This is to certify that the

thesis entitled
EUTYPA DIEBACK OF GRAPE (VITIS LABRUSCA L.)
CAUSED BY EUTYPA ARMENIACAE
I. INFECTION AND CONTROL STUDIES
II. FLUORESCENT ANTIBODY STUDIES

presented by

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has been accepted towards fulfillment
of the requirements for

M.S. degree in Botany and
Plant Pathology

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Major professor

Date Feb. 25, 1982

EUTYPA DIEBACK OF GRAPE (*VITIS LABRUSCA* L.) CAUSED BY *EUTYPA ARMENIACAE*

I. INFECTION AND CONTROL STUDIES

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By

Elie Hy Gendloff

A THESIS

submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1982

ABSTRACT

EUTYPA DIEBACK OF GRAPE (VITIS LABRUSCA L.)
CAUSED BY EUTYPA ARMENIACAE

I. INFECTION AND CONTROL STUDIES

II. FLUORESCENT ANTIBODY STUDIES

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Fungicide field trials were conducted for two years in a commercial vineyard at Lawton, MI. Benlate^{R} 50%WP sprays of 1.2 to 9.6 g/L gave significant control of Eutypa armeniacae ascospore infection of pruning wounds made on two-year-old wood. Other treatments gave little or no control.

Artificially induced frost injury and simulated mechanical harvester injury sites were not susceptible to E. armeniacae infection.

Differences in infection were found among treatments varying in temperature regimes after inoculation.

Antisera were made to whole cell and cell wall preparations of E. armeniacae. Specificity of these antisera was fairly poor when various fungi were stained on glass slides with rhodamine isothiocyanate-conjugated antisera, but was improved by cross-adsorption with Phomopsis viticola.

Cross sections of grape wood, inhabited by various fungi, were stained directly and indirectly with these antisera. Hyphae in the indirectly stained wood sections fluoresced brighter than hyphae in directly stained wood sections.

To my parents

ACKNOWLEDGEMENTS

I would like to express my sincere appreciation to Dr. Donald Ramsdell for his guidance, support, encouragement and friendship during this work.

I would also like to thank Dr. Clyde Burton for his support and suggestions.

I would also like to acknowledge the aid of Dr. Burton, Dr. Alfred Saettler and Dr. Wayne Weidlich for serving on my guidance committee.

To Dr. Leo Mericle and especially Dr. James Asher, I extend my appreciation for the use of their equipment. Also, thanks go to Dr. Karen Baker for aid in fungal culture identification.

I would also like to acknowledge the Michigan Grape Growers Association for their interest and support.

Finally, I would like to thank all my friends and colleagues for making my Master's research a valuable and enjoyable experience.

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INFECTION AND CONTROL STUDIES

INTRODUCTION

Eutypa dieback, also referred previously as "dying arm" (31) or "dead arm" (37,41), is an important disease of grapevine worldwide, causing cankering and necrosis of woody tissue. It has been reported in New York (2), Japan (17), Michigan (46), New Zealand (13), Australia (in 13), Ontario (48), California (26), Greece (19), Mexico (44) and France (in 44). Eutypa armeniacae Hansf. and Carter has only recently been proven to be the causal agent (27,30), due to early studies which had associated the leaf and cane spotting organism Phomopsis viticola with that role (12,41).

In Michigan, Eutypa dieback has been estimated to occur in about 10% of the mature grapevines (45) and in California, disease levels of 81% have been reported (31).

The purpose of this study is to follow up on the work of Trese (45) by conducting fungicidal control trials with compounds that were found in that work to be inhibitory to E. armeniacae in vitro, and by repeating experiments conducted in that report that assessed frost damage and mechanical harvester injury as sites of E. armeniacae infection. These studies also incorporate assessments of E. armeniacae infection and control factors determined to be important in other situations. Included here is an assessment of the biotic agent Fusarium lateritium Nees (shown effective on

apricots in Australia) and determinations of wound susceptibility variation that may be influenced by variations in time of inoculation, age of wound and temperature after inoculation.

LITERATURE REVIEW

The ascomycete Eutypa armeniacae Hansf. and Carter is described in detail by Carter (3). Briefly, the perfect stage is found on dead grape or apricot wood in the form of many perithecia imbedded in a carbonaceous black stroma. Numerous asci develop through a centrum of disintegrating pseudoparenchymatous tissue. Paraphyses are numerous but obscured in the mature perathecia by the very numerous asci. Eight allantoid, nonseptate ascospores with a pale yellowish brown color, 7 to 11 by 1.5 to 2μm, are produced in each ascus. After a wetting period, these spores are forceably discharged up to 1 mm as an octad, all eight held together by the mucilaginous contents of the ascus (3). Muller and von Arx (34) place Eutypa in the Diatrypaceae of the Sphaeriales.

The imperfect stage of E. armeniacae is Cytosporina sp. of the family Sphaeropsidaceae in the order Sphaeropsidales. The pycnidia, which are produced in culture as well as on wood, are black and subglobose with a very short ostiole or apical pore. Conidia (scolescospores) are very numerous, hyaline, bent to arcuate, filiform, and 18-25 by 1 μm (3).

Eutypa armeniacae has been found infecting many woody plant species. Beside being an important pathogen of grape and apricot, E. armeniacae has been found to cause a dieback of Ceanothus sp. (22,32), western choke cherry (14), prunes (3), lemon (18), and

manzanita (36). Experimental mycelial inoculations have caused disease in peach, nectarine and plum by Carter (3), and almond and peach by English and Davis (20). Ascospore inoculations have caused disease in peach and almond (8). Tamarix sp., almond and apple have been found bearing the perithecial stage (4).

The grapevine disease referred to as "dead-arm," "dying arm," or "dieback" is most noticeable during early spring growth, when the affected leaves take on a dwarfed, cupped, and chlorotic appearance. In subsequent years, the arms on which these symptoms occur die. Cutting through the wood of infected branches reveals a brown canker with a distinct margin between cankered and healthy tissue.

These symptoms were first observed by Reddick who, in 1914 (41) concluded that this disease was caused by the fungus Cryptosporella viticola. He included as symptoms caused by this fungus "small reddish brown or black spots on the green shoots, petioles, peduncles, and leaf veins" which may become very deep and extensive on the canes. Coleman (12) found a constant association of the dieback symptoms with pruning wound lesions but his inoculation studies were inconclusive.

Goidanich (in Pine [37]) renamed the pathogen Phomopsis viticola based on the presence of α and β spores, which are typical of the genus Phomopsis. Pine (37) agreed and further elucidated the cultural characteristics of P. viticola.

The first association of E. armeniacae with the grape dieback was made by Carter (4), who could isolate E. armeniacae from

margins of cankers on infected vines. Willison, et al. (48) noted that P. viticola could easily and rapidly infect green tissue but pruning wound infections with P. viticola occurred in only 25% of the inoculations, and these infections did not become large lesions. Further indications of a separation of the leaf and cane symptoms with the dieback symptoms were reported by Moller, et al. (31) in 1974, and Dye and Carter (13) and Braun, et al. (2) in 1976. These three papers reported consistent isolation of E. armeniacae from margins of cankers of infected vines.

Finally in 1978 (27) and again in 1981 (30) Moller and Kasimatis reported completion of Koch's postulates, proving that E. armeniacae causes the dying arm symptoms. In both cases, an E. armeniacae culture isolated from a dieback infected grapevine was used to make mycelial inoculations to fresh pruning wounds of Vitis vinifera cv. Grenache. Typical spring dieback symptoms as well as cankers were induced after at least two and a half years by this treatment. Isolation of E. armeniacae from the cankers followed.

Contrary to the relatively recent discovery that E. armeniacae (Cytosporina) causes a dieback of grape, Cytosporina has been recognized as the causal agent of a disease of similar etiology on apricot since 1938 when Adam (1) reported on extensive research done on this "gummosis" or "dieback" disorder. This research included the demonstration that pruning wounds were necessary for infection. Subsequently, Carter (3) showed that the perfect stage was E. armeniacae. That same paper also demonstrated

that the scolecospores were non-infectious and that ascospore discharge only occurs after a stomatal wetting. Since that time there has been extensive research in Australia and California on infection and control of E. armeniacae on apricot.

English and Davis (15,16) conducted histopathological studies of apricot infection by E. armeniacae. They found that the fungus first invades the young xylem, then moves out through the cambium and bark, at which point a canker develops. They also demonstrated that cambial inoculations were generally ineffective, due to an apparent inactivation of the fungus in the cambial tissues. Phloem inoculations were ineffective.

Ascospore trapping studies in Australia (23) and California (40) showed a seasonal periodicity in spore release, with a large reduction occurring in late fall and early winter (May to July in Australia). Related to these studies, Carter (3) and Ramos, et al. (40) noted that in dry apricot growing areas (less than about 30 cm annual rainfall) perithecia are absent. Ramos, et al. were able to trap spores in these areas, however, and eliminated the possibility that alternate hosts of the pathogen (i.e., grape and Ceanothus sp.) were the source of these spores. They concluded that the ascospores were dispersed over long distances via prevailing winds from areas where perithecia were present to the drier areas where they induced a great deal of disease.

Studies to determine the amount of inoculum needed to cause infection on apricot were also conducted in Australia (8) and California (29). In Australia it was found that 10 ascospores per

wound could infect 43% of pruning wounds inoculated whereas 100 ascospores could infect 83%. This is in contrast to the California study which reported that both 10 and 100 ascospores could infect 98% to 100% of wounds inoculated. The same California workers (39) also found that the California isolate was significantly more virulent than the Australian isolate, with single ascospore inoculations capable of causing more infection and more extensive discoloration.

Reduction in susceptibility of wounds over time was studied initially in Australia (7) and then California (39). In Australia, wounds were found to be susceptible for less than two weeks in early winter if not protected from rain but more than one month if covered with a rainproof shelter. It was hypothesized that the reduction in susceptibility was due to natural microflora colonizing the wound and taking up the sites that the pathogen would otherwise infect. Price (38) followed up on this hypothesis and determined that there was a peak in microbial colonies on the apricot wound surface at 12 days on unsheltered trees and 15 days on sheltered trees, although the final amount of microbial colonies was the same.

The California study (39) revealed a seasonal variation in length of susceptibility of pruning wounds, with susceptibility in fall longest (at least 42 days after pruning) and susceptibility in spring shortest (less than 14 days). Length of susceptibility was also found to vary by temperature (resistance to infection is acquired much quicker at 20°C than at 3°C) and humidity (wounds subjected to higher humidity became resistant faster). These

workers postulated physiological factors relating to wound healing as the reason for decrease in susceptibility over time.

Control studies of E. armeniacae infection on apricot were mainly done in Australia. They have taken a number of approaches, including escape of inoculum, preventive fungicide treatment, and biological control agents.

As discussed before, there is a lull in ascospore release in late fall. Presumably for this reason, it was found (6,21) that natural infection was greatly reduced over other times of the year when pruning was done in early winter (June in Australia). This recommendation was made (22), although infection was still possible, especially with large wounds (6). Therefore, studies aimed at preventing infection through fungicide treatment of fresh pruning wounds were initiated.

By this time, copper containing chemicals had already been found to be ineffective in preventing infection (4). Therefore, evaluations were made on the efficacy of 12 different fungicides in preventing infection (24). Only benomyl gave significant control when compared to the unsprayed check treatment. Benomyl again demonstrated control in a follow-up trial (25) when applied to pruning wounds by hand held sprayer. However, separate studies in Australia (11) and in California (33) revealed that benomyl applied by high volume orchard sprayers was ineffective in preventing infection. The only feasible control method found in the California study was hand application of benomyl at high concentration (2.4% w/v) using a paint brush or hand atomizer.

Biological control studies were also conducted in Australia as a follow-up of the previously postulated idea that microbial colonization prevents establishment of E. armeniacae on pruning wounds (7). Fusarium lateritium was isolated from a pruning wound in which E. armeniacae failed to become established after inoculation with 100 ascospores (5). Other studies in that paper demonstrated some prevention of establishment of E. armeniacae on fresh pruning wounds by F. lateritium. Subsequent studies (9) established that F. lateritium produced a non-volatile, diffusable inhibitor of E. armeniacae germination and growth in agar plates; was especially effective against E. armeniacae establishment on pruning wounds when introduction of the pathogen was delayed; and was about ten times as tolerant to benzimidazole fungicides as E. armeniacae. Finally (10), workers found that when benzimidazole fungicides and F. lateritium were used together, the former would prevent establishment of inoculum arriving to the infection site early, whereas the latter would prevent establishment of late arriving (six days or later after pruning) ascospores.

Another agent that may hold promise for E. armeniacae control is Gliocladium roseum, which has been reported to be mycoparasitic on E. armeniacae (42).

Compared to the extensive infection and control work done with E. armeniacae on apricot, there has been little work done dealing with infection and control of this pathogen on grapevine. This is because it has only recently been established that E. armeniacae is the cause of the grape dieback disease (27,30).

Spore release studies in Michigan (46) and in New York (35) strongly established that a lull in ascospore release occurs during the summer months in those areas, in contrast to the late fall lull observed on apricot in California (40) and Australia (23). This would preclude adjustment of the pruning schedule in Michigan and New York (as was partially successful with apricots in Australia [22]) to a time of low inoculum presence due to the need for dormant season pruning. Pruning schedule adjustment was suggested as a control measure in California by Petzoldt, et al. (36) who found that V. vinifera pruning wounds were more susceptible and susceptible for a longer period of time in December than in March. Trese (45), however, found greater susceptibility in February than in December in Michigan.

Susceptibility of pruning wounds on different age wood was investigated by Moller and Kasimatis (28). They found that one-year-old wounds were significantly less susceptible to infection than older wounds. In another California study (36) and in a Michigan study (45), however, this difference was not apparent.

One investigation (29) demonstrated protection of grape pruning wounds using 0.2 lb/gal. (active ingredient) benomyl paint. The report stated that a lower concentration was ineffective for this purpose. In another report, Trese (45) found that the compound Benlate^{R} 50%WP strongly inhibited mycelial growth of a Michigan isolate of E. armeniacae at the concentration of 0.1 µg/ml in poison agar tests, and the compounds Difolatan^{R}, Bravo^{R}, Captan^{R}, and

Phaltan^{R} strongly inhibited ascospore germination at the same concentration.

Clearly, a number of studies are needed to elucidate more completely parameters of E. armeniacae infection and possible control measures on grapevine. One cannot assume that the etiology of this pathogen is the same on grape as on apricot, especially in such areas as Michigan and New York where there are no commercial apricots to serve as a source of inoculum and where climatic conditions are very different than in apricot growing areas. As stated before, differences in spore release patterns have already been found between the two areas, supporting this assertion.

MATERIALS AND METHODS

Fungicidal Control Trials, Year 1

To examine the possibility of using fungicides on fresh pruning wounds of grapevine, Vitis labrusca L. 'Concord,' to prevent the establishment of Eutypa armeniacae ascospores, fungicide field trials were conducted in December, 1979 and repeated in January, March and April of 1980.

A 10-year-old commercial vineyard located about three miles south of Lawton, Michigan was used for these trials. No evidence of previous infection with Eutypa dieback could be found in this study or by Trese (45) in this vineyard. The vines were pruned since establishment into a bilateral cordon system, which facilitated mechanical harvesting.

Twenty pruning wounds per vine were made just above a node or branch on two-year-old wood. Any vines so small that twenty pruning wounds on two-year-old wood could not be obtained were eliminated from the trials.

After pruning, each vine was sprayed with 0.5L of a suspension of either Benlate^{R} 50%WP (benomyl) at the rate of 1.2 or 4.8 g/L (1 or 4 lb/100 gal.) water, Difolatan^{R}4F (captafol) at the rate of 10 or 20 ml/L (4 or 8 qt./100 gal.) water, or a water control (equivalent to 79.26 gal./acre or 741.3 L/ha.). In the March and April trials, a sixth treatment of 500 macroconidia per pruning

wound of Fusarium lateritium suspended in 5 µl distilled water was applied as a possible biological control agent. The F. lateritium culture was provided by M.V. Carter (Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide, Glen Osmond, South Australia). This inoculum was scraped off cultures growing in potato dextrose agar (Difco Laboratories, Detroit, MI 48201) and suspended in distilled water. Concentrations were determined using a hemacytometer. There were five vines per treatment randomly chosen along the rows.

Benlate^{R} was selected for these trials because tests conducted by Trese (45) demonstrated a strong inhibition of E. armeniacae mycelial growth on agar incorporated with Benlate^{R}. Benlate^{R} also has shown some control of E. armeniacae in previous studies on apricot (24,25,33) and grape (29). Difolatan^{R} was selected because Trese (45) also demonstrated a strong inhibition of E. armeniacae ascospore germination on agar incorporated with Difolatan^{R}. Fusarium lateritium was selected because studies by Carter and Price (9,10) demonstrated some control of E. armeniacae on apricot using F. lateritium in field trials in Australia.

After treatment, 10 of the 20 pruning wounds per vine were inoculated with 500 ascospores of E. armeniacae suspended in 5 µl distilled water. The other 10 pruning wounds were inoculated 14 days later when possible. In the January trial, however, it was necessary to make the second inoculation 35 days after pruning instead of 14 days due to the absence of above freezing temperatures during that period. An inoculum of 500 ascospores was chosen

because Trese, et al. (46) were only able to obtain an overall infection of 14.3% on two-year-old wood when inoculated at various times of the season with 250 ascospores.

Ascospores were obtained for these inoculations by soaking mature stroma of E. armeniacae in distilled water for 10 minutes then, one day later, making freehand sections of the stroma with a razor blade, cutting through numerous perithecia, and suspending these freehand sections in a few ml of distilled water. After a few seconds of shaking followed by a one hour wait, the suspension was again shaken and the ascospore concentration was determined with a hemacytometer. In no cases were spores other than those of E. armeniacae seen during microscopic examination of the spore suspension. Germination of these ascospores was always betwen 90 and 97%.

In the December, 1979 and January and March, 1980 trials the temperature was always between 0 and 5°C during the pruning, spraying and inoculating and in the April, 1980 trial the temperature was about 10°C during these events.

Isolations and identifications of E. armeniacae taken from the pruning sites were made from 9 to 13 months after inoculation after the method of Trese (45). Each cane was cut off 6 to 10 cm below the original pruning wound. Using aseptic techniques, the canes were then split lengthwise and ten small wood chips were removed from 1 to 5 cm below the pruning would and placed on 2% potato dextrose agar amended with 100 µg/ml streptomycin sulfate. After 3 to 6 days, any fungal colonies resembling E. armeniacae

were transferred to fresh potato glucose agar (an extract of 200 g potatoes, 8 g glucose, and 20 g agar in 100 ml distilled water). These plates were placed under cool white fluorescent light (GE F15T8CW) and soft black light (GE F30T8SB) with a 14 hour daylength. After about one month, the presence of E. armeniacae cultures were confirmed by the microscopic presence of scolecospores.

Fungicidal Control Trials, Year 2

Based on preliminary results from the first year trials, the year 2 trials were started on February 17, 1981. The same vineyard was used as in the year 1 trials. The trial was conducted as a 4 by 4 factorial experiment. Benlate^{R} 50%WP (benomyl) at the rate of 1.2, 4.8, or 9.6 g/L (1, 4, or 8 lb/100 gal.) water and a water control was applied after pruning as before. There were only 15 pruning wounds per vine in this trial, however. The pruning wounds were then inoculated as before on day 1, 14, 28, or 56 after pruning and spraying. The temperature was between -2 and 5°C for the first three inoculation periods and 22°C for the fourth inoculation period. There were three randomly selected vines per treatment. Isolation and identification of E. armeniacae was made as before, six to seven months after pruning and spraying.

Spring Frost Damage as a Possible E. armeniacae Infection Site

Sixty potted four-year-old vines of V. labrusca L. 'Concord' were placed in a growth chamber (Sherer-Gillett Co., Marshall, MI 49068) held at a constant 16°C on May 18, 1980, when new spring growth on these vines was from 5 to 20 cm long. Over the next

seven days the temperature was slowly brought down to near 0°C. The vines were then subjected to a temperature of -5°C for 2 hours, followed by a rise to 18°C over a 2 hour period. Ten control plants were also subjected to the same temperature regime except for the temperatures below 0°C. These control plants and ten plants that were subjected to freezing temperatures were then inoculated with a spore suspension containing 1000 E. armeniacae ascospores per ml distilled water (prepared as previously described). Inoculations were made by misting the vines with a DeVilbis No. 15 hand atomizer, applying 3 ml of the spore suspension uniformly to each vine. After inoculation, a plastic bag containing a wet paper towel was put over the plants for 24 hours to maintain free moisture and was then removed. Inoculations as just described were then done on ten plants each on 1, 3, 7 and 14 days after freezing. Areas of the plant were tagged where the new spring growth had died as a result of the freezing.

Isolations were made after about 12 months by examining ten sites per vine, selected to obtain samples from all ages of wood. All sites where new spring growth had died as a result of the freezing were included here. Five wood chips from each site were removed using aseptic technique and assessed for E. armeniacae as previously described.

Simulated Mechanical Harvester Induced Injury as a Possible E. armeniacae Infection Site

The easternmost (most downwind) row of a 17-year-old, 0.2ha. vineyard of V. labrusca L. 'Concord' in which about 5% of the vines

bore mature stromata of E. armeniacae was used for this experiment. The vines were beaten by hand with a 1m long metal bar on October 9, 1980, which was at the same time that mechanical harvesting was being done in Michigan. Two hundred wounds from this treatment, which resembled wounds made by mechanical harvesters, were tagged. One hundred of these wounds were inoculated with 500 ascospores of E. armeniacae as described previously. The other one hundred wounds were uninoculated. Rain sufficient to release ascospores of E. armeniacae (45) occurred 2 and 5 days after wounding.

Isolations to determine the presence of E. armeniacae near the site of inoculations were made about seven months later. They were done as described for the fungicide trials.

Effect of Various Environmental Conditions on Infection of Potted Vines with E. armeniacae

Forty-two potted vines as described previously were pruned at three sites just above a node or branch on two-year-old wood in early 1981. Treatments 1 and 2 were pruned on January 18, Treatment 3 on January 20, Treatment 4 on January 29, and Treatment 5 on February 28. Just after pruning, the pruning wounds were inoculated with 500 ascospores of E. armeniacae as described before. After inoculation, a plastic bag containing wet paper towel was put over the vines for 24 hours to maintain free moisture and then removed. The vines were then subjected to the following environmental conditions:

Treatment	Number of Vines
1. Held at 3-8°C until April 17, 1981 then put outside	8
2. Held at 1-3°C until April 17, 1981 then put outside	10
3. Held at -5°C seven days, then 1-3°C until April 17, 1981 then put outside	8
4. Held at -5°C 30 days, then 1-3°C until April 17, 1981 then put outside	8
5. Held at 10°C seven days, then 1-3°C until April 17, 1981 then put outside	8

Seven to eight months after inoculation, isolation and identification of E. armeniacae from near the pruning wounds were made as described for the fungicide trials.

Isolations from the Vicinity of a Canker on Infected Vines

This experiment was conducted to determine if E. armeniacae is present in healthy tissue adjacent to cankered wood. This knowledge would help the grower determine where to cut off infected vines to remove all traces of E. armeniacae.

Four mature vines, which in the previous spring had exhibited typical symptoms of Eutypa dieback, were cut off near ground level on October 10, 1981. They were then sawed into numerous pieces, exposing the discolored canker on the mature wood. After surface sterilization with 0.5% sodium hypochlorite, 10 wood chips were aseptically transferred as described previously from the margins of the canker and each 0.5 cm away from the canker to the edge of the

surface. The presence of E. armeniacae from each of these areas was determined as previously described.

RESULTS

Fungicidal Control Trials, Year 1

Table 1 shows cumulative totals of infection by E. armeniace over all four blocks (times of replication of the experiment). Included here is percent infection, percent reduction of infection compared to the control, and a X^2 value indicating the significance of the difference in infection of the various treatments vs. the control. Part A shows these parameters for both inoculation times summed together. Part B shows the data for the inoculation on the day of pruning and spraying. The data for the second inoculation are not included here because, as stated previously, the second inoculation was conducted at a different time after pruning and spraying (35 days instead of 14 days when the experiment was conducted in January) than when the experiment was conducted the other three times. Because of this, Table 2 is included, showing the same parameters as Table 1 with the January trial excluded. Part A again shows the data for both inoculation times and part B shows the totals for the second inoculation time (14 days after pruning and spraying).

Table 3 shows an analysis of variance of the data for all four blocks, analyzed as a randomized block, split plot design (43), with the time of inoculation after pruning and spraying as the split. This analysis was possible because Bartlett's test (43) showed no

TABLE 1.--Cumulative Totals, Fungicide Trials, Year 1[a]. Eutypa armeniacae Pruning Wound Infection (Lawton, MI). All Four Blocks Combined.

Treatment	Total Infected/Inoculated	% Infected	% Reduction of Infection Compared to Control	X^2 vs Control
A. Inoculation on day of pruning and spraying or day 14 or 35 after pruning and spraying, inclusive.				
Control	85/353	24.08	-	-
Benlate® 50%WP 1.2 g/L	53/366	14.48	39.87	10.06[b]
Benlate® 50%WP 4.8 g/L	26/347	7.49	68.90	31.61
Difolatan® 4F 10 ml/L	67/362	18.51	23.13	2.91
Difolatan® 4F 20 ml/L	57/364	15.66	34.97	7.99
F. lateritium	29/182	15.93	33.85	4.27
B. Inoculation on day of pruning and spraying.				
Control	64/173	36.99	-	-
Benlate® 50%WP 1.2 g/L	33/186	17.74	52.04	15.89
Benlate® 50%WP 4.8 g/L	17/179	9.5	74.32	35.93
Difolatan® 4F 10 ml/L	43/185	23.24	37.17	7.42
Difolatan® 4F 20 ml/L	39/187	20.86	43.61	10.68
F. lateritium	22/93	23.66	36.04	4.33

[a]This experiment was conducted in a healthy 10-year-old commercial vineyard of V. labrusca L. 'Concord.' Twenty pruning wounds were made per vine on two-year-old wood. There were five vines per treatment. Each vine except for the F. lateritium treated vines was sprayed after pruning with 0.5 L of its respective treatment. Each wound on the F. lateritium treated vines was inoculated with 500 macroconidia of F. lateritium. Ten of the 20 pruning wounds per vine were inoculated with 500 ascospores of E. armeniacae in 5 µl distilled water on the day of pruning and spraying. The other 10 pruning wounds per vine were inoculated as described above on day 14 or day 35 (in the case of Block 2) after pruning and spraying. The four dates of pruning and spraying were December 5, 1979 (Block 1), January 15, 1980 (Block 2), March 13, 1980 (Block 3) and April 17, 1980 (Block 4). Positive infections were determined by tissue isolations onto PDA 9-13 months after treatment.

[b]Critical X^2 values: $P = 0.10 = 2.71$; $P = 0.05 = 3.84$; $P = 0.01 = 6.63$; $P = 0.005 = 7.88$.

TABLE 2.--Cumulative Totals, Fungicide Trials, Year 1. Eutypa armeniacae Pruning Wound Infection (Lawton, MI). Block 2 Excluded.

Treatment	Total Infected/Inoculated	% Infection	% Reduction of Infection Compared to Control	X^2 vs Control
A. Inoculation on day of pruning and spraying or day 14 after pruning and spraying, inclusive.				
Control	60/162	22.90	-	-
Benlate® 50%WP 1.2 g/L	33/271	12.18	46.81	9.14[a]
Benlate® 50%WP 4.8 g/L	19/268	7.09	69.04	24.89
Difolatan® 4F 10 ml/L	54/271	19.93	16.97	<1.00
Difolatan® 4F 20 ml/L	44/277	15.88	30.66	3.82
F. lateritium	29/182	15.93	30.44	2.83
B. Inoculation 14 days after pruning and spraying.				
Control	16/138	11.59	-	-
Benlate® 50%WP 1.2 g/L	10/133	7.52	35.12	<1.00
Benlate® 50%WP 4.8 g/L	4/131	3.05	73.68	5.94
Difolatan® 4F 10 ml/L	14/135	10.37	10.53	<1.00
Difolatan® 4F 20 ml/L	14/138	10.14	12.51	<1.00
F. lateritium	7/89	7.87	32.14	<1.00

[a]Critical X^2 values: $P = 0.10 = 2.71$; $P = 0.05 = 3.84$; $P = 0.01 = 6.63$; $P = 0.005 = 7.88$.

TABLE 3.--Analysis of Variance, Fungicide Trials, Year 1. *Eutypa armeniacae* Pruning Wound Infection (Lawton, MI). All Four Blocks, Excluding *Fusarium lateritium* Treatment.

Source	Mean Square	F Value	Probability of F Value
Block	111.8529	1.99	.169
Treatment	301.8054	5.38	.010
Error (1)	56.1102		
Inoculation Time	1191.3723	11.86	.004
Treatment x Inoculation Time	129.3704	1.29	.310
Error (2)	100.4794		
Nonadditivity	106.6920	2.00	NS
Residual	53.3300		

significant violation of the assumption of homogeneity of the treatment variances. Also, as indicated in Table 3, there was no significant nonadditivity, indicating a lack of block- treatment interaction. The Fusarium lateritium treatment was excluded to facilitate ease in analysis (because it was only in two, rather than all four of the blocks) and because it demonstrated less control than the chemical treatments (Tables 1 and 2). A highly significant difference is shown among the treatments, as well as a highly significant difference between the inoculation times.

Table 4 shows a Duncan's multiple range test of the differences between the treatments at both inoculation times, again excluding the F. lateritium treatment. Significant differences from the control are shown in both rates of Benlate^{R} as well as the 20 ml/L rate of Difolatan^{R}, although the 4.8 g/L rate of Benlate^{R} was the only treatment to show control at the 1% level of significance.

Because the second inoculation of block 2 (the January trial) was conducted at a later time after pruning and spraying than the second inoculation of the other three blocks, an analysis of variance and Duncan's multiple range test was performed, excluding this block (Tables 5 and 6). The analysis of variance (Table 5) again shows a highly significant difference between the two inoculation times. A marginally significant difference in treatments (significant at the 5.5% level) was also found. The Duncan's multiple range test (Table 6) shows that, with only these three blocks, the 4.8 g/L

TABLE 4.--Duncan's Multiple Range Test, Fungicide Trials, *Eutypa armeniacae* Pruning Wound Infection (Lawton, MI). All Four Blocks, Excluding *Fusarium lateritium* Treatment. Difference Between Treatments at Both Inoculation Times.

Treatment	Mean % Infection
Control	24.08 A[y]
Benlate[R] 50%WP 1.2 g/L	14.48 BC
Benlate[R] 50%WP 4.8 g/L	7.49 C[z]
Difolatan[R] 4F 10 ml/L	18.51 AB
Difolatan[R] 4F 20 ml/L	15.66 BC

[y]Treatments followed by a common letter are not significantly different at $P = 0.05$.

[z]Significantly different from Control at $P = 0.01$.

TABLE 5.--Analysis of Variance, Fungicide Trials, Year 1. *Eutypa armeniacae* Pruning Wound Infection (Lawton, MI). Block 2 and *Fusarium lateritium* Treatment Excluded.

Source	Mean Square	F Value	Probability of F Value
Block	152.6290	2.40	.153
Treatment	234.7022	3.69	.055
Error (1)	63.6582		
Inoculation time	1383.1230	16.60	.002
Treatment x Inoculation time	56.3872	.68	.623
Error (2)	83.3023		

TABLE 6.--Duncan's Multiple Range Test, Fungicide Trials, Year 1. *Eutypa armeniacae* Pruning Wound Infection (Lawton, MI). Block 2 and *Fusarium lateritium* Treatment Excluded. Difference Between Treatments at Both Inoculation Times.

Treatment	Mean % Infection
Control	22.90 A[z]
Benlate[R] 50%WP 1.2 g/L	12.18 AB
Benlate[R] 50%WP 4.8 g/L	7.09 B
Difolatan[R] 4F 10 ml/L	19.93 A
Difolatan[R] 4F 20 ml/L	15.88 AB

[z]Treatments followed by a common letter are not significantly different at $P = 0.05$.

rate of Benlate[R] was the only treatment that showed a statistically significant difference from the control.

Neither analysis of variance showed a significant difference among the blocks (times of the dormant season the experiment was conducted). Therefore, there was no significant difference in infection (susceptibility) of the grape wood inoculated at these different times of the dormant season.

Fungicidal Control Trials, Year 2

Table 7 shows the overall results of these trials, summed over the three vines of each treatment/inoculation date combination.

Table 8 shows an analysis of variance of these data, analyzed as a four by four factorial experiment (43). A significant difference is shown here among the treatments but not the inoculation dates. Since a significant difference was found among the treatments, a Duncan's multiple range test was conducted to determine the treatments that were significantly different from the control (Table 9). Here, Benlate^{R} at both 1.2 g/L and 9.6 g/L showed a statistically significant reduction in infection compared to the control.

Spring Frost Damage as a Possible *E. armeniacae* Infection Site

Frost injury to young spring growth was considered to be a possible infection site of *E. armeniacae*. A total of 540 artifically injured and inoculated sites were examined from a total of 54 vines (including control vines). No *E. armeniacae* was isolated from any of these sites, including areas where visible frost damage occurred (a total of 16 sites).

Simulated Mechanical Harvester Induced Injury as a Possible *E. armeniacae* Infection Site

Sixty-six uninoculated and 69 inoculated sites of simulated mechanical harvester injury were assessed for *E. armeniacae* infection. None of the uninoculated sites and one of the inoculated sites (1.4%) showed *E. armeniacae* infection.

TABLE 7.--Cumulative Totals, Fungicide Trials, Year 2[a]. Eutypa armeniacae Pruning Wound Infection (Lawton, MI).

Rate of Benlate[R] 50%WP (g/L)	Day of Inoculation after Pruning and Spraying	Total Infected / Inoculated	% Infected
0	1	6/42	14.29
0	14	5/41	12.20
0	28	3/42	7.14
0	56	1/40	2.05
1.2	1	2/40	5.00
1.2	14	2/41	4.88
1.2	28	1/41	2.44
1.2	56	0/43	0.00
4.8	1	4/44	9.09
4.8	14	1/41	2.44
4.8	28	3/44	6.82
4.8	56	1/41	2.44
9.6	1	0/44	0.00
9.6	14	2/43	4.65
9.6	28	1/42	2.38
9.6	56	0/43	0.00
0	all[b]	15/165	9.09
1.2	all	5/165	3.03
4.8	all	9/170	5.29
9.6	all	3/172	1.74
all	1	12/170	7.06
all	14	10/166	6.02
all	28	8/169	4.73
all	56	2/167	1.20
all	all	32/672	4.76

[a]This experiment was conducted on a healthy 11-year-old commercial vineyard of V. labrusca L. 'Concord.' The date of pruning and spraying was February 17, 1981. Fifteen pruning wounds were made per vine on two-year-old wood. There were three vines per treatment-inoculation date combination. Each vine was sprayed with 0.5 L of its respective treatment. Inoculations were made by placing 500 ascospores of E. armeniacae in 5 µl distilled water on each pruning wound. Positive infections were determined by tissue isolations onto PDA 6-7 months after treatment.

[b]all = cumulative total from this heading.

TABLE 8.--Analysis of Variance, Fungicide Trials, Year 2. *Eutypa armeniacae* Pruning Wound Infection (Lawton, MI).

Source	Mean Square	F Value
Treatment	129.36	3.16 *[a]
Inoculation Day	81.39	1.99 NS[b]
Treatment x Inoculation Day	25.4035	< 1 NS
Error	40.88	-

[a] * = There are significant differences at $P = 0.05$.

[b] NS = There are no significant differences at $P = 0.05$.

TABLE 9.--Duncan's Multiple Range Test, Fungicide Trials, Year 2. *Eutypa armeniacae* Pruning Wound Infection (Lawton, MI). Differences Between Treatments.

Rate of Benlate[R] 50% WP (g/L)	% Infection
0 (Control)	9.09 A[z]
1.2	3.03 B
4.8	5.29 AB
9.6	1.74 B

[z] Treatments followed by a common letter are not significantly different at $P = 0.05$.

Effect of Various Environmental Conditions on Infection of Potted Vines with E. armeniacae

Inoculated pruning wounds were subjected to various environmental conditions to determine if some environmental conditions subsequent to inoculation are more conducive to E. armeniacae infection than others. Table 10 shows the totals of each treatment along with percent infection and differences based on individual X^2 comparisons. The only comparison that showed a difference at the 5% level of significance was Treatment 3 (-5°C for 7 days then 1-3°C until spring) compared to Treatment 4 (-5°C for 30 days then 1-3°C until spring), although Treatment 2 (1-3°C until spring) compared to treatment 4 showed a marginally significant difference (at the 10% level).

Isolations from the Vicinity of a Canker on Infected Vines

Isolations were made from the margins of cankers on eleven cut surfaces on four Eutypa dieback infected vines. In addition, isolations were made from areas adjacent to the canker margins in 0.5 cm increments progressing into the noncankered xylem tissue to determine if E. armeniacae was present in areas away from the canker. Out of a total of eleven surfaces from which isolations were made, E. armeniacae was isolated from the margins of the exposed canker on ten of them (90.9%). Of these eleven surfaces, E. armeniacae was also isolated 0.5 cm away from the canker margin in one case (9.1%). Six of these eleven surfaces had enough healthy

TABLE 10.--*Eutypa armeniacae* Infection of Inoculated Pruning Wounds Subjected to Various Environmental Conditions in Growth Chambers.

Treatment[y]	Total	Infected / Inoculated	% Infection
1. 3-8°C until April 17, 1981		6/23	26.09 AB[z]
2. 1-3°C until April 17, 1981		12/30	40.00 AB
3. -5°C for 7 days then 1-3°C until April 17, 1981		10/22	45.45 A
4. -5°C for 30 days then 1-3°C until April 17, 1981		3/24	12.50 B
5. 10°C for 7 days then 1-3°C until April 17, 1981		7/20	35.00 AB

[y]Treatments 1 and 2 were pruned and inoculated on January 18, 1981, Treatment 3 on January 20, 1981, Treatment 4 on January 29, 1981, and Treatment 5 on February 28, 1981. Just after pruning, all treatments were inoculated with 500 ascospores of *Eutypa armeniacae* then placed in growth chambers for treatment as indicated above.

[z]Treatments followed by the same letter are not significantly different at $P=0.05$ by individual X^2 comparisons.

tissue away from the canker to be able to make isolations 1.0 cm away from the canker margin. *Eutypa armeniacae* was isolated in one case here (16.7%), which was not the same surface from which *E. armeniacae* was isolated at 0.5 cm. In both cases where *E. armeniacae* was isolated at a distance from the canker margins (one at 0.5 cm and the other at 1.0 cm), only one of the ten wood chips taken from that distance away from the canker margin yielded *E. armeniacae*. In contrast, in the ten cases where *E. armeniacae* was isolated from the canker margins, an average of 2.8 wood chips from the ten taken yielded *E. armeniacae*.

DISCUSSION

It is apparent from the fungicidal control trials that a Benlate^{R} spray is effective in preventing establishment of E. armeniacae on pruning wounds. The most effective rate was not established, however. In the first year, a rate of 4.8 g/L gave the greatest control whereas in the second year the lower rate (1.2 g/L) was more effective, although the rate of 9.6 g/L was best.

Also important is prevention of infections introduced two weeks or later after pruning and spraying. Here, the higher rates were more effective. This is most apparent in the observation that, in the first year's trials, the 4.8 g/L rate of Benlate^{R} showed statistically significant control at the second inoculation time (table 2B) whereas the low rate did not.

The F. lateritium treatment was not effective, even though the organism consistently became established in more than one half of the pruning wounds to which it was inoculated (unpublished data). Apparently, F. lateritium is not inhibitory to the Michigan isolate of E. armeniacae, either, due to the observation that, in the stems that were inoculated with F. lateritium, more than one-half of the stems that E. armeniacae was isolated from also contained F. lateritium (unpublished data). This was true for either trial in which F. lateritium was included and either time of E. armeniacae

inoculation, as well. This is in contrast to the inhibition of E. armeniacae by F. lateritium found in Australia on apricots (5,9).

In the present study, susceptibility of pruning wounds decreased with time after pruning. This change in susceptibility with time was found to be highly significant statistically in the first year's trials (Tables 3 and 5). Although the differences in susceptibility were not statistically significant in the second year's trial (Table 8), the trend was again very strong and consistent (Table 7). Similar findings have also been found on apricots in Australia (7) and California (40) and on grape in California (36).

Results of the spring frost damage and simulated mechanical harvester injury experiments agreed with the findings of Trese (45) in which no infection was found associated with either type of damage.

The experiment dealing with the effect of various environmental conditions on infection of inoculated plants was somewhat inconclusive. Some differences between extended cold periods and warmer periods after inoculation are indicated (Table 10) and the statistically significant difference found agreed with Trese, et al. (47), who suggested that the cold period necessary for reduction of infection would have to be two weeks or greater. That study revealed a lower infection rate (2-6%) with the extended cold period than was found here, however. If the important parameters could be identified more clearly, some spray treatments might be avoided. Therefore, more work is needed on this point.

In the canker isolation experiment, contamination was seemingly responsible for the two cases where E. armeniacae was isolated away from the canker. In both cases only one wood chip out of ten yielded E. armeniacae, whereas almost three out of ten isolations from the canker margin yielded E. armeniacae. Also, the stem surface where E. armeniacae was isolated 1.0 cm away from the canker margin was not the same surface where the fungus was isolated 0.5 cm away from the canker. One would think that if E. armeniacae were present at 1.0 cm that it would also be present at 0.5 cm away from the area from which it had undoubtedly originated. Therefore, the sodium hypochlorite treatment probably failed to disinfest small wood chips containing E. armeniacae that were moved away from the canker when the stem was sawed in two.

During many years of research on E. armeniacae infection of apricot and grape, it has become apparent that many disease factors associated with E. armeniacae are quite variable, depending on the crop and geographic location where the infection occurs. The most obvious difference between infection of grape and apricot is the time interval between infection and occurrence of disease expression. This occurs very quickly on apricot (1,3) but takes two and a half years or longer on grapevine (27,30). This long incubation time is one of the reasons for the previous conclusion as to the causal agent of the grape disease (30).

On apricot, differences have also been found in virulence (39) and amount of inoculum required to cause infection (40) between a California isolate and an Australian isolate. Spore trapping

studies have also shown a difference in seasonal discharge of ascospores in the eastern United States (35,46) when compared with ascospore discharge in Australia (23) and California (40).

The studies reported in this thesis have also revealed some differences occurring between E. armeniacae infection and control in Michigan on grape vs. these parameters occurring in other situations. Benomyl sprays were found effective in protecting grape pruning wounds in Michigan whereas they were found ineffective on apricot in Australia (11) and California (33). Also, F. lateritium was found ineffective for this purpose as well as not inhibitory to E. armeniacae, whereas it was found both effective in apricot pruning wound protection and inhibitory to E. armeniacae in Australia (5,9 10). Finally, no differences in dormant season susceptibility of fresh grapevine pruning wounds were found in this work whereas a sharp drop in susceptibility during March has been found in California (36).

Clearly, therefore, E. armeniacae is variable from place to place. This variation is probably due to differences in predominant hosts in the area as well as differences in climate encountered.

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PART II

FLUORESCENT ANTIBODY STUDIES

INTRODUCTION

In histological studies of wound pathogens in which hyphae are stained in wood sections and observed, it is necessary that they be conducted in environments in which saprophytes that would normally also invade the wounds are excluded. This prevents confusion as to the identity of the hyphae observed. When these saprophytes are excluded, however, growth of the studied organism may be quite different than when interactions between the two fungi occur. As well, the necessary sterility of the environment is very difficult to maintain for long periods of time. Therefore, the length of time that these studies can be conducted is limited. This can put restrictions on studies with fungal pathogens in which there is a long latency period between infection and symptom expressions. Other problems in working with pathogens with long latency periods are discussed by Savage and Sall (48).

The fluorescent antibody (FA) technique is a possible solution to these problems. With a FA stain specific to the fungus of interest, it can be distinguished from other fungi observed in tissue. This would eliminate the constricting need for sterility of the environment and also allow effects of competing fungi to be studied along with the fungus of interest. Also, natural infections in which saprophytes are present could also be studied histologically. In addition, a long period of infection could be allowed to

occur before observation, without concern about confusion occurring due to contaminating fungi.

Eutypa armeniacae infection of grapevine is a good candidate for studies of this type. It is a wound pathogen with a long latency period (39). Also, previous serological work with this organism (17,46) indicates that an antiserum can be produced with good specificity. The work described represents attempts to produce this antiserum and to stain wood sections using the FA technique, thus visualizing the organism in situ.

LITERATURE REVIEW

The earliest studies employing fungal serology were conducted with yeasts, human pathogens, and other non-plant pathogenic fungi. They were conducted around the turn of the century and are reviewed by Link and Wilcox (30). Among the first workers using serology with plant pathogenic fungi were Coons and Strong (13) who, in 1928, distinguished between various Fusarium spp. and, in some cases, could separate varieties of the same species by using the complicated and exacting complement fixation test. The authors also investigated the anaphylaxis reaction induced by Phoma sp., leading to many dead guinea pigs. In the same year, Matsumoto (36) reported on serologic differences found among various Aspergillus spp., also using the complement fixation test. Link and Wilcox (30) investigated the usefulness of the precipitin ring test and reported serological differentiation between some, but not all of 22 Fusarium spp. Nelson (41) also used the precipitin ring test and compared antiserum made to whole mycelial extracts of Fusarium lini with antiserum made to a protein purified from the mycelium. He found that the antiserum made to the purified protein had a higher titer than the whole mycelium antiserum, even when tested against the whole mycelium antigen. Continuing work with the precipitin ring test, Beck (6) reported in 1938 on investigations with antisera

made to many smut species. In this case, the tests could differentiate various genera and species within the same family, and also different isolates within the same species. Beck also employed the cross-adsorption procedure in attempts to further differentiate some antisera.

These studies helped establish the difficulties inherent in fungal serology, namely, the frequently low or variable specificity of antisera made to fungi, and the large mixture of antigens that these fungi contain, making it necessary to make a choice of the type of antigen to be used for antiserum production.

During the 1940s and 1950s essentially all studies using fungal serology were conducted with fungi that are human pathogens. Seelinger (53) and Kaplan and Kaufman (27) present reviews of representative work during that period. Badami (4) gives a review of serological methods with plant pathogens used up through that time. Serology with fungal pathogens of man has now progressed to the point where serological tests have become routine laboratory procedures (28).

The most important advances in serology which occurred during that period were the introduction of the gel diffusion technique of Oucterlony (42), which allowed individual antigen-antibody reactions to be identified, and the fluorescent antibody (FA) technique, which allowed a specific antigen to be visualized. Both of these techniques have been utilized extensively in serological studies involving plant pathogens.

The FA technique was introduced by Coons, et al. in 1941 and 1942 (14,15). In the latter work, fluorescein isocyanate-labeled antipneumococcal-3 rabbit antiserum was used to detect the pneumococcal antigen in infected mouse tissue.

A major advance in the development of the FA technique was the introduction of the "indirect" method of FA staining, in which the antigen of interest is treated with its homologous unlabeled antiserum and then stained with labeled antiserum that was made against the immunoglobins of the homologous antiserum using a different animal. This technique was introduced in 1954 by Weller and Coons (58) working with antisera from human patients infected with herpes zoster and vericella viruses as the homologous antisera and fluorescent antihuman gamma globulin as the staining conjugate. The advantages of the indirect method over the original "direct" method are twofold. First, the degree of fluorescence is greater, due to the fact that, unlike directly stained material, there is more than one fluorescent molecule per antigenic site. This is because more than one fluorescent antiglobulin molecule will react with its homologous immunoglobin (20). Second, this method makes it unnecessary to label the antisera of each antigen of interest. All that is necessary is a labeled antiserum made against the immunoglobins of the animal in which the primary antiserum was made.

Another major advance in the development of the FA method was the introduction of isothiocyanate compounds as fluorescent dyes by Riggs, et al. in 1958 (48). These compounds, fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC), are much

more stable in storage than the corresponding isocyanate compounds previously used. Synthesis is much easier and safer, as well.

The FA technique has now developed to the point where commercial preparations are readily available. Equipment, including proper filters, designed specifically for use with particular fluorochromes is also available from a number of commercial sources. A more extensive discussion of the development of the FA technique is given by Goldman (20). The works of Paton (43), Trinick (55), and Bohlool and Schmidt (8) demonstrate the types of studies that the FA technique is useful for in phytobacteriological investigations.

Since 1960 there has been a great deal of work employing fungal serology that is phytopathologically related. The studies that have found fungal serology useful fall into three general categories: (1) taxonomy and serological differentiation of closely related species or strains; (2) morphology, anatomy, and physiology of fungi; and (3) detection and/or identification of certain fungal species on slides, in soil, or in tissue. These three categories will now be covered separately in detail.

Taxonomy and Differentiation

Since differentiation of closely related species and strains using serology is dependent upon differences within the antisera and/or antigens of the fungi being compared, the antisera produced must have a great deal of specificity to the homologous fungus. As will become apparent, this specificity need not be

complete, but there must be at least one antigenic difference between the two species or strains being compared.

A number of groups of fungi have been studied taxonomically using serology. A large part of these studies dealt with Fusarium species or the Oomycete genera Phytophthora and Pythium.

Fusarium.--Members of the genus Fusarium are frequently quite difficult to distinguish on the basis of cultural characteristics (30). In addition, the taxonomy can be quite difficult, especially considering the various numbers of forma speciales of some species, which can only be distinguished on the basis of host range. This is why Fusarium is a good candidate for serology as a means of differentiation of taxa. Also, the early papers using various Fusarium species as antigens for serology (13,30) indicated good specificity of antisera.

The work of Tempel (54) also demonstrated good specificity, since F. oxysporum f. lupini could be distinguished from F. oxysporum f. pisi using gel diffusion tests, on the basis of different precipitin lines exhibited in the agar between the homologous and heterologous strains. Likewise, Buxton (11) was able to distinguish between F. oxysporum f. cubense and F. oxysporum f. pisi and even between two races of each of these formae speciales using agglutination and gel diffusion tests. Cross-adsorption methods allowed differentiation among other forma speciales of F. oxysporum. Similarly, Madhosingh (33) could differentiate between F. oxysporum, F.

moniliforme, and F. solani using gel diffusion and Hornok and Jagicza (25) produced a very specific antiserum to a F. culmorum strain that allowed differentiation of that strain from other F. culmorum strains using FA staining.

These studies indicate that antiserum made to a Fusarium species is consistently quite specific. They also show the usefulness of the gel diffusion technique in these types of studies. Even though a serum reacted with a heterologous strain, the heterologous strain could be distinguished from the homologous strain by number and placement of bands in the agar.

Phytophthora and Pythium.--These organisms have been singled out for study largely because of the time consuming and difficult procedures necessary for taxonomic differentiation of species isolated (10). Burrell, et al. (10) made antisera against the Phytophthora species P. cinnamomi, P. cactorum, and P. erythroseptica. The three antisera proved to have variable specificity, with species differentiation possible with two of the three antisera using gel diffusion only through cross-adsorption procedures. Morton and Dukes (40) similarly could distinguish between Pythium aphanidermatum and two varieties of Phytophthora parasitica with antiserum to either P. aphanidermatum and P. parasitica var. nicotianae but could not distinguish between the two strains of P. parasitica with either antisera using gel diffusion tests. Gill and Powell (19) also produced an antiserum that indicated species specificity but not race

specificity using Phytophthora fragariae as the antigen. Species specificity was also demonstrated in Phytophthora cinnamomi antiserum produced by Malajczuk, et al. (34).

In contrast to these studies, a number of reports indicate a lack of species specificity in antisera made to fungi in this group. Merz, et al. (38), working with antisera to six Phytophthora species, could only obtain enough specificity to place them in two serological groups using gel diffusion. Similarly, White (59) could not distinguish between Pythium graminicola and Pythium aristosporum based on intensity of fluorescence in immunofluorescence tests with an antiserum to P. graminicola. Finally, MacDonald and Duniway (31) could not distinguish between Phytophthora megasperma and Phytophthora cinnamomi with immunofluorescent antiserum of P. megasperma. There is also evidence that this antiserum reacts with Pythium ultimum and Aphanomyces eutyches (Susan Cohen, personal communication).

Clearly, in contrast to the strong and consistent species and even variety specificity found with antisera made to Fusarium species, antisera made to Pythium and Phytophthora are much more variable and generally less specific.

Other Fungi.--Various other fungi have been tested serologically for differentiation purposes, generally with good results. Using gel diffusion tests, Madhosingh (33) could find differences between the taxonomically difficult Fomes roseus and F. subroseus.

Also, Adams and Butler (1) were able to distinguish between anastomosis groups of Rhizoctonia solani and Gooding and Powers (21) could distinguish between three Cronaritum species using cross-adsorption. Bahn (5), working with various Puccinia spp. uredospores, could distinguish between various species and races within species using the complement fixation and precipitin tests. Other serological differentiation of spores was done successfully with Ceratocystis spp. (3). Finally, Holland and Choo (24) used the sensitive immunoelectrophoresis technique with antisera to Ophiobolus graminis isolates to establish that the serological relationships between two wheat and two oat isolates of O. graminis are closer than the between the wheat and oat isolates. Using the FA technique with these isolates (12), the heterologous reaction could not be distinguished from the homologous reaction, indicating that the FA technique is less sensitive than immunoelectrophoresis.

Serology is apparently frequently successful in studies where closely related species must be distinguished. Because of its ability to distinguish between individual antigen-antibody reactions, the gel diffusion technique is more appropriate than the FA technique for these studies. Immunoelectrophoresis, as a more sensitive variant of the gel diffusion technique, seems to hold promise in making distinctions where gel diffusion is not sensitive enough.

Morphology, Anatomy, and Physiology

Two papers will be described as an example of the use of serology, specifically the FA technique, in determining sites of growth of fungi. May (37), working with Schizosaccharomyces pombe, treated cells with a homologous rabbit antiserum. The treated cells were allowed to grow and then stained with goat anti-rabbit FITC in a modification of the indirect staining procedure. The cell wall extension sites could be determined by where the fluorescence did not occur. This technique made use of the fact that antibody attachment does not kill the cell. Similarly, Goos and Summers (22), studying Candida albicans and Fusarium oxysporum f. cubense, stained the fungi with directly-conjugated homologous antisera, then allowed growth. Again, areas where new material was used in growth were non-fluorescing. With both of these studies, the antiserum could be completely non-specific, since the areas where fluorescence did not occur were simply areas that never came in contact with the antibody.

In the next three studies described, specificity is essential for the determinations desired. Raper and Esser (47) found serological differences between the homokaryon and dikaryon of Schizophyllum commune using gel diffusion that could only be attributable to biochemical activities of the incompatability factors. This demonstrates the use of serology in analysis of physiological phenomena. Fultz and Sussman (18) demonstrated a clever modification of the FA technique. Antiserum was prepared against the whole mycelium (rhizoids and hyphae) of Allomyces macrogynus. The

antiserum was then cross-adsorbed with hyphal cell wall extracts. A. macrogynus plants were incubated in this adsorbed serum and then treated with FITC-conjugated unadsorbed antiserum. Only the rhizoids fluoresced, indicating antigenic differences between the hyphae and rhizoids. Marchant and Smith (35) also made use of the FA technique and cross-adsorption procedure to demonstrate antigenic differences between mature hyphae and hyphal tips of Fusarium culmorum. Antiserum made to the hyphal tips, when cross-adsorbed with mature hyphae, allowed an (indirect) immunofluorescent reaction of the tips only. Also, the conjugated antiserum against the mature hyphae imparted fluorescence to the mature hyphae and not the tips. This indicated that the antiserum made to the tips has an additional antibody not found in the mature hyphae.

The papers described above should demonstrate the use of serological techniques to answer morphological, anatomical, or physiological questions that would be difficult or impossible to answer in other ways. Also, as indicated, in some cases the gel diffusion and in other cases the FA technique is most appropriate for the questions at hand.

Detection and Identification

As with the taxonomic studies, specificity of antisera is important in detection and identification studies because the species in question must be distinguished from other fungi present. The FA technique is usually the method of choice, especially when the hyphae is observed in situ.

Schmidt and Bankole (50,51) did the pioneering work with these types of studies. Using antiserum to Aspergillus flavus, they used a buried slide technique to isolate various fungi from soil, among them A. flavus. The specificity of the antiserum was not perfect; some other Aspergillus spp. and occasionally other species tested fluoresced similarly to A. flavus. The same group (52) also made antisera to two species of ectomycorrhizal fungi. They made one rather specific and the other quite specific with the aid of cross-adsorption procedures, and successfully stained and visualized them in pine root sections. Similar work was done by other workers with Polyporus tomentosus in pine (29). Other work with FA staining of fungi in tissue was done by Warnock (56, 57) who used quite specific FA preparations made to Alternaria alternata, Aspergillus flavus, and Penicillium cyclopium to stain hyphae of these fungi in barley lemmas and paleae.

In work done with Botrytis cinerea, both Preece and Cooper (45) and Savage and Sall (49) developed specific antisera to this fungus. The latter workers developed a highly sensitive radioimmunosorbant technique in attempts to detect small amounts of antigen in grape tissue.

Two past studies describe work done with antisera to Australian apricot isolates of Eutypa armeniacae. Francki and Carter (17) made two antisera, one using ascospores and the other using mycelium as antigens. The FA technique demonstrated that the ascospores did not react with the mycelial antiserum and vice versa.

Similarly, Price (46) showed good specificity of E. armeniacae antiserum made against whole mycelium using soluble fractions of the antigen in gel diffusion plates.

Serology with fungal plant pathogens, despite the relatively limited amount of work done, has been useful in solving a number of problems that would have been difficult or impossible to solve in other ways. Lack of specificity has sometimes proved to be a limitation, which may be solvable in part by using various fractions of the whole mycelium as antigens or by using sophisticated techniques, such as immunoelectrophoresis, in separating antigen-antibody reactions in gel plates. These techniques will undoubtedly continue to be employed, with improvements and new applications inevitable.

MATERIALS AND METHODS

Antigen Production

The *Eutypa armeniacae* isolate used for antigen production was isolated from infected grape wood (*Vitis labrusca* L. 'Concord') from a pruning wound inoculated with ascospores of *E. armeniacae* (from a Michigan source) about one year previous to the isolation. Mycelium was grown in potato dextrose broth (Difco Laboratories, Detroit, MI 48201) on a rotary shaker at 80 RPM at room temperature for about one week. The mycelium was then collected onto Whatman #1 filter paper through a Buchner funnel, washed extensively with distilled water, frozen with liquid nitrogen, and ground to a fine powder with a mortar and pestle. Each 3 g of this material was suspended in 25 ml 0.01 M phosphate buffered saline (PBS) pH 7.4 and sonicated for 20 minutes at 3.5 amperes with a Branson Sonifier^{R} Model No. 5125 (Branson Instruments, Inc., Shelton, CT 06484). Microscopic inspection indicated that the mycelium from this treatment was broken up sufficiently so that no pieces had more than one cross wall. Two types of antigen were prepared from this material:

1. Protein concentration was adjusted to 3 mg/ml with 0.01 M PBS pH 7.4 as determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA 94804) based on the method of Bradford (9). Lyophilized bovine gamma globulin was used as a protein standard. This antigen was called the "whole cell antigen."

2. The sonicated material was centrifuged at 4000 RPM for 4 minutes at 4°C. The supernatant was then discarded and the pellet resuspended in 20 ml 0.01 M PBS pH 7.4. This procedure was repeated until the supernatent was clear by visual inspection. Microscopic inspection of the pellet showed no cytoplasmic material within the cell walls of the mycelial pieces. The protein concentration was adjusted to 2 mg/ml as described before. This antigen was called the "cell wall antigen."

Immunization and Bleeding Schedule

Two New Zealand white doe rabbits were bled approximately 15 ml from the marginal ear vein to collect preinjection serum (normal serum). Each rabbit was then injected subcutaneously with 1.5 ml of either the whole cell or the cell wall antigen emulsified with an equal volume of Freund's complete adjuvant (Difco). The rabbits were then injected weekly as above with 1.5 ml of the respective antigen emulsified with an equal volume of Freund's incomplete adjuvant.

When the titer had increased to a reasonable level (1/16 to 1/64), determined according to the titering procedure described below, the rabbits were bled up to 40 ml when possible from the marginal ear vein weekly for 5 weeks for the rabbit injected with the whole cell antigen and 8 weeks for the rabbit injected with the cell wall antigen. The bleeding of the latter rabbit was carried on so long because in some weeks very little blood could be extracted from it.

Fresh blood was placed in a 37°C water bath for 2 hours to allow clot formation then refrigerated overnight. The serum

fraction was then drawn off with a pasteur pipette and stored at 4°C with a few crystals of chlorobutanol (Sigma Chemical Co., St. Louis, MO 63178) as a preservative.

Determination of Titer

The antigen (whole cell or cell wall) to the serum being tested was diluted 1:20, 1:40, and 1:50 (v/v) with 0.01 M sodium phosphate, pH 7.2. Two-tenths ml of each of these preparations was placed in test tubes with an equal volume of serum diluted with 0.85% saline to the dilution being tested. The same treatment was also done with the rabbit normal serum. These tubes were then immersed in a 37°C water bath for 6 hours. They were then refrigerated up to 2 days until observed. Observations were made by shaking the tube in question with the analogous tube made with the normal serum in front of a fluorescent light bar. A positive reaction was readily observed by the particulate fraction in the tube not going into homogeneous suspension easily when compared with the tube made with the normal serum.

Preparation of Fluorescent Antisera

Purification of Gamma Globulin

The gamma globulin was precipitated from the serum by mixing 10 ml serum with an equal volume of 80% saturated ammonium sulfate, pH 7.2 (SAS). This mixture was slowly stirred for 20 minutes then allowed to stand overnight at 4°C. It was then centrifuged at 9750 xg at 4°C for 15 minutes. The supernatant was then discarded and the pellet resuspended in 10 ml 0.85% saline plus 10 ml SAS.

After again stirring slowly for 20 minutes, the mixture was allowed to stand for 1 to 2 hours. These last centrifugation and resuspension steps were repeated three times except the final pellet was dissolved in 5 ml 0.85% saline. The ammonium sulfate from this solution was removed by dialysis against several changes of saline at 4°C for about 2 days. Absence of sulfate was confirmed by adding a small amount of saturated $BaCl_2$ to an equal volume of dialysate.

Conjugation of Gamma Globulin to Rhodamine Isothiocyanate (RITC)

The protein concentration of the purified gamma globulin was adjusted to 1% (v/v) with 0.85% saline as determined using the Bio-Rad Protein Assay detailed before. Three mg of RITC (United States Biochemical Corporation, Cleveland, OH 44128) was dissolved in 4 ml 0.1 M PBS pH 8.0 on a magnetic stirrer and then added to 10 ml of the 1% gamma globulin solution to which 4 ml 0.15 M PBS, pH 9.0 had been added. The pH of this solution was adjusted to 9.0 with NaOH. This solution was stirred slowly for 3 hours at room temperature.

The free RITC was separated from the RITC conjugated to gamma globulin on a Sephadex G-50-80 (Sigma) column equilibrated and eluted with 0.005 M sodium phosphate buffer, pH 7.2, containing 0.1 M NaCl. Conjugated gamma globulin was stored in 4 ml aliquots at -20°C.

Cross-adsorption of Fluorescent Antibody with Other Fungi

In order to increase the specificity of the fluorescent antibody prepared above, that preparation was cross-adsorbed with a

partial cell wall preparation of Phomopsis viticola and/or Epicoccum nigrum made as with the E. armeniacae cell wall antigen above. These preparations were called partial cell wall preparations because the sonification step failed to break up the hyphae of these fungi enough so that there was one or less cross wall in all the hyphal pieces. Either 0.25 g or 0.5 g of the partial cell wall preparation was added to 4 ml of the fluorescent antibody that was prepared from the serum made by injection of the cell wall antigen. This mixture was stirred slowly for 6 hours or overnight. The hyphae was then removed by a 10 minute centrifugation at 500 RPM.

Fluorescent Antibody Staining of Fungi on Glass Slides

Mycelium of various fungi was grown as was E. armeniacae and was stored at 4°C until used. Staining was conducted similar to the method of Schmidt and Bankole (50). A small piece of the mycelial colony of the test fungus was rinsed extensively with distilled water and spread out on a glass slide. After this mycelial smear had air-dried at room temperature, it was then mildly heat fixed and flooded with labeled antiserum that had been two-fold serially diluted (1:4 to 1:32, depending on the fluorescent antibody preparation) as much as possible while still retaining maximum fluorescence when E. armeniacae was stained. The slide was then placed in a humid chamber at 37°C. After 30 minutes, the slide was dipped in distilled water and then immersed in 1 liter of 0.85% saline circulating in a tub with a magnetic stir bar. After 30

minutes of this rinse, the slide was again dipped in distilled water and mounted in FA Mounting Fluid (Difco). As a control, each test fungus was also "stained" and rinsed as above with the elution buffer of the sephadex column described before.

Staining of Fungi in Wood Sections

Sectioning Procedure

Two-year-old *V. labrusca* 'Concord' vines were pruned above a node or branch and inoculated on the pruning wound with 500 *E. armeniacae* ascospores in 5 µl distilled water. In some cases, the pruning wound was also inoculated with 500 macroconidia of an Australian isolate of *Fusarium lateritium*. Isolations from these vines were made from 9 to 13 months after inoculation to identify fungi inhibiting the canes by aseptically transferring 10 wood chips from inside the cane near the pruning wound onto potato dextrose agar (Difco) that had been amended with 100 ppm streptomycin sulfate. Any fungi growing out of the wood chips were assumed to have been inhabiting the wood.

Forty-µm wood sections were made from these canes near the point of inoculation using a sliding microtome (AO Spencer, Buffalo, NY 14215). These sections were fluorescent antibody stained using the direct method, modified from Malajczuk, McComb, and Parker (34) or the indirect method, modified from Warnock (56). The modifications reflect adaptations to greater thickness of the stained sections than those in the references and also efforts to eliminate non-specific binding of the RITC-labeled gamma globulin to the wood.

Direct Staining

Wood sections were incubated for 30 minutes or 1 hour in a 37°C humid chamber in the presence of RITC-labeled gamma globulin prepared as described previously. They were then dipped in distilled water and immersed for 90 minutes in a tub with 1 liter of circulating 0.85% saline to which acetone was added to a final concentration of 10% (v/v) at pH 7.0. The sections were then dipped in distilled water again and mounted in FA mounting fluid. The presence of acetone in the rinse solution was necessary to eliminate nonspecific binding of the fluorescent antibodies to the wood.

Indirect Staining

A dilution of serum diluted with 0.85% saline was used which was at the greatest dilution possible (1:50) that gave maximum fluorescence of hyphae observed in wood sections from a stem in which all fungi isolated was E. armeniacae. This diluted serum was incubated with the wood section for 25 minutes in a 37°C humid chamber. Care was taken to keep the wood section wet with the serum for this period. The sections were then placed in a tub with 1 liter of circulating 0.01 M PBS pH 7.2 for 20 minutes at room temperature. After this rinse, the sections were incubated with goat anti-rabbit RITC (United States Biochemical) diluted as above (1:100) with 0.02 M PBS pH 7.3 for 30 minutes in a 37°C humid chamber. Again, care was taken to keep the section wet with the

goat anti-rabbit RITC solution. This was followed by a distilled water dip, then a 2 hour acetone-saline rinse as detailed before.

Observation of Material

All fluorescence was observed at 250X with a Zeiss universal microscope equipped with epifluorescence and a HBO 50W mercury lamp, a KG1 heat absorbing filter, a BP 546/7 green interference excitation filter, an FT 580 chromatic beam splitter, and a LP 590 barrier filter.

RESULTS

Antiserum Titer Levels

The antiserum made to the whole cell antigen reached a titer of 1/64 four weeks after the first injection. This increased to 1/128 two weeks later and stayed at this level with subsequent bleedings. The antiserum made to the cell wall antigen reached a titer of 1/16 seven weeks after the first injection, increased to 1/64 two weeks later, and also stayed at this level through the rest of the bleeding period.

Specificity of Fluorescent Antibody to Fungi on Glass Slides

Table 1 shows the reaction of various genera and species of fungi on glass slides to conjugates made with the two antisera as well as the conjugate made with the antiserum from the cell wall antigen cross-adsorbed with *Phomopsis viticola*. In this case, 0.12 g of the *P. viticola* partial cell wall preparation was allowed to react for 6 hours then centrifuged out as described previously. This process was then repeated to the fluorescent antibody solution remaining with 0.15 g of the partial cell wall preparation. The same dilution of this cross-adsorbed fluorescent antibody solution as with the fluorescent antibody solution it was derived from (1:32) was effective in giving full fluorescence to *E. armeniacae*. All

TABLE 1.--Fluorescence of Fungi on Slides.[a]

Fungus	Control	Conjugate from Whole Cell Antigen	Conjugate from Cell Wall Antigen	Cell Wall Conjugate Cross-adsorbed with *P. viticola*
EUTYPA ARMENIACAE ISOLATES				
Michigan grape	-[b]	+++[e]	+++	+++
California grape	-	+++	+++	+++
California apricot	-	+++	+++	++[d]
Washington grape	-	+++	+++	+++
Australia apricot	-	+++	+++	+++
Michigan grape ascospores	-	NT[f]	++	NT
Michigan grape scolecospores	-	NT	++	NT
FUNGI COMMONLY ISOLATED FROM GRAPE WOOD				
Alternaria sp.	-	+++	+/-	NT
Aureobasidium sp. (spores)	-	+++	+++	+++
Cytospora sp.	-	++	+++	++
Epicoccum nigrum	-	+++	+++	+++
Penicillium sp.	-	-	-	NT
Phomopsis sp. (?)[g]	-	+++	+++	+++
Phomopsis viticola	-	+++	+++	+/-
Sphaeropsis sp.	-	+++	+++	+[c]
OTHER FUNGI				
Botrytis cinerea	-	++	+	NT
Colletotrichum gloeosporioides	-	+	+	NT
Endothia parasitica	-	+++	+++	+/-
Fomes annosus	-	+++	+++	+
Fusarium lateritium	+/-	+/-	+/-	NT
Gloeosporium amphophalagum	-	+	+	NT
Guignardia bidwellii	-	-	-	NT
Hypoxylon mammatum	-	+++	+++	+
Phytophthora megasperma var. *glycinea* (mycelium)	-	-	-	NT
(oospores)	+++	+++	+++	NT
Poria placenta	-	+++	+++	++
Pythium ultimum	++	+++	++	++
Schizophyllum commune	-	+++	+++	++
Sclerotinia sclerotiorum	-	+++	+++	+

[a]See text for staining procedure. Observed at 250X. Scored on fluorescence of individual hyphae in area of smear where individual hyphae were discernable. Results represent two replications of each fungus.

[b]- = no hyphal fluorescence

[c]+ = hyphae barely visible

[d]++ = hyphae readily visible but with dull fluorescence

[e]+++ = hyphae fluoresced brightly

[f]NT = not tested

[g]Tentatively identified as *Phompsis* sp. No beta spores were found, however.

hyphae stained with equal intensity along all areas observed. See Figures 1-8.

An attempt was made to cross-adsorb the conjugated cell wall serum with 0.5 g of a partial cell wall preparation of Epicoccum nigrum for 6 hours. This did not succeed in reducing the reactivity of E. nigrum on glass slides relative to E. armeniacae. An attempt was also made to cross-adsorb the conjugated cell wall serum with 0.5 g of a partial cell wall preparation of E. nigrum plus 0.5 g of a partial cell wall preparation of P. viticola overnight. Again, this did not reduce the reactivity of E. nigrum relative to E. armeniacae although reactivity of P. viticola was eliminated.

All fungi that reacted with either a '++' or a '+++' to the cell wall conjugate that was cross-adsorbed with P. viticola (Aureobasidium sp. (spores), Cytospora sp., E. nigrum, E. armeniacae, Phomopsis sp., Poria placenta, and Schizophyllum commune) were stained with goat anti-rabbit RITC to determine if this staining was due to nonspecific binding of the conjugate to the fungus. In all cases no fluorescence was observed, indicating the reactions with the cross-adsorbed preparation were due to specific binding.

Staining of Hyphae in Wood

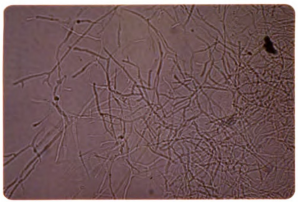
As observed previously (34), autofluorescence of the wood sections was very bright. This was especially true here since these sections were 40 μm thick, much thicker than previously used (34). The 40 μm thickness was necessary to obtain a good section of grape wood on the sliding microtome without resorting to paraffin

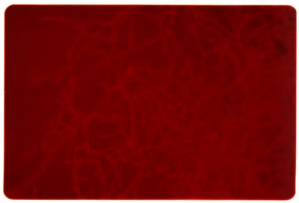
FIGURE 1.--Transmitted light view of *Eutypa armeniacae* mycelium stained with RITC-conjugated whole cell antiserum.

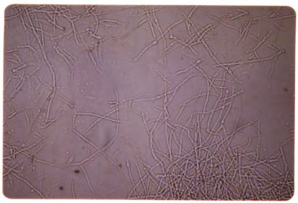
FIGURE 2.--Epifluorescence under fluorescence filters of *Eutypa armeniacae* mycelium stained with RITC-conjugated whole cell antiserum.

FIGURE 3.--Transmitted light view of *Fusarium lateritium* mycelium stained with RITC-conjugated whole cell antiserum.

FIGURE 4.--Epifluorescence under fluorescence filters of *Fusarium lateritium* mycelium stained with RITC-conjugated whole cell antiserum.







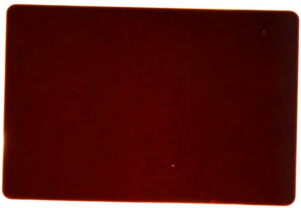
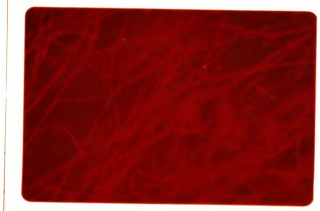


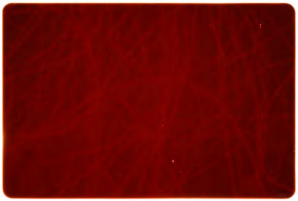
FIGURE 5.--Epifluorescence under fluorescence filters of Alternaria sp. mycelium stained with RITC-conjugated whole cell antiserum.

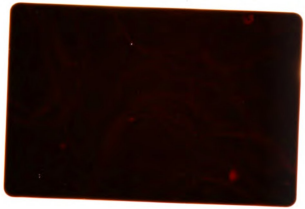
FIGURE 6.--Epifluorescence under fluorescence filters of Alternaria sp. mycelium stained with RITC-conjugated cell wall antiserum.

FIGURE 7.--Epifluorescence under fluorescence filters of Phomopsis viticola mycelium stained with RITC-conjugated cell wall antiserum.

FIGURE 8.--Epifluorescence under fluorescence filters of Phomopsis viticola mycelium stained with RITC-conjugated cell wall antiserum that has been cross-adsorbed with Phomopsis viticola.







imbedding due to the large vessel elements of grape (44). The RITC stain was selected instead of the almost universally used fluorescein isothiocyanate (FITC) stain because the autofluorescence of the grape wood was much less under the filters used for RITC fluorescence detection than under the filters used for FITC fluorescence detection. See Figures 9 and 10.

Hyphae in wood not stained or in wood stained with normal serum using the indirect method sometimes showed slight fluorescence. See Figures 11-14.

With the direct staining procedure, some specificity of hyphal staining was observed (Figures 15 and 16). However, the brightness of fluorescence of the most intensely stained hyphae was frequently not great enough to stand out over the autofluorescence of the wood when some wood tissue, for example a pith cell wall, was covering the hyphae. Hyphae in wood stained for 30 minutes exhibited the same intensity of fluorescence as hyphae in wood stained for one hour.

With the indirect staining method, hyphae that reacted strongly to the stain fluoresced in wood with much greater intensity than the analogous hyphae stained with the direct method (Figures 17 and 18). This intensity was great enough so that the autofluorescence of the wood did not interfere with the visualization of the hyphae, even if the tissue was in front of the hyphae (Figures 19 and 20).

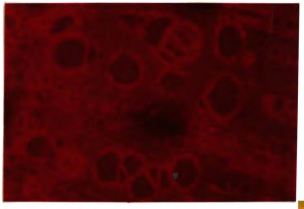
An attempt was made to determine if the specificity of hyphal fluorescence when stained in wood by the indirect method is

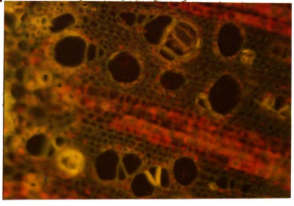
FIGURE 9.--Autofluorescence of cross section of grape wood under RITC fluorescence filters (as described in Materials and Methods).

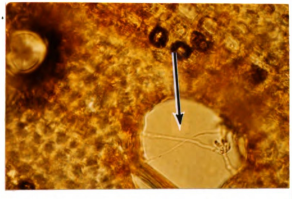
FIGURE 10.--Autofluorescence of cross section of grape wood under FITC fluorescence filters (blue interference 455-490 nm excitation filter set, FT 510 chromatic beam splitter and LP 520 barrier filter).

FIGURE 11.--Transmitted light view of cross section of grape wood not stained. (Arrow shows hypha.)

FIGURE 12.--Autofluorescence under fluorescence filters of same unstained cross section of grape wood as in Figure 11.







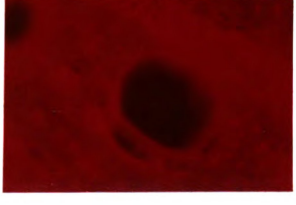
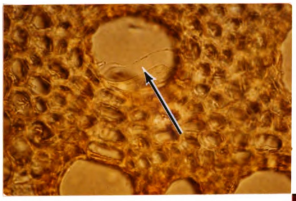


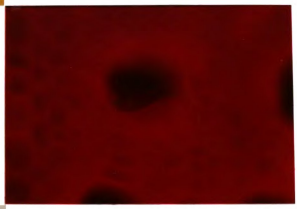
FIGURE 13.--Transmitted light view of cross section of grape wood indirectly stained with rabbit normal serum. (Arrow shows hypha.)

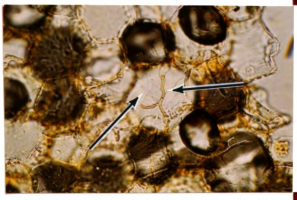
FIGURE 14.--Autofluorescence under fluorescence filters of same normal serum-stained cross section of grape wood as in Figure 13.

FIGURE 15.--Transmitted light view of cross section of grape wood directly stained with RITC-conjugated cell wall antiserum. (Arrow shows hyphae.)

FIGURE 16.--Autofluorescence under fluorescence filters of same directly stained cross section of grape wood as in Figure 15.







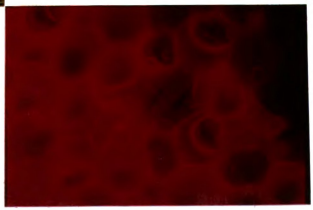
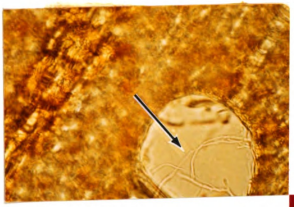


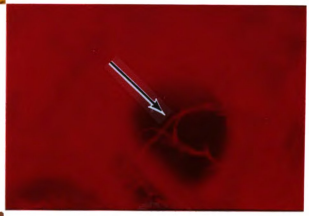
FIGURE 17.--Transmitted light view of cross section of grape wood indirectly stained with cell wall antiserum. (Arrow shows hyphae.)

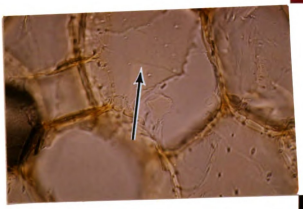
FIGURE 18.--Autofluorescence under fluorescence filters of same indirectly stained cross section of grape wood as in Figure 17. (Arrow shows hyphae.)

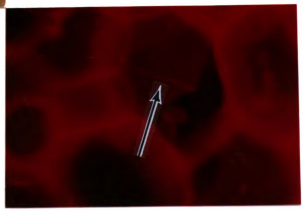
FIGURE 19.--Transmitted light view of cross section of grape wood indirectly stained with cell wall antiserum. (Arrow shows hypha behind pith cell wall.)

FIGURE 20.--Autofluorescence under fluorescence filters of same indirectly stained cross section of grape wood as in Figure 19. (Arrow shows hypha behind pith cell wall.)









the same as when stained on slides with the direct method. This was done by staining wood sections of stems from which the fungi isolated was primarily or entirely of one or two species. The limitation with this method is that there was a very small percentage of stems that had this "pure culture" of fungi as determined by isolation. Out of about 600 stems isolated from, only nine were judged to be appropriate to use. The results are on Table 2.

In the stem from which essentially all fungi isolated was E. armeniacae, hyphae was observed in the xylem vessels and pith, but in no other tissues.

TABLE 2.--Fluorescence of Fungal Hyphae in Grape Wood--Indirectly Stained.[a]

Stem Isolation Result[b]	Serum Made to Whole Cell Antigen	Serum Made to Cell Wall Antigen	Normal Serum
Eutypa armeniacae 8 Alternaria sp. 1 Phomopsis sp. 1	+++	+++	- to +
Epicoccum nigrum 10 Alternaria sp. 3	- to +++*	- to +++	- to +
Fusarium lateritium 7	- to +	- to +	- to +
Alternaria sp. 10 E. nigrum 1	- to +++*	- to +++	- to +
Penicillium sp. 10 F. lateritium 2 E. armeniacae 1	- to +++	- to +++	- to +
F. lateritium 5 E. nigrum 7	NT	- to +++	- to +
Cytospora sp. 10[c]	- to +++*	- to ++*	- to +
Alternaria sp. 10 Aureobasidium sp. 1[d]	NT	- to +	- to +

[a]See text for staining procedure. See Table 1 for explanation of symbols.

[b]Numbers after name of fungus denotes number of wood chips (out of 10) from which that fungus was isolated onto PDA.

[c]Two stems were used with this isolation result.

[d]Very little hyphae could be observed in sections made from this stem.

*Indicates results not explainable by specificity of fungi on slides with direct method (Table 1).

DISCUSSION

As stated under Results, the RITC stain was more appropriate here than the more commonly used FITC stain due to reduced autofluorescence of the wood tissue. This reduced autofluorescence was because the barrier filter used for RITC eliminates all wavelengths less than 590 nm whereas the barrier filters used for FITC must be ones that eliminate wavelengths no higher than 520 nm, which is the wavelength of maximum fluorescence of excited FITC-labeled globulin (20). Therefore, the RITC system has the advantage of eliminating all autofluorescence that occurs between 520 and 590 nm. This may explain why no autofluorescence was detected during this work in unstained hyphae on slides in most cases whereas a faint blue autofluorescence was often detected in previous studies (10,34,45,50). Craig, et al. (16) also attributes high autofluorescence of pea tissue under FITC filters to intense autofluorescence of protein bodies at between 490 and 530 nm, which is approximately the same emission maxima of FITC-labeled globulin.

The titer achieved for both types of antisera was similar to that achieved with E. armeniacae in the past as determined by gel diffusion methods with the soluble portion of a whole cell antigen (46).

Specificity of both antisera was fairly poor, as determined with the direct staining method on slides (Table 1). This is

apparently in contrast to the good specificity found with antiserum made to a whole cell preparation of E. armeniacae, again using a gel diffusion assay (46). Only the soluble antigens were used in these tests, however, as stated before. Also, there is some indication that fluorescent antibody staining is different in the detection of a reaction than gel diffusion methods (2,10). In other work (17), antiserum made to a whole cell E. armeniacae preparation did not react with ascospores of E. armeniacae using the indirect immunofluorescent technique. This would indicate greater specificity than the conjugate tested here, which showed some reaction to E. armeniacae ascospores.

In previous papers where some specificity has been found (34,49,51,52), the specificity was along taxonomic lines, with species that are closely related to the species used as antigen reacting and species less related not reacting. This was not the case here. This can be seen most readily on Table 1 by noting that the basidiomycetes Poria placenta, Schizophyllum commune, and Fomes annosus reacted strongly to the stain whereas the ascomycetes Fusarium lateritium (perfect genus: Gibberella) and Guignardia bidwellii did not.

There is some indication that the conjugated antisera tends to react more with fungi that are wood inhabitants (like Eutypa) than with fungi that are not wood inhabitants. The problems with making this assertion with the data at hand are twofold. First, there is little or no information in the literature as to whether

some of the fungi tested are wood inhabitants or not. For example, it is known that the Michigan isolate of Colletotrichum gloeosporioides may overwinter on blighted blueberry twigs (23) but it is not known if this organism will persist on secondary wood (John Hartung, personal communication). Second, there were very few fungi tested (Table 1) that are not wood inhabitants or putative wood inhabitants. However, if one assumes that the "+" scoring is essentially no reaction and that C. gloeosporioides, G. bidwellii, Gloeosporium amphophalagum, and Phytophthora megasperma var. glycinea are the only fungi on Table 1 that are not wood inhabitants, then there is a highly significant difference ($X^2 = 8.41^a$) with the whole cell conjugate and a significant difference ($X^2 = 5.11$) with the cell wall conjugate between reactivity of wood inhabitants and non-wood inhabitants (Ho: there is no significant difference in reactivity to the FA conjugate between wood inhabitants and non-wood inhabitants). Even if one considers C. gloeosporioides a wood inhabitant and the other three fungi mentioned above as non-wood inhabitants, there is still a significant difference ($X^2 = 5.14$) with the whole cell conjugate and a marginally significant difference ($X^2 = 3.03$) with the cell wall conjugate in reactivity of wood inhabitants vs. non-wood inhabitants.

The conjugate from the antiserum made to the cell wall antigen was somewhat more specific than the conjugate made from the

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[a] X^2 critical values: $P = 0.005 = 7.88$; $P = 0.010 = 6.63$; $P = 0.050 = 3.84$; $P = 0.10 = 2.71$.

whole cell antigen, since Alternaria sp. and Botrytis cinerea were reduced in reactivity. The increase in specificity of an antiserum made to a cell wall antigen over an antiserum made to a whole cell antigen is consistent with some findings in the literature (12) but not others (49). The difference in specificity is rather small, though, and may just be due to variations within the individual rabbits used.

The cross-adsorption procedure using Phomopsis viticola was quite successful, reducing reactivity of half (6 out of 12) of the fungi that previously reacted strongly to negligible levels and moderately reducing reactivity of another four. This procedure is used very commonly and has suceeded at other times in increasing specificity without reducing activity of the conjugate relative to its homologous antigen (52). The failure of Epicoccum nigrum to be cross-adsorbed out of the E. armeniacae cell wall conjugate implies that E. nigrum and E. armeniacae have many antigens in common. This is surprising, considering that E. nigrum is less closely related to Eutypa than P. viticola (Epicoccum is in the Moniliales whereas Eutypa [imperfect: Cytosporina] and Phomopsis are both in the Sphaeropsidales) and may also indicate a lack of taxonomic relationships among fungi with antigens in common with E. armeniacae.

Strong oospore fluorescence was also found in Pythium spp. by White (59). Very little mycelial autofluorescence was found there, however, compared to the moderate autofluorescence of Pythium hyphae found here. This difference may be due to different mycelial

culture methods. Oospore fluorescence in Phytophthora megasperma var. glycinea was not observed in another study (Susan Cohen, personal communication) but this difference has been attributed to the use of a tungsten halogen 12V 100W lamp in that study, which has only a fraction of the luminescence of the HBO 50W mercury lamp used in this work at the wavelength of RITC excitation.

The greater specific fluorescence of hyphae when stained with the indirect method over directly stained hyphae is consistent with other work where this comparison was made (12,16,20,25).

The use of acetone in the final rinse to eliminate nonspecific binding of the labeled antisera to grape wood seemed to be quite effective in this case and much simpler than other methods used (7,16,20,43). The lengthy rinse was necessary since nonspecific fluorescence of hyphae stained indirectly using normal serum sometimes occurred when rinsed for one hour.

The cases (marked with an * in Table 2) where the fungal species stained indirectly in wood sections reacted differently than they did when stained directly on slides (Table 1) were all of the type that all fungi observed in wood should have fluoresced whereas only some did. For example, both Alternaria sp. and E. nigrum (the only fungi found in two stems by isolation--Table 2) reacted to the RITC-conjugated whole cell antiserum. Therefore, all hyphae stained indirectly in sections from these stems should have fluoresced, but some did not. Also, all hyphae of Cytospora sp. stained directly on slides fluoresced, but only some did when stained indirectly in the stems. These inconsistencies are

probably not due to the inability of the stain to penetrate the wood and stain the hyphae because of consistent staining of hyphae in the stem that E. armeniacae was almost exclusively isolated from. They could have been due to failure to isolate the fungal species in these stems that did not react to the stain. Alternatively, the inconsistencies could have been due to variation in reactivity of the hyphae in the wood. This would imply that the hyphae in the wood is variable as to antigenic makeup. This is consistent with some studies which demonstrate different reactivities to a fluorescent antibody stain of different segments of hyphae, frequently with the most reactivity being at the growing hyphal tips (2,10,50, 59). This is also consistent with the observations of Jacobi and MacDonald (26) who observed variations of size of hyphae of Ceratocystis fagacearum in vessels and tracheids of oak. Wilson, working with the same organism (60), also found this large variation in hyphal size. In addition, he found that conidia produced in vessels and tracheids of oak were smaller than those produced in culture. This suggests a difference in culture-grown compared to wood-grown fungi.

The fluorescent antibody technique could become an effective histological tool in observing hyphae of interest in wood sections contaminated with other fungi or in studying ecological relationships of mixed fungal populations in wood. What is necessary is an antiserum specific to the fungus of interest. If possible, this specificity should be demonstrated with fungi grown in culture as well as with fungi grown in wood.

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