Behavioral adaptations of the Eastern spruce budworm (*Lepidoptera: Tortricidae*) to natural White spruce (*Picea glauca* (Moench)) resistance

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ABSTRACT

Behavioral adaptations of the Eastern spruce budworm (*Lepidoptera: Tortricidae*) to
natural White spruce (*Picea glauca* (Moench)) resistance
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The purpose of this study is to have a better understanding of how individual behaviors of the spruce budworm (*Choristoneura fumiferana*) (Clem.) (*Lepidoptera: Tortricidae*) are influenced by the natural resistance of its host in a white spruce (*Picea glauca* (Moench)) plantation. In this system, there are trees resistant to this insect and others more susceptible to its attacks. Second-instar spruce budworm dispersal experiments did show a significant difference between the two tree types when the experiment was conducted outdoors, whereas indoors no difference was found. Feeding choice experiments with sixth-instar larvae showed no preference for susceptible foliage. However, female adult spruce budworm reared on resistant foliage preferred to lay most of their eggs on susceptible foliage when given a choice between both types of foliage. Moreover, this deterrence was correlated with certain monoterpenes we measured in the foliage of the host trees. Our results suggest that adult female responses to thesemonoterpenes present in the waxes of the resistant foliage depend on their feeding experience as larvae. Our findings could contribute to a new pest management strategy by using a mixed plantation of the two phenotypes of white spruce we used in this study.

Key words: Spruce budworm, dispersal, feeding choice, oviposition, white spruce, pest management

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Introduction

The Canadian forestry industry contributes more than 450 000 jobs and the export of forest products generated 1.7% of Canada's gross domestic product in 2010. Yet this industry is subject to important losses due to several natural factors such as weather, fire and insect defoliators (NRC 2010).

With 11.5 million hectares disturbed, insects play a sizeable role in the forest industry yield. The eastern spruce budworm (*Choristoneura fumiferana*) (hereafter SBW) on its own, was responsible for 1 million ha of forest damage in Canada in 2008 and 765 740 ha in Quebec in 2010 (NRC 2010; Rapport TBE 2010, Ressources naturelles et faune Québec). Since 1992, the beginning of the current outbreak in Quebec, these numbers have risen continuously and this is in part due to a lack of knowledge of the outbreak dynamics of the insect.

Until now, the control of spruce budworm outbreaks has been achieved mainly with the application of *Bacillus thuringiensis* (Bt) spray (Moreau and Bauce 2003; Frankenhuyzen *et al.*, 1997). Even if a resistance to Bt has not yet been documented in SBW, it has occurred in several other pests such as diamondback moth (*Lepidoptera: Plutelidae*) (Tabashnik *et al.*, 1990). Because of possible resistance adaptations to Bt spray which would result in decreased efficiency of control, and because of possible negative effects on beneficial insects (James *et al.*, 1993), one might want to consider other pest management strategies.

The co-evolution between an insect and its host plant leads the latter to develop

many characteristics to decrease the negative impacts that the insect will have on its fitness. Resistance is here defined as the characteristic of a plant that reduces the damage inflicted by herbivores (Futuyma, 2000). There is an array of mechanisms that allow a tree to defend itself against herbivory ranging from phenology (Alfaro *et al.*, 2000; Lawrence *et al.*, 1997; Quiring, 1994) to production of chemical compounds (Alfaro *et al.*, 2002; King *et al.*, 2004; Chapman, 2003) and mechanical defenses (Levin, 1973; Burr and Clancy, 1993).

The chemical characteristics of foliage have been shown to be of major importance in the resistance of various conifer trees: Douglas-fir (*Pseudotsuga menziesii*) (Clancy *et al.*, 1993), Balsam fir (*Abies balsamea*) (Bauce *et al.*, 1994) and White spruce (*Picea glauca*) (Albert *et al.*, 1983; Daoust *et al.*, 2010; Despland *et al.*, 2011) to different herbivorous insects. Among these chemicals, secondary metabolites have been the subject of several literature reviews: in particular, phenols (Mattson and Scriber, 1987), tannins (Bernays, 1981; Mueller-Harvey, 1999) and terpenes (Gershenzon and Croteau, 1991; Tiberi *et al.*, 1999). In the case of the spruce budworm, studies on monoterpenes have been yielded contradictory results: they are phagodeterrent to the larvae (Chen *et al.*, 2002) but can also act as oviposition stimuli to adults (Grant *et al.*, 2007). Young balsam fir are more resistant than mature trees, and exhibit higher monoterpene levels compared to mature trees (Bauce *et al.*, 1994).

White spruce trees that suffer lower levels of defoliation have been identified (Daoust *et al,*. 2010). This natural resistance can be used as a form of insect pest control. In British Columbia, Canada, the use of genetically resistant trees is currently being tested to supplant spraying as the means of control of white pine weevil on Sitka spruce

(Alfaro *et al.*, 2008; King and Alfaro, 2009)**.**

Other than chemical defenses, phenology can also be considered a form of natural resistance: budburst in trees depends both on genetic factors and on environmental cues and can vary from year to year (Nienstaedt and King, 1969). An asynchrony in budburst compared to other trees in the population can allow a tree to escape attack from herbivores (Quiring, 1994). For the spruce budworm, emergence from diapause is generally in advance of conifer budburst (Thomson *et al.*, 1984; Nealis and Nault, 2005). The optimal synchrony for SBW is when emergence occurs 2 weeks prior to host budbreak. It has been shown that when this time is extended (larvae emerging earlier than 2 weeks before budbreak), the budworm population suffers a higher mortality rate. Indeed, during this period, young larvae occupy pollen cones and feed on previous years' growth which is a lower quality food source (Shepherd, 1992).

Our study took place in a fast growing white spruce plantation located in a zone of severe infestation (>50 larvae / 45 cm long branches) of spruce budworm in Drummondville, Quebec, Canada (45°53'0"N, 72°29'0"W). In this plantation two distinct types of spruce trees have been highlighted according to their level of defoliation. The two types were named as per Clancy *et al.*, (1993) susceptible and resistant to spruce budworm attack. Resistant trees showed less than 10% defoliation whereas up to 80% of foliage from susceptible trees was defoliated (Bauce and Kumbasli, 2007). Previous studies have attempted to explain the resistance at different levels of the plant-insect interactions. Daoust *et al.* (2010) showed an increase in monoterpene and condensed tannin concentrations in the needles, and a reluctance by sixth-instar larvae to feed on resistant needles. Removal of epicuticular waxes increased feeding on the resistant

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needles, suggesting a difference in phagostimulation from waxes of susceptible and resistant trees. Longer pauses between meals were also observed on resistant needles, suggesting a toxic effect of resistant foliar contents (Despland *et al.*, 2011). Other than tannins and monoterpenes, the presence of simple phenolics (notably the compounds pungenol and piceol) has been observed in resistant but not in susceptible trees. These two compounds have been shown to reduce larval survival and pupal mass and to extend the development time of the insect (Delvas *et al.*, 2011).

The eastern spruce budworm (*Choristoneura fumiferana*) is a univoltine insect from the order Lepidoptera. It passes through six different larval stages before undergoing complete metamorphosis to become an adult moth. Young larvae are very small and light yellow-green. They become darker with a dark brown head capsule in later instars. The larvae pupate within webbed foliage from late June to mid-July. The female moths lay eggs in masses on the underside of needles. The eggs hatch in July; the larvae immediately undergo a first moult and begin searching for a hibernaculum to pass the winter. The following spring, the second instar larvae emerging from diapause usually mine into the previous year`s needles until bud flush. Older larvae prefer recent foliage but will feed on older foliage if the current needles are depleted (Royama, 1984).

Larvae are oligophagous: the preferred hosts are, in order, the balsam fir (*Abies balsamea*), white spruce (*Picea glauca*), red spruce (*Picea rubens*) and the black spruce (*Picea mariana*) (Albert, 1980). The last SBW outbreak engendered a loss of 180 million m^3 of trees in North America. Lately, a new outbreak has started in Quebec with the area defoliated more than doubling every year (MNRF, 2010).

In order to control the budworm's highly damaging outbreaks, we need to

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understand the close relationship that exists between the budworm and its host better. Several individual behaviors and traits influence this insect's population dynamics. We used foliage from the resistant and susceptible trees to see if we can relate plant chemistry to behavior at three different stages in the budworm life-cycle. In this study we investigated several traits of the SBW life history: 1) dispersal and survival of second-instar larvae, 2) feeding of sixth-instar larvae and 3) oviposition by adults.

Dispersal of forest insect defoliators results in redistribution of the population within and between tree crowns and stands (Beckwith and Burnell, 1982). The early larval and the adult stages are the two points of its life cycle when the spruce budworm disperses. While the latter has been studied for years and its importance is well-known (Greenbank *et al.*, 1980; Dobesberger *et al.*, 1983), the former still needs further investigation. Early-stage larval dispersal can be split into two phases. The first comes immediately after hatching when the young larvae search for a hibernation site (late August). The second comes during the following spring when the second-instar larva is searching for a feeding site (Jennings *et al.*, 1983). In 2003, Nealis *et al.,* introduced the concept of 'Risk of dispersal' as the product of two processes: (i) the propensity for budworm larvae to move in search of suitable feeding sites and the (ii) likelihood of mortality resulting from that movement. This concept suggests that we should observe greater dispersal from resistant than from susceptible trees.

Spruce budworm caterpillars are able to discriminate between phytochemical components of host plants (Albert and Parisella, 1985). In our system, Daoust *et al.* (2010) have recently shown a significant difference in monoterpene concentrations in the

needles of resistant and susceptible trees. Chemical analyses were conducted only on the two most different trees in their study (the most resistant and the most susceptible) and they found that these chemical differences influence the pattern of feeding of SBW sixth-instar larvae. On susceptible foliage, insects are more likely to transition from probing to feeding behavior and have more frequent feeding bouts. We tested if their results can be generalized to the greater number of trees used in our study, and whether they can be correlated with behavior of young larvae and adult females.

In selecting her oviposition site the female is making a crucial choice for the survival of her offspring (Mader *et al.*, 2012). To make this decision the female uses visual and olfactory and chemical cues (see Wallace *et al.*, 2004). When hatching occurs, usually a few days after oviposition, the female has chosen the initial feeding site for her offspring. The female SBW has been shown to be able to discriminate between different host species (Rivet and Albert, 1990). Compared to several species (balsam fir, red and black spruce), white spruce was always the preferred host species in both lab and field experiments (Städler, 1974). Overall, we investigated if the female moth SBW is able to discriminate between two hosts of different quality within the same species.

We examined SBW behavior between resistant and susceptible trees at three stages in the life-cycle: young larvae, older larvae, and adult females. Our hypothesis for these experiments was that, overall, susceptible trees are preferred by SBW. The hypotheses for each experiment were: (1) second-instar larvae disperse more from resistant trees than from susceptible trees, (2) sixth-instar larvae choose to eat susceptible needles more often and in higher quantities compared to resistant needles, (3) SBW female moths choose to lay more eggs and more egg masses on susceptible branches than

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on resistant ones. Foliar chemistry was analyzed for each study tree to correlate with budworm behavior.

General methodology

The trees used in the experiments were selected by assessing the level of defoliation to determine their type as susceptible or resistant. All foliage was collected in the field during spring and summer of 2010 and 2011. The selected trees were used for all of the following experiments. Thirteen trees were used (6 susceptible and 7 resistant) in 2010 and 25 trees (13 resistant and 12 susceptible trees), including the 13 from 2010, in 2011. The branches collected had their cut ends placed in containers of water and were stored at 5º C for a maximum of two days before being used in experiments.

Caterpillars were obtained as diapausing second-instar larvae from the Forest Pest Management Institute, Sault Ste Marie, Ontario, Canada and were stored at a temperature of 5°C until used. Each larva was reared on foliage from a single tree in an incubator under a 16L:8D photoperiod at 22°C and 60% relative humidity.

In 2010, all the trees were used as rearing trees. Each insect used in the different experiments was tested on trees that excluded their rearing tree. In 2011 however, in order to reduce the variables, we used only 1 resistant tree and 1 susceptible tree as rearing trees.

The larvae used for the dispersal experiment were maintained on the same tree they had been tested on until sixth instar. Some of them were used in the oviposition experiment (but not in the feeding choice test) and tested on trees chosen randomly excluding their rearing tree.

All the data generated during the different experiments were analyzed using the

software R (R Development Core Team, 2008).

Chapter 1: Spruce budworm second instar larvae dispersal

Introduction

Outbreaks of the spruce budworm occur at irregular intervals of approximately 30-40 years (Royama, 1984; Jardon *et al.*, 2003). These outbreaks can have a significant impact on the structure and composition of forest stands (Nealis and Régnière, 2004). Several behaviors are associated with the success and survival of the spruce budworm, one of which is dispersal. The most important dispersal event occurs during the moth stage where adults, mostly egg laying females, can travel up to 600 km (Dobesberger *et al.*, 1983). At smaller spatial scales, some sort of dispersal occurs during all stages of the budworm's life cycle.

Field studies during the latest outbreak (Nealis and Régnière, 2004; Royama *et al.*, 2005; Régnière and Nealis, 2007) confirmed Royama's (1984) hypothesis that population densities fluctuate over large regions synchronously, irrespective of local tree mortality. Nevertheless, Régnière and Nealis (2007) suggested that larval dispersal was responsible for decreasing early-stage budworm larval survival over the length of an outbreak. Thus, this phenomenon plays a sizeable role in the dynamics of budworm populations.

Larval dispersal occurs mainly at two of the six instars the larvae experience in

their development (Beckwith and Burnell, 1982). In the summer, just after hatching, the first instar larvae (L1) seek a suitable site to make their hibernacula. The fact that the eggs are laid in masses of 15-20 eggs, and hatching occurs in a short period of only a few hours (Régnière, 1987) can lead to a high level of competition with kin to find an overwintering site. The emerging larvae descend on silk threads and are redistributed within the canopy, mostly to the interior and middle of the crown (Moody and Otvos, 1980; Régnière & Fletcher, 1983). This dispersal event takes place only over a short distance and has to occur rapidly because larval mortality increases rapidly as temperature decreases (Han *et al.*, 2000).

The second dispersal event occurs right after emergence of the larvae in the following spring before budbreak. At this time, larvae temporarily mine old needles (Trier and Mattson, 1997) or feed within pollen cones (when available) before finally penetrating fresh buds (Blais, 1952). This results in a high displacement rate of larvae seeking a feeding site into the crown of the host tree but also between crowns of different trees. These movements are associated with highly variable losses and are difficult to measure in a forest setting (Miller, 1958; Morris and Mott, 1963; Royama, 1984).

The present chapter tests two different apparati for measuring second instar larval dispersal and compares dispersal rates from susceptible and resistant white spruce trees.

Methods 2010

The insects were taken out of diapause on the day of the experiment and placed at ambient room temperature (20°C) for 2 hours before the experiment. One branch, measuring 10 cm in length, had its cut end placed into an Eppendorf tube filled with water to keep the needles fresh for the duration of the experiment. This tube was attached to a retort stand, 15 cm from the base. This stand was placed on a 25x15 cm tray filled with water to a depth of 2 cm. This was in order to prevent any larvae that dispersed from the branch from leaving the tray. Ten caterpillars were placed on each branch and branches from all 13 trees were tested simultaneously. This experiment was conducted outdoors on the Loyola campus of Concordia University (between the 7th and the 21st of May 2011), on four sunny days, from the morning to the late afternoon to maintain a relatively consistent natural light and temperature pattern. Every hour, trays were checked for dispersing larvae. Each tree was tested four times over the course of the experiment.

Figure 1: Experimental set-up to test dispersal activity during the summer of 2010

After 6 hours, surviving caterpillars were reared to pupation stage on the foliage from the tree they were tested on for use in the two experiments described below. Pupal weight was monitored on all 13 rearing treatments.

The final number of larvae which dispersed at the end of the experiment between both foliage types was compared using an unpaired Student's t-test. The effect of foliage type, time, tree and replication on the dispersal was tested using a Poisson regression.

Results 2010

The number of second-instar SBW larvae dispersing varied considerably between the different trees within each foliage type. Despite a tendency for more ballooning from resistant trees when comparing the mean number of dispersing insects from each foliage type (Fig. 2), our results did not show any significant difference in the final number of larvae which dispersed between foliage types (t-test, t = 1.5517, df = 11, p-value = 0.1490).
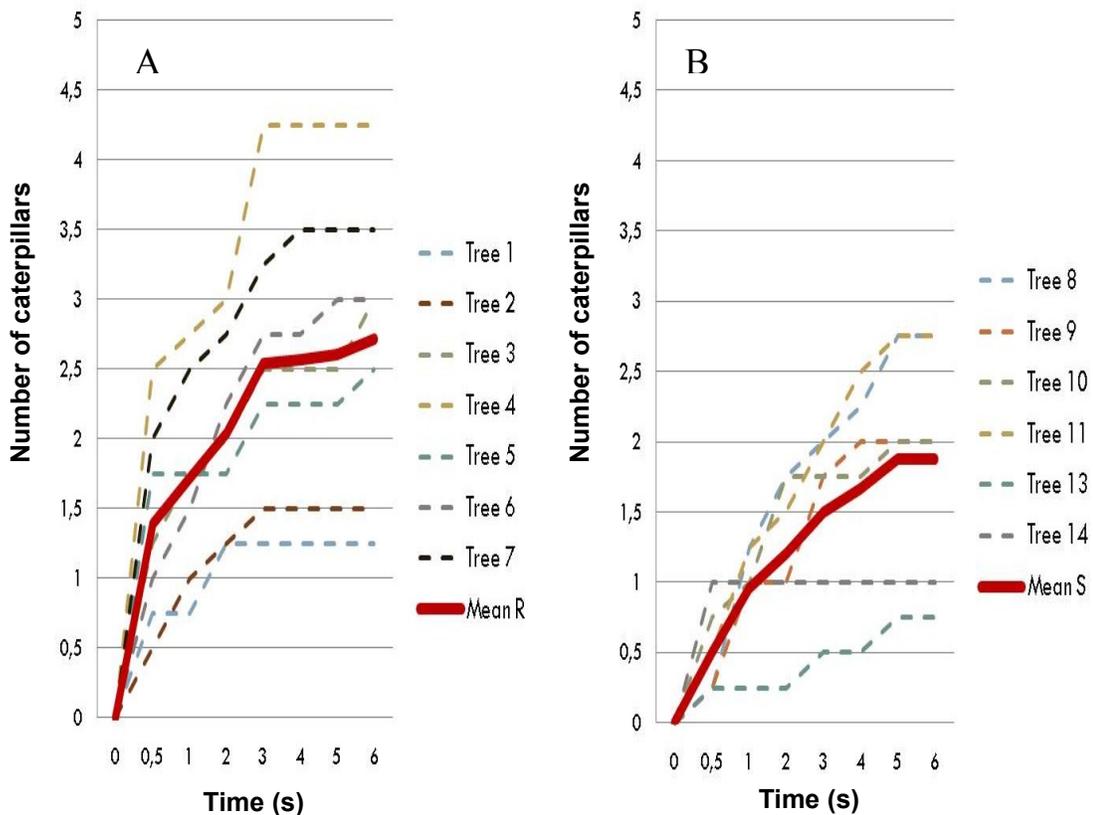
Figure 2: Mean (between the four different dates on which the experiments were done) number of caterpillars dispersing from resistant trees (A) and susceptible trees (B) over time. Each dashed line represents one tree; the red line represents the mean of all the trees of one type.

A Poisson regression revealed a significant effect of both the foliage type and the time on the number of larvae dispersing whereas the effect of tree and replication (the different dates on which the experiments were conducted) on dispersal was not significant (Appendix 1 (A)).

We did not find any consistent pattern in dispersal rate from any of the trees between the different dates on which the experiments were performed. Fig. 3shows results from 4 of the 13 trees.
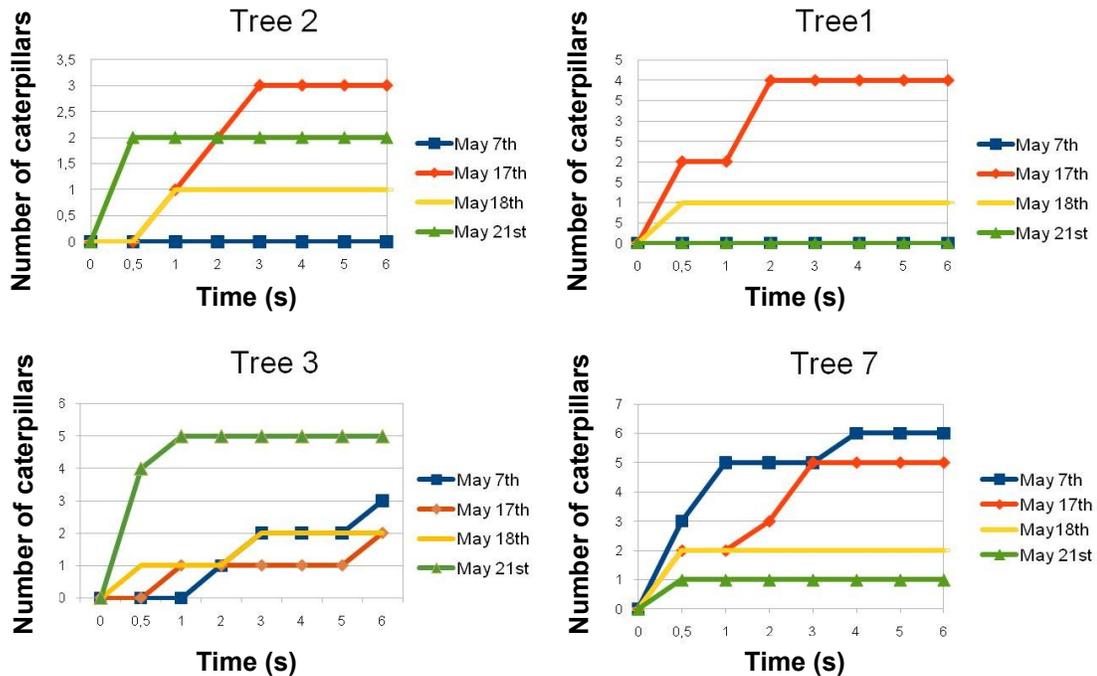
Figure 3: Comparison of numbers of caterpillars dispersing from each tree on the different dates on which the experiments were performed. There was no pattern or clear effect of a given date on any of the 13 trees tested so data from only 4 trees are presented.

Methods 2011

After assessing the results we obtained in the summer of 2010, we decided to change the experimental set-up for the summer of 2011. External conditions, even if they best represent what happens in nature, had too many variables. We believe that the wind could have dispersed some larvae farther than the limits of the tray we used to quantify the dispersal and so have removed few larvae from the system. Moreover, it is likely results in this setup are dependent on the weather conditions and we were able to conduct our experiment only when the weather was suitable.

We then decided to use closed boxes (Fig. 4) equipped with a light in the lid section, large enough to accommodate 10 branches. The light in each box is separated by a pane of thermal glass from the rest of the box to minimize any change in temperature. For a similar reason, a fan was fitted in the lid alongside the light. Four holes in the boxes allow air circulation without wind. The bottom of the box was lined with double sided tape to capture the dispersing larvae. In each box we placed 7 branches from each type of foliage, each coming from a different tree. There was enough space between each branch so that without wind the dispersing larvae fell directly on the area under the branch they were placed on. The boxes were placed in a climate-controlled room to maintain a relatively constant temperature and humidity level and the lights in the boxes were kept on during the experiment. Experiments were conducted between May 15th and 23rd 2011.

The 10 cm long branches were placed randomly in each box. Each of them had their cut end placed into an Eppendorf tube filled with water to keep the needles fresh for the duration of the experiment. The top of each Eppendorf was covered with tree-

tanglefoot, an organic pest barrier effective against crawling insects, to prevent larvae escaping by the base of the branches. Compared to the summer 2010 experiment (described above), we used 5, rather than 10, larvae per branch to reduce the competition between them. In nature at the second instar, one dispersal event has already occurred and the larvae are more spread out than at the first instar when they have just emerged from the egg masses.

At the end of the experiment, all branches were removed from the boxes and each one of them was placed in an individual Petri dish sealed with parafilm. The Petri dishes were placed in a growth chamber at 20°c with a 16L:8D photoperiod. After 72 hours, we counted the number of larvae established on each branch. This delay allowed us to determine whether the larvae that did not disperse established successfully on the branch and were actually feeding on it.

We had 12 replicates for each tree which makes a total of 156 replicates for the resistant type of foliage and 144 for the susceptible foliage. We counted the number of dispersing larvae every hour for 8 hours. The final number of larvae which dispersed at the end of the 8 hours was compared between both foliage types using an unpaired Student's t-test. The effect of foliage type, time, tree and replication on the dispersal was tested using a Poisson regression.

Figure 4: Experimental set-up to test dispersal activity during the summer of 2011

Results 2011

Dispersal of larvae from the different trees varied both between and within foliage types (Fig. 5). We found no significant differences in the final number of larvae dispersed at the end of the experiment between the two foliage types (t-test: $t = 1.2024$, $df = 314$, p-value = 0.2301).
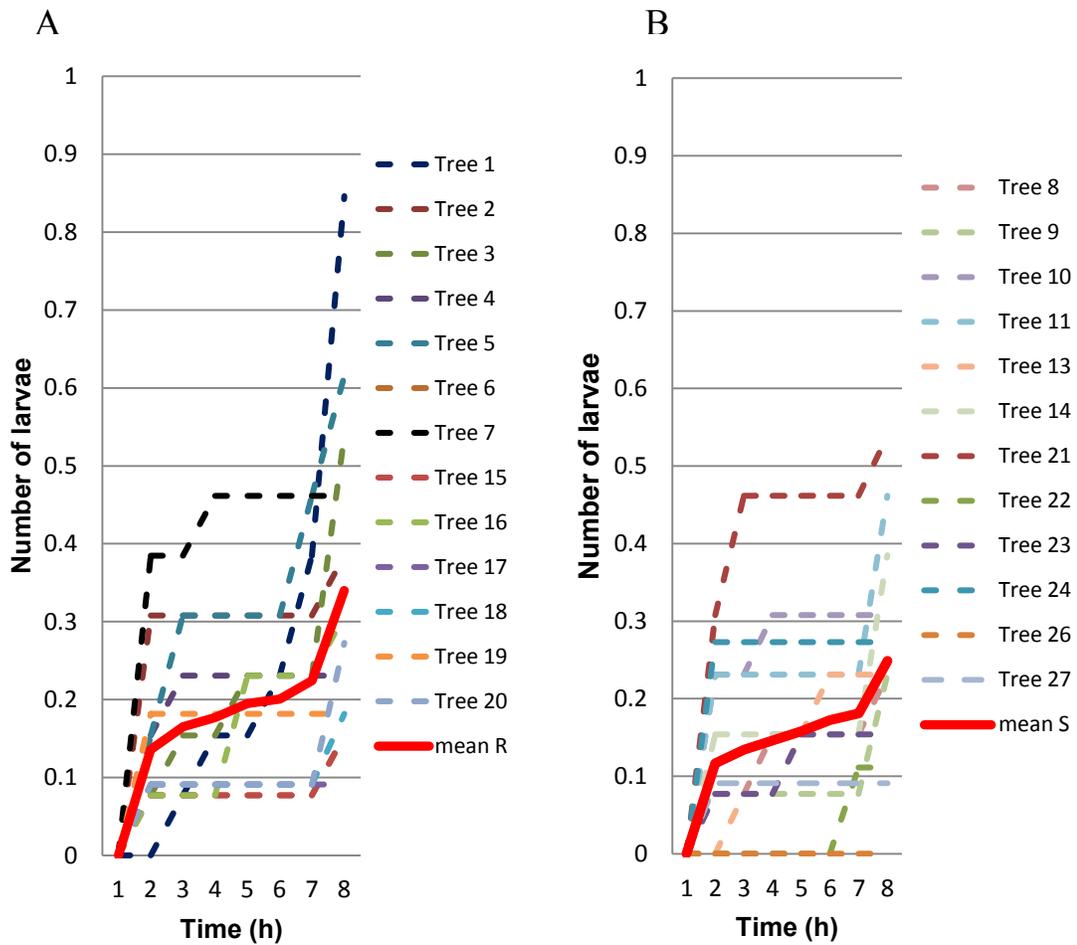
Figure 5: Mean number (between the different replicates) of caterpillars dispersing from resistant trees (A) and susceptible trees (B) over time. Each dashed line represents one tree; the red line represents the mean of all the trees of one type.

The Poisson regression showed that the effect of foliage type on dispersal was not significant whereas time, tree and replication had significant effects (see Appendix 1 (B)).

Discussion

Both years' results show a slight tendency for greater dispersal from resistant trees than from susceptible ones (Fig. 2 and 5). The 2010 experiment demonstrated a significant effect of the foliage type on the number of larvae dispersing. This result suggests that early stage larvae were able to distinguish between two conspecific hosts of different quality. By increasing the number of replicates and removing the effect of the environment in the 2011 experiment, we still found significant differences in dispersal between individual trees, although these did not always correspond to the trees' classification as susceptible or resistant. This might be due to high variability, both between replicates for a given tree but also between trees for a given type of foliage (Appendix 1).

Moreover, it seems that the environmental conditions play an important role in the dispersal phenomenon. In the 2010 outdoor experiment, not only did we find a difference between foliage types but the dispersal observed was also higher than in the 2011 indoor experiment. By conducting the experiment outside on sunny days, we selected the ideal conditions for budworm dispersal which could have lowered their 'Risk of dispersal' (Nealis *et al.*, 2003). This is confirmed by Miller (1958) who found greater larval dispersal during the sunniest days and at the warmest hours of the day in an outdoor experiment.

These results suggest that second instar larvae do distinguish between the trees used in this experiment, but that the criteria they use do not always reflect those that confer the resistance to defoliation observed in the field. There is, to my knowledge no evidence that

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second instar larvae use chemical cues to direct dispersal. Régnière and Nealis (2008) showed that losses during this phase was linked to previous defoliation but did not investigate the proximal reasons that cause the larvae to disperse. This issue will be explored further in Chapter 5.

Régnière and Nealis (2008) demonstrated a density-dependent relationship between survival of early-instars and host tree conditions where defoliation-induced damage to the trees resulted in increased losses of spring-emerging larvae that are dispersing in search of feeding sites. Indeed, throughout the outbreak, the defoliation of the trees increases, which lowers the quantity of food available to the larvae. This process has direct consequences on the dispersal of emerging larvae where a higher defoliation of the host increases the dispersal of larvae (Régnière, personal communication). In our system, susceptible trees have higher SBW defoliation-induced damage than resistant ones (Bauce *et al.*, 2006). Therefore, the increased dispersal we were expecting from resistant trees due to their chemical characteristics could have been counter-balanced by a higher defoliation on the susceptible trees. Unfortunately, we did not measure defoliation on the trees we used for this experiment and cannot confirm this hypothesis.

On the other hand, budworms hibernating near host foliage disperse very short distances whereas larvae that hibernate in non-foliated portions of host trees must disperse to survive (Régnière and Nealis, 2008). In our experiment the insects were obtained from a commercial supplier so their parents had been fed on artificial diet. The larvae we used overwintered in artificial conditions but we can still expect the dispersal rate to be low because they are placed on suitable hosts.

Our results suggest that the resistant trees, which suffer less defoliation from SBW, can reduce their insect load by increasing dispersal of young larvae but that this resistance is likely to be linked to other processes such as environmental conditions. A smaller quantity of eggs laid on their branches by adult females and/or a higher mortality at other stages due for example to post-ingestive toxic mechanisms on the older larvae might also be responsible for the insect load reduction.

Chapter 2: Feeding choice test on sixth-instar larvae

Introduction

Food source quality has consequences for the whole life cycle of insects. It can affect their survival, growth rate, mating success and even the survival of their offspring (Noseworthy and Despland, 2006; Carisey and Bauce, 2002). Nevertheless, insects can compensate for low quality food sources, either physiologically (Despland and Noseworthy, 2006) or behaviorally (Simpson *et al.*, 1988). Here we focused more on the latter kind of compensation. Insects have been shown to increase the quantity of food they would ingest when the quality of the food source decreases (Simpson *et al.*, 1988; Lavoie and Oberhauser, 2004). This phenomenon, called compensatory feeding, has been observed in the SBW (Albert and Bauce, 1994; Toufexis *et al.*, 1996).

When selecting their food source, insects use a range of different cues including the chemical composition of plant tissue (Chapman 2003, Wright *et al.*, 2003). In our system there are known feeding behavior differences: larvae feeding on needles from resistant trees were shown to have fewer feeding bouts and shorter duration of meals than those on needles from susceptible trees. Also, the number of insects that transitioned from probing to feeding was higher (79% vs. 34%) on susceptible foliage than on resistant (Daoust et al., 2010). Transition from probing to feeding behavior is likely to be due to surface

chemicals since the needle is not pierced yet. Daoust et al. (2010) found more monoterpenes in the needles of the resistant trees compared to the susceptible ones and concluded that because of their volatility these chemicals should be absorbed into the epicuticular waxes and are therefore hypothesized to be the cues responsible for the differences in behavior observed. In this experiment, we hypothesized that when given a choice between resistant and susceptible foliage: (1) sixth-instar larvae should choose to eat more of the susceptible foliage; (2) larvae reared on resistant foliage should be more selective than the ones reared on susceptible foliage.

Methods 2010

Experiments were conducted between June 18th and 25th, 2010, the time of year when the foliage is the same as that which sixth-instar SBW feeds on in the field. A 2 cm branch of current year foliage from each type (S and R) was placed at opposite ends of a plastic arena 15 cm long and 3 cm wide. The arena edges were covered with petroleum jelly to prevent escape by the caterpillar. Eight plastic arenas (15 cm x 3 cm) were placed on two cylindrical rubber stoppers (approx 3 cm in height) to create 'bridges'. Eight bridges were placed side by side on a plastic tray (35 cm x 45 cm) and the tray was filled with water to a depth of several millimeters.

Naïve insects were reared on one of the 7 resistant or 7 susceptible trees until sixth-instar and food-deprived for 4 hours at room temperature in individual plastic cups before the experiment. Four sixth-instar caterpillars reared on susceptible foliage and 4 caterpillars reared on resistant foliage were placed on each tray. One caterpillar was positioned at the middle of each bridge, and all bridges were identified according to the rearing diet of the caterpillar. Each bridge had foliage from a resistant and a susceptible tree placed at opposite ends. For each replicate, two trees (one of each type) were chosen randomly but excluding the rearing tree of the insect.

The experiment was recorded for 20 hours using a Canon, GL2 Video Camcorder, 3CCD Camera System, 20X/100X professional fluorite lens, 1.7 mega pixels and recorded onto a computer using Virtual Dub software (1.5.10, 19998-3003, Avery Lee) set at 1 frame/second.

We used a consumption scale to rate the amount of foliage eaten by the

caterpillars, as follows: 0: did not feed, 1: started feeding but ate less than half the amount of foliage present, 2: ate more than half the foliage but did not finish it and 3: finished all the needles. We compared the following variables between the two foliage types in the assay: time to first contact and first foliage type contacted, index of consumption (range from 0 = intact to 3 = completely eaten) and number of contacts with each type of foliage. Rearing treatment (S vs R) was also included in the analysis.

The videos were analyzed using the software The Observer developed by Noldus. When we started to analyze the data, we discovered out that one of the cameras had malfunctioned, which reduced our number of replicates to 48 out of the 96 expected. Of the 48 larvae from which we actually have the videos, only 18 stayed on their bridge and fed for the duration of the experiment. The other larvae were able to get over the petroleum jelly and either died in the water or crawled up on to another bridge. Out of the 18 useable replicates, half of them had been reared on resistant foliage and the other half on susceptible foliage.

The data did not satisfy the conditions for normality so we used a Wilcoxon signed rank test to compare the consumption indices between foliage types. We performed student t-tests to compare the time before first contact and on the number of switches between foliage types. A chi-square test of independence was used to compare the proportion of larvae that contacted each type of foliage first. In total 18 caterpillars were tested.

Results 2010

The results, on the 18 replicates we have, do not show any difference in mean consumption of one foliage type over the other (W = 161, p-value = 0.9867) despite a tendency for higher consumption of susceptible foliage (Fig. 6). Considering the rearing type of foliage did not change the outcome of the test. Neither larvae reared on resistant (χ^2 = 0.287, p = 0.866), nor those on susceptible foliage (χ^2 = 0.350, p = 0.839) showed a departure from the expected equal consumption of both foliage types presented.
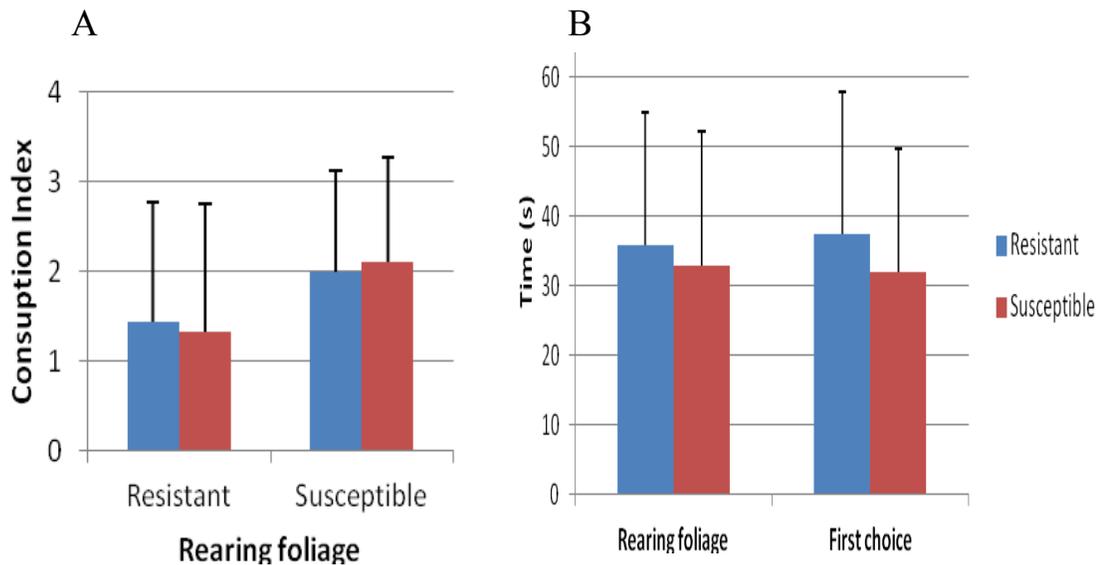
Figure 6: (A) Mean (+/- SE, type I) consumption index of each foliage type according to the rearing foliage. (B) Mean (+/- SE, type I) time before contacting the first foliage according to the rearing foliage and the first foliage type contacted. n=9 larvae reared on resistant, n=9 larvae reared on susceptible.

Combining both rearing diets, there was no difference between the two foliage types in the first choice made by the larvae (Chi² = 0.186, p = 0.911). Seven larvae out of the eight

which contacted the resistant foliage first had moved at least once to the other foliage in the first 10 minutes following that contact. In the same time period, 8 of the 10 larvae which contacted the susceptible foliage first had also switched to the other foliage type.

The time to first contact with one of the needles was not significantly different when comparing between the two rearing diets (t-test, t = 0.332, df = 16, p-value=0.744).

Methods 2011

We performed a similar experiment but in a different smaller, enclosed arena. The larvae were reared on foliage from the two rearing trees. Each larva was given a choice between two branches of different type of foliage selected among the 25 trees used in the behavioral experiments. The first experiment (in 2010) had a distance of 15 cm between the two food sources, in the second experiment we used large Petri dishes (15cm diameter) and set up a distance of 5 cm between the food sources. An external observer recorded the position of the larvae in each dish every 2 minutes for the first ten minutes, then every 5 minutes for the next 20 minutes, every 10 minutes for the following 30 minutes and every 20 minutes for the following hour and every hour for the next 5 hours and finally at 24, 32, 48 and 72 hours after the start of the experiment. The position of the insect was noted as on resistant, on susceptible or neither of them. Experiments were conducted between June 20th and 29th 2011.

We tested a total of 114 larvae in this experiment with an equal number reared on each foliage type, but we had to discard from the dataset the larvae that did not feed as well as the ones that started their metamorphosis before the end of the experiment. This reduced our final number of replicates to 33 larvae reared on resistant and 29 reared on susceptible foliage. As in the 2010 tests, we used: Wilcoxon signed rank test for the consumption indexes, student t-test for the time before first contact and the number of switches, and a chi-square test of independence for the proportion of larvae that contacted each type of foliage first.

Results 2011

We did not find any statistically significant difference in mean consumption indices of the two foliage types (W = 141, p-value = 0.387) (Fig. 7). Including the rearing foliage in the analysis did not make any difference (larvae reared on resistant: W = 72, p-value = 0.297; larvae reared on susceptible: W = 94, p-value = 0.896).

In both years, the larvae reared on susceptible foliage had a higher average consumption index than the larvae reared on resistant foliage (2010: 1.39 vs. 2.06; 2011: 1.56 vs. 1.88) but this was not significant in either of the two years.
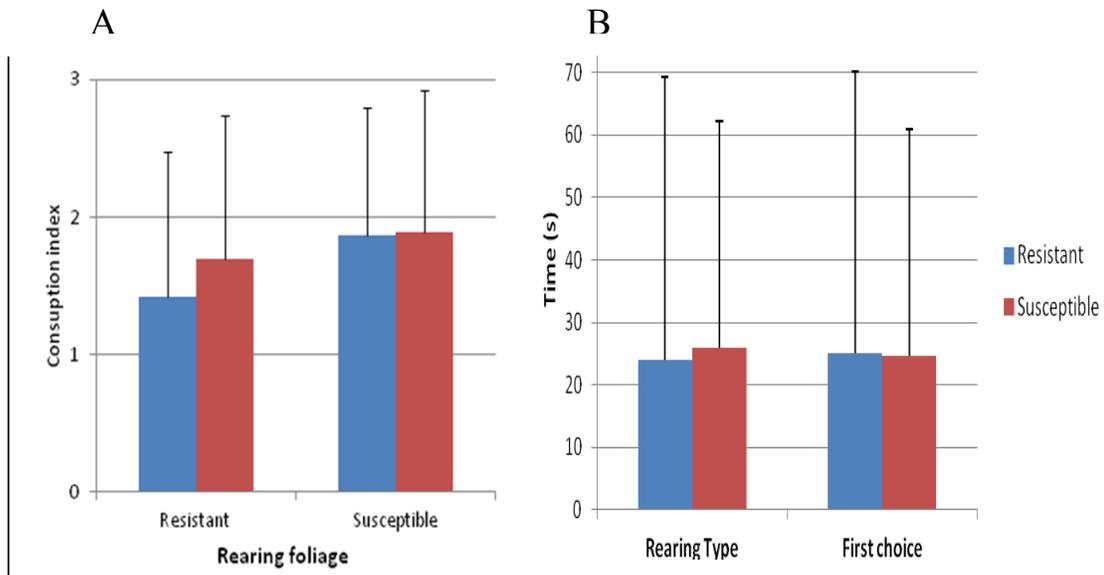

Figure 7: (A) Mean (+/- SE, type I) consumption index of each foliage type according to the rearing foliage. (B) Mean (+/- SE, type I) time before contacting the first foliage according to the rearing foliage and the first foliage type contacted. n=33 larvae reared on resistant, n=29 larvae reared on susceptible.

Of the 114 larvae tested, 50% chose the susceptible foliage first. No difference was noted when taking into account the rearing foliage type. Of the larvae which contacted the resistant foliage first, 75% of them had switched from their first choice to the other foliage at least once 10 minutes after the start of the experiment. This proportion was 63% for the larvae that contacted the susceptible foliage first. This difference was not significant (χ^2 = 3.70, p = 0.157). The average total number of switches observed was not different between the two rearing foliage types (1.71 for susceptible reared and 1.79 for resistant reared, t-test: t = 0.827, df = 61, p-value = 0.713); nor did it differ according to the foliage contacted first (1.80 after contacting susceptible first, and 1.68 for those that contacted resistant first, t-test: t = 0.827, df = 61, p-value = 0.512). There was no correlation between the first choice and the number of switches (Pearson correlation coefficient: r = -0.06).

The larvae reared on resistant foliage found the food source faster than the ones reared on susceptible foliage (25.07 vs. 46.15 seconds) but not significantly so (t-test, t = 0.612, df = 14, p-value = 0.550).

Discussion

Previous work indicates that SBW can compensate when subjected to low quality food by increasing their consumption (Albert and Bauce, 1994) which is the reason why we expected budworms reared on resistant foliage to behave differently in their choice than larvae reared on susceptible foliage. However, this effect was not observed.

Previous studies in the same plantation found no differences between the two foliage types in phagostimulatory power of internal leaf contents when evaluating meal duration and probing behavior when using different types of leaf extracts (S and R) in a choice test (Daoust *et al.*, 2010; Despland *et al.* 2011). The present experiment confirms that there do not appear to be differences in phagostimulatory power between resistant and susceptible foliage. These results are in agreement with Mader *et al.* (2012) who found no difference in the number of feeding events and pauses between meals by sixth instar spruce budworms between the two types of foliage in a no-choice assay.

Subtle behavioral differences were nonetheless observed between these foliage types. Daoust *et al.* (2010) highlighted what could be considered the first line of defence of the resistant trees: on resistant needles, fewer larvae transitioned from probing to feeding which resulted in fewer feeding bouts and a shorter first meal duration. After removing the epicuticular waxes of the resistant trees, which contain high levels of monoterpenes, this deleterious effect on the larvae decreased. In parallel, intermeal intervals have been shown to be twice as long on the resistant foliage leading to lower food consumption by the larva (Despland *et al.*, 2011) which is consistent with our results. This result suggests a post-ingestive second line of defense which might be due to

the presence of two phenolics, pungenol and piceol, which have been identified in the resistant but not in the susceptible white spruce. They have been shown to reduce growth and development of budworm as well as increasing mortality (Delvas *et al.*, 2011). Neither of these defense mechanisms would necessarily be detected in the choice experiment presented here.

Most of the previously cited work which exhibits differences between the two foliage types has been done using leaf extracts from a few extremely different trees and/or larvae fed on artificial diet. Larvae reared on artificial diet have been shown to have completely different patterns of feeding than those reared on foliage (Ennis, Unpublished data) which is why we decided to rear our insects on foliage and test them on non-manipulated foliage from many different trees.

Our results confirm previous work showing little or no difference between budworm feeding behavior on susceptible and resistant trees. The subtle effects documented by Daoust *et al.* (2010) and Despland *et al.* (2011) would not show up as clearly in the choice assay used here as in the long-term no-choice observations made in those studies. These results suggest that the use of resistant trees in future plantation may increase the yield of these plantations due to a higher mortality in the budworm population from selective pressure exerted by the resistant trees.

Chapter 3: Oviposition preferences of adult spruce budworm are influenced by past larval experience

Introduction

The specific oviposition preferences exhibited by insects may be genetically determined and/or based on larval conditioning. In the latter case, when selecting her oviposition site the female has two choices according to what she experienced as a larva: choosing the foliage on which she developed (Hopkins' host selection principle, Hopkins, 1917) or avoiding it if it contained noxious compounds (Mader *et al.*, 2012)

Female spruce budworm usually emerge in the late afternoon and start signaling for a mate at dusk by emitting odor plumes (Mader *et al.*, 2012). Odor plumes attract adult males and cause them to disperse within the stand to find a mate (at the 100 meters scale). They mate only once and disperse the following day in the stand to lay their eggs (100 to 200 in total) in several clusters on different needles. Both adults are strong flyers and can sometimes undergo long-range dispersal (Saunders, 1987).

Previous work (Honek, 1993) has shown that female pupal mass is a good predictor of fecundity. In our system, Bauce and Kumbasli (2007) have shown that females reared on resistant trees in the field were heavier than females reared on susceptible trees at the pupal stage.

The female spruce budworm usually oviposits in late July and early August

(Morris and Mott, 1963). First-instar larvae are known to graze the surface of the needle and ingest some epicuticular waxes (Retnakaran *et al.*, 1999) but most of the feeding occurs during the following spring after second instar larvae have overwintered. Spruce budworm larvae have limited mobility, and dispersal of larvae is associated with high losses (Régnière and Nealis, 2008). Therefore the female is making an important choice when selecting her oviposition site and the better the food source she chooses, the greater will be the survival of her progeny.

To make this decision the female uses visual, tactile and olfactory cues as well as chemicals present in the foliar waxes. Prior to laying, females tap the substrate with pro- and mesothoracic legs. The sensilla on the tarsi and on the ovipositor are likely to detect chemicals in the waxes (Wallace *et al.*, 2004). Several monoterpenes present in white spruce foliage have been shown to stimulate spruce budworm oviposition on neutral substrates (Grant *et al.*, 2007). Egg laying females are able to discriminate between several oviposition substrates (Rivet and Albert, 1990) and when given a choice between several acceptable hosts such as balsam fir and other spruce species, white spruce was the most preferred (Wallace *et al.*, 2004, Städler, 1974).

We investigated if the female SBW moth is able to discriminate between two hosts of different quality within the same species and if their experience as larvae influenced this preference.

Methods

We followed the protocol described in Wallace *et al.* (2004) to mate the adult SBW. The matings were performed within tree type between one to five days after metamorphosis. The copulating couples were removed from cages and the females were used the following day. Each female was placed in a 473 ml plastic container (Solo Cup Company, Urbana, Illinois) in which 2 cm long branches of previous year foliage from both tree types and a free water source had been placed. For each replicate, two trees (one of each type) were chosen randomly but excluding the rearing tree of the insect. Experiments were conducted between July 3rd and 12th in 2010 and July 5th and 15th in 2011.

Female behavior was recorded by an external observer. To perform this choice experiment, we made sure that all females contacted both types of foliage prior to laying. We recorded the number of eggs and the number of masses laid on each type of foliage. Each egg mass laid represented a separate oviposition event and so a separate choice made by the laying female. The number of masses laid was used to determine if the females were choosing one of the two foliage types more often than the other.

The data for the number of eggs and the number of masses laid did not satisfy the condition for normality due to a high number of females laying only on one type of foliage. We therefore used a Wilcoxon ranked test for paired data to investigate any difference in the number of eggs laid between the two foliage types. Finally, female pupal masses and fecundity were compared between the two rearing foliage types using a student t-test.

Results

Summer 2010

Overall, females showed a strong preference for the susceptible foliage when laying their eggs (2.68 times more eggs on S than on R, Wilcoxon test: W = 246.5, p-value = 0.003). This difference is due to the females reared on resistant foliage which laid on average 5.47 times more eggs on susceptible than on resistant foliage (Wilcoxon test: W = 217, p-value = 0.012) (Table 1). There were no significant difference for females reared on susceptible foliage (Wilcoxon test: W = 187.5, p-value = 0.094) despite a tendency for more eggs on susceptible foliage (1.87 times more). Thus, females reared on resistant foliage laid a greater proportion of their eggs on susceptible foliage compared to females reared on susceptible foliage (Fig. 8 A).

Table 1: Mean number of eggs laid on each type of foliage by females reared on susceptible and resistant foliage in the 2010 experiment.

		Rearing foliage		
		Resistant	Susceptible	Total
Laying	Resistant	7.64	26.54	34.18
foliage	Susceptible	41.83	49.85	91.68
	Total	49.47	76.39	125.86
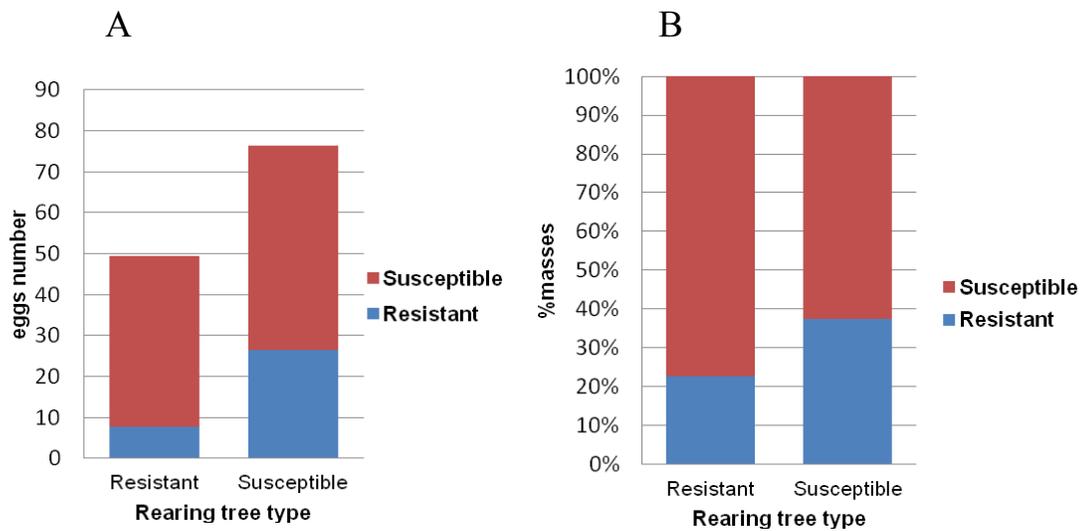
Figure 8: Mean of total number of eggs laid (A) and proportion of total number of masses (B) laid by females from each rearing tree type on each kind of foliage

Overall, the females lay more egg masses on susceptible foliage than on resistant foliage (Wilcoxon test W = 269.5, p-value = 0.01551) (Fig. 8 B). Only 12% (3/25) of the females tested failed to lay any eggs on the susceptible foliage whereas 40% (10/25) failed to lay eggs on the resistant foliage (χ^2 = 11.04, p-value = 0.004).

We observed a significant difference in mean pupal mass of females reared on the two foliage types (Females reared on resistant = 56.34 mg, n = 56; reared on susceptible = 48.18 mg, n = 64; t-test, t = 12.805, df = 1, p-value = 0.04961). However, of the females used in the oviposition experiment, we found that females reared on resistant foliage had lower fertility thanfemales reared on susceptible foliage (Table 1) (resistant reared: 49.47 eggs/female, n = 12; susceptible reared: 76.39 eggs/female, n = 13) but this result was not significant (t-test: t = -1.7243, df = 22.887, p-value = 0.09813).

This result would suggest that despite a bigger mass which is supposed to enhance

their fertility, females reared on resistant foliage suffer reduced fitness from being fed on resistant foliage. Another possibility is that the eggs laid during the experiment did not represent the female's full fecundity and that some remained within the ovary. This possibility was examined in the following field season.

Summer 2011

We increased the number of replicates (n=76 females tested: 30 reared on resistant foliage and 46 reared on susceptible) compared to 2010 where we tested only 25 females (12 reared on resistant and 13 reared on susceptible).

We found no significant difference in fertility (t = -0.1659, df = 59.104, p-value = 0.8688) despite a slight difference in the average number of eggs laid (70.02 eggs laid by females reared on resistant foliage (n=30) vs. 71.69 eggs laid by females reared on susceptible foliage (n=46)) (Table 2). We dissected the abdomen of 10 females chosen randomly (5 reared on each type of foliage) and found only one egg in one of them, confirming that the number of eggs laid does represent total fecundity. This is reinforced by the fact that the pupal weight of the females does not differ between the two rearing foliage types (resistant reared= 50.18 mg, n = 177; susceptible reared= 48.35 mg, n = 192; t-test: t = 1.049, df = 294.571, p-value = 0.295).

Overall, the females exhibit a preference for the susceptible foliage over the resistant (mean=31.70 eggs/female laid on resistant foliage vs 39.35 eggs/female laid on susceptible foliage) (Fig. 9). This preference was significant (Wilcoxon test: W = 248.5, p-value = 0.002) among the females reared on resistant foliage who laid more eggs on susceptible foliage, whereas no difference was observed among the females reared on

susceptible foliage (Wilcoxon test: W = 1051.5, p-value = 0.691).

Table 2: Mean number of eggs laid on each type of foliage by females reared on susceptible and resistant foliage in the 2011 experiment.

		Rearing foliage		
		Resistant	Susceptible	Total
Laying	Resistant	24.56	36.25	60.81
foliage	Susceptible	45.46	35.44	80.90
	Total	70.02	71.69	141.71

There was no significant difference in the mean number of masses laid on each type of foliage despite a tendency for more masses on susceptible foliage (3.72 masses on resistant vs 4.38 masses on susceptible, Wilcoxon test: W = 2715, p-value = 0.5215). No significant differences in number of egg masses on each foliage type appear when splitting the analysis by rearing foliage (3.53 masses on resistant vs. 5.13 masses on susceptible from females reared on resistant, Wilcoxon test: W = 373, p-value = 0.2527, 3.84 masses on resistant vs. 3.89 masses on susceptible from females reared on susceptible, Wilcoxon test: W = 1080.5, p-value = 0.8625).
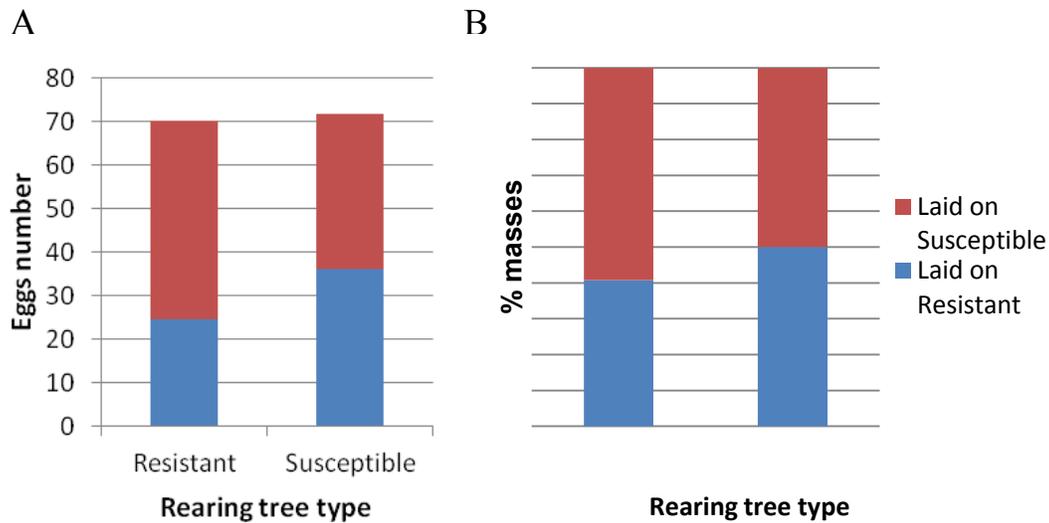
**Figure 9: Mean of total number of eggs laid (A) and proportion of total number of masses (B) laid on each rearing tree type on each kind of foliage**

Nevertheless, when examining the mean number of eggs per mass laid by the females we found that overall the females chose to invest more eggs per mass on susceptible foliage compared to resistant (Wilcoxon test: W = 1983.5, p-value = 0.055). This difference is due to the females reared on resistant foliage which laid on average 40% more eggs in each mass laid on susceptible foliage compared to the ones laid on resistant foliage (Wilcoxon test: W = 257, p-value = 0.029). The females reared on susceptible foliage showed no difference in the number of eggs per mass on any of the two foliage types (Wilcoxon test: W = 788, p-value = 0.4124).

Of the total number of females tested 3.8% of them failed to lay any eggs on susceptible foliage whereas 14.2% did not lay any eggs on resistant foliage (Chi² = 8.94, p-value = 0.03). Similarly, 76% of the females reared on resistant foliage invested more eggs on susceptible foliage than on resistant compared to 54% for the females reared on susceptible foliage (Chi² = 14.79, p-value = 0.002). Overall, these results are consistent

with those from 2010.

There is a strong positive correlation between the female pupal mass and the fertility, using the data from both years combined (Pearson R = 0.689 (t = 4.5566, df = 23, p-value = 0.0001) for females reared on resistant foliage and Pearson R = 0.828 (t = 7.0819, df = 23, p-value <0.0001) for females reared on susceptible foliage) (Fig. 10 A). The slope of the two best fit lines is similar: equation for S reared is y=2.11x -60.92 compared to R reared y=1.91x -51.49.

The correlation between the female pupal mass and the proportion of eggs laid on susceptible foliage can be seen as an index of selectivity for each female. These correlations are negative meaning that when the pupal mass increases the selectivity of the female decreases (Fig. 10 B). This relationshipis significant for females reared on susceptible foliage (Pearson R = -0.553 (t = -3.1834, df = 23, p-value = 0.004)), but not for those reared on resistant foliage (Pearson R = -0.164 (t = -0.7996, df = 23, p-value = 0.432)).
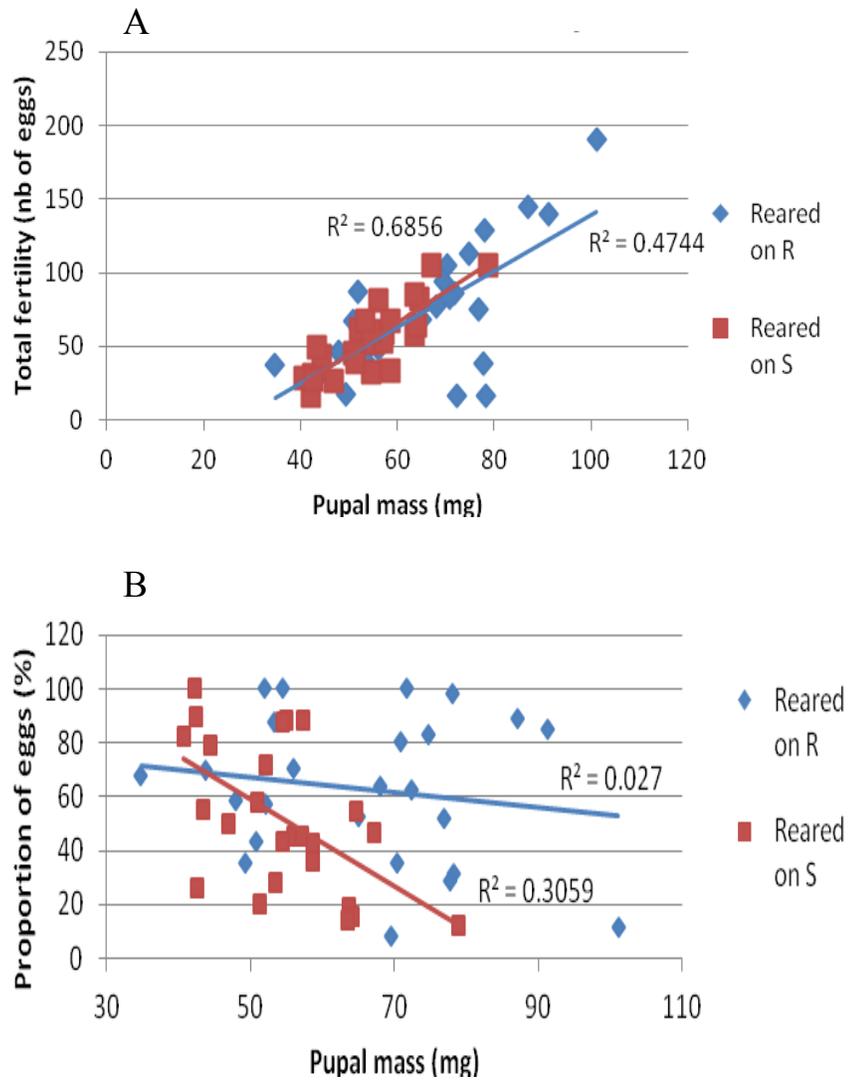
Figure 10: (A) Correlation between female pupal mass and fertility for moths reared on resistant and susceptible foliage from both years combined. (B) Index of selectivity: correlation between female pupal mass and proportion of eggs laid on susceptible foliage for moths reared on resistant and susceptible foliage from both years combined.

Discussion

Oviposition behavior was analyzed to determine if females were able to discriminate between intraspecific differences in host quality. The females could have behaved in four different ways in this experiment: (1) no preference shown between the two types of tree, (2) female spruce budworm select one type of host over the other no matter what they experience as larvae, (3) females preferentially lay on the foliage type they experienced as larvae (Hopkins host selection principle (HSP)), or finally (4) the choice made by the female is determined by what she experienced as a larva: the adults reared on resistant foliage recognize a less suitable host and chooses the opposite.

The results from both years demonstrate that female spruce budworm moths modified their choice according to their experience as larvae. The adults that were fed on resistant foliage preferentially selected the susceptible foliage to lay most of their eggs whereas the females reared on susceptible foliage did not show any preference between the two foliage types.

Our results show that larval nutritional experience has an impact on adult behavior and it seems that this acts in an adaptive way rather than following Hopkins HSP. The resistant trees have been shown to increase mortality of spruce budworm (Bauce *et al.*, 2006) and adult females that had been reared on them seem to be able to recognize them as a less suitable host. The resistant foliage contains more tannins, monoterpenes and simple phenolics which have been suggested to explain, at least in part, the tree resistance (Daoust *et al.*, 2010, Delvas et al 2011). It seems likely that memory of these compounds experienced as larva helps the adult to detect and discriminate against the resistant

foliage.

Monoterpenes in the wax layer have already been shown to diminish oviposition of other insect species (Muller and Riederer, 2005, Städler, 1986). In our system, however, it seems that previous experience is needed to cause a preference in the adult between two conspecific hosts with different monoterpene profiles. Indeed, no preference was noticed in the females reared on susceptible foliage when selecting a substrate to lay their eggs on.

We found similar results to Mader *et al.* (2012) who carried out an analogous experiment. Compared to their protocol, we almost doubled the number of adults tested and used 25 different trees when they used only the most susceptible and most resistant trees, and still had a strong evidence for a choice in favor of the susceptible foliage for the females previously reared on resistant foliage.

We showed no difference in female pupal mass or fertility between the two rearing diets, contrary to previous work showing that insects captured in the field on resistant trees had higher pupal mass (Bauce and Kumbasli 2006). This discrepancy might be due to differential mortality occurring in the field, but not under the more favourable laboratory conditions. We also show that larger females show a less strong preference for susceptible foliage, and appear to exhibit bet-hedging by spreading their eggs more among the two hosts available to them.

Chapter 4: Do differences in monoterpene profiles contribute to budworm defoliation resistance in white spruce?

Intoduction

White spruce is one of Canada's most commercially important tree species (OMNR, 1995). Trees can grow up to 40 meters tall and have trunk diameter of up to one meter. They possess long, needle-shaped leaves with a thick and complex epicuticular wax layer (Campbell and Reece, 2002). The surface of the needle represents a highly complex environment with waxes composed of a mixture of long-chain aliphatic and cyclic compounds (Muller and Riederer, 2005). The concentration of secondary metabolites in the waxes is of major importance for host-plant discrimination by insects (Woodhead and Chapman, 1986) as well as for plant protection against herbivory. The defensive compounds produced by the plants are usually grouped into two different categories: toxic compounds and digestibility-reducing compounds (Chown and Nicolson, 2004) to which we could add a third: the phagodeterrent compounds.

In our system, higher concentrations of some tannins and monoterpenes are present in the resistant trees than in susceptible trees (Daoust *et al.*, 2010). Moreover, Daoust *et al.* (2010) found a deterrent effect of the epicuticular wax of resistant trees on

sixth instar larvae, suggesting that the waxes play a role in the defense process of these trees. As dispersal and oviposition choices are made without biting and therefore depend on surface cues, this monoterpene difference might play a role in the dispersal of young larvae and the oviposition preferences of the adult females.

Conifers contain and produce monoterpenes for their defense but we know that the composition of the different monoterpenes and their concentration vary in relation to insect herbivory and in response to stress such as drought (Cates and Redak, 1988). Even though many studies have shown that most resistant (and susceptible) trees in a given year remained resistant (and susceptible) the following years, they have also shown that the monoterpenes that conferred the resistance changed between years (Cates and Redak, 1988; Sadof and Grant, 1997). We analyzed the needles of 25 trees from our system to quantify their monoterpenes and have also analyzed the waxes of these trees to see if we could find any noticeable difference in the chemical makeup of these trees with regards to resistance to the spruce budworm.

Methods

Chemical analyses of the foliage of our trees were performed in the laboratory of Eric Bauce (Université Laval, Québec, QC, Canada) in February 2011 and 2012. We first analyzed the whole foliar chemistry using the same protocol as in Daoust *et al.* (2010). These results gave us the chemical concentration of the whole needle for foliage collected at 4 different dates (May 5th and 18th, June 14th and July 12th 2010 and May 7th and 20th, June 15th and July 12th 2011). Each date corresponds respectively to the presence of second, fourth and sixth larval instar caterpillars and adults of the spruce budworm.

We then analyzed the epicuticular waxes, from foliage collected on date, modifying the protocol developed by Albert and Parisella (1992) to extract epicuticular waxes. Fifty needles were put into 1 ml of hexane-tetradecane (1l-120µl) solution for 30 seconds under Vortex agitation. This solution was then analyzed using a Varian model 3900 gas chromatograph equipped with a flame ionization detector and a SPB-5 fused silica capillary column (30 m ×0.25 mm) (Varian, Inc., Palo Alto, California, USA). The needles used were then dried in an incubator and weighed to estimate the amount of waxes analyzed.

Using both analysis methods allowed us to compare the distribution of the monoterpenes in both the needles and the waxes. If the monoterpenes play a role in repelling insect at non-feeding stadia, those monoterpenes will have to be contained in the waxes. To increase the power of our analysis we almost doubled the number of trees used in the second year of the experiments: In 2011, we used the same trees as in 2010 to investigate if there were any variations in the chemical concentrations of the different

compounds and we added 12 more trees.

These results allowed us to assess if any differences observed in the behavior of the insect between different trees can be correlated with the changes in the chemical make-up of these trees.

To counter the high correlation observed between the various monoterpenes (see Appendix 1), we chose to analyze them with principal component analysis (PCA). PCA is a multivariate technique that analyzes a data table in which observations are described by several inter-correlated quantitative dependent variables. It is used to extract the important information from the table in order to represent it as a set of new orthogonal variables called principal components. We can then use this new reference to display the pattern of similarity of the observations and of the variables as points and arrows in maps. Then we used a logistic regression to test the probability of a tree to exhibit resistance, based on its monoterpene content. We used the first three components from the two PCAs as explanatory variables in the regression, to avoid the correlation between the different monoterpenes.

Results 2010

The total concentration of monoterpenes increases gradually in the needles of the resistant trees with time whereas it decreases in the waxes (Fig. 11). Even if the general pattern is similar on the susceptible trees, the fluctuations with time are smaller.
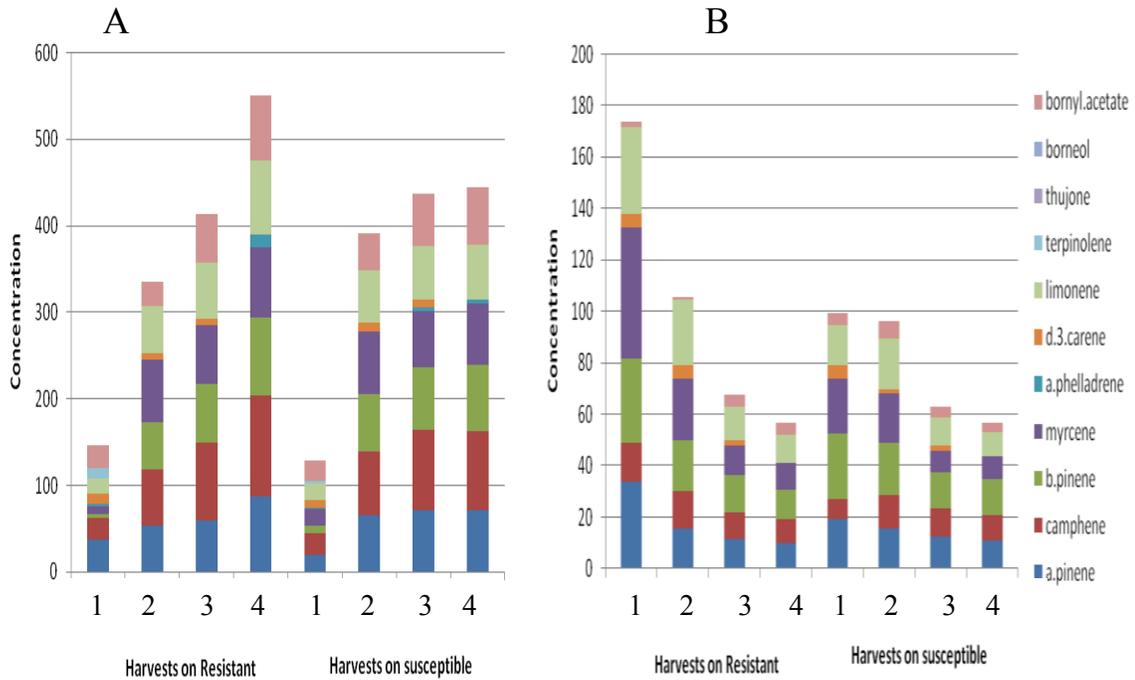
Figure 11: Concentration (ng/mg) of monoterpenes at four dates during the growing season 2010: Concentration in the needles (A) and concentration in the waxes (B). Both graphs show the mean concentration of each monoterpene, in the 7 resistant trees (left) and 6 susceptible trees (right). The numbers on the x-axis correspond to the four harvest dates described in the methods.
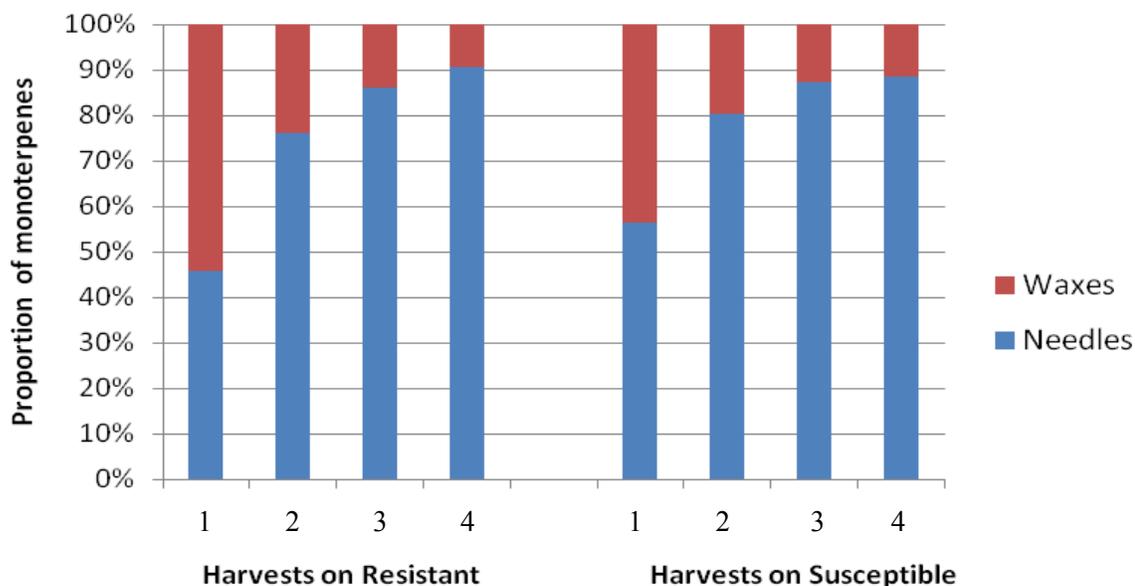
Figure 12: Proportion of total monoterpenes in the needles and in the waxes according to the four harvest dates (mean of the 7 different trees for each type of foliage).

When we examine the partition of the monoterpenes between the needle and the waxes, we see a similar pattern between foliage types (Fig. 12). Indeed, the total monoterpene content is distributed almost evenly between the needle and the wax layer at the beginning of the season and the balance goes toward the needle with almost 90% of the monoterpenes present in the needle at this end of the season.

Results 2011

The same general pattern is observed as in 2010 in both foliage types: the total monoterpene content increases during the season in the needles whereas it decreases in the waxes (Fig. 13). However the total concentration, particularly in the resistant needles, is lower than it was in 2010.
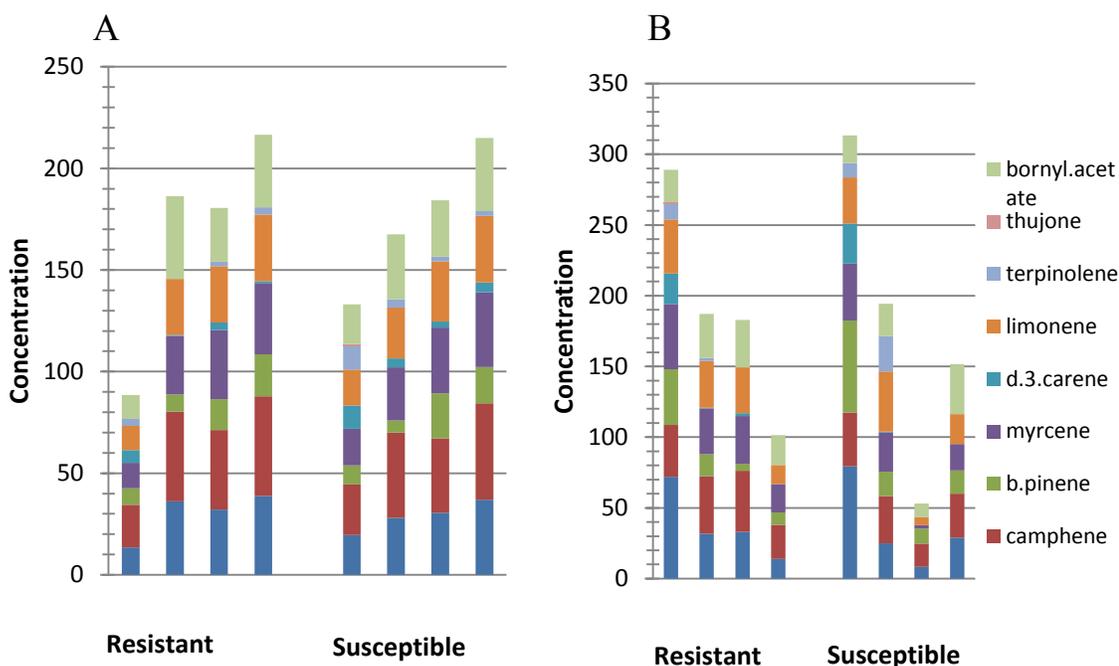
Figure 13: Concentration (ng/mg) of monoterpenes at four dates during the growing season 2011: Concentration in the needles (A) and concentration in the waxes (B). Both graphs show the mean concentration of each monoterpene, in the 13 resistant trees (left) and 12 susceptible trees (right).

We have represented the different samples according to their scores in the analysis on the two first components of the PCA, which combined explain almost 75% of the variation observed (Appendix 3). The first three components account for 84.5% of the

variance. Keeping all the variables make the results difficult to interpret, so we pooled the multiple values for each tree (2 years, 4 sampling dates, needles and waxes) and used the mean concentration for each tree in a second PCA. Each tree is represented in the space of the three first components (Fig. 14)

In this new PCA, the first three components explain 81% of the variance. The first component is composed mostly by the monoterpene variables that are highly correlated (Appendix 2): limonene, alpha pinene and camphene. The second component opposes bornyl acetate to alpha pinene, and the third one is borne by beta pinene and terpinolene (Table 3).

Table 3: Loadings of the three first components from the PCA calculated with the mean monoterpene concentration for each tree.

	Comp.1	Comp.2	Comp.3
Alpha pinene	0.464	0.627	-0.318
Camphene	0.405	-0.234	-0.186
Beta pinene	0	0	0.539
Myrcene	0.357	0	-0.231
D3carene	0.110	0	0.138
Limonene	0.523	0.192	0.255
Terpinolene	0.263	0	0.653
Bornyl acetate	0.372	-0.710	0
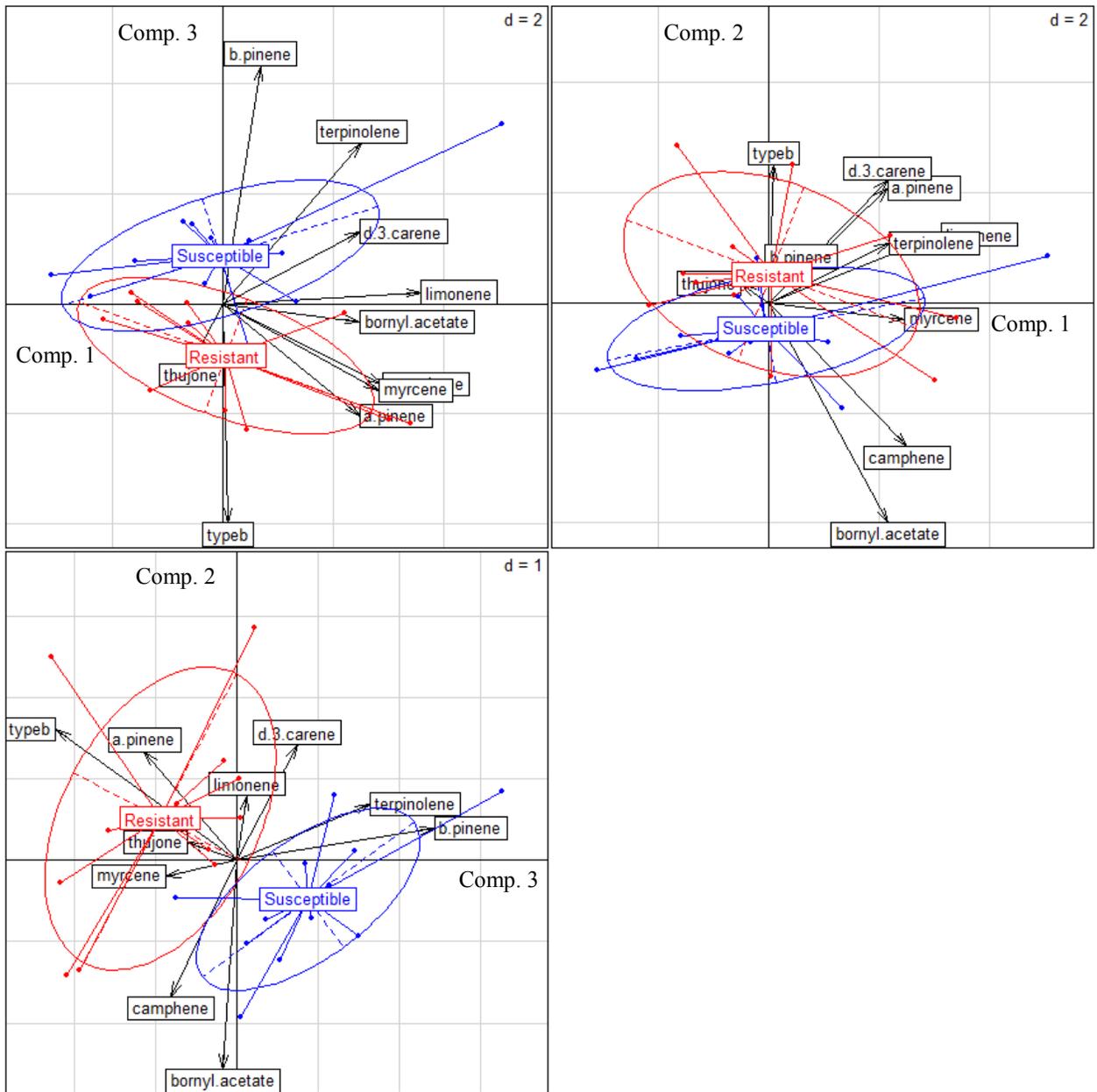
Figure 114: Representation of each tree and each monoterpene variable on the first three principal components of the second PCA. On the top left, components 1 vs 3, on the top right, components 1 vs 2 and on the bottom, components 2 vs 3. The position of each tree is used to build an ellipse characterizing each foliage type (resistant foliage in red and susceptible in blue).

None of the three first components in any of the two models significantly

influences the probability to be resistant (Appendix 4). Nevertheless, the logistic regression with the PCA components from the complete data set has a higher Akaike information criterion (AIC) than the one computed with the PCA components of the mean of each tree (346.89 vs 37.82), so the latter is a better model.

The correlation coefficient between the mean concentrations of monoterpenes in 2010 and 2011 show a stronger correlation between the concentrations in the waxes (r=-0.78) than in the needles (r=-0.47) (Fig. 15). In both years, the correlations are negative, contrary to expectations.
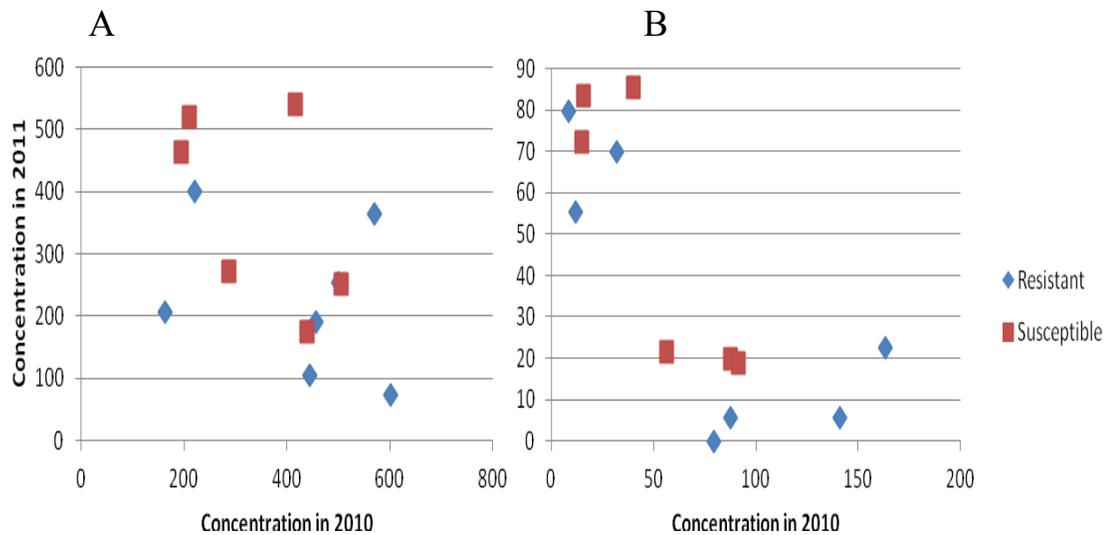
Figure 15: Correlation in total monoterpene concentration (ng/mg) between the two years (A) in the needles and (B) in the waxes for the 13 trees (7 resistant trees in blue and 6 susceptible trees in red) that were used in both years.

Discussion:

For a long time now, we have known that within a population of host trees, individual trees can differ in their vulnerability to budworm attack (Fleming, 1983). Here our goal was to test if this difference in vulnerability between the different trees could be explained by differences in monoterpenes between these trees.

We have based our analysis on a group of chemicals widely used in tree defense against herbivory: the monoterpenes. These secondary plant compounds have been shown to act as feeding and oviposition deterrents to a variety of herbivores including the spruce budworm (Bauce *et al.*, 1994; Clancy *et al.*, 1993), which is why we focused our analysis on this group.

Nevertheless, we did not find any consistent significant differences between the susceptible and resistant trees. We actually found a lot of variation between trees within a type of foliage but also within trees during the season and between years.

The partition of the monoterpenes between the waxes and the needles (Fig. 8 B) suggests an adaptation of the trees to the life cycle of the insect. Indeed, we see that around 50% of the monoterpenes are present in the waxes when dispersal occurs, when the insects are not yet feeding and in contact only with the waxes. We can expect that the resistance at this stage is mostly due to the wax composition since the larvae are not yet feeding and only come into contact with the exterior of the needles. In the later larval stages, the bigger insects are feeding on the entire needle and so the monoterpenes are more efficient as a resistance mechanism throughout the needle.

This does not seem to be the best strategy of defense against oviposition by the females. Indeed, during oviposition the female contacts only the surface of the needle without any cues on its' internal content. Therefore, it seems likely, if the monoterpenes actually act as oviposition deterrent, that the best strategy for the plant would be to maximize the monoterpene content of the waxes. Yet monoterpenes have also been shown to stimulate oviposition in other plant-insect systems (Leather, 1987). In our study organism, certain monoterpenes have been shown to stimulate oviposition on neutral substrates (Grant *et al.*, 2007).

The results of our study show that monoterpenes on their own do not seem to explain the resistance to defoliation observed in the field. It might be that the resistance implies several levels of action in which the monoterpenes might or might not be included. Moreover, Figure 15 suggests that monoterpene content is not stable between years: trees with high concentrations in 2010 had low concentrations in 2011 and *vice versa*.

Chapter 5: Correlation between chemical make-up of the trees and behavioral experiments

Introduction

Insect-host plant co-evolution is a reciprocal evolutionary interaction between a plant and one or more of its natural enemies that occurs in cycles (Rausher, 2001). In the first phase, natural selection imposed by enemies causes the evolution of a new plant resistance that reduces the enemy attacks. Because this resistance reduces the survival of natural enemies, its evolution generates the evolution of counter-resistance by those enemies. Plant enemies exhibit a wide range of physiological, morphological and behavioral characters that seem to have evolved in order to circumvent the newly evolved plant resistance (Rausher, 2011; Thompson, 1988). Insect-plant co-evolution is believed to have generated much of the Earth's biological diversity (Grimaldi and Engel, 2005), and it plays a key role in the design of control programs for insect pest species.

Many factors influence host selection by spruce budworm, but ultimately plant acceptance is determined by chemical characteristics of the host and is very specific (Chapman, 2003; Schoohoven and Van Loon, 2002). In our study so far, we have on one hand the behavior of the larvae on the different trees at different stages of their life cycle and on the other hand the chemical make-up of the trees used in the experiments at three dates of the season that correspond to the stages tested in the behavioral experiments.

Here we examined whether any correlation between the total monoterpene content of the trees and the behavior of the insect on the trees could be detected. For each behavioral experiment, we used the chemical make-up of the trees corresponding to the date at which the insect were tested.

In addition to the correlations we used a generalized linear model following a Poisson regression to test for effects of individual monoterpenes on the number of eggs laid by the females. We used only the concentration in the waxes of the 9 monoterpenes present at the oviposition time in both years (alpha-phelladrene, terpinolene and thujone were removed because they were absent from these samples). The oviposition variable was the number of eggs laid on each tree with the two years of data pooled together.

Results 2010

Despite relatively low R^2, we found a slight negative correlation between both behavioral experiments and the concentration in monoterpenes in the waxes (Fig. 16).

None of these two correlations are significant: r=- 0.50, and r=-0.49, for the correlation between the monoterpenes content in the waxes and respectively the mean number of eggs laid (t = -1.4114, df = 6, p-value = 0.2078) and the mean number of larvae dispersing from the tree tested (t = -1.3777, df = 6, p-value = 0.2175).

Due to a low number of replicates we were not able to discriminate between tree types in the different correlations.
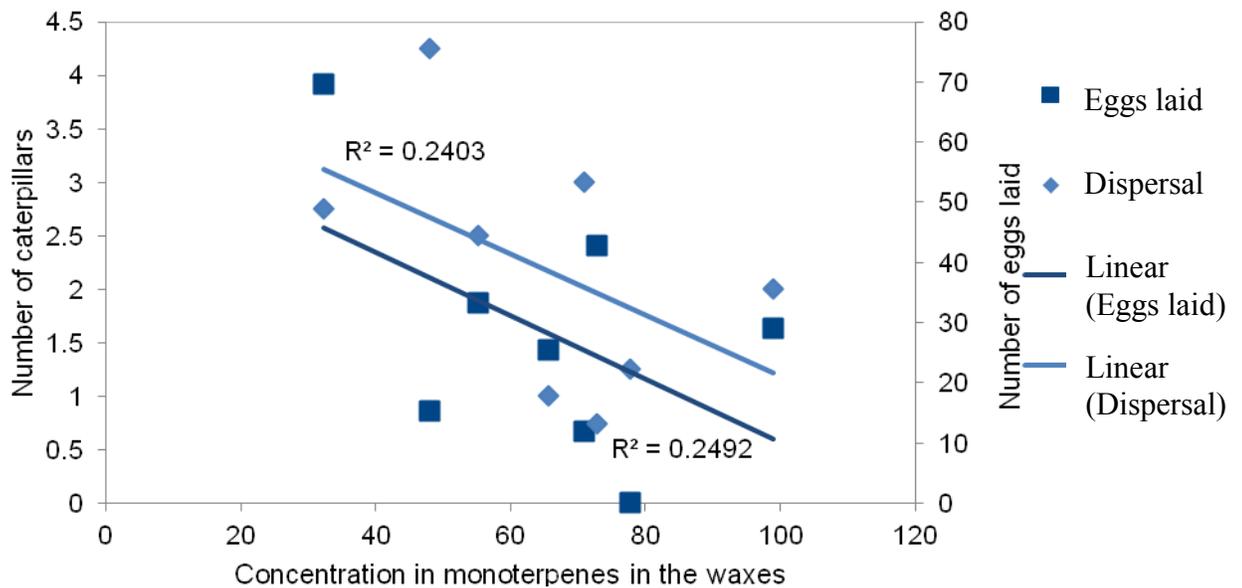
Figure 16: Correlation between behavioral experiments and monoterpene concentration (ng/mg) in the waxes from previous year growth at the appropriate date (May 5th for dispersal and July 12th for oviposition) for the 8 trees tested in the dispersal and oviposition experiments 2010. Not enough oviposition replicates for results on all trees.

Results 2011

There is a significant positive correlation between the number of larvae which dispersed from the susceptible trees and the total concentration of monoterpenes in the waxes (r= 0.66, t = 2.372, df = 7, p-value = 0.049) whereas from the resistant trees, this correlation is not significant (r= -0.22, t = -0.717, df = 10, p-value = 0.489) (Fig. 17).
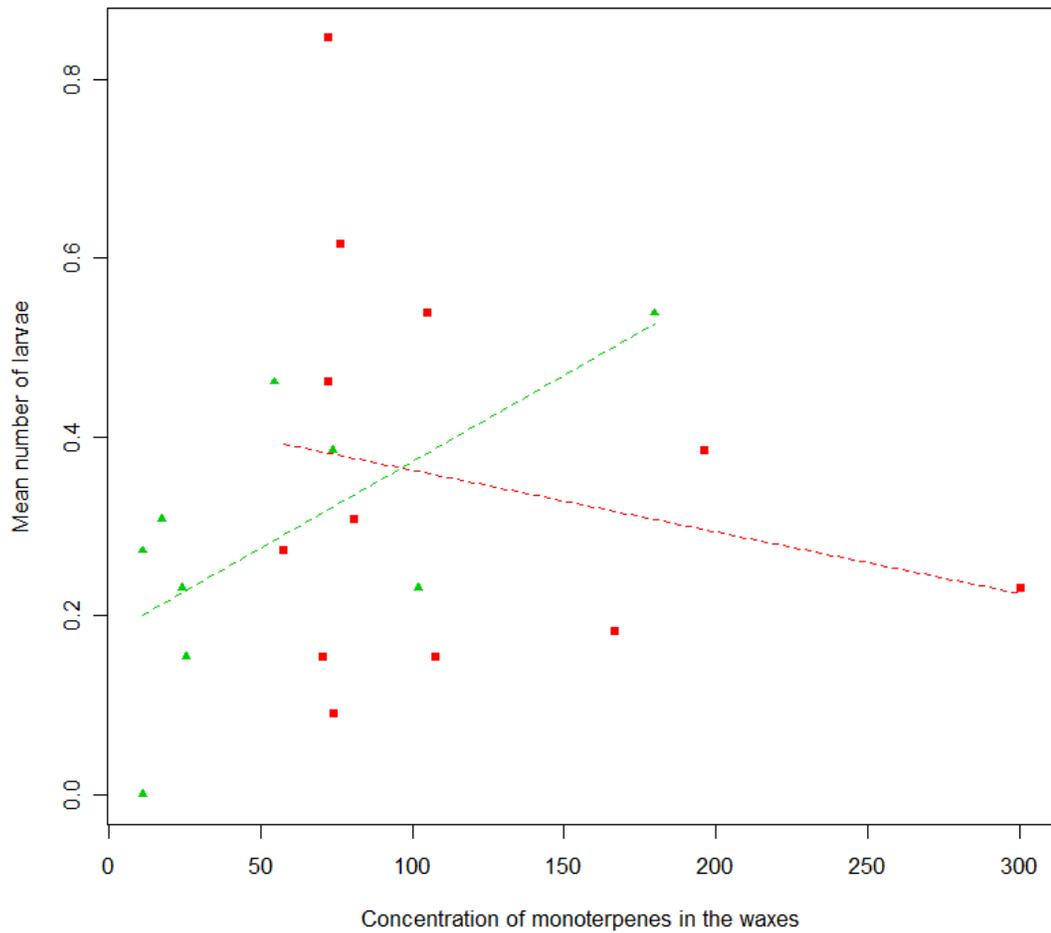
Figure 17: Correlation between the number of larvae which dispersed and the monoterpene content (ng/mg) in the waxes on the previous year's growth at the appropriate date (May 7th 2011). Each point represents the average of the different replicates for each tree. Data from resistant trees are in red and susceptible are in green.

There is no significant correlation between the total concentration of monoterpenes in the waxes and the mean number of eggs laid in any of the foliage types (resistant trees: r=0.05, t = 0.137, df = 7, p-value = 0.894; susceptible trees: r= 0.18, t = 0.568, df = 9, p-value = 0.583) (Fig. 18).
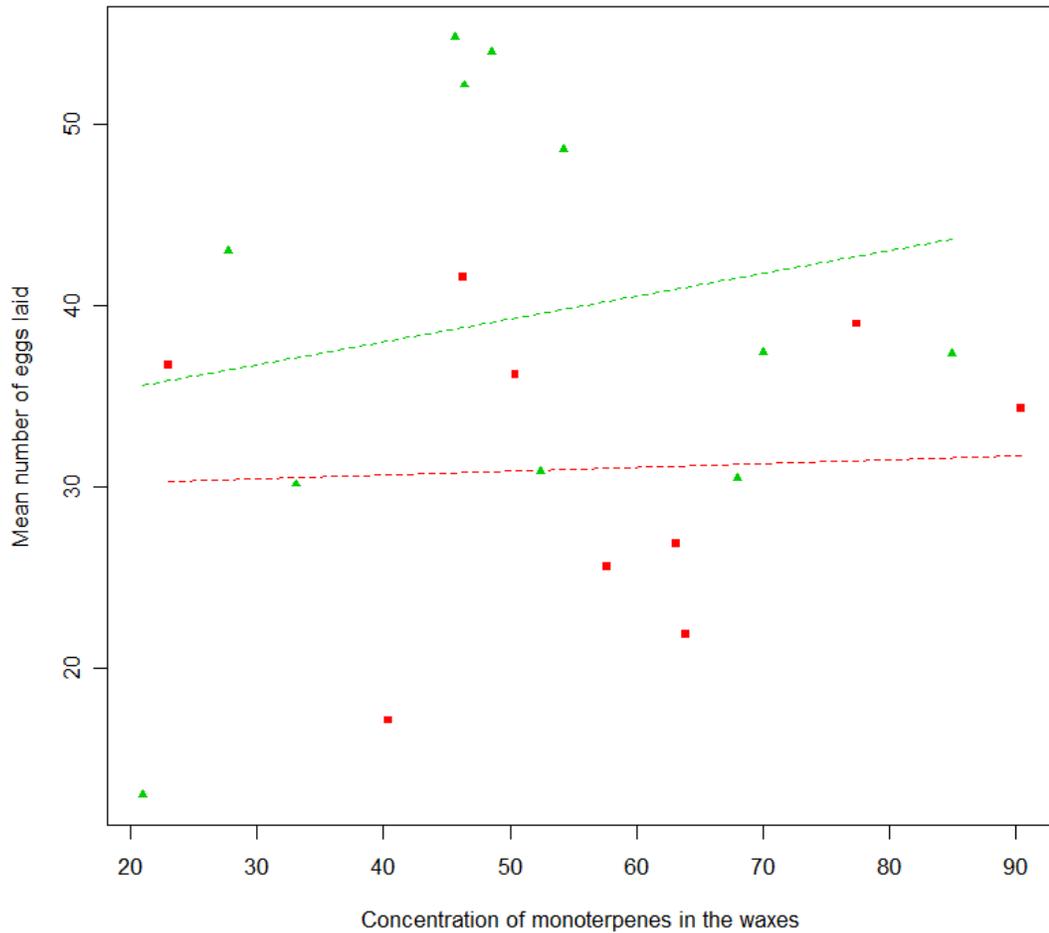
Figure 18: Correlation between the number of eggs laid and the total monoterpene content (ng/mg) in the waxes from the previous year's growth on July 12th 2011 for the two types of foliage. Each point represents the average of the different replicates for each tree. Resistant trees are in red and susceptible trees are in green.

A generalized linear model following a Poisson regression for the number of eggs laid by the females showed that five monoterpenes affected the results of oviposition. Alpha pinene, limonene and bornyl acetate increased the number of eggs laid, with the strongest effect for bornyl acetate. Camphene and myrcene were the two monoterpenes with a repulsive effect, diminishing the number of eggs laid on foliage (Table 4).

Table 4: Results of the Poisson regression on the mean number of eggs laid on each tree. Significance codes: 0.001 '*', 0.01 '**', 0.51 '*'**

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	22.90315	4.85330	4.719	2.37e-06 ***
a.pinene	1.06377	0.51711	2.057	0.03967 *
camphene	-0.77822	0.31137	-2.499	0.01244 *
b.pinene	-0.04056	0.38606	-0.105	0.91633
myrcene	-0.92175	0.33903	-2.719	0.00655 **
d.3.carene	1.57740	1.70659	0.924	0.35533
limonene	0.77957	0.39475	1.975	0.04829 *
bornyl.acetate	1.85093	0.57063	3.244	0.00118 **

A similar analysis, on the effect of the different monoterpenes on the number of larvae dispersing did not show any significant results.

Discussion:

Here we examined whether the monoterpene profiles of the different trees could explain, on their own, the behavior of the insect on these trees. Our 2010 results tend to show that monoterpenes have different effects on the insect at two stages of its life cycle. Indeed an increase in monoterpenes in the waxes seems to be attractive to the young larvae with lower dispersal, whereas it seems to be repulsive to the adults with fewer eggs laid. Nevertheless these results should be interpreted with care due to a low number of replicates and to the low correlations indexes that we have. With more replicates (2011) we found no evidence of such a pattern for the oviposition experiment. The dispersal experiment however demonstrates a significant effect of the monoterpene contents of the susceptible trees on the dispersal of young larvae whereas no relationship is shown on the resistant trees.

Monoterpenes have been shown to have various contradictory effects on host preference by insects, according to their concentration and the specific monoterpenes concerned. In spruce budworm, as early as 1974, Städler demonstrated that D-α and L-β pinene applied on paper substrates stimulated oviposition. This was confirmed by Grant *et al.* in 2007 who tested several monoterpenes and show that most of them were behaviorally active and promoted oviposition on the treated surfaces. However Tiberi *et al.* (1999) showed that application of limonene to host trees inhibited oviposition by the adult females. White spruce waxes contain a variety of different monoterpenes; in our analysis we found 12 different monoterpenes in the waxes of our trees, including α and β pinene as well as limonene. Of these 12 monoterpenes 9 were present in the waxes at the

oviposition time from which 5 were shown to affect the number of eggs laid by the female. Three of them, namely alpha-pinene, limonene and bornyl acetate were attractive and increased the number of eggs laid by the females whereas the other two, myrcene and camphene diminished the number of eggs laid.

The young larvae have been shown to graze some of the waxes before deciding whether to establish and feed or disperse from the tree. Figure 15 does not show any clear relationship between the larvae's decision and the monoterpenes present in the waxes. The direct effect of monoterpenes on larval dispersal has, to my knowledge, still to be tested.

When determining if the preference and behavior of an insect on different hosts can be explained by a specific plant trait, previous research often tested this specific trait under artificial laboratoryl conditions to avoid other characteristics of the hosts which could obfuscate the relationship under study. For instance, to test the effect of certain chemicals, leaf extracts are often used instead of actual foliage (Grant *et al.*, 2007; Städler, 1974; Wallace *et al.*, 2004 among others). Yet these results are often hard to generalize and to apply in the field due to the interplay of many factors. On the other hand, field experiments are subject to many uncontrollable factors and it is often hard to explain their results. As a compromise, we carried out our experiment using actual foliage in controlled lab conditions.

In accord with the literature (Städler, 1974, Grant *et al.*, 2007, Tiberi *et al.* 1999), we found that different monoterpenes in the foliage have contradictory effect on the laying females. If alpha pinene has been found to be attractive to the female by several of these authors and in our study, the other monoterpenes involved in this study were found to

have conflicting effects, being attractive in some studies and repulsive in others.

Chapter 6: General discussion

Insect-host tree association has important implications for forest management strategies (Lawrence *et al.*, 1997). Blum (1988) suggested that breeding for tree characteristics of white spruce could be an effective way to increase the resistance to spruce budworm attacks. Several examples of tree selection to resistance of insects exist among which the most advanced in Canada is the selection of Sitka spruce (*Picea sitchensis*) to resist attacks from the white pine weevil, *Pissodes strobi* (Peck) (Coleoptera: Curculionidae) in British Columbia (Alfaro *et al.*, 2007; King *et al.*, 2004). In this system, they conducted a series of screening trials to search for spruce with resistance to the white pine weevil. Using the weevil population augmentation method (adding reared insects to the local insect population) to maintain an attack rate of 50%, the authors were able to obtain genetically resistant trees in as little as 4 years. These genotypes are now being used in British Columbia to construct tree populations that have strong and durable resistance to white pine weevil.

Bernays and Chapman (1994) have suggested that an adaptation to the resistant characteristics of the trees may be possible over several generations assuming genetic variability within the population of insects. When designing durable management strategies a specific attention should be turned to avoiding fast adaptation.

Based on the results from this study and from the other studies on this system (Daoust *et al.*, 2010, Despland *et al.*, 2011, Delvas et al. 2011, Mader *et al.*, 2012), a

plantation strategy using the resistant trees may be worthwhile. Spruce budworm has been shown to develop poorly on the resistant trees with a high mortality rate (Bauce *et al.*, 2006). A plantation strategy using a majority of resistant trees and a few susceptible trees would have a higher yield than the current plantations. Indeed, resistant trees suffer less defoliation by the spruce budworm which would lower the negative impacts on tree growth and survival engendered by the spruce budworm. The presence of a few susceptible trees in the plantation would slow budworm adaptation and protect the plantation. The susceptible trees, selected preferentially by egg laying females reared on resistant trees, would reduce the strong selective pressure placed by resistant trees on budworm populations. This last finding, seen in Mader *et al.* (2012) on only one extremely resistant and on extremely susceptible trees, and generalized in the present study using 25 different trees allows a continuous mixing of the population preventing budworm adaptation to resistant trees to occur.

Further research investigating both individual and parental experience should be done before this plantation strategy can be implanted. Evaluation on behavior over many generations would help to evaluate how fast the budworm population could actually adapt to the resistance if this plantation strategy were deployed. Further research on tree resistance clarifying the complex roles of the monoterpenes should also been conducted.

Conclusions

Despite a slight tendency for more dispersal from the resistant trees, our results do not show any consistent difference between the two tree types at this stage of the life cycle of spruce budworm. High variability was noticed both between trees within the same foliage type but also between the different replicates for a given tree. Second instar larvae are capable of distinguishing between the trees, but the criteria they use are not always the same ones as confer the resistance to defoliation observed in the field. Environmental conditions also seem to play a key role in this phenomenon.

Sixth-instar budworm do not show behavioral modifications in response to rearing resistant foliage, nor do they exhibit preference for feeding on susceptible foliage..

We were able to show that the natural white spruce resistance to the spruce budworm was efffective at the adult stage of the insect. Females laying their eggs showed a strong preference for the susceptible trees when reared on resistant trees during their development, whereas females reared on susceptible foliage showed no preference. Larval experience, likely retained through metamorphosis, seem to be influencing adult oviposition preference in an adaptive way. Moreover, this deterrence was correlated with certain monoterpenes we measured in the foliage, and is likely linked to other compounds in the waxes.

Currently, the results of this study encourage us to advise a use of mixed plantation in forestry management. Indeed, a mixed phenotypes plantation of white

spruce is expected to have a higher yield than a normal plantation. Resistant trees have a much lower defoliation by SBW, and the susceptible trees should slow down budworm adaptation to the resistant tree phenotype. It is possible that mixed plantations could allow for a smaller number of susceptible trees to act as herbivory 'sinks' in order to reduce selection pressure to overcome the resistance exhibited by resistant trees. While it may not be possible to reduce feeding on resistant trees it might be possible to reduce their insect load via the attractive effect of susceptible trees on ovipositing moths. Budworm dispersal at young larval stages is associated with high mortality, (Jennings et al. 1983) so if the tendency for more dispersal from the resistant trees is confirmed, that would also help to decrease the population size of the spruce budworm on resistant trees.

Appendixes

Appendix 1: Results of the Poisson regression on dispersal of second instar larvae from susceptible and resistant foliage in the 2010 experiment (A) and the 2011 experiment (B). Significance codes: 0.001 '***' 0.01 '**' 0.05 '*'

(A)

	Estimate	Std. Error	z value	Pr (>|z|)
(Intercept)	-1.217404	0.361860	-3.364	0.000767 ***
Type	0.690653	0.342864	-2.014	0.043971 *
Tree	0.030003	0.042408	0.707	0.479270
Time	0.128121	0.045873	2.793	0.005223 **
Replicate	-0.008799	0.016149	-0.545	0.585856

(B)

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-1.304	0.264	-4.929	8.25e-07 ***
Type	0.145	0.250	0.582	0.560
Tree	-0.043	0.016	-2.698	0.006 **
Time	0.029	0.012	2.363	0.018 *
Rep	-0.356	0.044	-8.028	9.93e-16 ***
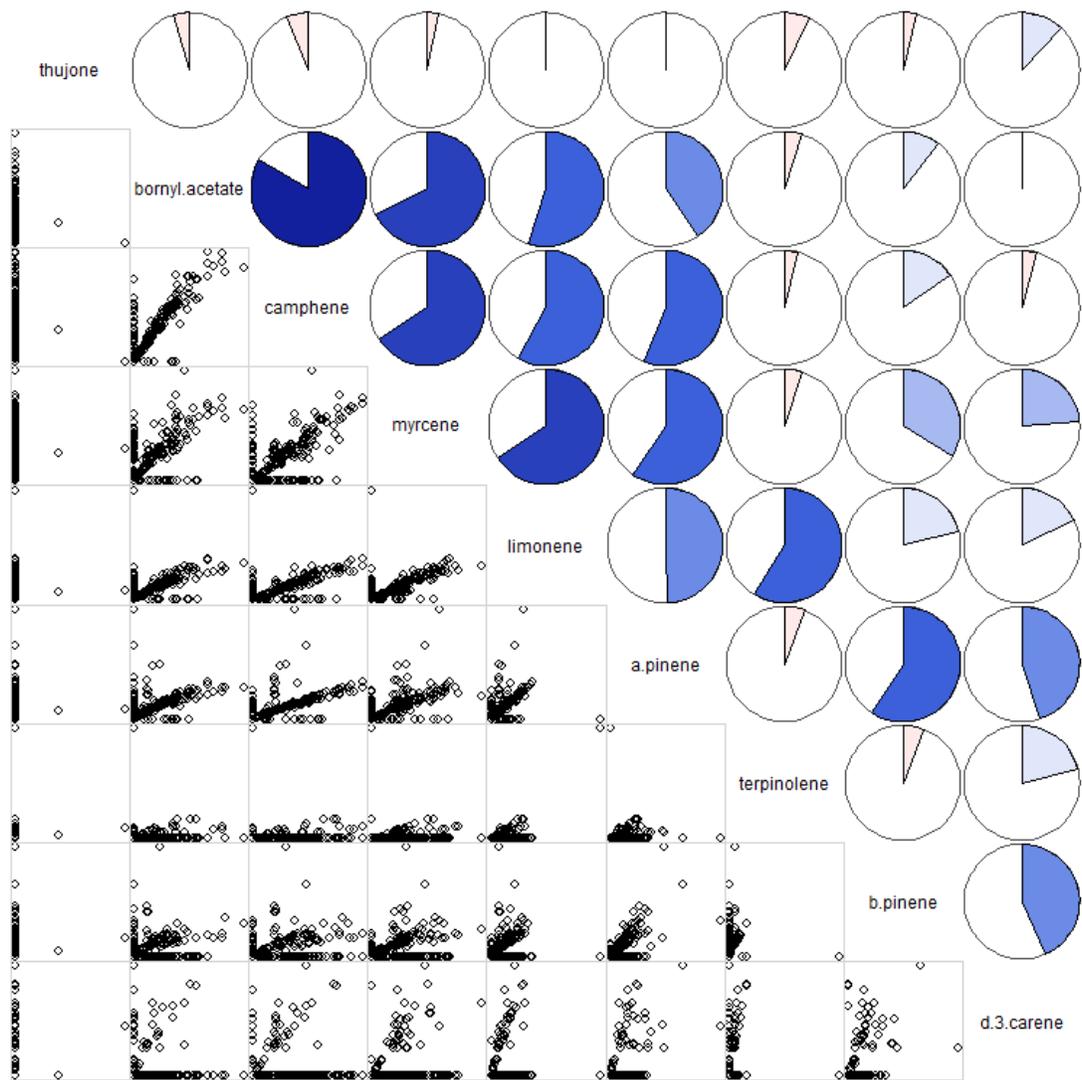
Appendix 2: Correlation between the concentrations of the different monoterpenes in the foliage (overall averages). The filled portion of the pie indicates the magnitude of the correlation.
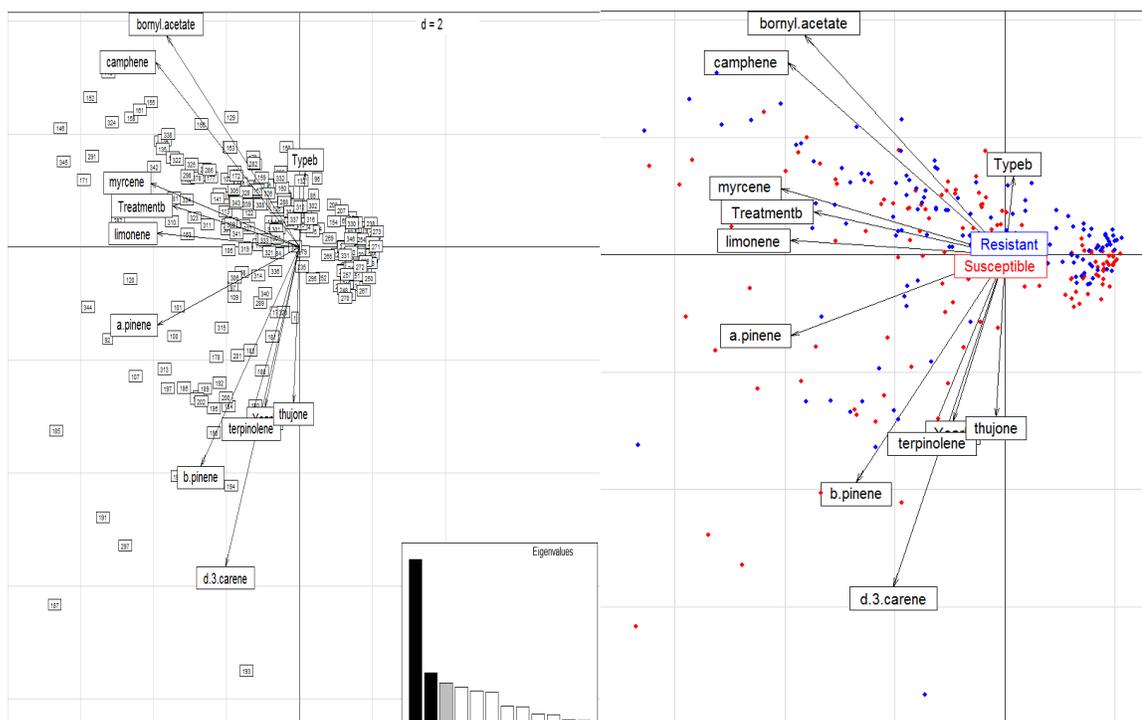
Appendix 3: Representation of the individuals and variables on the first two principal components of the first PCA. On the left each number represents a sample corresponding to a specific date of harvest, tree and year of foliage. On the right, for more clarity, each sample is represented only by the type of foliage (resistant foliage in blue and susceptible in red). Each individual dot represents a tree sample. The labels are the different monoterpenes present in the foliage and the vectors show the contribution of the different monoterpenes to the axes.

Appendix 4: Logistic regression results

(A) Logistic regression using PCA computed with means per tree

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-0.031958	0.459222	-0.070	0.945
Comp.1	-0.003401	0.032371	-0.105	0.916
Comp.2	0.071754	0.060668	1.183	0.237
Comp.3	-0.113156	0.082616	-1.370	0.171

(B) Logistic regression using PCA computed with complete data

	Estimate	Std. Error	z value	Pr(>|z|)
Intercept	-0.0683153	0.1283279	-0.532	0.594
Comp.1	-0.0009679	0.0021469	-0.451	0.652
Comp.2	0.0028006	0.0037428	0.748	0.454
Comp.3	0.0053316	0.0064379	0.828	0.408

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