PhD THESIS

Young forest stands protection in a climate change context

(SUMMARY OF Ph.D. THESIS)

PhD student **Amelia Augusta Tudoran**

Scientific coordinator **Prof.univ. Dr. Ion Oltean**

Co-coordinator **Lecturer Dr. Adriana Puentes**

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INTRODUCTION

Through their structural and functional complexity, forests are an indispensable ecosystem that maintain balance in nature and contribute to the proper functionality of the biosphere. Forest regeneration insect pests cause several international economic problems. In particular, the pine weevil, Hylobius abietis L., can cause damage that costs millions of euros annually by attacking freshly planted seedlings and on some occasions also attacking nursery seedlings.

Climate change and global warming have and will continue to bring forth a series of challenges that require adapting current forest management practices and forest protection strategies. Climate change can manifest itself in various forms depending on the location, with some of the main alternations happening in air and water temperature.

To be able to tackle some of these challenges it is necessary to adapt our plant protection strategies, with the main focus being on the prevention of pest attacks and keeping damage levels under control. In this context, this thesis explores an understudied method of pest damage prevention and control, known as associational effects. Associational effects focus on protecting the focal plant by associating it with a non-preferred plant that can offer protection from the pest in question.

Associational effects have been investigated at a large scale in adult forest sands but their effects on the regeneration stage have received less attention. To evaluate their effect on the pine weevil we chose to associate one of the preferred food source of the pine weevil (*Picea* abies, Norway spruce) with a less preferred one (*Fagus* sylvatica, European beech) to answer the following question: If Norway spruce seedlings have European beech as a neighbor would they suffer a less intense attack than if they have another Norway spruce as a neighbor?

Another ecological method of pest control that has also been little investigated in forest systems is utilizing entomopathogenic bacteria such as *Bacillus thuringiensis* (Bt). In this context, this thesis evluates the susceptibility of the pine weevil to three Bt strains: Bt ssp. *tenebrionis*, Bt *ssp. galleriae* and Bt *ssp. israeliensis*.

1. Structure of the doctoral thesis

The doctoral thesis includes a total of 123 pages and was structured in two parts:

- Current state of knowledge - part I (38 pages)
- Personal contribution – part II (85 pages)

The second part totals a number of 85 pages, structured in 6 chapters, which describe the objectives followed, the environment used in the experiments, materials and methods, results obtained, discussions, conclusions and recommendations based on the results obtained as well as the originality and innovative contribution of the thesis. The thesis contains a total number of 22 tables, 35 figures and 234 bibliographical titles.

2. Research objectives

In young spruce forest stands, insect pests can strongly influence the vegetative period of the stand, sometimes leading to dryness and death of the stand. Therefore, regular monitoring is necessary to adopt a proper control strategy in order to maintain the insect populations at low levels.

Reducing the number of attacked seedings by the pine weevil, *H. abietis*, is a constant challenge for forest owners. Current methods of control include utilizing insecticides, silvicultural methods, and in some cases genetically resistant material, but due to pesticide restrictions, there is a need to explore complementary and sustainable novel ecological means of control.

The research objectives of this thesis are:

1. Monitoring of insect pests in young forests located in D.S. Cluj

For this objective we monitored two pest species:

- Gregarious spruce sawfly, *Pristiphora abietina* Crist
- Pine weevil, *Hylobius abietis* L

2. Utilizing associational effects to mitigate attack by the pine weevil, *Hylobius abietis* L. on Norway spruce seedlings

In order to carry out this objective, we proceeded to:

- Identify and select the species used as a non-focal species to minimize damage to the focal species we want to protect,

We utilized two species of seedlings: Norway spruce (focal species) and European beech (non-focal species). The tests were done in the lab and in the field

- Testing associational effects in the lab,

In the lab we tested 3 experimental treatments:

V1 = Norway spruce seedling + Norway spruce seedling;
V2 = European beech + European beech;
V3 = Norway spruce + European beech.

- Testing associational effects in the field,

In the field we tested two experimental treatments in 5 blocks:

V1 = block planted only with Norway spruce;
V2 = block planted with Norway spruce and European beech.

3. Examining the effects of the entomopathogenic bacteria *Bacillus thuringiensis* on the pine weevil, Hylobius *abietis* L.

To carry out this objective we tested the toxicity of commercial products containing different *Bacillus thuringensis* strains: *galleriae, israeliensis* and *tenebrionis*.

- Testing product toxicity after ingestion,
- Testing various combinations of strains from different commercial products,
- Testing treated food avoidance versus non-treated.

4. Examining buffer effects on the pine weevil *Hylobius abietis* L.

To dilute Bt proteins, protein buffers are needed. Thus it is necessary to test if these buffers themselves can be toxic to the pine weevil.

For this experiment we tested 7 buffer treatments:

- V1. Sodium carbonate buffer 25 mM, pH 10.5
- V2. Sodium carbonate buffer 25 mM, pH 9.2
- V3. Sodium carbonate buffer 50 mM, pH 10.5

- V4. Sodium carbonate buffer 50 mM, pH 9.2
- V5. Buffer HEPES 50 mM, pH 7,5
- V6. Buffer TRIS-HCI 50 mM, pH 8
- V7. Buffer PBS X1, pH 7,4

3. Material and methods

3.1. Monitoring of young forest pests in D.S. Cluj

In order to monitor the gregarious spruce fly *Pristiphora abietina* Crist we made observations in 2019 in three forest administrative units of the forest department Cluj (O.S. Cluj, O.S. Dej and O.S. Gherla). These observations were carried out in both state-owned forests and privately owned forests that were administrated by forest administrative units of the forest department Cluj. In each subplot (u.a.) we checked for species presence and attack frequency.

If the species was found in one of the subplots (u.a.) and at one control point we considered the subplot as attacked (infested) and the species in latency.

Similarly, we monitored for the presence of the pine weevil, i L., in three forest administrative units: O.S. Beliş, O.S. Dej and O.S. Turda.

3.2. Utilizing associational effects to mitigate attack by the pine weevil, *Hylobius abietis* L. on Norway spruce seedlings

In order to examine the associational effects of European beech on attack and damage intensity of the pine weevil, we conducted a choice experiment in the laboratory, where the pine weevils could choose their food source by utilizing a circular arena. In the arena, we placed the plants in pairs belonging to three treatments in six different positions, treatments were as follows: Norway spruce + Norway spruce, European beech + European beech and Norway spruce + European beech. We starved 24 pine weevils of mixed sexes for 48 hours and then we placed them in the middle of the arena where they could choose between different food sources. The pine weevils were left in the arena for 48 hours. The observations were taken two times a day, where we noted the number of weevils present in the perimeter of a position and if they were feeding or not on the plants. Once the weevils were removed from the arena, we measured the damage inflicted on each plant by measuring adding up the sizes of each individual wound on the stem and branches. We replicated the experiment in 14 rounds (two arenas ran simultaneously per round) from March through July 2019.

The studies were carried out in the “Snyttbagge lab” of the Ecology Department at the Swedish Univerisity of Agricultural Sciences (SLU), Uppsala, Sweden.

To study the associational effects in the field, we planted a total of 640 seedlings (out of which: 160 European beech seedlings, *Fagus sylvatica* L. and 480 Norway spruce seedlings, *Picea abies* L) in five experimental blocks located in different areas of a clear cut located near Tierp, central Sweden. In each experimental block, we placed two treatments. One treatment contained Norway spruce alone and for the second treatment, we planted Norway spruce mixed with European beech. Each block had a size of 8 x 8 meters and the seedlings were planted in rows within 50cm distance from one another, resulting in a number of 64 seedlings per block. In the mixed blocks (European beech seedlings mixed with Norway spruce) the seedlings were planted alternating the species for each position, avoiding having neighbors of the same species. To be able to establish the attack/damage caused by the pine weevil we made four observations between July through September 2019. For each observation, we registered if the seedling had been attacked or not, the area debarked, if the seedling was alive or not and if the seedling was girdled or not.

3.3. Examining the effects of the entomopathogenic bacteria *Bacillus thuringiensis* on the pine weevil *Hylobius abietis* L.

To carry out this objective we tested the toxicity of commercial products containing different *Bacillus thuringiensis* strains: *galleriae*, *israeliensis,* and *tenebrionis*.

For these strains, we evaluated the toxicity of each product, of combined products and we tested for product avoidance.

Each mixture was tested at two concentrations and we checked for mortality at 3, 7, 14 and 21 days after each treatment.

3.4. Examining buffer effects on the pine weevil *Hylobius abietis* L.

To test the toxicity of bacterial proteins, they have to be mixed with buffers due to their insolubility in water. While experimenting with different bacterial strains for pest control, the buffer must not interfere with the strains or cause unwanted effects on the pest we are trying to control. We tested 7 buffers for which we evaluated their effects on the pine weevil feeding and mortality of pine weevil adults. The spruce stem pieces were treated and examined for toxicity to the pine weevil after 14 days. The experiment was replicated in time by being repeated 8 times. Pine weevils in each petri dish were weighed individually before the start of the experiment, 7 days after feeding and after 14 days we noted: survival rate, pine weevil weight, and amount of food consumed.

4. Results and discussions

4.1. 4.1. Results with respect to the monitoring of pests in the young stands of D.S Cluj.

The surfaces infested with *Pristiphora abietina* Crist. in the young stands of D.S. Cluj, are relatively little compared to the total area of the forest stand.

Within the monitored areas, the areas with the highest infestation were located in private forests relative to state-owned forests.

The degree of attack in stands infested with *Pristiphora abietina* Crist. in both private and state-owned forests is very low, the species being in the latency phase.

In the private forests of D.S. Cluj, the percentage of attacked surfaces from the total inventoried surface (depending on the age of the stand) varied between 54.9% in the 25-year-old stands and 76% in the 30-year-old stands. In state forests, it varied between 22.8% in 25-year-old stands and 54% in 40-year-old stands. In each age category, the percentage of attacked surfaces was higher in private forests.

Of the 139.9 ha with young conifer stands in private ownership, the *Hylobius abietis* species was reported on 18.7 ha in 2018, and in 26.4 ha in 2019. Every year the intensity of the attack on the saplings in private property was very low in O.S. Huedin and O.S. Turda (there were a maximum of 4 adults/100 seedlings planted), and in O.S. In Beliş, the intensity was also low (there were a maximum of 9 adults/100 seedlings planted).

In the state-owned young conifer stands aged 1-4 years, the pine weevil was monitored on an area of 518.6 ha. From this area, the species was reported on 147.6 ha in 2018, and in 2019 the infested area was 195.2 ha. Each year the attack intensity was very low in O.S. Beliş (there were a maximum of 4 adults/100 seedlings planted), and in O.S. Huedin, O.S. Someşul Rece and O.S. Turda intensity was also low (there were a maximum of 8 adults/100 seedlings planted).

4.2. Results regarding the use of associational effects to to mitigate the attack by the pine weevil, *Hylobius abietis* L. on Norway spruce seedlings

The associational effects as a method of protection for the Norway spruce seedlings against the attack produced by the pine weevil were studied both in laboratory conditions and in field conditions.

In the lab experiment, the pine weevil attack intensity on the Norway spruce seedlings was different depending on the identity of the neighboring plant. Norway spruce seedlings had 54% less bark consumed per plant when paired with a European beech seedling compared to those paired with another Norway spruce seedling.

Moreover, the average number of pine weevils located on the Norway spruce seedlings in the Norway spruce + European beech treatment was 54% less than for the Norway spruce + Norway spruce treatment.

On the other hand, we found no significant differences in the amount of food consumed on the European beech seedlings when they were planted together with another European beech seedling, compared to the mixed variant, Norway spruce + European beech.

Unlike the experiment in the lab, in the field, we found no statistically significant difference in the attack produced by the pine weevils.

Spruce seedlings from the spruce + spruce block and those from the spruce + beech block were similarly attacked throughout the experiment. The attack increased steadily from July to September in both blocks until all plants in the experimental plots were attacked.

However, we could see some trends in attack (whether the plant was attacked or not) and girdling rate (whether the plant was gnawed all around or not), even if these were not statistically significant.

During the first assessment, attack and girdling rates were relatively lower (7.3% and 4.5% lower) for Norway spruce seedlings in the Norway spruce + European beech treatment than those in the Norway spruce + Norway spruce treatment, however, this difference disappeared over time. With regards to the total mortality of Norway spruce seedlings (averaged over 4 assessments), we observed a reduction in mortality for seedlings in the Norway spruce + European beech treatment compared to those in Norway spruce + Norway spruce treatment, but this difference was not statistically significant.

Besides possible inter-plant communication, another factor that differs between the lab and the field experiment is the pressure exerted by the pine weevil density present in the field, which is substantially greater than in the laboratory.

In our experiment, we did not measure or estimate the density of *Hylobius abietis* directly, but given that the site where we placed the experiment was a fresh, unscarified clear cut, it attracted a large number of pine weevils. The frequency of attacked plants was very high 10 days after planting. It is possible that this pressure exerted by the pine weevils has lessened any benefit that the association with the beech would have brought.

4.3. Results regarding the efficacy of the entomopathogenic bacteria *Bacillus thuringiensis* on the pine weevil *Hylobius abietis* L.

All three *Bacillus thuringiensis* strains were toxic to *Hylobius abietis* adults, however, the weight, mortality and feeding were affected differently. In terms of total food intake, all Bt strains (except low-concentration Bt *galleriae*) reduced total food intake at 3 and 7 consecutive days of exposure to treated food compared to the control group. On average, the reduction in the amount of food consumed (for both low and high concentrations) during the 7 days varied considerably between treatments, with a reduction of approximately 30% for Bt *galleriae*, 42% for Bt *tenebrionis* and 70 % for Bt *israeliensis*.

The reduction in feeding was maintained even for the subsequent 7 days when the pine weevils were fed non-Bt-treated food. Again, the amount of bark consumed during the 7 days of feeding untreated food followed the same decline as in the previous 7 days, the highest decline was observed in Bt *israeliensis*, followed by Bt *tenebrionis* and Bt *galleriae* (82%, 38% and 11% reduction respectively). These differences were statistically significant only for Bt *israeliensis* and Bt *tenebrionis*.

In terms of mortality, all treatments (except Bt *galleriae* low concentration, BL) showed that a greater number of individuals died compared to the control group throughout the experiment and the observation period (7 days of Bt-treated feed + 7 subsequent days of untreated food + 7 subsequent days of observation in a separate box with untreated food). The highest mortality rate was reported for Bt *israeliensis*, 70-82% when the mixture was used in high concentration, statistically significant.

When applying combinations of Bt products, we observed that *Hylobius abietis* did not show greater susceptibility compared to the individual products. Treatments that contained Bt *israeliensis* were the most toxic and lethal compared to the individual products.

4.3. Results regarding the buffer assessments on the pine weevil *Hylobius abietis* L.

As far as the mortality is concerned, we found that the buffers based on sodium carbonate increased pine weevil mortality between 10% and 20% relative to controls after 14 days of feeding. In these treatments, the pine weevil consumed the least amount of food, and a decrease in weight was reported relative to the initial weight. In all other treatments, the pine weevils survived and no mortality was observed.

The only treatment where the amount of food consumed was higher relative to the control is the HEPES buffer 50 mM, pH 7.5 treatment where after 7 days of feeding they consumed 357.4 mm2, respectively 311.12 mm2 in the second week. In this treatment, the highest increase in weight of the pine weevils was reported, 0.01g.

5. Conclusions and recommendations

1. The surfaces infested with *Pristiphora abietina* Crist. in D.S. Cluj are very small relative to the total area of the forest.

2. In all monitored stands, the most infested areas are located in privately owned forests, compared to state-owned forests.

3. The attack intensity is low in both state-owned and privately-owned stands infested with *Pristiphora abietina* Crist., the species being in the latency phase.

4. Following this study, we determined that the identity of the neighboring species influences the attack of the pine weevil *Hylobius abietis* on the Norway spruce seedlings.

5. Under controlled environmental conditions, the pine weevil has a tendency of avoiding the group of mixed seedlings (Norway spruce + European beech).

6. The amount of food consumed in the mixed treatment (Norway spruce + European beech) on the Norway spruce seedlings was less than in the case of the Norway spruce + Norway spruce treatment.

7. In natural conditions, associational effects lose their efficacy. The distance between plants in the natural environment may be an important factor mediating effects.

8. Associational effects lose their efficacy when the pine weevil pressure is large.

9. *Bacillus thuringiensis* is shown to have toxic and lethal capabilities on the pine weevil.

10. *Bt israeliensis* caused 65-82% mortality and 70-82% reduction in feeding compared to the control group in adult pine weevils, which has the potential to be used in the management of *Hylobius abietis*.

11. Sodium carbonate buffers adversely affect the pine weevils in terms of both feeding and survival rate. These buffers are not recommended for use in experiments that require the use of buffers such as testing toxic bacterial proteins that are solubilized in the buffer.

12. The HEPES 50 mM, pH 7.5 is the most promising. Based on the results of this experiment, this buffer yields similar effects on the pine weevil as the control for all the studied variables.

Recommendations

- In order to be able to better understand the associational effects and to be able to improve the methods of protecting newly planted seedlings against *Hylobius abietis*, future studies should examine different distances between plants.
- Different combinations with non-preferred species that could mediate associational effects should be tested (focal and non-focal plants).
- *Bacillus thuringiensis* spp. *israliensis* strains could be utilized in forestry as a management strategy for *Hylobius abietis*.
- For the protection of conifer stands, continuous monitoring of all surfaces is required in order to develop a correct forecast to prevent the numerical increase of pest populations.

6. Originality and innovative contributions of the thesis

- Associational effects to protect against pest damage have been little studied in coniferous forest systems.
- Testing the preference of the pine weevil on European beech has been previously under-examined, and this thesis indicates that the European beech is not a preferred species for the pine weevil.
- *Bacillus thuringiensis* has been tested mainly in agriculture, and this is the first study to test the effects of Bt on *Hylobius abietis* adults.
- This study shows that a non-Coloptera-specific Bt spp. *israliensis* toxic protein is the most lethal to *Hylobius abietis* adults.
- The effect of some protein buffers was tested for the first time on *Hylobius abietis* adults.

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