SUMMARY OF RELATIONSHIPS AMONG SWOLLEN SUPERFICIAL CANKERS, SURVIVAL OF AMERICAN CHESTNUT TREES, AND HYPOVIRULENCE IN ENDOTHIA PARASITICA AT SOUTHEASTERN FOREST EXPERIMENT STATION

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ABSTRACT.--Sixty-seven *blight cankers on American and European chestnut trees yielded Endothia parasitica with hypovirulence (H) from 3 percent of the 1,240 isolates. One isolated American chestnut from near Bonair, Tennessee, yielded 28 of the 37 H isolates. American chestnut sprouts were killed 15 months after inoculation by virulent isolates but usually healed over after inoculations with H isolates. Bark patches from swollen superficial (SS) cankers used to inoculate American chestnuts have caused normal, lethal cankers 79 percent of the time after two growing seasons. Single compatible H isolates, combinations of H isolates including a compatible one, and 28 randomly selected H isolates when applied to wounded cankers have enhanced healing and prolonged tree survival in comparison to wounded check trees. Nonwounded cankers sprayed with conidia from compatible H isolates are healing as well as wounded cankers painted with mycelium in agar. Wounded and nonwounded check trees have 86 percent mortality in contrast to 29 percent tree mortality in the H treatments.*

Objectives

Three aspects of the research on hypovirulence being carried out at the Southeastern Forest Experiment Station are presented. The objectives were (1) to determine the frequency of occurrence of isolates of *Endothia parasitica* with and without hypovirulence (H) in swollen, superficial (SS) cankers ("healing cankers"); (2) to compare the effects of inoculation of American chestnut trees with virulent (V) and H isolates of *E. parasitica;* and (3) to increase survival of American chestnut trees by control of canker development with H isolates. An introduction, methods, results, and discussion sections are presented for each objective

Association of H Isolates with SS Cankers

Introduction

Subjective observations by European workers (Grente and Berthelay-Sauret 1978; Turchetti 1979; Turchetti and Marinelle 1979) form the basis for the hypotheses that natural intensification of H in *E. parasitica* is responsible

for increased survival of blight-infected European chestnut *Castanea sativa* in Italy and introduction of H isolates is providing biological control of chestnut blight in France. Survival of European chestnut in Italy is characterized by the presence of healthy bark callus beneath cankers according to Grente and Berthelay-Sauret (1978). If callus causes swelling and necrotic tissue is superficial, the canker is SS. Turchetti (1979) classified cankers as normal, intermediate, hypovirulent, or strongly hypovirulent. Normal cankers are depressed because the young xylem, cambium, and phloem are killed and these tissues collapse. Grente and Berthelay-Sauret (1978) state there was a direct relationship between the "degree to which the canker healed" and the number of white (presumably H) isolates of *E. parasitica* present. Recovery of white isolates varied from 5 to 100 percent. Completely healed cankers either yielded no isolates of *E. parasitica* or only white isolates from 10 to 20 percent of the samples. No details of sample size or sampling method were given. Many factors contribute to the disease cycle and therefore to disease expression. The disease pyramid consists of host, pathogen, environment, and time. Objective experiments should consider all four of these factors in relationship to any changes in disease expression.

Hypovirulent isolates of *E. parasitica* have been recovered from American chestnuts in the eastern United States (Day et al. 1977; Elliston et al. 1979; Jaynes and Elliston in press); however, the relative occurrence of H isolates and their association with SS and normal cankers have not been documented.

Methods

One study area near Buchanan, Virginia, was selected because trees with burrs were present, large SS cankers were abundant, and an H isolate was recovered from one canker in 1979. Trees near Robbinsville and Franklin, North Carolina, were sampled because they had swollen infections and at the latter site several trees were unusually large and had burrs. A tree at Bonair, Tennessee, was a source of H isolates in earlier reports (Elliston et al. 1979; Jaynes and Elliston in press). This tree was remote from other chestnuts. Two cankers on European chestnuts from Italy were sent by Turchetti in response to a request for superficial cankers to be used in grafting experiments; however, these cankers were normal in appearance.

At Buchanan, 42 cankers on 19 trees were sampled by removing 20 chips per canker. At Bonair, six cankers on one tree were sampled by removing 20 chips per canker. At Buchanan and Bonair, half the chips were from the canker margin and half were from the center of the canker. Eight SS cankers on five trees near Robbinsville were sampled by removing 25 chips from each canker. At Franklin, 10 SS cankers were sampled by removing 12 chips per canker. The two Italian cankers were sampled by removing 30 chips per canker. Chips were selected across the entire canker surface from the latter three sources,

Chips were surface-sterilized in 65 percent ethyl alcohol and plated on Difco potato dextrose agar (dPDA). Isolates of *E. parasitica* were subcultured on dPDA under white fluorescent lights to subjectively separate V and H isolates. Vegetative compatibility (v-c) groups were determined with the method of Anagnostakis (1977). Potential H isolates were paired with compatible V isolates on dPDA with methionine and biotin (dPDAmb) in a modification of Anagnostakis and Day's method (1977) to confirm the presence of a

cytoplasmic factor that was infectious and affected growth of V isolates. No cellophane is used and 5 mm agar discs with V and H isolates are placed 10 mm apart near the center of the plate. Plates are incubated at 24 C with a 16-hr-day (white flourescent light).

Results

Endothia parasitica grew from 85 percent of the sample chips (1,240:1,460) (Table 1). Subcultures were made of 910 *E. parasitica* isolates to study culture morphology, v-c groups, and capacity of suspected H isolates for conversion of V isolates. Thirty-seven isolates have been confirmed as having H present. The H isolates were common only in the Tennessee tree and were present in all six cankers. The canker margins yielded fewer H isolates (8:32) than did the canker centers (20:41). At Buchanan the four H isolates occurred in four different cankers. Three of these were SS and one was normal. One of the Italian cankers yielded five H isolates.

Table 1. Occurrence of hypovirulent (H) isolates of *Endothia parasitica* in normal (N) and swollen superficial (SS) cankers on American and European chestnut.

Location	Cankers (No.)		D.B.H. range of trees (cm)	Sample chips (No.)	*E. parasitica* Present with H	
	N	SS			(No.)	(No.)
Robbinsville, NC	0	8	3-8	200	187	0
Franklin, NC	0	10	5-30	258	176	0
Buchanan, VA	11	30	3-28	820	739	4
Bonair, TN	0	6	43	120	78	28
Italy	2	0	4-6	62	60	5
Total	13	54		1,460	1,240	37

Discussion

The intensive sampling of 65 cankers in the southern Appalachians indicates that H isolates are not commonly associated with SS cankers. In this region, environment, host response, or time (season of infection) may be causing the SS cankers.

At Buchanan, two of the four trees with H isolates died during 1981, whereas 17 of 37 with no H isolates present died. The tree at Bonair with 36 percent H isolates (28:78) appeared less vigorous in 1981 than it had in 1980. Since no trees with normal cankers were present in the Bonair area, no comparison of recovery from normal and SS cankers could be made. The presence of H isolates in one normal canker from Italy further confounds the association of H isolates with healing cankers.

The senescence syndrome Oinks 1966) and H have many similar characteristics including a change in growth habit and transmission by cytoplasmic exchange. The senescence syndrome appears more frequently in old cultures of some fungi (Jinks 1966). This suggested that H isolates might be more common in cankers on trees that had been dead for several years. However, isolation of *E. parasitica* from 50 dead trees in Virginia and North Carolina did not yield H isolates. Nor did the frequency of H isolates in the Virginia trees with H in 1980 increase in 1981 after they died.

Inoculation of American Chestnut Trees with H Isolates

Introduction

Previously Kuhlman (1981) suggested white or H isolates were essentially nonpathogenic. Koch's postulates require a regular association of the pathogen with any given symptoms, recovery of the pathogen in pure culture, inoculation of the host, subsequent development of the same symptoms, and reisolation of the pathogen. Published results have confirmed only the occasional recovery of a debilitated pathogen, the H isolates.

The SS symptom may be the result of a balanced ratio of V and H mycelium in the outer bark. Initially I hoped to graft bark from SS cankers to healthy American chestnut stems to maintain this balance as well as the live host response in the cambium. However, the thick cambium and phloem from SS cankers did not match up with the thin bark on healthy trees, and the live host effect from the donor could not be maintained. Therefore, instead of grafts, inoculations were made.

The hypotheses for this group of experiments were: (1) SS cankers are the result of infection by H isolates of *E. parasitica.* (2) SS cankers are the result of a critical balance between V and H isolates which can be maintained in bark patches from SS cankers. (3) Colonization and sporulation by H isolates will be enhanced if the host substrate is weakened (a stem section rather than an intact tree).

Methods

Mycelium as inoculum. American chestnut saplings 1.3 to 2.5 cm in diameter and free of blight symptoms were inoculated by removing 7-mm bark discs with a cork borer and inserting dPDA discs of mycelium of V or H isolates. Inoculation points were covered with masking tape for 6 weeks. To facilitate colonization and sporulation by H isolates, the midpoint of 15-cm long segments of American chestnut stems (1.3 to 2.0 cm in diameter) were inoculated as above. In 1979 the experiment was installed at two locations (Buchanan, Virginia and Franklin, North Carolina), three inoculation dates (June to September at 6-week intervals at each location), and four V and four H isolates randomly selected from our collection for each location. In 1980 at Franklin two additional inoculation dates (April and June) and four Italian H isolates (selected as intermediately pathogenic by J. E. Elliston) were included with eight isolates used in 1979 at this location. Symptom development and sporulation was observed periodically through September 1981.

Bark patches as inoculum. In 1980 near Buchanan, 24 SS cankers were used as donors. Inoculations on nondiseased areas of 10 trees with SS cankers were

possible. These were called selfed inoculations. Bark patches from all 24 were put on healthy neighbors in a nearby stand, and on healthy trees in a stand near Black Mountain, North Carolina. Bark patches were also taken from the six SS cankers from near Bonair, Tennessee, and from the two normal Italian cankers and used for inoculations at Black Mountain. Bark patches from Virginia and Tennessee consisted of 2.5-cm squares, with one square (replication) from each canker inoculated at each location. Bark patches from the Italian cankers were disc extracted with a 9-mm diameter cork borer with 10 replications per canker.

In May 1981, 10 bark plugs were taken with an increment hammer (4-mm diameter) from each of the six SS cankers on the tree near Bonair, and from a large SS canker and two normal cankers on trees near Franklin, North Carolina. These plugs were used to inoculate healthy trees near Franklin. Treatments (bark plug source) were randomly assigned within each of 10 replications. A second study included bark plugs from nine SS cankers from Buchanan, and three SS cankers near Franklin. Bark plugs were collected with a 7-mm diameter cork borer. Treatments were randomly assigned within six replications. Canker development was observed in September 1981.

Results

Mycelium as inoculum. Chestnut sprouts were highly susceptible hosts for V isolates but were generally resistant to infection by H isolates regardless of season of inoculation (Table 2). September inoculations with V isolates

Table 2. Percentage of American chestnut sprouts on two sites with various symptoms in September 1981 after inoculations with virulent (V) and hypovirulent (H) isolates of *Endothia parasitica* on four dates

Symptoms	Month of inoculation							
	April[a]		June[b]		July[c]		Sept.[c]	
	V	H	V	H	V	H	V	H
Infected								
Dead	83	0	95	7	94	6	52	4
Alive	8	4	2	4	4	0	13	4
Swollen callus	4	8	0	17	0	8	0	0
Not infected								
Dead	0	0	4	8	2	17	6	19
Healed	4	88	0	64	0	69	29	73

[a] Percentages based on 24 samples (four isolates x six reps inoculated in 1980).

[b] Percentages based on 72 samples (four isolates x six reps x three sites, two in 1979, one in 1980).

[c] Percentages based on 48 samples (four isolates x six reps x two sites in 1979).

were less successful than those in April, June, or July. Sprouts were killed within 15 months after inoculation by V isolates.

H isolates from Italy selected for intermediate pathogenicity have produced some swollen callus and an orange color on 42 percent of the sprouts 13 to 15 months after inoculation. Further observations will be needed to determine if SS cankers result from this symptom. These isolates have produced normal symptoms on 21 percent of the sprouts and avirulent symptoms on 37 percent.

Colonization of segments of chestnut stem, as determined by sporulation, occurred readily following inoculations in June and July with V isolates but was infrequent following inoculations with H isolates or September inoculations with V isolates (Table 3). Similar stem-section inoculations near Franklin produced fewer infections.

Table 3. Colonization and sporulation by virulent and hypovirulent (H) isolates of *Endothia parasitica* on chestnut stem sections in April 1980 following inoculations on three dates in 1979 in Virginia

	Virulent			Hypovirulent		
	6/12	7/18	9/12	6/12	7/18	9/12
Colonized (percent of 24)	96	96	13	17	4	0
Length of sporulation (mm)	143	120	2	7	<1	0

Bark patches as inoculum. The 84 bark patches from Virginia, Tennessee, and Italy in 1980 have initiated lethal or girdling cankers on 79 percent (33 to 92 percent) of the inoculated trees (Table 4). Even bark patches put on nondiseased portions of the same tree (selfed) had 60 percent dead or girdled. Heavy callus with color, a possibly favorable symptom, is present on only 5 (0 to 33) percent of the inoculations. The 1981 inoculations were only 4 months old when these readings were made and are included to show the early trend toward low incidence of infection and high incidence of healing callus or no symptoms from SS sources contrasted with high infection (95 percent) from normal cankers.

Discussion

Neither mycelium nor bark patch inoculations have produced SS infections. Most H isolates have failed to produce symptoms of infection during the second year following inoculation. This generally avirulent condition explains in part the infrequent occurrence of H isolates in the southern Appalachians.

Bark patch inoculations have the possibility of maintaining a delicate balance between the amount of V and H mycelium in SS cankers. In 1980, single replications were used from 30 SS cankers from Virginia and Tennessee. The original intent was to place patches on the same tree, a neighbor tree, and a tree on a distant site. This design should differentiate among host and environmental effects on development of SS cankers provided that this symptom developed at least on the same tree (selfed). Our hypothesis was that if the host was responsible for SS cankers, these would develop only

Table 4. Symptom development in September 1981 on American chestnut trees in Virginia (VA) and North Carolina (NC) following inoculation with bark patches from swollen superficial (SS) and normal (N) cankers from NC, VA, Tennessee (TN), and Italy (IT)

Canker inoculation				Dead	Infected	Type of callus	
Source	Type	Site	Bark patches	or with sprouts	other symptoms	Heavy orange	Healing or no symptoms
			No.	Percent			
1980							
VA	SS	Selfed	10	60	0	20	20
VA	SS	Neighbors	24	92	8	0	0
VA	SS	NC	24	79	17	0	4
TN	SS	NC	6	33	17	33	17
IT	N	NC	20	85	5	0	10
1981							
TN	SS	NC	60	0	32	17	52
NC	SS	NC	28	4	29	25	43
VA	SS	NC	54	0	31	19	50
NC	N	NC	20	0	95	0	5

on the same tree, if local environment was responsible, SS cankers would develop on selfed and neighbors. If SS cankers developed on selfed, neighbors, and a distant host, then only the pathogen was responsible for the symptom. The limited number of trees available for selfing and the low frequency of H weakened this design. In 1981, the cankers with H isolates present were used as the major source of bark patches and 6 to 10 replications were used to increase the possibility for SS canker development. After 4 months, canker development is much less frequent from SS sources than from normal canker sources.

The Italian H isolates selected for intermediate pathogenicity seem to have potential for producing SS cankers since 42 percent of these inoculations have swollen callus and orange color after two growing seasons. However, less debilitated H isolates must not be needed for optimizing survival and spread in Europe because Grente, with years of field experience, furnished highly debilitated H isolates for the initial work in the United States (Day et al. 1977; Van Alfen et al. 1975). Furthermore, highly debilitated H isolates were recovered from SS cankers on American chestnut in this and other studies (Day et al. 1977; Elliston et al. 1979; Jaynes and Elliston in press), indicating that these isolates are capable of surviving under some circumstances.

Control of Canker Development with H isolates

Introduction

The goal of all research on hypovirulence in *E. parasitica* is to prolong the life of American chestnut trees. Reports from Connecticut have indicated

that treatment of cankers with H isolates slows canker development and reduces tree mortality after two growing seasons (Jaynes and Elliston 1978; Jaynes and Elliston 1980; Van Alfen et al. 1975). Studies were established in North Carolina and Virginia to follow tree survival for several years after H treatments.

Methods

In each of the four studies, cankers initiated by five V isolates were treated 1.5 months after inoculation with H isolates.

Study 1978-F. Near Franklin, North Carolina, in July 1978, half the cankers were wounded by cutting through the bark with an ax in several places prior to treatment of all cankers with an agar slurry (check) or an agar slurry of a culture of a compatible H isolate. Cankers on all nongirdled trees were wounded and treated again in May 1979.

Study 1979-F. Treatments were applied 1.5, 10, and 24 months after June 1979 inoculations near Franklin to ax wounds through the canker. Treatments included an agar slurry check, a compatible H slurry for each V isolate, the five compatible H isolates in a slurry, and a random selection of 28 H isolates in a slurry.

Studies 1979-Va. and 1980-F. Agar slurries were applied to wounded cankers and conidial suspensions or water to nonwounded cankers 1.5 and 10, and 24 months after inoculation near Buchanan in 1979 and 1.5 and 10 months after inoculations near Franklin in 1980.

Results

In every experiment where H isolates were used to treat young cankers initiated by V isolates, survival of the trees has been improved over the check treatment(s) (Tables 5 and 6). The oldest experiment, 1978-F, has gone through four growing seasons (37 months). In this experiment all check trees died on the average of 19 months after inoculation, whereas 45 percent (18:40) of the treated trees are still alive. Although the treatment has kept the trees alive, some cankers are still active. During the 1981 growing season, five trees died, two others have sprouts below the cankers, and seven have signs of the fungus.

In study 1979-F, wounded cankers treated with combinations of H isolates have improved tree survival. In studies 1979-Va. and 1980-F, trees with nonwounded cankers treated with conidia from H isolates have better survival than trees in the check treatments (Table 6).

Discussion

Trees with cankers survive longer if treated with H isolates than do the check treatments. The average percentage of trees dead in the six check treatments is 86 percent (Tables 5 and 6), whereas only 29 percent of the trees in eight H treatments have died. In the oldest experiment, 45 percent of trees with treated cankers are still alive after 37 months which is nearly double the survival time after inoculation for all trees with check cankers

Table 5. Effect of wounding and hypovirulent (H) isolates in agar treatments on symptom development after inoculation of American chestnut trees with virulent isolates of *E. parasitica*

Study	Duration	Trees per treatment	Symptoms	Treatment: Agar check	Compatible H in agar	5 H's in agar	28 H's in agar
	Months			Percent affected			
1978-F	37	40	Infected:				
			Dead	100	55	--	--
			Girdled	0	5	--	--
			Not girdled	0	25	--	--
			No symptoms	0	15	--	--
1979-F	25	25	Infected:				
			Dead or w/sprouts	60	28	28	16
			Not girdled	36	28	16	44
			Not infected:				
			Healed	0	28	48	24
			No symptoms	0	12	4	4
			Dead	4	4	4	8

Table 6. Comparison of wounded and nonwounded treatments before hypovirulence (H) treatments on survival of American chestnut following inoculations with virulent isolates of *E. parasitica*

Study	Duration	Trees per treatment	Symptoms	Treatment: Wounded, agar check	Non-wounded, water check	Wounded, H in agar	Non-wounded, H conidia
	Months			Percent affected			
1979-Va.	25	25	Infected:				
			Dead	92	92	27	12
			Not girdled	4	0	27	48
			Not infected:				
			Healed	4	4	31	28
			Dead	0	4	15	12
1980-F	15	40	Infected:				
			Dead or w/sprouts	73	88	3	15
			Not girdled	28	8	33	43
			Not infected:				
			Healed	0	5	65	35
			Dead	0	0	0	7

(Table 5). Slurries of a random selection of 28 H isolates were as effective in reducing tree death due to inoculations as were slurries of a compatible H or five H's including the compatible one. Most interestingly, conidial suspensions from compatible H isolates have increased tree survival even though the cankers were not wounded prior to treatment.

Enhancing survival of American chestnut trees seems to be an appropriate area for additional research. In the 1979-F experiment 28 randomly selected H isolates were used in one treatment. This number was selected because earlier results had suggested 25 to 34 percent of any confrontations between any virulent and any H isolate would result in conversion of the virulent isolate to H (Anagnostakis and Day 1979; Kuhlman 1978). To ensure conversion of 95 percent of the virulent isolates, a conservative 20 percent conversion rate required 14 H isolates which was then doubled.

Jaynes and Elliston (1978) have reported reduced canker growth four months after treatment with conidial suspensions but the reduction was less than that provided by agar slurries to wounds. Conidia were more effective on wounded cankers than on nonwounded cankers (Jaynes and Elliston 1978). The present study indicates that H isolates compatible with V isolates can retard canker development and increase tree survival for several seasons. If conidial suspensions from a random selection of H isolates can provide similar control of natural cankers, control may be feasible for orchards and high-value recreational areas.

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