Video Article

A Technique to Screen American Beech for Resistance to the Beech Scale Insect (*Cryptococcus fagisuga* Lind.)

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Abstract

Beech bark disease (BBD) results in high levels of initial mortality, leaving behind survivor trees that are greatly weakened and deformed. The disease is initiated by feeding activities of the invasive beech scale insect, *Cryptococcus fagisuga*, which creates entry points for infection by one of the *Neonectria* species of fungus. Without scale infestation, there is little opportunity for fungal infection. Using scale eggs to artificially infest healthy trees in heavily BBD impacted stands demonstrated that these trees were resistant to the scale insect portion of the disease complex[1]. Here we present a protocol that we have developed, based on the artificial infestation technique by Houston[2], which can be used to screen for scale-resistant trees in the field and in smaller potted seedlings and grafts. The identification of scale-resistant trees is an important component of management of BBD through tree improvement programs and silvicultural manipulation.

Video Link

The video component of this article can be found at http://www.jove.com/video/51515/

Introduction

Beech bark disease (BBD) has had a detrimental impact on American beech in North America since the introduction of the invasive beech scale insect, *Cryptococcus fagisuga*, in the Canadian province of Nova Scotia in the late 1890s[3]. This insect-disease complex is initiated when the beech scale insect inserts its feeding stylet into the bark creating small fissures that provide entryway for infection by one of the *Neonectria* species of fungus (*Neonectria ditissima* or *Neonectria faginata*). As the fungal mycelia grow, large areas of tissue may die, eventually completely girdling the tree. The damage from the disease weakens the tree, making it prone to snapping in high winds[4]. Mortality levels in the first wave of the disease have been reported to be as high as 50%[5]. Surviving trees are often severely deformed as cankers form reducing value of the tree as a wood product. Such trees have a propensity for root-sprouting which leads to the formation of “beech thickets” that prevent other more desirable species from establishing, reducing the economic and ecological value of the stand[6]. Although beech bark disease is not likely to lead to extinction of the American beech, it alters stand composition and health leading to a decrease in food and habitat for wildlife[7,8].

In stands affected by BBD for many years, trees that remain free of any symptoms of the disease have been reported. Artificial inoculation trials have shown that these trees are resistant to the scale insect[2]. Without scale infestation, there is little opportunity for *Neonectria* infection, minimizing the impact of the fungus. Large scale mortality in American beech due to *Neonectria* infection in the absence of prior scale infestation has never been reported, so resistance to the beech scale insect results in resistance to BBD.

Recent research on management of BBD has focused on the identification, propagation, breeding, and retention of American beech trees with resistance to the beech scale insect. Genetic studies have shown that resistance to the scale insect is heritable and careful selection and breeding of resistant trees can result in significant improvement in a single generation[9]. This finding has fueled efforts by state and national forest managers in the United States to establish regional seed orchards of resistant American beech to provide a source of genetically diverse BBD-resistant seed for restoration plantings[10,11]. Research has also indicated that silvicultural manipulation of stand genetics by the removal of susceptible trees and retention of resistant trees can result in stand improvement[9,12].

Management of BBD through tree improvement activities or through carrying out silvicultural prescriptions requires the ability to select for and distinguish between beech scale-resistant and susceptible trees. The methods presented here have been adapted from a method first introduced by Dave Houston to artificially inoculate seedlings with beech scale eggs[1]. The method can be used as a screening tool to identify quantitative trait loci (QTL) associated with resistance or to distinguish between resistant and susceptible potted seedlings or grafted ramets in genetic studies. Alternatively, it can be used for screening mature trees in the field to identify resistant trees for seed orchard development, or retention in the field. Susceptible trees can be identified and removed to minimize disease impacts.

Protocol

1. Plant Material: Mature Field Trees, Potted Seedlings, or Potted Grafts

1. For field testing, select mature healthy American beech trees that show no signs of scale infestation or disease for testing for possible resistance. Visibly susceptible trees will also need to be identified to be used as a control (**Figure 1**).
2. For testing potted seedlings or grafts, collect and germinate beechnuts as described in Koch & Carey, 2004 or graft scion as described in Carey *et al*, 2013.
3. Grow seedlings or grafts in potting soil mix amended with 47 g micronutrients, 477 g slow release fertilizer 15N-3.9P-9.9K, 700 g coarse perlite and 75 g aluminum sulfate per 2.8 cu. ft. bag. If needed later in the growing season, fertilize plants weekly with soluble 17N-1.3P-14.1K at 200 ppm nitrogen.
4. During the growing season, keep plants in a shade house. Allow plants to go dormant outside in the fall prior to moving them to a controlled temperature storage facility (~4 °C) from November through April.

2. Collection of Beech Scale Eggs

1. In a BBD-infested stand, inspect heavily infested trees (easily identified by their “whitewashed” appearance, **Figure 1A**) with a hand magnifying glass to confirm the presence of eggs, typically abundant from mid-July to mid-August.
2. Use a paintbrush to gently brush the white waxy clumps of adult scale insects, eggs and other debris, into a plastic sealable one gallon collection bag (**Figure 1B**). Collect from a minimum of three different trees at least 12 m apart.
3. If needed, store eggs in the sealed collection bag for up to two weeks at 4 °C. Tape a small piece (2.5 cm square) of dampened polyethylene foam to the inside of the bag to prevent the eggs from drying out.
4. To separate the scale eggs (0.15 x 0.25 mm) from the adults (0.60 mm) and debris, construct a sieve using a short piece of 2’ PVC pipe and a coupling to support a square piece of 250 micron nylon mesh (**Figure 2**).
5. Empty the mixture of adults, wax, eggs, and debris from the collection bag onto the sieve and use a small paint brush to gently encourage the eggs to pass through the mesh into a glass Petri dish below. Plastic Petri dishes should be avoided because they hold more static electricity, making it difficult to move the eggs. Eggs before and after sieving are shown in **Figure 3**.
6. Purified eggs can be stored in the Petri dish at 4 °C for at least a week. To prevent the eggs from drying out, tape a damp piece of foam to the lid and seal with Parafilm.

3. Egg Viability Assays

1. To assess egg viability, use a 10 cc syringe to apply a thin ring of petroleum jelly around the circumference of the bottom of a 60 mm glass Petri dish (**Figure 4A**).
2. Transfer about 100 eggs to the center of the ring, place the lid on the Petri dish and seal with Parafilm. Allow the sealed Petri dish to remain undisturbed at room temperature for 2 weeks, or 3 weeks if eggs were stored at 4 °C prior to beginning the assay.
3. Hatched nymphs will become stuck in the petroleum jelly and can be easily counted, and empty eggs are easily distinguished from unhatched eggs by their color and sheen (**Figure 4B**). Calculate viability by dividing the number of nymphs by the sum of the empty eggs plus the remaining full eggs. Good viability should be in the 75% to 90% hatched eggs range.

4. Scale Resistance Screening of Large Mature Beech Trees in the Field

1. For a quantitative test, count out 500 eggs using a dissecting microscope and using a small spatula gently sprinkle them across the center of a predampened, open-cell 10 x 15 x 1.3 cm rectangle of polyethylene foam. To dampen foam, wet it then squeeze out as much water as possible. For a qualitative test, 500 eggs can be counted out and placed in a small glass vial and a “fill” line drawn that can be used to measure additional batches of approximately 500 eggs.
2. Place the foam pad onto the test tree with the egg side facing against the bark. Hold the pad in place with rope, string, twine, or plastic coated hardware wire. Plastic or metal based material should be used rather than natural fiber materials which are more readily scavenged by wildlife.
3. Line the top and sides of a precut 23 x 30 cm piece of vapor permeable waterproof house wrap with acetate based silicone adhesive and place it over the foam test pad. Press the edges of the house wrap to the tree to create a waterproof seal. Place nylon twine or plastic coated hardware wire around the tree and house wrap, to hold it in place while the adhesive sets (**Figure 5**).
4. Place a minimum of two test pads on each test tree, preferably on opposite sides of the bole. At each site, place test pads on at least 2 susceptible trees (with obvious natural scale infestation) as a control. Prior to placing the test pads on control trees, remove any naturally occurring scale insects or eggs using a firm bristled brush.
   Note: Foam pads with eggs can also be placed on trees for the purpose of rearing scale eggs, which can be particularly helpful in areas where the infestation level is low.

5. Scale Resistance Screening of Potted Seedlings or Grafts

1. For testing, select potted trees with a minimum diameter of 1 cm (caliper 5 cm above soil line) that are tall enough to have at least 2 separate test pads placed on them. Prune small side branches when necessary to make room for the test pads.
2. Use a dissecting microscope to count out 150 eggs and scatter them over a precut and dampened 2.5 x 7.6 x 1.3 cm open-cell polyethylene foam pad.

3. Affix the foam pad to seedling with the egg side against the bark, using plastic-coated wire. Wrap a small square of house wrap around the seedling just above the foam and seal it with acetate-based silicone (see **Figure 6A**).
4. Include known susceptible seedling families or grafts as controls.

6. Data Collection

Note: Approximately 52 to 57 weeks after placing the foam pads and eggs on the test trees, data can be collected. It is important that this is not done until after adults have begun to lay eggs so that their ability to reproduce can be determined. On some resistant trees it is not unusual to see a small number of adults establish but with no reproduction occurring.

1. Carefully remove the foam pad and count the number of adult scale insects established on the bark using a hand lens or magnifying glass (10X).
2. When the foam pad is removed, it is not uncommon for some adults and most egg clusters to be pulled off of the tree with the foam (see **Figure 7**). Using a dissecting microscope, count the egg clusters and adults that have remained attached to the foam pad.

Representative Results

Figure 6 shows a resistant seedling (**C**) and two susceptible seedlings (**D**, **E**) exhibiting different degrees of susceptibility. An example of what a susceptible mature tree looks like 57 weeks after the artificial infestation test was set up is shown in **Figure 5B**. When the foam is peeled away from the tree, it is not uncommon for scale insects and their egg clusters to remain stuck to the foam as shown in **Figure 7**, which is why it is essential to collect data from both the tree and the foam pad.

Figure 8 illustrates the average number of adult insects established per 100 eggs on artificially infested trees in two different stands, one in the Allegheny National Forest (ANF) in Pennsylvania and the other in Ludington State Park (LSP), Michigan. Both stands were infested with BBD and in each stand, a cluster of healthy trees was identified (LSP Resistant and ANF Resistant) and determined to be clonal based on DNA analysis (data not shown). A clonal cluster of BBD-susceptible trees was also identified at LSP (LSP Susceptible) but in the ANF five distinct susceptible trees were used (ANF Susceptible). Within and between clonal clusters, size varied with DBH ranging 2.1 to 12.8 inches. **Figure 8** shows that although there is a lot of variation in the number of adults established on the susceptible trees, clear differences are detected between susceptible and resistant trees. There was less variation in the number of adults in both resistant clusters relative to the susceptible trees. In the ANF Resistant cluster, all 12 clones that were tested had no adults, while in the LSP Resistant cluster some clones had a small number of adults. However, there was no overlap in the standard deviation from the mean of the resistant and susceptible trees at both sites.

The variation in the number of adults on clonally related trees in LSP (LSP Susceptible) is greater than observed in the ANF susceptible trees (ANF Susceptible) despite the fact that these trees are not clonal and are genetically different. Viability tests (data not shown) indicate this variation is not due to large differences in the viability of the eggs. The variation is likely due to both genetic and environmental differences and illustrates the importance of having susceptible controls for reference at every test site.

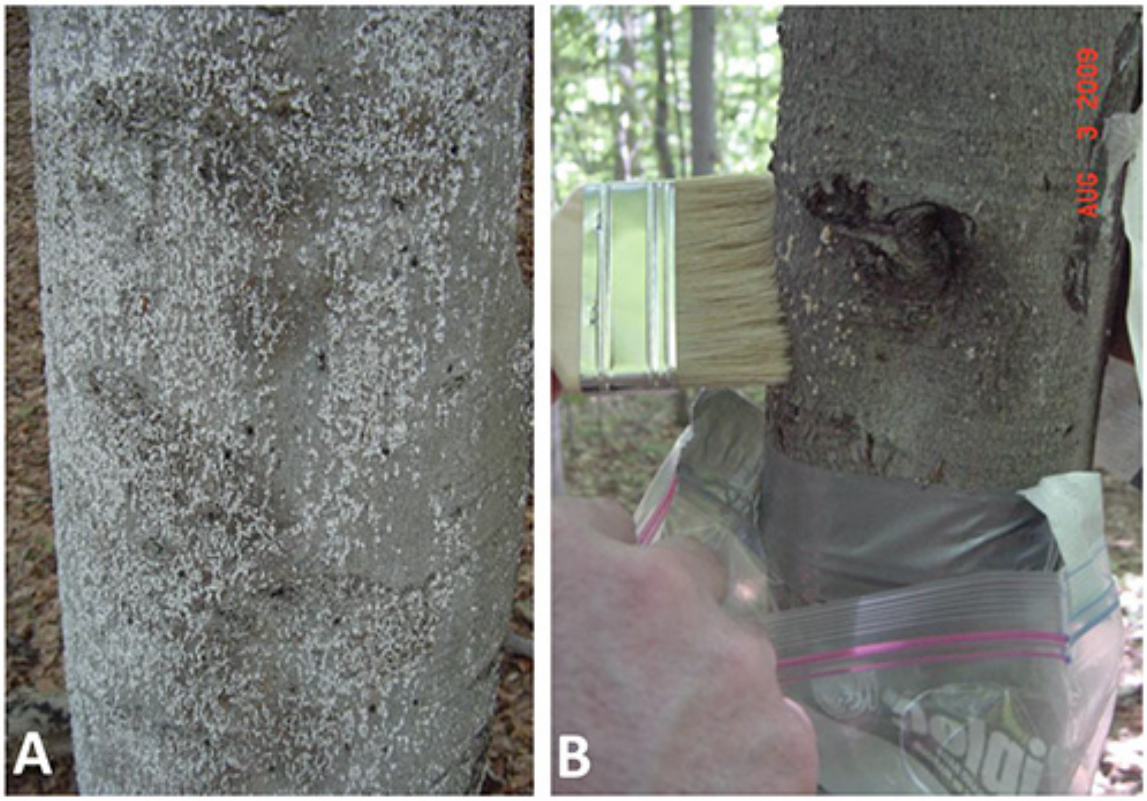

Figure 1. Collection of scale eggs from scale-infested American beech tree. A. Heavily scale-infested American beech tree. **B**. A paintbrush is used to brush adult scale insects and their eggs into a sealable storage bag below.

Figure 2. Construction of sieve to separate scale eggs from adults and other contaminants. A 4 x 4 inch pre-cut square of 250 micron nylon mesh is placed between a 4 inch piece of 2" diameter PVC pipe and a coupling to form the sieve.

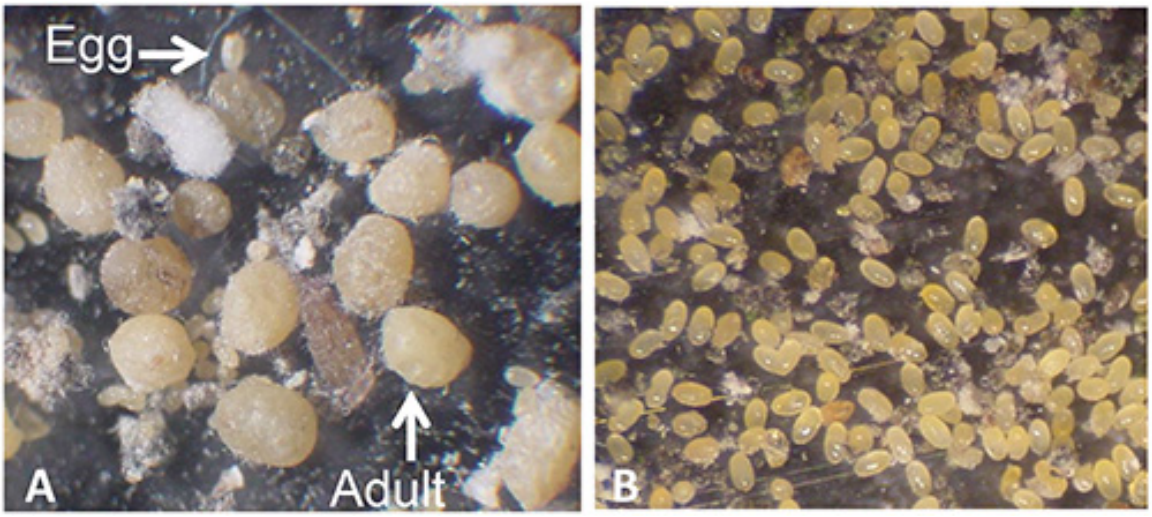

Figure 3. Scale eggs before (A) and after (B) sieving. A. Mixture of adult scale insects, eggs, and debris viewed under dissecting microscope after collection from tree. **B**. Purified eggs obtained after passing through sieve.

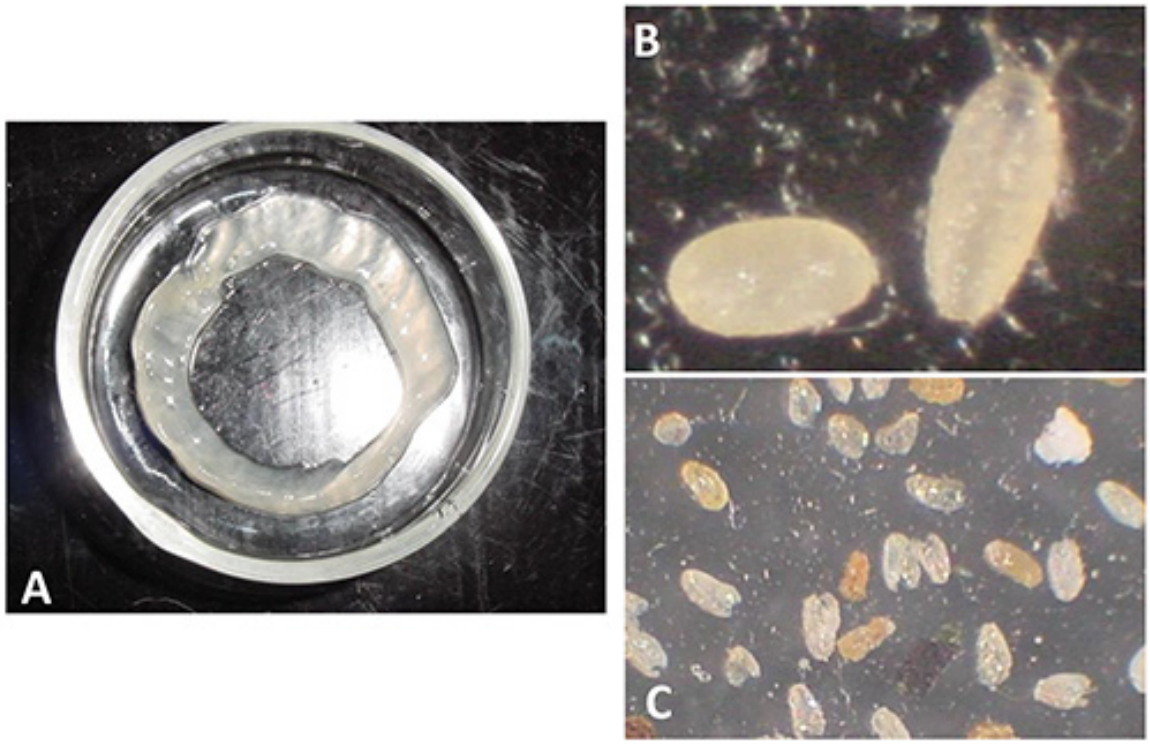

Figure 4. Egg viability test. A. Ring of petroleum in Petri dish to trap nymphs after hatching. **B**. Form I nymph on the right of an un-hatched egg. **C**. Hatched eggs are lighter in color and translucent relative to opaque yellowish eggs prior to hatching.

Figure 5. Artificial infestation of mature American beech in the field. A. House wrap affixed with acetate-based silicone to prevent excess moisture build up on foam pad with eggs underneath. **B**. House wrap has been removed and foam pad peeled back 52 weeks after placement to reveal scale colonization beneath on this susceptible tree.

Figure 6. Artificial infestation of potted American beech seedlings. A. Foam with eggs attached to stem. **B.** House wrap covering foam. **C.** Foam removed after 56 weeks on a resistant seedling, **D.** a susceptible seedling, **E.** and a highly susceptible seedling. Please click here to view a larger version of this figure.

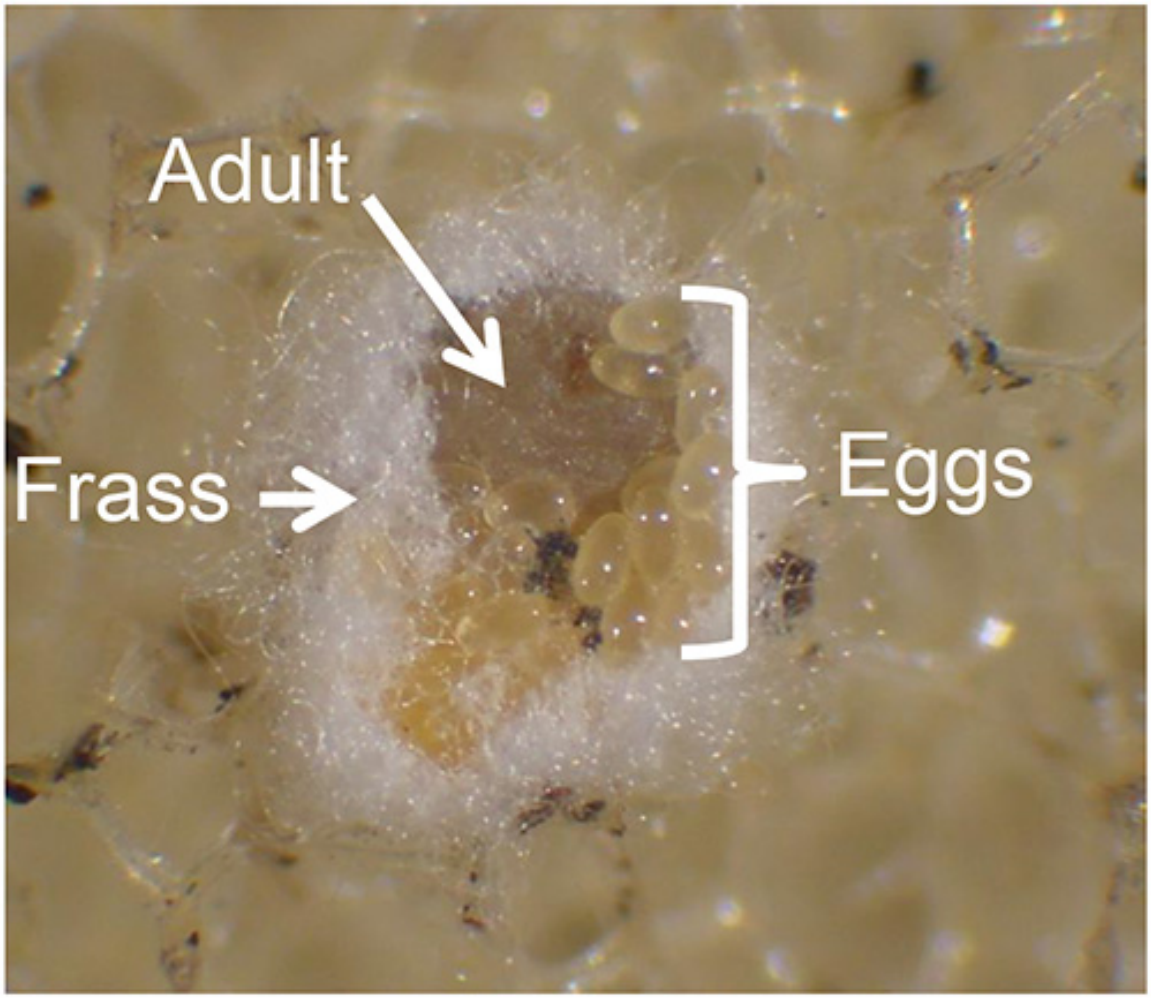

Figure 7. Adult scale and egg cluster on foam pad. The adult scale insect is surrounded and partially covered by white, waxy frass. The smaller, smooth and shiny eggs are on top of the adult insect and inside the wax. This would be scored as one adult and one egg cluster.

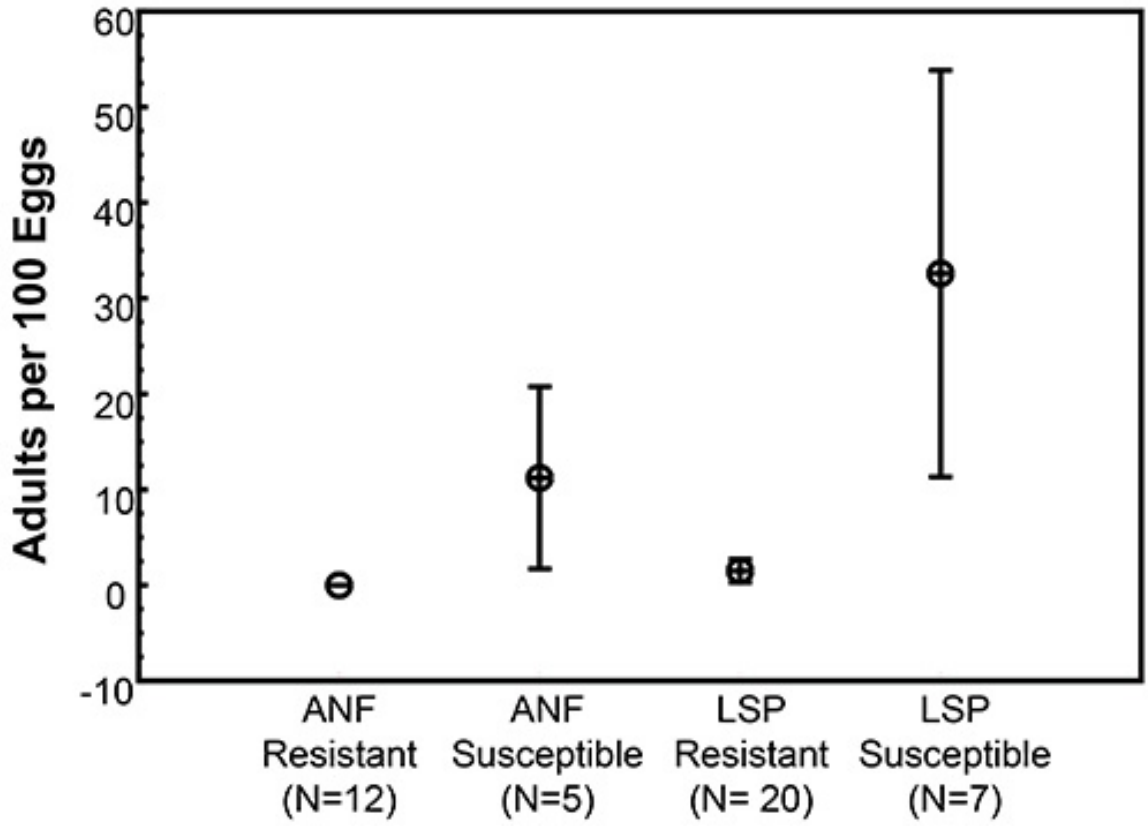

Figure 8. Comparison of the average number of adult insects established on artificially infested trees in two separate stands. Resistant trees in the Allegheny National Forest, PA (ANF) and in Ludington State Park, MI (LSP) are compared to susceptible control trees. The number of trees tested in each group is listed in parentheses below, and adult counts per 100 eggs applied were averaged across all trees within the group. The bar represents one standard deviation from the mean.

Discussion

The critical steps required for success of this assay include performing a viability test on the eggs and the use of susceptible controls at each site tested, and with potted materials. It is also important to use more than one test pad per tree or seedling. We have found that both in the field and in more controlled tests on potted plants, a frequent source of error can be pad failure. For example, in the field, even with our modification of covering the test pad with house wrap to prevent excess moisture, water flowing down the stem can favor one side of a tree and result in loss of a test pad.

In order to obtain accurate results, it is important to score both the tree surface under the pad and the foam pad itself. In addition to adults and egg clusters, the only mobile phase in the scale life cycle called form I nymphs, may also be observed. These mobile nymphs eventually insert their feeding stylet into the bark of the tree, becoming a form II nymph. Both types of nymphs are very similar in size and appearance to the small, oval shaped eggs, while the larger adults have a more spherical shape. The form I nymph is distinguishable from eggs because it has red eyes and is moving, but this movement makes getting an accurate count very difficult. The form II nymph can easily be mistaken for an egg, especially when recording data in the field with a hand lens or magnifying glass and not a microscope. For these reasons, we recommend counting only the adults and the number of egg clusters, which we have found gives us reproducible data on the ability of a test tree to support self-sustaining scale infestation. Previous work has shown that the adult count is highly correlated with the egg cluster number[9]. In many cases it may be sufficient to just count adults; however, we have observed some instances where adults are capable of establishing on certain genotypes of trees, but do not produce eggs. Without the ability to reproduce, the scale infestation would not be sustainable on these trees.

An unexpected downfall of this assay when performed in the field is the attractiveness of the test pads to bears. Future modifications to the protocol should focus on using materials that may be less attractive to bears or placing the test pads at different heights. A limitation to this method of testing is that it should not be used in areas that do not already have at least a light beech scale infestation, to avoid the risk of spreading beech bark disease.

The utility of this technique is that it can be used to assess parent performance as part of a breeding program. For such progeny tests stringent criteria for scale-resistance are usually followed, allowing only one adult and zero egg clusters as criteria for resistance. Using these guidelines to select parents, the proportion of resistant progeny should be approximately 50%, which is a considerable improvement over open-pollinated

seed[15]. However, for land management purposes it may be desirable to use less stringent criteria for retaining trees since trees that support a low level of scale insects may remain relatively healthy.

Disclosures

The authors declare that they have no competing financial interests.

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