ABSTRACT

NEWTON, LESLIE PHELPS. Novel Approaches for Artificial Infestations and Early Resistance Screening of Fraser fir against the Balsam Woolly Adelgid. (Under the direction of Fred P. Hain and John Frampton).

The balsam woolly adelgid (BWA) is a tiny, piercing-sucking insect that was introduced into North America around 1900 and into the Southern Appalachians in the 1950s. It is a major pest in natural Fraser fir stands and Christmas tree plantations. It has caused extensive mortality in native stands and is expensive to the Christmas tree industry. The development of BWA-resistant Fraser fir trees would be a relatively inexpensive solution to a difficult pest problem. The work described here was conducted to assist in this endeavor.

Studies were conducted at constant and ramping temperatures to determine whether young seedlings (1-, 2-, 3-, and 6-year old) could be artificially infested with BWA and whether the infestations could be maintained over a period of time. The studies resulted in at least some of the trees having developed egg-laying adults by the end of the study period. The infestations were sustained over time and supported multiple generations of BWA.

Historically, the technique often utilized for artificial infestations involved cutting pieces of bark from infested trees and attaching the bark to uninfested trees. Because this is a very time consuming technique, studies were developed to test this method with a novel technique mimicking natural dispersal. The new technique involves suspending infested logs over uninfested seedlings and allowing the crawlers to drop onto the material below. The technique was tested on 2- and 7-year old Fraser fir trees. Although there were no differences in infestation levels between the old and new techniques, it took substantially less time to cut the logs and hang them over the trees. An additional study looking at the density distribution

of crawlers dropping from the logs revealed that there can be over 50 crawlers per cm^2 falling from the log and that there is a relative level of drift in crawler distribution. Suspending logs (10 cm diameter) approximately 30 cm apart may provide ample crawlers to infest trees.

To determine whether seedlings of different fir species showed the same level of BWA susceptibility as mature trees, a study was conducted in which seedlings of 12 fir species of equal age and grown under the same conditions were infested (using the suspended log technique described above) with BWA collected at one time from a single source. Species were categorized into four *a priori* susceptibility groups (susceptible, tolerant, resistant, unknown). With few exceptions, results for each species fell within expectations for *a priori* resistance classifications. European silver fir and Veitch fir appear to be somewhat more susceptible in the seedling class than mature trees, however, they generally ranked as less susceptible than Fraser fir and other highly susceptible species. Turkish fir appears to be somewhat resistant to BWA and Trojan fir appears highly susceptible.

A separate infestation study was conducted to compare a (balsam x Veitch) x balsam backcross with other species. The backcross (3-year old seedlings) appeared to be more resistant to BWA infestation than the balsam fir of equal age and grown under the same conditions.

A clonal Fraser fir seed orchard in Avery County was found naturally infested with BWA. Each tree was assessed for infestation level and other variables to determine if there were clonal differences in susceptibility. Over 96% of the trees were infested with BWA and, although there were significant difference noted among clones, every clone was infested at some level. There were highly significant clone effects for infestation level, bark appearance, bark thickness, lichen coverage, apical dominance, crown health, and tree diameter.

Heritability calculations were low and it is believed that the practice of spraying the orchard with insecticides each year may be masking any genetic effects associated with BWA resistance.

Although most mature Fraser fir trees in native stands were killed by the balsam woolly adelgid during the first wave of mortality, many trees on mountain peaks in the Great Smoky Mountains National Park survived and are actively growing. Ten sites were visited and data collected on the survivor trees and younger regenerated trees for comparison, to investigate the potential resistance of the survivor trees to BWA by evaluating bark characteristics and to characterize site factors that may aid in the understanding of their survival. Survivor trees exhibited thicker and rougher bark than regeneration trees, and the depth of the outer bark was also greater in the survivor trees. The ratio of bark thickness to diameter at breast height was constant, and the ratio of impermeable tissue to bark thickness was constant, indicating that regeneration trees were developing impermeable tissue at a similar rate as the older survivor trees. This could be linked to genetic similarities between the regeneration and survivor trees.

Novel Approaches for Artificial Infestations and Early Resistance Screening of Fraser fir against the Balsam Woolly Adelgid

by
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DEDICATION

To Jim: my husband, my friend.

BIOGRAPHY

Leslie Carol Phelps Newton was born in Asheville, North Carolina, the middle of five children. Her formative years included living on a secluded mountain top surrounded by fir and spruce trees, moving to the Coastal Plains, and spending summers on the Outer Banks. She always loved the woods and when the time came for pursuing a formal education, Forestry seemed a good fit. She graduated from North Carolina State University with a Bachelor of Science in Forest Management in 2000 and a Master of Science in Forestry in 2003. She began her doctoral studies in Forest Entomology in 2003. When coursework, field research and prelims were completed in 2008, she began an internship with USDA APHIS and in 2009 accepted a position as a risk analyst in the Plant Epidemiology and Risk Analysis Laboratory within APHIS' Plant Protection and Quarantine, where she is currently still happily employed. Leslie lives in Raleigh, North Carolina with her husband (Jim) and her cat (Atticus) and enjoys gardening, traveling, and spending time with friends and family.

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I thank the Department of Entomology for funding assistance and for the support provided in so many ways. I want to thank the Christmas Tree Genetics Program for funding assistance as well, and the North Carolina Christmas Tree Association. Additionally, we were awarded a grant from the USDA Cooperative State Research, Education, and Extension Service within the Regional Integrated Pest Management Competitive Grants Program, and I am grateful for the assistance.

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I thank all of my Forest Entomology lab mates for technical assistance as mentioned above, but also for moral support and friendship over the years. My friends and colleagues in PERAL have provided much moral support during so many months of working and finishing a dissertation during off hours. I want to thank them and especially thank Heather Hartzog for using her creativity and fancy software to help with a particularly difficult graphic for one of the chapters. Last, but never least, I want to thank my family. My husband and our sons, Chris and Jimmy, helped with so many aspects of the project and I am grateful. Thanks, Fig. Thanks, boys.

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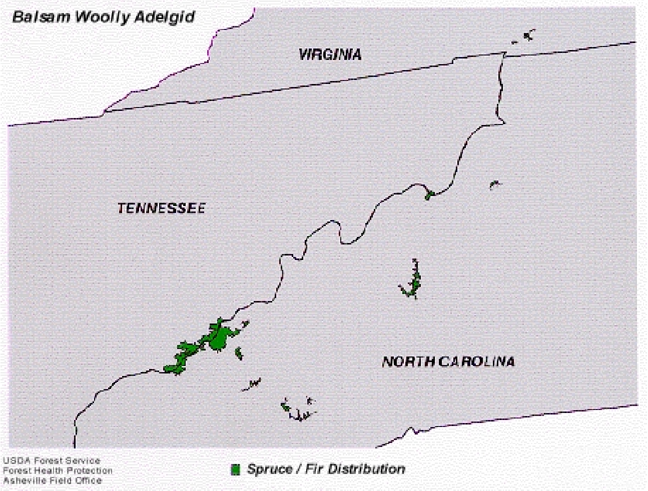
CHAPTER 1. INTRODUCTION

Fraser fir (*Abies fraseri* [Pursh] Poiret) is endemic to the spruce-fir forests of the Southern Appalachians where it obtains dominance above 1800-1900m (Cain 1935, Ramseur 1960, Busing et al. 1993). Natural stands are found in eastern Tennessee (Great Smoky Mountains and part of Roan Mountain), western North Carolina (Great Smoky Mountains, Plott Balsam Mountains, Black Mountains, Roan Mountain, Grandfather Mountain), and southwest Virginia (Mount Rogers) (Amman and Spears 1965). These native populations have declined, although many seem to be recovering at the present time. Extensive logging in the early 1900s (Pyle and Schafale 1988) and air pollution (White 1984, Hain 1986) have contributed to the decline, but these stands have been particularly decimated by the balsam woolly adelgid (BWA) (*Adelges piceae* Ratz.) (Hemiptera: Sternorryncha: Adelgidae). The balsam woolly adelgid is an exotic insect introduced from central Europe into Maine in 1908 (Kotinsky 1916) and discovered on Mount Mitchell in 1955 (Boyce 1955, Amman 1966). During the first wave of mortality following the introduction of BWA, over 90% of old-growth or mature overstory Fraser fir trees were killed (Hay and Johnson 1980, Wentworth et al. 1989, Hollingsworth and Hain 1991, Nicholas et al. 1999). Although regeneration has been good in many of the native



stands of Fraser fir, successive cycles of regeneration and mortality may result in decreasing populations over time (Potter et al. 2005, Ragenovich and Mitchell 2006).

Fraser fir is one of the most popular Christmas trees in North America and the Christmas tree industry provides an important economic resource for mountain communities. In North Carolina, there are 50 million Fraser fir trees growing on over 25,000 acres, providing annual cash receipts of well over $100 million for trees, wreaths, ropes, and greenery. There are approximately 1600 growers; the majority are small operations under ten acres (NCCTA 2005, Sidebottom 2003). Virtually all Fraser fir Christmas trees require treatment for BWA one or more times during a 5 to 10-year rotation. The threshold for treatment is a single infested tree and chemical insecticides are currently the only effective means for controlling this pest. Treatments require insecticides to be applied with a high-pressure sprayer using 3400 to 9000 liters per hectare (300 to 800 gallons per acre). The entire tree must be wetted and only two or three rows can be treated at a time. Only about half the Christmas tree growers own the type of equipment required and others must hire someone to treat the trees at a cost of $300 to $500 per acre. Treatments for BWA cost the Christmas tree industry over $1.5 million per year (Potter et al. 2005).

Balsam woolly adelgid infestations often result in the death of susceptible host trees; however, there are reports of full recovery from severe infestations in some species, e.g., noble and white firs (*Abies procera* Rehd. and *Abies concolor* (Gord. et Glend.) Lindl.) (Mitchell 1966), European silver fir (*Abies alba* Mill.) (Kloft 1957), West Virginia Canaan fir (*Abies balsamea* L. Mill. var. *phanerolepis* Fern.), and Fraser fir (Hain et al. 1991). This, coupled with the presence of numerous old-growth survivor Fraser fir in the high elevation forests of Great Smoky Mountains National Park (Kloster 2001; Johnson et al. 2005), suggests there may be some element of resistance or tolerance within Fraser fir. The hypothesis under evaluation is that there is a genetic basis for BWA resistance in Fraser fir. The utilization of genetically resistant Fraser fir planting stock for Christmas tree production would be a relatively inexpensive solution to a difficult pest problem and would minimize adverse effects from the pest and related management strategies. In addition, resistant trees could be utilized in the restoration of native Fraser fir stands. Although many of the native stands have recovered from the initial wave of devastation through regeneration from wild seed, the endemic population is shrinking and their long-term prospects are uncertain. Outplantings of BWA-resistant Fraser fir could increase the likelihood of the continued survival of this ecologically and recreationally important ecosystem.

The work detailed here supports the long term objective to develop BWA-resistant Fraser fir trees. Before this work could begin in earnest, there were a number of outstanding issues that needed to be addressed. The critical question of whether it was possible to artificially infest Fraser fir seedlings with BWA and maintain the infestations over a period of time, which had not been done when this project began, is addressed in Chapter 3. The second issue, whether we could develop a more time-efficient way to artificially infest the trees than the current method of attaching infested bark pieces to the uninfested material, is addressed in Chapter 4. Because young seedlings are often utilized as surrogates for mature trees, it is important to know whether seedlings from multiple fir species spanning the range of known BWA susceptibilities would respond to BWA infestation (a) as would older trees and (b) in keeping with their susceptibility ratings. Chapter 5 details this research.

Backcrosses involving susceptible and resistant fir species are addressed in Chapter 6. Chapters 7 and 8 focus on research designed to consider BWA resistance within Fraser fir.

It is my hope that this research will provide useful information for future work in developing BWA-resistant Fraser fir trees.

CHAPTER 2. LITERATURE REVIEW

Taxonomy of the balsam woolly adelgid

Adelgids are characterized by complex polymorphous life cycles and alternate hosts; the primary host is always a spruce and the secondary host always some other species of conifer. The typical species has five forms (Balch 1952, Havill and Foottit 2007): *sexuales* (winged, bisexual form on spruce, females lay only one egg) produce the *fundatrix* (wingless form, overwinters on spruce), which gives rise to the *gallicolae* (develop on spruce in a gall initiated by the *fundatrix*). The *gallicolae* generally migrate to the secondary host. The wingless offspring of the *gallicolae* on the secondary host are of two types: the *exsulis sistens* ("sistens") (three molts, characterized by a period of diapause after stylets insertion during the first instar) and the *exsulis progrediens* ("progrediens") (four molts; generally does not enter into any period of diapause). In the typical species, the sistens produces a winged, parthenogenetic form called *sexuparae* (Latin: 'those that bear the sexual generation'), which flies back to the spruce and produces the *sexualis* generation. In some species, continuous paracyclic generations can occur in which the insect remains on either the primary or the secondary host.

Of the eleven species of adelgids that infest true firs worldwide, only one (*Pineus abietinus* Underwood and Balch) is native to North America and two (*Adelges piceae* Ratz. and *Adelges nusslini* (Borner)) are introduced. *Pineus abietinus* infests grand fir (*Abies grandis* (Dougl.) Lindl.) and Pacific silver fir (*A. amabilis* Forbes) and causes no real damage to its hosts (Bryant 1974a). *Adelges nusslini* damages European firs but in North America has limited distribution and is of no economic importance. *Adelges piceae* is generally innocuous in Europe but has caused extensive damage in North America. J.T. Ratzeburg first named the balsam woolly adelgid *Chermes piceae* in 1844, after observing a species of *Chermes* producing ‘wool’ on the bark of silver fir (*Abies pectinata* D.C. [= *Abies alba* Mill.]) in Germany (Balch 1952). Over the next few decades, researchers found that there appeared to be two forms of *C. piceae* on fir: one that migrated to the spruce and the other which infested only fir. C.V. Börner (1908) recognized morphological differences in what were originally considered one species and separated the species into two, naming the form with the complete cycle *Dreyfusia nusslini* and placing both species into the genus *Dreyfusia* (Balch 1952). The wingless species that remained on fir continued to be called *Chermes piceae* or *Dreyfusia piceae* until Annand (1928) grouped the two species within the genus *Adelges*. Complete agreement between taxonomists has yet to be achieved. In Europe the true fir infesting adelgids are placed in the genus *Dreyfusia* and in North America all adelgids are placed in the subfamily Adelginae, in one of two genera, *Pineus* and *Adelges*. Thus, the balsam woolly adelgid is known as *Adelges piceae* in North America and *Dreyfusia piceae* in Europe. The name *Chermes* is no longer in use.

Three geographic subspecies of *A. piceae* Ratz. have been identified in North America (Foottit and Mackauer 1983): *A. piceae piceae* (Ratzeburg 1844) has been observed in British Columbia, Oregon and Washington, and in the Southern Appalachians. This subspecies corresponds morphologically to Pschorn-Walcher and Zwolfer’s forma *typica* (1956) and the ‘intermediate group’ of Foottit and Mackauer (1980). Greenhouse studies in North Carolina have supported the hypothesis that *A. piceae piceae* in the Southern Appalachians may be represented by two or more biotypes (Hollingsworth and Hain 1994), but more research is required to support this hypothesis. *Adelges piceae canadensis* (Merker and Eichhorn 1956) is found in Quebec and the maritime provinces of Canada, and in the northeastern United States. *Adelges piceae occidentalis* has been observed in British Columbia. The subspecies are based on the morphological differences of first instars and adults between the three subspecies (e.g., body shape, length, dorsal plates, fusion of pleural and mesial plates, shape of pore fields of mesial plates, number and range of wax pores).

Discovery and distribution of the balsam woolly adelgid

The balsam woolly adelgid is believed to be an indigenous species on European silver fir in the mountains of western and central Europe (Pschorn-Walcher 1964, Eichhorn 1968) that migrated to other areas in Europe and to North America on infested nursery stock. Its range now encompasses fir forests and plantations throughout Europe, from Sicily to Sweden and from France to Russia, the maritime provinces of eastern Canada, the northeastern United States, the Southern Appalachians, British Columbia, the Pacific Northwest (Pschorn-Walcher 1964), including eastern Oregon (Keen 1952, Overhulser 2004), and Idaho (Gast et al. 1990, Livingson et al. 2000), California (Annand 1928), and Chile (Cerda and Gara 1977).

Introduced into Maine and Nova Scotia around 1900 (Balch 1952), BWA was established on balsam fir in Maine by 1908 (Kotinsky 1916). It was first reported on Fraser fir in the Southern Appalachians on Mount Mitchell in 1955 (Amman 1966) and quickly spread throughout the natural range of Fraser fir. It was detected on bracted balsam fir (*A. balsamea* var. *phanerolepis* Fern.) in the Shenandoah National Park in Virginia in 1956 (McCambridge and Kowal 1957). On the West Coast, it was first reported on ornamental firs near San Francisco (Annand 1928) and in the Pacific Northwest, it was first reported on grand fir in the Willamette Valley of Oregon (Keen 1952). There, the most frequent hosts are Pacific silver fir and subalpine fir (*Abies lasiocarpa* (Hook.) Nutt.) at high elevations and grand fir in valleys (Mitchell et al. 1961). The adelgid was reported in British Columbia on Pacific silver fir and on Vancouver Island on grand fir by the late 1950s (Silver 1959).

Damage to North American fir forests

The balsam woolly adelgid has caused considerable damage to North American fir forests, to the point of eliminating some and reducing many of the native stands of Fraser fir (Pyle and Schafale 1988, Dull et al. 1988, Pauley et al. 1996, Rabenold et al. 1998, Smith and Nicholas 1998), and altering the composition of the surviving stands (Jenkins 2003). The balsam fir forests of New England and Canada and the mixed fir forests of the Pacific Northwest have been continually plagued by the insect for almost 100 years and the natural stands of Fraser fir in the Southern Appalachians for over 50 years. Grand fir is gradually being eliminated from low elevation landscapes in the Pacific Northwest and subalpine fir is being removed as a pioneer tree species in the Cascades (Mitchell and Buffam 2001, Ragenovich and Mitchell 2006). Balsam woolly adelgid populations appear to be on the rise in native stands and are predicted to be one of the biggest challenges to forest management in the natural range of balsam fir over the next decade (Quiring et al. 2008).

Adelgid infestations follow a general trend: The initial infestation begins with a few large trees, generally with deep fissures and spreads to nearby trees. The population peaks and when many trees have been killed or are damaged, the population diminishes. This second period may go on for an indefinite period of time and is characterized by increasing gout and gradual dying of trees. Some trees may actually recover. The first outbreak is generally the most severe (Balch 1952), but the infestations can persist for decades and many stands gradually succumb to the stress (Mitchell and Buffam 2001). Stem attack is generally observed in continental climatic zones and twig attack in maritime zones (Greenbank 1970, Schooley and Bryant 1978). Mass stem infestations can cause tree mortality within two to three years. With a crown infestation, the tree will suffer branch dieback and general deterioration, and it can take as long as 10 to 20 years for mortality to occur (Bryant 1974a).

Trees damaged by BWA often become more susceptible to other pests and pathogens. The incidence of Armillaria root rot increases in proportion to adelgid damage (Hudak and Singh 1970, Hudak and Wells 1974), and damaged trees are also more susceptible to attack by the hemlock looper (*Lambdina fiscellaria fiscellaria* Guen.) (Warren and Singh 1968, Bryant 1974a, Hudak *et al.* 1978), woodwasps in the family Siricidae (Stillwell 1966), bark beetles (Coleoptera: Curculionidae: Scolytinae) (Rudinsky 1956), and the western spruce budworm (*Choristoneura occidentalis* Freeman) (Ragenovich and Mitchell 2006). Crown-damaged trees are often more susceptible to wind damage than healthy trees (Schooley and Bryant 1978). Adelgid damage decreases the value of lumber (Balch 1956) and pulp value (Hunt 1968) and the aesthetic value of trees in recreational areas and on Christmas tree plantations is severely reduced by adelgid damage (Bryant 1974a).

The balsam woolly adelgid attacks natural fir stands as young as six years of age, but very young stands are often able to recover from attack (Schooley and Oldford 1974). The most severe damage has occurred in overmature stands (Warren, Parrott and Cochran 1967). It appears that once the adelgid has infested a stand of fir trees, although population levels may fluctuate, BWA is there to stay (Mitchell and Buffam 2001).

The decline of the spruce-fir forests in the Southern Appalachians has had, and continues to have, a negative effect on multiple species that live in these forests. For example, a study of birds present on Mount Guyot in 1967 was repeated in 1985 and it was revealed that two of the territorial species were no longer present on the mountain, although three new early successional species had arrived, and of the eleven species that were present in both years, density had decreased (Alsop 1991). On Mount Collins, where fir has been almost eliminated, over one-half of the territorial avian species have declined by more than 50% over the last 25 years and some species are near local extinction (Rabenold et al. 1998).

Mode of dispersal

In North America, the balsam woolly adelgid is passively dispersed. Crawlers and eggs may be carried on the bodies of animals (Woods and Atkins 1967), but the primary mode of dispersal is via wind (Crystal 1925, Balch 1952, Lambert and Franklin 1967, Greenbank 1970, Schooley and Bryant 1978).

Life history and life cycle of the balsam woolly adelgid

In Europe and North America, BWA exhibits the continuous paracyclical (anholocyclic) mode of generation on fir and does not migrate to spruce. It has been suggested that this characteristic may have come about when European silver fir retreated into the Mediterranean area during the glacial periods (Pschorn-Walcher 1964).

The sistens form is wingless and develops on the stem (near lenticels, crevices in bark, callus tissue), branches or bases of buds (Crystal 1925, Balch 1952). The number of sistens generations per year depends upon temperature and host conditions. The overwintering generation of the sistens is called *hiemosistens* and the summer generation *aestivosistens*. There is only one hiemosistens generation per year and as many as three or four aestivosistens generations. The sistens (plural = sistentes) develops through four phases after emerging from the egg: first instar (crawler, neosistens), second and third instar, adult. The neosistens is oval, flattened ventrally, moderately convex dorsally, 0.35 to 0.47 mm in length, with stylets over four times as long as the body, and a 3-segmented antenna. Each successive instar is larger and more rounded, with shorter antennae and legs. Adults are 0.70 to 0.86 mm in length (Balch 1952).

The progrediens form (plural = progredientes) can be wingless (aptera) or winged (alata) and develops on the needles of fir (Crystal 1925, Balch 1952, Varty 1956). These forms are considered rare; there is only a 2% progrediens:sistens ratio on twig infestations and 0% ratio on stem infestations (Eichhorn 1969). The progrediens form is observed only in the subspecies *A. piceae canadensis* in North America (Foottit and MacKauer 1983); it arises from the first eggs laid by the hiemosistens and differs morphologically from the sistens, particularly in the presence of a fourth instar, shorter stylets, and the development of wings in the progrediens alata (Balch 1952). The progrediens aptera is somewhat smaller (adult length 0.6 to 0.7 mm) than the sistens and the progrediens alata somewhat larger (adult 0.9 to 1.25 mm) with 5-segmented antennae, more clearly defined body structure (head, thorax, abdomen), and wings (Balch 1952, Schooley and Bryant 1978). Additionally, the progrediens form has a shorter embryonic developmental period than the sistens form (Eichhorn 1969). Progredientes have been observed only in Europe and Canada (Tunnock and Rudinsky 1959, Mitchell et al. 1961).

In central Europe there are often three generations per year (Pschorn-Walcher 1964) and in central and northern Italy there can be four to five generations per year (Binazzi and Francardi 2001). In eastern Canada there are generally two generations per year (Balch 1952), and in British Columbia there are two (exceptions being only a partial second at the highest elevations and a partial third at low elevations during certain years) (McMullen and Skovsgaard 1972). In the Pacific Northwest there can be as many as four generations, depending on the elevation (Tunnock and Rudinsky 1959, Mitchell et al. 1961). Many natural stands in the Southern Appalachians have been observed to support two generations (Amman 1962, Arthur and Hain 1984). Cultivated sites in the Southern Appalachians such as Christmas tree plantations at lower elevations, may experience as many as three generations, influenced possibly by both the warmer climate and the application of fertilizers to the trees (Arthur and Hain 1984).

The time required for generation development from egg to adult varies with temperature and the generation. At the highest elevations, with low temperatures and harsh conditions, fewer generations develop and there are clear distinctions between generations; at lower elevations and warmer temperatures, the generations often overlap and it is difficult to distinguish one from another (Mitchell et al. 1961). Hiemosistentes take two to four weeks to mature and aestivosistentes can take three to eight weeks (Balch 1952). Greenbank (1970) estimated that it takes about 650 degree-days (daily degrees above 5.5ºC) to complete one entire generation. Considerable variation in development can exist within the same tree: in natural stands of balsam fir, adelgids at staminate buds develop earlier than those at the proximal end of the shoot (Bryant 1971). Development takes place more rapidly in vigorous and healthy trees. Oviposition commences when the adult is two or three days old and can continue for up to five or more weeks. The oviposition period averaged 66 days at 8.9ºC and only 10 days at 26.7ºC (Greenbank 1970). The hiemosistens (spring) generation exhibits higher fecundity than aestivosistens (summer generations) and can often lay more than twice the number of eggs (100 per egg mass versus 50). Progrediens alata generally do not lay eggs and progrediens aptera either lay no, or very few, eggs (Balch 1952). The latter have been observed to lay eggs directly on needles (Bryant 1971).

Freshly laid eggs incubate for about 12 days, depending on the temperature (Balch 1952). The stage that generally suffers the highest mortality is the egg. A temperature of -8ºC or lower kills eggs (Balch 1952, Amman 1967) and eggs incubated at low temperatures produce weak crawlers which live only a short time (Amman 1968). The upper lethal temperature for incubation of eggs is 32ºC (Greenbank 1970). Amman (1968) found that the optimum range for development and hatching on Fraser fir was between 5º to 7ºC and 25ºC. Post-eclosion storage of eggs has a marked effect on crawler vigor and mobility. Crawlers held at warmer temperatures (21º to 28ºC) show a much more rapid decline in vigor than those held at 17ºC (Atkins and Hall 1969).

Crawlers generally settle within a few hours but can live from one to eight days at diurnal temperatures fluctuating between 10ºC and 22.2ºC (Balch 1952). Some (approximately 4%) become tangled in the waxy threads of the egg mass and die, and others (approximately 20%) settle near the original egg clutch, but they all tend to wander somewhat before settling (Greenbank 1970). Under natural conditions, they can crawl 100 feet or more (Balch 1952). On small balsam fir, crawlers tend to go to the ends of branches and settle on new shoots. They have been observed to fall off branch tips on warm sunny days (Edwards 1966). Rain washes crawlers from the tree (Karafiat and Franz 1956 in Amman 1970) and they are often blown by the wind to areas where no fir trees exist (Lambert and Franklin 1967). Laboratory tests indicate that as many as 80% of the crawler population drop or are blown off the original tree (Greenbank 1970). On larger trees most of the remaining population will settle on the stem and branches but some will go to the tips of branches (Balch 1952). Crawlers, particularly young ones, tend to be photopositive and go toward the light (Atkins and Hall 1969) except for strong sunlight (Balch 1952) and will not settle on bark exposed to the midday sun. Crawlers appear to hatch more during the night (in the dark) and the crawlers drop from the tree as the environment becomes warmer and lighter (Atkins and Hall 1969).

The crawlers respond to photic, chemical and tactile stimuli (Balch 1952, Varty 1956). The insect receives chemical stimuli through antennae touching the substrate, and is prompted to stop moving when several parts of its body receive tactile stimuli. On exposed bark, the insect often needs high numbers of other crawlers to create ‘mock’ crevices to stimulate settling (Bryant 1976). Feeding sites are chosen for accessibility to parenchyma cells (callous tissue, new shoots, lenticels, crevices in bark, sites previously infested where hypertrophy has occurred) (Balch 1952). The crawler inserts its stylets into the bark and generally undergoes a period of dormancy for 2 to 8 weeks while undergoing a pronounced change in appearance without actually molting. The legs and antennae atrophy, a grid of wax is secreted, the coloring changes from amber to purple-black, and the insect is now truly a ‘neosistens’ (Amman 1970). Neosistentes suffer high mortality. Most crawlers that remain on the tree successfully settle, entering their period of winter dormancy or summer aestivation, but most never come out of that stage to continue their development. Varty (1956) hypothesizes that it is due to some element of resistance in the tree (e.g., grand fir or white fir (*Abies lowiana* [Gordon])) and that the resistance lies in some nutrient deficiency, toxic substance, or pH unsuitable for adelgid development. However, some overwintering neosistentes can withstand temperatures below -34ºC (Balch 1952, Amman 1967).

Upon emerging from the period of aestivation, the adelgid passes through three molts before becoming a wingless adult. The adelgid remains effectively in the same location on the bark during the course of its lifetime, although neosistentes have been observed moving from their original feeding sites soon after settling or immediately after molting into the second instar (Carrow and Graham 1968). Atkins (1972) found that neosistentes reared from eggs of the final generation of the year developed only under fluctuating regimes (13ºC to 24ºC) but progeny from the spring generation developed under both constant and fluctuating temperatures. In these studies, alternating temperatures appeared to be required to terminate the dormant period and permit normal development to proceed (Atkins 1972).

Greenbank (1970) found that nymphal development is relatively unaffected by relative humidity (representing continental and maritime conditions) and the rate of development varies with temperature; at 7°C, duration of the nymphal period is 36 days and at 22ºC only 9 days. High mortality has been associated with high rearing temperatures: at 29ºC only 5% of the feeding population reached the adult stage and at 32ºC, none reached the adult stage; below 5.5ºC, no appreciable development occurred (Greenbank 1970).

In studies relating to BWA survival on cut logs, Atkins and Woods (1968) found that active stages of BWA can survive up to three weeks on logs moving in rafts or booms with sea water splashing the logs regularly. Egg laying adults protected by crevices or very thick layers of wax can survive total immersion in sea water for four days. On grand fir trees cut in the forest and left where they fell, BWA continued to develop on the cut logs for up to 10 weeks; egg viability and crawler vigor differed very little from the standing trees (Atkins and Woods 1968).

Initiation of damage to individual trees

The balsam woolly adelgid has piercing-sucking mouthparts (stylets) and feeds on cortical parenchyma within the outer 1.5 mm of bark or on twigs at the base of buds (Balch 1952). The stylets are inserted through the epidermis into the cortex, probing between cells, until a suitable feeding site can be located. The phloem is rarely entered except in very young stems. Salivary secretions, which can flow into intercellular spaces, are exuded from the tip of the maxillae to form a sheath that lines the path of the stylets. The neosistens inserts its stylets full length before entering diapause. Feeding occurs through repeated partial withdrawal and reinsertion of the stylets in a new direction. The stylets are withdrawn and completely renewed at each molt, with the new stylets inserted near the original point of entry. The tracks are branched and affect tissue in a 360^{o} pattern (Balch 1952). Aphids feed in a similar pattern and are able to assess feeding sites by intermittently ejecting and sucking back up a watery saliva along with soluble material from the host; this watery saliva is diffused into surrounding tissue and can be transported within the host plant (Miles 1965, 1999). Because of the close relationship between aphids and adelgids, and the many similarities in feeding patterns and capabilities, it is likely that adelgids also secrete the watery saliva, which is then transported throughout the tree through the phloem.

The adelgid's salivary secretions appear to create a chemical imbalance within the tree and disrupt the development of normal tissue. Balch et al. (1964) hypothesized that, while it is possible that growth hormones are directly present in the adelgid's saliva, it was more probable that a synergistic response was created between enzymes present in the saliva and chemicals already present in the tree. They also hypothesized that the greater the vigor of the part attacked, the greater the effect of the salivary injections (i.e., the more vigorous trees and parts of trees, the more saliva produced by the adelgid and thus the more damage to the tree) (Balch 1952). A susceptible tree responds immediately upon full insertion of a neosistens' stylets, even when the insect has entered a resting phase and has not yet begun to actively feed (Balch 1956). The gel-like stylet sheath may often form a barrier around damaged parts of parenchyma cells, preventing the disintegration and rupture of cell vacuoles, which would normally be followed by the production of autotoxic defensive compounds in surrounding cells (Miles 1999). The stylet sheath can also slow down or prevent the production of the phenolics that arise from and promote necrosis, by adsorbing and immobilizing the phenolics (Miles 1999).

Response of *Abies* species to balsam woolly adelgid attack

All *Abies* species appear to be susceptible, in varying degrees, to some species of adelgid throughout the world and susceptibility to BWA attack varies. Firs native to North America (subalpine fir, balsam fir, Fraser fir) are highly susceptible and firs native to central Europe (silver fir) tolerate infestation (Varty 1956, Mitchell 1966). Some Asian species

(Veitch fir [*Abies veitchii* Lindl.]) appear to be immune to attack, at least in North America (Hall et al. 1971). That said, it should be noted that Varty (1956) successfully infested seedlings of Veitch fir under laboratory conditions in Scotland (although both the trees and the adelgids would certainly have been of a different genotype than those on this continent), and Mitchell (1969) observed a light to moderate infestation by BWA of Veitch fir in the Pacific Northwest.

All ages of fir in plantations are susceptible, particularly Fraser fir, while in natural stands, older, mature trees are more susceptible. This begs the question as to whether there is something in younger trees—physical or chemical—that protects them from severe infestations.

Infestations are generally classified as ‘stem’ or ‘crown’ infestations. The damage, both microscopic and macroscopic, is fairly consistent among all susceptible firs. With a crown infestation, gouting (abnormal growth resembling a gall) is apparent. Growth of wood and bark is stimulated at the point of stylets insertion and both hypertrophy and hyperplasia occur: an enlargement of parenchyma cells (up to 6 or 7 times the size of normal cells), including cell walls and nuclei, occurs and these large cells are continually produced, causing swelling of the twigs and in the bark (Balch 1952). Pock-like swellings are often observed on the bark of young trees (Rudinsky 1956). The number of rays increase (Doerksen and Mitchell 1965), as do the number of parenchyma strands (Smith 1967), and ray parenchyma cells often have abnormally shaped nuclei (Saigo 1976). Large reductions in carbohydrate reserves of needles and twigs have been observed (Puritch and Talmon-De L’Armee 1971). Increasing numbers of these enlarged parenchyma cells disrupt the phloem channels and interfere with the metabolic pathways in the bark (Bryant 1971). Bud growth often ceases and the twig begins to die back from the ends; a flattened top is often observed in infested trees (Balch 1952, Mitchell 1967). In fact, this loss of apical dominance (observed in stem infestations as well as crown infestations) is often the first symptom looked for in Fraser fir Christmas tree plantations when scouting for BWA infestation (Sidebottom 2004). In addition to gouting, branch and stem growth are slowed or cease (Balch 1952, Schooley 1974).

With a stem infestation, although the stylets of the adelgid can be up to 5 mm from the vascular cambium, xylem production is often compromised resulting in abnormal wood called ‘rotholz’ or ‘redwood.’ Rotholz is anatomically similar to compression wood in conifers (Balch 1952). These resultant tracheids are circular rather than rectangular (Timell 1986), short, thick walled, and highly lignified with small lumens (Doerksen and Mitchell 1965), have a reduced number of conducting pits (Puritch and Petty 1971), higher specific gravity, greater fibril angle (Foulger 1968), and often have encrusted pit membranes such as those found in heartwood (Puritch and Johnson 1971). Rotholz wood has been found to have 13% higher lignin content and more than five times the amount of galactans than that of uninfested wood (Balakshin et al. 2005). Traumatic resin ducts may form in the xylem (Saigo 1976). Sapwood permeability in grand fir has been reduced to 5% of that of normal sapwood (Puritch 1971). Ultimately, these changes throw the tree into physiological drought, reducing the flow of water within the tree and compromising photosynthesis, transpiration, and respiration (Puritch 1973). Heavy infestations can result in tree mortality in one to two years (Balch 1952). Additionally, xylem formed during adelgid infestations contain decreased amounts of total sugars and exhibit increased longitudinal shrinkage as compared with uninfested material (Founger 1968). Adelgid infestation appears to contribute to premature heartwood formation (Puritch 1977). Rotholz production, which can be detected in wood by the application of perchloric acid (Hain et al. 1991), has also been associated with a significant increase in the proportional area of heartwood (Hollingsworth et al. 1991) and higher percentages of latewood (Doerksen and Mitchell 1965, Mitchell 1967, Smith 1967, Foulger 1968).

The changes that take place in the bark of the fir trees initially create a more favorable environment for adelgid development, and in the early stages of an infestation fecundity is at its peak; as the trees weaken and the population begins to dwindle, fecundity also decreases (Pschorn-Walcher and Zwolfer 1958, Amman 1970).

Host responses that may aid in resistance

Host resistance can often be attributed to a combination of factors rather than any single factor. Painter (1951) and Zobel and Talbert (2003) recognize essentially three types of resistance to insects: (1) *nonpreference,* in which the insect is not attracted to, or is repelled from, feeding and ovipositing on the tree, (2) *antibiosis*, in which the insect is killed, injured, or prevented from completing its normal life cycle after feeding on the tree, and (3) *tolerance*, in which the tree recovers from insect attack by a population equal to that which would damage a susceptible tree. Host characteristics that may contribute to resistance are listed as color, pubescent or waxy epidermis, protective cellular structures (high lignin content), vigor, growth habit, pH concentration, and specialized chemicals such as tannins, proteins, and alkaloids (Varty 1956). Amman (1970) stated that the host tree was the most important factor regulating BWA. Other factors would include inherent differences in wound healing mechanisms, and environmental conditions.

Some responses to adelgid attack may aid in host resistance or tolerance to infestation. For example, many conifers have the ability to form a secondary periderm, consisting of necrophylactic tissue, around a wound (Mullick 1975, Hain et al. 1991). This wound healing mechanism can isolate an area of bark occupied by the adelgid and can effectively protect the underlying bark from further attack for years (Balch 1952, Mullick 1971). The formation of impervious tissue at the site of injury has been shown to be an integral step in the process of periderm formation. It has been hypothesized that the formation of the protective secondary periderm may be inhibited or compromised by chemicals secreted by the adelgid's stylets (Mullick 1975, 1977). *Adelges piceae* saliva has been found to contain auxin-like compounds (Balch et al. 1964) and pectinase (Adams and McAllan 1958, Forbes and Mullick 1970). Hay and Eagar (1981) injected indole-3-acetic acid (IAA) or naphthalene acetic acid (NAA) into Fraser fir trees in the Great Smoky Mountains and found that the auxin-like compounds appeared to delay formation of impervious tissue by 11 days. Arthur and Hain (1985) artificially wounded 86 fir trees (76 Fraser fir and 10 European silver fir) at three different locations in the Southern Appalachians with naphthalene acetic acid (NAA), pectinase, or todomatuic acid, but none of these chemicals delayed formation of the impervious tissue.

Juvabione (a sesquiterpenoid which acts as an insect juvenile hormone analogue [Slama and Williams 1965]) or juvabione-like compounds may be produced in response to BWA attack. Puritch and Nijholt (1974) found that healthy specimens of grand fir and

Pacific silver fir did not produce the juvabione-like compound todomatuic acid, but infested trees of the same species did produce the compound in the vicinity of BWA infestation. Fowler et al. (2001) found higher concentrations of juvabione in the wood of infested Fraser fir, and the highest concentrations were found in trees that had maintained their apical dominance.

Some firs, particularly grand fir, produce copious amounts of resin in response to wounds. This may serve as a means of resistance, as grand fir is one of the more resistant species of American firs, with only 20-30% mortality associated with BWA infestation (Mitchell 1966). Many conifers have evolved resin-based defenses, such as oleoresin—a mixture of terpenoids consisting of a turpentine and rosin fraction—to deter insect pests and their symbiotic fungal pathogens. Turpentine contains insect and microbial toxins in addition to other biologically active agents that act in unison to discourage insect infestation (Raffa and Berryman 1983). True firs store only small amounts of primary resin in bark blisters, but respond to wounding by producing oleoresins in nonspecialized, adjacent tissues.

Other factors that may aid resistance

The onset of water stress (physiological drought), one of the chief factors in susceptibility to damage, differs among fir species and those exhibiting a more rapid onset are more susceptible to intense damage (Varty 1956, Mitchell 1967). It follows that species or individual trees within a species that exhibit high levels of drought resistance would also be more resistant or tolerant of BWA.

Production of thick outer bark, as found in European silver fir, has been associated with resistance to or recovery from adelgid attack (Pschorn-Walcher and Zwolfer 1958, Schooley and Bryant 1978). The texture of the bark may also be an important factor in resistance or susceptibility. Rough or flaky bark may be more preferable for BWA for a number of reasons, including the interception of airborne eggs and crawlers, stereotropism (the insect is stimulated to grow or change in response to touch), and the more easily accessible nutritive areas (e.g., lenticels and bark crannies) composed of young parenchyma (Varty 1956). Lenticel development has been observed to be the best single predictor for BWA population levels on Fraser fir in the Great Smoky Mountains (Hay and Eager 1981).

Some provenances of Fraser fir appear to respond to adelgid infestation differently than others. For example, BWA infestation was not discovered on Mount Rogers until 1979, although there was evidence that some trees had been under attack for up to 17 years (Haneman et al. 1981), and high mortality was not observed until the 1990s. Mount Rogers trees become heavily infested but do not appear to suffer rapid mortality (Hollingsworth and Hain 1991, Nicholas et al. 1992). The fir trees on Mount Rogers have been shown to be genetically different (unique allele frequencies) from other natural populations of Fraser fir (Ross 1988), but these differences have not been correlated with susceptibility to adelgid infestation (Hain et al. 1991). Nonetheless, Mount Rogers trees have been shown to form lower levels of rotholz and higher levels of secondary periderm in response to adelgid attack when compared to trees from Mount Mitchell (Hollingsworth and Hain 1992). It has been suggested that these trees may possess the ability to develop secondary periderm more rapidly than those in other locations—this characteristic may aid in adelgid tolerance (Hay and Eagar 1981). Additionally, the trees on Mount Rogers may be less likely to suffer water stress when infested partly due to environmental factors (deeper soil, less wind) (Hollingsworth and Hain 1994).

Within the natural range of Fraser fir, although the majority of mature fir trees have been killed, there are remnant populations that have survived multiple decades of adelgid attack. It remains to be seen as to whether these trees have survived as a result of genetic resistance or whether the environment (most are at the highest elevations) plays a more important role.

Among and within all *Abies* species, even those highly susceptible to BWA, trees have been observed time and again to tolerate and even recover from adelgid attack. It is not uncommon for balsam fir to recover from adelgid attack after a few years, with a replacement of the original leader and relatively small stem deformities from gouting (Balch and Carroll 1956, Schooley 1976, Schooley and Bryant 1978). Although Mount Mitchell Fraser firs suffered high mortality during the initial wave of infestation, and have been shown to be highly susceptible to adelgid damage (Dull et al. 1988), individual trees cored at Mount Mitchell showed production of rotholz in the past, but the trees had recovered, were uninfested, and otherwise appeared healthy (Hain et al. 1991).

Breeding trees for host resistance

Tree improvement techniques and an ever-increasing understanding of forest genetics have been utilized throughout the Southeast over the last 30 to 50 years in the growth and management of loblolly pine (*Pinus taeda* L.), to an extent that both productivity and wood quality has improved to a remarkable degree. With some programs entering their fourth generation of selection and breeding, virtually all of the loblolly pine seedlings planted per year throughout the South have been genetically improved (McKeand et al. 2003). Similar work is being conducted with Fraser fir and, while the results are not yet as dramatic as those seen in loblolly pine management, genetic testing of Fraser fir holds within it the potential of solving the problem of pests and pathogens threatening the species, as well as improving growth rates and desirable traits for the Christmas tree industry.

A geographic variation study was conducted by NCSU in 1983, wherein progeny from open-pollinated trees from five geographic locations of Fraser fir were planted on three different sites in the North Carolina Mountains. Significant family within elevation class within seed source differences in height were observed after only one growing season (Li et al. 1988). Significant source and family within seed source differences were observed in every measured trait after 8 years. Family heritabilities were moderate to strong in certain traits, suggesting a high potential for genetic improvement (Arnold et al. 1994). A wide-range cone collection was made by NCSU in 1994, wherein seed from over 500 open pollinated parent trees were collected from different elevations of all six natural sources of Fraser fir (McKeand et al. 1995). Seedlings from this collection were grown in a greenhouse in 1997 (Frampton 1998) and another progeny test series, with the objective of determining genetic variation among seed sources and estimating genetic parameters for various traits, was established on eight sites throughout western North Carolina in 2000. An analysis conducted after four growing seasons in the field revealed highly significant differences among seed sources and families within seed sources for height and overall tree quality (Emerson 2004). Heritability values for height at age 4 ranged from 0.15 to 0.67, with an overall heritability value of 0.44 for height (Emerson 2004).

Breeding for resistance to diseases and insects has been a prime target in breeding operations with trees and important steps in the process include the development of reliable and repeatable resistance testing techniques and achieving high correlation between nursery tests and field trials (Namkoong 1991). Tree breeders often try to capture easily identifiable resistance mechanisms and select for these characteristics in the hope that these characteristics will endure in subsequent generations. This approach sometimes focuses on single-gene effects and the genes must be present in the breeding populations or incorporated by backcrossing in classic breeding programs or through transgenesis (Namkoong 1991). The work conducted with the American chestnut (*Castanea dentata* [Marsh.] Borkh) since the late 1970s has now resulted in the development of chestnut progeny that appear to be resistant to the chestnut blight (*Cryphonectria parasitica* [Murrill] Barr) but are 15/16ths American chestnut and 1/16th Chinese chestnut (*Castanea mollissima* Blume) and thus exhibit primarily American chestnut traits (ACF 2010). Young trees are currently being outplanted into the environment in an attempt to restore the American chestnut to its native range.

Zobel and Talbert (2003) outline important steps in the construction of a breeding program against pests. The initial step is to understand the economic worth of the host; potential economic losses from the pest; biology and genetic variation within the host species; biology and genetic variation of the pest; interaction of the environment with tolerance of the host and virulence of the pest; and the interactions between the pest and the host. Selection of resistant trees should come from heavily infested stands and resistance trials should expose the trees to at least moderate levels of the pest. Another important consideration is the age of the trees to be screened – resistance genes may be turned on or off depending on the age of the tree, resulting in physiological or morphological changes that may alter resistance.

Control of the balsam woolly adelgid

Climatic

All stages of BWA can survive temperatures as low as -1°C. Low temperatures (-17°C) will kill all life stages except the overwintering neosistens. Extremely cold temperatures (-33°C) will destroy overwintering hiemosistens (Balch 1952). High temperatures (up to 39°C) do not appear to be detrimental in general, but mortality will occur if the insect is in direct sunlight (Balch 1952).

Biological

Although there exist many species of native predators and many more were introduced, attempts at biological control ultimately proved fruitless in the control of the balsam woolly adelgid. Biological control began to appear attractive to North American forest managers as early as the 1930's (Pschorn-Walcher 1964) and over the next few decades multiple species were introduced into the Maritime Provinces of Canada, British Columbia and both the east and western forests of the United States.

Native predators in Canada were reviewed by Balch (1952). They included the families Trombiidae (mites, 1 sp.), Hemerobiidae (brown lacewings, 3 spp.), Miridae (plant bugs, 1 sp.), Anthocoridae (1 sp.), Coccinellidae (lady beetles, 5 spp.), Syrphidae and Octhiphilidae (flies, 3 spp. and 2 spp. respectively). These were believed to be ineffective, so a number of additional predators were introduced: *Leucopis obscura* (Octhiphilidae) became naturalized over time. Other introduced predators included two species of brown lacewings, but the introductions were unsuccessful. By the 1960's, predators had been introduced from Switzerland, Germany, Sweden, Slovakia, Pakistan, India, Japan, Austria, and Great Britain (Pschorn-Walcher 1964). The introduced predators did not survive, with the exception of the Chamaemyiid fly *Leucopomyia obscura*, *Laricobius erichsonii, Cremifania nigrocellulata, Aphidoletes thomsoni*, and *Pullus impexus* (Pschorn-Walcher 1964). Eichhorn (1967) suggested infesting trees with a less damaging adelgid (i.e.*, A. nusslini*) to effect interspecific competition.

Of the predators released during the 1960s in British Columbia, by 1978 only two (*Laricobius erichosonii* Rosenh. and *Scymnus impexus* Muls.) had become established and none had any significant effect on reduction of adelgid-caused forest damage (Harris and Dawson 1979). A study from the early 1990s confirmed the establishment in British Columbia of *Cremifania nigrocellulata* and *Aphidecta obliterata* as well, and found an additional predator, *Leucopis atratula* (not deliberately introduced) on *A. nordmanniana* (Humble 1994). A new species of false spider mite (Acarina: Tenuipalpidae) has been observed in association with BWA on grand fir in the Pacific Northwest (Mitchell 1975).

Three beetles (two Coccinellids [*Aphidecta obliterata, Pullus impexus*] and one Derodontid [*Laricobius erichsonii*]), and one Cecidomyiid (*Aphidoletes thompsoni* Mohn.) were introduced into the Mount Mitchell area in 1959 and appeared to have established themselves fairly well within a few years (Amman and Speers 1965); however, the predators had little effect on adelgid populations due primarily to the high reproductive potential of the adelgids (Amman 1970). A later examination revealed that on Mount Mitchell, of the 22 introduced predators and numerous native predators, only 3 native mites and one introduced species (*Laricobius erichsonii*) could be found (Fedde 1972).

In the Pacific Northwest, 23 species of adelgid predators were released into Oregon and Washington; five species were well established in the early years of adelgid outbreak, yet high numbers of firs were killed and continued to be killed for the next 40 years in spite of the presence of predators (Mitchell and Buffam 2001).

Fungal diseases were studied and considered as a possible means for biological control of BWA. *Fusarium larvarum* was the most often-observed of the fungal species and the only one that was considered to be of any potential use, and then only in combination with sublethal doses of chemical insecticides (Smirnoff 1971).

Ultimately, biological control was not effective in reducing the onslaught of destruction brought about in North America fir forests by the balsam woolly adelgid. In a plant's native range, pests are generally kept in check through a combination of phenology, natural enemies, and host resistance.

Chemical

The use of insecticides has long been an effective means of insect control, and a number of insecticides were tried in the early days of BWA infestation, both in Canada and the United States (Randall et al. 1967). Insecticidal soaps were found to be effective biocides (Puritch et al. 1974, Puritch 1975, Puritch et al. 1980, Hastings et al. 1986). Except for nursery treatments and a few small-scale natural areas (e.g., Grandfather Mountain in North Carolina), insecticides proved to be ineffective in controlling the insect. Insecticide applications for adelgid control are commonplace in Christmas tree plantations (Potter et al.

2005); however they are expensive and lower the effectiveness of integrated pest management (IPM) strategies.

Topical application of juvenile hormone analogues has been considered as a possible means for controlling the balsam woolly adelgid (Retnakaran et al. 1979) and experiments with topical application of juvabione have resulted in inhibition of adeglid fecundity (Fowler 1999).

Silvicultural

Silvicultural treatments such as cutting and cleaning spot infestations were tried in the early days of BWA infestation, but did little to control the spread of this insect. However, it has been reported that in western Newfoundland, where precommercial thinning is widely used, there is less damage to balsam fir stands (Milne 1990). Converting to nonsusceptible tree species (e.g., Douglas fir) has also been used (Hall et al. 1971). Passive dispersal by loggers of infested material was recognized fairly early and warnings given to cut only during winter months and process the material before March (Balch 1956, Rudinsky 1956).

Nitrogen fertilization of the host tree has been suggested as a possible means of controlling BWA. Foliar applications of 1% ammonium nitrate have been shown to inhibit the settling of crawlers on *A. amabilis* (decreasing populations by 23%), most likely by upsetting the balance of amino acids (particularly by increasing the concentrations of arginine and completely eliminating the concentrations of ornithine) in the tissue (Carrow and Graham 1968), while applications of 1% urea increased populations by 30-35% (Carrow 1971). Carrow (1971) artificially infested 4-year old grand fir and assessed adelgid establishment and development under various foliar fertilization regimes. Crawler establishment appeared to be promoted by applications of urea and inhibited by ammonium nitrate. Survival of neosistens was lowest with ammonium nitrate. Adults appeared one week earlier on trees fertilized with urea, and the adult lifespan was prolonged with these agents. Reproductive rates were enhanced by urea fertilization. Fertilization with ammonium nitrate may compromise neosistens survival and adult fecundity by promoting deficiencies of phenylalanine and/or asparagines; creating an amino acid imbalance, either inducing a deficiency of essential amino acids or promoting arginine toxicity. Fertilization with urea may promote the appearance of asparagines and phenylalanine or by inducing a balance in dietary amino acids. These results demonstrate the importance of the free amino acid pool in the host tree and nutritive diet of the insect – phagostimulation of true aphids depends on the levels of sugars and certain amino acids in the tissue, and differences in amino acids and sugars are known to exist between resistant and susceptible plants (Mittler and Dadd 1965).

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CHAPTER 3. PRELIMINARY INFESTATION STUDIES OF FRASER FIR SEEDLINGS AT CONSTANT AND RAMPING TEMPERATURES

Abstract

Fraser fir seedlings of various ages were artificially infested with the balsam woolly adelgid in two controlled settings, the first at a constant temperature and the second at ramping temperatures. Both studies successfully resulted in at least some of the trees having developed egg-laying adults by the end of the study period. The infestations were sustained over time and supported multiple generations of BWA.

Introduction

The balsam woolly adelgid (*Adelges piceae* Ratz.; BWA) is a tiny (adults 0.8-1.0 mm in length) piercing-sucking insect adapted to specifically feed on species of the genus *Abies*. In North America, where it is an exotic species, the insect is wingless and entirely female, reproducing through parthenogenesis, and in the southern Appalachians there are generally two generations per year. Development includes three life stages (egg, three larval instars, adult). The only motile stage is the first instar and dispersal is primarily via wind, phoresy and gravity. Fraser fir (*Abies fraseri* Pursh Poiret) is endemic to the southern Appalachians, limited to high elevation forests where it generally obtains dominance above 1800-1900 meters. Following the introduction of BWA in the 1950s, mature Fraser fir stands experienced high levels of mortality, in some cases up to 95%. Fraser fir is economically important as one of the most widely utilized species for Christmas trees. Currently, the only effective means for controlling BWA is through the use of chemicals, burdening the growers with high costs and diminishing the effectiveness of integrated pest management (IPM) schemes. The development of BWA-resistant Fraser firs would be advantageous for restoration of native stands and for easing the financial burden of Christmas tree growers. To this end, resistance bioassays, particularly artificial infestations under controlled conditions, are critical for evaluating resistance among different fir species and for evaluating putative resistance with grafted material.

There was some question as to the most effective technique for achieving artificial infestations. From the early literature, it appeared that most experiments on potted trees were done by placing eggs taken from field infestations (e.g., Balch 1952, Varty 1956, Pschorn-Walcher 1964, Greenbank 1970). Although the exact method of ‘placing the eggs’ on trees is rarely mentioned, it was generally agreed that the more eggs placed on the tree (500+), the greater chance of successful infestation (e.g., 30 eggs may produce 1 adult, 500 eggs 10 adults, and 1200 eggs may result in a moderately heavy infestation [Balch 1952]). The most popular method of infestation involved cutting small pieces of infested bark from one tree and pinning, taping or tying it, with the woolly masses facing the bark of the new host (Tunnock and Rudinsky 1959).

Young fir seedlings had been successfully infested with the balsam woolly adelgid, including 4-year old Pacific silver fir (*A. amabilis*) (Carrow and Graham 1968) and 4-year old grand fir (*A. grandis*) (Carrow 1971), but relatively little success had been achieved with Fraser fir seedlings. Hollingsworth and Hain (1994a) infested 5-year old Fraser fir seedlings in an air-conditioned greenhouse in the Piedmont of North Carolina by pinning infested pieces of bark directly to the stems of the target trees (5 to 20 woolly masses per tree). The seedlings were later moved to the mountains and outside in an attempt to improve infestation levels. Approximately one-half of the seedlings became minimally infested and rarely produced multiple generations. In a separate study spanning a 13-week period, 3-year old Fraser fir seedlings were infested with BWA (by wrapping infested bark pieces around the stem) and just under one-half (44%) became infested (Hollingsworth and Hain 1994b). It was hypothesized that the minimal infestation levels of Fraser fir seedlings could be attributed to: (1) acquired resistance (Mount Mitchell seed source—through natural selection), (2) a negative impact from the fertilizer used in the studies, or (3) the production of juvabione in response to infestation (Hollingsworth and Hain 1994b).

Our objectives were to determine if we could artificially infest very young Fraser fir seedlings with BWA under a variety of environmental conditions and maintain those infestations over time, producing multiple generations. Past studies with other fir species suggested that ramping temperatures (13ºC to 24ºC) with relative humidity greater than 50% were preferable for achieving successful infestations (Carrow 1971; Atkins 1972).

Methods and Materials

We conducted two studies, both in controlled settings, the first (Study I) at a constant temperature (18ºC) and the second (Study II) at temperatures ramping from 13ºC to 55ºC.

Study I: Infestation of 2- and 3-year-old Fraser fir at 18ºC

A small study was initiated in the NCSU Insectary in July 2004. Temperatures were set to a constant 18ºC with even light days (12:12); relative humidity was not controlled but was estimated to be around 55%. Two ages of Fraser fir (3-year-old seedlings from a progeny test (Roan Mountain provenance) and 2-year-old seedlings (also from Roan Mountain) were utilized. Each age was represented by 13 seedlings, planted in 7.6 x 7.6 x 22.9 cm tree pots and placed in separate trays (Figure 1). Bark pieces with 3 woolly masses, i.e., egg clutches, (at least 10 eggs per clutch) were attached to the bole of each tree with a straight pin and the boles covered with translucent cloth. The seedlings were watered at ground level once a week for 17 months. The trees were assessed for infestation (presence of egg-laying adults, yes/no) at the end of the study period. This study did not include uninfested controls.

Study II: Infestation of 1- 3- and 6-year-old Fraser fir at ramping temperatures (13-23ºC)

A study was initiated in the NCSU Phytotron in July 2005 with Fraser fir trees of three different ages (1-, 3- and 6-years). The 1-year old trees were obtained from the North Carolina Forest Service Nursery and planted in 7.6 x 7.6 x 22.9 cm tree pots. The 3-year old trees had been grown from seed in a greenhouse at NCSU; these trees had been treated with a foliar insecticide three months prior to the initiation of this study. The 6-year old trees were 3-1 seedlings obtained from the NC Division of Forest Resources in 2003, transplanted into 11.4 liter pots, and grown in a shade or plastic covered pad (depending on the season) at the NCSU Horticultural Field Lab in Raleigh.

Walk-in chambers with identical environmental conditions were utilized. Temperatures were set to ramp between 13ºC and 24ºC within a 24-hour period. Radiant energy included full light (fluorescent and incandescent) with long days (16:8 light:dark). Relative humidity was set at 75%. The experimental design included the three ages of Fraser fir trees, divided equally and randomized by age in two blocks (separate Phytotron chambers). Adelgids were brought to the Phytotron from the field on six occasions

(8/18/2005, 8/24/05, 8/31/05, 9/6/05, 10/16/05, 10/23/05) and, for each transport, attached to 3 trees per age/block/collection date. For the first three transports, pieces of infested bark were cut from the trees and brought back as pieces; for the latter three, infested logs were brought back to the lab (with bark pieces cut from the logs in the lab). Bark pieces (each with approximately 10 eggs per woolly mass) were attached to uninfested trees with horticulture ties. Bark pieces with 2 egg masses were attached to the 1-year old trees, 8 egg masses to the 3-year old trees, and 18 egg masses were attached to the 6-year old trees. There were six control trees per age/block, three were treated with a foliar insecticide and three were untreated. There were 108 treatment trees and 36 controls, for 144 total study trees. After 11 months (July 2006), the trees were assessed for BWA infestation (egg-laying adults, yes/no), signs of gouting response (yes/no), and general health (good, fair, poor; new growth).

Results and Discussion

Study I: 2- and 3-year-old Fraser fir at constant 18ºC temperature

Three (23%) of the 2-year-old trees became infested, two of which died (Figure 1). A fourth 2-year-old tree died but there was no sign of infestation. Ten (77%) of the 3-year-old seedlings were infested (Table 1), and three of these died during the course of the study (Figure 2). A number of seedlings experienced bud death (including apical buds) and, indeed, death of entire branch sections (Figure 4). Gouting was apparent at many of the feeding sites (Figure 3). BWA egg masses were located on the bole and in the crown, particularly at the nodes, and along branches (Figure 5) under old bud scales, on needle cushions, and at the base of buds.

Since this study lasted 17 months, we can be certain that multiple generations of BWA developed during this time. Generation times, from egg to reproducing adult, vary. The egg stage may last from 6.41 (Atkins 1972) to 12 days (Balch 1952). Crawlers may live for 1 to 8 days (Balch 1952). Settled neosistentes may overwinter for months in that stage for months while overwintering, while the summer aestivation period may range from three to eight weeks (Balch 1952; Atkins 1972). The second and third instars last approximately 4.1 and 4.7 days, respectively (Atkins 1972). Adult oviposition begins after two to three days and continues for five or more weeks (Balch 1952). If we estimate a generation including one week in the egg stage, three days as a crawler, six weeks as a settled neosistens, four days as a second instar, five as a third instar, then three days before egg-laying begins as an adult, a generation may take 64 days, or a little over two months. Within the 17 months during this study, eight or more generations may have developed. Overlapping generations were observed on the infested trees, with multiple egg masses near and often on top of one another (Fig. 5).

Study II: Infestation of 1- 3- and 6-year-old Fraser fir at ramping temperatures (13-23ºC)

The level of infestation (development of adults with egg masses) across the three ages was low, with only 12% (13 of 108 treatment trees) exhibiting infestation (Tables 2 and 4). Three of the 1-year old trees supported egg-laying adults, as did ten of the 6-year old trees. None of the 3-year old trees became infested. The limited number of infested trees in Study II could be attributed to a variety of causes. The 1-year old trees were very small and a very limited number of bark pieces could be attached to these small trees; additionally, the crawlers, once hatched and freed from the egg mass, may have crawled off and away from the trees. The lack of infestation on any of the 3-year old trees was likely the result of these trees having been treated with a foliar insecticidal spray some months prior to the initiation of the study. The differences in infestation levels are likely related to the transport times and methods (bringing back bark pieces cut from trees in the field versus bringing back whole infested logs). Successful transports (meaning those transports that resulted in at least one tree infested with egg-laying adults) included the second (8/24), fourth (9/6), fifth (10/16), and sixth (10/23) (Table 2).

Gout production was observed in 15 of the 108 treated trees (ten 1-year old [Fig. 7] and five 6-year old [Fig. 8] trees). Only five of the trees (two 1-year and three 6-year old trees) supported adults with eggs; the other eleven appeared to be responding to the presence of settled instars. Almost half (48%) of the trees exhibited apical dominance.

By the end of the study, most of the treatment trees were in fair to good health (Table 3). Six (five 1-year and one 3-year old) trees were dead, but none of these showed signs of BWA infestation. Sixty percent the trees were actively growing and producing new growth (Table 4).

None of the non-infested controls exhibited any level of infestation (Table 4) and only one 1-year old tree exhibited any level of gouting. One 1-year old (not the one infested with adult BWA) and two 6-year old control trees had died. Many (47%, or 17 of the total 36) were actively growing and producing new growth (Table 4). Thirty-nine percent exhibited apical dominance.

Conclusion

These studies show that young Fraser fir seedlings can be successfully infested with BWA in both constant and ramping temperatures. These infestations can be sustained over time and the infested trees can support multiple generations of BWA. The limited number of 1- and 6-year old trees infested in Study II was likely the result of having too few adelgid crawlers applied to the seedlings. Bringing back the infested material as whole logs is a more effective method than bringing back pieces of bark cut in the field. A more efficient, less labor intensive method of exposing putatively resistant firs to BWA is needed.

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Table 1 (Study I). Number of infested and uninfested seedlings (and percentages of totals) 17 months following initial infestation. N = 26.

	-------Living Trees-------		-------Dead Trees-------		Total
Age	Infested	Uninfested	Infested	Uninfested	No. Trees
2	1 (8%)	9 (69%)	2 (15%)	1 (8%)	13
3	7 (54%)	2 (15%)	3 (23%)	1 (8%)	13
Totals	8 (31%)	11 (42%)	5 (19%)	2 (8%)	26

Table 2 (Study II). Number of trees supporting development of adult BWA with egg masses after the 11-month study period, relative to the date of exposure. N = 144 (108 study trees, 36 uninfested controls).

		----------------Age[1]----------------			
Date	Transport	1-year	3-years	6-years	Totals
(Controls)	0	---	---	---	---
08/18/2005	1	---	---	---	---
08/24/2005	2	1	---	2	3
08/31/2005	3	---	---	---	---
09/06/2005	4	---	---	5	5
10/16/2005	5	2	---	---	2
10/23/2005	6	---	---	3	3
	Totals	3	0	10	13

[o1] Note: The study lasted 11 months, so the trees were one year older than shown here. The ages reflect the age of the trees at the beginning of the study.

Table 3 (Study II). Comparison between uninfested controls and treatment trees in health rating. (Numbers of trees of each age and percentages of the totals are provided).

		Health								
	Age	-----Dead-----		-----Poor-----		-----Fair-----		-----Good-----		Total
		No.	%	No.	%	No.	%	No.	%	
Controls	1	1		2		8		1		12
	3	0		2		10		0		12
	6	2		5		5		0		12
		3	(8%)	9	(25%)	23	(64%)	1	(3%)	36
Treated	1	5		4		25		2		36
	3	0		4		25		7		36
	6	1		9		26		0		36
		6	(5%)	17	(16%)	76	(70%)	9	(8%)	108
Totals		9	(6%)	26	(18%)	99	(69%)	10	(7%)	144

Table 4 (Study II). Numbers (and percentages) of uninfested controls and treatment trees exhibiting the presence of adults with eggs, presence of gouting response, apical dominance, and active growth.

	Age		Adults w/eggs		----Gout---		Apical dominance		Active growth	
	(years)	N	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Controls	1	12	0	---	1	8%	7	58%	10	83%
	3	12	0	---	0	---	3	12%	3	12%
	6	12	0	---	0	---	4	33%	4	33%
		36	0	---	0	---	14	39%	17	47%
Treated (with BWA)	1	36	3	8%	10	28%	16	44%	27	75%
	3	36	0	0%	0	0%	15	42%	16	44%
	6	36	10	28%	5	14%	21	58%	17	47%
		108	13	12%	15	14%	52	48%	60	56%

Figure 1. Two-year old trees at the beginning (top) and end (bottom) of the study. After 17 months, many of these young seedlings were still living and had supported multiple generations of BWA.

Figure 2. Three-year old trees at the beginning (top) and at the end (bottom) of the study.

Figure 3. Infested 3-year old tree. BWA egg masses can be observed along the branches and stem. Terminal death has occurred on some branches.

Figure 4. Infested bud. Gouting is apparent and there are multiple egg masses present.

Figure 5. Close-up of an infested branch. Egg clutches overlap and are found at every leaf cushion and along the branch toward the bud. In the lower left quadrant of the photograph, settled neosistentes can be observed at the base of the needles. Multiple generations were observed on the branches, with old egg masses underneath current, active ones.

Figure 6. Experimental design for the Study II, Phytotron.

Figure 7. 2-year old seedling exhibiting gouting and stem deformity at first node where BWA have settled and are feeding.

Figure 8. Base of an infested 6-year old tree. Gouting is apparent at the base and at the first internode where the BWA have settled and are feeding.

CHAPTER 4. TWO NOVEL TECHNIQUES TO SCREEN *ABIES* SEEDLINGS FOR RESISTANCE TO THE BALSAM WOOLLY ADELGID, *ADELGES PICEAE*[1]

Abstract

Since its introduction into the Southern Appalachians in the 1950s, the balsam woolly adelgid, *Adelges piceae*, has devastated native populations of Fraser fir, *Abies fraseri*, and has become a major pest in Christmas tree plantations requiring expensive chemical treatments. *Adelges piceae*-resistant Fraser fir trees would lessen costs for the Christmas tree industry and assist in the restoration of native stands. Resistance screening is an important step in this process and four studies directed toward the development of time- and cost-efficient techniques for screening are reported here. In the first study, three methods to artificially infest seedlings of different ages were evaluated in a shade-covered greenhouse. Two-year-old seedlings had much lower infestation levels than 7-year-old seedlings. Placing infested bark at the base of the seedling was less effective than tying infested bark to the seedling or suspending infested bolts above the seedling. Although the two latter techniques resulted in similar densities on the seedlings, they each have positive and negative considerations. Attaching bark to uninfested trees is effective but very time consuming. The suspended bolt method mimics natural infestation and is more economical than attaching bark, but care must be taken to ensure an even distribution of crawlers falling onto the seedlings. The second study focused on the density and distribution of crawlers falling from

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suspended bolts onto paper gridded into 7.6 x 7.6 cm cells. Crawler density in a 30 cm band under and to each side of the suspended bolt ranged from 400 to over 3000 crawlers per cell (1 to 55 crawlers per cm^2). In the third study, excised branches from 4-year-old *A. fraseri* and *Abies vetchii* seedlings were artificially infested with *A. piceae* to determine whether this technique may be useful for early resistance screening. The excised *A. fraseri* branches supported complete adelgid development (crawler to egg-laying adult), and very little adelgid development occurred on *A. vetchii* branches. The fourth study compared infestation levels and gouting response on excised versus intact branches of 4-year-old *A. fraseri* seedlings from three different seed sources, and excised branches from 4-year-old and 25-year-old trees. There were no differences in infestation levels between excised versus intact branches nor in very young versus mature trees; a gouting response was observed only on intact branches.

Introduction

The balsam woolly adelgid, *Adelges piceae* Ratz., (Hemiptera: Adelgidae), a tiny, piercing-sucking insect specific to the genus *Abies*, is a pest native to central Europe that has been introduced to North America. Since its introduction into the Southern Appalachians in 1955 (Amman 1966), *A. piceae* has contributed to the decline of Fraser fir (*Abies fraseri* [Pursh] Poir.) throughout its native range, and has become a major pest in Fraser fir Christmas tree plantations. There are 50 million Fraser fir trees growing on over 10,000 hectacres in North Carolina alone, providing annual cash receipts of well over $100 million to the Christmas tree industry (Sidebottom 2008). Chemical insecticides are the only effective means for controlling this pest and treatments exceed $1.5 million per year (Potter et al. 2005). The frequent need to spray plantations for *A. piceae*, in addition to being expensive for growers, minimizes the effectiveness of integrated pest management (IPM) practices. Insecticides reduce populations of natural enemies, thus, the populations of otherwise minor pests (e.g., spruce spider mites [*Oligonychus ununguis*]) increase dramatically (Sidebottom 2009), requiring more chemical input into the system.

In North America, *A. piceae* reproduces through parthenogenesis and completes two or more generations per year (Balch 1952, Arthur and Hain 1984). Life stages consist of eggs, three instars and adults; the early phase of the first instar (crawler) is the only motile stage. The crawlers are primarily wind or gravity dispersed. Feeding sites are chosen for accessibility to parenchyma cells (Balch 1952). The crawler inserts its stylets into the bark and the adelgid remains in that location for the remainder of its life. White, ribbon-like threads are secreted from wax glands (Balch 1952), eventually covering the adelgid and giving the appearance of cottony or woolly tufts. This ‘woolly mass’ is the most obvious sign of infestation.

Susceptibility to *A. piceae* within the genus *Abies* varies considerably. Firs native to North America, e.g., subalpine fir, *Abies lasiocarpa* (Hook) Nutt., balsam fir, *Abies balsamea* (Linn.) Mill., and Fraser fir, are highly susceptible and firs native to central Europe, e.g., European silver fir, *Abies alba* Mill., are able to tolerate infestation for years with relatively few ill effects (Varty 1956; Mitchell 1966). Some Asian species, e.g., Veitch fir, *Abies veitchii* Lindl., appear to be almost immune to attack (Hall et al. 1971). Host resistance mechanisms, while not yet fully understood, appear to include a thick outer bark (Balch 1952; Varty 1956), the rapid formation of secondary periderm around the feeding site

(Amman and Fedde 1971, Hollingsworth and Hain 1992), and an accumulation (constitutive or induced) of chemicals (e.g., monoterpenes or sesquiterpenoids) at the site of attack that may interfere with adelgid development (Hain et al. 1991; Balakshin et al. 2005). Mature trees in natural stands appear to be more susceptible to *A. piceae* attack (Mitchell 1966), although saplings (5.1 cm diameter at breast height [dbh]) of various fir species have been observed with infestations (Harris 1973) and even the youngest trees in Fraser fir Christmas tree plantations are vulnerable to infestation.

Although *A. piceae* infestations often result in the death of susceptible host trees, there are reports of full recovery from severe infestations in some species, e.g., noble and white firs, *Abies procera* Rehd. and *Abies concolor* (Gord. et Glend.) Lindl., respectively, (Mitchell 1966), European silver fir (Kloft 1957), West Virginia Canaan fir, *Abies balsamea* L. Mill. var. *phanerolepis* Fern., and even Fraser fir (Hain et al. 1991). This, coupled with the presence of numerous old-growth survivor Fraser fir in the high elevation forests of the Great Smoky Mountains National Park (Kloster 2001; Johnson et al. 2005), suggests there may be some element of resistance or tolerance within Fraser fir. The predominant hypothesis under evaluation is that there is a genetic basis for host resistance in fir.

The utilization of genetically resistant Fraser fir planting stock for Christmas tree production would be a relatively inexpensive solution to a difficult pest problem and minimize adverse effects from the pest and related management strategies. In addition, resistant trees could be utilized in the restoration of native Fraser fir stands, which exist only as mountain top populations in Virginia, Tennessee, and North Carolina. Over 95% of mature trees were killed after the initial infestation of *A. piceae* in the 1950s and, although many of these stands have recovered from the initial wave of devastation through regeneration from wild seed, the endemic population is shrinking and their long-term prospects are uncertain. Outplantings of *A. piceae*-resistant Fraser fir could increase the likelihood of the continued survival of the ecologically and recreationally important ecosystem.

Resistance screening is an important step in the process of discovering and breeding resistance and this requires an effective, time- and cost-efficient technique for infesting large numbers of trees at the same time, under the same conditions. The most commonly reported infestation technique involves cutting bark pieces with egg masses from infested field trees and attaching the bark pieces to the stems of uninfested trees or seedlings (with the egg masses facing the new host) (Tunnock and Rudinsky 1959; Carrow and Graham 1968; Carrow 1971; Hollingsworth and Hain 1994a). This is a reliable, yet time-consuming, technique. Prior artificial infestations of Fraser fir seedlings in a laboratory or greenhouse setting have met with limited success, possibly because temperatures were too high or too constant, or because too few eggs and crawlers were utilized in the studies (Hollingsworth 1990; Hollingsworth and Hain 1994a & b). Balch (1952) suggests that 30 eggs may produce 1 adult, 500 eggs 10 adults, and 1200 eggs may result in a moderately heavy infestation. Settled neosistentes (first instar larvae) suffer high mortality; most crawlers will settle and enter into a period of rest, but most never come out of that stage to continue their development. Optimum temperatures reported for maximizing development and minimizing mortality on *A. grandis* are cycles between 13ºC and 24ºC (Atkins 1972). Our preliminary studies found that both constant (17ºC) and ramping (13-24ºC) temperatures and attaching bark with 20 to 180 eggs resulted in successful infestation of 1- to 6-year-old Fraser fir seedlings (Newton, unpublished data). These studies proved that Fraser fir seedlings of various ages could be successfully infested artificially with *A. piceae*, but the amount of time required to cut and attach bark pieces to each seedling was felt to be excessive for utilization in resistance screening trials. Thus, a new technique was developed that mimics natural infestation and reduces implementation time. This new technique involves suspending bolts of heavily infested Fraser fir over seedlings, allowing the hatching crawlers to fall onto the uninfested trees below. This technique was tested on 2- and 7-year-old trees and is described and reported in Studies I and II below.

Host resistance screening trails are often accomplished with young trees grown from seed (Nielsen et al. 2002, Alfaro et al. 2008) or as clonal material (Foster and Anderson 1989). These approaches require three or more years before the material can be utilized in a screening trial. The ability to screen trees of any age, either from existing grafted clone banks, plantations, or natural stands, would enable researchers to test for resistance using a wider range of genetic material in a more time-efficient manner. Fraser fir is well known for maintaining freshness after harvest and exhibits excellent needle retention even 35 days post-harvest (Bates et al. 2004). With this in mind, a new technique for artificially infesting excised branch tips with *A. piceae* has been developed and is being tested for its potential to serve as a means to pre-screen Fraser fir trees for resistance trials. The early work conducted with excised branches is reported in Studies III and IV.

Methods and Materials

Study I: Comparison of three artificial infestation techniques.

Two ages of Fraser fir were utilized, 2- and 7-years, both from the Roan Mountain seed source. The 2-year-old bareroot seedlings were obtained from the North Carolina Division of Forest Resources (NC DFR) in May 2006 and transplanted into 7.6 x 7.6 x 22.9 cm tree pots. The 7-year-old trees were 3-1 Fraser fir seedlings obtained from the NC DFR in 2003, transplanted into 11.4 liter pots, and grown at the North Carolina State University Horticultural Field Lab in Raleigh until June 2006, at which time they were transported to Laurel Springs. Potting medium consisted of mulched pine bark. The trees were fertilized with Osmocote 19-5-8 slow-release formula; nitrogen sources in this formula are provided equally as nitrate, ammonium nitrate, and urea. An impact-head sprinkler system was installed in the greenhouse to provide irrigation and the screen over the frame allowed rain as well.

The study was conducted at the North Carolina Department of Agriculture Upper Mountain Research Station in Laurel Springs, North Carolina, in a shade-covered 6.1 m x 12.2 m greenhouse. The greenhouse was divided into four blocks each containing 6 treatment plots. Each plot measured 0.9 m x 1.2 m x 1.2 m high, with the perimeter of each surrounded by organza cloth supported by 1.3 cm diameter PVC pipe to provide a barrier to prevent dispersal of crawlers between treatments. Each plot contained 12 potted Fraser fir, consisting of an equal number of 2- and 7-year-old seedlings arranged so that their crowns were level. One seedling of each age served as an uninfested control for two of the treatments. Seasonal effect was examined by infesting three of the treatment plots in the summer and three in the fall, with each plot receiving one of three infestation techniques.

Two of the three infestation techniques used pieces of bark containing *A. piceae* egg masses taken from infested *A. fraseri* trees cut from an abandoned Christmas tree plantation in Avery County. The bark pieces averaged 10 eggs per woolly mass. Sufficient bark pieces were attached to total 50 egg masses on the 7-year-old trees and 10 egg masses on the 2-year-old trees. One treatment (Attach Bark) consisted of attaching the bark pieces along the stems of the uninfested seedlings with horticultural ties. Another (Place Bark) placed the infested bark pieces at the base of the uninfested seedlings. The third treatment (Suspend Bolt) suspended 1.2 meter-long bolts of BWA-infested Fraser fir over uninfested seedlings so that hatching crawlers would fall off the bolt and onto the seedlings below.

The infestation treatments were applied to the seedlings three times, for a duration of one week each time. During the summer season the fir seedlings were infested on 7/5/2006, 7/12/06, and 7/19/06; during the fall season the seedlings were infested on 9/20/06, 9/27/06, and 10/11/06. The total number of eggs using the bark pieces was 300 and 1500, respectively, for each 2- and 7-year-old seedling. For the Suspend Bolt treatment, one bolt was suspended on each of the three dates, the first time across the center of the treatment plot, the second diagonally from corner to corner, and the third diagonally in the opposite direction.

The infestations were allowed to develop through winter dormancy and into the spring. The greenhouse was covered with white plastic between December and April. The study was dismantled in June 2007; each tree was cut at the base, placed individually in polyethylene bags, transported to North Carolina State University, and stored in a cooler (at 4ºC) pending assessment. Each tree was assessed and measured variables included height, diameter (2.5 cm above base), and the total number of *A. piceae* woolly masses per tree. There were 288 trees utilized in this study, including 256 treatment trees and 32 controls. The Attach Bark and Place Bark treatments included untreated controls; the Suspend Bolt treatment did not include controls. Because of a treatment application problem, all 12 trees from Block 4, Treatment 2 (Place Bark), Season 2 (fall) were deleted from the analysis. Another 101 trees were lost through death or mold problems in storage. A total of 157 treatment trees and 18 controls were analyzed.

Study II: Density distribution of crawlers dropping from bolts.

In July 2007, logs from heavily infested Fraser fir trees in an abandoned Christmas tree plantation in Ashe County were cut and transported back to North Carolina State University. The following day, two logs (10.2 cm in diameter and 1.2 m or 1.5 m length) were suspended over paper gridded into 7.6 x 7.6 cm cells, and sprayed with Tanglefoot® to trap the falling crawlers. The logs were suspended 0.91 m above the grids. One log was suspended in a relatively windless environment in the laboratory and one was suspended in an air-conditioned space within a greenhouse.

The logs were left suspended for five days. On the sixth day, each grid was covered with clear transparencies and the grids cut into squares (7.6 x 7.6 cm cells) for assessment. A subsampling scheme was developed that consisted of selecting cells representative of all levels of crawler densities and counting the crawlers under a microscope. A total of 18 samples were counted which had crawler densities of 24, 51, 73, 115, 243, 330, 418, 550, …, 3035). For the remaining cells (174 and 240, respectively, for the 2 logs), crawler densities were estimated by finding the most similar cell within the counted samples and using that known quantity to approximate the number of crawlers.

Study III: Infestation of excised Fraser fir and Veitch fir branches from 4-year-old seedlings.

In May 2007, four branches were taken from each of three trees of Fraser and Veitch fir. The excised branches (cuttings) were placed individually in glass tubes filled with wet sand. Bark discs (8-10 egg masses, each with 10-15 eggs) were taken from *A. piceae*-infested Fraser fir and attached to each branch with wire. The samples were split into two groups (randomized in trays with 6 cuttings per species per group) and stored in two incubators, both held at a constant temperature of 17ºC. Fluorescent grow lights were placed in each incubator and a timer installed to provide light for the cuttings (long days: 16 hours on, 8 hours off). Humidity was not controlled. The cuttings were watered twice a week and assessed for *A. piceae* development 4, 7, and 9 weeks following infestation. Measured variables included the numbers of crawlers, settled instars, adults, and eggs. The study included 12 branches per species.

Study IV: Infestation of intact and excised branches of 4-year-old Fraser fir seedlings and excised branches of 25-year-old Fraser fir trees.

This study included 4-year-old Fraser fir from three seed sources (Richland Balsam, Mount Mitchell, and Roan Mountain) and 25-year-old Fraser fir (Roan Mountain seed source). The seedlings were grown in a greenhouse in Raleigh, NC, and the mature trees were growing in a plantation in Ashe County, NC. The design included five seedlings per seed source for the 4-year-old trees and branches from five mature (25-year-old) trees. In September 2007, 3 branches were cut from each 4-year-old seedling and the mature field trees. The excised branches placed into sand-filled plastic centrifuge tubes, randomized in trays, and placed into plastic tubs. To keep the cuttings hydrated, the tubes and trays had small holes cut in the bottom and water was added to completely saturate the sand. The trees were watered three times per week and water was added to the tub with cuttings three times per week. Water was also squirted into each centrifuge tube to keep the sand moist. The seedlings and excised branches were held at a constant temperature of 17ºC. Humidity was not controlled and artificial lighting (long days) was provided with fluorescent grow lights. Excised branches from each age class and two to three intact branches from the 4-year-old seedling were infested with *A. piceae*. Bark discs (with 8-10 egg masses, each with 10-15 eggs and a few crawlers) were taken from *A. piceae*-infested Fraser fir and attached to the branches with wire. The discs were attached either at the branch node or at the tip of the branch (near the bud). Infestation levels were assessed after ten weeks, represented by the numbers of settled first instars, adults, and eggs. The condition (health or quality) of each branch specimen was assessed (poor [chlorotic or brown], fair [somewhat chlorotic], or good [deep green]) and whether or not gouting (swollen areas at the site of infestation) had taken place (yes/no). Also noted was the location that the crawlers settled in relation to the placement of the infested bark discs. The study included a total of 45 excised and 39 intact branches from the 4-year-old seedlings and 15 excised branches from the mature trees.

Statistical Analysis

Responses in each of the four studies were tested for normality (Shapiro-Wilk) using the Univariate procedure (PROC UNIVARIATE) of SAS version 9.1 (SAS Institute 2003). Transformations were applied as necessary to bring the distributions closer to normality and to make the variances more homogeneous. For each study, an analysis of variance was performed using the General Linear Model procedure (PROC GLM) and, where appropriate, pairwise comparisons were analyzed by the Tukey-Kramer Multiple Comparison Test (SAS Institute 2003). Data are presented as least squared means ± standard error; if a transformation was utilized, the results were back-transformed for reporting. Details specific to each study are provided as follows:

Study I: The GLM procedure incorporated the following effects: block, infestation technique, age, season, technique x age interaction, and season x age interaction. Block was considered a random effect; all others were fixed. A logarithmic transformation (log * (1 + x)) was applied to the response (number of woolly masses [adults with egg clutches]). An additional ANOVA was performed comparing the untreated controls with the treated trees from the Attach Bark and Place Bark treatments.

Study II. Data for each cell were entered into a spreadsheet and contour plots showing crawler density were created utilizing SigmaPlot for Windows Version 10.0 (© 2006 Systat Software, Inc.). The two repetitions were assessed separately.

Study III. The main effect was species (Fraser fir versus Veitch fir).

Study IV. Two analyses were performed, the first (to compare excised and intact branches) with the 4-year-old trees from different seed sources. The main effects were form (intact versus excised branches) and seed source (Richland Balsam, Mount Mitchell, and Roan Mountain). The first instar counts were transformed (square root (x)) for analysis and back-transformed for reporting. The second analysis (to compare young seedlings with mature trees) was performed with excised branches from the 4-year-old seedlings (Roan Mountain only) and the 25-year-old trees (also Roan Mountain). The main effect was age.

Results

Study I: Comparison of three artificial infestation techniques.

Attaching infested bark pieces and suspending infested bolts were equally effective, resulting in higher numbers of *A. piceae* infestations than placing bark at the base of tree (Table 1). All treatment effects included in the general linear model were significant ($p < 0.006$) (Table 2). There were significant infestation technique by age ($p < .001$) and infestation technique by season ($p < .0221$) interactions. The data may best be understood by examining the means for these interactions (Table 3). The 2-year-old seedlings averaged < 1 woolly mass per seedling and the 7-year-old trees averaged 30.6 woolly masses per tree. Placing the bark at the base of the tree was significantly less effective and resulted in far fewer egg masses than the other two methods. The means for the older trees ranged from 6.5 for the Place Bark treatment to 57.3 and 80.5 woolly masses, respectively, for the Attach Bark and Suspend Bolt methods. The 2-year-old seedlings exhibited a mean height of 14.5 cm (± 0.69) and diameter of 3.11 mm (± 0.34), whereas the measurements for the 7-year-old trees were 65.3 cm (± 0.91) and 16 mm (± 0.45), respectively. The older trees were infested with five times more eggs/crawlers than the smaller trees; however, at the end of the test some of the larger trees averaged more than fifty times the number of adult adelgids.

Infesting in the fall resulted in an average of 8.6 adults per tree versus 5.4 adults on trees infested during the summer. For both seasons, placing bark at the base of the seedling resulted in fewer adult adelgids on the seedling.

The standard error for the Suspend Bolt treatment was similar to those of the other two treatments (Tables 1 and 3). However, because the Suspend Bolt treatment is novel and offers less control over the number of adelgids applied, a look at the raw data is warranted. The number of woolly masses on 7-year-old trees from the Attach Bark treatment ranged from 0 to 183. The number from the Suspend Bolt treatment ranged, with one exception, from 33 to 219. The exception was one tree with a total of 445 woolly masses; this outlier was removed from the dataset and was not included in the analysis reported above.

Study II: Density distribution of crawlers dropping from bolts.

Over 82,000 crawlers dropped from each of the bolts suspended over the paper grids in these studies. Over the entire grid, crawler abundance within each cell (58 cm^2) ranged from 0 to over 3000 per cell or 0 to 50 crawlers per cm^2 (Figure 1). Just under the bolt, crawler abundance ranged from 500 to over 3000 per cell (9 to 51 crawlers per cm^2). The highest quantities of crawlers were found directly beneath the suspended logs, but the crawlers also drifted or fanned out away from the bolt as they fell. Hundreds of crawlers fell into each cell up to 10 cm on either side of the bolt, with the numbers rapidly dwindling in the cells approaching the opposite edges of the grid. Five hundred to one thousand eggs or crawlers have been suggested to achieve spot infestations on mature fir trees (Hollingsworth 1990) and successful infestations of Fraser fir seedlings have been achieved with 100 eggs (by attaching infested bark pieces) (Newton, unpublished data). Thus, a heavily infested bolt 10 cm in diameter could be expected to produce enough crawlers to infest fir seedlings or branches within a 30 cm diameter band underneath and on either side of the suspended bolt.

Study III: Infestation of excised branches from 4-year-old Fraser fir and Veitch fir seedlings.

After 4 weeks the excised branches from both species were in good condition. Settled neosistentes were found on almost all of the branches close to the attached bark disc. After 7 weeks, the cuttings were still healthy and 9 Fraser fir branches contained adults with eggs. No *A. piceae* development was observed on the Veitch fir branches. After 9 weeks, the excised branches were still relatively healthy and the 10 Fraser fir branches had adults (total of 47) with eggs (total of 686). Fecundity ranged from 0-53 eggs per adult. Egg masses were located primarily at the base of buds, but also under old bud scales and at the base of needles along the cutting. On Veitch fir, only one branch had any sign of *A. piceae* development, with one adult (no eggs) and one third instar. There were significant differences between species in all measured variables (Table 4). By the end of the 9 weeks, most of the Veitch fir cuttings had broken bud, while the Fraser fir buds had not yet elongated.

Study IV: Artificial infestation of intact and excised branches of 4-year-old Fraser fir seedlings and excised branches of 25-year-old Fraser fir.

After 10 weeks, intact branches from all 15 seedlings were in good condition. Of the excised branches, 30 of the 45 branches taken from 4-year-old seedlings were in good condition; only 2 of the 15 branches from mature trees were in fair condition and the remaining 13 branches were in poor condition. In comparing the intact and excised branches from the seedlings, all intact branches and all but one of the excised branches contained settled first instars (Figure 2). Three (3) intact and 9 excised branches produced adults; 2 of the excised branches (both from the Roan Mountain seed source) produced adults with eggs. Both source and form were significant for measured variables (Table 5). Trees from the Roan Mountain seed source produced significantly more adults than Mount Mitchell trees (Table 6). There were no source x form interactions and source rankings for each measured variable were the same for intact and excised branches. There were more settled instars on the excised branches than on the intact branches. The intact branches responded to the presence of settled *A. piceae* instars in the form of gouting (swelling) at the site of infestation and the excised branches exhibited no gouting response. Settled instars were found primarily at the base of buds, regardless of whether the infested bark discs had been placed near the bud or at the branch node.

A comparison between excised branches from 4-year-old seedlings and 25-year-old trees revealed no significant differences in the numbers of settled neosistentes or adults, but at the end of the trial, the material from the mature trees was in significantly poorer condition (Table 7). No gouting was observed on any of the excised branches.

Discussion

Attaching bark and suspending infested bolts (Study I) are both effective techniques for artificially infesting fir trees with *A. piceae*. It is important to note that in this study each treatment was applied on three separate occasions to ensure a high number of crawlers. Thus, with the attach bark technique, each 7-year-old tree had 500 eggs applied three times for a total of 1500 eggs, and each 2-year-old tree had 100 eggs applied three times for a total of 300 eggs. Ideally, one application would provide sufficient numbers of adelgids to effect an infestation, but this is often difficult to achieve with the attach bark technique. Because the bark pieces must be very small to allow for a connection between the bark and the uninfested tree, there are limitations in the number of egg masses that can be introduced at one time. Additionally, the relative health of each egg clutch on infested source Fraser fir trees varies. With the exception of the earliest spring generation, there are multiple overlapping generations of *A. piceae* found on infested trees and it is difficult to gauge how healthy a given population will be when the pieces are cut and applied to uninfested material.

The age effect noted in the infestation techniques study (I) was not surprising. The most obvious explanation lies in the size differences between the two age classes. Fewer eggs were applied to the younger trees and interception size may influence the Suspend Bolt technique. However, young seedlings in native stands of Fraser fir often appear resistant to *A. piceae* infestation (Hain 1988) and, although 1- and 2-year-old Fraser fir can be artificially infested with *A. piceae* (Newton, unpublished data), it is more difficult to accomplish than with older trees. Resistance in these young seedlings may include both morphological and chemical mechanisms. *Adelges piceae* frequently settles underneath old bud scales, on lenticels, in cracks and crevices in bark (Balch 1952) and at the base of buds. In very young seedlings, the stems are smooth and there are few internodes. Attaching bark pieces is difficult because physical damage can occur to the delicate stems in the process of handling the bark, and there is less area for the bark to contact. Thus, crawlers that emerge may simply fall off the bark piece rather than crawling onto the seedling, and those that crawl from the bark pieces onto the tree may crawl off of it entirely. Additionally, young trees may produce chemicals that inhibit adelgid development.

A suspended bolt can provide thousands of crawlers. Bark pieces dry out in a matter of days whereas the bolt provides suitable habitat for continued adelgid development for up to two or three weeks after the tree is cut. Hence, crawlers are continually produced from a bolt over a longer period of time than from a piece of bark. In this study, fresh bolts were suspended weekly (three weeks) over the treatment plots. Varying the placement of each bolt during the treatment period ensured a relatively even distribution of falling crawlers onto the trees below, and the successful infestation of trees throughout each plot provides evidence that this was effective. Cutting bark discs and attaching them to 40 uninfested trees took approximately 4 to 5 hours with two experienced technicians. Suspending the infested bolt was a simple process accomplished by tying each end to a frame and it took approximately 30 minutes with two technicians to suspend four bolts over 4 sets of 10 seedlings. The suspended bolt technique provides a method by which entire tables of uninfested seedlings or cuttings can be exposed to *A. piceae* with minimal time and effort, thus increasing the numbers of seedlings that can be utilized in future host resistance studies and maximizing the value of material brought in from the field. This technique may be adapted to screening trials both large and small, provided a sufficient quantity of infested material is available.

The numbers of *A. piceae* crawlers that fall from suspended bolts of heavily infested Fraser fir, as indicated in Study II, are more than sufficient to produce an infestation in susceptible fir trees. Because crawler densities were highest within about a 30 cm diameter band underneath the suspended bolt, it is advisable to place the bolts about 30 cm apart to ensure good coverage. Additionally, because the infestation levels on a given log will naturally be greater in some areas than others, the numbers of crawlers falling onto the uninfested material will vary. Evenly infested seedlings or trees is a desirable component within host resistance screening trials and care should be taken to ensure an even distribution pattern when utilizing the suspended bolt technique. Further, appropriate replication and randomization of genetic entities (species, families, clones, etc.) in resistance screening trials would also ameliorate variation problems. Improvements to the method could include fans to circulate air, thus widening the natural drift in the dispersal of crawlers, and rotating or moving the bolts to increase coverage and reduce over-application of crawlers under ‘hot spots’ of infestation. Even with the potential for variance in the numbers of falling crawlers, the suspend bolt technique appears to be more effective (on a tree-by-tree basis) for infesting the older (7-year-old) trees, than the attach bark technique. With the attach bark technique, although great care was taken to attach bark pieces with an equal number of *A. piceae* egg masses to each tree, there were still a number of trees with zero woolly masses at the end of the study.

The early attempts to infest excised branches with *A. piceae* and maintain health of those branches for a period of time sufficient to allow development and reproduction were successful (Studies III and IV), and this technique is promising on a number of levels. It is encouraging that in each case, within a few weeks an entire generation of *A. piceae* was produced. In Study III, the vast majority of Fraser fir (10 of 12 cuttings) produced adults with eggs. The difference in infestation levels between Fraser and Veitch fir was expected because Veitch fir is known to exhibit marked resistance to *A. piceae* infestation (Mitchell 1966; Hall et al. 1971).

The results from Study IV (intact versus excised branches) provide some interesting insights, particularly the lack of gout production at the infestation site of excised branches despite their higher frequency of settled instars. Gouting, the production of abnormal (larger and in greater quantities than normal tissue) parenchyma cells at the site of infestation, is a response to the presence of *A. piceae* by many fir species (e.g., Fraser fir, balsam fir). There appears to be a growth stimulating substance in *A. piceae* saliva that prompts this response in susceptible trees (Balch 1952). The saliva appears to contain a substance that induces similar effects as the auxin indolyl-3-acetic acid and disturbs the normal hormonal balance within the tree (Balch, Clark, and Bonga 1964). The absence of gouting in the excised branches may call into question the validity of utilizing this method as an early resistance screening tool because here there appears to be a clear difference between the intact and excised branches. In an ideal situation, there would be no difference between the two. Adelgids would find the excised branches (cuttings) no more or less attractive than intact tissue, would develop at the same rate and level of fecundity, and the cuttings would respond to the presence of adelgids as would a tree with roots. However, this ideal is not likely possible. Excised branches are placed under physiological stress and the absence of roots alters normal active growth, at least to the extent that the gouting response is inhibited. It appears that the roots are involved in the production of gout, but this is not clearly understood and has not been researched. Although it may be unreasonable to expect excised branch tips to serve as perfect surrogates for whole trees, this technique may be useful in helping to sort out the mechanisms of host resistance. Constitutive defenses may be present in excised branches while induced defenses may be slowed or not expressed at all.

Conclusion

The suspended bolt technique mimics natural dispersal and appears to be effective in producing *A. piceae* infestations in a time-efficient manner. This technique can be utilized for large or small studies and can be adapted for infestations of other wind-dispersed insects. Bolts of heavily infested Fraser fir are capable of producing tens of thousands of *A. piceae* crawlers and could be hung for a period of time over one set of trees and moved to another set of trees, thus economizing the number of bolts required for infestations. However, care should be taken to ensure an even distribution of crawlers. The time-honored technique of tying individual pieces of infested bark to trees is effective, although time consuming, and can be utilized in small studies or studies requiring infestation of specific areas of a tree rather than the entire tree.

Excised branches from two fir species spanning the range of resistance to *A. piceae* were successfully infested and adults with eggs were produced on many of the Fraser fir specimens and almost none of the Veitch fir specimens. These results are consistent with what would be expected from infestation of a highly susceptible and a highly resistant species of fir. Infestation of intact and excised branches from trees of three Fraser fir seed sources revealed no differences between the two forms in the numbers of adult adelgids that had developed on each branch by the end of the study. The excised branches appear incapable of producing a gouting response to the presence of the adelgid, a marked difference from intact branches; although this is an indicator that excised branches cannot serve as perfect surrogates for intact branches, it may help to further our understanding of defense mechanisms and physiological responses to the feeding of *A. piceae*. Lastly, excised branches taken from mature Fraser fir trees were shown to be capable of being infested, although the quality of the older specimens declined more rapidly than the younger material. The excised branch technique requires further study, but is promising for providing a nondestructive technique for initial host resistance screening trials.

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Table 1. (Study I) Lsmean (± SE) number of woolly masses (NoWM) per tree, by infestation technique.

Infestation Technique	**Number of woolly masses (LS mean ± SE)**
Attach bark to bole of tree	9.85^{a} (0.14)
Place bark at base of tree	2.86^{b} (0.13)
Suspend bolt over trees	10.53^{a} (0.13)

Data were transformed prior to the analysis (log (1 + NoWM)) and back-transformed for reporting. Means that share the same superscript letter are not significantly different from one another.

Table 2. (Study I). Analysis of variance for the mean (lsmean) number of *A. piceae* woolly masses per tree.

Source of variation	**df**	**Sum of Squares**	**F-value**	**Pr > F**
Model	11	347.25	44.22	< .0001
Block	3	3.52*	1.17	.1817
Season	1	5.88*	8.24	.0047
Infestation Technique	2	35.80*	25.07	< .0001
Age	1	267.06*	374.06	< .0001
Infestation Technique x Age	2	39.31*	27.53	< .0001
Infestation Technique x Season	2	5.59*	3.91	.0221
Error	144	102.81		

*Type III Sum of Squares

Data were transformed (log (1 + x)) prior to the analysis.

Table 3. (Study I) Lsmean (± SE) number of woolly masses per tree, by age and season.

	Age		**Season**	
Infestation Techniques	**2 yr**	**7 yr**	**Summer**	**Fall**
Attach Bark	1.02^{a} (0.18)	57.27^{a} (0.23)	7.50^{a} (0.20)	13.63^{a} (0.20)
Place Bark	0.99^{a} (0.17)	6.47^{b} (0.20)	1.59^{b} (0.18)	4.76^{b} (0.20)
Suspend Bark	0.84^{a} (0.14)	71.28^{a} (0.22)	11.06^{a} (0.18)	10.02^{a} (0.17)
Across treatments	0.95 (0.09)	30.58 (0.12)	5.42 (0.10)	8.58 (0.11)

Data were transformed prior to the analysis (log (1 + NoWM)) and back-transformed for reporting. Means within a column that share the same superscript letter are not significantly different from one another.

Table 4. (Study III) Least squared means (± SE) for excised branches of Fraser and Veitch fir.

	Crawlers	**Settled Instars**	**Adults**	**Eggs**
Species				
Fraser fir	1.75^{a} (0.49)	3.67^{a} (0.83)	3.92^{a} (0.86)	57.59^{a} (13.64)
Veitch fir	0.00^{b} (0.49)	0.75^{b} (0.83)	0.08^{b} (0.86)	0.00^{b} (13.64)

Means within a column that share the same superscript letter are not significantly different from one another.

Table 5. (Study IV, 4-year-old seedlings only; intact versus excised branches). ANOVA p-values of the numbers of settled first instars, presence/absence of adults, presence/absence of gouting, and an estimate of condition.

Source of variation	**df**	**Settled instars Pr > F**	**Adults Pr > F**	**Gouting Pr > F**	**Condition Pr > F**
Model	5	.0097	.0921	.0063	.0008
Source	2	.4119	.0354	.2068	.4298
Form	1	.0010	.2495	.0007	<.0001
Source*Form	2	.3207	.5464	.2068	.4298
Error	78				

The main effects are Source (Richland Balsam, Mount Mitchell, Roan Mountain) and Form (intact versus excised branches). Condition refers to the quality of the branch at the end of the study.

Table 6. (Study IV, 4-year-old seedlings only) Least squared means (± SE) for the main effects Source (Richland Balsam, Mount Mitchell, Roan Mountain) and Form (intact branch versus cutting).

	Settled Instars	**Adults**	**Gouting**	**Condition**
Source				
Richland Balsam	14.76^{a} (0.05)	0.07^{ab} (0.06)	0.04^{a} (0.05)	2.77^{a} (0.13)
Mount Mitchell	14.43^{a} (0.05)	0.03^{a} (0.06)	0.17^{a} (0.05)	2.53^{a} (0.13)
Roan Mountain	17.74^{a} (0.05)	0.24^{b} (0.06)	0.12^{a} (0.05)	2.70^{a} (0.13)
Form				
Intact	12.09^{a} (0.04)	0.08^{a} (0.05)	0.21^{a} (0.04)	3.00^{a} (0.11)
Excised	19.58^{b} (0.04)	0.16^{a} (0.05)	0.00^{b} (0.04)	2.33^{b} (0.10)

The settled first instar counts were transformed (square root (x)) prior to analysis and backtransformed for reporting. The variables ‘adults’ and ‘gouting’ are expressed binomially (present=1, absent =0). Condition was expressed as poor (=1), fair (=2), or good (=3). Means that share the same superscript letter are not significantly different from one another.

Table 7. (Study IV) Least squared means (± SE) for excised branches of 4-year-old Roan Mountain seedlings (N=15) and mature trees (N=15).

	Settled Instars	**Adults**	**Condition**
Age			
4-years	24.9^{a} (2.9)	0.33^{a} (0.12)	2.4^{a} (0.18)
25-years	26.3^{a} (2.9)	0.20^{a} (0.12)	1.2^{b} (0.18)

The variable ‘Adults’ is expressed binomially (present=1, absent=0). Condition is expressed as poor (=1), fair (=2), or good (=3). Means within a column that share the same superscript letter are not significantly different from one another.

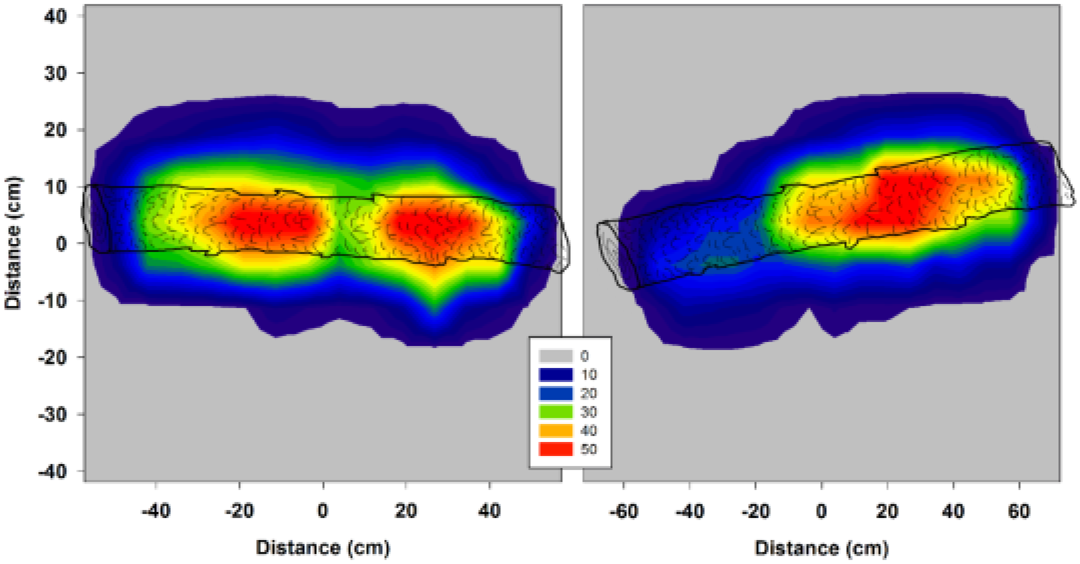

Figure 1. (Study II) Crawler density and distribution under the two logs. The image on the left reflects crawler densities under the 1.2 m log with little air flow and on the right the 1.5 m log in the air conditioned greenhouse space. The legend reflects the number of crawlers per square centimeter. The crawlers fell in higher densities directly under the logs, but they drifted an additional 10 cm on either side, providing good coverage within a 30 cm area. The differences in densities are a reflection of 'hot spots' of infestation where population densities were very high.

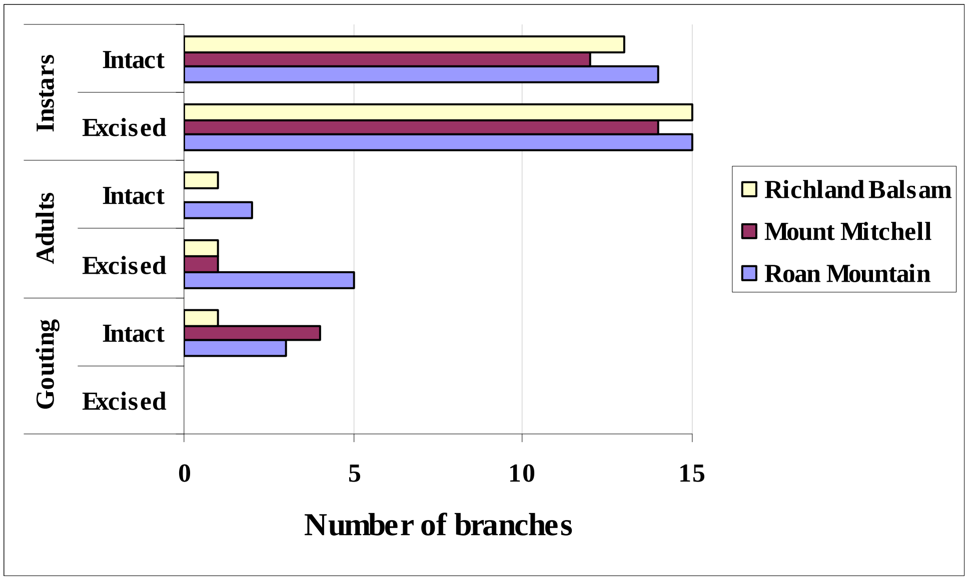

Figure 2. (Study IV) A comparison of intact versus excised branches from 4-year-old Fraser fir seedlings from three different seed sources. This figure presents the number of branches from each branch form (intact or excised) that contained settled first instars or adults, and also the number of branches within each form that responded to the presence of *A. piceae* through gouting (swelling at the site of infestation). Branches from the mature trees are not included here.

CHAPTER 5. HOST RESISTANCE SCREENING FOR BALSAM WOOLLY ADELGID: A COMPARISON OF SEEDLINGS FROM 12 FIR SPECIES

Abstract

Our objective for this study was to screen for resistance across multiple fir species of equal age, grown under the same conditions and infested with BWA collected at one time from a single source, and to observe the responses of both host and insect. We utilized the rain-down method for infesting the trees, suspending infested bolts of Fraser fir above the treatment plots and allowing the crawlers to drop onto the uninfested trees. Crawlers settled at the base of branches, at nodes under old bud scales, on needle cushions along branches or the bole, and at the base of buds (lateral and apical), showing a preference for the base of buds.

Species were categorized into four susceptibility groups (susceptible, tolerant, resistant, and unknown). With a few exceptions, results for each species fell within expectations for *a priori* resistance classifications. Turkish fir, one of the three species without any *a priori* resistance classification, appears to be somewhat resistant to BWA, ranking more often with the resistant and tolerant species in measured responses rather than with the susceptible species. Alternately, Trojan fir ranked most often with susceptible species and exhibited the highest proportion of infested trees and the highest fecundity levels. Turkish fir and Trojan fir showed relatively little gouting in response to BWA feeding. Fraser fir ranked consistently with other susceptible species (e.g., balsam fir) and there were some significant differences noted among responses of the three Fraser fir provenances, e.g., adults and eggs per cm branch and fecundity.

Introduction

The introduced balsam woolly adelgid (BWA; *Adelges piceae* Ratz.) was first reported on Fraser fir (*Abies fraseri* [Pursh] Poiret) in the Southern Appalachians on Mount Mitchell in 1955 (Boyce 1955, Amman 1966). Native stands of Fraser fir experienced severe mortality within the first few years of infestation and in some areas over 90% of mature overstory trees were killed (Hay and Johnson 1980, Wentworth et al. 1989). Fraser fir is restricted to high elevations in eastern Tennessee, western North Carolina and southwest Virginia. Although regeneration in many of the damaged stands has been good, successive cycles of regeneration and mortality may result in decreasing populations over time (Potter et al. 2005, Ragenovich and Mitchell 2006). Fraser fir is one of the most popular Christmas trees in North America and the Christmas tree industry is an important economic resource for mountain communities. The balsam woolly adelgid is a major pest in plantations (the threshold for treatment is one infested tree) and the use of chemical insecticides is currently the only effective means for controlling BWA. Nearly all Fraser fir Christmas trees produced in North Carolina need to be treated one or more times during their 5- to 10-year rotation to prevent or lessen damage caused by this adelgid. Chemical treatments for BWA cost the industry over $1.5 million per year (Potter et al. 2005) and may compromise the effectiveness of integrated pest management (IPM) systems (Sidebottom 2009).

The balsam woolly adelgid is a piercing-sucking insect specific to the genus *Abies*. In the United States, the insect reproduces through parthenogenesis and completes two or more generations per year (Balch 1952; Arthur and Hain 1984). Life stages consist of eggs, three instars and adults; the early phase of the first instar (crawler) is the only motile stage.

Crawlers are dispersed passively via wind, gravity, and phoresy. Feeding sites are chosen for accessibility to parenchyma cells (Balch 1952). The crawler inserts its stylets into the bark and the adelgid remains in that location for the remainder of its life. White, ribbon-like threads are secreted from wax glands (Balch 1952), eventually covering the adelgid and giving the appearance of cottony or woolly tufts. This 'woolly mass' is the most obvious sign of infestation.

Species within the genus *Abies* appear to vary in susceptibility to BWA. Firs native to North America (e.g., Fraser fir, subalpine fir [*Abies lasiocarpa* (Hook) Nutt.], and balsam fir [*A. balsamea* (Linn.) Mill.) are highly susceptible, suffering damage to the point of mortality, and firs native to central Europe (e.g., European silver fir [*A. alba* Mill.]) can tolerate moderate to heavy infestations while still remaining relatively healthy (Varty 1956, Mitchell 1966). Some Asian species (e.g., Veitch fir [*A. veitchii* Lindl.]) are highly resistant to BWA infestation (Hall et al. 1971).

Responses in susceptible trees often include gouting (abnormal cell growth resembling a gall) at the feeding site, a flattened crown (loss of apical dominance), and the production of abnormal xylem (similar to compression wood in conifers) called 'rotholtz' or 'redwood' (Balch 1952). The production of abnormal phloem and xylem disrupt metabolic pathways in the bark and cast the tree into a state of physiological drought, reducing the flow of water and compromising photosynthesis, transpiration, and respiration (Purich 1973). Heavy infestations may result in tree mortality in one to two years (Balch 1952). Responses that aid in BWA tolerance or resistance include thick outer bark (Pschorn-Walcher and Zwolfer 1958, Schooley and Bryant 1978), a rapid wound-healing response (production of necrophylactic tissue around the wound) (Mullick 1975, Hain et al. 1991), production of copious amounts of resin (Mitchell 1966), and the presence or production of chemicals (e.g. juvabione) that may inhibit or alter insect development (Puritch and Nijholt 1974, Fowler et al. 2001).

The development of BWA-resistant Fraser fir trees would be a relatively inexpensive solution to a difficult pest problem and would minimize adverse effects from management strategies. In addition, resistant trees could be utilized in the restoration of native Fraser fir stands. Our long-term objective is to develop BWA-resistant Fraser fir trees for native stand restoration and the Christmas tree industry.

BWA has been in North America for over 100 years and has been the focus of a considerable amount of research. Our knowledge of its biology and host interactions is rooted in studies conducted in Canada, the Pacific Northwest, and the Southern Appalachians on balsam fir, grand fir (*A. grandis* [Dougl.] Lindl.), subalpine fir, white fir (*A. concolor* [Gord. and Glend.] Lindl.), European silver fir, Fraser fir, Veitch fir, and others, with trees of all ages ranging from 2-year old seedlings to saplings to mature trees. Additionally, three subspecies of *Adelges piceae* have been identified in North America, *A. piceae piceae* on the West Coast and in the Southern Appalachians, *A. piceae canadensis* in Canada, and *A. piceae occidentalis* in British Columbia, (Footit and Mackauer 1983), all of which likely have been utilized in various studies. We have a general understanding of the resistance level of many fir species (e.g., Mitchell 1966), considered primarily on a mature-tree basis. Because seedlings are often utilized as surrogates for mature trees in resistance screening studies (Nielsen et al. 2002; Alfaro et al. 2008), an important step in the process of developing

BWA-resistant Fraser fir trees may be to determine the reaction of seedlings from multiple fir species representing the range of BWA resistance (susceptible, tolerant, resistant). Here, 'susceptible' refers to hosts that experience heavy infestations of BWA and are damaged by the presence/feeding of the adelgid. 'Tolerant' species may become infested with the adelgid, even heavily infested, but exhibit reduced symptom severity despite infestation. 'Resistant' species are those that reflect a reduced infestation level even under advanced infestation pressure (e.g., trees surrounded by heavily infested trees or those that are otherwise exposed to BWA eggs and crawlers). It is important to know whether the seedlings from species known to be resistant to BWA will show resistance and whether seedlings from susceptible species show the same levels of susceptibility.

Thus, our short-term objective was to screen for resistance across multiple fir species of equal age, grown under the same conditions and infested with BWA collected at one time from a single source, and to observe the responses of both host and insect. We tested species of known and unknown susceptibility levels.

Methods and Materials

Thirteen fir species, spanning the range of BWA susceptibility (Table 1), were sown from seed in 2003 provided by the NC State Christmas Tree Genetics Program and grown in tubular containers (3.8 x 30 cm) at the North Carolina State University Horticultural Field Lab in Raleigh for 4 years. Susceptible species included Fraser fir (3 seed sources: Roan Mountain, Richland Balsam, Mount Mitchell), balsam fir (*A. balsamea* [a New England source]), West Virginia Canaan fir (also called intermediate balsam fir) (*A. balsamea* [Linn.] var. *phanerolepis* Fern.), Korean fir (*A. koreana* Wils.), and corkbark fir (*A. lasiocarpa*

[Hook.] Nutt. var. *arizonica* [Merr.] Lemm.). Tolerant species included noble fir (*A. procera* Rehd.), white fir, and European silver fir. Resistant species included momi fir (*A. firma* Sieb. & Zucc.) and Veitch fir. Species representing unknown susceptibilities included Turkish fir (*A. bornmuellariana* Mattf.), Trojan fir ([*A. equi-trojani* Asch. & Sint. ex Boiss.] Coode et Cullen), and West Himalayan or pindrow fir (*A. pindrow* [Lamb.] Royle).

In 2007 the seedlings were transplanted into 7.6 x 7.6 x 23 cm tree pots and transported to an open greenhouse in Ashe County, NC. Potting medium consisted of mulched pine bark. The trees were fertilized with Osmocote 19-5-8 slow-release formula; nitrogen sources in this formula are provided equally as nitrate, ammonium nitrate, and urea. A misting system was installed in the greenhouse to provide irrigation and a screen over the frame allowed rain to fall onto the trees during the growing season (May through December). During winter months the greenhouse was covered with plastic.

The greenhouse was divided into four blocks, each containing two treatment plots. Each plot measured 0.9 m x 2.5 m x 1.2 m high, with the perimeter of each surrounded by organza cloth wrapped around 1.3 cm diameter PVC pipe to provide a barrier against dispersal of crawlers between plots. Each plot contained 90 trees, placed in 6 rows with 1 treatment tree per species within each row (randomly assigned) and 1 control per species (randomly assigned) throughout the plot. Seasonal effect was examined by infesting the trees in one treatment plot per block in August 2007 and the second plot in September 2007. There were 600 treatment trees and 120 controls utilized in the experiment, for a total of 720 study trees. Control trees were treated with a foliar insecticide (0.3% pyrethrins and 3.0% piperonyl butoxide). Trees within the study were exposed to BWA by suspending 3 logs (2.5 m in length) of BWA-infested Fraser fir over each treatment plot. This technique (Newton et al., 2011) mimics natural dispersal, allowing crawlers to drop onto the trees. The logs remained suspended above the trees for one month for each seasonal treatment.

The study was split into two groups, based on when that portion of the study was dismantled. In December 2007, one half of the study was dismantled (Group I), with treatment trees from rows 1, 3 and 5 in each plot cut at ground level and placed in a polyethylene bag. The bagged trees were stored in cardboard moving boxes (one box [45 trees] per treatment plot), transported back to NCSU and stored in a walk-in freezer at -2ºC pending assessment. Control trees were taken from Season 1 in Blocks 1 and 3 and Season 2 in Blocks 2 and 4. In May 2008, the remaining half of the study was dismantled (Group II), with each tree placed in a polyethylene bag and stored as described above.

Data collected from the first set of trees (Group I, December 2007) included the number of settled first instars (neosistentes) and the location of the neosistentes (nodes, leaf bases, base of buds). Data from the second set of trees (Group II, May 2008) included assessment of the number of BWA adults with eggs, the number of eggs, fecundity (eggs per BWA adult), whether settled instars were present (yes/no), and whether old woolly masses were present (yes/no)[1]. Parameters assessed for trees from both groups (December 2007 and May 2008) included height and diameter, general health (poor=1, fair=2, good=3), signs of tree response (gouting – yes/no), signs of development (e.g., exuviae, second or third instars – yes/no), length of primary, secondary, and tertiary branches, and length of bole. Because

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[1] The presence of old woolly masses was a sign that considerable BWA development had taken place between the initiation of the study in August or September and the dismantling of the study the following May. There were a number of trees with old woolly masses that had at some point clearly contained female(s) and eggs, but it was impossible to count either the number of females or estimate the number of eggs that had been present.

there was a significant difference in height between species, settled neosistentes, adult BWA, and egg counts are expressed as the number per centimeter branch or bole. For branch counts, a subsampling technique was developed from 100% counts of 61 trees (3 to 5 trees per species). We tested four schemes and chose the one that provided the best r^2 values (with raw data, the chosen scheme had an r^2 of 0.75 for adults and 0.86 for eggs; transformed (log(x + 1)), the r^2 was 0.78 for adults and 0.80 for eggs). The scheme that was most predictive was to take the first three branches from the top of the tree with secondary or tertiary branching, i.e., the bushiest branches from the second and third whorls. We measured the length of each branch (primary, secondary, tertiary) and counted every adult and egg on the sampled branches. For boles, 100% counts were done for every tree.

Data were analyzed on a per tree basis (sum of the three sampled branches). An analysis of variance was performed on height, diameter, health ratings and apical dominance using the General Linear Model procedure (proc glm) of SAS version 9.1 (SAS Institute 2003) and, where appropriate, pairwise comparisons were analyzed by the Tukey-Kramer Multiple Comparison Test (SAS Institute 2003) with an alpha level of 0.05. Main effects include block, time, species and pesticide treatment. Data are presented as least squared means ± standard error. Variables representing count data (number of settled neosistentes per cm branch or bole, number of adults per cm branch or bole, number of eggs per cm branch or bole, number of eggs per adult [fecundity]) were analyzed (proc genmod) using a zero inflated Poisson distribution with a logarithmic link function; the offset was the log-transformed length of branch, bole, or (in the case of eggs per female) the total number of egg-laying adults per branch or bole. Variables with a binary (yes/no) distribution (presence of settled neosistentes on overwintered trees, infested trees [presence of adults with eggs], early BWA development, and gouting) were analyzed using logistic regression (proc logistic). If there was a quasi-complete separation of data points detected in the logistic procedure, this was corrected by adding an option to the model (Firth bias-correction) that uses a penalized likelihood estimation method to correct for the separation issues (UCLA n.d.).

Results

There were a total of 470 trees (161 in Group 1 and 309 in Group 2) analyzed from the initial 720 study trees. All 48 trees representing noble fir were removed from the study because most died[2] soon after the study was initiated. A few (approximately 10 trees of various species) were removed from the analysis because of confusion in labeling datasheets and the remaining 192 trees (with representatives of all species) were lost to storage molds[3] in the freezer.

There were significant ($p < .0001$) differences in height between the 12 remaining species (Table 2). Canaan fir was the tallest with a mean height of 50.9 cm, followed by the three Fraser fir provenances (Mount Mitchell, Richland Balsam, and Roan Mountain, respectively). The shortest, with means under 20 cm, were Trojan fir, pindrow fir, Turkish fir, and European silver fir. Pairwise comparisons did not reveal any significant height differences among these shorter species. There were no differences in diameter, with an

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[2] The noble fir seedlings grew faster than the other species and were exceptionally pot-bound by the time we transplanted the set for the study. Although the healthiest-looking trees were chosen for the study, they did not appear to recover from transplant shock.

[3] We were able to salvage many of the overwintered trees despite mold growth by utilizing a gentle vacuuming technique to remove mycelia. We tested for a ‘vacuum’ effect, but, because it was not significant, ultimately removed it from the model.

overall mean of 9.21 mm. All trees were rated in good health (mean 2.98 [out of 3] ± 0.15) at the beginning of the study, but by the end of the study overall health had declined somewhat (mean 2.67 ± 0.46). Most of the trees within each species exhibited apical dominance (Table 2). All three provenances within Fraser fir exhibited excellent health at the beginning of the study and good to excellent health at the end. All provenances exhibited apical dominance, at 99% for Mount Mitchell and Richland Balsam trees and 96% for Roan Mountain trees.

Group 1 (December 2007, 3 to 4 months after initial infestation with crawlers)

Proportion of trees with settled neosistentes

There were no significant differences (p = 0.3853) among species in the proportion of trees with settled neosistentes present on the subsampled branches. Percentages ranged from 66% in European silver fir to 98% in corkbark fir. The tolerant and resistant species ranked somewhat lower than the susceptible species.

Location of settled neosistentes

On branches, crawlers settled at the base of the branches (adjacent to the bole), at the nodes under old bud scales, at the base of leaves along the branch stem, and at the base of buds (lateral and apical) (Table 3). The crawlers appeared to show a preference for the base of buds, with a higher percentage (ranging from 54 to 71%) of neosistentes settled on the buds than at other locations. There were no species differences in location preferences.

There appeared to be less preference shown on the boles, with the neosistentes present in relatively high percentages underneath old bud scales at nodes, at the base of needles and also at the base of buds (Table 4). As with branches, there were no differences shown among species.

Number of settled neosistentes per cm branch or bole

There were highly significant differences ($p = < .0001$) among species in the number of settled neosistentes per cm branch and bole (Table 5), with the resistant and moderately resistant species showing fewer numbers of these settled instars on the branches. Although there were significant differences in the numbers of settled neosistentes per cm bole, the differences in susceptibility groups was not as obvious.

There were, over all species, more settled neosistentes per cm branch in Time 2 (Time 1 [August] raw mean = 1.47, lsmean = 0.15; Time 2 [September] raw mean = 2.05, lsmean = 0.52 (± 0.01); $p < .0001$). Additionally, although there were still some, there were significantly fewer settled instars on the pesticide treated trees than on the nontreated trees (e.g., on branches, the raw mean for nontreated trees was 1.77 [lsmean = 0.14], versus 1.35 for treated [lsmean 0.54]); $p < .0001$).

Group 2 (May 2008, dismantled eight to nine months after initial infestation with crawlers)

Presence of settled instars

While we did not count each settled instar on the overwintered trees, we noted whether or not there were settled instars present on each subsampled branch and on the boles. We assumed that, because of the basic experimental design (6 rows, 1 tree per species per row) and because when we removed the trees in Group 1 we did so by altering the rows selected, we could expect similar numbers of settled instars (as found in Group 1 above) on the remaining trees in Group 2. There were no species differences in the mean proportion of trees with settled instars; percentages ranged from 83% in Turkish fir to 100% in momi fir (Table 6).

Proportion of infested trees

There were highly significant ($p = 0.0005$) differences among species in the proportion of trees that supported development of adults with eggs (= 'infested') (Table 6). The mean percentages of infested trees ranged from a low of 29% in Turkish fir to a high of 93% in Trojan fir. Almost all (93%) of the Trojan fir trees supported development of egg-laying adults and less than a third (28%) of Turkish fir trees, with the other species falling in between (Table 6). With a few exceptions, species rankings fell more or less within expectations (i.e., susceptible species exhibited a higher proportion of infested trees, and resistant or tolerant species exhibited a lower proportion). Momi fir and European silver fir ranked somewhat higher than would have been expected for resistant or tolerant species (87% and 68%, respectively), and Korean fir ranked lower than would be expected for a susceptible species. Korean fir, while classified as moderately susceptible, ranked near Veitch fir, one of the more resistant fir species, with just over half (52%) of the trees supporting the development of egg-laying adults. Similarly, almost half (47%) of the Veitch fir trees were infested with egg-laying adults.

There was a significant time effect in the proportion of infested trees ($P < .0001$); more of the trees in Time 2 (September, 78% of trees) exhibited egg-laying adults than in Time 1 (August 50%).

Mean number of BWA adults and eggs per cm branch and bole

There were highly significant species differences ($p < .0001$) in the numbers of adults and eggs per cm branch (Table 7). Trojan fir ranked highest with the most adults and the most eggs. Turkish fir and Korean fir exhibited the fewest adults and Veitch fir and pindrow fir exhibited the fewest numbers of eggs per cm branch. For the most part, particularly in the egg response, species ranked as would be expected with susceptible species showing more eggs per cm branch and moderate or resistant species showing fewer. The results from bole measurements were somewhat similar, with Trojan fir ranking highest and Veitch and Korean firs ranking lowest (Table 8).

Fecundity (mean number of eggs per female)

Mean fecundity levels (Table 9) may be considered a more important indicator of host suitability for the adelgid than the counts reported in the prior section. There were highly significant differences among species, with the raw means ranging from 5.79 in pindrow fir to 18.04 in European silver fir. There were some rank changes from the Poisson regression, with the highest fecundity exhibited by Turkish fir, Trojan fir and European silver fir (no differences among these three) and the least exhibited by Korean and pindrow fir.

Here, with the exception of European silver fir and Korean fir, each species group with an *a priori* susceptibility rating ranked as would be expected, with susceptible species exhibiting higher fecundities than the resistant or tolerant species. Species with unknown susceptibilities were vastly separated with Turkish fir and Trojan fir ranking highest, and pindrow, as mentioned above, exhibiting very low fecundity levels.

Pairwise comparisons showed significant different fecundity levels for the three provenances of Fraser fir (Table 9), with Richland Balsam ranking highest, then Roan Mountain coming next, and the Mount Mitchell trees showing the lowest levels of fecundity.

Bole fecundity levels (9) were similar, with (raw) means ranging from 0 eggs per female for Korean fir (there were some egg-laying females, but so few it resulted in a near-zero mean) to 24 eggs per female for Trojan fir. Turkish fir ranked fairly high, with 14 eggs per female, but significantly lower than Trojan fir. Veitch fir ranked low with 5.4 eggs per female, along with white fir (5.9). The three Fraser fir provenances ranked toward the middle with no differences shown between Roan Mountain and Richland Balsam, but Mount Mitchell trees ranked lower than the other two in bole fecundity levels. It is interesting to note that the susceptible species, with the exception of Korean fir, show a fairly similar ranking for both branch and bole measures, and all in the middle of the range. Momi fir ranked with balsam fir and Fraser fir (Mount Mitchell).

Early BWA development

Many trees from each species showed signs of early BWA development in that there were a number of woolly masses with the remnants of females and indications that eggs had been laid and hatched during the winter months, but the material was in no condition to be properly assessed. On our overwintered trees, most egg clutches (as reported above) contained either fresh eggs or recently hatched eggs that could be counted without any difficulty. These ‘old woolly masses’ appeared to have developed during the late fall and winter months and may provide additional information on susceptibility. Percentages of all trees within each species exhibiting this early development ranged from 12% in Turkish fir to

65% in Trojan fir (Table 10). Although there were significant ($p = 0.0205$) species differences, most species ranged between 20 and 40%. We also looked at this response in only those trees that were infested with egg-laying adults (infested trees). The proportions ranged from 27% in Veitch fir to 75% in Korean fir. There were no significant species differences ($p = 0.2819$).

Gouting

Gouting at the BWA feeding site is a symptom frequently observed in susceptible fir species and may be observed soon after crawlers settle, prior to the development of adults with eggs. Because this symptom may be an important indicator of tolerance or resistance, we analyzed this response for all trees and for infested trees. There were relatively few differences and, while we show the results from both analyses in Table 11, we report here only the results for infested trees. Balsam fir exhibited the highest percentage (85%) of trees with gouting response and Korean fir the lowest (17%) (Table 11). The species effect for gouting response was highly significant ($p < .0001$), with susceptible species ranking highest, tolerant and resistant species lowest, and the unknowns in the middle – the one notable exception is Korean fir, which showed the least amount of gouting.

Discussion

The data on settled neosistentes collected from Group I, harvested before winter dormancy, provide evidence that a BWA crawler can choose a feeding site, insert its stylets and settle on both susceptible and resistant tree species, at least at this young age. Additionally, these results suggest that the trees were exposed to a sufficient number of crawlers to produce at least a moderate level of infestation. The rankings were consistent with *a priori* resistance classifications, i.e., susceptible species ranked higher (the mean number of neosistentes were higher) than resistant or tolerant species. This may reflect some constitutive defense that inhibits settling by the crawler.

The preference for buds exhibited by the crawlers may indicate the presence of higher nutritive values in that region or may be a reflection of the adelgids settling on a substrate more physically suitable (softer, easier to penetrate) than in other areas. Alternately, the slight differences in the numbers of settled neosistentes per cm branch or bole could be the result of variability in the number of crawlers that fell onto any given tree. Although care was taken to choose and cut logs from trees that were both heavily and evenly infested, it would be impossible to find multiple trees with exactly the same level of BWA development all along an eight-foot section of the bole. BWA experiences three or more generations during the growing season and generations often overlap. Also, because this was a field study in a screen-covered cold frame on top of a mountain, environmental factors (e.g., wind or heavy rain) could have blown or washed some of the crawlers off the trees before they had a chance to settle.

Looking at the proportion of infested trees (supporting egg-laying adults) appears to separate the species of unknown resistance, Trojan fir and Turkish fir, and places them in different susceptibility groups. Here, Trojan fir appears to be highly susceptible to BWA infestation and Turkish fir appears resistant. This is an unusual result, given that both Trojan fir and Turkish fir originate in Turkey (Liu 1971; Linares 2011) and both belong to the *Abies nordmanniana* Spach fir complex, a genetically-related complex of Mediterranean fir species (Kaya et al. 2008). However, recent phytophthora root rot (*Phytophthora cinnamomi* Rands.)

resistance trials conducted with Trojan and Turkish fir showed a greater level of resistance to Phytophthora root rot within Turkish fir (35% mortality) as compared with Trojan fir (56% mortality) (Frampton et al. 2012). Here, the two species ranked at almost opposite ends of the spectrum in the numbers of adults and eggs per cm branch (Table 7), which supports the idea that they may be exhibiting different BWA resistance levels. However, the results from the fecundity calculations places them side-by-side in terms of ranking, with both exhibiting higher fecundity levels (in the Poisson regression) than any other species in the study (Table 9). That pindrow fir ranked almost last in fecundity levels suggests that this species may be resistant to BWA. If true, this could be promising for the Christmas tree industry because pindrow fir is also resistant to the root rot *Phytophthora cinnamomi*, one of the most serious diseases afflicting fir Christmas tree production (Frampton and Benson 2012).

Because the BWA egg clutches developed where the crawlers had settled and were found at specific locations (under old bud scales at nodes, at the base of buds and leaves) rather than evenly dispersed along the branches, and because the total branch length (including primary, secondary, and tertiary branches) were relatively high, the counts of adults and eggs (on a per cm branch basis) were surprisingly low, ranging from 0.02 to 0.17 for adults and 0.10 to 3.09 for eggs. It is somewhat surprising that 46% of the Veitch fir trees became infested with egg-laying adults. Mature Veitch fir trees exhibit a marked resistance to BWA (Hall et al. 1971; Mitchell 1966) and even young seedlings (4-5 years old) have been shown to exhibit resistance to BWA infestation (Newton et al. 2011).

It is interesting that European silver fir was one of the highest ranking species in fecundity levels. European silver fir can become heavily infested with BWA but can maintain an infestation for many years without showing symptoms of decline. Mature trees exhibit thicker outer bark and this characteristic is thought to serve as protection from severe BWA damage (Pschorn-Walcher and Zwolfer 1958; Schooley and Bryant 1978). Additionally, European silver fir may produce secondary periderm at the feeding site to effectively cloister off the insect and prevent it from damaging unaffected tissues. The young trees utilized in this study have thin bark and may not be able to resist adelgid infestation.

While most natural fir stands in the Southern Appalachians are reported to experience two BWA generations per year and Christmas tree plantations may experience three (Arthur and Hain 1984), it is not unusual for lowland fir plantations in the Pacific Northwest to experience up to four generations per year (Mitchell et al. 1970). In these low elevation areas, the adelgid may break hibernation as early as mid-January and the first adults appear in February (Mitchell et al. 1970). Most species ranked similarly between the measure of trees that were infested and the proportion of trees that exhibited early development, i.e., Trojan fir ranked highest in infested trees (96%) and also ranked highest in early BWA development (65%), Turkish fir ranked lowest in both infestation levels (29%) and early BWA development (12%). The others ranked somewhere in between. It may be worth noting that the two species known to be resistant to BWA, momi and Veitch fir, were at opposite ends of the spectrum. Fifty percent of the momi fir trees exhibited early development but only 14% of the Veitch fir trees did so. When we look at the percentages of trees that were infested with adults and eggs and that also showed early BWA development, the results (Table 10), while not necessarily surprising, are interesting. Just over half (52%) of the Korean fir trees were infested with egg-laying adults (Table 6); of these, 75% showed signs of early BWA development (Table 10). This begs the question as to whether, if we were to have harvested these trees earlier in the season (than in May), we would have seen more adults or greater fecundity on this species which has elsewhere been reported as being susceptible to BWA, yet here consistently ranks with tolerant and resistant species. It is important to also keep in mind that the *a priori* ratings by Mitchell were assessed in the field in the Pacific Northwest, with a different BWA genotype and environment. The fact that 27 to 75% of the infested trees exhibited early BWA development suggests, if nothing else, that BWA is active and developing before the fir trees—seemingly, regardless of species or susceptibility levels—fully break dormancy. While the trees may have entered into quiescence, bud-break was not yet evident on any of the trees in this study.

It is interesting to note that, with the exception of corkbark fir, which is native to the western United States, the highest levels of gouting were exhibited by species native to eastern North America (balsam fir, Fraser fir, Canaan fir). The Mediterranean and Asian species (Table 1) all exhibited relatively low (27% in Veitch down to 17% in Korean) levels of gouting response. Less than one third of the European silver fir and white fir trees exhibited any gouting. These are both known to be highly tolerant of BWA infestation and this low level of gouting response may be an important indicator of why they do not suffer more from BWA infestations.

Within Fraser fir, gouting responses for the three provenances ranged from 67% (Richland Balsam) to 76% (Roan Mountain). Because Fraser fir is known to be highly susceptible to BWA damage and gouting is one of the first responses to BWA feeding, the fact that 25% or more of the infested trees did not show any gouting response may be an indication of some level of tolerance within this species.

These results show that young trees representing a variety of fir species can become infested with BWA. While seedlings cannot serve as perfect surrogates for mature trees, here we show that results generally fall within expectations for known resistance classifications. This will allow for further comparative studies into BWA interactions with a variety of hosts and assist in the goal of developing BWA-resistant Fraser fir.

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Table 1. *Abies* species representing the range of known and unknown susceptibility ratings to the balsam woolly adelgid (adapted from Mitchell 1966).

Species	Common Name	Origin	Susceptibility Rating[1]
Abies balsamea (New England)	Balsam fir	Eastern North America	S
Abies balsamea var. *phanerolepis*	Canaan fir	Eastern U.S.	S
Abies fraseri,	Fraser fir	Eastern U.S.	S
Abies koreana	Korean fir	South Korea	S
Abies lasiocarpa var. *arizonica*	Corkbark fir	Western U.S.	S
Abies alba	European silver fir	Western Europe	M
Abies concolor	White fir	Western U.S.	M
Abies firma	Momi fir	Japan	R
Abies veitchii	Veitch fir	Japan	R
Abies bornmuelleriana	Turkish fir	Turkey	U
Abies equi-trojani	Trojan fir	Turkey	U
Abies pindrow	Western Himalayan fir	Nepal	U

[1] The parameter 'Sus' reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; M = moderately susceptible (tolerant); R = resistant; U = unknown.

Table 2. Mean (lsmean ± SE) height and diameter (Diam) by species; health rating (1 = poor, 2 = fair, 3 = good) at the beginning and end of the study; and apical dominance (ApDom) (0 = no, 1 = yes) at the beginning of the study. N = 468.

				Health		
Species	Sus[1]	Height (cm)	Diam (mm)	Beginning	End	ApDom
Balsam fir	S	32.1 (1.0)^{d}	8.2 (0.8)	3.00 (.03)^{a}	2.49 (.09)^{ab}	0.78 (.05)^{ab}
Canaan fir	S	50.9 (0.9)^{a}	9.9 (0.7)	3.00 (.02)^{a}	2.77 (.07)^{a}	0.96 (.04)^{ab}
Fraser fir (Mt. Mitch.)	S	39.6 (1.0)^{bc}	8.8 (1.0)	3.00 (.03)^{a}	2.65 (.08)^{ab}	0.99 (.05)^{a}
Fraser fir (Rich. Bals.)	S	44.1 (1.0)^{b}	9.2 (0.8)	3.00 (.03)^{a}	2.53 (.09)^{ab}	0.99 (.05)^{a}
Fraser fir (Roan)	S	41.8 (0.9)^{bc}	11.6 (0.7)	3.00 (.02)^{a}	2.70 (.08)^{ab}	0.96 (.04)^{ab}
Korean fir	S	34.6 (0.8)^{cd}	9.8 (0.7)	3.01 (.02)^{a}	2.75 (.07)^{a}	0.98 .(04)^{a}
Corkbark fir	S	22.9 (1.1)^{e}	8.3 (0.9)	2.93 (.03)^{ab}	2.34 (.09)^{b}	0.98 (.05)^{ab}
European silver fir	M	15.0 (1.0)^{f}	7.6a (0.8)	2.97 (.03)^{ab}	2.75 (.09)^{ab}	0.87 (.05)^{ab}
White fir	M	37.7 (1.0)^{c}	9.3 (0.8)	3.00 (.03)^{a}	2.78 (.09)^{a}	0.78 (.05)^{ab}
Momi fir	R	33.1 (0.9)^{d}	8.7 (0.7)	2.85 (.02)^{b}	2.47 (.07)^{ab}	0.89 (.04)^{ab}
Veitch fir	R	30.1 (1.0)^{d}	9.0 (0.8)	2.94 (.03)^{ab}	2.55 (.08)^{ab}	0.76 (.05)^{b}
Turkish fir	U	16.6 (0.9)^{f}	8.6 (0.8)	3.00 (.02)^{a}	2.83 (.08)^{a}	0.87 (.04)^{ab}
Trojan fir	U	19.5 (0.9)^{ef}	8.8 (0.8)	3.00 (.03)^{a}	2.82 (.08)^{a}	0.93 (.04)^{ab}
Pindrow fir	U	17.7 (1.0)^{f}	9.6 (0.8)	3.01 (.03)^{a}	2.75 (.08)^{ab}	0.92 (.05)^{ab}
Overall lsmean from general linear model		31.28 (5.3)	9.21 (4.4)	2.98 (.15)	2.67 (.46)	0.92 (0.26)

ANOVA p-values from general linear model

Source of variation	df	Pr > F	Pr > F	Pr > F	Pr > F	Pr > F
Model	18	< .0001	< .0001	0.0658	< .0001	< .0001
Block	3	0.5480	0.2787	0.2295	< .0001	0.0194
Time	1	0.5767	0.0772	0.1998	< .0001	0.0001
Species	13	< .0001	0.0723	< .0001	< .0001	0.0001
Pesticide treatment	1	0.0562	0.0985	0.4912	0.5414	0.1142

[1] The parameter ‘Sus’ reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; M = moderately susceptible (tolerant); R = resistant; U = unknown.

Table 3 (Group 1). Mean (± S.E.) number of settled neosistentes on the sampled branches with locations and the percentages of the total number at that location. An analysis of variance was performed on lsmeans but there were no species differences and few differences in other main effects. The overall means from the general linear model are shown for each location as well as p-values from the general linear model.

Species	Sus	N	Number of Neosistentes	Branch Base	Under Scales	Base of Needle	Base of Bud
				Percentages of settled neosistentes			
Balsam fir	S	10	190.3 (56.7)	0.0 (0.0)	15.7 (3.8)	15.7 (3.3)	68.6 (6.2)
Canaan fir	S	17	264.9 (55.5)	0.8 (0.8)	18.9 (2.5)	20.4 (3.2)	60.6 (4.4)
Fraser fir (Mt Mit)	S	10	164.0 (43.4)	0.8 (0.8)	15.1 (3.9)	22.3 (5.4)	62.6 (2.9)
Fraser fir (Rich Bal)	S	9	143.4 (27.9)	3.3 (2.4)	15.2 (3.0)	15.6 (3.6)	69.2 (3.9)
Fraser fir (Roan M)	S	17	217.2 (42.0)	0.0 (0.0)	16.2 (2.0)	21.8 (1.8)	62.0 (2.7)
Korean fir	S	21	169.8 (20.8)	0.0 (0.0)	20.7 (2.5)	17.8 (2.4)	61.6 (2.7)
Corkbark fir	S	5	92.2 (35.0)	0.0 (0.0)	10.6 (4.4)	29.1 (4.1)	60.2 (3.5)
European silver fir	M	10	100.3 (35.0)	0.0 (0.0)	15.2 (4.3)	27.0 (7.0)	57.8 (6.5)
White fir	M	7	45.9 (19.3)	0.0 (0.0)	22.9 (10.2)	14.0 (5.0)	63.1 (9.2)
Momi fir	R	16	119.7 (39.0)	0.1 (0.1)	19.3 (4.4)	26.4 (5.1)	54.2 (6.2)
Veitch fir	R	9	135.1 (51.8)	0.0 (0.0)	13.7 (4.2)	15.0 (4.3)	71.3 (7.6)
Turkish fir	U	12	109.8 (32.3)	0.0 (0.0)	9.8 (2.8)	20.0 (4.2)	70.1 (6.1)
Trojan fir	U	11	157.3 (44.9)	0.0 (0.0)	10.2 (2.6)	20.5 (3.3)	69.3 (4.3)
Pindrow fir	U	7	136.6 (49.7)	0.0 (0.0)	28.3 (7.8)	12.1 (4.2)	60.0 (5.2)
Overall lsmeans from general linear model				0.3 (2.1)	16.8 (12.4)	20.1 (13.8)	63.1 (16.6)
ANOVA p-values from general linear model							
Source of variation		df		Pr > F	Pr > F	Pr > F	Pr > F
Model		18		0.0163	0.0160	0.2335	0.0515
Block		3		0.0089	0.2903	0.0842	0.0810
Time		1		0.4375	0.0178	0.7912	0.0470
Species		13		0.0579	0.1720	0.3076	0.3671
Pesticide treatment		1		0.1405	0.0726	0.8223	0.1276
Error		139					

Table 4 (Group 1). Mean (± S.E.) number of settled neosistentes on boles with locations and the percentages of the total number at that location. An analysis of variance was performed on lsmeans but there were no species differences and few differences in other main effects. The overall means from the general linear model are shown for each location as well as p-values from the general linear model.

Species	Sus	N	Number of Neosistentes	Under Scales	Base of Needle	Base of Bud
				-----Percentage of settled neosistentes-----		
Balsam fir	S	10	22.0 (5.5)	39.2 (13.6)	21.4 (6.5)	39.3 (11.6)
Canaan fir	S	17	31.8 (8.4)	39.5 (9.1)	23.1 (5.6)	37.3 (9.1)
Fraser fir (Mount Mitchell)	S	10	22.2 (6.1)	19.6 (9.1)	16.6 (5.0)	63.8 (10.9)
Fraser fir (Richland Balsam)	S	9	28.4 (3.8)	34.3 (9.2)	15.9 (6.0)	49.9 (8.5)
Fraser fir (Roan Mountain)	S	17	37.7 (7.7)	28.8 (6.1)	28.5 (4.8)	44.3 (8.2)
Korean fir	S	21	34.8 (6.2)	32.1 (5.7)	21.4 (4.4)	46.4 (6.1)
Corkbark fir	S	5	14.2 (8.0)	50.7 (21.3)	33.7 (20.6)	15.1 (8.0)
European silver fir	M	10	11.6 (3.9)	40.2 (15.8)	47.6 (14.1)	12.2 (4.4)
White fir	M	7	8.6 (3.1)	43.0 (16.0)	19.3 (9.5)	37.6 (14.4)
Momi fir	R	16	27.2 (7.3)	34.1 (6.9)	20.0 (5.9)	45.9 (8.1)
Veitch fir	R	9	16.3 (6.4)	39.9 (13.0)	25.2 (10.8)	34.9 (13.9)
Turkish fir	U	12	7.6 (1.7)	28.8 (14.5)	35.6 (8.5)	35.6 (9.0)
Trojan fir	U	11	22.0 (6.9)	43.6 (13.4)	27.2 (8.7)	29.2 (6.9)
Pindrow fir	U	7	42.6 (11.9)	39.3 (12.5)	14.3 (7.6)	46.4 (11.9)
Overall lsmeans from general linear model				35.3 (27.7)	24.3 (21.4)	40.6 (28.4)
ANOVA p-values from general linear model						
Source of variation		df		Pr > F	Pr > F	Pr > F
Model		18		< .0001	0.0002	0.0016
Block		3		< .0001	0.0003	0.0001
Time		1		0.1137	0.0057	0.5095
Species		13		0.8197	0.3006	0.0507
Pesticide treatment		1		0.7058	0.1049	0.4184
Error		143				

Table 5 (Group I). Mean (± S.E.) number of neosistentes (settled first instar adelgids) per cm branch and bole. We are showing the means (averages) and also the lsmeans from the Poisson regression (proc genmod), with pairwise comparisons made in proc genmod. The first set of means reflect the actual means from the sampled branches for each tree (across all blocks, etc., one value per tree calcuated from three sampled branches). The analysis of variance was performed using the genmod procedure in SAS. Means within a column that share the same superscript letter are not significantly different from one another. (N = 161)

Species	Sus[1]	Number of Neosistentes per cm branch	Number of Neosistentes per cm branch	Number of Neosistentes per cm bole	Number of Neosistentes per cm bole
		[Means]	[Lsmeans]	[Means]	[Lsmeans]
Balsam fir	S	2.1 (0.4)	0.53 (0.03)^{bc}	0.7 (0.2)	-0.50 (0.07)^{e}
Canaan fir	S	1.8 (0.3)	0.50 (0.02)^{c}	0.6 (0.2)	-0.55 (0.05)^{e}
Fraser fir (Mt. Mitchell)	S	1.8 (0.4)	0.45 (0.03)^{cd}	0.6 (0.1)	-0.50 (0.07)^{e}
Fraser fir (Rich. Bals.)	S	2.1 (0.4)	0.62 (0.03)^{ab}	0.7 (0.1)	-0.40 (0.07)^{de}
Fraser fir (Roan Mtn.)	S	2.4 (0.4)	0.59 (0.02)^{b}	0.9 (0.2)	-0.25 (0.04)^{d}
Korean fir	S	1.8 (0.2)	0.40 (0.02)^{d}	1.0 (0.2)	-0.15 (0.04)^{cd}
Corkbark fir	S	2.3 (1.0)	0.69 (0.05)^{a}	0.6 (0.4)	-0.47 (0.12)^{de}
European silver fir	M	1.1 (0.3)	0.08 (0.03)^{f}	0.8 (0.3)	-0.04 (0.10)^{c}
White fir	M	0.5 (0.2)	-0.92 (0.06)^{h}	0.2 (0.1	-1.65 (0.13)^{f}
Momi fir	R	1.7 (0.4)	-0.04 (0.02)^{g}	0.9 (0.2)	-0.16 (0.05)^{cd}
Veitch fir	R	1.4 (0.4)	0.38 (0.03)^{d}	0.6 (0.2)	-0.48 (0.09)^{e}
Turkish fir	U	1.3 (0.4)	0.20 (0.03)^{e}	0.4 (0.9)	-0.47 (0.11)^{de}
Trojan fir	U	1.7 (0.4)	0.63 (0.02)^{ab}	1.1 (0.3)	0.43 (0.07)^{b}
Pindrow fir	U	2.2 (0.5)	0.61 (0.03)^{ab}	2.2 (0.5)	0.69 (0.06)^{a}

Results from Poisson regression

		------------Branch------------		------------Bole------------	
Source of variation	df	Chi square	Pr > Chi sq	Chi square	Pr > Chi sq
Block	3	527.64	< .0001	50.84	< .0001
Time	1	660.89	< .0001	171.78	< .0001
Species	13	1886.17	< .0001	581.08	< .0001
Pesticide treatment	1	372.31	< .0001	67.22	< .0001

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[1] The parameter ‘Sus’ reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; M = moderately susceptible (tolerant); R = resistant; U = unknown.

Table 6 (Group 2). Means (± S.E.) for presence/absence (1/0) of settled instars (Settled) and presence/absence (1/0) of adults with egg clutches (Infested). N = 309.

Species	Sus[1]	N	Proportion of Trees with Settled Instars	Proportion of Trees with Egg-laying Adults
Balsam fir	S	20	0.90 (0.07)	0.65 (0.10)
West Virginia Canaan	S	23	0.91 (0.06)	0.78 (0.09)
Fraser fir (Mount Mitchell)	S	21	0.90 (0.07)	0.62 (0.11)
Fraser fir (Richland Balsam)	S	21	0.86 (0.08)	0.71 (0.10)
Fraser fir (Roan)	S	21	0.95 (0.05)	0.81 (0.09)
Korean fir	S	23	0.87 (0.07)	0.52 (0.11)
Corkbark fir	S	21	0.90 (0.06)	0.57 (0.11)
European silver fir	M	19	0.95 (0.05)	0.68 (0.11)
White fir	M	23	0.87 (0.07)	0.56 (0.10)
Momi fir	R	24	1.00 (0.00)	0.87 (0.07)
Veitch fir	R	22	0.86 (0.07)	0.50 (0.10)
Turkish fir	U	24	0.83 (0.08)	0.29 (0.09)
Trojan fir	U	23	0.96 (0.04)	0.96 (0.04)
Pindrow fir	U	24	0.87 (0.07)	0.54 (0.10)

Results from logistic regression					
Type 3 Analysis of effects		------------Settled[2]------------		------------Infested[3]------------	
Effect	df	Wald Chi-Square	Pr > ChiSq	Wald Chi-Square	Pr > ChiSq
Block	3	7.9601	0.0480	32.8262	< .0001
Time	1	0.0273	0.8688	30.9175	< .0001
Species	13	5.1628	0.9714	36.2598	0.0005
Pesticide Treatment	1	1.1258	0.2887	0.0697	0.7918

[1] The parameter 'Sus' reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; M = moderately susceptible (tolerant); R = resistant; U = unknown.

[2] (Proc Logistic): Model = binary logit; Optimization technique = Fisher's scoring; Likelihood penalty = Firth's bias correction; Probability modeled: Settled = 1; Convergence criterion satisfied.

[3] (Proc Logistic): Model = binary logit; Optimization technique = Fisher's scoring; Probability modeled: Infested = 1; Convergence criterion satisfied.

Table 7 (Group 2). Mean (± S.E.) number of adults and eggs per cm branch. We are showing the means (averages) and also the lsmeans from the Poisson regression (proc genmod), with pairwise comparisons made in proc genmod. The first set of means reflect the actual means from the sampled branches for each tree (across all blocks, etc., one value per tree calculated from three sampled branches). Pairwise comparisons were calculated using the genmod procedure in SAS. Means within a column that share the same superscript letter are not significantly different from one another. (N = 309).

Species	Sus[1]	Number of adults per cm branch	Number of adults per cm branch	Number of eggs per cm branch	Number of eggs per cm branch
		[Means]	[Lsmeans]	[Means]	[Lsmeans]
Balsam fir	S	0.05 (0.02)	-3.57 (0.10)^{cd}	0.64 (0.21)	-0.74 (0.03)^{g}
Canaan fir	S	0.10 (0.03)	-2.96 (0.07)^{b}	1.33 (0.54)	-0.06 (0.02)^{c}
Fraser fir (Mt. Mitchell)	S	0.09 (0.05)	-2.86 (0.08)^{ab}	0.97 (0.50)	-0.00 (0.02)^{b}
Fraser fir (Rich. Bals.)	S	0.06 (0.02)	-3.56 (0.09)^{cd}	1.00 (0.52)	-0.33 (0.02)^{f}
Fraser fir (Roan Mtn.)	S	0.14 (0.07)	-3.10 (0.07)^{b}	2.38 (1.67)	-0.18 (0.02)^{d}
Korean fir	S	0.02 (0.01)	-5.01 (0.18)^{f}	0.10 (0.07)	-1.83 (0.07)^{j}
Corkbark fir	S	0.05 (0.02)	-3.80 (0.14)^{d}	0.83 (0.38)	-0.69 (0.04)^{g}
European silver fir	M	0.09 (0.04)	-3.63 (0.10)^{cd}	1.81 (0.81)	-0.28 (0.02)^{ef}
White fir	M	0.07 (0.04)	-3.48 (0.09)^{c}	0.53 (0.28)	-0.84 (0.03)^{h}
Momi fir	R	0.07 (0.02)	-3.51 (0.09)^{c}	0.60 (0.18)	-1.08 (0.03)^{i}
Veitch fir	R	0.06 (0.02)	-3.78 (0.10)^{d}	0.47 (0.19)	-1.14 (0.03)^{i}
Turkish fir	U	0.02 (0.01)	-4.41 (0.15)^{e}	0.46 (0.33)	-0.26 (0.03)^{e}
Trojan fir	U	0.17 (0.05)	-2.76 (0.07)^{a}	3.09 (1.35)	0.40 (0.02)^{a}
Pindrow fir	U	0.07 (0.03)	-3.44 (0.12)^{c}	0.49 (0.23)	-1.04 (0.05)^{i}

Results from Poisson regression[2]

		------------Adults------------		------------Eggs------------	
Source of variation	df	Chi square	Pr > Chi sq	Chi square	Pr > Chi sq
Block	3	1385.39	< .0001	13156.80	< .0001
Time	1	1049.95	< .0001	8797.91	< .0001
Species	13	490.26	< .0001	6320.51	< .0001
Pesticide treatment	1	53.70	< .0001	795.65	< .0001

[1] The parameter 'Sus' reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; M = moderately susceptible (tolerant); R = resistant; U = unknown.

[2] Analyzed using proc genmod; distribution: zero inflated Poisson; log link function; offset: log(branch length); algorithm converged for adults and also for eggs.

Table 8 (Group 2). Mean (± S.E.) number of adults and eggs per cm bole. We are showing the means (averages) and also the lsmeans from the Poisson regression (proc genmod), with pairwise comparisons made in proc genmod. The first set of means reflect the actual means from the of each tree (across all blocks, etc.). Pairwise comparisons were calculated using the genmod procedure in SAS. Means within a column that share the same superscript letter are not significantly different from one another. (N = 309).

Species	Sus[1]	Number of adults per cm bole	Number of adults per cm bole	Number of eggs per cm bole	Number of eggs per cm bole
		[Means]	[Lsmeans][2]	[Means]	[Lsmeans]
Balsam fir	S	0.04 (0.02)	---	0.28 (0.12)	-1.95 (0.08)^{j}
Canaan fir	S	0.05 (0.03)	---	0.37 (0.26)	-1.37 (0.07)^{h}
Fraser fir (Mt. Mitchell)	S	0.05 (0.04)	---	0.43 (0.30)	-0.90 (0.07)^{g}
Fraser fir (Rich. Bals.)	S	0.04 (0.02)	---	0.85 (0.68)	-0.31 (0.05)^{e}
Fraser fir (Roan Mtn.)	S	0.07 (0.06)	---	1.32 (1.22)	-0.35 (0.06)^{e}
Korean fir	S	0.00 (0.00)	---	0.00 (0.00)	-22.9 (3762)^{k}
Corkbark fir	S	0.04 (0.02)	---	0.52 (0.31)	0.36 (0.08)^{b}
European silver fir	M	0.11 (0.06)	---	2.01 (1.16)	-0.13 (0.07)^{d}
White fir	M	0.06 (0.04)	---	0.37 (0.21)	-1.63 (0.07)^{i}
Momi fir	R	0.13 (0.05)	---	1.07 (0.47)	-0.77 (0.05)^{f}
Veitch fir	R	0.01 (0.00)	---	0.05 (0.03)	-3.17 (0.18)^{k}
Turkish fir	U	0.05 (0.04)	---	0.75 (0.65)	0.13 (0.08)^{c}
Trojan fir	U	0.27 (0.11)	---	5.98 (3.12)	1.30 (0.05)^{a}
Pindrow fir	U	0.22 (0.15)	---	2.14 (1.89)	0.30 (0.06)^{b}

Results from Poisson regression[3]

		------------Adults[2]------------		------------Eggs------------	
Source of variation	df	Chi square	Pr > Chi sq	Chi square	Pr > Chi sq
Block	3	---	---	5923.12	< .0001
Time	1	---	---	3079.31	< .0001
Species	13	---	---	7648.59	< .0001
Pesticide treatment	1	---	---	95.37	< .0001

[1] The parameter 'Sus' reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; M = moderately susceptible (tolerant); R = resistant; U = unknown.

[2] For this response variable, the zero inflated Poisson regression model did not converge (the negative of the Hessian was not positive definite) and the results from the analysis are questionable. Thus, the lsmeans and p-values from this procedure for the number of adults per cm bole are not reported.

[3] Analyzed using proc genmod; distribution: zero inflated Poisson; log link function; offset: log(bole length). The algorithm converged for the egg response.

Table 9 (Group 2). Mean (± S.E.) number of eggs per female on branches and boles. We are showing the means (averages) and also the lsmeans from the Poisson regression (proc genmod), with pairwise comparisons made in proc genmod. The first set of means reflect the actual means from the sampled branches for each tree (across all blocks, etc., one value per tree). Pairwise comparisons were calculated using the genmod procedure in SAS. Means within a column that share the same superscript letter are not significantly different from one another. (N = 154 for branch fecundity and 77 for bole fecundity).

Species	Sus[1]	Fecundity on branches	Fecundity on branches	Fecundity on bole	Fecundity on bole
		[Means]	[Lsmeans]	[Means]	[Lsmeans]
Balsam fir	S	12.06 (1.55)	2.37 (0.03)^{d}	7.61 (1.91)	2.03 (0.08)^{ef}
West Virginia Canaan fir	S	11.52 (1.15)	2.46 (0.02)^{c}	6.80 (1.59)	2.22 (0.06)^{d}
Fraser fir (Mount Mitchell)	S	11.01 (1.78)	2.13 (0.03)^{e}	12.95 (2.68)	2.06 (0.07)^{ef}
Fraser fir (Richland Balsam)	S	13.13 (1.51)	2.61 (0.02)^{b}	11.42 (3.87)	2.64 (0.05)^{bc}
Fraser fir (Roan Mountain)	S	13.98 (2.01)	2.50 (0.02)^{c}	11.14 (3.16)	2.69 (0.06)^{b}
Korean fir	S	7.20 (3.43)	1.57 (0.07)^{h}	---	---
Corkbark fir	S	14.06 (4.10)	2.47 (0.04)^{c}	15.44 (3.67)	2.96 (0.08)^{a}
European silver fir	M	18.04 (3.12)	2.70 (0.03)^{a}	20.42 (7.13)	2.76 (0.07)^{b}
White fir	M	9.06 (2.26)	1.96 (0.03)^{f}	5.88 (0.63)	1.47 (0.07)^{g}
Momi fir	R	8.12 (1.15)	1.86 (0.03)^{g}	7.91 (0.89)	1.96 (0.05)^{f}
Veitch fir	R	7.59 (1.53)	2.01 (0.03)^{f}	5.42 (2.39)	1.43 (0.18)^{g}
Turkish fir	U	10.13 (3.89)	2.75 (0.04)^{a}	14.19 (2.49)	2.52 (0.08)^{c}
Trojan fir	U	16.69 (3.29)	2.71 (0.02)^{a}	24.04 (5.76)	3.06 (0.05)^{a}
Pindrow fir	U	5.79 (0.72)	1.70 (0.05)^{h}	5.10 (1.51)	2.15 (0.06)^{de}

Results from Poisson regression[2]

		-----Fecundity on branches----		------Fecundity on bole------	
Source of variation	df	Chi square	Pr > Chi sq	Chi square	Pr > Chi sq
Block	3	398.48	< .0001	68.52	< .0001
Time	1	36.97	< .0001	508.20	< .0001
Species	13	2478.43	< .0001	112.70	< .0001
Pesticide treatment	1	35.24	< .0001	1776.00	< .0001

[1] The parameter 'Sus' reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; M = moderately susceptible (tolerant); R = resistant; U = unknown.

[2] Analyzed using proc genmod; distribution: Poisson; log link function; offset: log(number of adults); algorithm converged for both models (branch fecundity and bole fecundity).

Table 10 (Group 2). Mean (± S.E.) proportion of trees exhibiting early BWA development[1]. The first column of data reflects all trees (N = 309) and the second reflects the proportions of infested trees exhibiting early BWA development (N = 200).

Species	Sus[2]	N	All trees	N	Infested trees
Balsam fir	S	20	0.25 (0.10)	13	0.38 (0.14)
West Virginia Canaan	S	23	0.30 (0.10)	18	0.39 (0.12)
Fraser fir (Mount Mitchell)	S	21	0.29 (0.10)	13	0.46 (0.14)
Fraser fir (Richland Balsam)	S	21	0.33 (0.10)	15	0.47 (0.13)
Fraser fir (Roan)	S	21	0.33 (0.10)	17	0.41 (0.12)
Korean fir	S	23	0.39 (0.10)	12	0.75 (0.13)
Corkbark fir	S	21	0.24 (0.09)	12	0.42 (0.15)
European silver fir	M	19	0.47 (0.12)	13	0.69 (0.13)
White fir	M	23	0.39 (0.10)	13	0.69 (0.13)
Momi fir	R	24	0.50 (0.10)	21	0.57 (0.11)
Veitch fir	R	22	0.14 (0.07)	11	0.27 (0.14)
Turkish fir	U	24	0.12 (0.07)	7	0.43 (0.20)
Trojan fir	U	23	0.65 (0.10)	22	0.68 (0.10)
Pindrow fir	U	24	0.25 (0.09)	13	0.46 (0.14)

Results from logistic regression[3]

Type 3 Analysis of effects		--------------All trees--------------		-----------Infested trees-----------	
Effect	df	Wald Chi-Square	Pr > ChiSq	Wald Chi-Square	Pr > ChiSq
Block	3	22.2431	< .0001	9.4119	0.0243
Time	1	3.0119	0.0827	0.8865	0.3464
Species	13	25.3880	0.0205	15.4197	0.2819
Pesticide Treatment	1	0.0162	0.8986	0.5460	0.4600

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[1] Early BWA development refers to BWA activity (egg-laying and hatching of crawlers) during the winter months. These woolly masses could not be assessed for the numbers of eggs, but it was clear that development had occurred.

[2] The parameter 'Sus' reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; M = moderately susceptible (tolerant); R = resistant; U = unknown.

[3] (Proc Logistic): Model: binary logit; optimization technique: Fisher's scoring; probability modeled: early development = 1; convergence criterion satisfied.

Table 11 (Group 2). Mean (± S.E.) proportion of trees exhibiting gouting. The first column of data reflects all trees (N = 309) and the second reflects only infested trees (N = 200).

Species	Sus[1]	N	All trees	N	Infested trees
Balsam fir	S	20	0.85 (0.08)	13	0.85 (0.10)
West Virginia Canaan	S	23	0.70 (0.10)	18	0.83 (0.09)
Fraser fir (Mount Mitchell)	S	21	0.71 (0.10)	13	0.69 (0.13)
Fraser fir (Richland Balsam)	S	21	0.57 (0.11)	15	0.67 (0.13)
Fraser fir (Roan)	S	21	0.81 (0.09)	17	0.76 (0.11)
Korean fir	S	23	0.13 (0.07)	12	0.17 (0.11)
Corkbark fir	S	21	0.71 (0.10)	12	0.75 (0.13)
European silver fir	M	19	0.21 (0.10)	13	0.30 (0.13)
White fir	M	23	0.26 (0.09)	13	0.31 (0.13)
Momi fir	R	24	0.29 (0.09)	21	0.29 (0.10)
Veitch fir	R	22	0.36 (0.10)	11	0.27 (0.14)
Turkish fir	U	24	0.29 (0.09)	7	0.43 (0.20)
Trojan fir	U	23	0.30 (0.10)	22	0.32 (0.13)
Pindrow fir	U	24	0.29 (0.09)	13	0.23 (0.12)

Results from logistic regression[2]

Type 3 Analysis of effects		--------------All trees--------------		-----------Infested trees-----------	
Effect	df	Wald Chi-Square	Pr > ChiSq	Wald Chi-Square	Pr > ChiSq
Block	3	9.0883	0.0281	5.5168	0.1376
Time	1	0.4595	0.4979	0.0153	0.9015
Species	13	58.8272	< .0001	41.1515	< .0001
Pesticide Treatment	1	0.5846	0.4445	0.0120	0.9126

[1] The parameter ‘Sus’ reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; M = moderately susceptible (tolerant); R = resistant; U = unknown.

[2] (Proc Logistic): Model: binary logit; optimization technique: Fisher’s scoring; probability modeled: gouting = 1; convergence criterion satisfied.

Figure 1. The suspended bolt technique applied to one of the treatment plots. Infested logs were cut and placed about 1 foot (1/3 meter) apart above the seedlings on sawhorses. Organza cloths were attached to the plot frames to prevent BWA crawlers from being blown from one plot to another.

Figure 2. The greenhouse after the installation of the first half of the study (time treatment #1 [August application of adelgids]). Trees were watered with a misting system (see above the plots) and also with rain coming through the screen.

CHAPTER 6. ARTIFICIAL INFESTATION OF PUTATIVE (VEITCH X BALSAM) X BALSAM FIR BACKCROSS SEEDLINGS

Abstract

Fir species native to eastern North America are highly susceptible to damage from the balsam woolly adelgid and some Asian fir species are resistant. An artificial infestation study consisting of 2- to 5-year old fir species was installed in an indoor growth chamber to test the responses of susceptible (balsam fir and Fraser fir) and resistant (Veitch fir) species, and trees representing a putative backcross between Veitch x balsam and balsam fir. The study lasted three months, during which time extensive mortality occurred. All species of fir showed evidence of settled BWA instars, but there was very little development from settled instars to adults. Ten of 12 balsam fir trees produced egg-laying adults and only one (Veitch x balsam) x balsam backcross exhibited any sign of BWA development.

Introduction

The balsam woolly adelgid (*Adelges piceae* Ratz.) is a major pest in natural fir (*Abies*) stands and plantations throughout North America. This tiny, piercing-sucking insect was introduced into Maine and Nova Scotia around 1900 (Balch 1952), most likely on imported nursery stock of European silver fir (*Abies alba* Miller) and was established on balsam fir (*Abies balsamea* [L.] Mill.) in Maine by 1908 (Kotinsky 1916). It quickly spread through eastern Canada and the northeastern United States and was first reported on Fraser fir (*Abies fraseri* [Pursh] Poiret) on Mount Mitchell in 1955 (Boyce 1955, Amman 1966). Fraser fir is endemic to the Southern Appalachians and is a foundation, or keystone, species in sensitive high elevation spruce/fir forests (Ellison et al. 2005, Bergmann et al. 1997). It is also one of the most popular Christmas tree species in North America. Fraser fir is highly susceptible to BWA damage and the use of chemical insecticides is currently the only effective means for controlling the adelgid in Christmas tree plantations. Chemical treatments for BWA are expensive and minimize the effectiveness of integrated pest management systems (Sidebottom 2009).

Fir species native to eastern North America (balsam fir, Fraser fir) are highly susceptible to BWA attack and can be killed by the insect. Damage from BWA is fairly consistent among susceptible species: gouting (swollen and abnormal growth of cells at the BWA feeding site) is one of the first responses (Balch 1952) and over time can lead to the disruption of phloem channels and metabolic pathways in the bark (Bryant 1971). Abnormal xylem development may occur, often distal to the feeding site, which casts the tree into a state of physiological drought, reducing the flow of water within the tree and compromising photosynthesis, transpiration, and respiration (Puritch 1973).

Some fir species exhibit marked resistance to BWA and are able to withstand intense infestation pressure, often without ever showing signs of BWA infestation or, if they do become infested, without suffering any damage from the adelgid. Veitch fir (*Abies veitchii* Lindley), an Asian fir species, is highly resistant to BWA infestation (Hall et al. 1971), although the resistance mechanisms are not yet fully understood.

Breeding for resistance to diseases and insects has been a prime target in breeding operations with trees; while classic breeding programs are time-consuming and care must be taken to preserve genetic diversity, this is a viable option for selecting pest-resistant trees. The development of BWA-resistant Fraser fir would be valuable for both the Christmas tree industry and for restoration of native stands. Interspecific hybrids between resistant and susceptible species may lead to higher resistance in fir and this method is currently being evaluated in an effort to combat *Phytophthora cinnamomi* (Rands) in the Southern Appalachians (Stejskal et al. 2011).

Our objective was to test the responses of susceptible (balsam fir and Fraser fir) and resistant (Veitch fir) species, and trees representing a putative backcross between Veitch x balsam and balsam fir to BWA infestation.

Methods and Materials

The study was initiated in an indoor growth chamber in the NCSU Insectary in October 2007. Temperatures were set to a constant 18ºC with even light days (12:12); relative humidity was not controlled but was estimated to be around 55%. The study utilized three fir species (*Abies veitchii* [Veitch fir], *A. balsamea* [balsam fir], *A. fraseri* [Fraser fir]) and one backcrossed fir ((*A. veitchii* x *A. balsamea*) x *A. balsamea*). The original parent trees for the Veitch x balsam backcross were grown by Les Corkum from Falmouth Nova Scotia. Putatively, the female parent was Veitch x balsam and the male parent was balsam fir. The seedlings were of three ages: (Veitch x balsam) x balsam backcross (3 years), Veitch (2 and 5 years), balsam (3 and 5 years), and Fraser fir (5 years). The backcross seedlings, the 2-year old Veitch fir seedlings, and the 3-year old balsam fir seedlings were obtained from Bob Giardin (New Hampshire), shipped from Itasca Greenhouse in Minnesota. The older Veitch, balsam, and Fraser fir trees were sown from seed provided by the NC State Christmas Tree Genetics Program and grown in a greenhouse at the NCSU Horticultural Field Lab in Raleigh for five years. The trees were all transplanted into 7.6 x 7.6 x 23 cm tree pots in medium consisting of finely chopped pine bark, peat moss and perlite. There were a total of 72 study trees, 12 of each species and age. The Fraser fir trees were representative of three seed sources (Mount Mitchell, Roan Mountain, Richland Balsam), four trees per seed source. The experiment utilized a completely randomized design.

Logs heavily infested with BWA were cut from an abandoned Christmas tree plantation in Ashe County and transported to NC State. Two logs, approximately 8 inches apart, were suspended over the study trees (Figure 1) for one month (October 7 to November 7, 2007). At the end of three months (January 2008), the study was dismantled and each tree assessed for height, diameter, the presence of settled instars (yes/no), instars, health (dead, poor, fair, good), signs of gouting (yes/no), and the presence of adults with egg masses (yes/no).

An analysis of variance was performed on height, diameter, and the number of settled instars using the General Linear Model procedure (PROC GLM) of SAS version 9.3 (SAS Institute 2010) and, where appropriate, pairwise comparisons were analyzed by the Tukey-Kramer Multiple Comparison Test with an alpha level of 0.05. Species was the main effect and we used age as a covariant. Variables with binary (yes/no) distribution (presence of adults with eggs) were analyzed using logistic regression analysis (PROC LOGISTIC or PROC GENMOD). To compare the differences between trees of the same age grown under the same conditions representing a known susceptible species and the unknown putative backcross, we created a new dataset with only 3-year old balsam and (Veitch x balsam) x balsam backcross trees and repeated the analyses as above dropping age from the model.

Results

There were highly significant height differences among the species and age groups (Table 1). The (Veitch x balsam) x balsam backcross trees were shorter than other species and the 2-year old Veitch fir trees were shorter with smaller diameters than older ones of other species (Table 1). With the exception of two of the 2-year old Veitch fir trees, all trees had BWA instars settled (at the base of buds, along the branch at the base of needles, at the nodes), but there were no significant age or species differences in the numbers of settled instars (Table 2). The logistic regression revealed significant differences (both species and age effects) in the presence of adults with egg masses (Table 3). An analysis of simple means revealed that only balsam fir (primarily the 3-year old trees) and the (Veitch x balsam) x balsam backcrosses supported development of adults with egg masses (Table 3).

A separate series of analyses were performed on a dataset consisting only of the 3-year old balsam and (Veitch x balsam) x balsam backcross trees. The balsam fir trees were significantly taller than the backcross trees but with a smaller diameter (Table 4).There were no differences in the numbers of settled instars (Table 5). There were highly significant differences in the presence of adults with egg masses (< .0001), with 83% of the balsam fir trees (10 of the 12 trees) supporting adults with eggs, and only one (of 12) of the (Veitch x balsam) x balsam backcross trees (Table 6). One-third (33%) of the balsam fir trees exhibited signs of gout production but none of the backcrosses did (Table 6).

Of the 72 total study trees, after the 3-month infestation period, only 26 trees were still alive (Table 7). All of the 2-year old and most (7 of 12) of the 5-year old Veitch fir trees died, as did the majority of trees representing the other species.

Discussion

A mortality rate of 64% during the course of this study is both unusual and noteworthy. This high mortality rate may be attributed primarily to transplant shock. All of the trees used in the study had been transplanted into tree pots only two weeks prior to the initiation of the study and exposure to BWA crawlers. Additionally, while the literature reports only two to three generations of BWA in the Southern Appalachians (e.g., Arthur and Hain 1984), in sheltered areas it is not unusual to find active BWA development even into December (Newton, unpublished data). This study was initiated in October, and the logs utilized as sources of BWA crawlers were heavily infested; bringing the logs into the controlled and warm experimental environment allowed BWA development to continue taking place longer than it may have occurred in nature. Allowing the logs to remain suspended over the trees for a full month, with the resultant steady downfall of active crawlers is likely to have increased the stress on the trees. However, the fact that there were two trees that did not exhibit any settled BWA instars (both 2-year old Veitch fir trees) and the fact that both of these trees died during the course of the study, adds credence to the idea that mortality may be due primarily to causes not related to infestation levels. We did not track mortality rates over time so it is unknown whether all trees died at the same time or whether some died earlier than others.

Why adults developed primarily only on the younger balsam fir trees is difficult to explain. Balsam fir is known to be susceptible to BWA (e.g., Mitchell 1966), but so is Fraser fir (e.g., Balch 1952). None of the Fraser fir trees and only two of the 5-year old balsam fir trees in this study supported BWA development to the adult stage. The Fraser fir trees were taken from a cool greenhouse in early October and were not in a period of active growth. The 3-year old balsam fir trees had also been growing in a greenhouse (in Minnesota) and it is unknown whether it was heated. The seedlings themselves appeared very healthy, green, and succulent. No adults developed on either the 2- or 5-year old Veitch fir trees. Because Veitch fir is known to be resistant to BWA, a low infestation rate is not surprising. However, we have been able to support BWA development on Veitch fir seedlings (see Chapter 5) and at least a small level of development was expected, especially on the older trees. Obviously, those trees that had died early in the course of the study could not be expected to develop any gouting response or support BWA development, but many (almost half) of the 5-year old Fraser fir trees were still living and appeared to be in relatively good health. It may be that these 5-year old Fraser fir trees were essentially dormant and this may have had an effect on BWA development as well. The stimuli that would cause a BWA first instar to come out of its resting phase and continue development may have been lacking in the 5-year old trees, but, because the 3-year old balsam fir trees were still in an active state, this stimuli may have been present. Similarly, only four balsam fir trees, all 3-year old and all of which produced egg-laying adults, showed any signs of gouting. Gouting results from the production of abnormally large parenchyma cells in amounts greater than normal and occurs primarily during active tree growth. Again, because this study was initiated in October and most of the trees were not actively growing, this lack of gouting response to the presence of settled instars may not be unusual for late season infestations.

Our objective for this study was to determine whether a backcross developed from a resistant species (Veitch fir) and a susceptible species (balsam fir) exhibits resistance to

BWA infestation. Ten of 12 balsam fir trees (83%) produced egg-laying adults and only one (Veitch x balsam) x balsam backcross exhibited any BWA development. The balsam fir trees and the (Veitch x balsam) x balsam backcrosses were the same age and grown under the same conditions. The Veitch fir on average would contribute 50% of the genes to the F1 hybrids and 25% to the backcrosses. The significant difference between infestation levels between the two species indicates that the mechanisms controlling resistance to BWA in Veitch fir may be genetically controlled and may be heritable. Further studies are warranted.

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Table 1. Means and least squares means (± S.E.) for height and diameter. Includes all species and age groups. Overall lsmeans and p-values (Type III sums of squares) from the general linear model are also included. Lsmeans within a column that share the same superscript letter are not significantly different from one another.

			----------Mean (± S.E.)----------		---------Lsmean (± S.E.)---------	
Species	Sus[1]	N	Height (cm)	Diameter (mm)	Height (cm)	Diameter (mm)
Veitch fir	R	24	21.5 (1.7)	6.0 (0.7)	23.4 (0.9)^{a}	7.2 (0.3)^{a}
Balsam fir	S	24	31.2 (0.7)	5.9 (0.5)	30.2 (0.9)^{b}	5.3 (0.3)^{b}
Veitch x balsam fir	U	12	24.0 (1.2)	4.3 (0.2)	26.0 (1.3)^{ab}	5.5 (0.5)^{b}
Fraser fir	S	12	34.3 (1.5)	8.6 (0.4)	30.4 (1.4)^{b}	6.2 (0.4)^{ab}
Overall LSMeans from general linear model					27.3 (3.9)	6.1 (1.2)
ANOVA p-values from general linear model						
Source of variation	df				Pr > F	Pr > F
Model	4				< .0001	< .0001
Age	1				< .0001	< .0001
Species	3				< .0001	< .0001
Error	67					

[1] The parameter 'Sus' reflects the *a priori* susceptibility rating for each species: S = highly susceptible; R = resistant; U = unknown.

Table 2. Means and least squares means (± S.E.) for the total number of settled instars. Includes all species and age groups. Overall lsmeans and p-values (Type III sums of squares) from the general linear model are also included.

Species	Sus[1]	N	Mean (± S.E.) No. settled	Lsmean (± S.E.) No. settled
Veitch fir	R	24	113.2 (34.0)	147.5 (81.4)
Balsam fir	S	24	389.0 (114.1)	371.8 (78.9)
Veitch x balsam fir	U	12	236.7 (85.1)	271.0 (112.7)
Fraser fir	S	12	354.2 (100.3)	285.6 (119.7)
Overall LSMeans from general linear model				265.9 (382.1)
ANOVA p-values from general linear model				
Source of variation		df		Pr > F
Model		4		0.0674
Age		1		0.1448
Species		3		0.2936
Error		67		

[1] The parameter 'Sus' reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; R = resistant; U = unknown.

Table 3. Means and least squares means (± S.E.), and results from logistic regression for adults with eggs (presence=1, absence=0). Includes all species and age groups.

Species	Sus[1]	N	Mean[2] (± S.E.) No. Adults	Lsmean[2] (± S.E.) No. Adults
Veitch fir	R	24	0.00 (0.00)	- 0.04 (0.06)
Balsam fir	S	24	0.50 (0.10)	0.52 (0.06)
Veitch x balsam fir	U	12	0.08 (0.08)	0.04 (0.09)
Fraser fir	S	12	0.00 (0.00)	0.09 (0.10)

Results from the Logistic Regression[3]

Type 3 analysis of effects

		----------Adults----------	
Effect	df	Wald Chi-Sq	Pr > ChiSq
Species	3	10.8232	0.0127
Age	1	7.6774	0.0215

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[1] The parameter 'Sus' reflects the *a priori* susceptibility rating for each species: S = highly susceptible; R = resistant; U = unknown.

[2] The means were calculated using the means procedure and least squares means from the glm procedure.

[3] (Proc Logistic): Model = binary logit; Optimization technique = Fisher's scoring; Likelihood penalty = Firth's bias correction; Probability modeled: Adults=1; Convergence criterion satisfied.

Table 4. Means and least squares means (± S.E.) for height and diameter. Includes only 3-year old balsam and (Veitch x balsam) x balsam fir backcross. Overall lsmeans and p-values (Type III sums of squares) from the general linear model are also included. Lsmeans within a column that share the same superscript letter are not significantly different from one another.

			----------Mean (± S.E.)----------		---------LSMean (± S.E.)---------	
Species	Sus[1]	N	Height (cm)	Diameter (mm)	Height (cm)	Diameter (mm)
Balsam fir	S	12	31.3 (0.7)	3.6 (0.2)	31.3 (1.0)^{a}	3.6 (0.2)^{a}
Veitch x balsam fir	U	12	24.0 (1.2)	4.3 (0.2)	24.0 (1.0)^{b}	4.3 (0.2)^{b}
Overall LSMeans from general linear model					27.7 (3.4)	4.0 (0.7)
ANOVA p-values from general linear model						
Source of variation	df				Pr > F	Pr > F
Model	1				< 0.0001	0.0351
Species	1				< 0.0001	0.0351
Error	22					

[1] The parameter 'Sus' reflects the *a priori* susceptibility rating for each species: S = highly susceptible; R = resistant; U = unknown.

Table 5. Means and least squares means (± S.E.) for the total number of settled instars. Includes only 3-year old balsam fir and (Veitch x balsam) x balsam backcrosses. Overall lsmeans and p-values (Type III sums of squares) from the general linear model are also included.

Species	Sus[1]	N	Mean (± S.E.) No. settled	LSMean (± S.E.) No. settled
Balsam fir	S	12	313.5 (82.0)	313.5 (83.6)
Veitch x balsam fir	U	12	236.7 (85.1)	236.7 (83.6)
Overall LSMeans from general linear model				275.1 (289.5)
ANOVA p-values from general linear model				
Source of variation		df		Pr > F
Model		1		0.5223
Species		1		0.5223
Error		22		

[1] The parameter 'Sus' reflects the *a priori* susceptibility rating (Mitchell 1966) for each species: S = highly susceptible; R = resistant; U = unknown.

Table 6. Means and Least squares means (± S.E.) for adults with eggs (presence=1, absence=0) and signs of gouting (presence=1, absence=0). Includes only 3-year old balsam fir and (Veitch x balsam) x balsam backcrosses.

			-------Mean (± S.E.)-------		-------Lsmean (± S.E.)-------	
Species	Sus[1]	N	Adults	Gouting	Adults	Gouting
Balsam fir	S	12	0.83 (0.11)	0.3 (0.1)	0.83 (0.1)	0.11 (0.05)
Veitch x balsam fir	U	12	0.08 (0.08)	0.0 (0.0)	0.08 (0.1)	0.00 (0.10)

Results from the Logistic Regression[3]

Type 3 analysis of effects

		----------Adults----------		----------Gouting---------	
Effect	df	Chi-Sq	Pr > ChiSq	Chi-Sq	Pr > ChiSq
Species	1	15.41	< .0001	6.35	0.0117

[1] The parameter ‘Sus’ reflects the *a priori* susceptibility rating for each species: S = highly susceptible; R = resistant; U = unknown.

[2] Least squares means from general linear model.

[3] (Proc Genmod): Binomial distribution, link = Logit; Probability modeled: Adults=1, Gouting=1; Convergence criterion satisfied.

Table 7. Health and infestation level (presence of adults with eggs) of trees.

Species	Age	Health Living	 Dead	Number of trees with adults
Veitch fir	2	0	12	0
Veitch fir	5	3	9	0
Balsam fir	3	6	6	10[1]
Balsam fir	5	5	7	2[2]
Veitch x balsam fir	3	5	7	1[3]
Fraser fir (Richland Balsam)	5	2	2	0
Fraser fir (Mount Mitchell)	5	3	1	0
Fraser fir (Roan Mountain)	5	2	2	0

[1] Four of the trees with adults were dead, and six were still living.

[2] Both of the trees with adults were dead.

[3] The one tree with adults was still alive.

Figure 1. Artificial infestation of Veitch, balsam, putative (Veitch x balsam) x balsam backcrosses, and Fraser fir seedlings. Randomized design. Trees were infested utilizing the rain-down method, mimicking natural dispersal.

CHAPTER 7. ASSESSING BALSAM WOOLLY ADELGID RESISTANCE IN A CLONAL FRASER FIR SEED ORCHARD

Abstract

A clonal Fraser fir seed orchard in Avery County, North Carolina was naturally infested with the balsam woolly adelgid (BWA), an exotic, soft-bodied insect. The orchard contained 59 clones with over 1200 individual trees. Our objective was to rate the infestation level of each tree and assess resistance to BWA among the 59 Fraser fir clones. We also measured other variables, including bark thickness, bark appearance, crown health, apical dominance, and bryophyte or lichen coverage. Over 96% of the individual trees were infested with BWA and, although we found significant differences among clones, every clone was infested at some level. Heritability calculations for infestation level were initially low (0.083) and remained low after accounting for spatial effects (0.052). The orchard has been routinely treated with an insecticide each year and we believe these treatments may be masking any genetic effects associated with BWA resistance. Most of the trees appeared healthy and, while many were heavily infested with BWA, they appeared to be tolerating the infestations.

Introduction

Fraser fir (*Abies fraseri* Pursh Poiret) is endemic to the Southern Appalachians and is one of the most popular Christmas trees in North America. The balsam woolly adelgid (BWA; *Adelges piceae* Ratz.), a tiny, piercing-sucking insect introduced from Central Europe and first reported on Mount Mitchell in 1955 (Boyce 1955, Amman 1966), killed up to 95% of native Fraser fir stands during the first wave of mortality and is still one of the most damaging and expensive pests for Christmas tree growers. The threshold for treatment is one infested tree and chemical insecticides are currently the only effective means for controlling this pest. These treatments cost the industry over $1.5 million per year (Potter et al. 2005) and may compromise the effectiveness of integrated pest management systems (Sidebottom 2009).

Fir species vary in their susceptibility to BWA attack. Highly susceptible species (e.g., Fraser fir) experience heavy infestations of BWA and are damaged by the presence of the feeding adelgid. Host responses may include gouting or swelling of buds, twigs and branches at the feeding site, the production of abnormal xylem (often called rotholz), and loss of apical dominance (Balch 1952). Moderately susceptible or tolerant species (e.g., European silver fir [*Abies alba* Miller]) may become infested with the adelgid, even heavily infested, but exhibit reduced severity of symptoms even under advanced infestation. Resistant species (e.g., Veitch fir [*Abies veitchii* Lindl.]) exhibit no infestation or a reduced infestation level even under advanced infestation pressure.

Host responses that may aid in the resistance or tolerance to BWA infestation include the production of thick outer bark (Pschorn-Walcher and Zwolfer 1958, Schooley and Bryant 1978) or the formation of a secondary periderm (Mullick 1975, Hain et al. 1991), which isolates the BWA infestation site and protects the underlying area. Juvabione or juvabione-like compounds may be produced in response to BWA attack (Puritch and Nijholt 1974, Fowler et al. 2001); these compounds mimic insect juvenile hormone and inhibit or disrupt normal development in some insects (Slama and Williams 1965). Some firs produce copious amounts of resin in response to wounds, a characteristic that may aid in BWA resistance

(Mitchell 1966). Bark texture may influence susceptibility, in that rough or flaky bark may provide a more suitable substrate for BWA infestation than smooth bark.

Among and within all *Abies* species, even those highly susceptible to BWA infestation, trees have been observed to tolerate and even recover from BWA attack. Balsam fir (*Abies balsamea* (L.) Mill.) often recovers from adelgid attack after a few years, frequently replacing original leaders (Balch and Carroll 1956, Schooley 1976). Within the natural range of Fraser fir, there are remnant populations that survived the initial wave of destruction and these trees have survived multiple decades of adelgid attack. Mount Mitchell Fraser fir stands suffered high mortality during the initial wave of infestation; however, years later these stands contained trees that, when cored, showed the production of roltholz at some point but were uninfested and otherwise appeared healthy (Hain et al. 1991).

The development of BWA-resistant Fraser fir trees would be an effective and relatively inexpensive solution to a difficult pest problem and would be useful for both the Christmas tree industry and for restoration of native stands of Fraser fir. An important step in this process is to determine whether there are resistant trees within the existing population of Fraser fir. This chapter details a study of BWA infestation in a clonal Fraser fir seed orchard; our objective for the study was to assess resistance to the balsam woolly adelgid among 59 Fraser fir clones.

Methods and Materials

Our study site was a Fraser fir clonal seed orchard at the Linville River Nursery near Crossnore, North Carolina. The orchard was established in 1983, 1984 and 1985 with grafts taken from The Lodge Orchard, established in the late 1960s and consisting of superior trees selected and transplanted from growers' fields. Most of the 312 trees in the original Lodge Orchard are believed to be from the Roan Mountain seed source.

The clonal seed orchard consists of three separate plantings (1983, 1984 and 1985) of 488, 421 and 422 individuals, respectively. Thirty-eight (38) clones were planted each year, on a 3 x 3 meter spacing. The balsam woolly adelgid is widespread throughout the southern Appalachians (the closest natural stand of Fraser fir trees is on Grandfather Mountain, less than ten kilometers from the orchard), thus placing the orchard under pressure for natural BWA infestations. The site is managed by the North Carolina Department of Forest Resources and trees within the orchard are sprayed with an insecticide each year in late summer (August or September). The insecticides are rotated each year: Thionex (non-systemic, chlorinated cyclic sulfurous acid ester insecticide) 160 mL / 100 L (21 oz. / 100 gal.) + 470 mL (16 oz.) dimethoate (systemic); Safari (dinotefuran 20% SG) 60 mL / 100 L (8 oz./100 gal.) + 470 mL (16 oz.) dimethoate.

In October 2006 each tree within the orchard was assessed for: level of BWA infestation (rated 0 to 3, none, light, moderate, heavy [Figures 1 to 3]); bark appearance (as a measure of bark response to BWA infestation; rated 1-5 representing smooth to rough); bark thickness (measures with a bark gauge in cm); crown health (rated 1-5: healthy to chlorotic); apical dominance (yes or no); lichen coverage (including bryophytes and lichens, rated 0 to 3: none, light, moderate, heavy [Figure 4]); height (estimated); and diameter at breast height (dbh, measured with a diameter tape).

Spatial and environmental effects

To account for spatial and possible environmental effects, we created a matrix of the orchard (in Microsoft Excel) with each cell representing a 3 x 3 meter area. We coded each cell with a number from 0 to 9 to represent the infestation level of trees within rows, missing trees within rows, orchard roads, gravel roads and surrounding forest (Figure 5). We created an ascii file from the Excel matrix and imported the data into ArcGIS ArcMap (Version 9.1.3) as a raster (grid) dataset. We converted the raster to a point shapefile and used spatial statistics calculate an average nearest neighbor index, incorporating all cells (trees and neighboring roads, forest, etc.). The ArcGIS software automatically created a raster from the average nearest neighbor analysis, with each cell containing a value representing the neighbor index. We then used that raster to create a new one containing only cells representing trees and converted the new raster to a point shapefile (using ArcGIS Version 10). We created three separate datasets (using the methodology described above, viz. convert Excel matrix to ascii file and import into ArcMap as a raster, convert the raster to a point shapefile) representing trees: clones, infestation levels, and the neighbor metric. We then joined the three datasets into one and exported that table into Excel for additional statistical analysis. We calculated Morans Index (as a measure of spatial autocorrelation, in ArcGIS Version 10) using the dataset representing trees only. We also conducted a hot spot analysis (in ArcGIS Version 10) on the infestation levels (only the trees, values 0 to 3) to determine if there were areas with significantly high (hot) infestation levels or low (cold) infestation levels. The hot spot analysis tool calculates a statistic for each feature in a dataset (in our case, infestation levels). The resultant z-scores and p-values show where features (infestation levels) with high or low values cluster spatially. The tool works by looking at each feature (infestation level of a given tree) within the context of neighboring features (infestation levels of neighboring trees).

Statistical analysis

Statistical analyses were conducted utilizing SAS software, version 9.3, and all data (all clones across all three planting years). An analysis of variance was conducted utilizing a general linear model (proc glm). The full model incorporated the following effects: year, clone, neighbor statistic, clone x year interaction. Year was the only fixed effect. Class variables included year and clone; the neighbor statistic was used as a covariant in the model. Least squares means were calculated (lsmeans statement, Tukey's test, $p < .05$).

Broad-sense heritability was calculated for infestation level, bark appearance, and bark thickness utilizing the following equation:

$$h^2_i = \sigma^2_c / (\sigma^2_c + \sigma^2_{y*c} + \sigma^2_e),$$

where, σ^2_c is the variance due to clone differences, σ^2_{y*c} is the variance due to year x clone interactions, and σ^2_e is the error variance. The variance components of random effects were estimated (proc varcomp) by the restricted maximum likelihood (REML) method. The nearest neighbor analysis described above provided a neighbor value for each tree. We used this value as a covariant and re-ran the general linear model for infestation level under a variety of modeling regimes (Table 3), including all clones over all years (59 clones), only clones present in all three years (17 clones), full model (*y = year clone year*clone*), partial model (*y = year clone*), with and without the neighbor value. We took into account both the

r^2 value for the model itself and the ultimate heritability calculation. Because there were no year*clone interactions, we dropped the full models from Table 3 and present only the partial models.

Clone means were calculated (‘proc means’) and correlations (‘proc corr’) made between the means for the following variables: infestation level, bark appearance, bark thickness, lichen coverage, crown health, apical dominance, and diameter at breast height (dbh).

A new variable, ‘percent infested,’ was calculated to assess the overall resistance of each clone. The values were ‘0’ (for any infestation level equaling zero) and ‘100’ (for any infestation level above zero). This variable was added to the general linear model for the analysis of all clones in all years.

The orchard contains 59 Fraser fir clones. Thirty-eight (38) clones were planted each year, some of the 59 total clones were present in all years, and others for only one or two years. Seventeen (17) clones are represented in all three planting years, 21 represented in 1984 and 1985, and 21 represented in 1983 only.

Results

Over 96% of the trees in the orchard were infested with BWA (Table 1). Although there were a few individual trees with no visible BWA infestations, every clone contained infested trees and there were no significant clone effects in the percent of infested trees. There were highly significant clone effects for infestation level, bark appearance, bark thickness, lichen coverage, apical dominance, crown health, and tree diameter. Year effects are confounded by the fact that not all clones were present in all years. A separate analysis of the 17 clones that were present in all three planting years reduced or, in some cases, eliminated significant year effects. There were no year*clone interactions (Table 2).

Clone mean heritability calculations from the initial analysis were low (Table 3) for infestation level ($h^2 = 0.084$), bark appearance ($h^2 = 0.149$) and bark thickness ($h^2 = 0.113$). Because infestation level was our primary variable of interest and because we believe that there is a basis for genetic resistance to BWA, we conducted additional analyses on the infestation levels to account for spatial effects (as described in Methods and Materials above).

We determined spatial autocorrelation for infestation levels throughout the orchard (Figure 5) by calculating Moran's I index. The results (Figure 6) show a clustered pattern and indicate that there is a less than 1% likelihood this pattern could be the result of random chance. We then calculated average nearest neighbor values; the overall results also showed a clustered pattern.

Although including the neighbor value as a covariant did improve the r^2 of the general linear model by 3-4 times, the heritability calculations (Table 3) were not improved by accounting for spatial effects, but rather, reduced (e.g., 59 clones, full model without neighbor value, $h^2 = 0.0827$; 59 clones, full model with neighbor value as covariant, $h^2 = 0.0518$).

Modeling regime #2 (all 59 clones, partial model with the neighbor value) (Table 3) provided a reasonably good fit for the overall model ($r^2 = 0.387$) and, although exceedingly low, a reasonable heritability calculation as compared with the others. Results from this analysis show a range of means in clonal infestation levels from 1.65 (± 0.2) in Clone 102 to

3.0 (± 0) in Clones 305 and 278 (Table 4). The results from the hot spot analysis (Figure 7) show significantly 'cool' spots (clustered areas with lower infestation levels) and 'hot' spots (clustered areas with significantly higher infestation levels).

Correlations among variables were made on a year by year basis, but because there were no clone x year interactions, and because the correlations were similar in each year, these results detail correlations between the means for the 59 clones over all three years rather than year by year (Table 5). There is a positive correlation between infestation level and bark appearance (the higher the infestation level, the rougher the bark), and infestation level and bark thickness (the higher the infestation level, the thicker the bark). There is a negative correlation between infestation level and apical dominance (the higher the infestation level, the greater the degree that apical dominance has been lost), and infestation level and lichen coverage (heavier infestations are found on trees with less lichen coverage). There was a negative correlation between crown health and apical dominance (the more healthy the crown, the less likely the tree exhibited apical dominance). Correlations between percent infested and other variables were similar to infestation levels and other variables.

Discussion

The balsam woolly adelgid has been present in the Southern Appalachians since the mid 1950s and is now widely spread throughout the region. Infestation pressure is intense on Fraser fir stands, whether natural or planted, and this orchard has clearly been under infestation pressure for some time. Although there are significant differences in infestation levels among the clones in this orchard, it is difficult to gauge whether these differences are due to genetic resistance in some clones or whether the differences are due to the effect of routine insecticide applications in the management of the orchard. While the insecticide treatments have not prevented BWA infestation, they may have been enough to mask any true genetic differences in BWA susceptibility. Heritability calculations were expected to be higher. The techniques employed to produce the neighbor statistic were exploratory and there may be a better method of accounting for spatial effects.

The clustered patterns shown in the hot spot analysis (Fig. 7), clearly reveal patterns of greater and lesser infestation levels in different points in the orchard. The cool spots (colored blue, Fig. 7) are on edges and near orchard roads. These areas may be slowing lower infestation levels because they can be reached more readily with spraying equipment and are chemically more protected than other areas, or because they are protected from wind-blown BWA crawlers by the surrounding mixed forest. The hot spots (colored red, Fig. 7) are often within the interior of the rows and within the interior of the planted areas for each year, but not always. For example, in the 1984 planting area (middle of the orchard, to the right of the grey gravel road, Fig. 7), there are relatively few cool spots and the hot spots are very near the road leading from the 1984 to the 1985 planting (far right, Fig. 7). It could be that infestation pressure is higher in that area due to infested natural stands (e.g., Grandfather Mountain), because surrounding trees are heavily infested, or because the trees may be more susceptible. We cannot make a final determination on the genetic effect on infestation level because of the uncertainty related to insecticide treatments.

The positive correlations between increases in infestation levels and most other measured variables were within expectations. Because one of the responses to BWA infestation is the production of thick outer bark (Schooley and Bryant 1978) and the production of larger, more numerous cells than normal at the site of infestation (Balch 1952), these effects are understandable. Interestingly, thick outer bark may protect trees from BWA damage or enable them to tolerate infestation; this is one of the traits thought to protect European silver fir (Pschorn-Walcher and Zwolfer 1958), enabling it to be one of the more tolerant fir species. The formation of secondary periderm consisting of necrophylactic tissue around a wound may help to cloister the wounded tissue from healthy tissue, thus protecting the tree from further damage (Mullick 1975; Hain et al. 1991). The rough texture of the bark may be the result of a wound healing mechanism in these trees. There is no question that these trees have been heavily infested for some time with BWA and that many of them appear to be quite healthy.

The negative correlation between infestation level and apical dominance is expected. Because one of the first symptoms of BWA infestation in Fraser fir is the loss of apical dominance (Sidebottom 2004), it is natural that the higher the level of infestation, the more likely apical dominance has been lost. The correlation between crown health and apical dominance (clones with healthier crowns have a higher percentage of trees exhibiting the loss of apical dominance) (Table 5) is more difficult to account for. One explanation is that healthy trees may be able to transmit the signal from the site of infestation to the apex better than unhealthy trees with rotholz (abnormal xylem) slowing the signal. The loss of apical dominance in some trees could be due to the fact that the orchard is at a relatively high elevation in the mountains and is exposed to extreme weather conditions. Some of the tops may have broken off in a storm.

Bryophytes are the small green plants commonly known as mosses, liverworts and hornworts. They have primitive tissues for conducting food and water. Because they lack roots, they obtain their water through direct surface contact with their environment. Lichens are dual organisms consisting of a fungus and an algae or cyanobacterium. Collectively known as cryptogams, bryophytes and lichens are important components of forest ecosystems (Hutton and Woodward 2002). Many bryophyte species grow on the bark of Fraser fir (Smith 1984) and Fraser fir is the preferred substrate tree of approximately 20 of the 100 epiphytic lichen species that occur in the spruce-fir forests of the Southern Appalachians (Dey 1984). We found that trees with heavy bryophyte and lichen coverage were less likely to be heavily infested. This is likely the result of the species combination on any given fir tree – many species grow tightly attached to the bark of the fir trees (see, for example, Figure 4). If the lichens provide a patchlike cover over much of the bark, it may be difficult for the adelgid to insert its stylets into the bark to feed, or there may be some inhibitory effect. Alternately, if the cryptogams are loosely attached to the bark, adelgids may be able to crawl underneath and use the material as an additional protective cover.

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Table 1. Overall means (± S.E.) & ANOVA p-values of means (from general linear model). Dataset includes all 59 clones across all three planting years. Model: *y = year clone year*clone*. N=1280.

	Percent Infested	*Infestation Level*	*Bark Appearance*	*Bark Thickness*	*Lichen Coverage*	*Apical Dominance*	*Crown Health*	*dbh*
	-------Means-------							
	96.6 (17.9)	2.47 (0.8)	3.75 (1.0)	0.52 (0.1)	2.02 (0.8)	0.65 (0.4)	2.3 (0.8)	21.0(3.4)
ANOVA P values (Type III SS)								
Year	0.0006	< .0001	0.1163	< .0001	< .0001	< .0001	< .0001	< .0001
Clone	0.2166	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001
Year*Clone	0.6093	0.5244	0.0428	0.3429	0.0102	0.2568	0.5102	0.3184

Table 2. Overall means (± S.E.) & ANOVA p-values of means (from general linear model). Dataset includes only the 17 clones present in all three planting years. Model: _y = year clone year*clone_. N=544.

	Percent Infested	*Infestation Level*	*Bark Appearance*	*Bark Thickness*	*Lichen Coverage*	*Apical Dominance*	*Crown Health*	*dbh*
	Means							
	95.6 (20.4)	2.40 (0.9)	3.63 (1.1)	0.51 (0.1)	2.05 (0.8)	0.67 (0.4)	2.2 (0.8)	20.3 (3.5)
ANOVA P values (Type III SS)								
Year	0.0095	< .0921	0.4222	< .0001	< .0001	0.0008	< .0001	0.0132
Clone	0.3435	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001
Year*Clone	0.6631	0.4804	0.2203	0.2976	0.0904	0.9107	0.6203	0.6432

Table 3. General linear model r^2 values and heritability calculations for infestation levels under various modeling regimes. Variance components were obtained using the ‘varcomp’ procedure (restricted maximum likelihood method) in SAS. There were a total of 59 clones present across all planting years and 17 clones that were present in all planting years. Where ’59 clones’ is cited, this reflect that all clones across all planting years were used in the analysis (N = 1282); where ’17 clones’ is cited, this reflects that only clones present in all three planting years were used (N = 548).

			Variance components		Heritability
	Modeling regime	r^2	σ^2_{clone}	σ^2_{error}	h^2
1)	59 clones, partial model without neighbor value *(infest = year clone)*	0.1374	0.06062	0.65677	0.084501
2)	59 clones, partial model with neighbor value *(infest = year neighbor clone)*	0.3870	0.02248	0.37735	0.056224
3)	17 clones, partial model without neighbor value *(infest = year clone)*	0.0952	0.05134	0.75885	0.063368
4)	17 clones, partial model with neighbor value *(infest = year neighbor clone)*	0.3951	0.02248	0.37735	0.056224

Table 4. Clonal means and least squares means (from general linear model) for infestation levels. Includes all clones across all years. The model fitted here is $y = year\ clone\ neighbor$ (Regime #2 from Table 3). Clones are listed from least to most susceptible (according to these results). Overall means and p-values (Type III sums of squares) from the general linear model are also included.

		---------Infestation level---------	
Clone	N	Mean (± S.E.)	Lsmean (± S.E.)
102	23	1.65 (0.17)	1.70 (0.14)
270	15	1.80 (0.31)	2.02 (0.18)
227	23	1.87 (0.23)	2.08 (0.14)
236	33	1.82 (0.17)	2.08 (0.12)
266	10	1.90 (0.41)	2.11 (0.22)
2	32	2.12 (0.17)	2.16 (0.12)
273	39	2.23 (0.15)	2.19 (0.11)
295	11	2.00 (0.27)	2.21 (0.21)
45	35	2.17 (0.17)	2.22 (0.12)
289	34	2.15 (0.16)	2.23 (0.12)
191	28	2.25 (0.16)	2.28 (0.13)
234	25	2.12 (0.18)	2.30 (0.14)
252	11	2.18 (0.26)	2.30 (0.21)
208	22	2.45 (0.21)	2.33 (0.15)
151	20	2.25 (0.19)	2.33 (0.15)
296	15	2.07 (0.25)	2.37 (0.18)
241	25	2.52 (0.17)	2.38 (0.14)
23	25	2.68 (0.16)	2.40 (0.14)
213	27	2.52 (0.18)	2.43 (0.13)
205	34	2.47 (0.16)	2.44 (0.12)
279	31	2.55 (0.15)	2.44 (0.12)
62	30	2.57 (0.16)	2.45 (0.13)
105	25	2.56 (0.13)	2.47 (0.14)
238	24	2.58 (0.18)	2.47 (0.14)
312	10	2.50 (0.27)	2.49 (0.22)
153	32	2.53 (0.15)	2.50 (0.12)
276	13	2.38 (0.27)	2.51 (0.19)
162	26	2.58 (0.14)	2.51 (0.14)
223	32	2.56 (0.12)	2.52 (0.12)
219	37	2.51 (0.15)	2.53 (0.11)
24	23	2.52 (0.18)	2.56 (0.14)
265	12	2.67 (0.18)	2.56 (0.20)
182	20	2.50 (0.17)	2.56 (0.15)
40	23	2.61 (0.17)	2.58 (0.14)
39	18	2.78 (0.13)	2.59 (0.16)
249	9	2.67 (0.24)	2.61 (0.23)
90	35	2.54 (0.12)	2.62 (0.12)
245	12	2.50 (0.23)	2.63 (0.20)
262	16	2.56 (0.18)	2.64 (0.18)
134	26	2.85 (0.09)	2.64 (0.14)
104	34	2.59 (0.15)	2.64 (0.12)
81	18	2.67 (0.20)	2.66 (0.16)
61	18	2.94 (0.06)	2.68 (0.16)
225	31	2.77 (0.10)	2.69 (0.12)
250	13	2.69 (0.17)	2.69 (0.19)
304	13	2.61 (0.21)	2.70 (0.19)

Table 4 Continued

Clone	N	Infestation level Mean (± S.E.)	Infestation level Lsmean (± S.E.)
280	11	2.81 (0.18)	2.71 (0.21)
282	23	2.61 (0.17)	2.72 (0.14)
248	13	2.69 (0.21)	2.73 (0.19)
237	36	2.72 (0.10)	2.74 (0.11)
293	13	2.69 (0.24)	2.74 (0.19)
307	13	2.61 (0.21)	2.75 (0.19)
277	9	2.89 (0.11)	2.77 (0.23)
148	19	2.89 (0.07)	2.80 (0.16)
291	10	2.70 (0.21)	2.81 (0.22)
128	24	2.87 (0.09)	2.83 (0.14)
203	23	2.87 (0.09)	2.84 (0.14)
305	11	3.00 (0.00)	2.96 (0.21)
278	9	3.00 (0.00)	3.13 (0.23)
Overall Lsmeans from general linear model			2.48 (0.68)
ANOVA p-values from general linear model			
Source of variation	df		Pr > F
Model	61		< .0001
Year	2		0.0123
Clone	58		< .0001
Neighbor	1		< .0001
Error	1220		

Table 5. Pearson Correlation Coefficients (based on means) for all clones across all three planting years.

Pearson Correlation Coefficient N = 114
Prob > \r\ under HO: Rho=0

	Infestation level	Bark appearance	Bark thickness	Lichen coverage	Crown health	Apical dominance	Percent infested
Infestation level	1	0.7484 < .0001	0.5902 < .0001	-0.5845 < .0001	0.2505 0.0072	-0.2769 0.0029	0.5598 < .0001
Bark appearance	0.7484 < .0001	1	0.5340 < .0001	-0.4396 < .0001	0.2225 0.0173	-0.3447 0.0002	0.2695 0.0037
Bark thickness	0.5902 < .0001	0.5340 < .0001	1	-0.5468 < .0001	0.4829 < .0001	-0.4057 < .0001	0.3473 0.0002
Lichen coverage	-0.5845 < .0001	-0.4396 < .0001	-0.5468 < .0001	1	-0.0146 0.8771	0.1278 0.1755	-0.3503 0.0001
Crown health	0.2505 0.0072	0.2225 0.0173	0.4829 < .0001	-0.0146 0.8771	1	-0.3552 0.0001	0.1530 0.1042
Apical dominance	-0.2769 0.0029	-0.3447 0.0002	-0.4057 < .0001	0.1278 0.1755	-0.3552 0.0001	1	-0.2023 0.0308
Percent infested	0.5598 < .0001	0.2695 0.0037	0.3473 0.0002	-0.3503 0.0001	0.1530 0.1042	-0.2023 0.0308	1

Figure 1. Light BWA infestation on Fraser fir stem.

Figure 2. Moderate BWA infestation on Fraser fir stem.

Figure 3. Heavy BWA stem infestation on Fraser fir.

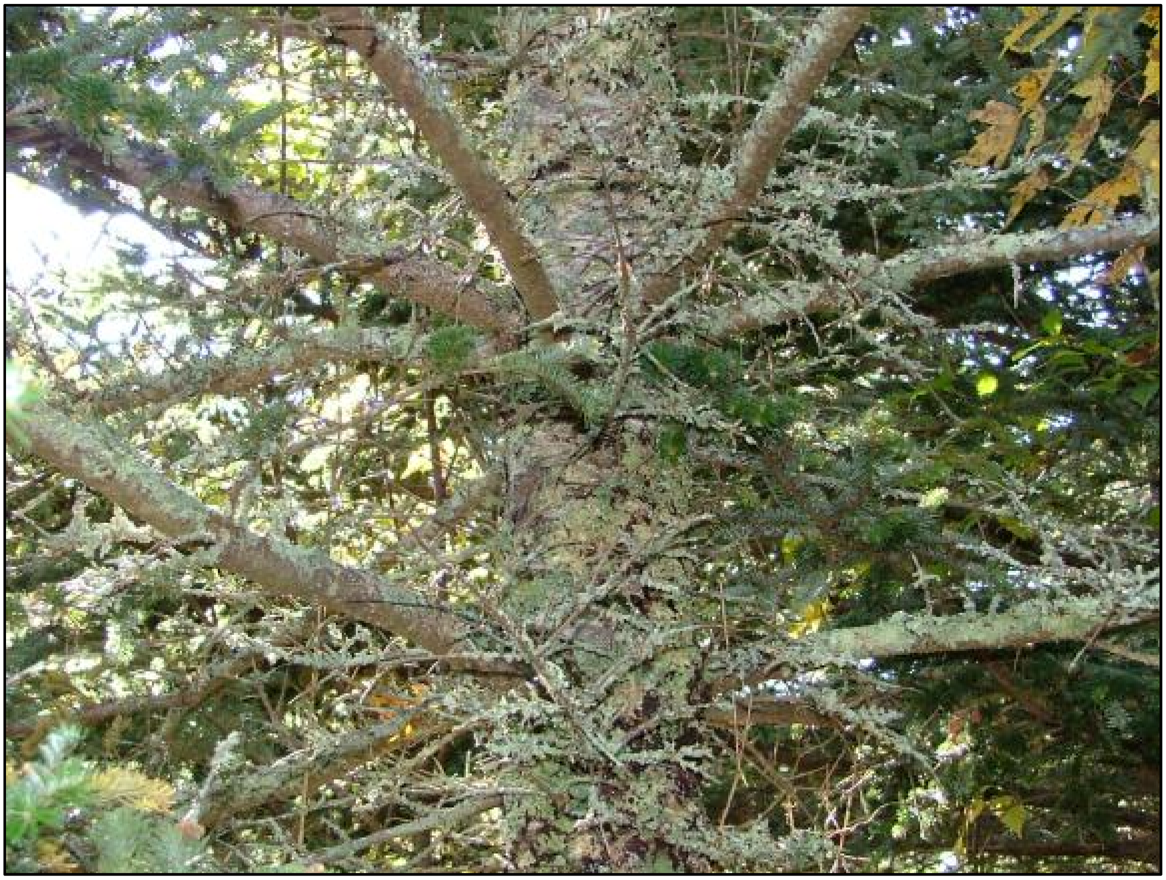
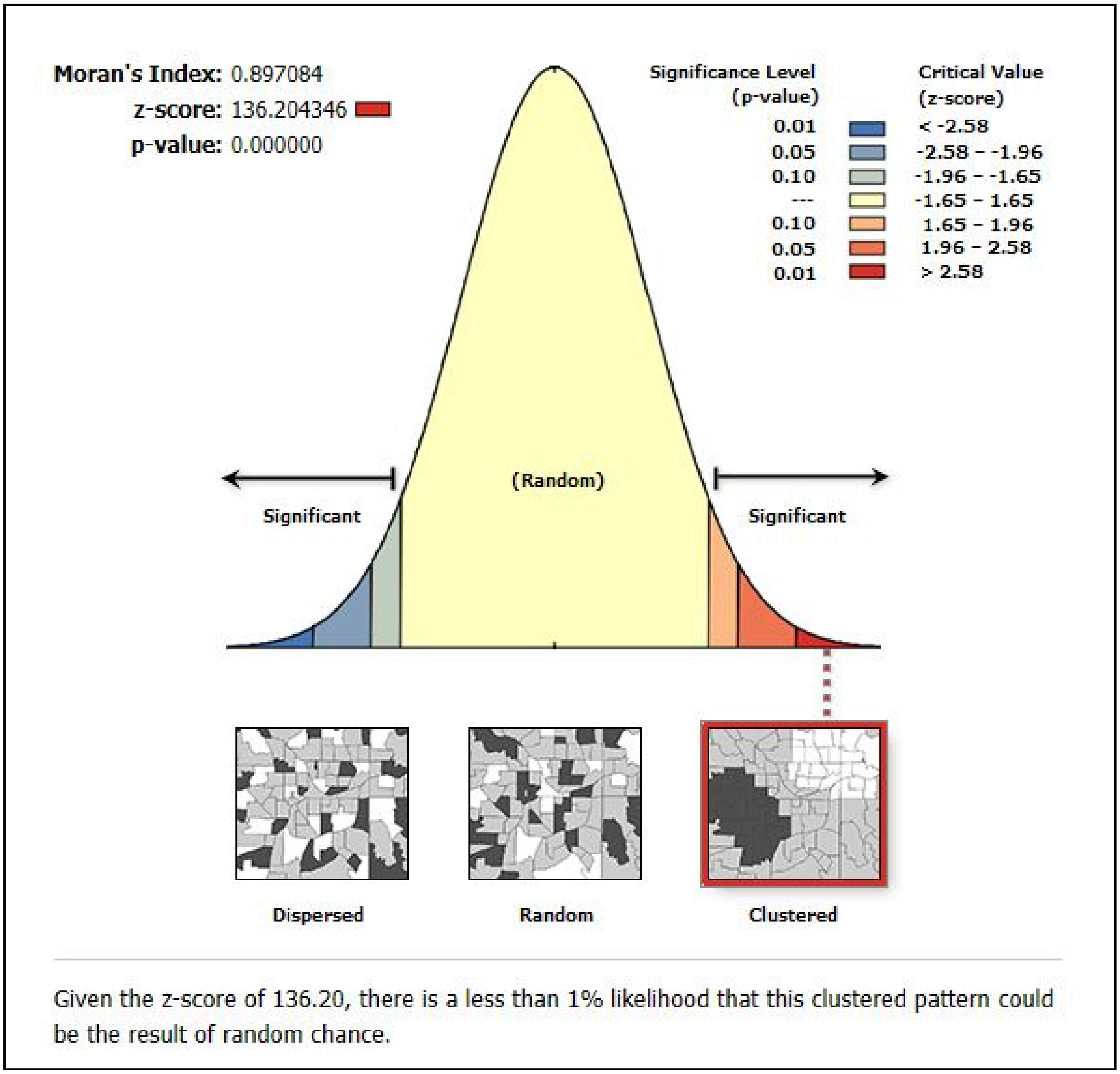


Figure 4. Heavy lichen coverage on Fraser fir.

Figure 5. Matrix representing a birds-eye view of the clonal seed orchard. The grey cells represent gravel roads. To the left of the vertical road are the trees planted in 1983; immediately to the right of the vertical road are trees planted in 1984; on the far right (just below the horizontal road) are the trees planted in 1985. The blue, yellow, orange, and red cells represent rows of living Fraser fir trees and are colored to represent BWA infestation levels: blue = none; yellow = light; orange = moderate; and red = heavy. The tan areas are within orchard rows but no trees are present. Light green cells represent orchard roads and the dark green area represents the surrounding mixed species forest.



Moran's Index: 0.897084
z-score: 136.204346 🟥
p-value: 0.000000

Significance Level (p-value)		Critical Value (z-score)
0.01	🟦	< -2.58
0.05	🟦	-2.58 - -1.96
0.10	🟩	-1.96 - -1.65
---	🟨	-1.65 - 1.65
0.10	🟧	1.65 - 1.96
0.05	🟧	1.96 - 2.58
0.01	🟥	> 2.58

Given the z-score of 136.20, there is a less than 1% likelihood that this clustered pattern could be the result of random chance.

Figure 6. Results of an analysis of infestation levels of each tree in entire orchard to calculate Moran's Index, a global measure of spatial autocorrelation.

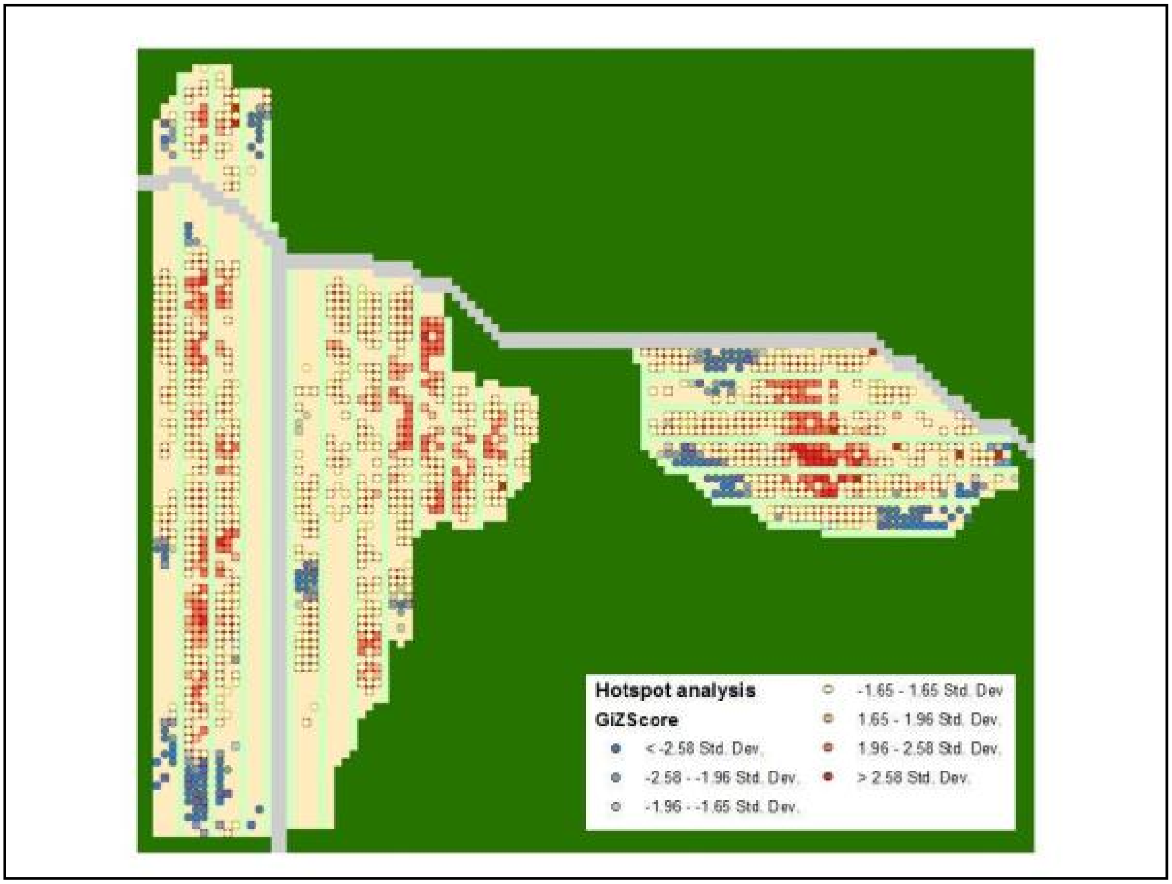


Figure 7. Results from hotspot analysis (ArcGIS) of infestation levels, overlaid onto a colored matrix of the entire orchard. The blue points represent areas with significant low values (cool spots) and the red points represent areas with significant high values (hot spots).

CHAPTER 8. SURVIVOR FRASER FIR IN THE GREAT SMOKY MOUNTAINS NATIONAL PARK

Abstract

Although most mature Fraser fir trees in native stands were killed by the balsam woolly adelgid during the first wave of mortality, many trees on mountain peaks in the Great Smoky Mountains National Park survived and are actively growing. Ten sites were visited and data collected on the survivor fir trees and younger, regenerated trees for comparison. Our objectives were to investigate the potential resistance of these trees to BWA by evaluating bark characteristics and to characterize site factors that may aid in the understanding of their survival. The bark of survivor trees was thicker and rougher than bark of regeneration trees, and the depth of the outer bark (impermeable tissue composed primarily of rhytidome or necrophylactic tissue) was also greater than that of the regeneration trees. The ratio of bark thickness to diameter at breast height was constant between the age classes (survivor and regeneration), and the ratio of impermeable tissue to bark thickness was similar, indicating that the regeneration trees were developing impermeable tissue at a similar rate as the older trees. These stands are at very high elevations and the extreme climatic conditions may help limit population development of BWA.

Introduction

Fraser fir (*Abies fraseri*) is the only fir species endemic to the Southern Appalachian Mountains and, together with red spruce (*Picea rubens*), comprises one of the most unique and increasingly fragile ecosystems in the Blue Ridge Mountains. Red spruce and Fraser fir occur together between 1300 and 1800 meters, and Fraser fir dominates above 1890 m

(Whittaker 1956). Remnants of the last ice age, spruce-fir forests once covered vast areas of land, but heavy logging in the early 1900s (Pyle and Schafale 1988) and the combined effect of air pollution and an exotic insect pest have now reduced these high elevation forests to a fraction of their former range. The balsam woolly adelgid (*Adelges piceae*) was introduced into the Southern Appalachians around 1955 (Amman 1966).

The balsam woolly adelgid, native to the silver fir (*Abies alba*) forests of central Germany and other areas, is a piercing-sucking insect specific to the genus *Abies*. Life stages include egg, three instars, and adult; the first instar ('crawler') is the only motile stage. Instars and adults produce threads of a waxy substance appearing as woolly or cottony tufts and remain under this 'woolly mass' protected from predators and desiccation. The insect inserts its stylets into the bark of true fir and feeds on parenchyma cells within the outer 1.5 mm of bark (Balch 1952). The adelgid's salivary secretions appear to create a chemical imbalance within the tree and disrupt the development of normal tissue. Balch et al. (1964) hypothesized that, while it is possible that growth hormones are directly present in the adelgid's saliva, it was more probable that a synergistic response was created between enzymes present in the saliva and chemicals already present in the tree. In addition to the production of abnormal phloem parenchyma cells, xylem production is often compromised and the resulting wood, called 'rotholz' or 'redwood', exhibits characteristics similar to compression wood (Balch 1952) or heartwood (Puritch and Johnson 1971) and has a 13% higher lignin content than non-infested wood (Balakshin et al. 2005). Ultimately, these changes result in physiological drought within the tree, reducing the flow of water, compromising photosynthesis, transpiration, and respiration (Puritch 1973), and resulting in tree mortality.

Fraser fir is highly susceptible to attack and damage by the balsam woolly adelgid and the introduction of BWA into the native range of Fraser fir resulted in a massive wave of mortality, particularly among mature, overstory trees. In some areas up to 95% of old growth or mature Fraser fir trees were killed (Hay and Johnson 1980, Dull et al. 1988, Wentworth et al. 1989, Hollingsworth and Hain 1991, Nicholas et al. 1999). However, some older trees survived this initial wave of mortality, and are growing in small populations throughout the native range. Additionally, there are reports of full recovery from severe infestations in a number of susceptible host species, e.g., noble and white firs (*Abies procera* and *A. concolor*) (Mitchell 1966), European silver fir (*A. alba*) (Kloft 1957), West Virginia Canaan fir (*A. balsamea* var. *phanerolepis*), and Fraser fir (Hain et al. 1991). This information, combined with the presence of the survivor firs, supports the hypothesis that there may be some level of BWA resistance within Fraser fir.

Possible resistance mechanisms to BWA-induced damage include the production of thick outer bark and the production of wound periderm around the feeding site. The term 'bark' refers to all tissues outside the vascular cambium, including the inner living phloem and dead outer tissue (rhytidome) (Kozlowski and Pallardy 1997). The living tissues are known as 'inner bark' (Eames and MacDaniels 1947). The rhytidome is comprised of alternating layers of periderms and associated tissues and is also known as 'outer bark' (Eames and MacDaniels 1947, Esau 1965). Periderm is a protective tissue formed to replace the epidermis in stems and roots, often within the first year of growth; as trees age, sequent periderms may arise and cause an accumulation of dead tissues on the surface of the stem, contributing to the formation of rhytidome on rough-barked species or outer bark on smooth-barked species (Biggs 1992).

Most pathogens are not able to penetrate the corky, suberized tissues of the outer bark and these tissues provide constitutive anatomical defenses or barriers against pathogen introduction (Biggs 1992). Production of thick outer bark, as found in European silver fir, has been associated with resistance to or recovery from adelgid attack (Pschorn-Walcher and Zwolfer 1958, Schooley and Bryant 1978). The texture of the bark may also be an important factor in resistance or susceptibility. Rough or flaky bark may be more preferable for BWA for a number of reasons, including the interception of airborne eggs and crawlers, stereotropism (the insect is stimulated to grow or change in response to touch), and the more easily accessible nutritive areas (e.g., lenticels and bark crannies) composed of young parenchyma (Varty 1956). In fact, lenticel development has been observed to be the best single predictor for BWA population levels on Fraser fir in the Great Smoky Mountains (Hay and Eager 1981).

Many woody species have the ability to form wound periderms in response to an injury. Natural (including first and sequent periderms) and wound periderms are essentially alike in origin and growth, but the difference involves timing of origin and restriction of the wound periderm to the place of injury (Biggs 1992). Mullick and Jensen (1973) provided the first evidence that natural and wound periderms may differ biochemically. To distinguish the two types, they proposed a new nomenclature: 1) exophylactic periderms include the first and sequent periderms and protect living tissues from the environment; 2) necrophylactic periderms include wound periderms, pathological periderms and secondary protective layers. Interest in periderms arose from an effort to understand defense mechanisms in conifers, particularly in connection with attack by the balsam woolly adelgid (Mullick 1975).

In the course of these studies, Mullick (1975) discovered that osmium tetroxide fixative failed to penetrate the necrophylactic periderm surrounding BWA feeding zones, but in some cases also failed to penetrate feeding zones where the necrophylactic periderm was absent or only partially present. He questioned whether this non-periderm impermeability could be responsible for cutting off abnormal BWA-induced tissues from healthy tissues and thus be a defensive response of the host. Subsequent studies showed that non-periderm impermeability was produced by wounding and that it arose from a tissue formed prior to wound periderm formation (Mullick 1975). These impervious tissues are thought to protect living tissues from the adverse effects of cell death (Biggs 1992). The wound healing mechanism in response to injury by the balsam woolly adelgid can isolate an area of bark occupied by BWA and can effectively protect the underlying bark from further attack for years (Balch 1952, Mullick 1971).

The Great Smoky Mountains National Park (GSMNP) contains the majority (74%) of existing spruce-fir forests in the Southern Appalachians (Dull et al. 1988). Mapping projects were conducted during the 1990s and early 2000s in a concerted effort to document the location of surviving mature Fraser fir throughout the park (Durr and Peine 1990, Johnson and Taylor 1997, Kloster 1998, Kloster 2001). Originally, park personnel searched for healthy fir stands at least 900 m^2 in size and comprised of at least 50% mature fir trees. After visiting each of the stands, patches of smaller mature stands and a few very large individual old fir trees were also found and mapped (Kloster 2001). In 2000-2001, park personnel mapped and permanently tagged a total of 34 mature Fraser fir within 10 stands, and began collecting data on the following peaks: Mount Buckley, Clingman's Dome, Mount Love, Mount Black, Mount Guyot (three sites), Mount Chapman, Mount Sterling, and Mount LeConte (Kloster 2001).

The objectives of this study were: 1) to investigate the potential resistance of these survivor Fraser fir trees to BWA by evaluating total bark thickness and depth of impermeable tissue (rhytidome, necrophylactic periderm, or the non-periderm impermeable tissue) in the bark, and 2) to characterize site factors that may aid in the understanding of how these trees were able to survive.

Methods and Materials

The ten survivor fir peaks in the GSMNP (Mt. Guyot – 3 sites; Mt. Chapman; Old Black; Clingmans Dome; Mt. Buckley; Mt. Love; Mt. LeConte; Mt. Sterling) (Figure 1) were visited during the summers of 2003 and 2004. Four (4) survivor trees per site (except Mt. Buckley & Mt. Love, with 1 survivor tree per site) were marked for this study, with an equal number of younger (regeneration) trees per site.

The study utilized a block design with the 10 mountain peaks ('sites') as blocks:

56	4 trees/class (Survivor/Regeneration) x 7 sites (Mt. Guyot [center, northeast, south], Mt. Chapman, Clingmans Dome, Mt.LeConte, Mt. Sterling)
8	5 survivor trees and 3 regeneration trees x 1 site (Old Black)
4	1 trees/class x 2 sites (Mt. Buckley and Mt. Love)
68	Total study trees

Measured variables included tree diameter at breast height (dbh, with a logger's diameter tape), slope (with a clinometer), aspect (with a compass), and elevation (latitude/longitude coordinates mapped with a geographic information system). Each tree was given a health rating (excellent, good, poor), assessed for new growth (yes/no), and assessed for presence/absence of BWA. Four bark samples (5 x 5 cm, cut with a knife) were collected from each tree for chemical analysis; two bark discs were cut from each tree with a brass punch (3.2 cm) from outer bark to cambium for measuring the thickness of the impermeable tissue. Bark pieces were assessed for color (rated 1 – 4, light grey to dark brown/black), roughness (rated 25-100, from a smooth to a very cobbled appearance), BWA woolly masses (yes/no), mites (yes/no), lichens (percent cover, rated 0-3: none, light, moderate, heavy). Overall bark thickness was estimated by taking the mean of two bark gauge measurements in the field (east/west) plus the total thicknesses of the bark samples (total of 6 measurements per tree). Because the two classes of trees were different ages, to enable a more direct comparison between the classes, ratios of bark thickness to dbh (BT/dbh) and depth of impermeable tissue to bark thickness (I/BT) were calculated.

The thickness (depth) of the impermeable tissue was determined by utilizing the ferric chloride-potassium ferricyanide (F-F) test described by Mullick (1975), as follows: The tops of the 3.2 cm discs were ringed with melted paraffin. The discs were placed on glass rods in petri dishes (Figure 2) and soaked for 3 days in 2% ferric chloride solution, rinsed, then soaked for another 3 days in 4% potassium ferricyanide solution. The reaction of Fe^{3+} with $Fe(CN)_6^{3-}$ stains living tissue a Prussian blue while the dead tissue (outer bark or necrotic tissue formed in response to wounding) remains brown.

Most responses were analyzed utilizing the General Linear Model procedure (proc glm) of SAS version 9.3 (SAS Institute 2010) with the model including as main effects site and class. The ANOVA for the variable I/BT (ratio of impermeable tissue to bark thickness) included dbh as a covariate. Pairwise comparisons (least squares means) were analyzed by the Tukey-Kramer Multiple Comparison Procedure. Correlations between measured variables over all sites were made utilizing the correlation procedure (proc corr). Data with a binomial distribution (presence of old woolly masses, presence of mites) were analyzed using logistic regression (proc genmod).

Results and Discussion

A review of observed site characteristics did not reveal any obvious differences – the condition of most trees, both survivor and regeneration, was classified as 'excellent,' and new growth was observed on almost all of the trees. The sites were primarily open, with slopes ranging from 5 to 45%. Aspects varied, with the majority facing S-SW or NW. Regeneration was good on Mts. Buckley, Guyot, LeConte, and Love, with both seedlings and saplings present, but poor on Mts. Chapman and Sterling, Clingmans Dome, and Old Black. All sites were at high elevations.

The bark samples revealed no differences in color, but there were site and class differences in roughness (Table 1), with the survivor trees exhibiting a rougher or more cobbled appearance than the regeneration trees. This could be an effect of age but also a response to repeated attack by BWA. There were no obvious signs of active BWA infestation on either survivor or regeneration trees, however, microscopic evaluation of the bark samples revealed what appeared to be remnants of old woolly masses on many of the bark samples.

The wax-like threads are long lasting and were present only in small amounts. The site effect was significant for old woolly masses but there were no differences in the survivor versus regeneration trees (Table 2). There were only two bark samples (both from Mount LeConte, one survivor and one regeneration tree) with egg masses, each containing a few eggs. Mites were present on survivor and regeneration trees, with more found on survivor trees (Table 2). There was a positive correlation between the presence of mites and the roughness of the bark (Table 3). The mites were not identified to species, but there are predatory mites known from the spruce-fir regions of the Great Smoky Mountains and natural enemies may be playing a part in keeping adelgid populations low; for example, *Allothrombium mitchelli* Davis, initially observed on Mount Mitchell in 1959 as a predator of the balsam woolly adelgid (Davis 1961), has been found in the GSMNP on beech (*Fagus grandifolia*) growing near spruce-fir stands (Wiggins et al. 2001). Lichens were present on the bark of most trees and there were both site and class differences, with more lichen coverage on the survivor fir trees (Table 1).

The survivor trees are significantly larger in diameter, and have thicker outer bark and layers of impermeable tissue than the younger trees (Table 4). For dbh and bark thickness, the main effects of site and class are highly significant ($p < .0001$), and there is a strong site x class interaction ($p < .0001$) (Table 4). The mean depth of impermeable tissue ranged by site, from 0.11 mm to 1.35 mm for the regeneration trees and 0.20 mm to 5.10 mm for survivor trees (Table5).

Pairwise comparisons reveal that for each of these measured variables, the trees on Mts. Buckley and Love, and possibly Clingmans Dome, were responsible for the strength of the response because they were substantially larger in size than the others (Table 5). Removing the trees associated with these peaks (Buckley, Love, Clingmans) from the analysis, however, did not alter the fact that the survivor firs have significantly greater diameter and bark thickness, and thicker layers of impermeable tissue. Because the survivor trees are older than the regeneration trees, probably by a few decades, these results are not surprising.

As would be expected, there is a strong positive correlation between each of these variables (Table 6). On a site-by-site basis, there is generally a strong relationship between dbh and bark thickness, but the relationship between impermeability and both dbh and bark thickness varies. There is no correlation among these traits for the southern peak of Mt. Guyot, Mt. Chapman, or Mt. LeConte.

There is no difference in the ratio of bark thickness to dbh (BT/dbh) between the survivor and regeneration trees, nor, after accounting for the size differences by including dbh as a covariate, is there any difference in the ratio of impermeable tissue to bark thickness (I/BT) (Table 4). The similarity in BT/dbh suggests a constant relationship over time in the lateral growth of the tree and bark development, which would be expected. The similarity between the age classes in the ratio of I/BT (after including dbh in the model statement) suggests that the size of the tree (represented by dbh) is an important factor in the depth of the impermeable tissue. In the analysis of covariance, site and dbh were significant ($p = .0007$ and $.0021$, respectively).

This analysis suggests that the two age classes are developing impermeable tissue at similar rates. The harsh climatic conditions of these high elevations may prompt the accelerated development of secondary periderms in general, regardless of the age of the tree, and it should not be forgotten that the regeneration trees, growing in close proximity to the survivors, may be genetically related to the survivors. The presence of BWA may have also stimulated the development of wound periderm and necrophylactic tissue around the feeding sites in both age classes. Perhaps these trees have a greater propensity to develop wound periderm, thus preventing the adelgids from causing long-term damage. Because BWA requires living cells upon which to feed, trees with thick outer bark and layers of necrophylactic tissues are likely to provide less suitable habitat for this insect. The depth of impermeable tissue of the survivor firs, with only two exceptions (Mts. Chapman and LeConte), exceeds 1.5 mm, the maximum length of BWA stylets (Balch 1952). This thick outer bark would provide a very poor substrate for the adelgid and it is unlikely that BWA populations could increase to any appreciable levels on this tissue.

Conclusion

Aside from some broken tops, most of the trees appeared to be in excellent health. There were relatively few site differences and the bark pieces from survivor and regeneration trees exhibited similar signs of past BWA infestation and the presence of mites and lichens. The bark of survivor trees was rougher than the regeneration trees, possibly an effect of age and possibly an effect of past BWA infestation. Diameter at breast height (dbh) and total bark thickness of the survivor firs is significantly greater than that of the younger trees, which is not surprising because of the age difference between the two classes. The depth of the outer bark (impermeable tissue composed primarily of rhytidome or necrophylactic periderm,) is also greater in the survivor fir trees. The ratio of bark thickness to dbh was constant, not only between the age classes (survivor/ regeneration) overall, but also between individual trees. The ratio of impermeable tissue to bark thickness was similar, after accounting for the differences in size, indicating that the regeneration trees are developing impermeable tissue at a similar rate as the older survivor trees. This could be linked to genetic similarities between the regeneration and survivor trees.

It is expected that older trees will have thicker bark in general than younger trees and it seems reasonable that older trees may have thicker outer bark, having had more opportunity to develop and slough off dead tissues. Because in most cases outer bark thickness on the tree bole exceeds the 1.5 mm length of BWA stylets, it is likely that the adelgids cannot find sufficient quantities of living cells upon which to feed. Mortality of settled instars is likely high and, subsequently, the adelgids have not been able to build their populations to a level that would kill the trees. Additionally, the extreme climatic conditions of the high elevations for each peak may also help to limit the population development of the balsam woolly adelgid. The harsh conditions at the high elevations (lower temperatures, high winds) may have protected the trees from BWA population explosions.

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Table 1. Least squares mean (± S.E.) value for color (rated 1-4, light grey to dark brown/black), bark roughness (rated 25-100, from smooth to very cobbled appearance), and percent lichen (and other bryophytes) coverage. Because the means and the lsmeans were so similar, we are showing only the lsmeans from the general linear model (proc glm). (N = 65).

Tree Class	N	Bark color	Bark roughness	Lichen coverage
Survivor fir	34	3.11 (0.09)	83.90 (2.44)	2.53 (0.10)
Regeneration	32	3.15 (0.09)	74.45 (2.48	2.15 (0.10)
ANOVA p-values from general linear model				
Source of variation	df			
Model	19	0.3261	0.0001	0.0002
Site	9	0.3038	< .0001	< .0001
Class	1	0.7251	0.0093	0.0063
Site * Class	9	0.2904	0.4372	0.5319

Table 2. Mean (± S.E.) value for signs of old BWA egg masses and presence of mites (binary data, yes=1, no=0). (N = 66).

Tree Class	N	Old egg masses	Mites
Survivor	33	0.47 (0.09)	0.28 (0.12)
Regeneration	33	0.53 (0.09)	0.37 (0.26)

Results from logistic regression[1]

		--------Old egg masses--------		------------Mites[2]------------	
Source of variation	df	Chi square	Pr > Chi sq	Chi square	Pr > Chi sq
Site	9	22.20	0.0083	---	---
Class	1	0.20	0.6581	---	---

[1] Analyzed using proc genmod; binomial distribution; link = logit. The algorithm converged only for the old egg masses response.

[2] For this response variable, the model did not converge (the negative of the Hessian was not positive definite, i.e. there was a quasi-complete separation of data) and the results from the analysis are questionable. Thus, the p-values from this procedure for the presence of mites are not reported.

Table 3. Pearson’s correlation coefficients for color, roughness, old woolly masses, mites, lichen coverage. These data represent only the survivor fir trees.

Pearson Correlation Coefficients (N = 34)
Prob > |r| under H0: Rho=0

	Color	Roughness	OldWM	Mites	Lichens
Color	1.0000	0.0014	-0.0618	0.1369	0.1461
		0.9492	0.7283	0.4400	0.4096
Roughness	0.0114	1.0000	0.2239	0.4046	-0.1082
	0.9492		0.2030	0.0176	0.5423
OldWM	-0.0618	0.2239	1.0000	0.0625	-0.2010
	0.7283	0.2030		0.7255	0.2543
Mites	0.1369	0.4046	0.0625	1.0000	0.1034
	0.4400	0.0176	0.7255		0.5607
Lichens	0.1461	-0.1082	-0.2010	0.1034	1.0000
	0.4096	0.5423	0.2543	0.5607	

Table 4. Least squares means (± S.E.) for diameter at breast height (dbh), bark thickness from the outside of the tree to the vascular cambium (6 measurements per tree), the ratio of bark thickness to dbh (BT/dbh), the depth of impermeable tissue, and the ratio of the impermeable layer to bark thickness (I/BT), by class (survivor or regenerated tree).

Class	Dbh (cm)	BT (mm)	BT/Dbh	I (mm)	I/BT[1]
Survivor	35.51 (0.82)	9.34 (0.23)	0.26 (0.006)	2.24 (0.22)	0.07 (0.05)
Regeneration	20.70 (0.84)	5.39 (0.23)	0.26 (0.006)	0.60 (0.22)	0.17 (0.03)

ANOVA p-values from general linear model

Source of variation	df	Pr > F	Pr > F	Pr > F	Pr > F	Pr > F
Model	20	< .0001	< .0001	0.0002	< .0011	0.0018
Site	9	< .0001	< .0001	< .0001	0.0151	0.0007
Class	1	< .0001	< .0001	0.9687	< .0001	0.2133
Site * Class	9	< .0001	< .0001	0.0681	0.2577	0.6647
dbh[2]	1					0.0021
Error	47					

[1] The least squares means expressed here for I/BT are those calculated after accounting for size differences by including dbh as a covariate in the model statement.

[2] This source (dbh) was included only in the model related to I/BT.

Table 5. Site least squares means (± S.E.) for diameter at breast height (dbh), overall bark thickness (mm), and the thickness of impermeable outer tissue (mm) between the survivor and regenerated fir trees in the Great Smoky Mountains National Park.

	--------dbh (cm)--------		Overall bark thickness		Impermeable outer layer	
Site	Survivor	Regen.	Survivor	Regen.	Survivor	Regen.
Guyot C	25.85 (2.06)	20.98 (1.84)	6.94 (0.57)	5.40 (0.51)	1.93 (0.55)	1.35 (0.50)
Guyot NE	28.26 (2.06)	18.99 (2.06)	7.67 (0.57)	5.49 (0.57)	2.74 (0.55)	1.29 (0.55)
Guyot S	28.13 (2.06)	16.51 (2.06)	8.33 (0.57)	5.41 (0.57)	2.35 (0.55)	1.21 (0.55)
Chapman	24.32 (2.06)	17.21 (2.06)	6.90 (0.57)	5.14 (0.57)	0.64 (0.55)	0.30 (0.55)
Old Black	28.85 (1.84)	21.08 (2.38)	8.55 (0.51)	5.73 (0.66)	2.23 (0.50)	0.63 (0.64)
Clingmans	37.46 (2.06)	21.00 (2.38)	9.31 (0.57)	6.53 (0.66)	1.95 (0.55)	0.12 (0.64)
Buckley	66.04 (4.12)	25.15 (4.12)	16.13 (1.15)	5.82 (1.15)	5.10 (1.11)	0.30 (1.11)
Love	63.25 (4.12)	31.24 (4.12)	16.33 (1.15)	5.98 (1.15)	3.35 (1.11)	0.55 (1.11)
LeConte	27.69 (2.06)	18.73 (2.06)	6.10 (0.57)	4.21 (0.57)	0.20 (0.55)	0.11 (0.55)
Sterling	25.21 (2.06)	16.13 (2.06)	7.14 (0.57)	4.19 (0.57)	1.93 (0.55)	0.11 (0.55)

Site means (± S.E.) for the ratios of bark thickness to dbh (BT/Dbh) and impermeable tissue to bark thickness (I/BT) between the survivor and regenerated fir trees.

	------------BT/Dbh-----------		------------I/BT[1]------------	
Site	Survivor	Regen.	Survivor	Regen.
Guyot C	0.27 (0.01)	0.26 (0.01)	0.25 (0.07)	0.23 (0.06)
Guyot NE	0.28 (0.01)	0.29 (0.01)	0.35 (0.07)	0.22 (0.07)
Guyot S	0.29 (0.01)	0.33 (0.01)	0.30 (0.07)	0.20 (0.07)
Chapman	0.28 (0.01)	0.30 (0.01)	0.09 (0.07)	0.05 (0.07)
Old Black	0.30 (0.01)	0.27 (0.01)	0.24 (0.06)	0.11 (0.08)
Clingmans	0.25 (0.01)	0.32 (0.01)	0.20 (0.07)	0.02 (0.08)
Buckley	0.24 (0.03)	0.23 (0.03)	0.32 (0.14)	0.05 (0.14)
Love	0.29 (0.03)	0.19 (0.03)	0.21 (0.14)	0.09 (0.14)
LeConte	0.22 (0.01)	0.23 (0.01)	0.03 (0.07)	0.03 (0.07)
Sterling	0.28 (0.01)	0.26 (0.01)	0.25 (0.07)	0.02 (0.07)

Table 6. Pearson's correlation coefficients for diameter at breast height (dbh), bark thickness (BT), and impermeable tissue (I). These data reflect only the survivor fir trees

	Pearson Correlation Coefficients (N = 35) Prob > |r| under H0: Rho=0		
	dbh	BT	I
dbh	1.0000	0.9234 < .0001	0.5161 0.0015
BT	0.9234 < .0001	1.0000	0.6161 < .0001
I	0.5161 0.0015	0.6161 < .0001	1.0000

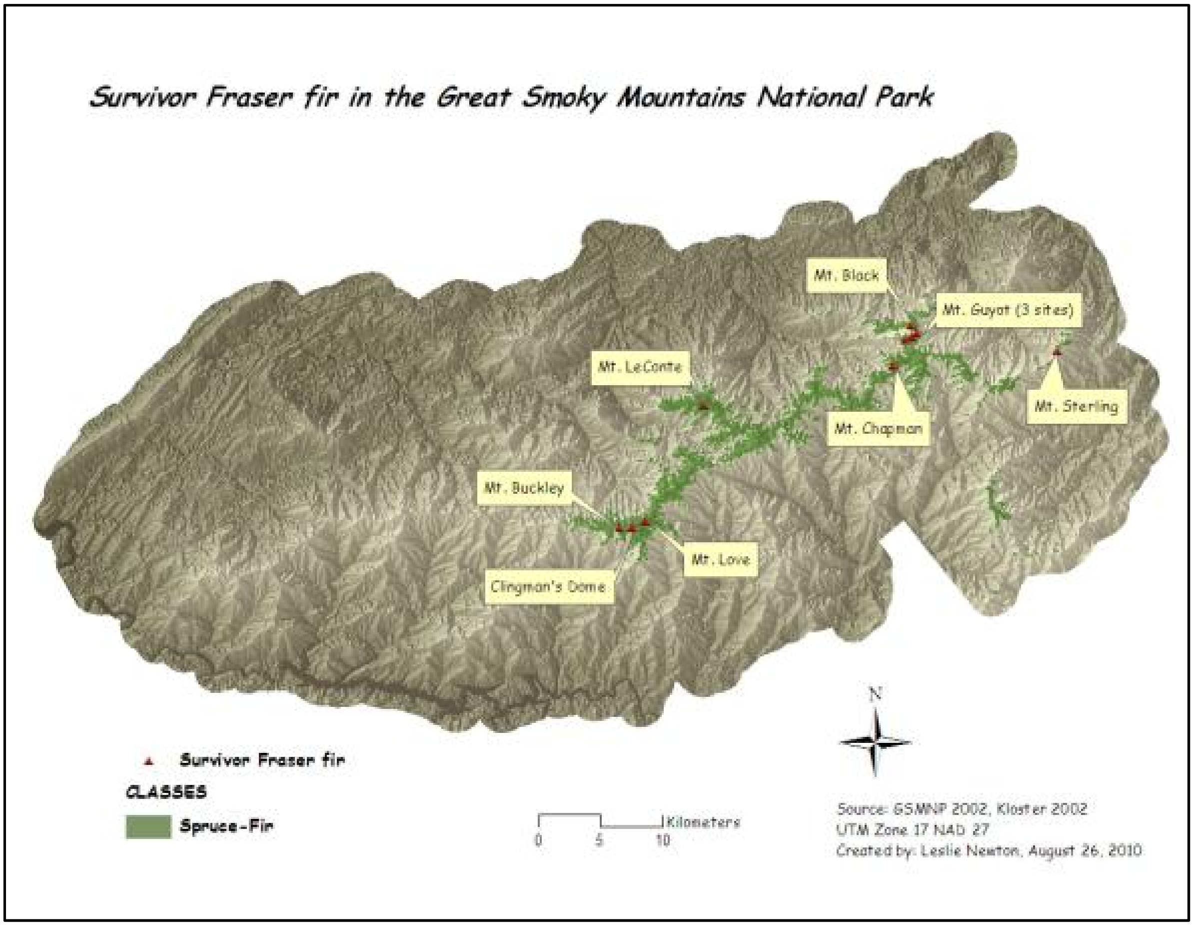

Figure 1. Mountain peaks in the Great Smoky Mountains National Park with mature Fraser fir that survived the first wave of mortality from the introduction of the balsam woolly adelgid.

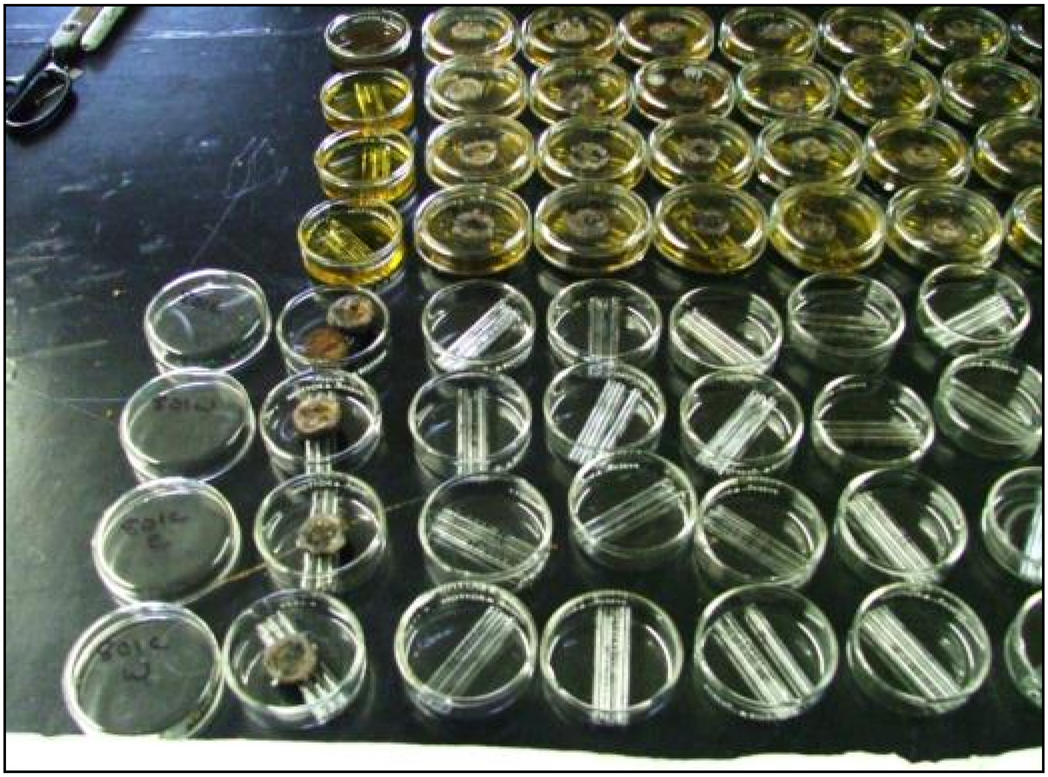

Figure 2. Setting up for the F-F test. Bark discs were ringed with melted parafin wax and placed on two glass rods within small petri dishes.

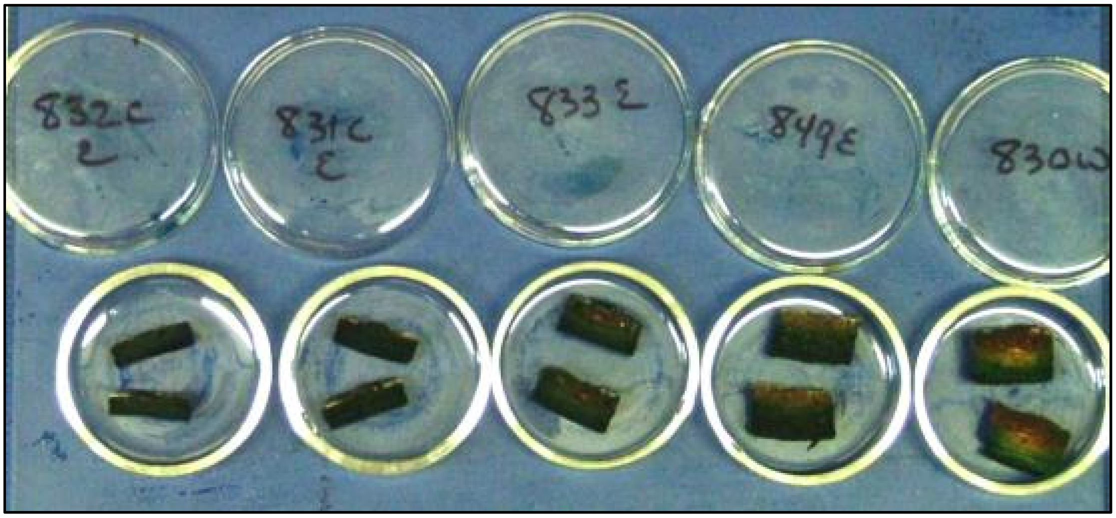

Figure 3. Results from the F-F test, showing the range in layers of impermeable tissue between the survivor trees (833, 849, 830) and the regeneration trees (832C, 831C). The 'E' and 'W' reflect whether the bark disc was cut from the east or west side of the tree. As the thickness of the bark disc increases, to does the depth of impermeable tissue from the outside of the tree (top of disc) in toward the cambial region. The two discs from regeneration trees (832C and 831C) look black (they are actually deep blue) while the differences between the impermeable tissue (brown) and permeable tissue (blue or green/blue) are more readily observed in the samples from survivor trees 849E and 830W.

CHAPTER 9. DISSERTATION CONCLUSIONS

The balsam woolly adelgid was introduced into North America over 100 years ago and into the Southern Appalachians over 50 years ago, but remains a significant pest in natural stands and in Christmas tree production systems. Fir species that evolved with this adelgid have shown resistance or tolerance to infestation and generally suffer less damage than most North American fir species, especially Fraser fir. The development of BWA-resistant Fraser fir trees would be a relatively cost effective way to combat the ubiquitous presence of BWA. Much is known about the biology of BWA and its effect on host species, but the mechanisms of host resistance are not fully understood. Before the intensive research on a large scale could begin, there were some very basic questions and issues that needed to be answered or addressed. The overarching objective of this project was to address some of these issues and we were successful in answering some very basic questions.

The critical question of whether it was possible to artificially infest very young Fraser fir seedlings, and to maintain those infestations over a period of time, was addressed in Chapters 3, 4, 5, and 6. We successfully infested seedlings from 1- to 2-years old to 7-years old, and maintained some of the infestations for up to a year and a half. We infested seedlings under constant temperatures, ramping temperatures, and natural environmental conditions. Although we did not formally compare infestation levels in these different environments, it appears that controlled environments provide a greater level of infestation. Natural conditions include heavy winds and rains, and it is likely that infestation levels are affected. Although very young (1- to 2-year old) trees could become infested, not all individuals did become infested. Whether that was due to the quantity or quality of crawlers applied to the tree, or whether it is due to some inherent resistance in the seedling is not clear. In natural stands, very young trees are rarely infested and it is this characteristic that has allowed natural Fraser fir stands to recover from the initial wave of mortality in the 1960s and 1970s. Older seedlings (3+) can support hundreds of BWA egg masses and up to an estimated eight generations of BWA (within a year and a half), and still remain alive. Their health was declining, obviously, but many of the trees were still alive at the conclusion of each of the studies.

The technique historically utilized for artificially infesting seedlings or trees was to cut pieces of infested bark from a mature tree and tie, pin, or otherwise attach it to the uninfested tree. This is effective, but is very time consuming and often requires multiple applications of bark pieces to achieve an adequate level of infestation. We developed a novel (yet logical) technique (Chapter 4) for infesting seedlings by suspending infested material (logs) over the uninfested trees, thus mimicking the natural dispersal mechanism utilized by BWA. The crawlers hatch out naturally and simply drop off the infested log onto the material below. This method has come to be known as the ‘rain down’ method. If the uninfested material is strategically placed, it is possible to have branches evenly distributed under the logs to receive the air-borne crawlers. We found that placing heavily infested logs (10 cm in diameter) about 30 cm apart would provide good crawler coverage. Because it is impossible to find infested trees with exactly the same quantity and quality of BWA egg clutches, with BWA in similar stages of development, all along the length of the bole, it is important to consider where the areas of exceptionally high BWA development may be so the log or plant material can be moved around to evenly distribute the crawlers. The question of how long to keep the infested log suspended over the uninfested material depends on how much infestation pressure (the level of crawler onslaught) is desired.

We developed another unique approach for testing BWA resistance (also outlined in Chapter 4), which involves cutting the tips of branches from living trees and infesting the excised branches. There are logistical difficulties in keeping the branch tips properly hydrated; I used wet sand, but there are other ways this could be accomplished. We found that Fraser fir, which is known to be susceptible to BWA, developed egg-laying adults and Veitch fir, which is resistant, did not. We also found that, while cut and intact Fraser fir branches became infested at a similar level, the cut branches did not produce any gouting as a response to BWA presence but the intact branches did. Although this is an indicator that excised branches cannot serve as perfect surrogates for whole trees, it may help us understand the physiological responses to BWA feeding. This technique requires further study, but is promising for providing a nondestructive method for conducting initial host resistance screening trials.

The issue of whether seedlings can be used as surrogates for mature trees could be a dissertation topic in and of itself. We know that there are many differences between juvenile and mature trees, so a seedling can never be a perfect surrogate. Within the context of screening for host resistance to BWA, however, one of the critical questions is whether seedlings exhibit the same basic tendencies in response to BWA as their mature counterparts. We screened seedlings from 12 different fir species representing the range of BWA susceptibility, all of which were the same age, grown under the same conditions, and infested with BWA from the same source at the same time, in an effort to at least begin the process of answering this important question. This is detailed in Chapter 5. We found that, with very few exceptions, results for each species fell within expectations for *a priori* resistance classifications. Susceptible species became more heavily infested than resistant species and generally exhibited greater BWA fecundity levels. Fraser fir consistently ranked as susceptible. One exception was European silver fir, a species that has long been associated with BWA (the adelgid is indigenous to Central Europe, which exhibited high fecundity rates. Mature trees within this species are known to be tolerant to BWA infestation and suffer less damage than susceptible species. It may be that seedlings of European silver fir are more susceptible than mature trees. This appears to be the case with Veitch fir. It is very rare to see BWA infestations on mature Veitch fir trees and in our studies (in Chapter 5, but also in Chapter 4), while the seedlings became less infested than susceptible trees, they did become infested. Fecundity levels were low, however. Korean fir was the final exception. It has been reported as being fairly susceptible (not at the level of Fraser fir but more than European silver fir), but here the seedlings appeared somewhat resistant to infestation.

Hybrids developed between Fraser fir and resistant species could be one solution to the current BWA problem. We were able to obtain 3-year old seedlings from a (balsam x Veitch) x balsam fir backcross and artificially infested the backcrosses at the same time we infested similarly aged seedlings from other species (detailed in Chapter 6). Although there was a high mortality rate in this study which compromised some of our results, it was very apparent that, as compared with balsam fir of the same age and grown under the same conditions, the backcross was more resistant to infestation. This is promising.

We believe that there is some level of genetic resistance within Fraser fir. A naturally infested clonal seed orchard in Avery County exhibited differing levels of BWA infestation. We conducted a study to determine whether there were clonal differences (Chapter 7) and found that, indeed, there was a significant clonal effect, but we could not draw firm conclusions because the trees have been sprayed almost every year with an insecticide. Heritability calculations were low (0.083) and remained low after accounting for spatial effects (0.052). We believe these treatments may be masking any genetic effects associated with BWA resistance.

There are very old survivor trees in native stands in the Great Smoky Mountains National Park. We visited ten mountain peaks, took measurements, and collected bark samples to consider differences in these survivor fir trees and younger trees that regenerated nearby (Chapter 8). The differences in diameter at breast height and bark thickness were expected because of age differences. There were also significant differences in the depth of impermeable tissue. However, the ratio of impermeable tissue to bark thickness was similar between survivor and regeneration trees, indicating that regeneration trees were developing impermeable tissue at a similar rate as the older trees. How much of their survival is due to genetic resistance or to environmental conditions is not yet fully understood.

This work sheds light on some of the issues involved in the arduous process of finding or developing BWA-resistant Fraser fir trees and it is my hope that it will prove useful to future researchers who are dedicated to ensuring the survival of this important and beloved tree.