To the Graduate Council

I am submitting herewith a thesis written by Brenda A. Rutherford entitled "Identification, Life Cycle, and Presence of *Nectria* Species Associated with Beech Bark Disease in the Great Smoky Mountains National Park and the Effectiveness of *Nematogonum ferrugineum* as a Biocontrol." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science.

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IDENTIFICATION, LIFE CYCLE, AND PRESENCE OF *NECTRIA* SPECIES ASSOCIATED WITH BEECH BARK DISEASE IN THE GREAT SMOKY MOUNTAINS NATIONAL PARK AND THE EFFECTIVENESS OF *NEMATOGONUM FERRUGINEUM* AS A BIOCONTROL

A Thesis

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Degree

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ABSTRACT

Two species of *Nectria*, *N. galligena* Bresadola and *N. coccinea* var. *faginata* Lohman, Watson, and Ayers, are associated with beech bark disease (BBD) in North America. In 1993, beech bark disease was discovered in the Great Smoky Mountains National Park (GSMNP).

Ten sites for permanent plots were selected for incidence of the disease at different elevations in the GSMNP. These plots were rated four times in 1994 and 1995 for the presence of two scale insects, *Cryptococcus fagisuga* Lindinger and *Xylococculus betulae* (Pergande) Morrison, and the presence of perithecia of *Nectria* spp. The beech trees in each plot were also rated for mortality, bark condition, and crown class. A significant negative correlation (-0.80691, p=0.0048) between the presence of *Nectria* spp. and the presence of *X. betulae* was observed, but no significant correlation was observed between the presence of *Nectria* spp. and the presence of *C. fagisuga*.

Differentiation of *Nectria* spp. was accomplished morphologically and by ascospore measurement. Of the ten plots, four contained *N. galligena,* and five contained *N. coccinea* var. *faginata*. Both *Nectria* spp. were present at one plot, the Chimneys. *Nectria* spp. from two plots could not be identified due to nonviable ascospores.

The life cycle of the pathogen was studied in the laboratory and in the field. Four trees, two at the Chimneys and two at Forney Ridge, were examined bimonthly from March 1994 to August 1995. In the field, perithecia were observed from March, 1994, through August, 1995, with the exceptions of February and April, 1995, when sites were inaccessible. Sporodochia were observed in the summer of 1995.

The mycoparasite, *Nematogonum ferrugineum* (Persoon) Hughes (*Gonatorrhodiella highlei* A. L. Smith), was studied in the laboratory for its effectiveness as a biocontrol agent of *Nectria* spp. In examining the growth rates of *N. ferrugineum* and the *Nectria* spp. on 1% water agar medium, it was found that the mycoparasite grew twice as fast as the *Nectria* spp. An experiment demonstrated that a thin film of water covering perithecia of *Nectria* spp. prevented hyphal contact between *N. ferrugineum* and the perithecia. The results of another experiment established that *N. ferrugineum* did not produce substances toxic to *Nectria* spp.

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CHAPTER I

PERMANENT PLOTS ESTABLISHED IN THE GREAT SMOKY MOUNTAINS NATIONAL PARK TO OBSERVE INCIDENCE OF BEECH BARK DISEASE

i. INTRODUCTION

The American beech, *Fagus grandifolia* Ehrlich, is a tall tree, as high as 49 m, a diameter at breast height (dbh) up to 1.3 m, and distinctive smooth gray bark. In summer, it is recognized by two-ranked leaves with dentate margins and acuminate tips. Beech is monoecious, blooms in April and May, and produces triangular nuts in September and October. In winter, it is recognizable by persistent dead leaves and golden terminal buds. The range of beech is from Nova Scotia to eastern Wisconsin and south and west to northern Florida and Texas (Duncan and Duncan 1988, Petrides 1972). In the Appalachian mountains, beech is found at elevations of up to 1800 m (Duncan and Duncan 1988).

Although the wood of beech is hard and strong, it is not durable. Its commercial uses include flooring, furniture, and fuel. Beech is sometimes used as an ornamental tree, but its most important use is as a food source for wildlife (birds, bears, deer, raccoons, foxes, rabbits, squirrels, porcupines, and opossums) (Duncan and Duncan 1988, Petrides 1972).

In the Great Smoky Mountains National Park (GSMNP), beech is an important part of the ecosystem. Before the formation of the park in 1934, 65 percent of the land within present day park boundaries was logged for hardwoods, such as black cherry, sugar maple, and chestnut. Other parts of the GSMNP were cleared by settlers for cattle grazing and crops (Stevenson 1967, Stupka 1964). This clearing of hardwoods has produced some distinct forest types (burn forests, lowland beech-hemlock forests, and beech gaps) (Russell 1953). Beech may occur as a dominant species in cove hardwood, northern hardwood, and hemlock forests (Stupka 1964). Beech gaps are defined as beech forests which occur as small stands, along with the dominant spruce-fir forest, occurring above 1500 m, and are apparent along the ridge of the Tennessee-North Carolina border, at high elevations, usually on the south-facing slopes (Russell 1953).

Since 1849, European beech (*Fagus sylvatica* L.) has been reported to be under attack in western Europe by a disease complex comprised of a beech scale, *Cryptococcus fagisuga* Lindinger, and a fungal pathogen, *Nectria coccinea* (Persoon) Fries. At the turn of this century, the beech scale, initiator of this disease complex of beech, was introduced into North America on infected European beech stock (Ehrlich 1934).

The initial sign of this complex is the appearance of the scale infesting the bark of the beech tree. The intracellular feeding of scale on the periderm of the tree predisposes the tree to infection by *Nectria* spp. by breaching the bark barrier (Ehrlich 1934, Lonsdale and Sherriff 1983). The pathogen then proceeds to kill the tree by disrupting the flow of nutrients. Eventually, the tree trunk will break from environmental pressures, strong winds or ice storms (Shigo 1972, Houston and O'Brien 1983).

The discovery of beech bark disease (BBD) in the GSMNP in 1993 was considered a sufficient threat to the mast source to warrant the establishment of permanent plots to estimate incidence and severity of BBD. The primary objectives of this research were to: (1) monitor the insect-fungal pathogen complex (*Cryptococcus fagisuga* Lindinger and *Nectria galligena* Bresadola or *Nectria coccinea* var. *faginata* Lohman, Watson, and Ayers) as it becomes established in the plots, and the association of *Xylococculus betulae* (Perg.) Morrison, a second scale insect, with the disease complex, (2) monitor and document beech mortality at different geographical and elevational sites within the GSMNP, and (3) establish a baseline for future comparison of conditions within the plots. Personnel with the GSMNP also planned to monitor regeneration and recovery of beech following infestation and infection by BBD.

ii. MATERIALS AND METHODS

Permanent Plot Research. Nine new permanent plots (20 m x 20 m) were established for long-term observations of progress and effects of beech bark disease in the GSMNP. Plots were established in areas believed to have zero, low, and high incidence of *C. fagisuga* infestations and *Nectria* spp. infections. One permanent plot from earlier research (Site 11) was added to the list for data collection (Table 1).

At each plot, site characteristics (slope, aspect, and elevation) were recorded and all beech trees with a diameter at breast height (dbh) greater than 3.5 cm. were tagged and dbh data collected. Each beech tree was evaluated for mortality, crown class, and crown condition (Durr et al. 1988). Diameter of each tree was taken using a flexible, aluminum, metric measuring tape (Forestry Suppliers, Inc., Jackson, MS), while the aspect, slope, and elevation were measured using a clinometer, a compass, and an altimeter, respectively (Table 2). Bark condition was rated on a scale of smooth, coarse, and rough. Each beech tree was also examined for incidence of *C. fagisuga*, *X. betulae*, and *Nectria* spp. Beech scale and *Nectria* spp. ratings were taken on the north and south sides of each tree with a rating square (33 cm x 33 cm) centered at 122 cm from the ground. An overall rating of the trunk, from the base of tree to approximately one meter above dbh, was also taken

Table 1. Permanent plots established in the Great Smoky Mountains National Park for beech bark disease research.

Location	Plot	*C. fagisuga*[1]	*Nectria* spp.
Gregory Bald	A	None	Yes
Jenkins Knob	3	Low	Yes
Forney Ridge	4	High	Yes
Trillium Gap	6	None	No
Deep Creek	7	None	No
Indian Gap	9	Low	No
Chimneys	10	Low	No
Sweat Heifer	11	High	Yes
Fork Ridge	14	High	No
Newfound Gap	16	Low	Yes

[1] None=insect not present, Low=insect present in low numbers, High=insect present in high numbers

Table 2. Permanent plot site characteristics used in beech bark disease studies in the Great Smoky Mountains National Park in 1994 and 1995.

Plot	Location	Aspect	Slope	Elevation(m)
A	Gregory Bald	358°	25.5%	1420
3	Jenkins Knob	10°	16.0%	1341
4	Forney Ridge	258.5°	44.5%	1670
6	Trillium Gap	277°	38.0%	1451
7	Deep Creek	178°	43.0%	1378
9	Indian Gap	193°	58.0%	1596
10	Chimneys	315.5°	21.0%	1109
11	Sweat Heifer	*	*	1778
14	Fork Ridge	171°	52.0%	1451
16	Newfound Gap	187°	63.5%	1596

* data not taken

for each tree. Incidence of *Nectria* spp. and beech scale were rated using ratings of 0 to 6 (0=no beech scale or *Nectria*, 1=low infestation or infection and scattered, 2=low infestation or infection and uniform, 3=moderate infestation or infection and scattered, 4=moderate infestation or infection and uniform, 5=high infestation or infection and scattered, 6=high infestation or infection and uniform). Permanent plots were first rated in the spring/summer, 1994, and a second rating was taken in fall, 1994. All of the plots were rated in the spring, 1995. Seven of the ten plots were rated in the fall of 1995.

iii. RESULTS

Permanent Plot Research. The percent incidence of *C. fagisuga* was relatively high (near or above 60%) in six of the ten plots (Figure 1). Two plots, Trillium Gap and Gregory Bald, had no beech scale infestations. The Deep Creek plot (Site 7) had 6.4% incidence of beech scale at the first rating, and increased to 16.1% at the second rating. There was no directional preference by the scale insect for the north or south side of the beech tree.

The Forney Ridge plot (Site 4) is located in an area with high levels of beech scale, but the percent incidence of beech scale is relatively low inside of this plot. This low incidence level, 29.8 percent at first rating and 22.0

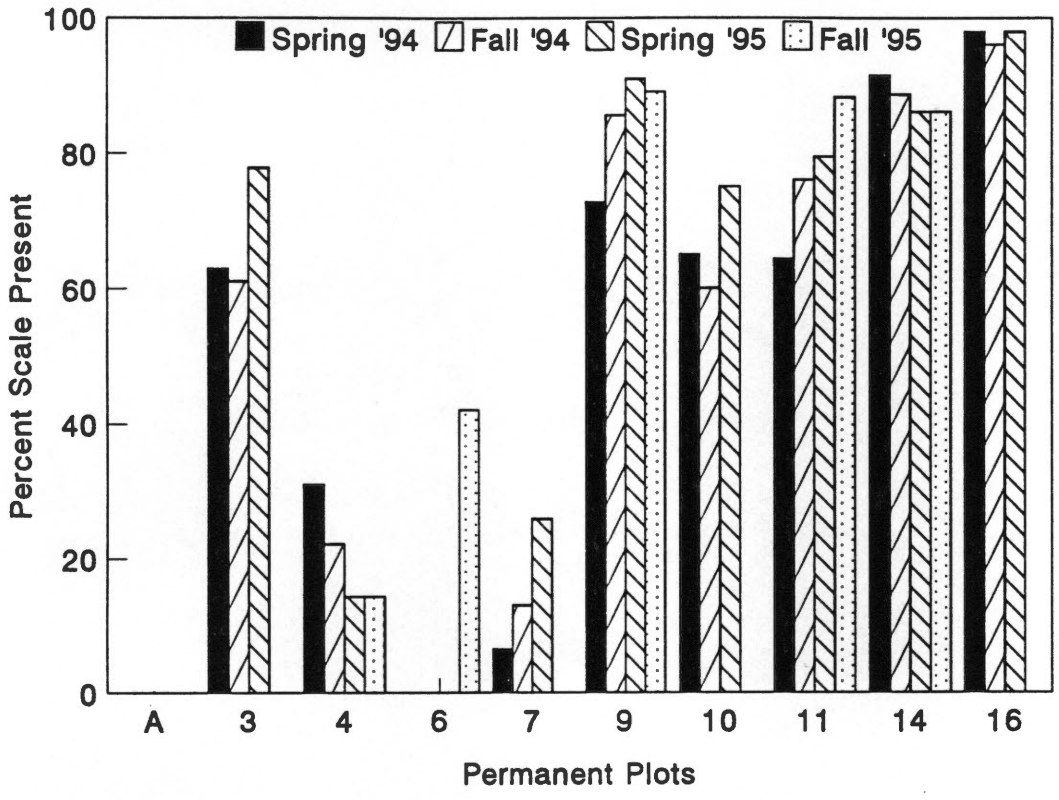


Figure 1. Percent incidence of the scale insect, *C. fagisuga*, in the permanent plots established in the Great Smoky Mountains National Park in 1994 and 1995.

percent incidence in the fall of 1994, is due in part to the high level of tree mortality.

Percent incidence of *Nectria* spp. was relatively low at the first rating, except for Forney Ridge (Site 4) (Figure 2). Incidence of infection by *Nectria* spp. gradually increased. Diameter at breast height, slope, aspect, and elevation had no apparent effect on incidence of BBD in the GSMNP. The mean dbh for *Nectria*-infected trees in the spring of 1994 was 7.5 cm, and increased to 11.0 cm in the spring of 1995. Sixty percent of the trees infected with *Nectria* spp. (n=45) were dead in early 1994, and 71.4% of infected trees (n=84) were dead by spring, 1995. Thirty-eight percent of the *Nectria*-infected trees (n=45) were infested with *C. fagisuga*, and 13% were infested with *X. betulae* in early 1994. The *C. fagisuga* decreased to 26.2% for *Nectria*-infected trees 1995; the *X. betulae* increased to 25% on the *Nectria*-infected trees. A significant negative correlation was found between the occurrence of *Nectria* spp. and *X. betulae* (Table 3), but not between *Nectria* spp. and *C. fagisuga*. *Nectria* infection increased from spring, 1994, to spring, 1995, but *C. fagisuga* infestations changed little (Table 4). Overall mortality rating steadily increased from spring, 1994 to fall, 1995, though only sevem of the ten plots were rated in the fall of 1995. *Xylococculus betulae* also increased from spring,

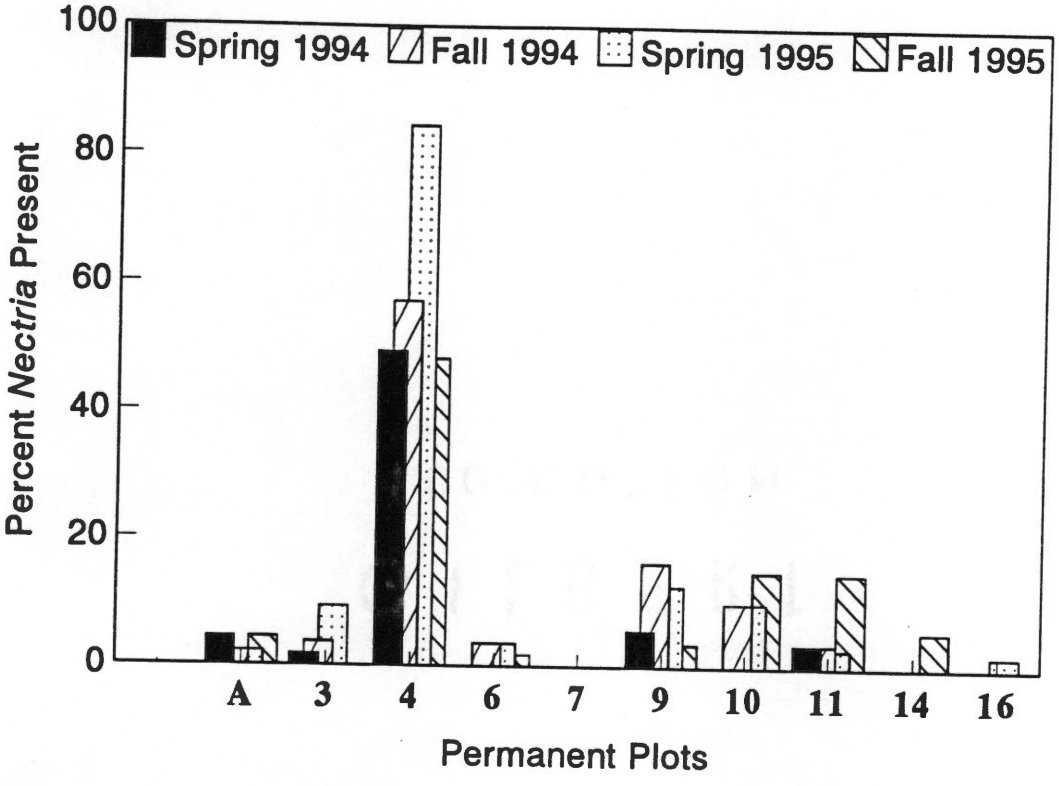


Figure 2. Percent incidence of *Nectria* spp. established in permanent plots in the Great Smoky Mountains National Park in 1994 and 1995.

Table 3. Correlation coefficients of the occurrence of *Nectria* spp., *C. fagisuga*, and *X. betulae*.

	C. fagisuga	*X. betulae*
Nectria spp.	-0.16823	-0.80691
P-value	0.64220	0.00480

Table 4. Permanent plot data taken for presence of *C. fagisuga*, *X. betulae*, *Nectria* spp., and mortality of beech trees, 1994 and 1995.

	Spring '94 (n=437)	Fall '94 (n=455)	Spring '95 (n=455)	Fall '95 (n=351)
C. fagisuga	48%	48%	51%	48%[1]
X. betulae	49%	64%	60%	59%
Nectria spp.[2]	10%	13%	19%	15%
Mortality	17%	18%	20%	29%[1]

[1] n=300
[2] Perithecia present

1994 to fall, 1995, but did not change during the 1995 ratings.

iv. DISCUSSION

The incidence of BBD in the GSMNP appears to be increasing. Two plots (Trillium Gap and Deep Creek) initially thought to be free of infection and infestation are slowly becoming infested by *C. fagisuga*. *Nectria galligena* has been found inside the plot at Trillium Gap, while *N. coccinea* var. *faginata* was found outside the margin of the plot at Deep Creek.

The incidence of BBD in the GSMNP is comparable to its establishment in the northeastern United States. Of the ten plots, two (Trillium Gap and Gregory Bald) were free of beech scale, *C. fagisuga*, when they were first established. Deep Creek became infested with beech scale not long after it was established. Approximately half of the trees at Trillium Gap are infested with both *C. fagisuga* and *X. betulae* as of fall, 1995. Incidence of scale insects in plots at Trillium Gap and Gregory Bald appear to typical of an "advancing front" (an area recently invaded by beech scale) (Shigo 1972). The "killing front" (Shigo 1972) is an area with high infestations of scale, severe *Nectria* attacks, and heavy tree mortality. Forney Ridge (Plot 4) had a high beech mortality rate (86%, n=77) inside of the plot, and a high scale population outside the plot. The

"aftermath zone" , characterized by heavy beech mortality in the past (Shigo 1972), has not been found in the GSMNP.

It was thought that the leading edge of the disease front was in West Virginia (Mielke et al 1982). Now, it is obvious that the front is advancing. The ten permanent plots established in the GSMNP will be monitored regularly over the next five years by park personnel. Their collection of data will be valuable in assessing the BBD situation.

CHAPTER II

IDENTIFICATION, LIFE CYCLE, AND PRESENCE OF *NECTRIA* SPECIES ASSOCIATED WITH BEECH BARK DISEASE IN THE GREAT SMOKY MOUNTAINS NATIONAL PARK

i. INTRODUCTION

Although the signs and symptoms of beech bark disease (BBD) are the same for both European and American beech (*F. sylvatica* L. and *F. grandifolia* Ehrh.), neither of the pathogens associated with the disease in North America, *Nectria galligena* Bres. and *Nectria coccinea* var. *faginata* Ehrh., appears to be the same fungal pathogen found in Europe, *Nectria coccinea* (Pers.) Fries (Houston et al. 1979).

Nectria galligena, a native of North America, causes cankers on many hardwood trees (red maple, sugar maple, black oak, yellow birch, and beech) (Spaulding et al. 1936, Lohman and Watson 1943, Twery and Patterson 1984), and has the ability to spread among different species of trees in a hardwood stand (Spaulding et al. 1936). This fungus has been reported as the primary pathogen in BBD (Houston 1994). The origin of *N. coccinea* var. *faginata* is uncertain. Mahoney (1991) proposed that *N. coccinea* var. *faginata* and *N. coccinea* are closely related genetically.

The BBD complex has slowly advanced south and west in North America since its introduction into this country

(Shigo 1972, Towers et al. 1974, Houston et al. 1979, Mielke et al. 1982). BBD was discovered in the GSMNP in 1993 (K. D. Johnson, National Park Service, personal communication).

To determine if the disease cycle of BBD and life cycles of *Nectria* spp. and beech scale follow a similar pattern as previously described in the northeastern United States, the life cycles of the pathogen and insect were monitored in the GSMNP. The life cycle of *C. fagisuga* in the GSMNP has been previously reported (Vance 1995). Therefore, the objectives of this research were to: 1) determine the species of *Nectria* present in the GSMNP, and 2) to observe seasonal development of new perithecia, mature perithecia, length of sporulation period, and sporodochia formation.

ii. MATERIALS AND METHODS

Identification of *Nectria* Species. To determine the identity of the *Nectria* spp., two or three perithecia with cirri were excised from bark tissue samples taken from trees inside permanent plots or very near plots (Table 5) and placed on a glass slide containing one drop of lactophenol with anilin blue. Perithecia were macerated to release asci and ascospores. After a coverslip was applied, the slide was air-dried for 24 hours, then the coverslip was sealed with fingernail polish. Twenty-five free ascospores on each slide from each plot location were measured at 1,000x with a

Table 5. Trees used to identify *Nectria* spp. and to determine pathogen life cycle in the beech bark disease complex in the Great Smoky Mountains National Park.

Plot	Tree No.	DBH (cm)	*Nectria*[1] Rating	Bark[2] Condition	Crown[3] Class
A[4]	A-0-29	10.4	1	Smooth	Suppressed
A	A-0-35	4.2	1	Smooth	Suppressed
3	3-0-33	8.3	1	Rough	Suppressed
4	4-2-1	10.3	3	Coarse	Suppressed
4	4-2-2	12.5	5	Rough	Suppressed
6	6-0-21	12.2	1	Coarse	Suppressed
7	Untagged[5]	>10	1	Rough	Codominant
9	Untagged	<3.5	5	Rough	Suppressed
10	10-2-1	52.2	3	Rough	Dominant
10	10-2-2	21.2	3	Coarse	Dominant
11	11-2-1	23.5	1	Rough	Dominant
11	11-116	10.8	3	Rough	Suppressed
14	Untagged	<10	5	Rough	Suppressed
14	Untagged	>10	1	Rough	Dominant

[1] 0=none, 1=low, scattered, 2=low, uniform, 3=moderate, scattered, 4=moderate, uniform, 5=high, scattered, 6=high, uniform
[2] Smooth=no damage to bark, coarse=raised areas with minute cracks, rough=cracks and crevices clearly visible
[3] From Durr et al. 1988
[4] See Table 1
[5] Untagged trees outside of plots

Filar micrometer eyepiece (American Optical Corp., Buffalo, NY). Measurement of ascospore length was accomplished with the scale recommended by Cotter and Blanchard (1981).

To culture fungal isolates associated with beech bark disease in the different plots, petri dishes containing 1.5% malt extract agar (MEA) were seeded with ascospores. The plates were sealed with Parafilm™ (American National Can, Greenwich, CT) and placed into an incubator at 20 C with a photoperiod of 8-10 hours.

Life Cycle of *Nectria* Species. The life cycle of *Nectria* spp. associated with BBD was studied at the Chimneys (Site 10) and Forney Ridge (Site 4). These plots were chosen because of differences in elevation, easy accessibility, and high incidence of the pathogen. Two trees located outside the margin of each permanent plot were tagged (4-2-1 and 4-2-2 at Forney, and 10-2-1 and 10-2-2 at Chimneys) and used for sample acquisition. Between March 11, 1994 and August 18, 1995, two bark samples were taken from each tagged tree bimonthly during the summers of 1994 and 1995, and whenever possible in the fall and spring. A total of 227 samples was collected. In the laboratory, each sample was microscopically examined and transferred to a moist chamber for induced sporulation of mature perithecia. Microscopic observations of the bark samples for sporodochia formation and parasitism by a mycoparasite or other organisms were done bimonthly.

iii. Results

Species Identification. Identification of the *Nectria* species within or near 8 of the 10 plots was accomplished either by morphological characteristics in culture or by measuring the mean length of 25 ascospores or both (Table 6). Both *Nectria* spp., *N. galligena* and *N. coccinea* var. *faginata*, were cultured from samples acquired at the Chimneys (Site 10). *Nectria* spp. could not be determined by ascospore measurement due to germinating ascospores present on the samples from Gregory Bald (Site A). The morphological characteristics of mycelia resulting from germination of ascospores from Trillium Gap (Site 6), Deep Creek (Site 7), and Fork Ridge (Site 14) could not be used for identification due to contamination of the fungal isolates. The plots at Indian Gap and Newfound Gap produced nonviable ascospores from desiccated perithecia.

Life Cycle of *Nectria* Species. Of the 65 samples taken from trees 4-2-1 and 4-2-2 (Forney Ridge) between March 23,1994 through August 4, 1995, immature perithecia were observed March, May, July and August, 1994, and January, 1995 (Figure 3). Mature perithecia were observed March, May, June, July, August, September, October and November, 1994, and January, May, June, and August, 1995. Cirri were noted on samples taken May, June, July, August, and October, 1994, and May, June, and August, 1995. Sporodochia of *Cylindrocarpon* spp. were observed on beech

Table 6. Mycelium color, mean ascospore length, and species identification in ten plots in the Great Smoky Mountains National Park.

Plot	Mycelium Color	Ascospore Length (μm)	*Nectria* spp.[1]	
			N. g.	*N. c. f.*
A	Rose	NA	-	+
3	Rose	11.6[2] 1.9[3]	-	+
4	Buff	15.2 1.6	+	-
6	NA	15.2 1.7	+	-
7	NA	10.6 0.8	-	+
9	NA	14.1 1.2	-	-
10	Buff	15.5 1.1	+	-
10	Rose	9.1 0.9	-	+
11	Buff	15.7 1.2	+	-
14	NA	9.0 0.9	-	+
16	NA	NA	-	-

[1] *N. g.*= *Nectria galligena*, *N. c. f.*= *Nectria coccinea* var. *faginata*
[2] Mean length of 25 ascospores
[3] Standard deviation

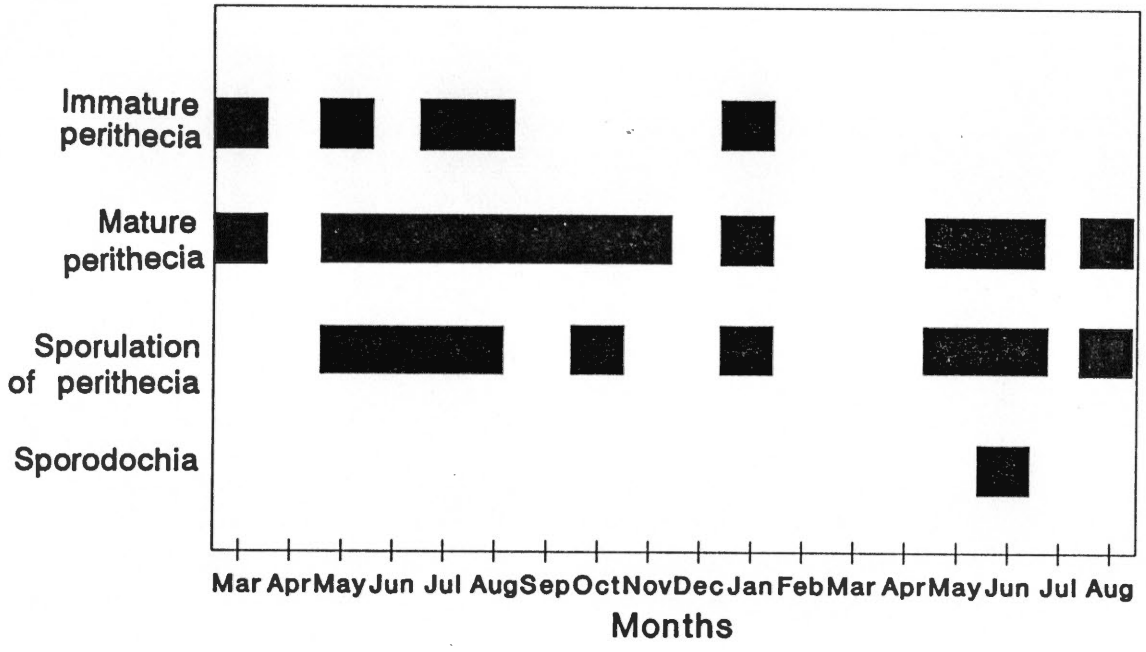
bark June, 1995 (Figure 3). The mycoparasite, *Nematogonum ferrugineum* (Persoon) Hughes, was not noticeable until samples were placed into moist chambers.

At the Chimneys, 86 bark samples were taken from beech trees, 10-2-1 and 10-2-2, between March 11, 1994 and August 11, 1995. Immature perithecia were observed from May through December, 1994, and March and April, 1995 (Figure 4). Mature perithecia were observed March, April, June, July, November, and December, 1994, and March, May, June, July, and August, 1995. Recent sporulation was noted March, April, May, July, August, October, November, and December, 1994, and May, June, and July, 1995. Sporodochia were observed June, July, and August, 1995 (Figure 4). The mycoparasite, *N. ferrugineum*, was present on all but three samples in 1994. No parasitism was noted on samples taken in 1995.

iv. DISCUSSION

In 1910, a *Nectria* sp. was identified as causing cankers on black walnut near Balsam, North Carolina and later in Sarah, Georgia (Gravatt 1933). The fungus was later identified as *N. galligena* (Lohman and Watson 1942). *N. galligena* is indigenous to this country and has been identified as the primary pathogen in the MNF with *N. coccinea* var. *faginata* isolated from two sites (Houston 1994). In the GSMNP, *N. galligena* was isolated from four

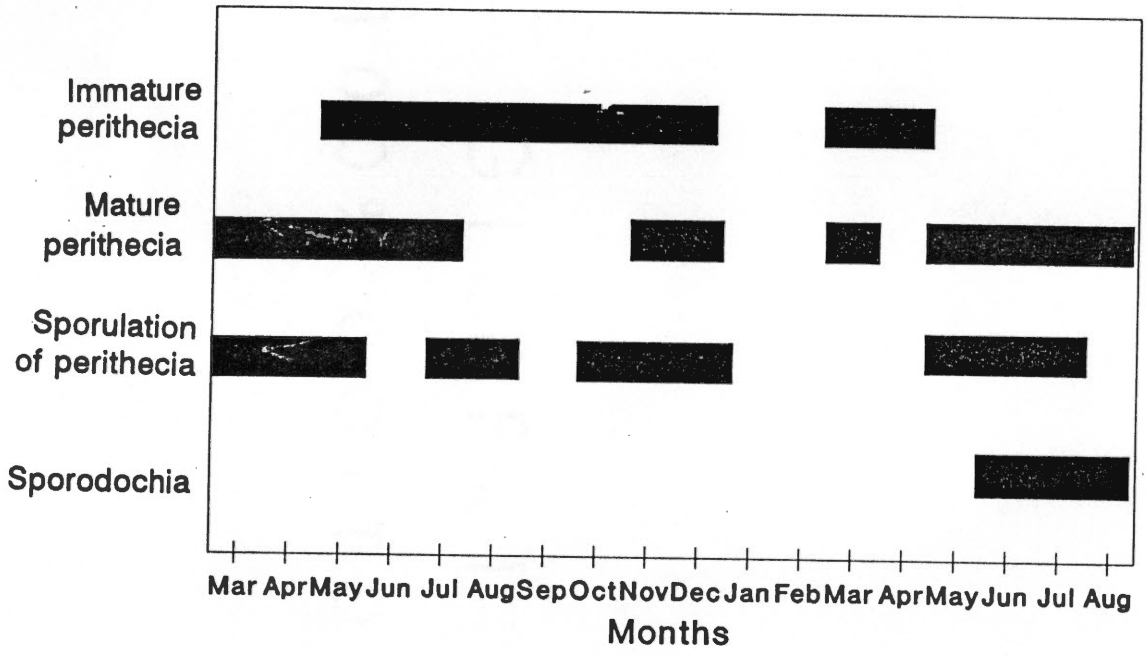
Figure 3. Presence of immature perithecia, mature perithecia, recent sporulation of perithecia, and sporodochia of *Nectria* spp. at permanent plot 4 (Forney Ridge) located in the Great Smoky Mountains National Park, 1994 and 1995.


Immature perithecia
Mature perithecia
Sporulation of perithecia
Sporodochia
Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug
Months

sites, and *N. coccinea* var. *faginata* was isolated from five sites. From the data taken in the last two years, BBD has probably been established in the park for at least three years. According to Houston (1994), *N. galligena* is the primary pathogen, while *N. coccinea* var. *faginata* comes into the complex after the trees are heavily infested by *C. fagisuga*, approximately three years after infestation. Half of the permanent plots produced viable ascospores of *N. coccinea* var. *faginata*. At the Chimneys, both species were present which suggests that the secondary pathogen is moving into the plot and taking over from the primary pathogen.

Life cycles of both species are similar to those reported for northern climates (Ehrlich 1934, Spaulding et al. 1936, Mielke et al. 1982). The sexual stage of the life cycle occurred at both the Chimneys and Forney Ridge for approximately fifteen months. The sites were not checked during February and April 1995 due to severe weather. The asexual form, *Cylindrocarpon* sp., did not appear on sample trees until the summer of 1995. This has been previously reported by Shigo (1964), who found the asexual stage in midsummer.

Figure 4. Presence of immature perithecia, mature perithecia, recent sporulation of perithecia, and sporodochia of *Nectria* spp. at permanent plot 10 (the Chimneys) located in the Great Smoky Mountains National Park, 1994 and 1995.


Immature perithecia
Mature perithecia
Sporulation of perithecia
Sporodochia
Mar Apr May Jun Jul Aug Sep Oct Nov Dec Jan Feb Mar Apr May Jun Jul Aug
Months

CHAPTER III

PARASITISM OF *Nectria* spp. BY *Nematogonum ferrugineum*

i. INTRODUCTION

The contact biotrophic mycoparasite, *Nematogonum ferrugineum* (Persoon) Hughes (=*Gonatorrhodiella highlei*, A.L. Smith), has been in association with *Nectria coccinea* (Pers.) Fries in the United States since 1933 (Ayers 1941, Gain and Barnett 1970). In nature, it appears to be host-specific on *Nectria* spp. (Ayers 1941, Blyth 1949, Walker et al 1982, Houston 1983). In culture it will parasitize species of *Chaetomella, Cladosporium, Graphium, Tritirachium* and *Verticillium* (Gain and Barnett 1970).

Parasitism by *N. ferrugineum* is accomplished by contact cells which connect to the host hyphae and establish a link between the two. It is not known whether a specific nutrient is absorbed from the host cell or whether there is competition between the host and the mycoparasite for nutrients extracted from beech bark which causes a decrease in growth rate of the host, *Nectria* spp. (Walker and Minter 1982, Gain and Barnett 1970). In earlier observations of samples after being placed in moist chambers with excess water, it was noted that the hyphae of the mycoparasite did

not come in contact with the fruiting bodies of *Nectria* spp. while perithecia were wet.

The objectives of this research were to: (1) compare growth rates between *N. galligena* and *N. ferrugineum*, (2) determine whether excess water affects parasitism by the mycoparasite, and (3) determine if the mycoparasite produces a toxin(s) that inhibits the growth of *N. galligena*.

ii. MATERIALS AND METHODS

Growth Rates of *N. galligena* and *N. ferrugineum*. Eight cultures of *N. galligena* and *N. ferrugineum* were randomly selected from stock cultures obtained in studies described in Chapter 2. Agar plugs (diameter 4.7 mm) containing fungal thalli, that were obtained from stock cultures, were placed on 1% water agar plates (WA). The plates were sealed with Parafilm™ (American National Can, Greenwich, CT) and incubated in the laboratory at room temperature. Diameters of fungal colonies were measured daily under a dissecting microscope with a metric ruler. Six measurements were taken over a 7-day period.

Inhibition of Parasitism by *N. ferrugineum* Caused by Excessive Amounts of Water. Ten beech bark samples (2.5 cm^2) with perithecia were collected from trees at the Forney Ridge site. The samples were microscopically examined to confirm the presence of *Nectria* spp. and *N. ferrugineum*. All samples had perithecia present in immature and mature

stages. After incubation for 2 days in moist chambers, samples were reexamined for *N. ferrugineum*. All samples supported the antagonist in varying amounts, and partial hyphal contact with perithecia on two bark samples was observed. Bark samples (n=20) were halved and labeled A or B. Each half was randomly placed in a rectangular plastic container (0.36 L) which was labeled 'Dry' or 'Wet'. To insure low relative humidity in the dry containers, ten to twelve granules of a desiccant, Drierite™ (W. A. Hammond Drierite Co., Xenia,OH) were placed in the containers. Samples placed in the 'Wet' containers were saturated with water. All containers were covered with fitted lids. Samples were observed three times each week for 4 weeks.

Toxin Production by *N. ferrugineum* as Inhibitor of *Nectria* Growth. Seventy-five ml of modified Czapek-Dox broth (substituting 20 g/l of glucose for 30g/l sucrose) was placed in each of twelve sterilized 125 ml flasks with nonabsorbent cotton stoppers. The broth in six of the flasks was autoclaved, and the broth in the other six flasks was filter sterilized using Nalgene™ Disposable Filterware (Nalge Co., Rochester, NY). One conidiophore, with conidia intact, of *N. ferrugineum* was placed in each flask after the broth had been sterilized. The flasks were placed in a shaker and the shaker was covered with a black plastic bag

to keep out light. The shake-cultures were incubated at room temperature. After 4 weeks growth, fungal thallus of *N. ferrugineum* consisted of orange spheres (diameters of approximately 4 cm) in the autoclaved broth and loose white mycelial strands tinged with orange in the filtered broth. Two cultures of *N. ferrugineum* growing in each medium treatment (autoclaved vs. cold filtered) were selected for good growth. After the fungal thallus was removed by filtration, the broth was sterilized again via autoclaving or using cold filtration. Sterilized broth was labeled AA (broth autoclaved before *N. ferrugineum* was added and after fungal thallus grew in the flask), labeled AF (broth autoclaved before *N. ferrugineum* was added and the broth cold filtered after thallus grew in the flask), labeled FA (broth was cold filtered before *N. ferrugineum* was added and the broth was autoclaved after thallus grew in the flask), or labeled FF (broth was cold filtered before *N. ferrugineum* was added and after fungal thallus grew in the flask). Broth of each treatment (AA, AF, FA, and FF) was subdivided into 4 aliquots and diluted with filtered, deionized water to form dilutions of 100, 50, 10, and 1% of the original broth.

Using a no. 3 Humbolt cork borer (0.8 cm)(Baxter Diagnostics Inc., McGraw Park, IL), 8 agar plugs were removed to form wells around 10 petri dishes containing 1.5%

MEA. The underside of each plate was labeled with type of broth treatment (AA, AF, FA, or FF) and concentrations (100, 50, 10, or 1%). Location of the broth/dilution treatments within wells in each plate was randomized by drawing slips of paper from a container. In each well, an aliquot (0.5 ml) of each broth dilution was placed into one of the wells. Five replicates were made for each treatment. After the broth treatments were placed in the plates, an agar plug (0.8 cm in diameter) of *N. galligena* was placed in the center of each prepared 1.5% MEA plate containing the wells with the broth treatments. The plates were sealed with Parafilm™ and carefully placed in a large Ziplock™ freezer bag (DowBrands, Indianapolis, IN) to prevent contamination. The plates were kept in the laboratory at room temperature for approximately two weeks. Each day, growth of the *N. galligena* colonies was observed.

iii. RESULTS

Growth Rates of *N. galligena* and *N. ferrugineum*. The rate of hyphal growth of *N. ferrugineum* was twice the rate of the *N. galligena* for the first few days (Figure 5). After initial growth, *N. ferrugineum* growth increased exponentially.

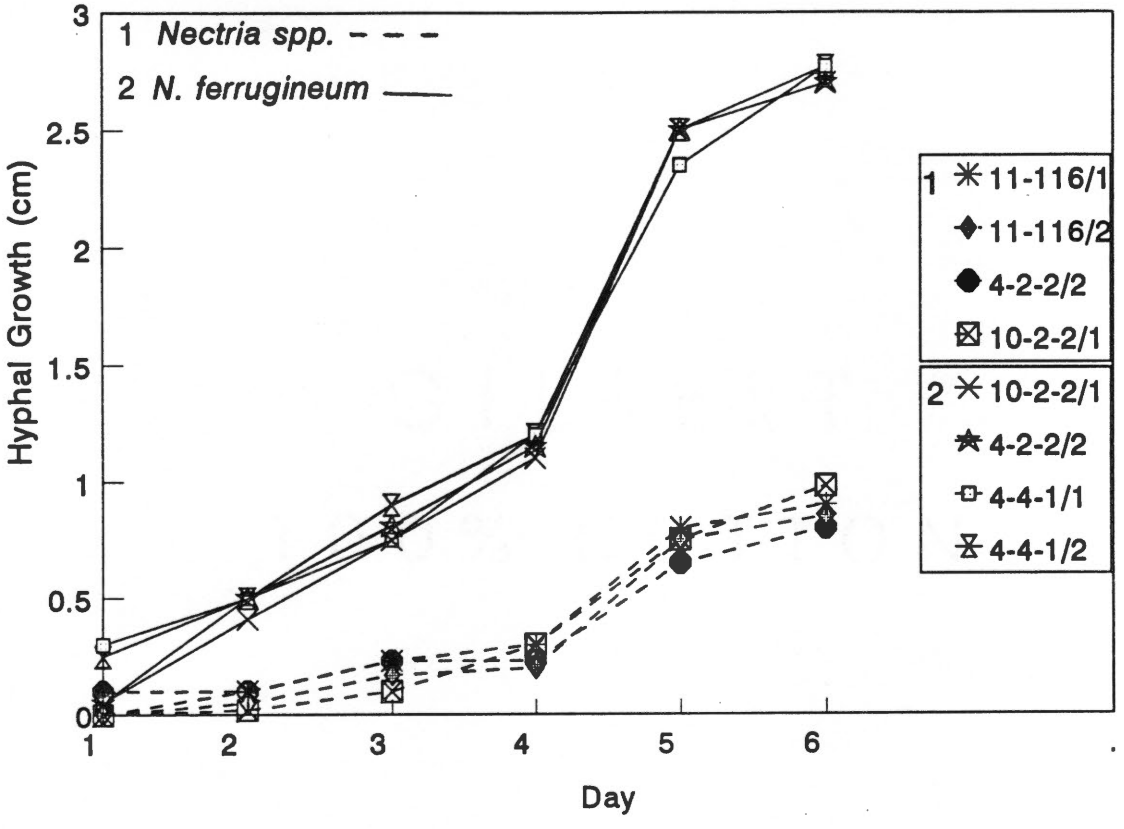


Figure 5. Comparison of growth of the mycoparasite, *N. ferrugineum*, and *N. galligena*, the initial pathogen of beech bark disease in the Great Smoky Mountains National Park.

Inhibition of Parasitism by *N. ferrugineum* Caused by Excessive Amounts of Water. By the end of the second week, seven of the ten dry samples had 86% contact between the perithecia of *Nectria* spp. and the hyphae *N. ferrugineum*. Of the ten wet samples, the hyphae of the mycoparasite came in contact with the perithecia of the pathogen as the samples began to dry (Figure 6). There was no reduction in sporulation of perithecia in either treatment.

Toxin Production by *N. ferrugineum* as an Inhibitor of *Nectria* Growth. After 7 days, hyphae of the *Nectria* plug had grown to the edge of all prepared plates. By the end of the second week, hyphae were growing at the bottom of all the wells, and perithecia primordia were beginning to form. There was no inhibition of growth of *Nectria* spp. on any of the prepared plates.

iv. DISCUSSION

Nematogonum ferrugineum is classified as a contact biotrophic mycoparasite (Barnett and Binder 1973). It apparently does not harm its host other than by slowing growth rate (Shigo 1964, Houston 1983). In previous reports, the mycoparasite was said to grow under specific conditions (without light, beech bark present) (Gain and Barnett 1970, Walker and Minter 1981). In the growth rate studies, the mycoparasite grew rapidly compared to the *N.*

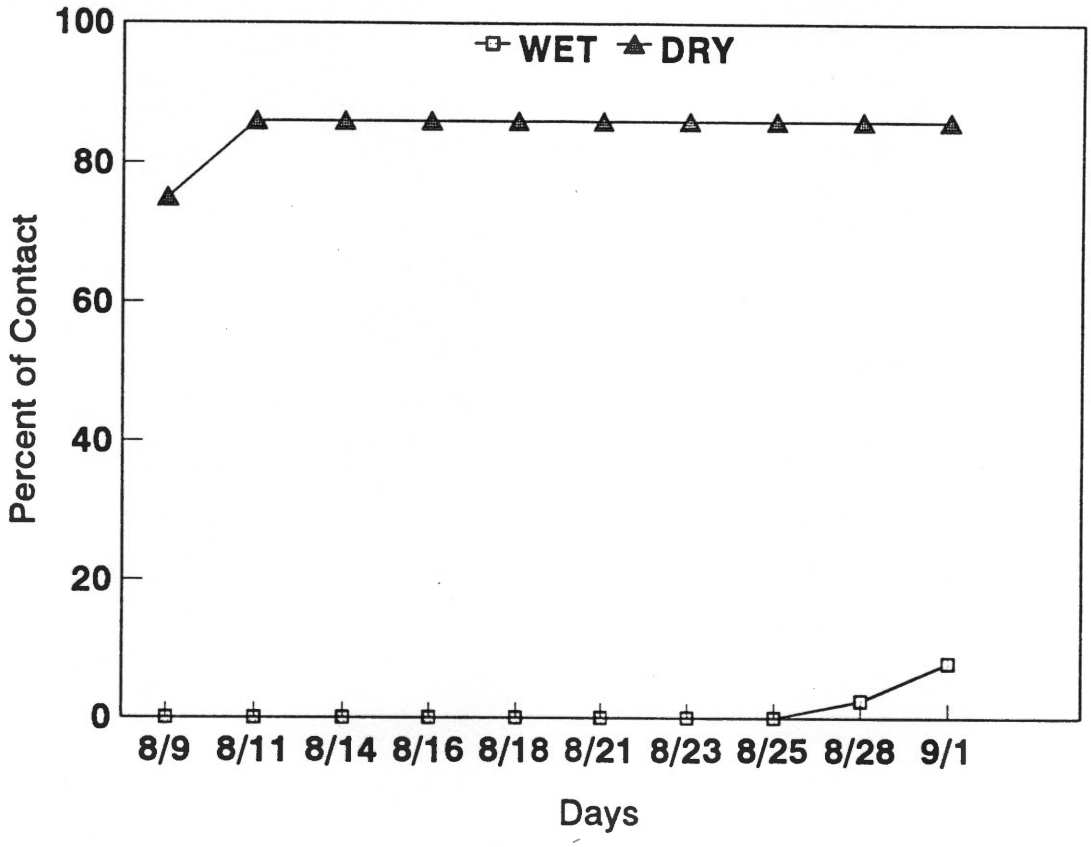


Figure 6. Percent of hyphal contact between *N. galligena* and *N. ferrugineum* for 'Dry' and 'Wet' treatments of infected beech bark.

galligena. In the water experiment, the interaction between the pathogen and the mycoparasite in both the 'Dry' and 'Wet' samples was as expected. The mycoparasite grew rapidly over perithecia in the 'Dry' samples. However, no reduction in sporulation of mature perithecia was noted in either treatment. Seven days after the experiment ended, cirri from perithecia became orange in color and hard. Observations of the 'Wet' samples showed no hyphal interaction between the perithecia and the mycoparasite while the perithecia were wet. As the 'Wet' samples dried, contact between mycoparasite hyphae and *Nectria* thalli increased. As a contact biotrophic mycoparasite, *N. ferrugineum* does not produce toxins (Barnett and Binder 1973). To confirm this, fungal extracts of *N. ferrugineum* were tested for toxins against *Nectria* spp. The results of this experiment were similar to the results of Barnett and Binder (1973). In conclusion, these data do not support using *N. ferrugineum* as a biological control agent for *Nectria* spp. associated with BBD. It did not prevent sporulation of mature perithecia. Its distribution at only two GSMNP sites (the Chimneys and Forney Ridge) suggests that it is not widely distributed in the park.

CHAPTER IV

CONCLUSIONS

In the northeastern U. S., the progression of the beech bark disease complex (BBD) was initiated by infestations of the beech scale, *C. fagisuga*, followed two to three years later by infection of scale infested trees with *N. coccinea* var. *faginata* (Houston 1973). The rate of spread of *C. fagisuga* has been estimated as 15 km/year (Sinclair et al. 1987). Before the discovery of BBD in the GSMNP in 1993, the last reported incidence of BBD was found in the Monongahela National Forest (MNF), West Virginia in August 1981 (Mielke and Haynes 1982). At the estimated rate of spread 15 km/year, the beech scale theoretically should have spread approximately 180 km. However, the GSMNP is located 644 km from MNF and is outside the expected range of BBD. The unexpected occurrence of BBD in the GSMNP suggests that movement of *C. fagisuga* was by means other than wind-blown crawler. Both the MNF and the GSMNP are visited by thousands of tourists yearly which could explain the appearance of the disease in areas favored by day-hikers. Unless another explanation is found, estimate of the rate of movement of the scale insect should be 53.6 km/year instead of the 15 km/year.

Data taken from the ten permanent plots in the GSMNP suggest that the "advancing front" is in or past some of the

areas (Deep Creek and Trillium Gap) where the plots were established. Data also suggest that one plot (Forney Ridge) is in an area that could be considered in the "killing front" (Shigo 1964, Houston and O'Brien 1983). There were no significant correlation between the presence of *Nectria* spp. and site factors or the presence of *C. fagisuga*. However, there was a significant negative correlation between the presence of *Nectria* spp. and the presence of a second scale insect, *Xylococculus betulae*.

Nectria galligena is indigenous to the U. S., and has been identified as the primary pathogen in the MNF with *N. coccinea* var. *faginata* isolated from two sites (Houston 1994). In the GSMNP, *N. galligena* was isolated from four sites, and *N. coccinea* var. *faginata* was isolated from five sites. *Nectria galligena* was considered the primary pathogen in the plot sites because of its frequency on more than four trees per site. Whereas, *N. coccinea* var. *faginata* was isolated from only one or two trees per site. An exception to this frequency of fungal isolation was at Trillium Gap. Two trees recently infected with beech scale had infections of only *N. galligena*. Two years of observation of the life cycle of both *Nectria* spp. in the GSMNP concluded with no obvious deviations from the normal cycle. According to Lohman and Watson (1943), perithecia of *Nectria* spp. can produce viable spores up to 30 months. Sporodochia are present on beech trees during the summer

months (Shigo 1964). Sporulation by mature perithecia can happen any time there is a sufficient amount of water (Ehrlich 1934, Lohman and Watson 1943).

Nematogonum ferrugineum, a reported antagonist of *Nectria* species (Ayers 1941, Blyth 1949), was found at two sites in the GSMNP. Experiments completed in the past 18 months with both the antagonist and *Nectria* spp. have been inconclusive as to the mechanism of parasitism. Increased amounts of water on bark samples did prevent hyphal contact between the two fungi. *Nematogonum ferrugineum* did not produce substances toxic to *N. galligena* in liquid culture. At present, the presence of the antagonist does not appear to lessen the impact of BBD in the GSMNP. At the Forney Ridge site, incidence of *N. ferrugineum* was observed as well as a high mortality rate of beech. At the second site, the Chimneys, whether the mycoparasite will inhibit either of the *Nectria* species is unknown. Other considerations for biocontrol are a *Verticillium* sp. found at Deep Creek, a species of *Hypoxylon* found at Fork Ridge, and a species of lichen (unidentified) found at Indian Gap which appeared to be overtaking a perithecium.

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