol to reverse the fungitoxicity suggests the existence of other sites. However, growth inhibition by high concentrations of pyrimidin-5-ylmethanol, not reversible by GA, perhaps involves action at sites outside the biosynthesis pathway (Sisler et al. 1984). They suggested from several studies of the mechanisms of fungitoxicity and growth regulation that any undetected primary targets of the pyrimidin-5-ylmethanols are likely to be haem enzymes, similar to the cytochrome P-450 involved in sterol C-14 demethylation.

Electron microscopic studies of fungicide-treated sporidia of U. avenae (Hippe et Grossman 1981 and 1982) and similar results obtained in the research of cell pathology in various organisms treated with triazole, pyrimidine or imidazole derivatives have been explained on the basis of similar modes of action. Depending on the toxicity and incubation period of the compounds, different stages of degradation up to complete cell lysis have been described (de Nollin and Borgers 1974 and 1976; Stiers et al. 1980). Results obtained through electron microscopy studies may add further evidence to the dependence of the structure of intracellular components upon the membrane composition. In addition, correlations may exist between membrane destabilization due to a lack of ergosterol and cell physiology, and indicate morphological and fine structural changes. Relatively little is known about the causal relationship between biochemical and structural changes in phytopathogenic fungi after fungicide treatment. Therefore, the purpose of this study is to examine the effects of the SIF, fenarimol, on the organization of a cell system of the Spilocaea state of V. inaequalis in vitro.

MATERIALS AND METHODS

Isolates of Venturia inaequalis were collected via single spore isolation from leaves of Red Delicious trees, located at the Winchester Fruit Research Center, and transferred to antibiotic potato dextrose agar (PDA) plates. Propagation of conidia of V. inaequalis was via transfer of mycelial plugs obtained from cultures grown on PDA plates. For this, 20-30ml of 4% malt extract solution was added to 8oz flat sided prescription bottles fitted with screw caps. Cheesecloth was placed against the inside of the front face of the bottle and then autoclaved for 20 min at 15 psi. The cheesecloth was inoculated with mycelial plugs from PDA plates 2 cm above the medium line when the bottle was on its side. The bottle was then placed flat with the cheesecloth immersed in the broth for fours days at 19°C followed by a turn on its side for an additional ten days. Approximately 100,000 conidia/ml were collected via filtering through cheesecloth and subsequently exposed to the SIF, Rubigan® 1E (fenarimol), at 0.117 ml/L of water for 2, 4 and 12 hours.

Controls and conidia treated with the fungicide for 2, 4 and 12 hours, were then processed for electron microscopy. This involved the sequential fixation of specimens in 4%

paraformaldehydeglutaraldehyde-acrolein and 2% osmium tetroxide. Specimens were placed in 4% PGA in 0.1M sodium cacodylate buffer (pH 6.8) for five hours at -4°C, washed in three changes of 0.1M sodium cacodylate buffer for a total of one hour, and postfixed in sodium cacodylate buffered 2% osmium tetroxide overnight. Following osmium fixation, specimens were washed in three changes of 0.1M sodium cacodylate buffer for a total of one hour and then in distilled water for 20 minutes. Specimens were dehydrated in a graded ethanol series (20 minutes each) followed by acetone (two changes of 10 minutes each). The specimens were infiltrated overnight and embedded in Spurr's low-viscosity resin (Spurr 1969) the next day. Blocks were cured at 70°C for 24 hours. Sections cut on a Sorvall MT2B ultramicrotome with a Dupont diamond knife were collected on 200-mesh copper grids and double stained with 2% uranyl acetate for 30 minutes and Reynold's lead citrate (Reynolds 1963) for eight minutes.

RESULTS

Cytoplasmic organization and structural integrity of various cellular components of Spilocaea pomi in vitro are similar to that previously described (Corlett et al. 1976; Hoch and Szkolnik 1979; Maeda 1970). Cytoplasm of conidia is uniformly dense with ribosomes and ground cytoplasm surrounded by an undulating plasmalemma and thick cell wall (FIGS. 6.1 & 6.2). Spherical to elongated mitochondrial profiles with electron dense matrices and plate-like cristae were also present (FIGS. 6.1 & 6.2). The endoplasmic reticulum was primarily of the granular type and located randomly throughout the cytoplasm (FIG. 6.1). The conidia contained lipid and vacuoles with electron-dense material (FIG. 6.1). Occasionally, septate conidia were accompanied by mesosome-like structures derived from the plasmalemma by invagination (FIG. 6.2).

Conidia treated with the SIF, fenarimol, for 2 hours revealed moribund areas throughout the cytoplasm accompanied by an increase in vacuolization (FIGS. 6.3 & 6.4). These areas were more electron dense than the surrounding cytoplasm. The undulating plasma membrane was no longer distinct and appeared to be degrading (FIGS. 6.3 & 6.4).

The conidial cytoplasm also appeared to be shrinking and separated from the adjacent cell wall (FIGS. 6.3, 6.4 & 6.5). An increased number of lipid bodies and multivesicular complexes (MVC) containing numerous vesicles in varying stages of electron densities were observed within the cytoplasm (FIGS. 6.3 & 6.4). The structural integrity of the organelles were such that they are difficult to recognize (FIGS. 6.3, 6.4 & 6.5). Occasionally, possible mitochondrial profiles were discernible (FIG. 6.4). Numerous MVCs appeared throughout the cytoplasm in conidia exposed for 4 hours to the fungicide (FIG. 6.5). Again, structural integrity of the organelles were such that they were not readily discernible. Eventually, the entire fungal cell became necrotic (FIGS. 6.6 & 6.7) after 12 hours, accompanied by the degradation of the cell wall (FIG. 6.7).

DISCUSSION

The organization of conidial cytoplasm and structural integrity of various cellular organelles of S. pomi are

similar to that previously described (Corlett et al. 1976; Hoch and Szkolnik 1979; Maeda 1970). However, numerous changes occur in the fine structure of conidia of S. pomi with exposure to the SIF, fenarimol.

Conidia treated with fenarimol for 2 and 4 hours showed increases in the degree of vacuolization and the number of lipid bodies. Richmond and Pring (1969) reported an increase in vacuolation in germinating conidia of Botrytis fabae treated with benomyl. Richmond (1984) also observed an increase in vacuolation and in the number of lipid vesicles in triadimefon-treated germinating conidia of Botrytis allii. Nuarimol-treated sporidia of Ustilago avenae were characterized by vacuolation and accumulation of lipid bodies (Hippe and Grossman 1982). Naftifine produced accumulations of lipid bodies in Candida parapsilosis (Meingassner and Sleytr 1982). These effects are consistent with the mode of action of SIFS, resulting in an interference with membrane synthesis and resulting accumulation initially of sterol intermediates and then FFAs (Siegel 1981).

In the present study, there appeared to be some effect of the SIF, fenarimol, on the plasma membrane. In a study by Hippe and Grossman (1982) on sporidia of Ustilago avenae following treatment with the fungicides nuarimol and

imazalil nitrate, changed composition and structure of the plasmalemma appeared to be directly correlated to effects on membrane bound transport systems. Changes in permeability are connected to a variety of cytopathological symptoms which may be seen as invaginations and intramembrane modifications of the plasma membrane. In addition, an increase in the number of lipid bodies may be correlated with increased lysis of the membranes (Hippe 1984).

The effects of fenarimol also revealed the localization of necrotic areas and the accumulation of cytoplasmic vesicles within MVCs. This was reported previously by Hoch and Szkolnik (1979) in V. inaequalis following treatment with the SIF, fenarimol. Multivesicular complexes, which may function as autophagosomes, increased in number after 4 hours suggesting further degradation of the cell. The accumulation of vesicles within such structures is similar to cytoplasmic responses toward toxic chemicals (Aist 1976). These responses may represent a cytoplasmic compartmentalization of the fungicide as suggested by Hoch and Szkolnik (1979).

Even after a 2 hour exposure to the fungicide, the structural integrity of the organelles was such that they were difficult to recognize and after 12 hours, the entire fungal cell was necrotic. In the transition period from 4

to 12 hours, lipoidal material may escape through the membrane resulting in cellular shrinkage and final necrosis. The cell wall was also observed to be breaking down. However, differences in cell wall structure cannot be satisfactorily explained, because cell wall synthesis in fungi is a complicated process. Protein biosynthesis is of primary importance for the regulation of growth processes in addition to cell wall formation. Correlations between physiological properties and cell wall irregularities found in U. avenae can be deduced from biochemical studies that suggest indirect influence of ergosterol biosynthesis inhibitors on protein and nucleic acid metabolism as well as respiration processes (Buchenauer 1977a; Buchenauer 1977b; Siegel and Ragsdale 1978; Ragsdale and Sisler 1973). There are similar correlations between physiological processes and membrane structure and activity.

In conclusion, numerous effects of the SIF, fenarimol, have been observed on the fine structure of S. pomi in vitro. Depending on the toxicity and incubation period of the applied substance different stages of degradation up to complete lysis may be observed. Untreated conidia remained viable throughout the incubation periods indicating that the ultrastructural changes were due to the fungicide.

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FIG. 6.1. Untreated Spilocaea pomi. 1. Transmission electron micrograph of an untreated conidium of Spilocaea pomi showing internal arrangement of organelles and a thick external cell wall. Note undulating plasma membrane; cell wall (CW), mitochondrion (M), plasma membrane (PM), rough endoplasmic reticulum (rER), vacuole (V), x20,000.

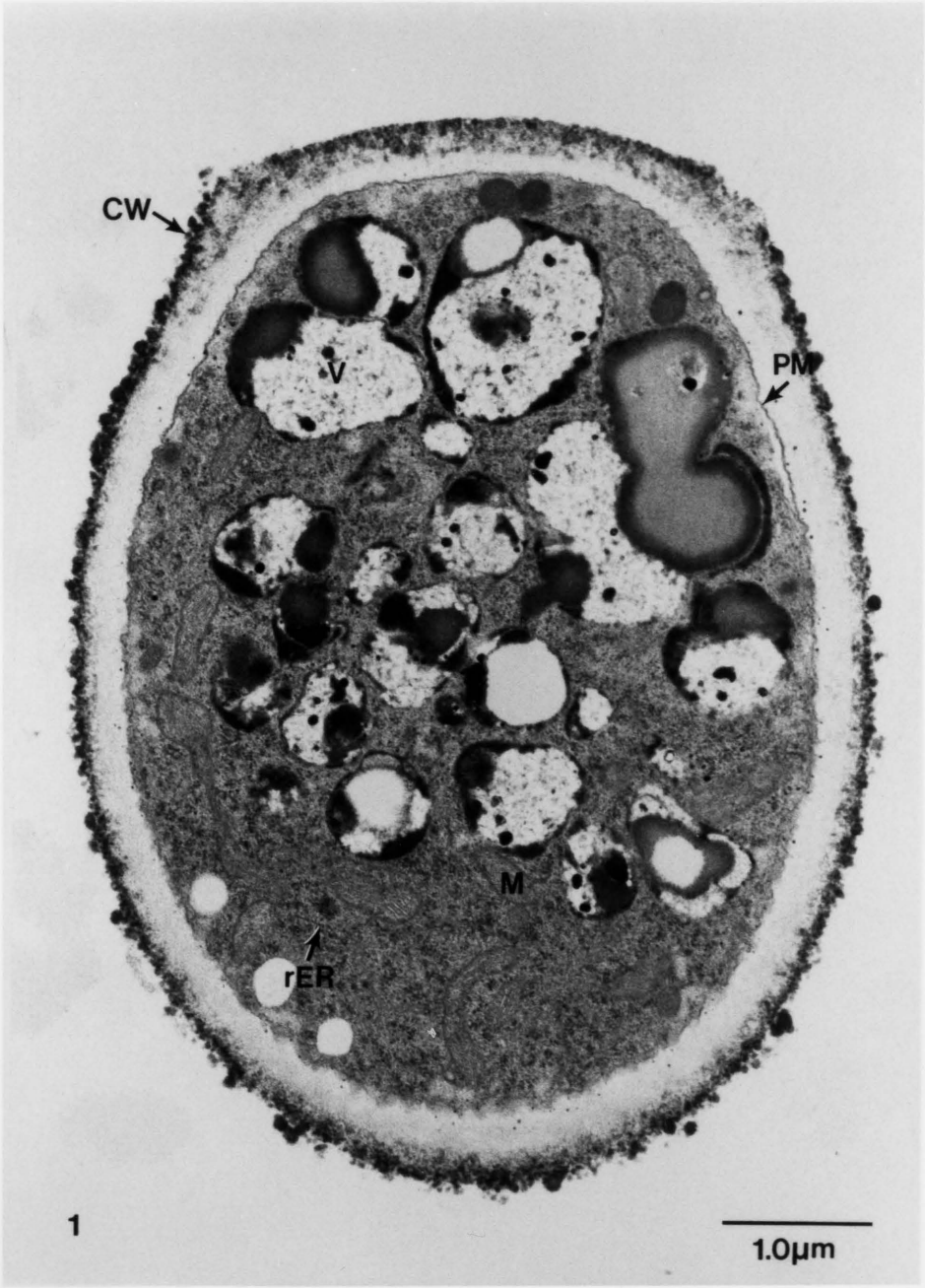

CW
V
PM
M
rER
1
1.0µm

FIG. 6.2. Untreated Spilocaea pomi. 2. Untreated septate conidium showing internal organelle arrangement. Note mesosome-like structure adjacent to septum; mitochondrion (M), mesosome-like structure (Mls), plasma membrane (PM), septum (S), x20,000.

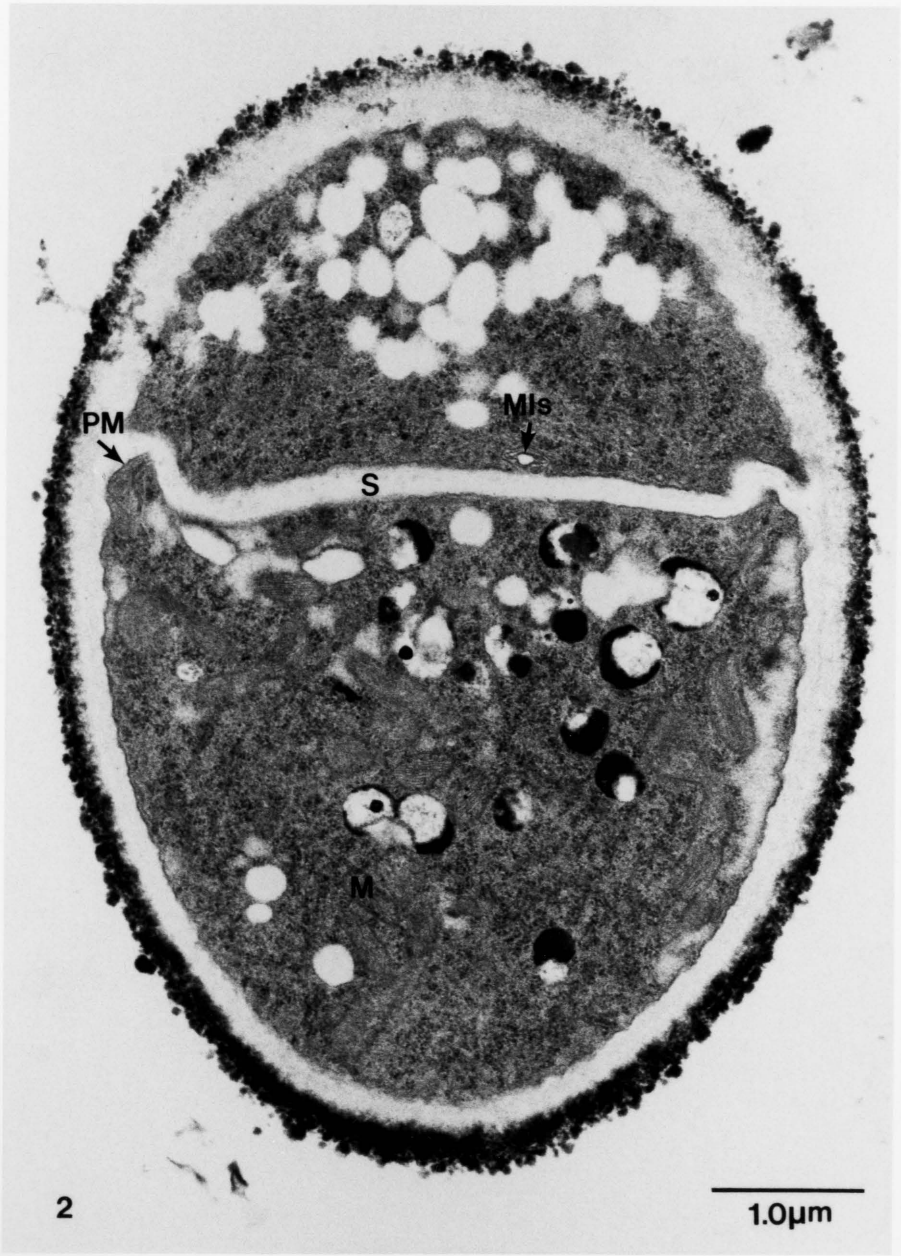
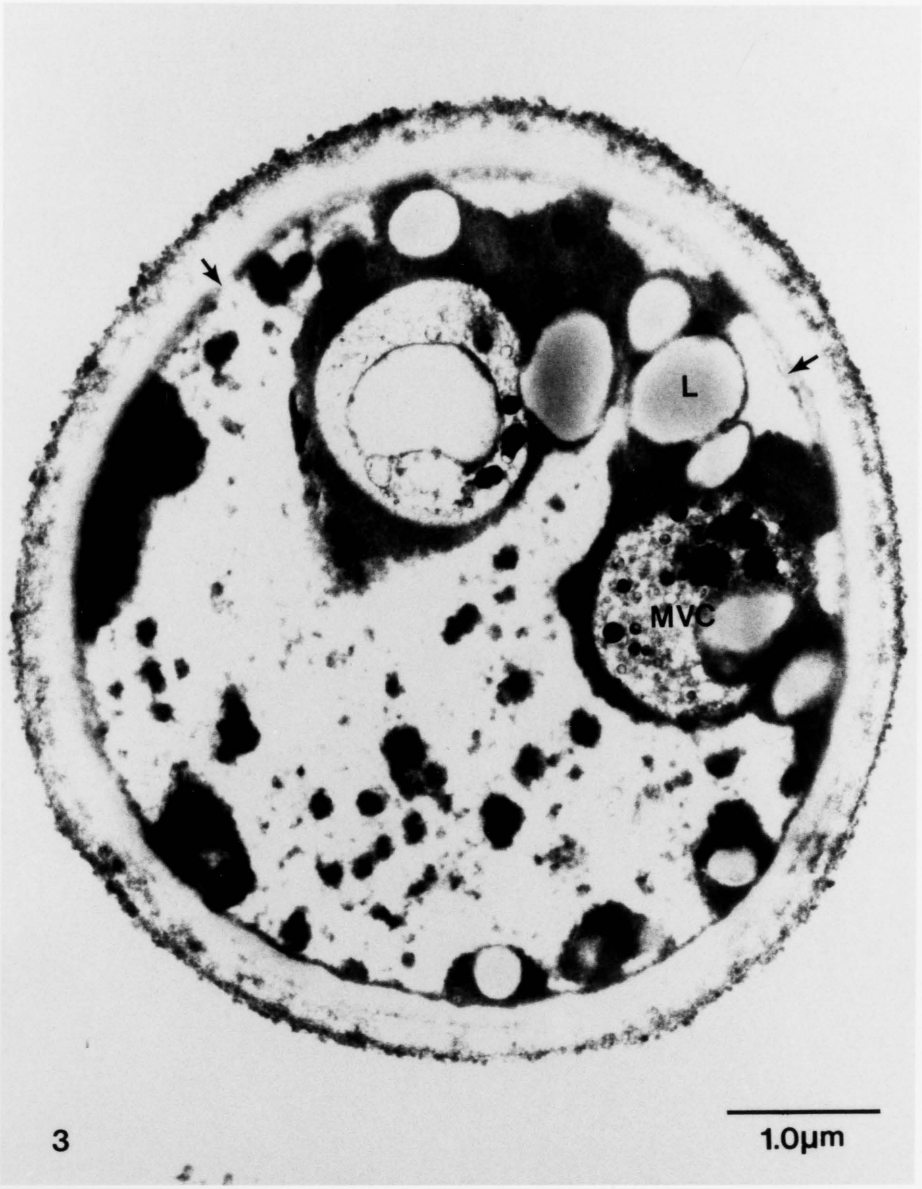
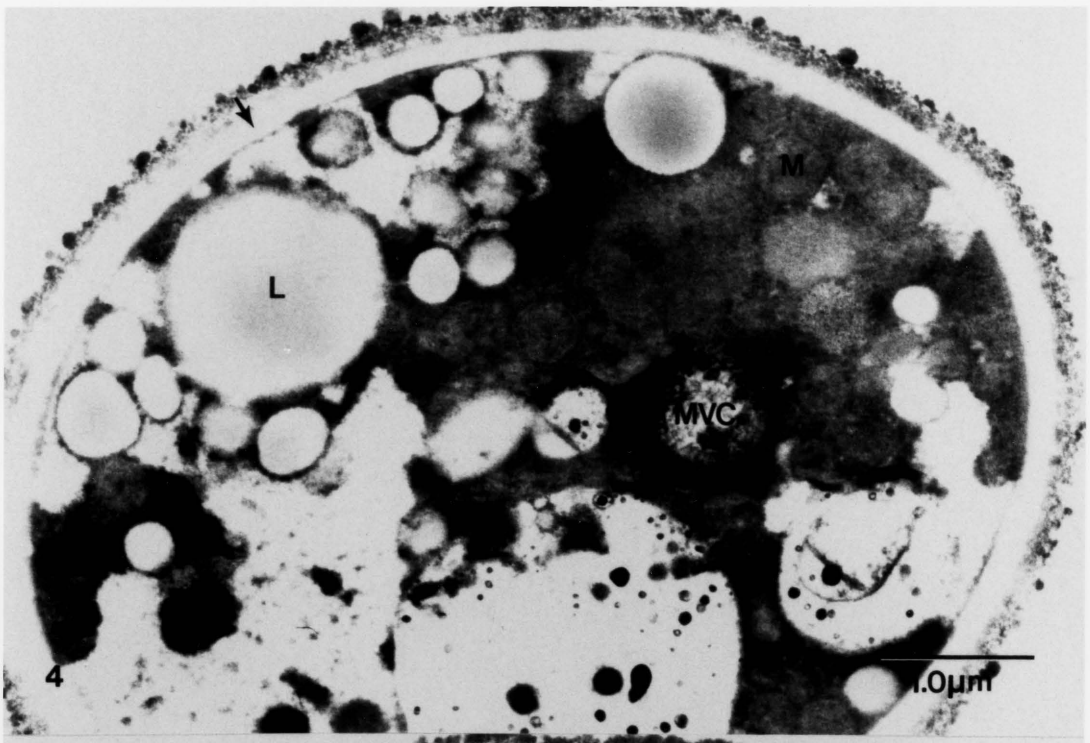
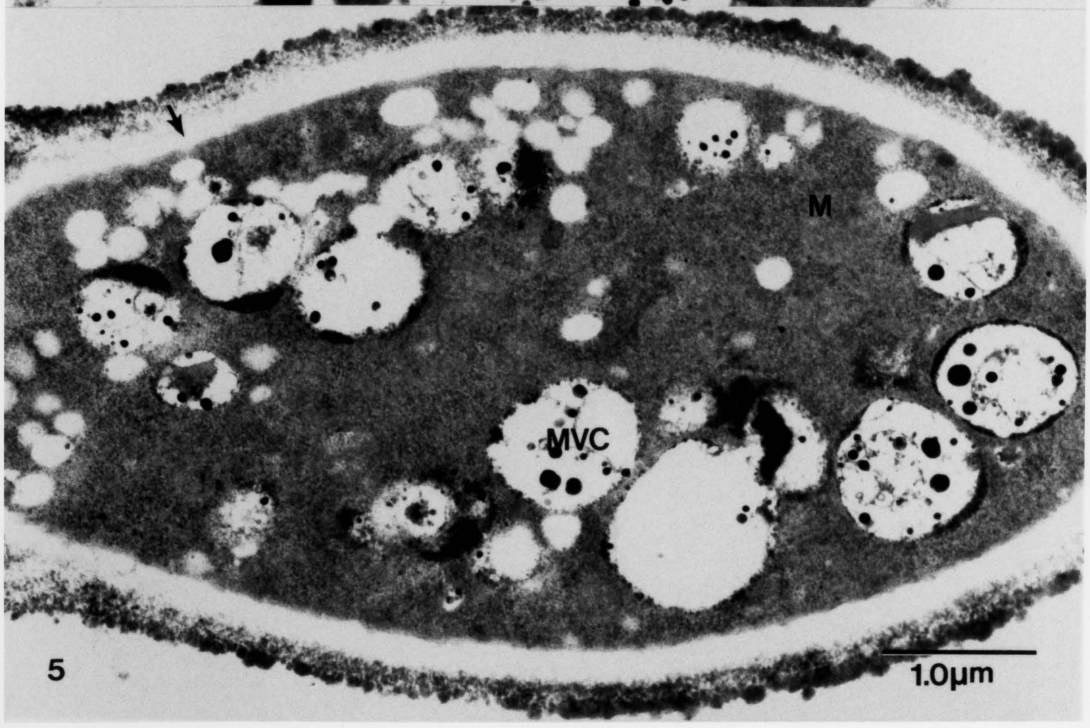

PM
MIs
S
M
2
1.0µm

FIG. 6.3. Spilocaea pomi treated for 2 hours. 3. Conidium treated for 2 hours with the SIF, fenarimol. Note swelling of the plasmalemma (arrows) as well as the formation of a multivesicular complex; lipid (L), multivesicular complex (MVC), x20,000.

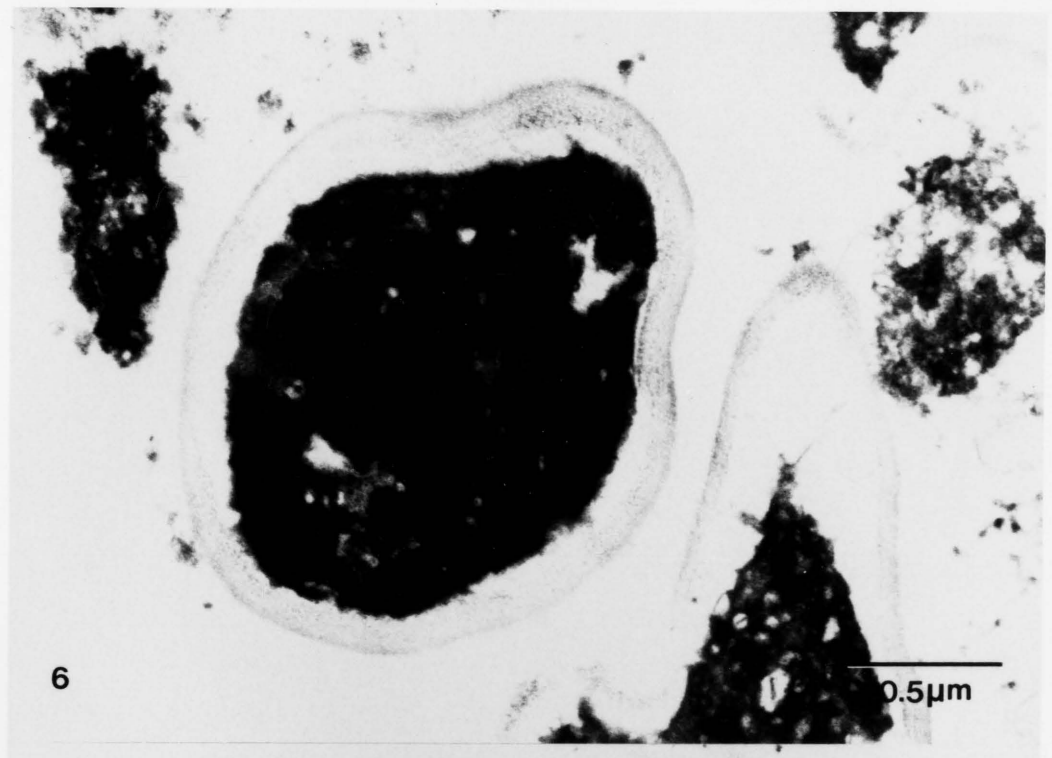

L
MVC
3
1.0µm

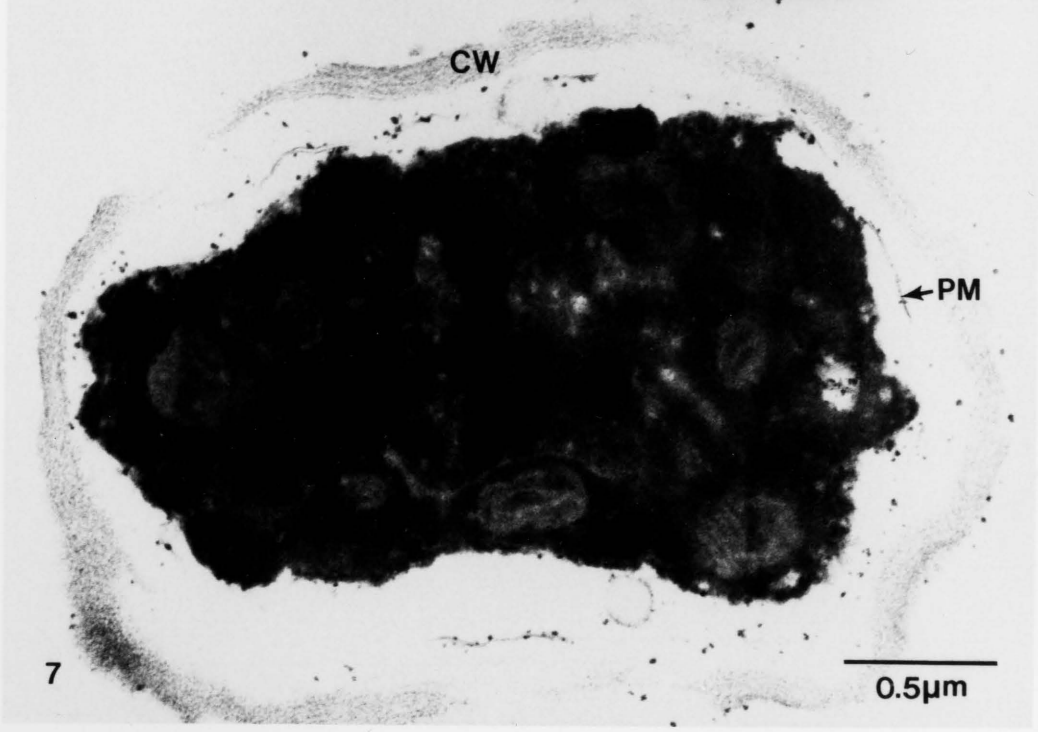
FIGS. 6.4-6.5. Spilocaea pomi treated for 2 and 4 hours with fungicide. 4. Fenarimol-treated conidium for 2 hours. Note separation of the thick cell wall from the cytoplasm (arrow) and formation of numerous lipid bodies. Structural integrity of organelles is difficult to recognize; lipid (L), mitochondrion (M), multivesicular complex (MVC), x20,000. 5. Transmission electron micrograph of a conidium treated for 4 hours. Note separation of cell wall from cytoplasm (arrow) as well as formation of numerous multivesicular complexes; mitochondrion (M), multivesicular complex (MVC), x20,000.


M
L
MVC
4
1.0µm


M
MVC
5
1.0µm

FIGS. 6.6-6.7. Spilocaea pomi treated for 12 hours with fungicide. 6. Fenarimol-treated conidium for 12 hours. Note necrotic cytoplasm and loss of structural integrity of organelles; x40,000. 7. Conidium treated for 12 hours. Note degradation of both plasma membrane and cell wall; cell wall (CW), plasma membrane (PM), x40,000.


6
0.5µm


CW
PM
7
0.5µm

CHAPTER 7

DETECTION OF STEROL-INHIBITING FUNGICIDES IN APPLES

ABSTRACT

Detection of a selected number of sterol-inhibiting fungicides (SIFs) in apple leaf tissue using bioassay procedures were evaluated. As leaves mature and senesce, an Alternaria sp. colonizes the leaf tissue. The leaf disk bioassay was unsuitable for determining the presence of SIFs, and the leaf extract bioassay offered no improvement. The SIFs either may be degrading before inhibition of the slow-growing fungus, Spilocaea pomi, can occur, or they may have diffused so rapidly through the apple tissues that relatively little of the compound remained to be detected via bioassay. In conclusion, the bioassays examined in this study were ineffective in determining the presence of SIFs in apple leaves.

INTRODUCTION

Sterol-inhibiting fungicides are characterized by uptake of their active material by the plant followed by translocation of the compound within the plant system so that it produces a protective action against a causal organism also in plant parts distal to their application. The degree of uptake and translocation is dictated by the chemistry of the compound (structure, polarity, lipophilic and hydrophilic groups), plant anatomy (surface structure, wax layer, cuticle, age of plant, degree of lignification) and environmental conditions (temperature, humidity, rate of transpiration).

Although foliarly-applied systemic fungicides need to be absorbed into the leaf tissue, the quantitative aspects of active ingredient uptake have been investigated in only a few cases. In a report on the biological activity of fenarimol against the scab and powdery mildew fungi on apples, fenarimol was absorbed by apple leaves within 30 min after a foliar spray (Anonymous 1979); however, quantitative data and methods were not given. Data on the SIF, triadimefon, uptake so far have been published only with respect to its absorption by barley and cucumber leaves

(Brandes et al. 1978; Fuhr et al. 1978). Therefore, the purpose of this study was to determine quantitatively the presence of a selected number of SIFs into apple leaf tissue using bioassay techniques.

MATERIALS AND METHODS

Effect of time on uptake and translocation:

During 1985 and 1986, Red Delicious, Earligold and Rome apple trees grown near Blacksburg, VA were treated with 3 different concentrations of each of the SIFs, fenarimol, triadimefon and triforine, or with a non SIF, metiram, in addition to untreated controls. Rates of application and formulation are listed in Table 7.1. Red Delicious and Earligold trees were approximately 5 to 10 yrs old, while Rome trees ranged in age from 15 to 20 yrs. Three treatments of fungicides of different concentrations were randomly applied to each tree at 7 AM. Fungicides were suspended in water and applied until runoff by a plant and garden sprayer.

Four tests were conducted per cultivar with leaf samples collected at 12, 24 and 72 hrs following application in September, 1985 and July, 1986. For each treatment, 7 leaves exhibiting no signs of disease or phytotoxicity and chosen for uniform size and age, were removed from each tree. Samples were placed in Whirl-Pac bags, kept on ice throughout the collection, and then stored at 0°C until needed.

Leaf disk bioassay:

Leaf samples were surface sterilized with 5% chlorox for 5 min, and three 13 mm leaf disks therefrom were placed on a potato-dextrose extract agar plate amended with penicillin and streptomycin (100 ug/ml each). The plate was first incubated at 4°C for 24 hrs to allow diffusion of the fungicide, then surface-seeded with approximately 100,000 conidia/ml of Spilocaea pomi and subsequently incubated at 19°C for 10 to 14 d to allow growth of the fungus. Zones of inhibition (ZOI) were then examined. There were 5 replications/treatment.

Leaf extract bioassay:

Leaf samples were surface sterilized as previously mentioned. The leaf extract bioassay consisted of removing a 13 mm plug from the center of an antibiotic amended potato-dextrose extract agar plate and surface-seeded with approximately 100,000 conidia/ml of S. pomi. A drop of molten agar was added to seal the well. Leaf samples weighing about 100 mg (fresh wt.) were ground with a mortar and pestle in a mixture of acetone and abs. ethanol (1:1) and filtered through Waltman #1 filter paper into a 50 ml beaker. The filtrate was transferred to a test tube, air dried and resuspended in 200 ul acetone:ethanol (1:1) which was then added to the well. The plate was incubated at 19°C for 10 to 14 d to allow growth of the fungus. Zones of inhibition (ZOI) were then examined.

RESULTS

In the leaf disk bioassay of September, 1985 a fungus identified as an Alternaria sp. grew from approximately 99% of the treated leaf disks, covering any ZOI which may have

existed. The leaf extract bioassay, containing each of the fungicide concentrations, exhibited negligible ZOI. To eliminate possible resident microorganisms, fungicide application and collection of samples were conducted earlier in the season in July, 1986. However, tests with leaves treated with the highest concentration of fungicide yielded similar results. The Alternaria sp. grew from approximately 50% of the leaf disks and the leaf extract bioassay exhibited negligible ZOI.

DISCUSSION

Rainfall was negligible during fungicide applications and sample collection times in both 1985 and 1986. This suggests that very little if any of the fungicide was lost from exposure to weather influences such as rainfall or dew.

Both leaf disk bioassays of 1985 and 1986 demonstrated fungal growth from the majority of leaf disks, thereby masking any ZOI that may have existed. The fungus which was identified as an Alternaria sp. was more prevalent in leaf

tissue later in the season. This suggests that as the leaves mature and senesce, microorganisms such as the Alternaria sp. become endemic to the leaf tissue, thus making bioassays such as this unsuitable for determining the presence of SIFs.

In leaf extract bioassays of 1985 and 1986, degradation of the SIFs may have occurred before the slow growing fungus covered the plate, thereby resulting in very little or no ZOI. Because these fungicides are translocated apoplastically, the fungicide may have diffused so rapidly through the leaves and subsequent apple tissues that very little of the compound remained to be detected via bioassay.

In a separate experiment, the benzimidazole fungicide, thiabendazole, was injected full strenght beneath selected branches of Red Delicious trees. Leaf samples were removed from these branches the following day to determine the presence of the fungicide via the bioassays used in this study. The fungicide was not detected suggesting that the compound may have been translocated elsewhere, possibly to the roots.

A more effective means of determining the presence of these SIFs may be via radiolabelled isotopes. Kraus (1981) found that [^{14}C] Bayleton® 5 WP very quickly becomes fixed in the surface of grape leaves and is thus removed from

exposure to weather influences such as rainfall, dew, etc., and from this site it is translocated in successive phases into the leaf tissue. Following application of [^{14}C] Bayleton® 5 WP to young stem tissue, he determined that the active ingredient was translocated into the leaves above the site of application. Autoradiography revealed that radioactivity was present primarily in the vascular system although it was also observed to be evenly distributed in the intercostal spaces of the leaves indicating that the active ingredient had been translocated in lateral direction. Translocation from the vascular system to the adjacent tissue was more pronounced in young leaves than in old ones. Following application to the leaf surface, the active ingredient moves rapidly into the uppermost epidermal layers (wax layer, cuticle) from where it is absorbed successively into the tissue. As a result, washing with water only 45 min after foliar application allowed recovery of not more than half of the applied dose.

Although the detection of SIFs in apple leaf tissue could not be determined in the present study, there have been reports on uptake and translocation of systemic fungicides involving other plants. Following treatment of bean leaves (Glycine max L. cv. Evans) with RH 2161, 50% of the compound was absorbed by the leaf after one day (Martin and Edgington

1978). Hisada et al. (1976) found that following application of procymidone to the foliage of cucumber plants, so much of the product had penetrated into the leaf tissue after one day that 45% of the applied dose could not be removed by washing with acetone. However, two days after application of metalaxyl to tomato leaves, so much of the product could still be removed by washing with water that its activity against Phytophthora infestans was greatly reduced (Staub et al. 1978).

Barak et al. (1983) reported that lignin preparations, prepared from stems of pepper (Capsicum annuum L. cv. Pelle California) and cotton (Gossypium hirsutum L. cv. SJ-1) plants, adsorbed the SIFs carbendazim, triadimefon, nuarimol, triarimol, fenarimol and the herbicide, fluometuron, more effectively than bovine serum albumin, cellulose, ethylcellulose or sodium polygalacturonate. They also determined that lignin is the main component which adsorbs pesticides in the apoplast of plants. The more lipophilic fungicides triarimol and fenarimol were adsorbed to the greatest extent while fluometuron, triadimefon and nuarimol were moderately adsorbed (Barak et al. 1983). In barley (Hordeum vulgare L. cv. Fergus), the imidazole fenapanil and several closely related analogs exhibited systemic translocation, which was limited to apoplastic transport (Martin and Edgington 1982).

In conclusion, the bioassays used in this study were unsuitable for the determination of the presence of SIFs in apple leaf tissue.

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Table 7.1. Fungicide treatment rates applied to apple cultivars.[a]

Fungicides	Common Names	Formulated Amt (x)/L[b]
Rubigan® 1EC	Fenarimol	0.12 ml
Bayleton® 50W	Triadimefon	112.35 mg
Funginex® 1.6EC	Triforine	0.78 ml
Polyram® 80W	Metiram	1.95 g

[a]Red Delicious, Earligold and Rome.

[b]Concentrations also included 1/2x and 2x.

CHAPTER 8

SUMMARY

The sterol-inhibiting fungicides (SIFs) etaconazole, fenarimol, bitertanol and triadimefon have been shown to be potent inhibitors of 14α-demethylation in ergosterol biosynthesis. The effects of SIFs on the free sterol and free fatty acid composition of apple leaves of Red Delicious and Jonathan cultivars were examined throughout the growing season over a 2 year period. Trees were treated in mornings vs evenings and samples collected after 24 and 72 hours. Early in the growing season, SIFs appeared to have an effect on the free sterol composition of apple leaves after 24 hours, but the concentrations of free sterol appeared to return to normal after 72 hours in both Red Delicious and Jonathan leaves. Generally, morning versus evening application had no effect on the free sterol levels of apple leaves. No consistent differences existed among the SIFs and the non sterol-inhibiting fungicide (NSIF), metiram.

Early in the season, there were increases in unsaturated and total fatty acid concentrations in Red Delicious leaves 24 hours following applications of the SIF, etaconazole, and the NSIF, metiram. The SIFs etaconazole, fenarimol and triadimefon and the NSIF, metiram, appeared to have similar effects on the fatty acid composition of Red Delicious leaves even though their modes of action are different. There were also increased concentrations of linoleic,

linolenic and total free fatty acids of fenarimol and triadimefon-treated Jonathan leaves 72 hours after treatment. Although these SIFs may have an effect on the free fatty acid concentrations of Red Delicious and Jonathan leaves early in the season, the free fatty acid concentration of the plant appeared to return to normal later in the year. There also appeared to be a faster response of the Red Delicious cultivar to the SIFs.

Early in the growing season, when the plant's metabolism is at its highest, the SIF, fenarimol, caused an increase in linolenic acid in both Red Delicious and Jonathan leaves 72 hours after either morning or evening applications. No significant differences were observed later in the season. Generally, both the Red Delicious and Jonathan leaves exhibited a decrease in saturation following morning application, whereas, there was an increase in saturation following evening application. The plant may be compensating for the increase in membrane viscosity by synthesizing more polyunsaturated fatty acids overnight to enhance membrane fluidity and maintain the integrity of the membrane. The large reduction in the ratio of saturated to unsaturated fatty acids in fenarimol and metiram-treated Red Delicious leaves was due to decreases in both saturated fatty acids as a percent of the total, and also to increases

in all three of the unsaturated fatty acids. In fenarimol- and triadimefon-treated Jonathan leaves, the reduction was seen as decreases in the saturated fatty acids as a percent of the total, and increases in linolenic acid. The increase in different unsaturated fatty acids, resulting in reduced saturated to unsaturated ratios and higher double bond indices (DBIs) in both cultivars, appeared to be cultivar related. Although SIFs may have had an effect primarily on the unsaturated fatty acids, particularly linolenic acid, early in the season, the fatty acid composition of the plant appeared to return to normal later in the season.

It appeared that the SIF, bitertanol, may function as a photosynthetic inhibitor initially after application. This was evident by the swollen and irregular thylakoid membranes and loss in chloroplast integrity observed 12 hours following treatment of Red Delicious leaves with bitertanol. The electron transport system of chloroplasts leading to the reduction of $NADP^+$ (photosystem II) and the generation of a proton gradient linked to an associated phosphorylation system is located within these chloroplast thylakoid membranes. However, the chloroplasts appeared to have recovered from the initial effect of the fungicide and returned to normal after 24 and 72 hours. This suggests that the SIF, bitertanol, had an immediate effect upon the

fine structure of chloroplasts which may have contributed to the accumulation of free fatty acid, particularly linolenic acid, of Red Delicious and Jonathan leaves after 24 and 72 hours. In the present study, there was no accumulative effect of the fungicides on the same trees later in the year. However, one must be aware that SIFs may have an effect on the plant's metabolism after continued use over an extended period of time (i.e. years) or perhaps on other parameters such as hormone synthesis.

Infection of bitertanol-treated Red Delicious leaves by Spilocaea pomi was also examined at the fine structural level. Fungal hyphae of S. pomi encased within an extracellular matrix or slime layer were observed in infected Red Delicious leaves not treated with the SIF, bitertanol. This structure may contain and provide translocation of cellular contents which may possess a depolymerizing agent required for the initial attack on cellulose. Extracellular matrices which enclosed the hyphae of S. pomi in untreated Red Delicious leaves, were not observed in infected leaves treated with the SIF, bitertanol. This suggests that the fungicide is effective against the structure which may house a depolymerizing agent necessary for the initial attack on cellulose as well as providing nutrients for growing hyphae.

Nuclear and mitochondrial profiles of fungal hyphae both appeared to be affected 12 and 72 hours following the application of the fungicide to infected Red Delicious leaves. Nuclear envelopes were not well defined and may have been breaking down. Mitochondrial matrices appeared washed-out together with the dissolution of their plate-like cristae and accumulation of minute electron dense bodies around their periphery. Biochemical studies have previously suggested indirect influence of ergosterol biosynthesis inhibitors on protein and nucleic acid metabolism as well as respiration processes.

Sterol-inhibiting fungicides rapidly curtail the biosynthesis of ergosterol. However, mycelial growth and various aspects of metabolism (respiration, protein and nucleic acid synthesis) are only mildly affected for a period of time after synthesis of the sterol has ceased. Ergosterol levels in treated mycelium do not decline rapidly which indicates that the rate of use of the sterol in membrane synthesis is slower than its biosynthesis. The ultrastructural modifications observed in the nuclei and mitochondria may be the initial stages which influence protein and nucleic acid metabolism and respiration processes resulting eventually in growth inhibition.

The plasmalemma exhibited both invaginations and proliferations accompanied by increased vacuolization 12 and 72 hours following the application of bitertanol. Changes in permeability are connected to a variety of cytopathological symptoms which may be seen as invaginations and intramembrane modifications of the plasma membrane. These effects were consistent with the mode of action of SIFs, resulting in an interference with membrane synthesis and resulting accumulation initially of sterol intermediates and then free fatty acids.

Further electron microscopic observations were made of the conidial state of Venturia inaequalis following the application of the SIF, fenarimol. Conidia treated with the fungicide for 2 hours revealed necrotic areas throughout the cytoplasm. The plasmalemma was not well defined and perhaps in the process of degradation. Increased vacuolization was also observed as well as numerous lipid bodies and multivesicular complexes (MVCs) which contained vesicles of varying electron densities. These effects are also consistent with the mode of action of SIFs, resulting in an interference with membrane synthesis and resulting accumulation initially of sterol intermediates and then free fatty acids. The accumulation of vesicles within MVCs is similar to cytoplasmic responses toward toxic chemicals.

These responses may also represent a cytoplasmic compartmentalization of the fungicide. Numerous effects of the SIF, fenarimol, were observed on the fine structure of S. pomi in vitro. Depending on the toxicity and incubation period of the applied substance different stages of degradation up to complete lysis may be observed.

Detection of several SIFs in apple leaf tissue were evaluated using bioassay procedures. However, the bioassays examined in this study were ineffective in determining the presence of SIFs in apple leaves. A more effective means of determining the presence of SIFs may be through the use of radiolabelled isotopes.

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