ABSTRACT

KAUR, NAVDIP. Developing Artificial Rearing Techniques for Hemlock Woolly Adelgid, *Adelges tsugae* and Balsam Woolly Adelgid, *Adelges piceae;* Artificial Infestation and Epicuticular Wax Study of Carolina Hemlock, *Tsuga caroliniana*, Provenances. (Under the direction of Fred P. Hain.)

Adelges tsugae Annand, the hemlock woolly adelgid (HWA), is an exotic pest of hemlocks that is a threat to the health and sustainability of hemlocks in the eastern United States. Hemlocks (*Tsuga spp.)* are one of the most important ecological, economic and aesthetic tree species of the eastern US. In order to save hemlocks an intensive research effort on HWA is underway. The objective of this study was to develop an artificial diet for HWA and to determine, if there are differences in adelgid infestation rates and fecundity among eight provenances of Carolina hemlock. Sixteen diets were prepared and were tested in liquid, solid and cellular forms to determine their suitability for adelgid development. In addition, feeding and delivery systems were developed to provide fresh diet continuously. All the liquid based diets showed no significant differences and no diet uptake except the MDB-1 and MDB-3 diets. BWA crawlers were able to survive for two weeks on these two diets. The 1st instars survived for 10-12 days on these diets whereas their survival was not more than 3-4 days on other diets. Solid based diets did not show any encouraging results and all the crawlers stopped their activity in 3-4 days on these diets. Digestive track dissection of the HWA suggested the cellular nature of their diet and subsequent cellular based diets allowed 10-15% of the crawlers to develop to 2nd instars, and the2nd instar survival was also high. Amylase tests on the HWA saliva showed very week amylase activity probably from the presence of microorganisms in HWA gut.

Variation among Carolina hemlock was observed with respect to infestation rate and fecundity. The infestation level (number of eggs/woolly mass) for the provenances from Caeser head Campground and Crabtree was significantly higher than Wildcat and Table rock, and no infestation was seen in Bluff Mountain, Linville and Cradle provenances. Insects respond to many chemical cues for feeding that can be responsible for susceptibility and resistance of the host. The epicuticular wax was analyzed by GC/MS and linked to the host preference of HWA. Hexacosanol was observed in all the provenances, however the concentration of hexacosanol with other chemicals seems to be dependent on insect infestation. Some other chemicals are also found, which are either deterrents or stimulants in other insects but their role in HWA and hemlock species is not known.

Developing Artificial Rearing Techniques for Hemlock Woolly Adelgid, *Adelges tsugae* and Balsam Woolly Adelgid, *Adelges piceae;* Artificial Infestation and Epicuticular Wax Study of Carolina Hemlock, *Tsuga caroliniana*, Provenances

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BIOGRAPHY

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CHAPTER 1

Introduction and Literature Review

Life history, impact and control of hemlock woolly adelgid in the Eastern U.S.

Eastern hemlock, *Tsuga canadensis* (**L.)**, is a dominant forest tree species in the eastern United State, and adjacent Canadian provinces, and is the third most common tree species in southern New England (Brooks et al 1993). They account for 7% of timberland in the northeastern states and 57% of timberland in the north central states (Powell et al 1993). In the northern United States, hemlock is estimated to occur on 7.6 million hectares of timberland (Schmidt and McWilliams1996). Hemlock-dominated forests are characterized by dark conditions and acidic soils that control and limit fundamental ecosystem characteristics such as composition, productivity, nutrient cycling, decomposition, and succession dynamics (Evans 2004).

The health of hemlocks is threatened by the introduction and spread of an introduced forest pest, the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Brooks 2001). HWA is a serious pest of hemlock forests causing severe decline and mortality of hemlock trees. Infestations by exotic pathogens and pests are an important economic, ecological (changing nutrient cycling, susceptibility to other diseases, disasters like fire, changing microenvironments), and evolutionary process that changes the structure and function of the ecosystem with devastating impact on the natural resources and their aesthetic value (McKnight 1993). Over the last 20 years, HWA has killed native stands of eastern hemlock,

resulting in serious changes to the landscape, as well as, widespread decline, and mortality of the hemlock in the Eastern United States (Young et al 1995, Playfoot and Ward 2005).

HWA was originally introduced to the Eastern U.S. in 1924 (Mcclure1987) and was discovered near Richmond, Virginia in 1954 (Havill 2005). Since then it has spread north and south to North Carolina, South Carolina, Georgia, West Virginia, Maryland, Delware, Pennsylvania, New Jearsy, New York, Rhode Island, Massachusetts, and Connecticut (Cheah and Mclure 1998). HWA has spread at a rate of approximately 12.5 km per year from its established eastern range (Evans and Gregoire 2007). According to Margaret *et al.* (2003) temperature and moisture are prime limiters in influencing the range of this pest. Gradual decline in survival of HWA occurs when temperature decreases; the low temperature survival threshold for North American adelgid populations is -30° C (Parker *et al.* 1999). The range of eastern hemlock extends well into Canada but the northern spread of this insect may be slowed or hindered by cold temperature. However, Skinner *et al*. (2003) point out that it is possible that over time this insect may develop greater cold tolerance thereby permitting it to expand its northern range.

Life History of Hemlock Woolly Adelgid

HWA covers itself with a white, waxy secretion for most of its life cycle (Cheah and Mclure1998) and is 1.3 to 1.5 mm in size with piercing sucking mouthparts. HWA deposits its eggs on hemlock twigs in the white woolly secretions. Eggs are brownish-orange but darken as they mature. The woolly mass surrounds the female and protects her and her eggs from various environmental elements (Wallace 2005). All stages of HWA spend their life at the base of a hemlock needle except the first instar crawlers that move around the branches

(McClure *et al* 1996). First instar nymphs are black with a white fringe around the edge and down the center of the back. HWA develop through four instars and eventually mature as adults in June. Reproduction in HWA is parthenogenetic in North America, and females produce two generations (spring and winter generation) a year with overlap among all life stages. Two phenotypes occur in spring generation, winged sexupura and wingless progrediens. Some of the adults produced in the spring generation are winged and are called sexupura. The sexupura leave the hemlocks in search of spruce trees, which are the adelgid's primary host (McClure *et al.* 2001). However, due to the absence of suitable species of spruce in North America, the sexupura dies before sexual reproduction occurs (McClure1987). Spruce is the primary host for all adelgids but they can survive in its absence. The wingless progrediens initiate new generations on hemlock. Adult progrediens lay their eggs soon after reaching maturity in late June, and hatching nymphs enter an aestival diapause that lasts until October (Stadler *et al*. 2005). Progrediens progeny are called sistens. This second generation feeds and develops through late autumn and matures in late February (McClure and Cheah 1999). The main reasons behind the proliferation of HWA are its ability to produce two parthenogenic generations, the lack of natural enemies and host resistance, and their ability to be spread by wind, birds, and human activity (McClure 1990).

Feeding Behavior of Hemlock Woolly Adelgid

HWA feeds on the young branches and twigs of hemlock, usually near the base of the needles, and is easily recognized by the presence of woolly secretions around egg masses. Adult HWA feed on the sap of young shoots in late winter and early spring and may also

inject toxic spittle into the branches (McClure1987). HWA feeds on the ray parenchyma cells with the help of long stylets (Young *et al* 1995).

Severe adelgid feeding prevents the spring flush of new growth, causes desiccation of existing needles, discoloration, and needle drop from the branches. Heavily infested trees show poor crown conditions, and reduced shoot growth. That, in combination with other environmental stresses, can result in rapid tree decline and death (Orwig 2002). In cases of a heavy infestation tree recovery without treatment is impossible. Main tree limbs are lost in the first summer, and the entire tree may be 3-4 years. Hemlocks infested with HWA have deficiencies in microelements, necessary to produce and maximize new growth (Pais and Demko 2005). Rates of decline vary but mortality can occur in as few as 4 years (McClure 1991). Smaller infested trees may have higher mortality rates (Brooks 2001). However, some stands remain living more than a decade after infestation. Adelgids insert their stylets into the xylem ray parenchyma cells of hemlock trees to extract carbohydrates (McClure 1991), this reduces the vitality of trees as these microelements and carbohydrates are important for new growth, vigor, reproduction, and defense. Moreover reduction in vitality of hemlock branches also restricts translocation (Doccola *et al*. 2003).

Ecological Importance of Hemlock

Hemlock is an ecologically important, long-lived, shade tolerant tree, and is present in either pure stands or in combination with white pine or deciduous trees. It plays an important and unique role in the forest ecosystem, which cannot be quantified. Hemlock creates structural diversity and provides cover for numerous terrestrial wildlife species (Godman and Lancaster 1990). Destruction of hemlock by HWA leads to disturbed

ecosystems due to alterations in habitats resulting from tree loss, which, in turn, affects understory vegetation composition, ecological processes, as well as the structure, and function of forest stands. Eastern hemlock has unique structural characteristics that provide important habitat for numerous bird species in northeastern United States, and removal of hemlock stands by HWA has profound effects on avian communities. Several species of songbirds, black throated green warbler, blackburnian warbler and blue-headed vireo are hemlock dependent species during their breeding season (Ross *et al.* 2004). Several species of hemlock dependent mammal, and amphibians are also affected (Brooks 2001). A study conducted within an area extending from southern Connecticut up to the Berkshire Plateau shows the disturbances by HWA infestations led to black birch (*Betula lenta*) dominated stands (Kizlinski 2002), and homogenization of forest lands (Jenkins *et al*. 2000) resulting in a decline in the overall biodiversity (Tingley *et al*. 2002). Trees destroyed by the HWA lose their needles and create canopy gaps over a period of several years (Yorks *et al*. 2003,Kizlinski 2002). Large gaps in the forest canopy allow more light to reach the ground or bodies of water, which increases the soil or water temperature (Orwig and Foster 1998), causing significant impact on the soil or aquatic life. Jenkins *et al.* (1999) reported higher N mineralization and nitrification rates at sites infested by the HWA and suggested that the accelerated turnover rates could result in NO_3^- leaching from stands with *T. canadensis* mortality. Most of the hemlock stands found in or near moist areas, such as streams and swamps, readily change the soil nutrient complex, and also affect the nutrient complex of stream waters. Hemlock mortality can result in increasing ion concentration in soil and stream water, leaching of NO_3^- and other anions associated with depletion of soil nutrient

cations (Ca^{2+} , Mg^{2+}) , mobilization of toxic elements (e.g. Al), and acidification of soil and stream water (Stoddard 1994). Yorks *et al*. (2003) reported that mortality of *T. canadensis* resulted in elevated concentration of NO_3^- cations in the soil water and increasing densities of *Betula alleghaniensis* seedlings. Kizlinski (2002) stated that damaged sites had significantly larger inorganic N pools and greater potential for N leaching than undamaged forests.

Control and Management of Hemlock Woolly Adelgid

Control of HWA on ornamental hemlocks is relatively easy compared to controlling it in forest areas. Hemlocks growing in nurseries and ornamental landscapes can be saved by carefully monitoring for the presence of adelgids, and then by implementing various cultural practices to enhance tree vigor and to discourage pest invasion, or by using mechanical and chemical measures (McClure 1987). A twice-yearly application of horticultural oil is the standard treatment for HWA, but efficacy depends on foliar coverage of the contact insecticide, and the spray is subject to aerial drift (Doccola *et a*l. 2003). Most of the year the HWA is covered in a woolly mass that interferes with the direct contact of insecticides. Soil injections of systemic insecticides are also a solution where the insecticide can be taken up by the roots and distributed throughout the tree (McClure 1997). Insecticide leaching into the soil is a potential problem with soil injections near waterways (Daccola *et al*. 2003). Tree injections of insecticides are a solution to the leaching problem. Concentrated pesticide is injected directly into the tree, which results in significant HWA mortality. This tactic is best for early infestations or low densities of HWA as movement of injected chemicals depends upon the health of the tree's transport tissue (Daccola *et al*. 2003). There is no effective large

scale method of controlling *A. tsugae* in forested areas because the use of chemicals is not feasible, and there are no effective indigenous natural enemies or host resistance to curb the adelgid's proliferation (Wallace and Hain 2000). Most hemlock forests are found in inaccessible locations or along riparian zones where chemical controls are neither reasonable nor allowed (Flowers *et al*. 2005).

The other option to control *A. tsugae* is classical biological control, or importation, mass rearing, release and establishment of non-native natural enemies (Cheah and McClure 1996). Several predatory beetles have been evaluated and imported from Asia and Japan, among these, *Sasajiscymnus tsugae, Scymnus ningshanensis, Laricobius nigrinus* and *Harmonia axyridis* seem to provide promising control results. *Sasajiscymnus tsugae* (is native to Japan, and very prey specific, exclusively feeding on all stages of HWA. Millions of *Sasajiscymnus tsugae* have been mass reared and released into the eastern United States forests. (Cheah and McClure 2000). Field surveys following augmentative release of *S. tsugae* indicate the successful overwintering, reproduction and local dispersion both laterally and vertically into the upper canopy (Cheah and McClure 2002).

No known parasitoids are associated with family Adelgidae (Montgomery and Lyon 1996). An insect killing fungus, *Verticillium lecanii,* also has the potential to diminish the growth of HWA without affecting the predator *S. tsugae* (Parker *et al.* 2004).

A considerable amount of research has been done or is still in progress relating to the chemical and biological control of HWA on eastern and Carolina hemlock, however, comparatively little work has been done on the resistance of hemlock species to the insect.

According to Painter (1951) the hereditary resistance of plants against infestation by insects may depend on three ecological principles: 1) the tolerance of the host plant to feeding; 2) antibiosis, that is, on the check of growth or reproduction of the pest by insufficient nutrients, or by toxic effects caused by special properties of the plant; and 3) preference, that is individual choice of the insects. The uppermost layer of cuticle, the cuticular wax, is hydrophobic and comprised of a multiple homologous series of long chain lipid molecules, principally hydrocarbons, alcohols, fatty acids, sterols, ketones and aldehydes (Bianchi 1995). The primary function of this wax layer is to protect the plant from dehydration, but also act as a first defense of the plant's protection system from pests and pathogens (Jeffree 1986). The development of a thick cuticle, the production of highly reflective wax and the accumulation of flavonoids prevent UV-B radiation from reaching physiological tissues (Caldwell and Tevini 1997). Wax crystals can also help to maintain stomatal gas exchange (Brewer and Smith 1997). Some of these compounds (wax layer) also have secondary roles as contact pheromones (Blomquist *et al*. 1993.). The chemical composition of both the polymeric and extractable lipids varies among species, among genotypes or within species and among parts within the plant (Eigbrode 1995). In many cases, visual factors such as shape, color, and size were significant to protect plant from insect pests (Renwick and Chew, 1994), but chemical cues play the major role in host selection (Udayagiri and Mason, 1995). Surface lipids may also act as a protecting and selecting barrier, contributing to the plant's defense by adversely affecting insects through direct toxicity, or by interfering with their movements (Knoll 1914). After touching the plant's surface, insects evaluate the plant as a food source, or oviposition site. Epicuticular

lipids, extracts, or individual lipid components enhance or deter the insect's movement, feeding and oviposition (Eigenbrode and Espele 1995). Plant chemistry is probably the most important source of information contributing to the final decision by an insect to oviposit. The balance of opposing positive and negative cues evoked by phytochemicals determines whether a plant is accepted or rejected by a herbivore (Renwick and Chew1994). Pentane leaf extracts of corn containing n-alkanes stimulate oviposition of *Ostrinia nublanis*, but methanol extracts received less oviposition (Udayagiri and Mason, 1997) and act as deterrents for insects.

Cameron et al studied the characteristics of the cuticle's influence on pest and pathogen susceptibility in plant feeding in 2002. Morphological and chemical studies carried out on the epicuticular wax of some higher plants have been used to correlate the nature and chemical composition of wax with susceptibility of plants to insect attack.

The present study concerns the development of a synthetic diet for the hemlock woolly adelgid (HWA). Such a diet would enable the bioassay of specific chemical components isolated from resistant and susceptible species or genotypes within the same species. This will help in determining which chemicals in the tree are directly involved in resistance which eventually may help us to breed trees with the chemical characteristics leading to HWA resistance.

Our objective in this research was to develop an artificial diet and feeding system for rearing the hemlock woolly adelgid (HWA) and the balsam woolly adelgid (BWA). A successful rearing diet and technique will prevent the insect from entering diapause and make it available for other laboratory experiments. In addition to this, it will reduce the cost of

rearing *Sasajiscymnus tsugae,* a predator of HWA, which requires a year round supply of HWA. Forestry practices to control HWA in United States rely mainly on the biological control of HWA. This seems practically impossible because millions of beetles are required each year to maintain the continuous effect on HWA.

HWA predator rearing in sufficient numbers for release at appropriate times is expensive and difficult. *Sasajiscymnus tsugae* are reared on their natural host, which is a very expensive and inefficient approach. Artificial rearing of HWA is needed for the economical production of consistent populations of natural enemies, so that healthy predators can be reared round the year.

Artificial Insect Rearing History

Very early unsuccessful attempts at artificially rearing insects using leafhoppers as the target insect can be dated back to 1927. Since then, many investigators have tried artificial rearing but the first success was achieved with an aphid ingesting sugary fluids (synthetic liquid diet) through a parafilm membrane (Mittler and Dadd 1962). Later this diet was modified and tried on several other insects in the same family with some success. These efforts enhanced the research in the field of nutritional requirements of insects, and opened new possibilities in the artificial rearing of insects.

Intensive studies on the rearing of insects and artificial diets have led to three laws proposed by House (1966): the rule of sameness, the principle of nutrient proportionality, and the principle of cooperative supplements. These laws simply state that the all insects essentially feed on the same food with small differences in nutritional proportions and with

additions of some stimulants. In addition to these basic laws, investigators also studied the mode of feeding and other factors that enhance or inhibit feeding. For example, aphids may depend upon the natural or optimum turgor pressure in order to feed normally (Kennedy and Mittler 1953). Also the ratio of nutrients in the diet and the mode of consuming it (solid, liquid or slurry) are different and these factors need to be studied for different insects in order to achieve successful rearing.

The greatest difficulty in insect dietetics lies in the art of getting the insect to eat an unfamiliar foodstuff. The intake of nutrients is only possible if a suitable choice of food is available (Abisgold et al 1994)**.** The problem is difficult because it must be solved for each species (House, 1966). In artificial rearing the insect doesn't get its diet in the natural way, and to make this acceptable to an insect a series of studies are needed e.g., nature of insect, life history, etc. Furthermore, in a comparative analysis based on the composition of chemically defined diets of 117 insect species, the nutrient requirements changed within the life history of the insect (Simpson and Raubenheimer 1993).

Artificial Rearing in Suborder Homoptera

The most important factor in developing and using successful artificial diets is a thorough understanding of insect's feeding biology. Feeding biology is the combination of many factors, such as the type of mouthparts, feeding stimuli, and digestive enzymes (Cohen 2004). The suborder Homoptera includes aphids, cicadas, leaf hoppers, scale insects and adelgids. The majority of the Homoptera are xylem sap or phloem sap feeders with some exceptions that feed on mesophyll cell sap. Saliva and stylets are important factors in

homopteran feeding. With complex stylet movements, insects locate the xylem and phloem bundles that are far from the plant surface. Saliva is continuously secreted during phloem sap ingestion (Tjallingii 1995) and is responsible for damaging plant cells.When watery saliva is ejected and sucked back with the soluble components of the selected substrate, it is assumed that aphids are able, by the action of hydrolytic enzymes that occur in the saliva, to assess the nature of the tissue through which the stylet passes (Miles 1999).

Significance of stylet and stylet sheaths

Stylets are the functional mouthparts of Homoptera that reach tissue far from the body; they penetrate all the way to the phloem without ingesting (Kloft and Ehrhardt 1962). The majority of cells that show punctures by stylet sheaths are vascular bundles (Tjallingii & Hogen Esch1993). Stylet sheaths are mostly made of proteins (Srivastava 1987). Fundamental knowledge about the working of stylet sheaths is very important to comprehend the feeding behavior of insects. Early work indicated that stylets do not contain nerves, and the central mandibular duct is empty (Weber 1928), but later morphological evidence has found nerves within the central duct of mandibular stylets (Forbes 1966). After inserting its stylets the insect may withdraw and then move forward again on a new path, branching to one side or the other of the old path but the resultant branches seem always to be more or less in the same dimension throughout feeding (Miles1968).

Penetration of tissue does not necessarily mean an acceptable food source. An aphid may insert its stylet all the way to the phloem of an unacceptable plant and even ingest measurable quantities of sap before terminating its attempt to feed (Ehrhardt1961). Aphids sample the substrate by sucking up liquids during penetration and thus accept or reject the

tissue or plant as food (Hennig 1966, McLean & Kinsey 1965). Entry of stylets depends not only upon the extent of acceptance of the diet but also on the nature of tissue through which it is passing. There are three steps in the process of food acceptance: 1) ignorance or 'no recognition' of sieve tube (element of phloem an help in transformation of food material) identity which is element of phloem an help in transformation of food material in fod, 2) recognition but no ingestion, 3) acceptance, sap ingestion and concurrent salivation (Tjallingii & Hogen Esch1993). But it is still unclear whether this acceptance is due to different motivational changes of aphids like previous experience or starvation.

Feeding also depends upon some other factors. Most plant-sucking insects feed passively on sap, which is sucked into the mouthparts by the turgor pressure in the sieve tubes (Tjallingii 1995). Aphids depend less on their own sucking power than on the turgor pressure in the plant (Kennedy and Mittler in 1953). Thus wilting leaves are less acceptable to insects than turgid leaves.

Stylet sheaths help prevent sap leakage from the stylet food canal (Arnaud 1918, these results were later corroborated by Crews et al 1998). The external sheath material has been termed a flange (Nault and Gyrisco 1966. Miles 1972). The flange forms a benign impermeable barrier around damaged parts of the cell wall, may actively absorb toxic biochemicals that may be generated by the plant, and prevents the damaged parenchyma cells from leaking into the intercellular space. Toxic substances released in the vicinity of the stylet sheath are either immobilized or absorbed by the stylet sheath (Urbanska & Leszczynski 1997). The lipoprotein nature of the stylet sheath makes it stick to the plant surface and prevents the stylet tips from slipping while penetrating the substrate (Pollard

1973). The stylet sheaths may lubricate the passage of the stylet and promote the sliding action of the stylets (Smith 1985).

Importance of Saliva and Salivary Enzymes

The saliva of Homoptera plays important roles as a physiochemical agent during the penetration of plant tissue, and in moistening food and mixing it with hydrolytic enzymes. All Homoptera and Heteroptera discharge two different kinds of saliva; one that solidifies rapidly upon ejection (gelling component) and another non-gelling component that always remains watery (Miles1972). One function of watery saliva may be to keep the sieve tubes open (Dixon1975, Miles 1999). Other functions of saliva are lubrication of the mouthparts, dilution of food, help in stylet penetration, excretion of some metabolites, initiation of food digestion, and prevention of deleterious changes in the food source. The Hemipteran stylet has a double canal that helps send saliva and suck fluid from the plant. Saliva is pumped down one side, and fluids are sucked up the other (Miles 1968)

The function of some enzymes in insect saliva is rather ambiguous. The entire enzyme complex present in saliva are not helpful in processing food but also promote other functions like the gelling of stylet sheath that facilitates intercellular stylet penetration (Peng and miles1998) and detoxifying functions (Peng and miles1991).

Damage symptoms from feeding by piercing–sucking insects may be caused by the injection of salivary phytotoxins into plants (Burd et al in 1998). Amylase is one of the key enzymes involved in digestion and carbohydrate metabolism in insects
(Horie and Watanabe, 1980). A physiochemical agent, catechol-oxidase, present in the saliva of a rose aphid, *Macrosiphum rosae* converts a deterrent (produced by the plant and

presumably toxic), o-diphenols, to a phagostimulant (presumably non toxic) products (Peng and Miles1998). In most of the sap sucking insects the salivary enzymes added by stylets dissolve or soften the pectin layers of mid-lamellae (McAllan and Adams 1961). Pectin is present in the middle lamellae and acts as an intercellular adhesive in plants. Pectinase present in the saliva splits the methyl groups from the naturally occurring pectin and makes them more susceptible to polygalacturonase, an enzyme present in saliva. (Campbell and Dreyer, 1985). Polygalacturonase is responsible for solubilizing pectin in the middle lamella (Squire 1947). The role of pectinase in the saliva of some aphids may be to allow stylet penetration through the primary cell wall (McAllen & Adams 1961). The enzyme polyphenol oxidase in the salivary gland of Aphididae helps overcome the natural defenses of plants (Peng and Miles1998). Polyphenol oxidase neutralizes the phenolic compounds produced by plants (Peng and miles 1991), and converts them into phagostimulatory products (Jiang1996)

Nutrient Requirement of Insects

The most important aspect in the artificial rearing of insects is understanding their nutritional requirements. Intensive studies have addressed nutritional requirements; the most important nutrients required by insects are discussed below.

Importance of Sugars

Auclair 1969 reviewed the importance of carbohydrates in aphid artificial rearing. Auclair (1965) did preference tests for sucrose in which aphids were given access simultaneously to diets containing 0%, 10%, 20%, and 40% sucrose. The rate of nymphal production, feeding and survival of green peach aphid, *Myzus persicae* was increased when

they fed on a 10-20% sucrose solution. Older nymphs preferred higher concentrations of sucrose when producing progeny, whereas younger nymphs preferred diets with a lower sucrose concentration (Srivastava and Auclair1962). Diptera and Hymenoptera favored 20%-50% of either glucose, sucrose, fructose or maltose, whereas Lepidoptera favored a 9%-17% solution of sucrose (Sotavalta et al 1962). Some insects also imbibed sugar solutions with concentrations as high as 60% -70%. It may be possible that at a higher concentration sucrose provides an optimal amount of some unidentified beneficial substance for aphid growth (Srivastava and Auclair1971). The main carbohydrate in sieve-tube sap was sucrose and the concentration of sugars in the sievetube exudates of trees usually varied between 10% and 25% (Zimmerman 1960) or 10%-30 % (Ziegler 1968). Reproduction and growth rates are higher when aphids grow on a diet with a sucrose concentration of 30-35% (Auclair 1965).

Replacing sucrose with other sugars such as fructose, glucose, trehalose or melezitose significantly reduces the growth of pea aphids (Auclair 1965). The reason behind this seems to be the stimulant function of sucrose. Sucrose is not only essential for growth and development but also acts as phagostimulant, which makes sucrose more preferable over other sugars (Mittler and Dadd 1963a). At least 10% sucrose was necessary for the absorption of the artificial diet in aphids (Srivastava and Auclair 1971). We conclude that sucrose is a crucial factor in the preparation of synthetic diets for the artificial rearing of sap sucking insects.

Importance of Amino acids

Insects require eight to ten essential amino acids in their food: methionine, threonine, tryptophan, valine, isoleucine, leucine, phenylaline, lysine, arginine and histidine (Cohen

2004). Other amino acids are also important in building proteins, but the insect can synthesize them. Amino acids are used as respiratory substrates by aphids (Wilkinson et al. in 2001). Adult pea aphids prefer diets with lower amino acid concentrations, and second to fourth instars prefer diets with high amino acid concentrations. The growth rate and survival of pea aphids is highest at 4.3% amino acid concentration but decreases significantly when amino acid concentration decrease to 3% or increase to 4.9% (Auclair 1965).

Excessive amounts of amino acids are excreted in honeydew without any deleterious effect on aphids (Mittler 1958). The performance of first instar aphids was substantially influenced by sugar and amino acid content, as the concentrations of both increased, growth also increased, and at low levels they performed poorly (Abisgold et al 1994). The ratio of sucrose to amino acids in the diet is more important than the absolute concentration of either (Simpson & Raubenheimer 1993).

Importance of Lipids

Lipids can also act as a part of a nutritive diet for insects. It is possible that some insects require lipids for metabolic function or as sterols. Lipids in insects usually occur in the form of glycerides (Strong 1963a). All insects studied have shown a requirement for sterol but not necessarily the same sterol (Friend 1958). *Myzus persicae* may require some linoleic acid in their diet (Strong 1963b), This fatty acid was abundant in sugar beet leaf juice but absent from the honeydew emitted by aphids feeding on sugar beet leaves**.** Gluten and gliadin are two lipids that are moderately effective in eliciting feeding responses in confused flour beetles (Loschiavo 1965). Not all insects require lipids as part of a nutritive diet. For example, there was no growth retardation with cholesterol omission as a source of soluble

fatty acid and sterol in artificial diets of green peach aphid, *Myzus persicae* (Dad and Mittler 1965).

Importance of Inorganic salt

Many trace minerals are required in a diet in the long run, because many universally vital processes depend upon their presence in basic enzyme systems. These elements act as activators or inhibitors of enzymatic activity and their lack may be detrimental to the growth and reproduction of insects. However, some of these elements occur ubiquitously as contaminants in other chemicals and so their availability in artificial diets is still not clear and very little information is available on the mineral requirements of plant –sucking insects. In the artificial diet of pea aphids seven inorganic elements are essential. Four of them; potassium, magnesium, phosphorus and sulphur are needed in substantial amounts and three others; iron, zinc and manganese, in trace amounts. Sodium and chlorine are also important but they end up in diets as contaminants in sufficient quantities (Dadd 1966). In addition to specific concentration, combination is also critical. Two salts, MgCl26H2O(2%) and K3PO4(5%) play important roles in the growth of the pea aphid *Acyrthosiphon pisum*, a slight increase or decrease in the concentration of these salts significantly reduced growth (Auclair 1965)**.** The same concentration of salts is also used in a diet fed to *Myzus persicae* (Mittler and Dadd 1962**)**. But optimum growth in *Tenebrio moliter* occurs with .82% of K_3PO_4 and 0.2% of $MgCl_26H_2O$ (Fraenkel 1959).

An element that is necessary for the growth of one insect can be a detriment for another insect. Peptidase present in the alimentary canal of *A. pisum* was activated by Mn^{++} and Co^{++}, and inhibited by Zn^{++} (Srivastava and Auclair 1963). Amylase activity from the

midgut of *Tenebrio molitor* was inhibited by Hg^{++}, Cu^{++} and ascorbic acid but stimulated by Ca^{++} and Cl^{-} (Applebaum et al 1961). The supply of trace elements is also important for the normal maintenance of symbionts in the insect gut (Auclair and Srivastava 1972). Magnesium is essential to *Blattella germanica* for transmitting beneficial bacteria to succeeding generations, and zinc is needed as a synergist for magnesium (Brooks1960).

Importance of Vitamin C

Vitamins have great importance in insect feeding. Ascorbic acid (vitamin C) is known to serve as a phagostimulant for phytophagous insects (Ave 1995). It is essential for the growth and development of most plant feeding insects and protects other labile dietary constituents due to its reducing and acidic properties. Ascorbic acid plays a role in enzymatic reactions associated with molting. Excess quantities of vitamins are not excreted in the honeydew of aphids; hence they are toxic when their concentration is too great (Cress and Chadda 1971). In those insects that do not require the vitamin, ascorbic acid is still ubiquitously present in insect tissue (Chippendale 1975). Bacteria are responsible for synthesizing ascorbic acid in the cockroach foregut and midgut (Raychaudhury and Banerjee 1968).

Role of pH

A pH of 7.4-7.5 is best for the growth of aphids, slight decreases and increases outside of this range seriously affect the rate of feeding (Auclair1965). Leafhoppers locate phloem in the sugar beet by following the pH gradient. When beet petioles were exposed to CO_2, which changes the pH gradient of the phloem, only a 12% success rate in phloem finding was observed as compared to a normal rate of 56% (Fife and Frampton, 1936). Pea

aphid, *Acyrthosiphon pisum* prefer a more alkaline pH and choose a pH of 7.8-8 (Auclair 1963). A decrease in the honeydew excreted by pea aphids was reported when they fed on diet with a pH of less than 7 or more than 8. This indicates that pH is very important for optimal growth, development, survival and reproduction (Auclair 1965).

Effects of Environmental Conditions on Amount Ingested

Environmental conditions also play an important role in insect feeding. Different Environmental conditions act differently in an insect's diet. In nature insects are free to move through their environment that contains gradients of temperature, light, electromagnetic energy and chemical agents (such as plant aromas and pheromones (Cohen2004). These conditions are impossible to simulate in the laboratory but care should be taken to ensure diet acceptance.

When brown leafhopper, *Orosius argentatus* ingested a diet under different environmental conditions of light, temperature and humidity different results were obtained (Day and Mckinnon, 1950). Results showed that insects fed readily, and were more active in dark and lower humidity conditions**.** Similarly environmental disturbances, such as light or temperature changes, promoted activity in general, including feeding (Blaney et al., 1973). Environmental conditions also affect the developmental processes differently in different stages of the insect's life cycle. The development rate of the balsam woolly adelgid from egg to adult is largely dependent on temperature; in warm areas there are chances of four or more generations per year (Mitchell et al, 1961). Similarly in most regions adelgids over winter in a dormant phase of the first instar nymph, the neositens (Balch 1952). This stage can survive

temperatures well below 0^{o} F, whereas temperatures near freezing kill other stages, including the egg. Artificial feeding systems need to take environmental conditions into account when trying to successfully raise insects.

Phagostimulants

Another factor that impacts insect feeding is phagostimulation. A phagostimulant is a reagent that may or may not have nutritive value in an insect's diet but helps the insect in the selection and consumption of a particular food. Knowledge of phagostimulants in a particular insect is critical in the successful artificial rearing or feeding of that insect. Initiation of feeding is strongly influenced by stimulants and deterrents in the host plant. Feeding stimulants are frequently nutritionally important substances (Beck, 1965). In aphid artificial diets, the requirements of sugars, amino acids, vitamins and trace metals which could act as stimulants for insects have been studied (Srivastava and Auclair1974). Nutritive material and odd plant substance both combine to play either an important or crucial role in insect feeding habits (Schoonhoven 1968).

But the role of these stimulants is not very clear. Chemical and physical characteristics of plants may influence host plant selection, but the role of these factors in regulating feeding behavior is only partially understood for a few species (Bernays 1985). Investigators are studying the influence of these stimulants in feeding behavior and also trying to establish the mechanisms by which these stimulants work. Artificial diets for crucifer feeders increase their acceptability considerably with the addition of mustard oil glucosides (Nayar and Thorsteinson 1963).

Feeding behavior depends on more than nutritional factors in aphid rearing. In the past successful artificial diets for insects were thought to satisfy the physical, chemical and nutritional demands of these organisms (Friend 1958). Currently the most successful diets contain not only nutritional substances but also stimulants. It is important to realize that the proportion of these stimulants in diet can cause them to act as either inhibitory or stimulatory (Gaugler and Molloy 1980). Even with all of this knowledge the creation of artificial insect diets is still a mix of art and science and getting an insect to accept an artificial diet is a long road littered with failures. We cannot neglect the importance of non-nutritive chemicals that act as phagostimulants to induce insects to feed.

Phago-amino

Amino acids are instrumental in initiating feeding in some insects. Feeding responses in insects are intensified by the combination of several amino acids (Davis 1965). All amino acids are not equally preferred, and some times they are required to combine with sugars. For example, in spruce budworm, a mixture of sucrose plus L-proline was preferred and less preferred were mixtures with other related amino acids like hydroxyl-L-proline and glutamic acid.(Heron 1965). Methionine in particular has been found to be an important phagostimulant and nutrient, but histidine is a deterrent in aphid feeding (Mittler 1967). The concentrations of these phagostimulants are also very important. High concentrations of catechin (natural compound in rose tissue) acts as a feeding deterrent but at low concentrations it acts as a phagostimulant in artificial diets (Peng and Miles 1998). So the determination of the specific concentration of these phagostimulants is needed. Gluten and

gliadin are two lipids that are moderately effective in eliciting feeding responses in the confused floor beetle (Loschiavo 1965).

Phago sucrose

Most researchers consider sucrose a phagostimulant**.** A mixture of sugars has a stimulating effect on the feeding pattern of insects (Starks 1966). Sucrose is a powerful feeding stimulant (Mittler and Dadd 1963). A concentration of 35% sucrose was necessary for the proper growth and development of *A. pisum* (Srivastava and Auclair 1971), but a sucrose concentration of only 5-10% and above was phagostimulatory (Srivastava and Auclair 1974). Glycosides can be phagostimulants in low concentrations, but in the same insects at higher levels they can be inhibitors (Heron 1965). Comprehensive screening of mono-, di-, and tri-saccharides demonstrated that glucose, fructose, sucrose, maltose, turanose and raffinose were significant phagostimulants for fire ant workers (Vander et al 1995). The combination of sucrose with other chemicals also acts as a stimulant and this is also a subject of interest for many investigators. The average rate of diet uptake by *A. pisum* on amino acid diets with sucrose was 2-5 times more than that on the diet lacking amino acids (Heron 1965). The synergic effect of amino acids and sucrose is a strong phagostimulant to the pea aphid. The phagostimulary activity of sugar is especially strong for phytophagous insects, various sugars like fructose, raffinose, arabinose, rhaminose, galactose, maltose and melistoze induce feeding behavior (Davis 1961).

Other stimulants

In aphid rearing colors are important factors in responding to feeding. Pea aphids *Acyrthosiphon pisumr* lived longer and grew rapidly on orange or yellow colored diets as

compared to the aphids reared on blue or white diets . (Cartier and Auclair 1964). Insect antennae and some maxillary palpi are loaded with miniaturized highly efficient maser (microwave amplification by stimulated emission of radiation) like structures tuned in on a very definite molecular vibration associated with the odors of a host plant **(**Callahan 1965). Flavors derived from pollen appear to play an important role in the feeding behavior of honey bees (Hanna and Schidmt 2004). In the absence of feeding stimulants nutritional superiority regulates food selection (Carteir 1966). Sinigrin in low concentrations act as phagostimulant to cabbage aphid (Emden 1978).

CHAPTER 2

Developing Artificial Rearing Techniques for Hemlock Woolly Adelgid, *Adelges tsugae* and Balsam Woolly Adelgid, *Adelges piceae*

Introduction

Artificial rearing and nutritional studies of insects have been subjects of great interest, for over 90 years. Artificial rearing is the process of raising insects on any diet that is not their natural food (Vanderzant 1966). Rearing insects in the absence of their host plants has become very important and could be used to rear insect parasites, predators, sterile insects, insects as feed for other animals (Versoi and French 1992), and as bioreactors for production of pharmaceuticals and other recombinant proteins (Hughes and wood 1998). Rearing of insects on artificial diet will help not only in the study of their nutrition but also their biochemistry, behavior and other biological processes. Control of insect pests with insecticides, while highly effective, has lost some of its appeal because of their threat to human welfare and wildlife (Carter 1966). Moreover insecticides are not always a solution, especially when the target is a forest insect. Controlling forest pests with insecticides is not usually economically feasible. Therefore developing an ecologically acceptable strategy to control these pests is needed which leads to the interest in the artificial rearing of insect predators and parasites. Although artificial rearing of insects was started earlier in the last century, its use for rearing insects for biological control has progressed more recently.

Feeding insects on its natural host is time consuming because it demands fresh and regular plant material, which requires effort and manpower. Frequent handling of the culture may result in high mortality of the insects. Therefore, artificial rearing of insects is economically advantageous over natural rearing because it is cheaper and the artificial diet is available all year.

A nutritionally complete diet for most insects must contain all or most of the following: protein or amino acids, carbohydrate, fatty acids, cholesterol, choline, inositol, pantothenic acid, nicotinamide, thiamin, riboflavin, folic acid, pyridoxine, biotin, vitamin B_{12}, β-carotene or vitamin A, α-tocopherol, ascorbic acid, several minerals and water (Vanderzant 1974). The basic requirement in artificial rearing is to understand the relationship between the plant and the insect. To develop perfect artificial diets, detailed knowledge about the actual needs of insects is very important, which not only include the nutritional requirements but also the physical feeding requirements and environmental conditions. In addition, feeding stimulants are also important. Almost all insects have similar nutritional requirements; the only difference is the proportion in which these nutrients are added to the diet, and the stimulants that are needed for the insect to begin feeding (Dadd 1966). The fact that insects eat the food that humans raise for themselves and their animals clearly indicates that insects use some of the same nutrients, undoubtedly the proteins, carbohydrates, and fats (Hoskins and Craig 1964,Vanderzant 1974).

Strict sanitation is required to control microorganisms and diseases in the insect's food. Various methods can be used such as heat, filtration, chemical treatment (antimicrobial agents), radiation or a combination of these, to either control the microorganisms or to

suppress their growth. But sometimes sterilized methods may affect the nutritional value or change the nature of the food. For example, most of the nutrients for flies are provided by microorganisms (Vanderzant 1974), and foreign grain beetle, *Ahasverus advena,* would not grow on food sterilized with propylene oxide, but did grow on autoclaved food (Hill 1962). Antimicrobial agents can be toxic to insects. Each insect has a different level of toxicity depending on the kind and concentration of the agent and the diet.

Hemlock plays an important and unique role in forest ecosystems that cannot be quantified. Destruction by the invasive hemlock woolly adelgid (HWA), *Adelges tsugae* Annand, leads to disturbed ecosystems, habitats, understory vegetation, ecological processes and structure and functions of forest stands. Several species of songbirds, black-throated green warbler, black burnian warbler and blue headed vireo are hemlock dependent species during their breeding season (Ross et al 2004). Eastern hemlocks have unique structural characteristics that provide important habitats for numerous bird species in the northeastern US. The removal of hemlock stands by HWA has profound effects on avian communities. Not only the avian species that are restricted to hemlock stands, but several species of mammals and amphibians are also affected (Brooks 2001).

The present study concerns research directed at developing a synthetic diet for the hemlock woolly adelgid (HWA) and the balsam woolly adelgid, *Adelges piceae* Ratzeburg One of the problems with developing diet was that HWA were available only for six months of the year. Both Hemlock Woolly Adelgid (HWA) and Balsam Woolly Adelgid (BWA) are in same genus *Adelges* and thus are very similar in many aspects. So in order to maintain the

continuity of diet development when HWA was not available we replaced HWA rearing with BWA rearing.

The objective was to develop a feeding system and evaluate various artificial diets for rearing HWA and BWA. The aim of this research was to produce uniform laboratory insects for future studies, to investigate aspects of the insect's nutrition, and to investigate feeding behavior and other aspects of the biology of the insect. A successful rearing diet and technique will prevent the insect from entering diapause, and make it available for other laboratory experiments. In addition, it will reduce the cost of rearing predators, which require a year round supply of adelgids. Forestry practices to control HWA in United States rely mainly on the biological control since chemical treatments need repeated applications and are restricted to individual trees. Rearing predators in sufficient numbers for release at appropriate times is expensive and difficult because millions of beetles are required each year to maintain the continuous affect on HWA.

Carbohydrates are essential energy-producing nutrients required for both optimal larval growth and for the maintenance of adult longevity for the majority of insects (Dadd, 1985). The nutritive value of carbohydrates depends on a number of factors, one being the availability of digestive enzymes available to reduce complex carbohydrates to their constituent monomers (Dojnov et al 2008). Digestive enzymes secreted by insects also reflect the type of food eaten. α-amylase plays an important role in the survival of insects under temperature or nutritional stress (Nenadović et al., 1994).

The presence of amylase has been demonstrated in larval midguts of many insects including members of Orthoptera, Hymenoptera, Diptera, Lepidoptera and Coleoptera (Terra

and Ferreira, 1994). But not much information about nature of enzymes and their presence in adelgids is available. The present study also aimed to investigate the presence of amylase in adelgid saliva.

To achieve these goals we developed and improved the designs of the feeding system, and we tested a number of diets in three different forms (liquid, solid and cellular based). We also gained insight into the adelgid diet by dissecting the insects to determine what type of food they have in their intestine.

Material and Methods

Hemlock branches infested with hemlock woolly adelgid (HWA) were collected from different sites near Laurel Springs, NC and taken to the NC State University insectory where they were stored at 65^0 F and 57% relative humidity. The cut branches of hemlock were kept in water that was changed every 2^{nd} day. If branches were stored for a longer period, then urea was added at 1 tablespoon per gallon in order to keep the plant material fresh for adelgid growth.

Artificial diets for HWA and BWA were prepared and sent by Dr. Allen Cohen, Director Insect Diet and Rearing Company, LLC. Raleigh, NC. The diets were in powdered form and were stored in a refrigerator.

Liquid Diet Feeding Unit

The feeding unit plays a very important role in the rearing of any insect, but for small insects like adelgids the structure and efficiency of the unit is critical. The feeding unit (Fig. 2.1) in our study consisted of two chambers. The lower chamber stored the diet and the upper

chamber held the adelgids. The diet chamber was 3mm deep, 13mm wide and the bottom was concave to store the diet (Fig. 2.1). The upper chamber was covered with a Plexiglas sheet with a hole in the center to circulate air, and was plugged with muslin cloth. Woolly masses in the upper chamber were laid directly on stretched parafilm that separated the chambers.

The membrane was sterilized in a solution of 70% ethanol and was stretched to four times its original area, thus a crawler could easily penetrate the parafilm. The woolly masses used for rearing were not disturbed and were laid on the membrane without being separated from the twigs to avoid any desiccation. The whole feeding system was developed so the adelgids could feed without disturbance for weeks. The diet chamber had holes on both sides that were attached to stopcocks. One of the stopcocks, the in-knob, was used to introduce diet while the other, the out knob, was used to adjust the volume of diet (Fig. 2.2). The out-knob also helped to maintain the turgor pressure of the diet. 3-4 ml of diet was introduced to the diet chamber from a delivery unit with the help of a syringe.

Diet Delivery Unit

The delivery unit (Fig. 2.3).was a flask which had knobs on both the top and bottom ends. The top knob was used to maintain nitrogen pressure in the flask and also had a 0.2 micro filter for filtering microbes. The bottom knob was used to control the amount of diet entering the feeding unit.

Diet Preparation

The diets, sent by Dr. Cohen, were in powder form for ease of storage and transportation, but were fed to insects in liquid form. 250ml of liquid diet was prepared at

one time. 15-16 gm of powdered diet was put in 250 ml of deionized water and mixed thoroughly with a magnetic stirrer, this mixture was than filtered with 0.02 micro filters into a sterile flask and stored under nitrogen pressure of 10 psi in a refrigerator. The nitrogen pressure removed air, and thus inhibited the growth of bacteria and fungi and keeping microbial activity to a minimum. Since the diet was a rich source of nutrients and it could be easily denatured by the propagation of microorganisms at normal temperature. We prepared a new batch of diet every week because prolonged storage of a liquid diet would decrease its nutritional value.

Diet Renewal and Cycling

Although all the glassware and other equipment used in preparing the diet were sterilized, the diets themselves were not. These diets were a great source of nutrients and had a high rate of contamination. To avoid any risk of microbial contamination, the diet was refilled three times a week under sterile conditions and without disturbing the adelgids. In place of using a delivery unit for diet renewal, a syringe assembly was used. The assembly created the desired pressure in the feeding chamber. A 10ml syringe, with 1.5mm gauge hypodermic needle, was used for this purpose. The diet was changed in the following sequence:

(1) Removed all old diet, opened both the out- and the in-knobs and allowed the diet to flow out.

(2) Flushed diet chamber twice with separate rinses of distilled-deionized water.

(3) Used a syringe to fill the diet chamber with fresh sterile diet. Disinfected the syringe assembly with chlorox (10%), ethanol (70%), and distilled water. Treated the needle with a hot flame to complete the disinfection.

10-15 ml of diet was poured out of the pipe of the delivery unit. Now the syringe was put in, and diet was sucked out from the delivery pipe. Then this diet was pumped out into the feeding unit through the in-knob. The sucking in and pumping out of the diet was done over a hot flame to avoid contamination.

Quality Control

The successful rearing of insects on artificial diets depends on the freshness of the diet and the absence of microbial contamination. Close observations were taken in order to maintain high standards of quality control. Quality controls in this experiment were maintained in three areas: diet, equipment and environment. In the diet and environment(fume hood) area, contamination was checked 3 times a week. This was done by a microbial contamination test, in which sterile cotton swaps were moistened with sterilized saline water and rotated around the in-knob, out-knob, and hood. Different swabs were used for different places. The Sabouroud Dextrose Agar, SDA (for fungal test) and Nutrient Agar, NA (for bacterial test) media in Petri dishes were then swabbed separately and incubated at 32.5 C for 48 hrs. The same procedure was used for the diets in the feeding unit and delivery unit. The plates were observed after 48 hrs to check the microbial contamination.

For diet freshness a FRAP (ferric reducing antioxidant power) test (Cohen 2004) was also performed. For this test 10ml of "working" TPTZ/$FeCl_3$ solution was made. During each renewal of the diet a sample was taken from the feeding unit and the delivery unit to run the

freshness test. 250 μL of sample and standard were diluted with 9750 μL of water. This solution was then mixed with 1000μL of working reagent and incubated. Then absorbance was read at 593 nm. For each diet, the standard was found and deviation from the standard was an indicator of freshness. The cotton swabs and FRAP test tubes were autoclaved at 120 ^{o}C for 1 hour. Feeding units, delivery units and all other equipments were sanitized with 10% chlorox for one minute followed by 90% ethanol and sterilized water for one minute each.

pH Test of Liquid Diets

In order to determine the best pH value of the diet, MDB-1 diet was tested over a range of pH. Diets were prepared that differed from each other by 1pH unit from pH 4.2 to 8.2. Adding microliter amounts of potassium hydroxide and hydrogen chloride altered the pH of the diets. HWA were reared from the egg stage on each of the prepared diets.

Rearing of insects on Solid Diet

Tests were also run to rear HWA and BWA on a solid or gel based diet. In order to prepare a solid diet, the powder diet (3g) was mixed with lukewarm water (25ml) with a magnetic stirrer. Gel 812 and Lucas bean gel were mixed at a ratio of 3:1 and then 0.75g of this mixture was slowly mixed with the diet solution on a magnetic stirrer to avoid clumping. Warm liquid gel was placed on a slide and cooled, and when it became solid it was covered with a parafilm membrane. Woolly masses of HWA or BWA were then put on the parafilm to feed. The gel area was surrounded by Vaseline to restrict insects to the gel.

Preparation of cellular based diets

In this type of feeding, the feeding unit (Fig 2. 4) consisted of a straw pipe though which diet was transferred. Three to four cuts were made in the middle of the straw pipe for

absorption of diet. An absorbent sheet surrounded the straw (Fig 2.4A). A pore was made in a cotton wick and then the straw pipe (along with absorbent sheet) was placed in the cotton wick pore (Fig. 2.4 B).

The diet constituents were mixed together using a magnetic stirrer and then this mixture was heated for two minutes. The cotton wick (feeding unit) was dipped into this warm gel mix. The cotton sheath absorbed the cellular diet, and when the gel cooled the whole arrangement was covered by a parafilm membrane (Fig 2. 4C). Finally HWA and BWA egg masses were attached to the parafilm membrane with a thread or sticky tape. Because these diets were open they were more exposed to microbes and ascorbic acid degraded very fast. So 1.5 g potassium sorbate and 3.5g ascorbic acid in 50 ml of water was added to these units through a straw pipe on alternating days. An absorbent sheet absorbed these components through the cuts made on the straw. Potassium sorbate and ascorbic acid were delivered to the cellular based feeding with a pipe attached to the straw pipes (Fig 2.4D)

The cellular based feeding unit was kept in the upright position in order to give the adelgids a natural posture (Fig 2. 5).

Rearing Conditions

Rearing was carried out at 25 ^{o}C with exposure to 16 hours of light per day. The light was provided by fluorescent tubes.

Dissection of Hemlock Woolly Adelgid

The gut of HWA was examined under both compound and stereomicroscopes. The woolly masses of HWA were removed and the adults were taken out. The adults were then put on slides and were dissected in ice-cold distilled water under a dissecting microscope. A

sharp pointed needle was used to puncture the insect's head and then the insect was cut longitudinally from anterior to posterior. After the cut, the specimen was opened with the help of needles. The gut of the insect was carefully pulled out through the head by cutting the rectum, and separating the alimentary canal from the adhering tissues. This alimentary canal was carefully dissected in ice-cold water. The freshly separated gut was treated with a drop of glycerin and then examined at high magnification.

Amylase Test

Saliva can be obtained for chemical analysis directly from mouth parts (Strong 1970), or after stimulating the insects to salivate by starving them (Mikes and Slowiak1970). The small size of adelgids made it difficult to follow either of these methods.

Whole HWA were used instead of salivary glands or heads because dissecting and collecting uninjured salivary glands from the minute adelgids (<1 mm) is extremely difficult. We used 100 adelgids as a sample for the enzyme assays. The woolly masses attached to these adelgids were removed with brushes. Then the adelgids were homogenized or crushed using a microtube pestle. The homogenate was prepared in ice- cold water (glycerol has some inhibitory effect on the amylase activity, Srivastava 1959). The homogenated adelgids were centrifuged at 4°C for 15 min, the supernatant was removed from the tube and the basal residue was also recovered. This test was performed in three parts:

1) A control using human saliva.

2) With supernatant

3) With basal residual

Starch and gel were mixed at a 1:1 ratio and then the mixture was poured into warm water again at 1:1. Pouring was done very slowly to avoid clogging the magnetic stirrer. The starch-gel solution was then poured into a small tube and after hardening, was cut into 1-2 mm slices. Human saliva, supernatant and basal residual were put into small test tubes and 5 slices of prepared starch-gel were added to each test tube. After 2 hours one slice was taken out from each test tube and was treated with a 1% iodine solution. In the presence of amylase (a digest starch) the color of iodine changes from blue to white This procedure was performed four more time at 4, 6, 8 and 16 hrs and the color change was observed.

A second test was performed to confirm the presence of amylase in HWA saliva. 4 g of dry starch substrate (10g agrose, 10g starch, 20g confectioner's sugar, 0.5g streptomycin sulphate, 0.5g chlortetracycline and 0.5g potassium sorbate) was mixed with 100 ml of water, stirred, heated to the boiling point and then mixed with the gels. This mixture was poured into a test tube and when the gel became solid, it was covered with a parafilm membrane. The woolly masses of HWA and BWA were placed on a parafilm membrane. The test tubes with HWA were incubated for more than 24hrs at 37°C. The starch substrate was covered by stretched parafilm and the attempts of the adelgids to feed on the substrate by puncturing the stretched membrane caused their saliva to directly contact the substrate. After incubation, three thin slices from the feeding surface were cut out. Subsequent analysis of these thin slices with 1% iodine revealed the presence or absence of amylase.

Diets Composition

Composition of the all diets sent by Dr. Allen Cohen are summarized in Tables 2.1 Modified diets with Betaine HCL (MDB's) are summarized in Table 2.2. The constituents and the ratio of cellular based diets are presented in Table 2.3

Results and Discussion

To determine the optimal artificial rearing diet for HWA and BWA, a systematic and progressive study was done with a number of diets. These diets were tested in liquid, solid and cellular based forms. A number of other tests/experiments were also performed to ensure a systematic approach and quality of the experiments. Observations on mortality, longevity, growth and fecundity were recorded every 24h.

In liquid diets the feeding results showed very little difference among diets. The performance of adelgids on all diets was similar; 98% of the eggs were hatched into crawlers. There was negligible diet uptake and no settlement was seen on any diet except the MDB-1 and MDB-3 diets (Table 2.2), which gave the best survival to BWA. On MDB-1 and MDB-3 diets, the crawlers were able to survive for more than 2 weeks. 10-15% of the original eggs became healthy looking 1st instars that survived for a longer period of 10-12 days but no further growth was observed after this, whereas the survival period on other diets were not more than 3-5 days. These 1st instars appeared to settle on the membrane and feed on the diet by inserting their stylets, which indicated an attempt to feed. Ingestion of the liquid diet was also assumed because yellow honey dew was produced and the adelgids survived for 15-21 days on the MDB-1 and MDB-3 series. These two diets contain Betaine hydrochloride, a non-essential nutrient and a source of hydrochloric acid, in combination with soy proteins

and wheat whey. These combinations were absent in other diets. Betaine helps in the digestion of proteins and other nutritive substances by activating digestive enzymes that break down food into small particles for absorption, so it might be possible that adelgids need some HCl source in their diet. Soy and wheat whey are both highly nutritious substances used in most insect diets. They have well balanced protein, mineral, vitamin and lipid contents. So it might be possible that these two components must contain something that is similar to what adelgids get from their natural host.

Several attempts with different nutrients, with addition and deletion of several sugars, proteins or mixtures of several amino acids were performed to increase the acceptability of diets but none of them was acceptable to the adelgids.

On solid diets none of the adelgids settled and the activity of crawlers stopped in 3-4 days. While feeding, 98% of the HWA on both liquid and solid based diets didn't stay on the diet but moved to the upper side of the feeding unit in an inverted position. On the natural host plant, they feed in an upside down position on the lower surface of the needle. Therefore, we modified our feeding unit according to the adelgids preference.

In cellular based diets the eggs of the adelgids were attached to the diet in an inverted and angular position as they would be on hemlock needles (for HWA) and vertical as they would be on Fraser fir (for BWA). On this diet BWA showed more development than they did on liquid based diets. Not all, but 10-20% of the crawlers developed to the 2nd instar and woolly mass production was also seen in some 2nd instars. But again no further development was seen. We discovered that the production of stylet sheaths and honeydew droplets were

related to ingestion. Honeydew produced by the insects reared on artificial diets was very sticky and viscous compared to insects reared on the natural host.

We tested a number of diets; these included 16 each of liquid, solid and cellular based diets. We concluded that until a more acceptable artificial diet was available for adelgids, experiments using artificial diet were of uncertain value. In our investigations we were unable to successfully rear insects to adult hood on any of the diets; this could be for a number of reasons.

One of the likely reasons for failure was the imperfection of the diets. Even after providing a range of diets we were not able to rear insects successfully. Although the adelgids did penetrate (Fig 2.6) the membrane, insert their stylet sheaths in the artificial diet and make some attempt to feed on the diet and even showed development to the 2nd instar, none developed to maturity.

The penetration of the membrane does not necessarily show its acceptability as food; an aphid may insert its stylet all the way to the phloem of an unacceptable plant and even ingest measurable quantities of sap before terminating its attempt to feed (Ehrhardt 1961). It appears that the hemlock (natural host) supplies some elements that we lacked in our artificial diets. All insects need some basic nutrients for their growth and development, e. g., sucrose, essential amino acids, vitamins, mineral, cholesterol fatty acids and many more. While preparing diets we considered these requirements very carefully. But some insects also require extra nutrients that could be trace elements, nucleic acids or other components. *Drosophila melanogaster* can only grow normally if externally supplied with purine and pyrimidines (Sang 1955). Similarly, folic acid is essential for optimal growth and the

successful completion of metamorphosis in the housefly; additionally neither adenine nor guanine will satisfy the folic acid requirement (Brookes and Fraenkel 1958). Every insect can have different requirements for normal growth and development so further studies are required to know whether this is the case with HWA and BWA. The radioisotope method can also be used to determine the nutrients required in insect diets. In this method diets are labeled with radioisotopes and by tracing these radioactive components it can be determined whether diet ingested by insects is metabolized and what the end products are. Another possibility is that some of the ingredients in the diet are imbalanced, for example the failure of *A. pisum* to grow on some diets was due to the imbalance between calcium and phosphate ions (Retnakaran and Beck 1967).

We also observed the development of insects was delayed on artificial diets. Insects lived up to the 2nd instar but they were apparently under stress. The reason for this stress might be the lack of some important nutrient in the diet. Under stressed conditions they were not able to make proteins or other chemicals required for the next stage of life. An indirect method can be used to discover the missing nutrient/ nutrients in the diet. This method is the carcass analysis. Carcass analysis was done to develop a meat based diet in *Dicyphus tamaninii* by Zapata et al. in 2005. In this method the dead insect is examined and the cause of death determined by examining the lack of protein or any other chemical in the insect's body. We can thus know the cause of death or absence of specific proteins/chemicals by comparing the dead insect with healthy insects of that stage (2nd instar in our case). After this, different diets can be prepared such that the insect can fulfill its requirement for that protein/chemical. These diets can be labeled with radioactive elements and traced to know

whether our proposed diet allows the insect to make a specific protein or chemical. (Wilinkson et al 1997).

There is also a remote possibility that the nutritional requirements of the early stages of the HWA are different from the later ones. Adult aphids prefer diets with slightly lower concentrations of free amino acids compared to second to fourth instars (Auclair 1965). Some insects do need different nutrients in different stages of life. Our experiments showed that that on certain diets the insects can live up to the second instar before dying. It might be possible that the insect has different nutritional requirements after this stage. Simpson and Raubenheimer (1993) conducted a comparative analysis based on the composition of the chemically defined diet of 117 insect species; they found that the estimated position of the intake target on the nutritional plane for carbohydrate and protein varied with life history characteristics. This type of shortcoming can be dealt by applying the concept of preference diets. This means that the insect is not fed on a single diet, but rather the insect chooses from a variety of diets. . Small compartments of the diet can be constructed and the insect is free to move to any diet and if it has different requirements after a certain stage of the life cycle, it can move to a different diet partition. Chamber feeders used by Cartier and Auclair (1964) can also be used in diet-preference tests.

Most insects make their own amino acids, fatty acids and other chemicals from essential amino acids, fatty acids and other basic elements like vitamins and microelements. These elements were provided in our experiments. It is possible that the conversion of basic or essential elements to other bio-chemicals in insects takes so long that the insect doesn't revive from this deficiency and insect growth is retarded. This could be a possible cause of

insect death. Jones et al (1979) conducted a study that proved that a series of polyunsaturated fatty acids must be provided in the diet. These fatty acids can be made from palmitic acid but the rates of these reactions appears too slow to meet the larval requirements for essential fatty acids.

The diet should be based on insect hemolymph and honeydew for successful artificial rearing. This could be another effective approach to prepare a suitable diet. In the experiment conducted by Auclair (1960) the qualitative and quantitative dietary composition of amino acids on which pea aphids were reared for 3-4 generations was based primarily on two biological fluids of the pea aphid, dew and hemolymph combined. Wilkinson in 1997 stated that the honeydew of aphids reared on an antibiotic diet contains oligosaccharide sugars of up to 16 hexose units while aphids reared on natural host contain monosaccharide (mainly glucose and fructose). These experiments demonstrate that insects show differences in byproduct composition when fed on different diets. In our experiments we observed physical differences (viscosity, color etc.) in honeydew produced by the insects reared on artificial diets and natural hosts. So we are suggesting that hemolymph and honeydew of insects, reared on artificial and natural diets, should be investigated quantitatively and qualitatively and by comparing these results we might be able to determine the possible deficiency in the insect diet.

In addition to this, there is a need to determine the effects of humidity and light on the amount digested. It is possible that the temperature, light and humidity, which are suitable in field conditions, might not be suitable in the incubator. Different responses were observed in the artificial rearing of *Jassid orosius* when feeding at different environmental conditions of

light, temperature and humidity (Day & McKinnon 1950). We conducted our experiments at a constant temperature but this is not the case in nature. There is evidence that fluctuations in temperature results in good growth for aphids. Blaney et al. (1973) noted that environmental disturbances, such as light or temperature changes, promoted activity in general, including feeding. Photoperiod requirements could also be different for different stages of life. These environmental conditions should also be studied in greater depth.

Sometimes insect requirements change throughout the life cycle. Photoperiod requirements can be one of them. It has been shown that in some cases insects show better growth when photoperiod changes. By transferring 5th instar wheat bug females (*Nisius huttoni*) to an increased photoperiod, all the females laid eggs (Wang and Carpenter 2002). The fluctuation in photoperiod can help growth and development of insects. Stimulants like colors, moisture and physical contact are also very important for diet acceptance. Feir & Beck 1963 stated that the initiation of feeding was associated with simple stimuli of moisture and physical contact (the indentation and stretching of the parafilm membrane was also a stimulus to feed)

Quality Control

To ensure the freshness and microbial activity in the diet a FRAP test and microbial contamination tests were performed. We rarely observed any microbial activity in the diet, either in the delivery unit or on the instruments we were using. The microbial test on the feeding unit diet sometimes showed contamination, although none of the stop knobs, feeding units, or hood showed any contamination. The results of FRAP test were also very close to our standard diet, which indicated freshness of the diet. Most of the diets (98%) maintained

the appropriate freshness; this assured us that microbial contamination and freshness were not the reasons artificial rearing of adelgids failed.

Longevity of Adelgids at Different pH Values

The pH of the diet plays an important role in the feeding and growth of the insect, and also in the success of artificial rearing. The greatest rate of feeding, survival and development of HWA was observed at 7.2 pH (Table 2.4).

As diets get older, even if they were fresh (as shown in microbial and FRAP tests), the pH decreases and the diets become acidic, which could be a possible cause of growth retardation in insects. Our diets had potassium sorbate to kill bacteria or other microbes, which explains why we didn't get contamination, but byproducts of these microbes might lead to decreases in pH. We conducted a small experiment Table 2.5, in which we simultaneously ran two feeding units, one with adelgids and one without adelgids.

The feeding unit with adelgids showed more of a decrease in pH but the other also had a decrease in pH. This indicates that even under sanitary conditions, after some period, the diet itself produces some metabolic components that decrease the pH of the diet. These factors are very hard to control manually. Akey and Beck (1975) designed an automated feeding unit for the efficient mass rearing of pea aphids. A pH meter can be attached to this type of feeding unit and diets can be changed whenever a difference in pH is observed. Moreover when we change the diet manually, we disturb the feeding unit, and thus disturbed the insects. And it could be possible that these insects are sensitive of this type of disturbance and could be a reason for our failure. So introduction to this type of automated feeding unit to our rearing experiment could minimize a number of problems.

Dissection of Hemlock Woolly Adelgid

For the successful rearing of insects on artificial diets, knowledge of their physiology, feeding biology, digestive structure and organization is as important as the chemical and nutrient balance of the diet. The length, shape and the inner construction of the gut are key components in determining the organizational matrix or dispersion of diets. The alimentary canal (Fig. 2.7) of the HWA looked simple and consisted of foregut, midgut and hindgut.

The foregut consists of an esophagus (a thin tube at the anterior end), and a group of spherical salivary glands. These salivary glands are connected to the buccal cavity via the salivary ducts. The foregut leads to the midgut, which was the longest section of gut leading to the hindgut. The hindgut resembles a large rectal pad at the posterior end. A food slurry is ingested through the food canal and passes into the alimentary canal where it is further digested and absorbed (Cohen 2000).

Contents of the midgut appeared to be particulate (Fig 2.8), which suggests that adelgids feed on cellular debris such as organelles; moreover the length of the gut was also not as long as with fluid feeders (very long gut as compared to body size of insect).

The length, shape and inner matter of gut are the key components in determining the organizational matrix or dispersion of diets (Cohen 2004). However, the small size of these insects made definitive conclusions difficult. On the basis of these observations we fed the insects cellular diets. BWA did survive on these diets for a while and 6-8% developed into 2^{nd} instars and produced woolly masses but after this we didn't see any further development. Our results show a need for detailed studies of the physiology and anatomy of the adelgid. Before now we knew that HWA and BWA feeds on plant parenchyma cells but we did not

know where exactly they feed in the cell. Whether they feed on cell sap, vacuoles or cell organelles. This knowledge, which can be gained by studying the contents of midgut under electron microscopy, will help us determine the best way to feed insects and develop a better diet. Further studies of the feeding targets of adelgids can also be done by tracing the salivary sheath and using clearing and staining techniques under an electron microscope.

Amylase Test

The ability of a consumer to use plant and animal parts depends upon the presence or absence of enzymes and its mouthpart activity (Miles 1972). We have very little information available concerning the enzymatic activity of HWA. Results from amylase tests are tabulated below (Table 2.6).

In the first test the results were negative (no digestion of starch material) which indicates that amylase is not present in the insect's saliva. To assure that the insect produced saliva when it was crushed, a second test for amylase was performed.

In the second test, an experiment was set up such that saliva production was facilitated in HWA by feeding. This time the result was positive. Very weak amylase activity is seen when they fed on gel for a 24 hr incubation period. The weak amylase activity was not from outside microorganisms because the control gel (without HWA) doesn't indicate any amylase activity. There is also the possibility that microorganisms present in the gut of the adelgid could be responsible for the positive results. These bacteria could hydrolyze the starch and also use many of the mono and disaccharides (Auclair 1963). This experiment was performed again but potassium sorbate was also added to the gel as an anti- microbial agent. This time in both control and the experiment, the results of the amylase test were negative

and showed no evidence of amylase, this increases the possibility that the microorganisms present in the gut of adelgid could be responsible for positive (amylase) results or starch digestion. Amylase production from certain yeasts present in the gut of the leaf hopper, *Empoasca solana*, Delong have been recorded (Hereford 1935).

The other possibility is that there is very low concentration of amylase in the saliva, and that level could hardly play any role in digestion. Duspiva 1953 observed very weak amylase activity in certain aphids and concluded that these enzymes do not play any significant role in digestion.

Symbiosis plays an important role in insect growth and development. Endosymbionts are present in the heamocyle of all adelgids, nymphs, adults, oocytes and even in eggs (Shields and Hirth 2005). Homopterous insects that feed on plant vascular tissue contain endosymbiotic organisms that are essential for development and reproduction. (Buchner 1965). These bacteria get food from the insect and in return help the insect make amino acids or other nutrients essential for the insect's growth and can also help the insect metabolize food. Endosymbionts play an important role in the synthesis of amino acids (Ishikawa 1989). Srivastava and Auclair (1963) suggested that gut bacteria in the pea aphid may contribute to the hydrolysis of ingested sucrose. Here we were careful not to provide all the amino acids (provided only essential amino acids) in our diet since we expected the insects to make some of them. It is possible that these amino acids are prepared by these endosymbiotic bacteria. Reduction in the symbionts can result an overall decreased rate of this type of metabolism. Absence or removal of these endosymbionts from the insect body results in reduced growth, death, or lack of reproduction (Mittler 1971). We put potassium sorbate in the diet to control

microbial growth in the diet. This antibacterial agent at concentrations effective in inhibiting fungi, was strongly inhibitory to both hatching and nymphal development of whitefly *Bemisia argentifolii* (Davidson et. al.2000). This quite possibly kills or affects the symbiotic bacteria present in the insect. Costa et al. (1927) suggested that these types of chemical agents were apparently toxic to eggs and may have acted as antibiotics against the symbiotic microorganisms that are necessary for whitefly development. There is also a need to study this type of toxic effect in order to prepare ideal diets.

Table 2.1 Composition of different liquid diets for *Adelges tsugae and Adeges piceae* feeding. All weights are in milligrams (mg) and Diets are named as 'D' with a number representing slight differences in composition

Component	**Diet D-5***	**Diet D-6***	**Diet-5**	**D-1**	**D-2**	**D-3**	**D-4**	**D-5**	**D-6**	**D-7**	**D-8**	**D-9**	**D-10**
Tryptone	20		20	2	2	2	2	2	2	2	2	2	2
Cholesterol	0.1	0.1	0.1	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Wesson Salts	0.5	0.5	0.5	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Vanderzant	0.5	0.5	0.5	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Fructose	40	40	20				3	2	1	2	1	2	3
Alcolec FF100	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Water	1000	1000	1000	100	100	100	100	100	100	100	100	100	100
Soy peptone		20											
Sucrose			20	1	2	3	1	2	3	2			

Table 2.2: Composition of different modified liquid diets with Betaine HCl for *Adelges tsugae and Adeges piceae* feeding. All weights are in milligrams(mg).

Component	**M DB-1**	**MDB-2**	**MDB-3**	**MDB-4**	**MDB-5**
Soy Peptone	20				
Yeast Extract		20			
Whey			20		
Potassium caseinate				20	
Calcium caseinate					20
Betaine, HCl	0.1	0.1	0.1	0.1	0.1
Cholesterol	0.1	0.1	0.1	0.1	0.1
Wesson Salt	0.5	0.5	0.5	0.5	0.5
Vanderzant(Sigma)	0.5	0.5	0.5	0.5	0.5
Fructose	40	40	40	40	40
Alcolec FF100	0.1	0.1	0.1	0.1	0.1
Potassium sorbate	0.2	0.2	0.2	0.2	0.2
Water	1000	1000	1000	1000	1000

Table 2.3 Composition of cellular diet for *Adelges tsugae and Adeges piceae* feeding.

Component	**Cellular Based Diet**
Spirulina	1.0 g
Gelatin 812	1.0 g
Sucrose	5.0 g
Wesson Salts	0.5 g
Vanderzant	1.0 g
Methylene blue	0.1 g
Wheat germ oil	1.0 ml
Ascorbic acid	0.1 g

Table 2.4 Longevity of *Adelges tsugae* at different pH values

Days	**No of live insects (pH 4.2)**	**No of live insects (pH 5.2)**	**No of live insects (pH 6.2)**	**No of live insects (pH 7.2)**	**No of live insects (pH 8.2)**
1	32	41	36	38	44
3	11	25	22	30	28
5	4	10	11	25	19
7	0	0	2	19	12
9	0	0	0	21	3
11	0	0	0	15	1
13	0	0	0	13	0
15	0	0	0	9	0
17	0	0	0	5	0
19	0	0	0	1	0

Table 2.5 Testing pH of diet in feeding unit with or without *Adelges tsugae*

No. of days	**Feeding Unit with HWA (pH)**	**Feeding Unit w/o HWA (pH)**
2	7.1	7.2
3	7.0	7.2
4	6.5	6.9
5	6.0	6.7
6	5.5	6.4
7	4.9	6.1
8	4.8	6.1

Table 2.6 Starch Iodine test for amylase activity in *Adelges tsugae* saliva

Test	**Results**
Amylase Test 1 (Grinded adelgids)	
Basal residual	Negative
Supernate	Negative
Control (Human Saliva)	Positive
Amylase Test 2A (Gel only)	
Adelgids Feeding on Gel	Weak positive
Control (no adelgids)	Negative
Amylase Test 2B.(Gel + potassium sorbate)	
Adelgids feeding on gel	Negative
Control (no adelgids)	Negative

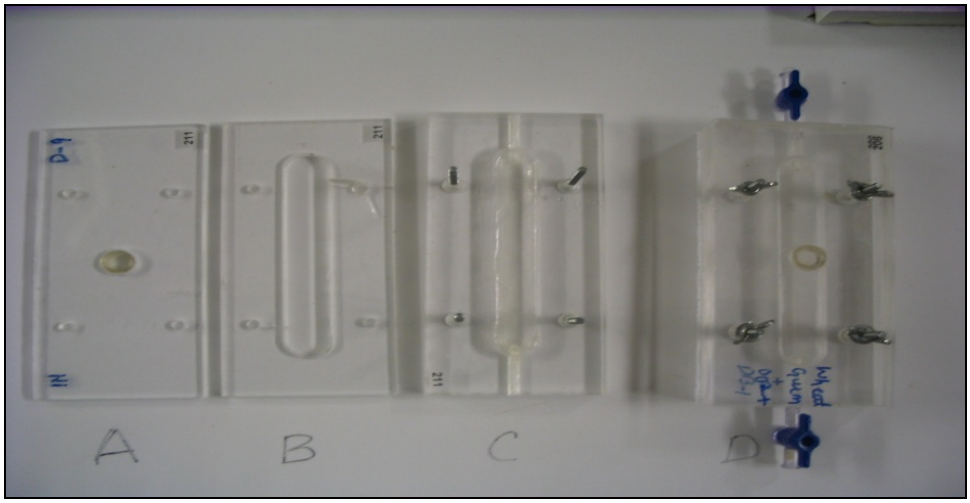

Figure 2.1 Components of feeding unit – A) cover of upper chamber, B) upper chamber, C) lower chamber, D) assembled feeding unit

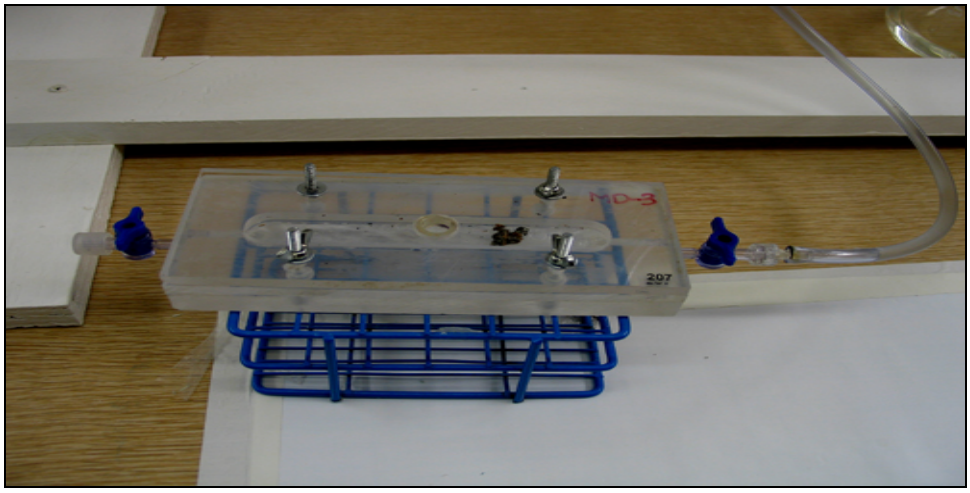

Figure 2.2 Feeding unit used for liquid diets

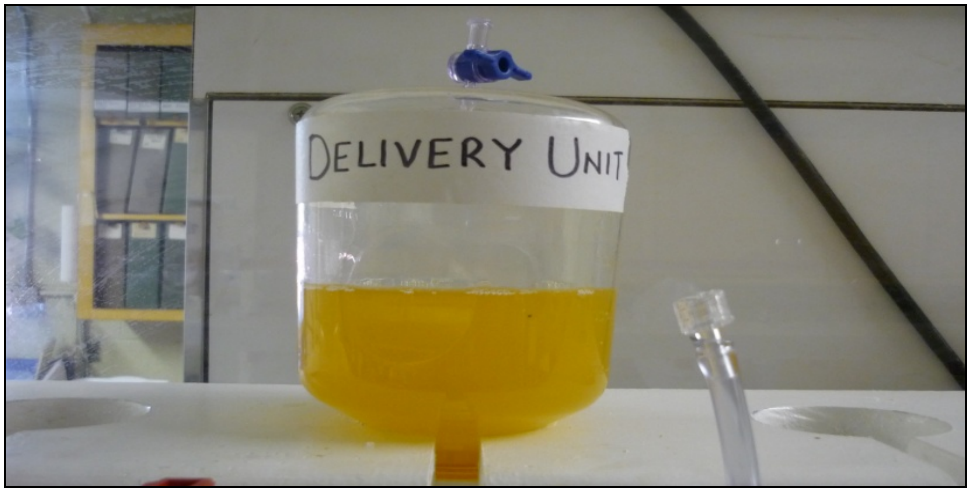

Figure 2.3 Delivery Unit used in liquid diets.

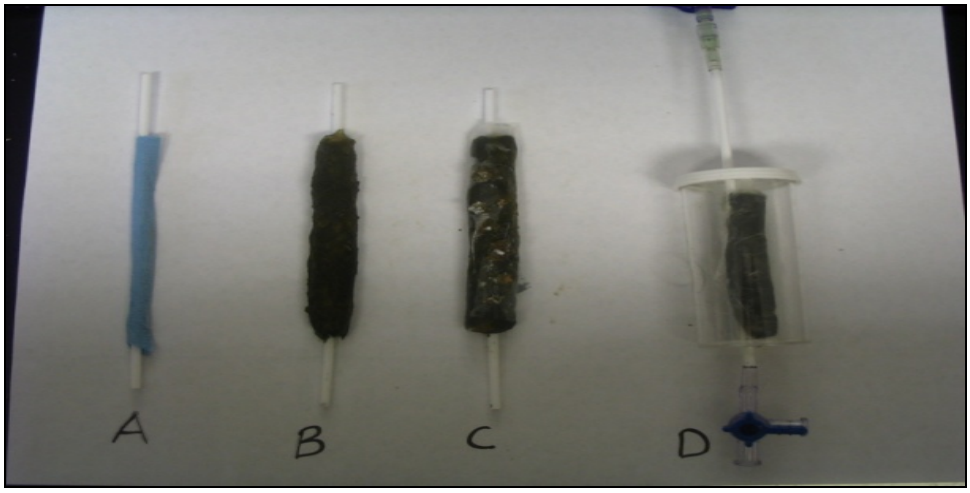

Figure 2.4 Components of Feeding Unit used in cellular based diet. A) Straw covered with an absorbent sheet. B) Cotton wick with gel mix. C) Gel mix covered with Parafilm. D) Full covered assembly with pipes attached to the straw.

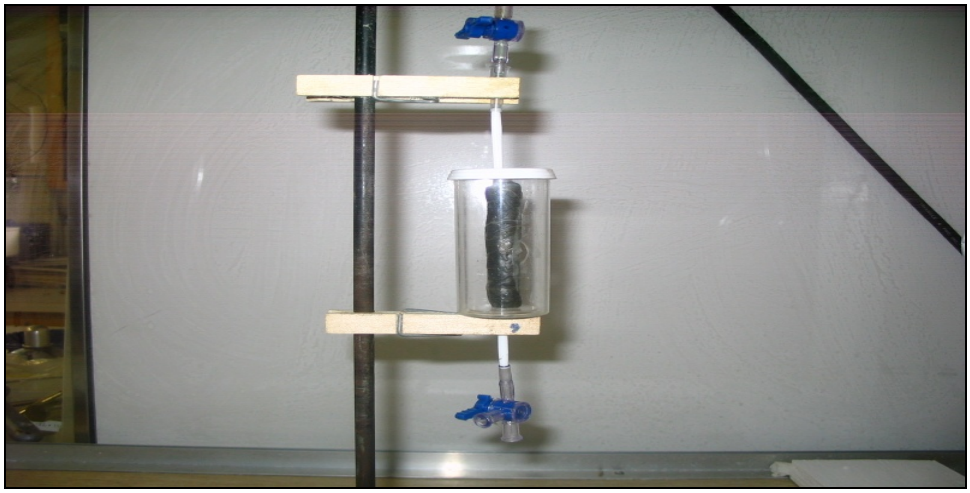

Figure 2.5 Feeding unit used for cellular based diets

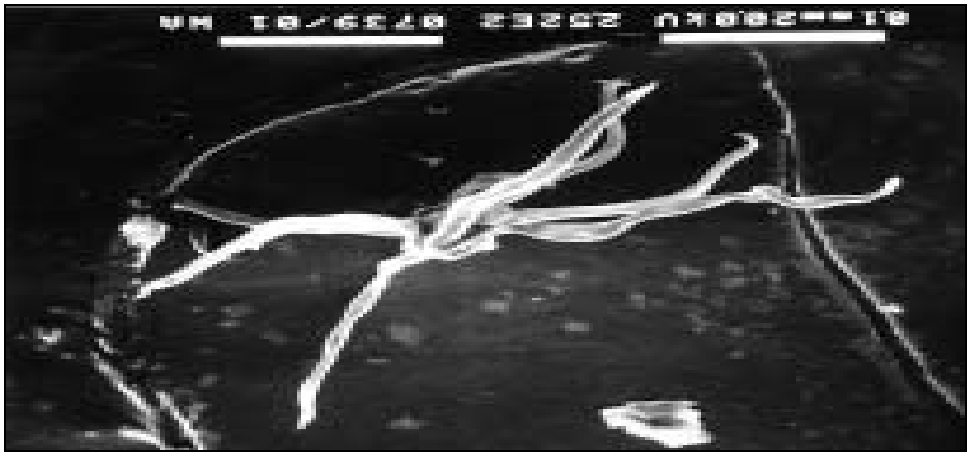

Figure 2.6 Penetration of *Adelges tsugae* stylet through parafilm membrane

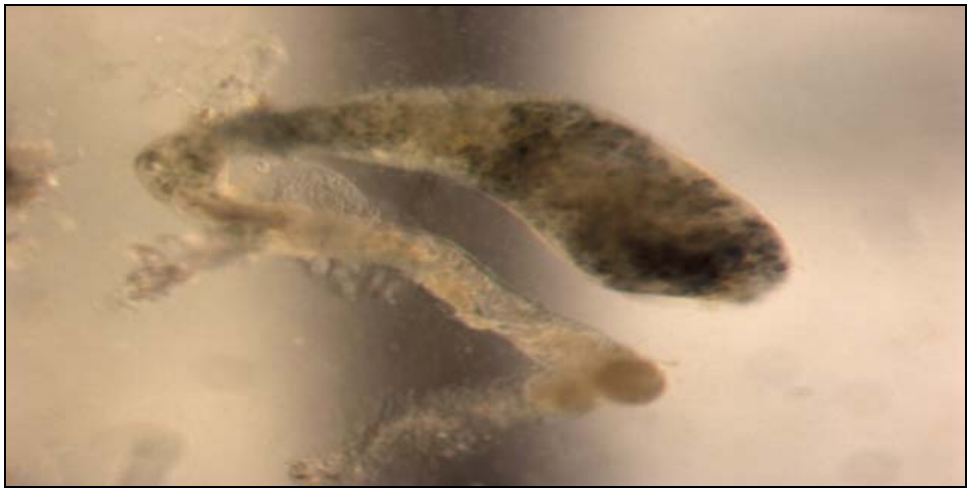

Figure 2.7 Alimentary canal of *Adelges tsugae*

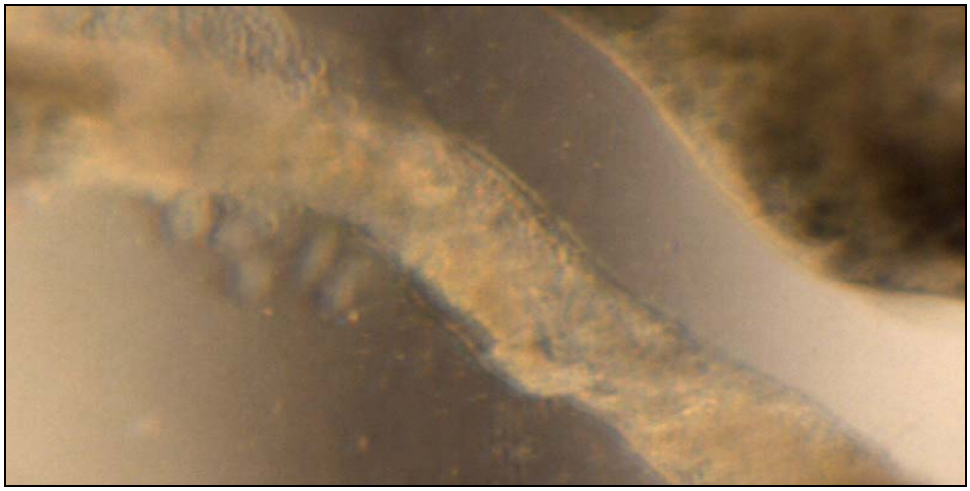

Figure 2.8 Particulate matter in the alimentary canal of *Adelges tsugae*

CHAPTER 3

Artificial infestation and epicuticular wax extraction of Carolina hemlock, *Tsuga caroliniana* provenances.

Introduction

Hemlocks (*Tsuga spp.*) are conifer trees found in the cooler climates of North America and eastern Asia. *Tsuga caroliniana* (Carolina hemlock) is one of the four hemlock species found in North America. Carolina hemlock is a rare species discovered by Dr. Lewis Gibbs in 1837 in Pickens County, South Carolina and was named later by George Engelmann in 1881 (James 1958). Carolina hemlock is native to the southern Appalachians and upper Piedmont regions of the southeastern United State (Jetton 2008). It is largely present in North Carolina (Fig. 3.1).(along with Eastern hemlock (*Tsuga canadensis*) but also distributed in South Carolina, Virginia, Georgia and Tennessee (Little 1970). Carolina hemlock, with a stress tolerant life strategy, is normally found on dry, steep and exposed slopes (usually poor in nutrients), but occasionally it is also found in moist valleys and ravines (Rentch et al 2000). Rarity of this species makes it undesirable for the timber industry but it has important ecological and aesthetic value. Many cultivars have been selected for ornamental and hedgerow use (Swartley, 1984). Stress conditions, low in species diversity, mark hemlock dominated stands. The under-stories of Carolina hemlock are dominated by evergreen shrubs that suppress oaks and other broad leaf species but don't effect the germination of shade tolerant *Tsuga caroliniana.*(Humphrey, 1989). It is commonly associated with red maple

(*Acer rubrum*), chestnut oak (*Quercus montana*), service berry (*Amelanchier spp*.), *Rhodendron spp*. and mountain laurel (*Kalmia latifolia*) (Schafale and Weakley 1990).

The health of hemlocks is threatened by the introduction, and spread of an introduced forest pest, the hemlock woolly adelgid (HWA), *Adelges tsugae* Annand (Brooks 2001). HWA is a serious pest of hemlock forests causing severe decline and mortality. Infections and infestations by exotic pathogens and pests, respectively, can be an important economic, ecological, and evolutionary process that can change the structure and function of ecosystems with devastating impact on the natural resources and their aesthetic value (McKnight 1993). Over the last 20 years, HWA has killed native stands of eastern and Carolina hemlock. Hemlocks are long lived species, sometimes more than 800 years, but in the mild climate of the southern mountains this pest can cause the mortality of eastern and Carolina hemlocks in 3 – 10 years. These two hemlock species are now in serious danger of extinction in the Southern Appalachians.

HWA was originally introduced to the Eastern U.S. in 1924 (McClure1987) and discovered near Richmond, Virginia in 1954 (Havill 2005). Since then it has spread north and south into North Carolina, South Carolina, Georgia, Tennessee, West Virginia, Maryland, Delaware, Pennsylvania, New Jersey, New York, Rhode Island, Massachusetts, and Connecticut (Cheah and McClure 1998). HWA has spread at a rate of approximately 12.5 km per year from its established eastern range (Evans and Gregoire 2007). According to Margaret *et al.* (2003) temperature and moisture are prime limiters in influencing the range of this pest. Gradual decline in survival of hemlock woolly adelgid occurs when temperature decreases; the low temperature survival threshold for North American adelgid populations is

-30° C (Parker *et al.* 1999). The range of eastern hemlock extends well into Canada but the northern spread of this insect may be slowed or hindered by cold temperature. However, Skinner *et al*. (2003) point out that it is possible that over time this insect may develop greater cold tolerance thereby permitting it to expand its northern range.

HWA feeds on the young branches and twigs of hemlock, usually near the base of the needles, and is easily recognized by the presence of woolly secretions around egg masses. Adult HWA feed on the sap of young shoots in late winter and early spring and may also inject toxic spittle into the branches (McClure1987). With their long stylets HWA feeds on ray parenchyma cells (Young *et al* 1995).

Severe adelgid feeding prevents the spring flush of new growth, causes desiccation of existing needles, discoloration, and needle drop. Heavily infested trees show poor crown conditions, and reduced shoot growth. That in combination with other environmental stresses can result in rapid tree decline and death (Orwig 2002). In cases of heavy infestation tree recovery without treatment is impossible. Main tree limbs are lost in the first summer, and the entire tree may be dead in one year. Hemlocks infested with HWA had deficiencies in microelements, necessary to produce and maximize new growth (Pais and Demko 2005). Rates of decline vary but mortality can occur in as few as 1 to 4 years (McClure 1991). Smaller infested trees have higher mortality rates (Brooks 2001). However, some stands remain living more than a decade after infestation. Adelgids insert their stylets into the xylem ray parenchyma cells of hemlock trees to extract carbohydrates (McClure 1991), this reduces the vitality of trees as these microelements and carbohydrates are important for new growth,

vigor, reproduction, and defense. Moreover reduction in vitality of hemlock branches also restricts translocation (Doccola *et al*. 2003).

To date none of the control measures taken for HWA have proven very effective. In order to minimize the attack of HWA, which can cause up to 100% damage to hemlock stands, there is a need to grow resistant or tolerant species of hemlock.

Increasing the number of resistant trees and reducing susceptible trees seems to be the best management approach to minimize the impact of HWA. Our study of artificial infestations will evaluate the tolerance of eight Carolina hemlock provenances to the attack by HWA. The process of natural selection operating over a long period of time has favored variants which are well adapted to the many diverse sites found within the hemlocks range. The various ecotypes that have evolved in this manner are referred to as provenances (Collins 1971). Evaluation of various provenances makes it possible to identify which seed source is best suited for a particular area. Plant breeders have often used the artificial infestation techniques to screen for the affect of insects on plants. Artificial infestations under favorable conditions for the pest are most reliable, but also the most expensive means of obtaining desirable levels of pest pressure (Calhoun and Oard 1999). Breeding for disease resistance has progressed more rapidly than breeding for insect resistance (de Ponti 1992), this is due in large part to availability of technologies to economically mass culture pathogens on artificial media and plant culture (Russel 1978). The artificial infestation technique was used to distinguish resistance from the suceptible varieties at the seedling stage. The use of seedlings allows screening of a greater number of genotypes, in a limited amount of time and space, and can be a very economical means of evaluation (Ruberson 1999). The method is cheap

and effective; however it provides only a general ranking of resistance level, with only limited information on the mechanisms involved. Selection of Carolina provenances that are not preferred as host plants for oviposition by the HWA will be valuable for the development of insect-resistant hemlocks.

Many insect species prefer to feed and oviposit on glossy leaves or leaves with wax removed over leaves with normal or heavy epicuticular wax loads (Stork 1980, Bodnaryk, 1992; Brennan et al., 2001; Cervantes et al., 2002). For example, oviposition elicitation in *Ostrinia nubilalis* is influenced by the presence of n-alkanes in the host plant epicuticle (Udayagiri and Mason 1997). Chemical composition of epicuticular waxes also influenced *Hippodamia convergens* attachment to *Brassica oleracea*. The increased proportion of primary alcohols lowers the attachment of this insect to the host plant (Eigenbrode and Jetter 2002).

Epicuticular wax may play a role in *A. tsugae* success, and subsequent hemlock decline. If wax chemistry plays a part in resistance, one would expect to see inherent differences in wax composition between resistant and susceptible Carolina hemlock provenances. The objectives of this study were to screen for resistance in Carolina provenances by artificial infestation, and, by GC Mass Spectrumetry, analyze the epicuticular wax of the provenances.

Material and Methods

Artificial infestation of Carolina Hemlock Provenances in Greenhouse

This experiment was conducted on hemlock specimens from eight Carolina hemlock provenances. All seedlings were 1 year old and grown from seeds source collected by Camcore, North Carolina State University in 2003. The seedlings remained in a greenhouse at a controlled temperature range of 70-80 F, under relative humidity of 55-75% and natural light conditions. The seedlings were arranged in 4 randomized blocks with one seedling from each provenance per block. Also a control eastern hemlock seedling was included and placed with the other seedlings. The artificial infestation took place on April 12, 2006. To perform artificial infestation hemlock branches infested with hemlock woolly adelgid (HWA) were collected from different sites in Laural Spring, NC and taken to the lab, where the cut ends of the branches were placed in water and refrigerated. Before using them, the branches were examined under a dissecting microscope, and 10-11 infested branches that were about two-to-three inches in size and with 10-11 healthy woolly masses per branch were collected for each seedling. The HWA infested branches were clipped to the lower surface of seedling branches using paper clips so that each seedling would have 100-120 woolly masses. Two or three clips were used for each infested branch to facilitate the proper attachment of infested branches to the seedlings. Eastern hemlock was used as the control and was not infested with woolly masses. To avoid any mechanical injury to plants, clips were loosened before using them. In this way, new crawlers from the infested branch could easily crawl and attach to the host tree.

Slow release Osmocote fertilizer was applied before infestation, and no fertilizer treatment was given after infestation. While irrigating, proper care was taken to minimize the disturbance to the seedlings and woolly masses. After applying the treatments, we waited until the progrediens crawlers had emerged, settled on the branch, and the adults had formed the woolly masses typical of mature insects. On June 25 and 26, 2006, we collected the infested branches from each seedling and bought them to laboratory for further evaluation. We counted and recorded the number of woolly masses per seedling and the number of eggs in each woolly mass on the recipient host branches.

Wax Extraction of Epicuticular Layer of Carolina Hemlock Provenances

In order to determine if there is any difference in wax load and composition of epicuticular wax (extracted from the needles of various provenances of Carolina hemlock), a GC mass spectrometry analysis was conducted. Seedlings which were used in this study were the same as those of the artificial infestation study. We randomly selected one seedling from the provenances which didn't show any infestation. In those provenances where all the seedlings were infested one of the infested seedlings was randomly selected. And in the provenances where half of the seedlings were infested, one of the infested seedlings was selected. One set of needles with stems (4cm) were cut from each seedling. Epicuticular wax was extracted from freshly cut hemlock needles attached to the branches by immersing the stem in 4ml of CH_2Cl_2 for 30 sec at room temperature. Prior to GC analysis the samples were prepared for the silyation process. For silyation, 1000 µl of wax sample was mixed with 10 µl of internal standard (n-tetracosane), and put in scintillation glass vials. The mixture was dried under a nitrogen stream. Then 300ul of BSTFA in pyridine was added to a dried wax

sample to transform hydroxyl-containing compounds into the corresponding trimethylsilyl derivatives. The wax mixture sample in scintillation vial with a Teflon-coated cap was than heated in a water bath at 70^0 C for 30 min. The sample was again dried under a nitrogen stream. Before shooting the sample to the GC, 100µl of hexane was added to the dried sample mixture, and 1µl of this mixture was injected in the GC/MS for a qualitative and quantitative analysis of the wax. The qualitative composition was analyzed with a DB-5 capillary column (30m) using helium gas as the carrier. An initial temperature of 50^0 C was set for 2 min and raised by 40^0C per min to 200^0 C, held for 2min at 200^0C, raised by 3^0C per min to 320^0C and remained at 320 ^{0}C for 30 min. Individual wax components were identified by comparison of their spectral range with a search of the WILEY 275 library.

The area under each peak on the chromatogram represented the concentration of a wax component in the extract. Each provenance sample containing known amounts of the internal standard tetracosanol from which the concentration of the other major wax components can be calculated.

Result and Discussion

Greenhouse Infestation Study

The results from the artificial infestation study are shown in Table 3.1. We were able to categorize the variation in infestation of Carolina provenance as highly infested, moderately infested, and uninfested.

A seedling with even a single woolly mass could be considered infested, so fecundity of HWA and level of infestation should be taken into account. Fecundity was measured with

number of eggs per woolly mass in each provenance (Fig. 3.2). And level of infestation was measured by number of woolly masses per seedling (Fig.3.3).

The results showed that Ceaserhead and Campground had 100 % infestation with an average of 16.9 and 16.5 eggs per woolly mass respectively. But other provenances like Crabtree, Wildcat and Table Rock showned 50% infestation, with Wildcat and Table Rock showing an especially low number of eggs per woolly mass (1.9 eggs/woolly mass for Wildcat and 2eggs/woollymass for Table Rock). Linville, Cradle and Bluff Mountain showed zero growth of woolly masses (Fig. 3.2).

Number of woolly masses per seedling is shown in Figure2.2. Ceaserhead and campground had 10.5 and 8.75 number of woolly masses per seedling respectively, whereas Crabtree, Wildcat and Table Rock had an average of 6.25, 2.5 and 1.5 woolly masses per seedling respectively. Linville, Cradle and Bluff Mountain showed no woolly masses.

The level of infestation was categorized into three parts: infested, moderately infested, and uninfested (Table 3.2). Provenances with averaging more than 8 woolly masses/seedling were considered as infested. Whereas seedlings with 1-3 and less than 1 woolly mass/seedling were considered to be moderately infested and uninfested, respectively.

These results suggest that Ceaserhead and Campground are infested. Wildcat and Table Rock are moderately infested and Linville, Cradle and Bluff are uninfested. Crabtree falls in between first two categories and if we look into individual seedlings the no. of woolly masses / seedling and no. of eggs/ woolly mass were high in this provenance but only 50% seedlings had shown infestation; so the avg. no. of woolly mass/ seedling decreases. Wildcat

and table rock also showed 50% infestation but the number of eggs/woolly mass were very low in these two provenances (Fig. 3.2).

We also ran a single factor ANOVA and lsd (t-test). The results from the ANOVA are shown in Table 3.3 and lsd means are in Table 3.1

The P-value is less than the level of significance ($\alpha = 0.05$). Moreover the calculated F-value is bigger than that of F-table value, which shows there is significant difference between at least 2 provenances. So least significant difference (lsd) was calculated in order to look at the significance difference between all the provenances. The provenances with different letters (Table 3.1) are significantly different from each other. Accordfing to the lsd test we divided the provenances into three groups. Ceasarhead and Campground were not significantly different from each other and there is no significant difference between Bluff Mountain, Linville, and Cradle. It also shows that there is no significant difference between Wildcat and Table Rock. And these groups are significantly different from each other.

The specimens from the Linville provenance were uninfested in our experiments, but are infested in their natural habitat. This could be the case with other provenances. One reason for this type of observation could be that our study was conducted in the greenhouse with seedlings, but ecological conditions at the sites of the various provenances were not simulated in the greenhouse, instead they were infested under the same conditions. Most of the genetic variability in tree species is distributed by geographic patterns (Collins 1971). A complex suite of biological, chemical, and environmental variables governs the suitability of a host tree. The seedlings were from different provenances and they could have different environmental, climatologically and topographical conditions, as well as different microsite

characteristics. We usually manipulate the environmental conditions as a tool to manage the pest population in an indoor experimental system. Varying temperature and other factors can enhance or mask the expression of resistance in indoor conditions so it is necessary to not rely fully on results from indoor screenings unless they reflect the field performance (Salim 1991). Some environmental variables may modify the expression of genetic resistance and occasionally increase host susceptibility to a degree unsuitable for management objectives (Hanover 1975). Shortcomings of this kind can be overcome by running the experiments in the provenances themselves. Another possible reason for resistance in the Linville seedlings is the "age factor". It is possible that with age this Linville provenance looses its resistance. Eastern hemlocks that were used as a control in the study didn't show any infestation, which is understandable as the dispersion of HWA is mainly through wind and human activity and not by the adelgid itself.

From the Table 3.1 it's clear that half of the Crabtree seedlings showed a high level of infestation, while the other half were uninfested. The following may be possible explanations for this.

1. Quality of the eggs: It might be possible that the egg masses we used in our study were not of good quality. Although we took great care while infesting the seedlings, we are dealing with such small delicate insects that it is hard to track hatched eggs, numbers of survivors, or how many lived long enough to feed and crawl out to go to the seedling.
2. Seedlings with no infestation were resistant: This hypothesis leads us to the idea of variation within the provenances.

3. Also extremely small sample size of seedling to represent a provenance.

All these reasons can be met by looking into the variation in topography within the provenance and running the experiments in different topographical parts of the provenance. It is also possible that the arrangement of leaves and branches might have a relationship with the preference of the insect. Some leaf surface features are known to influence insects. The small size of the insect herbivores means that they face the problems of attachment and access to the leaf even before they can ingest the desired leaf tissue (Peters 2002).

Eastern hemlocks, which are more susceptible to adelgid attack, have needles that are more open with a wider spread-out arrangement and give a smooth appearance to the branch. But in Carolina hemlock the needles are much more whorled about the twig giving it a rougher, less flat appearance. We have observed that the leaf pattern of the Campground seedlings is more similar to Eastern hemlock. Leaf structural traits including surface features, anatomy and morphology have the potential to impede insect feeding and may ultimately influence the host choice of insect herbivores (Peter 2002). Critchfield (1957) also related anatomical differences in the needles to the geographical distribution of the seed source, and some of these differences may reflect the physiological adaptation to specific site conditions.

Epicuticular wax extraction study

There is a great deal of evidence that diverse plant epicuticular lipids affect the behavior of herbivorous insects. In some instances, the insects accept or reject plants due to variations in plant surface lipids (Chapman 1977). The objective of this work was to study the epicuticular waxes from the needles of Carolina hemlock and their influence on HWA and hemlock interaction. The chemicals present on the upper surface are responsible for

mediating many of the behavioral steps involved in recognizing their host and rejecting unsuitable plants (Jaenike 1990). Thus in order to gain a better understanding of the chemical factors involved in the host feeding selectivity by *Adelges tsugae*, epicuticular wax was extracted from Carolina hemlock needles and studied.

Different wax esters were observed using GC/MS. The analysis of wax extract showed two large peaks (peak1: Tetracosanol and peak 2 : Hexacosanol)) and many small peaks. The large peaks were common in all provenances. Qualitative analyses were done and individual wax components were identified from the WILEY 275 library. The small peaks with areas of less than 2% were not analyzed, as we assumed that this concentration of the wax esters would be too low to be detected by the insects. Only peaks with an area of 2% or greater were analyzed. The chemical components corresponding to these peaks were identified and their impact on insect physiology and behavior was studied. GC/MS analysis indicated the variability in the profile of 20-45 peaks examined between provenances.

Qualitative Analysis of Epicuticular Wax Extraction of Eight Carolina Hemlock Provenances

In all the wax samples, two peaks were common: Tetracosane (internal standard) and Hexacosanol. Hexacosanol and Octacosanol are present in the leaves of the mulberry, *Morus alba* and are responsible for stimulation of feeding in the larvae of the silkworm, *Bombyx mori* (Mori, 1982).

Linville (Uninfested): Besides the two main peaks in fig. 3.4, three other small peaks were identified:

1 2-Furanmethanol(34.90): a volatile compound in protein insect bait PIB-7 (Buttery et al, 1983). Insects are attracted to these hydrolyzed protein baits. The hydrolyzed

protein mixtures are assumed to be related in composition to the "honeydew" extruded by aphids which, in nature, can apparently supply a suitable diet for both the adult and larva of certain insects (Hagen et al., 1976).

2 Cyclohexanecarboxylic acid(38.26): acts as a growth promoter of *Macrosiphum euporbiae*, the potato aphid (Chawla et. Al 1974).

3 6,7-Dimethoxy-1-isquinolinyl (38.99): there is very little information found in the literature about the relationship of this chemical with insects in general.

Cradle of Forestry (Uninfested): Other than the main peaks, two additional ones were found (Fig. 3.5).

1 Arsenous acid(34.81): it is responsible for causing damage to epithelial cells of the anterior midgut of *Periplaneta americana*, the American cockroach (Chaudhury & Chaudhury 1972).

2 Hexahydropyridine, 1-methyl-4-[4,5](38.44): No information is available in the literature about the relationship of this chemical with any insect.

Campground (Infested): Three other peaks were identified (Fig. 3.6)

1 2- Furanmethanol(34.85): The concentration of furanmethanol was higher in the Campground as compared to Linvile (Table 3.4), which seems to be resistant in the greenhouse study; therefore it might be possible that the higher concentration of furanmethanol is responsible for an attraction of HWA in Campground.

2 1,1,1,3,3,5,5-Heptamethyltrisiloxane(36.92)

3 Thymol(38.75): found in very low concentration in hemlock needles, thymol is responsible for a slight reduction in feeding damage by acting as anti-ovipositants

for *Frankliniella occidentalis* and *Thrips tabaci* (Sedy & Koschier 2003).

Crabtree (Infested): There are many other small peaks that are present but no other peak except the two main peaks tetracosane and hexacosanol were identified from WILEY 275 library. The concentration of hexacosanol (feeding stimulant) is less in crabtree but it showed susceptibility in the greenhouse study. We didn't find any growth retarder in this provenance, hence it might be possible that susceptibility of this provenance is due to the absence of insect growth retarder chemicals in epicuticular wax layer of this provenance (Fig. 3.7).

Table Rock (Moderately Infested): Besides two main peaks four new peaks were identified(Fig.3.8)

1 Eicosane(26.34): one of the aliphatic components that serve as epicuticular lipids in plants and are structurally very similar, even being identical in some cases, to those found in the epicuticular lipids of insects (Kolattkudy et al 1989).
2 Tricosane(28.49): it is a kairomonal compound present in the scales of moths. It also acts as an attractant for many parasitoids (Report 2007).
3 Octacosane(30.61): this compound positively affects *Bemisia tabaci* by acting as a growth promoter in cucumber (Leite 2006).
4 Arsenous acid(34.70): the peak area of arsenous acid is comparatively less in table rock as compared to cradle of forestry (Table 3.4). The presence of insect growth promoters and less concentration of arsenous also justifies the susceptible nature of Table Rock.

Bluff Mountain (ModeratelyInfested): One other main peak was identified besides hexacosanol and tetracosane peaks(Fig. 3.9).

1 Galactitol(36.99): a carbohydrate which reduced the survival of *Pimpla turionellae* eggs (Ozalp & Emri, 2001). The peak area of galactitol is more in Bluff Mountain as compared to Caeserhead (Table 3.4).

Wildcat (ModeratelyInfested): Only tetracosane and hexacosanol were identified (Fig. 3.10)

Ceaserhead (Infested): Three other main peaks were identified (Fig. 3.11).

1. ctanamide(34.66)
2. Galactitol(36.92): concentration of galactitol is less in caeserhead than Bluff Mountain which also explains the susceptible nature of caeserhead.
3. Cyclotrisiloxane(38.76).

Quantitative analysis of the wax ester, hexacosanol, common in all eight Carolina hemlock provenances

The area under each peak on the chromatogram represented the concentration of a wax component in the extract. The concentration of hexacosanal, which was observed in all the seedlings, was calculated by comparing the area under each peak with that of the internal standard (Table3.6). Each provenance sample containing known amounts of the internal standard were injected three times in the GC/MS to determine the ratio POI R.F/IS R.F (peak area of hexacosanol /peak area of internal standard)

The peak at retention time 32, which corresponds to hexacosanol, is more pronounced in Campground and Caeserhead but reduced in other Carolina provenances (Fig. 3.12).

Campground, Crabtree and Caeserhead provenances were found to be highly susceptible to HWA infestations in the preliminary greenhouse studies, which could be associated with the presence and abundance of the chemicals in the epicuticular wax. The

activity of chemical defenses can affect an insect's taste where several different types of gustatory receptors detect both qualitative and quantitative differences in the chemical content of the plant tissues tested (Hanson 1983). Hexacosonal, which acts as a feeding stimulant for silkworms, was found in high concentration in two highly susceptible provenances (Campground and Ceaserhead). Its lowest concentration was found in the provenance that was resistant in the greenhouse studies. But a Crabtree provenance, which is also susceptible, didn't seem to be dependent on hexacosanol concentration so it might be possible that hexacosanol in combination with other wax components is responsible for resistancein Carolina hemlock.

There are other factors that might be responsible for the susceptibility of hemlock trees. It might be possible that the concentration of hexacosanol in combination with other wax components plays an important role in host selection. Walker (1988) reported that the amount, or type, of epicuticular wax on a leaf can affect the ability of insects to remain attached to the plant or to move from one part of the plant to another. The finding of the present investigation revealed that the leaf extract of eight provenances of Carolina hemlock possess many compounds that have been demonstrated to significantly influence insect-plant relationships in other plants. These compounds may jointly or independently contribute to susceptibility and resistance against HWA. Their actual affects in HWA-hemlock interactions have not yet been demonstrated. Further research is required to investigate the possible role of these chemicals in host-plant selection in hemlock. These compounds were identified using the mass spectral database, and only compounds with a similarity index (SI) greater than 50% (which is low) were considered as positive identifications. Hence, their

identity should also be confirmed using other methods. It is necessary to isolate these compounds in large quantities and to identify them through more precise spectroscopic methods, as well as to conduct bioassays with the compounds. The chemical composition of the epicuticular wax on the aerial surface of a plant varies among species, within a species, among plant parts, and among different stages of development (Stadler 1986); so the old and new needle growth, abaxial and adaxial needle surface should be considered in future studies. Minor components in the wax extraction that we have not covered need to be considered in future studies. Moreover identification of the peaks that we could not identify should be identified from a larger database. Analyzing these minor components will be useful in checking resistance or host suitability in hemlock

Table 3.1 Number of woolly masses (*n*) and number of eggs (mean±s.d.) per woolly mass collected on seedlings of Carolina provenances.

	Seedling 1		Seedling 2		Seedling 3		Seedling 4			
Provenances (State)	**n**	**Mean ± s.d.**	**n**	**Mean ± s.d.**	**n**	**Mean ± s.d.**	**n**	**Mean ± s.d.**	**Mean No. of woolly masses/seedling**	**lsd (infestation)**
Ceaser Head(SC)	16	18.81±4.10	9	18.56±5.46	10	17.40±6.36	7	14.71±5.96	10.50	A
Campground NC)	10	11.20±5.80	12	13.75±5.30	8	24.00±6.45	5	21.40±2.88	8.75	A
Crabtree(NC)	13	11.58±4.28	12	11.88±6.94	0	0	0	0	6.25	A B
Wildcat (SC)	6	1.83±0.75	4	2.76±0.82	0	0	0	0	2.50	B C
Table Rock (SC)	4	2.25±1.25	2	1.50±0.71	0	0	0	0	1.25	B C
Linville (NC)	0	0	0	0	0	0	0	0	0	C
Cradle (NC)	0	0	0	0	0	0	0	0	0	C

Table 3.1 Continued

	Seedling 1		Seedling 2		Seedling 3		Seedling 4			
Provenances (State)	**n**	**Mean ± s.d.**	**n**	**Mean ± s.d.**	**n**	**Mean ± s.d.**	**n**	**Mean ± s.d.**	**Mean No. of woolly masses/seedling**	**lsd (infestation)**
BluffMountain(NC)	0	0	0	0	0	0	0	0	0	C
EasternHemlock	0	0	0	0	0	0	0	0	0	C

n= no. of woolly masses/seedling

Table 3.2 The three levels of HWA infestation based on average no. of woolly masses/seedling in Carolina hemlock provenances.

Average number of woolly masses/seedling	**Definition**
> 8	Infested
1-3	Moderately Infested
< 1	Uninfested

Table 3.3 ANOVA test of Woolly masses/seedling for Carolina hemlock provenances

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Between provenances	502.375	7	71.76785714	6.463146609	0.0002418	2.422629
Within provenances	266.5	24	11.1041666			
Total	768.875	31				

Table 3.4 Relative ratio of wax esters in Carolina provenances (where POI is Peak of Interest and IS is Internal Standard).

Sample	**I.S**	**POI(furanmethanol)**	**POI (Arsenous)**	**POI(Galactitol)**	**POI/IS(Relative ratio)**
Linville/U	29.33	5.67	*	*	5.172839506
Campground/I	33.67	5.03	*	*	6.693836978
Cradle/U	35.89	*	3.2	*	11.215625
Table Rock/MI	29.66	*	2.52	*	11.76984127
BluffMountai/U	37.19	*	*	2.95	12.60677966
Casearhead/I	33.23	*	*	3.61	9.20498615
Crabtree/I	44.54	*	*	*	*
*	*	*	*	*	*

Table 3.5 Quantitative analysis of hexacosanol in eight Carolina hemlock provenances {where (a)Internal standard (IS) Area; (b) Internal Standard (IS) Injected (Inj) volume (Vol) in µl; (c) Peak of Interest (POI) Area; (d) Peak of interest (POI) Injected (Inj) volume (Vol.) in µl; (e) Internal standard reference factor (RF); (f) Peak of Interest reference factor; (h) Internal standard concentration in µg/ µl; (g) peak of interest (POI) concentration in µg/ µl}

Provenances	**IS Area (a)**	**IS Inj. Vol.(b)**	**POI. Area (c)**	**POI Inj. Vol.(d)**	**IS R. F.(e)**	**POI R. F.(f)**	**POI / IS RF. (g)**	**IS Conc.(h)**	**POI Conc.(i)**
Linville	3748576	2	5157991	50	1874288.00	103159.82	0.055039471	0.003	0.000165118
Linville	3398133	2	4053561	50	1699066.50	81071.22	0.047715154	0.003	0.000143145
Linville	3741612	2	4845852	50	1870806.00	96917.04	0.051804965	0.003	0.000155415
Cradle	4007639	2	6919738	100	2003819.50	69197.38	0.034532741	0.003	0.000103598
Cradle	3949906	2	7145867	100	1974953.00	71458.67	0.036182466	0.003	0.000108547
Cradle	4660274	2	9361084	100	2330137.00	93610.84	0.040173964	0.003	0.000120522

Table 3.5 Continued

Provenances	IS Area (a)	IS Inj. Vol.(b)	POI. Area (c)	POI Inj. Vol.(d)	IS R. F.(e)	POI R. F.(f)	POI / IS RF. (g)	IS Conc.(h)	POI Conc.(i)
Campground	6640092	2	9842518	15	3320046.00	656167.87	0.197638185	0.003	0.000592915
Campground	6545307	2	8615276	15	3272653.50	574351.73	0.175500319	0.003	0.000526501
Campground	6172902	2	8060362	15	3086451.00	537357.47	0.174102057	0.003	0.000522306
Crabtree	3516252	2	5307810	50	1758126.00	106156.20	0.060380314	0.003	0.000181141
Crabtree	4269548	2	5653011	50	2134774.00	113060.22	0.052961213	0.003	0.000158884
Crabtree	4000941	2	5372319	50	2000470.50	107446.38	0.053710555	0.003	0.000161132
Table Rock	4641494	2	5494695	25	2320747.00	219787.80	0.094705627	0.003	0.000184117
Table Rock	3853738	2	2392432	25	1926869.00	95697.28	0.049664653	0.003	0.000148994
Table Rock	3758081	2	2368050	25	1879040.50	94722.00	0.050409770	0.003	0.000151229
Bluff	4315863	2	5080653	50	2157931.50	101613.06	0.047088177	0.003	0.000141265

Table 3.5 Continued

Provenances	IS Area (a)	IS Inj. Vol.(b)	POI. Area (c)	POI Inj. Vol.(d)	IS R. F.(e)	POI R. F.(f)	POI / IS RF. (g)	IS Conc.(h)	POI Conc.(i)
Bluff	4342659	2	5396826	50	2171329.50	107936.52	0.049709876	0.003	0.000149130
Bluff	5049897	2	6771199	50	2524948.50	135423.98	0.053634353	0.003	0.000160903
Wildcat	4101715	2	5122444	50	2050857.50	102448.88	0.049954168	0.003	0.000149863
Wildcat	4099207	2	5336387	50	2049603.50	106727.74	0.052072384	0.003	0.000156217
Wildcat	4204828	2	5012247	50	2102414.00	100244.94	0.047680875	0.003	0.000143043
Ceaser Head	5736813	2	8796418	15	2868406.50	586427.87	0.204443780	0.003	0.000613331
Ceaser Head	6246693	2	9648870	15	3123346.50	643258.00	0.205951533	0.003	0.000617855
Ceaser Head	5668056	2	8592216	15	2834028.00	572814.40	0.202120233	0.003	0.000606361

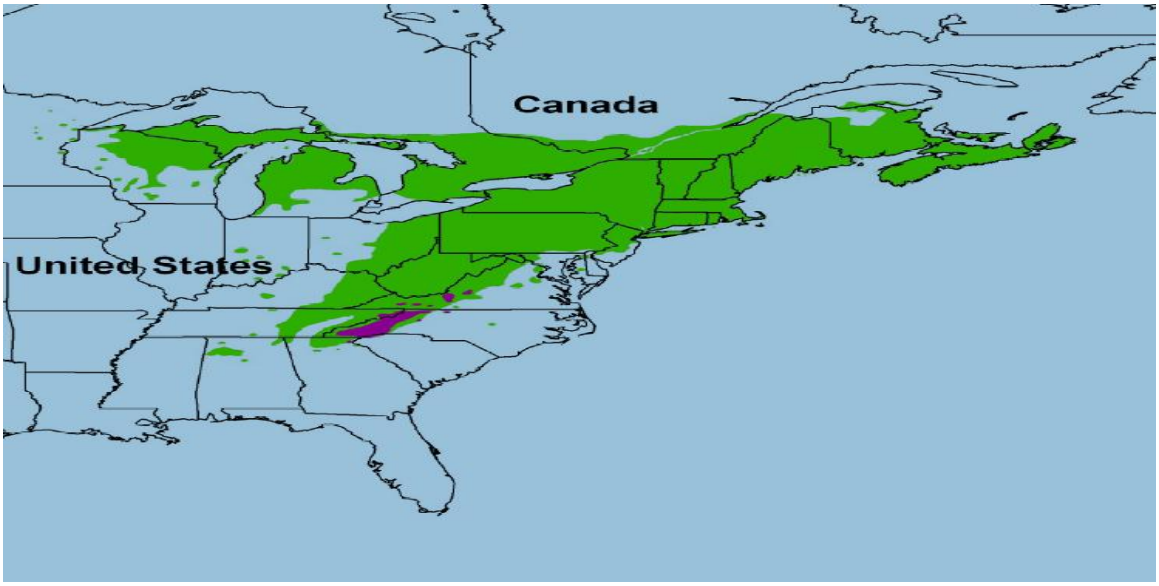

Figure 3.1. Native range of *T. canadensis* (green) and *T. caroliniana* (red) in eastern North America. Map produced by Camcore, Department of Forestry and Environmental Resources, N.C. State University.

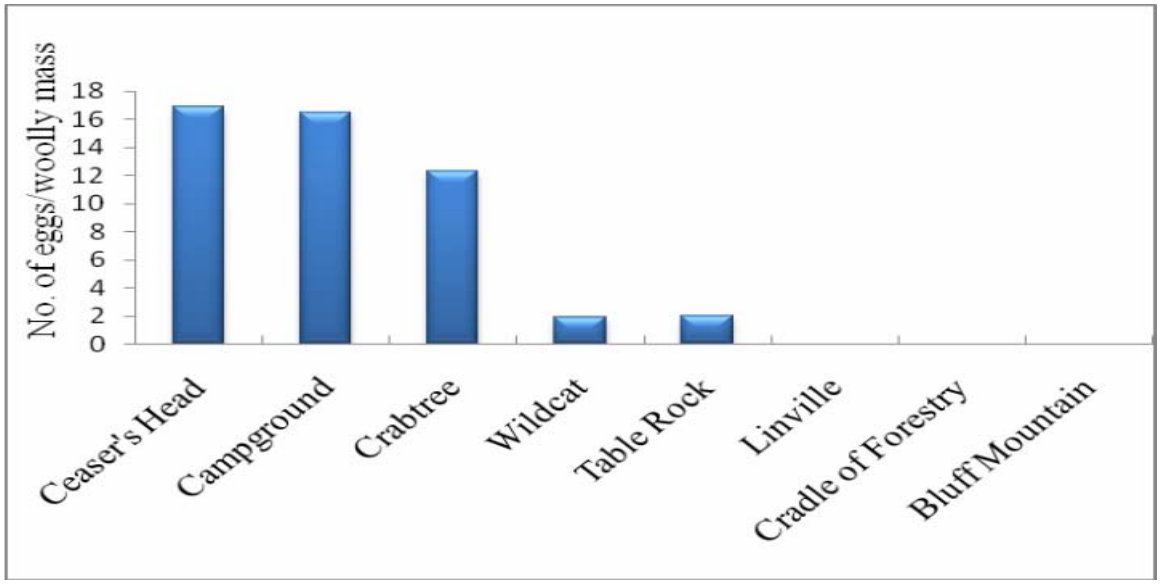

Figure 3.2 Adelgid Fecundity in eight Carolina hemlock provenances

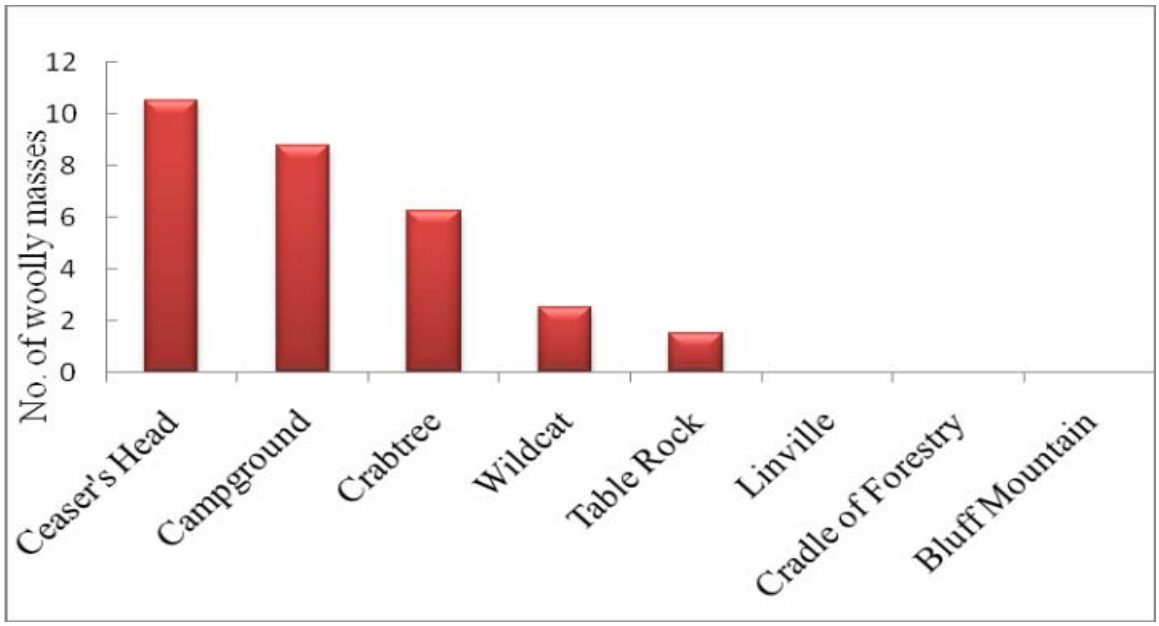

Figure 3.3 Level of Infestation in eight Carolina hemlock provenances

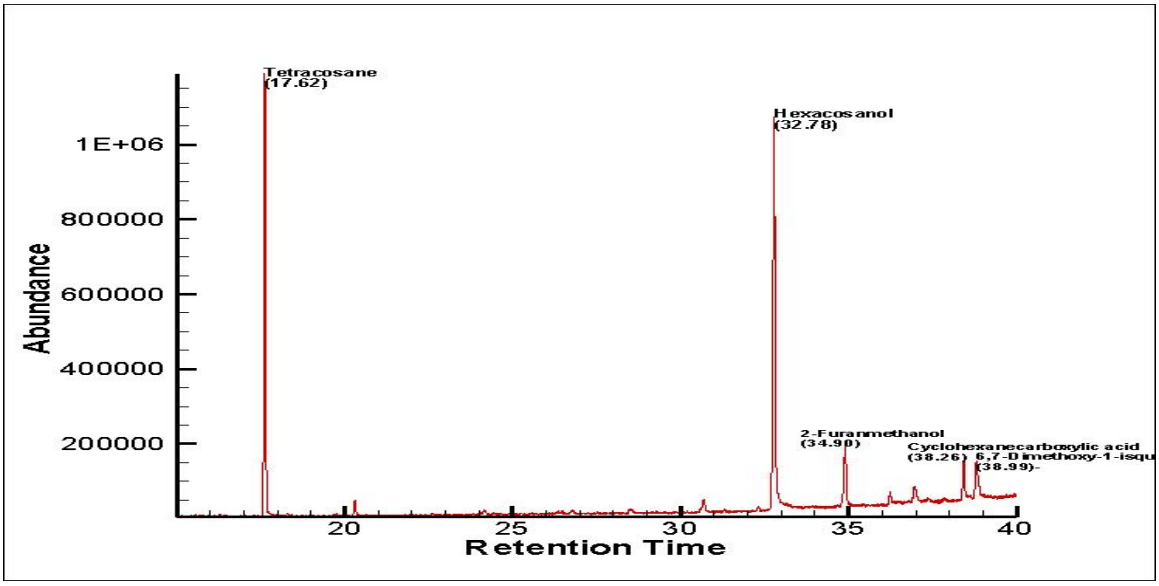

Figure 3.4 Chromatogram of the wax ester fraction of Linville provenance

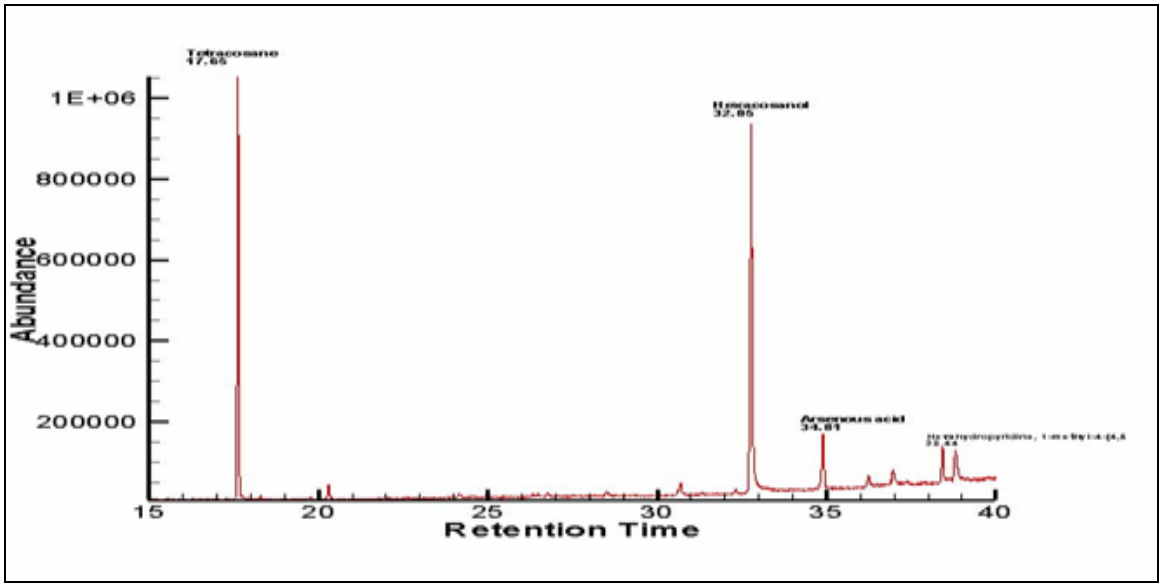

Figure 3.5 Chromatogram of the wax ester fraction of Cradle of Forestry provenance

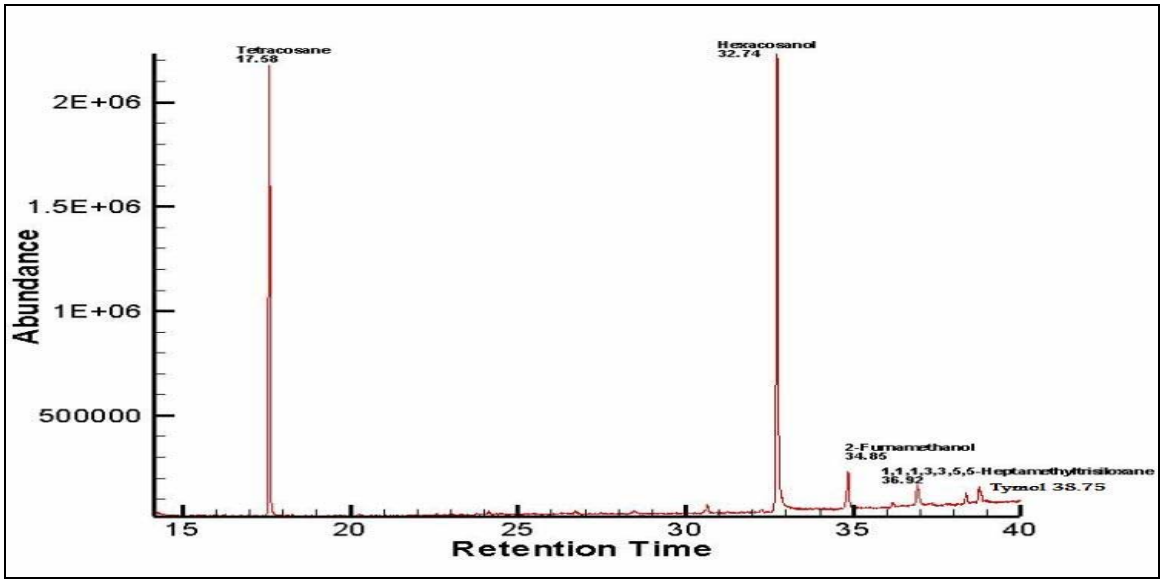

Figure 3 6 Chromatogram of the wax ester fraction of Campground provenance

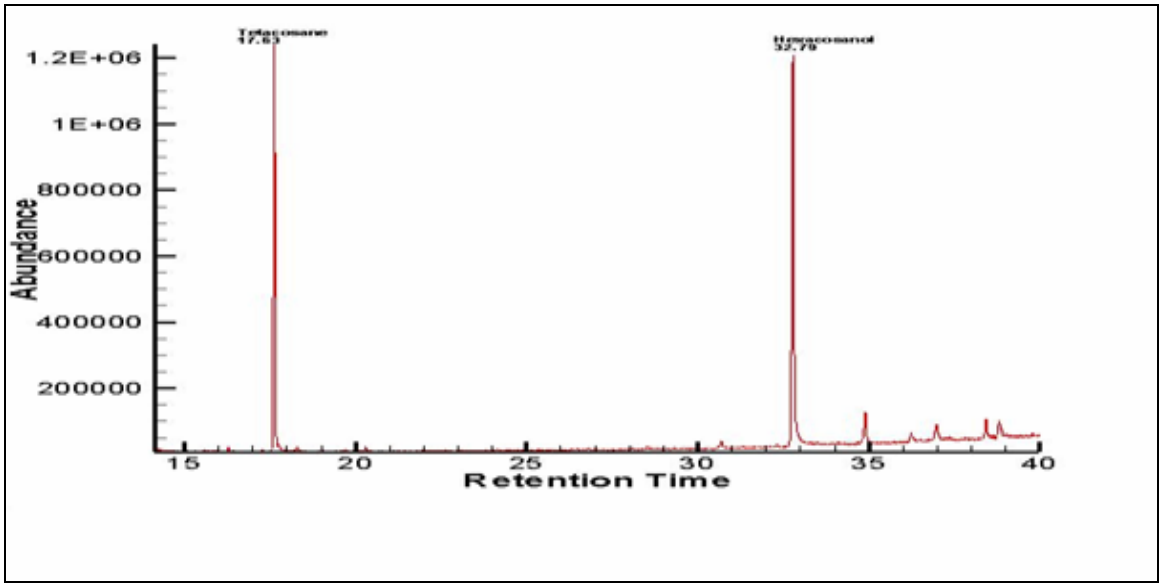

Figure 3.7 Chromatogram of the wax ester fraction of Crabtree provenance

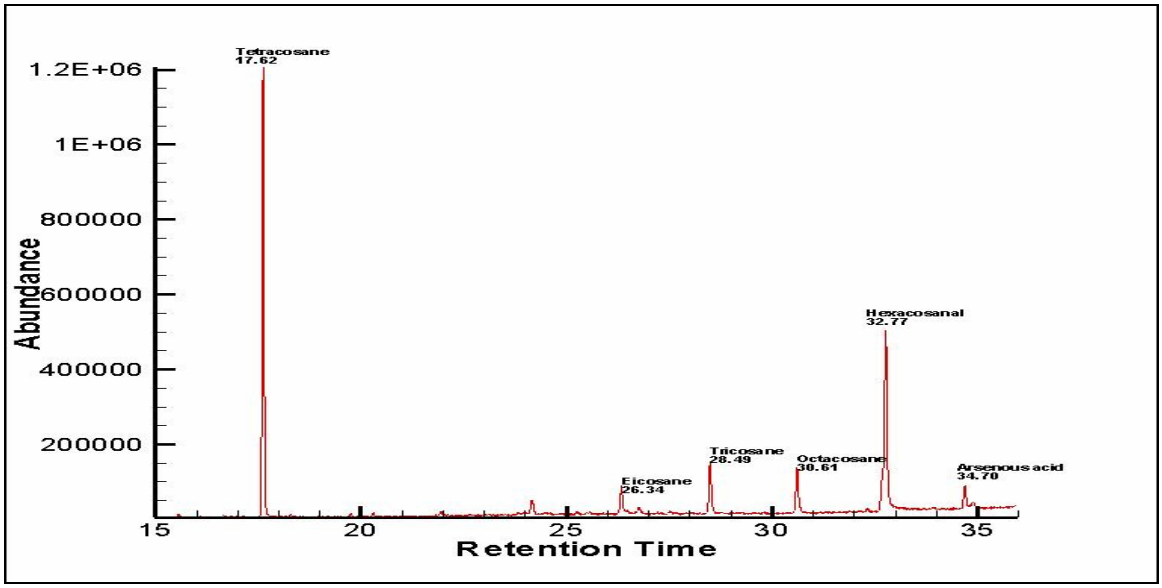

Figure 3.8 Chromatogram of the wax ester fraction of Table Rock provenance

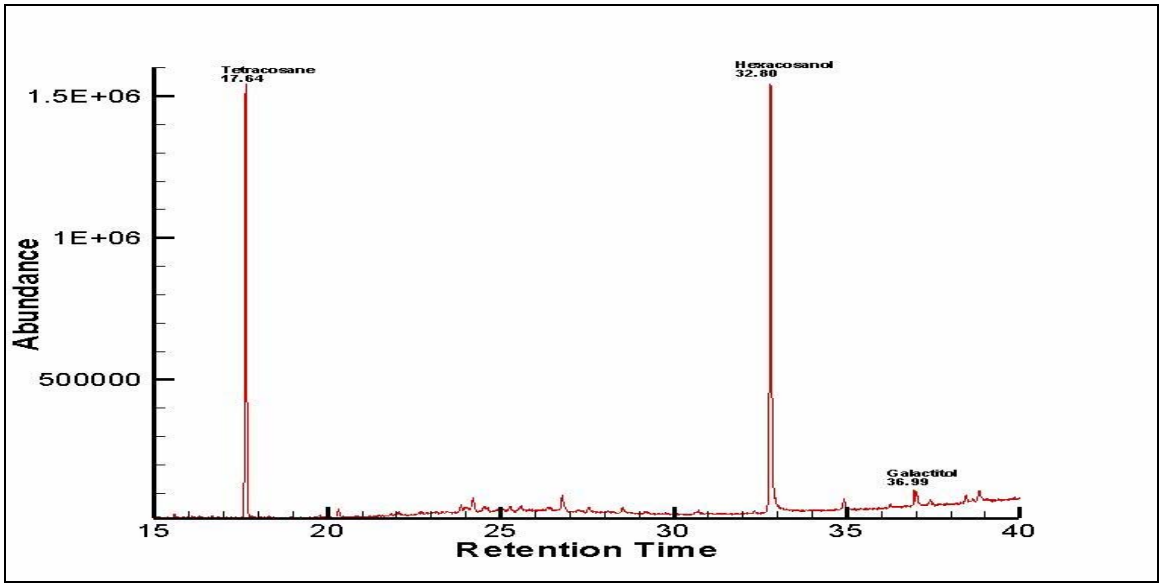

Figure 3.9 Chromatogram of the wax ester fraction of Bluff Mountain provenance

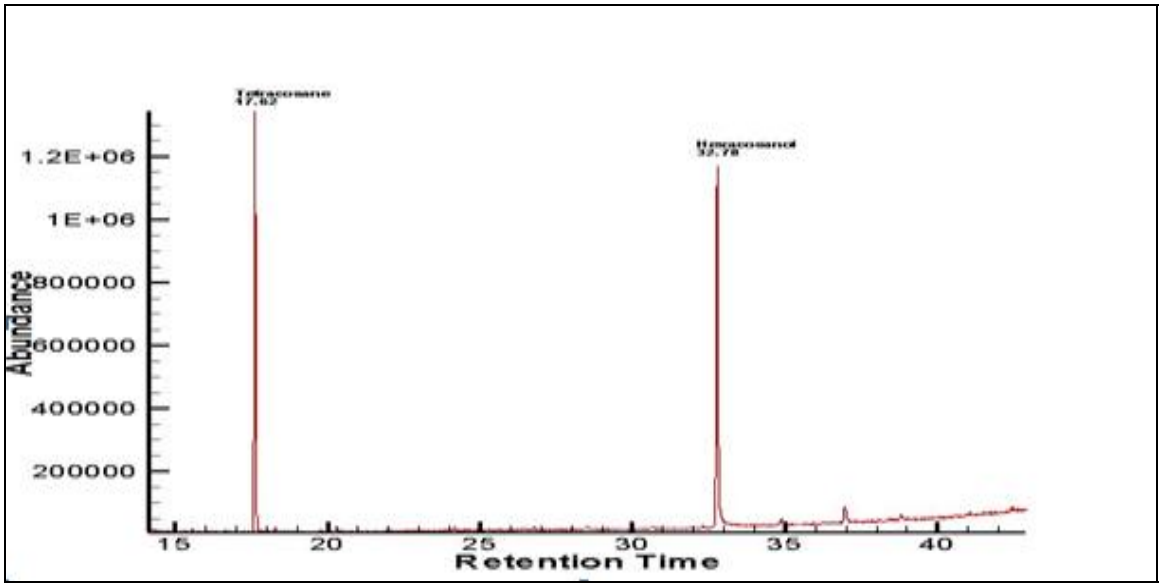

Figure 3.10 Chromatogram of the wax ester fraction of Wildcat provenance

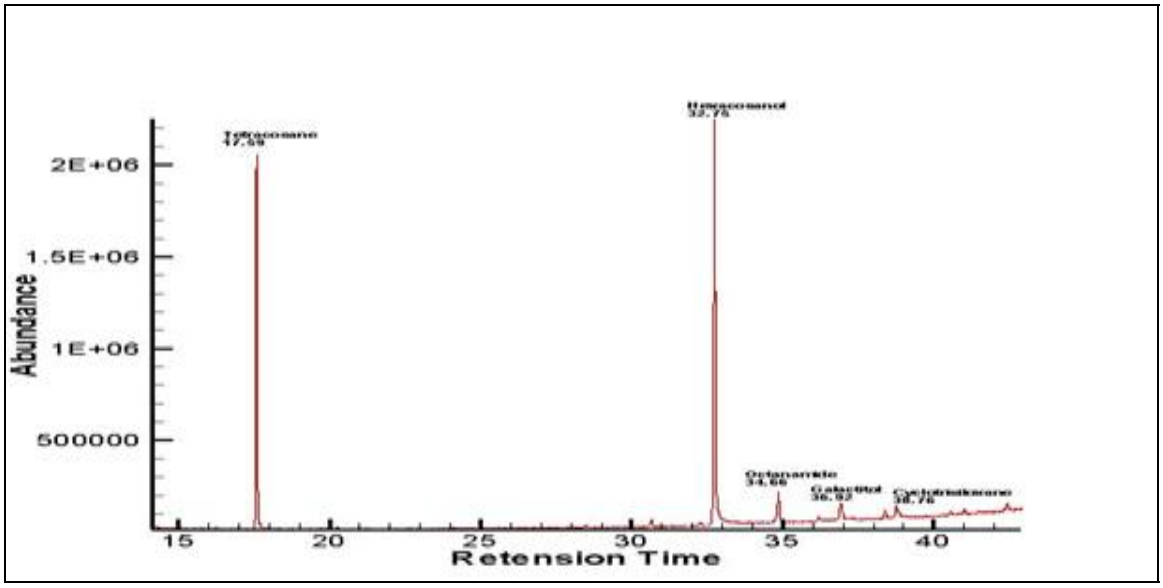

Figure 3.11 Chromatogram of the wax ester fraction of Ceaserhead provenance

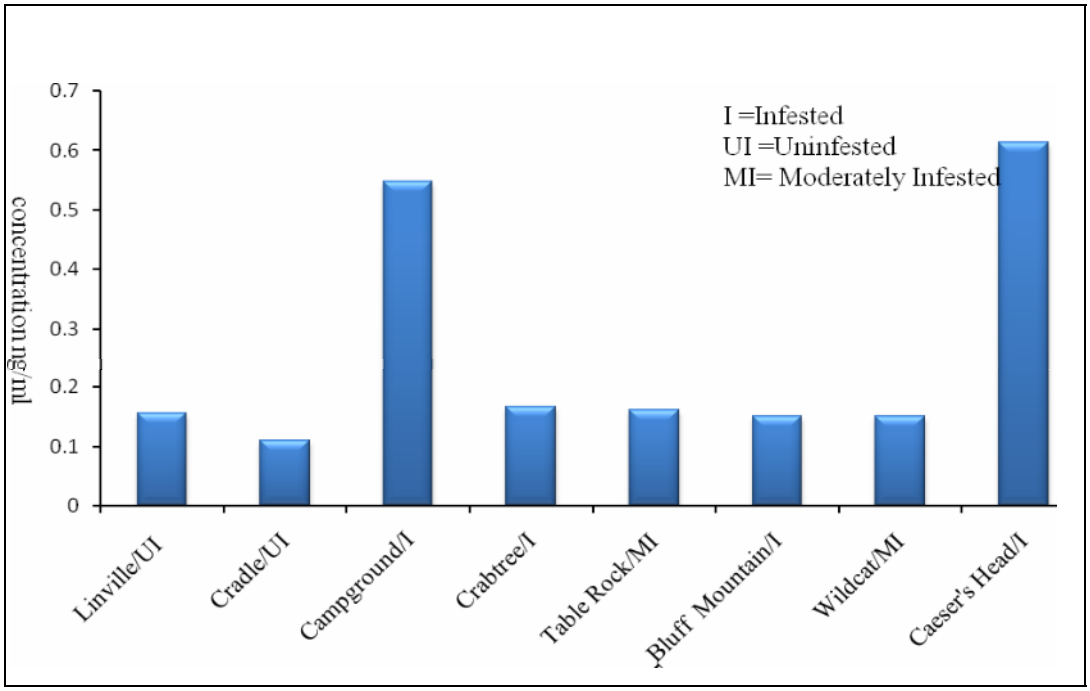

Figure 3.12 Concentration of hexacosanol in eight Carolina provenances.

SUMMARY AND CONCLUSION

While our diet did not enable HWA or BWA to successfully develop to adults, we were able to discover important factors in their feeding preferences and dietary requirements. Future efforts towards the artificial production of adelgids will benefit from these studies. The feeding apparatus that we have developed seems to be appropriate, once we determine the phagostimulant requirements and proper nutrient makeup of the diets. A successful artificial rearing setup is the combination of nutrients, stimulants, environmental requirements, and many other factors. While it is currently unclear what is missing from our approach, we have made great strides in developing an artificial rearing framework.

The conclusion of our artificial infestation of Carolina provenances in the green house study is that Ceaserhead, and Campground were highly infested, the Crabtree provenance was a mix of infested and uninfested, Wildcat and Table Rock were moderately infested with low numbers of eggs per woolly mass, and Linville, Cradle and Bluff were uninfested. The study conducted, was preliminary and gave us only an idea of the susceptibility of the Carolina provenance seedlings, and further studies need to be done. More reliable and definitive findings can be obtained with larger numbers of seedlings and repeated infestations on the same seedlings.

Epicuticular waxes play an important role, as plant surface waxes are the first contact after visual and olfactory cues. Our limited sample analysis using greenhouse seedlings of Carolina hemlock provides information about the role of epicuticular wax in the infestation preferences; the variations in apparent susceptibility could have wider implications for

populations in the wild. It is not certain the extent to which hydrocarbons may be influenced by genetic, ecological and other geographical differences particularly in the wild. Different wax components from the eight Carolina provenances were identified and quantified. One compound hexacosanol was present in all provenances. Twelve other compounds were identified, and some of these compounds are common between one or two provenances. Highest concentrations of hexacosanol were found in the two most susceptible provenances, but not in the Crabtree provenance. Hexacosanol, in combination with other wax components, might play a role in host suitability.

It is generally agreed that host selection is based on insect responses to both nutritional and non-nutritional phytochemicals. The relative importance of nutrition versus secondary chemistry may vary from insect to insect (Harborne 1988). Further analysis of samples of Carolina hemlock drawn from different populations, especially in areas where different Carolina provenances coexist, will help provide a clearer picture.Moreover smaller unidentified peaks, which we ignored in our study, need to be analyzed to get precise results. To some extent, plant surface chemicals of Carolina hemlock may help HWA to determine if the plant is suitable as a host, but the results are not conclusive and further research is required. Knowledge of the stimulants and deterrents in the epicuticular wax may be useful in preparing diets; this can provide some clues to manage HWA populations, and to genetically manipulate host plants to create resistant trees

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