Defence activation in strawberry and pine
- Epigenetic changes in treated plants

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- Epigenetic changes in treated plants

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plantor

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Summary

Strawberry plants (Fragaria x ananassa) and Scots pine (Pinus sylvestris) represent species, within agriculture and forestry respectively, that are traditionally protected by utilization of pesticides including neurotoxic insecticides. More environmentally friendly protection strategies are therefore highly desirable. Treating plants with specific metabolites naturally occurring in their tissues might alter epigenetic mechanisms, which in turn may strengthen plants self-defense against diseases and weevil attacks. F. x ananassa and P. sylvestris seeds were treated with 2,5 mM nicotinamide and 2,5 mM nicotinic acid in order to investigate possible epigenetical effects by analyzing changes in the level of the DNA methylation. The epigenetic changes, for both plants, were analyzed on the global DNA level. Reduction in the DNA methylation level in strawberry leaves as well as the DNA methylation increase in pine needles were observed by means of LUMA-analysis when HpaII restriction enzyme was used in the analysis. Further investigation is required in order to understand if NIC and NIA may have a significant impact on pathogen attack in strawberry plants and Scots pine. More research may also unveil if nicotinamide and nicotinic acid can play a potential role in more sustainable defense strategies of plants.
Sammanfattning

Jordgubbsplanter (Fragaria x ananassa) och tallar (Pinus sylvestris) representerar växter inom jord- och skogsbruk som traditionellt skyddas genom användning av bekämpningsmedel, detta inklusive neurotoxiska insekticider. Mer miljövänliga skyddsstrategier är därför mycket önskvärda. Behandling av växter med specifika naturligt förekommande metaboliter genererade av växterna kan påverka epigenetiska mekanismer. Förändringar på den epigenetiska nivån kan, i sin tur, bidra till förstärkning av växternas eget självförsvar mot sjukdomar och insektsangrepp. Frön av både F. x ananassa och P. sylvestris behandlades med 2,5 mM nikotinamid och 2,5 mM nikotinsyra i syfte att undersöka eventuella epigenetiska effekter. Detta genom att analysera förändringar i graden av DNA metylering i de behandlade plantorna. De epigenetiska förändringarna för jordgubbsplanter och tall analyserades på den globala DNA-nivån. Minskad DNA-metylering i jordgubbsblad samt ökad DNA-metylering i tallbarr observerades med hjälp av restriktionsenzymet HpaII och LUMA-analys. Ytterligare undersökningar behövs för att kunna förstå om NIC och NIA kan ha en inverkan på patogenangrepp i jordgubbsplanter och tall. Mer forskning kan också avslöja om nikotinamid och nikotinsyra kan ha en betydande roll inom hållbara försvarsstrategier för växter.
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1. Introduction

Plants are physically tied to the environment that they are living in. Due to the constant exposure to many stress factors, plants have developed various defense strategies [1] [2]. When occupying different habitats, plants are challenged with abiotic stresses like variations in temperature, light intensity as well as access to water and nutrients [2] [3]. Additionally, plants are exposed to biotic stress factors and forced to interact with insect herbivores as well as fungal and bacterial pathogens [2] [4]. Those interactions have a significant impact on plant populations that may concern many organizations and institutions from different sectors, among others, forestry and agriculture.

Plant defense against herbivores and microorganisms is an important issue from both ecological and economical point of view. Despite defense mechanism that plants evolved against biotic stresses, the balance of co-evolution between plants and plant-eating insects or fungi has been disturbed due to anthropogenic actions. The usage of pesticides, as a defense strategy against insects and pathogens, has its strong negative effects on environment that are well documented since the 1960’s [5]. Pesticides have toxic effects not only on sprayed plants but also on humans, via inhalation, oral and dermal routes. Aquatic organisms are also affected due to leakage via soil and water [6] [7] [8]. The consequences of exploitation of these toxic compounds may have a long-term effect on the environment [5]. Moreover, high susceptibility of plants to biotic stresses that may result in diseases has its economical consequences. Actions caused by herbivores and pathogens ingest a significant part of agricultural and forestry recourses [9] [10] [11].

Therefore, sustainable alternatives are desirable in order to protect plants against insect herbivore and fungi attacks. One of the possible possibilities is to improve plants stress tolerance through treatment with the compounds that naturally occurred in plants during exposure to biotic stress or with physical methods creating abiotic stress [10] [12]. The process, which makes it possible, called priming, is a defense strategy that enables plants to “remember” and “react” stronger and faster to stresses during their second exposure to stress factors where compared to non-primed plants [2] [12] [13] [14] [15] [16]. Furthermore, epigenetics allows studying if seed treated plants are affected by the treatment by investigating changes in the level of DNA methylation. Changes in genomic DNA methylation is a mechanism that is being associated with plant memory and plays a crucial role in expression of genes, like the ones activated during defense responses.

The goal of this project is to evaluate if two of the most heavily sprayed plants within agriculture and forestry, strawberry (Fragaria x ananassa) and pine (Pinus sylvestris) respectively, are susceptible to seed treatment with nicotinic acid (NIA) and nicotinamide (NIC). In other words, to analyze if changes in the level of DNA methylation occurs in F. ananassa and P. sylvestris plants. It should be mentioned that the study presented in this paper is the first attempt to estimate possible changes on the epigenetical level in the chosen plants that were seed treated with NIC and NIA. One should note that this project might give a foundation in further studies like estimating the grade of weevil attacks on the investigated plants.

In order to study epigenetic mechanisms several methods were applied. To perform analysis, samples were homogenized under liquid nitrogen followed by the extraction of DNA achieved with a DNA-isolation kit for plant tissues. LUMA (Luminometric Methylation Assay) was a method used for studying the changes of the DNA methylation level [17]. LUMA is a two-phase process, which involves DNA cleavage with restriction enzymes as well as pyrosequencing.
2. Background

2.1 Epigenetics

According to the recent literature, epigenetic mechanisms seem to lie behind the ability to preserve stress memories in plants [2] [3]. Epigenetic mechanisms can be defined as: “those genetic mechanisms that create phenotypic variation without altering the base-pair nucleotide sequence of the genes” or, in other words, mechanisms that “cause variation by altering the expression of genes” rather than changing the DNA sequence [18].

2.1.1 Epigenetic mechanisms – from the molecular perspective

Epigenetic changes that include the level of DNA methylations, histone modifications and long-term modifications of chromatin regulate the activity of the set of genes – a genome. In each eukaryotic cell nucleus, DNA, together with histone proteins, is densely packed in form of compact filament, a chromatin. The main component of the chromatin is a nucleosome that contains histone octamers on which 147 base pairs of DNA are wrapped around 1.75 times, see figure 1. Histone octamers are structures composed of 8 histone proteins with a common characteristic feature in form of tail. Histones forming octamer core are duplicates of H2A, H2B, H3 and H4. [14]

Chromatin, in the “compressed” state, has nucleosomes condensed with one another that form a chromatin fiber. In this state, genes are not available for being activated for transcription. [10] [14] [19]. However, in the “open” state, the distance between nucleosomes expands and DNA is exposed which makes activation for gene transcription more susceptible. As mentioned before histone modifications, like acetylation, phosphorylation and ubiquitination, have been correlated with gene activity [14] [20] [21] [22]. Changes in the level of DNA methylation, a well-studied epigenetical mechanism, are described below.

![Figure 1. Chromatin structure](image)

**Figure 1. Chromatin structure**

DNA is wrapped around nucleosomes; structures build from different histone proteins. Chromatin in its “closed” state has nucleosomes condensed to one another. Figure adapted from *Ecological Developmental Biology* by S.F. Gilbert and D. Epel [19]
2.1.2 DNA methylation

As previously stated, chromatin, in its “compressed” state, makes it impossible for RNA polymerase, together with transcription factors, to express genes. One of the known mechanisms that keep genes inactive is cytosine methylation. This methylated form of cytosine, 5-methylcytosine is, in other words, being correlated with gene silencing [19] [23]. Demethylation of DNA lowers the repression of chromatin and enables gene transcription [24] [19]. DNA cytosine methylation in plants appears in CG and non-CG sites [25]. Target for DNA methylation in non-CG sites can occur at both symmetric CNG and non-symmetric CNN sequences (where N stands for A, T, C). When changes in the level of DNA methylation occur, gene expression can be altered by either hypomethylation (decrease in methylation level) or hypermethylation (increase of DNA methylation level) [19] [20] [23].

2.1.3 Correlation between DNA methylation and stress factors in plants

Changes in the methylation of the DNA in plants due to abiotic factors have been well documented. Alteration in the level of DNA methylation as a result of various plants being exposed to stress factors like salt, cold and drought has been reported over the past 20 years. In a study from 2002, roots of maize seedlings exposed to cold stimuli, showed activated gene expression coupled to DNA hypomethylation of nucleosome core [26]. Prior to analysis, the seedlings were exposed to 4 °C cold for about six days. Although the seedling were then returned to normal growth conditions for another 7 days, the level DNA methylation continued to decline. This study is one of various investigating the change in the DNA methylation level. Table 1 illustrates more examples.

<table>
<thead>
<tr>
<th>Abiotic factor</th>
<th>Plant</th>
<th>The change in the DNA methylation</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical stress</td>
<td>White bryony (Bryonia dioica)</td>
<td>Hypermethylation</td>
<td>1993</td>
<td>[27]</td>
</tr>
<tr>
<td>Osmotic stress</td>
<td>TBY-2, tobacco (Nicotiana tabacum)</td>
<td>Hypermethylation</td>
<td>1997</td>
<td>[28]</td>
</tr>
<tr>
<td>Drought</td>
<td>Pea (Pisum sativum)</td>
<td>Hypermethylation</td>
<td>2002</td>
<td>[29]</td>
</tr>
<tr>
<td>Heavy metals Cr, Ni Cd</td>
<td>White clover (Trifolium repens)</td>
<td>Hypomethylation</td>
<td>2004</td>
<td>[30]</td>
</tr>
<tr>
<td>Salt</td>
<td>Crystalline iceplant (Mesembryanthemum crystallinum)</td>
<td>Hypermethylation</td>
<td>2006</td>
<td>[31]</td>
</tr>
<tr>
<td>Salt, cold, aluminum and oxidative stress</td>
<td>Tobacco (Nicotiana tabacum)</td>
<td>Hypomethylation</td>
<td>2007</td>
<td>[32]</td>
</tr>
<tr>
<td>Reactive oxygen species (ROS)</td>
<td>TBY-2, tobacco (Nicotiana tabacum)</td>
<td>Hypomethylation</td>
<td>2015</td>
<td>[33]</td>
</tr>
</tbody>
</table>

* Table adapted and extended from [23]
Alterations in the level of the DNA methylation has also been observed when plants when challenged with biotic factors. Tobacco leaves, in a series of experiment conducted by Wada et al. in 2004, were infected with tobacco mosaic virus and a reduction in methylation in the investigated pathogen-responsive gene (NtAlix1) was observed 24 hours later [34].

Hypomethylation at specific gene levels was also observed in further generations of rice plants (Oryza sativa) when plants were infected with bacteria, Xanthomonas oryzae pv. oryzae. In this study, seeds were treated with the methylation inhibitor 5-azadeoxycytidine whereas the cultivated progeny was investigated for about 10 years. Moreover, authors point out that not only hypomethylation but also disease resistance towards pathogen infection was inherited. [35]

2.2 Nicotinamide & nicotinic acid

2.2.1 Nicotinamide and nicotinic acid as NAD constituents

Nicotinamide (NIC) and nicotinic acid (NIA) are mostly known as precursors for the synthesis of nicotinamide adenine dinucleotide (NAD(H)) and nicotinamide adenine dinucleotide phosphate (NADP(H)) - NAD’s phosphorylated derivative [36]. According to Penberthy and Kirkland [37], NAD(P)(H) is essential in hundreds of reactions where at least 470 various proteins are involved. NAD(H) and NADP(H) are mostly related with redox reactions and their role as a cofactor [37]. Although NAD(H) and NADP(H) serve a crucial role in hundreds of oxidation-reduction reactions, various enzymes utilize NAD as a substrate [37]. These biological catalysts come from different protein families; poly(ADP-ribose) polymerases (PARPs), sirtuins (SIRTs) - NAD-dependent deacetylases, ADP cyclases (CD38 and CD157), and indoleamine 2,3-dioxygenases (IDO) [36] [37] [38]. In several reactions NAD participates even as a neurotransmitter acting as a ligand [37].

2.2.2 The pyridine nucleotide cycle.

The pyridine nucleotide cycle in plants, proposed by Ashihara, explains synthesis and possible metabolic pathways of nicotinamide and nicotinic acid and can be considered as a basis to understand synthesis and metabolism of nicotinamide and its metabolites, see figure 2 [39] [40] [41].

2.2.2.1 The source of NIC

As mentioned above, the level of NAD concentration is being controlled by several enzymes. Three of them; PARP, NAD-dependent deacetylases and ADP cyclases participate in reactions where nicotinamide is released. In plant cells, NAD-substrate is considered to be a source of nicotinamide via two pathways [39].

The first route involves PARP or NAD glycohydrolase in one-step reaction that results in NIC release in cell nucleus [1] (see step 1, figure 2). The release of NIC by nucleus enzyme PARP is considered to be primary source of nicotinamide. In the cell nucleus poly(ADP-ribose) polymerase is activated when DNA damage occurs. In cases of mild DNA damages, strand break initiates formation of ADP-ribose from its substrate NAD by PARP [36]. The product, ADP-ribose is further formed in to polymers that organize DNA repair process on a cellular level. The other product due to ADP-ribosylation is nicotinamide [36] [38] [42]. Nicotinamide can also be synthesized in a series of reactions. This second route of NIC formation is through nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) (see step 2-4, figure 2) [39].
Nicotinamide is converted to nicotinic acid (NIA) by the enzyme nicotinamidase (see step 5, figure 2). Further conversion of nicotinic acid includes 3 different pathways (step 6, figure 2). Regeneration of NAD via nicotinic acid mononucleotide (NaMN) is one of them. The non-regenerated fraction of NIC is converted, via other two routes, to trigonelline and nicotinic acid N-glucoside (NaG) respectively (see step 5a, 5b in figure 2). Conversion of NIC to both trigonelline and NaG is very rare and in most of the studied plants the production of one or the other substance only occurs. [41] [43]

Figure 2. The pyridine nucleotide cycle
The picture shows possible pathways for nicotinamide (NIA), nicotinic acid (NIC) and NIC’s metabolites. Enzymes for each of the presented numerals are following: (1) poly (ADP-ribose) polymerases; (2) NAD pyrophosphatase; (3) 5-nucleotidase; (4) nicotinamide riboside nucleosidase; (5) nicotinamidase; (5a) nicotinate N-glucosyltransferase; (5b) trigonelline synthase (nicotinate N-methyltransferase); (6) nicotinate phosphoribosyltransferase; (7) nicotinic acid mononucleotide adenylyltransferase; (8) NAD synthase. Metabolites: NaAD - nicotinic acid adenine dinucleotide; NaG - nicotinic acid N-glucoside; NaMN - nicotinic acid mononucleotide; NMN - nicotinamide mononucleotide; NR - nicotinamide riboside. Figure adapted from [39] [41] [49].

2.2.2 Metabolic pathways in the pyridine nucleotide cycle
Nicotinamide is converted to nicotinic acid (NIA) by the enzyme nicotinamidase (see step 5, figure 2). Further conversion of nicotinic acid includes 3 different pathways (step 6, figure 2). Regeneration of NAD via nicotinic acid mononucleotide (NaMN) is one of them. The non-regenerated fraction of NIC is converted, via other two routes, to trigonelline and nicotinic acid N-glucoside (NaG) respectively (see step 5a, 5b in figure 2). Conversion of NIC to both trigonelline and NaG is very rare and in most of the studied plants the production of one or the other substance only occurs. [41] [43]

2.2.3 The role of NIC and NIA in plant defense
Although nicotinamide has been viewed in some literature as a side product during controlling NAD-levels, its potential role as a stress signal compound was considered by Berglund, in his hypothesis in 1994 [44]. In his paper, Berglund suggested that NIC may be an important link but also an early stress signal itself in plant defense metabolism. The nicotinamide hypothesis was based on, among other things, several studies conducted in previous years by him and his colleagues regarding effects of NIC. In a series of research NIC showed to have ability to accumulate secondary metabolites like anthocyanins and alkaloids [1] [45]. Moreover, the increase in
concentration of NIC in plant tissues had been related with oxidative stress [1] [46]. Berglund in his hypothesis suggested that NIC might alter the level of DNA methylation and lean toward NIC’s hypomethylating effect in plants [44]. In other words, it had been suggested that NIC might be involved in regulation of gene expression.

Berglund’s theory about the role of nicotinamide in stress signaling but also in pathways regarding gene expression has been strengthened by him and his colleagues in a study regarding new protection strategy of Norway spruce (Picea abies (L.) Karst.) against pine weevil (Hylobius abietis L., Coleoptera Curculionidae). The results of the research confirmed that naturally occurring organic compounds such as NIC and NIA can contribute to decreasing of insect herbivore attack. In this study, 6-month old spruce seedlings, previously seed treated with 2.5 mM NIC and planted in a Swedish reforestation area, were less attacked by pine weevils compared to control seedlings. A parallel field-test, which involved watering spruce seedlings with 2 mM NIC or 2 mM NIA for 2.5 months, did reduce pine weevil attacks in 18-month-old spruce seedlings as well. [10]

2.3 Fundamentals of LUMA

LUMA stands for Luminometric Methylation Assay [17], which can be perceived as a two-phase assay for genomic DNA methylation (see figure 3).

In the first phase a DNA cleavage by restriction enzymes HpaII or MspI (phase 1) followed by pyrosequencing (phase 2). In a second phase, a conducive reaction between a nucleotide (or mix of nucleotides) causes conversion of released inorganic pyrophosphate (PPi) in ATP by ATP-sulfurylase. ATP, together with luciferase, converts luciferin to a visible light in form of oxyluciferen. The quantity of the light is proportional to the extent of restriction made by restriction enzymes in phase 1. The level of the methylation of genomic DNA can be determined. Figure adapted from [17].

In order to be capable to determine the level of DNA methylation, HpaII and MspI, two restriction enzymes are usually used for the analysis. Both enzymes have a common target sequence of nucleotides: CCGG. What both

![Figure 3. LUMinometric Methylation Assay (LUMA)](image)

LUMA showed as a two-phased process. DNA cleavage by restriction enzymes HpaII or MspI (phase 1) followed by pyrosequencing (phase 2).

In a second phase, a conducive reaction between a nucleotide (or mix of nucleotides) causes conversion of released inorganic pyrophosphate (PPi) in ATP by ATP-sulfurylase. ATP, together with luciferase, converts luciferin to a visible light in form of oxyluciferen. The quantity of the light is proportional to the extent of restriction made by restriction enzymes in phase 1. The level of the methylation of genomic DNA can be determined. Figure adapted from [17].
HpaII and MspI have in common is they are able to cleave DNA when CCGG sites are unmethylated and both are inhibited if the outer C is methylated (mCCGG). The distinction between the enzymes is the ability of MspI to cut DNA when the inner C is methylated (CmCGG). HpaII is said to be a methylation-sensitive restriction enzyme due to its susceptibility also towards methylation of internal cytosine. MspI can cleave DNA regardless the presence of 5-methylcytosine at the inner C. [17][33]

In the second phase, a stepwise addition of dNTPs in a pyrosequencing reaction is conducted. In this bioluminometric polymerase extension assay, after each successful extension of nucleotides, inorganic pyrophosphate (PPi) is released triggering a cascade of reactions. This binding of dNTPs to the DNA's overhangs results ultimately in a production of visible light that is proportional to the amount of overhangs executed by restriction enzymes. The sequence of first added dNTPs includes; step 1 dATPαS; step 2: a mixture of dCTP and dGTP; step 3: dITTP; step 4: a mixture of dCTP and dGTP. Nucleotide dATPαS, the derivative of adenosine triphosphate (dATP) was used due to its insensitivity to enzyme, luciferase. Otherwise dATP would react with the luminescent enzyme. [17]

When binding between a nucleotide and the previously cut restriction site of DNA occurs, the released PPi converts to ATP by enzyme ATP-sulfurylase. ATP is then used in the next reaction, together with luciferase. The product of these enzymes is oxyluciferen, which generates bioluminescence, a visible light. The light, detected by a charge coupled device (CCD) camera, is directly proportional to the extent of cleavage of the sample's DNA [17].

2.4 Fragaria x ananassa

2.4.1 Origin and cultivation

Strawberry is a small fruit crop, of the genus Fragaria that belongs to the Rosaceae plant family [47] [48]. The most cultivated species is the dessert strawberry Fragaria x ananassa Duchesne, one of the 23 species within Fragaria [47]. F. x ananassa is an octoploid meaning it has eight sets of chromosomes with a total number of 56 chromosomes [47] [48]. F. x ananassa is a hybrid of native species Chilean strawberry Fragaria chiloensis and an American type, Fragaria virginiana [49]. This incidental cross, which appeared in France in the mid-1700s where F. chiloensis and F. virginiana were planted next to each other, began to gradually replace commercial species cultivated in Europe [49].

Strawberry is a well-appreciated fruit crop throughout the world. The world strawberry production has been steadily increasing with annual growth production rate around 4% over the past 20 years. In 2013 the worldwide production reached over 7,7 million metric tones (Mt) where F. x ananassa represents around 60% of the world production [47] [50]. In 2013, according to Food and Agriculture Organization of the United Nations (FAO), nearly 50 % of production came from Asia whereas Americas and Europe were on the second and third place with approximate numbers 25 % and 19 % respectively [50]. Considering that in 2003 the strawberry production was around 5Mt and in 1993 was 3,2 Mt makes strawberry an economically important fruit crop. [50]

Strawberries are propagated in two major ways on a commercial scale. The first technique – the matted row system - utilizes runners as the main yield component. The matted row system prevails in continental Europe and North America. The hill system is another method, which is based on dividing and replanting of crowns. In regions with warm winters and hot to moderate summers the hill system is preferred. [49]
2.4.2 Pests

*F. x ananassa*, as well as other cultivars within *Fragaria* genus, is a crop susceptible to many diseases and attacks by fungi or insects [48] [49]. Pests and common diseases have a negative impact on strawberry fruit quality and plant yield production where, in some cases, crop loss may approach 100% [47] [48]. When it comes to fungi, the global-scale diseases are red stele or red core and *Verticillium* wilt. The former is caused by *Phytophthora fragariae*, the latter by *Verticillium albo-atrum* and *Verticillium dahlia* [49]. Soil pathogens of greater threat in Sweden are *Botrytis cinerea* that causes grey mold and *Podosphaera aphanis*, the source of powdery mildew in strawberry plants [51]. Both grey mold and powdery mildew are recurrent problems on strawberry fields all across the country [51].

Strawberry blossom weevil, *Anthonomus rubi*, is one of the most harmful insects to *F. x ananassa* in Europe [52] [53]. Caterpillars belonging to the family of tortrix moths (*Tortricidae*) like the strawberry tortrix, *Acleris comariana* causes also damage in strawberry plants in northern and central Europe [53]. Two-spotted spider mite (*Tetranychus urticae*) or Cyclamen mite (*Polyphagotarsonemus latus*) are other pests that have a negative impact on quality and yield production due to the damage caused on leaves that they are feeding on [53]. In Sweden as well as in other parts of northern Europe, the most common capsid bugs that can be found are *Lygus rugulipennis* and *Plagiognathus arbustorum* [53] [54]. Rather low resistance levels have been observed to other species i.e. nematodes e.g. *Longidorus elongatus* and *Pratylenchus* and snails like *Arion vulgaris* or *Deroceras reticulatum* [54].

2.4.3 Pesticides

Protection against pests and diseases threatening strawberry plants is highly based on pesticides utilization. The high susceptibility to various species may require handling plants with different types of chemicals. Several types of pesticides are aimed for specific pests or group of pests and accessibility of chemicals can also vary depending on the type of pesticide. For instance, availability of various chemicals against grey mold, excluding fungicides, is quite wide on the Swedish market whereas fungicides for grey mold are narrowed to only two [51]. Fungicides have, in general, been considered to have low to moderate toxicity on mammals [55] [56]. However, certain fungicides may have negative effects on water organisms due to leakage via soil and water [57] [58]. The other issue is fungicides toxicity to decreasing- in-amount pollinating insects or resistance to fungicides in mites [59] [60].

The biggest concern though, is the usage of pyrethroids, neurotoxic insecticides [54]. Pyrethroids have been reported to have a severe impact to water organisms but also on non-target insects [61] [62] [63] [64]. The observed toxicity is rather acute, partly because the nervous system is the main target for pyrethroids [65]. Some non-target species seem to recover if the protection strategy is terminated, however the range of pyrethroids impact should not be ignored and further investigations regarding long-term effects should be performed [8] [61]. Pyrethroid-type insecticides are also toxic to mammals with no exception to humans [6] [65] [66]. Unfortunately, pyrethroids are still broadly used on strawberry plants because of lack of other alternatives on the market [54].
2.5 Pinus sylvestris

2.5.1 The Pinaceae family in the Northern and Baltic countries as a biomass energy supply.

Scots pine, *Pinus sylvestris*, together with Norway spruce, *Picea abies* belong to a rich conifer family, Pinaceae. Both species are most dominant but also native to all of the Nordic and Baltic countries except Denmark [67]. Scots pine covers more than 30 million ha, around 44 % of the total forest area in the Northern Europe. The distribution of Norway spruce is not as far-stretched as of Scots pine, 26% of the total forest area in the above-mentioned part of Europe. [67]

*P. sylvestris* as well as other species distributed in Northern Europe can play a vital role as a biomass energy source. The energy sector is dominated by fossil fuels, which contribute to constantly increasing greenhouse gases. One of the actions to diminish the impact of the climate change is to take Mitigation Actions, initially mentioned in United Nations Framework Convention on Climate Change from 1992 and later stated in the Kyoto Protocol. Climate change mitigation can be done by limiting emission of greenhouse gases by utilizing renewable energy sources. Reforestation is one of the possibilities in order to gain an alternative energy source in form of forest biomass [67] [68]. Demand of forest biomass in Europe has been increasing and according to the estimations done in 2010 where demand of woody biomass was to be around 800 m$^3$, it will reach almost 1400 m$^3$ in 2030 [69]. Unfortunately, according to the same report, the demand in 2030 will exceed the potential supply of forest biomass estimated to be around 1100 m$^3$ [69].

Scots pine is one of the trees in the Northern Europe that has had a potential to be forest biomass resource [67]. In order to produce sufficient amounts of forest-derived biomass one have to consider, along with the properties of the regenerated site (soil temperature and moisture, access to nutrients etc.) and preparation of the regenerated area, planting material. Seedlings are susceptible to various stresses because they are in active growth during plantation. Effective defense against herbivores is therefore desirable in seedlings and is one of the factors providing a successful regeneration process [67]. The amount of *P. sylvestris* seedlings planted in Sweden in 2013 was 138 million of the total 381 million seedlings [70].

2.5.2 Pests and pesticides

The biggest threat to Scots pine as well as other conifers is the large pine weevil *Hylobius abietis* (L.) [71]. *H. abietis* is a serious problem and economically important pest mainly in the Northern and Baltic countries. According to the estimations, the losses caused by pine weevils would cost Europe 140 million euros each year if insecticides or other kinds of protection methods had not been used [72]. One of the most sensitive regions is southern Sweden, where losses in conifer seedlings could reach even 100% without appropriate defense strategy [72]. Other weevils, of minor importance are *Hylobius pinastri* (Gyll.) and *Hylobius piceus* (DeG.) and their impact on conifers damage is said to be low [72].

Utilization of insecticides together with herbicides is the most common strategy against pine weevils. Only in Sweden 17,1 tones of pesticides were sold to forestry where insecticides and herbicides made 5,2 and 8,2 tones respectively [70]. Although certain pyrethroids are no longer in use because of their known neurotoxic effects, products containing lambda-cyhalothrin are still accepted [73] [74]. Other insecticides, widely used in agriculture are products with an active substance called imidacloprid [73]. Study conducted in 2000 revealed that imidacloprid have higher acute toxicity (LD50) than insecticides considered being most toxic: dimethoate and cypermethrin. The LD50 value for imidacloprid varied between 5 to 60 ng/bee. Even if the higher value is taken into consideration (60 ng/bee), the acute dose is much lower compared to those for dimethoate and cypermethrin, where LD50 value reached 152 ng/bee and 160 ng/bee respectively [75]. Therefore, new substances providing sufficient protection against weevils but at the same time being harmless to non-target organisms are desirable.
3. Material and methods

3.1 Plant material
Strawberry seeds of cultivated *Fragaria x ananassa*, ('Temptation’) purchased from Impecta (Impecta Fröhandel AB, Sweden), were used in all experiments described in this report.
Seeds of Scots pine (*Pinus sylvestris* L.) – collected in the orchard of Hade were used in this study.

3.2 Seed treatment and growth conditions
Seed treatments of both *Pinus sylvestris* and *Fragaria x ananassa* had a similar protocol with few modulations in each experiment described below.

3.2.1 Seed treatment
Two separate experiments were carried out on seed treated strawberry plants; study of the germination of treated seeds as well as investigation regarding the level of methylation of seed treated plants. For both experiments, however, seed treatments had a similar protocol regarding used solutions and the process of incubation. Moreover, treatment and incubation of seeds of *P. sylvestris* had a similar procedure as for *F. x ananassa*.

Treatment of strawberry seeds, for seed germination study, took place on 12 April 2016. Seeds were divided in four groups. Three of them were treated with Milli Q water, nicotinamide - NIC (2,5 mM) and nicotinic acid - NIA (2,5 mM) respectively whereas the fourth group of seeds received no treatment. Seeds were incubated in the solutions in the dark at 23 °C for four hours. Treated seeds were under gentle shaking (123 rpm) during the incubation process.

Seed treatment of plants used in the second experiment was accomplished on 18 March 2016, before this project started. For the purpose of this research seeds of *F. x ananassa* were treated in seven different ways. Three groups of seeds received the same substances as in the study on plant growth; Milli Q water, NIC (2,5 mM) and NIA (2,5 mM). Furthermore, three additionally treatments were performed; a combination of the surfactant Tween 80 (0,24 µl ml⁻¹) with NIC, NIA and water respectively. The last, seventh, group contained seeds without any treatment. Incubation was conducted under the same conditions just as the seed treatment performed on 12 April.

On 17 February, *P. sylvestris* seeds were treated with Milli Q water, NIC (2,5 mM) and NIA (2,5 mM) respectively. Conditions for incubation were similar to those for strawberry plants explained above. The treatment and sowing of seeds were carried out before this project started.

3.2.2 Growth conditions
Directly after seed treatment, the strawberry seeds were sown separately. Seeds for the study on plant growth were sown in pots (5 cm in diameter) filled with peat, “P-jord” from Kekkilä Garden (Kekkilä Group, Finland).
Totally 64 pots were put on one of four trays, with 16 pots apiece. Each tray, covered with plastic lid, corresponded to the treatment method that seeds received. The trays were placed in the dark at 23 °C for ten days. From the 22 April 2016, when germination started, trays were transferred to a 18h light/ 6 h dark system.
Lightning was implemented by fluorescent lamps (Philips TL-D Master 80 14W 840). The germination process was observed during five weeks.

Strawberry seeds for the second study (DNA methylation) were sown individually in 3-cm-square seed tray cells on the day of the seed treatment. The number of sown seeds was 84, 12 for each treatment. On 20 April germinated seedlings were re-potted to 5-cm in diameter pots. Plants were watered every second or third day from below. Pine seeds were sown in 3-cm-square seed tray cells. Seedlings were watered with solution of water
and a plant nutriment, Blomstra (Cederroth AB, Upplands Väsby, Sweden). The same growth conditions as well as lightning (Philips TL-D Master) and irradiation time (18 h light/6 h dark) were used under germinating and growth of P. sylvestris and strawberry plants utilized for measurements of DNA methylation.

3.3 Total DNA from F. x ananassa and P. sylvestris cells

3.3.1 Preparation of plant tissue

Materials used for DNA extraction were plant leaves and needles from F. x ananassa and P. sylvestris respectively. After harvesting strawberry leaves or stem with needles, the material was immediately transferred to a 1,5 ml microcentrifuge tube and frozen in liquid nitrogen.

Plant tissue was grounded to a fine powder using a mortar and pestle. Before grinding, mortar and pestle, as well as other instruments used in this process, were cooled with the liquid nitrogen. Whole leaves of F. x ananassa were ground whereas needles were separated from the pine trunk under liquid nitrogen before milling.

During powdering the plant tissue, small amounts of the liquid nitrogen were poured into the mortar in order to avoid the tissue to thaw. As soon as the sample was homogenized the tissue powder was re-transferred into a 1,5 ml tube and placed in liquid nitrogen.

3.3.2 DNA purification

Extraction of total DNA from the plant cells was performed using DNeasy® Plant Mini Kit from QIAGEN (Sollentuna, Sweden), see figure 4. Plant Tissue Mini Protocol was followed during the extraction process with one modification in the last step. 50 µl of RNase Free water from Thermo Scientific™ (Thermo Fisher Scientific Inc., Gothenburg, Sweden) was used to elute DNA from the column instead of Buffer AE given in the protocol [76]. DNeasy® Plant Kit is aimed for purification of the total cellular DNA in plant tissues.

3.3.3 Quantity and quality of DNA

NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, USA) was used to estimate both concentration and purity of extracted DNA. Concentration of extracted DNA was given in ng/µl. Purity of tested samples was determined by the calculated ratio between absorbance at 260 and 280 nm (A260/A280) as well as absorbance at 260 and 230 nm (A260/A230) (QIAGEN, June 2015). The A260/A280 ratio should lie within an interval 1,7 – 1,9. A symmetric peak absorbance at 260 nm, which confirms sample’s purity, can be verified by comparing values for ratios A260/A230 and A260/A230. The value for A260/A230 can be higher or equal to value of ratio A260/A280.

Figure 4.
DNA purification procedure
An overview of DNeasy® Plant Tissue Mini Protocol with it’s cardinal stages. [76]
3.4 Luminometric Methylation Assay (LUMA)

3.4.1 Materials, solutions and instruments

Two restriction enzymes; FastDigest HpaII and FastDigest MspI; RNase Free water from Thermo Scientific™ as well as inorganic pyrophosphate (PPi) purchased from MERCK (Germany) were utilized in LUMA -analysis.

PyroMark® Gold Q96 Reagents (5 x 96) and PyroMark® Annealing Buffer from QIAGEN were other solutions used in this part of experiment. Set with PyroMark® Reagents contained Enzyme Mixture, Substrate Mixture and four nucleotides (dNTPs) in aqueous solutions necessary to perform analysis. PyroMark® nucleotides were following: deoxyadenosine triphosphate (dATPαS), deoxyguanosine triphosphate (dGTP), deoxycytidin triphosphate (dCTP), deoxythymidin triphosphate (dTTP).

The samples for determination of level of DNA methylation were analyzed on a PyroMark® Q24 Plate, in a PyroMark® Q24 instrument (QIAGEN). PyroMark® Q24 Analysis Software was used for calculations.

3.4.2 LUMA - analysis

LUMA analysis [17] was performed in a modified manner as described by Poborilova et al. [33]. Genomic DNA of F. ananassa and P. sylvestris (500 and 250 – 500 ng respectively) were cleaved by Fast Digest restriction enzymes HpaII and MspI. With analysis of F. ananassa only HpaII was used whereas both restriction endonucleases were used in two independent DNA cleavage set-ups of P. sylvestris.

Aqueous DNA samples were cleaved with restriction enzymes for 30 min at 37°C. In the next step, Annealing Buffer (Qiagen) was added to incubated DNA digests with a volume ratio 1:1. 20 μl samples were later placed on the plate with 24 wells (PyroMark® Q24 Plate) and analyzed in the instrument.

Analysis was performed based on additions of 4 dNTPs (A, C + G, T, A) and five repeated additions of inorganic pyrophosphate as an internal standard, see figure 5. The peak heights received from the LUMA- analysis were utilized to estimate the level of DNA methylation in form of a ratio: \((C+G)/PPi\) - formula 1, where \(PPi\) is the average value for peak heights received in the last five PPI additions during pyrosequencing.

Figure 5. Pyrograph for one of the analyzed samples of Fragaria x ananassa

Pyrograph shows results from analysis performed on PyroMark® Q24 instrument in form of peak heights. X-axis displays a preprogrammed sequence order ACTCGAGGGGG, where A corresponds to amount of adenine, C is equal to the amount of both cytosine and guanine (C + G), T represents amount of thymine and G is the amount of pyrophosphate PPI.
4. Results

4.1 Germination of seed treated seedlings *Fragaria x ananassa*

The number of germinated seed was inspected during five weeks; see figure 6. Three groups of seeds were treated with NIC, NIA and water respectively. The fourth seed unit received no treatment prior to germination.

Based on the chart of observed seeds of *F. x ananassa* seems that both nicotinamide and nicotinic acid have less impact on the germination of seedlings comparing to the group treated with water. The number of water treated germinated seeds was 14 of totally 16 sown. The number of seedlings for those without any treatment, NIC and NIA was 8, 9 and 10 respectively. The amount of NIC-seedlings rose gradually over the time while a noteworthy positive alteration appeared in each of the additional groups. For the water treated seeds a significant increase could be observed after the 10th day whereas for NIC and NIA seeds after the 15th day of the experiment.

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*Fragaria x ananassa*

Growth of seedlings

Figure 6.

Chart over germination of seeds treated with water, NIC, NIA and with no pre-treatment.
4.2 DNA extraction

4.2.1 Choosing plant tissue material

Plants utilized in this experiment were previously seed treated in 7 different ways, see table 2. Due to difficulties with the DNA purification from mature leaves, a different strategy of collecting material was taken in the middle of this project.

Extraction of mature, medium/dark green plant tissue resulted in very low DNA yield and/or insufficient purity of the tested samples. On the other hand, brighter and less mature leaves gave better results, both in quantity and quality of DNA. Hence, only young, light green and undeveloped leaves were thereafter harvested. The decision was made based on the literature study [77] [78] [79] as well as on the series of tests with F. x ananassa leaves of different size, development stage and color, see figure 7.

Harvest of young tissues occurred three times over the span of several weeks. It was necessary to collect the material with around 6-day interval in order to obtain sufficient amount for DNA extraction. A small sheaf of light green, newly developed and wrinkled leaves had a fresh weight of about 35 mg, too little to carry out DNA purification. Only very young leaves, within a specific seed treatment, were therefore collected several times.

According to the Plant Tissue Mini Protocol, the wet weight of plant tissue should not exceed 100 mg (weight of grinded material). On the other hand, the weight of prepared material should be higher than 50 mg. Taking the minimum and maximum value into consideration, but also the fact that during grinding around 30 % by weight was lost, the weight of strawberry leaves below circa 90 mg was not enough for DNA purification. Too little plant tissue results in low DNA yield. Moreover, too low DNA concentration could have an impact on performing LUMA – analysis. Furthermore, not all repotted strawberry plants were available for this experiment, due to parallel investigations within the research group on the same material.

In table 2, one can find information about how many plants were repotted and how many were available for DNA purification. The quality and quantity of extracted DNA for two different groups of F. ananassa leaves, undeveloped and mature (see figure 8), are presented in 4.2.2 and 4.2.3 respectively.

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**Figure 7.**
Differences in color, size and maturity between a bright green sheaf of young leaves (in the middle) and fully developed leaves.

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**Figure 8.**
8a, 8b Strawberry leaves, representing a mature-leaves group, were coming from different plants. Some leaves from this group were differing in color, like 8a and 8b, but only slightly in the size or development stage.

8c depicts an example of undeveloped leaves, the other group of analyzed material.
Table 2. Chart over number of treated seeds, repotted plants and plants available for this experiment relating to the method of seed handling.

<table>
<thead>
<tr>
<th>No. treatment</th>
<th>Seed treatment</th>
<th>No. sown seeds</th>
<th>Repotted plants</th>
<th>Plants available for DNA extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None (Dry seeds)</td>
<td>12</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>H$_2$O</td>
<td>12</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>H$_2$O + Tween</td>
<td>12</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>NIC, H$_2$O</td>
<td>12</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>NIA, H$_2$O</td>
<td>12</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>NIC, H$_2$O + Tween</td>
<td>12</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>NIA, H$_2$O + Tween</td>
<td>12</td>
<td>7</td>
<td>6</td>
</tr>
</tbody>
</table>
4.2.2 DNA extraction. Mature leaves of *F. x ananassa*

Table 3 shows data for selected samples with highest DNA concentrations within one of five test series. Unfortunately, too low DNA yield and/or impurities were problems that affected all extracted dark-colored and mature strawberry leaves. Those issues causing poor results of DNA extraction, applied also to developed and light-in-color leaves. Totally around 20 tries with mature leaves of different size, age and color were conducted. Samples presented in table 3, were chosen to demonstrate the insufficiency regarding quantity and quality that characterizes DNA extraction of harvested mature leaves.

For the first four samples, the ratio $A_{260}/A_{280}$ is below the minimum value 1.7, indicating low purity of extracted DNA. The last presented sample from test series 7, despite having an acceptable ratio $A_{260}/A_{280}$, was still unsatisfactory due to other issues. One of the problems was too low DNA yield. A DNA concentration below 70 ng/μl is insufficient to carry out LUMA – analysis. Another issue with the sample was remarkable difference between ratios, $A_{260}/A_{230}$ and $A_{260}/A_{280}$. Low value for absorbance ratio $A_{260}/A_{230}$ effects in a high peak absorbance at 230 nm, which was a general problem for samples containing DNA from mature leaves. In other words, the lower $A_{260}/A_{230}$ value, the more impurities seems sample to contain, see figure 9.

**Table 3. Purity and concentration of DNA of mature strawberry leaves.**

<table>
<thead>
<tr>
<th>Test No</th>
<th>Sample</th>
<th>Plant tissue</th>
<th>DNA yield</th>
<th>Peak abs. $A_{260}$ nm</th>
<th>Abs. ratio $A_{260}/A_{230}$</th>
<th>Abs. ratio $A_{260}/A_{280}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>Ca. 50</td>
<td>89,83</td>
<td>1,797</td>
<td>1,31</td>
<td>1,66</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>Ca. 75</td>
<td>85,86</td>
<td>1,717</td>
<td>0,61</td>
<td>1,38</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>65</td>
<td>17,62</td>
<td>0,352</td>
<td>1,21</td>
<td>1,55</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>83</td>
<td>69,75</td>
<td>1,395</td>
<td>0,57</td>
<td>1,37</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>75</td>
<td>37,63</td>
<td>0,753</td>
<td>1,20</td>
<td>1,71</td>
</tr>
</tbody>
</table>

Purity and concentration of DNA of mature strawberry leaves. The $A_{260}/A_{280}$ ratio is below acceptable minimum value (1.7) except for the last sample. Too low $A_{260}/A_{230}$ ratio and too low DNA yield were other frequent problems, represented here in the last sample.

**Figure 9. Absorbance spectrums of mature leaves**
Absorbance spectrums for samples presented Table 3.
4.2.3 DNA extraction. Undeveloped leaves of *F. x ananassa*

Here, presents results for plant material coming from very small, light green, wrinkled leaves. After purification was performed, concentration and quality of DNA was estimated.

The series of samples can be considered as pure. The ratio $A_{260}/A_{230}$ lane within interval 1.7 – 1.9 for all samples, see table 4. The value for ratio absorbance $A_{260}/A_{230}$ was higher or equal to $A_{260}/A_{280}$ with an exception for sample 6. Though, the difference between those two ratios for this sample is insignificant (around 5%). A symmetric peak at 260 nm can be seen in figure 10.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample</th>
<th>No of plants presented in sample</th>
<th>Plant tissue [mg]</th>
<th>DNA yield [ng]</th>
<th>Peak abs. $A_{260}$ nm</th>
<th>Abs. ratio $A_{260}/A_{230}$</th>
<th>Abs. ratio $A_{260}/A_{280}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>1</td>
<td>3</td>
<td>81</td>
<td>110,94</td>
<td>2,219</td>
<td>2,29</td>
<td>1,83</td>
</tr>
<tr>
<td>$H_2O$</td>
<td>2</td>
<td>2</td>
<td>80</td>
<td>80,05</td>
<td>1,601</td>
<td>1,71</td>
<td>1,71</td>
</tr>
<tr>
<td>$H_2O$, Tween</td>
<td>$3_1$</td>
<td>5</td>
<td>83</td>
<td>80,05</td>
<td>1,601</td>
<td>1,79</td>
<td>1,76</td>
</tr>
<tr>
<td></td>
<td>$3_2$</td>
<td>2</td>
<td>50</td>
<td>72,44</td>
<td>1,449</td>
<td>1,90</td>
<td>1,76</td>
</tr>
<tr>
<td>NIC, $H_2O$</td>
<td>4</td>
<td>3</td>
<td>81</td>
<td>84,26</td>
<td>1,685</td>
<td>1,92</td>
<td>1,74</td>
</tr>
<tr>
<td>NIC, $H_2O,Tween$</td>
<td>5</td>
<td>2</td>
<td>83</td>
<td>95,36</td>
<td>1,907</td>
<td>2,03</td>
<td>1,78</td>
</tr>
<tr>
<td>NIC, $H_2O,Tween$</td>
<td>$6_1$</td>
<td>4</td>
<td>61</td>
<td>77,41</td>
<td>1,548</td>
<td>1,69</td>
<td>1,78</td>
</tr>
<tr>
<td></td>
<td>$6_2$</td>
<td>4</td>
<td>80</td>
<td>92,42</td>
<td>1,848</td>
<td>2,05</td>
<td>1,79</td>
</tr>
<tr>
<td>NIA, $H_2O,Tween$</td>
<td>7</td>
<td>5</td>
<td>83</td>
<td>134,98</td>
<td>2,700</td>
<td>2,04</td>
<td>1,74</td>
</tr>
</tbody>
</table>

Results are presented concerning seven different seed treatments. One of two factors confirming the purity, the value of absorbance ratio (last column to the right). Concentration of genomic DNA is given in a DNA-yield – column. The table displays also a plant tissue weight used for the extraction and the number of plants whose leaves were used (column: No. of plants presented in sample).

![Figure 10. Absorbance spectrums of undeveloped leaves](image)

Graph plotted by spectrophotometer of tested (see Table 4) DNA-samples of young strawberry leaves. Absorbance as a function of wavelength (nm), that gives a fine distributed absorbance spectrum with the peak at 260nm.
4.2.4 DNA extraction of *Pinus sylvestris*

High value for the absorbance ratio, ($A_{260}/A_{280}$), and a well-formed absorbance spectrum with an absorbance peak at $A_{260}$ were valid factors for all tested pine samples, see table 5 and figure 11.

**Table 5. Purity and concentration of DNA from *Pinus sylvestris* needles**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample</th>
<th>Plant tissue [mg]</th>
<th>DNA yield [μg]</th>
<th>Peak abs. $A_{260}$ nm</th>
<th>Abs. ratio $A_{260}/A_{230}$</th>
<th>Abs. ratio $A_{260}/A_{280}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>1</td>
<td>105</td>
<td>88.65</td>
<td>1,773</td>
<td>2.13</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>108</td>
<td>128.49</td>
<td>2,570</td>
<td>2.15</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>104</td>
<td>166.25</td>
<td>3,325</td>
<td>2.22</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>81</td>
<td>76.76*</td>
<td>1,535</td>
<td>1.96</td>
<td>1.72</td>
</tr>
<tr>
<td>NIC, H$_2$O</td>
<td>1</td>
<td>102</td>
<td>95.04*</td>
<td>1,901</td>
<td>1.87</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>103</td>
<td>113.69</td>
<td>2,274</td>
<td>2.13</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>117</td>
<td>87.95</td>
<td>1,759</td>
<td>2.05</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>109</td>
<td>129.67</td>
<td>1,685</td>
<td>1.98</td>
<td>1.84</td>
</tr>
<tr>
<td>NIA, H$_2$O</td>
<td>1</td>
<td>109</td>
<td>94.33</td>
<td>1,887</td>
<td>1.80</td>
<td>1.81</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>110</td>
<td>85.17*</td>
<td>1,703</td>
<td>1.97</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>106</td>
<td>82.69*</td>
<td>1,654</td>
<td>1.97</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>116</td>
<td>85.49</td>
<td>1,710</td>
<td>2.23</td>
<td>1.80</td>
</tr>
</tbody>
</table>

* Selected samples with data valid after the additional concentration procedure.

Purity factors ($A_{260}/A_{230}$ and $A_{260}/A_{280}$) and DNA yield of pine needles. *P. sylvestris* was treated in three different methods displayed in the table. Material was taken from four different plants within each treatment category. Four samples (marked with *) were concentrated in further step due to too low DNA yield (below 70 ng/µl). Those samples, after concentration, were again analyzed. The chart shows records after the concentrating procedure.

**Figure 11. Absorbance spectrums: *P. sylvestris* needles**

Pure absorbance spectrums of pine’s needles for water (11a), NIC (11b) and NIA (11c) treatment respectively. Numbers of samples showed on graphs (11 a-c) corresponds to those presented in Table 5.
4.3 DNA methylation

4.3.1 DNA methylation of *Fragaria x ananassa*

For two of the seed treatments, Water Tween and NIC Tween, additional, DNA-sample was available for analysis. For these seed treatments, calculations were made as an average value of methylation level of both samples. Fast Digest restriction enzyme *Hpa*II was used in this experiment and 500 ng sample DNA was used in the analysis (for more information regarding essentials of LUMA-analysis, see 2.3).

![Diagram](chart.png)

**Figure 12.**
Chart over changes in the level of the DNA methylation in seed treated *F. ananassa* young leaves. Each analyzed sample contained material from diverse plants within one of seven seed treatments. Only one sample represented each treatment, except for Water Tween and NIC Tween. Results for Water Tween and NIC Tween shown here are presented as mean values based on 2 samples for each treatment, error bars shows standard deviation.

The samples shown in figure 12 can be divided into three groups: None Treatment (sample first to the left), No Tween group (purple group with treatments: Water, NIC and NIA) and Tween group (turquoise group with treatments: Water Tween; NIC Tween and NIA Tween).

When comparing samples from the last two groups, No Tween and Tween group, one can observe a similar pattern. The peak height value, $\frac{(C+G)}{PPi}$, within those two groups is higher in the treated samples comparing with control samples within each group, Water and Water Tween respectively. This means that the DNA methylation level for treatments is lower than for control within those two groups. Moreover, the hypomethylating effect is stronger for NIA relative to NIC treatment within No Tween group. One can also observe a similar relation within Tween group-treatments although discrepancy in peak high values between NIA Tween and NIC Tween is smaller than for corresponding treatments in No Tween group. Another aspect when comparing No Tween and Tween groups with each other is that surfactant present in Tween group samples seemed to give stronger hypomethylating effect. Peak height value for Water Tween is higher than for Water sample in No Tween group. Analogous tendency can be seen for NIC/NIC Tween and NIA/ NIA Tween treatments.

A high DNA methylation level can be observed for control samples (Water as well as Water Tween) because of the low peak height value comparing with sample that received no treatment dry seeds.
4.3.2 DNA methylation of *Pinus sylvestris*

Concentration and quality of pine needles for the analyzed sample can be found in Table 5. In order to perform LUMA analysis for *P. sylvestris* 4 plants were chosen from NIC and NIA treatment each as well as 4 plants treated with only water as control. Results from LUMA-analysis are presented in figure 13 and figure 14 with restriction enzymes, *Msp*I and *Hpa*II respectively. Vertical axis is estimated with formula 1, peak height value. In both analyses 300 ng sample DNA was used.

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**Pinus sylvestris**
Analysis of DNA Methylation with *Msp*I

![Graph showing DNA methylation analysis with *Msp*I.](image)

**Figure 13.**
Chart over changes in the level of the methylation in DNA cleaved with *Msp*I in *P. sylvestris* plants from seeds treated with nicotinamide (NIC) and nicotinic acid (NIA). Mean values of 4 seedlings per treatment are shown. Standard deviation is presented in form of error bars.

**Pinus sylvestris**
Analysis of DNA Methylation with *Hpa*II

![Graph showing DNA methylation analysis with *Hpa*II.](image)

**Figure 14.**
Chart over changes in the level of DNA methylation using *Hpa*II enzyme in Scots pine plants from seeds treated with nicotinamide (NIC) and nicotinic acid (NIA). Mean values are based on 4 seedlings per treatment, error bars show standard deviation.

Cleavage with *Msp*I gave no significant difference in DNA methylation level in seedlings from NIA treated seeds, when compared to control seedlings (water treated seeds), see figure 13.

However, hypermethylation could be observed in seedlings from NIA-treated seeds when LUMA-analysis with *Hpa*II was performed, which resulted in about 10% change. The analysis of the level of the global DNA methylation in seedlings from seeds treated with NIC indicated an opposite effect – hypomethylation.
5. Discussion

In case seed treatment will be useful as a protection strategy in strawberry cultivation, it is important to have knowledge about effects on germination. Treatments of *F. x ananassa* seeds with NIC and NIA respectively did not have a negative impact on the amount of germinated seedlings when compared with no treatment, even though treating seeds with only water did increase the germination process compared to non-treated seeds, see figure 6.

DNA extraction of *F. x ananassa* leaves was challenging regarding mature and darker leaves. The impurities and, in many cases, low DNA-yield can be explained by the fact that mature leaves have high amounts of polysaccharides and phenolic compounds [77] [78]. These substances reduce the activity of enzymes, used during the extraction procedure, by attaching to DNA and interfering with purification reactions [78] [79]. Nonetheless, newly developed young leaves tend to contain lower levels of polysaccharides or polyphenols as well as higher concentrations of DNA [78]. Results of DNA extraction presented in table 3 and table 4 verifies the above stated opinions. Although DNA extraction of mature strawberry leaves is difficult but still possible, it requires another DNA extraction protocol. The modified CTAB DNA Extraction Protocol that involves surfactant hexadecyltrimethylammonium bromide (CTAB) and utilization of chloroform is time-consuming and not appropriate for this project due to project’s time limitations [77].

Based on the LUMA-analyzed samples of *Fragaria x ananassa*, all seedlings that previously were seed treated with NIC and NIA had been hypomethylated, compared to water treatment. However, nicotinic acid had a stronger effect than nicotinamide in plants, this implies samples with and without surfactant tween. An interesting observation is that water had a hypermethylating effect on control seeds. Water seemed to trigger some processes in plants.

Regarding *Pinus sylvestris*, no significant changes were observed with *Msp*I cleavage, whereas LUMA-analysis with *Hpa*II enzyme resulted in hypermethylation in samples treated with NIA. A minor change had even occurred in NIC-treated seedlings however with the opposite effect in form of hypomethylation. When discussing results, is important to take into account that analysis investigates DNA-methylation changes on the global level. In other words, NIC and/or NIA can generate a hypermethylating effect on certain genes when on other genes the same substance may have a hypomethylating outcome. The results in the changes of the total cellular DNA-methylation level, for both *F. ananassa* and *P. sylvestris* are, are resultant of local changes on gene level.

In order to perform DNA extraction of strawberry leaves, samples from each treatment contained pooled tissues from a number of different plants. The number of plants used for individual samples varied and was between 2 and 5; see table 4. Samples with *P. sylvestris* tissue were comprised of material coming from individual plants. Change in DNA methylation did occur in both plant species, which suggest that NIC and NIA may play important role in influencing the epigenetic memory of plants, since treatments on seed level had effects on three months old plants. Furthermore, epigenetic changes in the investigated plants, which belong to different taxonomic orders also implies that both nicotinic acid and nicotinamide may play general roles in various species within Plantae kingdom.

Results in the change of the level of DNA-methylation might also be a reference point in an additional discussion. Plants receiving higher amounts of substances related to plant defence mechanism, like NIC or NIA, may have an altered compilation of generated volatile compounds (VOC). This, in turn, may impact smell and, in case of strawberry plants, taste of the treated plants. Such possible effects may be significant in insect behaviour.
Norway spruce is, as Scots pine, a species within *Pinaceae* family, of which epigenetic effects with NIC or NIA seed treatment have been studied [10]. Seed treatment of strawberry plants with these two substances and analysis of epigenetic changes, which was conducted and presented by the author of this paper, is, so far, one of the first experiments of this kind performed. The number of analyzed plants was limited and varied between 4 and 2-5 for *P. sylvestris* and *F. x ananassa* respectively. Therefore, it is important to highlight that repetition of the studies is necessary, where more material from seed treated strawberry plants and Scots pine than this report conveys is required.

Further work, apart from analysis of more samples, could be investigation of insects’ behavior and the level of pathogen attack on examined plants. Additionally, the research could be conducted on several levels; seed treated plants and/or plants watered with the concerned substances; investigation of NIC and NIA with and without additional surfactant.

Another aspect regarding strawberries, as crops, cannot be forgotten. The most popular production system in Europe employs runners in order to propagate strawberry plants [49]. If protection strategy involving seed treatment and utilization of NIC and NIA would be effective a new crop production strategy would be needed. Strawberry propagation by seeds could be an alternative when growing seed treated plants.
6. Conclusions
The goal of this project was to investigate effects of seed treatment with nicotinamide and nicotinic acid on the level of DNA methylation in *F. x ananassa* and *P. sylvestris* plants. Epigenetic changes did occur in both plant species observed when using *Hpall* restriction enzyme in the analysis. Hypomethylation was observed in analyzed strawberry leaves, whereas analysis of Scots pine resulted in hypermethylation in tested needles. The susceptibility of the investigated plants to NIC and NIA suggests that those substances may have a stress-signaling role themselves as it was proposed by Berglund [44]. The results are in line with research, conducted by Berglund et al., on Norway spruce, which pointed at a positive connection between NIA and NIC treated spruce seeds as well as seedlings and a decrease in pine weevils attacks [10]. The present investigation can therefore provide a supplementary material in further research regarding more sustainable protection strategies than utilization of pesticides. However, considering that the experiments conducted in this project are one of the first attempts of evaluating epigenetic effects of NIC and NIA in the seed treated species presented in this report, repetition of this study is needed. An access to more plant material within each treatment is necessary in additional studies and should be applied for both *F. x ananassa* and *P. sylvestris*. 
7. References


